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CHEMILUMINESCENCE
ENZYME IMMUNOASSAY (CLIA)
ESTRADIOL (E2)

Estradiol

REF 9028-16

Enzyme Immunoassay for the Quantitative Determination of Estradiol (E2) in Human Serum or plasma

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 ELISAs that have been widely used in many research and diagnostic applications. Furthermore, with the methodological advantages, chemiluminescence immunoassay will play an important part in the diagnostic and research areas that ELISAs cannot 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.

INTRODUCTION OF E2 IMMUNOASSAY

Estradiol (E2) is a C18 steroid hormone with a phenolic A ring. This steroid hormone has a molecular weight of 272.4. It is the most potent natural Estrogen, produced mainly by the ovary, placenta, and in smaller amounts by the adrenal cortex, and the male testes. (1,2,3)
Estradiol (E2) is secreted into the blood stream where 98% of it circulates bound to sex hormone binding globulin (SHBG). To a lesser extent, it is bound to other serum proteins, such as albumin. Only a tiny fraction circulates as free hormone or in the conjugated form. (4,5) Estrogenic activity is affected via estradiol-receptor complexes, which trigger the appropriate response at the nuclear level in the target sites. These sites include the follicles, uterus, breast, vagina, urethra, hypothalamus, pituitary, and to a lesser extent, the liver and skin.
In non-pregnant women with normal menstrual cycles, estradiol secretion follows a cyclic, biphasic pattern with the highest concentration found immediately prior to ovulation. (6,7) The rising estradiol concentration is understood to exert a positive feedback influence at the level of the pituitary, where it influences the secretion of the gonadotropins, follicle stimulating hormone (FSH), and luteinizing hormone (LH), which are essential for follicular maturation and ovulation, respectively (8,9). Following ovulation, estradiol levels fall rapidly until the luteal cells become active, resulting in a secondary gentle rise and plateau of estradiol in the luteal phase. During pregnancy, maternal serum Estradiol levels increase considerably, to well above the pre-ovulatory peak levels, and high levels are sustained throughout pregnancy. (10)
Serum Estradiol measurements are a valuable index in evaluating a variety of menstrual dysfunctions, such as precocious or delayed puberty in girls(11) and primary and secondary amenorrhea and menopause.(12) Estradiol levels have been reported to be increased in patients with feminizing syndromes,(14) gynaecomastia (15) and testicular tumors. (16) In cases of infertility, serum Estradiol measurements are useful for monitoring induction of ovulation following treatment with, for example, clomiphene citrate, LH-releasing hormone (LH-RH), or exogenous gonadotropins.(17,18) During ovarian hyperstimulation for in vitro fertilization (IVF), serum estradiol concentrations are usually monitored daily for optimal timing of human chorionic gonadotropin (hCG) administration and oocyte collection.(19).
The Estradiol (E2) CLIA kits are designed for the measurement of total Estradiol in human serum or plasma.

PRINCIPLE OF THE TEST

The E2 CLIA is based on the principle of competitive binding between E2 in the test specimen and E2-HRP conjugate for a constant amount of rabbit anti-Estradiol. In the incubation, goat anti-rabbit IgG-coated wells are incubated with 25 µl E2 standards, controls, patient samples, 100 µl Estradiol-HRP Conjugate Reagent and 50 µl rabbit anti-Estradiol reagent at room temperature (18-25°C) for 90 minutes. During the incubation, a fixed amount of HRP-labeled E2 competes with the endogenous E2

in the standard, sample, or quality control serum for a fixed number of binding sites of the specific E2 antibody. Thus, the amount of E2 peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of E2 in the specimen increases. Unbound E2 peroxidase conjugate is then removed and the wells washed. A solution of chemiluminescence substrate is then added and read relative light units (RLU) with 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 E2 in the sample. By reference to a series of E2 standards assayed in the same way, the concentration of E2 in the unknown sample is quantified.

MATERIALS AND COMPONENTS

Materials Provided with Test Kit

1. Goat Anti-Rabbit IgG-coated microtiter wells, 96 wells
2. Estradiol Reference Standards: 0, 10, 30, 100, 300, and 1000 pg/ml. Liquid, 0.50 ml each, ready to use.
3. Rabbit Anti-Estradiol Reagent, 7.0 ml
4. Estradiol-HRP Conjugate Reagent , 12 ml
5. 50x Wash Buffer Concentrate, 15 ml
6. Chemiluminescence Reagent A, 6.0 ml
7. Chemiluminescence Reagent B, 6.0 ml
8. Controls, Level 1&2 (Optional)

Materials Required but not Provided

- Distilled water.
- Precision pipettes: 0.05ml, 0.1ml, 0.2ml
- Disposable pipette tips.
- Glass tube or flasks to mix Chemiluminescence Reagent A and B.
- Microtiter well luminometer.
- Vortex mixer or equivalent.
- Absorbent paper.
- Graph paper.

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.

STORAGE

1. Unopened test kits should be stored at 2-8°C upon receipt and the microtiter plate should be kept in a sealed bag with desiccants to minimize exposure to damp air. The test kit may be used throughout the expiration date of the kit (One year from the date of manufacture). Refer to the package label for the expiration date.
2. Opened test kits will remain stable until the expiring date shown, provided it is stored as prescribed above.

REAGENT PREPARATION

1. All reagents should be allowed to reach room temperature (18-25˚C) before use, 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 Concentrate (50x) with 49 volumes of distilled water. For example, dilute 15 ml of Wash Buffer Concentrate (50x) into 735 ml of distilled water to prepare 750 ml of washing buffer (1x). Mix well before use.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 25 µl of standards, specimens and controls into appropriate wells.
3. Dispense 50 µl of rabbit anti-Estradiol (E2) reagent to each well.
4. Dispense 100 µl of Estradiol-HRP Conjugate Reagent into each well.
5. ***Thoroughly mix for 30 seconds. It is very important to mix them completely***.
6. Incubate at room temperature (18-25°C) for 90 minutes.
7. Rinse and flick the microwells 5 times with washing buffer (1X).
8. Strike the wells sharply onto absorbent paper to remove residual water droplets.
9. Dispense 100 µl of 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 dispensing the substrates).

Important Note:

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

CALCULATION OF RESULTS

Calculate the average read relative light units (RLU) for each set of reference standards, control, and samples.

We recommend using proper software to calculate the results. The best fitting curve 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 E2 concentration in pg/ml on linear graph paper, with RLU on the vertical (y) axis and concentration on the horizontal (x) axis.

Using the mean absorbance value for each sample, determine the corresponding concentration of E2 in pg/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 to run the 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.

E2 (pg/ml)	Relative Light Units (RLU) (10^5)
0	15.2
10	12.5
30	9.6
100	6.7
300	3.6
1000	1.8

EXPECTED VALUES AND SENSITIVITY

Each laboratory should establish its own normal range based on the patient population. The Estradiol CLIA was performed on randomly selected outpatient clinical laboratory samples. The results of these determinations are as follows:

Males: < 60 pg/ml
Females: postmenopausal phase < 18 pg/ml
ovulating, early follicular 30-100 pg/ml
late follicular 100-400 pg/ml
luteal phase 60-150 pg/ml
pregnant, normal up to 35,000 pg/ml
prepubertal children, normal < 10 pg/ml

The minimum detectable concentration of the Estradiol CLIA assay as measured by 2 SD from the mean of a zero standard is estimated to be 5 pg/ml.

CLINICAL APPLICATION

1. Assessment of Female Menstrual Dysfunctions:

• Hyperestrogenism in girls:

Elevated E2 can be used in the evaluation of precocious puberty in girls. However, extensive ancillary aids are required for specific diagnoses.

• Hypoestrogenism in women:

E2 measurements are frequently utilized in the assessment of hypoestrogenism in cases of delayed puberty, primary and secondary amenorrhea, and menopause. In hypoestrogenism women, E2 concentrations are usually <30 pg/ml.

2. Assessment of Excessive Estrogen Production in Women:

In pregnant women, E2 concentrations will be >1,000 pg/ml**.** In non-pregnant women, excessive estrogen may indicate ovarian neoplasms.

3. Monitoring Ovulation:

E2 is often measured to monitor ovulation induction and for patient follow-up during infertility therapy, e.g. in vitro fertilization (IVF).

4. Estradiol Measurement in Male:

E2 measurement is used in the differential diagnosis gynecomastia, feminizing syndromes, hypogonadism and testicular tumors.

LIMITATIONS

There are some limitations of the assay.

1) As with all diagnostic tests, a definite clinical diagnosis should not be based on the results of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.
2) Studies have implicated possible interference in immunoassay results in some patients with known rheumatoid factor and antinuclear antibodies. Serum samples from patients who have received infusions containing mouse monoclonal antibodies for diagnostic or therapeutic purposes, may contain antibody to mouse protein (HAMA). Although we have added some agents to avoid the interferences, we cannot guarantee it will eliminate all the effects.
3) The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance. The use of tap water for washing could result in a higher background reading.

REFERENCES

1. Tsang, B.K., Armstrong, D.T. and Whitfield, J.F., Steroid biosynthesis by isolated human ovarian follicular cells *in vitro*, *J. Clin. Endocrinol. Metab.,* 1980; 51: 1407-1411.
2. Gore-Langton, R.E. and Armstrong, D.T., Follicular steroidogenesis and its control. In: Knobil, E., and Neill, J. et al., ed. *The Physiology of Reproduction*. Raven Press, New York; 1988: 331-385.
3. Hall, P.F., Testicular steroid synthesis: Organization and regulation. In: Knobil, E., and Neill, J. et al., ed. *The Physiology of Reproduction*. Raven Press, New York; 1988: 975-998.
4. Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure, W.J. and Kuhn, R.W., The serum transport of steroid hormones, *Rec. Prog. Horm. Res.,* 1982; 38: 457-510.
5. Baird, D.T., Ovarian steroid secretion and metabolism in women. In: James, V.H.T., Serio, M. and Giusti, G., eds. *The Endocrine Function of the Human Ovary.* Academic Press, New York; 1976: 125-133.
6. McNatty, K.P., Baird, D.T., Bolton, A., Chambers, P., Corker, C.S. and McLean, H., Concentration of oestrogens and androgens in human ovarian venous plasma and follicular fluid throughout the menstrual cycle, *J. Endocrinol.,* 1976; 71: 77-85.
7. Abraham, G.E., Odell, W.D., Swerdloff, R.S., and Hopper, K.,
8. Simultaneous radioimmunoassay of plasma FSH, LH, progesterone, 17-hydroxyprogesterone and estradiol-17β during the menstrual cycle, *J. Clin. Endocrinol. Metab.,* 1972; 34: 312-318.
9. March, C.M., Goebelsmann, U., Nakumara, R.M., and Mishell, D.R. Jr., Roles of estradiol and progesterone in eliciting the midcycle luteinizing hormone and follicle-stimulating hormone surges. *J. Clin. Endocrinol. Metab.,* 1979; 49: 507-513.
10. Simpson, E.R., and MacDonald, P.C., Endocrinology of pregnancy. In: Williams, R.H., ed., *Textbook of Endocrinology*. Saunders Company, Philadelphia; 1981: 412-422.
11. Jenner, M.R., Kelch, R.P., Kaplan, S.L. and Grumbach, M.M., Hormonal changes in puberty: IV. Plasma estradiol, LH, and FSH in prepubertal children, pubertal females and in precocious puberty, premature thelarche, hypogonadism and in a child with feminizing ovarian tumor. *J. Clin. Endocrinol. Metab.,* 197 2; 34: 521-530.
12. Goldstein, D., Zuckerman, H., Harpaz, S., et al., Correlation between estradiol and progesterone in cycles with luteal phase deficiency. *Fertil. Steril.*, 1982; 37: 348-354.
13. Kirschner, M.A., The role of hormones in the etiology of human breast cancer. *Cancer,* 1977; 39: 2716-2726.
14. Odell, W.D. and Swerdloff, R.S., Abnormalities of gonadal function in men. *Clin. Endocr*., 1978; 8: 149-180.
15. MacDonald, P.C., Madden, J.D., Brenner, P.F., Wilson, J.D. and Siiteri, P.K., Origin of estrogen in normal men and in women with testicular feminization, *J.Clin. Endocrinol. Metabl.*, 1979; 49: 905-916.

16. Fishel, S.B., Edwards, R.G., Purdy, J.M., Steptoe, P.C., Webster, J., Walters, E., Cohen, J., Fehilly, C. Hewitt, J., and Rowland, G., Implantation, abortion and birth after in vitro fertilization using the natural menstrual cycle or follicular stimulation with clomiphene citrate and human menopausal gonadotropin, *J. In Vitro Fertil. Embryo Transfer*, 1985; 2: 123-131.
17. Ratcliffe, W.A., Carter, G.D., Dowsett, M., et al., Oestradiol assays: applications and guidelines for the provision of a clinical biochemistry service, *Ann. Clin. Biochem*., 1988; 25:466-483.
18. Tietz, N.W. ed., *Clinical Guide to Laboratory Tests*, 3rd Edition, W.B. Saunders, Co., Philadelphia, 1995: 216-217. USA Center for Disease Control/National Institute of Health Manual, “Biosafety in Microbiological and Biomedical Laboratories”, 1984.
19. *ICN Guide to Endocrine Testing*. Diagnostic Division, ICN Biomedicals, Inc. pp. 2:15-19.

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