Tandem Affinity Purification of Protein Complexes in Mouse Embryonic Stem Cells Using In Vivo Biotinylation

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ABSTRACT

In dissecting the pluripotent state in mouse embryonic stem (ES) cells, we have employed in vivo biotinylation of critical transcription factors for streptavidin affinity purification of protein complexes and constructed a protein-protein interaction network. This has facilitated discovery of novel pluripotency factors and a better understanding of stem cell pluripotency. Here we describe detailed procedures for in vivo biotinylation system setup in mouse ES cells, and affinity purification of multi-protein complexes using in vivo biotinylation. In addition, we present a protocol employing SDS-PAGE fractionation to reduce sample complexity prior to submission for mass spectrometry (MS) protein identification. *Curr. Protoc. Stem Cell Biol.* 8:1B.5.1-1B.5.17. © 2009 by John Wiley & Sons, Inc.

Keywords: tandem affinity purification • in vivo biotinylation • protein-protein interaction • embryonic stem cells

INTRODUCTION

Vital cellular functions require the coordinated action of a large number of proteins that assemble into an array of multi-protein complexes of distinct composition and structure. The analysis of protein complexes and intricate protein-protein interaction networks is key to understanding complex biological systems including stem cell pluripotency. Proteins and other macromolecules of interest can be purified from crude extracts or other complex mixtures by a variety of methods. Affinity purification makes use of specific binding interactions between molecules and generally involves the following steps. First, incubate crude sample with the immobilized ligand support material to allow the target molecule in the sample to bind to the immobilized ligand. Second, wash away nonbound sample components from solid support. Third, elute (dissociate and recover) the target molecule together with its associated proteins from the immobilized ligand by altering the buffer conditions so that the binding interaction weakens or no longer occurs. Prominent among affinity purification strategies is tandem affinity purification involving two different affinity tags. The FLAG peptides DYKDDDDK and MDYKDDDDK are widely used affinity tags (Chubet and Brizzard, 1996) that can be placed at either the amino-terminus, carboxy-terminus, or in association with other tags such as the biotinylation peptide tag (see Background information).

The protocols in this unit are based on our earlier studies using in vivo biotinylation to perform affinity purification of pluripotency factors and construct a pluripotency network in mouse ES cells (Wang et al., 2006). The general strategy is summarized in Figure 1B.5.1 and Figure 1B.5.2. This unit begins with a method to establish an in vivo biotinylation system in mouse ES cells (see Basic Protocol 1), followed by a detailed protocol to perform tandem affinity purification of the biotinylated protein together with its associated protein complexes (see Basic Protocol 2). Finally, a detailed protocol for fractionation

Published online March 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/9780470151808.sc01b05s8

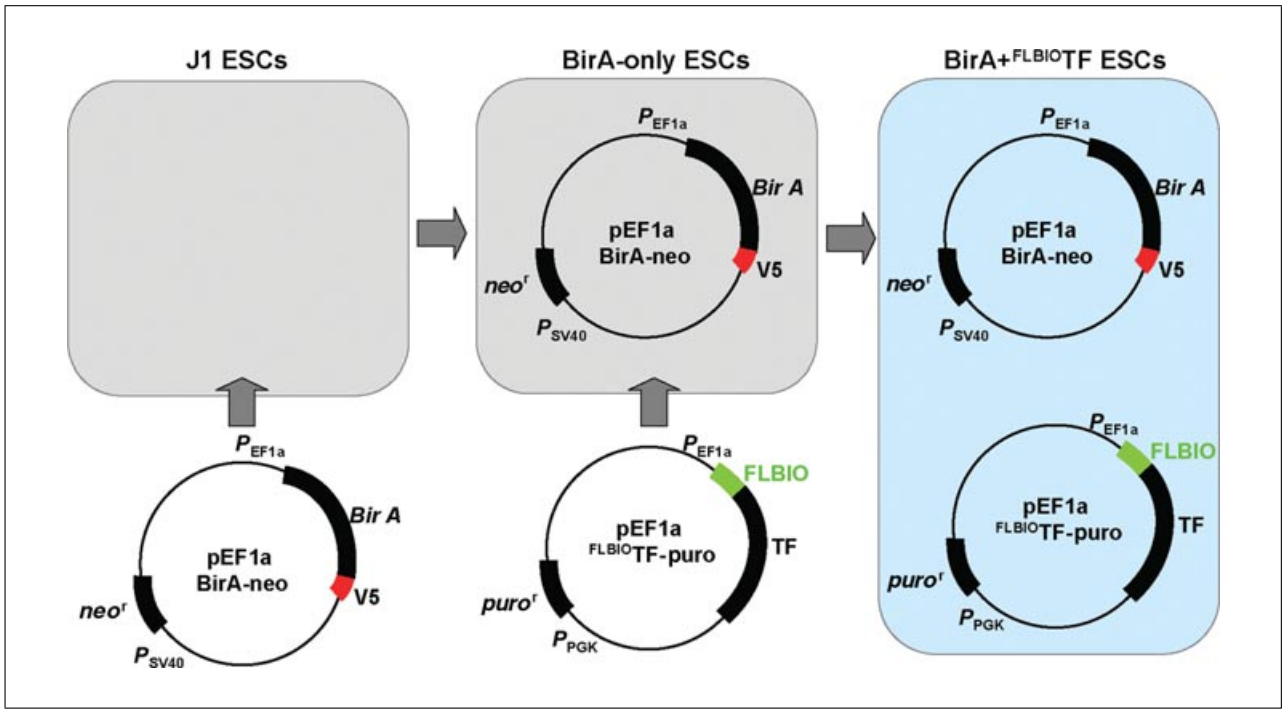

Figure 1B.5.1 Establishment of a biotinylation system in J1 ESCs. A stable ESC line expressing the bacterial BirA enzyme was first established by transfection with a BirA-expressing plasmid bearing the neomycin resistance (*neo*^{r}) gene and G418 selection. A second plasmid containing cDNA encoding a transcription factor (TF) of interest with an N-terminal Flag-biotin dual tag (FLBIO) and a puromycin resistance (*puro*^{r}) gene was introduced, and cells were selected with puromycin. The resulting stable line is resistant to both G418 and puromycin, and expresses FLAG-tagged, biotinylated TF (^{FLBIO}TF) that can be immunoprecipitated by anti-FLAG and streptavidin antibodies/beads.

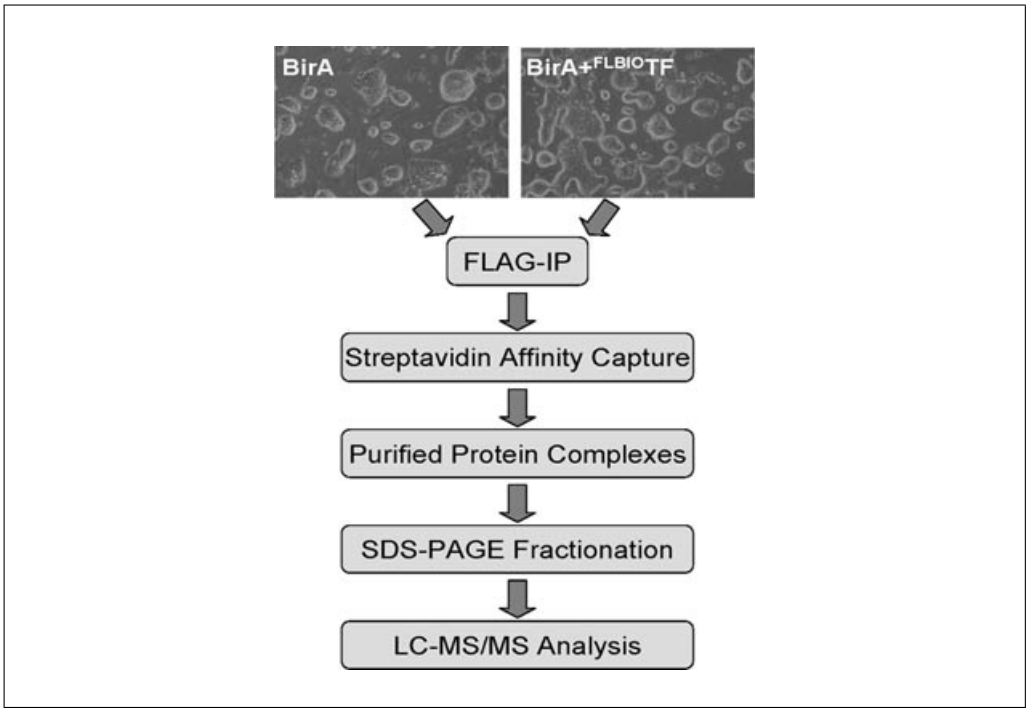

Figure 1B.5.2 A summary of the procedure for tandem affinity purification of multiprotein complexes in mouse ESCs. Following establishment of BirA-only and BirA+^{FLBIO}TF-expressing ES cell lines, immunoprecipitation is performed using anti-FLAG M2 agarose (FLAG-IP). The bound material is eluted with FLAG peptide and further purified by streptavidin affinity capture. The purified protein complexes are fractionated on SDS-PAGE, and subjected to LC-MS/MS to identify components of the protein complexes.

of purified protein complexes (to increase sample purity and reduce sample complexity) for downstream mass spectrometry analysis is presented (see Support Protocol).

NOTE: All ES cell cultures should be maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

NOTE: For tandem affinity purification, all reagents and solutions should be kept on ice unless otherwise specified.

ESTABLISHMENT OF ES CELL LINES EXPRESSING BirA AND SUB-ENDOGENOUS BIOTINYLATED PROTEINS

BASIC PROTOCOL 1

Two cell lines are generated in this protocol: BirA-only expressing ES cells are established first to serve as control cells for background signals during affinity purification of protein complexes. This cell line also serves as the recipient cells for subsequent introduction of genes dually tagged with Flag and biotin tags (FLBIO). The establishment of BirA-only ES cell lines first (using G418 selection) allows for future introduction of different FLBIO-tagged genes (using puromycin selection) and thus establishing multiple FLBIO-tagged cell lines in the presence of same amount of BirA expression. Nuclear extracts are prepared simultaneously from BirA-expressing cells with and without tagged genes of interest, and affinity purification is processed simultaneously (Basic Protocol 2).

Materials

J1 ES cells (ATCC, cat. no. SCRC-1010)
ES medium (see recipe)
IEF medium (see recipe)
0.05% (w/v) trypsin (Mediatech, cat. no. 25-052-CI)
0.25% (w/v) trypsin (Mediatech, cat. no. 25-053-CI)
Phosphate-buffered saline (PBS; Sigma, cat. no. D8537)
pEF1αBirAV5-neo plasmid (see Fig. 1B.5.1; available from the author upon request)
TE buffer (see recipe)
300 μg/ml G418 (from 300 mg/ml stock; see recipe)
2× freezing medium (see recipe)
RIPA buffer (Boston BioProducts, cat. no. BP-115)
anti-V5-HRP (Invitrogen, cat. no. 46-0708)
pEF1αFlagbiotin (FLBIO)-puro plasmid (see Fig. 1B.5.1; available from the author upon request) Puromycin (see recipe)
Streptavidin-HRP (Amersham, cat. no. RPN1231)

6-well, 24-well, 48-well, and 10-cm IEF plates (see recipe)
15-ml conical tubes (Corning, cat. no. 430791)
0.4-cm gap cuvette for electroporator (Bio-Rad, cat. no. 165-2008)
Gene Pulsor II (electroporation; Bio-Rad)
37°C, 5% CO_2 incubator
U-bottom 96-well plate
200-μl pipettor
Multi-channel pipettor (e.g., 12-channel pipettor)
Parafilm
Gelatin-coated cell culture plates (see recipe)

Establish cultures

1. Using standard cell culture procedures, thaw J1 ES cells (or ES cells of your choice) and expand to ~70% confluence in a well of a 6-well IEF plate containing 5 ml ES medium per well.

   It can take a few days to a week to expand the cells depending on the starting cell number.

2. One the day prior to electroporation, prepare a 10-cm IEF plate with 10 ml IEF medium (for use in step 10 to plate the transformed cells).

Mouse embryonic feeder cells (MEFs) are used in the culture of mouse ES cells. They provide both a substrate for the ES cells to grow on and secrete many factors necessary for ES cells to maintain pluripotency. Feeders are MEFs that have been mitotically inactivated by treatment with mitomycin C or by γ-irradiation (Conner, 2000; UNIT 1C.3). A unique quad-resistant DR4 feeder cell line can be purchased from Open Biosystems (cat. no. MES3948), which has been mitotically inactivated by treatment with mitomycin C. Alternatively, we also isolated primary embryonic fibroblast from DR4 mouse embryos, expanded them and inactivated them by γ-irradiation. These will be referred to as irradiated embryonic feeders (IEF) in this unit.

Prepare cells for electroporation

3. To harvest cells by trypsinization, aspirate ES medium and rinse the cells once with 0.05% trypsin. Then, add a sufficient amount of 0.25% trypsin to cover the ES cells and incubate 3 to 5 min at 37°C.

The ES cells should become detached from the vessels; if not, increase incubation time.

4. Collect cells by adding 3 vol ES cell medium to neutralize the trypsin and pipetting up and down to mix. Transfer to 15-ml conical centrifuge tubes.

5. Centrifuge 5 min at 200 × *g*, 4°C.

6. Wash harvested cells twice, each time with 5 to 10 ml PBS.

7. Count cells and then resuspend ES cells at 1.3×10^7/ml in PBS.

Electroporate cells to produce BirA cells

8. Add 20 to 30 μg of pEF1αBirAV5-neo DNA in no more than 50 μl TE buffer to make final total 0.75 ml of cell suspension (or ~10^7cells) for each electroporation.

Note that linearization of the plasmid prior to electroporation is not necessary.

9. To perform electroporation for J1 ES cells, use a 0.4-cm gap cuvette, 25 μF, 450 V, with the time constant for each electroporation reading around 0.6 to 0.8 msec. Incubate 5 min on ice.

Different ES cell lines may require different electroporation conditions.

Plate the cells and select transformed cells

10. Mix the electroporated cells with enough ES medium to bring the final cell suspension volume to 10 ml. Remove the medium from the 10-cm IEF plate prepared a day before and add the electroporated cell suspension. Rock the plates gently to mix and then incubate at 37°C, 5% CO_2.

11. On the second day (24 hr after initial plating), add 300 μg/ml (final) G418 drug directly to the ES medium on the cells. Rock the plate gently to mix drug completely with the medium and return to the incubator.

12. Feed the cells with fresh G418 drug and ES medium each day for the next 7 to 9 days. Swirl the plate gently to resuspend and remove the dead cells and debris by aspiration. Replace with 10 ml freshly made drug-containing ES medium mix to the plate.

After 3 or 4 days of this treatment, most of the ES cells will appear dead. By day 5 or 6, clones should start appearing and should be ready for picking by day 8 or 9.

Pick clones

13. A day prior to picking, prepare a 48-well IEF plate with 500 μl ES medium per well (for use in step 17) and a U-bottom 96-well plate containing 50 μl of 0.05% trypsin per well (for use in step 15).

It is wise to set up the 48-well plate and 96-well plate with matching wells for convenient multi-channel pipettor transfer.

14. On picking day, wash the 10-cm plate with 10 ml PBS. Replace the wash with 5 ml fresh PBS to protect the cells from drying out during picking.

15. Pick individual colonies with a 200-µl pipettor set at 10 µl and transfer each colony into a well of the U-bottom 96-well plate containing 50 µl of 0.05% trypsin.

16. After picking 24 to 48 clones or 30 min (whichever is first), incubate the 96-well plate 10 min at 37°C.

17. Using a multi-channel pipettor, transfer 150 µl ES medium from the 48-well IEF plate to the 96-well trypsin plate containing the colonies. Pipet up and down to mix and then transfer the entire cell suspension back to the 48-well feeder plate. Culture the cells in the 48-well plate overnight.

18. Replace old medium with 500 µl fresh ES medium without G418 every day.

G418 (300 µg/ml) may be added but is not necessary during the first few days.

Freeze cells

19. When the majority (>70%) of the wells are ready (i.e., cells reach near or over 70% confluency), wash with 500 µl PBS, add 35 µl of 0.05% trypsin, and incubate 10 min at 37°C. Meanwhile, prepare a 96-well plate containing 65 µl cold 2× freezing medium.

It is wise to set up the 96-well plate to match the 48-well plate (from step 17) for convenient multi-channel pipettor transfer.

20. Using a 12-channel pipettor, add 65 µl ES medium to each well of the 48-well plate, mix by pipetting up and down to neutralize the trypsin, and transfer 65 µl to the U-bottom 96-well plate containing 65 µl cold 2× freezing medium. Wrap the plate in Parafilm and store at −80°C for use in step 24.

21. Add 200 µl ES medium to the remaining cells in the IEF plate, and return the plate to the incubator. Replace old medium with fresh ES medium daily for 3 to 4 days.

Analyze clones

22. When the medium in most wells is yellow or cells are near confluence, aspirate medium and add 200 µl RIPA buffer to each well to make total lysate. Carry out SDS-PAGE and a standard western blot analysis with the lysate (20 µg) using anti-V5-HRP (1:5000 dilution is recommended).

Since BirA is V5-tagged, you should expect to see a band near 35 Kda in the positive clones (see Fig. 1B.5.3A). It is also recommended to make total lysate from the parental ES cells (e.g., J1) for use in western blotting as a negative control.

Expand positive clones

23. Prepare a 24-well IEF plate with 1 ml ES medium per well a day prior to step 24.

24. Thaw the positive clones (as determined by western blotting in step 22) by adding 100 µl of warm ES medium to the frozen wells (from step 20). Mix and transfer the thawed cell suspension to 24-well IEF plate prepared a day before (see step 23), repeat until all the remaining cells are thawed and transferred. Return to incubator and culture overnight.

25. On the second day, replace old medium with fresh ES medium containing 300 µg/ml G418. Let clones grow in ES cell selection medium (ES medium with 300 µg/ml G418) until they are 70% confluent. This takes ∼1 week.

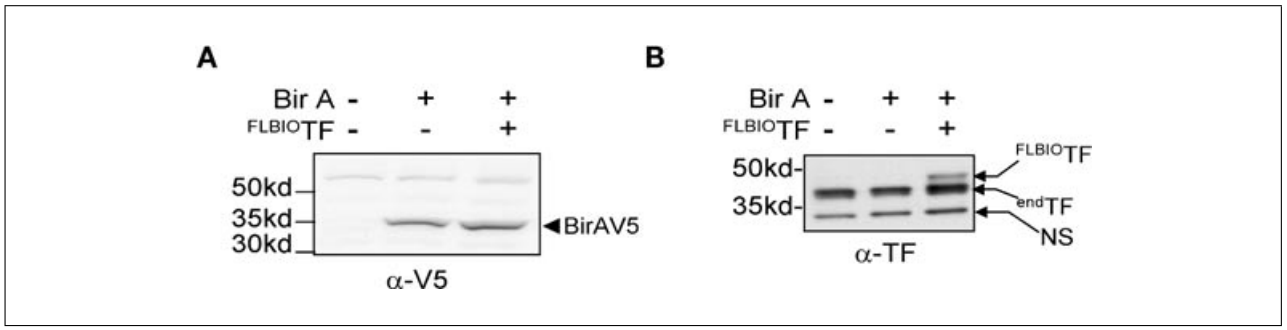

Figure 1B.5.3 Examples of western blot analyses. (**A**) A western blot with anti-V5-HRP to detect BirAV5 expression. (**B**) A western blot using native antibody against the TF of interest to detect both endogenous and biotinylated TF proteins. The sub-endogenous level of the biotinylated transcription factor (^{FLBIO}TF) and the expression level of the endogenous protein (^{end}TF) are indicated. NS denotes nonspecific signals.

26. Freeze down 60% of the cells as frozen stocks and grow the remaining 40% in 10-cm IEF plates until 70% confluent for use in step 27.

    Alternatively, all the cells can be frozen and stored at −80°C (short-term) or in liquid nitrogen (long-term) for later use.

Establish FLBIO(gene) cell line

27. Repeat steps 2 to 26 to establish ES cell lines expressing BirA and biotinylated proteins of interest at sub-endogenous levels.

28. Electroporate BirA-containing cells (from step 26) with pEF1αFlagbio-tagged plasmid containing a specific gene of interest and use ES medium containing 300 μg/ml G418 and 1 to 2 μg/ml puromycin to select for cells expressing both pEF1αBirAV5-neo and pEF1αFLBIO(gene)-puro plasmids (see Fig. 1B.5.1).

    Bacterial BirA will catalyze biotinylation of the FLBIO(gene), which has a biotinylation site.

29. Perform western analysis using a streptavidin-HRP conjugate (to detect the biotinylated protein) and a native antibody against the protein of interest if available (to detect both the biotinylated and endogenous versions of the protein; see an example in Fig. 1B.5.3B); thaw and expand the positive clones to make frozen cell stocks.

    Milk must not be used during incubation with streptavidin-HRP antibody (see Commentary).

30. Adapt the two cell lines established thus far to grow on gelatin-coated culture vessels without feeders by serially passaging them in complete ES medium. Freeze down gelatin-adapted cell stocks.

    We found that J1 ES cells could be efficiently adapted to grow on gelatin-coated culture vessels in the presence of LIF after three passages. Alternatively, it is advantageous to start with previously established, feeder-independent ES cells (e.g., E14 line) in the beginning.

BASIC PROTOCOL 2

TANDEM AFFINITY PURIFICATION OF PROTEIN COMPLEXES

Individual proteins often participate in the formation of a variety of different protein complexes that are a cornerstone of many biological processes. This protocol details large-scale expansion of mouse ES cells and nuclear extract preparation from the bulk culture for use in tandem affinity purification. This purification may require 20 to 40 culture dishes (15-cm) of ES cells grown to near 90% confluence, which corresponds to the yield of 50 to 100 mg of nuclear extract. It is advisable to adapt the two cell lines established in Basic Protocol 1 to grow in a feeder-independent condition (on gelatin) to minimize the cross-contamination by feeder cells and cost of the experiment.

Materials

Gelatin-coated ES cell culture dishes (see recipe)
BirA-only and BirA$^+$ Flagbiotagging ES cells (established in Basic Protocol 1)
ES medium (see recipe)
0.05% (w/v) trypsin (Mediatech, cat. no. 25-052-CI)
0.25% (w/v) trypsin (Mediatech, cat. no. 25-053-CI)
Phosphate-buffered saline (PBS; Sigma, cat. no. D8537)
Nuclear extract buffer A (see recipe)
Protease inhibitor cocktail (Sigma Mammalian Protease Inhibitor cocktail)
Trypan blue (Invitrogen, cat. no. 15250-061)
Nuclear extract buffer B (see recipe)
Bradford assay: Protein concentration Bio-Rad Dye kit (Bio-Rad, cat. no. 500-0006)
IP350 buffer with different NP40 concentrations (see recipes)
Protein G–agarose (Roche, cat. no. 11-243-233001)
FLAG M2-agarose beads (Sigma, cat. no. A2220-5ML)
FLAG peptide (Sigma, F-3290)
Streptavidin-agarose beads (Invitrogen, cat. no. 15942-050)
2× SDS sample buffer (see recipe)

250-ml conical plastic bottles (Corning, cat. no. 430776)
Centrifuge with a JS 4.2 rotor or equivalent
50-ml conical tubes (Corning, cat. no. 430829)
Glass Dounce homogenizer (40-ml size) with type B pestle (Wheaton, cat. no. 432-1273)
Drawn-out glass Pasteur pipet
Glass Dounce homogenizer (15-ml size) with type B pestle (Wheaton, cat. no. 432-1272)
NALGENE high-speed centrifuge tube (cat. no. 3114-0050)
Rotating wheel (Scientific Equipment Products, cat no. 60448)
15-ml conical tubes (Corning, cat. no. 430791)
1.5- and 2-ml screw-cap tube

Additional reagents and equipment for counting cells using a hemacytometer (Phelan, 2006), determining cell viability using trypan blue staining (Strober, 1997), and determining protein concentration using the Bradford assay (Siu et al., 2008)

Expand the cell lines

1. Culture ES cells as follows:

   a. Begin with a 10-cm gelatin-coated dish containing 1×10^6 cells/cm^2 in 10 ml ES medium for both control BirA and biotinylated protein (from step 30 in Basic Protocol 1). Incubate dishes.

   b. When cells reach 90% confluence, trypsinize and split at a 1:1 ratio into a 15-cm gelatin-coated dish. Incubate dishes.

   c. When these cells reach 90% confluence, trypsinize and split the 15-cm dishes at a 1:4 or 1:5 ratio into fresh 15-cm gelatin-coated dishes. Incubate dishes.

   d. Expand cell culture until you have 20 to 40 15-cm gelatin-coated dishes of cells for each cell line.

      Drug selection is needed only during initial culture and can be omitted during the scale-up culture to reduce costs. Optimal starting material has not been determined, however, you want to have enough cells to make 50 to 100 mg of nuclear extracts (see below).

2. Grow cells until they reach 80% to 90% confluency with constant medium renewal.

   Ideally, you want to renew the medium on a daily basis; however, you can renew the medium every other day before cells reach 70% confluence to reduce costs. When medium turns very yellow within a day after medium renewal, cells have likely reached near 80% to 90% confluence, and you should go directly to step 3.

Harvest the cells

3. To trypsinize cells, rinse each dish with 5 ml of 0.05% trypsin, then add 7 ml of 0.25% trypsin per dish and incubate the plate 5 min at 37°C.

   Process the dishes ten dishes at a time.

4. Add 10 ml of fresh ES medium to the cells to neutralize the trypsin, resuspend well, and transfer cells to a 250-ml conical plastic bottle. Rinse the dishes with an additional 10 ml of ES medium, collect the residual cells, and combine with the cells in the bottle.

   RPMI or low glucose DMEM with 10% FBS can be used instead of ES cell culture medium to neutralize trypsin. LIF is not required in the neutralization medium.

5. Centrifuge the cells in 250-ml conical plastic bottles 15 min at 2400 × *g* (using a JS 4.2 rotor or equivalent), 4°C.

6. Carefully decant the supernatant, resuspend the cell pellet in 50 ml of ice-cold PBS, and transfer to 50-ml conical tubes. Count cell numbers using a hemacytometer (Phelan, 2006).

   Pool pellets if multiple tubes are used for harvesting the same samples (BirA versus BirA+^{FLBIO}TF).

7. Centrifuge the 50-ml conical tubes 10 min at 2400 × *g*, 4°C in a JS 4.2 rotor.

8. Remove the supernatant carefully. Estimate the packed cell volume (PCV). Resuspend in ∼5 PCV volume of ice-cold PBS. Centrifuge again 10 min at 2400 × *g*, 4°C in a JS 4.2 rotor.

Isolate the nuclei

9. Remove the supernatant, and rapidly resuspend in ∼5 PCV of ice-cold nuclear extract buffer A with freshly added DTT (1 mM final), PMSF (0.2 mM final), and 1:1000 protease inhibitor cocktail.

10. Centrifuge 5 min at 2400 × *g*, 4°C in a JS 4.2 rotor.

11. Aspirate the supernatant carefully. Add ∼3 PCV of ice-cold nuclear extract buffer A and additives described in step 9 (except use 1:100 protease inhibitor cocktail). Incubate on ice for 10 min to swell cells.

12. Transfer by pouring into a glass Dounce homogenizer (40-ml size) with type B pestle prechilled on ice and prerinsed with nuclear extract buffer A. Homogenize up and down 10 times, slowly.

    Check for cell lysis by examination of small aliquot with trypan blue stain (Strober, 1997) under microscope (>80% of cells should be lysed).

13. Centrifuge 15 min at 4300 × *g*, 4°C using a JS 4.2 rotor.

Extract the nuclei

14. Carefully remove and discard the supernatant using a drawn-out glass Pasteur pipet. Add to the nuclei pellet 3 ml/1 × 10^9 starting number of cells of ice-cold nuclear extract buffer B containing freshly added DTT (1 mM final), PMSF (0.2 mM final), and protease inhibitor cocktail (1:1000).

15. Dislodge the pellet and transfer to a glass Dounce homogenizer (15-ml size) with type B pestle, prechilled and rinsed with nuclear extract buffer B. Homogenize up and down 10 times slowly to resuspend the pellet.

16. Transfer the homogenate to a NALGENE centrifuge tube, prerinsed with nuclear extract buffer B and rotate for 30 min on a rotating wheel in the cold room (4°C).

17. Centrifuge 30 min at 25,000 × *g*, 4°C using a JA-25.50 rotor to remove insoluble material. Meanwhile, prepare for the Bradford assay according to manufacturer's instructions.

18. Carefully transfer the supernatant (contains nuclear extract) to 50-ml conical tubes.

Determine protein concentration

19. Determine the protein concentration of an aliquot of each nuclear extract (NE) using the Bradford assay (Siu et al., 2008; e.g., the Bio-Rad Dye kit).

Preclear nuclear extract

20. Use equal amounts (~100 mg) of NE from the BirA (control) sample and BirA + biotinylated protein sample. Add an appropriate amount of cold IP350 buffer (0.3% or 0.5% v/v NP-40) to each sample so that the final NE concentration of each sample is ~2 mg/ml containing ~0.2% NP-40.

    Addition of DTT/PMSF/protease inhibitor cocktail to the IP350 buffer is preferred but not necessary.

21. Preclear supernatant with Protein G–agarose (100 µl of 50% slurry per 10 mg protein) for 1 to 2 hr at 4°C with continuous mixing.

22. Centrifuge samples 5 min at 300 × *g*, 4°C. Transfer precleared supernatant to new 50-ml conical tubes.

Affinity purify FLAG-tagged proteins

23. Equilibrate FLAG M2 agarose beads (100 µl of 50% slurry per 10 mg protein) in two 50-ml tubes containing 15 ml cold IP350/0.3% NP-40 buffer. Rotate in cold room for 5 min and centrifuge 4 min at 300 × *g*, 4°C. Aspirate. Repeat this once.

24. Carefully transfer the precleared nuclear extract prepared in step 21 to pre-equilibrated FLAG M2 resin from step 23. Divide into more 50-ml tubes if necessary to ensure complete mixing during immunoprecipitation. Place on end-over-end rotating wheel at 4°C overnight.

25. Centrifuge tubes from overnight incubation 4 min at 300 × *g*, 4°C. Remove supernatant (nonbound material).

    Make sure no obvious protein precipitate (whitish clumps) is formed after overnight incubation.

26. Wash FLAG-agarose beads four times, each time with 20 ml of ice-cold IP350/0.3% NP-40 buffer. Place on rotating wheel at 4°C for 15 min per wash. Centrifuge 4 min at 300 × *g*, 4°C between washes.

27. After the final wash, remove most of the supernatant. Use IP350/0.3% NP-40 buffer to transfer beads to a 15-ml tube. Pool beads, if multiple tubes were used for the incubation and washes from previous steps. Centrifuge 4 min at 300 × *g*, 4°C to pellet beads. Remove the supernatant carefully.

28. Elute beads four times, each time with 10 ml of IP350 with 0.1 mg/ml FLAG peptide. For each elution, place sample tubes on rotating wheel for 1 to 1.5 hr at 4°C. Centrifuge 4 min at 300 × *g*, 4°C, and carefully transfer the supernatant (contains

eluted proteins) to a new 50-ml conical tube. Pool the eluates for each sample for a total 40 ml of each sample. Discard beads.

Affinity purify biotin-labeled protein

29. Prepare streptavidin agarose, as described for FLAG M2 agarose in step 23.

30. Add the FLAG-eluate (40 ml each) from step 28 into equilibrated streptavidin–agarose and place on end-over-end rotating wheel at 4°C overnight.

31. Centrifuge tubes from overnight incubation 4 min at 300 × *g*, 4°C. Remove the supernatant (nonbound material).

    Make sure no obvious protein precipitate (whitish clumps) is formed after overnight incubation.

32. Wash streptavidin-agarose beads four times, each time with 20 ml of ice-cold IP350/0.3% NP-40 buffer. Place on rotating wheel for 15 min at 4°C per wash. Centrifuge as described in step 31.

33. After the final wash, remove most of the supernatant. Use a cut-off pipet tip and remaining IP350 buffer to transfer beads into a 1.5-ml screw-cap tube. Pool beads, if multiple tubes were used for the incubation and washes. Wash out original tubes and pipet tips with IP350 buffer and pool with sample. Centrifuge using tabletop microcentrifuge 2 min at 300 × *g*, 4°C to pellet beads.

34. Remove as much supernatant as possible from the beads using a drawn-out Pasteur pipet. Add 500 µl of 2× SDS sample buffer. Vortex gently and heat at 95° to 100°C for 5 min and vortex again. Allow to cool to room temperature.

35. Centrifuge 1 min at maximum speed, room temperature, to repellet beads. Carefully transfer supernatant to a new 1.5-ml screw-cap tube. Add 500 µl of 1× SDS sample buffer to residual beads and vortex gently. Recentrifuge 1 min at maximum speed, room temperature. Pool the sample with the first eluate in a 1.5-ml screw-cap tube.

36. Repeat washing of beads one more time with 400 µl of 1× SDS sample buffer and combine with samples (should now have 1.4 ml total).

    Repeated washing of the beads after boiling (and combining the supernatants) improves the yield of proteins.

37. Centrifuge 2 min at maximum speed, room temperature (to pellet any residual agarose beads that were carried over). Carefully transfer sample into new 2-ml screw-cap tubes.

    Carryover of residual agarose beads will block the Centricon filter (see Support Protocol). Samples can be frozen and stored at −80°C for future use.

SUPPORT PROTOCOL

SDS-PAGE FRACTIONATION OF PROTEIN COMPLEXES

This protocol details the concentration of the protein eluates and fractionation of the purified protein complexes on a SDS-PAGE gel to reduce the complexity of samples for downstream mass spectrometry analysis.

Materials

Affinity-purified complexes with biotinylated FLAG-tagged protein (Basic Protocol 2)
30% Acrylamide/Bis solution (37.5:1) (Bio-Rad, cat. no. 161-0158)
Colloidal Coomassie stain (Invitrogen, cat. no. 46-7015 and 46-7016)
HPLC-grade water (American Bioanalytical)

YM-10 Centricon (10,000 MWCO; Amicon Bioseparations, cat. no. 4205)
37°C water bath
Avanti J25 centrifuge using a JA-25.50 fixed-angle rotor
1.5-ml microcentrifuge tubes
Bio-Rad Protean II xi basic unit with casting stand (Bio-Rad, cat. no. 165-1834)
Scalpel or razor blade

Additional reagents and equipment for preparing a large denaturing polyacrylamide gel (Gallagher, 2006)

1. Transfer all eluate (from step 37, Basic Protocol 2) into the chamber of a YM-10 Centricon (10,000 MWCO) device. If just thawed from the freezer, warm samples in a 37°C water bath for 5 min to dissolve SDS.

   Undissolved SDS will block the Centricon filter.

2. Centrifuge in the Avanti J25 centrifuge using a JA-25.50 fixed-angle rotor ∼2 to 3 hr (until as much filtrate runs through the chamber as possible) at 5000 × *g*, 25°C .

   After centrifugation there should be ∼100 μl of concentrated material.

   This centrifugation step needs to be performed at room temperature to avoid precipitation of the SDS in the samples.

3. Remove and discard filtrate chamber first. Attach collection vial and invert quickly. Re-centrifuge inverted chamber (with collection vial) 2 min at 800 × *g*, 25°C. Transfer to a fresh 1.5-ml microcentrifuge tube.

4. Prepare a large denaturing polyacrylamide gel: Use Bio-Rad Protean II xi basic unit with casting stand. Prepare gel with fresh APS and TEMED. Pour lower running gel (10%) first and seal with ethanol or butanol for polymerization**.** Next, pour upper stacking gel (4%) with comb positioned in an angle, and then reposition the comb horizontally to avoid air bubbles in the comb wells. Pull out the bottom gel blocker carefully before use (Gallagher, 2006).

5. Load samples from step 3 and run the gel for ∼1 hr at 120V in stacking gel and run samples till the bromphenol blue dye reaches ∼2.5 cm into the separating (lower) gel (it takes ∼1 hr under 120V).

   Fill in empty wells with the same volume of 1× protein sample buffer and a similar salt concentration to prevent "smiling" effect from sample wells. Also, protein ladder can be loaded on left and right sides of the gel; the ladders help to cut out gel slices with an expected size range of proteins for mass spectrometry (see step 8).

6. Disassemble gel apparatus and stain gel overnight with Colloidal Coomassie stain.

7. Destain gel with multiple rinses of HPLC-grade water. Destain until background is very clear (can take 1 to 2 days) on rocker platform at room temperature.

8. Cut out the whole lane and separate into four to eight slices for mass spectrometry analysis. Using a scalpel or razor blade, cut into ∼1-mm cubes and transfer to clear 1.5-ml microcentrifuge tubes. Add a small amount of HPLC-grade water so that gel slices do not dry out.

   Do not spin tubes to avoid samples leaching out of gels. Cut-out gel slices can be stored at 4°C (or −20°C for prolonged storage).

9. Send samples to a MS facility of your choice to perform liquid chromatography coupled with tandem MS (LC-MS/MS) for protein identification.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

ES medium

Dulbecco's modified Eagle's medium (DMEM)
15% (v/v) fetal bovine serum (FBS; Hyclone, cat. no. SH30071.03)
0.1 mM 2-mercaptoethanol
2 mM L-glutamine
0.1 mM non-essential amino acid
1% (v/v) nucleoside mix (100× stock, Sigma)
1000 U/ml recombinant leukemia inhibitory factor (LIF; Chemicon)
50 U/ml penicillin/50 μg/ml streptomycin (Invitrogen, cat. no. 15070-063)
Store up to 1 month at 4°C

Each lot of fetal bovine serum needs to be prescreened for the ability to support optimal ES cell growth.

Freezing medium, 2×

20% (v/v) dimethyl sulfoxide (DMSO)
80% (v/v) fetal bovine serum (FBS; Hyclone, cat. no. SH30071.03)
Store up to 1 month at 4°C

G418

Also known as geneticin, G418 (Invitrogen, cat. no., 11811) is a broad-spectrum antibiotic that will select mammalian cells expressing the neomycin protein (encoded by the neomycin gene). Make a 300 mg/ml stock solution in PBS. Store up to 6 months at −20°C.

Gelatin, 0.1%

Dissolve 5 g of gelatin (Bacto; Difco, cat. no., 0143-15-1) in 500 ml distilled water and autoclave (1% stock). Store the solution at room temperature indefinitely. Before use, dilute 1:10 (to make 0.1% working solution) with sterile dH_2O and filter through a 0.45-μm filter apparatus.

Gelatin-coated tissue culture plates

Add a sufficient amount of 0.1% gelatin (see recipe) to each well of the culture plates, let stand for 20 min to 1 hr. Aspirate the gelatin solution and air dry the plates. Store the gelatin-coated plates at room temperature indefinitely.

IEF medium

86% (v/v) DMEM (high glucose)
10% fetal bovine serum (FBS; heat inactivated)
2% (v/v) penicillin/streptomycin 5000 U penicillin/5000 μg/ml streptomycin; Invitrogen, cat. no. 15070-063
1% of 200 mM L-glutamine
1% of 100 mM sodium pyruvate (Invitrogen, cat. no. 11360-070)
Store up to 1 month at 4°C

IMPORTANT NOTE: *It is extremely important that the sodium pyruvate is fresh. Using an expired lot will decrease your yields by 50% or more.*

IEF plates

Normally, 3×10^6 IEF cells are frozen down as 1× stock. When thawed, these cells are used to seed one full gelatin-coated tissue culture plate (i.e., $1 \times$ 96-well plate—3×10^4 cells/well, $1 \times$ 48-well plate—6.25×10^4 cells/well, $1 \times$ 24-well plate—1.2×10^5 cells/well, $1 \times$ 12-well plate—2.4×10^5 cells/well, $1 \times$ 6-well plate—4.8×10^5 cells/well, or $1 \times$ 10-cm dish—3×10^6 cells/dish).

IP350 buffer (0.3% NP-40)

350 mM NaCl
20 mM Tris·Cl, pH 7.5
0.3% (v/v) NP-40
1 mM disodium EDTA
10% (v/v) glycerol
Store up to several months at 4°C
Just before use, add fresh 1 mM DTT, 0.2 mM PMSF, protease inhibitor cocktail (1:1000; Sigma Mammalian Protease Inhibitor cocktail)

IP350 buffer (0.5% NP-40)

350 mM NaCl
20 mM Tris·Cl, pH 7.5
0.5% (v/v) Nonidet P40 (NP-40)
1 mM disodium EDTA
10% (v/v) glycerol
Store up to several months at 4°C
Just before use, add fresh 1 mM DTT, 0.2 mM PMSF, protease inhibitor cocktail (1:1000; Sigma Mammalian Protease Inhibitor cocktail)

Nuclear extract buffer A

20 mM HEPES
10 mM KCl
1 mM disodium EDTA
0.1 mM Na_3VO_4
0.2% (v/v) Nonidet P40 (NP-40)
10% (v/v) glycerol
Store up to several months at 4°C
Just before use add fresh 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (1:1000; Sigma Mammalian Protease Inhibitor cocktail)

Nuclear extract buffer B

20 mM HEPES
10 mM KCl
1 mM disodium EDTA
0.1 mM Na_3VO_4
350 mM NaCl
20% (v/v) glycerol
Store up to several months at 4°C
Just before use, add fresh 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail (1:1000)

NaCl concentration can vary from 150 mM to 500 mM, which needs to be determined empirically.

Puromycin

Make 1 mg/ml puromycin (Sigma, cat. no., P8833) in PBS. Filter sterilize using a 0.22-μm filter, divide into aliquots, and store up to 6 months at −20°C. Freeze/thaw stock no more than 5 or 6 times.

SDS sample buffer, 2×

1% (v/v) glycerol
3% (w/v) SDS
0.5 M Tris·Cl, pH 6.8
0.004% (w/v) bromphenol blue
Store up to 6 months at −20°C

TE buffer

10 mM Tris·Cl, pH 7.5
1 mM disodium EDTA
Store up to 12 months at room temperature (25°C)

COMMENTARY

Background information

Mammalian protein complexes have been studied by combining protein affinity purification with mass spectrometry (MS) and bioinformatics. Particularly useful among the affinity-based purification methodologies is the biotin-avidin system. Protein biotinylation is a powerful technique for molecular biology and biomedical applications due to the high affinity and specificity of the biotin-avidin interaction (Cronan, 1990; Beckett et al., 1999). For the purpose of protein purification, biotinylation offers a number of advantages. First, the high affinity of biotin for avidin allows purification of the biotinylated protein under high stringency conditions, thus reducing background binding that may be observed with other affinity tags or native antibodies. Second, there are few naturally biotinylated proteins, thus reducing the chance for cross-reactions. Third, the approach obviates the need for generation of protein-specific antibodies, which may often cross-react with other cellular proteins. Biotinylation can occur either by the cell's endogenous protein-biotin ligases, or through the coexpression of an exogenous biotin ligase (e.g., the bacterial BirA enzyme), as described in this protocol. Biotinylation of a tagged transcription factor mediated by an exogenous biotin ligase BirA has been demonstrated by others to maintain the factor's protein interactions, DNA binding properties in vivo, and subnuclear distribution (de Boer et al., 2003).

Recently, we tested the utility of in vivo biotinylation of transcription factors in mouse embryonic stem (ES) cells. First, we established an approach for the single-step and tandem purification of transcription factor complexes based on specific in vivo biotinylation mediated by BirA (Wang et al., 2006). Second, we demonstrated the feasibility of in vivo biotinylation for mapping global/chromosomal targets of many different transcription factors (Kim et al., 2008). A notable point is that the same cells expressing a biotin-tagged version of a given transcription factor can be utilized for the construction of both protein-protein and protein-DNA interaction networks (unpub observ.). Although we performed all of our study in mouse ES cells, our approaches should be readily applicable to other cellular systems.

Critical Parameters

In Basic Protocol 1, gelatin adaptation to make ES cells feeder-independent is important for the following two reasons: (1) it eliminates contamination by feeder cells in subsequent purification; (2) it greatly reduces the experimental cost incurred by the large-scale culture of ES cells required for affinity purification of protein complexes. Be aware that not all ES cells are favorable for gelatin adaptation and feeder-independent growth, so selection of ES cell lines to start with that can be gelatin adapted (e.g., J1 ES cells) or grow without feeders (e.g., E14 cells) is advantageous.

To screen for the positive clones expressing biotinylated protein, it is critical not to add milk during streptavidin-HRP antibody incubation, since the milk may contain biotin-related species that can interfere with the streptavidin antibody. Ideally, western analysis with

the native antibody should be performed to detect relative expression level of the biotinylated protein versus endogenous protein, and only the clones with sub-endogenous expression levels should be selected for affinity purification (see an example in Fig. 1B.5.3). The selection of sub-endogenous expression levels of tagged protein ensures minimal interference with endogenous protein complexes by the tagged protein and thus allows for affinity purification of the bona fide interacting partners. However, the native antibody is not always available for your protein of interest; in this case, several clones with medium- or low-level expression of biotinylated proteins should be used for affinity purification.

During affinity purification of protein complexes (Basic Protocol 2), a sufficient amount of the starting nuclear extract (NE) is important to obtain enough final material for MS analysis. A good starting point is 50 to 100 mg of NE protein. In addition, the salt concentration in nuclear extraction buffer B is critical for solubility and integrity of protein complexes. A salt concentration that is too low can result in protein precipitation and aggregation during overnight incubation of nuclear extracts with affinity agarose beads; a salt concentration that is too high may disrupt weak protein-protein interactions. The salt concentration used in affinity purification can vary from 150 mM to 500 mM; therefore, the optimal salt concentration for your protein of interest has to been determined empirically.

In the Support Protocol, due to the high sensitivity of mass spectrometry, it is critical to use high-quality HPLC-grade water to stain and destain the SDS-PAGE gel. Always wear gloves when handling gels, and keep gels in a dish covered with Saran wrap to avoid contamination. In addition, an experienced, reliable MS facility is critical to ensure the success from this expensive and time-consuming experiment**.**

Like all the biological experiments, the same affinity purification procedure should be repeated twice or three times to ensure reproducibility, and to increase the likelihood of identifying bona fide protein-protein interactions.

Troubleshooting

See Table 1B.5.1 for troubleshooting tips.

Anticipated Results

For in vivo biotinylation system setup, we usually screen 24 to 48 G418-resistant clones for BirAV5 expression (using anti-V5-HRP antibody); over half of the clones will show a varying amount of BirA expression. Since BirA is an active biotin ligase, we found that low, medium, and high BirA-expressing cells all mediate efficient biotinylation of the Flagbiotin (FLBIO)-tagged gene product. We screen a similar number of BirA+Flagbiotag clones, and a quarter to a third of the clones should be positive for FLBIO tag (using streptavidin-HRP antibody).

For tandem affinity purification, we usually observe minimal background binding signals in BirA control cells and few endogenously biotinylated proteins in BirA+Flagbiotag cells. The detailed information on the BirA control background signals and the endogenously biotinylated proteins have been described elsewhere (de Boer et al., 2003; Wang et al., 2006). The total number of potential interacting partners of the tagged protein varies according to individual proteins and ranges from a few to more than ten candidates. These candidate proteins are usually of high confidence, although further validation by reverse tagging followed by affinity purification and/or co-immunoprecipitation is required.

Time Considerations

In vivo biotinylation system setup

This is the most time-consuming process, which needs to be planned carefully and executed in a timely fashion. Overall, it takes over a month (~50 days) to set up the system as described in this unit. However, you may be able to shorten the set up process by establishing the BirA-only and BirA+Flagbiotag ES cell lines simultaneously (~30 days). In this case, you will select for the former line with G418 only after pEF1αBirAV5-neo electroporation, and the latter line with both G418 and puromycin after co-electroporation with the pEF1αBirAV5-neo plasmid and pEF1αFLBIO-tagged gene of interest plasmid.

Tandem affinity purification

This depends on the scale of culture and starting ES cell numbers. From a 10-cm culture dish, it takes over a week (~10 days) to expand to twenty 15-cm dishes. It takes another full day to make nuclear extracts. In general, we prepare nuclear extract, determine protein concentration, and set up Flag M2-agarose or streptavidin-agarose incubation all on the same day, to avoid prolonged storage and/or freezing/thaw cycles that will potentially disrupt multiprotein complexes.

Table 1B.5.1 Troubleshooting Guide for Tandem Affinity Purification of Complexes from ES Cells

Problem	Possible reasons	Solution
Weak or no positive signals for the biotinylated proteins (Basic Protocol 1, step 22)	Milk contains high levels of biotin, which block the interaction between streptavidin and the biotinylated protein	Western blot analysis with streptavidin-HRP should be done without milk
	No true positive clones	Screen more clones
Low yield of nuclear extract from both control BirA and BirA+Flagbiotin-tagged samples (Basic Protocol 2, step 19)	Cell lysis is not complete	Make sure cell lysis is complete at step 12
	Low cell numbers	Optimize cell culture and increase the starting material
High nonspecific binding present in both BirA only and BirA+Flagbiotin-tagged samples	The NE concentration is too high	Dilute/adjust the NE concentration to ~2 mg/ml
	% of NP-40 in IP350 is too low	Increase % of NP-40 in IP350
Protein precipitates after overnight incubation (Basic Protocol 2, steps 25 and 31)	The salt concentration is not optimal	Adjust NaCl concentration in nuclear extraction buffer B. It can vary from 150 mM to 500 mM. The optimal salt concentration has to be determined empirically.
The final volume does not go down to 100 µl (Support Protocol 1, step 2)	There are still residual resins in the eluate, which clogs the Centricon device	Briefly centrifuge the eluate, recover the supernatant carefully, and extend the centrifuge time at step 2
	SDS precipitates	Warm up the eluates before Centricon filtration

An extra day is required for a second affinity purification with streptavidin agarose. Therefore, allow ~2 weeks for executing the full protocol of tandem affinity purification.

SDS-PAGE fractionation of protein complexes for MS spectrometry

The concentration of the protein eluates using Centricon takes a few hours, and the SDS-PAGE fractionation takes another few hours. Once the samples are fractionated on gels, the staining is done overnight, and the de-staining takes from one to a few days. The gel slices can be cut out and stored at 4°C for short term before submitted for mass spectrometry analysis.

Acknowledgements

This work is supported by Seed Grant from the Harvard Stem Cell Institute Cell Reprogramming Program to J.W. A.B.C. is supported by NIH Grant R01 HL075705. S.H.O. is an Investigator of Howard Hughes Medical Institute.

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