Chapter 3

High-Efficiency Isolation of Nuclear Envelope Protein Complexes from Trypanosomes

Samson O. Obado, Mark C. Field, Brian T. Chait, and Michael P. Rout

Abstract

Functional understanding of the nuclear envelope requires the identification of its component proteins and their interactions. Trypanosomes cause human and livestock diseases worldwide but are so divergent from animals and fungi that in silico searches for homologs of proteins are frequently of low value. Here we describe a strategy for the straightforward identification of nuclear envelope proteins from trypanosomes that classifies proteins and their interaction networks in the nuclear pore complex. Milling frozen whole cells into a powder and rapid screening of buffer conditions for optimization of complex isolation is described. The method is inexpensive and potentially applicable to many organisms, providing fast access to functional information.

Key words Nuclear envelope, Nuclear pore complex, Affinity isolation, Cryomilling, *Trypanosoma*, Proteomics, Molecular evolution

1 Introduction

Trypanosomes are pathogenic Protozoa and may be one of the earliest branching taxa following radiation of the eukaryotes from the last eukaryotic common ancestor [1, 2]. Extreme evolutionary divergence represents a challenge to functional understanding of proteins, as many are novel and lack obvious homologs in model organisms [3]. The trypanosome nucleus harbors several novel systems, including polycistronic transcription of most protein-coding genes, and a lamina and kinetochores comprised of completely distinct proteins to the functionally analogous complexes of higher eukaryotes [4–6]. Extreme sequence divergence in the case of the nuclear pore complex (NPC) proteins and the lamina demands direct approaches for identification of proteins and protein–protein interactions.

We describe procedures that together allow the rapid isolation of protein complexes from African trypanosomes (Fig. 1). Similar approaches have been successfully applied to a broad range of

Sue Shackleton et al. (eds.), *The Nuclear Envelope: Methods and Protocols*, Methods in Molecular Biology, vol. 1411,
DOI 10.1007/978-1-4939-3530-7_3,

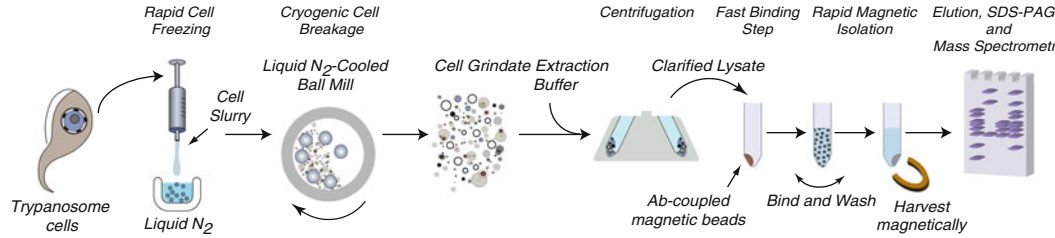

Fig. 1 Overall schematic of biophysical cell lysis and proteomics. Cells are harvested, flash frozen in liquid nitrogen, and added to precooled steel jars containing steel balls. As the jar is centrifuged in a planetary ball mill, it rotates, causing the balls to cascade with high force and mill the frozen cells into a powder. Various extraction solutions can then be tested to find the most suitable conditions for the protein complex of interest. The cell powder is resuspended in an extract solution and clarified by centrifugation. Affinity capture is performed in the presence of anti-GFP antibody coupled to magnetic beads and then eluted and fractionated by SDS–PAGE. Protein bands are excised, digested with trypsin, and identified by mass spectrometry. This figure is adapted and modified from Oeffinger et al. [7]

organisms, and we believe that these methods have major utility in deciphering the nuclear and cellular biology of trypanosomes. This protocol exploits the ability to grow parasites in semi-defined media, routine genetic modification, and specifically facile introduction of an epitope tag into one allelic copy of the diploid genome. The epitope tag is typically GFP, although a wide range of common epitopes and/or ectopic expression can also be used successfully. Parasites are frozen in liquid nitrogen and milled to a powder using a Retsch Planetary Ball Mill, providing highly efficient breakage of cells without a need for solubilization or denaturing reagents. This powder can be conveniently stored at −80 °C for several months. Individual aliquots of this stock powder can then be tested in affinity isolation procedures using magnetic beads with covalently coupled antibody allowing rapid screening of buffers, detergents, and additives, to arrive at an optimal, or acceptable, set of conditions suitable for electrospray ionization mass spectrometry. The cryomilling approach can provide rapid and inexpensive access to a large constellation of interaction data.

2 Materials

It is crucial to consult and follow the Material Safety Data Sheets and your institute's health and safety procedures for the appropriate handling of equipment and potentially hazardous materials used in these protocols. If in doubt, we advise contacting the authors for advice, which will be gladly given.

2.1 Preparation of Frozen Trypanosome Pellets

1. Refrigerated centrifuge and rotor for spinning large cell volumes (e.g., Beckman JLA 8.100 rotor).

2. Phosphate-buffered saline (PBS) if working with procyclic form trypanosomes or trypanosome dilution buffer (TDB) if working with bloodstream form trypanosomes. TDB—pH 7.4: 5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4$, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 20 mM glucose.
3. Resuspension buffer: Ice cold 1× PBS or TDB containing 100 mM dithiothreitol (DTT) and protease inhibitors without EDTA.
4. 50-mL polypropylene Falcon-type tubes.
5. 20-mL syringe and syringe caps.
6. Liquid nitrogen.
7. Styrofoam container.
8. Pipettor.
9. 10- and 20-mL pipettes.

2.2 Conjugation of Magnetic Beads

1. Rotator for microfuge tubes at 30 °C.
2. Vacuum aspirator or equivalent.
3. Anti-GFP antibody (*see* **Note 1**).
4. 0.1 M sodium phosphate buffer ($NaPO_4$)—pH 7.4: 2.62 g $NaH_2PO_4 \times H_2O$ (MW 137.99) (2 mM) and 14.42 g $Na_2HPO_4 \times 2H_2O$ (MW 177.99) (20 mM). Adjust pH if necessary.
5. 3 M ammonium sulfate (stock solution): 39.6 g $(NH_4)_2SO_4$ (MW 132.1). Dissolve in 0.1 M sodium phosphate buffer (pH 7.4) and adjust to 100 mL.
6. Phosphate-buffered saline (PBS).
7. PBS + 0.5 % Triton X-100: Include 0.5 % (w/v) Triton X-100 in 100 mL PBS solution.
8. 100 mM glycine–HCl pH 2.5—make fresh.
9. 10 mM Tris–HCl pH 8.8.
10. 100 mM triethylamine—make fresh.
11. 1× PBS/50 % glycerol.

2.3 Cryomilling

1. Liquid nitrogen.
2. Retsch Planetary Ball Mill PM100.
3. Retsch steel jar, lid, and 20-mm steel balls (*see* **Note 2**).
4. Teflon puck (custom made) and Teflon insulating jar (available from Retsch, a division of Verder Scientific that manufactures equipment for homogenizing laboratory samples) (Fig. 2a).
5. Cryoprotective gloves.
6. Safety goggles.

Fig. 2 Requirements and setup of the cryomilling procedure. (**a**) *Panels* showing required items: *A*, tweezers; *B*, liquid nitrogen scoop for pouring 2–15 mL (made by making an incision on a polypropylene tube using a razor blade and then inserting the spatula to create a handle); *C*, 20-mm steel balls; *D*, 50-mL steel jar; *E*, steel jar lid; *F*, Teflon puck; *G*, Teflon jar; *H*, spatula; *I*, cryo-gloves; *J*, liquid nitrogen dewar; *K*, goggles; *L*, face mask; *M*, Retsch PM100 Planetary Ball Mill; and *N*, *inset* showing a recommended safety adaptation to prevent accidental use of the PM100 without clamping the assembled milling apparatus. (**b**) A step-by-step guide to assembly of components

7. Large tweezers.
8. Spatula.
9. Liquid nitrogen scoop. This can be generated by fusing the handle to a 50-mL polypropylene tube (Fig. 2a).

2.4 Affinity Capture

1. Magnetic beads conjugated with anti-GFP (or other tag used) antibody.
2. Magnetic rack for microcentrifuge tubes.
3. Extraction buffers for affinity capture that contain a buffering agent such as Tris or HEPES, salt for ionic strength such as NaCl/KCl, detergents such as Triton, and protease inhibitors. Divalent cations such as Magnesium may be added as required.
4. Microcentrifuge.
5. Cryomilled powder.
6. Elution buffer: protein gel loading dye buffer without reducing agents added or 40 mM Tris–HCl pH 8.0 and 2 % SDS.
7. Reducing agent: 50 mM DTT.

3 Methods

3.1 Preparation of Frozen Trypanosome Pellets

1. For optimal results, we use 2×10^{10} cells as the minimum number of cells for efficient cryomilling. Harvest cells by pelleting at $3000 \times g$ in a centrifuge at 4 °C (*see* **Note 3**).
2. Discard the supernatant and resuspend the cell pellet in 40 mL of ice-cold 1× PBS for procyclic form trypanosomes or TDB for bloodstream form trypanosomes.
3. Pellet the resuspended cells at $1500 \times g$ for 5 min at 4 °C.
4. Discard supernatant and add ice-cold resuspension buffer equivalent to the volume of the cell pellet.
5. Remove the piston/plunger from a 20-mL syringe and add a cap to the nozzle to prevent flow through of the cell suspension (Fig. 3).
6. Transfer resuspended cells into the 20-mL syringe and place in a 50-mL polypropylene Falcon-type tube as illustrated (Fig. 3).
7. Pellet the resuspended cells at $5000 \times g$ for 5 min at 4 °C to compact the cells and minimize buffer carryover.
8. During **step 7**, fill a Styrofoam box with liquid nitrogen and cool a pre-labeled 50-mL polypropylene Falcon-type tube to liquid nitrogen temperature.
9. Discard the supernatant from the pelleted cells and remove the syringe cap.
10. Fill the cooled 50-mL tube with liquid nitrogen.

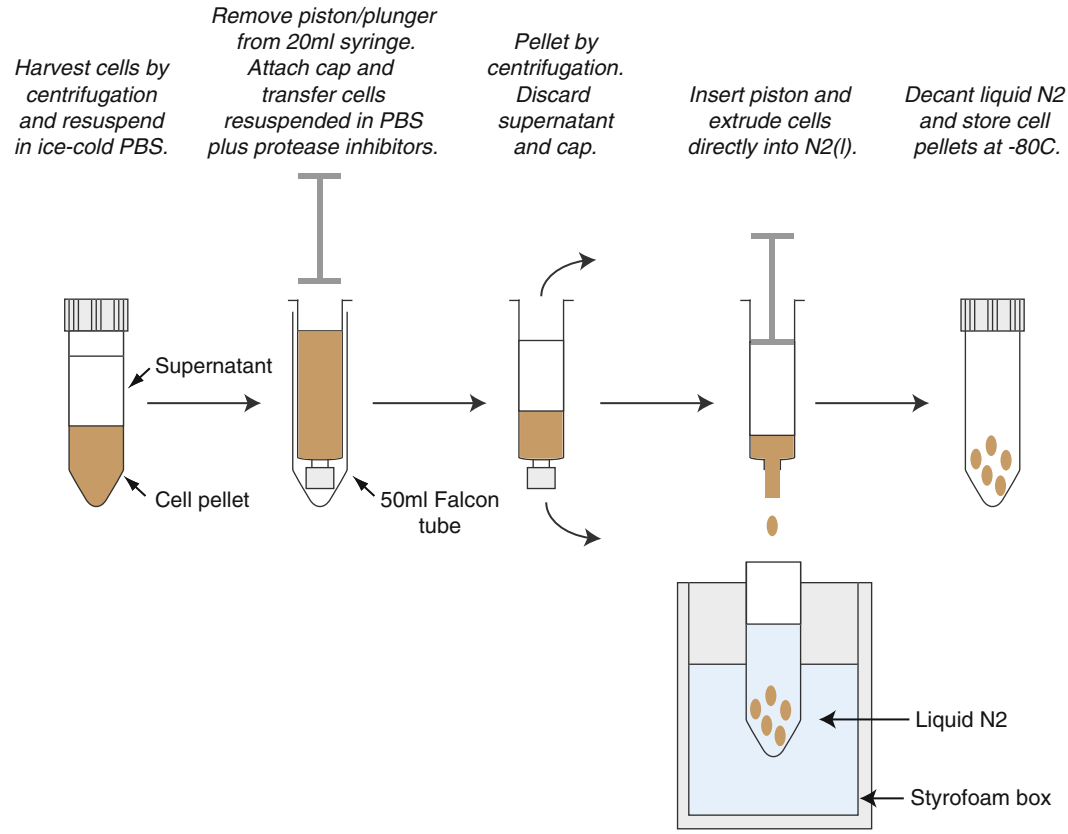

Fig. 3 Illustration of the making of frozen cell pellets. *Highlighted* are the key steps to facilitate the making of frozen cell pellets. Additional information can be found on www.ncdir.org/public-resources/protocols

11. Gently place the piston/plunger back onto the syringe that contains the cell pellets and apply very gentle pressure extruding the trypanosome cell slurry into the 50-mL tube containing the liquid nitrogen (Fig. 3).
12. Decant the remaining liquid nitrogen from the 50-mL tube that contains the frozen cell pellets/noodles and store at −80 °C until required for cryomilling (*see* **Note 4**).

3.2 Cryomilling Frozen Trypanosome Pellets

This protocol is heavily modified from an earlier version [7]. A modification of the earlier protocol has been shown to greatly facilitate mammalian cell affinity isolation procedures [8]. Here we describe a modified form for trypanosomes.

Extensive notes and description of any modifications and instructions that facilitate the cryomilling technique are available on the website of the National Center for Dynamic Interactome Research (www.ncdir.org/public-resources/protocols). It is important to visit the website for the latest updates and additional information,

particularly with respect to safety procedures, prior to attempting the cryomilling procedure. *Protective clothing, goggles, and cryoprotective gloves must be used at all times during cryomilling.*

1. Precool the steel jar, lid, Teflon puck, and Teflon insulating jar and steel balls in a Styrofoam box containing liquid nitrogen.
2. Once cooled to liquid nitrogen temperature (very little bubbling), carefully remove all cooled units from the liquid nitrogen using tweezers.
3. Assemble as shown in Fig. 2b.
4. Put frozen cells into the assembled and cooled steel jar (Fig. 2).
5. Place the cooled lid and then the cooled Teflon puck on top of the steel jar (Fig. 2).
6. Set the milling setup into the PM100 (Fig. 2).
7. Clamp down the milling jar as per manufacturers' instructions (*be sure to clamp down the jar or serious injury may occur*).
8. Adjust the setting on the PM100 to mill your frozen material at 400 rpm for 3 min, with a 1-min interval (the PM100 will pause briefly after each minute and switch direction to allow counterrotation of the milling jar). Cool the jar after each 3-min cycle by adding 10–15 mL of liquid nitrogen in the space between the Teflon jar and the 50-mL cryomilling steel jar. Allow the liquid nitrogen to evaporate completely (10 s) and repeat. Alternatively, the whole setup can be unclamped and cooled in a Styrofoam box with liquid nitrogen (do not submerge the jars completely as this will result in the loss of cell material/powder) for about 30 s before re-clamping and resuming the milling procedure. *It is important for the operator to follow good standard practice and take extreme care when handling liquid nitrogen.*
9. Repeat **step 8** three times (*see* **Note 5**).
10. Unclamp the steel jar and add 2–5 mL of liquid nitrogen into the grinding jar using the liquid nitrogen scoop described in Fig. 2 (*see* **Note 6**). The liquid nitrogen helps dislodge any compacted powder and facilitates liquid cryomilling that results in a finer millate. *It is important for the operator to follow good standard practice and take extreme care when handling liquid nitrogen.*
11. Re-clamp and cool the jar by adding 10–15 mL of liquid nitrogen in the space between the Teflon jar and the 50-mL cryomilling steel jar.
12. Mill the cell powder for 1–2 min at 400 rpm (*see* **Note 7**). Cool the jar immediately as in **step 11** while it is still clamped on the PM100 by pouring about 10–15 mL of liquid nitrogen using the liquid nitrogen scoop into the space between the Teflon

jacketed insulating jar and the 50-mL cryomilling steel jar. The liquid nitrogen will vaporize while cooling the cryomilling jars but simultaneously enables any residual evaporated nitrogen gas to cool down and lower the internal pressure in the steel milling jar. *Thus, this step is important to lower the risk of injury or sample loss when opening the jar. Nonetheless, unclamp the lid very slowly to enable a slow pressure release to prevent a sudden venting of gas that can lead to powder loss or injury.* Add 2–5 mL of liquid nitrogen into the grinding jar and re-clamp. Re-clamp and cool the jars once again as in **step 11**.

13. Repeat **step 12** ten times. While the repetitive nature of these steps are somewhat arduous, these conditions have been optimized: attempting to engage fewer but longer grinding cycles results in pressure buildup and sample warming that are unacceptable.
14. Cool the milling jars as in **step 12** prior to unclamping and carefully transfer the milled powder into a precooled and labeled 50-mL polypropylene tube that is being maintained cooled in a Styrofoam box containing liquid nitrogen.
15. Store milled powder until needed at −80 °C (*see* **Note 4**).

3.3 Conjugating Proteins to Magnetic Beads

This protocol is designed for the use with Life Technologies Dynabeads with epoxy chemistry and is modified from an earlier protocol [9]. Other approaches can be used, but we have found that the combination of speed and avoiding centrifugation steps provided by magnetic beads is optimal of those approaches we have tried. Each vial contains 300 mg of Dynabeads which correspond to the amount sufficient for the analysis of ~120 g of cell powder. Do not prepare the whole batch unless you plan to use it within 6 months (*see* **Note 8**).

1. Weigh out the appropriate amount of Dynabeads required (*see above*) in a 15-mL Falcon-type tube.
2. Add 5 mL of 0.1 M sodium phosphate buffer, pH 7.4, and vortex the tube for 30 s.
3. Insert the tubes with beads into the magnet holder. Wait until all are attached to the magnet. The solution will appear clear. Aspirate off the buffer, taking care not to touch the magnetic beads.
4. Resuspend the beads in fresh 5 mL of 0.1 M sodium phosphate buffer, pH 7.4.
5. Agitate slowly for 10 min on a shaking platform or rotator.
6. Harvest the beads magnetically as in **step 3** and discard supernatant.
7. Setup the conjugation reaction once the incubation in **step 5** has been started. Add the reagents sequentially in the order as follows.

8. For 100 mg of beads (1/3 of a Dynabeads vial) the ideal reaction volume is 2 mL. Add the following reagents to a 5-mL round bottomed tube with a snap cap or an equivalent reaction vessel in the order indicated:
   (a) 10 μg of affinity purified polyclonal anti-GFP antibody per mg of magnetic beads is required for optimal antibody conjugation. For other epitope tags, the amount required for conjugating beads needs to be tested on a small scale (i.e., use 10 mg of beads for each test reaction).
   (b) Depending on the volume of the antibody used, add 0.1 M sodium phosphate pH 7.4 to make the volume up to 1 mL (1/2 of the total reaction volume of 2.0 mL).
   (c) Add 1 mL (based on a 2 mL reaction volume) of 3 M ammonium sulfate to a final concentration of 1.5 M (*see* **Note 9**). Add slowly while shaking the tube on a slow-speed vortex. If testing conditions for other epitopes, it is advisable to test a range of Ammonium Sulfate concentrations to optimize this step.
   (d) Transfer the antibody/Sodium Phosphate/1.5M Ammonium Sulfate solution onto the magnetic beads.
9. Wrap the 5-mL tube snap cap with Parafilm to prevent leaking and evaporation.

   Allow the conjugation/reaction to proceed on a rotating platform at 30 °C for 24 h (*see* **Note 10**).

Following day—wash the Dynabeads. Perform all washes as described above after transferring the Dynabead suspension to a 15 mL Falcon-type tube and by inserting the tubes into the magnet holder. You can aspirate the supernatant using a vacuum aspirator.

Wash once with 5 mL of 100 mM glycine–HCl pH 2.5—add and then remove as fast as possible. Prolonged exposure to low pH may denature your antibody.

Wash once with 5 mL of 10 mM Tris–HCl pH 8.8.

Wash once with 5 mL of fresh 100 mM triethylamine—add and then remove as fast as possible. Prolonged exposure to high pH may denature your antibody.

Wash the coated beads—a total of 4 washes with 1× PBS in 5-mL tubes with a 5-min incubation for each wash.

Wash once with PBS + 0.5 % Triton X-100 for 5 min.

Wash again with PBS + 0.5 % Triton X-100 but incubate 15 min on rocker.

Wash one last time with 1× PBS.

Resuspend all beads in a total of 0.7 mL of 1× PBS + 50 % glycerol for 100 mg of beads.

Store the coated beads at –20 °C.

3.4 Affinity Capture of Tagged Proteins

This method can be used with any affinity tag as long as good affinity isolation antibodies are available. We describe GFP-tagged proteins in this instance. This protocol is modified from an earlier protocol [7]. For any individual protein–protein complex being investigated, a trial with smaller volumes should be conducted comparing different affinity isolation buffers. The range of buffers we typically test is given in Table 1. It is recommended that an aliquot of the input, flow through, eluate, and cell pellet be saved for analysis by Western blotting to make sure that your affinity handle is efficiently captured from your sample. If not, adjust the amount of beads and/or length of affinity capture accordingly.

1. Remove the cryomilled powder from the −80 °C freezer and place it in a Styrofoam box containing liquid nitrogen with a rack to keep the Falcon-type storage tube cold and upright.
2. Weigh out 50 mg to 1 g of cell powder, depending on the abundance of your target protein, into a precooled tube. The type of tube depends on the amount weighed—1.5- or 2-mL microcentrifuge tubes for powder of 50–200-mg and larger tubes for greater powder amounts (up to a 50-mL Falcon-type tube for 1 g, rather than a 15-mL tube for ease of powder resuspension) (*see* **Note 11**).

Table 1
Buffer compositions for affinity isolation experiments

1.	20 mM HEPES, pH 7.4, 100 mM NaCl
2.	20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 % CHAPS
3.	20 mM HEPES, pH 7.4, 100 mM NaCl, 0.5 % Triton
4.	20 mM HEPES, pH 7.4, 250 mM NaCl
5.	20 mM HEPES, pH 7.4, 250 mM NaCl, 0.1 % CHAPS
6.	20 mM HEPES, pH 7.4, 250 mM NaCl, 0.5 % Triton
7.	20 mM HEPES, pH 7.4, 100 mM NaCitrate, 0.1 % CHAPS
8.	20 mM HEPES, pH 7.4, 100 mM NaCitrate, 0.5 % Triton
9.	20 mM HEPES, pH 7.4, 250 mM NaCitrate, 0.1 % CHAPS
10.	20 mM HEPES, pH 7.4, 250 mM NaCitrate, 0.5 % Triton
11.	20 mM HEPES, pH 7.4, 250 mM NaCl, 0.5 % Triton, 0.5 % deoxy Big CHAP
12.	20 mM HEPES, pH 7.4, 250 mM NaCitrate, 0.5 % Triton, 0.5 % deoxy Big CHAP
13.	40 mM Tris, pH 8.0, 250 mM NaCl, 0.5 % Triton, 0.5 % deoxy Big CHAP
14.	40 mM Tris, pH 8.0, 250 mM NaCitrate, 0.5 % Triton, 0.5 % deoxy Big CHAP

3. Transfer weighed powder to an ice bucket and sit on ice for 30 s, until it gets close to thawing. *Do not let it thaw as rapid protein degradation may occur*.
4. Resuspend powder in room temperature extraction buffer of choice, at a ratio of 1:9 (powder-extraction buffer), by vortexing and pipetting up and down. Place on ice.
5. Once all aliquots are resuspended, sonicate on ice with a microtip sonicator to break apart aggregates that may be invisible to the eye. For example, we use a Misonix Ultrasonic Processor XL at Setting 4 (~20-W output) for 2× 1 s.
6. Clarify the cell lysate by centrifugation in a microcentrifuge at 20,000 × *g* for 10 min at 4 °C.
7. During the centrifugation in **step 6**, wash the antibody-conjugated magnetic beads once with the affinity isolation buffer of choice. Harvest the beads magnetically and discard buffer.
8. Add the clarified cell lysate to the magnetic beads and rotate at 4 °C for 1 h (or longer if necessary).
9. Wash the magnetic beads 3× in the affinity isolation buffer used during affinity capture (*see* **Note 12**).
10. After the third wash, transfer the magnetic beads to a fresh tube. This ensures that cellular protein that may have been adsorbed onto the original tube prior to elution is discarded.
11. Harvest the beads magnetically and discard the buffer.
12. Spin down the magnetic beads, and aspirate residual buffer trapped between the beads during the magnetizing process.
13. Elute your affinity captured protein complex with nonreducing SDS–PAGE loading buffer or equivalent (*see* **Note 13**).
14. Harvest the beads magnetically and transfer the eluate to a fresh tube.
15. If appropriate add reducing agent and heat at 70 °C for 10 min.

3.5 Downstream Analysis

Depending on the experiment, the isolated protein complexes may be taken directly to mass spectrometry using standard methods. If so desired, the complexes may also be analyzed by SDS–PAGE. We routinely analyze complexes using precast gel systems that provide highly reproducible results. This provides a quality control prior to mass spectrometric analysis or can be used to identify/isolate individual protein bands. All of these methods are standard.

An example of the affinity isolation of most of the trypanosome nuclear pore complex—a huge structure that has stable, as well as dynamic, components. Isolating protein complexes from different cell compartments requires buffer optimization [10]. As an example of the flexibility of the cryomilling and affinity

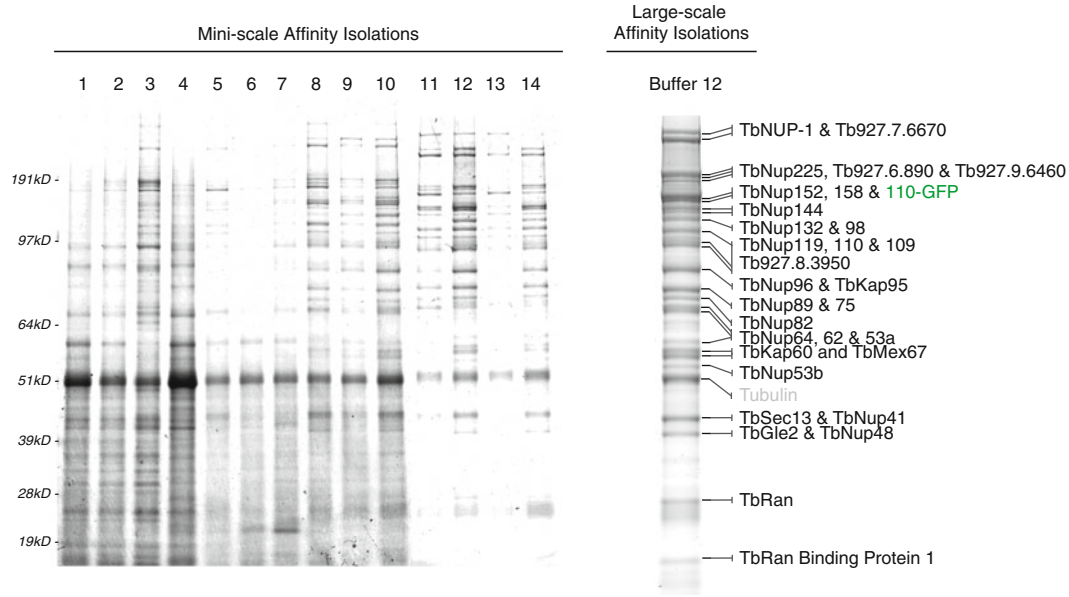

Fig. 4 Testing of various affinity isolation solvents on the nuclear basket protein TbNup110. 50 mg of TbNup110–GFP cell lysate powder was weighed out into 2-mL tubes and affinity purified in 14 different buffers containing various concentrations of salt and detergents (Table 1). Nup110 maintains the highest number of its interaction network in buffer 12. This condition was chosen for further exploration in a larger-scale pullout. Protein bands were excised out of the SDS–PAGE gel and identified by mass spectrometry. 18 of the original 22 Nups identified by DeGrasse et al. [11] in a classical biochemical fractionation and proteomic screen [11], as well as new Nups were identified with this method, demonstrating the power of this approach. Additionally, we observe interactions with the lamin-like NUP-1 [4] as well as transport factors

techniques, we highlight the ability to use low-stringency to high-stringency solutions to affinity isolate the trypanosome NPC (Fig. 4; Table 1). These methods are also applicable to dynamic protein complexes.

4 Notes

1. Antibody must not be stored in glycerol or Tris buffer or any solutions that contain amino, hydroxyl, or sulfhydryl groups, as these react with the epoxy groups on the magnetic beads. Dialyze to exchange buffer if this is the case.
2. The lid of the grinding jar we use has been modified with O-rings that are custom made by Marco Rubber (http://www.marcorubber.com) and consist of a stainless steel coil ensheathed in Teflon (PFA–PTFE-encapsulated spring-energized seal). These seals better withstand the extreme temperatures associated with liquid nitrogen use. To make these alterations, contact Marco Rubber and send them the original rubber O-ring that comes as standard with the steel lid

so that they may have the correct dimensions to manufacture the PFA–PTFE-encapsulated spring-energized seal. Pressure valves may be added to the lid of the cryomilling jar to relieve excess pressure buildup. We recommend pressure valves from the Lee Company (http://www.leeimh.com/). Specifically we use forward-venting valves that relieve pressures that exceed 5 bar/500 KPA. These are installed as to manufacturers' instructions (http://www.leeimh.com/metal/check-valves-axial/check-valve-558F.htm). Typically we install one valve in the center of a 50-mL jar lid or two valves in a 125-mL jar lid (Fig. 2).

3. A minimum volume of cell pellet is required to enable efficient cryomilling and recovery as there are losses to the walls of the jar and the steel balls. We find that 3 L of cells at $2–2.5 \times 10^7$ cells/mL generates a good-sized pellet.
4. Milled powder should be stored with a loosened cap at −80 °C in the first 24 h to allow any trapped nitrogen to escape or else tubes may explode.
5. It is necessary to do this initial "dry" milling to allow the cell pellets to be broken down to a coarse powder. If there is liquid in the jar, this initially coarse grinding is less or ineffective.
6. Do not add liquid nitrogen above the level of the steel balls, as this will cause excessive pressure during the cryomilling.
7. During liquid cryomilling, the balls should not make a rattling sound but rather should be quiet. If the balls make a loud rattling sound, then it means that the liquid in the grinding jar has evaporated into nitrogen gas.
8. It is not recommended to conjugate more magnetic beads than will be used within 6 months. Beads can be stored at −20 °C in 50 % glycerol to extend shelf life. However, there is a slow bleeding of the antibody from the beads which increases over time due to reversibility of the disulfide bridges in the antibody heavy and light chains.
9. Ammonium sulfate enhances the conjugation reaction by binding and excluding water, thus reducing reaction volume and promoting hydrophobic interactions between the bead surface and the antibody.
10. The coupling of the antibodies to the beads can be allowed to proceed up to 48 h without significant loss of antibody-binding activity. Indeed, if the antibodies being coupled are labile, it is recommended that the coupling reaction be performed at 4 °C for 48 h.
11. The amount of powder weighed out depends on the abundance of the tagged protein. Typically for mini-IP tests, 50 mg is sufficient initially. Due to difficulty weighing out several

small amounts of frozen powder, the use of metallic spice spoons is recommended. By testing out different sized spoons, on milled powder, one can have a standard spoon for weighing out fixed amounts of powder for each IP.

12. When affinity-isolating membrane proteins, it is recommended that the washes should be in the absence of detergent. The presence of detergent significantly reduces the yield of membrane proteins.
13. Reducing agents will cause antibody leakage into your eluate and must be avoided at this step.

References

1. Simpson AG, Roger AJ (2004) The real "kingdoms" of eukaryotes. Curr Biol 14:R693–R696
2. Dacks JB, Walker G, Field MC (2008) Implications of the new eukaryotic systematics for parasitologists. Parasitol Int 57:97–104
3. Berriman M, Ghedin E, Hertz-Fowler C et al (2005) The genome of the African trypanosome *Trypanosoma brucei*. Science 309: 416–422
4. DuBois KN, Alsford S, Holden JM et al (2012) NUP-1 Is a large coiled-coil nucleoskeletal protein in trypanosomes with lamin-like functions. PLoS Biol 10:e1001287
5. Akiyoshi B, Gull K (2014) Discovery of unconventional kinetochores in kinetoplastids. Cell 156:1247–1258
6. Holden JM, Koreny L, Obado SO et al (2014) Nuclear pore complex evolution: a trypanosome MLp analogue functions in chromosomal segregation but lacks transcriptional barrier activity. Mol Biol Cell 25:1421–1436
7. Oeffinger M, Wei KE, Rogers R et al (2007) Comprehensive analysis of diverse ribonucleoprotein complexes. Nat Methods 4:951–956
8. Domanski M, Molloy K, Jiang H et al (2012) Improved methodology for the affinity isolation of human protein complexes expressed at near endogenous levels. Biotechniques 0:1–6
9. Cristea IM, Williams R, Chait BT et al (2005) Fluorescent proteins as proteomic probes. Mol Cell Proteomics 4:1933–1941
10. Hakhverdyan Z, Domanski M, Hough LE et al (2015) Rapid, optimized interactomic screening. Nat Methods 12:553–560
11. DeGrasse JA, DuBois KN, Devos D et al (2009) Evidence for a shared nuclear pore complex architecture that is conserved from the last common eukaryotic ancestor. Mol Cell Proteomics 8:2119–2130