High-precision lentivirus titer determination and protein profiling

PA 800 Plus pharmaceutical analysis system

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Introduction

In this technical note, we introduce a high-performance sodium dodecyl sulfate-capillary gel electrophoresis based assay, using the SCIEX PA 800 Plus pharmaceutical analysis system in conjunction with the SDS-MW protein analysis kit for highly precise lentivirus titer determination and profiling. This instrument with the associated kit provides automated separation of proteins in the size range of 10 kD to 225 kD with high resolution, excellent quantification capability and great reproducibility. Along with the separation method, we also describe an easy sample preparation protocol including the denaturing and labeling steps for quantitative determination of the lentivirus P24 protein using a pyrylium dye. This dye readily reacts with the primary amines of the polypeptide backbone of proteins and also features very low (<1%) fluorescence in its unconjugated format, thus decreasing baseline noise and increasing detection limit.

Lentivirus, a member of the retrovirus family [1], is capable of integrating large DNA molecules into host cells, thus representing one of the most efficient gene delivery vehicles for transduction [2]. For this particular application, lentivirus possesses several advantages, including its ability to infect both non-dividing and dividing cells. For clinical therapeutic applications, the stable and long-term transgene expression of these retroviruses represent a great advantage [3]. The proteome of the lentivirus is composed of five important structural and several non-structural proteins. The vital structural proteins are as follows: gp120 surface envelope protein (120 kDa), gp41 transmembrane envelope protein (41 kDa), p24 capsid protein (24 kDa), p17 matrix protein (17 kDa) and the p7/P9 nucleocapsid protein (7-11 kDa). The two envelope proteins of gp120 and gp41 are highly glycosylated. This post translational modification (PTM) apparently conceals important antigenic sites, thus helps the virus to avoid recognition by the immune system of the host. The p24 protein is the building block of the lentivirus capsid and approximately 2000 of them assemble the full capsid.

The various challenges during lentivirus production include virus stability, toxicity of the virus to the host cell lines, and limited ability to properly characterize the active virus product. As a result, monitoring product quality is critical. Titer is one of the critical quality attributes (CQA) for lentivirus production from lab scale to GMP manufacturing.

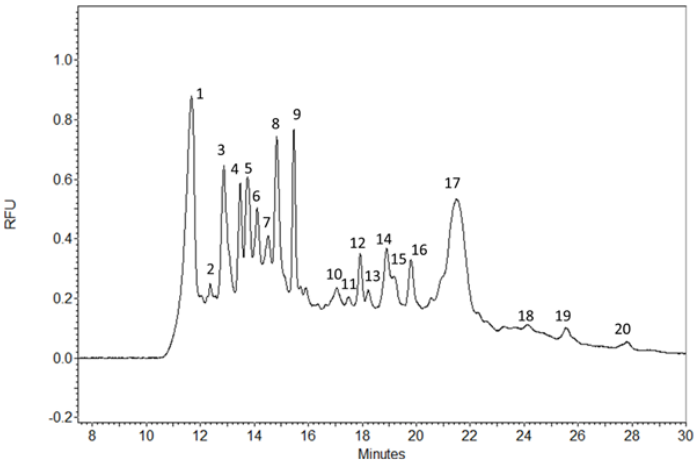

Figure 1. Sodium dodecyl sulfate capillary gel electrophoresis of the pyrylium dye tagged lentivirus proteome. Conditions: EZ-CE cartridge with 30 cm total length (20 cm to the detection point, 50 µm ID) fused silica capillary filled with the SDS-MW gel-buffer, E=500 V/cm, temperature: 25°C, Injection parameters: water pre-injection for 0.4 min at 20 psi followed by sample injection for 1 min at 5kV.

Key features

- Sensitive titer determination based on precise quantification of the separated p24 protein peak
- Easy batch-to-batch reproducibility and purity assessment based on the resulting proteome profile
- Efficient two-step sample preparation protocol including denaturation and covalent fluorophore labeling of the lentivirus proteome with no sample cleanup requirement
- Excellent repeatability with 0.20% RSD on migration time, and less than 0.91% on Corrected Peak Area%
- Good titer determination detection linearity of (r^2 = 0.9953) in the concentration range of 1-10 µg/mL
- The method offers the LOQ of 8 ng/mL for p24

Measuring the functional titer of a recombinant lentivirus is not only dependent on how efficiently the transfer vector is packaged but also the transduction efficiency of the cell line used [4]. Quantification (titer determination) of a lentivirus to ensure efficient expression is usually based on the quantification of the p24 protein. ELISA assay is the traditional method for lentiviral titer determination, aiming for this particular protein. However, this method measures virus-associated p24 along with possible false positives with similar epitopes, resulting in overestimation of the lentivirus titer. This calls for an orthogonal method to be used in combination with the regularly used screening ELISA test. The orthogonal method can be a separation-based technique, in which case it can be used to recognize false positives [5], to provide more accurate titer, and concomitantly utilized for profile-based purity assessment.

To address these issues, we describe an easy-to-use SDS-CGE method for high precision lentivirus titer determination along with its protein profiling-based purity assessment. The approach offers automated analysis of the lentiviral proteome with high resolution, excellent quantification capability and great reproducibility. An easily applicable two-step sample preparation and fluorescent labeling protocol is also described.

Materials and methods

Materials: Pre-made lentivirus (LV-CAG-GFP, PN SL100270) was from SignaGen Laboratories (Rockville, MD) and the HIV1 p24 protein (PN ab127888) was purchased from Abcam (Cambridge, UK). The Chromeo P503 dye (P/N 15106) was from Active Motif (Carlsbad, CA). Phosphate Buffered Saline (PBS) 10x bioreagent suitable for cell culture (P/N P5493-1L) was from Sigma-Aldrich (St. Louis, MO). The SDS-MW Analysis Kit (P/N 390953) was from SCIEX (Framingham, MA), including the SDS-MW gel buffer, the acidic and basic wash solutions (0.1 N HCl and 0.1 N NaOH) and the SDS-MW sample buffer of 100 mM Tris-HCl (pH 9.0) with 1% SDS.

Sample preparation: Preparation of the Chromeo P503 working solution: 1 mg of Chromeo P503 dye (lyophilized powder) was reconstituted in 1 mL of methanol. After reconstitution, the dye label can be stored at 2-8°C for six months according to the manufacturer's instructions. Sample preparation: 5 µL of lentivirus sample solution (1.5 x 10^9 TU/mL) was mixed with 5 µL of sample preparation solution provided in the SDS-MW Analysis Kit and incubated at 70°C for 10 minutes followed by mixing with 0.5 µL of Chromeo P503 dye working solution and incubated again for another 10 minutes at 70°C. After cooling down to room temperature, 39.5 µL of DI water was added to the reaction mixture and the diluted sample was transferred to the sample vial for SDS-CGE-LIF analysis.

Capillary electrophoresis: A PA 800 Plus pharmaceutical analysis CE system (SCIEX) equipped with a laser-induced fluorescence (LIF) detector with a 488 nm solid state laser and a 600 nm emission filter was used for all separations. The pre-assembled EZ-CE capillary cartridge (SCIEX, P/N A55625) had a bare fused-silica capillary with 50 µm I.D. and 30 cm total length (20 cm effective length). The SCIEX universal vials (P/N A62251), universal vial caps (P/N A62250) and PCR vials (P/N 144709) were used for sample loading with the following injection parameters: water stacking at 20.0 psi for 0.40 min and injection at 5kV for 60 s. The detailed information of condition, separation and shutdown methods are shown in Figure 2-6. Data acquisition and analysis were performed using 32 Karat software version 10.

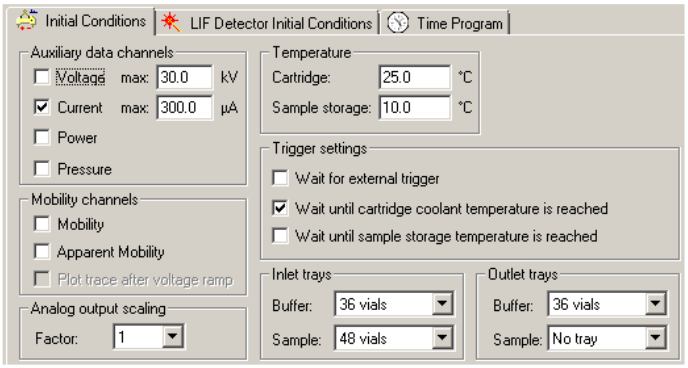

Figure 2. Initial conditions on the PA 800 Plus.

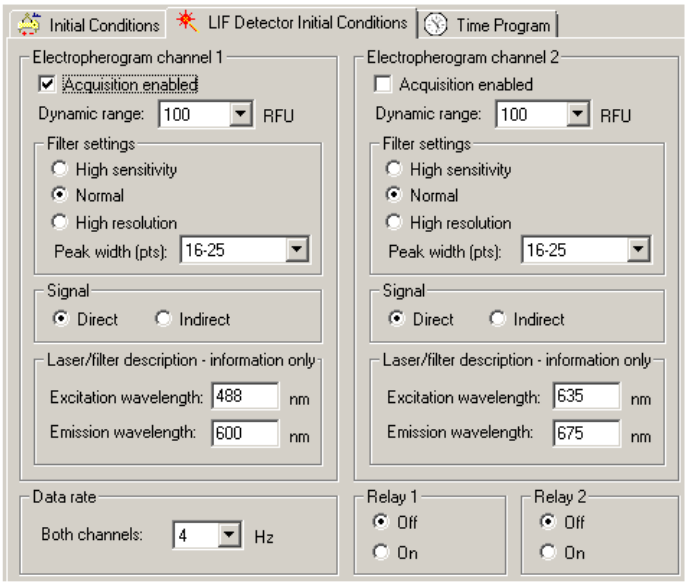

Figure 3. LIF detector initial conditions on the PA 800 Plus.

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	10.00 min	BI:D	BO:D1	forward	0.1 N NaOH rinse to clean capillary surface
2		Rinse - Pressure	20.0 psi	5.00 min	BI:E	BO:E1	forward	0.1 N HCl rinse to neutralize capillary surface silanol group
3		Rinse - Pressure	20.0 psi	2.00 min	BI:F1	BO:F1	forward	ddH2O rinse to remove the acid residue
4		Rinse - Pressure	70.0 psi	10.00 min	BI:B	BO:B1	forward	SDS Gel rise to fill the capillary
5	0.00	Separate - Voltage	15.0 KV	10.00 min	BI:C	BO:C1	5.00 Min ramp, reverse polarity, both	SDS Gel for voltage equilibration
6								

Figure 4. Time program for conditioning on the PA 800 Plus**.**

Initial Conditions | LIF Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	70.0 psi	3.00 min	BI:D	BO:D1	forward, In / Out vial inc 8	0.1 N NaOH rinse to clean capillary surface - Automatic increment every 8 runs
2		Rinse - Pressure	70.0 psi	1.00 min	BI:E	BO:E1	forward, In / Out vial inc 8	0.1 N HCl rinse to neutralize capillary surface silanol group - Automatic increment every 8 runs
3		Rinse - Pressure	70.0 psi	1.50 min	BI:F1	BO:F1	forward, In / Out vial inc 8	Water rinse to remove the acid residue - Automatic increment every 8 runs
4		Rinse - Pressure	70.0 psi	10.00 min	BI:B	BO:B1	forward, In / Out vial inc 8	SDS Gel rinse to fill the capillary with SDS gel - Automatic increment every 8 runs
5		Wait		0.00 min	BI:A	BO:A1	In / Out vial inc 8	ddH2O, use for dipping to clean capillary tip - Automatic increment every 8 runs
6		Wait		0.00 min	BI:A	BO:A4	In / Out vial inc 8	ddH2O, use for dipping to clean capillary tip - Automatic increment every 8 runs
7		Rinse - Pressure	20.0 psi	0.40 min	BI:C	BO:A1	forward, In / Out vial inc 8	
8		Inject - Voltage	5.0 KV	60.0 sec	SI:A	BO:C1	Override, reverse polarity	Sample injection
9		Wait		0.00 min	BI:B	BO:B4	In / Out vial inc 8	ddH2O, use for dipping to avoid sample carry over - Automatic increment every 8 runs
10	0.00	Separate - Voltage	15.0 KV	30.00 min	BI:C	BO:C1	1.00 Min ramp, reverse polarity, both, In / Out vial inc 8	SDS Gel for separation - Automatic increment every 8 runs
11	5.00	Autozero						
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Figure 5 Time program for separation on the PA 800 Plus.

Results and discussion

Lentivirus is a spherical enveloped virus with an average diameter of 80–100 nm and approximately 8 nm long spikes at the surface (Figure 6). Inside the envelope is an isometric capsid, assembled from p24 proteins to hold a rod-shaped nucleic acid payload possessing gag, pol and env genes, coding for all viral proteins [6]. Quantification of this p24 protein was used in this study for high precision lentivirus titer determination. Albeit, the sensitivity and specificity of immunoassay-based methods have been increasing in the past decades, cross reactivity based overestimation of the analyte molecules still represent a problem [7]. Introducing an orthogonal separation-based titer determination method alleviates many of the limitations of the ELISA assays [8].

The titer determination workflow started with an efficient two-step sample preparation protocol, including the denaturation and covalent fluorophore labeling steps as described in the Experimental section. The resulting pyrylium dye tagged lentivirus proteome was then subject to quantitative sodium dodecyl sulfate-capillary gel electrophoresis analysis. The resulting electropherogram is shown in Figure 1, featuring 18 peaks. Since most of the lentivirus proteins possess various post-translational modifications (PTMs), such as glycosylation, standard curve based molecular mass assessment using the sizing ladder was not considered to be adequate, thus was not pursued in this study.

The peak corresponding to the p24 protein for titer determination was determined by spiking the sample with a commercially available standard. Figure 7 compares the original separation trace with six spiked SDS-CGE electropherograms reflecting the effect of the increasing amount of the p24 protein from 0.05 µg to 0.3 µg. This experiment actually served three purposes: 1) Peak 9 was identified as the p24 protein, later used for titer determination, 2) the resulting profile can be readily used for batch-to-batch reproducibility and purity assessment, and 3) the excellent reproducibility of the CGE-SDS method was demonstrated, even with various amounts of p24 in the samples, proving that the quantitative variation of the p24 protein content did not affect profiling repeatability. The relative standard deviation of the migration time and peak areas were less than 0.20% RSD and 0.91% RSD, respectively.

Figure 6. Lentivirus structure. Titer determination is accomplished by quantifying the p24 capsid protein depicted by the orange dots inside the green viral envelope.

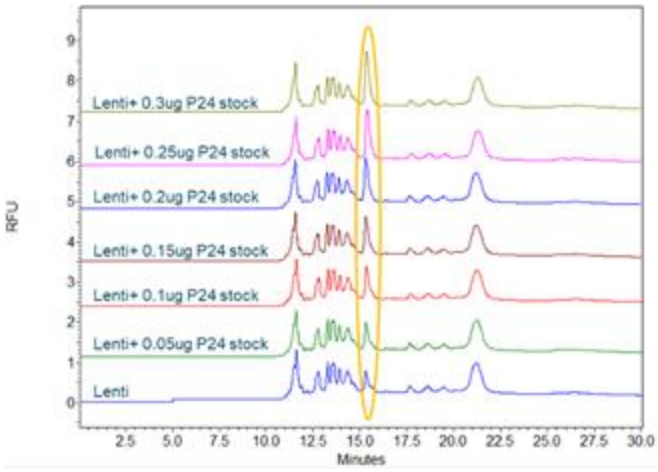

Figure 7. Reproducibility of the SDS-CGE analysis of the pyrylium dye labeled lentivirus proteome. Peak 9: p24 protein for titer determination. The increasing amounts of spiked p24 are shown at the corresponding traces. Separation conditions were the same as in Figure 1.

Based on the apparently good detection response during the spiked reproducibility study, the limit of quantification (LOQ) values were determined to be 8 ng/mL for p24 by injecting a series of 2x dilutions of standalone p24 standard into the SDS-MW gel-filled capillary column.

For 6 consecutive injections of the lentivirus sample solution, the RSD% of MT and corrected peak area of P24 peak were calculated to be 0.20% and 0.91%, respectively, as shown in Table 1.

Table 1. Repeatability of 6 replicate injections of lentivirus sample solution. Separation conditions were the same as in Figure 1.

Replicate	Migration Time	Corrected Peak Area
1	*15.57*	*3594174*
2	*15.6*	*3615001*
3	*15.57*	*3621318*
4	*15.61*	*3597266*
5	*15.62*	*3565088*
6	*15.65*	*3535125*
Ave	***15.60***	***3587995***
SD	***0.03***	***32508***
RSD%	**0.20**	**0.91**

For lentivirus titer determination, Figure 8 compares the superimposed resulting traces of the runs from 1 µg/mL to 10 µg/mL concentrations (A) and the calibration plot (r^2=0.9953) using the average of three 3 replicate runs with the corresponding error bars (B).

The titer of the lentivirus sample—the quantity of the p24 protein in Figure 1—was determined by using the detection linearity plot in Figure 3 and found to be 1.45 x 109 TU/mL, 1.45 x 109 TU/mL and 1.46 x 109 (Table 2) during three consecutive days of analysis. The titer value from the vendor for this particular sample was 1.5 x 109 TU/mL (also shown in Table 1), measured by ELISA assay. As a first approximation, we consider this apparent difference in the titer values between the SDS-CGE and ELISA assays was the overestimation of the latter, as ELISA is prone to detect similar epitope proteins in addition to the target protein. This assumption was supported by the complicated profile of the lentivirus proteome (Figure 1), showing the presence of all other proteins in the mixture possibly interfering with the ELISA assay. The SDS-CGE assay, on the other hand, provided the actual titer value of the sample based solely on quantifying the peak corresponding to the p24 protein (peak 9 in Figure 1), without any interference of other proteins in the sample. In addition, the resulting profile can be readily used for batch-to-batch purity assessment.

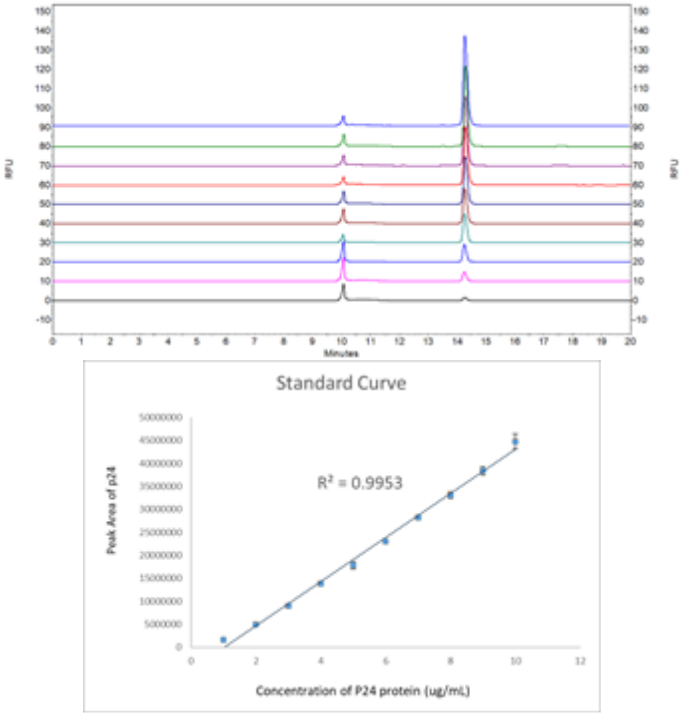

Figure 8. Lentivirus titer determination standard curve based on the SDS-CGE analysis of the p24 protein. (A) Superimposed electropherograms with increasing amounts of p24 injected, (B) calibration curve using the average of three 3 replicate runs with the corresponding error bars. Separation conditions were the same as in Figure 1.

Table 2. Titer of the lentivirus sample in 3 different days.

Titer measurement	Titer (TU/mL)
Titer measured by SDS-CGE_Day1	*1.45 x10^9*
Titer measured by SDS-CGE_Day2	*1.45 x 10^9*
Titer measured by SDS-CGE_Day3	*1.46 x 10^9*
Titer from vendor (ELISA P24 kit)	*1.5 x 10^9*

Conclusions

- In this technical note we introduced a rapid, two-step sample preparation protocol, along with the optimized separation parameters for the fluorophore labeled lentivirus proteome for capillary electrophoresis analysis with laser induced fluorescent detection
- Quantification of the p24 protein was used for precise lentiviral titer determination
- Comparing the ELISA and SDS-CGE data revealed a probably incorrect number for the former, possibly due to some cross-reactivity during the immunoassay-based method
- The inherent titer overestimation of the ELISA kit can be readily alleviated with the use of the SDS-CGE method described here
- The method introduced here features excellent migration time and corrected peak area repeatability of 0.20% RSD and 0.91% RSD, respectively, with excellent titer determination detection linearity of r^2 =0.9953 in the concentration range of 1 µg/mL-10 µg/mL
- The LOQ of the method was 8 ng/mL for the p24 capsid protein

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