Epitomics: Serum screening for the early detection of cancer on microarrays using panels of tumor antigens

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Abstract

Approximately 25,000 women in the United States are diagnosed each year with ovarian cancer. The disease presents with largely nonspecific symptoms during the initial stages of cancer progression and there is currently no adequate screening or diagnostic test for early stage detection of ovarian cancer. Consequently, most ovarian cancers are diagnosed only when later stage symptoms are presented. At this time, the cancer has usually metastasized to other parts of the body and the prognosis is extremely poor. The five-year survival rate for late stage diagnosis is less than 20%. In contrast, there is an approximate 90% survival rate if the disease is identified at the earliest stages when surgical intervention is usually successful. The inability to detect ovarian cancer in its early stages earmarks the disease as a "silent killer". Efforts toward the development of early detection assays for cancers have traditionally depended on single biomarker molecules. Current technologies have been disappointing and have not resulted in diagnostic tests suitable for clinical practice. Using a high throughput cloning method we have isolated large numbers of antigens reacting with autoantibodies to tumor proteins in the serum of the patients having ovarian cancer. The sequences that we identify using our new technology will lead to the discovery of novel disease-related proteins that have diagnostic value for the pre-symptomatic detection of ovarian cancer. The validation and characterization of these serum antitumor antibodies using microarrays and advanced bioinformatics tools led to the development of a diagnostic antigen panel. We refer to this technology of global antigen profiling as "Epitomics".

Introduction:

When epithelial ovarian cancer (EOC) is identified at the earliest stage, the cure rates tend to be very high (80-90%) and therefore early detection tests are crucial to reducing the morbidity and mortality of this disease. However, of the 25,000 women in the U.S. who will be diagnosed this year with ovarian cancer the vast majority, more than 75%, will be diagnosed at stages III and IV where cures are frequently less than 20%. The goal of this project is to develop a simple non-invasive screening test for early stage ovarian cancer and determine the most feasible form for full implementation of this test. We need to detect ovarian cancer at a curable stage, Stage I, when the cancer has not left the ovary. Implementation of this diagnostic test will allow surgeons to routinely cure women with their scalpels.

Markers for Ovarian Cancer

A variety of ovarian tumor markers have been studied and the most extensively investigated of these is CA125. This antigen was first recognized in 1981, using a murine monoclonal antibody developed in response to immunological challenge with an ovarian cancer cell line [1]. CA125 levels were found to be increased in 50% of stage I and 90% of stage II ovarian cancers [1-4]. Although sensitivity for stage I disease using a simple cut-off of 35U/ml was limited, it was apparent that CA125 was capable of detecting some ovarian cancer preclinically.

A variety of other tumor markers have also been studied in ovarian cancer (Table 1). Many of these have been shown to be of insufficient sensitivity or specificity regarding epithelial ovarian tumors. Among them, carcinoembryonic antigen (CEA) has been reported to be elevated in 30%-65% of epithelial tumors, mainly in patients with advanced stage disease [5]. CA19-9 is another carbohydrate antigen that can be found elevated in only 17%-25% of patients with epithelial malignancies [5]. Lipid associated sialic acid (LSA) can be detected in serum of about 60% of patients with advanced stage

disease and many other cancers as well [6]. The interleukins, IL-6 and IL-10, have been shown to be present in high levels in the ascites and serum of women with advanced stage epithelial cancer [7,8]. Measurement of serum levels of tumor-associated antigen CA125 [9], in conjunction with ultrasound screening as a second-line test, confers high specificity [10] but detects only approximately one half of early stage cases [3].

Other new markers such as tetranectin or Cancer associated serum antigen have not provided additional discriminative value [11]. Serum levels of EGF and its receptor are significantly different between ovarian cancer patients and healthy women and they may provide a potential diagnostic and/or prognostic marker useful for the management of recurrence and late stage cancer [12]. HOXB7 was recently found to be a tumor antigen whose up-regulated expression could play a role in promoting growth of ovarian carcinomas [13].

Use of multiple serum markers may provide a more sensitive test. Urban et al. have characterized the behavior of five serum tumor markers in a large cohort of healthy women [14]. Serial measurements of CA125, HER2/neu, urinary gonadotropin peptide, lipid-associated sialic acid, and Dianon marker 70/K during 6 years of follow-up of 1257 healthy women at high risk of ovarian cancer showed that the individual-specific tumor markers behaved independently with substantial heterogeneity among high-risk but cancer-free women. The use of a combination of markers to increase sensitivity and specificity has been extensively investigated and the marker that appears to exhibit the most complementarity to CA125 is OVX1, a monoclonal antibody developed using sequential immunization with three different ovarian cancer cell lines [15]. OVX1 is elevated in 70% of patients with clinically evident ovarian cancer. In addition, 59% of patients with normal CA125 levels have increased OVX1, suggesting complementarity between these two markers. Although these results indicate improvement in

sensitivity, preliminary data from different laboratories suggest that OVX1 may be unstable unless serum is rapidly separated, which could complicate its use in population screening if samples are sent by post. Another serum marker, macrophage colony stimulating factor, macrophage colony stimulating factor (M-CSF) [16] and is complementary to both OVX1[15] and CA125. Among 25 patients with clinically evident tumors and a negative CA125, 56% had an elevated M-CSF serum level [17]. M-CSF and OVX1 identify a percentage of patients with persistent ovarian cancer who had normal CA125 levels prior to second look surgical staging procedures. Recently Mor et al. [18] have reported the identification of four analytes (serum proteins/antigens) namely leptin, prolactin, osteopontin and insulin-like growth factor-II. They identified these markers using antibody microarray analysis to detect the levels of 169 proteins in the serum from 28 healthy women, 18 women newly diagnosed with EOC, and 40 women with recurrent diseases. ELISA assays of those four proteins were chosen that could discriminate between healthy women and patients with EOC (stage I-II) with a sensitivity, specificity and accuracy of 95%. Although the ELISA assays of those four proteins showed significant differences in the serum protein levels between controls and cancer patients, these markers must still be validated in sera from patients with other cancers or benign conditions. Studies on new technology involving generation of 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 to screen for early stage ovarian cancer have been reported by Petricoin et al. (2002)[19]. In such a proteomic assay on 116 serum samples (50 women with ovarian cancer, 66 women free of such malignancy), ovarian cancer was detected with sensitivity of 100% and specificity of 95%. 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 [20-25]. Three biomarkers namely, apolipoprotein, transthyretin, inter-∝-trypsin inhibitor heavy chain H4 from the serum proteomic analysis were identified for the detection of early stage ovarian cancer. The proteomic expression

profiling using the Proteinchip® biomarker system, a platform for surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy, showed that in an independent validation to distinguish early stage invasive EOC from healthy controls. The sensitivity of a multivariate model combining the three biomarkers and CA125 (74%) and it was higher that that of CA125 alone (65%) at a matched specificity of 97% [26].

Cancer Antigens

Cancer antigens are useful targets for diagnostics based on the immune responses of the host to various classes defined by mutations and/or changes in expression. SEREX is an antigen identification method that allows the screening of cDNA expression libraries using autologous sera derived from patients with cancer [27]. The SEREX approach has been used since 1995 to discover multiple novel tumor antigens in various types of cancers. The repertoire of SEREX-identified antigens can be found in a Cancer Immunome Database [28] that includes more than 1000 antigen entries. The utility of the markers for diagnostic purposes is under evaluation but SEREX antigens have yet to be proven to have definitive diagnostic value in clinical practice.

Arrays of antibodies and antigens are being used for research diagnostic tests. Shi et al., have used a modified SEREX approach to clone antigens and then study their utility as biomarkers of serum antibodies in the sera of patients with hepatocellular carcinoma. 2D liquid chromatography has been used to separate cell extracts into over 1000 fractions, which were then arrayed for an ELISA-type assay [29,30]. Most of the antibodies identified by reacting with the immunoglobulins in the sera of patients with cancer by this method are directed to carbohydrate portions of the antigens.

Cloning of Genes Coding for Antigens as Cancer Biomarkers of Serum Antibodies

Our technology detects antibodies that are produced by patients in reaction to proteins overexpressed or mutated in their ovarian tumors and uses them as diagnostic biomarkers. The serum antibodies have been detected by screening of large numbers of potential epitope targets on protein microarrays. We chose to direct discovery of biomarker panels in an unbiased fashion using a high throughput method to clone the cDNAs for large numbers of epitopes, which can indicate the presence of cancer. Epitopes in this context are sequences of amino acids within proteins that react with the antibodies present in the serum of ovarian cancer patients. These epitope biomarkers were cloned without a previous notion of their function. We screen large numbers of potential markers on protein microarrays spotted with proteins expressed on these phage cDNA clones. The T7 cDNA clones are genetically engineered to display the amino acid epitopes on their surface. The essential feature of the approach is the acknowledgment of the heterogeneous nature of any specific type of cancer. Departing from the reliance on any single marker for detection, we employ specialized data informatics analysis to interpret the results. This unbiased high throughput identification of tumor-related antigens is unique in the field and employs the patient's serum antitumor immunoglobulin-G molecules (IgGs) both as the bait in cloning and to detect cancer. Although the concept employing recognition of a pattern of immunologic response as a diagnostic strategy in not entirely novel, this is a new technological platform for cancer detection and ovarian cancer is the first target disease to which we are applying it. The identification of a panel of diagnostic antigens will lead us to other applications of this novel form of molecular profiling such as discovery of genes/proteins in cancer etiology.

We have prepared cDNA T7 phage display libraries to isolate cDNAs coding for epitopes reacting with antibodies present specifically in the sera of patients with ovarian cancer. The isolation of these antigens was achievable by our differential biopanning technology using human sera collected both

from healthy controls and patients having ovarian cancer. We screened phage display OVCA tumor cell expression libraries for proteins that could bind to IgGs present in OVCA patients' sera and could not bind to IgGs from normal sera (Figure 1). The serum reaction with large numbers of these epitopes is detected in a highly parallel assay on protein microarrays (Figure 2). The plan was to clone epitopes specifically reacting with IgG in patients' sera and to use the cloned epitopes that bind antibodies in subjects' sera to discriminate cancer and healthy subjects. This approach identifies antigen biomarkers without a preconceived notion as to their function.

A major advantage of our system is that we detect specific IgGs in serum. The IgG is likely the most stable, functional protein in serum. If a test uses a panel of serum proteins other than IgGs, it is likely that the different analytes will have different stabilities or half-lives in serum and therefore be subject to processing variations. Serum analytes that are labile are subject to inter-laboratory variations due to sample handling differences. There should be no inter-analyte stability variation among our markers because they are all IgGs. We have successfully used sera from many other laboratories and serum banks. In the future, we also anticipate that these biomarkers will lead to an integrated approach to cancer detection and treatment using specific immunotherapy or imaging reagents personalized to each patient. These latter issues are outside the scope of this review but are important future applications of this technology.

Antigen Discovery on Microarrays

The process of antigen discovery and detection is rapid and the evaluation of immunoglobulin reactivity is objective. Robotic production of these microarrays containing 100 μm spots of each potential antigen biomarker in quadruplicate on microarrays on nitrocellulose coated glass slides permits us to perform many assays in parallel on each patient's serum. A dilution of the serum being

tested is applied to the microarray and the excess fluid is removed after 1 hour. We employ a Cy5 (red fluorescent dye) to label an antibody to human IgG to detect patient serum IgGs binding to clones on the microarrays. We then compare that red fluorescent color representing the binding of the subject IgG molecules to a spot of phage proteins with a Cy3 (green fluorescent dye) labeled antibody to the T7 phage capsid protein (Figure 2A). This latter reagent binds to every T7 phage clone and serves to normalize for variations in the amount of each antigen clone at each spot (Figure 2B). When we analyze the dye ratio values of the antigen clones using multiple patient serum samples, we find a consistent set of antigen clones are identified in microarrays. We perform these microarray assays at a 1:300 dilution of each patient's serum. We only analyze the digitized dye ratio data derived from the images by IMAGENE^{TM} software. We do not choose clones by visual inspection.

Informatics for Antigen Microarrays

The central idea of our approach is that cancer is a complex illness whose evolution over time is determined by a complex set of factors that depend both on the etiology of the illness and its interaction with the immune system of the specific patient. Our main hypothesis is that any single marker will not have the ability to capture the complexities of this interaction whereas a set of markers will have a much better potential to do so. We investigated several approaches able to construct classifiers using multiple markers. Three such approaches were compared in detail: decision trees, voted perceptrons and neural networks. For each such paradigm, we used the training set to build a number of 10 classifiers, which were subsequently tested on a separate testing set. We found with 90% confidence intervals for these performance indicators and that the neural network paradigm performs better for this application. The neural network used in this case was a 3-layer backpropagation network. We also explored other neural network approaches such as support vector machines (SVMs) and radial basis functions (RBFs) but so far, we could not see any significant performance difference between these.

Therefore our subsequent studies were performed using this 3-layer network trained with backpropagation.

Building a Classifier using Multiple Markers

In order to estimate the performance of the ovarian cancer protein classifier, we used training and validation sets using sera from subjects not used in any of the phases that led to the fabrication of the array (i.e. not involved in the biopanning or clone selection as patients or as controls). The performance on this validation data is reported in terms of specificity, sensitivity, accuracy, positive predictive value and negative predictive value. Because these performance indicators are calculated on data from sera not previously used, they provide a good indication of the performance of the test for screening purposes in the general population. We first demonstrated that our current neural network trained on 480 clones provides high accuracy by using 10 independent partitions of the data and averaging the results. In these analyses we used the top antigens of the 480 total antigens. In each partition, we selected those top antigens significant at $p<0.1$. This was done using sera from 110 OVCA patients (~30% early stage patients) and 110 healthy controls. Each of the 10 partitions had a 66%:34% split between the training and the test sets. We define our results as the averages of the 10 partitions. (Sensitivity 92%, Specificity 93%, Accuracy 92.5%, Positive Predicted Value 91.6%, and Negative Predicted Value 93.5%), (Figure 3). As expected in our randomization scheme, each patient was found in a test set 3.4 times, on average.

This approach led to 10 sets of antigens, each set containing the clones significant in each partition. To identify a single, unique set of antigens we next selected 166 antigen clones that appeared in 9 out of the 10 lists of antigens. We tested this unique set of antigens in ten rounds of training. Each round of training was performed by dividing the 220 samples into random training sets and validation sets as

above. In each case, we constructed a neural network using the training set and tested it using the validation set. Performing this 10 times ensured that the results are not influenced by any specific choice of samples between the training and validation sets and offered the basis for estimating mean and variances for the chosen performance parameters (Sensitivity 89%, Specificity 92%, Accuracy 91%, Positive Predicted Value 90%, and Negative Predicted Value 91%) (Figure 3).

We further improved the specificity by using an architecture involving two neural networks in a cascade in which the first neural network was trained for sensitivity (to avoid false negatives, missing a true cancer) and then a second neural network trained for specificity (to avoid false positives, calling a healthy woman as cancer). Again, each experiment was performed on 10 independent random partitions of the samples to the training and test sets. This method increased the accuracy by over 4% to 95%. Using a partial area under the ROC curve we found the optimal values of 3 markers: RCAS1, Nibrin and EIF5A. We found a sensitivity of 75% and specificity of 86% but if the thresholds were optimized for specificity at 100% we found our sensitivity was 56%.

This reproducible level of performance exceeds those of PSA (65% sensitivity and 15.6-26.7% specificity) and NMP22 (62.5% sensitivity and 65.9% specificity) (Figure 3), which are the only FDA-approved biomarkers for serum testing for cancer of the prostate and bladder, respectively. CA125 is not currently FDA-approved for presymptomatic screening for ovarian cancer and its performance for early stage ovarian cancer suffers from low sensitivity and poor specificity in that it often identifies patients with other ailments including benign gynecological conditions. Most researchers agree that the early detection of cancer will require a panel of molecular markers.

Building a classifier epitope marker set able to distinguish stage I from late stage ovarian cancer

We next developed a training set using sera from 10 stage 1 patients and 20 healthy controls and identified 78 antigen clones, using a t-test, that were selective for cancer vs healthy with a p-value <0.01. We trained a neural network using these antigen clones and validated it on an independent test set of 14 stage 1 patients and 10 controls. This neural network was 100% accurate. Therefore our antigen clones can identify stage 1 ovarian cancer.

Phage-coded Antigens: Identification Through DNA Sequence Analysis.

We PCR amplified and DNA sequenced the inserts from clones that reacted with OVCA sera. The DNA sequences were checked for homology to the GenBank databases using BLASTn. These gene sequences were next translated to determine predicted amino acids in frame with the T7 surface capsid protein using EXPASY TRANSLATE TOOL. The gene products that were in the correct orientation and in the correct reading frame with the T7 gene 10 capsid protein indicated that the region of IgG binding was localized to a portion of the natural open reading frame of the protein.

Overexpression of Antigens in Ovarian Cancers

One of the genes that we identified four times is a cancer antigen, RCAS1, a protein that was also described as EBAG9 as it binds estrogen receptor-binding sites and is itself regulated at the transcriptional level by estrogen. Alone this marker has a sensitivity and specificity of more than 80%. The fact that our unbiased screening method was able to retrieve a known cancer antigen confirmed the efficacy of our approach. RCAS1 was found by others to be overexpressed in 51.1% of ovarian cancers [31]. This shows that our technology is indeed identifying overexpressed tumor antigens. We confirmed this observation using immunohistochemistry with commercially available antibodies to some of the antigen proteins. We found that RCAS1 was highly expressed in 23 out of 30 early stage

and 27 out of 30 late stage tumors. Likewise another antigen biomarker EIF5A (isolated twice) was also highly expressed in 13 out of 30 early stage and 27 out of 30 late stage tumors. One marker, p95 Nibrin, was positive in the sera of early and late stage ovarian cancer patients in our microarray analysis but the immunohistochemistry showed that elevated expression was specific to late stage cancers. This marker was expressed in only 1 out of 30 early stage but 15 out of 30 late stage tumors. The one early stage patient that had a positive immunohistochemical reaction was a stage 1C patient who was never off therapy and died 2.5 years after diagnosis. All of these antibodies have no positive staining on normal ovaries or ovarian cysts. These data confirm our hypothesis that these proteins are overexpressed in ovarian tumors and it is likely that overexpression is the mechanism by which they become immunogenic. These data also indicate that our approach to identifying serum biomarkers may reveal tissue markers that are stage-specific at the immunohistochemistry level.

Five-Year View

Further testing will be necessary to verify our technology and to validate the accuracy of our two-color fluorescence microarray assay using sera from larger test groups. These bioassays may be reduced to standard ELISA tests of a limited panel of antigens. Beyond expansion of the primary subgroups, i.e., early stage OVCA, late stage OVCA, and healthy controls, that comprised the initial ovarian cancer test set, we must demonstrate that women who develop ovarian cancer due to the inheritance of a mutation in BRCA1 or BRCA2 are detected as true positives in our bioassay. This segment of the female population will be the first group in which this test would be implemented. Another important validation will be to determine how far in advance one can detect ovarian cancer in a set of sera collected prospectively from generally healthy postmenopausal women between the ages of 50 to 80 who later developed ovarian cancer. The specificity of the test on patients must avoid false positives in patients with other cancers,

nonmalignant gynecologic disease or autoimmune conditions. Another significant challenge to those using immunoassays of serum antibodies will be obtaining positive controls for each antigen being used as a biomarker. Antigen specific antibody clones from phage display libraries may provide an approach to resolve this issue.

Key Issues:

The main goals for a diagnostic bioassay are that the technology employed is easily implemented and that the analytes are easily quantified with minimal complications due to handling. The field of serum diagnostics for the early detection of cancer will have to develop a consensus of what types of analytes can be reliably detected in bioassays. Although the field of serum proteomics using mass spectroscopy is a powerful technique, it has yet to be demonstrated that this technology can be implemented in a fashion suitable for routine testing in clinical laboratories. Therefore the penultimate bioassays for early detection of cancer need to be formulated so that they can be implemented on standard hospital and clinical laboratory platforms. Most agree that the comprehensive early detection of cancer will require that panels of the analytes be used. Panels of serum proteins of varying stability in serum will present a conundrum. Therefore targeting a panel of serum immunoglobulins with invariant stabilities is likely to be the most reliable for the presymptomatic screening for cancer.

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Table 1: Sensitivity and Specificity of Ovarian Cancer Biomarkers

Biomarker	Cutoff threshold	Stage	Sensitivity	Specificity	References
CA125 alone	35.0 U/ml	I-II, invasive epithelial ovca vs healthy controls	65%	97%	26
(Apolipoprotein A1+Inter-α-Trypsin inhibitor heavy chain H4 + Transthyretin)		I-II, invasive epithelial ovca vs healthy controls	74%	97%	26
(Apolipoprotein A1+Inter-α-Trypsin inhibitor heavy chain H4 + Transthyretin) + CA125 **(These CA-125 results were obtained in combination with SELDI)**		I-II, invasive epithelial ovca vs healthy controls	74%	97%	26
CA125 **(Using Radioimmunoassay)**	35.0 U/ml	I-IV, primary ovca vs benign pelvic masses	78.1 %	76.8%	32
OVX1	12.1 U/ml	I-IV, primary ovca vs benign pelvic masses	40.1 %	82.7%	32
M-CSF	31. ng/ml	I-IV, primary ovca vs benign pelvic masses	66.2%	76%	32
LASA	200.0mg/ml	I-IV, primary ovca vs benign pelvic masses	52.1%	87.8%	32
CA19-9	39.0 U/ml	I-IV, primary ovca vs benign pelvic masses	24%	88.2%	32
sEGFR	707 fmol/ml	I-IV epithelial ovca vs healthy controls	60.0%	94.4%	12
YKL-40	61 ng/ml	I-IV, epithelial ovca vs healthy controls	72%	90%	33
Human Kallikrein 6	4.4 μg/L	Primary ovarian carcinoma vs healthy controls	47%	95%	34
Leptin, Prolactin, Osteopontin, Insulin like growth factor -2		Stage I-II ovarian cancer patients vs healthy controls	95 %	95 %	18

Figure 1: Schematic Diagram of Biopanning Technology

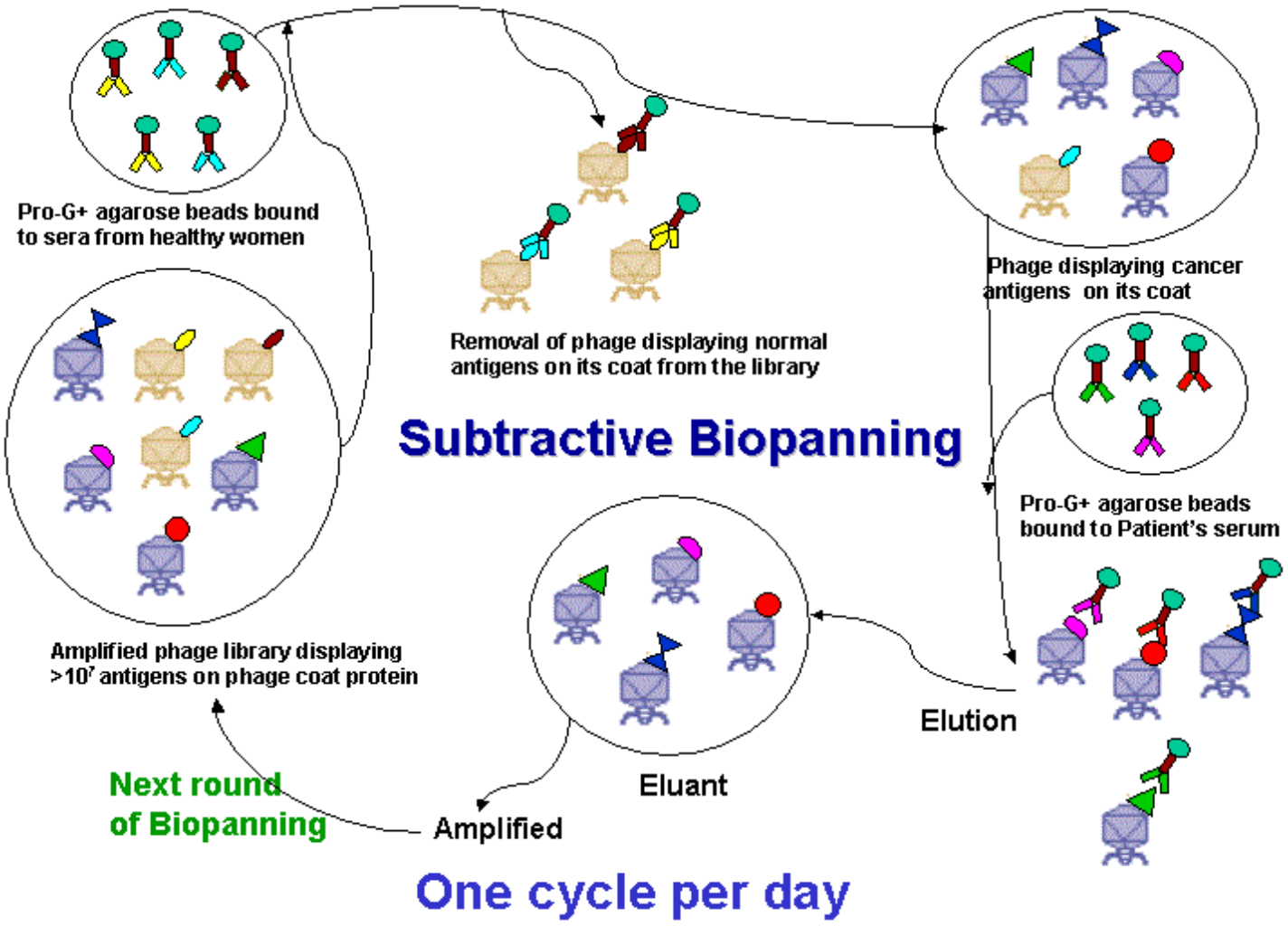
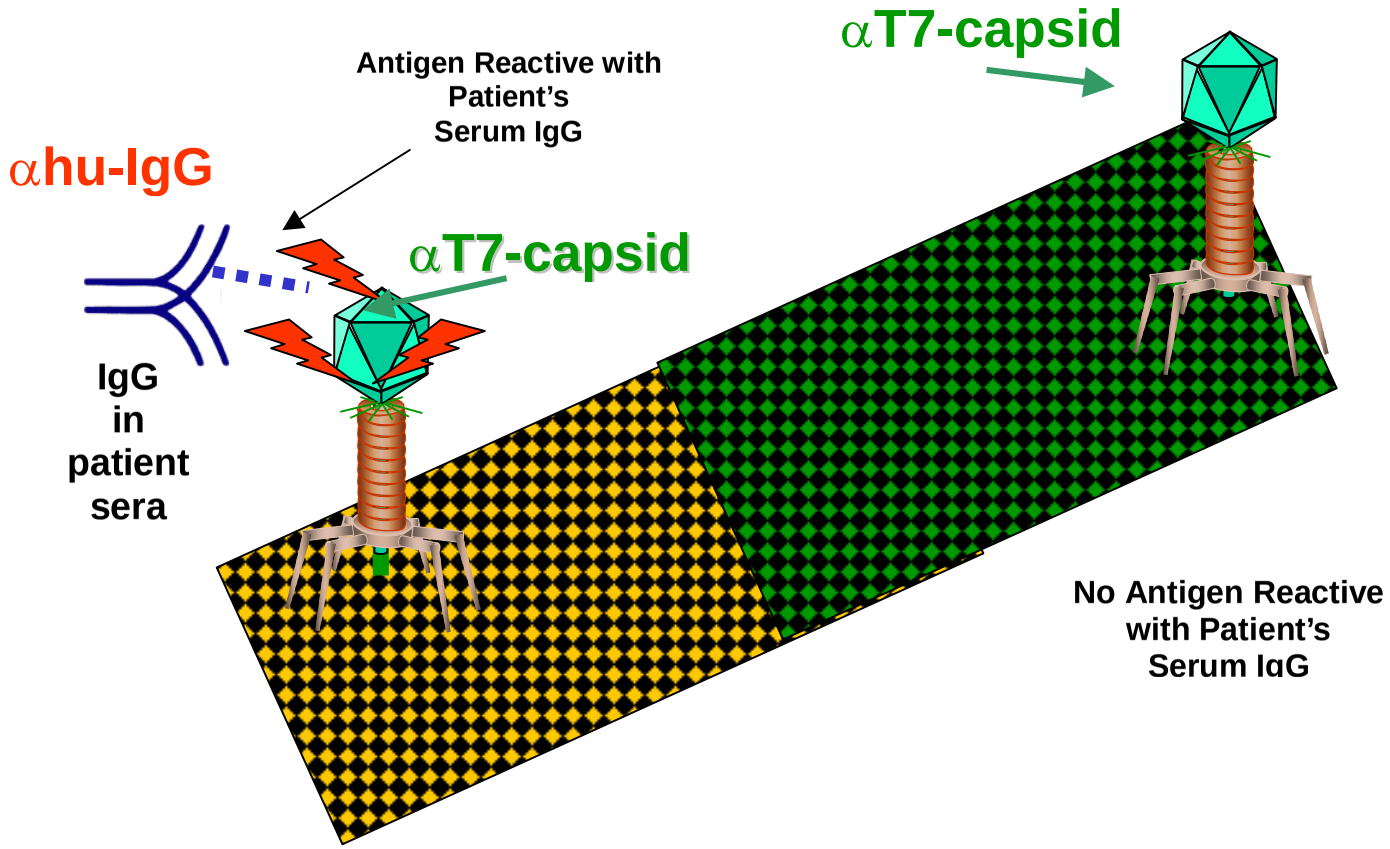
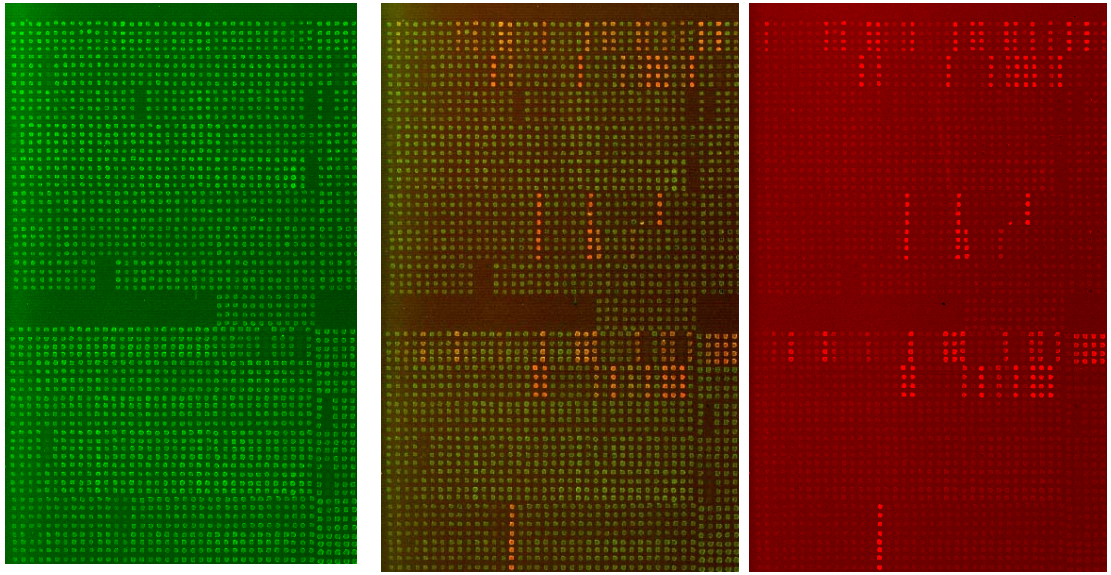


Figure 2A: Schematic Diagram of Two-Color Antigen Microarray



αhu-IgG indicates an antibody to human immunoglobulin G which is conjugated with a Cy5 red fluorescent dye. αT7capsid indicates an antibody to the T7 gene 10 capsid protein which is conjugated with a Cy3 green fluorescent dye. The protein antigen amino acid sequences expressed on the surface of the phage are indicated by the red zigzag line.

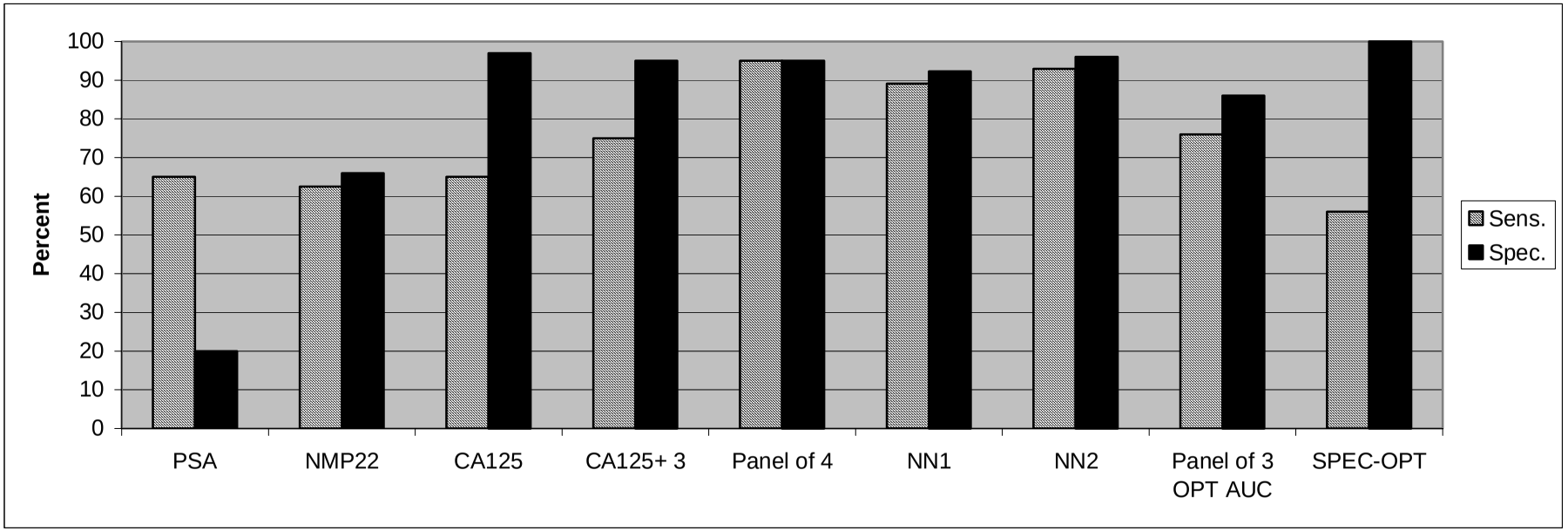
Figure 2B: Two-Color Detection of Antigens on a Microarray:



T7 Ab at 532 nm Composite image α-Hu-IgG at 635 nm

The right panel shows the reaction of the Cy5-labeled anti-human-IgG. Left panel is the reaction of the Cy3-labeled anti-mouse-IgG with a T7 capsid protein monoclonal antibody at each spot on the microarray, which provides individual spot normalization for the dye ratio. The center panel shows the composite image of the two wavelengths scanned individually. Antigen-bearing phage were robotically spotted in quadruplicate.

Figure 3



PSA:	prostate specific antigen
NMP22:	nuclear matrix protein 22
CA125:	cancer antigen 125
CA125+3:	cancer antigen 125 using in combination with apoplipoprotein A1+inter-a-trypsin inhibitor H4+transthyretin
Panel of 4:	leptin, prolactin, osteopontin, and IGF-2
NN1:	OVCA epitomics markers using a single neural network
NN2:	OVCA epitomics markers using a cascade of two neural networks
Panel of 3OPT AUC:	OVCA epitomics markers using 3 markers RCAS1, EIF5A, and Nibrin using optimized area under the ROC curve
SPEC OPT:	OVCA epitomics markers using 3 markers RCAS1, EIF5A, and Nibrin using specificity optimized for sensitivity

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