3.0 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 the 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 competitive or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

\[ \text{Ab}^{b} + \text{Ag} + \text{Ab}^{e} \rightarrow \text{Ab}^{b} - \text{Ag} - \text{Ab}^{e} \]

\( \text{Ab}^{b} \): Biotinylated Monoclonal Ab
\( \text{Ab}^{e} \): Enzyme labeled Monoclonal Ab (Excess Quantity
\( \text{Ag} \): Native Antigen (Variable Quantity

Note 3: Above reagents are for a single 96-well microplate. For other configurations, refer to the chart at the end of the instructions.

4.1 Required But Not Provided:
1. Pipette(s) capable of delivering 0.050 & 0.100 ml (50 & 100µl) volumes with a precision of better than 1.5%.
2. Dispensers (for repetitive deliveries of 0.10 & 0.350 ml (100 & 300µl) volumes with a precision of better than 1.5%.
3. Disposable wash or deionizer bottle (optional).
4. Microplate Reader with 450ml and 620nm wavelength absorbance capability (The 620nm filter is optional)
5. Absorbent Paper for top well 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.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

All products that contain insulin have been found to be non-reactive for Hepatitis B Surface antigen, HIV 1&2 and HIV 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, “Biologicals in Microbiological Laboratories”, 1981, NIH Pub.

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

6.0 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 a complete preparation to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain test tube (without anticoagulant) and stored at room temperature up to 24 hours. If stored at room temperature for more than 24 hours, the sample should be refrigerated. The blood should be centrifuged at 1000xg for 5 minutes at 20°C, and the supernatant separated and stored at 2-8°C.

The specimen is diluted with tris buffer at 2-8°C prior to assay. The diluted sample must be used within the time specified in Example 1 and Figure 2.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before starting the assay, bring all reagents, reference calibrators and controls to room temperature (20 ± 2°C).

Test procedure should be performed by a skilled individual or team proficient in immunoassay.

1. Format the microtubes’ 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 0.050ml (50µl) of the appropriate calibrators, controls and samples into the assayed wells.
3. Add 1.00ml (100µl) of C-Peptide Enzyme Reagent to each well. It is very important to dispense all reagents into the microwell bottle(s).
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°C-27°C).
6. Discard the contents of the microplate by decantation or aspiration, if decanting, tap and blot the plate dry with absorbent tissue.
7. Add 0.35ml (350µl) of wash buffer (see “Reagent Preparation”), 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 instructions for proper usage. If a squeeze bottle is used, put the empty bottle to the top by securing the container. Avoiding air bubbles. Decant the wash and repeat two (2) times more.
8. Add 0.100ml (100µl) of working substrate solution to all wells (see “Reagent Preparation”).
9. No shake the plate after substrate addition

Incubate at room temperature for fifteen (15) minutes.

10. Add 0.050ml (50µl) of stop solution to each well and mix gently for 30 seconds, then read the absorbances at 450nm, 630nm and 490nm to minimize reaction time differences between wells.
11. Record the absorbance at each well in 450nm using a reference wavelength of 600-650nm to minimize well imperfections in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

11.0 CALCULATION OF RESULTS

A. Calculations: To concentrate the amount of Insulin or C-Peptide in unknown specimens.
1. Record the absorbance obtained from the printout of the microtiter plate as the 
2. Plot the absorbance for each calibrator to the 
3. To determine the concentration of Insulin or C-Peptide for an unknown, locate the average absorbance of the duplicates for each concentration that most closely matches the unknown sample.
4. To determine the concentration of Insulin or C-Peptide for an unknown, locate the average absorbance of the duplicates for each concentration that most closely matches the unknown sample.
5. To determine the concentration of Insulin or C-Peptide for an unknown, locate the average absorbance of the duplicates for each concentration that most closely matches the unknown sample.
6. To determine the concentration of Insulin or C-Peptide for an unknown, locate the average absorbance of the duplicates for each concentration that most closely matches the unknown sample.

Note 1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

The specimen is diluted with tris buffer at 2-8°C prior to assay. The diluted sample must be used within the time specified in Example 1 and Figure 2.

Note 2: Do not use reagents that are contaminated or have bacteria growth.
4. If more than one (1) plate is used, it is recommended to repeat the assay using the same dose response curve and a different batch of reagents. 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 tests were conducted.

13.0 PERFORMANCE CHARACTERISTICS
14.1 Precision

The within and between assay precision of the C-Pep/Ins VAST® AccuBind® ELISA Test System were determined by analyzing on different days with four random pool control sera. The number, mean value, standard deviation and coefficient of variation for each of these control sera are presented in Table 1 and Table 2.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>o</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>24</td>
<td>10.7</td>
<td>1.43</td>
<td>0.09 (9.11)</td>
</tr>
<tr>
<td>Pool 2</td>
<td>24</td>
<td>10.1</td>
<td>0.87</td>
<td>0.07 (4.35)</td>
</tr>
<tr>
<td>Pool 3</td>
<td>24</td>
<td>10.0</td>
<td>1.30</td>
<td>0.07 (9.11)</td>
</tr>
</tbody>
</table>

As measured in ten experiments in duplicate over ten days.

14.2 SENSITIVITY

The sensitivity (detection limit) was ascertained by determining the variability of the 0 µIU/ml serum calibrator and using the 2a (95% certainty) statistic to calculate the minimum dose. The assay sensitivity was found to be 0.182 µIU/ml for insulin and 0.020 µIU/ml for C-Peptide.

14.3 ACCURACY

The C-Pep/Ins VAST® AccuBind® ELISA Test System was compared with a reference method. Biological specimens from non-diabetic individuals (normal and non-obese) were used. The specimens ranged from non-detectable values ranging from 0.01µIU/ml to 120ng/ml for insulin with a total number of 104 specimens. The specimens ranged from 0.2ng/ml to 11.8ng/ml for C-Peptide. The total number of 104 specimens. The data obtained is displayed in Tables 3 and 4.

Table 4 – C-Peptide

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean</th>
<th>Least Square</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>1.068</td>
<td>y = 0.2079 + 0.8036(x)</td>
<td>0.996</td>
</tr>
</tbody>
</table>

15.0 REFERENCES