
بسم الله الرحمن الرحيم

kingdom of Saudi Arabia
Ministry of Higher Education
king Saud University
College of Science
Biochemistry Department

Practical Note

Biomarkers in Health & Diseases

(BCH 473)

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1. Introduction to biomarkers

1.1 Identification:

Biomarkers are pharmacological and physiological measurements, or specific biochemicals in the body, that have a particular molecular feature that makes them useful for measuring the progress of disease or the effects of treatment.

1.2 Clinical significance:

Ultimately, the main goals of researches on human diseases are to cure the disorders or to increase the length and quality of life of those affected. Novel biomarker identification of different disorders will facilitate the achievement of these goals, first, by providing sensitive and selective clinical correlates for the evaluation and diagnosis of those affected and, second, by providing insights into disease mechanisms that can be used to identify therapeutic targets and to develop efficacious compounds to target them.

1.3 Properties:

Biomarkers that will be useful for either disease prediction or treatment should have one or more of several properties, including: (i) specific and selective association with illness in a population; (ii) heritability; (iii) state independence and presence, whether or not the clinical phenotype of the disease is present; (iv) co-segregation with disease within families; and (v) presence in relatives of affected individuals at a higher rate than in the general population (1,2).

1.4 Classification:

Accordingly, biomarkers have been classified as:

- Antecedent biomarkers (Identifying the risk of developing an illness).

- Screening biomarkers (Screening for subclinical disease),

-Diagnostic biomarkers (Recognizing overt disease),

-Staging biomarkers (Categorizing disease severity),

-Prognostic biomarkers (Predicting future disease course, including recurrence and response to therapy, and monitoring efficacy of therapy. (Biomarkers Definitions Working Group, 2001 (3,4)

Different methods for biomarkers measurement:

- Assay of enzymes using diagnostic kits.

- ELISA technique for identification of biomarkers.

- Assay development for genomic biomarkers (microarrays, PCR, genotyping)

- Assay development for protein biomarkers (MS, arrays, immunoassays)

- Flow cytometry and cell-based assays

- Cancer biomarker assay development (CTCs, imaging, etc.)

- Assays for single metabolite vs. panels vs. signatures

- Drug/diagnostic

1.5 References:

1- Gershon, E.S. and Goldin, L.R. (1986) Clinical methods in psychiatric genetics. I. Robustness of genetic marker investigative strategies. ActaPsychiatr. Scand. 74, 113–118.

2- Leboyer, M. et al. (1998) Psychiatric genetics: search for phenotypes. Trends Neurosci. 21, 102–105.

3-Biomarkers Definitions Working Group, 2001. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin. Pharmacol. Ther. 69, 89–95.

4-Vasan, R.S., 2006. Biomarkers of cardiovascular disease: molecular basis and practical considerations. Circulation 113, 2335–2362.

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5-Hood, L., et al., 2004. Systems biology and new technologies enable predictive and preventative medicine. Science 306, 640–643.

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2. Estimation of fasting blood glucose and two hour postprandial glucose level

INTRODUCTION:

2.1 Diagnostic significance:

Determination of glucose concentration is important in the diagnosis and treatment of disorders of carbohydrate metabolism. Values higher or lower than the reference are of diagnostic significance. The levels are increased in diabetes mellitus, hyperthyroidism and in the hyperactivity of the pituitary gland. Decreased levels are observed in cases of overproduction of insulin by the pancreas, with tumors of the pancreas, as well as with hypofunction of the organs involved in glucose synthesis and carbohydrate metabolism.

2.2 Principle:

Glucose oxidase (GOD) converts the sample Glucose into gluconate. The Hydrogenperoxide (H2O2) produced in the reaction is degraded by peroxidase (POD) and gives a colored product Phenol and 4-Aminoantipyrine which is measurable using Trinder indicator reaction at 505 nm. The increase in absorbance correlates with the glucose concentration of the sample.

Glucose + O2 GOD > Gluconic acid + H2O2

2H2O2+ Phenol + 4-Aminoantipyrine POD > Red quinone+4H2O

2.3 MATERIALS:

1. Reagent (R1)

Phosphate buffer, pH:7.5 90 mmol/l

Phenol 0.5 mmol/l

2. Reagent (R2)

Glucose oxidase 10000 U/l

Peroxidase 1000 U/l

4-Aminoantipyrine 2.5 mmol/l

3. Reagent (R3)

Standard glucose 5.56 mmol/l (1 g/l)

Sample : Serum free of haemolysis.

Procedure

Preparation and stability of working reagent :

Dissolve one vial of (R2) in appropriate amount of (R1).

Stability (in brown vial):

at 20-25 °C: 14 days

at 2-8 °C: 30 days

If the absorbance of working reagent is higher than 0.1 at 492 nm the reagent can not be used.

Assay conditions:

Wavelength : 505 nm

Temperature : 37 °C

Cuvette : 1 cm light path

Method : endpoint (increasing)

Read against: reagent blank

2.4 METHOD (assay):

Dispense 1 ml working reagent into 3 cuvettes labeled as blank, standard and sample. This will be followed by the addition of 10 µl H_2O_2 in blank, 10 µl standard glucose solution and10 µl samples in standard and sample cuvettes respectively. Mix and measure the absorbance (A) after five-minute incubation at 37°C or after ten-minute incubation at room temperature.

2.5 CALCULATIONS:

A sample/ A standard x C standard = C sample

A = Absorbance

C = Concentration

2.6 DISCUSSION:

2.7 CONCLUSION:

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3. a- Estimation of glycosylated haemoglobin in normal and diabetic patients

3.1 INTRODUCTION:

Throughout the circulatory life of the red cell, glycohemoglobin is formed continuously by the addition of glucose to the N-terminal of the hemoglobin beta chain. This process which is non-enzymatic, reflects the average exposure of hemoglobin to glucose over an extended period. In a classical study, Trivelli et al(1) showed glycohemoglobin in diabetic subjects to be elevated 2-3 fold over the levels found in normal individuals. Several investigators have recommended that glycohemoglobin serves as an indicator of metabolic control of the diabetic since glycohemoglobin levels approach normal values for diabetics in metabolic control (2,3,4).

Glycohemoglobin has been defined operationally as the "fast fraction" hemoglobins (HbAla,Alc) which elute first during column chromatography with cation-exchange resins. The non-glycosylated hemoglobin which consists of the bulk of the hemoglobin has been designated as HbAo. The present glycohemoglobin procedure employs a weak binding cation-exchange resin for the rapid separation of glycohemoglobin (fast fraction) from non-glycosylated hemoglobin.

3.1.1 Principal:

A hemolyzed preparation of the whole blood is mixed continuously for 5 minutes with a weak binding cation-exchange resin. During this time, HbAo binds to the resin. After the mixing period, a filter is used to separate the supernatant containing the glycohemoglobin from the resin. The percent glycohemoglobin is determined by measuring the absorbance at 415 nm of the glycohemoglobin fraction and the total hemoglobin fraction. The ratio of the two absorbance gives the per sent glycohemoglobin.

3.1.1.1 REAGENTS:

40 test kits include:

1. Cation-exchange resin : (120 ml) 8 mg/ml Buffered at pH 6.9. Store at room temperature. Mix well before use and after addition to each tube.

2. Lysing reagent : (20ml) 10 mM Potassium cyanide. Surfactant added. Store at room temperature.

3. Glycohaemoglobin standard (1Vial): lyophilized Human blood with Glycohemoglobin values provided in each lot. Store at 2-8□ C.

4. Serum separators : 40 Units.

3.1.1.2 Reconstitution:

Reconstitute with 1 ml of distilled water and let stand for 30 minutes with occassional mixing. Reconstituted standard may be aliquoted for future use but reconstituted aliquots must be stored frozen at -20 □ C or below and should be used within 30 days.

3.1.1.3 STABILITY:

All reagents are stable upto expiration date indicated on individual bottle label when stored as directed. Reconstituted Standard is stable upto 30 days when stored frozen at -20 □ C or below.

3.1.1.4 Precautions:

Exercise the normal precautions required for the handling of all laboratory reagents. Pipetting by mouth is not recommended for any laboratory reagent.

Lysing reagent is toxic .

Wash hands after handling. If ingested, seek medical attention immediately. Glycohemoglobin standard has been tested and found to be non-reactive for HBsAg, HCVand HIV antibody. However, it should be handled carefully as if, potentially infectious. All specimens should be considered as infectious and handled appropriately.

3.1.2 MATERIALS:

1- Cation Exchange Resin, Lysing Reagent, Glycohemoglobin Standard and Serum Separators.

2. 20 □ l and 100 □ l micropipettes.

3. 500 □ l, 3 ml and 5 ml pipettes or dispensers.

4. 13 x 100 mm glass tubes.

5 Glass or plastic test tubes to hold 0.6 ml and 5 ml.

6. Vortex mixer.

7. Glycohemoglobin controls: Normal level , Elevated level.

Specimen collection and preparation:

Special preparation of the patient is unnecessary. Fasting specimens are not required. No special additives or preservatives other than the anticoagulants are required. Collect venous blood with EDTA using aseptic technique. Glycohemoglobin in whole blood collected with EDTA is stable for one week at 2-8□ C.

3.1.3 METHOD:

A. Hemolysate Preparation

1. Dispense 500 □ l Lysing Reagent into * labeled: standard,

control, sample 1, etc.

2. Place 100 □ l of the well-mixed blood sample , standard or

control into the appropriately labeled tube. Mix.

3. Allow to stand for 5 minutes.

B. Glycohemoglobin preparation:

1. Dispense 3.0 ml of Glycohemoglobin Cation-exchange Resin into 13 x 100 mm glass tube** labeled: Standard, Control, Sample 1, etc.

2. Add 100 □ l of the hemolysate (from Step A3).

3. Position the Filter Separators in the tubes so that the rubber sleeve is approximately 1 cm above the liquid level.

4-Place the tubes on the vortex mixer and mix continuously for 5 minutes .

5. Remove the tubes from the vortex mixer.

6. Push the Filter Separators into the tubes until the resin is firmly packed.

7. The supernatant may be poured into another tube or directly into a cuvette for absorbance measurement.

8. Adjust the instrument to zero absorbance at 415 nm with deionized water as the blank.

9. Read and record the absorbance values for Standard, Control, Sample 1, etc. These readings are for glycohemoglobin.

C. Total Hemoglobin Fraction:

1. Dispense 5.0 ml deionized water into tubes* labeled. Standard, Control, Sample 1, etc.

2. Place 20 □ l of the hemolysate (from Step 3A) into the appropriately labeled tube. mix.

3. Adjust the instrument to Zero absorbance at 415 nm with deionized water as the blank.

4. Read and record the absorbance values for Standard, Control, Sample 1, etc. These readings are for total hemoglobin.

Plastic or glass tubes of appropriate sizes are acceptable.

Expected values:

1. Of healthy people and stabilized diabetics 6.5 - 8%

2. Of diabetics with metabolic imbalance or of insufficiently controlled diabetics > 9.0 %.

3.1.4 References:

1. Trivelli, L.A., Ranney, P.H., and Lai, H.T., New Eng. J. Med.284, 353 (1971).

2. Gonen, B., and Rubenstein, A.H., Diabetologia 15, 1 (1978).

3. Gabbay, K.H., Hasty, K., Breslow, J..L., Ellison, R.C., Bunn,H.F., and Gallop, P.M., J. Clin Endocrinol. Metab. 44, 859 (1977).

4.Bates, H.M., Lab Manag., Vol. 16 (Jan. 1978).

3.2 b- Measurement of glycohemoglobin in whole blood using HPLC

3.2.1 Principal:

Glycated proteins differ from non-glycated proteins by the attachment of a sugar moiety(s) at various binding sites by means of a ketoamine bond. Glycohemoglobin (GHb) thus contains 1, 2-cis-diol groups not found in non-glycated proteins. These diol groups provide the basis for separation of glycated and non-glycated components by boronate-affinity chromatography (1–3). In this analytical technique, a boronate such as phenylboronic acid is bonded to the surface of the column support. When a solution of proteins (e.g. hemolysate) is passed through the column, the glycated component is retained by the complexing of its diol groups with the boronate. After the unretained non-glycated component elutes from the column, the glycated component is eluted from the column with a reagent that displaces it from the boronate. GHb is an index of average blood glucose levels for the previous 2–3 months (5, 6). It is widely used as an indicator of glycemic control in the care and treatment of patients with diabetes mellitus.

The Primus instrument is a fully automated glycohemoglobin analyzer which utilizes the principle of boronate-affinity high performance liquid chromatography (HPLC) (4).

The analytical column contains aminophenylboronic acid bonded to a porous polymer support (gel). The low- and high-pressure pumps transfer reagents through the analytical column, with reagent selection executed by a switching valve.

Hemolyzed samples are automatically injected onto the column during the flow of A-Elution Reagent #1. The glycated component binds to the boronate, while the non-glycated component passes through the column to the spectrophotometric detector, where it is detected at wavelength of 413 ± 2 nm.

After the elution of non-glycated component, the Primus instrument pumps B-Elution Reagent #2, which displaces the glycated component from the column. The glycated component then passes through the detector. In the final stage of each sample cycle, the column is re-equilibrated with Elution A-Reagent #1. All reagent selection occurs in a timed sequence designed to allow complete elution of non-glycated and glycated components.

All functions in the liquid chromatography and computing integrator are controlled by

microprocessors (Model CLC330) or PC computer (Model CLC385). The signal from the spectrophotometric detector is processed and the concentration of glycohemoglobin is calculated as a percentage of the total detected. Integration is by peak area in millivolt-seconds.

The chromatogram is plotted first as the signal is received by the detector. The raw %GHb is calculated when glycated hemoglobin peak area is divided by the total hemoglobin peak area. Primus HPLC uses two point calibrators with HbA1c assigned values to obtain a final standardized GHb. The Schiff base does not interfere with boronate affinity method. The report is then printed with the sample information, raw GHb and standardized GHb results.

Sample:

Whole blood with anticoagulant, preferably K3EDTA at a concentration of 1.5 mg/mL whole blood. The optimal amount of specimen is 0.5 mL; minimum amount is 100 μL (0.1 mL).

3.2.2 MATERIALS:

(1) All reagents and supplies listed below are supplied by Primus Corp (Kansas City, MO)

Primus I (CLC330)

Primus IV (CLC385)

GHb diluent/hemolysis reagent

Ultra-pure filtered HPLC Grade water with 0.001% Sodium Azide (DIL)

Ultra-pure filtered HPLC Grade water with 0.001% Sodium Azide and detergent (DIL2).

Elution reagent #1

Acetate – Salt buffer in 5% Alcohol plus 1% Urea, pH 9.

Acetate – Salt buffer in 5% Alcohol plus 1% Urea, pH 9.

Elution reagent #2

Polyol – Salt in 5% Alcohol, pH 7

Polyol – Salt in 5% Alcohol, pH 7

Wash solution

Ultra-pure filtered HPLC grade water with 0.001% sodium azide in 5%

Ultra-pure filtered HPLC grade water with 0.001% sodium azide in 5%

Software

workdisk

Windows based program

Analytical column

aminophenylboronic acid bonded to a porous polymer support

aminophenylboronic acid bonded to a porous polymer support

Sample vials

Glass vials with caps

plastic vials without caps

Procedure and Operating Instructions:

(1) Preliminaries:

(a) All reagents should be at room temperature before assay.

(b) Allow frozen calibrators, QC specimens, and any frozen blood samples to thaw. Mix all samples at least ten minutes on a rocker before preparing them.

(c) Use a permanent marker to label sample vials with the corresponding sample identification MU accession numbers.

(2) Sample preparation: Using the Bio-Rad autodiluter, prepare control and patient hemolysates by making a 5:1000 dilution (WB to diluent) for Primus I and 5:500 dilution for Primus IV. Use the following procedure:

(a) Make sure that appropriate volume control rod is selected for the autodiluter. Use DIL2 reagent for Primus IV and Primus I.

(b) Insert tip of autodilutor into blood specimen and press the button on top of the handle to draw 5 μL of specimen into the tip.

(c) Wipe the tip clean with wet gauze and insert the tip into the corresponding sample vial. Press button again to dispense sample and reagent into the sample vial.

(d) Repeat procedure for all NHANES specimens and QC samples.

(e) If Primus I is used, cap the vial and mix thoroughly too completely lyse cells. Primus IV vials do not require mixing.

(3) Instrument setup for the Primus CLC330 HPLC System (Primus I):

(a) Press the "System On" button on the front panel. The instrument will perform a self-test and display a series message. After a successful self-test, the following message will be displayed: "HP 1090L system with INET on".

(b) Press the power switch on the rear panel of the disk drives. Wait for the disk drive to finish its start-up routine.

(c) Press the power switch on the left rear panel of the integrator to the "On" position. When the integrator completes its self-test, "LOOP UP" will be printed.

(d) Enter the date: "DA mm/dd/yyyy".

(e) Enter the time: "TI hh:mm:ss".

(f) Enter BASIC mode by typing "BA".

(g) Set the top of the form by pressing [shift] + [Enter] until the paper perforation is about an inch above the print head. Then press [Ctrl] + [k].

(h) At the > prompt, type "run a:hplc". The integrator will print out the date and time with a banner of the program name and version of the software, the last shutdown date and time, and the methods that are available.

(i) The main menu will appear.

(j) The instrument will go through a 30 minute warm up period before any samples can be analyzed. Equilibrate the column by running four dummy samples before analyzing the first sample of the day.

(k) Place sample vials in the Primus I autosampler racks in the following order:

(l) One quality control specimen in position 1 in rack #1.

(m) Patient samples in subsequent racks with one QC specimen (alternating high and low

(n) QC) after every 19th patient specimen and at the end of the run.

Calculations:

(a) All calculations are performed by the Primus HPLC system.

(b) Calculation of the percentage of GHb in the sample is by the following formula:

Area of Peak 2 (GHb) x 100

(Area of Peak 1 (Non-GHb) + Area of Peak 2 (GHb))

(c) Retest any specimens with % Std GHB values less than 4.0 % or greater than 14%.

3.2.3 **DISCUSSION:**

3.2.4 **CONCLUSION:**

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4. Biomarkers of Diabetes mellitus

Determination of insulin level using Elisa

4.1 INTRODUCTION:

Insulin is the principal hormone responsible for the control of glucose metabolism. It is synthesized in the ß-cells of the islets of Langerhans as the precursor, proinsulin, which is processed to form C-peptide and insulin. Both are secreted in equimolar amounts into the portal circulation. The mature insulin molecule comprises two polypeptide chains, the A chain and B chain (21 and 30 amino acids respectively). The two chains are linked together by two inter-chain disulphide bridges. There is also an intra-chain disulphide bridge in the A chain.

Insulin concentrations are severely reduced in insulin-dependent diabetes mellitus (IDDM) and some other conditions such as hypopituitarism.

Insulin levels are raised in non-insulin-dependent diabetes mellitus (NIDDM), obesity, insulinoma and some endocrine dysfunctions such as Cushing's syndrome and acromegaly. These signs can be detected with the Insulin blood test.

4.2 INTENDED USE OF THE INSULIN ELISA:

The Insulin ELISA Kit is an enzyme immunoassay intended for the quantitative determination of insulin in human serum and plasma. Each kit contains all necessary reagents and one microplate for 96 tests. The assay is based on a flexible microplate strip format (8 x 1) and the kit comprises two monoclonal antibodies. The kit shows excellent precision, sensitivity and specificity, is easy to use and requires only non-dedicated instrumentation.

Objective of the Experiment:

Development of an ELISA for human insulin that utilizes monoclonal antibodies (mAbs).

4.3 Test Principle:

The Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with enzyme (HRP)-conjugated anti-insulin antibody and anti-insulin antibody bound to micro-titration well. A simple washing step removes unbound enzyme labeled antibody. In the insulin ELISA, the bound HRP complex is detected by reaction with TMB substrate. The reaction is stopped by adding acid to give a colorimetric endpoint that is read using ELISA reader.

This assay is a Sandwich ELISA based, sequentially, on:

1) capture of human insulin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of monoclonal insulin antibody and the binding of a second biotinylated monoclonal insulin antibody to capture insulin,

2) wash away of unbound materials from samples,

3) conjugation of horseradish peroxidase to the immobilized biotinylated antibodies,

4) wash away the free enzyme conjugates, and

5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish

peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm after acidification of formed products.

Since the increase in absorbency is directly proportional to the amount of captured human insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human insulin.

4.4 METHODS AND MATERIALS:

REAGENTS SUPPLIED Each kit is sufficient to run one 96-well plate and contains the following reagents:

A. Human Insulin ELISA Plate

Coated with Monoclonal Insulin Antibody

Quantity: 1 plate

Preparation: Ready to Use

Note: Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C.

B. Adhesive Plate Sealer

Quantity: 2 sheets

Preparation: Ready to Use

C. 10X HRP Wash Buffer Concentrate

10X concentrate of 50 mM Tris Buffered Saline containing Tween-20

Quantity: Two bottles containing 50 mL each

Preparation: Dilute 1:10 with distilled or deionized water

D. Human Insulin Standards

Human Insulin in Buffer: 2, 5 10, 20, 50, 100, and 200 μU/mL

Quantity: 0.5mL/bottle

Preparation: Ready to Use

E. Quality Controls 1 and 2

Purified Recombinant Human Insulin in Assay Buffer

Quantity: 0.5mL/bottle

Preparation: Ready to Use

F. Matrix Solution

Treated Human Serum

Quantity: 1.0 mL/vial

Preparation: Ready to Use

G. Assay Buffer

0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.05% ProClin300, and 0.1% Human Serum Albumin

Quantity: 10 mL/vial

Preparation: Ready to Use

H. Human Insulin Detection Antibody

Pre-titered Biotinylated Monoclonal Insulin Antibody

Quantity: 12 mL/vial

Preparation: Ready to Use

I. Enzyme Dilution Buffer

0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.05% ProClin300, and 0.1% Human Serum Albumin

Quantity: 12 mL/vial

Preparation: Ready to Use

J. Concentrated Enzyme Solution

Concentrated Streptavidin-Horseradish Peroxidase Conjugate

Quantity: 0.5 mL/vial

Preparation: Just prior to use, dilute Enzyme Solution 40 fold by mixing 0.3 mL Enzyme Solution with 11.7 mL Enzyme Dilution Buffer.

K. Substrate

3, 3',5,5'-tetramethylbenzidine in Buffer

Quantity: 12 mL/vial

Preparation: Ready to use.

Minimize the exposure to light.

L. Stop Solution

0.3 M HCl

Quantity: 12 mL/vial

Preparation: Ready to Use [Caution: Corrosive Solution]

4.5 STORAGE AND STABILITY:

Upon receipt, all components of the kit should be stored at 2-8 oC. For longer storage, freeze diluted Wash Buffer, Matrix Solution, Insulin Standards and Controls at ≤ –20 ^{o}C. Avoid multiple freeze/thaw cycles of the Insulin Standards and Matrix Solution. Unused microtiter strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

4.6 REAGENT PRECAUTIONS:

A. Human Serum Albumin

The Human Serum Albumin (HSA) used in the preparation of this product is made from human serum and has the potential for bloodborne pathogens; strict adherence to a Bloodborne Pathogen Exposure Control Plan must be followed. Do not get in eyes, on skin, on clothing. Personal protective equipment must be worn when handling this material. The area must be decontaminated with 10% bleach and alcohol after preparation.

B. Hydrochloric Acid

Hydrochloric Acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

4.7 MATERIALS REQUIRED BUT NOT PROVIDED:

1. Pipettes and Pipette Tips: 10µl - 20 µl or 20µl - 100 µl

2. Multi-Channel Pipettes and Pipette Tips: 5 ~ 50 µl and 50 ~ 300 µl

3. Buffer and Reagent Reservoirs

4. Vortex Mixer

5. Deionized Water

6. Microtiter Plate Reader capable of reading absorbency at 450 nm

7. Orbital Microtiter Plate Shaker

8. Absorbent Paper or Cloth

4.8 SAMPLE COLLECTION AND STORAGE:

1. To prepare serum samples, whole blood is directly drawn into a Vacutainer® serum tube that contains no anticoagulant. Let blood clot at room temperature for 30 min.

2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2C.

3. Transfer and store serum samples in separate tubes. Date and identify each sample.

4. Use freshly prepared serum or store samples in aliquots at ≤ –20C for later use. Avoid freeze/thaw cycles.

5. To prepare plasma samples, whole blood should be collected into Vacutainer® EDTA-plasma tubes and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.

6. If heparin is to be used as an anticoagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.

7. Avoid using samples with gross hemolysis or lipemia.

4.9 ASSAY PROCEDURE:

Pre-warm all reagents to room temperature immediately before setting up the assay.

1. Dilute the 10X concentrated Wash Buffer 10 fold by mixing the entire contents of both buffer bottles with 900 mL deionized or distilled water.

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should

be resealed in the foil pouch and stored at 2-8°C. Assemble strips in an empty plate holder and fill each well with 300 µL of diluted Wash Buffer. Incubate at room temperature for 5 minutes. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Wash the plate one more time with 300 µL of Wash Buffer. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.

3. Add 80 µL Assay Buffer to the blank wells and sample wells. Add 60 µL Assay Buffer to Standard wells, and QC1 and QC2 wells.

4. If samples to be assayed are serum or plasma, add 20 µL Matrix Solution to the Blank, Standard, and Control (QC1 and QC2) wells.

5. Add in duplicate 20 µL Human Insulin Standards in the order of ascending concentration to the appropriate wells.

6. Add 20 µL QC1 and 20 µL QC2 to the appropriate wells.

7. Add sequentially 20 µL of the unknown samples in duplicate to the remaining wells.

For best result all additions should be completed within one hour.

Cover the plate with plate sealer and incubate at room temperature for 90 minutes on an orbital microtiter plate shaker set to rotate at moderate speed (approximately 400 to 500 rpm).

8. Remove plate sealer and decant solutions from the plate. Tap as before to remove

residual solutions in the wells.

9. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and

tap after each wash to remove residual buffer.

10. Add 100 µL Human Insulin Detection Antibody to each well. Cover the plate

with sealer and incubate with moderate shaking at room temperature for 1 hour on the microtiter plate shaker.

11. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.

12. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

13. Add 100 µL of 40 fold diluted Concentrated Enzyme Solution to each well.

IMPORTANT: Dilute the concentrated enzyme solution just prior to use.

Cover the plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

14. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.

15. Wash wells 5 times with diluted Wash Buffer, 300 µL per well per wash. Decant and

tap after each wash to remove residual buffer.

16. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for approximately 20-30 minutes.

Blue color should be formed in wells of insulin standards with intensity proportional to increasing concentrations of insulin.

NOTE: Please be aware that the blue color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

17. Remove sealer and add 100 µL Stop Solution [CAUTION: CORROSIVE

SOLUTION] and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification.

Read absorbance at 450 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well.

The absorbance of highest insulin standard should be approximately 1.7- 3.0.

4.10 CALCULATIONS:

The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function. [Note: When sample volumes assayed differ from 20 µL,

an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 µL, compensate the volume deficit with either matrix solution or assay buffer, whichever is appropriate.

4.11 INTERPRETATION:

1. The run will be considered accepted when all Quality Control values fall within the calculated Quality Control Range, if any QC's fall outside the control range, review results with the supervisor.

2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.

3. The limit of sensitivity of this assay is 2 µU/mL human insulin (20 µL sample size).

4. The appropriate range of this assay is 2 µU/mL to 200 µU/mL human insulin (20 µL sample size). Any result greater than 200 µU/mL in a 20 µL sample should be repeated on dilution using either matrix solution or assay buffer as diluent, whichever is appropriate, until the results fall within range.

4.12 ASSAY CHARACTERISTICS:

A. Sensitivity The lowest level of Insulin that can be detected by this assay is 0.73 uU/mL when using a 20 µL sample size.

B. Specificity The specificity (also known as selectivity) of the analytical test is its ability to selectively measure the analytes in the presence of other like components in the sample matrix.

Human Insulin 100%

Human Proinsulin n.d.**

Human C-peptide n.d.*

n.d.: not detectable at concentrations up to * - 20 ng/mL; and ** - 2 ng/mL.

QUALITY CONTROLS:

The ranges for Quality Control 1 and 2 are provided on the card insert.

4.13 TROUBLESHOOTING GUIDE:

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.

2. Throughout the assay the operator should adhere strictly to the procedures with good

laboratory practice.

3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.

4. Avoid cross contamination of any reagents or samples to be used in the assay.

5. Make sure all reagents and samples are added to the bottom of each well.

6. Careful and complete mixing of solutions in the well is critical. Poor assay

precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.

7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.

8. Do not let the absorbancy reading of the highest standard reach 3.0 units or higher after acidification.

9. High absorbance in background or blank wells could be due to

1) cross well contamination by standard solution or sample

or 2) inadequate washing of wells with TBS.

Case studies:

- Hyperinsulinaemia and insulin resistance.

4.14 DISCUSSIONS and CONCLUSIONS:

4.15 QUESTIONS:

1-Can we keep antibody-coated micro-wells in storage? How long can they be stored and at what conditions?

2- What is the source of the enzyme conjugate used in ELISA?

3- What is the enzyme substrate used in ELISA?

4- Should the samples be stored at 4°C or should they be frozen?

5-How can you stop the enzyme reaction in the ELISA?

6- What temperature is used for the ELISA incubations?

4.16 References:

Megha S. Even, Chad B. Sandusky, Neal D. Barnard, Jehangir Mistry and Madhur K. Sinha. Development of a novel ELISA for human insulin using monoclonal antibodies produced in serum-free cell culture medium. Clinical Biochemistry, Volume 40, Issues 1-2, January 2007, Pages 98-103.

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5. Cardiovascular Biomarkers

Estimation of plasma insulin resistance

Quantitative determination of total cholesterol in serum / plasma by enzymatic color/endpoint method.

5.1 INTRODUCTION:

5.2 Diagnostic Significance:

Cholesterol is a fatty substance found in blood, bile and brain tissues. It serves as a precursor to bile acids, steriods and vitamin D. The determination of serum cholesterol is a major aid in the diagnosis and classification of lipemias(1). Other conditions such as hepatic and thyroid diseases influence cholesterol levels(2).

Expected Values: (3)

It is strongly recommended that each laboratory should establish its own normal range.

RISK CLASSIFICATION TOTAL CHOLESTEROL (mg/dL)

Desirable <200

Borderline High 200 - 239

High >240

5.3 Principal:

Enzymatic methods, involving cholesterol esterase and oxidase and Trinders color system have replaced older methodologies. Allain et al developed a total enzymatic technique in which hydrogen peroxide during the oxidation of cholesterol is used in conjunction with peroxidase, 4-aminoantipyrine and phenol to form a quinoneimine dye(4) that absorbs light which is measured at 505 nm.

The enzymatic reaction sequence employed in the assay of cholesterol is as follows:

Cholesterol Esters C. ESTERASE > Cholesterol + Fatty Acids

Cholesterol + O2 C. OXIDASE > Cholesten-3-one + H2O2

2 H2O2 + 4-Aminoantipyrine + Phenol PEROXIDASE > Quinoneimine + 4 H2O

(red dye)

Cholesterol Esters are hydrolyzed to produce cholesterol, Hydrogen peroxide is then produced from the oxidation of cholesterol by cholesterol oxidase. In a coupled reaction catalyzed by peroxidase, quinoneimine red colored dye is formed from 4-aminoantipyrine, phenol and hydrogen peroxide. The absorption of light at 505 + 5 nm of the solution of this dye is proportional to the concentration of cholesterol in the sample.

5.4 Reagents:

1. CHOLESTEROL (LIQUID) ENZYMATIC REAGENT :

4-Aminoantipyrine 0.6 mM, Cholesterol Esterase >150 U/L, Cholesterol Oxidase 200 U/L, Horseradish Peroxidase >1000 U/L, Phenol 25 mmol/L, Sodium Cholate 0.5 mmol/L, Surfactant, Non-reactive stabilizers and fillers.

2. CHOLESTEROL STANDARD (200 mg/dL or 5.17 mmol/L) :

Cholestrol in alcohol. Keep tightly capped and store at 2-8 □ C

WARNING AND PRECAUTIONS

1. For in vitro diagnostic use

CAUTION : Cholesterol (Liquid) Enzymatic Reagent contains Phenol. Avoid contact.

2. Specimen should be considered as infectious; handle appropriately.

3. Use distilled or deionized water where indicated.

SPECIMEN

SERUM / HEPARINIZED PLASMA.

1. Test specimen should be free from hemolysis.

2. Cholesterol in serum /plasma is reported as stable for seven (7) days at room temperature (18 - 25 □ C) and six (6) months when frozen and properly protected against evaporation(5).

Pipette into clean dry test tubes:

BLANK

STANDARD

TEST

Cholesterol(Liquid)Enzymatic

Reagent

1.0 ml

1.0 ml

1.0 ml

Pre-warm at 37^{o} C for 3 minutes and add :

Standard	Sample
--	--
0.01 ml	--
--	0.01 ml

Mix and incubate at 37 oC for 10 minutes. Read the absorbance of standard and test at 505 + 5 nm against blank.

NOTE :

If the spectrophotometer being used requires a final volume greater than 1.0 ml for accurate reading, use 0.025 ml (25 □ l) of sample to 2.5 ml of reagent. Perform the test as described above.

5.5 CALCULATIONS:

(A = Absorbance)

A(TEST) X Conc. of Standard = Conc. of TEST

A(Standard) (mg/dL) (mg/dL)

EXAMPLE : A(TEST) = 0.40, A(STANDARD) = 0.32,

Concentration of Standard = 200 mg/dL

5.6 References:

1. Beaumount, J.L., Crison, L.A., Cooper, G.R., Feifar, Z., Frederickson, D.S., and Strasser, T.; "Classfication of Hyperlipidemias and Hyperlipoproteinemias". Standard Methods of Clinical Chemistry vol. 9, Academic Press, New York, NY (1972).

2. Holvey, D.N., ed. The Merck Manual of Diagnosis and Therapy.

3. Naito, H.K., et al., Clin. Chem. 20:193 (1988).

4. Allain, C.C., et al., Clin Chem. 20:470 (1974).

6. Quantitative determination of the triglycerides in serum or plasma.

6.1 Diagnostic Significance:

Elevated levels of both cholesterol and triglycerides in plasma have been identified as risk factors related to atherosclerotic disease. The hyperlipidemias can be inherited traits or they can be secondary to a variety of disorders or diseases including diabetes-mellitus, nephrosis, biliary obstruction and metabolic disorders associated with endocrine disturbances. The Levels of cholesterol and triglycerides in plasma can vary independently. Therefore, an evaluation of hyperlipidemias should include the determinations for both of these lipids(1).

Range of expected values in serum (2):

Normal distributions vary with age and according to Fredrickson(2), the following concentrations if exceeded, clearly indicate hyperlipidemia.

0 - 29 years ..... 10 - 140 mg/dL

30 - 39 years..... 10 - 150 mg/dL

40 - 49 years..... 10 - 160 mg/dL

50 - 59 years ..... 10 - 190 mg/dL

As with all "normal" values these values should be checked in each laboratory

6.2 Principle:

Standard methods for the measurement of triglycerides concentration have involved either an enzymatic or an alkaline hydrolysis to liberate glycerol. This formulation makes use of the enzymatic hydrolysis and quantification since it is specific and not subject to interference by phospholipids(3).

The enzymatic reaction sequence employed in the assay of Triglycerides is as follows:

Triglycerides Lipase > Glycerol + Fatty Acids

Glycerol + ATP Glycerol Kinase > Glycerol-1-Phosphate + ADP

Glycerol-1-Phosphate + O2 GPO > DAP + H2O2

H2O2 + 4AAP + TOOS Peroxidase > Quinoneimine Dye + 2H2O

The present procedure involves hydrolysis of triglycerides by lipase. The glycerol concentration is then determined by enzymatic assay coupled with Trinder reaction that terminates in the formation of a quinoneomine dye. The amount of the dye formed, determined by its absorption at 546 nm is directly proportional to the concentration of triglycerides present in the sample(4, 5).

Reagents Composition:

R1. Triglycerides buffer reagent : Pipes 50 mM, pH 7.1 + 0.1. and stabilizers.

R2. Triglyceride enzyme reagent : ATP 3.3 mM, Magnesium Salt 3 mM, 4-Aminoantipyrine 0.7 mM, Toos 0.8 mM, Glycero-1-Phosphate Oxidase 7000 U/L, Sodium Azide 0.01% , Lipase 200,000 U/L, Glycerol Kinase 1000 U/L and Peroxidase 3,000 U/L.

R3. Triglyceride standard: (200 mg/dL as Triolein) : 2.258 mmol/L Glycerol with Surfactant. Sodium azide 0.01% added as a preservative.

Reagent preparation:

Prepare working reagent by adding 1 volume of Tg-Enzyme Reagent (R2) to 9 volumes of Tg-Buffer Reagent (R1). The prepared working reagent is stable for 21days when stored at 2-8 oC and protected from light.

6.3 Sample:

SERUM

1. Fresh, non-hemolyzed serum from fasting patients is recommended.

2. Triglycerides in serum appears stable for three (3) days when stored at 2-8 □ C(6).

3. Prolonged storage of the samples at room temperature is not recommended since other glycerol containing compounds may hydrolyze, releasing free glycerol with an apparent increase in total triglycerides content.

4. Blood collection devices lubricated with glycerin (glycerol) should not be used.

Procedure:

Pipette into clean dry test tubes:

BLANK

STANDARD

TEST

Working Reagent

1.0 ml

1.0 ml

1.0 ml

Pre-warm at 37 □ C and add

Standard

Sample

--

--

0.01 ml

--

--

0.01 ml

Mix and incubate at 37 □ C for 10 minutes. Read the absorbance of standard and sample at 546 + 5 nm against blank.

Note: Final color is stable for 15 minutes at room temperature.

6.4 CALCULATIONS:

A=Absorbance

A (Test) x Conc of st. = Conc of test

A (Standard) (mg/dL) (mg/dL)

Example : 0.24 x 200 mg/dL = 154. 8 mg/dL

0.31

NOTE : To obtain the results in SI unit (mmol/L) multiply the results in mg/dL by 10 to convert dL to liter and divide the value by 885, (the molecular weight of triglycerides as triolein).

Example : 154.8 mg/dL x 10 = 1.75 mmol/L

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7. Cardiac enzymes (creatine kinase and aspartate transaminase in serum)

Quantitative determination of creatine kinase (E.C.2.7.3.2) activity in serum/heparinized plasma or EDTA plasma using a UV kinetic method.

7.1 Diagnostic significance:

Creatine Kinase (CK) plays an important role in the energy storing mechanism of tissue by catalyzing the reversible reaction between creatine and ATP to form creatine phosphate and ADP. CK is distributed in various organs; the highest activities (in decreasing order) are skeletal muscle, heart and brain(1). Thus determination of CK is an aid to diagnosing muscular dystrophy and other diseases of the skeletal muscles, myocardial infarction, hypothyroidism, renal diseases and/or disfunction(2).

Range of expected values in serum:

It is strongly recommended that each laboratory should establish its own normal range.

7.2 INTRODUCTION:

The earlier procedure for determining CK was based on the rate of ATP formation(3). A modified method was described by Nielson by adding a sulfhydryl compound and AMP to assure maximum CK activity and inhibit adenylate kinase activity(4). Optimized conditions for measuring CK were published by Szasz in 1976 as well as by the Scandinavian Committee on enzymes(5, 6). The above procedure was modified again in 1979 to include EDTA(7). The UDI CK-NAC reagent is a modification of the above revision.

Reaction Sequence:

Creatine Phosphate + ADP < CK > Creatine + ATP

ATP + D-Glucose < HK > Glucose-6-Phosphate + ADP

Glucose-6-Phosphate + NADP < G-6-PDH > 6-Phosphogluconate + NADPH + H+

7.3 Reagents:

1. CK buffer : 100 mmol/L Imidazole buffer (pH 6.7 + 0.05 at 25 oC; also contains EDTA 2 mmol/L, Magnesium acetate tetrahydrate 20 mmol and Stabilizers. Must be kept tightly capped and protected from contamination.

2. CK- zyme- reagent : (concentration based upon reconstitution) 30 mmol/L Creatine phosphate, 20 mmol/L D- Glucose, 20 mmol/L N-Acetyl-L-Cysteine, 2 mmol/L Adenosine diphosphate, 2 mmol/L Adenosine monophosphate, 2 mmol/L NAD, > 2000 U/L Hexokinase

ASSAY TEMP

37 ^{o}C

Men

Women

24 -190 U/L

24 -170 U/L

from yeast, > 1000 U/L Glucose 6-phosphate dehydrogenase from L. mesenteroides and also contains Filler and Stabilizer. Keep tightly capped and protected from contamination.

7.4 Reconstitution:

Reconstitute with the amount of CK buffer specified on the individual bottle label. Stable for approximately 7 days after reconstitution when stored at 2-8 oC or for approximately one month when stored at -18 oC. However, this reagent should not be repeatedly frozen and thawed.

Procedure conditions:

Wavelength .... 340 nm

Reaction Type . Delta Kinetics with factor

Units ..... U/L

Factor .....6592 (Semimicro Method) & 4984 (Macro Method)

Incubation Time ... 120 Seconds

Interval Time.....60 Seconds

No. of Intervals 3

Temperature ..... 37 □ C

Reaction Slope ..... .Increasing

Reagent

Volume added

Reconstituted CK Zyme Reagent

3.0 ml

Pre-warm at 37 □ C, and add:

Sample

100 □ l

Mix and incubate at 37 □ C for 2 minutes and record the absorbance of the sample at 340 nm against reagent grade water. Read the absorbance again after exactly 1, 2 and 3 minutes and determine □ A/min.

7.5 CALCULATIONS:

□ A/min x 4984 = CK Activity (IU/L)

NOTE : If rates greater than 0.12 □ A/minute are observed, make an appropriate serum dilution with 0.9% saline and repeat the assay. Multiply the results by appropriate dilution factor.

Unit definition:

1 IU/L of CK activity is that amount of enzyme which transfers 1 □ mol of phosphate from creatine phosphate to ADP per minute per litre of sample with concurrent reduction of 1 □ mol of NAD under

IU/L = □ A/min x TV x 1000 x 1000

□ x d x SV

where:

□ A/min = Change in absorbance per minute,

TV = Total volume of reaction mixture (ml),

"1000" is a Conversion factor to convert "ml-to-liter"

and "1000" is a conversion factor to convert "millimoles-to-micromoles",

□ = Molar absorptivity of NADH at 340 nm = 6.22 x 103,

d = Light path in cm, SV = Sample volume (ml).

Example : If the □ A/min = 0.035, TV = 1.025 ml, d = l-cm, SV = 0.025 ml, absorbance measurement taken at 340 nm; then :

IU/L = □ A/min x 6592 = 0.035 x 6592 = 230.7 or

0.035 x 1.025 x 1000 x 1000

---------- = 230.7 U/L

6.22 x 103 x 1 x 0.025

II- Quantitative determination of Aspartate Aminotransferase (AST) in serum

7.6 DIAGNOSTIC SIGNIFICANCE

Serum aspartate aminotransferase (AST) also known as serum glutamic oxaloacetic transaminase (SGOT) is a tissue enzyme that catalyzes the exchange of amino and keto groups between alpha-amino acids and alpha-keto acids. AST is widely distributed in tissues, principally cardiac, hepatic, muscle and kidney. Injury to these tissues results in the release of the AST (SGOT) enzyme to general circulation. Following a myocardial infarction, serum levels of AST (SGOT) are elevated and reach a peak 48 to 60 hours after onset. Hepatobiliary diseases such as cirrhosis, metastatic carcinoma and viral hepatitis also will increase serum AST levels(1).

RANGE OF EXPECTED VALUES IN SERUM

ASSAY TEMP. 30 oC 37 oC

MEN upto 26 IU/L upto 37 IU/L

WOMEN upto 21 IU/L upto 31 IU/L

7.7 Principle:

The first kinetic assay of AST for diagnostic purposes was described by Karmen et al. in 1955, using a coupled reaction of malate dehydrogenase (MDH) and NADH(2) . This assay system was critically evaluated and optimized in 1960 by Henry et al (3).. In 1977 the International Federation of Clinical Chemistry recommended a reference procedure for the measurement of AST activity based upon Karmen's procedure(4). The AST reagent applies the formulation recommended by the IFCC.

The enzymatic reaction sequence employed in the assay of aspartate aminotransferase is as follows:

L-Aspartate + 2-Oxoglutarate AST > Oxaloacetate + L-Glutamate

Oxaloacetate + NADH + H+ MDH > L-Malate + NAD+ + H2O

AST catalyzes the transfer of an amino group between L-aspartate and 2-Oxoglutarate. The oxaloacetate formed in the first reaction is then reacted with NADH in the presence of malate dehydrogenase (MDH) to form NAD. AST activity is determined by measuring the rate of oxidation of NADH at 340nm. Lactate dehydrogenase is included in the reagent to convert endogenous pyruvate in the sample to lactate during the lag phase prior to measurement.

7.8 Reagent composition:

1. AST buffer : 100 mM Tris, 200mM Pot. Aspartate, 12 mM 2-Oxoglutarate, with Preservative; pH 7.8 + 0.05 (25 oC)

2. AST substrate(kinetic) : NADH 0.19 mM, LDH 800 U/L, MDH 600 U/L, Non-reactive Preservatives and Fillers.

Preparation of working reagent : Mix 1.5 ml of AST Substrate with 1 vial (13.5 ml) of AST Buffer. Swirl gently to dissolve. Don't agitate vigourously. Stable for 7 days if kept at 2-8 oC.

Materials provided:

AST Buffer, AST Substrate (Kinetic)

Sample: Serum

Procedure:

Pipette into a clean cuvette:

AST Working Reagent 1.0 ml

Pre-warm to 30 oC /37 oC, then add:

Sample 0.1 ml

Mix and incubate at 30 oC /37 oC for 1 minute and read the absorbance at 340 nm against dist.water. Take the absorbance every minute for the next 2 min. and determine □ A/Min.

7.9 CALCULATIONS:

□ A/Min X 1768 = AST ACTIVITY (IU/L)

Example : If the average absorbance change per minute of a sample at 37 □ C is 0.028, then its AST activity at 37 oC would be 0.028 x 1768 = 49.5 IU/L.

The following formula was used to calculate the factor.

AST activity in U/L

= □ A/min x TV x 1000

□ x SV x d

Where :

□ A/min = Averageabs. change per minute

TV = Total reaction volume (ml)

1000 = Conversion factor for IU/ml to IU/L

SV = Sample volume

d = Light path in cm.

□ = Molar absorbtivity of NADH at 340 nm (6.22 x 103 L x mole-1 cm-1)

i.e:

□ A/min x 1.1 x 1000 = □ A/min x 1768

6.22 x 0.1 x 1

7.10 References:

1. Henry, J.B.,: Clinical Diagnosis and Management by Laboratory Methods, W.B. Saunders and Co., Philadelphia, PA. p332-35 (1974).

2. Karmen, A., et al.: J. Clin. Invest, 34:126 (1955).

3. Henry, R.J. et al.: Amer. J. Clin. Path. 34:381 (1960).

4. The Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology. Scan. J. Clin. Lab. Invest. 32:291 (1974).

Name:

ID #:

Title of the experiment

8. Prostate Specific Antigen(PSA) by ELISA

8.1 INTENDED USE

The PSA ELISA Kit is intended for the measurement of PSA in human serum.

8.2 INTRODUCTION:

Prostate Specific Antigen (PSA) is a single chain glycoprotein produced by epithelial cells of the prostate

gland. PSA is useful in the management of patients with prostate cancer. The measurement of serum PSA

has become the most accepted test to indicate men who are at risk of having prostate cancer and who should be examined by other tests.

Using a cut-off of 4 ng/ml, 92% of men over 50 years of age with malignant prostatic tissues, 8% of healthy men and 28% of men with benign prostate hyperplasia (BPH) test positive for PSA.

Three major forms of PSA exist in the serum: free PSA, bound PSA and complex PSA.

Bound PSA is found in higher concentrations in patients with prostate cancer; whereas, free PSA is detected in higher concentrations in patients with BPH.

If the free PSA to total PSA ratio is >25%, it is unlikely that the patient has prostate cancer; whereas, if free PSA is <16% then prostate cancer is likely to be the cause.

Serial measurement of PSA concentration in the serum is an important tool in monitoring

patients with prostatic cancer and determining the potential and actual effectiveness of surgery or other therapies, or may allow for earlier discovery of residual or recurrent carcinoma after radical prostatectomy or radiotherapy.

Current indications suggest that men over 50 years should be screened with digital rectal

examination and PSA. Men with a high risk of prostate cancer, such as a family history or of African heritage, should begin annual testing at age 40 years. If both are normal, the patient can be followed with annual evaluations and monitoring to determine the rate of change.

Slight elevations in PSA (4.1 ng/ml to 10.0 ng/ml) warrant a transrectal ultrasound (TRUS) to evaluate prostate volume and echogenicity of the gland. Hypo-echogenic lesions should be biopsied. Elevated PSA density (>0.15 ng/ml/cc), very high PSA (>10 ng/ml) or a free-to-total PSA ratio of <16% warrants systemic biopsy.

8.3 PRINCIPLE OF THE TEST

The PSA ELISA test is a solid phase two-site immunoassay. An anti-PSA monoclonal antibody is coated on the surface of the microtiter wells and a mouse anti-PSA antibody labeled with horseradish peroxidase is used as the tracer. The PSA molecules present in the standard solution or sera are "sandwiched" between the two antibodies. Following the formation of the coated antibody-antigen-antibody-enzyme complex, the unbound proteins

and antibody-enzyme tracers are removed by washing. The horseradish peroxidase activity bound in the wells is then assayed by a colorimetric reaction. The intensity of the color

formed is proportional to the concentration of PSA present in the sample.

8.3.1 MATERIALS PROVIDED 96 Tests

1. Microwell coated with PSA MAb 12x8x1
2. PSA Standard: 6 vials (ready to use) 0.7ml
3. PSA Enzyme Conjugate: 1 bottle (ready to use) 12 ml
4. TMB Substrate: 1 bottle (ready to use) 12ml
5. Stop Solution: 1 bottle (ready to use) 12ml
6. 20X Wash concentrate: 1 bottle 25

8.3.2 MATERIALS NOT PROVIDED

1. Distilled or deionized water
2. Precision pipettes. Disposable pipette tips
3. ELISA reader capable of reading absorbance at 450nm
4. Absorbance paper or paper towel
5. Graph paper

8.3.3 STORAGE AND STABILITY

1. Store the kit at 2 - 8 C.
2. Keep microwells sealed in a dry bag with desiccants.

3. The reagents are stable until expiration of the kit.

4. Do not expose test reagents to heat, sun, or strong light.

8.4 WARNINGS AND PRECAUTIONS

1. Potential biohazardous materials:

The calibrator and controls contain human source components which have been tested and found non-reactive for hepatitis B surface antigen as well as HIV antibody with FDA licensed reagents.

However, as there is no test method that can offer complete assurance that HIV, Hepatitis B virus or other infectious agents are absent, these reagents should be handled at the Biosafety Level 2, as recommended in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories." 1984

2. This test kit is designed for Research Use Only. Not for use in diagnostic procedures.

3. Do not pipette by mouth. Do not smoke, eat, or drink in the areas in which specimens or kit reagents are handled.

4. The components in this kit are intended for use as an integral unit. The components of different lots should not be mixed.

5. It is recommended that serum samples be run in duplicate.

6. Optimal results will be obtained by strict adherence to this protocol. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from this may yield invalid data.

8.5 SPECIMEN COLLECTION HANDLING

1. Collect blood specimens and separate the serum immediately.

2. Specimens may be stored refrigerated at (2-8C) for 5 days. If storage time exceeds 5 days, store frozen at (-20 C) for up to one month.

3. Avoid multiple freeze-thaw cycles.

4. Prior to assay, frozen sera should be completely thawed and mixed well.

5. Do not use grossly lipemic specimens.

8.6 REAGENTS PREPARATION

Prepare 1X Wash buffer by adding the contents of the bottle (25 ml, 20X) to 475 ml of distilled or deionized water. Store at room temperature (18-26 C).

8.7 ASSAY PROCEDURE

Prior to assay, allow reagents to stand at room temperature.

Gently mix all reagents before use.

1. Place the desired number of coated strips into the holder

2. Pipette 50 l of PSA standards, control and patient's sera to selected wells.

3. Add 100 l of enzyme conjugate to all wells.

4. Mix the content of the plate, gently, for 30 seconds.

5. Cover the plate and incubate for 60 minutes at room temperature (18-26 C).

6. Remove liquid from all wells. Wash wells three times with 300 l of 1X wash buffer. Blot on absorbent paper towels.

7. Add 100 l of TMB substrate to all wells.

8. Incubate for 15 minutes at room temperature.

9. Add 50 l of stop solution to all wells. Shake the plate gently to mix the solution.

10. Read absorbance on ELISA Reader at 450 nm within 15 minutes after adding the stopping solution.

8.8 CALCULATION :

The standard curve is constructed as follows:

1. Check PSA standard value on each standard vial. This value might vary from lot to lot. Make sure you check the value on every kit. See example of the standard attached.

2. To construct the standard curve, plot the absorbance for the PSA standards (vertical axis) against its concentration in ng/ml (horizontal axis) on a linear graph paper. Draw the best curve through the points.

3. Use the absorbance for controls and each unknown sample to determine the corresponding

concentration of PSA from the standard curve.

Example of a Standard Curve

OD 450 nm Conc.

ng/mL

Std 1 0.01 0

Std 2 0.11 2

Std 3 0.32 5

Std 4 0.81 10

Std 5 2.07 25

Std 6 3.07 50

8.9 EXPECTED VALUES

It is recommended that each laboratory establish its own normal ranges based on a representative sampling of the local population. The following values for PSA may be used as initial guideline ranges only:

PSA Normal Range = Less Than 4 ng/ml.

Sensitivity

The minimum detectable concentration for the PSA ELISA as measured by 2 X SD from the mean of 20 zero standards is estimated to be 0.032 ng/ml.

8.10 LIMITATIONS OF THE TEST

1. The test results obtained using this kit serve only as an aid to diagnosis and should be interpreted in relation to the patient's history, physical findings and other diagnostic procedures.

2. Do not use sodium azide as preservative. Sodium azide inhibits HRP enzyme activities.

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9. α-Fetoprotein (AFP) Blood Test

9.1 INTRODUCTION:

The most widely used biochemical blood test for liver cancer - hepatocellular carcinoma (HCC) is alpha-fetoprotein (AFP), which is a protein normally made by the immature liver cells in the fetus. At birth, infants have relatively high levels of AFP, which fall to normal adult levels by the first year of life. Also, pregnant women carrying babies with neural tube defects may have high levels of AFP. (A neural tube defect is an abnormal fetal brain or spinal cord that is caused by folic acid deficiency during pregnancy.)

In adults, high blood levels (over 500 nanograms/milliliter) of AFP are seen in only three situations:

- HCC
- Germ cell tumors (cancer of the testes and ovaries)
- Metastatic cancer in the liver (originating in other organs)

Several assays (tests) for measuring AFP are available. Generally, normal levels of AFP are below 10 ng/ml. Moderate levels of AFP (even almost up to 500 ng/ml) can be seen in patients with chronic hepatitis. Moreover, many patients with various types of acute and chronic liver diseases without documentable HCC can have mild or even moderate elevations of AFP.

The sensitivity of AFP for HCC is about 60%. In other words, an elevated AFP blood test is seen in about 60% of HCC patients. That leaves 40% of patients with HCC who have normal AFP levels. Therefore, a normal AFP does not exclude HCC. Also, as noted above, an abnormal AFP does not mean that a patient has HCC. It is important to note, however, that patients with cirrhosis and an abnormal AFP, despite having no documentable HCC, still are at very high risk of developing HCC. Thus, any patient with cirrhosis and an elevated AFP, particularly with steadily rising blood levels, will either most likely develop HCC or actually already have an undiscovered HCC.

An AFP greater than 500 ng/ml is very suggestive of HCC. In fact, the blood level of AFP loosely relates to (correlates with) the size of the HCC. Finally, in patients with HCC and

abnormal AFP levels, the AFP may be used as a marker of response to treatment. For example, an elevated AFP is expected to fall to normal in a patient whose HCC is successfully removed surgically (resected).

There are a number of other HCC tumor markers that currently are research tools and not generally available. These include des-gamma-carboxyprothrombin (DCP), a variant of the gamma-glutamyltransferase enzymes, and variants of other enzymes (e.g., alpha-L-fucosidase), which are produced by normal liver cells. (Enzymes are proteins that speed up biochemical reactions.) Potentially, these blood tests, used in conjunction with AFP, could be very helpful in diagnosing more cases of HCC than with AFP alone.

Normal values:

Alpha-fetoprotein in blood

Men and nonpregnant women:

0–6.4 international units per milliliter (IU/mL)

0–20 nanograms per milliliter (ng/mL)

0–20 micrograms per liter (mcg/L)

Women 15–22 weeks pregnant:

19–75 IU/mL

7–124 ng/mL

7–124 mcg/L

In pregnant women, the amount of AFP gradually rises starting in the 14th week of pregnancy. It continues to rise until a month or two before giving birth, then it slowly decreases. Values are generally slightly higher for black women than they are for white women. Values are slightly lower for Asian women than they are for white women. An accurate estimate of the age of the baby is needed to understand the AFP value correctly.

High values

- High alpha-fetoprotein values in a pregnant woman can mean:

o The age (gestational age) of the baby is wrong.

o The woman is pregnant with more than one baby, such as twins or triplets.

o The baby has a neural tube defect.

o The baby's intestines or other abdominal organs are outside the body (called an abdominal wall defect or omphalocele). Surgery after birth will be needed to correct the problem.

o The baby is not alive.

 In a non pregnant adult ,a high alpha-fetoprotein value can mean:

o Cancer of the liver, testicles, or ovaries is present.

o Liver disease, such as cirrhosis or hepatitis, is present.

o Alcohol abuse is present.

Low values

In a pregnant woman, a low level of alpha-fetoprotein can mean:

 The age (gestational age) of the baby is wrong.

 The baby has Down syndrome.

In a nonpregnant adult, alpha-fetoprotein is not normally present.

9.2 METHOD:

The use of human Alpha-Fetoprotein ELISA Kit

9.2.1 Principle of test:

Enzyme-linked Immunosorbent Assays (ELISAs) combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration.

There are two main variations on this method: The ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen.

An ELISA is a five-step procedure:

1) Coat the microtiter plate wells with antigen

2) block all unbound sites to prevent false positive results;

3) Add antibody to the wells

4) Add anti-mouse or sheep IgG conjugated to an enzyme;

5) Reaction of a substrate with the enzyme to produce a colored product, thus indicating a positive reaction.

9.3 MATERIALS AND METHOD:

According to a specific ELISA kit provided commercially (see kit manual), a double antibody sandwich assay with sheep anti- AFP and alkaline phosphatase conjugate is generally used.

9.4 PROCEDURE:

• A plate with 96 wells is coated with a primary antibody, which recognizes the antigen of the target molecule and bonds with it.

• Test serum is incubated with antigen immobilized on the 96-well plate .

• The antigen-antibody complex is recognized by a secondary antibody that is joined to an enzyme that catalyzes the reaction mixture, yielding a specific color.

• Positive reaction is detected by the marker changing color when an appropriate substrate is added

• By measuring the optical density of this color, the presence and number of a specific molecule can be determined; the density of color is proportional to the advancement of the reaction or disease being tested

• If there are no antigen in the sample, the second antibody will not be able to stick and there will be no color change (no reaction)

Results and discussion:

9.5 Refferences:

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2. http://www.webmd.com/baby/alpha-fetoprotein-afp-in-blood

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Title of the experiment

10. Determination of Hepatitis A,B and C

10.1 ELISA Assay for Hepatitis B Virus (HBV):

For Hepatitis B testing, there are five related ELISA kits, which are intended to detect specific substances related to Hepatitis B infection, and all contribute to the diagnosis of Hepatitis diseases. Respectively they are HBsAg ELISA kits, HBsAb ELISA kits, HBcAb ELISA Kits, HBeAg ELISA kits and HBeAb ELISA kits.

HBsAg ELISA Kit:

Enzyme-linked immunosorbent assay for the detection of HBsAg in serum or Plasma

10.2 INTRODUCTION:

HBsAg ELISA is used for the qualitative determination of Hepatitis B surface antigen (HBsAg) in human serum or plasma. This test is indicated for the screening of blood and blood products to be used for transfusion and an aid for the diagnosis of existing or previous hepatitis B infection.

HBsAg is one of the earliest markers that appear in the blood following infection with Hepatitis B virus (HBV). This infection of the liver is transmitted by homosexual or heterosexual activity, blood borne exposure, mother - infant, close personal contact and by intake of contaminated water and food products. In the HBV infected people, the virus persists for the rest of their lives and can be passed on to others. Therefore Hepatitis B has become a global public health problem.

Infection with HBV results in the appearance of a number of serological markers and one of the first of such markers is Hepatitis B surface antigen (HBsAg). The HBV infection causes a wide variety of liver damages such as acute self-limiting infection, fulminating hepatitis, chronic hepatitis with progression to cirrhosis and liver failure, and a symptomatic chronic carrier state.

Hepatitis B surface antigen (HBsAg) appears 1-7 weeks before biochemical evidence of liver disease or jaundice. Three weeks after the onset of acute hepatitis almost half of the patients will still be positive for HBsAg. In the chronic carrier state, the HBsAg persists for long periods (6-

12 months) with no seroconversion to the corresponding antibodies. Therefore, screening for HBsAg

is highly desirable for all donors, pregnant women and people in high-risk groups.

10.3 PRINCIPLE:

The HBsAg EIA is a solid-phase simultaneous sandwich immunoassay, which employs monoclonal antibodies and polyclonal antibodies specific for HBsAg.

Microtiter well are coated with monoclonal antibodies specific for HBsAg. A serum pecimen is added to the antibody coated Microtiter wells together with enzyme conjugated polyclonal antibodies. HBsAg, if present, will form an antibody-HBsAg-antibody-enzyme complex. The plate is then washed to remove unbound material. Finally, a solution of substrate is added to the wells and incubated. A blue color will develop in proportion to the amount of HBsAg present in the specimen. The enzyme-substrate reaction can be stopped and the result is visualized by naked eye or read by EIA plate reader for absorbance at the wave length of 450 nm.

10.4 REAGENTS:

Materials provided with the kits:

1. Microtiter Well: 8x12 or 12x8, coated with monoclonal anti-HBs antibody

2. Negative Control: 0.5ml HBsAg negative serum.

3. Positive Control: 0.5ml HBsAg positive serum.

4. Enzyme Conjugate: 6 ml, Goat anti-HBsAg-HRP

5. Wash Buffer Concentrate (20x): 25 ml, The buffer should be diluted 20

times with distilled water before use.

6. Substrate Solution A: 6 ml Urea Peroxide

7. Substrate Solution B: 6 ml TMB solution

8. Stop Solution: 6 ml 2N Sulfuric Acid

Materials required but not provided:

1. Precision pipettes: 0.02, 0.05, 0.10, 0.15, 0.20, and 1.0 ml.

2. Disposable pipette tips.

3. Distilled water.

4. Humidified Box capable of maintaining 37°C

5. Absorbent paper or paper towel.

6. Microtiter plate or strip-well washer

7. Microtiter plate reader.

10.5 SPECIMEN COLLECTION AND PREPARATION:

- No special preparation of the patient is required prior to blood collection.
- Blood should be collected by approved medical techniques.
- Remove serum or plasma from the clot or blood cells as soon as possible to avoid hemolysis. Grossly hemolytic, lipidic or turbid samples should not be used.
- Plasma samples containing EDTA, heparin or oxalate may interfere with test procedures and should be avoided.
- Specimen with extensive particulate should be clarified by centrifugation prior to use.
- Covered specimens may be stored for up to 48 hours at 2°-8°C prior to assaying.
- Specimens held for a longer time can be frozen at-20°C for mix prior to testing.
- Avoid repeated freeze thaw.
- At least, two wells of negative and positive controls each should be run in every assay.

10.6 PRECAUTIONS:

1. Caution: Some components of this kit contain human serum. No known test method can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all blood derivatives should be considered potentially infectious. It is recommended that these reagents and human specimens be handled using established good laboratory working practices.

2. Wear disposable gloves while handling kit reagents and specimens and thoroughly wash hands afterwards.

3. Dispose off all specimens and materials used to perform the test as if they contained infectious agents.

4. Do not mix reagents from kits with different lot numbers.

5. Cross contamination between reagents will invalidate the test results.

6. All reagents and components except the conjugate must be equiliberated at room temperature prior to use.

10.7 STORAGE OF TEST KITS AND INSTRUMENTATION:

 Unopened test kits should be stored at 2°-8°C upon receipt.

 Micro titer plate, once opened, should be kept in a sealed bag with desiccants to minimize exposure to damp air.

 To remove the required number of strips from the micro titer plates, bring the sealed pouches to room temperature first and then open the pouches. This is very important because absorbed atmospheric moisture by cold plates significantly reduces their shelf life.

 Opened test kits will remain stable until the expiration date shownin 4°C, provided it is stored as described above.

 A micro titer plate reader with a bandwidth of 10 nm or less and an optical density range of

 0-2 OD or greater at 450 nm wavelength is acceptable for use in absorbance measurement.

10.8 WORKING REAGENT PREPARATION, STORAGE AND STABILITY:

No reagent preparation is required except for wash buffer, which is supplied as a

20 X concentrate.

WORKING WASH BUFFER:

Dilute the 20X wash buffer concentrate with deionized or distilled water 1:20. For

example, 5 ml of wash buffer concentrate should be diluted to a total volume of 100

mL with deionized or distilled water.

STABILITY OF OPENED KIT COMPONENTS AND DILUTED REAGENTS:

 The diluted wash buffer is stable for at least one week when stored at room temperature.

 Substrate is stable for the expiration date of the kit.

 The micro titer plates should be opened after they have been kept at room temperature for 20-30 minutes.

 After removing the required number of strips, the plates should be resealed in the foil pouch bags along with the desiccant and stored at 2°-8°C.

 Exposure of HBsAg plates to humidity drastically reduces the shelf life.

10.9 ASSAY PROCEDURE:

It is strongly advised to analyze each specimen and controls in duplicate. All the reagents should equilibrate to room temperature before use.

1. Dispense one drop (50 ul) of Positive Control as well as Negative Control in duplicate into respective wells. Set one blank well as background control, and 50ul of serum or plasma samples into respective test wells

2. Add one drop (50 ul) of Enzyme Conjugate to each well. Mix it gently by swirling the microtiter plate on flat bench for 1 minutes. Do not add Enzyme Conjugate to the blank well.

3. Place the microtiter plates into a humidified box, and incubate at 37°C for 30 minutes.

4. Wash each well 4 times by filling each well with diluted wash buffer, then inverting the plate vigorously to get all water out and blocking the rim of wells on absorbent paper for a few seconds.

5. Add one drop (50 ul) of Substrate Solution A (HRP-substrate) to each well, then add one drop (50 ul) of Substrate Solution B (TMB) to each well. Mix gently and incubate at 37°C for 15 minutes.

6. Add 1 drop (50 ul) of Stop Solution to each well to stop the color reaction.

Read O.D. at 450 nm with an EIA plate reader.

ASSAY VALIDITY:

EIA Plate Reader at 450 nm (using the OD value of the blank well to correct all the OD reading from all wells, OD value of Positive Control should be between 1.5- 2.5).

10.10 INTERPRETATION OF RESULTS

Positive: P/N value is equal to or greater than 2.1

Negative: P/N value is less than 2.1

P/N value = OD value of specimen .

Average OD value of Negative Control

If the OD value of the negative control is less than 0.05, it should be reported as 0.05. If it is more than 0.05, it should be reported as the actual OD value measured.

10.11 LIMITATIONS OF THE PROCEDURE:

1. HBsAg kit is used for the detection of HBsAg in human serum of plasma.

Based on a single reactive test result, a sample should not be considered HBsAg positive.

- Further testing, including confirmatory testing, should be
- performed before a specimen is considered positive for HBsAg.
- A nonreactive test result does not exclude the possibility of exposure to hepatitis B virus.
- Levels of HBsAg may be undetectedboth in early infection and late after infection.
- Specimens containing precipitate may give inconsistent test results.

2. As the other sensitive immunoassays, there is the possibility that non-repeatable reaction may occur due to inadequate washing. So do aspirate the well or get rid of entire content of wells completely before adding the washing solution.

3. The positive control in the test kit is not to be used to quantify assay sensitivity. The positive control is used to verify that the test kit components are capable of detecting a reactive specimen provided the procedure is followed as defined in the kit and the storage conditions have been strictly adhered to.

10.12 REFERENCES:

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immunology. Rose N. and Firiedman H. editors.

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surface antigen. J. Infect. Dis. 136:311 (1977)

4. Pderson, D.L.. Nath N and Gravilanes E Structure of hepatitis B surface

antigen. The J d Bid.Chern. 257 (17):10414-10410 (1982).

Manufacturer:

Biocare Diagnostics Ltd.

King Saud University

College of Science

Biochemistry Department

BCH 473

Name:

ID #:

Title of the experiment

11. Detection of AIDS

Quantitation Kit of HIV p24 ELISA

Catalog Numbers:

VPK-108-HIV-p24 96 tests

VPK-108-HIV-p24-5 5 x 96 tests

11.1 INTRODUCTION:

-An anti-HIV p24 monoclonal coating antibody is adsorbed onto a microtiter plate. p24 antigen present in the sample or standard binds to the antibodies adsorbed on the plate; a FITC-conjugated mouse antip24 antibody is added and binds to p24 antigen captured by the first antibody.

-Following incubation and wash steps, a HRP-conjugated mouse anti-FITC antibody is added and binds to the FITC conjugated anti-p24. Following unbound HRP-conjugated mouse anti-FITC antibody is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

-A colored product is formed in proportion to the amount of p24 antigen present in the sample.

-The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from recombinant HIV-1 p24 protein and sample p24 concentration is then determined

11.2 Kit Components:

1. Anti-p24 Antibody Coated Plate (Part No. 310801): one strip well 96-well plate.

2. FITC-Conjugated Anti-p24 Monoclonal Antibody (Part No. 310810): One 20 µL vial.

3. HRP-Conjugated Anti-FITC Monoclonal Antibody (Part No. 310811): One 20 µL vial.

4. Assay Diluent (Part No. 310804): One 50 mL bottle.

5. Triton X-100 Solution (Part No. 310805): One 30 mL bottle containing 5% Triton X-100 in TBS.

6. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.

7. Substrate Solution (Part No. 310807): One 12 mL amber bottle.

8. Stop Solution (Part. No. 310808): One 12 mL bottle.

9. Recombinant p24 Standard (Part No. 310809): One 100 µL vial of 10 µg/mL heat inactivated recombinant HIV1 p24 antigen in TBS plus BSA.

11.3 MATERIALS:

1. Lentiviral Sample: purified virus or unpurified viral supernatant

2. Cell Culture Centrifuge

3. 0.45 µm filter

4. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips

5. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips

6. Multichannel micropipette reservoir

7. Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)

Storage

Upon receiving, aliquot and store recombinant HIV-1 p24 Standard at -20ºC and avoid freeze/thaw. Store all other components at 4ºC until their expiration dates.

11.4 Preparation of Reagents

• 1X Wash Buffer: Dilute the 10X Wash Buffer Concentrate to 1X with deionized water. Stir to homogeneity.

• FITC-Conjugated Anti-HIV1 p24 Monoclonal Antibody and HRP-Conjugated Anti-FITC

Monoclonal Antibody: Immediately before use dilute the FITC-conjugated antibody 1:1000 and HRP-conjugated antibody 1:1000 with Assay Diluent. Do not store diluted solutions.

Safety Considerations:

Remember that your lentiviral samples contain infectious viruses before inactivation; you must follow the recommended NIH guidelines for all materials containing BSL-2 organisms.

11.5 Assay Sample Preparation:

HIV p24 Standard Curve:

1. Prepare a dilution series of recombinant HIV-1 p24 antigen in the concentration range of 100 ng/mL – 1 ng/mL by diluting the p24 stock solution in Assay Diluent

2. Transfer 225µL of each dilution to a microcentrifuge tube containing 25 µL of Triton X-100 Solution. Perform the assay as described in Assay Instructions.

11.6 Assay Instructions:

1. Prepare and mix all reagents thoroughly before use.

2. Each lentiviral sample, HIV p24 standard, blank, and control medium should be assayed in duplicate.

3. Add 110 µL of inactivated sample or p24 antigen standard to anti-p24 antibody coated plate.

4. Cover with a Plate Cover and incubate at 37 0C for at least 4 hours or 4 0C overnight.

5. Remove Plate Cover and empty wells. Wash microwell strips 3 times with 250 µL 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.

6. Add 100 µL of the diluted FITC-Conjugated Anti-p24 Monoclonal Antibody to each well.

7. Cover with a Plate Cover and incubate at room temperature for 1 hour on an orbital shaker.

8. Remove Plate Cover and empty wells. Wash the strip wells 3 times according to step 5 above.

9. Add 100 µL of the diluted HRP-Conjugated Anti-FITC Monoclonal Antibody to all wells.

10. Cover with a Plate Cover and incubate at room temperature for 1 hour on an orbital shaker.

11. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to step 5 above. Proceed immediately to the next step.

12. Warm Substrate Solution to room temperature. Add 100 µL of Substrate Solution to each well, including the blank wells. Incubate at room temperature for 5-20 minutes on an orbital shaker.

13. Stop the enzyme reaction by adding 100 µL of Stop Solution into each well, including the blankwells. Results should be read immediately (color will fade over time).

14. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.

11.7 CALCULATIONS:

Use the standard curve to calculate your results.

11.8 References

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3. Kahl C. A., Marsh J., Fyffe J., Sanders D. A., and K. Cornetta (2004) J Virol. 78:1421-30.

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5. Kafri T., van Praag H., Ouyang L., Gage F. H., and I. M. Verma (1999) J Virol. 73:576-84.

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College of Science

Biochemistry Department

BCH 473

Name:

ID #:

Title of the experiment

12. Estimation of T3 and T4 by ELISA

Total T3 (Triiodothyronine) ELISA assay

Catalog: IB69112

For In Vitro Diagnostic Use Only

12.1 INTRODUCTION AND INTENDED USE:

This ELISA assay is a quantitative solid phase enzyme linked immunsorbent assay. This test provides quantitative measurement of Total T3 (Triiodothyronine) in human serum to aid in the diagnosis of diseases affecting thyroid metabolism and function. (For in-vitro diagnostic use only)

12.1.1 SUMMARY :

The thyroid gland produces thyroxine T4, triiodothyronine T3 and calcitonin. The first two hormones are synthesized by the gland following entrapment of iodide, conversion to iodine, and coupling of iodine with tyrosine, followed by coupling of two iodinated tyrosine molecules. T4 and T3 so formed are attached to thyroglobulin for storage and are released, as needed, as protease splits them from globulin.

Measurement of serum levels of triiodothyronine (T3) is an important adjunct in the determination of thyroid function.

An elevated value of T3 in serum is a strong indication of hyperthyroidism. In patients who demonstrate clinical hyperthyroidism, an elevated serum triiodothyronine level is evidence for T3 thyrotoxicosis ,

Most patients with unequivocal clinical hypothyroidism, levels of triiodothyronine are below normal. However, in borderline cases, triiodothyronine levels are sometimes or often normal. Thus, a reduced serum level of triiodothyronine is not so clear an indicator of primary hypothyroidism as a reduced serum level of thyroxine or an elevated level of thyroid stimulating hormone.

12.1.2 PRINCIPLE:

The IBL-America T3 Quantitative method is based on the competitive binding principle of enzyme immunoassay. Test specimen and enzyme labeled T3 are incubated in an antibody coated microwell. The T3 in the specimen competes with the labeled T3 for a limited number of binding sites on the well. In the assay procedure, T3 standard or patient serum is incubated with T3 conjugated to horseradish peroxidase and T3 antibody coated well. In this solid-phase system the antibody-bound T3 will remain on the surface of the wall and free T3 will be removed by washing. Upon addition of chromogen substrate, color development will occur proportional to the amount of enzyme activity that in turn is inversely proportional to the amount of T3 in the sample. The intensity of the color is measured using a spectrophotometer equipped with a 450 nm filter. The concentrations of samples are obtained by reference to the standards.

12.1.3 MATERIALS :

1. Microwell Strips: Anti-T3 antibodies coated wells, 8 x 12 strips, 96 wells.

2. Enzyme Conjugate (11 mL): T3 conjugated to horseradish peroxidase.

3. TMB Solution (11 mL): containing H2O2 and TMB.

4. Reference Standard Set (0.75 mL each vial): Calibrated to 0, 50, 100, 250, 500 and 1000 ng/dL in human serum.

5. High & Low controls (0.75 mL); values indicated on vial labels.

6. Washing buffer concentrate 100x (10mL). Requires dilution prior to use.

7. Stop Solution: 2 N HCl.

8. Microplate frame for securing individual wells.

12.1.4 MATERIALS REQUIRED BUT NOT PROVIDED:

1. Distilled or deionized water.

2. Microplate spectrophotometer with 450nm filter

3. Micropipets to deliver 50 and 100 µL

4. 8-channel micropipette (variable delivery), or automated microplate washer

STORAGE AND STABILITY:

1. Store kits refrigerated at 2-8 0C.

2. Always store microwells refrigerated and sealed in a dry bag with desiccant.

3. The unopened reagents are stable until expiration of the kit. TMB solution should be colorless; if TMB turns blue, it must be replaced. Do not expose test reagents to strong light during storage or usage.

12.1.5 WARNINGS AND PRECAUTIONS:

1. This assay is designed for in vitro use only.

2. The components in this kit are intended for use as an integral unit. Do not mix components from different lot numbers.

12.1.6 SAMPLE COLLECTION AND HANDLING:

Collect blood by venipuncture, allow to clot and separate the serum by centrifugation at room temperature. If sera cannot be assayed immediately, they can be stored at 2-8 0C for a week or frozen for up to six months. Avoid repeated freezing and thawing of serum sample. The use of hemolyzed or lipemic samples is not recommended.

12.1.7 PREPARATION FOR ASSAY:

1. Prepare working dilution of wash buffer by adding 10mL of concentrate to 990mL distilled or deionized water. CAUTION: If wash buffer concentrate has crystallized, it must be warmed gently to redissolve prior to dilution. Diluted wash buffer is stable at 2-8 0 C for 30 days

2. Before beginning the test, bring all samples and reagents to room temperature (24± 3 0 C) and mix gently.

3. Have all reagents and samples ready before the start of the assay. Once the test has begun

it must be performed without interruption to yield the most reliable and consistent results.

4. Use new disposable tips for each specimen.

12.1.8 PROCEDURAL NOTES:

1. Sodium azide and thimerosal at concentrations higher than 0.01% interfere with this assay. Testing control sera or samples containing high levels of these compounds is not recommended.

2. It is very important to wash the microwells thoroughly and remove residual moisture by inverting the microplate and tapping it sharply onto absorbent material after the final wash repetition.

3. Pipet all reagents and samples into the bottom of the microwells. Vortex-mixing or shaking of wells after sample and reagent pipetting is not required.

4. Absorbance is a function of the time and temperature of incubations. It is recommended that all reagent and sample vial caps be removed and all microwells organized in the microplate frame prior to pipetting to minimize elapsed time for each pipetting step.

5. Noticeable assay drift can be an issue with any ELISA assay if excessive time elapses during the initial loading of standards controls and samples into the plate. Care should always be taken to load the plate as quickly/efficiently as possible and without interruption.

6. All standards, controls and unknown samples should be run in duplicate.

12.1.9 ASSAY PROCEDURE:

1. Secure the desired number of coated wells in the holder. Provide a well for the assay blank.

2. Dispense 50 µL of Standards, Controls or Serum samples.

3. Dispense 100 µL of enzyme conjugate into every well except the blank.

4. Incubate for 60 minutes at room temperature.

5. Remove incubation mixture and dispense 300ul of diluted wash buffer into every well. Remove the wash buffer and repeat for a total of five repetitions. After removal of the final wash buffer, invert the plate and tap sharply onto absorbent material to remove any residual moisture from the wells.

6. Dispense 100 µL of TMB Solution into each well including the blank well.

7. Incubate 30 minutes at room temperature.

8. Add 50 µL of Stop solution to each well.

9. Read O.D. at 450 nm with a spectrophotometer within ten minutes.

12.1.10 CALCULATION OF RESULTS:

Any microwell reader capable of determining at 450 nm may be used. The values from the blank well are subtracted from all remaining microwells. Patient T3 values are obtained as follows:

Plot the concentration (X) of each Reference Standards against its absorbance (Y) on graph paper. Obtain the value of patient T3 by reference to the Standard Curve. es banc

450

12.1.11 QUALITY CONTROL:

Control values should fall within their defined ranges. If the control values should fall outside of their defined ranges, that run should be considered invalid and patient values should not be reported.

12.1.12 LIMITATIONS:

1. Extrapolation of T3 values beyond the standard curve may yield unreliable results. Samples containing >1000 ng/dL T3 can be diluted with T3 free human serum (zero std) and retested.

1. Calibrators and controls from other manufacturers may contain serum preservatives

incompatible with reagents from this kit and should not be used.

2. Laboratory test results must be used only in conjunction with clinical findings and other test results, and should never be the sole determinant for disease-state diagnosis.

12.1.13 EXPECTED VALUES:

1. It is recommended that each laboratory establish their own normal and abnormal ranges.

2. Use of the IBL-America total T3 assay in a study of 75 euthyroid patients in one geographic location yielded a normal range of 60-200 ng/dL, which may serve as a preliminary guideline only until the laboratory establishes their own ranges.

3. In an individual having normal levels of TBG, the measurement of total T3 yields an accurate diagnosis of thyroid status. However, there are many circumstances in which the levels of TBG are not normal. For instance, pregnancy or estrogen therapy cause increased synthesis of TBG and concomitant increase in total T3, whereas androgenic steroids have the opposite effect. Because of this variation in TBG levels, interpretation of T3 results should be tempered by the determination of TBG binding capacity.

4. Depressed levels of T3 have been observed in a wide variety of serious, non-thyroidal illness such as hepatic cirrhosis, anorexia nervosa, chronic renal failure

and disseminated malignancy after surgery and during calorie restriction.

12.1.14 PERFORMANCE CHARACTERISTICS:

SENSITIVITY

10 ng and 20 ng/dL T3 concentrations were run in replicates of 12. The 10 ng/dL could not be distinguished from the zero standard, whereas the 20 ng/dL was the lowest level of T3 that can be distinguished from the zero standard at the 95% confidence limit.

THE IBL-America Total T3 ELISA kit was compared with INCSTAR Clinical Assays T3 RIA kit by analyzing 100 patient samples values from 44-409 ng/dL. The

regression analysis shows good correlation between these two methods

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12.2 Total T4 (Thyroxine) ELISA assay

Catalog: IB69113

For In Vitro Diagnostic Use Only

12.2.1 INTENDED USE:

This ELISA assay is a quantitative solid phase enzyme linked immunsorbent assay. This test provides quantitative measurement of Total T4 (THYROXINE) in human serum to aid in the diagnosis of diseases affecting thyroid metabolism and function.

(For in vitro diagnostic use)

12.2.2 INTRODUCTION:

Thyroxine is a highly active thyrometabolic hormone that exists in protein-bound and unbound forms. Since T4 can be measured more easily and with greater accuracy than T3, determination of total T4 by immunoassay is the most reliable marker for detecting thyroid disorders in man. Release of T4 and T3 from the thyroid is greatly influenced by pituitary-thyroid stimulating hormone (TSH) that in turn is influenced by hypothalamic thyrotropin-releasing hormone (TRH).

Normally, increased blood levels of T4 and T3 act to decrease the amount of TSH secreted, thereby reducing the production and release of T4 and T3. Decreased levels of T4 and T3 in blood produce the opposite effect, leading to increased production and secretion of T4 and T3 In this manner a normal circulating thyroid hormone balance is maintained.

Circulating T4 and T3 are bound largely to thyroxine binding globulin (TBG). To a lesser extent they are bound to thyroxine binding prealbumin (TBPA) and, when present in excess, to albumin. Usually the ratio of T4 to T3 is about 9:1; However T3 has considerably greater physiological activity. It is the small free fraction (approx. 0.1% of the total) that is physiologically active and determines the clinical thyroid status of the patient as hyperthyroid, euthyroid, or hypothyroid.

12.2.3 PRINCIPLE OF THE ASSAY:

The IBL-America T4 quantitative assay is based on the principle of competitive binding solid phase enzyme immunoassay. Test specimen and enzyme labeled T4 are incubated in an antibody coated microwell. The T4 in the specimen competes with the labeled T4 for a limited number of binding sites on the well. In performing the assay procedure, T4 standard or patient's serum and

T4 conjugated to horseradish peroxidase are added to interact with T4 antibody coated on the well. In this solidphase system the antibody-bound T4 remains on the surface of the well whereas free T4 is removed by washing. Upon addition of chromogen substrate, color development will occur proportional to the amount of enzyme activity that in turn is inversely proportional to the amount of T4 in the sample. The intensity of the color is measured using a spectrophotometer equipped with a 450nm filter. The concentrations of samples are obtained by reference to the standards.

12.2.4 MATERIALS:

1. Microwell Strips: Anti-T4 antibodies coated wells, 8 x 12 strips, 96 wells.

2. Enzyme Conjugate (11 mL): T4 conjugated to horseradish peroxidase.

3. TMB Solution (11 mL): containing H2O2 and TMB.

4. Reference Standard Set (0.75 mL each vial): Calibrated to 0, 1.5, 3.0, 6.0, 12 and 24 μg/dL in human serum.

5. High & Low controls (0.75 mL each); values indicated on vial labels.

6. Stop Solution: 2 N HCl.

7. Washing Buffer Concentrate (100X) (10 mL): Requires diluton prior to use.

8. Microplate frame for securing individual wells.

12.2.5 MATERIALS REQUIRED BUT NOT PROVIDED:

1. Distilled or deionized water.

2. Spectrophotometer equipped with 450nm filter

3. Micropipettes to deliver 25 and 100 μL 4. 8-channel micropipette (variable

delivery) or automated microplate washer.

12.2.6 STORAGE AND STABILITY:

1. Store kits refrigerated at 2-8 0C.

2. Always store microwells refrigerated and sealed in a dry bag with desiccants.

3. The unopened reagents are stable until expiration of the kit. TMB Solution should be colorless; if TMB turns blue, it must be replaced. Do not expose test reagents to strong light during storage or usage.

12.2.7 WARNINGS AND PRECAUTIONS:

1. This assay is designed for in vitro use only.

2. The components in this kit are intended for use as an integral unit. Do not mix components from different lot numbers.

12.2.8 SAMPLE COLLECTION AND HANDLING:

Collect blood by venipuncture, allow to clot and separate the serum by centrifugation at room temperature. If sera cannot be assayed immediately, they can be stored at 2-8 0C for a week or frozen for up to six months. Avoid repeated freezing and thawing of samples. The use of hemolyzed or lipemic samples is not recommended.

12.2.9 PREPARATION FOR ASSAY:

1. Prepare working dilution of wash buffer by adding 10mL of concentrate to 990mL distilled or deionized water. CAUTION: If wash buffer concentrate has crystallized, it must be warmed gently to re-dissolve prior to dilution. Diluted wash buffer is stable at 2-8˚C for 30 days.

2. Before beginning the test, bring all samples and reagents to room temperature (24± 3 0C) and gently mix.

3. Have all reagents and samples ready before the start of the assay. Once the test has begun it must be performed without any interruptions to get the most reliable and consistent results.

4. Use new disposable tips for each specimen.

12.2.10 ASSAY PROCEDURE:

1. Secure the desired number of coated wells in the holder. Provide a well for the assay blank.

2. Dispense 25 µL of Standards, Controls or Serum samples.

3. Dispense 100 µL of enzyme conjugate into every well except the blank.

4. Incubate for 60 minutes at room temperature.

5. Remove incubation mixture and dispense 300µl of diluted wash buffer into every well. Remove the wash buffer and repeat for a total of five repetitions. After removal of the final wash buffer, invert the plate and tap sharply onto absorbent material to remove any residual moisture from the wells.

6. Dispense 100 µL of TMB Solution into each well, including the blank well.

7. Incubate 30 minutes at room temperature.

8. Add 50 µL of stop solution into each well.

9. Read at 450nm with a spectrophotometer within 10 minutes.

12.2.11 CALCULATION:

Any microwell reader capable of determining at 450 nm may be used. The value from the blank well is subtracted from the absorbance of all remaining microwells. Patient T4 values are obtained as follows:

1. Plot the concentration (X) of each Reference Standards against its absorbance (Y) on graph paper.

2. Obtain the value of patient T4 by reference to the Standard Curve. Well No.

12.2.12 QUALITY CONTROL:

Control values should fall within their defined ranges. If the control values should fall outside of their defined ranges, that run should be considered invalid and patient values should not be reported.

12.2.13 LIMITATIONS:

1. Extrapolation of T4 values beyond the standard curve may yield unreliable results. Samples containing >24 μg/dL can be diluted with T4 free serum (zero standard) and retested.

2. Calibrators and controls from other manufacturers may contain serum preservatives incompatible with reagents in this kit and should not be used.

3. Laboratory test results must be used only in conjunction with clinical findings and other test results, and should never be the sole determinant for disease state diagnosis.

12.2.14 EXPECTED VALUES:

It is recommended that each laboratory establish their own normal and abnormal ranges. Use of IBL-America T4 ELISA reagents in a study of 75 euthyroid patients in one geographic location yielded a normal range of 4-12 μg/dL at the 95 % confidence limit. Which may serve as a preliminary guideline only until the laboratory establishes their own ranges.

12.2.15 CORRELATION STUDY:

The IBL-America Total T 4 ELISA kit was compared with INC STAR Clinical Assays T4 RIA kit by analyzing 99 patient samples values from

2.6 –25.5 μg/dL. The regression analysis, shows good correlation between these two methods.

INC STAR T4 RIAL-America ELISA ASSAY (Y)

12.2.16 REFERENCES:

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Case Studies:

- child with hypothyroidism.

12.2.17 QUESTIONS:

1- Explain the principle of the competitive immunoassay (ELISA)?

2- Compare between the principle of sandwich and competitive immunoassay (ELISA)?

3- What is the relationship between the concentrations of the measured samples and

their absorbance in the competitive enzyme linked immunsorbent assay (ELISA)? Explain why?

4- Can we add TMB solution and wash it before adding the enzyme conjugate? Explain why?

King Saud University

College of Science

Biochemistry Department

BCH 473

Name:

ID #:

Title of the experiment

13. Quality Control Estimation in the lab

Laboratory Quality Control (Experiment No 12)

13.1 Definition

 Quality Control- QC refers to the measures that must be included during each assay run to verify that the test is working properly.

 Quality Assurance- QA is defined as the overall program that ensures that the final results reported by the laboratory are correct.

 "The aim of quality control is simply to ensure that the results generated by the test are correct. However, quality assurance is concerned with much more: that the right test is carried out on the right specimen, and that the right result and right interpretation is delivered to the right person at the right time"

 Quality Assessment- quality assessment (also known as proficiency testing) is a means to determine the quality of the results generated by the laboratory. Quality assessment is a challenge to the effectiveness of the QA and QC programs.

 Quality Assessment may be external or internal, examples of external programs include NEQAS, HKMTA, and Q-probes.

13.2 Variables that affect the quality of results

 The educational background and training of the laboratory personnel

 The condition of the specimens

 The controls used in the test runs

 Reagents

 Equipment

 The interpretation of the results

 The transcription of results

 The reporting of results

Errors in measurement

 True value - this is an ideal concept which cannot be achieved.

 Accepted true value- the value approximating the true value, the difference between the two values is negligible.

 Error- the discrepancy between the result of a measurement and the true (or accepted true value).

Sources of Errors

 Input data required- such as standards used, calibration values, and values of physical constants.

 Inherent characteristics of the quantity being measured- e.g. CFT and HAI titre.

 Instruments used- accuracy, repeatability.

 Observer fallibility- reading errors, blunders, equipment selection, analysis and computation errors.

 Environment- any external influences affecting the measurement.

Theory assumed - validity of mathematical methods and approximations

13.3 Random Error

 An error, which varies in an unpredictable manner, in magnitude and sign, when a large number of measurements of the same quantity are made under effectively identical conditions.

 Random errors create a characteristic spread of results for anytest method and cannot be accounted for by applying corrections. Random errors are difficult to eliminate but repetition reduces the influences of random errors.

 Examples of random errors include errors in pipetting and changes in incubation period. Random errors can be minimized by training, supervision and adherence to standard operating procedures.

Random Error

Systematic Error

 An error which, in the course of a number of measurements of the same value of a given quantity, remains constant when measurements are made under the same conditions, or varies according to a definite law when conditions change.

 Systematic errors create a characteristic bias in the test results and can be accounted for by applying a correction.

Systematic errors may be induced by factors such as variations in incubation temperature, blockage of plate washer, change in the reagent batch or modifications in testing method.

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Systematic Errors

Internal Quality Control Program for Serological Testing

An internal quality control program depend on the use of internal quality control (IQC) specimens, Shewhart Control Charts, and the use of statistical methods for interpretation.

13.4 Internal Quality Control Specimens

IQC specimens comprises either (1) in-house patient sera (single or pooled clinical samples), or (2) international serum standards with values within each clinically significant ranges.

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Shewhart Control Charts

A Shewhart Control Chart depend on the use of IQC specimens and is developed in the following manner:-

 Put up the IQC specimen for at least 20 or more assay runs and record down the O.D./cut-off value or antibody titre (whichever is applicable).

- Calculate the mean and standard deviations (s.d.)
- Make a plot with the assay run on the x-axis, and O.D./cut-off or antibody titre on the y axis.
- Draw the following lines across the y-axis: mean, -3, -2, -2, 1, 2, and 3 s.d.
- Plot the O.D./cut-off obtained for the IQC specimen for subsequent assay runs
- Major events such as changes in the batch no. of the kit and instruments used should be recorded on the chart.

Westgard rules

- The formulation of Westgard rules were based on statistical methods. Westgard rules are commonly used to analyse data in Shewhart control charts.
- Westgard rules are used to define specific performance limits for a particular assay and can be use to detect both random and systematic errors.
- There are six commonly used Westgard rules of which three are warning rules and the other three mandatory rules.
- The violation of warning rules should trigger a review of test procedures, reagent performance and equipment calibration.
- The violation of mandatory rules should result in the rejection of the results obtained with patients' serum samples in that assay.

Warning rules

- Warning 12SD : It is violated if the IQC value exceeds the mean by 2SD. It is an event likely to occur normally in less than 5% of cases.
- Warning 22SD : It detects systematic errors and is violated when two consecutive IQC values exceed the mean on the same side of the mean by 2SD.
- Warning 41SD : It is violated if four consecutive IQC values exceed the same limit (mean 1SD) and this may indicate the need to perform instrument maintenance or reagent calibration.

Mandatory rules

- Mandatory 13SD : It is violated when the IQC value exceeds the mean by 3SD. The assay run is regarded as out of control.
- Mandatory R4SD : It is only applied when the IQC is tested in duplicate. This rule is violated when the difference in SD between the duplicates exceeds 4SD.
- Mandatory 10x : This rule is violated when the last 10 consecutive IQC values are on the same side of the mean or target value.

Follow-up action in the event of a violation

There are three options as to the action to be taken in the event of a violation of a Westgard rule:

- Accept the test run in its entirety- this usually applies when only a warning rule is violated.
- Reject the whole test run- this applies only when a mandatory rule is violated.
- Enlarge the greyzone and thus re-test range for that particular assay run - this option can be considered in the event of a violation of either a warning or mandatory rule.