Protein Kinase Connect (Small Format PEP)

Catalog #   AB000404

Complete kit for the systematic analysis of protein kinases from a proteome such as cell lysate, tissue or microorganisms.

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, protein kinases is a large family of enzymes involved in many signal transduction pathways, there are over 500 protein kinases in the human genome and many of them play important roles in cancer and metabolic diseases. This Protein Kinase Connect kit will allow the systematic analysis of the protein kinases in any proteome of interest and build the 3-D landscape of this important enzyme family. The information can be used for the further understanding of the biochemical process, identification of novel drug targets and study drug safety among the potential applications.

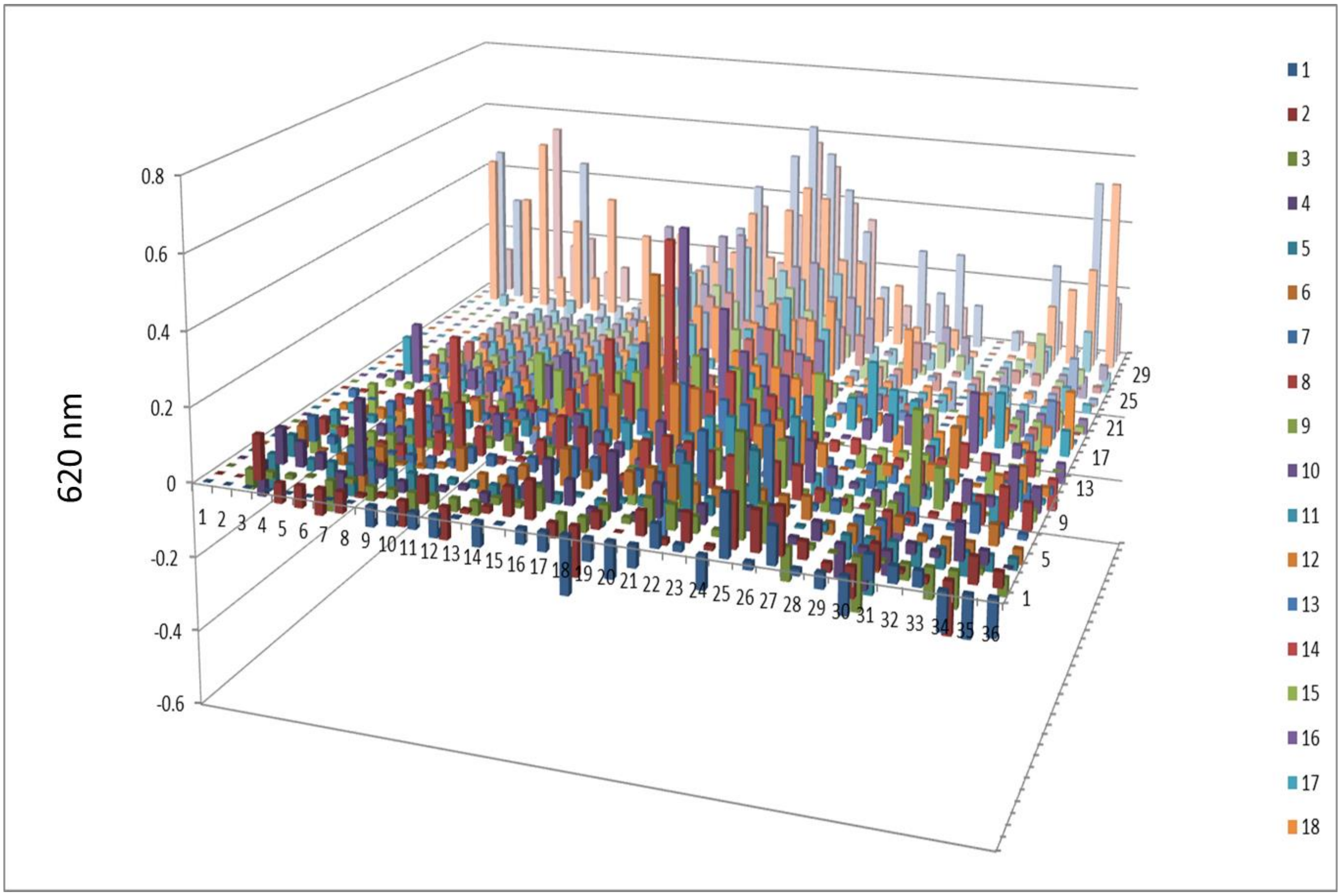
In the PEP technology, protein mixture is first separated by a modified two-dimensional gel electrophoresis, this modified procedure provides good resolution while still maintaining protein function. This is followed by an efficient protein transfer step to a specially designed 384-well Protein Elution 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

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 widely used 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.

Protein Kinase Profile from a Selected Proteome



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 deep-well 384-well polypropylene plate is provided for enzyme assay to identify which wells contain the protein of interest.

4. **384 Well Reading Plate** A standard 384-well plate is provided to read the absorbance from the samples in the Enzyme Assay Plate.

5. **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 to wet the filter papers for the transfer of proteins from the gel to the PEP plate.

6. **10x Tris-HCl Buffer (10 ml)** Buffer used for the pre-treatment of the Master Plate and fill in each well of the Master Plate with 50 µl of Tris-HCl.

7. **PEP Plate Protein Recovery Buffer (25 ml)** Solution used in the PEP plate to recover proteins eluted from the gel and prevent protein diffusion.

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

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:

Kinase Assay Kit: several manufacturers provide protein kinase assay kit, here the Universal Kinase Activity Kit from R&D System (Catalog Number: EA004) is used as an example.

Protein Kinase Substrate: customer will decide on what kind of kinase substrate will be used for this assay, it could be synthesized peptides or protein preparation from a specific source

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 Treatment.**

   High concentration of salt will interfere with the Isoelectric Focusing step. If the protein concentration is less than 5 mg/ml and the salt concentration is more than 100 mM, it is recommended to dialyze the samples in 5 mM phosphate buffer, pH 7.2 before use.

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

   2.1. 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 with up to 200 µg total protein, 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.

   2.2. 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.

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

   2.4. 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.

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

   2.6. 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.

2.7. 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.

2.8. 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.

2.9. 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).

2.10. 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.

3. Protein Transfer after the 2-D Gel

3.1. 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, there will be some overflow of the solution during this step, it is fine. 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.

3.2. 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).

3.3. 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.

3.4. 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).

3.5. 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.

3.6. While the gel is transferring, condition the 384-well deep-well plate by adding 100 µl Tris-HCl buffer in each well. 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 Tris-HCl buffer.

3.7. 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 , 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.

3.8. 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, 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.

4. Protein Kinase Analysis

4.1. **It is important to avoid phosphate buffer when using the Universal Kinase Activity Kit from R&D System, the following is adapted from this kit. Some other kinase assay kits (such as the Promega ADP-Glo Kinase Assay, catalog number: V9101 or the Kinase-Glo Luminescent Kinase Assay, catalog number: V6712).**

4.2. The Master Plate should be used immediately (preferred) for protein kinase analysis. Multiple enzymes (protein kinases, protein phosphatases etc.) 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).

4.3. For the protein kinase assay, transfer 20 µl of sample from each well of the Master Plate into an enzyme assay plate, followed by adding 20 µl of kinase substrate and ATP mixture (kinase substrate could be synthesized peptide mixture or cell extract of customer's choice at a concentration of 1 mg/ml; ATP is added to a final concentration of 2.5 mM).

4.4. Next, dilute the coupling phosphatases into 5 ml phosphatase buffer 4 (both components are provided in the R&D System kit), and add 10 µl to each well of the enzyme assay plate. Incubate the microplate for 60 min at room temperature.

4.5. Terminate the reactions by adding10 µl of Malachite Green Reagent A to each well. Mix by gently tapping the microplate.

4.6. Add 30 µl of deionized water to each well. Mix gently by tapping the microplate.

4.7. Add 10 µl of Malachite Green Reagent B to each well. Mix gently by tapping the microplate.

4.8. Incubate the microplate for 20 min. at room temperature to stabilize the color development.

4.9. Transfer 50 µl from each well to the Reading Plate and determine the optical density of each well using a microplate reader set to 620 nm.

5. Data Transformation and Analysis

5.1. Export the data set to an Excel file (if not already in this format).

5.2. Use Excel Insert function, and select the 3-D display to build the graph of this data set.

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.

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.