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CHEMILUMINESCENCE
ENZYME IMMUNOASSAY (CLIA)

C-Peptide

REF **9022-16**

Chemiluminescence Enzyme Immunoassay for the Quantitative Determination of Circulating C-Peptide Concentrations in Human Serum

INTRODUCTION OF CHEMILUMINESCENCE IMMUNOASSAY

Chemiluminescence Immunoassay (CLIA) detection using Microplate luminometers provides a sensitive, high throughput, and economical alternative to conventional colorimetric methodologies, such as Enzyme-linked immunosorbent assays (ELISA).

ELISA employs a label enzyme and a colorimetric substrate to produce an amplified signal for antigen, haptens or antibody quantitation. This technique has been well established and considered as the technology of choice for a wide variety of applications in diagnostics, research, food testing, process quality assurance and quality control, and environmental testing. The most commonly used ELISA is based on colorimetric reactions of chromogenic substrates, (such as TMB) and label enzymes.

Recently, a chemiluminescent immunoassay has been shown to be more sensitive than the conventional colorimetric method(s), and does not require long incubations or the addition of stopping reagents, as is the case in some colorimetric assays. Among various enzyme assays that employ light-emitting reactions, one of the most successful assays is the enhanced chemiluminescent immunoassay involving a horseradish peroxidase (HRP) labeled antibody or antigen and a mixture of chemiluminescent substrate, hydrogen peroxide, and enhancers.

The CLIA Kits are designed to detect glow-based chemiluminescent reactions. The kits provide a broader dynamic assay range, superior low-end sensitivity, and a faster protocol than the conventional colorimetric methods. The series of the kits covers Thyroid panals, such as T3, T4, TSH, Hormone

panals, such as hCG, LH, FSH, and other panals. They can be used to replace conventional colorimetric ELISA that have been widely used in many research and diagnostic applications. Furthermore, with the methodological advantages, Chemiluminescent immunoassay will play an important part in the Diagnostic and Research areas that ELISAs can not do.

The CLIA Kits have been validated on the **MPL2** microplate luminometer from Berthold Detection System, **Lus2** microplate luminometer from Anthos, **Centro LB960** microplate luminometer from Berthold Technologies, and **Platelumino** From Stratec Biomedical Systems AG. We got acceptable results with all of those luminometers.

SUMMARY AND EXPLANATION OF THE TEST

Diabetes is one of the leading causes of disability and death in the U.S. It affects an estimated 16 million Americans, about one third of them do not even know they have the disease. The causes of diabetes are not precisely known, but both genetic and environmental factors play a significant role. The disease is marked by deficiencies in the body's ability to produce and properly use insulin. The most common forms of diabetes are type 1, in which the body's ability to produce insulin is destroyed, and type 2, in which the body is resistant to insulin even though some amount of insulin may be produced.

In-vitro determination of insulin and *C-Peptide* levels help in the differential diagnosis of liver disease, acromegaly, Cushing's syndrome, familial glucose intolerance, insulinoma, renal failure, ingestion of accidental oral hypoglycemic drugs or insulin induced factitious hypoglycemia. Both insulin and *C-Peptide* are produced by enzymatic cleavage of proinsulin. Proinsulin is stored in the secretory granules of pancreatic β-cells and is split into a 31 amino acid connecting peptide (*C-Peptide;* MW 3600) and insulin (MW 6000). *C-Peptide* is devoid of any biological activity but appears to be necessary to maintain the structural integrity of insulin. Although insulin and *C-Peptide* are secreted into portal circulation in equimolar concentrations, fasting levels of *C-Peptide* are 5-10 fold higher than those of insulin owing to the longer half-life of *C-Peptide.* The liver does not extract *C-Peptide* however; it is removed from the circulation by degradation in the kidneys with a fraction passing out unchanged in urine. Hence urine *C-Peptide* levels correlate well with fasting *C-Peptide* levels in serum. The glucagon stimulated *C-Peptide* determination is often used for differential diagnosis of insulin-dependent from non-insulin-dependent diabetic patients.

TEST PRINCIPLE

The C-Peptide Quantitative Test Kit is based on a solid phase enzyme-linked immunosorbent assay. The assay system utilizes one anti-C- Peptide antibody for solid phase (microtiter wells) immobilization and another anti-C-Peptide antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The standards and test specimen (serum) are added to the C-Peptide antibody coated microtiter wells. Then anti-C-Peptide antibody labeled with horseradish peroxidase (conjugate) is added. If human C-Peptide is present in the specimen, it will combine with the antibody on the well and the enzyme conjugate resulting in the C-Peptide molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 1 hour incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. A solution of chemiluminescent substrate is then added and read relative light units (RLU) in a Luminometers. The intensity of the emitting light is proportional to the amount of enzyme present and is directly related to the amount of C-peptide in the sample. By reference to a series of C -peptide standards assayed in the same way, the concentration of C-peptide in the unknown sample is quantified.

PRECAUTIONS

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 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 red-top venipuncture tube without additives. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells. C-Peptide is not stable in serum basis. Samples should be used as fresh as possible. Refrigerated at 2-8oC for a maximum period of one day only. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20oC for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml of the specimen is required.

MATERIALS AND COMPONENTS

Materials provided with the test kits:
- Monoclonal anti C-Peptide antibody coated microtiter plate with 96 wells.
- Enzyme conjugate reagent, 12 ml.
- C-Peptide reference standards set ,containing; *0, 0.5, 1.0, 2.0, 5.0 and 10.0* ng/ml. (WHO 1st IRR 84/510). Lyophilized, ***DO NOT FREEZE and THAW***
- 50X Wash Buffer Concentrate, 15 ml
- Chemiluminescence Reagent A, 6.0 ml.
- Chemiluminescence Reagent B, 6.0 ml.

Materials required but not provided:
- Precision pipettes and tips, 0.05~0.2 ml, 1.oml
- Disposable pipette tips.
- Distilled water.
- Vortex mixer.
- Absorbent paper or paper towel.
- Microtiter plate luminometer.
- Graph paper.
- Glass tube or flasks to mix Reagent A and B.
- Quality Control Materials.

REAGENT PREPARATION

1. All reagents should be brought to room temperature (18-22°C) and mixed by gently inverting or swirling prior to use. Do NOT induce foaming.
2. To prepare substrate solution, make a 1:1 mixing of Reagent A with Reagent B right before use. Mix gently to ensure complete mixing. Discard excess after use.
3. Dilute 1 volume of Wash Buffer (50x) with 49 volumes of distilled water. For example, Dilute 15 ml of Wash Buffer (50x) into 735 ml of distilled water to prepare 750 ml of washing buffer (1x). Mix well before use.
4. Reconstitute each lyophilized standard and controls with distilled water (Reconstitute volume see labels). Allow the reconstituted material to stand for at least 20 minutes. Reconstituted standards and controls should be stored sealed at 2-8°C.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder. Dispense 50μl of C-Peptide standards, specimens, and controls into the appropriate wells. Gently but thoroughly mix for 10 seconds.
2. Dispense 100μl of enzyme conjugate reagent into each well. Mix gently for 30 seconds. It is very important to have a complete mixing in this setup. Incubate at room temperature for 60 minutes.
3. Remove the incubation mixture by emptying the plate content into a waste container. Rinse and empty the microtiter plate 5 times with 1 x washing buffer (300μl each well). Strike the microtiter plate sharply onto absorbent paper or paper towels to remove all residual water droplets.
4. Dispense 100 μl Chemiluminescence substrate solution into each well. Gently mix for 5 seconds.
5. Read wells with a chemiluminescence microwell reader 5 minutes later. (between 5 and 20 min. after dispensing the substrates).

Important Note:
1. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
2. If there are bubbles existing in the wells, the false readings will be created. Please use distilled water to remove the bubbles before adding the substrate.

CALCULATION OF RESULTS

1. Calculate the average read relative light units (RLU) for each set of reference standards, control, and samples.
2. We recommend using proper software to calculate the results. If the software is not available, construct a standard curve by plotting the mean RLU obtained for each reference standard against C-Peptide concentration in ng/ml on linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of C-Peptide in ng/ml from the standard curve.

EXAMPLE OF STANDARD CURVE

Results of a typical standard run are shown below. This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. It is required that running assay together with a standard curve each time. The calculation of the sample values must be based on the particular curve, which is running at the same time.

C-peptide Values (ng/ml)	Relative Light Units (RLU) (10^4)
0	0.1
0.5	5.2
1	12.4
2	26.5
5	70.7
10	133.0

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 C-Peptide concentrations above 10 ng/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

C-Peptide values are consistently higher in plasma than in serum; thus, serum is preferred. Compared with fasting values in non-obese non-diabetic individuals, C-Peptide levels are higher in obese non-diabetic subjects and lower in trained athletes.

Each laboratory is advised to establish its own ranges for normal and abnormal populations. These ranges are always dependent upon locale, population, laboratory, technique and specificity of the method.

Based on the clinical data gathered by Diagnostic Automation, Inc. in concordance with the published literature the following ranges have been assigned.

These ranges should be used as guidelines only:
  Adult (Normal) 0.7 – 1.9 ng/ml

PERFORMANCE CHARACTERISTICS

A. Sensitivity

The sensitivity (detection limit) was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2**SD** (95% certainty) statistic to calculate the minimum dose. The assay sensitivity was found to be 0.05 ng/ml.

B. Specificity

The cross-reactivity of the C-Peptide 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 C-Peptide needed to produce the same absorbance.

Substance	Cross Reactivity	Concentration
C-Peptide	1.0000	-
Proinsulin	0.81	200 ng/ml
Insulin	non-detectable	2.0 mIU/ml
Glucagon	non-detectable	200 ng/ml

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