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Tainsky et al.

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(54) **NEOEPITOPE DETECTION OF DISEASE
USING PROTEIN ARRAYS**

(75) Inventors: **Michael Tainsky**, West Bloomfield, MI
(US); **Sorin Draghici**, Troy, MI (US);
Madhumita Chatterjee, Lake Orion, MI
(US)

(73) Assignee: **Wayne State University**, Detroit, MI
(US)

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U.S.C. 154(b) by 114 days.

This patent is subject to a terminal dis-
claimer.

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continuation-in-part of application No. 10/004,587,
filed on Dec. 4, 2001, now Pat. No. 7,863,004.

(51) **Int. Cl.**

G01N 33/53 (2006.01)
G01N 33/00 (2006.01)
C40B 30/04 (2006.01)
C40B 40/10 (2006.01)
A61K 38/00 (2006.01)
C07K 14/00 (2006.01)

C07K 17/00 (2006.01)
C07K 16/00 (2006.01)

(52) **U.S. Cl.**

USPC **435/7.1**; 435/7.23; 435/7.9; 435/7.92;
506/9; 506/18; 530/300; 530/350; 530/391.3

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

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Primary Examiner — Peter J Reddig

(74) *Attorney, Agent, or Firm* — Kohn & Associates, PLLC

(57) **ABSTRACT**

A biosensor for use in detecting the presence of diseases, the biosensor comprising a detector for detecting a presence of at least one marker indicative of a specific disease. A method of determining efficacy of a pharmaceutical for treating a disease or staging disease by administering a pharmaceutical to a sample containing markers for a disease, detecting the amount of at least one marker of the disease in the sample, and analyzing the amount of the marker in the sample, whereby the amount of marker correlates to pharmaceutical efficacy or disease stage. Markers for gynecological disease selected from the list in Table 6. An immuno-imaging agent comprising labeled antibodies, whereby the labeled antibodies are isolated and reactive to proteins overexpressed in vivo. Informatics software for analyzing the arrays of claim 4, the software including analyzing means for analyzing the arrays.

7 Claims, 11 Drawing Sheets
(2 of 11 Drawing Sheet(s) Filed in Color)

FIG-1a

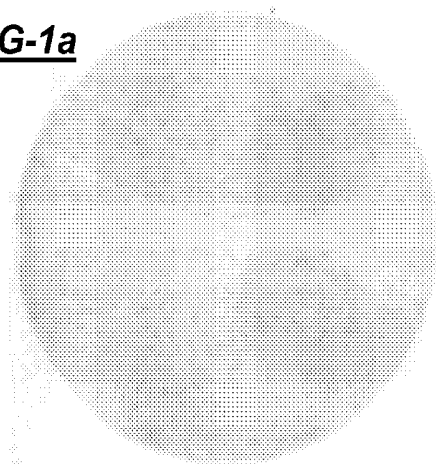


FIG-1b



FIG-1c

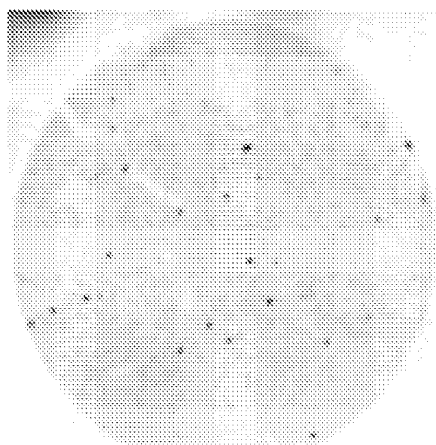


FIG-1d

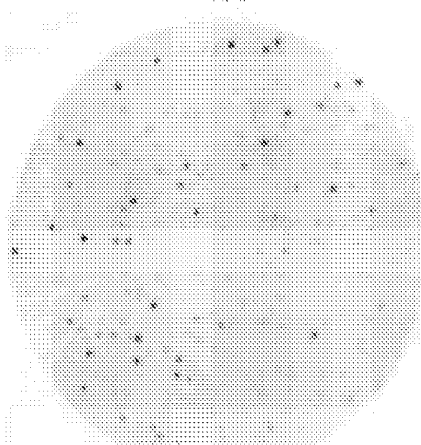
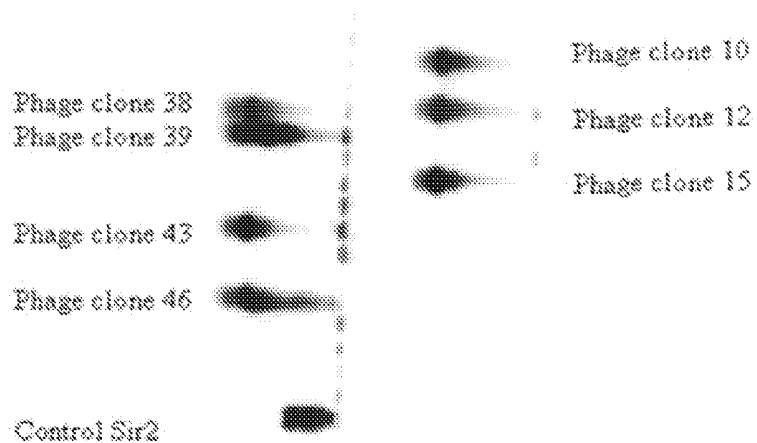
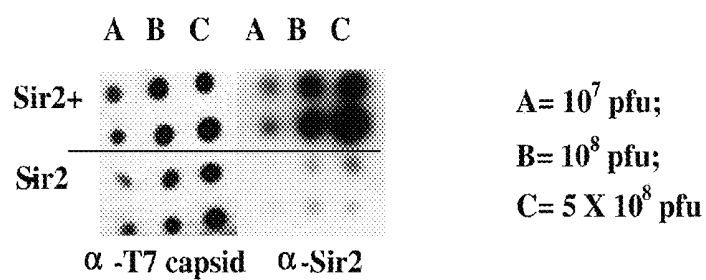
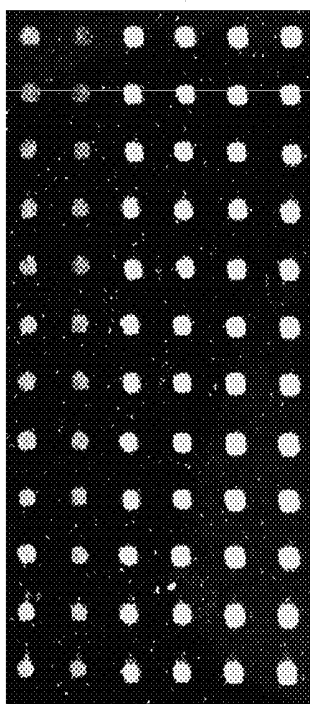


FIG-2



**FIG-3****FIG-4**

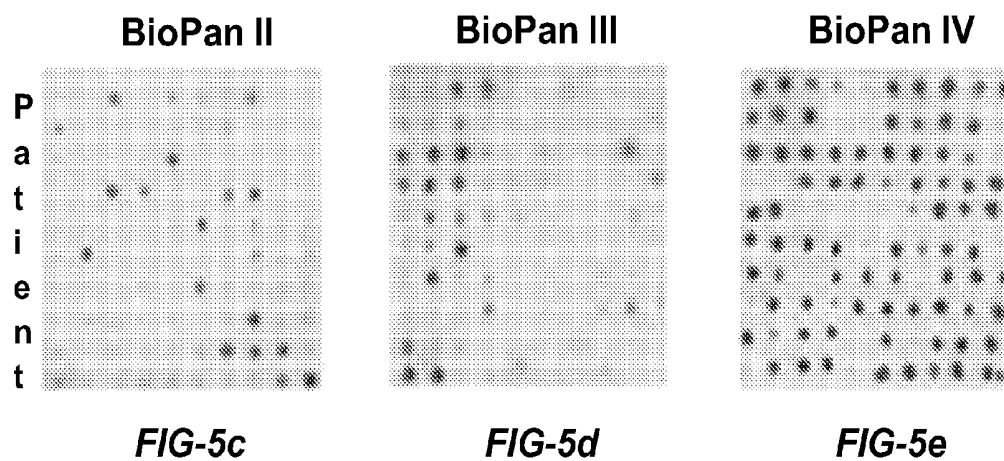
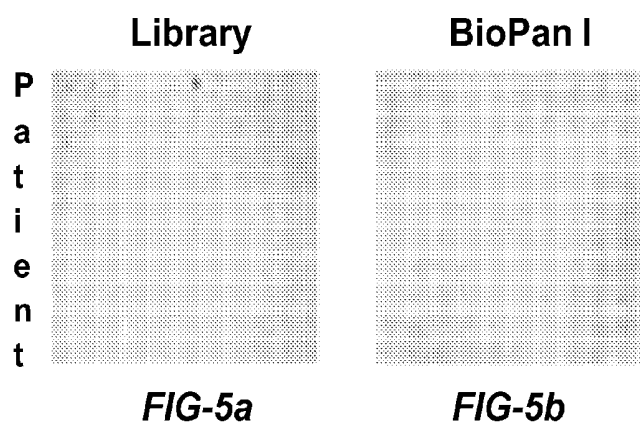


FIG - 6a

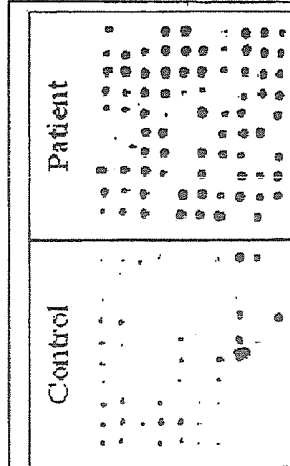
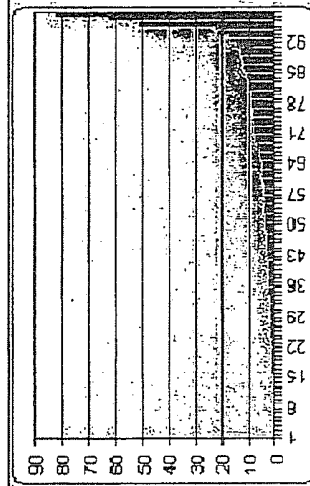


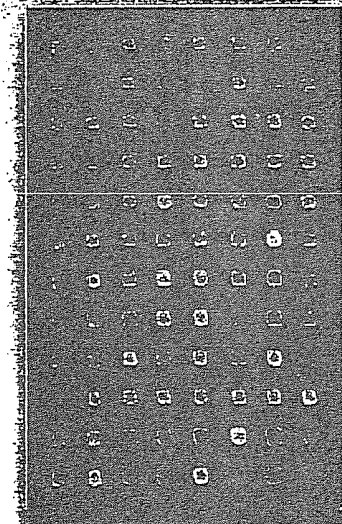
FIG - 6b

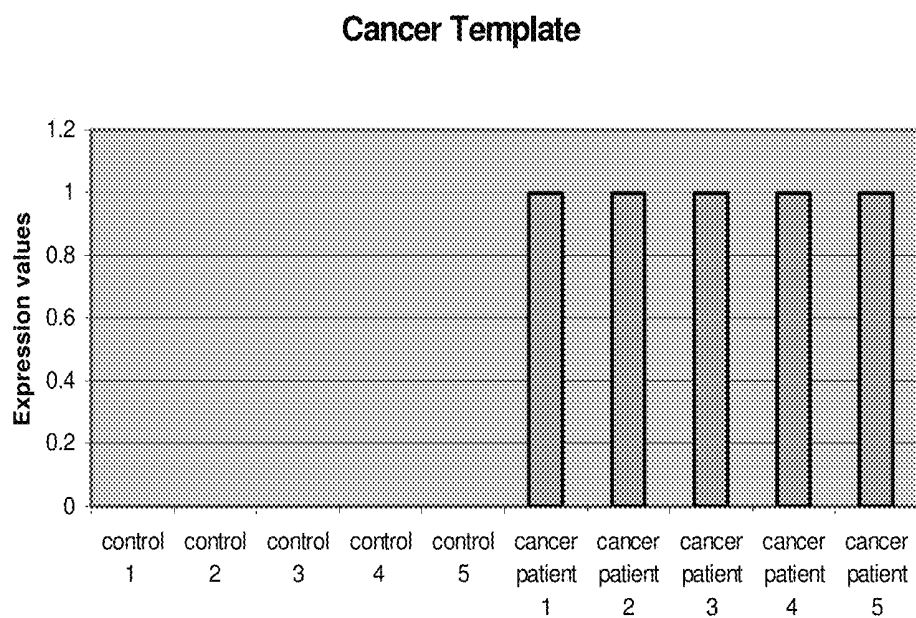
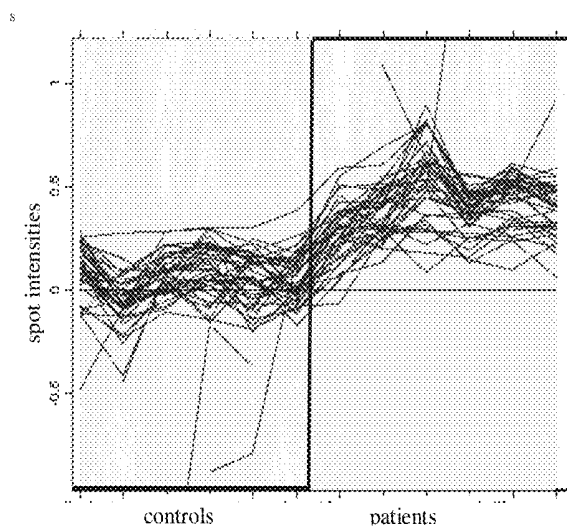


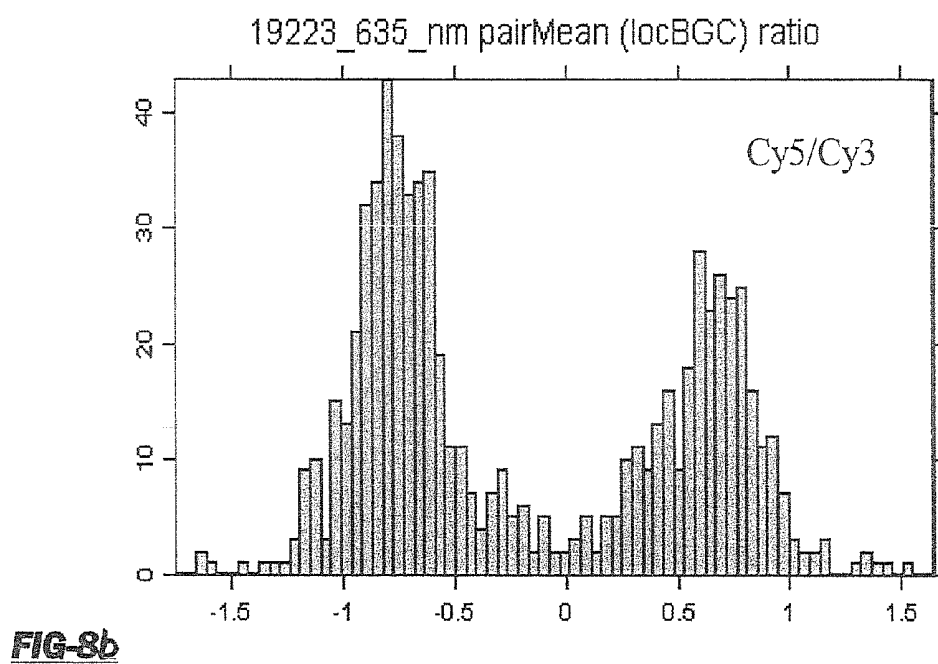
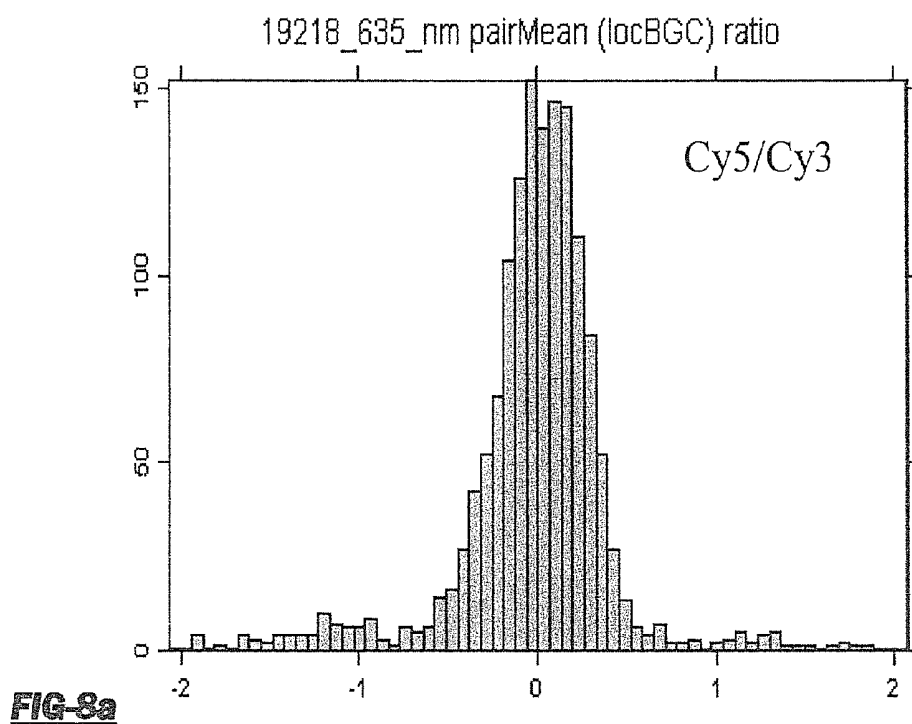
A

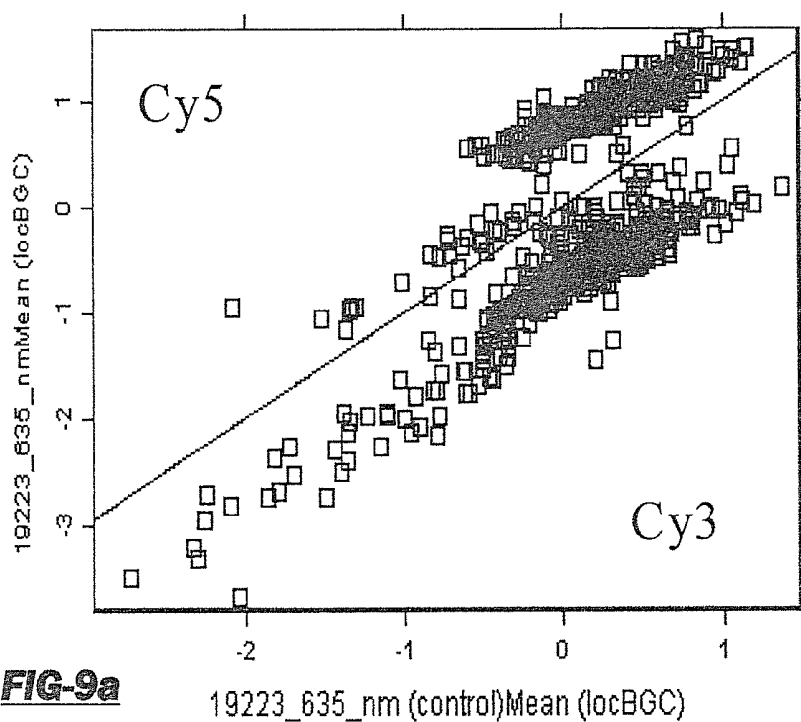
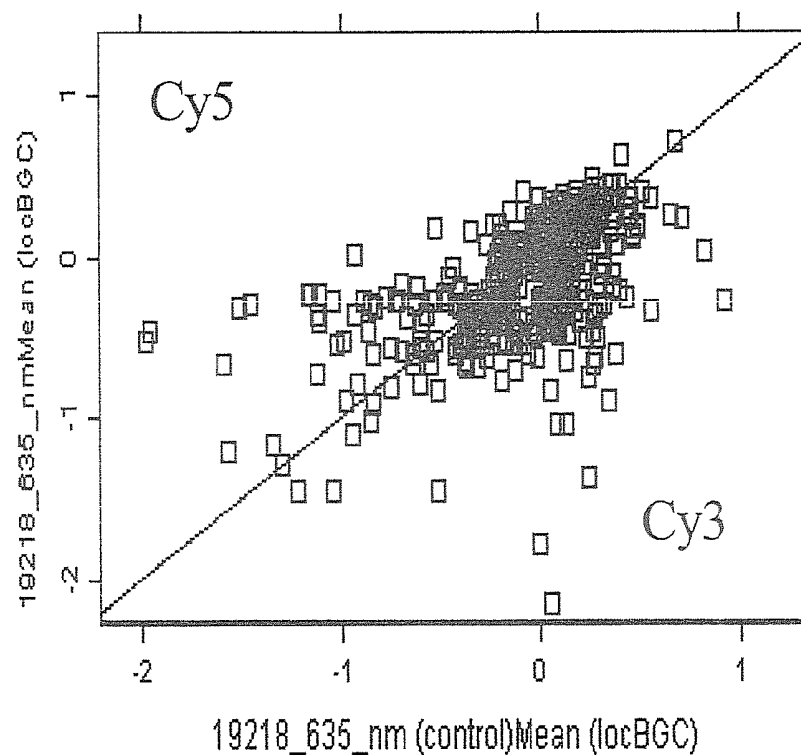
B

FIG - 6c



**FIG-7a****FIG-7b**



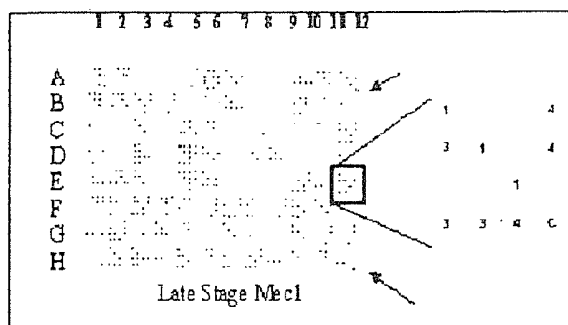
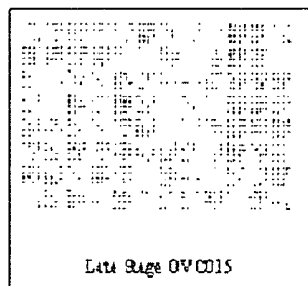
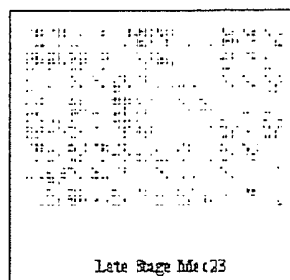
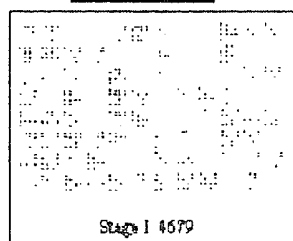
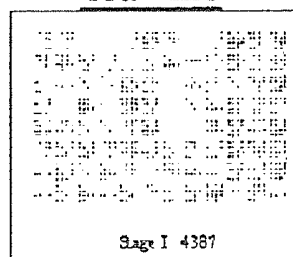
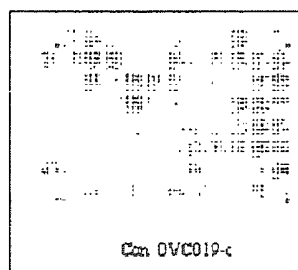
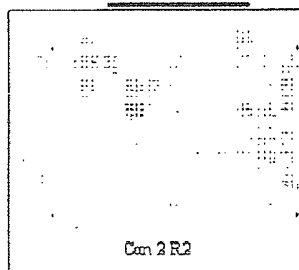
**FIG-9a****FIG-9b**

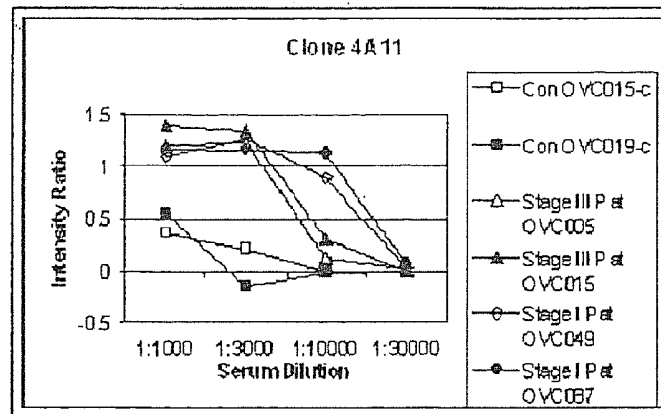
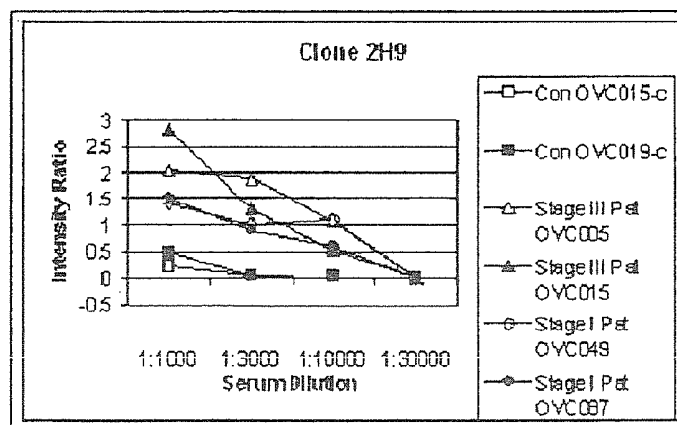
Clones from		Source of sera											
patient	1	2	3	4	5	6	7	8	9	10	11	12	
1	1												
2		1											
3			1										
4				1									
5					1								
6						1							
7							1						
8								1					
9									1				
10										1			
11											?		
12												?	

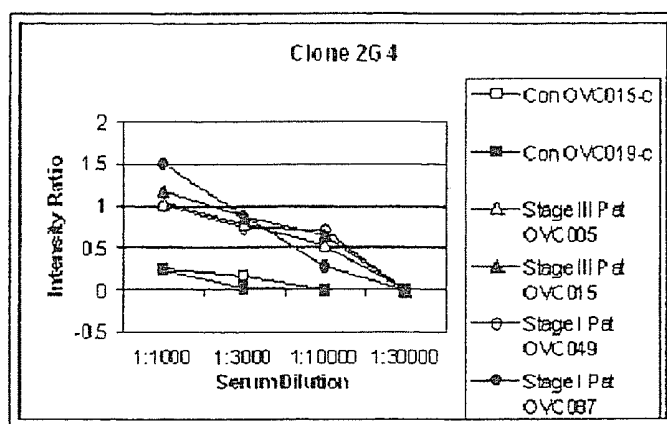
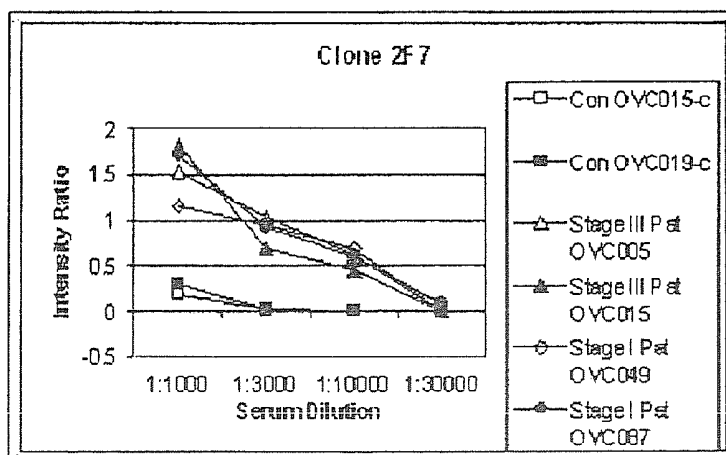
FIG - 10

Clones from					Source of sera					
patient	1	2	3	4	5	6	7	8	9	10
2		1		1				1		
3			1			1				1
5					1			1	1	
7							1			1
8								1		
9									1	
10										1
1	1									
4				1						
6						1				

FIG - 11

FIG - 12a**FIG - 12b****FIG - 12c****FIG - 12d****FIG - 12e****FIG - 12f****FIG - 12g**

***FIG - 13a******FIG - 13b***

***FIG - 13c******FIG - 13d***

NEOEPITOPE DETECTION OF DISEASE USING PROTEIN ARRAYS

Research in this application was supported in part by a grant from the National Institute of Health (NIH Grant No. IR21CA100740-01). The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Technical Field

The present invention relates to an assay and method for diagnosing disease. More specifically, the present invention relates to an immunoassay for use in diagnosing cancer.

2. Background Art

It is commonly known in the art that genetic mutations can be used for detecting cancer. For example, the tumorigenic process leading to colorectal carcinoma formation involves multiple genetic alterations (Fearon et al (1990) *Cell* 61, 759-767). Tumor suppressor genes such as p53, DCC and APC are frequently inactivated in colorectal carcinomas, typically by a combination of genetic deletion of one allele and point mutation of the second allele (Baker et al (1989) *Science* 244, 217-221; Fearon et al (1990) *Science* 247, 49-56; Nishisho et al (1991) *Science* 253, 665-669; and Groden et al (1991) *Cell* 66, 589-600). Mutation of two mismatch repair genes that regulate genetic stability was associated with a form of familial colon cancer (Fishel et al (1993) *Cell* 75, 1027-1038; Leach et al (1993) *Cell* 75, 1215-1225; Papadopoulos et al (1994) *Science* 263, 1625-1629; and Bronner et al (1994) *Nature* 368, 258-261). Proto-oncogenes such as myc and ras are altered in colorectal carcinomas, with c-myc RNA being overexpressed in as many as 65% of carcinomas (Erisman et al (1985) *Mol. Cell. Biol.* 5, 1969-1976), and ras activation by point mutation occurring in as many as 50% of carcinomas (Bos et al (1987) *Nature* 327, 293-297; and Forrester et al (1987) *Nature* 327, 298-303). Other proto-oncogenes, such as myb and neu are activated with a much lower frequency (Alitalo et al (1984) *Proc. Natl. Acad. Sci. USA* 81, 4534-4538; and D'Emilia et al (1989) *Oncogene* 4, 1233-1239). No common series of genetic alterations is found in all colorectal tumors, suggesting that a variety of such combinations can be able to generate these tumors.

Increased tyrosine phosphorylation is a common element in signaling pathways that control cell proliferation. The deregulation of protein tyrosine kinases (PTKs) through overexpression or mutation has been recognized as an important step in cell transformation and tumorigenesis, and many oncogenes encode PTKs (Hunter (1989) in *Oncogenes and the Molecular Origins of Cancer*, ed. Weinberg (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), pp. 147-173). Numerous studies have addressed the involvement of PTKs in human tumorigenesis. Activated PTKs associated with colorectal carcinoma include c-neu (amplification), trk (rearrangement), and c-src and c-yes (mechanism unknown) (D'Emilia et al (1989), *ibid*; Martin-Zanca et al (1986) *Nature* 3, 743-748; Bolen et al (1987) *Proc. Natl. Acad. Sci. USA* 84, 2251-2255; Cartwright et al (1989) *J. Clin. Invest.* 83, 2025-2033; Cartwright et al (1990) *Proc. Natl. Acad. Sci. USA* 87, 558-562; Talamonti et al (1993) *J. Clin. Invest.* 91, 53-60; and Park et al (1993) *Oncogene* 8, 2627-2635).

Mutations, such as those disclosed above can be useful in detecting cancer. However, there have been few advancements which can repeatably be used in diagnosing cancer prior to the existence of a tumor. For example, breast cancer, which is by far the most common form of cancer in women, is the second leading cause of cancer death in humans. Despite

many recent advances in diagnosing and treating breast cancer, the prevalence of this disease has been steadily rising at a rate of about 1% per year since 1940. Today, the likelihood that a women living in North America can develop breast cancer during her lifetime is one in eight.

The current widespread use of mammography has resulted in improved detection of breast cancer. Nonetheless, the death rate due to breast cancer has remained unchanged at about 27 deaths per 100,000 women. All too often, breast cancer is discovered at a stage that is too far advanced, when therapeutic options and survival rates are severely limited. Accordingly, more sensitive and reliable methods are needed to detect small (less than 2 cm diameter), early stage, in situ carcinomas of the breast. Such methods should significantly improve breast cancer survival, as suggested by the successful employment of Papinicolou smears for early detection and treatment of cervical cancer.

In addition to the problem of early detection, there remain serious problems in distinguishing between malignant and benign breast disease, in staging known breast cancers, and in differentiating between different types of breast cancers (eg. estrogen dependent versus non-estrogen dependent tumors). Recent efforts to develop improved methods for breast cancer detection, staging and classification have focused on a promising array of so-called cancer "markers." Cancer markers are typically proteins that are uniquely expressed (e.g. as a cell surface or secreted protein) by cancerous cells, or are expressed at measurably increased or decreased levels by cancerous cells compared to normal cells. Other cancer markers can include specific DNA or RNA sequences marking deleterious genetic changes or alterations in the patterns or levels of gene expression associated with particular forms of cancer.

The utility of specific breast cancer markers for screening and diagnosis, staging and classification, monitoring and/or therapy purposes depends on the nature and activity of the marker in question. For general reviews of breast cancer markers, see Porter-Jordan et al., *Hematol. Oncol. Clin. North Amer.* 8: 73-100, 1994; and Greiner, *Pharmaceutical Tech.*, May, 1993, pp. 28-44. As reflected in these reviews, a primary focus for developing breast cancer markers has centered on the overlapping areas of tumorigenesis, tumor growth and cancer invasion. Tumorigenesis and tumor growth can be assessed using a variety of cell proliferation markers (for example Ki67, cyclin D1 and proliferating cell nuclear antigen (PCNA)), some of which can be important oncogenes as well. Tumor growth can also be evaluated using a variety of growth factor and hormone markers (for example estrogen, epidermal growth factor (EGF), erbB-2, transforming growth factor (TGF) α), which can be overexpressed, underexpressed or exhibit altered activity in cancer cells. By the same token, receptors of autocrine or exocrine growth factors and hormones (for example insulin growth factor (IGF) receptors, and EGF receptor) can also exhibit changes in expression or activity associated with tumor growth. Lastly, tumor growth is supported by angiogenesis involving the elaboration and growth of new blood vessels and the concomitant expression of angiogenic factors that can serve as markers for tumorigenesis and tumor growth.

In addition to tumorigenic, proliferation and growth markers, a number of markers have been identified that can serve as indicators of invasiveness and/or metastatic potential in a population of cancer cells. These markers generally reflect altered interactions between cancer cells and their surrounding microenvironment. For example, when cancer cells invade or metastasize, detectable changes can occur in the expression or activity of cell adhesion or motility factors,

examples of which include the cancer markers Cathepsin D, plasminogen activators, collagenases and other factors. In addition, decreased expression or overexpression of several putative tumor "suppressor" genes (for example nm23, p53 and rb) has been directly associated with increased metastatic potential or deregulation of growth predictive of poor disease outcome.

Additionally, ovarian cancer has the highest mortality rate of all gynecological cancers and yet there is still no reliable and easy to administer screening test. Using the multimodality approach to treatment, including aggressive cytoreductive surgery in combination with chemotherapy, five-year survival rates diminish with increasing stage: Stage I (93%), Stage II (70%), Stage III (37%), and Stage IV (25%). Despite advances in molecular biology, surgical oncology, and chemotherapy, the overall prognosis for ovarian cancer patients diagnosed at Stages II-IV remains poor. The excellent survival rates for Stage I disease provide the rationale for efforts to detect early-stage ovarian cancer as a screening test. The first priority of any screening procedure for ovarian cancer is high specificity in order to minimize the number of false positive results and thereby ensuring an acceptable positive predictive value (PPV). There have been no effective and reliable tests developed to date.

Screening for ovarian cancer has been based on strategies using serum tumor markers or ultrasound imaging of the ovaries. The most extensively investigated biomarker is CA-125, whose serum levels are elevated in 50% of Stage I and 90% of Stage II ovarian cancer patients. However, elevated CA-125 levels have also been observed in healthy women during menstruation, in patients with other gynecological diseases, and other malignancies, which suggests that the false-positive rate of CA-125 can be high.

In contrast to detection of serum antigens, the detection of serum antibody responses to tumor antigens may provide a more reliable serum marker for cancer diagnosis because serum antibodies are more stable than serum antigens. Furthermore, antibodies may be more abundant than antigens, especially at low tumor burdens characteristic of early stages. Thirty percent of patients with ductal carcinoma in situ (DCIS) in which the protooncogene HER2/neu was overexpressed had serum antibodies specific to this protein. In addition, antibodies to p53 have been reported in patients with early-stage ovarian, and colorectal cancers. Antibodies against heat shock protein 90 (HSP90) were also found to be associated with patients' survival and tumor metastasis. Antibodies against ribosomal proteins may constitute a novel serological marker. The presence of antibodies to ubiquitin C-terminal hydrolase L3 in colon cancer has also been reported. Changes in the level of gene expression in cancer and aberrant expression of tissue-restricted gene products in cancer are factors in the development of a humoral immune response in cancer patients. In this respect, serological analysis of recombinant cDNA expression libraries (SEREX) of human tumors with autologous serum has identified some relevant tumor antigens. Among the gene products shown to be immunogenic are MAGE, SSX2, and NY-ESO-1, which are expressed in various tumor types, but not in normal tissues except testis.

Studies on new technology based on proteomic patterns in serum to screen for early stage ovarian cancer have been reported by Petricoin et al. (2002). The procedure involved generating proteomic spectra of serum proteins using Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) and surface-enhanced laser desorption and ionization time-of-flight (SELDI-TOF) mass spectroscopy. In independent validation to detect early stage invasive epi-

thelial ovarian cancer from healthy controls, the sensitivity of a multivariate model combining the three biomarkers and CA125 [74% (95% CI, 52-90%)] was higher than that of CA125 alone [65% (95% CI, 43-84%)] at a matched specificity of 97% (95% CI, 89-100%). When compared at a fixed sensitivity of 83% (95% CI, 61-95%), the specificity of the model [94% (95% CI, 85-98%)] was significantly better than that of CA125 alone [52% (95% CI, 39-65%)]. Due to the low prevalence of ovarian cancer in the general population, this level of specificity is unacceptable for a realistic ovarian cancer diagnostic test. Assuming that in a clinical setting with low-risk patients, ovarian cancer is present in approximately one per 2500 patients, the (MALDI/SELDI) approach would produce 125 false positives for every true cancer patient. Furthermore, some issues have arisen regarding the mass spectroscopy technology of protein profiling. It has been reported that the data obtained by this technology are difficult to reproduce and that they may be biased by artifacts in sample preparation, storage and processing, and patient selection.

In summary, the evaluation of proliferation markers, oncogenes, growth factors and growth factor receptors, angiogenic factors, proteases, adhesion factors and tumor suppressor genes, among other cancer markers, can provide important information concerning the risk, presence, status or future behavior of cancer in a patient. Determining the presence or level of expression or activity of one or more of these cancer markers can aid in the differential diagnosis of patients with uncertain clinical abnormalities, for example by distinguishing malignant from benign abnormalities. Furthermore, in patients presenting with established malignancy, cancer markers can be useful to predict the risk of future relapse, or the likelihood of response in a particular patient to a selected therapeutic course. Even more specific information can be obtained by analyzing highly specific cancer markers, or combinations of markers, which can predict responsiveness of a patient to specific drugs or treatment options.

Methods for detecting and measuring cancer markers have been recently revolutionized by the development of immunological assays, particularly by assays that utilize monoclonal antibody technology. Previously, many cancer markers could only be detected or measured using conventional biochemical assay methods, which generally require large test samples and are therefore unsuitable in most clinical applications. In contrast, modern immunoassay techniques can detect and measure cancer markers in relatively much smaller samples, particularly when monoclonal antibodies that specifically recognize a targeted marker protein are used. Accordingly, it is now routine to assay for the presence or absence, level, or activity of selected cancer markers by immunohistochemically staining tissue specimens obtained via conventional biopsy methods. Because of the highly sensitive nature of immunohistochemical staining, these methods have also been successfully employed to detect and measure cancer markers in smaller, needle biopsy specimens which require less invasive sample gathering procedures compared to conventional biopsy specimens. In addition, other immunological methods have been developed and are now well known in the art that allow for detection and measurement of cancer markers in non-cellular samples such as serum and other biological fluids from patients. The use of these alternative sample sources substantially reduces the morbidity and costs of assays compared to procedures employing conventional biopsy samples, which allows for application of cancer marker assays in early screening and low risk monitoring programs where invasive biopsy procedures are not indicated.

For the purpose of cancer evaluation, the use of conventional or needle biopsy samples for cancer marker assays is often undesirable, because a primary goal of such assays is to detect the cancer before it progresses to a palpable or detectable tumor stage. Prior to this stage, biopsies are generally contraindicated, making early screening and low risk monitoring procedures employing such samples untenable. Therefore, there is general need in the art to obtain samples for cancer marker assays by less invasive means than biopsy, for example by serum withdrawal.

Efforts to utilize serum samples for cancer marker assays have met with limited success, largely because the targeted markers are either not detectable in serum, or because telltale changes in the levels or activity of the markers cannot be monitored in serum. In addition, the presence of cancer markers in serum probably occurs at the time of micro-metastasis, making serum assays less useful for detecting pre-metastatic disease.

Previous attempts to develop non-invasive breast cancer marker assays utilizing mammary fluid samples have included studies of mammary fluid obtained from patients presenting with spontaneous nipple discharge. In one of these studies, conducted by Inaji et al., *Cancer* 60: 3008-3013, 1987, levels of the breast cancer marker carcinoembryonic antigen (CEA) were measured using conventional, enzyme linked immunoassay (ELISA) and sandwich-type, monoclonal immunoassay methods. These methods successfully and reproducibly demonstrated that CEA levels in spontaneously discharged mammary fluid provide a sensitive indicator of nonpalpable breast cancer. In a subsequent study, also by Inaji et al., *Jpn. J. Clin. Oncol.* 19: 373-379, 1989, these results were expanded using a more sensitive, dry chemistry, dot-immunobinding assay for CEA determination. This latter study reported that elevated CEA levels occurred in 43% of patients tested with palpable breast tumors, and in 73% of patients tested with nonpalpable breast tumors. CEA levels in the discharged mammary fluid were highly correlated with intratumoral CEA levels, indicating that the level of CEA expression by breast cancer cells is closely reflected in the mammary fluid CEA content. Based on these results, the authors concluded that immunoassays for CEA in spontaneously discharged mammary fluid are useful for screening nonpalpable breast cancer.

Although the evaluation of mammary fluid has been shown to be a useful method for screening nonpalpable breast cancer in women who experience spontaneous nipple discharge, the rarity of this condition renders the methods of Inaji et al. inapplicable to the majority of women who are candidates for early breast cancer screening. In addition, the first Inaji report cited above determined that certain patients suffering spontaneous nipple discharge secrete less than 10 μ l of mammary fluid, which is a critically low level for the ELISA and sandwich immunoassays employed in that study. It is likely that other antibodies used to assay other cancer markers can exhibit even lower sensitivity than the anti-CEA antibodies used by Inaji and coworkers, and can therefore not be adaptable or sensitive enough to be employed even in dry chemical immunoassays of small samples of spontaneously discharged mammary fluid.

In view of the above, an important need exists in the art for more widely applicable, non-invasive methods and materials to obtain biological samples for use in evaluating, diagnosing and managing breast and other diseases including cancer, particularly for screening early stage, nonpalpable tumors. A related need exists for methods and materials that utilize such readily obtained biological samples to evaluate, diagnose and manage disease, particularly by detecting or measuring

selected cancer markers, or panels of cancer markers, to provide highly specific, cancer prognostic and/or treatment-related information, and to diagnose and manage pre-cancerous conditions, cancer susceptibility, bacterial and other infections, and other diseases.

With specific regard to such assays, specific antibodies can only be measured by detecting binding to their antigen or a mimic thereof. Although certain classes of immunoglobulins containing the antibodies of interest can, in some cases, be separated from the sample prior to the assay (Decker, et al., EP 0,168,689 A2), in all assays, at least some portion of the sample immunoglobulins are contacted with antigen. For example, in assays for specific IgM, a portion of the total IgM can be adsorbed to a surface and the sample removed prior to detection of the specific IgM by contacting with antigen. Binding is then measured by detection of the bound antibody, detection of the bound antigen or detection of the free antigen.

For detection of bound antibody, a labeled anti-human immunoglobulin or labeled antigen is normally allowed to bind antibodies that have been specifically adsorbed from the sample onto a surface coated with the antigen, Bolz, et al., U.S. Pat. No. 4,020,151. Excess reagent is washed away and the label that remains bound to the surface is detected. This is the procedure in the most frequently used assays, or example, for hepatitis and human immunodeficiency virus and for numerous immunohistochemical tests, Nakamura, et al., *Arch Pathol Lab Med* 112:869-877 (1988). Although this method is relatively sensitive, it is subject to interference from non-specific binding to the surface by non-specific immunoglobulins that can not be differentiated from the specific immunoglobulins.

Another method of detecting bound antibodies involves combining the sample and a competing labeled antibody, with a support-bound antigen, Schuurs, et al., U.S. Pat. No. 3,654, 090. This method has its limitations because antibodies in sera bind numerous epitopes, making competition inefficient.

For detection of bound antigen, the antigen can be used in excess of the maximum amount of antibody that is present in the sample or in an amount that is less than the amount of antibody. For example, radioimmunoprecipitation ("RIP") assays for GAD autoantibodies have been developed and are currently in use, Atkinson, et al., *Lancet* 335:1357-1360 (1990). However, attempts to convert this assay to an enzyme linked immunosorbent assay ("ELISA") format have not been successful. The RIP assay is based on precipitation of immunoglobulins in human sera, and led to the development of a radioimmunoassay ("RIA") for GAD autoantibodies. In both the RIP and the RIA, the antigen is added in excess and the bound antigen:antibody complex is precipitated with protein A-Sepharose. The complex is then washed or further separated by electrophoresis and the antigen in the complex is detected.

Other precipitating agents can be used such as rheumatoid factor or C1q, Masson, et al., U.S. Pat. No. 4,062,935; polyethylene glycol, Soeldner, et al., U.S. Pat. No. 4,855,242; and protein A, Ito, et al., EP 0,410,893 A2. The precipitated antigen can be measured to indicate the amount of antibody in the sample; the amount of antigen remaining in solution can be measured; or both the precipitated antigen and the soluble antigen can be measured to correct for any labeled antigen that is non-specifically precipitated. These methods, while quite sensitive, are all difficult to carry out because of the need for rigorous separation of the free antigen from the bound complex, which requires at a minimum filtration or centrifugation and multiple washing of the precipitate.

Alternatively, detection of the bound antigen can be employed when the amount of antigen is less than the maxi-

imum amount of antibody. Normally, that is carried out using particles such as latex particles or erythrocytes that are coated with the antigen, Cambiaso, et al., U.S. Pat. No. 4,184,849 and Uchida, et al., EP 0,070,527 A1. Antibodies can specifically agglutinate these particles and can then be detected by light scattering or other methods. It is necessary in these assays to use a precise amount of antigen as too little antigen provides an assay response that is biphasic and high antibody titers can be read as negative, while too much antigen adversely affects the sensitivity. It is therefore necessary to carry out sequential dilutions of the sample to assure that positive samples are not missed. Further, these assays tend to detect only antibodies with relatively high affinities and the sensitivity of the method is compromised by the tendency for all of the binding sites of each antibody to bind to the antigen on the particle to which it first binds, leaving no sites for binding to the other particle.

For assays in which the free antigen is detected, the antigen can also be added in excess or in a limited amount although only the former has been reported. Assays of this type have been described where an excess of antigen is added to the sample, the immunoglobulins are precipitated, and the antigen remaining in the solution is measured, Masson, et al., supra and Soeldner, et al., supra. These assays are relatively insensitive because only a small percentage change in the amount of free antigen occurs with low amounts of antibody, and this small percentage is difficult to measure accurately.

Practical assays in which the free antigen is detected and the antigen is not present in excess of the maximum amount of antibody expected in a sample have not been described. However, in van Erp, et al., *Journal of Immunoassay* 12(3):425-443 (1991), a fixed concentration of monoclonal antibody was incubated with a concentration dilution series of antigen, and free antigen was then measured using a gold sol particle agglutination immunoassay to determine antibody affinity constants.

There has been much research in the area of evaluating useful markers for determining the risk factor for patients developing IDDM. These include insulin autoantibodies, Soeldner, et al., supra and circulating autoantibodies to glutamic acid decarboxylase ("GAD"), Atkinson, et al., PCT/US89/05570 and Tobin, et al., PCT/US91/06872. In addition, Rabin, et al., U.S. Pat. No. 5,200,318 describes numerous assay formats for the detection of GAD and pancreatic islet cell antigen autoantibodies. GAD autoantibodies are of particular diagnostic importance because they occur in preclinical stages of the disease, which can make therapeutic intervention possible. However, the use of GAD autoantibodies as a diagnostic marker has been impeded by the lack of a convenient, nonisotopic assay.

One assay method involves incubating a support-bound antigen with the sample, then adding a labeled anti-human immunoglobulin. This is the basis for numerous commercially available assay kits for antibodies such as the Syn ELISA kit which assays for autoantibodies to GAD65, and is described in product literature entitled "Syn^{ELISA} GAD II-Antibodies" (Elias USA, Inc.). Substantial dilution of the sample is required because the method is subject to high background signals from adsorption of non-specific human immunoglobulins to the support.

Many of the assays described above involve detection of antibody that becomes bound to an immobilized antigen. This can have an adverse affect on the sensitivity of the assay due to difficulty in distinguishing between specific immunoglobulins and other immunoglobulins in the sample, which bind non-specifically to the immobilized antigen. There is not only a need to develop an assay that avoids non-specific detection

of immunoglobulins, but there is also the need for an improved method of detecting antibodies that combines the sensitivity advantage of immunoprecipitation assays with a simplified protocol. Finally, assays that can help evaluate the risk of developing diseases are medically and economically very important. The present invention addresses these needs.

SUMMARY OF THE INVENTION

According to the present invention, there is provided a biosensor for use in detecting the presence of diseases, the biosensor comprising a detector for detecting a presence of at least one marker indicative of a specific disease. Also provided is a method of determining efficacy of a pharmaceutical for treating a disease or staging disease by administering a pharmaceutical to a sample containing markers for a disease, detecting the amount of at least one marker of the disease in the sample, and analyzing the amount of the marker in the sample, whereby the amount of marker correlates to pharmaceutical efficacy or disease stage. Markers for gynecological disease selected from the list in Table 8 are provided. An immuno-imaging agent comprising labeled antibodies, whereby the labeled antibodies are isolated and reactive to proteins overexpressed in vivo are provided. Informatics software for analyzing the arrays discussed above is provided, wherein the software includes analyzing means for analyzing the arrays.

DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Other advantages of the present invention are readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGS. 1A-D are photographs showing the identification of a phage displaying peptide sequence of Sirt2 by plaque lift;

FIG. 2 is a photograph showing the analysis of the PCR product of the plaques by Southern Blot hybridization;

FIG. 3 is a photograph showing the Dot Blot analysis of Sirt2 positive plaques;

FIG. 4 is a photograph showing green and red labeled detection of serum antibodies indicative of the antibody reaction to the protein;

FIGS. 5 A-E are photographs showing the ECL detection of phagotopes selected with a breast cancer patient's serum;

FIGS. 6 A-C are as follows: FIG. 6A is a photograph showing the comparison of serum reaction of control and breast cancer patient with phagotopes from BP4; and FIG. 6B is a graph of the BP4 filters which were scanned thereby showing the ratio of the pixel densities plotted in rank order;

FIG. 6C is a scan of a microarray demonstrating the binding a Cy5-labeled antihuman IgG to human IgG from patient #1's serum and the control Cy3-labeled antibody to phage T7 capsid protein to phage clones microarrayed on glass;

FIGS. 7A and 7B show the method of finding informative epitopes: The spot intensities are plotted on the vertical axis for 12 subjects (controls to the left and patients to the right) the template defined on the left (shown in blue) was used with a correlation distance, a correlation threshold of 0.8 selected the 46 epitopes shown here in red (out of the total of 4×96=384 shown here in yellow);

FIGS. 8A and 8B show an example comparison between the histogram of a control subject (19218) with a high but

non-specific reaction to the left, and the histogram of a patient (19223), to the right; the histograms are calculated on the ratios of the background corrected mean intensity of the human IgG labeled with Cy5 vs. the background corrected mean intensity of the T7 labeled with Cy3;

FIGS. 9A and 9B show a comparison between the scatterplot of a control subject (19218) with a strong but non-specific reaction and the scatterplot of a patient MEC1 (19223), the scattergrams plot the background corrected mean intensity of the human IgG labeled with Cy5 vs. the background corrected mean intensity of the T7 labeled with Cy3;

FIG. 10 shows the matrix of reactivity between sets of clones coming from patients 1-12 (in rows) and sera from same patients (in columns), at this point (step 2 of Procedure 2), the matrix contains the results of the self-reactions: patients 1-10 have a specific self-reaction whereas patients 11 and 12 do not, Patients 11 and 12 are eliminated from the clone selection procedure;

FIG. 11 shows a matrix of reactivity between sources of clones and different sera ordered by reactivity; the clones from patient 2 react with sera from self (column 2) and patients 4 and 8; the clones from patient 3 react with sera from self (column 3) and patients 6 and 10, etc, note that the union of the set of clones coming from patients 2, 3, 5, 7 and 1 ensures that the chip made with these clones reacts with all patients;

FIGS. 12A-G are filter microarrays showing antigen binding with IgGs in the serum of Stage I ovarian cancer patients; and

FIGS. 13A-D are graphs showing the determination of a titratable antigen-antibody binding in ELISA macroarray analysis.

DETAILED DESCRIPTION OF THE INVENTION

Generally, the present invention provides a method and markers for use in detecting disease and stages of disease. In other words, the markers can be used to determine the presence of disease without requiring the presence of symptoms.

The method and markers of the present invention can be used to diagnose the presence of a disease or a disease stage in a patient. The method of the present invention utilizes a detector device for detecting the presence of at least one marker in the serum of the patient. The benefit of such an analytical device is that the marker that is detected is one of a panel of markers. The panel of markers can include markers that are known to those of skill in the art and markers determined utilizing the methodology disclosed herein. The markers of the present invention can be used to detect diseases. Examples of diseases include, but are not limited to, gynecological sickness, such as endometriosis, ovarian cancer, breast cancer, cervical cancer, and primary peritoneal carcinoma. The method can also be used to identify overexpressed or mutated proteins in tumor cells. That such proteins are mutated or overexpressed presumably is the basis for the immune reaction to these proteins. Therefore markers identified using these methods could provide markers for molecular pathology as diagnostic or prognostic markers.

The method can also be used for immunotherapy targeted to a person's immunoprofile based on the arrays. For personalized immunotherapy, the reactivity to particular epitope clones can be correlated using sera from patients having cancer. Using a comprehensive panel of epitope markers that can accurately detect early stage ovarian cancer one can utilize these antigen as immuno-therapeutic agents personalized to the immuno-profile of each patient. When T-cells from the patient recognize antigen biomarkers, they get stimulated,

activated and therefore produce an immune-response. Such reactivity demonstrates the potential of each antigen as a component of a vaccine to induce a T cell-mediated immune response essential for generation of cancer vaccines. Individuals scoring positive in the presymptomatic testing for OVCA can then be offered an anti-tumor vaccine tailored to their immunoprofile against a panel of tumor antigens.

The detector includes, but is not limited to an assay, a slide, a filter, a microarray, macroarray, computer software implementing the data analysis methods, and any combinations thereof. The detector can also include a two-color detection system or other detector system known to those of skill in the art.

By "bodily fluid" as used herein it is meant any bodily fluid known to those of skill in the art to contain antibodies therein. Examples include, but are not limited to, blood, saliva, tears, spinal fluid, serum, and other fluids known to those of skill in the art to contain antibodies.

By "biopanning", it is meant a selection process for use in screening a library (Parnley and Smith, Gene, 73:308 (1988); Noren, C. J., NEB Transcript, 8(1); 1 (1996)). Biopanning is carried out by incubating phages encoding the peptides with a plate coated with the proteins, washing away the unbound phage, eluting, and amplifying the specifically bound phage. Those skilled in the art readily recognize other immobilization schemes that can provide equivalent technology, such as but not limited to binding the proteins or other targets to beads.

By staging the disease, as for example in cancer, it is intended to include determining the extent of a cancer, especially whether the disease has spread from the original site to other parts of the body. The stages can range from 0 to 5 with 0 being the presence of cancerous cells and 5 being the spread of the cancer cells to other parts of the body including the lymph nodes. Further, the staging can indicate the stage of a borderline histology. A borderline histology is a less malignant form of disease. Additionally, staging can indicate a relapse of disease, in other words the reoccurrence of disease.

The term "marker" as used herein is intended to include, but is not limited to, a gene or a piece of a gene which codes for a protein, a protein such as a fusion protein, open reading frames such as ESTs, epitopes, mimotopes, antigens, and any other indicator of immune response. The marker can also be used as a predictor of disease or the recurrence of disease.

The present invention further includes a random peptide epitope (mimotope) that mimics a natural antigenic epitope during epitope presentation. Such mimotopes are useful in the applications and methods discussed above. Also included in the present invention is a method of identifying a random peptide epitope. In the method, a library of random peptide epitopes is generated or selected. The library is contacted with an anti-antibody. Mimotopes are identified that are specifically immunoreactive with the antibody. Sera (containing anti antibodies) or antibodies generated by the methods of the present invention can be used. Random peptide libraries can, for example, be displayed on phage (phagotopes) or generated as combinatorial libraries.

"Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the various immunoglobulin diversity/joining/variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist, e.g., as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)_2'$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The $F(ab)_2'$ can be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the $F(ab)_2'$ dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (see Fundamental Immunology (Paul ed., 3d ed. 1993)). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill can appreciate that such fragments can be synthesized de novo either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized de novo using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (see, e.g., McCafferty et al., Nature 348:552-554 (1990)).

For preparation of monoclonal or polyclonal antibodies, any technique known in the art can be used (see, e.g., Kohler & Milstein, Nature 256:495-497 (1975); Kozbor et al., Immunology Today 4: 72 (1983); Cole et al., pp. 77-96 in Monoclonal Antibodies and Cancer Therapy (1985)). Techniques for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, can be used to express humanized antibodies. Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (see, e.g., McCafferty et al., Nature 348:552-554 (1990); Marks et al., Biotechnology 10:779-783 (1992)).

A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

The term "immunoassay" is an assay wherein an antibody specifically binds to an antigen. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen. In addition, an antigen can be used to capture or specifically bind an antibody.

The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant

amount to other proteins present in the sample. Specific binding to an antibody under such conditions can require an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to modified β -tubulin from specific species such as rat, mouse, or human can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive, e.g., with β -tubulin modified at cysteine 239 and not with other proteins. This selection can be achieved by subtracting out antibodies that cross-react with other molecules. Monoclonal antibodies raised against modified β -tubulin can also be used. A variety of immunoassay formats can be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g., Harlow & Lane, Antibodies, A Laboratory Manual (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically a specific or selective reaction can be at least twice background signal or noise and more typically more than 10 to 100 times background.

A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P , fluorescent dyes, iodine, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available, e.g., by incorporating a radiolabel into the peptide, or any other label known to those of skill in the art.

A "labeled antibody or probe" is one that is bound, either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds to a label such that the presence of the antibody or probe can be detected by detecting the presence of the label bound to the antibody or probe.

The terms "isolated" "purified" or "biologically pure" refer to material that is substantially or essentially free from components that normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, optionally at least 95% pure, and optionally at least 99% pure.

The term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

By "support or surface" as used herein, the term is intended to include, but is not limited to a solid phase which is typically

a support or surface, which is a porous or non-porous water insoluble material that can have any one of a number of shapes, such as strip, rod, particle, including beads and the like. Suitable materials are well known in the art and are described in, for example, Ullman, et al. U.S. Pat. No. 5,185, 243, columns 10-11, Kurn, et al., U.S. Pat. No. 4,868,104, column 6, lines 21-42 and Milburn, et al., U.S. Pat. No. 4,959,303, column 6, lines 14-31, which are incorporated herein by reference. Binding of ligands and receptors to the support or surface can be accomplished by well-known techniques, readily available in the literature. See, for example, "Immobilized Enzymes," Ichiro Chibata, Halsted Press, New York (1978) and Cuatrecasas, J. Biol. Chem. 245:3059 (1970). Whatever type of solid support is used, it must be treated so as to have bound to its surface either a receptor or ligand that directly or indirectly binds the antigen. Typical receptors include antibodies, intrinsic factor, specifically reactive chemical agents such as sulfhydryl groups that can react with a group on the antigen, and the like. For example, avidin or streptavidin can be covalently bound to spherical glass beads of 0.5-1.5 mm and used to capture a biotinylated antigen.

Signal producing system ("sps") includes one or more components, at least one component being a label, which generate a detectable signal that relates to the amount of bound and/or unbound label, i.e. the amount of label bound or not bound to the compound being detected. The label is any molecule that produces or can be induced to produce a signal, such as a fluorescer, enzyme, chemiluminescer, or photosensitizer. Thus, the signal is detected and/or measured by detecting enzyme activity, luminescence, or light absorbance.

Suitable labels include, by way of illustration and not limitation, enzymes such as alkaline phosphatase, glucose-6-phosphate dehydrogenase ("G6PDH") and horseradish peroxidase; ribozyme; a substrate for a replicase such as Q-beta replicase; promoters; dyes; fluorosceners such as fluorescein, isothiocyanate, rhodamine compounds, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, and fluorescamine; chemiluminescers such as isoluminol; sensitizers; coenzymes; enzyme substrates; photosensitizers; particles such as latex or carbon particles; suspendable particles; metal sol; crystallite; liposomes; cells, etc., which can be further labeled with a dye, catalyst, or other detectable group. Suitable enzymes and coenzymes are disclosed in Litman, et al., U.S. Pat. No. 4,275,149, columns 19-28, and Boguslaski, et al., U.S. Pat. No. 4,318,980, columns 10-14; suitable fluorosceners and chemiluminescers are disclosed in Litman, et al., U.S. Pat. No. 4,275,149, at columns 30 and 31; which are incorporated herein by reference. Preferably, at least one sps member is selected from the group consisting of fluorosceners, enzymes, chemiluminescers, photosensitizers, and suspendable particles.

The label can directly produce a signal, and therefore, additional components are not required to produce a signal. Numerous organic molecules, for example fluorosceners, are able to absorb ultraviolet and visible light, where the light absorption transfers energy to these molecules and elevates them to an excited energy state. This absorbed energy is then dissipated by emission of light at a second wavelength. Other labels that directly produce a signal include radioactive isotopes and dyes.

Alternately, the label may need other components to produce a signal, and the sps can then include all the components required to produce a measurable signal, which can include substrates, coenzymes, enhancers, additional enzymes, substances that react with enzymatic products, catalysts, activators, cofactors, inhibitors, scavengers, metal ions, specific

binding substance required for binding of signal generating substances, and the like. A detailed discussion of suitable signal producing systems can be found in Ullman, et al. U.S. Pat. No. 5,185,243, columns 11-13, which is incorporated herein by reference.

The label is bound to a specific binding pair (hereinafter "sbp") member which is the antigen, or is capable of directly or indirectly binding the antigen, or is a receptor for the antigen, and includes, without limitation, the antigen; a ligand for a receptor bound to the antigen; a receptor for a ligand bound to the antigen; an antibody that binds the antigen; a receptor for an antibody that binds the antigen; a receptor for a molecule conjugated to an antibody to the antigen; an antigen surrogate capable of binding a receptor for the antigen; a ligand that binds the antigen, etc. Binding of the label to the sbp member can be accomplished by means of non-covalent bonding as for example by formation of a complex of the label with an antibody to the label or by means of covalent bonding as for example by chemical reactions which result in replacing a hydrogen atom of the label with a bond to the sbp member or can include a linking group between the label and the sbp member. Such methods of conjugation are well known in the art. See for example, Rubenstein, et al., U.S. Pat. No. 3,817,837, which is incorporated herein by reference. Other sps members can also be bound covalently to sbp members. For example, in Ullman, et al., U.S. Pat. No. 3,996, 345, two sps members such as a fluorescer and quencher can be bound respectively to two sbp members that both bind the analyte, thus forming a fluorescer-sbp₁:analyte:sbp₂-quencher complex. Formation of the complex brings the fluorescer and quencher in close proximity, thus permitting the quencher to interact with the fluorescer to produce a signal. This is a fluorescent excitation transfer immunoassay. Another concept is described in Ullman, et al., EP 0,515,194 A2, which uses a chemiluminescent compound and a photosensitizer as the sps members. This is referred to as a luminescent Oxygen channeling immunoassay. Both the aforementioned references are incorporated herein by reference.

The analysis of mRNA expression in tumors does not necessarily reveal the status of protein levels in the cancer cells. Other factors such as protein half-life and mutation can be altered without an effect on mRNA levels thus masking significant molecular changes at the protein level. Serum antibody reactivity to cellular proteins occurs in cancer patients due to presentation of mutated forms of proteins from the tumor cells or overexpression of proteins in the tumor cells. The host immune system can direct individuals to molecular events critical to the genesis of the disease. Using a candidate gene approach, experience has shown that the frequency of serum positivity to any single protein is low. Therefore, to increase the identification of such autoantigens, a more global approach is employed to exploit immunoreactivity to identify large numbers of cDNAs coding for proteins that are mutated or upregulated in cancer cells.

In order to develop an effective screening test for early detection of ovarian cancer, cDNA phage display libraries are used to isolate cDNAs coding for epitopes reacting with antibodies present specifically in the sera of patients with ovarian cancer. The methods of the present invention detect various antibodies that are produced by patients in reaction to proteins overexpressed in their ovarian tumors. This is achievable by differential biopanning technology using human sera collected both from normal individuals and patients having ovarian cancer and phage display libraries expressing cDNAs of genes expressed in ovarian epithelial tumors and cell lines. Serum reactivity toward a cellular protein can occur because of the presentation to the immune system of a mutated form of

the protein from the tumor cells or overexpression of the protein in the tumor cells. The strategy provides for the identification of epitope-bearing phage clones (phagotopes) displaying reactivity with antibodies present in sera of patients having ovarian cancer but not in control sera from unaffected women. This strategy leads to the identification of novel disease-related epitopes for diseases including, but not limited to ovarian cancer, that have prognostic/diagnostic value with additional potential for therapeutic vaccines and medical imaging reagents. This also creates a database that can be used to determine both the presence of disease and the stage of the disease.

The series of experiments disclosed herein provide direct evidence that biopanning a T7 coat protein fusion library can isolate epitopes for antibodies present in polyclonal sera. This also showed that the technology can be applied to direct microarray screening of large numbers of selected phage against numerous patient and control sera. This approach provides a large number of biomarkers for early detection of disease.

More specifically, the methods of the present invention provide four to five cycles of affinity selection and biopanning which are carried out with biological amplification of the phage after each biopanning, meaning growth of the biological vector of the cDNA expression clone in a biological host. Examples of biological amplification include but are not limited to growth of a lytic or lysogenic bacteriophage in host bacteria or transformation of bacterial host with selected DNA of the cDNA expression vector. The number of biopanning cycles generally determines the extent of the enrichment for phage that binds to the sera of patient with ovarian cancer. This strategy allows for one cycle of biopanning to be performed in a single day. Someone skilled in the art can establish different schedules of biopanning that provide the same essential features of the procedure described above.

Two biopanning experiments are performed with each library differentially selecting clones between control and disease patient sera. The first selection is to isolate phagotopes clones that do not bind to control sera pooled from control women but do bind to a pool of disease patient serum. This set of phagotopes clones represent epitopes that are indicative of the presence of disease as recognized by the host immune system. The second type of screening is performed to isolate phagotopes clones that did not bind to a pool of control sera but do bind to an individual patient's serum. Those sets of phagotopes clones represent epitopes that are indicative of the presence of disease.

Subsequent to the biopanning, the clones so isolated can be used to contact antibodies in sera by spotting the clones or peptide sequences of amino acids containing those encoded by the clones. After spotting on a solid support, the arrays are rinsed briefly in a 1% BSA/PBS to remove unbound phage, then transferred immediately to a 1% BSA/PBS blocking solution and allowed to sit for 1 hour at room temperature. The excess BSA is rinsed off from the slides using PBS. This step insures that the elution step of antibodies is more effective. The use of PBS elutes all of the antibodies without harming the binding of the antibody. Antibody detection of reaction with the clones or peptides on the array is carried out by labeling of the serum antibodies or through the use of a labeled secondary antibody that reacts with the patient's antibodies. A second control reaction to every spot allows for greater accuracy of the quantitation of reactivity and increases sensitivity of detection.

The slides are subsequently processed to quantify the reaction of each phagotopes. Such processing is specific to the label used. For instance, if fluorophore cy3-cy5 labels are

used, this processing is done in a laser scanner that captures an image of the slide for each fluorophore used. Subsequent image processing familiar to those skilled in the art can provide intensity values for each phagotopes.

The data analysis can be divided into the following steps:

1. Pre-processing and normalization.
2. Identifying the most informative markers
3. Building a predictor for molecular diagnosis of ovarian cancer and validating the results.

The purpose of the first step is to cleanse the data from artifacts and prepare it for the subsequent steps. Such artifacts are usually introduced in the laboratory and include: slide contamination, differential dye incorporation, scanning and image processing problems (e.g. different average intensities from one slide to another), imperfect spots due to imperfect arraying, washing, drying, etc. The purpose of the second step is to select the most informative phages that can be used for diagnostic purposes. The purpose of the third step is to develop a software classifier able to diagnose cancer based on the antibody reactivity values of the selected phages. The last step also includes the validation of this classifier and the assessment of its performance using various measures such as specificity, sensitivity, positive predictive value and negative predictive value. The computation of such measures can be done on cases not used during the design of the chip in order to assess the real-world performance of the diagnosis tool obtained.

The pre-processing and normalization step is used for arrays using two channels such as Cy5 for the human IgG and Cy3 for the T7 control, the spots are segmented and the mean intensity is calculated for each spot. A mean intensity value is calculated for the background, as well. A background corrected value is calculated by subtracting the background from the signal. If necessary, non-linear dye effects can be eliminated by performing an exponential normalization (Houts, 2000) and/or LOESS normalization of the data and/or a piecewise linear normalization (see FIGS. 7 A-D). The values coming from each channel are subsequently divided by their mean of the intensities over the whole array. Subsequently, the ratio between the IgG and the T7 channels was calculated. The values coming from replicate spots (spots printed in quadruplicates) are combined by calculating mean and standard deviation. Outliers (outside $\pm$ two standard deviations) are flagged for manual inspection). Single channel arrays are pre-processed in a similar way but without taking the ratios. This preprocessing sequence was shown to provide good results for all preliminary data analyzed.

The step of selecting the most informative markers is used to identify the most informative phages out of the large set of phages started with. The better the selection, the better is the expected accuracy of the diagnosis tool.

A first test is necessary to determine whether a specific epitope is suitable for inclusion in the final set to be spotted. The selection methods to be applied follow the principles of the methods successfully applied in (Golub et al., 1999; Alizadeh et al., 2000) and can be briefly described in the following.

Procedure 1

The procedure is initiated defining a template for the cancer case (FIG. 8). Unlike gene expression experiments where the expression level of a gene can be either up or down in cancer vs. healthy subjects, here one is testing for the presence of antibodies specific to cancer were tested for. Therefore, epitopes with high reactivity in controls and low reactivity in patients are not expected and the profile to the left in FIG. 8 is sufficient. Each epitope can have a profile across the given set of patients (FIGS. 9 A and B). The profile of each

epitope is compared with the templates using a correlation-based distance. Those skilled in the art can recognize that the other distances may be used without essentially changing the procedure.

The epitopes are then ordered based on the similarity between the reference profile (FIG. 8) and their actual profile. FIG. 7 shows 46 epitopes found informative for a correlation threshold of 0.8. The final cutoff threshold is calculated by doing 1000 random permutations once the whole data set become available. Each such permutation moves randomly the subjects between the 'patient' and 'control' categories. Calculating the score of each epitope profile for such permutations allows us to establish a suitable threshold for the similarity (Golub et. al. 1999).

The technique follows closely the one used in (Golub, 1999). However, the technique can be further improved as follows. Firstly, this technique was shown to provide good results if most controls are consistent by providing the same type of reactivity. However, preliminary data showed that there are control subjects that show a non-specific reactivity with all clones (see FIG. 1b). While still clearly different from patients, FIG. 8 shows a comparison between the histogram profile of a control subject showing a non-specific reaction (19218) with and the profile of a patient (19223). FIG. 9 shows the scatterplots of the same subjects. While still clearly different from patients, such control subjects with a high non-specific reaction introduces spikes in the clone profile in the area corresponding to the control subjects (right left hand side of the template in FIG. 8). Such spikes decrease the score of the relevant clones making them more difficult to distinguish from the irrelevant ones. In order to reduce this effect, all control subjects with a non-specific response (i.e. a unimodal distribution such as in the left panel of FIG. 7) were eliminated from the analysis leading to the epitope selection.

A second essential modification is related to the set of epitopes selected. There are rare patients who might react only to a small number of very specific epitopes. If the selection of the epitopes is done on statistical grounds alone, such very specific epitopes can be missed if the set of patients available contains only few such rare patients. In order to maximize the sensitivity of the penultimate test resulted from this work, every effort was made to include epitopes which might be the only ones reacting to rare patients. In order to do this, the information content of the set of epitopes is maximized while trying to minimize the number of epitopes used using the following procedure.

Procedure 2

Assume there are m patients and k controls. Select n random patients from the m available. For each of the n patients used for epitope selection, amplify ($n \times 4$ biopannings) and do self-reactions. Eliminate those patients/epitopes that do not react to self.

Make a chip with all available, self-reacting epitopes printed in quadruplicates. React this chip with all patients and controls ($n+k$ antibody reactions). Eliminate controls with a non-specific reactivity. For the set of epitopes coming from a single patient, apply Procedure 1 to order the epitopes in the order of their informational content and select the ones that can be used to differentiate patients from controls.

Order the epitopes by their reactivity in decreasing order of the number of patients they react to. Scan this list from the top down, moving epitopes from this list to the final set. Every time a set of epitopes coming from a patient x is added to the final set, the patient x and all other patients that these epitopes react to are represented in the current set of epitopes. Repeat until all patients are represented in the current set of epitopes.

This procedure tries to minimize the number of epitopes used while maximizing the number of patients that react to the chip containing the selected epitopes.

The following example shows how this procedure works using a simple example. The matrix in FIG. 10 contains a row i for the clones coming from patient i and a column j for the serum coming from patient j . A serum is said to react specifically with a set of clones if the histogram of the ratios is bimodal (see subject 19218 in FIGS. 8 and 9). A serum is said to react non-specifically if the histogram of the ratio is unimodal (see subject 19223 in FIGS. 8 and 9). Furthermore, a serum might not react at all with a set of clones. If the serum from patient j reacts specifically with the clones from patient i , the matrix can contain a value of 1 at the position (i, j) . The element at position (i, j) is left blank if there is no reaction or the reaction is non-specific.

Each set of epitopes corresponding to a row of the matrix is pruned by sub-selecting epitopes according to Procedure 1. The rows are now sorted in decreasing reactivity (number of patients other than self that the clones react to). For instance, in FIG. 11, the clones from patient 2 react with sera from self (column 2) and patients 4 and 8. The clones from patient 3 react with sera from self (column 3) and patients 6 and 10, etc. The final set of clones was obtained from patients 2, 3, 5, 7 and 1 (reading top-down in column 1). Clones coming from patients 8, 9 and 10 are not included since these patients already react to clones coming from other patients. This set ensures that the chip made with these clones reacts with all patients in this example.

Procedure 3

Arrays using two channels such as Cy5 for the human IgG and Cy3 for the T7 control are processed as follows. The spots are segmented and the mean intensity is calculated for each spot. A mean intensity value is calculated for the background, as well. A background corrected value is calculated by subtracting the background from the signal. The values coming from each channel are normalized by dividing by their mean. Subsequently, the ratio between the IgG and the T7 channels are calculated and a logarithmic function is applied. The values coming from replicate spots (spots printed in quadruplicates) are combined by calculating mean and standard deviation. Outliers (outside $\pm$ two standard deviations) are flagged for manual inspection. Someone skilled in the art can recognize that various combinations and permutations of the steps above or similar could replace the normalization procedure above without substantially changing rest of the data analysis process. Such similar steps include without limitation taking the median instead of the mean, using logarithmic functions in various bases, etc.

The histogram of the average log ratio is calculated. If the histogram is unimodal (e.g. subject 19223 in FIG. 7), there is no specific response. If the histogram is clearly bimodal (e.g. subject 19218 in FIG. 7), there is a specific response. All 25 subjects analyzed so far fell in one of these two categories or had no response at all. A mixed probability model is used in less clear cases to fit two normal distributions as in (Lee, 2000). If the two distributions found under the maximum likelihood assumption are separated by a distance d of more than 2 standard deviations (corresponding to a p -value of approximately 0.05), there is a specific response. If the distance is less than 2 standard deviations, the response can be considered as not specific. The preliminary data analyzed so far showed a very good separation of the distributions for the patients.

Once the chosen clones are spotted on the final version of the array, a number of sera coming from both patients and controls can be tested. These sera come from subjects not

used in any of the phases that lead to the fabrication of the array (i.e. not involved in clone selection, not used as controls, etc.). Each test was evaluated using Procedure 3 above. The performance on this validation data can be reported in terms of PPV, NPV, specificity and sensitivity. Since these performance indicators are calculated on data not previously used, they provide a good indication of the performance of the test for screening purposes for the various categories of patients envisaged in the general population.

The present invention also provides a kit including all of the technology for performing the above analysis. This is included in a container of a size sufficient to hold all of the required pieces for analyzing sera, as well as a digital medium such as a floppy disk or CDROM containing the software necessary to interpret the results of the analysis. These components include the array of clones or peptides spotted onto a solid support, prewashing buffers, a detection reagent for identifying reactivity of the patients' serum antibodies to the spotted clones or peptides, post-reaction washing buffers, primary and secondary antibodies to quantify reactivity of the patients serum antibodies with the spotted array and methods to analyze the reactivity so as to establish an interpretation of the serum reactivity.

A biochip for detecting the presence of the disease state in a patient's sera is provided by the present invention. The biochip has a detector contained within the biochip for detecting antibodies in a patient's sera. This allows a patient's sera to be tested for the presence of a multitude of diseases or reaction to disease markers using a single sample and the analysis can be conducted and analyzed on a single chip. By utilizing such a chip this lowers the time required for the detection of disease while also enabling a doctor to determine the level of disease spread or infection. The chip, or other informatics system can be altered to weigh the results. In other words, the informatics can be altered to adjust the levels of sensitivity and/or specificity of the chip.

The present invention is well suited for providing useful information regarding the efficacy of pharmaceuticals at treating disease. Specifically, the present invention is well suited in measuring the effects of drugs and other medications based on the above-identified markers. The present invention determines the minimum level of a pharmaceutical needed to achieve therapeutic benefits. Thus, the present invention is useful in determining effective treatment of various diseases and illnesses. The results of the analysis can be utilized to determine if the treatment is effective or if such treatment needs to be altered.

Further, the treatment can be altered based upon the markers detected. For example, the treatment can be specifically designed based upon the markers identified. In other words, the therapy can be altered to most suitably treat the identified markers, such that the treatment is designed to most efficiently treat the identified marker. The ability to adjust the therapy enables the treatment to be tailored to the person being treated's needs. The treatments that can be used range from vaccines to chemotherapy.

The markers of the present invention can also be used for immuno-imaging. Immuno-imaging is a process in which antibodies to a specific antigen are labeled such that the label can be detected externally. Examples of external detectors include, but are not limited to, x-rays, MRI, CT scan, and PET scans. The imaging functions because an imaging reagent containing the labeled antibody is administered to a patient.

The above discussion provides a factual basis for the use of the combination of markers and method of making the com-

bination. The methods used with a utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

EXAMPLES

Example 1

The purpose of this study is to clone epitopes that are recognized by sera from women with ovarian cancer but not recognized by normal sera from unaffected women. As these epitopes are cloned, protein array assays are developed capable of detecting ovarian cancer at an early stage by analyzing antigens recognized in the sera of at risk women. Toward this end, individual sera were screened using these protein biochips to determine the antibody reactivity to each protein epitope. Antibody reactivity is detected that does not appear in control sera. The patients and control sera obtained for this study were used to calibrate the protein biochips and identify the most informative epitope-clones. The women were monitored for the appearance or reappearance of antibody reactivity and its correlation with tumor burden. By following the serum reactivity to tumor reactive new epitopes on the arrays of the phage display cDNA clones, the analysis of sera from women after their initial diagnosis and semi-annually thereafter allows the determination of the markers in predicting tumor recurrence.

Some of the markers can be predictive of recurrence, and thus can be used to correlate specific ovarian tumor types (using the World Health Organization Histological Classification of Ovarian Tumors), also the tumor grade (where appropriate, since not all tumors are graded), and the surgical stage. This can be done by review of the pathological material (glass slides, patient records, and surgical pathology reports). Certain currently accepted biomarkers of research interest such as Her-2 neu and other can also be included in the new protein biochips in order to compare the sensitivity and specificity of the new and existing immunohistochemical technologies. Testing for Her-2 neu and other biological markers is done by the immunoperoxidase method using formalin fixed, paraffin embedded tumor tissues.

For the purpose of comparison to the ovarian cancer patients, one can analyze serum markers in women in good health who do not have ovarian or any other type of cancer. These control subjects should not have a family history of ovarian cancer or breast cancer. Because some serum markers such as CA125 levels are increased in endometriosis, uterine leiomyoma, pelvic inflammatory disease, early pregnancy, and benign cysts, control subjects should be free of these conditions as well.

The series of experiments provides direct evidence that biopanning a T7 coat protein fusion library can isolate epitopes for antibodies present in polyclonal sera. This also showed that the technology can be applied to direct microarray screening of large numbers of the selected phage against numerous patient and control sera. This approach provides a large number of biomarkers for early detection of ovarian cancer. The likelihood of success of this approach is increased by the fact that the mRNA for human Sirt2 is present in cells at very low abundance in human brain RNA thus indicating that clones can be isolated for rare RNA transcripts by this approach.

To further demonstrate the feasibility of these methods for differential detection of epitopes between test and control sera, four cycles of biopanning of a commercial Novagen breast tumor cDNA library were performed using a serum sample from a breast cancer patient and a control serum

sample from a woman without cancer. 100 plaques were picked from each biopanning. Analysis of 100 plaques from the initial library and each successive biopanning were amplified in microtitre plates and the lysates cleared by centrifugation. One half microliter of each sample was spotted onto nitrocellulose filters and immunodetection performed using the breast cancer patient serum at 1:20,000 dilution (FIG. 5). Clear enrichment during biopanning is seen as was observed above with the anti-Sirt2 rabbit serum. As seen in FIG. 6 (using randomly picked plaques from BP 4) the filters contacted with the control serum on the left panels demonstrate weaker spot intensity as compared to a duplicate filter of the same clones on the right that was contacted with the patient serum. Approximately 65% of the phage selected for reactivity to the patient's serum were more than 3-fold more reactive with the patient's serum than with the control serum as determined by scanning densitometry.

FIG. 6A shows a comparison of serum reaction of control and breast cancer patient with phagotopes from BP4. FIG. 6B shows the BP4 filters that were scanned and the ratio of the pixel densities plotted in rank order.

This experiment demonstrates that one can differentially detect the epitopes for which the process is selecting, i.e. those bound to protein G-agarose beads in association with antibodies in the patient's serum and not the control serum. Someone skilled in the art can recognize that other solid supports for biopanning could replace the protein-G beads without substantively changing the biopanning process. These data also indicate that the selection is imperfect. Not all of the selected phagotopes are more reactive with the patient's serum than the control serum. Therefore, the identification of the most informative phagotopes requires analysis of the reactivity with multiple, individual patients' sera tested at various serum dilutions.

The immune reactivity to human tumors recognizes changes in the expression levels and mutation status of proteins in the tumor cells. These types of immunological reactivity are not observed in sera from control subjects. The antibody titer to tumor specific epitopes can be proportional to the tumor burden. The immune reactivity to human tumors can be used diagnostically and prognostically to predict the presence and behavior of human tumors such as tumor recurrence. Serum reactivity to single proteins tends to incompletely identify tumor bearing patients and therefore more robust methods are necessary to accurately identify tumor occurrence and recurrence. Whole genome-based proteomics such as the technology and data analysis methods embodied in the application can more comprehensively identify those proteins recognized by the host immune system.

Those of skill in the art are familiar with the construction of cDNA libraries and there are numerous published numerous papers on isolation of cDNAs from human cells in culture using this technology (Chiao, et al., 1992; shin et al., 1993; Buettner et al., 1993; Kim et al., 1996; Deyo et al., 1998; Bauer et al 1998). cDNA libraries can be prepared from ovarian cancer cell lines or from ovarian tumor tissue. Tumor tissue cDNA library can be prepared from a pool of mRNA preparations from each of the different stages of cancer to increase the diversity of clones in the library.

Methods

mRNA from one ovarian cancer cell line, SKOV3 and ovarian tumor tissues, was copied into cDNA and libraries prepared. Tumor tissue in excess of that needed for pathological evaluation was obtained by informed consent from ovarian cancer patients.

Sera was obtained from 1) ovarian cancer patients at the time of diagnosis and at six month intervals during the follow up physician visits; 2) unaffected women for control sera.

T7 cDNA phage display expression libraries are prepared for biopanning experiments, to select phage bearing epitopes ie phagotopes that are recognized by sera from women with ovarian cancer but not recognized by normal sera from unaffected women. For the biopanning process, sera from women in the control group was pooled to avoid individual variations unrelated to the presence of ovarian cancer.

The selection of the most informative epitopes was done by comparing the immune reaction profile of each individual epitope with templates defined for each disease stage. Several distances and information entropy measures were used. Several predictors were constructed based on three selected machine learning techniques using only a part of the available data. Specificity, sensitivity, positive predicted value and negative predicted value were calculated for each such classifier. The validation of the predictors and the selection of the best predictor was done by cross-validation on cases that have not been used during the predictor construction.

For example, to develop an effective screening test for early detection of ovarian cancer, cDNA phage display libraries were used to isolate cDNAs coding for epitopes reacting with antibodies present in the sera of patients with ovarian cancer. Screening of T7 phage cDNA library with serum containing polyclonal antibodies against a known protein, leads to the enrichment of one particular phage clone (which displays the peptide sequence recognized by the antibody on its coat) after several rounds of biopanning. Serum containing polyclonal antibodies were raised against a C-terminal 12 amino acid peptide from the human homologue of the yeast SIRT2 protein and screened against a T7 phage human brain cDNA library. This library was used because the Sirt2 transcript is expressed in human brain. Preimmune rabbit serum was bound to protein-G agarose beads and 6×10^{10} phage were added to the beads. The unbound phage were then bound to protein-G agarose beads to which the Sirt2p antibody was previously bound. The nonspecifically bound phage were washed away with PBS and the specifically bound phage eluted with 1% SDS. T7 phage is stable in this solution. These phage are diluted to reduce the SDS concentration and used to infect bacteria for amplification and another cycle of biopanning. Table 1 shows the value of the titer of the T7 phage library after each cycle of biopanning. This table reveals that the titer of the eluate after each round of biopanning increased with each successive cycle of antibody selection.

E. coli BLT5615 infected with amplified phage library after biopanning 1-4 were plated onto LB-Agar plates and plaque lifts were performed for all the individual plates. The plaque lift filter membranes were then hybridized with a P^{32} -labeled Sirt2 cDNA probe. The percentage of positive plaques (number of positive plaques/total number of plaques $\times 100$) as determined for each plates labeled BP1-4, FIG. 1 increased with each successive cycle of biopanning. For BP1 and BP2 the percentage of positive plaques was negligible. For BP3 and BP4, percentage of positive plaques was 1.7% and 8.6% respectively.

In order to confirm that those positive plaques contain phage clones displaying the peptide sequence of Sirt2, 50 plaques were randomly picked up and PCR amplified each insert using T7 coat protein forward primer (5'TCTTCGC-CCAGMGCTGCAG3' (SEQ ID NO: 1)) and T7 coat protein reverse primer (5'CCTCCTTTCAGCAAAAACCCC3' (SEQ ID NO: 2)). Filter hybridization was performed using the same Sirt2 cDNA probe as above. As shown in FIG. 2, 7 out of 50 plaques (14%) hybridized to the Sirt2 probe, a

frequency similar to that observed in the plaque lifts. Plaques positively reacting with the Sirt2 probe were picked and also hybridized on Southern Blots of PCR product.

Sirt2 positive plaques (upper two rows) and Sirt2-negative plaques (lower two rows) were chosen and 1 μ l (pfu indicated at left) of each amplified phage clone was spotted onto the nitrocellulose membranes which were then treated as if they were standard immunoblots using the rabbit polyclonal Sirt2 antibody (right panel) or a mouse monoclonal antibody to the T7 capsid protein (left panel). The rabbit polyclonal antibody provides a sample for testing as if it were a patient's serum using the Sirt2 protein as a model. The Sirt2 antibody in the rabbit polyclonal serum reacted specifically with the Sirt2 phage. The identity of the phage was confirmed by direct PCR sequence analysis of the cDNA inserts in two independent Sirt2 positive phage. Thus phage expressing the epitope to which the antiserum was directed were isolated and distinguished from other phage.

Microarrays were spotted using Sirt2 T7 clones and other T7 clones that do not express Sirt2. These arrays were used to analyze a mixture of Cy5-labeled (red) rabbit Sirt2-immunized serum and Cy3-labeled (green) T7 coat protein antibody (Novagen) added to the pre-immune rabbit serum. The scanned two-color image clearly shows specific detection of the Sirt2-expressing T7 clones by the anti-Sirt2 antibody. The Sirt2 expressing clones appear yellow because they bind both the red-labeled antibody to a rabbit immunoglobulin G protein and the green-labeled anti-T7 capsid 10B antibody. The non-Sirt2-expressing T7 clone are green as they only bind to the Cy3-labeled anti-T7 antibody. This development of detection of protein epitopes in bacteriophage bodies well for the applicability of phage arrays to the detection of low abundance species and weak binders. The spots in the image are approximately 100 microns in diameter.

The following is an example of the preparation of a tumor reactive cDNA expression library: Ovarian cancer cells were grown in monolayer culture. Cells or fresh tumors from patients were lysed by the addition of 3 ml of TRIZOL reagent and the homogenized sample was incubated for five minutes at room temperature. Chloroform, 0.6 ml, was added and the mixture was shaken vigorously for 15 seconds and then incubated at room temperature for 2-3 minutes. The extract was centrifuged at 12,000 $\times$ g for 30 minutes at 4° C. Following centrifugation, the mixture was separated a lower red, phenolchloroform phase, an interphase, and a colorless aqueous phase. Aqueous phase was transferred to a fresh tube and total RNA was precipitated by adding 1.5 ml of isopropanol. The mixture was incubated at room temperature for ten minutes and was centrifuged at 12,000 g for 30 minutes at 4° C. The supernatant was discarded and the RNA pellets were washed by adding 3 ml of 75% ethanol. The samples were centrifuged at 14,000 $\times$ g for 15 minutes. The RNA pellet was air-dried and was dissolved in RNase-free water.

mRNA was isolated from total RNA following Oligotex mRNA spin column protocol. Total RNA, 0.5 mg, was dissolved in 500 μ l of RNase-free water and 500 μ l of binding buffer and 30 μ l of Oligotex suspension was added. The contents were mixed thoroughly, incubated for three minutes at 70° C. in a water-bath, and then at room temperature for 10 minutes. The Oligotex:mRNA complex was pelleted by centrifugation for 2 minutes at 14,000 $\times$ g and the supernatant was discarded. The Oligotex:mRNA pellet was resuspended in 400 μ l washing buffer by vortexing and pipetted onto a spin column placed in a 1.5 ml microcentrifuge tube. The samples were centrifuged at maximum speed for one minute and the flow-through discarded. The spin column was transferred to a new RNase-free 1.5 ml microcentrifuge tube. Elution buffer

at 70° C. was then added to the column. Poly (A)⁺ mRNA was eluted, quantitated by UV spectroscopy and the process of poly A selection repeated one more time to further reduce contamination with ribosomal RNA. Twice poly A selected mRNA was stored at -70° C. for use in library preparation.

Novagen's OrientExpress cDNA Synthesis and Cloning systems were used for the construction of ovarian cancer cDNA T7 phage libraries. For first-strand cDNA synthesis, OrientExpress Random Primer System was used to ensure representation of both N-terminal and C-terminal amino acid sequences.

Ten ml of LB/carbenicillin medium were inoculated with a single colony of BLT5615 from a freshly streaked plate. The mixture was shaken at 37° C. overnight. Ten ml of the overnight culture was added to 90 ml of LB/carbenicillin medium and was allowed to grow until OD₆₀₀ reaches 0.4-0.5. IPTG (1 mM), M9 salts (1 $\times$) and glucose (0.4%) can be added and the cells were allowed to grow for 20 minutes. An appropriate volume of culture was infected with phage library at MOI of 0.001-0.01 (100-1000 cells for each pfu). The infected bacteria were incubated with shaking at 37° C. for one to two hours until lysis is observed. Glycerol (0.02%), PMSF (0.02M) was added to the cell lysate to block proteolysis of the capsid fusion proteins. The phage were centrifuged at 8000 $\times$ g for 10 minutes. The supernatant was collected and was stored at 4° C. The lysate was titered by plaque assay under standard conditions. The libraries are stored after purification by polyethylene-glycol precipitation and ultracentrifugation through a stepwise CsCl gradient.

Using this approach, applicants have constructed the first library. Using twice poly A selected mRNA from SKOV3 cells a T7 select cDNA library was prepared containing 1.8 $\times$ 10⁷ initial plaques after packaging. This representation is comparable to the clonal representation of the commercial libraries purchased. This library has been amplified and stored in aliquots in two -70° C. freezers.

Patients' sera were obtained from multiple institutions for this project. Three outside institutions have agreed to provide ovarian cancer patient sera and the associated medical record information in anonymized form. Dr. Steven Witkin from the Weill Medical College of Cornell University provided 46 patient serum samples and 27 controls. Dr. Karen Lu from the M. D. Anderson Cancer Center can provide 60 serum samples. Dr. David Fishman from the Northwestern University Comprehensive Cancer Center provided 35 serum samples of patients who have been followed from time of diagnosis.

The ideal sera for the clone biopanning studies come from women just before or after surgery and prior to chemotherapy. Follow up sera were obtained after chemotherapy and are important to determine whether the penultimate protein array technology can detect tumor recurrence.

In addition, a supply of tumor tissue was required for the preparation of mRNA for cDNA library production and gene expression studies using samples from patients. This tissue was harvested within 20 minutes of surgical excision from the patients. This requires the coordinated effort of the gynecologic surgeons and pathologists. Patients at the time of their original surgery or prior to chemotherapy were accrued for serum collection. If tumor tissue is available in excess of that needed for routine pathologic evaluation, that tissue was used for RNA preparation for mRNA expression studies associated with this study. Sections from tissue blocks were also acquired for the purpose of expression studies of proteins in the patients' tumors. Patients at follow up visits to the OB/GYN clinics were also subjects for serum acquisition. These latter patients can be at a time of recurrence or not. This

allows the observation of the reappearance of serum markers in the event of tumor recurrence. Serum was obtained from eligible patient-subjects during scheduled clinic visits. The initial serum acquisition occurs prior to surgery, if possible, or if post surgery, prior to chemotherapy. A single red top 7 cc vial of blood was obtained during normal phlebotomy and the serum isolated after clotting. Serum continues to be collected from these patients during follow up visits for up to five years or until ovarian cancer recurrence. Tumor tissue in excess of that required for pathological analyses were acquired at the time of surgery for the preparation of tumor RNA needed for antibody screening. Unaffected volunteers (controls) were be recruited through community outreach activities.

The Biopanning Process

Steps in the Biopanning Process:

Affinity selection with sera from normal individuals: Twenty-five μ l of Protein G Plus-agarose beads were taken in 0.6 μ l eppendorf tube and were washed two times with 1 $\times$ PBS. Washed beads were blocked with 1% BSA at 4° C. for one hour. The beads were then incubated at 4° C. for one hour with 250 μ l of pooled sera at a dilution 1:20 from 20 control women. After three hours of incubation, beads were washed three times with 1 $\times$ PBS and then incubated with phage library ($\sim 10^{10}$ phage particles). After incubation, the mixture was centrifuged at 3000 rpm for two minutes to remove phage nonspecifically bound to the beads and the supernatant (phage library) was collected for immunoscreening.

Fresh protein G Plus agarose beads were placed into a 0.6 ml eppendorf tube and were washed two times with 1 $\times$ PBS. Washed beads were blocked with 1% BSA at 4° C. for one hour. The beads were then incubated at 4° C. for three hours with 250 μ l of sera at a dilution 1:20 from patients with ovarian cancer. After this incubation, the beads were washed three times with 1 $\times$ PBS and were incubated with phage library supernatant from above (termed as Biopanning 1 (BP1)) collected for immunoscreening at 4° C. for overnight (shorter times of incubation have not proven successful using model antibody systems). After incubation, the mixture was centrifuged at 3000 rpm for two minutes and supernatant can be discarded. Beads were washed three times with 1 $\times$ PBS. To elute the bound phage 1% SDS was added to the washed beads and the mixture was incubated at room temperature for ten minutes. The bound phage were removed from the beads by centrifugation at 8000 rpm for seven minutes. Eluted phage were transferred to liquid culture for amplification (100 μ l elution to 20 ml culture). Four rounds of affinity selection and immunoscreening was carried out with amplified phage obtained after each biopanning. The number of biopanning cycles generally determines the extent of the enrichment for phage that binds to the sera of patient with ovarian cancer. This process allows for one cycle of biopanning to be performed in a single day.

In the past serum markers have been identified using SEREX technology that detected only a few gene products at a time. The biopanning approach developed can isolate large numbers of target epitopes. These epitopes are displayed on the surface of bacteriophage as in-frame fusion proteins with the T7 phage capsid protein and can be analyzed in large numbers by arraying the selected phage on filter paper or glass slides (protein microarrays). The method isolates large numbers of phage that react with antibodies from pooled patient sera but not with normal sera.

The titer of the T7 phage library obtained after amplification of each Biopanning (BP1-4) eluate was determined by plaque assay. *E. Coli* BLT 5616 were infected with the primary unamplified phage from biopanning (BP3-4) and plaged to limiting dilution onto LB/carbenicillin plates (150

mm $\times$ 15 mm petri dish) so that sufficient numbers of single plaques can be isolated to obtain 12 $\times$ 96 well plates for arraying. The plates were incubated at 37° C. for 3-4 hours until the plaques are visible and then picked for amplification in the 12 $\times$ 96 well plates. After two hours, lysis of the host bacteria occurs in the wells of the 96-well plates. One well of each plate was uninfected as a control. Five 96 well plates of 200 μ l phage lysates are clarified by centrifugation of the phage. The phage were cleared by whole plate centrifugation before robotic spotting in triplicate onto filters or glass slides. Excess reactivity in the surface area of the slide not spotted with phage is blocked using BSA, 1% solution in PBS for 60 minutes, followed by washing in water three times. After blocking the arrays on glass slides or filters were blocked with 1% BSA in PBS and incubated with a various dilutions of each of the individual controls and patient's sera spotted in triplicate or more for each dilution of serum. Serum antibodies binding to recombinant proteins expressed in the surface of the T7 bacteriophage were detected by incubation a Cy5-labeled anti-human IgG goat antiserum and visualized and quantified using GenePix and ImaGene software in a 4000B array scanner (AXON Instrument). As positive control for each spot a Cy3-labeled antibody for the T7 capsid protein was used. The ratio of the fluorescence intensity for the human antibodies were normalized to the T7 capsid antibody reactivity. Initial testing of phage solutions were performed on a spotting robot.

The optimal number of subtractive biopannings for each serum sample is determined by picking individual phage clones, and then testing the antibody reactivity for the serum used in the biopanning against those clones, (referred to as its self reaction). Plates of 96 clones were picked for each patient's biopanning at cycles 3, 4, and 5 which were then tested for the binding of the phage clones to antibodies in that serum, in a "self-reaction". Antibody binding is detected by spotting the filters with a 96 pin head on a Biomek robot or detected on glass slides of microarrays of phagotopes. The filters are then treated like a western blot by blocking with 1% dry milk powder in PBS and adding diluted serum. After rocking for 2 hours the filter is washed and reacted with an anti-human IgG antibody link to horseradish peroxidase (HRP) and detected by ECL. From the clones isolated from one patient, (designated patient #1) a total of 480 plaques were picked from that serum at biopanning 4. Biopanning four was chosen because about 35% of the clones bound antibodies from that patient's serum. Serum reactivity of the phagotopes with the patient's serum was detected at a 1:10,000 dilution indicating a very high titer of the IgG molecules that react with the epitopes (self reaction with 480 clones). Reactivity to these clones is detected at similar dilutions using the clones arrayed on glass slides as an alternative solid support.

When the serum reactivity with other patients (non-self reactions) was analyzed using replicates of the robotically spotted filters, reactivity was found in some patients again at a dilution of 1:10,000 (FIG. 1b). Other patients required a 1:3000 dilution of the serum for detection of the reactive clones Table 1). Patients #23 reacted quite strongly while patient #16 reacted more weakly (FIG. 1b and Table 1). Positivity was scored only when 3 out of 3 of the triplicates have similar intensity. In the subtractive biopanning scheme plaques binding to normal serum proteins nonspecifically were removed by loading protein-G beads with a pool of control sera. One can detect positive reaction on filters spotted with phage epitope clones on filter 13 of 21 other patients using 153 reactive clones of the original 480 clones. Filters were tested with control sera not used in the initial subtractive

step, and 5 of the 8 controls showed no reaction to the 480 phage on the filter arrays while a non-specific and even pattern of reactivity to all clones (without the typical triplicate pattern) was observed using 3 of the 8 different control sera (Table 1).

Patient's sera	# of phage Patient #1 BP4 clones reacted with each patient's sera at indicated dilution	
	1:10000	1:3000
PATIENT 1	153 (self reaction)	
PATIENT 2	None	142
PATIENT 16	NS	
PATIENT 20	70	
PATIENT 23	137	
PATIENT 29	NS	
PATIENT 30	NS	
PATIENT 33	NS	
PATIENT 35	NS	72
PATIENT 37	None	120
PATIENT 01-056	NS	
PATIENT 01-060	None	61
PATIENT 00-007	NS	
PATIENT 01-108	NS	
PATIENT 01-045	NS	
PATIENT 42501	40	
PATIENT 400162	120	
PATIENT 40036	Mostly NS	
PATIENT 42780	85	
PATIENT B755	NS	
PATIENT 40015	NS	
PATIENT 075	119	
PATIENT 015	155	
PATIENT 035	NS	
PATIENT 007	114	
PATIENT 005	133	
PATIENT 083	150	
PATIENT 054	92	
PATIENT 064	NS	
PATIENT 065	NS	

NS indicates Non-Specific reaction only:
None indicates No reaction detected.

The filter arrays are incubated with a patient's serum (pre-treated with 150 µg of bacterial extract to block nonspecific reactions with *E. coli* proteins for 2 hours at 4° C.) at various dilutions for 1 hour at room temperature. Bacterial extracts are used because some patients have antibodies to bacterial protein, and therefore pre-treatment with extracts of *E. coli* proteins blocks the nonspecific antibodies to bacterial protein present in the patient's serum. The membranes are then washed three times with TBST (0.24% Tris, 0.8% NaCl, and 1% Tween-20) for 15 minutes each. After washing is completed, the membranes are incubated with secondary antibody, goat-anti human IgG-HRP conjugated (Pierce) at 1:5000 dilution for 1 hour at room temperature. The membranes are again washed three times with TBST 15 minutes each. Finally, membranes are developed with Supersignal West Pico chemiluminescent substrate (Pierce) and the images were captured on a Kodak film.

Phagotome Microarrays on Glass Biochips Preparation of Arrays

Phage lysates are prepared as above. Phage lysates (usually five 96 well plates) from BP4 are transferred to 384-well plates, each lysate spotted in quadruplicate, using 10 µl per well. A robotic microarrayer is used to spot the phage in an ordered array onto FAST™ slides (Schleicher & Schuell) at a 350 µm spacing using 4 steel Micro-Spotting Pins. The arrays are dried overnight at room temperature.

Preparation of Fluorescent Antibody Probes

T7 monoclonal antibody and goat anti-human IgG are purchased from Novagen and Pierce respectively. Monofunctional NHS-ester activated Cy3 and Cy5 dyes are purchased from Amersham (PA33001 and PA35001). The antibodies are labeled in pH 8.0 sodium carbonate buffer as per the instructions from the manufacturer. Briefly, 100 µl of the protein solution with 5 µl of coupling buffer is transferred to the vial of reactive dye and mixed thoroughly. The reaction is incubated in the dark at room temperature for 30 minutes with additional mixing approximately every 10 minutes. The reaction solutions are then loaded into the gel filtration columns to separate the labeled protein from non-conjugated dye. T7 antibody is labeled by Cy3 and anti-human IgG is labeled by Cy5, respectively. The labeled protein is eluted and stored at 4° C. for future use. Reversing the dye-labeling scheme of the antibodies does not affect the results. The advantage of this strategy is that the same reagents were used on every phagotome array and the only variable is the patient's serum and therefore variations in labeling efficiency are not a factor.

Detection of Fluorescent Antibody Probes

The arrays are rinsed briefly in a 1% BSA/PBS to remove unbound phage, transferred immediately to 1% BSA/PBS as a blocking solution, and then incubated in this blocking solution for 1 hour at room temperature. The excess BSA is rinsed off from the slides using PBS. Without allowing the array to dry, 2 ml of PBS containing human serum at a dilution of 1:10,000 is applied to the surface in a screw-top slide hybridization tube. Multiple dilutions are tested per patient to obtain optimal detection. The arrays are incubated at room temperature for 1 hour with mixing. The arrays are rinsed in PBS to remove the serum, and then washed gently three times in PBS/0.1% Tween-20 solution 10 minutes each. All washes are performed at room temperature. After removing Tween-20 using PBS, the arrays are incubated with 2 ml of PBS containing Cy3-labeled-T7 anti-capsid antibody at a dilution of 1:50,000 and anti-human IgG labeled with Cy5 at a dilution of 1:10,000 as probes for 1 hour in the dark. The incubation solution is mixed every 20 minutes. Three washes are performed using PBS/0.1% Tween-20 solution with 10 minutes each. The array is then rinsed with filtered ddH₂O twice and dried using a stream of compressed air.

Analysis Phagotome Microarrays

The arrays are scanned in an Axon Laboratories scanner (Axon Laboratories, Palo Alto, Calif.) using 532 nm and 635 nm lasers. The ratio of anti-T7 capsid and anti-human IgG is determined by comparing the fluorescence intensities in the Cy3- and Cy5-specific channels at each spot. The location of each spot on the array is outlined using the image processing software. The background, calculated as the median of pixel intensities from the local area around each spot, is subtracted from the average pixel intensity within each spot. This normalized reactivity is entered into a database for analysis.

The information in this database can be analyzed in order to: i) select the most informative epitopes and ii) develop into a diagnostic test for tumor occurrence in high-risk women or tumor recurrence in women previously treated for ovarian cancer. The gene products thusly identified can provide insight into molecular changes recognized by the host immune system.

The human antibodies reacting at each spot are detected with Cy5-labeled human serum antibodies. The normalization of the fluorescence at each spot is compared to a reaction with a Cy3-labeled antibody to the T7 phage capsid protein. Only a small fraction of the phage capsid protein is substituted with the in-frame fusion of the human cDNAs of the library. The majority of the capsid protein is produced by the

host bacterium from an episomic T7-capsid gene. Therefore the majority of the each capsid protein is wild-type and can react with the anti-capsid antibody. An example of a Cy5 labeled anti-human IgG reacting with IgG in patients #1 serum bound to clones biopanned using patient #1 serum is shown in FIG. 6c.

The data analysis proceeds according to the following steps:

4. Pre-processing and normalization.
5. Identifying the most informative markers
6. Building a predictor for molecular diagnosis of ovarian cancer and validating the results.

The pre-processing and normalization step is used for arrays using two channels such as Cy5 for the human IgG and Cy3 for the T7 control. The spots are segmented and the mean intensity is calculated for each spot. A mean intensity value is calculated for the background, as well. A background corrected value is calculated by subtracting the background from the signal. If necessary, non-linear dye effects can be eliminated by performing an exponential normalization (Houts, 2000) and/or a piece-wise linear normalization of the data obtained in the first round. The exponential normalization can be done by calculating the log ratio of all spots (excluding control spots or spots flagged for bad quality) and fitting an exponential decay to the $\log(\text{Cy3/Cy5})$ vs. $\log(\text{Cy5})$ curve. The curve fitted is of the form:

$$y = a + b * \exp(-cx)$$

where a, b and c are the parameters to be calculated during curve fitting. Once the curve is fitted, the values are normalized by subtracting the fitted log ratio from the observed log ratio.

This normalization has been shown to obtain good results for cDNA microarrays but it relies on the hypothesis that the dye effect can be described by an exponential curve. The piece-wise linear normalization can be done by dividing the range of measured expression values into small intervals, calculating a curve of average expression values for each such interval and correcting that curve using piece-wise linear functions.

The values coming from each channel are subsequently divided by the mean of the intensities over the whole array. Subsequently, the ratio between the IgG and the T7 channels was calculated. The values coming from replicate spots (spots printed in quadruplicates) are combined by calculating mean and standard deviation. Outliers (outside $\pm$ two standard deviations) are flagged for manual inspection). Single channel arrays are pre-processed in a similar way but without taking the ratios. This preprocessing sequence was shown to provide good results for all preliminary data analyzed.

The step of selecting the most informative markers is used to identify the most informative phages out of the large set of phages started with. The better the selection, the better is the expected accuracy of the diagnosis tool.

A first test (Procedure 1 disclosed above) is necessary to determine whether a specific epitope is suitable for inclusion in the final set to be spotted.

Procedure 2 is used to maximize the information content of the set of epitopes while trying to minimize the number of epitopes used using the following procedure.

The arrays used in this example, (using two channels such as Cy5 for the human IgG and Cy3 for the T7 control) are processed as follows. The spots are segmented and the mean intensity is calculated for each spot. A mean intensity value is calculated for the background, as well. A background corrected value is calculated by subtracting the background from the signal. The values coming from each channel are normal-

ized by dividing by their mean. Subsequently, the ratio between the IgG and the T7 channels are calculated and a logarithmic function is applied. The values coming from replicate spots (spots printed in quadruplicates) are combined by calculating mean and standard deviation. Outliers (outside $\pm$ two standard deviations) are flagged for manual inspection.

The histogram of the average log ratio is calculated. If the histogram is unimodal (e.g. subject 19218 in FIG. 13), there is no specific response. If the histogram is clearly bimodal (e.g. subject 19223 in FIG. 13), there is a specific response. All 25 subjects analyzed so far fell in one of these two categories or had no response at all. The preliminary data analyzed so far showed a very good separation of the distributions for the patients.

Once the chosen clones are spotted on the final version of the array, a number of sera coming from both patients and controls can be tested. These sera come from subjects not used in any of the phases that lead to the fabrication of the array (i.e. not involved in clone selection, not used as controls, etc.). Each test was evaluated using Procedure 3 above.

Building the Predictor

A number of machine learning and statistical techniques have been considered for this task. The following algorithms were tested: CN2 (Clark, 1989), C4.5 (Quinlan, 1993; Breiman et al., 1984), CLEF (1998), 4.5 using classification rules (Quinlan, 1993), incremental decision tree induction (ITI) (Utgoff, 1989; quantization (LVQ) (Kohonen, 1988; Kohonen, 1995), induction of oblique trees (OC1) (Health and Salzberg, 1993; Murthy, 1993), Nevada backpropagation (NEVP); Rumelhart et al., 1987), Constraint Based Decomposition (Draghici, 2001), k-nearest neighbors with $k=5$ (K5), Q* and RBF's (Musavi et al., 1992; Poggio and Girosi, 1990).

The generalization abilities and the reliability of these techniques have been tested extensively on various problems and data sets from the UCI machine learning repository (Blake et al., 1998). This repository contains a large collection of mostly real world data from a large variety of domains (including biological and medical), and constitutes a benchmark on which various algorithms and techniques can be tested.

Table 2 presents the accuracies obtained by these techniques on the selected problems. Table 3 presents the standard deviation of each such algorithm on the same problems. Based on these tests applicant decided to start the tests by using constraint based decomposition (CBD), radial basis functions (RBFs) and decision trees (C4.5) as the three main candidates. The CBD was selected because it offers a high reliability across multiple trials (lowest standard deviation) and a good accuracy (second best). Furthermore, the CBD algorithm can also produce a logical expression describing the classifier produced. Such expressions allow one to understand the relative importance of various epitopes. The decision trees have been selected mainly because they can be mapped into logical expressions that can be compared to the one produced by the CBD. RBFs construct clusters by placing high dimensionality Gaussian functions on groups of given data points (one data point can be a set of expression values corresponding to a protein chip). This technique calculates automatically the number of clusters, their orientation (the eigenvectors of the correlation matrix of the expression vectors) and their widths. RBFs were expected to perform much better than k-means clustering and the other techniques already used in this context because RBFs avoid guessing (e.g. k in k-means clustering). Furthermore, extracting a

model from the trained RBF architecture is straightforward. Again, this model can be compared with the models provided by the CBD and C4.5

reducing the size of the training set even further by setting patterns aside for generalization testing could jeopardize the training. In such cases, the algorithm is used with only $n-1$ of

DATA-SET	C4.5	C4.5r	ITI	LMDT	CN2	LVQ	OC1	NEVP	K5	Q*	RBF	CBD
GLASS	70.23	67.96	67.49	60.59	70.23	60.69	57.72	44.08	69.09	74.78	69.54	68.37
IONO-SPHERE	91.56	91.82	93.65	86.89	90.98	88.58	88.29	83.8	85.91	89.7	87.6	88.17
LUNG	40.17	39.84	38.47	55.49	37.17	55.71	54.28	33.12	68.54	60	65.7	60
CANCER												
WINE	91.09	91.9	91.09	95.4	91.09	68.9	87.31	95.41	69.49	74.35	67.87	94.44
PIMA	71.02	71.55	73.16	73.51	72.19	71.28	50	68.52	71.37	68.5	70.57	68.72
INDIANS												
BUPA	65.14	65.39	63	71.54	64.31	64.13	65.57	77.72	66.43	61.43	59.85	62.32
TICTAC	83.52	99.17	92.89	89.61	98.18	65.61	78.56	96.91	84.32	65.7	72.19	75.1
TOE												
BALANCE	64.61	75.01	76.76	93.27	80.89	89.54	92.5	91.04	83.96	69.21	89.06	90.08
IRIS	91.6	91.58	91.25	95.45	91.92	92.55	93.89	90.34	91.94	92.1	85.64	96
ZOO	90.27	90	90.93	96.61	91.91	91.42	66.68	92.86	67.64	74.94	X	94.29
AVG	75.92	78.42	77.87	81.84	78.89	74.84	73.48	77.38	75.87	73.07	74.22	79.75

Table 2 shows a comparison of several classification techniques. The table presents the accuracies obtained in various problems from the UCI machine learning repository. Each accuracy is the average of 10 trials.

the n available patterns and tested on the remaining one. This is done n times, each time leaving out a different pattern. An average is calculated over the n experiments. This is known as the leave-one-out method. If more patterns are available, the

DATA-SET	C4.5	C4.5r	ITI	LMDT	CN2	LVQ	OC1	NEVP	K5	Q*	RBF	CBD
GLASS	7.23	6.28	7.96	11.25	8.34	10.24	9.1	6.29	7.81	6.98	7.35	2.08
IONO-SPHERE	2.82	2.58	2.71	3.51	3.29	3.36	2.21	3.81	4.14	4.7	6.45	2.56
LUNG	14.2	18.92	13.52	32.2	13.79	12.48	17.53	14.83	11.96	18.6	16.27	12.6
CANCER												
WINE	5.84	5.09	6.24	5.22	6.11	4.84	8.45	2.22	6.86	6.64	5.16	1.96
PIMA	2.1	3.92	2.16	4.3	2.36	4.46	22.4	3.19	3.67	8.19	2.39	3.02
INDIANS												
BUPA	5.74	6.05	4.23	6.63	7.99	7.14	8.45	11.97	7.22	4.25	7.92	2.05
TICTAC	2.44	1.05	2.38	8.79	0.95	2.99	5.88	1.32	2.7	3.16	3.35	9.43
BALANCE	3.35	3.98	3	2.95	3.38	4.39	2.07	7.12	7.53	19.09	2.38	3.03
IRIS	5.09	5.09	4.81	4.71	5.95	3.73	4.68	7.45	4.1	5.28	27.37	4.35
ZOO	7.59	7.24	6.11	1.56	5.95	6.26	30.36	4.62	20.03	23.8	X	2.13
AVG	5.64	6.02	5.312	8.112	5.811	5.989	11.11	6.282	7.602	10.07	8.738	4.321

Table 3 shows a comparison of several classification techniques. The table presents the standard deviations obtained in a set of 10 trials on various problems from the UCI machine learning repository.

Furthermore, one can also implement and try the predictors used in (Golub et al., 1999) and (Alizadeh et al., 2000) which were shown to work well in cancer diagnosis problems similar to applicant's. The selection of the final predictor was based on the validation results obtained in the last step of the data analysis.

Validating the Predictor

In order to validate the predictors, the classical method of cross-validation was used (Breiman et al., 1984). The idea behind cross-validation is that the predictor is tested, not based on its abilities to simply memorize the data presented during the training, but based on its abilities to generalize the knowledge acquired during the training to previously unseen cases. For this reason, the predictor must be checked on data that belongs to the same distribution but was not used during the training. This can be implemented in several ways depending on the number of examples available. If only few examples (such as stage I patients, ~40 total) are available,

pattern set can be divided into n different subsets of patterns. Then one subset can be left out of the training and used to test the generalization. Again, the value reported is an average of the n trials performed leaving out each of the n subsets. This method is known as n -fold cross validation. Finally, if the pattern set is very large (patients with stage III or IV cancer), it can simply be divided into a training set and a validation set. In this case, the generalization abilities of the technique can be characterized by its performance on the validation set.

For each predictor the specificity, sensitivity, positive predictive value and negative predicted value can be calculated using cross-validation data (i.e. values that have not been used in constructing the predictor itself). This ensures that the quality measures obtained in this study reflect the real world performance to be expected in the field.

Once informative phagotopes are found the gene encoding the phagotope was identified.

1. Identification Genes Encoding the Phagotopes.

Phage clones specifically reacting with patient sera, as determined by microarray immunoscreening, can be amplified by PCR using T7 capsid forward and reverse primers. PCR fragments were purified and 100 ng of fragment was

analyzed to determine the nucleotide sequence of the cDNA insets. Sequence alignments are performed using BLAST software and GenBank databases. The sequence information can be used in several ways. Initially, the DNA sequence information provides a database of the frequency of reactivity to a particular epitope.

Diagnostic Markers Derived from the Combined Processes Including Biopanning, Assay of Patients' Sera with Epitopes on Filters and Biochips, and Identifying the Best Predictor/Marker of Disease.

DNA Sequence Analysis of Phagotome Clones

PCR amplified DNA sequences from 96 phagotopes that reacted with patient #1 and at least one other OVCA serum are shown in the table below. Some clones were isolated multiple times and one clone was represented 23 times out of the 96 clones analyzed. This was the human homologue of the oncogenic gene Bmi-1, (GenBank NM005180.1) that inhibits the expression of p14ARF and cooperates with c-myc (Lindstrom et al., 2001). The insert sizes for the Bmi-1 phage clones varied in coding capacity depending on the isolate between 67-94 amino acids in length. Eight other clones were represented twice and one was isolated three times. One of these genes isolated twice was the heat shock protein 70, which has been shown to be overexpressed and antigenic in ovarian cancer tumors and was found to have been identified in the SEREX database 5 times. The size of the open reading frame in the HSP70 clone is 109 amino acids in length. Another clone isolated two times of the 96 sequenced is a known cancer antigen called RCAS1 which is overexpressed in 58% of ovarian cancer and many others as well (Sonoda et al., 1996) RCAS1 is an estrogen regulated gene which can inhibit the immune system from killing a tumor (Nakashima et al., 1999). This information clearly indicates that this technology is capable of detecting cancer antigens that can be used for diagnostic and immunotherapy purposes. If overbiopanning occurred, only a few different clones would be found. However, as the remaining clones were isolated once each, it is therefore convincing that 4-5 biopannings is appropriate. In this first group of 480 clones there were isolated clones that reacted with approximately 60% of the OVCA patients using the macroarray filters and more efficiently using the microarray technology. Additional epitope clones provide additional sensitivity for this assay.

Clone Name	GenBank ID
Clone found 23 times Bmi-1 (oncogene)	NM_005180.1
Clones found 2-3 times	
HSP-70	XM_050984.1
RCAS1 (EBAG9)	BC005249.1
A-kinase anchoring protein 220	XM_038666.1
G-protein gamma-12 subunit	NM_018841.1
Neuronal apoptosis inhibitory protein 6	AF242431.1
hypothetical protein DC42	XM_028240.1
WD repeat domain 1 (WDR1)	XM_034454.1
zinc finger protein 313	XM_009507.1
54 other clones isolated once each.	

Serum reactivity toward a cellular protein can occur for two possible reasons: 1) expression of a mutated form of the protein by the tumor cells and 2) overexpression of the protein in the tumor cells. Identification of proteins detected by the host immune system in this fashion therefore provides patient-specific information about protein(s) that can be mutated or overexpressed in ovarian cancer. Such information provides insight into the molecular targets and mecha-

nisms giving rise to ovarian cancer. Lastly, the sequences identified using the epitope-biopanning/phage microarray approach can be useful for early detection of cancer occurrence and recurrence by screening patients' sera and peritoneal fluids and providing immunogens for immunotherapy vaccines.

Example 2

A strategy was developed for serological detection of large numbers of antigens indicative of the presence of cancer, thereby using the humoral immune system as a biosensor. The high-throughput selection strategy involved biopanning of an ovarian cancer phage display library using serum immunoglobulins from an ovarian cancer patient as bait. Protein macroarrays containing 480 of these selected antigen clones revealed 44 clones that interacted with immunoglobulins in sera from all (32/32) ovarian cancer patients, but not with sera from either healthy women (0/25) or patients having other benign or malignant gynecological diseases (0/14). An informative subset of 26 antigen clones was chosen based on the criterion that the serum from each of a group of 16 patients interacted with at least one of the clones. When another, independent group of 16 serum samples was used, all 16 samples interacted with one or more of the 26 clones, and none from 12 healthy women. The process of globally profiling disease relevant epitopes is known as "epitomics".

In searching for a method for the early detection of ovarian cancer (OVCA), large numbers of potential diagnostic antibodies were identified and a high-throughput strategy was developed to clone antigen biomarkers. Because antibodies to any single antigen tend to detect only a small fraction of cancer patients, the necessity to screen a large panel of potential antigen markers was recognized. Therefore a differential biopanning technique was used to screen T7 phage display cDNA libraries to isolate cDNAs coding for epitopes binding with antibodies present specifically in the sera of patients with early or late stage ovarian cancer but not with antibodies in the sera of healthy women. Using a single OVCA patient's immunoglobulins (IgG) as bait, there were identified both established and novel antigen biomarkers. Large numbers of cancer-associated antigens can be found by this phage display technique more rapidly than using standard SEREX analysis. This is due to the power of repeated cycles of selective enrichment possible with viable phage display cDNA biopanning, especially when screening is performed with serum containing a complex mixture of low titer of IgGs, compared to the single step screening possible with SEREX, which is biased toward the identification of antigens that can be detected at a relatively high titer of IgGs.

The antigens that were identified through this process have diagnostic value with additional potential for development of therapeutic vaccines or imaging reagents. Since the host immune system can unravel molecular events (overexpression or mutation) critical to the genesis of ovarian cancer, this novel proteomics technology can identify genes with significant mechanistic involvement in the etiology of the disease. Our initial goal is to develop a serum-based test that can detect ovarian epithelial cancer at an early and curable stage.

Methods

Serum Samples. Blood samples from ovarian cancer patients (Stages I-IV) and healthy controls were obtained from the Barbara Ann Karmanos Cancer Institute. Processing of blood to extract serum was performed in the laboratory. Briefly, blood samples were centrifuged at 2500 rpm at 4° C. for 10-15 minutes and supernatant were stored at -70° C. until use.

Construction of T7 phage display cDNA library from ovarian cancer cell line, SKOV3. Isolation of mRNA from total RNA. Ovarian cancer cells were grown in monolayer culture. Total RNA was prepared using trizol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, Calif., USA). Total RNA, 0.5 mg, was used for the purification of Poly(A)⁺ mRNA following the method as suggested by the manufacturer (QIAGEN Inc, Valencia, Calif.). Poly(A)⁺ mRNA was quantitated by UV spectroscopy and the process of poly A selection was repeated once. Twice poly (A) selected mRNA was stored at -70° C. for use in library preparation.

Construction of T7 Phage Display cDNA Library.

Novagen's OrientExpress cDNA Synthesis and Cloning systems were used in the construction of the ovarian cancer T7 phage cDNA libraries (Novagen, cDNA manual, TB247). The OrientExpress Random Primer System was used to achieve orientation—specific cloning between EcoRI and HindIII sites. First and second strand cDNA synthesis were sequentially carried out in the presence of 5-methyl dCTP. After second strand synthesis, the cDNA was treated with T4 DNA polymerase to blunt the ends. The addition of EcoRI/HindIII Directional Linker d(GCTTGMTTCMGC) (SEQ ID NO: 3) at the d(A)_n:d(T)_n end created a HindIII site d(AAGCTT) (SEQ ID NO: 4) in which the two underlined bases were derived from cDNA. The two dT's were provided on the 5' end of each first strand by the HindIII random primer d(TTNNNNNN) (SEQ ID NO: 5). Excess linkers and small cDNAs (<300 bp) were removed by a gel filtration step as described in Novagen's manual TB 247. The digestion of the cDNA with both HindIII and EcoRI thus yielded cDNA molecules ready for directional insertion into EcoRI/HindIII vector T7Select 10-3 arms. After vector ligation and packaging using T7 packaging extracts, the phage were plated to determine the library titer. About 50 phage clones were randomly picked up and PCR was performed with the T7 forward primer (TCTTCGCCCAGAAGCAG) (SEQ ID NO: 6) and T7 reverse primer (CCTCCITTCAGCAAAAACCCC) (SEQ ID NO: 7), in order to determine the insert sizes. The insert size range was found to be between 300 bp-1.5 kb.

Amplification of Packaged Libraries by Liquid Culture Method.

10 ml of LB/carbenicillin medium was inoculated with a single colony of *E. coli* strain BLT5615 from a freshly streaked plate. The mixture was shaken at 37° C. overnight. Five ml of the overnight culture was added to 90 ml of LB/carbenicillin medium and was allowed to grow until the OD₆₀₀ reached 0.4-0.5. After obtaining the appropriate OD, 1 mM Isopropyl-β-D-thiogalacto-pyranoside (IPTG), (1×) M-9 Minimal salts and 0.4% glucose were added and the cells were allowed to grow for 20 minutes. An appropriate volume of culture was infected with phage library at multiplicity of infection (MOI) of 0.001-0.01 (100-1000 cells for each pfu). The infected bacterial culture was incubated with shaking at 37° C. for 1-2 hours until lysis was observed. After lysis, 0.02% glycerol and 0.02M phenyl-methyl sulphonyl fluoride (PMSF) and protease inhibitor cocktail (PIC) were added to the cell lysate to block proteolysis of the capsid fusion proteins. The phage lysate was centrifuged at 8000×g for 10 minutes. The supernatant was collected and stored at 4° C. The lysate was titered by plaque assay under standard conditions. The libraries were stored at -80° C. after purification by polyethylene-glycol precipitation and ultracentrifugation through a cesium chloride step gradient.

Selection of T7 Phage Displayed cDNA Libraries with Human Sera.

Affinity Selection with Sera from Normal Individuals.

Twenty-five μl of Protein G Plus-agarose beads were placed into a 0.6 ml microcentrifuge tube and washed twice with 1× phosphate buffered saline (PBS). The washed beads

were blocked with 1% bovine serum albumin (BSA) at 4° C. for 1 hour and then incubated at 4° C. for 1 hour with 250 μl of pooled sera from 20 healthy women at a 1:20 dilution. After 3 hours of incubation, beads were washed three times with 1×PBS and then incubated with phage library (~10¹⁰ phage particles) made from an ovarian cancer cell line, SKOV3. The mixture was centrifuged at 3000 rpm for 2 minutes to remove phage nonspecifically bound to the beads and the supernatant (phage library) was collected for immunoselection.

Immunoselection of the Phage Mixture with Serum from an Ovarian Cancer Patient.

Protein G Plus agarose beads were placed into a 0.6 ml microcentrifuge tube and washed two times with 1×PBS. The washed beads were blocked with 1% BSA at 4° C. for 1 hour and then incubated at 4° C. with 250 μl of a 1:20 dilution of serum from the ovarian cancer patient, MEC1. After 3 hours, the beads were washed three times with 1×PBS and incubated for immunoselection overnight at 4° C. with the phage library supernatant. After this incubation, the mixture was centrifuged at 3000 rpm for 2 minutes and the supernatant was discarded. The beads were washed three times with 1×PBS and the phage was eluted from the washed beads as per the manufacturers instructions. The bound phage was removed from the beads by centrifugation at 8000 rpm for 8 minutes. Eluted phage (200 μl) were transferred to liquid culture for amplification (100 μl elution to 20 ml culture). Four rounds of affinity selection were carried out on the amplified phage obtained for each series of biopannings. The number of biopanning cycles generally determines the extent of the enrichment for phage that binds to the sera of patient with ovarian cancer. Four other serum samples from ovarian cancer patients were also used for immunoselection of clones. MEC1 gave the strongest binding with its clones and therefore those clones were selected for the remainder of this study.

Macroarray Immunoscreening.

The titer of the T7 phage library obtained after amplification of each Biopanning (BP1-BP4) eluate was determined by plaque assay. *E. coli* BLT5615 was infected with the primary unamplified phage from biopanning (BP1-BP4) and plated to limiting dilution onto LB/carbenicillin plates (150 mm×15 mm petri dish) so that sufficient numbers of single plaques could be isolated to obtain 12×96 well plates for arraying. The plates were incubated at 37° C. for 3-4 hours until the plaques were visible and then picked for amplification in the 96-well plates. Lysis of the host bacteria generally occurred after 2 hours. After bacterial lysis, the plates were centrifuged at 3000 rpm for 20 minutes. The samples from the 96-well plates were arrayed onto a nitrocellulose membrane (Osmonics) using the Beckman Biomek 2000 liquid handling robot. This robot, equipped with a 96-pin printing head spotted the samples contained in 96 well plates onto nitrocellulose membranes. The patterns were printed in a 4×4 configuration. Position A1 contained 16 spots, each representing a phage sample (FIG. 12A). Triplicates were printed from well A1 of each of five different 96 well plates (15 spots) and the 16th spot contained a positive control of diluted human serum used in the 4 corners of the plate only as shown by black arrows (FIG. 12A). After each round of spotting, the pins were washed in 0.1% SDS, sterile water, and then ethanol. After the spotting was completed, nitrocellulose membranes were blocked with 5% non-fat dry milk for 1 hour at room temperature. The membranes were then incubated with a patient's serum (pretreated with 150 μg of bacterial extracts for 2 hours at 4° C.) at a dilution of 1:10000 or 1:3000 for 1 hour at room temperature. Bacterial extract was used because some patients and controls had antibody binding to bacterial

protein(s). The membranes were then washed three times with 0.24% Tris, 0.8% NaCl, 1% Tween-20 (TBST) for 15 minutes each and then incubated with secondary antibody, goat-anti human IgG-HRP conjugated (Pierce, Rockford, Ill., USA) at 1:5000 dilution for 1 hour at room temperature. The membranes were again washed three times with TBST for 15 minutes each, developed with Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill., USA) and the images captured on X-ray film.

Stability of Serum Specimens.

One source of error in the immunodetection on macroarrays could be variability in serum sample preparations or storage. Therefore, a test was performed to determine whether some common handling conditions adversely affect the usefulness of the sera for the assays. For this test, several aliquots of the same serum sample from one ovarian cancer patient were subjected to various treatments; repeated freeze-thaw cycles (10 times), incubation of the blood sample at 37° C. for 72 hours before processing the serum, extended storage at 4° C., treatment at room temperature overnight, and heat treatment at 65° C. for 10 minutes. Freshly thawed serum, processed normally, served as a control. Robotically printed nitrocellulose membranes containing the set of 480 clones were later processed with each of those treated and untreated serum samples.

ELISA Macroarray Analysis.

Forty-four Stage I-IV clones, in triplicate, were arrayed onto a nitrocellulose membrane (Osmonics) using the Beckman Biomek 2000 liquid handling robot. Nitrocellulose membranes were blocked with 5% non-fat dry milk for 1 hour at room temperature and then incubated with patient or control serum (pretreated with 150 µg of bacterial extract for 2 hours at 4° C.) at dilutions of 1:1000, 1:3000, 1:10000 and 1:30000 for 1 hour at room temperature. Immunoreactivity was performed with serum from patients or healthy controls. For one set, the immunoreactivity was also performed with a monoclonal antibody to the N-terminus of the T7 gene 10 protein at dilution 1:10000. This was performed as described in the macroarray immunoscreening. The intensity of each spot was measured using ImaGene software from BioDiscovery Inc, with background subtraction and calculated using the following equation:

$$\text{Intensity Ratio} = \frac{(\text{Mean of Clone}) / (\text{Mean of T7 for 12 replicates of that Clone}) - (\text{Mean of Blank Phage}) / (\text{Mean of T7 for 12 replicates of that Blank Phage})}{1}$$

The Intensity Ratio vs Serum concentration was plotted for each antigen clone.

Sequencing of Phage cDNA Clones.

Individual phage clones were PCR amplified using forward PCR primer 5' GTTCTATCCGCAACGTTATGG 3' (SEQ ID NO: 8) and reverse PCR primer 5' GGAG-GAAAGTCGTTTTTGGGG 3' (SEQ ID NO: 9). PCR products were purified on 1% agarose gels. The bands were excised from gels under UV light and DNA was extracted/purified using a Qiagen gel extraction kit (Qiagen Inc, Valencia, Calif., USA). Fifty ng of each purified PCR product was analyzed using forward Sequencing primer 5' TGCTAAG-GACMCGTTATCG 3' (SEQ ID NO: 10) by Wayne State University DNA Sequencing Core Facility.

Results

Differential Biopanning of T7 Phage cDNA Expression Libraries Employing Sera Obtained from Women with Ovarian Cancer and Healthy Controls

A method of differential biopanning to screen a T7 phage cDNA library prepared from an ovarian cancer cell line, SKOV3, was developed using a late stage ovarian cancer

patient's serum (MEC1) as the bait to isolate tumor-specific antigens. First the library was pre-adsorbed with sera pooled from 20 healthy controls so as to remove the antigen clones binding with common antibodies unrelated to cancer. The resulting phage were then bound to antibodies present in the serum of a cancer patient and the unbound phage removed. This selection procedure was repeated four times, amplifying the phage between cycles of biopanning. Groups of 96 clones were picked from the patient's biopanning at cycles 1, 2, 3 and 4. Amplified phage clones were spotted on nitrocellulose membranes, and useful phage clones were identified by their binding with patient IgG antibodies at a dilution of 1:10000. There was a significant enrichment for phage-bearing epitopes that bound serum IgGs after the fourth round of biopanning. Because about 35% of the selected phage clones interacted with MEC1 serum IgGs after the fourth round of biopanning, further biopanning was not performed to avoid reducing the diversity of phage clones.

Serological Detection of Antigens Using Macroarrays

The utility of such phage display antigen clone sets for the serological detection of cancer is best demonstrated by their interaction with sera from patients other than those used in the selection step. A set of 480 clones from the fourth round of biopanning was robotically spotted on nitrocellulose membranes. The binding of the cloned antigens with the IgGs in patients' sera was analyzed at a dilution of 1:10000. The strong positive interactions observed with the MEC1 serum indicated a relatively high titer of the IgG molecules that bound with the MEC1 clones (FIG. 12A). Several dilutions of the MEC1 serum were previously used for antigen detection and a dilution of 1:10000 produced the cleanest pattern of strong binding. Although 480 clones were identified from the biopanning with MEC1 serum as the bait, not all 480 clones interacted with the MEC1 serum (FIG. 12A). This can be explained by a non-specific interaction between phage clones and the Protein-G+ beads bearing the serum antibodies. When serum IgG-binding with sera from other patients (non-self reaction) was analyzed using replicates of these robotically spotted macroarrays, cross-reactivity was observed in most patients at a dilution of 1:10000 (FIG. 12B-E). Sera from other patients required either a 1:3000 or 1:30000 dilution to detect positive clones. Binding was scored positive only when 3 of the triplicates had similar intensity and when the intensity was significantly higher than the background intensity of other spots within the same patch. Sera from 71 individuals were tested; 10 were from women with early stage OVCA (Stage I and Stage I borderline), 22 from women with late stage OVCA, 14 from women with benign or other gynecological diseases, and 25 from healthy controls. Tumor histology and stage of all the patients' used for the study are listed in Table 4. Late stage patients OVC015 and MEC23 bound more intensely than the Stage I patients 4679 and 4387 (FIG. 12B-E). In the subtractive biopanning scheme, phage epitope clones binding IgGs were isolated in control sera even though these control sera were not used in the initial subtractive biopanning steps. As expected, a fraction of the 480 phage clones on the macroarrays interacted with approximately 10% of the controls. All clones that interacted with the control sera were eliminated from further consideration. One hundred and forty-nine clones interacted with sera from Stage I-IV ovarian cancer patients but with none of the 25 control sera. Forty-four out of 149 clones interacted specifically with these Stage I-IV sera. The remaining 105 clones interacted with sera from women who had benign tumors, endometrial cancers or other gynecological diseases and may represent biomarkers of gynecological sickness. These clones were excluded because these conditions are a common source of

false positive results in CA-125 clinical testing. A matrix summarizing the binding of the 44 Stage I-IV selected antigen clones to sera from patients and controls is shown in Table 5A. The derivation of this matrix was based on an agreement between two observers who analyzed the data independently, with 87% concordance.

Only 2/44 selected clones, 2G4 and 3B12, bound with MEC1 serum IgGs despite the fact that T7 cDNA library was biopanned with MEC1 serum as the bait. A large number of clones interacting with the MEC1 serum were eliminated because they bound with either healthy control or with patients having benign or other gynecological diseases. The best markers are those interacting with the most patients; these include such clones as 2H9 (13/32), 2G2 (13/32), 2B4 (12/32), and 2G4 (12/32) that had the highest frequency of IgG binding with sera from ovarian cancer patients. Three antigens, 2F7/2B4, 5C3/2G4, 2E1/4A3 were found in multiple clones resulting in a panel of 41 markers binding with IgGs in Stage I-IV ovarian cancer sera (Table 5A).

Although 41 antigens interacted with sera from all 32 patients, the number of clones in the set needed to detect all 32 ovarian cancer patients were reduced. The serum set from 32 patients was randomly divided into two groups. The first group (Group 1) consisted of 16 patients and 25 healthy women; and the second group (Group 2) consisted of the other 16 patients and 12 different healthy women. Group 1 was used to select the minimum number of clones necessary to detect all patients. The strategy of clone selection involved ranking of clones in order of decreasing binding with sera from ovarian cancer patients (Table 7A). Next, a combination of clones was selected for binding with IgGs in sera from all of the ovarian cancer patients in the set. Twenty-six clones of Group 1 detected all of the ovarian cancer patients (16/16) (Table 7A); all but one patient's serum bound with more than one of the selected clones. These 26 clones were then tested on sera from Group 2 (16 patients and 12 healthy controls), for antibody binding (Table 7B). Sera from all of the patients in Group 2, (16/16), bound with at least one of these clones and none of the sera from the healthy women (0/12) bound to these clones.

A second group of 21 clones was found to interact with (18/22) late Stage patients' sera but not with sera from early stage patients, with sera from 25 healthy women or with sera from 14 patients with either benign tumors, endometrial cancers or other gynecological diseases (Table 5B). Although 4 late stage patients were not detected by these 21 clones (Table 5B), they were detected by 44 Stage I-IV clones (Table 5A). Among these 21 clones, antigen 2B3 interacted with the greatest number of patients sera (10/22), clone 5A2 with 8/22, clones 2D7 and 2E7 with 5/22 sera. Although these clones did not detect women with early stage ovarian cancer, further analysis may show them to be useful as markers of recurrence.

Stability of Serum Specimen.

An important feature of a test for widespread clinical use is the stability of the analyte in the test sample. To identify any inaccuracy in detecting IgG molecules in this multianalyte assay due to serum sample preparation problems or serum storage, a test of the durability of the serum samples was carried out. Repeated freeze-thaw cycles (10 times), heated to 65° C. for 10 minutes, or left the unprocessed blood at 37° C.

for 72 hours were performed. Only heat treatments of the serum affected the positive signals on the macroarrays, because heat treatment is sufficient to denature immunoglobulins (IgG). Therefore, the complex set of IgG molecules in serum samples are very stable and provide a reliable analyte for clinical studies of diagnostic arrays of cloned antigens.

ELISA Macroarray Analysis.

The set of 44 (Stage I-IV) phage display cDNA clones listed in Table 5A, were printed robotically on nitrocellulose membrane and an enzyme-linked immunosorbent assay (ELISA)-like experiment was performed. For clones 4A11, 2H9, 2G4 and 2F7, the binding of antigens decreased with increasing dilution of serum (FIG. 13A-D). Although clones bound nonspecifically with control sera at high serum concentrations, their binding decreased to zero as the sera were diluted, whereas the interaction of the same clones with IgGs in patients' sera persisted at even 1:10000 serum dilution. This demonstrated that the interaction of antigen clones with patients' sera was indicative of a typical, titerable antigen-antibody interaction.

Phage-Coded Antigen Sequence Analysis

To identify the selected gene products, phage DNAs were amplified by PCR and the cDNA products sequenced. The DNA sequences were checked for homology to the GenBank databases using BLAST. The predicted amino acids in-frame with the T7 gene 10 capsid protein were determined. Eleven sequences were homologous to known gene products while other clones had no homology to any annotated sequences in the public databases (Table 6A). Among the gene products, 11 represented known gene products in the correct orientation and in the correct reading frame with the T7 gene 10 capsid protein indicating that the serum IgG binding region was localized to a portion of the natural open reading frame of the protein. Of the remaining 33 clones, 13 clones contained an open reading frame with the T7 10B gene with a frameshift within the natural reading frame of the gene; 7 clones contained portions of either 5' or 3' untranslated regions of known genes; 13 clones contained segments of genomic sequences. This in turn resulted in the formation of recombinant fusion proteins in which the predicted amino acid of the in-frame fusion with the T7 10B protein was not similar to the original protein coded by the gene. The size of the additional peptide sequences ranged from 5-48 amino acids. This result indicated that the recombinant gene products of these clones must be coding for proteins that mimic some other natural antigens, and hence can be termed mimotopes (Table 6A). BLASTp search of the SWISSPROT database for homology to each in-frame mimotope confirmed this observation. For example, clone 2H5 contained a nucleotide sequence homologous to the ATP synthase, H⁺ transporter. Using BLASTp, there was observed a sequence homology of (8/10) amino acids with the leukocyte common antigen precursor. Each mimotope had significant homology to a natural open reading frame (Table 6A).

Discussion

The early detection of cancer is a significant challenge in clinical oncology. Once accurate methods become available, early detection can result in a significant reduction in morbidity and mortality of these diseases. The detection of ovarian cancer at Stage I could result in a cure rate of 90%. To this end there has been devised an approach of high-throughput

selection of antigen biomarkers using phage display libraries and marker selection using a highly parallel analysis on macroarrays. The process began with a representative sample of 480 cloned markers from biopanning an ovarian cancer T7 phage display cDNA library with one patient's serum. There was first demonstrated that these clones bound to IgG molecules found in the sera of patients other than the one used for antigen selection. One hundred and forty nine markers that bound to IgGs in sera from OVCA patients showed no interaction with sera from cancer-free women. Forty-one of these antigen biomarkers had positive interactions with early (including cancers with borderline histology) and late stage ovarian cancer patients and there were no false positive interactions with IgGs in sera from either women having benign gynecological syndromes such as ovarian cysts and endometrial fibroids or sera from women with endometrial cancer. Because Stage I and Stage I borderline tumors can elicit a detectable immune response in this assay, this technology is sensitive to very small tumor burdens as (Table 5A). Sera from women with other cancers can be used to distinguish markers that are specific to ovarian cancer from those that bind to antibodies in sera from individuals with other cancers. Based on this representative sample of 480 clones from a single selection experiment, discovery of these markers to larger numbers of epitope clones were scaled up, cloning from additional libraries using sera from these and other women with ovarian cancer. Although the epitope markers were cloned using serum from a patient having the most common histologic type of ovarian cancer, serous adenocarcinoma, there has been shown that these markers are capable of detecting other histologic types of ovarian cancer, including endometrioid and clear cell tumors as well (Table 5A, Table 4). When the top ranking 26 (Table 7A) were applied, to the dataset comprised of 16 patients and 12 healthy women, these clones bound to IgGs in the sera from 16 out of 16 patients (Table 7B). As none of these 26 clones showed binding to IgGs in sera from 25 healthy women in Group 1 or 12 healthy women in Group 2, it is likely they represent a promising discriminator between the healthy and cancer sera. Larger studies with additional antigen biomarkers in other populations can be used to verify that the rate of diagnostic misclassification with this approach is small enough to justify its use in a clinical setting as screening test for ovarian cancer.

Knowledge regarding the immunogenicity and expression pattern of serologically-defined tumor antigens is critical in assessing the therapeutic and diagnostic potential of those antigens. The present study demonstrates that the use of T7 phage display selected clones is an effective technique for molecular profiling of the humoral immune response in ovarian cancer. Within this initial panel of 41 biomarkers, 8/9 contained large portions of open reading frames of the parental proteins; 1F6 is the receptor-binding cancer antigen expressed on SiSo cells (Human uterine adenocarcinoma cell line) (RCAS1); 3A9 is the signal recognition protein (SRP-19); 5C11 is the AHNK-related sequence; 2B4, nuclear autoantigenic sperm protein (NASP); 3C11 is the Ribosomal protein L4 (RPL4); 4H3 is the Nijmegen breakage syndrome 1 (nibrin) (NBS1); 2G4 is the eukaryotic initiation factor 5A (eIF-5A); and 5F8 is the *Homo sapiens* KIAA0419 gene product. With the exception of clone 4A11 that is the *Homo sapiens* chromodomain helicase DNA binding protein 1,

CHD1, all of the aforementioned gene products have a known or suspected etiological association with cancer. One of these markers, RCAS1, is overexpressed in many cancers such as uterine, breast and pancreatic cancer. As indicated by the broad overexpression of RCAS1 in human cancers, some of the antigens identified may not be specific to ovarian cancer. However, this does demonstrate that the epitomics profiling of the humoral immune response in cancer patients can identify serum antibody markers that are relevant to the etiology of their cancer (e.g. overexpressed or mutated) with diagnostic and therapeutic value. Interestingly, these 9 antigens with parental open reading frames are predicted to be intracellular products. This finding is in agreement with reports using the SEREX procedure, whereby the majority of those antigens are also intracellular, and their probable release by necrosis or cell lysis at the tumor site is an initiating factor in eliciting an immune response.

The remaining 32 clones are mimotopes, defined as peptides capable of binding to the paratope of an antibody, but are unrelated in sequence to the natural protein that the antibody actually recognizes. Such peptides are usually identified by testing combinatorial peptide libraries obtained by chemical synthesis or phage display for their ability to bind monoclonal antibodies specific for discontinuous epitopes. This is analogous to the previous studies that have selected randomized peptide libraries on serum from Hepatitis B patients. Peptide mimotopes can potentially be used as a novel form of immunotherapy to induce a beneficial antitumor response. A mimotope derived from a phage display library can induce specific inhibition of the binding between tumor-inhibitory antibody and the Erb-2 receptor. Such mimotopes may represent a superior form of immunotherapy that may not elicit side effects due to autoimmunity to a natural protein.

In conclusion, using a combination of high throughput selection and array-based serological profiling that are called Epitomics®, there was isolated a panel of 41 antigens, including 8 antigens previously associated with cancer. Further work with larger panels of antigens analyzed on macroarrays or microarrays provide a comprehensive set of markers that can be evaluated using sera from other cancers for the specificity of an ovarian cancer test. This epitomics approach to antigenic profiling has applications to cancer, autoimmune diseases, and infectious diseases for diagnostic, therapeutic, and epidemiologic studies.

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology that has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described.

TABLE 4

Blood Specimen ID #	Histology	Stage
MEC1*	serous adenocarcinoma	Unknown
MEC2	serous adenocarcinoma	IIA
MEC16	serous adenocarcinoma	IV
MEC20	serous adenocarcinoma	Unknown
MEC23	serous adenocarcinoma	IIIC
MEC35	serous adenocarcinoma	IIIC
MEC37	serous adenocarcinoma	IIIC
TB01-060	serous adenocarcinoma	IIIC
TB01-108	serous adenocarcinoma	IIIC
42501	adenocarcinoma NOS	late
400162	adenocarcinoma NOS	late
40036	adenocarcinoma NOS	late
42780	adenocarcinoma NOS	late
B755	adenocarcinoma NOS	late
40015	adenocarcinoma NOS	late
OVC075	serous adenocarcinoma	IIC
OVC015	serous adenocarcinoma	IIIC
OVC035	serous adenocarcinoma	IIIC
OVC007	mixed epithelial	IIIC
OVC005	Malignant Mixed Mesodermal Tumor	IIIC
OVC063	serous adenocarcinoma	III
OVC045	serous adenocarcinoma	IIIC
NW0629 (4387)	endometrioid adenocarcinoma	IC
NW0453 (4679)	adenocarcinoma NOS	IC
NW0046 (4555)	borderline serous cystadenofibroma	IA

TABLE 4-continued

	Blood Specimen ID #	Histology	Stage
5	NW1181 (4283)	endometrioid adenocarcinoma	IA
	OVC019	mixed epithelial	IC
	OVC087	clear cell	IA
10	OVC078	endometrioid	IC
	OVC070	borderline serous	IC
	OVC049	mixed epithelial	IA
	OVC079	borderline serous	I
	33-38	benign ovarian cyst	N/A
	92-96	uterine myoma	N/A
15	80-82	endometrial adenocarcinoma	IIIA
	79-62	endometrial adenocarcinoma	IIIA
	35-27	benign ovarian cyst	N/A
	30-141	benign ovarian cyst	N/A
	70-153	endometrial adenocarcinoma	IB
	81-80	endometrial adenocarcinoma	IA
20	31-55	benign ovarian cyst	N/A
	39-55	benign ovarian cyst	N/A
	36-11	endometrial polyp	N/A
	32-43	Benign, thickening of endometrium	N/A
	OVC068-1B	papillary serous adenoma (benign) & endometriosis	N/A
25	OVC054	benign serous cystadenoma	N/A

*Serum used for biopanning

TABLE 5A

Binding of 44 Clones with Late Stage and Stage I Ovarian Cancer Patient Sera

The binding of a panel of 44 clones with 22 Late Stage, 10 Stage I ovarian cancer patients was determined. These 44 antigens listed below bound exclusively with serum IgGs derived from both late stage and stage I ovarian cancer patients (including borderline histology) but not with serum IgG from normal control or patients with other gynecological diseases. The grey colored boxes represent positive binding of phage clones with patient's sera.

Late Stage Sera, Stage (II-IV)

[illegible]

TABLE 5A-continued

Binding of 44 Clones with Late Stage and Stage I Ovarian Cancer Patient Sera

The binding of a panel of 44 clones with 22 Late Stage, 10 Stage I ovarian cancer patients was determined. These 44 antigens listed below bound exclusively with serum IgGs derived from both late stage and stage I ovarian cancer patients (including borderline histology) but not with serum IgG from normal control or patients with other gynecological diseases. The grey colored boxes represent positive binding of phage clones with patient's sera.

2H9 (SEQ ID NO: 22)													
3A9 (SEQ ID NO: 15)													
3B12 (SEQ ID NO: 23)													
3C8 (SEQ ID NO: 47)													
3C11 (SEQ ID NO: 16)													
4A3 (SEQ ID NO: 35)													
4A4 (SEQ ID NO: 25)													
4A11 (SEQ ID NO: 17)													
4B2 (SEQ ID NO: 45)													
4C10 (SEQ ID NO: 28)													
4D9 (SEQ ID NO: 29)													
4G8 (SEQ ID NO: 36)													
4G9 (SEQ ID NO: 42)													
4H3 (SEQ ID NO: 18)													
4H4 (SEQ ID NO: 32)													
5A3 (SEQ ID NO: 26)													
5C3 (SEQ ID NO: 19)													
5C6 (SEQ ID NO: 46)													
5C9 (SEQ ID NO: 53)													
5C11 (SEQ ID NO: 20)													
5C12 (SEQ ID NO: 50)													
5D8 (SEQ ID NO: 24)													
5F8 (SEQ ID NO: 21)													
5F9 (SEQ ID NO: 54)													
5G9 (SEQ ID NO: 31)													
5H6 (SEQ ID NO: 39)													

Stage I sera

	OVC019	OVC087	OVC078	OVC070	OVC049	OVC079	TP
1B5 (SEQ ID NO: 44)							1
1B6 (SEQ ID NO: 11)							1
1H1 (SEQ ID NO: 48)							2
2A3 (SEQ ID NO: 27)							8
2B4 (SEQ ID NO: 12)							12
2C1 (SEQ ID NO: 40)							5
2C7 (SEQ ID NO: 33)							7
2D1 (SEQ ID NO: 38)							2
2E1 (SEQ ID NO: 34)							6
2E10 (SEQ ID NO: 37)							8
2E11 (SEQ ID NO: 30)							6
2E12 (SEQ ID NO: 43)							7
2F7 (SEQ ID NO: 13)							11
2F10 (SEQ ID NO: 49)							3
2F12 (SEQ ID NO: 52)							7
2G2 (SEQ ID NO: 41)							13
2G4 (SEQ ID NO: 14)							12
2H5 (SEQ ID NO: 51)							8

TABLE 5A-continued

Binding of 44 Clones with Late Stage and Stage I Ovarian Cancer Patient Sera

The binding of a panel of 44 clones with 22 Late Stage, 10 Stage I ovarian cancer patients was determined. These 44 antigens listed below bound exclusively with serum IgGs derived from both late stage and stage I ovarian cancer patients (including borderline histology) but not with serum IgG from normal control or patients with other gynecological diseases. The grey colored boxes represent positive binding of phage clones with patient's sera.

2H9 (SEQ ID NO: 22)						13
3A9 (SEQ ID NO: 15)						3
3B12 (SEQ ID NO: 23)						9
3C8 (SEQ ID NO: 47)						3
3C11 (SEQ ID NO: 16)						5
4A3 (SEQ ID NO: 35)						9
4A4 (SEQ ID NO: 25)						4
4A11 (SEQ ID NO: 17)						5
4B2 (SEQ ID NO: 45)						4
4C10 (SEQ ID NO: 28)						1
4D9 (SEQ ID NO: 29)						2
4G8 (SEQ ID NO: 36)						1
4G9 (SEQ ID NO: 42)						1
4H3 (SEQ ID NO: 18)						6
4H4 (SEQ ID NO: 32)						2
5A3 (SEQ ID NO: 26)						8
5C3 (SEQ ID NO: 19)						8
5C6 (SEQ ID NO: 46)						2
5C9 (SEQ ID NO: 53)						3

5C11 (SEQ ID NO: 20)						2
5C12 (SEQ ID NO: 50)						4
5D8 (SEQ ID NO: 24)						1
5F8 (SEQ ID NO: 21)						3
5F9 (SEQ ID NO: 54)						4
5G9 (SEQ ID NO: 31)						5
5H6 (SEQ ID NO: 39)						2

TP: Total number of patients whose serum IgGs bound to each phage clone.

•: Serum Dilution 1:3000; •: Serum Dilution 1:30000; for others 1:10000 serum dilution was used.

TABLE 5B

Binding of 21 Clones with Late Stage Ovarian Cancer Patient Sera

The binding of a panel of 21 clones with 22 Late Stage was determined on macroarrays. These 21 antigens listed below bound exclusively with serum IgGs derived from late stage ovarian cancer patients but not with serum IgG from normal control of patients with other gynecological diseases.

Late Stage Sera, Stage (II-IV)

	Mec1	Mec2	Mec16	Mec20	Mec23	Mec35 •	Mec37 •	TB01-060 •	TB01-108 •	42501	400162	40036
2B3 (SEQ ID NO: 56)												
2B9 (SEQ ID NO: 57)												
2C12 (SEQ ID NO: 58)												
2D7 (SEQ ID NO: 59)												
2D12 (SEQ ID NO: 60)												
2E7 (SEQ ID NO: 61)												
2G10 (SEQ ID NO: 62)												
2G11 (SEQ ID NO: 63)												
2H8 (SEQ ID NO: 64)												
3H1 (SEQ ID NO: 55)												
4C5 (SEQ ID NO: 65)												
4H6 (SEQ ID NO: 66)												
5A2 (SEQ ID NO: 67)												
5A4 (SEQ ID NO: 74)												
5A7 (SEQ ID NO: 68)												
5B9 (SEQ ID NO: 69)												
5B12 (SEQ ID NO: 70)												
5D6 (SEQ ID NO: 71)												
5E3 (SEQ ID NO: 72)												
5E7 (SEQ ID NO: 75)												
5H8 (SEQ ID NO: 73)												

	42780	B755 •	40015	OVC075	OVC015	OVC035	OVC007	OVC005	OVC063	OVC045	TP
2B3 (SEQ ID NO: 56)											10
2B9 (SEQ ID NO: 57)											3
2C12 (SEQ ID NO: 58)											4
2D7 (SEQ ID NO: 59)											5
2D12 (SEQ ID NO: 60)											3
2E7 (SEQ ID NO: 61)											5
2G10 (SEQ ID NO: 62)											2
2G11 (SEQ ID NO: 63)											5
2H8 (SEQ ID NO: 64)											1
3H1 (SEQ ID NO: 55)											1
4C5 (SEQ ID NO: 65)											4
4H6 (SEQ ID NO: 66)											3
5A2 (SEQ ID NO: 67)											8
5A4 (SEQ ID NO: 74)											3
5A7 (SEQ ID NO: 68)											2
5B9 (SEQ ID NO: 69)											1
5B12 (SEQ ID NO: 70)											1
5D6 (SEQ ID NO: 71)											2
5E3 (SEQ ID NO: 72)											2
5E7 (SEQ ID NO: 75)											2
5H8 (SEQ ID NO: 73)											2

•: Serum Dilution 1:3000; •: Serum Dilution 1:30000; all others were analyzed at a serum dilution of 1:10000;

TP: Total number of patients whose serum IgGs bound to each phage clone.

The mimotope sequences and the epitopes that are the real antigens that the antibodies were produced against based on ⁶⁵ the amino acid sequence homology similarity (see below Region of similarity of AA).

TABLE 6A

Description of Stage I-IV clones. Size range of the Mimotopes ≥5 amino acids						
Stage (I-IV) clones	Description of the genes that are in- frame with T7 10B gene	Peptide sequences of Epitopes, in-frame with T7 10 B gene	Size of the peptide	Unigene #	Region of similarity of AA	Antigen expression in any type of cancer
1F6	gi 18490914 gb BC022506.1 <i>Homo sapiens</i> , estrogen receptor binding site associated, antigen, 9 RCAS	AAWQAEVLRLQOKLADRE KRAAEQQ (SEQ ID NO: 11)	25 AA	Hs.9222	165-213	Overexpressed in ovarian, nonsmall cell lung carcinoma, pancreatic ductal cacinoma
2B4	gi 22042983 ref XM_032391.3 , <i>Homo sapiens</i> similar to nuclear autoantigenic sperm protein (histone- binding) (NASP)	EKGQEKQGEVIVSIEEK PKEVHEQPVVTLKQGT AVEVEAESLDPTVKPVDV GGDEPEKVVTSENEAGK AVLEQLVQGEVPPAEESP EVTTEAAEASAVEAGSEV SEKPGQEAPlVPKDGAVN GPSVVGDDQTPIEPQTSIE RLTETKDGSGLEEKVRK LVPSQERTKLSVESEAA GDGVDTKVAQGATEKSPE DKVQIAANEETQER (SEQ ID NO: 12)	212 AA	Hs.446206	258-469	Expression levels are higher in myelogenous leukemia and lymphoblastic leukemia cells.
2F7	gi 22042983 ref XM_032391.3 , <i>Homo sapiens</i> similar to Nuclear autoantigenic sperm protein (NASP)	EKGQEKQGEVIVSI (SEQ ID NO: 13)	15 AA	Hs.446206	256-270	Expression levels are higher in myelogenous leukemia and lymphoblastic leukemia cells.
2G4	gi 20987351 gb BC030160.1 , <i>Homo sapiens</i> , eukaryotic translation initiation factor 5A	MADLDLPETGDAGASATF PMQCSALRNGFVVLKGR PCKIVEMSTSKTGKHGHA KVHLVGIDIFTGKKYEDI CPSTHNMVDPNKRNDFO LIGIQDGYLSLLQDSGEV REDLRLPEGDLGKEIEHK FDCGEQILITVLSAMTEE ALVA (SEQ ID NO: 14)	148 AA	Hs.310621	1-148	eIF-5A2 sharing 82% identity of amino acid sequence with eIF-5A, is a candidate oncogene related to development of ovarian cancer.
3A9	gi 4507212 ref NM_003135.1 , <i>Homo sapiens</i> signal recognition particle 19 kDa (SRP19)	QKTGGADQSLQQEGSKK GKGKKK (SEQ ID NO: 15)	25 AA	Hs.2943	119-143	Transcript generated by alternative splicing between exon 14 of the Adenomatous polyposis coli gene and SRP19 is observed and it's expression is higher in Colorectal cancer
3C11	gi 16579884 ref NM_000968.2 <i>Homo sapiens</i> ribosomal protein L4	ALQAKSDEKAAVAGK KPVVGGKKGAAGV	68 AA	Hs.186350	360-427	over-expression of L7a and L37 mRNA is confirmed in prostate-cancer tissue samples.

TABLE 6A-continued

		Description of Stage I-IV clones.		Size range of the Mimotopes ±5 amino acids			
4A11	(RPL4)	gi 4557446 ref NM_001270.1 , Homo sapiens chromodomain helicase DNA binding protein 1 (CHD1)	KKQKKPLVGGKAAAT KKPSPEKKPAENKPT TEDNKPAA (SEQ ID NO: 16)	86 AA Hs.311553	107-192	Not associated with cancer	
4H3		gi 20543465 ref XM_045343.5 , Homo sapiens Nijmegen breakage syndrome 1 (nibrin) (NBS1)	QQQQQQHQASNSGSEE DSSSEDDDSSEVKRK KKHDEWQMSGGSPSQS GSDSESEEREKSSCDET ESDYEPKNKVKSRK (SEQ ID NO: 17)	101 AA Hs.25812	433-524	Three different mutations in NBS1 gene, generating truncated or aberrant NBS1 transcripts were observed in different cancer cell lines.	
5C3		gi 20987351 gb BC030160.1 , Homo sapiens, eukaryotic translation initiation factor 5A	MADDLPETGAGASATF PMQCSALRKNGFVLKGR PCKIVEMSTSKTRGHGA KVHLVGIDIFTGKKYEDI CPSTHNDVPNIKRNDFQ LIGIQDGYLSLLQDSGEV REDLPLPEGD (SEQ ID NO: 19)	118 AA Hs.310621	1-118	eIF-5A2 sharing 82% identity of amino acid sequence with eIF-5A, is a candidate oncogene related to development of ovarian cancer.	
5C11		gi 535176 emb X74818.1 HSAHNAKRS, H. sapiens mRNA of AHNAK-related sequence	PKFKMPDVHFKSPQISMS DIDLNLKPKTKGDMDIS VPKLEGLKGPVYKGP KVGIDTPDIDIHGPEGKL KGPFKMPDLHLKAPKIS MPEVDNLKGPVKGDMD ISLPKVEGDLKGP (SEQ ID NO: 20)	121 AA Hs.378738	393-512	Expression level of AHNAK is higher in melanoma, promyelocytic leukemia HL-60, osteosarcoma.	
5F8		gi 7662105 ref NM_014711.1 , Homo sapiens KIAA0419 gene product	GVCSKVVVGNKTSVKE DVLGKSNQVQSSGNHL ENKVTGGLVTVEGULTSD ERGAHIMNSTCAAMPKLH EPYASSQCIASFNFCTVS GLKPASMLEKNCLOTET NKSVDVKNPSPILLMQNQ XROQMDTPMVSCGNEQFL DNSFEK (SEQ ID NO: 21)	150 AA Hs.279912	434-583	mRNA expression level of another antigen KIAA1416 is up-regulated in colon cancer.	
1F6		gi 18490914 gb BC022506.1	AAWQAEVLRQOKLADRE	49 AA Hs.9222	165-213	Overexpressed in ovarian, nonsmall cell lung	

TABLE 6A-continued

		Description of Stage I-IV clones. Size range of the Mimotopes ±5 amino acids			
2B4	<i>Homo sapiens</i> , estrogen receptor binding site associated, antigen, 9 RCAS	KRAAEQQRKKMEKEAQRLL MKKEQNKGIVKLS (SEQ ID NO: 11)	212 AA Hs.446206	258-469	carcinoma, pancreatic ductal cacinoma
	<i>gi</i> 22042983 ref XM_0322391.3 , <i>Homo sapiens</i> similar to nuclear autoantigenic sperm protein (histone-binding) (NASP)	EKGQEQKQGEVIVSTEEK PKEVXEQPVVTLKQGT AVEVEAESLDPTVKFDV GGDEPEEKVTSENEAGK AVLEQLYQGEVPPAEESP EVTTEAAEASAVEAGSEV SEKPGQEPVLPKDGAVN GPSVVGDTPIEPQTSIE RLTETKDGSGLEEKVRAK LVPSQEETKLSVESEAA GDGVDTKVAQGATEKSPE DKVQIAANEETQER (SEQ ID NO: 12)	212 AA Hs.446206	258-469	Expression levels are higher in myelogenous leukemia and lymphoblastic leukemia cells.
2F7	<i>gi</i> 22042983 ref XM_0322391.3 , <i>Homo sapiens</i> similar to Nuclear autoantigenic sperm protein (NASP)	EKGQEQKQGEVIVSI (SEQ ID NO: 13)	15 AA Hs.446206	256-270	Expression levels are higher in myelogenous leukemia and lymphoblastic leukemia cells.
2G4	<i>gi</i> 20987351 gb BC030160.1 , <i>Homo sapiens</i> , eukaryotic translation initiation factor 5A	MADDLDFETGAGASATF PMQCSALRKNQGFVVLKGR PCKIVEMSTSKTGHGHA KVHLVGIDIFTGKKYEDI CPSTHNMMDVPNIKRNDFQ LIGTODGYLSLLQDSGEV REDRLPBGDLGKEIEHK FDCGEQILITVLSAMTEE ALVA (SEQ ID NO: 14)	148 AA Hs.310621	1-148	eIF-5A2 sharing 82% identity of amino acid sequence with eIF-5A, is a candidate oncogene related to development of ovarian cancer.
3A9	<i>gi</i> 4507212 ref NM_003135.1 , <i>Homo sapiens</i> signal recognition particle 19 kDa (SRP19)	QKTGGADQSLQOGEQSKK GKGGKKK (SEQ ID NO: 15)	25 AA Hs.2943	119-143	Transcript generated by alternative splicing between exon 14 of the Adenomatous polyposis coli gene and SRP19 is observed and it's expression is higher in Colorectal cancer
3C11	<i>gi</i> 16579884 ref NM_000968.2 <i>Homo sapiens</i> ribosomal protein L4 (RPL4)	ALQAKSDEKAAVAGK KPVVGGKGGKKAAGV KKQKKPLVGKKAAT KKPSPEKKPAENKPT TEDNKPAA (SEQ ID NO: 16)	68 AA Hs.186350	360-427	over-expression of L7a and L37 mRNA is confirmed in prostate-cancer tissue samples.
4A11	<i>gi</i> 4557446 ref NM_001270.1 , <i>Homo sapiens</i> chromodomain helicase DNA binding protein 1 (CHD1)	QQQQQQHQASNSGSEE DSSSESDSDSSSEVKRK KKHDEDQMSGSGSPSQS GSDSESEEREKSCDET	86 AA Hs.311553	107-192	Not associated with cancer

	Description of Stage I-IV clones.	Size range of the Mimotopes ≥5 amino acids			
			ESDYEPMKMKVRSRK (SEQ ID NO: 17)		
HH3	gi 20543465 ref XM_045343.5 , <i>Homo sapiens</i> Nijmegen breakage syndrome 1 (nibrin) (NBS1)		PTKLPSINKSKDRAS QOQTNSIRNPQPS TKKERDENQEMSS CKSARIETSCSLLEQ TQPATPSSLWKNKEQH LSENXPVDTXSXNL FTGYXFRXGXE (SEQ ID NO: 18)	101 AA Hs.25812	433-524 Three different mutations in NBS1 gene, generating truncated or aberrant NBS1 transcripts were observed in different cancer cell lines.
SC3	gi 20987351 gb BC030160.1 , <i>Homo sapiens</i> , eukaryotic translation initiation factor 5A		MADDLPFTGDAGSATF PMQCSALPRKNGFVLKGR PKTIEMSTSKTRGHGA KVHLVGIDIFTGKYVEDI CPSTHMDVPNIKRNDFQ LIIGIQDGYSLLQDSGEV REDLPLPRGD (SEQ ID NO: 19)	118 AA Hs.310621	1-118 eIF-5A2 sharing 82% identity of amino acid sequence with eIF-5A, is a candidate oncogene related to development of ovarian cancer.
SC11	gi 535176 emb X74818.1 HSAHNAKR, <i>H. sapiens</i> mRNA of AHNAK-related sequence		PKFKMPDVHFKSPQISMS DIDLNLKGPKIKGDMDIS VPKLEGDLKGPVDVKGP KVGIDTPDIDIHGPEGL KGPKFMPDLHLKAPKIS MPEVDNLKGPVKGDM ISLPKVEGDLKGP (SEQ ID NO: 20)	121 AA Hs.378738	393-512 Expression level of AHNAK is higher in melanoma, promyelocytic leukemia HL-60, osteosarcoma.
PF8	gi 7662105 ref NM_014711.1 , <i>Homo sapiens</i> KIAA0419 gene product		GVCSSKVVGNKTSEVKE DVVLGKSQVCOSSGNHL ENKVTHGLVTVEGLTSD ERGAHIMNSTCAAMPKLH EPYASSQCIAFPNFGTVS GLKPASMLEKNCQLTEL NKSYDVKNPSPLLMQNON XRQQMDTPMVSCNEOFL DNSFEK (SEQ ID NO: 21)	150 AA Hs.279912	434-583 mRNA expression level of another antigen KIAA1416 is up-regulated in colon cancer.
The above table shows two antigens and not mimotopes, the clones below are the mimotopes.					
	Peptide sequences of	Mimotopes, Size of in-frame with T7 10 B gene	Description of the sequences that peptide Mimotopes mimic	Unigene #	Region of similarity of AA Antigen expression in any type of cancer

TABLE 6A-continued

		Description of Stage I-IV clones.		Size range of the Mimotopes ≥5 amino acids		
2H9	gi 21619682 gb BC032762.1 , <i>Homo sapiens</i> optineurin, mRNA	5 AA	ELLRT (SEQ ID No: 22)	gi 20139301 sp Q9Y446 PKP3_HUMAN, Plakophilin 3	Hs.148074 407-411 Score = 18.9 bits (37), Expect = 827 Identities = 5/5 (100%), Positives = 5/5 (100%) Query b: 1 ELLRT ELLRT Sbjct c: 407 ELLRT	Immunohistochemical localization of plakophilins (PKP1, PKP2, PKP3, and p0071) 5 in primary oropharyngeal tumors 411
3B12	gi 21735624 ref NM_145690.1 , <i>Homo sapiens</i> tyrosine 3- monooxygenase/tryptophan 5- monooxygenase activation protein, zeta polypeptide (YHAZ), transcript variant	5 AA	QQTSM (SEQ ID No: 23)	gi 729143 sp P38936 CDN1_HUMAN, Cyclin- dependent kinase inhibitor 1 (p21) (CDK- interacting protein1) (Melanoma differentiation associated protein 6) (MDA-6) 2, mRNA.	Hs.370771 144-147 Score = 16.8 bits (32), Expect = 3595 Identities = 4/4 (100%), Positives = 4/4 (100%) Query: 2 QQTSM QTSM Sbjct: 144 QQTSM 147	mda-6 (p21) may function as a negative regulator of melanoma growth, 5 progression and metastasis 147
5D8	gi 22024583 gb AC087376.5 , <i>Homo sapiens</i> chromosome 11, clone RP11- 230019, complete sequence	5 AA	KKGPI (SEQ ID No: 24)	gi 20177863 sp Q9BXJ2 CQT7_HUMAN, Complement-clq and tumor necrosis factor- related protein 7 precursor	Hs.153714 102-106 Score = 18.5 bits (36), Expect = 1109 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 KKGPI KKGPI Sbjct: 102 KKGPI	TNF-alpha regulates expression of downstream components of 5 complement system and plays a 106 role in energy homeostatis where it is implicated in cachexia, obesity and insulin resistance.
4A4	gi 17028354 gb BC017483.1 BC017483, <i>Homo</i> <i>sapiens</i> , clone IMAGE: 3506553, mRNA.	6 AA	AKVIMR (SEQ ID No: 25)	gi 5921908 sp Q43174 CP26_HUMAN, Cytochrome P450 26 (Retinoic acid- metabolizing cytochrome) (P450RAI) (hP450RAI) (Retinoic acid 4-hydroxylase)	Hs.150595 138-142 Score = 20.6 bits (41), Expect = 255 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 2 KVIMR KVIMR Sbjct: 138 KVIMR	all-trans-Retinoic acid-induced expression and regulation of retinoic acid 4- hydroxylase 6 (CYP26) in human 142 promyelocytic leukemia
5A3	gi 15011541 gb AF397158.1 AF397158, <i>Homo</i> <i>sapiens</i> clone 11 pur alpha-	6 AA	YACLKD (SEQ ID No: 26)	gi 1170473 sp P42575 ICE2_HUMAN, Caspase-2 precursor (CASP-2) (ICH-1 protease)	Hs.433103 351-355 Score = 20.2 bits (40), Expect = 342 Identities = 5/5 (100%), Positives = 5/5 (100%)	CASP-3, CASP-4, CASP-2 heterogeneously coexpress in leukemic cell lines

TABLE 6A-continued

Description of Stage I-IV clones. Size range of the Mimotopes ≥5 amino acids						
	associated ribosomal RNA gene, partial sequence			Query: 1 YACLK Sbjct: 351 YACLK	5 355	
2A3	gi 23271193 gb BC036014.1 , <i>Homo sapiens</i> poly(A) polymerase alpha, mRNA	QILFMDP (SEQ ID NO: 27)	7 AA	gi 729597 sp P39086 GLK1_HUMAN, Glutamate receptor, ionotropic kainate 1 precursor	Hs.222405 242-246 Score = 21.4 bits (43), Expect = 142 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 QILFM Sbjct: 242 QILFM	Ionotropic and metabotropic glutamate receptor protein expression in glioneuronal tumours from patients with epilepsy 246 intractable
4C10	gi 24756892 gb AC008507.10 , <i>Homo sapiens</i> chromosome 19 clone CTC-448F2, complete sequence	LNTVNTLI (SEQ ID NO: 28)	8 AA	gi 13633936 sp Q9NPR2 SM4B_HUMAN, Semaphorin 4B	Hs.416077 440-445 Score = 21.8 bits (44), Expect = 106 Identities = 6/6 (100%), Positives = 6/6 (100%) Query: 1 NTVNTL Sbjct: 440 NTVNTL	Not associated with cancer 6 445
4D9	gi 21629397 gb AC099571.2 , <i>Homo sapiens</i> chromosome 1 clone RP11- 438H8, complete sequence	GNSILLIA (SEQ ID NO: 29)	8 AA	gi 2842764 sp Q99735 GST2_HUMAN, Microsomal glutathione S-transferase 2 (Microsomal GST-2)	Hs.81874 3-10 Score = 21.4 bits (43), Expect = 140 Identities = 7/8 (87%), Positives = 7/8 (87%) Query: 1 GNSILLIA Sbjct: 3 GNSILLIA	GST-pi has significance in the diagnosis of cancers as it is expressed 8 abundantly in tumor cells. 10
2E11	gi 22004067 dbj AP005356.2 , <i>Homo sapiens</i> genomic DNA, chromosome 8q23, clone: KB1198A4, complete sequence.	WDLKSE YS (SEQ ID NO: 30)	8 AA	gi 1710146 sp P49798 RGS4_HUMAN, Regulator of G-protein signaling 4 (RGS4) (RGP4)	Hs.386726 80-85 Score = 21.8 bits (44), Expect = 106 Identities = 6/6 (100%), Positives = 6/6 (100%) Query: 3 LKSEYS Sbjct: 80 LKSEYS	RGS4 is highly expressed in brain regions implicated in pathophysiology of schizophrenia 8 85
5G9	gi 20072204 gb BC026241.1 , <i>Homo sapiens</i> ubiquitin-protein isopeptide ligase (E3), mRNA	PGCSTTL S (SEQ ID NO: 31)	8 AA	gi 14423962 sp O94966 UBPJ_HUMAN, Ubiquitin carboxyl- terminal hydrolase 19	Hs.255596 940-947 Score = 18.9 bits (37), Expect = 827 Identities = 6/8 (75%), Positives = 7/8 (87%) Query: 1 PGCSTTLLS Sbjct: 940 PGCSTTLLS	Ubiquitin carboxyl- terminal-hydrolase L1 genes cause autosomal dominant familial Parkinson disease. 8 947

TABLE 6A-continued

		Description of Stage I-IV clones. Size range of the Mimotopes $\pm$ 5 amino acids					
4H4	gi 20072204 gb BC026241.1 , <i>Homo sapiens</i> ubiquitin-protein isopeptide ligase (E3), mRNA	8 AA	PRCSTTL S (SEQ ID NO: 32)	gi 6225843 sp O60760 PGD2_HUMAN, Glutathione-requiring prostaglandin D synthase	Hs.128433	156-160 Score = 18.9 bits (37), Expect = 827 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 3 CSTTL Sbjct: 156 CSTTL	Lipocalin-type prostaglandin D synthase (L- PGDS) has recently been 7 shown to be expressed in 160 human brain tumors, breast tumors and in ovarian cancer
2C7	gi 3152628 gb AC004744.1 AC004744, <i>Homo sapiens</i> BAC clone GS1- 465N13 from 7, complete sequence	9 AA	GDRSQL WRK (SEQ ID NO: 33)	gi 24211441 sp Q13443 AD09_HUMAN, ADAM 9 precursor (A disintegrin and metalloproteinase domain 9)	Hs.2442	720-725 Score = 20.2 bits (40), Expect = 342 Identities = 5/6 (83%), Positives = 5/6 (83%) Query: 3 RSQWLR R QLWR Sbjct: 720 RDQLWR	Expression of ADAM-9 mRNA and protein in human breast cancer 8 725
2E1	gi 16160856 ref XM_007763.5 , <i>Homo sapiens</i> myosin VA (heavy polypeptide 12, myoxin) (MYO5A), mRNA	9 AA	KKQSSW YQI (SEQ ID NO: 34)	gi 2498310 sp Q12882 DPYD_HUMAN Dihydropyrimidine dehydrogenase [NADP+] precursor (DPD) (DHPDHase) (Dihydrouracil dehydrogenase) (Dihydrothymine dehydrogenase)	Hs.1602	497-502 Score = 21.8 bits (44), Expect = 106 Identities = 5/6 (83%), Positives = 6/6 (100%) Query: 2 KQSSWY KQ+SWY Sbjct: 497 KQASWY	Higher DPD activity in gastric cancer is observed than in colorectal cancer 7 502
4A3	gi 16160856 ref XM_007763.5 , <i>Homo sapiens</i> myosin VA (heavy polypeptide 12, myoxin) (MYO5A), mRNA	9 AA	KKQSSW YQI (SEQ ID NO: 35)	gi 2498310 sp Q12882 DPYD_HUMAN Dihydropyrimidine dehydrogenase [NADP+] precursor (DPD) (DHPDHase) (Dihydrouracil dehydrogenase) (Dihydrothymine dehydrogenase)	Hs.1602	497-502 Score = 21.8 bits (44), Expect = 106 Identities = 5/6 (83%), Positives = 6/6 (100%) Query: 2 KQSSWY KQ+SWY Sbjct: 497 KQASWY	Higher DPD activity in gastric cancer is observed than in colorectal cancer 7 502
4G8	gi 15778776 gb AC012363.6 , <i>Homo sapiens</i> BAC clone RP11- 438012 from 2, complete sequence	9 AA	PEGGTD ASR (SEQ ID NO: 36)	gi 13634077 sp Q9Y493 ZAN_HUMAN, Zonadhesin	Hs.307004	1912-1919 Score = 18.9 bits (37), Expect = 827 Identities = 6/8 (75%), Positives = 7/8 (87%) Query: 2 EGGTDASR	zonadhesin functions during fertilization to anchor the acrosomal shroud 9 to the zona

Description of Stage I-IV clones.
Size range of the Mimotopes ≥ 5 amino acids

					Sbjct:	EGGT+ A R 1912 EGGTEAPR	pellucida 1919
2E10	gi 20521965 dbj AB051476.2 , <i>Homo sapiens</i> mRNA for KIAA1689 protein, partial cds	9 AA QS (SEQ ID NO: 37)	ASFTLKL RTSV (SEQ ID NO: 38)	gi 6226869 sp P34932 HS74_HUMAN, HEAT SHOCK 70 KDA PROTEIN 4 (HEAT SHOCK 70-RELATED PROTEIN APG-2)	Hs.90093 647-653 Score = 21.8 bits (44), Expect = 106 Identities = 6/7 (85%), Positives = 7/7 (100%) Query: 2 SFTLKQ SFTLKL+	Expression of HSP70 is observed in human hepatocellular carcinoma 8	
2D1	gi 4504522 ref NM_002157.1 , <i>Homo sapiens</i> heat shock 10 kDa protein 1 (chaperonin 10) (HSPe1), mRNA	10 AA GGGSNG RTSV (SEQ ID NO: 38)	GGGSNG RTSV (SEQ ID NO: 38)	gi 20137621 sp O95071 EDD_HUMAN, Ubiquitin-protein ligase EDD (Hyperplastic discs protein homolog) (hHYD) (Progesterin induced protein)	Hs.94262 140-148 Score = 21.8 bits (44), Expect = 105 Identities = 7/9 (77%), Positives = 9/9 (100%) Query: 1 GGSGNGRSTS GGGS+GR +S GGGSSGRSS	EDD, the human orthologue of the hyperplastic discs tumour suppressor gene, is amplified 9 and overexpressed in cancer 148	
5H6	gi 40849829 gb AA095625 NADH dehydrogenase subunit 4	11 AA NSFLMTS SKPR (SEQ ID NO: 39)	NSFLMTS SKPR (SEQ ID NO: 39)	gi 12643618 sp O60242 BAI3_HUMAN, Brain-specific angiogenesis inhibitor 3 precursor	Hs.334087 694-699 Score = 20.6 bits (41), Expect = 254 Identities = 5/6 (83%), Positives = 6/6 (100%) Query: 1 NSFLMT NS+LMT NS+LMT	BAl1 expression inhibit stromal vascularization in pulmonary adenocarcinoma 6	
2C1	gi 23958536 gb BC036216.1 , <i>Homo sapiens</i> cullin 4B, mRNA	11 AA ACSSTVS ETWI (SEQ ID NO: 40)	ACSSTVS ETWI (SEQ ID NO: 40)	gi 33112422 sp Q16827 PTPO_HUMAN Receptor-type protein-tyrosine phosphatase O precursor (Glomerular epithelial protein 1) (Protein tyrosine phosphatase U2) (PTPase U2) (PTP-U2)	Hs.160871 623-629 Score = 21.8 bits (44), Expect = 128 Identities = 6/7 (85%), Positives = 7/7 (100%) Query: 3 SSTVSFI SST+SFI SST+SFI	Functional involvement of PTP-U2L in apoptosis subsequent to 9 terminal differentiation of monoblastoid leukemia cells 629	
2G2	gi 25988997 gb AF541939.1 , <i>His-3</i> integration vector pJHAM007, complete sequence	14 AA KKKKKKK RVGGPL Q (SEQ ID NO: 41)	KKKKKKK RVGGPL Q (SEQ ID NO: 41)	gi 20532388 sp Q9NVF1 DD18_HUMAN, ATP-dependent RNA helicase DDX18 (DEAD-box protein 18) (Myc-regulated	Hs.363492 108-115 Score = 27.4 bits (57), Expect = 2.8 Identities = 8/8 (100%), Positives = 8/8 (100%) Query: 1 KKKKKKR	The expression of MrDb is induced upon proliferative stimulation of primary human 8 fibroblasts as well	

TABLE 6A-continued

Description of Stage I-IV clones. Size range of the Mimotopes ≥ 5 amino acids		DEAD-box protein) (MrDb)		Sbjct: 108 KKKKKR		as B cells and 115 down-regulated during terminal differentiation of HL60 leukemia cells	
4G9	gi 17136149 ref NM_014708.2 , GPVFICS <i>Homo sapiens</i> kinetochore associated 1 (KNTC1), mRNA	14 AA	gi 115892 sp P16870 CBPH_HUMAN, carboxypeptidase H precursor (CPH) (Carboxypeptidase E) (CPE) (Enkephalin convertase) (Prohormone processing carboxypeptidase)	Hs.75360	333-340 Score = 24.4 bits (50), Expect = 18 Identities = 7/8 (87%), Positives = 8/8 (100%) Query: 7 SSNCFKIT SSNCF+IT	Expression of the protein product of the PCPH proto- oncogene in human tumor cell lines	14
2E12	gi 22062543 ref XM_170670.1 , APFTCW <i>Homo sapiens</i> putative transmembrane protein; homolog of yeast Golgi membrane protein Yiflp (Yiflp- interacting factor) (54TM), mRNA.	15 AA	gi 128062 sp P08473 NEP_HUMAN, Neprilysin (Neutral endopeptidase) (NEP) (Enkephalinase) (Common acute lymphocytic leukemia antigen) (CALLA) (Neutral endopeptidase 24.11) (CD10)	Hs.307734	167-175 Score = 23.5 bits (48), Expect = 32 Identities = 7/10 (70%), Positives = 7/10 (70%), Gaps = 1/10 (10%) Query: 6 WPTVATNTWE WP VAT WE	Loss or decrease in expression of NEP has been reported in brain cancer, renal cancer and invasive bladder cancer.	15 175
1B5	gi 12654862 gb BC001275.1 BC001275, <i>Homo sapiens</i> annexin A1, mRNA	15 AA	gi 6707734 sp Q13077 TRAI_HUMAN, TNF receptor associated factor 1 (TRAF1)	Hs.531251	64-70 Score = 21.0 bits (42), Expect = 187 Identities = 6/7 (85%), Positives = 7/7 (100%) Query: 5 SISPGNR SISPG+R	Tumor necrosis factor receptor- associated factor 1 gene overexpression in B-cell chronic lymphocytic leukemia	11 70
4B2	gi 23272851 gb BC035645.1 , <i>Homo sapiens</i> , similar to RIKEN cDNA 3830613022 gene, clone	15 AA	gi 20139133 sp Q9BZF3 ORP6_HUMAN, Oxysterol binding protein-related protein 6	Hs.318775	906-912 Score = 21.8 bits (44), Expect = 104 Identities = 5/7 (71%), Positives = 6/7 (85%) Query: 8 QRETW IS QRE W+S	Oxysterols are potent signalling lipids that directly bind liver X receptors (LXRs). Oxysterol- regulated function control the expression of genes involved in	14 912

TABLE 6A-continued

Description of Stage I-IV clones.							
Size range of the Mimotopes ±5 amino acids							
5C6	gi 22797897 emb AL160171.27 , Human DNA sequence from clone RP11-256E16 on chromosome 1, complete sequence	ICGSWKG YNLWQS SSSK (SEQ ID NO: 46)	17 AA	gi 12644310 sp P53618 COPB_HUMAN, Coatomer beta subunit (Beta-coat protein) (Beta-COP)	Hs.3059	250-257 Score = 22.3 bits (45), Expect = 93 Identities = 7/8 (87%), Positives = 7/8 (87%) Query: 8 YNLWQSSS YNL QSSS Sbjct: 250 YNL LQSSS	reverse cholesterol transport, catabolism of cholesterol, and lipogenesis A major component of the coat of non-clathrin-coated vesicles, beta-15 COP, mediate membrane traffic through the Golgi complex
3C8	gi 24234687 ref NM_004134.3 , <i>Homo sapiens</i> heat shock 70 kDa protein 9B (mortalin-2) (HSPA9B), nuclear gene encoding mitochondrial protein, mRNA.	EILKPEG QHKLR SEETS (SEQ ID NO: 47)	18 AA	gi 2493676 sp Q12889 OGP_HUMAN, Oviduct-specific glycoprotein precursor (Oviductal glycoprotein)	Hs.1154	585-599 Score = 24.4 bits (50), Expect = 21 Identities = 10/15 (66%), Positives = 10/15 (66%), Gaps = 1/15 (6%) Query: 5 PEGQHKLRSEE-TS PEGQ M LR E TS Sbjct: 585 PEGQTM PLRGENTLS	Oviduct specific glycoproteins are involved in variety of roles during fertilization and early embryonic development 18
1H1	gi 22024587 gb AC103702.3 , <i>Homo sapiens</i> chromosome 17, clone RP11-357H14, complete sequence	AKARALA RRSEPCS TGKQLR (SEQ ID NO: 48)	21 AA	gi 12230848 sp O95049 Z03_HUMAN, Tight junction protein ZO-3 (Zonula occludens 3 protein)	Hs.25527	853-862 Score = 23.5 bits (48), Expect = 38 Identities = 8/10 (80%), Positives = 8/10 (80%) Query: 3 ARALARSEP A ALAR SEP Sbjct: 853 APALARS SEP	Occludin expression in microvessels of neoplastic and non-neoplastic human brain 12
2F10	gi 21166212 gb AC109584.2, <i>Homo sapiens</i> chromosome 3 clone RP11-674F14, complete sequence	VORGIGT IPSETIPV NRKRVN PP (SEQ ID NO: 49)	23 AA	gi 118206 sp P14416 D2DR_HUMAN, D(2) dopamine receptor	Hs.73893	264-270 Score = 22.7 bits (46), Expect = 56 Identities = 6/7 (85%), Positives = 7/7 (100%) Query: 14 PVNRKRV PVNR+RV Sbjct: 264 PVNRRRV	Expression of dopamine receptors and transporter in neuroendocrine gastrointestinal tumor cells 20
5C12	gi 24430032 emb AL939123.1 SC0939123,	VSWFPS WARSCG	28 AA	gi 3915660 sp Q16850 CP51_HUMAN,	Hs.512872	283-292 Score = 24.4 bits (50),	CYP2E1 protein is expressed in both

TABLE 6A-continued

Description of Stage I-IV clones.			
Size range of the Mimotopes ±5 amino acids			
2H5	Streptomyces coelicolor A3(2) complete genome; segment 20/29	ROTPLGA TYKDTLL PV (SEQ ID NO: 50)	Cytochrome P450 51A1 (CYPL1) (P450LI) (Sterol 14-alpha demethylase) (Lanosterol 14-alpha demethylase) (LDM) (P450-14DM)
	gi 18606292 gb BC022865.1 <i>Homo sapiens</i> ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein), mRNA	DLQPPG RRWLPQ QCPGSP GRCDAS VPLWSD HLPSL (SEQ ID NO: 51)	Expect = 17 Identities = 8/10 (80%), Positives = 8/10 (80%) Query: 14 QTPLGATYKD QT L ATYKD Sbjct: 283 QT LLDATYKD 292
2F12	gi 10443350 emb AL133264.10 AL133264, Human DNA sequence from clone RP3-369A17 on chromosome 6p22.1-22.3 Contains ESTs, STSs, GSSs and CpG islands	RGLGPLA AACGRS GGGGG GAGGTG SSNVNKK TPPN (SEQ ID NO: 52)	gi 8928460 sp O75962 TRIO_HUMAN, Triple functional domain protein (PTRPF interacting protein)
	gi 519209 2232-2244 Score = 31.2 bits (66), Expect = 0.22 Identities = 11/13 (84%), Positives = 13/13 (100%) Query: 13 SGGGGGGGAGGTG SGGGGGGG+GG+G Sbjct: 2232 SGGGGGGSGSG	Not associated with cancer	
5C9	gi 15072584 emb AL442003.8 , Human DNA sequence from clone RP11-324H6 on chromosome 10, complete sequence	PMRCS TMGEIQM QIHCGAR RRKAVPS SKDNVQ SSAH (SEQ ID NO: 53)	gi 34395516 sp O15085 ARHB HUMAN, Rho guanine nucleotide exchange factor 11 (PDZ-RhoGEF)
	gi 371602 409-417 Score = 23.1 bits (47), Expect = 72 Identities = 7/9 (77%), Positives = 7/9 (77%) Query: 8 MGEIQMQIH M EIQ QIH Sbjct: 409 MPRIEQIH	A novel gene at 11q23 named LARG for leukemia-associated Rho exchange factor 417 (GEF) has strong sequence homology to several members of the Rho family of GEFs. Further, LARG was found to be fused with	

TABLE 6A-continued

Description of Stage I-IV clones.									
Size range of the Mimotopes ≥5 amino acids									
5F9	gi 18693518 gb AC015911.8 , <i>Homo sapiens</i> chromosome 17, clone RP11- 1094M14, complete sequence	WRTTYISI LNLAQFY YSLITVLK TFNMPG TVVHACN PSTLGGQ GRRIT (SEQ ID NO: 54)	48 AA	gi 20139105 sp Q99959 PKP2 HUMAN, Plakophilin 2	Hs.25051	471-492 Score = 47.4 bits (111), Expect = 5e-06 Identities = 19/22 (86%), Positives = 19/22 (86%) Query: 27 WPGTVVHACNPSTLGGQG RRIT WPG V HACNPSTLGGQG RIT	MLL in a patient with primary Rho GEF, Bcr, has been implicated in leukemia through a recurrent chromosomal translocation.	Immunohistochemical localization of plakophilins (PKP1, PKP2, PKP3, and p0071) 48 in primary oropharyngeal tumors	
						Sbjct: 471 WPGAVAHACNPSTLGGQG GRIT			492

TABLE 6B

Description of Stage II-IV clones. Size range of the Mimotopes ≥5 amino acids									
Stage (II-IV) clones	Description of the genes that are in- frame with T7 10B gene	Peptide sequences of Epitopes in- frame with T7 10 B gene		Size of the peptide	Serex Y/N	Unigene #	Region of similarity of AA	Antigen expression in any type of cancer	Antigen expression in any type of cancer
3H1	gi 12654010 gb BC00805.1 <i>Homo sapiens</i> nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate, mRNA	DDSDYGSKKKN XKWKSKPERKE KKMPKRLKATVT PSFVKGGKVGSRP TASKASKEKTPSPK EEDPEPSPEKK TSISPPPEKSGDEG SEDEAPSGED (SEQ ID NO: 55)	103 AA		Hs.510265	N	140-243	The expressions of casein kinase II (CK2) is higher in neoplastic ovarian surface epithelium. Casein kinase II (CK II) is expressed at a higher level in lung tumours.	
2B3	gi 7023439 dbj AK001891.1 , <i>Homo sapiens</i> cDNA FLJ11029 fis, clone PLACE1004156	LSTSSPDEQN (SEQ ID NO: 56)	10 AA		Hs.528654	N	350-360	Not associated with cancer	

The table above are two antigens and not mimotopes, the clones below are the mimotopes.

Peptide sequences of the Mimotopes that are in- frame with T7 10 B gene								
Stage (II-IV) clones	Description of the genes that are in Mimotope clones	Size of the peptide	Description of the sequences that Mimotopes mimic	Unigene #	Region of similarity of AA	Antigen expression in any type of cancer		
2B9	gi 28837315 gb BC047588.1 Homo sapiens KIAA1363 protein, mRNA	VIVVLIATVISF 18 PONTWL AA (SEQ ID NO: 57)	gi 20141211 sp P18825 A2AC_HUMAN, Alpha-2C-adrenergic receptor (Alpha-2C adrenoceptor) (Subtype C4)	Hs.123022	172-185 Score = 24.8 bits (51), Expect = 16 Identities = 10/14 (71%), Positives = 10/14 (71%), Gaps = 3/14 (21%) Query: 2 IVV---LI-AVISFP IV LI AVISFP Sbjct: 172 IVAVWLISAVISFP 185	Stimulation of alpha2-adrenergic receptor inhibits cholangiocarcinoma growth through modulation of Raf-1 and B-Raf activities. Beta adrenergic receptor is overexpressed in pulmonary adenocarcinoma		
2C12	gi 15072584 emb AL442003.8 , PMRCSCTM	37	gi 2851534 sp Q13724	Hs.516120	4-12	Not associated		

TABLE 6B-continued

Human	DNA sequence from	AA	GEIQMGIHC	GCS1_HUMAN	Score = 24.0 bits (49), Expect = 40 Identities = 7/9 (77%), Positives = 8/9 (88%) Query: 18 GARRKAVP G RRR+AVP Spjct: 4 GERRRAVP	with cancer
2D7	gi 34882281 ref XM_236768.2 , Rattus norvegicus hypothetical LOC316116 (LOC316116), mRNA	14 AA	LRGTSVQP PEIEQ (SEQ ID NO: 59)	gi 32172435 sp P46934 Hs.1566 NED4_HUMAN, Ubiquitin-protein ligase Nedd-4	514-520 Score = 22.7 bits (46), Expect = 70 Identities = 6/7 (85%), Positives = 6/7 (85%) Query: 8 QPPEIEQ QP EIEQ Spjct: 514 QPSEIEQ	RING protein Trim32 associated with skin carcinogenesis 14 has E3-ubiquitin ligase properties 520
2D12	gi 34783327 gb BC022049.2 , Homo sapiens cDNA clone IMAGE: 4291567, partial cds	5 AA	ILHLH (SEQ ID NO: 60)	gi 128616 sp P23975 Hs.78036 S6A2_HUMAN, Sodium-dependent noradrenaline transporter (Norepinephrine transporter) (NET)	218-222 Score = 17.6 bits (34), Expect = 2425 Identities = 4/5 (80%), Positives = 5/5 (100%) Query: 1 ILHLH +LHLH Spjct: 218 VLHLH	NET is involved in neurotransmitter removal from neuronal synapses 222
2E7	gi 6330364 dbj AB033020.1 , Homo sapiens mRNA for KIAA1194 protein	32 AA	VLSALPEKN CNTVPPQPP EDLRVQHC SSRFLE (SEQ ID NO: 61)	gi 34395825 sp Q9H106 Hs.339789 PTL2_HUMAN Protein tyrosine phosphatase non- receptor type substrate 1-like 2 precursor	167-174 Score = 24.4 bits (50), Expect = 21 Identities = 7/8 (87%), Positives = 8/8 (100%) Query: 2 LSAALPEKN LSALPE+N LSALPERN Spjct: 167 LSAALPERN	Protein-tyrosine phosphatase (SAP-1) is overexpressed in gastrointestinal cancer 174
2G10	gi 16307467 gb BC010282.1 , Homo sapiens leucine-rich PPR-motif containing, mRNA	20 AA	WGFNERDR LSSILQQR VTL (SEQ ID NO: 62)	gi 13638201 sp P41214 Hs.274151 LIGA_HUMAN, Ligatin (Hepatocellular carcinoma-associated antigen 56)	523-528 Score = 24.0 bits (49), Expect = 29 Identities = 6/6 (100%), Positives = 6/6 (100%) Query: 12 ILQQR ILQQR Spjct: 523 ILQQR	CD15 and CD50 antigens are both overexpressed in hepatocarcinoma. 17
2G11	gi 7329921 emb AL117379.14 , HSJ563E14, Human DNA sequence from clone RP4-563E14 on chromosome 20 Contains the 5' of the DATF1 gene encoding the death	11 AA	VVSGFRSTP SL (SEQ ID NO: 63)	gi 1705762 sp P13569 Hs.521149 CFTR_HUMAN, Cystic fibrosis transmembrane conductance regulator (CFTR)	429-435 Score = 21.8 bits (44), Expect = 128 Identities = 6/7 (85%), Positives = 6/7 (85%) Query: 5 PFSFSL PFS PSL Spjct: 429 PFSNPSL	Mutation of CFTR is observed in Cystic Fibrosis 11 435

[illegible]

TABLE 6B-continued

5A7	gi 16508181 emb AL138765.18 , Human DNA sequence from clone RP11-34E5 on chromosome 10, complete sequence	7 KMWRTKM (SEQ ID NO: AA 68)	gi 30580423 sp Q8IX29 FX16_HUMAN, F-box only protein 16	Hs.511876	14-19 Score = 20.6 bits (41), Expect = 310 Identities = 5/6 (83%), Positives = 6/6 (100%) Query: 2 KMWRTKM KM+TKM Sbjct: 14 KMWRTKM	A high expression level of F-box protein, Skp2 is observed in 7 diffuse large cell B lymphoma.
5B9	gi 27469381 gb BC042411.1 , <i>Mus musculus</i> , clone IMAGE: 4014861, mRNA	17 QIDSSFSIP WVVVHGRS (SEQ ID NO: AA 69)	gi 126885 sp P08235 MCR_HUMAN, Mineralocorticoid receptor (MR)	Hs.331409	420-426 Score = 22.3 bits (45), Expect = 93 Identities = 6/7 (85%), Positives = 7/7 (100%) Query: 3 DSSFSIP DSSFS+P Sbjct: 420 DSSFSVP	Glucocorticoid and mineralocorticoid cross-talk with progesterone 9 receptor to induce focal adhesion and growth inhibition in breast cancer cells
5B12	gi 34996477 tpg BK001418.1 , TPA: <i>Homo sapiens</i> metastasis associated in lung adenocarcinoma transcript 1 long isoform, transcribed non-coding RNA, complete sequence	18 GGRSLRK POISFPLFER (SEQ ID NO: AA 70)	gi 34223735 sp Q08462 CYA2_HUMAN, Adenylate cyclase, type II (ATP pyrophosphate-lyase) (Adenyl cyclase)	Hs.414591	136-142 Score = 24.4 bits (50), Expect = 21 Identities = 6/7 (85%), Positives = 7/7 (100%) Query: 10 QISFPLF Q+SFPLF Sbjct: 136 QVSFPLF	In human Y-79 retinoblastoma cells, corticotropin-releasing hormone (CRH) stimulates 142 adenyl cyclase activity and increases cyclic AMP accumulation
5D6	gi 16741726 gb BC016660.1 , <i>Homo sapiens</i> heat shock 70 kDa protein 8, transcript variant 1, mRNA	12 GIRVEPPT TIS (SEQ ID NO: 71)	gi 6226869 sp P34932 HS74_HUMAN, HEAT SHOCK 70 KDA PROTEIN 4 (HEAT SHOCK 70-RELATED PROTEIN APG-2) (HSP70RY)	Hs.90093	316-328 Score = 21.0 bits (42), Expect = 229 Identities = 6/9 (66%), Positives = 8/9 (88%) Query: 3 RVEPPTRI RVEPP R++ Sbjct: 316 RVEPPLRSV	Expression of HSP70 is observed in human hepatocellular carcinoma 11
5E3	gi 40849693 gb AY495321.1 , <i>Homo sapiens</i> isolate V1-16 mitochondrion, complete genome	10 RNRYSTARE R (SEQ ID NO: 72)	gi 2501463 sp Q93008 FAFX_HUMAN, Probable ubiquitin carboxyl-terminal hydrolase FAF-X	Hs.77578	1356-1361 Score = 21.4 bits (43), Expect = 172 Identities = 6/6 (100%), Positives = 6/6 (100%)	Oxidative Modifications and Down-regulation of Ubiquitin

TABLE 6B-continued

5H8	gi 13273214 gb AAK17820 , cytochrome c oxidase subunit I [Homo sapiens]	GKRHIGTD Y (SEQ ID NO: 73)	10 AA	gi 41713338 sp Q9N690 D119_HUMAN Beta-defensin 119 precursor (Beta-defensin 19) (DEFB-19)	Corresponding 21-25 Unigene number Score = 19.3 bits (38), Expect = 550 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 GKRHI Sbjct: 21 GKRHI	10 Carboxyl-terminal 1361 Hydrolase L1 Associated with Idiopathic Parkinson's and Alzheimer's Diseases.
5A4	gi 17149463 gb AC068228.8 , Homo sapiens chromosome 8, clone RP11-539E17, complete sequence	VVSQLTAE RLE (SEQ ID NO: 74)	12 ID AA	gi 129825 sp P05164 PERM_HUMAN, Myeloperoxidase precursor (MPO)	23-29 Score = 22.7 bits (46), Expect = 71 Identities = 6/7 (85%), Positives = 7/7 (100%) Query: 5 LTAEMRL LTAEM+L Sbjct: 23 LTAEMKL	The expression of human beta-defensin genes in oral squamous cell carcinomas (SCCs) was demonstrated by in situ hybridization. Myeloperoxidase immunoreactivity is observed in adult acute lymphoblastic leukemia
5E7	gi 4885510 ref NM_005381.1 , Homo sapiens nucleolin (NCL), mRNA	RACQSTW KTEGNGQ TESSS (SEQ ID NO: 75)	21 AA	gi 25453064 sp Q9UPT6 JIP3_HUMAN, C-jun-amino-terminal kinase interacting protein 3 (JNK-interacting protein 3) (JIP-3)	192-199 Score = 23.1 bits (47), Expect = 51 Identities = 7/8 (87%), Positives = 7/8 (87%) Query: 13 GNGQTESS GN QTESS Sbjct: 192 GNSQTESS	JNK interacting protein (JIP) can inhibit JNK signaling pathway in NPC cell (nasopharyngeal carcinoma)
	gi 40849693 gb AY495321.1 , Homo sapiens isolate V1-16 mitochondrion, complete genome	RNRYSTARE R (SEQ ID NO: 72)	10 AA	gi 2501463 sp Q93008 FAFX_HUMAN, Probable ubiquitin carboxyl-terminal hydrolase FAF-X	1356-1361 Score = 21.4 bits (43), Expect = 172 Identities = 6/6 (100%), Positives = 6/6 (100%) Query: 5 STARE STARE Sbjct: 1356 STARE	Oxidative Modifications and Down-regulation of Ubiquitin Carboxyl-terminal Hydrolase L1 Associated with Parkinson's and Alzheimer's Diseases.

TABLE 6B-continued

gi 13273214 gb AAK17820 , cytochrome c oxidase subunit I [<i>Homo sapiens</i>]	GKRHIGTD Y (SEQ ID NO: 73)	10 AA	gi 41713338 sp Q8N690 D119_HUMAN Beta- defensin 119 precursor (Beta-defensin 19) (DEFB-19)	Corresponding 21-25 Unigene number is not found	Score = 19.3 bits (38), Expect = 550 Identities = 5/5 (100%), Positives = 5/5 (100%) Query: 1 GKRHI GKRHI Sbjct: 21 GKRHI	The expression of human beta- defensin genes in oral squamous cell 5 carcinomas (SCCs) was 25 demonstrated by in situ hybridization.
gi 17149463 gb AC068228.8 , <i>Homo sapiens</i> chromosome 8, clone RP11-539E17, complete sequence	VVSQLTAE RLE (SEQ ID NO: 74)	12 AA	gi 1259825 sp P05164 PERM_HUMAN, Myeloperoxidase precursor (MPO)	Hs.458272	23-29 Score = 22.7 bits (46), Expect = 71 Identities = 6/7 (85%), Positives = 7/7 (100%) Query: 5 LTAEMRL LTAEM+L LTAEMKL Sbjct: 23 LTAEMKL	Myeloperoxidase immunoreactivity is observed in adult acute lymphoblastic 11 leukemia 29
gi 4885510 ref NM_005381.1 , <i>Homo sapiens</i> nucleolin (NCL), mRNA	RACQSTWK TRKGGQTE SSS (SEQ ID NO: 75)	21 AA	gi 25453064 sp Q9UPT6 JIP3_HUMAN, C-Jun- amino-terminal kinase interacting protein 3 (JNK-interacting interacting protein 3) (JIP-3)	Hs.514335	192-199 Score = 23.1 bits (47), Expect = 51 Identities = 7/8 (87%), Positives = 7/8 (87%) Query: 13 GNGQTSS GN QTSS GNSQTSS Sbjct: 192 GNSQTSS	JNK interacting protein (JIP) can inhibit JNK signaling pathway in NPC cell cell 20 cell (nasopharyngeal 199 carcinoma)

TABLE 7A

Selection of most significant clones from Group 1 dataset

26 Clones ordered according to binding with the 16 patients in Group 1. None of the 25 healthy women's sera (belonging to Group 1) contained IgGs that any of these clones. Clones are shown in rows. Patients numbers are shown in the columns. The last column, TP, Total number of patients whose serum IgGs bound to each phage clone.

	Mec1	Mec2	Mec16	A	108A	400162	40015	OVC015	OVC035	OVC007	OVC005	NW 4679	NW 4283	OVC019	OVC087	OVC045	TP
2G2																	8
2G4																	8
2H9																	7
2B4																	6
4A3																	5
5A3																	5
3B12																	4
2H5																	4
2E12																	4
2E10																	3
2C7																	3
2F12																	3
2E11																	3
4H3																	3
4A11																	3
2A3																	2
3C11																	2
5G9																	2
4A4																	2
4B2																	2
5C12																	2
2C1																	1
5F9																	1
2F10																	1
3A9																	1
3C8																	1

A: Serum Dilution 1:3000; all others were analyzed at a serum dilution of 1:10000.

TABLE 7B

Binding of 26 clones with 16 Patients on a new dataset (Group 2)

The rows represent the 26 clones and the columns represent the 16 patients. As shown in this table, sera from 16 out of the 16 patients in Group 2 contained IgGs that bound at least one clone. None of IgGs in sera 12 healthy women interacted with any of these 26 clones.

	Mec20	Mec23	Mec37 ▲	060 ▲	42501	40036	42780	B755 ▲	OVC075	OVC063	NW4387	NW 4555	OVC078	OVC070	OVC049	OVC079	TP
2G2																	5
2G4																	4
2H9																	6
2B4																	6
4A3																	4
5A3																	3
3B12																	5
2H5																	4
2E12																	3
2E10																	5
2C7																	4
2F12																	4
2E11																	3
4H3																	3
4A11																	2
2A3																	6
3C11																	3
5G9																	3
4A4																	2
4B2																	2
5C12																	2
2C1																	4
5F9																	3
2F10																	2
3A9																	2
3C8																	2

A: Serum Dilution 1:3000; ▲: Serum Dilution 1:30000; all others were analyzed at a serum dilution of 1:10000.

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 SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 75

<210> SEQ ID NO 1

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: T7 coat protein forward primer

<400> SEQUENCE: 1

tcttcgccca gmgctgca

18

<210> SEQ ID NO 2

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: T7 coat protein reverse primer

<400> SEQUENCE: 2

cctcctttca gcaaaaaacc cc

22

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<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: EcoRI/HindIII Directional Linker

<400> SEQUENCE: 3

gcttgmttcm gc

12

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<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: HindIII site

<400> SEQUENCE: 4

aagctt

6

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<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: HindIII random primer

<220> FEATURE:

<221> NAME/KEY: misc_feature

<222> LOCATION: (3)..(8)

<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 5

ttnnnnnn

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<210> SEQ ID NO 6
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: T7 forward primer

<400> SEQUENCE: 6

tcttcgcca gaagcag 17

<210> SEQ ID NO 7
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: T7 reverse primer

<400> SEQUENCE: 7

cctccttcag caaaaacccc 20

<210> SEQ ID NO 8
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: forward PCR primer

<400> SEQUENCE: 8

gttctatccg caacgttatg g 21

<210> SEQ ID NO 9
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: reverse PCR primer

<400> SEQUENCE: 9

ggaggaaagt cgttttttgg gg 22

<210> SEQ ID NO 10
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: forward Sequencing primer

<400> SEQUENCE: 10

tgctaaggac mcgttatcg 19

<210> SEQ ID NO 11
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

Ala Ala Trp Gln Ala Glu Glu Val Leu Arg Gln Gln Lys Leu Ala Asp
1 5 10 15

Arg Glu Lys Arg Ala Ala Glu Gln Gln
20 25

<210> SEQ ID NO 12
<211> LENGTH: 212
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE

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<222> LOCATION: (23)..(23)

<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 12

Glu Lys Gly Gly Gln Glu Lys Gln Gly Glu Val Ile Val Ser Ile Glu
 1 5 10 15
 Glu Lys Pro Lys Glu Val Xaa Glu Glu Gln Pro Val Val Thr Leu Glu
 20 25 30
 Lys Gln Gly Thr Ala Val Glu Val Glu Ala Glu Ser Leu Asp Pro Thr
 35 40 45
 Val Lys Pro Val Asp Val Gly Gly Asp Glu Pro Glu Glu Lys Val Val
 50 55 60
 Thr Ser Glu Asn Glu Ala Gly Lys Ala Val Leu Glu Gln Leu Val Gly
 65 70 75 80
 Gln Glu Val Pro Pro Ala Glu Glu Ser Pro Glu Val Thr Thr Glu Ala
 85 90 95
 Ala Glu Ala Ser Ala Val Glu Ala Gly Ser Glu Val Ser Glu Lys Pro
 100 105 110
 Gly Gln Glu Ala Pro Val Leu Pro Lys Asp Gly Ala Val Asn Gly Pro
 115 120 125
 Ser Val Val Gly Asp Gln Thr Pro Ile Glu Pro Gln Thr Ser Ile Glu
 130 135 140
 Arg Leu Thr Glu Thr Lys Asp Gly Ser Gly Leu Glu Glu Lys Val Arg
 145 150 155 160
 Ala Lys Leu Val Pro Ser Gln Glu Glu Thr Lys Leu Ser Val Glu Glu
 165 170 175
 Ser Glu Ala Ala Gly Asp Gly Val Asp Thr Lys Val Ala Gln Gly Ala
 180 185 190
 Thr Glu Lys Ser Pro Glu Asp Lys Val Gln Ile Ala Ala Asn Glu Glu
 195 200 205
 Thr Gln Glu Arg
 210

<210> SEQ ID NO 13

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

Glu Lys Gly Gly Gln Glu Lys Gln Gly Glu Val Ile Val Ser Ile
 1 5 10 15

<210> SEQ ID NO 14

<211> LENGTH: 148

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

Met Ala Asp Asp Leu Asp Phe Glu Thr Gly Asp Ala Gly Ala Ser Ala
 1 5 10 15
 Thr Phe Pro Met Gln Cys Ser Ala Leu Arg Lys Asn Gly Phe Val Val
 20 25 30
 Leu Lys Gly Arg Pro Cys Lys Ile Val Glu Met Ser Thr Ser Lys Thr
 35 40 45
 Gly Lys His Gly His Ala Lys Val His Leu Val Gly Ile Asp Ile Phe
 50 55 60
 Thr Gly Lys Lys Tyr Glu Asp Ile Cys Pro Ser Thr His Asn Met Asp
 65 70 75 80

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Val Pro Asn Ile Lys Arg Asn Asp Phe Gln Leu Ile Gly Ile Gln Asp
85 90 95

Gly Tyr Leu Ser Leu Leu Gln Asp Ser Gly Glu Val Arg Glu Asp Leu
100 105 110

Arg Leu Pro Glu Gly Asp Leu Gly Lys Glu Ile Glu His Lys Phe Asp
115 120 125

Cys Gly Glu Gln Ile Leu Ile Thr Val Leu Ser Ala Met Thr Glu Glu
130 135 140

Ala Leu Val Ala
145

<210> SEQ ID NO 15
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

Gln Lys Thr Gly Gly Ala Asp Gln Ser Leu Gln Gln Gly Glu Gly Ser
1 5 10 15

Lys Lys Gly Lys Gly Lys Lys Lys Lys
20 25

<210> SEQ ID NO 16
<211> LENGTH: 68
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 16

Ala Leu Gln Ala Lys Ser Asp Glu Lys Ala Ala Val Ala Gly Lys Lys
1 5 10 15

Pro Val Val Gly Lys Lys Gly Lys Lys Ala Ala Val Gly Val Lys Lys
20 25 30

Gln Lys Lys Pro Leu Val Gly Lys Lys Ala Ala Ala Thr Lys Lys Pro
35 40 45

Ser Pro Glu Lys Lys Pro Ala Glu Asn Lys Pro Thr Thr Glu Asp Asn
50 55 60

Lys Pro Ala Ala
65

<210> SEQ ID NO 17
<211> LENGTH: 86
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 17

Gln Gln Gln Gln Gln Gln Gln His Gln Ala Ser Ser Asn Ser Gly Ser
1 5 10 15

Glu Glu Asp Ser Ser Ser Ser Glu Asp Ser Asp Asp Ser Ser Ser Glu
20 25 30

Val Lys Arg Lys Lys His Lys Asp Glu Asp Trp Gln Met Ser Gly Ser
35 40 45

Gly Ser Pro Ser Gln Ser Gly Ser Asp Ser Glu Ser Glu Glu Glu Arg
50 55 60

Glu Lys Ser Ser Cys Asp Glu Thr Glu Ser Asp Tyr Glu Pro Lys Asn
65 70 75 80

Lys Val Lys Ser Arg Lys
85

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<210> SEQ ID NO 18
<211> LENGTH: 101
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (80)..(100)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

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<400> SEQUENCE: 18

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Pro Thr Lys Leu Pro Ser Ile Asn Lys Ser Lys Asp Arg Ala Ser Gln
1          5          10          15
Gln Gln Gln Thr Asn Ser Ile Arg Asn Tyr Phe Gln Pro Ser Thr Lys
          20          25          30
Lys Arg Glu Arg Asp Glu Glu Asn Gln Glu Met Ser Ser Cys Lys Ser
          35          40          45
Ala Arg Ile Glu Thr Ser Cys Ser Leu Leu Glu Gln Thr Gln Pro Ala
          50          55          60
Thr Pro Ser Leu Trp Lys Asn Lys Glu Gln His Leu Ser Glu Asn Xaa
65          70          75          80
Pro Val Asp Thr Xaa Ser Xaa Xaa Asn Leu Phe Thr Gly Tyr Xaa Phe
          85          90          95
Arg Xaa Gly Xaa Glu
          100

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<210> SEQ ID NO 19
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 19

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Met Ala Asp Asp Leu Asp Phe Glu Thr Gly Asp Ala Gly Ala Ser Ala
1          5          10          15
Thr Phe Pro Met Gln Cys Ser Ala Leu Arg Lys Asn Gly Phe Val Val
          20          25          30
Leu Lys Gly Arg Pro Cys Lys Ile Val Glu Met Ser Thr Ser Lys Thr
          35          40          45
Gly Lys His Gly His Ala Lys Val His Leu Val Gly Ile Asp Ile Phe
          50          55          60
Thr Gly Lys Lys Tyr Glu Asp Ile Cys Pro Ser Thr His Asn Met Asp
65          70          75          80
Val Pro Asn Ile Lys Arg Asn Asp Phe Gln Leu Ile Gly Ile Gln Asp
          85          90          95
Gly Tyr Leu Ser Leu Leu Gln Asp Ser Gly Glu Val Arg Glu Asp Leu
          100          105          110
Pro Leu Pro Glu Gly Asp
          115

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<210> SEQ ID NO 20
<211> LENGTH: 121
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 20

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Pro Lys Phe Lys Met Pro Asp Val His Phe Lys Ser Pro Gln Ile Ser
1          5          10          15
Met Ser Asp Ile Asp Leu Asn Leu Lys Gly Pro Lys Ile Lys Gly Asp
          20          25          30
Met Asp Ile Ser Val Pro Lys Leu Glu Gly Asp Leu Lys Gly Pro Lys
          35          40          45

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-continued

Val Asp Val Lys Gly Pro Lys Val Gly Ile Asp Thr Pro Asp Ile Asp
 50 55 60

Ile His Gly Pro Glu Gly Lys Leu Lys Gly Pro Lys Phe Lys Met Pro
 65 70 75 80

Asp Leu His Leu Lys Ala Pro Lys Ile Ser Met Pro Glu Val Asp Leu
 85 90 95

Asn Leu Lys Gly Pro Lys Val Lys Gly Asp Met Asp Ile Ser Leu Pro
 100 105 110

Lys Val Glu Gly Asp Leu Lys Gly Pro
 115 120

<210> SEQ ID NO 21
 <211> LENGTH: 150
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <222> LOCATION: (127) .. (127)
 <223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 21

Gly Val Cys Ser Ser Lys Val Tyr Val Gly Lys Asn Thr Ser Glu Val
 1 5 10 15

Lys Glu Asp Val Val Leu Gly Lys Ser Asn Gln Val Cys Gln Ser Ser
 20 25 30

Gly Asn His Leu Glu Asn Lys Val Thr His Gly Leu Val Thr Val Glu
 35 40 45

Gly Gln Leu Thr Ser Asp Glu Arg Gly Ala His Ile Met Asn Ser Thr
 50 55 60

Cys Ala Ala Met Pro Lys Leu His Glu Pro Tyr Ala Ser Ser Gln Cys
 65 70 75 80

Ile Ala Ser Pro Asn Phe Gly Thr Val Ser Gly Leu Lys Pro Ala Ser
 85 90 95

Met Leu Glu Lys Asn Cys Ser Leu Gln Thr Glu Leu Asn Lys Ser Tyr
 100 105 110

Asp Val Lys Asn Pro Ser Pro Leu Leu Met Gln Asn Gln Asn Xaa Arg
 115 120 125

Gln Gln Met Asp Thr Pro Met Val Ser Cys Gly Asn Glu Gln Phe Leu
 130 135 140

Asp Asn Ser Phe Glu Lys
 145 150

<210> SEQ ID NO 22
 <211> LENGTH: 5
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 22

Glu Leu Leu Arg Thr
 1 5

<210> SEQ ID NO 23
 <211> LENGTH: 5
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 23

Gly Gln Thr Ser Met
 1 5

-continued

<210> SEQ ID NO 24
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 24

Lys Lys Gly Pro Ile
1 5

<210> SEQ ID NO 25
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

Ala Lys Val Ile Met Arg
1 5

<210> SEQ ID NO 26
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 26

Tyr Ala Cys Leu Lys Asp
1 5

<210> SEQ ID NO 27
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 27

Gln Ile Leu Phe Met Asp Pro
1 5

<210> SEQ ID NO 28
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 28

Leu Asn Thr Val Asn Thr Leu Ile
1 5

<210> SEQ ID NO 29
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 29

Gly Asn Ser Ile Leu Leu Ile Ala
1 5

<210> SEQ ID NO 30
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 30

Trp Asp Leu Lys Ser Glu Tyr Ser
1 5

<210> SEQ ID NO 31
<211> LENGTH: 8

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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 31

Pro Gly Cys Ser Thr Thr Leu Ser
1 5

<210> SEQ ID NO 32
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 32

Pro Arg Cys Ser Thr Thr Leu Ser
1 5

<210> SEQ ID NO 33
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 33

Gly Asp Arg Ser Gln Leu Trp Arg Lys
1 5

<210> SEQ ID NO 34
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 34

Lys Lys Gln Ser Ser Trp Tyr Gln Ile
1 5

<210> SEQ ID NO 35
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 35

Lys Lys Gln Ser Ser Trp Tyr Gln Ile
1 5

<210> SEQ ID NO 36
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 36

Pro Glu Gly Gly Thr Asp Ala Ser Arg
1 5

<210> SEQ ID NO 37
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 37

Ala Ser Phe Thr Leu Lys Leu Gln Ser
1 5

<210> SEQ ID NO 38
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

-continued

<400> SEQUENCE: 38

Gly	Gly	Gly	Ser	Asn	Gly	Arg	Thr	Ser	Val
1				5					10

<210> SEQ ID NO 39

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 39

Asn	Ser	Phe	Leu	Met	Thr	Ser	Ser	Lys	Pro	Arg
1				5					10	

<210> SEQ ID NO 40

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 40

Ala	Cys	Ser	Ser	Thr	Val	Ser	Phe	Ile	Trp	Ile
1				5					10	

<210> SEQ ID NO 41

<211> LENGTH: 14

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 41

Lys	Lys	Lys	Lys	Lys	Lys	Lys	Arg	Val	Gly	Gly	Pro	Leu	Gln
1				5					10				

<210> SEQ ID NO 42

<211> LENGTH: 14

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 42

Gly	Pro	Val	Phe	Ile	Cys	Ser	Ser	Asn	Cys	Phe	Lys	Ile	Thr
1				5					10				

<210> SEQ ID NO 43

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 43

Ala	Pro	Phe	Thr	Cys	Trp	Pro	Thr	Val	Ala	Thr	Asn	Thr	Trp	Glu
1				5					10				15	

<210> SEQ ID NO 44

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

Thr	Asp	Gln	Ser	Ser	Ile	Ser	Pro	Gly	Asn	Arg	Lys	Ala	Pro	Gly
1				5					10				15	

<210> SEQ ID NO 45

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 45

Arg	Ile	Met	Gly	Gly	Gly	Ile	Gln	Arg	Glu	Thr	Trp	Ile	Ser	Ser
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1	5	10	15
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<210> SEQ ID NO 46
 <211> LENGTH: 16
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 46
 Cys Gly Ser Trp Gly Lys Tyr Asn Leu Trp Gln Ser Ser Ser Ser Lys
 1 5 10 15

<210> SEQ ID NO 47
 <211> LENGTH: 18
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 47
 Glu Ile Leu Lys Pro Glu Gly Gln His Met Lys Leu Arg Ser Glu Glu
 1 5 10 15
 Thr Ser

<210> SEQ ID NO 48
 <211> LENGTH: 21
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 48
 Ala Lys Ala Arg Ala Leu Ala Arg Arg Ser Glu Pro Cys Ser Thr Gly
 1 5 10 15
 Lys Leu Gln Leu Arg
 20

<210> SEQ ID NO 49
 <211> LENGTH: 23
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 49
 Val Gln Arg Gly Ile Gly Thr Ile Pro Ser Glu Thr Ile Pro Val Asn
 1 5 10 15
 Arg Lys Arg Val Asn Pro Pro
 20

<210> SEQ ID NO 50
 <211> LENGTH: 28
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 50
 Val Ser Trp Phe Pro Ser Trp Ala Arg Ser Cys Gly Arg Gln Thr Pro
 1 5 10 15
 Leu Gly Ala Thr Tyr Lys Asp Thr Leu Leu Pro Val
 20 25

<210> SEQ ID NO 51
 <211> LENGTH: 35
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 51
 Asp Leu Gln Pro Pro Gly Arg Arg Trp Leu Pro Gln Gln Cys Pro Gly
 1 5 10 15
 Ser Pro Gly Arg Cys Asp Ala Ser Val Pro Leu Trp Ser Asp His Leu

-continued

20	25	30
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Pro Ser Leu
35

<210> SEQ ID NO 52
 <211> LENGTH: 36
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 52

Arg Gly Leu Gly	Pro Leu Ala Ala	Ala Cys Gly Arg Ser Gly Gly Gly
1	5	10 15

Gly Gly Gly Gly	Ala Gly Gly Thr	Gly Ser Ser Asn Val Asn Lys Lys
20	25	30

Thr Pro Pro Asn
35

<210> SEQ ID NO 53
 <211> LENGTH: 37
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 53

Pro Met Arg Cys	Ser Cys Thr Met	Gly Glu Ile Gln Met Gln Ile His
1	5	10 15

Cys Gly Ala Arg	Arg Arg Lys Ala	Val Pro Ser Ser Lys Asp Asn Val
20	25	30

Gln Ser Ser Ala His
35

<210> SEQ ID NO 54
 <211> LENGTH: 48
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 54

Trp Arg Thr Thr	Tyr Ile Ser Ile	Leu Asn Leu Ala Gln Phe Tyr Tyr
1	5	10 15

Ser Leu Ile Thr	Val Leu Lys Thr	Phe Asn Trp Pro Gly Thr Val Val
20	25	30

His Ala Cys Asn	Pro Ser Thr	Leu Gly Gly Gln Gly Arg Arg Ile Thr
35	40	45

<210> SEQ ID NO 55
 <211> LENGTH: 103
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (14)..(14)
 <223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 55

Asp Asp Asp Ser	Asp Tyr Gly Ser	Ser Lys Lys Lys Asn Xaa Lys Met
1	5	10 15

Val Lys Lys Ser	Lys Pro Glu Arg	Lys Glu Lys Lys Met Pro Lys Pro
20	25	30

Arg Leu Lys Ala	Thr Val Thr	Pro Ser Pro Val Lys Gly Lys Gly Lys
35	40	45

Val Gly Arg Pro	Thr Ala Ser	Lys Ala Ser Lys Glu Lys Thr Pro Ser
50	55	60

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Pro Lys Glu Glu Asp Glu Glu Pro Glu Ser Pro Pro Glu Lys Lys Thr
65              70              75              80

Ser Ile Ser Pro Pro Pro Glu Lys Ser Gly Asp Glu Gly Ser Glu Asp
85              90              95

Glu Ala Pro Ser Gly Glu Asp
100

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<210> SEQ ID NO 56
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 56

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Leu Ser Thr Ser Ser Phe Asp Glu Gln Asn
1      5              10

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<210> SEQ ID NO 57
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 57

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Val Ile Val Val Leu Ile Ala Val Ile Ser Phe Pro Gln Asn Tyr Thr
1      5              10              15

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Trp Leu

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```

<210> SEQ ID NO 58
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

```

```

<400> SEQUENCE: 58

```

```

Pro Met Arg Cys Ser Cys Thr Met Gly Glu Ile Gln Met Gln Ile His
1      5              10              15

```

```

Cys Gly Ala Arg Arg Arg Lys Ala Val Pro Ser Ser Lys Asp Asn Val
20              25              30

```

```

Gln Ser Ser Ala His
35

```

```

<210> SEQ ID NO 59
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

```

```

<400> SEQUENCE: 59

```

```

Leu Arg Gly Thr Ser Gly Val Gln Pro Pro Glu Ile Glu Gln
1      5              10

```

```

<210> SEQ ID NO 60
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

```

```

<400> SEQUENCE: 60

```

```

Ile Leu His Leu His
1      5

```

```

<210> SEQ ID NO 61
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

```

```

<400> SEQUENCE: 61

```

-continued

Val	Leu	Ser	Ala	Leu	Pro	Glu	Lys	Asn	Cys	Asn	Thr	Val	Pro	Phe	Gln
1				5					10					15	

Pro	Pro	Glu	Asp	Leu	Arg	Tyr	Gln	His	Cys	Ser	Ser	Arg	Phe	Leu	Glu
			20					25					30		

<210> SEQ ID NO 62
 <211> LENGTH: 20
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 62

Trp	Gly	Phe	Asn	Glu	Arg	Asp	Arg	Leu	Ser	Ser	Ile	Leu	Gln	Gln	Arg
1			5					10					15		

Cys	Val	Thr	Leu
			20

<210> SEQ ID NO 63
 <211> LENGTH: 11
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 63

Val	Val	Ser	Gly	Phe	Phe	Ser	Thr	Phe	Ser	Leu
1			5					10		

<210> SEQ ID NO 64
 <211> LENGTH: 9
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 64

Leu	Thr	Arg	Pro	Gly	His	Gly	Gln	Asp
1				5				

<210> SEQ ID NO 65
 <211> LENGTH: 11
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 65

Leu	Tyr	Ile	Asn	Glu	Met	Lys	Ser	Lys	Lys	Leu
1			5					10		

<210> SEQ ID NO 66
 <211> LENGTH: 10
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 66

Leu	Pro	Gln	Cys	Pro	Ser	Arg	Gly	Ser	Leu
1			5					10	

<210> SEQ ID NO 67
 <211> LENGTH: 23
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 67

Pro	Gly	Trp	Asp	Cys	Arg	Leu	Pro	Glu	Ala	Glu	Ser	Cys	Arg	Phe	Leu
1				5					10				15		

Leu	Ser	Ser	Arg	Gly	Glu	Asp
				20		

<210> SEQ ID NO 68

-continued

<211> LENGTH: 7
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 68

Lys Lys Met Arg Thr Lys Met
 1 5

<210> SEQ ID NO 69
 <211> LENGTH: 17
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 69

Gln Ile Asp Ser Ser Phe Ser Ile Pro Trp Val Val Val His Gly Arg
 1 5 10 15

Ser

<210> SEQ ID NO 70
 <211> LENGTH: 18
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 70

Gly Gly Arg Arg Ser Leu Arg Lys Pro Gln Ile Ser Phe Phe Leu Phe
 1 5 10 15

Glu Arg

<210> SEQ ID NO 71
 <211> LENGTH: 12
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 71

Gly Ile Arg Val Glu Pro Pro Thr Arg Thr Ile Ser
 1 5 10

<210> SEQ ID NO 72
 <211> LENGTH: 10
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 72

Arg Asn Arg Tyr Ser Thr Ala Arg Glu Arg
 1 5 10

<210> SEQ ID NO 73
 <211> LENGTH: 10
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 73

Gly Lys Arg His Ile Gly Gly Thr Asp Tyr
 1 5 10

<210> SEQ ID NO 74
 <211> LENGTH: 12
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 74

Val Val Ser Gln Leu Thr Ala Glu Met Arg Leu Glu
 1 5 10

-continued

<210> SEQ ID NO 75
 <211> LENGTH: 21
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 75

Arg Ala Cys Gln Arg Ser Thr Trp Lys Thr Lys Glu Gly Asn Gly Gln
 1 5 10 15

Thr Glu Ser Ser Ser
 20

What is claimed is:

1. A biosensor for use in detecting the presence of ovarian cancer, said biosensor comprising an immunoassay for detecting a presence of a serum antibody reactive with at least one marker indicative of ovarian cancer, said at least one marker being selected from the group consisting of a plurality of polypeptides, each of said polypeptides including one of SEQ ID NOS: 59, 62, 65, 66, 69, 70, 71, 72, and 74, each of said plurality of polypeptides being either an isolated phage display antigen clone or an isolated polypeptide, and a phage display antigen clone including a polypeptide display insert consisting of SEQ ID NO 75, wherein the immunoassay comprises said at least one marker.

2. The biosensor according to claim 1, wherein said immunoassay is selected from the group consisting of a microarray immunoassay, a macroarray immunoassay, a slide immunoassay, a bead immunoassay, an ELISA and a filter immunoassay.

3. A diagnostic tool for determining the efficacy of a pharmaceutical for treating ovarian cancer, said tool comprising: an immunoassay for detecting a presence of a serum antibody reactive with at least one marker indicative of ovarian cancer, and analyzing means operatively connected to said immunoassay, said analyzing means for determining fluctuations in amount of said serum antibody reactive with said at least one marker present in said immunoassay, whereby said fluctuations correlate to pharmaceutical efficacy, said at least one marker being selected from the group consisting of a plurality of polypeptides, each of said polypeptides including one of

SEQ ID NOS: 59, 62, 65, 66, 69, 70, 71, 72, and 74, each of said plurality of polypeptides being either an isolated phage display antigen clone or an isolated polypeptide, and a phage display antigen clone including a polypeptide display insert consisting of SEQ ID NO 75, wherein the immunoassay comprises said at least one marker.

4. An antigen array for use in detecting the presence of ovarian cancer, said array consisting of the set of polypeptides SEQ ID NOS: 59, 62, 65, 66, 69, 70, 71, 72, 74, and 75, each of said polypeptides being either an isolated phage display antigen clone or an isolated polypeptide.

5. Markers for detecting the presence of ovarian cancer selected from the group consisting of a plurality of polypeptides, each of said polypeptides including one of SEQ ID NOS: 59, 62, 65, 66, 69, 70, 71, 72, and 74, and a polypeptide consisting of SEQ ID NO: 75, each of said polypeptides being either an isolated phage display antigen clone or an isolated polypeptide.

6. An immuno-imaging agent comprising labeled antibodies, whereby said labeled antibodies are isolated and reactive to at least one marker selected from the group consisting of a plurality of polypeptides, each of said polypeptides including one of SEQ ID NOS: 59, 62, 65, 66, 69, 70, 71, 72, and 74, each of said plurality of polypeptides being either an isolated phage display antigen clone or an isolated polypeptide, and a phage display antigen clone including a polypeptide display insert consisting of SEQ ID NO 75.

7. The agent according to claim 6, wherein said antibodies are identified by the biosensor of claim 1.

* * * * *

一种用于检测疾病存在的生物传感器，该生物传感器包括用于检测指示特定疾病的至少一种标记物的存在的检测器。一种通过向含有疾病标记物的样品施用药物，检测样品中至少一种疾病标记物的量，以及分析标记物的量来确定药物治疗疾病或分期疾病的功效的方法在样品中，标记物的量与药效或疾病阶段相关。用于妇科疾病的标记物选自表6中的列表。包含标记抗体的免疫成像剂，其中标记的抗体被分离并对体内过表达的蛋白质具有反应性。用于分析权利要求4的阵列的信息学软件，该软件包括用于分析阵列的分析装置。

[illegible]