

# Chapter 2

## Identification of Mammalian Protein Complexes by Lentiviral-Based Affinity Purification and Mass Spectrometry

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### Abstract

Protein complexes and protein–protein interactions (PPIs) are fundamental for most biological functions. Deciphering the extensive protein interaction networks that occur within cellular contexts has become a logical extension to the human genome project. Proteome-scale interactome analysis of mammalian systems requires efficient methods for accurately detecting PPIs with specific considerations for the intrinsic technical challenges of mammalian genome manipulation. In this chapter, we outline in detail an innovative lentiviral-based functional proteomic approach that can be used to rapidly characterize protein complexes from a broad range of mammalian cell lines. This method integrates the following key features: (1) lentiviral elements for efficient delivery of tagged constructs into mammalian cell lines; (2) site-specific Gateway™ recombination sites for easy cloning; (3) versatile epitope-tagging system for flexible affinity purification strategies; and (4) LC-MS-based protein identification using tandem mass spectrometry.

**Key words:** Protein–protein interactions, Protein complexes, Affinity purification, Lentivirus, Gateway™ cloning, Mass spectrometry

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### 1. Introduction

Most cell type-specific functions and phenotypes are mediated by the activities of multiprotein complexes as well as other types of protein–protein interactions (PPIs) and posttranslational modifications. Accordingly, the formation and function of macromolecular protein complexes underpin the whole of cell biology, making the identification of PPI networks central – even fundamental – to understanding most molecular pathways and processes. Analysis of the variations of protein complex composition in different cell and tissue types is an important step toward understanding the relationship between gene products and cellular function and

phenotype in diverse physiological contexts. To date, most large-scale PPI studies have been performed in model organisms, primarily the budding yeast *Saccharomyces cerevisiae*, including analysis by yeast two-hybrid (Y2H) and tandem affinity purification (TAP). While the former approach provides information relating to interactions between two proteins, typically of binary nature, the latter is a type of affinity purification and mass spectrometry (AP-MS) approach, which can detect multimeric protein complexes. In this chapter, we focus on AP-MS for the identification of protein complexes in mammalian contexts and outline a detailed lentiviral-based AP-MS protocol.

AP-MS is one of the more commonly used techniques in studies of PPIs and protein complex compositions (1–4). AP-MS relies on the fusion of a protein-coding sequence of interest with an epitope tag that allows protein purification to be conducted in a generic and standardized fashion (4–8). We recently developed a lentivirus-based tagging system for the affinity purification of mammalian proteins from tissue culture cells (9). This system contains an N- or C-terminal triple affinity tag, termed the versatile affinity (VA) tag, which consists of 3×FLAG, 6×His, and 2×StrepII epitopes with the dual tobacco etch virus (TEV) protease cleavage site located between 3×FLAG and 6×His. This lentivirus-based tagging system also incorporates an efficient system for cloning open reading frames (ORFs) from Gateway™ entry ORF clones into the vectors (10–12). The VA tag and Gateway™ recombination elements were reconstructed into lentiviral vectors that can infect most human cell types, including both dividing and nondividing cells (13–15). Multiple promoters are available in this system to control tagged gene expression, including a doxycycline-inducible promoter for adjusting protein expression levels. A vector map and brief outline of the lentiviral-based tagging method are found in Fig. 1.

Collectively, this innovative lentiviral-based AP-MS approach for mammalian protein complex identification addresses key bottlenecks related to large-scale mammalian AP-MS experiments. Important features include the following: (1) applicability to most mammalian cell types; (2) compatibility with publicly and commercially available cDNA libraries; (3) an efficient epitope-tagging system; and (4) compatibility with multiple purification strategies. As an overview, the workflow involved in such a pipeline entails (1) cloning target ORFs into destination vectors containing lentiviral elements, (2) production and harvesting of lentiviruses encapsulating the tagged constructs, (3) infection and scale-up of mammalian cell lines stably expressing the tagged constructs, (4) two-step purification for enrichment of target protein complexes, and (5) proteolytic digestion and sequencing of the peptide mixtures by shot-gun tandem mass spectrometry.

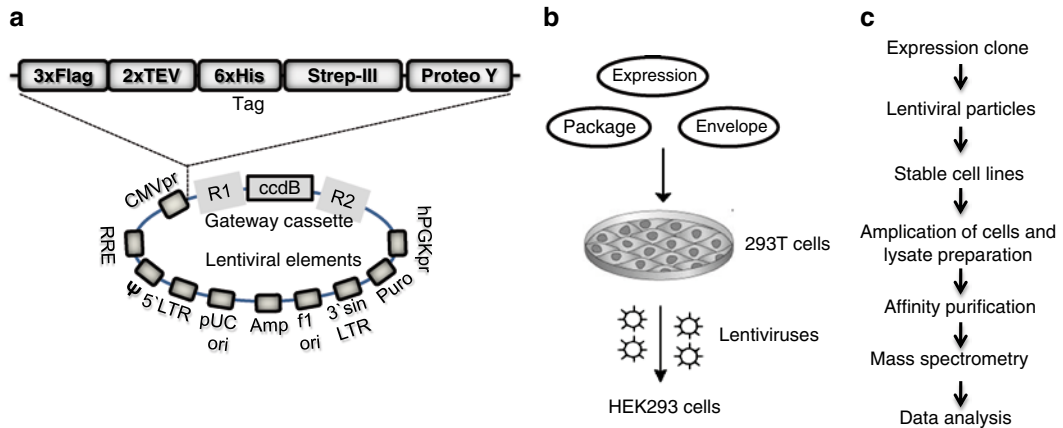


Fig. 1. MAPLE system. **(a)** Triple tag in gateway lentiviral system. The versatile affinity purification (VAP) tag consists of the following epitopes: 3×FLAG, 2× tobacco etch virus (TEV) protease cleavage site, 6×His, and 2× streptactin (Strep III). A yeast proteotypic peptide (Proteo Y) is included for bait quantitation. The VAP tag is in-frame with the Gateway cassette in a lentiviral transfer vector. Only N-terminal tag is shown here, and the tags at the C terminus are in reverse order. The key lentiviral elements are as follows: *RRE* Rev-responsive element;  $\Psi$  packaging element (Psi); *5'-LTR* 5'-long terminal repeats; *3'-sin LTR* HIV 3'-LTR with self-inactivating U3 deletion; *Puro* Puromycin resistance; *Amp* Ampicillin resistance. **(b)** Lentiviral infection. Expression plasmids with tagged constructs are transfected into HEK293T cells together with the package and envelop plasmids to produce lentivirus. Harvested lentiviruses are then infected into HEK293 cells. **(c)** MAPLE system pipeline. Adapted from (9).

## 2. Materials

### 2.1. BP Reaction

1. BP clonase enzyme mix (Invitrogen).
2. Gateway™ DONR plasmids pDONR221 or pDONR223 (Invitrogen).
3. GeneJET plasmid miniprep kit (Fermentas).
4. GeneJET PCR purification kit (Fermantas).
5. Kanamycin (Sigma) dissolved into H<sub>2</sub>O and stored at -20°C.
6. M13 sequencing primers. Forward: 5'-GTT TTC CCA GTC ACG AC-3', reverse: 5'-CAG GAA ACA GCT ATG AC-3'.
7. NucleoBond Xtra Maxi Plus (Macherey-Nagel).
8. Protease K (Sigma).
9. SOC Medium (Bioshop).
10. Subcloning Efficiency DH5α cells (Invitrogen).

### 2.2. LR Reaction

1. Ampicillin and spectinomycin (Sigma).
2. Destination vector: VA tag destination vector in lentivirus cassette can be obtained from Dr. Jason Moffat (University of Toronto).

3. Gateway™-compatible entry clones for human and other species are obtained from (1) Open Biosystems, (2) UltimateORF collection (Invitrogen), or (3) BP reaction (outlined below).
4. Gateway™ LR clonase II enzyme mix (Invitrogen).

### **2.3. Lentivirus Infection**

1. Dulbecco's modified Eagle's medium (DMEM; Wisent or LONZA).
2. Fetal Bovine Serum (FBS; Wisent or PAA).
3. FuGene (Roche).
4. Penicillin–Streptomycin (Wisent).
5. Glycerol for the storage of cell line (10% with DMEM media).
6. Opti-MEM (GIBCO).
7. Lentivirus packaging (Pax2) and envelope (MD2G) plasmids.
8. Polybrene (Sigma). Dissolve to a final concentration of 2 mg/ml in water and store at  $-20^{\circ}\text{C}$ .
9. Puromycine dihydrochloride (Sigma). Dissolve to a final concentration of 2 mg/ml in water and store at  $-20^{\circ}\text{C}$ .
10. Trypsin (0.05%)/EDTA (0.53 mM) (Wisent).

### **2.4. Protein Purification**

1. 3×FLAG peptide (Sigma).
2. AFC (high salt) buffer: 10 mM Tris–HCl (pH 7.9), 420 mM NaCl, 0.1% NP-40.
3. AFC (low salt) buffer: 10 mM Tris–HCl (pH 7.9), 100 mM NaCl, 0.1% NP-40.
4. Anti-FLAG M2 agarose from mouse (Sigma). In a 1:1 slurry with glycerol. Store at  $-20^{\circ}\text{C}$ .
5. Benzonase (Sigma).
6.  $\beta$ -Biotin (Sigma).
7. Bio-Spin Disposable Chromatography Column (small) (Bio-Rad).
8. Elution buffer (for Biotin): 50 mM ammonium bicarbonate, 2 mM biotin.
9. "Flat top" 1.5-ml microtube siliconized (BioCan Scientific).
10. Micro Bio-Spin Chromatography Columns (large, Bio-RAD).
11. Ni-NTA Agarose (Qiagen). Store at  $2^{\circ}\text{C}$ .
12. Phosphatase inhibitors: Make the stock solutions as follows: (1) 1 M sodium fluoride (NaF, Sigma). Dissolve 4.2 g into 100 ml  $\text{H}_2\text{O}$  and store at room temperature. (2) 100 mM sodium orthovanadate (NaOrth, Sigma). Dissolve 1.8392 g in 100 ml  $\text{H}_2\text{O}$  (pH is 12 when dissolved, and adjust to pH 10 with HCl). Then, place in boiling water and cool on ice. Repeat boiling and cooling cycles until no color remains. pH is

much lower than pH 10, so adjust with NaOH. Repeat boiling and cooling cycles until color has gone. Stabilize at pH 10. Aliquot and store at  $-20^{\circ}\text{C}$ . (3) 100 mM Sodium Pyrophosphate decahydrate (Fisher Scientific).

13. Protease inhibitors: First, make mixes A and B. Mix A: 250 ml DMSO, 240 mg chymostatin, 150 mg pepstatin A, and 8.7 g PMSF. Mix B: 200 ml  $\text{H}_2\text{O}$ , 50 mg leupeptin, 1.7 g aprotinin, and 31 g benzamidine. Next, mix A and B as follows: 1 ml lysis buffer, 2.5  $\mu\text{l}$  mix A, and 2  $\mu\text{l}$  mix B. Alternatively, the use of commercially available protease inhibitor cocktails is acceptable.
14. Rinsing buffer: 50 mM ammonium bicarbonate, 125 mM NaCl.
15. Silver staining kit: ProteoSilver plus (Sigma).
16. Strep-Tactin Sepharose, 50% suspension, 20 ml (IBA).
17. TEV buffer (streptavidin-binding buffer): 10 mM Tris-HCl (pH 7.9), 125 mM NaCl, 0.1% NP-40.
18. TEV protease (2 mg/ml).

## **2.5. Materials for LC-MS**

1. Orbitrap-Velos mass spectrometer (Thermo) and XCalibur software (Thermo), which is a software package and user interface for instrument operation and acquisition of mass spectra.
2. Trichloroacetic acid (TCA) (PIERCE) and acetone (PIERCE) for protein precipitation and washes, respectively. Keep ice cold prior to use.
3. Digestion buffer: 50 mM ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ), pH 8.0, 1 mM calcium chloride ( $\text{CaCl}_2$ ). Store at  $4^{\circ}\text{C}$  prior to use.
4. 100 mM Tris(2-carboxyethyl) phosphine (TCEP-HCl) (PIERCE) for cysteine reduction.
5. 0.5 M iodoacetamide (IAM) (Sigma) for alkylation of reduced cysteine residues.
6. Immobilized trypsin solution: Mix 18.7  $\mu\text{l}$  digestion buffer, 1.8  $\mu\text{l}$  PIERCE immobilized trypsin beads (PIERCE), 0.9  $\mu\text{l}$  immobilized trypsin beads (Applied Biosciences), and 0.06  $\mu\text{l}$  1 M  $\text{CaCl}_2$ . Make sure that the pH of the immobilized trypsin is 8.0 for optimal digestion.
7. 75- $\mu\text{m}$  fused silica (Polymicro Technologies).
8. Luna C18 reverse-phase packaging material (Phenomenex).
9. Solvent A: 5% ACN, 95%  $\text{H}_2\text{O}$ , 0.1% formic acid (all solutions HPLC grade or better).
10. Solvent B: 5%  $\text{H}_2\text{O}$ , 95% ACN, 0.1% formic acid (all solutions HPLC grade or better).
11. EASY-nLC nano LC pump (Proxeon).
12. P-2000 laser puller (Sutter Instrument Company).

### 3. Methods

#### **3.1. BP Reaction to Make Gateway™-Compatible ORF Clones**

The first step in lentivirus-based protein purification is to make the Gateway™ entry clone (see Note 1). Design PCR primers with attB1 forward primer (5'-GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-TNN-(gene-specific sequence)-3', attB1 site underlined) and attB2 reverse primer (5'-GGGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTN-(gene-specific sequence)-3', attB2 site underlined). Gene-specific sequences should map to the 5'- and 3'-ends of the ORF of interest.

1. Amplify the PCR product from cDNA templates and use PCR purification kit to purify the PCR product.
2. Prepare Gateway™ DONR plasmids (pDONR221 or pDONR223).
3. Mix 2 µl 75 ng/µl PCR fragment, 1 µl 75 ng/µl pD221 plasmids, 2 µl BP clonase, and 5 µl TE buffer in a total volume of 10 µl and incubate at room temperature for 1–2 h.
4. Add 1 µl protease K and incubate at 37°C for 10 min, followed by cooling on ice for 2–5 min.
5. Transform the BP reaction products into DH5-α or TOP10 competent bacteria by adding 5–10 µl BP reaction mixture into 50 µl competent bacteria, keeping on ice for 15–30 min, followed by heat shock at 42°C for 1 min. Next, put on ice for 10 min and add 0.75 ml of SOC media to incubate at 37°C for 1 h. Finally, streak the bacteria on 100 µg/ml kanamycin LB plate and incubate at 37°C overnight.
6. Pick four colonies and grow each overnight in 2 ml of LB with 100 µg/ml kanamycin at 37°C.
7. Isolate DNA by using miniprep kits.
8. Verify the cloned gene by either PCR, restriction enzyme digestion (e.g., by EcoRV), or sequencing (using M13 primers).

#### **3.2. Gene (ORF) Tagging by LR Reaction**

1. Streak bacteria that contain the entry ORF clone into LB agar plates with proper antibiotics (e.g., 150 µg/µl spectinomycin for the entry clones from OpenBiosystems in pDONR223 or 100 µg/ml kanamycin for the clones from Invitrogen in pENTR™ 221 or from OpenBiosystems in pDONR221). Incubate at 37°C overnight.
2. Pick two colonies and make DNA minipreps for each of the colonies.
3. Sequence the DNA (200–300 ng of each) using 10 µM of gene-specific or universal (such as M13) primers in the reaction mixture of 7.7 µl.

4. Check the sequencing results to ensure proper gene identity, length, and sequence.
5. Select correct sequence colonies for the LR reaction for tagging (see Note 3).
6. Make a mixture containing 75 ng entry clone DNA, 75 ng VA tag destination vector DNA, 6  $\mu$ l TE buffer, and 2  $\mu$ l Gateway™ LR clonase II enzyme mix in a total volume of 20  $\mu$ l.
7. Incubate the mixture at 25°C for 1–2 h.
8. Add 1  $\mu$ l protease K and incubate at 37°C for 10 min.
9. Transform the LR reaction products into 50  $\mu$ l DH5 $\alpha$  cells by incubating the DNA and 50  $\mu$ l DH5 $\alpha$  on ice for 30 min, then at 42°C for 90 s, and finally on ice for another 2 min. Then, add 250  $\mu$ l SOC and incubate at 37°C for 1 h followed by centrifugation at 12,000 $\times g$  for 1 min.
10. Spread all or half the mixture on an ampicillin (100  $\mu$ g/ml) LB agar plate and incubate overnight at 32 or 37°C (see Note 4).
11. Pick two colonies, grow each in 3 ml of LB with 100  $\mu$ g/ml ampicillin, and prepare miniprep DNA. Run the DNA on a 0.8% agarose gel to check for correctly recombineered clones and do diagnostic enzymatic digestions (the latter is optional).
12. Make maxi-, midi-, or minipreps of the DNA.

### **3.3. Making Lentiviruses and Cell Infection**

1. Split  $1 \times 10^6$  293T cells into each 60-mm dish with 6 ml low-antibiotic media (DMEM with 10% heat-inhibited FBS and 0.1 $\times$  penicillin–streptomycin) and incubate at 37°C overnight and 5% CO<sub>2</sub>.
2. Make transfection mixes A and B: Mix A contains 6  $\mu$ l FuGene and 90  $\mu$ l Opti-MEM. Mix B contains 900 ng packaging plasmid (Pax2), 100 ng envelope plasmid (MD2G), 2,000 ng lentivirus expression plasmid, and Opti-MEM to a total volume of 20  $\mu$ l.
3. Mix A and B dropwise, swirl the tube, and incubate the mix for 15–30 min at room temperature.
4. Carefully transfer (dropwise) the mix into the 293T cells (in the original 6 ml of 0.1 $\times$  penicillin–streptomycin DMEM) and incubate at 37°C in 5% CO<sub>2</sub> for 18 h or overnight.
5. Replace the medium with 6 ml high-serum medium (DMEM + 30% iFS + 1 penicillin–streptomycin) and incubate for 24 h.
6. Harvest the viral supernatant (~6 ml), add new media, and incubate for 24 h.
7. Harvest the supernatant again, combine the supernatants, and centrifuge at 2,000 $\times g$  for 5 min at 4°C. The virus in the supernatants can be stored at –80°C.

8. Transduce cells by first seeding  $3 \times 10^6$  HEK293 cells (or other cells) in 10-cm dishes with 10 ml medium (or  $0.5 \times 10^6$  cells in each well of 6-well plate) on day 0 and then culturing them at 37°C in 5% CO<sub>2</sub> overnight. On day 1, remove the medium and add 5 ml (2 ml for 6-well plate) of the virus-containing medium plus 8 µg/ml of polybrene. The virus-containing medium is replaced by regular medium (DMEM, 10% FBS, and 1× penicillin–streptomycin) on day 2. Finally, add 2 µg/µl puromycin on day 3 and grow the cells under selection for 2–3 days (see Notes 15 and 16).
9. Stably transduced cells can be stored in liquid nitrogen or at –80°C in 10% glycerol DMEM medium. Cell lysates are collected for anti-FLAG Western blots to examine the expression of tagged proteins.
10. Expand the cells to five 15-cm dishes, grow for approximately 12 days, and harvest the cell pellets (approximately 1 g) for purification (see Note 11).

### **3.4. FLAG-Strep Two-Step Protein Purification**

Two-step purifications usually yield cleaner products than do one-step purifications (4), although at the possible cost of losing some more weakly interacting proteins. In the lentivirus-based VA tag system, either FLAG-His or FLAG-Strep dual purification can be chosen for protein purification. Here, the latter protocol is described because it generally produces less background than is the case for FLAG-His purifications. All steps are carried out on ice or at 4°C unless indicated otherwise.

1. Thaw cell pellets with 5 ml of 1× high-salt AFC buffer for cell pellets from five 15-cm dishes (1 ml/dish), supplemented with protease inhibitors (2.5 µl/ml protease inhibitor A and 2 µl/ml protease inhibitor B) and phosphatase inhibitors (10 mM NaF, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate) (see Notes 5 and 6).
2. Perform three freeze–thaw cycles by quickly freezing cells on dry ice followed by thawing in a 37°C water bath, inverting frequently to prevent the temperature from rising above 4°C.
3. Add benzonase to a final concentration of 12.5–25 units/ml and incubate at 4°C for 30 min with rotation to digest RNA and DNA (see Note 7).
4. Transfer cell lysates to centrifuge tubes and spin at  $100,000 \times g$  for 1 h at 4°C to remove cell debris and precipitates.
5. Make 20 µl slurry (10 µl beads and 10 µl liquid) of M2 agarose beads.
6. Wash beads twice with 0.5–1 ml of 1× high-salt AFC buffer and aspirate off the supernatant.
7. Resuspend beads in one volume of 1× high-salt AFC buffer.



8. Add 20  $\mu$ l M2 agarose beads (1:1 slurry) to 5-ml extract and incubate for 4 h with rotation at 4°C.
9. Transfer the bead slurry into a column (Bio-Spin Disposable Chromatography Column).
10. Wash with 1 ml of 1 $\times$  low-salt AFC buffer through the column three times, waiting for 10 min between each wash.
11. Wash with 1 ml of TEV buffer through the column two times, waiting for 10 min between each wash.
12. Add 100  $\mu$ l TEV buffer and 5  $\mu$ l TEV protease (2 mg/ml) (final concentration of TEV 100  $\mu$ g/ml), and 2  $\mu$ l 3 $\times$ FLAG peptide, and rotate overnight at 4°C.
13. Wash 20  $\mu$ l Strep-Tactin bead slurry with 1 ml TEV buffer three times in a column.
14. Collect, through the column, the 100  $\mu$ l TEV buffer–protein mix in a siliconized microtube (the use of siliconized microtubes is optional). Otherwise, samples can be directly eluted into the next column that has the washed Strep-Tactin beads.
15. Add 100  $\mu$ l TEV buffer to the beads twice, each time incubate for 2–4 (3) h at 4°C with rotation, and wait for 10 min between each collection (for a total volume of 300  $\mu$ l).
16. Wash three times with 1 ml of TEV buffer, waiting for 10 min between washes.
17. Wash three times with 1 ml of rinsing buffer, waiting for 10 min between washes.
18. Resuspend beads in 150  $\mu$ l elution buffer and incubate for 15 min–1 h at 4°C with rotation. Repeat the elution with 150  $\mu$ l elution buffer for total volume of 300  $\mu$ l eluate.

### **3.5. TCA Precipitation**

1. Add 1 volume of ice-cold 100% TCA to 4 volumes of protein sample and incubate at 4°C overnight.
2. Centrifuge at 12,000 $\times g$  for 30 min at 4°C.
3. Discard supernatant.
4. Suspend pellet in 200  $\mu$ l ice-cold acetone, incubate for 30 min at –20°C, and centrifuge at 14,000 rpm for 30 min at 4°C.
5. Repeat step 4.
6. Dry pellet by placing open tube at room temperature for 5–10 min. Cover with a kimwipe.
7. Dissolve the sample in 40  $\mu$ l 50 mM  $\text{NH}_4\text{HCO}_3$ , pH 8.0.

### **3.6. Trypsin Digestion (Reduction/Alkylation)**

1. Add TCEP–HCl to a final concentration of 2 mM and incubate at room temperature for 45 min.
2. Add IAM to a final concentration of 10 mM and incubate at room temperature for 40 min. Protect from light.
3. Add  $\text{CaCl}_2$  to a final concentration of 1 mM.

4. Add trypsin (1:20–1:100 for trypsin:protein) and incubate at 37°C with shaking ( $1,000\times g$ ) overnight.
5. Add FA to a final concentration of 1% to terminate the enzymatic digestion, and store digests at 4°C.

### 3.7. LC-MS/MS

#### Analysis

1. For AP-MS experiments, we recommend using single-dimension reverse-phase chromatography. Prepare a spray tip using a 75- $\mu\text{m}$  inner diameter/350- $\mu\text{m}$  outer diameter fused-silica capillary column and a laser puller (program 19: heat=280, FIL=0, VEL=30, DEL=200). Pack 10–12 cm of C18 reverse-phase beads (Luna, 3  $\mu$ ; solubilized in MeOH) by pressure bomb.
2. Connect the packed spray column to an EASY-nLC quaternary pump (Proxeon) and assemble in-line with the LTQ Orbitrap-Velos. Ensure that the nLC pumps maintain a stable pressure read-out and are free of leaks. The HPLC gradient programs for alternating data acquisition and wash cycles should be as follows:

#### Data acquisition (total runtime = 105 min)

Time	Duration	Flow (nl/min)	% B
0:00	N/A	300	2
1:00	1:00	300	2
3:00	2:00	300	6
75:00	72:00	300	24
91:00	16:00	300	90
96:00	5:00	300	90
97:00	1:00	300	2
105:00	8:00	300	2

#### MeOH wash (total runtime = 30 min)

Time	Duration	Flow (nl/min)	% B
0:00	N/A	300	2
1:00	1:00	300	2
2:00	1:00	300	6
17:00	15:00	300	24
18:00	1:00	300	90
21:00	3:00	300	90
22:00	1:00	300	2
30:00	8:00	300	2

3. The data-dependent data acquisition parameters include the following: 1 centroid MS (mass range 400–2,000) followed by MS/MS on the 10 most abundant product ions. General parameters include the following:

<i>General parameters</i>	<i>Setting</i>
Activation type	CID
Isolation width	3
Normalized collision energy	32
Activation Q	0.25
Activation time	30 ms
<i>Data-dependent parameters</i>	<i>Setting</i>
Minimum threshold	1,000
Repeat count	1
Repeat duration	30 s
Exclusion list size	500
Exclusion duration	30 s
Exclusion mass width (by mass)	Low = 1.2, high = 1.5

4. Ionize peptides from the nanocolumn by applying a 2.5-kV spray voltage. In data-dependent acquisition mode, the mass spectrometer acquires MS and MS/MS spectra through consecutive cycles of 11 scans throughout the duration of the gradient. Specifically, an initial full mass scan of precursor ions which detects the  $m/z$  values of intact peptide ions is followed by ten successive tandem mass spectra acquisitions of the ten most intense precursor ions.
5. The acquired tandem mass spectra are then matched to theoretical spectra based on an *in silico* digestion of a human proteome reference database, such as UniProtKB/Swiss-Prot, which can be downloaded in FASTA format from <http://www.uniprot.org/downloads>.
6. Peptide spectra acquired during the LC-MS run are identified using the SEQUEST search engine and validated using the STATQUEST probabilistic scoring algorithm. Alternatively, MASCOT or X!Tandem, among other search engines, may be used. Confidence scores are assigned to all putative matches using the probabilistic STATQUEST scoring program (or comparable statistical tools with other search algorithm suites). Proteins detected with two or more high-confidence peptide matches (i.e., 90–99%) and with a minimum likelihood threshold cutoff of 90% or greater probability are considered positive identifications.

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## 4. Notes

1. *Gateway<sup>TM</sup> ORFs*. Large numbers of human, mouse, and rat ORFs have been cloned into Gateway<sup>TM</sup> cassettes and are commercially available from various sources, such as Invitrogen, OpenBiosystems, and GeneCopoeia. For these genes, the BP reaction can be skipped and the LR reaction can be directly carried out.
2. *ORF quality*. It is important to sequence the ORFs that are obtained to ensure that the sequence is correct and to know which isoform was cloned. Ensure that the ORF does not possess undesirable mutations, such as premature stop codons. For larger ORFs, multiple overlapping sequencing primers should be used for complete coverage of the coding sequence.
3. *LR reactions*. Run a 0.8% agarose gel to verify insertion of the gene and avoid incorrect clones from self-recombination. Since the lentiviral DNA contains repeats that are sometimes unstable and may recombine between repeats, we recommend using the following growth conditions to reduce the chances of recombination between direct repeats: (1) culture transformants at 25–30°C; (2) do not use “rich” bacterial media as they tend to give rise to a greater number of unwanted recombinants; and (3) if the plasmid confers chloramphenicol resistance, select and culture transformants using LB medium containing 15–30 µg/ml chloramphenicol in addition to the antibiotic appropriate for selection of plasmids.
4. *Selection*. It is important to determine the optimal concentrations of selection drugs for different cell lines. For example, the transduced cells were selected with puromycin at a concentration of 1 µg/ml for mouse R1 embryonic stem cells and 2 µg/ml for human HEK293 cells for a minimum of 48 h.
5. *High- and low-salt cell lysis buffers*. The advantage of carrying out cell lysis in a buffer with a high salt concentration (420 mM NaCl) is that it efficiently disrupts the nuclear membrane, thus permitting better identification of nuclear complexes. However, its disadvantage is the possible loss of some salt-sensitive interactions. Therefore, a buffer with a low salt concentration (approximately 100 mM NaCl) is the best choice for preparing cell lysates when studying cytoplasmic proteins or weak interactions.
6. *Protein sample dialysis*. To rescue and recover protein interactions disrupted by high salt concentrations, sample dialysis could be carried out to reduce the salt concentration after the cell lysis step. Dialysis of samples with a low-salt buffer reduces the salt concentration, resulting in the potential recovery of some interactions. However, dialysis may also increase the purification

background. Dialysis is carried out with 10 mM HEPES pH 7.9, 100 mM NaCl, 0.1 mM EDTA, and 20% glycerol in a Spectra/Por Membrane (MWCO 3500) for 4 h at 4°C, followed by centrifugation at  $100,000\times g$  for 30 min at 4°C.

7. *Treatment of cell extracts with nuclease.* For protein–RNA/DNA interaction studies, avoid the use of benzonase. Instead, when studying protein–RNA interactions, for example, add RNase inhibitors and carry out the purification in DEPC-treated water to keep the RNA intact.
8. *Tags interfering with protein interactions.* In some cases, the tag may interfere with protein interactions (9). One of the possible solutions is to add the tag at the other terminus of the ORFs (i.e., test clones with both N- and C-terminal tags). Alternatively, multiple subunits of a protein complex can be tagged.
9. *Tag choice for purifications.* The VA tag is compatible with single, dual, or triple affinity purification schemes. We have found that dual purification from HEK293 cell lysates by FLAG followed by Strep III yields less background and higher spectral counts for purified proteins than do the FLAG–His purifications.
10. *Tag affecting cell viability.* In rare cases, the tag itself could affect cell viability. For example, a tag that interferes with the essential function of a protein, resulting in loss of function, could have a dominant negative effect that reduces cell viability. Such cases may require the use of alternative tagging strategies, such as fusing the tag to the opposite end of the protein or using an inducible promoter system.
11. *Number of cells used for purification.* The choice of purification method affects the requirement of starting materials for purification. As well, the expression level of the tagged protein also affects the number of cells that are required, (i.e., the higher the expression, the fewer cells that are needed). The lentivirus-based system typically uses approximately  $10^8$  cells for starting material, similar to other methodologies described elsewhere (5).
12. *Inducible promoter.* One possible effect of bait overexpression is the formation of nonbiologically relevant interactions, that is, interactions that do not occur under endogenous contexts. Moreover, overexpression of certain genes can affect cell viability (e.g., negative regulators of the cell cycle). In such instances, an inducible promoter is a viable option, where the bait expression level can be modulated by the addition of an inducer, such as tetracycline for the tetracycline-inducible promoter (9). This type of system permits experimental control of bait expression, both in terms of amount and timing.
13. *Endogenous tagging.* As an alternative to an inducible promoter, an ideal solution is to insert the tag into the endogenous gene by homologous recombination (16). Although endogenous

tagging links the tagged construct to the endogenous promoter, resulting in more or less endogenous expression levels, the success rate for endogenous tagging in mammalian systems is extremely low.

14. *Cell type-specific promoter activities*. Certain promoters exhibit cell/tissue type-specific activation or restriction (17). For example, the cytomegalovirus (CMV) promoter is active in many mammalian cells, but not in human and mouse ES cells. In contrast, phosphoglycerate kinase (PGK) and human elongation factor-1 $\alpha$  (EF1 $\alpha$ ) promoters are transcriptionally active in ES cells (9, 17). Therefore, care should be taken to choose the optimal promoter.
15. Integration site, copy number, and lentivirus biosafety (see lentiviral shRNA screening).
16. *Multiplicity of infection (MOI)*. (See lentiviral shRNA screening.) Try to transduce a minimum number of lentiviruses into target cells. Low MOI keeps the expression levels lower and reduces the cell-to-cell variation of expression.

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