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IVD See external label 2°C-8°C
Σ=96 tests REF #1669-15

Digoxin (DIG) Microplate ELISA Cat. No. 1669-15

Intended Use: The Quantitative Determination of Digoxin Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay

SUMMARY AND EXPLANATION OF THE TEST

The clinical usefulness of the measurement of serum digoxin (DIG) is due to its low therapeutic ratio; a very small difference exists between therapeutic and toxic tissue levels. In addition, individuals may vary in their response to digoxin with an apparent increase in susceptibility to toxicity with age (1).

The action of digoxin is to increase the force and velocity of myocardial contraction. This is necessary in the treatment of congestive heart failure and arrhythmias such as atrial fibrillation and atrial flutter (2).

The myocardial concentrations of digoxin to serum levels remain relatively constant during normal renal function. This distribution ratio of digoxin is approximately 29 to 1 between the heart and serum (3). Thus, monitoring digoxin therapy by measurement of serum levels is feasible from the pharmacological standpoint, since serum levels are related to tissue levels following post-absorption equilibration (1). A practical and sensitive method of digoxin quantitation in serum is by enzyme immunoassay.

This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, patient specimen, or control is first added to a microplate well. Enzyme-digoxin conjugate is added, then the reactants are mixed. A competition reaction results between the enzyme conjugate and the native digoxin for a limited number of antibody combining sites immobilized on the well.

After the completion of the required incubation period, the antibody bound enzyme-digoxin conjugate is separated from the unbound enzyme-digoxin conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known digoxin concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with digoxin concentration.

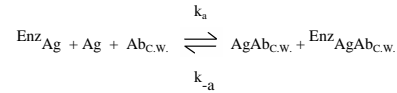
PRINCIPLE

Competitive Enzyme Immunoassay

The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a

limited number of insolubilized binding sites. The interaction is illustrated by the following equation:



Ab_{C.W.} = Monospecific Immobilized Antibody (Constant Quantity)

Ag = Native Antigen (Variable Quantity)

Enz_{Ag} = Enzyme-antigen Conjugate (Constant Quantity)

AgAb_{C.W.} = Antigen-Antibody Complex

Enz_{Ag} Ab_{C.W.} = Enzyme-antigen Conjugate -Antibody Complex

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Disassociation

K = k_a / k_{-a} = Equilibrium Constant

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

REAGENTS

Materials Provided:

A. Human Serum References -- 1ml/vial - Icons A-F

Six (6) vials of serum reference for digoxin at concentrations of 0 (A), 0.25 (B), 0.5 (C), 1.0 (D), 2.0 (E) and 4.0 (F) ng/ml. Store at 2-8°C. A preservative has been added.

B. Enzyme-antigen Conjugate -- 13 ml/vial - Icon E

One (1) vial of Digoxin-alkaline phosphatase (APase) conjugate in a buffer with dye. A preservative has been added. Store at 2-8°C.

C. Anti-digoxin Coated Microplate -- 96 wells - Icon

One 96-well microplate coated with rabbit anti-digoxin serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution Concentrate -- 20ml - Icon

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

E. p-NPP Substrate -- 10ml/vial - Icon B^S

One (1) bottle containing p-nitrophenyl phosphate (p-NPP). Store at 2-8°C.

F. End Reagent -- 6.0ml/vial - Icon END

One (1) bottle containing a strong base (1N NaOH). Store at 2-30 C.

I. Product Instructions

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.

Note 3: Above reagents are for a single 96-well microplate.

Required But Not Provided:

1. Pipette capable of delivering 25µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 405nm and 620nm wavelength absorbance capability.
5. Test tube(s) for mixing substrates A&B.

6. Absorbent Paper for blotting the microplate wells.
7. Plastic wrap or microplate cover for incubation steps.
8. Vacuum aspirator (optional) for wash steps.
9. Timer.
10. Quality control materials

PRECAUTIONS

*For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals*

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA licensed reagents. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

SPECIMEN COLLECTION AND PREPARATION

Collect sample(s) by venipuncture in ten (10) ml silicone evacuated tube(s) or evacuated tube(s) containing EDTA or heparin. The usual precautions in the collection of venipuncture samples should be observed. Separate the red blood cells by centrifugation. Use serum or plasma for the total DIG procedure. Specimen(s) may be refrigerated at 2-8°C for a maximum period of 48 hours. If the specimen(s) cannot be assayed within 48 hours, the sample(s) may be stored at temperatures of -20°C for up to 30 days. When assayed in duplicate, 0.05ml of the specimen is required.

The cross-reactivity of the Digoxin antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of digoxin needed to displace the same amount of tracer.

Substance	Cross Reactivity
Digoxin	1.000
Digitoxin	0.019
Digitoxigenin	0.017
Lanatoside A	0.016
Oubain	0.001
Spirinolactone	0.001
Prednisone	0.001
Pregnenolone	0.001
Digitoxose	0.001

Di-Acetyldigoxin, β-Methyl digoxin, α-Acetyldigoxin completely cross react in the assay.

REAGENT PREPARATION

1. **Wash Buffer**
Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature 20-27°C for up to 60 days

TEST PROCEDURE

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

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of Digoxin-enzyme conjugate solution to all wells.
4. Swirl the microplate gently for 20-30 seconds to mix and cover.

5. Incubate 30 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
7. Add 300µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
8. Add 0.100 ml (100µl) of p-NPP substrate to all wells **Always add reagents in the same order to minimize reaction time differences between wells.**
9. Incubate at room temperature for thirty (30) minutes.
10. Add 0.050ml (50µl) of end reagent to each well and gently mix for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
11. Read the absorbance in each well at 405nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. **The results should be read within thirty (30) minutes of adding the stop solution.**

Note: For reassaying specimens with concentrations greater than 4 ng/ml, pipet 12.5µl of the specimen and 12.5µl of the 0 serum reference into the sample well. Multiply the readout value by 2 to obtain the digoxin concentration.

RESULTS

A dose response curve is used to ascertain the concentration of follicle stimulating hormone in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding DIG concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of digoxin for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance 1.262 (intersects the standard curve at (0.93 ng/ml) digoxin concentration (See Figure 1).

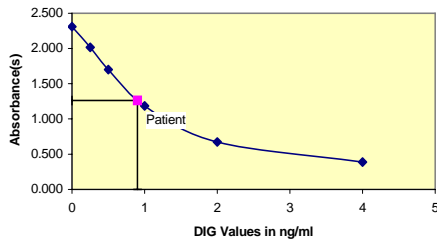
QUALITY CONTROL

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

EXAMPLE 1

Sample L.D.	Well Number	Abs (A)	Mean Abs (B)	Value (ng/ml)
Cal A	A1	2.306	2.311	0
	B1	2.316		
Cal B	C1	2.012	2.018	0.25
	D1	2.024		
Cal C	E1	1.717	1.701	0.5
	F1	1.685		
Cal D	G1	1.144	1.184	1
	H1	1.224		
Cal E	A2	0.662	0.672	2
	B2	0.683		
Cal F	C2	0.379	0.387	4
	D2	0.396		
Patient	E2	1.240	1.262	0.9
	F2	1.284		

Figure 1



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

Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator 0 ng/dl should be ≥ 1.3 .
2. Four out of six quality control pools should be within the established ranges.

LIMITATIONS OF PROCEDURE

A. Assay Performance

1. It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
2. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the end reagent. Therefore, the

addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time deviation during reaction.

3. Sample(s) that are contaminated microbiologically should not be used in the assay. Highly lipemic or hemolysed specimen(s) should similarly not be used.
4. Plate readers measure vertically. Do not touch the bottom of the wells.
5. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.

B. Interpretation

1. Certain disease states are known to increase a patient's susceptibility to digoxin toxicity (4). The following are examples of such disease states.
 - (a) Hypokalaemia
 - (b) Hypothyroidism
 - (c) Renal Failure
 - (d) Advance Heart Disease
2. A number of researchers have reported relatively high serum digoxin levels in infants. However, digoxin treated-children older than two years of age demonstrate serum digoxin levels more closely resembling adult values (3).
3. Patients receiving simultaneous quinidine and digoxin therapy should be monitored closely (5). Serum digoxin levels may rise to greater than twice the stabilized level within 24 hours after initiation of quinidine therapy and may remain higher for several days.
4. Patients receiving the diuretic furosemide may not display digoxin values that correspond to the clinical picture (6). When furosemide and digitalis preparations are used concurrently, monitoring patients is desirable (7).

EXPECTED RANGES OF VALUES

The usual therapeutic range of digoxin in adults is 0.5-2.0 ng/ml. However, there is an overlap of serum digoxin concentrations in groups of patients with and without clinical toxicity. A significant number of non-toxic patients have serum concentrations greater than 2.0 ng/ml and a correspondingly significant number of toxic patients have serum values in the range of 1.4-2.0 ng/ml (8). Also, patients with supraventricular arrhythmias may require higher doses to control their cardiac rate: these patients' digoxin concentrations range from 2.0-4.0 ng/ml without clinical toxicity. For these reasons, the physician should make a definite clinical diagnosis after all clinical and laboratory findings have been evaluated.

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precision of the DIG Microplate EIA Test System were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in ng/ml)

Sample	N	X	σ	C.V.
Low	12	0.66	0.05	7.6%
Normal	12	1.91	0.10	5.2%
High	12	3.20	0.17	5.3%

TABLE 3
Between Assay Precision* (Values in ng/ml)

Sample	N	X	σ	C.V.
Low	10	0.68	0.06	9.1%
Normal	10	1.83	0.12	6.6%
High	10	3.15	0.20	6.5%

*As measured in ten experiments in duplicate.

B. Sensitivity

The Digoxin procedure has a sensitivity of 2.5 pg. This is equivalent to a sample containing a concentration of 0.04 ng/ml.

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