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Mass spectrometry-based detection of protein acetylation

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Summary

Improved sample preparation techniques and increasingly sensitive mass spectrometry (MS) analysis have revolutionized the study of protein post-translational modifications (PTMs). Here, we describe a general approach for immunopurification and MS-based identification of acetylated proteins in biological samples. This approach is useful characterizing changes in the acetylome in response to biological interventions (1).

Keywords

Post-translational modification; sirtuin; immunopurification; immunoprecipitation; immunoblotting

Introduction

Reversible acetylation on the ϵ -amino group of lysines (acetylation; acK) has emerged as a PTM with a crucial role in regulating the biology of its target proteins, analogous to phosphorylation (2). This modification is the target of many sirtuins, NAD⁺-dependent deacetylases that regulate key physiologic processes involved in healthspan in mammals (3). There is great current interest in whole-proteome, MS-based approaches to identifying changes in acetylation occurring *in vivo* in response to modulation of sirtuin activity or that of other deacetylases, changes in diet, and other biological interventions. Many groups have published such characterizations (*e.g.* (1, 4–7)). Here we described a general method for generation and immunoaffinity purification (IAP) of acetylated peptides from complex biological samples for MS, analysis of the MS data, and validation of the results via immunoprecipitation (IP)/immunoblotting (IB). Although we describe this method specifically in the context of protein acetylation, the approach is generally useful for identification of a variety of different PTMs. Included among these methods are a series of immunoaffinity techniques using motif antibodies to enrich for a specific subset of post-translational modifications (acetylation or others such as serine-, threonine- and tyrosine-phosphorylation, methylation and ubiquitination) that can be utilized to probe specific areas of signal transduction or protein modification (http://www.cellsignal.com/services/discovery_overview.html). In addition, other immunoaffinity applications have been

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²¹There are many other routine methods for concentrating peptides using commercial products such as ZipTip® (<http://www.millipore.com/catalogue/item/ZTC18S096>) and StageTips (http://www.proxeon.com/productrange/sample_preparation_and_purification/stage_tips/index.html) that have been optimized for peptide desalting/concentration. Regardless of the particular method, we recommend that the method of choice be optimized for recovery and be amenable for peptide loading capacities of at least 10 μ g.

developed using site-specific antibodies to target particular signaling pathways (http://www.cellsignal.com/services/direct_overview.html). If the reader wishes simply to use IP/IB to confirm hits from previous MS or other studies, she/he can skip to protocol 3.12 “Validation of MS/MS results by IP/IB”.

Materials

2.1 Stock solutions for peptide preparation and IAP

Prepare solutions for cell lysis, Sep-Pak purification, IAP enrichment, and IP/IB with Milli-Q or equivalent grade water. Prepare MS solutions with HPLC grade water (*e.g.* Burdick and Jackson Water, Honeywell, AH365-4).

1. 200 mM HEPES/NaOH, pH 8.0: Dissolve 23.8 g HEPES (Sigma, H-4034) in approximately 450 mL water, adjust pH with 5 M NaOH to 8.0, and bring to a final volume of 500 mL. Filter through a 0.22 μ M filter, use for up to 6 months.
2. Sodium pyrophosphate: Make 50 $\times$ stock (Sigma, S-6422; 125 mM, MW = 446): 1.1 g/20 mL. Store at 4°C, use for up to one month.
3. β -glycerophosphate: Make 1000 $\times$ stock (Sigma, G-9891; 1 M, MW = 216): 2.2 g/10 mL. Divide into 0.10 mL aliquots and store at -20°C .
4. Sodium orthovanadate (Na_3VO_4): Make 100 $\times$ stock (Sigma, S-6508; 100 mM, MW = 184): 1.84 g/100 mL. Sodium orthovanadate must be depolymerized (activated) according to the following protocol. For a 100 mL solution, fill up with water to approximately 90 mL. Adjust the pH to 10.0 using 1 M NaOH with stirring. At this pH, the solution will be yellow. Boil the solution until it turns colorless and cool to room temperature (put on ice for cooling). Readjust the pH to 10.0 and repeat boiling until the solution remains colorless and the pH stabilizes at 10.0 (usually it takes two rounds). Adjust the final volume with water. Store the activated sodium orthovanadate in 1 mL aliquots at -20°C . Thaw one aliquot for each experiment; do not re-freeze thawed vial.
5. Trypsin-TPCK: Store dry powder (Worthington, LS-003744) for up to 2 years at -80°C . Parafilm cap of trypsin container (Worthington) to avoid collecting moisture, which can lead to degradation of the reagent. Prepare 1 mg/mL stock in 1 mM HCl. Divide into 1 mL aliquots, store at -80°C for up to one year.
6. Urea Lysis Buffer: 20 mM HEPES pH 8.0, 9 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate. The Urea Lysis Buffer should be prepared prior to each experiment (*see* Note 1). Aliquots of the Urea Lysis Buffer can be stored at -80°C for up to 6 months.
7. DTT solution: Make 1.25 M stock (American Bioanalytical, AB-00490; MW = 154): 19.25 g/100 mL. Divide into 0.20 mL aliquots, store at -20°C for up to one year. Thaw a fresh aliquot for each experiment.
8. Iodoacetamide solution: Dissolve 95 mg of iodoacetamide (Sigma, I-6125; MW = 184.96 g/mol) in water to a final volume of 5 mL. After weighing the powder, store in the dark and add water only immediately before use. The iodoacetamide solution should be prepared fresh prior to each experiment.

¹Dissolving urea is an endothermic reaction. Preparing the Urea Lysis Buffer can be facilitated by placing a stir bar in the beaker and by using a warm (not hot) water bath on a stir plate. 9 M urea is used so that upon lysis, the final concentration is approximately 8 M. The urea lysis buffer should be used at room temperature. Placing the urea lysis buffer on ice will cause the urea to precipitate out of solution.

9. IAP buffer: 50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl.
10. 20% trifluoroacetic acid (TFA): add 10 mL TFA to water to a total volume of 50 mL (CAUTION: TFA is a strong acid and is very toxic. Contact, inhalation, or ingestion may cause severe injury).
11. Solvent A (0.1% TFA): add 5 mL of 20% TFA to 995 mL water.
12. Solvent B (0.1% TFA, 40% acetonitrile): add 400 mL of acetonitrile (MeCN) and 5 mL of 20% TFA to 500 mL of water, adjust final volume to 1L.
13. Solvent C (0.1% TFA, 40% acetonitrile): add 0.1 mL TFA to 30 mL water, then add 60 mL acetonitrile, adjust the final volume to 100 mL by water.
14. Solvent D (0.1% TFA, 50% acetonitrile): add 0.1 mL TFA to 40 mL water, then add 50 mL acetonitrile, adjust the final volume to 100 mL by water.
15. Solvent E (0.1% TFA): add 0.1 mL TFA to 50 mL water, adjust the final volume to 100 mL.

2.2 Components for peptide preparation and IAP

1. TFA, Sequanal Grade (Thermo Scientific, 28903).
2. Acetonitrile (Thermo Scientific, 51101).
3. Sep-Pak® Classic C18 columns, 0.7 mL (Waters, WAT051910).
4. acK beads. To prepare acK beads, wash protein A agarose in a microfuge tube three times with 1 mL of 1× PBS, add acK antibody at 4 mg/mL beads, and incubate 4 hours at room temperature or overnight at 4°C. After binding, wash the beads three times with 1 mL of 1× PBS and then two times with IAP buffer. Resuspend the beads as 1:1 slurry in IAP buffer and store at 4°C for up to one week.

2.3 Components for liquid chromatography (LC) data acquisition

1. Standard protein mixture (Waters Corporation, MassPrep Digestion Standard-Mix1, catalog# 186002865).

2.4 Stock solutions for IP/IB validation of MS hits

1. acKIP buffer: 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 50 mM Tris (pH 7.4), 10% glycerol, with Complete protease inhibitor cocktail (Roche), 1 µM trichostatin A, and 10 mM nicotinamide (8).
2. Laemmli buffer (6×): 0.175 M Tris-base, pH 6.8, 30% glycerol, 10% (w/v) SDS, 9.3% (w/v) DTT, and ~10 mg of bromophenol blue (9). Vortex and warm to dissolve SDS. Aliquots should be frozen at -20°C.
3. Running Buffer (10×): 1% (w/v) SDS, 1.92 M glycine, 4 M Tris-base.
4. Transfer Buffer (10×): 1.92 M glycine, 4 M Tris-base. To make 1L of 1× transfer buffer, add 700 mL of water to 100 mL of 10× transfer buffer, and then add 200 mL of methanol. Store 1× transfer buffer at 4°C.
5. Tris buffered saline containing 0.1% Tween-20 (TBST; 10×): dissolve 0.2 M Tris-base, 1.4 M NaCl, pH to 7.6, add 0.1% (v/v) Tween-20.
6. Ponceau S Staining Solution: 0.1% (w/v) Ponceau S, 5% (v/v) acetic acid.

Methods

3.1 Preparation of protein lysate, suspension cells

1. Grow approximately $1\text{--}2 \times 10^8$ cells for each experimental condition (enough cells to produce approximately 10 – 20 mg of soluble protein). Alternatively, tissues may be used (1) (*see* Note 2).
2. Harvest cells by centrifugation at $130 \times g$ for 5 minutes at room temperature and then carefully remove supernatant. Wash cells with 20 mL of cold PBS, centrifuge and remove PBS wash. Add 10 mL Urea Lysis Buffer (at room temperature) to the cell pellet. Pipet the slurry up and down a few times (Do not cool lysate on ice as this may cause precipitation of the urea). At this point the harvested cells can be frozen and stored at -80°C for several weeks.
3. Using a microtip, sonicate at 15 W output with 3 bursts of 15 seconds each. Cool on ice for 1 minute between the bursts. Clear the lysate by centrifugation at $20,000 \times g$ for 15 minutes at 15°C or room temperature and transfer the protein extract (supernatant) into a new tube (*see* Note 3).

3.2 Preparation of cell lysate, adherent cells

1. Grow $1\text{--}2 \times 10^8$ cells for each experimental condition (enough cells to produce approximately 10 – 20 mg of soluble protein). The cell number corresponds to approximately three to ten 150 mm culture dishes (depending on the cell type), grown to between 70–80% confluence.
2. Take all 150 mm culture dishes for one sample, remove the medium from the first dish by decanting, and let stand in sharply tilted position for 30 seconds so that the rest of the medium flows to the bottom edge. Remove the remainder of the medium at the bottom edge with a P-1000 micropipettor. Wash each dish with 5 mL of cold PBS. Remove PBS as described above.
3. Add 10 mL of Urea Lysis Buffer (at room temperature) to the first dish, scrape the cells off in the buffer, let the dish stand in tilted position after scraping the buffer to the bottom edge of the tilted dish. Remove the medium from the second dish as above. Transfer the lysis buffer from the first dish to the second dish using a 10 mL pipette, then tilt the first dish with the lid on for 30 seconds and remove remaining buffer from the dish and collect. Scrape cells from the second dish and repeat the process until the cells from all the dishes have been scraped into the lysis buffer. Collect all lysate in a 50 mL conical tub (*see* Note 4). The yield will be approximately 9 – 12 mL lysate after harvesting all the culture plates. The cell lysate can be frozen and stored at -80°C for several weeks.
4. Using a microtip, sonicate at 15 W output with 3 bursts of 15 seconds each. Cool on ice for 1 minute between the bursts (*see* Note 5). Clear the lysate by centrifugation at $20,000 \times g$, for 15 minutes at 15°C or room temperature and transfer the protein extract (supernatant) into a new tube.

²Cells should be washed with $1 \times$ PBS before lysis to remove any media containing protein contaminants. Elevated levels of media-related proteins will interfere with the total protein determination.

³Centrifugation is performed at 15°C or room temperature to prevent urea from precipitating out of solution. Centrifugation should be performed in an appropriate container rated for at least $20,000 \times g$.

⁴DO NOT place Urea Lysis Buffer or culture dishes on ice during harvesting. Harvest cells using Urea Lysis Buffer at room temperature. During lysis, the buffer becomes viscous due to DNA released from the cells.

⁵As the viscous lysate material is sonicated, the DNA will be fragmented and the viscosity of the lysate will decrease. Ensure that the sonicator tip is submerged in the lysate. If the sonicator tip is not submerged properly, this may induce foaming and degradation of the sample (refer to instruction manual of the manufacturer of the sonication apparatus).

3.3 Reduction and alkylation of proteins

1. Add 1/278 volume of 1.25 M DTT to the cleared cell supernatant (e.g. 36 μ L of 1.25 M DTT for 10 mL of protein extract), mix well, place the tube into a 55°C incubator for 30 minutes.
2. Cool the solution on ice briefly until it has reached room temperature (tube should feel neither warm nor ice-cold by hand).
3. Add 1/10 volume of iodoacetamide solution to the cleared cell supernatant, mix well and incubate for 15 minutes at room temperature in the dark.

3.4 Dilution

1. Dilute lysate 3-fold with 20 mM HEPES pH 8.0 to a final concentration of 2 M urea, 20 mM HEPES, pH 8.0. For example, for 10 mL of lysate add 30 mL 20mM HEPES pH 8.0.
2. After dilution and mixing, remove a small aliquot (e.g. 30 μ L) for analysis on SDS-PAGE.

3.5 Trypsin digestion

1. Add 1/100 volume of 1 mg/mL trypsin-TPCK (Promega) stock in 1 mM HCl and digest overnight at room temperature with mixing.
2. Analyze the lysate before and after digest by SDS-PAGE to check for complete digestion.
3. Continue through the Sep-Pak, IAP and StageTip protocols prior to LC-MS analysis of enriched peptides.

3.6 Sep-Pak[®] C₁₈ Purification of Lysate Peptides (see Note 6)

1. Add 1/20 volume of 20% TFA to the digest for a final concentration of 1% TFA. Check the pH by spotting a small amount of peptide sample on a pH strip (the pH should be under 3). After acidification, allow precipitate to form by letting stand for 15 minutes on ice (*see* Note 7).
2. Centrifuge the acidified peptide solution for 15 minutes at 1,780 $\times$ g at room temperature to remove the precipitate. Transfer peptide-containing supernatant into a new 50 mL conical tube without dislodging the precipitated material.
3. Connect a 10 cc reservoir (remove 10 cc plunger) to the SHORTER END of the Sep-Pak column (*see* Note 8).
4. Pre-wet the column with 5 mL 100% MeCN (*see* Note 9).
5. Wash with sequentially with 1 mL, 3 mL and 6 mL of solvent A (0.1% TFA).

⁶Sep-Pak[®] C₁₈ purification entails reversed-phase (hydrophobic) solid-phase extraction. Peptides and lipids bind to the chromatographic material. Large molecules such as DNA, RNA and most protein, as well as hydrophilic molecules such as many small metabolites are separated from peptides using this technique. Peptides are eluted from the column with 40% acetonitrile (MeCN) and separated from lipids and proteins, which elute at approximately 60% MeCN and above.

⁷Before loading the peptides from the protein digest on the column, the digest must be acidified with TFA for efficient peptide binding. The acidification step helps remove fatty acids from the digested peptide mixture.

⁸The purification of peptides is performed at room temperature on 0.7 mL Sep-Pak columns from Waters Corporation, WAT051910. About 20 mg of protease-digested peptides can be purified from one Sep-Pak column. Peptides should be purified immediately after the proteolytic digest.

⁹Application of all solutions should be performed by gravity flow. Each time solution is applied to the column air bubbles form in the junction where the 10 cc reservoir meets the narrow inlet of the column. These must be removed with a gel-loader tip placed on a P-200 micropipettor, otherwise the solution will not flow through the column efficiently. Always check for appropriate flow.

6. Load acidified and cleared digest (*see* Notes 10–¹¹).
7. Wash with sequentially with 1 mL, 5 mL and 6 mL of solvent A (0.1% TFA).
8. Wash with 2 mL of 5% MeCN, 0.1% TFA.
9. Place columns above new 15 or 50 mL polypropylene tubes to collect eluate. Elute peptides with a sequential wash of 3×2 mL of solvent B (0.1% TFA, 40% acetonitrile).
10. Freeze the eluate on dry ice (or -80°C freezer) for two hours to overnight and lyophilize frozen peptide solution for a minimum of two days to assure all the TFA has been removed from the peptide sample (*see* Note 12).

3.7 Immunoaffinity purification (IAP)

1. Centrifuge the tube containing lyophilized peptide in order to collect all material to be dissolved. Add 1.4 mL IAP buffer. Resuspend pellets mechanically by pipetting repeatedly with a P-1000 pipette taking care not to introduce excessive bubbles into the solution. Transfer solution to a 1.7 mL Eppendorf tube (*see* Notes 13–¹⁴).
2. Clear solution by centrifugation for 5 minutes at $10,000 \times g$ at 4°C in a microcentrifuge (the pellet of insoluble matter may at times seem considerable, but this will not pose a problem since most of the peptide will be dissolved nonetheless). Cool on ice.
3. Transfer the peptide solution into the microfuge tube containing acK antibody beads (use 80 μL of a 1:1 slurry of beads:IAP buffer per IP). Pipet sample directly on top of the beads at the bottom of the tube to ensure immediate mixing. Avoid creating bubbles upon pipetting.
4. Incubate for 2 hours on a rotator at 4°C .
5. Centrifuge at $2,000 \times g$ for 30 seconds and transfer the supernatant with a P-1000 micropipettor to a labeled Eppendorf tube to save for future use. Flow-through material can be used for subsequent IAPs (*see* Note 15).
6. Add 1 mL of IAP buffer to the beads, mix by inverting tube 5 times, centrifuge for 30 seconds and remove supernatant with a P-1000 micropipettor (*see* Note 16).

¹⁰In rare cases, if the flow rates decrease dramatically upon (or after loading of sample), the purification procedure can be accelerated by *gently* applying pressure to the column using the plunger that was originally inserted, after cleaning it with organic solvent. Again make sure to remove air bubbles from the narrow inlet of the column before doing so. Do not apply vacuum (as advised against by the manufacturer).

¹¹The lysate digest may have a much higher volume than the 10 cc reservoir will hold (up to 50–60 mL from adherent cells) and therefore the peptides must be applied in several fractions. If available a 60 cc syringe may be used in place of a 10 cc syringe to allow all sample to be loaded into the syringe at once.

¹²The lyophilization should be performed in a standard lyophilization apparatus. **DO NOT USE A SPEED-VAC APPARATUS AT THIS STAGE OF THE PROTOCOL.** After the lyophilization step, the digested peptides are stable at -80°C for several months (seal the tightly closed tube with parafilm for storage). The procedure can be interrupted before or after lyophilization. Once the lyophilized peptide is dissolved in IAP buffer (*see* next step), continue to the end of the procedure.

¹³If the cells were directly harvested from culture medium without PBS washing, some of the Phenol Red pH indicator will remain (it co-extracts during the Sep-Pak[®] C18 purification of peptides) and color the peptide solution yellow. This coloration has no effect on the immunoaffinity purification step.

¹⁴After dissolving the peptide, check the pH of the peptide solution by spotting a small volume on pH indicator paper (the pH should be close to neutral, or no lower than 6). In the rare cases that the pH is more acidic (due to insufficient removal of TFA from the peptide under suboptimal conditions of lyophilization), titrate the peptide solution with a 1 M Tris base solution that has not been adjusted for pH; 5–10 μL is usually sufficient to neutralize the solution.

¹⁵In order to recover the beads quantitatively, do not spin the beads at lower *g*-forces than what is specified in this procedure. Avoid substantially higher *g*-forces as well, since this may cause the bead matrix to collapse. All centrifugation steps should be performed at the recommended speeds throughout the protocol.

¹⁶All subsequent wash steps are at $0-4^{\circ}\text{C}$. In all wash steps, the supernatant should be removed reasonably well. Avoid removing the last few microliters, except in the last step, since this may cause inadvertent carry-over of the beads.

7. Repeat step 6 once for a total of **TWO** IAP buffer washes.
8. Add 1 mL chilled water to the beads, mix by inverting tube 5 times, centrifuge for 30 seconds and remove supernatant with a P-1000 micropipettor (*see* Note 17).
9. Repeat step 8 three times for a total of **THREE** water washes. During the last water washes, the tube may need to be shaken while inverting in order to ensure efficient mixing (*see* Note 18).
10. Add 55 μ L of 0.15% TFA to the beads, tap the bottom of the tube several times (do not vortex) and let stand at room temperature for 10 minutes, mixing gently every 2–3 minutes (*see* Note 19).
11. Centrifuge 30 seconds at $2,000 \times g$ and transfer supernatant to a new 1.7 mL Eppendorf tube.
12. Add 50 μ L of 0.15% TFA to the beads, and repeat the elution/centrifugation steps. Combine both eluents in the same 1.7 mL tube. Briefly centrifuge the eluent to pellet and remaining beads and carefully transfer eluent to a new 1.7 mL tube taking care not to transfer any beads.

3.8 Concentration and purification of peptides for LC-MS analysis (*see* Notes 20–22)

1. Equilibrate the StageTip by passing 50 μ L of Solvent D through followed by 50 μ L of Solvent E twice.
2. Load sample by passing IP eluent through the StageTip. Load IAP eluent in two steps using 50 μ L in each step.
3. Wash the StageTip by passing 55 μ L of Solvent E through twice.
4. Elute peptides off the StageTip by passing 10 μ L of Solvent C twice, pooling the resulting eluent.
5. Dry down the StageTip eluate in a vacuum concentrator (Speed-Vac) and re-dissolve the peptides in an appropriate solvent for LC-MS analysis such as 5% acetonitrile, 0.1% TFA.

3.9 LC data acquisition

1. Resuspend vacuum dried, immunoaffinity purified acetylated peptides in 0.125% formic acid containing 100 fmoles of a standard five protein mixture (*e.g.* MassPrep Digestion Standard-Mix1) and separate on a reverse phase column (75 μ m inner diameter $\times$ 10cm) packed into a PicoTip emitter ($\sim$ 8 μ m diameter tip) with Magic C18 AQ (100 $\text{\AA}$ $\times$ 5 μ m). Elute acetylated peptides using a 72 min gradient of acetonitrile (5% – 40%) in 0.125% formic acid delivered at 280 nL/min.

3.10 MS/MS data acquisition

1. Tandem MS/MS is collected in a data-dependent manner with an LTQ-Orbitrap Velos (or Elite) mass spectrometer running XCalibur software (version 2.0.7 SP1)

¹⁷All steps from this point forward should be performed with solutions prepared with Burdick and Jackson or other HPLC grade water.

¹⁸After the last wash step, remove supernatant with a P-1000 micropipettor as before, then centrifuge for 5 seconds to remove fluid from the tube walls, and carefully remove all remaining supernatant with a gel loading tip attached to a P-200 micropipettor.

¹⁹In this step, the post-translationally modified peptides of interest will be in the eluent.

²⁰We recommend concentrating peptides using this protocol by Rappsilber *et al.* (10)

²²Solvents A–E contain volatile organic solvent. Tubes containing small volumes of these solutions should be prepared immediately before use and should be kept capped as much as possible, because the organic components evaporate quickly.

using a top twenty MS/MS method, a dynamic repeat count of one, and a repeat duration of 30 seconds. Real time recalibration of mass error is performed using lock mass with a singly charged polysilaxane ion ($m/z = 371.101237$).

3.11 MS/MS data analysis

1. MS/MS spectra is searched using SEQUEST 3G and the SORCERER2 platform from Sage-N Research (version 4.0). Peptide assignments are generated using the following SEQUEST parameters for ESI-CID MS/MS spectra: peptide precursor mass tolerance of 10 ppm and fragment ion tolerance of 1.0 Da. A maximum of four amino acids are allowed per modification and up to four internal missed cleavage sites are permitted. Neutral loss of water and ammonia from corresponding b and y ions are considered in the correlation analysis for the matching peptide assignment and the proteolytic enzyme was specified as trypsin. SEQUEST searches are performed against a FASTA formatted, species-specific database. Cysteine carboxamidomethylation is specified as a static modification, oxidation of methionine residues is allowed as a variable modification and acetylation is allowed on lysine residues. A reverse decoy databases is included for all searches to estimate false positive rates and filtered using a 5% false discovery rate in the Peptide Prophet module of SORCERER2. The final peptide assignment are further narrowed by applying a mass accuracy filter of ± 2 to 5 ppm of the calculated m/z ., depending on the distribution and symmetry of the XCorr versus ppm plot for a given search result (*see* Figs. 1A–B).
2. In a typical label-free LC-MS/MS study, replicate injections of each sample condition are collected to assess the analytical reproducibility of the LC-MS acquisition of the data set prior to conducting any further analysis. In addition, an external standard (*e.g.* Waters Corporation, MassPrep Digestion Standard-Mix1, catalog# 186002865) is included along with the enriched acetylated peptide samples to serve as a performance check during the data acquisition phase of the study to help verify that the LC and MS systems are performing with acceptable specifications. Significant deviations in retention time reproducibility, signal intensity, mass resolution and mass accuracy indicate that the LC-MS system should be inspected for system errors and/or recalibration. Typical performance metrics include retention time deviations of less than 2.5% relative standard deviation (RSD), intensity measurement variation of less than 30% RSD and mass measurement accuracy of within ± 2 to 5 ppm (Figs. 2A–B).
3. After confirming that the performance metrics for the LC-MS/MS data set are acceptable, the intensity measurements for all acetylated peptide assignments are evaluated between the analytical replicates. There are a variety of commercial and open source software platforms that can be used to extract the corresponding intensity measurements from the RAW LC-MS data files (Rosetta Elucidator System, Progenesis, mzMine, ProteinLynx Global Server, GeneData Expressionist, LC-MSWarp, MaxQuant, ProteoIQ, PEAKS, Skyline, COMPASS, Scaffold, APEX, Census and others). The main function of these software tools is to accurately extract peak height or intensity information for all identified peptides (or features) using the specificity afforded by the accurate mass measurements of the corresponding peptides along with their associated chromatographic retention times. The clustering algorithms associated with these software tools utilize the mass precision and accuracy of the mass spectrometer and the retention time reproducibility obtained from the chromatography to align identical peptides across all the samples in a particular study (Figs. 3A–D). Proper alignment is required to ensure that the correct intensity measurements are properly associated to the

originating peptides (or features) in order to accurately determine the relative quantitation of acetylated peptides (proteins and their associated sites of modification) among the different conditions (Fig. 4).

4. Although the sample preparation protocol for AcetylScan incorporates a means to normalize against any sample bias among different conditions by processing identical amounts of soluble protein from each sample condition, there are additional steps one can employ to control for slight changes in protein amounts and injection variability that may have been introduced by the LC autosampler or systematic attenuation of the MS signal. In instances when it is expected that a majority of the proteins/sites of modification will not change upon the applied treatment condition, a median normalization technique can be applied between replicate injections and between control and treatment conditions. If the perturbation causes changes in many proteins/peptides, this normalization strategy may not be the best technique and will result in attenuation of the overall quantitative changes between control and treatment conditions and will underestimate the total number of proteins/sites affected in the study. Once all the peptide assignment are clustered across all replicate injections and experimental conditions, the relative ratio for each replicate injection can be compared independently to determine the correct offset correction for each sample normalization. In the example below (Fig. 5), the raw intensity ratio for all detected peptides (or features) is converted to a $\log_2(\text{Ratio})$ and the corresponding median $\log_2(\text{Ratio})$ is determined for the entire data set between the two analytical replicates. An offset correction factor, equal to the anti-log value of the median offset, is applied to one of the replicate injections to force the newly calculated median $\log_2(\text{Ratio})$ of the entire data set to be centered at zero. This is performed for each replicate pairs in the study. To normalize against sample bias, the average intensity value for each peptide is calculated within each sample condition using the same normalization routine between each control and treatment condition in a binary manner (Fig. 6).
5. After data normalization is complete, those modified peptide showing significant fold changes between treated and control conditions can be identified as potential biomarkers associated with the perturbation and potentially related to the mechanism of action (*i.e.*, inhibitor treatment, overexpression of ligase or transferase, comparison of sensitive versus resistant cell lines or tissues).
6. One common strategy used to understand the biological significance of a given perturbation is to query the list proteins identified proteins from the set of enriched acetylated peptides using pathway analysis tools such as STRING, Cytoscape, Ingenuity or Reactome, to name a few examples. In Fig. 7, the proteins (or accession numbers) identified from the set of enriched acetylated peptides obtained from mouse liver samples to obtain the illustrated set of predicted protein interactions obtained from experimental and database evidence at EMBL. The same list of proteins was also submitted to Reactome's expression analysis data tool to provide a representative list of signaling pathways associated with those immunoaffinity enriched acetylated peptides (Table 1). PhosphoSitePlus is another commonly used bio-informatics resource that can provide comprehensive information related to the biological role of associated post-translational modifications (<http://www.phosphosite.org>) along with examples from a variety of sample data sets related to the characterization of acetylated and other post-translational proteins (<http://www.phosphosite.org/staticDownloads.do>).

3.12 Validation of MS/MS results by IP/IB (see Note 23)

1. Resuspend cells/organelles of interest in acKIP buffer in 1.7 mL tubes, gently pipetting up and down (*see* Note 24–25).
2. Rotate lysates for 20 minutes (*see* Note 26).
3. Spin at maximum speed in a refrigerated microcentrifuge at 4°C.
4. Remove supernatant to a new prechilled 1.7 mL tube.
5. Measure protein concentration (*see* Note 27), keeping samples on ice.
6. Dilute samples in acKIP buffer to a uniform 1–2 mg in 1 mL acKIP buffer (*see* Note 28).
7. Remove 50 µL of extract and set aside at 4°C for use later as a control.
8. Preclear extract for 2 hours at 4°C in a rotator with appropriate control beads (*see* Note 29).
9. Spin extracts at $2300 \times g$ for 5 minutes in a refrigerated microcentrifuge at 4°C to pellet the beads. Transfer the supernatant to a new tube.
10. Add the acK antibody (or antibody conjugate) to tubes with extract; incubate overnight with rotation at 4°C overnight (*see* Note 30). The optimal amount of antibody needed requires optimization for each different antibody and antigen, but as a rough guide, as a starting point, use 5 µg purified antibody/1 mg IP. For preconjugated acetyl-lysine beads (*see* Note 30), we use 25 µL slurry/1 mg IP (*see* Note 31).
11. The following day, if free antibody was used, add 25 µL Protein A- (for rabbit) or Protein G-conjugated agarose beads (*e.g.* EMD Millipore Corp. Protein A beads catalog #16–125; Roche Diagnostics Protein G beads catalog #1243233).
12. Incubate 1 h with rotation at 4°C.

²³In our experience, performing an IP reaction using an acK-directed antibody followed by IB for the protein of interest is a more robust approach for most proteins than the converse (*i.e.*, performing an IP reaction for the candidate protein followed by acK IB). This approach offers the additional advantage that the resulting immunoblots can be cut into several sections and probed for different acetylated proteins, thus minimizing the number of IP reactions that need to be performed. However, there are numerous examples of both approaches working in the literature.

²⁴All subsequent steps until boiling in Laemmli should be conducted at 4°C, ideally in the cold room and on ice whenever possible.

²⁵We have found that acK IPs are quite sensitive to the IP buffer used, and in our experience, do not work well in certain common IP buffers (*e.g.* RIPA). The acKIP buffer listed works well for immunopurification of acetylated proteins from cells, mitochondria, and tissue lysates.

²⁶If necessary, sonication can be performed at this point. For mitochondria, we have found this step to be unnecessary.

²⁷Make sure that whatever protein quantification method is used is insensitive to the detergent in the lysis buffer (*e.g.* DC Protein Assay, Bio-Rad DC Protein Assay Kit II, cat. # 500-0112). Also, it is critical that the blank and the standard reactions be performed in the same buffer as the samples.

²⁸acK antibodies tend to be of low affinity; hence the need for relatively large amounts of starting material. We have been uniformly unsuccessful at immunopurifying acetylated proteins by acK IP using less than 1–2 mg of starting material.

²⁹This step removes proteins from the extract that bind non-specifically to beads or immunoglobulin and produce artifactual results. For example, for subsequent IPs using pre-conjugated rabbit acK beads, we typically add 25 µL of rabbit IgG-agarose (Sigma A2909) for the preclearing step.

³⁰Different acK antibodies recognize different subsets of acetylation sites and proteins, so it is worth trying several different acK antibodies from different manufacturers at this step to optimize detection of acetylation on specific proteins of interest (*e.g.*, Cell Signaling catalog #s 9814, 9681, 9441; Immunechem catalog # ICP0380; Millipore catalog #3879, *etc.*). One product that works extremely well is acK antibodies pre-conjugated to agarose beads (Immunechem catalog #ICP0388). This product has the additional advantage that it obviates the need for adding protein A/G-conjugated beads subsequently. As the preconjugated beads also minimize the amount of free immunoglobulin heavy and light chain in the sample, they improve background in subsequent immunoblotting significantly.

³¹To allow accurate pipetting of agarose beads, cut the tip off of a 200 µL pipet tip to widen the bore, and pipet the beads up and down several times prior to adding them to the sample.

13. Spin the IP tubes at $2300 \times g$ for 5 minutes in a refrigerated microcentrifuge at 4°C to pellet the beads. Remove the supernatant (*see* Notes 32–33).
14. Add 1 mL acKIP buffer and tap tubes gently to resuspend beads. Rotate 5 minutes at 4°C .
15. Repeat steps 13–14 for a total of three washes.
16. Following removal of the last wash, centrifuge the pellets again at $2300 \times g$ for 5 minutes in a refrigerated microcentrifuge at 4°C . Then, using a 20 μL pipet tip, remove the last bit of liquid from the beads (*see* Note 34).
17. Add 80 μL 6 $\times$ Laemmli, boil for 5 minutes (*see* Note 35–36).
18. Fractionate samples by SDS-PAGE. Load 35 μL of each sample and each control, and 8 μL of protein standard ladder (*e.g.* Bio-Rad Precision Plus Kaleidoscope™ #1610375) on a 4–20% polyacrylamide pre-cast gel (*see* Note 36).
19. Run the gel at 200V in 1 $\times$ running buffer for roughly 50 minutes (until the dye just runs off of the bottom of the gel).
20. Pre-soak four pieces of Whatman paper and two transfer pads in 1 $\times$ transfer buffer for the transfer of fractionated proteins from the gel to a PVDF membrane (*e.g.* Fisher/Pierce catalog #0088518). The pre-soaked Whatman paper should be cut to the size of the corresponding transfer pads.
21. Hydrate the PVDF membrane (which should be cut to the approximate size of your gel) by soaking it in methanol for 1 minute and then rinse the membrane with 1 $\times$ transfer buffer. Use forceps only to handle the membrane.
22. On the black (internal) portion of the transfer cassette, place one pre-soaked transfer pad followed by two pieces of pre-soaked Whatman paper.
23. Remove the polyacrylamide gel from the casting rig and position it on the Whatman paper in the cassette so that the gel is oriented face-down toward the black side of the cassette where the top of the gel is nearest the cassette clamp and the bottom of the gel is nearest the cassette hinge (*see* Note 37).
24. Using forceps, place the PVDF membrane on top of the gel, followed by two pieces of pre-soaked Whatman paper. Gently run a small roller over the Whatman paper to remove any bubbles that may have been in between the gel and the PVDF membrane and assure uniform transfer.
25. Place the final pre-soaked transfer pad on top of the Whatman paper and close the cassette. There should be a snug fit when closing the clamp of the cassette.

³²If desired, a sample of the post-IP supernatant can be saved at this step and run alongside the IP reactions to check depletion of the antigen of interest by the IP. However, it is rare to observe such depletion, even with robust acK IPs.

³³Aspirate the supernatant using a 200 μL pipet tip attached to an aspirator hose. The narrow pipet tip minimizes bead losses, and the amount of vacuum can be lowered to achieve a slow rate of removal.

³⁴This last step is critical, since a significant amount of liquid remains after the last aspiration, which can dilute the sample.

³⁵To avoid the opening of tubes during boiling, lid-locks can be used.

³⁶It is possible to split each IP reaction onto two gels, loading 35 μL /well. If many different acetylated proteins are to be identified, it is desirable to load the IP reactions on gradient gels, enabling a better resolution across a wide range of molecular weights (*e.g.* Bio-Rad Criterion Gels, 4–20% Tris-HCl, 12-well, 1.0 mm, precast polyacrylamide gels, catalog #345-0032).

³⁷When removing the polyacrylamide gel from the cassette, the Bio-Rad Criterion cell that is used for running the gel has a built-in cassette opening wedge in the lid, but we prefer to crack the welded joints with a metal spatula. The lower half of the gel is thicker and easier to lift for removal without tearing the gel. If tearing of the gel still occurs, it can also be floated off in 1 $\times$ transfer buffer for gentle removal.

26. Insert the cassette into the blotting apparatus, aligning the black side of the cassette with the black side of the box; the other side will be either red or clear depending on the apparatus used. Fill to mark with 1× transfer buffer. Add a magnetic stir bar and an ice pack to the apparatus.
27. Run the transfer at 4°C at 350 mA for 1.5 hours on a stir plate. Alternatively, gels can be transferred at 150 mA overnight.
28. Remove the PVDF membrane with forceps and rinse in purified water by rocking in a small tray for 1 minute. Repeat 2 more times.
29. Remove water from the tray and add just enough Ponceau S staining solution to cover the membrane and rock for 5 minutes.
30. Wash in purified water until transferred proteins are visualized and the background is removed. Over-washing can remove the Ponceau stain entirely.
31. Place the membrane in clear plastic and make a digital scanned image of the stained membrane for later reference.
32. Rock the membrane in 20 mL of 5% milk in 1× TBST for 30 minutes to block non-specific binding sites.
33. Discard blocking solution and incubate blot in primary antibody (in 5% milk or 5% BSA in TBST – check manufacturer’s instructions appropriate starting concentration) for 1 hour at room temperature or overnight at 4°C. Remove the primary antibody for later use (*see* Note 38).
34. Wash the membrane in 20 mL of 1× TBST at room temperature for 5 minutes. Repeat two more times.
35. Incubate the membrane with appropriate HRP-conjugated secondary antibody diluted in 20 mL of 5% milk in 1× TBST for 1 hour at room temperature. Check manufacturer’s instructions for appropriate starting concentration of secondary antibody.
36. Wash membrane 3× for 10 minutes each in 20 mL 1× TBST.
37. Develop signal using appropriate substrate (*e.g* Fisher #WBKLS0500).

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³⁸The primary antibody can be saved and reused. Add sodium azide to 0.05% and store at 4°C for future use. Alternatively, antibody solutions can be frozen.

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Fig. 1.

Correlation of ppm Error and SEQUEST Cross Correlation Score, XCorr. (A) Scatter plot of ppm Error and XCorr value from SEQUEST showing 7886 peptide assignments (modified and unmodified, lysine acetylation) with 95% of the peptide assignments within ± 2 ppm error (in black) of the calculated monoisotopic mass. (B) Histogram plot showing the binned ppm Error for each peptide assignment.

Fig. 2.

System performance metrics for LC-MS Data Acquisition. **(A)** Extracted ion chromatograms for a control peptide, IGDYAGIK (Yeast, ADH1, m/z 418.7293, $z = 2$) among the duplicate injections of three experimental conditions. The relative standard deviation for the retention time and area measurements are 0.4% and 3.2%, respectively. **(B)** Mass spectrum of the yeast ADH1 control peptide, measured at 0.0 ppm mass accuracy.

Fig. 3. Alignment of Label-Free LC-MS Data. An overlay of two LC-MS runs represented as m/z (x-axis) and retention time (y-axis) before (**A**) and after (**B**) alignment using Progenesis LC-MS software from Nonlinear Dynamics. An overlay of two LC-MS runs showing the total ion chromatogram before (**A**) and after (**B**) alignment.

Fig. 4.
3D View of Aligned LC-MS Data. The top panel shows the three-dimension depiction (m/z , retention time and intensity corresponding to x-axis, z-axis and y-axis, respectively) of an isotopic cluster for duplicate injections of a peptide feature in a control (red panel) and treated (blue panel) sample set. Comparison of the corresponding peak heights (or areas) reflects the relative changes between control and treated sample conditions.

Fig. 5. Median Normalization between Analytical Replicates. The $\log_2(\text{ratio})$ of replicate injection 1 versus 2. The scatter plot before normalization shows a median $\log_2(\text{Ratio})$ off-set of 0.071 (red line) where the data is centered. After subtracting the median off-set from all the $\log_2(\text{Ratio})$ values, the normalized data is centered at zero (median $\log_2(\text{Ratio})$ after normalization is zero).

Fig. 6. Median Normalization between Sample Conditions. The $\log_2(\text{ratio})$ of treated versus control conditions. The scatter plot before normalization shows a median $\log_2(\text{Ratio})$ off-set of 0.370 (red line) where the data is centered. After subtracting the median off-set from all the $\log_2(\text{Ratio})$ values, the normalized data is centered at zero (median $\log_2(\text{Ratio})$ after normalization is zero).

Fig. 7. STRING Confidence Plot of Acetylated Peptides to Proteins from enriched from mouse Liver. The proteins associated to acetylated lysine peptides enriched from mouse liver were submitted to STRING (version 9.0, <http://string-db.org/>) to illustrate known protein-protein interactions based on direct and indirect association from experimental and database evidence high confidence score of 0.700. Peptides were enriched using the acetylated lysine motif antibody from Cell Signaling Technology (http://www.cellsignal.com/services/ptmscan_antibodies.html).

Table 1
Signaling Pathways Associated to Acetylated Peptides from Mouse Liver

The top 35 signaling pathways associated to acetylated lysine peptides obtained from immunoaffinity enrichment of mouse liver tissue. The list of signaling pathways were from Reactome's Expression Data tool, <http://www.reactome.org/ReactomeGWT/entrypoint.html>.

No.	Pathway name	Total number of proteins	Matching proteins in data	% in data
1	Activation of Chaperone Genes by ATF6-alpha	5	3	60.00%
2	Neurotransmitter uptake and Metabolism In Glial Cells	2	1	50.00%
3	Peroxisomal lipid metabolism	21	10	47.62%
4	alpha-linolenic (omega3) and linoleic (omega6)acid metabolism	12	5	41.67%
5	Metabolism of amino acids and derivatives	206	74	35.92%
6	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	124	43	34.68%
7	The citric acid (TCA) cycle and respiratory electron transport	165	57	34.55%
8	O2/CO2 exchange in erythrocytes	13	4	30.77%
9	Uptake of Carbon Dioxide and Release of Oxygen by Erythrocytes	13	4	30.77%
10	Uptake of Oxygen and Release of Carbon Dioxide by Erythrocytes	13	4	30.77%
11	Neurotransmitter Clearance In The Synaptic Cleft	7	2	28.57%
12	Bile acid and bile salt metabolism	44	12	27.27%
13	Abnormal metabolism in phenylketonuria	4	1	25.00%
14	Phase II conjugation	76	19	25.00%
15	tRNA Aminoacylation	44	10	22.73%
16	Eukaryotic Translation Elongation	137	30	21.90%
17	Metabolism of porphyrins	14	3	21.43%
18	Eukaryotic Translation Termination	134	27	20.15%
19	Fatty acid, triacylglycerol, and ketone body metabolism	189	37	19.58%
20	Metabolism of nitric oxide	21	4	19.05%
21	SRP-dependent cotranslational protein targeting to membrane	165	31	18.79%
22	Eukaryotic Translation Initiation	167	31	18.56%
23	Metabolism of vitamins and cofactors	54	10	18.52%
24	Metabolism of water-soluble vitamins and cofactors	54	10	18.52%
25	Nonsense-Mediated Decay	162	30	18.52%
26	Translation	207	38	18.36%
27	Metabolism	1671	284	17.00%
28	Mitochondrial Protein Import	36	6	16.67%
29	Biological oxidations	209	34	16.27%
30	Meiotic Synapsis	37	6	16.22%
31	Metabolism of nucleotides	88	14	15.91%
32	Cellular response to hypoxia	26	4	15.38%
33	Cellular responses to stress	26	4	15.38%

No.	Pathway name	Total number of proteins	Matching proteins in data	% in data
34	Regulation of Hypoxia-inducible Factor (HIF) by Oxygen	26	4	15.38%
35	Signaling by Hippo	20	3	15.00%