The Application of a Novel Nanovolume Capillary Electrophoresis-Based Protein Analysis System in Personalized & Translational Medicine Research

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Abstract

There is increasing evidence that abnormal protein synthesis and modification are associated with a variety of human diseases. In the coming era of personalized/precision medicine, it will be required to utilize a rapid, highly sensitive and quantitative method to analyze the proteins and related post-translational modifications in clinical specimens in order to better define specific therapies for patients. However, the current gold standard in proteomic analysis is still the traditional Western blot, which requires many manual steps with lower sensitivity and provides a semi-quantitative read-out. Here, in this manuscript, we present the first report of a novel fully automated Capillary Electrophoresis (CE)-based immunodetection technology, called the Simple Western size assay, which is run on the instrument, called the Simon™. This technology is based on nanovolume size-based protein separation that can be used to quantify proteomic profiles of clinical specimens for both biomarker discovery and diagnostics. Our results demonstrated that the Simple Western has higher sensitivity of target protein detection, a greater linear dynamic range of different molecular weight proteins, high reproducibility and the capacity for the higher-throughput screening of samples using small sample input volumes compared to traditional Western blot analysis. In addition, the quantitiveness and accuracy, the exquisite sensitivity and reduced background noise, has made the Simon Western highly versatile. This technology can quantitate the level of protein and related post-translational modifications in translational medicine research, such as specific biomarkers for diabetes and cancer research. These results based on several broad applications in this study suggest the Simple Western size assay will be a novel potential protein detection accelerator in the personalized and translational medicine era.

Keywords: Simple Western; Protein immunoblot; Biomarker diagnostics; Biomarker discovery; Biomarker validation; Capillary Electrophoresis; Personalized medicine; Ovarian Cancer; Saliva; Diabetes; Size assay

Introduction

Proteins belong to a large family of molecules that are involved in many biological functions necessary for sustaining life and as such, are essential components for organisms [1], which are synthesized from 20 amino acids and extends the range of functions by post-translational modifications with other biochemical functional groups, such as phosphorylation and ubiquitination. The rapid advances in science and technology have increased the understanding of health and disease at the molecular level with more and more evidence suggesting that abnormal protein synthesis and modifications are associated with a variety of disease states [1,2]. In the era of personalized and translational medicine, it is imperative for a novel streamlined process for protein and post-translational modification detection. This process will provide a more sensitive approach, enhancing the drug development process, earlier interventions and better medical outcome.

The Western blot, also called the protein immunoblot, is the most widely used analytical technique to detect specific proteins and related modifications in a given sample of tissue, homogenate or extract. It utilizes gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide [3]. It has been over 30 years since it was first described, and is still considered the “gold standard” for characterization of proteins and related modifications, although many challenges and shortcomings to this method still exist. The traditional Western blot usually consists of the following steps: sample preparation, gel electrophoresis, transfer, blocking and detection. Recently, there are many scientists that have started to streamline/integrate the individual process in Western blot [4]. Currently, there are four methods for image acquisition and

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Received February 09, 2013; Accepted March 20, 2013; Published March 23, 2013

Citation: Liu SB, Sardi S, Sonom B, Zocco D, McSweeney R, et al. (2013) The Application of a Novel Nanovolume Capillary Electrophoresis-Based Protein Analysis System in Personalized & Translational Medicine Research. J Bioanal Biomol 3: 004. doi:10.4172/1948-593X.S3-004

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analysis for Western blot; colorimetric detection, chemiluminescence detection, radioactive detection and fluorescence detection [5]. The traditional chemiluminescence Western blot is still considered the most useful quantitative technique, taking into consideration the intensity time and hands on time.

There have been recent developments of incorporating Capillary Electrophoresis (CE) in the Western blot process. CE is a technique designed to separate ionic species based on their size to charge ratio in the interior of a small capillary filled with electrolytes [5]. It is not only a powerful separation technique with high resolution and reproducibility, but has the capability to detect with high sensitivity detection even at low sample concentrations. CE has gained traction in a variety of areas from pharmacology, toxicology and/or pharmacokinetic profiles to the separations of drug compounds in pharmaceutical and biological media [6,7].

The Simple Western size assay developed by Protein Simple incorporates sample separation performed in glass capillaries followed by UV-triggered immobilization of the proteins directly onto the capillary walls, followed by immuno-probing and chemiluminescence detection. Simple Western size assays are run on the Simon, the instrument manufactured by Protein Simple, is fully automated, that integrates all the manual steps from the start of the experiment to the results associated with performing a traditional Western. Simon performs loading of the lysate, separation, immuno-probing, washing and detection steps all in nanoliter volume capillaries, making it a complete walk-away solution, providing reproducible and truly quantitative results. In this study, we report multiple clinical applications of the Simon system in translational medicine research as a rapid approach to quantitatively detect different protein biomarkers with high sensitivity.

Materials and Methods

Reagents and materials

The antibodies used for this study were purchased from a variety of companies. Antibodies against PTEN, β-catenin, β-actin, cytoplasmic-actin (c-actin), p-eIF2α and GADPH were purchased from Cell Signaling Technology (Beverly, MA). EPRS (Glutamyl-Proyl t-RNA Synthetase) and ghrelin antibodies were purchased from Cell Signaling Technology (Beverly, MA). EPRS (Glutamyl-Proyl t-RNA Synthetase) and ghrelin antibodies were purchased from Biovendor (San Diego, CA) and pLLGL2 (Ser653) antibody from Abgent (Cambridge, MA). CtBP2 antibody was purchased from BD Bioscience (San Jose, CA) and blocked with 5% nonfat dry milk. Polyvinylidene fluoride membrane (PVDF) was obtained from Millipore (Billerica, MA).

Cell culture and cell lysate preparation

Human ovarian cancer cells (MCAS, RMG1, Tov112D and RMUG1), human osteosarcoma cell line U2OS and mouse embryonic fibroblast (MEF) cells were obtained from ATCC (Manassas, VA). Normal human ovarian surface epithelium (HOSE) cells were collected by scraping the ovary surface of the control subjects who were undergoing the procedure of hysterectomy or oophorectomy for benign diseases from the Obstetrics/Gynecology Epidemiology Center at the Laboratory of Gynecologic Oncology at Brigham and Women’s Hospital (Boston, MA). All normal cells and cancer cells were established and grown in a mixture of 199 and MCD105 medium (1:1) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA). The MEF cells were grown in DMEM (Lonza, Allendale, NJ) and 2 mM glutamine supplemented with 4% FBS, non-essential amino acids (1 mM), insulin (1 µg/ml), transferrin (5.5 µg/ml) and penicillin/streptomycin (1 µg/ml). Cells were grown in 60 cm² dishes to 80% confluence at 37°C in an atmosphere of 5% CO₂ and humidified air. Cells were washed twice with ice cold phosphate-buffered saline (PBS) and lysed in Phosphate-Buffered RIPA (Radio Immuno Precipitation Assay Buffer, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40 (v/v), 0.25% Triton X-100 (v/v) and Halt Protease/Phosphatase Inhibitor Cocktail 1X (Thermo Scientific, Waltham, MA). The protein concentrations of cell lysates were determined by the BCA kit (Thermo Scientific, Waltham, MA).

PKC inhibitor cell screen model

U2OS cells were cultured in McCoy’s medium (Invitrogen) supplemented with L-Glutamine, 10% FBS, 25 mM HEPES, Bstactinin (100 µg/ml) and Zeocin (50 µg/ml). Briefly, sub-confluent U2OS cells were transfected with the mouse PKC-iota constructs using Lipofectamine Plus (Invitrogen, Carlsbad, CA) as previously described [8]. The transfected U2OS cells were stimulated with Doxycycline and employed the phosphorylated level of the substrate protein lethal giant larva protein homolog 2 (LLGL2) as functional readout indicator. In this study, the Doxycycline (10 µg/ml) stimulated U2OS cells for 24 hours to induce the expression of PKC-iota, which modified the expression of LLGL2 protein at higher than endogenous levels to facilitate detection, and these cells were prepared and using a BCA protein assay kit, the protein amount in each cell solution was measured and adjusted. The increase in the phosphorylation level of LLGL2 Ser653 was determined by Simple Western size assay and traditional Western blotting method separately.

Clinical tumor tissue collection

The ovarian cancer tissue samples were selected from the Obstetrics/ Gynecology Epidemiology Center and tumor bank at the Laboratory of Gynecologic Oncology at Brigham and Women’s Hospital (Boston, MA). The studies were approved by the Brigham and Women’s Hospital and Dartmouth Medical Center’s Institutional Review Boards. The samples were lysed with RIPA buffer and the protein concentrations were determined by the BCA kit. In Simple Western size assay, 2 µg per sample were loaded for each test.

Saliva sample preparation

The saliva samples from healthy individuals were treated with the ProteoMiner Protein Enrichment Kit (BioRad, Hercules, CA) to concentrate the protein content due to the complexity of saliva. The following fractions including flow through, wash and eluate were collected as described in the ProteoMiner manual and ran on the Simon. For these experiments, the protein of interest was ghrelin, a 4 kDa peptide of the 13 kDa premature protein breakdown product.

Traditional western blots

Cell lysates were separated by SDS/PAGE and transferred to polyvinylidene fluoride membrane (PVDF), and blocked with 5% milk in blocking buffer (10 mM Tris-HCl pH 7.4, 104 mM NaCl, 25 mM NaF, 8 mM NaN₃ and 0.1% Tween 20). The membranes were incubated with primary antibodies (1:1000) against PTEN, β-catenin, CtBP2, β-actin, EPRS, GADPH and c-actin at 4°C overnight. After incubation, the membranes were washed 5 times with TBS-T buffer (10 mM Tris-HCl pH 7.4, 104 mM NaCl and 0.1% Tween 20), and then the membranes were incubated with horse radish peroxidase-conjugated secondary antibodies (1:3000) and developed with the Super signal...
chemiluminescent kit (Thermo Scientific, Waltham, MA) and Kodak film. The scanned bands values were analysed manually in software by Bio-Rad, and normalized with internal loading control.

**Simple Western size assay methodology and protocol overview**

Simon is capable of performing loading, separation, washing, blocking, immunodetection and analysis on 12 samples simultaneously. The teflon-coated silica fused capillaries are 5 centimeters in length with a 100 µm diameter. Reagents including lysate, antibodies, substrate and the separation matrices are pipetted into an assay plate. In each capillary, the proprietary separation gel matrix is loaded, followed by the proprietary stacking matrix (lower ionic strength solution), and finally the samples are injected. This is performed using a precise vacuum manifold to draw the reagents into the capillaries based on defined assay protocol times. As shown in the figure 1A, similar in principle to separation on SDS-PAGE, a voltage is applied, the protein sample plug experiences high electric field (low conductivity buffer) causing the protein sample to migrate rapidly through the stacking matrix before slowing down as it enters the separation matrix. This stacking of the sample results in a condensed population of proteins at the boundary between sample plug and separation matrix [9].

The total volume in the capillary is approximately 400 nanoliters (nL) and the sample injection volume is typically 10% of total volume (40 nL). Proteins are separated based on their size in the capillary by applying voltage (250 volts for 40 minutes). The unique aspect of this technology is that the capillaries are pre-coated with proprietary capture chemistry that is active upon presentation to a UV light source within the instrument, thereby immobilizing the separated proteins to the walls of the capillary. The separated and immobilized proteins are then identified with a primary antibody and subsequent immunodetection using horseradish peroxidase (HRP)-conjugated secondary antibody and chemiluminescence substrate. All immunodetection steps take place within the confines of a capillary. The molecular weight for immunodetected proteins and signal intensity, peak intensity as well as signal to noise ratio are automatically reported by the accompanying system software (Compass). A maximum of twelve samples can be

![Figure 1](image-url)

**Figure 1:** The principle of the Simon Simple Western size assay system. (A) Principle and procedure of the Simon Simple Western size assay system. The workflow comparison (B) and the work time comparison (C) between Simple Western size assay and traditional Western blot. The target proteins used to verify the dynamic range of the Simon system (D).
analysed during one experimental run, which lasts between three and five hours, depending on the antibody incubation times.

The samples and reagents were prepared according to the Protein Simple’s manual. Briefly, the samples (cell lysates, clinic samples, or tissue lysates) were then mixed with 2x Simple Western Sample Master Mix containing 80 mM DTT, 2x Sample buffer, and 2x fluorescence standard (Protein Simple, Santa Clara, CA). Only 5 µl of the 1X cell lysate/Master mix solution will be loaded into the 384-well plate. After a denaturation step at 95°C for 5 mins, the prepared samples, the primary antibodies (1:50 dilution), the secondary antibodies (1:1000 dilution) and chemiluminescent substrate were dispensed into designated wells in a 384-well-assy plate. A biotinylated ladder provides molecular weight standards for each target protein. The prepared assay plate was placed into the Simon followed by the addition of the Simple Western assay buffers into the Simon’s system tray and the insertion of a capillary clip into the clip holder. Note that the capillary clip needs to be replaced after each run. All subsequent separation, immunodetection and analysis steps were performed automatically by the Simon. Using Compass software for the Simon, the resulting data can be reviewed in three different viewing formats. The image view provides visualization of the detected chemiluminescent signal recorded by the CCD camera across the capillaries. The intensity of the bands is directly proportional to the amount of target protein present in the respective capillary. The graph view generates an electropherogram image for each target protein which has an immunodetected signal. The area under the curve, peak height and peak width are all recorded for each named peak, which represents the signal intensity of the chemiluminescent reaction from the HRP-conjugated secondary antibody and chemiluminescent substrate, which directly correlates with the amount of target protein present in the capillary. Lastly, the lane view is a virtual Western blot interpretation of the electropherogram data which depicts the results for each capillary.

Applications using the Simple Western Size Assay Methodology

In this section we briefly describe four applications and their significance in which we utilized the Simple Western size assay methodology and the corresponding results to each application are explained in the results and discussion section.

Anti-Cancer kinase inhibitor screening model

The human osteosarcoma cell line, U2OS is one of the first generated cell lines broadly used in various areas for biomedical research including chromosomal instability, structural rearrangements and alterations. U2OS cells express two tumor suppressor genes p53 and Rb but lack p16 [8]. In addition, U2OS cells exhibit epithelial adherent morphology and without virus contamination. Protein kinase C is a family serine-threonine phosphorylation enzyme activated by various stimulations. The activated PKC phosphorylates the substrate protein to produce a predetermined cellular response and to exhibit a physiological function. Previous research indicated that LLGL2 is a substrate downstream of PKC-iota in the signal transduction pathway that regulates the life support in cancer cells, and the LLGL2 Ser653 phosphorylation level is dependent on the kinase activity of PKC-iota. In the overexpressing PKC-iota U2OS cell, we employed the LLGL2 Ser653 phosphorylation level as a readout indicator for evaluation of an inhibitor’s effect on the kinase activity of PKC-iota, and measured different gradient concentrations using the Simon system as compared with traditional Western.

Pharmacological research applied to traditional medicine

Traditional medicine, also known as indigenous or folk medicine, comprises knowledge systems that developed over hundreds of years within various societies before the era of modern medicine. Traditional medicine, as practiced by indigenous people today, relies on its own version of “clinical trials”, where natural products continue to be used only if they have been shown to be effective, although its pharmacologic mechanism is unclear [9]. People have become inspired by traditional medicine and what it can offer in reference to a novel process for drug discovery, combined with a reverse pharmacology approach [10].

Halofuginone is a halogenated derivative of febrifugine, a compound extracted from the plant Dichroa Febrifuga that has been used as an anti-malarial drug in traditional Chinese medicine. It has received orphan drug designation from the U.S. Food and Drug Administration (FDA) for Scleroderma [11]. In addition, halofuginone has been shown to target EPRS to promote amino acid starvation (stress) and induce phosphorylation of eIF2a [12]. The eIF2a protein is phosphorylated and activated in response to cellular stress, including endoplasmic reticulum, viral infections and, as with halofuginone, by amino acid starvation. Upon its activation, eIF2a acts as an inhibitor of translation to allow cells to adapt to stress [12]. We used the Simple Western size assay to monitor the expression level of EPRS in MEF cell lysates, and compared the performance and reproducibility with that of traditional Western.

Detection of ovarian cancer biomarkers

Ovarian cancer is the leading cause of death among gynecologic diseases in Western countries [13]. Early detection and early intervention is critical to improve the survival of ovarian cancer patients. The need to identify novel tumor biomarkers and key players involved in their pathogenic pathways are necessary and require accurate and sensitive detection methods that will have impact from a clinical diagnostic and therapeutic perspective. An increasing number of expression studies have indicated some tumor suppressors, such as PTEN [14-16], β-catenin [17] and CtBP2 [18], to be associated with embryonic development and tumorigenesis signaling pathways. We used the Simple Western size assay to quantitatively compare the protein expression patterns of three tumor suppressors: PTEN, β-catenin and CtBP2, in normal human ovarian epithelium and four human ovarian cell cancer lines: MCAS and RMUGL (two mucinous epithelial ovarian cancer types), RMG1 (Clear cell ovarian cancer type) and TOV112D (endometrioid ovarian cancer type) and compared their performance and reproducibility with that of traditional Western.

Clinical diabetes biomarker diagnostics

Ghrelin, a human growth hormone releasing peptide with a stimulatory effect on food intake, energy expenditure and fat accumulation, has been demonstrated to be produced and released by salivary glands, stomach, and placenta [10,19]. Recent studies have shown ghrelin levels to be associated with obesity, lipid metabolism disorder and anorexia nervosa [20]. More interesting, the level of ghrelin in saliva has the potential to be a diagnostic biomarker for type II diabetes and prostate cancer [21-23]. Proper sample collection, sample preparation and a sensitive detection method for ghrelin will have broad applications in clinical diagnostics. In this application, we
implemented the Simple Western size assay to quantitatively detect ghrelin in patient saliva samples.

Data analysis

The data from the Compass software are presented as means ± standard error of the mean (SEM). Significance of differences for the values were determined using the student t-test with the Prism software (GraphPad Software, Inc. San Diego, CA). A p-value less than 0.05 was considered a significant difference. We chose to measure data consistency by calculating the coefficient of variation (CV). The CV is defined as the variation among multiple measurements in proportion to their mean. We used % CV to measure the reproducibility among multiple experimental values [10].

Results and Discussion

The Simon system accelerates protein detection with broad dynamic range

The Simon technology is a benchtop instrument capable of processing 12 samples simultaneously with Simple Western size assays, which are size-based assays equivalent to SDS-PAGE (Figure 1A). The samples for Simple Western size assays are treated with SDS/DTT and heat denatured, and then loaded into capillaries, separated by molecular weight and immobilized to the capillary wall via a proprietary UV capture method. Target proteins are probed with a specific antibody followed by HRP-amplified chemiluminescent detection in order to obtain a quantitative signal for analysis. Unlike traditional Westerns, the Simon system has the capacity to use different IgG controls to normalize the background and allows for the screening of up to 11 different target antibodies individually during a single run without having to cut membranes and probe with respective antibodies. This attribute greatly reduces manual labor time and the time to carry out the experiment as well as potential user induced errors.

Reproducibility of results can be challenging using traditional Western methods mainly due to the “protein transfer” step. The workflow of the Simple Western size assay excludes the excessive manual steps and hands-on processing time of the traditional Western, reducing the ten-step traditional Western to three steps - to obtain a fully analyzed and quantitated report from the Simple Western size assay. The total run time for the Simon system is about 3-5 hours depending on the antibody incubation times. As shown in the figure 1B, compared with the traditional Western blot, Simon allows for the automation of the entire Western blot procedure, which results in increased reproducibility and reduced-cycle time in receiving the results (Figure 1C). The Simon system also has good extension ability and could be combined with robotics for sample preparation in 384 well plates and reduce the run time to 1.5 hours.

In this study, we evaluated the Simple Western size assay methodology in four different applications described above, which tested several different targets proteins that ranged from 4 kDa to 160 kDa (Figure 1D) to verify the technology’s dynamic range.

Enhanced sensitivity of anti-cancer kinase inhibitor screening model

An anti-cancer kinase inhibitor screening model was utilized to test different gradient concentrations (from 0.8 to 0.05 μg/ul) of U2OS (Dox induced) cell lysates by Simple Western size assay and traditional Western using phosphoLLGL2-specific primary antibody as a probe. As shown in the Simple Western lane view and accompanying electropherogram image (Figures 2A and 2B), the dynamic concentration range of cell lysate exhibited a good detection signal and the lower detection levels of phosphoLLGL2 protein were down to 0.05 μg/ul of total protein. The measured target peak area values showed a tight linear relationship with the total protein concentrations (R²=0.9655, Figure 2C). Furthermore, the measured values of target protein molecular weights showed little variation (Figure 2D). The same cell lysate titration experiments were performed in parallel by traditional Western analysis. Our results indicated that, for the traditional Western, the dynamic range for pLLGL2 in cell lysate was less linear with an R² value of 0.9003 (Figures 2E and 2F). These results indicate that the Simon system is a more sensitive and reliable detection method for LLGL2-Ser653 phosphorylation level readout in the U2OS cell based kinase inhibitor screen model.

Accelerating traditional medicine pharmacological research with the Simple Western size assay

To demonstrate the biological/clinical utility of the Simple Western size assay to monitor biological responses, MEF cells were treated for two hours in the presence or absence of halofuginone and the phosphorylation of eIF2α was examined. We utilized the Simple Western size assay to normalize the expression level of the EPRS protein, which has a molecular weight of approximately 160 kDa in MEF cell lysates. As shown in the figure 3A, EPRS is clearly visible and exhibited minimal variation as seen from the measured peak area and molecular weight values from multiple replicates (Figures 3B and 3C). To assess the reproducibility of experiments to that of a traditional Western, we employed the coefficient of variation (CV) of experiments as a quantitative metric. The % CV for signal intensity is 0.02%, which was calculated from eleven different MEF cell lysate sample preps that were probed with the EPRS antibody. In parallel, the expression level of EPRS was analysed by traditional Western from the same lysate batch, and the % CV was calculated to be 1.09% (Figures 3D and 3E, n=11). These results indicate that the Simple Western size assay is highly reproducible, approximately 65 fold greater than the traditional Western approach (Figure 3F, p<0.001). This notable reproducibility is attributed to the absence of a “protein transfer” step in the Simple Western workflow and in addition, the results are quantitative.

Five untreated samples were loaded along with six samples treated with halofuginone. A phospho-eIF2α specific antibody was used as the probe, and the results were automatically generated by the system software (Figure 4A). A 2.6-fold induction of phospho-eIF2α was observed upon treatment of MEF cells with halofuginone (Figure 4B).

Using the Simple Western as a smart tool for ovarian cancer biomarker discovery

In this application, it’s necessary to measure the typical tumor biomarker protein level in the patient’s tissue sample for clinical diagnostics. We used the Simple Western size assay to compare the protein expression pattern of three tumor suppressors, PTEN, β-catenin and CtbP2, in normal human ovarian epithelium and four human ovarian cancer cell lines: MCAS and RMUGL (two mucinous epithelial ovarian cancer types), RGM1 (Clear cell ovarian cancer type) and TOV112D (endometrioid ovarian cancer type). The PTEN expression pattern is shown in figure 5A. The MCAS, RGM1 and RMUGL cell lysates have shown to have significantly higher expression than normal ovarian epithelial cells. Protein expression levels for PTEN in the HOSE and Tov112D cell lysates, although lower compared to the other cell lysates, were visible. Upon analysis of the levels of β-catenin in the
Figure 2: The comparison results for the linear dynamic range of Simple Western size assay and Western blot in anti-cancer kinase inhibitor screen cell model. The U2OS cell lysate was gradient diluted and analysed using pLLGL2-specific primary antibody. (A) Simple Western size assay lane view. The numbers (1&2) mean the samples were loaded in duplicate. The un-stimulated U2OS cell lysate was used as negative control. (B) Electropherogram and system software generated peak area and molecular weight data linear analysis (D). The traditional Western blot result (E) and bands intensity linear analysis (C, F).

Figure 3: The comparison of EPRS detection via Simple Western size assay and Western blot. Eleven replicates (n=11) of MEF cell lysate were analysed by using EPRS-specific primary antibody. (A) Simple Western lane view with results automatically generated by system software. (B) Normalised peak area and (C) Normalised MW graphs for the Simple Western. (D) Western blot with results analysed manually using a scanner. (E) Band volume intensity graph for the Western blot. (F) % CV graph.
Figure 4: The application of Simple Western size assay in investigation of the traditional medicine halofuginone pharmacologic effect on MEF cells. The Simple Western lane view (A) and automatic quantitative analysis of phosphorylation level of eIF2α (B) by system software. ** present the p<0.001.

Figure 5: The normal and ovarian cancer cell lysates were analysed by Simple Western size analysis using the respective specific primary antibodies. The lane views, electropherogram and data analysis generated automatically by system software for PTEN (A), β-catenin (B) and CtBP2 (C).
cell lysates, the MCAS and Tov112D cell lysates displayed significantly higher levels of β-catenin than normal epithelial cells (Figure 5B). There are no notable differences between RMG1, RMUGL and normal. In the CtBP2 expression profiles, darker bands were seen in the MCAS, RMG1 and RMUGL cell lysates as compared to the normal HOSE cells, while in the Tov112D lanes a faint band was noted (Figure 5C). Using the current gold standard the traditional Western, only distinct bands were seen in the MCAS and Tov112D lanes for β-catenin, and in MCAS, RMG1 and RMUGL for PTEN, and fairly weak bands in MCAS, RMG1 and RMUGL for CtBP2 (Supplementary Figure 1). From these results we concluded that the Simple Western size assay provided a more sensitive measurement for the target proteins with lower expression levels as compared to the traditional Western. These data supported our hypothesis that the Simon system could provide a clearer, more sensitive and quantitative profile for the expression of the tumor suppressors.

However, the supply of clinical tissue samples is very limited and thus requires highly sensitive detection methods to analyze them. In this component of the study, 2 µg of each clinical tissue lysate (330 and RB088), which were prepared from clinic ovarian cancer patients, were loaded on the Simon system to test the protein expression patterns of tumor suppressors PTEN and β-catenin. Cell lysates of HOSE and MCAS were our controls. As shown in the figure 6A, PTEN bands were clearly visible at 57 kDa with different expression levels in two clinical samples (Figures 6A and 6B), and close to the characterized molecular weight (Figures 6C and 6D) in the HOSE and MCAS. Interestingly, we found that the β-catenin expression pattern of two clinical tissue samples were different from the cell lysates of HOSE and MCAS. The molecular weight of β-catenin detected in the sample 330 and RB088 were 60 and 68 kDa, respectively, while in the normal cells (HOSE) was at 87 kDa (Figures 6A and 6D). This result suggests that there are potential genetic deletions of β-catenin in these cancer patient tissue lysates, which matched the previous report that there were β-catenin gene deletions linked to some populations of ovarian cancer patients [19].

Taken together the results indicate that the Simple Western size assay protocol has the capability to measure protein expression with higher sensitivity and with minimal assay optimization compared to traditional Westerns and thus has broad applications in cancer biomarker discovery.

Applying the Simple Western in clinical diabetes biomarker diagnostics

In this application, the Simple Western size assay was used to detect levels of ghrelin expression from patient saliva samples. As demonstrated in the Simple Western lane view and accompanying electropherogram image (Figures 7A and 7B), a strong 4 kDa band for the ghrelin protein was detected in the elution fraction, which in this case of the Simple Western, is outside the lower limit of accurate size detection at 12 kDa. These results suggest that the Simple Western size

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**Figure 6**: The expression profiles of oncogenes β-catenin and PTEN in clinic ovarian cancer patient's tissue determined by Simple Western size assay. The lane view (A) and system software generated electropherogram (B), expression peak area (C) and molecular weight (D) analysis for the oncogene expression pattern in clinic patient tissue lysates 330 and RB088.
The Simple Western will serve as a potential accelerator for protein detection utilizing the novel Simon Simple Western technology. As shown and described in the results section, the Simple Western size assay has demonstrated its advantage over traditional Westerns. Advantages include a higher sensitivity of target protein detection, a greater linear dynamic range of different molecular weight proteins, high reproducibility and the capability for the higher-throughput screening of samples using small sample input amounts (in this study, the lowest total protein amount was 0.05 µg). The quantitativeness and accuracy, along with high sensitivity and less background noise, has made the Simon highly versatile as compared to results observed using traditional Western blot analysis. These unique features are particularly useful in the personalized and translational medicine era, which requires a higher standard for protein detection in a variety of diseases involving protein modifications and signalling pathways analysis. The Simple Western will serve as a potential accelerator for protein detection via immune detection. Furthermore, in clinical diagnostics, many highly biohazardous patient samples, such as blood and plasma from patients with HIV infection, hepatitis B infection, Lyme disease, bovine and spongiform encephalopathy (BSE), commonly referred to as ‘mad cow disease’, need verification/validation of their biomarkers by immune detection, which would be challenging utilizing traditional Western blot approach. The application of the automated Simon system which runs the Simple Western size assays will significantly lower the risk for medical research scientists when processing these samples, as well as increase the benefits to patients based on a faster and more accurate clinical diagnosis.

In summary, the broad applications of the Simon Simple Western size assay system will accelerate protein detection via immune detection in the personalized and translational medicine era providing a significant benefit to patient care and patient outcomes.

Acknowledgments

This work was conducted with support from Harvard Catalyst | The Harvard Clinical and Translational Science Center (National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health Award 8UL1TR000170-05 and financial contributions from Harvard University and its affiliated academic health care centers). The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, Harvard University and its affiliated academic health care centers, the National Center for Research Resources, or the National Institutes of Health. We would also like to thank Protein Simple and their team (Peter Fung and Michele Daht) for providing technical support, the Simon instrument, reagents and cartridges to complete this study.

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