ACTIN-TOOLKITS

Actin-Based Bioassays for
Functional & Structural Ligand Analysis

Analytical Biochemistry

Molecular Cell Biology

Proteomics

Structural Biology

Molecular Medicine

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Contents

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Handbook Actin-Toolkit Fluorometry

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Kit Content

Actin-Toolkit Fluorometry
Cat.# 8030-01

Pyrene Actin (10%)	8x1mg
Gelsolin	20µg
ATP-Actin Buffer	1x1000ml (supplied as concentrate components)
PolyMix [1]	1x1ml (10x)
$CaCl_2$ (0.1M)	1x500µl
Black Tubes & Tags	8x
Handbook	1x

[1] 1M KCl, 0.02M $MgCl_2$, 0.01M ATP, 0.1M imidazole pH 7.4

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 ++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 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 Fluorometry is used to determine the polymerization and depolymerization reaction of actin by measuring the fluorescence of pyrenyl coupled actin. By this means it also allows to analyze the interaction of actin with ligands or other components effecting the polymerization or depolymerization. The fluorescence of Pyrene Actin is increased by a factor of 22 upon polymerization. The Actin-Toolkit Fluorometry is suitable to measure the effect on actin polymerization of purified ligands, cell or tissue extracts.

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.).

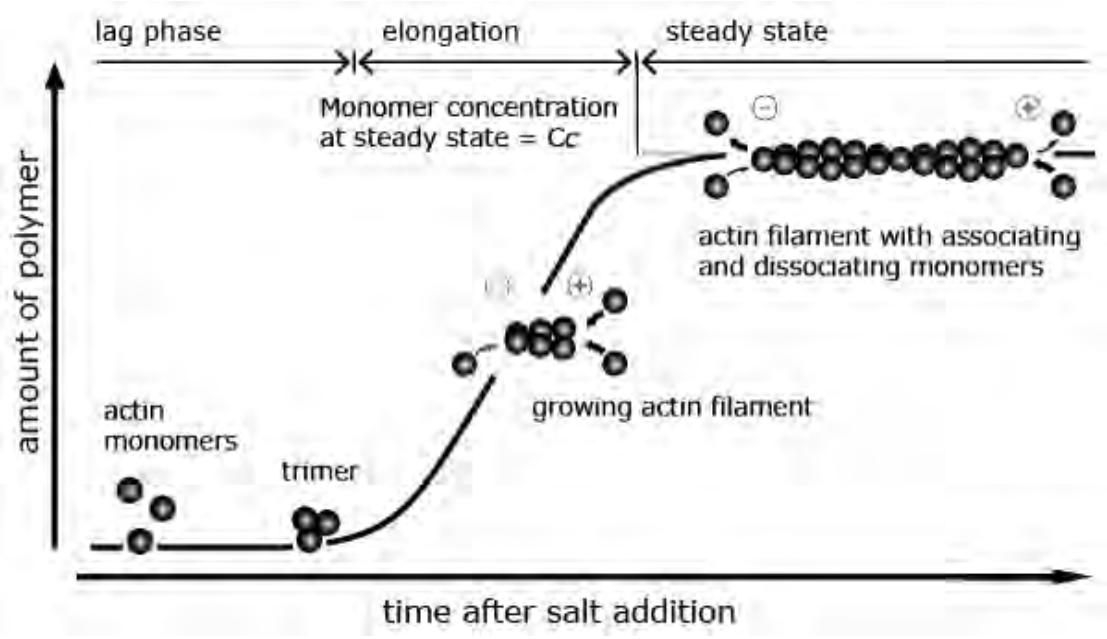
Due to its central function for the cell, it is not surprising, that the amino acid sequence of actin is highly conserved throughout evolution. Human skeletal muscle actin is identical in sequence to muscle actin in mouse, rat, rabbit, chicken, and beef.

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 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 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 hydrolyze 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 actin-binding proteins (short: ABPs).

At physiological salt concentration in the presence of ATP, two factors should be kept in mind that influence the actin polymerization in the absence of ABPs: the actin-concentration and the state of the bound nucleotide.

Fluorometry with Pyrene-Actin

Fluorometry allows the analysis of actin polymerization kinetics as well as the detection & quantification of ligands (e.g. ABPs), which affect the polymerization or depolymerization of actin. To measure actin polymerization by fluorometry, a chemically modified actin is used, which changes the fluorescence upon actin

Protocol Section

Protocol 1: Preparation and Handling of Actin Buffer

The actin buffer provided with this kit is a 10x concentrate Actin Buffer that has to be supplemented with the ATP included.

1.1. Preparation of G-Actin Buffer

Add 1ml Actin Buffer (10x) to the vial containing ATP and 1ml Actin Buffer (10x)

Protocol Section

Protocol 2: Reconstitution of Pyrene G-Actin

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

1. Calculate the final amount needed for the measurements and reconstitute the appropriate number of tubes. To obtain e.g. a 1.1mg/ml actin stock solution, add 0.9ml H_2O to each tube of Pyrene Actin, and vortex vigorously for 10secs.

Protocol Section

Protocol 3: Preparation of Pyrene F-Actin

This protocol is used for depolymerization assays.

1. Prepare a Pyrene Actin stock solution according to Protocol 1.
2. Add 1ml of H_2O to the tube containing PolyMix, and vortex vigorously until fully dissolved to obtain a 10x PolyMix stock solution.
3. To obtain a 1.0mg/ml Pyrene F-actin solution add 100µl of PolyMix (10x) to 0.9ml of the G-actin stock solution and mix immediately.

Protocol Section

Protocol 4: Reconstitution of Gelsolin

1. Prepare a 1mg/ml stock by adding 20µl H_2O to gelsolin and vortex slowly until fully dissolved.
2. Store gelsolin on ice. If possible, use within 3 days.

DO NOT FREEZE THE GELSOLIN SOLUTION.

Protocol Section

4. Start recording immediately after addition of KCl! Mix the sample with a pipette or spatula in parallel to the addition of KCl.

5. Measure at an excitation wavelength of 365nm and at an emission wavelength of 407nm. Run a time-based kinetics program with a recording time of 20-30min. Follow the polymerization until steady state is reached.

Sample Processing Scheme

Protocol Section

Protocol 6: Depolymerization Assay

Depolymerization assays can be very different, depending on the purpose of the assay. The following protocol describes an assay used to measure the depolymerization of actins alone, or in the presence of i.e. an actin ligand like capping proteins. In these assays F-actin is rapidly diluted below or close to the critical concentration. The dilution causes actin filaments to depolymerize.

Troubleshooting Guide

Bleaching	Pyrene is prone to bleaching upon intense illumination – for experiments longer than 30min chose a small excitation slit (e.g.: 1.5-3nm) and a larger emission slit (e.g.: 5-7nm). Store Pyrene Actin in dark tubes or wrap normal Eppendorf-

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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Actin-Toolkits and other products from Hypermol are exclusively and directly distributed by:

HYPERMOL
Schulenburger Landstr. 132A
D-30165 Hannover
Germany

mail: hypermol@hypermol.com
fon : ++49 52 19 87 62 26
fax : ++49 52 19 87 62 27

Mail Address: Hypermol | P.O. Box | 201025 D-33549 Bielefeld | Germany

D-33549 Bielefeld
Germany