

RECAF, A New Broad-Spectrum Cancer Marker.

Moro Ricardo^{*}, Tcherkassova, Janneta, Song Elizabeth^{**},
Shen George^{**}, Moro Rafael^{**}, Schmid Ralph^{**}, Hu
Xiaolong^{**}, Kummer Angela^{**}, Chen Chen^{**}

^{*} BioCurex Inc. and ^{**} Pacific Biosciences Research Centre, 215 – 7080 River Road, Richmond, BC, V6X 1X5, Canada. Correspondence: rmoro@compuserve.com

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Introduction:

One out of four people die of cancer. That amounts to half a million Americans every year and the USA represents only 5% of the world population. Approximately 50% of patients who get cancer die from it. These are sobering statistics and it is easy to understand why so much money and effort is spent in developing new methods to detect cancer at earlier stages, when therapy results in a much higher percentage of cures.

Among the diagnostic tools developed in the past few decades, tumor markers provide a unique combination of low cost and accuracy that makes them suitable for diagnosis, monitoring and screening. Despite a great deal of research in the area, only a handful of markers have found their way to clinical use. The most notorious ones are Alpha-fetoprotein (AFP) for primary liver cancer and some rare forms of testicular cancer, Carcino-Embryonic Antigen (CEA) for colorectal cancer, CA125 for ovarian cancer and Prostate Specific Antigen (PSA), which appears in cancers as well as in benign lesions of that organ.

In this article, we present data related to a new cancer marker, named RECAF, which exhibits high sensitivity and specificity on tissue sections and serum of patients with diverse types of malignancies.

RECAF Background:

RECAF is a receptor protein which binds and takes up a circulating fetal protein; Alpha-Fetoprotein (AFP). RECAF and AFP should not be confused; the latter is a

liver and testicular cancer marker known since 1963¹ whereas RECAF is an emerging broad-spectrum cancer marker.

AFP works in the fetus as albumin does in the adult: They both bind and carry other smaller molecules, including fatty acids². *In-vitro* experiments have shown that following binding to RECAF, AFP penetrates the cell via coated-pits^{3,4}, releases its load of fatty acids and then it leaves the cell immunologically intact⁵, probably ready to fetch another fatty acid molecule. The uptake of AFP and hence the expression of RECAF is related to the degree of cell differentiation^{6,7,8}: When a given fetal organ or tissue has reached a certain maturity, AFP is no longer taken up even if it is present in high concentrations in the extracellular fluid. Since cancer cells are poorly differentiated, it was first postulated⁹ and then demonstrated that cancer cells re-express RECAF, hence it behaves as an oncofetal antigen¹⁰. The AFP uptake by fetal cells occurs in most organs and tissues¹¹ and therefore RECAF, which mediates the uptake, has been shown to be re-expressed in many types of cancers^{12,13,14,15,16}.

RECAF is not a single molecule: There are two main membrane fractions with molecular weights of 18 and 31 kDa and two main soluble cytoplasmic components weighing ~50 and ~62/67 kDa respectively. The latter often appears as a doublet¹⁷. All of these fractions bind AFP. The AFP binding site is localized in the sugar moiety of these glycoproteins as shown by experiments in which the AFP binding activity is fully conserved following complete digestion of RECAF with pepsin whereas it is completely destroyed after treatment with sodium periodate (in preparation).

The presence of a membrane RECAF fraction can be used for several purposes, from specifically targeting cancer cells to tumor imaging. In reference to the latter, we have imaged breast tumors as small as 4 mm in mice¹⁸ and 1.5 cm in humans using radiolabeled AFP, which has the advantage over antibodies that it is a homologous protein and therefore it does not need to be humanized in order to prevent an immune response from the host.

The existence of soluble RECAF fractions opened the opportunity to detect them in the cytoplasm of tissue sections as well as in the serum of cancer patients.

RECAF in tissue sections:

We first developed a staining kit that uses peroxidase-labeled human AFP to bind and detect RECAF. The FDA has classified this assay, named Histo-RECAFTM, as a Class I staining device for paraffin sections of breast tissue. A variant of the test called Cryo-RECAFTM has been engineered to stain frozen section in approximately 15 minutes, while the patient is under anesthesia. Cryo-RECAFTM has not been yet cleared by the FDA and therefore it is only available "*for research purposes only*". Since the RECAF binding ligand is labeled, the staining is done in only one step followed by development with diaminobenzidine (DAB) which is included, as a ready to use solution in the package. Frozen sections require a 3 minute incubation in Carnoy's fixative (25% Acetic Acid in 95% ethanol) prior to staining. The kits contain all the reagents and solutions

required for staining. Histo-RECAF™ contains enough material for 60 slides whereas the Cryo-RECAF™ kit is designed for use on a single patient (10 slides). The large majority of cancers stain positively (breast cancers are all positive). Normal tissue and benign tumors are practically negative, with the odd RECAF positive cell. Figure 1 shows the staining, in brown, of breast tissues with Histo-RECAF™. Both ductal and lobular carcinomas are positive, whereas fibroadenomas and other benign lesions are negative. Figure 1D shows the staining of breast cancer cells in an infiltrated sentinel axillary lymph node. The contrast offered by the staining may prove useful in those cases in which the number of cancer cells is too low to be easily visualized. Figure 2 shows frozen sections of two breast cancers stained with Cryo-RECAF™. The staining should prove useful to distinguish the cancer cells in those cases in which the uncertainty of the diagnosis is compounded with the inevitable poor quality of frozen sections. Fine needle biopsies of the breast are another situation in which diagnosis can prove difficult due to the lack of tissue structure. Figure 3 shows the staining difference between benign and malignant cases using Cryo-RECAF™ with a higher dilution of AFP-Peroxidase to reduce the background and a longer incubation time. Figure 4 shows examples of other RECAF positive malignancies. The results obtained with anti-RECAF antibodies are similar, only that the staining is cleaner and the background is weaker.

Interestingly enough, there seems to be a correlation between the degree of cell malignancy and the staining: The more aggressive a tumor appears under the microscope, the more intense is the RECAF staining. This can also be noticed within a given sample: Loose, invading cords of cancer cells are usually more positive than cancer cells in organized structures such as neo-ducts, glands, etc.

RECAF in serum:

The soluble fractions of RECAF can be released from cancer cells either actively or after the cells die and therefore the circulating RECAF in cancer patients could be higher than in non-cancerous individuals. To test this hypothesis, we developed a competitive radio-immunoassay (RIA) using a polyclonal rabbit antibody reactive against both the 50 kDa and the 62/67 kDa soluble RECAF fractions. The purified IgG fraction of the antiserum is coated onto 96 well plates which are then blocked with 3% BSA. Soluble RECAF, purified from cancer cells in culture and labeled with ¹²⁵I, is mixed with the patients' sera (50 uL) and the mixture is incubated in the wells coated with the anti-RECAF antibody. After one hour, the wells are washed, detached from the plate's frame and the radioactivity is measured in a gamma counter. A calibrated cell extract containing RECAF is used as a standard and the determination is expressed in RECAF Units. The results presented herein were obtained with the 62/67 kDa fraction and they are similar to the results obtained using the purified 50 kDa fraction. Figure 5 shows the distribution of values for normal individuals, patients with benign lesions and cancer patients. The horizontal lines represent the cutoff values for 95% and 99% specificity (i.e. only 5% or 1% of the normal samples fall above the cutoff value). Table I shows the corresponding sensitivity value and the number of samples each type of malignancy or benign tumor. It is worth noting that a small

number of prostate tumors appear as false positives when the 95% specificity cutoff value is used. Increasing the cutoff value to yield 99% specificity greatly reduces the number of false positives at the expense of reducing by a small percentage the overall sensitivity of the test. Figure 6 shows the ROC analysis of all the cancer patients combined, against a combination of all the benign and normal cases. This graph gives an idea of what to expect when the Serum-RECAF™ test is used for screening. The individual ROC curves for each type of cancer are similar in shape and area (data not shown).

In ovarian cancers, we found a relatively high correlation between circulating CA125 and RECAF ($r^2 = 0.81$). On the other hand, we found no correlation with CEA in a small number of colon cancers (data not shown). The origin of that correlation is uncertain since CA125, which has a MW higher than 200 kDa appears to be unrelated to RECAF (MW < 70 kDa).

In Conclusion:

Most cancer markers have been discovered by making antibodies that react against cancer tissue but not against normal or benign tissue. RECAF is an exception in that it was first described as a part of a physiological mechanism during embryogenesis and then proposed as a new cancer marker. The final assessment of any assay comes from evaluating its performance on a large number of samples but it is worth noting that the available basic research data support the concept that RECAF behaves as a broad-spectrum oncofetal antigen.

Histo-RECAF™ is designed as a tool to pinpoint cancer cells under the microscope, particularly when searching for lymph node metastases or in frozen sections and fine needle biopsies. There is a clear difference in the staining of cancer cells as opposed to normal or benign cells. Histo-RECAF™ might also prove useful in other types of cancer, such as thyroid carcinomas, which are difficult to diagnose by needle biopsy. The test is of particular interest for automated systems using vision recognition where the computer time can be drastically reduced by examining only the brown “spots” (cells) on the slide. One of the most important automated applications is on cervical smears (PAP smears), of which approximately 50 million are carried out every year. Paraffin sections of cervical cancers are positive in contrast to the normal cervical epithelium, which is negative. This indicates that the test should work on smears. However, the intense background emerging from the poor conditions of the cells at the time of collection need to be addressed before this test can be used systematically for cervical cancer diagnosis. The same applies to sputum samples in relation to lung cancer.

In serum, RECAF values were elevated in all the types of cancer studied (breast, prostate, lung, stomach and ovary). Altogether, these represent 50% of all cancers in Occident. We have not yet found a consistently negative type of cancer and therefore it is safe to assume that the assay should also work in other cancers as well. The assay detects approximately 90% of lung and breast cancers which are the two prevalent types of malignancies and for which most

current markers perform poorly. The vast majority of the benign tumors studied were RECAF negative, which is advantageous for prostate cancer diagnosis since PSA has the drawback that it tends to be elevated in benign prostate tumors. Thus, the combination of a cancer specific marker such as RECAF with a tissue specific marker such as PSA could improve the specificity of the latter.

The fact that RECAF behaves as a rather sensitive and specific pan-cancer marker makes it a suitable candidate for routine screening and since only one test is required, the cost is minimal. Screening leads to earlier detection and this is, of course, closely linked to survival.

The results shown herein indicate that RECAF has the potential to become a cancer marker of clinical significance. To assess the full extent of that potential, more samples from these and other types of cancer, as well as benign lesions must be studied and therefore we welcome collaborations with colleagues interested in this field.

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TABLE I

Cancer type	Sensitivity with 95% Specificity	Sensitivity with 99% Specificity	Number
Ovarian Ca	96%	92%	162
Stomach Ca	90%	87%	31
Lung Ca	91%	87%	32
Breast Ca	93%	90%	88
Prostate Ca vs. Normal	99%	95%	20
TOTAL	94%	91%	333
Breast Benign*	0	0	22
Prostate Benign*	25%	5%	77

All samples in the table were compared with a set of 103 samples from individuals without tumors (“normal individuals”). The cutoff value was extracted from the “normal individuals” to include either 95% or 99% of cases. The sensitivity values in the columns above correspond to the percentage of known cancers with values over the corresponding cutoff values.

* At the 95% cutoff value, a small percentage of benign breast and prostate samples were positive. Increasing the cutoff value to include 99% of “normal individuals” reduces the percentage of false positives, at the expense of a slight decrease in sensitivity.

FIGURES

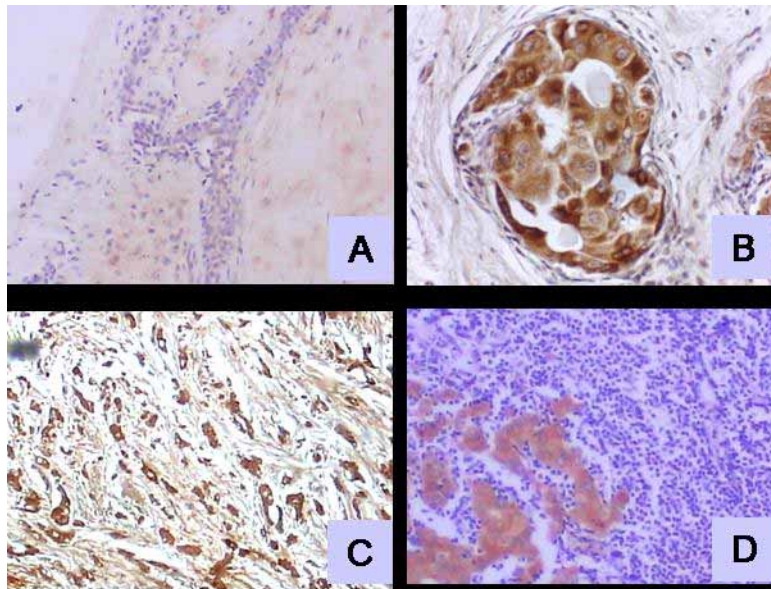


Figure 1. Histo-RECAFTM on breast tumors. (A) Fibroadenoma (negative), 100X; (B) Ductal carcinoma, 200X; (C) Lobular carcinoma, 100X; (D) Axillary Lymph node metastasized by a breast carcinoma, 100X.

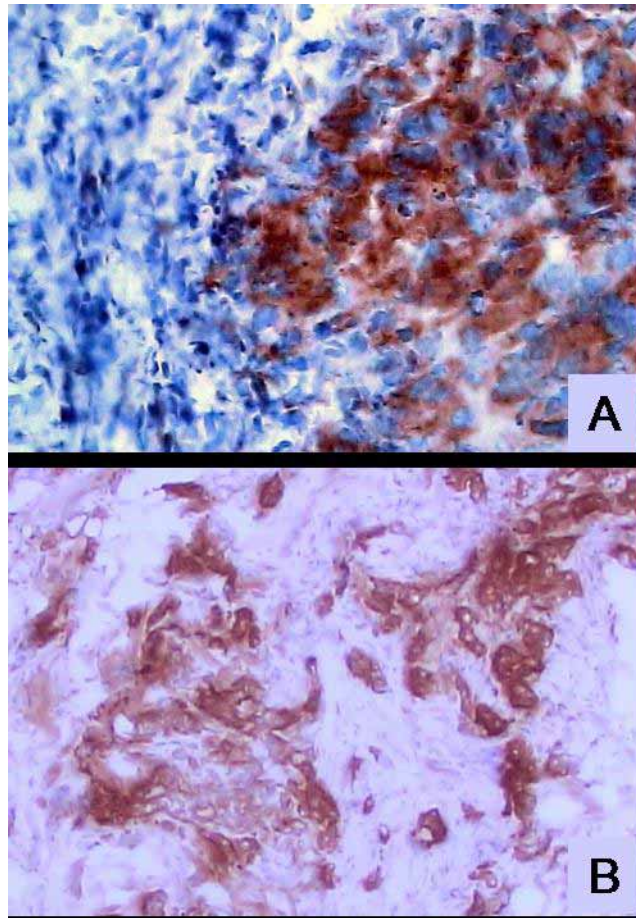


Figure 2. Frozen sections of two different breast ductal carcinomas stained with Cryo-RECAF™. (A) Counterstained with Haematoxylin, 100X; (B) No Haematoxylin, 200X.

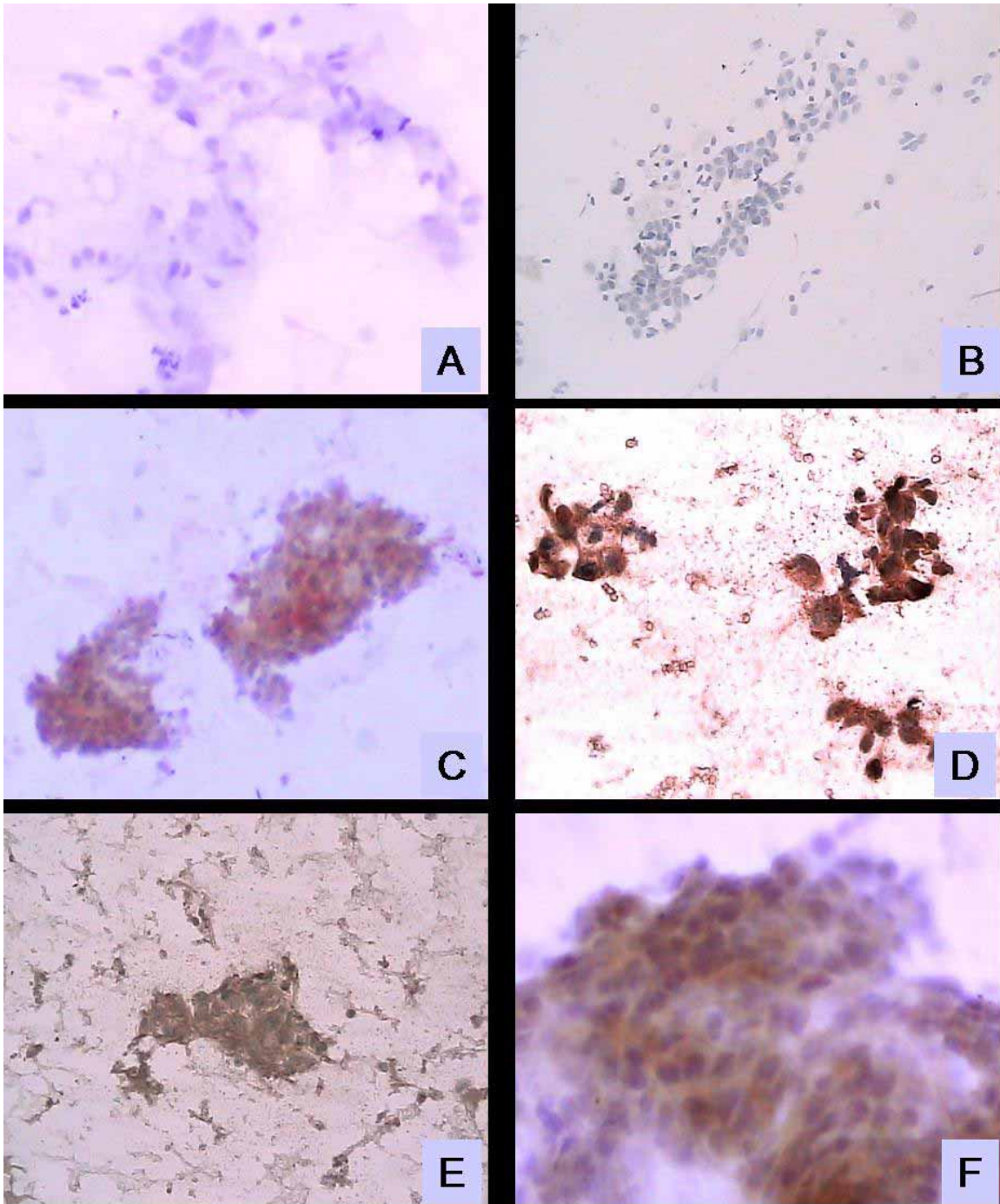


Figure 3. Breast fine needle biopsies stained for RECAF: (A and B) Fibroadenomas, 200X; (C-E) Carcinomas, 200X; (F) A carcinoma, 400X.

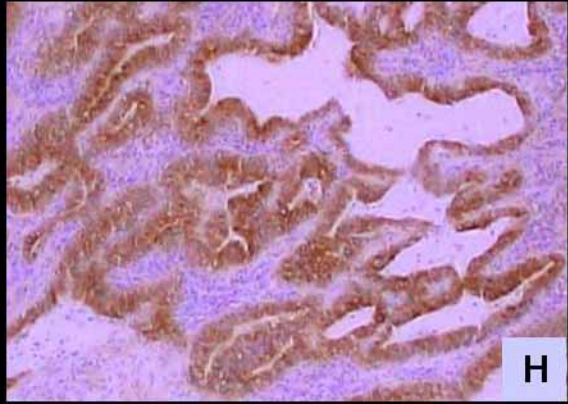
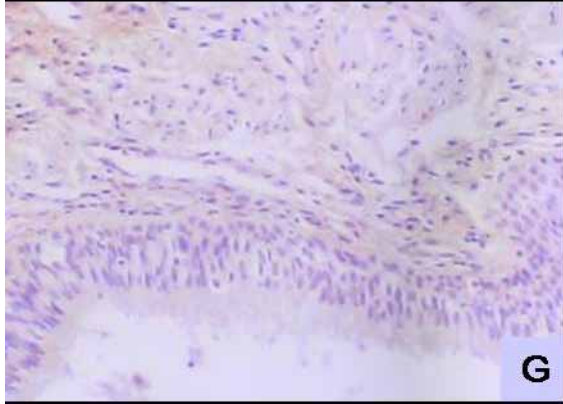
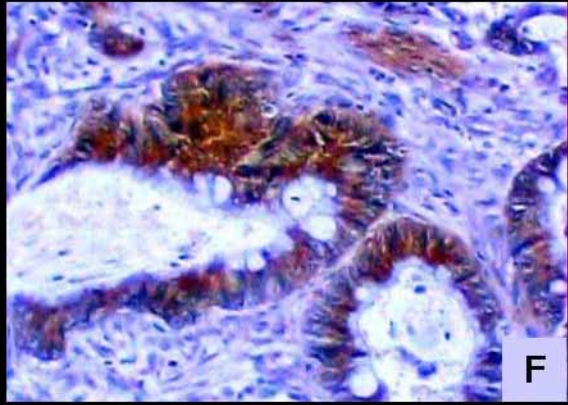
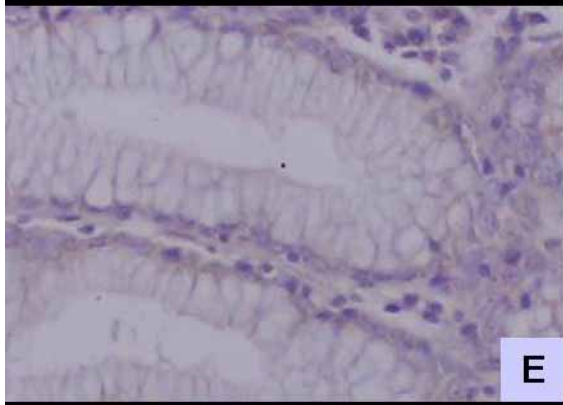
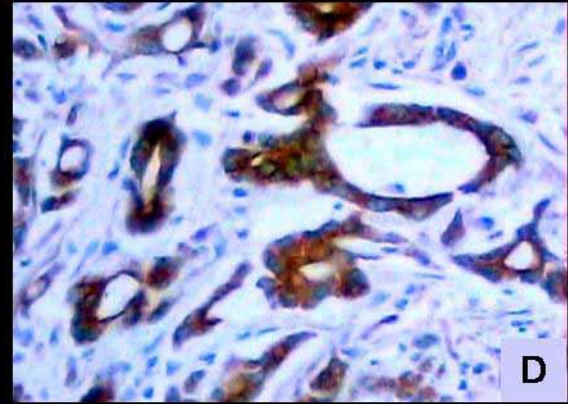
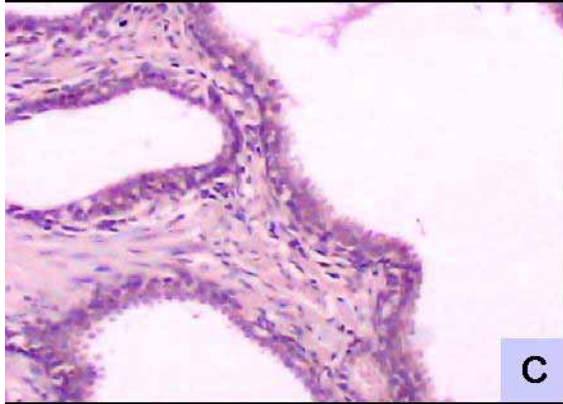
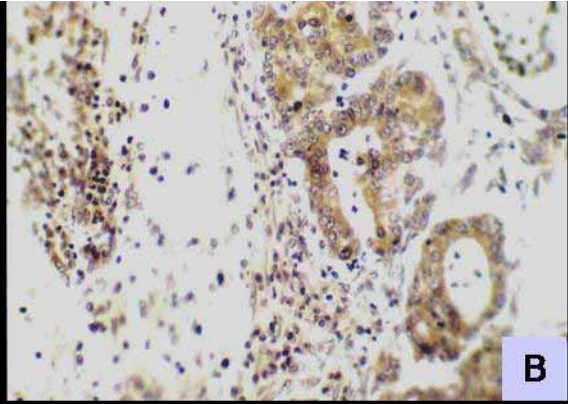
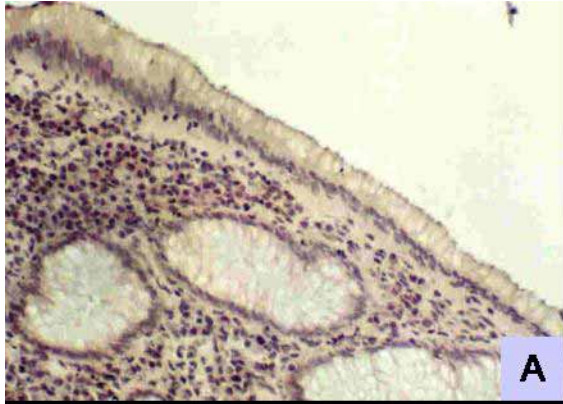


Figure 4. Different tissues stained with Histo-RECAF™ (A) Normal stomach 40X; (B) stomach cancer 100X; (C) Prostate adenoma, 40X; (D) Prostate adenocarcinoma, 100X; (E) Normal colon 200X; (F) Colon carcinoma 100x; (G) Normal lung 40X; (H) Lung cancer 40X.

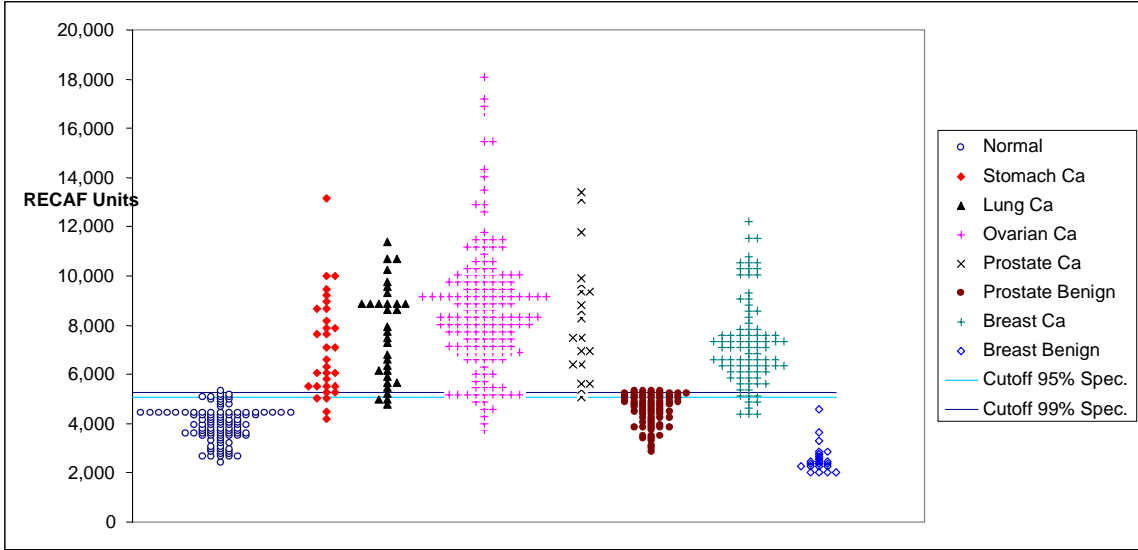


Figure 5. Distribution of RECAF values for normal, cancer and benign tumor samples. The horizontal lines mark the 95% and 99% specificity cutoff values.

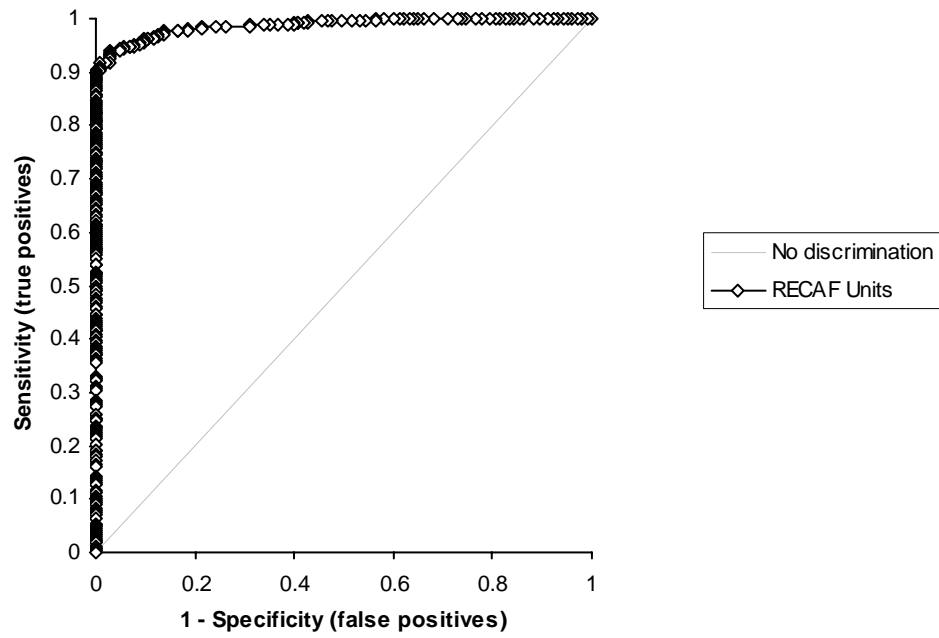


Figure 6. ROC analysis of 103 normal individuals and 333 assorted cancer samples. The area under the curve is 0.988. Overall sensitivity is 94% with 95% specificity or 91% with 99% specificity.