Colon-IC

ELISA Kit for the detection of Carcinoembryonic Antigen (CEA) Immune Complexes in Colorectal Carcinoma (CRC)

PRODUCT PROFILE

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FOR RESEARCH USE ONLY

rev.08/2008

Colon-IC
PRODUCT PROFILE
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COLORECTAL CARCINOMA (CRC): from diagnosis to treatment

Colorectal Carcinoma (CRC) is the third most commonly diagnosed cancer and the second leading cause of death in the United States (1). It represents approximately 11% of all new cases of cancer. The lifetime risk of developing colorectal cancer has been estimated at approximately 6%, while the estimated lifetime risk of colorectal cancer death is approximately 2.6% (1). Colorectal cancer affects men and women with nearly equal frequency and is the third most common cancer in both men (after lung and prostate cancer) and women (after lung and breast cancer) (1).

Several potential risk factors for the development of colorectal cancer have been identified, however it is important to note that approximately 75% of colorectal cancers occur in patients with no known predisposing factors for the disease (2), while only approximately 6% of colorectal cancers occur in patients with specific hereditary conditions that are known to be predisposing factors for colorectal cancer. Patients without predisposing factors are considered to be at average risk. All other patients are considered to be at moderate or high risk. Factors that have been associated with an increased risk for the development of colorectal cancer include older age, family history of colorectal cancer, individual history of colorectal cancer or adenomatous polyps, certain hereditary conditions, inflammatory bowel disease, diets that are high in saturated fat and/or low in fiber, excessive alcohol consumption, and sedentary lifestyle (3–14). Obviously, many of these risk factors cannot be changed. Furthermore, the majority of the controllable risk factors (eg, diet, physical inactivity) can require massive efforts to achieve substantial reductions in colorectal cancer risk (15). It is generally accepted that most cancers of the colon and rectum develop from adenomatous polyps (16,17). Fortunately, only a small percentage of all adenomatous polyps progress to cancer. The risk that a polyp harbours invasive cancer is related to its size: polyps smaller than 1 cm are associated with approximately a 1% risk; polyps between 1 and 2 cm, a 10% risk; and polyps larger than 2 cm, a risk of more than 25% (18,19,20). The risk of cancer is also related to the villous component of the polyps; in general, villous adenomas have approximately 10 times the risk of cancer compared with that of tubular adenomas of the same size (21).

In general, screening for any disease can be justified in the following circumstances: (*a*) The disease is common and is associated with clinically important morbidity and mortality; (*b*) screening tests are available, acceptable, feasible, and sufficiently accurate for the detection of early disease; (*c*) earlier diagnosis and treatment is associated with improved prognosis; and (*d*) the sum of the benefits associated with screening outweighs the sum of the potential harms and costs. Fortunately, colorectal cancer screening fulfills each of these criteria.

As described previously, colorectal cancer is both common and serious. Survival of patients with colorectal cancer depends largely on the stage of disease at diagnosis. Thus, in patients with localized disease, 5-year survival is approximately 90%, whereas in patients with regional spread of disease, 5-year survival decreases to approximately 60%. In patients with distant metastases, 5-year survival is less than 10% (1). Treatment of patients with advanced colorectal cancer is largely unsuccessful. Furthermore, evidence from a variety of clinical trials suggest that screening and/or removal of adenomatous polyps significantly reduces both the incidence of colorectal cancer and the mortality rate of the disease. A number of screening tests are available for the early detection of colorectal cancer. These include digital rectal examination, fecal occult blood testing, flexible and rigid sigmoidoscopy, barium enema examination, colonoscopy, and, most recently, computed tomographic (CT) colonography (TAB.1).

There is now substantial evidence that reductions in colorectal cancer mortality can be achieved through detection and treatment of early-stage cancers (22–27). However, the current diagnostic methods are ineffective or invasive and expensive.

It is therefore important to identify highly specific and sensitive markers in colorectal tissue, that can predict tumor and tumor staging in an identified at-risk population and in an early stage of development, in order to carry out a timely intervention.

METHODS	PROS	CONS
DIGITAL RECTAL EXAMINATION		Very low sensitivity
FECAL OCCULT BLOOD TESTING	The safest and least expensive	Low sensitivity
SIGMOIDOSCOPY	Moderate expense, moderate patient discomfort	Only the distal colon and rectum are examined; more proximal lesions may be missed
BARIUM ENEMA EXAMINATION	Permits examination of the entire colon; good sensitivity	Expensive, discomfortable
COLONOSCOPY	Permits examination of the entire colon; high sensitivity; can also be a therapeutic procedure	The most invasive and expensive screening test; high risk of perforation
COMPUTER TOMOGRAPHIC (CT) COLONOGRAPHY	Less invasive that Colonoscopy; good sensitivity on polyps larger than 10mm	Discomfortable; small polyps cannot be seen

***TAB.1**: Comparison among different current methods of CRC diagnosis.*

Thus, there is an increasing demand for biomarkers in colon cancer for risk assessment, early detection, prognosis, and surrogate end points. A number of biomarkers have been identified for early detection of colon cancer, although the risk factors have not been identified extensively. The major advances in understanding colorectal cancer include the identification and the involvement of *APC, p53,* and *Ki-ras* in the development and progression of the disease, the identification of the aberrant crypt foci as an early pre-invasive lesion, and its relation to the development of cancer. Detecting malignant neoplasms in the early stages offers great clinical advantages. In fact, treatment options (TAB.2) for colorectal cancer depend on the stage of the tumour, that is, how far it has spread or how deeply it is affecting the intestinal wall and other tissues. Treatment is also determined by the patient's age, medical history, overall health, and tolerance for specific medications and therapies.

TREATMENT	METHOD
PARTIAL COLECTOMY	The tumour and normal tissue on either side of the diseased area in the colon are removed.
LAPAROSCOPYC SURGERY	Small tube-like instruments and an extremely small camera are inserted into the abdomen through incisions made in the abdominal wall. A large section of the bowel and adjacent tissue are cut.
RADIATION THERAPY	High-energy radiation is used to kill cancer cells. May be used in conjunction with surgery as definitive therapy, or may be used to reduce the symptoms of colorectal cancer.
CHEMOTHERAPY	Drugs are given intravenously or orally to kill cancer cells. Is often given to decrease the chance of the tumor returning elsewhere in the body.

***TAB.2**: Treatment choices in CRC therapy.*

SEROLOGICAL TUMOR MARKERS FOR CRC DIAGNOSIS

Carcinoembryonic antigen (CEA) is a protein found in many types of cells but associated with tumors and the developing fetus. CEA is tested in blood. The normal range is <2.5 ng/ml in an adult non-smoker and <5.0 ng/ml in a smoker. CEA was one of the first oncofetal antigens to be described and exploited clinically. It is a complex glycoprotein of molecular weight 20,000, that is associated with the plasma membrane of tumor cells, from which it may be released into the blood. Although CEA was first identified in colon cancer, an abnormal CEA blood level is specific neither for colon cancer nor for malignancy in general. Elevated CEA levels are found in a variety of cancers other than colon, including pancreatic, gastric, lung, and breast. It is also detected in benign conditions including cirrhosis, inflammatory bowel disease, chronic lung disease, and pancreatitis (28). CEA was found to be elevated in up to 19 percent of smokers and in 3 percent of a healthy control population. Thus, the test for CEA cannot substitute for a pathological diagnosis.

As a screening test, the CEA is also inadequate. Since cancer prevalence in a healthy population is low, an elevated CEA has an unacceptably low positive predictive value, with excess false positives. Also, since elevated CEA occurs in the advanced stage of incurable cancer but is low in the early, curable disease, the likelihood of a positive result affecting a patient's survival is diminished (29). CEA has been suggested as having prognostic value for patients with colon cancer. Preoperative CEA values have been positively correlated with stage and negatively correlated with disease free survival (30). Although not satisfactory for screening a healthy population, CEA has been used to monitor recurrence. Early data suggested that CEA preceded clinical relapse by several months. Subsequently, several investigators have examined intensive, serial CEA monitoring as an indicator for second look surgery in the hope that relapse could be detected at a time when surgical resection for cure was still possible. Criteria for a subsequent operation included a significant rise of CEA above a base line level on serial determinations and absence of obvious unresectable disease on staging workup. Determinations of CEA should be done frequently: at a minimum of every 3 months and if possible every 1 month to 2 months. Elevations above baseline should be verified rapidly to exclude laboratory error. The CEA is of some use as a monitor in treatment. Usually the CEA returns to normal within 1 to 2 months of surgery, but if it returns elevated persistent disease may be indicated (29). The test is not infallible in patients treated with radiotherapy and chemotherapy but can be useful in those whose tumour is not measurable.

Colon-IC

ELISA Kit for the detection of Carcinoembryonic Antigen (CEA) Immune Complexes in Colorectal Carcinoma (CRC)

Colon-IC is a highly specific and sensitive ELISA assays for CRC detection designed to measure CEA immune complexes (CEA-IC) in patients sera. While all healthy controls are negative, Colon-IC allows to detect abnormal levels of CEA-IC, especially in early staging (Duke's stage A) colorectal cancer patient sera, resulting in a higher sensitivity than that provided by the analysis of serum free CEA (fCEA) levels (39% vs 8% respectively). Furthermore, combined determination of the two forms of circulating CEA (CEA-IC and fCEA) significantly increased the efficiency for discriminating CRC from normal individuals. By using a fCEA cut off value of 5 ng/mL, 33% to 100% (from Duke's stage A to C) of CRC patients are positive for at least one marker.

DETAILED SEROLOGICAL AND MOLECULAR STUDIES

A total of 72 sera from patients suffering from colorectal cancer and 34 sera from healthy donors were analyzed in parallel by CEA-IC and fCEA assays. The circulating free CEA concentration was 71±361 ng/mL (mean ± S.D.) in CRC group and 1.8±1.2 ng/mL in healthy donors group, while the concentration of circulating CEA immune complexes was 608±2922 AU/mL versus 92±79 AU/mL in CRC and healthy subjects groups respectively. In both cases, the mean serum biomarker concentration level was significantly higher in the colorectal cancer group (Fig.1). The generally accepted *cut-off* of 5 ng/mL allows fCEA quantification to discriminate healthy subjects from patients with a sensitivity of 43% and a specificity of 100%. The CEA-IC *cut-off* value was set to 200 AU/mL in order to clearly discriminate between the two groups. In terms of sensitivity, CEA-IC was above the cut-off in 27 of 72 patients (38%), with a specificity of 94%. Since no correlation was found between the serum levels of the two biomarkers (Fig. 1), combined determination of both markedly improved the indexes of diagnostic accuracy for the diagnosis of colon cancer. In fact, 64% (46/72) of CRC patients were positive for at least one marker (Tab. 3).

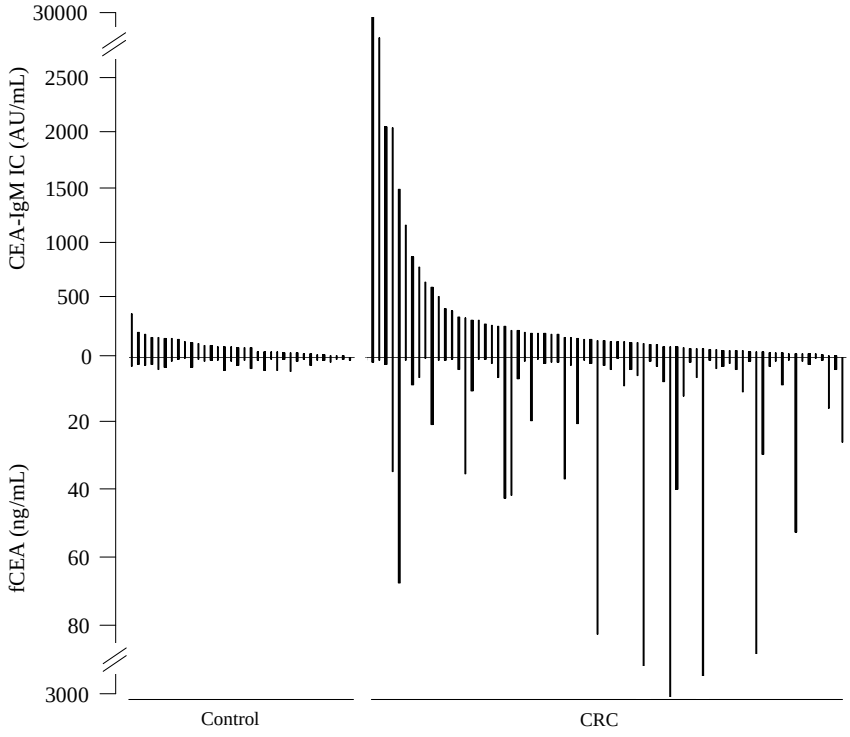

***Fig.1**: Serum levels of fCEA and CEA-IgM IC in patients with CRC (n=72) and in healthy subjects (n=34).*

Tab. 3: *Comparison of the indexes of diagnostic accuracy for CEA-IgM IC, fCEA and the combination of both in the differentiation of patients with CRC at different stage from healthy subjects (control).*

BIOMARKER	Sensitivity	Specificity	PPV	NPV	Efficiency
CEA-IgM IC: cut-off 200AU/mL					
CRC vs control	38		93	42	56
CRC (A) vs control	39		78	65	67
CRC (B) vs control	37	94	78	73	74
CRC (C) vs control	53		80	82	82
CRC (D) vs control	36		71	78	77
free CEA: cut-off 5ng/mL					
CRC vs control	43			45	61
CRC (A) vs control	8			61	62
CRC (B) vs control	37	100	100	74	77
CRC (C) vs control	73			89	92
CRC (D) vs control	79			92	94
CEA-IgM IC: cut-off 200AU/mL & free CEA: cut-off 5ng/mL					
CRC vs control	65%		96	55	74
CRC (A) vs control	38%		82	68	71
CRC (B) vs control	56%	94%	85	80	81
CRC (C) vs control	93%		88	97	94
CRC (D) vs control	86%		86	94	92

PPV (positive predictive value) = true positive (TP) / TP + false positive (FP); NPV (negative predictive value) = true negative (TN) / false negative (FN) + TN; efficiency = TP + TN / TP+ FP + TN + FN.

When the same data were correlated to the tumor staging according to Duke, it could be seen how better the CEA-IgM IC biomarker could discriminate the earliest stages of the pathology from the normal controls in respect to the fCEA determination (Fig. 2). The CEA-IC levels were above the *cut-off* level (200 AU/mL) in 29% of stage A patients (7/24) while circulating fCEA concentration were above 5 ng/mL only in 8% (2/24) of stage A colon cancer suffering patients (Table 3). The combination of the two biomarker led to a further increase in sensibility of cancer detection since the very early stages of the pathology, since 9 of 24 patients (38%) with colorectal cancer in stage A were positive for at last one marker (Table 1).

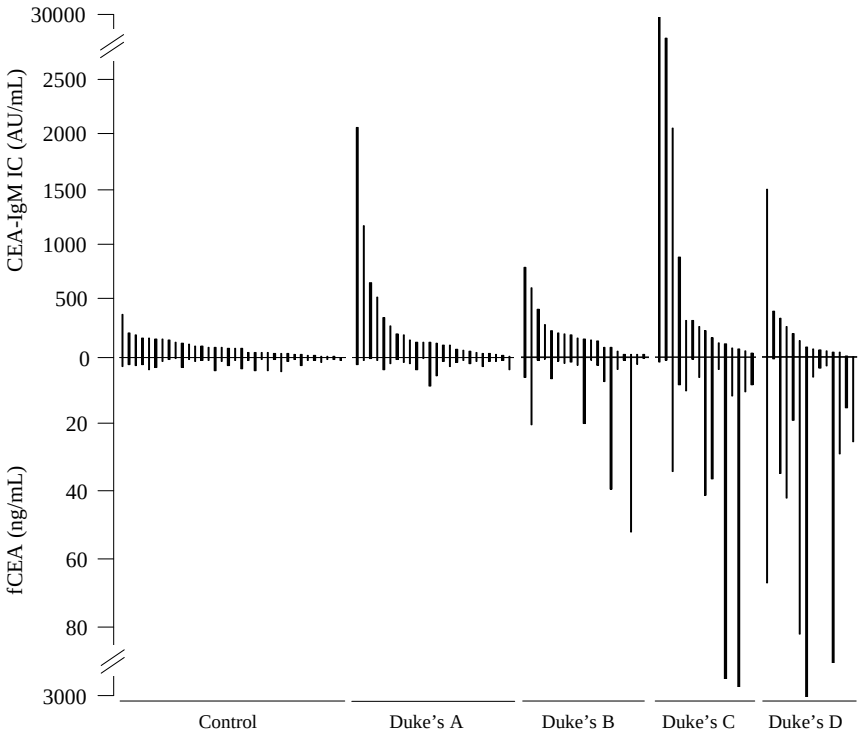

Fig. 2*: Correlation of serum levels of CEA-IgM IC and fCEA with the presence and the staging of the pathology.*

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Colon-IC

ELISA Kit for the detection of Carcinoembryonic Antigen (CEA) Immune Complexes in Colorectal Carcinoma (CRC)

INSTRUCTIONS FOR USE

Colon-IC: Instructions for use

PRODUCT CODE

Colon-IC XG006

INTENDED USE

Colon-IC is an enzyme linked immunosorbent assay (ELISA) for the quantitative measurement of Carcinoembryonic Antigen (CEA) immune complexes (CEA-IgM).

EXPLANATION

Colon-IC belongs to a novel generation of in vitro diagnostics based on the detection of CEA as circulating Immune Complex (IC). Colon-IC is a highly specific and sensitive ELISA assay for Colorectal cancer (CRC) detection designed to measure CEA-IgM in patient sera. The amount of CEA-IgM is expressed in Arbitrary Units (AU), using a CEA-IgM purified standard of human origin as a reference. The measurement of CEA-IgM offers an increased sensitivity of early stage (stage 1) CRC, without compromising specificity with respect to the well-established serum CRC biomarker assays such as the quantitative determination of free CEA (1). Furthermore, CEA-IgM is a complementary biomarker to free CEA, thus when used together the overall sensitivity increases to 64% (1).

PRINCIPLE OF THE TEST

Standard Calibrators and specimens are incubated in parallel with anti-CEA antibodies coated to the wells of a microtiter plate. The immune complexed CEA-IgM is detected by the addition of an enzyme conjugated secondary antibody and an enzyme substrate (ABTS). The developed color is proportional to the amount of the analyte in the sample.

REAGENTS AND MATERIALS PROVIDED

- **XG006-PL**: 96 wells multi-strip Assay-Plate, pre-coated with affinity purified rabbit anti-CEA
- **XG006-Calibrator**: lyophilized calibrator, two vials. See label for exact concentration. Each vial is intended for the construction of a calibration curve in duplicate.
- **XG-EA**: Enzyme-conjugated secondary antibody solution. Green cap
- **XG-CH4**: Chromogen Solution ABTS (2,2'-AZINO-bis(3-ETHYLBENZOTHIAZOLINE-6-SULFONIC ACID))
- **XG-SB**: Enzyme substrate solution (Blue cap)
- **XG-DB**: Dilution Buffer
- **XG-WB**: Washing Buffer

EQUIPMENT REQUIRED

- Microplate washer
- Microplate readers

STORAGE

Avoid repeated freeze and thaw cycles

Storage at 4°C:

- **XG006-PL**: 96 wells multi-strip Assay-Plate, pre-coated with affinity purified rabbit anti-CEA.
- **XG-CH4**: Chromogen Solution*†
- **XG-SB**: Enzyme substrate
- **XG-DB**: Dilution Buffer*
- **XG-WB**: Washing Buffer*

Storage at -20°C:

- **XG006-Calibrator**: lyophilized calibrator
- **XG-EA**: Enzyme-conjugated secondary antibody solution

(*) Must be used within one month of reconstitution
(†) Must be stored in a dark location

EXPIRATION DATE

SEE LABEL ON VIAL.

WARNINGS

POTENTIAL BIOHAZARDOUS MATERIALS:

The **XG006-Calibrator** contains proteins of **human origin.** The reference material was tested using an approved method of evaluation for the presence of the antibodies to HIV, antibodies to the hepatitis C virus and hepatitis B surface antigens, and found to be negative. **Since no test method can offer complete assurance that HIV, hepatitis B virus, hepatitis C virus, or other infectious agents are absent, all human sourced materials should be considered potentially infectious.** It is recommended that these reagents and human specimens be handled in accordance with the OSHA Standard on Bloodborne Pathogens (6). Biosafety Level 2 (7) or other appropriate biosafety practices (8, 9) should be used for materials that contain or are suspected of containing infectious agents.

SPECIMEN COLLECTION AND PREPARATION

The use of serum samples are recommended for the Colon-IC assay.
Serum specimens should be collected aseptically, avoiding hemolysis if possible.
Specimens should be stored at 2-8°C if the assay will be performed within 24 hours after collection. Specimens should be stored frozen if testing will occur after 24 hours.
If frozen, specimens should be mixed thoroughly after thawing to ensure consistency in the results. Avoid repeated freezing and thawing. Specimens showing particulate matter, erythrocytes, or turbidity must be clarified by centrifugation before testing.

PROCEDURAL NOTES

1. Allow samples and reagents to reach room temperature prior to testing. Do not use water baths to thaw samples or reagents.
2. Mix samples and all reagents thoroughly before use.
3. Avoid excessive foaming of reagents. Also avoid exposure of reagents to excessive heat or light during storage and incubation.
4. Avoid handling the tops of the wells both before and after filling.
5. Standards and samples should be assayed in duplicate.
6. Run a separate standard curve for each assay.
7. Use only coated wells from the same reagent batch for each assay. Also do not mix reagents from different kit lots.
8. Perform incubations in a sealed box containing a wet paper towel, in order to prevent evaporation.

INSTRUCTIONS FOR USE

Reagents preparation

- Reconstitute **XG0006-Calibrator** (lyophilized calibrator) with 440 µL of distilled water for each calibrator vial.
- Reconstitute **XG-CH4** chromogen solution with 20 mL of distilled water.
- Reconstitute **XG-DB** dilution buffer with 25 mL of distilled water.
- Reconstitute **XG-WB** washing buffer with 1 L of distilled water.
- Prepare the required amount of **XG-EA** enzyme-conjugated secondary antibody solution diluting 10-fold in reconstituted **XG-DB** dilution buffer.

Assay protocol

1. Prepare assay reagents as described above.
2. Set up the microtiter plate with sufficient wells to enable the running of all required standards and samples.
3. Remove excess microtiter plate strips from the frame and store in the re-sealable foil bag with the desiccant provided.
4. Wash the microtiter plate strips three times with **XG-WB** washing buffer (300 µL/well).
5. Dispense 100 µL/well of standard calibrators (in duplicate) starting from the reconstituted solution and performing in-plate 2-fold serial dilutions in order to obtain a seven-point calibration curve. Use **XG-DB** dilution buffer as diluent. For exact concentration of the reconstituted calibrator please refer to the concentration value (AU/mL) indicated on the **XG006-Calibrator** vial. Also dispense 100 µL/well of **XG-DB** dilution buffer as blank, in duplicate.
6. Dispense 100 µL/well of eight fold (1:8) dilution of samples (in duplicate). Use **XG-DB** dilution buffer as diluent.
7. Incubate 1h at room temperature.
8. Wash six times with **XG-WB** washing buffer (300 µL/well).
9. Add 100 µL/well of diluted **XG-EA** enzyme-conjugated secondary antibody solution.
10. Incubate 1 hour at room temperature.
11. Wash six times with **XG-WB** washing buffer (300 µL/well).
12. Prepare the required amount of chromogen-enzyme substrate solution adding 1 µL of **XG-SB** enzyme substrate solution per 3 mL of **XG-CH4** chromogen solution. The chromogen-enzyme substrate solution has to be used within 24 hours.
13. Apply 150 µL/well of freshly prepared chromogen-enzyme substrate solution. Allow color to develop for 20 min. at 37°C in the dark and measure OD values of each well using an ELISA plate reader set to 405 nm.
14. Plot the standard curve ΔOD values as described in the next section: Processing of the results.

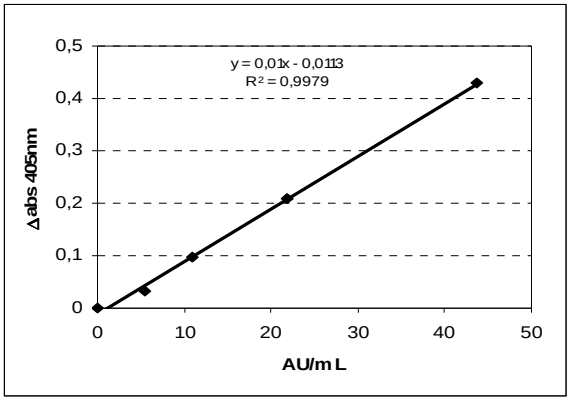

FIG. 1A: range of linearity (3.9 to 31.2 AU/mL) of a typical linear standard curve for CEA-IC after 20 minutes of substrate incubation at 37°C.

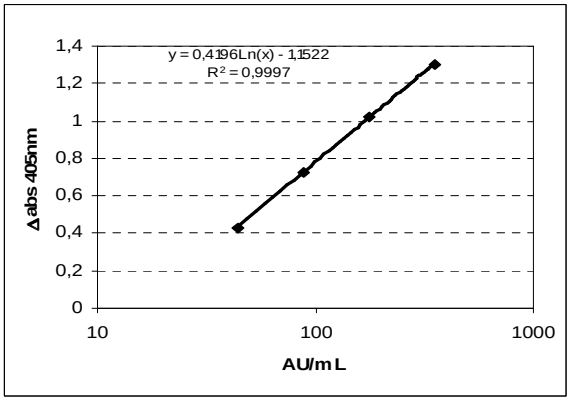

FIG. 1B: range of linearity (form 31.2 to 250 AU/mL) of a typical semi-logarithmic standard curve for CEA-IC after 20 minutes of substrate incubation at 37°C

PROCESSING OF THE RESULTS

Average the duplicate readings for each standard calibrator and sample and subtract the zero standard optical density.

The standard calibrators may be used to construct two distinct standard curves with values reported in AU/mL. Plot on a linear graph the OD readings corresponding to the CEA-IgM titers obtained diluting 1:8 to 1:64 the reconstituted standard calibrator. Plot on a semi-logarithmic graph the OD readings corresponding to the reconstituted standard calibrator and those corresponding to 1:2, 1:4, 1:8 dilutions.

Data deduction may be performed through computer methods using curve fitting routines or may also be manually deduced on paper.

The immune complexes concentration in the biological sample can be calculated directly from the appropriate standard curve, depending on the sample absorbance value, by interpolation. The value obtained has to be multiplied by the dilution factor. Samples with OD values exceeding the upper calibration limit should be further diluted and re-measured.

QUALITY CONTROL

The intra- and inter-assay coefficients of variation were determined on 4 typical standard curves and the results were less than 10 %.

For optimal performance, the absorbance of the zero standard should be < 0.2 OD_{405}.

It is recommended that each laboratory assay appropriate quality control samples in each run to ensure that all reagents and procedures are correct.

INTERPRETATION

The CEA-IgM cut-off value was 200 AU/mL for differentiating CRC from healthy subjects (1).

SPECIFIC PERFORMANCE CHARACTERISTICS

Hook effect

The Hook effect may occur for concentrations > 1000 AU/mL. The sample with values above 1000 AU/mL should be further diluted and re-measured.

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