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REF

Cat # 9003-16

Chemiluminescence

Enzyme Immunoassay (CLIA)

T4

Cat # 9003-16

Chemiluminescence Enzyme Immunoassay for the Quantitative Determination of Thyroxine (T4) Concentration 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 panels, such as T3, T4, TSH, Hormone panels, such as hCG, LH, FSH, and other panels. 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 **MPL1 or 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.

INTRODUCTION OF T4 IMMUNOASSAY

L-Thyroxine (T4) is a hormone that is synthesized and stored in the thyroid gland. Proteolytic cleavage of follicular thyroglobulin releases T4 into the bloodstream. Greater than 99% of T4 is reversibly bound to three plasma proteins in blood - thyroxine binding globulin (TBG) binds 70%, thyroxine binding pre-albumin (TBPA) binds 20%, and albumin binds 10%. Approximately 0.03% of T4 is in the free, unbound state in blood at any one time.

Diseases affecting thyroid function may present a wide array of confusing symptoms. Measurement of total T4 by immunoassay is the most reliable and convenient screening test available to determine the presence of thyroid disorders in patients. Increased levels of T4 have been found in hyper-thyroidism due to Grave's disease and Plummer's disease and in acute and subacute thyroiditis. Low levels of T4 have been associated with congenital hypothyroidism, myxedema, chronic thyroiditis (Hashimoto's disease), and with some genetic abnormalities.

Principle of the test

In the T4 CLIA, a certain amount of anti-T4 antibody is coated on microtiter wells. A measured amount of patient serum, and a constant amount of T4 conjugated with horseradish peroxidase are added to the microtiter wells. During incubation, the anti-T4 antibody is bound to the second antibody on the wells, and T4 and conjugated T4 compete for the limited binding sites on the anti-T4 antibody. After a 60 minutes incubation at room temperature, the wells are washed 5 times by wash solution to remove unbound T4 conjugate. A solution of chemiluminescent substrate is then added and read relative light units (RLU) in a Luminometer. The intensity of the emitting light is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled T4 in the sample. By reference to a series of T4 standards assayed in the same way, the concentration of T4 in the unknown sample is quantified.

Materials and components

Materials provided with the test kits:

1. Anti-T4 Antibody-Coated Microtiter Wells, 96 wells.
2. T4 HRPO Conjugate Concentrate (20x), 0.8 ml.
3. T4 HRPO Conjugate Diluent, 15 ml
4. Reference Standard, 1 set. Ready to use.
5. 50x Wash Buffer Concentrate, 15 ml
6. Chemiluminescence Reagent A, 6.0 ml.
7. Chemiluminescence Reagent B, 6.0 ml.

Materials required but not provided:

- Precision pipettes
- Disposable pipette tips.
- Distilled water.
- Glass tubes or flasks to mix Reagent A and B.
- Vortex mixer or equivalent.
- Absorbent paper or paper towel.
- Graph paper.
- Microtiter well luminometer

Specimen collection and preparation

Serum should be prepared from a whole blood specimen obtained by acceptable medical techniques. This kit is for use with serum samples without additives only.

Reagent preparation

1. All reagents should be brought to room temperature (18-25°C) before use.
2. To prepare T4-HRPO Conjugate Reagent, add 0.1 ml of T4-HRPO Conjugate Concentrate to 2.0 ml of T4 Conjugate Diluent (1:20 dilution), and mix well. The amount of conjugate diluted is depend on your assay size. The Conjugate Reagent is stable at 4°C at least for two weeks.
3. To prepare Chemiluminescence Substrate solution, make a 1:1 mixing of Reagent A with Reagent B right before use. Discard the excess after use.
4. 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.

Assay procedures

1. Secure the desired number of coated well in the holder. Make data sheet with sample identification.
2. Dispense 50 µl of standard, samples, and controls into appropriate wells.
3. Thoroughly mix for 10 seconds, then dispense 100 µl of Enzyme Conjugate Reagent into each well.
4. Thoroughly mix for 30 seconds. It is important to have complete mixing in this step.
5. Incubate at room temperature for 45 minutes.
6. Remove the incubation mixture by flicking plate contents into a waste container.
7. Rinse and flick the microtiter wells 5 times with washing buffer (1X).
8. Strike the wells sharply onto absorbent paper to remove residual water droplets.
9. Dispense 100 µl Chemiluminescence substrate solution into each well. Gently mix for 5 seconds.
10. Read wells with a chemiluminescence microwell reader 5 minutes later. (between 5 and 20 min. after dispensed 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 bobbles existing in the wells, the false readings will be created. Please use distilled water to remove the bobbles 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 to use a proper software to calculate the results. The best curve fitting used in the assays are 4-parameter regression or cubic spline regression. If the software is not available, construct a standard curve by plotting the mean RLU obtained for each reference standard against T4 concentration in ug/dl on linear graph paper, with RLU 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 T4 in ug/dl 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.

T4 (ug/dl)	Relative Light Units (RLU) (10 ⁵)
0.0	45.70
1.0	33.38
2.5	24.26
5.0	15.01
15.0	2.31
30.0	0.98

Expected values and sensitivity

T4 EIA was performed in a study of 200 euthyroid patients in one geographic location and yielded a normal of 5.0 to 13.0 µg/dl. This range corresponds to those suggested by other commercial manufacturers. It is recommended that laboratories adjust values to reflect geographic and population differences specific to the patients they serve.

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