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: ELISA

All products from HYPERMOL are made in Germany.

Kit Content

Actin-Toolkit ELISA

Cat. #: 8122-01 / Cat. #: 8122-02

4x 100µg Biotin-Actin (lyophilized powder)[1];
4x 50ml MonoMix (lyophilized);
1x 1ml PolyMix (10x, lyophilized)[2];
1x 50ml 10x Buffer F (liquid); 100mM ATP and 500mM DTT (lyophilized); [3]
1x 50mg BSA (fatty-acid free);
1x Handbook Actin-Toolkit ELISA;

[1] Contains (1x): 2mM Tris pH 8.2, 0.08mM $CaCl_2$, 0.4mM ATP, 0.02mM DTT;
[2] Contains (10x): 1M KCl, 100mM 20mM $MgCl_2$, 1mM ATP, 0.5mM DTT; imidazole pH 7.4;
[3] Contains (10x): 1M KCl, 100mM 20mM $MgCl_2$, 0.2mM ATP, 1mM DTT; 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 use in laboratory research only and not for use human or veterinary diagnostic or therapeutic 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

Characterization of actin-binding proteins is a major approach 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.

The Actin-Toolkit ELISA is used to analyze ligand interactions with G-Actin or F-actin by enzyme linked immunosorbent assay. Purified proteins or other ligands, semi purified ligands or crude extracts can be used for the assays. Thus the Toolkit is suited for quantitative analyses or to determine actin-binding at various concentrations e.g. with crude extracts.

This handbook for the Actin-Toolkit ELISA guides through direct ELISA assays, where the ligand is coated to the surface of the plate. You will learn to utilize Biotin G-actin or Biotin F-actin to directly interact with the ligand for successful detection of ligand-actin complexes. To detect Biotin-Actin bound to the ligand, either anti-biotin antibodies or Streptavidin (HRP or alkaline Phosphatase conjugated) can be employed.

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.

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.

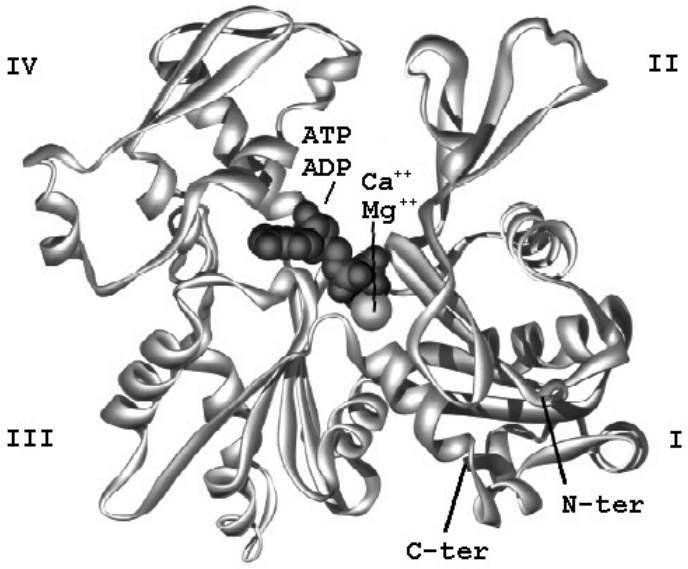
In addition to the filamentous actin incorporated into the cytoskeleton, cells have a rather variable pool of unpolymerized actin (30-50% of the total actin). Actin sequestering proteins like thymosin β4 take control of the G-actin pool, which would otherwise polymerize under intracellular conditions.

Beside of 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.

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 374 amino acids. Due to its central function for cellular life, it is not surprising, that the amino acid sequence of actin is highly conserved throughout evolution. The sequence of human skeletal muscle actin is even practically identical in sequence to muscle actin in mouse, rat, rabbit, and chicken. For the investigation of actin in cultured cells this unique degree of sequence conservation is advantageous, since interactions with skeletal muscle α-actin and cytoskeletal events can be investigated in any vertebrate and eukaryotic cell line.

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 between these domains nucleotides (ATP/ADP) and divalent cations (Ca^{++}, Mg^{++} et al.) are exchangeably bound. These two domains are subdivided further into two subdomains each. The small domain consists of subdomains 1 and 2, and the large domain of subdomains 3 and 4. Both, N- and C-term of actin are located in subdomain 1.



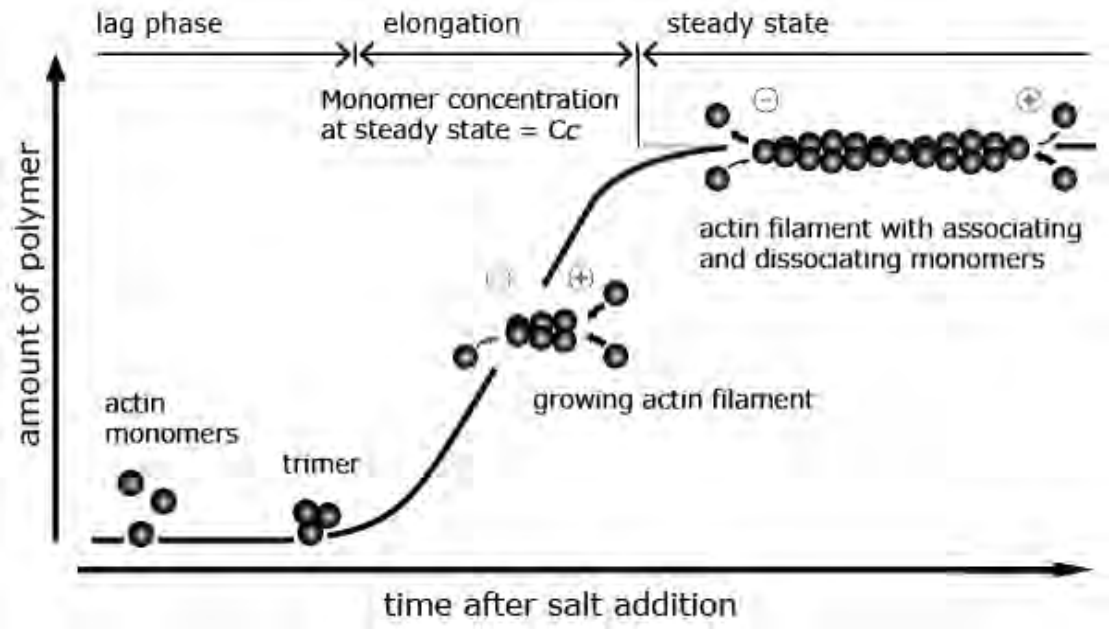
The four subdomains of actin are essentially stabilized by salt bridges and hydrogen bonds to the ATP phosphate groups and to their associated Ca^{++}. In the actin filaments the polarity of the actin molecules becomes obvious. Actin filaments possess a fast growing plus-end and a slow growing minus-end, as described below for F-actin. Beside this biochemical distinction of the 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

After microinjection of monomeric actin the polymerization of actin occurs as a result of the cytoplasmic buffer conditions. Single actin molecules 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. 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.

In vitro 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. Actin monomers associate to each other in a head-to-tail orientation when incorporated into the double helical filament.

The result of this polymerization is a polar, right handed double-stranded actin helix – the actin filament - with a pitch of 73.6nm per turn and a diameter of ~8nm.



The polymerization of actin can be described as a three step process. In 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.

The steady state is characterized by 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.

Protocol Section

Proteins and buffers from Hypermol are prepared with ultrapure water (Milli-Q™). We recommend using a similar quality to reconstitute these products.

Protocol 1: Reconstitution of Biotin-Actin

1. Prepare a 1.1mg/ml (26µM) Biotin-actin stock solution, by adding 90µl H_2O to the tube containing Biotin-actin, and vortex for 10secs.

Protocol Section

Protocol 3: Direct ELISA with Biotin G-actin

The ligand is coated to the surface of the plate and Biotin G-actin is applied directly for interaction with the coated ligand. To detect Biotin-Actin bound to the ligand, either anti-biotin antibodies or Streptavidin (HRP or alkaline Phosphatase conjugated) are employed.

Note: During all incubation steps the plate is covered with either a lid or foil!

Further components required (see Buffers & Solutions):

it does not interfere with the assay, even if the supplements are not required in the initial steps.

Always remove the buffer completely from the plate. Discard the buffer in the sink and place some overlaid sheets of tissue on a table. Knock out the plates - with the wells facing the tissue - on the layers of tissue, to drain the liquid completely. Do not allow the plates to fall dry! Immediately proceed with step 3.

3. Block the plate with 10mg/ml BSA in Buffer F at 37°C for 1h. Rows 1, 2, 3, 4, 6. Do not coat row 5 (positive control for Biotin Actin!).

Protocol Section

Protocol 4: Direct ELISA with Biotin F-actin

The ligand is coated to the surface of the plate and Biotin F-actin is applied to directly interact with the ligand. To detect Biotin-Actin bound to the ligand, either anti-biotin antibodies or Streptavidin (HRP or alkaline Phosphatase conjugated) are employed.

NOTE: During all incubation steps the plate is covered with either a lid or foil.

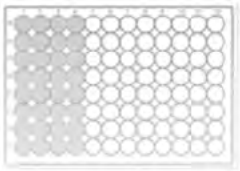
Further components required (see Buffers & Solutions):

6. Wash 3x with 2 50µl/well using Buffer F. Place the plate on 3D-rocker with slow motion. Do not use an ELISA-shaker, as vigorous shaking will disrupt the filaments. Remove the buffer from the plate completely.
7. Add 50µl of an anti-biotin antibody or Streptavidin-conjugate, diluted in Buffer F according to the manufacturer's instructions for 1h, 37°C.
8. Wash 3x with 250µl/well using Buffer F (see step 5.). Remove the buffer from the plate completely as described in step 2.
9. Add 50µl Donor-solution per well (e.g. OPD, ABTS – see Attachment) and proceed development according to your favourite ELISA protocol.

DIRECT ELISA SCHEME

Step 1.-2.
Coating the ligand

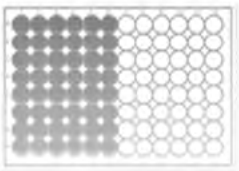
Row: 1,2,3,4
wash 3x



Step 3.-4.
Blocking with BSA

Step 7.-8.
Application of
Streptavidin-HRP

Row: 1-6
wash3x



Troubleshooting Guide

Low signal of ligand/G-actin complex	- Increase the ligand concentration for coating; - Vary the G-actin concentration (10nM, 30nM, 90nM) using a fixed quantity of ligand. - Stop the donor reaction after 8min instead of 4min.
No binding is observed with Biotin G-Actin	Actin filaments are dynamic and relatively fragile. It is strongly recommended to work at concentrations above 0.7mg/ml (best: 0.15mg/ml) to avoid depolymerization.

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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