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Short communication

# Titer measurement of HIV-1 envelope trimeric glycoprotein in cell culture media by a new tandem ion exchange and size exclusion chromatography (IEC-SEC) method

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ABSTRACT

An efficient and specific liquid chromatography (LC)-based assay was developed to monitor the production of recombinant HIV-1 trimeric envelope glycoprotein (HIV Env trimer), a candidate vaccine for HIV-1 infection, in cell culture media to support scale-up process development. In this method, titer measurement was achieved by coupling a weak anion exchange chromatography (IEC) column with a size exclusion chromatography (SEC) column. This assay was specific, accurate, precise, and has been qualified for its intended purpose, with a limit of quantification (LOQ) of 10 μg/mL. This tandem separation strategy offered a reliable and timely analytical support to directly monitor the titer of HIV Env trimer during cell growth, without any extra sample purification steps.

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## 1. Introduction

With 2 million people newly infected with HIV-1 every year, the development of effective HIV-1 vaccines is needed to protect public health [1]. Infection of human host cells by HIV-1 is mediated by an envelope glycoprotein trimeric spike on the surface of the virus. One of the vaccine candidates under development is a recombinant, non-pathogenic HIV-1 Env trimeric protein (HIV Env trimer), which functions by mimicking the HIV-1 virus' transmembrane protein, inducing virus-neutralizing antibodies to impede the virus-cell fusion process, and thereby preventing infection. HIV Env trimer is comprised of three heterodimers of gp120-gp41 subunits that non-covalently associate into a trimeric protein. Uniquely, HIV Env trimer is extensively glycosylated as there exists 28 potential N-linked glycans on each gp120-gp41 dimer subunit (up to 50% of its molecular weight is attributed to glycans), rendering a relatively polar surface with many hydroxyl groups. [2,3]. Upon growth in a mammalian host cell line (CHO-DG44) developed in-house, the HIV Env trimer co-existed with complex components that included host cell proteins (HCPs), host cell DNA (HCD) and other cell debris. To support the development of a scale-up process, the volumetric productivity (titer) needed to be monitored and studied for its correlation with multiple bioreactor parameters, such as temperature, pH, viable cell density, and nutrient feeding strategies.

Ideally, the titer of HIV Env trimer should be measured directly without any additional purification steps for quick feedback on protein production. However, conventional biological binding assays and custom affinity columns for titer measurement have limitations, such as high costs, long development times, and unknown specificities [4]. Traditional protein A/G immunoaffinity chromatography techniques for titer measurement of antibodies also do not apply for vaccine molecules. Therefore, we developed a new tandem chromatography approach for the titer measurement of HIV Env trimer in cell culture media.

HIV Env trimer is a glycoprotein (MW ~ 350 kDa) with a hydrodynamic radius of 9.6 nm [5,6] while the HCPs and cell culture impurities are generally less than 100 kDa [7]. Upon evaluation, however, HIV Env trimer co-eluted with interfering species using either individual SEC or IEC approaches and undermined the basic principle for chromatographic quantitation. To this end, we hypothesized that separation of HIV Env trimer could be achieved

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by combining IEC in tandem with SEC. Herein, we report the successful development of a tandem IEC-SEC assay to reach the goal of titer measurement by taking advantage of the characteristics of HIV Env trimer, as well as the dual separation modes based on charge and size. No prior purification step was needed for the assay, which ensured a quick turnaround time. The quality attributes of this new method were also assessed and reported, including specificity, linearity, accuracy and precision according to ICH recommendations [8].

## 2. Material and methods

For method development, all chemicals and reagents were purchased from VWR Scientific (Waltham, MA), unless otherwise stated. Purified HIV Env trimer was expressed from the CHO-DG44 cell line at the Vaccine Production Program Laboratory (Gaithersburg, MD). A CHO-DG44 expansion was lysed and clarified to obtain a HCP/HCD enriched harvest, which was a mock CHO-DG44 cell culture media and used as the negative control. A Waters AcQuity™ UPLC system with a FLR detector was used for the development and application of this assay. The precolumn PEEK filter with a 0.5 μm frit and one-piece PEEK column coupler were purchased from Sepax Technologies (Newark, DE).

For the final IEC-SEC assay, an IEC column (BioBasic™ AX, 4.6 × 100 mm, 5 μm particle size, 300 Å porosity, Thermo Scientific) and a SEC column (SRT SEC, 7.8 × 150 mm, 5 μm particle size, 300 Å porosity, Sepax) were coupled in tandem by a PEEK column coupler with the IEC column positioned first in the flow path. A PEEK pre-column filter was also installed in front of the IEC-SEC column set-up to capture any potential large particulates (Supplementary Fig. S1). 250 mM sodium phosphate (pH 6.0) was used for mobile phase A (MPA) while 1 M sodium chloride (NaCl) was used as mobile phase B (MPB). A simple step gradient at 1 mL/min was used: 0–10 min: 100% MPA (load/elute HIV Env trimer); 10–20 min: 100% MPB (strip any strongly bound species from the IEC column); then stepping back to 100% MPA from 20 to 30 min (re-equilibrate). The total run time was 30 min. An external calibration curve of known concentrations of purified HIV Env trimer in 2× PBS was used to quantify the titer of HIV Env trimer in cell culture media. For each test, the calibration standards and cell culture samples were filtered through 0.2 μm cellulose acetate filters (to prevent clogging of the LC system), and 50 μL of each sample/standard was directly loaded onto the tandem IEC-SEC columns for analysis. The column temperature was set at 30 °C. The excitation and the emission wavelengths of the FLR detector were set at 277 nm and 344 nm, respectively. After each run, the columns were washed with water for about 10 column volumes and stored in 10% ethanol.

## 3. Method development and results

### 3.1. Evaluation of single mode chromatography approaches

Different SEC columns (of varying chemistries and pore sizes) with different mobile phases were first assessed for the separation of HIV Env trimer in mock CHO-DG44 cell culture media. However, with SEC alone HIV Env trimer coeluted with the cell culture media components as illustrated for one of the evaluated conditions in Fig. 1. Different cation and anion exchange IEC columns were also evaluated, but none of these individual chromatography approaches were able to separate HIV Env trimer to achieve titer measurement.

### 3.2. Evaluation of a tandem IEC-SEC approach

Anion exchange IEC has been one of the key approaches in the downstream purification process of biopharmaceuticals. To this end, we speculated that by combining both charge (IEC) and size (SEC) separation capacities, HIV Env trimer may be well separated from the cell culture components. HIV Env trimer is heavily

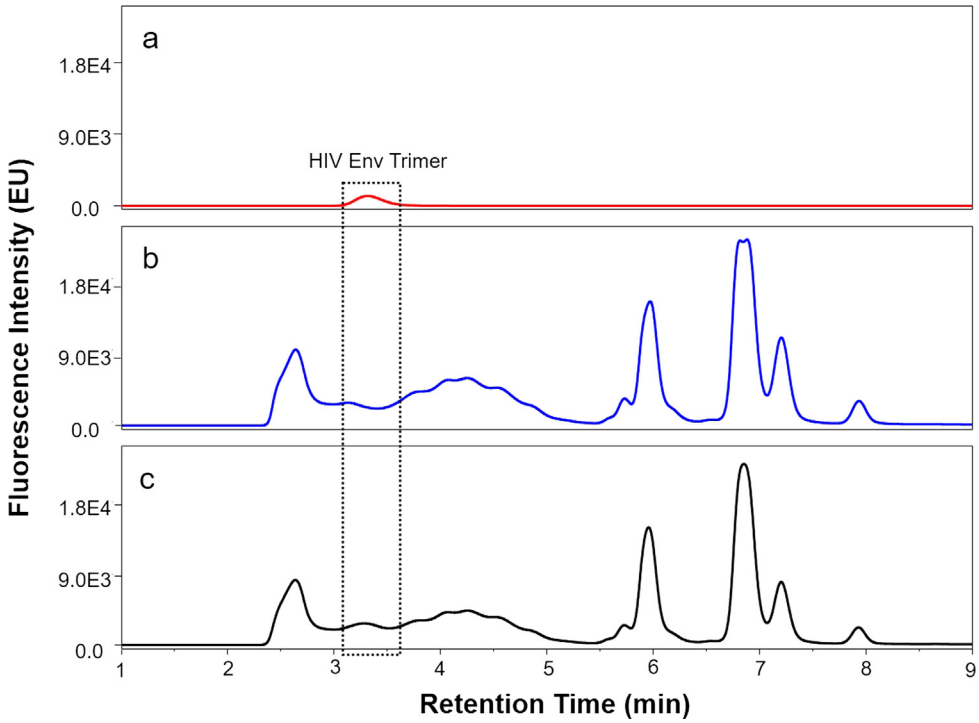


**Fig. 1.** SEC chromatograms of (a) HIV Env trimer spiked in 2× PBS (positive control, final: 100 μg/mL), (b) mock cell culture media (negative control), and (c) HIV Env trimer spiked into the mock cell culture (final: 100 μg/mL). Method conditions included an isocratic elution at 1 mL/min in 250 mM sodium phosphate, pH 6.0 using an SRT-300 SEC column (5 μm, 7.8 × 150 mm) with a 50 μL injection volume.

glycosylated; therefore, under acidic conditions (pH 6.0), protonation on the hydroxyl groups from the glycans could make HIV Env trimer positively charged. Choosing an anion exchange column with a positively charged stationary phase was thought to retain the cell culture media components that are less protonated compared to the highly glycosylated HIV Env trimer when under acidic conditions. HIV Env trimer was assumed not to interact with the initial IEC column and directly pass to the SEC column while less protonated species would retain longer. Choosing the correct stationary phase and mobile phase to exploit the separation capabilities from the IEC was critical to achieve initial separation of HIV Env trimer which could then be further sieved by SEC for a complete separation.

We found that the weak anion exchange column (BioBasic™ AX IEC, polyethyleneimine coated deactivated silica stationary phase) coupled with a SEC column (SRT-300 SEC) had optimal synergistic performance to separate HIV Env trimer. A wide range of mobiles phases with different salt compositions and acidities were tested. The optimized mobile phases A (MPA) and B (MPB) were 250 mM sodium phosphate (pH 6.0) and 1 M NaCl, respectively. With this tandem IEC-SEC setup, good separation of HIV Env trimer was achieved. As shown in Fig. 2, HIV Env trimer was well separated from the CHO-DG44 spent culture media by IEC-SEC, enabling reliable titer quantitation of HIV Env trimer.

### 3.3. Assay quality assessment

Linearity of the method was demonstrated by preparing calibration standards in both mock cell culture media and 2× PBS. Both sets of calibration curves generated good linear correlations ($R^2 > 0.99$) with similar slopes in a working range from 10 to 400 μg/mL (Supplementary Fig. S2). Titers calculated by the two calibration curves were also comparable; therefore, 2× PBS was selected as the diluent for the calibration curve.

The accuracy, precision and specificity of the method were demonstrated by spiking studies. Purified HIV Env trimer was spiked into mock media at three different concentrations (i.e., 50, 100, 150 μg/mL). The measured titer divided by the theoretical concentration was used for the evaluation of accuracy. The spike recoveries at three levels were approximately 90%, meeting the acceptable range of 80–120% for this in-process method. Good specificity was also observed for HIV Env trimer spiked into either PBS buffer or mock cell culture media (Supplementary Fig. S3).

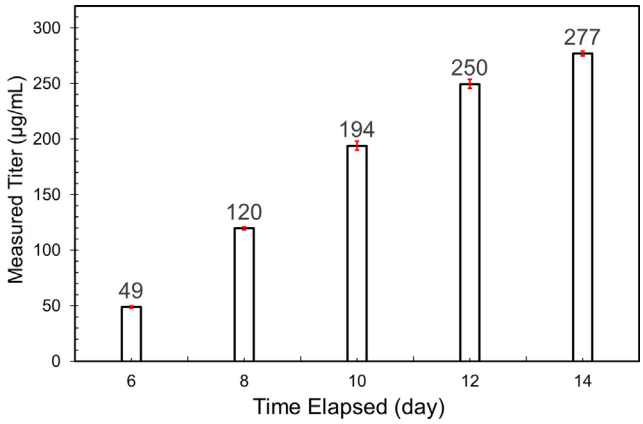


**Fig. 3.** Titer growth of HIV Env trimer in the bioreactor from day 6 to day 14. Error bars indicate the standard deviation of each test (n = 6).

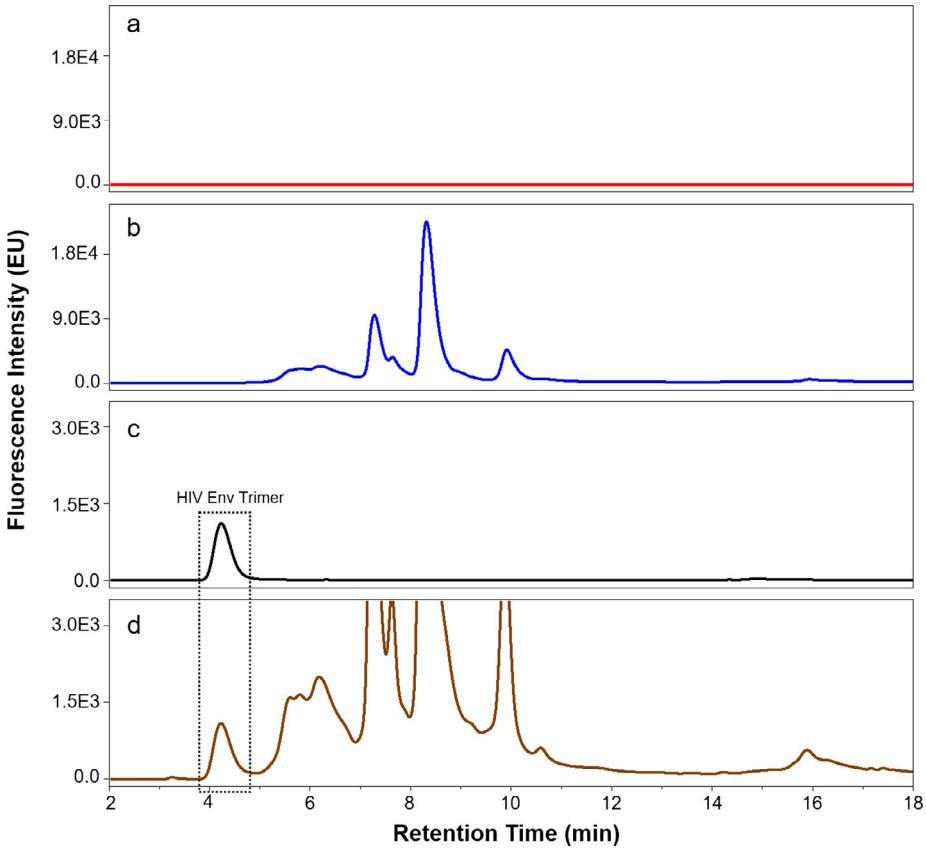


**Fig. 2.** Representative IEC-SEC chromatograms of (a) 2× PBS; (b) CHO-DG44 mock media (negative control); (c) HIV Env trimer spiked into 2× PBS (positive control, final: 100 μg/mL); (d) HIV Env trimer spiked into CHO-DG44 mock cell culture media (final: 100 μg/mL).

### 3.4. Assay application

In summary, this method has been routinely applied to quantitatively measure the titer of HIV Env trimer directly during cell growth and at harvest. As an example, Fig. 3 shows the titer growth monitoring of HIV Env trimer in a CHO-DG44 cell culture bioreactor from day 6 to day 14. This method has provided a timely and accurate measurement for titer growth and trending studies. Using this IEC-SEC assay, at least 300 injections of have been routinely achieved without noticeable column clogging or retention time shifting; thereby, demonstrating good robustness.

## 4. Conclusion

This quantitative IEC-SEC method demonstrated to be capable of accurately and directly measuring HIV Env trimer in cell culture samples. Notable advantages of this assay include limited sample handling steps, quick turnaround time and the capability of measuring HIV Env trimer down to 10 μg/mL. This work provided critical information for the evaluation of multiple bioreactor parameters to further enable cell culture development. Lastly, the generic nature of this approach also suggests that it can be applied to other vaccines and therapeutic proteins.

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## Conflict of interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.089.

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