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APPLICATION NOTE

BIOPROCESS CONTAMINANT DETECTION USING SIMPLE WESTERN

IDENTIFICATION AND QUANTITATION OF PROCESS-RELATED IMPURITIES

INTRODUCTION

The identification and quantitation of process-related impurities during biotherapeutic development is critical to demonstrating the quality, efficacy and safety of the therapeutic agent. These impurities are typically residuals originating from the production cell line, the reagents and components used throughout cell culture/maintenance or during downstream processing. Residual proteins that go undetected can be recognized as foreign antigens in vivo, triggering a potentially fatal immune response. Additionally, traces of process-specific reagents or components may alter the product stability and reduce therapeutic efficacy. Therefore, the accurate detection of impurities is imperative but complicated, given the range of potential impurity sources or types, their presence in trace amounts and the complex matrix of the sample in which they exist.

In this application note, we focus on the accurate detection of four candidate contaminants that may be present during various stages of the therapeutic protein and vaccine development processes: host cell protein (HCP), Protein A, green fluorescence protein (GFP) and bovine serum albumin (BSA). Current methods for assessing the concentration of such residuals, like enzyme-linked immunosorbent assays (ELISAs), flow cytometry and traditional Western blotting are labor-intensive and prone to error. These traditional methods can also be misleading due to a lack of specificity and flexibility required to obtain a comprehensive contaminant profile and may exclude relevant critical information like size-based results, oligomerization state and low limit of detection[1]. We'll show you how highly sensitive and analytical Simple Western™ immunoassays deliver where others come up short.

HOW SIMPLE WESTERN DOES IT BETTER

Current approaches may be adequate in their ability to determine if the concentration of a given residual analyte is below regulatory guidelines. However, Simple Western immunoassays get you there with far less time spent at the bench without compromising assay sensitivity or precision! You'll get orthogonal information from size- or charge-based separation plus separation of protein fragments or oligomers and improved assay linearity, coefficient of variation (CV), limit of detection (LOD) and quantitation (LOQ) that are sure to increase reproducibility. Simple Western assays also achieve reduced sample matrix interference due to their compatibility with denatured or reduced conditions. And you'll have more time for science by eliminating plate-washing and reagent/buffer-making steps that can eat up hours of time.

Simple Western immunoassays are automated, capillary-based protein analysis tools that solve many of the challenges that come with traditional Westerns. There are five systems to choose from: Jess™ (PN 004-650), Wes™ (PN 004-600), Peggy Sue™ (PN 004-800), Sally Sue™ (PN 004-700) and NanoPro™ 1000 (PN 004-109). For size-based separation, you'll only need to dilute 3 µL or 5 µL of sample in ProteinSimple master mix on Jess/Wes or Peggy Sue/Sally Sue, respectively, to get pg/mL-level sensitivity. Charge-based separation assays on Peggy Sue or NanoPro 1000 also only require 12 µL of sample. Compass for Simple Western software performs automatic analysis and generates quantitative results that are reproducible with intra-assay CVs less than 15%.

All are open platforms, so you can evaluate the presence of any contaminant as long as you have the primary antibody for detection. Jess and Wes give you 25 size-based data points in just three hours with only 30 minutes of hands-on time for setup. If you need higher throughput or want an instrument that can do both size and charge, Sally Sue (size only), NanoPro 1000 (charge only) and Peggy Sue (size and charge) all give you 96 data points overnight and only involve about an hour of hands-on time.

HOST CELL PROTEIN

HOST: MAMMALIAN CELLS

Chinese hamster ovary (CHO) cells are the most commonly used host for therapeutic protein production. This is in part because they quickly adapt and grow in large-scale serum-free and/or chemically-defined suspension cultures, and, in part, because they possess the necessary machinery to accommodate the posttranslational modifications required for a product to be bioactive[2]. When the therapeutic protein is secreted into the host cell culture supernatant for collection, so too are protein impurities specific to the host cell (HCPs), and in this example, those of CHO cells. No compulsory limit has been set for the HCP level permitted, but <100 ng of HCPs per 1 mg of the bioproduct is generally considered acceptable[3].

To demonstrate the utility of Simple Western size- and charge-based assays in detecting HCPs on Peggy Sue, we established an assay detection range using a CHO HCP control antigen concentrate and a biotinylated goat CHO HCP antibody (Cygnus Technologies, PN F553X and 3G-0016-AFB, respectively). A dilution series beginning at 1 µg/mL down to 0.037 µg/mL of the CHO HCP control antigen lysate in Premix G2, pH 3–10 separation gradient and pI Standard Ladder I (PN 040-968 and 040-644, respectively) was evaluated using default Peggy Sue isoelectric focusing (IEF) conditions. CHO HCP control antigen was detected using the Biotin Detection Module (PN DM-004), where biotinylated anti-CHO HCP primary antibody (1:50 dilution) was incubated for 60 minutes and the ready-to-use Secondary Streptavidin-Horseradish Peroxidase (HRP) conjugate for 30 minutes. **FIGURE 1A** shows a lane view of the charge-based separation of the CHO HCP control antigen lysate, generated in Compass for Simple Western software, where as little as 37 ng/mL was detected (LOD) with the anti-CHO HCP antibody. The concentration of each sample can be plotted by its peak area in Compass to generate a standard curve to assess the linearity of the relationship; **FIGURE 1B** depicts this output and a strong linear relationship, $R^2 \geq 0.99$. The assay LOQ was determined to be 104 ng/mL.

Charge distribution information, among other applications, is useful when analyzing the purity of your final product which might appear to degrade in the presence of impurities at various pH values. A charge separation profile of the 1-µg/mL CHO HCP sample is shown in **FIGURE 2**, in both lane and graph view. The IEF assay on Peggy Sue requires only a 12-µL sample. However, this does not compromise the high-resolution of the data generated. By running multiple cycles out of the same sample well (on Sally Sue, Peggy Sue or NanoPro 1000), you can detect multiple contaminants without wasting precious material. For example, analyzing 1 µg/mL on Peggy Sue is equivalent to loading just

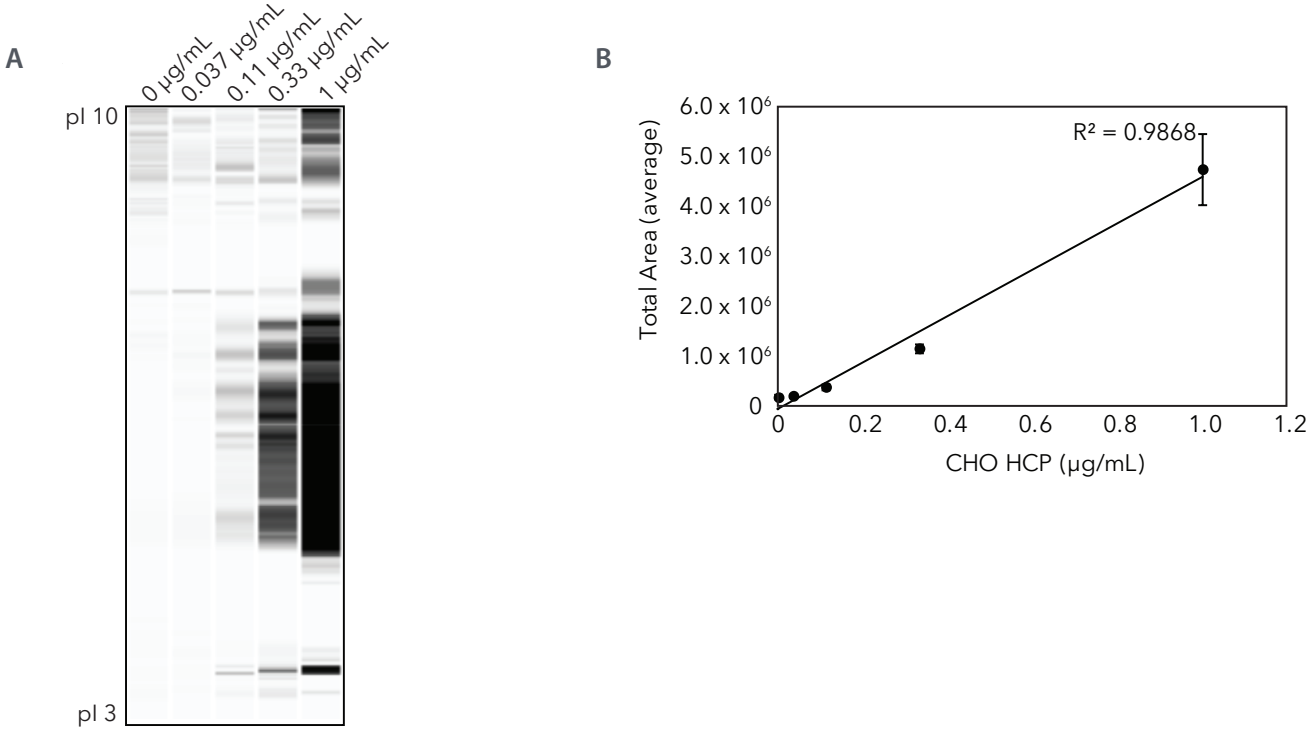

FIGURE 1. CHO HCP titration and IEF separation on Peggy Sue. Lane view of the CHO HCP serial dilution series (**A**). Standard curve of the serial dilution series (**B**). HCP antigen was diluted into G2 3-10 ampholytes and titrated from 1 µg/mL to 0.037 µg/mL. The HCP antigen was detected with a biotinylated CHO HCP antibody (1:50 dilution) and Secondary Streptavidin HRP conjugate.

400 pg into the capillary. In this way, charge-based separation on Peggy Sue can support product stability monitoring during progression through various parts of the development process that may alter its purity.

Size-based separation on Sally Sue and IEF assays on NanoPro 1000 can also be used to evaluate the presence of HCPs and other impurities during the purification process of a biotherapeutic with the same sample throughput as Peggy Sue, who can do both assay types. Researchers at Biogen recently quantitated the presence and clearance of residual host cell HSP70 through the production of a recombinant fusion-Fc protein, demonstrating R^2 values ≥ 0.99 and a detection range from 250 ng/mL to 1.25 µg/mL[4]. Compared with Western blotting, Biogen researchers noted the clear resolution of molecular weight differences between impurities and their fusion-Fc polypeptide that is achievable with Sally Sue but not possible with other methods. In another publication, analytical protein chemists at Genentech report on the application of NanoPro 1000 for monitoring charge variants in crude cell lysates where HCP presence is of concern[5]. By spiking harvested cell culture fluid with purified IgG proteins of varying pI values, researchers could accurately detect protein products with no matrix interference from the lysate with CVs less than 3%[5]. They demonstrate strong assay linearity (R^2 ≥ 0.99) when performing serial dilutions of the protein concentration in lysate from 4 µg/mL to 12.5 ng/mL and obtain a 6-ng/mL LOD for the charge variants analyzed. The ability to accurately evaluate protein products of ranging pI values in crude cell lysates makes NanoPro 1000 highly useful all throughout bioproduct development and production.

HOST CELL PROTEIN

HOST: BACTERIAL CELLS

Another commonly used expression system for the production of recombinant proteins is *Escherichia coli* (*E. coli*), from which HCPs also originate and must be quantitated and minimized. In this example, we utilize Wes to detect *E. coli* HCP using the 12–230 kDa Wes Separation Module (PN SM-W004). The *E. coli* HCP control antigen and anti-*E. coli* HCP antibody were purchased from Cygnus Technologies (PN F417 and F117-PA, respectively). Anti-*E. coli* HCP was detected using the Anti-Goat HRP Detection Module (PN DM-006). **FIGURE 3** shows the titration range (from 100 µg/mL to 1.23 µg/mL) of *E. coli* HCP, detected with the anti-*E. coli* HCP antibody (1:10 dilution). Linearity of the titration data (R^2 = 0.9659) and the assay LOD (15.7 ng/mL) were also determined based on a standard curve.

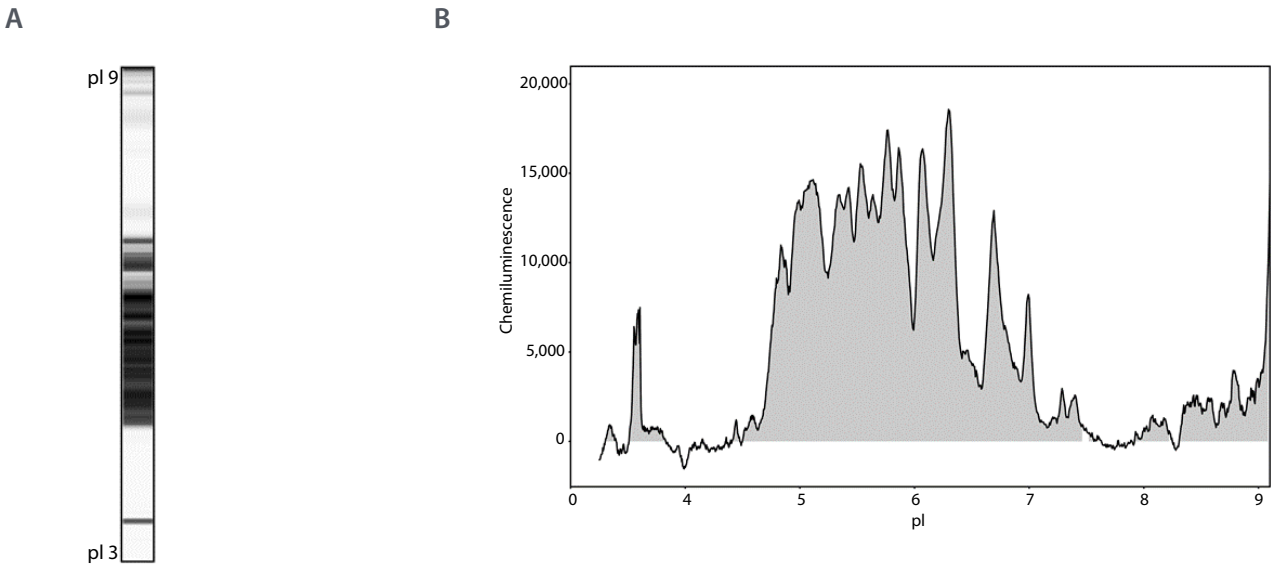

FIGURE 2. CHO HCP lysate (1 µg/mL) IEF separation on Peggy Sue. Lane view of the CHO HCP lysate (**A**) Graph view of the CHO HCP lysate with fitted peaks (**B**). The HCP antigen was detected with a biotinylated CHO HCP antibody (1:50 dilution) and Secondary Streptavidin HRP conjugate.

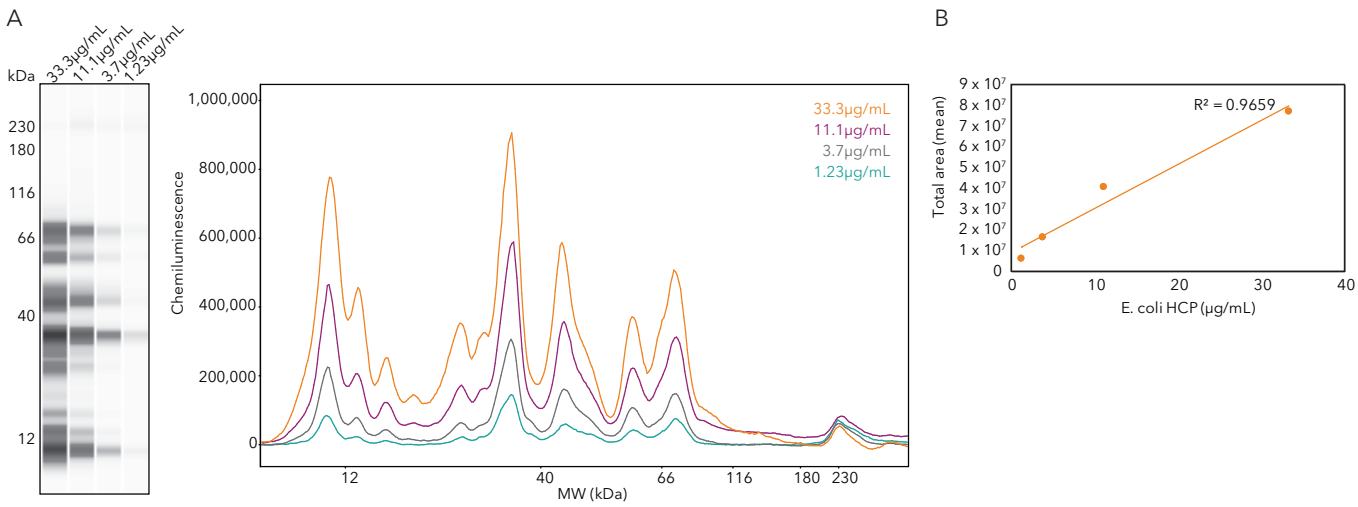

FIGURE 3. *E. coli* HCP antigen detection on Wes. Compass for Simple Western lane and graph views (**A**). The concentration of HCP lysate plotted against the average background-subtracted total peak area (**B**). *E. coli* HCP antigen was diluted in 0.1X Sample Buffer and titrated from 33.3 µg/mL to 1.23 µg/mL following default Simple Western protocols. Goat anti-*E. coli* HCP (1:10) was diluted in Antibody Diluent 2 (PN 042-203), followed by the anti-goat secondary HRP conjugate. All other instrument and assay conditions were standard settings.

PROTEIN A

Many therapeutic proteins are isolated from cell culture media using Protein A chromatography[6]. During purification, Protein A can leach from the solid phase, ending up in the purified product. This can be problematic as regulatory guidelines dictate that detection and reduction of residual Protein A are necessary to obviate potential immunogenic consequences in vivo. Protein A and Protein A-derived ligands can be especially tricky to detect since there are several potential IgG-binding regions, making quantitation difficult[7]. Although several approaches may be suitable, Wes is superior in his ability to deliver quantitative data that resolves your target protein from other impurities of similar molecular weight.

In **FIGURE 4** we demonstrate the sensitivity of Wes by evaluating the separation of a two-fold dilution series (from 400 pg/mL to 25 pg/mL) of recombinant Protein A, short and long forms (Novus Biologicals, NBP2-34964 and NBP2-34966, respectively) using the Wes 12–230 kDa Separation Module. A Protein A antibody from R&D Systems (PN MAB99761) and a donkey anti-rat HRP secondary antibody (Jackson ImmunoResearch Laboratories, PN 712-035-153) were diluted in Antibody Diluent 2 and used at a final concentration of 1.2 µg/mL and 13.5 µg/mL, respectively, for detection. Electropherograms and linearity results of the Protein A short form (**FIGURE 4**, left) and long form (**FIGURE 4**, right) antigen titrations are shown.

The Protein A antibody shows slight cross-reactivity with the internal fluorescent standard (230 kDa). This binding will not impact quantitation, as Protein A short and long forms have apparent molecular weights of approximately 42 kDa and 55 kDa, respectively, and are well-separated from the 230 kDa peak. To validate the sensitivity and reliability of Wes, LOD and LOQ values were determined using the linearity results for each assay. The Protein A short form assay LOD was 28.1 pg/mL, and the LOQ was 74.1 pg/mL. The Protein A long form assay LOD was 39.6 pg/mL, and the LOQ was 133.0 pg/mL.

To reflect a real-world scenario, the recombinant Protein A short form was serially diluted and spiked into a lysate background. We used the same CHO HCP Antigen Concentrate (0.1 mg/mL) from Cygnus Technologies and calculated the LOD/LOQ in the presence and absence of CHO HCP lysate. The linearity comparison shows a minor decrease in our ability to detect Protein A in the presence of CHO HCP (**FIGURE 5,** left). This translated to a slightly higher LOD/LOQ for Protein A (**FIGURE 5**, right). Nevertheless, the assay LOD amounted to as little as 28.1 pg/mL of Protein A detected, based on the standard curve. Even in the dense and complex environment of the CHO HCP lysate solution, a low LOD for Protein A can be achieved (35.3 pg/mL), which is in line with the implemented regulatory guidelines[8].

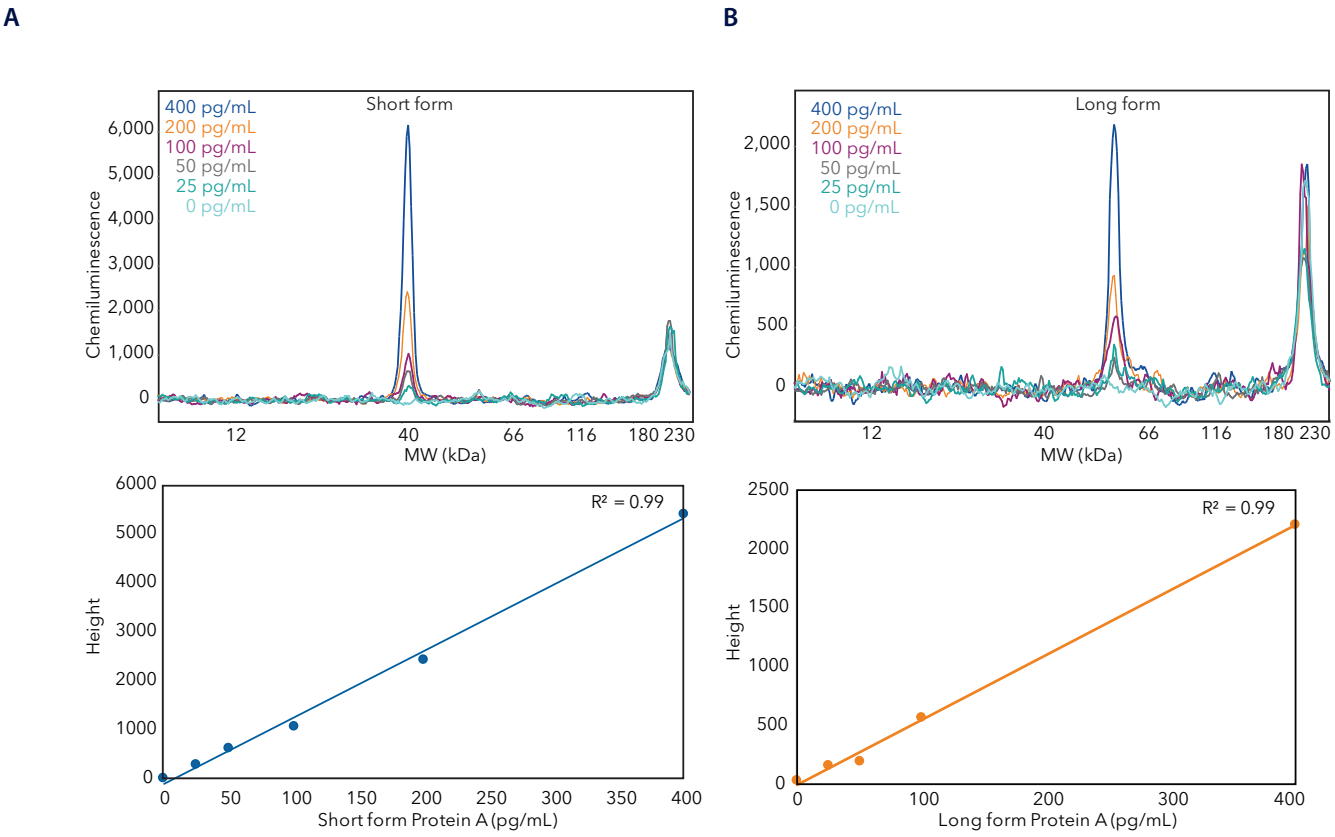

FIGURE 4. Protein A detection and assay linearity on Wes. Compass for Simple Western graph views of Protein A short form (**left**) and long form (**right**) were diluted into 0.1X Sample Buffer and titrated from 400 pg/mL to 25 pg/mL. Both forms of Protein A were detected using the same primary and secondary antibodies.

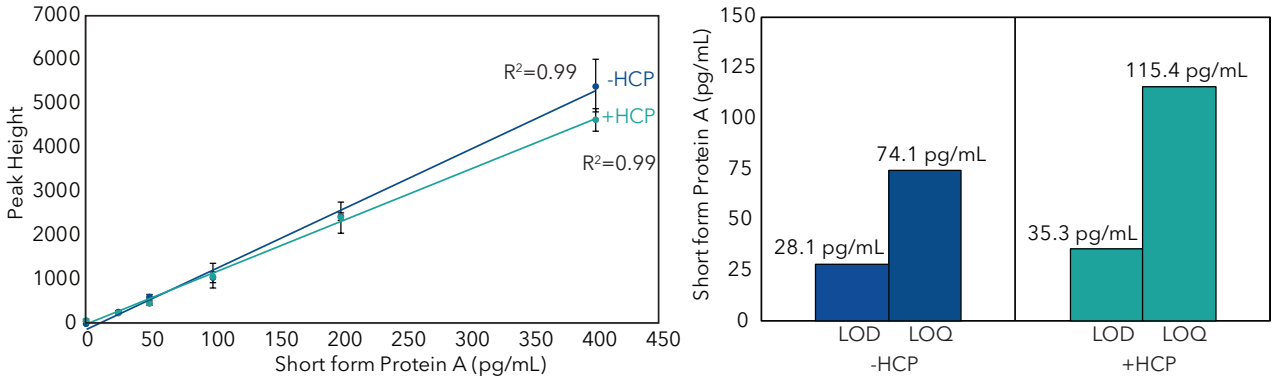

FIGURE 5. Linearity and LOD/LOQ data for Protein A spiked into a CHO HCP solution. Protein A (short form) was titrated in a CHO HCP control antigen (0.1 mg/mL). Data were compared with Protein A alone, diluted in 0.1X Sample Buffer. Linearity data (**left**) show only a minor decrease in the slope of the Protein A linearity curve, n=2. The LOD and LOQ increased slightly in the presence of CHO HCP antigen (**right**).

GREEN FLUORESCENCE PROTEIN

In upstream bioprocess, where mammalian cells are transfected with recombinant DNA encoding the target product, GFP is routinely co-transfected as a marker to measure transfection efficiency. Moreover, recombinant protein products are often genetically fused with GFP as a biosensor to monitor expression, promote solubility and facilitate purification. The detection of residual GFP requires a method sensitive enough to reveal degraded or aggregated GFP-tagged protein fragments.

Wes makes it easy to identify GFP contaminants with sensitivity below 1 ng/mL. Here we provide an exampleof how you can use a standard curve, like the one generated in **FIGURE 6A**, where we used 0.1X Sample Buffer to serially dilute recombinant jellyfish GFP (Novus Biologicals, NBC1-22949), to calculate the concentration of residual GFP in prepared samples. **FIGURE 6B** illustrates a scenario in which two discrete bands are separated in our HEK293-transfected cell lysates using the Wes 12–230 kDa Separation Module, the Anti-Goat Detection Module and an anti-GFP antibody (R&D Systems, PN AF4240) diluted in Antibody Diluent 2 (15 µg/mL) for detection. The observed lower-molecular-weight (30 kDa) and upper-molecular-weight (150 kDa) proteins are clearly resolved by Wes and represent the GFP-tagged target protein and residual free GFP, respectively. The concentration of free GFP in each sample was calculated using the standard curve in **FIGURE 6A** and is listed below each lane in the gel view of **FIGURE 6B**, in which lanes could be rearranged using Compass software to illustrate the low to high detection range of free GFP. As little as 0.23 ng/mL of residual GFP could be detected.

In the next example, we utilized the same host cell line, HEK 293, to express various recombinant proteins fused to GFP. Cells were lysed in Bicine/CHAPS Lysis Buffer (PN 040-764) with Aqueous Inhibitor Mix (PN 040-482) and DMSO Inhibitor Mix (PN 040-510) for 30 minutes on ice. Samples were spun for 10 minutes at 15,000 x *g*, and 0.1 mg/mL was analyzed on Wes using the previously mentioned goat anti-GFP antibody for detection (**FIGURE 7**). The target protein products are outlined in green boxes. Bands appearing below the target protein are likely the result of protein degradation, and those above may be oligomers or higher order aggregates—both undesirable events during clone selection and scale-up. Note that these circumstances typically go undetected using other assays like ELISA and flow cytometry since they cannot distinguish proteins of differing molecular weight. Wes, though, allows you to rapidly identify them and provides you with concentration and molecular weight information not achievableby traditional Western blot, flow cytometry or ELISA approaches—all without compromising sensitivity.

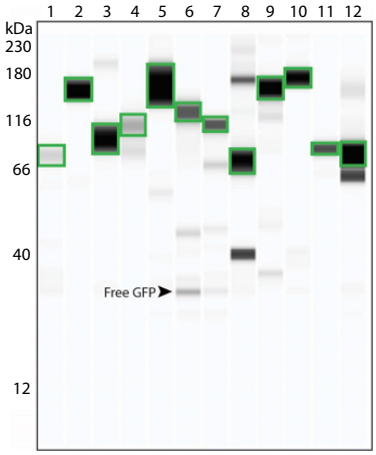

FIGURE 7. HEK 293 cells transfected with various GFP-tagged recombinant proteins. Cells were lysed in Bicine/CHAPS Lysis Buffer containing Aqueous and DMSO Inhibitor Mixes. 0.1 mg/mL was analyzed on Wes using a goat anti-GFP antibody for detection. Target protein products are outlined in the green boxes. Unfused GFP is indicated by an arrow.

BOVINE SERUM ALBUMIN

BSA is a commonly encountered contaminant during the preparation of vaccines because it is a major component of the culture medium used during vaccine production[8]. Residual BSA has the potential to elicit an allergic immune response in vivo and is thus firmly regulated by the World Health Organization[9]. To illustrate Simple Western's robust analytical capacity and to reiterate the proof-of-concept studies presented herein, we highlight a recent publication by vaccine development researchers at Merck who adopted both Wes and Peggy Sue for the detection of residual BSA in vaccine formulations[8].

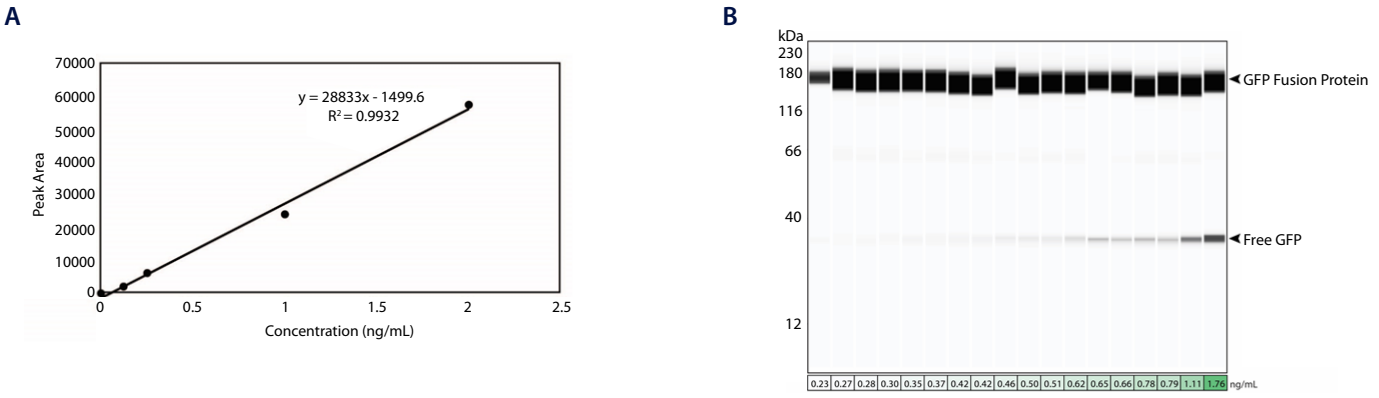

FIGURE 6. GFP detection on Wes. Line of fit calculated based on the plotted peak area for each sample concentration in a serial dilution series of recombinant GFP (from 2 ng/mL to 0.125 ng/mL) using Compass for Simple Western software (**A**). Lane view of free GFP, generated by Compass software (**B**). The 150 kDa band represents the GFP-tagged target protein, and the 30 kDa band is free (residual) GFP. Free GFP concentration in each sample was calculated using the linear equation generated in (**A**) and the shading of white to green is representative of low to high GFP concentration, respectively.

Using the 12–230 kDa Separation Module, researchers created a serial dilution series (from 600 ng/mL to 7.4 ng/mL) using ≥98% pure BSA protein reference standard ampules (ThermoFisher, PN 23209) and a rabbit anti-BSA antibody (Bethyl Laboratories, PN A10-127A) to generate a standard curve ($R^2 \geq 0.9994 \pm 0.0008$). This curve was applied to determine the BSA concentration in tested vaccine samples[8]. The Simple Western size-based assays performed allowed for the identification of dimers and higher order aggregates within the analyzed vaccine products, and the data included could be confidently repeated with reported CVs less than 10%. Notably, and as the authors point out, the reproducibility of manual Westerns was previously evaluated and determined to have CV values greater than 35%.

CONCLUSION

The precision, sensitivity and flexibility of Simple Western rivals traditional approaches for detecting process-related contaminants during bioproduction. This is supported by the achievable R^2, LOD, LOQ and CV values, both published and presented herein, which are within industry acceptance criteria. With minimal hands-on and assay times, Simple Western can provide you with information on molecular weight, protein charge, degraded products and oligomerization state, among other attributes, while also being compatible with denatured or reduced samples for analysis. The protocol is fully automated after sample preparation, and Compass for Simple Western software automatically quantitates the residual presence of impurities, minimizing time and labor. The methods outlined in this application note for the evaluation and characterization of HCP, Protein A, GFP and BSA are a proof-of-concept for adaptation as quantitative and reliable protocols throughout the development process of a biotherapeutic.

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