3) ***WASH*** the cells in a volume of 20 mM K-HEPES (pH 7.4) equal to the pellet size. Centrifuge yeast and bacteria cells at 2600 × *g* for 15 min at 4°C; or mammalian or insect cells at 500 × *g* for 10 min at 4°C. Discard the buffer after centrifugation.

Optional: Addition of protease inhibitors and 1 mM DTT to the K-HEPES buffer.

4) ***REMOVE*** as much buffer as possible. Centrifuge the pellet again at 2600 × *g* for 15 min at 4°C for yeast or bacteria, and at 500 × *g* for 5 min at 4°C mammalian and insect cells. Remove the remaining buffer and thoroughly resuspend the pellet.

5) ***FILL*** a syringe barrel with the cell sample using a spatula and press it into a 50 ml tube filled with liquid N_2 (**Figure 3**).

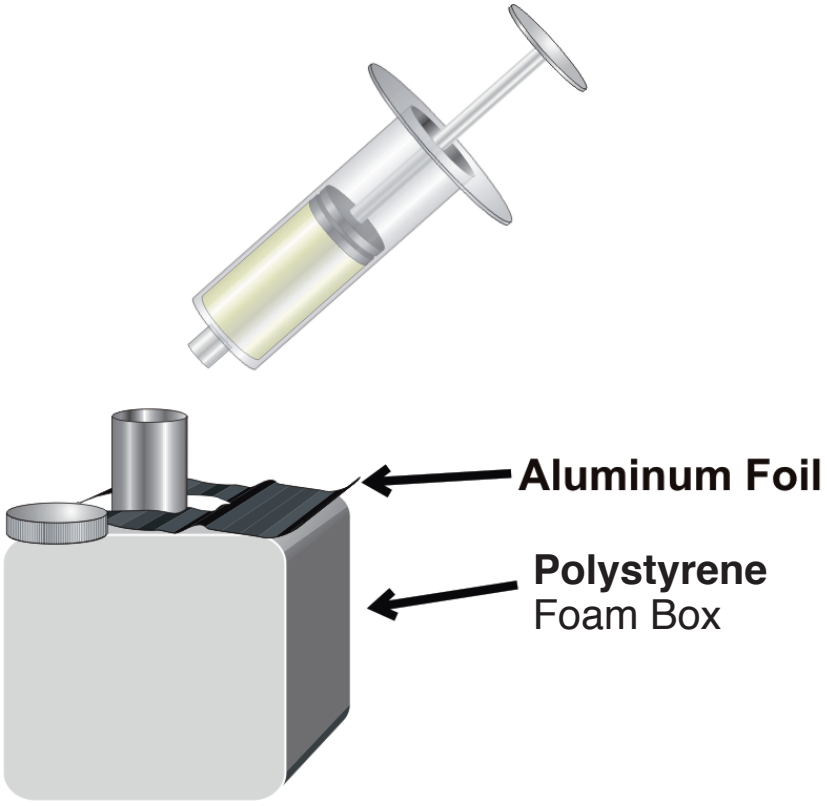
The cell sample should immediately freeze into noodles.

If this process is not feasible for certain mammalian cells, the cells can also be frozen in liquid N_2 as small lumps.

6) ***POUR*** out as much of the liquid N_2 as possible from the tube. Screw the cap on loosely and store at -80°C until use.

It is important that the cap is not screwed on tightly as residual liquid N_2 needs to evaporate during storage. The cells may be stored for months at this stage.

FIGURE 3



Fill a Styrofoam™ (Dow Chemical Company) box with liquid N_2, top with aluminum foil and place a 50 ml tube through a hole in the foil. Fill the tube with liquid N_2.

Press the cell sample through the syringe (without needle) into the tube. Decant the liquid N_2, loosely screw on the cap and store at -80°C.

5.3.2 CRYOLYSIS OF CELL SAMPLES BY GRINDING

A large batch of cell sample can be prepared for multiple co-immunoprecipitation experiments using this method. Prior to grinding, completely chill all tools in liquid N_2. Although cryogrinding may be done manually, **Retsch® Planetary Ball Mill PM100 or Retsch® Mixer Mill MM301** (both from Retsch GmbH, Haan, Germany) are strongly recommended for best results. The following procedure is adapted for Retsch Planetary Ball Mill or Mixer Mill. Approximately 8 L of liquid N_2 is necessary for this process. **Do not** substitute liquid N_2 with dry ice and Ethanol bath. The latter will not sufficiently chill the material during the grinding procedure.

1) ***CHILL*** the stainless steel grinding jar, lid, grinding balls, and the storage tube containing the cell sample in liquid N_2.

The tools are ready for use when the liquid N_2 boiling has almost ceased.
For the 125 ml and 50 ml grinding jars, 20 mm stainless steel balls are used.

2) ***LYSE*** frozen cell sample by cryogenic grinding in 8 sessions of 3 min at 400 RPM with 1 min reverse rotation for PM100, and in 8 sessions of 3 min at 30 Hz for the MM301.

The grinding jar should be approximately 2/3 filled with cell sample and grinding balls. PM100 is used for grinding 6 g – 20 g of cell sample and MM301 is used for grinding under 5 g cell sample.

Make sure you hear a constant rattling during the grinding. If the rattling stops, modify the number of balls until the rattling starts again.

3) ***CHILL*** the jar in liquid N_2 between the grinding sessions.

It is important that the entire jar is completely chilled between the session to counteract the heat development during grinding. If the cell sample should turn into paste during the grinding, the sample is considered ruined.

4) ***TRANSFER*** the grindate to a 50 ml tube that has been pre-chilled in liquid N_2.

The cell grindate should look like fine powder. Over 90% of cells should be disrupted by this procedure.

Sometimes the grindate is packed solid and difficult to remove from the jar. In such instance, a 30 sec grinding session will releases the grindate from the jar.

5) ***STORE*** the frozen yeast cell grindate at -80°C until use.

The grindate can be stored for months at this stage.

5.3.3 PREPARATION OF CELL GRINDATE FOR CO-IMMUNOPRECIPITATION

Make sure the antibody coupled beads from **Section 3** and the Co-IP buffers for **Section 6.1** or **6.2** are prepared prior to start.

1) ***PREPARE*** an **Extraction Buffer** of the desired stringency by mixing ingredients according to the table below just prior to use. If necessary, adjust the concentration of DTT and protease inhibitors (see **Section 4.4** for details).

Extraction Buffer A

Components	Final Conc.	Volume
5 x IP	1 x	
NaCl		
Protease Inhib.		
H_2O		

Extraction Buffer B

Components	Final Conc.	Volume
5 x IP	1 x	
$MgCl_2$	2 mM	
DTT	1 mM	
NaCl		
Protease Inhib.		
H_2O		

2) ***WEIGH*** an appropriate amount of cell grindate in a 50 ml tube that has been chilled in liquid N_2. Let the grindate thaw to an ice-cream like consistency.

Do not use more than 2.5 g cell grindate in one 50 ml tube. For cell samples exceeding 2.5 g (total volume including **Extraction Buffer** will exceed 25 ml), divide the sample into several tubes.

3) ***RESUSPEND*** the cell grindate in 1:9 ratio of cell to **Extraction Buffer A** or **B** by vortexing for 30 sec.

Example: 0.5 g grindate should be resuspended in 4.5 ml of **Extraction Buffer**.

Optional: The suspension can be homogenized using a Polytron PT-K (Kinematica, Switzerland) for 30 sec at 5.5 setting.

4) ***CENTRIFUGE*** the lysate at 2600 × *g* for 5 min at 4°C.

If your sample has been homogenized, centrifuge at 840 × *g* for 2 min at 4°C.

5) ***TRANSFER*** the supernatant to a clean tube. ***Use immediately for co-immunoprecipitation*** (**Section 6.1** or **6.2**).

6. CO-IMMUNOPRECIPITATION PROTOCOL

6.1 *CO-IMMUNOPRECIPITATION – DETECTION WITH WESTERN BLOTTING OR SILVER STAINING*

For co-immunoprecipitation that is to be analyzed by Western Blotting or Silver staining, 0.05 g to 1.5 g cell sample and 1.5 mg antibody coupled beads are recommended. Note that the same amount of beads is used despite varying the amount of cell sample.

1) ***PREPARE*** the co-immunoprecipitation buffers.

   a) Prepare **Extraction Buffer A** or **B** with the appropriate stringency (i.e. NaCl concentration) according to the below table. Approximately 2 ml is necessary per co-immunoprecipitation. An additional 2.7 ml will be necessary per co-immunoprecipitation if the beads have been stored in a solution containing NaN_3.

Extraction Buffer A

Components	Final Conc.	Volume
5 x IP	1 x	
NaCl		
Protease Inhib.		
H_2O		

Extraction Buffer B

Components	Final Conc.	Volume
5 x IP	1 x	
$MgCl_2$	2 mM	
DTT	1 mM	
NaCl		
Protease Inhib.		
H_2O		

b) Prepare **Last Wash Buffer (LWB)** according to the below Table. Approximately 200 µl is necessary per co-immunoprecipitation.

Last Wash Buffer		
Components	**Final Conc.**	**Volume**
5 x LWB	**1 x**	
Tween 20	**0.02%**	
H_2O		

2) ***TRANSFER*** 1.5 mg of antibody coupled Dynabeads to a fresh tube.

3) ***WASH*** the beads in 900 µl **Extraction Buffer**: place the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant.

If the antibody coupled beads have been stored in NaN_3, the beads need to be washed 3 times in 900 µl Extraction Buffer to completely remove the NaN_3.

4) ***RESUSPEND*** the antibody coupled Dynabeads in cell lysate (from **Section 5.2**) or cell grindate (from **Section 5.3**).

5) ***INCUBATE*** on a roller/rotator at 4°C.

Generally, incubations between 10 to 30 min are recommended. We do not recommend incubations longer than 1 hour due to increased risk of non-specific binding. However for antibodies with lower affinities, incubations in excess of 1 hour may be necessary.

6) ***PLACE*** the tube on a magnet, allow the beads to collect at the tube wall, then remove depleted supernatant.

7) ***WASH*** the Dynabeads in 200 µl **Extraction Buffer** by gentle pipetting.

8) ***PLACE*** the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant.

Do not vortex! Vortexing at this stage increases the risk of losing components of the complex.

9) ***REPEAT*** **Steps 6 & 7** twice for a total of three washes.

10) ***WASH*** the Dynabeads in 200 µl **LWB**. Mix by gentle pipetting and incubate on a roller/rotator at RT for 5 min.

11) ***TRANSFER*** the bead suspension to a clean labeled tube.

12) ***PLACE*** the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant.

13) ***RESUSPEND*** the beads in 60 µl **EB** and incubate on a roller/rotator at RT for 5 min.

14) ***PLACE*** the tube on a magnet, allow the beads to collect at the tube wall.

15) ***TRANSFER*** the supernatant to a clean tube. ***The supernatant contains your purified protein complex.***

6.2 CO-IMMUNOPRECIPITATION – DETECTION WITH COOMASSIE STAINING

For co-immunoprecipitation that is to be analyzed with Coomassie staining, 1 g to 15 g cell sample and 7.5 mg antibody coupled beads are recommended (see **Section 4.1**). Note that the same amount of beads is used despite varying amount of cell sample. Furthermore for such large scale experiments, it is recommended to elute in volatile 0.5 M NH_4OH and 0.5 mM EDTA (**HPH EB**) followed by drying in a SpeedVac to enable loading of the entire sample in a single well for gel analysis.

1) ***PREPARE*** the co-immunoprecipitation buffers.

a) Prepare **Extraction Buffer A** or **B** with the appropriate stringency (i.e. NaCl concentration) according to the table below. Approximately 4 ml is necessary per pull-down for the standard protocol. An additional 2.7 ml will be necessary per co-immunoprecipitation if the beads have been stored in a solution containing NaN_3.

Extraction Buffer A

Components	Final Conc.	Volume
5 x IP	1 x	
NaCl		
Protease Inhib.		
H_2O		

Extraction Buffer B

Components	Final Conc.	Volume
5 x IP	1 x	
$MgCl_2$	2 mM	
DTT	1 mM	
NaCl		
Protease Inhib.		
H_2O		

b) Prepare **Last Wash Buffer** according to the table below. Approximately 1 ml is necessary per co-immunoprecipitation. Additional **LWB** will be needed if **Step 13** Option 1 or 2 is used: Three ml 1 × LWB without Tween 20 is needed

for Option 1. 500 µl 1 × LWB with or without Tween 20 is needed for Option 2 (see **Step 13** for details).

Last Wash Buffer		
Components	**Final Conc.**	**Volume**
5 x LWB	**1x**	
Tween 20	**0.02%**	
H_2O		

c) Prepare **HPH EB** according to the below Table. One ml is necessary per co-immunoprecipitation. This buffer needs to be made fresh each time.

HPH EB		
Components	**Final Conc.**	**Volume**
14.8 N NH_4OH	**0.5 M**	**338 µl**
EDTA	**0.5 mM**	
H_2O		**to 10 ml**

2) ***TRANSFER*** 7.5 mg of antibody-coupled Dynabeads to a clean tube

3) ***WASH*** the beads in 900 µl **Extraction Buffer**. Place the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant.

4) ***RESUSPEND*** the antibody coupled Dynabeads in cell lysate (from **Section 5.2**) or cell grindate (from **Section 5.3**).

For cell samples exceeding 2.5 g (i.e. total volume including **Extraction Buffer** exceeding 25 ml), the sample should have been divided into equal volumes into several tubes in **Section 5.3.3**. Divide the beads accordingly into the samples.

5) ***INCUBATE*** on a roller/rotator for 30 min at 4°C.

Incubations between 10 to 30 min are recommended. However for antibodies with poor affinity, incubations up to 1 hour may be necessary. We do not recommend incubations longer than 1 hour due to increased risk of non-specific binding.

6) ***PLACE*** the tube on a magnet, allow the beads to collect at the tube wall, then remove depleted supernatant.

7) ***WASH*** the Dynabeads in 900 µl **Extraction Buffer**. Mix by gentle pipetting.

Do not vortex! Vortexing at this stage increases the risk of losing components of the protein complex.

8) ***PLACE*** the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant.

9) ***REPEAT*** **Steps 7 & 8** twice for a total of 3 washes.

10) ***WASH*** the Dynabeads in 900 µl **Last Wash Buffer**. Mix by gentle pipetting and incubate on a roller/rotator at RT for 5 min.

Do not extend the washing time beyond 5 min. Prolonged incubation will disrupt the protein complex.

11) ***TRANSFER*** the bead suspension to a fresh tube after the incubation.

12) ***PLACE*** the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant.

13) ***OPTION 1:*** For in-solution digestion of the isolated protein complex (e.g. for direct analysis by Mass Spectrometry), wash an additional 3 times in 900 µl **LWB without Tween 20**. Mix by gently pipetting. Immediately place the tube on a magnet and remove the supernatant. Continue to **Step 14**.

OPTION 2: For extraction of DNA or RNA from the isolated protein complex go to **Step 13** in **Section 6.3**.

14) ***RESUSPEND*** the beads in 500 µl **HPH EB** and incubate on a roller/rotator at RT for 20 min.

15) ***PLACE*** the tube on a magnet, allow the beads to collect at the tube wall. Transfer the eluate to a fresh tube. ***The supernatant contains your purified protein complex.***

16) ***REPEAT*** the elution: Resuspend the beads again in 500 µl **HPH EB** and incubate on a roller/rotator at RT for 10 min. Transfer the eluate to the tube containing the previous eluate.

Two serial elutions are necessary to ensure complete release of the protein complex.

17) ***DRY & PELLET*** the eluate using a centrifugal vacuum concentrator (e.g. SpeedVac®, Thermo Savant Inc.) *without heat or radiant cover* overnight. The lyophilized protein can be solubilized in SDS-PAGE Sample Loading Buffer prior to gel loading.

6.3 ISOLATION OF NUCLEIC ACIDS FROM ISOLATED DNA/RNA BINDING PROTEIN COMPLEXES

Continuation from **Step 13** in **Section 6.2**.

13) ***RESUSPEND*** the beads in 500 µl **Last Wash Buffer** (with or without Tween 20). Add 5 µl of 10% SDS and 5 µl Proteinase K (20 mg/ml). Incubate at 55°C for 30 min.

14) ***ADD*** 500 µl Phenol:Chloroform:Isoamyl Alcohol, vortex for 30 sec and centrifuge at 16,000 x *g* for 2 min.

15) ***TRANSFER*** the upper phase to a fresh tube. Add 2.5 volumes of 100% EtOH, 0.1 volume of 3M NaAc, and 5 µl of glycogen (20mg/ml). Precipitate for 1 hour at -20°C.

16) ***CENTRIFUGE*** at 16,000 × *g* for 15 min at 4°C.

17) ***REMOVE*** the supernatant carefully. Wash pellet in 500 µl 70% EtOH.

18) ***CENTRIFUGE*** at 16,000 × *g* for 5 min at 4°C.

19) ***REMOVE*** the supernatant carefully. Air-dry the pellet and resuspend in desired volume of RNase/DNase-free H_2O.

6.4 CO-IMMUNOPRECIPITATION BUFFER FORMULATIONS

	Stock concentration	Working concentration (1 ×)
5 × IP	Buffering Salts (pH 7.4) 550 mM KOAc 2.5% Triton X-100	Buffering Salts (pH 7.4) 110 mM KOAc 0.5 % Triton X-100
5 × LWB	Buffering Salts (pH 7.5)	Buffering Salts (pH 7.5)
EB		Buffering Salts (pH 2.8)
HPH EB (not supplied)		0.5 M NH_4OH 0.5 mM EDTA

7. REFERENCES

1. Oeffinger M, *et al.*, *Comprehensive analysis of diverse ribonucleoprotein complexes.* Nature Methods, 2007, 4(11); 951-956.
2. Alber F, *et al.*, *Determining the architectures of macromolecular assemblies.* Nature 2007, 450(7170); 683-694.
3. Alber F, *et al.*, *The molecular architecture of the nuclear pore complex.* Nature 2007, 450(7170); 695-701.
4. Cristea I, *et al.*, *Fluorescent proteins as proteomics probes.* Mol Cell Proteomics, 2005, 4(12): 1933-1941.

8. GENERAL INFORMATION

Dynal AS complies with the
Quality System Standards ISO 9001:
2000 and ISO 13485:2003.

8.1 Precautions

These products are designed to be used with very strong permanent magnets. People wearing a pacemaker or any other medical magnetizable implant should not use this product unless advised by a health professional; the implant could be affected or damaged by exposure to a strong magnetic field.

Keep magnetizable tools and objects out of the working area. This includes, but is not restricted to, credit cards and other products containing magnetic recording devices. Keep away from delicate instruments, watches, electronic equipment, displays and monitors.
Magnets may attract steel or other magnetic material with high mechanical forces. Take care during handling. Avoid contact between two magnets. Do not pull the magnets apart if contact has been made; twist off to prevent damage to the unit or fingers. The Health and Safety Officer should take all necessary steps and full responsibility to ensure that the precautions and statements are followed and adhered to. IN NO EVENT SHALL INVITROGEN DYNAL AS BE LIABLE FOR ANY SPECIAL, INCIDENTAL OR CONSEQUENTIAL DAMAGES.

8.2 Disinfection of DynaMag™ Magnets

The following materials have been tested for cleaning purposes. Spray and/or wipe the DynaMag unit with one of the following cleaning agents:

- 70% isopropyl alcohol
- 1% sodium hypochlorite solution (Bleach)
- 0.1N HCl solution

Other disinfectants have not been tested and may not be suitable. Do not submerge in aqueous solutions and avoid prolonged exposure to water or aqueous solutions.
Clean with a damp cloth and mild detergent when exposed to harsh solvents. Do not autoclave the DynaMag magnets.

8.3 Storage and Stability

All kit components can be stored between 2°C and room temperature.
Precautions should be taken to prevent bacterial contamination of the beads.
When stored in unopened vials at 2°C – Room Temperature, the Dynabeads and buffers provided in this kit are stable until the expiration date printed on the label.

Beads should not be autoclaved, but can be incubated with ethanol (70%, 1 hour) or gamma irradiated.

Coated beads may be stored at 2-8°C for several weeks or even months, depending on the stability of the immobilized ligand. Coated beads should be washed once for 5 min in PBS/BSA before use. Use the magnet to collect the beads according to the washing procedure.

If a preservative is needed for storage of coated beads, a final concentration of 0.02% (w/v) sodium azide (NaN_3) may be added to the storage buffer. Carefully remove before use by washing (see 3.2 above). Required safety precautions must be followed when handling this cytotoxic material.

8.4 Technical Support

Please contact Invitrogen Dynal® for further technical information (see contact details).

8.5 Warning and Limitations

This product is for research use only. Not intended for any animal or human therapeutic or diagnostic use unless otherwise stated.

Sodium azide is toxic if ingested. Avoid pipetting by mouth. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. When disposing through plumbing drains, flush with large volumes of water to prevent azide buildup.

This product guarantees optimum isolation of Dynabeads, not the isolation of a specific material. Recovery of biomolecules by magnetic isolation depends on the avidity of the antibodies or ligands on the surface of Dynabeads, as well as factors concerning the biomolecules themselves and the matrix from which they are to be isolated. Material Safety Data Sheet (MSDS) is available at http://www.invitrogen.com.

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