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

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INTRODUCTION

Purity is a critical quality attribute (CQA) that must be monitored during AAV manufacturing. Impurities in protein products can be dangerous and impact efficacy. For example, protein impurities in final drug products may 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, or more sensitive stains like SYPRO Ruby and 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.

Here, we present a new workflow that enables total protein detection of AAVs on the Simple Western capillary electrophoresis-based immunoassay platform with a sensitivity that exceeds SYPRO Ruby. While SYPRO Ruby requires at least 1 ng of protein for reliable detection, Simple Western can reliably detect as little as 150 pg. These findings should enable researchers who are currently using SDS-PAGE to monitor purity to apply the automated platform and sensitivity improvements enabled by this workflow to assess AAV purity using smaller AAV sample sizes.

MATERIALS AND METHODS

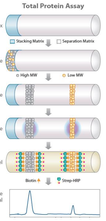
Instrumentation

Jess

FluorChem M

Reagents

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 monoclo-nal, 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



TOTAL PROTEIN ASSAY

The Total Protein Assay (shown on the left) works by labeling proteins with biotin after they've been separated and immobilized to the capillary wall, and then detecting the labeled proteins with Streptavidin-HRP. This allows for an easy workflow, that has high reproducibility sample to sample and run to run. The assay comes with a standard protocol. For this work, we have deviated from that protocol by increasing the concentration of the kit's labeling reagent.

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 and used as normal.

RESULTS

PUTTING THE 5X BIOTIN LABELING REAGENT TO THE TEST

We first looked at the LOD/LOQ improvements of the 5X labeling using a recombinant protein, rDnaK. We generated a 3-fold serial dilution series of rDnaK from 100, 33.3, 11.1, 3.7, 1.23, 0.41, 0.14, 0.046, 0.015 and included a buffer control (0 µg/mL) and ran on Jess using either 1X or 5X TP in the labeling step (**Figure 1**). These data show that there is an approximately 3-fold increase in both the LOD and LOQ using the 5X labeling reagent.

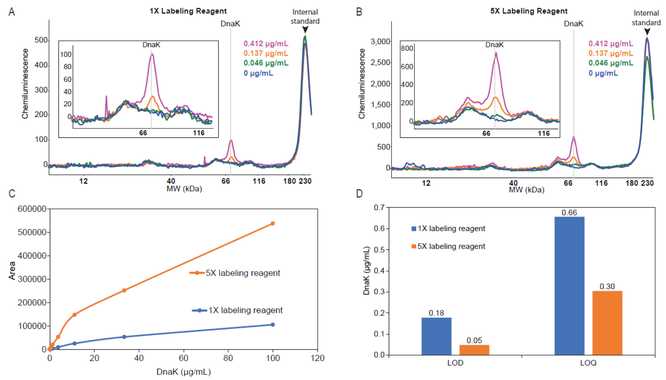


Figure 1. Labeling of DnaK with 5X labeling reagent increases assay signal 4- to 5-fold compared to 1X labeling reagent. Overlaid electropherograms of DnaK labeled with (A) 1X and (B) 5X labeling reagent. The insets are zoomed-in views of the DnaK signal. (C) Peak area by DnaK concentration and (D) LOD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

APPLYING THE 5X LABELING REAGENT TO AAV ANALYSIS

One of the main potential uses of this approach is for improved impurity detection. To assess the ability of the 5X approach to improve impurity detection, we used a pure AAV2 sample and added various amounts of recombinant RNase A. We saw that both approaches can detect similar levels of impurities, but the 5X approach enables detection of these impurities at higher levels (**Figure 2**). In addition, a lower point for RNase A (0.123 µg/mL) is quantifiable only using the 5X approach.

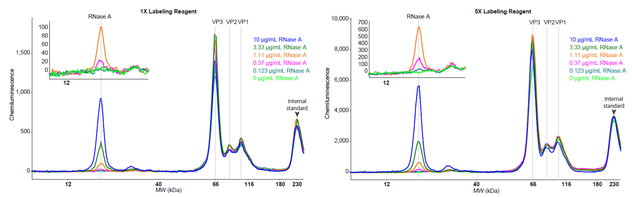


Figure 2. Recombinant RNase A impurity model in pure AAV2. AAV2 was spiked with RNase A (10, 3.33, 1.11, 0.37, 0.123 and 0 µg/mL) and prepared in 1X MM and denatured under reducing conditions for 10 minutes at 70 °C. Samples were then loaded onto a Jess and run with either 1X (left) or 5X total protein (right).

MEASURING IDENTITY AND PURITY IN AAV SEROTYPES

Simple Western can run total protein assays as well as immunoassays. We next applied the 5X TP approach in conjunction with the RePlex feature, where we looked at the total protein with either the standard or 5X TP approach after immunoassay. For this experiment, we obtained 4 unique serotype in-process samples (**Figure 3**). While all 4 AAV samples have significant total protein (shaded in grey), which are detected significantly better with the 5X TP approach, only two serotypes reacted were detected in our immunoassay. Based on the antibody specificity, this result is expected.

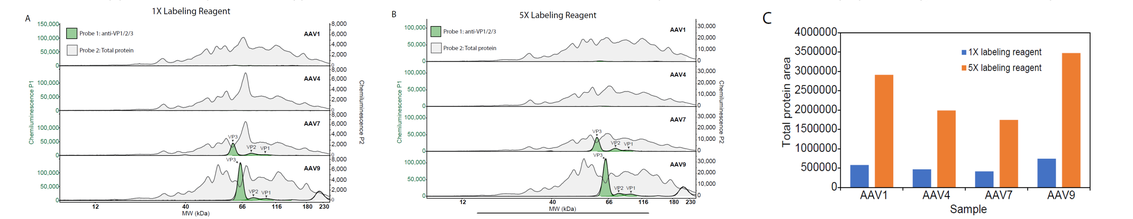


Figure 3. Evaluating 5X total protein in RePlex of AAV in-process samples. AAVs (1x10^9 GC/mL) were diluted 1:20 and analyzed on Jess using an anti-VP1/2/3 antibody (Progen) followed by RePlex with the (A) 1X or (B) 5X total protein. (C) Total protein area for each AAV sample were quantified.

GOING HEAD-TO-HEAD WITH SYPRO RUBY FOR AAV ANALYSIS

We next sought to compare the 5X approach with SYPRO Ruby, which is used frequently with SDS-PAGE for detection of impurities and other low concentration materials. SYPRO Ruby can detect typically 1 ng protein at the lower levels of detection. For this experiment, we used a commercially available AAV2 sample (1 x 10^{13} GC/mL), serially diluted from 1:5 to 1:160 fold before analysis on SDS-PAGE followed by SYPRO Ruby dye and imaging on a FluorChem M or analysis on Jess with the 5X approach. Comparing the results, Simple Western can easily detect the same amount of protein as SYPRO Ruby and can reliably detect even less material. Based on our calculations, we can detect as little as 150 pg of protein with this approach.

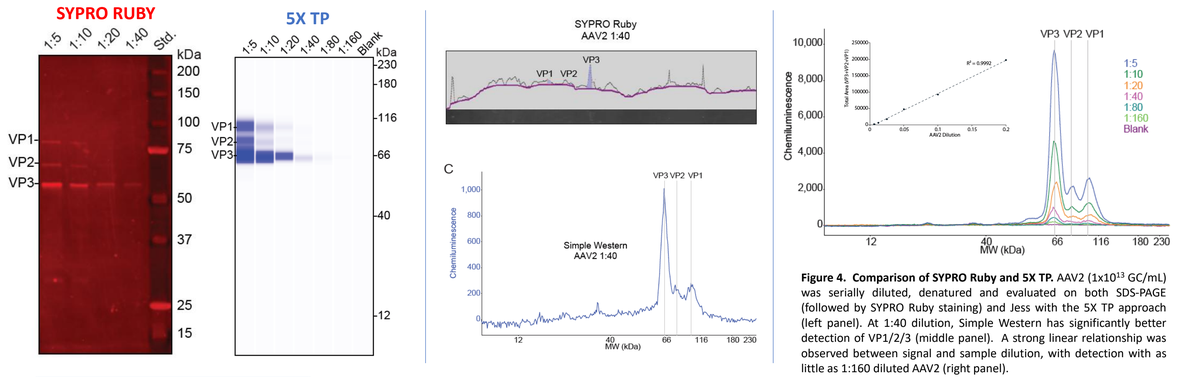


Figure 4. Comparison of SYPRO Ruby and 5X TP. AAV2 (1x10^{13} GC/mL) was serially diluted, denatured and evaluated on both SDS-PAGE (followed by SYPRO Ruby staining) and Jess with the 5X TP approach (left panel). At 1:40 dilution, Simple Western has significantly better detection of VP1/2/3 (middle panel). A strong linear relationship was observed between signal and sample dilution, with detection with as little as 1:160 diluted AAV2 (right panel).

CONCLUSIONS

- Impurity analysis requires ultrasensitive detection methods. For SDS-PAGE, silver stain and SYPRO Ruby are typically used.
- Increasing the sensitivity of the existing Simple Western Total Protein product is possible by increasing the concentration of the total protein reagent, affording a 3-fold improvement in LOD/LOQ, and detection of as little as 150 pg of protein.
- This approach exceeds the sensitivity of SYPRO Ruby, positioning this assay as suitable to replace laborious assays currently reliant on SYPRO Ruby or silver stain for detection.

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