bio-techne® | PROGEN

APPLICATION NOTE

AAV2 VP Protein Standards and Their Use in Quantifying Capsid Protein Ratio by Western Blot and Simple Western

Hüseyin Besir, Ph.D., Rene Wetzel, Ph.D., Dana Holzinger, Ph.D., Caroline Odenwald, Ph.D. and Katja Betts, EMSc, PROGEN, Heidelberg, Baden-Württemberg, Germany

Rachel Talis, Francisco Ramirez, Charles Haitjema, Ph.D., Kelly Gardner, Ph.D., and Chris Heger, Ph.D., ProteinSimple, a Bio-Techne brand, San Jose, CA, United States of America

ABSTRACT

Adeno-associated viruses (AAV) represent the most commonly used viral vector for the delivery of therapeutic transgenes. However, the gene therapy community is facing major challenges like the high demand for efficient production of high-quality AAV vectors in clinical scale quantities. Robust, quality protein standards and fast, automated highly sensitive analytical instrumentation are needed to address these challenges. Comprehensive monitoring of VP1, VP2, and VP3 protein concentrations and their molar ratios is an effective tool to ensure initial optimization and consistent quality of the final AAV gene therapy products. Here we introduce PROGEN´s recombinant VP standards used with Simple Western™ automated capillary electrophoresis in fit-for-purpose solutions for VP capsid protein ratio and viral protein titer measurements. We further show the establishment of the AAV2 VP protein standards by PROGEN as well as the assay performance data that demonstrates linearity and high sensitivity in an automated workflow optimized for AAV samples.

INTRODUCTION

AAV is the most commonly used viral vector for the delivery of genes into target cells. The non-pathogenic viruses induce only limited immunogenicity while having a high transduction efficiency, stable long-term expression, and selective tissue tropism. The use of recombinant AAV (rAAV) vectors in gene therapy applications has strongly increased. The high demand for rAAV vector production in quantities necessary for industrial scale production appears to be a major challenge for the AAV community to overcome. The optimization as well as a comprehensive monitoring of the production procedure is indispensable to ensure the production of high quantities and conserve optimal potency.

VP PROTEIN STOICHIOMETRY

In this context, the stoichiometry of the viral capsid proteins VP1, VP2, and VP3 represents a critical factor that has a strong influence on the quantity but also transduction efficiency of the final product. Comprehensive monitoring of the VP protein concentrations and ratios during AAV vector production is a continuously underestimated tool for proper optimization and prediction of the final AAV vector product.

It is commonly known that the AAV capsid proteins VP1, VP2, and VP3 are required trans elements for AAV capsid assembly. Though the standard formulation of an AAV capsid consists of 60 subunits in a VP1:VP2:VP3 ratio of 1:1:10,[1] however, the stoichiometry of the three proteins might differ strongly depending on the production system of choice which has an impact on the infectivity and stability of the capsids. While VP1 is not essential for capsid assembly itself, the absence or extensive incorporation of the protein has a major negative effect on the transduction efficiency.[2] In addition, insufficient VP2 and VP3 have been described to be the primary cause of ineffective packaging while excessive incorporation of VP3 simultaneously has been shown to decrease vector potency.[3,4]

Identifying the optimal ratio between the three AAV capsid proteins represents a major challenge during the optimization of the production process. Overcoming this is worth it to allow rAAV vector production with consistent quality in high quantities.

PROTEIN STANDARDS FOR EFFICIENT OPTIMIZATION OF rAAV VECTOR PRODUCTION

The use of suitable protein standards to ensure accurate viral capsid protein characterization is therefore indispensable. PROGEN established the recombinant production of suitable VP1, VP2 and VP3 protein standards that can be used as adequate control material to compare and adjust optimal capsid protein concentrations and ratios by either manual or automated analysis to allow efficient rAAV vector production.

MATERIAL AND METHODS

PRODUCTION OF THE AAV2 VP1, VP2, AND VP3 PROTEIN STANDARDS

The AAV2 VP1, VP2, and VP3 gene sequences were cloned and expressed by E. coli as His6-tagged proteins in inclusion bodies for preparation of the AAV2 protein standards (PROGEN, Cat. No. 72001). The His-tagged proteins VP1, VP2, and VP3 were purified under denaturing conditions using Ni-NTA resin and stored in PBS with 6M urea at -80°C.

MANUAL GEL-BASED ANALYSIS OF THE AAV2 VP PROTEIN STANDARDS

The recombinant proteins were analyzed using standard SDS-PAGE to determine the corresponding protein purity as well as traditional western blot using a VP protein-specific antibody (PROGEN clone B1, Cat. No. 690058) against a common C-terminal epitope. The western blot analysis with fluorescence detection was performed using the same antibody clone labeled with AFDye488 (PROGEN, Cat. No. 61058-488) and appropriate filters. For proper analysis, the amounts of loaded VP1, VP2 and VP3 in ng were adjusted according to the molecular weight (MW) difference of each protein to ensure analysis of the correct molar ratio in a mixture. The applied correction factors were 1.35 for VP1, 1.11 for VP2 relative to 1.00 for VP3.

AUTOMATED ANALYSIS OF THE AAV2 VP PROTEIN STANDARDS USING SIMPLE WESTERN

The VP protein standards were analyzed by a capillary electrophoresis (CE) based immunodetection system (Simple Western™, ProteinSimple, Bio-Techne Corp.). Simple Western experiments were performed on the Peggy Sue™ instrument with the 66-440 kDa Separation Module (Bio-Techne, PN SM-S002). Samples were prepared as described in the product insert of the separation module. To optimize for baseline resolution between the viral proteins, the separation time was set to 60 minutes, sample loading time was set to 6 seconds, and stacking time was set to 12 seconds. Target proteins were detected using the unconjugated mouse anti-VP1/VP2/VP3 monoclonal antibody (PROGEN, clone B1, Cat. No. 690058) diluted to 20 µg/mL in Antibody Diluent 2 and using the Anti-Mouse Detection Module (Bio-Techne, PN DM-002).

To determine the linear range of detection, we created a 2X serial dilution series of each protein from 50 ng/mL to 0.2 ng/mL (for VP1 and VP2) and 100 ng/mL to 0.2 ng/mL (for VP3), and a no sample blank was included as a negative control. Because only 3 µL of sample are loaded per well, these values correspond to 150 pg (for VP1 and VP2) and 300 pg (for VP3) to 0.59 pg of protein. Calibration curves were generated by plotting average peak area by protein loaded per well and linear regression analysis was performed. Signal noise was measured using the raw data from the no sample capillaries and a standard deviation for noise was calculated. The limit of detection (LOD) and limit of quantification (LOQ) are defined as the concentration at which the target signal is 3 times and 10 times the standard deviation of signal noise, respectively.

RESULTS

ESTABLISHMENT OF AN AAV2 VP1, VP2 AND VP3 PROTEIN STANDARD

AAV2 VP1, VP2 and VP3 standards were produced in E. coli as His6-tagged proteins and purified under denaturing conditions as described in the material and methods section. To analyze purity of the recombinant proteins, the proteins were separated by SDS PAGE and stained with Coomassie Brilliant Blue G250 (**FIGURE 1**). Each gel shows a single band at the corresponding MW of AAV2 VP1, VP2, and VP3 and demonstrates a purity of >90%.

To further validate AAV2 VP1, VP2, and VP3 identity, three different concentrations of the corresponding proteins were applied to an SDS-PAGE and analyzed by western blot using PROGEN´s highly specific AAV VP1/VP2/VP3 antibody that was fluorescently labeled with AF488 (PROGEN, clone B1, Cat. No. 61058-488) (**FIGURE 2A**). The results show specific detection of the AAV2 VP1 (90kDa), VP2 (68 kDa), and VP3 (62 kDa) for the corresponding samples. The western blot analysis further demonstrates a decrease of signal intensity according to the decreasing concentration applied to the SDS-PAGE.

To use the AAV2 VPs as standard in AAV capsid protein ratio analysis, the three AAV capsid proteins were tested as a protein mix (**FIGURE 2B**). The protein amounts used were adjusted to their MW difference in order to have a molar ratio of VP1:VP2:VP3 of 1:1:10, representing the distribution in native AAV2 capsids. The proteins were then detected with either the fluorescently labeled VP1/VP2/VP3 antibody (PROGEN, clone B1, Cat. No. 61058-488) or standard ECL detection method using the non-labelled VP1/VP2/VP3 antibody (PROGEN, clone B1, Cat. No. 690058) and an anti-mouse secondary antibody. Both methods showed highly specific detection of the capsid proteins with the highest signal intensity for VP3. Although VP1 and VP2 proteins were applied in a stoichiometric ratio of 1:1, these proteins showed different signal intensities. This could be due to a difference in blot transfer behavior of these proteins. To clarify the discrepancy of the signal intensities in the traditional western blot, we performed CE-based immunodetection using Simple Western.

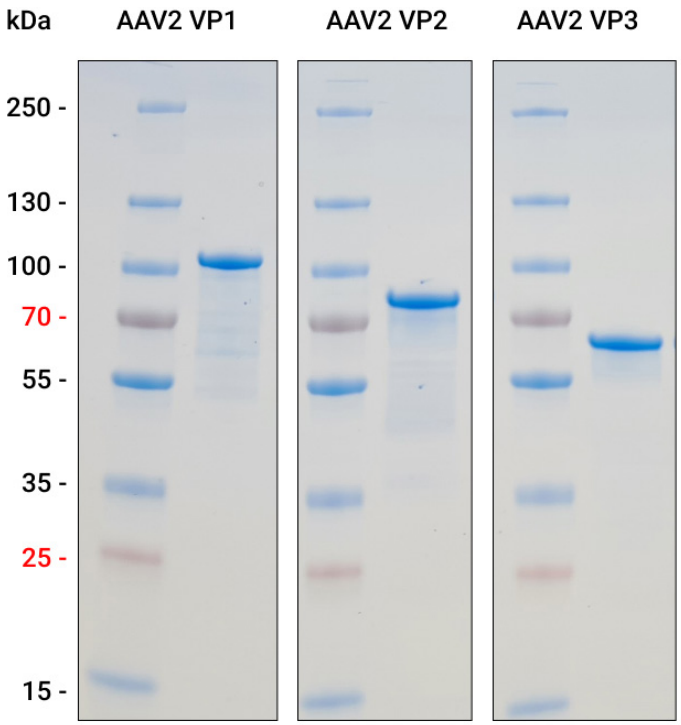

FIGURE 1. Coomassie-stained gel of purified AAV2 VP1, VP2, and VP3 standard proteins (1 µg each per lane) shows greater than 90% purity.

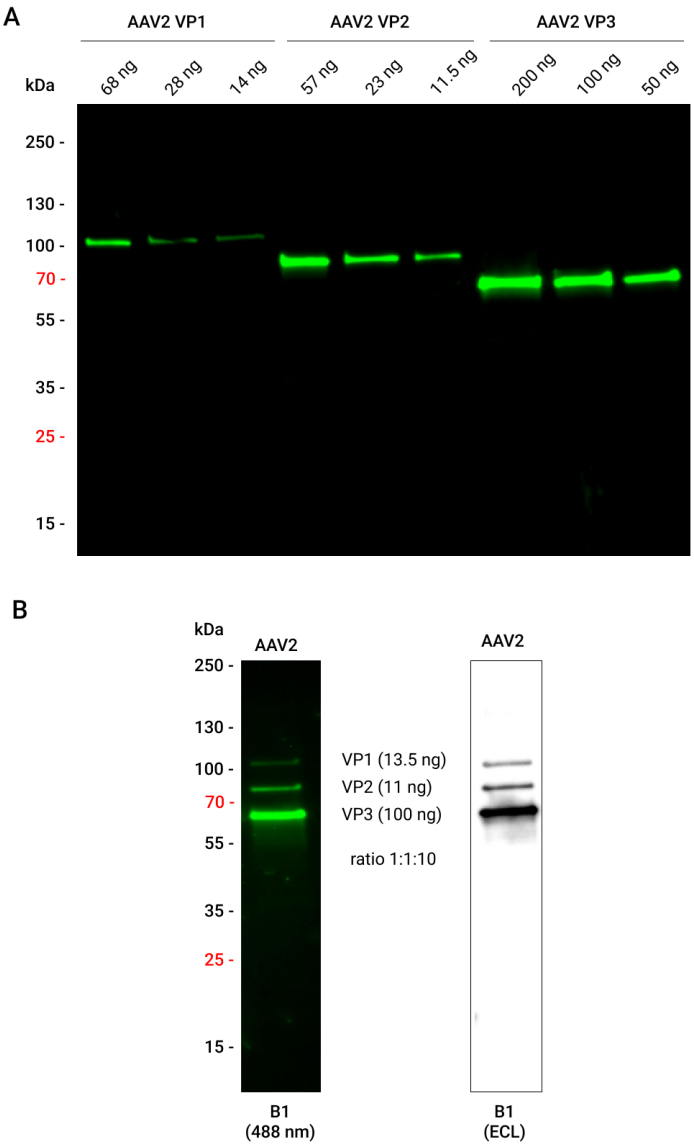

FIGURE 2. Western Blots of individual (A) and defined mix (B) of AAV2 VP1, VP2, and VP3 standard proteins, using the AF488 fluorescently labeled VP1/VP2/VP3 antibody (A, B left) or ECL (B, right) for detection. The amounts of loaded VPs in Fig. 2B were adjusted for the molecular weight difference in order to have a molar ratio of VP1:VP2:VP3 of 1:1:10.

AUTOMATED ANALYSIS OF VP1, VP2, AND VP3 FOR CAPSID PROTEIN RATIO AND VIRAL PROTEIN TITER MEASUREMENTS

While traditional SDS-PAGE is still commonly used in bioprocessing workflows, it is labor-intensive and difficult to scale. Furthermore, traditional western blot suffers from a large amount of sample material required, poor reproducibility, and results that are only semi-quantitative at best. By contrast, the CE-based immunoassay platform known as Simple Western requires as little as 3 µL of sample for low-pg detection.[5] Simple Western can perform total protein stains as well as immunoassays. The results Simple Western produces are fully quantitative, and Simple Western can automatically process 25 samples in approximately 3 hours or 96 samples overnight, so it is easier to scale. Because Simple Western is automated and only requires one manual sample preparation step, it has less variability compared to traditional western blot. Taken together, these advantages make Simple Western a promising solution in AAV manufacturing workflows.

The use of AAV VP protein standards on Simple Western has the potential to enable the generation of standard curves for quantification of VP protein concentration as a measure of viral protein titer as well as a measurement of VP capsid protein ratios which can impact AAV infectivity, an important contributor to potency. Thus, we asked how the use of the AAV2 VP standard developed by PROGEN compares by analysis on Simple Western versus traditional western blot in terms of assay sensitivity and accuracy of protein quantification.

First, we analyzed each capsid protein individually by creating a 2X serial dilution series of each protein, and each sample dilution was loaded for Simple Western immunoassay analysis on Peggy Sue. For each capsid protein, a clear signal was observed that decreased with decreasing protein concentration, and no signal was observed in the no sample control, as expected (**FIGURE 3-5**). Furthermore, the observed MW of each signal corresponded approximately with the predicted MW of each respective capsid protein. Interestingly, signals of larger MW were observed at higher concentrations of protein loading, particularly in the analyses of VP1 (**FIGURE 3**) and VP3 (**FIGURE 5**), indicating possible aggregation or oligomerization. These larger MW signals were only observable after a longer exposure time in the traditional western blot (**FIGURE 2A**), possibly due to the lower sensitivity of the traditional western blotting assay compared to Simple Western.

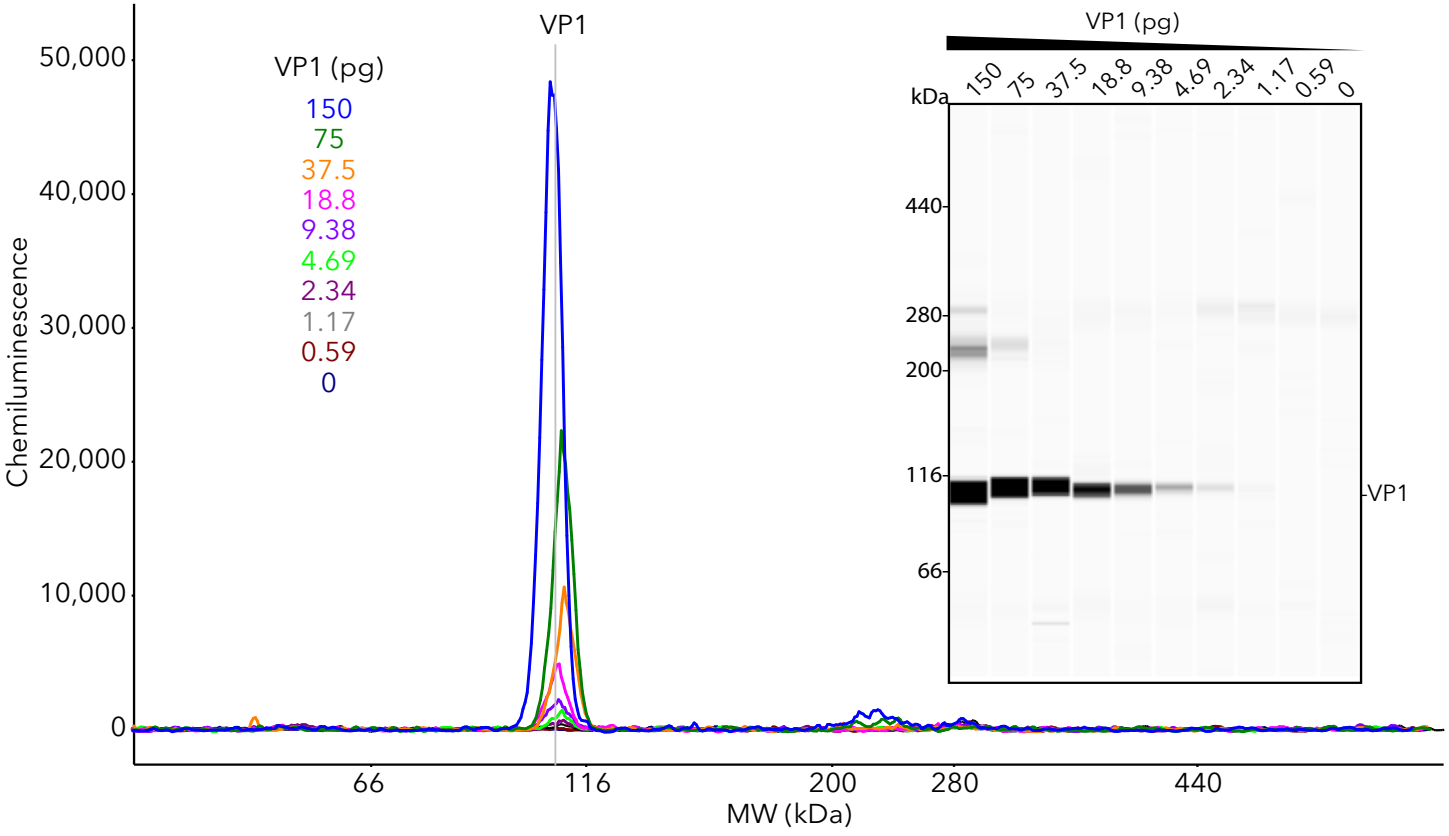

FIGURE 3. Overlaid electropherograms and lane view (inset) resulting from Simple Western analysis of denatured VP1 diluted in a 2X serial dilution series.

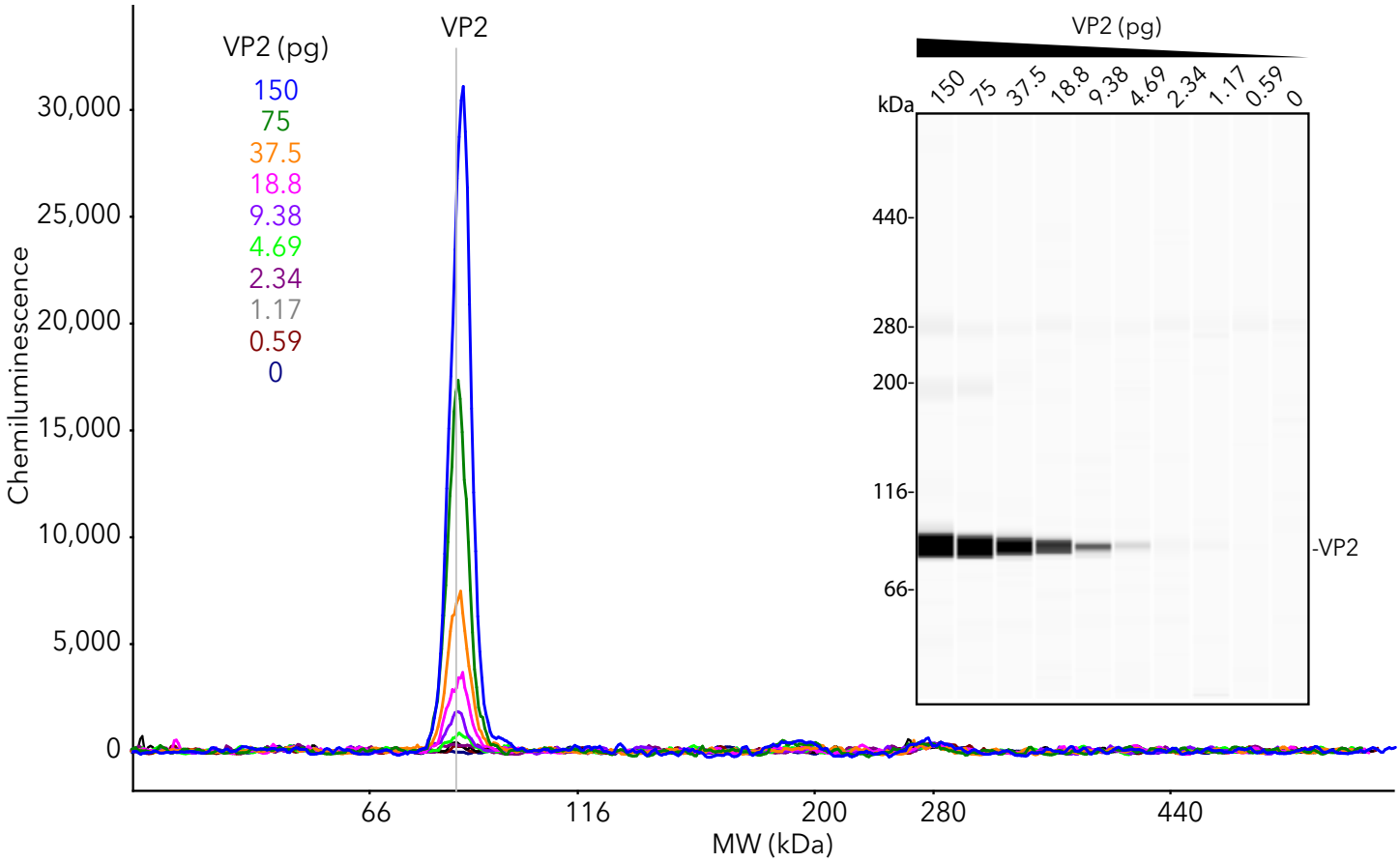

FIGURE 4. Overlaid electropherograms and lane view (inset) resulting from Simple Western analysis of denatured VP2 diluted in a 2X serial dilution series.

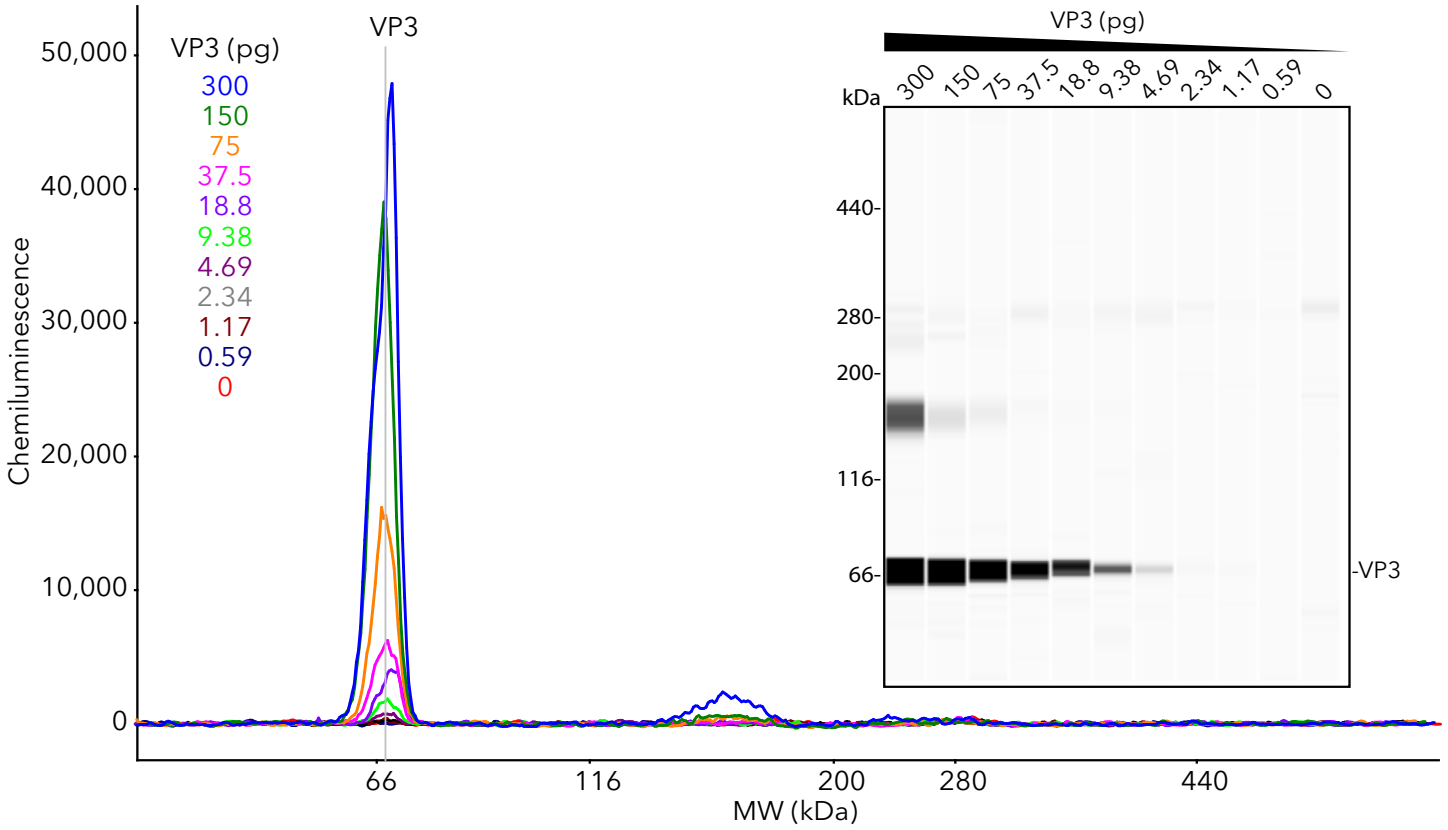

FIGURE 5. Overlaid electropherograms and lane view (inset) resulting from Simple Western analysis of denatured VP3 diluted in a 2X serial dilution series.

To determine the sensitivity of VP protein detection on Simple Western, we measured the limit of detection (LOD) and limit of quantification (LOQ) of each capsid protein as described in the materials and methods section. We generated calibration curves by plotting the average peak area of each protein signal by the amount of protein loaded per well. This analysis showed a linear range of detection between 75 pg and 0.59 pg of VP1, 150 pg and 1.17 pg of VP2, and 75 pg and 1.17 pg of VP3, with all R^2 values >0.995 (**FIGURE 6**). Next, we used these calibration curves to determine the LOD and LOQ for each protein, resulting in values that were similar across the 3 proteins (**TABLE 1**). Under the conditions tested here, VP3 showed the most sensitive detection, with an LOD of 2.04 and LOQ of 4.23 pg. Thus, it is reasonable to conclude that Simple Western detection is more sensitive than SDS-PAGE Coomassie staining as well as traditional western blot, which typically requires 50-100 pg or more for reliable detection.[6]

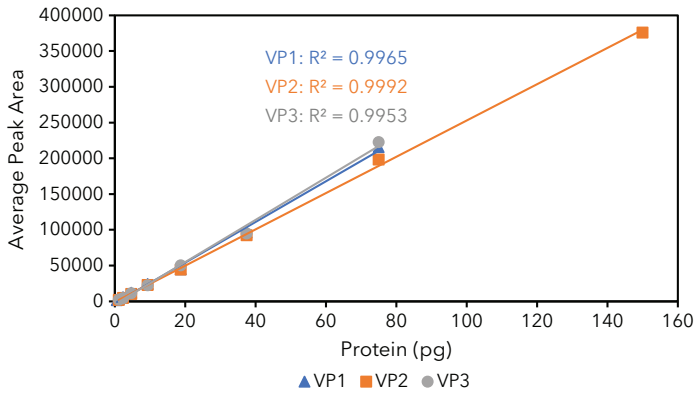

FIGURE 6. Linear regression analysis of Simple Western detection of AAV2 capsid proteins. These calibration curves were used to calculate the LOD/LOQ values listed in **TABLE 1**.

	LOD (pg)	LOQ (pg)
VP1	2.31	4.32
VP2	2.49	5.16
VP3	2.04	4.23

TABLE 1. Simple Western detection sensitivity of AAV2 capsid proteins represented by LOD/LOQ values.

EXPECTED	SIMPLE WESTERN	TRADITIONAL
VP1		
1.00	0.99	0.95
2.00	2.14	0.28
1.00	1.04	0.84
3.00	2.85	3.52
3.00	3.38	1.73
VP2		
1.00	1.00	3.28
2.00	2.13	4.39
1.00	1.15	3.01
3.00	2.82	8.52
3.00	3.14	3.39
VP3		
10.00	10.00	10.00
5.00	5.00	5.00
5.00	5.00	5.00
10.00	10.00	10.00
3.00	3.00	3.00

TABLE 2. Expected molar ratios and the measured molar ratios of VP1:VP2:VP3 by Simple Western and traditional western blot.

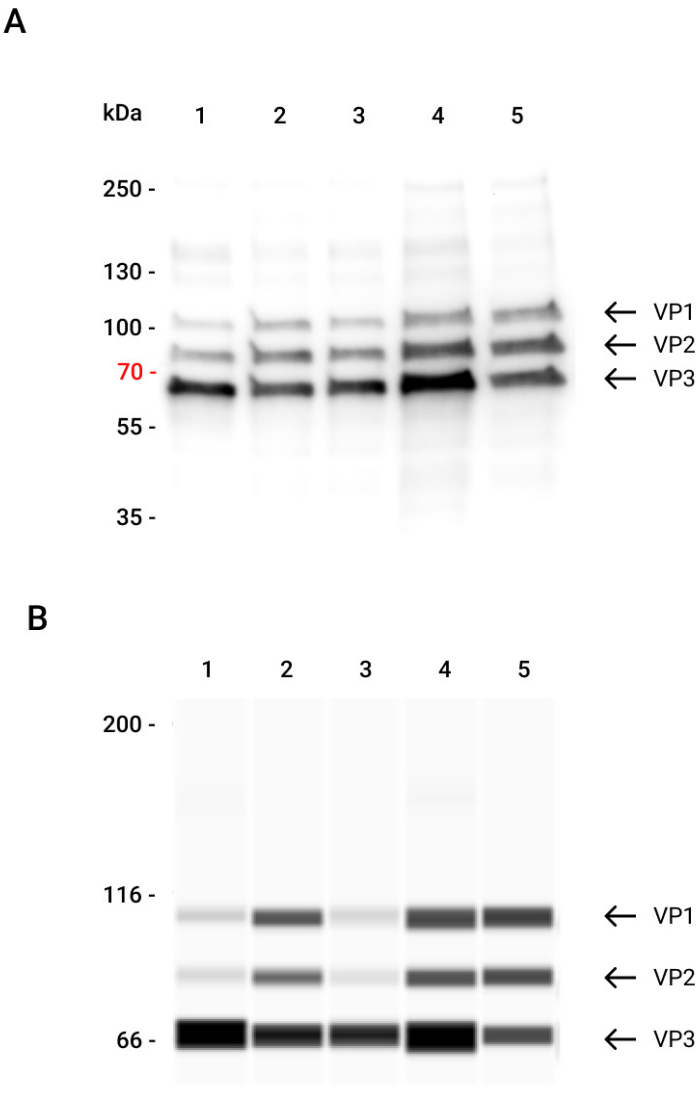

FIGURE 7. Traditional ECL western Blot of various mixtures of AAV2 VP1, VP2, and VP3 standard proteins.

Encouraged by the detection sensitivity of Simple Western, we next determined how the reproducibility and accuracy of AAV protein quantification compare with traditional western blot. To do so, we mixed different molar ratios of VP1, VP2, and VP3 capsid proteins (TABLE 2) and we analyzed these sample mixtures in parallel on Simple Western and traditional western blot (FIGURE 7). On Simple Western, this analysis showed no significant differences in the intensities of identical amounts in stoichiometric VP mixtures. By contrast, traditional western blot showed abnormal signal intensities for VP proteins loaded at identical molar ratios, especially the signals from VP1 and VP2 (TABLE 2, FIGURE 7). Therefore, we conclude that the blot-free method of Simple Western can eliminate the variability in transfer efficiency associated with traditional western blot and yield more accurate quantitative measurements of VP protein expression for AAV protein titer and VP capsid protein ratio measurements.

CONCLUSION

PROGEN's recombinant AAV2 VP1, VP2, and VP3 proteins (PROGEN, Cat. No. 72001) are useful reagents for highly accurate and sensitive analysis of VP content and VP ratios in AAV samples both in traditional western blot and CE-based Simple Western assays. In traditional western blots, differences in transfer efficiencies of different proteins can result in misleading signal intensities in a blot analysis as shown for VP1 vs. VP2 or VP3 where VP1 bands consistently show weaker signals than the other two VPs despite loading the same amount in the sample. The CE-based Simple Western analysis, however, shows no significant differences in the intensities of identical amounts in stoichiometric VP mixtures and confirms this artifact behavior of proteins in traditional western blots. Using reference proteins of known concentrations improves the correct assessment and comparability of VP ratios of different AAV preparations. This in turn helps to decide which preparation to take to the next step during the downstream manufacturing process or to control quality in the upstream process.

In addition to removing the variability associated with the gel-to-blot transfer of proteins, Simple Western offers other advantages over traditional western blot. For example, Simple Western is automated and has no manual steps after sample preparation, which in turn increases data reproducibility and dramatically reduces the time to results. Finally, the advantages of a small sample volume requirement (3 µL) and detection sensitivity in the low pg range make Simple Western assays particularly advantageous in the analysis of AAVs, which are difficult to manufacture, limiting supply.

In summary, the combination of the highly specific binding of the VP1/VP2/VP3 antibody (PROGEN, Clone B1, Cat. No. 690058) to a common epitope in all three VP subunits with the sensitive detection by both traditional and Simple Western enables accurate monitoring of AAV samples from crude cell lysates to the final steps of the purification process.

Learn more
bio-techne.com/instruments/simple-western

Learn more
progen.com/AAV/AAV-Standards/

REFERENCES

1. Structural proteins of adenovirus-associated virus type 3, F.B. Johnson, H.L. Ozer, and M.D. Hoggan, J Virol, 1971; 8(6):860-63.

2. A robust system for production of superabundant VP1 recombinant AAV vectors, Q. Wang, Z. Wu, J. Zhang et al., Mol Ther Methods Clin Dev, 2017; 7:146-156.

3. Optimization of viral protein ratios for production of rAAV serotype 5 in the baculovirus system, B. Bosma, F. du Plessis, E. Ehlert, et al., Gene Ther, 2018; 25(6):415-424.

4. Adeno-associated virus capsid assembly is divergent and stochastic, T. Wörner, A. Bennett, S. Habka et al., Nat Commun, 2021;12(1):1642.

5. Simple Western: Bringing the Western blot into the twenty-first century, C. Lück, C. Haitjema, and C. Heger, In: Methods in Molecular Biology, 2021; 2261:481–8.

6. Stellar NIR and IR Modules on Jess for Simple Western, Application Note, ProteinSimple, ProteinSimple, Bio-Techne Corp.

bio-techne® | PROGEN

Making gene therapy better. Together.

Contact Us

Contact Us

Trademarks and registered trademarks are the property of their respective owners.