V. 1.0

Rhodamine Non-Muscle Actin
(Human platelet, >99% pure)
Cat. # APHR

Upon arrival store at 4°C (desiccated)
See datasheet for storage after reconstitution

Material
Non-muscle actin purified from human platelets (Cat. # APHL99) has been modified to contain covalently linked rhodamine at random surface lysine residues. An activated ester of rhodamine is used to label the protein. The labeling stoichiometry has been determined to be 0.5 dyes per actin monomer. Rhodamine non-muscle actin has an approximate molecular weight of 43 kDa. Rhodamine non-muscle actin (10 µg of protein) is supplied as a pink lyophilized powder.

Storage and Reconstitution
Briefly centrifuge to collect the product at the bottom of the tube. The lyophilized protein is stable for 6 months when stored desiccated to <10% humidity at 4°C. The protein should be reconstituted to 10 mg/ml with 1 µl of distilled water; it will then be in the following buffer: 5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 5% (w/v) sucrose and 1% (w/v) dextran. The concentrated protein can then be snap frozen in liquid nitrogen and stored at -70°C where it is stable for 6 months. For working concentrations, further dilution of the protein should be made with General Actin Buffer (Cat. # BSA01) supplemented with 0.2 mM ATP (Cat. # BSA04) and 0.5 mM DTT. Rhodamine non-muscle actin is a labile protein and should be handled with care. Avoid repeated freeze-thaw cycles.

Purity
Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 12% polyacrylamide gel. Rhodamine non-muscle actin was found to be >99% pure (see Figure 1).

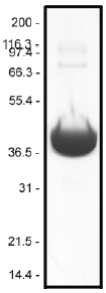

Figure 1. Rhodamine Non-Muscle Actin Protein Purity Determination. A 100 µg sample of rhodamine non-muscle actin (molecular weight approx. 43 kDa) was separated by electrophoresis in a 12% SDS-PAGE system, and stained with Coomassie Blue. Minor protein bands present at 86 and 120 kDa are dimers and trimers of rhodamine actin respectively, and constitute <1% of the total protein. Protein quantitation was determined with the Precision Red Protein Assay Reagent (Cat. # ADV02). Mark12 molecular weight markers are from Invitrogen.

Product Uses
- In vivo actin polymerization studies (micro-injection into non-muscle cells)
- In vitro motility studies using fluorescent F-actin and non-muscle myosins

Biological Activity Assays

1. Microinjection of rhodamine non-muscle actin into tissue culture cells

Reagents
1. Rhodamine non-muscle actin (Cat. # APHR)
2. General Actin Buffer (5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$; Cat. # BSA01)
3. Tissue culture cells seeded on glass coverslips

Equipment
1. Microinjector apparatus

Method
1. Resuspend rhodamine non-muscle actin to 2 mg/ml with General Actin Buffer supplemented with 0.2 mM ATP and 1 mM DTT. Leave on ice for 1 h to depolymerize actin oligomers. Note: rhodamine non-muscle actin can be resuspended in 5 mM Tris-HCl pH 8.0, 10 µM $MgCl_2$ if microinjecting $CaCl_2$ is detrimental to your cells.
2. Centrifuge the non-muscle actin solution at 14,000 x g at 4° C for 10 min. Remove the top 4 µl of the supernatant to a new tube and load the microinjection needle by capillary action.
3. Microinject rhodamine non-muscle actin into cells maintaining positive pressure in the needle to prevent blockage. Note: salt in the tissue culture media and in the cell cytoplasm can result in actin polymerization within the needle.

2. Fluorescent imaging of In vitro polymerized rhodamine labeled actin filaments

Reagents
1. Rhodamine non-muscle actin (Cat. # APHR)
2. General Actin Buffer (5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$; Cat. # BSA01)
3. Polymerization Buffer (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP; Cat. # BSA02)
4. 10 µM phalloidin

Equipment
1. Fluorescence microscope with an excitation filter at 535 nm and emission filter at 585 nm
2. Digital CCD camera

Method
1. Resuspend rhodamine non-muscle actin to 0.5 mg/ml with General Actin Buffer supplemented with 0.2 mM ATP and 1 mM DTT. Add $1/10^{th}$ the volume of Polymerization Buffer supplemented with 1 mM DTT and incubate at room temperature for 1 h.
2. Dilute the polymerized actin filaments 100 fold in 1x Polymerization Buffer containing 70 nM phalloidin and spot 1 µl into a drop of anti-fade mounting media on a microscope

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slide.
3. Place a coverslips over the drop and remove excess liquid with a tissue.
4. Observe rhodamine labeled actin filaments under a fluorescence microscope. A typical fluorescence image is shows in Figure 2.

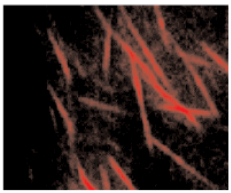


Figure 2. Fluorescence image of rhodamine non-muscle actin filaments. Rhodamine non-muscle actin was polymerized for 1 h, spotted onto a microscope slide and observed under a fluorescence microscope equipped with a Coolsnap digital CCD camera and 100 x objective.

Quality Control

1. Polymerization spin down assay

The biological activity of rhodamine non-muscle actin can be determined by its ability to efficiently polymerize into filaments in vitro and separate from unpolymerized components in a spin down assay. Stringent quality control ensures that >80% of the labeled non-muscle actin can polymerize in this assay.

Reagents

1. Rhodamine non-muscle actin (Cat. # APHR)
2. General Actin Buffer (5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$; Cat. # BSA01)
3. Polymerization Buffer (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP; Cat. # BSA02)
4. 100 mM ATP solution (Cat. # BSA04)
5. 1 M DTT solution
6. Precision Red Protein Assay Reagent (Cat. # ADV02)

Equipment

1. Microfuge at 4°C
2. Beckman Airfuge and Ultra-Clear™ centrifuge tubes (Cat. # 344718), Beckman ultracentrifuge and SW 55 Ti rotor with Ultra-Clear™ centrifuge tubes (Cat. # 344718) and adapters (Cat. # 356860), or other ultracentrifuge capable of centrifuging 200 µl at 100,000 x g.
3. Spectrophotometer capable of measuring absorbance at 600 nm.

Method

1. Resuspend the non-muscle actin to 0.4 mg/ml in General Actin Buffer supplemented with 0.2 mM ATP.
2. Incubate on ice for 1 h to depolymerize actin oligomers that form during storage.
3. Centrifuge the protein in a 4°C microfuge at 14k rpm for 15 min.
4. Transfer the supernatant to a new microfuge tube and determine the total protein concentration with the Precision Red Protein Assay Reagent.
5. Aliquot 200 µl of the actin solution to an ultracentrifuge tube.
6. Add 20 µl (1/10th the volume) of Polymerization Buffer to each airfuge tube and mix well.
7. Incubate at room temperature for 1 h.
8. Centrifuge the tubes at 100,000 x g for 1 h to pellet the polymerized actin.
9. Remove the top 90% of the supernatant of each tube to a clean microfuge tube.
10. Determine the concentration of the protein in the supernatant (unpolymerized monomer actin) with the Precision Red Protein Assay Reagent. This protein concentration is used to determine the efficiency with which actin polymerized and pelleted during centrifugation.

2. Stoichiometry of Rhodamine Labeling

Reagents

1. Rhodamine non-muscle actin (Cat. # APHR)

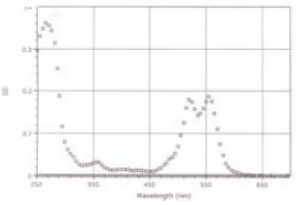
Equipment

1. SPECTRAmax 250 microplate spectrophotometer (Molecular Devices Corp.)
2. UV-compatible microtiter plate

Method

1. Rhodamine non-muscle actin was resuspended to 0.5 mg/ml with Milli-Q water and added into well of a UV-compatible 96 well microtiter plate.
2. The absorbancy was scanned every 5 nm between 250 and 650 nm.
3. The labeling stoichiometry was then calculated using the Beer-Lambert law: A = ε x path length x concentration, where ε is the extinction coefficient for tetramethyl rhodamine in $cm^{-1}M^{-1}$(85,800).
4. The absorption scan is shown in Figure 3.

Figure 3. Absorption scan of rhodamine actin in solution. Rhodamine non-muscle actin was diluted with Milli-Q water in a microtiter plate well according to the method and its absorbancy scanned between 250 and 650 nm. Labeling stoichiometry was calculated to be 0.5 dyes per actin monomer.



Advice for Working with Non-muscle Actin

1. Monomer actin is unstable in the absence of ATP, a divalent cation and dithiothreitol (DTT)
2. Monomer actin will polymerize at >2 mM K^+, Na^+, and in > 0.05 mM Mg^{2+}.
3. Monomer actin is unstable below pH 6.5, or above pH 8.5.
4. Polymerized actin is more resilient to adverse conditions than monomeric actin. Therefore, actin is preferably stored in the polymerized form at 4°C for several weeks. If filaments are to be stored for longer than 24 h, addition of an antibacterial agent such as 0.05% sodium azide or 100 µg/ml ampicillin and 10 µg/ml chloramphenicol is recommended.
5. Snap freeze actin in liquid nitrogen at 10 mg/ml to maintain high biological activity.

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