Small Format PEP Universal Protein Purification Kit

Catalog # AB000401

Complete kit for the fast purification of functional proteins from a protein mixture, preferentially a partially purified protein fraction.

Please read this insert completely prior to performing the assay.
This kit is intended for research use only. Not for use in diagnostic procedures.

Background information

Proteins play essential roles in numerous biological processes. It is always a challenge to purify a functional protein from a protein mixture because of their charge and size heterogeneity. Another challenge in protein purification is the limitation of the amount of material that can be used for the protein purification; sometimes only micrograms of proteins are available, making most of the current protein purification procedures less effective.

In the PEP technology, protein mixture is first separated by a modified one-dimensional or two-dimensional gel electrophoresis, this modified procedure provides good resolution while still maintaining protein function. This is followed by an efficient protein transfer step to a specially designed 384-well Protein Elution Plate (PEP). After further transfer of the samples from the PEP plate to a master 384-well microplate, functional assays can be performed using part of the sample from each well to generate an enzyme activity profile. The purity of the protein in each well can be tested by a standard SDS-PAGE (Polyacrylamide Gel Electrophoresis) after loading a portion of the sample from the master 384-well plate. If desired, Mass Spectrometry can be used to identify the protein from the well with pure protein or from the protein band on the SDS-PAGE gel. It is also possible to analyze multiple enzyme families in parallel to obtain functional profiles for each enzyme. The PEP technology can be applied to the systematic analysis of any proteins for which functional assays exist.

Technology Principle

One of two gel electrophoresis methods can be used for the functional protein purification depending on the complexity of the sample. If the fraction contains less than 20 proteins, it is

possible to purify the protein using a modified SDS-PAGE method. On the other hand, if there are more than 20 proteins in the sample, a small format 2-D gel may be used. If there are hundreds to thousands of proteins in the mixture, a large format 2-D gel (offered as different PEP Protein Purification kit) should be used.

For protein fraction with less than 20 proteins, the sample is first treated with 0.1% SDS in Tris-Glycine buffer at room temperature then loaded on a SDS-PAGE gel for protein separation.

For protein fraction with more than 20 protein species, 2-D gel should be used. Two-dimensional (2-D) Gel Electrophoresis is a powerful technology to separate complex protein samples. In the first dimension called Isoelectric Focusing (IEF), the proteins are separated based on their isoelectric points (pI), proteins with as little as 0.02 unit pI differences could be separated, making it a high resolution method. In the second dimension, the proteins are separated based on their molecular size. Because 2-D Gel Electrophoresis is using two orthogonal parameters (charge and size) for separation and displaying the proteins in a two-dimensional manner, it is one of the most powerful technologies in protein separation. In a large format gel, more than 10,000 proteins could be separated and detected with information on their relative abundance and post-translational modification acquired simultaneously. Because of these advantages, 2-D Gel Electrophoresis has been used widely in proteomics studies. However, in a typical 2-D Gel Electrophoresis, the proteins are denatured by the addition of reagents to disrupt disulfide bonds (DTT or β-mercaptoethanol), chemicals to prevent disulfide bond formation (iodoacetamide) and high concentration of SDS (typically 1%). To keep the proteins active in 2-D Gel Electrophoresis, a few modifications were made in the current PEP technology. First, no reducing reagent is used in the Isoelectric Focusing step, keeping the disulfide bonds in the proteins intact. Secondly, iodoacetamide is omitted from the process. Thirdly, much reduced SDS concentration is used in the SDS-PAGE (from 1% to 0.1%), again trying to maintain enzymatic activity. Recent studies indicated that many different enzyme families from a wide variety of organisms are active in the presence of SDS such as protein kinases, protein phosphatases, proteases, oxido-reductases, to just name a few (See References).

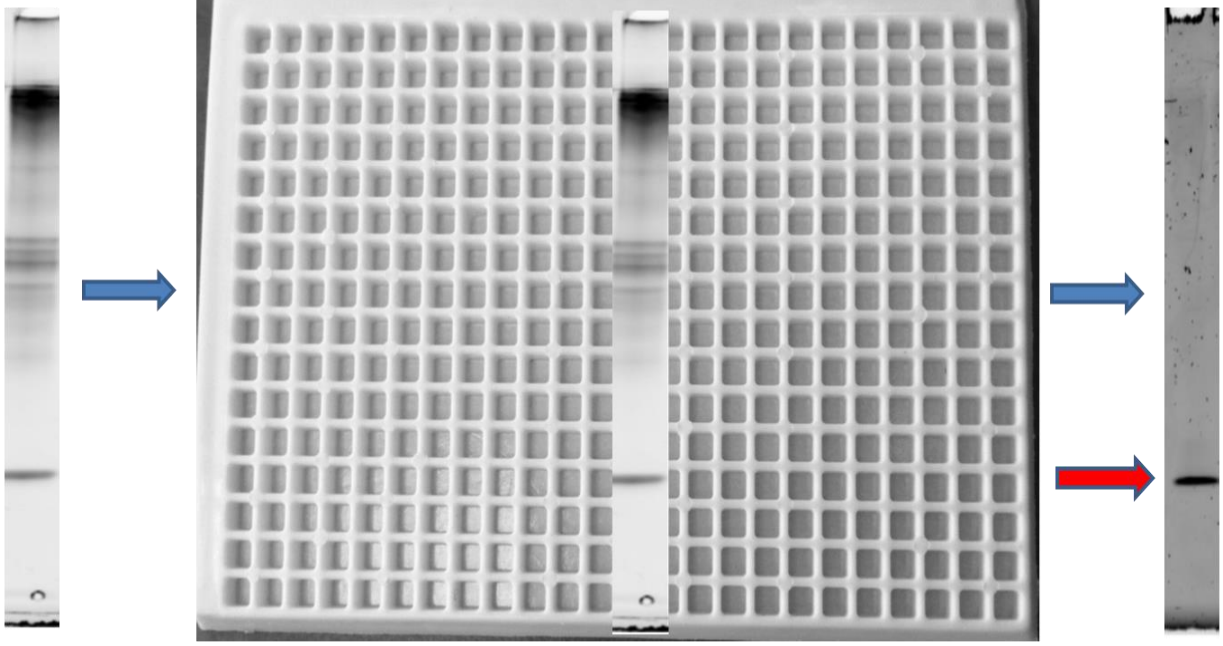
In addition of method modification, a high resolution Protein Elution Plate (PEP) was designed. The small format PEP has 384-wells matching the current 384-well microplate dimension for ease of sample processing. For the large format PEP, the plate is composed of 4 384-well PEP thus have 1536 wells. In both the large and small format PEP, a membrane with molecular cut off of 6,000 Dalton is attached that will allow the electric current and small charged molecules to pass through but collect proteins with molecular weight large than 6,000 Dalton in the PEP wells. Furthermore, a special solution was developed for the PEP to reduce protein diffusion after the proteins are transferred from the gel to the PEP. After transfer the solutions from PEP to a deep-well master plate, the enzyme activity or protein function can be analyzed using part of the sample from the master plate and purified protein can be verified using SDS-PAGE in standard condition and identified using Mass Spectrometry.

Purification of Functional Protein from Modified 1-D SDS-PAGE

Modified SDS-PAGE for separation

Fraction 4 electro-eluted to the PEP Plate after gel electrophoresis and recovered for enzyme assay and protein purity testing.

NADH Oxidase Purified by the PEP Plate



Fraction 4 from Mono Q column

Protein of interest is eluted into 2-3 adjacent PEP wells

Supplied Components:

1. **384 Well PEP Plate** A PEP plate is provided. The plate was treated with a special solution to reduce the binding of the transferred protein and increase the recovery efficiency.

2. **384 Well Master Plate** A deep-well plate is provided to contain samples recovered from the PEP plate.

3. **384 Well Enzyme Assay Plate** A standard 384-well polypropylene plate is provided for enzyme assay to identify which wells contain the protein of interest.

4. **10x Protein Transfer Buffer (50 ml)** Buffer used for running the modified SDS-PAGE or the second dimension of 2-D gel, and also used for the transfer of proteins from the gel to the PEP plate.

5. **10x PBS (10 ml)** Buffer used to pre-treat the deep-well plate before use and fill in each well with 50 µl of PBS.

6. **10x Modified SDS-PAGE Sample Buffer (1 ml)** Solution used in sample treatment for the modified SDS-PAGE.

7. **Standard SDS-PAGE Sample Buffer (0.5 ml)** Solution used in sample treatment for the standard SDS-PAGE to check enzyme fraction purity.

8. **PEP Plate Protein Recovery Buffer (50 ml)** Solution used in the PEP plate to recover proteins eluted from the gel and prevent protein diffusion.

9. **Plate Sealer** For sealing the Master plate and the enzyme assay plate during the purification process. Kit AB-00401 (two).

10. **Filter Papers** Used to form a sandwich in the protein transfer process.

Instruments and other Materials Required

Instruments:

Gel electrophoresis unit includes power supply and gel unit.

Isoelectric focusing unit that is capable of running IEF at different length, an example of such unit is the Bio-Rad PROTEAN IEF Cell (Catalog Number: 165-4000).

Spectrophotometer Plate Reader capable of reading 384-well plates with a wide wavelength selection and fluorescence reading.

Semi-Blot unit for protein transfer such as Bio-Rad's Trans-Blot SD Semi-Dry Transfer Cell (Catalog Number: 170-3940).

Materials:

SDS-PAGE gels: Customer can choose any format SDS-PAGE gel to run the sample. Preferentially the loading capacity of each well should be 15 µl or more. Gels from Bio-Rad (Criterion 10-20% 18-well Tris-HCl gel, catalog number: 345-0043; Criterion 10-20% IPG + 1 well Tris-HCl gel, catalog number: 345-0107), Invitrogen, etc. can be used for the protein separation.

Isoelectric Focusing strips: Immobilized pH gradient (IPG) strips to run IEF can be purchased from either Bio-Rad (Catalog Number: 163-2014 for 11 cm IPG strips and 163-2033 for 18 cm IPG strips) or GE Health Life Sciences (Catalog Number: 18101661 for 11 cm pH 3-10 Immobiline Dry Strips; 17123501 for 18 cm, pH 3-10 Nonlinear Immobiline Dry Strips).

Electrolyte: electrolyte used for the IEF gel can be purchased from either Bio-Rad (Bio-Lyte buffer, pH 3-10, catalog number: 163-2094) or GE Health (Pharmalyte pH 3-10, catalog number: 17-0456-01).

Protein staining components: if protein staining is required, the following conditions can be used: gels after electrophoresis first fixed in fixing solution (10% acetic acid, 10% ethanol in Mili-Q water) for one hour, then stained in SYPRO Orange (Invitrogen, catalog number: S6650) or other fluorescence dye overnight in Mili-Q water, dilute the fluorescence dye as recommended by the manufacturer.

Distilled or deionized water.

Single- and multi-channel micro-pipettes with disposable tips to accurately dispense volumes 5-250 µL.

Plastic tubes (i.e. 1.5 ml – 15 ml) for sample dilution

Reagent reservoirs for sample addition

Precautions

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

Procedural Notes

Allow diluted reagents and buffers to reach room temperature (18-25°C) prior to starting the assay. Once the assay has been started, all steps should be completed in sequence and without interruption. Make sure that required reagents and buffers are ready when needed. Prior to adding to the plate, reagents should be mixed gently (not vortexed) by swirling.

Avoid contamination of reagents, pipette tips and wells. Use new disposable tips and reservoirs, do not return unused reagent to the stock bottles / vials and do not mix caps of stock solutions.

Assay Protocol

1. Sample Evaluation.

1.1. It is recommended to check the protein complexity first (if not known) by standard gel electrophoresis. If it is a total protein preparation from specific cell types or tissues, large format PEP (AB000501) Protein Purification kit should be used. In the standard SDS-PAGE testing, if there are less than 20 protein species (bands) detected, the modified 1-D SDS-PAGE could be used (go to step-2 in this section) for the functional protein purification. If there are more than 20 protein species (bands) in the sample, an IEF (Isoelectric Focusing) with 11 cm IPG strip (pH 3 to pH 10, nonlinear) should be used first, followed by the modified SDS-PAGE method (go to step 3 in this section directly).

1.2. The recommended condition for running the modified SDS-PAGE is to treat the sample with 0.1% SDS in Tris-Glycine buffer without reducing reagent. Many proteins will retain some activity/function. However, there are also proteins that are highly sensitive to SDS treatment. In this case, SDS will not be used in the gel system; instead a non-denaturing gel will be used for the separation.

1.3. Before running the modified gel electrophoresis, it is recommended to test the sensitivity of the protein toward 0.1% SDS. Incubate the protein sample in the presence of 0.1% SDS in Tris-Glycine buffer at 37°C for 60 min followed by enzyme or protein function analysis. If there is residual but measurable enzyme activity left, then the modified SDS-PAGE can be used for protein separation. If the enzyme activity is completely lost under this condition, a non-denaturing gel will be used for the separation.

2. Running the Modified SDS-PAGE Directly

2.1. Sample treatment. For the Bio-Rad Criterion gel with loading capacity of 30 µl, 25 µl sample can be loaded including 22 µl protein sample and 3 µl of the 10x Modified SDS-PAGE Sample Buffer. Incubate the samples at room temperature for 5 min before loading.

2.2. Running the gel. The gel is run in two steps. In the first step, the constant voltage is set at 80 V and run for 15 min. In the second step, the voltage is increased to 120 V and run until the dye front is about 0.5 cm from the bottom of the gel.

3. Running the IEF Gel First Followed by Running the Modified SDS-PAGE

3.1. It is suggested to use the 11 cm IPG strip (Bio-Rad, catalog number: 163-2033) for the IEF. To rehydrate one IPG strip, 225 µl of solution is needed. It is suggested to use 200 µl of samples, add urea to a final concentration of 8 M, add 2 µl of Ampholyte such as Bio-lyte (Bio-Rad, catalog number: 163-2094). If the protein sample can be lyophilized, then the lyophilized sample can be dissolved into a sample solution with 8 M urea and 0.5% Bio-lyte.

3.2. The solution is first added to a rehydration tray, the IPG strip is taken out from the storage and the plastic cover is peeled off. The side with the dried gel surface is facing down to make contact with the sample solution. Please make sure to let the whole IPG strip making full contact to the sample solution. Add enough mineral oil to cover the IPG strip to prevent evaporation and rehydrate the sample overnight at room temperature.

3.3. After rehydration, the IPG strip is taken out from the rehydration tray and the attached mineral oil is carefully removed with a Kimwipe paper.

3.4. In the IEF tray, carefully wet two pieces of Electrode Wick (Bio-Rad, catalog. No. 165-4071) and put on the metal wire in one lane. Carefully lay down the IPG strip face down, and gently push the IPG strip so that it can make a close contact to the filter paper-covered metal wire. Add enough mineral oil to cover the IPG strip to prevent the evaporation.

3.5. Put the IEF tray cover to the try followed by close the IEF unit cover (Bio-Read Protean IEF Unit).

3.6. In the first step, Set the voltage gradient from 0 to 8,000 V for 4 hours, in the second step, set at constant voltage at 8,000 for 24 hours. The gel will actually run overnight, but the minimum voltage-hrs. is 30,000.

3.7. After the IEF is completed, turn off the unit, and carefully take out the IPG strip and use Kim wiper paper to remove the mineral oil from the IPG strip. Put the IPG strip into a rehydration tray and incubate in Tris-Glycine transfer buffer supplied in this kit, incubate for 10 min. to remove the urea and allow the SDS to bind to the proteins.

3.8. Take out a Bio-Rad Criterion gel and remove the plastic comb, use Mili-Q water to rinse the flat well. Put the gel into the running unit and fill both the lower and upper tank with Tris-Glycine-SDS buffer.

3.9. Carefully lay down the IPG strip in the IPG well with the acidic side always on the left side when facing the gel. Load 5 µl of unstained protein standard in the protein standard well (the well next to the acidic end of the IPG strip).

3.10. Run first at 80 voltages for 15 min followed by 120 voltages until the dye front from the protein standard is about 0.5 cm from the bottom of the gel.

4. Protein Transfer after either the Modified One-dimensional SDS-PAGE or the 2-D Gel

4.1. While the SDS-PAGE is still running, put the PEP plate in a tray and add 50 µl of the protein recovery solution to the plate with a multiple channel pipette. If an eight channel pipette is used, the solution could be dispensed every other row. For example, in the first round, add solutions to row A, C, E and so on, in the second round, add solution in the row B, D, F and so on. Cover the tray to minimize evaporation.

4.2. Carefully take out the gel from the gel cassette and rinse with Mili-Q water followed by adding 200 ml of the transfer buffer (supplied with the kit) in the tray. Wet two pieces of the transfer filter paper completely and lay down on the metal plate of the Semi-Dry Trans-Blot (Bio-Rad or similar Semi-Dry Trans-Blot from other manufacturers).

4.3. Lay the PEP plate on top of the filter paper followed by carefully lay the gel on top of the PEP plate and make sure the upper left corner of the gel align with the upper left corner of the PEP plate.

4.4. Wet another two pieces of transfer filter paper in the transfer buffer and lay on top of the gel to form a sandwich (from the bottom it should be filter papers, PEP plate, gel and filter papers again).

4.5. Cover the sandwich assembly with the other metal plate of the Semi-Dry Trans-Blot, and transfer the proteins with constant current at 120 mA for 60 min. It is shown that under this condition, the proteins in the gel will be efficiently transferred into the PEP plate, longer protein transfer is not recommended.

4.6. While the gel is transferring, condition the 384-well deep-well plate by adding 100 µl PBS in each well (if protein kinase or protein phosphatase assays are performed, a phosphate-free buffer such as Tris –HCl should be used to minimize interference from the buffer). This treatment will improve the protein recovery in later step for enzyme activity analysis and mass spectrometry protein identification.

After 30 min treatment, completely empty the solution from each well and refill the well with 50 µl PBS (for protein kinase assay or any other assay where phosphate is interfering, Tris-HCl buffer or other buffer of choice could be used).

4.7. When the protein transfer is completed, turn off the power, take off the Semi-Dry Trans-Blot cover and release the top metal plate. Wait for 10 seconds before lifting the top metal plate (this is important to let some air in so that the solutions in the PEP plate will not be sucked out to cause proteins in one well over flow to adjacent wells). After removing the metal plate, carefully lift the two pieces of filter paper followed by remove the gel (sometimes the filter papers and gel will stick together, in this case lift both parts together). When removing the gel, it is important to remove it from left to right (This is especially important for running the modified SDS-PAGE gel for less complex protein fraction because the proteins are separated into horizontal bands in the gel, if lifting the gel from left to right, some of the solutions could move horizontally into wells without sample, thus avoiding mixing with other proteins. It should be point out that the specific composition of the PEP transfer buffer will reduce the protein diffusion). Carefully take the PEP plate with the two transfer paper still on the bottom of the PEP plate and put in a tray.

4.8. If the 1-D modified SDS-PAGE is used and one or a few lanes contain the protein sample, only the samples from 3 columns underneath the corresponding protein sample lane will be collected. This can be accomplished using either a single channel pipette or multiple channel pipettes. When using the multiple channel pipettes, please set the transfer volume at 45 µl to make sure most of the solution in the well is transferred. The samples from the wells with odd numbers (row A, C, E and so on) is first transferred followed by transfer of the samples from the even number wells in the same column (rows B, D, F and so on).

4.9. If the 2-D gel is used, all the samples from the PEP plate will be transferred to the Master Plate. Use multiple channel pipettes to transfer the recovered protein solution from the PEP plate to the deep-well Master Plate in the corresponding columns. If using an eight channel pipette, please set the transfer volume at 45 µl to make sure most of the solution in the well is transferred. The transfer will start at column 1 from the left side of the PEP plate, and the wells with odd numbers (row A, C, E and so on) is first transferred followed by transfer of the wells from the even number wells in the first column (rows B, D, F and so on). Repeat the process until all the samples from the PEP plate are transferred to the 384-well Master Plate.

5. Enzyme or Functional Protein Analysis

5.1. After the transfer of samples from the PEP plate to the deep-well Master Plate, the Master Plate should be used immediately (preferred) for enzyme or functional protein analysis. Multiple enzymes can be analyzed from samples collected since the total volume in each well of the Master Plate is about 90 µl (50 µl buffer plus 40-45 µl sample transferred from the PEP plate). For the enzyme activity or other protein function assay, transfer 10-30 µl of sample from each well of the Master Plate into an enzyme assay plate, followed by adding up to 50 µl of enzyme assay solution including substrate. Use a spectrophotometer or any other suitable instruments to measure the enzyme activity or protein function. Before the assay readout, some wells of the enzyme assay plate may contain bubbles because of the SDS in the protein transfer buffer (one technique to avoid bubble is to set the dispensing volume smaller than the aspirating volume so that the pipette will not cause bubbles when dispensing). Use a transfer pipette tip to pinch the bubbles to get rid of them before the reading; this will reduce the interference from the bubble.

5.2. When reading the enzyme assay plate, use pipette to remove the solutions from the well P24 (lower right corner well in the 384-well plate) and use this well as blank for the reading (sometimes this is not necessary, the whole plate could be read). It is recommended to read at least 3 data points such as 10 min(starting point), 60 min and 120 min. and save the reading in separate file.

6. Protein Purity Confirmation

6.1. If the enzyme testing showed that some wells have the enzyme activity of interest, the next step is to test the purity of the protein in that well. Collect all the samples from the wells with enzyme activity in a siliconized microcentrifuge tube, dry down the solution and re-suspend into 20 µl of Milli-Q water. Take 10 µl and mixed with 10 µl of SDS sample buffer (this sample buffer is a 2X SDS-PAGE sample buffer with 20 mM DTT), incubate at 37°C for 60 min.

6.2. Load on a SDS-PAGE gel and run the gel as in Section 2 of this protocol.

6.3. Fix the gel in a gel fixing solution for at least two hours.

6.4. Rinse with distilled water and stain the gel in Sypro Ruby or other fluorescence dye overnight.

6.5. The next day, remove the staining solution; wash the gel twice with distilled water followed by incubation in the distilled water for 5 min with moderate shaking.

6.6. Take the gel image with a CCD camera such as the Bio-Rad ChemiDoc.

6.7. Save the image in tiff file for later image processing. The gel image will tell whether the protein is pure or not.

7. Mass Spectrometry to Identify the Protein of Interest

7.1. If the gel staining in Section 6 shows that the fraction with enzyme activity is pure, the 10 µl Milli-Q water resuspended sample in Section 6.1 can be submitted for Mass Spectrometry analysis (sometimes fraction with more than one protein bands can be submitted for MS analysis, and the identity of the protein can be assigned by bioinformatics effort based on protein homology, it is unlikely that more than one protein from the preparation share the same type of enzyme activity).

7.2. Alternatively, if there is enough protein to be seen in step 6.7 with the fluorescence staining, the protein band can be excised and sent for MS analysis.

8. Recommended Enzyme Assay Kit Suppliers:

1. Protein Kinase Kit-1: R&D System Universal Kinase Kit, catalog number: EA004 (www.RnDSystem.com)
2. Protein Kinase Kit-2: Promega ADP-Glo Kinase Assay Kit, catalog number: V9101 (www.promega.com).
3. Protein Kinase Kit-3: Promega Kinase-Glo Luminescent Kinase Assay Kit, catalog number: V6712 (www.promega.com).
4. Protein Kinase Kit-4: EMD Millipore TruLight Universal Kinase/Phosphatase Assay Kit, catalog number: 539714 (www.emdchemicals.com).
5. Serine/Threonine Phosphatase Assay Kit-1: Promega Serine/Threonine Phosphatase Assay System, catalog number: V2460 (www.promega.com).
6. Protein Tyrosine Phosphatase Kit-1: Sigma-Aldrich Protein Tyrosine Phosphatase Assay Kit, catalog number: PTP-101 (www.sigmaaldrich.com).
7. Protease Assay Kit-1: Sigma-Aldrich Protease Fluorescent Detection Kit, catalog number: PF0100 (www.sigmaaldrich.com).
8. NADH-dependent Oxidases Kit-1: Array Bridge NADH Oxidase Landscape, catalog number: AB000502 (www.arraybridge.com).
9. NADPH-dependent Oxidases Kit-1: Array Bridge NADPH Oxidase Landscape, catalog number: AB000503.

References

1. Bischoff, K. M.; L, Shi.; and P. J. Kennelly. 1998. The detection of enzyme activity following Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Analytical Biochemistry 260, 1-17.

2. Weber, K. and D. J. Kuter. 1971. Reversible denaturation of enzymes by Sodium Dodecyl Sulfate. The Journal of Biological Chemistry 246(14), 4504-4509.

3. Kameshita, I.; A. Ishida.; S. Okuno. And H. Fujisawa. 1997. Detection of protein phosphatase activities in Sodium Dodecyl Sulfate-Polyacrylamide Gel using peptide substrate. Analytical Biochemistry. 245, 149-153.

4. Biswas, A. and K. P. Das. 2004. SDS induced structural changes in α-crystallin and its effect on refolding. The protein Journal. 23(8), 529-538.

5. Vincenzini, M. T.; F. Favilli.; C. Treves. And P. Vanni. 1982. Specific interaction among some enzymes and Sodium Dodecyl Sulfate. Life Sciences 31, 463-470.

6. Anderson, N. G. and N. L. Anderson. 1996. Twenty years of two-dimensional electrophoresis: Past, present and future. Electrophoresis, 17, 443-453.

7. Langen, H.; D. Rader.; J-F. Juranville. and M. Fountoulakis. 1997. Effect of protein application mode and acrylamide concentration on the resolution of protein spots separated by two-dimensional gel electrophoresis. Electrophoresis, 18, 2085-2090.

8. Gorg, A.; G. Boguth.; C. Obermaier.; A. Posch and W. Weiss. 1995. Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients in the first dimension (IPG-Dalt): The state of the art and the controversy of vertical versus horizontal system. Electrophoresis, 16, 1079-1086.

9. Nestler, H. P. and A. Doseff. 1997. A two-dimensional, diagonal sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique to screen for protease substrates in protein mixtures. Analytical Chemistry, 251, 122-125.