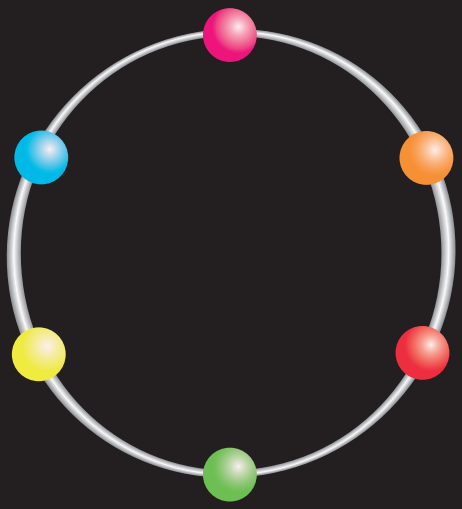
ACTIN-TOOLKITS

Actin-Based Bioassays for
Functional & Structural Ligand Analysis



Analytical Biochemistry

Molecular Cell Biology

Proteomics

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Content

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Kit Content

Actin-Toolkit G-Actin Binding (Cat.# 8020-01 / Cat.# 8200-01)

ActinBeads (50% slurry) [1]	4x250µl
Beads (control)	0.1ml
Profilin	1x10µg
MonoMix [1]	4x50ml
Needles & Syringes	1x
Handbook	1x

[1] Buffer: 5mM Tris pH 8.2, 0.1mM $CaCl_2$, 0.4mM ATP, 0.5mM DTT

Shipping, Storage and Handling Conditions

Actin-Toolkits are shipped at ambient temperature. Proteins and ATP-containing buffers are stable for at least 3 months when frozen at –20°C, and for at least 6 months when frozen at –70°C upon arrival. Avoid repeated freeze/thaw of ATP-containing components and of proteins. Solubilized proteins and ATP-containing solutions must be kept on ice and used as described in the Protocol Section.

Product Warranty

Hypermol guarantees quality and product performance as described in this handbook only, when products are frozen upon arrival as mentioned above. We do not take any guarantee for uses of our products other than described here. This product is designed for *in vitro* use only and not for use in therapeutic or diagnostic processes.

Should any product fail to perform as guaranteed due to reasons other than misuse or should not meet your expectations, please first contact our TechnicalService (techserv@hypermol.com or ++495219876230) within 5 working days and then return the product to Hypermol as advised. We reserve the right to test the performance of returned products in order to suggest replacement free of charge or refund of the purchase price. The buyer obtains a copy of our “Terms and Conditions of Sale” before ordering, and agrees to this by ordering.

Preface

Identification of an actin-binding protein is a major step forward in research. Today about two hundred proteins are known to either possess direct or cryptic binding sites for actin.

- Actin-Toolkits were developed to safely guide experiments with actin of highest quality.
- The handbook provides background information and protocols for successful and error-free handling.
- Actin-Toolkit proteins are fully biologically active.
- Actin-Toolkits are user-friendly all-in-one applications.

Actin Toolkits are invaluable tools to analyse the biological activity of protein ligands, especially for recombinant proteins, fragments or mutants. Identification and mapping of actin-binding sites in full length proteins or fragments are examples for the use of these assays.

The assays of the "Actin-Toolkit G-Actin Binding" are performed with G-actin coupled to Agarose (ActinBeads), with the option to use profilin as a reference protein. Binding of ligands to G-actin is highly specific, and thus, a positive proof of this interaction strongly indicates that G-actin binding is a biological function of the ligand. The mixture of ActinBeads and ligand is spun at low centrifugal forces. Only ligands bound to G-actin will be co-precipitated under these conditions.

Introduction to Actin and the Actin Cytoskeleton

Actin is one of the most abundant proteins in eukaryotic cells. Comprising 5 to 10% of the total cellular protein, actin turned out to be a key protein of cellular architecture and thus a keeper of cellular functions.

Today more than two hundred proteins are known to possess one or more actin binding sites. Some proteins can readily bind to actin, while some have to undergo ligand induced conformational changes prior to actin binding. Several dozens of proteins directly modulate either the state or the conformation of F- or G-actin.

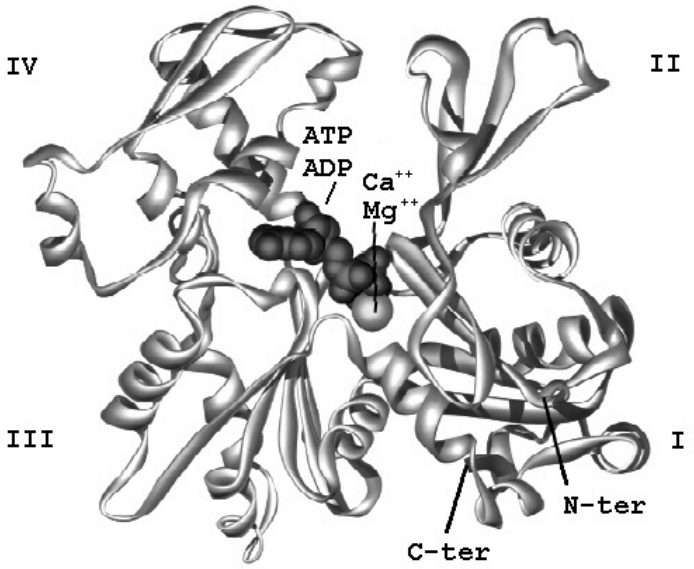
In addition to the filamentous actin incorporated into the cytoskeleton, cells have a rather variable pool of unpolymerized actin (30-50% of the total actin). Actin sequestering proteins like thymosin beta-4 take control of the G-actin pool, which would otherwise polymerize considering intracellular conditions.

Apart from these direct actions on actin - like polymerization, nucleation, capping, depolymerization, severing, bundling etc. - the function of many actin-binding proteins is to support the different states of the actin-cytoskeleton in order to follow the demands of cellular life (movement, cell division, signalling, etc.).

G-Actin

Monomeric actin (globular or G-actin) has a molecular mass of 42kD and is translated as a single polypeptide chain. Rabbit skeletal muscle actin consists of 375 amino acids. Due to its central function for the cell, it is not surprising, that the amino acid sequence of actin is highly conserved throughout evolution. The sequence of human skeletal muscle actin is practically identical to muscle actin in mouse, rat, rabbit, chicken, and beef. Structurally the actin molecule is divided into two domains, referred to as the *large domain* on the left side and the *small*

domain on the right side in the common representations. In between these domains nucleotides (ATP, ADP*P*i* and ADP) and divalent cations (Ca^{++}, Mg^{++}) are bound and can be exchanged. These two domains are subdivided further into two subdomains each. The small domain consists of subdomains I and II, and the large domain of subdomains III and IV. Both, N- and C-term of actin are located in subdomain I.



The four subdomains are stabilized essentially by salt bridges and hydrogen bonds to the ATP phosphate groups and to their associated Ca^{++}. In the actin filaments the polarity of the actin molecules becomes obvious. Actin filaments possess a fast growing plus-end and a slow growing minus-end, as described below for F-actin. Beside this biochemical distinction of either end of the molecule there is a phenotypical description which is often used - *barbed end* and *pointed end*. These terms describe the heavy meromyosin (HMM) decoration pattern of the actin filament reflecting the polar orientation of the actin molecules. The barbed end is identical with the plus-end and the pointed-end with the minus-end.

F-Actin

The most characteristic feature of single actin molecules is to polymerize into double helical filaments (filamentous or F-actin) of several micrometres' length *in vitro* and *in situ*. This polymerization process is readily initiated at physiological salt concentrations in the presence of ATP. The polymerization of actin can also be initiated by G-actin binding proteins with a nucleating function. While some cytoskeletal proteins specifically bind to F-actin, others bind exclusively to G-actin and some possess binding sites for F- and G-actin.

An increase in salt concentration of a low salt buffer (e.g. <5mM KCl) in the presence of 0.1mM ATP to a nearly intracellular salt concentration (~100mM KCl, 2mM $MgCl_2$) induces the polymerization of G-actin to F-actin. In biochemical terms, the polymerization of actin is a non-covalent association of actin

The polymerization of actin is divided into three steps. During the first step (nucleation), actin monomers form an unstable dimer, which becomes stabilized by the addition of another G-actin molecule to form a trimer. Actin-trimers strongly favour the elongation of the actin filament by further monomer addition. Both, temperature and concentration affect the polymerization kinetics.

G-actin is an ATPase that finally converts ATP in ADP*P*i*. Either ATP, ADP*P*i* or ADP is bound in the nucleotide binding pocket of the actin molecule. As the affinity of actin is higher for ATP than for ADP, the ADP is exchanged. Free actin monomers hydrolyse ATP at a very low rate, while upon polymerization the process of hydrolysis is significantly accelerated.

In the early stage of elongation the polarity of actin filaments becomes obvious. Actin polymerizes from both ends of the filament, but the rate of polymerization at either end is different. A fast growing end (plus-end or barbed end) and a slowly growing minus-end (pointed end) are distinguished. The polarity of actin filaments is the consequence of the head-to-tail association of the monomers. The critical concentration (C_c) for polymerization is ~0.1µM for the plus-end and ~0.6µM for the minus-end. As a consequence, addition of monomers to the plus-end occurs below the C_c of the minus-end and thus filaments are still growing. Below the C_c actin does not polymerize. At the C_c actin monomers and filaments are in steady state, and F-actin is preferentially ADP-actin.

The steady state is characterized by treadmilling, where monomers add to the plus-end while others dissociate from the minus-end at identical rates. This equilibrium is a dynamic exchange of monomers between the G-actin and the F-actin pool. This dynamic is modulated by ABPs (actin-binding proteins).

At physiological salt concentration in the presence of ATP, two factors should be noted influencing the actin polymerization in the absence of ABPs: the actin-concentration and the state of the bound nucleotide (ATP, ADP*P*i* or ADP).

Means for the choice of experimental conditions

✓ buffer control

Work at low salt concentrations (e.g. <5mM KCl). ATP-buffers must be kept on ice should be used within 5 days.

Protocol Section

Protocol 1: Reconstitution of ActinBeads

In this step, ActinBeads are reconstituted and activated with a G-actin buffer to remove cryoprotectants from freeze-drying. Since cryoprotectants can inhibit actin-ligand interactions, the activation procedure is a vital step for the success of the assays.

1. Prepare the G-actin buffer stock by adding 1ml of ultrapure H_2O to the

Protocol Section

4. After the third washing step, use the syringe with a 27 gauge needle. Remove the upper supernatant carefully. Place the needle at the very bottom of the tube and remove the supernatant completely. Let air bubbles appear in the syringe, to ensure complete removal of buffer from the ActinBeads.

5. After the washing buffer has been discarded, add 200µl of MonoMix to the ActinBeads and keep on ice.

ACTINBEADS
Reconstitution Scheme

Protocol Section

Storage of MonoMix

Keep reconstituted MonoMix on ice and use within 3-5 days. Freshly reconstituted MonoMix can be aliquoted (1.0ml) for flash freezing and stored at -70°C. Nevertheless, the quantity of MonoMix in this Toolkit should be sufficient to avoid freezing of aliquots.

Protocol 2: G-Actin Binding Assay

Protocol Section

be used in succession. Note: keep profilin on ice and use within 1-4 days.

Sample mix:

Sample (positive control)	ActinBeads	MonoMix/Ca^{++}	Profilin	Final volume
1. ActinBeads + profilin	50µl	400µl	50µl	500µl

Sample	ActinBeads	MonoMix	Ligand	Final volume
2. ActinBeads + your ligand	50µl	400µl	50µl	500µl

Optional:

8. Wash the pellet by adding 1ml of MonoMix to Sample 2 & 3, and 1ml of MonoMix/Ca^{++} to Sample 1. Vortex each sample for 3secs and spin at 6.000xg for 4min.

9. Remove the washing buffer carefully by pipette, without touching the ActinBeads. It is again not necessary to remove the washing buffer completely, since washing has to be repeated 3-4x with the appropriate buffers.

Troubleshooting Guide

No binding is observed in SDS-PAGE	- Ensure the salt concentration in MonoMix or an equivalent buffer is at or below 5mM. - If ligands have to be activated in order to bind to actin add the activating compounds to MonoMix used for washing the ActinBeads after incubation.

the ligand after gelfiltration or dialysis at least at 15.000xg, 1h.

- If the ligand is in high salt buffer you may concentrate the ligand first, and then add MonoMix to obtain the desired ligand concentration. A 5x concentration of ligand buffers containing ~50mM salt is sufficient to keep the final salt concentration of the sample at ~5mM.

Ordering Information

Related Toolkits

Product	Description	Cat. #	Size
Actin-Toolkit F-Actin Binding (α-skeletal muscle actin or α-cardiac actin)	Determination and quantification of F-actin binding or bundling by ligands in solution.	8010-01	4x1.0mg Rabbit skeletal muscle actin
Actin-Toolkit G-Actin Binding (α-skeletal muscle actin or α-cardiac actin)	Determination and quantification of ligands binding to ActinBeads* in solution.	8020-01	4x250µl Rabbit skeletal muscle actin
Actin-Toolkit Fluorometry (α-skeletal muscle actin)	Kinetic measurements of actin dynamics in solution based on pyrenyl fluorescence of actin.	8030-01	8x1.0mg Rabbit skeletal muscle actin
Actin-Toolkit TIRFM (α-skeletal muscle actin or α-cardiac actin)	Single molecule imaging of ligands interacting with ATTO-fluorescent G- or F-actin.	8093-01	4x100µg Rabbit skeletal muscle actin
Actin-Toolkit Crystallography (α-skeletal muscle actin)	Co-crystallization of a ligand with non-polymerizable, native G-actin.	8050-01	8x1.0mg Rabbit skeletal muscle actin
Actin-Toolkit SPR (α-skeletal muscle actin or α-cardiac actin)	A unique method to analyze ligand interactions with actin filaments by surface plasmon resonance.	8090-01	4x250µg Rabbit skeletal muscle actin
Actin-Toolkit ELISA (α-skeletal muscle actin or α-cardiac actin)	Molecular imaging analysis of ligands bound to monomeric actin, filaments or networks by TEM.	8070-01	4x0.5mg Rabbit skeletal muscle actin
Actin-Toolkit Fluorescence Microscopy (α-skeletal muscle actin or α-cardiac actin)	Identification of ligands bundling actin filaments or forming filament networks by using ATTO-fluorescent actin.	8080-01	4x100µg Rabbit skeletal muscle actin

* Rabbit skeletal muscle actin conjugated to NHS-activated Agarose

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