Application Note

Molecular Biology and Biochemical Research

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# AlphaLISA Quantitation of Human Insulin Using a Hybrid Agilent BioTek Synergy 4 Multimode Microplate Reader


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## Abstract

Diabetes mellitus is a disease that is reaching epidemic proportions in the United States. Insulin plays a critical role in the regulation of blood glucose levels. Its production and secretion by beta cells in the islets of Langerhans is tightly regulated under normal conditions. The quantitation of insulin levels in serum or plasma samples after treatment with potential drug compounds is an essential procedure in diabetes research. Here we describe the use of the Agilent BioTek Synergy 4 multimode microplate reader to quantitate insulin using an AlphaLISA assay.

## Introduction

The Amplified Luminescent Proximity Homogeneous Assay (AlphaLISA) uses active donor and acceptor beads that have been coated with a layer of hydrogel, which allows their conjugation with biological molecules (Figure 1). With excitation, a photosensitizer in the donor bead converts ambient oxygen to reactive singlet oxygen. A high concentration of the photosensitizer in the donor bead can result in the generation of as much as 60,000 singlet oxygen molecules per second and serves as a means for significant signal amplification. The singlet oxygen species reacts with thioxene compounds in the acceptor bead to generate a chemiluminescent signal that emits at 370 nm. The energy is immediately transferred to fluorophores contained in the same acceptor bead, which effectively shifts the emission wavelength to 520 to 620 nm. Because singlet oxygen is unstable, with an average lifetime of approximately 4 µsec, it can only diffuse a distance of 200 nm before it decays. The distance limitation insures that in the absence of a specific biological interaction between the two beads the singlet oxygen produced by the donor bead will go undetected. Acceptor beads that are not within this distance will not emit light.

The human insulin assay uses two different monoclonal antibodies against insulin to bring the donor and acceptor beads in close proximity. The AlphaLISA acceptor beads have been conjugated with 8E2 anti-human insulin antibodies, which can bind and capture any human insulin in the sample. The biotinylated 3A6 anti-human monoclonal antibody forms a bridge with the streptavidin coated donor bead by binding the insulin-acceptor bead complex and the donor bead (Figure 1).

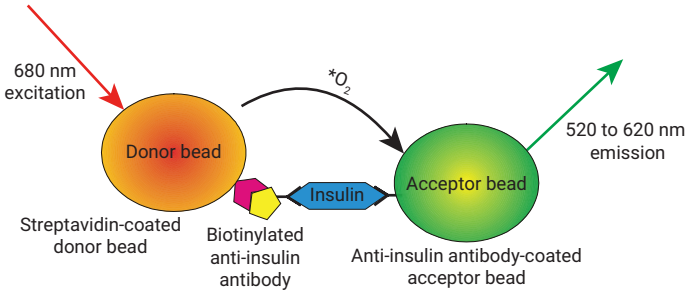


**Figure 1.** Schematic of human insulin AlphaLISA assay.

## Materials and methods

A human insulin detection kit, which contained AlphaLISA acceptor beads, AlphaLISA Donor-streptavidin beads, human insulin, and biotinylated anti-human insulin antibody, was obtained as a generous gift from Perkin Elmer (Boston, MA). Solid white 384-well microplates (part number 3705) were obtained from Corning (Corning, NY).

A series of dilutions ranging from 0 to 400 µIU/mL of human insulin were made using assay buffer as the diluent. Assay buffer consisted of 30 mM Tris pH 7.4, 0.5% Triton X-100, 0.1% BSA. A master mix that contained biotinylated anti-human insulin-3A6 antibody (2.5 nM) and Anti-insulin 8E2 antibody coated acceptor beads (25 µg/ml) was prepared using assay buffer as the diluent. Streptavidin coated donor beads were diluted 80 µg/mL using assay buffer. Reactions were prepared by adding 5 µL of insulin dilution to wells of a 384-well microplate in replicates of 8 followed by the addition of 20 µL of master mix to all wells.

This was allowed to incubate for 30 minutes at room temperature followed by the addition of 25 µL of diluted donor bead solution. The complete reaction mixture contained 1 nM biotinylated-3A6 antibody, 10 µg/mL acceptor beads and 40 µg/mL donor beads besides the human insulin dilution. The reaction was allowed to incubate in the dark for 60 minutes and the AlphaLISA signal determined using a Synergy 4 multimode microplate reader. The reader was controlled and the data collected and analyzed using Agilent BioTek Gen5 data analysis software.

## Results and discussion

Figure 2 demonstrates the relationship between insulin concentration and AlphaLISA signal. This relationship can be best described using a 4-parameter logistic fit of the data. This assay directly quantifies insulin concentration and as such the AlphaLISA signal increases with increasing insulin concentrations.

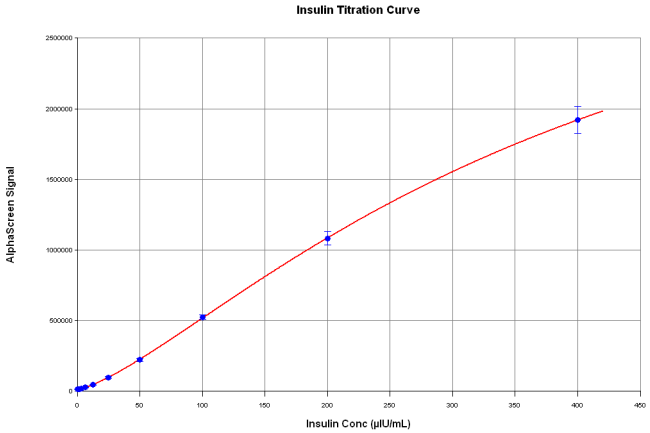


**Figure 2.** Human insulin concentration curve. AlphaLISA signal generated from a titration of Human insulin was plotted against insulin concentration and a logistic 4-parameter best fit applied to the data using the Agilent BioTek Gen5 data analysis software.

By using the "zoom" feature of the Gen5 data analysis software, one can examine the signal for low insulin concentrations. As seen in Figure 3, even at very low insulin concentrations the logistic fit of the data shows a very good relationship with the true data. The detection limit of the assay was determined from the calibration curve using the calculation of 3x the standard deviation above the zero standard. This value was then used to interpolate the calibration curve. An average value of 2.8 µIU/mL or 120 pg/mL was calculated as the lowest limit of detection.

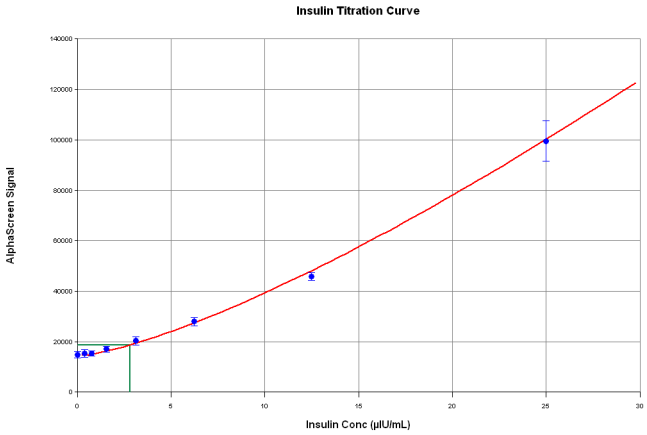


**Figure 3.** Titration of low insulin concentrations. Data presented in Figure 2 were expanded to demonstrate the calibration curve at low concentrations. The AlphaLISA signal and the concentration of the calculated detection limit are indicated by the green line.

This application note describes the quantitation of human insulin research using AlphaLISA assay technology. The AlphaLISA is a homogeneous assay, which unlike conventional ELISA, does not require any separation or washing steps. By not requiring any separation steps, the AlphaLISA assay can not only save considerable time and reagent expense, but also improve the ease and simplicity of the assay. Because they do not require wash steps, homogeneous assays can also be moved to higher matrix microplates (e.g.1536 well) much easier.

The AlphaLISA acceptor beads in conjunction with streptavidin coated donor beads provide an easy means by which any biomarker assay based on antibody detection can be developed. The basic requirement would be to have two antibodies specific towards the analyte. One antibody is covalently attached to the AlphaLISA acceptor bead and the other is biotinylated, both of which are straightforward and efficient procedures.

These data presented demonstrate the ability of the Agilent BioTek Synergy 4 hybrid reader to perform AlphaLISA assays. Typically, devices that have been designed to run AlphaLISA assays are very expensive dedicated instruments that utilize a laser for excitatory light. Monochromatic lasers provide continuous light output, as well as wavelength specificity, both of which are necessary for the AlphaLISA assay. Xenon flash lamps are typically used in multimode microplate readers because they can provide light output over a broad wavelength range, but also cease illuminating very rapidly as required for time resolved measurements. Unfortunately, the flash lamp does not provide the continuous illumination necessary for AlphaLISA assays. The time between flashes allows the excitatory reaction to diminish. Unlike most multimode microplate readers, the Agilent BioTek Synergy 4 has both a constant tungsten-halogen light source and a xenon-flash lamp. This multi-optic design has the capability to provide a continuous excitation light source, wavelength specificity, and high sensitivity necessary for these assays to be run.

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