



AccuBind® CA 15-3 ELISA

Product Code: 5625-300

Intended Use: The Quantitative Determination of Cancer Antigen (CA 15-3) Concentration in Human Serum by a Microplate Immunoenzymometric assay

SUMMARY AND EXPLANATION OF THE TEST

Although multiple serum based tumor markers have been described for breast cancer, such as CA 15-3, BR 27-29, carcinoembryonic antigen (CEA), tissue polypeptide antigen (TPA), tissue polypeptide specific antigen, and HER-2 (the extracellular domain), the most widely used are CA 15-3 and CEA. CA 15-3 is considered to be one of the first circulating prognostic factors for breast cancer⁽¹⁾. Preoperative concentrations thus might be combined with prognostic factors for predicting outcome in patients with newly diagnosed breast cancer⁽²⁾. At present the most important clinical application of CA 15-3 is in monitoring therapy in patients with advanced breast cancer that is not accessible by existing clinical or radiologic procedures⁽³⁾.

The CA 15-3 assay measures the protein product of *MUC1* gene. *MUC1* protein is a large transmembrane glycosylated molecule containing three main domains, a large extracellular region, a membrane spanning sequence, and a cytoplasmic domain⁽⁴⁾. Although the physiologic function of *MUC1* is unclear, the glycoprotein has been implicated in cell adhesion, immunity and metastasis. Compared with healthy breast tissue, *MUC1* is present in higher concentrations but less glycosylated in breast carcinoma⁽⁵⁻⁸⁾.

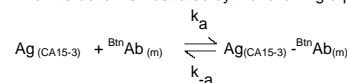
In this method, a prediluted CA15-3 calibrator diluted patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal antibody (specific for CA15-3) is added and the reactants mixed. Reaction between the CA15-3 antibodies and native CA15-3 forms complex that binds with the streptavidin coated to the well. The excess serum proteins are washed away via a wash step. Another enzyme labeled antibody specific for a different epitopic recognition of CA15-3 is added to the wells. The enzyme labeled antibody binds to the CA15-3 already immobilized on the well through its binding with the biotinylated monoclonal antibody. Excess enzyme is washed off via a wash step. A color is generated by the addition of a substrate. The intensity of the color generation is directly proportional to the concentration of the CA15-3 in the sample.

PRINCIPLE

Immunoenzymometric sequential assay (TYPE 4):

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, **in excess**, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-CA15-3 antibody.

Upon mixing monoclonal biotinylated antibody, and a serum containing the native antigen, a reaction results between the native antigen and the antibody, forming an antibody-antigen complex. The interaction is illustrated by the following equation:



$\text{B}^{\text{in}}\text{Ab}_{(\text{m})}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

$\text{Ag}_{(\text{CA15-3})}$ = Native Antigen (Variable Quantity)

$\text{Ag}_{(\text{CA15-3})} - \text{B}^{\text{in}}\text{Ab}_{(\text{m})}$ = Antigen-antibody complex (Variable Quantity)

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Disassociation

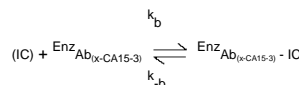
Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$\text{Ag}_{(\text{CA15-3})} - \text{B}^{\text{in}}\text{Ab}_{(\text{m})} + \text{Streptavidin}_{\text{CW}} \rightleftharpoons \text{Immobilized complex (IC)}$

$\text{Streptavidin}_{\text{CW}}$ = Streptavidin immobilized on well

$\text{Immobilized complex (IC)}$ = Ag-Ab bound to the well

After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation or aspiration. Another antibody (directed at a different epitope) labeled with an enzyme is added. Another interaction occurs to form an enzyme labeled antibody-antigen-biotinylated-antibody complex on the surface of the wells. Excess enzyme is washed off via a wash step. A suitable substrate is added to produce color measurable with the use of a microplate spectrophotometer. The enzyme activity on the well is directly proportional to the native free 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.



$\text{Enz}_{\text{Ab}_{(\text{X-CA15-3})}}$ = Enzyme labeled Antibody (Excess Quantity)

$\text{Enz}_{\text{Ab}_{(\text{X-CA15-3})}} - \text{IC}$ = Antigen-Antibodies Complex

k_b = Rate Constant of Association

k_{-b} = Rate Constant of Dissociation

REAGENTS

Materials Provided:

- A. **CA 15-3 Calibrators – 1.0 ml/vial - Icons A-F**
Six (6) vials of human serum based reference calibrators at concentrations of 0 (A), 10 (B), 40 (C), 100 (D), 200 (E) and 400 (F) U/ml. Store at 2-8°C. A preservative has been added.
Note 1: The calibrators are provided prediluted.
Note 2: The calibrators, human serum based, were made using a purified preparation of CA 15-3. The preparation was calibrated against Centocor CA 15-3 IRMA test.
- B. **CA15-3 Biotin Reagent – 12 ml/vial** ▽
One (1) vial contains biotinylated anti-human CA15-3 mIgG in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.
- C. **CA15-3 Enzyme Reagent – 12 ml/vial - Icon** ⓔ
One (1) vial contains horseradish peroxidase incorporated anti-human CA15-3 mIgG in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.
- D. **Streptavidin Coated Plate – 96 wells –Icon** ↓
One 96-well microplate coated with 1 µg/ml streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

- E. **Wash Solution Concentrate – 20ml - Icon** ⬇
One (1) vial contains surfactant in buffered saline. A preservative has been added. Store at 2-30°C.
- F. **CA 15-3 Dilution Matrix – 50 ml**
One (1) vial of serum diluent contains buffer salts, protein, surfactants. Store at 2-8°C.
- G. **Substrate Solution – 12ml/vial - Icon** S^N
One (1) bottle contains tetramethylbenzidine (TMB) and hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C.
- H. **Stop Solution – 8ml/vial - Icon** ⓧ
One (1) vial contains a strong acid. 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:

- Pipette capable of delivering 25 µl and 50 µl with a precision of better than 1.5%.
- Dispenser(s) for repetitive deliveries of 0.100ml and 0.350ml volumes with a precision of better than 1.5%.
- Pipette (1000µl) used for serum diluent in patient dilutions.
- Microplate washer or a squeeze bottle (optional).
- Microplate Reader with 450nm and 620nm wavelength absorbance capability.
- Absorbent Paper for blotting the microplate wells.
- Plastic wrap or microplate cover for incubation steps.
- Vacuum aspirator (optional) for wash steps.
- Timer.
- 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 required tests. 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

The specimens shall be blood; serum or heparinized plasma in type and taken with the usual precautions in the collection of venipuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a redtop (with or without gel additives) venipuncture tube or for plasma use evacuated tube(s) containing heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma 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 repetitive freezing and thawing. When assayed in duplicate, 0.050ml of the diluted specimen is required.

REAGENT 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 (20-27°C) for up to 60 days.

2. Patient Sample Dilution (1:21)

Dispense 25 µl of each control and/or patient specimen into 0.500 ml of CA 15-3 dilution matrix appropriately labeled, clean container(s) and mix thoroughly before use. Store refrigerated at 2-8°C for up to 48 hours.

TEST PROCEDURE

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

- 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.**
- Pipette 0.025 ml (25 µl) of the appropriate diluted serum reference, control or specimen into the assigned well.
- Add 0.100 ml (100µl) of the biotinylated labeled antibody to each well. **It is very important to dispense all reagents close to the bottom of the coated well.**
- Swirl the microplate gently for 20-30 seconds to mix and cover.
- Incubate 60 minutes at room temperature.
- Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
- Add 350µl 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.**
- Add 0.100 ml (100µl) of the CA15-3 Enzyme Reagent to each well.

DO NOT SHAKE THE PLATE AFTER ENZYME ADDITION

- Cover and incubate 60 minutes at room temperature.
- Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
- Add 350µl 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.**
- Add 0.100 ml (100µl) of substrate reagent to all wells. **Always add reagents in the same order to minimize reaction time. DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION**
- Incubate at room temperature for twenty (20) minutes.
- Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds.
- Read the absorbance in each well at 450nm (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.**

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.

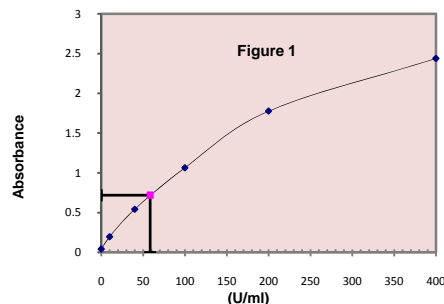
CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of CA15-3 in unknown specimens.

- Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
- Plot the absorbance for each duplicate serum reference versus the corresponding CA 15-3 concentration in U/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
- Connect the points with a best-fit curve.
- To determine the concentration of CA 15-3 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 U/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 (0.721) intersects the dose response curve at (58.4U/ml) CA 15-3 concentration (See Figure 1).

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (U/ml)
Cal A	A1	0.044	0.043	0
	B1	0.042		
Cal B	C1	0.204	0.198	10
	D1	0.191		
Cal C	E1	0.560	0.543	40
	F1	0.525		
Cal D	G1	1.103	1.064	100
	H1	1.024		
Cal E	A2	1.784	1.777	200
	B2	1.770		
Cal F	C2	2.431	2.438	400
	D2	2.445		
Patient	A3	0.737	0.721	58.4
	B3	0.705		



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

Q.C. PARAMETERS

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

- The absorbance (OD) of calibrator F should be ≥ 1.3 .
- Four out of six quality control pools should be within the established ranges.

Risk Analysis

A. Assay Performance

- It is important that the time of reaction in each well is held constant for reproducible results.
- If more than one (1) plate is used, it is recommended to repeat the dose response curve.
- Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time-deviation during reaction.
- Plate readers measure vertically. Do not touch the bottom of the wells.
- Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
- Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
- Use components from the same lot. No intermixing of reagents from different batches.
- Patient specimens (diluted) with CA 15-3 concentrations above 400 U/ml may be further diluted (1/10 or higher) with CA15-3 diluted serum diluent and re-assayed. The sample's concentration is obtained by multiplying the result by the dilution factor.
- It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used for using this device.
- Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

B. Interpretation

- 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.
- CA 15-3 has a low clinical sensitivity and specificity as a tumor marker. Clinically an elevated CA 15-3 value alone is not of diagnostic value as a test for cancer and should only be used in conjunction with other clinical manifestations (observations) and diagnostic parameters.

EXPECTED RANGES OF VALUES

The serum CA 15-3 is elevated in 2% of normal healthy women and 7% of patients with non-neoplastic conditions. Also, it has been reported to be elevated in cases of liver, lung, ovarian and colorectal cancers. No definitive ranges have been reported for those conditions.

TABLE I
Expected Values for the CA 15-3 Elisa Test System

Healthy Females ≤ 37 U/ml

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal"-persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precision of the CA 15-3 AccuBind™ ELISA test system were determined by analyses on three different levels of control sera. The number, mean value, standard deviation (σ) 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 U/ml)

Sample	N	X	σ	C.V.
Level 1	20	20.9	1.91	9.1%
Level 2	20	61.7	2.03	3.3%
Level 3	20	96.9	2.67	2.8%

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

Sample	N	X	σ	C.V.
Level 1	10	22.2	2.0	9.1%
Level 2	10	58.5	3.85	6.6%
Level 3	10	104.6	9.33	8.9%

*As measured in ten experiments in duplicate.

B. Sensitivity

The CA 15-3 procedure has a analytical sensitivity of 0.2 U/ml at three (3) SD from the zero calibrator. The functional sensitivity (20% CV) was found to be 1.25U/ml.

C. Accuracy

The Monobind CA 15-3 AccuBind™ ELISA procedure was compared with a reference Elisa method. Biological specimens from low, normal, and elevated concentrations were assayed. The total number of such specimens was 43. The least square regression equation and the correlation coefficient were computed for the CA 15-3 in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4
Least Square Regression Analysis
Correlation Coefficient

Method	Mean	Analysis	Correlation Coefficient
This Method (Y)	180.2	$y = -0.219 + 1.008 (x)$	0.999
Reference (X)	178.6		

D. Specificity

In order to test the specificity of the antibody pair used massive concentrations of possible cross-reactants were added to known serum pools and assayed in parallel with the base sera. No cross reaction was found. Percent cross-reactions for some of these additions are listed below in Table 5.

TABLE 5

Analyte	Concentration	Interference
CA 15-3	-	1.000
CA 125	10000 U/ml	0.001
CA 19-9	5000 U/ml	0.001
PSA	1000 ng/ml	0.026
AFP	30,000 ng/ml	ND*
CEA	5,000ng/ml	ND*
HCG	125,000mIU/ml	ND*
RF	12,500 IU/ml	0.001
Bilirubin	200 μ g/ml	ND*
Hemolysis	30 μ l/ml	ND*
Lipids	50 μ g/ml	-0.009

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Revision: 2 Date: 112009

Cat #: 5625-300

Size	96(A)	192(B)
Reagent (ml)	A) 1ml set	1ml set
	B) 1 (12ml)	2 (12ml)
	C) 1 (12ml)	2 (12ml)
	D) 1 plate	2 plates
	E) 1 (20ml)	1 (20ml)
	F) 1 (50ml)	2 (50ml)
	G) 1 (12ml)	2 (12ml)
	H) 1 (8ml)	2 (8ml)

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Instruments & Applications

Monobind's immunoassay products are designed to work in both manual and automated lab environments. AccuBind™ and AccuLite™ are compatible with any open-ended instrumentation, including chemistry analyzers, microplate readers and microplate washers. There may or may not be an application developed for your particular instrument, please visit the instrument section of our website, or contact techsupport@monobind.com