Modification of Peptides, Oligonucleotides and Other Small Biomolecules with Tide Quencher™ (TQ) Dye Succinimidyl Esters

Introduction

Although DABCYL has been used to develop a variety of FRET applications, its low quenching efficiency of longer wavelength dyes (such as fluoresceins, rhodamines and cyanines) has limited its use in the development of sensitive fluorogenic FRET probes. Additionally, the absorption spectrum of DABCYL is environment-sensitive. AAT Bioquest has developed the robust Tide Quencher™ (TQ) acceptor dyes for the development of longer wavelength FRET probes. These Tide Quencher™ dark FRET acceptors (such as TQ1, TQ2, TQ3, TQ4, TQ5, TQ6 and TQ7) are optimized to pair with our Tide Fluor™ dyes and the other fluorophores (such as Alexa Fluor® dyes, AMCA, EDANS, FAM, TAMRA, HEX, JOE, TET, ROX, Cy3, Cy5 and Cy7). Like our Tide Fluor™ (TF) donor dyes, our Tide Quencher™ acceptor dyes are much more cost-effective with comparable or even better performance for your desired biological applications than other similar products on the market.

Besides their broad applications in the development of real time PCR probes, our Tide Quencher™ dyes have also been used to develop various protease substrates such as HIV protease (see above), MMPs and secretases. In some cases, it has demonstrated greatly improved enzyme performance. This may be partly due to the red-shifted absorption spectrum that overlaps better with the emission spectrum of fluoresceins, rhodamines and cyanines. Tide Quencher™ dyes are great choice for you to eliminate the limitations of classic quenchers. They are excellent dark quenchers that are individually optimized to pair with all of the popular fluorescent dyes such as fluoresceins and rhodamines. Our TQ dye series are essentially nonfluorescent, and cover the full visible spectrum with unusually high efficiency. For example, TQ2 has absorption maximum perfectly matching the emission of FAM while TQ3 is proven to be the best quencher for TAMRA and Cy3. In summary, our Tide Quencher™ dyes have the following advantages:

- *Most Powerful:* enable you to explore the FRET potentials that might be impossible with other quenchers.
- *Versatile Reactive Forms:* convenient for self-constructing your desired FRET biomolecules.
- *A Complete Set of Dyes:* perfectly match your desired fluorescent donors.
- *Enhanced Value:* competitive price with the best performance.

Labeling Mechanism

Succinimidyl esters are proven to be the best reagents for amine modifications because the amide bonds that are formed are essentially identical to, and as stable as the natural peptide bonds. These reagents are generally stable and show good reactivity and selectivity with aliphatic amines. There are few factors that need be considered when SE compounds are used for conjugation reaction:

1). *Solvents:* For the most part, reactive dyes are hydrophobic molecules and should be dissolved in anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO).

2). *Reaction pH:* The labeling reactions of amines with succinimidyl esters are strongly pH dependent. Amine-reactive reagents react with non-protonated aliphatic amine groups, including the terminal amines of proteins and the ε-amino groups of lysines. Thus amine acylation reactions are usually carried out above pH 7.5. Protein modifications by succinimidyl esters can typically be done at pH 7.5-8.5, whereas isothiocyanates may require a pH 9.0-10.0 for optimal conjugations.

3). *Reaction Buffers:* Buffers that contain free amines such as Tris and glycine and thiol compounds must be avoided when using an amine-reactive reagent. Ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for protein precipitation must also be removed (such as viadinlysis) before performing dye conjugations.

4). *Reaction Temperature:* Most conjugations are done at room temperature. However, either elevated or reduced temperature may be required for a particular labeling reaction.

Storage and Handling

The dye labeled oligos should be stored at ≤–15°C, and kept from light. For longer storage, dye labeled oligo could be divided as single-used aliquot and stored at ≤–15°C. PROTECT FROM LIGHT.

Sample Protocols (FOR REFERENCE PURPOSE ONLY)

Always wear the protection apparatus (such as gloves). Warm all the components before opening them, and immediately prepare the required solutions before starting the conjugation. Avoid repeated freezing and thawing if possible. Any solutions containing the dye should be kept from light.

Label Amino-Modified Oligonucleotides with Tide Quencher™ Dyes

The following protocol has been optimized for labeling 200 µg (~6 $A_{260\ nm}$ units) of a proprietary oligonucleotide. You need modify the protocol to get the best results for your particular application by multiple experimentations. YOUR AMINO-MODIFIED OLIGO MUST BE TREATED TO REMOVE AMMONIA THAT RAPIDLY REACTS AND CONSUMES DYE SUCCINIMIDYL ESTERS.

1. Prepare Oligo Solution (Solution A)

Dissolve your amino-modified oligo (~200 µg) in a tetraborate buffer (100 µL, pH 8.5±0.5).

Note 1: The oligonucleotide must be synthesized with an amine group. See Appendix 1 for the purification of amino-modified oligos.

Note 2: Avoid buffers that contain primary amines, such as Tris, as these compete for conjugation with the amine-reactive compound.

2. Prepare Dye Solution (Solution B)

Dissolve 1 mg dye SE in 100 µL DMSO (>10 mg/mL if possible) by pipetting up and down. Centrifuge the solution stock on the sides of the vial to the vial bottom.

Note: prepare the DMSO dye solution before starting the conjugation. Extended storage of the dye solution may reduce the dye activity. Any solutions containing the dye should be kept from light. We do not recommend that you store the DMSO dye solution for future use.

3. Run Conjugation Reaction

3.1 To the dye solution (B, 20-50 µL) add the oligo solution (A, 100 µL) with stirring or shaking (keeping the reaction mixture from light).

3.2 Rotate or shake the reaction mixture for 4-6 hours at room temperature on a rotator or shaker.
Note: Gently vortex tap the vial every10 minutes for the first hour to ensure that the reaction solution remains well mixed. Do not mix violently, as material may be left on the sides of the vial. After six hours, 50–90% of the amine-modified oligonucleotide molecules should be labeled. The reaction might be incubated overnight if it is more convenient. However, overnight incubation will not result in a greater labeling efficiency in most cases.

4. Purify Dye-Oligo Conjugate

4.1 Preliminary purification by ethanol precipitation of labeled oligonucleotide

a. Add 20 µL (one-tenth reaction solution volume in general) of 3 M NaCl and 300 µL cold absolute ethanol (two and half reaction solution volume volumes in general) to the reaction vial.
b. Mix the solution well and place it at –20°C for 30 minutes.
c. Centrifuge the solution in a microcentrifuge at 10,000 to 15,000 × g for 30 minutes.
Note: Loss of sample may occur if the centrifugation is not long enough.
d. Carefully remove the supernatant, rinse the pellet 1-3 times with cold 70% ethanol and dry briefly.
Note: Some unreacted labeling reagent may have precipitated over the course of the reaction or may be stuck on the walls of the reaction vial. This material should be completely redissolved by extensive vortex mixing before centrifugation. Redissolving the labeling reagent ensures that the precipitated oligonucleotide will be minimally contaminated with unreacted label.

4.2 Final Purification by HPLC or by gel electrophoresis
See Appendix I

Label Peptides with Tide Quencher™ Dyes

The following protocol has been optimized for labeling 10 mg of a proprietary peptide (MW ~ 2000) that contains only a single free amino group. YOU NEED MODIFY THE PROTOCOL TO ARCHIE THE BEST RESULTS FOR YOUR PARTICULAR APPLICATION BY MULTIPLE EXPERIMENTATIONS.

1. Prepare Peptide Solution (Solution A)

Dissolve your peptide (~10 mg) in DMF (~1 ml).

Note 1: The peptide must be neutralized with a base such as triethylamine or potassium carbonate.

Note 2: Avoid buffers that contain primary amines, such as Tris, as these compete for conjugation with the amine-reactive compound.

2. Prepare Dye Solution (Solution B)

Dissolve 5 mg dye SE in 500 µL DMF (>10 mg/mL if possible) by pipetting up and down.

Note: prepare the DMF dye solution before starting the conjugation. Extended storage of the dye solution may reduce the dye activity. Any solutions containing the dye should be kept from light. We do not recommend that you store the DMF dye solution for future use.

3. Run Conjugation Reaction

3.3 To the dye solution (B, 500 µL) add the peptide solution (A, 1 mL) with stirring or shaking (keeping the reaction mixture from light).

3.4 Stir the reaction mixture for 4-6 hours at room temperature.

4. Purify Dye-Peptide Conjugate

The reaction solution was concentrated and purified on a C18 column to afford the desired conjugate. The fractions were analyzed by HPLC, and the fractions of >97% purity were pooled and lyophilized.

Note 1: HPLC Purification Conditions: TEAB buffer (triethyl ammonium bicarbonate, 0.25 mmol, pH=7.0-8.0) was used as buffer A and acetonitrile as buffer B. The HPLC was run from 0% B to 30% B in 60 min (flow rate: 100 mL/min).

Note 2: Avoid strong light during the operation.

References

1. Hermanson GT (1996). *Biocojugate Techniques*, Academic Press, New York.
2. Sambrook J., Fritsch E.F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory.
3. Brinkley M (1992). A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjug Chem* **3,** 2-13.

Appendix I. Physical and Spectral Properties of Tide Quencher™ Dyes

Labeling Dye	Extinction Coefficient[1] ($cm^{-1}M^{-1}$)	Abs (nm)	CF at 260 nm[2]	CF at 280 nm[3]	Molecular Weight Calculation[4]
TQ1	20,000	510	0.147	0.194	+ 272
TQ2	21,000	531	0.100	0.120	+ 364
TQ2WS	19,000	539	1.296	0.559	+ 504
TQ3	22,000	598	0.085	0.091	+ 453
TQ3WS	90,000	576	0.186	0.205	+ 779
TQ4WS	90,000	601	0.149	0.136	+ 781
TQ5WS	130,000	662	0.072	0.082	+ 758
TQ6WS	130,000	702	0.120	0.102	+ 806
TQ7WS	140,000	763	0.072	0.091	+ 783

Note I. Extinction Coefficient at their maximum absorption wavelength;2. CF at 260 nm is the correction factor used for eliminating the dye contribution to the absorbance at 260 nm (for oligo and nucleic acid labeling); 3. CF at 280 nm is the correction factor used for eliminating the dye contribution to the absorbance at 280 nm (for peptide and protein labeling); 5. The molecular weight of the desired conjugate = the molecular weight + the value listed in the table.

Note II. BHQ is the is the trademark of Biosearch Technologies, Inc. Texas Red®, QSY® and Alexa Fluor® are the trademarks of Molecular Probes. CyDye, Cy3®, Cy5®, Cy5.5® and Cy7® are the trademarks of GE Health Care. DyLight™ is the trademark of ThermoFisher Corp. IRDye® 700 and IRDye® 800 are the trademarks of Li-COR. Tide Quencher™ and Tide Quencher™ are the trademarks of AAT Bioquest.

- **Optimized to label peptides and nucleotides with superior FRET performance**
- **Optimized to pair with Tide Fluor™ donors to maximize FRET efficiency**
- **pH-insensitive and environment-insensitive fluorescence for developing robust assays**
- **Higher photostability to improve the quality of fluorescence imaging**
- **A variety of reactive forms available for conjugations**

Appendix II. FRET Selection Guide of Tide Quencher™ Dyes

Dark FRET Acceptor	λ_{max} (nm)	Features and Benefits	Ordering Information
Tide Quencher™ 1 (TQ1)	510	*Alternative to Dabcyl, QSY® 35 and BHQ®-0* • Best paired with Tide Fluor™ 1 (TF1) • Excellent FRET efficiency with coumarins	#2188 & #2189 (Click chemistry) #2190 (TQ1 acid); #2192 (TQ1 amine) #2193 & #2194 (TQ1 CPG, OH-reactive) #2196 (TQ1 maleimide, SH-reactive) #2198 (TQ1 phosphoramidite, OH-reactive) #2199 (TQ1 SE, NH_2-reactive)
Tide Quencher™ 2 (TQ2)	531	*Alternative to BHQ®-1* • Best paired with Tide Fluor™ 2 (TF2) Better matched with FAM, FITC and Alexa Fluor® 488 than other commercial quenchers	#2211 & #2212 (Click chemistry) #2200 (TQ2 acid); #2202 (TQ2 amine) #2203 & #2204 (TQ2 CPG, OH-reactive) #2206 (TQ2 maleimide, SH-reactive) #2208 (TQ2 phosphoramidite, OH-reactive) #2210 (TQ2 SE, NH_2-reactive)
Tide Quencher™ 2WS (TQ2WS)	539	*Alternative to BHQ®-1& QXL 520* Better matched with FAM, FITC and Alexa Fluor® 488 than other commercial quenchers	#2050 (TQ2WS acid) #2058 (TQ2WS, SE, NH_2-reactive)
Tide Quencher™ 3 (TQ3)	598	*Alternative to QSY® 7, QSY® 9 and BHQ®-2* • Best paired with Tide Fluor™ 3 (TF3) Excellent FRET efficiency with Cy3®, Alexa Fluor® 555 and TAMRA than other commercial quenchers	#2220 (TQ3 acid); #2222 (TQ3 amine) #2223 & #2224 (TQ3 CPG, OH-reactive) #2226 (TQ3 maleimide, SH-reactive) #2228 (TQ3 phosphoramidite, OH-reactive) #2230 (TQ3 SE, NH_2-reactive) #2231 & #2232 (Click chemistry)
Tide Quencher™ 3WS (TQ2WS)	576	*Alternative to QSY® 7, QSY® 9 and BHQ®-2* Excellent FRET efficiency with Cy3®, Alexa Fluor® 555 and TAMRA than other commercial quenchers	#2227 (TQ3WS acid) #2229 (TQ3WS SE, NH_2-reactive)
Tide Quencher™ 4 (TQ4)	647	*Alternative to QSY® 21 and BHQ®-3* Better FRET efficiency with ROX, Texas Red® and Alexa Fluor® 594 than other commercial quenchers	#2062 & #2063 (TQ4 CPG, OH-reactive)
Tide Quencher™ 4WS (TQ4WS)	601	*Alternative to QSY® 21 and BHQ®-3* • Best paired with Tide Fluor™ 4 (TF4) Better FRET efficiency with ROX, Texas Red® and Alexa Fluor® 594 than other commercial quenchers	#2060 (TQ4WS acid); #2061 (TQ4WS amine) #2064 (TQ4WS maleimide, SH-reactive) #2067 (TQ4WS SE, NH_2-reactive) #2068 & #2069 (Click chemistry)
Tide Quencher™ 5 (TQ5)	680	*Alternative to QSY® 21 and BHQ®-3* Better FRET efficiency with ROX, Texas Red® and Alexa Fluor® 594 than other commercial quenchers	#2077 & #2078 (TQ5 CPG, OH-reactive)
Tide Quencher™ 5WS (TQ5WS)	662	*Alternative to QSY® 21 and BHQ®-3* • Best paired with Tide Fluor™ 5 (TF5) Excellent FRET efficiency with Cy5®, DyLight™ 649 and Alexa Fluor® 647.	#2075 (TQ5WS acid); #2076 (TQ4WS amine) #2079 (TQ5WS maleimide, SH-reactive) #2081 (TQ5WS SE, NH_2-reactive) #2082 & #2083 (Click chemistry)
Tide Quencher™ 6WS (TQ6WS)	702	• Stronger absorption • Best paired with Tide Fluor™ 6 (TF6) Better FRET efficiency with Cy5.5®, IRDye® 700 and Alexa Fluor® 680 than other commercial quenchers	#2090 (TQ6WS acid); #2091 (TQ6WS amine) #2094 (TQ6WS maleimide, SH-reactive) #2096 (TQ6WS SE, NH_2-reactive) #2097 & #2098 (Click chemistry)
Tide Quencher™ 7WS (TQ7WS)	763	• Stronger absorption • Best paired with Tide Fluor™ 7 (TF7) Better FRET efficiency with Cy7® and Alexa Fluor® 750 than other commercial quenchers	#2105 (TQ7WS acid); #2106 (TQ7WS amine) #2109 (TQ7WS maleimide, SH-reactive) #2111 (TQ7WS SE, NH_2-reactive) #2112 & #2113 (Click chemistry)

Appendix III. HPLC Purification of Dye Oligonucleotide Conjugates

Ethanol Precipitation

Some commercial oligonucleotides often contain some interfering compounds, especially amines, such as triethylamine or Tris, and ammonium salts, we strongly recommend you to extract and precipitate the commercial oligo samples prior to initiating your labeling reaction. On the other hand, the labeling mixture contains labeled oligonucleotide, unlabeled oligonucleotide, hydrolyzed dye acid and unincorporated dye SE. The impurities of hydrolyzed dye acid and unincorporated dye SE resulted from the labeling reaction can be effectively removed by ethanol precipitation. The following protocol was optimized for the further purification of 0.1–1 mg commercial oligonucleotide sample that was purified by HPLC (3–30 A260 units).

1) Dissolve your target oligonucleotide in 100 µL of deionized water and extract three times with an equal volume of chloroform.
2) Precipitate the oligonucleotide by adding one-tenth volume (10 µL) of 3 M NaCl and two and a half volumes (250 µL) of cold absolute ethanol. Mix well and place at –20°C for 30 minutes.
3) Centrifuge the solution in a microcentrifuge at 10,000 to 15,000 g for 30 minutes.
4) Carefully remove the supernatant, rinse the pellet 1-3 times with cold 70% ethanol, and dry under a vacuum.
5) Dissolve the dry pellet in deionized water to achieve a final concentration of >50 µg/µL. This amine-modified oligonucleotide stock solution may be immediately used or stored frozen at ≤–15°C.

Purification by HPLC

Labeled oligonucleotides can be purified by reverse-phase HPLC using a standard analytical C8 or C18 column using an analytical or semi-preparative HPLC instrument. The following protocol was optimized for the further purification of 0.1–1 mg labeled oligonucleotide (3–30 A260 units).

1) Dissolve the pellet from the ethanol precipitation in 0.1 M triethylammonium acetate (TEAA).
2) Load the dissolved pellet onto the column in 0.1 M TEAA and run a linear 5–95% acetonitrile gradient over 30 minutes.
   Note 1: There will be peaks that correspond to the unlabeled oligonucleotide, the labeled oligonucleotide, and the free dye. The actual order and number of these peaks depends on the length of the oligonucleotide and the purity of the sample.
   Note 2: To determine the identity of the peaks, monitor the absorbance at both 260 nm and at the absorbance maxima (λmax) for the dye. For instruments with only one detector, two small samples should be run, each monitored at a different wavelength. Unlabeled oligonucleotide will show an absorbance at 260 nm only. Both the free dye and the labeled oligonucleotide will have absorbance at both 260 nm (A260 for oligo) and at the absorbance maximum of the dye (Amax for dye); The dye-labeled oligonucleotide will have a higher A260:Amax ratio than the dye or hydrolyzed dye.

Purification by Gel Electrophoresis

1) Pour a 0.5 mm–thick polyacrylamide slab gel.
   Note: For oligonucleotides less than 25 bases in length, use 19% acrylamide, for oligonucleotides 25–40 bases, 15% acrylamide, and for oligonucleotides 40–100 bases, 12% acrylamide.
2) Resuspend the pellet from ethanol precipitation in 200 µL of 50% formamide, and incubate at 55°C for 5 minutes to disrupt any secondary structure.
3) Load the warmed oligonucleotide onto the gel and load an adjacent well with 50% formamide plus 0.05% bromophenol blue. The bromophenol blue will migrate at approximately the same rate as the oligonucleotide.
   Note: You may need to use several wells.
4) Run the gel until the bromophenol blue indicator dye is two-thirds of the way down the gel.
5) Remove the gel from the glass plates and place on Saran Wrap.
6) Lay the gel on a fluorescent TLC plate.
7) Locate the labeled and unlabeled oligonucleotides by illumination with a handheld UV source.