Chapter 2

Tandem Affinity Purification Approach Coupled to Mass Spectrometry to Identify Post-translational Modifications of Histones Associated with Chromatin-Binding Proteins

Sophie Beyer*, Philippe Robin*, and Slimane Ait-Si-Ali

Abstract

Protein purification by tandem affinity purification (TAP)-tag coupled to mass spectrometry analysis is usually used to reveal protein complex composition. Here we describe a TAP-tag purification of chromatin-bound proteins along with associated nucleosomes, which allow exhaustive identification of protein partners. Moreover, this method allows exhaustive identification of the post-translational modifications (PTMs) of the associated histones. Thus, in addition to partner characterization, this approach reveals the associated epigenetic landscape that can shed light on the function and properties of the studied chromatin-bound protein.

Key words Histones, Epigenetics, Chromatin, Post-translational modifications, TAP-tag, Mass spectrometry

1 Introduction

Tandem affinity purification (TAP)-tag approach and subsequent mass spectrometry analysis allow the specific purification of a protein and its interaction partners. It is thus is a useful method to reveal entire protein complexes [1]. Additionally, the purification method described in this chapter allows the separation of cytoplasmic, nuclear soluble, and chromatin-enriched subcellular fractions. The protein of interest, containing a FLAG and HA tags, is either stably overexpressed in a cell line or, alternatively, the endogenous allele(s) can be tagged, thanks to the recent genome editing based [2] to avoid non-specific interactions due to the overexpression conditions.

The TAP-tag approach permits a quantitative and qualitative efficient purification, which is sufficient to detect the interaction partners of the protein of interest. Thus, this purification procedure was extensively used in the past to identify protein-protein interactions and to reveal protein complexes [1].

*Author contributed equally with all other contributors.

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The preparation of the cellular extracts for TAP-tag consists of two main steps: the separation of cytoplasmic and nuclear fractions and, second, dividing the nuclear fraction into soluble and chromatin-enriched subfractions. This separation additionally enables to identify unexpected interaction partners dependent on the subnuclear compartment [3]. With the focus on chromatin-bound or chromatin-modifying proteins, the complexes recovered from the chromatin-rich fraction could be of main interest. One of main interests here is the identification of the posttranslational modifications (PTMs) of the co-purified histones and of the coprecipitated nucleic acids [4, 5] (Fig. 1).

Thus, the added value of the TAP-tag approach is the possibility to identify PTMs of the purified protein itself and abundant identified interaction partners (originally described in [5]). Thereby, with this feature, the TAP-tag purification is suitable to identify not only new interaction partners but also new enzymatic functions associated with the protein of interest and/or its partners.

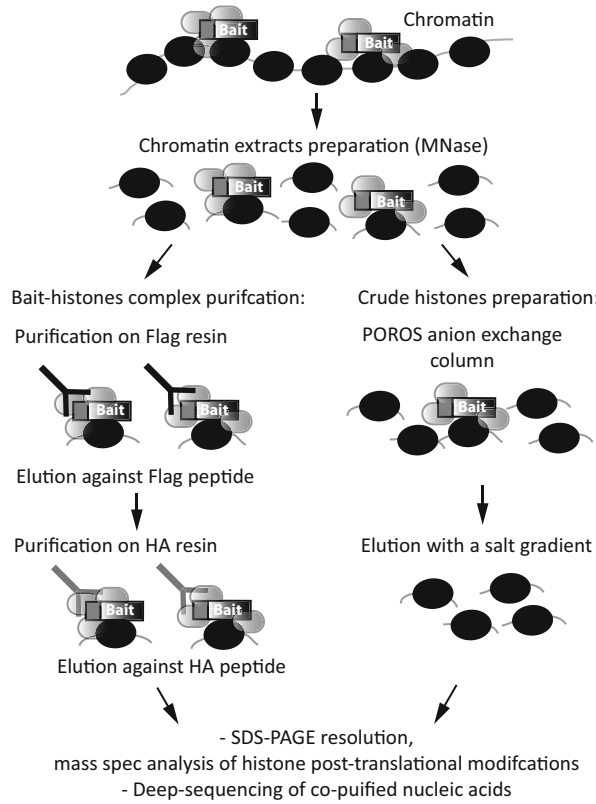

Fig. 1 Post-translational modifications of histones associated with a chromatin-binding protein. Schematic representation of the purification protocol used to purify a bait-histone complexes and crude histones. The latest being used to determine the enrichment compared to the level of a given histone PTM in the input material

In the case of a chromatin-binding protein, this method is thus adapted for identification of the associated "histone code." Indeed, the amino-terminal histone tails, which are exposed on the nucleosome surface, are subject to multiple covalent PTMs. These histone PTMs include lysine and arginine methylation, lysine acetylation, serine and threonine phosphorylation, ADP-ribosylation, ubiquitination, and sumoylation [6]. Histone PTMs confer a unique signature to the nucleosomes involved. Combination of the different modifications on histone N-terminal tails can thus alter chromatin structure to allow gene expression or to repress it, either reversibly or stably. The combinatory pattern of histone PTMs influences the binding and activities of other chromatin-associated proteins that regulate gene expression. Indeed, initial modifications of histones at a specific nucleosome could influence subsequent modifications. Thus, characterizing such modifications associated with a given protein could provide insights into the roles and mechanisms of action of the studied chromatin-binding proteins.

2 Material

2.1 Cells

Cell lines stably expressing FLAG-HA-tagged proteins transduced could be either established using protocol described in [1] or provided by any other mean. Alternatively, the endogenous allele(s) can be tagged using genome editing-based methods [2].

2.2 Buffers

All buffers are used cold if not indicated otherwise and must be supplied with protease inhibitors prior using (*see* **Note 1**).

1. Hypotonic buffer: 10 mM Tris–HCl, pH 7.65, 1.5 mM $MgCl_2$, 10 mM KCl.
2. Low salt buffer: 20 mM Tris–HCl pH 7.65, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM NaCl.
3. High salt buffer: 20 mM Tris–HCl pH 7.65, 25% glycerol (12.5 ml), 1.5 mM $MgCl_2$ (75 µl from 1 M), 0.2 mM EDTA, 900 mM NaCl.
4. TEGN: Tris–HCl 20 mM, EDTA 0.1 mM, Glycerol 10%, NaCl 150 mM, NP40 0.01%.
5. Sucrose buffer: 20 mM Tris–HCl, pH 7.65, 15 mM KCl, 60 mM NaCl, 0.34 M sucrose, 0.15 mM spermine, 0.5 mM spermidine.

3 Methods

3.1 Preparation of Cytoplasmic, Nuclear Soluble, and Chromatin-Enriched Fractions

3.1.1 Separating Nuclei and Cytoplasm

1. When working with frozen cell pellets, briefly defrost 20 g cell pellet in a water bath at 37 °C and resuspend first in 10 ml hypotonic buffer. Then add twice 5 ml of fresh hypotonic buffer and hereby wash the pipettes well to obtain the maximum amount of cells. The final volume of the lysate is approximately 40 ml.
2. Use a pre-chilled Dounce homogenizer (40 ml volume) with a tight pestle, and homogenize 20 ml of the lysate with 20 strokes (20 times in and out). Transfer the lysate to a 50 ml tube.
3. Use the second 20 ml lysate and proceed as described in Subheading 3.2.
4. To analyze the efficiency of the lysis, use 30 µl of lysate, mix it with 30 µl of 0.4% trypan blue, and analyze under the microscope. If lysis was efficient, all nuclei are blue. In case of inefficient lysis repeat steps 2 and 3 above.
5. Add 7 ml sucrose buffer (1/3 of the hypotonic buffer volume) supplemented with 0.15 mM spermine and 0.15 mM spermidine. Sucrose buffer preserves the nuclei. Spermine and spermidine avoid leakage by blocking the nuclear pore.
6. Centrifuge the lysate 7 min at 10,000 × *g* to get the nuclei, which are in the pellet. The supernatant is the cytoplasmic fraction.
7. In case of interest in the cytoplasmic fraction (CF), transfer the supernatant as mentioned in above step to a new tube and centrifuge again 7 min at 10,000 × *g*. The supernatant is the CF.

3.2 Preparation of Nuclear Soluble Fraction (NSF) Containing Proteins, Which Weakly Interact with Chromatin

1. Resuspend the nuclei pellet from 1.6 in 10 ml of low salt buffer (one volume equal to the pellet size).
2. Add 10 ml high salt buffer drop by drop while mixing systematically on a vortex. The final concentration of NaCl will be 300 mM.
3. Incubate for 30 min on ice and mix every 5 min.
4. Add 10 ml (1 nuclei pellet volume) of the sucrose buffer.
5. Centrifuge 10 min at 13,000 × *g*. The supernatant is the NSF (*see* **Note 2**).

3.3 Preparation of Nuclear Chromatin-Enriched Fraction (NCF), Containing Proteins Which Strongly Associate with Nucleosomes

1. Resuspend thoroughly the nuclear pellet from 2.5 in 7 ml of sucrose buffer (1 nuclei pellet volume).
2. Add $CaCl_2$ to a final concentration of 1 mM and mix. Starting from a 0.5 M $CaCl_2$ solution, take 28 µl for 14 ml of suspension.
3. Preheat the suspension for 1 min at 37 °C.

4. Add micrococcal nuclease (MNase) to get final concentration of 0.0025 U/µl and mix. Starting from a from 0.5 U/µl stock solution, take 70 µl.
5. Incubate precisely 12 min at 37 °C and mix every 4 min.
6. Immediately place the reaction on ice to stop MNase activity.
7. Add EDTA pH 8.0 to reach 4 mM as final concentration. Starting from a 0.5 M stock solution, take 112 µl EDTA.
8. Perform five cycles of sonication on high amplitude with 1 min each cycle. Between each cycle do 1 min break. The total sonication time will be 10 min.
9. Ultracentrifuge for 30 min at 85,000 × *g*. The supernatant is the NCF.
10. Take 50 µl aliquot of NCF and 100 µl of NSF, which is used as input later, and freeze in liquid nitrogen.

3.4 Protein Complex Purification

3.4.1 Protein Affinity Pulldown by FLAG-Tag

1. Use 600 µl of FLAG affinity resin from the commercial 50% stock for each experimental point (300 µl of pure FLAG resin). Transfer into 15 ml tube, wash with 13 ml of cold TEGN buffer (invert the tube 5 times), centrifuge for 2 min at 1000 × *g*, and remove supernatant. Repeat washing 5 times.
2. Resuspend total amount of FLAG resin in equal volume TEGN buffer, and distribute 600 µl to each experimental point in a 1.5 ml tube (*see* **Note 3**).
3. Incubate over night at 4 °C.
4. Centrifuge 2 min at 1000 × *g* at 4 °C. Keep the supernatant on ice for efficiency check. The FLAG-tagged proteins are in the pellet, bound to the FLAG resin.
5. Resuspend the FLAG resin in 1 ml TEGN buffer and transfer to a 15 ml tube. Repeat this **step 5** times. Use hereby always the same pipette tip for transfer to ensure efficient transfer of all beads.
6. Wash FLAG resin 7 times in the 15 ml tube by adding 13 ml TEGN buffer and inverting the tube 5 times. Do not resuspend the beads with the pipette to avoid losing material. Centrifuge after each washing step for 2 min at 1000 × *g* at 4 °C.
7. Resuspend FLAG resin of each experimental point in 1 ml TEGN buffer and transfer to 1.5 ml tube but keep the 15 ml tube. Centrifuge the 1.5 ml tube (2 min, 1000 × *g*, 4 °C) and remove supernatant.
8. To ensure complete carryover of all beads, rinse the 15 ml tube with 1 ml TEGN buffer, and transfer to the 1.5 ml tube from the previous step. Centrifuge and remove supernatant.
9. Add 200 µl of 4 mg/ml FLAG peptide solution (pH 7.5–8) to the FLAG resin of each experimental point. Add 200 µl TEGN

buffer. Mix by tipping the tube. To avoid losing FLAG resin, do not touch with the pipette tip. Incubate on a rotating wheel overnight (or at least 4 h) at 4 °C.

10. Spin tubes 2 min at 1000 × *g* at 4 °C. Use a flat-narrow pipette tip to transfer the supernatant (FLAG eluate). Do not carry over beads.
11. Centrifuge the FLAG resin again to recover leftover supernatant. Use flat-narrow pipette tips to avoid carryover of beads.
12. To ensure efficient elution from the FLAG resin, take the FLAG beads from **step 11** and repeat **steps 9** and **10**.
13. Combine supernatants from first and second elution.

3.5 Efficiency Test After FLAG Purification

1. Take 15 µl eluate obtained from previous step, add 5 µl of 4× loading buffer and 2 µl 10× reducing agent, and mix (if loading buffer has a different concentration, adjust volumes of buffer and reducing agent).
2. Boil samples 5 min at 95 °C, quick spin samples, and run on a SDS-PAGE.
3. For silver staining use a commercially available silver staining kit and follow the manufacturer's protocol (*see* **Note 4**).

3.6 Protein Affinity Pulldown by HA-Tag

1. Use 300 µl of HA affinity gel from the commercial 50% stock for each experimental point (150 µl of pure HA resin). Transfer into 15 ml tube, wash with 13 ml of cold TEGN buffer (invert the tube 5 times), centrifuge for 2 min at 1000 × *g*, and remove supernatant. Repeat washing 5 times.
2. Resuspend total amount of HA resin in equal volume TEGN buffer and distribute 300 µl to each experimental point in a 1.5 ml tube. Centrifuge 2 min at 1000 × *g* and 4 °C. Eliminate the maximum of the washing buffer by using the flat-narrow pipette tips.
3. Add the eluates from FLAG-based purification (from 3.4.1.13) to the HA resin.
4. Incubate overnight at 4 °C on a rotating wheel.
5. Centrifuge 2 min at 1000 × *g* at 4 °C. Keep the supernatant on ice for efficiency check. The HA-tagged proteins are in the pellet, bound to the HA resin.
6. Resuspend the HA resin in 0.5 ml TEGN buffer and transfer to a new 1.5 ml tube. Repeat this step once and transfer to the same tube. Use hereby always the same pipette tip for transfer to avoid losing beads.
7. Wash HA resin 8 times in the 1.5 ml tube by adding 1 ml TEGN buffer and inverting the tube 5 times. Do not touch the beads with the pipette to avoid losing material. Centrifuge after each washing step for 2 min at 1000 × *g* at 4 °C.

8. Transfer HA resin of each experimental point to a new 0.5 ml tube with the last washing step. Rinse the pipette tip, used for transfer with TEGN buffer, and collect as many beads as possible into the same 0.5 ml tube. Centrifuge the tube (2 min, 1000 × *g*, 4 °C) and remove as much supernatant as possible.
9. Add 100 μl of 4 mg/ml HA peptide solution to the HA resin of each experimental point. To avoid losing HA resin, do not touch with the pipette tip. Incubate on a rotating wheel overnight (or at least 4 h) at 4 °C.
10. Spin tubes 2 min at 1000 × *g* at 4 °C. Use a flat-narrow pipette tip to transfer the supernatant (HA eluate). Do not carry over beads.
11. Centrifuge the HA resin again to recover leftover supernatant. Use flat-narrow pipette tips to avoid carryover of beads.
12. To ensure efficient elution from the HA resin, take the HA beads from **step 11** and perform a second elution. Repeat **steps 9–11**.
13. Combine supernatants from first and second elution.

3.7 Efficiency Test After HA Purification

1. Repeat steps as described in Subheading 3.5.

3.8 Concentration of Eluates

1. Use centrifugal filter units with 10 kDA cutoff.
2. Concentrate the eluate to 30 μl by using the filter unit according to manufacturer's instructions.
3. Take ¼ of the eluate (7.5 μl), snap freeze in liquid nitrogen, and store at −80 °C. It will be used for western blot analysis to confirm the results obtained by mass spectrometry.
4. Take the remaining ¾ (22.5 μl) and prepare samples for mass spectrometry analysis (as in [5, 7]).

3.9 Nucleosomal Histone Preparation for Mass Spectrometry Analysis (See Note 5)

1. Run the purified protein complex from the chromatin-enriched nuclear fraction on a 4–12% acrylamide gradient SDS-PAGE gel.
2. Stain SDS-PAGE gel with Colloidal blue.
3. Cut gel bands corresponding to each histone and destain overnight in 50% acetonitrile, 50 mM NH_4HCO_3.
4. Subject histones to a propionylation-based modification method to study lysine modifications [7]. Propionic anhydride makes covalent bonds with non-modified or monomethylated lysines and with the N-termini of proteins.
5. Treat gel slices for 1 h at 37 °C with 100 ml of 30% propionic anhydride in methanol and 40 ml of 50 mM NH_4HCO_3 [7], followed by two 10-min washes in 100 mM NH_4HCO_3, one wash in 50% acetonitrile, 100 mM NH_4HCO_3, and one wash in acetonitrile.

6. Dry gel slices and digest at 37 °C overnight using 0.4 mg of sequencing grade trypsin.
7. Acidify the digests in 0.5 % TFA, lyophilize, resuspend in 40 ml of 50 mM NH_4HCO_3, and propionylate again in 100 ml of 30 % propionic anhydride in methanol for 1 h at 37 °C, lyophilized and resuspended in 20 ml of 0.1 % of formic acid. The second propionylation modifies the newly created N-terminal ends after trypsin digestion. These conditions give complete lysine and N-terminal propionylation, but also chemical methylations that can be detected using deuterated methanol (methanol-d4) for the propionic anhydride dilution.
8. Run the obtained peptide mixtures on a Nano C18 PepMap 100 pre-column (5 mm, 100 Å, 300 mm I.D. × 1 mm), coupled with a column of 75 mm I.D. × 15 cm with the same resin (LC Packings). The Nano-flow-High Pressure Liquid Chromatography LC (LC Packings) is directly coupled to an electrospray ionization system on an ion-trap mass spectrometer (ESI/MS-MS) (Thermo Finnigan LCQ Deca XP).
9. Proceed with mass spectrometry analysis to identify complex composition and PTM of histones (*see* **Note 6**).

4 Notes

1. All steps must be performed on ice if not indicated otherwise. Keep all buffers at 4 °C and perform all centrifugation steps at 4 °C throughout the entire procedure. FLAG and HA resins are centrifuged 2 min at 1000 × *g*. Use low binding tubes during all steps.
2. If the NSF will be analyzed, leave the supernatant from step in Subheading 3.2.5 on ice during preparation of chromatin-bound fraction and then treat both fractions simultaneously.
3. Take the NCF (Subheading 3.3) and add 600 µl washed FLAG resin to each experimental point. Do equally for NSF (Subheading 3.2) and CF (Subheading 3.2) if interested.
4. If the signal difference is clear between the cell line specifically overexpressing a FLAG-HA-tagged protein and the control cell line, proceed to step in Subheading 3.6.
5. Products and materials used to optimize mass spec analyses: ddH_2O or Milli-Q; change gloves very often; wear a lab coat all the time, use exclusively pre-cat 4–12 % SDS-PAGE gels to avoid extra-contamination of samples, especially the gradient gels and their buffers; use tubes with low adherence if possible to minimize the loss of material. Other precautions to avoid contamination of mass spec samples are the following: clean the bench, clean the pipettes with alcohol, use new pipette tip boxes, wash the gloves just after wearing them, and wash all

materials to be used (SDS-PAGE system, dounces, boxes, etc.) with detergents (e.g., cleaning solution 7×, ICN- Cat No 76-670-95) in distilled water (more than 10 times).

6. For mass spectrometry, the five most intense ions of the MS scan are subjected to fragmentation (MS-MS) without any data-dependent scan. The interpretation of the mass spectrometry data can be performed with the BioWorks software version 3.2 (Thermo Scientific). For example, for lysine methylation, a bank of peptides from the histones cut at arginine residues can be indexed with permanent add mass for the N-terminus and lysine of 56.025 Da and three modifications: K– 14.015 for acetylation or trimethylation, K+14.015 Da for a monomethylation, and K– 27.995 Da for a dimethylation. This set-up allows automation of analysis of the MS raw data. Each raw dataset can then be analyzed to check for combinations of modifications that might have been missed by the automated method.

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References

1. Nakatani Y, Ogryzko V (2003) Immunoaffinity purification of mammalian protein complexes. Methods Enzymol 370:430–444
2. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121):819–823. doi:10.1126/science.1231143, science.1231143 [pii]
3. Fritsch L, Robin P, Mathieu JR, Souidi M, Hinaux H, Rougeulle C, Harel-Bellan A, Ameyar-Zazoua M, Ait-Si-Ali S (2010) A subset of the histone H3 lysine 9 methyltransferases Suv39h1, G9a, GLP, and SETDB1 participate in a multimeric complex. Mol Cell 37(1):46–56. doi:10.1016/j.molcel.2009.12.017, S1097-2765(09)00921-6 [pii]
4. Ouararhni K, Hadj-Slimane R, Ait-Si-Ali S, Robin P, Mietton F, Harel-Bellan A, Dimitrov S, Hamiche A (2006) The histone variant mH2A1.1 interferes with transcription by down-regulating PARP-1 enzymatic activity. Genes Dev 20(23):3324–3336
5. Robin P, Fritsch L, Philipot O, Svinarchuk F, Ait-Si-Ali S (2007) Post-translational modifications of histones H3 and H4 associated with the histone methyltransferases Suv39h1 and G9a. Genome Biol 8(12):R270
6. Kouzarides T (2007) Chromatin modifications and their function. Cell 128(4):693–705
7. Bonaldi T, Imhof A, Regula JT (2004) A combination of different mass spectroscopic techniques for the analysis of dynamic changes of histone modifications. Proteomics 4(5):1382–1396