Revised 18 Feb. 2011 rm (Vers. 2.1) **USA: RUO**

This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

PRINCIPLE

Immunoenzymometric assay (TYPE 3):
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobile), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes palce during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-hCG 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 competition or steric hindrance to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(x\text{-}hCG)} + Ag_{hCG} + {}^{Btn}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightarrow}} {}^{Enz}Ab_{(m)} - Ag_{hCG} - {}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)
Ag_{hCG} = Native Antigen (Variable Quantity)
$^{Enz}Ab_{(x\text{-}hCG)}$ = Enzyme labeled Antibody (Excess Quantity)
$^{Enz}Ab_{(m)} - Ag_{hCG} - {}^{Btn}Ab_{(m)}$ = Ag-Anatibodies Sandwich complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$$^{Enz}Ab_{(x\text{-}hCG)}\text{-}Ag_{hCG}\text{-}{}^{Btn}Ab_{(m)} + Streptavidin_{cw} \rightarrow \text{immobilized complex}$$

$Streptavidin_{cw}$ = Streptavidin immobilized on well
Immobilized complex – sandwich complex bound to the well

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

A. hCG Calibrators – 1 ml/vial
Six (6) vials of references for hCG Antigen at levels of 0(**A**), 5(**B**), 25(**C**), 50(**D**), 100(**E**) and 250(**F**) mIU/ml. Store at 2-8°C. A preservative has been added.
Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the WHO 3rd IS (75/537).

B. hCG Enzyme Reagent – 13 ml/vial
One (1) vial containing enzyme labeled affinity purified antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Streptavidin Coated Plate – 96 wells
One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution Concentrate – 20 ml
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

E. Substrate A – 7 ml/vial
One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

F. Substrate B = 7 ml/vial
One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

G. Stop Solution – 8 ml/vial
One (1) bottle containing a strong acid (1N HCl). Store at 2-30C.

Note 1: Do not use reagents beyond kit expiry date.
Note 2: Open reagents stable for sixty (60) days when stored at 2-8°C.
Note 3: Above reagents are for a single 96-well microplate.

REQUIRED BUT NOT PROVIDED:
1. Pipette(s) capable of delivering 25 and 50 volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 ml and 0.350 ml volumes with a precision of better than 1.5%
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450 nm and 620 nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Quality control materials.

PRECAUTIONS

For Research Use Only
Not for Internal or External Use in Humans or Animals
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 Publication No. (CDC) 88-8395.

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 accurate comparison to established normal values, a fasting morning serum sample should be obtained.
The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants.
Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.
Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimens cannot be assayed within this time, the samples may be stored at temperatures of – 20°C for up to 30 days.
Avoid repeated freezing and thawing. When assayed in duplicate, 0.05 mL of the diluted specimen is required.

REAGENT PREPARATION

1. **Wash Buffer :** Dilute contents of Wash concentrate to 1000 mL with distilled or deionized water in a suitable storage container. Store at room temperature 20-27°C for up to 60 days.
2. **Working Substrate Solution** Pour the contents of the amber vial labeled Solution "A" into the clear vial labeled Solution "B". Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2-8°C.

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

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C.)

1. Format the microplates' wells for each serum reference, control and 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 0.025 mL (25 µL) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 mL (100 µL) of Working Reagent A, (T3U-enzyme solution) to all wells.
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 60 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7. Add 350 µL of wash buffer (see Reagent Preparation Section), 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 instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.
8. Add 0.100 mL (100 µL) of working substrate solution to all wells (see Reagent Preparation Section). *Always add reagents in the same order to minimize reaction time differences between wells.*
   Do not shake the plate after Substrate addition.
9. Incubate at room temperature for fifteen (15) minutes.
10. Add 0.050 mL (50 µL) of stop solution to each well and gently mix for 15-20 seconds.
    Always add reagents in the same order to minimize reaction time differences between wells.
11. Read the absorbance in each well at 450 nm (using a reference wavelength of 620-630 nm to minimize well imperfections) in a microplate reader.
    The results should be read within thirty (30) minutes of adding the stop solution.

QUALITY CONTROL

Each laboratory should assay controls at levels in the hypothyroid, euthyroid and hyperthyroid range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Human chorionic gonadotropic (hCG) in unknown specimens.

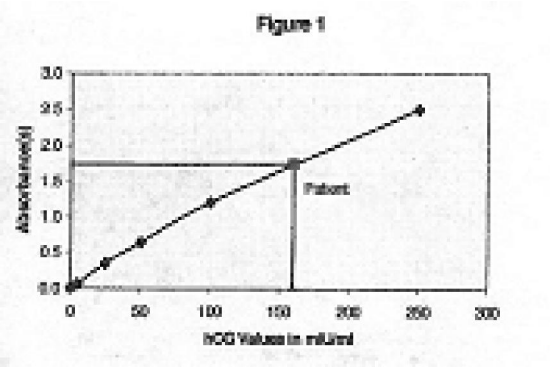
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the correspondence hCG concentration in mIU/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of hCG for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the reference response, and read the concentration (in mIU/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.745) intersects the reference curve at (157 mIU/ml) hCG concentration (See Figure 1).

Note: Computer data reduction software designed for IEMA/Elisa assays may also be used for the data reduction.

EXAMPLE I

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Absorbance
Cal A	A1 B1	0.002 0.005	0.004	0
Cal B	C1 D1	0.073 0.069	0.071	5
Cal C	E1 F1	0.340 0.360	0.350	25
Cal D	G1 H1	0.637 0.663	0.650	50
Cal E	A2 B2	1.223 1.199	1.212	100
Cal F	C2 D2	2.518 2.486	2.520	250
Ctrl 1	E2 F2	0.075 0.077	0.076	5.8
Ctrl 2	G2 H2	0.280 0.301	0.290	21.9
Sample	A3 B3	1.736 1.754	1.745	157

*The data presented in Example 1 and Figure 1 is for illustration only and should not be used in lieu of a dose response curve prepared with each assay.



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