Chapter 11

An Improved In Vivo Biotinylation Strategy Combined with FLAG and Antibody Based Approaches for Affinity Purification of Protein Complexes in Mouse Embryonic Stem Cells

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Abstract

The proteome in mouse embryonic stem cells has not been extensively studied in comparison to other cellular systems, limiting our understanding of multi-protein complex functions in stem cell biology. Several affinity purification techniques followed by mass spectrometry analysis have been designed and validated to identify protein–protein interaction networks. One such approach relies on in vivo biotinylation of a protein of interest and subsequent pull-down of its interacting partners using streptavidin-conjugated agarose beads. This technique takes advantage of the high affinity between biotin and streptavidin, allowing for high affinity purification of protein complexes without the use of antibodies. Here, we describe an improved large-scale purification of multi-protein complexes in mouse embryonic stem cells by in vivo biotinylation, complemented with standard antibody and/or FLAG based affinity captures. This combined strategy benefits from the high efficiency of the streptavidin pull-down and the validation of the most highly confident interacting partners through the two alternative approaches.

Key words ESC, Biotinylation, Streptavidin, Multi-protein complexes, Purification, Mass spectrometry, BirA

1 Introduction

Mouse embryonic stem cells (mESCs) have the capacity to self-renew indefinitely and to differentiate into all cell types [1]. They exert these properties through many ESC specific proteins, such as the core pluripotency factors Nanog, Sox2, Oct4, and many other ubiquitous factors, including Polycomb proteins and epigenetic modifiers [2–4]. They form many multi-protein complexes to regulate functions such as transcription, translation, cell cycle, DNA replication, and so on. Therefore, to truly comprehend the many aspects of ESC biology, purification, identification, and analysis of these multi-protein complexes are fundamental.

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In recent years, the enormous technical improvements of mass spectrometry (MS) techniques and the availability of numerous proteomic datasets have significantly facilitated the determination of the protein content of a given sample [5]. However, the purification of such multi-protein complexes remains relatively challenging and still represents a bottleneck for executing proteomic studies [6].

Several different affinity purification strategies have been utilized over the past few decades [6–8]. These include (1) epitope tagging of a protein of interest with tags such as HA or FLAG, (2) antibody based affinity purification, provided that an antibody against the protein of interest is available, (3) ion exchange and gel filtration chromatography for the sequential fractionation of nuclear extracts to preserve and separate multi-protein complexes, and (4) biotinylation of a protein of interest by the biotin ligase BirA at a biotinylatable peptide sequence tag, described herein (Fig. 1). All these approaches have advantages and disadvantages, and since an optimal universal technique has not yet been designed, successful purification and identification of multi-subunit complexes may be achieved only by a combination of at least two of these approaches, for instance, the epitope tagging of a transcription factor with two different epitopes to perform a two-step (or tandem) affinity purification [9]. However, tandem affinity purification, although very efficient in reducing background, has the drawback of possible loss of peripheral and weakly interacting proteins due to the prolonged purification process. One solution to this is the iterative tagging of a different subunit of the multi-protein complex identified in the first affinity purification, and then performing a second independent affinity purification [9]. This second procedure greatly improves the identification of *bone fide* components of multi-protein complexes, but is much more lengthy and costly. Alternatively, double tagging of a single protein of interest, followed by two independent affinity purifications coupled, whenever possible, with antibody based immunoprecipitation (IP)/Co-IP, also improves the analysis of multi-protein complexes (Fig. 1) [10]. Everything considered, successful purification and identification of protein complexes relies on the improvements of each single affinity purification step. Here, we describe an improved biotin-based affinity purification approach for the identification of transcription factor associated protein complexes in mESCs. This strategy uses biotinylation of a bio-tagged [11] protein of interest by the bacterial enzyme BirA, followed by purification using streptavidin (SA)-conjugated agarose beads (Fig. 1) [12]. This technique offers several advantages compared to the traditional affinity purifications based on tags such as FLAG and HA. The high affinity between biotin and SA ($K_d \sim 10^{-15}$ M) allows for more efficient and stable capture of the protein of interest and associated factors. In addition, in ESCs there are only a few endogenously biotinylated proteins such as

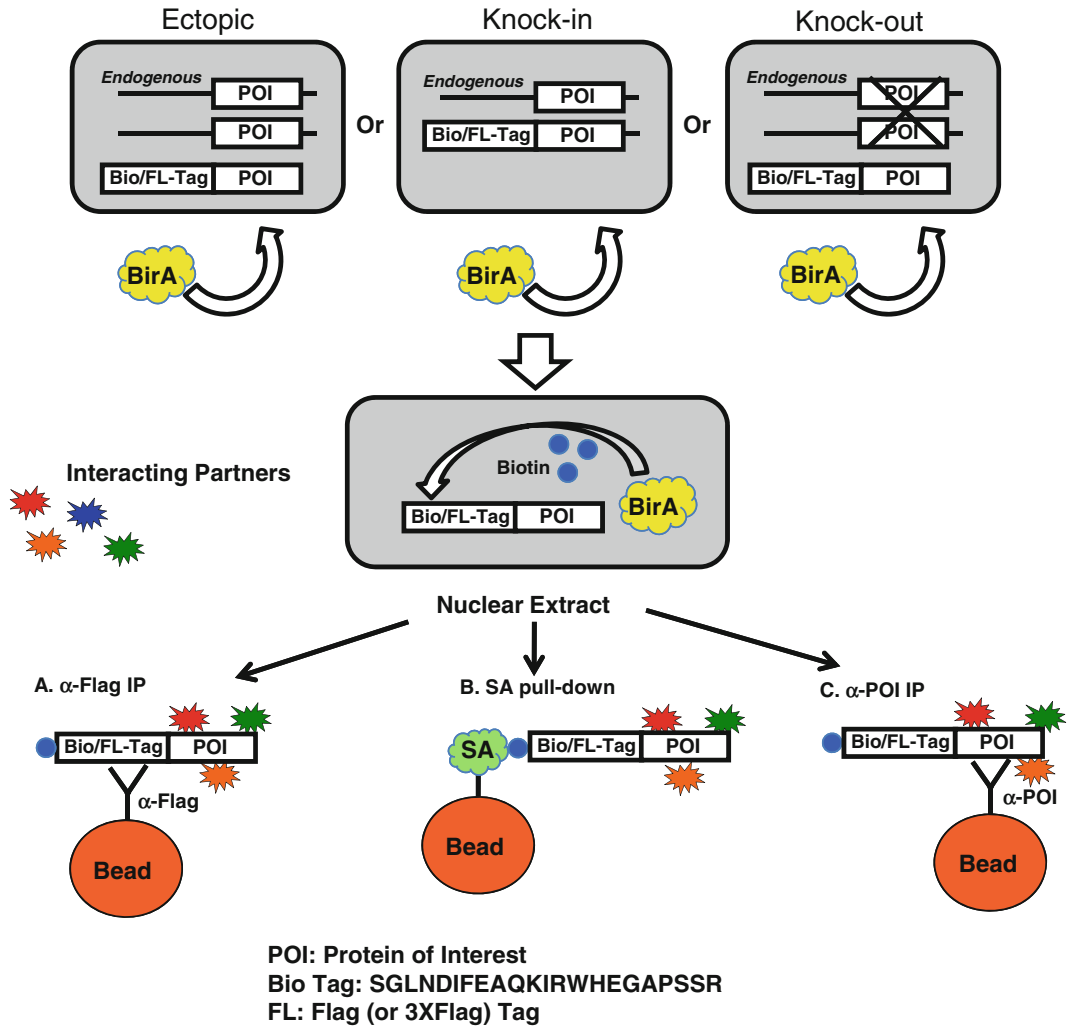

Fig. 1 Strategies for affinity purification of the protein of interest (POI) and associated complexes. The transgene expressing the POI with a Bio and FLAG dual tag, is incorporated by stable transfection in either wild type cells or when possible, knockout cells,. Alternatively, the tag is engineered on the endogenous allele(s) of the gene of interest. The BirA transgene is always present to biotinylate the POI at the Bio tag. Upon nuclear extract preparation, the biotinylated POI and its associated factors are IPed with either a FLAG antibody, or pulled-down by SA conjugated beads, or captured with an antibody against the POI

some carboxylases and ribosomal proteins, which have already been characterized and can be excluded in the final list of interacting partners [9]. This approach also avoids using antibodies for the affinity capture, which significantly reduces nonspecific binding due to antibody cross-reactivity. Significant improvements over our previously published method [12, 13] have been outlined in this current protocol. Since mESCs are very sensitive to the expression levels of several key factors such as Oct4 and Sox2 [14, 15], we rely whenever possible on over-expressing our bio-tagged

protein of interest at physiological levels, and often in lines where the endogenous protein is knocked out. In the latter case, if the cells do not present any abnormal behavior, it means that the tagged ectopic version of the protein of interest is expressed at physiological levels and can substitute for the endogenous one. This also eliminates spurious complex formation and allows for more efficient and complete purification of the protein of interest and its associated multi-protein complexes. Alternatively, we sometimes engineer knock-in lines where the protein of interest is endogenously bio-tagged and the only transgene stably integrated in the genome is the BirA biotin ligase (Fig. 1). Once again, the normal phenotype of the knock-in cells will assure that knocked-in allele(s) functions similarly to the wild type. We now also employ transposon-based integration vectors over standard over-expression plasmids to facilitate the generation and screening of transgenic lines. In addition, to ensure detection of more peripheral and/or weakly interacting factors, we utilize lower salt and detergent conditions during the affinity purification. We also add a FLAG (or 3XFLAG) tag to the protein of interest to have the option of performing a large scale FLAG based affinity purification, and when possible a capture with an antibody raised against the protein of interest, to complement the SA pull-down. In conclusion, although very confident about the many advantages of our biotin-SA based affinity purification, we believe that a combination of a few techniques performed independently significantly improves the identification of *bone fide* multi-protein complexes. Indeed, in our recently published article, we purified an extended Nanog interactome in mESCs by utilizing a combination of SA, anti-FLAG, and anti-Nanog based affinity purifications [16]. This technique, where two or more independent affinity approaches are employed in parallel to identify an interactome (Interactomes by Parallel Affinity Capture (iPAC)), has already been shown to improve the identification of bona fide interacting partners [17].

2 Materials

All solutions, buffers, and media are sterilized by filtration unless otherwise specified.

1. Difco gelatin: 1 % (v/v) gelatin in H_2O (autoclaved). Dilute 1:10 in H_2O to gelatinize dishes. Gelatinized 3.5 cm, 10 cm, 15 cm, 245 mm × 245 mm square, and 6-well dishes.
2. ESC Medium: DMEM high glucose, 15 % (v/v) FBS, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 % (v/v) nucleoside mix, 50 U/mL penicillin–streptomycin, 1,000 U/mL recombinant LIF.
3. STOP medium: DMEM, 10 % FBS.

4. Dulbecco's Eagle Medium (DMEM) with high glucose.
5. Fetal bovine serum (FBS).
6. Penicillin–streptomycin solution.
7. Phosphate buffered saline (PBS).
8. pPiggyBac-BirA-V5-HGR plasmid vector.
9. pPiggyBac-bio-FLAG-[cDNA of interest]-BSD plasmid vector.
10. pBASE (PiggyBac transposase).
11. Lipofectamine 2000 (Life Technologies).
12. Opti-MEM I (Gibco).
13. 0.05 % Trypsin–EDTA.
14. Hygromycin b solution at a concentration of 50 mg/mL.
15. Blasticidin S HCl.
16. Streptavidin agarose (SA) beads (Life Technologies).
17. Anti-FLAG M2 agarose beads (Sigma).
18. FLAG peptide (or 3XFLAG peptide) (Sigma).
19. Anti-POI antibody: antibody raised against protein of interest (POI).
20. IgG control antibody.
21. 2x Laemmli sample buffer.
22. Buffer A: 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT (add fresh), 0.2 mM PMSF (add fresh), 1× protease inhibitor cocktail (add fresh).
23. Protease inhibitor cocktail, 1,000× (Sigma).
24. Buffer C: 20 mM HEPES pH 7.9, 25 % (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT (add fresh), 0.2 mM PMSF (add fresh), 1× protease inhibitor cocktail (add fresh).
25. Dialysis Buffer D: 20 mM HEPES pH 7.9, 20 % (v/v) glycerol, 100 mM KCl, 1.5 mM MgCl, 0.2 mM EDTA, 0.5 mM DTT (add fresh), 0.2 mM PMSF (add fresh).
26. Slide-A-Lyzer dialysis cassette 15 or 3 mL (Thermo Scientific).
27. Benzonase (Novagen).
28. Protein G agarose beads (Roche).
29. IP Buffer DNP: Dialysis Buffer D supplemented with 0.02 % NP-40.
30. Protein LoBind 1.5- or 2-mL safe-lock microcentrifuge tubes (Eppendorf) or other low protein binding tubes.
31. Amicon Ultra centrifugal 3 K filters (Millipore) or other compatible spin filters.
32. 4–20 % gradient polyacrylamide gels.

33. BupH Tris-HEPES-SDS running buffer (Thermo scientific).
34. Gel code blue safe protein stain (Thermo scientific) or other similar gel stain.
35. Dounce homogenizer with type B pestle (or 3–10 mL syringes with 16G1½ or 20G1½ needles).

3 Methods

3.1 Bio Cell Line Generation, Cell Culture, and Pilot Scale Affinity Purification

All mESC lines are grown on gelatinized dishes and maintained in standard ES medium at 37 °C in 5 % CO_2. The following procedures apply to control lines as well as lines with tagged proteins of interest.

1. Grow mESCs (parental ESC lines such as J1, or specific knockout/knock-in lines) in a 3.5 cm dish to 70–80 % confluence.
2. Pre-incubate 1 μg BirA and 2 μg pBASE plasmids diluted in 250 mL Opti-MEM I Medium, with 3 μL Lipofectamine 2000 diluted in 250 mL Opti-MEM I medium for 10 min at room temperature.
3. Meanwhile, wash cells once with PBS and add 1 mL 0.05 % Trypsin–EDTA for 5 min at 37 °C or at room temperature.
4. Detach cells by gently tapping the plate and then resuspend them by gently pipetting up and down.
5. Add 1 mL STOP medium, mix, harvest, and pellet the cells by spinning for 5 min at 300 × *g* at 4 °C.
6. Aspirate medium and resuspend cells in the 500 μL DNA/Lipofectamine 2000 mixture. Let the cells rest for 10 min at room temperature.
7. Add cells dropwise to a 10 cm dish containing 10 mL of ESC medium. Incubate overnight at 37 °C.
8. The next day, replace the medium and add the drug Hygromycin b at a concentration of 200 μg/mL for positive selection of transfectants.
9. Select the cells for at least 1 week by replacing medium supplemented with the selection drug every other day (*see* **Note 1**).
10. When discernible colonies appear (*see* **Note 1**), pick 6–12 of them and expand on a 6-well dish (*see* **Note 2**).
11. When the cells reach 70–80 % confluence, trypsinize each clone, freeze one half (in at least two vials; keep one at −80 °C for the next several days), and run SDS-PAGE with the other half of the cells to check for BirA expression (*see* **Note 3**).
12. Choose one clone in which BirA is expressed and thaw the corresponding vial in one well of a 6-well dish (*see* **Note 4**).

13. Repeat **steps 2–7** using a PiggyBac vector encoding the FLAG-bio-tagged version of the protein of interest.
14. Select as in **step 8** but use 20 μg/mL of Blasticidin S.
15. Repeat **steps 9–11** above for the protein of interest (for western blot we use an endogenous antibody and/or a streptavidin-HRP antibody).
16. Choose one clone in which BirA and the FLAG-bio-tagged protein of interest are expressed and thaw the corresponding vial (thaw also BirA-only control cells or any other negative control line) in one well of a 6-well dish.
17. Expand to a 10 cm dish until cells are 80–90 % confluent.
18. When confluence is reached, harvest the cells and replate 10 % of them in a 15 cm dish with ESC medium supplemented with selection drugs.
19. Resuspend the leftover 90 % of cells in 1 mL Buffer A (*see* **Note 5**). Transfer to a microcentrifuge tube and spin at 10,000 × *g* for 10 min at 4 °C.
20. Discard the supernatant cytosolic extract and repeat **step 19**.
21. Discard the second supernatant cytosolic extract and resuspend the nuclei in 1 mL of Buffer C (supplemented with 0.02 % NP-40). Pipette up and down several times, and then rotate at 4 °C for about 30 min.
22. Centrifuge at maximum speed for 30 min at 4 °C and then transfer the supernatant nuclear extract (NE) into a new Eppendorf microcentrifuge tube.
23. Repeat **step 22** once more.
24. Measure the protein concentration of the NE and then slowly dilute 500 μL of NE in a 1:1 ratio with Buffer C (without NaCl but supplemented with 0.02 % NP-40), to adjust the salt concentration to about 200 mM. Mix and spin as in **step 22**.
25. Meanwhile, cut off the tip of a 200 μL pipette tip and pipette 40 μL of streptavidin-agarose (SA) beads (50 % slurry; equivalent to 20 μL of solid beads) into two different Eppendorf tubes (one for the control line) (*see* **Note 6**).
26. Wash the beads twice by adding 1 mL of Buffer C, resuspend them, and spin at 1,000 × *g* for 15–30 s at 4 °C. Remove most of the supernatant after each spin without touching the beads.
27. Perform a pilot-scale affinity purification by adding 900 μL of the diluted and spun NE in the Eppendorf tube with the 20 μL of pre-equilibrated streptavidin-agarose beads, and rotate at 4 °C for a minimum of 3 h-overnight (*see* **Note 7**).
28. Transfer the supernatant (unbound) to a different tube and save for later.

29. Wash the beads four times with Buffer C (with 200 mM NaCl and supplemented with 0.02 % NP-40) as in **step 26**. After the last wash add 10–15 μL of 2× Laemmli sample buffer, heat at 95 °C for 3–5 min, spin for a few seconds at maximum speed, and transfer the supernatant with a gel loading tip to a new Eppendorf tube.
30. Run SDS-PAGE with 1–5 % input extracts, pull-down samples, and 1–5 % unbound extracts. Probe with streptavidin-HRP, or an antibody against the protein of interest (*see* **Note 8**).
31. Once biotinylation and capture of the protein of interest are verified, and the amount of SA beads to use per mg of NE is estimated, proceed to the large scale pull-down.
32. The 15 cm dishes where the 10 % fraction of cells was plated should be confluent by now (*see* **Note 9**). Wash with 5 mL of PBS and add 5 mL of 0.05 % Trypsin–EDTA. Resuspend the cells by pipetting up and down several times and neutralize the trypsin with 5 mL of STOP medium. Collect cells in a 15 mL conical tube and rinse plate with an additional 5 mL of STOP medium. Collect and pool into the same conical tube.
33. Spin at 300 × *g* for 5 min at 4 °C, remove supernatant, and resuspend cells in ESC medium. Plate cells in four 15 cm dishes (*see* **Note 10**).
34. Grow until 70–80 % confluent (*see* **Note 11**). Then split the four dishes into twenty 15 cm dishes.
35. Grow for an additional 2–3 days and change medium accordingly.
36. When cells are near confluence, wash with 10 mL of PBS, add 5 mL of 0.05 % Trypsin–EDTA for about 5 min at room temperature, resuspend the cells, neutralize the trypsin with 5 mL of STOP medium, and harvest the cells into 250 mL plastic centrifuge bottles. Rinse the dishes with 5 additional mL of STOP medium and collect leftover cells.
37. Pellet the cells by spinning at 300 × *g* for 20 min at 4 °C. Carefully decant the supernatant.
38. Resuspend the cells in 50 mL of ice-cold PBS and transfer into a 50 mL conical tube.
39. Spin at 300 × *g* for 10 min at 4 °C and decant the supernatant. Proceed to nuclear extract preparation.

3.2 Nuclear Extract Preparation

1. Estimate the packed cell volume (PCV) (*see* **Note 12**) and resuspend in 5× PCV of ice-cold Buffer A.
2. Spin at 2,100 × *g* for 10 min at 4 °C and discard the supernatant by aspiration.
3. Add 3× PCV of ice-cold Buffer A to the pellet, resuspend, and let it sit for 5–10 min on ice.

4. Prechill a glass dounce homogenizer with type B pestle (the loose one) on ice and pre-rinse it with Buffer A. Add the resuspended pellet and slowly homogenize up and down for no more than 12 times. Keep everything on ice while homogenizing (*see* **Note 13**).
5. Transfer into a conical tube and spin at 4,300 × *g* for 10 min at 4 °C. Discard the supernatant (cytosolic extract) (*see* **Note 14**) and keep the pellet (nuclei).
6. Add 3–10 mL of ice-cold Buffer A and resuspend the nuclei. Transfer into a high speed centrifuge tube and spin at 25,000 × *g* for 20 min at 4 °C (*see* **Note 15**).
7. Remove the supernatant and add 3–10 mL of ice-cold Buffer C to the nuclei and resuspend (*see* **Note 16**).
8. Transfer into a glass dounce homogenizer with type B pestle (loose one) (*see* **Note 17**), and slowly homogenize on ice with no more than ten strokes (*see* **Note 13**).
9. Transfer homogenate into a 15 mL conical tube and rotate for 30 min at 4 °C.
10. Centrifuge at 4,300 × *g* for 10 min at 4 °C and transfer the supernatant nuclear extract to several Eppendorf tubes.
11. Centrifuge at maximum speed for 30 min at 4 °C.
12. Meanwhile, for each sample pour Dialysis Buffer D into a beaker (*see* **Note 18**). We use at least 100 volumes of dialysis buffer per volume of NE.
13. After spinning, transfer the supernatant into a dialysis cassette, pre-equilibrated according to the manufacturer's instructions, and incubate for 3–5 h at 4 °C with constant stirring to adjust the salt concentration to 100 mM.
14. Following dialysis, transfer the nuclear extract to a 15 mL conical tube and spin at 4,300 × *g* for 10 min at 4 °C (*see* **Note 19**).
15. After centrifugation, transfer the supernatant NE to several Eppendorf tubes and spin at maximum speed for 30 min at 4 °C in a microcentrifuge. Carefully transfer the supernatant to new Eppendorf tubes without touching the precipitate.
16. Repeat **step 15** once more and combine the supernatants.
17. Supplement the NE with 1× protease inhibitor cocktail, 0.02 % NP-40, and Benzonase (*see* **Note 20**).
18. For each sample, pipette 200 μL of Protein G agarose beads (400 μL of 50 % slurry) into a 15 mL conical tube (*see* **Note 6**).
19. Resuspend the beads with 10 mL of IP Buffer DNP (without protease inhibitor cocktail), spin at 1,000 × *g* for 1 min at 4 °C, and aspirate most of the supernatant without touching the beads.

20. Add the NE to the tube containing the equilibrated beads and pre-clear by rotating overnight at 4 °C.
21. The following morning, centrifuge at 1,000 × *g* for 1 min at 4 °C to pellet the beads.
22. Carefully remove most of the supernatant NE without touching the beads.
23. Aliquot the NE into several Eppendorf tubes and centrifuge for 30 min at maximum speed at 4 °C.
24. After spinning, combine the supernatants in a 15 mL conical tube and label as pre-cleared NE.

3.3 SA Affinity Purification

All steps are performed on ice or at 4 °C unless otherwise specified.

1. Measure protein concentrations and use the same amount (in mg) of NE for all samples for the affinity purification.
2. Equilibrate no more than 1 mL of 50 % slurry streptavidin agarose beads (500 μL of beads) per sample by pipetting the slurry into a 15 mL conical tube and resuspending with 10 mL of IP Buffer DNP (without protease inhibitor cocktail) (*see* **Note 21**).
3. Spin at 1,000 × *g* for 1 min at 4 °C to pellet the beads, and aspirate most of the supernatant without touching the beads.
4. Add the pre-cleared NE directly to the equilibrated beads (adjust the volume to 10 mL with IP Buffer DNP) and gently rotate for 3–5 h at 4 °C (*see* **Note 22**).
5. Centrifuge at 500 × *g* for 2 min at 4 °C and remove most of the supernatant (unbound material) without disturbing the beads (bound material). Transfer into a new 15 mL conical tube (aliquot also ~100–200 μL in an Eppendorf tube), flash-freeze in liquid nitrogen, and store at −80 °C (*see* **Note 23**).
6. Wash the beads and captured complexes by adding 10 mL of cold IP Buffer DNP (without protease inhibitor cocktail) and rotating 15 min at 4 °C. Afterwards, centrifuge at 500 × *g* for 2 min at 4 °C and discard most of the wash by aspirating without disturbing the beads.
7. Repeat **step 6** three more times.
8. After the last wash, leave behind about 200–300 μL of washing buffer and transfer the beads into a low protein binding Eppendorf tube (*see* **Note 24**).
9. Wash one more time by adding 1 mL of IP Buffer DNP (without protease inhibitor cocktail) and rotating 5–10 min at 4 °C.
10. Centrifuge at 1,000 × *g* for 1 min at 4 °C and aspirate most of the wash without touching the beads.

11. Elute captured protein complexes by adding 500 µL of 2× Laemmli sample buffer (*see* **Note 25**). Vortex briefly and heat at 95–100 °C for 4–5 min (vortex briefly from time to time), spin at 5,000 × *g* for 30 s at RT and transfer as much supernatant as you can with a gel loading tip into a new 2 mL Eppendorf tube.
12. Repeat **step 11** (this time use 1× Laemmli sample buffer: 50 % 2× Laemmli sample buffer and 50 % H_2O) twice and pool eluates (about 1.5 mL total volume). Spin once more and transfer supernatant to a new tube to get rid of any bead carryover (*see* **Note 26**).
13. Concentrate the eluate with Amicon ultra centrifugal filter according to the manufacturer's instructions (*see* **Note 27**). Keep a 50–100 µL aliquot of the unconcentrated eluate to check by western blot.

3.4 FLAG Affinity Purification

All steps are performed on ice or at 4 °C unless otherwise specified (*see* **Note 28**).

1. Perform **steps 1–10** as in Subheading 3.3 but use anti-FLAG M2 agarose beads instead of SA beads.
2. Elute captured protein complexes by adding 500 µL of 0.3 µg/mL FLAG peptide (or 3XFLAG peptide if your protein of interest has a 3XFLAG tag) in IP Buffer DNP (*see* **Note 29**). Rotate at least 1 h at 4 °C, then centrifuge at 1,000 × *g* for 1 min at 4 °C and transfer supernatant in a new 2 mL Eppendorf tube (*see* **Note 30**).
3. Repeat **step 2** three more times and combine all the eluates (*see* **Note 31**). Spin once more and transfer the eluate into a new tube to get rid of any bead carryover.
4. Perform **step 13** as in Subheading 3.3.

3.5 Anti-POI Affinity Purification

All steps are performed on ice or at 4 °C unless otherwise specified (*see* **Note 32**).

1. Equilibrate 150–200 µL of protein G agarose beads (300–400 µL of 50 % slurry) in IP Buffer DNP, as in **steps 25** and **26** in Subheading 3.1.
2. Pre-bind 30–50 µg of purified anti-POI antibody (or IgG raised in the same species) with the beads overnight at 4 °C by gentle rotation (*see* **Note 33**).
3. The morning after, incubate the pre-cleared and spun nuclear extract with the pre-bound antibody and rotate for about 3–4 h at 4 °C (*see* **Note 34**).
4. Perform **steps 5–13** as in Subheading 3.3 but wash in Eppendorf tubes, and use 150–200 µL 2× Laemmli sample buffer per elution.

3.6 SDS-PAGE/MS

Before proceeding to MS analysis, run SDS-PAGE to check the efficacy of the pull down. Use 10–20 μL of input pre-cleared NE (use the frozen aliquot you set aside in **step 5** of the affinity purification procedure), the same percentage of the unbound aliquot you set aside in **step 5** of the affinity purification, and 20–30 μL of the unconcentrated eluate aliquot set aside in **step 13** of the affinity purification. Also load on the gel the aliquots from the negative control affinity purification. Check by western blot with an antibody against the protein of interest or with a streptavidin-HRP antibody. Proceed to running the gel for MS (*see* **Note 35**).

1. Use a commercial pre-cast 4–20 % gradient polyacrylamide gel.
2. Load on the gel a protein ladder marker and the concentrated eluates (specific and control). You can use more than one well to load all the eluate for each sample. Keep at least one well empty between control and specific eluates.
3. Run at 60 V to 1/3 or 1/2 of the gel length and stain with gel code blue safe stain for at least 2 h or overnight, according to the manufacturer's instructions. De-stain with H_2O several times.
4. Proceed according to the guidelines from the MS facility of choice.

4 Notes

1. If after selection too many cells survived and colonies are not separated, trypsinize the cells and replate around 10,000 cells. Wait for colonies to appear and then pick them.
2. Use a microscope and a P200 pipet to pick colonies. If they are well separated, you can pick them in the hood. We generally pick colonies in PBS and transfer them into individual wells of a 96-well round bottom plate containing 50 μL trypsin per well to dissociate the cells in each colony. When the cells are separated, we transfer them to wells of a 24-well dish, then to wells of a 6-well dish.
3. We use an anti-BirA antibody or anti-V5-HRP antibody for western blot.
4. In our experience, screening six clones will yield several BirA lines from which to choose. Thaw the vial stored at −80 °C.
5. We generally resuspend the cells and extract the cytosolic portion by simply pipetting up and down.
6. Make sure you cut the bottom of the tip to facilitate accurate pipetting of the 50 % slurry.
7. It is better to incubate the lysate with the beads only for a few hours, but it can be done overnight if you start late in the afternoon.

8. The biotinylated version of the protein of interest should migrate slower than the unmodified endogenous form. Therefore, when using an antibody against that protein you should see two bands in the input and unbound lanes (unless you are using a knockout or knock-in line), but only one (the heavier) in the pull-down lane. This SDS-PAGE is to validate the affinity capture and estimate the amount of beads to use per mg amount of nuclear extract (by comparing input and unbound).
9. You should have one line with BirA plus the bio-tagged protein of interest and at least one control line: BirA only or bio-tagged protein only. Try to avoid using parental lines without either BirA or the bio-tagged protein of interest.
10. Alternatively, one 245 mm × 245 mm square dish can be used in place of three to four 15 cm dishes.
11. It should take 2–3 days. Change medium every other day, or daily if it becomes yellow.
12. You should have around 5 mL of pelleted cells.
13. If a dounce homogenizer is not available, you can use a syringe with a 16G1½ or a 20G1½ needle.
14. The cytosolic extract can be kept in case the protein of interest is cytosolic.
15. Alternatively, you can aliquot in several Eppendorf tubes and spin in a microcentrifuge at maximum speed for at least 10 min.
16. The more Buffer C you use, the more protein you will extract and the more Eppendorf tubes you will have to handle later on. It will also depend on how many cells you start with.
17. As in **step 4** above, the homogenizer is prechilled on ice and pre-rinsed with Buffer C.
18. We do not supplement the dialysis buffer with protease inhibitor cocktail.
19. This step will pellet most, but not all proteins precipitated during the dialysis.
20. We generally use 225 U of Benzonase per five 15 cm dishes worth of cells.
21. The optimal amount of beads should have been estimated in the pilot scale experiment. Using too many beads will increase the background; therefore, in case it is impossible to estimate the right amount of beads, use 200 μL of beads.
22. For the least concentrated NE, use everything except a few hundred μL (you will need these aliquots for SDS-PAGE later). For the more concentrated NE, pipette accordingly. It is not recommended to incubate for longer than 5 h with the beads to avoid nonspecific binding.

23. We keep the unbound fraction in case something is wrong and we need to repeat the capture. The small aliquot is for SDS-PAGE and western blot.
24. Use a 1,000 mL tip from which you cut off the bottom to make a bigger aperture to resuspend the beads up and down, and transfer into a microcentrifuge tube (set the pipet to 150 μL). Do not discard the tip. With another pipet add 200 μL of IP Buffer DNP to the original 15 mL conical tube (make sure you wash down the leftover beads form the bottom wall of the conical tube). Collect the leftover beads with the original bottom-cut 1,000 μL tip and transfer into the microcentrifuge tube. Repeat the 200 μL wash and harvest at least one more time to completely collect the beads from the conical tube and the 1,000 μL pipet tip.
25. Elute with one volume of Laemmli sample buffer per volume of beads. Therefore, if you originally used 200 μL of beads, elute with 200 μL of SDS-LB.
26. The eluates can be frozen at this time at –20 or at –80 °C for later use.
27. You may need to repeat the 30 min 4,000 × *g* centrifuge step more than once to reduce the volume to 50–100 μL.
28. For simplicity, the BirA only line can be used as negative control here as well.
29. Do not use the FLAG peptide to elute 3XFLAG tagged proteins and vice versa otherwise the elution process will not work.
30. It is not necessary to use a gel loading tip and to remove the supernatant completely.
31. At the end of the last elution step, use a gel loading tip to collect the supernatant completely.
32. Any wild type mESC line can be used for this purification. In this case five to ten 15 cm dishes worth of cells are enough for each antibody IP. To make nuclear extract, use 3 mL of Buffer C.
33. Make sure you have at least 800–1,000 μL volume in each Eppendorf tube. Protein G agarose beads can be replaced with Protein A beads, according to the antibody used.
34. Alternatively, incubate the nuclear extract with the antibody first, for at least 6 h to a maximum of 12–14 h. Then, centrifuge the extract at maximum speed for about 30 min in the cold to remove precipitates, and transfer into a new tube. After that, capture the immune-complexes with 150–200 μL pre-equilibrated protein G beads for 2 h at 4 °C.
35. It is fundamental to keep a very clean working environment to reduce background later on during the mass spectrometry analysis.

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