



# Protein Dephosphorylation Methods

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Phosphospecific antibodies are designed to differentiate between the phosphorylated and the non-phosphorylated states of a protein. The method to determine if or how well a phosphospecific antibody can distinguish between these different protein forms is evolving with new techniques using phosphatase enzymes. The protocols listed in Protein Dephosphorylation Methods show how one can dephosphorylate proteins when fixed to a membrane, while harvesting tissue culture cells, or after collecting animal organs. Additionally, protein dephosphorylation can be performed on frozen cells and organs if correctly preserved. Choosing the right phosphatase enzyme is crucial; knowing the specific activity and which amino acids the enzyme dephosphorylates are key components.

Determining phosphospecificity of phosphospecific antibodies is serious business to PhosphoSolutions. This step-by-step method is broken down into 2 major sections on how to dephosphorylate proteins the right way. Within each section there is a detailed list of steps, buffers, specific materials, and calculation examples to help researchers through this comprehensive process. Additionally, helpful technical tips are offered throughout the protocol to provide insight about various factors that should be considered when performing protein dephosphorylation. Our main goal is to provide essential techniques and tools to help researchers obtain publishable and reproducible results.

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## Dephosphorylation Protocols for:

- Proteins Fixed on a Membrane
- Organ and Tissue Culture Cell Proteins





## Dephosphorylation of Proteins Fixed on a Membrane

### Materials Required:

- ✓ **Protein membrane blot:**
  - PVDF membrane is recommended.
  - Refer to Western Blot Protocol (up to Protein Transfer) to prepare protein membrane blot.
- ✓ **Lambda Phosphatase Enzyme:** Sigma Aldrich product, includes 10X phosphatase buffer and 10X MnCl<sub>2</sub>, Catalog number: P9614
- ✓ **1X Incubation Buffer:** Lambda phosphatase buffer and MnCl<sub>2</sub> solution diluted to 1X with dH<sub>2</sub>O
- ✓ **Alkaline Phosphatase Enzyme:** Sigma Aldrich product, Catalog number: P0114
- ✓ **Conical tube or microtube with screwcap**
- ✓ **Deionized H<sub>2</sub>O (dH<sub>2</sub>O)**
- ✓ **Wash Buffer (1X TTBS):** 14 mM NaCl, 2 mM TRIS, 1%(w/v) Tween 20, pH 7.6
- ✓ **100% Methanol**
- ✓ **Ponceau S stain:** 0.2% Ponceau S stain, 3% Trichloroacetic Acid, 3% Sulfosalicylic Acid, 94% dH<sub>2</sub>O

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*This protein dephosphorylation method uses a two-step approach to focus on specificity and potency. The first treatment is done with a lambda phosphatase to specifically target phosphate groups from threonine, serine, and tyrosine amino acids for dephosphorylation. Most phosphatases only dephosphorylate serine and threonine amino acids. If you are testing the phosphospecificity of a tyrosine phosphospecific antibody, lambda phosphatase is essential. The second dephosphorylation treatment is done with an alkaline phosphatase, a significantly more potent enzyme than lambda phosphatase. The alkaline phosphatase only dephosphorylates phosphate groups from threonine and serine amino acids, and therefore is not intended to be utilized alone to determine the phosphospecificity of tyrosine phosphospecific antibodies. When using both lambda and alkaline phosphatases in conjunction with each other, it is best to separate the treatments so the alkaline phosphatase doesn't inhibit the activity of the lambda phosphatase. There are a variety of phosphatases one may use to dephosphorylate proteins; the ones listed in this protocol are recommended examples. The end user is highly encouraged to optimize their chosen phosphatase(s) before determining phosphospecificity of an antibody.*

*In this protocol, we refer to the phosphatase treated section of membrane (**Treated**) and a non-treated section of membrane (**Control**).*

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## Membrane Preparation

### A. Freshly Prepared Membrane

1. After protein transfer to PVDF is complete, rinse the membrane with dH<sub>2</sub>O.
2. Cut membrane and place each portion into a separate container containing dH<sub>2</sub>O.

Tech Tips:

- a. It is ideal to use a membrane prepared from a large one trough gel. Cut the membrane in half for each section.
  - b. If using multiple lanes, identify the lanes by staining the membrane with Ponceau S. Cut the fresh, wet membrane for each treatment. Place membrane sections back into container with water. Completely remove Ponceau S stain with 3 x 5 minute washes with wash buffer. Additional washes may be needed.
  - c. To distinguish between the two sections and orientation, make a small cut in the top right corner of each membrane and label the containers **Control** and **Treated**.
  - d. When labeling membrane sections a gel pen is needed so the ink does not wash away. However, the membrane can only be labeled once it is dry.
3. Rock the membrane in wash buffer for 5 minutes at room temperature. **DO NOT DRY MEMBRANE BEFORE TREATMENT.**

Tech Tip:

- a. Choose a container for each membrane section that minimizes the volume needed to completely immerse blot in wash buffer.
4. Proceed to 'Protein Dephosphorylation'.

### B. Deactivated Membrane

1. Cut and label dry deactivated membrane.

Tech Tips:

- a. When performing a single phosphospecificity test, strips can be cut and used instead of treating a large section of membrane. This is optimal for saving special, hard to prepare lysate.
  - b. When labeling membrane sections or strips a gel pen is needed so the ink does not wash away.
  - c. Membrane strips and sections can be labeled with "C" for **Control** and "T" for **Treated** before activating the membrane.
2. Activate the two halves of the PVDF membrane by immersing them in methanol for 30 seconds in selected containers, then thoroughly rinse the membrane halves with dH<sub>2</sub>O.

Tech Tip:

- a. Choose a container for each membrane section that minimizes the volume needed to completely immerse blot in wash buffer.
3. Wash membranes in wash buffer for 3 x 5 minutes.

Tech Tip:

- a. If the membrane was stained with Ponceau S, additional washes may be needed. Stain must be completely washed away from membrane before proceeding.





4. Calculate the total volume of 1X incubation buffer needed to completely immerse all **Control** and **Treated** membrane sections or strips in each container.
5. Proceed to 'Protein Dephosphorylation'.

### Protein Dephosphorylation

1. Prepare the calculated total volume of 1X incubation buffer needed to completely immerse all membrane sections or strips. Divide the 1X incubation buffer into 2 fractions: one labeled **Treated** and one labeled **Control**.
2. Add 5 uL of lambda phosphatase per mL of incubation buffer to the containers labeled **Treated**.
3. Remove wash buffer from membrane and add prepared **Control** and **Treated** solutions to respective membrane sections or strips. Incubate for 4 hours rocking at room temperature, or overnight rocking at room temperature.

*Tech Tip:*

- a. A sealed container is recommended to prevent evaporation.
4. Add 1 uL of alkaline phosphatase per mL of incubation buffer to the containers labeled **Treated**. Incubate for an additional 30 minutes, rocking at room temperature.
5. Discard 1X incubation solution. Rinse membrane thoroughly with dH<sub>2</sub>O.

*Tech Tip:*

- a. If needed, restain the membrane halves with Ponceau S to visualize proteins and remove excess membrane or cut lanes.
6. Deactivate membrane by rinsing with dH<sub>2</sub>O and air dry. The membrane is ready for blocking and antibody incubation (part of Western Blot protocol) once it is completely deactivated and the proteins are fixed to the membrane.

*Tech Tip:*

- a. To speed up the drying process, rinse the membrane with 100% methanol after rinsing with dH<sub>2</sub>O.





## Dephosphorylation of Organ and Tissue Culture Cell Proteins

### Materials Required:

- ✓ **10X Lysis Buffer:** 100 mM TRIS, 100 mM NaCl, pH 8.0
- ✓ **Detergent for Lysis Buffer:**
  - 10% (v/v) NP40-LB
- ✓ **10% SDS:** Phosphatase deactivating agent
- ✓ **Lambda Phosphatase Enzyme:** Sigma Aldrich product that includes 10X Phosphatase Buffer and 10X MnCl<sub>2</sub>, Catalog number: P9614
- ✓ **Alkaline Phosphatase Enzyme:** Sigma Aldrich product, Catalog number: P0114
- ✓ **1X Lysis/Incubation Buffer:** Prepared from 10X Lysis buffer, 10X Phosphatase Buffer, and 10X MnCl<sub>2</sub> solution
- ✓ **Spatula, plastic transfer pipette, and scissors**
- ✓ **Conical tube or microtube with screwcap**
- ✓ **Ice**
- ✓ **Deionized H<sub>2</sub>O (dH<sub>2</sub>O)**
- ✓ **Heat block:** set to 95°C
- ✓ **Sonicator:** Select probe and optimize strength based on lysis buffer volume and tube size to avoid foaming of the protein sample.

*This protein dephosphorylation method uses a two-step approach to focus on specificity and potency. The first treatment is done with a lambda phosphatase to specifically target phosphate groups from threonine, serine, and tyrosine amino acids for dephosphorylation. Most phosphatases only dephosphorylate serine and threonine amino acids. If you are testing the phosphospecificity of a tyrosine phosphospecific antibody, lambda phosphatase is essential. The second dephosphorylation treatment is done with an alkaline phosphatase, a significantly more potent enzyme than lambda phosphatase. The alkaline phosphatase only dephosphorylates phosphate groups from threonine and serine amino acids, and therefore is not intended to be utilized alone to determine the phosphospecificity of tyrosine phosphospecific antibodies. When using both lambda and alkaline phosphatases in conjunction with each other, it is best to separate the treatments so the alkaline phosphatase doesn't inhibit the activity of the lambda phosphatase. There are a variety of phosphatases one may use to dephosphorylate proteins; the ones listed in this protocol are recommended examples. The end user is highly encouraged to optimize their chosen phosphatase(s) before determining phosphospecificity of an antibody.*

*Calculations for the recommended examples can be found at the end of the protocol. It is important to know the specific activity of the phosphatase(s) chosen to determine the amount of enzyme needed. If dephosphorylating pure protein and the molecular weight is known, use this number to determine the amount of each phosphatase needed. If you are preparing a lysate with mixed proteins an assumption of the average molecular weight, aka highly educated guess, is best used. The calculated phosphatase volumes are a starting point to determine the amount needed to dephosphorylate the protein. Since the calculation is based on assumption, additional experimental optimization may be needed.*





## Lysate Preparation

1. Determine the protein mass of the organs or tissue culture cells.

Tech Tips:

- a. Organs: Brush off any debris from frozen organs. Wash fresh organs with 1X PBS and dry with Kimwipe. Weigh fresh or thawed organs. Estimate protein mass as 10% of total mass.
  - b. Suspension Cells: Make estimate based on cell count.
  - c. Adherent Cells: This protocol is not recommended for adherent cells due to non-optimal lysing conditions. The 'Dephosphorylation of Fixed Protein on membrane' protocol is recommended.
2. Based on the estimated protein mass for the samples, calculate a desired final concentration of lysate.

Tech Tip:

- a. A final concentration below 10 mg/mL is recommended for optimal lysing. Calculate concentration with formula below:

$$\text{Protein Concentration} = \frac{(\text{Estimated Protein Mass})\text{mg}}{(\text{Volume of Lysis Buffer})\text{ml}}$$

3. Determine the volume needed for the desired protein concentration and prepare 1X lysis/incubation buffer. Chill on ice for 30 minutes before lysing.

Tech Tips:

- a. Bring all lysis/incubation buffer components to a final concentration of 1% or 1X.
  - b. 10% NP40-LB or 10% Triton are recommended detergents to use in the preparation of 1X lysis buffer. These detergents preserve proteins and lyse most enzymes, but do not lyse phosphatase enzymes.
  - c. 1% SDS lyses everything, including phosphatase enzymes. It is important not to use 10% SDS prior to dephosphorylation treatment.
4. In an appropriate volume conical tube or microtube, add the calculated amount of freshly prepared chilled lysis buffer to organs/cells to reach the desired concentration. Keep sample chilled on ice. Make a note of the volume of lysis buffer added.

Tech Tips:

- a. Organs: For large organs, cut into 1/4" sections. For organs that are thick and dense, cut even smaller sections.
- b. Suspension Cells: Pipette media and cells into conical tube. Pellet cells by centrifugation (1 minute at 1800 x g) and remove media. Resuspend cells with 1-3 mLs of room temperature 1X PBS to wash. Repeat centrifugation to pellet and remove 1X PBS. Add desired volume of lysis buffer.





- Sonicate sample in 5-20 second intervals until solution is clear and can be easily pipetted without clogging. Keep sample on ice while sonicating and after sonication is completed.

*Tech Tips:*

- Organ and cell membranes will appear as white, stringy debris in lysate. It is essential to completely lyse this debris when studying transmembrane proteins.
  - To avoid puncturing/melting the plastic tube, minimize prolonged contact with the probe to the conical tube walls.
  - To avoid foaming, keep probe tip submerged in lysate until sonication is complete.
  - Pipette sample after sonicating. If the lysate clogs while pipetting, sonicate again until clog is no longer present.
- Divide lysate into 2 equal volumes: Label one the **control lysate**, which will not be treated with phosphatase enzymes. Label the other the **treated lysate**, which will be treated with the phosphatase enzymes. Make a note of the volume for each sample.
  - Spike the **control lysate** with 10% SDS to a final concentration of 1% SDS. Make note of volume of 10% SDS added to the control lysate. Heat control lysate at 95°C for 10 minutes. Set sample aside, leave at room temperature.

### Protein Dephosphorylation

- Add 1 ul of lambda phosphatase per 1 mg of protein to the **treated lysate**. Make a note of the volume of enzyme added to the **treated lysate**. Incubate in a water bath at room temperature for 30 minutes.

*Tech Tip:*

- Testing the dephosphorylated lysate is advised before using the lysate to determine phosphospecificity of an unknown phospho-antibody. Try using a known phosphospecific antibody that is phosphorylated at the same type of residue, i.e. Ser, Thr, or Tyr as your unknown. Since the original calculation was based on assumption further optimization of the amount of enzyme and incubation time may be required.
- Add 1 ul of alkaline phosphatase per 10 mg of protein to the **treated lysate**. Make a note of the volume of enzyme added to the treated lysate. Incubate in a water bath at room temperature for 10 minutes.
  - Add 10% SDS to the **treated lysate** to a final concentration of 1% SDS and place in a heat block at 95°C for 10 minutes to inactivate the phosphatase enzymes. Make note of volume of 10% SDS added to the treated lysate.
  - Add together the recorded volumes of reagents added to each lysate in previous steps. Adjust the lysates to equal volumes using the freshly prepared lysis buffer that contains 1% SDS.





5. Determine the protein concentration using the control lysate.

Tech Tips:

- a. The prepared 10X buffer for the enzyme used in this protocol contains DTT, this reagent will interfere with the BCA assay. It is recommended to use a different assay to determine protein concentration.
- b. It is not recommended to use the treated lysate to determine the protein concentration as the concentration will be higher than the control due to the presence of the phosphatase enzyme.

6. Lysate is ready for assay application or can be frozen at -80°C for long term storage.

Tech Tip:

- a. When producing a large preparation of treated cells or organs, aliquoting the lysates into small volumes for individual assay applications is recommended for convenience.

## Phosphatase Calculation

Important factors, physical constants, and assumptions:

- ✓ Avogadro's Number:  $6.023 \times 10^{23}$  molecules/mol
- ✓ Assumed average molecular weight of the protein lysate: 100,000 daltons (g/mol)
- ✓ The Specific Activity of the Phosphatases
  - Lambda Phosphatase: 1 Unit hydrolyzes 1 nmol of phosphate per minute; 1 ul of lambda phosphatase contains 400 units, so for every 1 ul of phosphatase 400 nmol of phosphate will be hydrolyzed per minute.
  - Alkaline Phosphatase: 1 Unit hydrolyzes 1 mmol of phosphate per minute; 1 ul of alkaline phosphatase contains 133 units, so for every 1 ul of phosphatase 133 mmol of phosphate will be hydrolyzed per minute.
- ✓ If you know the exact size and amount of that pure protein, use those numbers. If not, don't freak out! An assumption (aka highly educated guess), is best used in these scenarios. The phosphatase volumes calculated are starting points to determine the amount needed to dephosphorylate 10 mgs of protein. Since the calculation is based on assumption, additional experimental optimization may be needed.





## Lysate Calculation Example

The first calculation is to convert the protein lysate sample from milligrams to molecules. This amount is important in determining the amount of each phosphatase needed. Since the lysate is a mixture of proteins an assumed average molecular weight of 100,000 daltons (g/mol) will be used to simplify the calculation. In this example there are 10 mgs of protein (2mLs of lysate at 5 mg/mL) that will be treated with phosphatase enzyme.

Average Protein Molecular Weight: 100,000 g/mol

$$1 \text{ mol} = 100,000 \text{ g}$$

$$(10 \text{ mgs of protein}) \times \left(\frac{1 \text{ g}}{1000 \text{ mg}}\right) \times \left(\frac{1 \text{ mol}}{100,000 \text{ g}}\right) = 1 \times 10^{-7} \text{ mols of total protein}$$

$$(1 \times 10^{-7} \text{ mols of total protein}) \times \left(\frac{6.023 \times 10^{23} \text{ molecules}}{1 \text{ mol}}\right) = 6.023 \times 10^{16} \text{ molecules of protein}$$

There are  $\sim 6.023 \times 10^{16}$  molecules for 10 mgs of protein.

## Lambda Phosphatase Calculation

The second calculation will be converting the number of lambda phosphatase units into molecules/minute. It is important to note that the reaction time and volume of enzyme can be adjusted once this has been determined.

$$\left(\frac{1 \frac{\text{nmol}}{\text{min}}}{\text{Unit}}\right) \times \left(\frac{400 \text{ Units}}{\text{ul}}\right) = \left(\frac{400 \frac{\text{nmol}}{\text{min}}}{\text{ul}}\right)$$

$$\left(\frac{400 \frac{\text{nmol}}{\text{min}}}{\text{ul}}\right) \times \left(\frac{1 \text{ mol}}{10^9 \text{ nmol}}\right) \times \left(\frac{6.023 \times 10^{23} \text{ molecules}}{\text{mol}}\right) = \frac{2.41 \times 10^{17} \text{ molecules}}{\text{ul} \cdot \text{min}}$$

For every 1 ul of lambda phosphatase enzyme added to the reaction, it will hydrolyze  $2.41 \times 10^{17}$  phosphate molecules. This final calculation can now determine how long the reaction will take.

$$6.023 \times 10^{16} \text{ molecules of protein} \times \left(\frac{\text{ul} \cdot \text{min}}{2.41 \times 10^{17} \text{ molecules}}\right) = 0.25 \text{ minutes per 1 uL of lambda phosphatase}$$

It takes 15 seconds to completely dephosphorylate 10 mg of protein when using 1 ul of lambda phosphatase.





## Alkaline Phosphatase Calculation

The same steps will be done to calculate the amount of alkaline phosphatase needed for the reaction. Notice the specific activity of Alkaline Phosphatase is different than Lambda Phosphatase.

$$\left(\frac{1 \frac{\text{mmol}}{\text{min}}}{\text{Unit}}\right) \times \left(\frac{133 \text{ Units}}{\text{ul}}\right) = \left(\frac{133 \frac{\text{mmol}}{\text{min}}}{\text{ul}}\right)$$

$$\left(\frac{133 \frac{\text{mmol}}{\text{min}}}{\text{ul}}\right) \times \left(\frac{\text{mol}}{1000 \text{ mmol}}\right) \left(\frac{6.023 \times 10^{23} \text{ molecules}}{\text{mol}}\right) = \frac{8.01 \times 10^{22} \text{ molecules}}{\text{ul} \cdot \text{min}}$$

$$6.023 \times 10^{16} \text{ molecules} \times \frac{\text{ul} \cdot \text{min}}{8.01 \times 10^{22} \text{ molecules}} = 7.5 \times 10^{-4} \text{ minutes per 1 ul of Alkaline Phosphatase}$$

It takes 0.045 seconds to completely dephosphorylate 10 mg of protein using 1 ul of alkaline phosphatase.

