V. 3.8

G-actin / F-actin In Vivo Assay Kit

Cat. # BK037

New Kit Format:

Rabbit Polyclonal Ab has been replaced with a Mouse Monoclonal Ab in Lot # 105 (onward from 9/24/20)

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com

Manual Contents

I: Introduction

Background

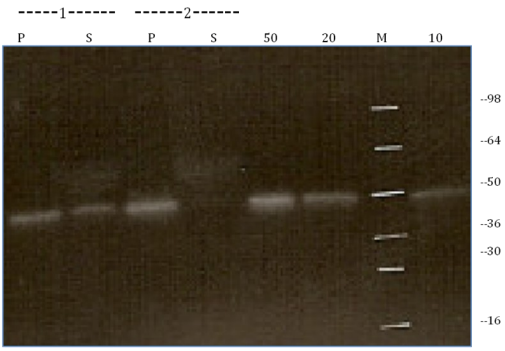
Actin protein contains 375 amino acids and migrates at 43kDa in an SDS-PAGE system. It is abundant, highly conserved and is present in all eukaryotes. Actin exists in cells in globular / monomer (G-actin) and filamentous (F-actin) forms. The filamentous form of actin exists as a cytoplasmic network of 7 - 10 nm wide microfilaments and is the major component of the actin cytoskeleton (1). In non-muscle cells, the actin cytoskeleton is a highly dynamic structure, the ratio of G– and F– actin can alter rapidly and is directly controlled via the activity of well over 100 actin - binding proteins (2), which in turn are regulated by a plethora of indirect actin regulating proteins.

The actin cytoskeleton is critical to many cellular functions, including the maintenance and regulation of cell shape, motility, intracellular communication, intracellular transport and the transduction of extracellular signals into the cell. Understanding the mechanisms that directly and indirectly regulate the actin cytoskeleton is an important area of research and quantitation of the G-actin / F-actin ratio is a useful metric in helping define these mechanisms.

Assay Principle

Cells are lysed in a detergent-based lysis buffer that stabilizes and maintains the G– and F– forms of cellular actin. The buffer solubilizes G-actin but will not solubilize F-actin. A centrifugation step pellets the F-actin and leaves the G-actin in the supernatant. Samples of supernatant and pellet are run in an SDS-PAGE system and actin is quantitated by western blot analysis. Typical assay results are shown in Fig.1.

Figure 1: Reorganization of the actin cytoskeleton in Swiss 3T3 cells after treatment with jasplakinolide



Legend: Swiss 3T3 cells were grown to 50% confluency in DMEM / 10% FBS at 37°C/5% CO_2. Cells were untreated (lanes 1P and 1S) or treated with 0.1 µM of the actin polymerizing drug jasplakinolide for 30 minutes at 37°C/5% CO_2 (lanes 2P and 2S). Cells were lysed and processed into supernatant (S) and pellet (P) fractions and analysed by western blot quantitation of actin protein according to the G-actin/F-actin In Vivo Assay Kit instructions.

Panel 1: In untreated Swiss 3T3 cells, 45% of actin is soluble G-actin (1S) and 55% is insoluble F-actin (1P). This agrees with published data (3).Panel 2: In Swiss 3T3 cells treated with the actin polymerizing drug jasplakinolide, only 5% of actin remains in the soluble G-actin fraction (2S) while 95% is found in the insoluble F-actin pellet fraction (2P). Lanes 50, 20 and 10 represents 50ng, 20ng and 10 ng of G-actin standard. M represents molecular weight markers (molecular weights are shown to the right of the blot).

II: Purchaser Notification

Limited Use Statement

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of product and components of product in research conducted by the buyer. The buyer cannot sell or otherwise transfer this product or any component thereof to a third party or otherwise use this product or its components for commercial purposes. Commercial purposes include, but are not limited to: use of the product or its components in manufacturing; use of the product or its components to provide a service; resale of the product or its components.

The terms of this Limited Use Statement apply to all buyers including academic and for-profit entities. If the purchaser is not willing to accept the conditions of this Limited Use Statement, Cytoskeleton Inc. is willing to accept return of the unused product with a full refund.

III. Kit Contents

This kit contains sufficient reagents to process 100 x 1ml lysates as described in Section VI: Assay Protocol: Detailed Method. There is sufficient primary antibody to make 125 ml of antibody solution. When properly stored, kit components are guaranteed stable for a minimum of 6 months. Table 1 summarizes kit contents.

Table 1: Kit Contents and storage upon arrival

Reagents	Cat.# or Part#*	Quantity	Storage Upon Arrival
Lysis and F-actin Stabilization Buffer	Part # LAS01	1 bottle, 100 ml	4°C
Protease Inhibitor Cocktail	Cat. # PIC02	1 tube, lyophilized	Desiccated 4°C
ATP	Cat. # BSA04	1 tube, lyophilized	Desiccated 4°C
F-actin Depolymerizing Buffer	Part # FAD02	1 bottle, powder	4°C
F-actin Enhancing Solution	Part # FAE01	1 tube, lyophilized	Desiccated 4°C
G-actin Protein Standard	Cat # AKL99	1 tube, 250 µg, lyophilized	Desiccated 4°C
Anti-Actin MAb (clone 7A8.2.1)	Cat # AAN02-S	1 tube, lyophilized	Desiccated 4°C
SDS Sample Buffer	Part # SDS01	2 tubes, 1.5 ml, 5x stock	4°C
DMSO	Part # DMSO	2 tubes, 1 ml	4°C

* Items with Part numbers (Part #) 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 are not supplied;

- Temperature controlled centrifuge capable of reaching 100,000 x g. Ideally accepts 100 µl sample volumes. The assay can be adapted for larger volumes, however, this may result in less assays per kit (see Section VI: Assay Protocol).
- Small homogenizer suitable for low milliliter volumes or 25G needle and syringe.
- SDS-PAGE and western blot apparatus and reagents, anti-mouse-HRP secondary Ab

IV: Reconstitution and Storage of Components

Many of the components in this kit have been provided in lyophilized form. Prior to beginning the assay you will need to reconstitute several components as detailed in Table 2.

Table 2: Kit Component Storage and Reconstitution

Reagents	Reconstitution	Storage
Lysis and F-actin Stabilization Buffer	No reconstitution necessary	4°C
Protease Inhibitor Cocktail	Reconstitute with 1ml of dimethyl sulphoxide (DMSO) for a 100x stock solution	4°C (solution will freeze at 4°C)
ATP	1) Reconstitute with 1ml of ice cold 100 mM Tris pH 7.5 to make 100 mM stock solution 2) Aliquot into 10 x 100 µl volumes 3) Store at –70°C or -20°C	-70°C Note: -20°C storage is also acceptable
F-actin Depolymerizing Buffer	1) Reconstitute in 100 ml of sterile water. 2) Allow powder to completely resuspend prior to storage.	4°C
F-actin Enhancing Solution	Reconstitute in 100 µl of DMSO for a 100x stock	-70°C Note: -20°C storage is also acceptable
G-actin Protein Standard	1) Reconstitute to 10 mg/ml with 25 µl of sterile water 2) Further dilute to 2.5 mg/ml with 75 µl of F-actin depolymerizing Buffer. 3) Mix well. 4) Aliquot into 25 x 4 ul volumes 5) Store at –70°C or -20°C. This protein is used as a western blot standard and as such it does not need to be snap frozen in liquid nitrogen prior to storage.	-70°C Note: -20°C storage is also acceptable
Actin Antibody	1) Reconstitute in 125 µl of 50% glycerol in room temperature sterile water 2) Stable at 4°C for 6 months 3) For long term storage, aliquot into 10 µl volumes and store at -20°C	4°C (up to 6 months) -20°C (up to 12 months)
SDS Sample Buffer	No reconstitution necessary	4°C
DMSO	No reconstitution necessary. Used to resuspend protease inhibitor cocktail and F-actin enhancing solution	4°C DMSO freezes at 4°C

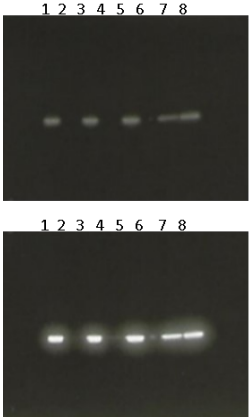
V: Important Technical Notes

A) **Notes on Updated Versions of Manual**

The following updates from version 2.2 should be noted:

1. The manual has been changed to a 5.5 x 8.5 format (version 3.0).
2. The F-actin depolymerizing Buffer has been changed from 1 mM cytochalaisin to 8M urea. The urea solution was found to give more consistent depolymerization and produces actin samples that are compatible with SDS-PAGE / western blot analysis (version 3.3).
3. The manual clarifies that cellular lysate cannot be frozen during Part 2 of the protocol (version 3.5).
4. Version 3.6 of the manual includes a change in actin detection antibody from a rabbit polyclonal antibody (AAN01) to a mouse monoclonal antibody (AAN02). Both antibodies recognize multiple actin isoforms (see individual item data sheets at www.cytoskeleton.com), however AAN02 is approximately 10x more sensitive than AAN01 (see Fig. 2).

Figure 2: Reorganization of the actin cytoskeleton in Swiss 3T3 cells after treatment with jasplakinolide: Comparison of anti-actin antibodies AAN01 & AAN02



Legend: Swiss 3T3 cells were grown to 50% confluency in DMEM / 10% FBS at 37°C/5% CO_2. Cells were untreated or treated with 0.1 µM of the actin polymerizing drug jasplakinolide for 30 minutes at 37°C/5% CO_2. Cells were lysed and processed into supernatant (S) and pellet (P) fractions and analyzed by western blot quantitation of actin protein according to the G-actin/F-actin In Vivo Assay Kit instructions.

Lanes 1,3 & 5 represent pelleted fractions from jasplakinolide treated cell lysate. Lanes 2,4 & 6 represent supernatants (soluble fractions) from jasplakinolide treated cell lysates. Lanes 7 & 8 represent the pellet and supernatant fractions respectively from untreated cell lysates.

The upper western was performed with AAN01 antibody. The lower western was performed using the new kit Mab antibody AAN02.

B) **Positive Control: F-actin Enhancing Solution**

The G-actin/F-actin kit contains 100 µl of an F-actin Enhancing Solution (100x phalloidin stock). This can be added to any given lysate at 1x final concentration prior to incubating the lysates at 37°C for 10 minutes (See Section VI: Assay Protocol, step 3). The phalloidin should drive actin polymerization to give >80% F-actin. This serves as a control to demonstrate the F-actin is efficiently pelleted during the centrifugation step. This control does not have to be run with every experiment. The kit contains sufficient F-actin stabilization solution to process 10 x 1ml lysate samples.

C) **Assay Sensitivity**

The assay can detect down to a 15% shift in G-actin to F-actin ratio. Each condition should be performed in duplicate and repeated several times as assay reproducibility can vary by 10-20% between experiments.

D) **Processing Tissue Samples**

Tissue samples that are to be used in this assay need to be freshly prepared or snap frozen in liquid nitrogen as soon as they are prepared and stored at –70°C for later processing.

Tissue samples can be more difficult to homogenize than cultured cells. The following tips can help with this process;

1) A hand-held homogenizer that can handle low milliliter volumes or 25G needle is often required to lyse cells in tissue samples.
2) In some cases a motor driven micro-homogenizer has been used to homogenize tissue, e.g. aorta (4).
3) In some cases a tissue is particularly difficult to homogenize, e.g. highly elastic artery samples. The snap frozen tissue can be pulverized to a frozen powder in a liquid nitrogen cooled mortar and pestle prior to addition of warm LAS2 buffer. NOTE: in such cases the tissue powder must remain frozen during processing. If the powder becomes wet and sticky prior to LAS2 buffer addition the actin ratio results will not be reliable. Take care when handling liquid nitrogen and follow recommended lab safety precautions.

E) **LAS2 Buffer and Cell Lysates**

Samples homogenized in LAS2 must be processed until PART 2 is completed. If the protocol needs to be halted and resumed at a later time this is best done after the completion of PART 2. F-actin depolymerizes upon freezing so it is necessary to separate the F-actin from the G-actin prior to freezing samples away to ensure an accurate measurement of F-actin and G-actin ratios.

Detailed Assay Method

PART 1: Assay Preparation

1. Warm a centrifuge and rotor to 37°C prior to beginning the assay. The centrifuge must be able to achieve rotor speed of 100,000 x g .
2. Determine the total volume of LAS2 buffer you require per experiment using the volumes given in Table 3 as a guide (see LAS2 recipe below) .
3. Make the required volume of LAS2 buffer as follows;

1 ml	LAS01 buffer (Lysis and F-actin Stabilization Buffer)
10 µl	BSA04 (100 mM ATP stock solution (remaining stock can be re-frozen)
10 µl	PIC02 (100x protease inhibitor cocktail stock)

Warm LAS2 buffer to 37°C 30 minutes prior to beginning the assay.

Table 3: Recommended volumes of LAS2 buffer

Culture Vessel	Vessel surface area (cm^2)	Volume of LAS2 (µl)
35 mm dish	8	100
60 mm dish	21	250
100 mm dish	56	500
150 mm dish	148	1500
6-well plate	9.5 / well	100
12-well plate	4 / well	100*
T-25 Flask	25	300
T-75 Flask	75	750
T-150 Flask	150	1500
Tissue samples	Per 0.1g of tissue	1000

* a minimum volume of 100 µl is recommended for this assay

PART 2: Lysate Collection

1. Lysis methods for suspension cells (A), adherent cells (B) or tissue samples (C) are given below.

A) Cells in suspension

a) Harvest 1 x 10^7 cells by centrifugation at 1,000 x g for 2 minutes.
b) Remove supernatant and discard.
c) Resuspend cell pellet in 500 µl of warm LAS2.
d) Proceed to step 2.

B) Adherent Cells

a) Aspirate media from dish. Incline dish to 30° angle to help remove of as much media as possible.
b) Add appropriate volume of warm LAS2 (see Table 3).
c) Harvest cells by scraping thoroughly with cell scraper, again keep the plate at a 30° angle to help collect all of the lysate.
d) Pipette cell lysate into tube and proceed to step 2.

C) Tissue Samples

a) Add 1000 µl of warm LAS2 per 100 mg (0.1g) of tissue sample.
b) Proceed to step 2.

2. Homogenize samples using a small hand held or motorized homogenizer suitable for low milliliter volumes or a 25G syringe (usually required for solid tissue samples, See also Section V: Important Technical Notes).
3. Incubate lysates at 37°C for 10 minutes.
4. Remove 100 µl volume from each lysate for further analysis. Any remaining lysate can be discarded or used for other purposes at this point.

   NOTE: If larger volumes of lysate are required, due to limitations of centrifuge equipment, make sure that the pellet is resuspended in a volume of F-actin depolymerization buffer that is equal to the centrifuged lysate volume, i.e. if you centrifuge 1 ml of lysate then pellets should be resuspended in 1 ml of F-actin depolymerization buffer.
5. Centrifuge the 100 µl volumes of lysates at 350 x g (approx. 2,000 rpm in a tabletop microfuge), room temperature for 5 minutes to pellet unbroken cells of tissue debris.
6. Pipette supernatants into clearly labeled ultracentrifuge tubes.
7. Centrifuge at 100,000 x g, 37°C for 1h. This step will pellet F-actin and leave G-actin in the supernatant.
8. Remove supernatants to fresh tubes designated as supernatant samples. Supernatants should be removed gently to avoid disturbing the F-actin pellet.
9. Add 100 µl of F-actin depolymerization buffer to each pellet and incubate on ice for 1h to allow actin depolymerization to occur. Pipette up and down several times every 15 minutes to help pellet resuspension.
10. Add 25 µl of 5X SDS sample buffer to each of the pellet and supernatant samples and mix well.
11. The samples are now ready for actin quantitation by SDS-PAGE and western blot analysis. Samples can be stored at –20°C prior to moving to PART 3: Actin quantitation by SDS-PAGE / Western blot analysis.

PART 3: Actin Quantitation by SDS-PAGE / Western Blot Analysis

1. Prepare the following G-actin protein standards as follows;
   a) Resuspend one 4 µl aliquot of frozen actin control protein (see Table 2) in 1 ml of F-actin depolymerization buffer. Label this TUBE 1 (actin is at 10 ng/µl).
   b) Aliquot 100 ul from TUBE 1 into a fresh tube and dilute with 900 µl of F-actin depolymerization buffer. Label this TUBE 2 (actin is at 1ng/µl).
   c) Prepare actin standards as detailed in Table 4.

Table 4: Preparation of G-actin standards

Tube 1 (10ng/µl) (µl)	Tube 2 (1ng/µl) (µl)	F-actin De-polymeriza-tion Buffer (µl)	5X SDS buffer (µl)	Final actin amount (ng)
0	10	2	3	10
2	0	10	3	20
5	0	7	3	50

2. Run lysate supernatant and pellet samples with G-actin protein standards on an SDS polyacrylamide gel (e.g. a 4-20% gradient gel or a 12% gel).

3. We recommend running 10 µl of each lysate sample as an initial test. In some cases a 10 µl sample of lysate will contain actin amounts that are out of the linear range of western blot detection. In these cases either more or less sample will need to be run. It is important not to discard lysate samples until an accurate actin quantitation has been obtained. Lysates can be stored at –20°C for up to 12 months.

4. Transfer the proteins from SDS-PAGE to a western blot membrane (nitrocellulose or PVDF) according to the manufacturers instructions.

5. After transfer, block the membrane in TBST/5% non-fat milk (10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.01% Tween 20/5% non-fat milk) for 30 minutes at room temperature.

6. Wash the membrane 3 x 10 minutes in TBST at room temperature.

7. Dilute the anti-actin mouse monoclonal antibody supplied in this kit 1:1000 in TBST/5% non-fat milk. This is 1 µl of antibody per ml of TBST/5% non-fat milk.

8. Incubate the membrane in primary antibody for 1h at room temperature.

9. Wash the membrane 3 x 10 minutes in TBST at room temperature.

10. Incubate the membrane in anti-mouse-HRP secondary antibody according to the manufacturers instructions. Generally a 1:5,000 -1:20,000 antibody dilution and membrane incubations of 0.5 -1h at room temperature are recommended.

11. Wash the membrane 5 x 10 min with TBST at room temperature.

12. Process the membrane for chemiluminescent detection of actin (43kDa). Use the G-actin standard curve to quantitate the amount of actin in your lysate supernatants (G-actin) and pellets (F-actin).

VI: Assay Protocol: Quick Method

This section is intended for users that are familiar with this assay protocol.

1. Scrape adherent cells or resuspend suspension cells or tissue sample in **LAS2** buffer. As general guideline, see Table 3.
2. Gently homogenize to lyse the cells; **LAS2** contains detergents which will disrupt the cell membrane.
3. Centrifuge the lysate at 350 x g (approx. 2,000 rpm in a tabletop microfuge) for 5 min, room temperature to pellet unbroken cells or tissue debris.
4. Centrifuge 100 µl of the supernatant from Step 3, at 100,000 x g, 1h, 37°C to separate F-actin from soluble G-actin.
5. Analyze supernatant for actin content (G-actin in the supernatant versus F-actin in the pellet) by Western blot with anti-actin mouse monoclonal antibody.
6. Scan G-actin/F-actin bands by densitometry and calculate the ratio of free G-actin versus that present as F-actin.

VII: Troubleshooting

Observation	Possible Cause	Possible Solution
Actin samples are not in the linear range of chemiluminescent detection	The G-actin / F-actin assay protocol provides guidelines for performing the assay. If there is a high level of actin polymer in any given sample then it could be overloaded on your first western analysis. Conversely if very little actin is polymerized you may need to load more onto SDS-PAGE.	Each new condition may require an initial assay followed by a fine tuning of sample loading to make sure you are in a good linear range for actin band quantitation.
Actin bands are not detected in any samples, including the G-actin standards	1) The actin antibody supplied in the kit is a mouse monoclonal IgG2b (clone 7A8.2.1). 2) Check that you have transferred your samples efficiently to the membrane. 3) The assay is designed to analyse low ng of actin protein. Make sure you are using a sensitive chemiluminescent reagent.	1) Make sure you are using an anti-mouse secondary Ab that recognizes mouse IgG2b. 2) Stain the membrane with Ponceau S or similar reversible protein detection stain to confirm efficient protein transfer. 3) We recommend Pierce West Dura reagent.
Actin bands are detected for the G-actin standards but not in tissue samples	Tissue samples are more difficult to homogenize than cell culture samples. Poor lysis may result in little or no actin signal.	1) An homogenizer or 25G needle is often required to lyse cells in tissue samples. 2) In some cases a motor driven micro-homogenizer is used to homogenize tissue, e.g. aorta (4). 3) In some cases a tissue is particularly difficult to homogenize, e.g. highly elastic artery samples. The snap frozen tissue can be pulverized to a frozen powder in a liquid nitrogen cooled mortar and pestle prior to addition of warm LAS01 buffer. NOTE: in such cases the tissue powder must remain frozen during processing. If the powder becomes wet and sticky prior to LAS2 buffer addition the actin ratio results will not be reliable.

VIII: References

1) Milligan R.A. et al. 1990. Molecular structure of F-actin and location of surface binding sites. *Nature* **348**, 217-221.

2) Dos Remedios C.G. et al. 2003. Actin binding proteins: regulation of cytoskeletal microfilaments. *Physiol. Rev*. **83**, 433-473.

3) Phillips D.R. et al. 1980. Identification of membrane proteins mediating the interaction of human platelets. *J. Cell Biol.* **86**, 77-86.

4) Kim H.R. et al. 2008. Cytoskeletal remodeling in differentiated vascular smooth muscle is actin isoform dependent and stimulus dependent. *Am. J. Physiol. Cell Physiol.* **295**, C768-C778.

NOTES:

NOTES:

NOTES:

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com