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APPLICATION NOTE

CONCENTRATING ON AAV IMPURITIES WITH ULTRASENSITIVE TOTAL PROTEIN DETECTION ON SIMPLE WESTERN

TOTAL PROTEIN DETECTION WITH SIMPLE WESTERN

Impurities in protein products can be dangerous and impact efficacy. For example, protein impurities in final drug products could lead to undesirable immune responses in patients, so detecting total protein is critical for revealing impurities in preparative protein production. Traditional methods for total protein detection rely on SDS-PAGE with dyes like Coomassie Blue, SYPRO Ruby, or silver stain. However, SDS-PAGE requires large sample volumes, a lot of hands-on time, and it is poorly reproducible. Also, the use of staining dyes often comes with a lot of waste and can require specialized imaging equipment to which not every researcher has access.

Conversely, Simple Western™ assays on instruments like Jess and Wes from ProteinSimple offer fully automated protein separation and quantification with small sample volumes, and sensitive chemiluminescent-based immunodetection and total protein detection. While immunoassays on Simple Western allow target-specific detection, the Total Protein Detection Module (**FIGURE 1**) allows for all proteins to be labeled and detected, which is ideal for monitoring impurities. Here, we show that total protein detection with Simple Western can be even more sensitive by using 5 times more concentrated biotin labeling reagent, resulting in protein detection that surpasses the sensitivity of protein stains like SYPRO Ruby. While SYPRO Ruby requires at least 1 ng of protein for reliable detection,[1-2] Simple Western can reliably detect as little as 150 pg. This SYPRO Ruby-beating sensitivity improvement makes Simple Western well suited for the analysis of precious samples such as Adeno-Associated Virus (AAV) samples used in Cell & Gene Therapy workflows.

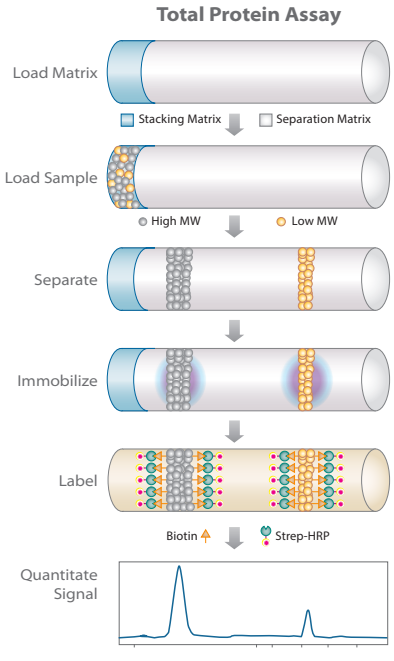


FIGURE 1. How the Total Protein Detection Assay works

SIMPLE WESTERN IS A MULTI-ATTRIBUTE METHOD

Identity and purity are among the critical quality attributes (CQAs) that must be monitored during AAV manufacturing. While ELISA can provide identity information, it cannot provide purity information in the same assay. In this regard, ELISA is a single-attribute method. By contrast, Simple Western is a multi-attribute method because it can deliver both identity and purity in the same assay. For example, identity can be achieved with antibodies specific to VP1/VP2/VP3 capsid proteins, while purity may be achieved with total protein detection, which now rivals the sensitivity of the best gel staining techniques. In this Application Note, we show how to achieve ultrasensitive total protein detection using the 5X biotin labeling reagent with a focus on AAV analysis.

MATERIALS AND METHODS

The reagents used in this study are listed in **TABLE 1**.

PREPARING THE 1X AND 5X LABELING REAGENT

To prepare 1X biotin labeling reagent, the default instructions provided with the Total Protein Detection Module were followed. To prepare the 5X biotin labeling reagent, 30 µL of Reconstitution Agent 1 were added per tube (instead of 150 µL for a 1X concentration). Then, this solution was mixed 1:1 with Reconstitution Agent 2. For RePlex analysis, the default instructions provided with the RePlex module were followed.

PREPARING THE SAMPLES

To analyze rDnaK (Enzo Life Sciences), a 3-fold serial dilution series was prepared in 1X master mixture (MM) and denatured under reducing conditions for 5 minutes at 95 °C. The concentrations in the dilution series were 100, 33.3, 11.1, 3.7, 1.23, 0.41, 0.14, 0.046, 0.015 and 0 µg/mL.

AAV2 (Vigene Biosciences) spiked with RNase A was prepared in 1X MM and denatured under reducing conditions for 10 minutes at 70 °C. The AAV2 (Vigene Biosciences) was diluted at 1:10 and the RNase A at 10, 3.33, 1.11, 0.37, 0.123 and 0 µg/mL. AAVs analyzed on RePlex were prepared at 1:20 concentration in 1X MM (with 2-40 kDa FI Standards) and denatured under reducing conditions for 10 minutes at 70 °C.

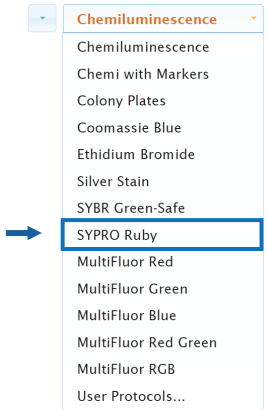
NAME	VENDOR	PART NUMBER
12-230 kDa Jess or Wes Separation Module	ProteinSimple	SM-W004
Total Protein Detection Module	ProteinSimple	DM-TP01
RePlex™ Module	ProteinSimple	RP001
Bicine/Chaps Lysis Buffer and Sample Diluent	ProteinSimple	040-764
DnaK (E. coli), (recombinant)	Enzo Life Sciences	ADI-SPP-630
AAV2-CMV-GFP	Vigene Biosciences	CV10004
RNase A	Sigma Aldrich	R5250
anti-AAV VP1 mouse monoclonal, A1, lyophilized, purified	Progen	61056
anti-AAV VP1/VP2/VP3 mouse monoclonal, B1, lyophilized, purified	Progen/Origene	61058/BM5015
10% Criterion™ TGX™ Precast Midi Protein Gel, 26 well, 15 µl	Bio-Rad	5671035
Precision Plus Protein Dual Standards Ladder	Bio-Rad	1610377
4X Laemmli Buffer	Bio-Rad	1610747
10x Tris/Glycine/SDS Buffer	Bio-Rad	1610732
SYPRO® Ruby Protein Gel Stain	ThermoFisher	S12000

TABLE 1. Reagents used in this study. The anti-AAV VP1 and anti-AAV VP1/VP2/VP3 antibodies were diluted 1:20 and 1:50 in Antibody Diluent 2, respectively.

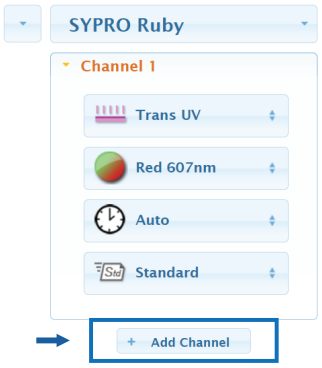
COMPARING 5X LABELING REAGENT TO SYPRO RUBY

AAV2 (1 x 10^{13} GC/mL) was prepared in 2X serial dilution series from 1:5 to 1:160. Each dilution was labeled with 5X labeling reagent and analyzed on Wes in duplicate using the 12-230 kDa Separation Module and the Total Protein Detection Module under default conditions. For SYPRO Ruby staining, the 1:5 through 1:40 dilutions were mixed 1:1 with 1X Laemmli Buffer (final) and Bicine/Chaps Lysis Buffer. Then, 3 µL of each sample were loaded on a 26-well 10% TGX gel and subjected to electrophoresis at 180 V for 40 minutes. Following electrophoresis, SYPRO Ruby staining was performed according to the manufacturer's basic protocol. Gel imaging was performed with ProteinSimple's FluorChem M, Software Version 4.1.1, with the settings below:

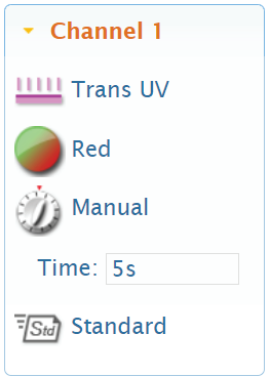
Step 1. Select "SYPRO Ruby"



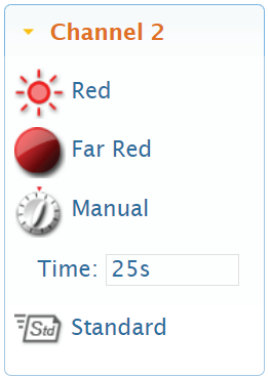
Step 2. Select "+ Add Channel"



Step 3. Enter the settings below for the respective channels.



SYPRO Ruby Stain AAV Sample



Precision Plus Dual Protein Standard Ladder

PUTTING THE 5X BIOTIN LABELING REAGENT TO THE TEST

To establish proof of concept, we compared 5X labeling reagent to 1X labeling reagent using a purified recombinant preparation of the molecular chaperone DnaK. DnaK was subjected to a 3-fold serial dilution series from 100 µg/mL to 0.123 µg/mL, and a 0 µg/mL concentration was included as a blank negative control. When this serial dilution series was analyzed on Jess using either 1X or 5X labeling reagent, the 5X labeling reagent resulted in a signal that was approximately 4- to 5-fold larger than the signal generated by the 1X labeling reagent (FIGURE 2A-C).

As expected, the 0 µg/mL sample concentration did not result in signal above background. The theoretical limit of detection and limit of quantification were compared between 1X and 5X labeling reagents, revealing an improvement in both metrics when the 5X labeling reagent was used (FIGURE 2D). Because Simple Western assays require as little as 3 µL of sample, the LOD of 0.05 µg/mL corresponds to just 150 pg of protein, which is only a fraction of what is reportedly required for SYPRO Ruby.[1-2]

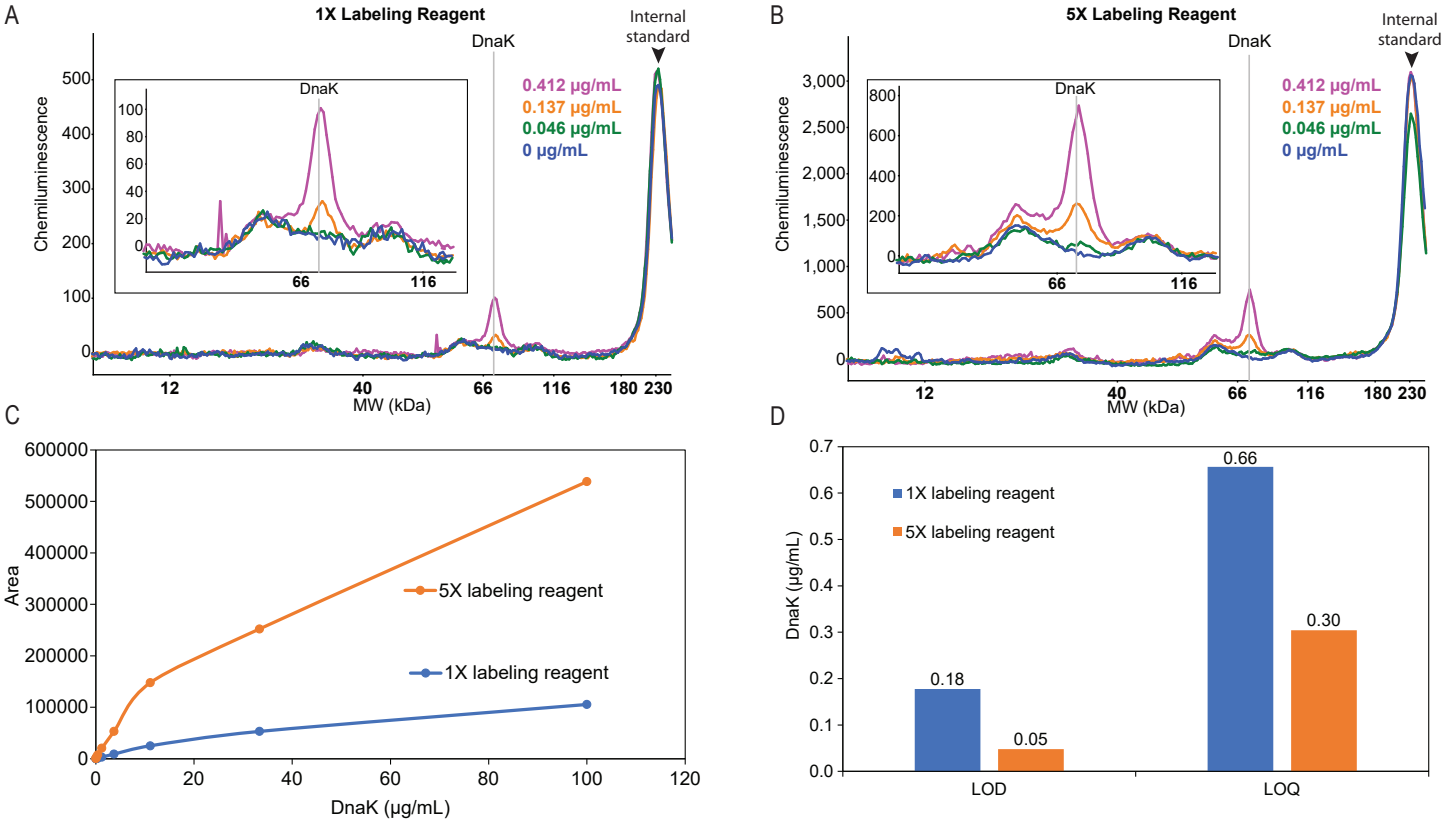


FIGURE 2. Labeling of DnaK with 5X labeling reagent increases assay signal 4- to 5-fold compared to 1X labeling reagent. Overlaid electropherograms of DnaK labeled with (A) 1X and (B) 5X labeling reagent. The insets are zoomed-in views of the DnaK signal. (C) Peak area by DnaK concentration and (D) LOD and LOQ determination of DnaK labeled with 1X and 5X labeling reagent.

APPLYING THE 5X LABELING REAGENT TO AAV ANALYSIS

Due to the tiny sample size requirements and enhanced sensitivity of this assay, it is particularly attractive for manufacturers of recombinant AAVs for gene therapy, which are difficult to manufacture and sample sizes are limiting. To mimic a real-world example, RNase A was spiked into a purified AAVs sample and analyzed with 1X and 5X total protein labeling reagent on Jess. The RNase A was serially diluted from 10 µg/mL down to 0.123 µg/mL, and a 0 µg/mL concentration was included as a blank negative control, and the AAV sample was held at a constant 1:10 dilution. As expected, the three AAV capsid proteins, VP1, VP2 and VP3, were clearly detected along with a singular RNase A peak that decreased with decreasing RNase A concentration (**FIGURE 3**). Under these conditions, the assay sensitivity for detecting RNase A increased about 3-fold with 5X labeling reagent when compared to 1X labeling reagent.

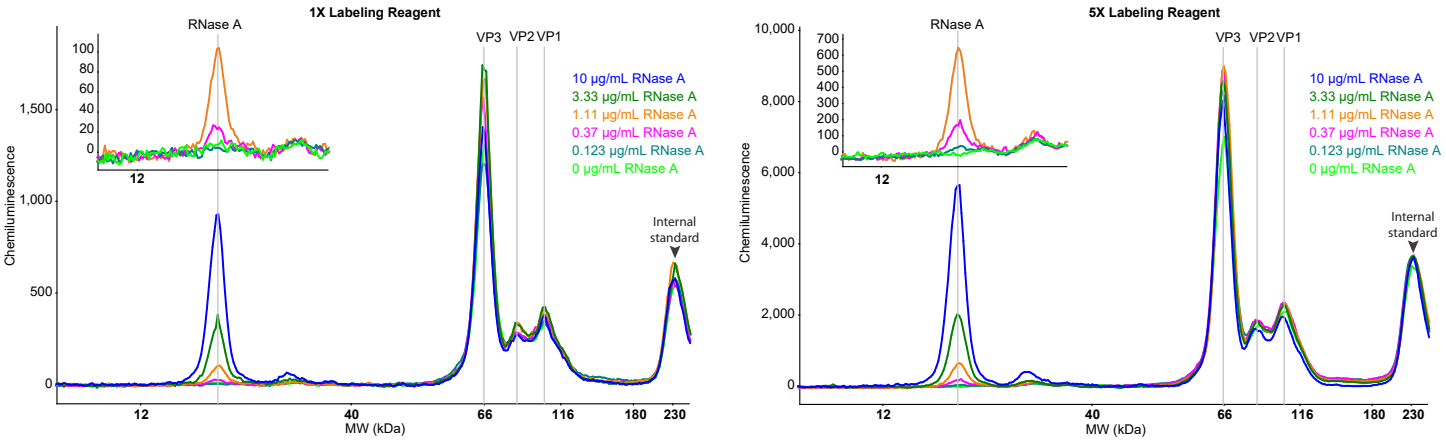


FIGURE 3. Overlaid electropherograms of AAV samples spiked with RNase A labeled with 1X (left) and 5X (right) labeling reagent. The insets are zoomed-in views of the RNase A signal.

MEASURING IDENTITY AND PURITY IN AAV SEROTYPES

A useful feature available on Simple Western systems is multiplex analysis by sequential immunoassays, known as RePlex™. It efficiently removes the antibodies from the first probing cycle for a second cycle of immunodetection without compromising assay robustness. The second probing round may be used to detect new targets, or it may be used for total protein normalization. Thus, we tested if 5X labeling reagent was compatible with the RePlex assay. In the first round, AAV capsid proteins were detected with anti-VP1 or anti-VP1/2/3 antibodies, and the second probing cycle was dedicated to total protein detection with 1X labeling reagent or 5X labeling reagent. The samples used in this analysis were crude, in-process samples and therefore a complex total protein profile is expected. From this analysis, the 5X labeling resulted in a ~5X increase in signal over the 1X label (**FIGURE 4**). While AAV1 and AAV4 did not have strong immunoreactivity with the anti-VP1/2/3 antibody, they did have similar total protein signals compared to AAV7 and AAV9 samples that do have strong immunoreactivity. This is expected as each sample was loaded at the same concentration of ~5 x 10^7 GC/mL. Taken together, these observations demonstrate that 5X labeling reagent is compatible for use in total protein normalization in RePlex assays.

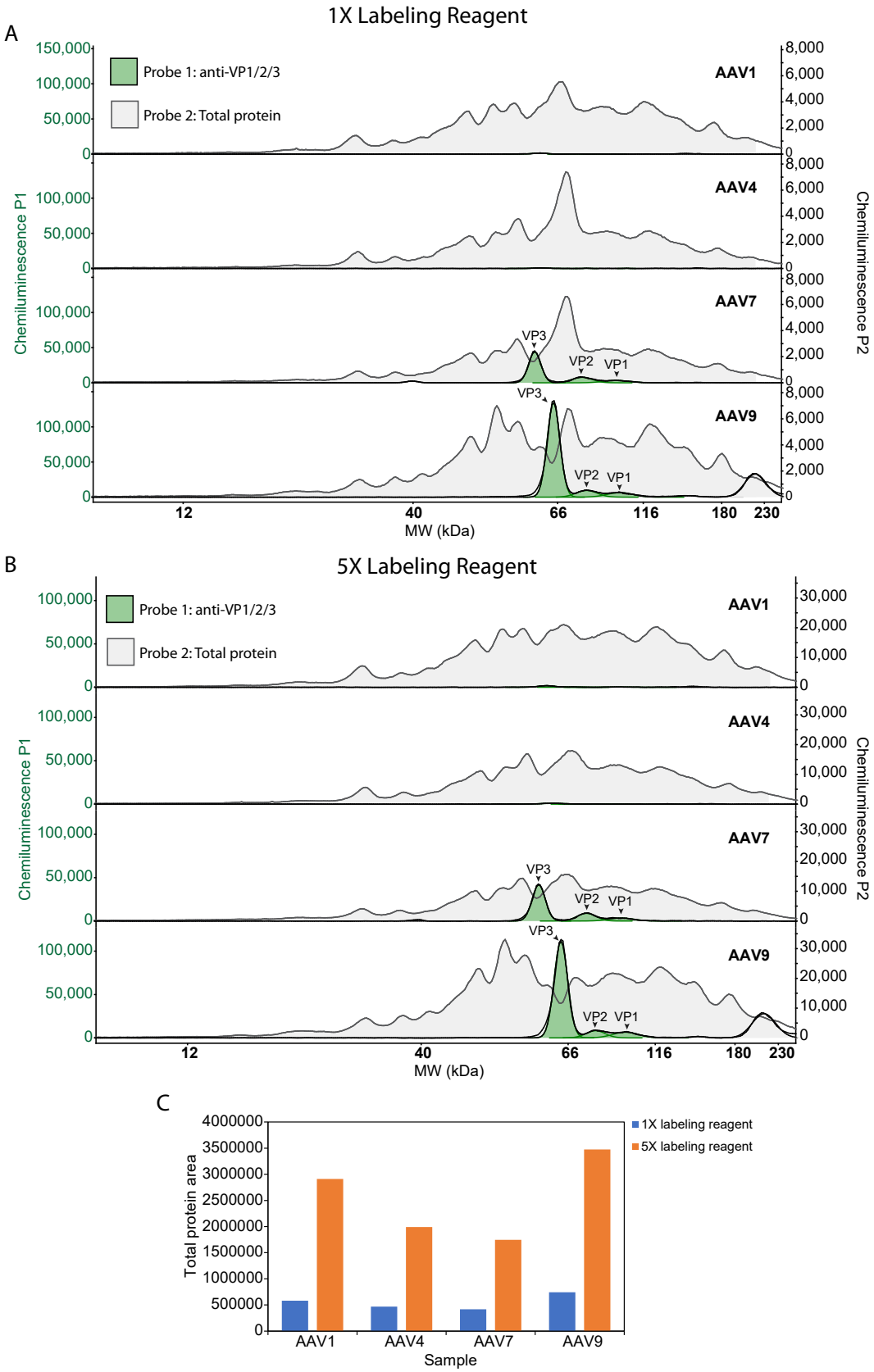


FIGURE 4. **AAV immunoassay analysis with RePlex on Jess.** Overlaid electropherograms of VP1/2/3 protein detection with the anti-VP1/2/3 antibody in the first probing cycle (green peaks) and total protein detection in the second probing cycle (gray peaks) with (**A**) 1X and (**B**) 5X biotin labeling reagent. All AAV samples were diluted 1:20 for a final concentration of 5 x 10^7 GC/mL. (**C**) Total protein area resulting from the second round of total protein detection with 1X and 5X labeling reagent.

SIMPLE WESTERN IS MORE SENSITIVE THAN SYPRO RUBY STAINING

SYPRO Ruby is among the most sensitive gel staining techniques, and many manufacturers of AAVs still rely on traditional SDS-PAGE with SYPRO Ruby staining to monitor purification. Unlike Simple Western, SYPRO Ruby staining is labor-intensive with many manual washing steps, generates large volumes of liquid waste, and requires special imaging equipment. Since AAV manufacturers could greatly benefit from replacing SYPRO Ruby staining with the automated Simple Western platform, we compared the sensitivity of the 5X labeling reagent on Wes with SYPRO Ruby staining by SDS-PAGE. To do so, we prepared AAV2 as described in the Materials and Methods and loaded equal volumes on SDS-PAGE with SYPRO Ruby staining and Wes with the 5X labeling reagent.

As expected, three signals corresponding to VP1, VP2, and VP3 appeared in both the SYPRO Ruby stain (**FIGURE 5A**) and by Wes analysis (**FIGURE 5B**). However, at the 1:40 dilution, the VP1 and VP2 peaks were hardly discernible above background levels on SYPRO Ruby staining (**FIGURE 5C**, right panel). On Wes, VP1 and VP2 peaks were clearly visible above background levels at the 1:40 dilution (**FIGURE 5C**, left panel). Furthermore, when peak area was plotted against sample dilution, a strong linear relationship was revealed on Wes, with R^2 = 0.9992, and the 1:40 dilution is within this linear dynamic range (**FIGURE 5B**, right panel). These results demonstrate that Simple Western analysis with 5X labeling reagent is more sensitive than SYPRO Ruby for the analysis of this AAV sample. In sum, Simple Western outperforms SYPRO Ruby in sensitivity in addition to improved automation and time to results.

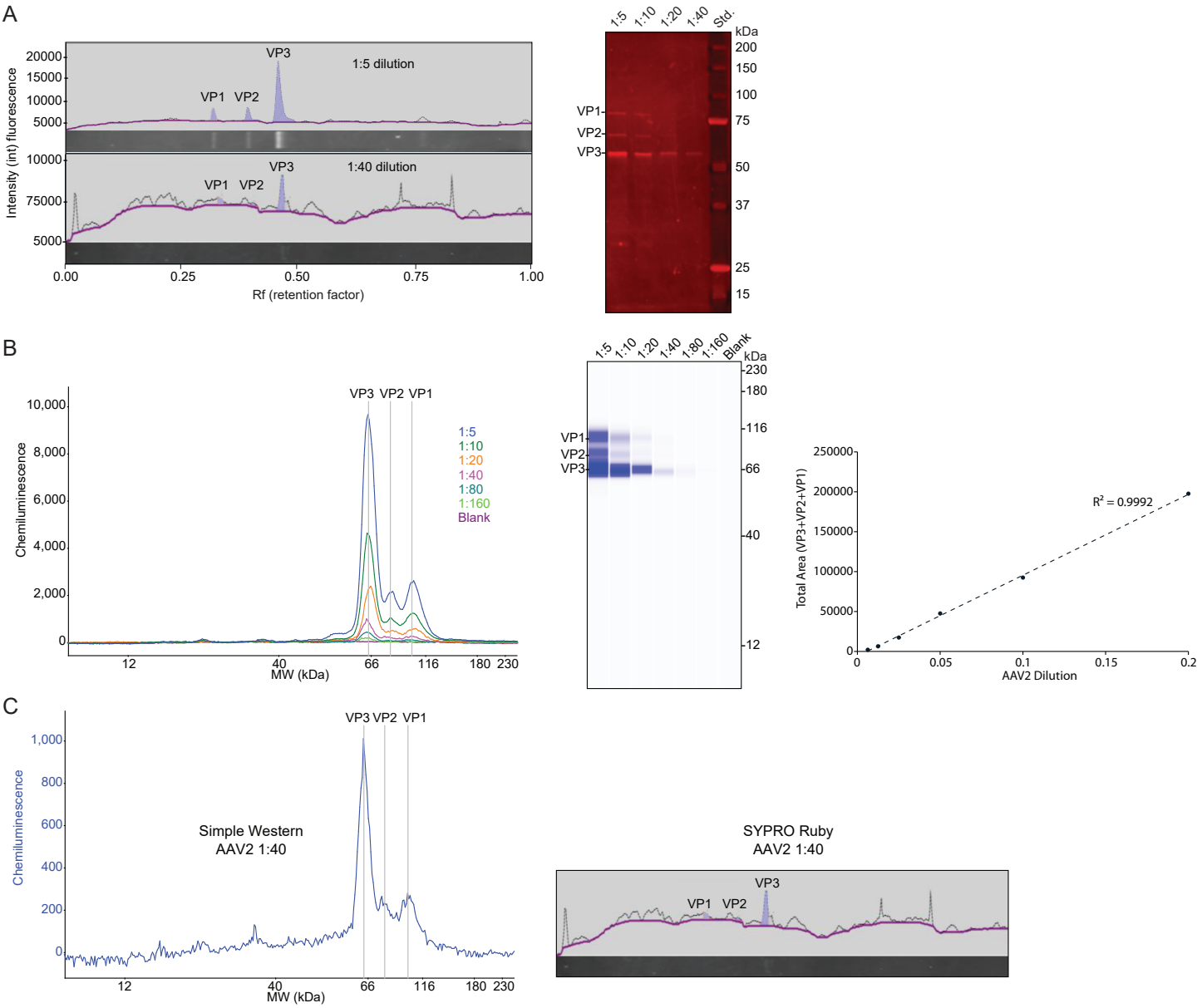


FIGURE 5. Comparison of AAV total protein detection with SYPRO Ruby and 5X labeling reagent with Wes. (**A**) SDS-PAGE stained with SYPRO Ruby (right) and densitometry analysis of the SYPRO Ruby stain (left); (**B**) Simple Western analysis graph view (left), lane view (middle), and linearity analysis (right); (**C**) Side-by-side comparison of the 1:40 dilution on Wes (left) and SYPRO Ruby (right).

LEADING SENSITIVITY COMBINED WITH SPEED AND AUTOMATION

With 5X biotin labeling reagent, Simple Western can achieve limits of detection in the upper picogram range. This is as good if not better than the best gel staining techniques like SYPRO Ruby and silver stain, which reportedly require lower nanogram levels for reliable detection.[3] Unlike messy gels, the Simple Western assay eliminates the hazardous waste generated by silver stain as well as all of the manual washing steps, with automated total protein detection in as little as 3 hours. And when it comes to AAV analysis, the use of 5X biotin labeling reagent gave results that were more sensitive than SYPRO Ruby. This is a major advantage for gene therapy because AAVs are difficult to manufacture and samples are extremely limiting. Also, the total protein concentration of AAV samples is often significantly lower than other biological therapeutics such as monoclonal antibodies. Therefore, a lower LOD may be required for AAV protein analytics. Finally, 5X total protein labeling reagent is compatible for use in RePlex, allowing users to get the most data out of their precious samples and normalize their protein expression data with confidence.

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