Phallotoxins

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Fluorescent phallotoxins	1 vial of 300 units of product, lyophilized solids *	• ≤–20°C • Desiccate • Protect from light	When stored as directed, product is stable for at least 1 year.
Biotin-XX phalloidin (B7474)	1 vial of 50 units of product, lyophilized solids *		
Unlabeled phalloidin (P3457)	1 vial of 1 mg of product, lyophilized solids †		

* Note that the vial contents are hardly visible. † ~1.3 micromoles.

Approximate Fluorescence Excitation and Emission, in nm: See Table 2.

Introduction

Molecular Probes offers several fluorescent and biotinylated phalloidin and phallacidin derivatives for labeling F-actin (Table 2). These phallotoxins, isolated from the deadly *Amanita phalloides* mushroom,[1] are bicyclic peptides that differ by two amino acid residues. They can be used interchangeably in most applications and bind competitively to the same sites in F-actin. Phalloidin and phallacidin contain an unusual thioether bridge between a cysteine and tryptophan residue that forms an inner ring structure. At elevated pH, this thioether is cleaved and the toxin loses its affinity for actin.

Fluorescent and biotinylated phallotoxins stain F-actin at nanomolar concentrations and are extremely water soluble, thus providing convenient probes for labeling, identifying, and quantitating F-actin in tissue sections, cell cultures, or cell-free experiments.[1-3] Labeled phallotoxins have similar affinity for both large and small filaments, binding in a stoichiometric ratio of about one phallotoxin molecule per actin subunit in muscle and nonmuscle cells from many different species of plants and animals. Unlike antibodies, the binding affinity does not change appreciably with actin from different species or sources. Nonspecific staining is negligible, and the contrast between stained and unstained areas is extremely large. It has been reported that phallotoxins are unable to bind to monomeric G-actin.[1] Phallotoxins shift the monomer/polymer equilibrium toward the polymer, lowering the critical concentration for polymerization up to 30-fold.[3,4] Phallotoxins also stabilize F-actin, inhibiting depolymerization by cytochalasins, potassium iodide, and elevated temperatures.

Because the phallotoxin conjugates are small, with an approximate diameter of 12–15 Å and molecular weight of <2000 daltons, a variety of actin-binding proteins—including myosin, tropomyosin, troponin, and DNase I—can still bind to actin after treatment with phallotoxins. Even more significantly, phallotoxin-labeled actin filaments remain functional; labeled

glycerinated muscle fibers still contract, and labeled actin filaments still move on solid-phase myosin substrates.[6,7] Fluorescent phallotoxins can also be used to quantitate the amount of F-actin in cells.[8,9] The unlabeled phallotoxins may be used as controls in blocking F-actin staining or in promoting actin polymerization. Our biotin-XX phalloidin allows researchers to visualize actin filaments by electron microscopy using standard enzyme-mediated avidin/streptavidin techniques. Eosin phalloidin can potentially be used for correlated fluorescence and electron microscopic studies. Deerinck and co-workers reported that when eosin conjugates are excited in the presence of diaminobenzidine (DAB), an insoluble, electron-dense DAB oxidation product is formed that is easily visualized with either light or electron microscopy.[10]

Even though phallotoxins have an LD_{50} of approximately 2 mg/kg when injected into the mouse, the toxins of *A. phalloides* that are responsible for most of the symptoms and fatalities associated with poisoning by this mushroom are the structurally related amatoxins. The major *in vivo* lesions produced by injected phallotoxin occur in the liver and are associated with stabilization of polymeric actin. Phallotoxins, however, tend not to be absorbed by the gastrointestinal tract even though they are stable between pH 3 and pH 9.

The approximate molecular weights (MW) of unlabeled phalloidin and phallacidin are 790 and 825, respectively. The approximate MWs of the labeled phallotoxins are given in Table 2. The peak excitation and emission wavelengths for each conjugate and the actin binding constants (K_d) for several of the conjugates are also listed in Table 2.

Table 2. Spectral characteristics and dissociation constants of phallotoxin probes.

Cat #	Conjugate	Ex *	Em *	Approximate MW	Kd (nM) †
A22281	Alexa Fluor® 350 phalloidin	346	442	1100	ND
C606	coumarin phallacidin	355	443	1100	24
N354	NBD phallacidin	465	536	1040	18
A12379	Alexa Fluor® 488 phalloidin	495	518	1320	ND
F432	fluorescein phalloidin	496	516	1175	18
O7466	Oregon Green® 488 phalloidin	496	520	1180	ND
B607	BODIPY® FL phallacidin	505	512	1125	38
O7465	Oregon Green® 514 phalloidin	511	528	1281	ND
E7463	eosin phalloidin	524	544	1500	ND
A22282	Alexa Fluor® 532 phalloidin	531	554	1350	ND
R415	rhodamine phalloidin	540	565	1250	40
A22283	Alexa Fluor® 546 phalloidin	556	570	1800	ND
A34055	Alexa Fluor® 555 phalloidin	555	565	1910	ND
B3475	BODIPY® 558/568 phalloidin	558	569	1115	ND
A12380	Alexa Fluor® 568 phalloidin	578	600	1590	ND
A12381	Alexa Fluor® 594 phalloidin	581	609	1620	ND
B3416	BODIPY® 581/591 phalloidin	584	592	1150	ND
T7471	Texas Red®-X phalloidin	591	608	1490	ND
A22284	Alexa Fluor® 633 phalloidin	632	647	1900	ND
A34054	Alexa Fluor® 635 phalloidin	633	647	1850	ND
B12382	BODIPY® 650/665 phalloidin	647	661	1200	ND
A22287	Alexa Fluor® 647 phalloidin	650	668	1950	ND
A22285	Alexa Fluor® 660 phalloidin	663	690	1750	ND
A22286	Alexa Fluor® 680 phalloidin	679	702	1850	ND
B7474	biotin-XX phalloidin	NA	NA	1300	10.5

* Approximate fluorescence excitation (Ex) and emission (Em) maxima, in nm. Complete spectra for most of these dyes are available at our website (probes.invitrogen.com). † Rhodamine phalloidin's fluorescence increases upon binding to actin, a phenomenon that allowed Molecular Probes researchers to determine the ligand's binding constant. The binding constants of the other conjugates were determined by competitive binding with rhodamine phalloidin.[10] All binding constants were determined on rabbit skeletal muscle actin. ND = Not determined. NA = Not applicable.

Once reconstituted in methanol, the stock solutions are stable for at least one year when stored frozen at ≤–20°C, desiccated, and protected from light. It appears that NBD phallacidin, and possibly all of these toxins, exhibit a small loss of activity when stored in aqueous solution at 2–6°C for over three weeks.

While the amount of toxin present in a vial could be lethal only to a mosquito (LD_{50} of phalloidin = 2 mg/kg), it should be handled with care.

Materials Required but Not Provided

- Methanol
- PBS
- Methanol-free formaldehyde
- Acetone or Triton X-100
- Fluorescent or enzyme-conjugated streptavidin, as prepared in step 1.9, for use with biotin-XX phalloidin only
- BSA, optional
- Image-iT™ FX Signal Enhancer (I36933), optional

Preparing the Stock Solution

Fluorescent Phallotoxins

The vial contents should be dissolved in 1.5 mL methanol to yield a final concentration of 200 units/mL, which is equivalent to approximately 6.6 µM.

One unit of phallotoxin is defined as the amount of material used to stain one microscope slide of fixed cells, according to the following protocol (see step 1.6), and is equivalent to 5 µL of methanolic stock solution for the fluorescent phallotoxins.

Biotin-XX Phalloidin

Staining cells with biotin-XX phalloidin (B7474) requires 1) the use of a higher concentration of the phallotoxin conjugate than when staining with fluorescent phallotoxins and 2) the addition of a fluorescent or enzyme-conjugated avidin or streptavidin detection reagent (see step 1.9). The vial contents should be dissolved in 0.5 mL methanol to yield a final concentration of 100 units/mL, which is equivalent to approximately 20 µM.

For biotin-XX phalloidin, 10 µL of the methanolic stock solution is equivalent to one unit of phallotoxin, which is defined as the amount of material used to stain one microscope slide of fixed cells according to the following protocol (see step 1.6).

Unlabeled Phalloidin

Solutions of this product should be prepared just like the fluorescent phallotoxins described in *Fluorescent Phallotoxins*, taking into account the larger quantity of material provided.

Experimental Protocol

The procedure below was originally developed for use with NBD phallacidin.[11] It has been successfully used with all of Molecular Probes phallotoxin conjugates. This procedure may not be optimum for a particular experimental system, but has yielded consistent results in most instances. The following protocol describes the staining procedure for adherent cells grown on glass coverslips.

Formaldehyde-Fixed Cells

1.1 Wash cells twice with prewarmed phosphate-buffered saline, pH 7.4 (PBS).

1.2 Fix the sample in 3.7% formaldehyde solution in PBS for 10 minutes at room temperature.

Note: Methanol can disrupt actin during the fixation process. Therefore, it is best to avoid any methanol containing fixatives. The preferred fixative is methanol-free formaldehyde.

1.3 Wash two or more times with PBS.

1.4 Place each coverslip in a glass petri dish and extract it with a solution of acetone at ≤–20°C or 0.1% Triton X-100 in PBS for 3 to 5 minutes.

1.5 Wash two or more times with PBS.

1.6 When staining with any of the fluorescent phallotoxins, dilute 5 µL methanolic stock solution into 200 µL PBS for each coverslip to be stained. To reduce nonspecific background staining with these conjugates, add 1% bovine serum albumin (BSA) to the staining solution. It may also be useful to pre-incubate fixed cells with PBS containing 1% BSA or with the Image-iT™ FX signal enhancer (I36933) for 20–30 minutes prior to adding the phallotoxin staining solution.

When staining with biotin-XX phalloidin (B7474), dilute 10 µL of the methanolic stock solution into 200 µL PBS for each coverslip to be stained.

When staining more than one coverslip, adjust volumes accordingly. For a stronger signal, use 2 or 3 units per coverslip.

1.7 Place the staining solution on the coverslip for 20 minutes at room temperature (generally, any temperature between 4°C and 37°C is suitable). To avoid evaporation, keep the coverslips inside a covered container during the incubation.

1.8 Wash two or more times with PBS.

1.9 When using biotin-XX phalloidin, incubate for 30 minutes with 100 µL of a 10 µg/mL solution of fluorescent or enzyme-conjugated streptavidin dissolved in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3% Triton X-100 and 1% BSA. Incubate for 15 minutes at room temperature. After incubation, wash the coverslip with PBS. To develop enzyme activity, follow a procedure recommended for the specific enzyme.

1.10 For long-term storage, the cells should be air dried and then mounted in a permanent mountant such as ProLong® Gold reagent or Cytoseal. Specimens prepared in this manner retain actin staining for at least six months when stored in the dark at 2–6°C.

Simultaneous Fixation, Permeabilization, and Fluorescent Phallotoxin Staining

The phallotoxins appear to be stable for short periods in 4% formaldehyde fixation buffers. This permits a rapid one-step fixation, permeabilization, and labeling procedure as follows.

2.1 Prepare a 1 mL solution containing 50 to 100 µg/mL lysopalmitoylphosphatidylcholine and 3.7% formaldehyde and then add 5–10 units of fluorescent phallotoxin (approximately 25 to 50 µL of methanolic stock solution).

2.2 Place this staining solution on cells and incubate for 20 minutes at 4°C.

2.3 Rapidly wash three times with buffer.

2.4 Mount coverslips and view.

Living Cells

Phallotoxins are usually not cell-permeant and have therefore not been used extensively with living cells. However, living cells have been labeled.[11,12] Pinocytosis appears to be the method of entry for some cells, although hepatocytes "avidly" take up the dye by an unknown mechanism.[5,13] In general, a larger amount of stain will be needed for staining living cells. Rhodamine phalloidin has been microinjected into fibroblasts without noticeable changes in shape or ruffling.[14,15] Injections of phalloidin into living cells appear to alter the actin distribution and cell motility.[16,17] Consult the literature to find procedures suitable for your experiments.

Fluorescence Microscopy

Photostability or resistance to photobleaching is a primary concern when making fluorescence measurements. Alexa Fluor®, Oregon Green®, BODIPY®, and rhodamine fluorophores (including Texas Red®-X) are significantly more photostable than NBD and fluorescein and will therefore enable more accurate photographic measurements. To further reduce photobleaching, minimize the exposure of fluorescently labeled specimens to light with neutral density filters and expose samples only when observing or recording a signal. Maximize collection of fluorescence by using a minimum of optics, high–numerical aperture objectives, relatively low magnification, high-quality optical filters, and high-speed film or high-efficiency detectors. Antifade reagents, including Molecular Probes *SlowFade*® Gold and ProLong® Gold antifade reagents, can extend the useful lives of many fluorescent probes. These reagents are also available in a formulation with DAPI. They can be used on fixed cell preparations but are not compatible with living cells. Cytoseal also appears to protect BODIPY® fluorophores from photobleaching.

References

1. Wieland, T. in *Phallotoxins*, Springer-Verlag, New York (1986); **2.** J Muscle Res Cell Motil 9, 370 (1988); **3.** Methods Enzymol 85, 514 (1982); **4.** Eur J Biochem 165, 125 (1987); **5.** J Cell Biol 105, 1473 (1987); **6.** Nature 326, 805 (1987); **7.** Proc Natl Acad Sci USA 83, 6272 (1986); **8.** Blood 69, 945 (1987); **9.** Anal Biochem 200, 199 (1992); **10.** J Cell Biol 126, 901 (1994); **11.** Proc Natl Acad Sci USA 77, 980 (1980); **12.** Nature 284, 405 (1980); **13.** CRC Crit Rev Biochem 5, 185 (1978); **14.** J Cell Biol 106, 1229 (1988); **15.** J Cell Biol 103, 265a (1986); **16.** Eur J Cell Biol 24, 176 (1981); **17.** Proc Natl Acad Sci USA 74, 5613 (1977).

Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat #	Product Name	Unit Size
A12379	Alexa Fluor® 488 phalloidin	300 U
A12380	Alexa Fluor® 568 phalloidin	300 U
A12381	Alexa Fluor® 594 phalloidin	300 U
A22281	Alexa Fluor® 350 phalloidin	300 U
A22282	Alexa Fluor® 532 phalloidin	300 U
A22283	Alexa Fluor® 546 phalloidin	300 U
A34055	Alexa Fluor® 555 phalloidin	300 U
A22284	Alexa Fluor® 633 phalloidin	300 U
A22285	Alexa Fluor® 660 phalloidin	300 U
A22286	Alexa Fluor® 680 phalloidin	300 U
A22287	Alexa Fluor® 647 phalloidin	300 U
A34054	Alexa Fluor® 635 phalloidin	300 U
B12382	BODIPY® 650/665 phalloidin	300 U
B3416	BODIPY® 581/591 phalloidin	300 U
B3475	BODIPY® 558/568 phalloidin	300 U
B607	BODIPY® FL phallacidin	300 U
B7474	biotin-XX phalloidin	50 U
C606	coumarin phallacidin	300 U
E7463	eosin phalloidin	300 U
F432	fluorescein phalloidin	300 U
I36933	Image-iT™ FX signal enhancer	10 mL
N354	*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (NBD phallacidin)	300 U
O7465	Oregon Green® 514 phalloidin	300 U
O7466	Oregon Green® 488 phalloidin	300 U
P3457	phalloidin	1 mg
P36930	ProLong® Gold antifade reagent	10 mL
P36931	ProLong® Gold antifade reagent with DAPI	10 mL
P36934	ProLong® Gold antifade reagent *special packaging*	5 x 2 mL
P36935	ProLong® Gold antifade reagent with DAPI *special packaging*	5 x 2 mL
S36936	SlowFade® Gold antifade reagent	10 mL
S36937	SlowFade® Gold antifade reagent *special packaging*	5 x 2 mL
S36938	SlowFade® Gold antifade reagent with DAPI	10 mL
S36939	SlowFade® Gold antifade reagent with DAPI *special packaging*	5 x 2 mL
R415	rhodamine phalloidin	300 U
T7471	Texas Red®-X phalloidin	300 U

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Alexa Fluor® 555 Dye—A Superior Alternative to Tetramethylrhodamine and Cy3.

Tetramethylrhodamine is a time-honored red-orange fluorophore for protein labeling, yet it is not without its limitations. The Alexa Fluor®, 555 dye, which exhibits fluorescence excitation and emission spectra that are almost identical to those of tetramethylrhodamine and the Cy3 dye (Figure 1), overcomes several of these obstacles:

- **Increased photostability.** The red-orange–fluorescent Alexa Fluor® 555 dye is significantly more photostable than both tetramethylrhodamine and Cy3, allowing more time for image capture.
- **High extinction coefficient.** The extinction coefficient of the succinimidyl ester of Alexa Fluor® 555 carboxylic acid (155,000 $cm^{-1}M^{-1}$) is significantly larger than that of the succinimidyl ester or isothiocyanate derivates of tetramethylrhodamine (92,000 $cm^{-1}M^{-1}$ and 99,000 $cm^{-1}M^{-1}$, respectively)
- **Brighter protein conjugates.** Alexa Fluor® 555 protein conjugates are appreciably brighter than both tetramethylrhodamine and Cy3 protein conjugates. This superior brightness is due in part to the observation that the absorption spectrum of tetramethylrhodamine-labeled proteins frequently splits into two absorption peaks, with the shorter-wavelength peak corresponding to a nonfluorescent dye aggregate (Figure 2). Protein conjugates of our Alexa Fluor® 555 dye show normal absorption spectra with correspondingly high extinction coefficients.

Since its introduction in 2002, the Alexa Fluor® 555 dye has been cited in close to 200 journal articles, many of which describe the direct substitution of the Alexa Fluor® 555 dye in experiments that formerly used either tetramethylrhodamine or Cy3. With a variety of reactive forms of the Alexa Fluor® 555 dye available, as well as a wide selection of protein conjugates (Table 1), we offer many choices for replacing tetramethylrhodamine in your research. And because the Alexa Fluor® 555 dye (excitation/emission maxima ~555/565 nm) is such an excellent spectral match to tetramethylrhodamine, you won't have to change the instrumentation or the other fluorescent parameters in your experiment.

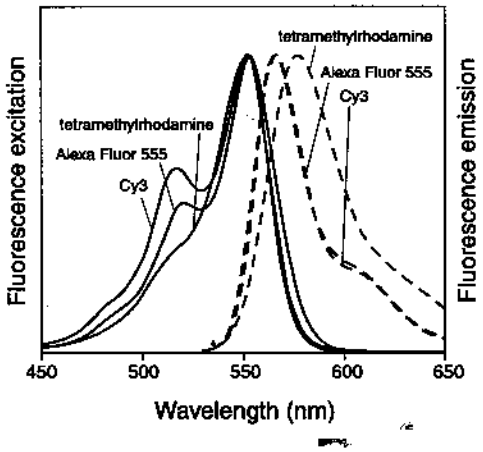

Figure 1. Fluorescence excitation and emission spectra for Alexa Fluor® 555 dye, Cy3, and tetramethylrhodamine.

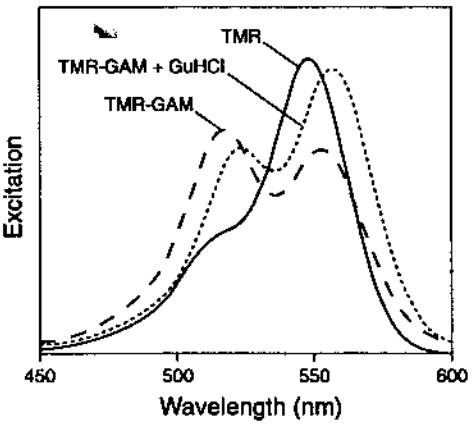

Figure 2. Effect of protein conjugation on the excitation spectrum of tetramethylrhodamine. The excitation spectrum of tetramethylrhodamine conjugated to goat anti–mouse IgG antibody (TMR-GAM) shows an additional peak at about 520 nm when compared with the spectrum of the same concentration of the free 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.

Table 1. If you are using tetramethylrhodamine probes, consider switching to these Alexa Fluor® 555 probes for enhanced brightness and photostability.

Fluorophore Derivative	Cat # of TMR Derivative	Cat # of Alexa Fluor® 555 Derivative
Amine-Reactive Derivatives	T490, T668, C1171, T1480, T1481, C2211, T6105, C6123	A20009, A20109, A32755, A32756
Protein Labeling Kits	F6163	A20174, A20187, A30007, Z25005, Z25105, Z25205, Z25305, Z25405, Z25605
Thiol-Reactive Derivatives	T6006, T6027, T6028	A20346, H30463
Amine-Containing Derivatives	A1318	A30677
Avidin and Streptavidin Conjugates	S870, A6373	S21381, S32355, I37162
Secondary Antibodies—goat anti-mouse IgG conjugates	T2762	A21127, A21137, A21147, A21157, A21422, A21424, A21425, A21427, I37152
Secondary Antibodies—goat anti-rabbit IgG conjugates	T2769	A21428, A21429, A21430, I37157
Transferrin Conjugate	T2872	T35352
Epidermal Growth Factor Conjugate	E3481	E35350
Phalloidin	R415	A34055
Bovine Serum Albumin (BSA)	A23016	A34786
Wheat Germ Agglutinin (WGA)	W849	W32464
Dextran	D1816, D1817, D1868	D34679
α-Bungarotoxin	T1175	B35451

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