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Identification of SUMO E3 Ligase-Specific Substrates Using the HuProt Human Proteome Microarray

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Abstract

The functional protein microarray is a powerful and versatile systems biology and proteomics tool that allows the rapid activity profiling of thousands of proteins in parallel. We have recently developed a human proteome array, the HuProt array, which includes ~80 % of all the full-length proteins of the human proteome. In one recent application of the HuProt array, we identified numerous SUMO E3 ligase-dependent SUMOylation substrates. For many SUMO E3 ligases, only a small number of substrates have been identified and the target specificities of these ligases therefore remain poorly defined. In this protocol, we outline a method we developed using the HuProt array to screen the human proteome to identify novel SUMO E3 ligase substrates recognized by specific E3 ligases.

Keywords

Microarray; SUMO; Proteomics; E3 ligases; Posttranslational modifications

1 Introduction

The functional protein microarray is a powerful and versatile systems biology and proteomics tool that allows the rapid activity profiling of thousands of proteins in parallel. Applications of functional protein microarrays range from the identification of protein-binding properties, to surveying targets of posttranslational modifications, to uncovering novel enzymatic activities. Since the development of the yeast proteome microarray over 10 years ago [1], more recent work has seen the development of complete and near-complete proteome arrays representing viruses, bacteria, and plants [2–4]. However, most existing human protein microarrays are comprised of only a minority of the human proteome [5–9]. We have recently developed a human proteome microarray, the HuProt array, which includes nearly 20,000 full-length human proteins [10]. The proteins used to generate this microarray were expressed in yeast and purified under native conditions. Expressing recombinant eukaryotic proteins in yeast improves the likelihood that proteins will retain their biological activity relative to prokaryotic and in vitro expression systems.

Numerous collaborations between our labs and others have so far harnessed the power of the HuProt array to profile a wide range of protein activities. The role of posttranslational modifications in regulating enzymatic activity is one area of investigation particularly well

suited for the HuProt array platform. A screen to define the S-nitrosylated proteome revealed an important regulatory role for this posttranslational modification in the control of ubiquitin E3 ligase activity [11]. In other work, phosphorylation and glycosylation states of the protein kinase CK2 were shown to affect its substrate specificity [12]. The HuProt array has also been used in two separate studies to link novel protein–RNA interactions to neurological disease, including an interaction between RNA splicing factors and a long noncoding RNA linked to schizophrenia [13] and an interaction between multiple RNA binding proteins and an expanded repeat-containing transcript implicated in amyotrophic lateral sclerosis [14]. Another ongoing project in our labs is the generation of monospecific monoclonal antibodies whose specificity can be quickly evaluated using the HuProt array [10]. The utility of the HuProt array further extends to exciting clinical applications including the identification of novel biomarkers that may be used as a diagnostic tool in primary biliary cirrhosis, an autoimmune disease of the liver [15].

Protein SUMOylation is an essential posttranslational modification in most organisms, including yeast, *C. elegans*, Arabidopsis, and mice [16]. The reversible SUMO-modification of target proteins involves an enzymatic cascade chemically similar to ubiquitylation, involving E1 activating, E2 conjugating, E3 ligating enzymes and SUMO proteases. As the only classes of SUMOylation enzymes for which multiple members have been identified, the SUMO E3 ligases and the SUMO proteases have been proposed to be the major factors determining substrate specificity. Recently, we have conducted SUMOylation assays using the HuProt microarray to identify numerous previously uncharacterized SUMO E3 ligase-dependent substrates using a subset of human SUMO E3 ligases. While our study focused on some of the best characterized SUMO E3 ligases, recently additional SUMO E3 ligases have been described [17–21] and it is likely that new SUMO E3 ligases await discovery [22]. The methods that we describe here could be used to identify substrates for these additional SUMO E3 ligases. For most SUMO E3 ligases, only a limited number of substrates are known. In this chapter, we will describe the on-chip SUMOylation protocol that we have developed so that the reader may conduct SUMOylation assays using the HuProt microarray with their SUMO E3 ligase of interest.

2 Materials

2.1 Equipment

1. HuProt human proteome microarray (CDI Laboratories, USA).
2. Bench-top centrifuge.
3. Four-well plate.
4. Humidity chamber (*see* Note 1).
5. Laboratory tissues (Kimwipes).
6. LifterSlip Coverslips for Microarray Slides (Thermo Scientific, USA).

[1]The humidity chamber is made by placing a folded paper towel in the bottom of an empty pipette tip box (we usually use a USA scientific stacked rack made for 200 μl tips, e.g., #1111-1206), adding 1 in. of ddH_2O, and replacing the tip holder on the box. The arrays will sit on the tip holder and be covered with the lid to maintain a humid environment.

7. Microscope slide box (25-slide size).
8. Microarray analysis software, GenePix Pro 6.0 (MDS Analytical Technologies, USA).
9. Orbital shaker.
10. GenePix 4000B Microarray Scanner (Molecular Devices, USA).

2.2 Purification of the SUMO E1 Enzyme

1. E1 Binding Buffer: 20 mM Tris–HCl, pH 8.0, 350 mM NaCl, 1 mM beta-mercaptoethanol, 10 mM imidazole.
2. E1 Wash Buffer: 20 mM Tris–HCl, pH 8.0, 350 mM NaCl, 1 mM beta-mercaptoethanol, 20 mM imidazole.
3. E1 Elution Buffer: 20 mM Tris–HCl, pH 8.0, 350 mM NaCl, 1 mM beta-mercaptoethanol, 400 mM imidazole.

2.3 Purification of the SUMO E2 Enzyme

1. E2 wash buffer: 1 mM DTT in PBS.

2.4 Common Reagents for Expression and Purification of SUMO Protein and SUMOylation Enzymes

1. General lysis buffer: 1 mg/ml lysozyme, 2 mM DTT, 1× Roche protease inhibitor cocktail (EDTA-free), 10 U/ml benzonase dissolved in PBS.
2. Enzyme dialysis buffer: 20 mM Tris–HCl, pH 8.0, 75 mM NaCl, 1 mM beta-mercaptoethanol.
3. PreScission protease (GE Healthcare).
4. 100 mM IPTG.
5. Glutathione sepharose (GE Healthcare).
6. Ni-NTA agarose (Life Technologies).

2.5 SUMO Antibody Labeling

1. SUMO-1 affinity-purified mouse monoclonal antibody (21C7) (#33-2400, Life Technologies).
2. DyLight 549 Antibody Labeling Kit (Pierce Biotechnology): DyLight NHS ester, 0.67 M borate buffer, purification resin, spin columns, microcentrifuge collection tubes.

2.6 On-Chip SUMOylation Assay

1. SUMO blocking buffer: 2 % BSA, 0.05 % Tween-20 in TBS.
2. 2× SUMO conjugation buffer: 40 mM HEPES, pH 7.3, 200 mM NaCl, 20 mM $MgCl_2$, 0.2 mM DTT.

3. SUMO reaction mix (for 200 μl reaction): 100 μl 2× SUMO conjugation buffer, 0.25–2.5 μM E1 enzyme, 0.07–7 μM E2 enzyme, 20 nM E3 ligase (*see* Note 2).
4. TBST: 0.05 % Tween-20 in TBS.
5. 1 % SDS.

3 Methods

3.1 Purification of SUMO Protein

1. Streak out colonies onto a LB + ampicillin agar plate from a glycerol stock of GST-SUMO in pGEX6p.1 in BL21 cells.
2. Pick a single colony and inoculate into 5 ml LB with ampicillin overnight at 37 °C.
3. Dilute the 5 ml culture into 50 ml LB with ampicillin culture overnight at 37 °C.
4. Dilute 50 ml culture into 1 l LB with ampicillin until the OD = 0.6, then drop the temperature to 20 °C and induce with 1 mM IPTG and shake overnight at 20 °C.
5. Freeze pellet at −80 °C until ready to proceed with purification.
6. Thaw pellet at 37 °C, resuspend with 25 ml general lysis buffer, rotate at RT for 15 min.
7. Centrifuge at 4 °C for 30 min at 20,000 × *g* to pellet insoluble material.
8. During centrifugation prepare glutathione sepharose by washing 2 ml 50 % glutathione sepharose with 1× PBS in 50 ml tube. Repeat twice.
9. Bind protein by mixing supernatant with glutathione sepharose, rotate at 4 °C for 1 h or longer.
10. Wash sepharose by spinning down sepharose, discard supernatant, resuspend sepharose in 1 ml of 1× PBS. Transfer sepharose to column; wash with 12 ml of 1× PBS.
11. Cleave SUMO from GST tag by transferring sepharose bound with GST-precission protease and GST-SUMO to the same column. Parafilm column to prevent leakage. Incubate with shaking at 4 °C overnight.
12. Allow sepharose to settle. Remove supernatant containing purified untagged SUMO. Apply 4 ml of 1× PBS and repeat.
13. Dialyze overnight against 2 l 1× PBS

[2]SUMOylation of many proteins occurs in the absence of E3 ligases at relatively high E1 and E2 concentrations; therefore E1 and E2 concentrations are usually used in the low end of this range when using an E3 ligase. However, it is advisable to establish the best SUMOylation conditions using a conventional tube-based assay, particularly E1 and E2 concentrations, with the SUMO E3 ligase of interest and a known substrate. The best result will allow clear visualization of enhanced SUMOylation in the presence of the E3 ligase, before proceeding with the assay on-chip.

14. Concentrate protein using a micron centrifugal filter (10 kDa MWCO) by spinning 20 min at 500 × *g*. Aliquot and freeze with liquid nitrogen and store at −80 °C.

3.2 Purification of SUMO E1 Enzyme

1. Streak out colonies onto an LB + ampicillin agar plate from a glycerol stock of His-hE1 (His-Aos1/Uba2) in BL21 cells.
2. Pick a single colony and inoculate into 5 ml LB with ampicillin overnight at 37 °C.
3. Dilute the 5 ml culture into 50 ml LB with ampicillin culture overnight at 37 °C.
4. Dilute 50 ml culture into 1 l LB with ampicillin until the OD = 0.6, then drop the temperature to 20 °C and induce with 1 mM IPTG and shake overnight at 20 °C.
5. Freeze pellet at −80 °C until ready to proceed with purification.
6. Thaw pellet at 37 °C, resuspend with 25 ml general lysis buffer, rotate at RT for 15 min.
7. Centrifuge at 4 °C for 30 min at 20,000 × *g* to pellet insoluble material.
8. During centrifugation prepare Ni-NTA agarose by washing 2 ml 50 % Ni-NTA agarose with E1 binding buffer in 50 ml tube. Repeat twice.
9. Bind protein by mixing supernatant with Ni-NTA agarose, rotate at 4 °C for 1 h or longer.
10. Wash agarose by spinning down agarose, discard supernatant, resuspend agarose in 1 ml of wash buffer. Transfer agarose to column; wash with 12 ml of E1 wash buffer.
11. Elute with 3 ml of E1 elution buffer. Collect fractions and analyze by SDS-PAGE. Pool fractions containing protein.
12. Dialyze overnight against 2 l enzyme dialysis buffer.
13. Concentrate protein using a micron centrifugal filter (10 kDa MWCO) by spinning 20 min at 500 × *g*. Aliquot and freeze with liquid nitrogen and store at −80 °C.

3.3 Purification of SUMO E2 Enzyme

1. Streak out colonies onto an LB + ampicillin agar plate from a glycerol stock of GST-Ubc9 in pGEX6p.1 in BL21 cells.
2. Pick a single colony and inoculate into 5 ml LB with ampicillin overnight at 37 °C.
3. Dilute the 5 ml culture into 50 ml LB with ampicillin culture overnight at 37 °C.
4. Dilute 50 ml culture into 1 l LB with ampicillin until the OD = 0.6, then drop the temperature to 20 °C and induce with 1 mM IPTG and shake overnight at 20 °C.

5. Freeze pellet at −80 °C until ready to proceed with purification.
6. Thaw pellet at 37 °C, resuspend with 25 ml general lysis buffer, rotate at RT for 15 min.
7. Centrifuge at 4 °C for 30 min at 20,000 × *g* to pellet insoluble material.
8. During centrifugation prepare glutathione sepharose by washing 2 ml 50 % glutathione sepharose with 1× PBS in 50 ml tube. Repeat twice.
9. Bind protein by mixing supernatant with glutathione sepharose, rotate at 4 °C for 1 h or longer.
10. Wash sepharose by spinning down sepharose, discard supernatant, resuspend agarose in 1 ml of E2 wash buffer. Transfer sepharose to column; wash with 12 ml of E2 wash buffer.
11. Cleave UBC9 from GST tag by transferring beads bound with GST-precission protease and GST-UBC9 to the same column. Parafilm column to prevent leakage. Incubate with shaking at 4 °C overnight.
12. Allow sepharose to settle. Remove supernatant containing purified untagged UBC9. Apply 4 ml of E2 wash buffer and repeat.
13. Dialyze overnight against 2 l enzyme dialysis buffer.
14. Concentrate protein using a micron centrifugal filter (10 kDa MWCO) by spinning 20 min at 500 × *g*. Aliquot and freeze with liquid nitrogen and store at −80 °C.

3.4 SUMO Antibody Labeling

1. Add 40 μl 0.67 M borate buffer to 0.5 ml of 0.5 mg/ml affinity-purified SUMO-1 antibody.
2. Add 0.5 ml of antibody in borate buffer to the vial of DyLight reagent and vortex gently.
3. Briefly centrifuge to collect the sample in the bottom of the tube.
4. Incubate the reaction mixture for 60 min at room temperature protected from light.
5. Mix purification resin to ensure uniform suspension and add 400 μl of the suspension into both spin columns. Centrifuge for 45 s at 1,000 × *g* to remove the storage solution. Discard the used collection tubes and place the columns in new collection tubes.
6. Add 250–270 μl of the labeling reaction to each spin column and mix the sample with the resin by vortexing.
7. Centrifuge columns for 45 s at 1,000 × *g* to collect the purified proteins. Combine the samples from both columns (0.5 ml total).
8. Aliquot and store the labeled antibody at −20 °C.

3.5 On-Chip SUMOylation Assay

1. Remove arrays from −80 °C freezer and immediately rinse by quickly dunking in a beaker of 300 ml of TBST.
2. Using forceps, transfer each array to a well of a four-well plate with 3 ml of SUMO blocking buffer per well.
3. Block protein microarray by gently shaking overnight at 4 °C (*see* Note 3).
4. Prepare the reaction mix and keep on ice. Add E1 and E2 enzymes and E3 ligase immediately before the end of the blocking step. Set up two control experiments, one using antibody only, and one without the E3 ligase.
5. Remove one array from blocking buffer and carefully wick off liquid by tapping the edge on a paper towel, give the bottom of the array a quick wipe with a kimwipe and place array on the top surface (protein side up) of a humidity chamber (*see* Note 4).
6. Add SUMO reaction mix to each slide carefully and place lifterslip on top, being careful to avoid bubbles (*see* Note 5).
7. Incubate at 37 °C for 90 min (depending on enzyme activity).
8. Immediately after start of incubation, pre-warm appropriate volume of 1 % SDS to 55 °C for later washing steps (*see* Note 6).
9. Remove coverslip by gently sliding off array (*see* Note 7).
10. Place arrays in a four-well plate and wash gently on orbital shaker 3× for 10 min at room temperature with 3 ml TBST per well.
11. Wash with 1 % SDS warmed to 55 °C 3× for 5 min each.
12. Wash once with 3 ml TBST.
13. Dilute labeled SUMO1 antibody in blocking buffer at 1:1,000 dilution. Apply 200 μl of the antibody mixture to each array and add fresh coverslip. Incubate array with labeled SUMO1 antibody for 1 h at room temperature.
14. Remove coverslip by gently sliding off array and wash slides three times for 10 min in 3 ml TBST in four-well plate.

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[3]To minimize evaporation and potential photobleaching of any fluorescently labeled protein beacons included on the protein microarray, it is advised to wrap four-well plate with a layer of saran wrap, then a layer of aluminum foil, before overnight incubation.
[4]It is critical to strike the right balance of removing excess liquid from the array while not over-drying at this step. The goal is to remove enough liquid so as not to dilute the reaction mix, while also not allowing the top (protein surface) to not become completely dry. The best results can be obtained by being ready to add reaction mix immediately, i.e., reaction mix tube open, pipet set to correct volume and already loaded with a pipet tip, after placing the chip on the surface of the tip holder.
[5]Ensure that bubbles are pushed out of the reaction mix while adding coverslip to the top surface of the array. One reliable strategy is to first rest one end of the coverslip on the edge of the array, then gradually lower the other end of the coverslip to push out the bubbles.
[6]1 % SDS is used in order to stringently wash the array and remove any non-covalently bound SUMO from the arrayed proteins.
[7]Removing the coverslip is best achieved by gently sliding the coverslip straight along the array with only the raised edges of the coverslip contacting the outside edges of the chip, where no proteins are printed. Allowing the raised edges of the coverslip to move across the array (contacting the region where proteins are printed) can scratch the surface of the chip, causing high background signal in the scratched region, resulting in lost information about proteins in the path of the scratch.

15. Wash the slides once in 50–100 ml milliQ water for 5 min to remove residual salts from the surface of the microarray (*see* Note 8).

16. Place each array horizontally into a micro slide box with kimwipes on the bottom. Centrifuge the box in a benchtop centrifuge for 3 min at 500 × *g*.

17. Scan the microarray with a GenePix 4000B scanner with 5-μm resolution detection at 532 nm with the appropriate gain and power settings (*see* Note 9). Save the scanned images as TIFF files. All slides that will be compared should be scanned using the same gain and power settings.

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[8]A wash with high purity water is essential to remove residual salts that can result in high background when scanning.
[9]To get the most meaningful information from your protein microarray experiment, it is important to scan the chip with the appropriate gain and power settings. A useful starting point using GenePix software would be PMT Gain = 600, Power = 100 %. Usually the gain value can then be raised or lowered depending on the resulting image obtained at these settings. Generally, it is desired to have the highest signal intensity values possible while avoiding signal saturation of important features.

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