UbiQ
targeting the ubiquitin system

DUB activity and DUB inhibitor profiling using SDS-PAGE based assays

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applicable for UbiQ probes
reference Leestemaker Y, De Jong A, Ovaa H. Profiling the activity of deubiquitinating enzymes using chemically synthesized ubiquitin-based probes. Methods In Molecular Biology (Manuscript accepted for publication)

materials

1. cell harvesting and lysis

1. Cell line of choice, cultured in appropriate medium, e.g. RPMI 1640 (Roswell Park Memorial Institute) medium for suspension cell lines and DMEM (Dulbecco's modified Eagle's medium) for adherent cell lines, supplemented with fetal calf serum (FCS).
2. Stock solution of inhibitor of choice, dissolved in dimethyl sulfoxide (DMSO), aqueous buffer or medium, in the appropriate concentration.
3. Phosphate-buffered saline (PBS), trypsin solution (0.05%, Gibco) for adherent cells, appropriate cell culture medium.
4. HR lysis buffer (see Note 3): 50 mM Tris–HCl (pH 7.4), 5 mM MgCl2, 250 mM sucrose. Optional supplements (see Note 4): 0.5% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), 0.1% NP40, 1 mM DTT (added fresh from a 1 M stock solution before use), 2 mM ATP (added fresh from a 0.5 M stock solution before use), protease inhibitors. Prepare HR buffer without DTT, ATP, or protease inhibitors, filter over a 0.22 mm filter, and store at 4°C.
5. Sonication equipment (e.g. Bioruptor, Diagenode).
6. Protein concentration determination assay reagents (e.g. Bio-Rad protein assay).

2. in vitro profiling of DUB activity in cell lysates

1. Cell lysate obtained in paragraph 1, HR lysis buffer (see Note 4 and 5).
2. Stock solution of 0.25 mg/mL TAMRA-Ub-VME probe in 50 mM sodiumacetate (pH 4.5, 5% DMSO) (see Note 6).
3. 50 mM NaOH.
4. 3x reducing sample buffer (see Note 7): 4X NuPAGE® LDS Sample Buffer, 2-Mercaptoethanol, MQ (75:17.5:7.5). Store at room temperature.

3. assessment of DUB inhibitor potency in cell lysates using TAMRA-Ub-VME

1. Cell lysate obtained in paragraph 1, HR lysis buffer, supplemented with 0.5% CHAPS and 0.1% NP40 (see Note 4 and 5). Optional supplements (see Note 4): 1 mM DTT (added fresh from a 1 M stock solution before use), 2 mM ATP (added fresh from a 0.5 M stock solution before use), protease inhibitors (e.g. Complete protease inhibitor cocktail, Roche). Prepare HR buffer without DTT, ATP, or protease inhibitors, filter over a 0.22 mm filter, and store at 4°C.
2. 20x stock solution of inhibitor of choice, dissolved in DMSO, aqueous buffer or medium.
3. Stock solution of 0.125 mg/mL TAMRA-Ub-VME probe in 50 mM sodiumacetate (pH 4.5, 5% DMSO) (see Note 6).
4. 50 mM NaOH.
5. 3x reducing sample buffer (see Note 7).

4. gel electrophoresis and in-gel fluorescence scanning

1. Precast gel system (NuPAGE, Invitrogen), 4-12% NuPAGE® Novex® Bis-Tris precast protein gel (1.0 mm) (Invitrogen).
2. NuPAGE® MOPS SDS Running buffer (Invitrogen).
3. NuPAGE® Antioxidant (Invitrogen).
4. SeeBlue® Pre-Stained Standard (Invitrogen) (see Note 8).
5. 3x Reducing sample buffer.
6. Power supply, e.g. PowerPac Basic Power Supply (Bio-Rad).
7. ProXPRESS 2D Proteomic imaging system (Perkin Elmer).
8. TotalLab analysis software.

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UbiQ
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method

1. cell harvesting and lysis

1. Culture cell line of choice in appropriate medium and under appropriate culture conditions. Suspension cells should be cultured until log-phase and adherent cells should be passaged when approximately 80% confluency is reached.
2. Seed cells in a multi well tissue culture plate and allow the cells to attach. Add the compounds to be tested, dissolved in DMSO (or medium if the compounds are water-soluble), in the desired concentrations to the cells. Make sure to have enough wells available to include all of the appropriate controls.
   : the final concentration of DMSO should not exceed 0.5% as this can interfere with the assay.
3. To harvest adherent cells by trypsinization, aspirate the medium, wash cells with PBS and aspirate. Add sufficient trypsin and wait for cells to detach. After the cells have detached, add medium supplemented with 10% FCS to the cells to inactivate the trypsin. Collect cells and pellet by centrifugation at 1300 rcf for 5 minutes at 4 °C. To harvest suspension cells, pellet cells by centrifugation at 1300 rcf for 5 minutes at 4 °C. Wash cells using 10-20 pellet volumes of PBS and pellet cells again centrifugation at 1300 rcf for 5 minutes at 4 °C. Discard the supernatant.

   Pause point: at this time, cell pellets can be snapfrozen in liquid nitrogen and stored at -20°C until further use (See Note 9).

4. Resuspend cell pellets in two pellet volumes of cold HR buffer (see Note 3). Optional (see Note 4): Supplement the HR lysis buffer with 0.5% CHAPS, 0.1% NP40, 1 mM DTT (add freshly from a 1 M stock solution before use), 2 mM ATP (add freshly from a 0.5 M stock solution before use), and/or protease inhibitors (add freshly, e.g. Complete protease inhibitor cocktail, Roche). Keep samples on ice.
5. Lyse cells by sonication using e.g. a Bioruptor (5 cycles of 30 seconds on and 30 seconds off).
6. Centrifuge cells at 14000 rcf for 15 minutes at 4 °C to remove cell debris. Transfer the supernatant to a fresh eppendorf tube and determine the protein concentration using e.g. the Bio-Rad protein assay or a comparable protein assay according to the manufacturer's instructions.

   Pause point: At this point, lysates can be snap-frozen in liquid nitrogen and stored at −20°C until further use.

   To label DUBs directly proceed to paragraph 2. To determine the effect of DUB inhibitors prior to DUB labelling, proceed to paragraph 3.

2. in vitro profiling of DUB activity in cell lysates using Ub-based DUB probe TAMRA-Ub-VME (See note 10).

1. Add 25 µg of cell lysate to an eppendorf tube and adjust the volume to 22 µL with HR buffer (a final volume of 25 µL and a final protein concentration of 1 mg/mL is obtained after addition of probe/NaOH, step 2 and 3) (see Note 4 and 5).
2. Add 1 µL of a 25 µM TAMRA-Ub-VME solution in sodium acetate buffer (50 mM NaOAc, 5% DMSO, pH 4.5, see Note 6) to the lysate.
3. Add 2 µL (double the volume compared to volume of probe solution) of 50 mM NaOH solution to adjust for the pH drop after addition of the acidic probe solution to the lysate (see Note 5 and 6). Vortex and spin samples briefly. Incubate for 30 minutes at 37 °C..
4. Add 12,5 µL of a 3x reducing sample buffer (e.g. 4x Invitrogen NuPAGE® LDS Sample Buffer, supplemented with 2-mercaptoethanol and MQ) to the reaction mixture and heat the samples for 10 minutes at 70 °C (see Note 7). Centrifuge at 14,000 x *g* for 1 min at room temperature to spin down condensed water droplets and gently vortex the sample.

   Pause point: At this point, reduced and heated samples can be snap-frozen in liquid N2 and stored at – 20 °C until further use.

   Proceed to paragraph 4

3. in vitro assessment of DUB inhibitor potency in cell lysates using TAMRA-Ub-VME

1. Add 25 µg of cell lysate to an eppendorf tube and adjust the volume to 20.75 µL with HR buffer (see Note 4 and 5) supplemented with 0,5% CHAPS, and 0,1% NP40 (for improved solubility of DUB inhibitors, see Note 4), so that a final volume of 25 µL and a final protein concentration of 1 mg/mL is obtained after addition of probe/NaOH (step 2 and 3).
   Critical: The presence of 1 mM DTT can improve inhibitory effect. Check beforehand whether the DUB inhibitor of choice is stable in the presence of DTT (see Note 4).

2. Add 1.25 µL of a 20x stock solution of the desired inhibitor in DMSO. Include a reference sample to which 1.25 µL DMSO, but no inhibitor is added. Vortex and incubate the samples for the desired time period at 37 °C. Typically, samples are incubated for 1h.
   Critical: The quality of labelling will decrease if more than 5% DMSO is present in the reaction mixture.
3. Add 1 µL of a 12.5 µM TAMRA-Ub-VME solution in sodiumacetate buffer (50 mM NaOAc, 5% DMSO, pH 4.5, see Note 6) to the lysate.
   Critical: When non-covalent inhibitors are used, the use of a lower concentration of TAMRA-Ub-VME solution and lower incubation temperatures are preferred (see Note 11), compared to standard DUB labeling (paragraph 2).
4. Add 2 µL (double the volume compared to volume of probe solution) of 50 mM NaOH solution to adjust for the pH drop after addition of the acidic probe solution to the lysate (see Note 5 and 6) Vortex and spin samples briefly. Incubate for 5 minutes at ambient temperature.
5. Add 12,5 µL of a 3x reducing sample buffer to the reaction mixture and heat the samples for 10 minutes at 70 °C (see Note 7). Centrifuge at 14,000 x *g* for 1 min at room temperature to spin down condensed water droplets and gently vortex the sample.

   Proceed to paragraph 4

4. gel electrophoresis and in-gel fluorescence scanning

The following instructions assume the use of the NuPAGE precast gel system and precast protein gels from Invitrogen.

1. Assemble the NuPAGE gel unit using a precast NuPAGE 4-12% Bis-Tris gel according to the manufacturer's instructions.
2. Add 1x MOPS SDS running buffer to both the inner and outer chamber of the gel unit.
3. Add 125 mL antioxidant to the inner gel chamber to keep the samples in a reduced state.
4. Load 10-30 µL (depending on of the size of the wells) of the reduced and heated samples into the wells of the gel.
   When reduced samples were frozen for storage, heat the samples again for 10 minutes at 70 °C, centrifuge at 14,000 x *g* for 1 min at room temperature to spin down condensed water droplets, and gently vortex the sample.
   Keep one well free and load this well with 10 µL of prestained protein molecular weight marker (e.g., SeeBlue® Pre-Stained Standard from Invitrogen, see Note 8).
5. Load 3x reducing sample buffer to remaining empty wells (use a volume of 1/3 of the sample volume).
6. Run the gel at 170–180 V for appropriate time (at least until the blue loading front is no longer visible) using a Power Supply (e.g. PowerPac Basic Power Supply, Bio-Rad)
7. Gently take the gel out from the cassette and image the gel using a fluorescence imager containing appropriate filter settings (λ (ex/em) = 550/590 nm for TMR). For imaging the bands of the protein molecular weight marker SeeBlue® Pre-Stained Standard, image the gel once more using the following filter settings: λ (ex/em) = 625/680 nm.
8. Analyse images using appropriate software.

notes

1. Depending on the reactive moieties present in the building blocks that will be coupled to the N-terminus of Ub, different methods can be used to synthesize Ub-based probes. The Ub sequence is built up from the C-terminus on solid phase using Fmoc-based solid phase synthesis. Method A describes the coupling of building blocks to the N-terminus of Ub directly on solid-phase using Fmoc-based solid phase synthesis, after which the C-terminal warhead is coupled in solution and protecting groups are removed. This applies in case the building blocks that will be coupled to the N-terminus of Ub contain only one free carboxylic acid and other reactive moieties, such as other carboxylic acids and amines, are protected. Method B describes the coupling of the C-terminal warhead to Ub in solution, after which N-terminal building blocks are coupled in solution, followed by deprotection of reactive moieties. Method B applies when the building blocks that will be coupled to the N-terminus of Ub contain an extra unprotected carboxylic acid. For the synthesis of TAMRA-Ub-VME method B is used, since 5-carboxytetramethylrhodamine (TMR) contains two unprotected carboxylic acids. If method A would be used, the free-amine containing warhead that should only be coupled to the C-terminus of Ub will be coupled to the other carboxylic acid of TMR as well.
2. Dry all Fmoc-protected amino acid building blocks overnight under high vacuum. Drying removes moisture, as well as traces of acetic acid (or other acids) that are present, which are detrimental for peptide synthesis.

3. Other lysis buffers en methods can be used. Nonetheless, DUB labelling efficiency should be determined experimentally using other lysis buffers and methods.
4. The use of non-supplemented HR lysis buffer in combination with sonication should be sufficient for lysis of the cells. However, the use of additives can increase lysis efficiency and/or labelling efficiency:
    - Detergents: Detergents CHAPS and NP40 will improve lysis of cells and increases solubility of DUB inhibitors. However, the use of detergents can decrease the quality of DUB labelling using DUB probe TAMRA-Ub-VME and should be determined experimentally. Though, the use of 0.5% CHAPS and 0,1% NP40 does not decrease labelling efficiency.
    - DTT: DUB inhibitory effect can be increased when DTT is used. However, the effect of DTT on the DUB inhibitor should be examined beforehand. The use of DTT does not affect labeling of DUBs in cell lysates. Add DTT freshly before use, since DTT is not stable in solution.
    - ATP: The effect of ATP on labelling is not thoroughly investigated and should be established experimentally.
    - Protease inhibitors: The addition of protease inhibitors is recommended, to protect deubiquitinating enzymes in cell lysate from degradation. Labelling efficiency in the presence of protease inhibitors should be tested beforehand. The use of Complete Protease Inhibitor Cocktail from Roche did not seem to negatively affect labelling efficiency. Add protease inhibitors freshly, e.g. from a 50x stock solution, before use. Manufacturer's instructions should be checked for storage conditions of stock solutions.
5. Different buffers than HR lysis buffer can be used for the incubation of cell lysate with DUB inhibitors and probe. When stronger incubation buffers are used, possibly the addition of NaOH, subsequent to the addition of the acidic probe buffer, becomes redundant. The effect of incubation buffers on the inhibition, labelling of DUBs and solubility of DUB inhibitors should be determined experimentally, however. In addition, other reaction volumes and other concentrations can be used. Changing probe and lysate protein concentrations will affect DUB labelling efficiency. We advise to use different conditions at first, to determine optimal labelling conditions.
6. Other buffers can be used to dissolve Ub-based DUB probes. When a buffer of neutral pH is used, the addition of 50 mM NaOH, after the addition of the acidic probe solution, becomes redundant. Probe solubility and labelling efficiency using other buffers should be experimentally established.
7. Heating samples at 70 °C for ten minutes is optimal for Invitrogen NuPAGE® LDS Sample Buffer containing buffers. When the NuPAGE precast gel system is used, also NuPAGE LDS Sample buffer (or other recommended buffers) should be used to prepare the 3x reducing sample buffer. The use of a different reducing sample buffer can result in improper running of the gel.
8. Other protein molecular weight marker than SeeBlue® Pre-Stained Standard from Invitrogen can be used. However, molecular weight markers that possess similar fluorescence properties to the dyes used in the DUB probe (in case of TRM: λ (ex/em) = 550/590) could contribute to high signal intensities of these markers during fluorescence imaging.
9. Freezing cell pellets could give rise to differential DUB labelling profiles compared to freshly lysed cells. This should be experimentally determined. Frozen cells cannot be taken into culture again. They will not survive, unless frozen in proper freezing medium.
10. Similar labelling conditions can be used for Ub-based DUB probes containing other dyes (e.g.Cy5) or visualization handles (e.g. HA-tag). Use appropriate fluorescence settings for other dyes or other appropriate visualization methods for other handles (e.g. Western Blotting for HAtag or Biotin).
11. Since the covalent binding of the TAMRA-Ub-VME probe to DUBs is very efficient, competition with a non-covalent DUB inhibitor is challenging. When non-covalent inhibitors are used, the use of a lower concentration of TAMRA-Ub-VME probe, shorter probe incubation times, and lower probe incubation temperatures are preferred, compared to the conditions used for standard DUB labelling (paragraph 3). In addition, a DUB inhibitor incubation temperature of 37 °C, and long DUB inhibitor incubation times are beneficial.