INTENDED USE
Insulin kit is a direct solid phase enzyme immunoassay for quantitative determination of insulin in human serum or plasma. Insulin kit is intended for laboratory use only.

1. CLINICAL SIGNIFICANCE
Insulin is a polypeptide hormone that regulates carbohydrate metabolism. Apart from being the primary effector in carbohydrate homeostasis, it has effects on fat metabolism and it can change the liver’s ability to release fat stores. Insulin is involved in: control of cellular intake of glucose in muscle and adipose tissue, increase of DNA replication and protein synthesis, modification of the activity of numerous enzyme (allosteric effect), increased glycogen, fatty acid synthesis, aminoacid uptake, decreased proteinolysis, lipolysis, gluconeogenesis. Beta cells release insulin in a glucose-dependent way.

In most humans blood glucose levels vary from about 70 mg/dL to perhaps 110 mg/dL (3.9 to 6.1 mmol/L) except shortly after eating when the blood glucose level rises temporarily. This homeostatic effect is the result of many factors, of which hormone regulation is the most important.

There are several conditions in which insulin disturbance is pathologic: diabetes mellitus, insulinoma, metabolic syndrome and polycystic ovary syndrome. There are two types of diabetes mellitus: type 1 (autoimmune-mediated destruction of insulin producing beta cells in the pancreas resulting in absolute insulin deficiency), and type 2 (multifactor syndrome with combined influence of genetic susceptibility and influence of environmental factors, the best known being obesity, age, and physical inactivity, resulting in insulin resistance in cells requiring insulin for glucose absorption. This form of diabetes is strongly inherited). In both cases the insulin production must be increased by medication or delivering insulin by intravenous method.

The quantitative determination of insulin can help to determine the dose to delivery.

2. PRINCIPLE
Insulin ELISA test is based on simultaneous binding of human insulin by two monoclonal antibodies, one immobilized on microwell plates and the other conjugates with horseradish peroxidase (HRP). After incubation, the bound/free separation is performed by a simple solid-phase washing. Then the enzyme HRP in the bound-fraction reacts with the Substrate (H₂O₂) and the TMB Substrate and develops a blue color that changes into yellow when the Stop Solution (H₂SO₄) is added. The colour intensity is proportional to the insulin concentration in the sample. The insulin concentration in the sample is calculated based on a calibration curve.

3. REAGENT, MATERIAL AND INSTRUMENTATION

3.1. Reagent and material supplied in the kit
1. Insulin Calibrators (6 vials)
   CAL0 (3 mL) REF DCE002/7606-0
   CAL1 (1 mL) REF DCE002/7607-0
   CAL2 (1 mL) REF DCE002/7608-0
   CAL3 (1 mL) REF DCE002/7609-0
   CAL4 (1 mL) REF DCE002/7610-0
   CAL5 (1 mL) REF DCE002/7611-0
2. Insulin Control (1 vial, 1 mL)
   Concentration of Control is Lot-specific and is indicated on Quality Control Report REF DCE045/7603-0
3. Conjugate (1 vial, 13 mL)
   Monoclonal antibody anti-insulin conjugated with horseradish peroxidase (HRP) REF DCE002/7602-0
4. Coated Microplate (1 microplate breakable)
   Monoclonal antibody anti- insulin adsorbed on microplate REF DCE002/7603-0
5. **TMB Substrate** (1 vial, 15 mL)

H$_2$O$_2$-TMB 0.26 g/L (avoid any skin contact)

**REF** DCE004-0

6. **Stop Solution** (1 vial, 15 mL)

Sulphuric acid 0.15 mol/L (avoid any skin contact)

**REF** DCE005-0

7. **50X Conc. Wash Solution** (1 vial, 20 mL)

NaCl 45 g/L; Tween-20 55 g/L

**REF** DCE006-0

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### 3.2. Reagents necessary not supplied

Distilled water.

### 3.3. Auxiliary materials and instrumentation

Automatic dispenser.

Microplates reader (450 nm).

**Note**

Store all reagents between 2°-8°C in the dark.

Open the bag of reagent 4 (Coated Microplate) only when it is at room temperature and close it immediately after use.

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### 4. WARNINGS

- This kit is intended for in vitro use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.
- Some reagents contain small amounts of Proclin 300$^8$ as preservative. Avoid the contact with skin or mucosa.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H$_2$O$_2$ to directed sunlight, metals or oxidants.
- This method allows the quantitative determination of insulin from 3 to 200 μIU/mL.

### 5. PRECAUTIONS

- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
- If you use automated equipment is your responsibility to make sure that the kit has been appropriately tested.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
- Maximum precision is required for reconstitution and dispensation of the reagents.
- Samples microbologically contaminated, highly lipemic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

### 6. PROCEDURE

#### 6.1. Preparation of the Calibrators (C$_0$…C$_5$)

The Calibrators are ready to use and have the following concentrations:

<table>
<thead>
<tr>
<th>μIU/mL</th>
<th>C$_0$</th>
<th>C$_1$</th>
<th>C$_2$</th>
<th>C$_3$</th>
<th>C$_4$</th>
<th>C$_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
<td>10</td>
<td>30</td>
<td>80</td>
<td>200</td>
</tr>
</tbody>
</table>

Once opened, the Calibrators are stable 6 months at 2°-8°C.
6.2. Preparation of Wash Solution
Dilute the contents of each vial of the "50X Conc. Wash Solution" with distilled water to a final volume of 1000 ml prior to use. For smaller volumes respect the 1:50 dilution ratio. The diluted wash solution is stable for 30 days at 2±8°C.

6.3. Preparation of the Sample
Follow Good laboratory procedures for handling blood products.
For accurate comparison to establish normal values, a fasting morning serum sample should be obtained.
To obtain the serum, the blood should be collected in a venipuncture tube without additives or anti-coagulants; allow the blood to clot; centrifuge the specimen to separate the serum from the cells.
Samples may be refrigerated at 2÷8°C for a maximum period of 5 days. If the specimens cannot be assayed within this time, they may be stored at -20°C for up to 30 days. Avoid repetitive freezing and thawing.
Patient specimens with insulin concentrations above 200 μIU/mL may be diluted (for example 1:10 or higher) with Calibrator zero and re-assayed. The sample’s concentration is obtained by multiplying the result by the dilution factor.
The Control is ready for use.

6.4. Procedure
- Allow all reagents to reach room temperature (22-28°C).
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C₀-C₅), two for each Control, two for each sample, one for Blank.

7. QUALITY CONTROL
Each laboratory should assay controls at levels in the low, medium and high ranges of the dose response curve 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.

8. RESULTS

8.1. Note
The optical densities (O.D.s) of some calibrators and samples may be higher than 3.0, in such a case, they could be out of the measurement range of the microplate reader. It is therefore necessary, for O.D.s higher than 3.0, to perform a reading at 405 nm in addition to 450 nm and 620 nm (reference filter for the subtraction of interferences due to the plastic).
For microplate readers unable to read the plate at 3 wavelengths at the same time,
It is advisable to proceed as follows:
- Read the microplate at 450 nm and at 620 nm.
- Read again the plate at 405 nm and 620 nm.
- Find out the wells whose ODS at 450 nm are higher than 2.0
- Select the corresponding ODS read at 405 nm and multiply these values at 405 nm by the conversion.
factor 3.0 (where OD 450/OD 405 = 3.0), that is: 

\[ OD_{450 \text{ nm}} = OD_{405 \text{ nm}} \times 3.0. \]

Warning: The conversion factor 3.0 is suggested only. For better accuracy, the user is advised to calculate the conversion factor specific for his own reader.

8.2. Mean Absorbance

Calculate the mean of the absorbance (Em) for each point of the calibration curve \((C_0-C_5)\) and of each sample.

8.3. Calibration curve – Automatic method

Use the smoothed cubic spline – preferred – or 4 parameters logistic function as calculation algorithm.

8.4. Calibration curve – Manual method

A dose response curve is used to ascertain the concentration of insulin in unknown specimens. Record the OD obtained from the printout of the microplate reader. Plot the average OD for each duplicate calibrator versus the corresponding insulin concentration in \(\mu\text{IU/mL}\) on linear graph paper. Draw the best-fit curve through the plotted points. 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 \(\mu\text{IU/mL}\)) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

9. REFERENCE VALUES

Insulin values are consistently higher in plasma than in serum; thus, serum is preferred. The following ranges have been assigned by Diametra in concordance with the published literature.

<table>
<thead>
<tr>
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<th>(\mu\text{IU/mL})</th>
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<tbody>
<tr>
<td>Children &lt;12 yrs</td>
<td>&lt;10</td>
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<tr>
<td>Adult (Normal)</td>
<td>0.7 – 9.0</td>
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<tr>
<td>Diabetic (Type II)</td>
<td>0.7 – 25</td>
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</table>

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacturer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision

10.1.1. Intra Assay Variation

Within run variation was determined by replicate (20x) determination of three different levels of serum in one assay. The within assay variability is \(\leq 5.0\%\).

10.1.2. Inter Assay Variation

Between run variation was determined by replicate measurements of three different level of serum in different lots. The between assay variability is \(\leq 10.0\%\).

10.2. Specificity

The cross reaction of the antibody calculated by deriving a ratio between dose of interfering substance to dose of insulin needed to produce the same absorbance:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>Insulin</td>
<td>100%</td>
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<tr>
<td>Proinsulin</td>
<td>N.D.</td>
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<tr>
<td>C-Peptide</td>
<td>N.D.</td>
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</table>

10.3. Accuracy

The recovery on three serum samples spiked with 7 - 14 - 28 - 56 \(\mu\text{IU/mL}\) of antigen gave an average value (\(\pm\) SD) of 101.02\% \(\pm\) 5.57\%.

The dilution test performed on three sera diluted 2 - 4 - 8 times gave an average value (\(\pm\) SD) of 96.94\% \(\pm\) 5.41\%.

10.4. Sensitivity

The lowest detectable concentration of insulin that can be distinguished from the Calibrator 0 is 0.25 \(\mu\text{IU/mL}\).

10.5. Correlation

Diametra Insulin ELISA was compared to a reference method Insulin assay. 30 serum samples were compared by linear regression analysis. The linear regression curve was calculated:

\[(\text{Insulin Diametra}) = 0.88\times(\text{Ref. method}) - 0.23 \quad r^2 = 0.963\]

10.6. Hook Effect

Diametra Insulin assay shows no Hook effect until 25000 \(\text{mIU/mL}\).

11. WASTE MANAGEMENT

Reagents must be disposed off in accordance with local regulations.
BIBLIOGRAPHY
- Boehm TM, et al Diabetes Care 479-490. (1079)
- Turkinton RW, et al Archive of Internal Med. 142

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Fax 0039–02–2133354.

Manufactury: Via Pozzulo 14, 06038 SPELLO (PG) Italy
Tel. 0039-0762–24864
Fax 0039–0762–316197
E-mail: info@diametra.com
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SUGGERIMENTI PER LA RISOLUZIONE DEI PROBLEMI/TROUBLESHOOTING

ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction
- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers
- contamination of pipettes, tips or containers
- insufficient washing (conjugates not properly removed) too high within-run
- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
- too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation