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A High-Throughput Screening Method for Identification of Inhibitors of the Deubiquitinating Enzyme USP14

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Abstract

Deubiquitinating enzymes (DUBs) reverse the process of ubiquitination, and number nearly 100 in humans. In principle, DUBs represent promising drug targets, as several of the enzymes have been implicated in human diseases. The isopeptidase activity of DUBs can be selectively inhibited by targeting the catalytic site with drug-like compounds. Notably, the mammalian 26S proteasome is associated with three major DUBs: RPN11, UCH37 and USP14. Because the ubiquitin ‘chain-trimming’ activity of USP14 can inhibit proteasome function, inhibitors of USP14 can stimulate proteasomal degradation. We recently established a high-throughput screening (HTS) method to discover small-molecule inhibitors specific for USP14. The protocols in this article cover the necessary procedures for preparing assay reagents, performing HTS for USP14 inhibitors, and carrying out post-HTS analysis.

Keywords

26S proteasome; USP14; deubiquitinating enzyme; high-throughput screening; small-molecule inhibitor

INTRODUCTION

In just a few decades, the ubiquitin-proteasome system (UPS) has come to permeate many areas of basic biology and clinical medicine. In this pathway, the covalent attachment of ubiquitin to proteins targets them for degradation by the 26S proteasome (Finley, 2009). The proteasome has recently emerged as an important drug target, as the proteasome inhibitor Bortezomib (PS-341 or Velcade), which inhibits the proteolytic active sites of the enzyme, is approved to treat multiple myeloma and mantle cell lymphoma. Another way to intervene in the UPS is by specifically targeting enzymes upstream of the proteasome, as is the case for MLN4924 (NAE-E1), Nutlin (E3-Hdm2), and TAME (E3-APC/C) (Cohen and Tcherpakov, 2010; Zeng et al., 2010). The DUBs represent another class of attractive drug targets due to their relevance to several human diseases (Cohen and Tcherpakov, 2010; Colland, 2010). For example, many proteotoxic proteins or oncoproteins are targeted by ubiquitin-mediated proteasomal degradation pathways. DUBs that antagonize the elimination of these proteins represent potential new drug targets. There are currently only a handful of published DUB inhibitors (Altun et al., 2011; Chen et al., 2011; Cohen and Tcherpakov, 2010; Colland, 2010; D’Arcy et al., 2011; Lee et al., 2010; Liu et al., 2003), but none have yet entered clinical trials. Because of the multiplicity of DUBs, it can be difficult to identify compounds

INTERNET RESOURCES

<http://iccb.med.harvard.edu> The web page of the ICCB screening facility at Harvard Medical School. This site provides general guidance and detailed information of small-molecule inhibitor screening.

<http://www.ncbi.nlm.nih.gov/books/NBK53196/> The web page of NIH Chemical Genomics Center. This site provides a general overview about how the screened inhibitors can be analyzed for their kinetic parameters.

that selectively inhibit a single DUB, and several of the published inhibitors clearly inactivate multiple DUBs.

The mammalian 26S proteasome contains three distinct classes of DUBs – USP14, UCH37, and RPN11/POH1. RPN11 belongs to the metalloenzyme JAMM family, whereas USP14 (the USP family) and UCH37 (the UCH family) are thiol proteases. Each of these DUBs appears to have distinct effects on proteasomal degradation. RPN11 may remove the ubiquitin chains *en bloc* from substrates and promote their degradation in an ATP-dependent manner (Finley, 2009; Reyes-Turcu et al., 2009). On the other hand, very little was known about USP14 and UCH37 until recently. In contrast to RPN11, both USP14 and UCH37 appear to disassemble the chain from its substrate-distal tip, thus progressively shortening the chains. Because UCH37 has much higher activity than USP14 against the model DUB substrate ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub-AMC) (Fig. 1A), it has been challenging to study the relative roles of USP14 and UCH37 on the proteasome. Recently, we developed a method to specifically analyze USP14 activity on the human proteasome, which is described below. To our surprise, USP14 can be activated up to 800-fold by association with proteasome, while free USP14 (USP14 in the absence of proteasome) has negligible activity (Lee et al., 2010) (Fig. 1B). The ‘chain-trimming’ activity of USP14 seems to suppress the proteasome’s degradative capability, probably by reducing the affinity of ubiquitin conjugates for the proteasome (Lee et al., 2010). We hypothesized that inhibiting this ‘enzyme brake’ may be beneficial in disease states that are associated with an increased load on proteasome capacity. Based on this rationale, we developed a high-throughput screening (HTS) method to identify inhibitors of proteasome-bound USP14. We found that a selective small-molecule inhibitor of USP14 stimulates protein degradation *in vitro* and in cells, and also attenuates toxic effects of oxidatively damaged proteins (Lee et al., 2010).

The protocols in this unit provide the critical procedures for identifying selective inhibitors of USP14. The first three protocols describe the assays and steps for small-molecule inhibitor screening of USP14: the DUB assay for proteasome-bound USP14 (Basic Protocol 1), the HTS procedure for identifying small molecule inhibitors of USP14 (Basic Protocol 2), and post-HTS analysis to identify selective USP14 inhibitors (Basic Protocol 3). The next three protocols describe methods for purifying human 26S proteasomes (Support Protocol 1), recombinant human USP14 (Support Protocol 2), and the substrate Ub-AMC (Support Protocol 3) (Fig. 2).

BASIC PROTOCOL 1 MEASUREMENT OF DEUBIQUITINATION ACTIVITY OF USP14

This protocol describes how to measure USP14 activity using Ub-AMC as a substrate. The assay is based on USP14’s catalytic cysteine-catalyzed liberation of a fluorogenic moiety (AMC) from its quenched state (Ub-AMC). Free USP14 has little activity but can be highly activated by association with the proteasome holoenzyme (Fig. 1B).

It is important to measure the kinetic parameters of the enzymes before performing HTS (Inglese et al., 2007b). The optimal concentrations of enzymes and substrates can be determined from the kinetics and will improve the assay performance and sensitivity for screening. Also, it is generally recommended to determine Z' -factor before performing HTS since this value is a useful indicator for quality assessment of assay conditions (Zhang et al., 1999).

Note: The scale in this protocol is highly miniaturized with a total volume of 20 μ l per well, employing the low-volume 384-well format.

Materials

Ub-VS-treated human proteasomes (VS-26S; approximately 200 ~ 400 nM) (See Support Protocol 1; Fig. 2A)

Recombinant USP14 (approximately 15 ~ 30 μ M) (See Support Protocol 2 or commercial; Fig. 2C)

Purified Ub-AMC (approximately 150 ~ 250 μ M) (See Support Protocol 3 or commercial; Fig. 2B)

Ub-AMC buffer (see recipe)

0.25 M ATP-MgCl₂ (see recipe)

384-well microplate, low-volume, flat bottom, non-binding, black (Corning, 3820)

Envision plate reader (Perkin Elmer)

1. On the day of the assay, estimate the required amount of materials and thaw appropriate amounts of VS-26S, USP14, and Ub-AMC. The total volume for each assay well will be 20 μ l, and the final concentration of USP14, VS-26S, and Ub-AMC is 15 nM, 1 nM, and 0.8 ~ 1 μ M, respectively. Put the reagents on ice.

Note: Plan for at least triplicate experiments per condition.

2. Prepare the assay buffer by freshly adding DTT and ATP-MgCl₂ (final concentration 1 mM each) to the Ub-AMC buffer.

ATP enhances stability of the proteasome during the assay, but is not required for USP14 activity per se.

3. Prepare a 30 nM solution of recombinant USP14 by diluting the stock protein in Ub-AMC assay buffer. Dispense 10 μ l of USP14 solution into each well of the 384-well plate.

Ovalbumin in the assay buffer serves as a carrier protein to prevent non-specific protein binding. We also found that a non-binding 384-well plate gives more consistent results by minimizing assay-to-assay and well-to-well variations.

4. Prepare a 2 nM solution of VS-26S containing 1.6 ~ 2 μ M of Ub-AMC substrate by diluting the stock proteins in Ub-AMC assay buffer.

Appropriate assay controls may include no USP14, no VS-26S, and no USP14/Vs-26S.

5. Dispense 10 μ l of the VS-26/Vs-AMC mixture into each well containing USP14 to initiate the reaction.

The total volume for each well is 20 μ l, and the final concentration of USP14, VS-26S, and Ub-AMC is 15 nM, 1 nM, and 0.8 ~ 1 μ M, respectively.

6. Monitor the Ub-AMC cleavage in a real time by measuring fluorescence at Ex365/Em460 with an Envision plate reader equipped with a mirror with an appropriate bandpass cutoff (e.g. LANCE/DELFI, 400 nm).

The Envision plate reader enables the researcher to read the fluorescence in real time. Fluorescence increases as Ub is cleaved from AMC. Under these assay conditions, USP14 activity is linear for ~90 min.

7. Determine the specific activity of proteasome-associated USP14-dependent Ub-AMC hydrolysis by subtracting the signal produced by VS-26S alone (e.g. no USP14 addition) in a given time. This corrects for any activity due to residual UCH37 activity in the VS-26S prep.

The increase in fluorescence signal should be linear over time. USP14 alone at 15 nM concentration should have virtually no Ub-AMC hydrolysis activity.

BASIC PROTOCOL 2 HIGH-THROUGHPUT SCREENING OF SMALL-MOLECULE INHIBITORS OF PROTEASOME ASSOCIATED USP14

We recommend that new screeners familiarize themselves with general principles of high throughput screening, using resources such as those posted here (<http://iccb.med.harvard.edu>). The schematic workflow for USP14 inhibitor screening is shown in Figure 3. We recommend performing a pilot screen of 2,000 ~ 3,000 compounds to ensure that assay conditions are sufficiently optimized. Replicates of each screening plate are also critical to minimize the assay variability and reduce imprecision (Malo et al., 2006). Compound libraries are available from various commercial sources and elsewhere including the National Institutes of Health.

Note: Small-molecule inhibitor screening for proteasome-associated USP14 was performed in the ICCB-Longwood screening facility at Harvard Medical School (<http://iccb.med.harvard.edu>). Twenty plates in duplicate (i.e. total 40 plates) in 384-well plate format were screened per day for a total of 63,052 library compounds (Lee et al., 2010).

Materials

- Ub-VS-treated human proteasomes (VS-26S; See Support Protocol 1; Fig. 2A)
 - Recombinant USP14 (See Support Protocol 2 or commercial; Fig. 2C)
 - Purified Ub-AMC (See Support Protocol 3 or commercial; Fig. 2B)
 - Ub-AMC buffer (see recipe)
 - 0.25 M ATP-MgCl₂ (see recipe)
 - 0.5 mM acetic acid (optional for Step 5, see recipe)
 - 2.5 M Sodium chloride (NaCl) (optional for Step 5, see recipe)
 - Compound libraries dissolved in dimethyl sulfoxide (DMSO) in 384-well plate format (various sources, see <http://iccb.med.harvard.edu>)
 - 384-well microplate, low-volume, flat bottom, non-binding, black (Corning, 3820)
 - Matrix WellMate liquid dispenser (Thermo Scientific) (Rudnicki and Johnston, 2009)
 - WellMate, 8-channel, small-bore disposable tubing cartridge, pre-sterilized (Thermo Scientific) (Rudnicki and Johnston, 2009)
 - Compound Pin Transfer Robot (Seiko, see <http://iccb.med.harvard.edu>) (Rudnicki and Johnston, 2009)
 - Envision plate reader (Perkin Elmer)
1. On the day of the screen, estimate the amount of materials required for assays. See Basic Protocol 1 “MEASUREMENT OF DEUBIQUITINATION ACTIVITY OF USP14” for details. Thaw VS-26S, USP14, and Ub-AMC, and put them on ice.

Compound library plates should be thawed in a desiccated container at room temperature.

You will need an additional amount of reagents to account for dead volumes in the liquid dispensing equipment. These should be calculated in advance of the screen (see Step 3).

2. Prepare the assay buffer by freshly adding DTT and ATP-MgCl₂ (1 mM final concentration each) to Ub-AMC buffer.
3. Prepare a 30 nM solution of recombinant USP14 by diluting the stock protein in Ub-AMC assay buffer. Dispense 10 μ l of USP14 solution into each well of small volume non-binding 384-well plates in duplicate using the Matrix WellMate liquid dispenser equipped with an 8-channel small-bore tubing cartridge.

The priming volume for filling the tubing cartridge is approximately 6 ~ 10 ml, which can be recovered, and an additional 0.1 ml of reagent between each microplate is an automated priming solution, which cannot be recovered.

4. Transfer 33.3 nl of each compound from the library into the replica plate A and B using a Seiko Pin Transfer Robotic system.

Generally the last two columns of each library plate are empty or filled with DMSO to allow placement of positive and negative assay controls in the destination plates. Assuming that each library compound is 5 mg/ml (approximately 10 mM) in DMSO, the final test compound concentration after transfer will be about 17 μ M (the final assay volume is 20 μ l).

Once compound is added, plates can sit up to 45 min at room temperature to allow batch processing of plates. Considering that it takes 45 to 60 sec per plate to add compound, a total of 40 plates can be processed in batch.

5. Prepare the reagent mixture containing 2 nM of VS-26S and 1.6 μ M of Ub-AMC. Initiate the enzyme reaction by dispensing 10 μ l of the reagent mixture into wells using a WellMate liquid dispenser. Incubate for 30 ~ 45 min at room temperature.

The final concentration of USP14, VS-26S, and Ub-AMC is 15 nM, 1 nM, and 0.8 μ M, respectively. Optionally, the reaction can be terminated by adding final 0.08 ~ 0.1 mM of acetic acid or final 0.3 ~ 0.5 M of NaCl per well.

6. Measure the Ub-AMC hydrolysis at Ex355/Em460 using an Envision plate reader. Once the primary screen is performed, data are processed for analysis. Define the primary hits by 'robust' Z-score analysis as described (Malo et al., 2006). Briefly, the raw measurements of each sample from two replicates are averaged, and the Z-score is calculated by $(X_i - X)/MADE$, where X_i is the averaged raw measurement of each sample, X is the median, and MADE is an adjusted median absolute deviation for each replicate plate. This normalization can be performed for each plate to adjust for possible plate-to-plate variation. The authors defined strong hits as Z-score < -10, medium hits as $-10 < Z < -7$, and weak hits as $-7 < Z < -3.5$.

A median-based normalization method was employed because many small-molecules in a compound library are autofluorescent within the wavelength range of the AMC fluorophore.

BASIC PROTOCOL 3 SECONDARY SCREENING AND ANALYSIS

Once active compounds are identified, the primary hits are analyzed in several ways: 1) validation of hits that reproduce USP14 inhibition and removal of false positives (the vast majority of hits will pass this test), 2) counter screening to identify selective USP14 inhibitors (the vast majority of hits will fail this test), and 3) analysis of potency. The protocol in this unit briefly describes the steps that we employed in characterization of USP14 inhibitors (Lee et al., 2010).

Note: Unless otherwise noted, all the assays in Basic Protocol 6 can be performed in the Ub-AMC assay buffer containing freshly prepared 1 mM DTT and 1 mM ATP-MgCl₂, and the final reaction volume is 20 μ l.

Materials

- Ub-VS-treated human proteasomes (VS-26S; See Support Protocol 1; Fig. 2A)
 - Recombinant USP14 (See Support Protocol 2 or commercial; Fig. 2C)
 - Purified Ub-AMC (See Support Protocol 3 or commercial; Fig. 2B)
 - Ub-AMC buffer (see recipe)
 - 0.25 M ATP-MgCl₂ (see recipe)
 - AMC amine (Sigma)
 - Cherry pick compounds in DMSO as the primary hits from the screening facility
 - IsoT/USP5, human (Boston Biochem)
 - Ubiquitin, bovine (Sigma)
 - 384-well microplate, low-volume, flat bottom, non-binding, black (Corning, 3820)
 - Envision plate reader (Perkin Elmer)
 - Scientific curve fitting software (GraphPad Prism or SigmaPlot)
1. To identify false positives that arise from quenching of AMC fluorescence by the test compound, dissolve the AMC amine in DMSO and aliquot 50 nM into the wells of 384-well plates. Add 17 μ M (final concentration) of each primary hit into the wells and measure the AMC fluorescence at Ex355/Em460 using an Envision plate reader. In parallel, test DMSO only for comparison.
 - Compounds that quench AMC can be scored as false positives and excluded from further analysis.
 2. Retest and confirm the primary hits for inhibition of proteasome-associated USP14 activity in a dose- and time-dependent manner as described in Basic Protocol 1.
 - For example, the potential USP14 inhibitors can be tested at 0, 8, 17, and 34 μ M for dose-dependent inhibition. This step will further eliminate the false positives (i.e. compounds without reproducible activity) and the remaining hits can be defined as true hits in the USP14 assay.
 3. To determine the specificity of USP14 inhibitors, perform secondary screening of each true hit against human IsoT (USP5), a closely related DUB. Place 1.5 nM of IsoT or 30 nM of USP14 side by side into wells of 384-well plates. Activate IsoT by adding 0.01 ~ 0.02 μ M of free ubiquitin to the reaction. Incubate each USP14 inhibitor with the enzymes in a dose-dependent manner (e.g. 0, 8, 17, and 34 μ M) and preincubate for 30 ~ 45 min.

4. Add 1 μ M of Ub-AMC for IsoT and 2.5 nM of VS-26S plus 1 μ M of Ub-AMC for USP14 to initiate the DUB reaction. Measure the AMC fluorescence at Ex355/Em460 every minute in a real time for 30 ~ 45 min using an Envision plate reader. To compare each inhibitor's selectivity for USP14 over IsoT, calculate the percent inhibition at a given time as described in Basic Protocol 1.

Only compounds having significant selectivity for Usp14 over IsoT should be considered for further counter screening, if necessary, with a panel of other DUBs. Note that VS-26S is only added in USP14 reaction since IsoT does not need proteasome to be activated.

5. For measuring IC₅₀ values, titrate 9 concentrations of each USP14 inhibitor (e.g. 100 μ M to 50 nM for proteasome-bound USP14 and 2 mM to 50 nM for IsoT). Preincubate the inhibitors with corresponding DUBs for 30 min. Initiate the reaction by adding 2.5 nM of VS-26S plus 1 μ M of Ub-AMC for USP14 and 1 μ M of Ub-AMC for IsoT as described in Step 4. Measure the fluorescence by monitoring the reactions every minute at room temperature for 30 ~ 45 min in real time using an Envision plate reader. Perform experiments in triplicate and represent the percent inhibition as mean $\pm$ standard deviation. Determine the IC₅₀ values by obtaining dose-response curves with the percent inhibition for each inhibitor and then fitting the curves by scientific graph analysis software (e.g. GraphPad Prism or SigmaPlot) according to the program's instructions.

The authors strongly recommend the screeners review the guidelines from NIH Chemical Genomics Center (<http://www.ncbi.nlm.nih.gov/books/NBK53196/>) for more information about kinetic analysis of the inhibitors.

SUPPORT PROTOCOL 1 PURIFICATION OF HUMAN 26S PROTEASOMES THAT LACK ENDOGENOUS USP14 AND ARE DEVOID OF UB-AMC HYDROLYSIS ACTIVITY

The purpose of this protocol is to prepare defined human proteasomes for selectively monitoring USP14 activity through an *in vitro* reconstitution system. Native human 26S proteasomes can be isolated from 293T cells that stably express epitope-tagged RPN11 (Wang et al., 2007). For proteasomal DUB assays, unless otherwise noted, the substrate of choice is Ub-AMC, which is the most universal commercially available DUB substrate (see Basic Protocols 1 and 2). Endogenous USP14 can be stripped off the proteasome by salt treatment during purification. This step eliminates interference from endogenous USP14 in the later DUB assay and also enables one to add back different recombinant USP14 proteins, such as wild type or a catalytically inactive mutant (Fig. 1B). After salt washing, two other DUBs remain associated with human proteasomes, RPN11 and UCH37. Of these, only UCH37 can cleave Ub-AMC. UCH37 needs to be inactivated because this UCH-type DUB is highly active for Ub-AMC hydrolysis, and therefore cannot be distinguished from USP14 activity in the reconstitution assay (Fig. 1A). To inhibit UCH37 activity, proteasomes on the beads are treated with Ub-VS, which is a commercially available active-site-directed inhibitor that irreversibly inactivates thiol protease DUBs. After removing residual Ub-VS, the 26S proteasome can be eluted from the beads. Purified 26S proteasomes can then be assessed for residual DUB activity from UCH37 by assaying Ub-AMC cleavage (see Basic Protocol 1). Furthermore, native gels can be performed to confirm the integrity and peptidase activity of the proteasome (Fig. 2A) (Elsasser et al., 2005).

Materials

293T cells stably expressing RPN11 appended with C-terminal TEV cleavage and biotinylation sites (Lan Huang, UC Irvine)

Ice-cold PBS buffer

Proteasome lysis buffer (see recipe)

0.25 M ATP-MgCl₂ solution (see recipe)

Protease inhibitor cocktail, complete tablet (Roche) or equivalent

Proteasome wash buffer (see recipe)

Proteasome elution buffer (see recipe)

Immobilized NeutrAvidin resin (Thermo Scientific)

Ubiquitin-vinyl sulfone (Ub-VS) (Boston Biochem)

TEV protease (Invitrogen)

Refrigerated centrifuge (Sorvall RC-5B or equivalent)

Cell scraper (BD Falcon)

50 ml polypropylene centrifuge tubes (Corning and Thermo Scientific)

Dounce homogenizer, type B pestle (Pyrex or Wheaton)

Econo-Column for chromatography (Bio-Rad)

30°C incubator or water bath

Note: All purification steps are conducted on ice or in the cold room at 4°C, unless otherwise noted.

Harvest and lyse 293 cells expressing tagged RPN11

- 1 Plate cells at an initial density of $1 \sim 1.5 \times 10^7$ cells per plate in 33 plates (150 × 25 mm). Grow cells ~72 hr until confluent. Wash cells once in ice-cold PBS, and then scrape cells in ice-cold PBS (3 ~ 5 ml per plate) in a 50 ml polypropylene centrifuge tube. Centrifuge the cells 15 min at $3,300 \times g$, 4°C in Sorvall RC-5B.

The total packed cell volume is about 15 to 16 ml from 33 plates at the density indicated above.

- 2 Resuspend the cell pellet in 2 volumes of chilled proteasome lysis buffer containing protease inhibitors, 1 mM DTT, and 5 mM ATP-MgCl₂, and transfer to Dounce homogenizer.
- 3 Lyse the cells by stroking 15 times up and down with a Dounce homogenizer (type B pestle). Transfer the lysate into a polypropylene centrifuge tube, and incubate the homogenate on ice for 10 min.
- 4 Clear the lysate by centrifuging 15 min at $20,000 \times g$, 4°C in the Sorvall RC-5B and collect the supernatant.
- 5 Prepare ~2 ml slurry of immobilized NeutrAvidin resin (i.e. about 1 ml bed volume) by washing with 20 bed volumes of chilled lysis buffer on Econo-chromatography column.

Instead of NeutrAvidin resin, regular streptavidin resin can be used (Wang et al., 2007). NeutrAvidin resin is supposed to cross-react less with nonspecific proteins, according to the manufacturer.

Isolate proteasome holoenzyme and treat with Ub-VS

- 6 Incubate the cleared lysate with washed resin in a polypropylene centrifuge tube for at least 2 hr by gently rotating in the cold room.
- 7 Pack the affinity resin into a chromatography column by gravity flow in the cold. Wash the beads with 20 bed volumes of proteasome lysis buffer containing protease inhibitors, 1 mM DTT, and 5 mM ATP-MgCl₂.

Proteasome lysis buffer contains 100 mM NaCl, which is sufficient to strip off most endogenous USP14 from the proteasome.

- 8 Wash the beads with 10 bed volumes of proteasome wash buffer containing 1 mM ATP-MgCl₂ in the cold, and then wash the beads with 5 bed volumes of proteasome elution buffer containing 1 mM ATP-MgCl₂ at room temperature.
- 9 Add about 0.5 to 1 bed volume of proteasome elution buffer containing 1 mM ATP-MgCl₂ and 1.5 ~ 2.5 μ M of Ub-VS (1.5 ~ 2 ml total volume accounting for beads). Incubate the Ub-VS containing resin for 2 hr at 30°C with occasional agitation.

This step is required to inhibit basal DUB activity from UCH37. The above concentrations of Ub-VS were determined empirically after purifying more than 100 batches of proteasomes. Ninety percent of basal DUB activity should be eliminated at a minimum. The inhibitory efficiency appears to depend on the purity of proteasome on the beads, each lot of commercial Ub-VS, and some technical variations from preparation to preparation.

- 10 Wash the beads with 30 bed volumes of proteasome elution buffer containing 1 mM ATP-MgCl₂.

This step removes residual Ub-VS. It is important that it is removed or it will interfere with USP14 DUB activity in the reconstitution assay.

Elute proteasome holoenzyme from beads

- 11 Prepare 2 bed volumes of proteasome elution buffer containing 1 mM ATP-MgCl₂ with 6 μ l of TEV protease (10 U/ μ l). Add to the beads and incubate for 1 hr at 30°C with occasional agitation.
- 12 Collect 4 ~ 5 fractions of about 0.5 ml each, measure the protein concentration, and pool the desired fractions.

Generally the second and third fractions contain most of the eluted proteasome. Alternatively the combined fractions can be concentrated by ultrafiltration.

- 13 Add glycerol to a final concentration of 10%, measure the protein concentration, prepare multiple aliquots, and snap freeze them in liquid nitrogen for storage at -80°C.

The proteasome is abundant in cells, constituting 0.2 ~ 0.5% of total protein. About 0.6 ~ 1 mg of pure 26S proteasome from the above scale prep can be obtained.

After purification, the yield of proteasome should be measured and compared from each batch. Prepare multiple aliquots of different volumes for single use. Generally, the proteasome is concentrated at about 200 ~ 400 nM (molecular weight as ~2.5 MDa), and can be stored at least for a year at -80°C without any detectable instability.

SUPPORT PROTOCOL 2 PURIFICATION OF RECOMBINANT HUMAN USP14 FROM *E. coli*

High quality recombinant USP14 can be easily purified from *E. coli*, yielding about 1 mg of USP14 protein per liter of bacterial culture (Fig. 2C). Free USP14 has little activity but can be activated several hundred fold by binding to the proteasome (Lee et al., 2010) (Fig. 1B). This protocol describes the method for purifying glutathione S-transferase (GST)-tagged USP14; GST can be removed through cleavage with thrombin. Recombinant USP14 is also commercially available (Boston Biochem and Enzo Life Sciences).

Materials

Competent *E. coli* strains suitable for GST-USP14 expression (e.g. BL21 or Rosetta 2 series from Novagen)

Isopropyl- β -D-1-thiogalactopyranoside (IPTG) (various commercial sources): prepare 1 M solution in sterilized water, and store it at -20°C

Ice-cold PBS buffer

Protease inhibitor cocktail, complete tablet (Roche) or equivalent

Lysozyme (various commercial sources): prepare 10 mg/ml in water or add directly to lysis buffer at 1 mg/ml as powder

Glutathione-Sepharose 4 Fast Flow resin (GE Healthcare)

Benzamidine-Sepharose 6B resin (GE Healthcare)

Thrombin cleavage buffer (see recipe)

Thrombin (R&D Systems)

Reduced glutathione (Sigma)

GST elution buffer (see recipe)

Temperature adjustable shaking incubator that can hold 2 ~ 2.6 L flasks (e.g. I 26 series, Fisher Scientific)

Refrigerated centrifuge (Sorvall RC-5B or equivalent)

French press or sonicator with microtip for cell lysis

Polypropylene centrifuge tubes and bottles (15 ml, 50 ml, and 1 L; Corning and Thermo Scientific)

Econo-Column for chromatography (Bio-Rad)

Disposable Micro Bio-Spin column (Bio-Rad)

Amicon Ultra centrifugation filter (Millipore)

Note: All purification steps are conducted on ice or in the cold room at 4°C , unless otherwise noted.

Purify recombinant human USP14 with GST tag removed

- 1** Grow 1 L of *E. coli* cells in 2 ~ 2.6 L flask transformed with a desired expression vector (e.g. pGEX-USP14; Lee et al., 2010) under antibiotic selection at 37°C until OD₆₀₀ reaches 0.6 ~ 0.8. Add IPTG to a final concentration of 1 mM to induce protein expression and grow for 12 ~ 15 hr at room temperature in a shaking incubator.
- 2** Collect the cells in 1 L polypropylene centrifuge bottles by centrifuging 15 min at 3,300 × g, 4°C. Discard the supernatant and resuspend the cell pellets with 30 ~ 40 ml of ice-cold PBS containing protease inhibitors and 1 mM DTT.
- 3** Lyse the cells with two passes through a French press at 1,000 ~ 1,200 psi.
- 4** (*Alternative lysis*) Add lysozyme to final concentration of 1 mg/ml and incubate on ice, 20 min. Sonicate the cells according to the sonicator manufacturer's instructions until the sample is no longer viscous.

Avoid frothing and overheating during sonication.
- 5** Clear the lysate by centrifuging 20 min at 20,000 × g, 4°C, in a 50 ml polypropylene centrifuge tube and collect the supernatant in a 50 ml polypropylene conical tube. Meanwhile, prepare 1 ml of Glutathione-Sepharose resin by washing with 20 bed volumes of PBS.
- 6** Combine the washed resin with the cleared lysates and incubate with rotation for 1 hr in the cold room.
- 7a** Pack the resin-containing lysate into a chromatography column by gravity flow in the cold room. After packing the column, wash the beads with 20 to 50 ml of cold PBS, then wash with 20 to 50 ml of cold PBS containing an additional 100 mM NaCl, and wash again with 10 ml of cold PBS. Finally, wash with 5 ml of thrombin cleavage buffer in the column at room temperature.
- 8a** Prepare 2 bed volumes of thrombin cleavage buffer containing 10 µl of 1 U/µl thrombin protease. Add to the beads and incubate at least 2 hr at room temperature with occasional agitation.
- 9a** Meanwhile, prepare 100 µl slurry of Benzamidine Sepharose by washing with 20 bed volumes of cold PBS. Keep on ice.
- 10a** From step 8a, elute the thrombin-cleaved USP14 in a few fractions of several drops each. Add 1 ml of PBS and collect several additional fractions. Measure the protein concentration of each fraction and pool desired fractions.

The first four fractions will contain most of USP14 proteins, ranging about 0.5 ~ 3.5 mg/ml. The combined fraction volume is approximately 1 ~ 1.5 ml.
- 11a** Add 100 µl of the washed Benzamidine Sepharose resin from step 9a to the pooled fractions and incubate for 30 min with gentle rocking in the cold room.

Benzamidine Sepharose resin will remove the residual thrombin.
- 12a** Remove the Benzamidine-Sepharose resin from the pooled fractions by passing through a disposable Micro Bio-Spin column by gravity or centrifugation.
- 13a** Collect the eluate and concentrate it by ultrafiltration with an Amicon Ultra centrifugation filter (30 KDa cut-off).

The eluate can be dialyzed or buffer exchanged into other buffers depending upon the purpose.

- 14a** Add glycerol to 10% final concentration, measure the protein concentration, prepare multiple aliquots, and snap freeze them in liquid nitrogen for storage at -80°C .

Generally, purified USP14 is concentrated at $15 \sim 30 \mu\text{M}$ (molecular weight as 56 KDa) and can be aliquot in different volumes (e.g. each about $10 \sim 30 \mu\text{l}$) for single use purpose.

The modified protocol below allows purification of intact GST-USP14 without thrombin cleavage

GST-USP14 can be activated by association with the proteasome to the same extent as its GST-cleaved form. Steps 1 to 6 can be performed as above.

- 7b** Pack the resin-containing lysate into the chromatography column by gravity in the cold room. First, wash the beads with 20 to 50 ml of PBS, then wash with 20 to 50 ml of PBS containing 100 mM NaCl, and again wash with 10 ml of PBS in the cold room. Finally, wash with 5 ml of GST elution buffer not containing reduced glutathione at room temperature.
- 8b** Prepare 2 bed volumes of GST elution buffer containing 10 mM of reduced glutathione. Add to the beads and incubate on-column for 10 min at room temperature.
- 9b** Elute the GST-USP14 in one fraction in a 15 ml polypropylene conical tube. Add 2 ml of PBS and collect 4×0.5 ml fractions. Measure the protein concentration of each fraction and pool the desired fractions.
- 10b** Concentrate the pooled fractions by ultrafiltration with an Amicon Ultra centrifugation filter (30 KDa cut-off).
- The eluate can be dialyzed or buffer exchanged into another buffer depending upon the purpose.
- 11b** Add glycerol to 10% final concentration, measure the protein concentration, prepare multiple aliquots, and snap freeze them in liquid nitrogen for storage at -80°C .

The molecular weight of GST-USP14 is 73 KDa. Desired concentration and amount of purified GST-USP14 are as described in Step 14a.

SUPPORT PROTOCOL 3 SYNTHESIS AND PURIFICATION OF UBIQUITIN-AMC

Ub-AMC is commercially available (e.g. Boston Biochem), but HTS requires a substantial amount of substrate, which may be unaffordable. The purpose of this protocol is to prepare a large amount of Ub-AMC with relatively inexpensive starting materials. The protocol is composed of two sections: first, generation of ubiquitin (Ub)-thioester using intein chemistry from *E. coli* (modified from the IMPACT-CN manual, New England Biolabs) and second, synthesis and purification of Ub-AMC after reacting purified Ub-thioester with Gly-AMC (Ovva et al., 2005; Wilkinson et al., 2005). Generally, about a 10% yield of highly pure Ub-AMC can be prepared by this method (Fig. 2B).

Materials

Competent *E. coli* strains, BL21 cells or Rosetta 2 cells (from Novagen), suitable for expressing pTYB2-Ub-Intein-chitin binding domain (CBD) or equivalent plasmid

pTYB2 vector (New England Biolabs, N6702S): Intein-fusion protein expressing pTYB vector series

IPTG (various commercial sources): prepare 1 M solution in sterilized water, and store it at -20°C

Ub-intein lysis buffer (See recipe)

Protease inhibitor cocktail, complete tablet (Roche) or equivalent

Chitin binding beads (New England Biolabs)

2-mercaptoethanesulfonic acid, sodium salt (MESNa) (Sigma)

Glycyl-7-amido-4-methyl coumarin (Gly-AMC), hydrobromide salt (Glycosynth or Golden Biotechnology)

N,N-Dimethylformamide (DMF), HPLC gradient (Sigma)

N-hydroxysuccinimide (NHS) (Sigma)

HEPES base, sodium salt (Sigma)

Hydrochloric acid (HCl) (various commercial sources)

Dialysis buffer (see recipe)

Temperature adjustable shaking incubator that can hold 2 ~ 2.6 L flasks (e.g. I 26 series, Fisher Scientific)

Refrigerated centrifuge (Sorvall RC-5B or equivalent)

French press or sonicator with microtip for cell lysis

Polypropylene centrifuge tubes and bottles (15 ml, 50 ml, and 1 L: Corning and Thermo Scientific)

Econo-Column for chromatography (Bio-Rad) Amicon Ultra centrifugation filter (Millipore)

15 ml or 50 ml borosilicate glass tube (Kimble Glass)

SnakeSkin Pleated Dialysis Tubing, 3.5 KDa cut-off (Thermo Scientific) or equivalent

AKTA fast protein liquid chromatography (FPLC) system (GE Healthcare)

HiTrap-SPHP ionic exchange column, 1 ml (GE Healthcare) or equivalent

Note: All purification steps were conducted on ice or in the cold room at 4°C , unless otherwise noted.

Generate ubiquitin-thioesters using intein system in *E. coli*

- 1 Grow 1 L of *E. coli* cells in 2 ~ 2.6 L flask transformed with a desired expression vector (e.g. pTYB2-Ub₇₅-Intein-CBD or equivalent one; this study) under appropriate antibiotic selection at 37°C until OD_{600} reaches ~ 0.6. Add IPTG to a final concentration of 0.5 mM to induce protein expression, and grow at 30°C , 2 ~ 3 hr.

- 2 Collect the cells in 1 L polypropylene centrifuge bottles by centrifuging 15 min at $3,300 \times g$, 4°C . Discard the supernatant and resuspend the cell pellets with 40 ml of Ub-intein lysis buffer containing protease inhibitors.
- Do not add DTT during purification since this will cleave off the intein fusion before the elution step.
- 3 Lyse the cells by performing French press two times at 1,000 ~ 1,200 psi or by sonication with a micro tip in a 50 ml polypropylene tube (see Steps 3 and 4 in Support Protocol 2).
- 4 Clarify the cell extracts by centrifuging 20 min at $20,000 \times g$, 4°C and collect the supernatant. Meanwhile, equilibrate 15 ml of chitin binding resin on the column with 10 bed volumes of Ub-intein lysis buffer.
- 5 Load the cleared supernatant onto the resin and allow it to flow slowly by gravity in the cold room.
- Roughly 0.5 ~ 0.8 ml/min of flow rate will be sufficient to bind most of Ub-intein fusion protein.
- 6 Wash the beads with 80 ml of Ub-intein lysis buffer containing protease inhibitors in the cold, and then wash quickly with 30 to 50 ml of Ub-intein lysis buffer containing 50 mM MESNa at room temperature.
- 7 Apply 1 bed volume of 50 mM MESNa-containing Ub-intein lysis buffer to the resin for on-column cleavage. Incubate at 37°C , overnight, without agitation.
- 8 The next day, elute the Ub₇₅-MESNa thiol ester by gravity. Apply 15 ml of lysis buffer for further elution. Concentrate the eluate with Amicon Ultra centrifugation filter (5 KDa cut-off) until the protein concentration reaches ~ 3 mg/ml. Snap freeze Ub thiol ester in liquid nitrogen for storage at -80°C until use or proceed directly to Step 9 as below.
- Roughly 10 mg of Ub thiol ester can be produced from 1 L of *E. coli* culture. Chitin binding beads can be regenerated and reused several times according to the manufacturer's instructions.

Synthesize and purify Ub-AMC

- 9 For the scale of about 1 ml of Ub-MESNa thioester (~ 3 mg), first dissolve ~ 54 mg of Gly-AMC salt in 4 ml of 70% DMF in borosilicate glass tube through vigorous vortexing. Sonication and/or heating at 37°C may help solubilize Gly-AMC. Next add 200 μl of 2 M NHS in DMF to the Gly-AMC solution, and mix well. Slowly add Ub-MESNa thioester drop-by-drop while vortexing to prevent precipitation. Finally, initiate the reaction by adding 250 μl of 2 M HEPES base (pH is approximately 10).

There can often be solubility or precipitation problems in Gly-AMC solution before and after adding Ub thiol ester. If there is massive precipitation, try to vary the conditions, such as reducing the amount of Gly-AMC, increasing the amount of DMF, or decreasing the amount of Ub thiol ester in each reaction. The precipitation may also depend on the purity of Ub thiol ester, Gly-AMC, and DMF. Use a water bath sonicator for solubilizing the materials. The final pH in the reaction mixture is around 8, and above that, Ub thiol ester may undergo more self-hydrolysis.

- 10 The next day, add 250 μ l of 5% HCl to solubilize any precipitates. Vortex vigorously.
- 11 Spin down 20 min at 20,000 $\times$ g, 4°C to remove any insoluble material. Recover the supernatant.
At this stage, the solution contains Ub-AMC, hydrolyzed Ub₇₅, and unreacted Gly-AMC.
- 12 Dialyze out unreacted Gly-AMC using dialysis tubing (3.5 KDa cut-off) with excess dialysis buffer (e.g. 4 $\times$ 4 L dialysis buffer and each for at least 2 hr) in the cold room.
Residual Gly-AMC after dialysis will be removed during the FPLC run.
- 13 Spin down the dialyzed solution for 30 min at 20,000 $\times$ g, 4°C before loading the FPLC column. Retrieve the supernatant and keep it on ice.
Alternatively, the dialyzed solution can be concentrated, if there is too much liquid volume (e.g. final 1 ~ 2 ml of 1 ~ 2 mg/ml).
- 14 Equilibrate a HiTrap-SPHP column with 10 bed volumes (e.g. 10 ml) of dialysis buffer using FPLC according to the manufacturer's instructions.
Our lab has also successfully purified Ub-AMC with SOURCE S column (GE Healthcare). In principle, any cationic exchange column should work.
- 15 Load the sample onto the column, wash the column with 5 bed volumes of dialysis buffer, and elute with dialysis buffer containing a 0 – 70% gradient of 1 M NaCl with a flow rate of 1 ml/min. Collect each 0.5 bed volume of fractions for a total of 40 ~ 45 fractions.
The major contaminant at this step is hydrolyzed Ub₇₅, which will elute earlier than Ub-AMC due to its C-terminal carboxyl group.
- 16 Measure the protein concentration, collect the desired fractions, concentrate by Amicon Ultra centrifugation filter (5 KDa cut-off), and snap freeze multiple aliquots for storage at –80°C.
Generally, our lab prepares pure Ub-AMC at 150 ~ 250 μ M as aliquots in different volumes (e.g. each about 10 ~ 50 μ l) for single use purpose. Approximately 10% yield of Ub-AMC can be expected from the starting amount of Ub thiol ester. Gel-based staining of purified Ub-AMC with known amounts of controls (e.g. commercial Ub or Ub-AMC) can be performed to check its purity (Fig. 2B). We recommend analyzing the final product by mass spectrometry. Molecular weights of Ub-AMC and hydrolyzed Ub₇₅ are 8725 Da and 8508 Da, respectively.

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes.

Proteasome lysis buffer

50 mM NaH₂PO₄, pH 7.5
100 mM NaCl
5 mM MgCl₂

10% Glycerol
0.5% NP-40
Store up to months at 4 °C

0.25 M ATP-MgCl₂ stock solution

For 10 ml solution, weigh 1.38 g of ATP disodium salt (Sigma, A3377). Add 4.4 ml of water, 2.5 ml of 2 M Tris Base, and 2.5 ml of 1 M MgCl₂ solution. Dissolve it by vortexing, prepare aliquots, and store up to months below –20 °C.

Proteasome wash buffer

50 mM Tris-HCl, pH 7.5
10% Glycerol
Store up to months at 4 °C

Proteasome elution buffer

50 mM Tris-HCl, pH 7.5
Store up to months at 4 °C

Thrombin cleavage buffer

50 mM Tris-HCl, pH 8.0
150 mM NaCl
2.5 mM CaCl₂
0.1% 2-mercaptoethanol
Store up to months at room temperature and add 2-mercaptoethanol just before use

GST elution buffer

50 mM Tris-HCl, pH 8.0
Store up to months at 4 °C

Ub-intein lysis buffer

50 mM HEPES, pH 6.5
100 mM NaOAc
Store up to months at 4 °C

Dialysis buffer

50 mM NaOAc, pH 4.5
Prepare fresh just before use

Ub-AMC buffer

50 mM Tris, pH 7.5
1 mM Ethylenediaminetetraacetic acid (EDTA)
1 mg/ml ovalbumin

1 mM MgCl₂

Store up to months at 4 °C

0.5 mM acetic acid

Dilute glacial acetic acid (approximately 17.5 M; various commercial sources) in water.

Prepare fresh just before use.

2.5 M NaCl

For 100 ml solution, dissolve 13.36 g of NaCl (Sigma, S9888) in water. Store up to months at room temperature

COMMENTARY

Background Information

Among the mammalian proteasome-associated DUBs, USP14 and UCH37 are thiol proteases. Although both enzymes may trim ubiquitin conjugates from the substrate distal tip, their relative contributions on the proteasome have been highly elusive (Lee et al., 2011). Some past studies may have underestimated proteasomal USP14's activity because 1) free USP14 has very little DUB activity, 2) UCH37 on the proteasome has predominant DUB activity over USP14 for the most common model substrate, Ub-AMC (Fig. 1A), a dominance that is not generally observed for physiological substrates such as ubiquitin-protein conjugates, and 3) there have been few efforts to measure specific activity of UCH37 and USP14 on the proteasome. DUB substrates with small leaving groups, such as Ub-AMC, may not reflect the relative physiological activity of DUB activities against ubiquitin-protein conjugates. Recent advances in purification techniques using cell lines expressing a tagged proteasome subunit have made it much more straightforward to purify mammalian proteasome holoenzymes. We developed a reconstitution system that enables USP14 activity to be assessed specifically. In this method endogenous proteasomal USP14 is removed by salt wash and UCH37 is irreversibly inhibited by the treatment of Ub-VS. This VS-26S proteasome, whose basal DUB activities are eliminated, surprisingly revealed that USP14 activity is highly enhanced by the association with proteasome (Lee et al., 2010) (Fig. 1B). Furthermore, this reconstituted system has enabled a high-throughput screening method for small-molecules that specifically inhibit proteasome-bound USP14. In screening 63,000 compounds, we found specific inhibitors for USP14 after counter screening against a panel of other DUBs including UCH37. Among these, compound IU1 was the most thoroughly characterized. IU1 clearly antagonizes the rate of USP14's trimming of ubiquitin chains bound to substrates and promotes their degradation by proteasome *in vitro*. Although direct comparisons of chain trimming by USP14 and UCH37 remain to be addressed, the IU1 data strongly suggest that at least for the substrates examined, USP14 trims the chain more rapidly to the degree that it may be dominant over UCH37. Thus, USP14 is a major chain trimming DUB on the mammalian proteasome, which is in sharp contrast to the observations from the DUB assays performed with the Ub-AMC substrate.

The DUBs are emerging as promising drug targets because many of them have been implicated in human diseases including cancers, infectious diseases, and neurodegeneration (Colland, 2010; Sacco et al., 2010). In principle, DUBs counteracting the turnover of harmful proteins may represent potential drug targets in many disease contexts. Our recent discovery of USP14 inhibitors clearly suggests that proteasomal DUBs can be valid pharmacological targets because USP14 inhibition may potentiate proteasome-mediated protein quality control (Lee et al., 2010). Interestingly, chemical inhibition of proteasomal

DUB activities has also been proposed to confer novel therapeutic strategies for cancer treatment (D'Arcy et al., 2011).

Critical Parameters and Troubleshooting

The quality of reagents—VS-26S, USP14, and Ub-AMC should be purified to high quality for good assay performance (Fig. 2). Careful optimization is especially important for preparation of VS-26S. Although more than 95% of basal proteasome Ub-AMC hydrolysis activity should be eliminated by the treatment with 1.5 ~ 2.5 μ M of Ub-VS, the screener should verify that the Ub-VS preparation indeed has low basal Ub-AMC hydrolysis activity. If the basal DUB activity is too high, it will limit the assay sensitivity, making it difficult to identify the compounds with low or moderate potency. The purity of 26S proteasome on the beads, different lots of commercial Ub-VS, and technical variations during the procedures may affect the efficiency of inhibition of the basal DUB activities. Also it is important that residual Ub-VS after proteasome treatment should be completely removed by applying a sufficient excess of proteasome wash buffer. Otherwise, the remaining Ub-VS will interfere with the USP14 add-back assays in the reconstitution experiments.

For Ub-AMC synthesis, careful attention should be given to the solubility of Gly-AMC in the Ub thiol ester buffer. Precipitation can be avoided by adjusting the ratio of Gly-AMC to DMF and the amount of Ub thioester per each reaction. Other important factors include the purity of Ub thiol esters and the pH of the reaction mixture (e.g. if the pH is too basic, the rate of self-hydrolysis of Ub thioesters will be increased).

Screening feasibility and efficiency—Ub-AMC is commercially available but also can be synthesized from Ub-intein chemistry. In-house production of Ub-AMC is recommended if use of the commercial reagent is cost-prohibitive. Small-volume 384-well plates were chosen for screening to reduce the amount of assay reagents per well by more than 60% (with a typical assaying volume of 15 ~ 30 μ l). This should allow screeners to scale up the number of library compounds for screening.

The Ub-AMC substrate has some limitations that are important to understand. Many library compounds can autofluoresce in the wavelengths of light used to measure AMC fluorescence, causing a potential false negative, or can absorb light and quench fluorescence to score as a false positive. Currently, there are several DUB substrates available from various commercial suppliers (e.g. Boston Biochem, Enzo Life Sciences, Life Sensors, or Invitrogen) that are less prone to these problems, including Ub-rhodamine and Ub-aminoluciferin. Fluorescence polarization-based isopeptide linkage substrates and time resolved-fluorescence resonance energy transfer-based fluorophore-labeled diubiquitins are also available. While these DUB substrates certainly have advantages over Ub-AMC, the starting materials for their synthesis are typically more expensive or the detailed synthesis methods have not been documented.

Well-designed assays and protocols will increase the success rate of USP14 inhibitor screening. The assays should be also validated for reproducibility and robustness to be suitable for HTS; the assay performance can be evaluated from Z' -factor measurement (Zhang et al., 1999; Inglese et al., 2007a). In planning a protocol for HTS, it must be kept simple, with a minimum of steps, because increased assay variability often accompanies extra protocol steps (Fig. 3).

It is helpful to perform test runs of automation steps using buffer alone to ensure proper functioning of liquid dispensing equipment. Also, it is useful to perform an initial pilot screen of 2,000 ~ 3,000 compounds to identify any problems during the screening procedure and to confirm the assay protocol for suitability in HTS. During HTS, it is critical to perform

the assays in replicates to minimize assay imprecision and variation. In addition, placing negative and positive controls in each plate provides an indication of the expected biological response and the efficacy of the assays. IU1 is now commercially available (e.g. Enamine and Boston Biochem) and so should serve as a positive control for future USP14 inhibitor screening. For accurate pin transfer, library plates should be completely thawed and desiccated at room temperature.

Importantly, after all HTS and post-HTS analysis, the final selective USP14 inhibitors should be re-ordered as dry powders from the original vendor of the relevant compound library and retested for their ability to inhibit USP14 activity; sometimes breakdown products or modified compounds (e.g. oxidized forms), are responsible for inhibition of USP14's DUB activity.

Anticipated Results

Support Protocols 1 to 3 are straightforward to perform and generate high purity reagents. One liter of monolayer culture of 293 cells containing tagged RPN11 typically yields up to 1 mg of pure VS-26S, and 1 L of bacterial culture transformed with USP14 plasmid will produce about 1 mg of recombinant protein. Ub-AMC synthesis is a rather challenging task because it includes multiple steps and produces a lower yield. Roughly 1 mg of pure Ub-AMC can be synthesized starting from 1 L of *E. coli* culture. Figures 2 and 4 summarize the typical results of representative preparations and the approximate amounts required for screens of different scales. Basic Protocol 1 enables screeners to pilot the USP14 DUB assay evaluate the performance of the reagents prepared in the previous protocols. Basic Protocol 2 generates the primary hits from the HTS compound screen (Lee et al., 2010). The hits can be verified through Basic Protocol 3 to identify selective and potent USP14 inhibitors. Figure 3 describes the workflow for carrying out USP14 inhibitor screening.

Time Considerations

The time required to prepare the amount of the reagents for HTS depends on the scale of the screen (Fig. 4). For a screen of 100,000 compounds, USP14 preparation is the fastest and will take a week or less. VS-26S and Ub-AMC together are more time consuming and labor intensive, and will likely require a few months to prepare, optimize, and validate. Once all assay reagents are prepared, the HTS will take a couple of weeks if 20 plates of compound libraries are screened each day in duplicate and the screening is carried out five days per week. It should be noted that, depending most likely on the DUB to be screened, the identification of potent and selective inhibitors may require screening of well over 100,000 compounds. Post-HTS analysis and secondary screening depend especially on the time required to obtain the larger amounts of each compound required for follow-up work; several weeks to months may be required to confirm the true hits and identify the selective USP14 inhibitors.

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A **B** **Figure 1.**

DUB assays with Ub-VS-untreated 26 proteasome (A, 26S) or Ub-VS-treated 26 proteasome (B, VS-26S) in the USP14 reconstitution system. 1 nM of 26S or VS-26S in the absence or presence of indicated concentrations of recombinant USP14 wild-type (WT) or catalytic inactive mutant (CA) was analyzed for the Ub-AMC hydrolysis in a given time. 1 μ M of Ub-AMC was added as substrate. Note that most of the DUB activity of 26S in (A) comes from proteasome-associated UCH37 since RPN11 does not cleave Ub-AMC. RFU indicates relative fluorescence unit. Detailed descriptions for the assay reagents and the DUB assay can be found in the Protocols.

Figure 2. Representative preparations of VS-26S (**A**), Ub-AMC (**B**), and recombinant USP14 (**C**). (**A**) Native gel analysis of 26S and VS-26S. 7 μ g of the proteasome was analyzed for in-gel peptidase activity with LLVY-AMC as the substrate (left) or for Coomassie Brilliant Blue staining (middle, CBB) on a 3.6% native gel. SDS-PAGE analysis of 7 μ g of purified proteasome is shown (right, CBB). (**B**) SDS-PAGE analysis and CBB staining of purified Ub thiol esters (2 μ g), and 1 μ g of purified Ub-AMC and HA-tagged Ub-AMC (HA-Ub-AMC). 1 μ g of free Ub was loaded for comparison. (**C**) SDS-PAGE analysis and CBB staining of purified USP14 WT and CA. A known amount of BSA was loaded for comparison. RP₂CP indicates doubly-capped proteasome holoenzyme.

Figure 3. Workflow of the HTS for small-molecule inhibitors of proteasome-associated USP14. The screening procedures were based on the equipment at the ICCB-Longwood Screening Facility, Harvard Medical School (<http://iccb.med.harvard.edu>).

Figure 4.
Schematic flowchart of the required amount of assay reagents for a given scale of screen.