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## Technical Tips:

# LYSATE PREPARATION

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# FOREWORD

In 1979, Jaime Renart et al. published an article entitled "Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure," the prelude to the modern Western blot (WB) technique.

Soon after, Harry Towbin et al. went one step further and published "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." With that, the WB technique was officially born.

Today, WB experiments are a cornerstone of biological research. Unfortunately, it is frequently a challenge to obtain good results. A wise man once said:

***"A successful WB relies upon***
***10% Reagents***
***10% Execution***
***10% Luck***
***And 70% protein extraction."***

Speaking of WB, as an original manufacturer of all our products, Proteintech's R&D staff test on average over 70 samples for each product in WB. Proteintech's senior R&D staff will share with you our years of experience with WB to ensure every WB is a successful one.

## What's Inside

# What Goes Into A LYSIS SOLUTION?

## What Goes Into A Lysis Solution?

### 1. The buffer system

The pH of the solution is critical. Proteins may precipitate or become unstable when the pH is outside of the physiological range. To avoid this situation, a buffer system such as Tris-HCl is recommended. Besides buffering solutions in this range, a Tris-HCl buffer preserves the physiological ionic strength and prevents the formation of insoluble products with other ions. A HEPES buffering system is another option. We recommend avoiding buffers with high concentrations of potassium, because these can precipitate proteins when sodium dodecyl sulfate (SDS) is present.

### 2. Salt ions

When the salt ion concentration is too high, some proteins may precipitate. Additionally, when ion concentration is too high, a "smiley face" of band migration may result.

### 3. Chaotropic agents

Chaotropic agents weaken the hydrophobicity of the proteins to solubilize them. There are two kinds of chaotropic agents in a lysis buffer:

a. Urea/thiourea. These molecules unravel hydrophobic regions by disrupting hydrogen bonding between amino acids. Usually when doing protein extraction for a WB, 6¬–8M urea and/or 2M thiourea can be used.

b. Detergents. These are a broad class of surfactants. The key to their solubilizing power is their amphiphilic structure. The hydrophobic end binds to the hydrophobic portions of proteins while the hydrophilic end interacts with water, resulting in solubilization.

Ionic detergents can be further divided into cationic, anionic, and amphoteric detergents. Common ionic surfactants are SDS, DOC (sodium deoxycholate), and SLS (sodium lauryl sarcosine). Common amphoteric surfactants are CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), and CHAPSO (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate). Common non-ionic surfactants are Triton X-100, Triton X-114, Tween-20, and NP40.

**Please Note: It is critical in a WB that the number of negatively charged SDS molecules that bind to a protein is proportional to the protein's mass, so that the migration rate is influenced only by mass. Adding cationic surfactants in the lysis buffer would disrupt the SDS-protein interaction and make the proteins migrate in the opposite direction.**

Due to the complexity of protein biochemistry, it is challenging to predict the optimal surfactant to extract a given protein. Thus, experimenting with different surfactants is recommended if you encounter issues. This is especially recommended for membrane proteins.

### 4. Protease inhibitors

Tissues and cells often contain large amounts of proteases. During lysis, these are released and, in turn, can digest the target protein. Therefore, protease inhibitors are critical for preserving the target protein. Common protease inhibitors are PMSF (phenylmethylsulfonyl fluoride), Aprotinin, Leupeptin, Pepstatin, and AEBSF-HCL (4-benzenesulfonyl fluoride hydrochloride). PMSF is highly effective and is the most popular choice for lysate preparation.

Many protease inhibitors require a divalent metal ion to function, so a sequestering agent is also often used to inhibit protease activity, such as EDTA. In addition, for phosphorylated target proteins, a phosphatase inhibitor such as sodium fluoride or sodium orthovanadate is needed to preserve the phosphorylated form of the protein. Sodium orthovanadate, in particular, is very effective, but needs to be activated by adjusting the pH of the solution to 10 and then boiling it until the solution is colorless. Other phosphatase inhibitors include sodium pyrophosphate and β-glycerol phosphate.

**Continue for step 5 on the next page.**

### 5. Reductant

Many proteins exist in multimers through disulfide bonds. Reductants disrupt these bonds so that the extracted proteins are present in monomeric form. Common reducing agents are DTT (dithiothreitol) and BME (beta-mercaptoethanol).

Keeping all of this in mind, RIPA buffer is the best choice for sample lysate preparation. We have validated over 13,000 antibodies in WB, and time and time again, experience the best results using RIPA buffer. Over the years we have refined the buffer and below you will find Proteintech's optimized version:

| RIPA buffer | For 1000 ml |
|---|---|
| 50 mM Tris•HCl, pH 7.4 | 50 ml |
| 150 mM NaCl | 8.76 g |
| 1% Triton X-100 or NP-40 | 10 ml |
| 0.5% Sodium deoxylcholate | 5 g |
| 0.1% SDS | 1 g |
| 1 mM EDTA (0.5 M stock) | 2 ml |
| 10 mM NaF | 0.42 g |
| Add dd$H_2O$ to 1000 ml | |
| Add PMSF to a final concentration of 1 mM and any other protease inhibitors immediately before use. | |

| 4X SDS sample buffer | For 1000 ml |
|---|---|
| 12% SDS | 120 g |
| 25% Glycerol | 250 ml |
| 150 mM Tris•HCl (pH 7.0•1 M stock) | 150 ml |
| 0.03% Bromophenol Blue | 300 mg |
| 20% β-mercaptoethanol | 200 ml |
| Add dd$H_2O$ to 50 ml, aliquot and store at -20°C. | |
| 20% β-mercaptoethanol, (or 500 mM DTT), should be added freshly before use. | |

**Please Note: If you are experiencing issues extracting the conventional lysate protein, we recommend reading the literature.**

# How Do I Optimize My EXTRACTION?

## Lysis

### 1. Cell Lysis

For suspension cell culture samples, cells can be collected directly by centrifugation and washed with PBS or with saline 2–3 times to remove the serum in the medium. For adherent cells, though trypsin treatment is popular, we do not recommend it since trypsin can also digest the protein of interest. Instead, we recommend scraping off adherent cells when possible.

Whether you are using suspension or adherent cells, the rest of the process is the same: lysis buffer is added, the sample is sonicated, and then centrifuged. Afterward, a small amount can be used to determine the concentration, with the remainder used for the WB.

### 2. Tissue Lysate

Tissue protein extraction is more complex. The first and most critical step is to perform a clean dissection of the desired sample. Once the sample is dissected, it is necessary to wash the sample with PBS to remove blood contamination, thereby preventing nonspecific signal from a secondary antibody binding to the organism's endogenous immunoglobulins. After washing, the sample is homogenized.

Following homogenization, the general procedure is similar to that of the cell culture process. Unlike cell culture samples, however, the tissue is often rich in connective tissue, and some is difficult to dissolve in conventional lysates and might require experimentation to optimize results.

## Preventing Protein Degradation

**As mentioned earlier, many tissues and cells contain proteases. Here are some methods to mitigate these enzymes during sample preparation:**

### 1. Use protease inhibitors

Both PMSF and EDTA are inexpensive yet highly effective inhibitors, and are therefore used in almost all WB experiments.

### 2. Perform the procedure at a low temperature

For the preparation of protein samples of common mammalian tissues or cells, all steps can be performed at low temperature, and all reagents should be precooled to reduce protease activity and prevent protein degradation. In particular, digestive system-related tissue samples should be processed as quickly as possible, and the preparation method should be grinding after flash freezing in liquid nitrogen to minimize sample degradation.

However, there are species such as zebrafish whose proteases are most active at low temperatures. In these cases, perform the procedure at a high temperature (50°C–60°C) where their activity is low.

### 3. When taking samples from multiple organs, order the dissections by protease activity

Digestive system-related organs and macrophage-rich tissues (e.g., lungs) should be dissected and snap frozen first. Then, reproductive tissues should be processed. Heart, spleen, kidney, brain, and other organs can be dissected last.

Some cell lines, such as Raw 264.7 and U-937, have high protease activity. In these cases, consider using a high concentration of SDS to accelerate the extraction process.

## Avoiding Impurities

**Tissues and cells contain many other substances besides proteins and these can interfere with your WB. Here are some strategies to mitigate impurities in your sample:**

### 1. Avoid cross-contamination

During extraction, use sterile, clean tools, especially equipment such as homogenizers, grinding pestle, and dissection tools. Avoid using proteases to improve your protein extraction yield.

### 2. Sonicate the sample to remove nucleic acids

Nucleic acids can bind to proteins and interfere with your analysis. If the prepared sample contains large amounts of nucleic acids, the target protein's mobility may be affected, or the complex may form large aggregates that are insoluble. The solution is to break down nucleic acids using a sonicator, which renders them incapable of forming intact protein-binding domains.

### 3. Remove as much fatty tissue as possible from your dissected sample

Like nucleic acids, lipids can bind to proteins and cause issues with your WB. If there is still some fatty tissue remaining after dissection, use of a silica column is recommended to adsorb lipids.

### 4. Maintain physiological salt ion concentration

As mentioned earlier, high concentrations of salt ions may result in a smiley band. Additionally, if salt concentration among the different lanes is not uniform, the migration of the same protein in different lanes may be different. Therefore, in the sample preparation process, try to maintain physiological ion concentration across samples.

## Fractionate Or Separate

### Fractionate or separate your sample to increase concentration of low-abundance proteins

Some proteins only reside in specific cells or organelles. Consequently, when using the whole lysate, its abundance may not reach the WB detection limit. In these cases, it is recommended to fractionate the desired cell subsets or specific organelles you need according to the literature.

## Concentration

### Concentration leveling and evaluation

After protein extraction is complete, it is important to determine the protein concentration in your sample. A quantitative protein assay such as BCA will yield the most accurate results. It is also recommended to run SDS-PAGE to evaluate the quality of the lysate to ensure that the lysis is thorough and not degraded.

To ensure consistent sample loading, it is necessary to adjust the concentration so that it is consistent across your samples. Once this is completed, 4X loading buffer can be added to the sample.

Despite a variety of methods to ensure error-free protein extraction and similar concentrations, it is still strongly recommended to include loading controls in your experiments.



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