Monobind Inc.
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AccuBind ELISA Microwells

INSULIN
Product Code: 2425-300

INTENDED USE:

Monobind Insulin Microplate Elisa test is intended to be used for the quantitative determination of insulin levels in human serum.

SUMMARY AND EXPLANATION OF THE TEST

Human insulin is a peptide produced in the beta cells of the pancreas and is responsible for the metabolism and storage of carbohydrates. As a result of biofeedback the insulin levels increase with intake of sugars and decline when sugar content is low for absorption. In the diabetic population the mechanism of insulin production is impaired because of genetic predispositions (***Type I***) or because of lifestyle and/or hereditary factors (***Type II***). In such cases either the insulin production has to be boosted by medication or it has to be supplemented by oral or intravenous methods. The quantitative determination of insulin can help in dose selection the patient has to be subjected to.

On the other hand the circulatory insulin can be found at much higher levels in patients with pancreatic tumors. These tumors secrete abnormally high levels of insulin and thus cause hypoglycemia. Accordingly, fasting hypoglycemia associated with inappropriately high concentrations of insulin strongly suggests an islet-cell tumor (insulinoma). To distinguish insulinomas from factitious hypoglycemia due to insulin administration, serum C-peptide values are recommended. *(Please see Monobind C-Peptide Microwell Elisa Cat#2525-300)*. These insulinomas can be localized by provocative intravenous doses of *tolbutamide* and *calcium*.

PRINCIPLE

Immunoenzymometric assay (TYPE 3):
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (Ab), (enzyme conjugated and immobilized), with different and distinct epitope recognition, **in excess,** and native antigen (Ag). In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal Insulin antibody.
Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(M)} + Ag_{Ins.} + {}^{Btn}Ab_{(M)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(M)}\text{-}Ag_{Ins}\text{-}{}^{Btn}Ab_{(M)}$$

$^{Btn}Ab_{(M)}$ = Biotinylated Monoclonal Ab (Excess Quantity)

Ag_{Ins} = Native Antigen (Variable Quantity)

$^{Enz}Ab_{(M)}$ = Enzyme labeled Monoclonal Ab (Excess Quantity)

$^{Enz}Ab_{(M)}\text{-}Ag_{Ins}\text{-}{}^{Btn}Ab_{(M)}$ = Antigen-Antibodies complex

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$$^{Enz}Ab_{(M)} - Ag_{Ins} - {}^{Btn}Ab_{(M)} + \underline{\text{Streptavidin}}_{C.W.} \Rightarrow \underline{\text{Immobilize complex}}$$

$\underline{\text{Streptavidin}}_{C.W.}$ = Streptavidin immobilized on well
Immobilized complex = sandwich complex bound to the solid surface

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface antigen, HIV 1&2 and HCV antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood; serum or plasma in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin.. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml of the specimen is required.

REAGENTS AND MATERIALS PROVIDED:

A. Insulin Calibrators – 2.0 ml/vial (Dried) – [Icons A – F]
Six (6) vials of references for Insulin antigen at levels of 0(**A**), 5(**B**), 25(**C**), 50(**D**), 100(**E**), and 300(**F**) µIU/ml. Reconstitute each vial with 2ml of distilled or deionized water. The reconstituted calibrators are stable for sixty (60) days at 2-8°C. A preservative has been added.
Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the WHO 1st IRP 66/304.

B. Insulin Enzyme Reagent —13ml/vial - Icon
One (1) vial containing enzyme labeled affinity purified monoclonal mouse x-insulin IgG, biotinylated monoclonal mouse x-insulin IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Streptavidin Coated Plate -- 96 wells – Icon
One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution – 20 ml [Icon
One (1) vial containing a surfactant in phosphate buffered saline. A preservative has been added. Store at 2-30°C.

E. Substrate A --7.0ml/vial – Icon S[A]
One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

F. Substrate B -- 7.0ml/vial – Icon S[B]
One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

G. Stop Solution -- 8.0ml/vial - Icon STOP
One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.

H. Product Instructions.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.
Note 3: Above reagents are for a single 96-well microplate.

Required But Not Provided:

1. Pipette(s) capable of delivering 50µl and 100µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5% (optional).
3. Microplate washer or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability (The 620nm filter is optional)
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Storage container for storage of wash buffer.
10. Distilled or deionized water.
11. Quality Control Materials.

REAGENT PREPARATION:

1. **Wash Buffer**

   Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature (20-27°C) for up to 60 days.

2. **Working Substrate Solution**

   Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

   Note: Do not use the working substrate if it looks blue.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27 C).

1. Format the microplates' wells for calibrator, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.050 ml (50µl) of the appropriate calibrators, controls and samples into the assigned wells.
3. Add 0.100 ml (100µl) of the Insulin Enzyme Reagent to each well. **It is very important to dispense all reagents close to the bottom of the microwell.**
4. Swirl the microplate gently for 20-30 seconds to mix. Cover with a plastic wrap.
5. Incubate for 120 minutes at room temperature (20-27°C).
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. Add 300µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is used, fill each well to the top by squeezing the container. Avoiding air bubbles. Decant the wash and repeat two (2) additional times.**
8. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section***)***.

   DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9. Incubate at room temperature for fifteen (15) minutes.
10. Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
11. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. **The results should be read within thirty (30) minutes of adding the stop solution.**

QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Insulin in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding Insulin concentration in µIU/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of Insulin for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µIU/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.624) intersects the dose response curve at 66.8 µIU/ml for the Insulin concentration (See Figure 1).

Note: Computer data reduction software designed for IEMA (ELISA) assays may also be used for the data reduction.

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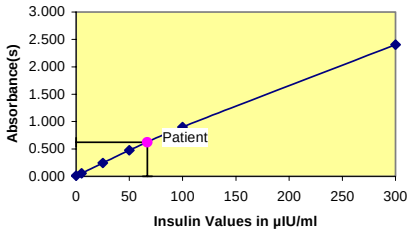
EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (µIU/ml)
Cal A	A1	0.011	0.010	0
	B1	0.009		
Cal B	C1	0.054	0.054	5
	D1	0.053		
Cal C	E1	0.244	0.243	25
	F1	0.241		
Cal D	G1	0.464	0.476	50
	H1	0.488		
Cal E	A2	0.882	0.902	100
	B2	0.922		
Cal F	C2	2.467	2.405	300
	D2	2.342		
Ctrl 1	E2	0.065	0.065	6.4
	F2	0.067		
Ctrl 2	G2	1.581	1`.587	188.0
	H2	1.593		
Patient 1	A3	0.597	0.624	66.8
	B3	0.651		

*The data presented in Example 1 and Figure 1 are for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay.

Figure 1



QC PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrators 0 µIU/ml should be ≤ 0.1.
2. The absorbance (OD) of calibrators 300 µIU/ml should be ≥ 1.3.
3. Four out of six quality control pools should be within the established ranges.

LIMITATIONS OF PROCEDURE

1. It is important that the time of reaction in each well is held constant for reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
4. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time deviation during reaction.
5. Plate readers measure vertically. Do not touch the bottom of the wells.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Highly lipemeic, hemolysed or grossly contaminated specimen(s) should not be used.
8. Patient samples with Insulin concentrations above 300µIU/ml may be diluted with the zero calibrator and re-assayed. Multiply the value obtained by the dilution factor to obtain the corrected value.
9. Use components from the same lot. No intermixing of reagents from different batches.

EXPECTED VALUES

Insulin values are consistently higher in plasma than in serum; thus, serum is preferred. Compared with fasting values in non-obese non-diabetic individuals, insulin levels are higher in obese non-diabetic subjects and lower in trained athletes. Although proinsulin cross reacts with most competitive insulin assays, there is less than 1% cross reaction found with proinsulin using Monobind Insulin Microwell Elisa.

Based on the clinical data gathered by Monobind in concordance with the published literature the following ranges have been assigned. **These ranges should be used as guidelines only:**

Children < 12 yrs	< 10 µIU/ml
Adult (Normal)	0.7 – 9.0 µIU/ml
Diabetic (Type II)	0.7 – 25 µIU/ml

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal"-persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precision of the Insulin AccuBind™ ELISA Microplate Test System were determined by analyses on three different levels of pool control sera. The number, mean value, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision
(Values in µIU/ml)

Sample	N	X	σ	C.V.
Pool 1	20	11.6	0.93	8.0%
Pool 2	20	39.8	1.96	4.9%
Pool 3	20	117.8	6.00	5.1%

TABLE 3
Between Assay Precision
(Values in µIU/ml)

Sample	N	X	σ	C.V.
Pool 1	10	10.3	1.01	9.8%
Pool 2	10	41.5	2.32	5.6%
Pool 3	10	122.1	8.32	6.8%

*As measured in ten experiments in duplicate over seven days.

B. Accuracy

The Monobind Insulin AccuBind™ ELISA was compared with a reference coated tube radioimmunoassay assay. Biological specimens from population (symptomatic and asymptomatic) were used. (The values ranged from 0.01µIU/ml – 129µIU/ml). The total number of such specimens was 104. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method	13.6	y = 2.6 + 0.91(x)	0.975
Reference	11.4		

Only slight amounts of bias between the Insulin AccuBind™ ELISA system and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

C. Sensitivity

The sensitivity (detection limit) was ascertained by determining the variability of the 0 µIU/ml serum calibrator and using the 2**σ** (95% certainty) statistic to calculate the minimum dose. The assay sensitivity was found to be 0.75 µIU/ml.

D. Specificity

The cross-reactivity of the Insulin AccuBind™ ELISA method to selected substances was evaluated by adding the interfering substance(s) to a serum matrix at the following concentration(s). The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of insulin needed to produce the same absorbance.

Substance	Cross Reactivity	Concentration
Insulin	1.0000	-
Proinsulin	0.0078	100 ng/ml
C-Peptide	non-detectable	75 ng/ml
Glucagon	non-detectable	150 ng/ml

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Cat# 2425-300

Size		96(A)	192(B)
Reagent (fill)	A)	2ml set	2ml set
	B)	1 (13ml)	2 (13ml)
	C)	1 plate	2 plates
	D)	1 (20ml)	1 (20ml)
	E)	1 (7ml)	2 (7ml)
	F)	1 (7ml)	2 (7ml)
	G)	1 (8ml)	2 (8ml)

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Instruments & Applications

Monobind's immunoassay products are designed to work in both manual and automated lab environments. AccuBind™ and AccuLite™ are compatible with any open-ended instrumentation, including chemistry analyzers, microplate readers and microplate washers. There may or may not be an application developed for your particular instrument, please visit the instrument section of our website, or contact techsupport@monobind.com

Monobind offers several instruments, including the Impulse 2 Luminometer CLIA Plate Reader designed hand-in-hand with our products and capable of 2-point calibration. Visit our website for more information.