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Profiling Protein Methylation with Cofactor Analogue Containing Terminal Alkyne Functionality

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Abstract

Enzymatic transmethylation from the cofactor *S*-adenosyl-*L*-methionine (SAM) to biological molecules has recently caught the increased attention, because of the diversity of substrates and their implications in normal biology and diseases. To reveal the substrates of protein methyltransferases (PMTs), the current protocol focuses on using an alkyne-containing SAM mimic, *Se*-adenosyl-*L*-selenomethionine (ProSeAM) and a cleavable azido-azo-biotin probe to profile the targets of endogenous PMTs in cellular contexts. Here we described the stepwise preparation of cell lysates containing active, endogenous PMTs and subsequent target labeling with ProSeAM. We then described the protocol of the enrichment of the ProSeAM-labeled proteins with the azido-azo-biotin probe as a pulldown reagent and the subsequent reductive elution with sodium dithionite for proteomic analysis. The current protocols were formulated for ProSeAM as a profiling reagent in particular but can be applied to other terminal-alkyne-containing SAM analogue cofactors in general.

Keywords

Se-adenosyl-*L*-selenomethionine; ProSeAM; azido-azo biotin probe; click chemistry

INTRODUCTION

Chemical modification of a protein after its translation (posttranslational modification, or PTM) is a well-adapted biochemical strategy to diversify protein functions (Luo, 2012; Walsh et al., 2005; Zhang et al., 2012). Among several dozen revealed PTMs, many involve the addition of small functional chemical moieties (*e.g.* glycosylation, phosphorylation, acetylation and methylation). These chemical transformations are often catalyzed by transferase enzymes, which render the functional moieties from donors (cofactors) to acceptor proteins (substrates) in a target-specific manner (Luo, 2012). For instance,

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glycosyltransferases, kinases, acetyltransferases and methyltransferases are categorized into this enzyme family according to their abilities to deliver glycosyl, phosphyl, acetyl and methyl groups from respective cofactors (glycosyl donors, adenosine-5'-triphosphate (ATP), acetyl coenzyme A and S-adenosyl-L-methionine or SAM) (Charron et al., 2009; Luo, 2012; Prescher and Bertozzi, 2006; Wang et al., 2011a; Yount et al., 2010).

The SAM-dependent enzymatic methylation of biological molecules has been of particular interest because of its wide variety of substrates and their implications in a plethora of biological processes and diseases (Luo, 2012; Walsh et al., 2005; Zhang et al., 2012). The transfer of SAM's sulfonium methyl moiety can occur on the side chains of various amino acids, such as Lys, Arg, His, Cys, Glu and Gln (Van Der Werf and Koshland, 1977; Walsh et al., 2005; Zhang et al., 2012). Given the increasing appreciation for the importance of protein methylation, new chemical tools for profiling proteome-wide substrates of protein methyltransferases (PMTs) are becoming critical for continued research.

The recent efforts of our laboratory and others have demonstrated the utility of SAM-mimetic cofactors in labeling substrates of native or engineered PMTs. Among several such applications are bulky alkyne/azido-containing sulfonium- β -sp² SAM derivatives for native MLL4 and engineered PRMT1, G9a, GLP (Islam et al., 2012; Islam et al., 2011; Peters et al., 2010), less-sterically-hindered sulfonium- β propargylic SAM for SETDB1 (Binda et al., 2011) and more stable *Se*-adenosyl-L-selenomethionine (ProSeAM) as a more general SAM surrogate (Bothwell et al., 2012; Willnow et al., 2012). As a common feature, these SAM mimetics carry an alkyne or azido handle, which will be enzymatically transferred to PMT substrates. In combination with azido/alkyne-containing biotin probes and established protocols for azide-alkyne cycloaddition (click) chemistry, the identity of the modified proteins can be readily revealed with streptavidin affinity pulldown and subsequent mass spectrometric analysis (Best, 2009; Yang et al., 2010; Yount et al., 2010).

In the previous article, we described a protocol for profiling the substrates of designed PMTs with an azido-containing SAM analogue, coupled with a commercially available alkyne-containing biotin reporter (Fig. 1). Given the specific need for engineered PMTs to process the azido-containing SAM analogue, the method described here is more general, relying on the utility of ProSeAM (Bothwell et al., 2012). The current protocol focuses on a reversed approach, using the alkyne-containing SAM mimic and azido-containing probes to reveal the targets of endogenous PMTs in cellular contexts. **BASIC PROTOCOL 1** described the stepwise preparation of the cell lysates with maintained activities of endogenous PMTs and the subsequent target labeling with ProSeAM. **BASIC PROTOCOL 2** describes the enrichment of the ProSeAM-labeled proteins with the cleavable azido-azo biotin probe as a pulldown reagent (Bothwell et al., 2012; Yang et al., 2010) and the subsequent reductive elution with sodium dithionite for proteomic analysis (Bothwell et al., 2012; Yang et al., 2010; Islam et al., 2012). The protocols described here were formulated for ProSeAM as a profiling reagent in particular but can also be generally applied to other alkyne-containing SAM analogue cofactors.

STRATEGIC PLANNING

Suitable SAM Analogues for Proteome-wide Profiling—Several SAM analogue cofactors have been characterized previously to label individual substrates of PMTs (Fig. 2a) (Binda et al., 2011; Bothwell et al., 2012; Peters et al., 2010). A more cautious approach should be taken upon implementing these compounds as SAM surrogates for proteome-wide profiling of PMT targets. The important parameters include (1) the stability of the SAM analogues at physiological pH; (2) the catalytic efficiency of the SAM analogues toward PMTs of interest under optimized conditions; (3) the cross reactivity of SAM analogues toward irrelevant endogenous PMTs. Here we recommended thoroughly evaluating these parameters *in vitro* prior to the proteome-wide profiling. For instance, ProSeAM was shown to be active toward a set of native PMTs *in vitro* (Bothwell et al., 2012). Such promiscuity allowed us to define the ProSeAM as a semi-general chemical probe of native PMTs (for ProSeAM synthesis see **SUPPORTING PROTOCOL 4**).

Treatments of Cell Culture—The protocols outlined below are not restricted to specific cell lines. Conditions for cultured cells can be modified according to the questions addressed. For instance, adenosine-2',3'-dialdehyde (Adox, sigma A7154) is an irreversible inhibitor of *S*-adenosyl-*L*-homocysteine (SAH) hydrolase. Treatment with this inhibitor causes intracellular accumulation of SAH, a pan-PMT inhibitor, and generate hypomethylated proteomes (Herrmann et al., 2005). The proteome-wide induction of hypomethylation may enhance the subsequent labeling signal with SAM analogue cofactors. In contrast, target-specific PMT inhibitors (*e.g.* UNC 0638 for G9a/GLP (Vedadi et al., 2011)) can also be used for generating, target-specific, hypomethylated proteome.

Rationale of Azido-azo-biotin Probe for Proteomic Enrichment—In the current protocol, the transferred terminal-alkyne functionality was used as a molecular handle for coupling with a terminal-azido-containing biotin probe, enabling target enrichment (Fig. 2b). The azido moiety of the latter is expected to rapidly react with the terminal alkyne of modified PMT targets via well-established copper-catalyzed azide-alkyne cycloaddition (CuAAC) chemistry, while the latter's biotin moiety interacts with streptavidin-sepharose for target enrichment. Although many commercially available biotin probes meet these criteria, our laboratory prefers the azido-azo-biotin probe developed by the Hang laboratory (Szychowski et al., 2010; Yang et al., 2010). This biotin probe is characterized by a cleavable linker buried between its azido and biotin moieties (Fig. 2b). This linker is readily subject to cleavage with sodium dithionite under mild reducing environment, thus avoiding harsh but inefficient disruption of biotin-streptavidin (boiling in SDS or incubating in highly acidic buffer) required for target release from streptavidin-sepharose beads (Yang et al., 2010; Yount et al., 2010). In addition, the characteristic colorimetric change after the cleavage can be used to indicate complete cleavage. Here we synthesized the cleavable azido-azo-biotin probe at gram scale using the published procedure with some modifications (Yang et al., 2010) (see **ALTERNATIVE PROTOCOL**).

BASIC PROTOCOL 1: LABELING SUBSTRATES OF ENDOGENOUS PMTS WITH ProSeAM

Although multiple native PMTs can process ProSeAM as a cofactor surrogate for substrate labeling, the poor membrane permeability of SAM analogues only allow ProSeAM to be used *in vitro* in contexts of cell lysates (Bothwell et al., 2012). The preparation of such cell lysates is expected to maintain the activities of the endogenous PMTs. We therefore developed a lysis protocol which does not require high concentrations of detergents for this purpose. The resultant cell lysates can be treated with ProSeAM in the absence or presence of PMT inhibitors. With the subsequent coupling with azido-containing fluorescent dyes, the efficiency and pattern of labeling were then readily evaluated and compared with in-gel fluorescence (*SUPPORTING PROTOCOL 1*).

Parameters cultured cells (medium type, cell confluency etc.) and the amount of harvested protein depend on the cell type. In the current protocol, HEK293T cells were used and the conditions were documented for this cell type. The set of parameters for other cell lines or patient samples need to be determined with small scale, pilot experiments, before full-scale labeling experiments are carried out.

Materials

Human embryonic kidney (HEK) 293T cells propagated in Dulbecco modified Eagle medium (DMEM, GIBCO) supplemented with 10 % (v/v) fetal calf serum (FCS).

Adenosine-2',3'-dialdehyde (Adox) (Sigma, A7154) stock solution, 1.5 mM in deionized distilled water (DD water). The HEK293T cells were pretreated with 25 μ M Adox for 48 h to induce hypomethylated proteome.

1 $\times$ PBS (GIBCO)

Tris-HCl Buffer (see **Recipe**)

ProSeAM stock solution, 8 mM stock in 0.01% (v/v) TFA and DD water 8 mM. The acidity of the storage solution is important for ProSeAM's stability. With this buffer, this material can be stored at – 80 °C for < 2 months (*see supporting protocol 4* for ProSeAM synthesis).

5'-Methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN) (Ibanez et al., 2010; Wang et al., 2011a)

Bradford assay reagent (Bio-Rad)

1.5 ml microcentrifuge tubes

37 °C, 5% CO₂ cell culture incubator

Refrigerated centrifuge (4 °C, 3,000 g capacity)

Sonicator (Misonix Ultrasonic Liquid Processor)

Refrigerated high speed centrifuge (4 °C, 21,000 g capacity)

Cell culturing and lysis

The size of cell culture flask is determined by the amount of protein required. A typical proteomic pulldown experiment, requires cell lysate containing around 10 mg total protein. We generally cultured HEK293T cells in a T-150 flask to approximately 50% confluency (BD Falcon™ 150 cm² Cell Culture Flask). The cells are then treated with 25 μM Adox for 24 h. The harvested total protein is approximately 10 mg.

1. HEK293T cells are grown in a T-150 flask containing DMEM and 10% (v/v) FBS to confluency (2×10^7 cells), and then treated with 25 μM Adox (1.5 mM stock) for 48 h to induce hypomethylation.
2. Harvest cells by repetitive pipetting and centrifuge them down at 4 °C at 1,000 g for 10 min.

Use of trypsin to harvest cells at this step is not recommended because the treatment may degrade cell surface proteins.

3. Remove the supernatant, wash the cells with PBS ($50 \times$ volume of cell pellet), and then centrifuge at 1,500 g at 4 °C for 10 min. Repeat the washing step 3 times.
4. Remove supernatant and then resuspend cell pellet in 4 mL Tris-HCl buffer.

The cell pellet can be flash frozen in liquid nitrogen and stored as pellet at –80 °C for < 2 months. The amount of buffer used for lysis should be adjusted proportionally to the amount of cells (e.g. 4 ml buffer for 2×10^7 cells). When a large volume of buffer is used, the samples should be split into several microcentrifuge tubes with < 500 μl for each tube. Since the detergent-low Tris-HCl lysis buffer was used here, a larger volume is required in comparison with the amount for conventional detergent-containing lysis buffers, such as RIPA buffer (Sigma), which contains SDS.

5. Sonicate the resuspended cells in an ice bath with a single pulse for 15 min at 65 % amplitude.

A solution of fully lysed cells is characteristically translucent. Adding 25 % extra Tris-HCl buffer (v/v) and another sonication cycle is occasionally required to reach translucency. Keep the samples on the ice bath during the sonication to prevent heat denaturing or protein degradation.

6. Centrifuge the cell lysates at 4 °C at 21,000 g for 30 min and combine the split fractions if necessary. Quantify the protein concentration with Bradford assay.
7. Dilute the cell lysates with Tris-HCl buffer to the final concentration of 2 mg protein/ml.

Once the cells are lysed, they should be used immediately to avoid loss of activity in endogenous PMTs.

8. Supplement the cell lysates with MTAN to the final concentration of 100 nM or other PMT inhibitors such as the pan-PMT inhibitor SAH, where appropriate.

5'-Methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) is expected to degrade SAH, a pan-PMT inhibitors, which may exist in the current protocol. This step is included to relieve potential SAH inhibition on native PMTs and thus enhance the subsequent labeling with ProSeAM. In contrast, the presence of PMT inhibitors in the following labeling process is expected to inhibit corresponding PMTs and cause the loss-of-signal labeling. The alternation due to the different treatments will correlate the absence of labeling to the corresponding PMT activities. The current protocol focuses on a single condition with 100 nM MTAN and can readily transformed to other scenarios.

9. Add ProSeAM to the cell lysates with the final concentration of 25 μ M and incubate the mixture for 1 h at ambient temperature (23 °C).

The concentration of ProSeAM and the incubation time have been optimized as described before (Bothwell et al., 2012). For any new SAM analogue cofactor, such reaction parameters need to be evaluated using in-gel fluorescence and optimizing for signal-to-noise ratios (Fig. 3a). A common method for optimizing is to start with a minimal concentration of cofactor and with a minimal reaction time and then elevate them gradually until desired signal-to-noise ratios are observed. Here we recommend using a small aliquot of 50 μ g protein, treating them with ProSeAM followed by the conjugation with a fluorescent probe via click chemistry, followed by visualization with in-gel fluorescence (*SUPPORTING PROTOCOL 1,2,3*).

10. Quench the reactions and precipitate the proteins with cold Methanol/Chloroform/Water mixture (*SUPPORTING PROTOCOL 2*).
11. Precipitated protein pellets can be stored at –80 °C for 3 d.

BASIC PROTOCOL 2: TARGET PULLDOWN AND ENRICHMENT

ProSeAM-labeled protein substrates obtained from *BASIC PROTOCOL 1* are subject to CuAAC with a cleavable azido-azo-biotin probe (see STRATEGIC PLANNING). The resultant biotinylated proteins were enriched with streptavidin-sepharose beads and then released from the beads with sodium dithionite, under mild conditions. The eluted fractions were concentrated and lyophilized for proteomic analysis.

Materials

10 mg air-dried protein pellet from the whole cell lysates of 2×10^7 cells labeled with ProSeAM (*BASIC PROTOCOL 1*).

Bradford Assay reagents (Bio-Rad)

Ice-cold 4% SDS (w/v) buffer supplemented with EDTA-free Roche protease inhibitors (see **Recipe**)

Dilution buffer (see **Recipe**)

5 mM azido-azo-biotin in DMSO (see STRATEGIC PLANNING and ALTERNATIVE PROTOCOL 1)

Freshly-prepared 50 mM and 200 mM Tris(2-carboxyethyl)phosphine (TCEP)
2 mM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMSO
Freshly-prepared 50 mM CuSO₄
Streptavidin-sepharose beads (GE healthcare, 17-5113-01)
Ice-cold 0.2 % SDS (w/v) PBS buffer (see **Recipe**)
Ice-cold ABC buffer (250 mM Ammonium Bicarbonate)
Ice-cold PBS buffer (GIBCO)
8 M Urea
Freshly-prepared 400 mM idoacetamide in DD water
Elution buffer (see **Recipe**)
β-Mercaptoethanol (Sigma)
Fluorescent protein molecular weight ladder (15 – 250 kDa, Bio-Rad)
1 × electrophoresis buffer MOPS (Bio-Rad)
50 ml conical polypropylene tubes
Turn-over-turn mixer
Refrigerated centrifuge (4 °C, 3,000 g capacity)
1.5 ml microcentrifuge tubes
2 ml Dolphin tubes
Amicon® ultra 3K 2 ml centrifugal filter unit (Millipore)
Lyophilizer
100 °C heating block
12-well 4% to 12% Tris-HCl protein gel (Criterion XT Precast Gel, Bio-Rad)
SDS-PAGE electrophoresis gel box for Criterion XT Precast Gel (Bio-Rad)

CuAAC with the azido-azo-biotin probe

1. Resolubilize the air-dried protein pellet in 4.45 ml freshly-prepared, 4% SDS (w/v) buffer.
Repetitive pipetting and brief sonication can facilitate the solubilizing process.
2. Prepare the following click cocktail by adding the following reagents in order in a separate tube: 100 µl 5 mM azido-azo-biotin probe (the final concentration 100 µM); 100 µl 50 mM TCEP (the final concentration 1 mM); 250 µl 2 mM TBTA (the final concentration 100 µM); 100 µl 50 mM CuSO₄ (the final concentration 1 mM).

3. Add 550 μ l of the click cocktail into the protein sample from Step 1 to a final volume of around 5 ml with final protein concentration of 2 mg/ml.
4. Incubate the reaction at ambient temperature (23 °C) for 90 min.
5. Quench the click reaction and precipitate the proteins with cold MeOH (see **SUPPORTING PROTOCOL 3**)

The purpose of this step is to remove the unreacted biotin probe, which may interfere with enrichment over streptavidin beads by saturating biotin binding sites. The precipitated proteins can be kept at -80°C for 3 d.

Protein pulldown with streptavidin beads

6. Resolubilize the precipitated protein pellets in 1 ml freshly-prepared, 4 % SDS (w/v) buffer supplemented with 10 mM EDTA.

EDTA chelates the residual Cu(II/I) of the click reaction to avoid high background labeling.
7. Add 2 ml of dilution buffer to each sample.

Diluting the concentration of SDS below 1.5% (w/v) is required for optimal binding between biotin and streptavidin.
8. Into a 50 ml conical tube, add 100 μ l streptavidin-sepharose beads (the amount for 10 mg protein input).
9. Wash the beads with 50 ml PBS buffer by inverting the tube several times, centrifuge the beads at 4,000 g at ambient temperature (23 °C) for 2 min, and then remove the supernatant. Repeat step 3 times.
10. Suspend the beads in 100 μ l 0.2 % SDS (w/v) PBS (the amount for 100 μ l beads).
11. Add 200 μ l of the bead suspension to each protein sample and mix (turn-over-turn) for 1 h.
12. Remove supernatant by centrifuging the beads at 4,000 g for 2 min.

Wash streptavidin beads

13. Resuspend the beads in 10 ml 0.2 % SDS (w/v) buffer, invert-mix the sample for several times and centrifuge them at 4,000 g at 4 °C for 2 min.
14. Discard the supernatant, wash the beads with 10 ml PBS, centrifuge the mixture at 4 °C at 4,000 g for 2 min and discard the supernatant. Repeat step twice.
15. Wash the beads with 10 ml ABC buffer, centrifuge the mixture at 4 °C at 4,000 g for 2 min, and discard the supernatant. Repeat step twice.

The extensive washing steps are needed to reduce amount of non-specifically bound proteins.

Cysteine reduction and iodoacetamide capping

- 16** Mix the beads with the freshly prepared reduction buffer containing 500 µl 8 M Urea, 25 µl of 200 mM TCEP (freshly prepared) and 25 µl of 400 mM Iodoacetamide (freshly prepared) in the dark at ambient temperature (23 °C) for 40 min.

The workup steps above result in the reduction and alkylation of cysteines and prevent formation of random disulfide bonds and protein aggregates.

- 17** Add 10 ml ABC buffer and centrifuge the sample at 4 °C at 4,000 g for 2 min.
- 18** Remove supernatant, suspend the beads in 1 ml ABC buffer and transfer the suspension to a 2 ml dolphin microcentrifuge tube. Centrifuge at 4 °C at 4,000 g for 2 min.

Protein elution from streptavidin beads

- 19** Remove the supernatant and resuspend the beads in 250 µl elution buffer (see Recipe).
- 20** Incubate the mixture at ambient temperature (23 °C) for 30 min and centrifuge the sample at ambient temperature (23 °C) at 4000 g for 2 min.
- 21** Carefully recover the supernatant. Repeat Step 20 twice and combine the three batches of the supernatant.

During the elution process, the sample color will gradually change from yellow to clear, indicating the cleavage reaction is complete. The elution step can be repeated until the yellow color disappears.

- 22** Equilibrate Amicon® ultra 3K 2 ml centrifugal filter unit with 500 µl 1 % (w/v) SDS 250 mM ABC by centrifuging the tube at 3,000 g for 30 min.
- 23** Add the supernatant eluted from the streptavidin beads into the pre-equilibrated Amicon® ultra centrifugal filter unit and concentrate by centrifugation at 3,000 g for 30 min.
- 24** Flip the Amicon® ultra 3K 2ml centrifugal filter unit into a new 1.5 ml microcentrifuge tube, and centrifuge the tube at 1,000 g for 2 min to collect the pulled down proteins.

Concentrating the pulled down proteins reduces the amount of residual sodium dithionate from the previous cleavage step.

- 25** Wash the filter membrane with 50 µl of 1 % SDS in water supplemented with 75 mM β-mercaptoethanol, flip and spin the membrane into the collection tube at 1,000 g for 2 min. Repeat step twice to reach a final volume of ~ 150 µl of the eluted protein sample.
- 26** Flash freeze the collected sample with liquid nitrogen and lyophilize to dryness.

- 27 Redissolve the lyophilized powder in 50 μ l of 1 $\times$ loading buffer, denature the sample at 100 $^{\circ}$ C. Load a small scale of 10 μ l per sample onto a SDS-PAGE gel and resolve via electrophoresis.
- 28 Stain gel with Coomassie Blue (Bio-Rad) according to the manufacturer's protocol.
- The small scale gel analysis is added to visualize the pulled down proteins as a step of quality control (see Fig. 3b).
- 29 Process the remaining samples for proteomic analysis according to the standard procedures (Bothwell et al., 2012).

SUPPORTING PROTOCOL 1: PROTEOME-WIDE TARGET VISUALIZATION USING IN-GEL FLUORESCENCE

The quality of the cell lysates labeled by ProSeAM (*BASIC PROTOCOL 1*) can be assessed by commercially available TAMRA-azide fluorescent probe (Invitrogen) and in-gel fluorescence prior to the coupling of the azido-azo-biotin probe for proteomic analysis. The in-gel fluorescence visualization protocol can also be implemented for a rapid optimization of the parameters of the enzymatic reaction such as buffer components, reaction time, and cofactor concentration.

Materials

- 50 μ g pellet of ProSeAM-treated cell lysates from *BASIC PROTOCOL 1*
- Ice-cold 4 % SDS (w/v) buffer supplemented with EDTA-free Roche protease inhibitors (see Recipe)
- 5 mM stock of TAMRA-Azide (Invitrogen, T10182) in DMSO
- Freshly-prepared 100 mM Tris(2-carboxyethyl)phosphine (TCEP) in DD water
- 5 mM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMSO
- Freshly-prepared 100 mM CuSO₄
- 1 $\times$ protein loading buffer (see Recipe)
- Fluorescent protein molecular weight ladder (15 – 250 kDa, Bio-Rad)
- 1 $\times$ electrophoresis buffer MOPS (Bio-Rad)
- Coomassie Blue staining reagent (Bio-Rad)
- Fluorescent gel destaining solution: 4:1:5 (v/v/v) methanol/acetic acid/DD water
- 1.5 ml microcentrifuge tubes
- 70 $^{\circ}$ C heating block
- 12-well 4% to 12% Tris-HCl protein gel (Criterion XT Precast Gel, Bio-Rad)
- SDS-PAGE electrophoresis gel box for Criterion XT Precast Gel (Bio-Rad)

Fluorescence gel scanner equipped with the excitation wavelength at 532 nm, < 580 nm cut-off filter and 30 nm band-pass (Amersham Biosciences Typhoon 9400)

1. Solubilize 50 µg of ProSeAM-labeled protein pellet in 50 µl 4% SDS buffer (see Recipe).
Vortexing and sonication can accelerate the solubilizing process.
2. Prepare the following cocktail for click reaction in the following order (the amounts are adjusted for 50 µg protein pellet and can be increased if more protein samples are used): 1 µl 5 mM TAMRA-Azide fluorescent dye (final concentration of 100 µM); 1 µl 5 mM TBTA ligand (final concentration of 100 µM); 1 µl 100 mM TCEP (final concentration of 2 mM); 0.5 µl 100 mM CuSO₄ (final concentration of 1 mM).
3. Add a total 3.5 µl of the click cocktail into each reaction tube and allow the reaction to proceed at ambient temperature (23 °C) in the dark with mild shaking for 60 min.
Keep the reaction mixture minimally exposed to light because TAMRA dye is light sensitive.
4. Quench the click reaction and precipitate the proteins with the methanol/chloroform/water mixture (**SUPPORTING PROTOCOL 2**).
5. Solubilize the precipitated protein pellets in 50 µl freshly-prepared, 1 × loading buffer (see Recipe) by vortexing, and then centrifuge the sample at 4,000 g for 1 min.
6. Denature the sample on a 70 °C heat block for 10 min.
Cover the sample tubes with tin-foil during the heating process to avoid degradation of TAMRA dye. Keep the temperature of the heat block at 70 °C in the denaturing step because the dye degrades at higher temperatures.
7. Vortex the sample and centrifuge it at 4,000 g for 1 min.
8. Load 25 µl of each sample, and the fluorescent protein ladder onto SDS-PAGE gel (1 × MOPS buffer).
Dilute the commercial fluorescent protein ladder at least 10-fold and load the minimal amount for visualization. We also recommend leaving an empty lane between the protein ladder and protein samples to minimize signal bleeding.
9. Run gel at 200 V for 55 min.
Place the gel box in the dark and cover it with tin-foil to minimize light-stimulated dye decomposition.
10. Scan the gel using a fluorescent gel scanner with the mode of excitation 532 nm, <580 nm filter and 30 nm band-pass (Amersham Biosciences Typhoon 9400 was used in our protocol).
11. Destain gel with the fluorescent gel destaining solution (4:1:5 (v/v/v) methanol/acetic acid/DD) at ambient temperature (23 °C) for 4 h to remove unspecifically

bound dye. Repeat this step multiple times until the desired signal-to-noise is obtained.

12. To confirm the protein loading after recording the in-gel imaging, stain the gel with Coomassie Blue (Bio-Rad) according to the manufacturer's protocol.

SUPPORTING PROTOCOL 2: PROTEIN PRECIPITATION WITH METHANOL/CHLOROFORM/WATER

The following is a standard protocol for protein precipitation with a mixture of methanol/chloroform/water. This protocol is implemented in the current protocol to remove the unreacted ProSeAM from the enzymatic reaction or the unreacted biotin probe after the click reaction (**BASIC PROTOCOL 2**). This protocol is formulated for the large scale of pulldown (**BASIC PROTOCOL 2**) and can be modified for a smaller scale.

Materials

5 ml cell lysates (2 mg/ml) in a 50 ml Falcon tube (**BASIC PROTOCOL 2**)

Ice-cold methanol

Ice-cold chloroform

Ice-cold DD water

Refrigerated centrifuge (4 °C, 3,000 g capacity)

1. To each sample, add 42.5 ml 5:2:3 (v/v/v) methanol:chloroform:water.
2. Vortex the sample for 10 s and centrifuge it at 4 °C at 3,000 g for 45 min.
3. Carefully remove as much as possible of the upper aqueous without disturbing the interface layer, where the protein precipitate is located.
4. Add another 40 ml ice-cold methanol, vortex the sample briefly and centrifuge at 4 °C at 3,000 g for 30 min.
5. Carefully remove the supernatant without disturbing the protein pellet at the bottom.
6. Repeat Steps 4 and 5.
7. Air dry the precipitated protein pellets at ambient temperature (23 °C) for 20 min.

It is important to avoid drying the pellet longer than 20 min, because it is difficult to redissolve fully-dried protein pellet in the subsequent procedures.

SUPPORTING PROTOCOL 3: PROTEIN PRECIPITATION WITH METHANOL

The following is a standard protocol for protein precipitation with methanol. This protocol can be used to remove the unreacted ProSeAM from the enzymatic reaction or the unreacted biotin probe after the click reaction (**BASIC PROTOCOL 2**). This protocol is exemplified for protein sample on large scale and can be modified proportionally for smaller scales.

Materials

5 ml cell lysates (2 mg/ml) in a 50 ml Falcon tube (**BASIC PROTOCOL 2**)

Ice-cold methanol

Refrigerated centrifuge (4 °C, 3,000 g capacity)

1. Add 45 ml ice-cold methanol into a 50 ml conical tube containing the 5 ml cell lysates.
2. Allow the proteins to precipitate at –80 °C overnight.
3. Collect the precipitated protein pellet by centrifuging the tube at 4 °C at 4000 g for 30 min.
4. Remove the supernatant carefully without disturbing the precipitated protein pellet.
5. Add an additional 45 ml ice-cold methanol into the tube, and gently vortex.
6. Centrifuge the tube at 4 °C at 4000 g for 30 min, and then remove supernatant carefully without disturbing the protein precipitated pellet.
7. Repeat Steps 5 and 6.
8. Air-dry the protein pellet at ambient temperature (23 °C) for 20 min.

Avoid drying the pellet more than 20 min, because it is difficult to redissolve fully-dried protein pellet in the subsequent procedures.

SUPPORTING PROTOCOL 4

The following protocol provides the detailed synthesis of *Se*-adenosyl-*L*-selenomethionine (ProSeAM) and its precursors as reported previously (Bothwell et al., 2012)

Selenohomocystine

Sodium metal (15 mg, 0.652 mmol) was added to a mixture of selenomethionine (Sigma-Aldrich, 50 mg, 0.255 mmol) dissolved in liquid ammonia (~2 mL) at –78 °C under an N₂ atmosphere. After 20 min, the reaction was exposed to air and brought to ambient temperature (23 °C). Following the evaporation of ammonia, the residue was dissolved in 1M HCl and purified over Dowex® 50WX8 ion exchange resin with the 1:4 (v/v) NH₂OH:H₂O eluting solvent. Volatile solvents were removed under reduced pressure to yield selenohomocystine as a pale yellow solid (20 mg, 43% yield). ¹H NMR (500 MHz; D₂O + 1% TFA): δ = 2.34 (m, 2H), 2.95 (m, 2H), 4.12 (t, 1H). ESI-MS (m/z): 364.96 [M]⁺, 181.74.

2',3'-O-Isopropylidene-5'-O-p-toluenesulfonyl adenosine

Into 15 mL anhydrous pyridine was added 2',3'-isopropylidene adenosine (Sigma-Aldrich, 1 g, 3.26 mmol). Into this mixture, cooled on ice, was slowly added *p*-toluenesulfonyl chloride (680 mg, 3.58 mmol). The dark yellow reaction mixture was allowed to warm up at ambient temperature (23 °C) and stirred for overnight. Water of 10 mL was then added, immediately followed by the addition of 100 mL ice-cold saturated NaHCO₃. The aqueous solution was

extracted with ice-cold chloroform (3 × 75 mL). Organic extracts were combined and washed with ice-cold saturated NaHSO₄ (2 × 50 mL), then ice-cold water (2 × 50 mL). Volatile solvents were first removed under reduced pressure and then by a high vacuum oil pump overnight. The resultant off-white solid was dissolved in 50 mL chloroform. After adding hexane of 150 mL, the solution was left at 4 °C overnight. After decanting the mother-liquor, the precipitate was washed with 100 mL hexane and dried under a high vacuum, affording the desired product (white solid, 625 mg, 41% yield). ¹H NMR (500 MHz; CDCl₃): δ = 1.29 (s, 3H), 1.52 (s, 3H), 2.33 (s, 3H), 4.20 (m, 2H), 4.41 (m, 1H), 4.98 (dd, 1H; J_a = 6.4 Hz, J_b = 3.0 Hz), 5.27 (dd, 1H; J_a = 6.5 Hz, J_b = 2.0 Hz), 5.97 (d, 1H, J = 2 Hz), 7.11 (d, 2H, J = 8.1 Hz), 7.56 (d, 2H, J = 8.40 Hz), 7.76 (s, 1H), 8.17 (s, 1H).

Se-(5'-deoxyadenosine-5')-L-selenohomocysteine (SeAH)

Sodium borohydride (6 mg, 0.17 mmol) was added to a mixture of selenohomocysteine (20 mg, 0.06 mmol) in 10 mL EtOH. After 30 min, 2',3'-O-isopropylidene-5'-O-p-toluenesulfonyl adenosine (56 mg, 0.12 mmol) was added and the reaction was allowed to proceed for 24 h at ambient temperature (23 °C). The reaction was quenched by addition of 1 M HCl to pH = 2.0 and neutralized with 1.0 M KOH. The mixture was then washed with dichloromethane (2 × 30 mL). The aqueous phase was collected and the solvent removed under reduced pressure. The resultant off-white residue was dissolved in 1.0 M H₃PO₄ and stirred for 24 h at ambient temperature (23 °C). The reaction mixture was then neutralized with saturated Ba(OH)₂ and the supernatant purified by preparative reversed-phase HPLC (XBridge Prep C18 5 μm OBD 19 × 150 mm) using a flow rate of 10 mL/min with acetonitrile in 0.1% aqueous trifluoroacetic acid with a linear gradient from 0% to 10% in 20 min and then to 70% in 5 min. The elute containing the product was collected and concentrated by lyophilization overnight. HPLC retention time = 10 min. ¹H NMR (500 MHz; D₂O): δ = 2.10 (m, 2H), 2.63 (m, 2H), 3.00 (m, 2H), 3.77 (t, 1H), 4.33 (m, 1H), 4.38 (m, 1H), 4.84 (m, 1H), 6.09 (d, 1H), 8.38 (s, 1H), 8.46 (s, 1H).

Synthesis of ProSeAM

SeAH (15 mg, 34.8 μmol) was dissolved in a mixture of 1:1 formic acid and acetic acid (1 mL) and cooled on ice. Propargyl bromide (1.74 mmol, 80% v/v in toluene) was then slowly added. After addition of AgClO₄ (5.4 mg, 34.8 μmol) dissolved in 0.5 mL of 1:1 formic and acetic acid mixture, the reaction was continued at ambient temperature (23 °C) for 8 h and then quenched 5 mL H₂O containing 0.1% TFA (v/v). The aqueous phase was washed with ice-cold diethyl ether (3 × 5 mL), centrifuged and then passed through a Nalgene 0.2 μm syringe filter to remove the precipitate. ProSeAM was purified by preparative reversed-phase HPLC (XBridge Prep C18 5 μm OBD 19 × 150 mm) using a flow rate of 10 mL/min with acetonitrile in 0.1% aqueous trifluoroacetic acid with a linear gradient from 0% to 10% in 20 min and then to 70% in 5 min (the HPLC retention time of ProSeAM is 7.5 min). The ProSeAM elute was then concentrated by lyophilization overnight. TFA was added to the concentrated solution to adjust its pH to 2.0. The concentration of the ProSeAM stock solution was determined by UV absorption (ε₂₆₀ = 15,400 L·mol⁻¹·cm⁻¹). The final isolation yield was 15%. ¹H NMR (500 MHz, D₂O + 0.1% TFA-d): δ = 2.34 (m, 2H), 3.15 (s, 1H), 3.52 (m, 2H), 3.86 (m, 1H), 3.9 (ddd, 2H; J_a = 34.3 Hz, J_b = 12.8 Hz, J_c = 3.2 Hz), 4.14 (m, 2H), 4.49 (m, 1H), 4.56 (t, 1H, J = 6.13 Hz), 4.75 (m, 1H), 6.09 (d, 1H, J = 3.85

Hz), 8.37–8.40 (m, 2H, arom.); ESI-MS (m/z): 471.11 [M]⁺, 369.99 [5'-(propargyl)-selenoadenosine + H]⁺, 249.91 [5'-deoxyadenosine + H]⁺, 135.73 [adenine + H]⁺. HRMS: 471.0871 calculated and 471.0880 observed for C₁₇H₂₄N₆O₅Se.

ALTERNATIVE PROTOCOL 1: LARGE-SCALE SYNTHESIS OF AZIDO-AZO-BIOTIN PROBE

The gram-scale preparation of the azido-azo-biotin probe (Fig. 2b) was developed on the basis of the previous synthesis with several minor modifications (Yang et al., 2010; Yount et al., 2010). When synthesizing (*E*)-4-((5-(2-azidoethyl)-2-hydroxyphenyl)diazenyl)benzoic acid precursor, the acidic reaction (6 M HCl) was neutralized with a solution of saturated sodium carbonate, instead of adding sodium carbonate powder as reported previously. The modified protocol is more efficient to quench the reaction. The final step, coupling the biotin-PEG-NH₂ (2.16 mmol) precursor with the activated (*E*)-2,5-dioxopyrrolidin-1-yl 4-((5-(2-azidoethyl)-2-hydroxyphenyl)diazenyl) benzoate (1.5 eq), was performed in 50 ml DMF under argon. The resultant mixture was stirred at ambient temperature (23 °C) overnight. After removing the DMF with high vacuum, 250 ml DD water was added to the pellet. This mixture was extracted 3 times with ethyl acetate (final total volume of 700 ml). The organic layers were combined and washed with brine, dried with sodium sulfate and concentrated. Purification of the product with silica flash chromatography (7:2:1 to 6:3:1 ethyl acetate:methanol:hexane) resulted in 824 mg of the azido-azo-biotin probe (Fig. 2b). Adding this chromatography step allowed the access to semi-pure azido-azo-biotin in a convenient manner. This material is readily subjected to final HPLC purification achieving high purity for the subsequent click reaction and streptavidin pulldown experiment.

REAGENTS AND SOLUTIONS (RECIPES)

Deionized distilled water (DD water) was used throughout the protocol unless otherwise mentioned.

0.2 % SDS (w/v) PBS buffer

GIBCO's PBS supplemented with 0.2 % (w/v) Sodium Dodecyl Sulfate (SDS).

1 × loading buffer

This buffer contains 40 mM Tris-HCl (pH 6.8), 69 mM sodium dodecyl sulfate (SDS), 10 % (v/v) β-mercaptoethanol, and 10 % (v/v) glycerol.

4 % SDS (w/v) buffer

This buffer contains 50 mM triethanolamine (1 M stock, pH 7.4), 150 mM NaCl (2 M stock), 4 % (w/v) sodium dodecyl sulfate (SDS). The buffer can be stored at ambient temperature (23 °C) up to 6 months. 1 × EDTA-free Roche protease inhibitor cocktail is added according to the manufacturer's instructions immediately prior to its use.

Dilution buffer

This buffer contains 50 mM Triethanolamine (1 M stock, pH 7.4), 150 mM NaCl (2 M stock), and 1 % (w/v) Brij97 (Sigma). The buffer can be stored up to 6 months at ambient temperature (23 °C). 1 × EDTA-free Roche protease inhibitor cocktail is added according to the manufacturer's instructions immediately prior to its use.

Elution Buffer

This buffer contains 250 mM ammonium bicarbonate and 1 % (w/v) sodium dodecyl sulfate (SDS). Freshly-prepared 25 mM sodium dithionite (Sigma) is added immediately prior to its use.

Tris-HCl Buffer

This buffer contains 50 mM Tris-HCl (1 M stock, pH 8.0), 50 mM KCl (1 M stock), and 10 % (v/v) glycerol. This mixture can be stored at ambient temperature (23 °C) up to 6 months. 2 mM freshly-prepared TCEP (the final concentration) and 1 × EDTA-free Roche protease inhibitor cocktail are added immediately prior to its use.

COMMENTARY

Background Information

The study of proteome wide methylation has advanced significantly in recent years. For conventional candidate-based approaches, the radioactive ^3H -SAM combined with recombinant PMTs is often used to label individual substrates *in vitro*. The radiolabeled substrates can then be resolved by SDS-PAGE and confirmed by autoradiography (Rathert et al., 2008a). Another candidate-based approach is to use arrayed peptide/protein libraries as substrate candidates and allow identification of novel substrates in a less unbiased manner (Levy et al., 2011; Rathert et al., 2008b). These methods may have certain limitations. For instances, the signals of radiolabeled proteins are low in general and thus not suitable for detecting less abundant methylation targets. The candidate-based array approach relies on the robust activities of PMTs *in vitro*, which may not reflect the situation within living cells or *in vivo*. The advent of SAM cofactor surrogates (Binda et al., 2011; Bothwell et al., 2012; Islam et al., 2012; Islam et al., 2011; Peters et al., 2010) and the improvements of bioorthogonal chemical probes (Bothwell et al., 2012; Szychowski et al., 2010; Yang et al., 2010; Yount et al., 2010) have allowed unprecedented implementation of new experimental methods to profile protein methylation. The utility of SAM analogue cofactors carrying transferable alkyne/azide moieties has enabled the identification of many methylation targets (Bothwell et al., 2012; Islam et al., 2012). Certain PMTs can promiscuously process SAM analogue cofactors containing a terminal alkynyl moiety. However, many bulky SAM analogue cofactors (*e.g.* (E) pent-2-en-4-ynyl SAM) only react with specific PMTs (otherwise requiring PMT engineering (Islam et al., 2012; Islam et al., 2011; Wang et al., 2011b)) and the less-sterically-hindered propargyl SAM has been shown to be unstable at physiological pH (Bothwell et al., 2012). In contrast, the current protocol documents the method relying on the more stable ProSeAM as a methylation reporter. This method enjoys multi-fold advantages by enhancing detection sensitivity over ^3H -SAM labeled proteins via target enrichment strategy and showing more general promiscuity of ProSeAM for native

PMTs (Bothwell et al., 2012). Upon combining ProSeAM with the cleavable azido-azobiotin probe for methylation profiling, the samples can be processed under mild conditions for proteomic analysis (Bothwell et al., 2012). We have previously demonstrated the utility of the set of reagents to identify novel methylation targets (Bothwell et al., 2012). We foresee potential application of the current protocol in dissecting the varied methylomes of cells in primary tumor samples. The corresponding protocol can also be modified for Bioorthogonal Profiling of Protein Methylation (BPPM) with bulky terminal-alkyne-containing SAM analogues as cofactors (Islam et al., 2011; Wang et al., 2011b).

Critical Parameters and Troubleshooting

It is essential to maintain or restore the activities of endogenous PMTs after cell lysis. Here we were able to address the need successfully by using the increased amount of detergent-free lysis buffer and implementing extended sonication step. In the current protocol, we did not examine whether a small amount of detergent such as 0.005% (w/v) Tween 20 (a condition of many PMT activity assays (Ibanez et al., 2012)) interferes the activities of endogenous PMTs in the context of cell lysates. In certain cases, large amounts of detergents have to be included for efficient lysis of the samples (*e.g.*, primary tumor samples). To address this specific need, a buffer exchange with a commercially available detergent removal spin column (*e.g.*, Pierce, cat. No: 87778) can be included as described previously (Islam et al., 2012). Tolerance to PMT activities and the efficiency of lysis need to be evaluated when developing the recipes for lysis buffer.

Particular care should be taken when handling the ProSeAM cofactor. Even though ProSeAM is more stable than parpargyl SAM (60 min $t_{1/2}$ versus < 2 min half $t_{1/2}$ at pH 8.0), ProSeAM is still relatively unstable in contrast to sulfonium-containing SAM analogues (Bothwell et al., 2012), in particular at ambient temperature (23 °C) and under neutral-to-basic conditions. We therefore recommended aliquoting the cofactor and using it immediately after thawing. We don't recommend frequent freeze/thaw of the cofactors and storing the cofactor for more than 2 months. If it is the case, the quality of ProSeAM should be evaluated by LC-MS analysis.

For performing a large-scale pulldown (**BASIC PROTOCOL 2**), the parameters for the enzymatic reaction with ProSeAM such as buffer components, cofactor concentration, and incubation time should be optimized *a priori*. Because the collection and level of endogenous PMTs can differ significantly between cell types (Beroukhi et al., 2010), the optimal parameters may alter accordingly. In general, we implemented in-gel fluorescence for rapid evaluation (**SUPPORTING PROTOCOL 1**), as demonstrated for labeling of HEK293T cell lysates with ProSeAM (Bothwell et al., 2012). For identification of methylation targets of low abundance in HEK293T context, an initial input of at least 10 mg lysate proteins for ProSeAM labeling should be used. Lower than 10 mg lysate may restrict the detection of lowly-expressed methylation targets. It is imperative to freshly prepare the solutions of Cu(II) and TCEP for the click reaction, otherwise vastly reducing the reaction efficiency.

It is important to wash the precipitated protein pellet with ice-cold methanol twice after each precipitation step. After the enzymatic reaction, the washing step removes the residual

cofactor, which would otherwise sequester the azido-azo-biotin probe in the subsequent step. After the click reaction, the washing step removes the unreacted biotin probe, which would otherwise compete with biotinylated proteins for streptavidin beads. The buffer containing 4% (w/v) SDS can effectively solubilize the precipitated protein pellet and is compatible with the click chemistry. Fully dissolving the precipitated protein pellet after each precipitation step is essential, and can be achieved by repetitive pipetting or mild sonication. However, the buffer containing 4% (w/v) SDS is not compatible with streptavidin beads. The concentration of SDS must be diluted below 1.5 % before mixing with streptavidin beads for target pulldown. The subsequent use of 8 M urea in **BASIC PROTOCOL 2** should be sufficient to disrupt noncovalent interactions between proteins and thus essential to suppress background proteins for MS analysis.

Anticipated Results

The signal efficiency of proteome-wide labeling with ProSeAM should correlate with concentrations of the cofactor, as reflected with in-gel fluorescence (Fig. 3a). Here the background labeling with no cofactor is barely detectable. In general, optimal labeling is achieved by using the minimal concentration of the cofactor that reaches the plateau of signal gain versus background labeling (*e.g.* 25 μ M for ProSeAM with HEK 293T cell lysates). The pulldown sample should display an array of protein targets comparable to the pattern shown by in-gel fluorescence (Fig. 3b). In certain situations, slight signal gain or loss may be observed because of unknown reasons. The cell lysates can also be labeled by ProSeAM in the absence or presence of different PMT inhibitors. The corresponding gain- or loss-of-signals are expected to reflect the effects and potency of the PMT inhibitors acting on their enzyme targets in cellular contexts. Regardless of the pattern of in-gel fluorescence of total cell lysates and Coomassie stain of pulldown samples, the pulldown samples should be subject to proteomic MS analysis given the much higher resolution and detection threshold for the latter. Implementing the current method readily yielded > 200 target proteins modified by ProSeAM.

Time Considerations

BASIC PROTOCOL 1 can be completed within 6 h after having cells ready and before the precipitation step. Collecting cells takes around 30 min; lysis and protein quantification around 2 h; the enzymatic reaction with ProSeAM for another 1.5 h. The workup can be stopped after quenching the ProSeAM-labeling with **SUPPORTING PROTOCOL 2** or 3. The precipitated protein pellets can be stored at -80°C for 3 d. It takes 2 working days to complete **BASIC PROTOCOL 2** with 6 h allocated in Day 1 and full-day work in Day 2. It takes around 2 h to wash the precipitated protein pellet from the enzymatic reaction, followed by 2 h to resolubilize the dried pellet; 2 h for the click reaction and protein precipitation. In Day 2, all the steps must be processed continuously. Washing the protein pellet takes around 2 h; resolubilization, bead enrichment and subsequent washing for 2 h; cysteine reduction/capping and subsequent washing for 1 h; reductive elution with sodium dithionite for 2 h. It takes 0.5 h to concentrate and lyophilize the protein samples. The lyophilized protein powder (or denatured proteins in 1xbuffer) can be stored at -80°C prior to MS-based proteomic analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

General principle and workflow with terminal-alkyne-containing SAM analogue for labeling and pulling down substrates of PMTs. In **BASIC PROTOCOL 1**, the cultured cells are lysed and then retreated with terminal-alkyne-containing PMT-promiscuous SAM analogues such as ProSeAM. The enzymatic incorporation of ProSeAM's progargyl moiety leads to terminal-alkyne-labeled products. In **BASIC PROTOCOL 2**, a cleavable azido-biotin probe is covalently conjugated to the progargyl handle of modified proteins via copper-catalyzed click chemistry. The modified proteins are then enriched by streptavidin sephrose beads. After multiple-step extensive washing, the selectively enriched proteins are then eluted mildly with sodium dithionite. The released proteins can then be subject to MS analysis.

Figure 2.

Key chemical entities discussed in the current protocol. **a.** Terminal-alkynyl-containing SAM analogue cofactors including (*E*)-pent-2-en-4-ynyl, (*E*)-hex-2-en-5-ynyl-SAM, Pro-2-ynyl SAM and ProSeAM (Binda et al., 2011; Bothwell et al., 2012; Islam et al., 2011; Peters et al., 2010; Willnow et al., 2012). ProSeAM is used in **BASIC PROTOCOL 1** for labeling substrates of endogenous PMTs in HEK293T cells. **b.** Reported azido-containing biotin probes for the click reaction with terminal-alkyne-labeled targets and the pulldown with streptavidin beads. Such molecule entities include commercially available PEG4 carboxamide-6-azidohexanyl Biotin (Invitrogen), a cleavable azido-azo biotin probe with an alkyl linker (Szychowski et al., 2010), and a cleavable azido-azo-biotin probe with a PEG linker. The latter was used in **BASIC PROTOCOL 2**.

Figure 3.

Anticipated results with ProSeAM as a protein methylation reporter. **a.** In the top panel, in-gel fluorescence (*SUPPORTING PROTOCOL 1*) of HEK293T cell lysates modified with the increasing concentrations of ProSeAM. Bottom panel is Commassie Blue (CB) staining of the same gel as a loading control. **b.** HEK293T cells treated with 25 μ M ProSeAM for 1 h, pulled down with streptavidin beads and eluted with the azo-diazo-biotin probe (*BASIC PROTOCOL 1, 2*). 10 μ l of pulldown sample was separated by SDS-PAGE and stained with Commassie Brilliant Blue.