Enzyme Immunoassay for the quantitative determination of Breast Cancer Antigen CA15-3 in Human Serum

FOR IN VITRO DIAGNOSTIC USE ONLY

Store at 2 to 8°C.

INTENDED USE

For the quantitative determination of the Cancer Antigen CA15-3 concentration in human serum.

INTRODUCTION

Breast cancer is the most common life-threatening malignant lesion in women of many developed countries today, with approximately 180,000 new cases diagnosed every year. Roughly half of these newly diagnosed patients are node-negative, however 30% of these cases progress to metastatic disease.
There are a number of tumor markers that can help clinicians to identify and diagnose which breast cancer patients will have aggressive disease and which will have an indolent course. These markers include estrogen and progesterone receptors, DNA ploidy and percent-S phase profile, epidermal growth factor receptor, HER-2/neu oncogene, p53 tumor suppressor gene, cathepsin D, proliferation markers and CA15-3. CA15-3 is most useful for monitoring patients post-operatively for recurrence, particularly metastatic diseases. 96% of patients with local and systemic recurrence have elevated CA15-3, which can be used to predict recurrence earlier than radiological and clinical criteria. A 25% increase in the serum CA15-3 is associated with progression of carcinoma. A 50% decrease in serum CA15-3 is associated with response to treatment. CA15-3 are more sensitive than CEA in early detection of breast cancer recurrence. In combination with CA125, CA15-3 has been shown to be useful in early detection of relapse of ovarian cancer. CA15-3 levels are also increased in colon, lung and hepatic tumors.

PRINCIPLE OF THE TEST

The CA15-3 ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a monoclonal antibody directed against a distinct antigenic determinant on the intact CA15-3 molecule is used for solid phase immobilization (on the microtiter wells). A rabbit anti-CA15-3 antibody conjugated to horseradish peroxidase (HRP) is in the antibody-enzyme conjugate solution. The test sample is allowed to react sequentially with the two antibodies, resulting in the CA15-3
molecules being sandwiched between the solid phase and enzyme-linked antibodies. After two separate 1-hour incubation steps at 37°C, the wells are washed with water to remove unbound labeled antibodies. A solution of TMB Reagent is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of Stop

Solution changing the color to yellow. The concentration of CA15-3 is directly proportional to the color intensity of the test sample. Absorbance is measured spectrophotometrically at 450 nm.

REAGENTS

Materials provided with the kit:

- Antibody-Coated Wells (1 plate, 96 wells)
  Microtiter wells coated with CA15-3 MoAb
- Reference Standard Set (1.0 ml/vial)
  Contains 0, 15, 30, 60, 120, and 240 Unit/ml of CA15-3 in bovine serum with preservatives; liquid, ready to use
  These standards have been pre-diluted 51-fold. Please do not dilute them again.
- CA15-3 Enzyme Conjugate Concentrate (22x), 1.0 ml
  Contains CA15-3 MoAb conjugated to horseradish peroxidase with preservatives
- CA15-3 Conjugate Diluent, 21 ml
  Contains bovine serum, tris buffer and preservatives
- CA 15-3 Sample Diluent, 100 ml
  Tris buffer with preservatives
- TMB Reagent (11 ml)
  Contains 3, 3', 5, 5' tetramethylbenzidine (TMB) stabilized in buffer solution
- Stop Solution -1N HCl (11 ml)
  Diluted hydrochloric acid

Materials required but not provided:

- Precision pipettes and tips: 20 µl, 100 µl, 200 µl, and 1 ml
- Distilled water
- Disposable pipette tips
- Vortex mixer
- Absorbent paper or paper towel
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an optical density range of 0-2 OD or greater
- Graph paper

STORAGE CONDITIONS

1. Store the unopened kit at 2-8°C upon receipt and when it is not in use, until the expiration shown on the kit label. Refer to the package label for the expiration date.
2. Keep microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

INSTRUMENTATION

A microtiter well reader with a bandwidth of 10 nm or less and an optical density range of 0 to 3 OD or greater at 450 nm wavelength is acceptable for absorbance measurement.

WARNINGS AND PRECAUTIONS

1. CAUTION: This kit contains human material. The source material used for manufacture of this component tested negative for HBsAg, HIV 1/2 and HCV by FDA-approved methods. However, no method can completely assure absence of these agents. Therefore, all human blood products, including serum samples, should be considered potentially infectious. Handling should be as defined by an appropriate national biohazard safety guideline or regulation, where it exists.[25]
2. Avoid contact with 1N HCl. It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.
3. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.
4. Replace caps on reagents immediately. Do not switch caps.

5. Do not pipette reagents by mouth.
6. For in vitro diagnostic use.

SPECIMEN COLLECTION AND PREPARATION

1. The use of SERUM samples is required for this test.
2. Specimens should be collected using standard venipuncture techniques. Remove serum from the coagulated or packed cells within 60 minutes after collection.
3. Specimens which cannot be assayed within 24 hours of collection should be frozen at –20°C or lower, and will be stable for up to six months.
4. Avoid grossly hemolytic (bright red), lipemic (milky), or turbid samples (after centrifugation).
5. Specimens should not be repeatedly frozen and thawed prior to testing. DO NOT store in "frost free" freezers, which may cause occasional thawing. Specimens which have been frozen, and those which are turbid and/or contain particulate matter, must be centrifuged prior to use.

PROCEDURAL NOTES

1. Pipetting Recommendations (single and multi-channel): Pipetting of all standards, samples, and controls should be completed within 3 minutes.
2. All standards, samples, and controls should be run in duplicate concurrently so that all conditions of testing are the same.
3. <u>It is recommended that the wells be read within 15 minutes following addition of Stop Solution.</u>

REAGENT PREPARATION

1. All reagents should be brought to room temperature (18-25°C) before use.
2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
3. To prepare working CA 15-3 Conjugate Reagent, add the entire 1.0 ml of Conjugate Concentrate (22x) to 21 ml of the Enzyme Conjugate Diluent (1:21 dilution) and mix well. The diluted Enzyme Conjugate Reagent is stable at 4° C for at least 4 months.

ASSAY PROCEDURE

1. **Patient serum and control serum should be diluted, 51 fold, before use. Prepare a series of small tubes (such as 1.5 ml microcentrifuge tubes) and mix 20 µl serum with 1.0 ml Sample Diluent. <u>PLEASE DO NOT DILUTE THE STANDARDS.</u>**
2. Secure the desired number of coated wells in the holder.
3. Dispense 200 µl of CA15-3 standards, **<u>diluted</u>** specimens, and **<u>diluted</u>** controls into the appropriate wells. Gently mix for 10 seconds.
4. Incubate at 37°C for 1 hour.
5. Remove the incubation mixture by emptying the plate content into a waste container.
6. Rinse and empty the microtiter plate 5 times with distilled or deionized water. (Please do not use tap water.)
7. Strike the microtiter plate sharply onto absorbent paper or paper towels to remove all residual water droplets.
8. Dispense 200 µl of Enzyme Conjugate Reagent into each well. Gently mix for 10 seconds
9. Incubate at 37°C for 1 hour.
10. Remove the contents and wash the plate as described in steps 6-7 above.
11. Dispense 100 µl of TMB Reagent into each well. Gently mix for 10 seconds.
12. Incubate at room temperature in the dark for 20 minutes.
13. Stop the reaction by adding 100 µl of Stop Solution to each well.
14. Gently mix for 30 seconds. **It is important to make sure that all the blue color changes to yellow color completely.**

15. Read the optical density at 450nm with a microtiter plate reader **within 15 minutes**.

QUALITY CONTROL

Good laboratory practice requires that quality control specimens (controls) be run with each calibration curve to verify assay performance. To ensure proper performance, control material should be assayed repeatedly to establish mean values and acceptable ranges.

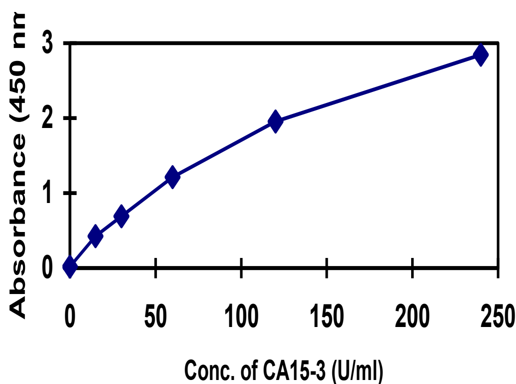
CALCULATION OF RESULTS

1. Calculate the average absorbance values (A450) for each set of reference standards, control, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in U/ml on linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of CA15-3 in U/ml from the standard curve.
4. Any diluted samples must be further corrected by the appropriate dilution factor.

EXAMPLE OF STANDARD CURVE

Results of a typical standard run with optical density readings at 450nm shown in the Y axis against CA15-3 concentrations shown in the X axis. This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her own data and standard curve.

CA15-3 Values (U/ml)	Absorbance (450 nm)
0	0.021
15	0.425
30	0.693
60	1.214
120	1.956
240	2.845



EXPECTED VALUES

Healthy women are expected to have CA15-3 assay values below 35 U/ml.

PERFORMANCE CHARACTERISTICS

1. Accuracy

A statistical study using patient samples demonstrated good correlation of results with the commercially available kits as shown below:

Comparisons between CA 15-3 ELISA and Truquant BR™ CA 27.29 RIA kits provide the following data:

N = 142
Correlation coefficient = 0.998
Slope = 1.055

Intercept = 14.48
CA 15-3 ELISA Test Mean = 197 U/mL
Truquant BR Mean = 222 U/mL

2. Sensitivity
The minimal detectable concentration of CA 15-3 by this assay is estimated to be 5 U/mL.

3. Precision

a. Intra-Assay Precision
Within-run precision was determined by replicate determinations of four different control sera in one assay. Within-assay variability is shown below:

Serum Sample	**1**	**2**	**3**	**4**
Number of Replicates	24	24	24	24
Mean CA 15-3 (U/mL)	12.5	52.9	146.2	273.5
Standard Deviation	1.10	4.28	12.76	27.54
C.V. (%)	8.8	8.1	8.7	10.1

b. Inter-Assay Precision
Between-run precision was determined by replicate measurements of four different control sera in several different assays. Between-assay variability is shown below:

Serum Sample	**1**	**2**	**3**	**4**
Number of Replicates	30	30	30	30
Mean CA 15-3 (U/mL)	17.0	72.7	165.6	267.3
Standard Deviation	1.16	7.25	8.01	20.66
C.V. (%)	6.8	10.0	4.8	7.7

4. Recovery and Linearity Studies
a. Recovery
Various patient serum samples of known CA 15-3 levels were mixed and assayed in duplicate. The average recovery was 96.1%.

	Expected Concentration (U/ml)	**Observed Concentration (U/ml)**	**% Recovery**
1	282.53	300.24	106.3
.	224.04	206.76	92.3
2	69.38	67.52	97.3

. 3 . 4 . 5 . 6 .	109.85 15.55 15.19	109.15 13.99 13.84	99.4 90.0 91.1
	Average Recovery = 96.1%		

b. Linearity

Four patient samples were serially diluted with the zero standard in a linearity study. The average recovery was 96.4%.

#	Dilution	Expected Conc. (U/mL)	Observed Conc. (U/mL)	% Recovery
1.	Undiluted	----	112.20	----
	1:2	56.10	62.64	111.7
	1:4	28.05	29.25	104.3
	1:8	14.03	13.53	96.4
	1:16	7.01	6.59	94.0
				Mean = 101.6 %
2.	Undiluted	----	165.50	----
	1:2	82.75	74.53	90.1
	1:4	41.38	39.43	95.3
	1:8	20.69	20.65	99.8
	1:16	10.34	9.32	90.1
				Mean = 93.8 %
3.	Undiluted	----	111.30	----
	1:2	55.65	56.47	101.5
	1:4	27.83	28.35	101.9
	1:8	13.91	12.90	92.7
	1:16	6.96	6.53	93.9
				Mean = 97.5%
4.	Undiluted	----	159.40	----
	1:2	79.70	74.87	93.9
	1:4	39.85	37.70	94.6
	1:8	19.93	18.08	90.8
	1:16	9.96	9.15	91.9
				Mean = 92.8%

5. Specificity

The following substances were tested for cross-reactivity:

Analyte Tested	Concentration	Produced Intensity Equivalent To CA 15-3 (U/mL)
PSA	250 ng/mL	0
	500 ng/mL	0
	1,000ng/mL	0

	2,500ng/mL 5,000ng/mL	0 0
CEA	2,500ng/mL 5,000ng/mL 10,000ng/mL	0 0 0
CA 125	1,000 ng/mL 2,500 ng/mL 5,000 ng/mL 10,000 ng/mL 50,000 ng/mL	0 0 0 15 47
CA 19-9	500 ng/mL 1,000 ng/mL 2,500 ng/mL 5,000 ng/mL 10,000 ng/mL	0 0 0 14 47
PAP	6,000 ng/mL 12,000 ng/mL	0 0
AFP	2,500 ng/mL 5,000 ng/mL 10,000 ng/mL 25,000 ng/mL 50,000 ng/mL	0 0 0 0 0

- The cross reactivity was probably resulting from unpurified commercial CA 125 and CA 19-9 Antigens which contained small amount of CA 15-3.

LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.
2. The results obtained from the use of this kit should be used only as an adjunct to other diagnostic procedures and information available to the physician.
3. Serum samples demonstrating gross lipemia, gross hemolysis, or turbidity should not be used with this test.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. Patient samples may contain human anti-mouse antibodies (HAMA) that are capable of giving falsely elevated or depressed results with assays that utilize mouse monoclonal antibodies. This assay has been designed to minimize interference from HAMA-containing specimens. Nevertheless, complete elimination of this interference from all patient specimens cannot be guaranteed. A test result that is inconsistent with the clinical picture and patient history should be interpreted with caution.

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