DRG® Digoxin ELISA (EIA-3268)

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Please use only the valid version of the package insert provided with the kit.

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. Digoxin coupled with enzyme horseradish peroxidase (HRP) is added, and the reactants are mixed. A competition reaction results between the enzyme conjugated digoxin 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. Excess unbound enzyme reagent is washed via a wash step. 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.

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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 insolubulized binding sites. The interaction is illustrated by the followed equation:

$$Enz_{Ag} + Ag + Ab_{C.W.} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{C.W.} + {}^{Enz}AgAb_{C.W.}$$
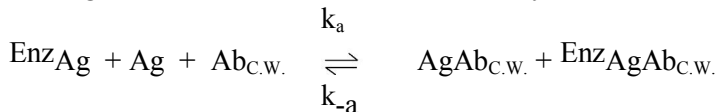
$Ab_{C.W.}$ = Monospecific Immobilized Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
Enz_{Ag} = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{C.W.}$ = Antigen-Antibody Complex
$Enz_{Ag} Ab_{C.W.}$ = Enzyme-antigen Conjugate -Antibody Complex
k_a = Rate Constant of Association
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 – 1 ml/vial**
   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. **Digoxin Enzyme Reagent – 6.0 ml**
   One (1) vial of Digoxin-Horseradish peroxidase (HRP) conjugate in a buffer with dye. A preservative has been added.
   Store at 2-8°C.

C. **Digoxin Biotin Reagent – 6.0 ml**
   One (1) bottle of reagent contains anti-digoxin biotinylated rabbit serum conjugate in buffer, dye and preservative.
   Store at 2-8°C.

D. **Streptavidin Coated Plate – 96 wells**
   One 96-well microplate coated with 1.0 µg/ml streptavidin and packaged in an aluminum bag with a drying agent.
   Store at 2-8°C.

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

F. **Substrate A – 7 ml/vial**
   One (1) vial contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

G. **Substrate B – 7 ml/vial**
   One (1) vial contains hydrogen peroxide (H_2O_2) in buffer.
   Store at 2-8°C.

H. **Stop Solution – 8 ml/vial**
   One (1) vial contains a strong acid (1N HCl).
   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. Pipettes capable of delivering 25, 50 & 100 µl volumes with a precision of better than 1.5%.

2. Dispenser(s) for repetitive deliveries of 0.100 ml and 0.350 ml volumes with a precision of better than 1.5%.

3. Microplate washers or a squeeze bottle (optional).

4. Microplate Reader with 405 nm and 620 nm wavelength absorbance capability.

5. Absorbent Paper for blotting the microplate wells.

6. Plastic wrap or microplate cover for incubation steps.

7. Vacuum aspirator (optional) for wash steps.

8. Timer.

9. Quality control materials

PRECAUTIONS

For 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.

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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. Avoid repeat freeze-thaw cycles.

When assayed in duplicate, 0.050 ml 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 doses 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
Ouabain	0.001
Spimolactone	0.001
Prednisone	0.001
Pregnenolone	0.001
Digitoxose	0.001

Di-Acetyidogoxin, β-Methyldigoxin, α-Acetyldigoxin completely cross react in the assay.

REAGENT PREPARATION

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

2. **Working Substrate Solution – Stable for 1 year**
Pour the contents of the amber vial labeled Solution 'A" into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2-8°C.
Note: Do not use the working substrate if it looks blue.

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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 (25 µl) of the appropriate serum reference, control or specimen into the assigned well.

3. Add 0.050 ml (50µl) of Digoxin Enzyme Reagent to all wells.

4. Swirl the microplate gently for 20-30 seconds to mix and cover.

5. Add 0.050 ml (50 µl) Digoxin Biotin Reagent to all wells.

6. Swirl the microplate gently for 20-30 seconds to mix and cover.

7. Cover and incubate for 30 minutes at room temperature.

8. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

9. Add 350 µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes.
    An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

10. Add 0.100 ml (100 µl) of working substrate solution to all wells. (see Reagent Preparation Section).
    Always add reagents in the same order to minimize reaction time differences between wells.
    DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

11. Incubate at room temperature for fifteen (15) minutes.

12. Add 0.050 ml (50 µl) of Stop Solution to each well and gently mix for 15-20 seconds.
    Always add reagents in the same order to minimize reaction time differences between wells.

13. Read the absorbance in each well at 450 nm (using a reference wavelength of 620-630 mn)
    The results should be read within thirty (30) minutes of adding the stop solution.

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

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QUALITY CONTROL

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

Calculation of RESULTS

A dose response curve is used to ascertain the concentration of digoxin 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 (0.884) intersects the standard curve at (1.56 ng/ml) digoxin concentration (See Figure 1).

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EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (ng/ml)
Cal A	A1	2.572	2.548	0
	B1	2.524		
Cal B	C1	2.090	2.086	0.25
	D1	2.083		
Cal C	E1	1.738	1.705	0.5
	F1	1.671		
Cal D	G1	1.250	1.255	1.00
	H1	1.260		
Cal E	A2	0.816	0.810	2.00
	B2	0.805		
Cal F	C2	0.474	0.470	4.00
	D2	0.467		
Patient	E2	0.867	0.878	**1.56**
	F2	0.889		

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

Figure 1

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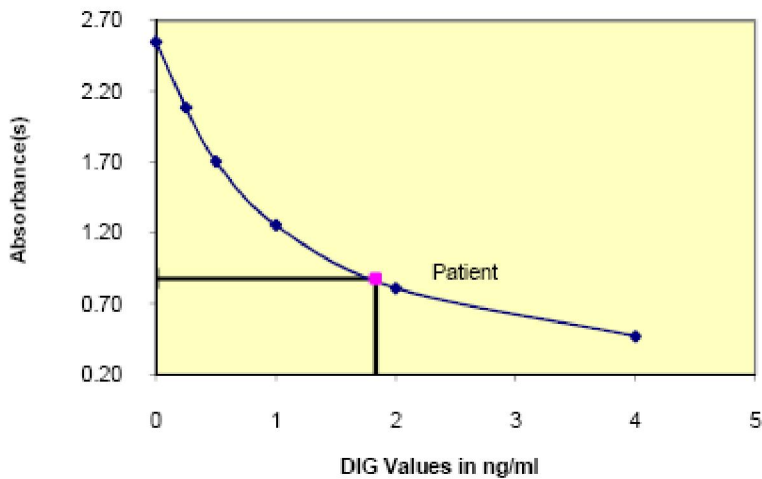
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Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:
1. The absorbance (OD) of calibrator 0 ng/ml should be ≥ 1.3.
2. Four out of six quality control pools should be within the established ranges.

Risk Analysis

Assay Performance
1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipaemic, hemolysed or grossly contaminated specimen(s) should not be used
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore the substrate and the stopping solution should be added in the same sequence to eliminate any time deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from this IFU may yield inaccurate results.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
11. It is important to calibrate al the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device and to perform routine preventive maintenance.
12. Risk Analysis – as required by CE Mark IVD Directive 98/79/EC - for this device, can be requested via email from corp@drg-international.com.

Interpretation
1. Laboratory results alone are only one aspect for determining patient care and should not be the sole basic for therapy, particularly if the results conflict with other determinants.
2. For valid results, adequate controls and other parameters must be within the listed ranges and assay requirements.
3. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, DRG shall have no liability.
4. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10 % of the assigned concentrations.

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5. 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
6. 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).
7. 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.
8. 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).
9. Individuals on large doses of biotin supplements should discontinue use one day before blood draw in order to eliminate possible interferences.

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

Precision

The within and between assay precision of the Digoxin ELISA were determined by analyses on three different levels of pool control sera. The number (N), mean values (X), standard deviation (σ) and coefficient of variation (C.V.) 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	0.48	0.04	9.00 %
Normal	12	1.67	0.11	6.60 %
High	12	3.14	0.16	5.00 %

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

Sample	N	X	σ	C.V.
Low	10	0.51	0.05	9.80 %
Normal	10	1.62	0.13	8.00 %
High	10	3.32	0.22	6.60 %

*As measured in ten experiments in duplicate.

Sensitivity

The Digoxin ELISA test system has an analytical sensitivity of 2.6 pg.
This is equivalent to a sample containing a concentration of 0.05 ng/ml.

The functional sensitivity (20% CV) was found to be 0.14 ng/ml.

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