A Solid-phase enzyme immunoassay for the quantitative determination of hCG in human blood serum or plasma.

**1.0 INDICATION**

A solid-phase enzyme immunoassay for the quantitative determination of hCG in blood serum or plasma. This kit is designed for measurement of hCG in blood serum or plasma. For possibility of use with other sample types, please, refer to Application Notes (on request). The kit contains reagents sufficient for 96 determinations and allows to analyze 41 unknown samples in duplicates.

Human chorionic gonadotropin (hCG) is a glycoprotein hormone secreted by trophoblastic cells of placenta. A molecule of hCG consists of two noncovalently bound subunits: alpha- and beta-hCG. Beta-subunit is specific for hCG hormone. Determination of hCG is widely used for early diagnosis of pregnancy. Multiple pregnancy results in correspondent elevation of serum hCG; while ectopic pregnancy and placental insufficiency cause decreased serum hCG levels. Determination of hCG in serum during second trimester is used for pregnancy monitoring, especially in screening for Down syndrome, along with other laboratory tests (AFP and Estriol). Serum hCG is also a laboratory marker of trophoblastic tumours – choriocarcinomas, some seminomas and theralomas. Serial determination of serum hCG can be used for therapy monitoring in these cancers. The present test system uses beta (β)-chain specific monoclonal antibody as the capture, and alpha (α)-chain specific monoclonal antibody as the tracer; therefore only the whole intact hCG molecule is detected.

**2.0 PRINCIPLE OF THE ASSAY**

This test is based on two-site sandwich enzyme immunoassay principle. Tested specimen is placed into the microwells coated by specific murine monoclonal to β chain of hCG-antibodies. By the antibodies coated onto the microwell surface. Second antibodies – murine monoclonal to α chain of hCG, labelled with peroxidase enzyme, are then added into the microwells. After washing procedure, the remaining enzymatic activity bound to the microwell surface is detected and quantified by addition of chromogen-substrate mixture, stop solution and photometry at 450 nm. Optical density in the microwell is directly related to the quantity of the measured analyte in the specimen.

**3.0 KIT CONTENT**

1. Reagent 1 – Microplate
   12x8 strips. Polystyrene microwells coated with murine monoclonal to β chain of hCG
2. Reagent 2 – Calibrators
   6 vials of 0.8 ml. The set contains 6 calibrators: 0; 15; 60; 125; 250; 500 IU/l.
3. Reagent 3 – Control serum (0.8 ml)
   1 vial of 0.8 ml. dilution of preselected human serum, with high content of hCG with casein solution; preservative -0,1% phenol, colourless.
4. Reagent 4 – Conjugate.
   1 vial of 11 ml. aqueous solution of murine monoclonal to α chain of hCG coupled with horseradish peroxidase diluted on phosphate buffered solution with casein from bovine milk and detergent (Tween-20), contains 0,1% phenol as preservative and red dye
5. Reagent 5 – EIA buffer ( assay buffer )
   1 vial of 12 ml. Phosphate buffered saline with casein from bovine milk and detergent (Tween-20), contains 0,1% phenol as preservative and blue dye
6. Reagent 6 – Substrate solution (TMB) .
   1 vial of 11 ml. ready-to-use single-component tetramethylbenzidine (TMB) solution.
7. Reagent 7 – Wash solution concentrated 21x.
   1 Vial of 22 ml. aqueous solution of sodium chloride and detergent (Tween 20), contains proClin300 as a preservative
8. Reagent 8 – Stop solution.
   1 vial of 11 ml. 5,0% vol/vol solution of sulphuric acid.
9. Component 9 – Plate sealing tape.
**4.0 MICROBIOLOGICAL STATE AND CLEANING GRADE**

1. All the materials of human origin resulted negative to HbsAg, HIV 1&2 and HCV FDA approved tests. Anyhow, as no test can guarantee the absolute absence of infective agents, handle reagents as potentially infected, especially standards, controls and samples. All objects come in direct contact with samples and all residuals of the assay should be treated or eliminated as potentially infected. Best procedures for inactivation are treatments with autoclave at 121°C for 30 minutes or with sodium hypochlorite at a final concentration of 2.5% for 24 hours. This last method can be used for treating the liquid washes that have to be neutralized before with sodium hydroxide.

2. Avoid any contact with skin and mucous membrane, in particular for Stop Solution.

3. Use protective disposable talk-free gloves.

4. Avoid contaminating reagents when taking them from the vials. We recommend to use automatic pipettes with disposable tips. When dispensing reagents, do not touch with tips the wall of wells in order to avoid cross-contaminations.

5. For the washing step, use only the Washing Solution provided in the kit and follow carefully the indications reported in "WASHING INSTRUCTION".

6. Avoid the substrate/chromogen to come in contact with oxidizing agents or metallic surfaces; avoid intense light exposure during incubation or reagent preparation.

**5.0 STORAGE AND STABILITY OF THE KIT**

1. The kit has to be stored at 2-8°C and used before the expiry date stated on the label.

2. Unused strips have to be placed in the bag containing the desiccant and firmly sealed before restore at 2-8°C. After opening the strips are stable up to the expiry date stated on the label.

3. All other reagents can be repeatedly used up to the expiry date stated on the label.

4. Avoid long interruptions between each step of the assay procedure.

**6.0 AUXILIARY MATERIALS**

- Semi automatic pipettes of 10, 200 and 1000 µl
- Vortex mixer and absorbent paper
- Chronometer
- Ultrapure Elisa grade water
- Photometric reader of microplates or microstrips, linear up to at least 2 OD and supplied with filter of 450 nm (620-630 nm).
- Automatic microplates washing device or manual apparatus capable of aspirating and dispensing volumes of 300 µl.

**6.0 SAMPLES**

This kit is intended for use with serum or plasma (ACD- or heparinized). Grossly hemolytic, lipemic, or turbid samples should be avoided. Specimens may be stored for up to 48 hours at 2-8°C before testing. For a longer storage, the specimens should be frozen at -20°C or lower. Repeated freezing/thawing should be avoided.

**7.0 REAGENTS PREPARATION**

- **WASHING SOLUTION**: dilute 1:21 with distilled or ELISA grade water (ex.: 22 ml of Reagent 7 + 462 ml of distilled water) and mix carefully before use. The diluted washing solution can be stored for one week at room temperature or 3 weeks at +2-8°C. It is recommended to store diluted washing solution at room temperature for immediate use.

**8.0 WASHING INSTRUCTION**

A good washing procedure is essential to obtain correct and precise analytical results. We therefore recommend to use a good quality ELISA microplate washer, maintained at a good level of washing mechanical performances.

Generally, 2-3 automatic washing cycles of 0.3 ml/well are sufficient to avoid false positive reactions and remove high background. Anyhow we recommend to calibrate the washing system on the kit itself so to match the declared analytical performances.

In case of manual washing, we suggest to perform 3 washing cycles, dispensing and aspirating 0.3 ml/well per cycle.

In any case the liquid washed out from the plates must be inactivated with a sodium hypochlorite solution at a final concentration of 2.5%, before being thrown away or autoclaved, as it must be considered as potentially infected.

**9.0 ASSAY PROCEDURE**

1. At least one hour before use, bring all reagents, standards and samples to room temperature (18-30°C), mixing them carefully on vortex.

2. Do not mix reagents from different lots.

3. We recommend to distribute standards and samples in duplicate.

4. Distribution and incubation times must be the same for all wells in the same analysis.

5. Avoid long interruptions between each step of the assay procedure.

6. It is suggested to eliminate the excess of washing solution from the microplate after washing by blotting it gently on an absorbent paper pad.

7. The colour developed in the last incubation is stable for a maximum of one hour. Otherwise, in case of reading after 10-15 min after dispensing stop solution, immediately place the strips in the dark.

8. We recommend to read the plate with an ELISA automatic reader able to subtract the background at 620-630 nm and to read the absorbance of samples and standards at 450 nm. The "blanking" of the instrument is to be carried out in the blank reagent well (well A1).
10.0 ASSAY SCHEME

Put the desired number of microstrips into the frame; allocate 14 wells for the calibrators CAL 1 - 6 and control samples CONTROL and two wells for each unknown sample. DO NOT REMOVE ADHESIVE SEALING TAPE FROM Unused STRIPS.

If suggested analyte concentration in the sample exceeds the highest calibrator, additionally dilute this sample accordingly, using DIL SPE (EIA buffer). Use of other buffers or reagents for sample dilution may lead to incorrect measurement.

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>Microplate wells coated with murine monoclonal to β chain of hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 4 (Conjugate)</td>
<td>Blank</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
</tr>
<tr>
<td>Calibrators, control</td>
<td>-</td>
</tr>
</tbody>
</table>

- Cover the strips with cardboard sealer
- Incubate 60 minutes at 37 °C

Washing

- Peel out the cardboard sealer and aspirate the reaction solution from all wells
- Rinse 5 times with 300 µl of diluted washing solution, carefully aspirating off the remaining liquid

Reagent 6 (Chromogen-Substrate) 100 µl

- Cover the strips with cardboard sealer
- Incubate 15 minutes at room temperature (22-28 °C), avoiding light exposure

Reagent 8 (Stop Solution) 100 µl

Read the absorbance of each well against Blank at 450

11.0 QUALITY CONTROL

It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. The test must be performed exactly as per the manufacturer’s instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable federal, state, and local standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test. The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications.

12.0 CALCULATION OF RESULTS

12.1. Calculate the mean absorbance values (OD450) for each pair of calibrators and samples.
12.2. Plot a calibration curve on graph paper: OD versus hCG concentration.
12.3. Determine the corresponding concentration of hCG in unknown samples from the calibration curve. Manual or computerized data reduction is applicable on this stage. Point-by-point or linear data reduction is recommended due to non-linear shape of curve.
12.4. Below is presented a typical example of a standard curve. Not for calculations!

<table>
<thead>
<tr>
<th>Calibrators</th>
<th>Value</th>
<th>AbsorbanceUnits(450nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL1</td>
<td>0IU/l</td>
<td>0.04</td>
</tr>
<tr>
<td>CAL2</td>
<td>15IU/l</td>
<td>0.15</td>
</tr>
<tr>
<td>CAL3</td>
<td>60IU/l</td>
<td>0.41</td>
</tr>
<tr>
<td>CAL4</td>
<td>125IU/l</td>
<td>0.81</td>
</tr>
<tr>
<td>CAL5</td>
<td>250IU/l</td>
<td>1.43</td>
</tr>
<tr>
<td>CAL6</td>
<td>500IU/l</td>
<td>2.36</td>
</tr>
</tbody>
</table>

13.0 EXPECTED VALUES

Therapeutical consequences should not be based on results of IVD methods alone – all available clinical and laboratory findings should be used by a physician to elaborate therapeutically measures. Each laboratory should establish its own normal range for hCG. Based on data obtained by XEMA, the following normal range is recommended (see below). NOTE: the patients that have received murine monoclonal antibodies for radioimaging or immunotherapy develop high titered anti-mouse antibodies (HAMA). The presence of these antibodies may cause false results in the present assay. Sera from HAMA positive patients should be treated with depleting adsorbents before assaying.
14.0 ANALYTICAL PERFORMANCES

14.1 Sensitivity. The lowest detectable concentration of hCG is 2.5 IU/l at the 95% confidence limit.

15.0 PRECAUTIONS IN USE

The reagents contain inactive components such as preservatives (Sodium azide or others), surfactants etc. The total concentrations of these components is lower than the limits reported by 67/548/EEC and 88/379/EEC directives about classification, packaging and labelling of dangerous substances. However, the reagents should be handled with caution, avoiding swallowing and contact with skin, eyes and mucous membranes. The use of laboratory reagents according to good laboratory practice is recommended.

Waste Management

Please refer to local legal requirements.

REFERENCES