AlphaScreen Insulin Detection Assay using AlphaLISA Acceptor beads

APPLICATION NOTE

ALPHASCREEN

Authors
Martin Boissonneault
Geneviève Pinard
Nathalie Bouchard
Soo-Hang Wong
Chantal Illy

PerkinElmer Life and Analytical Sciences
Montreal, Quebec,
H3J 1R4, Canada

Introduction

Insulin production and secretion by the beta cells in the islets of Langerhans in the pancreas is controlled by blood glucose levels. Insulin promotes the uptake of glucose by target tissues and, thus, prevents hyperglycemia by shuttling glucose into tissues for storage. Beta cell dysfunction and the concomitant decrease in insulin production can result in diabetes mellitus. Measurement and quantification of insulin in serum or plasma samples after treatment with compounds and under various pathophysiological conditions are essential steps in diabetes research.

Different methods exist on the market for insulin quantification, including chromatographic methods such as mass spectrometry and immunoassays. The most commonly used approach is ELISA, a two-site immunometric assay using a pair of antibodies against insulin. This technique offers great sensitivity but suffers from its non-homogenous nature, as the protocol involves several washing steps. Consequently, ELISA assays have a limited throughput. Furthermore, ELISA assays have a limited dynamic range and only perform well with high affinity antibodies.

The AlphaScreen® technology is well suited for biomarker detection, with distinct advantages over conventional ELISA techniques: high sensitivity, homogeneous nature (all-in-one well assays), high throughput capability and proven reliability. An AlphaScreen biomarker assay can be designed either as a sandwich assay with two antibodies or as a competition assay with one antibody and the biotinylated form of the analyte of interest.

This application note describes a human insulin AlphaScreen sandwich assay taking advantage of the new AlphaLISA™ Acceptor beads for measuring insulin in biological samples (serum and plasma). The assay was shown to present a high sensitivity, robustness and reliability and results correlated well with those generated in an ELISA insulin assay.

Principles of AlphaScreen technology

AlphaScreen (**A**mplified **L**uminescent **P**roximity **H**omogeneous **A**ssay) is a bead-based non-radioactive technology. When a biological interaction brings the beads into close proximity, a cascade of chemical reactions is induced that results in a greatly amplified signal. Upon laser excitation, a photosensitizer present in the Donor beads converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse and react with a thioxene

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precisely.

derivative in the Acceptor beads, generating chemiluminescence at 370 nm that further activates a fluorophore contained in the same beads. The fluorophore contained in the AlphaLISA Acceptor beads subsequently emits light at 615 nm. In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor beads go undetected without the close proximity of the Acceptor beads.

AlphaScreen provides a highly versatile, sensitive, time-resolved, homogeneous, and miniaturizable means for efficiently performing assay development and HTS resulting in higher throughput at lower costs. For further details on AlphaScreen technology, refer to *A Practical Guide to Working with AlphaScreen* (reference no. S4077).

In the assay described here, a pair of human monoclonal antibodies recognizing different epitopes of human insulin was used in a sandwich assay to measure insulin in biological samples. The first antibody (Ab1) was biotinylated and captured by the streptavidin coated Donor beads, while the second (Ab2) was directly conjugated to the AlphaLISA Acceptor beads. In the presence of insulin in samples, the two beads come into close proximity due to the recognition by the two antibodies, leading to the generation of a strong specific AlphaScreen signal. A calibration curve of insulin is then used to determine the level of insulin in unknown samples.

AlphaScreen Insulin detection assay

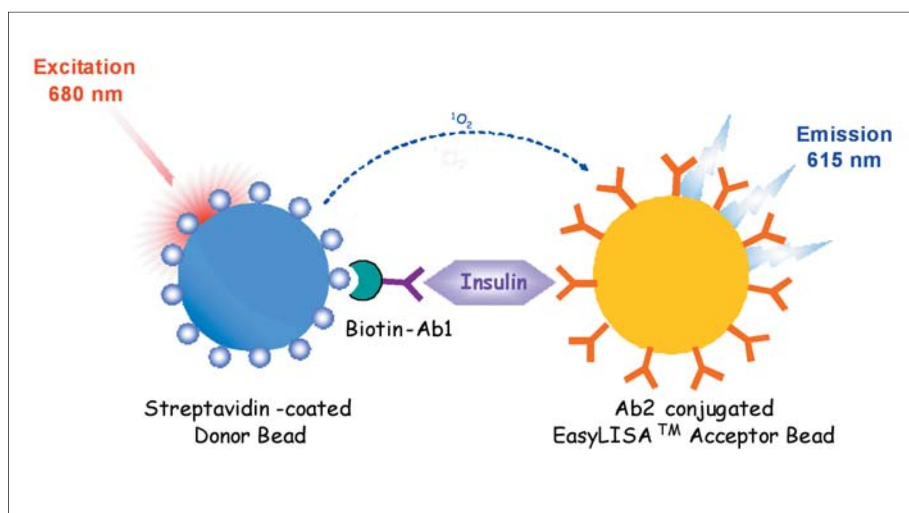

Figure 1. Principle of the AlphaScreen Insulin detection assay. A sandwich approach was used where one antibody (Ab2) is conjugated to the AlphaLISA Acceptor beads and the second antibody (Ab1) is biotinylated and captured by the streptavidin coated Donor beads.

Materials and methods

Materials

The AlphaLISA Acceptor beads (Cat. No. 6772001, 6772002, 6772003, 6772004) and the Donor-streptavidin beads (Cat. No. 6760002) are available from PerkinElmer. Anti-insulin Antibodies 3A6 and 8E2 were purchased from Fitzgerald (Cat. No. RDI-TRK2I1-3A6 and RDI-TRK2I1-8E2). N-hydroxysuccinimido-PEG-biotin was bought from Nektar Pharmaceuticals (Cat. No. 0H4M0F02). Human insulin standard (Cat. No. 8014) and human insulin ELISA kit (Cat. No. EZHI-14K) were purchased from Linco Research. The various serum and plasma samples used in this study were acquired from Fitzgerald, Innovative Research, Biomeda and Cambrex. Streptavidin-Sepharose beads were purchased from GE (Cat. No. 17-5113-01).

96-Optiplate™ microplates were from PerkinElmer (Cat. No. 6005290 & 6005299). The reader used in this study was the EnVision™ reader with the AlphaScreen standard option from PerkinElmer (Cat. No. 2101-0010).

Protocols

All protocols are thoroughly described in the Biomarker Detection Assay Development Guide available from PerkinElmer.

Coupling of 8E2 monoclonal antibody to AlphaLISA acceptor beads

The 8E2 insulin antibody was directly coupled to AlphaLISA Acceptor beads via the aldehyde reactive groups on the beads, as follows:

In an eppendorf tube, the following reagents were added and the tube was incubated for 48h at 37 °C.

- 2 mg of AlphaLISA acceptor beads
- 0.2 mg of the antibody (1:10 ratio)
- 0.5 mg of sodium cyanoborohydride (prepared fresh in water)
- Complete to 400 µL with 0.1M MES pH 6.0 + 0.1% Tween 20

Following the incubation, 20 µL of a 0.3 M solution of carboxymethoxylamine hemihydrochloride was added for 1h to block the unreacted aldehyde sites. The beads were then washed twice with 0.1M Tris, pH 8.0 by centrifugation in a microfuge

at 14,000 RPM, and finally resuspended at 5 mg/mL in 0.1 M Tris pH 8.0 supplemented with 0.05% Proclin-300 (preservative).

Biotinylation of 3A6 antibody

The 3A6 antibody (1.5 nmoles) was reacted with N-hydroxysuccinimido-PEG-biotin (15 nmoles) in a PBS buffer pH 7.2 for 15-18 hours at 37 °C. The biotinylated antibody solution was then used in the insulin detection assay.

AlphaScreen insulin detection assay

The assay was performed in 96-well Optiplates in a total volume of 50 µL (the assay can also be performed in 384-well Optiplates using the exact same protocol). The optimized assay buffer was composed of 25 mM Hepes pH 7.4, 1 mg/mL Dextran T-500, 0.5% Triton X-100, and 0.1% casein.

The order of addition was as follows:

- 5 µL of serum or plasma sample
- 20 µL of a mixture of biotin-3A6 (1 nM final) and AlphaLISA Acceptor-8E2 beads (10 µg/mL final)

30 min incubation at 23 °C

- 25 µL of Streptavidin-Donor beads (40 µg/mL final)

60 min incubation at 23 °C

Read at RT with EnVision reader using normal AlphaScreen settings

Preparation of the matrix solution

Insulin-depleted serum and plasma samples were prepared by incubating the samples (10 mL) overnight at 4 °C with the biotinylated-anti-insulin-3A6 antibody (200 nM) and streptavidin-Sepharose beads (5 mL). The next day, the tubes were centrifuged and the supernatants containing the depleted samples were collected. Alternatively, charcoal-treated serum or plasma samples can be used as matrix solutions (Myrick J.E. *et al.*, 1989).

Results

AlphaScreen Human Insulin Assay Development

A homogeneous human insulin sandwich assay was developed using the AlphaScreen technology. Different pairs of anti-insulin antibodies from commercial sources were tested and the optimal pair (3A6 and 8E2 antibodies) giving the highest AlphaScreen signals was selected for further assay optimization. Optimization of the concentrations of detection reagents (8E2-conjugated AlphaScreen AlphaLISA Acceptor beads, biotinylated 3A6 antibody and streptavidin coated AlphaScreen Donor beads) and order of reagent additions were performed in order to determine the highest assay window, widest dynamic range as well as the lowest detection limit. Furthermore, the composition of the reaction buffer was also optimized to minimize non-specific interactions. To overcome non-specific interference by the serum matrix, the insulin standards were prepared in the same matrix solution as the samples. A commercial human serum was depleted from insulin by affinity purification using the biotinylated 3A6 antibody captured by streptavidin-Sepharose beads and was used as the matrix solution for all experiments.

Insulin calibration curve

Figure 2 shows a representative AlphaScreen human insulin calibration curve performed with 5 µL of

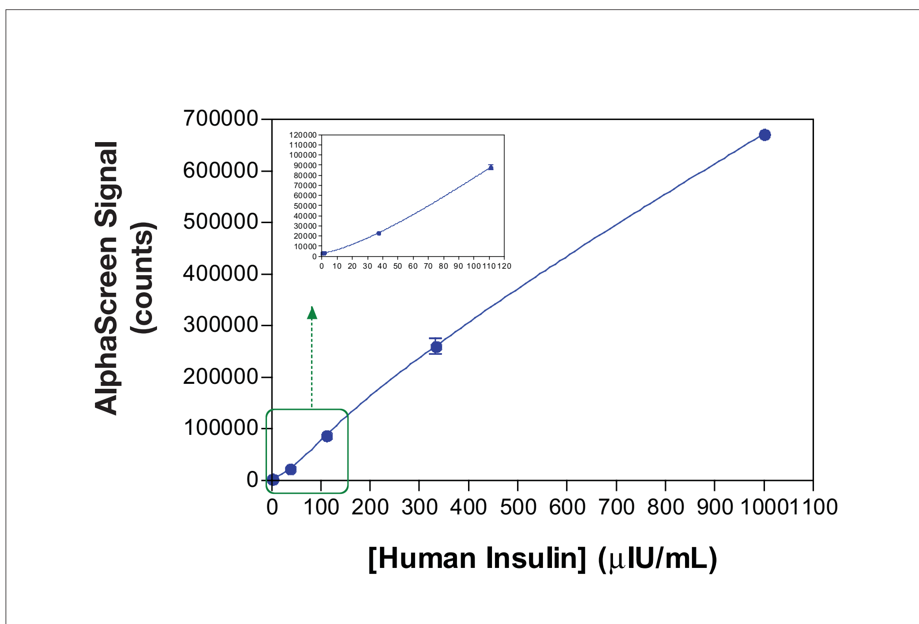

Figure 2. AlphaScreen Human Insulin calibration curve. Final concentrations of reagents in well were: 1 nM biotinylated 3A6 antibody, 10 µg/mL 8E2 antibody-conjugated AlphaLISA Acceptor beads, and 40 µg/mL streptavidin-Donor beads. Assay buffer was 25 mM Hepes pH 7.4, 1 mg/mL Dextran T-500, 0.5% Triton X-100, and 0.1% casein.

insulin standards ranging from 0.5 to 1000 µIU/mL under optimized assay conditions. The AlphaScreen signal obtained was plotted against the insulin concentrations tested.

The detection limit of the assay corresponding to the lowest level of insulin that can be detected was determined from the calibration curve using the calculation of 3xSD over the background (response at zero calibrator). An average value of 2 µIU/mL (85 pg/mL) was calculated as the lowest limit of detection. The assay has a wide dynamic range over 2 orders so that unknown samples containing as low as 2 µIU/mL and as high as 1000 µIU/mL insulin in the sample assay volume could be analyzed in the serum without the need to dilute the sample.

Assay precision (within and between assay variations)

The assay variation was studied on a serum sample spiked with varying concentrations of analyte (3, 18, 27, 48 and 85 µIU/mL). The intra- and inter-assay variability (intra-lot variability) was calculated from the levels of insulin calculated in these samples by interpolation from an insulin calibration curve.

To determine the intra-assay variation, the five spiked samples were assayed in six replicates (Table 1).

Table 1. Intra-assay precision

	Intra-assay variation (insulin content in µIU/ml)							
	Replicate							
[spiked insulin]	1	2	3	4	5	6	Average	**%CV**
85	74	76	84	85	86	81	81	**6**
48	41	44	46	52	51	48	47	**9**
27	23	23	24	29	29	26	26	**11**
18	15	17	17	21	20	20	18	**12**
3	3	5	7	5	4	5	5	**23**

Table 2. Intra-assay precision

	Intra-assay variation (insulin content in µIU/ml)							
	Experiment							
[spiked insulin]	n=1	n=2	n=3	n=4	n=5	n=6	Average	**%CV**
85	90	84	77	98	96	82	88	**9**
48	49	51	43	54	50	58	51	**10**
27	26	28	24	26	26	27	26	**6**
18	17	20	17	19	17	18	18	**7**
3	4	5	6	4	4	4	4	**17**

For most spiked samples, the coefficient of variation was less than 12%, indicating very good intra-assay reproducibility. Only very low insulin amounts (3 µIU/mL) produced CV higher than 20%. The precision of the AlphaScreen assay was shown to advantageously compare to that of the ELISA assay (4.6 to 11.4%).

The inter-assay precision was determined from six independent experiments performed on different days (Table 2). CV values below or equal to 10% could be obtained for the majority of the samples, except for the lowest amount of analyte, corresponding to the detection limit of the assay, showing a variation of 17%. These values are comparable to those obtained in ELISA (6 to 10.3%).

Overall, these data indicate that the observed intra-assay and inter-assay variations of the AlphaScreen insulin assay allows precise determination of physiological concentrations of the analyte (5-50 µIU/mL).

Matrix effect

Five different insulin-depleted human serum samples were spiked with 4 insulin concentrations of insulin. Serum 5 was used for the calibration curve in AlphaScreen. As shown in Figure 3 (right), the % recovery is excellent in serum 5 (from 96 to 100%). Recovery of insulin was calculated by dividing the concentrations interpolated from the calibration curve by the expected spiked concentrations. However, when other serum samples are correlated against the calibration curve performed in serum 5, the % recovery is lower (on average, recovery is 79%). This is especially true with lower

Table 3. Linearity of the AlphaScreen insulin assay

	Linearity (insulin content in µIU/ml)				
	Experiment				
[Dilution]	n=1	n=2	n=3	Average	% of expected
1	114	124	112	117	**117**
1/2	48	53	52	51	**102**
1/3	40	34	36	37	**112**
1/4	30	23	29	27	**109**
0	1	1	1	1	**n/a**

concentrations of insulin (recovery % from 56 to 72%). These results are comparable to the ELISA data obtained from a calibration curve generated with the matrix solution provided in the ELISA kit (recovery % from 69 to 135%). These results point out the importance of using the most representative calibration curve for the correlation of unknown samples.

Linearity of the assay (effect of serum dilution)

A human insulin-depleted serum was spiked with insulin and tested under different dilutions (1/1, 1/2, 1/3, 1/4). These dilutions, prepared in the matrix solution, corresponded to 5, 2.5, 1.66 and 1.25 µL sample volumes assayed.

Table 3 summarizes the results obtained from 3 independent experiments. The results are expressed as percentages of expected insulin concentrations, calculated using the following formula: observed / expected x 100%.

Correlation with the ELISA assay

A series of 25 serum or plasma samples spiked with insulin was assayed in AlphaScreen and ELISA in a side-by-side comparison. The calculated insulin concentrations found in both assays (average of two independent experiments) were plotted in a correlation graph. Correlation of the two kits was derived by linear regression analysis of paired results from each sample. As observed in Figure 4, a very good correlation ($r^2 = 0.94$) was observed between the two technologies, demonstrating the accuracy of the AlphaScreen test relatively to the ELISA test.

Conclusion

The present application note describes a highly sensitive and reliable AlphaScreen insulin detection assay. This homogeneous sandwich immunoassay uses the newly developed AlphaLISA Acceptor beads, streptavidin coated Donor beads and two specific anti-insulin antibodies. The AlphaScreen assay characteristics presented in this document compare advantageously to the performance of a human insulin ELISA assay. In addition to its excellent performance, the AlphaScreen assay does not need any separation or washing steps, thus improving the ease, rapidity, and simplicity of the assay. This

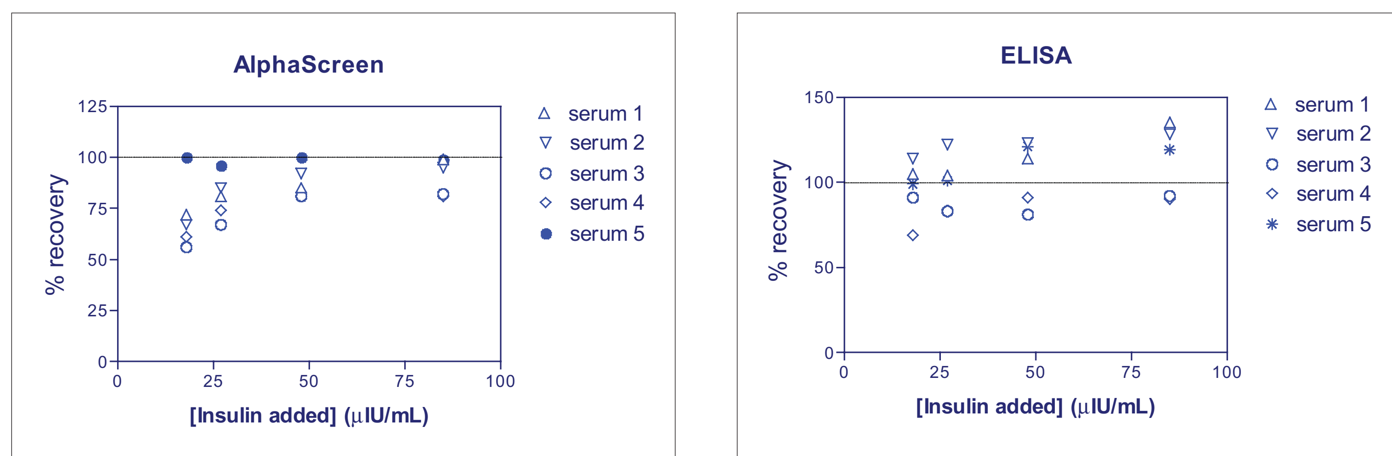

Figure 3. Matrix effect and recovery of spiked insulin in the AlphaScreen and ELISA insulin detection assay.

important feature allows an increased throughput of samples to analyze. Moreover, the assay is entirely scalable (96- and 384-formats) and easily automatable.

Using the new AlphaLISA Acceptor beads, any biomarker assay of choice for which antibodies are available will be easily developed. At the same time, if only one antibody specific to the analyte of interest is available, a competition binding approach involving a biotinylated form of the analyte could be advantageously used.

References

AlphaScreen Biomarker Assay Development Guide (AlphaLISA Acceptor beads)

A Practical Guide to Working with AlphaScreen (PerkinElmer guide).

Myrick, J.E. *et al.*, 1989, Clin. Chem., 35/1, pp 37-42: Charcoal-treated serum/plasma preparation

Ullman, E.F. *et al.*, 1996, Clin. Chem., 42/9, pp 1518-1526: Luminescent Oxygen Channeling Assay (LOCI™): sensitive, broadly applicable homogeneous immunoassay method.

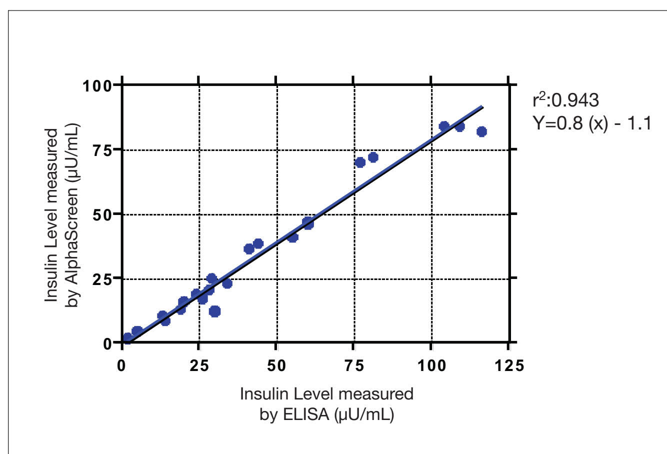

Figure 4. Correlation between AlphaScreen and ELISA

PerkinElmer Life and Analytical Sciences
710 Bridgeport Avenue
Shelton, CT 06484-4794 USA
Phone: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

For a complete listing of our global offices, visit www.perkinelmer.com/lasoffices

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