TECHNICAL NOTE

Developing a high-throughput AlphaLISA assay for screening activity of biologics produced by engineered probiotic microbes.

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Introduction

The aim of this project was to develop a functional assay to characterize the activity of a protein biologic delivered by an engineered microbe. This novel approach administering biologics was developed to meet key challenges in delivering traditional drug formulations to the mucosa. Due to limitations driven by circulatory half-life and drug target bioavailability, injected biologics often require injection of high doses which can result in patient discomfort, unwanted side effects, a limited therapeutic window, and higher costs. To sidestep these pain points, Tenza engineers probiotic microbes to synthesize and deliver protein therapeutics directly to the target tissue. The functional activity of the secreted protein biologic is assessed by its binding to a target protein relevant to its therapeutic indication. The pre-existing assay format for testing functional activity was a standard ELISA, which had limited dynamic range and throughput, required large sample volumes, and multiple tedious wash steps. As the main interest was to screen many microbial strain variants to assess the level of functional protein being produced, it was necessary to look for a new assay format. The assay needed to assess both the protein production titer and its function by capturing the same binding interaction observed in the ELISA. In addition, the assay would have to be tolerant of a complex medium required to grow various microbes, which includes high amounts of biotin and background fluorescence. To that end, an AlphaLISA® Assay was developed.

AlphaLISA technology is a fast, highly sensitive, homogeneous, no-wash assay platform that can be performed in a microplate format. AlphaLISA assays require two bead types that each bind a target of interest: Donor beads and Acceptor beads. The Donor beads generate singlet oxygen upon illumination at 680 nm. The singlet oxygen can diffuse approximately 200 nm in solution. If an Acceptor bead is within that distance, energy is transferred to the Acceptor bead, resulting in light production with a maximum emission at 615 nm. If an Acceptor bead is not in proximity of a Donor bead, little to no signal is produced over background. The AlphaLISA Signal is then read on a plate reader equipped with AlphaLISA detection capabilities like the EnVision® and VICTOR® Nivo™ multimode plate readers.

Assay development

A commercially available purified binding partner protein labeled with a FLAG-tag was purchased for testing functionality of the secreted protein of interest. The secreted protein (payload) was engineered for expression with a 6x-His tag. Anti-FLAG and Anti-6xHis AlphaLISA toolbox beads (Revvity) were used to measure the level of binding of purified protein of interest to the FLAG-tagged binding partner in raw media. Both combinations of the detection reagents were tested: Anti-FLAG Donor (#AS103D) with Anti-6xHis Acceptor (#AL178C) and the opposite configuration of Anti-6xHis Donor (#AS116D) with Anti-FLAG Acceptor (#AL112C) to see which bead pair gave the best Alpha signal with the lowest background. An example of the AlphaLISA assay format is shown in Figure 1A. All reagents (purified payload protein, binding partner protein, and AlphaLISA beads) were diluted in 1X Immunoassay Buffer (#AL000C) and dispensed into 96-well low volume white microplates (#66PL96025) for the assay. Early experiments involved a three-step addition protocol, splitting the addition and incubation steps of the Acceptor and Donor beads into two parts. For the final optimized two-step protocol used in the library screen, 10 µL of sample (His-tagged protein of interest) was added to 10 µL of binding partner protein and incubated for 30 mins. Next, 20 µL of Donor and Acceptor beads pre-mixed (20 µg/mL final concentration each) were added and incubated at Room Temperature (RT) in the dark for 60 mins. AlphaLISA signal was measured on a VICTOR Nivo plate reader (Figure 1B) equipped with an Alpha module using pre-programmed AlphaLISA detection settings.

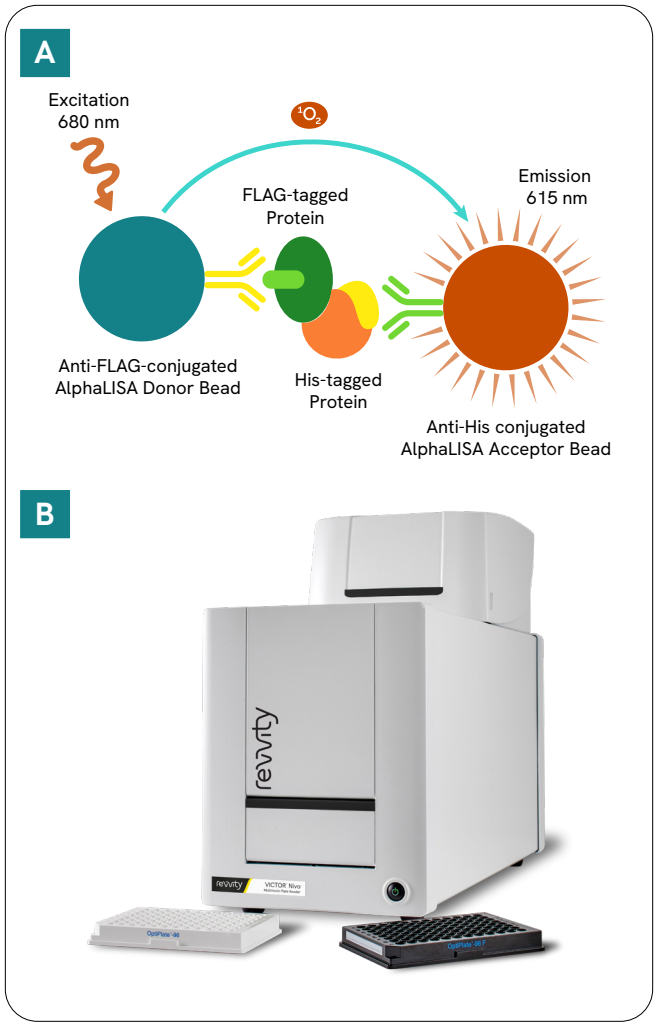

Figure 1: A) AlphaLISA schematic for a typical protein-protein interaction using tagged proteins and available toolbox donor and acceptor beads. B) The VICTOR Nivo plate reader, a small but powerful benchtop plate reader equipped with six technologies: Abs, FI, FP, Luminescence, TRF/HTRF, and Alpha.

To determine which bead pairing was appropriate, a concentration curve was generated using a set concentration of the binding partner (2.5 nM) and a varied concentration of the payload 6x-His tagged protein. The fixed starting concentration of 2.5 nM for the binding partner was chosen because the binding affinity of the payload and the binding partner is in the sub-nanomolar range. Figure 2 shows a representative result from the first set of experiments.

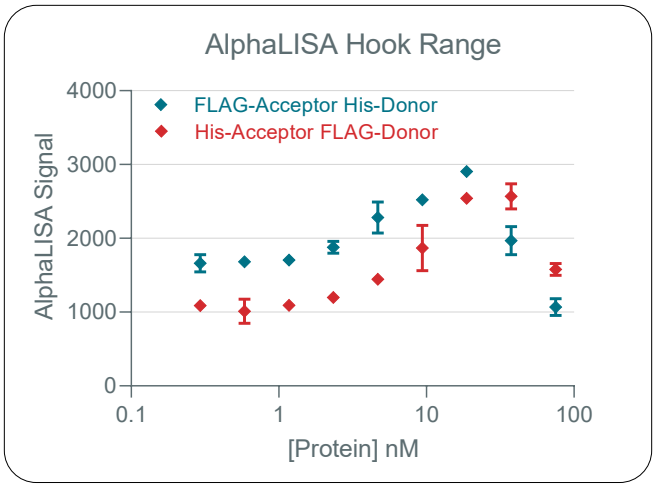

Figure 2: AlphaLISA assay detection range from minimum to maximum (the assay hook point) was determined for 2.5 nM of FLAG-tagged binding partner by titrating 6X-His tagged protein and testing both Donor and Acceptor bead pairs.

Based on these data, the anti-6x His Acceptor with anti-FLAG Donor bead setup was selected for further assay development because it had a lower background signal and a good signal-to-background ratio. The hook point of the payload protein (concentration producing maximal Alpha signal) was similar for both combinations of bead pairs. To further optimize the assay, a strain that yielded high levels of the payload protein according to the traditional ELISA assay was tested using several different concentrations of the FLAG-tagged binding partner in cell supernatant. In addition, the test sample was serially diluted 10-fold to gauge linearity of the assay with dilution.

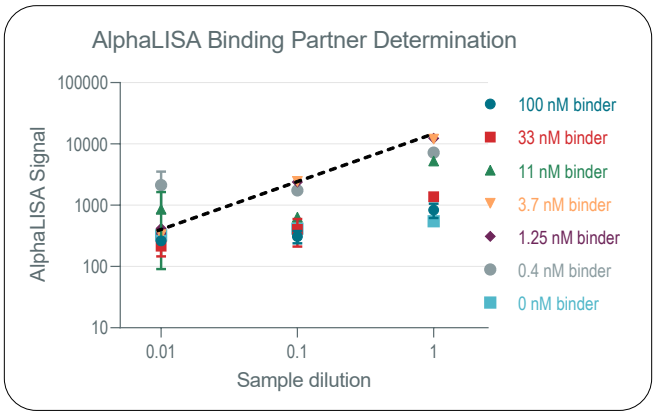

Figure 3: Determining the appropriate concentration of binding partner. Error bars show biological replicates.

As shown in Figure 3, the lower concentrations of the binding partner achieved a higher Alpha signal. Increasing the protein concentration of the binding partner resulted in a hook effect with reduced Alpha signal, indicating that the concentrations of the proteins exceeded the binding capacity of the beads. From these data the binding partner concentration of 2 nM was selected. Additionally, it was shown that a 10-fold dilution of the media before performing the assay increases the room at the top of the dynamic range and decreases background interference from the complex cell media without compromising the assay results (data not shown).

Assay validation and utilization

Upon optimization of the AlphaLISA assay, a library of 112 strains was generated and compared to an existing reference strain with a known quantity of payload produced. The AlphaLISA results from the library matched general expectations (Figure 4), with most strains resulting in relatively similar activity to the reference strain with a cutoff of +/- 20% activity. Overall, 50 strains produced a yield <80% of the reference while 28 strains produced a yield >120% of the reference. Some members of the strain library showed 3- to 4-fold higher activity over the reference strain which was correlated in further characterization via orthogonal ELISA assays.

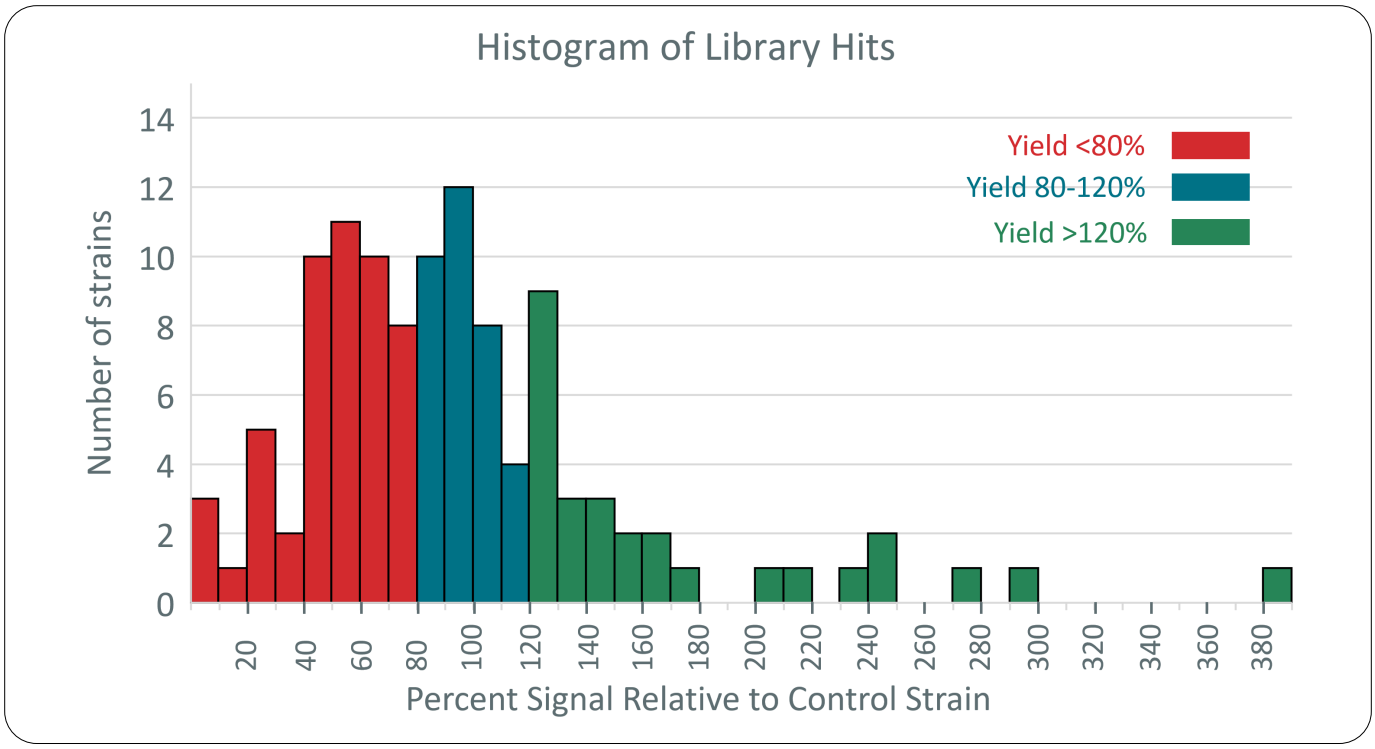

Figure 4: Library screen histogram of results. The reference strain used previously for binding partner concentration determination was used to normalize the graph (result set to 100). Results are colored based on percent yield relative to the reference strain, < 80%, 80-120%, or > 120%.

Conclusion

AlphaLISA no-wash immunoassays are a powerful method for easy, high-throughput detection of targets present in complex matrices. With a few rounds of assay optimization using the available AlphaLISA toolbox reagents, an assay detecting functional activity of the payload secreted by engineered microbial strains was rapidly developed. The high degree of sensitivity of the AlphaLISA format allowed for dilution of the samples during testing and helped eliminate any interference with the complex cell media. Additionally, the ease of use and higher throughput of the AlphaLISA assay relative to a traditional ELISA facilitated an increase in the number of strains tested in the library, enabling the identification of several new strains (or "hits") which produced the payload (therapeutic protein) above the level of one of the high-quality performing reference strains.

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