



Residual bovine serum albumin (BSA) quantitation in vaccines using automated Capillary Western technology



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ABSTRACT

Bovine serum albumin (BSA) is a major component of fetal bovine serum (FBS), which is commonly used as a culture medium during vaccine production. Because BSA can cause allergic reactions in humans the World Health Organization (WHO) has set a guidance of 50 ng or less residual BSA per vaccine dose. Vaccine manufacturers are expected to develop sensitive assays to detect residual BSA. Generally, sandwich enzyme-linked immunosorbent assays (ELISA) are used in the industry to detect these low levels of BSA. We report the development of a new improved method for residual BSA detection using the SimpleWestern technology to analyze residual BSA in an attenuated virus vaccine. The method is based on automated Capillary Western and has linearity of two logs, >80% spike recovery (accuracy), intermediate precision of CV <15%, and LOQ of 5.2 ng/ml. The final method was applied to analyze BSA in four lots of bulk vaccine products and was used to monitor BSA clearance during vaccine process purification.

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Bovine serum albumin (BSA)¹ is an acidic single polypeptide protein with molecular weight of 66.4 kDa that has been used extensively in the pharmaceutical industry for a variety of applications. It is used as a stabilizer, a blocking agent in various immunoassays, in microarray technologies, and in cell culture medium as a growth supplement for viral vaccine manufacturing [1–4]. Production processes for most viral vaccines require cell culture medium that contains fetal bovine serum (FBS), BSA of which is one of the major components. Numerous studies have shown BSA to be a trigger for allergic reactions [5–7]. Due to this safety concern, the World Health Organization (WHO) guidance requires BSA concentration be demonstrated to be no greater than 50 ng per human dose of vaccine [8]. Hence, sensitive analytical tools are required to adequately monitor this low level residual BSA in vaccine production.

Several immune-based techniques have been used to determine the concentration of BSA in vaccines. Detection of bovine proteins

initially employed techniques such as immunodiffusion [5], counter-current electrophoresis [9], and latex agglutination inhibition assay [10], all of which are very time-consuming. Later several enzyme-linked immunosorbent assays (ELISA) were developed to detect residual BSA [4,11–13]. More specialized techniques, such as label-free AC impedimetric [14] and quantum dots spectrofluorimetric methods [15], were used as well, but those had limited detection ranges. More recently, residual BSA analyses using ELISA with specialized detection techniques were developed to achieve 1–2 orders lower sensitivity [16,17]. Regardless, standard sandwich ELISA is still the most commonly used method in the biopharmaceutical industry to analyze residual BSA.

Manual Western blotting is another immune-based assay commonly used to detect BSA. This technique has the advantage of molecular weight-based separation in addition to the antigen specificity recognition that is a property of an immune-based assay. This technique is labor intensive and qualitative in nature. Koller and Wätzig evaluated the reproducibility of manual Westerns and reported a CV >35% [18]. More recently, an automated capillary electrophoresis (CE)-sized-based Western blot called SimpleWestern has been described [19,20]. We have recently evaluated this technology for qualitative and quantitative analysis for vaccine protein components [21]. This CE-based Western provides advantages of being fully automated in operation, except for sample preparation, and provides a CV <10% which is comparable to the standard ELISA technique. In this paper, we describe the development of a quantitative residual BSA method, using a size-based

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¹ Abbreviations used: BSA, bovine serum albumin; FBS, fetal bovine serum; WHO, World Health Organization; ELISA, enzyme-linked immunosorbent assays; CE, capillary electrophoresis; NIST, National Institute of Standards and Technology; HRP, horseradish-peroxidase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; HPSEC, high performance size-exclusion chromatography; mAb, monoclonal antibody; pAb, polyclonal antibody; SB, sample buffer; AD, antibody diluent; HS, histidine saline buffer; HSS, histidine saline sucrose buffer; LOQ, limit of quantitation; CV, coefficient of variation.

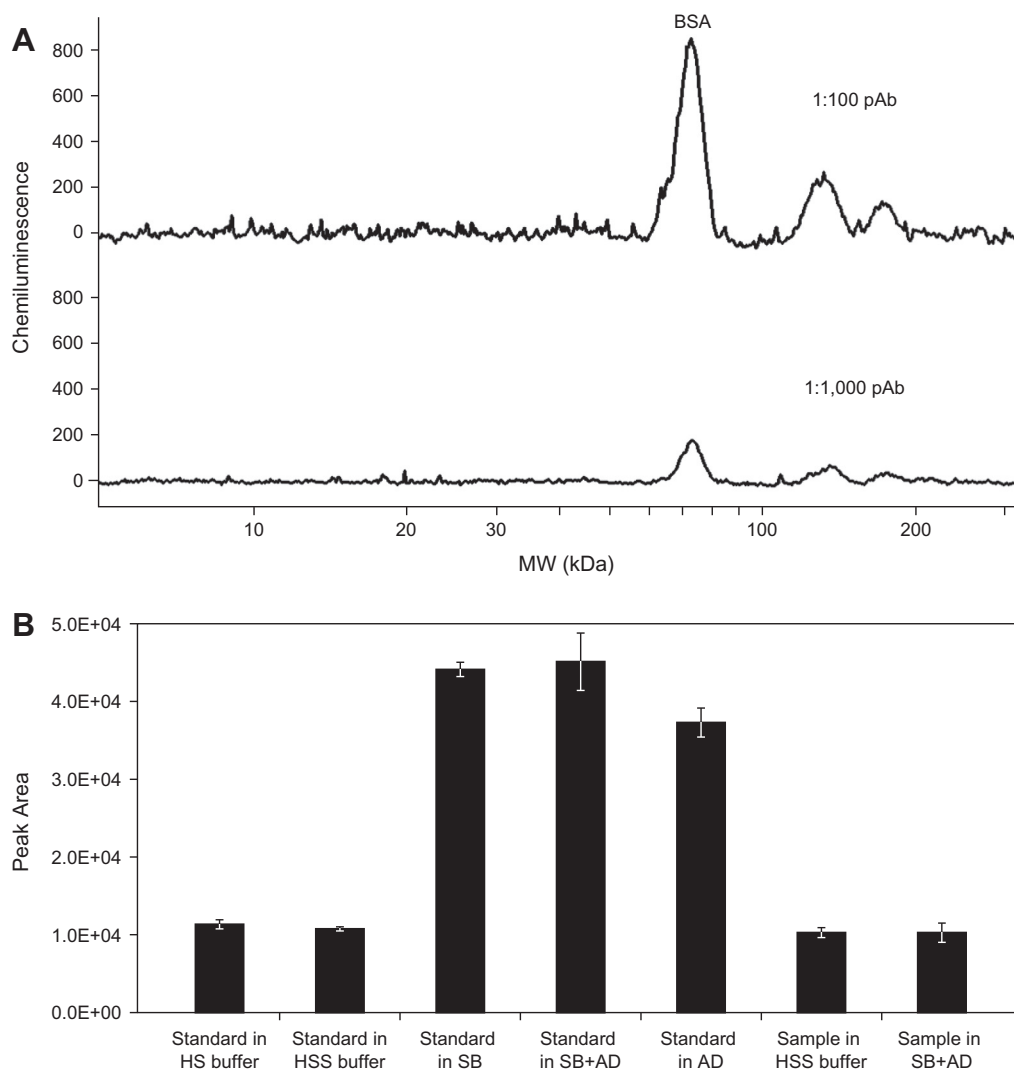


Fig.1. (A) Electropherogram traces comparing 1:100 and 1:1000 dilution of primary rabbit anti-BSA pAb with secondary goat anti-rabbit HRP conjugate antibody (from ProteinSimple) detecting 100 ng/ml BSA at 120-s exposure. (B) Comparison of peak area for 150 ng/ml BSA standard and vaccine sample diluted in histidine-saline buffer (HS), histidine-saline-sucrose buffer (HSS), 1X sample buffer (SB), 1X sample buffer in antibody diluent (SB + AD), and antibody diluent (AD).

Capillary Western technology, for one of our attenuated virus vaccine programs.

Materials and methods

Chemicals and reagents

BSA protein reference standards ($\geq 98\%$ pure) were obtained from Thermo Pierce Scientific (Rockford, IL) and National Institute of Standards and Technology (NIST) (Gaithersburg, MD). Primary antibodies rabbit anti-bovine albumin and sheep anti-bovine albumin horseradish-peroxidase (HRP) conjugate were obtained from Bethyl Labs (Montgomery, TX). Secondary antibody goat anti-rabbit HRP conjugate, dithiothreitol (DTT), molecular weight fluorescence standards, luminol-S, hydrogen peroxide, sample buffer, antibody diluent, running buffer, and stacking matrix, separation matrix, wash buffer, matrix removal buffers, and capillaries were purchased from ProteinSimple (Santa Clara, CA). SDS-PAGE reagents were from Invitrogen (Carlsbad, CA). Trichloroacetic acid (TCA) and Gelcode blue stain reagent were from Thermo Scientific (Waltham, MA). Buffers for HPSEC were analytical grade and

sodium phosphate, sodium chloride, and sodium azide were purchased from Sigma-Aldrich (St. Louis, MO).

Vaccine sample

All vaccine samples were produced from live attenuated virus. The vaccine was purified from human cell substrate by the Bioprocess Research and Development group at Merck Research Laboratories (Merck & Co., West Point, PA).

SDS-PAGE analysis

BSA reference standards were prepared in a final concentration of 1X reducing sample buffer (Invitrogen) at a high load (10 μ g) and a low load (5 μ g). The standards were incubated at 95 °C for 10 min and then separated using a 4–20% Tris-glycine 10-well mini-gel. Gels were fixed with 12% TCA for 1 h and stained overnight with Coomassie G-250 protein stain. Gels were scanned and peak quantitation was performed using Molecular Dynamic densitometer and Image Quant software (GE Healthcare).

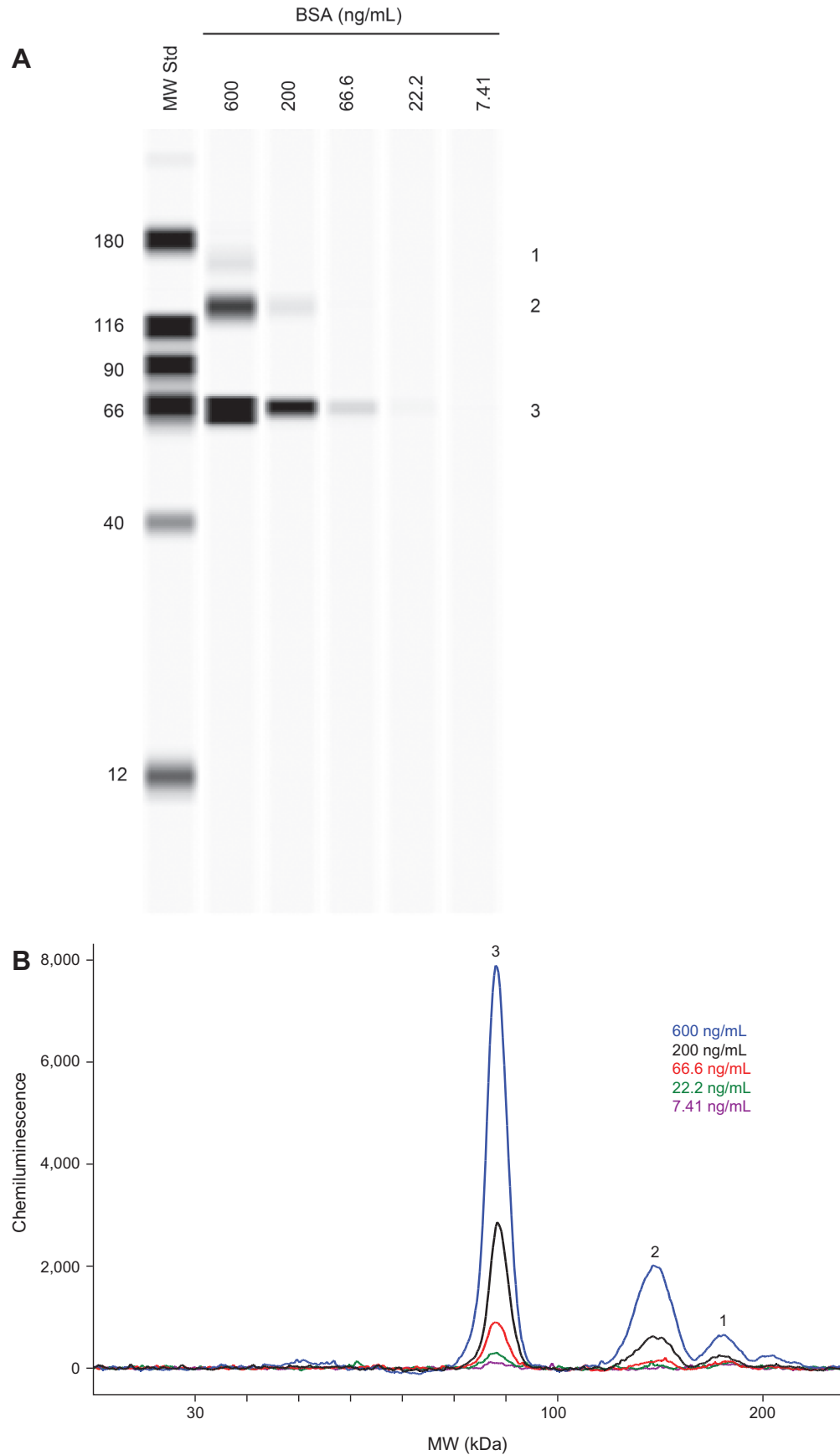


Fig.2. Detection of BSA reference standard showing aggregates (1), dimer (2), and monomer (3) ranging from 7.4 to 600 ng/ml total mass diluted in SB + AD using Capillary Western technology. (A) SimpleWestern image view of molecular weight ladder and BSA reference standard and (B) electropherogram traces of BSA reference standard.

Table 1
Percentages aggregates, dimer, monomer, and fragments in BSA reference standard tested by Capillary Western, SDS–PAGE, and HPSEC.

	Thermo BSA reference standard		
	Capillary Western	SDS–Page*	HPSEC*
% Aggregates	5.5	16	3
% Dimer	26	4	12
% Monomer	68.5	70	83
% Fragments	–	10	2

* The protein amounts used to determine percentages aggregates, dimer, monomer, and fragments were within the linear detection range for each technology.

High performance size-exclusion chromatography (HPSEC) analysis

BSA reference standards were analyzed on an Alliance 2695 separation module with a 2487 dual wavelength detector set to 280 nm (Waters Corporation, Milford, MA). Standards were diluted in mobile phase to 2 mg/ml and were held at 2–6 °C on the

autosampler until injection. The injection volume was 10 µl. The mobile phase contained 25 mM sodium phosphate, 150 mM sodium chloride, and 0.05% sodium azide at pH 7.3. Separation was performed on a TSK gel G3000SWxl 5 µm silica column (300 × 7.8 mM) from Tosoh biosciences (King of Prussia, PA). The column was maintained at room temperature with an isocratic flow of 0.5 ml/min for 30 min. Percentages aggregation, monomer, and fragment were calculated from the total peak area using Empower software (Waters Corporation, Milford, MA).

Automated Capillary Western assay

BSA reference standards, vaccine samples, and reagents were prepared according to the ProteinSimple manual with some minor modification as previously described [21]. Briefly, all vaccine samples and BSA standards were diluted to the appropriate concentration with sample buffer in antibody diluent. Then each sample was further diluted with master mix to a final concentration of 1X sample buffer, 1X molecular weight fluorescence standards, and

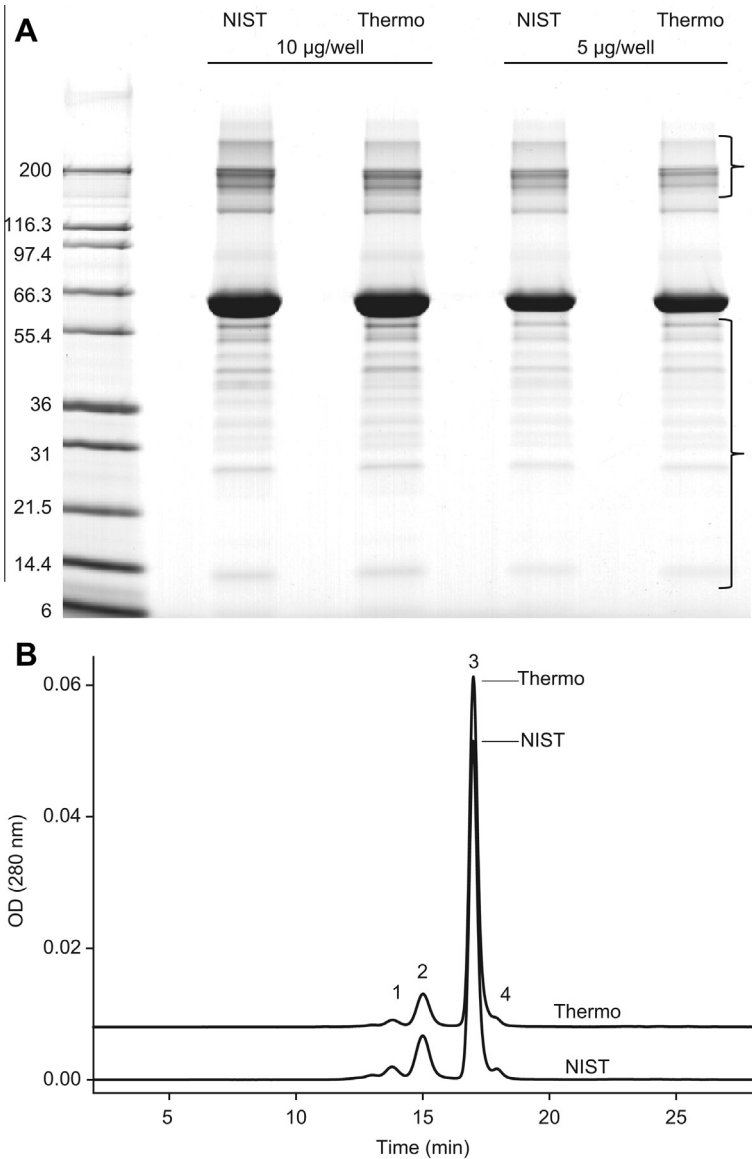


Fig. 3. Characterization of the BSA reference standard (≥98% pure) from two different suppliers to determine higher order aggregates (1), dimer (2), monomer (3), and degradates (4). (A) SDS–PAGE image of denatured and reduced BSA loaded at 10 and 5 µg per well and (B) HPSEC chromatogram traces of 20 µg native BSA injected onto column.

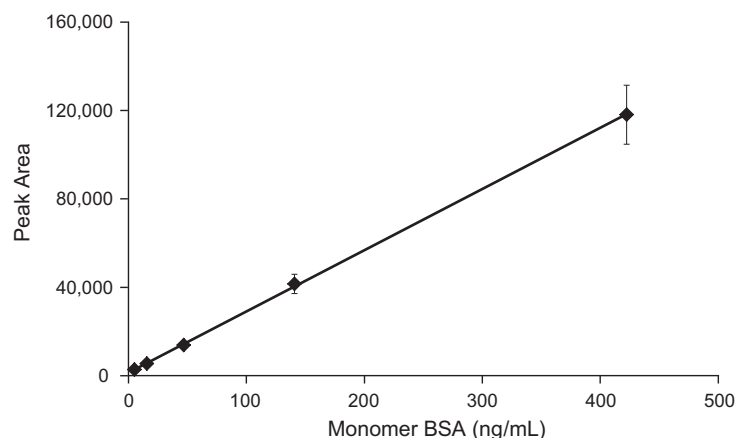


Fig. 4. Calibration curve of monomeric BSA detected on SimpleWestern, ranging from 5.2 to 420 ng/ml with error bars from 6 independent experiments.

100 mM DTT. Samples and standards were heated for 10 min at 95 °C. The 384-well plate setup is similar to the ProteinSimple manual, except 5 µl of standards and samples was transferred to the plate. Once the plate was loaded the automated separation and detection were performed using the default SimpleWestern settings, except that the primary antibody incubation time was decreased to 90 min and secondary antibody decreased to 30 min. The capillary images were analyzed with Compass software (ProteinSimple). The concentration of protein in the sample was determined by comparing peak area against a calibration curve of BSA of known concentration. The BSA calibration curve was calculated using a linear regression analysis.

Results and discussion

Method development

Using the SimpleWestern technology, a method was developed to measure residual BSA in virus vaccine samples. Two different commercial polyclonal antibodies (pAb) were evaluated: one from rabbit and the other from sheep. The sheep pAb was directly conjugated with HRP and did not produce signal, most likely because the HRP concentration was nonoptimized. Additionally, ProteinSimple does not provide a secondary anti-sheep HRP conjugate antibody. The sheep pAb was not further evaluated. The rabbit pAb at a final concentration of 10 µg/ml (1:100 dilution) provided strong signal with the secondary anti-rabbit HRP conjugate antibody from ProteinSimple. This pair of antibodies was used for all subsequent experiments. The incubation times for primary and secondary antibodies were tested from 120 to 90 and 60 to 30 min, respectively. The primary antibody was tested at two different dilutions: 1:100 and 1:1000. The 1:100 dilution had approximately 4-fold increase in signal compared to the 1:1000 dilution. Exposure times were evaluated from 30 to 960 s; 120 s was chosen to have adequate signal (Fig. 1A). The 120-s exposure time was used for quantitative data analysis in subsequent experiments.

BSA reference standard was diluted in several different buffers to determine if the matrix has an effect on quantitation. BSA was diluted in 1X sample buffer (SB) which contains a final concentration of 1% SDS, antibody diluent (AD), 1X sample buffer in antibody diluent (SB + AD), histidine-saline buffer (HS), and histidine-saline-sucrose buffer (HSS). There was no difference in terms of the amount of BSA standard signal when comparing HS to the HSS buffer; however, there was approximately a 4-fold increase in BSA standard signal when using a buffer containing sample buffer (Fig. 1B). The decrease seen in HS and HSS buffer was determined

to be BSA protein surface adsorption loss to plastic tubes and pipette tips. The amount of protein loss was significantly reduced when the standard was diluted in the presence of a detergent such as SDS. There was no increase of BSA signal when comparing the protein-rich vaccine samples diluted in SB + AD or HSS buffer. It is conceivable that the additional virus proteins in the vaccine sample prevent BSA adsorption to plastics. To keep the matrix consistent both the BSA standard and the vaccine sample dilutions were prepared in SB + AD to ensure the accuracy of the method. The loss of BSA protein without SDS is not unexpected since BSA is commonly used as a blocking reagent in ELISA and manual Western blots to reduce nonspecific binding.

Capillary Western quantitation of BSA

Serial dilutions of the Thermo BSA standard in SB + AD buffer ranging from 7.4 to 600 ng/ml were separated by size-based Capillary Western and quantitation was performed. Fig. 2A and B illustrate the Western image and electropherogram traces, respectively. The rabbit polyclonal antibody recognizes monomer, dimer, and higher order aggregates of BSA as indicated in Fig. 2A. The molecular weight of the three forms is measured as 66 kDa for monomer, 122 kDa for dimer, and 173 kDa for trimer by the Compass software. The Capillary Western data suggested that the BSA standard contains 68.5% monomer, 26% dimer, and 5.5% higher order aggregates (Fig. 2A). At first glance, the relatively large amount of dimer was a bit surprising for two reasons. First the BSA standard was reduced with DTT and there should be very little aggregates present. Second, typical HPSEC results for BSA standards from other suppliers show ≤12% dimer. To assess the purity of the reference standard used for the Capillary Western the BSA was further characterized by HPSEC and reducing SDS-PAGE (Table 1).

The result from the denatured and reducing SDS-PAGE reveals that the Thermo BSA standard was 70% monomer, 4% dimer, 16% higher order aggregates, and 10% fragments (Fig. 3A). In both SDS-PAGE and Capillary Western assays the sample is denatured by heat during sample preparation. This sample preparation may cause the formation of nonreducible covalent aggregates. Disulfide scrambling in BSA has been observed resulting in the formation of nonreducible thioether links [22]. However, since the samples are heated in both SDS-PAGE and Capillary Western the percentage difference in dimer between the SDS-PAGE and the Capillary Western is caused by the primary rabbit pAb that was used. The antibody may preferentially recognize dimer BSA versus monomer BSA and the intensity is not related to concentration only. We

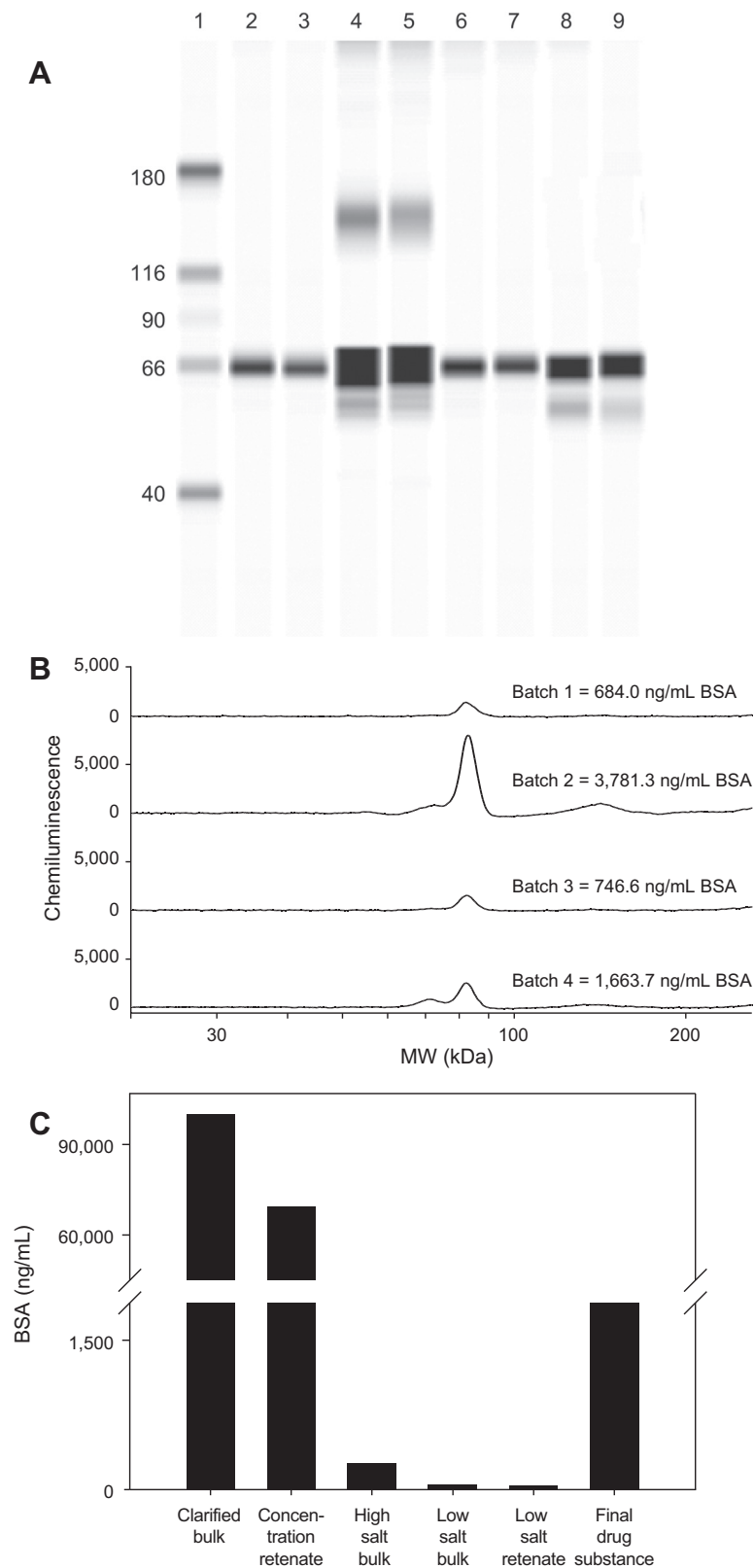


Fig.5. Quantitation of residual BSA in viral vaccine samples using size-based Capillary Western technology. (A) Image view of four vaccine final drug substance batches tested in duplicate (lanes 1, MW ladder; 2–3, Batch 1; 4–5, Batch 2; 6–7, Batch 3; 8–9, Batch 4) and (B) electropherogram traces of final drug substance batches 1–4 with dilution corrected concentrations of BSA (ng/ml). (C) Quantitative analysis of BSA concentration in process intermediate vaccine samples to demonstrate clearance of BSA.

observed similar profiles for BSA standard from other suppliers such as the NIST BSA. The HPSEC data also showed the presence of dimer and higher order aggregates; however, the levels of each

were slightly different than those observed via SDS–PAGE (Fig. 3B, Table 1). Since the BSA was analyzed under native conditions for HPSEC the different dimer levels observed suggest that the dimer

may exist in two forms, one that is covalently linked and a second which is noncovalently linked.

Because the Capillary Western can separate monomer, dimer, and higher order aggregates of BSA based on size, it could potentially be used to quantitate each form. However, since dimer and higher order aggregates were typically not observed in our tested vaccine samples, the following assay performance evaluation is focused on the monomeric form only. Monomeric BSA, the major BSA form, in both vaccine samples and reference standard was used as a surrogate for quantitating residual BSA. For the monomeric quantitation, the Thermo BSA standard was corrected for 70% of the total mass as determined by SDS–PAGE.

A standard calibration curve is illustrated in Fig. 4 and demonstrates linearity over the selected corrected concentration range from 5.2 to 420 ng/ml BSA with an excellent correlation coefficient ($R^2 \geq 0.9994 \pm 0.0008$) throughout 6 independent experiments. Reproducibility of the peak area for the calibration curve has a CV $\leq 10\%$, except for the lowest standard which has a CV $\leq 20\%$ ($n = 6$). The estimated limit of quantitation (LOQ) for BSA was adopted from the lowest point on the standard curve as 5.2 ng/ml. This estimated LOQ was prepared and analyzed over six independent experiments and has an average S/N of 20 ± 8 . Note that LOQ could certainly be further optimized in this assay by changing injection time, signal exposure time, primary antibody dilution, or different primary antibody. Measuring a lower LOQ was not needed for analyzing residual BSA to support our current vaccine development because all vaccine samples tested contain measurable amounts of BSA within the linear calibration curve. The accuracy was evaluated by spiking different concentrations of BSA standard into buffer and vaccine final drug substance lots. Spike recovery for the lowest spike level (60 ng/ml) was calculated to be $\geq 81\%$. The reproducibility (intermediate precision) was evaluated by testing two vaccine final drug substance lots in duplicate at least three independent times. The CV was $\leq 6\%$ for the sample containing 2540 ng/ml BSA and $\leq 15\%$ for the sample containing 703 ng/ml BSA.

Analysis of final product and process intermediates for vaccine samples

The concentrations of residual BSA in four vaccine final drug substance batches in HS or HSS buffer were tested by the developed Capillary Western method. All samples were diluted 1:10 in SB + AD and have measured values ranging from 684.0 to 3781.3 ng/ml BSA with CV between duplicates of $< 10\%$. Fig. 5A and B depict the Western image and electropherogram traces respectively of BSA detected in vaccine batches. The monomeric BSA peak is calculated at 66 kDa and consistent with the standard. Batch 2 contains the highest concentration of residual BSA and this is the only batch that contains both monomer and dimer BSA. In addition batches 2 and 4 show that BSA degradation formed below the main peak. Due to the separation power of CE-based methods, the electropherogram is able to give additional qualitative details about the samples. This method was also used to show the clearance of BSA in process intermediate vaccine samples (Fig. 5C). The process intermediate samples show a decrease in BSA concentration from clarified bulk to low salt bulk. Note: the final drug substance is concentrated from the low salt bulk.

The data illustrate the benefits of using the Capillary Western technology for quantitating BSA concentration in complex vaccine samples. In addition, we were able to successfully test intermediate vaccine samples with this method. This suggests the Capillary Western is less prone to traditional sample matrix interference compared to ELISA-based immunoassays. Lastly, the Capillary Western allows for increased specificity by separating proteins according to size. By separating monomer and aggregate forms of

BSA, we were able to accurately determine the concentration of BSA in vaccine samples compared to a dot blot or ELISA, which does not distinguish the antibodies affinity for the monomer BSA compared to dimer or trimer.

Conclusions

The optimized Capillary Western method for residual BSA assay in vaccine samples is developed with about two logs linearity and it is sensitive to fulfill the WHO guidelines (50 ng per human dose). The method LOQ, accuracy, and reproducibility are comparable to standard ELISA. However, the automated Capillary Western method provides three additional benefits: (1) additional specificity by size separation; (2) denaturing and reducing conditions of Westerns eliminate the possibility of matrix interference or BSA loss; (3) SimpleWestern technology is fully automated leading to an increased precision and reduced analyst bench work. Finally, this, to the best of our knowledge, is the first time Capillary Western technology using SimpleWestern is being used to quantitate residual proteins in vaccines. This technology has great potential to replace standard sandwich ELISA as the 96-well version of SimpleWestern is available. It is expected that this technology may provide both great analytical capacity (a large amount of information in a relatively short time) and product coverage for a wide spectrum of vaccine product development (discovery, pre-clinical, clinical, and commercialization) in the biopharmaceutical industry.

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References

- [1] K. Hirayama, S. Akashi, M. Furuya, K. Fukuhara, Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and FRIT-FAB LC/MS, *Biochem. Biophys. Res. Commun.* 173 (1990) 639–646.
- [2] S. Mirapurkar, U.H. Nagvekar, N. Sivaprasad, Polyreactivity of monoclonal antibodies produced against thyroid stimulating hormone (hTSH), *J. Immunoassay Immunochem.* 28 (2007) 119–126.
- [3] O. Gutmann, R. Kuehlewein, S. Reinbold, R. Niekrawietz, Fast and reliable protein microarray production by a drop-in-drop technique, *Lab Chip* 5 (2005) 675–681.
- [4] M. Granstrom, M. Eriksson, G. Edevag, A sandwich ELISA for bovine serum in viral vaccines, *J. Biol. Stand.* 15 (1987) 193–197.
- [5] O. Bonin, I. Schmidt, W. Ehrengut, Sensitization against calf serum proteins as a possible cause of allergic reactions after vaccination, *J. Biol. Stand.* 1 (1973) 187–193.
- [6] S. Tanabe, Y. Kobayashi, Y. Takahata, F. Morimatsu, R. Shibata, T. Nishimura, Some human B and T cell epitopes of bovine serum albumin, the major beef allergen, *Biochem. Biophys. Res. Commun.* 293 (2002) 1348–1353.
- [7] J. Vicente-Serrano, M.L. Caballero, R. Rodriguez-Perez, P. Carretero, R. Perez, J.G. Blanco, S. Juste, I. Moneo, Sensitization to serum albumins in children allergic to cow's milk and epithelia, *Pediatr. Allergy Immunol.* 18 (2007) 503–507.
- [8] WHO expert committee on biological standardization, WHO Technical Report Series 941, Geneva (2007).
- [9] A.R. Ter-Avest, G. Van-Steenis, A.D.M.E. Osterhaus, A comparison of an enzyme-linked immunosorbent assay and counter current electrophoresis for the detection of bovine serum albumin in virus vaccines, *J. Biol. Stand.* 15 (1987) 245–250.
- [10] S. Sladoljev, A. Trescec, M. Cvetkovic, M. Beck, M. Juzbasic, The development of a latex agglutination inhibition assay for the determination of foetal calf serum in viral vaccines, *Biologicals* 18 (1990) 45–48.
- [11] J. Wang, T. Masuko, H. Ueno, H. Hojo, Y. Hashimoto, Derivation and application of monoclonal antibodies recognizing several epitopes on bovine serum albumin, *Tohoku J. Exp. Med.* 172 (1994) 345–353.
- [12] S. Khamehchian, R. Madani, F. Golchinfar, M. Taghavian, Development of a sandwich enzyme-linked immunosorbent assay (ELISA) for determining of bovine serum albumin (BSA) in trivalent measles-mumps-rubella (MMR) vaccines, *Hum. Vaccin.* 4 (1998) 375–378.

- [13] K. Zhang, C. Song, Q. Li, Y. Li, Y. Sun, K. Yang, B. Jin, The establishment of a highly sensitive ELISA for detecting bovine serum albumin (BSA) based on a specific pair of monoclonal antibodies (mAb) and its application in vaccine quality control, *Hum. Vaccin.* 6 (2010) 652–658.
- [14] A.C. Barton, S.D. Collyer, F. Davis, G.Z. Garifallou, G. Tsekenis, E. Tully, R. O'Kennedy, T. Gibson, P.A. Millner, S.P.J. Higson, Labelless AC impedimetric antibody-based sensors with pg ml^{-1} sensitivities for point-of-care biomedical applications, *Biosens. Bioelectron.* 24 (2009) 1090–1095.
- [15] Y. Yu, Y. Lai, X. Zheng, J. Wu, Z. Long, C. Liang, Synthesis of functionalized CdTe/CdS QDs for spectrofluorimetric detection of BSA, *Spectrochim. Acta Part A* 68 (2007) 1356–1361.
- [16] X. Zhang, C. Song, L. Chen, K. Zhang, A. Fu, B. Jin, Z. Zhang, A novel immunoassay for residual bovine serum albumin (BSA) in vaccines using laser induced fluorescence millimeter sensor array detection platform, *Biosens. Bioelectron.* 26 (2011) 3958–3961.
- [17] P. Xue, K. Zhang, Z. Zhang, Y. Li, F. Liu, Y. Sun, X. Zhang, C. Song, A. Fu, B. Jin, K. Yang, Highly sensitive chemiluminescent analysis of residual bovine serum albumin (BSA) bases on a pair of specific monoclonal antibodies and peroxyoxalate-glyoxaline-PHPPA dimer chemiluminescent system in vaccines, *Appl. Biochem. Biotechnol.* 166 (2012) 1604–1614.
- [18] A. Koller, H. Wätzig, Precision and variance components in quantitative gel electrophoresis, *Electrophoresis* 26 (2005) 2470–2475.
- [19] U. Nguyen, N. Squaglia, A. Boge, P.A. Fung, The Simple Western (TM): a gel-free, blot-free, hands-free Western blotting reinvention, *Nat. Methods* 11 (2011) v–vi.
- [20] S. Liu, S. Sardi, B. Sonom, D. Zocco, R. McSweeney, A. Fraser, A. Halleck, H. Li, G. Smejkal, S. Munevar, J.G. Jin, T. Kawai, I. Ghiran, J.P. McGrath, M. Whitman, S. Ng, W.P. Kuo, The application of a novel nanovolume capillary electrophoresis based protein analysis system in personalized and translational medicine research, *J. Bioanal. Biomed.* S3 (2013) 1–10.
- [21] R.R. Rustandi, J.W. Loughney, M. Hamm, C. Hamm, C. Lancaster, A. Mach, S. Ha, Qualitative and quantitative evaluation of SimonTM, a new CE-based automated Western blot system as applied to vaccine development, *Electrophoresis* 33 (2012) 2790–2797.
- [22] H.R. Constantino, R. Langer, A.M. Klibanov, Solid-phase aggregation of proteins under pharmaceutically relevant conditions, *J. Pharm. Sci.* 83 (1994) 1662–1669.