Published in final edited form as:

Curr Protoc Stem Cell Biol. 2009 March ; CHAPTER: Unit1B.5. doi:
10.1002/9780470151808.sc01b05s8.

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.

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; and 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-

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COMPETING INTERESTS STATEMENTS

The authors declare no competing financial interests.

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 1 and Figure 2. This section 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 of purified protein complexes (to increase sample purity and reduce sample complexity) for downstream mass spectrometry analysis is presented (see Basic Protocol 3).

Note: All ES cell cultures should be maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

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

BASIC PROTOCOL 1

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

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. It 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 Flagbiotagged 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.

Materials

- J1 ES cells (ATCC cat. no. SCRC-1010)
- ES media (see recipe)
- pEF1αBirAV5-neo plasmid (see Figure 1) (available upon request)
- pEF1αFlagbiotin (FLBIO)-puro plasmid (see Figure 1) (available upon request)
- Gelatin-coated cell culture plates (see recipe)
- Irradiated mouse embryonic feeders (IEF) (see recipe)
- IEF plates (see recipe)
- IEF media (see recipe)
- PBS (Sigma cat. no. D8537)
- 15-ml conical tubes (Corning cat. no. 430791)
- 50-ml conical tubes (Corning cat. no. 430829)
- RIPA buffer (Boston BioProducts cat. no. BP-115)
- Gene Pulsor II (electroporation) (Bio-Rad)

0.4 cm gap cuvette for electroporator (Biorad cat. no. 165–2008)

anti-V5-HRP (Invitrogen cat. no. 46-0708)

Streptavidin-HRP (Amersham cat. no. RPN1231)

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 6-well IEF plate containing 5 ml ES media per well. Also, prepare a 10-cm IEF plate with 10 ml IEF media a day prior to electroporation (for use at Step 7).

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

2. 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 at 37°C for 3~5 min.

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

3. Collect cells by adding 3 x vol of ES cell medium to neutralize the trypsin and pipetting up and down to mix, transfer to 15-ml Falcon tubes. Centrifuge at 200 x g for 5 min.
4. Wash harvested cells twice with PBS, then resuspend ES cells at 1.3×10^7 /ml in PBS.
5. Add 20~30 µg of pEF1αBirAV5-neo DNA in TE to 0.75 ml of cell suspension (or ~ 10^7 cells) for each electroporation.

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

6. Perform electroporation: for J1 ES cells, use 0.4 cm gap cuvette, 25 µF, 450V, time constant for each electroporation should read around 0.6–0.8 msec. Incubate 5 min on ice.

Different ES cell lines may require different electroporation conditions.

7. Mix electroporated cells with enough ES medium to bring final cell suspension volume to 10 ml, and transfer them to replace the IEF media in the 10-cm IEF plate prepared a day before. Rock plates gently to mix. Then incubate at 37°C, 5% CO₂.
8. On second day (24 hours after initial plating), add G418 (final concentration 300µg/ml) drug directly to ES media. Rock the plate gently to mix drug completely with medium and return to incubator.
9. Feed the cells with fresh G418 drug and medium each day for the next 7–9 days. Swirl the plate gently to resuspend and remove the dead cells and debris by aspiration. Replace with 10 ml freshly made drug/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.

10. A day prior to picking, prepare a 48-well IEF plate with 500 µl ES medium per well (for use in Step 13) and a U-bottom 96-well plate containing 50 µl of 0.05% trypsin per well (for use in Step 12).

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

11. On picking day, wash the 10-cm plate with 10 ml of PBS. Replace the wash with 5 ml of fresh PBS to prevent the cells drying out during picking.

12. Pick individual colonies with a P200 set at 10 μ l and transfer each colony into a well of the U-bottom 96-well plate containing 50 μ l of 0.05% trypsin. After 24–48 clones or 30 minutes (whichever is first), incubate the 96-well plate at 37°C for 10 min.
13. Using a multi-channel pipette, transfer 150 μ l of ES media from the 48-well IEF plate to the 96-well trypsin plate containing the colonies. Pipette up and down and then transfer the entire cell suspension back to the 48-well feeder plate. Culture the cells in 48-well plate overnight.
14. Replace old medium with fresh ES media without G418 every day.
G418 (300 μ g/ml) may be added but not necessary during the first few days.
15. When the majority (>70%) of wells are ready (i.e., cells reach near or over 70% confluence), wash with PBS, add 35 μ l of 0.05% trypsin and incubate at 37°C for 10 min. Meanwhile, prepare a 96-well plate containing 65 μ l cold 2 x freezing media (see Reagents).
It is wise to set up the 96-well plate to match the 48-well plate (from Step 13) for convenient multi-channel pipette transfer.
16. Using a 12-channel pipette, add 65 μ l ES media to each well of the 48-well plate, mix by pipetting up and down to neutralize the trypsin, and transfer 65 μ l to the U-bottom 96-well plate containing 65 μ l cold 2 x freezing media prepared above. Wrap the plate in parafilm and store at –80°C until for use at Step 20.
17. Add 200 μ l media to the remaining cells in the IEF plate, and return the plate to incubator. Replace old media with fresh media daily for 3–4 days.
18. When the medium in most wells is yellow or cells are near confluence, aspirate medium and add 200 μ l of RIPA buffer to each well to make total lysate. Carry out a standard Western blot analysis with the lysate (20 μ g) using anti-V5-HRP (Invitrogen).
Since BirA is V5-tagged, you should expect to see a band near 35Kd in the positive clones (see Figure 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.
19. Prepare a 24-well IEF plate with 1 ml ES media per well a day prior to Step 20.
20. Thaw positive clones (as determined by Western blotting in step 18) by adding 100 μ l of warm media to frozen wells (from Step 16). Mix and transfer the thawed cell suspension to 24-well IEF plate prepared a day before (see Step 19); repeat until all the remaining cells are thawed and transferred. Return to incubator and culture O/N.
21. On second day, replace old media with fresh ES media containing 300 μ g/ml G418. Let clones grow in ES cell selection media (300 μ g/ml G418) until they are 70% confluent. This takes approximately a week.
22. 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 23. Alternatively, all the cells can be frozen and stored at –80°C (short-term) or in liquid nitrogen LN₂ (long-term) for later use.
23. Repeat Steps 2–22 to establish ES cell lines expressing BirA and biotinylated proteins of interest at sub-endogenous levels: electroporate BirA containing cells (from Step 22) with pEF1 α Flagbio-tagged plasmid containing a specific gene of interest; use media containing 300 μ g/ml G418 and 1–2 μ g/ml puromycin to select

for cells expressing both pEF1 α BirAV5-neo and pEF1 α FLBIO(gene)-puro plasmids (see Figure 1); 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 Figure 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)

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

We found that J1 ES cells can 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 a previously established, feeder-independent ES cells (e.g. E14 line) in the beginning.

BASIC PROTOCOL 2

TANDEM AFFINITY PURIFICATION OF PROTEIN COMPLEXES

This protocol details large-scale expansion of mouse ES cells and nuclear extract preparation from the bulk culture for use in tandem affinity purification. A requirement of 20~40 culture dishes (15-cm) of ES cells grown to near 90% confluence, which corresponds to the yield of 50~100 mg of nuclear extract, may be necessary. It is advised to adapt the two cell lines established in Basic Protocol 1 to grow in a feeder-independent condition to minimize the cross contamination by feeder cells and cost of the experiment.

Materials

BirA-only and BirA+Flagbiotagging ES cells (established in Basic Protocol 1)

ES media (see recipe)

Gelatin-coated ES cell culture plates (see recipe)

Hemocytometer

NALGENE® high-speed centrifuge tube (cat. no. 3114-0050)

250-ml conical plastic bottles (Corning cat. no. 430776)

15-ml conical tubes (Corning cat. no. 430791)

50-ml conical tubes (Corning cat. no. 430829)

Rotating wheel (Scientific Equipment Products cat no. 60448)

glass Dounce homogenizer (40-ml size) with Type B Pestle (Wheaton cat. no. 432-1273)

glass Dounce homogenizer (15-ml size) with Type B Pestle (Wheaton cat. no. 432-1272)

Nuclear Extract Buffer A (see recipe)

Nuclear Extract Buffer B (see recipe)

Protease inhibitor cocktail (Sigma Mammalian Protease Inhibitor cocktail)

Trypan Blue (Invitrogen cat. no. 15250-061)

Bradford assay: Protein concentration Bio-Rad Dye kit (Biorad cat. no. 500-0006)

IP350 buffer with different NP40 concentrations (see recipe)

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)

1. Begin with 10-cm dish culture (10 ml ES medium) for both control BirA cells and cells expressing BirA and biotinylated protein (from Step 24 in Basic Protocol 1). Expand the culture until you have 20–40 × 15-cm dishes (gelatin-coated) 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 50–100 mg of nuclear extracts (see below).

2. Grow cells till they reach 80–90% confluence with constant media renewal.

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

3. 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 at 37°C for 5 min.

10 dishes should be processed at a time.

4. Add 10 ml of fresh medium to the cells above 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 medium, collect the residual cells and combine with the cells in the bottle.

RPMI or low glucose DMEM with 10% FCS 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 at 2,400 × g for 15 min using a JS 4.2 rotor (Beckman) at 4°C.

6. Carefully decant the supernatant, resuspend the cell pellet with 50 ml of ice-cold PBS and transfer to 50-ml conical tubes. Count cell numbers using a hemocytometer.

7. Centrifuge the 50-ml conical tubes at 2,400 × g for 10 min at 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 at 2,400 × g for 10 min at 4°C in a JS 4.2 rotor.

9. Remove supernatant, and rapidly resuspend with ~5 × PCV of ice-cold Nuclear Extract Buffer A with 1M DTT (1μl/ml)/0.2 M PMSF (5μl/ml) and 1:1000 protease inhibitor cocktail (Sigma Mammalian Protease Inhibitor cocktail; add fresh).

10. Centrifuge at 2,400 × g for 5 min at 4°C in a JS 4.2 rotor.

11. Aspirate the supernatant carefully. Add ~ 3 x 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 glass Dounce homogenizer (40-ml size) with Type B Pestle pre-chilled on ice (pre-rinse 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 under microscope (>80% of cells should be lysed).
13. Centrifuge at 4,300 x g at 4°C for 15 min in JS 4.2 rotor.
14. Carefully remove and discard the supernatant using a drawn-out glass Pasteur pipette. Add to the nuclei pellet with 3 ml (per 1×10^9 starting number of cells) of ice-cold Nuclear Extract Buffer B with 1M DTT (1 μ l/ml)/0.2 M PMSF (5 μ l/ml) and protease inhibitor cocktail (1:1000).
15. Dislodge the pellet and transfer to a glass Dounce homogenizer (15-ml size) with Type B Pestle (pre-chilled and rinsed with Nuclear Extract Buffer B). Homogenize up and down 10 times slowly to resuspend the pellet. Transfer homogenate to NALGENE® centrifuge tube (pre-rinse with Nuclear Extract Buffer B) and rotate for 30 min on a rotating wheel in the cold room (4°C).
16. Centrifuge at 25,000 x g in a JA-25.50 rotor at 4°C for 30 min to remove insoluble material. Meanwhile, prepare for the Bradford assay according to manufacturer's instructions.
17. Carefully transfer the supernatant (contains nuclear extract) to 50-ml conical tubes.
18. Determine the protein concentration of each nuclear extract (NE) using the Bradford assay (Siu et al., 2008) (e.g., the Bio-Rad Dye kit).
19. 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% (vol/vol) 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.
20. Preclear supernatant with Protein G–agarose (100 μ l of 50% partial slurry per 10 mg protein) for 1–2 hr at 4°C with continuous mixing.
21. Spin samples at 1,200 x g for 5 min at 4°C. Transfer pre-cleared supernatant to new tubes.
22. Equilibrate FLAG M2 agarose beads (100 μ l of 50% partial 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 at 1,200 x g for 4 min. Aspirate. Repeat this once.
23. Carefully transfer the precleared nuclear extract prepared from Step 21 to pre-equilibrated FLAG M2 resin above. 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.
24. Centrifuge tubes from overnight incubation at 1,200 x g for 4 min at 4°C. Remove supernatant (non-bound material).

Make sure no obvious protein precipitate is formed after overnight incubation.

25. Wash FLAG-agarose beads 4 times 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 at 1,200 x g for 4 min at 4°C between washes.
26. After final wash, remove most of supernatant. Use IP350 (0.3% NP-40) buffer to transfer beads to 15-ml tube. Pool beads, if multiple tubes were used for the incubation and washes from previous steps. Centrifuge at 1,200 x g for 4 min at 4°C to pellet beads. Remove supernatant carefully.
27. Elute beads 4 times with 10 ml of IP350 with 0.1 mg/ml FLAG peptide (Sigma F-3290). For each elution, place sample tubes on rotating wheel for 1~1.5 hours at 4°C. Centrifuge at 1,200 x g for 4 min at 4°C, and carefully transfer supernatant (contains eluted proteins) to a new 50-ml conical tube. Combine total 40 ml of each sample. Discard beads.
28. Prepare streptavidin agarose, as described for FLAG M2 agarose in Step 22.
29. Add the FLAG-eluate (40 ml each) from Step 27 into equilibrated streptavidin-agarose, place on end-over-end rotating wheel at 4°C overnight.
30. Centrifuge tubes from overnight incubation at 1,200 x g for 4 min at 4°C. Remove supernatant (non-bound material).
- Make sure no obvious protein precipitate is formed after overnight incubation.
31. Wash streptavidin-agarose beads 4 times with 20 ml each of ice cold IP350 (0.3% NP-40) buffer. Place on rotating wheel for 15 min at 4°C per wash. Centrifuge as above.
32. After final wash, remove most of supernatant. Use cut-off pipette 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 pipette tips with IP350 buffer and pool with sample. Centrifuge using tabletop microcentrifuge at 1,200 x g for 2 min at 4°C to pellet beads.
33. Remove as much supernatant as possible from the beads using drawn-out Pasteur pipette. Add 500 µl of 2 x SDS sample buffer. Vortex gently and heat at 95–100°C for 5 min and vortex again. Allow to cool to room temperature. Centrifuge at maximum speed for 1 min at room temperature to re-pellet beads. Carefully transfer supernatant to a new screw-cap tube. Add 500 µl of 1 x SDS sample buffer to residual beads and vortex gently. Re-centrifuge at maximum speed at room temperature for 1 min. Pool sample with the 1st eluate in screw-cap tube. Repeat washing of beads one more time with 400 µl of 1 x 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.
34. Centrifuge at maximum speed for 2 min at room temperature (to pellet any residual agarose beads that were carried over). Transfer sample into new 2-ml screw-cap tubes.

Carryover of residual agarose beads will block the Centricon filter (see Basic Protocol 3). Samples can be frozen and stored at –80°C for future use.

BASIC PROTOCOL 3

SDS-PAGE FRACTIONATION OF PROTEIN COMPLEXES

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

Materials

Bio-Rad Protean II xi Basic Unit with casting stand (cat. no. 165–1834)

YM-10 Centricon (10,000 MWCO, Amicon Bioseparations cat. no. 4205)

HPLC-grade water (American Bioanalytical)

30% Acrylamide/Bis solution (37.5:1) (Biorad cat. no. 161-0158)

Colloidal Coomassie Stain (Invitrogen cat. no. 46–7015 and 46–7016).

1. Transfer all eluate (from Step 34, Basic Protocol 2) into chamber of YM-10 Centricon (10,000 MWCO) device. If just thawed from the freezer, warm up samples at 37°C water bath for 5 min to dissolve SDS.

Undissolved SDS will block Centricon filter.

2. Centrifuge in the Avanti J25 centrifuge using JA-25.50 fixed angle rotor at 25°C at 5,000 x g until as much filtrate runs through the chamber as possible (~2–3 hrs). After centrifugation, there should be about 100 µl of concentrated material.

This centrifuge step needs to be performed at room temp 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) at 800 x g for 2 min at 25°C. Transfer to a fresh 1.5-ml Eppendorf tube.
4. Prepare 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, seal with ethanol or butanol for polymerization, then pour upper stacking gel (4%) with comb positioned in an angle, then reposition the comb horizontally to avoid air bubbles in the comb wells. Pull out the bottom gel blocker carefully before use.

5. Load samples from Step 3 and run the gel for approximately 1 hour at 120V in stacking gel and run samples till the bromophenol blue dye reaches ~2.5 cm into separating (lower) gel (it takes approximately 1 hour under 120V).

Fill in empty wells with same volume of 1 x 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, which helps 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 (Invitrogen).
7. Destain gel with multiple rinses of HPLC-grade water (American Bioanalytical). Destain until background is very clear (can take 1–2 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 scalpel or razor blade, cut into ~1 mm cubes and transfer to clear

1.5-ml Eppendorf 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. Can store cut out gel slices 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

ES Media

Dulbecco's modified Eagle's medium (DMEM)

15% fetal calf serum*

0.1 mM β -mercaptoethanol,

2 mM L-glutamine

0.1 mM non-essential amino acid

1% nucleoside mix (100 x stock, Sigma)

1000 U/ml recombinant leukemia inhibitory factor (LIF; Chemicon)

50 U/ml Penicillin/Streptomycin

Store at 4°C. *Fetal calf serum (Hyclone cat. no. SH30071.03) needs to be prescreened batch to batch for supporting optimal ES cell growth.

Irradiated Mouse Embryonic Feeder cells (IEF)

Mouse embryonic feeder cells (MEFs) are used in the culturing 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, 2001). A unique quad-resistant DR4 feeder cell line can be purchased from Open Biosystems (cat. no. MES3948), which have 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.

IEF plates

Normally 3 million IEF cells were frozen down as 1 x stock. When thawed, these 3 million cells will be used to seed one full tissue culture plate coated with 0.1% gelatin (i.e., 1 x 96-well plate, 1 x 48-well plate, 1 x 24-well plate, 1 x 12 well plate, 1 x 6-well plate, or 1 x 10-cm dish/plate).

IEF Media

86% DMEM (high glucose)

10% FCS (heat inactivated)

2% PEN/STREP (5,000 i.u./ea/ml)

1% L-GLUTAMINE (200 mM)

1% **SODIUM PYRUVATE (100mM) (GIBCO 11360-070)

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

G418 (GIBCO cat. no. 11811)

Also known as geneticin, 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.

Gelatin (Bacto[®], DIFCO cat. no. 0143-15-1)

Dissolve 5 g of gelatin in 500 ml distilled water and autoclave (1% stock). Store at room temperature indefinitely. Before use, dilute 1:10 (to make 0.1% working solution) with sterile dH₂O and filter through 0.45 micron filter apparatus.

Gelatin-coated tissue culture plates

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

Puromycin (Sigma, cat. no. P8833)

Make 1 mg/ml in sterile distilled water. Filter sterilize, aliquot, and store at -20°C. Freeze/thaw stock no more than 5 or 6 times.

0.05% (wt/vol) trypsin (Mediatech cat. no. 25-052-CI)

0.25% (wt/vol) trypsin (Mediatech cat. no. 25-053-CI)

2 x freezing media

20% DMSO

80% FCS

Nuclear Extract Buffer A

20 mM HEPES 10 mM KCl 1 mM EDTA

0.1 mM Na₃VO₄

0.2% Nonidet P40 (NP-40)

10% glycerol

add fresh 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail (1:1000). Store at 4°C.

Nuclear Extract Buffer B

20 mM HEPES, 10 mM KCl

1 mM EDTA

0.1 mM Na₃VO₄

350 mM NaCl*

20% glycerol

add fresh 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail (1:1000). Store at 4°C. NaCl concentration can vary from 150 mM to 500 mM, which needs to be determined empirically.

IP350 Buffer (0.5% NP-40)

350 mM NaCl
20 mM Tris-HCl, pH 7.5
0.5% NP-40
1 mM EDTA
10 % glycerol (vol/vol)
add fresh 1 mM DTT, 0.2 mM PMSF, Protease inhibitor cocktail (1:1000). Store at 4°C.

IP350 Buffer (0.3% NP-40)

350 mM NaCl
20 mM Tris-HCl pH 7.5
0.3% NP-40
1 mM EDTA
10 % glycerol (vol/vol)
add fresh 1 mM DTT, 0.2 mM PMSF, Protease inhibitor cocktail (1:1000). Store at 4°C.

2 x SDS sample buffer

1% vol/vol glycerol
3% SDS
0.5 M Tris-Cl pH 6.8
0.004% bromophenol blue

TE buffer

10 mM Tris-Cl pH 7.5
1 mM EDTA
Store 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 (Beckett et al., 1999; Cronan, 1990). 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). 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 (Kim et al., Nature Protocol in preparation). 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 vs. endogenous protein, and only the clones with sub-endogenous expression levels should be selected for affinity purification (see an example in Figure 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 50~100 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 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 be determined empirically.

In Basic Protocol 3, 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 increase the likelihood of identifying bona fide protein-protein interactions.

Troubleshooting

See table 1 for troubleshooting tips.

Anticipated results

For *in vivo* biotinylation system setup, we usually screen 24~48 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 screened 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 setup 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 pEF1aBirAV5-neo electroporation and the latter line with both G418 and Puromycin after co-electroporation with pEF1aBirAV5-neo plasmid and pEF1aFLBIO-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 plate, it takes over a week (~10 days) to expand to 20 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 in the same day, to avoid prolonged storage and/or freezing-thaw cycles that will potentially disrupt multiprotein complexes. 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 day to a few days. The gel slices can be cut out and stored in 4°C for short term before submitted for mass spectrometry analysis.

Acknowledgments

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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Figure 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 selected with puromycin. The resulting stable line is resistant to both G418 and puromycin and express FLAG-tagged, biotinylated TF ($^{FLBIO}TF$) that can be immunoprecipitated by anti-FLAG and streptavidin antibodies/beads.

Figure 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 are 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.

Figure 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 non-specific signals.

Table 1

Troubleshooting guide

Problem	Possible reasons	Solution
Weak or no positive signals for the biotinylated proteins (Basic Protocol 1, Step 23)	Milk contains high levels of biotin, which block the interaction between streptavidin and the biotinylated protein. No true positive clones.	Western blot analysis with streptavidin-HRP should be done without milk. Screen more clones.
Low yield of nuclear extract from both control BirA and BirA+Flagbiotin-tagged samples (Basic Protocol 2, Step 18)	Cell lysis is not complete. Low cell numbers.	Make sure cell lysis is complete at Step 12. Optimize cell culture and increase the starting material.
High non-specific binding present in both BirA only and BirA+Flagbiotin-tagged samples	The NE concentration is too high % of NP-40 in IP350 is too low	Dilute/adjust the NE concentration to approximately 2 mg/ml Increase % of NP-40 in IP350
Protein precipitates after overnight incubation (Basic Protocol 2, Steps 24 and 30)	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 μ l (Basic Protocol 3, Step 2)	There are still residual resins in the eluate which clogs the Centricon device. SDS precipitates.	Briefly centrifuge the eluate, recover the supernatant carefully. Extend the centrifuge time at Step 2. Warm up the eluates before Centricon filtration.