ACTIN-TOOLKITS

Actin-Based Bioassays for
Functional & Structural Ligand Analysis

Analytical Biochemistry

Molecular Cell Biology

Proteomics

Structural Biology

Molecular Medicine

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Content

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Handbook Actin-Toolkit: Fluorescence Microscopy

All products from HYPERMOL are made in Germany.

Kit Content

Actin-Toolkit Fluorescence Microscopy

ATTO-Actin	4x100µg
Actin (0.5mg)	1x0.5ml
PolyMix [1]	1x1.0ml (1ml, 10x stock)
MonoMix [2]	5x50ml (1ml, 50x stock)
$MgCl_2$ (1M)	1x0.5ml
Tubes (light protective)	10x
Handbook	1

[1] Content: 1M KCl, 20mM $MgCl_2$, 0.1M imidazole pH 7.4, 10mM ATP

[2] Content: 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 the quality and product performance 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 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 +49 521 987 623 0) 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 analyze 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 Actin-Toolkit Fluorescence Microscopy was developed to analyse and demonstrate the effect of ligands on actin polymerization, or ligand-induced formation of actin bundles and networks. In the assays ATTO-Actin – lysine-labeled actin by esterification - is mixed with the ligand to observe the effects on either G- or F-actin. In contrast to the staining with fluorochromated phalloidin, which can interfere with ligand binding sites on the actin molecule, no such observations were made with ATTO labeled actin.

Introduction to Actin and the Actin Cytoskeleton

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

The most characteristic feature of single actin molecules (globular or G-actin, Mr=42kD, single polypeptide chain) is to polymerize into double helical filaments (filamentous or F-actin) of several micrometers length *in vitro* and *in situ*. This polymerization process is readily initiated at physiological salt concentrations in the presence of ATP.

Today more than two hundred proteins are known to possess one or more actin binding sites. Some proteins can readily bind to actin, some have to undergo ligand induced conformational changes to bind to actin. 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 β4 take control of the G-actin pool, which would otherwise polymerize considering intracellular conditions.

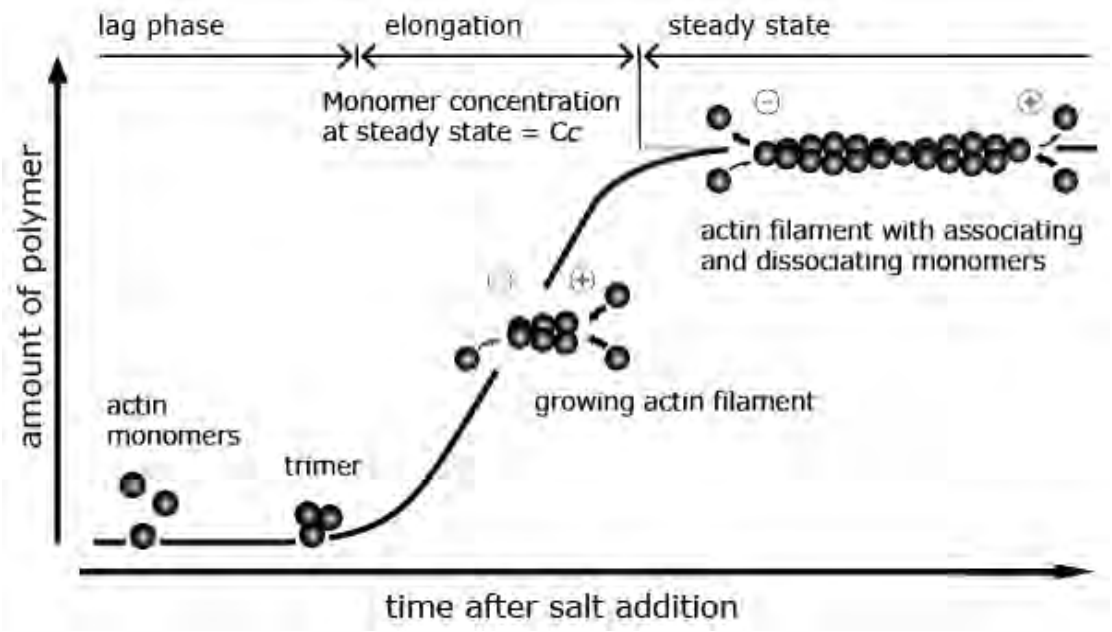
Apart from these direct actions on actin - like polymerization, nucleation, capping, depolymerization, severing, bundling etc. - the function of many actin-binding proteins is to support the different states of the actin-cytoskeleton in order to follow the demands of the cellular life (movement, cell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

Due to its central function for the cell, it is not surprising, that the amino acid sequence of actin is highly conserved throughout evolution. Human skeletal muscle actin is practically identical in sequence to muscle actin in mouse, rat, rabbit, chicken, and beef.

In Vitro Polymerization of Actin

An increase of the 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 monomers. Each actin monomer (G-actin) has two actin binding sites and is incorporated into the double helical filament via head-to-tail association. The result of this polymerization is a polar, right handed double-stranded actin helix (synonym. actin filament) with a pitch of 73.6nm per turn and a diameter of ~8nm.

The polymerization of actin can be 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 alter polymerization kinetics.



G-actin is an ATPase converting ATP in ADP*Pi. Either ATP 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. 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.

A typical phenomenon of the steady state is treadmilling, where monomers add to the plus-end while others dissociate from the minus-end. For actin alone, the 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.

Means for the choice of experimental conditions

✓ **buffer control**

Add salt and ATP. Polymerization buffers should contain about 0.1M KCl, an excess of 1mM ATP, and 2mM $MgCl_2$. ATP-buffers must be kept on ice should be used within 5 days.

✓ **time control**

Wait 15-30min until polymerization reaches steady state.

✓ **temperature control**

Work at room temperature. Polymerization kinetics is usually monitored at 25°C.

Protocol Section

Protocol 1. Preparation of ATTO G-Actin

For the preparation of ATTO G-actin or F-actin, the ATTO G-actin powder has to be reconstituted to make a stock solution.

Protocol Section

Protocol 2. Preparation of ATTO F-Actin

1. Prepare the ATTO G-actin stock solution according to Protocol 1.
2. Add 1.0ml of H_2O to the tube containing PolyMix, and vortex for ~30secs to fully dissolve the PolyMix (10x PolyMix stock solution). Aliquot PolyMix

Protocol Section

Protocol 3. Preparation of ATTO F-Actin for Bundles

In the following bundling assay, a positive control is prepared by adding a final concentration of 50mM $MgCl_2$ to the ATTO F-actin sample. Upon addition of high $MgCl_2$ concentrations F-actin forms bundles, the so called paracrystals, which are

Protocol Section

Control sample	*Actin*	*1x PolyMix*	*$MgCl_2$*	*Final volume*
4. ATTO F-actin + $MgCl_2$	6µl	13µl	1µl	20µl

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Protocol 4. Ligand-Induced Polymerization and Bundling

Proteins that bundle F-actin are often capable to polymerize G-actin into F-actin. Using a laser-type microscope, actin filaments and can easily be resolved, while also less equipped epifluorescence microscopes resolve bundle formation. In the following approach bundles should be observed under non-polymerizing

Protocol Section

5. Use 8µl of sample # 2., drop onto the slide, immediately place a clean coverslip on top, and use for microscopy. Do not allow the samples to dry.

   Note: for time dependent observations addition of the ligand should be performed at the microscope. Start the timer upon addition of each component to the tube, mix with a pipette and prepare the slide for microscopy. Observe

Troubleshooting Guide

Protein-concentration of ligand is low	Low ligand concentrations often require the addition of large volumes to the F-actin stock solution. An easy way to compensate low ligand concentrations is to prepare a ATTO G-actin stock of 2 or 3mg/ml, by adding 34µl or 22.5µl of H_2O to G-actin according to Protocol 1, then polymerize. After

Bundling is not observed	Perform the control experiments described in Protocol 3. In the presence of 50mM $MgCl_2$ bundles are formed and seen under the fluorescence microscope. Inspect the samples slowly to match the layer with bundles. For help, please contact our TechnicalService.

Some ligands polymerize G-actin, but do not bundle F-actin:

Toolkit Ordering Information

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

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