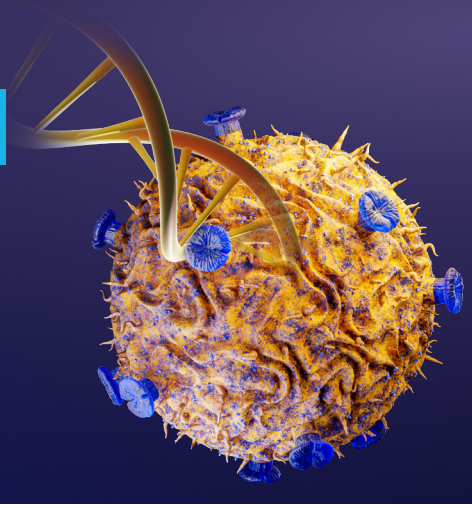
APPLICATION NOTE



Lentiviral Vector Analysis for Cell and Gene Therapy Made Simple

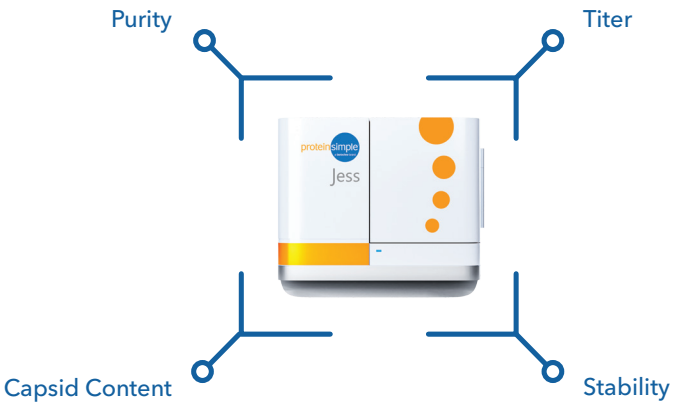
An Automated Multi-Attribute Platform for Scalable At-Line Lentiviral Analytics

As lentiviral vectors (LVV) are increasingly used for *in vivo* and *ex vivo* gene delivery in the creation of therapies for common genetic and acquired diseases, large-scale biomanufacturing of clinical-grade LVV products must meet strict lentiviral manufacturing, product characterization, and regulatory requirements.[1] However, there is a lack of standardized analytical assays that can meet scale-up demands to ensure safety, efficacy, quality, and lot-to-lot consistency.[2] Typically, these assays include thorough characterization of LVV titer, stability, purity, capsid content, and transduction efficacy.[3] To save on precious LVV samples and get to market faster, what if you could measure all these parameters with one platform?

Current technologies are borrowed from other industries and they are unscalable and inflexible to the needs of cell and gene therapies (C&GT). By contrast, Simple Western™ is an established and rapidly growing capillary electrophoresis immunodetection platform that is revolutionizing analytical development for C&GT.

- **Multi-attribute method.** The ability to measure 4 or more critical quality attributes (CQAs) in one automated bench-top instrument like Jess™ reduces the upfront capital, time, labor, and overall cost required to develop new LVV-based C&GT.
- **At-line protein analysis.** As a compact benchtop instrument with automated runtimes of 25 samples in 3 hours or 96 samples overnight, Simple Western enables at-line protein analysis that scales with process development.
- **Minimal sample expenditure.** Using only 3 µL of starting material, Jess can multiplex in chemiluminescence and Stellar™ high-sensitivity NIR/IR fluorescence, or perform RePlex™ with sequential immunoassays and Total Protein Detection.

We previously demonstrated that Simple Western assays are a multi-attribute method for analytical-grade protein expression potency quantification and other CQAs for AAVs. Here, we demonstrate Simple Western's analytical capacity to simultaneously characterize multiple parameters of LVVs, including viral titer, stability, purity, and capsid content. Finally, we show an example of Simple Western in downstream vector transduction analysis to measure LVV-mediated gene knockout in iPSC-derived macrophages.



Why Choose Simple Western Over Other Methods for LVV Analysis?

	TEM	Western Blot	ELISA	CE-SDS/icIEF	**Simple Western**
Fully quantitative data	No	No	Yes	Yes	**Yes**
Charge or Size separation profiles	No	Yes	No	Yes	**Yes**
Multiplex across Chemi/IR/NIR channels and Total Protein Detection	No	No	No	No	**Yes**
Complex sample types like whole cell lysates and tissue homegenates	No	Yes	Not Ideal	No	**Yes**
Automated sample processing	No	No	No	Yes	**Yes**
High throughput (96 samples per run)	N/A	No	Yes	Yes	**Yes**

Materials and Methods

Unless otherwise noted, all experiments were performed on the Simple Western instrument Jess and all materials are listed in TABLE 1.

Titer

The capsid protein p24 is commonly used to quantify LVV titer.[1] All samples were prepared in 10 µg/mL HEK293T whole-cell lysate. HEK293T cells are common hosts for the biomanufacturing of LVVs.[1] A 3X serial dilution series of recombinant p24 was prepared from 300 ng/mL to 3.7 ng/mL. A 3X serial dilution series of purified LVV sample at a stock concentration of 10^9 transducing units(TU)/mL was created from a dilution factor of 1.25X to 922.35X, corresponding to 8 x 10^8 TU/mL to 1.1 x 10^6 TU/mL. All samples were analyzed on Jess using the RePlex assay. RePlex removes antibodies from the first probing cycle for a second probing cycle with fresh antibodies or total protein detection. Because the samples are covalently bound to the capillary wall, RePlex completely and reproducibly removes antibodies between cycles without loss of signal intensity. Samples were probed with the anti-HIV-1 Gag p24 mAb at 2 µg/mL (1:250) and with the 5X Total Protein Detection labeling reagent in Probe 1 and 2 of RePlex, respectively.

To analyze non-p24 targets, LVV samples were probed with anti-HIV-1 Gag p24 antibody at 2 µg/mL, anti-HIV-1 p17 antibody at 20 µg/mL, anti-SV40 T-Antigen antibody at 66.7 µg/mL, and the anti-VSV-G antibody at 13.3 µg/mL. All samples were loaded at a concentration of 10^9 TU/mL, except for the detection of VSV-G, which was performed using a 10^6 TU/mL sample concentration.

Stability

Purified LVV particles were diluted to 1.1 x 10^9 TU/mL in PBS. Then, one aliquot was immediately snap-frozen on dry ice as a frozen control and the other aliquots were incubated at 4 °C, room temperature, and 37 °C for 24 hours, 48 hours, and 72 hours. These conditions were chosen based on a previous study.[4] The samples were collected at the appropriate times and snap-frozen on dry ice. On the day of analysis, all samples were thawed on ice and double-inactivated in 1.1% SDS for 10 minutes at room temperature, followed by 90 °C for 10 minutes.

After samples were double-inactivated, they were prepared for analysis at a final concentration of 10^8 TU/mL. Samples were probed with the anti-dsDNA antibody at 2 µg/mL (1:100) and with the anti-HIV-1 Gag p24 antibody at 2 µg/mL (1:250) in Probe 1 and 2 of RePlex, respectively.

Materials Used in this Study

Name	Vendor	Part Number
12-230 kDa Separation Module	Bio-Techne	SM-W001
Anti-Mouse Detection Module	Bio-Techne	DM-002
Anti-Rabbit Detection Module	Bio-Techne	DM-001
Total Protein Detection Module	Bio-Techne	DM-TP01
Recombinant HIV-1 Gag p24 His-tag Protein, CF	Bio-Techne	11243-HV
Ms anti-HIV-1 Gag p24 mAb	Bio-Techne	MAB73602
Recombinant S. marcescens Benzonase Nuclease/NucA	Bio-Techne	10038-NA-100
Rb anti-dsDNA mAb	Bio-Techne	NBP3-07302
Rb anti-VSV-G pAb	Bio-Techne	NB100-2485
Rb anti-HEK293T HCP pAb	Canopy Biosciences	HCP021-0.5
Ms anti-HIV-1 p17 mAb	Santa Cruz Biotechnology	sc-69723
Ms anti-SV40 T-Antigen mAb	Abcam	ab16879
LVV sample: rLVV.EF1. ZsGreen1-9, 1.1 x 10^{10} TU/mL	Takara Bio	0010VCT
HEK293T cell lysate (untransfected)	Origene	LY500001
10X DNase I Buffer	NEB	B0303S

TABLE 1. Materials used in this study. Prior to analysis, LVV samples were double inactivated in 1.1% SDS for 10 minutes at room temperature followed by 90 °C for 10 minutes.

Capsid content

LVV (10^8 TU/mL) was treated with nuclease (NucA) diluted in a range of concentrations from 0 to 20 µg/mL in 1X DNase I Buffer. Treatment was performed directly in the capillary by adding the nuclease in the blocking step for 30 minutes. A buffer-only sample was included as a negative control. Samples were probed with the anti-dsDNA antibody at 2 µg/mL (1:100).

Purity

All samples were prepared in the presence of a constant concentration of LVV particles of 10^7 TU/mL. The HEK293T lysate was prepared in a 3-fold serial dilution series from 100 µg/mL to 0.0051 µg/mL. Samples were probed with the anti-HEK293T HCP antibody at 66.7 µg/mL (1:15) with NIR detection and by 5X Total Protein Detection with chemiluminescence detection in Probe 1 and 2 of RePlex, respectively.

Results of Multi-Attribute LVV Analysis by Simple Western

Titer

To develop a Simple Western p24 assay for LVV titer quantification, a 3X serial dilution series of recombinant p24 from 300 ng/mL to 3.7 ng/mL was prepared in a constant concentration of 10 µg/mL HEK293T whole-cell lysate. Each sample in this serial dilution series was analyzed on Simple Western and probed using an anti-p24 antibody. The results from this analysis showed a strong peak corresponding to p24 that decreased in signal intensity with decreasing sample concentration (**FIGURE 1A**). To generate a standard curve, the p24 peak area was plotted against p24 concentration and a 4-parameter logistic (4-PL) model provided a point-to-point curve fitting (**FIGURE 1A, inset**). Because only 3 µL of the sample are loaded for analysis on Simple Western, the lowest concentration in this range of 3.7 ng/mL corresponds to 11 pg of p24 loaded for analysis.

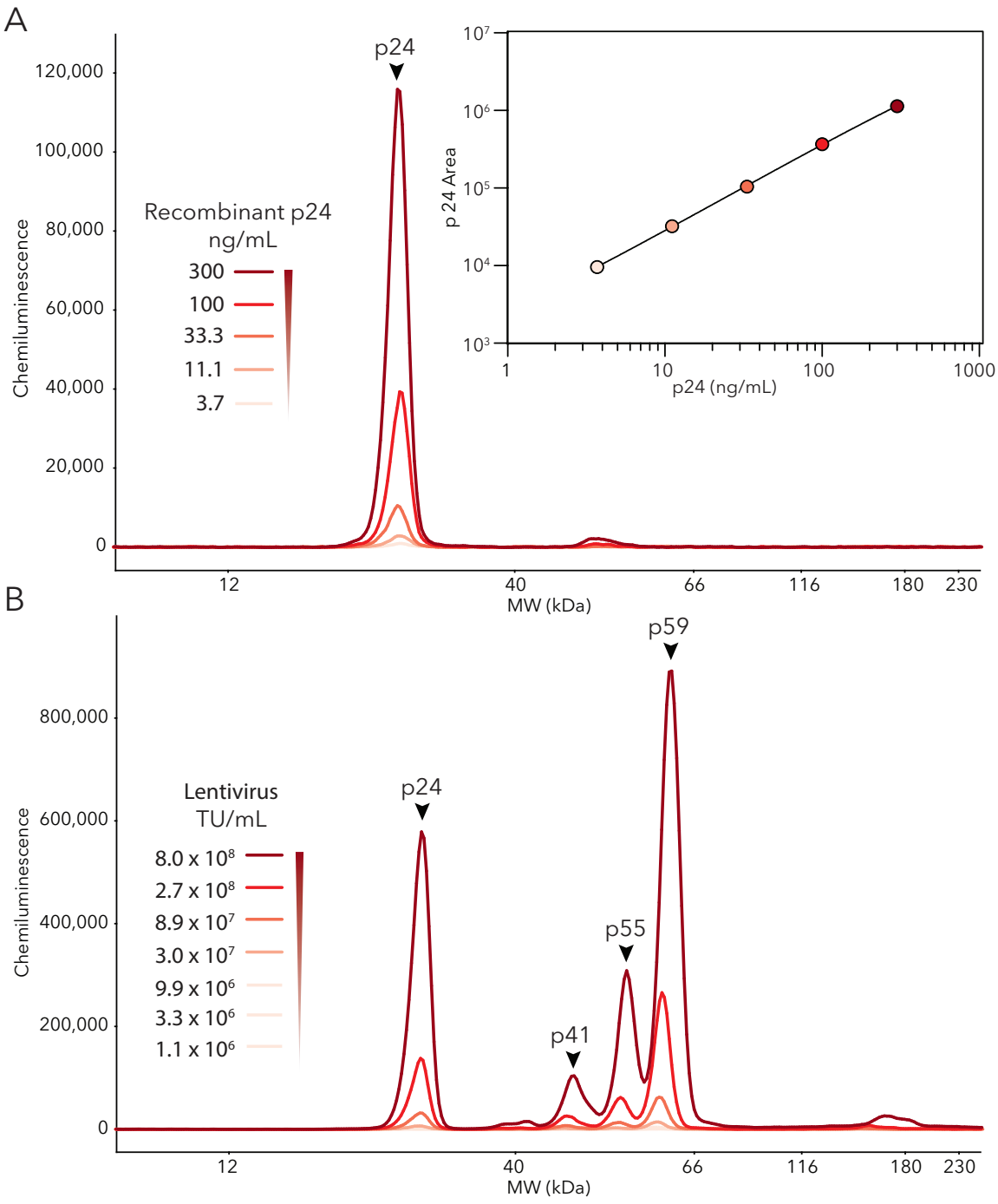


FIGURE 1. The Simple Western p24 assay for lentivirus titer quantification. (**A**) Simple Western analysis of a serial dilution series of recombinant p24 prepared in HEK293T whole-cell lysate. The inset shows the 4-PL standard curve resulting from this analysis. (**B**) Simple Western analysis of a serial dilution series of purified LVV prepared in HEK293T whole-cell lysate.

Next, we sought to apply this standard curve to quantify p24 concentrations in LVV samples. To do so, a stock LVV sample with a functional titer of 10^9 TU/mL was prepared in a 3X serial dilution series with dilution factors from 1.25X to 911.25X (corresponding to 8.0 x 10^8 TU/mL to 1.1 x 10^6 TU/mL). Then, each sample in this serial dilution series was analyzed by Simple Western using the anti-p24 antibody. The results from this analysis showed a signal peak corresponding to p24 that decreased with decreasing sample concentration (**FIGURE 1B**). In addition to the p24 peak, 4 more peaks appeared that corresponded to p41, p55, and p59, indicating the cross-reactivity of the anti-p24 antibody with these viral capsid proteins (**FIGURE 1B**).

Next, the p24 peak area was plotted against the LVV sample concentration (**FIGURE 2A**). The middle three samples in this serial dilution series had p24 peak areas that fell within the recombinant p24 standard curve detection range, which are shown in a solid shade of red (**FIGURE 2A**). Therefore, we quantified the p24 concentrations in these three samples using the standard curve generated previously. Importantly, when these three samples were corrected for their dilution factor from the stock concentration, the quantification of p24 remained constant, with an average of 800 ng/mL (**FIGURE 2B**). Therefore, it is reasonable to conclude that 800 ng/mL of p24 corresponds to 10^9 TU/mL in the sample. Because p24 alone does not provide a measure of functional titer, these results provide the ability to convert the p24 peak area to LVV concentration.

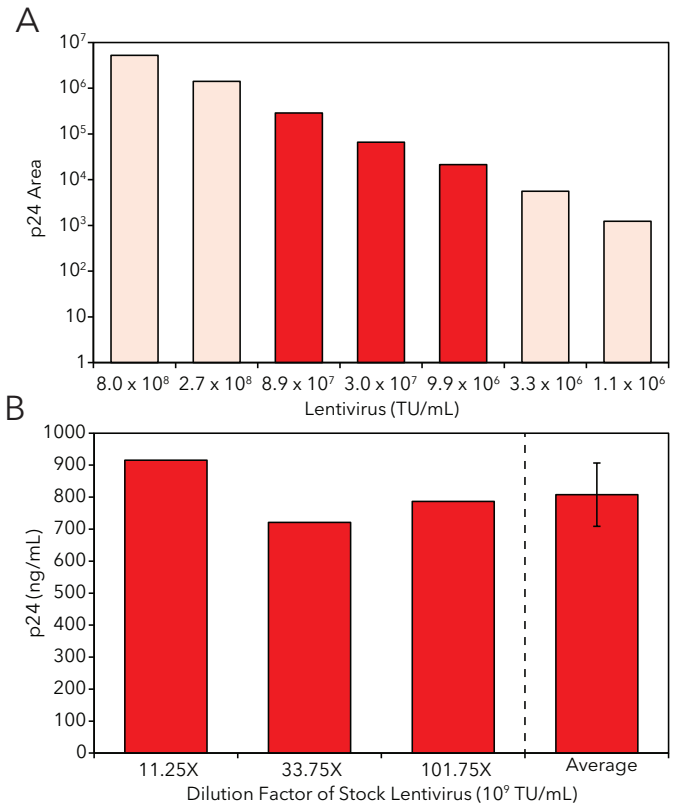


FIGURE 2. The Simple Western p24 assay for LVV titer quantification. (**A**) Peak area of p24 by LVV concentration. The three middle samples shaded solid red fell within the p24 standard curve range. (**B**) Quantification of p24 in LVV sample dilutions corrected by the dilution factor from the stock concentration.

Traditionally, p24 titer is determined by ELISA, which offers high-sensitivity immunodetection. However, a disadvantage of ELISA is the lack of protein separation profiles. By contrast, the size-based separation profile of the Simple Western assay showed that the p24 antibody cross-reacts with other LVV capsid proteins (**FIGURE 1B**). These cross-reactions would go unnoticed by ELISA, resulting in an overestimation of p24 in the sample.

While CE-SDS systems like the PA 800 plus offer separation profiles, they lack immunodetection, relying on direct UV or native fluorescence detection instead. Therefore, the disadvantages of traditional CE-SDS systems are low sensitivity and the need for purified samples. By contrast, Simple Western offers sensitive and specific immunodetection of LVV in complex sample types, like HEK293T whole-cell lysate as shown here.

As an open platform, any antibody may be used for target detection. Thus, we tested if more targets in addition to p24 can be analyzed in LVV samples by Simple Western. Thus far, we have shown that Simple Western detects SV40 T-antigen, HIV-1 p17, and VSV-G (**FIGURE 3**). Simple Western analysis of these additional targets can provide important insight into LVV identity, tropism, and production. Because CE-SDS PA 800 lacks immunodetection, these targets would be difficult to identify, even if purified recombinant proteins are spiked in the sample. On Simple Western, other proteins of interest may be analyzed by simply swapping in corresponding primary antibodies for detection.

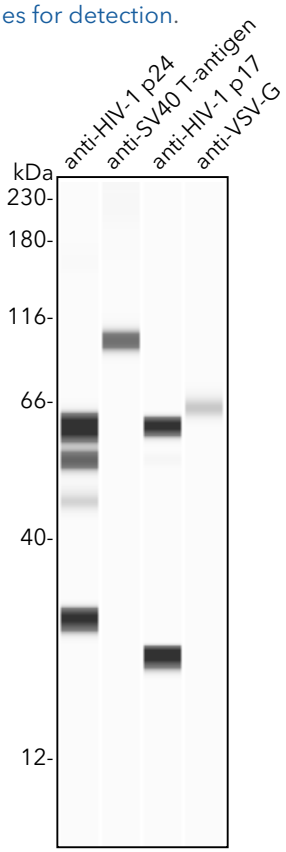


FIGURE 3. Simple Western analysis of p24 and non-24 LVV targets. Virtual lane view of LVV samples probed with an anti-HIV-1 p24 antibody, an anti-SV40 T-antigen antibody, an anti-HIV-1 p17 antibody, and an anti-VSV-G antibody.

Stability and capsid content

Stability and capsid content are important metrics in LVV characterization because they directly impact infectious titers. Previously, we showed that Simple Western can measure the empty/full content ratio of AAV samples using antibodies that individually target VP1/2/3 capsid proteins and DNA capsid content. Here, we applied a similar strategy to monitor LVV stability and capsid content using an anti-p24 antibody and an anti-DNA antibody which also detects RNA.

For stability testing, purified LVV particles were incubated at a range of times and temperatures. Then, these samples were analyzed on Simple Western using antibodies for p24 and RNA detection. The results from this analysis showed that time and temperature do not have a significant impact on p24 signal intensity, while RNA signal intensities begin to decrease at 37 °C after 24 hours (**FIGURE 4**). Collectively, these results demonstrate Simple Western's ability to monitor the stability of LVVs by quantification of p24 and RNA components.

For capsid content analysis, empty LVV particles were not available for analysis. To provide a proof-of-concept of empty/full analysis in the absence of empty LVV particles, full LVV samples were treated with nuclease at a range of concentrations directly in the capillary before probing with an antibody that detects RNA. The results from this analysis showed an RNA signal that decreased with increasing nuclease concentration, with an RNA signal that was virtually undetectable at 20 µg/mL nuclease (**FIGURE 5**). These data represent a promising proof-of-concept that Simple Western can similarly measure content ratio to the Simple Western empty/full assay for AAV samples.

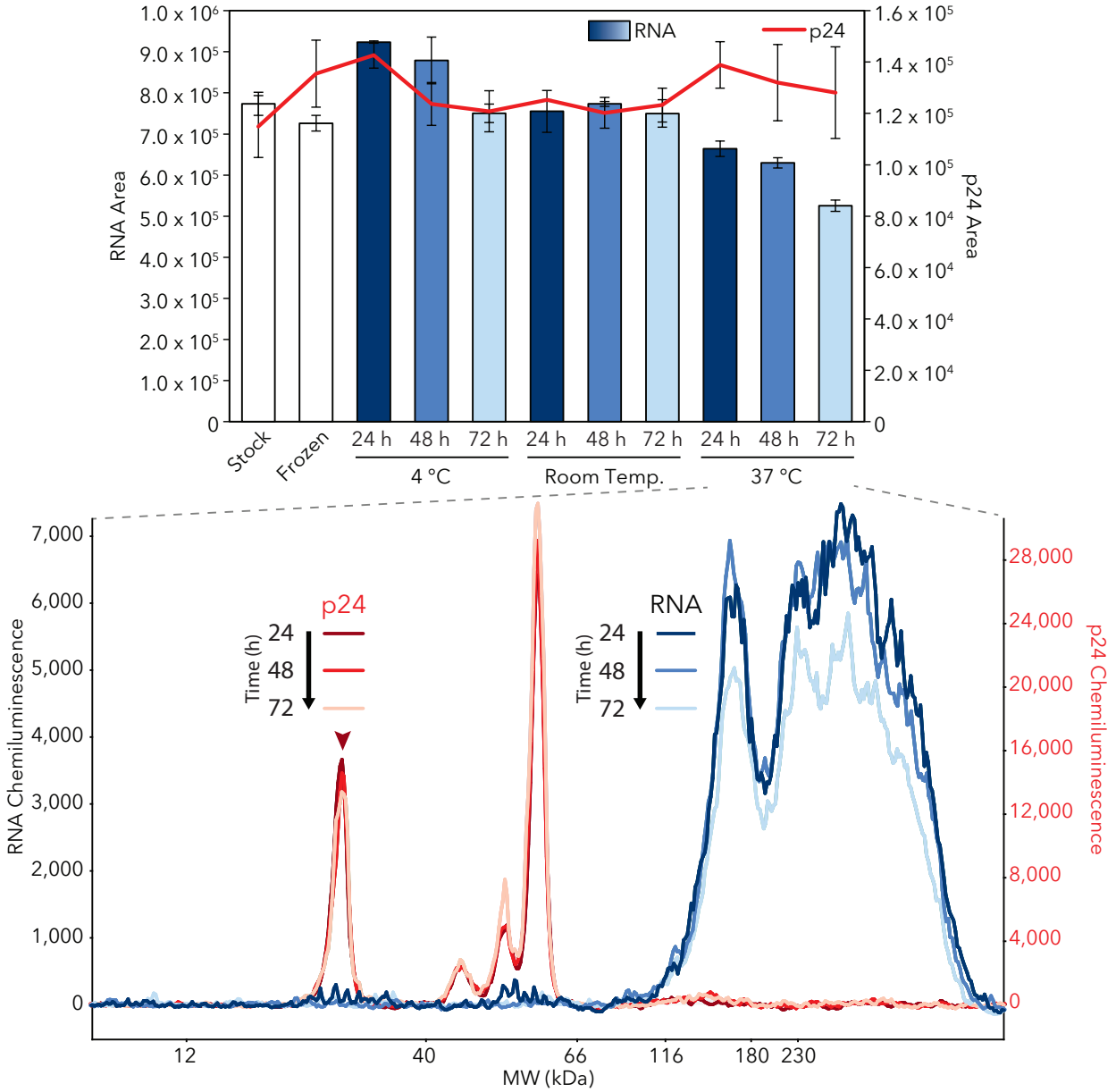


FIGURE 4. Stability analysis of LVV by Simple Western. (Top) Time course of p24 and RNA detection by Simple Western compared to frozen and stock controls. (Bottom) Overlaid electropherograms of p24 and RNA detection by Simple Western from a 3-point time course at 37 °C.

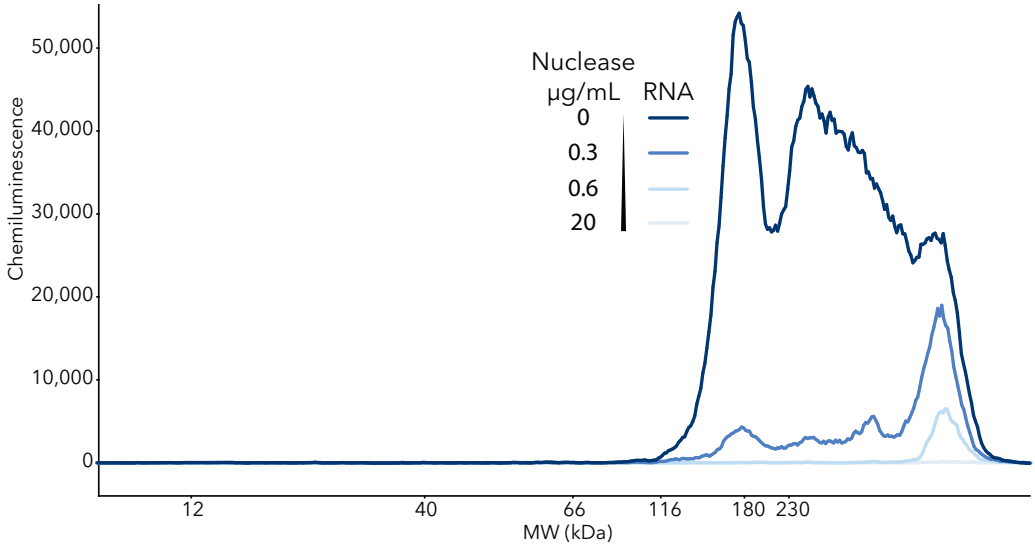


FIGURE 5. Simple Western LVV capsid content analysis. LVVs samples were treated with nuclease at concentrations from 0 to 20 µg/mL directly in the capillary before analysis using an antibody that detects RNA.

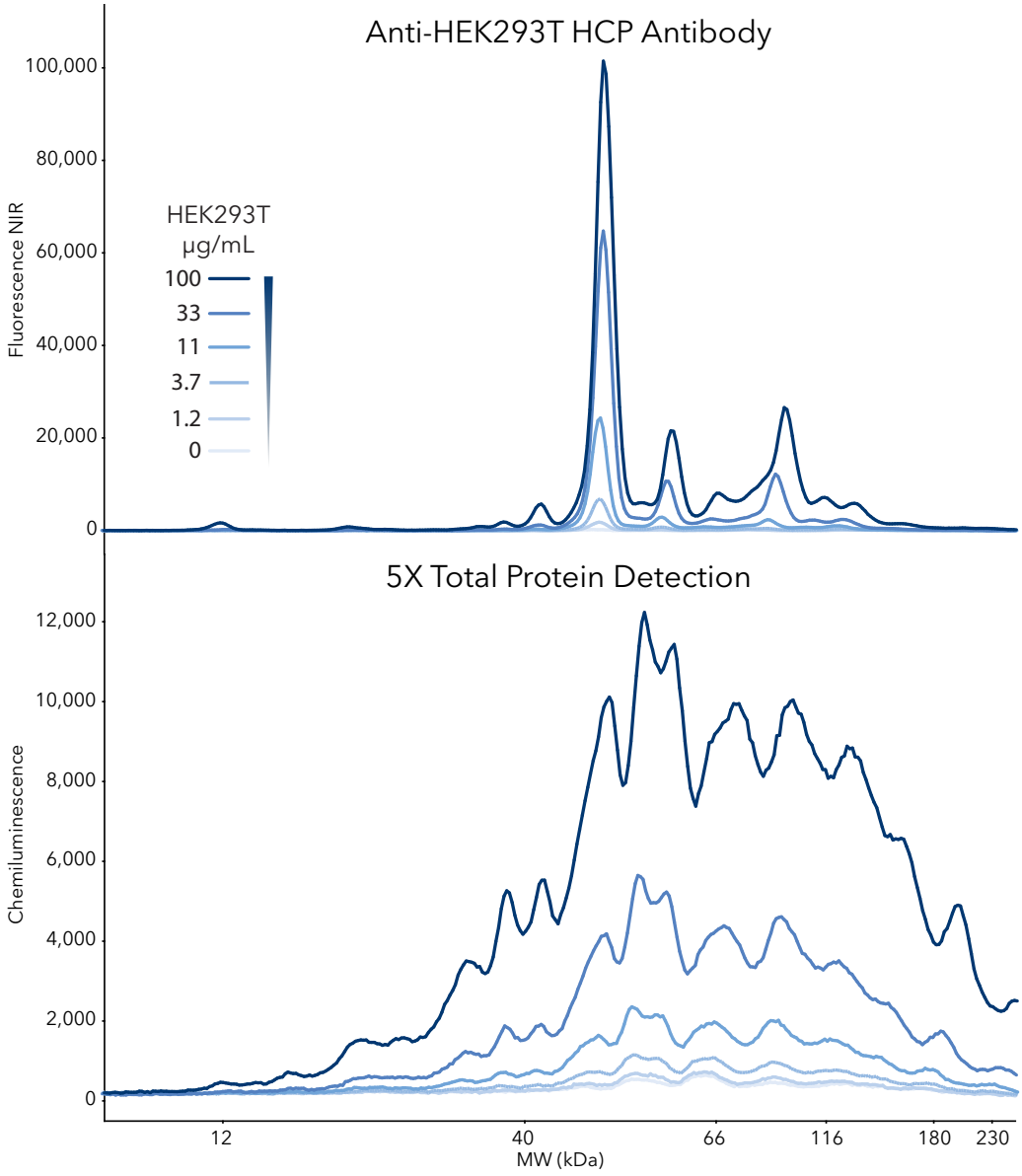


FIGURE 6. Simple Western LVV purity analysis. Overlaid electropherograms of a HEK293T whole-cell lysate dilution series detected by an anti-HEK293T antibody (top) and the 5X Total Protein Labeling Reagent (bottom).

Purity

Process-related impurities like host cell proteins (HCP) must be monitored and eliminated during LVV manufacturing. Traditionally, purity is monitored by ELISA using an antibody targeting HCP or by SDS-PAGE with total protein staining. Anti-HCP antibodies may be used on Simple Western with the added advantage over ELISA of size separation profiles and minimal matrix interference. Additionally, Simple Western has total protein detection with the 5X Total Protein Labeling Reagent with a sensitivity that rivals SYPRO Ruby.

Here, we compared the two total protein detection methods on Simple Western by an anti-HEK293T HCP antibody and the 5X Total Protein Labeling Reagent. To do so, we created a serial dilution series of HEK293T from 100 µg/mL to 0 µg/mL in a 10^7 TU/mL LVV particle background and analyzed each sample on Simple Western by the two detection methods. For both detection methods, a complex protein separation profile was observed that decreased in signal intensity with decreasing protein concentration, as expected (**FIGURE 6**). Interestingly, the separation profile from the 5X Total Protein Detection showed a broader apparent separation pattern, possibly indicating more complete coverage of the HEK293T proteome (**FIGURE 6**). When we plotted the total protein area resulting from the two detection methods against each other, a linear relationship was revealed, with an R^2 value of greater than 0.99 (**FIGURE 7**). Thus, the anti-HEK293T HCP antibody and 5X Total Protein Detection titrate linearly, despite having apparent differences in size separation profiles.

A major advantage of the 5X Total Protein Labeling Reagent on Simple Western is that it can label any protein regardless of host cell origin. Therefore, Total Protein Detection on Simple Western eliminates the need for an anti-HCP antibody to detect impurities and may offer more complete coverage of the host cell proteome.

Transduction analysis

Once LVV introduces a genetic modification, phenotypic validation at the protein level is often required to confirm viral infectivity, and this has traditionally been accomplished by Western blot. However, to analyze non-proliferating cells like iPSC-derived macrophages, traditional Western blot is severely limited because it requires a relatively large amount of starting material.

By contrast, Simple Western can analyze very small sample volumes, as little as 3 µL, with pg-level sensitivity. This makes analyzing limited sample types like iPSC-derived macrophages possible. For this reason, researchers at the University of Oxford used Simple Western to measure LVV-mediated gene knockout in iPSC-derived macrophages.[5] The results from their analysis showed the absence of the 3 non-essential proteins, HPRT1, PPIB, and CDK4, that were the targets of their knockout studies (**FIGURE 8**). These results represent the first demonstration of targeted genome editing in iPSC-derived macrophages by CRISPR/Cas9 LVV transduction, opening the door to the systematic exploration of macrophages with great therapeutic potential.[5]

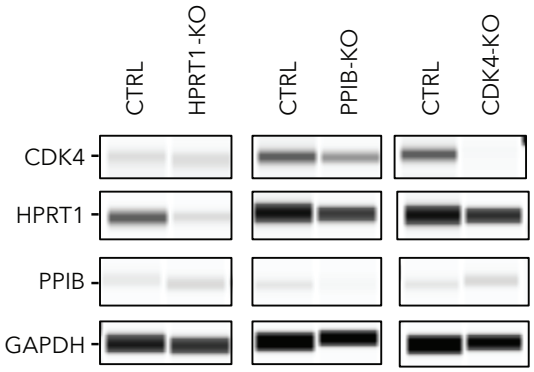


FIGURE 8. Simple Western LVV transduction analysis. LVV and CRISPR/Cas9 knockout (KO) and control (CTRL) in iPSC-derived macrophages. Adapted under license CC BY 4.0.[5]

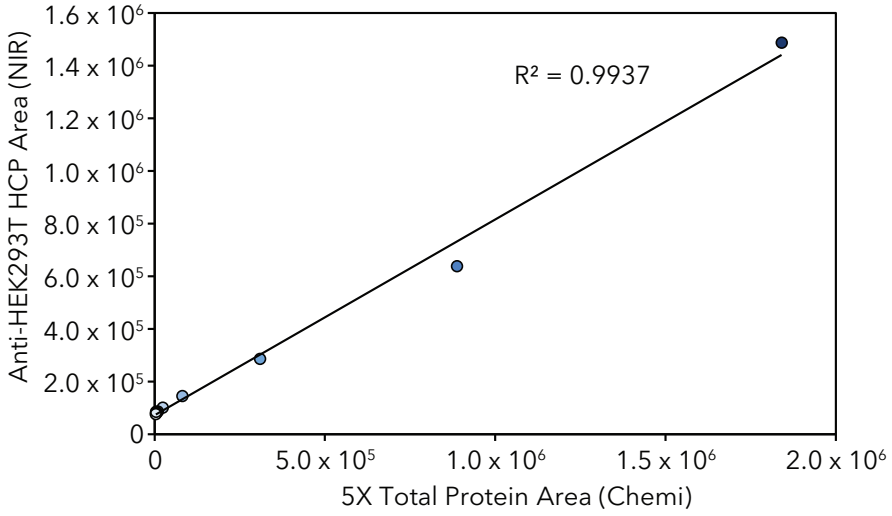


FIGURE 7. Simple Western LVV purity analysis. Linearity of detection by the anti-HEK293T antibody and the 5X Total Protein Labeling Reagent.

Advanced Lentiviral Characterization from All Angles

The demand for clinical-grade LVV for C&GT continues to grow rapidly, yet analytical solutions for thorough vector characterization to meet regulatory standards and ensure lot-to-lot consistency remain a stringent bottleneck. Now, Simple Western alleviates this bottleneck by providing high-throughput multi-attribute analysis for LVV characterization that scales with manufacturing workflows. Simple Western's automation and fast time to results enable at-line protein analysis for more efficient analytical process development. Because Simple Western offers an all-in-one multi-parameter analysis which would typically require multiple instruments, Simple Western can reduce upfront capital expenditure and overall costs, including time and labor, to bring new C&GT to market.

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Additional Resources

- Simple Plex HIV-1 Gag p24 Assay
- Simple Plex HEK 293 HCP 3G Assay
- Assessing Your AAV Product Quality? Get the Confidence You Need with Maurice
- Next-Generation Analytical Solutions for C&GT

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