USER GUIDE

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Dynabeads® Co-Immunoprecipitation Kit

Catalog number 14321D

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For Research Use Only. Not for human or animal therapeutic or diagnostic use.

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Contents

Kit Contents and Storage

Storage

Upon receipt, store all components of the Dynabeads® Co-Immunoprecipitation Kit at 2°C to 8°C or room temperature.

When stored in unopened vials at 2°C to room temperature, the Dynabeads® M-270 Epoxy and buffers provided in this kit are stable until the expiration date printed on the label.

Precautions should be taken to prevent bacterial contamination of the beads. In the event contamination occurs, do not autoclave Dynabeads® M-270 Epoxy, but incubate beads with ethanol (70%, 1 hour) or gamma irradiate the beads.

Kit Contents

The components included in the Dynabeads® Co-Immunoprecipitation Kit are listed in the following table. Sufficient reagents are supplied for 40 co-immunoprecipitation (Co-IP) reactions using up to 1 g of cells or 8 Co-IP reactions using up to 7.5 g of cells. All kit components are analytical grade and are compatible with protease & phosphatase inhibitors.

Component	Amount
Dynabeads® M-270 Epoxy	>60 mg
C1	20 mL
C2	8 mL
HB	15 mL
LB	15 mL
SB	40 mL
5X IP	120 mL
5X LWB	24 mL
EB	4 mL

Product Use

For Research Use Only. Not for human or animal therapeutic or diagnostic use.

Description of the System

About the Kit

Product Description

The Dynabeads® M-270 Epoxy and buffers provided in this kit enable covalent immobilization of antibodies of your choice onto the Dynabeads® surface and use the antibody coated beads for co-immunoprecipitation. This kit is to be used for the co-immunoprecipitation (Co-IP) of proteins, intact protein complexes, and intact protein-nucleic acid complexes.

The protein to bead interaction occurs directly on the bead surface enabling the ultra rapid procedure that permits isolation of even labile composites, and allows isolation of complexes of all sizes.

This kit is designed for and tested with cultured yeast and mammalian cells and is compatible with tissue, insect, bacteria, and other lysates.

Advantages of the Dynabeads® Co-Immuno-precipitation Kit

- Highly efficient covalent binding of most common antibody species to Dynabeads® M-270 Epoxy.
- Antibody-coated Dynabeads® M-270 Epoxy exhibit ultra-low background binding eliminating the need for blocking.
- Magnetic separation facilitates washing, buffer changes, and elution.
- Other protein ligands (e.g., lectins, enzymes) are covalently coupled to the surface of Dynabeads® M-270 Epoxy using the same beads, buffers, and protocol provided in this kit.

Methods

Antibody Coupling Considerations

Antibody Selection

The choice of antibody is the most important factor for successful target capture.

Note: Not all antibodies are suitable for all applications.

While a particular antibody may recognize and bind to its target antigen in some applications such as western blotting, there is no guarantee that the same antibody, will function well in immunoprecipitation or Co-IP. Refer to the manufacturer's recommendations regarding your antibody.

Dynabeads® M-270 Epoxy Coupling Guidelines

- Use low quantities of antibody per mg beads
- Optimal coupling is achieved with purified antibody
- Coupling of antibodies stabilized in glycerol is not recommended

Additives

Common additives in antibody preparations may affect coupling to Dynabeads® M-270 Epoxy (see **Antibody Additives**, page **34**).

- Sodium Azide (NaN_3)
- Antibody Stabilizing Proteins such as Bovine Serum Albumin (BSA) or Gelatin
- Glycerol

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Antibody Coupling Considerations, continued

Antibody Use

- Optimal coupling at 5–10 µg antibody per mg of beads (see **Antibody Binding Curve** page **33**). Low affinity antibodies may require increasing the amount of input antibody.
- "Optimal coupling range" is antibody dependent and should be empirically determined.
- Using excess antibody significantly increases antibody consumption. If antibody cost is not a factor, we recommend using 20–30 µg antibody per mg Dynabeads® M-270 Epoxy.
- Saturation binding of the Dynabeads® surface may be desirable. Excess coupled antibody may potentially further reduce non-specific binding.
- Using excess antibody increases the potential for "leakage" of non-covalently adsorbed antibody in downstream assays. Additional bead washing steps after coupling reduces "leakage".

Antibody Dependent Variability

Different antibodies have different characteristics. Different antibody clones raised in the same species against the same antigen can vary greatly in pI, antigen binding affinity, and stability. Consequently, the coupling efficiency varies slightly between different antibodies. Furthermore, some coupled antibodies retain their function for months or even years when stored properly, while others lose their function within several weeks. These are entirely ligand dependent.

Antibody Aggregates and Antibody Leakage

The presence of antibody aggregates in the antibody stock used for coupling can result in antibody leakage during the downstream assay. To help reduce this, we recommend removing antibody aggregates from the antibody stock by centrifugation at 16,000 × *g* for 10 min at 4°C.

Antibody Coupling Protocol

Introduction

The following protocol may be used for coupling antibodies to the included Dynabeads® M-270 Epoxy. The quantity of Dynabeads® M-270 Epoxy used for antibody coupling depends upon the subsequent number and scale of Co-IP samples to be run.

Required Materials

Components required but not supplied:

- Magnet: e.g., DynaMag™-2 (see **www.lifetechnologies.com/magnets**).
- Mixer allowing rotation or tilting of tubes.
- Antibodies of your choice.
- Centrifuge capable of achieving >16,000 × *g*.

Components supplied with the kit:

- **Dynabeads® M-270 Epoxy**
- **C1**
- **C2**
- **HB**
- **LB**
- **SB**

Scale of Coupling Reaction

Antibody coupling reactions should be scaled as outlined in **Calculation of Antibody and C1 Volumes** (page **5**). For best Co-IP results:

- Coupling of 5–7 µg antibody per mg beads is recommended for high affinity antibodies.
- Coupling of more antibody per mg beads may be necessary if the antibody affinity is poor.
- Typically, for Co-IP of protein complexes with analysis by silver staining or western blotting, 1.5 mg of antibody-coupled beads are used.
- For detection by Coomassie™ Brilliant Blue staining, 7.5 mg of antibody-coupled beads are used. (For more information see **Co-Immunoprecipitation Protocol**, page **23–25**).

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Antibody Coupling Protocol (Day 1)

Important: Moisture on unused beads deactivates the reactive groups necessary for covalent antibody coupling. To avoid condensation on unused beads, ensure the beads are at room temperature prior to opening the bottle.

1. Disinfect the magnet to prevent accidental sample contamination (see page **35**).
2. Weigh out the appropriate amount of Dynabeads® M-270 Epoxy (see **Calculation of Antibody and C1 Volumes** in the following table).
3. Wash the beads with 1 mL of **C1** and mix by vortexing or pipetting.
4. Place the tube on a magnet for 1 min and allow the beads to collect at the tube wall. Remove the supernatant.
5. Add the appropriate volume of antibody + **C1** (see **Calculation of Antibody and C1 Volumes** in the following table) to the washed beads and mix by gentle vortexing or pipetting.

   Example: If you are coupling 5 mg Dynabeads® M-270 Epoxy and your required quantity of antibody has a volume of 100 µL, you need to add 150 µL of **C1** (i.e., 250 µL **C1** – 100 µL Ab = 150 µL).
6. Add the appropriate volume of **C2** and mix by gentle vortexing or pipetting.

Calculation of Antibody and C1 Volumes

Rule of Thumb: The **C1** + Ab volume is equal to **C2** volume. The total reaction volume (**C1** + µL Ab + **C2**) should be 100 µL per mg beads.

	Volume (µL)		
Beads (mg)	**C1**	**C2**	**Total Volume**
5	250 – vol. Ab	250	500
20	1000 – vol. Ab	1000	2000
60	3000 – vol. Ab	3000	6000

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Antibody Coupling Protocol, Day 1, continued

7. Incubate on a roller at 37°C overnight (16–24 hours). Make sure the fluid in the tube is mixing well.

   Important: Make sure the beads do not settle because this results in inefficient antibody coupling.

Antibody Coupling Protocol (Day 2)

Note: We recommend including 0.01%–0.1% Tween®-20 in **HB** and **LB** wash buffers for improved stringency.

1. Place the tube on a magnet for 1 min and allow the beads to collect at the tube wall. Remove the supernatant.
2. **HB** wash: Add the appropriate volume of **HB** and mix by vortexing or pipetting.

Beads (mg)	Volume HB (µL)
<20	800
≥20	1600

3. Place the tube on a magnet for 1 min and allow the beads to collect at the tube wall. Remove the supernatant.
4. **LB** wash: Add the appropriate volume of **LB** and mix by vortexing or pipetting.

Beads (mg)	Volume LB (µL)
<20	800
≥20	1600

5. Place the tube on a magnet for 1 min and allow the beads to collect at the tube wall. Remove the supernatant.

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Antibody Coupling Protocol (Day 2), continued

6. Short **SB** wash: Add the appropriate volume of **SB** and mix by vortexing or pipetting.

Beads (mg)	Volume SB (µL)
<20	800
≥20	1600

7. Place the tube on a magnet for 1 min and allow the beads to collect at the tube wall. Remove the supernatant.
8. Repeat **Short SB** wash once more.
   Note: If antibody leakage is determined to be a problem repeat this step one or two more times.
9. Long **SB** Wash: Add the appropriate volume of **SB** and mix by vortexing or pipetting.

Beads (mg)	Volume SB (µL)
<20	800
≥20	1600

10. Incubate on a roller/rotator at room temperature for 15 min.
11. Place the tube on a magnet for 1 min and allow the beads to collect at the tube wall. Remove the supernatant.
12. Resuspend antibody-coupled beads in 100 µL **SB** per mg beads and store at 2°C to 8°C until use.

    The final bead concentration is 10 mg/mL antibody-coupled beads.
13. If desired, antibody-coupled beads may be concentrated up to 30 mg/mL by reducing the storage buffer volume. Your beads are now covalently coupled with antibody and ready for IP, Co-IP, or other assays.

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Long Term Storage

Coated beads may be stored at 2°C to 8°C for several weeks or even months, depending on the stability of the immobilized antibody.

If a preservative is needed for long term storage of coated beads, a final concentration of 0.02% (w/v) sodium azide may be added to the storage buffer.

Wash coated beads once for 5 min in PBS with 0.1% BSA before use.

Important: Not all coupled antibodies retain their function in long term storage. Verify your coupled antibody stability by testing in small scale.

WARNING! Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

Co-Immunoprecipitation Considerations

Scale of Co-IP

The abundance and method of detection for your protein of interest dictates the scale of the Co-IP reaction required. Suggested amounts of starting materials are provided in the following table when using 5–7 µg antibody/mg beads as recommended (see **Dynabeads® Binding Capacity**, page **33**)

Detection Method	Sample Size (g)	Ab Coupled Dynabeads® (mg)
Western Blot Silver Stain	0.05–1.5	1.5
Coomassie™ Stain Mass Spectrometry	1–15*	7.5

*Rare cases up to 25 g

For high-abundance proteins, use the lower scale of the cell sample recommended. For low-abundance proteins, use the upper scale of the cell sample recommended.

Sample Preparation

Detergent lysis- appropriate for mammalian cell culture samples.

Cryolysis-recommended for isolation of large and/or labile complexes. Applicable to all sample types including:

- Mammalian cell culture samples
- Tissue samples
- Yeast
- Bacteria
- Whole organisms i.e., *Drosophila*

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Co-Immunoprecipitation Considerations, continued

Stringency

To isolate intact protein complexes while minimizing non-specific binding, adjust the stringency of the Extraction Buffer. Components that can be varied to increase or decrease stringency of the Co-IP include;

- Salts
- Detergents
- Dithiothreitol (DTT)
- Protease Inhibitors

See **Co-immunoprecipitation Optimization,** page **30**.

Wash Buffer

Bead-bound Co-IP protein complexes are washed following isolation for added stringency or buffer exchange prior to downstream processing (e.g., in solution proteolytic digestion or nucleic acid extraction).

Elution Buffer

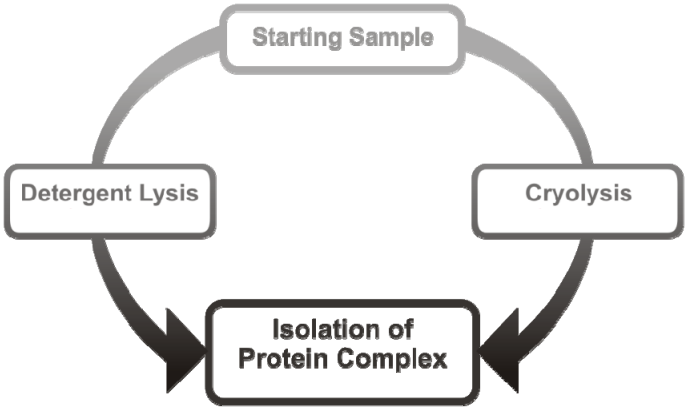
For the elution of the isolated protein complex, the buffer EB is supplied in the kit. However, for large scale Co-IP from which the isolated protein complex requires concentration or is to be analyzed by mass spectrometry, elution in NH_4OH based buffer (**HPH EB**, see **Required Materials** page **12**) followed by lyophilization in a centrifugal vacuum concentrator is strongly recommended.

Cell Sample Preparation

Introduction

The most appropriate method for the preparation of samples for co-immunoprecipitation depends upon the type of sample to be used and the types of complexes to be isolated.

In many instances cell disruption using detergent lysis is adequate. However, for isolation of large and/or labile complexes, whole cell cryolysis is strongly recommended. Liquid nitrogen stabilizes labile complexes during the cryolysis process. Cryolysis protocols are applicable for all cell types. For further information on the recommended cryolysis procedure, see **References** (1–4) on page **39**.



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Required Materials

Components required but not supplied:

- Centrifuge capable of achieving >16,000 × *g*
- Optional: Buffer modifiers
  - Sodium Chloride (e.g., 1 M NaCl)
  - Dithiothreitol (e.g., 1 M DTT)
  - Magnesium Chloride (e.g., 1 M $MgCl_2$)
  - Potassium Acetate (e.g., 1 M KOAc)
  - Tween®-20
  - Triton® X-100
  - Protease inhibitors without EDTA
- **HPH EB**, (0.5 M NH_4OH, 0.5 mM EDTA)

Components supplied with the kit:

- **5X IP**
- **5X LWB**
- **EB**

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Extraction Buffer Considerations

For co-immunoprecipitation, the cell sample needs to be resuspended in an Extraction Buffer. Different protein complexes require different Extraction Buffer composition for successful Co-IP. Currently there are no rules allowing prediction of buffer composition needed for isolation of particular protein complexes.

- To isolate intact protein complexes while minimizing non-specific binding, adjust the stringency of the Extraction Buffer. Determine the optimal buffer composition empirically (see **Co-Immunoprecipitation Optimization**, page **30**) for each target antigen.
- The extraction buffer (**5X IP**) is supplied as a 5X concentrate formulated to achieve isolation of intact protein complexes while minimizing non-specific binding.

  Dilute **5X IP** buffer to 1X prior to use.
- Modify **1X IP** extraction buffer with various buffer modifiers to achieve optimal stringency. For instance, some protein complexes require the presence of DTT and/or $MgCl_2$ to remain intact, while in other complexes these modifiers disrupt the complex and/or increase non-specific binding. Increasing concentrations of salt and detergent in the Extraction Buffer generally increases the stringency of the Co-IP.
- EDTA is incompatible with many protein complexes. Avoid protease inhibitors that contain EDTA. For most applications, a 1:200 to 1:500 dilution of 0.1 M phenylmethylsulphonyl fluoride (PMSF) will suffice.

Note: Several different buffer compositions can be used in the same experimental set up (see **Co-IP Optimization** on page **30** for details).

Detergent Lysis Method

Before starting

Use cell samples prepared using detergent lysis immediately for Co-IP. Make sure the antibody-coupled beads from **Antibody Coupling Protocol** (page **5**) and the Co-IP buffers for the **Co-Immunoprecipitation Protocol** (page **23**) are prepared prior to start.

Detergent Lysis Method

1. Prepare (~2 mL per Co-IP) Extraction Buffer of the desired stringency by mixing ingredients according to the following table just prior to use.

   Note: 5X IP diluted to 1X for use in Extraction Buffer already contains 0.5% Triton® X-100 (see **Recipes** page 36).

   Approximately 2.7 mL Extraction Buffer per Co-IP is necessary if the Ab-coupled beads are stored in a solution containing sodium azide. If necessary, adjust the concentration of salt, DTT, and protease inhibitors (see **Co-IP Optimization**, page **30).**

	Extraction Buffers A & B							
	Component	**5X IP**	**NaCl**	**KOAc**	**$MgCl_2$**	**DTT**	**Protease Inhibitors**	**Water**
A	**Concentration**	1X	*	*	*	*	**	—
	Volume							TBD
B	**Concentration**	1X	*	*	*	*	**	—
	Volume							TBD

*User determined concentration; see Co-Immunoprecipitation Optimization page 30.
**See Protease Inhibitors page 30.

2. Weigh the empty tube in which the sample is to be collected. Do not assume an average weight of the tube.
3. Transfer the cells to the pre-weighed tube and collect by centrifuging at 200–500 × *g* for 5 min at 4°C. Discard the supernatant.

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Detergent Lysis Method, continued

Detergent Lysis Method, continued

4. Wash harvested cells once in PBS by centrifuging at 200–500 × *g* for 5 min at 4°C. Remove as much of the liquid as possible from the cells.
5. Weigh the tube again. Calculate the weight of the cell pellet.

   (Weight of tube + Cells) – (Weight of empty tube) = (Weight of Cells)
6. Resuspend cells in 1:9 ratio of cell mass to Extraction Buffer with protease inhibitors.

   Example: 50 mg cells are lysed in 450 µL buffer. For reproducible results use a 1:9 ratio of cell sample to Extraction Buffer for the Co-IP each time (cells should be weighed with as much liquid removed as possible).
7. Incubate on ice for 15 min.
8. Centrifuge at 2600 × *g* for 5 min at 4°C to remove large cell debris and nuclei.
9. Transfer the supernatant to a fresh tube. Use immediately for co-immunoprecipitation (pages **23** or **25**).

Cryolysis Method

Introduction

Cryolysis is applicable to all sample types. Cryolysis stabilizes large and/or labile complexes facilitating their isolation. Additionally, large sample sizes can be prepared for use in multiple Co-IP experiments using this method.

Required Materials

In addition to the materials required for Detergent Lysis the following components are required but not supplied:

- Liquid nitrogen (~8 L required for this procedure)
- Retsch® Planetary Ball Mill PM100
  Or
  Retsch® Mixer Mill MM301
- 50 mL Syringe
- 20 mM Potassium-HEPES buffer pH 7.4

Sample Size

- Approximately 6 L yeast or bacterial culture grown to OD_{600} ~0.8 is needed to produce ~20 g cells.
- Approximately 8 L of the mammalian cell line HEK 293 grown to late log phase is needed to produce ~12 g cells.
- For tissue samples (e.g., liver) or whole organisms (e.g., *Drosophila*), weigh the sample directly and freeze in liquid nitrogen.

Before starting

Make sure all buffers and cells are kept on ice during the procedure. For the cryogenic freezing of the harvested cell sample, approximately 1–2 L liquid nitrogen is needed.

WARNING! Use appropriate protective gear when working with liquid nitrogen. Contact your local environmental health work place safety officer for more information.

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Cryolysis Method, continued

Prepare Sample for Cryolysis

1. Harvest 6 L yeast or bacterial culture or 8 L (or equivalent amount) mammalian or insect cell culture.
2. Centrifuge the yeast or bacteria culture at 4000 × *g*, and mammalian or insect cell culture at 500 × *g* for 10 min at 4°C. Discard the culture media. For mammalian and insect cells, skip Step 3; go directly to Step 4.
3. Wash the yeast/bacterial cells in 50 mL water. Centrifuge yeast and bacteria cells at 2600 × *g* for 5 min at 4°C, then discard the supernatant.
4. 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. Remove as much buffer as possible after centrifugation.

   Optional: Add protease inhibitors and 1 mM DTT to the K-HEPES buffer.
5. 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 as much of the remaining supernatant as possible and resuspend the pellet in the residual buffer.
6. Proceed immediately to **Freeze Sample in Liquid Nitrogen** on the next page.

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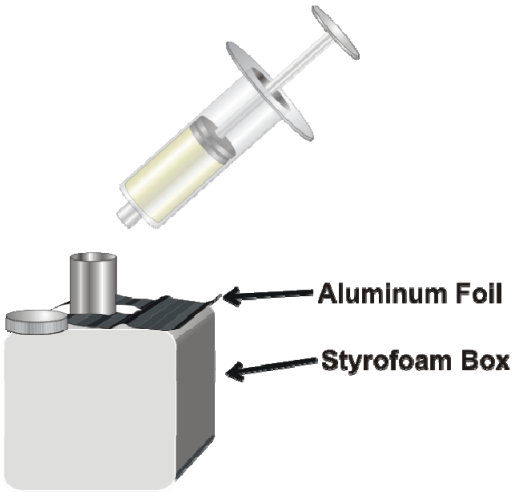
Freeze Sample in Liquid Nitrogen

1. Fill a Styrofoam box with liquid nitrogen, cover with aluminum foil and place a 50-mL tube through a hole in the foil (see figure below). Fill the tube with liquid nitrogen.
2. Transfer the sample to the barrel of a 50-mL syringe.
3. Press the cell sample through the syringe (without needle) into the tube filled with liquid nitrogen.

   Note: 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 nitrogen as small lumps.
4. Decant as much of the liquid nitrogen as possible from the tube. Screw the cap on loosely and store the sample –80°C until use.

   Note: It is important that the cap is not screwed on tightly as residual liquid nitrogen needs to evaporate during storage. The cells may be stored for months at –80°C.



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Cryolysis of Samples by Grinding

Although cryo-grinding may be done manually, the following procedure is adapted for using a Retsch® Planetary Ball Mill PM100 (6–20 g of cell sample) or Retsch® Mixer Mill MM301 (under 5 g cell sample). Use of either Mill is strongly recommended for best results.

Approximately 8 L of liquid nitrogen is necessary for this process.

Important:

- Prior to grinding, and between sessions, chill the entire grinding jar in liquid nitrogen to counteract heat buildup during grinding. If the sample turns into paste during grinding, the sample is considered ruined.
- **Do not** substitute liquid nitrogen with dry ice and ethanol bath as this 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 nitrogen.

   Note: Mill components are ready for use when the liquid nitrogen boiling has almost ceased. For the 125-mL and 50-mL grinding jars, 20-mm stainless steel balls are used.

2. Fill grinding jar approximately 2/3 filled with frozen sample ("noodles" from step 4, page 18) and grinding balls.
3. Perform cryogenic grinding in 8 sessions
   - 3 min each at 400 RPM with 1 min reverse rotation for PM100
   - 3 min each at 30 Hz for the MM301.

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

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Cryolysis of Samples by Grinding, continued

4. Transfer the frozen ground sample to a pre-chilled 50-mL tube.

   Note: Over 90% of cells should be disrupted by this procedure. The sample should look like fine powder. If the sample is solid and tightly packed it may be difficult to remove from the grinding jar. In such cases a 30 second grinding session releases the frozen ground sample from the jar.

5. Store the frozen ground sample at –80°C until use. The frozen ground sample is stable at –80°C for months at this stage.

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Prepare Cell Lysate for Co-immuno-precipitation

Make sure the antibody-coupled beads and buffers for Co-IP are prepared prior to start.

1. Prepare (~2 mL per Co-IP) Extraction Buffer of the desired stringency by mixing ingredients according to the following table just prior to use. **Note:** Approximately 2.7 mL Extraction Buffer per Co-IP is necessary if the antibody-coupled beads are stored in a solution containing sodium azide. If necessary, adjust the concentration of salt, DTT, and protease inhibitors (see **Co-Immunoprecipitation Optimization**, page 30).

Extraction Buffers A & B								
	Component	**5X IP**	**NaCl**	**KOAc**	**$MgCl_2$**	**DTT**	**Protease Inhibitors**	**Water**
A	**Concentration**	1X	*	*	*	*	**	—
	Volume							TBD
B	**Concentration**	1X	*	*	*	*	**	—
	Volume							TBD

*User determined concentration, See Co-Immunoprecipitation Optimization pages 30.
**See Protease Inhibitors page 30.

2. Weigh an appropriate amount of the frozen ground cell sample in a pre-chilled 50-mL tube and thaw to an ice-cream-like consistency.

   Note: Do not use more than 2.5 g frozen ground cell sample 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.

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Prepare Cell Lysate for Co-immuno-precipitation, continued

3. Resuspend the ground cell sample in 1:9 ratio of cell sample to Extraction Buffer A or B by vortexing for 30 seconds.

   Example: Resuspend 0.5 g ground cell sample in 4.5 mL of Extraction Buffer.

   Optional: The suspension can be homogenized using a Polytron® homogenizer for 30 sec at a setting of 5.5.
4. Centrifuge the sample 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 (pages **23** or **25**).

Co-Immunoprecipitation Protocol

Co-IP Protocol for Western Blot or Silver Stain Analysis

For samples that are 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: The same amount of beads is used despite varying the amount of cell sample.

1. Prepare the co-immunoprecipitation buffers.
   - Prepare Extraction Buffer with the appropriate stringency (see **Co-IP Optimization**, page **30**), ~2 mL per Co-IP. An additional 2.7 mL is needed per Co-IP if the beads have been stored in a solution containing sodium azide.
   - Prepare Last Wash Buffer (**LWB**) according to the following table, 200 µL is required per Co-IP.

Last Wash Buffer (LWB)			
Component	**5X LWB**	**Tween®-20**	**Water**
Concentration	1X	0.02%	—
Volume	—	—	TBD

2. Transfer 1.5 mg of antibody-coupled Dynabeads® Epoxy 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 for 1 min, and then remove the supernatant.

   Note: If the antibody-coupled beads have been stored in sodium azide, wash the beads three times in 900 µL Extraction Buffer to completely remove the sodium azide.
4. Resuspend the antibody-coupled Dynabeads® Epoxy in cell lysate prepared by detergent lysis or cryolysis.

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Co-IP Protocol for Western Blot or Silver Stain Analysis, continued

5. Incubate on a roller/rotator at 4°C. Generally, incubations between 10–30 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 for 1 min, and then remove depleted supernatant.
7. Wash the Dynabeads® Co-IP complexes in 200 µL Extraction Buffer by gentle pipetting.

   Important: Do Not Vortex.
8. Place the tube on a magnet, allow the beads to collect at the tube wall for 1 min, and then remove the supernatant.
9. Repeat Steps 7 and 8 twice for a total of three washes.
10. Wash the Dynabeads® Co-IP complexes in 200 µL 1X **LWB**. Mix by gentle pipetting and incubate on a roller/rotator at room temperature 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 for 1 min, and 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 for 1 min.
15. Transfer the supernatant to a clean tube. The supernatant contains your purified protein complex.

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Co-IP Protocol for Coomassie™ Stain Analysis

For samples that are to be analyzed with Coomassie™ Brilliant Blue staining after Co-IP, 1 g to 15 g cell sample (up to 25 g cell sample in rare cases) and 7.5 mg antibody-coupled beads are recommended

Note: 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, 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.
   - Prepare Extraction Buffer with the appropriate stringency (see **Co-IP Optimization**, page **30**), ~4 mL per Co-IP. An additional 2.7 mL is needed per Co-IP if the beads have been stored in a solution containing sodium azide.
   - Prepare Last Wash Buffer (**LWB**) according to the following table, 1 mL per Co-IP. Additional **LWB** is required for Step 13.

     Option 1: 3 mL 1X **LWB** without Tween®-20 per Co-IP.

     Option 2: 500 µL 1X **LWB** with or without Tween®-20 per Co-IP (see Step 13 for details).

Last Wash Buffer (LWB)			
Component	**5X LWB**	**Tween®-20**	**Water**
Concentration	1X	0.02%	—
Volume	—	—	TBD

Continued on next page

Co-IP Protocol for Coomassie™ Stain Analysis, continued

- Prepare **HPH EB** according to the following table, 1 mL per Co-IP. This buffer needs to be made fresh each time.

HPH EB			
Component	**NH_4OH (14.8 N)**	**EDTA**	**Water**
Final Conc.	0.5 M	0.5 mM	—
Volume	338 µL	—	To 10 mL

2. Transfer 7.5 mg of antibody-coupled Dynabeads® M-270 Epoxy 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 for 1 min, and then remove the supernatant.

   Note: If the antibody-coupled beads have been stored in sodium azide, wash the beads three times in 900 µL Extraction Buffer to completely remove the sodium azide.
4. Resuspend the antibody-coupled Dynabeads® Epoxy in cell lysate prepared by either detergent lysis or cryolysis.

   Note: For cell samples ≥2.5 g (i.e., total volume including Extraction Buffer ≥25 mL), divide the sample into equal volumes into several tubes (see **Note** page **21**). Divide the beads accordingly into the samples.
5. Incubate on a roller/rotator for 30 min at 4°C.

   Note: Incubations between 10–30 min are recommended. However for antibodies with poor affinity, incubate for up to 1 hour. 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 for 1 min, and then remove depleted supernatant.

Continued on next page

Co-IP Protocol for Coomassie™ Stain Analysis, continued

7. Wash the Dynabeads® Co-IP complexes in 900 µL Extraction Buffer. Mix by gentle pipetting.

   Important: Do Not Vortex.
8. Place the tube on a magnet, allow the beads to collect at the tube wall for 1 min, then remove the supernatant.
9. Repeat Steps 7 and 8 twice for a total of 3 washes.
10. Wash the Dynabeads® Co-IP complexes in 900 µL **LWB**. Mix by gentle pipetting and incubate on a roller/rotator at RT for 5 min.

    Important: Do not extend the washing time beyond 5 min. Prolonged incubation disrupts 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 for 1 min, and 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 proceed immediately to Step 1**, Extract DNA/RNA**, page **29**.

    Option 3: For direct analysis by Coomassie™ Brilliant Blue stained gels continue to Step 14.
14. Resuspend the beads in 500 µL **HPH EB** and incubate on a roller/rotator at room temperature for 20 min.

Continued on next page

Co-IP Protocol for Coomassie™ Stain Analysis, continued

15. Place the tube on a magnet, allow the beads to collect at the tube wall for 1 min. Transfer the eluate to a fresh tube.
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.

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

    The supernatant contains your purified protein complex.
17. Lyophilize the eluate using a centrifugal vacuum concentrator without heat or radiant cover overnight.
18. Solubilize the lyophilized protein in SDS-PAGE sample loading buffer prior to gel loading.
19. Perform analysis by SDS-PAGE followed by Coomassie staining.

Extraction of Nucleic Acids from Isolated DNA/RNA Binding Protein Complexes

Extract DNA/RNA

Extraction of nucleic acids from Co-IP bound nucleoprotein complexes is a continuation from step 13 (Option 2**) Co-IP Protocol for Coomassie™ Stain Analysis**, page **27**.

1. Resuspend the nucleoprotein containing Dynabeads® Co-IP complexes in 500 µL 1X **LWB** (with or without Tween®-20).
2. Add 5 µL of 10% SDS and 5 µL Proteinase K (20 mg/mL).
3. Incubate the tube at 55°C for 30 min.
4. Add 500 µL Phenol:Chloroform:Isoamyl Alcohol, vortex for 30 sec, and centrifuge at 16,000 × *g* for 2 min.
5. Transfer the upper phase to a fresh tube. Add 2.5 volumes of 100% ethanol, 0.1 volume of 3 M sodium acetate, and 5 µL of glycogen (20 mg/mL).
6. Incubate for 1 hour at –20°C.
7. Centrifuge at 16,000 × *g* for 15 min at 4°C.
8. Remove the supernatant carefully. Wash nucleic acid pellet in 500 µL 70% ethanol.
9. Centrifuge at 16,000 × *g* for 5 min at 4°C.
10. Remove the supernatant carefully. Air-dry the pellet and resuspend in desired volume of RNase/DNase-free water.

Co-Immunoprecipitation Optimization

Introduction

For simple co-immunoprecipitation, use Extraction Buffer A with 100 mM NaCl (see the following table). To isolate intact protein complexes while minimizing non-specific binding, it is necessary to adjust the stringency of the Extraction Buffer. The larger and/or more unstable a protein complex, the more important it is to optimize the Extraction Buffer. The following section describes a method for Extraction Buffer optimization.

Most salts and detergents are compatible with this kit and may be used to modulate the Extraction Buffer. Increasing concentrations of salt and detergent in the Extraction Buffer generally increase the stringency of the co-immunoprecipitation.

Extraction Buffer

Follow the Co-IP Protocol (page **23**), using two different variants of Extraction Buffer (A and B).

Note: Initially both buffers are modified with 100 mM NaCl. Extraction Buffer B is further modified with 2 mM $MgCl_2$ and 1 mM DTT.

	Extraction Buffers A & B						
	Component	**5X IP**	**NaCl**	**$MgCl_2$**	**DTT**	**Protease Inhibitors**	**Water**
A	**Concentration**	1X	100 mM	—	—	optional	—
	Volume						TBD
B	**Concentration**	1X	100 mM	2 mM	1 mM	optional	—
	Volume						TBD

Protease Inhibitors: Although very little protease inhibitor is needed, it is still recommended as an additive. This kit is compatible with most protease inhibitors.

Note: EDTA is incompatible with many protein complexes. Avoid protease inhibitors that contain EDTA.

Continued on next page

Co-Immunoprecipitation Optimization, continued

Extraction Buffer, continued

Dithiothreitol (DTT): DTT (reducing agent) is compatible with this procedure when used in physiological concentrations (up to 1 mM). Concentrations above 1 mM DTT increase the risk of destabilizing the antibodies bound on the bead surface (i.e., breaking disulfide bridges between the antibody heavy and light chains, and the covalent bond between the antibody and the bead surface). The risk for disrupting protein complexes also increases.

Stringency

Based on the results from the previous section, choose the Extraction Buffer, A or B, that yields the most numerous and distinct bands for further optimization (provided that your complex consists of several proteins). If there is no difference between the two buffers, then use both in parallel.

1. If you see too many bands and/or smeared bands, the buffer stringency is too low. Use Extraction Buffer A or B (or both) with several different NaCl concentrations ranging from 50–200 mM in 50 mM increments (i.e., test buffers with 50 mM, 100 mM, 150 mM, and 200 mM NaCl).

   or

   If you see just your target protein and no other or very faint bands, the buffer stringency is too high. Use Extraction Buffer A or B (or both) with several different NaCl concentrations ranging from 25–125 mM in 25 mM increments (i.e., test buffers with 125 mM, 100 mM, 75 mM, 50 mM, and 25 mM NaCl).
2. Perform SDS-PAGE analysis of the resulting Co-IP complexes obtained.

Continued on next page

Co-Immunoprecipitation Optimization, continued

Stringency, continued

3. Determine the optimal NaCl concentration from the gradient of lanes varying from too few and/or faint bands to too many and/or smeared bands.
4. If no gradient is observed continue NaCl optimization until such a gradient is seen.

   Note: In most cases NaCl concentrations of ≤200 mM will be required. Only in very rare cases will the NaCl concentration exceed 200 mM.

Detergent

For most protein complexes, optimization of detergent is not necessary. However, detergent optimization may be necessary for certain membrane protein complexes. In such case, increasing concentrations of Triton® X-100 is recommended as a first choice

Note: 5X IP diluted to 1X already contains 0.5% Triton® X-100.

Salts

After performing **Extraction Buffer, Stringency,** and **Detergent** optimizations, the following should have been determined:

- Choice between Extraction Buffer A or B.
- Optimal NaCl concentration.
- Optimal detergent concentration for membrane proteins.

For further optimization, the potassium acetate concentration may be examined in addition to NaCl. Since Extraction Buffer A or B already contains 110 mM potassium acetate, test potassium acetate increments of 10 mM (e.g., 110 mM, 120 mM, 130 mM, and 140 mM).

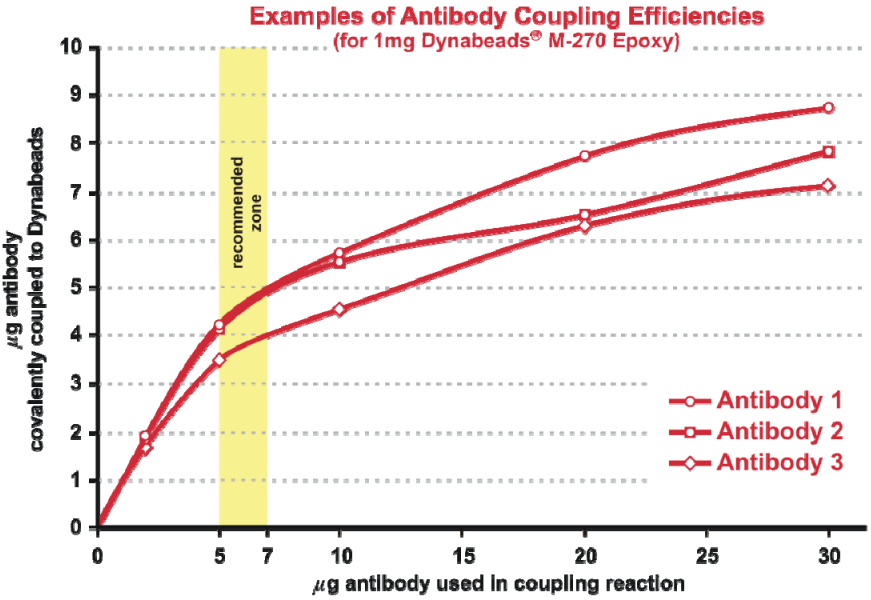
The co-immunoprecipitation results with Extraction Buffers with different potassium acetate concentrations should show a gradient of lanes varying from too few and/or faint bands to too many and/or smeared bands from which the optimal condition is determined.

Appendix

Dynabeads® Binding Capacity

Ligand Binding Curve

Antibody coupling to Dynabeads® M-270 Epoxy is most efficient when using low quantities of antibody per mg beads (5–10 µg antibody per mg of beads. This same general rule applies when coupling other proteins (e.g. lectins, enzymes, etc.) although the "optimal coupling range" may differ for different ligands.



Antibody Additives

Sodium Azide

Many commercially available antibodies contain sodium azide as preservative. The presence of sodium azide can lead to a small decrease (<10%) in antibody coupling efficiency which is not a problem for most applications.

This can be easily compensated by slightly increasing the quantity of antibody used in the coupling reaction.

Alternatively the sodium azide can be removed prior to coupling by standard gel filtration chromatography or dialysis.

Antibody Stabilizing Proteins

Some commercially available antibodies (proteins and enzymes) contain protein additives such as:

- BSA
- Gelatin

Protein additives present during the coupling reaction are coupled to the bead surface along with antibody (or other input protein). The presence of protein additives does not affect antibody coupling efficiency if the total quantity of protein (antibody + protein additive) in the coupling reaction does not exceed the capacity of the beads. In the case of BSA or gelatin, the coupled proteins may provide a beneficial blocking effect but may also result in the co-isolation of BSA or gelatin interacting proteins.

Glycerol

Coupling of antibodies or other proteins stabilized in glycerol is not recommended. Although it is possible to couple such ligands, the antibody (or protein) function may be severely negatively affected.

DynaMag™ Magnet Disinfection

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)
- 0.1 N 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.

Accessory Products

Related Products

The following products may be used with the Dynabeads® Co-Immunoprecipitation Kit. For details, visit **www.lifetechnologies.com** or contact **Technical Support** (see page **37**).

Product	Quantity	Cat. no.
DynaMag™-2	each	12321D
SampleRack (for DynaMag™-2)	each	12322D
DynaMag™-5	each	12303D
Dynabeads® Antibody Coupling Kit	1 Kit	14311D
Dynabeads® M-270 Epoxy	60 mg	14301
Dynabeads® M-270 Amine	2 mL	14307D
Dynabeads® Oligo(dT)$_{25}$	5 mL	61005

Technical Support

Obtaining Support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetechnologies.com**)
- Search for user documents, Safety Data Sheets (SDSs), vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**.

Certificate of Analysis

The Certificate of Analysis is available at **www.lifetechnologies.com/support**. Search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited Product Warranty

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General Information

Life Technologies AS complies with the Quality System Standards ISO 9001:2008 and ISO 13485:2003.

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