

## In Vitro Characterization of Chain Depolymerization Activities of SUMO-Specific Proteases

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### Abstract

SUMO-specific proteases, known as Ulp in baker's yeast and SENPs in humans, have important roles in controlling the dynamics of SUMO-modified proteins. They display distinct modes of action and specificity, in that they may act on the SUMO precursor, mono-sumoylated, and/or polysumoylated proteins, and they might be specific for substrates with certain SUMO paralogs. SUMO chains may be dismantled either by *endo* or *exo* mechanisms. Biochemical characterization of a protease usually requires purification of the protein of interest. Developing a purification protocol, however, can be very difficult, and in some cases, isolation of a protease in its pure form may go along with a substantial loss of activity. To characterize the reaction mechanism of Ulp, we have developed an in vitro assay, which makes use of substrates endowed with artificial poly-SUMO chains of defined lengths, and *S. cerevisiae* Ulp enzymes in crude extract from *E. coli*. This fast and economic approach should be applicable to SUMO-specific proteases from other species as well.

**Key words** SUMO chain, Ulp/SENP, Protease, In vitro assay, Desumoylation

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### 1 Introduction

Proteomic studies have identified hundreds of cellular proteins that are covalently modified with SUMO [1]. For most of these SUMO substrates, the function of their sumoylation is not known, yet. The SUMO modification of a substrate alters certain parameters such as its localization, interactions with other polypeptides, or DNA binding, to name just a few [2, 3]. Which parameter is affected depends on the respective substrate. Also, like for ubiquitin, both mono- and poly-modifications are possible, and whether a substrate is decorated with only single units or with chains has a different outcome [4, 5]. The precise physiological role of SUMO chains in *Saccharomyces cerevisiae*, even though detected, is largely elusive, since yeast cells expressing a mutant version of SUMO that does not form chains do not exhibit any obvious phenotype except for a meiotic defect [6–8]. One function of SUMO chain formation is to provide a proteolytic control of sumoylated forms of a protein by

directing them into the ubiquitin/proteasome system [9]. If recognized and targeted by SUMO-targeted ubiquitin ligases (Uls), substrates carrying poly-SUMO chains are further modified by attachment of ubiquitin [5, 10–13]. This can then lead to proteasomal degradation [9]. SUMO molecules are synthesized as inactive precursors, which require processing to expose a diglycine motif at the C-terminus, thereby becoming conjugation competent [14]. Sumoylation is reversible. Deconjugation as well as precursor maturation are carried out by specialized cysteine proteases. Two classes of SUMO-specific cysteine proteases have been identified. The first one is the ubiquitin-like protein-specific protease (Ulp/SENp) group. The second one has only recently been found when the mammalian desumoylating isopeptidase (DeSI-1) protein was identified as a SUMO-specific protease, whose active cysteine residue resides in a papain-like fold that is structurally distinct from the Ulp fold [3, 15]. In *S. cerevisiae*, to date only two SUMO-specific proteases have been identified, namely Ulp1 and Ulp2 [16–18]. In humans, there are six UlpS termed sentrin-specific proteases (SENp-1, -2, -3, -5, -6, -7) catalyzing de-sumoylation [19–24]. One reason for an increased complexity of the mammalian Ulp equipment is the existence of multiple conjugated mammalian SUMO paralogs. SUMO1 shares only ~45% sequence identity with SUMO2 and SUMO3, while the latter two are nearly identical [3]. SUMO2/3 conjugation is induced by various forms of stress, and chains form efficiently. By contrast, SUMO1 modification dominates under non-stressed conditions, and formation of SUMO1 chains is inefficient [25–27]. SENp enzymes display distinct specificities or preferences for the different SUMO paralogs as well as for single SUMO moieties or SUMO chains [19–24].

In general, a biochemical characterization of the activity of a protease requires a purification strategy. Once the hurdle of expressing the protein in a soluble state has been overcome, it is usually separated from the pool of other components present in the expression host, commonly *Escherichia coli*. However, purification is often not only tedious but also, for some proteins, comes along with severe loss of activity. Here we describe an *in vitro* assay to characterize the chain depolymerization activity of SUMO-specific proteases that works with the enzymes in crude extracts from *E. coli*. The approach can also be used to test whether a certain form of a protein represents a sumoylated form of it. Instead of purified Ulp, this assay only requires the enzyme to be expressed actively in *E. coli*, hence avoiding purification, which can be costly, both in terms of time and resources.

We developed this assay for the yeast ubiquitin-like protein-specific protease 2 (Ulp2) [28]. Ulp2 is a 1034-amino acid protein with an important function in controlling cellular levels of SUMO chains [8, 17, 18]. As other studies showed, Ulp2 does not lend itself well to *in vitro* analysis, as it is poorly expressed in *E. coli*, coming out with just little activity [17]. As we were interested in the mechanism

by which Ulp2 dismantles SUMO chains, we needed an assay in which the cleavage reaction can be observed until completion. Marginal activity was not enough. In brief, we designed an artificial substrate with a chain consisting of five Smt3 moieties linked to enhanced GFP (eGFP) [28]. The most distal Smt3 moiety is a full-length Smt3, whereas all subsequent units were N-terminally truncated by 17 residues. This design was chosen to closely mimic the linkage pattern of native Smt3 chains, whose units are commonly linked via an isopeptide bond connecting the terminal glycine of one Smt3 molecule to one of several possible lysine residues (in most cases K11, K15, or K19) in a flexible N-terminal extension of the next Smt3 unit. It is nearly impossible to isolate native poly-SUMO chains of defined length from yeast. We verified the integrity of our strategy by performing the assay with a substrate linked to natural lysine-linked poly-SUMO chains generated in a reconstituted sumoylation system in *E. coli* [28, 29]. Therefore, our artificial substrate chains create a suitable model, and also allow for testing protease activity/affinity towards chains of defined length and composition [28].

We have chosen green fluorescent protein (GFP) as a mock substrate because of its stability conferred by the beta-barrel fold [30]. Additionally, its green color conveniently allows tracing the fusion protein throughout the purification process.

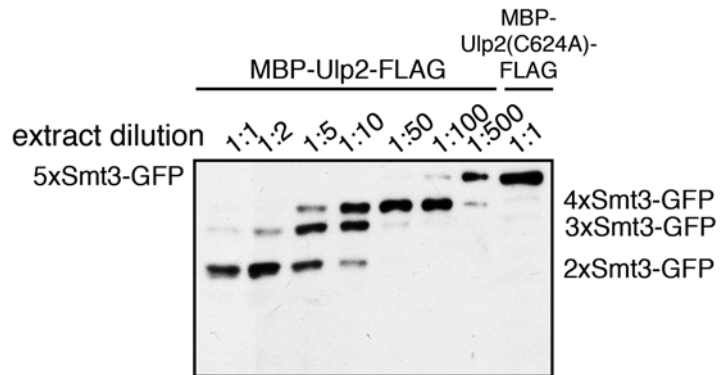
Here we describe the approach for Ulp2, but the assay has been successfully employed for analyzing Ulp1, as well [28]. Using this assay, we were able to show that Ulp1 acts on Smt3 chains by an *endo* mechanism, meaning that it stochastically cleaves any of the bonds between an Smt3 moiety and the polypeptide it is linked to, irrespective of whether it is another Smt3 moiety or any other polypeptide. Ulp2, by contrast, acts by an *exo* mechanism [28]. It disassembles Smt3 chains from their distal end by releasing single Smt3 moieties (Fig. 1). It requires a minimum of three Smt3 moieties to bind, and therefore stops when only two SUMO moieties are left on the substrate [28]. Using defined linear substrates, which form the basis for the method described here, and either enzyme dilutions or time courses of their action, allows to readily distinguish between the *endo* and *exo* modes of Ulp enzymes (Fig. 2).

If applied to other SUMO proteases with specificity for distinct SUMO orthologs or paralogs, the substrates should be chosen accordingly. We successfully cloned, expressed, and purified chains of SUMO1 and SUMO2 using the same procedure as described below for poly-Smt3 chains.

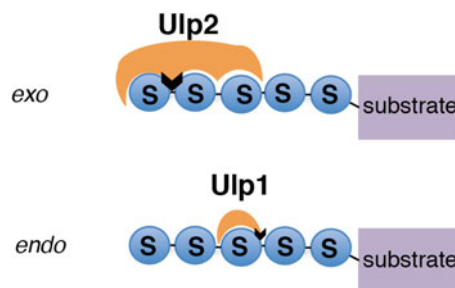
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## 2 Materials

Prepare all solutions using deionized ultrapure water. It is not necessary to filter any of the buffers prior to usage. Use sterile (autoclaved) LB medium and glucose solution. Pass additive stocks



**Fig. 1** Example of cleavage assay analysis. 5xSmt3-GFP substrates were incubated with *E. coli* lysates containing Ulp2 diluted in activity test buffer (“1:1” = undiluted lysate) for 2 h at 30 °C. As a control, a lysate was used that contained the inactive (inact.) variant of Ulp2(C624A). Reaction products were then analyzed by SDS-PAGE and anti-HA Western blotting. The full-length substrate is indicated on the *left-hand side* of the blot, and cleavage products are indicated on the *right*. “This research was originally published in the *Journal of Biological Chemistry*. J. Eckhoff and R.J. Dohmen. In vitro studies reveal a sequential mode of chain processing by the yeast SUMO (Small Ubiquitin-related Modifier)-specific protease Ulp2. 2015; 290:12268-12281. © the American Society for Biochemistry and Molecular Biology.” [28]



**Fig. 2** Schematic representation of *exo* and *endo* cleavage mechanisms exemplified for Ulp2 and Ulp1. Ulp2 binds to three Smt3 units and works by cleaving single Smt3 units off the end of a chain (*exo*). Ulp1 requires only a single Smt3 molecule to bind and can cleave randomly after any Smt3 moiety inside the chain (*endo*). S = Smt3. “This research was originally published in the *Journal of Biological Chemistry*. J. Eckhoff and R.J. Dohmen. In vitro studies reveal a sequential mode of chain processing by the yeast SUMO (Small Ubiquitin-related Modifier)-specific protease Ulp2. 2015; 290:12268-12281. © the American Society for Biochemistry and Molecular Biology.” [28]

(except for chloramphenicol) through sterile filter before usage. Make sure to subject any waste that had contact with bacteria to autoclaving before disposal.

## **2.1 Expression of Ulp2 and Substrate Chains**

1. *E. coli* BL21-CodonPlus cells.
2. LB agar plates: 10 g/l Tryptone, 5 g/l yeast extract, 10 g/l NaCl, 2 % agar.
3. 500 ml Erlenmeyer flasks, sterilized.
4. LB medium: 10 g/l Tryptone, 5 g/l yeast extract, 10 g/l NaCl.
5. 50 % Glucose stock solution, sterilized.
6. 30 mg/ml Chloramphenicol stock solution (in EtOH).
7. 100 mg/ml Ampicillin stock solution, sterilized.
8. 1 M Isopropyl- $\beta$ -d-thiogalactopyranoside (IPTG) stock solution, sterilized.
9. An autoclave.
10. A temperature-controlled shaker/incubator that can accommodate 15-ml glass tubes and 500 ml flasks and can be set to either 20, 30, or 37 °C.
11. A spectrophotometer and cuvette to measure absorbance at 600 nm.
12. A refrigerated centrifuge with rotor fitting 50-ml conical tubes capable of spinning at 2800  $\times g$ .

## **2.2 Substrate Chain Purification**

1. A refrigerated room set at 4–6 °C.
2. Cell lysis buffer (substrate chains): 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 % glycerol, 1 mM MgCl<sub>2</sub>, 1 $\times$  protease inhibitor cocktail (cOmplete, EDTA-free; Roche), 2.5 mg/ml lysozyme, 0.8 mg/ml DNaseI, 1 mM PMSF.
3. 1.5 and 2 ml reaction tubes.
4. Glass beads with a diameter of 0.1–0.11 mm.
5. Vortex.
6. A high-speed centrifuge for 1.5- or 2-ml reaction tubes (e.g., Eppendorf refrigerated centrifuge).
7. Ni purification buffer: 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10 % glycerol, 1 mM MgCl<sub>2</sub>.
8. 1 M Imidazole stock solution.
9. Ni Sepharose™ High Performance (GE Healthcare).
10. Disposable drop column.
11. Rotating device (wheel or roller mixer).
12. Buffer exchange system (e.g., PD-10 column (GE Healthcare), spin concentrator, or dialysis equipment).
13. FLAG purification buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 % glycerol, 1 mM MgCl<sub>2</sub>.
14. Anti-FLAG M2 resin.

15. FLAG peptide.
16. PCR tubes.
17. Liquid nitrogen.

### **2.3 Preparation of Cell Extract Containing Ulp2**

1. A refrigerated room set at 4–6 °C.
2. Extract buffer: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM DTT, 4 mM MgCl<sub>2</sub>, 1 mM EDTA, 2.5 mg/ml Lysozyme, 1× protease inhibitor cocktail (e.g., complete, EDTA-free; Roche), 0.8 mg/ml DNaseI, 1 mM PMSF.
3. 1.5- and 2-ml reaction tubes.
4. Glass beads with a diameter of 0.1–0.11 mm.
5. Vortex.
6. A high-speed centrifuge for 1.5- or 2-ml reaction tubes (e.g., Eppendorf refrigerated centrifuge).

### **2.4 Ulp2 Activity Assay**

1. Protein LoBind tubes (Eppendorf).
2. Activity test buffer (ATB): 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10 mM DTT, 1 mM EDTA.
3. An incubator set at 30 °C.
4. 6× Laemmli buffer: 380 mM Tris–HCl, pH 6.8, 60% glycerol, 12% SDS, 0.015% bromophenol blue.
5. PCR tubes (preferably strips).
6. A thermocycler.

### **2.5 Ulp2 Activity Assay**

1. SDS-PAGE equipment.
2. Blotting paper.
3. Nitrocellulose membrane.
4. Western blot device.
5. PBS: 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 137 mM NaCl, pH 7.4.
6. Nonfat milk powder.
7. PBS-T: PBS + 0.1% Tween 20.
8. A shaking device to incubate blots on.
9. 3F10 anti-HA antibody.
10. Horseradish peroxidase-coupled goat anti-rat IgG.
11. An ECL detection system.

## 3 Methods

### 3.1 Cloning

The gene encoding the enzyme of interest is expressed as a maltose-binding protein (MBP) fusion construct from the “tac” promoter. This is achieved by cloning the target gene into the multiple cloning site of the commercially available pMALc2x vector (NEB) using restriction enzymes. If desired, a TEV protease recognition site (ENLYFQG) can be included in the primer design. Even though pMALc2x contains a factor X cleavage site to remove the MBP-tag, one might want to opt for the more specific TEV protease if considering cleaving off the N-terminal appendix at some point. In addition, a C-terminal tag can be introduced via a suitable reversed primer. In our hands, FLAG tag proved to be a good choice for this purpose. Our expression plasmid for MBP-ULP2 is called pJE12 and will be referred to like that in the following description.

The ORF for the substrate chain is generated in a multistep procedure: First, a fusion of sequences encoding ubiquitin (UBI) and an enhanced version of GFP (EGFP) is prepared via overlap extension PCR, introducing an NsiI restriction site in between the two genes. The resulting fragment, containing the sequencing encoding an EcoRI site followed by a FLAG tag plus a subsequent SacI site at the N-terminus, and an HA tag followed by a KpnI site at the C-terminus, is then ligated into a derivative of pET11a. This vector adds a C-terminal 6xHis tag to the ORF. Since SUMO contains an EcoRI restriction site, it is necessary to first introduce UBI into the construct, and then substitute it with SMT3 via digestion by SacI/NsiI. To this end, generate a fragment encoding SMT3 with a N-terminal SacI site and a C-terminal NsiI site.

To generate a clone encoding a SUMO chain, make use of the NsiI site: generate amplicons encoding N-terminally truncated Smt3 and bearing a 5' NsiI site as well as a 3' PstI site. These can now be introduced into the construct one by one, utilizing the compatibility of NsiI- and PstI-generated sticky ends. The final construct is expressed from the T7 promoter. For simplicity, our substrate expression plasmid (pFLAG-Smt3-4x $\Delta_{17}$ Smt3-eGFP-HA-6xHis) will be referred to as pJE10 in the protocol.

### 3.2 Protein Overexpression in *E. coli*

Apart from the substrate and extract containing Ulp2, a control extract lacking Ulp activity is needed for the desumoylation assay. To this end, transform BL21-CodonPlus cells with the MBP-Ulp2(C624A)-FLAG fusion protein expression vector and proceed as described below for the active variant. Ulp/SENp enzymes are cysteine proteases, and the C624A mutation hits the active cysteine of Ulp2, thereby rendering the enzyme inactive.

1. Transform competent *E. coli* BL21-CodonPlus cells with the MBP-Ulp2-FLAG fusion protein expression vector (pJE12). Do the same with the vector containing the ORF of the substrate chain 5xSmt3-GFP (pJE10). Select transformants on LB

- agar plates supplemented with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol. Incubate the plates for ~16 h at 37 °C.
2. Pick cells from one colony of the pJE12-transformation, and transfer them to 5 ml LB medium containing 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, and 1% glucose. Pick cells from one colony of the pJE10-transformation, and transfer them to 5 ml LB medium containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol. Grow both cultures overnight shaking at 180 rpm and 37 °C.
  3. The next morning, inoculate 50 ml of the same media as used for the overnight cultures in 500-ml Erlenmeyer flasks with 0.5 ml of the saturated overnight cultures.
  4. Grow the cells at 37 °C with shaking to mid-log phase ( $OD_{600} \sim 0.5-0.6$ ).
  5. Briefly cool down the cultures by placing flasks on ice.
  6. Add IPTG:
    - (a) For Ulp2 expression to a final concentration of 1 mM.
    - (b) For substrate expression to a final concentration of 0.5 mM.
  7. Allow expression for:
    - (a) ~20 h while shaking at 160–200 rpm at 20 °C.
    - (b) 3.5 h while shaking at 160–200 rpm at 30 °C.
  8. Measure the  $OD_{600}$  of the cultures.
  9. Transfer the entire volume of each culture to a 50-ml conical centrifuge tube and pellet the cells by centrifugation ( $2800 \times g$ ) for 10 min at 4 °C.
  10. Discard the supernatant and resuspend each pellet in 25 ml ice-cold ddH<sub>2</sub>O.
  11. Pellet cells again ( $2800 \times g$ , 10 min, 4 °C).
  12. Discard the supernatant and store pellets at –20 °C for one night or longer (until further processing). This freezing will aid breaking the cells in subsequent cell lysis steps.

### 3.3 Substrate Chain Purification

Perform all steps at 4 °C. This also applies to centrifugation steps. Ideally, work in a 4 °C room. Unless stated differently, all steps can be regarded to have the addition “at 4 °C.” Even though it is possible to pause the procedure by snap-freezing the eluate of the first purification and storing it at –80 °C, the quality of the final product is higher if the purification is done in one go.

1. Thaw a cell pellet from *E. coli* cells expressing FLAG-Smt3-4xΔ<sub>17</sub>Smt3-eGFP-HA-6xHis (pJD12) on ice.
2. Add 15 µl lysis buffer per 1  $OD_{600}$  of cells.



3. Resuspend cells by gentle manual shaking. Avoid protein degradation by foaming.
4. Incubate cell suspension for 5 min on the bench, and then for another 5 min on ice.
5. To each 1 ml of suspension, add 500  $\mu$ l glass beads.
6. Subject the lysate to vigorous vortexing for 1 min, followed by 1-min incubation on ice. Repeat three times.
7. Pellet the insoluble cell debris (and proteins) by centrifuging the lysate at  $30,000\times g$  for 20 min.
8. Transfer the supernatant to a 15-ml conical tube. Make sure not to transfer any pelleted material.
9. Add NaCl to a final concentration of 500 mM and imidazole to a final concentration of 20 mM.
10. Dilute 1:3 in Ni purification buffer containing 20 mM imidazole.
11. Add  $\sim 300$   $\mu$ l Ni sepharose beads.
12. Incubate on a rotating device for 30 min.
13. Transfer resin to drop column.
14. Wash with 15 ml Ni purification buffer containing 20 mM imidazole.
15. Transfer resin to 1.5-ml reaction tube.
16. Add 500  $\mu$ l Ni purification buffer containing 200 mM imidazole to the resin.
17. Incubate on a wheel for 5 min.
18. Sediment the resin beads by centrifuging at  $100\times g$  for 1 min.
19. Carefully transfer the supernatant to a fresh tube. Make sure not to transfer any resin! Rather do not take the entire volume to avoid accidentally transferring beads along with the supernatant. It helps to let the resin settle for a few minutes after centrifugation.
20. Repeat **steps 16–19** four times. Pool all eluates.
21. Exchange the buffer to FLAG purification buffer using your favorite procedure. Several methods are possible: PD-10 columns, dialysis, repeated dilution and concentration in spin concentrators. In the latter case, make sure not to reduce the volume of the sample. The protein might precipitate.
22. Add  $\sim 50$   $\mu$ l equilibrated anti-FLAG M2 resin to the protein solution.
23. Allow specific binding by incubation on a wheel for 1.5 h.
24. Sediment the beads by centrifugation ( $100\times g$ , 1 min).
25. Discard the supernatant.

26. Add 1 ml FLAG purification buffer.
27. Sediment the beads by centrifugation ( $100 \times g$ , 1 min).
28. Discard the supernatant.
29. Repeat **steps 26–28** five times. In the process, transfer the resin to a fresh tube twice (e.g., after the second and the fourth washing steps). This helps to get rid of unbound proteins.
30. Add 400  $\mu$ l FLAG purification buffer containing 150  $\mu$ g/ml FLAG peptide.
31. Incubate on a wheel for 3 h.
32. Sediment the resin beads by centrifugation ( $100 \times g$ , 1 min).
33. Carefully transfer the supernatant to a fresh tube. Again: Make sure not to transfer any resin.
34. Aliquot the eluate into 200- $\mu$ l tubes (PCR tubes) (*see Notes 1 and 2*).
35. Snap-freeze.
36. Store at  $-80$  °C until usage.

### **3.4 Preparation of Cell Extract Containing Ulp2**

Perform all steps at 4 °C. This also applies to centrifugation steps. Ideally, work in a 4 °C room. Unless stated differently, all steps can be regarded to have the addition “at 4 °C.” To obtain control extract, use a pellet of a MBP-Ulp2(C624A)-FLAG expression culture and follow the procedure described below.

1. Thaw a cell pellet of *E. coli* cells expressing MBP-Ulp2-FLAG (pJD12) on ice.
2. Add 15  $\mu$ l extract buffer per 1 OD<sub>600</sub> of cells.
3. Resuspend the cells by very gentle manual shaking. Make sure to touch the tube as little as possible to avoid warming. Avoid foaming.
4. Incubate cell suspension for 5 min on the bench, and then for another 5 min on ice.
5. Subject the lysate to vigorous vortexing for 1 min followed by 1-min incubation on ice. Repeat three times.
6. Pellet the insoluble cell debris (and proteins) by centrifuging the lysate at  $30,000 \times g$  for 20 min.
7. Transfer the supernatant to a fresh reaction tube. Make sure not to carry over any pellet material. You will probably not need much of the extract, so rather take only ~50% of the total volume than to risk disturbing the pellet.
8. Keep the extract on ice until usage.

### **3.5 Ulp2 Activity Assay**

It is best to prepare the activity assay in a 4 °C room. If no such facility is available, do it on ice.

1. Prepare one protein LoBind tube for each test you want to do. Usually, it is sufficient to test three different concentrations of Ulp2 extract: undiluted extract, a 1:10 dilution, and a 1:100 dilution. Add one control sample containing extract containing inactive Ulp enzyme for each substrate you test.
2. Prepare serial dilutions of Ulp2 extract in activity test buffer.
3. Thaw one aliquot of the substrate preparation.
4. Prepare a suitable dilution of the substrate solution (*see Note 3*).
5. Add 12  $\mu\text{l}$  ATB to each tube.
6. Add 2  $\mu\text{l}$  of the substrate dilution to each tube.
7. Add 6  $\mu\text{l}$  of the extract or the appropriate extract dilution to each tube.
8. Mix by pipetting up and down (*see Note 4*).
9. Incubate at 30 °C for 2 h.
10. Spin down the reactions at 30,000 $\times g$  for 5 min at 4 °C.
11. For each reaction, transfer 17  $\mu\text{l}$  to a fresh tube (*see Note 5*). Discard the rest.
12. Add 3  $\mu\text{l}$  6 $\times$  Laemmli buffer. Mix.
13. Boil for 2–5 min at 100 °C.
14. If not directly subjected to analysis, samples can be stored at –20 °C.

### 3.6 Assay Analysis

1. Boil the samples briefly.
2. Spin samples down at maximum speed for 1 min.
3. Load the entire volume of each sample on a 10% SDS polyacrylamide gel.
4. Separate the samples by SDS-PAGE.
5. Transfer the proteins to a nitrocellulose membrane using your favorite system (*see Note 6*).
6. Block the membrane by incubation in 5% nonfat milk powder in PBS with gentle shaking at room temperature for at least 1 h.
7. Incubate the blot in a 1:5000 dilution of rat anti-HA antibody in PBS-T containing 5% nonfat milk powder with gentle shaking overnight at 4 °C.
8. Wash the blot by incubating it 3 $\times$  in an excess amount of PBS-T with gentle shaking for 10 min at room temperature.
9. Incubate the blot in a 1:5000 dilution of horseradish peroxidase-coupled goat anti-rat IgG in PBS-T containing 5% nonfat milk powder with gentle shaking for 50 min at room temperature.
10. Repeat **step 8**.
11. Detect the signal by ECL (*see Note 7*).

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## 4 Notes

1. It is not necessary to get rid of the FLAG peptide in the eluate. It does not interfere with the assay.
2. We find it convenient to aliquot the substrate solution into PCR tubes. The small volume allows fast thawing (several seconds on ice). The substrate chains are not suitable for storage at 4 °C once they have been thawed.
3. How much substrate you want to apply in each assay depends on the sensitivity of your detection system. You want to have a clear but not too strong signal upon anti-HA Western blotting (see Fig. 1). We recommend to estimate the appropriate dilution by subjecting several dilutions of the final protein solution obtained from FLAG tag purification to SDS-PAGE followed by Western blot detection of HA tag.
4. It is sufficient to pipette up and down 2–3 times after adding the 6 µl of extract. The reaction is very slow when the tubes are kept on ice, so you should work fast but there is no rush.
5. It is most economic to use PCR tubes for this, and do the subsequent boiling step in a thermocycler. Additionally, we find it convenient to first put the Laemmli loading buffer into the tubes and then add the reaction solution once it has been spun down.
6. We routinely apply semidry blotting, but any other blotting system should work, as well.
7. If the signal is too weak, incubate the blot for another night in primary antibody and develop it again the next day. In our hands, this has worked very well on many occasions.

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