Universal Magnetic Co-IP Kit

(version A3)

Catalog No. 54002

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Introduction

Co-Immunoprecipitation (Co-IP) is a powerful method used to study protein/protein interactions. In Co-IP, one antibody is used to immunoprecipitate a target antigen, which at the same time co-precipitates any other proteins that are bound to/interacting with the first protein. These protein complexes are then captured by antibody binding beads (such as Protein A or G). This is followed by elution of the antibody-bound proteins, SDS-PAGE and Western blot using a second antibody that is targeted against one of the bound, interacting proteins.

However, traditional methods for performing Co-IP are not optimal for studying DNA-binding protein complexes as the complexes are often disrupted during the extraction process. And, high background has often necessitated the use of high-stringency buffers that, while reducing background, can destroy the protein complexes before they have been immunoprecipitated. In addition, traditional high-salt extraction procedures do not remove histone proteins from DNA.

Because of this, Active Motif's Universal Magnetic Co-IP Kit has been developed to enable the preparation and immunoprecipitation of protein complexes from both nuclear and whole-cell extracts, beginning from either cell or tissue samples. The kit utilizes protein G-coated magnetic beads that improve Co-IP by simplifying the IP and wash steps while providing a much lower background. This reduced background enables the use of a low-stringency Co-IP/Wash Buffer that helps maintain the protein complexes. To further improve Co-IP of nuclear complexes, the kit's proprietary Enzymatic Shearing Cocktail uses DNA digestion to release undissociated protein complexes from the DNA. Even histone complexes are released, and they remain intact. Moreover, all extraction and IP steps, both nuclear and whole-cell, are performed in buffers supplemented with Protease, Phosphatase and Deacetylase Inhibitors, which limits further protein modifications (expression, proteolysis, dephosphorylation, *etc.*), thereby preserving the *in vivo* states of the immunoprecipitated protein complexes.

Each Universal Magnetic Co-IP Kit provides sufficient reagents to perform 25 nuclear and 25 whole-cell extractions, and then to perform 25 co-immunoprecipitations. The kit includes optimized nuclear and whole-cell protein complex extraction buffers, inhibitor solutions, immunoprecipitation/wash buffer, Magnetic Protein G Beads, a powerful Bar Magnet for creating a magnetic stand and a comprehensive protocol to provide a simplified method to capture and detect protein complexes.

product	format	catalog no.
Universal Magnetic Co-IP Kit	25 rxns	54002

Kit Performance

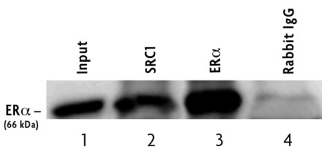


Figure 1: Nuclear Co-IP of SRC-1 and ERα.

The Universal Magnetic Co-IP Kit was used to make nuclear extract from MCF-7 cells induced 1 hour with 10 nM Estradiol. IP was performed on 300 µg samples using 2 µg of SRC-1 pAb, ERα pAb and rabbit IgG (as a negative control). Western blot was then performed using the ERα pAb on 10 µg Input Extract, SRC-1 IP, ERα IP and the rabbit IgG IP.

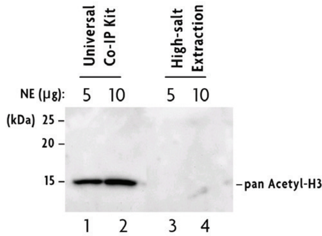


Figure 2: Detection of acetylated Histone H3.

HeLa nuclear extracts were made using the Universal Magnetic Co-IP Kit and a traditional high-salt extraction protocol, supplemented with 1 µM trichostatin A, a deacetylase inhibitor. Five and ten µg samples of each extract were used in Western with Histone H3 acetyl rabbit pAb (Cat. No. 39139). Protein was detected only in samples made using the kit's nuclear extraction procedure, as it was designed to release histone and other protein complexes from DNA while preserving modifications.

Kit Components and Storage

Kit components can be stored at -20°C prior to first use. Then, we recommend storing each component at the temperatures recommended in the table below:

Reagents	Quantity	Storage / Stability
10X PBS	80 ml	4°C for 6 months
10X Hypotonic Buffer	50 ml	4°C for 6 months
Whole-cell Lysis Buffer	10 ml	-20°C for 6 months
Co-IP/Wash Buffer	100 ml	4°C for 6 months
Detergent	3 ml	4°C for 1 year
Phosphatase Inhibitors	40 ml	4°C for 6 months
Protease Inhibitor Cocktail	500 µl	-20°C for 6 months
Deacetylase Inhibitor	5 ml	-20°C for 6 months
100 mM PMSF	500 µl	-20°C for 6 months
0.5 M EDTA	200 µl	4°C for 6 months
Digestion Buffer	10 ml	4°C for 6 months
Enzymatic Shearing Cocktail	50 µl	-20°C for 6 months
Protein G Magnetic Beads*	650 µl	4°C for 6 months
Bar Magnet	1	Room temperature
Mini Glue Dots	1 sheet	Room temperature

* The Protein G Magnetic Beads are shipped on dry ice, but should not be re-frozen by the customer. Upon receipt, the beads should be stored at 4°C.

Additional materials required

- 5 and 10 ml pipettes and Pipettors
- Cell scraper
- 15 ml conical tubes
- Microcentrifuge tubes
- Centrifuge (with swinging buckets adapted to 15 ml conical tubes) and microcentrifuge pre-cooled to 4°C
- Rocking platform
- Distilled water
- Apparatus to rotate tubes end-to-end at 4°C (for co-immunoprecipitation)
- Antibodies for IP and Western blot
- 37°C water bath
- Reagents and equipment for performing SDS-PAGE and Western blot

Protocols – Nuclear Extraction

Buffer Preparation and Recommendations

Note: The Phosphatase Inhibitors solution should be a clear yellow color. It may precipitate during storage and become turbid in appearance. If this occurs, heat at 50°C for 10 minutes before using it in the solutions below. Phosphatase Inhibitors, PMSF and PIC lose activity 24 hours after dilution. Therefore, use all of the solutions below on the same day prepared, then discard any remaining solution.

Preparation of PBS/Inhibitors (8 ml/plate)

To make nuclear extract from a 100 mm plate of cells, prepare 8 ml of PBS/Inhibitors solution as follows: mix 0.8 ml 10X PBS in 6.72 ml distilled water, then add 0.4 ml Phosphatase Inhibitors and 80 µl Deacetylase Inhibitor. Vortex to mix and place on ice. (This buffer is used for both nuclear and whole-cell extractions.)

Preparation of Complete Hypotonic Buffer (500 µl/plate)

To make nuclear extract from a 100 mm plate of cells, prepare 500 µl Complete Hypotonic Buffer as follows: mix 50 µl 10X Hypotonic Buffer, 415 µl distilled water, 25 µl Phosphatase Inhibitors, 5 µl Deacetylase Inhibitor, 2.5 µl Protease Inhibitor Cocktail (PIC) and 2.5 µl 100 mM PMSF. Vortex to mix and place on ice.

Preparation of Complete Digestion Buffer (100 µl/plate)

To make nuclear extract from a 100 mm plate of cells, prepare 100 µl of Complete Digestion Buffer as follows: mix 93 µl Digestion Buffer with 5 µl Phosphatase Inhibitors, 1 µl Deacetylase Inhibitor, 0.5 µl Protease Inhibitor Cocktail (PIC) and 0.5 µl 100 mM PMSF. Vortex to mix and place on ice.

Quick Chart for Preparing Nuclear Extraction Buffers

Reagents to Prepare	Components	60 mm plate or 3.2 x 10^6 cells	100 mm plate or 8.8 x 10^6 cells	150 mm plate or or 2 x 10^7 cells
PBS/Inhibitors	10X PBS	0.4 ml	0.8 ml	1.6 ml
	Distilled water	3.36 ml	6.72 ml	13.44 ml
	Phosphatase Inhibitors	0.2 ml	0.4 ml	0.8 ml
	Deacetylase Inhibitor	40.0 µl	80.0 µl	160.0 µl
	TOTAL REQUIRED	**4.0 ml**	**8.0 ml**	**16.0 ml**
Complete Hypotonic Buffer	10X Hypotonic Buffer	25.0 µl	50.0 µl	100.0 µl
	Distilled water	207.5 µl	415.0 µl	830.0 µl
	Phosphatase Inhibitors	12.5 µl	25.0 µl	50.0 µl
	Deacetylase Inhibitor	2.5 µl	5.0 µl	10.0 µl
	Protease Inhibitor Cocktail	1.25 µl	2.5 µl	5.0 µl
	100 mM PMSF	1.25 µl	2.5 µl	5.0 µl
	TOTAL REQUIRED	**250.0 µl**	**500.0 µl**	**1.0 ml**
Complete Digestion Buffer	Digestion Buffer	46.5 µl	93.0 µl	186.0 µl
	Phosphatase Inhibitors	2.5 µl	5.0 µl	10.0 µl
	Deacetylase Inhibitor	0.5 µl	1.0 µl	2.0 µl
	Protease Inhibitor Cocktail	0.25 µl	0.5 µl	1.0 µl
	100 mM PMSF	0.25 µl	0.5 µl	1.0 µl
	TOTAL REQUIRED	**50.0 µl**	**100.0 µl**	**200.0 µl**

Vortex/mix thoroughly and keep the solutions on ice.

Nuclear Extraction from Cells

The following protocol is based samples of approximately 8.8 x 10^6 cells, which corresponds to HeLa cells grown to confluence in a 100 mm tissue culture plate. Each sample is one extraction reaction. Prepare PBS/Inhibitors, Complete Hypotonic and Complete Digestion Buffers as described above. Adjust the volumes accordingly using the chart above if using plates of different sizes. Place buffers and any tubes needed on ice before beginning.

Step 1: Cell Collection

1. After growing and inducing/treating your cells as desired, aspirate the media out of the plate. Wash with 5 ml ice-cold PBS/Inhibitors. Aspirate solution out, then add 3 ml ice-cold PBS/Inhibitors.
2. Remove cells from dish by gently scraping with cell lifter. Transfer cells to a pre-chilled 15 ml conical tube.

3. Centrifuge cell suspension for 5 minutes at 1500 rpm in a centrifuge pre-cooled at 4ºC.

4. Discard supernatant. Keep cell pellet on ice. At this point the protocol can be continued or the pellet can be frozen at -80°C.

Step 2: Isolation of the Nuclei

1. Gently resuspend cell pellet in 500 µl freshly prepared (same day) Complete Hypotonic Buffer by pipetting up and down several times. Transfer to a pre-chilled microcentrifuge tube. Incubate on ice for 15 minutes.

2. Add 25 µl Detergent and gently pipet up and down 3-5 times to mix.

3. Centrifuge suspension for 30 seconds at 14,000 x *g* in a microcentrifuge pre-cooled at 4ºC.

4. Discard supernatant (cytoplasmic fraction). Keep the pellet (nuclear fraction) on ice.

Step 3: Nuclear Fraction Digestion and Collection

1. Resuspend nuclear pellet in 100 µl freshly prepared (same day) Complete Digestion Buffer by pipetting up and down 3-5 times.

2. Add 0.5 µl of the Enzymatic Shearing Cocktail. Vortex gently for 2 seconds.

3. Incubate suspension for 10 minutes at 37°C water bath. Vortex the sample gently every 2-3 minutes during this incubation step.

4. Add 2 µl 0.5 M EDTA to stop the reaction. Vortex gently on a low setting for 2 seconds and place on ice for 5 minutes.

5. Centrifuge for 10 minutes at 14,000 x *g* in a microcentrifuge pre-cooled at 4ºC. Transfer the supernatant into a pre-chilled microcentrifuge tube.

6. Save a small aliquot for protein quantification by a Bradford-based assay. Aliquot and store at -80ºC. Avoid freeze/thaw cycles.

   Note: The presence of certain detergents may interfere with protein determination assays, such as Bradford or BCA. We therefore recommend using the Complete Digestion Buffer as the blank and performing a 1:50 or 1:250 dilution of your samples. Alternatively, try Active Motif's ProStain™ Protein Quantification Kit, which is ideal for quantifying extracts because it offers greater sensitivity and resistance to many contaminating reagents used in extract preparation.

Nuclear Extraction from Tissue

Note: We recommend using only fresh tissue samples, as nuclear extraction with frozen tissue is very inefficient. If you have frozen tissue, we suggest you use the Whole-cell Extraction from Tissue protocol on page 8.

Step 1: Tissue Homogenization

1. Weigh tissue and dice into very small pieces using a clean razor blade. Collect pieces in a pre-chilled, clean Dounce homogenizer.
2. On ice, add 3 ml ice-cold Complete Hypotonic Buffer per gram of tissue and homogenize in the Dounce. Incubate on ice for 15 minutes.
3. Centrifuge for 10 minutes at 850 x *g* at 4°C.
4. At this point, the tissue is homogenized. However, most of the cells are not yet lysed. Therefore, discard the supernatant and continue with the pellet at Step 2, No. 1 of the Nuclear Extraction Procedure from Cells (page 5), based on a 150 mm plate (2 x 10^7 cells).

Protocols – Whole-cell Extraction

Buffer Preparation and Recommendations

Note: The Phosphatase Inhibitors solution should be a clear yellow color. It may precipitate during storage and become turbid in appearance. If this occurs, heat at 50°C for 10 minutes before using it in the solutions below. Phosphatase Inhibitors, PMSF and PIC lose activity 24 hours after dilution. Therefore, use all of the solutions below on the same day prepared, then discard any remaining solution.

Preparation of PBS/Inhibitors (8 ml/plate)

To make whole-cell extract from a 100 mm plate of cells, prepare 8 ml of PBS/ Inhibitors solution as follows: mix 0.8 ml 10X PBS in 6.72 ml distilled water, then add 0.4 ml Phosphatase Inhibitors and 80 µl Deacetylase Inhibitor. Vortex to mix and place on ice. (This buffer is used for both whole-cell and nuclear extractions.)

Preparation of Complete Whole-cell Lysis Buffer (100 µl/plate)

To make whole-cell extract from a 100 mm plate of cells, prepare 100 µl Complete Whole-cell Lysis Buffer as follows: mix 93 µl Whole-cell Lysis Buffer, 5 µl Phosphatase Inhibitors, 1 µl Deacetylase
Inhibitor, 0.5 µl Protease Inhibitor Cocktail (PIC) and 0.5 µl 100 mM PMSF. Vortex to mix and place on ice.

Quick Chart for Preparing Whole-cell Extraction Buffers

Reagents to Prepare	Components	60 mm plate or 3.2 x 10^6 cells	100 mm plate or 8.8 x 10^6 cells	150 mm plate or or 2 x 10^7 cells
PBS/Inhibitors	10X PBS	0.4 ml	0.8 ml	1.6 ml
	Distilled water	3.36 ml	6.72 ml	13.44 ml
	Phosphatase Inhibitors	0.2 ml	0.4 ml	0.8 ml
	Deacetylase Inhibitor	40.0 µl	80.0 µl	160.0 µl
	TOTAL REQUIRED	**4.0 ml**	**8.0 ml**	**16.0 ml**
Complete Whole-cell Lysis Buffer	Whole-cell Lysis Buffer	46.5 µl	93.0 µl	186.0 µl
	Phosphatase Inhibitors	2.5 µl	5.0 µl	10.0 µl
	Deacetylase Inhibitor	0.5 µl	1.0 µl	2.0 µl
	Protease Inhibitor Cocktail	0.25 µl	0.5 µl	1.0 µl
	100 mM PMSF	0.25 µl	0.5 µl	1.0 µl
	TOTAL REQUIRED	**50.0 µl**	**100.0 µl**	**200.0 µl**

Vortex/mix thoroughly and keep the solutions on ice.

Whole-cell Extraction from Cells

The following protocol is based on samples of approximately 8.8 x 10^6 cells, which corresponds to HeLa cells grown to confluence in a 100 mm tissue culture plate, and will yield approximately 0.20-0.35 mg of protein. Each sample is one extraction reaction. Prepare PBS/Inhibitors and Complete Whole-cell Lysis Buffer as described above. Adjust volumes accordingly using the chart above if using plates of different sizes. Place buffers and all tubes needed on ice before beginning.

Step 1: Cell Collection

1. After growing and inducing/treating your cells as desired, aspirate the media out of the plate. Wash with 5 ml ice-cold PBS/Inhibitors. Aspirate solution out, then add 3 ml ice-cold PBS/Inhibitors.
2. Remove cells from dish by gently scraping with cell lifter. Transfer cells to a pre-chilled 15 ml conical tube.
3. Centrifuge cell suspension for 5 minutes at 1500 rpm in a centrifuge pre-cooled at 4ºC.
4. Discard supernatant. Keep cell pellet on ice. At this point the protocol can be continued or the pellet can be frozen at -80°C.

Step 2: Whole-cell Extraction

1. Resuspend the cell pellet in 100 µl freshly prepared (same day) Complete Whole-cell Lysis Buffer and vortex vigorously for 10 seconds.
2. Incubate on ice for 30 minutes, or on a rotating platform at 4°C for 30 minutes.
3. Vortex vigorously for 10 seconds.
4. Centrifuge for 10 minutes at full speed in a in a centrifuge pre-cooled at 4ºC. Transfer the supernatant into a pre-chilled microcentrifuge tube.
5. Save a small aliquot for protein quantification by a Bradford-based assay. Aliquot and store at -80ºC. Avoid freeze/thaw cycles.

   Note: The presence of certain detergents may interfere with protein determination assays, such as Bradford or BCA. We therefore recommend using the Complete Whole-cell Lysis Buffer as the blank and performing a 1:50 or 1:250 dilution of your samples. Alternatively, try Active Motif's ProStain™ Protein Quantification Kit, which is ideal for quantifying extracts because it offers greater sensitivity and resistance to many contaminating reagents used in extract preparation.

Whole-cell Extraction from Tissue

1. Weigh tissue and dice into very small pieces using a clean razor blade. Collect pieces in a pre-chilled 15 ml conical tube.
2. On ice, disrupt and homogenize tissue in 3 ml ice-cold Complete Whole-cell Lysis Buffer per gram of tissue with a pre-chilled, clean Dounce homogenizer or a Polytron device. Maintain temperature at 4°C throughout all procedures. Incubate on ice for 30 minutes.

   Note: Frozen tissue can be sliced very thinly and thawed in this buffer prior to homogenization with the Dounce. When using a mechanical homogenizer, begin homogenization at slow speeds until the tissue is broken into smaller pieces and then increase the speed to the maximum for 45-60 seconds. Avoid the generation of excess heat or foam.

3. Transfer to pre-chilled microcentrifuge tubes, centrifuge at 10,000 x *g* for 10 minutes at 4°C.
4. Transfer supernatants to new pre-chilled tubes and centrifuge again. Pool supernatants in a single tube. The supernatant fluid is the whole-cell lysate. Sometimes a longer centrifugation is necessary to obtain a clarified lysate.
5. Save a small aliquot for protein quantification by a Bradford-based assay. Aliquot and store at -80ºC. Avoid freeze/thaw cycles.

   Note: The presence of certain detergents may interfere with the Bradford or BCA assay, thus use the Complete Whole-cell Lysis Buffer as the blank and perform a 1:50 or 1:250 dilution of your samples. As an alternative, try Active Motif's ProStain™ Protein Quantification Kit, which offers greater sensitivity and resistance to many contaminating agents.

Buffer Preparation and Recommendations

Preparation of Complete Co-IP/Wash Buffer (500 µl/sample for Co-IP; 2 ml/sample for washes)

Complete Co-IP/Wash Buffer is used for both the Co-IP and washing steps, and this same buffer is used whether your sample is nuclear or whole-cell extract. Because the Phosphatase Inhibitors, PMSF and PIC lose their activity 24 hours after dilution, you must use this buffer on the same day it is made. Therefore, plan your experiment so that the Co-IP incubation and wash steps can be performed on the same day. Use the chart below to prepare sufficient Complete Co-IP/Wash Buffer for the number of samples being processed:

Reagents to Prepare	Components	1 Co-IP	5 Co-IP's
Complete Co-IP/Wash Buffer	Co-IP/Wash Buffer	2.325 ml	11.625 ml
	Phosphatase Inhibitors	125.0 µl	625.0 µl
	Deacetylase Inhibitor	25.0 µl	125.0 µl
	Protease Inhibitor Cocktail	12.5 µl	62.5 µl
	100 mM PMSF	12.5 µl	62.5 µl
	TOTAL REQUIRED	**2.5 ml**	**12.5 ml**

Vortex to mix thoroughly and keep the Complete Co-IP/Wash Buffer on ice.

Increasing stringency (optional)

The Protein G Magnetic Beads included in the Universal Magnetic Co-IP Kit were chosen because they exhibit a very small degree of non-specific binding. This helps ensure very low background, enabling the Complete Co-IP/Wash Buffer used in the kit to be of low stringency, which better preserves fragile protein complexes.

Particular cell lines, stimulations or antibodies may result in higher background. If your experiment has more background than is optimal, increase the number of washes performed from 4 to 6.

In extreme cases, increase the stringency of the Complete Co-IP/Wash Buffer used during the Wash steps. Keep in mind, however, that this may disrupt the protein complexes, causing loss of co-immunoprecipitated proteins. (Do not increase the stringency of this buffer for the Co-IP.)

You can increase the stringency of the Complete Co-IP/Wash Buffer by adding 5 M NaCl (not supplied) and/or additional Detergent (included). Add up to a maximum final concentration of 150 mM NaCl and/or up to a maximum final concentration of 0.5% Detergent. Co-IP/Wash Buffer as supplied contains 50 mM NaCl and 0.1% Detergent (IGEPAL CA-630, an NP-40 equivalent). We recommend that you begin at the lower end of the ranges and increase concentrations as needed.

Co-Immunoprecipitation Protocol

IMPORTANT: Please read the previous section, Co-IP – Buffer Preparation and Recommendations before starting the assay. It is highly recommended that all Co-IP incubation and wash steps be performed on the same day. When possible, all IP steps should be performed on ice. The protocol below provides volumes sufficient for one IP experiment. It is recommended that you perform, in addition to your samples, a Negative Control IP with Rabbit or mouse IgG, (use the same species as that of the primary antibody you are using for IP). This will help identify non-specific binding.

Step 1: Preparation of Antibody/Extract mixture

1. Combine 100-500 µg nuclear or whole-cell extract and 1-5 µg antibody in a final volume of 500 µl Complete Co-IP/Wash Buffer in a pre-chilled 1.5 ml microcentrifuge tube. Keep on ice.
2. Incubate for 1-4 hours at 4°C on a rotator or a rolling shaker (*e.g.* a Labquake from Barnstead/Thermolyne with a tube holder for microcentrifuge tubes).
3. Centrifuge for 30 seconds at 4000 rpm in a in a centrifuge pre-cooled at 4ºC to collect liquid from the inside of the cap.

Step 2: Addition and Incubation of Magnetic Protein G Beads

Note: Protein G Magnetic Beads are shipped on dry ice but should not be re-frozen. Upon receipt, store the beads at 4ºC. The beads are ready to use following complete resuspension to a homogeneous slurry. For best results, gently shake and roll the tube. The beads settle quickly, thus resuspend just before pipetting. If performing a large number of reactions, you may need to resuspend the beads more than one time while adding them to your samples.

4. Add 25 µl Protein G Magnetic Beads to each tube and incubate for 1 hour at 4°C on a rotator.
5. Centrifuge for 30 seconds at 4000 rpm in a in a centrifuge pre-cooled at 4ºC to collect liquid from the inside of the cap.

Step 3: Washing the Magnetic Protein G Beads

See Appendix A for more complete information on washing the magnetic beads and assembling a magnetic stand using the Bar Magnet and Sticky Dots that are supplied in the kit.

6. Place tube on magnetic stand to pellet beads on the side of the tube.
7. Carefully remove and discard supernatant.
8. Add 500 µl Complete Co-IP/Wash Buffer and resuspend pellet completely by pipetting up and down several times. Take care that the beads are not clinging to the pipet tips after pipetting. You may need to move the tubes away from the magnet before resuspension.
9. Carefully remove and discard supernatant.
10. Add 500 µl of Complete Co-IP/Wash Buffer. Repeat this wash step for a total of 4 washes. After final wash, remove as much supernatant as possible using a P200 Pipetteman.

11. Resuspend each bead pellet in 20 µl of 2X Reducing Loading Buffer (130 mM Tris pH 6.8, 4% SDS, 0.02% Bromophenol blue, 20% glycerol, 100 mM DTT). You can vortex the samples to mix thoroughly. Samples can be stored at -20°C, or you can run Westerns immediately.

Western Blotting Protocol

For your convenience, a brief Western blotting protocol is provided below. When performing Western blot, individual optimization of antigen and antibody quantities may be required in order to detect the protein of interest, and you may have Western/blotting protocols that are more appropriate for you antibodies. It is recommended that in addition to your samples (and the negative control IP you performed) that you run include an Input control lane that contains 5 µg of the nuclear or whole-cell extract sample that was used for Co-IP. Resuspend the 5 µg of extract in a final volume of 10 µl distilled water, then add 10 µl of 2X Reducing Loading Buffer.

Electrophoresis

1. Boil all samples at 95-100°C for 3-5 minutes. Centrifuge for 30 seconds at full speed. Transfer to a fresh tube.
2. Load 10 µl of each sample and a molecular weight marker onto an appropriate SDS-PAGE gel and run until migration front reaches bottom of gel. (For weaker interactions, it may be necessary to load all 20 µl of the sample in order to see the protein band.)

Transfer & Blocking

3. Just before the end of electrophoresis, incubate a polyvinylidene difluoride (PVDF) membrane in 100% methanol for 30 seconds. Rinse the membrane in distilled water, then incubate it in Transfer Buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 15% methanol).
4. Transfer protein to the PVDF membrane (1.5 hours at 30 V, 170 mA max) using Transfer Buffer.
5. Incubate membrane 30 seconds in 100% methanol, then wash in distilled water for 5 minutes.
6. Incubate the membrane in PBS + 0.1% Tween 20 (PBST) + 3% dry, non-fat skim milk powder for 1 hour at room temperature.

Incubation with Primary & Secondary Antibodies

7. Dilute the primary antibody (at the appropriate dilution) in PBST + 3% milk for 1.5-2 hours at room temperature. Wash the membrane 2 times for 10 minutes each in PBST.
8. Incubate membrane with HRP-conjugated secondary antibody for 1 hour at room temperature. The dilution of the secondary antibody conjugate will vary according to manufacturer's specifications. Wash the membrane 3 times for 10 minutes each in PBST.

Chemiluminescence Reaction

9. Prepare and use the chemiluminescent substrate according to the manufacturer's instructions.

Appendix

Section A. Use of Magnetic Beads and Included Bar Magnet

Caution: The included neodymium bar magnet is extremely powerful and is easily broken if handled incorrectly.

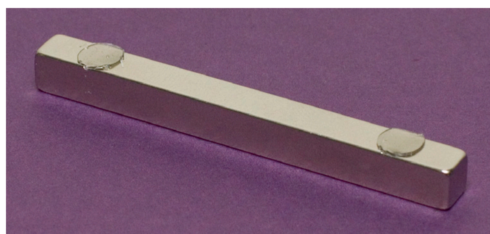
1. The magnet should be stored in the provided tube.
2. Be careful when working near metal objects or surfaces. A free magnet will jump great distances onto nearby metal objects with surprising speed/force. This can break the magnet.
3. If the magnet becomes attached to a flat metal surface, it should be removed by sliding it off the edge of surface. The magnet may be broken if you attempt to pull one end away from the metal.

Assembly of Magnetic Stands

The provided Mini Glue Dots can be used to attach the bar magnet to an empty tip box to create an effective magnet stand. Commercially available magnetic stands can also be used.

Creating a magnetic stand for microcentrifuge tubes:

1. Remove the covering tape from one side of two glue dots.
2. Place two microcentrifuge tubes in the wells of an empty tip box (1000 µl) and place the magnet directly against the tubes. This is the way the magnet will be positioned when the glue dots are used to affix it to the box.
3. Attach the glue dots on the bar magnet (the uncovered face of the dot is placed on the magnet) as shown below.



4. Remove the covering tape from the exposed side of the glue dot. Fix the magnet to the tip box so that it is against the tubes. The magnetic stand is now ready for use.

   Note: The microcentrifuge tubes are held less securely in this assembled tube stand than in a typical commercially available magnetic stand. This is not a problem if the below washing protocol is followed. That is, work with 1 tube at a time, and keep the tubes in the standard tube rack unless you are holding the tube next to the magnet.

Washing the Protein G Magnetic Beads

Washing is best performed one tube at a time, and should be performed as follows:

1. Place the tube in a standard microcentrifuge tube rack and open the cap.
2. Place the opened tube in the assembled magnetic stand. The beads will pellet more rapidly if the bottom of the tube is held against the magnet, as shown below, and then slowly lowered into the well. This will pellet the beads up onto the side of the tube.

3. Allow the beads to pellet completely and remove supernatant with a 1000 µl pipetteman. You can either leave the tube in the rack or pull it out when you remove the buffer. The beads will remain on the side of the tube, even when not next to the magnet.
4. Return the tube to the standard microcentrifuge tube back, add 500 µl Complete Co-IP/Wash Buffer and resuspend pellet completely by pipetting up and down several times.
5. Repeat steps 2-4 until desired washing steps are complete. After the final wash has been removed, the last traces of wash buffer should be removed with a 200 µl pipetteman.

Section B. Troubleshooting Guide

Problem/question	Possible Cause	Recommendation
No or low signal of IP'd proteins by Western blot	The level of expression of the proteins of interest are very low.	Confirm presence of proteins of interest in the extract. Alternatively, use more extract in the IP reaction (up to 500 µg per IP).
	Target proteins lost or destroyed during sample preparation.	Confirm presence of proteins of interest in the extract.
	The protein/protein interaction of interest is dependent on specific induction conditions.	Optimize conditions of culture and induction.
	The protein/protein interaction of interest is not stable and dissociates during the extraction process.	Perform the Enzymatic Shearing Cocktail incubation (Step 3, No. 3) for 90 minutes at 4°C instead of 10 minutes at 37°C.
	Amount of antibody used in the assay is too low.	Use more antibody in IP and/or WB; perform a titration to determine how much.
	Antibodies used in IP and/or WB are not suitable for these applications.	It is important to perform IP and WB controls, as antibodies raised against denatured antigen and native epitopes may not be recognized. For an IP Positive Control, use the same antigen of interest for IP and WB. For a WB Positive Control, perform a direct WB analysis of the extract.
	Large number of competing proteins present in the sample.	Centrifuge the extract at 20,000 x *g* for 20 minutes before adding antibody. This should remove insoluble aggregates.
	Interfering substances present in the extract.	Use the appropriate amount of inhibitors when performing the assay.
High background signal	Too much antibody used in IP and/or WB.	Titrate antibody concentrations and decrease primary antibody dilution or incubation time.
	Non-specific binding of other proteins to the Magnetic Protein G Beads or antibodies.	Preclear extracts with Magnetic Protein G Beads prior to addition of the primary antibody.
	Wash steps are not sufficiently stringent.	See "Increasing stringency (optional)" on page 9 for details.
	Aggregated proteins in extracts.	Spin extracts at 20,000 x *g* for 20 minutes and discard pellet prior to addition of antibody.
	IgG heavy chains appear at 55 kDa, light chains appear at 28 kDa.	Use different antibody origins for IP and WB.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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