

Phallotoxins

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Fluorescent phallotoxins	1 vial of 300 units of product, lyophilized solids *	<ul style="list-style-type: none"> • ≤-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 phalloidin 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 phalloidin 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 phalloxin 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 phalloxin 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, phalloxin-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 LD₅₀ 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 phalloidin 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 phalloidin	355	443	1100	24
N354	NBD phalloidin	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 phalloidin	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.

Before You Begin

Once reconstituted in methanol, the stock solutions are stable for at least one year when stored frozen at $\leq -20^{\circ}\text{C}$, desiccated, and protected from light. It appears that NBD phalloidin, and possibly all of these toxins, exhibit a small loss of activity when stored in aqueous solution at $2-6^{\circ}\text{C}$ 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 phalloxin 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 phalloxin 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 phalloxin, 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 phalloidin.¹¹ It has been successfully used with all of Molecular Probes phalloxin 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 $\leq -20^{\circ}\text{C}$ 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 phalloxins, 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 phalloxin 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 $\mu\text{g}/\text{mL}$ 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 $2-6^{\circ}\text{C}$.

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 phalloidin	300 U
B7474	biotin-XX phalloidin	50 U
C606	coumarin phalloidin	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)phalloidin (NBD phalloidin)	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

Contact Information

Molecular Probes, Inc.

29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:

6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:

Order Phone: (800) 438-2209
Order Fax: (800) 438-0228

Technical Service:

8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probestech@invitrogen.com

Invitrogen European Headquarters

Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Assistance Department in Eugene, Oregon.

Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Limited Use Label License

For research use only. Not intended for any animal or human therapeutic or diagnostic use. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541) 465-8300. Fax: (541) 335-0504.

Several Molecular Probes products and product applications are covered by U.S. and foreign patents and patents pending. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2006, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.