

# Chapter 16

## Applications of an Automated and Quantitative CE-Based Size and Charge Western Blot for Therapeutic Proteins and Vaccines

Richard R. Rustandi, Melissa Hamm, Catherine Lancaster, and John W. Loughney

### Abstract

Capillary Electrophoresis (CE) is a versatile and indispensable analytical tool that can be applied to characterize proteins. In recent years, labor-intensive SDS-PAGE and IEF slab gels have been replaced with CE-SDS (CGE) and CE-IEF methods, respectively, in the biopharmaceutical industry. These two CE-based methods are now an industry standard and are an expectation of the regulatory agencies for biologics characterization. Another important and traditional slab gel technique is the western blot, which detects proteins using immuno-specific reagents after SDS-PAGE separation. This technique is widely used across industrial and academic laboratories, but it is very laborious, manual, time-consuming, and only semi-quantitative. Here, we describe the applications of a relatively new CE-based western blot technology which is automated, fast, and quantitative. We have used this technology for both charge- and size-based CE westerns to analyze biotherapeutic and vaccine products. The size-based capillary western can be used for fast antibody screening, clone selection, product titer, identity, and degradation while the charge-based capillary western can be used to study product charge heterogeneity. Examples using this technology for monoclonal antibody (mAb), Enbrel, CRM197, and *Clostridium difficile* (*C. difficile*) vaccine proteins are presented here to demonstrate the utility of the capillary western techniques. Details of sample preparation and experimental conditions for each capillary western mode are described in this chapter.

**Key words** Capillary western, Western blot, Mab, *Clostridium difficile* vaccine, CRM197

---

### 1 Introduction

Since the introduction of the western blot by Towbin and Renart [1, 2], it has become one of the most important methods in protein characterization, especially in complex cell lysates. The use of the western blot has increased tremendously in many laboratories worldwide over the last 30 years. The western blot can be considered as a pseudo two-dimensional analytical technique where in the first dimension protein is separated by either size or charge using gel electrophoresis and in the second dimension the

separated protein of interest is immuno-blotted. During this immuno-blot the protein of interest is probed using a specific antibody (primary) and detected by a labeled secondary antibody against the Fc-region of the primary antibody. The secondary antibody is usually labeled with an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) or other fluorescence agents [3]. Even after three decades, traditional westerns are still performed manually, are labor intensive, only moderately reproducible, and qualitative or semi-quantitative at best [4, 5].

In recent years, CE has emerged as an indispensable tool to analyze proteins due to its high resolving power, quantitative nature, relative speed, and the potential to automate. One of the many contributions of CE in the biopharmaceutical industry is the replacement of traditional, labor-intensive SDS-PAGE (size) and IEF (charge) slab gel electrophoresis by CE-SDS (CGE) and CE-IEF methods, respectively [6–13]. In 2006 the first capillary IEF-based western was introduced [14] and subsequently in 2011 the capillary SDS-PAGE-based western was introduced with the first commercial instrument called Simon™ from ProteinSimple [15, 16]. Over a 3-year period, several versions of this technology have come out with various names: Sally, Peggy, Sally Sue, Peggy Sue, and Wes, all collectively called Simple Western. Simple Western technology is a size and charge CE-based western blot system that provides advantages of being fully automated in operation, except for the sample preparation, quantitative and reproducible with CV <10% [16, 17]. Technical details of the Simple Western technology have been described in several publications [15–18]. Briefly, proteins are separated in a capillary by either molecular weight (size) or pI (charge). The capillaries contain a proprietary UV-induced cross-linked chemical that covalently binds proteins to the wall of the capillary after exposure to UV light. After proteins are bound, the capillary is washed to remove the separation matrix and each capillary is incubated with the target-specific primary antibody followed by the species-specific secondary HRP-labeled antibody. Lastly the capillaries are incubated with a luminol and peroxide mixture to develop the chemiluminescence signal which is then recorded with a charge-coupled device (CCD) camera across all capillaries. All the above operations are fully automated including the electropherogram signal integration. We have recently evaluated this western technology for qualitative and quantitative analysis of vaccine protein components, residual bovine serum albumin (BSA), dot blot for 15-valent pneumococcal conjugate vaccine, and enzymatic activity for PARP-1 [16, 19–21]. Here we provide detailed protocols for the Simple Western method and examples of its application such as clone selections, rapid identity test, heat stress stability, and acidic variants biological activity in vaccine and therapeutic proteins. A monoclonal antibody, a heavily sialylated Fc-fusion glycoprotein for joint inflammation pain (Enbrel), CRM197, and four toxin proteins from *C. difficile* vaccine will be discussed.

## 2 Materials

### 2.1 Size-Based Western

1. Sally, Sally Sue, Peggy, Peggy Sue, Wes (ProteinSimple) (*see Note 1*).
2. UV-activated clear Teflon coated bare fuse silica capillary 5 cm long with 100  $\mu\text{m}$  ID and 375 OD  $\mu\text{m}$  (ProteinSimple) (*see Note 2*).
3. SDS-containing separation matrix (ProteinSimple) (*see Note 3*).
4. Stacking matrix (ProteinSimple) (*see Note 3*).
5. Running buffer (ProteinSimple) (*see Note 3* and *Note 4*).
6. Matrix removal buffer (ProteinSimple) (*see Note 4*).
7. Concentrated wash buffer (ProteinSimple) (*see Note 4*).
8. Luminol S (ProteinSimple) (*see Note 5*).
9. Peroxide (ProteinSimple) (*see Note 5*).
10. Sample buffer 10 $\times$  (ProteinSimple) (*see Note 5*).
11. Streptavidin HRP (ProteinSimple or Jackson Immuno Research) (*see Note 5*).
12. Goat anti-rabbit HRP (ProteinSimple) (*see Note 6*).
13. Goat anti-mouse HRP (ProteinSimple) (*see Note 6*).
14. Antibody Diluent (ProteinSimple) (*see Note 7*).
15. Fluorescent standard markers (ProteinSimple) (*see Note 8*).
16. Biotinylated molecular weight markers (ProteinSimple) (*see Note 9*).
17. D,L-Dithiothreitol (DTT).
18. Iodoacetamide.
19. 20% SDS solution.

#### 2.1.1 Master Mix (2 $\times$ ) Reducing Solution Preparation (Final Volume 120 $\mu\text{L}$ ) (See Note 10)

1. One vial lyophilized fluorescent protein markers, 2% SDS, 2 $\times$  sample buffer, 200 mM DTT.
2. Vortex and place at 22–25  $^{\circ}\text{C}$  for up to 1 day only.

#### 2.1.2 Molecular Weight Standards Preparation (Final Volume 20 $\mu\text{L}$ ) (See Note 9)

1. One vial lyophilized biotinylated molecular weight markers, 1 $\times$  sample buffer, 100 mM DTT.
2. Vortex until all dissolved.
3. Place in 90  $^{\circ}\text{C}$  heating block for 10 min.
4. Equilibrate at room temperature for 10 min and centrifuge it to collect all liquid.
5. Transfer 5  $\mu\text{L}$  of sample into a 384-well plate, avoid bubbles while transferring.
6. Store remaining MW markers at  $-70^{\circ}\text{C}$  for up to 1 year.

### 2.1.3 Reducing Condition Sample Preparation

1. Place 4  $\mu\text{L}$  sample in 500  $\mu\text{L}$  Eppendorf Tube.
2. Add 4  $\mu\text{L}$  of 2 $\times$  prepared master mix solution.
3. Place in 90 °C heating block for 10 min (*see Note 11*).
4. Equilibrate at room temperature for 10 min and centrifuge it to collect all liquid.
5. Transfer 5  $\mu\text{L}$  of sample into 384-well plate, avoid bubbles while transferring.

## 2.2 Charge-Based Western

1. NanoPro 1000, Peggy, and Peggy Sue (ProteinSimple) (*see Note 12*).
2. UV-activated clear Teflon coated bare fuse silica capillary 5 cm long with 100  $\mu\text{m}$  ID and 375  $\mu\text{m}$  OD (ProteinSimple) (*see Note 2*).
3. Anolyte (100 mM NaOH).
4. Catholyte (10 mM Phosphoric acid).
5. Wash Concentrate (ProteinSimple).
6. Pharmalyte Ampholyte, pH 5–8 (GE Healthcare).
7. Pharmalyte Ampholyte, pH 8–10.5 (GE Healthcare).
8. Luminol (ProteinSimple) (*see Note 13*).
9. Peroxide XDR (ProteinSimple) (*see Note 13*).
10. Bicine Chaps (ProteinSimple) (*see Note 14*).
11. Antibody Diluent (ProteinSimple).
12. pI Standard Ladder 1 (ProteinSimple) (*see Note 15*).
13. pI Standard Ladder 3 (ProteinSimple) (*see Note 15*).
14. pI Standard 8.4 (ProteinSimple) (*see Note 16*).
15. pI Standard 9.7 (ProteinSimple) *see Note 16*.
16. Premix Ampholyte Free (ProteinSimple) (*see Note 17*).
17. Premix Ampholyte 3–10, G2 (ProteinSimple) (*see Note 17*).
18. Premix Ampholyte 4–7, G2 (ProteinSimple) (*see Note 17*).
19. Premix Ampholyte 4–9, G2 (ProteinSimple) (*see Note 17*).

### 2.2.1 Preparation of Premix

1. Combine 1000  $\mu\text{L}$  premix ampholyte of pI range that is appropriate with 60  $\mu\text{L}$  pI standard ladder (*see Note 18*).
2. Vortex thoroughly.

### 2.2.2 Preparation of Antibodies

1. Dilute primary antibody in antibody diluent as appropriate (*see Note 19*).
2. Dilute secondary antibody in diluent as appropriate (*see Note 19*).

### 2.2.3 Sample Preparation

1. Dilute samples in bicine chaps buffer to the predetermined concentration within the linear range (*see Note 20*).

2. Urea may also be added to the sample for a denatured condition.
3. Mix premix and sample in a 3:1 volumetric ratio (*see Note 21*).
4. Vortex thoroughly (*see Note 21*).

---

## 3 Methods

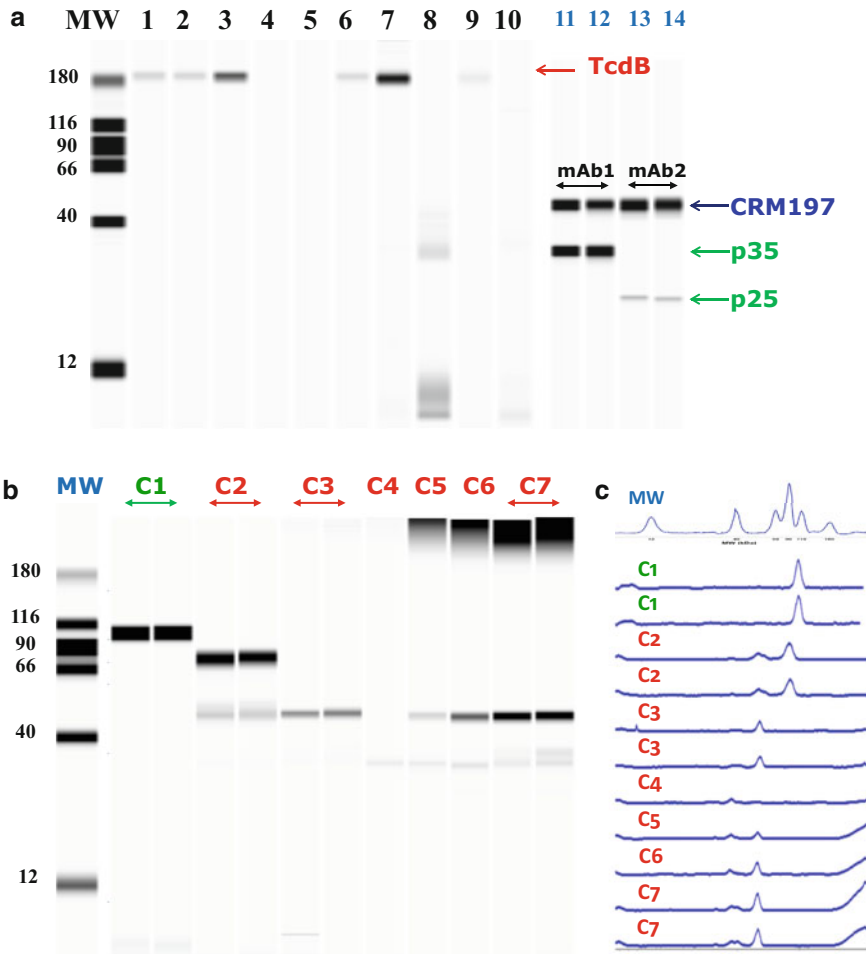
### 3.1 Size-Based Western

The size-based Western blot is performed under denaturing and reducing or nonreducing conditions. The conditions provide the advantage of less matrix interference when analyzing proteins in crude cell lysate/culture compared to standard ELISA which is run under native conditions. Furthermore, the protein identification in a size-based western is much more specific due to the additional dimension of separation by molecular weight compared to ELISA which is one dimension (batch mode). Here we present first the general analysis protocol and then demonstrate several important applications for the use of this automated and quantitative capillary size-based western technology in vaccine and therapeutic protein development.

1. Prepare a sample plate containing luminol S/peroxide, primary antibody, secondary antibody, antibody diluent, stacking matrix, separation matrix, running buffer, and samples according to individual instrument user's manual.
2. Perform self-test check (*see Note 22*).
3. Prepare Simple Western instrument parameters according each system user's manual (*see Note 23*).
4. Click run.
5. Wait till the fluorescent image appears indicating samples are injected.
6. Analyze data using Compass software (*see Note 24*).
7. If results are not satisfactory, then perform CE and western optimizations
8. Optimize standard CE parameters (*see Note 25*).
9. Optimize standard western blot parameters if necessary (*see Note 26*).

The first example of the utility of this technique is in antibody reagent screening (Fig. 1a) and clone screening (Fig. 1b). Figure 1a illustrates antibody screening for toxin B (TcdB), one of the four protein antigens from a *C. difficile* vaccine [22], which has MW ~270 kDa (*see Note 27*). Ten different monoclonal antibodies obtained commercially (lane 1–10) are screened for the best response. The antibody in lane 7 clearly has the best signal among all antibodies screened and therefore is chosen for further use in

this capillary western. Another antibody screening example for a different protein, CRM197 (MW ~58 kDa), which is a nontoxic form of diphtheria toxin used as a carrier protein in many polysaccharide conjugate vaccines [23], is also shown in Fig. 1a. Two antibodies (mAb1 and mAb2) are evaluated against CRM197 and the figure shows that mAb1 also detects CRM197 fragment (p35) that is different from that detected using mAb2 (CRM197 fragment

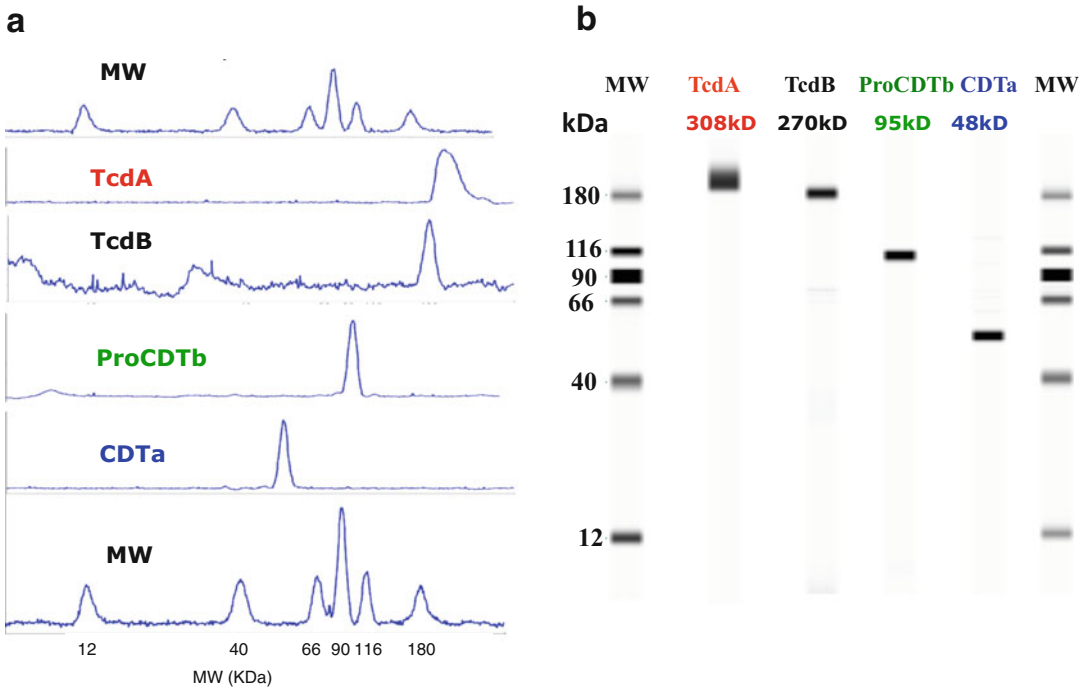


**Fig. 1** (a) Virtual gel image of ten antibodies screened for TcdB (43  $\mu\text{g}/\text{mL}$ , MW ~270 kDa) in lanes 1–10. All antibodies were obtained from different commercial sources. The antibody in lane 7 provides the strongest response and was subsequently used for Western blot assays. In order to observe the high MW protein band, the stacking and sample loading times were adjusted from default conditions to 16 and 8 s, respectively. Similarly, two different mAbs were evaluated for CRM197 (50  $\mu\text{g}/\text{mL}$ , MW 58 kDa) in lane 11–14. Each mAb was tested in duplicate. Both mAbs detect the main peak of CRM197 but they are specific for different epitopes as demonstrated by the two different CRM197 fragments (p35 and p25) observed. All antibodies used were from mouse with 1:25 dilution; goat anti-mouse HRP was used as the detection secondary antibody. (b) Virtual gel image from proCDTb protein clone selection experiment in cell culture indicating clones that produce significantly different protein product (C1 is intended, while other clones (C2–C7) provide either fragments and/or aggregates, no protein). ProCDTb-specific rabbit pAb with 1:1000 dilution was used. (c) The actual electropherograms of (b)

(p25)). Hence these two antibodies detect two different epitopes on CRM197 and both become very useful for CRM197 degradation characterization. The antibody screening process can be completed in one run using the Simple Western platform that can take as little as 3 h, while with a manual western this would take at least 1–3 days and significant manual labor. Clone selection is commonly performed on a small scale and the quantity available for testing at this stage is often low. Therefore we need a sensitive assay to screen for the best product quality and titer. Simple Western is an ideal method for this purpose as it requires low sample volume (5–10  $\mu\text{L}$ ), sensitivity is high, and results are obtained quickly compared to traditional methods. Figure 1b demonstrates the use of this technology for clone selection for binary toxin B (proCDTb), another one of the four protein antigens from the *C. difficile* vaccine which has MW  $\sim 95$  kDa. Seven different clones obtained from *Baculovirus* constructs (C1-C7) are screened to determine which clone produced the highest yield and best quality. Clone 1 shows the intended product quality while clone 2 and 3 produce degradation products with no intact proCDTb. Clone 4 shows no product at all, while clones 5, 6, and 7 have increasing aggregation and degradation products. Therefore, clone 1 is chosen to move forward for development.

The second example showing the usefulness of this technology is for identity testing and antigen stability in the final drug product of our tetravalent *C. difficile* vaccine. Figure 2 shows the identity test using Simple Western for the final drug product containing four antigens in our *C. difficile* vaccine. TcdA and TcdB have MW  $\sim 308$  and 270 kDa, respectively. Both large MW proteins are not resolved using ProteinSimple gel matrix; consequently they appear above the highest MW standard of 180 kDa (*see Note 27*) but the MW is not accurate. The binary toxins, proCDTb and CDTa, appear at their respective MW  $\sim 95$  and 48 kDa. This result demonstrates that Simple Western technology can perform identity analysis in the *C. difficile* vaccine drug product containing four different proteins potentially replacing the manual western blot.

Additionally we have used this technology for assessing heat-stressed stability of our *C. difficile* vaccine samples. The samples are tested by both Simple Western and ELISA. ELISA is the typical method used as a stability indicating assay for final drug product but the results are sometimes difficult to understand and they can be misleading due to the one-dimensional nature of the assay. Western blots have the potential of providing further information about each antigen's stability because of the additional dimension of size or charge separation. Figure 3 illustrates the use of the size-based simple western to generate stability profiles of individual antigens in control and heat-stressed (37 °C) final container *C. difficile* vaccine. Both TcdA and TcdB aggregate upon stress, indicated by the appearance of high MW bands, while CDTa and proCDTb are very stable. The slight decrease in proCDTb

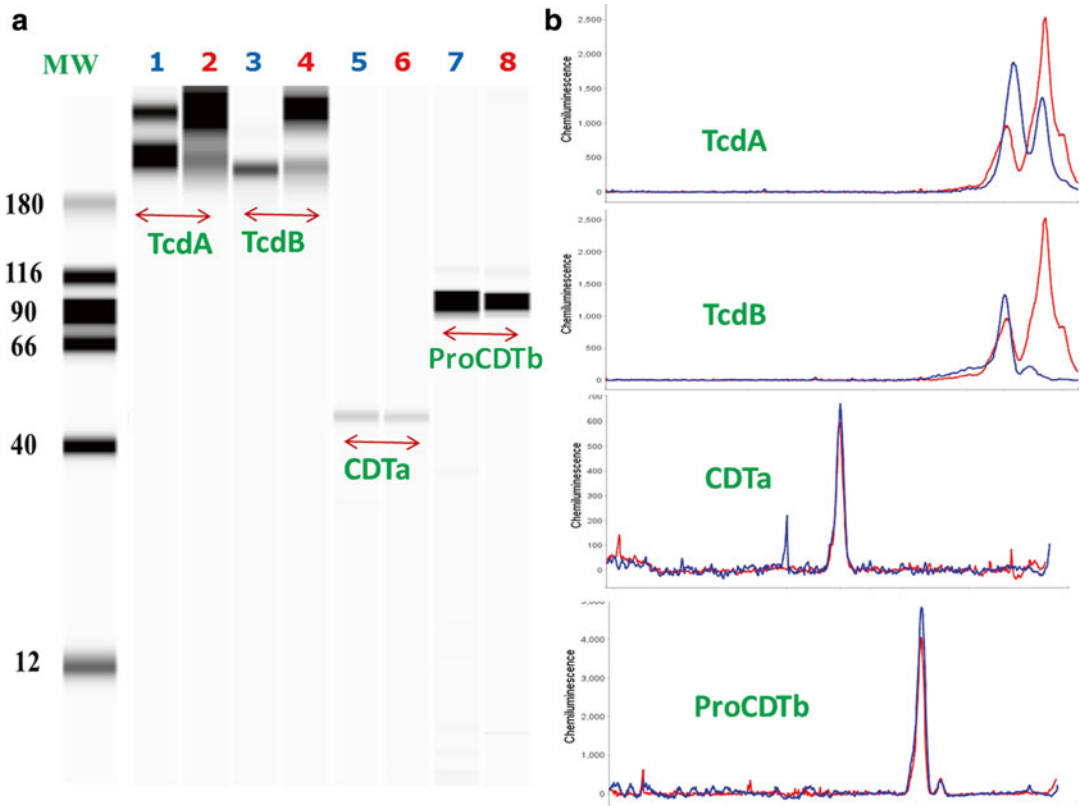


**Fig. 2** Identity test for tetraivalent *C. difficile* vaccine drug product using rabbit pAb (1:1000 dilution) specific for TcdA, TcdB, CDTa, and proCDTb with final concentration of 5, 2.5, 1.4, and 1.4  $\mu\text{g}/\text{mL}$ , respectively (see **Note 27**). (a) Electropherograms and (b) virtual gel image

intensity at 37 °C is attributed to sample preparation. Hence, the capillary western blot provides an extra layer of information about stability by detecting both degradation and aggregation products as well as a quantifiable mass.

The third example where this technology has been applied is in measuring titer for early cell culture samples. We use Simple Western for concentration measurements in both binary toxin CDTa (*C. difficile* vaccine) and for a therapeutic monoclonal antibody. Figure 4 shows typical CDTa electropherograms for titer optimization in various cell culture conditions. The quantitation is done by comparing with purified standard CDTa from 0.45 to 7.5  $\mu\text{g}/\text{mL}$ . Furthermore CDTa concentrations measured by Simple Western correlate well with RP-HPLC [16, 24] (the original method which is used before Simple Western exists, Fig. 4c). Monoclonal antibody upstream production and quality can also be monitored by this Simple Western as depicted in Fig. 5. Electropherograms and virtual images by Simple Western are shown for mAb production from day 6 through day 14. This result indicates an increase in measured mAb (IgG) over time under the nonreducing condition and also confirms good quality (no fragments; only HC and LC) by reducing western. This capillary western can be used as alternative method to the standard protein A HPLC for measuring IgG production.

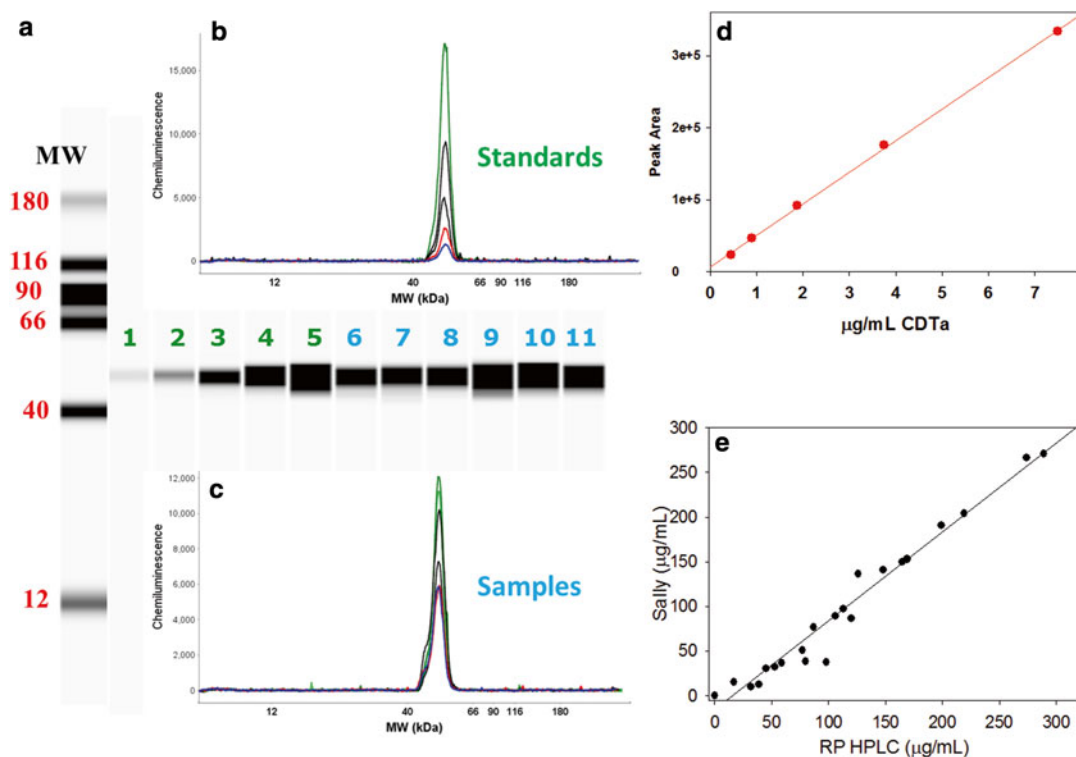




**Fig. 3** Stability indicating assay for tetraivalent *C. difficile* vaccine drug product. **(a)** The virtual gel image comparing drug product at  $-70\text{ }^{\circ}\text{C}$  (blue) and 14 days at  $37\text{ }^{\circ}\text{C}$  (red), TcdA (lane 1 vs. 2), TcdB (lane 3 vs. 4), CDTa (lane 5 vs. 6), and proCDTb (lane 7 vs. 8). **(b)** The electropherograms of 3A. Note that TcdA initially has some high MW aggregation that increases at  $37\text{ }^{\circ}\text{C}$  for 14 days, while TcdB has no aggregation initially but becomes aggregated upon heat stress. The aggregation intensity is in most cases much higher than monomer in an immuno-type of assay; hence the decrease in monomer intensity is not proportional to the increase in aggregation intensity. No clipping or aggregation was observed for CDTa and proCDTb. A slightly lower intensity observed for proCDTb peak at  $37\text{ }^{\circ}\text{C}$  was attributed to sample preparation (see also Fig. 6 for charge-based western data)

### 3.2 Charge-Based Western

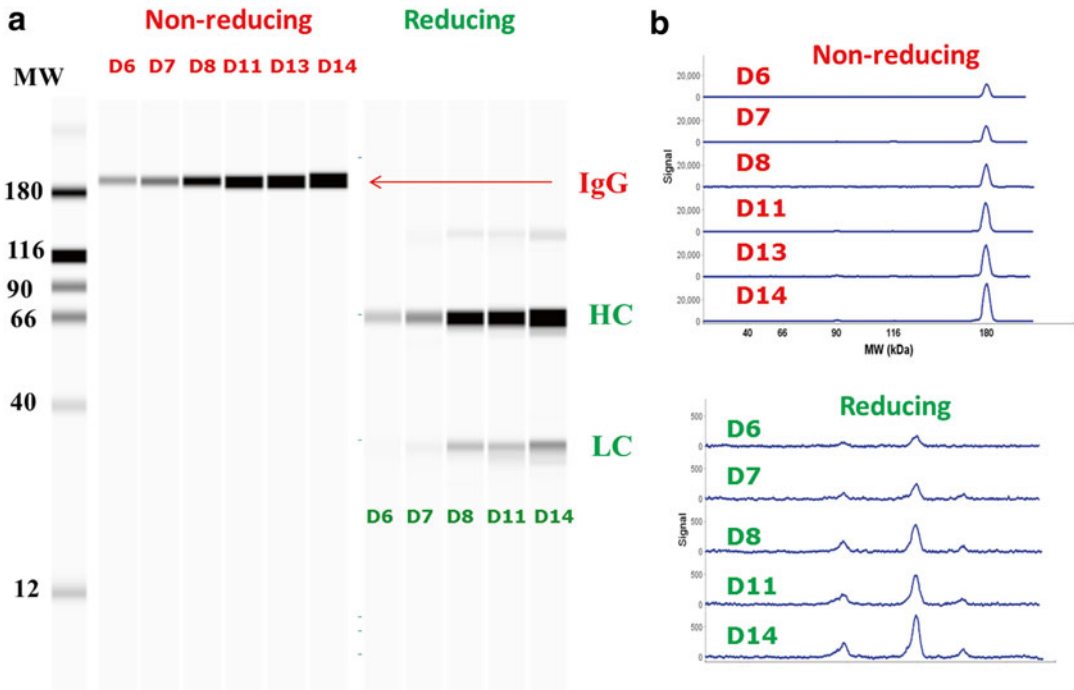
The charge-based western separates proteins by their pIs in a pH gradient matrix (capillary IEF) and the pI-separated protein is blotted in a similar manner as in standard size-based western blots. This experiment can be performed either native or denatured (by adding various concentrations of urea). Although the charge-based western is seldom used, it can provide complementary information to the size-based western (*see Note 28*). Here we present first a detailed protocol of the method and provide several examples of how this charge-based technology can be a useful tool; as a potential stability indicating method for multivalent vaccine products, as a method for evaluating biosimilar candidates, and in evaluating mAb charge heterogeneity in cell culture.



**Fig. 4** An example of CDTa titer screening in various cell culture conditions and using Simple Western. A five-point standard curve was run together with many cell culture samples on a 96-capillary Sally. (a) Gel image of standard curve (lane 1–5) and samples (lane 6–11); (b) Electropherograms of standard curve in (a); (c) Electropherograms of samples in (a); (d) Linearity of standard curve from 0.45 to 7.5 µg/mL with  $R^2 > 0.990$ ; (e) CDTa titer correlation curve between Sally and the traditional RP HPLC method [16, 24] with  $R^2 > 0.967$

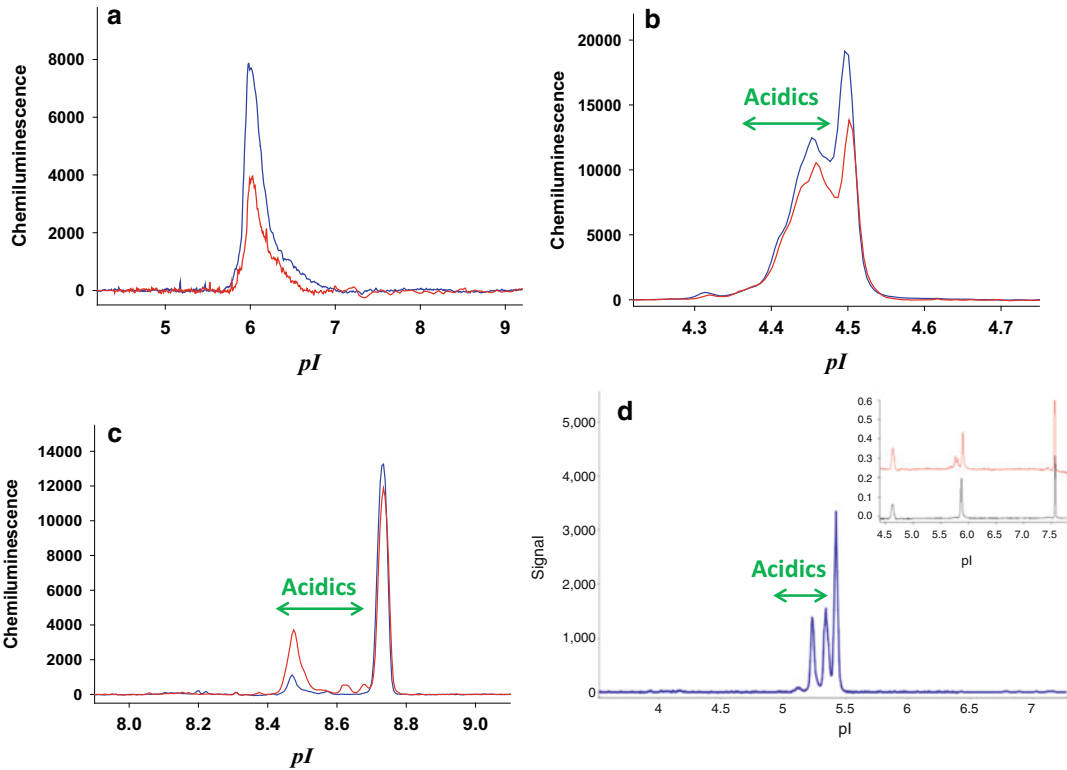
1. Prepare sample plate by adding 15 µL of prepared samples, primary antibody, secondary antibody, and antibody diluent according to individual instrument user's manual.
2. Prepare Simple Western instrument parameters according to system user's manual (see Note 23).
3. Perform self-test check (see Note 22).
4. Click run.
5. Analyze data using Compass software (see Note 24).
6. If results are not satisfactory, then perform CE and western optimizations.
7. Optimize standard CE parameters (see Note 28).
8. Optimize standard western blot parameters if necessary (see Note 29).

The first example where the charge-based western shows utility is in analyzing heat-stressed stability samples of final container drug product of tetravalent *C. difficile* vaccine. Figure 6 illustrates



**Fig. 5 (a)** Virtual gel image of therapeutic mAb production in CHO cell culture from day 6 to 14 as monitored under both nonreducing and reducing conditions with Simple Western. A 20× dilution of human anti-(HC + LC)-HRP obtained from Jackson Immuno Research was used. Iodoacetamide was added in the nonreducing condition. An increase of IgG production is observed from day 6 to 14 without any clipping or misfolding between HL-LC and HC-HC observed. The method can be used as an alternative to both Protein A HPLC IgG titer assay and IgG product quality (see also Fig. 7b for charge-based western data); **(b)** Electropherograms of **(a)**

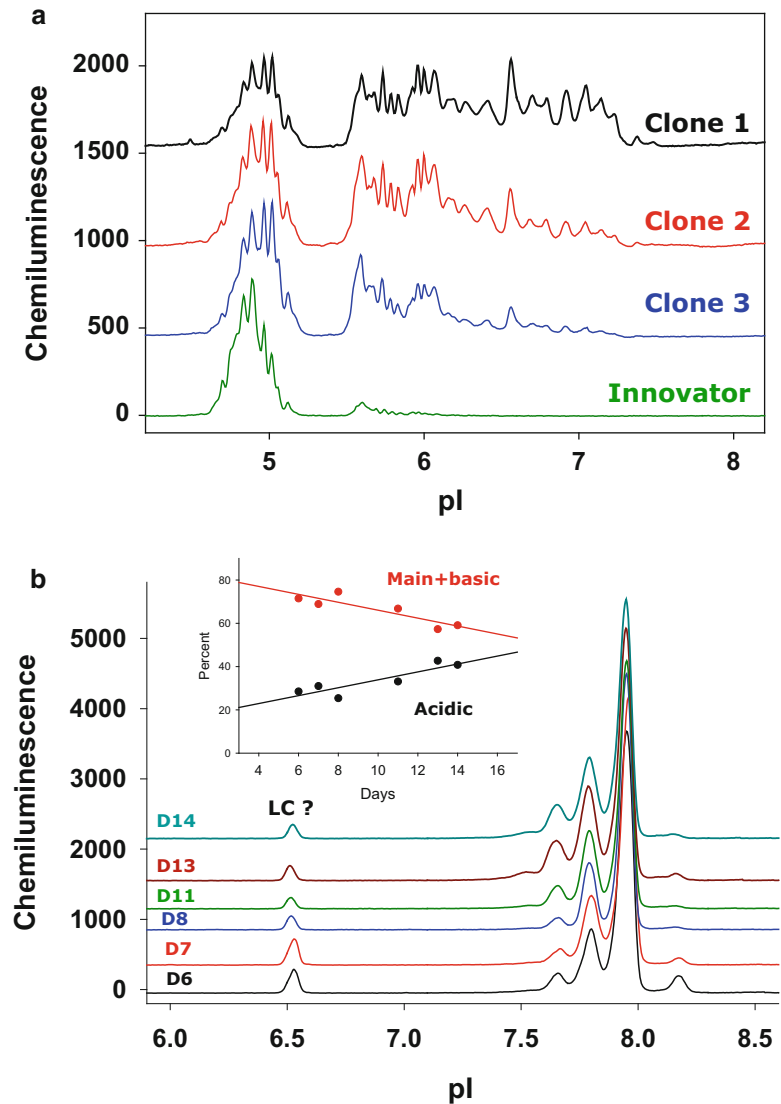
the capillary IEF western for TcdA (pI = 5.9), CDTa (pI = 8.7), and proCDTb (pI = 4.5) final drug product before (blue) after (red) an accelerated heat stress at 37 °C study. We are not able to obtain a reproducible IEF profile for TcdB antigen. There is a decrease in intensity for TcdA after heat stress, while there is a slight increase in intensity for TcdB after heat stress. For CDTa two peaks are present of which the acidic peak increases when heat-stressed. For CDTa and proCDTb, the size-based western shows one peak with no change after heat stress while the charge-based western detected charge heterogeneity, with the acidic peak increasing upon stress, demonstrating how both techniques are complementary. Understanding acidic variants of proteins and the impact of heat stress on these variants is important and often this can be caused by deamidation. It has been demonstrated for some mAb products that their acidic variants are important for biological activity [25, 26]; therefore the acidic variant in these cases becomes a critical quality attribute. The analysis of these acidic variants is very labor intensive involving multiple analytical methods including HPLC, MS, cIEF, and ELISA [27]. The native charge-based western can be used as a fast surrogate biological activity assay for acidic



**Fig. 6** Stability indicating assay for tetraivalent *C. difficile* vaccine drug product evaluated using charge-based western at  $-70\text{ }^{\circ}\text{C}$  (blue) and 14 days at  $37\text{ }^{\circ}\text{C}$  (red). (a) TcdA decreases in intensity at  $37\text{ }^{\circ}\text{C}$ . Ampholytes 3–10 (1000  $\mu\text{L}$ ) range was used with pI ladder 3 (60  $\mu\text{L}$ ); (b) ProCDTb shows a slight increase in acidic variants. Note that a slightly lower intensity observed for proCDtB peak at  $37\text{ }^{\circ}\text{C}$  was attributed to sample preparation. Ampholytes 4–7 (1000  $\mu\text{L}$ ) range was used with pI ladder 3 (60  $\mu\text{L}$ ); (c) CDTa shows two charge variants and the acidic portion has increased while the main peak decreases. Ampholyte-free solution (888  $\mu\text{L}$ ) was mixed with pharmalytes 8–10.5 (88  $\mu\text{L}$ ) with pI ladder 3 (60  $\mu\text{L}$ ); (d) Charge profile of CRM197 stressed material with final concentration  $\sim 5\text{ }\mu\text{g}/\text{mL}$ . The ampholyte 3–10 (700  $\mu\text{L}$ ) range was used with pI ladder 3 (50  $\mu\text{L}$ ). Four peaks are observed by simple western after 3 months at  $25\text{ }^{\circ}\text{C}$  indicating that all three acidic variants are immunoreactive and hence biologically active. The inset shows an imaged cIEF profile of CRM197 control (black) and stressed 3 months  $25\text{ }^{\circ}\text{C}$  (red) material which also showed four variants (one main and three acidic peaks) after stress [23]. Peggy instrument default focusing parameters were employed for all charge-based western

variants as demonstrated in Fig. 6d. The imaged capillary IEF of CRM197 is illustrated on the inset of Fig. 6d showing the IEF profile of CRM197 ( $\text{pI} = 5.8$ ) and that acidic variants increase after 3 months at  $25\text{ }^{\circ}\text{C}$  [23]. When this CRM197 is probed by its biologically relevant mAb in a charge-based simple western, the three acidic variants are detected indicating that all are biologically active.

The second example for charge-based western used is during biosimilar development of the heavily sialylated Fc-fusion protein, Enbrel. This technique is used to quickly screen cell culture samples from various clones for production of Enbrel produced in a *Pichia pastoris* expression system and compared to the



**Fig. 7** (a) The charge-based western (Peggy) was used to analyze samples as part of biosimilar development of Enbrel produced from *Pichia pastoris*. Charge profiles are shown for three clones compared to the innovator. All sample final concentrations were 0.5  $\mu\text{g}/\text{mL}$ . Ampholyte 4–9 (1050  $\mu\text{L}$ ) range was used with pI ladder 1 (23  $\mu\text{L}$ ). (b) The same mAb production samples as in Fig. 5 were monitored for charge heterogeneity. Electropherograms from day 6 to 14 are shown and the percent acidic variants increase during production (see inset graph). The extra peak at pI=6.5 is likely LC since there is always more LC than HC during cell culture production (note that this was not observed in the size-based western in Fig. 5 and was likely caused by sensitivity, since it is known that in IEF the sample is concentrated at least 10 $\times$  or higher after focusing). The final mAb concentration was 2  $\mu\text{g}/\text{mL}$  and the ampholyte 3–10 (500  $\mu\text{L}$ ) range was used with pI ladder 1 (20  $\mu\text{L}$ ). Peggy instrument default focusing parameters were employed for all charge-based western

innovator. The charge distribution profiles are shown in Fig. 7a. Although Enbrel from clone 3 looks slightly better than clone 2 or 1 in terms of its charge variant distribution, it is still far cry from its innovator profile. The method has helped the evaluation of various clones early in cell culture to match the innovator charge variant profile. This demonstrates the use of this technique as a quick screen of low volume, upstream, cell culture samples (prior to purification) for development of a biosimilar therapeutic protein. The method can be applied to other heavily sialylated proteins such as erythropoietin (EPO).

The third example where the charge-based western has shown usefulness is for monitoring charge heterogeneity in therapeutic mAb production during cell culture fermentation. Figure 7b illustrates the capillary IEF western electropherograms from day 6 to day 14 of mAb production. The inset graph demonstrates that the acidic variants increase during mAb production. This early information could become very important if the acidic variants are critical and the quantity of these variants can be controlled by varying the harvest time. Again this demonstrates that the charge-based Simple Western is a complementary tool to the size-based western as no size heterogeneity was detected during fermentation.

---

## 4 Notes

1. ProteinSimple has come out with five different instruments for the size-based Simple Western in the last 3 years. All instruments use the same technology but vary by modes of separation, number of capillaries, and sensitivity. The first generation is called Simon which runs 12 capillaries and is being discontinued. The first 96-capillary version for size is called Sally, and was followed by Peggy, which can run both size- and charge-based capillary westerns. Later they launched Sally Sue and Peggy Sue which are more sensitive versions of Sally and Peggy. They are planning to eventually phase out Sally and Peggy, but the new reagents for Sally Sue and Peggy Sue can be used for Sally and Peggy, which will increase sensitivity without the need for upgrading instruments. Similarly, reagents from Sally and Peggy can be used on the new Sally Sue and Peggy Sue but sensitivity will decrease compared with using the Sue platform reagents. Finally, Wes is the most recently introduced instrument and is a 25 capillary version of Simple Western. The reagents for Wes are identical to Sally Sue or Peggy Sue, but most reagents for Wes are preplated and one only needs to prepare and add samples, luminol S/peroxide mixture, antibody diluent, primary and secondary antibodies, and wash buffer. The benefit of Wes is that sensitivity is as high as Sally Sue and Peggy Sue and it runs much faster than the other four instruments.

2. The total volume of a 5 cm capillary is approximately 400 nL. When the default vacuum injection is used for each instrument, the total volume taken up in the capillary is about 40 nL for the size-based western. In the charge-based western, the whole capillary is filled with sample (400 nL), then focused by a pH gradient after applying voltage. It is not recommended to use size capillary for charge or vice versa.
3. Although the SDS-containing separation matrix and stacking matrix are labeled differently for Sally/Peggy and Sally Sue/Peggy Sue, both can be used interchangeably without noticing any differences. The running buffer is the same for all instruments. These three reagents are already in the prefilled plate for Wes.
4. The matrix removal buffer and concentrated wash buffer are only for Sally/Peggy. For Sally Sue/Peggy Sue, matrix removal becomes lower running buffer and running buffer becomes upper running buffer, while wash buffer is the same as in Sally/Peggy. This change was recently implemented by Protein Simple. There is no matrix removal buffer in Wes and concentrated wash buffer can be used in Wes by diluting it three times.
5. Luminol S, peroxide, sample buffer 10 $\times$ , and Streptavidin-HRP are the same for all size-based instruments. Sample buffer 10 $\times$  can be substituted by preparing 100 mM Tris, 10% SDS pH 7.5. Streptavidin-HRP can be purchased from Jackson Immuno Research and when diluted three times, it will give about a similar intensity as Streptavidin-HRP from ProteinSimple.
6. Although both goat anti-rabbit and goat anti-mouse mAbs are different for Sally/Peggy and Sally Sue/Peggy Sue/Wes instruments, the ones that are designated for Sally Sue/Peggy Sue can be used for Sally/Peggy and the sensitivity will increase significantly. Different antibodies such as anti-donkey-HRP, anti-sheep-HRP, anti-monkey-HRP, and anti-human-HRP can be purchased from Jackson Immuno research and work well with some optimization such as dilution and incubation time.
7. ProteinSimple came out with three different types of antibody diluents (proteins-containing blocking reagent similar to milk or casein for manual western). These three antibody diluents (antibody diluent, antibody diluent plus, and antibody diluent II) can be used on all instruments but sensitivity may vary.
8. Fluorescent protein markers for size-based western contain three different proteins labeled covalently with a fluorescent dye that function mainly for alignment (registration) using the 1 kDa protein and coarse molecular weight standards (if these standards are used instead of the biotinylated MW markers, MW determination in the samples will be inaccurate) in Sally/Peggy and Sally Sue/Peggy Sue. The first generation fluorescent standard markers contain 1, 29, and 180 kDa, while the

second generation has 1, 29, and 230 kDa. Both generations will work on all size-based instruments. It is also very important to perform a negative control experiment of your primary antibody against these fluorescent protein markers for possible cross reactivity. Cross reactivity has been observed for various antibodies especially with the high MW protein markers, 180 and 230 kDa.

9. A large batch of these MW markers can be prepared, and they can be aliquoted at 8  $\mu$ L each for single use and stored at  $-70^{\circ}\text{C}$  for up to 1 year. Custom biotinylated MW markers can be made by labeling the proteins of interest with biotin molecule; however, any residual biotin needs to be removed because biotin will be separated and detected on this Simple Western system. Though surprising, we speculate the separation mechanism is not by MW but rather pseudo-MEKC [21].
10. The master mix (2 $\times$ ) reducing solution preparation is not the same as ProteinSimple's recommendation because we found that increasing the amount of DTT and SDS in final sample buffer yields better reproducibility especially for crude lysate samples or membrane containing proteins. Increasing DTT helps in removing higher order disulfide-linked oligomers sometimes observed for membrane proteins which are very critical for accurate quantitation. Master mix (2 $\times$ ) nonreducing solution can be prepared similarly by replacing the 200 mM DTT with 200 mM Iodoacetamide (this minimizes the disulfide shuffling during sample denaturation and heating).
11. The sample heating condition should be optimized for each protein. For example, mAbs are well known to degrade, forming a thioether bond between LC-HC, and increase disulfide shuffling if it is heated above  $70^{\circ}\text{C}$  [6, 9, 28]. However, some membrane proteins containing coiled-coil motif anchored and  $\beta$ -strands or  $\beta$ -barrel structures need at least  $95^{\circ}\text{C}$  or above to be efficiently denatured and bind SDS [29].
12. The charge-based western was actually the first instrument that came to the market in 2006 and it was called the FireFly<sup>TM</sup> 3000 blotless immunoassay which then later became the CB1000 and finally was called the NanoPro 1000. This NanoPro 1000 could only do the IEF-based western, but later it got upgraded to Peggy and Peggy Sue which can run both charge- and size-based westerns. Unlike size-based western, there is no difference in terms of performance in charge-based westerns between Peggy and Peggy Sue.
13. Do not mix luminol/peroxide reagents for charge and size, as they are not identical.
14. Bicine chaps is used for dilution but primarily for crude cell lysate to help cell lysis. However, for native condition, it is preferred to remove bicine chaps if possible.



15. Each pI standard set consists of five different fluorescently labeled pI markers (these are very similar to the fluorescently labeled protein standards in size-based western). The pI ladder 1 has pI 4.0, 4.9, 6.0, 6.4, and 7.3; while pI ladder 3 contains pI 4.9, 6.0, 6.4, 7.0, and 7.3.
16. There are individual fluorescently labeled pI markers that are added together with the pI ladder when very basic or acidic proteins are measured since all pI ladder sets do not contain very high pI markers.
17. Premix ampholyte-free solution is used by mixing it with other commercial ampholytes such as pharmalytes, servalytes, or biolytes. ProteinSimple also provides ready to use premix with ampholyte added at varying ranges.
18. pI ladder and ampholyte premix are chosen as appropriate to bracket the sample of interest. Combinations of these and conditions may be optimized for the given application but a 20:1 (premix:pI ladder) ratio is a good starting point.
19. Antibody dilution must be optimized for each application. A 1:100 dilution for primary and secondary antibody is a good starting point but a range of dilutions should be tested (*see Note 26*).
20. The linear range of the assay needs to be determined for each analyte separately and will be dependent on sample type, antibodies, and other factors. For a purified protein or vaccine typically 1 µg/mL of protein was within the linear range for most applications so this may be a good starting place. For crude upstream samples, the dilution will vary depending on the amount of antigen present. However, some dilution in detergent or lysis buffer may be needed to solubilize cells and extract proteins of interest. Bicinchoninic acid is a standard diluent but ProteinSimple has other recommended dilution and lysis buffers that can be used.
21. It is important to use pipettes suitable for pipetting very viscous solutions accurately. In addition it is important to carefully add the sample solution to the vial so as to avoid the introduction of air bubbles into the solution. Air bubbles in the sample solution injected into the separation capillary will result in noise spikes in the electropherogram. Lastly, mixing by vortexing is critical as the viscous premix and sample must be thoroughly mixed for accurate quantitation.
22. It is important to perform self-test under the instrument tab on the main menu especially when the instrument has been idle for a long period of time.
23. Since Simple Western is a combination between CE and western blot, both portions of the assay should be optimized for each protein. Default conditions from the manufacturer can

be used but these conditions may be suboptimum for the proteins of interest.

24. Although the instrument software Compass is adequate in most cases, it is still highly recommended to transfer data and analyze using a standard HPLC software package such as Empower.
25. Standard CE optimization steps can be applied here such as varying stacking and sample injection times (sample volume) to obtain resolution and sensitivity. Resolution is usually the least important in a western blot since in most cases we are only interested in one protein, and hence improving sensitivity is more critical. A field amplified stacking, as is in the Simple Western system, can yield a tenfold or more improvement in sensitivity by varying stacking times to up 20 s and by longer sample injection times to up 15–20 s. When the stacking time is increased significantly, the separation time may also need to be reduced or the 1 kDa marker could come off from the capillary and the ability to align the sample will be lost. If greater resolution between two proteins is needed, then a higher stacking to sample loading ratio is preferred especially in the region of the higher MW proteins. Other CE parameters that can be optimized are separation time and voltage to reduce the capillary gel electrophoresis separation time. Since resolution is not typically important in western blot, separation time can be decreased by either increasing the voltage or reducing separation run time. When voltage is increased, the current needs to be monitored and maintained  $<150 \mu\text{A}$  for Sally/Peggy and Sally Sue/Peggy Sue, and  $<400 \mu\text{A}$  for Wes to reduce Joule heating.
26. Western blot optimization is very similar to a manual western with respect to optimizing primary antibody dilution and primary and secondary antibody incubation times. In general, decreasing antibody incubation times from the default will reduce signal intensity and background; however if sensitivity is not required reducing antibody incubation times as much as 50% can be done. Primary antibody dilution depends significantly on how well it binds its target protein. Dilution can be varied between 10 $\times$  and 1000 $\times$  dilution from a standard 1 mg/mL concentration of mAb. Generally lower mAb dilution gives both higher signal and background similar to longer antibody incubation times; therefore the balance between the signal to noise ratio needs to be considered when adjusting these parameters. Polyclonal antibodies produced in rabbits usually provide higher signal than monoclonal produced in mice; however, polyclonals can have more cross reactivity with other proteins especially if the source of the polyclonal is from a commercial vendor. If a polyclonal reagent is generated by immunizing rabbits using antigens with  $>90\%$  purity, the poly-

clonal serum reagent usually has low/zero cross reactivity with other proteins and a 1000 $\times$ –10,000 $\times$  pAb dilution can be achieved. Two types of secondary antibody are available from ProteinSimple, anti-rabbit and anti-mouse (rarely dilution optimizations are needed). Finally, another parameter that can be changed to decrease the total run time is the chemiluminescence detection times. The instrument default usually provides 6 different detection times varying from 5 to 960 s. In most cases longer detection time such as 960, 480, and 240 s are not needed especially when the assay produces strong signal. If these portions of the default method detection times are eliminated, it reduces the total run time by almost 30 min for each cycle. Overall, with these adjustments, the total run time can be reduced from 22 h for all 96 capillaries to about 16–18 h, while for Wes the total run time can be decreased from 3 to 2 h.

27. The TcdA (MW ~308 kDa) and TcdB (MW ~270 kDa) bands appear above 180 kDa MW marker because these MWs are beyond the range of the separation gel matrix of 12–180 kDa (stacking and sample injection times were adjusted accordingly; see figure legend). The stock separation gel matrix could be diluted to 80% with water which might give better separation, but ProteinSimple has just recently come out with a separation matrix gel for high MW protein ranging from 66 to 440 kDa.
28. In general, method development of capillary IEF is much more difficult than CE-SDS gel (CGE) methods for protein because protein isoelectric focusing depends on many parameters such as ampholyte mixture concentration, low ionic buffer concentration (<20 mM), and focusing time (5–30 min). Additionally, a potential protein precipitation exists at its pI which may cause a requirement for additives such as a non-ionic surfactant, urea, or glycerol to prevent it. Furthermore, when signal is not detected in charge-based western, it is sometimes difficult to troubleshoot the root cause (i.e., protein is not focused or primary antibody is not good).
29. Instrument running conditions may be optimized outside of the vendor recommended defaults. For instance, the antibody incubation time, exposure times, wash times, and other conditions may be changed to optimize each assay similar to the size-based western (see **Note 26**).

---

## Acknowledgments

The authors would like to thank our colleagues in Bioprocess Fermentation and Purification for providing us with material and technical support. We also thank Dr. David Thiriot and Bei Wang for providing us with stressed samples of the tetravalent *C. difficile* drug product. We gratefully thank Carrie Anderson and Brian

Peklansky for icIEF experimental support and Drs. Annegret Boge, Erik Gentalen, Chris Heger, Robert Gavin, and Chris Conway from ProteinSimple for technical support. Finally, we thank our management support, Drs. Sha Ha and Richard Peluso.

## References

- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to NC sheets: procedure and applications. *Proc Natl Acad Sci U S A* 76:4350–4354
- Renart J, Reiser J, Stark GR (1979) Transfer of proteins from gels to diazobenzoyloxymethyl paper and detection with anti-sera: a method for studying antibody specificity and antigen structure. *Proc Natl Acad Sci U S A* 76:3116–3120
- Kurien BT, Scofield RH (2006) Western blotting. *Methods* 38:283–293
- Ghosh R, Gilda JE, Gomez AV (2014) The necessity of and strategies for improving confidence in the accuracy of Western Blots. *Expert Rev Prot* 11:549–560
- Koller A, Wätzig H (2005) Precision and variance components in quantitative gel electrophoresis. *Electrophoresis* 26:2470–2475
- Hunt G, Nashabeh W (1999) Capillary electrophoresis sodium dodecyl sulfate nongel sieving analysis of a therapeutic recombinant monoclonal antibody: a biotechnology perspective. *Anal Chem* 71:2390–2397
- Ma S, Nashabeh W (2001) Analysis of protein therapeutics by capillary electrophoresis. *Chromatographia* 53:S75–S89
- Rustandi RR, Washabaugh MW, Wang Y (2008) Application of CE SDS gel in development of biopharmaceutical antibody-based products. *Electrophoresis* 29:3612–3620
- Rustandi RR, Wang Y (2011) Use of CE-SDS gel for characterization of monoclonal antibody hinge region clipping due to copper and high pH stress. *Electrophoresis* 32:3078–3084
- Rustandi RR, Anderson CL, Hamm M (2013) Application of capillary electrophoresis in glycoprotein analysis. In: Beck A (ed) *Glycosylation engineering of biopharmaceuticals methods and protocols*, vol 988. Humana Press, Totowa, NJ, pp 181–197
- Wu J, Wu XZ, Huang T, Pawliszyn J (2004) Analysis of proteins by CE, CIEF and microfluidic devices with whole-column-imaging detection. In: Strege MA, Lagu AL (eds) *Methods in molecular biology: capillary electrophoresis of proteins and peptides*, vol 276. Humana Press, Totowa, NJ, pp 229–252
- Anderson C, Wang Y, Rustandi RR (2012) Applications of imaged capillary isoelectric focusing technique in development of biopharmaceutical glycoprotein-based product. *Electrophoresis* 33:1538–1544
- Rustandi RR, Wang F, Hamm C, Cuciniello JJ, Marley ML (2014) Development of imaged capillary isoelectric focusing method and capillary zone electrophoresis in hepatitis B vaccine RECOMBIVAX HB®. *Electrophoresis* 35:1072–1078
- O'Neill RA, Bhamidipati A, Bi X, Deb-Basu D, Cahill L, Ferrante J, Gentalen E, Glazer M, Gossett J, Hacker K, Kirby C, Knittle J, Loder R, Mastroieni C, MacLaren M, Mills T, Nguyen U, Parker N, Rice A, Roach D, Suich D, Voehringer D, Voss K, Yang J, Yang T, Vander Horn PB (2006) Isoelectric focusing technology quantifies protein signaling in 25 cells. *Proc Natl Acad Sci U S A* 103:16153–16158
- Nguyen U, Squaglia N, Boge A, Fung PA (2011) The simple Western™: a gel-free, blot-free, hands-free Western blotting reinvention. *Nat Methods* 8:v–vi
- Rustandi RR, Loughney JW, Hamm M, Hamm C, Lancaster C, Mach A, Ha S (2012) Qualitative and quantitative evaluation of Simon™, a new CE-based automated western blot system as applied to vaccine development. *Electrophoresis* 33:2790–2797
- Chen J-Q, Heldman MR, Herrmann MA, Kedei N, Woo W, Blumberg PM, Goldsmith PK (2013) Absolute quantitation of endogenous proteins with precision and accuracy using a capillary Western system. *Anal Biochem* 442:97–103
- Liu S, Sardi S, Sonom B, Zocco D, McSweeney R, Fraser A, Halleck A, Li H, Smejkal G, Munevar S, Jin JG, Kawai T, Ghiran I, McGrath JP, Whitman M, Ng S, Kuo WP (2013) Application of a novel nanovolume capillary electrophoresis based protein analysis system in personalized and translational medicine research. *J Bioanal Biomed* S3:1–10
- Loughney JW, Lancaster C, Ha S, Rustandi RR (2014) Residual bovine serum albumin (BSA) quantitation in vaccines using automated capillary Western technology. *Anal Biochem* 461:49–56

20. Hamm M, Ha S, Rustandi RR (2015) Automated capillary Western dot blot method for the identity of 15-valent pneumococcal conjugate vaccine. *Anal Biochem* 478: 33–39
21. Rustandi RR, Hamm M, Loughney JW, Ha S (2015) Detection of ADP-ribosylation in PARP-1 and bacterial toxins using a capillary-based western system. *Electrophoresis* 36: 2798–2804. <http://onlinelibrary.wiley.com/doi/10.1002/elps.201500173/pdf>
22. Wang B, Wang S, Rustandi RR, Wang F, Mensch CD, Hong L, Kristopeit A, Secore S, Dornadula D, Kanavage T, Heinrichs JH, Mach H, Blue JT, Thiriot DS (2015) Detecting and preventing reversion to toxicity for a formaldehyde-treated *C. difficile* toxin B mutant. *Vaccine* 33:252–259
23. Rustandi RR, Peklansky B, Anderson CL (2014) Use of imaged capillary isoelectric focusing technique in development of diphtheria toxin mutant CRM197. *Electrophoresis* 35:1065–1071
24. Lancaster C, Pannizzo P, Ha S, Rustandi RR (2014) A reserve phase HPLC mass assay for *C. difficile* binary toxin vaccine. HPLC 2014 Poster P-W-2005, New Orleans
25. Harris RJ, Kabakoff B, Macchi FD, Shen FJ, Kwong M, Andya JA, Shire SJ, Bjork N, Totpal K, Chen AB (2001) *J Chromatogr A* 752:233–245
26. Vlasak J, Bussat MC, Wang S, Wagner-Rousset E, Schaefer M, Klinguer-Hamour C, Kirchmeier M, Corvaia N, Ionescu R, Beck A (2009) Identification and characterization of asparagine deamidation in the light chain CDR1 of a humanized IgG1 antibody. *Anal Biochem* 392:145–154
27. Wang F, Peklansky B, Anderson C, Wang Y, Rustandi RR (2012) Improved ion-exchanged HPLC method in mAb using pH gradient and its comparison with cIEF. *J Liq Chrom Rel Tech* 35:1259–1269
28. Cohen SL, Price C, Vlasak J (2007)  $\beta$ -Elimination and peptide bond hydrolysis: two distinct mechanisms of human IgG1 hinge fragmentation upon storage. *J Chem Am Soc* 129:6976–6977
29. Commanducci M, Bambini S, Brunelli B, Adu-Bobie J, Arico B, Capecchi B, Giuliani MM, Massignani V, Santini L, Savino S, Granoff DM, Caugant DA, Pizza M, Rappuoli R, Mora M (2002) NadA, a novel vaccine candidate of *Neisseria meningitidis*. *J Exp Med* 195: 1445–1454