V. 2.1

Rhodamine Phalloidin
(*Amanita phalloides*)
Cat. # PHDR1
Lot: 069 Amount: 1 x 500 µl
Upon arrival store at 4°C (desiccated)
See datasheet for storage after reconstitution

Background

Phalloidin is a seven amino acid peptide toxin from the mushroom *Amanita phalloides*, which binds specifically and with high affinity (Kd 20 nM) to the polymerized form of actin (F-actin). Phalloidin lowers the critical concentration of actin polymerization to less than 1 µg/ml, thereby acting as a polymerization enhancer. Phalloidin has been labeled with tetramethylrhodamine B isothiocyanate (1) and it is widely used as an alternative to actin antibodies for specifically labeling actin filaments in tissue cultured cells and tissue sections (2, see Fig. 1) and cell-free preparations. Rhodamine phalloidin-labeled actin filaments retain many functional characteristics of unlabeled actin including their ability to interact with myosin.

Material

Rhodamine phalloidin is supplied as an pink solid, mol. wt. 1306. A 1x working stock of PHDR1 gives sufficient reagent to stain cells on 300-350 coverslips (22 x 22mm) (Fig. 1).

Note: Phalloidin is toxic and must be handled with care (LD50 human = 2mg/Kg).

Storage and Reconstitution

Shipped at room temperature. Briefly centrifuge to collect the product at the bottom of the tube. Reconstitute with 500 µl of 100% methanol to create a 14 µM solution. It is recommended that the solution be aliquoted into 10 x 50 µl amounts and stored in the dark at -20°C, where it is stable for 6 months. The lyophilized product is stable at 4°C desiccated (<10% humidity) for 1 year.

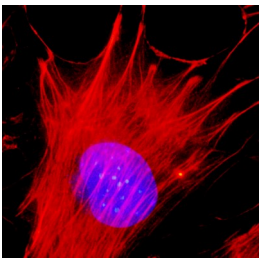
Application 1: Immunofluorescence

There are several methods that are used for fluorescent staining of actin filaments in tissue culture cells. The fixation procedure is critical for obtaining faithful representation of the F-actin distribution within the cell. The fixation method should be selected on the basis of the experimental requirements. Fixing tissue culture cells in paraformaldehyde or glutaraldehyde results in excellent actin filament staining and good lamellipodia preservation.

Reagents

1. Rhodamine Phalloidin (Cat. # PHDR1)
2. Semi-confluent Swiss 3T3 cells grown on glass coverslips
3. Either obtain the F-actin staining kit from Cytoskeleton, Inc. (Cat. # BK005) or prepare Reagents 4 thru 7 below
4. Phosphate-buffered saline (PBS, 50 mM potassium phosphate pH 7.4, 50 mM NaCl)
5. Fixative solution (4% paraformaldehyde in PBS, pH to 7.0 is necessary)
6. Permeabilization buffer (0.5 % Triton X-100 in PBS)
7. Antifade mounting medium (Fluka BioChemika, Cat. # 10981)
8. 100 nM DAPI (4' 6-diamidino-2-phenylindole) in PBS
9. Glass microscope slide (25 x 75 x 1 mm)
10. Coverslip sealing solution (clear nail polish)

Figure 1: ***Actin Stress Fibers in a Swiss 3T3 cell***



Legend: Swiss 3T3 cells were grown to semi-confluency on a glass coverslip and fixed and stained with rhodamine phalloidin as described in the method. Cells were observed under a fluorescent microscope equipped with a digital CCD camera and 100x objective. Note the abundance of actin stress fibers (red) stained throughout the cell. The cell nucleus is counterstained with DAPI (blue).

Equipment

1. Fluorescent microscope with excitation filter at 535 +/- 20 nm and emission filter at 585 +/- 20 nm for rhodamine, and an excitation filter at 355 +/- 20 nm and emission filter at 460 +/- 20 nm for DAPI.
2. Digital CCD camera.

Method

1. Grow tissue culture cells on glass coverslips until semi-confluent.
2. Prepare 100 nM working stock of rhodamine phalloidin by diluting 3.5 µl of 14 µM labeled stock rhodamine phalloidin into 500 µl of PBS. Keep at room temperature in the dark.
3. Remove culture media and gently wash the cells once with PBS at 37°C.
4. Fix the cells in fixative solution for 10 min at room temperature.
5. Wash the cells once with PBS at room temperature for 30 s.
6. Permeabilize the cells in permeabilization buffer for 5 min at room temperature.
7. Wash the cells once with PBS at room temperature for 30 s.
8. Move the coverslip to a piece of parafilm in a humid chamber and add 200 µl of 100 nM rhodamine phalloidin. Incubate at room temperature in the dark for 30 min.
9. Wash the coverslip three times in PBS.
10. Counterstain the DNA for 30 s with 200 µl of 100 nM DAPI in PBS.
11. Rinse the coverslip in PBS and invert on a drop of antifade mounting media on a glass slide. Gently remove the excess media with a tissue and seal each side with nail polish.
12. Store the slides in the dark at 4°C.
13. Typical F-actin staining results are shown in Figure 1.

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Application 2: Preparation of stabilized fluorescent actin filaments

Stabilized fluorescent actin filaments are an excellent substrate for *in vitro* actin motility assays used in the study of myosin motor proteins (2). Rhodamine phalloidin binding has no effect on actin activation of myosin ATPase *in vitro.*

Reagents

1. Actin protein (250 µg, Cat. # AKL99-A)
2. General Actin Buffer (5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$; Cat. # BSA01)
3. 10x Polymerization Buffer (100 mM Tris pH 7.5, 500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP; Cat. # BSA02)
4. Rhodamine Phalloidin (Cat. # PHDR1)

Equipment

1. Fluorescence microscope with excitation filter at 535 +/- 20 nm and emission filter at 585 +/- 20nm and 63X—100X oil immersion lense.
2. Digital CCD camera.

Method

1. Resuspend rabbit muscle actin (Cat. # AKL99-A) to 1 mg/ml with 250 µl of General Actin Buffer supplemented with 0.2 mM ATP and 1.0 mM DTT. Mix well and leave on ice for 1 h.
2. Polymerize the actin with 1/10th the volume of Polymerization Buffer for 1 h at room temperature.
3. Dilute the polymerized actin filaments 100 fold in 1x Polymerization Buffer containing 70 nM rhodamine phalloidin (2.5 µl of 14 µM stock plus 500 µl of polymerization buffer).
4. Spot 1 µl of the labeled actin into a drop of anti-fade mounting media on a microscope slide (Note: Anti-fade for motility assay should be compatible with movement and ATPase activity i.e. glucose oxidase system).
5. Place a coverslip over the drop and remove excess liquid with a tissue.
6. Examine the fluorescent filaments by microscopy. Actin filaments will have an average length of 5-40 µm and are stable at 4°C in the dark for 1 week.

Product Uses

- Fluorescent staining of actin filaments in fixed tissue sections and tissue culture cells preparations. **Note:** Unlike many actin antibodies, rhodamine phalloidin binds only to F-actin resulting in low background fluorescence. Furthermore, actin staining by is not appreciably different between species.

- Preparation of stabilized fluorescent actin filaments *in vitro.*

References

1. Wulf, E. et al. (1979). *Proc Natl Acad Sci USA*. 76(9):4498-4502.
2. Kron, S.J. et al. (1991). *Meth. Enzmol.* 196: 399-416.

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