Application Note

Comparison of ELISA and AlphaScreen™ Assay Technologies for Measurement of Protein Expression Levels

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Introduction

The epitope tag, FLAG™, is a 1013 Dalton, eight amino acid peptide that is commonly used to tag proteins. It is useful to have a generic quantitative assay method to measure levels of expression and purification of FLAG-tagged proteins. A competitive ELISA was developed to quantify FLAG-tagged protein levels using a coated-well assay, but it showed poor sample linearity (CV's > 50%). An AlphaScreen™ homogeneous assay was chosen as an alternative for comparison for a number of reasons. In theory, a homogeneous assay, which requires fewer steps in general, and requires no separation steps, should have superior sample linearity. In addition, the ability to develop assays easily and miniaturize to save on reagent costs are added potential benefits of this technology. The purpose of this study was to compare the AlphaScreen assay to the traditional ELISA method in terms of dynamic range and lowest detectable limit, precision, accuracy and linearity. The goal was to determine if the AlphaScreen method should replace the ELISA method for this routine assay.

Materials and Methods

ELISA Assay Method

Enzyme linked immunosorbant assay (ELISA) is a traditional method used to quantify proteins[1]. Anti-FLAG M2 monoclonal antibody was left overnight at 2-3°C and washed out. Unknown samples of FLAG-tagged protein were incubated for one hour and then biotinylated-FLAG-DR4-Fc was sequentially incubated for one hour without wash, so that the two could compete for the coated antibody. After washing, horseradish peroxidase (HRP) conjugated streptavidin was incubated for one hour as shown in Figure 1. Color was then developed and stopped. The plate was read on a standard fluorescence reader at 450 nm excitation and 620 nm emission. The resulting decrease in absorbance is proportional to the amount of FLAG-protein in the sample.

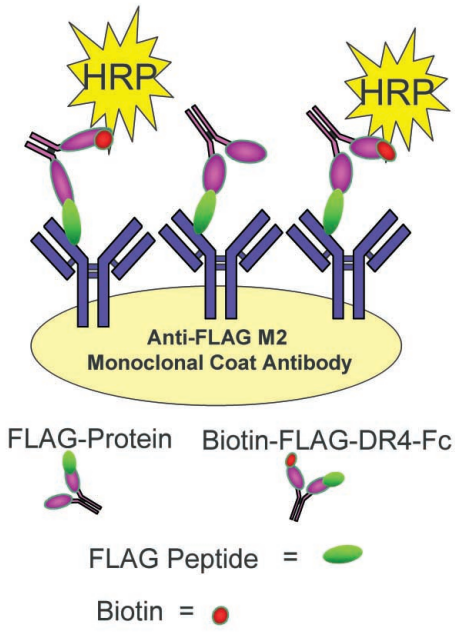

Figure 1: *ELISA Assay Method*

The optimized assay parameters gave the standard dynamic range, with the following components: anti-FLAG M2 monoclonal antibody (1 µg/mL), biotin-FLAG-DR4-Fc (68 ng/mL), FLAG-DR4-Fc (standard), and horseradish peroxidase conjugated streptavidin (200 ng/mL). The standard dynamic range is 156 ng/mL - 5000 ng/mL with a signal to noise ratio of ten. All assays were run in PBS. Once the reagent concentrations were optimized, five basic assay performance parameters were evaluated and compared to that of the AlphaScreen competition assay: assay dynamic range, lowest detectable limit (LDL), precision, accuracy, and linearity.

AlphaScreen Assay Method

AlphaScreen is a bead-based non-radioactive Amplified Luminescent Proximity Homogeneous Assay. When a biological interaction brings the beads together, a cascade of chemical interactions act to produce a greatly amplified signal. On laser excitation, a photosensitizer in the Donor bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a thioxene derivative in the Acceptor bead, generating chemiluminescence at 370 nm that further activates fluorophores contained in the same bead. The fluorophores subsequently emit light at 520-620 nm.

In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. As a result only a very low background signal is produced.

AlphaScreen provides a highly versatile, sensitive, time-resolved, homogeneous and miniaturizable means to efficiently perform assay development and high throughput screening (HTS) resulting in higher throughput at lower costs.

To maximize the AlphaScreen signal detection, the AlphaQuest®-HTS instrument and Fusion™α Multilabel Readers were developed with the capability to measure assays in multi-well plates. These instruments use a highly efficient laser diode emitting at 680 nm, fiber optics and specially optimized photomultiplier tubes. Use of the OptiPlate-384 NEW microplates is also recommended for best performance. Fusionα [3], AlphaQuest-HTS instruments and OptiPlate-384 NEW microplates are available from PerkinElmer Life Sciences company.

In this particular AlphaScreen assay (FLAG (M2) detection kit, cat. no. 6760612C, PerkinElmer Life Sciences), biotin-FLAG-DR4-Fc competes with unlabeled FLAG-Protein for binding to the anti-FLAG M2 coated acceptor bead. The bound biotin-FLAG-DR4-Fc was detected with streptavidin conjugated donor beads that were added simultaneously in a 384-well white OptiPlate (cat. no. 6005214, PerkinElmer Life Sciences). Plates were incubated for one hour at room temperature in the dark and then read using AlphaQuest-HTS at an excitation wavelength of 680 nm and emission wavelength of 520-620 nm.

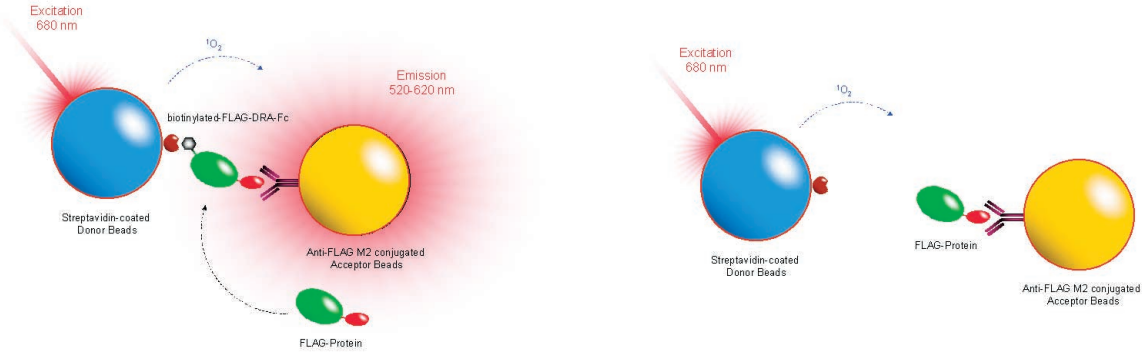

Figure 2: *AlphaScreen Assay Method*

The optimized assay parameters gave the standard dynamic range, with the following components: biotinylated FLAG-DR4-Fc (750 ng/mL) and FLAG-DR4-Fc (standard). The standard dynamic range is 156 ng/mL - 5000 ng/mL with a signal to noise ratio of 20-25 after a one hour incubation. The signal to noise ratio increases and plateaus after an incubation time of 1-3 hours (data not shown). All assays were run in PBS containing 0.5% BSA. The acceptor and donor beads were used each at a final concentration of 16 µg/mL. Once the reagent concentrations were optimized, five basic assay performance parameters were evaluated and compared to that of the ELISA: assay dynamic range, lowest detectable limit (LDL), precision, accuracy, and linearity.

Results

Dynamic Range and Lowest Detectable Limit are similar for ELISA and AlphaScreen Assays

Figure 3a and 3b show the standard curves for the AlphaScreen assay and the ELISA. The dynamic range and LDL were determined by evaluating the accuracy and precision of the standard (FLAG-DR4-Fc) from 19 ng/mL - 20,000 ng/mL for the AlphaScreen assay and 78 ng/mL - 5000 ng/mL for the ELISA assay. The dynamic range for both assay formats is limited to 156 ng/mL - 5000 µg/mL with a LDL of 156 ng/mL. At concentrations below and above the dynamic range, the accuracy and precision is poor.

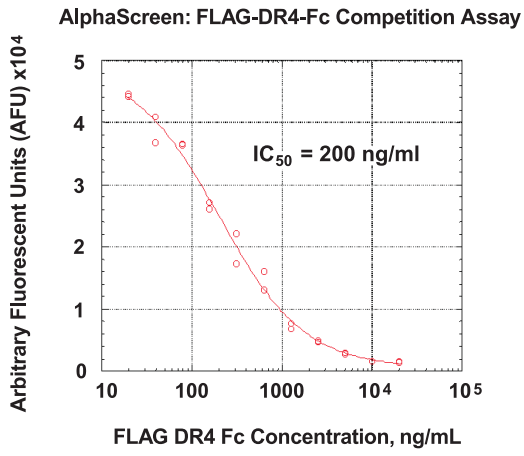

Figure 3a: *AlphaScreen assay standard curve*

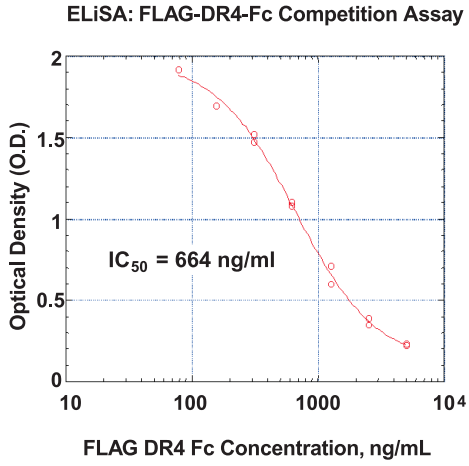

Figure 3b: *ELISA standard cuve*

Precision of AlphaScreen Assay

The inter and intra assay precision were estimated by ANOVA analysis of three levels of control assayed in duplicate in three independent assays. The inter and intra assay precision for the AlphaScreen assay are summarized in table 1. A precision analysis for the ELISA could not be conducted due to erratic control quantitation.

Control Level	Mean (µg/mL)	Inter Assay	Intra Assay
Low	216	10.5%	13.6%
Mid	948	2.4%	13.7%
High	5872	4.3%	11.9%
N=6			

Table 1: *AlphaScreen Precision*

AlphaScreen Performed with Better Accuracy than ELISA

To determine the accuracy of the AlphaScreen assay and the ELISA, four unknowns were combined 1:1 with three levels of controls and assayed. In addition, each unknown and control was assayed alone. The % recovery was determined by taking the observed value for the combined sample (unknown + control) divided by the average of the expected value for the unknown and control assayed alone ([control alone + sample alone]/2). For the accuracy of the AlphaScreen assay, the data in Table 2a shows an average recovery of 102.9% with a range of 81.2 to 114.4%. For the accuracy of the ELISA, the data in Table 2b shows an average recovery of 121.9% with a range of 85.1 to 163.1%. Based on these results, the AlphaScreen assay has better accuracy than the ELISA. It should also be noted that the values for control alone quantitation in the ELISA were significantly above expected values.

Sample Alone (ng/mL)	Control Alone (ng/mL)	Expected ([control alone + sample alone]/2) (ng/mL)	Observed (control + sample combined 1:1) (ng/mL)	% Recovery ([Observed/ Expected]* 100 (ng/mL)
199.5 (Sample 1)	3201 722 152	1700.3 460.8 175.8	1861 510 201	109.5 110.7 114.4
124 (Sample 2)	3201 722 152	1662.5 423.0 138.0	1784 404 112	107.3 95.5 81.2
185 (Sample 3)	3201 722 152	1693.0 453.5 168.5	1880 507 170	111.0 111.8 100.9
564 (Sample 4)	3201 722 152	1882.5 643.0 358.0	2090 639 296	111.0 99.4 82.7
			Average	102.9
			Range	81.2 - 114.4

Table 2a: *Accuracy of the AlphaScreen Assay*

Sample Alone (ng/mL)	Control Alone (ng/mL)	Expected ([control alone + sample alone]/2) (ng/mL)	Observed (control + sample combined 1:1) (ng/mL)	% Recovery ([Observed/ Expected]* 100 (ng/mL)
153.025 (Sample 1)	8342.2 2180.1 695.8	4247.6 1166.6 424.4	3839.1 1454.2 525.3	90.4 124.7 123.8
923.791 (Sample 2)	8342.2 2180.1 695.8	4633.0 1551.9 809.8	4115.3 1943.1 1179.8	88.8 125.2 145.7
1064.522 (Sample 3)	8342.2 2180.1 695.8	4703.4 1622.3 880.2	4002.2 1922.5 1242.8	85.1 118.5 141.2
1978.679 (Sample 4)	8342.2 2180.1 695.8	5160.5 2079.4 1337.3	5539.8 3100.1 2180.7	107.4 149.1 163.1
			Average	121.9
			Range	85.1 - 163.1

Table 2b: *Accuracy of the ELISA*

Linearity is Better with AlphaScreen

The corrected concentrations (µg/mL) from the same sample in the ELISA and AlphaScreen are compared with the expected concentration in Figure 4. To determine the linearity of the two assays, a sample was serially diluted and the concentrations were determined in the assays. Values are plotted as the dilution factor versus the corrected concentration of serially diluted samples. The values of the corrected concentrations from both assays are compared to that of the expected. When these corrected concentrations were compared to that of the expected value, the AlphaScreen shows significantly less deviation from the expected and therefore has better linearity.

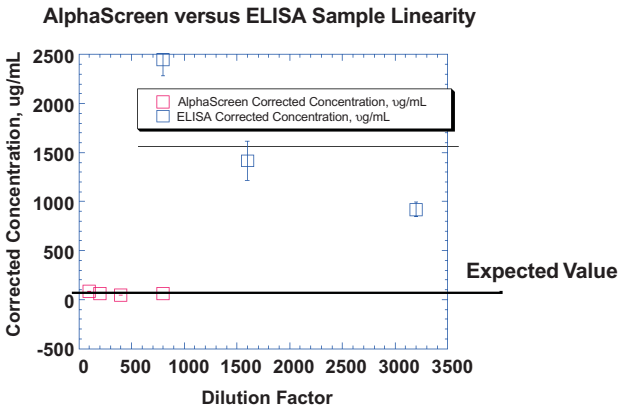

Figure 4: *Corrected concentrations from the same samples in ELISA and AlphaScreen assays are compared with expected concentrations.*

AlphaScreen Technology Improves Productivity

A resource comparison between the ELISA and the AlphaScreen assay shows an important difference between the technologies. Table 3 shows that AlphaScreen technology increased assay efficiency by reducing the assay time by more than a factor of four and by decreasing reagent volume by a factor of ten as compared to the ELISA. Furthermore, there were no wash and coat steps required for the AlphaScreen assay.

	ELISA	AlphaScreen
Time	4 hrs	1 hr
# washes	12	0
Volume per reagent	100 µL	10 µL

Table 3: *Productivity Comparison*

Conclusions and Discussion

AlphaScreen Assay was Chosen to Compare with ELISA Method

The ELISA method was developed for routine analysis of FLAG-tagged proteins. The poor linearity (CV>50%) led us to investigate other assay technologies. AlphaScreen assays are homogeneous and uniquely suited for assay development for eventual high throughput screening due to the amplified signal that is produced. Samples can be miniaturized without loss of sensitivity and without additional optimization. Two instruments are available for detection of AlphaScreen chemistry and both are designed to take advantage of the miniaturization of AlphaScreen assays down to 1536-well format. Fusion α Multilabel Reader for assay development provides versatility in detection methods so that it can be used for other assay technologies in addition to the AlphaScreen chemistry. The AlphaQuest-HTS is a four-detector system for screening large compound libraries with assays developed with AlphaScreen chemistry.

AlphaScreen Assay Outperformed ELISA

A FLAG-Protein competitive binding assay was easily developed using AlphaScreen technology. Due to the speed of the assay (one hour for competition reaction and bead binding), assay optimization was very efficient and it was possible to perform as many as three experiments per day. Evaluation of assay performance showed that the AlphaScreen assay has superior precision and accuracy compared to the ELISA. In addition, sample linearity improved significantly. The improvement in linearity is most likely due to the homogeneous assay format. Therefore, the AlphaScreen FLAG competitive assay will replace the ELISA for routine analysis of FLAG-tagged proteins.

References

1. Wild, D. (1994) The Immunoassay Handbook. Stockton Press New York, NY.

2. Bossé, R. *et al.* (2001) Principles of AlphaScreen. Application Note ASC-001, PerkinElmer Life Science, Inc., Montreal, Canada.

3. Gaidar, l., Prasauckas, K., Carter-Allen, K., and Upham, L. (2001) Comparison of Performance between AlphaQuest™-HTS and the Fusion™ α Universal Microplate Analyzer for Detection of cAMP. Application Note FAN-004, PerkinElmer Life Science, Inc., Downers Grove, Ill.

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