Chapter 15

Affinity Proteomic Analysis of the Human Exosome and Its Cofactor Complexes

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Abstract

In humans, the RNA exosome consists of an enzymatically inactive nine-subunit core, with ribonucleolytic activity contributed by additional components. Several cofactor complexes also interact with the exosome—these enable the recruitment of, and specify the activity upon, diverse substrates. Affinity capture coupled with mass spectrometry has proven to be an effective means to identify the compositions of RNA exosomes and their cofactor complexes: here, we describe a general experimental strategy for proteomic characterization of macromolecular complexes, applied to the exosome and an affiliated adapter protein, ZC3H18.

Key words Exosome, Cofactors, Affinity capture, Mass spectrometry, Affinity proteomics

1 Introduction

The RNA exosome is a multisubunit protein complex harboring 3′-5′ exoribonucleolytic and endoribonucleolytic activities [1]. It consists of a nine-subunit core, which associates with the exonuclease Rrp6p in budding yeast/EXOSC10 in human, and the exonuclease and endonuclease Dis3p/DIS3, respectively. The exosome core is evolutionarily conserved, including an orthologous complex in archaea [2] (*see* Chapter 4). However, there are species-dependent differences in the use of the catalytic subunits and cofactors (reviewed in [3–6]). For example, in the budding yeast *S. cerevisiae*, Rrp6p associated exosomes are restricted to the nucleus [7], whereas exosomes with Dis3p can be found throughout the cell [8]. In mammalian cells, EXOSC10 is prevalent in the nucleus and several reports also demonstrate its presence in the cytoplasm [9–11]. DIS3 is present in the nucleus and the cytoplasm but excluded from the nucleolus and the DIS3 homologue DIS3L associates with the exosome core only in the cytoplasm [11, 12]—an extended description of the organism specific differences in exosome-cofactor associations is provided in Chapter 11. The

John LaCava and Štěpánka Vaňáčová (eds.), *The Eukaryotic RNA Exosome: Methods and Protocols*, Methods in Molecular Biology, vol. 2062, https://doi.org/10.1007/978-1-4939-9822-7_15,

exosome has been shown to have a role in decay of virtually all classes of RNA, in the nucleus and cytoplasm alike, partaking in complete degradation of some transcripts and partial trimming/processing of the others. Such prolific yet controlled activity is thought to be achieved by cooperation of the exosome with its cofactor complexes and adaptor proteins, which modulate its activity.

S. cerevisiae has been the predominant workhorse of exosome research for the same reasons budding yeast has, generally, been an indispensable tool for genetic and biochemical research. However, presumably as a result of greater complexity in RNA processing, mammalian exosomes associate with cofactor complexes lacking obvious, direct orthologs in *S. cerevisiae*. Recently characterized examples include the *n*uclear *ex*osome *t*argeting (NEXT) complex and the *p*oly(*A*) e*x*osome *t*argeting (PAXT) complex [13, 14]. NEXT is composed of the exosome RNA helicase MTR4 (MTREX; this helicase robustly coprecipitates with the human exosome as well as with the NEXT complex during affinity capture), zinc finger CCHC domain-containing protein 8 (ZCCHC8), and RNA-binding protein 7 (RBM7). NEXT was shown to mediate exosomal degradation of promoter-upstream transcripts (PROMPTs) and enhancer RNA (eRNA) as well as processing of small nuclear/nucleolar RNAs (sn/snoRNA) [13, 15, 16]. PAXT contains MTREX, zinc finger C3H1 domain-containing protein (ZFC3H1), and poly(A)-binding protein 2 (a.k.a. nuclear poly(A)-binding protein 1; PABPN1). PAXT is implicated in degradation of polyadenylated RNA [14, 17]. Hence, for biomedical researchers, there is a strong motivation to shift exosome/cofactor research toward human cells (*see* Chapter 1).

In this chapter, we provide protocols for (1) establishing cell lines expressing a 3xFLAG affinity-tagged exosome component, EXOSC10, as well as a recently described cofactor ZC3H18, (2) their affinity capture, (3) proteomic analyses by mass spectrometry (MS), and (4) assessment of exosome enzymatic activity [18, 19] (Fig. 1). We use Flp-In T-REx 293 cells for stable genomic integration of the gene of interest (GOI) via Flp recombinase. To avoid overexpression artifacts, controlled expression of the GOI at a near-endogenous level is induced with tetracycline (Tet; or an appropriate analog). Cells expressing the tagged GOI are cryomilled, offering thorough breakage and other favorable properties for affinity capture (many described within refs. 20–22). The cell powder can be stored for at least several years at −80 °C (or colder) without apparent alterations in results obtained (in cases we have observed thus far), permitting comparisons of samples across many batches, for example, for quality control and the reproduction of historical experiments from the same sample. For affinity capture, we use antibody-coupled superparamagnetic beads; these beads provide a number of advantages over porous resins, including the

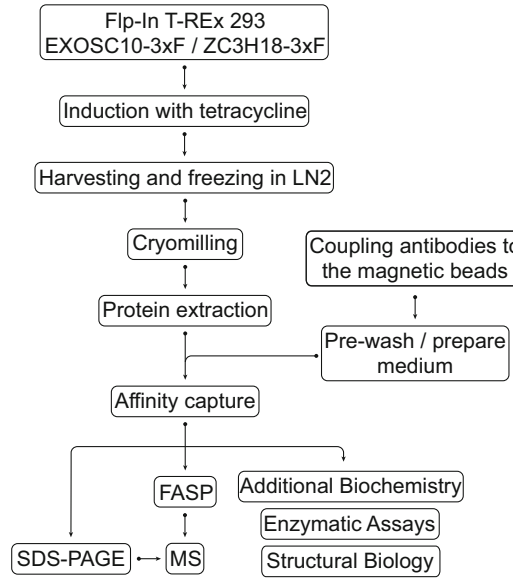

Fig. 1 A schematic diagram presenting a workflow for affinity capture and downstream analyses of native complexes isolated from Flp-In T-REx 293 cell lines stably expressing C-terminally 3xFLAG-tagged exosome component, EXOSC10, or exosome cofactor protein, ZC3H18

ability to bind complexes that may be excluded from pores and reduced nonspecific binding (further described in refs. 20–23). Exosomes isolated via these affinity capture protocols have been analyzed by mass spectrometry, in vitro ribonuclease assays, and negative stain electron microscopy [18, 24, 25]. Applying the same approach to ZC3H18-3xFLAG led to the discovery of its membership in compositionally and functionally distinct complexes [19].

2 Materials

In some cases we have indicated a specific reagent supplier or instrument manufacturer because we have worked with that particular company's product. In many such cases an alternative reagent or instrument of equivalent quality/functionality will suffice.

2.1 Producing Tagged Cell Lines in Flp-In T-REx 293 System

1. DMEM medium.
2. Tetracycline-free FBS.
3. 100× Pen/Strep; P/S (10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin).
4. Phosphate-buffered saline (PBS).
5. Trypsin solution suitable for cell culture.
6. Transfection reagent (e.g., Lipofectamine 2000).

7. RPMI medium.
8. Hygromycin.
9. Blasticidin.
10. Tetracycline.
11. Dimethyl sulfoxide (DMSO).
12. Liquid nitrogen (LN_2).
13. SDS extraction solution: 40 mM Tris–Cl pH 8.0, 2 mM EDTA, 1% (w/v) SDS.
14. Protease inhibitors.
15. Ethanol.
16. Cell culture plates and flasks.
17. 1.5 mL microtubes and 15 mL conical tubes.
18. Cryotubes.
19. Cell lifter/scraper.
20. Laminar flow hood.
21. Cell culture CO_2 incubator set to 37 °C.
22. Water bath set to 37 °C.
23. Centrifuge with appropriate rotor.

2.2 Cryogenic Cell Milling

1. Ice pan.
2. Ice-cold PBS.
3. Large cell scraper.
4. 50 mL conical tubes.
5. Centrifuge equipped with a 50 mL conical tube rotor.
6. 20 mL Luer-lock syringes with end caps.
7. Styrofoam box.
8. LN_2.
9. RETSCH Planetary ball mill PM 100.
10. RETSCH Stainless steel milling balls 20 mm diameter.
11. RETSCH Stainless steel "comfort" milling jars 50 mL and/or 125 mL.
12. PTFE jar insulator.
13. Stainless steel spatulas.
14. Large stainless steel tweezers.
15. Stainless steel measuring spoons for small amounts.

2.3 Affinity Capture of the Exosome and Cofactors

1. Magnetic medium (Dynabeads M-270; Thermo Fisher Scientific).
2. Anti-FLAG M2 antibodies (Millipore Sigma).

Table 1
Composition of solutions used in AC of EXOSC10-3xFLAG and ZC3H18-3xFLAG

Bait protein	Solution composition	Solution name	AC characteristic
EXOSC10-3xFLAG	20 mM HEPES-Na pH 7.4, 300 mM NaCl, 1% (v/v) Triton X-100, 1× protease inhibitor cocktail	ExoI	DIS3− exosomes
EXOSC10-3xFLAG	20 mM HEPES-Na pH 7.4, 150 mM NaCl, 5 mM CHAPS, 1× protease inhibitor cocktail (*see* **Note 1**)	ExoII	DIS3+ exosomes
EXOSC10-3XFLAG	20 mM HEPES-Na pH 7.4, 100 mM $MgCl_2$, 1% (v/v) Triton X-100, 1× protease inhibitor cocktail	ExoIII	ZCCHC8 enriched
ZC3H18-3xFLAG	20 mM HEPES-Na pH 7.4, 100 mM NaCl, 0.5% (v/v) Triton X-100, 1× protease inhibitor cocktail, 1× phosphatase inhibitor	Low salt	NEXT
ZC3H18-3xFLAG	20 mM HEPES-Na pH 7.4, 300 mM NaCl, 0.5% (v/v) Triton X-100, 1× protease inhibitor cocktail, 1× phosphatase inhibitor	Medium salt	NEXT and histone proteins
ZC3H18-3xFLAG	20 mM HEPES-Na pH 7.4, 500 mM NaCl, 0.5% (v/v) Triton X-100, 1× protease inhibitor cocktail, 1× phosphatase inhibitor	High salt	Histone proteins

3. Minicentrifuge.
4. Magnetic separator for microcentrifuge tubes.
5. Vortex.
6. Nutating mixer or orbital shaker.
7. 0.1 M sodium phosphate buffer pH 7.4.
8. 3 M ammonium sulfate, prepared in 0.1 M sodium phosphate pH 7.4
9. 100 mM glycine–HCl pH 2.5.
10. 10 mM Tris–Cl pH 8.8.
11. 100 mM triethylamine solution (freshly prepared).
12. 0.5% (v/v) Triton X-100 in PBS.
13. Affinity medium storage solution A: 50% (v/v) glycerol in PBS.
14. Affinity medium storage solution B: 0.5 mg/mL BSA, 50% (v/v) glycerol in PBS.
15. Balance.
16. Stainless steel spatula.
17. Protein extraction solutions (*see* Table 1).
18. Protease inhibitors (EDTA-free).
19. Phosphatase inhibitors.
20. Microprobe sonicator.

21. Benchtop centrifuge with a microtube rotor.
22. Rotator, rocker, nutator, mixer, or other agitation device.
23. 4× LDS sample buffer (Thermo Fisher Scientific).
24. 500 mM dithiothreitol (DTT).
25. 1 M iodoacetamide (IAA).
26. 3xFLAG peptide.

2.4 SDS-PAGE and Staining

1. NuPAGE 4–12% Bis-Tris protein gel (Thermo Fisher Scientific).
2. X-Cell SureLock Electrophoresis system (Thermo Fisher Scientific).
3. 1× MOPS running buffer.
4. Protein marker.
5. Sturdy spatula or "gel knife."
6. 0.1 M HCl.
7. Blue Silver protein stain.
8. Orbital shaker.

2.5 Basic Mass Spectrometry Analysis

Solutions and reagents used in this section should be of the highest grade available. Mass spectrometry grade solutions must be used for all manipulation using digested peptides.

1. Acetic acid.
2. 5.5 M ammonium hydroxide.
3. Centrifugal filter unit Microcon-30 kDa (Millipore Sigma).
4. UA∗: 8 M urea, 0.1 M Tris–Cl pH 8.5, 0.5% PEG 35.000.
5. UA: 8 M urea, 0.1 M Tris–Cl pH 8.5.
6. IAA solution: 50 mM iodoacetamide in UA.
7. 50 mM ammonium bicarbonate (ABC).
8. Trypsin, mass spectrometry grade.
9. 0.5 M NaCl.
10. 20% (w/v) trifluoroacetic acid (TFA).
11. 15 cm diameter petri dishes with lid.
12. 0.1 M HCl.
13. Blue silver protein stain.
14. Clean razor blades.
15. Destaining solution: 50 mM ammonium bicarbonate in 50% (v/v) acetonitrile, prepared in LC-MS grade water.
16. Acetonitrile (ACN).
17. OMIX tips (C18, 100 μL).

18. Vacuum centrifuge (e.g., "SpeedVac").
19. Humidified incubator with a rack for microfuge tubes.
20. Thermal shaker (e.g., Thermomixer).
21. Vortex (equipped with multitube holder—optional).
22. Orbital shaker.
23. Incubator set to 37 °C.

2.6 RNase Activity Assay

1. Gradient solution "light": 10% (v/v) glycerol, 20 mM HEPES-Na pH 7.4, 100 mM NaCl.
2. Gradient solution "heavy": 40% (v/v) glycerol, 20 mM HEPES-Na pH 7.4, 100 mM NaCl.
3. Syringes.
4. 0.45 μm syringe filters.
5. Stainless steel cannula.
6. Gradient Master and accessories for SW 55 Ti (BioComp).
7. Centrifuge tubes (13 × 51 mm; Seton, preferentially obtained from BioComp).
8. Balance.
9. Ultracentrifuge.
10. SW 55 Ti rotor (Beckman Coulter).
11. Piston Gradient Fractionator and accessories for SW 55 Ti (BioComp).
12. Sensitive protein stain (e.g., silver or SYPRO Ruby).
13. Recapture solution: 20 mM Tris–Cl pH 8.0, 100 mM NaCl, 0.01% (v/v) Triton X-100.
14. Anti-FLAG magnetic beads.
15. 2× reaction solution: 20 mM Tris–Cl pH 8.0, 20 mM DTT, 0.5 mM $MgCl_2$, 5% (v/v) RNasin® ribonuclease inhibitor, 0.4 pmol/μL RNA oligo substrate.
16. The RNA oligo substrate: HPLC-purified, 34-mer with the sequence: 5′-CCUAUUCUAUAGUGUCACCUAAAUGCUAGAGCUC-3′ (a 5′ -truncated form of the generic substrate presented in [26]) synthetized with a 5′-6-FAM (fluorescein amidite) group to enable fluorescence detection [18] (*see* **Note 2**).
17. 2× RNA loading buffer: 95% formamide, 20 mM EDTA, 1% (v/v) DNA loading dye.
18. UreaGel 29:1 Denaturing Gel System (National Diagnostics).
19. 1× TBE buffer: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA.
20. Syringe with a bent needle.
21. Rotator, rocker, nutator, mixer or other agitation device.

22. Magnetic separator for microcentrifuge tubes.
23. Thermal shaker.
24. Fluorescein detecting imager (e.g., SYBR green setting on Fuji LAS series).

3 Methods

3.1 Producing Tagged Cell Lines in Flp-In T-REx 293 System

The Flp-In T-REx 293 system allows the generation of stable mammalian cell lines exhibiting tetracycline-inducible expression of a gene of interest from a specific genomic location. It is based on three main components: (1) the Flp Recombinase Target (FRT) site; (2) the Tet repressor (TetR); and (3) integration of an expression vector containing the gene of interest (GOI) under the control of a tetracycline-inducible promoter into the genome via Flp-mediated DNA recombination at the FRT site [27]. Currently, Thermo Fisher Scientific offers a ready-to-use (HEK)293 cell line bearing the FRT site and the Tet repressor gene (Flp-In T-REx 293) and we have successfully used it to generate the EXOSC10-3xFLAG and ZC3H18-3xFLAG stable cell lines [15, 21]. The resulting cell lines possess an additional copy of the affinity-tagged GOI at the FRT locus, whose expression is suppressed in the absence of Tet and induced in the presence of it [28]. If desired, virtually any cell line can be adapted to work with this system following the manufacturer's protocol. In this section, we provide protocols for establishing and maintaining Flp-In T-REx 293 stably expressing a GOI.

3.1.1 Cell Culture

1. *Before starting.*

   The Flp-In T-REx 293 is an adherent cell line. For most routine work, we grow it in standard cell culture conditions at 37 °C and 5–8% CO_2. Methods are summarized here; refer to standard cell culture texts for detailed descriptions [29, 30]. Cells are maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Tet-free fetal bovine serum (FBS) and 1× Penicillin–Streptomycin (P/S). Phosphate-buffered saline (PBS) is used to rinse cell layers and trypsin is used to detach adherent cells from the bottom of the flask. Growth media, PBS and trypsin are stored at 4 °C and warmed up to 37 °C before adding to the cells (DMEM-FBS should be used within 2–4 weeks when stored at 4 °C). All solutions and equipment that come in contact with the cells must be sterile. Therefore, always work with cells in a laminar flow hood and sterilize everything that goes inside the hood (bottles of media, flasks with cells, plastics, pipettes, your gloved hands, etc.) by spraying with 70% ethanol.

2. *Thawing cells.*
   (a) Prepare a 15 mL tube with ~5 mL prewarmed DMEM-FBS-P/S.
   (b) Thaw a frozen cell aliquot at 37 °C and quickly transfer it to the media-containing tube.
   (c) Centrifuge at 200 × *g* for 5 min to pellet the cells.
   (d) Discard the supernatant (*see* **Note 3**).
   (e) Resuspend the cell pellet in ~5 mL of fresh DMEM-FBS-P/S.
   (f) Transfer the cell solution to a T-75 (75 cm^2) flask.
   (g) Add ~5 mL of DMEM-FBS-P/S to the flask to make the total volume of 10 mL.
   (h) Mix well by moving the flask up and down, right and left several times to distribute cells evenly in the flask (*see* **Note 4**).
   (i) Incubate the cells at 37 °C and check them daily until they are 80–100% confluent (2–7 days).
3. *Passaging and plating cells.*

   Cells are ready to be split when they reach ~90–100% confluence, which is when they fully cover the bottom of the flask. It is recommended to split cells two to three times a week. Generally, cells are split 1:10, but this can be adjusted depending on the needs.

   (a) Remove all medium from the flask.
   (b) Add ~5 mL of PBS to one corner of the flask and rinse cells by gently tilting the flask (*see* **Note 5**).
   (c) Remove PBS.
   (d) Add 1 mL of trypsin and distribute evenly over the bottom of the flask.
   (e) Incubate the cells at 37 °C until they are detached but no longer than 5 min (*see* **Note 6**).
   (f) Add 9 mL of DMEM-FBS-P/S to inhibit trypsin activity.
   (g) Rinse the bottom of the flask with the added medium to collect all the cells and transfer the cells to a 15 mL tube.
   (h) Centrifuge at 200 × *g* for 5 min to pellet the cells and discard supernatant (medium with trypsin).
   (i) Add 10 mL of DMEM-FBS-P/S and pipette the solution up and down a few times to break cell aggregates.
   (j) If necessary, count cells using your method of choice (e.g., a hemocytometer).

(k) Transfer a desired amount of the cell suspension to a new flask or dish (1 mL when splitting 1:10), add ~9 mL of fresh medium and mix well (*see* **Note 7**).

(l) Incubate at 37 °C and split again when cells reach full confluence.

4. *Harvesting cells.*

(a) Remove medium from the culture dish.

(b) Wash cells 1× with ice-cold PBS and remove waste (~5 mL of PBS per ~50 cm^2 surface area).

(c) Add 1 mL of ice-cold PBS to the dish, scrape cells using a cell lifter and collect them on one side of the dish.

(d) Transfer the cell suspension to a tube of appropriate size.

(e) Centrifuge at 2500 × *g*, for 5 min at room temperature.

(f) Remove supernatant and flash-freeze the cell pellet in LN_2 (for harvesting large quantities of cells *see* Subheading 3.2.1).

5. *Freezing cells.*

(a) Trypsinize cells as above.

(b) Transfer resuspended cell suspension to a 15 mL tube.

(c) Centrifuge at 200 × *g* for 5 min to pellet the cells.

(d) In the meantime, prepare five labeled 2 mL cryotubes and FBS + 10% (v/v) DMSO (e.g., 9 mL FBS + 1 mL DMSO).

(e) After centrifugation, remove the supernatant and resuspend the cell pellet in 7.5 mL FBS-DMSO.

(f) Quickly aliquot 1.5 mL of the cell suspension into each cryotube.

(g) Make sure the tubes are tightly closed, put them into a cryobox and move to −80 °C freezer.

(h) Next day, transfer the cell stocks from the −80 °C freezer to the liquid nitrogen (LN_2) tank for long-term storage.

3.1.2 Preparing Plasmids

Stable integration of the GOI in the genome of the Flp-In T-REx 293 cell line requires cotransfection of two plasmids: (1) pOG44, which encodes the Flp recombinase and (2) an expression plasmid pcDNA5/FRT/TO bearing the coding sequence of the GOI. Both the pOG44 and the backbone pcDNA5/FRT/TO plasmids are available from Thermo Fisher Scientific. The sequence of the GOI can be cloned in the pcDNA5/FRT/TO vector using standard cloning techniques. However, it does not contain any purification tag, therefore, the sequence of the tag of choice must be cloned in together with the GOI. Alternatively, available on Addgene (https://www.addgene.org), there is a wide selection of

pcDNA5/FRT/TO based vectors already containing sequences of various tags and adapted for use with alternative cloning systems such as the Gateway system [31].

3.1.3 Transfection and Selection

This section presents our optimized protocol for transfection using Lipofectamine 2000 (Thermo Fisher Scientific).

1. Plate ~10^6 cells in 5 mL DMEM-FBS without P/S in a 6 cm plate (*see* **Note 8**).
2. 24 h later prepare transfection mixtures:
   (a) Add 10 μL Lipofectamine 2000 to 500 μL RPMI 1640 medium and mix by flicking the tube.
   (b) Add 9 μg of pOG44 and 1 μg of pcDNA5/FRT/TO plasmids to 500 μL RPMI 1640 medium and mix by flicking the tube.
3. Incubate the mixtures for 5 min at room temperature.
4. Combine two mixtures and pipette up and down several times to mix them well.
5. Incubate for 15–20 min at room temperature.
6. Add dropwise 1 mL of the transfection reaction to the cells and mix by rocking the plate.
7. 24 h post-transfection, trypsinize the cells and place them in a T-175 flask (175 cm^2) without antibiotics. It is important to resuspend cells well before plating in order to avoid cell aggregates (cells grown in aggregates are less susceptible to the antibiotics).
8. Approximately 3 h later, when cells are attached to the bottom of the flask, remove antibiotic-free medium and add medium containing 100 μg/mL hygromycin B and 10 μg/mL blasticidin (and 1× P/S).
9. Incubate cells at 37 °C, changing medium every 4–5 days (this removes dead cells and supplies fresh antibiotics).
10. After approximately 2 weeks white-colored colonies should appear.
    (a) *Bulk population vs. single clones.*

        At this point, one can pool all colonies together and grow them in a mixed, so called, bulk population. It is also possible to pick single colonies and transfer them to separate dishes to grow monoclonal populations. Because the Flp-In T-REx 293 cells contain a single integrated FRT site, the pcDNA5/FRT/TO expression vector should integrate into the same genomic locus in every clone and all of the hygromycin-resistant foci that are obtained after cotransfection with the pcDNA5/FRT/TO expression vector and pOG44 should be identical. In our experience,

pooling clones together is sufficient to obtain an apparently homogenous population of cells.

- Add 2 mL of prewarmed trypsin to the flask and incubate for 5 min at 37 °C.
- Tap gently on the bottom of the flask to help colonies detach.
- Add 15 mL of DMEM-FBS-P/S to stop trypsinization.
- Pipette the solution up and down several times to ensure decomposition of colonies into a single cell suspension.
- Grow continuously until the flask is confluent and cells can be used in downstream manipulations.

3.1.4 Tetracycline Induction

In the Flp-In T-REx 293 system, ectopic expression of the GOI is suppressed by TetR in the absence of Tet and induced upon addition of Tet or Tet derivatives, such as doxycycline (Dox), to the media [28]. To limit potential artifacts from ectopic expression, such as the formation of spurious nonphysiological complexes with the tagged bait protein, its expression should be limited to near-physiological quantities [32]. This is achieved by titrating induction of the GOI.

While some Tet-based systems respond better to Dox [33], we have observed comparable results using both Tet and Dox as inducers of T-REx-driven transgenes, when used in the 1–5 ng/mL range. Dox is reported to have greater stability in stock solutions and a longer half-life in tissue culture medium than Tet [34, 35]. Others have reported Tet to permit finer tuning of expression from T-REx promoters than Dox; this effect was reported for concentrations in excess of 5 ng/mL [36].

This protocol describes the Tet titration experiment which is performed in order to find the Tet concentration that induces physiological expression of the GOI. Increasing concentrations of tetracycline are added to the cells over the fixed amount of time, typically 24 h. After this time cells are collected and protein extracts are prepared for WB analysis comparing levels of endogenous protein and its tagged-counterpart. The success of this approach relies mostly on two factors: (1) good separation of native and tagged proteins on SDS-PAGE in order to convincingly distinguish the two forms of the protein and (2) availability of a good antibody recognizing both endogenous and tagged proteins.

1. Plate ~10^5 cells in 5 mL of DMEM-FBS-P/S media in each of the six 6 cm dishes.
2. Next day, add tetracycline at the following concentrations: 0, 2, 10, 50, 100, 500 ng/mL (*see* **Note 9**).

3. After 24 h, collect cells by scraping them and moving to 1.5 mL tubes.
4. Extract proteins with SDS extraction solution supplemented with 1× protease inhibitors.
   (a) Add 100 µL of SDS extraction solution and incubate on ice for 10 min.
   (b) Centrifuge at max speed, for 10 min at 4 °C.
   (c) Transfer the supernatant (clarified protein extract) to a fresh tube.
5. Prepare SDS-PAGE samples by adding 10 µL of 4× LDS buffer and 4 µL of 10× Reducing Agent (or 500 mM DTT) to 26 µL of the protein extract (total sample volume is 40 µL). Heat the samples for 10 min at 70 °C.
6. Load 20 µL of the samples on a NuPage 4–12% Bis-Tris gel and run it at 200 V until the protein marker reaches the bottom of the gel cassette (50–60 min).
7. Transfer proteins on the membrane using your method of choice.
8. Perform WB analysis suitable for your protein of interest.

3.2 Cryogenic Cell Milling

This protocol is optimized for up to 10 grams of cells (~10^9 cells) grown on 8–16 large 500 cm^2 dishes [21, 22, 37]. To produce endogenous human RNA exosomes at large-scale, we conditioned normally adherent Flp-In T-REx 293 cells expressing tetracycline inducible 3xFLAG-tagged EXOSC10 to suspension growth, which yielded 10 g wet cell weight (WCW) per 400 mL of culture medium [37, 38].

3.2.1 Harvesting and Freezing Cells

1. Plate ~10^7 cells on each of 16 × 500 cm^2 square dishes in 90 mL DMEM-FBS-P/S.
2. When the cells reach ~90% confluency, induce the expression of the GOI by adding fresh DMEM-FBS-P/S supplemented with tetracycline at a desired concentration identified in the titration experiment.
3. 24 h post induction, remove the growth medium by pouring it off into a large beaker.
4. Place the culture dish on ice in a large rectangular ice pan.
5. Add 20 mL of ice-cold PBS to the dish and release the cells from the dish using a large cell scraper; transfer the cells to a 50 mL tube, prechilled on ice; hold the tube on ice (*see* **Note 10**).
6. Add an additional 10 mL of ice-cold PBS to the same dish. Collect the remaining cells and transfer them to the 50 mL tube.

7. Repeat for each dish; cell suspensions from different dishes may be combined to reduce sample number and plastic waste (*see* **Note 11**).
8. Centrifuge at 1000 × *g*, for 5 min at 4 °C.
9. Carefully pour off the supernatant. Resuspend each pellet in 10 mL ice-cold PBS. Consolidate the resuspended pellets, up to 5 per 50 mL tube, to reduce sample number.
10. Centrifuge at 1000 × *g*, for 5 min at 4 °C.
11. Carefully pour off the supernatant. Resuspend the pellet in 10 mL ice-cold PBS.
12. Remove the plunger from a 20 mL syringe and set it aside. Cap the nozzle of the syringe and transfer the cell suspension to it.
13. Place the syringe inside a 50 mL tube and centrifuge at 1000 × *g*, for 5 min at 4 °C.
14. Aspirate the supernatant. This results in a wet cell pellet.
15. Uncap the syringe, insert the plunger and drip the cells directly into a 50 mL tube filled with LN_2, held in an LN_2 bath in a Styrofoam box. Forcibly plunge the remaining cells from the syringe (*see* **Note 12**).
16. Loosely cap the tubes to allow excess LN_2 to evaporate; hold them overnight at −80 °C. Tighten caps fully the next day. The frozen cell pellet may be stored in this way at −80 °C until cryomilling (*see* **Note 13**).

3.2.2 Milling

We cryomill cells under LN_2 in a Retsch PM 100 planetary ball mill; model PM 100 CM is also suitable. Depending on the quantity of cells to mill, we either use a 50 mL jar (~1–8 g cells) or a 125 mL jar (~5–30 g cells). We consider 1 g WCW to be the effective minimum quantity of cells for milling and retrieval of powder because ~300 mg may be lost to caking on the surfaces of the balls and jar; to offset these losses and maximize the percentage of input material recovered, we aim to produce at least 1.5–3 g WCW. Custom-made polytetrafluoroethylene (PTFE) insulators minimize warming of the sample during milling and improve safety and performance—different insulators and their relative merits have been previously described [22]– the below protocol assumes the use of "puck" insulators. We use a homemade LN_2 decanter made using a spatula and 50 mL conical tube to pour LN_2 into and over the milling jars [21]. Pressure from gaseous N_2 can build up to high levels within the jar during milling; an optional additional safety precaution is to install a commercially available 5 bar/500 kPa pressure valve on the milling jar lid.

1. Pre-clean milling jar, lid, balls, two small steel spatulas, and large tweezers using Windex glass cleaner or similar. For a

50 mL jar, use two 20 mm diameter balls. For a 125 mL jar, use five 20 mm diameter balls.

2. Weigh the jar+insulators+balls and adjust PM 100 counterbalance accordingly.
3. Precool the jar, balls, spatulas, tweezers, LN_2 decanter, and PTFE insulators in a clean Styrofoam box or ice pan containing LN_2, until the LN_2 stops boiling. Set up a second ice pan for working in, also with LN_2.
4. Transfer the frozen cell pellets into the milling jar.
5. Fill with LN_2 to within ~0.5–1 cm of the top. Cover with the lid.
6. Transfer the cold PTFE base to the grinder.
7. Move the assembled jar and lid onto the mill.
8. Position the Teflon top insulator on the lid and clamp in place.
9. Pour LN_2 over the jar using the decanter.
10. Cryomill with three cycles of the following program: 400 rpm, 3 min, reverse rotation every 30 s, no interval breaks. Between milling cycles, cool the jar as below:
    (a) Pour LN_2 over the jar using the decanter to cool the lid and top.
    (b) Some pressure will have built up during the milling. The jar may be gently hissing as pressure escapes: this is normal. Carefully remove the jar and transfer to the pan of LN_2 to recool.
    (c) Remove the lid and use a spatula to scrape any adhered powder back into the jar. Do not scrape the gasket with a spatula: it will damage the PTFE seal. Submerse the lid to cool.
    (d) Use a spatula to scrape around the lower corners of the jar, dislodging any packed cells.
    (e) Refill the jar with LN_2, reassemble and repeat the cycle.
11. Carefully remove the jar and transfer it to the pan of LN_2.
12. Put a prelabeled 50 mL tube in a rack in the LN_2 pan. Remove steel balls with tweezers, scraping caked on powder back to the jar.
13. Transfer powder to conical tubes by pouring or with chilled spatulas or spoons. Once the sample is fully transferred, cap the tube loosely and move to a rack inside the Styrofoam box.
14. Store vertically at −80 °C overnight with the caps loose to allow LN_2 to evaporate, and then seal and store.

3.2.3 Storage and Handling of Cell Powder

Cell powder can be stored at −80 °C essentially indefinitely, without affecting performance.

1. Before taking the cell powder out from the freezer:
   (a) Prepare a container filled with LN_2 and place there a rack appropriate to hold the cell powder tube.
   (b) Precool a metal spatula, metal tweezers, and tubes used for weighting out the powder.
2. Take the tube with powder out of the freezer and place it in the rack in LN_2.
3. Weigh empty tubes—using cold tweezers, grab a tube and place it on the balance, note down its weight and place it back in LN_2 (*see* **Note 14**).
4. With a precooled metal spatula add a scoop of powder to the tube and place it back on the balance.
5. After the measurement is taken, put the tube with the powder back to the LN_2 to cool it down. If necessary, add more powder and remeasure (*see* **Note 15**).

3.3 Affinity Capture of the Exosome and Cofactors

In this chapter, we present a general protocol for affinity capture (AC) of 3xFLAG-tagged EXOSC10 and ZC3H18. The main difference in AC of these target proteins is the use of different protein extraction solutions, which were experimentally identified previously [18, 19]. Depending on the extraction solution used, AC of the same bait protein may yield different compositions within affiliated macromolecular complexes and different degrees of purity. We use a parallelized AC screen to identify working conditions for differential AC, the protocol for which can be found in [25].

3.3.1 Coupling Antibodies to the Beads

This protocol is for conjugating 150 μg of antibody to 15 mg of Dynabeads, resulting in 100 μL of slurry [21]. The amount of antibodies and magnetic beads can be scaled up or down without changing the volume of extraction solutions during the washing steps. Briefly, prepare 20 μL of 0.5 μg/μL antibody, in a sodium phosphate (pH 7.4) buffered 1 M ammonium sulfate solution, per mg of magnetic beads to be antibody-conjugated (i.e., 10 μg of antibody, in 20 μL solution, will be used per 1 mg of beads). All steps are performed at room temperature (except the conjugation which is performed at 37 °C).

1. Weight out 15 mg of Dynabeads M-270 Epoxy in 2 mL round bottom tube (*see* **Note 16**).
2. Wash beads with 1 mL of sodium phosphate buffer pH 7.4, vortex for 30 s and mix for 15 min on a nutating mixer or rotator.

3. Pulse-spin the tube in a minicentrifuge to collect all the solution at the bottom and place the tube on a magnetic tube rack. Wait until beads are collected at the side of the tube and remove the supernatant (using a pipet or an aspirator).
4. Add again 1 mL of sodium phosphate buffer, vortex for 30 s and remove buffer.
5. Prepare antibody mixture: For the conjugation, use approximately 20 μL total volume per mg beads. This includes the antibody solution, the sodium phosphate buffer, and ammonium sulfate. The ammonium sulfate should be added last. For example, to conjugate 15 mg beads, a total volume of approximately 300 μL is appropriate. For this:
   (a) Add sodium phosphate buffer to the antibody solution so that the final volume is 200 μL.
   (b) Add 100 μL of 3 M ammonium sulfate (to final concentration of 1 M). To avoid antibody precipitation, keep the tube with the antibody mixture in motion on a gentle vortex and add ammonium sulfate drop-wise.
6. Incubate antibody–beads mixture at 37 °C for 16–24 h with constant mixing.
7. Place the tube on a magnet, remove supernatant and wash beads with 1 mL of:
   (a) Sodium phosphate buffer (fast wash—add solution, vortex, place on a magnet, and discard supernatant).
   (b) 100 mM glycine pH 2.5 (fast wash).
   (c) 10 mM Tris–Cl pH 8.8 (fast wash).
   (d) Freshly prepared 100 mM triethylamine solution (fast wash).
   (e) PBS (4 washes, each 5 min with mixing).
   (f) 0.5% (v/v) Triton X-100 in PBS (15 min wash with mixing).
   (g) PBS (fast wash).
8. After the last wash, spin the beads down, place on a magnet and remove supernatant carefully.
9. Resuspend beads in 100 μL of affinity medium storage solution A or B (*see* **Note 17**).

3.3.2 Affinity Capture

This is a general protocol for affinity capture from 100 mg cell powder. It can be scaled up or down within 50–250 mg range. Using less than 50 mg may obscure the reproducibility of results due to the weighing errors. 250 mg is the maximum amount to be conveniently processed in a 2 mL microcentrifuge tube. Should you

use >250 mg, we recommend to perform multiple purifications in parallel and combine them before the downstream analysis.

1. Precool a metal spatula and 1.5 mL microcentrifuge tube in LN_2.
2. Weight out 100 mg of cell powder (*see* **Note 18)**.
3. Place the samples in the rack, open the caps, and let them stand at room temperature for 1 min.
4. Add 500 μL of the extraction solution supplemented with protease and phosphatase inhibitors (*see* Table 1).
5. Vortex at maximum speed to fully resuspend the cell powder (should not require more than 30 s), and immediately place the samples on ice (*see* **Note 19)**.
6. Briefly sonicate cell lysate to homogenize the cell powder. Use four pulses, 2 s each (~30 J total per 250 mg sample) [22] (*see* **Note 20)**.
7. Centrifuge at 20,000 × *g*, for 10 min at 4 °C to clarify the extract.
8. While the samples are in the centrifuge, add 10 μL of anti-FLAG beads slurry into a 1.5 mL microcentrifuge tube and wash twice with 1 mL of the extraction solution without inhibitors to thoroughly remove the storage solution (*see* **Note 21)**.
9. Add the clarified protein extract to the prewashed beads.
10. Incubate for 1 h with rotation at 4 °C (cold room).
11. Collect the beads on a magnet, remove the supernatant, and wash three times with 1 mL of ice-cold extraction solution. Move the sample to a fresh tube during the last wash (*see* **Note 22)**.
12. After the third wash, spin the sample briefly in a minicentrifuge. Place again on a magnet and carefully remove any remaining liquid.

3.3.3 Elution of the Affinity Captured Proteins from the Beads

During elution, the 3xFLAG-tagged protein of interest is released from the beads together with bound interactors. This can be achieved in many ways. In this chapter we cover a few examples: (1) by adding SDS-PAGE sample buffer (e.g., LDS), which will compromise most interactions, releasing the captured proteins into the eluate (described below), (2) by adding an excess of 3xFLAG peptide, which will compete for the binding sites on the magnetic beads, releasing the protein into the eluate (described below), or (3) by adding an acidic solution to compromise interactions and release proteins (*see* Subheading 3.5.1).

1. *Elution in denaturing conditions (1.1x LDS buffer).*
   (a) Add 26 μL of 1.1× LDS buffer to the beads and incubate at room temperature for 5–10 min with agitation (*see* **Note 23**).
   (b) Place the tube on a magnet and transfer the eluate to a fresh tube.
   - Samples may be frozen at −20 °C for brief storage (a few days) or at −80 °C for extended storage until analysis is desired.

   (c) Add 1.5 μL of 500 mM DTT to the eluate and incubate at 70 °C for 10 min.
   - If protein bands or gel regions are to be analyzed by mass spectrometry (MS) following the SDS-PAGE, e.g., [39] and as described in Subheading 3.5.2, add 3 μL of 1 M IAA (final concentration 100 mM) and incubate in the dark at room temperature for 30 min. Proceed to electrophoresis.

2. *Native elution with 3xFLAG peptide.*
   (a) Add 18 μL of 1 mg/mL 3xFLAG peptide, diluted from the stock in extraction solution, to the tube with beads.
   (b) Incubate the sample at room temperature for 15 min with gentle mixing.
   (c) Place on a magnet and transfer the eluate to a fresh tube.
   (d) Add 8 μL of 4× LDS.
   - Samples may be frozen at −20 °C for brief storage (a few days) or − 80 °C for extended storage until analysis is desired. If native assays such as glycerol gradient sedimentation and/or RNase assays will follow, *do not add LDS nor freeze the sample*. Hold on ice or 4 °C and proceed to the next manipulation.

   (e) Add 1.5 μL of 500 mM DTT and incubate at 70 °C for 10 min.
   - As outlined above, if MS will follow, add 3 μL of 1 M IAA (final concentration 100 mM) and incubate in the dark at room temperature for 30 min. Proceed to electrophoresis.

3.4 SDS-PAGE and Staining

To separate proteins, we routinely use the NuPAGE SDS polyacrylamide gel system (Thermo Fisher Scientific), which offers excellent stability and separation of the protein during electrophoresis. Another advantage of this system is a wide selection of precast gradient gels and buffer systems, which can be applied for better separation of proteins of variable sizes. To visualize proteins in the gel, we use the Blue Silver protein stain, which exhibits high

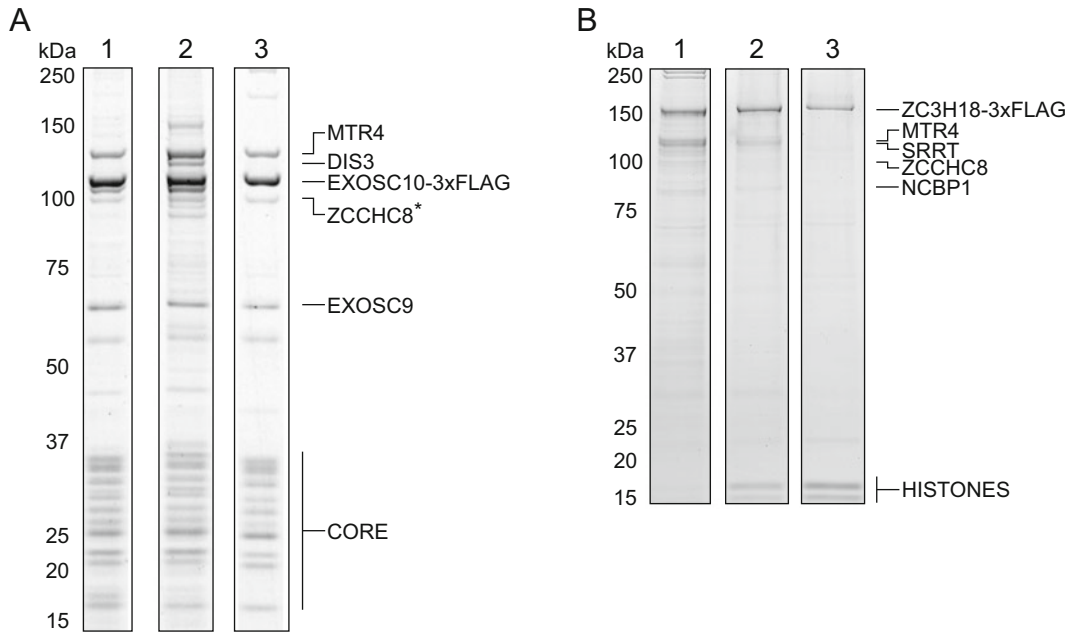

Fig. 2 Representative results of EXOSC10-3xFLAG and ZC3H18-3xFLAG affinity capture from Flp-In T-REx 293 cells. (**a**) SDS-PAGE analysis of proteins copurified with EXOSC10-3xFLAG in ExoI (lane 1), ExoII (lane 2), and ExoIII (lane 3) extraction solutions. The bait and select abundant coprecipitated proteins are labeled. Proteins were visualized by the Blue Silver staining. For details of extraction solutions composition, *see* Table 1. This figure panel modified from copurification.org [25]. ∗ ZCCHC8 was detected in the position labeled after affinity capture in solutions of 100 mM $MgCl_2$ (ExoIII) and 300 mM NaCl (ExoI), exhibiting greater abundance, as estimated by mass spectrometry, in the $MgCl_2$ condition. ZCCHC8 was not detected at 200 mM NaCl, in an otherwise similar solution to ExoI, and is therefore unlikely to be present in the band at the same position in lane 2 (ExoII). (**b**) SDS-PAGE analysis of proteins coprecipitated with ZC3H18-3xFLAG in a low, medium and high salt extraction solutions (lanes 1, 2, and 3, respectively). The bait and copurified components of the CBC-NEXT complex as well as histone proteins are labeled. Proteins were visualized by Blue Silver staining. For details of extraction solutions composition, *see* Table 1. This figure panel modified from [19]

sensitivity, revealing proteins down to the ~1–10 ng per band range [40] (composition and the recipe for the stain provided therein). We have added a gel prewashing procedure, carried out before adding the stain, useful for NuPAGE Bis-Tris gels, described below. For more sensitive detection of low abundance proteins, SYPRO Ruby or silver staining are also suitable (mind the latter if mass spectrometry compatibility is needed). Below, we present a protocol for SDS gel electrophoresis followed by the Blue Silver staining and show representative results of the EXOSC10- and ZC3H18-3xFLAG affinity captures under differential extraction conditions (Fig. 2).

1. Assemble a 4–12% Bis-Tris gel in the XCell SureLock apparatus according to the manufacturer's protocol and fill the tank with

1× MOPS running buffer (~200 mL in the inner chamber until the wells of the gel are fully submersed); ~600 mL in the outer chamber).

2. Load 30 μL of the sample and a protein marker.
3. Run the gel at 200 V until the protein marker reaches the bottom of the gel (50–60 min).
4. After electrophoresis, remove the gel cassette from the apparatus, disassemble the cassette and, using a clean spatula or gel knife, transfer the gel to a clean container with an excess of deionized water (≳50 mL) (*see* **Note 24**).
5. Incubate for 10 min at room temperature with gentle agitation.
6. Replace water with 0.1 M HCl (≳50 mL) and incubate for 10 min at room temperature with gentle agitation.
7. Discard the HCl and wash with deionized water (≳50 mL) for 10 min at room temperature with gentle agitation.
8. Remove water, add the Blue Silver stain and incubate for at least 2 h or overnight (*see* **Note 25**).
9. To destain the gel, discard the Blue Silver dye and wash several times with deionized water over the course of few hours, until the background staining is removed—the gel will appear essentially clear, with many protein bands readily discerned by eye.

3.5 Basic Mass Spectrometry Analysis

Mass spectrometry may be used to characterize the sample composition. Individual protein bands may be excised for identification or the entire sample may be characterized in a single analysis. There are many ways to approach such analyses [32] for example, by electrophoresing the sample only briefly (4–6 mm into the gel) and processing all the proteins in the sample together as a "gel plug," or by *f*ilter *a*ided *s*ample *p*reparation (FASP) [41].

3.5.1 Filter Aided Sample Preparation

FASP protocol combines the solubilization of proteins with SDS and their release from the SDS-protein complex with urea, which is a strong chaotropic reagent, thus, facilitating tryptic digestion of peptides and subsequent MS analysis. The procedure is performed on the membrane surface of an ultra-filtration device. We adapted this method for label-free quantitative MS characterization of protein complexes engaged with the human exosome and mRNA cap-binding complexes [15]. The original FASP protocol requires detergent removal from proteins, which may alter its effectiveness amongst different users and/or samples. In order to avoid this step, we performed elution using low pH (acetic acid) in conjunction with a modified version of the FASP protocol [42] utilizing polyethylene glycol (PEG), which allows for higher recovery of low abundance proteins.

1. Add 50 μL of 0.5 M acetic acid to a tube with washed beads and incubate for 10 min at room temperature with shaking.
2. Place on a magnet and transfer the eluate to a fresh tube.
3. Add 5 μL of 5.5 M ammonium hydroxide to neutralize the eluate.
4. Concentrate the sample to 30 μL in a vacuum centrifuge (SpeedVac) (*see* **Note 26**).
5. Add 200 μL of UA∗ solution to the eluate and transfer everything to the filter unit (*see* **Note 27**).
6. Centrifuge at 14,000 × *g* for 15 min.
7. Add 200 μL of UA solution to the filter unit and centrifuge at 14,000 × *g* for 15 min.
8. Discard the flow-through from the collection tube.
9. Add 100 μL IAA solution, vortex for 1 min and incubate in dark without mixing for 20 min.
10. Centrifuge at 14,000 × *g* for 10 min.
11. Wash twice by adding 100 μL of UA solution to the filter unit and centrifuging at 14,000 × *g* for 15 min.
12. Wash twice by adding 100 μL of 50 mM of ammonium bicarbonate (ABC) to the filter unit and centrifuging at 14,000 × *g* for 10 min.
13. Add 40 μL of ABC with trypsin (enzyme to protein ratio 1:100) and vortex for 1 min.
14. Incubate the filter units in a humidified incubator at 37 °C for 4–18 h.
    (a) Place all the tubes with filter units in a plastic rack. Thoroughly moisten a few (~3) papers towels and place underneath the rack, inside of a zip lock bag. Seal the bag and place in the incubator.
15. Transfer the filter units to new collection tubes.
16. Centrifuge the filter units at 14,000 × *g* for 10 min.
17. Add 50 μL of 0.5 M NaCl and centrifuge the filter units at 14,000 × *g* for 10 min.
18. Acidify the eluate with TFA to 0.2% (w/v); proceed to Subheading 3.5.2, **step 16**.

3.5.2 Gel Plugs

This procedure is essentially a modification of that described in Subheading 3.4 with the goal of obtaining MS data for the entire affinity captured sample (the same rationale as FASP). Gel plugs (a region of a polyacrylamide gel in which a whole sample has been immobilized) provide the beneficial qualities of gel-based sample preparation and allow removal of interfering substances during

electrophoresis and subsequent washing of the excised plug [32]. To produce a gel-plug, the whole sample is loaded on an SDS polyacrylamide gel and run only 4–6 mm into the gel, stained, and then excised—producing a small region of gel containing all the proteins present in the fraction and facilitating typical processing by in-gel digestion. A modified version of this approach, "gel-aided sample preparation," which skips the electrophoresis, has also been published [43].

1. Electrophorese the samples until they migrate ~6 mm into the gel as assessed by gel loading dye at the sample migration front (*see* **Notes 28** and **29**).
2. After opening the cassette, with the gel still stuck to one side, cut away the pieces of gel protruding from the wells and the body of the gel below the position of the loading dye.
3. Bring the segmented upper portion of the gel forward to Coomassie staining, essentially as described in Subheading 3.4, but with modified solution volumes to accommodate a 15 cm diameter petri dish.
   (a) Transfer the upper portion of the gel to a clean plastic 15 cm diameter petri dish and incubate for 10 min in 40 mL of deionized water with gentle agitation on an orbital shaker.
   (b) Remove the water and replace it with 40 mL of 0.1 M HCl; incubate for 10 min.
   (c) Remove the 0.1 M HCl and replace it with 40 mL of deionized water; incubate for 10 min.
   (d) Remove the water and replace it with 40 mL of Blue Silver stain; incubate for 2 h to overnight (*see* **Note 25**). In this case, the gel only needs to be stained long enough to clearly visualize the bounds of the material within the plug—hence a relatively short length of staining (and destaining) is permissible. Longer staining durations are permissible as a matter of convenience.
   (e) You may choose to take an image of the gel. Always clean surfaces used to image gels that will contribute sample to mass spectrometry analyses.
4. Using a clean razor blade, cut the regions of gel containing the samples into 1 mm^3 cubes, and transfer the gel pieces into a 1.5 mL tube (*see* **Notes 30** and **31**).
5. Destain the proteins by adding 500 μL of destaining solution. Mix with the gel pieces by vortexing. If the color of the solution changes to dark blue immediately, discard it and add another 500 μL of destaining solution. 1 mL may be used

here instead of 500 μL; repeat multiple times if the solution becomes blue quickly.

6. Incubate at 37 °C for 1 h with shaking in a Thermomixer (≥1000 rpm); discard the supernatant.
7. Add another 500 μL of destaining solution if the gel pieces are still blue. Destain as much as needed (this step can go overnight).
8. Remove destain when the gel pieces no longer exhibit blue staining. They should appear semi-transparent with no obvious blue remaining.
9. Dehydrate the gel pieces by adding 500 μL of acetonitrile, vortex, let the tubes sit at room temperature for 2 min or more. Gel pieces will appear opaque and thoroughly white.
10. Remove acetonitrile from all samples.
11. Dry samples in a vacuum centrifuge (SpeedVac) for 10 min.
12. Samples can be stored at −20 °C or proceed to trypsin digest.
    - *In-gel digestion of proteins to peptides with trypsin.*
13. Prepare all tubes on ice.
    (a) Prepare trypsin working solution: 12.5 ng/μL in 50 mM ammonium bicarbonate. Our trypsin stock is 100 ng/μL in 1 mM HCl and kept in small aliquots at −80 °C.
    (b) Add enough 12.5 ng/μL trypsin to cover gel pieces (*see* **Note 32**).
    (c) Let samples swell for 45 min on ice.
    (d) After swelling, add 50 mM ammonium bicarbonate to cover gel pieces if necessary.
14. Move the tubes to a 37 °C incubator to digest overnight (or at least 6 h).
15. Terminate the digest; extract and recover peptides.
    (a) Produce aliquots of 2% and 0.1% (w/v) TFA from the 20% stock.
    (b) Add 1/3rd the digestion volume of 2% (w/v) TFA to the digestion mixture (0.5% (w/v) TFA final concentration) to stop digestion.
    (c) Incubate at room temperature for 5 min with shaking on Thermomixer (1000 rpm) or vortex mixer equipped with multitube holder (typically setting 2 or 3 out of 10).
    (d) Remove the acidified digest solutions to fresh tubes; hold at room temperature or 4 °C while pieces are further extracted (*see* **Note 33**).

(e) Extract with 50 μL of 0.1% (w/v) TFA at room temperature for 45 min on Thermomixer (1000 rpm) vortex mixer equipped with multitube holder.

(f) Remove extractant, combine with digest solution (total volume may be ≳100 μL for each gel plug).

16. Desalt sample using OMIX tips (C18, 100 μL) following the manufacturer's instructions using the following solutions (*see* **Note 34**).

(a) Tip wet: 0.1% (w/v) TFA, 50% (v/v) acetonitrile; 200 μL per gel plug.

(b) Tip wash: 0.1% (w/v) TFA; 600 μL per gel plug.

(c) Elution 1: 0.5% (v/v) acetic acid, 40% (v/v) acetonitrile; 80 μL per gel plug.

(d) Elution 2: 0.5% (v/v) acetic acid, 80% (v/v) acetonitrile; 80 μL per gel plug.

(e) Proceed samples by sample. Only expel solutions to the first stop until the final elution. Do not draw or push air onto the resin bed.

(f) Elute each sample twice; once into a tube containing Elution 1 solution and once into a tube containing Elution 2 solution. After elution, these solutions can be combined and evaporated in a centrifugal speed vacuum system to remove the acetonitrile prior to mass spectrometry.

3.5.3 Liquid Chromatography, Tandem Mass Spectrometry (LC-MS/MS)

MS methods will vary depending upon the liquid chromatography (LC) setup, the model of MS instrument, and the operator. We provide the following only as guidelines for conducting shotgun proteomic analyses of affinity enriched samples using an Orbitrap-based hybrid mass spectrometer for LC-MS/MS [44]. We typically resuspend our dried samples in 10 μL of 0.5% (v/v) acetic acid (or, samples are initially concentrated to this volume). On a first run, ~1/3rd of the sample will be loaded onto the LC column for MS analysis (permitting double the quantity to be used in a second run if needed). The column bound peptides are eluted in an acetonitrile gradient (typically reaching ≥40% (v/v) final concentration of acetonitrile over ~1 h), in the presence of an ion pairing reagent such as acetic acid (0.1 M) or formic acid (0.1% v/v) at a flow rate of 200–300 nL/min. Data dependent MS^2 fragmentation spectra (e.g., of the top 20 most abundant MS^1 ions) are acquired via collision induced dissociation (CID) or high-energy collisional dissociation (HCD). The resulting mass spectra then require processing in a suitable software package to interpret the proteins present as well as relative quantities and specificities thereof. MaxQuant is a widely used, freely available MS processing software

package with detailed protocols available [45]. Quantitative comparisons between groups of samples may be made, but require additional data processing [46, 47].

3.6 RNase Activity Assay for EXOSC10 Containing Exosome

The RNase activity assay described below was previously conducted on glycerol gradient purified EXOSC10-3xFLAG-containing exosomes as the point of entry into the assay [38], however, the method should be applicable to any sufficiently pure and concentrated samples, utilizing in vitro conditions comparable to those previously described [24, 26, 48]. If affinity isolated exosomes will be further purified across a density gradient, we suggest a scale-up to ~1 g of cell powder as starting material. A control substrate or catalytic point mutant (or both) will be important to ensure the observed activity results from the action of the exosome—trace contamination can confound interpretation [1, 8]. A 3′-PO_4 RNA nucleotide at the 3′-end will block the activity of EXOSC10, but will not block the activity of DIS3 containing exosomes [18, 49].

3.6.1 Rate Zonal Centrifugation

The following settings and parameters are optimized for an SW 55 Ti rotor in a Beckman Optima L series ultracentrifuge. For additional background, refer to [38, 50].

1. Prepare 10 mL each of the "light" and "heavy" gradient solutions and filter them through a 0.45 μm syringe filter.
2. Prepare two 10–40% (v/v) glycerol gradients using the BioComp Gradient Master instrument or other appropriate method.
   (a) Mark halves of the centrifugation tubes with the marker block.
   (b) Add 10% (v/v; light) glycerol solution to the tube until a few mm above halfway mark.
   (c) Using a syringe with attached cannula, deposit the 40% (v/v; heavy) solution at the bottom of the tube until it reaches the halfway mark.
   (d) Cap the tubes (using "short caps"), making sure not to trap any air bubbles.
   (e) Form gradients by running the 10–40% (v/v) "short cap" program on the Gradient Master instrument.
3. Precool gradients to 4 °C (typically ≥1 h in a cold room).
4. Precool the ultracentrifuge to 4 °C.
5. Load the sample eluted with 3xFLAG peptide (Subheading 3.3.3, **steps 6–8**) by gently pipetting it onto the top of the gradient.

6. Balance the tubes before loading them into the ultracentrifuge. Keep the weights of gradients within ±100 mg of one another by adding solution as needed.
7. Run the gradient using the following settings: minimum acceleration; no brake; 4 °C; 50,000 rpm; 6 h 36 min.
8. After the run is completed, collect the fractions using a BioComp Piston Gradient Fractionator (consult the manufacturer's instructions), or other appropriate method. We used the following settings with manual fraction collection: distance: 2 mm, speed: 0.3 mm/s (*see* **Note 35**).
9. Analyze an aliquot (typically ~5–10%) of each fraction by SDS-PAGE and sensitive protein staining (e.g., silver or SYPRO Ruby) to visualize the components and localize the peak fraction.

3.6.2 RNase Assay

1. Pool together up to two fractions constituting the peak of intact EXOSC10-containing exosomes (up to ~450 μL total volume).
2. Add an equal volume of the recapture solution.
3. Prewash 10 μL of the anti-FLAG magnetic slurry (Subheading 3.3.1) twice with 1 mL of recapture solution.
4. Add fractions to a 1.5 mL tube with prewashed beads.
5. Incubate for 30 min with rotation at 4 °C (cold room).
6. Collect beads on the magnet and remove the supernatant.
7. Wash once with 1 mL of the recapture solution.
8. Resuspend the affinity medium (magnetic beads with immobilized exosomes) in 25 μL of the recapture solution.
9. Add 25 μL of the 2× reaction solution containing 0.4 pmol/μL RNA oligo.
10. Incubate at 37 °C with mixing at 1000 rpm.
11. Produce a time-course for the reaction by taking 10 μL aliquots at different time points (e.g., 0, 2.5, 5, 15, and 30 min).
12. Stop the reactions by adding 10 μL 2× RNA loading buffer (*see* **Note 36**).
13. Collect beads on the magnet and transfer the supernatant to a fresh tube; hold on ice.
14. Cast a 20% urea–polyacrylamide 10-well gel accordingly to the manufacturer's instructions (*see* **Note 37**).
15. Prerun the gel in 1× TBE at 15 W for 15–20 min (*see* **Note 38**).
16. Heat the samples at 80 °C for 30 s and then place on ice. Spin down briefly before loading (*see* **Note 39**).

17. Wash the urea out from the wells using a syringe with a bent needle.
18. Load on the gel the entire volume of each sample (20 μL) per well.
19. Run at 15 W until the bromophenol blue tracking dye reaches the first line from the bottom of the plastic gel cassette.
20. Open the cassette and image the gel directly using an imager appropriately configured to detect fluorescein (SYBR green setting on Fuji LAS series).

4 Notes

1. Table 1 lists 150 mM NaCl for the cocapture of DIS3 with EXOSC10-3xFLAG (profile displayed in Fig. 2). 100 mM yields a greater proportion of DIS3 than 150 mM, but at lower overall exosome purity. Various banding profiles can be observed at http://copurification.org.
2. 2′-*O*-methylation at the 3′-end of this substrate is optional, but doing so allows the facile production of a control substrate that is also uniformly 3′-phosphorylated (we used Integrated DNA Technologies for this synthesis). 3′-phosphorylation blocks EXOSC10 activity.
3. The supernatant contains DMSO, which is used for freezing cells. DMSO is toxic and has to be removed immediately after thawing.
4. Do not mix cells by circular motion as this will make cells go to the edges of the flask.
5. Although HEK293 are adherent cells, they attach to surfaces quite loosely. Addition of solutions straight on the cells can cause detaching.
6. To check if cells are detached, tap the bottom and sides of the flask. If a white layer of cells starts moving, cells are ready to be collected.
7. When growing cells on 500 cm^2 square plates we typically use 100 mL total medium volume per plate (90 mL + 10 mL Tet-containing medium added 24 h prior harvesting).
8. Although the penicillin and streptomycin, present in media at standard concentrations, are not toxic to mammalian cells, most transfection protocols recommend using antibiotic free media. Lipid transfection reagents increase cell permeability, which may also increase the amount of antibiotics delivered into the cells, resulting in cytotoxicity and lower transfection efficiency.

9. Tetracycline is sensitive to light. We typically prepare a Tet stock solution at 10 mg/mL in ethanol and store it at −20 °C in a tube wrapped in aluminum foil. We then use it to prepare working concentration by serial dilutions in the growth medium.
10. For all cell handling steps use an electropipettor set to "low" and 25 mL pipettes to avoid excessive shearing of cells during transfer manipulations. Arrange 50 mL collection tubes and 1× PBS in an ice bucket prior to initiating the procedure.
11. Because the cells themselves will not constitute a large proportion of the suspension volume, three plates worth of cell suspension can be combined into two 50 mL tubes. Because 50 mL tubes actually hold more than the nominal volume, 16 plates can typically be spread across eight such tubes.
12. LN_2 is capable of causing severe cryogenic burns. Wear protective clothing and exercise appropriate handling precautions.
13. Do not tightly close the tubes before all the LN_2 has visibly evaporated, otherwise excessive pressure may cause the tube to explode. Not closing the tubes after the LN_2 has dissipated may result in the accumulation of frost on the cells material, adding excess water weight and effectively reducing the protein concentration upon milling.
14. To minimize risk of cryoburns caused by prolonged contact with cold metal tweezers and spatulas, we recommend wearing cotton gloves over the standard rubber gloves.
15. It is convenient and a time-saver to prepare several tubes with certain amount of powder (e.g., 50, 100, 200 mg) in advance and store them at −80 °C ready to be used when necessary.
16. Weight Dynabeads without gloves—wearing gloves make Dynabeads stick to the walls of the tube.
17. The affinity medium in solution A can be stored at −20 °C for several months without any noticeable loss of performance. The affinity medium in solution B can be stored at −20 °C for at least 1 year. Alternatively, one can resuspend the affinity medium in 0.02% (w/v) NaN_3 in PBS and store at 4 °C up to 2–3 weeks.
18. It is important to keep the cell powder frozen. Leave the tubes in LN_2 until the next step is initiated.
19. Once the cell powder is resuspended, the sample should be held on ice throughout the procedure unless otherwise stated.
20. If clumps are visible, the sonication step should be repeated until no clumps are obvious by visual inspection.

21. To wash the beads, add 1 mL of the extraction solution to 10 μL of the anti-FLAG bead slurry and vortex briefly to fully resuspend the beads. Spin the tube briefly to collect all the solution at the bottom and then place the tube on a magnet until the beads are collected at the side of the tube. Remove the supernatant using a pipet or an aspirator, and repeat the washing step one more time. Perform both washing steps at room temperature. After washing, the beads may be held on ice and are ready for use.
22. Moving the sample (beads resuspended in extraction solution) to a fresh tube minimizes contamination of the eluate. Cell extract proteins nonspecifically adsorbed to the internal surfaces of the tube used for affinity capture may be released into the sample during subsequent manipulations.
23. Do not add reducing agent (DTT) to the buffer to mitigate the release of antibody chains from the beads. More persistent antibody-antigen interactions may benefit from elevated temperature for release (typically 70 °C is sufficient). In our experience, anti-FLAG effectively releases 3xFLAG tagged protein complexes within ~5 min when incubated at room temperature with mixing in the presence of 1× LDS. Other antibodies may be more tenacious. Elevating the temperature may increase IgG contamination of the eluate.
24. If the gel bands are to be analyzed by MS, it is crucial to work clean, always wear gloves and the lab coat, use a clean apparatus for running gels, clean containers for staining gels (e.g., 15 cm tissue culture dishes) and new scalpels for cutting bands.
25. The Blue Silver exhibits an 80% uptake during the first hour of staining but continues to accumulate intensity over long incubations; for the visualization minor sample constituents, overnight incubations are suggested. If the gel has been properly prewashed, the Blue Silver will remain a deep, translucent green color, after combining it with the gel, due to maintenance of the low pH dye solution. If the washing is incomplete, residual buffering capacity within the gel may turn the stain bluish and the gel may develop uneven background staining. Changing the stain and incubating in several hours to overnight in fresh (green-hued) Blue Silver will typically resolve this problem and result in mild, uniform background staining regions of high protein concentration clearly visible above background. Extending the final water wash to 15 min (rather than 10 min) may also help if you frequently encounter uneven staining and color shift from green to blue upon adding the stain to the gel.

26. To reduce a chance of contaminating the sample, clean the inside walls and the rotor of the vacuum centrifuge. Cover the opening of the sample tube with a piece of Parafilm M and perforate it with a clean needle to allow evaporation of the solvent.
27. UA∗, UA and IAA must be freshly prepared and used within a day.
28. Prior to electrophoresis, draw a horizontal line at a distance 6 mm below the sample well using an indelible marker. Run the gel until the sample loading dye migrates to the same position as the line has been drawn. Usually ~5 min. Remove the gel and proceed.
29. Flanking the sample with molecular mass markers will assist in later gel trimming steps. Loading quantity several standards, such as BSA, will allow an estimate of the total protein within the gel plugs to be made.
30. This depends on plug size, but for a 10 well gel, this may be 4–5 vertical slices, crossed by 4–5 five horizontal slices. Producing a collection of loosely conglomerated ~1 mm cubes. These can be scooped up all together using the razor blade and transferred into the tube.
31. Remove the area of gel above the highest mass marker (typically ~250 kDa) and the area just below the dye front. Then, segment the regions containing sample into rectangles (plugs), removing the gel between them. This minimizes the amount of gel within the extraction that does not contain protein sample.
32. Typically ~40 μL per tube for 15-well gel plugs or 90 μL for 10-well gel plugs; presuming gels are 1 mm thick.
33. One may opt to use protein "low binding" tubes. This has been shown to affect final peptide yield [51].
34. Tubes that will be exposed to 40% + acetonitrile should be prewashed with pure acetonitrile. This prewash reduces the potential for polymer contamination of the sample by plasticizers or other components of the tube manufacture by first solubilizing them in high acetonitrile and removing them with the wash. We use 100 μL acetonitrile to wash 0.65 mL tubes and 300 μL to wash 1.5 mL tubes.
35. This collection regime will produce a meniscus fraction, ~20 fractions of ~225 μL, and a bottom fraction. Some variations will be observed depending on the specific set-up implemented, however, using the described procedure and setup we retrieved the peak of sedimented EXOSC10-3xFLAG-tagged

exosome (DIS3−, *see* Table 1) in fraction 11. Isolating DIS3+ exosomes by glycerol gradient following these procedures requires additional manipulations [38].

36. This assay should ideally include two controls set up as independent reactions: (a) as above, but using the blocked substrate, and (b) a mock reaction with affinity medium and reaction solution containing the generic substrate, but no RNA exosomes. Both controls should reveal the presence of intact substrate across the time course, indicating that the preparation and solutions are free from interfering RNase contamination. DIS3 is capable of degrading the 3′-PO_4 blocked substrate.
37. We use the National Diagnostics system but the gels can be prepared using standard materials and methods for urea-polyacrylamide gel electrophoresis [52].
38. The prerunning step clears excess free ions from the gel which affect the electrical current and heat generation. The cassette will warm during the prerun and run; typical operating temperatures are between 45 and 60 °C. The heat produced may help maintain the RNA sample in a denatured, single stranded form; this is not expected to be a major variable for small oligo substrates lacking strong secondary structure.
39. Heating the sample denatures the RNA and dissociates RNA-protein assemblies.

Acknowledgments

This work is supported in part by National Institutes of Health grant R01GM126170. We gratefully acknowledge Ms. Hua Jiang and Ms. Kelly Molloy for additional proofreading.

References

1. Mitchell P, Petfalski E, Shevchenko A, Mann M, Tollervey D (1997) The exosome: a conserved eukaryotic RNA processing complex containing multiple 3′-->5′ exoribonucleases. Cell 91(4):457–466
2. Koonin EV, Wolf YI, Aravind L (2001) Prediction of the archaeal exosome and its connections with the proteasome and the translation and transcription machineries by a comparative-genomic approach. Genome Res 11(2):240–252
3. Kilchert C, Wittmann S, Vasiljeva L (2016) The regulation and functions of the nuclear RNA exosome complex. Nat Rev Mol Cell Biol 17(4):227–239
4. Zinder JC, Lima CD (2017) Targeting RNA for processing or destruction by the eukaryotic RNA exosome and its cofactors. Genes Dev 31 (2):88–100
5. Ogami K, Chen Y, Manley JL (2018) RNA surveillance by the nuclear RNA exosome: mechanisms and significance. Noncoding RNA 4(1)
6. Schmid M, Jensen TH (2018) Controlling nuclear RNA levels. Nat Rev Genet 19 (8):518–529

7. Allmang C, Petfalski E, Podtelejnikov A, Mann M, Tollervey D, Mitchell P (1999) The yeast exosome and human PM-Scl are related complexes of 3′ --> 5′ exonucleases. Genes Dev 13(16):2148–2158
8. Dziembowski A, Lorentzen E, Conti E, Seraphin B (2007) A single subunit, Dis3, is essentially responsible for yeast exosome core activity. Nat Struct Mol Biol 14(1):15–22
9. Lejeune F, Li X, Maquat LE (2003) Nonsense-mediated mRNA decay in mammalian cells involves decapping, deadenylating, and exonucleolytic activities. Mol Cell 12(3):675–687
10. van Dijk EL, Schilders G, Pruijn GJ (2007) Human cell growth requires a functional cytoplasmic exosome, which is involved in various mRNA decay pathways. RNA 13 (7):1027–1035
11. Tomecki R, Kristiansen MS, Lykke-Andersen S, Chlebowski A, Larsen KM, Szczesny RJ, Drazkowska K, Pastula A, Andersen JS, Stepien PP, Dziembowski A, Jensen TH (2010) The human core exosome interacts with differentially localized processive RNases: hDIS3 and hDIS3L. EMBO J 29 (14):2342–2357
12. Staals RH, Bronkhorst AW, Schilders G, Slomovic S, Schuster G, Heck AJ, Raijmakers R, Pruijn GJ (2010) Dis3-like 1: a novel exoribonuclease associated with the human exosome. EMBO J 29(14):2358–2367
13. Lubas M, Christensen MS, Kristiansen MS, Domanski M, Falkenby LG, Lykke-Andersen S, Andersen JS, Dziembowski A, Jensen TH (2011) Interaction profiling identifies the human nuclear exosome targeting complex. Mol Cell 43(4):624–637
14. Meola N, Domanski M, Karadoulama E, Chen Y, Gentil C, Pultz D, Vitting-Seerup K, Lykke-Andersen S, Andersen JS, Sandelin A, Jensen TH (2016) Identification of a nuclear exosome decay pathway for processed transcripts. Mol Cell 64(3):520–533
15. Andersen PR, Domanski M, Kristiansen MS, Storvall H, Ntini E, Verheggen C, Schein A, Bunkenborg J, Poser I, Hallais M, Sandberg R, Hyman A, LaCava J, Rout MP, Andersen JS, Bertrand E, Jensen TH (2013) The human cap-binding complex is functionally connected to the nuclear RNA exosome. Nat Struct Mol Biol 20(12):1367–1376
16. Lubas M, Andersen PR, Schein A, Dziembowski A, Kudla G, Jensen TH (2015) The human nuclear exosome targeting complex is loaded onto newly synthesized RNA to direct early ribonucleolysis. Cell Rep 10 (2):178–192
17. Ogami K, Richard P, Chen Y, Hoque M, Li W, Moresco JJ, Yates JR 3rd, Tian B, Manley JL (2017) An Mtr4/ZFC3H1 complex facilitates turnover of unstable nuclear RNAs to prevent their cytoplasmic transport and global translational repression. Genes Dev 31 (12):1257–1271
18. Domanski M, Upla P, Rice WJ, Molloy KR, Ketaren NE, Stokes DL, Jensen TH, Rout MP, LaCava J (2016) Purification and analysis of endogenous human RNA exosome complexes. RNA 22(9):1467–1475
19. Winczura K, Schmid M, Iasillo C, Molloy KR, Harder LM, Andersen JS, LaCava J, Jensen TH (2018) Characterizing ZC3H18, a multidomain protein at the Interface of RNA production and destruction decisions. Cell Rep 22 (1):44–58
20. Cristea IM, Williams R, Chait BT, Rout MP (2005) Fluorescent proteins as proteomic probes. Mol Cell Proteomics 4 (12):1933–1941
21. Domanski M, Molloy K, Jiang H, Chait BT, Rout MP, Jensen TH, LaCava J (2012) Improved methodology for the affinity isolation of human protein complexes expressed at near endogenous levels. BioTechniques 0 ((0)):1–6
22. LaCava J, Jiang H, Rout MP (2016) Protein complex affinity capture from Cryomilled mammalian cells. J Vis Exp 118
23. Oeffinger M, Wei KE, Rogers R, DeGrasse JA, Chait BT, Aitchison JD, Rout MP (2007) Comprehensive analysis of diverse ribonucleoprotein complexes. Nat Methods 4 (11):951–956
24. Domanski M, LaCava J (2017) RNA degradation assay using RNA exosome complexes, affinity-purified from HEK-293 cells. Bio Protoc 7(8)
25. Hakhverdyan Z, Domanski M, Hough LE, Oroskar AA, Oroskar AR, Keegan S, Dilworth DJ, Molloy KR, Sherman V, Aitchison JD, Fenyo D, Chait BT, Jensen TH, Rout MP, LaCava J (2015) Rapid, optimized interactomic screening. Nat Methods 12(6):553–560
26. Januszyk K, Liu Q, Lima CD (2011) Activities of human RRP6 and structure of the human RRP6 catalytic domain. RNA 17 (8):1566–1577
27. O'Gorman S, Fox DT, Wahl GM (1991) Recombinase-mediated gene activation and

site-specific integration in mammalian cells. Science 251(4999):1351–1355

28. Yao F, Svensjo T, Winkler T, Lu M, Eriksson C, Eriksson E (1998) Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. Hum Gene Ther 9(13):1939–1950
29. Helgason CD, Miller CL (2013) Basic cell culture protocols. Methods in molecular biology, vol 946, 4th edn. Humana Press, Totowa
30. Freshney RI (2015) Culture of animal cells: a manual of basic technique and specialized applications, 7th edn. Wiley-Blackwell, Hoboken
31. Katzen F (2007) Gateway((R)) recombinational cloning: a biological operating system. Expert Opin Drug Discov 2(4):571–589
32. LaCava J, Molloy KR, Taylor MS, Domanski M, Chait BT, Rout MP (2015) Affinity proteomics to study endogenous protein complexes: pointers, pitfalls, preferences and perspectives. BioTechniques 58 (3):103–119
33. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H (1995) Transcriptional activation by tetracyclines in mammalian cells. Science 268(5218):1766–1769
34. Okerman L, Van Hende J, De Zutter L (2007) Stability of frozen stock solutions of beta-lactam antibiotics, cephalosporins, tetracyclines and quinolones used in antibiotic residue screening and antibiotic susceptibility testing. Anal Chim Acta 586(1–2):284–288
35. Inducible Protein Expression - T-Rex™ System. ThermoFisher Scientific
36. Szczesny RJ, Kowalska K, Klosowska-Kosicka-K, Chlebowski A, Owczarek EP, Warkocki Z, Kulinski TM, Adamska D, Affek K, Jedroszkowiak A, Kotrys AV, Tomecki R, Krawczyk PS, Borowski LS, Dziembowski A (2018) Versatile approach for functional analysis of human proteins and efficient stable cell line generation using FLP-mediated recombination system. PLoS One 13(3):e0194887
37. Taylor MS, LaCava J, Dai L, Mita P, Burns KH, Rout MP, Boeke JD (2016) Characterization of L1-Ribonucleoprotein particles. Methods Mol Biol 1400:311–338
38. Domanski M, LaCava J (2017) Affinity purification of the RNA degradation complex, the exosome, from HEK-293 cells. Bio Protoc 7 (8)
39. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1(6):2856–2860
40. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG (2004) Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25(9):1327–1333
41. Wisniewski JR, Zougman A, Nagaraj N, Mann M (2009) Universal sample preparation method for proteome analysis. Nat Methods 6 (5):359–362
42. Wisniewski JR, Ostasiewicz P, Mann M (2011) High recovery FASP applied to the proteomic analysis of microdissected formalin fixed paraffin embedded cancer tissues retrieves known colon cancer markers. J Proteome Res 10 (7):3040–3049
43. Fischer R, Kessler BM (2015) Gel-aided sample preparation (GASP)—a simplified method for gel-assisted proteomic sample generation from protein extracts and intact cells. Proteomics 15(7):1224–1229
44. Gillet LC, Leitner A, Aebersold R (2016) Mass spectrometry applied to bottom-up proteomics: entering the high-throughput era for hypothesis testing. Annu Rev Anal Chem (Palo Alto, Calif) 9(1):449–472
45. Tyanova S, Temu T, Cox J (2016) The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc 11(12):2301–2319
46. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, Mann M, Cox J (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 13(9):731–740
47. Armean IM, Lilley KS, Trotter MW (2013) Popular computational methods to assess multiprotein complexes derived from label-free affinity purification and mass spectrometry (AP-MS) experiments. Mol Cell Proteomics 12(1):1–13
48. Greimann JC, Lima CD (2008) Reconstitution of RNA exosomes from human and Saccharomyces cerevisiae cloning, expression, purification, and activity assays. Methods Enzymol 448:185–210
49. Zinder JC, Wasmuth EV, Lima CD (2016) Nuclear RNA exosome at 3.1 a reveals substrate specificities, RNA paths, and allosteric

inhibition of Rrp44/Dis3. Mol Cell 64 (4):734–745

50. Fernandez-Martinez J, LaCava J, Rout MP (2016) Density gradient ultracentrifugation to isolate endogenous protein complexes after affinity capture. Cold Spring Harb Protoc 2016(7)
51. Kraut A, Marcellin M, Adrait A, Kuhn L, Louwagie M, Kieffer-Jaquinod S, Lebert D, Masselon CD, Dupuis A, Bruley C, Jaquinod M, Garin J, Gallagher-Gambarelli M (2009) Peptide storage: are you getting the best return on your investment? Defining optimal storage conditions for proteomics samples. J Proteome Res 8(7):3778–3785
52. Sambrook J, Russell DW (2006) Preparation of denaturing polyacrylamide gels. CSH Protoc 2006(1)