Mouse Insulin ELISA Kit  
(Code No.: AKRIN-011T)  
March 2, 2010 Ver.3  

For in vitro laboratory use only  

Please, read this instruction carefully before use.

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This kit is manufactured by Shibayagi Co., Ltd.  
Use only the current version of Instruction Manual enclosed with the kit!

**1. Intended use**

Mouse Insulin ELISA Kit is a sandwich ELISA system for quantitative measurement of mouse insulin.  

**Features**  
(1) This is intended for research use only.  
(2) Rapid assay (total reaction time: 3 hours).  
(3) This kit is for insulin in mouse serum, plasma, culture medium and tissue extract.  
(4) A small sample volume of serum or plasma is needed.  
(5) Assay format is 96 wells.  
(6) Standard insulin is derived from mouse pancreas extract.  
(7) All reagents are provided in liquid form and ready to use.

**2. Storage and expiration**

When the complete kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box.  
Opened reagents should be used as soon as possible to avoid less than optimal assay performance caused by storage environment.

**3. Introduction**

Insulin is a peptide hormone secreted from B cells of islet of Langerhans in the pancreas with a molecular weight of about 5,800 and pl 5.4. It is consisted of 2 chains, A and B. It has 3 disulfide bonds formed between A6 and A11, A7 and B7, and A20 and B19. Insulin exists as a dimer molecule in acidic to neutral solution without Zn ion, and as a hexamer including two Zn ions in neutral solution if Zn ions are present.  
Main targets of insulin are liver, muscle, and adipose tissue. Insulin actions in these targets are as follows.  
In the liver, it promotes glycogenesis, protein synthesis, fatty acid synthesis, carbohydrate utilization, and inhibition of gluconeogenesis.  
In the muscle, it promotes membrane permeability for carbohydrates, amino acids and K ion, glycogenesis, protein synthesis, while inhibits protein degradation. In the adipose tissue, it promotes membrane permeability for glucose and fatty acid synthesis.  
A precursor of insulin, called proinsulin with a single polypeptide chain, is first synthesized in the cell, then sulfide bonds are formed, and finally by enzymatic cutting at two sites, active insulin and c-peptide (connecting peptide) are formed.  
Potency of an insulin preparation was originally determined by bioassay. However, whole body bioassay inevitably shows poor precision owing to individual variation. To avoid any variation, international standard preparation has been used. The 4th international standard preparation is a mixture of purified preparations of bovine (52%) and porcine (48%) insulin, and 1 mg of the standard is defined as 24 IU (=0.04167 mg/IU).
Following further purification of insulin, WHO issued 1\textsuperscript{st} International Standard for human insulin in 1986 which has the potency of 26 IU/mg (0.038 mg/IU). In the same year, 1\textsuperscript{st} International Standard of bovine insulin, the potency of which is 25.7 IU/mg, and Porcine insulin 1\textsuperscript{st} International Standard, 26 IU/mg, were provided. Before these standards, in 1974, 1\textsuperscript{st} International Reference Preparation of human insulin for immunoassay was provided as 3 IU/ampoule.

Based on the above data, if the biological activity of insulin per molecule is the same among various animal species, potencies of animal insulin might be calculated from their molecular weights. But, so far, we do not have sufficient data for this. As the molecular weights of insulin of various animals are nearly the same, and the differences are within 1%, there may be no critical fault if we think that the general potency of insulin is 26 IU/mg.

Rat and mouse have two molecular species of insulin, type 1 and type 2. Amino acid sequences of these molecular species are same between rat and mouse. But as their ratios are different between these two animal species, it is recommended to use standard preparation derived from each animals.

4. Assay principle

In Shibayagi’s Mouse Insulin ELISA Kit, biotin conjugated anti insulin, and standard or sample are incubated in monoclonal anti-insulin-coated wells to capture insulin bound with biotin conjugated anti insulin. After 2 hours incubation and washing, HRP (horse radish peroxidase) conjugated streptavidin is added, and incubated for 30 minutes. After washing, HRP conjugated streptavidin remaining in wells are reacted with a substrate chromogen reagent (TMB) for 20 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to insulin concentration. The standard curve is prepared by plotting absorbance against standard insulin concentrations. Insulin concentrations in unknown samples are determined using this standard curve.

5. Precautions

- For professional use only, beginners are advised to use this kit under the guidance of experienced person.
- Do not drink, eat or smoke in the areas where assays are carried out.
- In treating assay samples of animal origin, be careful for possible biohazards.
- This kit contains components of animal origin. These materials should be handled as potentially infectious.
- Be careful not to allow the reagent solutions of the kit to touch the skin, eyes and mucus membranes. Especially be careful for the reaction stopper because it is 1 M sulfuric acid. The reaction stopper and the substrate solution may cause skin/eyes irritation. In case of contact with these wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate solution containing hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- The materials must not be pipetted by mouth.
- Unused samples and used tips should be rinsed in 1% formalin, 2% glutal aldehyde, or more than 0.1% sodium hypochlorite solution for more than 1 hour, or be treated by an autoclave before disposal.
- Dispose consumable materials and unused contents in accordance with applicable regional/national regulatory requirements.
- Use clean laboratory glassware.
- In order to avoid dryness of wells, contamination of foreign substances and evaporation of dispensed reagents, never forget to cover the well plate with a plate cover supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25°C strictly. Avoid airstream velocity over 0.4 m/sec. (including wind from air conditioner), and humidity less than 30%.

6. Technical tips

- In manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best not to mix the reagents with those that have been preserved for some period.
- Be careful to avoid any contamination of assay samples and reagents. We recommend the use of disposal pipette tips, and 1 tip for 1 well.
- Optimally, the reagent solutions of the kit should be used immediately after reconstitution. Otherwise, store them in a dark place at 2-8 °C.
- Time the reaction from the pipetting of the reagent to the first well.
- Prepare a standard curve for each assay.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The substrate chromogen reagent (TMB) should be almost colorless before use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.
- To avoid denaturation of the coated antibody, do not let the plate go dry.
- As the anti-insulin-coated plate is module type of 8wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.
- When ELISA has to be done under the airstream velocity of over 0.4 m/sec. and the humidity of less than 30%, completely close each well in addition to cover the well plate with a plate cover in each step of incubation. (Ex.) Cover the well plate with parafilm, and put the plate cover on it. Or place the well plate with the plate cover in an incubator, or in a styrofoam box. Take the best way depending on situation of each laboratory.

7. Reagents supplied

<table>
<thead>
<tr>
<th>Components</th>
<th>State</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Anti-Insulin-coated plate</td>
<td>Use after washing</td>
<td>96 wells/1 plate</td>
</tr>
<tr>
<td>(B) Standard Mouse Insulin solution (200ng/ml)</td>
<td>Concentrated. Use after dilution</td>
<td>25µl/1 vial</td>
</tr>
</tbody>
</table>
8. Equipment or supplies required but not supplied

- Purified water (distilled water)
- Test tubes for preparation of standard solution series.
- Glassware for dilution of washing buffer (a graduated cylinder, a bottle)
- Pipettes (disposable tip type). One should be able to deliver 10 µl precisely, and another for 100-200 µl.
- Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50-100 µl.
- Paper towel to remove washing buffer remaining in wells.
- A vortex-type mixer.
- A shaker for 96 well-plate (~800rpm)
- An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle.
- Software for data analysis, if available. Shibayagi is proposing the use of assay results calculation template for EXCEL. Please check our website (http://www.shibayagi.co.jp/en/tech_003.html).

9. Preparation of reagents

- Bring all reagents of the kit to room temperature before use.
- Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.
- Do not use the reagent after expiration date.
- Reagents ready for use after return to room temperature
  [Anti-Insulin-coated plate]
  Storage and stability
  If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8 °C. The strip will be stable until expiration date.

  [Buffer solution]
  [Substrate chromogen reagent]
  Storage and stability
  If not opened, store at 2-8 °C. It maintains stability until expiration date. Once opened, we recommend using as soon as possible to avoid influence by environmental condition.

  [Reaction stopper (1 M H2SO4)]
  Storage and stability
  Close the stopper tightly and store at 2-8 °C. It maintains stability until expiration date.

- Concentrated reagents
  [Washing buffer concentrate (10x)]
  Dilute 1 volume of the washing buffer concentrate (10x) to 10 volume with deionized water to prepare working solution. Example: 100 ml of washing buffer concentrate (10x) and 900ml of dionized water.
  Storage and stability
  The rest of undiluted buffer: if stored tightly closed at 2-8 °C, it is stable until expiration date. Dispose any unused diluted buffer.

  [Standard Mouse Insulin solution (200ng/ml)]
  Make a serial dilution of master standard (200ng/ml) solution to prepare each standard solution (0.313-10 ng/ml).

<table>
<thead>
<tr>
<th>Volume of standard solution (µl)</th>
<th>Buffer solution (µl)</th>
<th>Concentration (ng/ml)</th>
<th>Concentration (µIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original solution: 10µl</td>
<td>190µl</td>
<td>10</td>
<td>260</td>
</tr>
<tr>
<td>10 ng/ml solution: 100µl</td>
<td>100µl</td>
<td>5.0</td>
<td>130</td>
</tr>
<tr>
<td>5 ng/ml solution: 100µl</td>
<td>100µl</td>
<td>2.5</td>
<td>65</td>
</tr>
<tr>
<td>2.5 ng/ml solution: 100µl</td>
<td>100µl</td>
<td>1.25</td>
<td>32.5</td>
</tr>
<tr>
<td>1.25 ng/ml solution: 100µl</td>
<td>100µl</td>
<td>0.625</td>
<td>16.3</td>
</tr>
<tr>
<td>0.625 ng/ml solution: 100µl</td>
<td>100µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>