



Cytoskeleton, Inc.

Manual

V 5.0

F-Actin Visualization Biochem Kit™

(Rhodamine-Phalloidin Based)

Cat. # BK005

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I: Introduction

Background

Conventional actins have a relative molecular mass of approximately 43 kDa. Monomeric actin (G-actin) can self assemble (polymerize) into microfilaments (F-actin), the fundamental unit of the actin cytoskeleton. Actins are highly conserved within the eukaryotic kingdom and exist in higher eukaryotes as multigene families. The different actin isoforms can show distinct cellular and sub-cellular expression and localization. It has been demonstrated that different isoforms have subtly different biochemical properties *in vitro* which supports functional diversity within isoforms *in vivo* (1).

The actin cytoskeleton is a highly dynamic structure, a property under the tight regulation of more than 50 actin binding proteins (ABPs) (2). It is involved in a large number of cellular processes, including muscle contraction, lamellipodial extrusion, cell locomotion, cytokinesis, intracellular transport and cytoplasmic streaming (1). The morphology of the actin cytoskeleton changes rapidly in response to a wide variety of internal and external stimuli, for example figure 1 shows that calpeptin stimulation of serum starved 3T3 cells results in a rapid accumulation of actin stress fibers. This response is due to the activation of the small GTPase Rho (3). As a further example, many pathogenic bacteria and viruses harness the host actin cytoskeleton for their intracellular spread, resulting in characteristic actin comet tails (4).

Fluorescent phalloidins selectively stain filamentous actin at nanomolar concentrations (5). They are the reagent of choice for F-actin staining of fixed cells for several reasons:

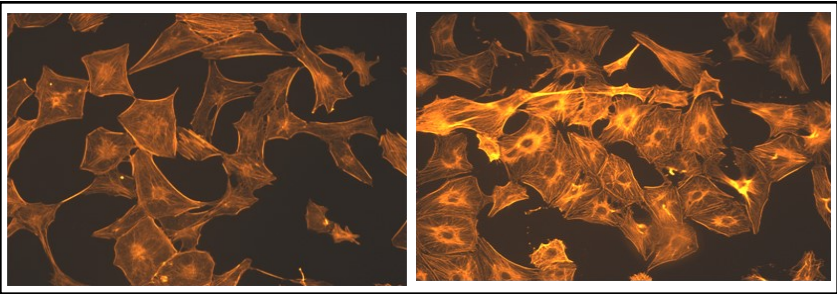
- Bind in a stoichiometric ratio of one phalloidin to one actin monomer
- Do not bind to monomeric G-actins (unlike many actin antibodies) which results in cleaner filament staining
- Binding properties do not change with actins from a wide variety of species
- Binding properties do not change between different actin isoforms
- Non-specific staining is negligible

Figure 1 below shows phalloidin stained 3T3 cells under serum starvation conditions (A) and calpeptin stimulated conditions (B). It can be seen that most actin stress fibers disappear under serum starvation conditions, due to the de-activation of the Rho signaling pathway (3).

I: Introduction (Continued)

Figure 1: Rhodamine Phalloidin Stained Serum Starved and Calpeptin Treated Cells.

Swiss 3T3 cells were grown at 37°C, 5% CO₂ and 95% humidity in 100 mm dishes containing a small glass coverslip. Cells were grown in Dulbecco's modified Eagle's medium plus 10% calf bovine serum to 60% confluency, media was changed to 0.5% calf bovine serum for 24 h then to 0% calf bovine serum for a further 24 h. After this time one dish was treated with calpeptin (0.1 mg/ml final) for 30 min, the other dish treated with carrier only (DMSO). After incubation, the coverslips were removed from the dishes and stained with rhodamine phalloidin (A, serum starved cells; B, calpeptin treated cells).



II: Kit Contents

This kit contains enough reagents for approximately 300 assays when using a standard 12 x 12 mm coverslip; more assays can be performed with smaller coverslips such as the circular 12 mm diameter type. Table 1 summarizes the kit contents.

Table 1: Kit Contents

Reagents	Cat. # or Part # *	Quantity	Storage
Rhodamine Phalloidin	Cat. # PHDR1	1 tube, lyophilized	Desiccated at 4°C. Protected from light
Wash Buffer	Part # WB33	1 tablet	4°C
Permeabilization Buffer	Part # PB123	1 bottle, lyophilized	4°C
Fixative Stock	Part # FX577	4 tubes, 1.5 ml each	4°C
Mounting Medium	Part # MM100	4 tubes, 1.5 ml each	Room temperature
Rhodamine Phalloidin Dark Box	Part # BX376	1 box	4°C or room temperature

*** Items with Part numbers are not sold separately and available only in kit format. Items with catalog numbers (Cat. #) are available separately.**

The reagents and equipment that you will require but not supplied are:

- Coverslips for cell culture growth (12 x 12 mm square or 12 mm diameter round). Thickness of 1 – 1.5.
- Tweezers (e.g., Dumont #7) to handle coverslips.
- Filter paper (approximately 5" x 3") or tissue paper for lining the dark box.
- Fluorescence microscope (excitation filter 535 nm, emission filter 585 nm).
- Microscope camera or video image recording system for data collection.

III: Reconstitution and Storage of Components

Prior to beginning the assay you will need to reconstitute the components shown in Table 2:

Table 2: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage Conditions
Rhodamine Phalloidin	Reconstitute with 500 μ l of methanol to make a 14 μ M Rhodamine Phalloidin Stock Solution	Store at -20°C. Protected from light. Stable for 6 months
Wash Buffer	Reconstitute tablet in 1000 ml of sterile distilled water	Store at room temperature
Permeabilization Buffer	Reconstitute with 100 ml of sterile water	

IV: Important Technical Notes

A: Handling of Fixative Stock and Rhodamine Phalloidin Stock

Fixative Stock contains Formaldehyde solution (37%), a substance known to cause eye, skin and respiratory tract irritation. Formaldehyde may affect behavior, eyes, blood, kidneys, and liver, metabolism, and genetic material, respiratory, nervous, urinary, immunological and gastrointestinal systems. It can cause cancer and may cause birth defects.

Avoid contact with eyes, skin and clothing. Avoid breathing mist or vapor. Avoid prolonged or repeated exposure. Use with adequate ventilation. Wash thoroughly after handling.

In case of contact, flush affected area with plenty of water for at least 15 min. Remove contaminated clothing and or contact lenses if worn. If inhaled, remove to fresh air. If irritation persists or if swallowed, call a physician.

Phalloidin (from *Amanita phalloides*) is very toxic and may be fatal by inhalation, in contact with skin or if swallowed. Target organs are liver and kidneys. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). Wear suitable protective clothing, gloves and eye/face protection. Do not breathe dust. In case of contact, flush affected area with plenty of water for at least 15 min. Remove contaminated clothing and / or contact lenses if worn. If inhaled, remove to fresh air. Call a physician.

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. Cytoskeleton Inc. shall not be held liable for any damage resulting from handling or from contact with the above mentioned products.

B: Pre-treatment of coverslips

Coverslips may be cleaned by washing in ethyl acetate or 10% acetic acid for 20 min followed by washing in 100 % ethanol for 20 min. Coverslips can be air dried and stored for use.

C: Microscope filter requirements

The maximum excitation and emission wavelengths for rhodamine phalloidin are 547 nm and 576 nm respectively. An excitation filter of 535 nm and an emission filter of 585 nm are recommended.

V: Assay Protocol

Method

1. The following method is for a single 12 x 12 mm coverslip, reagent volumes will need to be adjusted for multiple coverslips and for coverslips of different sizes.
2. Remove the Wash Buffer and Permeabilization Buffer from 4°C to room temperature.
3. Prepare a working solution of Fixative by diluting 20 µl of Fixative Stock into 180 µl of Wash Buffer. The 1X working solution can be kept at room temperature.
4. Prepare a 100 nM working stock of Rhodamine Phalloidin by diluting 1.5 µl of Rhodamine Phalloidin Stock Solution into 200 µl of Wash Buffer in a microfuge tube. Cover the microfuge tube with foil or place in the provided Dark Box. Store at room temperature.
5. Take a piece of standard laboratory filter paper (not supplied). Cut to approximately 5" x 3" and dampen it with water. Place the damp filter paper in the Dark Box. This creates a humid environment for staining and also makes handling the coverslips easier.
6. Carefully remove coverslip/s from growth medium using tweezers and place with cell side up on the filter paper in the Dark Box.
7. Wash coverslip once with 200 µl of Wash Buffer for 30s at room temperature. Remove Wash Buffer by wicking action by touching the side of the coverslip to dry filter paper. Do not use a blotting action to remove buffer from the coverslip as this will damage the cells.
8. Add 200 µl of Fixative solution to the coverslip (diluted from stock as in step #3) and incubate for 10 min at room temperature. Remove Fixative with dry filter paper as in step 7.
9. Wash the coverslip once with 200 µl of Wash Buffer for 30s at room temperature. Remove Wash Buffer with dry filter paper as in step 7.
10. Permeabilize cells by adding 200 µl of Permeabilization Buffer to the coverslip and incubate for 5 min at room temperature. Remove Permeabilization Buffer with dry filter paper as in step 7.
11. Wash once with 200 µl of Wash Buffer for 30 s at room temperature. Remove Wash Buffer with dry filter paper as in step 7.
12. Add 200 µl of working stock Rhodamine Phalloidin (diluted from stock as in step #4) to the coverslip and incubate for 30 min at room temperature. This step is carried out in the dark so close the Dark Box lid. Remove the Rhodamine Phalloidin with dry filter paper as in step 7.
13. Wash **three times** with 200 µl of Wash Buffer for 30s each at room temperature. Remove Wash Buffer with dry filter paper as in step 7.

V: Assay Protocol (Continued)

14. Add 20 μ l of Mounting Medium to the center of the coverslip and carefully invert onto a microscope slide. Allow the coverslip to set for 30 min in the dark at room temperature.
15. View actin filaments by fluorescence microscopy (excitation filter 535 nm, emission filter 585 nm).
16. If slides are to be stored, the coverslip should be sealed at the edges with clear nail polish (not supplied) and stored at 4°C in the dark. Under these conditions actin morphology can be maintained for several weeks.

1.

VI: Troubleshooting Guide

Observation	Possible cause	Remedy
High Background	Insufficient Washing after Rhodamine Phalloidin staining	Wash thoroughly three times with 200 μ l of Wash Buffer.
No visible actin filaments	<ol style="list-style-type: none"> 1) A step in the protocol was omitted 2) Fixative Stock has become acidic 3) Rhodamine Phalloidin has bleached due to light exposure during staining 4) Rhodamine Phalloidin has bleached due to long exposure under microscope 5) Incorrect filters are being used 	<ol style="list-style-type: none"> 1) Read protocol carefully 2) If the kit is stored for greater than 6 months, the Fixative Stock can become acidic. This will result in poor actin staining. Adjust the pH of the Fixative Stock to 7.0 with 5 M sodium hydroxide. 3) Make sure that the Rhodamine Phalloidin solution is kept in the dark and that the Dark Box is closed during the Rhodamine Phalloidin staining steps. 4) The Mounting medium contains an anti-bleaching component; however, prolonged exposure to bright light will result in bleaching of the signal. Neutral density filters can be used to reduce bleaching. 5) Rhodamine (TAMRA) phalloidin has maximal excitation and emission wavelengths of 547 nm and 576 nm respectively. An excitation filter of 535 nm and an emission filter of 585 nm are suitable for visualizing rhodamine phalloidin treated actin filaments.

VII: References

- 1) Sheterline, P., Clayton, J., & Sparrow, J.C (Eds.). Protein Profile: Actin. 4th edition. Oxford University Press (1998)
- 2) Kreis, T., Vale, R. (Eds.). Guidebook to the cytoskeleton and motor proteins. Oxford University Press. (1993)
- 3) Ridley, A.J. & Hall, A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**: 389-399 (1992)
- 4) Merz, A.J. and Higgs, H.N. Listeria Motility: Biophysics Pushes Things Forward. *Curr. Biol.* **13**: R302 – R304 (2003).
- 5) Wulf, E., et al. Fluorescent phalloxin: a tool for the visualization of cellular actin. *Proc. Natl. Acad. Sci. USA* **76**: 4498 – 4502 (1979).

