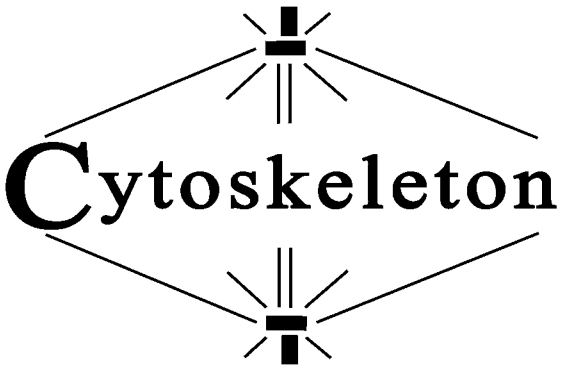

G-actin / F-actin In Vivo Assay Kit

BK037

ORDERING INFORMATION

To order by phone: (303) - 322 - 2254
To order by Fax: (303) - 322 - 2257
Technical assistance: (303) - 322 – 2254 or tservice@cytoskeleton.com
World Wide Web: www.cytoskeleton.com
Write to us: Cytoskeleton, Inc., 1830 S. Acoma Street, Denver, CO 80223. U.S.A.

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Section I: F-actin / G-actin *in vivo* Assay Introduction

The most reproducible and accurate method of determining the amount of filamentous actin (F-actin) content versus free globular-actin (G-actin) content in a cell population is to use Western blot quantitation of F-actin and G-actin cellular fractions (ref. 1-4). The general approach is to homogenize cells in F-actin stabilization buffer, followed by centrifugation to separate the F-actin from G-actin pool. The fractions are then separated by SDS-PAGE and actin is quantitated by Western blot. The final result gives the most accurate method of determining the ratio of F-actin incorporated into the cytoskeleton versus the G-actin found in the cytosol (ref. 5, see figure 7c).

Uses of the kit

1. To study the effects of pharmaceutical compounds on the ratio of G-actin to F-actin.
2. To study the effects of mutated cell lines versus their parent cell line for the change in ratio of G-actin to F-actin.
3. To study the effects of physical alterations of environment on the ratio of G-actin to F-actin.

Typical Assay Results

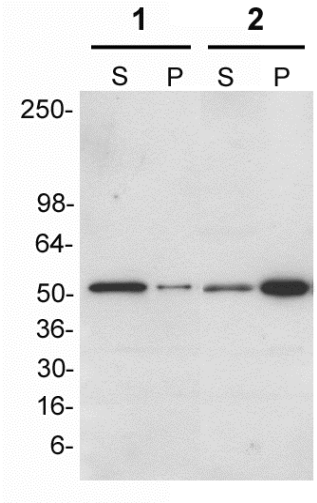

Figure 1. ***Reorganization of actin in Swiss 3T3 cells after treatment with Jasplakinolide.*** Changes in the amount of G-actin and F-actin were investigated in Swiss 3T3 cells treated with the actin polymerizing drug jasplakinolide, using the G-actin/F-actin in vivo assay kit (Cat. # BK037). Panel 1. In untreated Swiss 3T3 cells, 80% of actin is soluble G-actin, and is found within the supernatant fraction (S), 20% of actin is filamentous F-actin and is found in the pellet fraction (P). Panel 2. In Swiss 3T3 cells treated with jasplakinolide, 80% of actin is reorganized into F-actin and is found in the pellet fraction (P).
Method: Semi-confluent Swiss 3T3 cells were treated with (panel 2) or without (panel 1) 0.1 μM jasplakinolide for 30 min at 37°C. Cells were scraped into 2 ml of LAS2 buffer at 37°C and homogenized with an 25 Gauge needle. Cell lysates were then cleared of unbroken cells with a low speed (2000 rpm) centrifuge. Cleared lysates were then centrifuged at 100,000 x g to separate soluble G-actin (S) from insoluble F-actin (P). Supernatant (S) and pellet (P) samples were then proportionally loaded on a polyacrylamide gel, separated by electrophoresis and transferred to Nitrocellulose membrane for probing with an anti-actin antibody.

Section II: Important Technical Notes

The following technical notes should be carefully read prior to beginning the assay

1. The F-actin / G-actin *in vivo* assay requires a constant cells-to-buffer volume ratio. Essentially the lysis step has to dilute the cellular extract so that the free G-actin does not polymerize onto existing F-actin. This ratio is roughly 10 volumes of buffer to 1 volume of cell pellet; larger volumes of buffer are fine e.g. 50 volumes of buffer per volume of cells. A general guide is to use 0.3 ml for 3 cm plate, 1 ml for 6 cm plate and 2 ml for 9 cm plate.

2. F-actin populations are sensitive to temperature. Therefore, pay particular attention to temperature detail, for example you can measure the exact temperature inside a centrifuge tube by performing a test run with water in the tube and then placing a thermometer in the water before and after the run. **Warm all apparatus rotors and centrifuge tubes to culture temperature before starting the assay.**

Required Equipment

1. Temperature controlled centrifuge capable of reaching 100,000 x g with volumes ranging between 100 ul to 2 ml. Suitable systems are:

    a. Beckman tabletop ultracentrifuge with type 100 Ti rotor.
    b. Beckman Ultracentrifuge, SW50.1 rotor with tube adaptors.
    c. Beckman Airfuge, A-100/30 rotor and Ultra-Clear ™ tubes (Beckman, Cat. # 344718)

2. Small homogenization device; either a pestle for 1.5 ml tubes, 25-Gauge 1 ml syringe, or fine tip 200 ul pipette tip.

3. Gel electrophoresis and Western blot apparatus.

Section III: Kit Contents

This kit contains sufficient reagents for approximately 100 assays of 1 ml volume.

KIT COMPONENT	AMOUNT	DESCRIPTION
Lysis and F-actin stabilization buffer (LAS1)	1 x 100 ml	50 mM PIPES pH 6.9, 50 mM NaCl, 5 mM $MgCl_2$ 5 mM EGTA, 5% (v/v) Glycerol, 0.1% Nonidet P40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% 2-mercapto-ethanol, 0.001% AntifoamC
Protease inhibitor cocktail (100x stock) (Cat. # PIC02)	1 x 1 ml	0.4 mM Tosyl arginine methyl ester, 1.5 mM Leupeptin, 1 mM Pepstatin A, 1 M Benzamidine when reconstituted
ATP (Cat. # BSA04-001)	1 x 1 ml	100 mM ATP solution when reconstituted
F-actin enhancing solution	1 x 100 ul	100 uM Phalloidin when reconstituted
F-actin depolymerization solution	1 x 200 ul	1 mM Cytochalasin when reconstituted
G-actin Control Standard (Cat. # AKL99)	1 x 250 ug	10 mg/ml rabbit muscle actin when reconstituted
G-actin antibody (Cat. # AAN01).	1 x 100 ug	0.5 mg/ml rabbit polyclonal G-actin antibody when reconstituted
SDS sample buffer	1 x 1.5 ml	5x stock solution.
Anhydrous DMSO	2 x 1.5 ml	Solvent for F-actin enhancing and depolymerizing solutions

Section IV: Things to do Prior to Beginning the Assay

Prior to beginning the assay you will need to reconstitute several components as follows:

Kit Component	Reconstitution	Storage Conditions
ATP Stock	1) Reconstitute in 1 ml of ice cold Milli-Q water. This stock will take up to 5 minutes to completely resuspend so keep on ice for 5 minutes with occasional pipetting to help the resuspension. 2) Aliquot into 100 ul volumes. 3) Snap freeze in liquid nitrogen and store at -20°C.	Store at -20°C. Stable for 1 year under these conditions.
F-actin enhancing solution	1) Reconstitute with 100 ul of DMSO. 2) Aliquot into 10 ul volumes. 3) Freeze and store at -20°C.	Store at -20°C. Stable for 1 year under these conditions.
F-actin depolymerization solution	1) Reconstitute with 200 ul of DMSO. 2) Aliquot into 20 ul volumes. 3) Freeze and store at -20°C.	Store at -20°C. Stable for 1 year under these conditions.
G-actin Control Standard	1) Reconstitute with 25 ul of Milli-Q water. 2) Aliquot into 5 ul volumes. 3) Snap freeze in liquid nitrogen and store at -20°C.	Store at -20°C. Stable for 1 year under these conditions.
G-actin antibody	1) Reconstitute with 200 ul of 30 % glycerol in Milli-Q water. 2) Aliquot into 50 ul volumes. 3) Snap freeze in liquid nitrogen and store at -20°C.	Store at -20°C. Stable for 1 year under these conditions.
Lysis and F-actin stabilization buffer (LAS1)	None required.	Store at 4°C. Stable for six months under these conditions.
Protease inhibitor cocktail	Reconstitute with 1 ml of DMSO.	Store at -20°C. Stable for one month under these conditions.
Anhydrous DMSO	None required.	Store at room temperature. Stable for 1 year under these conditions.

In addition to preparing the above components, you will need to:

1. Prepare active growing cell cultures, usually one 3, 6 or 9 cm plate of cells.
2. Prepare **LAS2** buffer as follows:
   2 ml of LAS1 buffer
   20 ul of 100 mM ATP
   20 ul of protease inhibitor cocktail
   Store LAS2 buffer at 37°C or cell culture temperature for a maximum of 2 h.

Section V: Quick Method

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

1. Scrape adherent cells or resuspend suspension cells in **LAS2** buffer. As general guideline, use 0.3 ml for a 3 cm plate, 1 ml for a 6 cm plate and 2 ml for a 9 cm plate.
2. Gently homogenize to lyse the cells; **LAS2** contains detergents which will disrupt the membrane.
3. Centrifuge the lysate at 2000 rpm for 5 min to pellet unbroken cells.
4. Centrifuge the supernatant from Step 3, at 100,000 x g 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 antibody.
6. Scan G-actin/F-actin bands by densitometry and calculate the ratio of F-actin versus that present as free G-actin.

Section VI: Detailed Assay Protocol:

1. Warm the centrifuge rotor to 37°C or culture temperature in an incubator or centrifuge for at least 2 h before starting the experiment. **Having all equipment and reagents at culture temperature is critical for this assay.**
2. Determine the required volume of lysis buffer (**LAS2**) to make. Use 0.3 ml for a 3 cm plate, 1 ml for a 6 cm plate, and 2 ml for 9 cm plate.
3. Prepare **LAS2** buffer by pipetting the total required volume of LAS1 into a clean tube or bottle at room temperature. Add the following supplements per ml of LAS1.

   a) 10 µl 100 mM ATP (Cat. # BSA04)
   b) 10 µl. Protease inhibitor cocktail (Cat. # PIC02)
   Mix all the components until homogenous.

4. Warm LAS2 buffer to 37°C or culture temperature (allow at least 30 min for temperature equilibrium).
5. Prepare and label centrifuge tubes equivalent to the number of samples being tested. Use tube numbers 1, 2 and 3 for "normal", "positive" and "negative" controls respectively. Add $1/100^{th}$ the sample volume of F-actin enhancing solution (1 uM final concentration) to tube 2, and $1/100^{th}$ the sample volume of F-actin depolymerization solution (10 uM final concentration) to tube 3.
6. Harvest the cells using of the following methods:

   For cells in suspension:
   i) Pipette cells into culture temperature centrifuge tubes with enough cells per tube for each test.
   ii) Centrifuge cells for 1 min at 2000 x g in a culture temperature rotor.
   iii) Add the appropriate volume of LAS2 buffer per tube. (see Step 2). Record the time of buffer addition.

   For cells on a culture dish:
   i) Aspirate media from the bottom of the inclined dish. Add the appropriate volume of LAS2 buffer (see Step 2). A larger volume of LAS2 buffer can be used if required to cover the cells. Record the time of buffer addition.
   ii) Scrape cells off the dish surface and collect them at the bottom of the inclined dish.
   iii) Pipette into the pre-labeled tubes.

7. Homogenize cells with the appropriate volume of warm LAS2 (see Step 2) using a pestle, 25G syringe with a bent-over tip, or 200 ul pipette tip with a fine orifice (syringe or pipette up and down eight times). Homogenize quickly because the samples should be treated for a similar time. Incubate at culture temperature for a total of 10 min, beginning after cell suspension in LAS2. During this time, determine cell lysis by taking a 2 ul sample and diluting into 10 volumes of an exclusion dye like Trypan blue or Neutral red, check under the light microscope. Greater than 95% cell rupture is required, if this has not been obtained, homogenize more vigorously and incubate for a longer time. Keep a 10 ul aliquot of each sample for determining the total protein concentration (see the Precision Red Advanced Protein Assay Reagent Cat. # ADV02 for a rapid and accurate procedure).
8. Centrifuge the lysate at 2000 rpm for 5 min to pellet unbroken cells.
9. Centrifuge the homogenate at 100,000 x g for 1 h at 37°C.
10. Label tubes S1, S2 and S3 etc for the supernatants place on ice. Place 10 ml of Milli-Q water on ice.

11. When rotor stops, immediately pipette the supernatants into appropriately labeled tubes and place on ice.
12. Resuspend pellet to the same volume as the supernatant using ice cold Milli-Q water plus 1/100th the volume of F-actin depolymerizing solution (10 μM cytochalasin-D) and leave on ice for 1 h to dissociate F-actin; every 15 min pipette up and down once with a fine 200 ul pipette tip. **It may be necessary to shear the pellet in 20 ul of Milli-Q water for 15 min prior to resuspending it to the same volume as the supernatant and continuing with the 1 h incubation.**
13. Dilute each sample ten fold into Milli-Q water, mix well and pipette 16 ul into a tube containing 4 ul of SDS sample buffer. Mix and heat to 95°C for 2 min.
14. Load equivalent volumes (e.g. 20 ul) onto a 12% SDS-polyacrylamide gels in duplicate. Electrophorese to separate the samples according to molecular mass. Use the sample of actin (250 ug) to create a standard curve (2, 5, 10, 20 and 50 ng per lane).
15. Blot the gel onto a nitrocellulose membrane, and perform an actin immunoblot as described in section VIII.
16. Determine ratio of G-actin to F-actin by scanning densitometry from the X-ray film.

Important considerations

1. The assays should be performed twice in duplicate for valid significant results. Experiments should be performed at least on different days and best on different weeks for the highest accuracy. Samples may vary by up to 20% even in the best experiments so the duplicates and two experimental days are crucial for accurate results.
2. The results can be presented either as total G-actin or as a ratio of G-actin versus F-actin, both are valid. In addition the exact actin concentration (as G- and F-actin) can be calculated by knowing the amount of total cellular protein loaded onto the wells (from Detailed Method Step 12) and the calculated mass of actin therein using the actin standard curve, and by assuming total cellular protein concentration inside the intact cell is 80 mg/ml.

3. A useful equation is: Total cellular actin = G-actin + F-actin.

   This equation is useful to determine if all the actin is being analyzed, if 100% of expected actin can be accounted for in the supernatant and pellet fractions, then your assay becomes more significant than if 30% of protein is lost in the SDS-sample buffer spins for example. Try running samples without an SDS-sample spin to see if the results differ. Also run a sample of total protein from Detailed Method Step 7 to see if the amount of actin in this sample equates with the G- and F-actin fractions.

Interpretation of results:

The phalloidin control sample is an example of enhanced actin polymerization; you should easily determine that G- to F-actin ratio has changed from 50:50 to 10:90. Conversely, the cytochalasin treatment is an example of decreased F-actin content causing a G- to F-actin ratio of 70:30. The remaining G-actin in this sample may come from G-actin in complex with other proteins (5) and the G-actin left at the equilibrium of G- to F-actin in the presence of this drug, also called the critical concentration of G-actin. In the case of the cytochalasin sample the ratio of G- to F-actin may not change as much as the phalloidin sample because depolymerization is dependent on the natural dissociation of actin from the ends of actin filaments. To increase this affect one can sonicate the sample with a probe sonicator; this will break actin filaments, creating more F-actin ends for dissociation.
As an example of the range of G-actin to F-actin ratio that can be determined in diverse cells types, cardiomyocytes may have a 10:90 ratio, whereas non-activated platelets may have a 80:20 ratio.

These control results are usually extreme with respect to physiological conditional responses. There may be a 15 or 30% difference in G-actin between your samples containing for example different hormones in the culture media, or low concentrations of drug compounds, or mild mutations. The lower the difference between control cells and your test samples the more replicates you will need to show a significant difference. Generally, less than 10% difference cannot be picked out by this or any other presently accepted method.

If there are concerns about your results you can see the Troubleshooting section in this manual or call 303-322-2254 for technical assistance.

Section VII: References

1. Yassin R, Shefcyk J, White JR, Tao W, Volpi M, Molski TFP, Naccache PH, Feinstein MB and Sha'Afi RI. 1985. Effects of chemotactic factors and other agents on the amounts of actin and a 65,000-mol-wt protein associated with the cytoskeleton of rabbit and human neutrophils. *JCB*, **101**, 182-188.
2. White JR, Naccache PH. and Sha'Afi RI. 1983. Stimulation of chemotactic factor of actin association with the cytoskeleton in rabbit neutrophils. Effects of calcium and cytochalasin B. *JBC*, **258**, 14041-14047.
3. Hartwig JH. 1992. An ultrastructural approach to understanding the cytoskeleton. In The Cytoskeleton, A practical approach Ed. KL Carraway and CAC Carraway, Oxford University Press.
4. Rao KMK, Betschart JM and Virji MA. 1985. Hormone induced actin polymerization in rat hepatoma cells and human leucocytes. *Biochem J*. **230**, 709-714.
5. Tu Y, Wu S, Shi X, Chen K, and Wu C. 2003. Migfilin and Mig-2 link focal adhesions to filamin and the actin cytoskeleton and function in cell shape modulation. *Cell*. **113**, 37-47.

Section VIII: Actin Immunoblot Method:

1. Transfer your protein samples onto a nitrocellulose membrane (6).
2. Block the membrane for 20 min in 5% non-fat milk in TBST (20 mM Tris-HCl pH8.0, 50 mM NaCl, 0.01% Tween20).
3. Wash the membrane 3 x 10 min with TBST.
4. Incubate the membrane in a 1:500 dilution of anti-actin antibody (Cat. # AAN01) in **0.1%** non-fat milk in TBST for 1 h at room temperature.
5. Wash the membrane 3 x 10 min with TBST.
6. Incubate the membrane in a 1:25,000 dilution of anti-rabbit HRP (Cat. # RG04, not provided) in **0.1%** non-fat milk in TBST for 1 h at room temperature.
7. Wash the membrane 3 x 10 min with TBST.
8. Process the membrane for chemiluminescent detection of actin. We recommend the SuperSignal® West Dura chemiluminescent reagent (Pierce Biotechnology, Inc. Cat. # 34075) for 5 sec to 5 min to obtain a visible band in the 5 ng G-actin standard lane.

Section VIII: Troubleshooting:

Observation	Possible cause	Correction
In the control G-actin is >80% of the total	1. This could be a valid result, some cell lines may have >80% free G-actin. 2. Assay performed at incorrect temperature 3. Excessive proteolysis	1. Try correction steps 2 and 3. 2. Perform a temperature inventory to see if your equipment and solutions are at the expected temperature. 3. Add extra protease inhibitors to your sample. (e.g. aprotinin, chymostatin, trypsin inhibitor).
Cannot detect bands on the blot	1. Poor protein transfer 2. Low activity chemiluminescence assay	1. Include 0.01% SDS in transfer buffer to increase mobility of protein. 2. Use ECL (Amersham Biosciences), or SuperSignal® West Dura (Pierce Biotechnology, Inc) chemiluminescence reagents.
Cells are not lysed. This kit will not work on yeast or plant or similar cells that have a hard cell wall.	1. Homogenization not abrasive enough to disrupt cells.	1. Use a tight fitting homogenization device that shears cells. **Do not use sonication which will break-up F-actin resulting in rapid disassembly and low F-actin values.**