Video Article

Method for Measuring the Activity of Deubiquitinating Enzymes in Cell Lines and Tissue Samples

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Abstract

The ubiquitin-proteasome system has recently been implicated in various pathologies including neurodegenerative diseases and cancer. In light of this, techniques for studying the regulatory mechanism of this system are essential to elucidating the cellular and molecular processes of the aforementioned diseases. The use of hemagglutinin derived ubiquitin probes outlined in this paper serves as a valuable tool for the study of this system. This paper details a method that enables the user to perform assays that give a direct visualization of deubiquitinating enzyme activity. Deubiquitinating enzymes control proteasomal degradation and share functional homology at their active sites, which allows the user to investigate the activity of multiple enzymes in one assay. Lysates are obtained through gentle mechanical cell disruption and incubated with active site directed probes. Functional enzymes are tagged with the probes while inactive enzymes remain unbound. By running this assay, the user obtains information on both the activity and potential expression of multiple deubiquitinating enzymes in a fast and easy manner. The current method is significantly more efficient than using individual antibodies for the predicted one hundred deubiquitinating enzymes in the human cell.

Video Link

The video component of this article can be found at https://www.jove.com/video/52784/

Introduction

The ubiquitin-proteasome system (UPS) serves as one of the major degradation pathways in the mammalian cell. Substrates bound for degradation in the proteasome are covalently tagged with polymers of ubiquitin (Ub)[1]. Before the targeted substrate enters the proteasome for degradation, the poly-ubiquitin tag must be removed. A class of enzymes known as deubiquitinating enzymes (DUBs) is responsible for the removal and recycling of ubiquitin molecules[2]. It has been predicted from the human genome that there are nearly a hundred DUBs working in the cell[3]. With such a large number of DUBs controlling Ub-mediated cellular processes, studying these enzymes presents a challenge since mRNA techniques do not give information on activity and western blotting only gives information on expression levels.

The use of influenza hemagglutinin (HA) tagged, Ub-derived active site directed probes allows for a covalent modification of the functional DUBs and therefore gives a direct visualization of the activity of these enzymes on a western blot[4]. The probes have a C-terminal thiol reactive group that serves as a suicide substrate for the active site cysteine residue[5]. With these probes, it is possible to study the activity and potential expression of many DUBs under both pathological and physiological states of the cell.

Changes in DUB activity have been implicated in a range of pathological conditions such as Parkinson's, Alzheimer's, anemia and various cancers[6-10]. This technique provides a powerful tool for the study of disease. In the present paper, we show the application of this technique in HeLa and M17 cells that have been lysed using glass beads. Additionally, we outline how to use this method in mouse spinal cord tissue samples. The information obtained from this technique can be used as a starting point for identifying therapeutic targets as well as establishing models for the study of different disease conditions. The true utility of this technique lies in its ability to provide information on multiple DUBs in a single assay.

Protocol

1. Lysis Buffer Preparation

1. Dissolve sucrose in deionized (DI) water to make a 2 M stock solution. Filter a 2 M solution of sucrose using a vacuum driven 0.22 µm polyvinylidene fluoride (PVDF) filter.
2. Dissolve dithiothreitol (DTT) in DI water to make a 500 mM stock solution and store under anaerobic conditions. Dissolve magnesium chloride ($MgCl_2$) in DI water to make a 100 mM stock solution. Dissolve adenosine triphosphate (ATP) disodium hydrate in DI water to make a 50 mM stock solution.
3. Make up a Tris solution at pH 7.4 by dissolving Trizma hydrochloride in DI water and adjusting the pH using sodium hydroxide (NaOH).
4. Combine portions of the stock solutions in DI water to make a lysis buffer with a sucrose concentration of 250 mM, a DTT concentration of 1 mM, a $MgCl_2$ concentration of 5 mM, an ATP concentration of 1 mM and a Tris concentration of 50 mM. For example, mix 535.3 µl of tris, 500 µl of $MgCl_2$, 1.25 ml of sucrose, 20 µl of DTT, 200 µl of ATP and 7.4947 ml of DI water to make 10 ml of lysis buffer. This can be used for approximately 20 experiments.

2. Culturing Cells for the Experiment

1. Make media by aliquoting 30 ml of DMEM + 10% fetal bovine serum (FBS) into a 50 ml conical tube.
2. Transfer frozen M17 or HeLa cells from liquid nitrogen storage to a small beaker with lukewarm water.
3. Transfer the cells to the 50 ml conical tube. First, add some media to the cell vial. Then transfer the resuspended cells within 5 sec of thawing.
4. Centrifuge the cell suspension at 450 x g for 5 min to obtain a cell pellet. Add 20 ml of media into a T75 flask.
5. Remove the media from the cells using suction. Add 5 ml of fresh media to the cell pellet and resuspend.
6. Transfer the 5 ml cell suspension to the 20 ml of media in the T75 flask and incubate at 37 °C. Change the media every 3 days.

3. Cell Harvesting

1. Remove the media via suction, being careful not to touch the cells.
2. Wash the cells with 5 ml of phosphate buffered saline (PBS). Remove PBS using suction.
3. Add 3 ml of trypsin-ethylenediaminetetraacetic acid (EDTA) to the T75 flask and incubate at 37 °C for 3 min.
4. Add 6 ml of fresh media to the flask and resuspend the cells.
5. Transfer the suspension to a 15 ml conical tube and centrifuge at 290 × g for 5 mins.
6. Remove the supernatant and add 5 ml of PBS. Gently tap the bottom of the tube to break up the pellet. Centrifuge at 290 x g for 5 min.
7. Repeat step 3.6 three times.
8. Remove PBS and resuspend the cells in 1 ml of fresh PBS. Transfer to the Eppendorf tube (1.5 ml). Spin at 290 x g for 5 min.

4. Cell Lysis

1. Measure the approximate volume of the pellet using a pipette and weigh out glass beads in a mass:volume ratio of 1:1. Add twice the volume of the pellet in lysis buffer.
   Note: Add the buffer before adding the glass beads.
2. Lyse the cells in the Eppendorf tube at the maximum agitation for 30 min at 4 °C by vortexing in a cold room.
3. Centrifuge briefly at 200 x g for 30 sec to settle the beads. Collect the supernatant.
4. Add the same volume of glass beads as before to the supernatant.
5. Vortex once again at maximum agitation for 30 min at 4 °C.
6. Centrifuge briefly at 200 x g for 30 sec to settle the glass beads. Collect the supernatant.
7. Centrifuge at 5,030 x g for 5 min to remove the nuclei, membranes and unbroken cells. Collect the supernatant, which is the cell lysate.

5. Tissue Homogenization

1. Make a mass:volume of 1:9 solution of mouse spinal cord tissue to lysis buffer.
   Note: This method is applicable to a variety of different tissue samples.
2. Homogenize the tissue using a setting of level 2 on the homogenizer for 30 sec to ensure that there are no chunks remaining.
3. Spin down at 5,030 x g for 3 min to remove the nuclei, membranes and unbroken cells.
4. Collect the supernatant, which is the lysate.

6. Sample Derivatization

1. Perform a bicinchoninic acid (BCA) in a 96-well plate analysis to determine the protein concentration of the tissue lysate using a colorimetric 96-well plate reader as specified by the Pierce BCA Protein Assay kit protocol.
2. Bring an aliquot corresponding to 20 µg of total protein to 50 µl using de-ionized (DI) water.
3. Add 2 µl of 1.35 µM of HA-Ub-Vinyl sulfone (VS) to the solution and incubate for 1 hr at 37 °C. This results 20 µg of lysate to 50 nM of probe in the final reaction mixture. This is in large molar excess to ensure tagging of functional DUBs.

4. Incubate the sample in Laemmli's sample buffer for 5 mins at 95 °C. For a 52 µl reaction volume use 26 µl of 2x sample buffer, 13 µl of 4x sample buffer or 8.87 µl of 6x sample buffer.
5. Cool on ice before loading the gel.

7. Western Blot

1. Load a 12-well 4-20% tris-glycine gel with 40 µl of the prepared sample
2. Run the gel at 95 V until the ion front reaches the bottom.
3. Carry out an overnight transfer of the gel onto a polyvinylidene difluoride (PVDF) membrane.
4. Incubate the membrane in the amido black stain and scan the membane to obtain an image showing the amount of protein in each lane.
5. Destain the membrane and incubate in 5% milk in PBS for 1 hr to block.
6. Incubate the membrane in the primary anti-HA antibody at a concentration of 1:10,000 in 5% milk in PBS either overnight at 4 °C or for 4 hr at room temperature.
7. Perform 3 washes for 5 min each in PBS with 0.1% Tween-20 detergent.
8. Incubate the membrane in a mouse horseradish peroxidase at a concentration of 1:10,000 in 5% milk in PBS for at least 2 hr.
9. Perform 3 washes for 5 min each in PBS with 0.1% Tween-20 detergent.
10. Perform 1 wash for 5 min in PBS.
11. Incubate the membrane in a chemiluminescent detection reagent for 10 min and detect using a chemiluminescent detector. Use the automatic exposure setting on the detector.

Representative Results

Cultured M17 and HeLa cells were harvested using the method detailed in the protocol (3. Cell Harvesting) and mouse spinal cord tissue was obtained. The cell pellet/spinal cord tissue was placed in a tube with the lysis buffer described in the reagent preparation section. Cell pellets were lysed using glass beads (**Figure 1A**) and mouse spinal cord tissue samples were homogenized using the homogenizer (**Figure 1B**). After lysis or homogenization, the sample was then centrifuged at 5,030 x g to remove the glass beads and/or the unbroken membranes and organelles (**Figure 2**). Both of these methods are mechanical means of lysis to preserve enzymatic activity. The protein concentration of the cells was then determined using the BCA method. 20 µg of total protein was incubated with 2 µl of 1.35 µM probe for 1 hr at 37 °C (**Figure 3**). The samples were then incubated in 4x Laemmli's sample buffer at 95 °C to quench the reaction. The prepared samples were loaded on a 4-20% tris-glycine gel and run at 95 V until the ion front reached the bottom. The proteins were transferred overnight onto a PVDF membrane. The protein loading was checked using the amido black stain (**Figure 4**). This control step ensures that the differences in activity are due to actual cellular processes and not unequal protein loading. Equal protein amounts were used in the first experiment (**Figure 4A**). In the second experiment, unequal protein amounts were loaded due to the expected differences in the activity profiles of the different cell lines (**Figure 4B**). This was done to ensure the detection of an activity profile for the M17 cell lysates incubated with *N*-ethylmaleimide (NEM), which is a cysteine inhibitor and should lead to a reduced signal on the western blot. The membranes were incubated in anti-HA primary antibody, a mouse horseradish peroxidase and then detected using chemiluminescent imaging (**Figure 5**). Successful tagging of the DUBs using the probes results in a profile in each lane of the membrane (**Figure 5A, C**). The intensity of the bands at different molecular weights corresponds to the activity level of the DUB enzyme. The differences in activity levels of various DUBs across cell lines become evident when similar proteins such as UCHL1 are compared in the HeLa and M17 cells (**Figure 5**). The chemiluminescent visualization of the gel is critical because unlike the amido black stain, which shows the amount of protein loaded, this shows the amount of active protein that has reacted with the probes. The light image of the molecular weight standards (**Figure 5B**) serves as the guide for identifying the various DUBs. The mass of the probe needs to be added to the size of the protein during the analysis of the results to characterize the DUBs at the correct molecular weights.

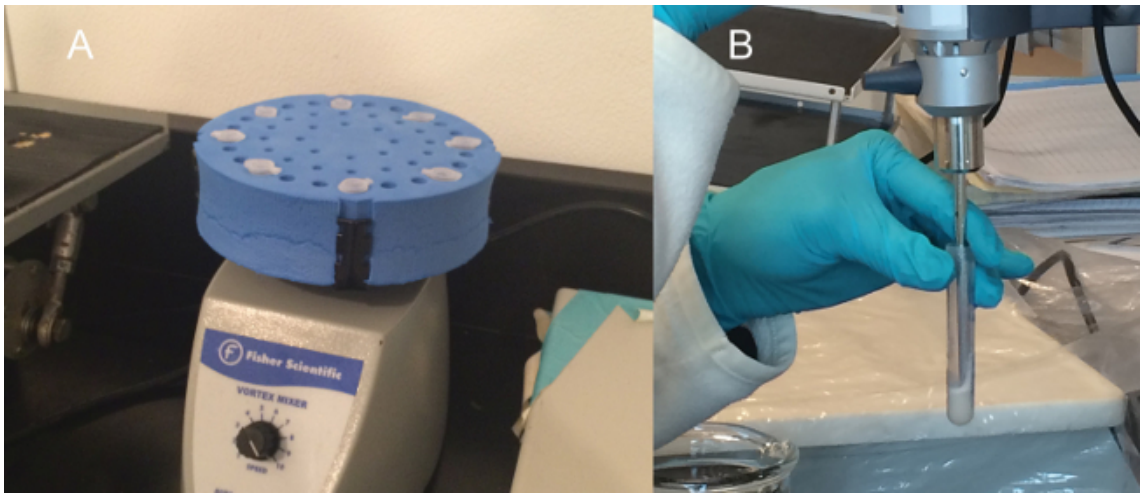

Figure 1: Lysis Methods for Cells and Tissue. Picture showing cell lysis using glass beads in (**A**) versus polytron lysis in (**B**).

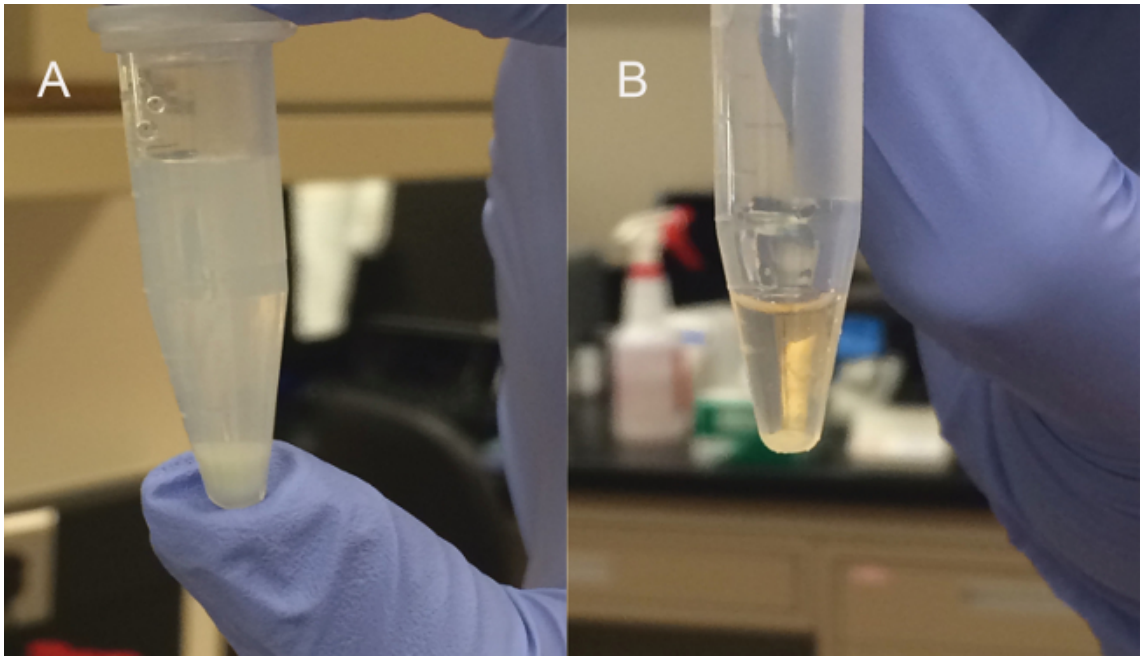

Figure 2: Centrifugation Products of Cells and Tissue. Cell lysate showing the settled glass beads and unbroken membranes and organelles at the bottom after lysis and centrifugation in (**A**). Tissue lysate showing the unbroken membranes and organelles at the bottom after homogenization and centrifugation in (**B**).

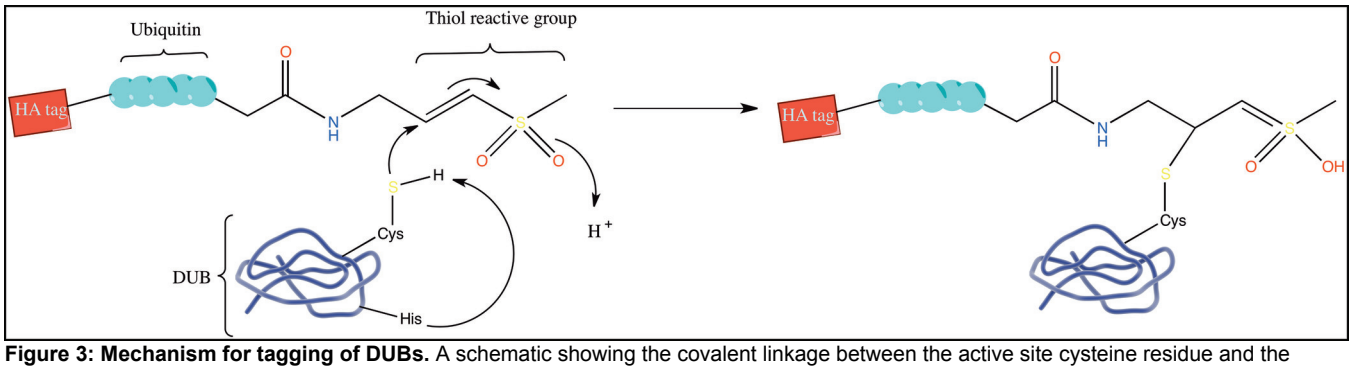

Figure 3: Mechanism for tagging of DUBs. A schematic showing the covalent linkage between the active site cysteine residue and the functional group of the HA-Ub-VS. Please click here to view a larger version of this figure.

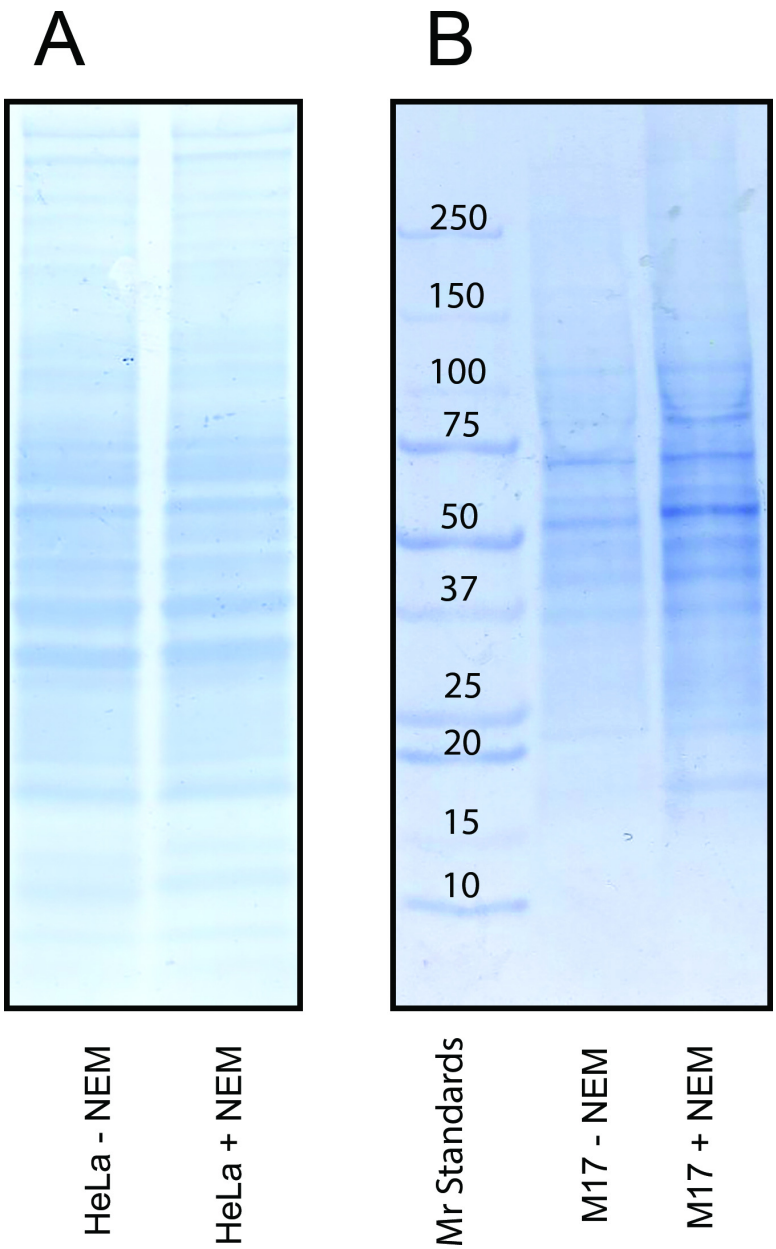

Figure 4: Amido black stains of the membranes. Membranes showing the amido black staining done on lysates incubated with *N*-ethylmaleimide (NEM), tagged with HA-Ub-VinylSulfone (VS) and run on a western blot. The samples are as follows from left to right – (**A**) HeLa - NEM, HeLa + NEM; (**B**) Mr Standards, M17 - NEM, M17 + NEM. Please click here to view a larger version of this figure.

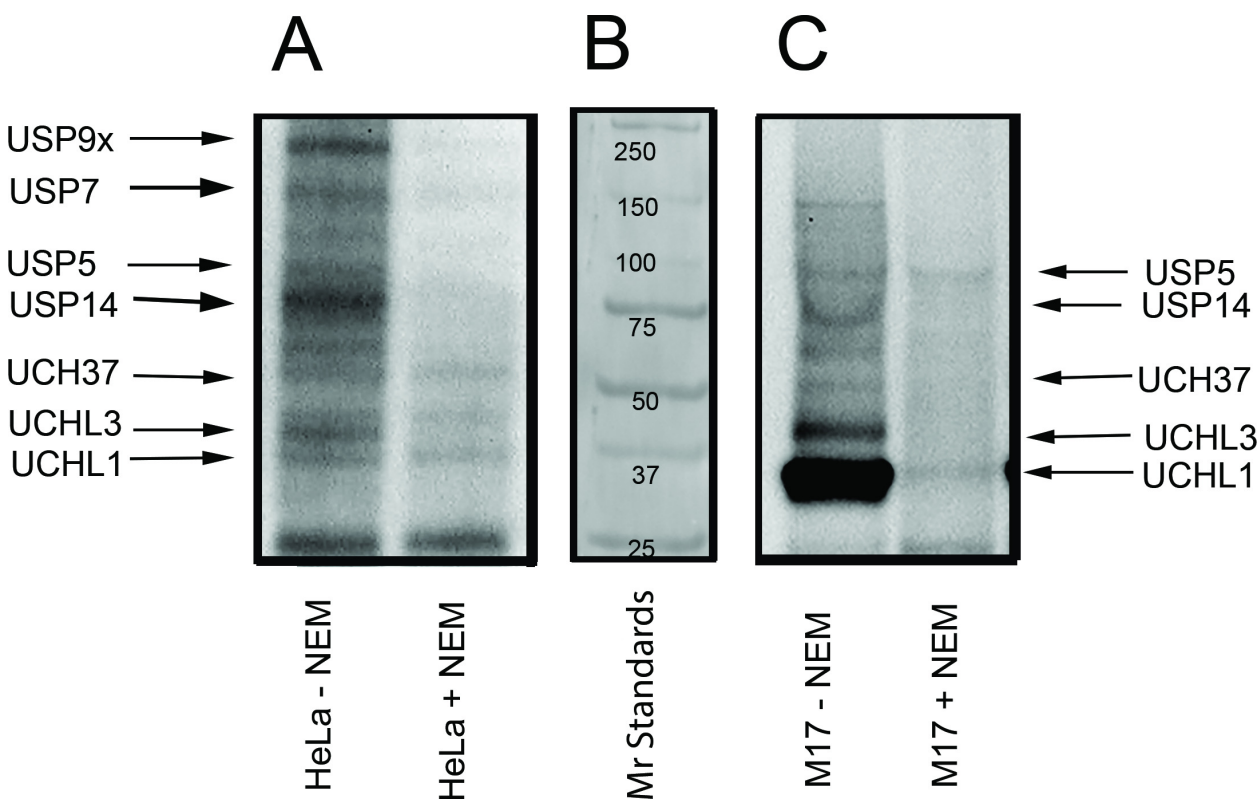

Figure 5: Western blots results. Membranes showing the activity levels of the various DUBs as determined by probing for Anti-HA. The samples are as follows from left to right – (**A**) HeLa - NEM, HeLa + NEM; (**B**) Mr Standards; (**C**) M17 - NEM, M17 + NEM. Please click here to view a larger version of this figure.

Discussion

Since ubiquitination is a fundamental cellular activity, understanding the regulatory mechanisms could be the key to unearthing the processes of disease and pathology. The use of HA tagged Ub-derived active site directed probes reported here provides an easy, but highly applicable method for studying Ub-mediated protein degradation. This method is faster and less expensive than studying each one of the DUBs individually.

In this method, the lysis of the cells is achieved via mechanical means – using the glass beads. This gentle method of lysis preserves a metabolic intracellular picture. However, a major drawback of this lysis method is its low efficiency of about 60-70%. This means a lot of cells are required to ensure lysates are concentrated enough for experiments. T75 flasks with 80 - 90% confluent cell layers should be used to produce a concentrated lysate. Furthermore, the trypsinization step of the experiment is critical because most of the attached cells must be suspended in solution. Check the flask under a microscope to make sure that a significant portion of the cells in culture are floating before transferring to the 50 ml tube. Failure to properly trypsinize adherent cells will result in a smaller pellet and by extension less lysate at a lower concentration. Lysates that are not in use should be stored at -80 °C, transferred to -20 °C 24 hr before use and thawed on ice the day of use. This allows the lysate to thaw evenly and reduces damage to the enzymes from rapid thawing.

To apply this method to tissues either a dounce or polytron homogenizer can be used in place of the glass beads[11]. The obtained lysate can be tagged with the probes immediately or stored for use at -80 °C. If the dounce or polytron is used to obtain the lysate then glass beads should not be used. This leads to a reduction in the total protein concentration of the lysate. Alternatively, using primary cell culture methods the solid tissue samples could be cultured first and then lysed with the glass beads.

A major limitation of this method is that even though it allows the user to visualize the activity of the enzymes, it doesn't give accurate information on the expression patterns of the individual DUBs. This method uses functional homology in the enzymes not structural homology. Therefore, further experiments with the individual antibodies will have to be done to explain whether the reduction in activity is a result of defects in the active site or an actual loss of the enzyme. Additionally, the lysis method used in this protocol does not break open the nuclei of the cells. DUBs in the nucleus could provide further critical information about disease processes.

Nevertheless this method is unique in its ability to provide information that cannot be obtained from any other source. Both RNA methods and the use of individual antibodies do not provide functional information about enzymes. In the future, the potential exists for the application of this method to immunohistochemical (IHC) staining. IHC staining will not only give information about the activity of the enzymes, but it will also provide insights into the location in tissue where the various DUBs are active. Also, this method could be coupled with a subcellular fractionation technique such as the one detailed by Colla *et al.* in 2012 to study the activity of the DUBs in membrane bound organelles[12]. Although this is a relatively new technique in molecular biology, there is a huge potential to expand the applications. Using active site directed molecular probes might hold the key to elucidating the etiology of many diseases.

Disclosures

The authors have nothing to disclose.

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