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Sedimentation and Immunoprecipitation Assays for Analyzing Complexes that Repress Transcription

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Summary

Co-repressor proteins function as platforms for the assembly of multi-subunit complexes that mediate transcriptional repression. Common components of such complexes are histone deacetylases, which catalyze the removal of acetyl groups from the tails of histones within nucleosomes, resulting in chromatin compaction and gene repression. In addition, co-repressor complexes generally interact with sequence-specific DNA-binding proteins that direct association with regulatory elements in the genome. Thus, identifying proteins that stably associate with co-repressors can provide insights regarding the biochemical function and target gene specificity of these molecules. Here, we describe a density gradient fractionation method for determining whether a co-repressor is incorporated into high-molecular complexes within cells and for identifying potential constituents of these complexes. We also describe a co-immunoprecipitation assay for confirming and further studying interactions between co-repressors and other proteins that may represent functional partners.

Keywords

Sucrose gradient sedimentation; co-immunoprecipitation; immunoblots; co-repressor proteins; DNA-binding proteins; histone deacetylases

1. Introduction

A central role for transcriptional activation in regulating gene expression has long been appreciated, but more recently it has become clear that transcriptional repression is equally important. The application of biochemical methods has greatly advanced our understanding of the factors and mechanisms that mediate transcriptional repression (1–4). This approach helped establish that proteins called co-repressors function as “nucleators” of complexes containing enzymes which alter chromatin conformation or histone modification state, resulting in transcriptional repression (5–7). For example, common constituents of co-repressor complexes are histone deacetylases (HDACs), a class of enzymes that remove acetyl groups from the tails of histones within nucleosomes. In turn, the absence of acetylated histones within a gene locus is a hallmark of a transcriptionally repressed state.

We have studied the Gon4-like protein, which appears to be a novel co-repressor that is required for B cell development (8). To gain insight into the function of this molecule, we

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used biochemical tools to demonstrate that Gon4-like associates with another co-repressor, Sin3a, and with HDAC1 (9). One method we employed was separation of cell lysates in a continuous sucrose gradient followed by immunoblot analysis of gradient fractions. This approach separates proteins and complexes through a density gradient as driven by centrifugation. Individual molecules and stable complexes are resolved in the gradient based on sedimentation velocities, which are dictated by mass, shape, density and hydration state (10). Following centrifugation, gradient fractions are collected and those containing the target protein are identified. Comparison to control gradients in which protein standards were separated determines whether a target protein has migrated into fractions containing high-molecular species, signifying association with other factors. The relevant fractions are then analyzed for the presence of other proteins that may stably interact with the target. For co-repressors, such proteins would likely be HDACs. In terms of methodology, sucrose gradient fractionation involves five major steps. First, an ultracentrifuge tube is filled with a continuous sucrose density gradient generated using a gradient maker. Second, cell lysate is prepared and loaded on top of the gradient. Third, the sample is centrifuged to separate lysate components. Fourth, gradient fractions are collected. Lastly, fraction aliquots are analyzed by immunoblot.

A complementary biochemical method for defining the composition of protein complexes is immunoprecipitation. In this assay, antibodies specific for a target protein are linked to a bead support and then incubated with cellular lysate containing the target. Bead-bound antibodies will bind and capture the target and other proteins stably bound to it, which are then recovered for analysis. Co-immunoprecipitation of other proteins with the target supports the hypothesis that these molecules interact in functionally relevant manner. This assay involves four major steps. First, whole cell lysates or nuclear extracts are prepared. Second, control or protein-specific antibodies are bound to beads and then incubated with protein samples. Third, the bead-antibody-protein complexes are separated from unbound lysate components and washed to remove material bound non-specifically. Finally, the recovered material is analyzed by immunoblot to confirm target recovery and to detect other proteins that may associate with it.

2. Materials

Make all solutions using ultrapure, deionized water with a resistivity of 18M Ω .cm at 25°C. Sterilize solutions by autoclaving or filtering through a 0.2 μ pore-size filter. Wash and autoclave all glassware prior to use. Unless indicated otherwise, store all reagents at room temperature (RT). Adhere to all federal and local regulations for disposal of waste materials.

2.1. Cell Culture Reagents

1. Tissue culture incubator for maintaining 37°C temperature and 5% CO₂ atmosphere.
2. Tissue culture grade filter screw cap flasks or plates.
3. Transformed cell line: For our studies, we use the mature B cell line M12.4.1 (11).
4. Appropriate culture media.
5. Eppendorf 5804C centrifuge, or equivalent, for spinning down cells.
6. Delbuccho's Phosphate-buffered Saline (PBS) pH 7.4, lacking Mg²⁺ and Ca²⁺.
7. Hemocytometer and coverslip.

2.2. Reagents for Determining Protein Concentrations

1. Bradford Assay reagent (Biorad, Hercules, CA, USA) prepared and used according to manufacturer's instructions.
2. A spectrophotometer capable of measuring visible light absorbance.

2.3 Sucrose Gradient Components

1. 10X Phosphate-buffered Saline (PBS) pH 7.4, lacking Mg^{2+} and Ca^{2+} : 100 mM $Na_2HPO_4 \cdot 2 H_2O$, 17.6 mM KH_2PO_4 , 1.37 M NaCl and 27 mM KCl.
2. Sucrose gradient cell lysis buffer (Buffer S): PBS containing 1 mM EDTA, 0.5% Triton X-100 and EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) (*see* Note 1). Store at $-20^{\circ}C$; use ice-cold.
3. 70% (w/v) sucrose stock solution: Dissolve 350 gms of sucrose in water added up to 500 mL and filter-sterilize. The stock can be stored at $4^{\circ}C$ for up to 3 months.
4. 20% (w/v) sucrose solution: For 100 mL, mix 60.73 mL of water, 10 mL of 10X PBS, 28.57 mL of 70% sucrose stock solution, 0.2 mL of 500 mM EDTA pH 8 and 0.5 mL of Triton X-100. Chill to $4^{\circ}C$ prior to use.
5. 50% (w/v) sucrose solution: For 100 mL, mix 17.87 mL of water, 10 mL of 10X PBS, 71.43 mL of 70% sucrose stock solution, 0.2 mL of 500 mM EDTA pH 8 and 0.5 mL of Triton X-100. Chill to $4^{\circ}C$ prior to use.
6. Hoefer SG 15 sucrose gradient maker (Fisher Scientific, Dallas, TX, USA), tubing to connect to outlet of gradient maker for pouring the gradient, glass capillary tubes that fit snugly into the end of the tubing, Vaseline for use as a sealant.
7. Stir plate and 1/2-inch stir bar.
8. Molecular weight standards: Mix 100 μ L of Precision-plus standards (Biorad, Hercules, CA, USA) with 100 μ L Buffer S and load onto a gradient. Resuspend Thyroglobulin (Sigma Chemical Company, St. Louis, MO, USA) to 20 mg/mL in a reconstitution solution that contains 50 mM Tris-HCl pH 7.5, 100 mM KCl and H_2O . Add 50 μ L of this stock to 150 μ L Buffer S and load onto a gradient. Store Thyroglobulin stock at $-20^{\circ}C$.
9. Centrifuge tubes: 14 $\times$ 89 mm polyallomer tubes (Beckman, Brea, CA, USA).
10. SW41Ti swinging bucket rotor (Beckman). Chill to $4^{\circ}C$ prior to use.
11. Free-standing ultracentrifuge (Beckman). Chill ultracentrifuge chamber to $4^{\circ}C$ prior to use.
12. Ring stand and clamp for holding a 14 $\times$ 89 mm polyallomer centrifuge tube.

2.4. Whole Cell Lysate Reagents

1. 200 mM sodium orthovanadate ($NaVO_4$) stock solution: the tyrosine phosphatase inhibitor $NaVO_4$ (Sigma Chemical Company) must be depolymerized prior to use. Add 368 mg of $NaVO_4$ to 8 mL of water and incubate at $37^{\circ}C$ while occasionally mixing until dissolved. Adjust the pH to 10 by adding drops of 1N NaOH, which will generate an orange-colored solution. Seal the tube and incubate in boiling water until the solution becomes colorless (five to ten min) and then cool to RT.

¹Add protease inhibitor cocktail to buffers immediately before use. Avoid subjecting buffers containing the inhibitors to multiple (>2) freeze/thaw cycles.

Repeat the cycle of pH adjustment to 10 followed by boiling and cooling to RT until a colorless liquid at pH 10 is generated (*see Note 2*). Usually, two cycles are required. Add water to 10 mL, filter-sterilize and store as aliquots at -20°C .

2. 200 mM phenylmethylsulfonyl fluoride (PMSF) stock solution: dissolve 348.4 mg of the serine protease inhibitor PMSF (Sigma Chemical Company) in 10 mL of ethanol (or isopropanol or methanol). Incubate at 37°C while occasionally mixing to dissolve. Store as aliquots at -20°C and warm to RT immediately before use (*see Note 3*).
3. 0.5 M sodium fluoride (NaF) stock solution: Add 210 mg of the phosphatase inhibitor NaF (Sigma Chemical Company) to 10 mL of water. Incubate at 37°C while occasionally mixing until dissolved. Filter-sterilize and store as aliquots at -20°C .
4. Whole cell lysis (WCL) Buffer: 50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40 (Roche Applied Science), 0.2 mM NaVO_4 , 50 mM NaF, 1 mM PMSF (add immediately before use) and EDTA-free protease inhibitor cocktail (Roche Applied Science). Store WCL Buffer lacking PMSF at -20°C (*see Note 4*). Use ice-cold.

2.5. Nuclear Extract Reagents

This methodology is based on that originally described by Dignam and Roeder (12).

1. Buffer A: 20 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA pH 8, 1 mM DTT, 10% glycerol and EDTA-free protease inhibitor cocktail (Roche Applied Science). Store at -20°C ; use ice-cold.
2. Buffer B: 20 mM Hepes pH 7.5, 10 mM KCl, 420 mM NaCl, 1.5 mM MgCl_2 , 1 mM EDTA pH 8, 1 mM DTT, 25% glycerol and EDTA-free protease inhibitor cocktail (Roche Applied Science). Store at -20°C ; use ice-cold.
3. Buffer D: 20 mM Hepes pH 7.5, 100 mM KCl, 1.5 mM MgCl_2 , 0.2 mM EDTA pH 8, 0.5 mM DTT and 25% glycerol. Prepare immediately before use and cool to 4°C (*see Note 5*).
4. Kontes glass dounce with Kontes Pestle “B” (Fisher Scientific). Chill on ice prior to use.
5. DNase I, grade II (Roche Applied Science): Resuspend to 10 mg/mL in 10 mM Tris-HCl pH 7.5, 0.5 mM CaCl_2 , 0.5 mM MgSO_4 and 50% (v/v) glycerol. Store at -20°C .
6. Slide-A-Lyzer G2 Dialysis cassettes with 7 kDa mass cut-off (Thermo Scientific/Pierce, Rockford, IL, USA).
7. 10 mL syringes and 1.5 inch, 18-gauge needles.
8. 1 L glass beaker.
9. Stir plate and 1.5-inch stir bar.

²In our hands, addition of NaOH is required to adjust the pH of NaVO_4 stock solution to 10. In some cases, it may be necessary to add 1N HCl to adjust the pH.

³When stored at -20°C , PMSF will fall out of solution. Prior to adding to lysis buffer, redissolve the PMSF by incubating at 37°C while mixing occasionally.

⁴PMSF is unstable in aqueous solutions. Always add PMSF to lysis buffer immediately before use. Discard any unused lysis buffer containing PMSF.

⁵Use pre-chilled water to prepare cold Buffer D as dialysis buffer. Prior to making nuclear extract, prepare 1 L bottles of autoclaved water and store at 4°C overnight or longer.

2.6. Immunoprecipitation Components

1. Antibodies: polyclonal or monoclonal antibodies specific for target protein and species-matched control IgG for negative control samples (*see Note 6*).
2. Dynal® magnetic beads conjugated to Protein A or G (Invitrogen, Carlsbad, CA, USA) (*see Note 7*).
3. DynaMag-2™ magnet for pulling out magnetic beads from solutions in 1.5–2.0 mL tubes.
4. 1X PBS lacking Ca^{2+} and Mg^{2+} .
5. Wash buffer: WCL buffer, prepared exactly as described in *section 2.3*, is used as high-stringency wash buffer. Low-stringency wash buffer is of the same composition except that NP-40 is added to a final concentration of 0.1% (rather than 0.5%).
6. Rotator that can hold 1.5 mL tubes for mixing samples.

2.7. SDS Polyacrylamide Gel Components

This methodology is based on information in the NuPAGE® Technical Guide, which can be obtained as a PDF from the Invitrogen website. Consult the guide for additional details.

1. XCell SureLock® mini-cell electrophoresis system (Invitrogen): includes a chamber for running gels, a 2-gel holder (Buffer Core) with electrodes, Gel Tension Wedge to lock gels in place and a lid with wire leads for connecting to a power supply.
2. Power supply: Biorad PowerPac (300 V maximum) or equivalent.
3. SDS polyacrylamide gels: 1.0 mm, 10-well, NuPAGE® Novex 3–8% Tris-Acetate gels (Invitrogen).
4. Running Buffer: For 1L, add 50 mL of 20X NuPAGE® Tris-Acetate SDS Running Buffer (Invitrogen) into 950 mL of water and mix thoroughly.
5. NuPAGE® antioxidant reagent (Invitrogen).
6. NuPAGE® reducing reagent (Invitrogen).
7. 10 mL syringe and a 1.5 inch 18-gauge needle.
8. 1X Sample Buffer: For 100 μL (enough for one sample), mix 25 μL of 4X NuPAGE® LDS Sample Buffer (Invitrogen), 10 μL of 10X NuPAGE® Reducing Agent and 65 μL of water.
9. Heat block or water bath set at 70°C.
10. Gel-loading tips (Invitrogen).

⁶Polyclonal antibodies are considered to be optimal for immunoprecipitation analysis because they likely recognize multiple epitopes on the target protein. However, in general monoclonal antibodies also work well. As a negative control, obtain “non-specific” IgG isolated from the same species (usually rabbit or mouse) in which the target-specific antibodies were raised.

⁷The choice of Protein A or Protein G beads depends on the species of origin and isotype of the target-specific antibodies. Consult Invitrogen’s “Product Selection Guide for Immuno-precipitation” for more information. In most cases, Protein G is the correct reagent to use.

2.8. Protein Transfer to Membrane Components

This methodology is based on information in the Mini Trans-blot® Electrophoretic Transfer Cell Instruction Manual, which can be obtained as a PDF from the Biorad website. Consult the manual for additional details.

1. Wet transfer apparatus: Mini Trans-Blot Cell System (Biorad) (*see* Note 9).
2. Power supply: Biorad PowerPac (300 V maximum) or equivalent.
3. Blunt-end tweezers (Millipore, Billerica, MA, USA) for handling PVDF membranes (*see* Note 8).
4. Methanol (ACS grade).
5. Transfer Buffer: for 1 L, mix 50 mL of 20X NuPAGE® Transfer Buffer (Invitrogen) and 100 mL of methanol into 849 mL of water. Chill prior to use.
6. NuPAGE® antioxidant reagent (Invitrogen).
7. Sponge blotting pads: provided with Biorad's Mini Trans-Blot® Cell system.
8. 3 mm-thick filter paper cut into 7 cm × 8 cm pieces. Pre-cut paper can be obtained from Invitrogen or Biorad. Alternatively, large sheets of paper can be obtained from Whatman/GE Healthcare (Piscataway, NJ, USA) and cut prior to use.
9. Immobilon-P PVDF transfer membrane with 0.45µm pore size (Millipore). Cut membrane into 7 cm × 8 cm pieces prior to use.

2.9. Immunoblot Components

1. Blunt-end tweezers (Millipore)(*see* Note 8).
2. Methanol (ACS grade).
3. 3 mm-thick filter paper (Whatman/GE Healthcare) cut into 10 cm × 10 cm squares.
4. 10X Tris-buffered saline (TBS): 250 mM Tris HCl, pH 7.4, 1.5 M NaCl, 20 mM KCl. Dilute to 1X with water.
5. Blocking Buffer: add 2.5 gms non-fat dry milk powder into 25 mL of 1x TBS. Mix well and adjust volume to 50 mL. Incubate at 37°C while mixing occasionally until dissolved.
6. TBS-T: Add 0.5ml Triton X-100 to 1L of 1X TBS (0.05% Triton X-100). Mix using a stir bar and stir plate. Use at RT and discard after 3 days (microbes will grow within this solution).
7. 8 cm × 10 cm plastic boxes with lids.
8. Primary antibodies specific for target proteins that have been verified to work for immunoblot analysis. These can be obtained from several vendors.
9. Secondary (or detection) antibodies specific for immunoglobulin G constant regions from mouse or rabbit and conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

⁹We find that wet (rather than semi-dry) transfer of proteins from SDS-polyacrylamide gels provides optimal immunoblot results, particularly when proteins of relatively large mass (>100 kDa) are being analyzed. Some investigators may find that semi-dry transfer is suitable for their studies.

⁸Never directly touch PVDF membranes with gloves or any other materials except those specified in **Methods**; this will preserve the protein-binding capacity of the membrane.

10. Enhanced chemiluminescent HRP substrate mixes: SuperSignal West Pico Chemiluminescent Substrate (Pierce/Thermo Scientific). To increase assay sensitivity, obtain SuperSignal West Femto Chemiluminescent Substrate (Pierce/Thermo Scientific) (*see* Note 10).
11. Two sheets of overhead transparency film, taped together down the length of one side.
12. Glogos II Autorad Markers (Agilent Technologies, Santa Clara, CA, USA).
13. X-ray film cassette, X-ray film and a film developer.

2.10 Coomassie® Blue Staining Reagents

This methodology is similar to that described in Molecular Cloning: A Laboratory Manual (13).

1. Glacial acetic acid (ACS grade).
2. Methanol (ACS grade).
3. Glycerol.
4. Coomassie® Brilliant Blue R-250 powder (Biorad)
5. Coomassie® Staining Buffer: dissolve 0.1gms of Coomassie® Brilliant Blue R-250 powder in a solution consisting of 10mL of glacial acetic acid, 30 mL of methanol and water up to 50 mL. Stir the solution with a stir bar on a stir plate for at least 1 hr. Adjust the final volume to 100 mL with water and filter through Whatman filter paper.
6. Destaining Buffer: mix 200 mL of methanol, 100 mL of glacial acetic acid and water to a final volume of 1000 mL.
7. Glycerol Fix Solution: mix 50 mL of methanol and 15mL of glycerol with water up to a final volume of 500 mL.
8. Gel drying frame, membranes and clips for frame (Research Products International, Mount Prospect, IL, USA).

3. Methods

All procedures can be carried out at RT. However, keep all reagents and protein-containing samples on ice as much as possible.

3.1. Culture of Cells in Suspension for Whole Cell Lysate or Nuclear Extract Preparation

1. M12 cells are grown in suspension at 37°C in 5% CO₂. Stocks are maintained in T-25 cm² flasks and split 1:10 (cell suspension:fresh medium) every 2–3 days as judged by cell concentration and media color.
2. To expand cells for harvest, transfer 5 mL of stock at high concentration to a T-75 cm² flask and add 25 mL of fresh medium. Depending on the numbers of cells needed, T-75 cm² flasks are split 1:3 every 2–3 days by transferring into fresh T-75 cm² flasks.

¹⁰In some instances, a weak ECL signal may be obtained when the “conventional” SuperSignal West *Pico* substrate mix is used. To amplify the signal, add 100 ul (50 ul of each solution) of SuperSignal West *Femto* substrate per 2 mL of SuperSignal West *Pico* substrate.

3. A single T-75 cm² flask of M12 cells grown to high density will yield $\sim 1 \times 10^7$ cells.

3.2. Cell Harvest

1. Decant or transfer cells into 50 mL polypropylene conical tubes.
2. Pellet the cells by centrifugation at $270 \times g$ for 10 min.
3. Remove as much medium as possible by aspiration.
4. Resuspend cells in 40 mL of cold PBS and pellet by centrifugation at $270 \times g$ for 10 min.
5. Resuspend cells in 5 or 10 mL of cold PBS, pool into one 50 mL polypropylene conical tube and pellet the cells by centrifugation at $270 \times g$ for 10 min.
6. Resuspend total cells obtained in 10 mL of PBS and count using a hemacytometer.
7. Pellet the number of cells required for the method to be used (*see* first line in Sections 3.3, 3.4 and 3.5).

3.3. Sucrose Gradient Cell Separation of Protein Lysates Prepared from Cells

1. Prepare a cell pellet containing $\sim 1 \times 10^7$ cells.
2. Resuspend the cell pellet in 0.5 mL of Buffer S by gentle pipetting.
3. Incubate cells on ice for 20 min, inverting occasionally.
4. Spin the sample at $16,100 \times g$ for 15 min in a microfuge at 4°C.
5. Recover supernatant.
6. Measure the protein concentration using Bradford assay. The ideal concentration is ~ 5 mg/mL, allowing 1 mg of protein in a 200 μ L volume to be loaded onto a gradient.
7. Place sample on ice until sucrose gradient is ready for loading.
8. Put the gradient maker onto a stir plate placed on a shelf or other solid support that is ~ 2 feet higher than the lab bench, allowing sufficient flow of sucrose solution. Make sure that the gradient maker is level.
9. Attach tubing to the outlet connector of the gradient maker. To the other end of the tube, attach a glass capillary tube and insert it vertically to the bottom of an ultracentrifuge tube in a rack on the bench. Apply Vaseline to the outside of the capillary tube to seal the connection with the tube (*see* Note 11).
10. Add water to both chambers of the gradient maker. Open the connector-stopcock and the outlet stopcock. Let water flow through, removing bubbles. Determine the flow rate, which should be ~ 0.5 to 1 mL/min; this can be adjusted by elevating or lowering the gradient maker. Afterward, allow water to drain out of the chambers.
11. Add 6 mL of 20% sucrose solution to the mixing chamber (chamber 1, which is directly connected to the outlet stopcock) of the gradient maker. Open the outlet-connector stopcock and fill the tubing with the 20% gradient solution to prevent bubble formation. Drain almost all of the solution from the chamber.

¹¹The diameter of the glass capillary tube and the tubing must be very similar to minimize turbulence at the interface between them, which can disrupt gradient formation. Sealing with Vaseline will prevent airflow and bubble formation, minimizing turbulence.

12. Place 6.4 mL of the 20% and 50% sucrose solution in chamber 1 and 2, respectively. Place a stir bar in chamber 1 and set a stir of < 300 revolutions per minute. High-speed stirring may cause bubbles and disrupt gradient formation.
13. Open the connector stopcock between the two chambers.
14. Open the outlet stopcock allowing the solution to flow into the ultracentrifuge tube. After tube is nearly full (~7 mm from the top), slowly remove the glass capillary tube. The poured gradient can be stored at 4°C up to two hours before being loaded and centrifuged.
15. Rinse the chambers with water and repeat the process as needed. At least four tubes will be required per experiment (one for the sample, two for each of the mass markers, and another for balance).
16. Ensure that all tubes are the same weight, adding 20% sucrose solution to the top of the gradient as necessary.
17. Gently load the lysate in Buffer S onto the top of the gradient solution using a P200 pipette. Place the tip of the pipette against the wall of the tube just above the gradient surface and slowly expel the sample.
18. Load protein markers diluted to 200 µL with Buffer S onto identical gradients.
19. Confirm that the loaded gradients are balanced, dispensing 20% sucrose solution at the gradient-sample interface as needed (*see* Note 12).
20. Place the tubes into the pre-cooled buckets of a pre-cooled SW41Ti swinging bucket rotor. Seal the buckets and centrifuge the samples for 16 hours at $288,000 \times g$ in an ultracentrifuge pre-cooled to, and maintained at, 4°C.
21. After centrifugation, transfer all the rotor buckets containing samples to a rack and store at 4°C. Minimize shaking of the buckets during transport.
22. Prepare a set of 1.5 mL tubes on ice to hold the gradient fractions to be collected. Typically, 25 tubes, each filled with a 500 µL fraction, is required to collect a single gradient.
23. Set up a support stand holding a clamp that can securely hold a polyallomer centrifuge tube containing a gradient in a vertical position while minimizing movement. Remove a tube from the centrifuge bucket by pulling out with a blunt-end tweezer and secure it with the clamp.
24. Use a 1 mL pipette and tips to collect gradient fractions. Place the tip just beneath the surface of the gradient and slowly withdraw 500 µL. Transfer the aliquot to a tube on ice. Repeat this process until the entire gradient is collected.
25. Store sample tubes at -80°C until you are ready to perform SDS-PAGE and immunoblot analysis (*see* Sections 3.7–3.9). To minimize protein degradation, avoid subjecting samples to multiple freeze-thaw cycles.
26. Aliquots (15 to 18 µL) of gradient fractions containing protein standards will be resolved on SDS-polyacrylamide gels and then stained with Coomassie Blue.
27. Aliquots (15 to 18 µL) of gradient fractions containing cell lysate components will be resolved on SDS-polyacrylamide gels and subjected to immunoblot analysis (*see* Fig. 1).

¹²It is critical to fill the centrifuge tube with liquid to within 5 mm or less from the top of the tube. Otherwise, the tube may collapse during ultracentrifugation.

3.4. Making Whole Cell Lysate for Immunoprecipitation and Immunoblot Analysis

- 1 Prepare a cell pellet containing $\sim 2 \times 10^7$ cells.
- 2 Resuspend cells in 800 μL of WCL Buffer (200 μL per 5×10^6) by pipetting. Incubate cells on ice for 20 min, inverting occasionally.
- 4 Spin the sample at $16,100 \times g$ for 15 min in at 4°C .
- 5 Recover the supernatant.
- 6 Determine the protein concentration using the Bradford assay.
- 7 Set aside an aliquot of lysate equivalent to $\sim 200 \mu\text{g}$ of protein as a positive control for immunoblot analysis.
- 8 Immediately begin immunoprecipitation assay as described below (*see Note 13*).

3.5. Making Nuclear Extract for Immunoprecipitation and Immunoblot Analysis

1. Prepare a cell pellet containing $\sim 3 \times 10^7$ cells.
2. Determine the packed cell volume (PCV) of the cell pellet and resuspend cells in 5X PCV of Buffer A.
3. Spin cells at $270 \times g$ for 5 min and remove the supernatant.
4. Resuspend cells in 3X PCV of Buffer A and incubate on ice for 10 min.
5. Add NP40 to 0.25% (2.5 μL of 100% NP40 per mL) mix by gentle pipetting and incubate ice for 10 min.
6. Transfer cells to an ice-cold Kontes dounce homogenizer that was pre-rinsed with Buffer A.
7. Perform 25–50 strokes with Kontes Pestle B to lyse cells (*see Note 14*).
8. Transfer sample to a 15 mL conical tube. Spin the sample at $700 \times g$ for 10 min to pellet nuclei.
9. The resulting supernatant should be cloudy, signifying efficient cell lysis. Remove all of the supernatant by pipetting or aspiration.
10. Determine packed nuclei volume (PNV) and resuspend cells in 3X PNV of Buffer B.
11. Transfer nuclei to Kontes Dounce homogenizer that has been pre-rinsed with Buffer B and perform 50 strokes with Kontes Pestle B to shear nuclei.
12. Transfer sample to a 1.5 mL tube.
13. Place tube on rotator in cold room and rotate for 1 hr. Visible aggregates of insoluble material will be present at the end of the incubation.
14. Spin the sample at $16,100 \times g$ for 15 min in a microfuge at 4°C and the recover supernatant.
15. Add 2.5 μL of DNase (10 mg/mL) per mL of supernatant and place on ice.

¹³Freezing and thawing can alter the structure of proteins, causing loss of functional conformation and thus affecting the outcome of immunoprecipitation experiments. To prevent this, perform the assay using freshly prepared whole cell lysates or nuclear extracts. If time is required, store protein samples on ice in an insulated bucket with a lid in a cold room.

¹⁴The number of strokes required depends on cell size, with more strokes needed to lyse smaller cells. Cell lysis can be judged by using a microscope to view 5 μL aliquots of cells taken before and after douncing. A large amount of debris with few whole cells apparent signifies efficient lysis. If this is not observed, perform more strokes.

16. Prepare a Slide-A-Lyzer G2 dialysis cassette for incubating in 1 L of chilled Buffer D for 10 min. Afterward, open the top of the cassette and use a 10 mL syringe capped with an 18-gauge needle to withdraw all the air from the dialysis chamber.
17. Load the protein sample into a 10 mL syringe capped with an 18-gauge needle. Insert the needle into the loading port of the dialysis cassette and gently expel the sample.
18. Seal the dialysis cassette and place in 1 L of chilled Buffer D. Dialyze the extract for a minimum of 3 hrs at 4°C while stirring the Buffer D using a stir bar and stir plate.
19. Use a 10 mL syringe capped with an 18-gauge needle to recover the extract. Place the recovered material in a 1.5 mL tube or divide between 2 tubes if necessary.
20. Spin the sample at $16,100 \times g$ for 15 min in a microfuge at 4°C and recover the supernatant.
21. Determine the protein concentration using the Bradford assay.
22. Set aside an aliquot of lysate equivalent to ~200 µg of protein as a positive control for immunoblot analysis.
23. Immediately begin immunoprecipitation assay as described below (*see* Note 13).

3.6. Immunoprecipitation Assay

1. Completely resuspend Dynabeads conjugated to Protein A or G by pipetting.
2. Remove 50 µL (~1 mg) of beads for each immunoprecipitation (*see* Note 15) plus one extra sample and transfer to a 1.5 mL tube
3. Wash the beads twice with 1 mL of PBS: add 1 mL of PBS to the bead slurry, place the tube in the DynaMag-2™ magnet and let stand for ~ 1 min. The beads will concentrate on the wall of the tube facing the magnet. Aspirate the PBS, avoiding contact with the beads. Repeat.
4. Resuspend the beads in 200 µL of PBS per immunoprecipitation and transfer 200 µL to the appropriate number of 1.5 mL tubes.
5. Add 5 µg of isotype control or target-protein-specific antibody to each tube and adjust the volume of the sample to 500 µL using PBS.
6. Incubate the samples at 4°C for 20 min while rotating.
7. Wash beads twice with 1 mL PBS.
8. Resuspend the beads in 200 µL of the Wash Buffer to be used for the experiment (*i.e.* high or low stringency buffer).
9. Add to the bead suspension a volume of whole cell lysate or nuclear extract containing a total of 500 to 1000 µg of protein.
10. Adjust the final volume of the sample to 1 mL by adding Wash Buffer.
11. Incubate the samples at 4°C for 2–16 hrs while rotating (*see* Note 16).

¹⁵Each immunoprecipitation experiment should consist of a sample in which lysate/extract is incubated with isotype control antibodies bound to beads (*i.e.* a negative control sample) and a sample in which lysate/extract is incubated with protein-specific antibodies bound to beads. For all subsequent steps, these samples should be processed in parallel. If the isotype control produces a confounding signal, try a control antibody mix from a different vendor.

12. Place the samples in the DynaMag-2™ magnet and let stand for ~ 1 min, allowing the beads to concentrate on the wall of the tube.
13. Recover the supernatant and save for immunoblot analysis if desired (*see* Note 17).
14. Remove the samples from DynaMag-2™ magnet and place on ice. Gently resuspend the beads in 1 mL of Wash Buffer and incubate on ice for 1 to 5 min.
15. Place the samples in the DynaMag-2™ magnet, allowing the beads to concentrate on the wall of the tube facing the magnet.
16. Repeat steps 14 and 15 so that the bead-protein complexes are washed a total of 3 or 4 times.
17. Perform a final wash with 1mL of PBS and remove as much of the supernatant as possible.
18. Resuspend beads in 100 µL of 1X Sample Buffer and proceed to SDS-PAGE and immunoblot analysis (*see* Sections 3.7.–3.9.).

3.7. SDS-PAGE Analysis

1. Prepare 1L of 1X Running Buffer.
2. Transfer 200 mL of 1X Running Buffer to a separate container. To this, add 500 uL of NuPAGE® antioxidant reagent and mix thoroughly just prior to loading samples onto a gel.
3. Remove 3–8% gradient gel from packaging. Remove the plastic strip from the bottom of the gel and the comb from the top of the gel. Gently rinse the wells with 1X running buffer dispensed from a syringe capped with an 18-gauge needle.
4. Place the gel onto the “Buffer Core” so that the well side of the gel (notched plate) faces inward. On the other side, place either another gel or the “Buffer Dam”.
5. Insert the gel/Buffer Core assembly into the Xcell Mini-Cell (the clear plastic container that holds the gel(s) and buffer) and lock the assembly into place using the Gel Tension Wedge.
6. Fill the inner chamber formed by the gel/Buffer Core assembly with 1X Running Buffer containing antioxidant reagent. Inspect the assembly to ensure buffer is not leaking out.
7. Fill the outer chamber with 1X Running Buffer lacking antioxidant reagent. The buffer level must exceed that of the gel wells.
8. Heat tubes containing protein samples at 70°C for 10 min immediately prior to loading.
9. Insert tubes containing Dynabeads into the DynaMag-2™ magnet and let stand for 1 min to remove beads from the sample.
10. In one well of the gel, load pre-stained protein standards of choice.
11. Load 20 to 25 uL of protein samples into wells of the gel.

¹⁶Although immunoprecipitations are commonly incubated overnight, we find that a 2 to 4 hr incubation is effective and may help preserve protein integrity.

¹⁷Immunoblot analysis of supernatant aliquots in parallel with material recovered by immunoprecipitation provides an assay for judging the immunoprecipitation efficiency.

12. Attach the lid for the Mini-Cell and attach the wire leads from the lid to a power supply set to provide a constant voltage of 150.
13. Depending on the mass of the proteins to be detected by immunoblot, the gel run may take between 50 and 120 min.

3.8. Protein Transfer to Membrane for Immunoblotting

1. Prepare 1L of 1X NuPAGE® Transfer Buffer chilled to 4°C.
2. Turn off and disconnect the power supply and disassemble the Mini-Cell to obtain the gel(s).
3. Using a gel knife, carefully pry apart the gel plates. Ensure that the gel sticks to one plate; if not, soak the gel and plates in 1X Transfer Buffer to help separate.
4. Remove the top of the gel spanning the wells and then soak in 1X Transfer Buffer for ~10 min.
5. Completely submerge the PVDF membrane in 100% methanol. Decant the methanol and add water. Incubate with gentle agitation for 5 min, exchange the water for fresh and incubate again. Exchange the water for Transfer Buffer and incubate with gentle agitation for >5 min.
6. Soak four pieces of filter paper and the blotting sponges in Transfer Buffer.
7. Place the electrode module into the buffer tank and pour 500 mL of Transfer Buffer into the tank.
8. Assemble a “sandwich” on black half of the gel holder cassette by placing the listed materials on top of each other in this order: a) sponge pad; b) two pieces of filter paper; c) gel; d) PVDF membrane; e) two pieces of filter paper; f) sponge pad. When the PVDF is laid on top of the gel, make sure to remove all bubbles between them. Close and lock the gel holder.
9. Immediately slide the gel holder into the electrode module in the tank so that the clear half of the holder faces the red half of the electrode module.
10. Fill the tank near to the top with Transfer Buffer and attach the lid so that the appropriate leads and electrodes are connected. Transport the assembly to a cold room and place on a stir plate. Set the plate so that the stir bar within the chamber spins slowly to moderately.
11. Attach wire leads from the lid to a power supply set to provide a constant voltage of 50.
12. Allow transfer to proceed from 4 hrs to overnight.
13. Recover the PVDF membrane, wash with water and then methanol. Allow membrane to dry at RT.

3.9. Immunoblot Analysis of Protein Samples

1. Prepare TBS-T and Blocking Buffer.
2. Rehydrate the membrane by submerging in 100% methanol. Decant the methanol and add water. Incubate with gentle agitation for 5 min, exchange the water for fresh and incubate again. Exchange the water for TBS-T and incubate with gentle agitation for >5 min.

3. Decant the TBS-T and add 10 mL of Blocking Buffer. Incubate at RT for 1 hr with modest shaking.
4. Decant the Blocking Buffer and rinse the membrane twice with TBS-T. Keep the membrane in TBS-T while preparing primary antibody solution.
5. Dilute 1mL of Blocking Buffer into 9 mL of TBS-T. Add protein-specific primary antibody to the concentration recommended by the manufacturer.
6. Add the TBS-T/antibody solution to the membrane and incubate with modest shaking. Incubate at either RT for 1 hr or 4°C overnight.
7. Remove the TBS-T/antibody solution and wash the membrane three times with TBS-T, incubating for 5–10 min with modest shaking for each wash.
8. Dilute 1 mL of Blocking Buffer into 9 mL of TBS-T. Add species-specific secondary anti-IgG antibodies conjugated to HRP to the concentration recommended by the manufacturer.
9. Add the TBS-T/antibody solution to the membrane and incubate at RT for 1 hr with modest shaking.
10. Wash the membrane as described for Step 7 above.
11. Add ECL substrate mix (*see* Note 10) to the membrane and incubate for 1 min.
12. Transfer the membrane to a piece of filter paper and allow to briefly dry.
13. Insert the membrane between two pieces of overhead transparency film and place into an X-ray cassette containing a Glogos Autorad II Marker fixed to a site, thus serving as a position marker for the X-ray film.
14. In a dark room, overlay a piece of X-ray film onto the transparency film, incubate for 10 to 60 seconds and develop.
15. After the desired exposure has been attained, use Glogos Autorad II Marker to position the developed film over the membrane. Mark the positions of pre-stained protein standards on the film so that the mass of species detected by ECL can be estimated.

3.10. Coomassie® Blue Staining of Gels

1. After the electrophoresis, put the gel into a plastic container (the top from a box of 1mL pipette tips works well) containing Staining Buffer and incubate with gentle shaking for 1 hr at RT.
2. Decant the Staining Buffer into a bottle and store for reuse.
3. Add Destaining Buffer so that the gel is submerged. Incubate with gentle shaking for at RT until bands are visible and background staining is minimal. If the Destaining Buffer becomes dark blue during incubation, replace with with fresh buffer and continue incubating.
4. Wash the gel with water twice for 5 min at RT.
5. Submerge the gel in Glycerol Fix Solution and incubate 60 min at RT.
6. Mount and dry the gel using a drying frame and membrane according to the manufacturer's instructions.

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Fig. 1.

Detection of the putative co-repressor protein Gon4-like and potential interacting factors in sucrose gradient fractions containing high molecular weight species. Whole cell lysates were prepared from M12 B cells and separated through a 20–50% continuous sucrose gradient via ultracentrifugation as described in **Methods**. After centrifugation, the fractions indicated across the top of the panel (odd numbers only are shown) were collected and analyzed by immunoblot using the antibodies listed on the right side of the panels. M12 whole cell lysate (WCL) was used as a positive control for immunoblots (left side of panels). Protein standards were separated in parallel gradients and fractions containing these are indicated at the top of the panel. The box with the asterisk on top denotes fractions that contain Gon4-like, the co-repressor Sin3a, HDAC1 and the DNA-binding protein YY1, suggesting these factors associate as components of high-molecular weight complexes. Data shown are representative of 3 independent experiments.

Fig. 2.

Co-immunoprecipitation of Gon4-like, Sin3a and HDAC1 from M12 cell nuclear extracts (NE). Nuclear extracts were prepared from M12 B cells and used for immunoprecipitation experiments as described in **Methods**. Affinity-purified rabbit polyclonal antibodies specific for Sin3a or non-specific rabbit IgG (both obtained from Santa Cruz Biotechnology) were used to perform immunoprecipitations. Recovered material was immunoblotted with antibodies specific for Gon4-like (top panel), Sin3a (middle panel), or HDAC1 (bottom panel). M12 nuclear extract (NE) was used as a positive control for immunoblots. Data shown are representative of 3 independent experiments.