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Application Note

Determination of Insulin Levels in Human Serum

Using the ELx405™ Microplate Washer and the Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader to Perform an Insulin ELISA

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Type 2 diabetes mellitus is a disease that is reaching epidemic proportions in the United States. Insulin plays a critical role 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 samples after treatment with potential drug compounds is an essential procedure in diabetes research. Here we describe the use of the ELx405™ Microplate Washer and a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader to automate the washing and reading steps of a human insulin ELISA.

Introduction

Insulin is a polypeptide hormone with a molecular weight of approximately 5800 that plays a critical role the regulation of blood glucose levels. Its production and secretion by beta cells in the islets of Langerhans is tightly regulated under normal conditions. Type I diabetes is caused by the destruction of the beta cells with a subsequent lack of insulin. Treatment involves the administration of exogenous insulin. In type 2 diabetes, which is usually associated with obesity, insulin resistance plays a critical role. After a period of time with elevated insulin levels, beta-cell failure may occur; requiring insulin administration. It is this form of diabetes that is reaching epidemic proportions in the US and the rest of the world. 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 ELx405™ Microplate Washer (Figure 1) in combination with the Synergy Mx Multi-Mode Microplate reader to semi-automate the washing and reading steps respectively of a quantitative ELISA for human Insulin.

The human insulin assay used is a sandwich ELISA. The solid phase microplate is coated with a blend of monoclonal antibodies directed against distinct epitopes of the human insulin peptide. Samples, standards and controls are pipetted into these wells, along with a conjugate monoclonal antibody against human insulin, which has horseradish peroxidase (HRP) covalently linked to it. During the incubation the insulin antigen binds to the immobilized capture antibodies. At the same time the conjugate antibody is binding to the insulin present in the well forming the sandwich.

After washing to remove unbound materials, a chromogenic-substrate for HRP, consisting of tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) is added. The substrate is converted to a colored compound by the action of HRP. The reaction is halted by the addition of H_2SO_4 and the microplate is read spectrophotometrically. The absorbance of the solution is proportional to the amount of insulin present in the original sample.

Figure 1. ELx405™ 96-/384-well Microplate Washer.

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Materials and Methods

Human insulin ELISA kits, catalog number KAQ1251, and recombinant human insulin, catalog number 12585-014, were purchased from Invitrogen (Carlsbad, CA). Pooled human normal serum was purchased from Innovative Research (Novi, MI). Pooled human serum was spiked with known amounts of exogenous insulin prior to quantitation.

Samples and standards were prepared off line and 50 µL of each was pipetted manually into the assay plate. After loading samples and standards, 50 µL of assay conjugate was added to all wells. The plates were incubated at room temperature for 30 minutes on a microplate rotary shaker. After incubation the plates were transferred to ELx405 Automated Microplate Washer and washed three times with 400 µL of washer buffer with a 15 second soak period between wash cycles. After washing, 100 µL of substrate solution was added. Plates were returned to the microplate shaker and the color development allowed for 7.5 minutes. After color development, 100 µL of Sulfuric acid stop solution was added and the absorbance of each well at 450 (650 nm reference wavelength) was determined using Synergy™ Mx Multi-Mode Microplate Reader (BioTek Instruments). See Figure 2.

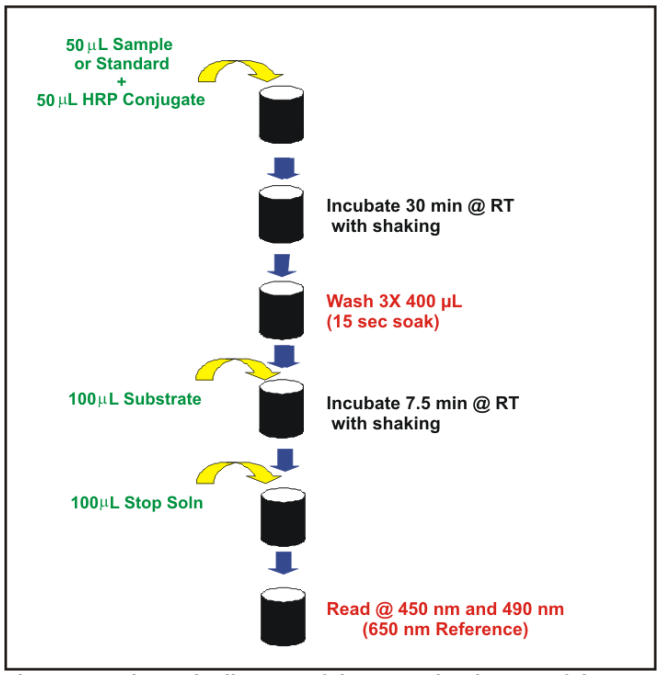

Figure 2. Schematic diagram of the procedural steps of the human Insulin ELISA reaction. Processes carried out by the ELx405 Washer or the Synergy Mx Reader are indicated in Red.

Results

Figure 3 demonstrates the relationship between insulin concentration and the absorbance values obtained from the ELISA reaction. This direct sandwich assay results in increasing absorbance signal with increasing analyte concentration. Like many ELISA analyte concentration curves, the response of the data is sigmoidal in nature, which can be best described using a 4-parameter logistic fit of the data.

A detection limit of 5 µIU/mL can be calculated using the mean of 24 replicates of the 0 standard plus 3 times its standard deviation and interpolating the curve.

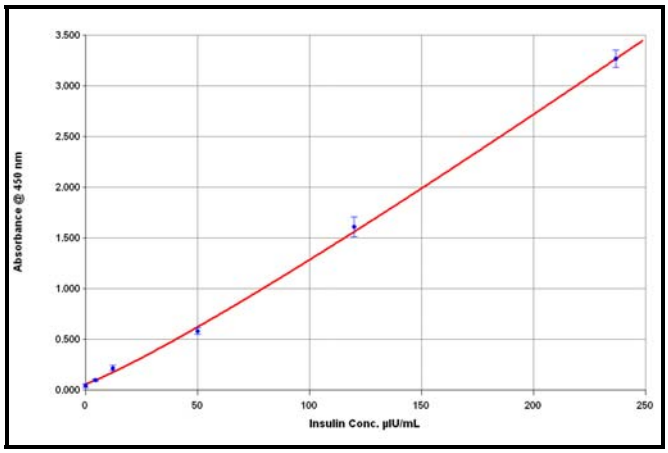

Figure 3. Human Insulin Calibration Curve

Removal of unbound contaminates through plate washing is a prerequisite for good results. Tight CVs of replicate standards, low well to well variation for sample replicates and low blank values are good indicators of sufficient washing. As demonstrated in Figure 4, the determined insulin concentration is very repeatable when using the ELx405 microplate washer. Likewise the standard deviation of the standards-replicates used in the calibration curve demonstrates the high degree of repeatability (Figure 3).

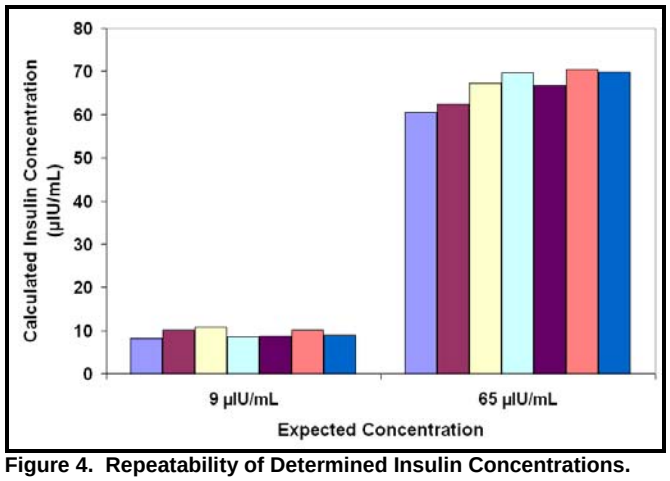

Figure 4. Repeatability of Determined Insulin Concentrations.

The calibration curve can be used to calculate insulin concentrations from unknown samples. Several different concentrations of human insulin were added to pooled human serum samples and their insulin levels measured. After subtracting the determined endogenous insulin levels, as measured from the unspiked controls, the calculated spike concentrations were compared to the expected values. As seen in Table 1, the results agree well with the expected values. The greatest discrepancy occurred at the highest spike concentrations.

Spike Expected (µIU/mL)	Total (µIU/mL)	Spike (µIU/mL)
0	54.9	--------
10	66.9	12.0
25	79.8	24.8
50	107.4	52.5
100	167.8	112.9
250	321.5	266.6

Table 1. Spiked Samples

Discussion

These data indicate that the combination of ELx405™ Microplate Washer with Synergy™ Mx Multi-Mode Microplate Reader can be used to automate some of the steps of a human insulin ELISA. The assay kit instructions indicate that the initial 30-minute and the second 15-minute incubations are both performed with shaking throughout their duration. In our hands the color development step occurred more rapidly than described in the assay kit instructions. We routinely halted the color development at 7.5 minutes rather than allowing the reaction to run for 15 minutes.

Semi-automation of ELISA procedures such as the human insulin ELISA assay allows for improved efficiency in the processing of assay plates. While the assay is not completely “walk-away”, the time consuming liquid handling step of washing has been automated. Attaching a BioStack plate stacker to the washer allows the unattended washing of multiple plates in succession. After processing, the plates are read automatically using a Synergy Mx reader. Once the absorbance has been measured, the generation of a standard curve and the interpolation of the curve for the determination of sample concentrations are automatically performed by Gen5™ Data Analysis Software.

The ELx405™ has several hardware features that make in emenable for use in automating ELISA reactions. The washer has an option for a built in sonicator. This allows for automated cleaning procedures to remove protein and salt crystal buildup. The 96- / 384-well washer manifold is provided with a buffer switching module. This allows for different wash buffers to be used within the assay protocol or a cleaning cycle to be run at the completion of the run.

The Synergy™ Mx reader and the accompanying Gen5™ Data Analysis Software provide complete data analysis. Besides controlling reader function, Gen5 will generate a number of different calibration curve types including linear regression, nonlinear 4-parameter and 5-parameter parametric analysis, and polynomial curve fitting. The calibration curve is then interpolated and concentrations of unknown samples determined automatically.