Isoelectric Western Blotting (iWB™) after Digital ProteomeChip (dPC™) Focusing

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Introduction

Protein Forest has developed a rapid parallel isoelectric focusing method based on a chip technology. The digital proteome chip (dPC™) has 41 gel features each present at a unique pH. In its typical presentation, this would be a gradient from 4.20-6.20, pH 6.00-8.00 or pH 7.20-9.20, with each gel feature separated by 0.05 pH units. Complex proteomic samples can be separated in 30 to 45 minutes using this technology. We present a method of transferring the contents of the dPC™ to PVDF membranes to allow the immunodetection of the contents of the dPC™, isoelectric western blotting (iWB™). This method is designed to prevent blow-through of protein through the trapping membrane and therefore is more quantitative than conventional Western blotting. Using rat liver lysates, dPC™ separation followed by iWB™ was used to fractionate and detect protein isoelectric point changes. Using this strategy, it is possible to detect potential post-translational modifications (PTM) on a number of examined proteins, including fructose 1,6 bisphosphatase (F1,6 BPase) and HSP 90.

Technology

The Heart of the System: the dPC™ chip

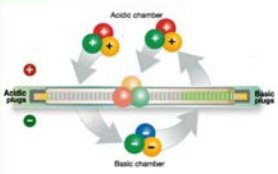

Figure 1: The dPC™ separates complex biological samples into fractions suitable for mass spectroscopy, chromatography and blotting using the principle of parallel isoelectric focusing. Proteins or peptides are charged in the cathode or anode chamber that is either above or below their isoelectric point allowing them to migrate in an electric field. Charged proteins/peptides will migrate back and forth through the dPC™ between the acidic and basic sides of the chip until they encounter a gel plug that is at or near their isoelectric point where they will become neutral. The uncharged protein or peptide will no longer migrate and will become concentrated and separated from other proteins with different isoelectric points.

Protein Forest's parallel fractionation technology platform differs from conventional IPG electrophoresis by allowing proteins to access the entire pH range until they become trapped. Due to the parallel nature of the separation and design of the specialized chamber, a high electrical field strength is generated allowing the proteins to be quickly focused in 30-45 minutes.

Workflow

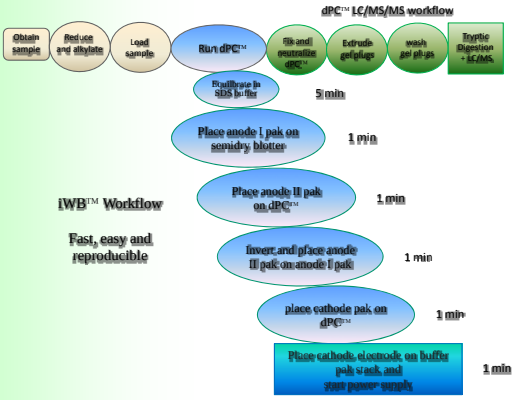

Figure 2: Workflow for dPC™ and iWB™ of protein samples. The dPC™ is a parallel isoelectric focusing technology that separates proteins by pI. The iWB™ quantitatively transfers the contents of the dPC™ onto a PVDF membrane. After transfer to PVDF, proteins are detected by standard immunodetection procedures.

Materials and Methods

Materials:

Semi-dry blotter with two plate electrodes and power supply. Currently we use a blotter with a 10^2 cm surface. Buffer paks of Anode I, II and cathode buffers are packaged by Protein Forest, Inc. Equilibration buffer contains 3M urea, 2% SDS, 25 mM Tris 40 mM Glycine.

Lysates of rat liver or biotinylated ovalbumin samples were reduced and alkylated. Duplicate samples of 30 μg of each lysate or 25 ng of ovalbumin were loaded into a dPC™ running chamber containing a dPC™ (pH 4.20-6.20). After dPC™ running, the dPC™ are briefly rinsed in dI H_20.

Method:

The iWB™ is assembled and run as follows:

1. After dPC™ is run, it is briefly rinsed in dI H_20 and then placed in 35 mL of equilibration buffer for 5 minutes at 70° C.
2. Bottom electrode of semi-dry blotter is Anode (+). All iWB™ components are loaded on top of this electrode
3. Remove Anode I filters from Anode Pak I using forceps and place on Anode electrode. Remove any air bubbles using small roller or 12 x 75 mm culture tube.
4. If running 2 chips, position Anode I filters at the extreme ends of the anode electrode surface to ensure that the upper cathode electrode will rest evenly. If using 3 or more dPC™, space them equidistantly on the surface.
5. Remove Anode II filters and membrane from Anode Pak II using forceps and place on dPC™ with dPC™ handle facing up. Use roller or culture tube to assure that air bubbles are removed.
6. Flip dPC™ and Anode II filters and membrane over and place on top of Anode I filters. Remove bubbles with roller.
7. Remove cathode filters from Cathode Pak using forceps and place on dPC™. Remove any air bubbles using small roller or 12 x 75 mm culture tube.
8. When all chips have been assembled and positioned on the Anode electrode, place the Cathode electrode on top, careful to ensure that the plate is resting evenly over all chips. When Cathode electrode is resting evenly, place a full 1L bottle of water over the center of the plate to ensure complete contact between all layers.
9. Attach + and – leads to a power source and run for 15 minutes with current limited to 15mA and voltage limited to 50V.
10. Once run is complete, remove paper layers and carefully separate PDVF from dialysis membrane. Membrane is ready for blocking and development.

Results

TABLE 1: % PROTEIN BLOW-THROUGH USING RADIOACTIVE PROTEINS: STANDARD WESTERN BLOT VS. QUANTITATIVE METHOD

	Standard Western Blot blow-through	Protein Forest technology to eliminate blow-through
Radioactive ovalbumin (Mr 40-50,000)	23.1%	0.1%
Radioactive myoglobin (Mr 15-20,000)	43.9%	0.1%

Proteins were separated on standard PAGE gels and then transferred to PVDF in presence or absence of a specialized trapping layer that prevents proteins from transitting through the PVDF layer (blow-through). After transfer, sections of the PVDF membrane were excised and counted compared to a second layer of PVDF membrane.

Figure 3: Reproducibility of dPC™ Western Analysis. 0.05 μg biotinylated ovalbumin was fractionated on pH 4.20 to 6.20 dPC™. The ovalbumin was transferred to PVDF membrane using the tank method. After blocking, the blot was probed with HRP-strepavidin and ECL substrate.

Figure 4. dPC™ Western Analysis of F1,6 BPase from a liver lysate fractionated on two pH 4.20 to 6.20 dPC™ for each treatment group. The lysates were transferred to PVDF membrane using the semi-dry blotter. After blocking, the blot was probed with a F1,6 BPase antibody and followed with a anti-Ig HRP conjugate. Blots were visualized using ECL chemiluminescent substrate.

Discussion

Separation of proteins by isoelectric focusing has long been a preferred method in protein characterization. However, the current methods of IEF separation are hampered by lack of reproducibility, long run time and difficulty to automate. We present a novel parallel isoelectric trapping method that is rapid and separates the protein samples into discrete fractions. The iWB™ procedure allows the quantitative transfer of the separated dPC™ contents onto PVDF for analysis by immunodetection methods. The method for quantitative transfer is based on placing a specialized membrane under the PVDF layer of the iWB™. The effectiveness of the membrane at eliminating blow-through is demonstrated in Table 1.

Biotinylated ovalbumin was used as a test compound to work out elution conditions for proteins from the dPC™. Parameters examined included time, voltage and current, in addition to the need for pre-equilibration in a buffer containing SDS. It was found that efficient transfer of proteins to the PVDF membranes was accomplished in less than 20 minutes at low voltage. The use of a specialized retention membrane allowed quantitative capture of protein that normally passes through the PVDF membrane (blow-through). As little as 1 ng of separated and iWB™ transferred protein can be detected.

The efficient transfer of complex protein samples from the dPC™ to PVDF membrane has been demonstrated using mammalian cell lysates (see Figure 4). In this example, fructose 1,6 bisphosphatase, a phosphoprotein, was found to change its pattern of staining in liver lysates from treated animals compared to normal lysates. This is suggestive of changes in charge, which may be due to phosphorylation changes in this protein.

Human plasma and E.coli lysates have also been evaluated using the dPC™ and iWB™ methods (data not presented).

Conclusions

- The dPC™ technology allows for 30 minute fractionation of proteins by pI.
- dPC™ can be directly coupled with LC/MS/MS by in-gel digestion of discrete pH features.
- dPC™ is highly reproducible giving a researcher the opportunity to increase throughput.
- dPC™ followed by iWB™ allows rapid isoelectric separation and detection of proteins.
- iWB™ technology is able to quantitatively eliminate protein blow-through.
- Potential PTM can be observed after the dPC™ separation followed by iWB™ transfer and immunodetection.