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UbiQ protocol P005 _ Quantitative analysis of DUB activity in vitro

description:

- purification of USPs
- determination of a well-behaved USP catalytic domain construct
- general purification protocol for USPs
- USP activity on a minimal substrate
- measuring deubiquitination of a natural target 301
- regulation of USP activity
- quantitative USP interaction analysis

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UbiQ Bio BV
Chamber of Commerce 50023438
VAT NL822502136B01

Science Park 408
1098 XH Amsterdam
The Netherlands

t +31 20 303 1970
e info@ubiqbio.com
i www.ubiqbio.com

Rabobank
IBAN NL86RABO0150658907
BIC/SWIFT RABONL2U

CHAPTER THIRTEEN

Quantitative analysis of USP activity in vitro

Shreya Dharadhar, Robbert Q. Kim[†], Michael Uckelmann[‡], Titia K. Sixma*

Division of Biochemistry and Oncode Institute, Netherlands Cancer Institute, Amsterdam, The Netherlands
*Corresponding author: e-mail address: t.sixma@nki.nl

Contents

Abstract

Ubiquitin-specific proteases (USPs) are an important class of deubiquitinating enzymes (DUBs) that carry out critical roles in cellular physiology and are regulated at multiple levels. Quantitative characterization of USP activity is crucial for mechanistic understanding of USP function and regulation. This requires kinetic analysis using in vitro activity assays on minimal and natural substrates with purified proteins. In this chapter we give advice for efficient design of USP constructs and their optimal expression, followed by a series of purification strategies. We then present protocols for studying USP activity quantitatively on minimal and more natural substrates, and we discuss how to include possible regulatory elements such as internal USP domains or external interacting proteins. Lastly, we examine different binding assays for studying USP interactions and discuss how these can be included in full kinetic analyses.

[†] Current address: Department of Cell and Chemical Biology and Oncode Institute, Leiden University Medical Center, Leiden, The Netherlands.
[‡] Current address: Department of Biochemistry and Molecular Biology, Monash Biomedicine Discovery Institute, Monash University, Melbourne, Victoria, Australia.

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1. Introduction

Ubiquitination of proteins has become one of the most widely studied aspects of cellular physiology in eukaryotes. This is due to its crucial role in regulating a plethora of cellular pathways ranging from DNA damage responses to cell migration. The (de)ubiquitinating enzymes orchestrating the ubiquitination cycle were first described in the 1980's (Hershko, Heller, Elias, & Ciechanover, 1983; Pickart & Rose, 1985), and since then considerable progress has been made in understanding their role as essential components of many, if not all cellular pathways. Deubiquitinating enzymes (DUBs) are proteases that cleave ubiquitin from their target substrates, and sometimes can also remove closely related ubiquitin-like proteins such as NEDD8. They play a role in the formation of mature ubiquitin monomers by processing C-terminally extended ubiquitin precursors, and they maintain a free ubiquitin pool by recycling unanchored polyubiquitin chains into free ubiquitin. Apart from being important for ubiquitin maintenance, DUBs also cleave ubiquitin marks from their target proteins, which counteracts the activities of ubiquitin-ligating enzymes. This leads to distinct roles for DUBs depending on the type of ubiquitin modification and the nature of substrate being cleaved. Cleavage of Lys-48 linked ubiquitin chains prevents proteasome-mediated degradation of the target proteins, while cleaving "nondegradative" ubiquitin linkages turns off the signal created by particular ubiquitin-substrate attachments. Finally, DUBs can also partially trim ubiquitin chains, which leads to modification of ubiquitin chain architecture and changes in downstream signaling (Reyes-Turcu, Ventii, & Wilkinson, 2009).

There are approximately 100 DUBs encoded in the human genome, which are subdivided into smaller families based on their sequences and catalytic mechanisms (Leznicki & Kulathu, 2017). Seven families of DUBs are characterized by structurally distinct catalytic folds, six of which are cysteine proteases and one a metalloprotease, the so-called JAMM domain (Hermanns et al., 2018; Hewings et al., 2018; Kwasna et al., 2018; Mevissen & Komander, 2017). The ubiquitin-specific proteases (USPs) form the largest family of DUBs, and in this chapter we focus on this group.

USPs contain a conserved catalytic core which has a papain-like fold that is comprised of approximately 350 residues. This catalytic domain adopts a conformation which resembles an extended open hand, subdivided into fingers, palm, and thumb subdomains (Hu et al., 2002). USPs have a catalytic

triad composed of cysteine, histidine, and aspartate/asparagine residues that come from regions remote in the primary sequence. Many USPs have insertions of various sizes in their catalytic domains (Ye, Scheel, Hofmann, & Komander, 2009), as well as substantial N- and C-terminal extensions. These additional regions can play major roles in the catalysis and regulation of the USPs. A well-studied example is USP7 in which an N-terminal TRAF domain is crucial for interaction with its substrates (Holowaty, Sheng, Nguyen, Arrowsmith, & Frappier, 2003; Sheng et al., 2006), while the C-terminal region is important for regulating its catalytic activity as well as substrate binding (Cheng et al., 2015; Faesen et al., 2011; Fernández-Montalván et al., 2007; Pfoh et al., 2015).

The physiological functions of USPs are slowly emerging. Many USPs are involved in pathways that are dysregulated in human diseases such as cancer and neurodegenerative diseases. (Clague, Coulson, & Urbe, 2012; Heideker & Wertz, 2015). For example, USP1, USP3, USP11, USP16, USP28, USP47, USP48 are involved in DNA damage repair pathways; USP2, USP4, USP15, USP34 participate in Wnt signaling; and USP8, USP15, USP30, USP32 are implicated in the autophagy of mitochondria (mitophagy) (Bingol et al., 2014; Cornelissen et al., 2014; Durcan et al., 2014; Fraile, Quesada, Rodríguez, Freije, & López-Otín, 2012; Wang et al., 2015). How most USPs select their respective substrates is unclear, which makes it hard to infer any specific function from their sequence or structure; this is further complicated by their tendency to function on multiple substrates. A quantitative analysis of USP activity on different substrates (especially natural substrates) can yield deeper insights into how specific USP targets are selected.

Since USPs are essential biological regulators, they themselves have to be tightly regulated to ensure proper functioning. Different modes of regulation exist, affecting catalytic activity, subcellular localization, or cellular abundance of these enzymes. Regulation can be orchestrated by internal factors (domains within the USPs), external factors (binding partners, substrate, posttranslational modifications) as well as transcriptional control; many different modes of regulation may contribute to activity of a single USP (Leznicki & Kulathu, 2017; Mevissen & Komander, 2017; Sahtoe & Sixma, 2015). Continuing with the example of USP7, substrate binding and catalytic activity are regulated by its internal domains but it can be further modulated by an external protein called GMPS that enhances its activity and affects its subcellular localization (Faesen et al., 2011; Reddy et al., 2014; Van Der Knaap et al., 2005). There are many examples where multiple

modes of regulation are employed for a single USP and these have been extensively reviewed elsewhere (Leznicki & Kulathu, 2017; Mevissen & Komander, 2017; Sahtoe & Sixma, 2015).

To understand how internal and external regulatory factors modulate catalytic activity of USPs it is important to perform quantitative analysis of USP activity. In vitro analysis can be very valuable here, as it allows separating individual functions by performing assays in the presence and absence of the regulatory elements. These kinds of analyses shed light on the mechanism of activity modulation and in some cases inform us on how concerted action of multiple regulatory elements brings about changes in USP function.

In this chapter we give a detailed workflow for quantitative analysis of USP function. We discuss expression systems and present a series of examples of USP purification. We then describe how USP catalytic activity can be quantitatively analyzed on different substrates, i.e., minimal substrates and natural substrates. We also present a detailed workflow for generating fluorescently labeled ubiquitinated substrate for quantitative analysis of DUB activity. Furthermore, we review the use of activity assays to study how internal and external factors modulate USP catalytic activity. Finally, we discuss the importance of studying USP interactions with their substrates and/or cofactors quantitatively in vitro and highlight the advantages and limitations of commonly used binding assays.

2. Purification of USPs

For the in vitro characterization of USPs it is important to carefully purify the USP in question. The first step is expression of the protein, but as yields vary in a protein-specific manner we do not present a general protocol, but rather suggest testing different expression systems. As USPs are intracellular proteins, either bacteria or insect cells usually work well for expression. In bacterial expression, there will be no eukaryote-specific post-translational modifications taking place, whereas this may happen during expression of USPs in insect cells. As these may affect activity, this needs to be carefully examined. Moreover, DUBs purified from insect cells may bind tightly to regulatory proteins that are hard to remove; a notable example is USP1, that carries along its activator UAF1 (WDR48). A related problem occurs when purifying the activators: it is difficult to purify GMPS from insect cells without carrying along a fraction of USP7.

To efficiently test different affinity tags and expression systems we make use of a coherent set of ligation-independent expression vectors (Luna-Vargas et al., 2011), most of which are available from Addgene (www.addgene.org). Unless posttranslational modifications are important, we prefer bacteria for expression as procedures are faster. Therefore, we first test if the protein of interest can be expressed in bacteria in small-scale expression tests under a set of different conditions. In these assays we vary the choice of affinity tag, expression strain, growth medium, and induction conditions. If none of these conditions yield soluble protein, then we try insect cells, where we again start with small scale tests. Expression is read out after the initial affinity purification by analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

If specific protein domains have to be expressed, or if soluble full-length protein is not expressed in any of the tested conditions, one needs to design the specific region to be expressed. In the following sections we will reflect on the design of the expression construct that can aid in obtaining pure USP proteins or protein fragments and describe three case studies to show what a USP purification protocol can look like.

2.1 Determination of a well-behaved USP catalytic domain construct

Protocol

1. Look up the USP of interest in, e.g., UniProt. Here protein sequences are stored, including splicing variants, as well as additional information from the literature: scroll through the page and follow links to published papers or second party websites. Especially when there is structural information known, we recommend checking the linked page of the Protein Databank (PDB) and if applicable the accompanying paper(s) for expression constructs and domain information.
2. If the literature assessment has not yielded defined construct boundaries, we highly recommend looking at the sequence analysis by Ye et al. (2009). Here the catalytic domains (CDs) of all (human) USPs have been aligned and annotated in the supplemental information. Search for the USP of interest and note the active-site residues as well as the presence of any zinc-binding motifs. This information is useful for the actual purification and analysis.
3. The alignment in Ye et al. (2009) shows boundaries for the catalytic core. These boundaries have been obtained by sequence comparison and in our experience, they give a good starting point to design a

construct that yields a soluble catalytic domain. However, the actual domain boundaries vary more than expected from the sequence alignments alone and may require optimization.

4. We use sequence conservation and secondary structure to define the borders of the constructs. As a tool we make use of the Crystallographic Construct Designer (CCD, https://ccd.rhpc.nki.nl) (Mooij, Mitsiki, & Perrakis, 2009) as this shows the results from multiple analyses and suggests potential cloning primers.
5. Usually the N-terminal end of the catalytic construct does not need a lot of adaptation, but the C-terminal boundaries may vary. We generally extend the construct by up to 40 residues as this can improve heterologous expression. As an example, USP7 has a large α-helix just C-terminal of the CD. Inclusion of this region was helpful for soluble bacterial expression (Kim, Van Dijk, & Sixma, 2016). As an extra check, one can run a protein structure prediction program on the final construct (e.g., Phyre2 (Kelley, Mezulis, Yates, Wass, & Sternberg, 2015)). The designed construct should yield the USP domain, possibly with small N- and C-terminal extensions.
6. Order primers and clone the designed constructs into expression vectors. We recommend testing several constructs, with varying start and end points.
7. Using a codon-optimized sequence should also be considered. Codon-optimization can increase the chances of successful heterologous expression. It can be done for multiple expression systems through various suppliers (e.g., GenScript, Integrated DNA technologies).

Note: Remember that any protease used to cleave off an affinity tag requires an unobstructed recognition sequence: a slightly extended N- or C-terminus in the construct can help this.

2.2 General purification protocol for USPs

For the expression and purification of the designed USP constructs we refer the reader to the many handbooks available (e.g., Strategies for Protein Purification by GE Healthcare). In general, the protocols supplied by manufacturers suffice for initial purification trials, and optimization is done from this starting point. Here we describe three case studies to give examples of purification of USPs in bacteria, insect cells and copurification of a USP with its regulator in insect cells.

Note: The practical guidelines mentioned in the notes should be applied for all the purification protocols described in this chapter.

Note: Since USPs are cysteine proteases, it is important that all purification buffers should contain reducing agents because oxidation by reactive oxygen species (ROS) has been shown to inactivate a large number of USPs (Cotto-Rios, Békés, Chapman, Ueberheide, & Huang, 2012; Lee, Baek, Soetandyo, & Ye, 2013).

2.2.1 Case study—(a) expression of a USP in E. coli: USP7

The full length codon-optimized USP7 sequence is cloned into a pGEX-6p vector containing a 3C protease-cleavable N-terminal GST tag (Faesen et al., 2011). Transform the sequence-verified expression construct into *Escherichia coli* BL21 Rosetta2 (DE3) T^{1R} cells and grow the transformed cells in 4 L of Terrific Broth growth medium, which allows growing to high density. When cells reach an $O.D._{600nm}$ of 1.8 add 0.2 m*M* IPTG to induce and incubate overnight at 18°C.

Buffers

- Lysis buffer—50 m*M* HEPES (pH 7.5) + 150 m*M* NaCl + 1 m*M* EDTA + 1 m*M* DTT + 0.1 m*M* PMSF + DNase I (Roche)

Note: DNAse should be omitted if the USP will be tested for binding or activity against DNA.

- GST Wash buffer—50 m*M* HEPES (pH 7.5) + 250 m*M* NaCl + 1 m*M* EDTA + 1 m*M* DTT
- GST Elution buffer—50 m*M* HEPES (pH 7.5) + 250 m*M* NaCl + 1 m*M* EDTA + 1 m*M* DTT + 15 m*M* glutathione
- IEX buffer A—10 m*M* HEPES (pH 7.5) + 50 m*M* NaCl + 1 m*M* DTT
- IEX buffer B—10 m*M* HEPES (pH 7.5) + 1 *M* NaCl + 1 m*M* DTT
- Gel filtration buffer—25 m*M* HEPES (pH 7.5) + 100 m*M* NaCl + 1 m*M* DTT

Protocol

1. Harvest cells by centrifugation at 5300 × *g* for 15 min and resuspend in Lysis buffer. For expressions in TB we use 30 mL of buffer per expressed liter.
2. Lyse the resuspended cells by homogenization using a cell homogenizer (e.g., Avestin Emulsiflex-C5) which is precooled to 4°C.

Note: We use the emulsiflex and sonicator both. Results are mostly interchangeable, but occasionally a protein seems to respond better to one or the other treatment.

3. Spin down the lysed cell suspension at $53{,}000 \times g$ for 40 min in a precooled centrifuge at 4°C and collect the supernatant. Collect samples of the supernatant and pellet and dilute them tenfold for analysis by SDS-PAGE along with samples from step 6 to 7.
4. Add 5 mL of GST-Sepharose beads (GE Healthcare) to a gravity flow column (Bio-Rad) and equilibrate with five column volumes (CV) of GST Wash buffer at 4°C. Determine the amount of GST beads to use based on protein yields from the first small scale prep. We use 1 mL of GST beads for 10 mg of GST tagged protein but this might differ for individual proteins depending on their size.
5. Add the lysate and incubate by rotating at 4°C for 30 min.
6. Allow the lysate to pass through the column by gravity flow and collect the flowthrough. Take a sample of the flowthrough for SDS-PAGE analysis.
7. Wash the column with 20 CV of Wash buffer and elute with 5×0.8 CV of Elution buffer. Collect wash and elution fractions and take samples for SDS-PAGE.
8. Perform SDS-PAGE with all the collected samples to determine which fractions contain USP7. If all the steps are performed properly then protein should be in the elution fractions.
9. Pool elution fractions containing USP7 and set aside a sample for comparison with the 3C protease postcleavage sample by SDS-PAGE.

Note: Estimate the absolute amount of protein after every step during the purification. This will help in identifying if unusual amounts of protein are lost in any step.

10. Add 3C protease to the pooled USP7 sample and transfer the mixture to a dialysis tube, Incubate at 4°C while dialyzing overnight against 2 L of IEX buffer. Make sure the molecular weight cut-off does not allow the 3C protease to go through (MWCO <20 kDa).

Note: Cleavage times can vary depending on the sample and the amount of enzyme added; it is usually most convenient to incubate the sample overnight. Adding 1μg of 3C protease for 100μg of protein is sufficient for complete cleavage under these conditions.

11. Equilibrate a 10 mL POROS Q anion exchange column with sequential washes of 2 CV IEX buffer A, 2 CV IEX buffer B and 2 CV IEX buffer A at 4°C. The size of the column can vary depending upon the purification scale.
12. Check if 3C protease cleavage is complete by analyzing samples from before and after cleavage on SDS-PAGE. If it is already known that cleavage is complete under these conditions, then this step can be skipped.

13. Spin down the cleaved protein sample and load the supernatant on the column followed by washing with 3 CV of IEX buffer A.
14. Elute USP7 by using a salt gradient of 20 CV from 50 m*M* to 1 *M* NaCl; full-length human USP7 typically elutes around 150–250 m*M* NaCl.
15. Collect samples corresponding to all the significant peaks as measured by UV absorbance at 280 n*M* (UV280) and analyze them by SDS-PAGE.
16. Equilibrate a Superdex 200 10/300 size exclusion column (GE Healthcare) with Gel filtration buffer at 4°C. The size of column is based on the amount of protein being purified (e.g., if USP7 $\geq$ 10 mg then we use the 16/60 column and for $\leq$10 mg we use the 10/300 column).

Note: If a larger column is used, then make sure that equilibration is started earlier so that it is ready to use as soon as the sample is concentrated.

17. Concentrate the pooled fractions from the anion-exchange column at 4°C using a Amicon Ultra-15 centrifugal filter unit with a 30 kDa cutoff (Merck) until a final volume of 500 μL is reached.
18. Load the concentrated sample on the preequilibrated size exclusion column.
19. Collect samples corresponding to the UV280 peaks and analyze them by SDS-PAGE.
20. Combine fractions containing pure USP7 (128 kDa) and concentrate at 4°C in a Amicon Ultra-15 centrifugal filter unit with a 30 kDa cutoff (Merck) to a final concentration of 10 mg/mL.
21. Aliquot the concentrated protein and flash freeze in liquid nitrogen for long term storage at −80°C. If protein is to be used for functional studies, then make aliquots of 10 μL; if protein is to be used for crystallography, then make aliquots of 40 μL.

Note: It is not recommended to refreeze the protein aliquot after assays as this can influence the activity and stability.

2.2.2 Case study—(b) expression of a USP in insect cells: USP46

Many USPs are not expressed in bacteria or have very low expression and solubility levels. In such cases, expression and purification in insect cells can be a good alternative, frequently improving expression levels as well as solubility. We employ *Spodoptera frugiperda* (Sf9) insect cells for protein expression using the baculovirus expression system. Sf9 cells are more sensitive to variations in culture conditions compared to bacterial cells; our reproducibility improved when we appointed a single person to maintain the insect cell cultures. This helps to avoid contamination, makes it possible to

efficiently expand cultures for protein expression, and ensures that new stock cultures are started before the passage limit is reached.

Production of recombinant bacmids and baculoviruses is carried out based on protocols in the Invitrogen manual (Bac-to-Bac® Baculovirus Expression Systems) for insect cell expression. The titer of the P1 viral stock is not calculated and it is assumed to be in the range of 1×10^6–1×10^7. Optimal infection conditions for large-scale expression vary for each recombinant baculovirus. Therefore, small-scale expression tests are performed, varying virus amounts. Additionally, different expression times are tested at constant virus levels and only after determining these two parameters is large-scale expression initiated.

The full length USP46 cDNA is cloned into a pFastbac vector with a cleavable N-terminal His tag. Transform the sequence-verified construct into DH10Bac bacterial cells for bacmid preparation. Purify the recombinant bacmid and use it for transfection of Sf9 insect cells to produce the recombinant baculovirus. We do not normally determine virus titer, but rather rely on small-scale expression tests to decide on the necessary amount of USP46 P2 viral stock to add. Here we use 4 mL of the P2 virus per 2 L of Sf9 culture at 2×10^6 cells/mL and harvest after 72 h.

Buffers

- Lysis buffer—50 m*M* Tris–HCl (pH 7.5) + 200 m*M* NaCl + 2 m*M* Tris(2-carboxyethyl)phosphine (TCEP) + Pierce™ Protease Inhibitor Mini Tablets, EDTA Free (1 tablet/50 mL)
- His wash buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 2 m*M* TCEP + 50 m*M* Imidazole (pH 8.0)
- His elution buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 2 m*M* TCEP + 500 m*M* Imidazole (pH 8.0)
- IEX dilution buffer—50 m*M* Tris–HCl (pH 7.5) + 50 m*M* NaCl + 2 m*M* TCEP
- IEX buffer A—20 m*M* Tris–HCl (pH 7.5) + 50 m*M* NaCl + 2 m*M* DTT
- IEX buffer B—20 m*M* Tris–HCl (pH 7.5) + 1 *M* NaCl + 2 m*M* DTT
- Gel filtration buffer—20 m*M* HEPES (pH 7.5) + 150 m*M* NaCl + 2 m*M* DTT

Protocol

1. Harvest Sf9 cells by spinning them down at $750 \times g$ for 15 min at room temperature and resuspend the cells in 50 mL of Lysis buffer. We generally use 25 mL of Lysis buffer to harvest 1 L of Sf9 cells (2×10^6 cells/mL).
2. Add the resuspended cells to a 125 mL metal beaker immersed in ice, and lyse by sonicating with a precooled sonicator (Qsonica Q700 with 12.7 mm probe). The lysis conditions are as follows: Amp—50; Pulse on—15 s; Pulse off—45 s; Total Time—2 min.

3. Spin down the lysed cell suspension at 53,000 × *g* for 40 min in a precooled centrifuge at 4°C and collect the supernatant. Collect and dilute samples of the supernatant and pellet for analysis by SDS-PAGE along with samples from step 6 to 7.
4. Add 2 mL of Ni^{2+}-Sepharose beads (GE Healthcare) in a gravity flow column and equilibrate with 4 CV of Lysis Buffer (without protease inhibitors) at 4°C.

Note: Nickel and Talon beads are most commonly used for His affinity purification. They have somewhat different affinity and this can be optimized for individual constructs.

Note: The amount of beads used depends on the estimated yields based on small scale expression tests. It is important to note that Nickel and Talon beads can act as ion exchangers and can bind to random proteins. It is therefore important to limit the quantity of beads and better to underestimate than to overestimate. This may result in not binding all the protein of interest, but what is purified is much cleaner.

5. Add the lysate and incubate with rotation at 4°C for 30 min.
6. Allow the lysate to pass through the column by gravity flow and collect the flowthrough. Take a sample of the flowthrough for SDS-PAGE analysis.
7. Wash the column with 50 CV of Wash buffer and elute with 5 × 0.8 CV of Elution buffer. Collect wash and elution fractions and take aliquots for SDS-PAGE.

Note: Some His-tagged proteins start eluting at 50m M imidazole therefore always check if this is the case before using 50 mM imidazole in the wash buffers.

8. Perform SDS-PAGE with all the collected samples to determine which fractions contain USP46.
9. Equilibrate a 10 mL POROS Q anion exchange column with sequential washes of 2 CV IEX buffer A, 2 CV IEX buffer B and 2 CV IEX buffer A at 4°C.
10. Pool elution fractions containing USP46 and dilute the pooled sample with an equal volume of IEX dilution buffer.

Note: The final salt concentration of the buffer should be significantly lower than the salt concentration at which the protein is expected to elute from the column. Do not dilute more than necessary as some proteins might precipitate.

11. Load the diluted USP46 sample on the column followed by washing with 3 CV of IEX buffer A.
12. Elute USP46 by using a salt gradient of 20 CV from 50 m*M* to 1 *M* NaCl.
13. Collect samples corresponding to all the significant UV280 fractions and analyze them by SDS-PAGE. USP46 typically elutes around 200–300 m*M* NaCl.

14. Equilibrate a Superdex 200 16/60 size exclusion column (GE Healthcare) with Gel filtration buffer at 4°C.
15. Concentrate the pooled fractions at 4°C using a Amicon Ultra-15 centrifugal filter unit with a 10 kDa cutoff (Merck) until a final volume of 500 μL is reached.
16. Load the concentrated sample on the preequilibrated size exclusion column. A single UV280 peak with a leading shoulder will be obtained. The shoulder contains USP46 bound to its interacting proteins from insect cells such as UAF1.
17. Collect fractions corresponding to the shoulder and peak for analyzing them by SDS-PAGE.
18. Combine peak fractions containing pure USP46 and concentrate at 4° C in a Amicon Ultra-15 centrifugal filter unit with a 10 kDa cutoff (Merck) to a final concentration of 10 mg/mL.
19. Aliquot the concentrated protein and flash freeze in liquid nitrogen for long term storage at −80°C. If protein is to be used for activity assays, then make aliquots of 10 μL, and if protein is to be used for crystallography, then make aliquots of 40 μL.

2.2.3 Case study—(c) coexpression of a USP with a regulatory protein in insect cells: USP1–UAF1

USP1 was cloned into a pFastBac vector with a cleavable N-terminal His tag, UAF1 into a pFastBac vector with a cleavable N-terminal Strep II tag. Transform the sequence-verified constructs separately into DH10Bac cells for bacmid preparation. Purify the recombinant USP1 and UAF1 bacmids and use each for transfection of *S. frugiperda* (Sf9) insect cells to produce recombinant baculoviruses. Perform small-scale expression tests with different ratios of USP1 and UAF1 viruses and check if both proteins are expressed equally. Based on the small-scale expression tests, select appropriate amounts of P2 viral stock. Here we use 4 mL of USP1 P2 viral stock and 2 mL of UAF1 P2 viral stock in 2 L of Sf9 culture at 2×10^6 cells/mL and harvest after 72 h.

Buffers

- Lysis buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 2 m*M* TCEP + Pierce™ Protease Inhibitor Mini Tablets, EDTA Free (1 tablet/50 mL)
- His wash buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 2 m*M* TCEP + 50 m*M* Imidazole (pH 8.0)
- His elution buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 2 m*M* TCEP + 500 m*M* Imidazole (pH 8.0)

- Strep wash buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 2 m*M* TCEP
- Strep elution buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 2 m*M* TCEP + 2.5 m*M* Desthiobiotin
- Gel filtration buffer—20 m*M* HEPES (pH 7.5) + 150 m*M* NaCl + 2 m*M* DTT

Protocol

1. Harvest cells by spinning them down at 750 × *g* for 15 min at room temperature and resuspend the cells in 100 mL of Lysis buffer.
2. Add the resuspended cells to a 125 mL metal beaker immersed in ice, and lyse by sonicating with a precooled sonicator (Qsonica Q700 with 12.7 mm probe). We use lysis conditions as follows: Amp—50; Pulse on—15 s; Pulse off—45 s; Total Time—2 min.
3. Spin down the lysed cell suspension at 53,000 × *g* for 40 min in a precooled centrifuge at 4°C and collect the supernatant. Collect and dilute samples of the supernatant and pellet for analysis by SDS-PAGE along with samples from step 7 to 8.
4. Add 2 mL of Ni^{2+}-Sepharose beads (GE Healthcare) to a gravity flow column (Bio-Rad) and equilibrate with 4 CV of Lysis Buffer (without protease inhibitors) at 4°C.
5. Load the lysate and incubate by rotating at 4°C for 30 min.
6. Allow the lysate to pass through the column by gravity and collect the flowthrough. Take a sample of the flowthrough for SDS-PAGE analysis.
7. Wash the column with 50 CV of His wash buffer and elute with 5 × 0.8 CV of His elution buffer. Collect wash and elution fractions and take aliquots for SDS-PAGE.
8. Perform SDS-PAGE with all the collected samples to determine which fractions contain USP1–UAF1.
9. Add 5 mL of Streptactin sepharose beads (IBA Life Sciences) to a gravity flow column and equilibrate with 5 CV of Strep wash buffer at 4°C. The amount of beads used depends on the estimated protein yields. Generally 1 mL of Streptactin sepharose beads bind up to 100 nmol of strep-tagged protein.
10. Combine and load the USP1–UAF1 fractions on the column without disturbing the Streptactin beads. Allow the sample to pass through the column slowly by gravity and reload the flowthrough at least once on the column. Collect the flowthrough and take a sample for SDS-PAGE.
11. Wash the column with 2 CV of Strep wash buffer and elute with 6 × 0.8 CV of Strep elution buffer.

Note: Excessive washing of the Streptactin column should be avoided as contaminating proteins get easily washed away in 1–2 CV due to lack of nonspecific interactions. More importantly, excessive washing will lead to loss of the strep tagged protein since the affinity between the strep tag and the streptactin beads is in the low micromolar range.

12. Perform SDS-PAGE with all the collected samples to determine which fractions contain the USP1–UAF1 complex.
13. Combine the elution fractions and take a sample for comparison with the postcleavage sample by SDS-PAGE.
14. Add His-tagged 3C protease to the pooled USP1–UAF1 sample and incubate overnight at 4°C. Save a sample and check for cleavage on SDS-PAGE.
15. Add 4 mL of Ni^{2+}-Sepharose beads (GE Healthcare) to a gravity flow column and equilibrate with 4 CV of Lysis Buffer (without protease inhibitors) at 4°C.
16. Perform a reverse Ni^{2+}-affinity purification by adding the cleaved sample to the column. The cleaved USP1–UAF1 should not bind, while the His-tagged protease will bind to the column.
17. Allow the sample to go through the column slowly by gravity flow and reload the flowthrough at least once on the column. Collect the flowthrough and take a sample for SDS-PAGE.
18. Wash the column with 5×1 CV of His wash buffer and elution is done with 3×0.8 CV of His elution buffer.
19. Perform SDS-PAGE with all the collected samples to determine which fractions to collect for the next step; pure USP1–UAF1 should be in the flowthrough and wash fractions.
20. Equilibrate the Superdex 200 10/300 size exclusion column (GE Healthcare) with Gel filtration buffer at 4°C.
21. Pool and concentrate the fractions at 4°C using a Amicon Ultra-15 centrifugal filter unit with a 50 kDa cutoff (Merck) until a final volume of 500 μL is reached.
22. Load the concentrated sample on a preequilibrated size exclusion column.
23. Collect fractions corresponding to the UV280 peak and analyze them by SDS-PAGE.
24. Combine fractions containing pure USP1–UAF1 and concentrate at 4°C in a Amicon Ultra-15 centrifugal filter unit with a 50 kDa cutoff (Merck) to a final concentration of 5 mg/mL.
25. Aliquot the concentrated protein and flash freeze in liquid nitrogen for storage at −80°C.

3. Characterization of USPs

After purification of the USP of interest, or a USP domain or USP-regulator complex, one would like to assess the quality of the particular protein preparation. Methods to assess protein stability and stoichiometry have been described extensively elsewhere (Senisterra & Finerty, 2009; Wen, Arakawa, & Philo, 1996); for this analysis of USPs, we focus on the enzymatic activity.

Besides using an activity assay for quality control, these assays can also provide insight into the mechanisms of USP activity by yielding key enzymatic parameters. Here we describe our protocols to determine parameters such as K_M and k_{cat}, using a minimal substrate and a more "realistic" one. Furthermore, we indicate how these activity assays could help to understand the effect of ancillary domains or interactors on the USP protein.

3.1 USP activity on a minimal substrate

Ubiquitin-specific proteases specifically cleave ubiquitin (Ub) from targets, with ubiquitin being the minimally recognized entity as a substrate. As such the minimal substrate to use in activity assays consists of ubiquitin with its C-terminus conjugated to a readout molecule, usually a quenched fluorophore that increases in fluorescence when released or a moiety that can trigger a secondary, luminescent signal (Orcutt, Wu, Eddins, Leach, & Strickler, 2013). For an activity assay, the ubiquitin needs to have a peptide(-like) linkage to the C-terminal leaving group to allow processing by the USP. One possibility is assessment using a fluorescence polarization assay (FP, see Section 3.4.4) in which the release of fluorescently tagged ubiquitin from a substrate is measured by the change in polarization.

Here we use two commonly used quenched fluorophores, rhodamine (Rho), or 7-amino-4-methylcoumarin (AMC), which are conjugated to ubiquitin. Ubiquitin rhodamine (UbRho) or Ubiquitin 7-amino-4-methylcoumarin (UbAMC) are commercially available and often used in the DUB-field (Hassiepen et al., 2007). As the released compound from these model substrates is a direct readout of cleaved product, we can use the time-resolution of a plate reader to determine the enzymatic parameters.

Here, we use a PheraStar plate reader (BMG LabTech), but other machines could be suitable as well. Make sure that the plate reader has the required filters or monochromators to measure rhodamine (Rho) or 7-amino-4-methylcoumarin (AMC) fluorescence and that it can measure

the fluorescence intensity (FI) over an extended period of time. Some plate readers, like the PheraStar, also have an injection option, which sometimes can be useful. The injector allows detection of early events of the reaction, which is necessary for very active USPs or when they display unexpected behavior ((Clerici, Luna-Vargas, Faesen, & Sixma, 2014; Haahr et al., 2018; Kim et al., 2019). For enzymes with normal Michaelis–Menten behavior, the procedure described here, where we prepare the plates manually, leaving a delay time between the addition of substrate or enzyme and the start of the measurement, is sufficient.

3.1.1 Deubiquitination assay as a quality control step

For a simple check of deubiquitinating activity for a new protein construct, one would simply add the newly purified enzyme to the minimal substrate and monitor the fluorescence increase. If this is a new purification of an already analyzed USP, take into account the assay conditions (concentration, buffer) already established for the protein (see Section 3.1.3).

Materials

– Freshly thawed protein, make sure it has reduced cysteines

Note: Using fresh DTT ensures the cysteines (including the active site) are reduced, which is essential for the enzyme to be active.

Note: Try not to refreeze enzymes as they lose activity. It is better to make small aliquots and thaw only once.

– UbRho (from, e.g., Boston BioChem (US), or UbiQ Bio (Europe)); for these quality control assays, we use an 8 μ*M* stock.

Note: Upon 1:1 dilution in the assay, this will result in an end concentration of 4μM, which is around the K_D for many USPs.

– Protein storage buffer: the exact composition depends on the USP of interest. Generally a buffer containing 20 m*M* HEPES (pH 7.5); 150 m*M* NaCl and 1 m*M* DTT suffices.
– Low Volume 384-Well Black Flat Bottom Polystyrene NBS™ Microplate (Corning, catalog number: 3820).

Protocol

1. Add 10 μL UbRho to two wells of the plate.
2. At the plate reader, check the settings and the program, take care to select the proper wells. Time-wise the result of the experiment will be clear within 10 min, but we usually measure longer.

Note: Rhodamine: excitation maximum—485 nm, emission maximum—530 nm. AMC: excitation maximum—380 nm, emission maximum—480 nm.

3. Add 10 μL buffer to one well (blank) and 10 μL of protein solution to the other.
4. Insert the plate and start the program, monitoring the fluorescence increase over time.
5. If correct, the blank will show a steady, low baseline fluorescent signal over time, indicating no cleavage is taking place. The other well will show, if there is an active DUB, an increasing fluorescence intensity signal, leveling off as the substrate is consumed.

3.1.2 Preparatory analysis for quantitation of UbRho cleavage

To convert the obtained fluorescence units into concentration (μ*M*) a calibration curve for the batch of UbRho has to be determined. Keep in mind that such a calibration curve differs with the machine and "gain" settings used. We use the same machine with one particular gain setting per batch of minimal substrate.

Determine optimal gain

1. Decide on the highest concentration of UbRho you are planning to use. 30 μ*M* is generally sufficient for K_M determination for most USPs.
2. Prepare UbRho at this concentration and add a known active DUB, preferably at a high concentration so hydrolysis proceeds quickly. We generally use USP7, but other very active USPs such as USP21 will work too and are available at reasonable price from a supplier such as Boston BioChem.
3. Add the sample to a microwell plate and measure fluorescence in the plate reader.
4. If the signal reaches the detection limit, restart the measurement using a lower gain setting. Wait until the signal increase levels off, all substrate is now hydrolyzed.
5. Use the plate reader's auto-gain function, setting the FI signal of the well to 90%.
6. Make a note of the gain setting, or alternatively save this in a method file to be used by the plate reader.

Note: UbRho concentrations higher than 30 μM are tricky to work with. It can give unreliable readings, possibly due to aggregation of the fluorophore.

Determine a calibration curve of Rhodamine fluorescence as a function of substrate concentration

Once the optimal gain has been determined, one can run the calibration that gives the conversion factor to calculate product concentrations from the fluorescence readings.

1. Make a dilution range of your UbRho stock in the range of 0.25–30 μ*M*.
2. Add an active DUB to each well.
3. Monitor the fluorescence using the plate reader and wait until the signal is stable.

Note: The optimal gain can also be determined here by using the plate readers autogain function on the highest concentration of substrate once the fluorescence signal is stable. Alternatively, one can also use a stock of free rhodamine to determine the optimal gain.

4. After the measurements, assess the reactions using a spectrophotometer (e.g., Nanodrop) at their excitation wavelength. Using Beer's law and the extinction coefficient of the cleaved fluorescent product, one can determine the product (and thus the starting substrate) concentration.
5. Get the FI readings of the plateau (in AU) for each UbRho dilution and plot them against the used substrate concentration.
6. Fit the points linearly to get a conversion factor, converting AU to μ*M*.

3.1.3 Optimal USP concentration for kinetic analysis of enzyme activity

In order to quantify USP kinetics, the experiment must be performed under optimal sample conditions. To determine enzyme parameters such as K_M and k_{cat}, the enzyme and substrate concentrations have to be within the Michaelis–Menten domain ([E] << [S]; Michaelis & Menten, 1913). In general, this means that the enzyme concentration should be at least two orders of magnitude lower than the substrate concentration. Due to the detection limits of the released fluorophore, the substrate concentration must be between 100 n*M* and 30 μ*M*. This places initial constraints on the enzyme concentration in the assay. Moreover, the assay needs to sample the full range of activity, if there is too much enzyme, we cannot get the initial velocity (V0) of the reaction; if there is too little enzyme it will take too long before the signal appears.

Materials

- The purified protein, with a known concentration (UV280 or Bradford assay)
- UbRho, for this optimization assay we use an 8 μ*M* stock
- Protein storage buffer

Note: Make sure to have fresh reducing agent in both the buffer and protein stock.

- Low Volume 384 Well Black Flat Bottom Polystyrene NBS™ Microplate (Corning, catalog number: 3820)
- PCR tubes, preferably in a strip for easy use with a multichannel pipette

Protocol

1. Make a concentration range of the USP protein in an eight strip of PCR tubes. We usually make a 10-fold dilution range going down from 2 μ*M* to 2 n*M* or even p*M* with very active enzymes.
2. For every concentration to be tested add 10 μL UbRho stock into a well.
3. At the plate reader, check the settings and the program, take care to select the proper wells. We generally run this experiment for 1 h.
4. Using a multichannel pipette, add 10 μL of the protein samples into the wells.
5. Insert the plate and start the measurement.

Analysis

1. Observe the FI increase over time. For the highest concentration the signal will probably level off within seconds: with this concentration no V0 can be determined. Try to find the curve with the optimal protein concentration; this curve will preferably reach a plateau right before the end of your measurement (see note).
2. Note down the optimal concentration or fine-tune it by repeating the experiment with a narrower dilution range.

Note: If the signal reaches a plateau it gives you an internal experiment control within this one measurement. When converting the plateau value (in AU) to μM using the calibration curve (Section 3.1.2), it should yield the used UbRho concentration.

3.1.4 Determining steady-state enzymatic parameters using minimal substrate

Here we describe steady-state kinetic analysis of USP7 activity on a minimal substrate. The protocol can be used for other USPs as well, but buffer conditions and protein concentrations may have to be optimized (see Section 3.1.3).

Materials

- Reaction buffer: 20 m*M* HEPES 7.5; 100 m*M* NaCl; 1 m*M* DTT; 0.05% Tween-20
- UbRho stock (1 m*M*, in DMSO)
- USP7 protein stock
- Low Volume 384 Well Black Flat Bottom Polystyrene NBS™ Microplate (Corning, catalog number: 3820)
- PCR tubes, preferably in a strip for easy use with a multichannel pipette
- Data fitting software: GraphPad Prism 7

Protocol

1. Prepare reaction buffer, using fresh DTT.

Note: Using fresh DTT ensures the cysteines (including the active site) are reduced, which is essential for the enzyme to be active. The Tween-20 is included to prevent proteins or compounds from sticking to the walls of the plate.

2. Thaw stocks and determine the protein concentration (For USP7: 1 OD280 = 7.96 μ*M*).
3. Make a 2 n*M* USP7 stock. Make sure to make enough volume-wise, for one single measurement, we make 100 μL.

Note: Do not take too large dilution steps. We usually do sequential dilutions starting with a 1 μM dilution (measure for certainty using a NanoDrop), then dilute 25-fold (40 nM) and then 20-fold (2 nM). This helps prevent aggregation.

4. Make serial twofold dilutions of UbRho (starting at 16 μ*M*) in an eight-strip of PCR tubes. Make sure the minimal amount is 10 μL for each reaction. For example make 25 μL of 16 μ*M* UbRho, then do a serial dilution transferring 12.5 μL to the next well with 12.5 μL buffer.
5. Add 10 μL of 2 n*M* USP7 into the wells of the assay plate—note down the wells.
6. At the plate reader, check the settings and the program, take care to select the proper wells.

Note: Rhodamine: excitation maximum—485 nm, emission maximum—530 nm. AMC: excitation maximum—380 nm, emission maximum—480 nm. Gain setting as determined in Section 3.1.2.

7. Using the multichannel pipette, add 10 μL of the substrate into the wells.
8. Start the program, and monitor the fluorescence increase.

Analysis

1. Get the data from the machine (.xls or .csv file) and copy them into Prism 7.
2. Using the determined conversion rate (Section 3.1.2), convert the AU values into μ*M* and plot them (as converted substrate vs time).
3. Examine the plot to assess the quality of the experiment. Are there curves where the signal increase does not seem to follow the dilution steps and do the plateaus reached (see note in Section 3.1.3) match the substrate concentrations used? Also find the time range to determine the initial velocity (V0); this range encompasses the linear part of the curve right after the start.
4. If the linear range cannot be properly determined, look into optimizing the experiment (Section 3.1.3).
5. Use the determined time range to linearly fit that part of the data and acquire the V0 for each substrate concentration tested. Then plot these velocities against substrate concentration to obtain a Michaelis–Menten

plot. The data can also be fitted using the built-in Michaelis–Menten equation to obtain the K_M and V_{max}.

Note: Modern computers do not require linearization of the data for fitting Michaelis–Menten data. Linearization creates large artifacts and should be avoided.

6. The curve and the fit statistics will indicate whether you have sufficient plateaus at the top and bottom of the curve. Take this into account in the next experiments for the tested enzyme, extending the substrate dilution range if necessary.
7. Convert the V_{max} value to k_{cat} by dividing it with the enzyme concentration used and calculate the catalytic efficiency k_{cat}/K_M.

3.2 Measuring deubiquitination of a natural target

Minimal substrates such as ubiquitin-AMC or ubiquitin-rhodamine are convenient for initial enzymological characterization of DUBs. However, activities on minimal substrates do not always translate directly to activities on a physiological target (Kim et al., 2019; Uckelmann et al., 2018), and substrates may affect the reaction itself. Since regulation of deubiquitinating enzymes is often achieved through modulating their activities (Mevissen & Komander, 2017; Sahtoe & Sixma, 2015), it is ultimately necessary to study the enzymology of DUBs on their physiological targets. The identification of physiological targets for some DUBs and advanced tools for modeling enzyme kinetics allow one to study target-specific deubiquitination in some systems.

Quantitative analysis is assisted by a well-defined singly modified substrate. To make such a substrate, requires a properly characterized ubiquitination site, known to be used in vivo, knowledge of the natural ubiquitin modification (monomer or polymer and if the latter what linkages), and a way to consistently produce labeled substrate. In our USP7 case study (Kim et al., 2019), we investigated its interaction with p53, for which the ubiquitination sites and USP7 recognition sites were well defined in literature. This protein sequence and the advanced methods in chemical ubiquitin synthesis allowed the generation of a fluorescently labeled, homogeneous, peptide substrate. We could use this tool, along with the kinetic modeling program KinTek (Johnson, Simpson, & Blom, 2009), to quantitatively study the effect of target recognition on the enzyme kinetics of USP7.

Here we describe a different approach to measuring substrate-specific deubiquitination. We employ a gel-based setup, enzymatically ubiquitinated target proteins (H2A in nucleosomal core particles) with a fluorescently labeled ubiquitin and a laser-based fluorescence gel scanner for

quantification of the signal. In this experimental system we exploit the fact that ubiquitination of H2A by BRCA1/BARD1 is unusually site-specific. This allows tracking of H2A deubiquitination by the BRCA1-site-specific DUB USP48. The three steps necessary for measuring specific deubiquitination of nucleosomes are described in detail:

1. Labeling of ubiquitin
2. Generation of the ubiquitinated nucleosome substrate
3. Measurement of site-specific deubiquitination

Note: Here we make use of a well characterized system that allows tracking specific ubiquitination and deubiquitinating events. For every new experimental system, it is worth considering some very basic questions:

What exactly constitutes the substrate?

- *Are there different distinct ubiquitination sites (different lysines ubiquitinated) on the target protein?*
- *Should different sites be treated as distinct targets?*
- *Can the experimental setup distinguish between different sites?*
- *Are the sites mono-ubiquitinated or will ubiquitin chains be formed?*

Once the substrate is identified, can it be produced in high purity?

- *Can the ubiquitinated substrate be produced synthetically or enzymatically?*
- *Are there side products of the ubiquitination reaction that need to be considered (e.g., formation of nonspecific ubiquitin chains)?*
- *Can the product be purified after the ubiquitination reaction?*
- *Will substrate preparation yield a homogeneous substrate?*

Finally, does the selected assay readout report on the deubiquitination of the site of interest?

3.2.1 Labeling ubiquitin

To enable labeling of ubiquitin with cysteine-reactive fluorescent dyes, a mutant ubiquitin construct carrying a cysteine as the second residue (^{Cys}ubiquitin) is used; ubiquitin has no native Cys residues. Any dye carrying a cysteine-reactive moiety can in principle be used. In the following example we used a tetramethylrhodamine dye (TAMRA) with a Cys-reactive maleimide moiety to generate ^{TAMRA}ubiquitin which was suitable for our specific purpose and economical when considering the large amounts of fluorophore used in the study.

Purification of ^{Cys}ubiquitin

The ^{Cys}Ubiquitin construct is cloned into the pETNKI-His-SUMO2-kan vector (Luna-Vargas et al., 2011). Transform the sequence-verified expression construct into *E. coli* BL21 (DE3) T^{1R} cells and grow the

transformed cells in 1 L of Lysogeny Broth (LB) growth medium. When cells reach an $O.D._{600nm}$ of 0.8 add 0.2 m*M* IPTG to induce and incubate at 37°C for 4 h.

Buffers

- Lysis buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 1 m*M* TCEP + 5 m*M* Imidazole + Pierce™ Protease Inhibitor Mini Tablets, EDTA Free (1 tablet/50 mL)
- Wash buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 1 m*M* TCEP + 20 m*M* Imidazole
- Elution buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 1 m*M* TCEP + 350 m*M* Imidazole
- Dialysis buffer 1–50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 1 m*M* TCEP
- Dialysis buffer 2–50 m*M* ammonium acetate (pH 4.5) + 1 m*M* DTT
- IEX buffer A—50 m*M* ammonium acetate (pH 4.5) + 2 m*M* DTT
- IEX buffer B—50 m*M* ammonium acetate (pH 4.5) + 500 m*M* NaCl + 2 m*M* DTT
- GF buffer—50 m*M* Tris–HCl (pH 7.5) + 150 m*M* NaCl + 5 m*M* DTT

Protocol

1. Spin down cells at 5300 × *g* for 15 min at room temperature and resuspend in 50 mL lysis buffer.
2. Lyse cells by sonicating with a precooled sonicator (Qsonica Q700 with 12.7 mm probe). The lysis conditions are as follows: Amp—80; Pulse on—15 s; Pulse off—45 s; Total Time—4 min.
3. Centrifuge the lysate at 53,000 × *g* for 30 min at 4°C.
4. Add 8 mL chelating sepharose beads (Roche) charged with Ni^{2+} to the supernatant, load onto a sealed gravity-flow column (Bio-Rad), let the beads settle by force of gravity, then open the column and let the supernatant flow through.
5. Wash the beads with 3 × 80 mL of wash buffer.
6. Elute the sample in 25 mL of elution buffer.
7. Add His-tagged SENP2 protease to a final concentration of 3 μg/mL, transfer sample to a 3500–5000 Da cutoff Spectra/Por dialysis tube (Spectrum) and dialyze against 2 × 2 L of dialysis buffer 1 at 4°C. After 4 h replace the buffer with 2 L of fresh dialysis buffer 1 and let this second dialysis step proceed overnight.
8. To remove the His-SUMO, uncleaved protein and SENP2, add 8 mL chelating sepharose beads charged with Ni^{2+} to the dialysed sample and load on a gravity-flow column. Let the beads settle by force of gravity

and collect the flow-through containing ubiquitin (His-SUMO, uncleaved SUMO-^{Cys}Ubiquitin and His-SENP2 will remain bound to the beads). Wash beads with 8 mL of dialysis buffer 1 and collect in the same tube.

9. While stirring the sample on ice, add perchloric acid dropwise until a final concentration of 2% v/v is reached. Most proteins will precipitate at this stage, while ^{Cys}Ubiquitin will stay in solution.
10. Centrifuge at 53,000 × *g* at 4°C for 30 min and collect the supernatant.
11. Dialyze the supernatant against 2 L Dialysis Buffer 2 overnight at 4°C.
12. Load sample on a 5 mL HiTrap SP HP ion exchange column (GE healthcare) in IEX-buffer A at 4°C.
13. Elute sample using a linear gradient ranging from 0% IEX-buffer B to 100% IEX-buffer B in 12 column volumes.
14. Combine fractions containing ^{Cys}Ubiquitin and load on a Superdex 75 16/60 column (GE healthcare) preequilibrated in GF buffer.
15. Combine fractions containing ^{Cys}Ubiquitin, concentrate in an Amicon Ultra-15 centrifugal filter unit with a 3 kDa cutoff (Merck) to a concentration of ~1 m*M*, snap-freeze 50 μL aliquots in liquid nitrogen and store at −80°C.

Materials

- Fresh DTT
- Labeling buffer: 50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl
- Purified ^{Cys}ubiquitin
- TAMRA-maleimide
- Storage buffer: 25 m*M* HEPES, 150 m*M* NaCl, 1 m*M* DTT
- Spectra/Por 3500—5000 D cutoff dialysis tubing (Spectrum)

Protocol

Note: Throughout the protocol, wherever possible, keep the fluorophore and the fluorescently labeled protein protected from direct light.

1. Thaw the desired amount of ^{Cys}ubiquitin to be labeled.
2. Add 5 m*M* of fresh DTT to the sample to reduce the cysteine residue for labeling.

Note: It is critical the cysteine residues are reduced, otherwise labeling will not be possible.

3. Dialyze reduced ^{Cys}ubiquitin against at least three changes of 2 L labeling buffer to remove residual DTT which would interfere with labeling. Ideally the second dialysis step should be carried out overnight.

Note: Removal of residual DTT is critical as it would otherwise react with the maleimide group and compete with cysteine labeling. TCEP should not interfere with maleimide-dependent labeling reactions as it does not contain thiols. However, we

have never tested labeling in the presence of TCEP and the reader is advised to conduct their own pilot experiments if the reducing agent is to be changed. For faster removal of DTT, a buffer exchange column or desalting spin column can be used.

4. For the labeling reaction, add a fivefold molar excess of TAMRA-maleimide over ubiquitin and incubate for 2 h at room temperature, followed by overnight incubation at 4°C.

Note: The ratio of fluorophore over ubiquitin is critical for high labeling efficiency. The optimal ratio can vary between batches of protein and fluorophore. When feasible, the reader is advised to conduct small-scale pilot experiments, varying the ratio incrementally between 2:1 and 8:1 and assess labeling efficiency as described later on. Similarly, different incubation times and temperatures can be tested. We had good success with the suggested ratios and incubation.

5. Quench the labeling reaction with a threefold molar excess of DTT over fluorophore.
6. Centrifuge the sample at $20{,}000 \times g$ to remove aggregated material and collect the supernatant.
7. Dialyze the supernatant against three changes of 2 L storage buffer to remove excess dye.
8. To remove residual dye that might be present even after extensive dialysis, purify the sample on an appropriate size-exclusion column (Superdex S75 or comparable) using FPLC or a commercially available dye-removal spin column.

Note: Removal of residual dye is a crucial step in order to be able to estimate concentrations of labeled ubiquitin accurately. The suggested procedure using FPLC allowed us to obtain exceptionally pure sample. If purification by FPLC is not an option, or if the protocol needs speeding up, commercially available dye-removal spin columns are a good alternative. High amounts of free fluorophore might necessitate the use of two successive column steps.

9. If using FPLC, be aware that fluorescent dyes can be very sticky and adhere to the column resin, especially when present in high concentration during size exclusion chromatography. Extensive column washes are necessary to remove residual dye.
10. Concentrate sample to the desired concentration; the concentration is best calculated using the fluorophores absorbance at 542 nm and the corresponding extinction coefficient of $101{,}000\,\mathrm{cm}^{-1}\,M^{-1}$.

Note: This combination of maximum absorbance and extinction coefficient is true for the particular fluorophore used in this study. Make sure you use the right absorbance and extinction coefficient for the fluorophore you are using. This might differ from the stated values here, even if the fluorophore is a TAMRA-based fluorophore.

11. Assess labeling efficiency by determining concentration of dye and protein. To determine the dye concentration measure absorption at the appropriate wavelength. Estimation of ubiquitin concentration is best done on a SDS gel. As a standard, run a titration series of known amounts of unlabeled ubiquitin. To determine the ubiquitin concentration in your labeled sample, run several dilutions on the same gel and compare to the standard. Divide this estimate of the ubiquitin concentration by the dye concentration to estimate the labeling efficiency.
12. Flash freeze sample aliquots and store at −80°C.

Note: The labeled ubiquitin can be used not only for measuring deubiquitination, but is equally suited for E3 ligase ubiquitination reactions. It can further be used for binding assays reliant on fluorescence polarization.

3.2.2 Generating ubiquitinated nucleosomes

We describe here the ubiquitination of nucleosome core particles (NCPs) using truncated BRCA1/BARD1 as the E3 ligase. If other E3-substrate combinations are used, concentrations of components may vary and pilot experiments need to be conducted to establish reaction conditions. However, in our experience, the stated conditions are appropriate for all H2A-specific E3 ligases and should provide a reasonable starting point for other systems as well.

Materials

- E1: hUBA1 purified from *E. coli* (Uckelmann et al., 2018), also commercially available (Boston Biochem)
- E2: UBCH5C purified from *E. coli* (Uckelmann et al., 2018), also commercially available (Boston Biochem)
- E3: $BRCA1^{1–306}$/$BARD1^{26–302}$ purified from *E. coli* (Uckelmann et al., 2018)
- Purified recombinant *Xenopus laevis* H2A, H2B, H3 and H4 (Luger, Rechsteiner, & Richmond, 1999)
- Purified 147 bp DNA for nucleosome reconstitution (Vasudevan, Chua, & Davey, 2010)
- Labeled ubiquitin
- EDTA, pH 8 (500 m*M*)
- ATP, pH 7.5 (100 m*M*)
- Ubiquitination reaction buffer: 25 m*M* HEPES (pH 7.5) + 150 m*M* NaCl + 3 m*M* $MgCl_2$ + 1 m*M* DTT (fresh)
- Gel filtration buffer: 25 m*M* HEPES (pH 7.5) + 150 m*M* NaCl + 1 m*M* DTT
- 50 kDa cutoff Amicon ultra-15 centrifugal filter unit (Merck)

Protocol

Note: Throughout the protocol, wherever possible keep the fluorophore and the fluorescently labeled protein protected from direct light.

1. Reconstitute recombinant nucleosome core particles (NCP) according to a previously published protocol (Luger et al., 1999).
2. For the ubiquitination reaction, combine 0.5 μ*M* of hUBA1, 1 μ*M* UBCH5C, 1 μ*M* $BRCA1^{1–306}$/$BARD1^{26–302}$, 5 μ*M* NCP and 40 μM^{TAMRA}ubiquitin in Ubiquitination reaction buffer.
3. Start the reaction by adding ATP to a final concentration of 3 m*M*.
4. Incubate at 30°C for 45 min.
5. Stop the reaction by putting the sample on ice and adding EDTA to a final concentration of 5 m*M*.
6. Purify the ubiquitinated nucleosomes by size exclusion on a Superose 6 increase column (GE Healthcare) in gel filtration buffer.

Note: This step removes excess ubiquitin, ATP, E1 and E2 enzymes but ubiquitinated NCP cannot be resolved from nonubiquitinated NCP. However, the reaction conditions of the ubiquitination reaction have been optimized to yield a mix of mono-, di-, and triubiquitinated NCP and a negligible amount of nonubiquitinated NCP. This heterogeneous mix of different ubiquitination states is appropriate for the following USP48 analysis as mono-, di-, and triubiquitinated species can be resolved on a SDS-PAGE gel, which allows to discern individual catalytic rates for different ubiquitination states.

7. Check for ubiquitination by running a SDS-PAGE gel and combine fractions that contain ubiquitinated nucleosomes. Concentrate sample to ~10 μ*M* using a 50 kDa cutoff concentrator.
8. Ideally the ubiquitinated nucleosomes are used immediately. However, storage of up to a week at 4°C leads to only minor deterioration in sample quality. Never freeze reconstituted nucleosomes.

3.2.3 Quantitative analysis of USP activity: Deubiquitination of nucleosome core particles by USP48

To enable quantitation of kinetic parameters, full data sets are needed under Michaelis–Menten conditions. However, an interesting alternative approach is the modeling of kinetic parameters (with, e.g., Kintek explorer (Johnson et al., 2009)) based on reaction velocities while sampling over a wide range of substrate and enzyme concentrations. A 4 × 3 grid of enzyme and substrate concentrations (Table 1) was sufficient to reliably estimate kinetic parameters for NCP deubiquitination by USP48. It will become

Table 1 Combinations of enzyme and substrate concentrations used to determine kinetic parameters for NCP deubiquitination by USP48

USP48 12.5 n*M* NCP 500 n*M*	USP48 25 n*M* NCP 500 n*M*	USP48 50 n*M* NCP 500 n*M*	USP48 100 n*M* NCP 500 n*M*
USP48 12.5 n*M* NCP 2000 n*M*	USP48 25 n*M* NCP 2000 n*M*	USP48 50 n*M* NCP 2000 n*M*	USP48 100 n*M* NCP 2000 n*M*
USP48 12.5 n*M* NCP 3000 n*M*	USP48 25 n*M* NCP 3000 n*M*	USP48 50 n*M* NCP 3000 n*M*	USP48 100 n*M* NCP 3000 n*M*

obvious during the fitting process how much data is necessary for proper restraint of the model parameters for the particular system studied (for details see (Johnson et al., 2009)).

For the sake of clarity, we describe the experimental setup used to record a single time course for only one combination of enzyme and substrate concentration (25 n*M* USP48, 2000 n*M* NCP). For the complete grid of different combinations, the reader is referred to Table 1.

Material

- Full length USP48 purified from Sf-9 insect cells (Uckelmann et al., 2018); 80 μ*M*
- Purified NCP ubiquitinated with ^{TAMRA}ubiquitin; 15 μ*M*
- DUB-reaction buffer: 25 m*M* HEPES, 150 m*M* NaCl, 2 m*M* DTT (fresh)
- 4 × Laemmli sample buffer

Protocol

Note: Throughout the protocol, wherever possible keep the fluorophore and the fluorescently labeled protein protected from direct light.

1. Prepare a 30 μL aliquot containing 50 n*M* USP48 in DUB-reaction buffer.
2. Prepare a 27.5 μL aliquot of 4 μ*M* ubiquitinated NCP (NCP^{Ub}) in DUB-reaction buffer.

Note: Make sure DTT in the reaction buffer is added freshly

3. Prewarm USP48 and NCP^{Ub} to 30°C.

Note: Assays are best done using thin-walled PCR tubes and a PCR machine set to incubate at 30°C.

4. Time points will be taken after 0, 1, 2, 4, 8, 16, and 45 min. The reaction will be stopped at each time point by transferring 5 μL of the reaction to a tube prefilled with 1.7 μL of 4 × Laemmli sample buffer. While samples are prewarming, prepare "stop-tubes" for each time point by adding 1.7 μL 4 × Laemmli sample buffer to 200 μL PCR tubes.

5. For the time point 0, add 2.5 μL reaction buffer and 2.5 μL of the preincubating NCP^{Ub} dilution to the appropriate stop-tube.
6. Start the reaction by adding 25 μL from the USP48 dilution to the now 25 μL of the NCP^{Ub} dilution and incubate at 30°C.

Note: In experimental systems where the fluorophore tends to aggregate and form precipitate, addition of 0.05% Tween20 v/v can help prevent aggregation

7. At each time-point, take 5 μL from the reaction mixture and add to the appropriate stop-tube.

Note: Several reactions can easily be streamlined by using a multichannel pipette so that different enzyme-substrate combinations can be recorded simultaneously.

8. Boil the samples and run 5 μL on a 4%–12% gradient SDS-gel.
9. For quantitative readout a laser-based fluorescence gel scanner is suggested.
10. Quantify the bands in each lane using programs such as ImageJ. Each band represents a fraction of the total concentration of labeled ubiquitin (and thus ubiquitinated substrate) used in the reaction. Note, however, that this represents the molar amounts of ubiquitin present in each band. If the interest lies with the molar amounts of substrate, the quantification needs to be corrected for the number of ubiquitins present on each substrate species. For example, to calculate molar amounts for the species corresponding to diubiquitinated H2A, the molar amounts quantified from the fluorescence signal need to be divided by two, as there are two ubiquitins on the diubiquitinated H2A.

Note: More details on analysis and interpretation of the kinetic data generated this way can be found in (Johnson et al., 2009; Kim et al., 2019; Uckelmann et al., 2018).

3.3 Regulation of USP activity

USPs are usually large multidomain proteins where the accessory domains can play a vital role in regulation of the USPs either by modulating their catalytic rates or by altering their affinity for ubiquitin or their natural substrates. Additionally, USP activity can be regulated upon binding to regulatory proteins in *trans*. To investigate the potential effects of any regulatory element on DUB activity, a first step is to assess differences in USP activity in the presence and absence of the regulatory element.

3.3.1 Estimation of K_D based on activity assays

For determining kinetic parameters of USP activity in the presence of regulatory factors it is important to estimate the K_D between the USP and the

regulatory factor. This gives us an idea of the concentration required to saturate the binding so that the effect caused by this regulatory binding can be observed. Here we describe a general protocol for estimating K_D that is based on change in USP catalytic activity upon binding to its regulator.

Note: Activation assays done with domains added in trans will yield an apparent K_D for the in trans interaction, in a covalently linked setting the affinities can differ.

Materials

- Purified regulatory protein (e.g., UAF1) or a domain (e.g., UBL domains of USP7)
- Purified USP full length or catalytic domain
- UbRho (8 μ*M* stock)
- Reaction buffer (20 m*M* HEPES (pH 7.5) + 100 m*M* NaCl + 5 m*M* DTT + 0.05% Tween 20)
- Low Volume 384 Well Black Flat Bottom Polystyrene NBS™ Microplate (Corning, catalog number: 3820)

Protocol

1. Determine the protein concentration of the USP and the regulatory protein.
2. Prepare a USP stock (refer to Section 3.1.3) in reaction buffer.
3. Prepare a twofold dilution series of the regulatory protein in reaction buffer. Each dilution should have a volume of 10 μL.
4. Add 10 μL of the USP stock to each dilution and let the sample incubate for 10–15 min at room temperature.
5. Prepare an 8 μ*M* stock of UbRho in reaction buffer.
6. Add 10 μL of USP + regulator into the wells; each dilution goes into a separate well.
7. Using a multichannel pipette, add 10 μL of the substrate into the wells.
8. Start the program, and monitor the fluorescence increase.
9. If the regulatory effect is saturated at the lowest concentrations of regulator or if saturation is not reached even at the highest concentration then adjust the concentration range of the regulator and perform the experiment again.
10. Determine the apparent K_D by calculating the initial rates and plotting them against the concentration of regulator.

Note: Since this assay includes ubiquitin, the K_D obtained here could be different for the ubiquitin-USP intermediate then for the USP alone. For nonubiquitin-based affinity experiments refer to Section 3.4.

3.3.2 Quantitative assessment of activity modulation

Here we describe the case study of USP1–UAF1, in which we determine the steady-state kinetic parameters for this complex. The protocol can be used for other USP complexes as well, although the buffer and protein concentrations may have to be optimized (see Section 3.1.3).

Materials

- Purified USP1 (65 μ*M*) and UAF1 (75 μ*M*)
- UbRho stock (1 m*M*, in DMSO)
- Reaction buffer (20 m*M* HEPES 7.5 + 100 m*M* NaCl + 5 m*M* DTT + 0.05% Tween 20)
- Low volume 384 Well Black Flat Bottom Polystyrene NBS™ Microplate (Corning, catalog number: 3820)
- PCR tubes, in a strip
- Data fitting software: GraphPad Prism 7

Note: Follow the same guidelines for sample and buffer preparation provided in the notes of Section 3.1.4.

Protocol

1. Determine the concentration of USP1 and UAF1.
2. Prepare a 40 n*M* USP1 stock and a 400 n*M* UAF1 stock in reaction buffer. Make at least 60 μL of each stock solution.

Note: The K_D for USP1–UAF1 binding is around 10n M , but we still use 10 times more UAF1 to USP1 to ensure all of the USP1 is bound. For every USP complex, perform the experiment at varying concentrations of regulator (Section 3.3.1) to determine the concentration of regulator to use.

3. Mix 50 μL of the USP1 stock with 50 μL of the UAF1 stock, and incubate at room temperature for 10 min.
4. Make a twofold dilution range of UbRho (starting at 60 μ*M*) in an eight-strip of PCR tubes. Make sure the minimal amount is 10 μL for each reaction. For example, prepare 25 μL of 60 μ*M* UbRho, then do a serial dilution transferring 12.5 μL to the next well containing 12.5 μL buffer.
5. Add 10 μL of USP1/UAF1 stock solution into the wells of the assay plate—note down the wells.
6. At the plate reader, check the settings and the program, take care to select the proper wells.
7. Using the multichannel pipette, add 10 μL of the substrate into the wells.
8. Start the program, and monitor the fluorescence increase.
9. Analyze the data by following the analysis protocol outlined in Section 3.1.4.

3.3.3 Case study: Mapping substrate recognition sites on USP with effects on activity assays

While the activation assay is designed for use with a minimal ubiquitin substrate (UbRho), some domains within a USP could aid specifically in the recognition or cleavage of a physiological ubiquitinated target. By omitting the domain of interest in the protein construct, one can investigate its effect in comparison to the full-length protein. We have used such a system to study the effect of the TRAF domain of USP7 on the recognition and activity of USP7 toward a ubiquitinated p53-peptide, which mimics a physiological substrate of the enzyme (Kim et al., 2019).

3.4 Quantitative USP interaction analysis

Large scale proteomic studies carried out specifically on DUBs have uncovered large protein interaction networks (Sowa, Bennett, Gygi, & Harper, 2009). These DUB interaction networks can be examined and simplified further by using in vitro binding assays to identify and quantify protein interactions. Most USPs are part of multiprotein complexes where the members of the complex are either substrates of the USP or regulators of USP function. Binding studies can be used to simplify these complicated interaction networks by examining which members are responsible for direct interactions with the USP in question. Furthermore, once the binding partner has been identified, the stoichiometry of binding can also be determined using certain binding assays. Additionally, the equilibrium dissociation constant (K_D) and binding kinetics data obtained from such analyses give an indication of the lifetime of a protein interaction with its binding partner. This can then be compared with its other binding partners to obtain relative abundance of protein complexes for the protein in question. This kind of information is very useful for understanding mechanisms of USP regulation, especially when multiple regulatory factors are involved. For example, information obtained from binding studies can be used in activity assays to quantitatively analyze the effect of interactors on USP activity (refer to Section 3.3.2).

Here we describe a number of in vitro binding assays which can be performed to study protein–protein interactions. We do not go into the details of any of these assays as they are available in the published literature. Instead, we highlight the important features and limitations of each binding assay so that an informed decision can be made by the reader before performing these assays (Table 2).

Table 2 Features of commonly used binding assay for studying protein–protein interactions

	Pull down	Analytical gel filtration	FP	SPR	MST	ITC
K_D quantification	−	−/+	+	+	+	+
Measurable K_D range	▮	▮	▮▮	▮▮▮	▮▮	▮▮
Quantification of kinetic parameters	−	−	−/+	+	−	−/+
Sample size	▮	▮▮	▮	▮▮	▮	▮▮▮
Sample labeling	+	−	+	+	+	−
Measurement time	▮▮	▮▮	▮	▮▮▮	▮▮	▮▮▮

3.4.1 In vitro pull-down assays

In vitro pull-down assays are a fast and inexpensive way of identifying protein–protein interactions as these experiments do not require highly specialized instrumentation or a large amount of material. Pull-down assays are a type of affinity purification where one of the proteins is immobilized to a surface using a specific antibody or an affinity tag. The potential binding partner is incubated with the immobilized protein and interaction is confirmed if both proteins coelute from the surface. Many proteins interact nonspecifically with the immobilization surface which leads to false positive results. Thus, control experiments should be performed to confirm lack of nonspecific interactions. Pull down assays are usually not quantitative and low affinity interactions or interactions with a fast off rate cannot be detected using this method. Additionally, if the interacting region on the immobilized protein is masked by the surface then no binding will be observed.

3.4.2 Analytical gel filtration

This technique is an easy way of determining if two proteins interact with each other. The biggest advantage here is that the proteins do not have to contain any tag and also the amount of protein required is relatively low. The time required for a typical size exclusion run means that complexes with fast off-rates will be poorly detected. This technique is primarily used for

qualitative purposes, but multiple runs at varying concentrations, with sensitive read-out (e.g., western blot) would allow quantification of binding parameters.

3.4.3 Surface plasmon resonance

Surface plasmon resonance (SPR) is a spectroscopic method which is used to detect protein interactions by immobilizing the ligand on a thin metal film and measuring the change in refractive index upon binding of the analyte. SPR experiments allows quantification of the K_D, and if the binding process is well defined, also makes it possible to determine kinetic parameters (k_{on} and k_{off}). High-affinity interactions of less than 1 nM (depending on the system) and low-affinity interactions upto 500 μM can both be analyzed in this label-free setup with the only requirement being that one of the proteins has to be tagged so that it can be immobilized on a complementary surface. The starting material required to carry out binding measurements is not very high unless the affinity is very low, in which case the amount of analyte required will increase considerably. The disadvantage of this system is that one of the protein partners has to be immobilized on a solid surface which can prevent binding due to steric hindrance or in some cases lead to more binding than what is actually observed in solution. Moreover, the instrument and sensors are expensive and running the instrument requires some expertise.

3.4.4 Fluorescence polarization

Fluorescence polarization (FP) measures protein binding based on change in polarization of emitted light upon excitation of a fluorescent molecule with plane-polarized light. Therefore, when an interacting protein binds to the fluorescently labeled protein there is a change in the polarization of emitted light due to slower tumbling of the fluorescent molecule. FP is a quantitative technique and the amount of sample required for these assays is lower compared to SPR but that again depends on the affinity of the interaction being measured. FP depends on the size of the interacting proteins; thus, it usually only works well when the size of the labeled protein is much smaller compared to the interacting protein. The other limitation of FP arises when measuring low-affinity interactions because for such cases the concentration of the unlabeled protein is very high which can lead to artificial crowding effects. Finally, the choice of label can also lead to nonspecific interactions as some of the labels are very hydrophobic.

3.4.5 Microscale thermophoresis

Microscale thermophoresis (MST) measures protein interactions in solution based on the diffusion of a fluorescently labeled molecule along a laser-induced local temperature gradient (Wienken, Baaske, Rothbauer, Braun, & Duhr, 2010). This technique requires labeling of one protein but unlike FP there is no limitation on the relative size of the labeled and unlabeled proteins. Other advantages of MST are that the amount of sample required for obtaining a K_D is lower than any of the techniques described here and also the range of binding affinities that can be measured is very high. MST is a very sensitive method and because of that small changes in buffer or sample preparation can lead to changes in signal which hamper reproducibility. Additionally, kinetic parameters cannot be determined with this method and hydrophobicity of the label can lead to nonspecific interactions.

3.4.6 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) measures the heat generated in an interaction. Upon titration of one of the partners in the interaction, it allows determination of the thermodynamic properties of protein interactions in solution and gives K_D and stoichiometry. This is a robust method which gives very solid data. It enables measuring protein interaction in a label-free environment. The biggest disadvantage of this method is the large sample quantities required, and this is particularly important in the case of USPs because many of these enzymes are not easily produced in large quantities. Other disadvantages of ITC are that measurement times are longer, it requires high concentrations, it requires stirring of the sample, and also that it does not measure kinetic parameters of binding.

4. Conclusions

The prerequisite for in vitro characterization of USPs is pure and stable protein. USPs are usually large proteins and often with several unstructured regions which makes their expression and purification difficult. Purification of USPs from bacterial and insect cells presents distinct challenges which have been discussed in this chapter. The purification protocols outlined in this chapter serve as a starting point for purification of any USP but they might require modifications depending on the choice of expression construct and the USP being purified.

In vitro activity studies are a great way for studying USP function. These assays are also used to probe the role of regulatory factors in modifying USP catalytic activity. Similarly, when performing binding assays all the quality control measures mentioned above should be taken into consideration. The protocols described in this chapter in combination with several quality control measures will enable one to obtain reliable quantitative data on USP activity and regulation.

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