

# A comparative study of two immunoassay platforms to determine lentivirus titer for CAR-T development.

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## Introduction

Lentiviral transduction is an effective method of gene transfer used to generate cell lines expressing a gene of interest. Lentivirus can infect both dividing and non-dividing cells, which makes it a useful tool for difficult to transfect cell lines and provides long-term stable expression of a transgene.<sup>1</sup> One commonly utilized lentiviral vector backbone originates from human immunodeficiency virus (HIV). In order to use the HIV as a vector, essential genetic and respective phenotypic components are critical to combine with supporting elements from packaging and envelope plasmids. Splitting the HIV sequence across multiple vectors followed by co-transfection minimizes the likelihood of a single replication competent, pathogenic lentivirus, enabling safer production and handling in the laboratory. The p24 capsid protein of HIV remains on the lentivirus vector which facilitates viral titer determination via an immunoassay such as an ELISA (enzyme-linked immunosorbent assay). Lentivirus can be generated by transiently transfecting the helper plasmids along with the gene of interest into a packaging cell line (typically 293T cells) and harvesting the cell supernatant which contains the lentivirus product.<sup>2,3</sup>

Lentivirus is frequently used to deliver DNA to T-cells to generate CAR T-cell (chimeric antigen receptor T-cell) products.<sup>4</sup> CAR T-cells are one approach to immunotherapy wherein T-cells are removed from a patient and then genetically modified to contain CARs to target surface antigens on tumors for eradication. CAR T-cells are expanded ex vivo before injecting back into the patient for treatment. CAR design consists of an extracellular domain, typically a scFv from a monoclonal antibody to recognize the tumor antigen, and a linker or spacer followed by a transmembrane domain (ex CD3 $\zeta$ ) and an intracellular signaling domain that acts in a stimulatory fashion to the T-cells (ex CD28, or 4-1BB) to facilitate destruction of the cancer cells.<sup>5</sup>



When producing lentivirus, measurement of the viral titer enables the determination of the efficiency of the transient co-transfection as well as normalization of the amount of lentivirus used across experiments. The p24 protein pool that is quantified contains a variable amount of free p24 as well as p24 from non-functional vector particles.<sup>2</sup> The Alliance HIV-1 p24 Antigen ELISA kit (for Research Use Only, Revvity, Inc.) is a commonly used p24 immunoassay in the HIV field<sup>6,7</sup> to determine viral titer.<sup>8,9,10</sup> An ELISA is a sandwich immunoassay designed to detect the presence of a target ligand (generally a protein) in a liquid sample (Figure 1). In the Alliance HIV-1 p24 ELISA, an antibody specific for HIV-1 p24 is coated on the assay plate and used to capture the p24 present in the sample. After incubation with the sample and multiple wash steps, a second biotinylated antibody against HIV-1 p24 is added for detection. Excess unbound antibody is washed away and then streptavidin conjugated to horseradish peroxidase (HRP) binds the biotin of the secondary detection antibody. After additional washing, the HRP is treated with an ortho-phenylenediamine-HCl (OPD) substrate to produce a color change. In the termination step facilitated by the addition of acid, the amount of target in the sample can be quantified by absorbance (optical density, OD) as determined from the standard curve.

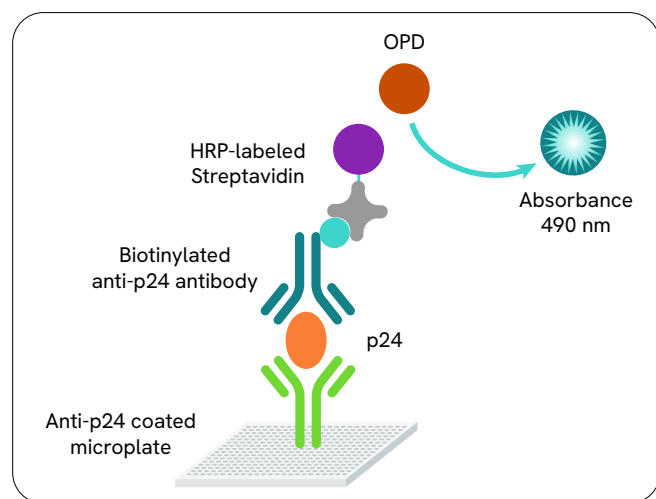


Figure 1. Alliance HIV-1 p24 Antigen ELISA assay principle. Refer to text for details.

The AlphaLISA<sup>®</sup> (Revvity, Inc.) bead-based sandwich immunoassay (similar to ELISA) is used to study biomolecular interactions in a microplate format (Figure 2). Alpha technology offers flexibility as well as higher throughput capability since it utilizes a higher density microplate format and is automation friendly. The technology was originally developed for drug discovery screeners and has been adopted as an immunoassay alternative. The acronym

“Alpha” stands for Amplified Luminescent Proximity Homogeneous Assay. Binding of antibodies captured on donor and acceptor beads and subsequent binding to the analyte form a complex. Excitation of the donor results in energy transfer from the donor bead to the acceptor bead in the complex producing a luminescent/fluorescent signal. The AlphaLISA HIV-1 p24 detection assay (for Research Use Only, Revvity, Inc.) can also be used to assess viral titers in a high throughput format. In this study, we demonstrate a comparative quantification of the p24 titer in a GFP control lentiviral sample using both the Alliance HIV-1 p24 Antigen ELISA and the p24 AlphaLISA assay platforms.

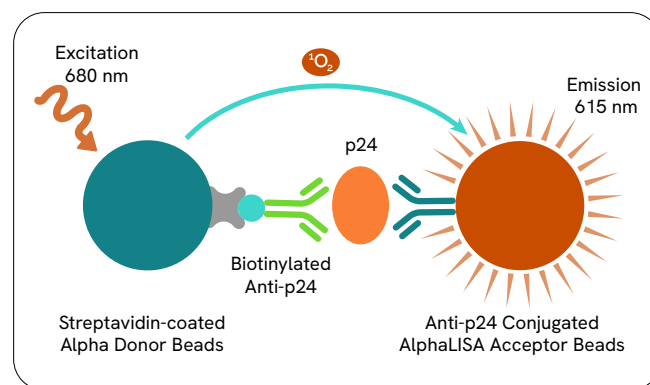


Figure 2. AlphaLISA p24 Assay Principle. A biotinylated anti-p24 antibody is bound by the Streptavidin-coated AlphaLISA Donor beads, while another anti-p24 antibody is directly conjugated to AlphaLISA Acceptor beads. In the presence of p24, the donor and acceptor beads both bind the target and come into proximity. Excitation of the donor beads at 680 nm provokes the release of singlet oxygen molecules that trigger a cascade of chemical reactions in the acceptor beads in proximity, resulting in maximum emission at 615 nm. AlphaLISA signal is proportional to the amount of p24 present in the sample.

## Materials and methods

### Reagents and consumables

- GFP control Lentivirus (Genecopoeia, #L303-100)
- AlphaPlate-384, light gray (Revvity, #6005350)
- AlphaLISA kit – p24 High Sensitivity (Revvity, #AL291C)
- Alliance HIV-1 p24 Antigen ELISA (Revvity, #NEK050001KT)
- Fetal bovine serum (FBS), heat inactivated (Thermo Fisher, #10082-147)
- Dulbecco’s Modified Eagle’s Medium, DMEM (ATCC, #30-2002)

## Data collection and analysis

Control GFP lentivirus was thawed from an aliquot on each day of testing and carefully diluted into cell culture media (DMEM + 10% FBS) to simulate lentivirus production from 293T cells. Serial dilutions of the mock lentivirus were generated across a wide span of concentrations for testing.

The AlphaLISA p24 assay was performed following Revvity's recommended protocol. Each assay required 5  $\mu$ L of test sample in a 50  $\mu$ L reaction. AlphaLISA signal was measured on a Revvity EnVision<sup>®</sup> 2105 Multilabel plate reader using default values for Alpha detection of the luminescent/fluorescent label. MyAssays Desktop<sup>®</sup> software was used to graph the standard curve and interpolate the unknown values using a four-parameter logistic curve with  $1/y^2$  weighting.

The Alliance HIV-1 p24 Antigen ELISA was performed following Revvity's recommended protocol. The assay required 200  $\mu$ L of test sample. Absorbance at 490 nm was measured on a Revvity EnVision 2105 Multilabel plate reader using the appropriate settings. MyAssays Desktop<sup>®</sup> was used to graph the standard curve and interpolate the unknown values using linear regression curve fitting.

## Results

In order to determine a viral titer, the amount of p24 in the lentiviral stock is quantified. To do this, standard curves using purified p24 are tested to determine the reference signal at each concentration in each assay. Standard curves were run for both the AlphaLISA p24 and Alliance p24 ELISA assays using the purified p24 protein included in each kit (Figure 3). The first assay tested with lentivirus samples was the AlphaLISA due to the simple, rapid assay workflow and generally wide dynamic range of the technology. Figure 3 compares the standard curve from each assay technology and demonstrates the broader dynamic range of the AlphaLISA assay relative to the ELISA assay.

A very broad initial dilution series of the GFP lentivirus (4-fold to 50,000-fold) was generated for the initial AlphaLISA assessment due to the unknown titer of the concentrated GFP control lentiviral stock. Figure 4 shows the average AlphaLISA signal of each dilution point. There is a clear hook effect from high titer of p24 present in the first four dilution points in the series due to an excess of target molecules oversaturating the Donor and the Acceptor beads, which inhibits their association.

The AlphaLISA assay was repeated and run in parallel with the Alliance p24 ELISA using a starting dilution of 1000-fold with serial 2-fold dilutions to fall within the linear range of both assays. All 12 AlphaLISA dilutions were linear and the interpolated concentration of p24 (pg/mL) is shown in Figure 5. The average p24 titer of the GFP lentiviral stock as determined by AlphaLISA assay (correcting for dilution factor) was  $6.14 \times 10^6$  pg/mL.

For the Alliance p24 ELISA, only dilutions 5 through 12 were tested in the assay with results plotted along with the AlphaLISA data in Figure 5. The top two dilutions and bottom three dilutions were outside the dynamic range. Therefore, only three data points fell within the linear range of the ELISA assay. For best accuracy when performing an ELISA, the OD of the unknown samples should fall within the dynamic range of the standard curve. The average p24 titer of the GFP lentiviral stock as determined by the samples within the standard curve range (correcting for dilution factor) using the Alliance HIV-1 p24 Antigen ELISA was  $5.32 \times 10^6$  pg/mL. The quantified p24 for both assays correlate well, showing the utility and accuracy of both methods for determining viral titer.

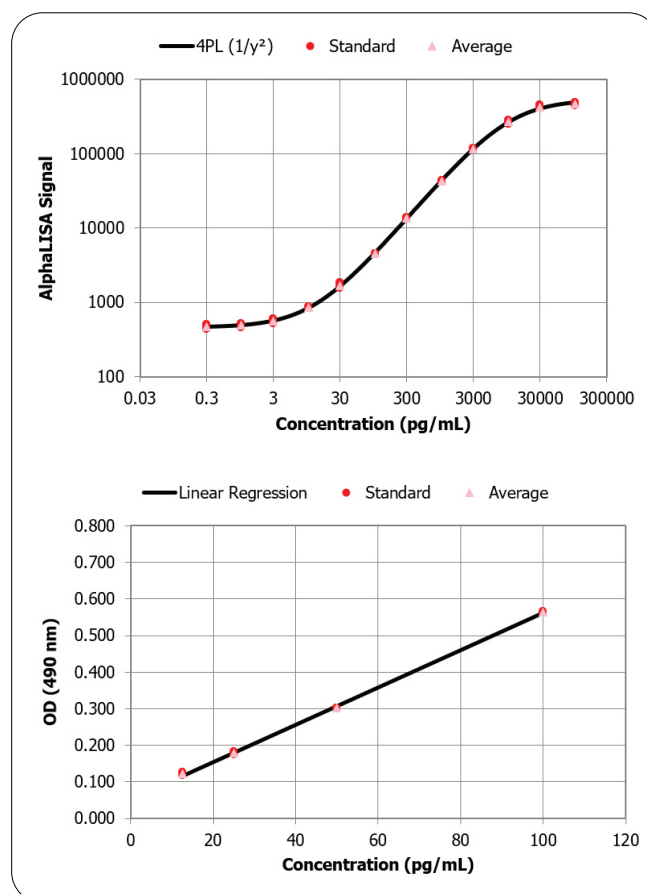


Figure 3. p24 Standard Curve from each assay technology. (Top) AlphaLISA standard curve provides ~ 4 logs of dynamic range (Bottom) ELISA standard curve provides ~2 logs of dynamic range.

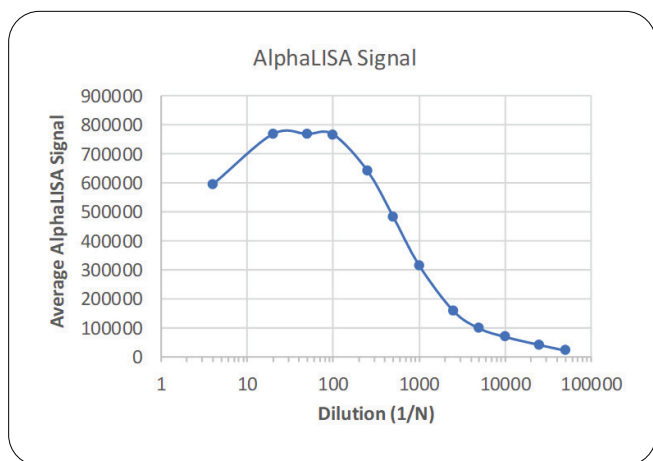


Figure 4. Average p24 AlphaLISA counts at each dilution point. Dilution point #1 equals 4-fold whereas dilution point #12 equals 50,000-fold from the stock GFP Lentivirus. The AlphaLISA signal of dilutions #2 through #4 are above the top values of the standard curve that was run in parallel and should not be used. Dilution #1 (4-fold) is a hooked data point which also should not be factored when interpolating the unknown viral titer.

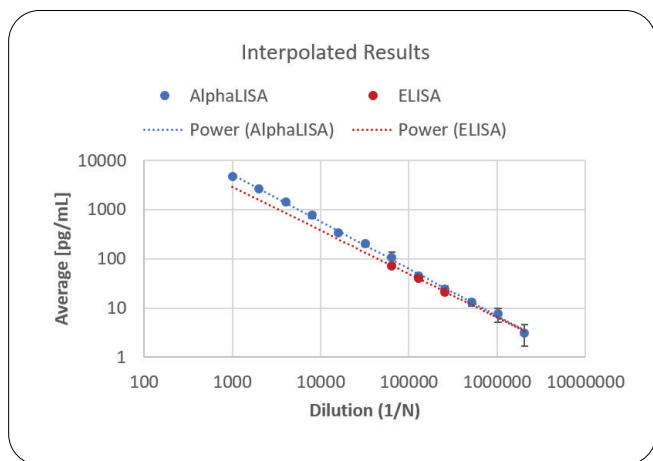


Figure 5. p24 AlphaLISA and ELISA assay results. Interpolated pg/mL of p24 from unknown samples within the linear range of each assay. R<sup>2</sup> for AlphaLISA is 0.998 and for ELISA is 0.998.

## Conclusion

As stated above, determination and control of the viral titer is a critical step during the use of viral vectors for cell and gene therapy applications. When using lentiviruses, for example HIV based constructs, the capsid protein p24 is an important marker for measurement of the titer using immunoassays. In the current study, we demonstrated the use of two immunoassay platforms to determine the viral titer by quantitation of p24. The Alliance HIV-1 p24 ELISA kit (Research Use Only, Revvity, Inc.) is widely cited and is considered to be a standard for lentiviral titer.<sup>8,9,10</sup> The AlphaLISA HIV-1 p24 detection assay (Research Use Only, Revvity, Inc.) is an ELISA alternative technology and uses a platform originally developed for drug discovery screeners. The two immunoassay platforms were used to measure p24 concentration from a mock lentiviral supernatant (concentrated lentivirus control stock diluted into cell culture media). Based on the laboratory infrastructure and workflow volumes, two immunoassay platform options are now available to measure p24 as a titer marker for lentivirus driven CAR T-cell generation.

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