Video Article

Highly Sensitive and Quantitative Detection of Proteins and Their Isoforms by Capillary Isoelectric Focusing Method

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Abstract

Immunoblotting has become a routine technique in many laboratories for protein characterization from biological samples. The following protocol provides an alternative strategy, capillary isoelectric focusing (cIEF), with many advantages compared to conventional immunoblotting. This is an antibody-based, automated, rapid, and quantitative method in which a complete western blotting procedure takes place inside an ultrathin capillary. This technique does not require a gel to transfer to a membrane, stripping of blots, or x-ray films, which are typically required for conventional immunoblotting. Here, proteins are separated according to their charge (isoelectric point; pI), using less than a microliter (400 nL) of total protein lysate. After electrophoresis, proteins are immobilized onto the capillary walls by ultraviolet light treatment, followed by primary and secondary (horseradish peroxidase (HRP) conjugated) antibody incubation, whose binding is detected through enhanced chemiluminescence (ECL), generating a light signal that can be captured and recorded by a charge-coupled device (CCD) camera. The digital image can be analyzed and quantified (peak area) using software. This high throughput procedure can handle 96 samples at a time; is highly sensitive, with protein detection in the picogram range; and produces highly reproducible results because of automation. All of these aspects are extremely valuable when the quantity of samples (*e.g.*, tissue samples and biopsies) is a limiting factor. The technique has wider applications as well, including screening of drugs or antibodies, biomarker discovery, and diagnostic purposes.

Video Link

The video component of this article can be found at https://www.jove.com/video/56794/

Introduction

Capillary isoelectric focusing (cIEF) is an automated, capillary-based immunoassay that resolves proteins on the basis of their charge[1,2,3]. It is highly reproducible and capable of resolving proteins and their post-translationally modified isoforms rapidly and quantitatively. It presents an alternative to conventional methods such as western blotting. While western blotting is very good for confirming the presence of abundant proteins in readily accessible samples; variability, time consumption, and accurate quantitation all present challenges, in particular when examining biological tissue samples. Indeed, variability is an inherent problem in western blotting, as there are numerous steps involved, such as loading and running of SDS-PAGE gels, transfer of proteins onto membrane, incubation with various reagents (*e.g.*, primary and secondary antibodies, ECL), and development onto X-ray film[4]. Presently, the western blotting technique is improving with the implementation of digital recording of chemiluminescent signals (digital westerns). Recently, an automated western blotting system has been developed, namely the capillary western, which is a more hands-free and gel-free system. The entire assay is automated following the loading of a sample plate (samples with all necessary reagents) into the system[3,4]. The instrument will perform all the steps such as protein separation, immobilization of proteins onto capillary wall, antibody incubations, washes between different steps, and development and quantification of the chemiluminescent signals. Thus, the cIEF procedure presented here provides higher resolution and sensitivity.

This method is sensitive, as signals can be generated and quantified from picograms of proteins[1]. The high sensitivity with excellent reproducibility makes this technology very useful for the analysis of clinical samples. It can detect as well as distinguish post translational modification (*e.g.,* different phosphorylated protein isoforms) of proteins. This technology has been successfully used to dissect different signaling pathways[4,5] in clinical studies aiming to develop new therapeutics in cancer[3], and it has great potential for protein biomarker and drug discovery.

Protocol

1. Cell Culture, Stimulation, and Lysis

Note: This method can be used with many cell types. To illustrate the method, an example using human umbilical vein endothelial cells (HUVECs) is described.

1. Culture HUVECs on gelatin-coated, 10 cm Petri dishes in endothelial cell basal medium with appropriate supplementation (see **Table of Materials**), and also containing 5% FCS, epidermal growth factor (5 ng/mL), vascular endothelial growth factor (VEGF: 0.5 ng/mL), basic FGF (10 ng/mL), insulin-like growth factor (20 ng/mL), hydrocortisone (0.2 µg/mL), and ascorbic acid (1 µg/mL).
2. Starve cells overnight with endothelial cell basal medium supplemented with 1% FCS and no growth factor supplement.
3. Aspirate all medium, add 2 mL of endothelial cell culture medium without growth factors (starvation medium) in one dish (control), then add 50 ng/mL VEGF in 2 mL of starvation culture medium in a second dish for 7 min.
4. Aspirate the medium and wash cells 2 times with 10 mL room temperature PBS.
5. Ensure the Petri dishes are on ice and keep them there from this step onwards.
6. Add 250-400 µL of ice cold lysis buffer (Bicine/CHAPS; see **Table of Materials**) containing aqueous protease inhibitor mix and DMSO inhibitor mix (Phosphatase inhibitors; see **Table of Materials**) to each 10 cm plate. Swirl the plate to ensure proper coverage and keep it for 10 min on ice.
   NOTE: The final concentration of protease and the DMSO inhibitor mix should be 1x.
7. Scrape the cells from the dish with a cell scraper and transfer to a pre-chilled microfuge tube. Pipette up and down 5 times to lyse cells.
8. Briefly sonicate the cells at 4 °C. Set the sonicator as follows: number of cycles = 5, power = LOW, ON = 5 sec, OFF = 30 s. This step is included to break nucleic acids, and not to lyse cells.
   NOTE: Sonication should be mild to prevent denaturation of proteins.
9. Vortex the tube for 5 s (not continuously), 2 s at a time (3 times), and keep on ice.
10. Clarify lysate by centrifugation at 14,000 x g for 15 min at 4 °C, then immediately transfer the supernatant to a clean pre-chilled microfuge tube.
11. Measure the protein concentration using a Bicinchoninic Acid (BCA) protein assay kit[4].
12. Make 10 µL aliquots, snap freeze in dry ice or liquid nitrogen, and store at -80 °C until use.

2. Sample Mix Preparation (Calculation for 150 µL Sample Mix)

1. First, prepare a sample diluent mix by adding 1 µL of DMSO inhibitor mix (stock 50x) and 2 µL of protease inhibitors (stock 25x) to 47 µL of sample diluent (see **Table of Materials**), so that the final concentration of DMSO inhibitors and protease inhibitors becomes 1x. Next, dilute the protein lysates using the sample diluent mix to obtain the desired concentrations (see step 2.3).
2. Prepare ampholyte/ladder/protease inhibitor/DMSO inhibitor mix by adding 3.325 µL standard ladder (see **Table of Materials**; stock 60x), 6 µL of protease inhibitor (25x), 3 µL of DMSO (50x) inhibitor to 137.675 µL ampholyte premix (see **Table of Materials**). Vortex the tube at least 15 s total, 5 s at a time (3-4 times), and keep on ice.
3. Mix the solutions from steps 2.1 and 2.2 in a 1:3 ratio, so that the final concentrations of DMSO, protease inhibitors, and the pI standard ladder become 1X, and the proteins in the capillary reach the final desired concentrations (*e.g.*, 50 µg/mL was used for **Figure 2**).
   NOTE: Protein concentration in the capillary and well are the same.
4. Load 10 µL of sample mix into the appropriate well, according to the 384-well plate assay template layout (see step 3 and **Figure 1**) and keep the plate on ice.
5. Dilute the primary (pERK1/2, 1:50; ERK1/2, 1:100; HSP 70, 1:500) and secondary antibodies (1:300) with antibody diluent (see **Table of Materials**) and pipette 10 µL of primary and 15 µL of secondary antibodies into each well.
   NOTE: In general, higher concentration of antibodies are required for cIEF assays as compared to conventional western blots.
6. Mix Luminol and peroxide XDR (1:1 ratio; see **Table of Materials**) together and pipette 15 µL into each well.
   NOTE: Mix these solutions fresh every time before use and keep on ice.
7. Once samples and all reagents are pipetted into the plate, centrifuge the plate at 2,500 x g for 10 min at 4 °C to spin down the liquid and remove the bubbles. If bubbles still exist, remove them manually using a thin pipette tip.
   NOTE: Do not load more than 20 µL of sample or reagents per well; otherwise, the instrument pipette cannot be washed properly. Make sure to follow the instruction manual from the manufacturer to prepare all reagents.

3. Designing a New Assay Template with System Software (Figure 1)

1. Open the system software and click "new" from the File menu.
2. Go to the layout pane and assign locations for reagents and samples in a 384 well-plate. Use up to a maximum of 96 wells per 384-well plate.
3. Make a reagent assignment by clicking a well anywhere in the block.Select a row block of 12 wells each. Wells, *e.g.*, from 1-12 or 13-24, are assigned as a block by default.
4. Go to the layout pane tool bar and insert either an empty row block or a sample, primary, secondary, or luminol row block. Each block is assigned a different color, so they are easy to differentiate.
   NOTE: When clicking a well, a black border can be seen around the block where the new block will be inserted in the pane. A row block can also be deleted.
5. Go to the protocol pane and select a reagent location in the plate. Then, click a cell in the sample column and select the reagent from the dropdown menu.
6. In this same fashion, select the reagent locations for the primary, secondary, and luminol for each cycle.
7. Click the cells, one at a time, in the primary or secondary antibody column, and change the incubation times, if necessary.

NOTE: Entry notes for each cycle can also be added.

8. Add cycles by clicking the 'add' button in the protocol section. 1 cycle, 4 cycles, or all cycles (a maximum of 8 cycles can be added to the protocol).
   NOTE: It is possible to copy and paste cycle information in this step: select a cycle, go to edit, and copy and paste.
9. Enter information pertaining to the sample, such as the identity of the primary and secondary antibodies, their catalog numbers and dilutions, *etc.*, in the template pane. This is an optional step that is useful during the post-run analysis.
10. Save the new assay file. Before clicking the 'start' button, quickly check the layout to make sure that everything is correct.

4. Protocol for cIEF Instrument Setting

1. Set capillary isoelectric focusing electrophoresis separation conditions. These should be 15,000 µW and 40 min, and the UV immobilization time should be 80 s. However, the UV immobilization time can be set within the range of 80-140 s, and may need to be optimized for the protein of interest.
   NOTE: A time point for UV immobilization can be set for each cycle, but not for each capillary.
2. Set wash 1 to 2 washes, for 150 s each wash (default), and primary antibody incubation for 120 min.
3. Set wash 2 to 2 washes, for 150 s each wash (default), and secondary antibody incubation for 60 min.
4. Set wash 3, which should be 2 washes, for 150 s each wash (default).
5. Set exposure times when chemiluminescence will be detected; these should be 30, 60, 120, 240, 490, and 960 s. All steps (1-8) will be carried out in a single capillary.

5. Running the Assay File

1. Once everything is ready, click start to begin the run. The software will prompt you to remove the waste, to refill the water and the capillary box, to add anolyte and catholyte to the resource tray, to add wash buffer and finally to load the plate into the cooled sample tray.
   NOTE: Remove the lid from the capillary box, but do not remove the lid from the assay plate. The lid from the plate can be removed automatically by the instrument when necessary. This helps to prevent reagent evaporation.
2. A couple of minutes after the run has been started, an instrument status bar will be displayed on the computer screen. Status messages and progress bars can be seen corresponding to the instrument's current stage.
   NOTE: Initially, the instrument will check if everything is correct before proceeding to pipette the anolyte and catholyte into the separation tray, picking the first block of 12 capillaries, removing the lid of the plate, and placing the samples from the sample plate into the capillaries, and then finally into the separation chamber for electrophoresis.
3. Click on the run summary screen to see two panes: status and separation. Users can view the run progress, and movies of the separation (the 12 capillaries of each cycle) can be stored when the electrophoresis step of each cycle is completed. To observe the electrophoresis separation in a capillary, play the separation movie for that capillary by clicking the desired cycle and then the play button in the control panel.
4. The run file can be stored automatically upon the completion of the run.

6. Analyze the Data (Figure S1)

1. Open the run file and select the analysis screen tab. In **Figure S1A**, the data shown (peaks) in the sample graph is from the selected capillary (*i.e.*, 4th capillary from cycle 1).
2. Click edit and then analysis (**Figure S1B**). At this stage, users can change the pI range, apply the appropriate pI standard for a given experiment, add a peak fit, and add a peak name.
   NOTE: When using a pH 5-8 ampholyte premix (as is the case here), the recommended standard ladder to use is one with fluorescently labeled peptides with pIs of 4.9, 6.0, 6.4, 7.0, and 7.3, and when using a pH 3-10 premix, the recommended ladder is one with pIs of 4.0, 4.9, 6.0, 6.4, and 7.3.[2]
3. Results are displayed in the Peaks tab; the values displayed for the samples are Position, pI, peak Height and Area, % Area, peak Width, and signal to noise (S/N) (**Figure S1C**). Choose which data to use, and export the data for further analysis.
   NOTE: Data can only be exported after labeling the peak (**Figure S1C**).

Representative Results

Design of a new assay: An assay plate layout is shown in **Figure 1A**. Maximally, 96 wells can be used from the 384-well plate in blocks of 12 wells for each condition (antibody). Each block of 12 wells can start either from A1-A12 or A13-A24. Color coded rows allow one to distinguish samples or reagents from one another. In the assay template (**Figure 1B**), relevant information for that particular assay can be stored, which can be used at later stages of result analysis. **Figure 1C** shows the summary of the protocol.

Detection of phosphorylated and unphosphorylated extracellular regulated kinase (pERK1/2 and ERK1/2) protein in HUVEC lysate stimulated with VEGF: In general, post translationally modified proteins, such as phosphoproteins, are difficult to detect because of their low quantity and transient nature, and a lack of specific antibodies. Vascular Endothelial Growth Factor (VEGF) stimulation following serum starvation activates receptor tyrosine kinase (VEGFR2) that leads to activate the MAPK pathway and results in ERK phosphorylation. For experiments using HUVECs, lysates from cells stimulated with or without VEGF for 7 min can be used, as shown in **Figure 2**. **Figure 2A** shows the electropherogram of pERK1/2 from ± VEGF-stimulated lysates. Clearly, with VEGF there is very high induction of phosphorylated proteins (peaks outlined in red). The inset shows the endogenous loading control HSP 70 (**Figure 2A inset**), indicating similar loading of samples for both untreated and treated samples. In conventional immunoblot (**Figure 2C, left panel**) only two bands were detected (phosphoERK1; pERK1 and pERK2). However, pERK1/2 proteins were resolved into 4 peaks for ppERK2, pERK2, ppERK1, and pERK1 by cIEF analysis (**Figure 2A**). Similarly, **Figure 2B** shows the electropherogram of ERK1/2 from ± VEGF-stimulated lysates. The inset shows the same loading control used in parallel. A conventional immunoblot shows only two bands corresponding to ERK1 and ERK2 (**Figure 2C, right panel**) whereas with the cIEF, the pERK1/ERK2 was resolved into 6 peaks (**Figure 2B**) corresponding to all 4 different phosphorylated peaks and 2 unphosphorylated peaks, ERK1 and ERK2. This demonstrates one of the advantages with cIEF, namely that protein isoforms can be resolved and assessed both qualitatively and quantitatively. In addition, it is possible to get quantitative information of phosphorylated isoforms from an antibody directed to the protein core, rather than the posttranslational modification.

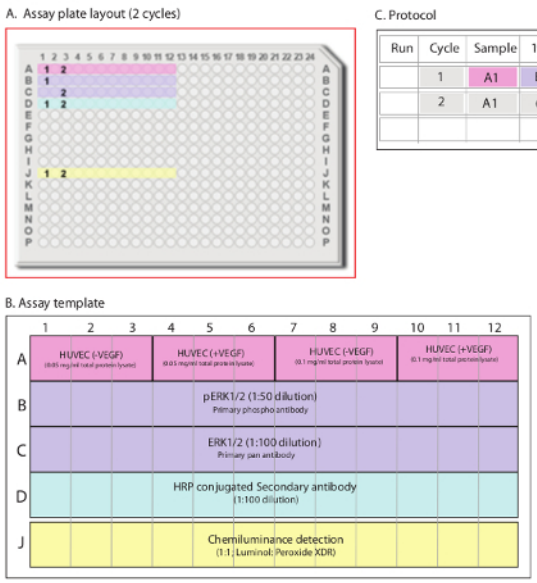

Figure 1: Designing of assay plate layout in software. (**A**) 384-well plate layout defining the position of all reagents. Color-coded 12 well rows showing the location for samples, antibodies, and chemiluminescent reagents. (**B**) An assay template showing relevant information with individual well. (**C**) Protocol showing all the relevant information for cycle 1 and 2. In cycle 1 (12 capillaries as shown in colored box), the instrument takes sample from A1 (wells from A1-A12) followed by primary antibody from B1 (wells from B1-B12), secondary antibody from D1 (wells from D1-D12), and finally luminol:peroxide XDR from J1 (wells from J1-J12). In the 2nd cycle, everything is the same as the first cycle, except it takes primary antibody from C1 (wells from C1-C12). This figure has been modified with permission from Aspinall-O'Dea *et al.* 2015[2]. Please click here to view a larger version of this figure.

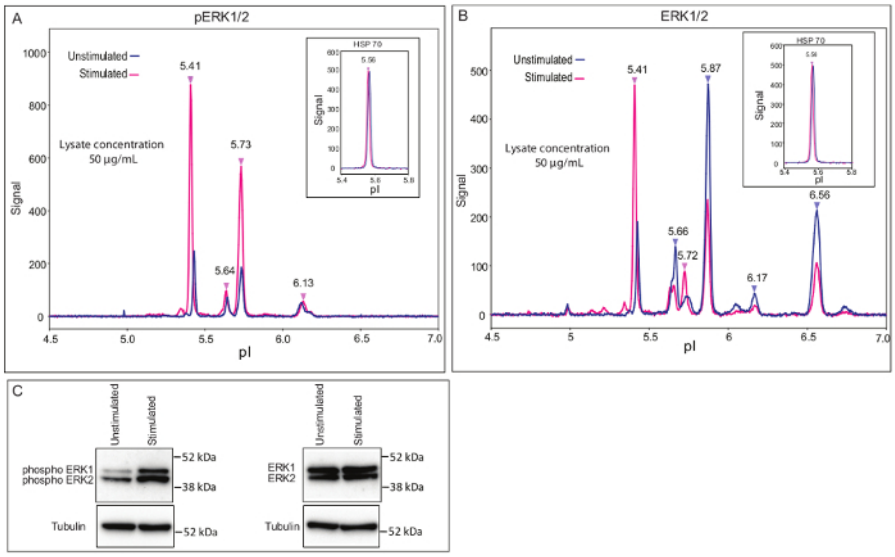

Figure 2: Detection of phosphorylated and total Mitogen-Activated Protein Kinase (ERK 1/2) proteins by cIEF assay and traditional immunoblot assay. (**A**) Representative electropherogram for pERK1/2 and proteins in HUVECs treated ±VEGFA followed by cIEF assay, probed for pERK1/2 antibodies. Blue line (unstimulated) and red line (stimulated). Peaks show the differently phosphorylated pERK/2 isoforms separated on the basis of their isoelectric points. The inset shows the endogenous control HSP70 run in parallel with same lysate. **(B**) Representative electropherogram showing ERK1/2 ± VEGFA-stimulated lysate. The inset shows the endogenous control HSP70 that was run in parallel. For electropherogram, 50 µg/mL lysate concentrations were used, which is equivalent to 20 ng of total proteins in the capillary. (**C**) Conventional immunoblotting for pERK1/2 and ERK1/2 proteins on the ±VEGFA (50 ng/mL) stimulated HUVEC cell lysate. 10 µg of total lysate per lane was used for immunoblotting. Please click here to view a larger version of this figure.

Supplemental Figure S1: Stages of result analysis
(A) View the results from a capillary of interest. (**B**) Edit/add different parameters to the assay file. (**C**) It is possible to add peak names to the peaks before exporting data for further analysis. Please click here to download this figure.

Discussion

Sensitivity and resolution of proteins are crucial for proteomic research on biological samples. There is great value in being able to detect proteins which are present in minute quantities in cells. cIEF can offer improved sensitivity and resolution for the detection of proteins and their isoforms[4].

This technology has been successfully used in many proteomic research reports[5,6,7]. Still, several limitations are associated with it, such as the fact that not all primary antibodies can give a signal even if they work nicely in conventional immunoblots. There is, as yet, no list of validated antibodies shown to function in cIEF, since this technique is quite new. Finally, the instrument cannot handle fewer than 12 samples or more than 96 samples at a time.

cIEF is an alternative technique for conventional immunoblotting, but it may not give identical results. cIEF has been shown to be far superior to conventional immunoblotting in terms of sensitivity and resolution. Moreover, cIEF is high throughput, robust, automatic, robotized, and highly sensitive, yielding signals from fewer than 25 cells depending on the protein analyzed[1]. It is quantitative and highly reproducible, as handling is minimized. The detection of various protein isoforms of similar sizes can easily be resolved. Thus, the same antibody can give quantitative information on phosphorylated and unphosphorylated protein forms from nanogram quantities of sample[4]. Although both conventional immunoblot and cIEF rely on antibody based chemiluminescent detection, there are some important differences, such as in conventional immunoblotting, proteins are separated on the basis of their molecular weight whereas in cIEF, separation is based on the protein charge. Finally, proteins are denatured in immunoblot, whereas they are preserved in their native state in cIEF. This last point is the reason why the same antibody may or may not work in both methods.

Many different studies have demonstrated the application of the cIEF to study protein phosphorylation from human patient samples[3], human colon cancer samples[4,8],mouse tumor tissue[9], or various cell lines[10]. Adoption of this method could promote rapid progress in proteomic research in many fields such as drug discovery, diagnostic purposes, biomarkers discovery, and signaling studies.

Disclosures

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