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Global Proteomics Analysis of Protein Lysine Methylation

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Abstract

Lysine methylation is a common protein post-translational modification dynamically mediated by protein lysine methyltransferases (PKMTs) and demethylases (PKDMs). Beyond histone proteins, lysine methylation on non-histone proteins play substantial roles in a variety of functions in cells, and is closely associated with diseases such as cancer. A large body of evidence indicates that the dysregulation of some PKMTs lead to tumorigenesis via their non-histone substrates. However, more studies on other PKMTs have made slow progress owing to the lack of the approaches for extensive screening of lysine methylation sites. Recently a series of publications to perform large-scale analysis of protein lysine methylation have emerged. In this unit, we introduce a protocol for the global analysis of protein lysine methylation in cells by means of immunoaffinity enrichment and mass spectrometry.

Keywords

Post-translational modification; methylation; Immunoaffinity enrichment; Mass spectrometry; Non-histone; proteomics

Introduction

Recent advances in generation of highly specific and efficient pan methyl-lysine antibodies allow the identification of hundreds of protein lysine methylation sites in cells (Cao et al., 2013; Guo et al., 2014; Wu et al., 2015). In this unit we describe a proteomic approach for global analysis of protein lysine methylation. The entire procedure (step-by-step from sample preparation to mass spectrometry analysis) can identify more than 1000 methylation sites in a single experiment. During this procedure, the most crucial steps are the immunoaffinity enrichment of methylated peptides and high-resolution/accurate mass (HR/AS) mass spectrometry analysis. This approach can be used to uncover novel non-histone substrates of protein lysine methyltransferases (PKMTs) and demethylases (PKDMs), by combining the platform with enzyme inhibition through specific inhibitors and/or knockdown by RNAi technology. Additionally, protocols for antibody purification and analytical-column construction for acquiring high-quality data are also described in this unit.

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Basic Protocol

Large-scale analysis of protein lysine methylation in cells

This protocol describes all steps required for robust and large-scale detection of protein lysine methylation sites *in vivo*. The entire procedure workflow is outlined in Figure 1. Briefly, proteins are extracted from mammalian cells, reduced and alkylated, and then digested by trypsin. Peptides were subject to prefractionation with strong cation exchange (SCX) chromatography. Immunoaffinity enrichment was performed in pooled fractions and lastly methylated peptides underwent nanoLC-MS/MS analysis. Among them, immunoaffinity enrichment by pan methyl lysine antibody is the key point for the success of the protocol. This protocol can be also combined with SILAC (Stable isotope labeling by amino acids in cell culture) to quantify the change of the methylation sites under different conditions (Ong et al., 2002).

Materials

Trypsin solution
Phosphate-buffered saline (PBS)
Tris base
1 M Tris-HCl, pH 8.3
Urea lysis buffer (see recipe)
Bradford protein assay solution
1 M DTT stock solution (see recipe)
1 M IAA solution (see recipe)
Trypsin
pH indicator strip, 1–14.
Trifluoroacetic acid
Waters Sep-Pak C18 cartridges
Menthol, HPLC grade
Acetic acid
Formic acid
Acetonitrile
PolySULFOETHYL A™ column (9.4 mm i.d. × 250 mm, PolyLC)
SCX Buffer A (see recipe)
SCX Buffer B (see recipe)
Protein A Mag Sepharose (GE healthcare)
Empore C18 disk (3M)

Reverse-phase HPLC Buffer A (see recipe)
Reverse-phase HPLC Buffer B (see recipe)
Reverse-phase HPLC column (see Support protocol 2)
Probe sonicator
QIAvac vacuum manifold
HPLC with UV-detector
SpeedVac concentrator
Magnetic rack
Tube rotator

Preparing cell lysate

1. Trypsinize cells and spin in centrifuge for 4 min at 1000 rpm at 4°C. *We recommend 30 mg proteins as start material for one experiment. Fewer protein amounts can be tried, but the identification of methylation sites is probably much less. Generally the protein amount in mammalian cell is about 200 pg/cell, so about 150 million of cells are needed for extraction of 30 mg proteins. For HeLa cells, it may need about seven 15-cm cell dishes.*
2. Wash cells with PBS three times.
3. Add chilled urea lysis buffer to the cell pellet (5:1 volume) and suspend. *The volume of HeLa cell pellet is normally about 10 μ L per million cells. According to this principle and the principle described in step 1, the protein concentration is expected to about 4–5 mg/mL. A lower concentration like 1–2 mg/mL is normally not recommended since the proteins will be too diluted during trypsin digestion.*
4. Sonicate 5 $\times$ 10 s with 10 s rest between sonication on ice, 30% amplitude.
5. Centrifuge for 20 min at 5000 $\times$ g at 4 °C and transfer the supernatant into a new tube.
6. Measure protein concentration using Brad-ford protein assay.
7. Take about 30 mg proteins to go to the following steps. Store the rest solution at –80°C.

Protein reduction and alkylation

1. Add DTT from 1 M stock solution to a final concentration of 10 mM to disulfide bonds. Incubate for 30 min at 56 °C.
2. Cool the proteins to the room temperature. Add iodoacetamide from 1 M freshly made solution to a final concentration of 50 mM to alkylate cysteine residues. Incubate for 40 min at room temperature in the dark.

Trypsin digestion

1. Dilute the protein solution 1:4 with 50 mM Tris-HCl, pH 8.3, to reduce the urea concentration to 1.6 M.
2. Add trypsin to a final ratio of 1:200 (wt/wt) enzyme to substrate. Incubate overnight at 37 °C with rotating.
3. Quench trypsin digestion by acidification with TFA to 0.4% (vol/vol). Make sure the pH is < 2 using a pH indicator strip.
4. Centrifuge for 20 min at 5000 × g at room temperature and transfer the supernatant into a new tube.

Peptide desalting for fractionation

1. For desalting 30 mg peptides, use a 1 g tC18 Sep-Pak cartridge with a vacuum manifold to desalt peptide samples. *The capacity of this cartridge is about 5% of the resin mass, and the bed volume is about 1.6 mL. Since the column (cartridge) volume is about 60%–70% of the void (bed) volume, the buffer volumes used below are at least 5 mL–10 mL, which will be 5 × 10 × column volumes. As an alternative, a 500 mg Oasis HLB cartridge that has the capacity of the up to 10% of the resin mass is recommended, and the following used volumes will be cut to half.*
2. Condition the cartridge with 15 mL acetonitrile and followed by 10 mL 50% (vol/vol) acetonitrile/0.1% (vol/vol) FA. *The cartridge should be kept wet during the whole process.*
3. Equilibrate the cartridge with 15 mL 0.1% (vol/vol) TFA.
4. Load peptide sample onto the cartridge. Keep the flow rate about 1–2 mL/min by adjusting the vacuum valve position. *Low flow is recommended to minimize peptides loss during loading.*
5. Wash the cartridge with 15 mL 0.1% (vol/vol) TFA.
6. Wash the cartridge with 15 mL 0.1% (vol/vol) FA.
7. Elute peptides with 10 mL 50% (vol/vol) acetonitrile/0.1% (vol/vol) FA. *This percentage of acetonitrile is strong enough to elute most of the peptides. A higher percentage of acetonitrile is normally not recommended since it might wash off other non-peptide stuffs like lipids from the cartridge.*
8. Freeze the eluate with liquid nitrogen and dry using SpeedVac. *You can aliquot about 5 µg peptides for checking the trypsin digestion and desalting by mass spectrometry.*

SCX (Strong-Cation Exchange) chromatography

1. Wash the SCX column by a blank run. Set up a gradient outlined in Table 1 for peptide fractionation.

2. Equilibrate the SCX column with SCX Buffer A for 35 min at a flow rate of 3 mL/min. *The column volume of this column is about 10 mL. A 10-fold volume for equilibration should be used here for acquiring good reproducibility. It is important because we need to combine 3 runs.*
3. Reconstitute peptide sample in 1.5 mL SCX Buffer A. Centrifuge for 3 min at $15000 \times g$ at room temperature to discard the insoluble material.
4. Inject 500 μ L sample onto the column and run the gradient in Table 1. *The capacity of the column used here is about 16 mg peptides, so multiple injection and run is necessary.*
5. Collect 1-min fractions.
6. Repeat step 4–5 twice. Combine the same time fractions.
7. Freeze the fractions with liquid nitrogen, and dry using SpeedVac until the volume in each tube is decreased by at least 30% (acetonitrile volume).

Peptide desalting for immunoaffinity enrichment

1. Pool the fractions to 10 fractions based on equal chromatography areas. Use a 100 mg tC18 Sep-Pak cartridge with a vacuum manifold to desalt peptides in each fraction. *As an alternative, a 60 mg Oasis HLB cartridge is recommended.*
2. Condition the cartridge with 1 mL acetonitrile and followed by 1 mL 50% (vol/vol) acetonitrile/0.1% (vol/vol) FA. *The cartridge should be kept wet during the whole process.*
3. Equilibrate the cartridge with 2 mL 0.1% (vol/vol) TFA.
4. Load peptide sample onto the cartridge. Keep the flow rate about 1–2 mL/min.
5. Wash the cartridge with 2 mL 0.1% (vol/vol) TFA.
6. Wash the cartridge with 2 mL 0.1% (vol/vol) FA.
7. Elute peptides with 1 mL 50% (vol/vol) acetonitrile/0.1% (vol/vol) FA.
8. Freeze the eluate with liquid nitrogen and completely dry using SpeedVac. *You can aliquot about 5 μ g peptides from each fraction before SpeedVac as “input” for checking SCX fractionation and desalting.*

Immunoaffinity enrichment of methylated peptides

- 1 Resuspend Protein A Mag Sepharose stock solution (20% (vol/vol)) and transfer 200 μ L to a 1.5-mL tube.
- 2 Wash the beads with PBS three times.
- 3 Add 400 μ g pan anti-monomethyl lysine antibody to the beads. *Support protocol 1 described how to purify antibodies from homemade antisera. You*

can buy commercial antibody beads from Cell signaling Technology (PTMScan® Pan-Methyl Lysine Kit #14809)

- 4 Incubate the antibodies and the beads for 4 h at 4 °C on a rotator.
- 5 Wash the beads with PBS three times.
- 5 Reconstitute peptide sample in 0.8 mL PBS. Make sure the pH is close to neutral using a pH indicator strip. If not, adjust pH using saturated Na₂HPO₄ in PBS. Centrifuge for 3 min at 15000 × g at 4 °C to discard the insoluble material.
- 6 Incubate the peptide sample and the antibody-protein A beads overnight 4 °C on a rotator.
- 7 Transfer the peptide sample solution into a new tube. Store at –80 °C or sequentially incubate protein A-pan anti-dimethyl (trimethyl) lysine antibody beads. Wash the beads with PBS three times.
- 8 Wash the beads with 0.8 M NaCl in PBS three times.
- 9 Wash the beads with water, and transfer the beads into a new tube.
- 10 Wash the beads with water twice.
- 11 Add 100 µl 0.1% (vol/vol) TFA to the beads. Incubate for 10 min at room temperature on a Vortex.
- 12 Fast spin to let the liquid and the beads go to the tube bottom.
- 13 Transfer the eluate into a new tube.
- 14 Repeat step 11–13 twice. Pool the eluates.
- 15 Dry the eluates to less than 50 µl.

Peptide desalting for LC-MS/MS

1. Pack Stage-Tip: Pouch out a small (~1 mm diameter) piece of Empore C18 disk using a pipette tip with the end cut off and transfer it into a 200 µl tip. Carefully use a fused silica capillary with a large outer diameter to push the disk out and jam it into the tapered part of the tip. *The loading capacity of a piece of Empore C18 disk (1 mm diameter, 0.5 mm length) is about 25 µg peptides (Rappsilber et al., 2003). Therefore two pieces of disk should be enough for each fraction sample.*
2. Condition Stage-Tip with 50 µl methanol. *The Stage-Tip should be kept wet during the whole process.*
3. Equilibrate the cartridge with 50 µl 0.1% (vol/vol) FA.
4. Load peptide sample onto the cartridge at a flow rate of 20 µl/min.
5. Re-load the sample if desired.
6. Wash the cartridge with 50 µl 0.1% (vol/vol) FA twice.

7. Elute peptides with 50 μ l 50% (vol/vol) acetonitrile/0.1% (vol/vol) FA.
8. Freeze the eluate in -80°C and dry to less than 5 μ l using SpeedVac.

nanoLC-MS/MS analysis

1. Reconstitute immunoaffinity-purified peptide sample to 8 μ l with 0.2% (vol/vol) FA.
2. Centrifuge for 3 min at $15000 \times g$ at room temperature and carefully transfer sample into a HPLC autosampler vial.
3. Inject 4 μ l sample to the reverse-phase HPLC column, and acquire mass spectrometry data via Q Exactive mass spectrometer coupled with EASY-nLC 1000 HPLC system. The parameters of HPLC and mass spectrometer are outlined in Table 2 and Table 3.

Database searching

pFind (Chi et al., 2015) or Maxquant (Cox and Mann, 2008) is used to search the UniProt human protein database for peptide and protein identification. The searching parameters are shown in Table 4. *pFind seems more sensitive to identify peptides for the HR-AM raw data acquired from Q Exactive. However, it doesn't have the function to evaluate the PTM location. An additional program with the algorithm similar to Ascore should be also used to locate the methyl groups if pFind is used* (Beausoleil et al., 2006).

Support protocol 1

This protocol described the procedure performing affinity purification of polyclonal pan methyl-lysine antibodies from rabbit antisera. Considering no reliable commercial pan methyl antibodies to be applied to enrich methylated peptides, we made a custom production of rabbit polyclonal antisera from the antibody company, and carried out affinity purification of antibodies in the lab. By imitating the way to purify anti-phosphosite antibodies, we performed tandem affinity purification from the antisera. Firstly we let the antisera go through the resins immobilized with the non-methyl lysine peptides, and then go through the affinity resins immobilized with the methyl lysine peptides. Although technically a more strictly specific purification for individual type of methylation should be done by going through the columns made by the other two different methylation types, we don't think it is necessary since mass spectrometry can distinguish the methylation types.

Antisera generation and affinity purification of antibody

Materials

PBS
TCEP
5 M NaCl
50 mM L-cysteine

SulfoLink™ Coupling Resin
Resin Coupling Buffer (see recipe)
Econo-Pac Chromatography Columns
Tube rotator

Pan methyl-lysine antisera generation

This part of work is done in the antibody company through custom production. Generally, peptide libraries are synthesized to serve as immunogens. They have the sequence CXXXXXXK(me)XXXXXX, where X is any common amino acid residue except cysteine and K(me) can be mono-, di- or tri-methyl-lysine residue. The immunogens were crosslinked to KLH carrier protein via N-terminal cysteine and injected to rabbits, respectively. The whole immunization takes about three month and the antisera are taken after that. Meanwhile, the peptide library without methyl lysine was synthesized for tandem purification of antibodies.

Making affinity columns

1. Stir the bottle to evenly suspend SulfoLink™ Coupling Resin and then transfer 10 mL of resin slurry to a 15-mL conical tube. Centrifuge for 1min at 1000 rpm at 4 °C and discard the supernatant. *SulfoLink Coupling Resin has an iodoacetyl group that allows covalent immobilization of sulfhydryl-containing peptides to agarose beads. It is supplied as a 50% (vol/vol) slurry.*
2. Wash the resin with 10 mL Coupling Buffer twice.
3. Dissolve 5 mg of peptide library in 10 mL Coupling Buffer and add TCEP to a final concentration of 10 mM. Mix it with the resin. *The purpose of TCEP addition is to reduce peptides and TCEP won't react with the resin.*
4. Wrap the tube in aluminium foil. Incubate for 40 min at room temperature with gentle end-over-end rotation.
5. Centrifuge for 1 min at 1000 rpm at 4 °C and discard the supernatant.
6. Add 5 mL 50 mM L-cysteine solution to the resin and incubate for 30 min at room temperature with gentle end-over-end rotation.
7. Wash the resin with 10 mL 1M NaCl three times.
8. Add 10 mL PBS with 0.05% (w/v) sodium azide. Store for up to six months at 4 °C. *The resin can be re-used, but no more than six times.*

Tandem purification of antibodies from antisera

- 1 Take 1 mL resin immobilizing with non-methyl-lysine peptides in a 50 mL conical tube. Centrifuge for 1 min at 1000 rpm at 4 °C and discard the supernatant.
- 2 Wash the resins with 10 mL PBS.

- 2 Thaw the antisera on ice and aliquot 10 mL antisera in 50 mL conical tube. Store at -20°C if not use.
- 3 Add 20 mL PBS. Mix and centrifuge for 10 min at $3500 \times g$ at 4°C .
- 4 Transfer the supernatant into the resin tube.
- 5 Incubate the antisera and the beads for 4 h at 4°C with gentle end-over-end rotation.
- 6 Transfer the antisera and the beads to a 10 mL gravity-flow chromatography column.
- 7 Collect the flow-through and mix it with the beads immobilizing with methyl-lysine peptides prepared with step 1–2. For the beads immobilizing with non-methyl lysine peptides, add 20 mL PBS, pH 2.5 to clean, and then go to step 17–18.
- 8 Incubate overnight at 4°C with gentle end-over-end rotation.
- 9 Transfer the antisera and the beads to a 10 mL gravity-flow chromatography column.
- 10 Collect the flow-through for next use. *We normally re-utilize the flow-through one more time. Store for up to four week at 4°C or for one year at -20°C , if not use immediately.*
- 11 Wash the resin with 10 mL PBS twice.
- 12 Wash the resin with 10 mL 0.8 M NaCl in PBS twice.
- 13 Wash the resin with 10 mL PBS, pH 5.0.
- 14 Check the protein concentration of the effluence using protein A280 assay. Wash with more buffers until no proteins are washed out.
- 14 Elute the antibody with 15 mL PBS, pH 2.5. Collect 1 mL eluate in tubes containing 50 μL saturated Na_2HPO_4 in PBS. *Antibodies are unstable in the acidic buffer, so they are needed to neutralize immediately after eluted.*
- 15 Check the protein concentration of the effluence in each tube using protein A280 assay, and pool the elution solutions with antibodies.
- 16 Measure antibody concentration using Brad-ford assay. Aliquot 400 μg antibody in 1.5 mL tube, and store at -20°C . *Concentrate antibody using ultrafiltration tubes if the concentration is lower than 0.5 mg/mL. You also can dilute the antibodies with glycerol to a final concentration of 50% glycerol, and store them at -20°C , so the antibodies won't be frozen.*
- 17 Re-equilibrate the beads with 10 mL PBS twice.
- 18 Transfer the beads into a new tube with PBS containing 0.05% (wt/vol) sodium azide. Store at 4°C for next use.

Support protocol 2

This protocol described how to make analytical columns for nano-LC-MS/MS analysis. An analytical column with high resolution and high peak capacity is necessary to identify low-abundant PTM peptides. Here we made columns with integrated emitter tips and packed them by 3- μ m C18 resin. Compared to the commercial columns without integrated tips, this kind of columns can increase the electrospray signals, provide better chromatographic resolution and increase peak capacities. They are also low cost to replace, and flexible to adjust the length and change the packing materials. As an alternative, you can buy PicoFrit columns (New Objective) to pack.

Material

360- μ m o.d. $\times$ 75 μ m i.d. fused silica tubing
Methanol
Acetonitrile
HF acid
Reprosil-Pur C18-AQ resin (3 μ m; Dr. Maisch GmbH, Germany)
BSA digests
Lighter
Lamp
Laser puller P-2000
Helium tank and its accessories
Pressure bomb
2 mL HPLC glass vial
Stirrer
Microscope
HPLC instrument

Making Capillary Columns with Integrated Emitter Tips

- 1 Cut approximately 60-cm 360- μ m o.d. $\times$ 75 μ m i.d. fused silica tubing using a ceramic scoring wafer.
- 2 Use a lighter to remove about 2 cm polyimide coating in the middle of the tubing. Wipe off the burnt coating completely to expose the silica using a methanol-wet Kimwipe. *Don't over-burn and Let the tubing straight when burn.*
- 3 Place the tubing inside of the laser puller with the exposed silica centered in the path of the laser. Attach the puller's clamps and start the puller method to pull a 10- μ m tip. *The setting parameters on our laser puller are listed in the*

Table 5. However, it should be noted that the optimum settings for individual laser puller always differ. Our setting just gives you a guideline.

- 4 Inspect the tip under a microscope. If the tip size is too small or even closed, go to step 5; otherwise go to step 6.
- 5 In the fume hood, transfer 30 μ L HF to a tube and 1 mL 0.2 M ammonium formate to another tube. Dip the tip straight in HF for 1–2 min. Immerse the tip in ammonium formate for 30 s to quench etching. *The time for HF etching should be adjusted to get the desired tip diameter.*
- 6 Place a glass vial containing 1 mL methanol into the pressure bomb.
- 7 Insert the bottom of a column with integrated emitter tip through the ferrule on the top of the high-pressure bomb. Tighten the screws on the bomb. Push the column into the vial until it touches the bottom of the vial, and then pull it up about 2 mm from the bottom of the vial. Tighten the nut around the ferrule. *Wipe the tubing and cut about 5-mm tubing in the front after the tubing goes through the ferrule, in case the tube end carries plastic particles from the ferrule to clog the packing.*
- 8 Turn on the helium gas tank, and regulate the pressure to about 100 psi. Turn on the valve.
- 9 Rinse the column with methanol about 30 s. Turn off the valve.
- 10 Add 1 mL acetonitrile and about 0.5 mg of 3- μ m C18-AQ resin into another glass vial with a stir bar. Suspend with a pipette tip and sonicate in water bath for 1 min for dispersing resin particles.
- 12 Open the pressure bomb and place the vial containing the resin slurry into the bomb. Turn on the stir.
- 13 Slowly turn on the valve to start packing. Check the packing under a microscope.
- 14 Regulate the pressure to about 1000 psi after packing about 1-cm tubing.
- 15 Allow the column to pack until it reaches the desired length. *Use a lamp placed beside the column to visually monitor the packing. We normally pack analytical columns with about 30 cm-long resins.*
- 16 Turn off the helium gas tank, and then slowly close the valve to release the pressure. *If the pressure is released too quickly the resin will flow back. It is hard to avoid. That's why we normally pack a little longer than desired. Don't worry about the gaps generated when it happens because the column will be re-packed after connected to the HPLC instrument.*
- 17 Make a fitting to connect the column to Easy nLC 1000 HPLC instrument. Set 600-bar pressure to equilibrate the column using Buffer A for 5 min. *Use the maximal pressure you set in your LC method. Use a light source to check for any gap in the column. Normally the gaps will disappear under this high*

pressure. If gaps are still present (it indicates somewhere may be clogged) but the flow rate under the set pressure is within the reasonable level, we normally keep the column until it doesn't pass the test using the standard samples. If your HPLC doesn't have the option to output the pressure, adjust the flow rates to reach a maximal pressure you normally use.

- 18 Turn off the pump to slow release the pressure to less than 100 bars. Disconnect the column, cut it to the desired length and reconnect it back.
- 19 Run a couple of 100-fmol BSA digests to block non-specific binding sites on silica columns. *Normally in our hands, the chromatography peaks of BSA peptides will get normal in three runs. Check if the peak tailing is present and if the peak widths (full width at half maximum, FWHM) are within the desired values, then decide if you want to keep it. If the peak tailing is present, check the fitting and make a new fitting if necessary.*

Reagents and Solutions

5 M NaCl

143.75 g NaCl

Milli-Q water to 500 mL

Store up to 1 year at room temperature.

1M Tris-HCl, pH 8.3

121.14 g Tris base

Milli-Q H₂O to 1 L

pH 8.3 with HCl

Dissolve Tris base in 800 mL water, adjust pH to 8.3 using HCl, and then supplement water to 1L. Store up to 1 year at room temperature.

Urea lysis buffer (8 M urea, 75 mM NaCl, 50 mM Tris-HCl, pH 8.3)

Always make fresh buffer before use and keep it on ice. To make 10 mL, add 4.8 g urea, 0.15 mL 5M NaCl and 0.5 mL Tris-HCl, pH 8.3 to a 15 mL conical tube, supplement water to 10 mL and rotate on a rotator until urea is totally dissolved. Supplement water to 10 mL. Transfer the needed volume into a new tube and add 1x protease inhibitor cocktail before use.

1M DTT

15.4 mg Dithiothreitol

50 mM Tris-HCl, pH 8.3 buffer to 0.1 mL.

Make fresh buffer before use.

1M IAA

92.5 mg Iodoacetamide

50 mM Tris-HCl, pH 8.3 buffer to 0.5 mL.

Make fresh buffer before use.

SCX Buffer A (10 mM KH₂PO₄, pH 2.65, 30% acetonitrile (vol/vol))

2.72 g KH₂PO₄

1.4 L HPLC water

0.6 L LC-MS grade acetonitrile

pH 2.65 with H₃PO₄

Filter the buffer using a pharmaceutical-grade 0.45-μm membrane filter to remove any insoluble material. The pH adjustment should be made before adding acetonitrile because organic solvents interfere with the pH reading. Store up to three months at room temperature.

SCX Buffer B (10 mM KH₂PO₄, 500 mM KCl, pH 2.65, 30% acetonitrile (vol/vol))

1.36 g KH₂PO₄

37.28 KCl

0.7 L HPLC water

0.3 L LC-MS grade acetonitrile

pH 2.65 with H₃PO₄

Filter the buffer using a pharmaceutical-grade 0.45-μm membrane filter to remove any insoluble material. The pH adjustment should be made before adding acetonitrile because organic solvents interfere with the pH reading.

Saturated Na₂HPO₄ in PBS

5g Na₂HPO₄

40 mL PBS

Add Na₂HPO₄ to PBS, Vortex for 5min. Store up to three months at room temperature.

Reverse-phase HPLC buffer A

0.1% (vol/vol) FA

1 mL LC-MS grade FA

LC-MS grade water to 1 L

Store up to one month at room temperature. Sonication for degassing in water bath for 15 min before use.

Reverse-phase HPLC buffer B

0.1% (vol/vol) FA in acetonitrile

1 mL LC-MS grade FA

LC-MS grade acetonitrile to 1 L

Store up to one month at room temperature. Sonication for degassing in water bath for 15 min before use.

Resin Coupling Buffer

50 mM Tris-HCl, 5mM EDTA-Na, pH 8.3

5 mL 1M Tris-HCl, pH 8.3

1mL 0.5 M EDTA-Na, pH 8.5

Milli-Q water to 100 mL

Make fresh before use.

50 mM L-Cysteine

30 mg L-Cysteine

Milli-Q water to 5 mL

Make fresh solution before use.

Commentary**Background Information**

Protein lysine methylation was once thought as an irreversible post-translational modification until the first demethylase LSD1 was discovered about ten years ago (Shi et al., 2004). To date, it has been reported that there are more than fifty protein lysine methyltransferases (PRMTs) and more than thirty protein lysine demethylases (PKDMs) in human cells (Copeland et al., 2009; Rotili and Mai, 2011). A large number of studies on these PKMTs and PKDMs have focused mainly on their regulation of histone methylation. However, it has been revealed that the protein targets of many of these enzymes extend beyond histones. An increasing number of non-histone proteins, such as p53, VEGFR1, Rb1 and STAT3, have been reported to serve as the substrates of some well-known PKMTs (Autiero et al., 2003; Chuikov et al., 2004; Huang et al., 2006; Saddic et al., 2010). A recent review summarized the molecular functions of non-histone protein lysine methylation, and particularly its involvement in cancers (Hamamoto et al., 2015). A variety of methylation sites catalyzed by different PKMTs have been explored to be strongly associated with cancers. For instance, it was recently discovered that the K260 methylation on MAP3K2 by SMYD3 increased MAP kinase signaling and promoted the formation of Ras-driven carcinomas (Mazur et al., 2014). Consequently more anticancer drugs against PKMTs and PKDMs have been developed in recent years (Hojfeldt et al., 2013; Wagner and Jung, 2012). Thereby it is critical and pressing to clarify the non-histone substrates of these enzymes, so as to deeply understand the roles of PKMTs in cancers and the mechanism of action of the

anticancer drugs. However, owing to the lack of highly effective methods for large-scale screening of methylation *in vivo*, the majority of PKMT substrates identified are mainly based on indirect or *in vitro* methylation assays (Huang et al., 2006; Nakakido et al., 2015; Piao et al., 2014; Saddic et al., 2010). It brought slow progress on studies in this field.

Most of the proteins containing PTMs in cells are low abundant and the PTM occurrence is normally at low-stoichiometry, except for very few proteins. Therefore, enrichment is necessary for larger-scale analysis of these protein PTMs *in vivo*. A general methodology is to combine immunoaffinity enrichment by pan PTM antibody and mass spectrometry analysis. By means of this strategy, hundreds and thousands of protein lysine acetylation, ubiquitylation and other PTM sites have been identified (Choudhary et al., 2009; Kim et al., 2011; Lundby et al., 2012; Tan et al., 2014). However, owing to the poorly specific and efficient commercial antibodies, the identification of methylation sites remains challenging (Bremang et al., 2013; Ong et al., 2004; Pang et al., 2010). Therefore, few methylated proteins were identified, and furthermore due to the enrichment performed previously at the protein level, scarce actual methylation sites were uncovered. Recently, a new strategy that utilized the methyl-lysine reading domain, MBT domain, to capture methylated proteins has been shown useful to identify hundreds of methylated proteins (Moore et al., 2013). However, it should be noted that only a actual few methylation sites were detected for the same reason described above (i.e. enrichment at protein level). We previously reported our initial work toward the first global comprehensive large-scale identification of protein lysine methylation sites in cells using our homemade pan methyl antibodies (Cao et al., 2013). Since that publication other labs also published their work on identification of protein lysine methylation by generating new pan methyl-lysine antibodies (Guo et al., 2014; Wu et al., 2015). These studies basically followed the same strategy, acquiring methylated peptides by immunoaffinity enrichment and then identifying them utilizing HR-AS mass spectrometry. However, it should be noted that specificities of polyclonal antibodies generated in different sources might differ, which might lead to a low overlap of identified methylation sites among the results acquired in different labs.

Critical Parameters and troubleshooting

Peptide prefractionation may be necessary to hunt for low-abundant methylation sites. Here we described the procedure of peptide SCX fractionation. Other HPLC fractionation can be performed, such as high-pH RP-LC, which may bring more even peptide distribution in fractions according to the description in a recent publication (Mertins et al., 2013). Post-immunoprecipitation fractionation was applied in other PTM identification studies such as with lysine acetylation (Lundby et al., 2012). However, considering that very high-abundant methylation occurrences exist on a few proteins like histone proteins and translation-related proteins in cells. These high abundant methylated peptides may interference with the capture of the other low-abundant methylated peptides, so we do not recommend strategy, although we have utilized it previously (Cao et al., 2013). We also do not recommend protein fractionation either, considering that protein solubility may be an issue in organic HPLC buffer.

The immunoaffinity enrichment of methylated peptides is the key point during this protocol. Protein A Mag Sepharose from GE Healthcare is used to conjugate the antibodies here. We have tried a few protein A agarose and magnetic beads from different vendors, and found Protein A Mag Sepharose to be the best in our hands. It has some advantages compared to the protein A agarose beads. Firstly, the entire process is much easier using a magnetic rack to settle the beads instead of centrifuging. Secondly, bead loss is minimized to decrease the loss of the methylated peptides. The most important reason is that it dramatically decreases the contamination of non-specific binding of non-methylated peptides. Compared to the other brands' magnetic beads, Protein A Mag Sepharose is more hydrophilic. This characteristic makes it not stick to the tube wall and much easier to disperse the bead aggregation without addition of any detergent, so as to decrease both the non-specific peptide binding and the sample loss. We did not try chemically immobilizing antibodies directly to the beads since most of the magnetic beads for this purpose have pretty low-capacities. We did not crosslink antibodies to the protein A beads either, so the antibodies are also eluted from the beads using 0.1% TFA. Our concern is that crosslinking may de-active some portions of antibodies. Such high molecular-weight proteins are not bound to the Stage-Tips, and are therefore separated away from methylated peptides during the desalting process. If the antibody contamination is concerned, crosslinking can be performed following the instructions of the beads provided by the vendors. Any detergent should be avoided in immunoprecipitation buffers. Detergents like NP-40, Tween-20 cannot be completely washed off from Stage-Tip and other C18 cartridges. This brings polyethyleneglycol (PEG) contamination that can interfere with peptide signals in mass spectrometry analysis. Here we simply use PBS as the binding buffer. It may introduce non-specific binding of non-methylated peptides. Two kinds of washing reagents are sequentially used to minimize the non-specific binding in our protocol, 0.8 M sodium chloride and pure water. Another aspect that should be kept in mind is to transfer the beads into a new tube before elution, to eliminate the contamination of non-methylated peptides that strongly stick to the tube wall but can be liberated by acid elution.

High-sensitive nanoLC-MS/MS is critical to improve the identification of methylated peptides, since most of this modified peptides are of low abundance even after the enrichment. Ultra-high-performance nano-LC setups on analytical columns and the LC instrument can produce optimal chromatography for improving peptide identification. Here we use 25 cm-long analytical columns with integrated emitter tips and packed with 3- μ m C18 particles. The integrated emitter tip decreases the post-column volume, so as to improve the electrospray signals of peptides. The long-length column and small-size particle packing is recommended, since these can also improve chromatographic resolution of peptide peaks and increase the peak capacity, though it also increases the system pressure. The column heater can be used to decrease the pressure if available. Start-of-the-art mass spectrometers such as Q Exactive or Fusion Orbitraps are strongly recommended to run enriched peptide samples since they have fast duty cycles and high sensitivity to detect low-abundant peptides. The peptide standards like BSA digests should be analyzed in advance to block the new columns and evaluate the performance of the nanoLC-MS/MS settings.

Anticipated Results

Using the protocol described above, we have been able to routinely identify more than 1000 mono-methylation sites in a single experiment. However, depending on the antibody quality in different batches, cell types and the protein amounts used, the values might be variable ranging from 400 to 1500. A representative result is shown in Figure 3. In this experiment, we identified a total of 1246 monomethylation, 59 dimethylation and 53 trimethylation sites from one cell sample, respectively. Identification of dimethylation and trimethylation sites is much lower compared to monomethylation sites, but might reflect the real distribution of three different degrees of lysine methylation in cells (Guo et al., 2014).

Time Considerations

Construct affinity columns and antibody purification (2 days)—Making affinity columns may take 2 h. The preparation of antisera and the first round of incubation may take about 5–6 h. The second round of incubation is overnight. The column washing and elution takes about 2 h.

Construct analytical columns with integrated emitter tips (1 day)—The column generation takes about 10 min, and the packing may take a few hours. We normally leave a column packing overnight.

Sample preparation and trypsin digestion (2 days)—It may take about 2–3 h to harvest cells and for protein extraction. Protein reduction and alkylation may take 1.5 h and the trypsin digestion is performed overnight. Peptide desalting may take about 1 h and drying peptide samples down may be variable from 3 h to 6 h depending on the performance of the SpeedVac.

SCX fractionation and desalting (3 days)—Multiple SCX fractionation may take 7 h and the desalting may take about 2 h. Dryness take totally 2 days. SCX fractionation should be finished in one day, and then half of the collection tubes are subjected to dryness overnight, and the next day continue to dry the other half of the tubes. The desalting and sample drying is done on the third day. The dried peptides can be stored at –80 if not used immediately.

Immunoaffinity enrichment of methyl peptides and desalting (2 days)—Incubation of protein A beads and antibody takes about 5 h, and the incubation with peptide samples is done overnight. The washing and elution of the peptides from beads take about 1 h, and peptide concentration and desalting may take 3–4 h. More time is needed if performing enrichment of dimethylated or trimethylated peptides.

nanoLC-MS/MS analysis (2 days)—Each sample may take 3.5 h to analyze using a 180-min gradient, so 10-fraction samples will take about 1.5 days of instrumentation time for identification of monomethylation. The time will be doubly or triple if also analyzing dimethylation and/or trimethylation. The mass spectrometry experiments for peptide standards and a few cycles of washes in between samples take about 5 h.

Database searching (1 day)—Typically can be performed in one day. The further comprehensive analysis may take a few more days.

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Significance Statement

Accumulating evidence indicates that lysine methylation on non-histone proteins plays a substantial role in a variety of functions in cells, and is highly relevant to diseases such as cancer. However, unlike histone methylation, current studies on non-histone protein lysine methylation have remained challenging since the approaches for systemic analysis of lysine methylation sites have lacked for a long time. This protocol introduces procedures for the global analysis of protein lysine methylation in cells. It will be advantageous to extensively study the function of non-histone methylation and its catalytic enzymes, methyltransferases and demethylases, under different physiological and pathological conditions, particularly in cancers. Moreover, this protocol can be also used for reference in studies of other post-translational modifications.

Figure 1.
The stepwise procedure for global proteomics analysis of protein lysine methylation.

Figure 2. Chromatogram of the SCX fractionation of peptides
A 60-min gradient (Table 1) was run to separation peptides.

Figure 3. An example of identification of methylated unique peptides and sites from a single experiment

The numbers of monomethylated (A), dimethylated (B) and trimethylated (C) unique peptides across 10 SCX are presented, respectively. (D) The identification number of three degrees of methylation sites.

Table 1
Gradient used for fractionation of trypsin-digested peptides by SCX chromatography

The flow rate is constant at 2.5 mL/min.

Time (min)	Duration (min)	Gradient (% B)
0	N/A	0
5	5	0
10	5	10
46	36	40
50	4	100
55	5	100
56	1	0
60	4	0

Table 2
Gradient setting on Easy nLC 1000 instrument for nano-LC-MS/MS analysis of peptides

The flow rate is constant at 300 nL/min.

Time (min)	Duration (min)	Gradient (% B)
0	N/A	5
160	160	30
170	10	95
180	10	95

Table 3

Mass spectrometry parameter settings on Q Exactive for acquiring LC-MS/MS data.

MS parameter	Value
Full MS	
Microscans	1
Resolution (at m/z 200)	70,000
Automatic gain control target	1.00E+06
Maximum ion time	50 ms
Scan range	350–1600
dd-MS2	
Microscans	1
Resolution (at m/z 200)	17,500
Automatic gain control target	1.00E+05
Maximum ion time	250 ms
Loop count	12
Isolation window	3 m/z
Fixed first mass	100 m/z
Normalized collision energy	24
dd settings	
Underfill ratio	5%
Intensity threshold	1.00E+04
Charge exclusion	Unassigned, 1, 5
Peptide match	on
Exclude isotopes	on
Dynamic exclusion	20 s

Table 4

Database searching parameter settings for pFind and Maxquant programs.

	pFind	Maxquant
Database	UniProt human protein database	
Mass tolerance	5 ppm	6 ppm
MS/MS tolerance	0.02 Da	50 ppm
Maximum missed cleavages	3	
Fixed modification	Carbamidomethyl[C]	
Variable modification	Acetyl[ProteinN-term] Oxidation[M] Methyl[K] Dimethyl[K] Trimethyl[K]	
FDR filter	0.01 peptide 0.01 protein	0.01 PSM 0.01 protein 0.01 site

Table 5

Laser-puller P-2000 settings

These parameters were used to pull 10-µm tips from 360-µm o.d. × 75 µm i.d. fused silica tubing on our laser puller. The parameters on individual laser puller should be optimized following the instruction of the vendor.

Line	HEAT	FIL (Filament)	VEL (Velocity)	DEL (Delay)	PULL
1	300	0	30	128	0
2	250	0	20	128	0
3	175	0	12	128	0
4	155	0	9	128	0