invitrogen™ | DYNAL®
invitrogen bead separations

TABLE OF CONTENTS

1. PRODUCT DESCRIPTION

1.1 INTENDED USE

The Dynabeads® and buffers provided in this kit will enable you to a) covalently immobilize antibodies of your choice onto the surface of Dynabeads, and b) use the antibody coated beads for co-immunoprecipitation. This kit is intended to be used for the co-immunoprecipitation of proteins, intact protein complexes, intact protein-nucleic acid complexes, etc. The antibody coated Dynabeads surface has ultra-low background binding, thus does not require blocking prior to use. The protein to bead interaction occurs directly on the bead surface; this enables the ultra rapid procedure that permits isolation of even labile composites, and allows isolation of complexes of all sizes (i.e. no upper size limit). This kit is designed for and tested with cultured yeast and mammalian cells and is compatible with tissue, insect, bacteria, and other lysates. ***For best results, please read through the manual carefully prior to start.***

1.2 PRINCIPLE

Antibodies (Ab) of your choice are covalently coupled to Dynabeads. Once antibodies have been coupled to the Dynabeads, the bead-bound antibody may then be used for co-immunoprecipitation experiments. Captured proteins and protein complexes are easily separated from lysate using magnetic separation properties of Dynabeads. Magnetic separation facilitates washing, buffer changes, and elution.

Antibody-to-bead coupling works optimally with purified antibodies, although the coupling reaction also works well with antibodies in storage buffers that include protein additives (e.g. BSA) and/or sodium azide (NaN_3). **This kit is not recommended for**

use with antibodies that have been stabilized in glycerol (See **Section 2.3** for more information).

1.3 MATERIALS SUPPLIED

Reagents are analytical grade and are compatible with protease & phosphatase inhibitors. **All kit components can be stored between 2 °C and room temperature (RT).**

- **Dynabeads® M-270 Epoxy** (> 60 mg)
- **C1** (20 ml)
- **C2** (8 ml)
- **5 × IP** (120 ml)
- **5 × LWB** (24 ml)
- **LB** (15 ml)
- **HB** (15 ml)
- **SB** (40 ml)
- **EB** (4 ml)

1.4 ADDITIONAL REQUIRED MATERIALS

- Magnet: e.g. DynaMag™-2 (see www.invitrogen.com/magnets).
- Mixer allowing rotation or tilting of tubes (www.invitrogen.com/magnets).
- Antibodies of your choice.
- Optional: Device for Cryolysis (see **Section 5.3.2**).
- Optional: Buffer Modifiers: Sodium Chloride (e.g. 1M NaCl)
  Dithiothreitol (e.g. 1M DTT)
  Magnesium Chloride (e.g. 1M $MgCl_2$)
  Potassium Acetate (e.g. 1M KOAc)
  Tween®-20
  Triton™ X-100
  Protease inhibitors without EDTA

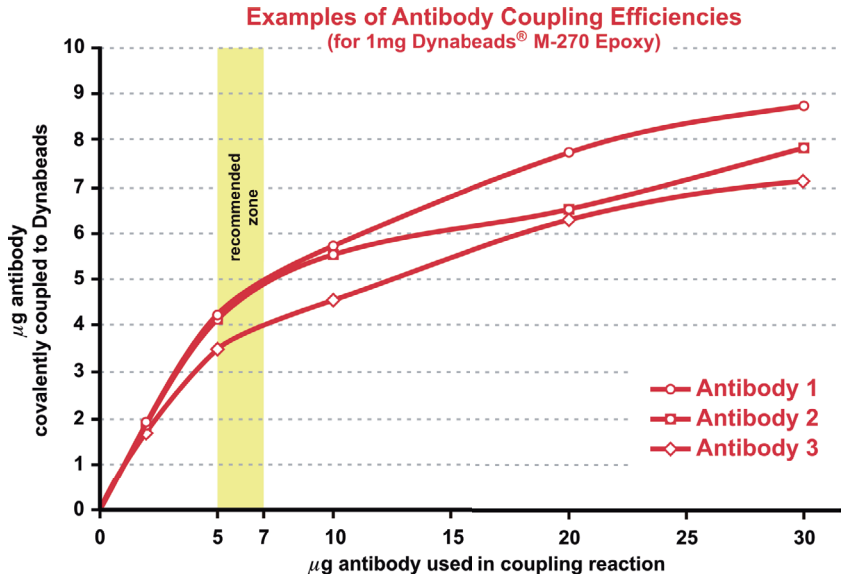
2. ANTIBODY COUPLING CONSIDERATIONS

2.1 OPTIMAL ANTIBODY USE

Antibody coupling to Dynabeads is most efficient when using low quantities of antibody per mg beads (**Figure 1**). For applications such as immunoprecipitation and co-immunoprecipitation, the recommended amount of antibody for a coupling reaction is typically 5 to 7 µg Ab/mg Dynabeads.

For some assays saturation of beads with antibodies may be necessary. In such cases the excess antibodies may provide an increased blocking effect on the bead surface, potentially reducing nonspecific binding of certain proteins. However, excess antibodies will also increase nonspecific antibody interaction with non-target proteins. Moreover, antibody consumption will be significantly increased, along with the potential for (non-covalently) adsorbed antibody to “leak” off the beads. Therefore the optimal amount of antibody coupled per mg bead will depend upon the need for functional antibody balanced against the increased chance for non-specific antibody interaction and non-covalently bound antibody to leak off during the assay.

FIGURE 1



2.2 ANTIBODY SELECTION

The choice of antibody is the most important factor for successful target capture. **Note that 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-immunoprecipitation. Please refer to the antibody manufacturer's recommendations regarding your antibody.

2.3 ANTIBODY ADDITIVES

- **SODIUM AZIDE (NaN_3)**

  Many commercially available antibodies contain NaN_3 as preservative. The presence of NaN_3 can lead to a small decrease (<10%) in antibody coupling efficiency. This will not be a problem for most applications. Furthermore, if desired this can be easily compensated by slightly increasing the quantity of antibody used in the coupling reaction. Alternatively the NaN_3 can be removed prior to coupling by standard gel filtration chromatography or dialysis.

- **ANTIBODY STABILIZING PROTEINS**

  Some commercially available antibodies contain protein additives such as BSA or Gelatin. The presence of protein additives will not reduce antibody coupling efficiency if the total quantity of protein (antibody + protein additive) in the coupling reaction does not exceed the capacity of the beads. Protein additives present during the coupling reaction will be coupled to the bead surface along with antibody. 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 stabilized in glycerol is not recommended. Although it is possible to couple such antibodies, the antibody function may be severely affected.

2.4 FUNCTION AND STABILITY OF ANTIBODIES COUPLED TO DYNABEADS

Different antibodies have different characteristics. Even different antibody clones raised in the same species against the same antigen can vary in pI, antigen binding affinity, and stability. Consequently the coupling efficiency will vary between different antibodies. Furthermore, some coupled antibodies will retain their function for months, even years when stored properly, while others will lose their function within weeks. This is entirely antibody dependent.

2.5 ANTIBODY QUANTITY

The amount of Dynabeads used for antibody coupling at any one time will depend upon the subsequent number and scale of co-immunoprecipitation samples to be run. Antibody coupling reactions should be scaled as outlined in **Table 1**. For best co-immunoprecipitation results, coupling of 5 µg to 7 µg antibodies per mg Dynabeads is recommended for high affinity antibodies. Coupling of more antibodies per mg Dynabeads may be necessary if the antibody affinity is poor. Typically, for co-immunoprecipitation of protein complexes with analysis by silver staining or Western blotting, 1.5 mg of antibody-coupled beads are used. For the detection by Coomassie staining, 7.5 mg of antibody coupled beads are used. (For more information on co-immunoprecipitation, see **Section 4**)

2.6 ANTIBODY AGGREGATES AND ANTIBODY LEAKAGE

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

3. ANTIBODY COUPLING PROTOCOL

Day 1

1) ***WEIGH*** out the appropriate amount of Dynabeads® M-270 Epoxy (according to **Table 1**).

Moisture on unused beads will deactivate the reactive groups necessary for covalent antibody coupling. **To avoid condensation on unused beads, make sure the beads are at RT prior to opening the bottle.**

TABLE 1: CALCULATION OF ANTIBODY AND C1 VOLUMES

Beads (mg)	Volumes in µl			
	Antibody	C1	C2	Total Reaction Volume
5	V	250 – V	250	500
10	W	500 – W	500	1000
20	X	1000 – X	1000	2000
40	Y	2000 – Y	2000	4000
60	Z	3000 – Z	3000	6000

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

2) ***WASH*** the beads: Add 1 ml of **C1** to the beads and mix by vortexing or pipetting.

3) ***PLACE*** the tube on a magnet and allow the beads to collect at the tube wall. Remove the supernatant.

4) ***ADD*** the appropriate volume of antibody + **C1** to the washed beads (**Table 1**) and mix by vortexing or pipetting.

Example: If you are coupling 5 mg Dynabeads and your required quantity of antibodies has a volume of 100 µl, you will need to add 150 µl of C1 (i.e. 250 µl C1 – 100 µl Ab = 150 µl.)

5) ***ADD*** the appropriate volume of **C2** and mix by vortexing or pipetting.

6) ***INCUBATE*** on a roller at 37°C overnight (16-24 hours). Make sure the fluid in the tube is mixing well.

Make sure the beads do not settle. Beads settling during the overnight incubation will result in inefficient antibody coupling.

Day 2

7) ***PLACE*** the tube on a magnet. Allow the beads to collect at the tube wall and remove the supernatant.

TABLE 2: REQUIRED BUFFER VOLUMES

Beads (mg)	Volumes in µl			
	HB	LB	SB	SB (for storage)
5	800	800	800	500
10	800	800	800	1000
20	1600	1600	1600	2000
40	1600	1600	1600	4000
60	1600	1600	1600	6000

8) ***HB WASH:*** Add 0.8 or 1.6 ml (**Table 2**) of **HB** and mix by vortexing or pipetting. Place the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant.

9) ***LB WASH:*** Add 0.8 or 1.6 ml (**Table 2**) of **LB** and mix by vortexing or pipetting. Place the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant.

10) ***SHORT SB WASH:*** Add 0.8 or 1.6 ml (**Table 2**) of **SB** and mix by vortexing or pipetting. Place the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant. Repeat the wash once more.

11) ***LONG SB WASH:*** Add 0.8 or 1.6 ml (**Table 2**) of **SB** and mix by vortexing or pipetting. Incubate on a roller/rotator at RT for 15 minutes. Place the tube on a magnet, allow the beads to collect at the tube wall, then remove the supernatant.

12) ***RESUSPEND*** beads in the same volume of **SB** as was the total coupling reaction volume and store at 4°C until use. The final bead concentration is **10 mg antibody coupled beads/ml.** ***Your beads are now covalently coupled with antibody and ready for Co-IP.***

If desired, antibody coupled beads may be concentrated up to 30 mg/ml by reducing the storage buffer volume.

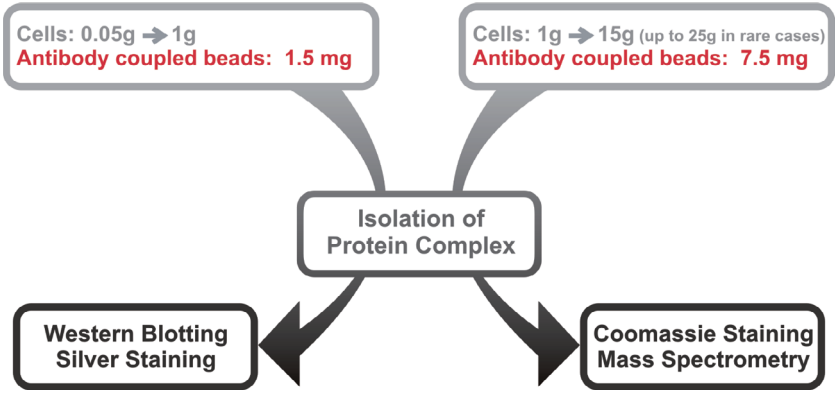
For long term storage, a final concentration of 0.02% NaN_3 may be added to the antibody coupled beads.

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

4. CO-IMMUNOPRECIPITATION CONSIDERATIONS

4.1 SCALE OF CO-IMMUNOPRECIPITATION

Co-immunoprecipitation for detection with Western blotting or silver staining methods requires 0.05 g to 1.5 g of cell sample and 1.5 mg of antibody coupled Dynabeads (when using 5 to 7 µg antibodies per mg beads as recommended in the **Antibody Coupling Considerations** in **Section 3**). For co-immunoprecipitation for detection with Coomassie staining or for Mass Spectrometry analysis, 1 g to 15 g (and in some rare cases up to 25 g) of cell sample and 7.5 mg of antibody coupled Dynabeads are necessary. **Note that the same amount of antibody-coupled beads is used despite varying amount of cell sample.** For high abundant proteins, use the lower scale of the cell sample recommended. For low abundant proteins, use the upper scale of the cell sample recommended.



4.2 CO-IMMUNOPRECIPITATION BUFFER SYSTEM

The co-immunoprecipitation buffer system provided in this kit has been optimized to achieve isolation of intact protein complexes while minimizing non-specific binding.

- **5 × IP**

  For co-immunoprecipitation, the cell sample needs to be resuspended in an **Extraction Buffer** (see **Section 4.4**) from which the desired protein complex is isolated. The **Extraction Buffer** is made with **5 × IP** supplied in the kit. The **5 × IP** is supplied as 5 × concentrate and in sufficient quantity to allow for 40 co-immunoprecipitation reactions using up to 1 g cells, or 8 reactions using up to 7.5 g cells. **For reproducible results, it is imperative that a 1:9 ratio of cell sample to Extraction Buffer is used for the co-immunoprecipitation each time**; e.g., 0.5 g cells needs 4.5 ml **Extraction Buffer.** (Note that cells should be weighed with as much liquid removed as possible)

Example: Weigh the empty tube in which the cell sample is to be collected. Do not assume an average weight of the tube. Centrifuge the cells in the tube, remove as much of the liquid as possible from the cells. Weigh the tube again. Calculate the weight of the cell pellet. **[Weight of tube + Cells] – [Weight of empty tube] = Weight of Cells**

Different protein complexes require different **Extraction Buffer** composition for successful co-immunoprecipitation. Currently there are no rules allowing prediction of buffer composition needed for isolation of particular protein complexes. **The optimal buffer composition needs to be empirically determined**. For instance, some protein complexes require the

presence of DTT and/or $MgCl_2$ to remain intact, while in other complexes these modifiers will disrupt the complex and/or increase non-specific binding. Increasing concentrations of salt and detergent in the **Extraction Buffer** will generally increase the stringency of the co-immunoprecipitation. To isolate intact protein complexes while minimizing non-specific binding, it is necessary to adjust the stringency of the **Extraction Buffer. The 5 × IP buffer must be diluted to 1 × prior to use**. At this stage, the buffer can be modified with various buffer modifiers to different stringencies. Note that several different buffer compositions can be used in the same experimental set up (see **Section 4.4** for more details).

- **5 × LWB**

  For added stringency, the bead-bound co-immunoprecipitated protein complex is washed once in the **Last Wash Buffer** (**LWB**) prior to elution. **5 × LWB** supplied in this kit is a 5 × concentrate of the **LWB**. **The 5 × LWB concentrate needs to be diluted to 1 × prior to use**. A final concentration of 0.02 % Tween 20 should be also added to the **LWB** for optimal performance.

  The **LWB** buffer also enables in-solution digestion of isolated protein complexes for direct Mass Spectrometry analysis (**Section 6.2**), or the extraction of nucleic acids (NA) after co-immunoprecipitation of DNA or RNA binding protein complexes (**Section 6.3**).

- **EB**

  For the elution of the isolated protein complex, the buffer **EB** is supplied in the kit. However, for large scale co-immunoprecipitations from which the isolated protein complex is to

be analyzed by Mass Spectrometry, elution in NH_4OH based buffer (**HPH EB**) followed by drying in a centrifugal vacuum concentrator (e.g. SpeedVac®, ThermoSavant Inc.) is strongly recommended (**Section 6.2**).

4.3 NOTES ON BUFFER ADDITIVES

- **SALTS AND DETERGENTS**

  Most salts and detergents are compatible with this kit, therefore may be used to modulate the **Extraction Buffer** (see **Section 4.4** for details).

- **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 that **EDTA is incompatible with many protein complexes**. Therefore, protease inhibitors that contain EDTA should be avoided. For most applications, a 1:200 to 1:500 dilution of 0.1 M phenylmethylsulphonyl fluoride (PMSF) will suffice.

- **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 di-sulfide 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.

4.4 CO-IMMUNOPRECIPITATION OPTIMIZATION

For simple co-immunoprecipitation, use **Extraction Buffer A** with 100 mM NaCl (see below Table). However 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.

1) Co-immunoprecipitate, using two different variants of **Extraction Buffer** (**A** and **B**).

Extraction Buffer A

Components	Final Conc.	Volume
5 x IP	1 x	
NaCl	100 mM	
Protease Inhib.		
H_2O		

Extraction Buffer B

Components	Final Conc.	Volume
5 x IP	1 x	
NaCl	100 mM	
$MgCl_2$	2 mM	
DTT	1 mM	
Protease Inhib.		
H_2O		

Note that initially, both buffers are modified with 100 mM NaCl. **Extraction Buffer B** is further modified with 2 mM $MgCl_2$ and 1 mM DTT.

Buffer Modifier Calculations:

$$\frac{[\ ]\text{ mM Final Conc.} \times [\ ]\text{ ml Total Buffer Vol.}}{[\ ]\text{ mM Stock Conc.}} = [\ ]\text{ ml Stock Used.}$$

Example: To make 10 ml **Extraction Buffer** with 100 mM NaCl final concentration using a 2.5 M stock NaCl, the formula should look like following:

100 mM NaCl × 10 ml total Buffer / 2500mM NaCl stock = 0.4 ml NaCl stock

2) Based on the results from **Step 1**, a choice between **Extraction Buffer A** or **B** can be made. The buffer that yields the most numerous and distinct bands should be chosen for further optimization (provided that your complex consists of several proteins). If there is no difference between the two buffers, then use both in **Step 2** in parallel.

   a) If you see too many bands and/or smeared bands, the buffer stringency is too low. In **Step 2**, use **Extraction Buffer A** or **B** (or both) with several different NaCl concentrations ranging from a 50 mM increment lower to several 50 mM increments higher (i.e. buffers with 50 mM, 100 mM, 150 mM, and 200 mM NaCl should be tested).

   b) If you see just your target protein and no other or very faint bands, the buffer stringency is too high. In **Step 2**, use **Extraction Buffer A** or **B** (or both) with several different NaCl concentrations ranging from a 25 mM increment higher to several 25 mM increments lower (ie buffers with 125 mM, 100 mM, 75 mM, 50 mM and 25 mM NaCl should be tested).

      Based on the results from the **Step 1**, the abundance of your target protein can also be determined and the cell sample adjusted for subsequent immunoprecipitations.

3) The co-immunoprecipitation results obtained using **Extraction Buffers** with varying NaCl concentrations should, when examined on gels, show a gradient of lanes varying from too few and/or faint bands to too many and/or smeared bands. The optimal **Extraction Buffer** can thus be identified. If not, then continue NaCl optimization until such a gradient is seen.

In most cases NaCl concentrations of 200 mM and less will be required. Only in very rare cases will the NaCl concentration exceed 200 mM.

For most protein complexes, optimization of detergents 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 that the **5 × IP** diluted to 1 × already contains 0.5 % Triton X-100 (detailed buffer formulations in **Section 6.4**).

4) After **Steps 2** and **3**, the following should have been determined:

   a) Choice between **Extraction Buffer A** or **B**

   b) Optimal NaCl concentration

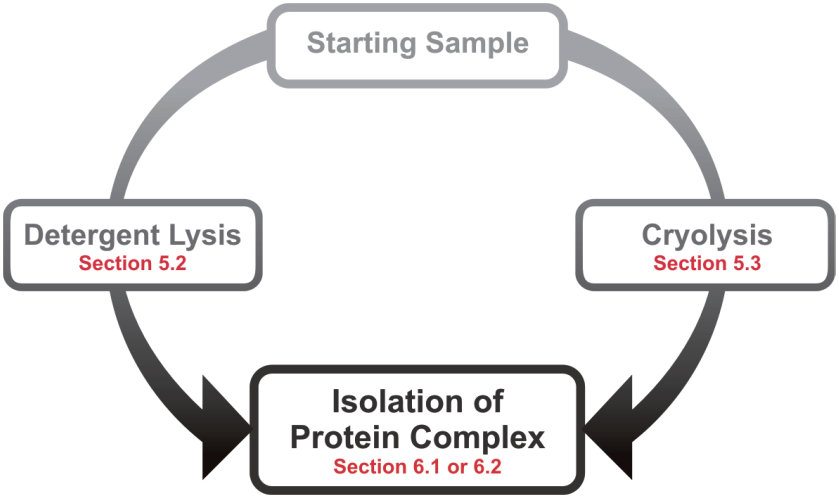
   For further optimization, the NaCl may be exchanged for KOAc. Since **Extraction Buffer A** or **B** already contains 110 mM KOAc (detailed buffer formulations in **Section 6.4**), KOAc increments of 10 mM should be tested (e.g. 110 mM, 120 mM, 130 mM and 140 mM). Once again, the co-immunoprecipitation results with **Extraction Buffers** with different KOAc concentrations should show a gradient of lanes which varies from too few and/or faint bands to too many and/or smeared bands from which the optimal condition may be identified.

5. CELL SAMPLE PREPARATION

5.1 PREPARATION OF SAMPLES FOR CO-IMMUNOPRECIPITATION

The most appropriate method for the preparation of samples for co-immunoprecipitation will depend 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 N_2 will stabilize labile complexes during the cryolysis process. Cryolysis protocols are applicable for all cell types. For further information on the recommended cryolysis procedure, please see **References 1 to 4**.

FIGURE 2



5.2 DETERGENT LYSIS METHOD – MAMMALIAN CELL CULTURE SAMPLES

Cell samples prepared using detergent lysis should be used immediately for co-immunoprecipitation. Make sure the antibody coupled beads from **Section 3** and the co-immunoprecipitation 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 tables below. 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) ***WASH*** harvested cells once in PBS by centrifuging at 200 to 500 × *g* for 5 min at 4°C.

3) ***RESUSPEND*** cells in 1:9 ratio of cell mass to **Extraction Buffer** with protease inhibitors. Incubate on ice for 15 min. See **Section 4.2** for details for calculation.

Example: 50 mg cells are lysed in 450 µl buffer.

4) ***CENTRIFUGE*** at 2600 × *g* for 5 min at 4°C to remove large cell debris and nuclei.

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

5.3 CRYOLYSIS METHOD – ALL TYPES OF CELL SAMPLES

A large batch of cell sample that can be used for multiple pull-down experiments can be prepared at once using this method. Approximately 6 L yeast or bacteria 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. Make sure all buffers and cells are kept on ice during the procedure. For the cryogenic freezing of the harvested cell sample, ~ 1 to 2 L liquid N_2 is needed. For tissue samples (e.g. liver) or whole organisms (e.g. Drosophila), weigh the sample directly and freeze in liquid N_2.

Use appropriate protective gear when working with liquid N_2. Contact your local environmental health work place safety officer for more information.

5.3.1 PREPARATION OF CELL SAMPLES FOR CRYOLYSIS

1) ***CENTRIFUGE*** 6 L yeast or bacteria culture or 8 L (or equivalent amount) mammalian or insect cell culture. Pellet 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 2; go directly to Step 3.**

2) ***WASH*** the cells in 50 ml H_2O. Centrifuge yeast and bacteria cells at 2600 × *g* for 5 min at 4°C, then discard the supernatant.