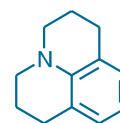


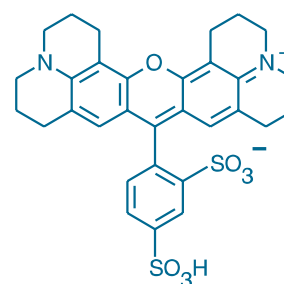
1.6 Dyes with Absorption Maxima Above 520 nm

Our long-wavelength light-emitting dyes (Table 1.6) are among the most photostable fluorescent labeling reagents available. Moreover, spectra of most of the dyes are not affected by changes in pH between 4 and 10, an important advantage over the fluoresceins for many biological applications. Dyes in this spectral range are important for certain multicolor applications, such as DNA sequencing, detection on microarrays and fluorescence *in situ* hybridization, which demand a greater number of fluorophores with distinct spectra. The most common members of this group have been the tetramethylrhodamines — including the reactive isothiocyanate (TRITC) and carboxylic acid (TAMRA) derivatives — as well as the X-rhodamines. The X prefix of the X-rhodamines, which include Texas Red derivatives, refers to the fluorophore's extra julolidine rings (Figure 1.65). These rings prevent rotation about the nitrogen atoms, resulting in a shift in the fluorophore's spectra to longer wavelengths and usually an increase in its fluorescence quantum yield. Our unique patented diarylrhodamine derivatives — the QSY 7, QSY 9 and QSY 21 dyes — are essentially nonfluorescent compounds that have strong absorption in the visible spectrum (Figure 1.66). They are probably the best chromophores available for use as nonfluorescent quenchers in many bioassays.

This section includes dyes that have absorption maxima beyond about 520 nm, extending to nearly 800 nm. Significant exceptions, however, are the long-wavelength Alexa Fluor dyes, which are all discussed in Section 1.3, the long-wavelength BODIPY dyes — BODIPY TMR, BODIPY TR, BODIPY 630/650 and BODIPY 650/665 — which are described in Section 1.4 and the 2',4',5',7'-tetrabromofluorescein (eosin), 2',4',5',7'-tetraiodofluorescein (erythrosin), TET, JOE and HEX dyes, which also absorb maximally beyond 520 nm but are discussed with other fluoresceins in Section 1.5. The versatile Alexa Fluor and BODIPY dyes provide demonstrably superior performance in many applications.



Julolidine



Sulforhodamine 101

Figure 1.65 The amine substituents of X-rhodamine, sulforhodamine 101 and Texas Red dyes are rigidified in a julolidine ring structure.

Table 1.6 Amine-reactive orange- and red-fluorescent fluorophores in this section.

Fluorophore (Abs/Em) *	Succinimidyl Ester	Other	Protein Labeling Kits	Notes
Lissamine rhodamine B (570/590)		L-20 (SC) L-1908 (SC)		<ul style="list-style-type: none"> Optimal for 568 nm excitation Photostable
Naphthofluorescein (602/672)	C-653 (SE) ^M	C-652 (COOH) ^M		<ul style="list-style-type: none"> Very long-wavelength excitation and emission pH-sensitive fluorescence, with a high pK_a (~7.6)
Rhodamine 6G (525/555)	C-6127 (SE) ⁵ C-6128 (SE) ⁶ C-6157 (SE) ^M			<ul style="list-style-type: none"> Excited by the 514 nm spectral line of the argon-ion laser Spectra intermediate between those of fluorescein and TMR
Rhodamine Red-X (580/590)	R-6160 (SE, X) ⁵		F-6161 (F)	<ul style="list-style-type: none"> Conjugates of Rhodamine Red-X are generally more fluorescent than those of Lissamine rhodamine B, and the succinimidyl ester is more stable in H₂O
Tetramethylrhodamine (555/580)	C-2211 (SE) ⁵ C-6123 (SE) ⁶ C-1171 (SE) ^M T-6105 (SE, X) ^M	C-6121 (COOH) ⁵ C-6122 (COOH) ⁶ C-300 (COOH) ^M T-1480 (ITC) ⁵ T-1481 (ITC) ⁶ T-490 (ITC) ^M	F-6163 (F)	<ul style="list-style-type: none"> pH-insensitive fluorescence Good photostability Conjugates are prone to aggregation The succinimidyl ester derivative (6-TAMRA, SE; C-6123) is widely used for automated DNA sequencing
Texas Red dye (595/615)	T-6134 (SE, X) ^M T-20175 (SE, X) ⁵	T-353 (SC) T-1905 (SC) T-10125 (STP)	F-6162 (F) T-10244 (P)	<ul style="list-style-type: none"> Good spectral separation for green fluorophores Texas Red-X succinimidyl ester typically yields greater fluorescence per attached dye than Texas Red sulfonyl chloride and is more stable in H₂O
X-rhodamine (580/605)	C-6125 (SE) ⁵ C-6126 (SE) ⁶ C-1309 (SE) ^M	C-6124 (COOH) ⁵ C-6156 (COOH) ⁶ X-491 (ITC) ^M		<ul style="list-style-type: none"> Succinimidyl ester derivative (6-ROX, SE; C-6126) is widely used for automated nucleic acid sequencing

* The numbers in parentheses reflect the absorption (Abs) and fluorescence emission (Em) maxima, in nm, of the goat anti-mouse IgG antibody or dextran conjugates in aqueous buffer. These values were obtained from the Molecular Probes data tables. (SE) = Succinimidyl ester. (SC) = Sulfonyl chloride. (ITC) = Isothiocyanate. (X) = Aminohexanoyl spacer separating the dye and the SE. (5) = 5-Isomer. (6) = 6-Isomer. (M) = Mixture of 5- and 6-isomers. (S) = Single isomer. (STP) = 4-Sulfotetrafluorophenyl ester. (F) = FluoReporter Protein Labeling Kit (Section 1.2). (P) = Easy-to-Use Protein Labeling Kit (Section 1.2).

Tetramethylrhodamine

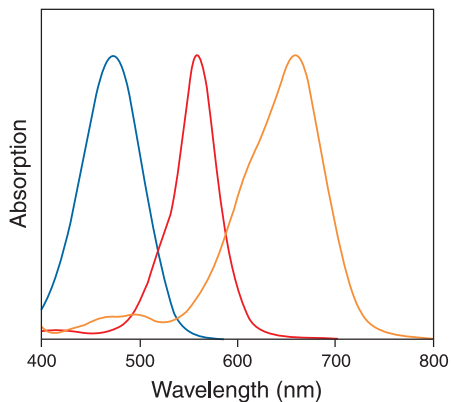


Figure 1.66 Normalized absorption spectra of the QSY 35 (blue), QSY 7 (red) and QSY 21 (orange) dyes. The QSY 7 and QSY 9 dyes have essentially identical spectra.

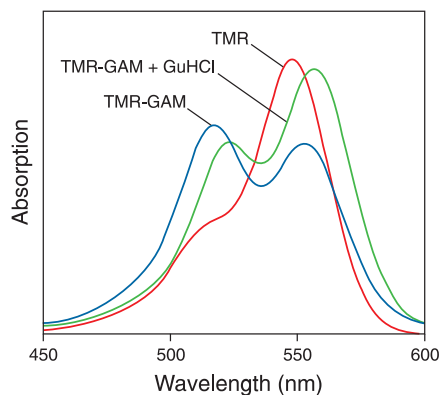


Figure 1.67 Effect of protein conjugation on the absorption spectrum of tetramethylrhodamine. The absorption spectrum of tetramethylrhodamine conjugated to goat anti-mouse IgG antibody (TMR-GAM) shows an additional peak at about 520 nm when compared to the spectrum of the same concentration of the free dye (TMR). Partial unfolding of the protein in the presence of 4.8 M guanidine hydrochloride (TMR-GAM + GuHCl) results in a spectrum more similar to that of the free dye.

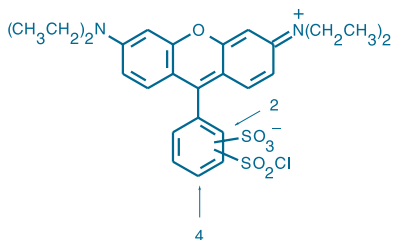


Figure 1.68 L-20 Lissamine rhodamine B sulfonyle chloride.

Tetramethylrhodamine (TMR) has been an important fluorophore for preparing protein conjugates, especially fluorescent antibody and avidin derivatives used in immunochemistry, although we now strongly recommend using conjugates of our Alexa Fluor 546 and Alexa Fluor 555 dyes (Section 1.3) instead of the tetramethylrhodamine conjugates for applications in this spectral range. Under the name TAMRA, the carboxylic acid of TMR has also achieved prominence as a dye for oligonucleotide labeling and automated DNA sequencing applications¹⁻³ (Section 8.2, Table 8.10). Because it can be prepared in high purity, the 5-isomer of TAMRA (C-6121) is one of the five dyes in our Reference Dye Sampler Kit (R-14782, Section 24.1). The detection limit of TMR-labeled amino acids by capillary electrophoresis is reported to be ~600 molecules.⁴ The fluorescence quantum yield of TMR conjugates is usually only about one-fourth that of fluorescein conjugates. However, because TMR is readily excited by the intense 546 nm spectral line from mercury-arc lamps used in most fluorescence microscopes and is intrinsically more photostable than fluorescein, TMR conjugates often appear brighter than the corresponding fluorescein conjugates. TMR is also efficiently excited by the 543 nm spectral line of the green He-Ne laser, which is increasingly being used for analytical instrumentation. TMR conjugates are not as well excited by the 568 nm spectral line of the Ar-Kr mixed-gas laser used in many confocal laser-scanning microscopes.

A significant limitation of the TMR dyes TAMRA and TRITC as protein-labeling reagents is that the absorption spectrum of TMR-labeled proteins is frequently complex (Figure 1.67), usually splitting into two absorption peaks at about 520 and 550 nm,⁵ so that the actual degree of labeling is difficult to determine. Excitation at wavelengths in the range of the short-wavelength peak fails to yield the expected amount of fluorescence, indicating that it arises from a nonfluorescent dye aggregate. Furthermore, when the TMR-labeled protein conjugate is denatured by guanidine hydrochloride, the long-wavelength absorption increases, the short-wavelength peak mostly disappears and the fluorescence yield almost doubles⁶ (Figure 1.67). This change in the absorption spectrum indicates that the extinction coefficient of TMR probably decreases upon conjugation to proteins. The absorption spectra of TMR-labeled nucleotides and of other probes such as our rhodamine phalloidin (R-415, Section 11.1) do *not* split into two peaks, indicating a labeling ratio of one dye molecule per biomolecule. The emission spectrum of TMR conjugates does not vary much with the degree of labeling.⁵ An improved method for estimating the degree of substitution of TRITC conjugates has been described.⁶ Unlike TMR-labeled proteins, protein conjugates of our Alexa Fluor 546 and Alexa Fluor 555 dyes (Section 1.3) show normal absorption spectra (Figure 7.7, Figure 7.8) and are also more fluorescent than either TMR or Cy3 protein conjugates (Figure 1.19).

Mixed-Isomer and Single-Isomer TRITC Preparations

Our tetramethylrhodamine isothiocyanate (TRITC) is of the highest quality available from any commercial source. Both our mixed-isomer (T-490) and single-isomer (T-1480, T-1481) TRITC preparations typically have extinction coefficients above 80,000 cm⁻¹M⁻¹, whereas some competitive TRITCs have extinction coefficients reported to be below 50,000 cm⁻¹M⁻¹. TRITC is widely used by other companies to prepare most of their so-called “rhodamine” immunoconjugates; however, they also often employ reactive versions of rhodamine B or Lissamine rhodamine B, which have somewhat different spectra, resulting in some confusion in matching the product name to the correct fluorophore.

Succinimidyl Esters of Carboxytetramethylrhodamine (TAMRA)

Almost all of Molecular Probes' TMR conjugates are prepared using succinimidyl esters of carboxytetramethylrhodamine (TAMRA), rather than TRITC, because bioconjugates from succinimidyl esters are more stable and often more fluorescent. We offer the mixed-isomer (C-300) and single-isomer (C-6121, C-6122) preparations of TAMRA, as well as the corresponding mixed-isomer (C-1171) and single-isomer (C-2211, C-6123) TAMRA succinimidyl esters. The single-isomer preparations of TAMRA are most important for high-resolution techniques such as DNA sequencing² and separation of TAMRA-labeled carbohydrates by capillary electrophoresis.⁷ 6-TAMRA is one of the traditional fluorophores (5-FAM, 6-JOE, 6-TET, 6-HEX, 6-TAMRA and 6-ROX) used in automated

DNA sequencing^{1-3,8} (Section 8.2, Table 8.10). Our FluoReporter Tetramethylrhodamine Protein Labeling Kit (F-6163, Section 1.2) supplies the mixed-isomer 5(6)-TAMRA succinimidyl ester for preparing TMR-labeled proteins.

We have also prepared the mixed-isomer TAMRA-X succinimidyl ester (5(6)-TAMRA-X, SE; T-6105), which contains a seven-atom aminohexanoyl spacer (“X”) between the reactive group and the fluorophore. This spacer helps to separate the fluorophore from its point of attachment, potentially reducing the interaction of the fluorophore with the biomolecule to which it is conjugated and making it more accessible to secondary detection reagents.⁹⁻¹¹ Polyclonal anti-tetramethylrhodamine and anti-Texas Red dye antibodies that recognize the tetramethylrhodamine, Rhodamine Red-X, X-rhodamine and Texas Red fluorophores are available (Section 7.4).

Lissamine Rhodamine B and Rhodamine Red-X Dyes

Lissamine Rhodamine B Sulfonyl Chloride

Lissamine rhodamine B sulfonyl chloride (L-20, L-1908; Figure 1.68) is much less expensive than Texas Red sulfonyl chloride (see below), and the fluorescence emission spectrum of its protein conjugates lies between those of tetramethylrhodamine and Texas Red conjugates (Figure 1.69). Although the absorption spectral shift relative to tetramethylrhodamine is not large, it is sufficient to permit conjugates of Lissamine rhodamine B to be excited by the 568 nm spectral line of the Ar-Kr mixed-gas laser used in many confocal laser-scanning microscopes. Furthermore, the protein conjugates of Lissamine rhodamine B are easier to purify and more chemically stable than are the conjugates of tetramethylrhodamine. Like Texas Red sulfonyl chloride, Lissamine rhodamine B sulfonyl chloride is actually a mixture of isomeric sulfonyl chlorides.

Rhodamine Red-X Succinimidyl Ester

Lissamine rhodamine B sulfonyl chloride is unstable, particularly in aqueous solution, making it somewhat difficult to achieve reproducible conjugations using this dye. Unlike Lissamine rhodamine B sulfonyl chloride, which is a mixture of isomeric sulfonyl chlorides, our patented Rhodamine Red-X succinimidyl ester (R-6160, Figure 1.70) is isomerically pure. Rhodamine Red-X succinimidyl ester is resistant to hydrolysis at the pH typically used for conjugation and provides a spacer between the fluorophore and the reactive site. Moreover, we have found that protein conjugates of the Rhodamine Red-X dye are frequently brighter than those of Lissamine rhodamine B (Figure 1.71), and are less likely to precipitate during storage.¹² Rhodamine Red-X succinimidyl ester is used in our FluoReporter Rhodamine Red-X Protein Labeling Kit (F-6161). See Section 1.2 for further information on this kit.

X-Rhodamine

The derivatives of carboxy-X-rhodamine (ROX) — a dye originally developed at Molecular Probes in 1986 — are widely used for oligonucleotide labeling and automated DNA sequencing applications (Section 8.2, Table 8.10). Conjugates of this dye and of the similar isothiocyanate (5(6)-XRITC, X-491; Figure 1.72) have longer-wavelength spectra (Figure 1.73) than the spectra of Lissamine rhodamine B, but somewhat shorter-wavelength spectra than those of Texas Red conjugates (Figure 1.74). Both the pure 5-isomer (C-6124) and 6-isomer (C-6156) of ROX are available, as are mixed-isomer (C-1309, Figure 1.75) and single-isomer (C-6125, C-6126) preparations of the succinimidyl ester of ROX.

Texas Red and Texas Red-X Dyes

The Texas Red fluorophore emits at a longer wavelength than do either tetramethylrhodamine or Lissamine rhodamine B (Figure 1.69), making Texas Red conjugates among the most commonly used long-wavelength “third labels” in fluorescence microscopy and flow cytometry (Figure 11.7, Figure 11.10, Figure 11.12). Unlike the other rhodamines, the Texas Red fluorophore exhibits very little spectral overlap with fluorescein

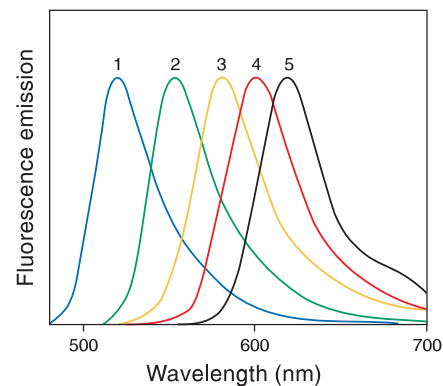


Figure 1.69 Normalized fluorescence emission spectra of goat anti-mouse IgG antibody conjugates of 1) fluorescein, 2) rhodamine 6G, 3) tetramethylrhodamine, 4) Lissamine rhodamine B and 5) Texas Red dyes.

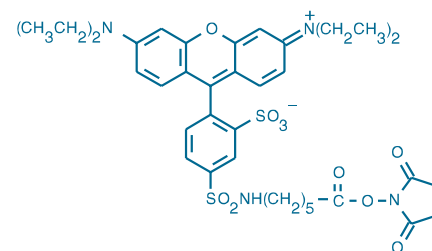


Figure 1.70 R-6160 Rhodamine Red-X, succinimidyl ester.

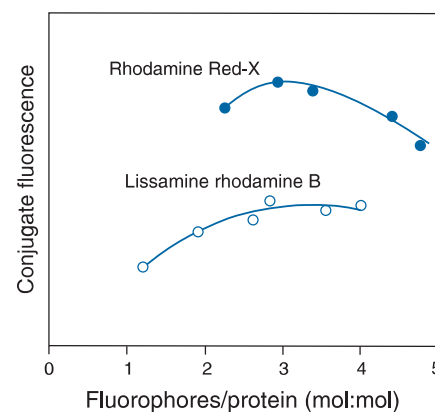


Figure 1.71 Comparison of the relative fluorescence of goat anti-mouse IgG antibody conjugates of Rhodamine Red-X succinimidyl ester (R-6160, ●) and Lissamine rhodamine B sulfonyl chloride (L-20, L-1908, ○). Conjugate fluorescence is determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of the free dye and multiplying by the number of fluorophores per protein. Higher numbers of fluorophores attached per protein are attainable with Rhodamine Red-X dye due to the lesser tendency of this dye to induce protein precipitation (Bioconjug Chem 7, 482 (1996)).

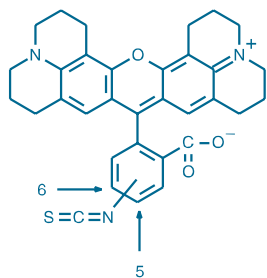


Figure 1.72 X-491 X-rhodamine-5-(and-6)-isothiocyanate (XRITC).

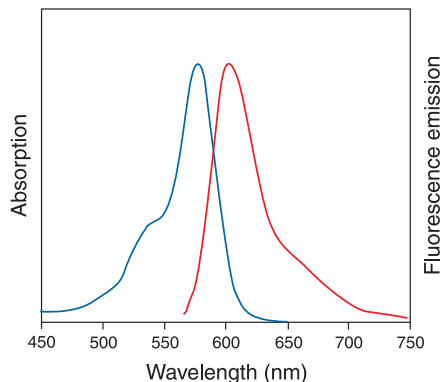


Figure 1.73 Absorption and fluorescence emission spectra of 5-carboxy-X-rhodamine (5-ROX, C-6124) in pH 7.0 buffer.

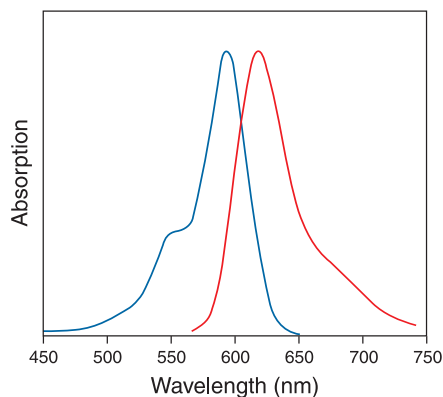


Figure 1.74 Absorption and fluorescence emission spectra of the Texas Red conjugate of bovine serum albumin (A-23017) in pH 7.0 buffer.

Texas Red dyes were developed while Molecular Probes was located in Plano, Texas, from 1978 to 1982. Texas Red is a registered trademark of Molecular Probes.

cein (Figure 1.69), and its fluorescence can be distinguished from that of phycoerythrins.^{13–15} Moreover, the fluorescence quantum yield of Texas Red conjugates is usually higher than that of tetramethylrhodamine or Lissamine rhodamine B conjugates. When the correct optical filter sets are used (Section 24.5, Table 24.8), Texas Red conjugates are brighter and have lower background than conjugates of the other commonly used red-fluorescent dyes, except the Alexa Fluor 594 dye. Texas Red conjugates are particularly well suited for excitation by the 568 nm spectral line of the Ar–Kr mixed-gas laser now used in many confocal laser-scanning microscopes, or the 594 nm spectral line of the orange He–Ne laser.

Texas Red Sulfonyl Chloride

Texas Red sulfonyl chloride is Molecular Probes' trademarked mixture of isomeric sulfonyl chlorides (Figure 1.76) of sulforhodamine 101.¹⁶ This reagent is quite unstable in water, especially at the higher pH required for reaction with aliphatic amines. For example, dilute solutions of Texas Red sulfonyl chloride are totally hydrolyzed within 2–3 minutes in pH 8.3 aqueous solution at room temperature.¹² Protein modification by this reagent is best done at low temperature. Once conjugated, however, the sulfonamides that are formed (Figure 1.4) are extremely stable; they even survive complete protein hydrolysis. Because Texas Red sulfonyl chloride rapidly degrades upon exposure to moisture, Molecular Probes offers this reactive dye specially packaged as a set of 10 vials (T-1905), each containing approximately 1 mg of Texas Red sulfonyl chloride for small-scale conjugations. We also offer the 10 mg unit size packaged in a single vial (T-353) for larger-scale conjugations. Each milligram of Texas Red sulfonyl chloride modifies approximately 8–10 mg of protein. Polyclonal anti-tetramethylrhodamine and anti-Texas Red antibodies that recognize tetramethylrhodamine, Rhodamine Red, X-rhodamine and Texas Red fluorophores are available (Section 7.4, Table 4.2).

Texas Red-X Succinimidyl Ester

Texas Red sulfonyl chloride's susceptibility to hydrolysis and low solubility in water may complicate its conjugation to some biomolecules. To overcome this difficulty, Molecular Probes has developed and patented Texas Red-X succinimidyl ester, which contains an additional seven-atom aminohexanoyl spacer ("X") between the fluorophore and its reactive group.¹² The single-isomer preparation of Texas Red-X succinimidyl ester (T-20175, Figure 1.77) is preferred over the mixed-isomer product (T-6134) when the dye is used to prepare low molecular weight peptides, oligonucleotides and receptor ligands that are to be purified by high-resolution techniques. Also, because isomers of a reactive dye may differ in their binding geometry, certain applications such as fluorescence resonance energy transfer (FRET, see Section 1.3) may benefit from the use of single-isomer reactive dyes.¹⁷ Thiol-reactive Texas Red derivatives that are based on a similar synthetic approach are described in Section 2.2. Texas Red-X succinimidyl ester offers significant advantages over Texas Red sulfonyl chloride for the preparation of bioconjugates:

- In the absence of amines, greater than 80% of Texas Red-X succinimidyl ester's reactivity is retained in pH 8.3 solution after one hour at room temperature.¹²
- Much less Texas Red-X succinimidyl ester (usually half or less of the amount of Texas Red sulfonyl chloride) is required to yield the same degree of labeling, making the effective costs of these two reagents about the same.
- Conjugations with Texas Red-X succinimidyl ester are more reproducible.
- Unlike Texas Red sulfonyl chloride, which can form unstable products with tyrosine, histidine, cysteine and other residues in proteins, the Texas Red-X succinimidyl ester reacts almost exclusively with amines.
- Protein conjugates prepared with Texas Red-X succinimidyl ester have a higher fluorescence yield than those with the same labeling ratio prepared with Texas Red sulfonyl chloride (Figure 1.78).
- We have noted a decreased tendency of Texas Red-X protein conjugates to precipitate during the reaction or upon storage.

Texas Red-X STP Ester

Molecular Probes has prepared the water-soluble 4-sulfo-2,3,5,6-tetrafluorophenyl (STP) ester of the Texas Red-X dye¹⁸ (T-10125). STP esters, which are prepared by

coupling a carboxylic acid and 4-sulfo-2,3,5,6-tetrafluorophenol (S-10490, Section 3.3), react rapidly with amines on proteins (Figure 1.3) under the same conditions as succinimidyl esters but are much more water soluble. STP esters are also available for several of our BODIPY dyes (Section 1.4).

Texas Red-X Conjugates and Texas Red-X Labeling Kits

Because of the advantages of Texas Red-X succinimidyl ester, we have converted some of our Texas Red conjugates to the Texas Red-X conjugates. Consequently, we have prepared Texas Red-X conjugates of:

- Antibodies (Section 7.3, Table 7.3)
- Streptavidin (S-6370, Section 7.6, Table 7.17)
- Wheat germ agglutinin (W-21405, Section 7.7)
- dUTP (C-7631, Section 8.2)
- Phalloidin (T-7471, Section 11.1, Table 11.1)
- Polymyxin B (P-13237, Section 15.2)
- Methotrexate (M-23273, Section 15.6)
- α -Bungarotoxin (B-7489, Section 16.2)

Protein conjugates of the Texas Red-X dye are readily prepared using our FluReporter Texas Red-X Protein Labeling Kit (F-6162) and Texas Red-X Protein Labeling Kit (T-10244). See Section 1.2 for further information on these kits. Our exclusive Zenon One Texas Red-X Mouse IgG₁ Labeling Kit (Z-25045, Section 7.2) permits the rapid and quantitative labeling of intact mouse or rat IgG₁ antibodies with the Texas Red-X dye. Polyclonal anti-tetramethylrhodamine and anti-Texas Red antibodies that recognize tetramethylrhodamine, Rhodamine Red, X-rhodamine and Texas Red fluorophores are available (Section 7.4, Table 7.13).

Reactive Texas Red-X dyes and their conjugates are patented by Molecular Probes.¹⁹ They are offered for research purposes only. We welcome inquiries about licensing these products for resale or other commercial uses.

Naphthofluorescein

Naphthofluorescein carboxylic acid and its succinimidyl ester (C-652, C-653; Figure 1.79) have emission maxima of approximately 660 nm in aqueous solution at pH 10 (Figure 1.80). However, their fluorescence is pH dependent ($pK_a \sim 7.6$), requiring a relatively alkaline pH for maximal fluorescence.

Carboxyrhodamine 6G

The excitation and emission spectra of carboxyrhodamine 6G (CR 6G) fall between those of fluorescein and tetramethylrhodamine (Figure 1.69). With a peak absorption at ~ 520 nm, conjugates prepared from the mixed-isomer (C-6157) or single-isomer (C-6127, C-6128) preparations of CR 6G succinimidyl esters are an excellent match to the 514 nm spectral line of the argon-ion laser. They also tend to exhibit a higher fluorescence quantum yield than tetramethylrhodamine conjugates, as well as excellent photostability. As with the Rhodamine Green dyes, the carboxyrhodamine 6G dyes are more suitable for preparing nucleotide and oligonucleotide conjugates than for preparing protein conjugates. Oligonucleotide conjugates of the CR 6G dye have spectroscopic and electrophoretic properties that are superior to the JOE dye (C-6171, Section 1.5) that is often used for DNA sequencing (Section 8.2, Table 8.10).

One of our reactive BODIPY dyes (BODIPY R6G, D-6180, D-6186; Section 1.4) has spectra similar to carboxyrhodamine 6G but with narrower absorption and emission spectra (Figure 1.37), which may be advantageous for multicolor applications.

QSY Dyes: The Best Fluorescence Quenchers

Dyes that quench the fluorescence of visible light-excited fluorophores are increasingly important for use in proximity studies (see Fluorescence Resonance Energy Trans-

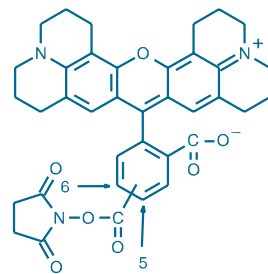


Figure 1.75 C-1309 5-(and-6)-carboxy-X-rhodamine, succinimidyl ester (ROX, SE).

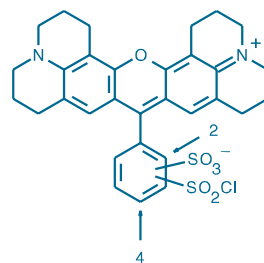


Figure 1.76 T-353 Texas Red sulfonyl chloride.

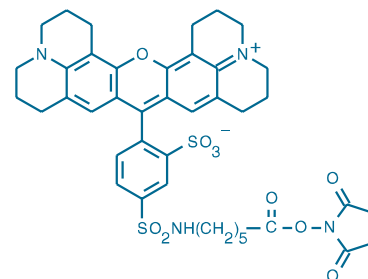


Figure 1.77 T-20175 Texas Red-X, succinimidyl ester.

Trademarks

Trademarks are important to any company that uses them and aid the customer in recognition of the original source of a product. Molecular Probes has a vast number of trademarks that are listed at the end of this book. We also attempt to appropriately honor the trademarks of all other companies.

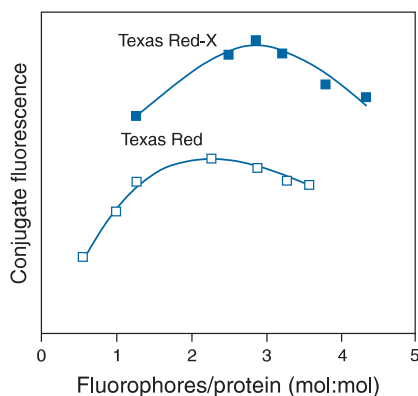


Figure 1.78 Comparison of the relative fluorescence of goat anti-mouse IgG antibody conjugates of Texas Red-X succinimidyl ester (T-6134, ■) and Texas Red sulfonyl chloride (T-353, □). Conjugate fluorescence is determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of the free dye and multiplying by the number of fluorophores per protein. Higher numbers of fluorophores attached per protein are attainable with the Texas Red-X dye due to the lesser tendency of this dye to induce protein precipitation (Bioconjug Chem 7, 482 (1996)).

fer (FRET) in Section 1.3) and in a wide variety of assays, such as those based on DNA hybridization (Section 8.5). Our QSY 7, QSY 9 and QSY 21 dyes (Table 1.7) are diaryl-rhodamine derivatives that have several properties that make them superior to the commonly used dabcyll chromophore (Section 1.8) when preparing bioconjugates for use in energy transfer-based assays:

- Broad absorption in the visible-light spectrum, with an absorption maximum near 560 nm for both the QSY 7 and QSY 9 dyes and near 660 nm for the QSY 21 dye (Figure 1.66)
- Extinction coefficients that are typically in excess of $90,000 \text{ cm}^{-1}\text{M}^{-1}$
- Absorption spectra of the conjugates that are insensitive to pH between 4 and 10
- Fluorescence quantum yields typically <0.001 in aqueous solution
- Efficient quenching of the fluorescence emission of donor dyes by the QSY 7 and QSY 9 dyes, including blue-fluorescent coumarins, any of our green- or orange-fluorescent dyes, and conjugates of the Texas Red and Alexa Fluor 594 dyes
- Quenching of all red-fluorescent dyes by the exceptionally long-wavelength light-absorbing QSY 21 dye (Table 1.8)
- Quenching of most green and red fluorophores that is more effective at far greater distances than is possible with dabcyll quenchers (Table 1.8, Figure 8.49)
- Residual fluorescence of the conjugates, at close spatial separations, that is typically lower than in conjugates that use dabcyll as the quencher
- High chemical stability of the conjugates and very good resistance to photobleaching

The distance at which energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated by fluorescence resonance energy transfer) is defined by the Förster radius (R_0). The magnitude of R_0 is dependent on the spectral properties of the donor and acceptor dyes. Förster distances (R_0) calculated for energy transfer from various Alexa Fluor dyes to QSY and dabcyll quenchers are listed in Table 1.8.

For preparing bioconjugates of the QSY dyes, Molecular Probes offers the amine-reactive QSY 7 (Figure 1.81), QSY 9 and QSY 21 succinimidyl esters (Q-10193,

Table 1.7 Molecular Probes' nonfluorescent quenchers and photosensitizers.

Dye	Abs *	Extinction Coefficient †	Amine-Reactive ‡	Notes
Dabcyll	453	32,000	D-2245 (SE)	<ul style="list-style-type: none"> • Broad and intense visible-wavelength absorption • Efficient energy transfer acceptor from blue- and green-fluorescent dyes in FRET applications
Dabsyl	466	33,000	D-1537 (SC)	<ul style="list-style-type: none"> • Sulfonyl chlorides form very stable conjugates • Broad and intense visible-wavelength absorption
Malachite green	628	76,000	M-689 (ITC)	<ul style="list-style-type: none"> • Nonfluorescent photosensitizer
QSY 7	560	92,000	Q-10193 (SE)	<ul style="list-style-type: none"> • Nonfluorescent quencher • Broad visible-wavelength absorption • Efficient energy transfer acceptor from UV light-excited green- and orange-fluorescent dyes in FRET applications
QSY 9	562	86,000	Q-20131 (SE)	<ul style="list-style-type: none"> • Nonfluorescent quencher • Spectrally similar to QSY 7, but with enhanced water solubility • Efficient energy transfer acceptor from UV light-excited green- and orange-fluorescent dyes in FRET applications
QSY 21	660	89,000	Q-20132 (SE)	<ul style="list-style-type: none"> • Nonfluorescent quencher • Long-wavelength absorption • An efficient energy transfer acceptor from red- and near IR-fluorescent dyes in FRET applications
QSY 35	472	23,500	Q-20133 (SE)	<ul style="list-style-type: none"> • Nonfluorescent quencher • Spectrally similar to dabcyll • An efficient energy transfer acceptor from blue- and green-fluorescent dyes in FRET applications

* Absorption (Abs) maxima, in nm. † Molar extinction coefficient in $\text{cm}^{-1}\text{M}^{-1}$ determined at the wavelength listed in the column headed Abs. These values were obtained from Molecular Probes' data tables and may vary with the environment, particularly for the QSY 35 dye. ‡ (SE) = succinimidyl ester; (SC) = sulfonyl chloride; (ITC) = isothiocyanate.

Q-20131, Q-20132), a thiol-reactive QSY 7 maleimide (Q-10257, Section 2.2) and a QSY 7 aliphatic amine (Q-10464, Section 3.3) that can be coupled to carboxylic acids and other functional groups. We also have prepared a QSY 7 derivative of α -Fmoc lysine (Q-21930, Section 9.5) for automated synthesis of peptides that contain this important quencher.

These QSY dyes, their conjugates and their use as fluorescence quenchers are patented by Molecular Probes, Inc. We welcome inquiries about licensing these products for resale or other commercial uses. Oligonucleotide conjugates of these QSY dyes are available only through our licensees.

In addition to the QSY 7, QSY 9 and QSY 21 dyes, Molecular Probes has available other essentially nonfluorescent quenchers that absorb maximally below 500 nm, including the QSY 35 dye, dabcy1 and dabcy1 dyes (Table 1.7). These products are described in Section 1.8.

Nonfluorescent Malachite Green

Malachite green is a nonfluorescent photosensitizer that absorbs at long wavelengths (Figure 1.82) (~630 nm). Its photosensitizing action can be targeted to particular cellular sites by conjugating malachite green isothiocyanate (M-689, Figure 1.83) to specific antibodies. Enzymes and other proteins within ~10 Å of the binding site of the malachite green-labeled antibody can then be selectively destroyed upon irradiation with long-wavelength light.^{20,21} Studies by Jay and colleagues have demonstrated that this photoinduced destruction of enzymes in the immediate vicinity of the chromophore is apparently the result of localized production of hydroxyl radicals, which have short lifetimes that limit their diffusion from the site of their generation.²² Earlier studies had supported a thermal mechanism of action.^{23–25}

NANOGOLD Sulfosuccinimidyl Ester

In collaboration with Nanoprobes, Inc., Molecular Probes offers NANOGOLD and Alexa Fluor FluoroNanogold particles, small metal cluster complexes of gold particles for research applications in light or electron microscopy.²⁶ The NANOGOLD and Alexa Fluor FluoroNanogold clusters are discrete chemical compounds, not gold colloids. NANOGOLD mono(sulfosuccinimidyl ester) (N-20130) permits attachment of these very small (1.4 nm) yet uniformly sized gold particles to biomolecules in the same way that one reacts a succinimidyl ester of a dye (Figure 1.84). This product, which is supplied as a set of five vials of a powder that has been lyophilized from pH 7.5 HEPES buffer, is resuspended with the protein in deionized water at room temperature or below, then any excess NANOGOLD mono(sulfosuccinimidyl ester) is removed by gel filtration and the conjugate is stored frozen. 100 nmol of NANOGOLD mono(sulfosuccinimidyl ester) is sufficient to label about 100 μ g of a protein with a MW of 100,000. Excess reagent

Table 1.8 R_0 values for QSY and dabcy1 quenchers.*

Donor	Acceptor			
	QSY 35	dabcy1	QSY 7 and QSY 9	QSY 21
Alexa Fluor 350	47	50		
Alexa Fluor 488	44	49	64	
Alexa Fluor 546	25	29	67	
Alexa Fluor 555			45	
Alexa Fluor 568			56	75
Alexa Fluor 594				77
Alexa Fluor 647				69

* R_0 values in angstroms (Å) represent the distance at which fluorescence resonance energy transfer from the donor dye to the acceptor dye is 50% efficient. Values were calculated from spectroscopic data, as outlined (see Fluorescence Resonance Energy Transfer (FRET) in Section 1.3).

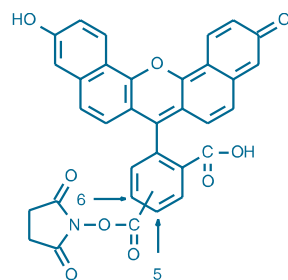


Figure 1.79 C-653 5-(and-6)-carboxynaphthofluorescein, succinimidyl ester.

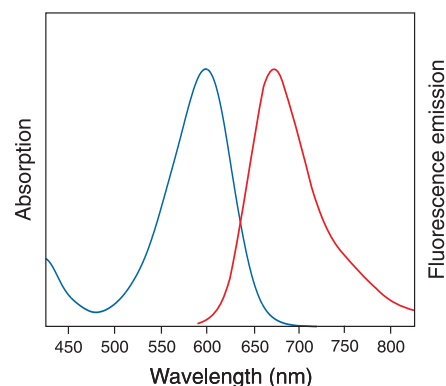


Figure 1.80 Absorption and fluorescence emission spectra of 5-(and-6)-carboxynaphthofluorescein (C-652) in pH 10.0 buffer.

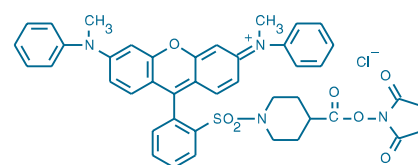


Figure 1.81 Q-10193 QSY 7 carboxylic acid, succinimidyl ester.

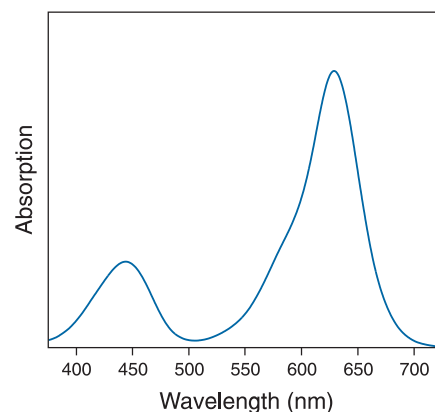


Figure 1.82 Absorption spectrum of malachite green isothiocyanate (M-689) in acetonitrile.

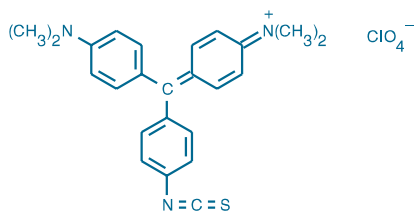


Figure 1.83 M-689 malachite green isothiocyanate.

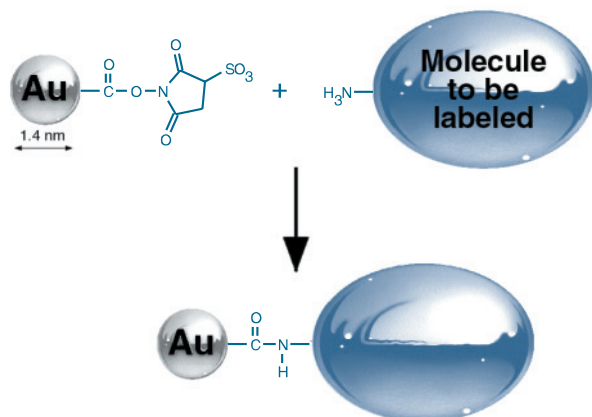


Figure 1.84 Reaction of NANOGOLD mono(sulfosuccinimidyl ester) (N-20130) with a primary amine. Image courtesy of Nanoprobes, Inc.

should not be stored, and the conjugation mixture must be free of thiols or amine-containing buffers. NANOGOLD and Alexa Fluor FluoroNanogold conjugates of antibodies and streptavidin are described in Section 7.3 and Section 7.6, respectively, along with reagents and methods for silver enhancement to amplify electron microscopic detection. In addition to being used for ultrastructural studies, NANOGOLD conjugates are extremely effective excited-state energy transfer quenchers with an enhanced ability to detect single-base mismatches in beacon technology²⁷ (Figure 8.101). We also supply NANOGOLD monomaleimide (N-20345, Section 2.2, Figure 2.17).

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Data Table — 1.6 Dyes with Absorption Maxima Above 520 nm

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
C-300	466.92	L	DMF, DMSO	540	95,000	565	MeOH	1
C-652	476.44	L	pH >6, DMF	598	49,000	668	pH 10	2
C-653	573.51	F,D,L	DMF, DMSO	602	42,000	672	pH 10	2
C-1171	527.53	F,D,L	DMF, DMSO	546	95,000	576	MeOH	1, 3
C-1309	631.68	F,D,L	DMF, DMSO	576	80,000	601	MeOH	1
C-2211	527.53	F,D,L	DMF, DMSO	546	95,000	579	MeOH	1, 3
C-6121	430.46	L	pH >6, DMF	542	91,000	568	MeOH	1
C-6122	430.46	L	pH >6, DMF	540	103,000	564	MeOH	1
C-6123	527.53	F,D,L	DMF, DMSO	547	91,000	573	MeOH	1, 3
C-6124	635.80	F,L	pH >6, DMF	567	92,000	591	MeOH	1
C-6125	631.68	F,D,L	DMF, DMSO	574	78,000	602	MeOH	1
C-6126	631.68	F,D,L	DMF, DMSO	575	82,000	602	MeOH	1
C-6127	555.59	F,D,L	pH >6, DMF	524	108,000	557	MeOH	
C-6128	555.59	F,D,L	DMF, DMSO	524	102,000	550	MeOH	
C-6156	534.61	F,L	pH >6, DMF	570	113,000	590	MeOH	1
C-6157	555.59	F,D,L	DMF, DMSO	524	92,000	552	MeOH	
L-20	577.11	F,DD,L	DMF, MeCN	568	88,000	583	MeOH	4
L-1908	577.11	F,DD,L	DMF, MeCN	568	88,000	583	MeOH	4
M-689	485.98	F,DD,L	DMF, DMSO	629	75,000	none	MeCN	5
Q-10193	791.32	F,D,L	DMSO	560	90,000	none	MeOH	
Q-20131	951.43	F,D,L	H ₂ O, DMSO	562	88,000	none	MeOH	6
Q-20132	815.34	F,D,L	DMSO	661	90,000	none	MeOH	
R-6160	768.90	F,D,L	DMF, DMSO	560	129,000	580	MeOH	
T-353	625.15	F,DD,L	DMF, MeCN	588	84,000	601	CHCl ₃	4
T-490	443.52	F,DD,L	DMF, DMSO	544	84,000	572	MeOH	3, 5
T-1480	443.52	F,DD,L	DMF, DMSO	543	99,000	571	MeOH	3, 5
T-1481	443.52	F,DD,L	DMF, DMSO	544	90,000	572	MeOH	3, 5
T-1905	625.15	F,DD,L	DMF, MeCN	587	85,000	602	CHCl ₃	4
T-6105	640.69	F,D,L	DMF, DMSO	543	92,000	571	MeOH	1, 3
T-6134	816.94	F,D,L	DMF, DMSO	583	112,000	603	MeOH	

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
T-10125	969.97	F,D,L	H ₂ O, DMSO	585	92,000	605	MeOH	7
T-20175	816.94	F,D,L	DMF, DMSO	587	96,000	602	MeOH	
X-491	547.67	F,DD,L	DMF, DMSO	572	92,000	596	MeOH	5

For definitions of the contents of this data table, see "How to Use This Book" on page viii.

Notes

1. Abs and Em for TAMRA and ROX dyes in pH 8 buffer are red-shifted approximately 8 nm compared to MeOH, with EC lower by ~10%.
2. Absorption and fluorescence of naphthofluorescein derivatives are pH dependent. Both the absorption and emission spectra shift to much shorter wavelengths at pH <8. Fluorescence quantum yield ~0.14 at pH 9.5 (Cytometry 10, 151 (1989)).
3. Tetramethylrhodamine protein conjugates often exhibit two absorption peaks at about 520 and 545 nm. The 520 nm peak is due to nonfluorescent dye aggregates (J Immunol Methods 143, 263 (1991); J Phys Chem B 102, 1820 (1998)).
4. Do NOT dissolve in DMSO.
5. Isothiocyanates are unstable in water and should not be stored in aqueous solution.
6. This sulfonated succinimidyl ester derivative is water soluble and may be dissolved in buffer at ~pH 8 for reaction with amines. Long-term storage in water is NOT recommended due to hydrolysis.
7. This sulfotetrafluorophenyl (STP) ester derivative is water soluble and may be dissolved in buffer at ~pH 8 for reaction with amines. Long-term storage in water is NOT recommended due to hydrolysis.

Product List — 1.6 Dyes with Absorption Maxima Above 520 nm

Cat #	Product Name	Unit Size
C-652	5-(and-6)-carboxynaphthofluorescein *mixed isomers*	100 mg
C-653	5-(and-6)-carboxynaphthofluorescein, succinimidyl ester *mixed isomers*	25 mg
C-6127	5-carboxyrhodamine 6G, succinimidyl ester (5-CR 6G, SE) *single isomer*	5 mg
C-6128	6-carboxyrhodamine 6G, succinimidyl ester (6-CR 6G, SE) *single isomer*	5 mg
C-6157	5-(and-6)-carboxyrhodamine 6G, succinimidyl ester (5(6)-CR 6G, SE) *mixed isomers*	5 mg
C-6121	5-carboxytetramethylrhodamine (5-TAMRA) *single isomer*	10 mg
C-6122	6-carboxytetramethylrhodamine (6-TAMRA) *single isomer*	10 mg
C-300	5-(and-6)-carboxytetramethylrhodamine (5(6)-TAMRA) *mixed isomers*	100 mg
C-2211	5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA, SE) *single isomer*	5 mg
C-6123	6-carboxytetramethylrhodamine, succinimidyl ester (6-TAMRA, SE) *single isomer*	5 mg
C-1171	5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (5(6)-TAMRA, SE) *mixed isomers*	25 mg
C-6124	5-carboxy-X-rhodamine, triethylammonium salt (5-ROX) *single isomer*	10 mg
C-6156	6-carboxy-X-rhodamine (6-ROX) *single isomer*	10 mg
C-6125	5-carboxy-X-rhodamine, succinimidyl ester (5-ROX, SE) *single isomer*	5 mg
C-6126	6-carboxy-X-rhodamine, succinimidyl ester (6-ROX, SE) *single isomer*	5 mg
C-1309	5-(and-6)-carboxy-X-rhodamine, succinimidyl ester (5(6)-ROX, SE) *mixed isomers*	25 mg
F-6161	FluoReporter [®] Rhodamine Red [™] -X Protein Labeling Kit *5–10 labelings*	1 kit
F-6163	FluoReporter [®] Tetramethylrhodamine Protein Labeling Kit *5–10 labelings*	1 kit
F-6162	FluoReporter [®] Texas Red [™] -X Protein Labeling Kit *5–10 labelings*	1 kit
L-20	Lissamine [™] rhodamine B sulfonyl chloride *mixed isomers*	1 g
L-1908	Lissamine [™] rhodamine B sulfonyl chloride *mixed isomers* *special packaging*	10 x 10 mg
M-689	malachite green isothiocyanate	10 mg
N-20130	NANOGOLD [®] mono(sulfosuccinimidyl ester) *special packaging*	5 x 6 nmol
Q-10193	QSY [®] 7 carboxylic acid, succinimidyl ester	5 mg
Q-20131	QSY [®] 9 carboxylic acid, succinimidyl ester	5 mg
Q-20132	QSY [®] 21 carboxylic acid, succinimidyl ester	5 mg
R-6160	Rhodamine Red [™] -X, succinimidyl ester *5-isomer*	5 mg
T-6105	6-(tetramethylrhodamine-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (5(6)-TAMRA-X, SE) *mixed isomers*	10 mg
T-1480	tetramethylrhodamine-5-isothiocyanate (5-TRITC; G isomer)	5 mg
T-1481	tetramethylrhodamine-6-isothiocyanate (6-TRITC; R isomer)	5 mg
T-490	tetramethylrhodamine-5-(and-6)-isothiocyanate (5(6)-TRITC) *mixed isomers*	10 mg
T-353	Texas Red [®] sulfonyl chloride *mixed isomers*	10 mg
T-1905	Texas Red [®] sulfonyl chloride *mixed isomers* *special packaging*	10 x ~1 mg
T-10244	Texas Red [®] -X Protein Labeling Kit *3 labelings*	1 kit
T-10125	Texas Red [®] -X, STP ester, sodium salt *mixed isomers*	5 mg
T-6134	Texas Red [®] -X, succinimidyl ester *mixed isomers*	5 mg
T-20175	Texas Red [®] -X, succinimidyl ester *single isomer*	2 mg
X-491	X-rhodamine-5-(and-6)-isothiocyanate (5(6)-XRITC) *mixed isomers*	10 mg
Z-25045	Zenon [™] One Texas Red [®] -X Mouse IgG ₁ Labeling Kit *50 labelings*	1 kit