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.

**TEST PRINCIPLE**

Competitive Enzyme Immunoassay (TYPE 7):
The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing biotinylated 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 antibody binding sites. The interaction is illustrated by the following equation:

\[
\text{Ab}_{\text{inv}} + \text{Ag} + \text{Ab} = \text{AgAb} + \text{EnzAgAb}_{\text{inv}}
\]

\[
\text{K} = \frac{k_a}{k_{-a}}
\]

Ab_{inv} = Biotinylated Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
EnzAg = Enzyme-antigen Conjugate (Constant Quantity)
AgAb = Antigen-Antibody Complex
EnzAgAb_{inv} = Enzyme-antigen Conjugate-Antibody Complex
k_{a} = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation
K = k_{a} / k_{-a} = Equilibrium Constant

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation or aspiration.

AgAb_{inv} + EnzAgAb_{inv} + Streptavidin_{w} \Rightarrow \text{immobilized complex}

Streptavidin_{w} = Streptavidin immobilized on well

Immobilized complex = sandwich complex bound to the solid surface

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.

**SPECIMEN COLLECTION AND PREPARATION**

The specimens shall be blood, serum in type and taken with the usual precautions in the collection of venipuncture samples. The blood should be collected in a redtop (with or without gel additives) venipuncture tube. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50 µl) of the specimen is required.
MATERIALS AND COMPONENTS

Materials provided with the test kits

1. Human Serum References – 1 ml/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.

2. 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.

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

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

5. Wash Solution Concentrate - 20 ml
   One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

6. Substrates - 7 ml/vial
   One (1) vial contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

7. Substrates - 7 ml/vial
   One (1) vial contains hydrogen peroxide H2O2 in buffer. Store at 2-8°C.

8. Stop Solution - 8 ml/vial
   One (1) vial contains a strong acid (1N HCl). Store at 2-8°C.

9. Plastic wrap or micro plate cover for incubation steps.

10. Vacuum aspirator (optional) for wash steps.

11. Timer

12. Quality control materials

ASSAY PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C). **Test Procedure should be performed by a skilled individual or trained professional**

1. Format the microplates’ wells for each serum reference, calibrator, 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, calibrator, control or specimen into the assigned well.

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

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

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

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

7. Cover and Incubate 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 0.350 ml (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.

11. Incubate at room temperature for thirty (30) 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 450nm (using a reference wavelength of 620-630nm). The results should be read within thirty (30) minutes of adding the stop solution.

Note: For re-assaying specimens with concentrations greater than 4 ng/ml, pipette 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.

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 Digoxin 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 (1.224) intersects the standard curve at (1.06 ng/ml) digoxin concentration (See Figure 1).

Preparation

Wash Buffer

Dilute contents of Wash Solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at room temperature (2-30°C) for up to 60 days.

Working substrate solution – Stable for 1 year

Pour the contents of the amber vial labeled as 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:

1. Do not use the working substrate if it looks blue.
2. Do not use reagents that are contaminated or have bacteria growth.
Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

**EXAMPLE 1**

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>Well Number</th>
<th>Abs (A)</th>
<th>Mean Abs (B)</th>
<th>Value (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>2.510</td>
<td>2.465</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>2.420</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal B</td>
<td>C1</td>
<td>2.107</td>
<td>2.088</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>D1</td>
<td>2.070</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal C</td>
<td>E1</td>
<td>1.832</td>
<td>1.805</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>1.779</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal D</td>
<td>G1</td>
<td>1.262</td>
<td>1.232</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>H1</td>
<td>1.202</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal E</td>
<td>A2</td>
<td>0.835</td>
<td>0.798</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>0.762</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal F</td>
<td>C2</td>
<td>0.434</td>
<td>0.425</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>D2</td>
<td>0.415</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>E2</td>
<td>1.214</td>
<td>1.224</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>1.233</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

**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.

**EXPECTED 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. 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.

**TABLE 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>12</td>
<td>0.048</td>
<td>0.04</td>
<td>9.0</td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>1.67</td>
<td>0.11</td>
<td>6.6</td>
</tr>
<tr>
<td>High</td>
<td>12</td>
<td>3.14</td>
<td>0.16</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*As measured in ten experiments in duplicate over a ten day period.

**B. Sensitivity**

The Digoxin ELISA test system has an analytical sensitivity of 0.072 ng/ml. The sensitivity was ascertained by determining the variability of the ‘0’ calibrator and using the 2σ (95% certainty) statistic to calculate the minimum concentration.

**Accuracy**

The DIG ELISA test system was compared against a predicate Digoxin method. Biological specimens from a general population were used. The values ranged from 0.5 – 2.917 ng/ml. The correlation is presented in Table 4.

**TABLE 4**

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (X)</th>
<th>Least Square Regression Analysis</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAI</td>
<td>1.249</td>
<td>y = 0.9702x + 0.1384</td>
<td>0.9288</td>
</tr>
<tr>
<td>Reference</td>
<td>1.144</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient also indicates excellent method agreement.
“Heterophilic antibodies: a problem for all immunoassays” Clin.Chem. 1988;34:27-33). For diagnostic purposes, the results from this assay should be used in combination with clinical examination, patient history and all other clinical findings.

4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.

5. 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, DAI shall have no liability.

6. 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.

7. Certain disease states are known to increase a patient’s susceptibility to digoxin toxicity. The following are examples of such disease states: hypokalemia, hypothyroidism, renal Failure, and advanced heart Disease.

8. 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.

9. Patients receiving simultaneous quinidine and digoxin therapy should be monitored closely. 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.

10. Patients receiving the diuretic furosemide may not display digoxin values that correspond to the clinical picture. When furosemide and digitalis preparations are used concurrently, monitoring patients is desirable.

11. Individuals on large doses of biotin supplements should discontinue use one day before blood draw in order to eliminate possible interferences.

QUALITY CONTROL

Each laboratory should assure 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.

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.

REFERENCES

7. Swidler, G., HANDBOOK of drug interactions, Wiley-Interscience, New York,