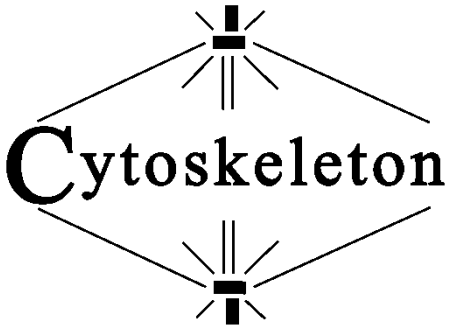

Actin Binding Protein Biochem Kit

Non-Muscle Actin
(Cat. # BK013)

ORDERING INFORMATION

To order by phone: (303) - 322 - 2254
To order by Fax: (303) - 322 - 2257
Technical assistance: (303) - 322 - 2254
Customer Service cserve@cytoskeleton.com
World Wide Web: www.cytoskeleton.com

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Section I: Introduction

The Non-Muscle Actin Binding Protein Spin-Down Biochem Kit (Cat. # BK013) is an extremely quick and economical way to obtain an answer concerning binding affinity for monomer (G-) or polymer (F-) actin. If you are new to the field you may not know that actin requires ATP and a divalent cation for stability. Without this knowledge it is easy to obtain incorrect data, which can lead to inappropriate experimental interpretation. This kit is designed to guide you through the process of studying actin binding proteins

The Actin Binding Protein Spin-Down Biochem Kit provides G- or F-actin plus positive (α-actinin) and negative (Bovine Serum Albumin, BSA) binding control proteins. Actin binding occurs when there is an affinity for any site of actin. F-actin binding can be measured by using a spin down assay where centrifugation is used to separate F-actin from G-actin by differential sedimentation. F-actin binding proteins will co-sediment with actin filaments and form a pellet at the bottom of the centrifugation tube. F-actin severing proteins, G-actin binding proteins or non-actin binding proteins will stay in the supernatant. Actin severing proteins will result in more G-actin remaining in the supernatant compared to the negative control sample. This activity should be further tested by measuring F-actin length distributions before and after adding the test protein. G-actin binding proteins can be measured by adding the test protein to G-actin and inducing polymerization. If the test protein sequesters G-actin, more actin will remain in the supernatant compared with the control.

Actin can exist in two forms: **G**lobular subunit (G-actin) and **F**ilamentous polymer (F-actin) (See Appendix 1 for more information about actin properties). Both forms of actin interact with a plethora of proteins in the cell. To date there are over 50 distinct classes of **A**ctin-**B**inding **P**roteins (**ABPs**) and the inventory is still far from complete (1). Actin Binding Proteins allow the actin cytoskeleton to respond rapidly to cellular and extracellular signals and are integral to cytoskeletal involvement in many cellular processes. These include cell shape and motility (2), muscle contraction (3), intracellular trafficking (4), cell pathogenesis (5) and signal transduction (6).

A comprehensive review of the huge body of literature concerning the structure and functions of ABPs is beyond the scope of this general introduction and we direct the reader to several excellent review articles and references therein (7-10). In this introduction to ABPs we will briefly outline the major recognized classes of ABPs and the experimental procedures that are currently used to study ABP activity.

Example Results:

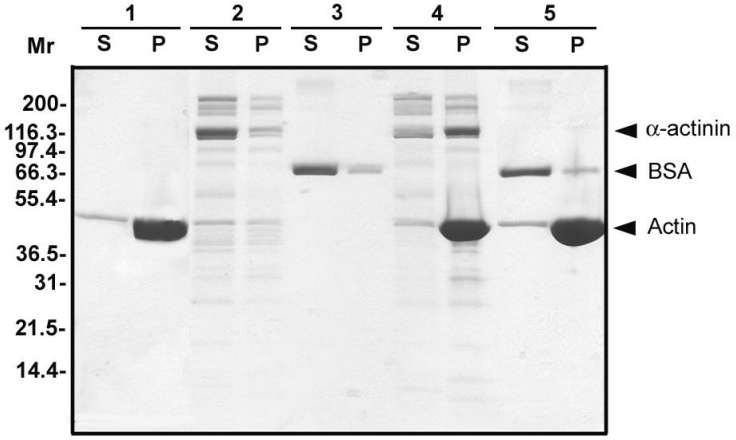


Figure 1. F-Actin Binding Assay. An F-actin binding assay was performed as described in Section VI Binding Assay (method 1). Samples of the supernatant (S) and pellet (P) fractions were collected for each reaction Samples were separated on a 4-20% SDS-gel and stained with 0.1% Coomassie blue. Reaction 1, F-actin alone. Reaction 2, α-actinin alone. Reaction 3, BSA alone. Reaction 4. α-actinin and F-actin. Reaction 5, BSA and F-actin. Note how in the presence of F-actin filaments greater than 70% of the ABP α-actinin is found in the pellet fraction (4 P). In contrast, greater than 90% of BSA remains in the supernatant fraction when incubated with F-actin filaments (5 S)

Classification of ABPs

ABPs can be classified into one (or more) groups of proteins depending upon their activity towards actin:

1. Monomer Binding Proteins

These proteins bind to G-actin. Their major function is thought to be the sequestration of G-actin from the monomer pool and the subsequent prevention of polymerization. Examples of monomer binding proteins include profilin (11), thymosin (12) and ABP-50 (13). These proteins will be detected with this kit by sequestering a greater amount of actin in the supernatant and no test protein will be present in the pellet.

2. Capping Proteins

These proteins cap one end of an actin filament and affect actin dynamics. The majority of capping proteins characterized so far cap the barbed (fast growing) end of actin filaments. These include Cap Z (14) and insertin (15). These proteins will reduce the polymerization rate of F-actin and therefore create a greater amount of actin in the supernatant and an F-actin stoichiometric amount of test protein in the pellet.

3. Severing Proteins

Many of the proteins classified as severing proteins are also barbed end capping proteins. These include proteins in the gelsolin family (16) (Cat. # HPG6). A severing protein that does not show a capping activity is depactin (17). These severing proteins will result in more actin in the supernatant compared to the control, and the test protein will be present in the pellet.

4. Side Binding Proteins

These proteins bind to the sides of actin filaments; they can be subdivided into two major groups, those proteins that bind to one actin filament and those that are able to crosslink filaments by binding to two filaments. The former group includes tropomyosin (19) while the crosslinking proteins include α- actinin (20) (Cat. # AT01). The bundling activity of these proteins can be assayed by actin co-sedimentation assays (see Figure 1); bundling activity can also be directly visualized by microscopic methods. These proteins may cause actin to polymerize to a greater extent leaving more actin in the pellet compared to the control. The test protein will be present in the pellet.

5. Membrane Associated ABPs

The actin cytoskeleton is intimately linked to the cell membrane where it is involved in cell locomotion and signal transduction mechanisms. The proteins that mediate this attachment include the ERM proteins (21) and ponticulin (22). The activity of these proteins can be studied by actin co-sedimentation assays and *in vitro* reconstitution experiments. The test protein will be present in the pellet.

6. Actin Motor Proteins

These proteins are members of the myosin family (2); they include the classical muscle myosin, responsible for muscle contraction (3) and the non-muscle myosins that are implicated in many cellular processes including cell motility, endocytosis and intracellular trafficking (4). Actin motor proteins are studied by ATPase assays and *in vitro* motility assays (23). Actin binding of these proteins is usually sensitive to the presence of magnesium and ATP and/or GTP, therefore removal these nucleotides will result in the motor proteins being present in the pellet.

Section II: Important Technical Notes

Equipment Required

1. Ultracentrifuge capable of centrifuging 50-200 µl volumes at 150,000 x g at 4°C and 24°C.
   Examples are: 1. Beckman Airfuge with Ultraclear tubes (Beckman, Cat. # 344718).
   2. SW50 ultracentrifuge rotor with adapters for Ultraclear tubes (Beckman, Cat. # 344718).
   3. Tabletop ultracentrifuge (Beckman) with TLA-100 rotor.
2. Laemmli reducing-sample buffer (1x stock, 62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue and 5% β-ME)
3. SDS-PAGE equipment.
4. Protein detection system (Coomassie blue is suitable for purified test proteins; antibody for Western blot or silver stain for less pure or low abundance test proteins).
5. Gel scanner for densitometry determinations.

Section III: Kit Contents

This kit contains sufficient reagents for approximately 30-100 assays.

Reagent	Cat. # Part #	Quantity	Description
Non-Muscle Actin	APHL99	2	Lyophilized. >99% pure actin (43 kDa), 1 mg per tube.
α-actinin	AT01	2	Lyophilized. Positive control protein (116 kDa), 50 µg per tube.
BSA	BSAB-200	1	Lyophilized. 3.4 mg/ml negative control protein (68 kDa). 3.4 mg per tube.
General Actin Buffer	BSA01-001	1	Lyophilized. 5 mM Tris-HCl pH 8.0 and 0.2 mM $CaCl_2$.
Actin Polymerization Buffer	BSA02-001	1	Lyophilized. 500 mM KCl, 20 mM $MgCl_2$, and 10 mM ATP. **10X strength.**
F-actin Cushion Buffer	BSA03-001	1	Liquid. 5 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, and 10% glycerol
ATP stock	BSA04-001	1	Lyophilized. 100 mM ATP when resuspended
EGTA solution	BSEG-01	1	Liquid. 0.5 M EGTA
$MgCl_2$ solution	BSMG-01	1	Liquid. 100 mM $MgCl_2.6H_2O$
Tris-HCl pH 7.5	BSTR-01	1	Lyophilized. 100 mM Tris-HCl pH 7.5 when resuspended
Tris-HCl pH 6.5	BSTR-02	1	Liquid. 1.0 M Tris-HCl pH 6.5

Section IV: Reconstitution and Storage of Components

Many of the components of this kit have been provided in lyophilized form. Prior to beginning the assay it is necessary to reconstitute several components as described in Table 3. When stored and reconstituted as described, reagents are guaranteed to be stable for a minimum of 6 months.

Table 3. Kit Component Reconstitution and Storage.

Component	Reconstitution	Storage
Non-Muscle Actin	1) Reconstitute each tube to 10 mg/ml with 100 µl of 4°C water. 2) Aliquot into 4 x 25 µl volumes. 3) Snap freeze aliquots in liquid nitrogen.	Store resuspended protein at -70°C
α-actinin	1) Reconstitute to 1 mg/ml with 50 µl of 4°C water. 2) Aliquot into 5 x 10 µl volumes. 3) Snap freeze in liquid nitrogen.	Store resuspended protein at -70°C.
BSA	1) Reconstitute to 3.4 mg/ml with 1 ml of 4°C water. 2) Aliquot into 10 x 100 µl volumes. 3) Snap freeze in liquid nitrogen.	Store resuspended protein at -70°C.
General Actin Buffer	Reconstitute with 10 ml of Milli-Q water.	Store at 4°C.
Actin Polymerization Buffer	1) Reconstitute with 1.8 ml of 100 mM Tris pH 7.5. 2) Aliquot into 100 µl volumes. 3) Snap freeze in liquid nitrogen.	Store resuspended buffer at -70°C.
F-actin Cushion Buffer	No reconstitution necessary. Required only if non-specific protein interaction with F-actin is suspected.	Store at 4°C.
ATP	1) Reconstitute with 1.0 ml of 100 mM Tris pH 7.5. 2) Aliquot into 100 µl volumes. 3) Immediately store at -70°C.	Store resuspended buffer at -70°C.
0.5 M EGTA	No reconstitution necessary. Required only if the test protein requires buffer modification for binding.	Store at 4°C.
100 mM $MgCl_2$	No reconstitution necessary. Required only if the test protein requires buffer modification for binding.	Store at 4°C.
1.0 M Tris-HCl pH 6.5	No reconstitution necessary. Required for α-actinin binding.	Store at 4°C.
100 mM Tris-HCl pH 7.5	Reconstitute with 5 ml of water. Required for ATP and Actin Polymerization Buffer resuspension.	Store at 4°C.

Section V: Things to do Prior to Beginning the Assay

Prior to beginning the assay you will need to prepare and organize several components as follows:

Buffers and control protein preparation:

1. Rapidly defrost one aliquot of Actin Polymerization Buffer, ATP, α-actinin, and BSA by placing each tube in a room temperature water bath and then place on ice. After thawing, spin each tube for 5 s in a microfuge to collect the liquid at the bottom of the tube. Place on ice.
2. Centrifuge the BSA solution at 150,000 x g for 1 h at 4°C. Remove the supernatant and place on ice; this is your **negative control stock**. This clarification spin can be done in conjunction with your test protein (see below).
3. Place General Actin Buffer (Cat. # BSA01) on ice.

Test protein preparation:

1. Make the test protein at the highest possible concentration in an actin compatible buffer (see Appendix 1). The high concentration (preferably >20 μM) is necessary to give you every chance of detecting actin interactions. The final test protein concentration will be $1/5^{th}$ of this stock solution. In some cases the Kd (binding affinity) of an ABP/actin interaction may be so low as not to be detectable by protein assay of the pellet, however the affect on the actin in the reaction mix may be enough to be detected by differences in the amount of actin in the pellet and supernatant versus the negative control.
2. Centrifuge the test protein at 150,000 x g for 1 h at 4°C.
3. Remove supernatant and place on ice, this is your **test protein stock.**

F-actin preparation for Method 1 only:

1. Thaw out one 100 µl aliquot of 100 mM ATP stock and place on ice.
2. Dilute to 10 mMATP with 900 µl of ice cold sterile water.
3. Aliquot 250 µl of General Actin Buffer and supplement with 5 µl of 10 mM ATP to give 0.2 mM final ATP.
4. Thaw one 250 µg aliquot of actin in a room temperature water bath.
5. Transfer to ice immediately after the protein has thawed.
6. Dilute the actin to 1 mg/ml with 225 µl of supplemented General Actin Buffer. Pipette up and down 5 times to ensure complete resuspension.
7. Leave on ice for 30 min.
8. Defrost one aliquot of Actin Polymerization Buffer and keep on ice (this should be used within 4h and any remaining buffer should be discarded as it is sensitive the freeze/thaw cycles).
9. Pipette 25 µl of the Actin Polymerization Buffer into the actin protein.
10. Mix well by pipetting up and down.
11. Incubate the actin at room temperature (24°C) for 1 h. This is your **F-actin stock** at 21 μM actin.

G-actin preparation for Method 2 only:

1. Thaw out one 100 µl aliquot of 100 mM ATP stock and place on ice.
2. Dilute to 10 mMATP with 900 µl of ice cold sterile water.
3. Aliquot 250 µl of General Actin Buffer and supplement with 5 µl of 10 mM ATP to give 0.2 mM final ATP.
4. Thaw one 250 µg aliquot of actin in a room temperature water bath.
5. Transfer to ice immediately after the protein has thawed.
6. Dilute the actin to 1 mg/ml with 225 µl of supplemented General Actin Buffer. Pipette up and down 5 times to ensure complete resuspension.
7. Leave on ice for 30 min.
8. Aliquot an additional 25 µl of supplemented General Actin Buffer into the actin.
9. Mix well by pipetting up and down.
10. Leave on ice for 1h. This is your **G-actin stock** at 21 μM actin.

Section VI: Binding Assays

The most common use for this kit is the spin-down assay used to detect F-actin binding proteins. This assay involves incubating actin and the test protein and then pelleting the F-actin by differential sedimentation. F-actin will pellet at 150 000 x g for 1.5 h. If the protein of interest is an ABP, it should pellet with the F-actin.

Different actin binding proteins (ABPs) often require different conditions for actin binding, some may even bind G-actin and F-actin under the same or different conditions so one should be aware of all possibilities when investigating a new test protein for the first time.
There are three preliminary "Methods" and a "Results Interpretation Section" which should be completed before more detailed studies are performed, these are described below:

Method 1 can determine whether a test protein binds F-actin or affect the equilibrium between G-actin and F-actin as many ABPs usually do.
Method 2 can indicate whether a test protein has G-actin sequestering or F-actin polymerization enhancing activity.
Method 3 can determine whether a test protein of interest can bundle F-actin

There are more details for further characterizing your binding activity after the Methods section. It should be noted that even if there is no indication of an actin interaction from the preliminary methods, one should read the "Interpretation of Results" section because many ABPs will only bind under their unique conditions.

Method 1: Detecting F-actin Binding and Perturbations in G- to F-Actin Equilibrium.

F-Actin Binding Reactions:
1. Prepare 700 µl of F-actin buffer as follows: Add 630 µl of General Actin buffer and 70 µl of Actin Polymerization buffer in a centrifuge tube. Place on ice and discard the remaining Actin Polymerization Buffer.
2. Label six centrifuge tubes (1-6) and place on ice.

    Tube 1 will be the F-actin only control sample
    Tube 2 will be the α-actinin only control sample
    Tube 3 will be the α-actinin and F-actin positive control sample
    Tube 4 will be the BSA and F-actin negative control sample
    Tube 5 will be the "test protein" only sample
    Tube 6 will be the "test protein" and F-actin sample

3. Pipette 40 µl of F-actin buffer into tubes 2 and 5
4. Pipette 40 µl of **F-actin stock** in each of tubes 1, 3, 4, and 6
5. Pipette 10 µl of **test protein stock** into tubes 5 and 6 and mix by pipetting slowly up and down three times.
6. Pipette 10 µl of α-actinin, 2 µl of 1 M Tris-HCl pH 6.5 into tubes 2 and 3. The final concentration of α-actinin is 2 µM.
7. Pipette 2 µl of BSA and 9 µl of Milli-Q water into tube 4. The final concentration of BSA is 2.0 µM.
8. Pipette 10 µl of the buffer the **test protein stock** is dissolved in into to tube 1.
9. Incubate all tubes at room temperature for 30 min.

F-actin separation:
1. Centrifuge all tubes at 150,000 x g for 1.5 h at 24°C.
2. Carefully remove the supernatants and place them into individually labeled tubes on ice.
3. Add 10 µl of 5x Laemmli reducing-sample buffer (not included) to each supernatant containing tube.
4. Resuspend the pellets in 30 µl of Milli-Q water. Pipette up and down for 2 min, leave on ice for 10 min and repeat pipetting. Add 30 µl 2x Laemmli reducing-sample buffer (not included).
5. Run 20 µl of each sample on a 4-20% SDS-gel until the dye front is at the bottom of the gel.
6. Stain the gel with Coomassie blue or silver stain. Alternatively, the gel can be processed for Western blot analysis.

7. Scan all lanes of the gel/blot with a densitometer.

Method 1 Results:

Tabulate the results from Method 1 in terms of area under the protein peaks obtained from the densitometric scan (visual interpretation of Coomassie band intensity can also be used as a rough indicator). Follow the flow-text below to determine what detailed assays should be performed next as described in Section VI: Interpretation of Results.

1. If there is "test protein" in the pellet of tube 6, go to 2, if not go to 3.
2. If there is less actin (go to point A, Section VII), more actin (go to point B, Section VII) or the same actin (go to point C, Section VII) in the pellet of tube 6 compared to the control pellet of tube 1
3. If there is less actin (go to point D, Section VII), more actin (go to point E, Section VII) or the same actin (go to point F, Section VII) in the pellet of tube 6 compared to the control pellet of tube 1.

Method 2: Detecting Monomer Sequestering or Polymerization Enhancement Activity.

Prepare and organize all buffer components, positive and negative control proteins, test protein and G-actin stock as described in Section V.

G-actin Binding Reactions:

10. Label six centrifuge tubes (1-6) and place on ice.

    Tube 1 will be the actin only control sample
    Tube 2 will be the α-actinin only control sample
    Tube 3 will be the α-actinin and G-actin control sample
    Tube 4 will be the BSA and G-actin negative control sample
    Tube 5 will be the "test protein" only sample
    Tube 6 will be the "test protein" and G-actin sample

11. Pipette 40 µl of General Actin Buffer (Cat. # BSA01) into tubes 2 and 5
12. Pipette 40 µl of **G-actin stock** in each of tubes 1, 3, 4, and 6
13. Pipette 10 µl of **test protein stock** into tubes 5 and 6 and mix by pipetting slowly up and down three times.
14. Pipette 10 µl of α-actinin, 2 µl of 1 M Tris-HCl pH 6.5 into tubes 2 and 3. The final concentration of α-actinin is 2 µM.
15. Pipette 10 µl of the buffer the **test protein stock** is dissolved in into to tube 1.
16. Pipette 2 µl of BSA and 9 µl of Milli-Q water into tube 4. The final concentration of BSA is 2.0 µM.
17. Incubate all tubes at room temperature for 30 min.

Actin polymerization:

1. Add 2.5 µl of 10X Actin Polymerization Buffer (Cat. # BSA02) to each tube and incubate at room temperature (24°C) for exactly 30 min.

Spin-down assay:

1. Centrifuge all tubes at 150, 000 x g for 1.5 h at 24°C.
2. Carefully remove the supernatants and place them into individually labeled tubes on ice.
3. Add 10 µl of 5x Laemmli reducing-sample buffer (not included) to each supernatant containing tube.
4. Resuspend the pellets in 30 µl of Milli-Q water. Pipette up and down for 2 min, leave on ice for 10 min and repeat pipetting. Add 30 µl 2x Laemmli reducing-sample buffer (not included).
5. Run 20 µl of each sample on a 4-20% SDS-gel until the dye front is at the bottom of the gel.
6. Stain the gel with Coomassie blue or silver stain. Alternatively, the gel can be processed for Western blot analysis.
7. Scan all lanes of the gel/blot with a densitometer.

Method 2 Results:

Tabulate the results from Method 2 in terms of area under the protein peaks obtained from the densitometric scan (visual interpretation of Coomassie band intensity can also be used as a rough indicator. Then follow the flow-text below to determine what detailed assays should be performed next in Section VI: Interpretation of Results.

1. If there is test protein in the pellet of tube 6, go to 2, if not go to 3.
2. If there is less actin (go to point A, Section VII), more actin (go to point B, Section VII) or the same actin (go to point C, Section VII) in the pellet of tube 6 compared to the control pellet of tube 1.
3. If there is less actin (go to point D, Section VII), more actin (go to point E, Section VII) or the same actin (go to point F, Section VII) in the pellet of tube 6 compared to the control pellet of tube 1

Method 3: Detecting F-actin bundling activity

F-actin bundling activity can be determined by modifying Method 1 so that a low speed centrifugation is performed after sample incubation.

1. Set up all reaction tubes (1-6) as described in Method 1, F-actin Binding Reactions, Steps 1-9.

F-actin separation:
1. Centrifuge all tubes at 14,000 x g for 1 h at 24°C.
2. Carefully remove the supernatants and place them into individually labeled tubes on ice.
3. Add 10 µl of 5x Laemmli reducing-sample buffer (not included) to each supernatant containing tube.
4. Resuspend the pellets in 30 µl of Milli-Q water, then add 30 µl 2x Laemmli reducing-sample buffer (not included).
5. Run 20 µl of each sample on a 4-20% SDS-gel until the dye front is at the bottom of the gel.
6. Stain the gel with Coomassie blue or silver stain. Alternatively, the gel can be processed for Western blot analysis.
7. Scan all lanes of the gel/blot with a densitometer.

Method 3 Results:

Tabulate the results from Method 3 in terms of area under the protein peaks obtained from the densitometric scan (visual interpretation of Coomassie band intensity can also be used as a rough indicator). Follow the flow-text below to determine what detailed assays should be performed next as described in Section VI: Interpretation of Results.

1. If there is "test protein" in the pellet of tube 6, go to 2, if not go to 3.
2. If there is less actin (go to point A, Section VII), more actin (go to point B, Section VII) or the same actin (go to point C, Section VII) in the pellet of tube 6 compared to the control pellet of tube 1
3. If there is less actin (go to point D, Section VII), more actin (go to point E, Section VII) or the same actin (go to point F, Section VII) in the pellet of tube 6 compared to the control pellet of tube 1.

Section VII: Interpretation of Results

The following points will help to establish the actin binding nature of the test protein as determined from the results obtained for Method 1 and 2.

A. Test protein is binding to F-actin and causing a depolymerization of F-actin. The test protein could be a severing or capping protein. The putative severing protein activity should be investigated by polymerizing actin and then adding test protein; the F-actin lengths can be determined over time by electron microscopic observation of negatively stained samples. The capping activity will be clarified by the Method 2 result which should show much less actin in the pellet versus the Method 1 result. Capping can be further investigated by using F-actin populations of different lengths. F-actin of different lengths can be obtained by polymerizing actin at different concentrations (e.g. at 1 mg/ml the average F-actin length is approx. 2-3 μm, at 0.4 mg/ml the average F-actin length is 10-20 μm and at 0.2 mg/ml the average F-actin length is 20+ μm). Binding affinity can be estimated from the spin-down assay by titrating the test protein concentration between 1 and 20 μM and finding the concentration where half the original protein is in the pellet. Phalloidin can be used to stabilize F-actin if the test protein depolymerizes too much F-actin for the results to be reproducible. Polymerization inhibition can be quantitated by using the pyrene muscle actin fluorescence polymerization assay (see Appendix 2 and the Actin Polymerization Biochem Kit, Cat. # BK003 and non-muscle actin Cat. # APHL99).

B. Test protein is binding to F-actin and enhancing its polymerization. Method 2 should show greater enhancement over its control than Method 1. If not, then the significance of your result should be checked by repeating the experiment (go to C if the result is still insignificant). Electron microscopy could be used to observe the binding and whether or not the test protein causes bundling of F-actin. Binding affinity can be estimated from the spin-down assay by altering the test protein concentration and finding the concentration where half the original protein is in the pellet. Polymerization enhancement can be quantitated by using the pyrene muscle actin fluorescence polymerization assay (see Appendix 2 and the Actin Polymerization Biochem Kit, Cat. # BK003 and non-muscle actin Cat. # APHL99).

C. Test protein is binding to F-actin. Electron microscopy could be used to observe the binding and whether or not the test protein causes bundling of F-actin. Binding affinity can be estimated from the spin-down assay by titrating the test protein concentration and finding the concentration where half the original protein is in the pellet. The F-actin bundling assay (Method 3) should be used to determine if the test protein has bundling activity.

D. Test protein could be G- or F-actin binding and have depolymerizing activity depending on the amount of actin in the pellet. If there is no actin in the pellet then the protein could be an F-actin binding protein which has depolymerized all the actin. If there is actin in the pellet then the protein is not likely to be F-actin binding (see point F). Monomer actin binding can be determined by size exclusion chromatography, DNase 1 inhibition assay (see Appendix 2), use of biotin actin (Cat. # AB07) on streptavidin-agarose beads or inhibition of pyrene muscle actin fluorescence during polymerization (see Appendix 2 and the Actin Polymerization Biochem Kit, Cat. # BK003 and non-muscle actin Cat. # APHL99).

E. This is an unlikely scenario but conceivably an F-actin nucleating factor could bind monomer and fall off after nucleation. An increase in F-actin in the pellet under these circumstances may only be observable during early time points of polymerization. Later on in polymerization the polymer mass may be identical to the control (see Appendix 2). Actin Polymerization can be followed with enhanced fluorescence of pyrene labeled muscle actin (see Cat. # AP05 and BK003).

F. Test protein does not appear to interact with G- or F-actin. There are many reasons why a putative ABP does not appear to interact with G- or F-actin in this assay, these are listed below:

1. A test protein cofactor may be missing. Add possible cofactors to the test protein and repeat the binding reaction.

2. A factor in the assay could be actively inhibiting interactions. Possible inhibitors include calcium and magnesium ions. Add reagents to remove the inhibitors such as EGTA (Part #BSEG-01) for calcium and EDTA (not supplied) for magnesium. In the case of motor proteins the ATP/Mg^{2+} combination will dissociate the motor from the F-actin. In this case you can use phalloidin to stabilize F-actin in the absence of ATP and Mg^{2+}; alternatively you could use Mg^{2+} (Part # BSMG-01) only in the polymerization reaction.
3. Test protein could have a preference for muscle actin. Try the Muscle Actin Binding Protein Kit (Cat. # BK001)
4. Test protein may be at a low concentration in tissue extracts. Use an actin affinity column to concentrate and isolate more of the test protein.

Section VIII: Troubleshooting:

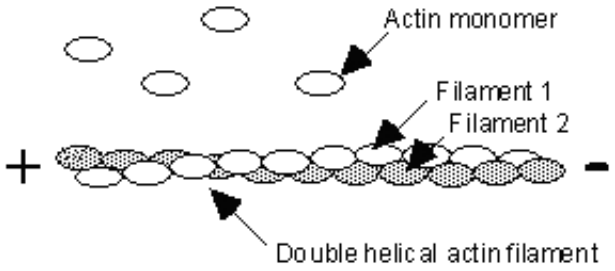
Observation	Possible cause	Correction
Actin in the BSA control tube does not spin-down to form a pellet.	1. Filaments are too short. 2. Centrifugation problem. 3. Denatured or monomer actin.	1. Reduce concentration of G-actin before polymerization. 2. Increase centrifugation speed and time. 3. Missing polymerization agent or actin stabilizing agent (see Appendix 1).
Positive control protein (α-actinin) does not spin-down in the pellet.	1. pH of reaction is incorrect.	1. Add Tris-HCl pH 6.5 to adjust pH.
F-actin is trapping non-specific protein in the pellet.	1. Bundling and crosslinking may create voids in the polymer network which traps non-specific protein.	1. Use Actin Cushion Buffer (Cat. # BSA03) to tighten the pellet. Two volumes of cushion buffer per volume of reaction mixture should be sufficient. Pipette cushion buffer into centrifuge tube then carefully load reaction mixture on top; centrifuge as normal.
Protein / F-actin does not pellet reproducibly.	1. Bundling and crosslinking between F-actin causes loose pellet and which may smear down the side of a fixed angle centrifuge rotor tube.	1. Use the Actin Cushion Buffer as described above.
Protein smears on the gel.	1. Too many contaminants in the binding reaction (e.g. DNA or other polymeric material). 2. Proteolysis of protein.	1. Add DNase or perform partial purification to reduce the contaminants. 2. Add protease inhibitor cocktail.
Test protein fails to bind to actin	1. Salt concentration is too high. 2. Test protein concentration is too low 3. Test protein requires a cofactor 4. pH is incorrect for binding	1. Use 20 mM $MgCl_2$ (Part # BSMG-01) and 10 mM ATP as a "low salt" actin polymerization buffer at 10X strength. 2. Concentration the test protein 10 fold before adding to reaction; 20 μM is sufficient in most cases. 3. Add single cofactors in separate reactions 4. Test different pH conditions (eg. 6.5, 7.5 and 8.5

Appendix 1

G-actin polymerizes to form F-actin

Globular-actin (G-actin) readily polymerizes under physiological conditions to form Filamentous-actin (F-actin) with the concomitant hydrolysis of ATP. F-actin is a double-helical filament as shown below:

Figure 1. Double-helical structure of actin filaments.



Actin can polymerize from both ends *in vitro*, however, the rate of polymerization is not equal. This results in an intrinsic polarity in the actin filament. It has therefore become the convention to term the rapidly polymerizing end the plus-end (see Figure 1) or barbed-end while the slow growing end is called the minus-end or pointed-end.

The propensity of actin to polymerize is dependent upon the affinity of actin monomers for filament ends. Thus, there is an actin monomer concentration below which actin will not polymerize; this value has been termed the Critical Concentration (CC). At monomer concentrations above the CC, the actin will polymerize until the free monomer concentration is equal to the CC. When one is working with actin in vitro the extent of actin polymerization depends upon the conditions used. For example, at 4°C muscle actin has a CC of 0.03 mg/ml in the presence of Mg^{2+} (2 mM) and KCl (50 mM), but when these ions are absent, the CC is approximately 3.0 mg/ml. Thus, by altering the ionic type and strength one can alter the amount of polymer formed. Non-muscle actin has its own CC values, for example, at 4°C in the presence of Mg^{2+} (2 mM) and KCl (50 mM) the CC is approximately 0.15 mg/ml. If Mg^{2+} and KCl are replaced with Ca^{2+}, the CC will increase to nearly 3 mg/ml. Finally, the CC of non-muscle actin can be reduced to 0.03 mg/ml by increasing the temperature to 30°C (Gordon D.J., Boyer J.L. and Korn E.D. 1977. Comparative biochemistry of non-muscle actins (*Journal of Biological Chemistry*, **252**, 8300-8309).

Conditions in which actin is stable

G-actin is stable upon thawing for two days at 4°C. F-actin is not stable to freezing but can be stored at 4°C for one month. F-actin can be transferred to a variety of buffers (e.g. HEPES, phosphate, etc) without detrimental effects. Actin requires a divalent cation, pH 6.5 - 8.0 and ATP for stability. F-actin also requires Mg^{2+} for stability.
If you have any further questions about purified actins please call our technical assistance at 303-322-2254.

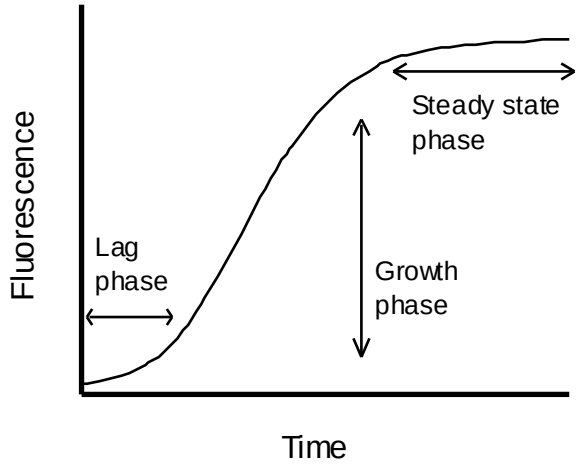
Appendix 2

Assays for Actin

Actin Polymerization Assay:

The most versatile, sensitive and easiest actin polymerization assay consists of pyrene conjugated muscle actin (Cat. # AP05) and a fluorescence spectrophotometer. Fluorescence of pyrene muscle actin is enhanced two to twenty fold by the association of actin monomer into the polymer form (Kouyama and Mihashi, 1981. Fluorimetry study of N-(1-pyrenyl) iodoacetamide-labeled F-actin. (*Eur. Jo. Biochem*, **114**, 33-38, see Figure 2).

Figure 2. Polymerization of actin as measured by pyrene actin fluorescence.



Actin polymerization follows three phases, similar to microtubule assembly; these are nucleation, growth and steady state as depicted in Figure 2. The extent of polymerization is indicated by the steady state level of fluorescence, an upper limit can be measured by adding phalloidin (an actin stabilizing compound) which pushes the CC down to <0.01 mg/ml. This assay is greater than 95% accurate and the sample is not disturbed during the assay. Further details of setting up this assay are supplied with pyrene muscle actin (Cat. # AP05).

Actin Monomer Assay:

DNase inhibition assays are based on the high affinity interaction between G-actin and DNase 1 which results in inhibition of DNase activity (Blikstad et al. 1978. Selective assay for monomeric and filamentous actin in cell extracts, using inhibition of deoxyribonuclease 1. *Cell*, **15**, 935-943). The value of this assay is that it can distinguish between G- and F-actin. In this regard, it has been used to selectively assay the amounts of monomeric and filamentous actin in cell extracts. This assay has also been successfully adapted to study actin binding proteins that generate G- actin from actin filaments; an example of this is gelsolin protein.

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