CA 15-3 ELISA
Direct immunoenzymatic determination of CA 15-3 in human serum or plasma

INTENDED USE
Immunoenzymatic colorimetric method for quantitative determination of CA 15-3 concentration in human serum or plasma.
CA 15-3 is intended for laboratory use only.

1. CLINICAL SIGNIFICANCE
CA-15-3, is an abbreviation for cancer antigen 15-3. CA 15-3 levels are most useful in following the course of treatment in women diagnosed with breast cancer, especially advanced breast cancer. CA 15-3 levels are rarely elevated in women with early stage breast cancer. Cancers of the ovary, lung, and prostate may also raise CA 15-3 levels. Elevated levels of CA 15-3 may be associated with non-cancerous conditions, such as benign breast or ovarian disease, endometriosis, pelvic inflammatory disease, and hepatitis. Pregnancy and lactation can also cause CA 15-3 levels to rise. CA 15-3 is most useful for monitoring patients post-operatively for recurrence, particularly metastatic diseases. 96% of patients with local and systemic recurrence have elevated CA 15-3, which can be used to predict recurrence earlier than radiological and clinical criteria. A 25% increase in the serum CA 15-3 is associated with progression of carcinoma. A 50% decrease in serum CA 15-3 is associated with response to treatment. CA 15-3 is more sensitive than CEA in early detection of breast cancer recurrence. In combination with CA-125, CA 15-3 has been shown to be useful in early detection of relapse of ovarian cancer. CA 15-3 levels are also increased in colon, lung and hepatic tumours.

2. PRINCIPLE
CA 15-3 ELISA test is based on simultaneous binding of human CA 15-3 to two monoclonal antibodies, one immobilized on microwell plates, the other conjugated with horseradish peroxidase (HRP). After incubation, the separation bound-free is obtained with a simple solid-phase washing. Then, the enzyme in the bound-fraction reacts with the Substrate (H₂O₂) and the TMB Substrate and develops a blue color that changes into yellow when the Stop Solution (H₂SO₄) is added. The colour intensity is proportional to CA 15-3 concentration in the sample.

CA 15-3 concentration in the sample is calculated using a calibration curve.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit
1. CA 15-3 Calibrators (6 vials, 2 mL each)
   - CAL0
   - CAL1
   - CAL2
   - CAL3
   - CAL4
   - CAL5

2. CA 15-3 Control (1 vial, 2 mL) Concentration of Control is Lot-specific and is indicated on Quality Control Report
   - REF DCE045/5503-0

3. Serum diluent (1 vial, 100 mL)
   - Phosphate buffer 50 mM pH 7.4; BSA 1 g/L
   - REF DCE018/5518-0

4. Conjugate (1 vial, 22 mL)
   - Monoclonal antibody Anti CA 15-3 conjugated with horseradish peroxidase (HRP)
   - REF DCE002/5502-0

5. Coated Microplate (1 breakable microplate)
   - Monoclonal antibody anti-CA 15-3 adsorbed on microplate
   - REF DCE002/5503-0

6. TMB Substrate (1 vial, 15 mL)
   - H₂O₂-TMB 0.26 g/L (avoid any skin contact)
   - REF DCE004-0

7. Stop Solution (1 vial, 15 mL)
   - Sulphuric acid 0.15 mol/L (avoid any skin contact)
   - REF DCE005-0

8. 20X Conc. Wash Solution (1 vial, 50 mL)
   - NaCl 9 g/L; Tween-20 22 g/L
   - REF DCE007-0

3.2. Reagents necessary not supplied
Distilled water.

3.3. Auxiliary materials and instrumentation
Automatic dispenser.
Microplates reader (450 nm)

Note
Store all reagents at 2-8°C in the dark.
Open the bag of reagent 5 (Coated Microplate) only when it is at room temperature and close it...
immediately after use; once opened, the microplate is stable until the expiry date of the kit.

4. WARNINGS

• This kit is intended for in vitro use by professional persons only. Not for internal or external use in Humans or Animals.
• Use appropriate personal protective equipment while working with the reagents provided.
• Follow Good Laboratory Practice (GLP) for handling blood products.
• Some reagents contain small amounts of Proclin 300\(^{\text{a}}\) as preservatives. Avoid the contact with skin or mucosa.
• The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
• The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
• Avoid the exposure of reagent TMB/H\(_2\)O\(_2\) to directed sunlight, metals or oxidants. Do not freeze the solution.
• This method allows the determination of CA 15-3 from 0.5 to 240.0 U/mL.

5. PRECAUTIONS

• Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
• All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
• Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
• Do not interchange kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
• If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
• The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
• 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 minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.
• Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
• Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
• Maximum precision is required for reconstitution and dispensation of the reagents.
• Samples microbiologically contaminated, highly lipemic or haemolysed should not be used in the assay.
• Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibrators (\(C_0\)…\(C_5\))

The Calibrators have the following concentrations:

<table>
<thead>
<tr>
<th>U/mL</th>
<th>(C_0)</th>
<th>(C_1)</th>
<th>(C_2)</th>
<th>(C_3)</th>
<th>(C_4)</th>
<th>(C_5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>15</td>
<td>30</td>
<td>60</td>
<td>120</td>
<td>240</td>
</tr>
</tbody>
</table>

Once opened, the Calibrators are stable 6 months at 2-8°C.

CA 15-3 Calibrators and Control have already been pre-diluted and are ready for use.

6.2. Preparation of Wash Solution

Dilute the content of each vial of the “20X Conc. Wash Solution” with distilled water to a final volume of 600 mL prior to use. For smaller volumes respect the 1:20 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C.

6.3. Preparation of the Sample

The specimens can be human serum or plasma and the usual precautions in the collection of venipuncture samples should be observed.

For accurate comparison to established normal values, a fasting morning serum sample should be obtained.

To obtain the serum, the blood should be collected in a venipuncture tube without additives or anti-coagulants; allow the blood to clot; centrifuge the specimen to separate the serum from the cells.

Specimen can be stored at 2-8°C for at least a maximum of five days. For longer storage the specimen should be frozen at -20°C (max 30 days). Avoid repeated freezing and thawing.

All the samples must be diluted 1:50 with the Serum diluent, as in the following example:

<table>
<thead>
<tr>
<th>Serum Sample</th>
<th>Serum Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 (\mu)L</td>
<td>980 (\mu)L</td>
</tr>
</tbody>
</table>

Mix gently. The Contol is ready to use.

NB: for samples with concentration over 240 U/mL, dilute further the sample with the Sample diluent (attention: consider this dilution in the calculation of final result).

6.4. Procedure

• Allow all reagents to reach room temperature (22-28°C).
• Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
• To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
• As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve ($C_0$-$C_5$), two for each Control, two for each sample, one for Blank.

7. QUALITY CONTROL
Each laboratory should assay controls at normal, high and low levels range of CA 15-3 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. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the calibration curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. 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.

8. RESULTS

8.1. Mean Absorbance
Calculate the mean of the absorbance (Em) for each point of the calibration curve ($C_0$-$C_5$) and of each sample.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Samples/Control</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted samples/Control</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>$C_0$-$C_5$</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
</tr>
</tbody>
</table>
| Incubate for 1 hour at 37°C without covering the plate. Remove the content from each well. Wash the wells 5 times with 300 µL of diluted Wash Solution.
| Conjugate | 200 µL | 200 µL |
| Incubate for 1 hour at 37°C without covering the plate. Remove the content from each well. Wash the wells 5 times with 300 µL of diluted Wash Solution.
| TMB Substrate | 100 µL | 100 µL | 100 µL |
| Incubate at room temperature (22±28°C) for 15 minutes in the dark.
| Stop Solution | 100 µL | 100 µL | 100 µL |
| Shake gently the microplate. Read the absorbance (E) at 450 nm against Blank.

8.2. Calibration curve
Plot the mean value of absorbance (Em) of the Calibrators ($C_0$-$C_5$) against concentration. Draw the best-fit curve through the plotted points. (Es: Four Parameter Logistic).

8.3. Calculation of Results
Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in U/mL.

9. REFERENCE VALUES

<table>
<thead>
<tr>
<th>CA 15-3</th>
<th>Non-pregnant healthy women</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 35 U/mL</td>
<td></td>
</tr>
</tbody>
</table>

Please pay attention to the fact that the determination of a range of expected values for a "normal" population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacturer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision
10.1.1. Intra Assay Variation
Within run variation was determined by replicate measurements (15x) of three different control sera in one assay. The within assay variability is ≤ 7.8%.

10.1.2. Inter Assay Variation
Between run variations was determined by replicate measurements (10x) of five different control sera in different lots of kit. The between assay variability is ≤11.4%.

10.2. Accuracy
The recovery on three serum samples spiked with 25 - 50 - 100 U/mL of antigen gave an average value (±SD) of 106.61% ± 8.86%.

10.3. Sensitivity
The cross reaction of the antibody are shown in the table:

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Concentration</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA 15-3</td>
<td>---</td>
<td>100.0%</td>
</tr>
<tr>
<td>CA 125</td>
<td>1,000 U/mL</td>
<td>N.D.</td>
</tr>
<tr>
<td>CA 19-9</td>
<td>1,000 U/mL</td>
<td>N.D.</td>
</tr>
<tr>
<td>PSA</td>
<td>1,000 ng/mL</td>
<td>N.D.</td>
</tr>
<tr>
<td>PAP</td>
<td>1,000 ng/mL</td>
<td>N.D.</td>
</tr>
<tr>
<td>AFP</td>
<td>10,000 ng/mL</td>
<td>N.D.</td>
</tr>
<tr>
<td>CEA</td>
<td>1,000 ng/mL</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

The dilution test performed on three sera diluted 2 - 4 - 8 - 16 times gave an average value (±SD) of 103.71% ± 8.56%.

10.3. Sensitivity
The lowest detectable concentration of CA 15-3 that can be distinguished from the Calibrator zero is 0.5 U/mL at the 95 % confidence limit.

10.4. Specificity
The cross reaction of the antibody are shown in the table:
10.5. Correlation
CA 15-3 Diametra kit was compared with a commercial reference method. 54 serum samples were analysed according in both test system. The linear regression curve was calculated:

\[ y = 0.86x + 2.66 \]
\[ r^2 = 0.838 \]

\( y = \text{CA 15-3 Diametra} \)
\( x = \text{CA 15-3 MODULAR (Roche)} \)

11. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
<table>
<thead>
<tr>
<th></th>
<th>DE</th>
<th>ES</th>
<th>FR</th>
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<td>Producto sanitario para diagnóstico</td>
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<td>In vitro Diagnostic Medical Device</td>
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<td>Elaborado por</td>
<td>Fabriqué par</td>
<td>Manufacturer</td>
<td>Produttore</td>
<td>Produzido por</td>
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<tr>
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<td>Achtung, Begleitdokumente</td>
<td>Precaución, consulte los documentos adjuntos</td>
<td>Caution, consult accompanying documents</td>
<td>Attention, consultare la documentazione allegata</td>
<td>Atenção, consultar os documentos de acompanhamento</td>
<td>Attention, veuillez consulter les documents d'accompagnement</td>
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<tr>
<td><strong>yyyy-mm</strong></td>
<td>yyyy-mm-dd</td>
<td>Verwendbar bis</td>
<td>Estable hasta (usar antes de último día del mes)</td>
<td>Utiliser avant (dernier jour du mois indiqué)</td>
<td>Use by (last day of the month)</td>
<td>Utilizzare prima del (ultimo giorno del mese)</td>
</tr>
<tr>
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<td>Consultar las instrucciones</td>
<td>Consulter le mode d’emploi</td>
<td>Consult instructions for use</td>
<td>Consultare le istruzioni per l’uso</td>
<td>Consultar instrucciones para uso</td>
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<tr>
<td><strong>Σ = xx</strong></td>
<td>Ausreichend für “n” Tests</td>
<td>Contenido suficiente para “n” tests</td>
<td>Contenu suffisant pour “n” tests</td>
<td>Contains sufficient for “n” tests</td>
<td>Contenuto sufficiente per “n” saggi</td>
<td>Contém o suficiente para “n” testes</td>
</tr>
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<tr>
<td><strong>Min</strong></td>
<td></td>
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</tbody>
</table>
SUGGERIMENTI PER LA RISOLUZIONE DEI PROBLEMI / TROUBLESHOOTING

ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction
- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers
- contamination of pipettes, tips or containers
- insufficient washing (conjugates not properly removed) too high within-run
- reagents and/or strips not pre-warmed to CV % Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
- too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation