NADPH Oxidase Landscape (Large Format PEP)

Catalog # AB000503

Complete kit for the systematic analysis of NADPH-dependent Oxidases from a proteome such as cell lysate, tissue or serum.

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, NADPH-dependent Oxidases is a large family of enzymes involved in many metabolisms including cancer and metabolic diseases. This NADPH Oxidase Connect kit will allow the systematic analysis of the NADPH-dependent Oxidases 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.

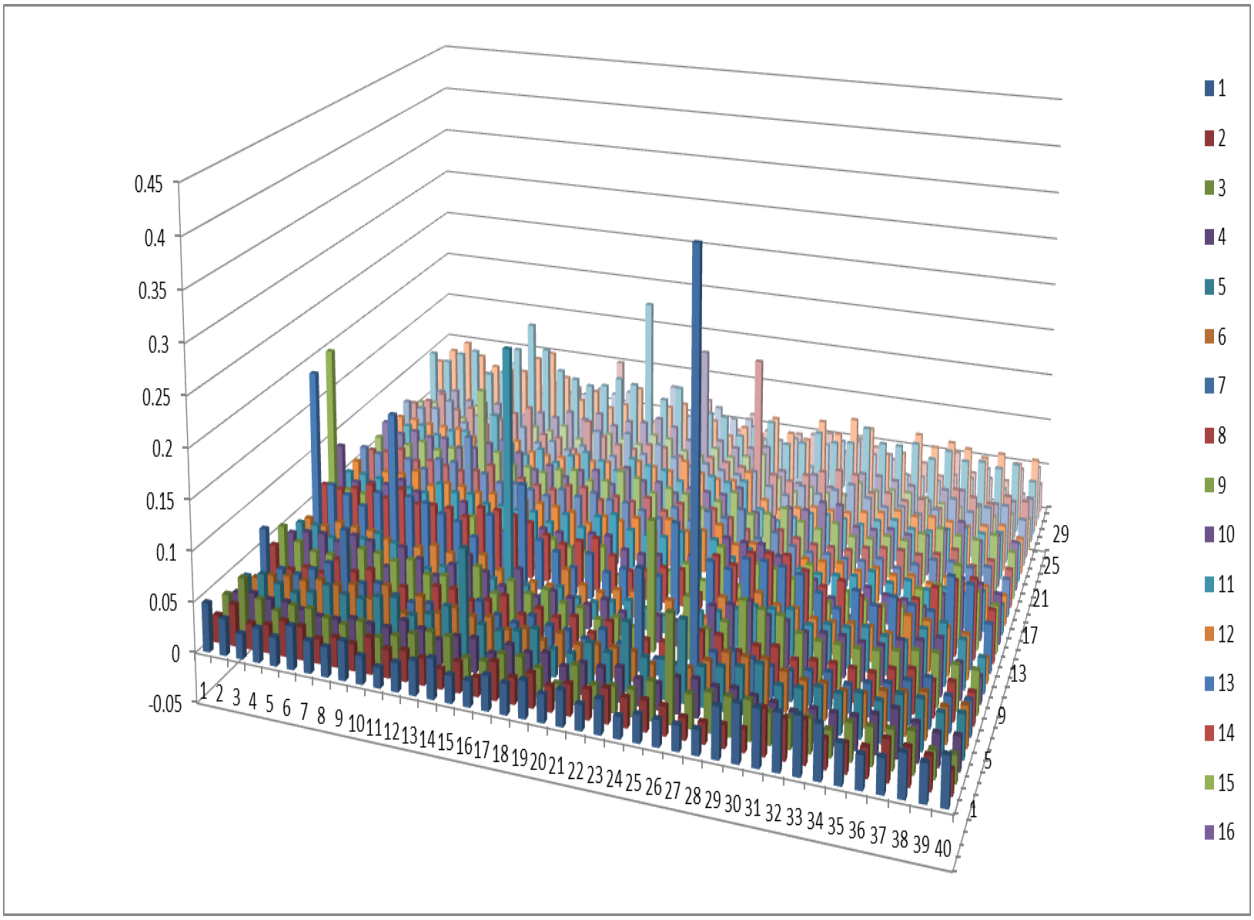
In the PEP technology, protein mixture is first separated by a modified two-dimensional gel electrophoresis, this modified procedure provides good resolution while still maintaining protein function. This is followed by an efficient protein transfer step to a specially designed 384-well Protein Elution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

NADPH-dependent Oxidase from a Selected Proteome



Supplied Components:

1. **1536 Well PEP Plate** An 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 Mater Plates** Four (4) deep-well plates is provided to contain samples recovered from the PEP plate.

3. **384 Well Enzyme Assay Plate** Four (4) standard 384-well polyproperlene plates is provided for enzyme assay to identify which wells contain the protein of interest.

4. **NADPH Oxidase Substrate (100 ml)** A proprietary substrate for the systematic analysis of NADH Oxidases from a proteome, mixed with the NADH stock before use.

5. **NADPH Stock Solution (0.8 ml)** NADH stock at 36 mM, mix with the NADH Oxidase substrate right before the enzyme assay.

6. **20x Protein Transfer Buffer (100 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.

7. **10x PBS (20 ml)** Buffer used for the pre-treatment of the Master Plates and fill in each well of the Master Plate with 50 µl of PBS.

8. **IEF Sample Buffer (0.5 ml)** Solution used to dissolve lyophilized protein samples for the IEF step.

9. **PEP Plate Protein Recovery Buffer (100 ml)** Solution used in the PEP plate to recover proteins eluted from the gel and prevent protein diffusion.

10. **Standard SDS-PAGE Sample Buffer (1 ml)** Solution used in sample treatment for the standard SDS-PAGE to check enzyme fraction purity.

11. **Plate Sealer** For sealing the Master plate and the enzyme assay plate during the purification process. Kit AB-00501 (eight pieces).

12. **Filter Papers** Used to form a sandwich in the protein transfer process (four pieces).

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 that can run at least 18 cm wide IPG strips or to run the sample. One gel supplier is Jule Inc., the 12% Tris-Glycine gel with 1.5 mm thickness was used (Catalog Number: 12D1.5BLC1G)

Isoelectric Focusing strips: Immobilized pH gradient (IPG) strips to run IEF can be purchased from either Bio-Rad (Catalog Number: 163-2033 for 18 cm IPG strips)) or GE Health Life Sciences (Catalog Number: 18101661 for 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.

Incubation time can affect results. All wells should be handled in the same order for each step.

When making additions to the plate, be careful to avoid damaging the antibody coating, for example by scratching the bottoms or the sides of the wells. One technique to avoid this is to make additions (for a right-handed person) from left to right across the plate, supporting the pipette tips on the right edge of the well with each addition and thus avoiding contact with the bottom or sides of the wells.

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. **For running the IEF first followed by running the modified SDS-PAGE**

   2.1. It is suggested to use the 18 cm IPG strip for the IEF. To rehydrate one IPG strip, 325 µl of solution is needed. It is suggested to use 300 µl of samples with up to 2 mg of total protein for the IEF gel, add urea to a final concentration of 8 M, add 2 µl of Biolyte. If the protein sample can be lyophilized, then the lyophilized sample can be dissolved into a sample solution with 8 M urea, 0.5% Biolyte.

   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 to make full contact to the sample solution. Add enough mineral oil to cover the IPG strip to prevent evaporation and incubate 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 Kimwiper paper.

   2.4. In the IEF tray, carefully wet two pieces of electrode wicks (Bio-Rad) and cover the metal wire in lanes where the IPG strips will be run. Carefully lay down the IPG strip face down, and use a tweezer to 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 10,000 V for 4 hours, in the second step, set at constant voltage at 10,000 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 Kimwiper paper to remove the mineral oil from the IPG strips. 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 from the IPG strip and allow the SDS to bind to the proteins.

2.8. Take out a large dimension gel (for example the large format gel from Joule Inc.) and remove the plastic comb, use Mili-Q water to rinse the flat loading well. Put the gel into the running unit (Bio-Rad PROTEAN II xi) 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. If the IPG strip is longer than the dimension of the gel, about 1 cm IPG strip will be removed from the acidic end and the rest of the cut will be from the basic end. The reason for this is that the first 1 cm of the IPG strip at the acidic end is on the outside of the electrode, therefore no proteins are focused in this area. In the basic end, since most of the cellular proteins in a typical proteome have their pI below 8, there are very few proteins close to the basic end of the IPG strip. If however, the protein of interest is a strong basic protein, then more cut will be from the acidic end of the IPG strip.

2.10. Run first at 80 volt for 15 min followed by 150 volt until the dye front from the protein standard is about 0.5 cm from the bottom of the gel.

3. Protein Transfer after the large format modified SDS-PAGE

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 each well with a multiple channel pipette (there will be some solution overflow 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. If a 12 channel pipette is used, the solution could be dispensed every other column. For example, in the first round, add solution to column 1, 3, 5 and so on; in the second round, add solution to column 2, 4, 6 and so on.

3.2. When the gel electrophoresis is completed, carefully take out the gel from the gel cassette and immediately cut a small piece of gel from the upper left corner to make a mark on the orientation of the gel. Rinse the gel with Mili-Q water followed by adding 500 ml of the protein transfer buffer (supplied 10x 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-Blot (Bio-Rad or similar Semi-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-Blot, and transfer the proteins with constant current at 200 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, fill the Master Plate wells with 50 µl PBS (for protein kinase assay or any other assay where phosphate is interfering, Tris-HCl buffer or other buffer without phosphate could be used).

3.7. When the protein transfer is completed, turn off the power, take off the Semi-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 to overflow to adjacent wells). After removing the metal plate, carefully lift the two pieces of filter paper followed by remove the gel. When removing the gel, it is important to remove it from left to right .

3.8. Carefully take the PEP plate together with the two transfer papers still on the bottom of the PEP plate and put in a try or on a glass plate. Use multiple channel pipette to transfer the recovered protein solution from the PEP plate to the deep-well Master Plate in the corresponding columns. The large format PEP plate is marked with P1, P2, P3 and P4 on the left and right side of the plate respectively to indicate which Master Plate the fractions will be transferred to. For example, the upper left part of PEP is marked P1, the fractions from P1 will be transferred to Master Plate-1. This is followed by fraction transfer for Master Plate 2, 3 and 4. If

using an eight channel pipette, please set the transfer volume at 40 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 four 384-well Master Plates. If using a 12 channel pipette, follow the instructions detailed in 3.1 of this section to complete the whole sample transfer process.

4. NADPH Oxidase Analysis

4.1. After the transfer of samples from the PEP plate to the four deep-well Master Plates, the Master Plates can be sealed and stored at 4°C for later use or used immediately (preferred) for NADPH Oxidase analysis. For the NADPH Oxidase activity assay, transfer 30 µl of sample from each well of the Master Plates into the corresponding enzyme assay plates (supplied and marked with enzyme assay plate-1, 2, 3 and 4 respectively), followed by adding 50 µl of enzyme assay substrate in each well. Use a spectrophotometer to measure the enzyme activity at 340 nm, a positive NADPH Oxidase fraction is indicated by the decrease of absorbance at 340 nm at a rate faster than the most of the fractions because only a small portion of the fractions will have NADPH Oxidase activity. Before the assay readout, some wells of the enzyme assay plate may contain bubbles because of the SDS in the protein transfer buffer. Use a transfer pipette tip to pinch the bubbles to get rid of them before the readings, this will reduce the interference from the bubble. (Depending on the purpose of the experiment, multiple enzyme assays can be performed on the same enzyme or different enzymes since each well in the Master Plate has about 80-90 µl solution (50 µl PBS plus 30-45 µl transferred from the PEP plate).

4.2. 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. It is recommended to read at least 3 data points such as 20 min, 60 min and 120 min. and save the reading in separate file.

5. Data Transformation and Analysis

5.1. Convert (or export) the reading from the enzyme assay plate into a text file followed by copy and paste the text file into a Microsoft Excel spreadsheet (some of the plate readers generate the data set in Microsoft Excel directly). Repeat the same process for all the 3 data points: 20 min, 60 min and 120 min. There will be 12 data sets at this time because each enzyme assay plate will have three time point readings.

5.2. For the results from enzyme assay plate-1, in Microsoft Excel, subtract the 60 min readings in each well from the corresponding readings at 10 min to obtain the data set for the 340 nm absorbance difference which reflect the NADPH Oxidase activities from the proteome. Use Excel Insert function, and select the 3-D display to build the graph of this data set, name it: Plate-1 20 min-60 min.

5.3. Subtract the 120 min readings from each well from the corresponding readings at 60 min to obtain the data set for the 340 nm absorbance difference which reflect the NADPH Oxidase activities from the proteome for the second data set. Use Excel Insert function, and select the 3-D display to build the graph of this data set, name it: Plate-1, 60 min – 120 min. This data set and graph will be used to confirm the findings from the first data set in step 5.2.

5.4. Repeat 5.2 and 5.3 for the data sets from enzyme assay plate-2, 3 and 4.

5.5. Build a combined graph by copy and paste the data set obtained in 5.2 and 5.3 for the 4 enzyme assay plates into one combined data set. For example, for the 20 min-60 min data point, copy and paste plate-1 data set in the area of column 1-24, row 1-16; plate-2 data set in the area of column 25-48 and row 1-16; plate-3 data set in the area of column 1-14 and row 17-32; plate-4 data set in column 25-48 and row 17-32. Highlight the whole data set and use Microsoft Excel 3-D graph function to build the combined graph for the proteome.

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

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