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Native Co-immunoprecipitation Assay to Identify Interacting Partners of Chromatin-associated Proteins in Mammalian Cells

Afzal Husain[1, *], Nasim A. Begum[2], Maki Kobayashi[2] and Tasuku Honjo[2, *]

[1]Deptartment of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, India; [2]Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan

*For correspondence: honjo@mfour.med.kyoto-u.ac.jp; afzalh01@gmail.com

[Abstract] Protein-protein interactions play key roles in nuclear processes including transcription, replication, DNA damage repair, and recombination. Co-immunoprecipitation (Co-IP) followed by western blot or mass spectrometry is an invaluable approach to identify protein-protein interactions. One of the challenges in the Co-IP of a protein localized to nucleus is the extraction of nuclear proteins from sub-nuclear fractions without losing physiologically relevant protein interactions. Here we describe a protocol for native Co-IP, which was originally used to successfully identify previously known as well novel topoisomerase 1 (TOP1) interacting proteins. In this protocol, we first extracted nuclear proteins by sequentially increasing detergent and salt concentrations, the extracted fractions were then diluted, pooled, and used for Co-IP. This protocol can be used to identify protein-interactome of other chromatin-associated proteins in a variety of mammalian cells.

Keywords: Co-immunoprecipitaion, Nuclear proteins, Protein-protein interaction, Topoisomerase 1, Chromatin

[Background] Co-IP is extensively used to unravel the intricate relationship between protein complexes and various chromatin transactions during replication, transcription, and genome maintenance. However, it is challenging to keep labile protein-protein interactions intact during extraction, immunoprecipitation and washing steps of a Co-IP experiment. One way to stabilize labile protein interactions is to treat cells with cell-permeable reversible chemical cross-linker such as dithiobis-succinimidyl propionate prior to cell lysis (Smith *et al.*, 2011). As this approach is associated with shortcomings such as inefficient extraction and nonspecific protein trapping, Co-IP without crosslinking (native-IP) is preferred.

A nuclear protein can be distributed to various sub-nuclear compartments or chromatin regions that require varying degree of stringency for its extractions and solubilization. For example, TOP1 is present in nucleoplasm, associated with chromatin, and also localized to nucleolus. Extraction of such proteins into multiple sub-nuclear fractions using buffers with narrow incremental stringencies would preserve more labile interactions than extracting them in either a single fraction with high stringency buffer or multiple fractions using buffers with large changes in stringencies (Figure 1A). For proteome-wide interactome analysis the key to successful Co-IP of labile interacting partners is to keep small differences in stringencies of the buffers used for sequential extraction to prevent unnecessary exposure of protein complexes that can be extracted with low stringency buffers to high stringency buffers. We applied such

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a sequential nuclear extraction strategy to efficiently extract, Co-IP, and identify proteins that interact with TOP1 (Figures 1B and 1C) (Husain *et al.*, 2016).

TOP1 is an enzyme that relieves superhelical tension when two DNA strands are separated during transcription and replication, and prevent accumulation of negative supercoils, which if not relieved, may facilitate the formation of non-canonical DNA secondary structures. The non-canonical DNA secondary structures are implicated in transcription-associated mutagenesis, triplet repeat instability and activation induced cytidine deaminase-dependent immunoglobulin gene diversification (Kim *et al.*, 2012; Kobayashi *et al.*, 2009 and 2011). To understand the molecular mechanisms involved in TOP1-associated genomic instability, we performed Co-IP and identified proteins that interact with TOP1 (Figure 1). For these Co-IP experiments, we used transfectants of the TOP1-deficient mouse B-lymphocytic leukemia cell-line P388/CPT45 that stably expresses either GFP (green fluorescent protein)-tagged human TOP1 (GFPhTOP1) or GFP alone. Nuclear lysates from these cells were immunoprecipitated using the GFP-Trap® system (Figure 1C). Although this protocol was developed for Co-IP of a GFP-fused proteins, it also worked successfully with FLAG®-tagged proteins. The protocol described here is easy to perform and can be used as described or with minor modifications to Co-IP interacting partners of most chromatin-associated protein.

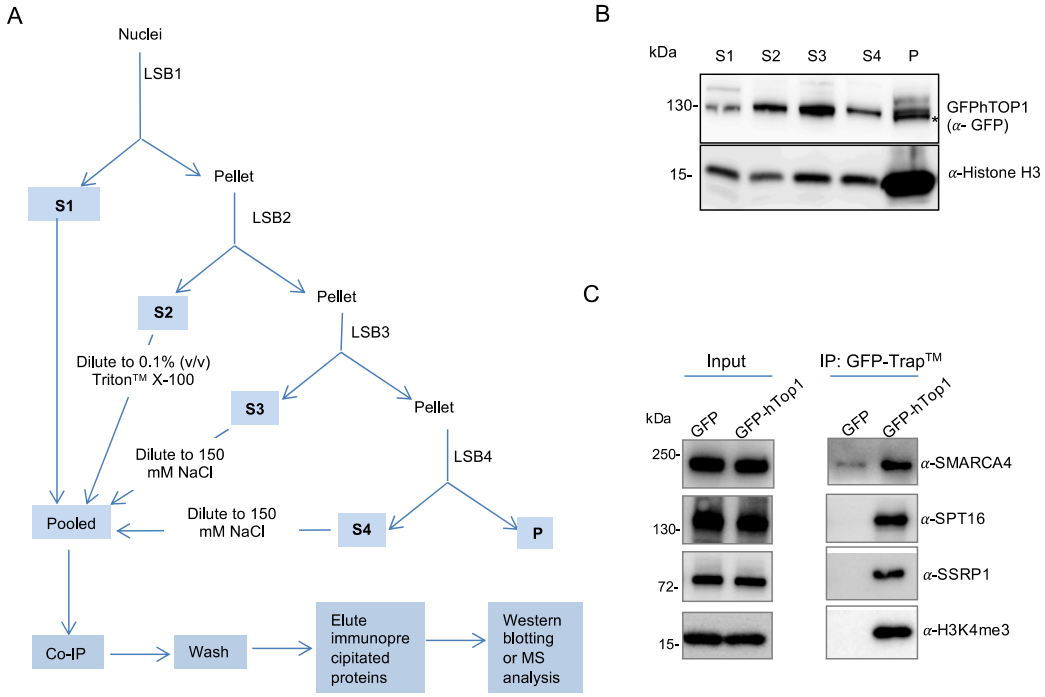

Figure 1. Native co-immunopreipitaion of TOP1-associated proteins. A. Schematic representation of sequential extraction of TOP1 protein complexes from P388/CPT45-GFPhTOP1 cells. P388/CPT45 cell expressing GFP alone was used as control. B. Analysis of TOP1 in soluble fractions (S1 to S4) and insoluble pellet (P) by western blotting with TOP1 and H3 antibodies. Asterisk (*) indicates a non-specific protein band. C. Co-immunoprecipitation of SMARCA4, subunits of histone chaperone FACT (SSRP1 and SUPT16H), and H3K4me3 with TOP1. The nuclei from

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P388/CPT45 transfectants expressing either GFP (P388/CPT45-GFP) or GFP-TOP1 (P388/CPT45-GFPhTOP1) were immunoprecipitated with GFP-Trap® agarose, and 5-10% of the co-immunoprecipitated proteins were electrophoresed on 4-20% SDS–PAGE gradient gel, followed by western blot with indicated antibodies. The positions of nearest MW marker bands and their size are also shown on the left.

Materials and Reagents

1. Pipette tips (any brand)
2. 100 mm cell culture dishes (IWAKI, catalog number: 4020-010)
3. 50 ml tubes (FALCON, catalog number: 352070)
4. 1.5 ml tubes (BIO-BIK, catalog number: RC-0150)
5. Tris (hydroxymethyl) aminomethane (Tris·HCl) (NACALAI TESQUE, catalog number: 35409-45)
6. Triton™ X-100 (Sigma, catalog number: T8787)
7. Phosphate buffered saline without Ca^{2+} and Mg^{2+} [PBS (-)] (NACALAI TESQUE, catalog number: 1148215)
8. Protein assay dye reagent (Bio-Rad, catalog number: 500-0006)
9. Sodium dodecyl sulfate (SDS) (Thermo Fisher Scientific, catalog number: BP166500)
10. β-mercaptoethanol (Thermo Fisher Scientific, catalog number: BP176-100)
11. HEPES (NACALAI TESQUE, catalog number: 17546-05)
12. Sodium chloride (NaCl) (NACALAI TESQUE, catalog number: 31320-05)
13. Glycerol (NACALAI TESQUE, catalog number: 17018-25)
14. 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, catalog number: 4561093)
15. SilverQuest™ silver staining kit (Thermo Fisher Scientific, catalog number: LC607)
16. Protein markers (Bio-Rad, Precision Plus Protein™ Standards-Dual Color, catalog number: 1610374)
17. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10270106)
18. RPMI 1640 (Thermo Fisher Scientific, catalog number: 11875093)
19. Benzonase® Nuclease (Novagen, catalog number: 70746-3)
20. cOmplete™, EDTA-free Protease Inhibitor Cocktail Tablets (Roche, catalog number: 4693132001)
21. GFP-Trap® Agarose (Chromotek, catalog number: gta-20)
22. ANTI-FLAG® M2 Agarose (Sigma, catalog number: A1205)
23. 3x FLAG™ Peptides (Sigma, catalog number: F4799)
24. Magnesium chloride hexahydrate (NACALAI TESQUE, catalog number: 7791-18-6)
25. Dithiothreitol (DTT) (NACALAI TESQUE, catalog number: 14112-52)
26. Bromophenol blue (Sigma-Aldrich, catalog number: B5525)
27. Glycine (NACALAI TESQUE, catalog number: 17109-35)
28. Methanol (Wako Pure Chemical Industries, catalog number: 136-09475)

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29. Quick CBB-stain (Wako Pure Chemical Industries, catalog number: 299-50101)
30. Reticulocyte Standard Buffer (RSB) (see Recipes)
31. HEPES-Glycerol-NaCl (HGN165) (see Recipes)
32. Protease Inhibitor Cocktail (25x) (see Recipes)
33. Lysis Buffers 1 (LSB1) (see Recipes)
34. Lysis Buffer 2 (LSB2) (see Recipes)
35. Lysis Buffer 3 (LSB3) (see Recipes)
36. Lysis Buffer 4 (LSB4) (see Recipes)
37. Washing buffer (see Recipes)
38. FLAG™ elution buffer (see Recipes)
39. 2x-SDS sample buffer (see Recipes)
40. 4x-SDS sample buffer (see Recipes)
41. 10x-SDS-PAGE running buffer (see Recipes)
42. Semi-dry Western blot buffer (see Recipes)

Equipments

1. Pipettes (any brand)
2. Refrigerated swing-bucket centrifuge (Tomy, model: EX-136)
3. Refrigerated fixed-angle centrifuge (Tomy, model: MX-301)
4. Shaking incubator (TAITEC, model: BR-23FP)
5. Rotator (TAITEC, model: RT-50)
6. Sonicator (Diagenode, model: Bioruptor® Standard UCD-200)
7. Vortex mixer (Thermo Fisher Scientific, catalog number: 128101)
8. Block heater (any brand)
9. Magnetic stand (Thermo Fisher Scientific; DynaMag™-2, model: 12321D)
10. Western blotting apparatus (Bio-Rad, model: Trans-Blot® SD Semi-Dry Transfer Cell, catalog number: 1703940)
11. Protein electrophoresis system (Bio-Rad, The Mini-PROTEAN® Tetra cell, catalog number: 1658005EDU)
12. Imaging of Western blots (GE Healthcare, model: ImageQuant LAS 4000)

Procedure

A. Preparation of nuclear lysate

1. Collect 2 x 10^7-3 x 10^7 cells with culture medium in 50 ml conical tube and centrifuge at 800 *x g* for 3 min at 4 °C.
2. Discard the supernatant and suspend the cells in 10 ml of pre-chilled PBS and centrifuge at 800 *x g* for 3 min at 4 °C.

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3. Discard the supernatant, suspend the cells in 1 ml of pre-chilled PBS, and transfer to 1.5 ml tube and centrifuge cells at 800 *x g* for 3 min at 4 °C.
4. Discard the supernatant, suspend the cells in 950 µl of pre-chilled RSB containing 1x protease inhibitor cocktail, and incubate at ice for 15 min.
5. Add 20 µl of 10% Triton™-X100 (v/v), invert tube 6-10 times, and centrifuge at 1,200 *x g* for 5 min at 4 °C.
6. Discard the supernatant (cytoplasmic fraction), suspend the nuclear pellet in 200 µl of pre-chilled LSB1 and mildly sonicate at Bioruptor® for 5 min at low power (pulse 30 s, rest 60 s). Keep on ice for 5 min, and repeat sonication for 5 min at low power (pulse 30 s, rest 60 s). Rotate end-over-end for 1 h at 4 °C.
7. Centrifuge at 8,000 *x g* for 10 min at 4 °C and collect supernatant in fresh tube (S1).
8. Immediately suspend the pellet with the help of pipette tip in 150 µl of pre-chilled LSB2, and rotate end-over-end for 30 min at 4 °C.
9. Centrifuge at 8,000 *x g* for 10 min at 4 °C and collect supernatant in fresh tube. Add 50 µl of RSB containing 1x protease inhibitor cocktail to S2 and keep at ice (S2).
10. Immediately suspend the pellet with the help of pipette tip in 150 µl of pre-chilled LSB3, and rotate end-over-end for 30 min at 4 °C.
11. Centrifuge at 8,000 *x g* for 10 min at 4 °C and collect supernatant in fresh tube. Add 50 µl of RSB containing 1x protease inhibitor cocktail to S3 and keep at ice (S3).
12. Immediately suspend the pellet with the help of pipette tip in 150 µl of pre-chilled LSB4, and rotate end-over-end for 30 min at 4 °C.
13. Centrifuge at 8,000 *x g* for 10 min at 4 °C and collect supernatant in fresh tube. Add 100 µl of RSB containing 1x protease inhibitor cocktail to S4 and keep at ice (S4).
14. Pool extracts S1, S2, S3 and S4. Invert few times to mix and centrifuge at 3,000 *x g* for 5 min at 4 °C and collect supernatant in a fresh tube. Save 30 µl of pooled extract, add 30 µl of 2x SDS-sample buffer, and heat for 10 min at 95 °C. This will be used as input in western blotting.

B. Co-immunoprecipitation

1. Transfer 30 µl of GFP-Trap® agarose for GFP-fused proteins or 30 µl FLAG®-M2 agarose for FLAG®-tagged proteins to 1.5 ml tube.
2. Add 500 µl of pre-chilled RSB, invert 4-6 times, and centrifuge at 800 *x g* for 3 min at 4 °C. Discard the supernatant carefully without disturbing the resin.
3. Transfer pooled nuclear lysates to the washed GFP-Trap® agarose or FLAG®-M2 agarose, and rotate end-over-end for 1-2 h at 4 °C.
4. Centrifuge at 80 *x g* for 3 min at 4 °C, and discard supernatant. Low speed centrifugation at this stage prevent precipitation of insoluble proteins aggregates that may have formed during incubation lysates with GFP-Trap® or FLAG®-M2 agarose at 4 °C. Discard the supernatant carefully without disturbing the resin.

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5. To wash resin, add 800 µl of pre-chilled WB, invert 8-10 times, centrifuge at 800 *x g* for 3 min at 4 °C, and discard the supernatant carefully without disturbing the resin.
6. Repeat washing 3 more times, and discard the supernatants carefully without disturbing the resin. Proceed to elution of the bound proteins.

C. Elution
1. Elution by boiling
   a. Suspend resin in 100 µl 2x SDS-sample buffer, and heat for 10 min at 95 °C.
   b. Centrifuge at 1,800 *x g* for 3 min at 25 °C and collect supernatant in a fresh tube.
2. Elution by competition
   a. Suspend resin in 75 µl of 3x-FLAG® elution buffer containing 500 µg/ml of 3x-FLAG® peptides at 25 °C for 20 min with mild shaking.
   b. Centrifuge at 1,800 *x g* for 3 min at 25 °C and collect supernatant in a fresh tube.
   c. Add 25 µl of 4x SDS-sample buffer, and heat for 10 min at 95 °C.

D. Analysis of the immunoprecipitated proteins
1. Run 5-10 µl of input lysates and 10 µl of eluates (with SDS sample buffer) on SDS-PAGE gels followed by western blotting.
2. For silver staining, run 2-3 µl of input lysates and 10 µl of eluates (with SDS sample buffer) on SDS-PAGE gels and stain with SilverQuest™ Silver Staining Kit.
3. For identification of proteins by mass spectrometry, eluates from 4-10 Co-IP experiments can be pooled. To load the pooled eluates in one single lane of SDS-PAGE, pooled eluates can be concentrated using a method that utilizes MS-compatible reagents such as methanol/chloroform based method. Run concentrated proteins on 4-20% SDS-PAGE gradient gel SDS-PAGE gels and stain with Quick-CBB stain. Cut the gel lane into 8-10 slices and process for MS analysis.

Notes

1. In a very first experiment, check the extraction of the protein that you want to immunoprecipitate in extracted fractions (S1 to S4) as well as insoluble pellet (Figure 1). If you do not see sufficient extraction, add additional extraction steps with higher Triton™ X-100 and/or NaCl concentrations. Similarly, extractions with higher stringency buffer can be omitted if the protein is sufficiently extracted with low stringency buffers.
2. Pooled lysates prepared in this protocol can also be used for Co-IP using primary antibodies against the protein target of interest.
3. The pH of the each buffer was measured at room temperature.

Recipes

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1. Reticulocyte Standard Buffer (RSB)
   10 mM Tris-HCl, pH 7.4
   5 mM $MgCl_2$
   10 mM NaCl
2. HEPES-Glycerol-NaCl (HGN165)
   10 mM HEPES-NaOH, pH 7.4
   10% Glycerol (v/v)
   165 mM NaCl
3. Protease Inhibitor Cocktail (25X)
   Dissolve 1 tablet of cOmplete™, EDTA-free Protease Inhibitor Cocktail in 0.4 ml of water
4. Lysis Buffer 1 (LSB1)

HGN165	2 ml
Triton™ X-100 (10% v/v)	20 µl
$MgCl_2$ (0.5 M)	8 µl
DTT (0.5 M)	4 µl
Benzonase® (250 units/µl)	4 µl
Protease Inhibitor cocktail (25x)	130 µl

5. Lysis Buffer 2 (LSB2)

LSB1	500 µl
Triton™ X-100 (10 % v/v)	10 µl

6. Lysis Buffer 3 (LSB3)

LSB1	500 µl
NaCl, 1 M	26.5 µl

7. Lysis Buffer 4 (LSB4)

LSB1	500 µl
NaCl, 1 M	53 µl

8. Wash buffer (WB)

HGN165	6 ml
Protease Inhibitor cocktail (25x)	240 µl

9. FLAG™ elution buffer
   50 mM Tris-HCl, pH 7.5
   150 mM NaCl
   0.1% Triton™ X-100 (v/v)
10. 2x-SDS sample buffer
    120 mM Tris-HCl, pH 6.8
    20% glycerol (v/v)
    2% SDS (w/v)
    0.04% bromophenol blue
    10% β-mercaptoethanol (v/v)

11. 4x-SDS sample buffer
    120 mM Tris-HCl, pH 6.8
    40% glycerol (v/v)
    4% SDS (w/v)
    0.08% bromophenol blue
    20% β-mercaptoethanol (v/v)
12. 10x-SDS-PAGE running buffer

Tris base	30.3 g
Glycine	188 g
SDS	10 g
Water	to 1 liter

13. Semi-dry Western blot buffer

Tris base	6 g
Glycine	14.4 g
SDS	1 g
Methanol	200 ml
Water	to 1 liter

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Competing interests

The authors declare no competing financial interests.

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