Analysis of protein phosphorylation using Phos-tag gels

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Significance statement

Protein phosphorylation is a versatile post-translational modification which can alter the activity, localization or stability of proteins by modifying their conformation or interaction potential. The Phos-tag technology provides a simple small-scale approach to study protein phosphorylation and is compatible with traditional vertical slab minigel systems. In addition, by offering information on the ratio of phosphorylated versus non-phosphorylated protein species, Phos-tag gels enable comprehensive characterization of protein phosphorylation. The technology is becoming increasingly popular because it can complement studies using phosphorylation site-specific antibodies or phosphoproteomics, where information on phosphorylation site occupancy is not readily available.

Abstract

Phos-tag gels are recent tools to dissect protein phosphorylation which operate by inducing a shift in the electrophoretic mobility of phosphorylated proteins compared to their non-phosphorylated counterparts. This unit describes the preparation and electrophoresis of Zn^{2+}-Phos-tag gels (Basic Protocol 1) along with electrotransfer of the separated phospho-, and non-phosphoproteins onto a PVDF membrane using either wet-tank (Basic Protocol 2) or semidry transfer (Alternate Protocol 1). We also discuss the theory behind the technology with critical parameters to keep in mind for its successful application.

Keywords

Phos-tag, phosphorylation, phosphate-affinity electrophoresis, protein kinase

Introduction

Phos-tag gels are used to analyze the phosphorylation status of proteins. Phos-tag is a dinuclear metal complex which binds to phosphate monoesters with high affinity under neutral pH conditions in aqueous solution (Kinoshita, Takahashi, Takeda, Shiro, & Koike, 2004). Phos-tag acrylamide, a derivative of the phosphate capture molecule conjugated to acrylamide, can be included into polyacrylamide gels to reduce the electrophoretic mobility of phosphorylated proteins compared to their non-phosphorylated counterparts. The polyacrylamide-immobilized Phos-tag complex leads to slower migration of phosphoproteins during electrophoresis by reversibly binding to phosphate moieties on them.

Differentially phosphorylated protein species can be detected simultanously as multiple migration bands in the same lane, provided that specific pan (phosphorylation independent) antibodies are available. Even phosphoprotein species containing the same number of phosphate groups on different sites can be separated from each other. A great advantage of Phos-tag technology is that it provides information on the quantity of phosphorylated species relative to non-phosphorylated protein (Figure 1), which is not available with the traditional [^{32}P]orthophosphate incorporation method, phosphorylation site-specific antibodies or mass spectrometry based phosphoproteomics. Further benefits of phosphate-affinity electrophoresis and immunoblotting are that they do not require phosphorylation site-specific antibodies, chemical, or radioactive labels, and can be performed with any traditional vertical slab minigel electrophoresis units; thus, provide a relatively simple way to study protein phosphorylation.

This unit contains procedures for preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Zn^{2+}-Phos-tag gels (Basic Protocol 1). Subsequent wet-tank electroblotting of proteins onto a membrane is described in Basic Protocol 2. The Alternate Protocol 1 presents procedures for transfer in semidry system. Instead of electroblotting, other downstream procedures can be also performed, such as gel staining or mass spectrometic analysis which are not discussed in this unit.

Basic Protocol 1 – Zn^{2+}-Phos-tag SDS-PAGE under neutral pH conditions

As the Zn^{2+}-Phos-tag SDS-PAGE is superior to the Mn^{2+}-Phos-tag protocol (see Background Information), we only discuss the Zn^{2+}-Phos-tag version here. The following procedure details the preparation of Phos-tag gels using a dizinc(II) complex of Phos-tag acrylamide with a neutral pH gel system buffered with 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol (Bis-Tris) and hydrochloric acid (HCl). This is followed by a protocol describing one-dimensional electrophoresis of Phos-tag gels.

Zn^{2+}-Phos-tag SDS-PAGE uses a discontinuous buffer system in which Bis-Tris (+) and chloride (–) ions are provided by the gel buffer and Tris (+), 3-(N-morpholino) propane sulfonic acid (MOPS; –) and SDS (–) ions are provided by the running buffer. Chloride and MOPS ions serve as leading and trailing ions, respectively, whereas Bis-Tris is the common ion present in the gel resulting in an operating pH of ~7 during electrophoresis. Phosphate-affinity electrophoresis is performed under denaturing conditions using the anionic detergent SDS which unfolds and covers proteins, resulting in highly negatively charged SDS-protein complexes. Importantly, SDS is omited from the gel itself and is present only in the sample, running and transfer buffers. Under these conditions, the dizinc(II) complex of Phos-tag acrylamide is able to reversibly bind to negatively charged phosphate moieties in phosphoproteins and reduce their migration speed. In addition, electrophoresis is performed under reducing conditions using beta-mercaptoethanol or dithiothreitol to reduce disulfide bonds between cysteine residues. Of note, because of the neutral pH environment, these reducing agents do not migrate into the gel from the sample buffer, thus to prevent re-oxidation and maintain proteins in a reduced state during electrophoresis, it is essential to include another antioxidant which migrates with proteins. For this purpose, sodium bisulfite is included in the running buffer and sulfite ion serves as a reducing agent which inhibits the oxidation of reduced proteins in the gel.

Materials

Acrylamide/Bis-acrylamide, 30% solution, 29:1

Phos-tag solution (see recipe)

0.5 M Sodium bisulfite solution (see recipe)

2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol (Bis-Tris)

Hydrochloric acid (HCl), 6M solution
Bis-Tris-HCl buffer, 4 X (see recipe)
2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris) base
3-(N-morpholino) propane sulfonic acid (MOPS)
Sodium dodecyl sulfate (SDS), 10% (w/v) solution
10 mM $ZnCl_2$ solution (see recipe)
100% methanol
Tetramethylethylenediamine (TEMED)
Ammonium persulfate (APS), 10% (w/v) solution (see recipe)
2 X and 1 X SDS sample buffer
Running buffer, 1 X (see recipe)
Ultrapure water (to be used for preparation of all solutions)
Protein sample to be analyzed in SDS sample buffer

Vertical slab minigel unit with clamps, glass plates and casting stand
Power supply (constant voltage and constant current)

Prepare Phos-tag gels

1. Assemble the glass plate sandwich for gels according to manufacturer's instructions by using clean glass plates and 1.5-mm spacers.

2. Prepare separating gel solution (8% (w/v) Acrylamide, 350 mM Bis-Tris HCl, 25 μM Phos-tag, 50 μM $ZnCl_2$, 0.1% (v/v) TEMED, 0.1% (w/v) APS) which contains the dizinc(II) complex of Phos-tag acrylamide as follows. In a 50 ml centrifuge tube, mix 6.67 ml of 30% acrylamide/0.8% bisacrylamide solution with 6.25 ml of 4× Bis-Tris HCl solution (1.4 mM stock), then add 125 μl Phos-tag solution (5 mM stock) and 125 μl $ZnCl_2$ solution (10 mM stock). Subseqently, add 11.58 ml H_2O, 25 μl TEMED and start polymerization by adding 250 μl of 10% APS. Mix by vortexing the tube. Use the solution immediately to pour gels.

In the present protocol an 8% (w/v) acrylamide separating gel is used, however this should be adjusted according to the desired gel pore size. The following recipes produce

25 ml of separating gel, sufficient to cast two 1.5 mm gels in most vertical slab minigel units, but volumes can be adjusted based on experiental needs and to other gel formats.
It is important, that 2 equivalents of $ZnCl_2$ are added to Phos-tag acrylamide. Vortexing the tube at the end is crucial to achieve a homogenous solution, which is a prerequisite for reproducible gels and sharp bands.

3. Use a pipet to load the separating gel solution into the assembled glass plate sandwich. Gently overlay the top of the gel with ~1 ml of H_2O. Be careful not to disturb the gel surface. This ensures that oxygen, which inhibits polymerization has no access to the solution. Allow the gel to polymerize 45 to 60 min at room temperature, then pour off H_2O from the gel.

4. Prepare stacking gel solution. (4% (w/v) Acrylamide, 350 mM Bis-Tris HCl, 0.1% (v/v) TEMED, 0.4% (w/v) APS). For 20 ml stacking gel solution, mix 2.67 ml of 30% acrylamide/0.8% bisacrylamide solution with 5 ml of 4× Bis-Tris HCl solution (1.4 mM stock) in a 50 ml centrifuge tube. Subseqently, add 11.33 ml H_2O, 20 µl TEMED and start polymerization by adding 800 µl of 10% APS. Mix by vortexing the tube. Use the solution immediately.

5. Use a pipet to pour stacking gel solution onto the Zn^{2+}-Phos-tag separating gel until the height of the solution in the sandwich is ~1 cm from the top of the plates. Insert a 1.5-mm comb with the desired number of teeth into the layer of stacking gel solution. Allow the gel to polymerize 30 to 45 min at room temperature, then carefully remove the comb without damaging the wells. Rinse wells with H_2O and if well walls are not straight, they should be corrected with a gel-loading tip.

Unused gels can be wrapped in wet paper and cling film to protect it from drying and stored at 4 °C for a few weeks.

Perform electrophoresis of Phos-tag gels

6. Prepare 1 X Running buffer by diluting 5 X stock solution and adding sodium bisulfite solution immediately before use.

7. Place the gel into the electrophoresis unit according to manufacturer's instructions and fill upper and lower buffer chambers with 1 X Running buffer. Wells of the stacking gel should be covered by the buffer.

8. Add SDS sample buffer containing 2-mercaptoethanol or dithiothreitol to lysates and solubilize proteins by boiling for 5 minutes at 100°C.

Omit EDTA from lysis and sample buffers as it chelates Zn^{2+} ions from Phos-tag gels. Some phosphatase or protease inhibitor cocktails do contain EDTA. If it is not possible to avoid using EDTA, add equimolar amounts of $ZnCl_2$ to the sample buffer. Avoid using phosphate-containing buffers, such as PBS during lysate preparation (e.g. washing steps) as phosphate ions can bind to the Phos-tag complex which would result in less efficient separation of phospho-proteins.

9. Load samples into the wells by gel-loading tips.

Do not use molecular weight ladders with Phos-tag gels as these gels cannot be used to make molecular weight assignments. Prestained molecular weight markers migrate anomalously in Phos-tag gels, hence identity of proteins should be based on previously confirmed immunoreactivity. Load mock samples of the same volume and SDS sample buffer concentration as real samples into empty wells.

10. Connect the power supply to the unit and run gels under constant current 30 mA/gel until the leading front reaches the bottom of the gel.

Control the temperature of the tank buffer during the run. Attach tubing to the electrophoresis unit and start the flow of coolant (e.g. run tap water). It is reasonable to run Phos-tag gels longer in some cases, e.g. for high molecular weight proteins which would lead to larger shifts in mobility.

11. Disconnect the power supply and remove gel from the unit. Disassemble the gel sandwich and proceed with electroblotting of the gel onto membrane. Alternatively, proteins in the gel may be stained directly.

Place gel sandwich on paper towels with a known orientation, open the glass plate and mark gel by cutting off a small piece from one corner to enable tracking lane positions. Carefully remove gel from plate.

Basic Protocol 2 – Wet-tank electroblotting of Zn^{2+}-Phos-tag gels

This protocol describes wet-tank transfer of proteins from Phos-tag gels onto PVDF membranes, which is the preferred method over semidry transfer (Alternate Protocol 1). An important step before the transfer is to remove Zn^{2+} ions from the gel as phosphorylated proteins remain bound to Zn^{2+}-Phos-tag complexes which would impede efficient transfer of these proteins. Zn^{2+} ions are removed by washing the gel in transfer buffer containing the chelating agent EDTA. The rest of the electroblotting protocol is similar to the one used for traditional electroblotting (*UNIT 10.7*).

Materials

1 X transfer buffer (see recipe)
2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris) base
Glycine
0.5 M EDTA solution, pH 8.0
Phos-tag gel (see Basic Protocol 1)
100% methanol

Wet-tank electroblotting apparatus
Polyvinylidene difluoride (PVDF) transfer membrane
Whatman filter paper
Power supply

Remove Zn^{2+} ions from Phos-tag gels

1. Soak gel in 50 ml methanol-free 1X transfer buffer containing 1 mM EDTA (100 µl 0.5 M EDTA solution into 50 ml 1X transfer buffer) for two times 10 minutes to remove Zn^{2+} ions.

    Handle Phos-tag gels with care as these gels are easier to rip than normal ones.

2. Soak gel in 50 ml methanol-free 1 X transfer buffer without EDTA for 20 minutes.

    EDTA also reduces electrotransfer efficiency so it is necessary to remove it from Phos-tag gels by soaking them in normal tranfer buffer prior to electrotransfer.

Assemble the Phos-tag gel/membrane sandwich

3. Wet the polyvinylidene difluoride (PVDF) membrane by immersing in 100% methanol then place it in transfer buffer.

4. Fill a vessel with 1 X transfer buffer and place plastic pad into the vessel. Submerge one piece of foam pad into transfer buffer and drive out air bubbles with a glass rod. Place three layers of filter paper onto the foam pad and gently drive out air bubbles.

5. Place Phos-tag gel onto the filter papers upside down.

6. Place PVDF membrane onto the gel and drive out air bubbles.

7. Place three layers of filter paper onto the membrane and drive out air bubbles. Place foam pad onto the sandwich and drive out air bubbles. Secure sandwich with clamps and place it into the tank.

8. Fill tank with the appropriate volume of 1 X transfer buffer to cover the gel/membrane sandwich and place cooling block into the tank. Place the unit on ice.

Transfer proteins from Phos-tag gel to membrane

9. Put the lid onto the electroblotting apparatus and start tansfer.

10. Transfer conditions must be optimized for the protein of interest. As a starting point we recommend the following conditions, voltage: 130V, constant current: 380 mA, duration: 3h.

11. Following electroblotting, allow PVDF membranes to dry completely and subsequently wet them again using methanol. Proceed with immunodetection the same way as for traditional immunoblotting (UNIT 10.10).

Alternate Protocol 1 – Semidry electroblotting of Zn^{2+}-Phos-tag gels

This protocol describes semidry transfer of proteins from Phos-tag gels onto PVDF membranes, which usually has lower efficiency than wet-tank transfer (Basic Protocol 2), but requires significantly shorter transfer times and can be more convenient. In semidry electroblotting systems, the gel lies horizontally on top of the membrane. Removing Zn^{2+} ions from the gel before electrotransfer is a crucial step as described in Basic Protocol 2.

Additional Materials *(also see Basic Protocol 2)*

Semidry electroblotting apparatus

Razor blade

Remove Zn^{2+} ions from Phos-tag gels

1. Soak gel in 50 ml methanol-free 1X transfer buffer containing 1 mM EDTA (100 µl 0.5 M EDTA solution into 50 ml 1X transfer buffer) for two times 10 minutes to remove Zn^{2+} ions.

    In case of inefficient transfer (depends on the protein of interest) add more wash steps with methanol-free 1X transfer buffer containing 1 mM EDTA.

2. Soak gel in 50 ml methanol-free 1 X transfer buffer without EDTA for 20 minutes.

Assemble the Phos-tag gel/membrane sandwich

3. Wet the PVDF membrane by immersing it in 100% methanol then place it in transfer buffer.

4. Fill a small tray with 1 X transfer buffer and submerge three layers of filter paper into transfer buffer, place them on the anode of the transfer unit and drive out air bubbles with a glass rod.

5. Place PVDF membrane on top of filter papers and drive out air bubbles.

6. Gently place the Phos-tag gel on top of the membrane and to be able to track orientation, label one corner by cutting off a small triangle with a razor blade.

    The gel might also be immersed together with the PVDF membrane in transfer buffer in the same tray. The gel should then be placed above the membrane and both are removed

together from the transfer buffer and placed on top of filter papers. This procedure can help to avoid introducing air bubbles between gel and membrane.

7. Submerge three layers of filter paper into transfer buffer, place them on top of the gel and drive out air bubbles.

Transfer proteins from Phos-tag gel to membrane

8. Attach the cathode unit to the apparatus and connect leads to the power supply and start transfer.

9. Transfer conditions must be optimized for the protein of interest. As a starting point we recommend the following conditions, voltage: 30 V, constant current: 250 mA, duration: 3h.

To prevent overheating the transfer should be performed in a cold room and an ice pack can be placed on top of the transfer apparatus.

10. Following electroblotting, allow PVDF membranes to dry completely and subsequently wet them again using methanol. Proceed with immunodetection the same way as for traditional immunoblotting (UNIT 10.10).

Reagents and Solutions

1.4 M Bis-Tris-HCl buffer, pH 6.8, 4 X

Bis-Tris 29.3 g

6.0 M HCl 10 ml

Dissolve the Bis-Tris in about 70 ml of ultrapure water, then add HCl and make up to 100 ml with water. Keep at 4 ºC. This buffer solution is used for both separating and stacking gels.

10 mM $ZnCl_2$ solution

$ZnCl_2$ 0.70g

Dissolve in 500 ml water and filter.

Because $ZnCl_2$ is a deliquescent salt, this solution should be prepared by using a fresh product.

Phos-tag solution, 5 mM Phos-tag acrylamide, 3% (v/v) methanol

Add 100 µl methanol to 10 mg Phos-tag acrylamide (Phos-tag™ Acrylamide AAL-107, Wako Pure Chemical Industries) and vortex thoroughly. Dilute solution with 3.2 ml distilled water and vortex until the white cloudy material dissolves. Protect from light as the reagent is light-sensitive and keep at 4 ºC for a few weeks.

10% (w/v) Ammonium persulfate (APS) solution

Ammonium persulfate 1.0 g

Dissolve in 10 ml water. This solution should be freshly prepared.

0.5 M Sodium bisulfite solution

Sodium metabisulfite 4.75 g

Make up 100 ml with water. Keep at 4 ºC.

Sodium metabisulfite becomes sodium bisulfite upon dissolving in water. Use 91.3g of sodium metabisulfite as a substitute for 100 g sodium bisulfite. Use fresh sodium bisulfite solution to prepare running buffer.

Running buffer, 0.5 M Tris, 0.5 M MOPS, 0.5% SDS, pH 7.8, 5 X

Tris base 30.3 g

MOPS 52.3 g

10 % SDS solution 25 ml

First dissolve Tris and MOPS in about 400 ml of water, then add SDS and make up to 500 ml with water. Do not adjust pH. Keep at 4 ºC.

Running buffer, 0.1 M Tris, 0.1 M MOPS, 5 mM Sodium bisulfite, 0.1% SDS, pH 7.8, 1 X

Add 5 ml of 0.5 M sodium bisulfite solution to 100 ml of 5 X running buffer and dilute with 395 ml water prior to use.

Transfer buffer, 250 mM Tris, 1.92 M glycine, 10 X

Glycine 144 g

Tris base 30.2 g

Dissolve Tris and glycine together in 900 ml water, then add water up to 1 l.

Transfer buffer, 25 mM Tris, 192 mM glycine, 10% methanol, 1 X

Dilute 100 ml of 10X transfer buffer in 800 ml of water and add 100 ml methanol.

Addition of 0.05-0.2 % SDS (final concentration) can help to increase transfer efficiency.

Commentary

Background Information

In 2004, an alkoxide-bridged dinuclear zinc(II) complex (1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex) was identified as a highly selective phosphate-binding tag (Phos-tag) under neutral pH conditions in aqueous solution (Kinoshita et al., 2004). Initial Phos-tag SDS-PAGE protocols used buffers with alkaline pH (adopted from Laemmli's method), however under these conditions the Zn^{2+}-Phos-tag complex was unable to separate phosphoproteins, as a consequence of the high pH during electrophoresis, which was not optimal for the Zn^{2+}-Phos-tag complex to trap phosphate groups. Subsequently, a manganese(II) (Mn^{2+}) complex of Phos-tag acrylamide was identified as a useful alternative that is compatible with the Laemmli buffer system (Kinoshita, Kinoshita-Kikuta, Takiyama, & Koike, 2006), and has been used to determine the phosphorylation status of numerous proteins. Mn^{2+}-Phos-tag SDS-PAGE, however, had some limitations and was unable to separate certain phosphoproteins. Eventually, these limitations were overcome by the development of an improved protocol involving the dizinc(II) complex of Phos-tag acrylamide and neutral pH conditions, which resulted in increased affinity between phosphate groups and the Zn^{2+}-Phos-tag complex (Kinoshita & Kinoshita-Kikuta, 2011).

The advantage of Phos-tag technology over other current techniques used to dissect protein phosphorylation (e.g. [^{32}P]orthophosphate incorporation method, phosphorylation site-specific antibodies and phosphoproteomics) is that it enables stoichiometric determination of the phospohrylation status of proteins (Figure 1). Using fluorescently-labeled secondary antibodies with an Odyssey Imaging System (Licor) can yield even more quantitative results. Combining the Phos-tag method with phosphorylation site-specific antibodies (Gegenbauer, Nagy, & Smolenski, 2013; Zhou et al., 2015), [^{32}P]orthophosphate incorporation (Kane et al., 2014) or mass spectrometry (Swaffer, Jones, Flynn, Snijders, & Nurse, 2016) in combination with phosphorylation-site mutants can provide comprehensive information on the differentially phosphorylated protein species.

Critical Parameters and Troubleshooting

Since the different phosphorylated species along with the non-phosphorylated protein can potentially generate several bands, it is vital that a specific antibody is available with minimal background staining to allow for a straightforward analysis of phosphorylation. The lack of mono-specific antibodies could be overcome with an enrichment step of the lysate, e.g. with immunoprecipitation or a pull-down assay of the protein of interest (Nagy, Wynne, von Kriegsheim, Gambaryan, & Smolenski, 2015). It is likely that the number of phosphorylation sites that can be resolved simultaneously and without difficulties is limited.

For analysis of lysates on Phos-tag gels it is critical not to wash cells with a phosphate-containing buffer, such as PBS. EDTA or high salt concentrations in the sample can lead to distortion of bands. The recommended final concentration of Phos-tag acrylamide in the separating gel is used for complex cell lysates which can be doubled (along with that of $ZnCl_2$) for purified proteins to enable their efficient separation. To obtain quantitative results it is necessary that the electrotransfer of phosphoproteins is as efficient as that of non-phosphoproteins, hence removing Zn^{2+} ions from Phos-tag gels after electrophoresis is of crucial importance. An extensive characterization of different transfer conditions for Phos-tag gels has been described (Kinoshita-Kikuta, Kinoshita, Matsuda, & Koike, 2014). The protocol presented above might be inefficient to resolve phosphorylation of higher molecular weight proteins. This issue could be addressed by using Zn^{2+}-Phos-tag SDS-PAGE with a low-concentration polyacrylamide gel strengthened with agarose and a neutral pH gel system buffered with Tris and acetic acid (Tris-AcOH) (Kinoshita, Kinoshita-Kikuta, & Koike, 2012).

Anticipated Result

With the protocol described here, one can detect the phosphorylation of single and multiple serine, threonine or tyrosine residues of any protein using purified proteins or cell lysates. It is possible to detect phosphorylation at endogenous protein levels depending on the availability of suitable antibodies against the total protein. There is no requirement for phosphorylation site-specific antibodies. The method will provide information on the proportions of differentially phosphorylated protein species relative to their non-phosphorylated counterpart. The phosphorylation-induced shift can affect all copies of a given protein (e.g. PKA phosphorylation of ArhGAP17, Figure 1C) or only a smaller or larger fraction of the total protein (e.g. PKA phosphorylation of CCS and Cullin-4A, Figure 1A and B, respectively). As higlighted in Basic Protocol 1 and represented in Figure 1, Phos-tag gels do not provide molecular weight information of proteins. Phos-tag gels can be used to establish protein phosphorylation time courses in response to different stimuli or inhibitors (English et al., 2015).

Time Considerations

Preparing Phos-tag gels takes ~2 hr 30 min. Electophoresis of Phos-tag gels can be completed in 3 – 5 h. Preparation of gels for electroblotting takes 40 min and the electroblotting itself takes 3 h – overnight using wet-tank transfer unit and 3 h using semidry transfer unit.

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Figure Legend

Figure 1. CCS, Cullin-4A and ArhGAP17 are phosphorylated by PKA in intact cells to various extent. HEK293T cells expressing (A) FLAG-CCS (copper chaperone for superoxide dismutase, 29 kDa - delivers copper to copper zinc superoxide dismutase SOD1), (B) Cullin-4A-FLAG (88 kDa - scaffold component of multiple ubiquitin E3 ligase complexes), (C) FLAG-ArhGAP17 (120 kDa - member of the Rho GTPase activating protein (RhoGAP) family) were left untreated or incubated with 10 µM forskolin, an activator of adenylate cyclase, for 10 minutes to stimulate cAMP production and to activate cAMP-dependent protein kinase (PKA) and lysed. Samples were subjected to Zn^{2+}-Phos-tag SDS-PAGE and wet-tank transfer as described in this protocol followed by western blotting using an anti-FLAG antibody (A, B) or an anti-ArhGAP17 antibody. (A-C) The shifted, phosphorylated forms of CCS, Cullin-4A and ArhGAP17 are indicated as p-CCS, p-Cullin-4A and p-ArhGAP17, respectively. Data are representative of three independent experiments. Note the different ratios of phosphorylated and non-phosphorylated species.