Chapter 20

Characterization of L1-Ribonucleoprotein Particles

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Abstract

The LINE-1 retrotransposon (L1) encodes two proteins, ORF1p and ORF2p, which bind to the L1 RNA in *cis*, forming a ribonucleoprotein (RNP) complex that is critical for retrotransposition. Interactions with both permissive and repressive host factors pervade every step of the L1 life cycle. Until recently, limitations in detection and production precluded in-depth characterization of L1 RNPs. Inducible expression and recombinant engineering of epitope tags have made detection of both L1 ORFs routine. Here, we describe large-scale production of L1-expressing HEK-293T cells in suspension cell culture, cryomilling and affinity capture of L1 RNP complexes, sample preparation for analysis by mass spectrometry, and assay using the L1 element amplification protocol (LEAP) and qRT-PCR.

Key words LINE-1, Ribonucleoprotein, Affinity purification, Protein complexes, Interactomics, Cryomilling, Mass spectrometry, Metabolic labeling

1 Introduction

LINE-1 is the only active protein-coding transposon in humans. Hundreds of thousands of copies of L1 make up ~20 % of the human genome and are a source of genetic structural variation and between humans and instability in cancer [1–4]. Although most L1s are truncated or mutated, ~90 copies per cell are capable of replication [5]. As a streamlined DNA parasite with which we have coevolved since early eukaryotic existence [1], L1 encodes only two proteins and coopts cellular machinery in order to replicate. To defend against L1-mediated mutagenesis, our cells possess multiple mechanisms to suppress its activity [6–18]. Although teleological roles of retrotransposition in evolution are somewhat controversial [19–21], critical interactions with the host functions are highly conserved; for example, the PIP box, a motif required for L1 ORF2p-PCNA binding and retrotransposition, is conserved from corn to humans [6].

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L1 ORFs bind the L1 mRNA to form a ribonucleoprotein (RNP) complex [22, 23]. ORF1p is a trimeric nucleic acid binding protein that is highly expressed in human cancers and cell culture models and thought to have chaperone activity; it is required for retrotransposition but its precise role is unknown [24–29]. ORF2p possesses both endonuclease and reverse transcriptase activities [30, 31] and, in contrast to ORF1p, is expressed at such low levels even after overexpression (due to an unconventional translation mechanism) that detection was a technical barrier in the field until recently [6, 32, 33]. However, the combination of a tetracycline-inducible promoter and improved epitope tagging now makes detection and purification of both ORFs routine [6], reviewed in [34]. A synthetic codon-optimized L1, *ORFeus*-Hs [35, 36], produces ~40-fold more L1 RNA and ORF2p and was critical to establishing protocols for purification of ORF2p; interactors were similar to native L1RP [6].

Here, we outline our methods for suspension production, cryomilling, and affinity capture of L1 RNPs with subsequent characterization by mass spectrometry, the L1 element amplification protocol (LEAP) [23], and quantitative real-time reverse transcription PCR (qRT-PCR). Suspension production improves cell densities as compared to adherent cell culture, and allows for sufficient cell material for solid-phase lysis under liquid N_2 by cryomilling. This approach has a number of practical advantages over liquid-phase lysis including reduced background in coimmunoprecipitation and the ability to store the milled cell powder at –80 °C, allowing repeated experiments to be performed from the same sample without the need to produce more starting material [37, 38].

We express inducible L1 in Tet-On HEK-293_{LD} cells using pCEP4-based episomal vectors and either large-scale transient transfection or quasi-stable episomal puromycin-selected cell pools [6]. Because no Tet-On HEK-293T cells were available commercially, we produced this line using a linearized pTet-On Advanced (Clontech), modified to contain blasticidin resistance instead of neomycin (pLD215). Cells are maintained in square bottles on an orbital shaker [39]. Alternative systems include GNTI-HEK293S cells, HEK-293F cells (Life Technologies), and the T-REx system (Life Technologies), and can be achieved in spinner flasks, conical flasks, and wave bags [40, 41].

For L1 affinity isolation, we couple antibody to functionalized micron-scale paramagnetic beads with relatively inert surfaces (Dynabeads, Life Technologies). After coupling, antibody is immobilized on the surface of the bead. For the isolation of protein complexes, these beads provide a number of advantages over agarose and porous synthetic resins (which both contain antibody bound within the pores) including the ability to bind and release larger complexes, faster binding and release, and reduced background [37, 38, 42]. Switching to this medium was critical for the successful characterization of L1 RNPs.

L1 RNPs purified by affinity capture provide the purest, most active L1 elements reported to date and are excellent starting material for assay by mass spectrometry, LEAP, and qRT-PCR [6]. We provide several of our working protocols for L1 sample preparation and analysis.

2 Materials

2.1 Suspension Cell Culture

1. Humidified CO_2-controlled tissue culture incubator.
2. Orbital shaker platform at 130 rpm fitted with racks.
3. 20 mm 40-place test tube racks.
4. Diagonal cutting pliers, flat wood file, bandsaw (for modifying racks).
5. Corning Pyrex 1 L glass bottles.
6. 7×® Cleaning solution (Bellco Glass).
7. Hybridoma SFM medium (Life Technologies).
8. Freestyle 293 Medium (Life Technologies).
9. Opti-MEM Reduced Serum Medium.
10. TrypLE Express (Life Technologies).
11. Certified tetracycline-Free FBS (Tet-free FBS).
12. DMEM medium.
13. Phosphate-buffered saline (PBS).
14. PEI Max (MW 40,000), Polysciences: Two grams is enough to transfect >600 L.
    (a) To prepare working 1 mg/mL PEI Max solution:
    (b) Dissolve 100 mg PEI Max in 90 mL ddH_2O.
    (c) Adjust pH to 7.0 using 1 M NaOH.
    (d) Adjust volume to 100 mL, filter sterilize, and store at 4 °C.
    (e) *NEVER FREEZE PEI working stock*. Working stocks can be used for up to 6 months if stored at 4 °C.

2.2 Cell Harvest

15. Large-volume floor centrifuge with appropriate rotor (e.g., 4×1 L, 6×500 mL).
16. 16ga needles.
17. Luer-lock syringes, 5 mL, 10 mL, or 30 mL.
18. Luer-lock syringe end caps (BioRad).
19. Liquid nitrogen and Dewar flask.
20. Gloves for handling liquid nitrogen.
21. Small Styrofoam box.

2.3 Cryomilling

22. Ice pan (Fisher).
23. RETSCH Planetary ball mill PM 100 or PM 100 CM.
24. RETSCH Stainless steel grinding balls 20 mm diameter.
25. RETSCH Stainless steel "comfort" grinding jars 50 mL and/or 125 mL.
26. Stainless steel measuring spoons for small amounts (e.g., "hint, pinch, dash").
27. Stainless steel spatulas.
28. Extra-large forceps.

2.4 Coupling of Magnetic Medium (Dynabeads)

29. Dynabeads M270 Epoxy (Life Technologies).
30. Anti-Flag M2 Antibody (Sigma). *See* **Note 1.**
31. Anti-ORF1 Antibody 4H1 (obtained from Kathleen H. Burns).
32. Magnetic separator for microcentrifuge tubes (Dynamag 2, Life Technologies).
33. Magnetic separator for 15 mL conical tubes (Dynamag 15, Life Technologies). *See* **Note 2**.
34. Zeba Spin Desalting Columns 7 K MWCO (Thermo) or chromatography system with preparatory-scale desalting column.
35. Nutating mixer or orbital shaker.
36. Rotating test tube wheel in a 37 °C environment.
37. 100 mM Sodium phosphate buffer pH 7.4 (makes 1 L):
    (a) 2.62 g Sodium phosphate monobasic monohydrate.
    (b) 14.42 g Sodium phosphate dibasic dihydrate.
    (c) Dissolve in 900 mL ddH_2O, adjust pH if necessary with HCl and NaOH, and adjust to 1 L.
38. 3 M Ammonium sulfate (in phosphate buffer) (makes 100 mL):
    (a) 39.6 g Ammonium sulfate.
    (b) Dissolve in 0.1 M sodium phosphate buffer (pH 7.4) and adjust to 100 mL.
39. 10× PBS—pH 7.4. *See* **Note 3** (makes 1 L):
    (a) 2.62 g Sodium phosphate monobasic monohydrate.
    (b) 14.42 g Sodium phosphate dibasic dihydrate.
    (c) 87.8 g Sodium chloride.
    (d) Dissolve in 900 mL with ddH_2O, adjust pH if necessary with HCl and NaOH, and adjust to 1 L.
40. Resuspension buffer (PBS, 50 % glycerol, 0.5 mg/mL BSA; makes 10 mL):
    (a) 1.0 mL 10× PBS.
    (b) 6.3 g glycerol (place tube + rack on a balance, tare, and pipet).

(c) 5 mg BSA.

(d) ddH_2O to 10 mL.

41. PBS + 0.5 % Triton X-100 (w/v) in 100 mL.
42. 100 mM Glycine-HCl, pH 2.5 (adjust pH with HCl).
43. Triethylamine.
44. 10 mM Tris–HCl, pH 8.8.
45. 10 % (w/v) Sodium azide (NaN_3).

2.5 Affinity Capture (See Note 4)

46. HEPES buffer, pH 7.4.
47. Sodium chloride.
48. Triton X-100.
49. Protease inhibitor cocktail: Complete EDTA-free (Roche).
50. Ultrasonic liquid processor with micro tip (Branson Sonifier or similar).

2.6 LEAP (L1 Element Amplification Protocol) and Real-Time Reverse Transcription PCR (qRT-PCR)

51. LEAP reaction mixture (Table 1), shown for ten reactions.
52. Primers for LEAP and qRT-PCR (*see* Table 2).
53. SuperScript III Reverse Transcriptase (Life Technologies) or similar.
54. FastStart Taq DNA Polymerase (Roche Applied Science) or similar.
55. TRIzol Reagent (Life Technologies).
56. RNA from GFP-transfected human cells, purified (*see* **Note 5**).

Table 1
LEAP reaction mixture for ten reactions

Sample	Final concentration	Stock concentration	µL each	×10
RNP prep			2	
Tris pH 7.5	50 mM	1 M	2.5	25
KCl	50 mM	1 M	2.5	25
$MgCl_2$	5 mM	1 M	0.25	2.5
DTT	10 mM	1 M	0.5	5
3′ Anchor primer	0.4 µM	10 µM	2	20
RNAsin	20 U/rxn	40 U/µL	0.5	5
dNTPs	0.2 mM	10 µM	0.5	5
Tween 20	0.05 %	10 %	0.25	2.5
ddH_2O			39	390

Table 2
Primers for LEAP and qRT-PCR

Name	Sequence
JB11560	5′-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTT-3′
JB11564	5′-GCGAGCACAGAATTAATACGACTC-3′
JB14067	5′-GGATCCAGACATGATAAGATACATTGATGA-3′
JB13415	5′-GCTGGATGGAGAACGACTTC-3′
JB13416	5′-TTCAGCTCCATCAGCTCCTT-3′
JB13417	5′-CTGATCAGCCGCATCTACAA-3′
JB13418	5′-TGGTCTTGATCTGCATCTCG-3′
JB13766	5′-ACGTAAACGGCCACAAGTTC-3′
JB13767	5′-AAGTCGTGCTGCTTCATGTG-3′

57. StepOne Plus Instrument or similar (Life Technologies) and appropriate reaction plates.
58. Fast SYBR Green Master Mix (Life Technologies) or similar.
59. RNAseZap (Life Technologies) or similar.

3 Methods

3.1 Suspension Culture of HEK-293T Cells

Suspension culture allows production of large amounts of human cell pellets with minimal work, waste, and cost when compared to monolayer adherent growth. We use a square bottle system in an orbital shaker, modified from [39] (Fig. 1a). For comparison, one confluent 15 cm plastic adherent culture dish provides about 100 mg wet cell weight (cell pellet, WCW), corresponding to 20–30 million cells, and uses 20 mL of media. To produce 1.5 g of cell pellet, 15 disposable dishes and 300 mL of media are required, and scale up, induction, and harvest are laborious. In suspension, cell densities of 4–5 million cells/mL are readily achieved in log phase using reusable 1 L glass bottles, yielding 4–5 g WCW from 333 mL media. Thus, each bottle is the equivalent of approximately 50 culture plates. The total yield from 48 culture bottles (Fig. 1a) was approximately 250 g WCW and was harvested in 90 min by one person; an entire incubator full of plates (Fig. 1b) produced 16 g WCW and was harvested in 4 h by two people.

We culture Tet-On HEK-293T$_{LD}$ cells in Freestyle 293 medium supplemented with 1 % tetracycline-free FBS and 2 mM l-glutamine (suspension medium, *see* **Note 6**). Basic shaker setup and maintenance are described below. Initially, for each construct we

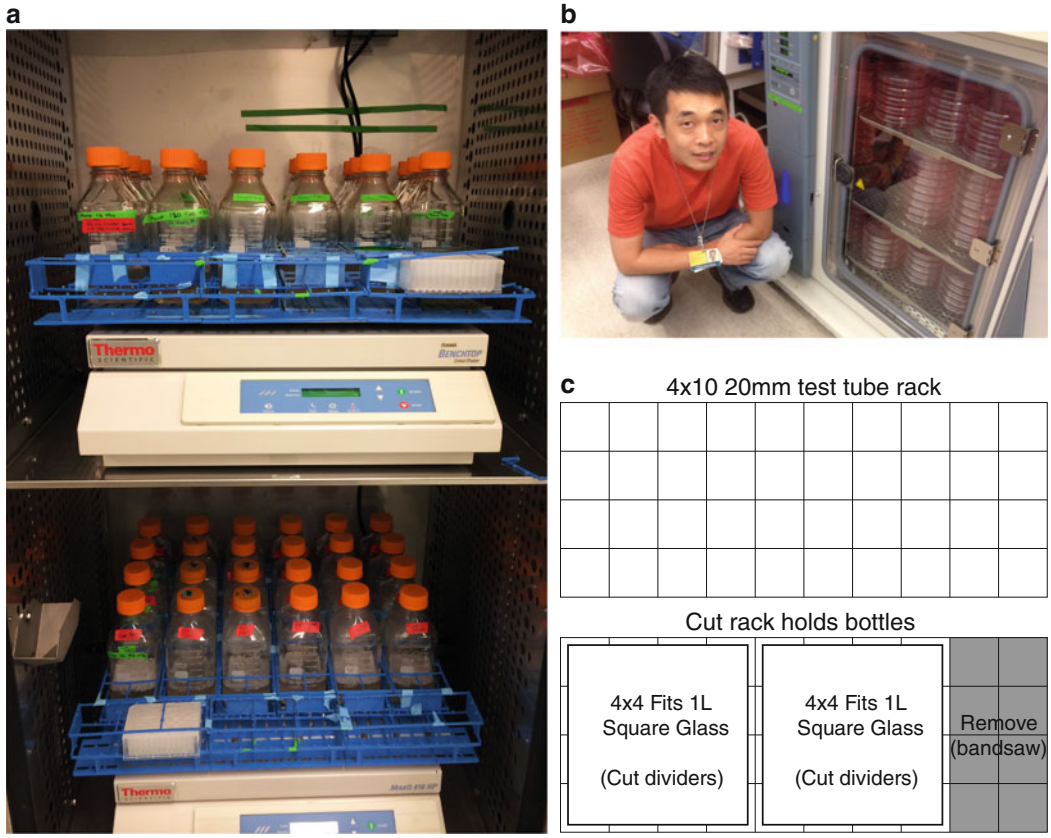

Fig. 1 Suspension culture of HEK-293T$_{LD}$ cells using square glass bottles. (**a**) Orbital shaker bottle setup in a CO_2-controlled humidified incubator cabinet. Total yield from 48 bottles was approximately 250 g wet cell weight (WCW), the equivalent of 2500 15 cm adherent culture plates. (**b**) Dr. Lixin Dai proudly showcases an incubator full of adherent culture plates for harvest. Harvesting this entire incubator full of cells yielded 16 g WCW, which can now be accomplished in three to four suspension bottles. (**c**) Diagram of modifications to Nalgene 4 × 10 configuration 20 mm test tube racks to fit square glass bottles. Dividers are cut with diagonal cutting pliers and smoothed with a file. Sections of rack can be removed with a bandsaw. The diversity of available rack sizes and shapes allows adaptation of this strategy for many size bottles

transfected, puromycin-selected, and scaled up adherent cultures to suspension. This protocol is effective but time consuming. Subsequently, we found that large-scale transient transfection using PEI-MAX (Polysciences) provides equivalent per-cell yields with much less work. Both protocols are described in separate sections below (*see* **Note 7**).

3.1.1 Square Bottle Orbital Shaker Setup

We use a Thermo Forma Model 416 orbital shaker in a 5 % CO_2 humidified incubator cabinet, shaking at 130 rpm. After repeated inconsistency issues with failed transfections and poor growth in plastic bottles made of polypropylene, polyethylene, and other plastics, we can only recommend culture in square Corning Pyrex

glass bottles. Bottles are held in place using autoclavable test tube racks, cut to size. The racks last for a number of years in regular use before needing to be replaced.

1. Cut tube racks to fit bottle configurations (Fig. 1c). Use diagonal cutting pliers to remove undesired dividers and then a flat wood file to smooth sharp edges. To shorten racks, cut to length with a bandsaw. We start with 10×4 racks and cut most racks to fit two 1 L glass bottles, a configuration with two 4×4 openings as shown. Other configurations or other size starting racks would allow use of alternate or smaller bottles.
2. Clean and autoclave racks before installation.
3. Install racks to shaker platform using stainless steel flat-head machine screws and, if needed, washers. The bottom of the racks may need to be drilled.
4. Use paper tape between the racks to hold weak sections together.

3.1.2 Bottles and Volumes

1. Clean freshly gloved hands with 70 % ethanol before going into the shaker cabinet.
2. Media is maintained at up to 33 % of the indicated capacity for optimal mixing and gas exchange.
   (a) Minimum volume is 5 % of indicated capacity (50 mL for 1 L bottles).
   (b) Cell shearing is inversely proportional with bottle size; too little volume can result in excessive shearing.
3. Bottle caps are kept loose to allow gas exchange, but not loose enough to easily fall off.
4. The outsides of bottles are wiped clean before returning to the shaker.
5. After use, bottles must be immediately rinsed and filled with tap water. Bottles are left soaking until they are ready to clean. A small number of "beached" cells stuck to the side of the glass at the media-air interface is normal for bottles that have been used for a number of weeks.
6. When a batch of bottles is ready for sterilization, bottles are thoroughly scrubbed with a brush cleaned using 1 % solution of 7× cleaner, rinsed six times using tap water, and finally rinsed with deionized water.
7. Bottles are sterilized by autoclaving using a dry cycle at 121 °C, with 45-min sterilization and 15-min drying. *See* **Note 8**.
8. In the event of contamination, bottles are soaked in 10 % bleach for at least 2 h and then washed and autoclaved as above. *See* **Note 9**.

3.1.3 General Cell Line Maintenance

1. Most operations are done by pouring. Be careful to consider that only the threads of the bottle are sterile.
2. Do not allow non-sterile bottle sides to be positioned over the mouth of an open bottle. If a bottle is to be totally emptied, we pipet the last ~40 mL.
3. Caps are stored face-down on the hood. Remember that the hood surface and the rim of the cap are never sterile but the cap threads and inside are sterile.
4. This media has no pH indicator dye, but one can be added if desired.
5. Cells are generally maintained at a density of ~0.2–4 million/mL (*see* **Note 10**). Cells will grow to ~7 million/mL [39] but growth slows after ~5 million/mL.
6. We do not spin the cells down to fully exchange media. Passaging is done by dilution. Centrifugation is time consuming, risks contamination, and is generally not helpful.
7. A typical 1:5 split of a 333 mL culture is done as follows:
   (a) Count the cells.
   (b) Fill to 1 L (there is an indentation in the bottles at exactly this volume).
   (c) Cap tightly and gently shake to mix.
   (d) Pour 200 mL of diluted culture into each bottle.
   (e) Fill each bottle to 333 mL.
8. Antibiotics and/or antimycotics can only be used if cells are not to be transfected in suspension because the combination of PEI and Pen-strep is toxic. Most of our cultures are antibiotic free.

3.1.4 Counting Suspension HEK-293Ts with a Hemocytometer

HEK-293Ts grow in small clumps of 1–30 cells in suspension. Accurate counting requires dissociation of the clumps by gentle trituration using a pipet. Due to differences in light scattering by different clump sizes, optical density is not an accurate measure of cell number. We visualize the cells before and after dissociation because shearing in the dissociation protocol lyses a small fraction of the cells.

1. Using a 1 mL serological pipet, aliquot 200 µL (*see* **Note 11**) of culture to a clean microcentrifuge tube (*see* **Note 12**).
2. Mix by flicking, and pipet 10 µL onto one side of the hemocytometer.
3. With a 200 µL pipet, set the volume to ~150 µL and triturate 30 times to break up clumps. Try not to foam.
4. Pipet 10 µL onto the remaining half of the hemocytometer.

3.1.5 Suspension Transient Transfection Using PEI Max

Transfection is done using 1 μg DNA/mL media. Transfection complexes are prepared in 1/20th the culture volume hybridoma SFM with a 3:1 ratio of PEI Max to DNA (*see* **Note 13**). A transfection protocol for 1 L culture (three 1 L bottles, each containing 333 mL culture) is outlined below.

1. Prep DNA. High-quality endotoxin-free DNA is critical to success. We have had best results with PureLink HiPure Maxi and Giga prep kits (Life Technologies) using the manufacturer's protocol (*see* **Note 14**).
2. Day 1:
   (a) Grow cells to 2.5–3.5 million/mL. Count the cells.
   (b) Warm 50 mL of hybridoma SFM to room temperature (*see* **Note 15**).
   (c) Dilute 50 μg DNA in the hybridoma media. Mix well (*see* **Note 16**).
   (d) Add 150 μL 1 mg/mL PEI Max (pH 7.0). Mix well.
   (e) Incubate for 15 min at room temperature to allow DNA-PEI complex to form.
   (f) Pipet 16.7 mL into each 333 mL culture and return to the incubator.
3. Days 2–3 or 2–5:
   (a) Option 1: Induce cells by adding 1 μg/mL doxycycline. Harvest 24 h later on day 3 (*see* **Note 17**).
   (b) Option 2: Split cells 1:3 on day 2, induce on day 4, and harvest 24 h later on day 5 (*see* **Note 18**).

3.2 Adherent Transfection, Selection, and Adaptation to Suspension

Transfection is done on 6-well plates and followed by a puromycin selection done simultaneously with scale-up and transition to suspension medium. Adherent cells are maintained in DMEM supplemented with 10 % tetracycline-free FBS and penicillin-streptomycin (adherent medium).

1. Transfection (*see* **Note 19**):
   (a) Day 1: Plate 300,000 cells per well 6-well plates. Plate four wells per construct. Plate an additional four wells for a killing control.
   (b) Day 2: Prepare transfection mixtures of 400 μL Opti-Mem, 4 μg of DNA, and 12 μL Fugene HD (3:1 reagent:DNA ratio), following the manufacturer's protocol. After complex formation, add 100 μL of transfection mixture to each well and mix by rocking the plate.
   (c) Day 3:
      i. Dissociate the cells by banging or by using TrypLE Express (*see* **Note 20**).

ii. Pool the four wells used for each construct. Plate on 10 cm dishes or T-75 culture flasks in a 50:50 mixture of suspension medium (*see* **Note 21**) and adherent medium, supplemented with 1 μg/mL puromycin; higher concentrations may also be used [43] (*see* **Note 22**). For a good transfection, >80 % of cells will survive this harsh transition.

(d) Day 5–6: Control (untransfected) cells should be almost completely dead, with few cells adhered. Once transfected cells are >80 % confluent, split onto 2 × 15 cm dishes using an 80:20 mixture of suspension medium and adherent medium, supplemented with puromycin (*see* **Notes 23** and **24**).

(e) Days 7–9: Once the cells are confluent, adapt to suspension:

i. Prepare 50 mL of a 90:10 mixture of suspension medium and adherent medium (*see* **Note 25**).

ii. Dissociate and count the cells. Remove the media.

iii. Resuspend the cells in 10 mL media and triturate aggressively to break up clumps. Increase volume to 50 mL (typically ~1 million/mL), transfer to a 1 L bottle, and immediately put in the shaker.

(f) Days 9–11: Check the cells after 2 days in suspension. Typical density should be >2 million per mL. Cultures are typically very clumpy at this point, but healthy and doubling every 24–36 h. Sometimes the clumps are large enough to see by the naked eye. The goal of the following steps is to reduce the serum concentration and select non-clumping cells.

i. Transfer 25 mL of the culture (50 mL) into each of the two 50 mL conical tubes.

ii. Triturate five times.

iii. If the cells are very clumpy, vortex three pulses of 5 s (maximum intensity) to break up clumps.

iv. Transfer tubes to a rack in the hood. Count the cells. While counting, allow the cells to settle for a total of 5 min; large clumps will settle rapidly.

v. Split the cells to 0.5 million per mL, adding only suspension medium.

vi. Remaining cells may be used for a test induction at this point.

(g) Expand cells to desired volume and induce at 3.5–4.5 million/mL with 1 μg/mL doxycycline. Harvest 24 h later (*see* **Note 26**).

3.3 Harvest of Suspension Cells to Make Liquid Nitrogen "BB's" (See Note 27)

Cells are spun down in syringes and injected into tubes containing liquid nitrogen [38]. A video protocol of this process done in yeast cells is available at http://www.ncdir.org/public-resources/protocols/. The below protocol is modified for human cells.

1. Gather an ice bucket and clean centrifuge bottles.
2. Count cells. Counts are useful for normalization and blotting.
3. Transfer a 1 mL aliquot(s) of culture for western blotting to a microcentrifuge tube. Spin at 500 × *g* for 30 s, aspirate the media, and store on ice until freezing is convenient.
4. Spin the cultures at 1000 × *g* for 10 min at 4 °C to pellet. We use both 4 × 1 L and 6 × 500 mL centrifuge rotors (*see* **Note 28**). Pour off the media.
5. Resuspend the pellets in a minimal volume of PBS (approximately equal volume to the pellet). Pool resuspended cells.
6. Pellet cells inside syringes.
   (a) Select syringe(s) for cell pelleting. Five milliliter syringes are ideal for small samples. Use 10 mL or 30 mL syringes for large samples.
   (b) Remove the plungers and set aside.
   (c) Securely cap syringes with luer-lock end caps and place inside 50 mL conical tubes (*see* **Note 29**).
   (d) Spin at 200 × *g* for 10 min at 4 °C. Spinning harder risks breaking the syringes. If cells are not well pelleted, spin again. Transfer syringes to an ice bucket.
   (e) Aspirate the PBS, leaving wet cells in the syringe (*see* **Note 30**).
7. Insert a conical tube rack in a small Styrofoam box. Fill with liquid N_2 (LN_2) to the top of the rack (*see* **Note 31**). Pre-label 50 mL conical tubes, transfer to the rack, and fill with LN_2 (*see* **Note 32**) (Fig. 2).
8. Pre-chill a small stainless steel spatula, standing it vertically in the liquid nitrogen.
9. Punch a number of holes in the caps of the tubes using a 16ga needle. This allows drainage of the LN_2 without loss of cells.
10. Inject the cells gradually into the tube containing LN_2 (Fig. 2) (*see* **Note 33**). If injected too fast, they will form large clumps. Use the pre-chilled spatula as needed to break apart any clumps.
11. Cap the tube using the punched lid. Careful! Pressure will build up and liquid can shoot out. Decant into a sink. Replace the punched lid with a new, intact lid and store tubes at −80 °C until cryomilling (*see* **Note 34**).

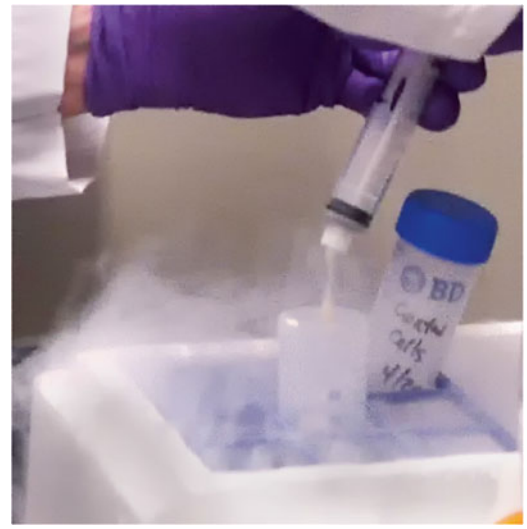

Fig. 2 Injection of pelleted human cells into liquid nitrogen to make "BB's." After centrifugation in a capped syringe, cells are injected into 50 mL conical tubes containing liquid nitrogen. Injection at a moderate rate prevents over-clumping of the cells. Use a pre-chilled spatula to break up any clumps

3.4 Cryomilling

We cryomill cells under liquid nitrogen in a Retsch PM 100 planetary ball mill; model PM 100 CM is also suitable. A newer model, the CryoMill, may be suitable for smaller samples. This protocol was initially developed for yeast, which are harder to break than mammalian cells due to the tough cell wall, and then adapted for mammalian cells with two stages of grinding [38]. Depending on the amount of cells to grind, we either use a 50 mL jar (~1–8 g cells) or a 125 mL jar (~5–30 g cells, Fig. 3a) (*see* **Note 35**). We present here a simplified protocol, after finding that the second stage of grinding was not necessary (Fig. 3b). Custom-made PTFE insulators (Fig. 3a) minimize warming of the sample during grinding and improve safety and performance (*see* **Note 36**). We use a homemade LN_2 decanter made using a spatula and 50 mL conical tube to pour LN_2 into and over the grinding jars [38].

1. Pre-clean grinding jar, lid, balls, two small steel spatulas, and large forceps using Windex glass cleaner or similar. Inspect the PTFE gasket for signs of damage. For a 50 mL chamber, use two 20 mm diameter balls. For a 125 mL chamber, use five 20 mm diameter balls.
2. Weigh the jar + insulators + balls and adjust PM 100 counterbalance accordingly.
3. Precool the jar, balls, spatulas, forceps, LN_2 decanter, and PTFE insulators in a clean Styrofoam box containing liquid LN_2 until the LN_2 stops boiling (*see* **Note 37**). Set up a working pan with LN_2 (*see* **Note 38**).

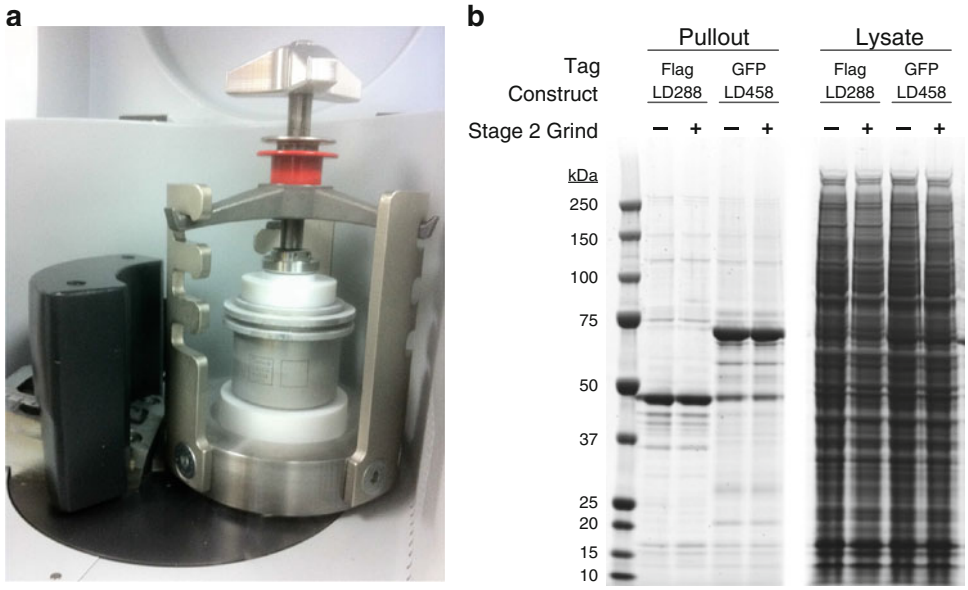

Fig. 3 Cryomilling setup for human cells. (**a**) Cryomilling apparatus in the Resch PM 100. 125 mL grinding jar is shown with custom PTFE insulators above and below. (**b**) Comparison of simplified one-stage and prior two-stage grinding protocols on protein extraction and affinity capture reveals equivalent results. HEK-293T_{LD} cells expressing full-length L1 (ORFeus*HS* background) with ORF1p tagged with Flag (LD288) or GFP (LD258) were affinity captured (pullout) using respective epitope tag antibody-conjugated Dynabeads in our standard extraction solution and eluted under denaturing conditions. Total extracted lysate (lysate) before affinity capture is shown

4. Transfer the cold PTFE base to the grinder.
5. Transfer the frozen cell BB's into the grinding jar.
6. Fill with LN_2 to within ~0.5–1 cm of the top. Cover with the lid and Teflon top insulator, move jar plus lids *en bloc* onto the grinder, and clamp in place (*see* **Note 39**).
7. Pour LN_2 over the jar using the decanter.
8. Grind with three cycles of the following program: 400 rpm, 3 min, reverse rotation every 30 s, no interval breaks (*see* **Note 40**). Between grinding cycles, cool the jar as below:
   (a) Pour LN over the jar using the decanter to cool the lid and top.
   (b) Some pressure will have built up during the grinding. The jar may be gently hissing as pressure escapes: this is normal. Carefully remove the jar as in **step 9** below 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 chamber. 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 and reassemble as in **steps 6** and **7**.

9. To remove the jar, slowly release clamping pressure (*see* **Note 41**). Transfer jar assembly to the pan of LN_2.
10. Put a pre-labeled 50 mL tube in a rack in the LN_2 pan. Remove steel balls with forceps, dislodging large chunks of grindate with a spatula.
11. Transfer grindate 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.
12. Store vertically at −80 °C overnight with the caps loose to allow LN_2 to evaporate, and then seal and store (*see* **Note 42**).

3.5 Conjugation of Dynabeads with Anti-Flag or Anti-ORF1 Antibody

This protocol was originally developed for bulk rabbit IgG [37] and has been adapted for use with precious/expensive antibodies, like anti-Flag M2. See also [44] for a general protocol. Nucleophilic side chains and N-termini on the antibody react with epoxide functional groups on the bead surface. It is critical that all other nucleophiles are absent from the buffer, or these will react with the beads and prevent antibody coupling. This includes tris, glycerol, azide, and other common antibody buffer components. It is safest to buffer exchange antibodies from commercial sources unless the absence of nucleophiles can be assured.

3.5.1 Antibody Buffer Exchange with Microcentrifuge Desalting Columns

We exchange the buffer twice to remove as much contaminant as possible. For large-scale couplings, we use a fast protein liquid chromatography (FPLC) system (ÄKTA, GE Healthcare) with a preparative-scale desalting column, but in the absence of this equipment, and for small, precious antibody samples, we use the below protocol. Alternative methods include ion exchange and dialysis.

1. Pre-equilibrate Zeba™ Spin Desalting columns in PBS three times according to the manufacturer's instructions.
   (a) Zeba™ columns have a maximum volume of 130 μL. Because we desalt twice, equilibrate two columns for every 130 μL. For example, for 250 μL antibody solution, equilibrate four columns.
2. Load, centrifuge, and recover exchanged antibody solution according to the manufacturer's instructions. Repeat once on fresh columns for a total of two exchanges (*see* **Note 43**).

3.5.2 Antibody Coupling

This protocol is for coupling of 300 mg Dynabeads. At the last step the beads are slurried by the addition of 2 mL buffer (*see* **Note 44**). We also routinely couple with 60 mg Dynabeads, slurried by the addition of 400 μL. For a 60 mg coupling, scale volumes in the

protocol linearly. Smaller volumes can also be used [44]. A commercial coupling kit for Dynabeads is available from Life Technologies; this uses proprietary components but is effective.

Day 1: Coupling

1. Calculate how much antibody to use. One milligram of Dynabeads M-270 Epoxy has been estimated to immobilize 7–8 μg of antibody during coupling (*see* **Note 45**). Coupling is not 100 % efficient and excess antibody appears to help drive the reaction [37]. For commercially available antibodies we use 10 μg antibody/mg Dynabeads (3 mg α-Flag for a 300 mg Dynabead coupling, *see* **Note 46**). We use 5 μg/mg for precious custom antibodies, and 15 μg/mg when we have the hybridoma.
2. Resuspend an entire bottle of 300 mg Dynabeads M270 Epoxy with 10 mL of 100 mM phosphate buffer, pH 7.4. Directly add buffer to the bottle and vortex bottle.
3. Transfer to a 15 mL falcon tube and wash the bottle twice with 2 mL phosphate buffer (*see* **Note 47**).
4. Shake bead suspension for 10 min on a nutating mixer or orbital shaker.
5. While the beads mix, prepare the *antibody mixture* (20 μL/mg Dynabeads, 6 mL total).
   (a) 2 mL 3 M Ammonium sulfate.
   (b) 3 mg antibody, double buffer-exchanged or supplied in phosphate buffer or PBS, free of glycerol and interfering species (see the manufacturer's instructions).
   (c) 0.1 M Phosphate buffer to 6 mL.
6. Transfer beads to a magnetic separator (*see* **Note 48**). Aspirate the buffer.
7. Wash again with 10 mL 100 mM phosphate buffer. Add buffer, vortex for 15 s, apply magnet, and aspirate; no incubation is necessary.
8. Add the *antibody mixture* to the beads. Seal and Parafilm the tube, and mix well.
9. Incubate overnight (18–24 h) on a rotating wheel at 37 °C (*see* **Note 49**).

Day 2: Bead Washing

1. Separate beads from the antibody mixture with a magnet. Carefully remove the antibody mixture and set aside in a clean tube: it still contains 30–50 % of the antibody, unreacted, which can be recovered for reuse.
2. Wash beads once with 12 mL 100 mM glycine pH 2.5. Add buffer, vortex briefly, and take it off as fast as possible.

3. Wash once with 12 mL 10 mM Tris–HCl, pH 8.8.
4. Prepare fresh 100 mM triethylamine: Add 168 μL stock to 11.8 mL ddH_2O. Apply, mix, remove, and proceed to the next step as fast as possible.
5. Wash the beads with 12 mL 1× PBS, incubating for 5 min on the nutator. Repeat a total of four times.
6. Wash twice with 12 mL PBS + 0.5 % Triton X-100, incubating each wash for 10 min on the nutator.
7. Resuspend beads in 2 mL *resuspension buffer:* PBS, 50 % glycerol, and 0.5 mg/mL BSA (*see* **Note 50**).
8. Mix well and aliquot 100 μL each into Eppendorf tubes. Store at −20 °C (*see* **Note 50**).

Reuse and Optional Antibody Recovery Using Protein G affinity (*see* **Note 51**). Approximately 30–50 % of antibody is unbound after coupling. We routinely save antibody mixtures for use in other assays such as immunoblotting and immunofluorescence. Alternatively, recovered antibody can easily be re-captured using Protein G affinity. Recovery also allows concentration of antibody and transfer into a more permanent storage buffer. We use Protein G Sepaharose Fast Flow (GE Healthcare) (*see* **Note 52**) following the manufacturer's instructions. The antibody sample should be diluted with an equal volume of PBS for binding. Based on 50 % antibody-Dynabead binding, ~125 μL resin is needed for recovery after a 300 mg Dynabead coupling. After elution, add 50 % glycerol for storage or dialyze or desalt into the buffer of choice.

3.6 Affinity Capture of RNPs Using Conjugated Magnetic Medium

For a comprehensive review of considerations affecting expression systems, epitope tagging, and affinity medium choice see [45]. In brief, we find that Dynabeads, when conjugated to high-quality antibodies, provide for excellent quality recovery of endogenous protein complexes from human cells [37, 38]. We have successfully applied this approach to L1 RNPs [6]. For purifying 3× Flag-tagged constructs, α-Flag Dynabeads are necessary, prepared as above. When combined with neodymium magnet racks, antibody-conjugated magnetic medium (beads) is rapidly separated from the buffer and immobilized on the side of the tube. This allows near-complete aspiration of buffer without the risk of aspirating the beads.

1. Prepare clarified cell extracts:
   (a) Weigh out 200 mg of cell powder into a microcentrifuge tube—hold on LN_2 (*see* **Note 53**).
      i. Repeat (a) for as many purifications as you will carry out.
      ii. Multiple purifications can be pooled after elution if larger scale is required.
   (b) Move the tubes to room temperature for 1–2 min (*see* **Note 54**).

(c) Add 800 μL of extraction solution (*see* **Note 55**) (20 mM Na-HEPES pH 7.4, 500 mM NaCl, 1 % v/v Triton X-100; plus protease inhibitors) to each tube, vortex for ~30 s until powders are resuspended, and then place the crude extracts on ice. Some membrane aggregates may be observed (*see* **Note 56**).

(d) Sonicate each tube with a micro-tip probe on a low-power setting using 2 × 2 s pulses. Membrane aggregates should no longer be visible (*see* **Note 57**).

(e) Centrifuge for 10 min at full speed (20–30,000 RCF) in a refrigerated microcentrifuge at 4 °C.

i. During this step the affinity medium can be pre-washed (**step 2a**).

(f) Remove supernatant—this is your clarified extract—and add to the tube containing α-Flag Dynabeads (**step 2b**).

i. Set a fraction aside before combining with beads to compare pre- and post-bead binding (**step 2ci**) in order to assess the efficacy of the affinity capture.

2. Affinity capture:

(a) Prepare beads:

i. Pipette 20 μL of α-Flag Dynabeads slurry into a 1.5 mL microcentrifuge tube (*see* **Note 58**).

ii. Repeat for each affinity purification to be carried out.

iii. Wash the beads twice with 500 μL of extraction solution.

iv. Remove the supernatant, and hold the beads on ice until needed.

(b) Combine the clarified extract (**step 1f**) with the beads.

i. Incubate at 4 °C for 30 min (*see* **Note 59**).

(c) Separate beads on a magnetic separator. Set a fraction aside to compare with input (**step 1fi**), and aspirate the remainder.

(d) Wash the beads with 1 mL of extraction solution and then remove the supernatant. Wash protocol for beads (used throughout):

i. Add buffer, and vortex at full power for 2–3 s.

ii. Pulse-spin in a benchtop microcentrifuge to remove any magnetic beads from the cap.

iii. Separate beads on a magnetic separator, and remove buffer using a vacuum aspirator.

(e) Resuspend the beads in 1 mL of extraction solution, transfer to a fresh microcentrifuge tube, and then remove the supernatant (*see* **Note 60**).

(f) Wash again with 1 mL of extraction solution.

(g) Elute the L1 RNPs from the beads with 26 μL of 1 mg/mL 3× Flag peptide in extraction solution (native) or using 1× SDS-PAGE loading buffer without reducing agent (*see* **Note 61**).

i. Native elution: Incubate for 15–30 min at room temperature with gentle agitation (just enough to mix and suspend the beads).

ii. Denaturing elution: Incubate for 5–10 min at 70 °C with moderate agitation (*see* **Note 62**).

iii. If native elution is used, remove the eluate and then perform a second denaturing elution to assess the efficacy of the native elution.

3. Natively eluted samples can be carried forward to other assays (see LEAP and RT-PCR below) and/or subsequently prepared for SDS-PAGE (*see* **Notes 63–65**).

4. For samples to be analyzed by mass spectrometry, reduce and alkylate with iodoacetamide:

(a) Add SDS-PAGE loading dye to 1× and DTT to 20 mM (*see* **Note 66**).

(b) Incubate for 10 min at 70 °C.

(c) Cool to room temperature.

(d) Add iodoacetamide to 0.1 M and incubate in the dark at room temp for 30 min.

(e) Load directly on a gel.

3.7 Sample Preparation for Mass Spectrometry

Proper preparation of samples is critical for mass spectrometry as a number of interfering species can reduce sensitivity and compromise protein identification (for discussion and advice, see [45]). For identification of the most prominent species, readily observed by standard protein staining techniques (reviewed in [46, 47]), we excise regions of the gel containing stained protein bands and use MALDI-MS peptide mass fingerprinting (PMF, [48, 49]; example protocols that we use can be found in the supplement of [38]). For more sensitive detection and identification of the complement of proteins within the sample we use liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) approaches (reviewed in [50, 51]). If proteins are first metabolically labeled with stable isotopes prior to affinity capture, then isotopic differentiation of interactions as random or targeted (I-DIRT) can be implemented. This MS-based analysis provides statistical discrimination between interactors likely to have originated in vivo and those likely to be in vitro artifacts, and can be accomplished by modifying the procedures outlined in this chapter [6, 52, 53]. Whether MALDI-MS or LC-MS/MS will be used, we prefer gel-based peptide sample

work-up [54]. Because different MS-based analytical approaches may require different sample work-up procedures, we recommend adopting appropriate procedures based on the preferences of your proteomics core facility or collaborator.

3.8 LEAP (L1 Element Amplification Protocol)

This protocol uses ORF2p reverse transcriptase and an anchor primer to reverse transcribe bound RNA [23]. cDNA is then amplified by PCR for visualization on a gel or quantification by real-time PCR. A boiled sample is used for a negative control. cDNA is made with commercial reverse transcriptase as a positive control.

1. Thaw RNPs on ice. Clean bench and work area using RNAseZap or similar. Open a clean box of pipet tips.
2. Prepare sufficient LEAP reaction mix for twice the number of samples (see recipe, Subheading 3).
3. Prepare LEAP + SuperScript reaction mixture:
   (a) Pipet half of the LEAP buffer into a clean tube.
   (b) Add SuperScript III Reverse Transcriptase (0.25 μL per reaction, 50 U), and mix well.
4. For the negative control, add 5 μL of one RNP sample to a clean tube. Boil for 5 min at 100 °C.
5. Aliquot LEAP and LEAP + SuperScript reaction mixtures into reaction tubes.
6. Add 2 μL of each RNP to both LEAP and LEAP + SuperScript mixture. Do not forget to add the boiled negative control.
7. Incubate LEAP reactions for 1 h at 37 °C. Incubate LEAP + SuperScript reactions for 1 h at 50 °C (*see* **Note 67**).
8. Optional pause point: Snap-freeze LEAP products.
9. Amplify LEAP products for visualization and/or sequencing:
   (a) Use 1 μL LEAP product in a 50 μL PCR reaction using FastStart Taq DNA Polymerase with primers JB11564 and JB14057.
10. Measure LEAP products by qRT-PCR using a StepOne Plus instrument or similar:
    (a) Use 0.5 μL of each LEAP product in triplicate 20 μL reactions with SYBR Green 2× master mix.
    (b) Measure the ORF1 region of the L1 RNA with primers JB13415 and JB13416 (*see* **Note 68**).
    (c) Measure the ORF2 region of the L1 RNA with primers JB13417 and JB13418.

3.9 RNA Isolation and qRT-PCR of L1 RNPs

Total RNA is isolated from L1 RNPs after spiking in purified GFP-transfected HEK-293T RNA (*see* **Note 4**). This serves as both a carrier and as an internal control for normalization. Alternatively, in vitro-transcribed GFP mRNA may be used along with glycogen

as a carrier. A practical review of qRT-PCR methods can be found in [55].

1. Aliquot 2 μg purified GFP-transfected control RNA into each tube.
2. Add 10 μL of each RNP sample, and mix well.
3. Purify RNA using TRIzol Reagent according to the manufacturer's protocol.
4. Resuspend RNA in 20 μL RNase-free water.
5. Quantify by qRT-PCR as in Subheading 3.8, **step 10**, using 0.5 μL resuspended RNA in triplicate. Primers for GFP (JB13766 and JB13767) are used for normalization of each sample.

4 Notes

1. This affinity-purified version performs as well or better than the non-affinity-purified F3165, and at the time of writing is approximately half the cost.
2. The Dynamag 15 rack is useful for couplings starting with 300 mg Dynabeads. We often do smaller test couplings with 60 mg Dynabeads, which can be done with 2 mL tubes. To reduce cost, high-quality neodymium magnets from MAGCRAFT are widely distributed and available in a variety of shapes. These can be fitted into homemade racks or attached to tubes using rubber bands; arc-shaped magnets are particularly useful with rubber bands.
3. The pH of 10× PBS is between 6.6 and 6.7 at 10× but is 7.4 when diluted to 1×.
4. Buffer components for affinity capture and LEAP should be RNase free, and handled with precautions for cleanliness appropriate for RNA work. This includes buffers used for dissolution of 3× Flag peptide, etc..
5. We transfect HEK-293TLD cells with pCAG-eGFP (Addgene # 11150) and purify total RNA using an RNeasy mini kit (Qiagen).
6. Life Technologies reports that Freestyle 293 provides serum-free growth of their engineered HEK-293-F cells. In our experience with other HEK-293 and HEK-293T cells, 1 % tet-free FBS adds little cost to the medium and increases reliability. Similarly, supplemental l-glutamine, added fresh, provides more reliable growth than their GlutaMax alternative alone. We make a 50:50 mix of serum and 200 mM l-glutamine, filter, and add 20 mL per liter.

7. We have been unable to establish an effective protocol for puromycin selection after suspension transfection.
8. Always autoclave with loose bottle caps. The tops of the bottles can be covered in foil for additional protection. After the autoclave finishes, transfer bottles to a culture hood to cool, and then tighten caps for storage.
9. If any bottle is found to be contaminated, all bottles in the incubator must be checked for signs of contamination.
10. Until a suspension line is well established or after steps that can be toxic to cells, we find a minimum density of 0.5 million/mL is safer than 0.2 million/mL.
11. Do not directly transfer from the serological pipet to the hemocytometer.
12. Never use a micropipettor in suspension bottles: this risks contamination.
13. This ratio should be optimized for different cell lines and DNA preparation methods.
14. After column purification, precipitate with isopropanol and wash with ethanol. DNA prepared with their precipitator modules has been less effective in our experience.
15. To save time, hybridoma SFM aliquots can be left at room temperature overnight.
16. For small numbers of transfections, prepare DNA-PEI complex in conical tubes. For larger volumes, use a glass bottle.
17. ORF2p expression peaks 24 h after induction and falls thereafter. ORF1p expression peaks at ~18 h after induction and is constant for at least 4–5 days. Harvest time should be optimized for your protein of interest.
18. We have found that with episomal pCEP4-based plasmids, a 1:3 "split-back" minimally reduces per-cell yield but allows the use of threefold less DNA per gram WCW.
19. We find that transfection efficiency is higher in 6-well wells than larger scales, so we transfect a number of wells and then pool the cells later.
20. If protease is used, quench with serum-containing media and centrifuge for 5 min at 200 RCF to remove the protease.
21. Recipe for suspension medium is as described above, but when following the protocol for adherent transfection, selection, and adaptation to suspension it may include antibiotics.
22. Cells can be adapted into higher puromycin concentrations of 2–10 μg/mL, resulting in higher per-cell expression but slower growth and longer time needed for adaptation.
23. Suspension cells are more sensitive to puromycin than adherent cells.

24. It can be helpful to perform a test induction at this point. Plate 0.5 million cells in each of the two 6-well wells. Add 1 μg/mL doxycycline to induce one. Lyse 24 h later for western blotting and freeze lysate for later comparison.
25. Note that this 90:10 medium contains ~2 % FBS.
26. Once cells stably transfected with a construct of interest are growing in suspension, it is advisable to freeze aliquots for later use. We freeze 20 million cells per vial in suspension medium supplemented with 5 % DMSO and 20 % FBS.
27. A "BB" is a small round steel ball, like those used in toy guns. Cells frozen in this way form spheroid shapes (and globules of spheroids).
28. Pellets should appear approximately uniform. If centrifuged too hard, cells will be crushed, forming two different-colored layers.
29. Depending on the syringes used, it may be necessary to trim the finger grips so the syringe fits inside the tube. Thirty milliliter syringes are taller than 50 mL conical tubes. Make sure that these have enough clearance in your swinging bucket centrifuge before adding cells.
30. In order to thoroughly remove the liquid, we commonly suck off the very top layer of cells in order to remove the PBS. We use a vacuum aspirator and glass Pasteur pipet.
31. Use care and best practices in handling liquid nitrogen. Appropriate protective gear and goggles should be used to prevent injury.
32. Each tube will hold approximately 15–20 g BB's. For very large samples, we use polypropylene bottles.
33. A pair of pliers may be needed to remove the luer-lock syringe cap after centrifugation.
34. Storage with a punched cap is acceptable for a few days; however frost will form on cells over longer periods.
35. 1 g WCW is approximately the minimum amount of cells for grinding. A small amount of material (up to ~500 mg) is lost on the surface of the jar and balls, so practically we aim to produce at least 1.5–3 g WCW.
36. The custom PTFE insulators are not required for this protocol [38]. However, we find that grinding is greatly aided by the inclusion of LN within the jar ("wet grinding"). The insulators prevent warming of the jar, evaporation of the nitrogen, and pressure buildup, resulting in faster, more reliable grinds. To have insulators made at the Rockefeller University machine shop, contact the Rout Lab.
37. All tools for grinding should be chilled during use.

38. We use two containers: a Styrofoam box filled with enough N2 to completely submerge the jar, and a pan for sample prep and intermediate cooling steps in which the jar is not completely submerged. When grinding multiple samples, we put a tube rack in the Styrofoam box for storage of BB's and grindate and cover the box with its lid.
39. Clamping force should be firm, but not excessive such that removal of the jar becomes a problem.
40. Grinding should make a distinct clunking noise as the balls collide. This noise may stop at some point during a rotation, but should resume when rotation is reversed. If these sounds are not heard, inspect between cycles to make sure that grinding is occurring.
41. Releasing too quickly can cause rapid depressurization and loss of grindate. A controlled release allows gentle depressurization, which can be heard as a gentle hiss.
42. Grindate can be stored at −80 °C essentially indefinitely, without affecting performance.
43. Protein recovery can be verified by Bradford assay or SDS-PAGE with Coomassie, comparing input and output. For Bradford use gamma globulin as the standard to get an accurate concentration.
44. This results in ~2.2 mL final volume, and a slurry of approximately ~10–15 % by volume.
45. Measurements of coupling yield were done using rabbit polyclonal IgG. The apparent capacity of the beads may vary depending upon the coupling conditions, including the concentration of salt, ammonium sulfate, pH, and temperature of coupling.
46. With anti-Flag M2, beads conjugated at 8 μg antibody/mg Dynabeads do not perform as well as beads conjugated at 10 μg/mg. There is only a marginal improvement in going from 10 to 12.5 μg/mL; we use 10 μg/mg as a cost-effective compromise.
47. Sometimes resuspension requires more volume. Simply remove the buffer from the beads using a magnetic separator and wash the bottle with fresh buffer.
48. The addition of BSA increases the long-term stability of M2-Flag antibody-conjugated Dynabeads.
49. 30 °C is also effective.
50. With proper storage, coupled Dynabeads may be used without loss of performance for >1 year. Alternatively, if the beads will be completely consumed within ~8 weeks, storage at 4 °C is suitable—resuspend with PBS, 0.5 mg/mL BSA, and 0.02 % sodium azide and store at 4 °C.

51. Depending on the antibody species and subtype, protein A may be more appropriate than protein G.
52. A variety of Protein G preparations, including magnetic, are commercially available. At this scale, magnetic medium is much more expensive.
53. Use a small Styrofoam rack on a microbalance and pre-chill tubes and weighing instruments on LN2. We have found that this is easiest using inexpensive small stainless steel measuring spoons designed for culinary use.
54. Allowing the tube to briefly warm prevents extraction solution from flash freezing on the side of the tube.
55. Determination of the optimal extraction solution for each complex is critical but beyond the scope of this chapter. For more information see LaCava et al. (45).
56. Hold tubes on ice between each subsequent manipulation—working at room temperature is otherwise acceptable.
57. The power should be adjusted such that the minimum amount of energy is used that will disperse the aggregates. On a Branson Sonifier with Microtip, this is power setting 3.
58. The ratio of beads to lysate can be optimized. For α-ORF1p pullouts 50 μL Dynabeads were needed to deplete the ORF1p from extracts. For α-ORF2p pullouts, 10 μL was sufficient and background increased with larger amounts.
59. This time can be optimized. For α-ORF1p pullouts, 5 min was as effective as 30 min. For α-ORF2p pullouts using α-Flag Dynabeads, 1 h was more effective than 30 min.
60. We find that transfer to a fresh tube at this step reduces background because some protein nonspecifically sticks to the tube.
61. Reducing agent is omitted to reduce the release of IgG from the beads. It should be added after separation from the magnetic medium, before denaturation for SDS-PAGE.
62. We use a Thermomixer (Eppendorf) at full speed.
63. The advantage of native elution, even if SDS-PAGE is the next step, is its tendency to release only specific interactors of the tagged protein, reducing nonspecific contamination.
64. Regardless of the elution method, all samples should be reduced and alkylated prior to loading on the gel to maximize sensitive detection of cysteine-containing peptides during subsequent MS.
65. For storage of purified RNPs, for example for future LEAP, we dilute 1:1 with 50 % glycerol (25 % final), flash freeze with N2, and store at −80 °C.
66. This is 40 % of the typical 50 mM DTT concentration in reducing SDS-PAGE loading dye.

67. Incubation at 50 °C favors the SuperScript III reaction over the ORF2p reaction. At 37 °C both reactions occur simultaneously.
68. These primers are specific for ORFeusHS; appropriate primers should be chosen for other L1 elements.

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