SDIA 510(k) Filing

A "Mock 510(k)" for a "An Immumological Array Platform For Simultaneous Assay Of Multiple Glycoprotein Isoforms"

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The author gratefully acknowledges contribution of the Clinical Performance study by Dr. Steve Skates.

Commented [A1]: FDA comment:

Depending on your final intended use (which would appear to be novel and currently unclassified), de novo down-classification could be considered if there are special controls that can be identified to mitigate the risks. At this time, considering that the intended use has not been finalized, we do not have sufficient information on whether the risk associated with the use of this device can be mitigated with special controls, or whether a device would need to be a Class III and reviewed as a PMA.

Currently, patients whose mammogram result falls into BI-RADS Category 4 are referred for a biopsy. Therefore, if we understand your proposed indications for use correctly, the impact of this blood test would be to reduce the number of biopsies based on the test results. Considering the potential harm to the patient due to a false negative result, this type of test would most likely be a PMA.

CAVEATS:

Fictitious data was used to generate this "mock document". The glycoproteins described as biomarkers have not been validated to be biomarkers although they have been reported to be elevated in breast cancer patients as described in the INTRODUCTION.

Some of the features in the instrument platform described are not found in commercial instruments, although they have been used and reported in laboratory instruments described in peer reviewed publications.

The objective of this "mock 510k" application was to deal with some of the issues in producing commercial tests that will be encountered in going to i) the assay of multiple antigen panels of markers, ii) assaying single isoforms of multiple antigens, and iii) the need in the future immunological assays for high levels of antibody specificity analysis and validation through mass spectrometry based proteomic analysis of antibody bound antigens.

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A. INTRODUCTION.

The blood test described here was designed to determine isoforms of the glycoproteins histidine-rich glycoprotein, plasminogen, vitronectin, proteoglycan-4, clusterin, fibrinogen, kininogen-1, platelet factor 4, and serum amyloid A protein in plasma that bear sialyl Lewis (sLe^x) antigen, Lewis x (Le^x) antigen, or both; all of which are elevated three fold or more above levels found in breast cancer free subjects.

The intended use of this test is for subjects whose mammograms have been classified by the Breast Imaging Reporting and Data System[1] (BI-RADS) as being of category 4 risk. The vast majority of screening mammograms are classified in the BI-RADS 1 and 2 categories. Seven percent of mammograms classified as BI-RADS category 3 require some patient follow-up. Approximately 2% for BI-RADS category 3 subjects actually have breast cancer. According to the original publication, subjects in the BI-RADS 4 category have roughly a 14% chance of having breast cancer. More recently it is being suggested that 25% of BI-RADS 4 patient could have breast cancer.

Subjects with a category 5 mammogram have a 95% chance of having breast cancer based on features in the mammograms such as masses with spiculated margins and/or irregular shape, as well as calcifications with linear morphology and/or segmental distribution. For this reason, there is little value in running the blood test described here on this population. BI-RADS 5 classified subjects will have a biopsy. BI-RADS 6 subjects have already had a positive biopsy indicating breast cancer.

This immunological array platform and the assays being run differ from conventional immunological assays and mass spectrometry based proteomics approaches in that i) multiple (8 to over 100) intact protein biomarkers are being determined in 20 or more plasma samples in parallel within an hour, ii) isobaric isoforms of protein biomarkers are being distinguished and assayed that do not ionize well in mass spectrometry, iii) both peptide epitopes and epitopes resulting from post-translational modifications on biomarkers are being targeted in the same assay, iv) 10 fold measurement redundancy is used with each analyte to increase measurement accuracy and provide “at-use” quality assurance, v) non-specific binding is determined and auto-subtracted, vi) internal and external calibration standards are used to achieve absolute and relative quantification, and vii) all “wet-chemical” components of the system are disposable, precluding sample carry-over between analyses.

B. MEASUREMENTS.

The measurements described here use and immunological assay strategy to identify multiple glycoproteins that bear both a polypeptide epitope unique to the specific protein and either the Le^x or sLe^x glycan epitope. The scientific evidence that these glycoproteins are markers, the mechanism of cancer metastasis, and execution of assays are discussed below.

1. Background.

Aberrations of cellular glycosylation involving a small number of biosynthetic pathways are common phenotypes in cancer[2]. ***Moreover, cancer triggers the formation of oligosaccharide structures on glycoproteins not normally produced in human subjects.*** These structural changes alter the function of tumor cells, their

antigenic and adhesive properties, their potential to invade peripheral tissues, and ultimately their ability to metastasize. Lewis x (Le^x), sialyl-Lewis x (sLe^x), sulfosialyl-Lewis x (sLe^x), and sialyl-Lewis a (sLe^a) antigens (Figure 1) in various combinations are elevated in the glycoproteins of breast cancer patients along with increased β 1,6-branching and the addition of fucose[3,4], galactosamine[5], sialic acid[6] and lactosamine oligomers to glycans on glycoproteins[7]. Up-regulation of the glycosyl transferases required to synthesize these deviant oligosaccharide appendages has been reported as well[8]. These changes in N- and O-linked glycosylation patterns of proteins are commonly observed on the surface of malignant cells during cancer progression. Tumor-associated glycans have been connected to tumor grade, metastasis, and poor prognosis[9]. Aberrations in glycosylation at the periphery of protein conjugated glycans appear to promote metastasis by diminishing cellular adhesion in the primary tumor[10] and enhancing the binding of metastasized cells at remote sites[11].

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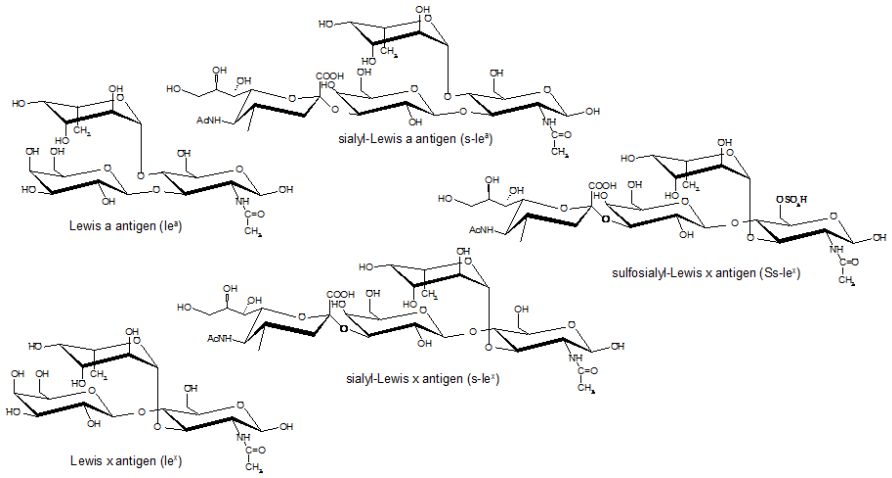

Figure 1. Lewis antigens associated with cancer.

Subsequent to tumor initiation and proliferation at a primary site, metastasis often occurs by i) dissociation of malignant cells from the tumor, ii) invasion of the basal membrane to which the tumor is attached, iii) migration through tissue into the circulatory or lymphatic system, iv) transport to remote sites, and v) extravascular invasion at those sites. A critical element of metastasis is that malignant cells find a favorable site at which to bind and proliferate in remote organs. Without this, migrating tumor cells die. Attachment at distant sites is greatly facilitated by the selectin family of lectins[12,13,14]. Expression of sLe^x and Le^x on cell-surface glycoproteins gives malignant cells the ability to adhere to L-selectin on leukocytes, E-selectin on the vascular endothelium, and P-selectin on platelets [15,16,17,18,19,20,21,22].

Extracellular communication plays an important role in adhesion and extravascular invasion in the case of breast cancer[23]. Tumor cells induce the expression of E-selectin on endothelial cells through release of cytokines, such as IL-

beta. Following sLe^x mediated adhesion of tumor cells through E-selectin on the vascular endothelium, adjacent endothelial cells are induced to release cytokines, such as HB-EGF that stimulate transmigration of tumor cells into extravascular tissues.

There can be a mechanical component to metastasis as well. Sialyl-Lewis x modulates the rolling of leukocytes and tumor cells on the vascular endothelium[24]. Activated endothelia, platelets, and leukocytes increase the probability of tumor cells forming clusters with other cells that are sufficiently large to lodge in the microvasculature of distant organs. This allows the extracellular communication process noted above to stimulate transmigration of cancer cells through adjacent vascular walls.

Lewis antigens can be linked to glycoproteins in multiple ways. Although Lewis antigens can be bound at either N- or O-glycosylation sites, core 2-O-glycans modified with sLe^x (C2-O-sLe^x) confer highest selectin binding affinity to glycoproteins. Formation of C2-O-sLe^x glycans is enhanced by expression of the core 2 β(1,6) N-acetylglucosaminyltransferase (C2GnT) enzyme[25]. C2GnT forms β1,6 branched C2-O-glycans for sLe^x modification. C2-O-sLe^x glycans are ligands for all three selectins[26]. mRNA levels of these enzymes have also been found to be elevated in tumor cells and to positively correlated with metastasis.

The concentration of sLe^x bearing isoforms of histidine-rich glycoprotein, plasminogen, apolipoprotein A-I, vitronectin, proteoglycan-4, clusterin, Ig gamma-2 chain C region, Ig mu chain C region, and inter-alpha-trypsin inhibitor heavy chain H4 glycoprotein have all been shown to change three fold or more in breast cancer patients[27]. Similar findings have been reported with Le^x bearing glycoforms of clusterin, fibrinogen alpha chain, fibrinogen beta chain, kininogen-1, platelet factor 4, serum amyloid A protein, and vitronectin[28].

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2. Immunological assay for Lewis x bearing isoforms of a protein.

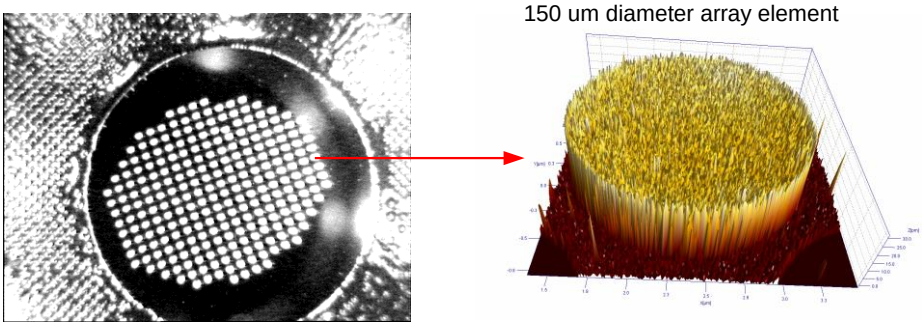
Glycoproteins exist in large numbers of glycoforms, many in which the glycans are isobaric. Affinity selection is a valuable asset in distinguishing between isobaric isoforms. Glycoforms carrying either Le^x or sLe^x antigens are being determined in this immunological assay platform with a sandwich assay using an immobilized capture antibody (-Ab_1) targeting a peptide epitope that selects all forms of the antigen (Ag) from a plasma sample. After washing away unbound proteins antigens are quantified in two ways. Non-biomarker antigens present at 10 ng/ml or higher are determined by spinning disc interferometry. This is true of clusterin, plasminogen, and fibrinogen. At low concentration, as in the case of glycoprotein isoforms it is necessary to use a fluorescent sandwich assays with laser induced fluorescence (LIF) detection. A sandwich is created when a fluorescence labeled secondary antibody (Ab_{2Lex}* or Ab_{2sLex}*) targeting Le^x or sLe^x antigens in the captured antigen (Ag) is added to form an Ab_1:Ag:Ab_2* complex. Two types of sandwiches will be formed, sometimes with the same protein. In one the immunological complex is Ab_1:Ag:Ab_{2Lex}* while in the second the complex is Ab_1:Ag:Ab_{2sLex}*. Quantification is based on laser induced fluorescence (LIF) of the fluorescing second (Ab_2*) antibody.

The IgM type monoclonal antibody TG-1 binds with high selectivity to Le^x antigens whether they are coupled to 0- or N-linked glycans. The IgM type monoclonal antibody CHO-131 binds to sLe^x glycans, preferentially to core 2-O-glycans but also to

I-type N-linked glycans. With glycoproteins carrying multiple glycoforms, Le^x and sLe^x can bind at more than one site. It is important to note that this assay lacks the specificity to differentiate between glycoforms that bear one or several of these Lewis antigens. Immunological assays in general lacking the ability to differentiate between proteins with a single and redundant epitopes.

Assays of single protein isoform(s) bearing the Le^x or sLe^x antigens are achieved at a 150 μm diameter individual antibody array element in a 1 mm diameter sample well containing 128 array elements (Figure 2). Each array element has a single immobilized antibody species. Individual antibodies immobilized on the array elements are directed against either a) a peptide epitope in one of the glycoprotein cancer markers taken from the group clusterin, plasminogen, fibrinogen, kininogen-1, platelet factor 4, serum amyloid A protein, histidine rich protein, proteoglycan-4, or vitronectin, b) an internal standard protein, c) a glycoprotein in plasma being used as a reference, or d) a protein in plasma being used to assess degradation during storage. Monoclonal antibodies targeting a peptide epitope in histidine-rich glycoprotein, plasminogen, apolipoprotein A-I, vitronectin, proteoglycan-4, clusterin, fibrinogen, kininogen-1, platelet factor 4, or serum amyloid A protein are being used in all cases.

A human plasma sample is added to the sample well in Figure 2 and allowed to incubate at room temperature (~27 ºC) for 30 min during which time antigen is captured at a specific array element (or elements) to which an antibody targeting a peptide epitope on the antigen has been immobilized. Proteins not bound and incorporated into a high affinity immunological complex on an antibody array element are eluted from the well in a washing procedure to be described in more detail below.



sample well containing an immunological array

high resolution AFM of a single array element with immobilized antibody

Figure 2. Immunological array format to be used in glycoprotein isoform assays.

A solution containing 10 μg/mL of fluorescent labeled, Le^x or sLe^x targeting second antibody is added to the sample well and allowed to incubate at room temperature (~27 ºC) for 30 min. During this incubation period the sLe^x or Le^x targeting second antibody in solution stoichiometrically binds to the Lewis x moiety (or moieties) of glycoprotein antigen isoforms captured by the first antibody. Because some antigens have more than one glycosylation site it is possible they will contain multiple

Le^x or sLe^x antigens or both antigens on the same glycoprotein. Second antibody not involved in antigen:antibody complex formation is removed by extensive washing.

Assaying the amount of an antigen bound at a specific array element is achieved through a LIF determination of the amount of fluorescent labeled second antibody bound at the array element.

Because each sample well has 128 array elements, each of the cancer biomarkers is assayed at 10 identical array elements. Array elements bearing any particular first antibody for a specific antigen are randomly distributed throughout the immunological array to preclude the possibility of positional bias. The objective of doing this is two fold. One is to assess manufacturing reproducibility and/or antibody stability after shipping and storage. If the relative standard deviation between these identical array elements is greater than 30%, the data system informs the analyst the array failed field quality control tests and the assay should be repeated. The second reason for running multiple assays of an antigen is to increase measurement accuracy. Assay redundancy relative to accuracy will be discussed at greater length below.

Commented [A5]: This seems very high for replicates of identical elements on the same array and in the same well.

It is also important to note that 5 array elements in each well carry immobilized immunoglobulin G and immunoglobulin M from mouse since all of the antibodies used in the tests described here are from mouse. These array elements are used to detect non-specific binding human plasma proteins to mouse antibodies. This is particularly important in the mass sensitive SDI detection mode. Signal produced by non-specific binding is subtracted from all other array elements.

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The sensitivity of the LIF detector used in these studies was not as high as is generally the case with LIF detectors. This is because the low power laser was used in SDI detection was also used in LIF detection to reduce the sensitivity of this detector. Sensitivity is increased 100 to 1000 fold in another model of the Quadraspec Reader by using a separate laser for LIF detection, increasing the power of the laser, and refining the optics.

3. Multiple biomarker assays for Lewis x and sialyl Lewis x bearing isoforms. The assay of individual Le^x or sLe^x antigen bearing glycoproteins has been described above. The objective of the test described in this document is to examine plasma samples for the presence of at least eight glycoprotein breast cancer biomarkers that according to the scientific literature are involved in metastasis[29,,29]. The first antibody for each of these breast cancer marker proteins is immobilized on 10 randomly distributed array elements. The specific position in the immunological array and identity of the 110 antibodies used in this multiple biomarker assay process is known and recorded in the fluorescent reader data system.

Le^x or sLe^x bearing isoforms of all the proteins noted above have been found to be elevated 3 fold or more in all breast cancer patients of stage 2 and beyond[29,31].

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4. Internal standard proteins. A troublesome aspect of quantifying proteins in plasma is that blood volume is not constant and antigen concentration will vary accordingly. A simple way to compensate for this variable is to relate cancer marker measurements to the mean concentration of a few abundant proteins in plasma that are not disease associated and vary little between patients. Transferrin, haptoglobin, α_1-antitrypsin, and α_2-macroglobulin were chosen as internal standards and measured by

spinning disc interferometry (SDI). SDI is mass sensitive, measuring the amount of antigen bound to an antibody without the use of secondary reagents, such as a second antibody. There is excellent correlation between SDI response and mass spectrometry based quantification by multiple reaction monitoring (MRM) as seen in Figure 3 with clusterin. Samples used to carry out this correlation study were obtained by stripping clusterin from the NIST plasma standard and adding clusterin back at the indicated concentrations. Clusterin was obtained from Sigma and characterized by mass spectrometry based proteomics. Quantification of clusterin in samples was achieved by the multiple reaction monitoring (MRM) method. The concentration of a protein is determined in the MRM method by using at least three synthetic carbon-13 labeled tryptic peptides of the protein that serve as internal standards. These internal standard peptides were heavy isotope labeled to facilitate their differentiation from tryptic peptides obtain upon proteolysis of the natural protein. The internal standard peptides were added to tryptic digests of sample aliquots at a fixed concentration and the isotope ratio of the internal standard to sample peptides determined by tandem mass spectrometry using at least three fragment ions in the second dimension of mass spectrometry for quantification of each tryptic peptide.

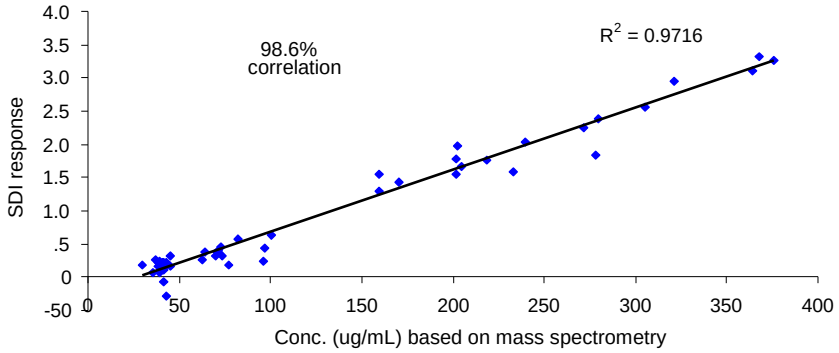

Figure 3. Correlation of interferometric and mass spectrometry based quantification of clusterin. .

Dose response curves with transferrin, haptoglobin, α_1-antitrypsin, and α_2-macroglobulin in the SDI (Figure 4) detection mode were constructed using proteins obtained from Sigma. These internal standard proteins were also identified and quantified in samples by the MRM method. Again internal standard peptides were heavy isotope labeled to facilitate their differentiation from tryptic peptides and added to tryptic digests of sample aliquots at a fixed concentration. The isotope ratio of the internal standard to sample peptides was determined by tandem mass spectrometry in the same manner as described above.

Haptoglobin and α_2-macroglobulin are the better of the four proteins to use as plasma standards because they fall in a linear range of the calibration curve.

Transferrin and α_1-antitrypsin are at the top of the dose-response curve and will respond minimally to changes in protein concentration among patients. Dilution can be used to bring all the standards into the linear range of the SDI detector but several of the breast cancer protein markers would be at too low a concentration for accurate detection.

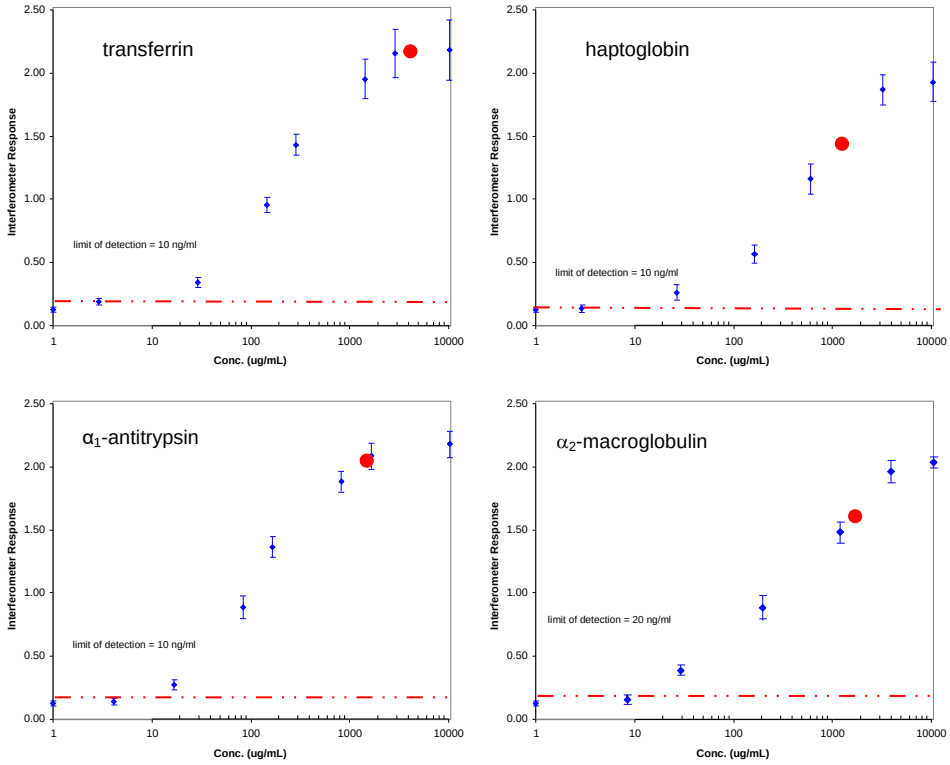

Figure 4. Protein dose response curves of several abundant reference proteins with the Quadraspec Integra SDI detector. The red dots on the curves are the mean concentration of these proteins reported in plasma[30]. The dotted red line at the bottom of the curves is the lower limit of detection.

Clusterin, fibrinogen, and plasminogen also occur in plasma at total concentration levels detectable by SDI. Concentrations of these three proteins are 1.08 x 10^{-4} g/ml, 2.72 x 10^{-3} g/ml, and 1.08 x 10^{-4} g/ml, respectively. The SDI dose response curve for these proteins is seen in Figure 5. Again these proteins were obtained from commercial suppliers and quantified by MRM based tandem mass spectrometry using synthetic carbon-13 labeled internal standard peptides. These three proteins are routinely measured in plasma by SDI as part of this test. Glycoproteins used in this dose-response experiment are a mixture of all the natural glycoforms. Values for these

three proteins are determined in both control and breast cancer patients by SDI during the course of a patient sample assay. These three proteins also occur in plasma as isoforms carrying Le^x and sLe^x antigens that constitute a small fraction of the total parent protein. The Le^x and sLe^x isoforms of these proteins are measurable by LIF with a fluorescent sandwich assay as will be shown below.

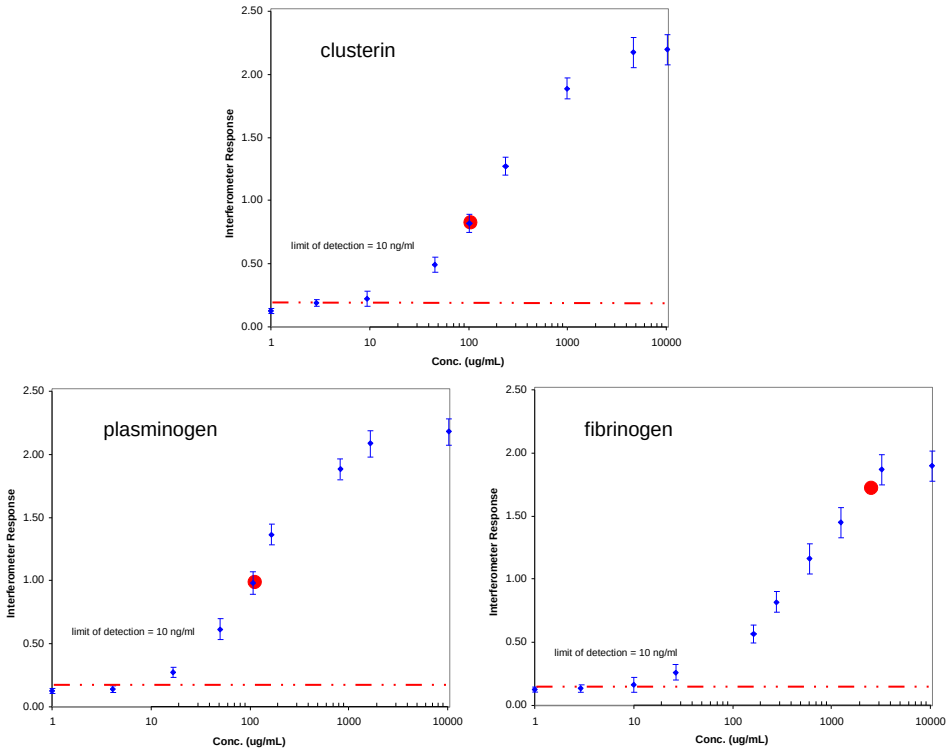

Figure 5. Dose response curve for the clusterin, plasminogen, and fibrinogen. The red dots on the curves in Figure are the mean concentration of these proteins reported in plasma. The dotted red line at the bottom of the curves is the lower limit of detection.

A calibration mixture of transferrin, haptoglobin, α_1-antitrypsin, and α_2-macroglobulin along with human clusterin, plasminogen, and fibrinogen are provided with assay kits at concentrations found in normal plasma. [*These concentration values were noted above.*] The glycoprotein standards supplied with the kit are a mixture of all the natural glycoforms. This mixture is used on a daily basis to calibrate the Integra Reader. The calibration mixture is stable at 4 ^oC for a week after the kit is opened. The ratio of SDI measurements between the internal standard proteins transferrin, haptoglobin, α1-antitrypsin, and α2-macroglobulin and total amount of clusterin, fibrinogen, and plasminogen in groups of 100 normal subjects and 20 breast cancer

patients is seen in Table 1. The normal samples were taken from BI-RADS 1 and 2 category patients whereas the cancer patient samples were from BI-RADS 6 patients. Each of the subjects in the cancer group had a positive breast cancer biopsy. Values used in calculating these ratios were the mean of measurements from 10 different array elements targeting the same antigen in a well. In the SDI detection mode each array element is read individually.

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Please note that mammography is not a perfect test, which means that there is a chance that subjects with BI-RADS 1 and 2 may have cancer.

Table 1. SDI measurement ratios between the internal standard proteins transferrin, haptoglobin, α1-antitrypsin, and α2-macroglobulin and total amount of the cancer associated proteins clusterin, fibrinogen, and plasminogen.

Non-cancer subjects (n-100)[1]	ratio[3]	Breast cancer patients (n-20)[1,2]	ratio[3]
$SDI_{transferrin}/SDI_{clusterin}$	3.7±0.41	$SDI_{transferrin}/SDI_{clusterin}$	3.1± 0.96 **0.83**
$SDI_{haptoglobin}/SDI_{clusterin}$	1.2±0.28	$SDI_{haptoglobin}/SDI_{clusterin}$	0.81±0 **0.65**
$SDI_{\alpha1\text{-}antitrypsin}/SDI_{clusterin}$	1.3±0.41	$SDI_{\alpha1\text{-}antitrypsin}/SDI_{clusterin}$	1.1±0.19 **0.85**
$SDI_{\alpha2\text{-}macroglobulin}/SDI_{clusterin}$	1.7±0.61	$SDI_{\alpha2\text{-}macroglobulin}/SDI_{clusterin}$	1.1±0 **0.64**
$SDI_{transferrin}/SDI_{fibrinogen}$	1.5±0.33	$SDI_{transferrin}/SDI_{fibrinogen}$	1.2±0 **0.80**
$SDI_{haptoglobin}/SDI_{fibrinogen}$	0.46±0.19	$SDI_{haptoglobin}/SDI_{fibrinogen}$	0.35±0.12 **0.76**
$SDI_{\alpha1\text{-}antitrypsin}/SDI_{fibrinogen}$	0.52±0.18	$SDI_{\alpha1\text{-}antitrypsin}/SDI_{fibrinogen}$	0.41±0 **0.79**
$SDI_{\alpha2\text{-}macroglobulin}/SDI_{fibrinogen}$	0.66±0.25	$SDI_{\alpha2\text{-}macroglobulin}/SDI_{fibrinogen}$	0.57±0.17 **0.86**
$SDI_{transferrin}/SDI_{plasminogen}$	3.8±1.7	$SDI_{transferrin}/SDI_{plasminogen}$	2.7±0.51 **0.71**
$SDI_{haptoglobin}/SDI_{plasminogen}$	1.1±0.19	$SDI_{haptoglobin}/SDI_{plasminogen}$	0.94±0.23 **0.85**
$SDI_{\alpha1\text{-}antitrypsin}/SDI_{plasminogen}$	1.2±0.43	$SDI_{\alpha1\text{-}antitrypsin}/SDI_{plasminogen}$	1.3±0.31 **1.08**
$SDI_{\alpha2\text{-}macroglobulin}/SDI_{plasminogen}$	1.9±0.51	$SDI_{\alpha2\text{-}macroglobulin}/SDI_{plasminogen}$	1.4±0.27 **0.74**

[1]Values reported are the average of measurements taken from 100 subjects.
[2]Subjects used in these measurements had tested positive in a breast cancer biopsy.
[3]Ratio of breast cancer values/non-cancer subjects.

There is some difference in the SDI ratios between normal subjects and cancer patients in Table 1 but the variations are so small they lack diagnostic significance. This is interpreted to mean there is little difference in the expression levels of clusterin, fibrinogen, and plasminogen between control and cancer patients. This is further supported by the fact that all the ratios of difference between control and cancer patients are near one. ***The difference ratios between cancer and control subjects in Table 2 are five or more in all cases compared to Table 1 where difference ratios were near one of slightly below***. This shows that the glycoprotein markers carrying the Le^x or sLe^x antigens have increased substantially in breast cancer patients. The fact that the total amount of clusterin, fibrinogen, and plasminogen increased little according to the SDI measurements while the amount of the glycoforms increase many fold means there was almost no change in the expression of clusterin, plasminogen, and fibrinogen while changes in Lewis antigen synthesis on these proteins were very large.

Table 2. The ratio of LIF/SDI measurements between the cancer associated Le^x and sLe^x isoforms of clusterin, fibrinogen, and plasminogen determined by LIF and the total amount of the individual internal standard proteins transferrin, haptoglobin, α1-antitrypsin, and α2-macroglobulin determined by SDI.

Non-cancer subjects (n-100)[1]		Breast cancer patients (n-100)[1,2]		ratio
$LIF_{clusterin}/SDI_{transferrin}$	1.7±0.57	$LIF_{clusterin}/SDI_{transferrin}$	15±6	**8.82**
$LIF_{clusterin}/SDI_{haptoglobin}$	5.4±2.1	$LIF_{clusterin}/SDI_{haptoglobin}$	43±19	**7.96**
$LIF_{clusterin}/SDI_{\alpha1\text{-}antitrypsin}$	4.8±1.4	$LIF_{clusterin}/SDI_{\alpha1\text{-}antitrypsin}$	39±11	**8.13**
$LIF_{clusterin}/SDI_{\alpha2\text{-}macroglobulin}$	3.7±1.2	$LIF_{clusterin}/SDI_{\alpha2\text{-}macroglobulin}$	41±15	**11.72**
$LIF_{fibrinogen}/SDI_{transferrin}$	0.83±0.42	$LIF_{fibrinogen}/SDI_{transferrin}$	6.6±2.7	**7.95**
$LIF_{fibrinogen}/SDI_{haptoglobin}$	2.7±1.0	$LIF_{fibrinogen}/SDI_{haptoglobin}$	36±12	**13.30**
$LIF_{fibrinogen}/SDI_{\alpha1\text{-}antitrypsin}$	2.4±0.75	$LIF_{fibrinogen}/SDI_{\alpha1\text{-}antitrypsin}$	21±5	**8.75**
$LIF_{fibrinogen}/SDI_{\alpha2\text{-}macroglobulin}$	1.8±0.71	$LIF_{fibrinogen}/SDI_{\alpha2\text{-}macroglobulin}$	19±6	**10.55**
$LIF_{plasminogen}/SDI_{transferrin}$	1.3±0.4	$LIF_{plasminogen}/SDI_{transferrin}$	7.9±2.1	**6.07**
$LIF_{plasminogen}/SDI_{haptoglobin}$	4.1±0.9	$LIF_{plasminogen}/SDI_{haptoglobin}$	24±4.8	**5.85**
$LIF_{plasminogen}/SDI_{\alpha1\text{-}antitrypsin}$	3.8±1.1	$LIF_{plasminogen}/SDI_{\alpha1\text{-}antitrypsin}$	26±4.9	**6.84**
$LIF_{plasminogen}/SDI_{\alpha2\text{-}macroglobulin}$	2.9± 0.7	$LIF_{plasminogen}/SDI_{\alpha2\text{-}macroglobulin}$	19±4.7	**6.55**

[1]Values reported are the average of measurements taken from 100 subjects.
[2]Subjects used in these measurements had tested positive in a breast cancer biopsy.
[3]Ratio of breast cancer values/non-cancer subjects.

Relative concentration differences between glycoprotein markers in the normal versus cancer patient group using the fluorescent sandwich assay method described above are seen in Figures 6 A and B. The difference ratio between control and cancer subjects seen with the clusterin, plasminogen, and fibrinogen isoforms in Table 2 based on LIF/SDI measurements is close to the amount of change seen in Figures 3 and 6 with the same groups of subjects.

Figure 6 shows the fluorescence sandwich assay dose response curves for nine protein maker proteins using the Quadraspec Integra Reader. The mean fluorescence was computed from 10 immunological array elements bearing an antibody targeting the protein being measured. The values reported in the Table are based on sandwich assays using a fluorescent labeled second antibody targeting either the Le^x or sLe^x. antigen. The second antibody in the assay was a mixture of Le^x and sLe^x targeting antibodies. Antibody from clone TG-1 targeted Le^x while antibody from clone CHO-131 targeted sLe^x. Both antibodies were obtained from Santa Cruz Biotechnology Inc. Samples from100 subjects with BI-RADS 1 and 2 category mammograms were used as the normal control samples. Samples of 20 patients with BI-RADS 5 mammograms with a breast cancer positive biopsy were use as the cancer positive samples. The cutoff

values in the figures were set at a value three times higher than the mean value for normal patients. This cutoff value will be used until a statistically derived value from a larger 2000 patient population is developed. Assay values for cancer patients are in red while those of control subjects are in light blue. The lower limit of detection is indicated by a doted red line. Again protein concentration of proteins used to establish these standard curves was determined by a tandem mass spectrometry based MRM approach using carbon-13 labeled internal standards. Details of this procedure have been described above.

Commented [A9]: This will need to be from the actual IU population (BI-RADS 4, not 1, 2 and 5).

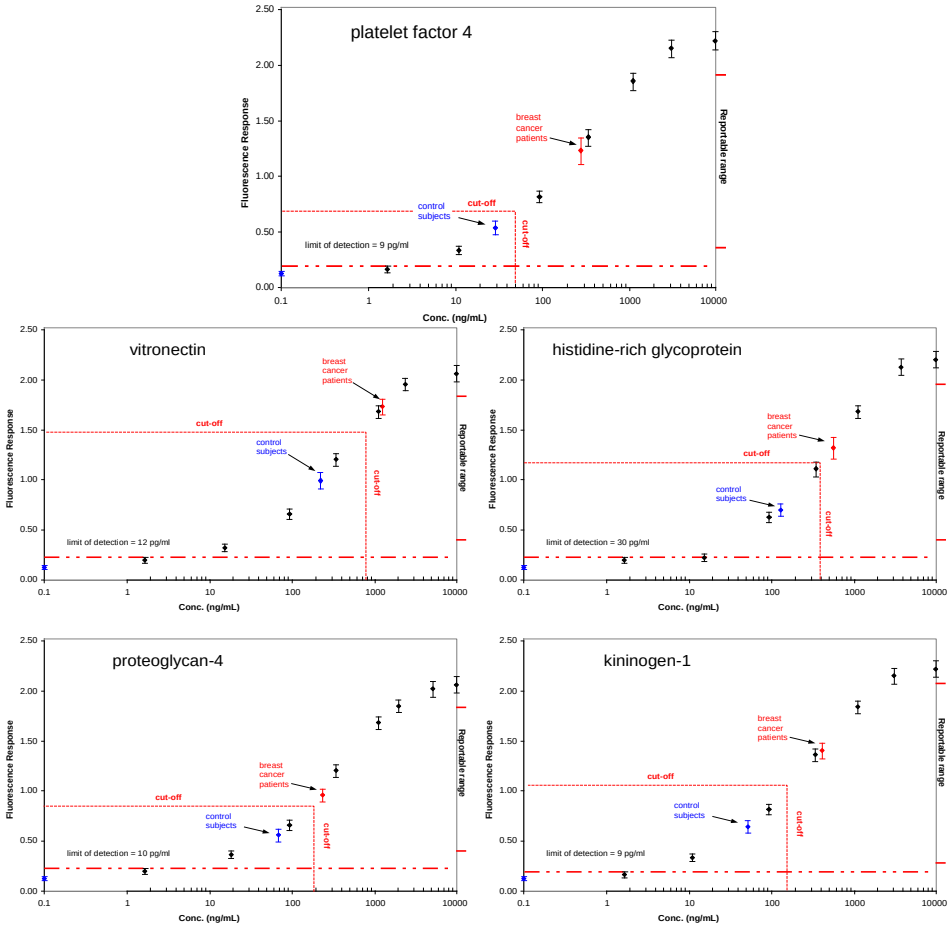

Figure 6A. Fluorescent sandwich assays targeting Lewis antigen containing isoforms of proteins in non-cancer and cancer subjects.

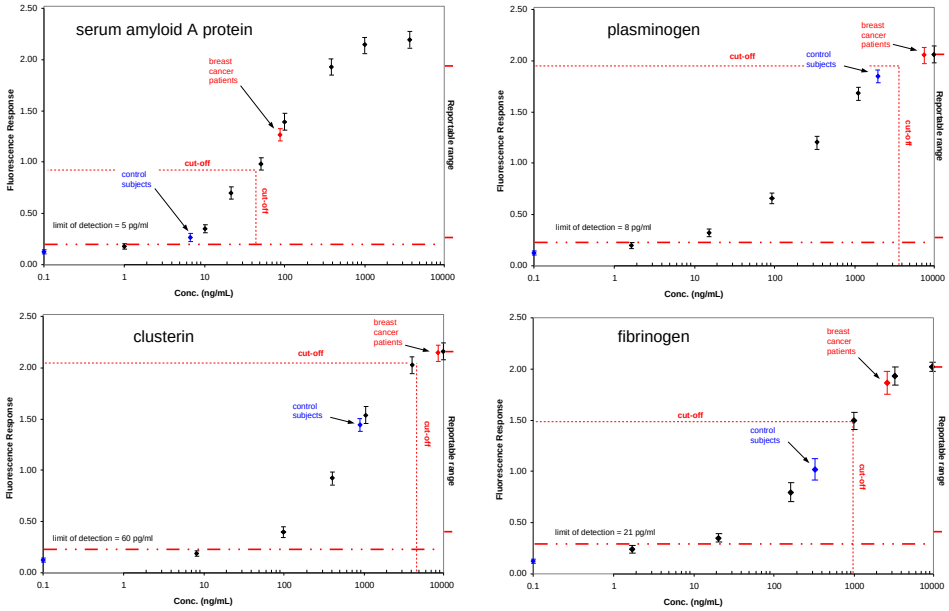

Figure 6B. Fluorescent sandwich assays targeting Lewis antigen containing isoforms of proteins in non-cancer and cancer subjects.

5. Interpretation and analysis of data.

A feature of this test is that transferrin (Tr), haptoglobin (Ha), α_1-antitrypsin (An), and α_2-macroglobulin (Ma) are being used as internal standards to compensate for changes in blood volume between subjects. These proteins vary little between subjects and are easily measured by spinning disc interferometry (SDI). Another is the use of immobilized mouse IgG and IgM on array elements as a reference for non-specific binding (NSB) in both the SDI and LIF detection modes. NSB measured in the SDI and LIF detection modes at these array elements is subtracted from other array elements.

Simplifying the nomenclature used in Tables 1-2, SDI readings for the calibration mixture of transferrin (Tr), haptoglobin (Ha), α_1-antitrypsin (An), α_2-macroglobulin (Ma), plasminogen (Pl), clusterin (Cl), and fibrinogen (Fi) are designated as $[Tr]_S$, $[Ha]_S$, $[An]_S$, $[Ma]_S$, $[Pl]_S$, $[Cl]_S$, and $[Fi]_S$, respectively. These readings are stored in the data system each time the instrument is calibrated and used in the computations described below. SDI readings for transferrin, haptoglobin, α_1-antitrypsin, α_2-macroglobulin, plasminogen, clusterin, and fibrinogen in patient samples are indicated by the symbols $[Tr]_P$, $[Ha]_P$, $[An]_P$, $[Ma]_P$, $[Pl]_P$, $[Cl]_P$, and $[Fi]_P$, respectively. Data from LIF measurements of

Commented [A10]: In the actual product, interpretation should ideally be simple and straightforward, without many additional explanations.

Elements used as either in-process or any other type of controls should have listed outputs and what would they mean (e.g. if certain control value is outside of the specific range, the assay is invalid), basically what is described in wording below.

plasminogen (Pl), clusterin (Cl), and fibrinogen (Fi) in patient samples is represented as $\{Pl\}_p$, $\{Cl\}_p$, and $\{Fi\}_p$.

First, the internal standard proteins haptoglobin, α_1-antitrypsin, α_2-macroglobulin, and transferrin generally occur in a ratio of 1.00:1.12:1.44:3.20, respectively. Any time one of these proteins deviates from this ratio by more than 30% it is eliminate as an internal standard. Large deviations in a single protein such as transferrin could occur if a subject has a disease other than breast cancer which causes a large change in the concentration of the internal standard. When this occurs it is automatically eliminated.

Commented [A11]: This seems to be somewhat unreliable standard if this would be the case – maybe some different proteins would be better standards? Practice of disregarding a standard if it behaves unexpectedly is in most cases inappropriate.

Second, corrections for non-specific binding are made based on the binding of proteins at the mouse IgG and IgM array elements.

Third, using data from the calibration mixture and the patient sample the following ratios are calculated;

$$R_{Tr} = \frac{[Tr]_s}{[Tr]_p}, \quad R_{Ha} = \frac{[Ha]_s}{[Ha]_p}, \quad R_{An} = \frac{[An]_s}{[An]_p}, \quad R_{Ma} = \frac{[Ma]_s}{[Ma]_p}$$

Ideally all of the ratios would be one, indicating that the concentration of the internal standard proteins in the patient sample are identical to those in the calibration mixture and equal to averages found in plasma. This is generally not the case. Compensation for dilution and slight differences in internal standard concentration are made by averaging the ratios R_{Tr}, R_{Ha}, R_{An}, *and* R_{Ma} to calculate a correction ratio (R_c)

Commented [A12]: This may be plausible, but would have to be shown that it works.

$$R_c = \frac{R_{Tr} + R_{Ha} + R_{An} + R_{Ma}}{4}$$

All SDI and LIF values obtained on patient samples are multiplied by R_c to obtain a corrected concentration value. The exception would be if one of these internal standards has been rejected because it deviates from normal. The process for doing this is described above.

Commented [A13]: See comment above.

Fourth, the ratios between SDI measurements of patient samples and the standard calibration mixture are calculated for clusterin, plasminogen and fibrinogen

$$R_{Cl} = \frac{[Cl]_p}{[Cl]_s}, \quad R_{Pl} = \frac{[Pl]_p}{[Pl]_s}, \quad R_{Fi} = \frac{[Fi]_p}{[Fi]_s}$$

As seen in Table 1, the ratio is near one or slightly below in all cases. This measurement has no diagnostic value and is made only to assure that the SDI measurement and immunological assay component are working correctly.

Fifth, ratios between the fluorescent sandwich assays of patient samples and the standard calibration mixture are calculated for clusterin, plasminogen, and fibriginogen where

$$R_{Cl} = \frac{\{Cl\}_p}{\{Cl\}_s}, \quad R_{Pl} = \frac{\{Pl\}_p}{\{Pl\}_s}, \quad R_{Fi} = \frac{\{Fi\}_p}{\{Fi\}_s}$$

Subjects exhibiting a ratio between 0.5 and 1.5 are considered to be normal. Those in which the ratio is 3 and above are considered to have a positive indication of cancer. A value of 3 is used based on the fact that during the discovery phase of this work cancer markers were always elevated 3 fold or more in breast cancer patients.

Commented [A14]: This would be an important part of the results interpretation; might be useful to put it in a tabular format.

Commented [A15]: This would need to be evaluated in the pivotal study; it would ultimately be a part of the submission, not necessarily in the interpretation part.

Sixth, the fluorescent sandwich assay for clusterin, plasminogen, and fibrinogen versus SDI measurements of the four standards is computed using the equations below

$$R_{Pl} = \frac{\{Pl\}_p/[Tr]_p}{\{Pl\}_s/[Tr]_s}, \quad R_{Cl} = \frac{\{Cl\}_p/[Tr]_p}{\{Cl\}_s/[Tr]_s}, \quad R_{Fi} = \frac{\{Fi\}_p/[Tr]_p}{\{Fi\}_s/[Tr]_s}$$

$$R_{Pl} = \frac{\{Pl\}_p/[Ma]_p}{\{Pl\}_s/[Ma]_s}, \quad R_{Cl} = \frac{\{Cl\}_p/[Ma]_p}{\{Cl\}_s/[Ma]_s}, \quad R_{Fi} = \frac{\{Fi\}_p/[Ma]_p}{\{Fi\}_s/[Ma]_s}$$

$$R_{Pl} = \frac{\{Pl\}_p/[Ha]_p}{\{Pl\}_s/[Ha]_s}, \quad R_{Cl} = \frac{\{Cl\}_p/[Ha]_p}{\{Cl\}_s/[Ha]_s}, \quad R_{Fi} = \frac{\{Fi\}_p/[Ha]_p}{\{Fi\}_s/[Ha]_s}$$

$$R_{Pl} = \frac{\{Pl\}_p/[An]_p}{\{Pl\}_s/[An]_s}, \quad R_{Cl} = \frac{\{Cl\}_p/[An]_p}{\{Cl\}_s/[An]_s}, \quad R_{Fi} = \frac{\{Fi\}_p/[An]_p}{\{Fi\}_s/[An]_s}$$

Ratio values between 0.5 and 1.5 are considered to be normal while those of 3 or more are considered to be positive indicators of breast cancer.

Commented [A16]: It is not clear how results for 5th that are above 3 would be interpreted if below 3 for 6th.

Seventh, the ratio for fluorescent sandwich assay for vitronectin, histidine-rich glycoprotein, proteoglycan-4, kininogen-1, platelet factor 4, serum amyloid A protein versus SDI measurements of the four internal standards is computed using the equations below. Fluorescence assay values for these proteins are available from the dose response curves in Figure 6.

$$R_{Vi} = \frac{\{Vn\}_p}{0.697\{Fi\}_s}, R_{Hi} = \frac{\{Hi\}_p}{0.421\{Fi\}_s}, R_{Pf} = \frac{\{Pf\}_p}{0.055\{Fi\}_s}, R_{\Pr} = \frac{\{\Pr\}_p}{0.215\{Fi\}_s}, R_{Kii} = \frac{\{Ki\}_p}{0.164\{Fi\}_s},$$
$$R_{Sap} = \frac{\{Sap\}_p}{0.026\{Fi\}_s}$$

$$R_{Vi} = \frac{\{Vn\}_p}{0.223\{Cl\}_s}, R_{Hi} = \frac{\{Hi\}_p}{0.135\{Cl\}_s}, R_{Pf} = \frac{\{Pf\}_p}{0.0174\{Cl\}_s}, R_{\Pr} = \frac{\{\Pr\}_p}{0.068\{Cl\}_s}, R_{Kii} = \frac{\{Ki\}_p}{0.052\{Cl\}_s},$$
$$R_{Sap} = \frac{\{Sap\}_p}{0.0082\{Cl\}_s}$$

$$R_{Vi} = \frac{\{Vn\}_p}{0.18\{Pl\}_s}, R_{Hi} = \frac{\{Hi\}_p}{0.106\{Pl\}_s}, R_{Pf} = \frac{\{Pf\}_p}{0.014\{Pl\}_s}, R_{\Pr} = \frac{\{\Pr\}_p}{0.054\{Pl\}_s}, R_{Kii} = \frac{\{Ki\}_p}{0.041\{Pl\}_s},$$
$$R_{Sap} = \frac{\{Sap\}_p}{0.0065\{Pl\}_s}$$

Again, ratio values for the marker proteins between 0.5 and 1.5 are considered to be normal. Values greater than 3 are considered to be a positive indicator of cancer.

Commented [A17]: Would any of the values above 3 (from 5, 6, and 7) be positive? Or would 5 and 6 have some kind of algorithm considering they are looking at the same 3 analytes?

Summary. All the data from these measurements is interpreted in terms of a series of ratio values based on known concentrations of transferrin (Tr), haptoglobin (Ha), α_1-antitrypsin (An), α_2-macroglobulin (Ma), plasminogen (Pl), clusterin (Cl), and fibrinogen (Fi) considered to be normal in plasma. These concentration values are obtained from a standard calibration mixture run on a daily basis and stored in the data systems to be used in generating the standard (control) to patient ratios. Ratio values between 0.5 to 1.5 are normal. Those of 3 and above are interpreted as positive indictors of breast cancer.

Commented [A18]: For any one of the analytes?

C. SYSTEM DESCRIPTION.

The principles involved in the fluorescence sandwich assay for specific isoforms of glycoproteins are described above. This section describes how the assay is executed.

1. Definition of Terms.

The terms “platform”, “kit”, and “glycoprotein calibration standard” as used here are defined in the following way. The ***wet chemistry platform*** is a circular plate on which immunological array discs are placed and rotated at speeds up to hundreds of rpm during sample application, washing, sequential addition of reagents, and drying (Figure 7). The ***Reader platform*** is another device with a circular plate on which immunological assay discs are placed for reading after the wet chemistry component of the assay is complete (Figure 8). An ***assay kit*** contains a i) disposable disc with multiple immunological assay wells with each well containing an immunological array of 128 array elements, ii) sample and washing buffers, and iii) fluorescent labeled secondary antibodies for detection.

2. Assay protocol.

An assay is achieved in several steps. The first is a wet chemistry component in which antigens are captured from samples and immunological complexes are assembled followed by spin drying of the disc. The second is the reading process in which antigens are detected at array elements and quantified.

a. Wet chemistry component of the assay.

Glycoproteins exist in large numbers of glycoforms. Glycoforms carrying either Le^x or sLe^x antigens are being determined in this immunological assay platform with a sandwich assay using an immobilized capture antibody (-Ab_1) targeting a peptide epitope that selects all glycoforms of the antigen (Ag) from a plasma sample. After washing away unbound proteins, a fluorescence labeled secondary antibody (Ab_{2Lex}* or Ab_{2sLex}*) targeting Le^x or sLe^x antigens in the captured antigen (Ag) is then added to form an Ab_1:Ag:Ab_2* sandwich. Assays for the Le^x or sLe^x antigens will be carried out in separate immunological array wells. Two types of sandwiches will be formed, sometimes with the same protein. In one the immunological complex is Ab_1:Ag:Ab_{2Lex}* while in the second the complex is Ab_1:Ag:Ab_{2sLex}*. Quantification is based on laser induced fluorescence (LIF) of the second (Ab_2*) glycan targeting antibody.

The work flow involved in the wet chemistry component of this array is shown below.

- The loading of samples, controls, diluent, and secondary antibodies in addition to sample barcoding, sample dilution on microtiter plates, bulk washing solutions, and nature of test discs is described in the "**INSTRUMENT DESCRIPTION**" section below.
- Sample diluent is pipetted from a source vial to a microtiter plate (all tips aspirate a bulk quantity and dispense a metered quantity into eight wells at a time). This process is followed by a tip wash.
- Pipette patient sample.
    - Approximately 50 µL of patient samples are aspirated into pipettes and transferred to microtiter plate wells containing 100 µL sample diluent.
    - Samples are dispensed into the plate and mixed by a series of aspirate/dispense cycles of the tips.
    - Tips are washed in the wash station by a flush with the system liquid (DI water). The liquid is sprayed into cups directly below the tips causing the water to bubble up around the tip and thus also washes the outside.
    - Repeat the previous three steps for the remaining patient samples.
- Sample transfer from plate to 24 sample well disc. Sample well are distributed around the periphery of the disc.
    - Fifty uL of diluted patient samples are aspirated into pipettes.
    - A flap opens in the center of the humidity chamber lid to allow tips to access the disc surface.
    - Fifteen uL of each samples is dispensed into a disc sample well.
    - Tips are washed in the wash station (see above)
    - Repeat the previous steps for the remaining diluted samples.

- Incubate samples on the disc for 30 min maintaining humidity surrounding the disc to prevent evaporation from sample wells.
- A global wash to remove remaining unbound antibody is achieved by rapidly accelerating the disc to 1000 rpm as a stream of wash buffer is added at the center of the spinning disc for 30 sec. Centrifugal force carries the liquid across the face of the disc and washes unbound proteins from the disc. The disc is spun dry to remove any remaining wash liquid.
- Second antibody application
    - A bulk quantity of the fluorescent labeled second antibody is aspirated into pipettes and used for detection. Each well is dosed with 15 uL of second antibody solution. [*The secondary antibody in this case is an equimolar mixture of two monoclonal antibodies, CHO-131 and TG-1. The former targets sLe^x while the latter Le^x. Both are IgM antibodies.]*
    - At the conclusion of this process, the tips are washed in the wash station
- The secondary antibody solution incubates on the disc for 30 minutes. As with the sample incubation, humidity is maintained at a high level in a sample assay chamber to prevent dry-down.
- A global wash removes unbound secondary antibody in a manner identical to that described above.
- The disc receives a final spin at 1000 rpm for one min to remove any remaining liquid from the surface.

b. The reading process.

Sample discs are read as seen in Figure 7 and 8. The surface of the rotating disc is interrogated with a laser beam that rasters across the face of the disc. Readings from each array element are then integrated to produce the final, averaged signal.

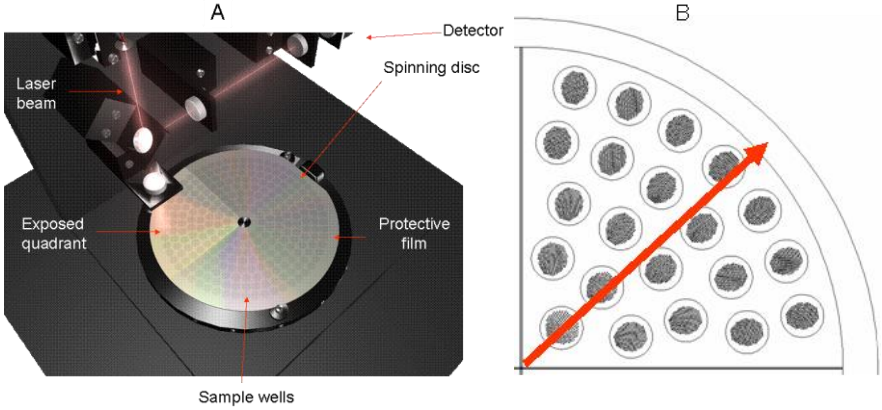

Figure 7. Spinning disc reader platform and disc. (A) This photograph shows the basic elements of the Reader with the disc placed on the rotating platform where discs are read. (B) A multiple well disc is illustrated here. **It is important to note that**

discs used in assaying the cancer markers discussed here will only have wells around the periphery of the disc. This is important because washing is achieved by centrifugal force carrying wash buffers across the surface of wells. Wash buffers from inner wells will not pass over outer wells during washing when all wells are equidistant from the center of the disc.

3. Multiple biomarker assays for Le^x and sLe^x bearing isoforms.

The assay of individual Le^x or sLe^x antigen bearing glycoproteins has been described above. Assays for Le^x and sLe^x bearing isoforms of histidine-rich glycoprotein, plasminogen, vitronectin, proteoglycan-4, clusterin, fibrinogen alpha chain, kininogen-1, platelet factor 4, and serum amyloid A protein simultaneously is achieved in the same general manner. The first antibody for each of these proteins is immobilized on 10 randomly distributed array elements. The specific position in the immunological array and identity of the 110 antibodies used in this multiple biomarker assay process is recorded in the data analysis system.

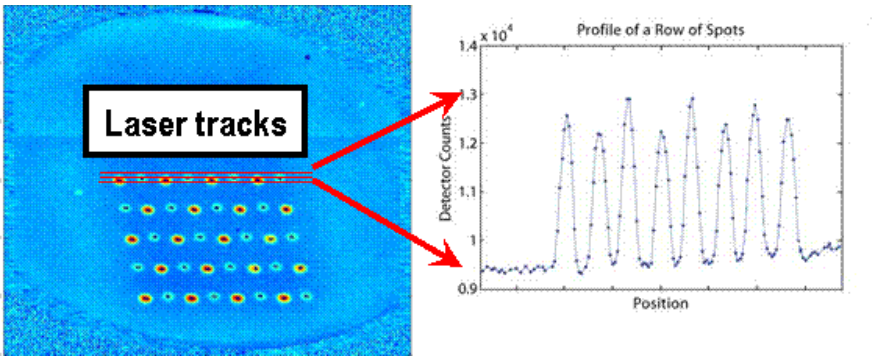

Figure 8. A sample well showing loaded immunological array elements and laser tracks across the surface of the well. The panel on the right shows signal derived from a laser scan of a sample well. Each Ab spot is 125 µm in diameter whereas the laser beam is 20 µm in diameter. At least 5 traces are obtained across each spot as the laser beam rasters in 20 µm steps toward the center of the disc. The laser beam is held at each radial position for 3-10 revolutions to allow multiple readings of each array element. The number of readings is designated by the user.

D. COMPONENTS OF AN ASSAY KIT.

1. Reagents and test components.

Disposable components of the assay kit are the multiple sample well disc, washing buffers, and the fluorescent secondary antibodies that target the Le^x and sLe^x antigens.

a. Immunological array disc.

Assay discs carry immobilized capture antibodies targeting histidine-rich glycoprotein, plasminogen, vitronectin, proteoglycan-4, clusterin, fibrinogen alpha chain,

fibrinogen, kininogen-1, platelet factor 4, and serum amyloid A protein to carry out glycoform assays of these proteins. These antibodies were obtained from the following sources.

Commented [A19]: There might be some additional information about purity, QC, binding properties etc that we would ask to see for the actual submission.

i. *Clusterin monoclonal antibody*.

Santa Cruz Biotechnology, Inc. Catalog number of clone number: Clusterin (1A11): sc-73415: This is a mouse monoclonal IgG_1 antibody at an estimated concentration of 100 µg/ml. Raised against amino acids 1-333 of recombinant Clusterin of human origin.

Santa Cruz Biotechnology, Inc. 2145 Delaware Avenue. Santa Cruz, California 95060. U.S.A. Phone: 831.457.3800. Fax: 831.457.3801. e-mail: scbt@scbt.com

Chromosomal location. Genetic locus: CLU (human) mapping to 8p21-p12; Clu (mouse) mapping to 14 D1.

Source: Clusterin (1A11) is a mouse monoclonal antibody raised against amino acids 1-333 of recombinant Clusterin of human origin.

Target: Lewis x antigen on clusterin dimer. Molecular Weight of Clusterin precursor: 70 kDa. Molecular Weight of Clusterin-α: 36-39 kDa. Molecular Weight of Clusterin-β: 34-36 kDa. Positive Controls: SK-BR-3 cell lysate: sc-2218, human breast tumor or human brain tissue.

Secondary reagents used in QC: The following support (secondary) reagents are used in QC: 1) Western Blotting: use goat anti-mouse IgG-HRP: sc-2005 (dilution range: 1:2000-1:32,000) or Cruz Marker™ compatible goat antimouse IgG-HRP: sc-2031 (dilution range: 1:2000-1:5000), Cruz Marker™ Molecular Weight Standards: sc-2035, TBS Blotto A Blocking Reagent: sc-2333 and Western Blotting Luminol Reagent: sc-2048.

Storage: Store at 4° C, **DO NOT FREEZE**. Stable for one year from the date of shipment. Non-hazardous. No MSDS required.

ii. *Vitronectin monoclonal antibody*.

BioPorto Diagnostics. BioPorto Cat.No. CSI 003-23. Mouse Anti-Human Vitronectin Monoclonal Antibody, Unconjugated, Clone number HV23. **BioPorto** Grusbakken 8 DK-2820 Gentofte Denmark. Phone: (+45) 4529 0000 Fax:(+45) 4529 0001. info@bioporto.com. Subclass IgG1/k

Antigen: Vitronectin is a plasma glycoprotein that circulates in the blood. Vitronectin is circulating as a mixture of both 75 kDa and 65 kDa forms. Vitronectin is a major cell adhesive glycoprotein and is a common component of extracellular matrix and plasma. It competes effectively with other plasma proteins and is often involved in cell attachment, regulation of blood coagulation and immune responses. It has similar tissue distribution to fibronectin and also its integrin receptor recognises fibronectin (2).

Immunogen: Human vitronectin purified from plasma by heparin-affinity chromatography.

Specificity: CSI 003-23 is highly specific for vitronectin. There is no evidence for cross-reactivity with other connective tissue proteins (fibronectin, elastin,

collagen,laminin). CSI 003-23 cross-reacts with vitronectin from cat, dog, goat, cow and to a lesser extent with horse.
Epitope specificity: Epitope is located in the somatomedin B domain. CSI 003-23 binds nearly as well to native vitronectin as to denatured. CSI 003-23 is a potent inhibitor of integrin-mediated cell adhesion to vitronectin and a moderate inhibitor of PAI-1 binding. CSI 003-23 also binds to vitronectin in ELISA when vitronectin is coated directly onto the microtiter well.
Storage: In the dark at 4-8ºC,

iii. *Platelet factor 4 monoclonal antibody*.

Santa Cruz Biotechnology, Inc. Catalog number of clone number: PF-4 (L14Z): sc-74256. **Santa Cruz Biotechnology, Inc.** 2145 Delaware Avenue. Santa Cruz, California 95060. U.S.A. Phone: 831.457.3800 Fax: 831.457.3801. e-mail: scbt@scbt.com
Target: PF-4 (L14Z) is recommended for detection of PF-4, also designated platelet factor-4, of human origin as detected by Western Blotting (starting dilution 1:200, dilution range 1:100-1:1000) and solid phase ELISA (starting dilution 1:30, dilution range 1:30-1:3000). Suitable for use as control antibody for PF-4 siRNA (h): sc-39364. Molecular Weight of PF-4: 10 kDa. Positive Controls: human platelets or human serum.
Secondary reagents used in QC. The following support (secondary) reagents were used in QC: 1) Western Blotting: use goat anti-mouse IgG-HRP: sc-2005 (dilution range: 1:2000-1:32,000) or Cruz Marker™ compatible goat anti-mouse IgG-HRP: sc-2031 (dilution range: 1:2000-1:5000), Cruz Marker™ Molecular Weight Standards: sc-2035, TBS Blotto A Blocking Reagent: sc-2333 and Western Blotting Luminol Reagent: sc-2048.
Background: Platelet factor 4 (PF-4 or PF4) is a 70 amino acid protein that is released from the α-granules of activated platelets and binds with high affinity to heparin. Platelets secrete low molecular weight PF-4, which binds to and neutralizes heparin and related sulfated glycosaminoglycans (GAGs). Its major physiologic role appears to be neutralization of heparin-like molecules on the endothelial surface of blood vessels, thereby inhibiting local antithrombin III activity and promoting coagulation. As a strong chemoattractant for neutrophils and fibroblasts, PF-4 probably has a role in inflammation and wound repair. Both PF4 and eotaxin, a specific chemoattractant for eosinophils, have been shown to exhibit stronger expression in spleens of adult NOA mice (an animal model of allergic or atopic dermatitis) than in younger mice, parallel to the increase in ulcerative skin lesions in older mice. This suggests that PF-4 and eotaxin may play important roles in the etiology of atopic dermatitis. PF-4 is encoded by a small inducible gene (SIG), so called because of its small size and its stimulation with platelet activation. The gene encoding PF-4 maps to human chromosome 4q12-q21.
Chromosomal location: Genetic locus: PF4 (human) mapping to 4q12-q21.
Source: PF-4 (L14Z) is a mouse monoclonal antibody raised against full length recombinant PF-4 of human origin.

Storage: Store at 4° C, **DO NOT FREEZE**. Stable for one year from the date of shipment. Non-hazardous. No MSDS required.

iv. *Kininogen-1 monoclonal antibody*.

Santa Cruz Biotechnology, Inc. Catalog number of clone number: Kininogen LC (14J09): sc-80524. **Santa Cruz Biotechnology, Inc.** 2145 Delaware Avenue. Santa Cruz, California 95060. U.S.A. Phone: 831.457.3800. Fax: 831.457.3801. e-mail: scbt@scbt.com

Background: Kininogen is a 644 amino acid precursor protein that is expressed by the KNG1 gene and is secreted into blood plasma. Due to alternative splicing events, several Kininogen protein derivatives exist, including Kininogen LC (light chain) and Kininogen HC (heavy chain), both of which are produced from the Kininogen precursor and exhibit different functions throughout the cell. Kininogen HC plays an important role in blood coagulation by helping to ensure that prekallikrein and Factor XI (both of which are involved in blood coagulation) are properly situated for interaction with Factor XII. Additionally, Kininogen HC releases a smaller, active protein known as bradykinin, which plays a role in smooth muscle contraction, induction of hypotension, regulation of blood glucose levels, stimulation of nociceptors and overall mediation of inflammatory responses throughout the cell. In contrast to Kininogen HC, which is involved in blood clotting, Kininogen LC is primarily associated with inhibition of thrombocyte aggregation and also functions as a strong inhibitor of cysteine proteinases.

Storage: Store at 4° C, **DO NOT FREEZE**. Stable for one year from the date of shipment. Non-hazardous. No MSDS required.

Chromosomal location: Genetic locus: KNG1 (human) mapping to 3q27.3.

Source: Kininogen LC (14J09) is a mouse monoclonal antibody raised against amino acids 438-531 of recombinant Kininogen LC of human origin.

Target: Kininogen LC (14J09) binds Kininogen LC of human origin by Western Blotting (starting dilution 1:200, dilution range 1:100-1:1000) and immunoprecipitation [1-2 µg per 100-500 µg of total protein (1 ml of cell lysate)]. Suitable for use as control antibody for Kininogen siRNA (h): sc-40723, Kininogen shRNA Plasmid (h): sc-40723-SH and Kininogen shRNA (h) Lentiviral Particles: sc-40723-V. Molecular Weight of Kininogen HC: 64 kDa. Molecular Weight of Kininogen LC: 53 kDa. Positive Controls: ECV304 cell lysate: sc-2269.

Secondary reagents for QC: The following support (secondary) reagents were used in QC: 1) Western Blotting: use goat anti-mouse IgG-HRP: sc-2005 (dilution range: 1:2000-1:32,000) or Cruz Marker™ compatible goat antimouse IgG-HRP: sc-2031 (dilution range: 1:2000-1:5000), Cruz Marker™ Molecular Weight Standards: sc-2035, TBS Blotto A Blocking Reagent: sc-2333 and Western Blotting Luminol Reagent: sc-2048. 2) Immunoprecipitation: use Protein A/G PLUS-Agarose: sc-2003 (0.5 ml agarose/2.0 ml).

v. *Amyloid A protein monoclonal antibody*.

Santa Cruz Biotechnology, Inc. Catalog and clone number: **SAA (115): sc-59679. Santa Cruz Biotechnology, Inc.** 2145 Delaware Avenue. Santa Cruz,

California 95060. U.S.A. Phone: 831.457.3800. Fax: 831.457.3801. e-mail: scbt@scbt.com

Target: SAA (115) binds natural and recombinant SAA of human origin by Western Blotting (starting dilution 1:200, dilution range 1:100-1:1000) and immunoprecipitation [1-2 µg per 100-500 µg of total protein (1 ml of cell lysate)]; non cross-reactive with other human cytokines or growth factors tested such as IL-1β, IL-8, MCAF, TGFβ and EGF. Suitable for use as control antibody for SAA siRNA (h): sc-40817, SAA shRNA Plasmid (h): sc-40817-SH and SAA shRNA (h) Lentiviral Particles: sc-40817-V. Molecular Weight of SAA: 12 kDa. Positive Controls: Caki-1 cell lysate: sc-2224.

Secondary reagents used n QC: The following support (secondary) reagents were used in QC: 1) Western Blotting: use goat anti-mouse IgG-HRP: sc-2005 (dilution range: 1:2000-1:32,000) or Cruz Marker™ compatible goat antimouse IgG-HRP: sc-2031 (dilution range: 1:2000-1:5000), Cruz Marker™ Molecular Weight Standards: sc-2035, TBS Blotto A Blocking Reagent: sc-2333 and Western Blotting Luminol Reagent: sc-2048. 2) Immunoprecipitation: use Protein A/G PLUS-Agarose: sc-2003 (0.5 ml agarose/2.0 ml).

Background: The serum Amyloid A (SAA) family of proteins is encoded by muliple genes, which display allelic variation and a high degree of homology in mammals. The four members of the SAA gene family are clustered on human chromosome 11p15.1. Three SAA genes are differentially expressed and encode small apolipoproteins. SAA1 and SAA2 encode the acute phase SAAs (A-SAAs) and SAA4 encodes the constitutively expressed SAA (C-SAA). A fourth locus, SAA3, is a pseudogene that contains two C/EBP-binding sites and a third site, which interacts with SAA3 enhancer factor. Human SAA proteins are a group of apolipoproteins found predominantly in the high-density lipoprotein (HDL) fraction of plasma. SAA is a major acute-phase protein and precursor to Amyloid A protein, which is the major constituent of the fibril deposits of reactive amyloidosis. SAA is secreted in large amounts by the liver during microbial infections or inflammatory diseases.

Chromosomal location: Genetic locus: SAA1 (human) mapping to 11p15.1.

Source: SAA (115) is a mouse monoclonal antibody raised against highly purified recombinant SAA of human origin.

Storage: Store at 4° C, **DO NOT FREEZE**. Stable for one year from the date of shipment.

vi. Fibrinogen alpha chain monoclonal antibody.

Santa Cruz Biotechnology, Inc. Catalog and clone number: Fibrinogen a(FgA): sc-59525. **Santa Cruz Biotechnology, Inc.** 2145 Delaware Avenue. Santa Cruz, California 95060. U.S.A. Phone: 831.457.3800. Fax: 831.457.3801. e-mail: scbt@scbt.com

Background: The plasma glycoprotein Fibrinogen is synthesized in the liver and comprises three structurally different subunits: α, β and γ. Fibrinogen is important in platelet aggregation, the final step of the coagulation cascade (i.e. the formation of Fibrin) and determination of plasma viscosity and erythrocyte aggregation. It is both constitutively expressed and inducible during an acute

phase reaction. Hemostasis following tissue injury deploys essential plasma procoagulants (Prothrombin and Factors X, IX, V and VIII), which are involved in a blood coagulation cascade leading to the formation of insoluble Fibrin clots and the promotion of platelet aggregation. Following vascular injury, Fibrinogen is cleaved by Thrombin to form Fibrin, which is the most abundant component of blood clots. The cleavage products of Fibrinogen regulate cell adhesion and spreading, display vasoconstrictor and chemotactic activities, and are mitogens for several cell types.
Chromosomal location: Genetic locus: FGA (human) mapping to 4q32.1.
Source: Fibrinogen α (FgA) is a mouse monoclonal antibody raised against full length Fibrinogen α of human origin.
Storage: Store at 4° C, **DO NOT FREEZE**. Stable for one year.

vii. *Fibrinogen beta chain monoclonal antibody*.

Santa Cruz Biotechnology, Inc. Catalog and clone number: Fibrinogen β (C-20): sc-18029. **Santa Cruz Biotechnology, Inc.** 2145 Delaware Avenue. Santa Cruz, California 95060. U.S.A. Phone: 831.457.3800. Fax: 831.457.3801. e-mail: scbt@scbt.com
Background: The plasma glycoprotein Fibrinogen is synthesized in the liver and comprises three structurally different subunits: α, β and γ. Fibrinogen is important in platelet aggregation, the final step of the coagulation cascade (i.e. the formation of Fibrin) and determination of plasma viscosity and erythrocyte aggregation. It is both constitutively expressed and inducible during an acute phase reaction. Hemostasis following tissue injury deploys essential plasma procoagulants (Prothrombin and Factors X, IX, V and VIII), which are involved in a blood coagulation cascade leading to the formation of insoluble Fibrin clots and the promotion of platelet aggregation. Following vascular injury, Fibrinogen is cleaved by Thrombin to form Fibrin, which is the most abundant component of blood clots. The cleavage products of Fibrinogen regulate cell adhesion and spreading, display vasoconstrictor and chemotactic activities and are mitogens for several cell types.
Target: Fibrinogen β (C-20) binds Fibrinogen β of mouse, rat and human origin by Western Blotting (starting dilution 1:200, dilution range 1:100-1:1000), immunoprecipitation [1–2 µg per 100–500 µg of total protein (1 ml of cell lysate)], immunofluorescence (starting dilution 1:50, dilution range 1:50-1:500) and solid phase ELISA (starting dilution 1:30, dilution range 1:30-1:3000). Suitable for use as control antibody for Fibrinogen β siRNA (h): sc-37096 and Fibrinogen β siRNA (m): sc-37097; and as shRNA Plasmid control antibody for Fibrinogen β shRNA Plasmid (h): sc-37096-SH and Fibrinogen β shRNA Plasmid (m): sc-37097-SH. Molecular Weight of Fibrinogen β: 67 kDa.
Secondary reagents in QC: The following support (secondary) reagents are used for QC: 1) Western Blotting: use donkey anti-goat IgG-HRP: sc-2020 (dilution range: 1:2000-1:100,000) or Cruz Marker™ compatible donkey anti-goat IgG-HRP: sc-2033 (dilution range: 1:2000-1:5000), Cruz Marker™ Molecular Weight Standards: sc-2035, TBS Blotto A Blocking Reagent: sc-2333 and Western Blotting Luminol Reagent: sc-2048. 2) Immunoprecipitation: use Protein

A/G PLUS-Agarose: sc-2003 (0.5 ml agarose/2.0 ml). 3) Immunofluorescence: use donkey anti-goat IgG-FITC: sc-2024 (dilution range: 1:100-1:400) or donkey anti-goat IgG-TR: sc-2783 (dilution range: 1:100-1:400) with UltraCruz™ Mounting Medium: sc-24941.

Chromosomal location: Genetic locus: FGB (human) mapping to 4q32.1; Fgb (mouse) mapping to 3 E3.

Source: Fibrinogen β (C-20) is an affinity purified goat polyclonal antibody raised against a peptide mapping near the C-terminus of Fibrinogen β of human origin.

Storage: Store at 4° C, **DO NOT FREEZE**. Stable for one year from the date of shipment. Non-hazardous. No MSDS required.

b. Secondary fluorescent antibodies.

i. Fluorescent labeling of antibodies.

The Molecular Probes' Alexa Fluor® 532 Protein Labeling Kit was used to label secondary antibodies with Alexa Fluor 532 dye. Alexa Fluor 532 dye–labeled proteins have absorption and fluorescence emission maxima of approximately 530 nm and 554 nm, respectively. [http://probes.invitrogen.com/media/pis/mp10236.pdf] The Alexa Fluor 532 reactive dye has a succinimidyl ester moiety that reacts efficiently with primary amines of proteins to form stable dye–protein conjugates. The purified protein was placed in phosphate-buffered saline (PBS) for derivatization. **Step one:** 1 M solution of sodium bicarbonate was prepared by adding 1 mL of deionized water (dH2O) to the provided vial of sodium bicarbonate (Component B). After vortexing to dissolve the bicarbonate the solution will have a pH of ~8.3 and can be stored at 4°C for up to two weeks. **Step two:** involves making the antibody solution up to a concentrsyion of 2 mg/mL PBS or 0.1 M sodium bicarbonate. **Step three:** 50 µL of 1 M bicarbonate (prepared in step 1.1) is added to 0.5 mL of the 2 mg/mL protein solution. [*Bicarbonate, pH ~8.3, is added to raise the pH of the reaction mixture, since succinimidyl esters react efficiently at pH 7.5–8.5].* **Step four:** a vial of reactive dye is warmed to room temperature and the protein solution from step three is transferred to the vial of reactive dye. This vial contains a magnetic stir bar. Cap the vial and invert a few times to fully dissolve the dye. Stir the reaction mixture for 1 hour at room temperature.

Antibodies thus labeled were purified by size exclusion chromatography on a G-100 Sephadex column.

ii. Secondary antibody source.

***1. Sialyl-Lewis x directed monoclonal antibody**.

Santa Cruz Biotechnology, Inc. Calalog number: (CHO131): sc-32243. **Santa Cruz Biotechnology, Inc.** 1.800.457.3801 831.457.3800 fax 831.457.3801 **Europe** +00800 4573 8000 49 6221 4503 0 **www.scbt.com**

Target: CD15s (CHO131) is recommended for detection of CD15s (sialyl-Lewis X) of human origin. CD15 (also known as embryonic stage-specific antigen, SSEA-1, Lewis X or Lex) is found in embryonal carcinoma cells of mouse and human origin and n some preimplantation stage mouse embryos. This stage-specific antigen (SSEA-1) is first detected on blastomeres of 8-cell stage embryos. Trophectodermal cells are transitorily positive; however, each cell in

the inner cell mass eventually expresses CD15. CD15 has been implicated as having a role in mediating compaction of the mouse embryo at the morula stage. Additionally, CD15 functions as an adhesion molecule capable of calcium-mediated homotypic binding. Cells with high surface expression of CD15 therefore exhibit strong self-aggregation (based on CD15-CD15 interaction) in the presence of calcium.
Chromosomal location: Genetic locus: FUT4 (human) mapping to 11q21
Source: CD15s (CHO131) is a mouse monoclonal antibody raised against alpha-1-3-Fucosyltransferase product.
Storage: Store at 4° C, **DO NOT FREEZE**. Stable for a year.

***2. Lewis X bind monoclonal antibody**.
Catalog and clone numbers: CD15 (TG-1): sc-19595. **Santa Cruz Biotechnology, Inc.**: 1.800.457.3801 831.457.3800. fax 831.457.3801 **Europe** +00800 4573 8000 49 6221 4503 0 **www.scbt.com**
Source. CD15 (TG-1) is a mouse monoclonal antibody raised against glycoprotein fraction of human lymphocytes.
Target: CD15 (TG-1) binds CD15 of human origin.
Storage. Store at 4° C, **DO NOT FREEZE**. Stable for one year. Non-hazardous. No MSDS required.
Source. CD15 (TG-1) is a mouse monoclonal antibody raised against the glycoprotein fraction of human lymphocytes.

c. Calibration reagents not provided in the kit.

Commented [A20]: There is a required calibrator information that would need to be provided, including stability, etc.

i. Glycoprotein standard.

Based on the specificity of the antibodies used in peptide and glycan epitope selection, the use of high resolution separation methods to further fractionate affinity captured species, and mass spectrometry sequencing methods to characterize their polypeptide backbone, identification of the glycoproteins being used as biomarker standards in this platform brings an unprecedented level of structure elucidation to these standards. That does not mean however that the full structure of the several glycoforms of each protein standard has been determined or is known. Methods for total characterization of all the glycoforms of a specific glycoprotein have not yet been described.

Glycoprotein isoforms required to determine the dose response curves in Figure 6 and for supplying internal standards in assay kits were obtained by preparative chromatography. Cancer patient plasma was passed through a 1 x 5 cm column packed with 20 um particle diameter POROS Al packing to which a mixture of CHO-131 and TG-1 antibodies were immobilized by Schiff base formation, after which the –C=N- bond was reduced with sodium cyanoborohydride. Ten ml plasma samples were used in each run. Le^x and sLe^x antigens thus captured were eluted with 0.5 M glycine (pH 2.0) containing 5% ethylene glycol. The eluted fraction was dialyzed against 0.1 M Tris (pH 7.5) and stored at 4 ^oC until used. Within 24 hr the Le^x and sLe^x fraction was applied to a 0.46 x 5 cm immunoaffinity column targeting one of the marker proteins. These columns were prepared in a manner identical to the one used in the preparation

of the CHO-131/TG-1 column. Purity of these double affinity selected proteins was assessed by bottom up proteomics in which samples were tryptic digested, the peptide fragments separated by reversed phase chromatography (RPC), and the individual fractions were examined by tandem MALDI and ESI mass spectrometry with an ABI-4800 and LTQ-Orbit trap, respectively. As part of this process they were quantified by MRM based tandem mass spectrometry as well.

Purification of the Lewis antigen containing glycoforms used as assay standards for calibration will be described in detail below. All the Lewis antigen bearing glycoforms were selected from plasma samples (obtained from NIST) with an immunosorbent column. Ten ml of plasma was processed at a time on a 1 x 5 cm CHO-131/TG-1 antibody column to capture Le^x and sLe^x bearing glycoproteins. Glycoproteins thus selected were released from the affinity column, the eluent fraction neutralized, the sample dialyzed, and either lyophilized or used directly in a second dimension of affinity selection.

The affinity selected Lewis antigen glycoform (ASLAG) fraction was used in the purification of specific glycoproteins. Individual POROS Al immunosorbent columns (1 x 1 cm) targeting clusterin, plasminogen, fibrinogen, vitronectin, proteoglycan-4, serum amyloid protein A, kininogen-1, and histidine rich glycoprotein were connected in series and loaded with the ASLAG fraction. The affinity columns were connected in series to maximize utilization of the ASLAG fraction. After loading the columns were disconnected and eluted individually with pH 2 glycine mobile phase as described above. These double affinity selected glycoprotein fractions were individually dialyzed, identified after trypsin digestion and tandem mass spectrometry of the peptide fragments, and quantified by MRM quantification as described above.

ii. Interferometry calibration standards.

A solution of standard proteins is supplied with kits for instrument calibration. The proteins and their concentration are as follows; transferrin (4×10^{-3} g/ml), haptoglobin (1.25×10^{-3} g/ml), α_1-antitrypsin (1.4×10^{-3} g/ml), α_2-macroglobulin ($1.8\ 10^{-3}$ g/ml), clusterin (1.08×10^{-4} g/ml), fibrinogen (2.72×10^{-3} g/ml), and plasminogen (1.08×10^{-4} g/ml). Clusterin, fibrinogen, and plasminogen are present as multiple isoforms.

All these protein standards were obtained from commercial suppliers. The concentration of each standard in the mixture was determined by mass spectrometry methods using multiple reaction monitoring. At least three peptides were used to quantify each protein.

ii. Le^x and sLe^x bearing glycoprotein calibration standards.

Glycoprotein standards are not supplied with the kit but can be purchased separately. Standards and their concentration are as follows; clusterin (890 ng/ml), plasminogen (1.0 ug/ml), fibrinogen (131 ng/ml), vitronectin (121 ng/ml), proteoglycan-4 (69 ng/ml), serum amyloid protein A (6.8 ng/ml), kininogen-1 (52 ng/ml), and histidine rich protein (110 ng/ml) glycoforms has been described above.

d. Controls.

Commented [A21]: Additional positive and negative controls that laboratories need to use per CLIA, local, and/or state requirements would need to be recommended and also run as a part of your analytical and clinical study.

A pooled subject, control plasma sample from NIST was used in developing this assay. It is not clear whether NIST will supply control plasma samples of this type in the future.

2. Characterization of the active reagents and supplies.

a. Disc manufacturing process.

The manufacturing processes for discs follows the requirements laid out in 21CFR820. Discs are manufactured in-house using qualified manufacturing equipment and following standardized production records. However, the manufacturing facility has not yet been audited or reviewed by FDA.

Process Outline: The following is a generic outline of the process used to produce a test disc.

- Raw silicon discs with a thin silicon dioxide layer are received from a qualified supplier. Discs are sampled and tested for conformance of the oxide layer thickness to the specification.
- The discs undergo a surface activation process that opens silanol groups on the surface and prepares the disc for a deposition of a functionalized silane. Activation is achieved by a combination of plasma etching and standard washing processes employed in the semi-conductor industry. Activation time, salt concentration in the washing solutions, and strength of the plasma field employed (as measured by gas flow rate and power output) are important parameters in the activation process.
- Once activated, the discs undergo a vapor-phase deposition with an amine-functionalized silane. Critical parameters for this process are the deposition time and temperature.
- Silanized discs are sampled and tested for the presence and uniformity of the silane deposition. This is done using contact angles to test surface hydrophobicity and by binding a fluorescent dye to the surface and measuring the intensity and variability of the fluorescent response.
- After the silane is applied, a linker is bound to the surface in a liquid phase deposition. This linker allows the surface to bind proteins. The critical parameters for this process are concentration of the linker and time.
- Discs with the linker are sampled and tested in a similar fashion to the silanized discs in order to assess the presence and uniformity of linker deposition.
- Fully functionalized discs (amine + linker) are printed with a hydrophobic well mask to create test wells to contain individual test samples.
- Discs after well mask application are sampled and visually inspected to ensure that mask alignment marks are clear and that no breaches are present in well barriers that could cause cross-contamination between individual patient samples.

- Discs with the well mask applied proceed to step where the target proteins are applied to each test well. This is accomplished by printing a micro-array of spots into each well. A number of spots are printed for each of the analytes included in the multiplex. Spot presence and alignment is checked during printing by the printer operator. Once the spots are printed, the disc is incubated with all the proteins on the array elements to allow chemical binding at the surface. Critical parameters for this process are protein print concentration and incubation time.
- Protein printed discs undergo a series of post-print processing steps:
    - Washing: removes any unbound protein from the print spots.
    - Chemical blocking: chemically deactivates the functional surface in order to prevent serum proteins binding to the well background during the assay.
    - Passivation: floods the well background with a small protein to cover any functional groups on the surface that survived chemical blocking.
    - Stabilization: coats the disc and the protein spots with a stabilizing agent to keep the proteins active during storage.
- **At this stage, the discs are sampled and tested using the actual customer assay protocol to assess performance of the test.**
- **After passing the final QC step, discs are packaged and labeled:**
    - Each disc is individually barcoded.
    - Discs are packaged in individual or bulk holders and placed in plasticized foil pouches with desiccant. The packaging is designed to protect the discs from impact and humidity.
    - Labels are applied to the pouches.
    - The discs are packaged along with the remaining assay reagents and a package insert before shipping.
    - Note that all labels and inserts comply with the regulations set forth in 21CFR809.10.
- **Cross-reactivity issues**. Described in section G.5.a below.

Commented [A22]: Where is this section? Cross-reactivity would need to be appropriately assessed.

Any carryover should be assessed in appropriate studies.

b. Disc storage.

After immunological array fabrication antibodies immobilized on disc arrays are blanketed with nitrogen and then covered with a gas impermeable plastic film. This plastic film is left in place until the disc is used. It is recommended that assay discs be stored in a refrigerator (at approximately 4 ^{o}C until used to guarantee retention of antibody activity although discs are not refrigerated when shipped. Assays are carried out at room temperature.

c. Secondary fluorescent antibodies.

i. Biochemical and immunochemical purity.

***$_1$. CHO131 monoclonal antibody targeting sLe^x.**

Purity of the antibody was determined by 1) affinity chromatograpy on an sLe^x column with reversed phase chromatography of the non-retained material, 2) immunoprecipitation using sLe^x containing neoglycoprotein described below.

***2. TG-1 monoclonal antibody targeting Le^x.**

Purity of the antibody was determined by 1) Immunoprecipitation: use Protein L PLUS-Agarose: sc-2336 (0.5 ml agarose/2.0 ml). 2) Immunofluorescence: use goat anti-mouse IgM-FITC: sc-2082 (dilution range: 1:100-1:400) or goat anti-mouse IgM-TR: sc-2983 (dilution range: 1:100-1:400) with UltraCruz™ Mounting Medium: sc-24941.

ii. Cross-reactivity.

Described in section G.5.a below.

iii. Reagent stability.

Immobilized antibodies on discs and secondary fluorescent labeled antibodies in assay kits are stable for one year when stored at 4 ºC.

Commented [A23]: Data to support this would need to be provided.

3. Instrument.

Commented [A24]: Instrumentation needs to be manufactured under GMP/QSR.

a. Sample Processor.

Immunological array discs are read with the Quadraspec Inspira Sample Processor which is built on the basis of Sias' (Hombrechtikon, Switzerland) Xantus platform with certain modifications to allow processing of sample discs. The sample Processor is used to carry out all liquid handling operations from receipt of patient serum to the point where the disc is ready for scanning and analysis. This includes scanning of sample ID barcodes, sample transfer from tubes/plates, sample dilutions, sample and conjugate antibody application to the disc, disc incubation, and disc washing. The operation is batched to allow the use of the 24 wells of the disc. The 24 wells on the disc are distributed around the periphery of the disc, all of which are equidistant from the center. The device is attached to a local computer network through the user software in order to allow for patient results to be directly uploaded into LIMS software.

The rationale for using this device is based on 1) the reduced variability resulting from automated transfer/mixing compared to manual pipetting, 2) the improved data integrity resulting from samples being tracked by software from first scan to final result, and 3) the improved throughput characteristics of an automated system.

The Inspira Sample Processor (Figures 9 and 10) consists of a robotic pipetting arm with linear motion capabilities in the x, y, and z dimensions, a spinning stage for the disc enclosed in a sealed, humidified chamber, a disc washing system capable of drawing up to three unique wash solutions, and sufficient deck space to hold a variety of different reagents. The sample Processor uses eight washable, reusable pipette tips that are cleaned in a wash station between all patient samples. Carry-over studies have been performed to ensure that no remnant of a previously pipetted sample will contaminate a following sample. The tips have level sensing capability to control

submersion depth and to flag samples that have insufficient volume. The following images show the unit with the major components marked.

The following is a description of a standard sample processing sequence:

- Load tube racks with patient samples and scan sample barcodes
- Load control, sample diluent, and detector antibody vials
- Load microtiter plate for patient sample dilution (optional)
- Load bulk wash solutions (and empty waste receptacle if necessary)
- Load test disc, scan disc barcode, and close humidity chamber lid
- Push “Start Sequence” to launch the process through the software user interface

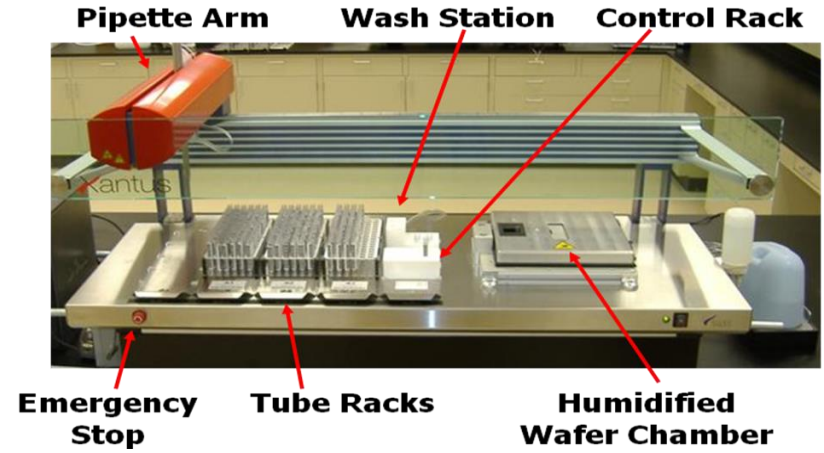

Figure 9: Inspira Sample Processor

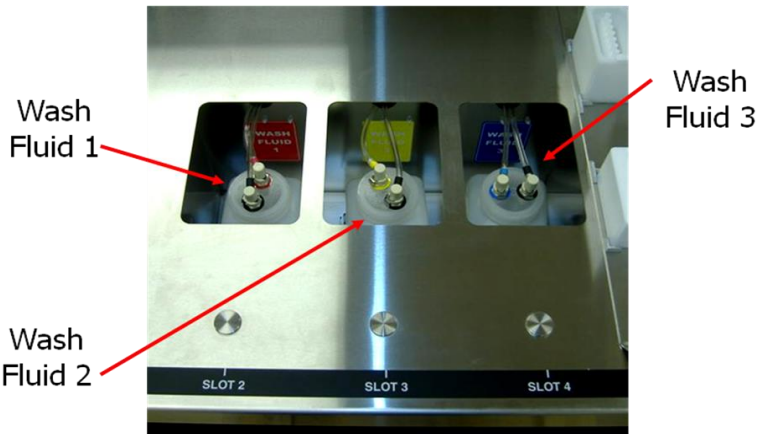

Figure 10: Close-Up of Bulk Fluids (located under tube racks)

- Sample diluent is pipetted from source vial to microtiter plate (all tips aspirate a bulk quantity and dispense a metered quantity into eight wells at a time). This process is followed by a tip wash.
- Patient sample pipetting
  - Patient samples 1 – 8 are aspirated at one time by each of the eight pipette tips and transferred to the microtiter plate containing the sample diluents.
  - Samples are dispensed into the plate and mixed by a series of aspirate/dispense cycles of the tips
  - Tips are washed in the wash station by a flush with the system liquid (DI water). The liquid is sprayed into cups directly below the tips causing the water to bubble up around the tip and thus also washing the outside.
  - Repeat the previous three steps for the remaining patient samples.
- Sample transfer from plate to disc
  - Diluted patient samples 1 – 8 are aspirated at one time by each of eight pipette tips and transferred to the disc.
  - A flap opens in the center of the humidity chamber lid to allow access for the tips to the disc surface.
  - Samples are dispensed into wells on the disc surface.
  - Tips are washed in the wash station (see above)
  - Repeat the previous steps for the remaining diluted patient samples.
- Samples incubate on the disc for the required time. During this step humidity surrounding the disc is maintained at a high level to prevent drying down of the sample drops (Figure 11).
- A global wash removes any remaining unbound antibody. This wash consists of a stream of bulk wash solution being dispensed onto the center

of the disc while the disc spins. Centrifugal force carries the liquid towards and off the edge of the disc.

- The disc is spun dry to remove any remaining wash liquid.
- Detector antibody application
    - All eight tips aspirate a bulk quantity of the labeled detector antibody
    - Each well is dosed with a quantity of the detector
    - At the conclusion of this process, the tips are washed in the wash station
- The detector antibody incubates on the disc for the required time. As with the sample incubation, humidity is maintained at a high level to prevent dry-down.
- A global wash removes the unbound detector unbound. Since the same detector is used for all wells, no blotting step is used in this case since cross-contamination is not a concern.
- The disc receives a final spin dry to remove any remaining liquid from the surface.
- The disc is now ready to be transferred to the Reader.

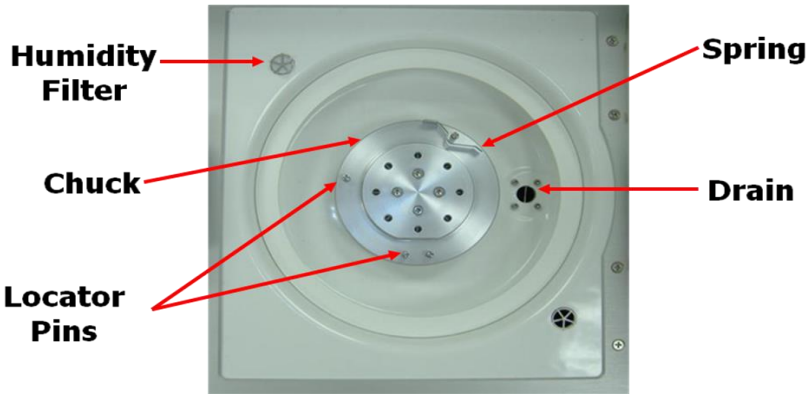

Figure 11: Close-up of Humidified Disc Chamber

Any remaining reagents and patient samples are removed from the sampler to ready the unit for the next test run.

Units are calibrated by Quadraspec prior to shipment. This includes setting targets for x, y, and z motion and checking accuracy of pipetting volumes. The liquid detection capabilities are also verified. No customer calibration of these variables is required. Instruments are installed calibrated by Quadraspec personnel.

Commented [A25]: Once calibrated, how long can the instrument be used before re-calibration is required?

b. Quadraspec Inspira Reader.

The Quadraspec Inspira Reader (Figure 12, 13) is used to scan the disc both interferometrically and fluorescently. After completion of sample handling, incubation, and washing, the test disc is mounted in the Reader for scanning. Data collected by the

Reader is analyzed using Quadraspec's proprietary image processing algorithms. The device is attached to a local computer network through the user software in order to allow for patient results to be directly uploaded into the customer's LIMS software. The network connection also allows several sample Processors to be coupled to a single Reader in order to maximize testing efficiency since scanning is generally quicker than sample processing.

The rationale for using this device is based on 1) the benefits derived from dual channel detection in both the interferometric and fluorescent realms, 2) the ability to multiplex multiple analytes without concern for cross-reactivity and cross-talk, and 3) the improved throughput obtained from the platform.

The Inspira Reader consists of a rotating platform which holds the disc, a laser to scan the protein patterns on the disc, a series of lenses to polarize, filter, and focus the laser beam, an interferometric detector for reading mass, and a fluorescence detector for reading fluorescence. The laser, optics, and detectors are mounted on a stage that moves laterally across the top of the disc, thus enabling radial scanning of the different "tracks" of data. This whole assembly is contained in a sealed case to prevent accidental exposure of the laboratory technician to laser radiation. The system is locked anytime the laser is powered on, except when Quadraspec personnel perform maintenance on the system.

Figure 12. Quadraspec Inspira Reader

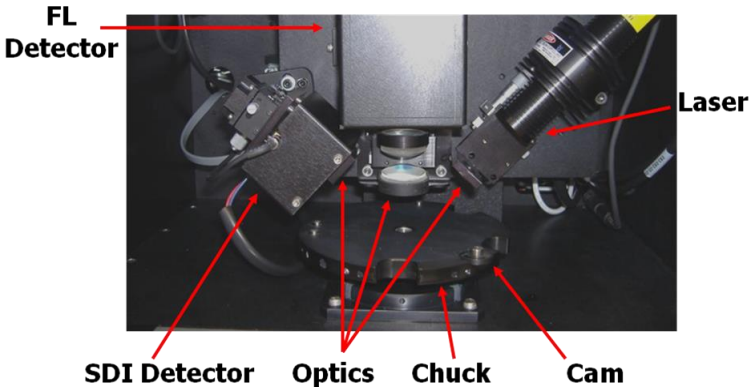

Figure 13. Close-up of Internal Components

A reader scan consists of the following steps:

- Mount the disc on the chuck of the Reader and scan the disc barcode
- Close the Reader door
- Push “Start Sequence” to launch the process through the user interface
- The laser scans the disc track by track
- After completion of the scan, the disc is removed from the Reader and stored in case a rescan is required.

c. Quadraspec Workstation and BioCD Software.

i. Computer Workstation.

Each Quadraspec Inspira Reader and Sample Processor requires a dedicated computer workstation (Dell, HP, or similar) running Windows XP or Vista with Quadraspec’s proprietary BioCD software. This computer is connected to a local network in order to allow for multiple samplers and Readers to communicate with one another and enable the software to directly upload test results to the customer’s LIMS software. Furthermore, a customer-initiated remote network connection allows Quadraspec technical service personnel to perform initial equipment troubleshooting remotely rather than immediately requiring a site visit.

ii. Communications Systems.

Each computer is connected to either a sample Processor or a Reader using a USB cable. Computers controlling Quadraspec hardware are networked locally to enable multiple sample Processors to feed a single Reader. Finally, a variety of connections are available to the customer to enable data transfer directly from the BioCD software to the customer’s LIMS software.

iii. Software Versions.

The current version of BioCD is v2.9.x. BioCD interfaces with firmware in the sample Processor provided by Sias. The current firmware versions are v.1.00 build 2 for Sias' BB boards (control y and z-motors and pipette pumps) and x-motor board and v.05 build 1 for the Sias I/O board.

iv. Assay Types.

Quadraspec's BioCD platform is designed to allow the user to run a variety of assays. Each assay is characterized in the software by a unique set of processing parameters to be used on the sample Processor and Reader. These parameters are currently stored on the workstation's hard drive in unencrypted xml format; they are hidden from the user but not actually access-controlled. Each assay is associated with a unique barcode that, when entered into the system, triggers the appropriate parameter set to be launched.

v. User Definable Parameter.

All parameters critical to the performance of the assay are hardcoded into the software, triggered by the assay barcode, and unchangeable by the user. However, a few data management related parameters may be edited by the user if necessary. These include turning on and off the LIMS export function, enabling the use of duplicate patient sample barcodes, and the ability to modify the length of the patient sample barcode.

vi. User Interface:

Quadraspec's BioCD software was written specifically to control Quadraspec's hardware and to analyze the data obtained from the Reader's scan of the disc. The program consists of a graphical user interface (GUI) that guides the user through the steps of the assay process, beginning with the scanning of patient samples and ending with the patient results.

The first screen a user sees upon launching the software prompts the technician to launch a disc (Figure 14).

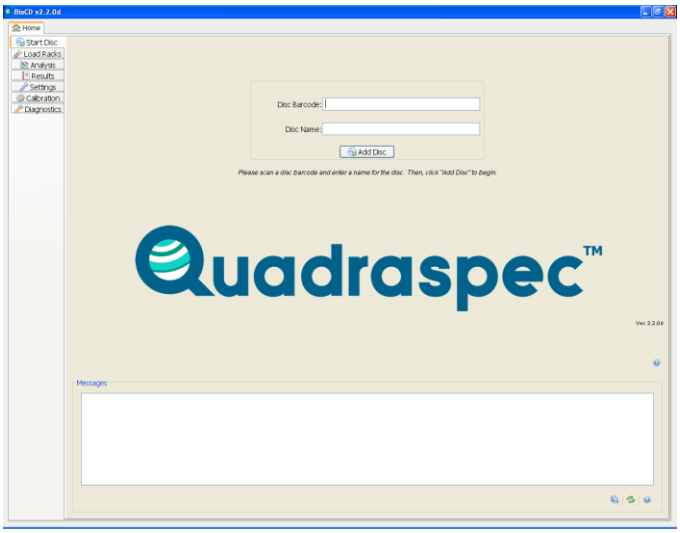

Figure 14. BioCD Software “Launch Disc” Screen.

The user scans the disc’s barcode and enters a name for the disc. The barcode contains information used by the software to launch the correct process sequence for the assay being performed. The barcode is also used to track the disc through the remainder of the process in order to ensure that sample identity is properly matched to test results. The disc name is used for reference by the technician only.

The next step for the operator is to load patient sample tubes into racks. This process can be run offline and in parallel with another disc being processed on the sample Processor in order to maximize the efficiency and throughput of the system. To this end, Quadraspec supplies several sets of racks each labeled with unique barcodes (for example: set 1 = A1, A2, A3; set 2 = B1, B2, B3; etc.). These barcodes are scanned prior to loading the racks and again when the racks and the disc are loaded onto the sample Processor. The following screen is displayed during the process of loading racks (Figure 15).

As samples are loaded into the rack and scanned using the manual barcode scanner attached to the computer, the software illuminates the rack positions of the next sample to be loaded. An audible cue from the scanner indicates a successfully scanned barcode. Furthermore, a text log at the bottom of the GUI tracks each sample and its rack location as a final check. Semi-automated and fully automated sample barcode scanning systems are available as optional accessories to the system if the customer prefers that approach.

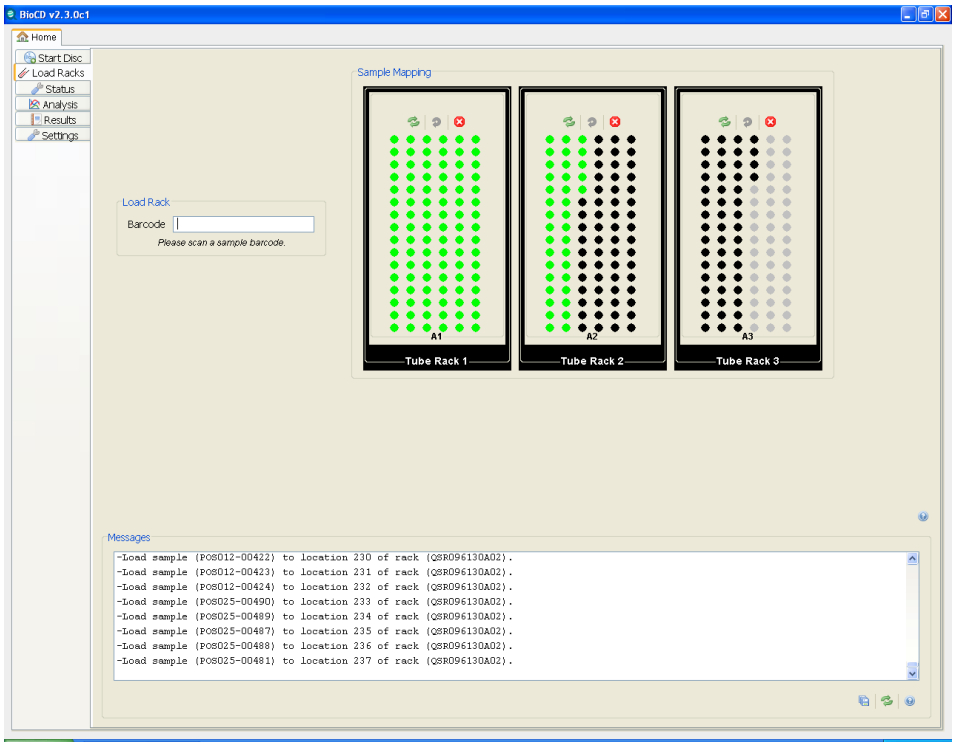

Figure 15. BioCD Software “Load Racks” Screen.

Once the user is ready to run the assay, all components of the test including samples, controls, diluents, wash solutions, and the disc are loaded into the sample Processor. All critical reagents (disc, control tubes, diluent tubes, and detection antibody tubes) are barcoded and scanned into the system. The barcodes contain information about the identity of the assay to which the reagents belong, and the system will not allow the user to proceed with the test unless the components match. The barcodes also contain unique a serial component in order to prevent a used disc or an empty reagent tube from being reloaded into the system. The sample tube rack barcodes are also scanned again at this stage in order to link the sample identities to the specific test disc being run. The following screen is displayed to the user at this stage of disc process (Figure 16).

Since wash solution, system liquid, humidity system fluid, and waste containers have sufficient volumes for multiple runs and thus don’t require filling, emptying, or replacing for every assay, the software reminds the user to check these fluid levels prior to launching the assay. The software provides the following screen as a quick reference for fluid liquid levels (Figure 17).

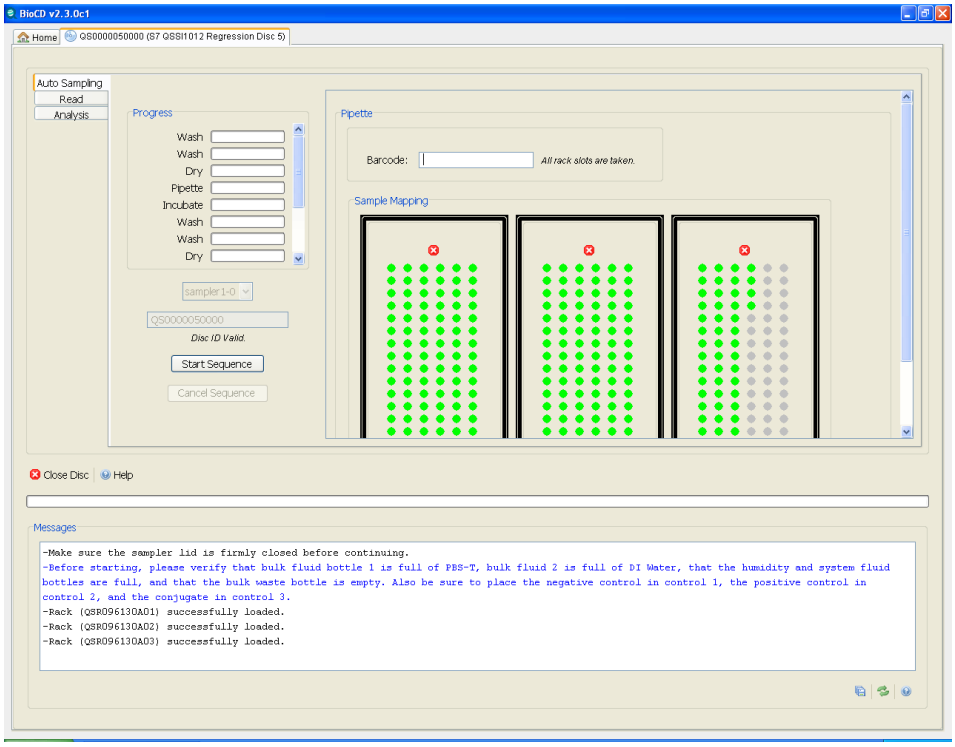

Figure 16. BioCD Software “Disc Processing” Screen

The system does not provide actual liquid sensing capabilities; however, liquid levels are updated on the screen based on the nominal flow rates and process times. When a bulk fluid reservoir is refilled or emptied, the user must manually push the appropriate button on the screen to reset the levels. If the levels drop below a pre-determined threshold and the user attempts to launch an assay, the software will warn the operator before initiating the process that fluid levels may be too low (or high in the case of the waste reservoir) to complete the process.

Once the user has loaded all components and verified the bulk fluid levels, he or she simply pushes the “Start Sequence” button, and the remaining steps of the process are completed automatically be the sample Processor. Status bars for each processing steps as well as the overall process illustrate the progress of the assay.

Several checks exist in the software to ensure that test result integrity is maintained. If any of these checks fail, the software takes appropriate steps to flag the problem. The first check is to see whether the pipette tips detect liquid prior to any sample aspiration operation. If no liquid is detected, the sample is automatically invalidated and no results are reported at the conclusion of the assay. This check is also displayed visually in the GUI. A sample that is appropriately detected turns blue in

the rack schematic; a sample that is not detected turns red. If liquid detection fails for a control aspiration, the software automatically cancels the run since proper classification of the controls during analysis is a prerequisite for valid sample test results. Any mechanical failure in the system included pump blockages or failures to activate the disc chamber flap or the spinner motor also automatically cancel a run. If humidity in the disc chamber remains below a predetermined threshold for the duration of the assay, the user is warned at the conclusion of the run that sample dry-down may have occurred and to check the assay results for anomalies. Low humidity does not automatically invalidate the run.

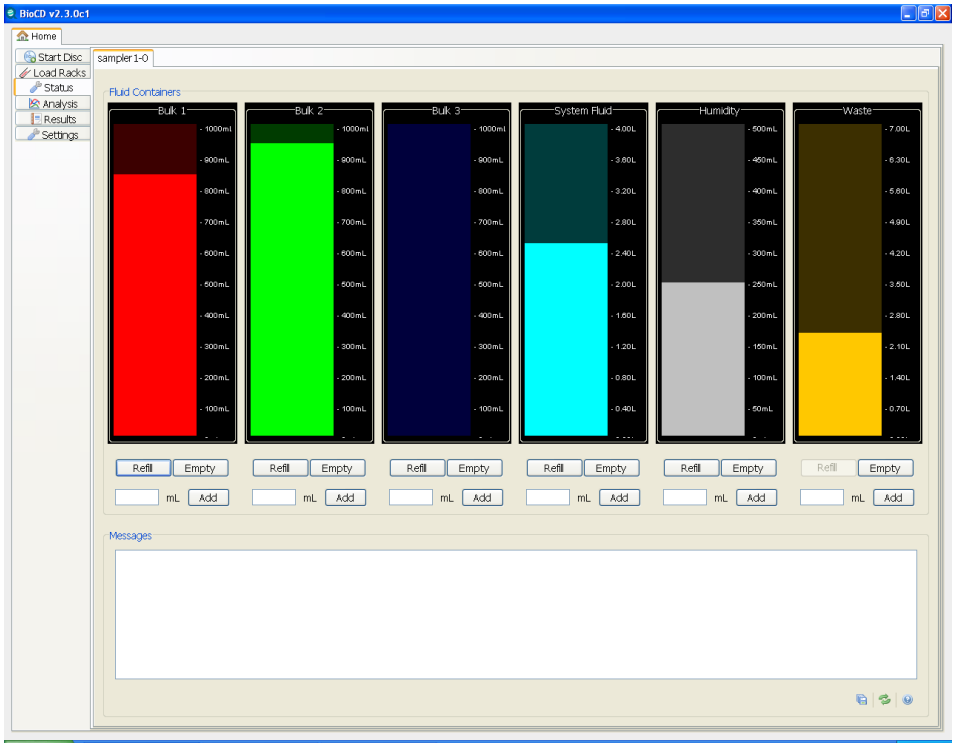

Figure 17. BioCD Software "Bulk Fluid Level" Screen

Upon completion of the sample processing sequence, the software prompts the user to transfer the disc from the sample Processor to the Reader. Since multiple discs can be processed through the system in parallel, the user is required to rescan the disc barcode at this point in time in order to maintain the integrity of the data flow. The following screen in the GUI is used during the Reader scanning process (Figure 18).

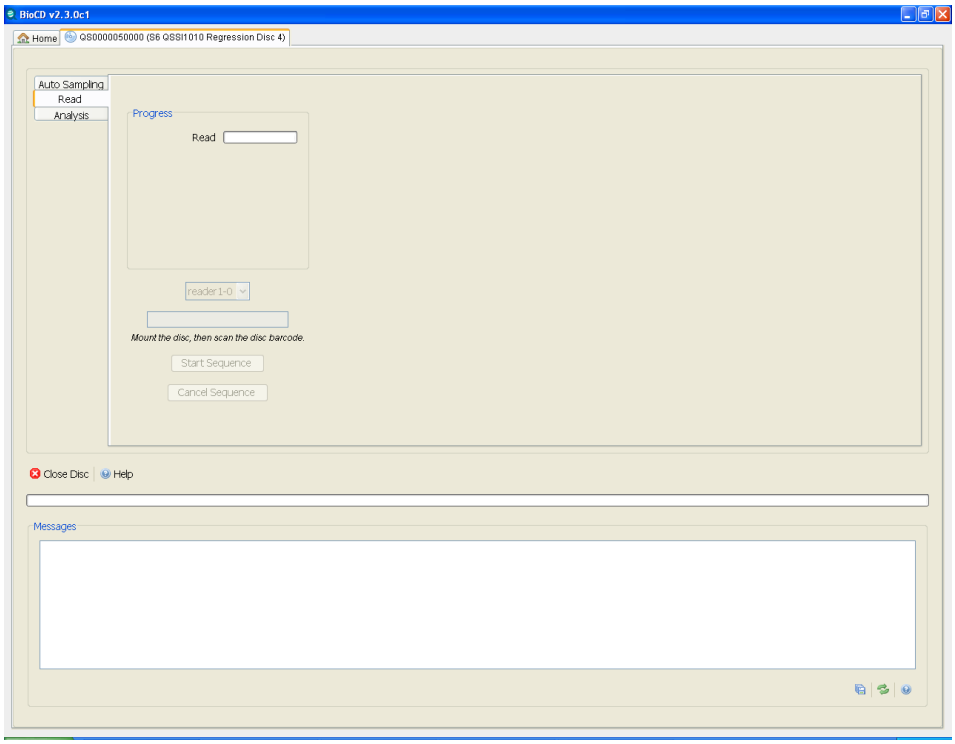

Figure 18. BioCD Software “Read” Screen

Once the disc has been mounted in the Reader and the disc barcode scanned, the user simply pushes the “Start Sequence” button to begin the scanning process. The “Start Sequence” button does not become available to the user until the barcode has been scanned. A status bar indicates the progress of the read.

After completion of the scan, the user is prompted that the disc can now be removed from the Reader and stored per the customer’s standard procedures. Simultaneously, the software automatically initiates the analysis process for the data (Figure 19). Several checks are performed early in the analysis process in order to streamline the process. If any of these checks fail, the process is terminated rather than wasting time by completing the remainder of the analysis. First, the data is checked for proper identification of the alignment marks on the disc well pattern. Failure to detect these alignment marks causes a failure to properly identify the test wells and thus leads to a complete disc failure; no results are reported, and the user would have to rerun the samples. Next, the software checks for proper classification of the positive and negative controls. Since proper control classification is a prerequisite for valid assay results, a failure in the controls also results in a full-disc failure. Again, no results are

reported. Assuming these initial checks pass, the analysis algorithm proceeds to analyze each individual test result.

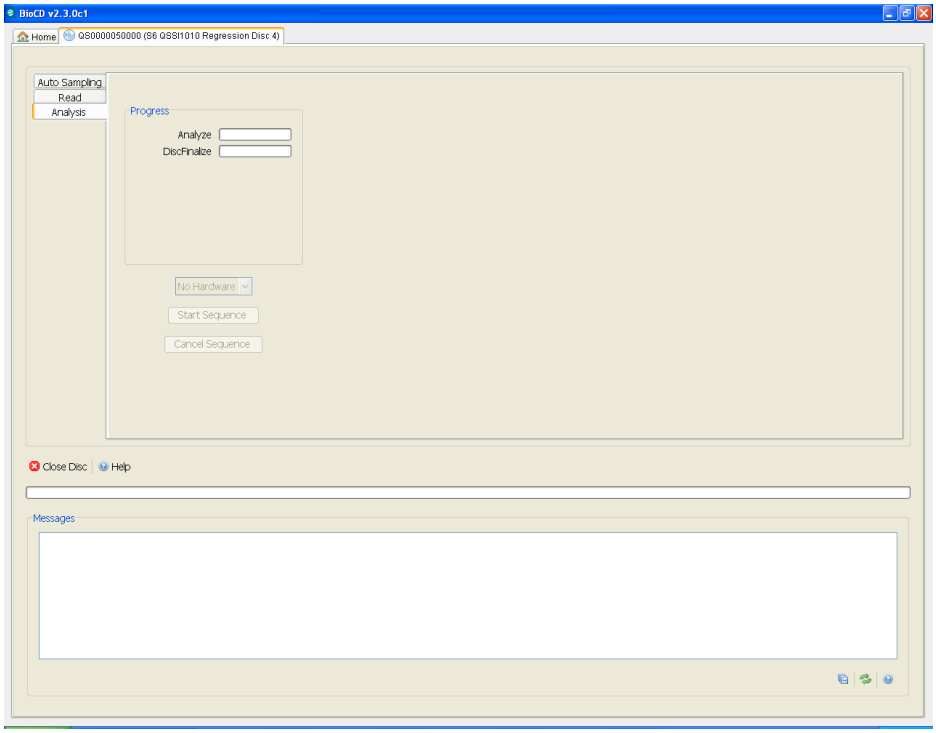

Figure 19. BioCD Software "Analysis" Screen

Once the analysis is completed, the system automatically displays the results on the screen in tabular form as a positive or negative for each of the glycoproteins assayed. The table will include the sample ID (as scanned from the barcode when the sample racks were loaded), the sample rack location, and the glycoproteins that were positive or negative in each sample test well. An example of the result panel is seen in Figure 20.

The results screen allows the user to review the results of the current test disc as well as those from discs run previously. This screen also gives the user the option to print the results or to export them to a csv file for further analysis.

vii. Concerns.

An error in sample tracking during the course of sample processing, scanning, and analyzing could cause a result to be reported for the wrong patient, which could potentially result in improper diagnosis and treatment. Extensive testing has been performed to ensure that the sample tracking throughout the process is accurate. At present 2 million assays have been performed with the system without such an error.

Commented [A26]: How will this be mitigated, or prevented from happening?

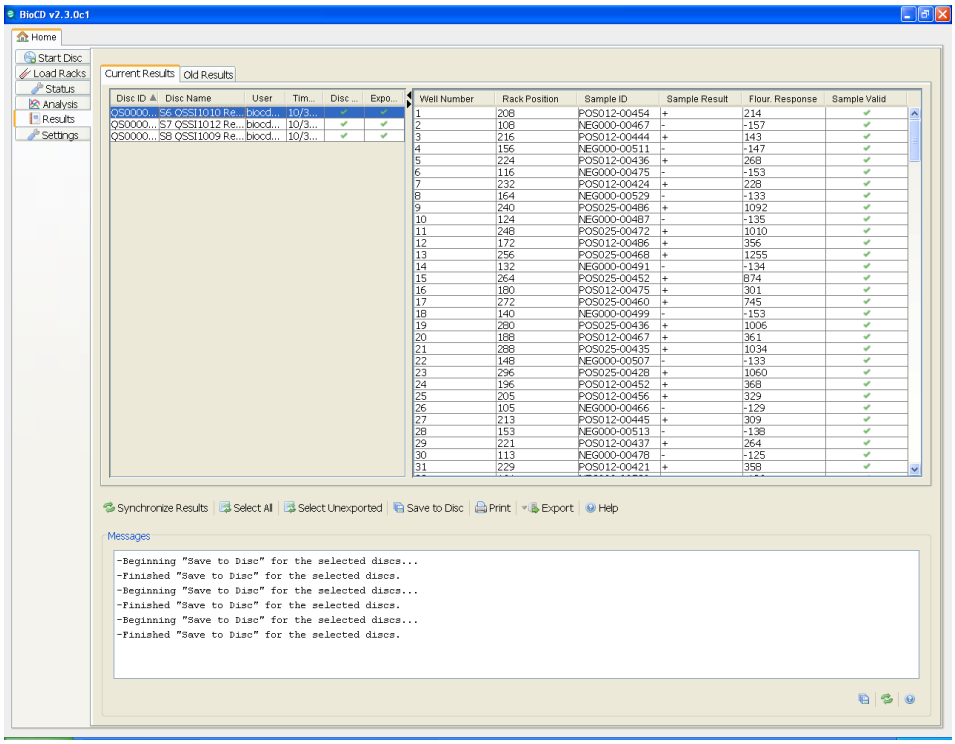

Figure 20. BioCD Software "Results" Screen

Errors in the image analysis algorithm could cause faulty classifications of the presence or absence of one or more of the markers included in the assay. This could also lead to a flawed diagnosis and improper treatment. Several features are included in the system to mitigate the risk of this, including the aforementioned checks for proper control classification and proper well pattern identification in the analysis process. Further features include the presence of control spots inside each test well and the ability to detect anomalies in the spot patterns. Validation of the image analysis algorithm has included a variety of method including analyzing simulated image files, deliberately causing flaws to actual test discs and ensuring proper classification or invalidation of test results, and running true assays with patient samples.

4. Describe the principles of operation for the system.

a. Systems design.

Optical detection is very fast, giving the opportunity to take multiple readings during a short time to increase measurement accuracy. Coupling this with the fact that it is very easy with immunological arrays to execute an assay for an antigen multiple

times within a single well using very small sample volume there is the opportunity to both assay redundancy and over sampling as a means to increase assay accuracy.

A second issue is how to increase sample throughput. High throughput immunological assay systems generally have multiple sample wells within the same assay plate, as in the case of microtiter plates. An issue with multiple well plates is how to read them quickly and accurately.

The Quadraspec Inspira Sample Processor, Sample Reader, and compact discs (BioCD) address these two issues by using a circular disc with multiple assay wells placed around the periphery. Each of up to several hundred samples wells on a single disc contains a 128 elements antibody array. Because of the large number of array elements within a sample well, each antigen is assayed at least 10 times with the same antibody within a single well. Immunological complex formation is carried out in a fashion similar to that used with a microtiter plate except that washing is achieved by flooding the disc with buffer while spinning the disc at high speed. Washing buffer is transported across the surface of the disc by centrifugal force, washing all wells simultaneously.

Antigens bound at antibody array elements are detection by placing the disc on a platform that is rotated at 6000 rpm, bringing array elements under a detection head composed of a laser having a 20 μm laser beam and a detector for detecting light emerging from the disc. Array elements pass under the detection head on a μsec time frame during which time a portion of the 150 μm diameter array element is irradiated by the laser beam. Multiple array elements are irradiated during a single revolution of the disc. The laser beam is left at a radial position for 3-10 revolutions of the disc. The degree of over sampling is user specified. The laser beam and detector are rastered toward the center of the disc in a stepwise fashion until all array elements on the disc are read. The system is capable of reading more than 10^7 array elements in 30 min.

b. Physics of interferometric detection.

Spinning disc Interferometry (SDI) is a relatively new detection method compared to fluorescence. For this reason a more in depth discussion of the technique is presented.

i. Interferometric Quadrature

Interferometry compares two light waves. One light wave is the reference wave. It acts like a high-precision meter stick. The other light wave is the signal wave. It is identical to the reference, with the single exception that it has passed through a thin layer of material, such as biomolecules bound to a surface. The peaks and troughs of the signal wave field are compared with the peaks and troughs or the reference wave. Relative shifts of the peaks and troughs (known as the phase of the wave) can be measured with picometer resolution. These shifts provide the information about how many molecules are in the surface-bound layer. The key to interferometric sensitivity is the condition known as phase quadrature. This is the condition where the signal and the reference waves have a 90º relative phase shift. In this condition, a shift in the phase of the signal is transduced linearly to a change in intensity at the detector. Furthermore, in phase quadrature, the conversion factor of phase-to-intensity is a maximum. Clearly, for high-precision interferometry, it is necessary to stabilize the phase between the

signal and the reference waves to high accuracy. In many interferometers this phase stabilization is very difficult, requiring expensive vibration isolation systems to shield the interferometer from mechanical disturbances. However, there is a class of interferometers known as common-path interferometers. In these systems the signal and the reference waves share common paths through the entire system. Only in a microscopic portion of the system, in the sample region, do their paths vary. Because of the common path, these interferometers require no path length stabilization and hence are ultra stable and inexpensive. The system uses common path interferometers. The common-path architecture makes it possible to make high-precision surface height measurements down to a picometer on the surface of the spinning disc, even though the disc surface may be wobbling by many microns.

ii. The reason for spinning discs.

The act of spinning is of fundamental importance to achieving high interferometric sensitivity. The advantage of spinning over stationary interferometric detection comes from the advantage of high-frequency sampling in the presence of 1/f noise. Almost all measurement systems are dominated by 1/f noise, in which the noise spectrum increases at lower sampling frequency. Static measurements, also known as DC measurements, occur at the very peak of the 1/f noise spectrum and are hence the least advantageous to achieving good signal-to-noise ratios. On the other hand, if the sampling frequency is high, it significantly reduces the noise. It is not uncommon in optical systems to suppress the noise floor of the detection at high frequency by 50 dB. Conversely, it is extremely difficult to engineer 50 dB of gain into a system. Therefore, the very simple act of spinning is the key to the advantage of the BioCD over other systems like SPR.

To get a sense of the signal-to-noise advantage of spinning disc interferometry, it is instructive to perform a back-of-the-envelope calculation of signal to noise ratios, comparing static detection to spinning detection. We assume equal-time measurements for this comparison to give the two cases equal signal values $S : T_{m2}$, where Tm is the total measurement time for a single pixel within a protein spot. The difference is that the static measurement makes a single measurement in the time Tm, while the spinning measurement makes n-measurements accumulated over n rotations. The power-spectrum noise of the measurement scales according to

$$N : \frac{T_m}{f_{samp}} BW$$

where f_{samp} is the sampling frequency, and BW is the detection bandwidth. For a static measurement, $f_{samp} = 1/T_m$ and $BW = 1/T_m$. Conversely, for spinning $fsamp = n/T_m$, where n is the total number of sub-measurements that combine to equal Tm, and the bandwidth is again $BW = 1/T_m$. Therefore, the signal-to-noise ratios of these two cases are

$$\left.\frac{S}{N}\right|_{spin} : nT_m$$

$$\left.\frac{S}{N}\right|_{stat} : T_m$$

which gives spinning an n-fold increase in the signal-to-noise ratio.

This result is best understood by thinking of noise as drifting signal strength. The act of spinning takes n samples spread out over many rotations. The total time of these n samples is the same as when only a single measurement is made in the static case. However, for the single static measurement, the signal could be acquired during a statistical excursion from the mean value. The same is true for any one measurement out of the n for the spinning case. However, in the spinning case, by spreading out the measurements in time, the excursions from the mean value fluctuate randomly from measurement to measurement and hence tend to cancel out. An equivalent way of understanding this is simply that the higher sampling frequency of the spinning case moves the detection farther away from 1/f noise. This simple analysis illustrates the immediate advantage obtained by high-speed spinning detection when the system noise is dominated by 1/f noise.

ii. In-line Quadrature

In-line quadrature takes its name from the planar optical configuration that places a partially-reflecting reference surface in the same line as the signal beam. For example, a dielectric layer on top of a reflecting surface provides a partial reflection. Furthermore, if the optical thickness of the layer is an eight-wavelength, then the wave reflected from the top surface has a 90-degree phase offset from the lower high-reflecting surface. The thin film is mechanically stable, and thus the interferometry is stable, without the need for any phase or path stabilization.

To analyze the performance of in-line quadrature, we begin with the normalized two-dimensional intensity distribution of the incident Gaussian beam

$$I(\rho) = \frac{1}{\pi\sigma^2} e^{-\rho^2/\sigma^2}$$

where $\rho^2 = x^2 + y^2$, $\sigma = \frac{\sqrt{2}}{2} w_o$, and w_o is focal spot radius. The corresponding dimensionless electric field is

$$g(\rho) = g(x, y) = \frac{1}{\sigma\sqrt{\pi}} e^{\frac{\rho^2}{2\sigma^2}}$$

The dimensional diffraction problem is considered in the Fraunhofer regime. The reflect near field is

$$E(x,y) = r'(x,y)g(x,y) = r[i\phi(r)h(x-\nu t,y)]g(x,y)$$

where

$$\phi(r) = \left[\frac{(r_p - r)(1 - rr_p)}{r(1 - r_p^2)}\right]\frac{4\pi\eta_p}{\lambda}$$

is a complex-valued function that plays the role of phae, where η_p is the protein refractive index and l is the free-space wavelength of the light. In this expression rp is the reflectivity of the air-protein interface, and r is the original surface reflectivity.

The surface topology, including the motion of the disc, is contained in the real-valued height function $h(x + \eta, y)$, here $\eta = -vt$, and v is the linear speed of the disc at the radius of the probe beam. Performing the integral yields the expression

$$i_d^{II}(\eta) = 2|r|^2 \phi_{\text{Im}} g^2(\eta) \Theta h(\eta)$$

which is the convolution of the beam shape with the protein profile. The in-line signal depends on the imaginary (90-degree phase) component of the phase. This component is maximized when the thickness of the dielectric layer is nearly an eighth wavelength, establishing the quadrature condition.

iii. Oxide on Silicon.

Silicon is one of the most common materials available because of its importance to the electronics industry. It therefore is a good substrate choice for economic reasons, as well as for its compatibility to thermal oxide coatings. Thermally-grown silicon dioxide on silicon is one of the most nearly perfect dielectric structures. It also provides a good refractive index difference between both air/oxide and oxide/silicon interfaces. The intensity response to immobilization of a monolayer of antibody is shown in Figure 21 as a function of the oxide thickness. Two channels are shown: differential phase contrast, and in-line, including a quadrature sum of these two channels. Differential phase contrast is a maximum for an oxide thickness near 100 nm. However, the in-line channel passes through zero at this condition. The in-line channel is maximized on either side of the phase-contrast peak, at conditions near eight-wave. In practice, the in-line maximum on the high-thickness side is used, because a protein layer leads to an increase in signal. This makes it possible to distinguish between thin layers (with positive signal) and light loss from scattering (with negative signal). For in-line BioCD applications, we operate at an oxide thickness of approximately 120 nm.

The sensitivity of interferometry to surface-bound mass is a function of the number of statistical samples that are acquired. This means that often-quoted values of mass sensitivity in units of mass per area are not intrinsic properties of the detection system. However, it is possible to derive a scaling surface mass sensitivity that is an intrinsic property.

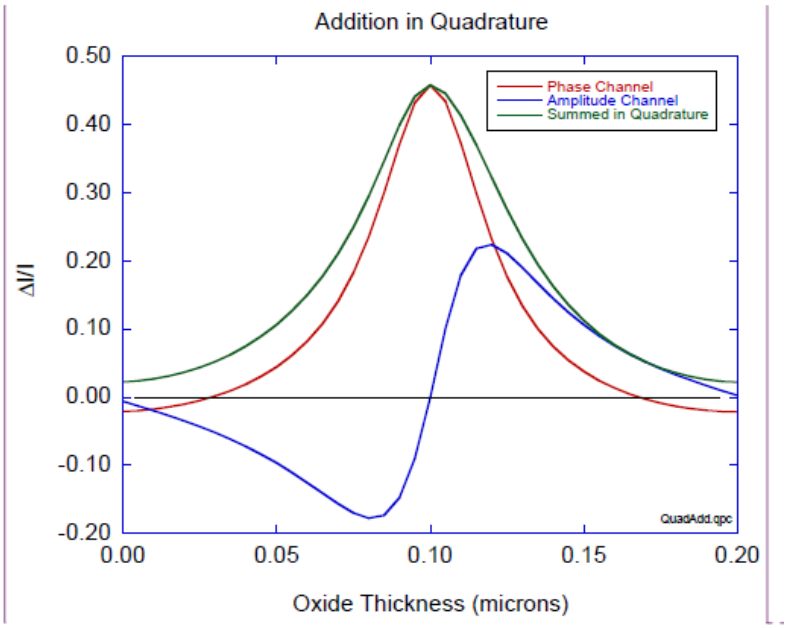

Figure 21. Intensity modulation in response to an 8 nm monolayer of antibody showing the response of the phase and intensity channels and their summation in quadrature.

To derive the experimental scaling mass sensitivity of the BioCD, we performed an experiment on a protein-spotted disc in which the disc was washed for 20 hours in a PBS solution containing 10 ng/ml casein. The disc was scanned prior to and after the wash, and the scans were differenced to measure the change in protein mass in addition to noise contributions. A histogram of the root variance of the data differenced between the two scans is shown in Figure 22 below for a region containing approximately 1000 antibody spots. The maximum root variance of the height difference is 46 pm. This is the root-mean-squared height measurement error per focal spot area. It is dominated by the mass variability caused by the 20 hour wash and also by mechanical performance of the system (repositioning error between scans). The root variance of 46 pm in the surface height corresponds to 5 femtograms of protein per focal spot with a diameter of 15-20 microns. Assuming Gaussian random statistics, the surface height sensitivity at the scale of 1 mm is given by

$$\Delta h_{mm} - \Delta h_{meas} \sqrt{\frac{a_{foc}}{1mm^2}}$$

where a_{foc} is the area of the focused laser spot and Δh_{meas} is the root variance in the height difference. For Δh_{meas} = 46 pm and a_{foc} = 200 μm^2 this gives Δh_{mm} = 0.65 pm. The mass associated with this protein height is $\Delta m_{mm} = \Delta h_{mm} \rho_m 1mm^2$ which, for Δh_{mm} = 0.65 pm gives Δm_{mm} = 0.25 pg. The appropriate scaling mass sensitivity is therefore

$$S = \frac{\Delta m_A}{\sqrt{A}} = \rho_m \Delta h_{meas} w_{meas} = 0.25 pg / mm$$

which has the units of mass per length. To obtain the minimum detectable surface mass density the scaling sensitivity is divided by the square-root of the sensing area. For a square millimeter this is

$$S_{mm} = \frac{S}{\sqrt{1mm}} = 0.25 pg / mm^2$$

This area-dependent sensitivity is better than the best values determined by SPR. This sensitivity is gained without the need for resonance and hence is much more robust and easy to manufacture than other interferometric or resonance approaches.

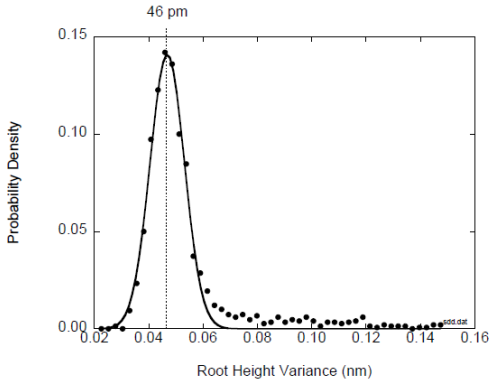

Figure 22. Histogram of the root height variance between two scans of the same disc before and after a 20 hour buffer wash. The maximum is at 46 picometers.

c. Laser induced fluorescence detection.

The low power laser used for SDI detection in the Quadraspec Integra Reader also allows for laser induced fluorescence (LIF) detection, albeit at low sensitivity. A second model of this instrument uses a separate, high power laser for high sensitivity detection in addition to SDI detection.

Laser-induced fluorescence (LIF) is the result of optical emission from molecules that have been excited to higher energy levels by absorption of electromagnetic radiation from a laser. The main advantage of LIF as opposed to absorbance is that detection is achieved at a different wavelength than excitation. This means that very high excitations energies can be

used because the fluorescence emission wavelength is easily differentiated from excitation wavelength through optical filtering or monochronometers and there will be very low background from the laser beam at the detection wavelength. As the Reader is switched from the SDI to LIF detection mode a wavelength filter is imposed between the array element and photo multiplier that allows only the emission wavelength to pass. Being of a different wavelength the laser excitation wavelength is blocked.

The problem with fluorescence detection of proteins is that most do not fluoresce strongly. This is overcome by labeling proteins that must be detected with a tag of high fluorescence yield. The Alexa Fluor 532 dye is such a molecule, being of high fluorescence yield at 532 nm. Molecules of high fluorescence yield give higher emission energy relative to excitation energy than most other light absorbing species.

Second antibodies are labeled with the Alexa Fluor 532 dye from Invitrogen. This allows them to be detection in the immunological complex at array elements with great efficiency.

d. Reading a disc.

(See Section D.3.a – D.3.c.)

Additional FDA comments and questions about device description can be found in the review memorandum.

E. INTENDED USE.

The intended use of the test described here is to provide a semiquantitative assay for 8 breast cancer associated glycoproteins in the plasma of subjects with BI-RADS category 4 mammograms to be used as a second source of data in evaluating the need for a biopsy. Two percent of BI-RADS category 3 subjects are subsequently found to have breast cancer while 14% of subjects classified at category 4 will give a breast cancer positive biopsy. The objective of this blood test is to reduce the number of breast cancer free individuals with BI-RADS category 4 mammograms from needlessly undergoing a biopsy. In contrast, this test will be of little clinical value to subjects classified as having BI-RADS 5 mammograms because they have a 95% chance of having breast cancer. A blood test will be of little additional value to these subjects.

1. **Analytes to be measured.**

Two types of proteins are being measured with this assay; a set of reference proteins that are of high abundance and relatively constant concentration in plasma and a set of breast cancer biomarkers proteins. Several high abundance proteins in plasma are not associated with cancer; among them being transferrin, haptoglobin, α_1-antitrypsin, and α_2-macroglobulin. These proteins will be used as internal standards to assess variations in the concentration of biomarkers. Of the breast cancer associated proteins in plasma, there is a set of glycoproteins elevated 3 fold or more in breast cancer patients that bear Lewis x (Le^x) or sialyl-Lewis x (sLe^x) antigens. Histidine-rich glycoprotein, plasminogen, vitronectin, proteoglycan-4, clusterin, fibrinogen, kininogen-1, platelet factor 4, and serum amyloid A protein are members of this group.

Commented [A27]: The meaning of "semi-quantitative" is unclear. The understanding is that if at least one of the glycoproteins is above the cutoff then the subject is considered test positive. Every subject has the result as the number of the glycoproteins above the cutoff (s). Then the value of the test are 0,1,2,..8. A patient is positive if result of the test is above or equal to 1. If this understanding is correct then we need a further discussion.

Commented [A28]: Why would this assay be semi-quantitative?

Commented [A29]: Would these glycoproteins be interpreted separately, using some kind of algorithm, or as a "signature"?

Commented [A30]: Would be more suitable to replace this wording with "as an aid".

Commented [A31]: Might need to modify the wording.

Commented [A32]: This is background information that should not be a part of Intended Use.

Commented [A33]: Not necessary to be a part of IU; justification and background information.

Commented [A34]: At what stage of breast cancer are these markers elevated?

a. The qualitative and quantitative nature of the test.

The assay being performed here is to determine a set of cancer associated proteins through immunological assays. As with all immunological assays, antibodies are being used to target a single glycoprotein from blood plasma. The fact that a specific protein of known structure is being targeted at each array element makes this a qualitative assay. The fact that other molecular species could bear some of the same structural elements being targeted is a problem that is addressed through multiple levels of *orthogonal* targeting. A high level of orthogonality is achieved by targeting a polypeptide epitope in one dimension and a highly specific glycan sequence in the second dimension.

Antigen capture is also being quantified. Quantification is being achieved by either interferometry or laser induced fluorescence. Both of these detectors provide quantitative measurements and linear dose-response curves when calibrated with standard proteins (Figures 3-6). Although these assays provide quantitative determinations of 10-15% relative standard deviation, any concentration three times higher than that of the control (breast cancer free) population is considered to be a positive. This makes antigen quantification semi-quantitative.

b. Specimen type(s).

See section F below.

c. Conditions for use.

Prescription use in a hospital laboratory.

2. Indications for use.

The intended use of this blood test is for subjects with a BI-RADS 4 category mammogram; the intent being to reduce the number of breast cancer free individuals with BI-RADS category 4 mammograms who needlessly undergo biopsy. The objective of the test is to determine which of the eight glycoprotein biomarkers in the plasma of BI-RADS 4 patients are above the concentration cutoff level, i.e. in the concentration level seen in cancer patients.

F. SAMPLE COLLECTION.

Blood plasma obtained by venipuncture (see Section E) is used in this assay. Approximately 6 mL of blood is drawn in a lavender top vial, inverted gently, and centrifuge at 3500 rpm for 30 minutes. The supernatant is divided between three (3) cryovials and frozen immediately at -70 ^{o}C until it is shipped to the analytical laboratory. If a -70 ^{o}C freezer is not available plasma samples may be sotred at -20 ^{o}C until shipped. Samples that have thawed before arrival at the analytical laboratory should be discarded.

Pre-printed cryovial labels are provided with the sampling kits. Sample collection vails are labeled with a permanent ink. The patient ID#, date, and time of collection should be recorded on the label with a Sharp permanent marker.

Commented [A35]: To determine? Or to measure because they were shown to be associated with malignancy in breast cancer?

Commented [A36]: This is an incorrect definition of "qualitative assay".

Commented [A37]: This will have to be proven to work in your analytical and clinical studies.

Commented [A38]: It is very important to clarify what is the output of the test.
Sometimes the term "semi-quantitative" is used for the test with ordinal outputs.

Commented [A39]: This seems to be a cutoff determination, which would make this assay type qualitative. For a quantitative assay, you would have to provide evidence that higher values obtained for certain glycoprotein would somehow be correlated with e.g. different cancer risk.

Commented [A40]: See comments on the IU. In most cases IU and IFU are the same.

Samples are placed in a ZipLock bag along with a Sample Submission Form for shipping. Samples are then placed in the provided container in a minimum of 5 pounds of dry ice and shipped Monday through Thursday by FedEx. The analytical laboratory should be contacted about the shipment by email when shipped.

G. STUDY DESIGN AND SAMPLE SIZE CONSIDERATIONS.

Clinical effectiveness of this test was determined using blood samples derived from 1) a population of 100 subjects with positive mammograms but who were not receiving therapy and 2) 100 disease free control subjects, many of whom were a relative. The mean concentration and relative standard deviation of each cancer marker from the mean in the cancer free population was determined. These values are referred to here as "normal values".

Commented [A41]: ? Women can have a "positive" mammogram but this does not mean they are diagnosed with breast cancer at that point and therefore would not be receiving therapy.

Commented [A42]: This may be useful population for signature determination, but would not be good for a pivotal clinical study, where individuals/patients should be representative of the IU population.

Commented [A43]: It appears that there is confusion between the reference interval ("normal values") and the pivotal study. The pivotal study should include consecutive patients with BI-RADS 4. Ideally all subjects should have biopsy results. Subjects with malignancy are used for calculation of sensitivity and ALL other subjects (not-malignant) are used for calculation of specificity. Subjects with BI-RADS 1 and 2 are NOT used for calculation of specificity because these subjects are not part of the intended use population.

The reference interval for each cancer marker can be used for the determination of the cutoff for this marker. For calculation of the reference interval, non-parametric methods are recommended (calculation of 2.5th and 97.5th percentiles). We recommend you follow CLSI C28-A3.

Commented [A44]: Is this supposed to be a type of expected values study?

H. PERFORMANCE CHARACTERISTICS.

1. Pre-analytical (specimen related)

a. Specimen type, collection, storage, handling, stability and requirements.

Blood plasma is obtained by venipuncture (see Section E). Protocols generally used by the Cancer Proteomics Technology Assessment for Cancer (CPTAC) consortium have been chosen for this test as outlined in Section F.

b. Specimen purification, enrichment, fractionation, digestion requirements.

Purification and enrichment in the test describe here is by solid phase immunoselection. Although antibodies give a high degree of purification, it is often the case that one or two other species with the same epitope will be captured as well. We deal with the problem in two ways. The first is to qualify antibodies for their selectivity through proteomics methods. Before a first (capture) antibody is used in an assay it is immobilized in an affinity chromatography column and used to capture antigens from plasma. The POROS column method described above is used in this process. All proteins captured by the antibody are released, identified, and their relative concentration quantified by stable isotope labeling and mass spectrometry. Antibodies capturing the least number of proteins in addition to the targeted antigen are selected. The second antibody is put through this same qualification process as well in which all captured antigens are identified and quantified. **A critical feature of this process is that the only glycoprotein captured by both antibodies can be the targeted antigen isoform**. Combinations of antibodies that capture the same antigen are eliminated from use.

No form of digestion is used in the assay.

2. Platforms and technologies.

The assay instrumentation (platform) is composed of two components; a wet chemistry unit in which antigens are selected from samples with immobilized antibodies and a Reader that quantifies antigen binding to antibodies. The wet chemistry unit is described in section C2a above. The Reader is described in section C2b (Figures 3 and 4).

a. Characteristics of instruments.

Quantification is achieved through optical measurements described extensively above.

b. Separation methods.

Antigens are separated from plasma in a batch mode, i.e. immunoselection. During the course of incubating samples in a well with immobilized antibodies, antigens diffuse to the surface of antibody array elements and bind. Unbound proteins and those bound non-specifically with low affinity are removed by extensive washing of sample wells with buffer.

g. Stability.

Immobilized antibodies on discs and secondary fluorescent labeled antibodies in assay kits are stable for one year when stored at 4 ºC. Following calibration the Reader is stable for a 24 hour period of use as seen in section K1.

Commented [A45]: This should be shown and a protocol for stability studies provided.

Commented [A46]: K1 seems to be repeatability. There appears to be no data there related to reader stability.

h. Software.

The system software is described in section D3c.

3. **Analytical (instrument related).**

a. Repeatability/precision/intra-lab variation (runs, days, operators, on/off cycles, etc.) and acceptable variation limits. Precision of Qualitative/Semi Quantitative Assays.

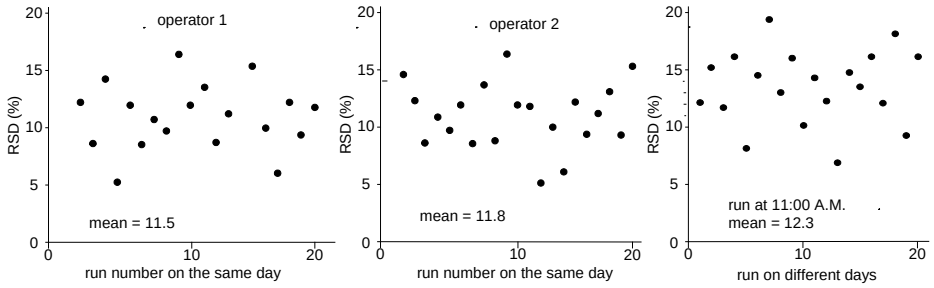

Figure 23. Relative standard deviation in the clusterin fluorescence sandwich assay. A normal plasma sample was split into 100 ul fractions and frozen at -80 ºC until analyzed. Sample analysis began at 9:00 A.M. each day. Samples run on different days were examined each day at 11:00 A.M.

Commented [A47]: What are the mean obtained assay values for this assay? It is not clear from this description how the study was performed, but it seemed to be assessing a single source of variability – different operators. We recommend that you design and perform the study and provide the results in tabular form according to the studies recommended in CLSI EP5-A2.

It is concluded from the results presented above that changing operators has little impact on variability in measurements. This is attributed to the fact that both the wet chemistry and reading components of the analysis are automated. Beyond putting samples in place for analysis and triggering the various stages of analysis through the

computer key board, the operator has little direct role in sample analysis. The same is true of runs on different days. With daily calibration there is little variation in sample measurements.

Disc manufacture could be another contributor to variability. This possibility was assessed by running sandwich assays on the same sample in all 24 wells on a disc and determining the relative standard deviation for each of the 8 proteins being targeted. One should obtain identical results for a glycoprotein in all 24 wells. The fact that the results differ is probably due to differences in antibody immobilization and perhaps non-specific binding in the sample wells. Results from this inter-well and inter-array element heterogeneity are presented in Figures 24 and 25, respectively. Data in Figure 25 was obtained by spotting the same antibody targeting a specific glycoprotein marker on 33 array elements in a well and quantifying the amount of the targeted protein bound.

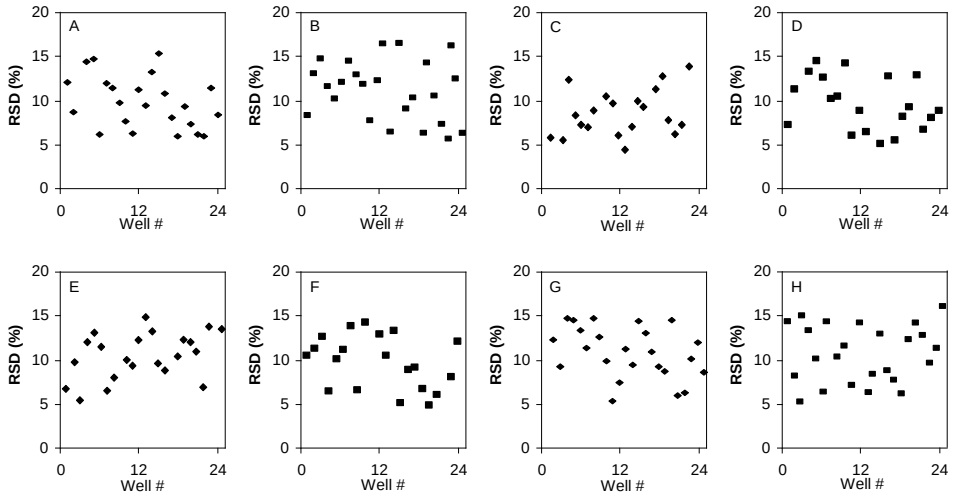

Figure 24. Interwell homogeneity based on spinning disc interferometry. Pannels A-H show results for the following proteins in the fluorescent sandwich assay: A = clusterin; B = plasminogen; C = kininogen-1; D = serum amyloid protein A; E = histidine rich glycoprotein; F = proteoglycan-4; G = vitronectin; H = platelet factor 4.

Although there is substantial heterogeneity, the differences between control and disease state samples is so large that variations of 50% or more are tolerable in most assays without compromising the assay.

b. Interferences.

Interferences were miminized in the design and construction of assays, as opposed to trying to eliminate interference with additives and computation during an assay. This was achieved by selecting antibodies and immobilization chemistries that showed the least sensitivity to interferences.

Immunological assays and immunosorbent affinity chromatography (IAC) are very similar in that they use an immobilized antibody to capture an antigen. The

Commented [A48]: This would have to be shown. In general EP5-A2 recommends a 20-day in-house precision study, while a 3-site study to cover the real-life expected use of the assay is recommended. For novel assays like yours, we might need to consider a slightly different precision study design, while making sure that all possible sources of variability are adequately evaluated.

Commented [A49]: This should be evaluated and accounted for.

Commented [A50]: Please also provide means, and CVs for well-to-well and array to array. Were there any trends across different well positions?

Commented [A51]: This would need to be evaluated and shown to be the case.

Commented [A52]: It is possible that this expectation is too optimistic. Do we understand correctly that you compared BI-RADS 4 cancer cases to BI-RADS 1 and 2 cases?

Commented [A53]: Will need to be shown in interference study.

difference between the two is that in IAC the captured proteins are desorbed from the solid phase and may be structurally characterized. Every antibody used in this immunological array platform was analyzed by IAC of a NIST control plasma sample before it is used in a test. All interfering proteins bound both specifically and non-specifically were identified. Approximately 65 glycoproteins were identified, of which some were truly cross-reactive. Others were bound to the antigen being selected. In addition, the concentration of interfering species relate to antigens was quantified. Concentrations of these interfering proteins were close to those of the antigens. With a single antibody selection assay cross-reactivity and non-specific binding could be a large problem.

Commented [A54]: Will need to show and justify why would this not be a problem for this multiplex assay.

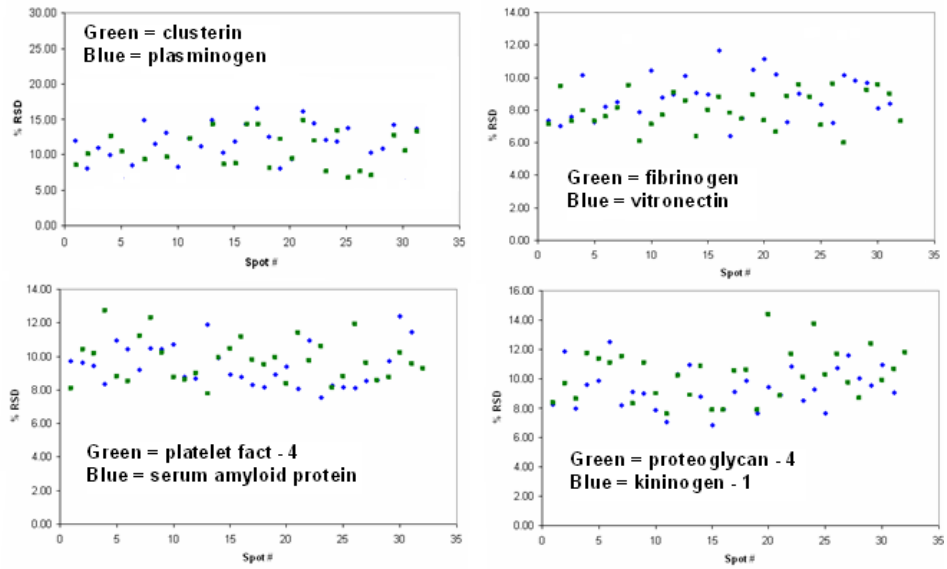

Figure 25. Inter-array element variation in sandwich assays. Although data from two proteins is plotted together in each panel, the binding of each protein was examined in a separate well.

Commented [A55]: Please describe exactly how the study was performed, present results in tabular format, and clarify how results obtained prove the conclusions below that seem to be drawn from it.

But even more important, in no case were antibodies used in kit fabrication that cross-reacted with the same antigen species. This means that the antibody combinations used in the test did not (could not) demonstrate non-specific or cross-reactive binding of the same antigen.

Commented [A56]: What about other antigens?

c. Reproducibility/portability/inter-lab variation (all sources of variation across sites).

Reproducibility across sites is addressed in Figure 26. These tests were carried out using discs from the same manufacturing run that had been distributed to the same

location. Twenty control samples were each divided into four fractions and shipped to the four sites where they were analyzed at 10:00 A.M. on March 4, 2009 after the respective instrument platforms had been calibration and run at least one set of samples prior to the set reported in the Figure.

Commented [A57]: In general, a reproducibility study is performed over 5 non-consecutive days.

Also, different concentrations of the analyte/sample need to be evaluated; depending on whether assay is qualitative or quantitative these specimens may need to span different range and encompass relevant assay decision points.

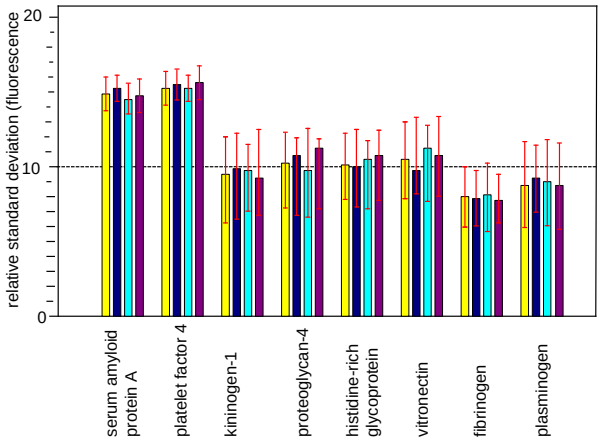

Figure 26. Reproducibility across sites. Yellow – New York City; dark blue – Chicago; light blue – Los Angeles; burgundy – Huston. Twenty samples were each divided into four lots and shipped to all four sites where they were examined on March 4, 2009.

c. Assay reportable range.

Reportable ranges are seen in Figures 6 A and B. In general the reportable range is far greater than range needed for the assay of patient samples.

d. Detection limit.

Detection limits in both SDI and LIF assays with the various antigens are seen in Figures 3, 4, and 6.

Commented [A58]: Study design and analysis of data for determination of LoD should be provided. We recommend you follow CLSI EP17-A.

e. Analytical sensitivity, normal range and cut off limits.

Assay sensitivity and cutoff limits with the various antigens are seen in Figure 3, 4, and 6.

The intended use of this test is to provide a semiquantitative assay for breast cancer associated glycoproteins in the plasma of subjects with BI-RADS category 4 mammograms. Two percent of BI-RADS category 3 subjects are subsequently found to have breast cancer while 25% of subjects classified at category 4 will give a breast cancer positive biopsy. Figure 27 illustrates a frequency distribution with which breast cancer marker glycoproteins are identified in subjects whose mammograms were classified as BI-RADS 4. Clearly there is a bimodal distribution showing two different populations of subjects. The standard deviation is larger in the population of subjects with breast cancer and is also skewed to a small extent, but the two population are totally isolated in the frequency distribution. Depending on the cancer marker being

Commented [A59]: If you would like to compare the distributions, a non-parametric method should be used.

measured, the mean of the frequency distribution was at a concentration 6-14 fold higher in the breast cancer population than in the normal population.

As breast cancer develops in an individual their breast cancer marker profile will shift from that of a cancer free subject to that of a breast cancer subject. Unfortunately large numbers of individual longitudinal studies are not available to determine the manner and rate with which this occurs but it is reasonable to assume that some individuals will be in this intermediate region at the time of blood sample analysis. In the absence of longitudinal data it is assumed breast cancer biomarker synthesis is proportion to tumor mass, e.g. the rate of increase is exponential. If true, there is both good and bad news in this fact. The good news is that the population of subjects of intermediate biomarker concentrations between these two extremes will be small. The bad news is that this makes early detection more difficult. At the earliest stages of cancer development the concentration of biomarkers will be too small to detect.

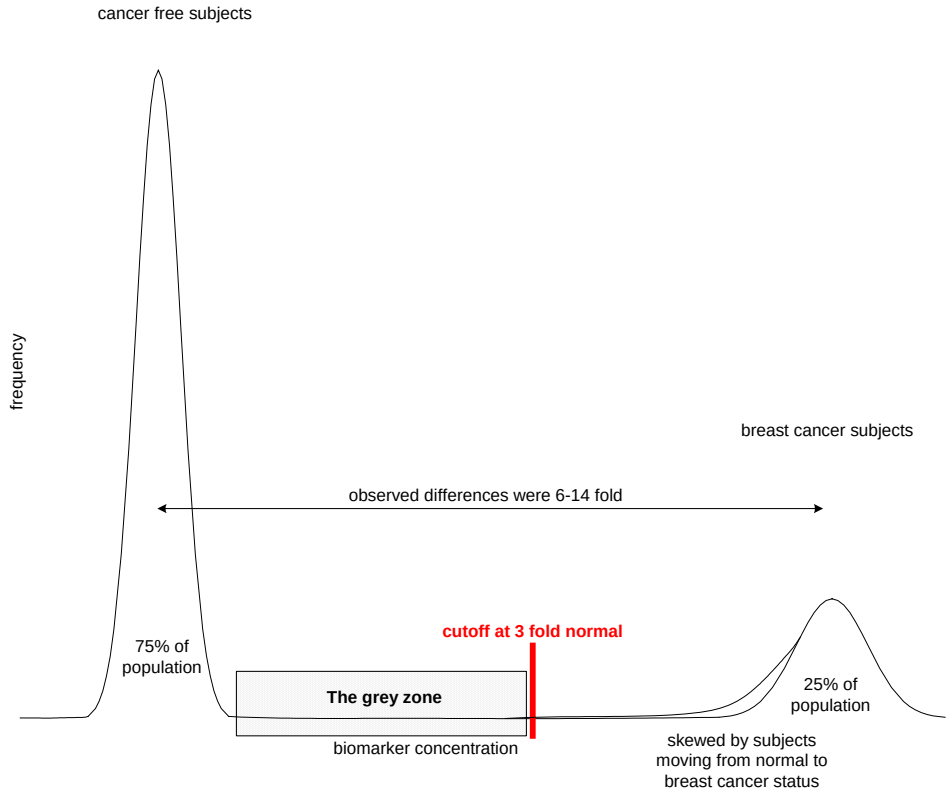

Figure 27. Frequency distribution of cancer markers in a BI-RADS 4 category population.

The cutoff value for biomarker detection was set at a concentration 3 times higher than the median of normal subjects to preclude the possibility of a false positives

Commented [A60]: Please divide this section into 3 sections. Section A: limit of blank, limit of detection, analytical sensitivity – we recommend CLSI EP17-A. Section B: reference interval, show reference intervals for the pre-menopausal and post-menopausal subjects separately. For the reference interval, you need to have at least 120 pre-menopausal and 120 post-menopausal subjects. Non-parametric methods should be used. We recommend you follow CLSI C28-A3. Section C: describe cutoff (s) for each glycoprotein; how the overall result should be calculated; how the result will be reported to a physician, what is the cutoff for the overall result.

Commented [A61]: It should be easy to determine from the results of your pivotal clinical study whether this is the case and in what percentage of subjects.

Commented [A62]: This would need to be shown if claimed to be the case.

Commented [A63]: Please note that you should have a very high level of sensitivity (very high level of NPV). Therefore, you should have a cutoff (s) such that if the overall result is negative then subjects without cancer will be excluded with very high probability. This would need to be further discussed, but it might be acceptable to consider subjects with intermediate results as "positive".

while keeping it far enough below the mean of the population of breast cancer patients to greatly reduce false negatives. This cutoff value will be used until a statistically derived value from a larger 2000 patient population is developed. This larger study is currently underway.

Although false negatives can be reduced, they can not be eliminated. The grey zone in Figure 27 is for individuals at an early stage of tumor development whose biomarker concentrations fall between 5σ or 3 fold above the mean of the cancer free population. At this very early stage of breast cancer development no more than 1% of the total population would be expected to fall in this zone. Based on the fact that 25% of the BI-RADS 4 population has breast cancer and the large difference in maker concentration between the two populations it is expected that with a cutoff of 3 fold above the cancer free mean the false positive rate will be zero and the false negative rate will be `1% of all BI-RADS 4 category patients examined.

So what is recommended for subjects who show one or more markers in the gray zone of Figure 27. We know there is the possibility of making a false negative diagnosis and that maker concentrations will rapidly move beyond this zone. The recommendation is for the subject to be retested in six months. At this time biomarker concentrations in false negatives should have more upward beyond the gray zone.

f. Signal saturation, max/min sample volume/mass.

The maximum signal from an antigen assay never exceeded 60% saturation of the SDI or LIF detectors in any case.

Maximum sample volume of assay wells was 25 ul. Fifteen ul of sample was assayed in all cases. Minimum sample volume was approximately 10 ul.

g. Analytical specificity.

Analytical specificity in the case of immunological assays is generally describes the ability of a test to detect a single molecular species. That is not the case in the assay platform being described here. As noted in the "Background", a hallmark of cancer is the attachment of certain glycan structures at the periphery of oligosaccharide structures of glycoproteins. The assay described here uses a first antibody to capture a particular protein and second antibodies to detect the presence of either Lex and/or sLe^x antigen attached to this protein. The assay does not detect the position(s) on the protein to which these glycans are attached. Moreover, many of the structures being detected may not be present in normal (breast cancer free) subjects. ***An important component of the test is the presence of large amounts of these cancer associated glycans in specific proteins, not their position in the protein matrix.*** This makes this type of immunological assay distinctly different from other types of immunological assays.

j. Linearity over the measuring interval.

Reportable detection ranges are seen in Figure 6A and 6B for the eight biomarkers.

Commented [A64]: You might want to consider the percentile approach described in Kondratovich (2005).

Commented [A65]: This will be assessable from pivotal study results.

Commented [A66]: We assume this is a made-up data. It is possible that this difference will be smaller in reality.

Commented [A67]: The pivotal data will be used for estimation of the test performance. Among the 2000 subjects with BI-RADS 4 risk, there might be approximately 500 cancer cases. Please address the problem of verification bias; among these 2000 subjects with BI-RADS 4 risk, some will not have a biopsy.

Commented [A68]: This does not seem to fit with the current clinical practice and follow-up that would be recommended for a patient.

Commented [A69]: You should probably consider a gray zone result as a positive result – please note that high level of sensitivity is important.

Commented [A70]: Please show study results

Commented [A71]: If only 15ul was assessed, that is what should be recommended – if performance is unavailable with different volumes.

Commented [A72]: How is this paragraph related to analytical specificity?

Commented [A73]: We recommend you follow CLSI EP7-A.

Commented [A74]: Please clarify whether the quantitative values for each glycoprotein will be reported to physicians? The lower limit of quantitation and the upper limit of quantitation should be estimated if your assay is quantitative.

k. **High dose hook effect.**

The hook effect is not seen in our assays. We have never encounter antigen concentrations in any of our assays that would take us past the top of the dose response curve where the hook effect occurs.

Commented [A75]: Some data showing this would help back these statements.

Commented [A76]: The data demonstrating that there is no hook effect should be provided.

l. Recovery.

We have no evidence that the any of the proteins we are assaying are lost between drawing the sample and analysis. The basis for this comment is that result from immediate analysis of a portion of a sample are within experimental error identical that those on a frozen fraction of the sample many months later.

Commented [A77]: This is not what recovery study generally is about.

4. **Data analysis/computational.**

Interpretation and analysis of data is discussed in section B.5.

a. How are data handled to deliver a result?

See section B.5.

Commented [A78]: It is not clear from Section B.5 whether you plan to provide the quantitative value for each protein or only a negative/positive result; how should the overall result should be interpreted (for example, the result is positive if at least one of proteins is above the corresponding cutoff or if at least 2 proteins are above the corresponding cutoff etc).

b. What statistical approaches are required?

i. Basis: statistical measures.

Computations involving determination of the mean, standard deviation, coefficient of variation, and relative standard deviation were used in identifying variables in the assay platform. Statistical measures were not used in the analysis of individual samples.

Commented [A79]: Why were these statistical measures not listed in the precision studies above?

Commented [A80]: Why not?

ii. Normalizations.

Two types of normalization were used. One was the normalization of all assay measurements to the concentration of several abundant internal standard proteins in plasma that are not associated with breast cancer. If there is an exception and one of the internal standards is higher or lower than expected, it is detected by the platform and the value for the standard is rejected. SDI values for these proteins were acquired from a calibration mixture run at the beginning of each day. Alternatively the calibration standards can be run in a non-sample well on the disc and used as a reference for computation. The data system will accommodate either case.

Commented [A81]: How many of these values are needed in order to interpret assay results as valid?

The second type of normalization was against control values for glycoprotein marker isoforms.

Commented [A82]: Please provide more details.

iii. Computational effects.

None were noted.

iv. Software validation.

Commented [A83]: Need hazard analysis etc

Calibration mixtures are routinely analyzed in place of patient samples in all 24 wells of a disc to determine whether the software functions properly. Bar coding errors and blurring of bar codes are routinely introduced to see if the software catchs them. At each point where an input value from the system is needed to proceed faulty values are introduced to see if the system detects them. Discs having array elements that did not

meet specification were used to see if they were detected by the software. In all cases, the software worked as designed.

Fifty Quadraspec Integra Readers and Processors are being used to carry out animal diagnostics at 10 different sites. More than 3 million canine heartworm assays have been run on this system with no single recurring problem noted with the software.

5. Interpretation.

Interpretation and analysis of data are discussed in section B.5.

Commented [A84]: See our comments in section B5.

J. ANALYTICAL SPECIMENS AND CONTROLS.

1. What is needed to demonstrate validation, what varies, what matters?

Ideally, a single plasma sample would be available of sufficient size and stability to last for years and supply the international clinical community. The closest thing to this would be a large pooled plasma sample from normal subjects that was split into one ml lot and stored at -80 ºC until used. The closest thing to this is the pool plasma sample from NIST that is described below.

Commented [A85]: This would satisfy some but not all requirements for validation.

2. Controls: their needed, how they are used, qualified, and quantified.

A series of calibration standards are made available to users that are not provided in the assay kit. [See section D1c.] Among these are the Le^x and sLe^x isoforms of clusterin, plasminogen, fibrinogen, proteoglycan-4, serum amyloid protein A, vitronection, histidine rich glycoprotein, and platelet factor 4. These reagents are provided in a test solution at a concentration equivalent to their concentration in normal plasma and are applied to a non-sample well on the disc as an external calibration standard and quantified with a LIF sandwich assay. Values obtained in non-sample wells are then compared to those in sample wells.

Commented [A86]: If these "standards" in non sample wells are contributing to the result calculation, for some type of e.g. normalization, then they cannot be considered controls.

Glycoforms of marker proteins were selected from cancer patient plasma samples (obtained from Asterand; Detroit, Michigan) with an immunosorbent column targeting clusterin as described in section D1c. The clusterin standard is qualified by mass spectrometry based proteomics using tryptic digests of the protein. Glycosylation sites are determined by selecting glycopeptides from the digest with the lectin concanavalin A, deglycosylated with PNGase F, and deglycosylated peptides sequenced by collision induced dissociation in an ABI 4800 tandem MS instrument.

1. **Controls.**

A pooled subject, control plasma sample from the National Institute of Standards and Testing (NIST) was used in developing this assay. This control sample is currently available from NIST.

Commented [A87]: Would this represent an IU population sample? "Positive" or "negative" for breast cancer?

a. Reference materials.

The calibration standards described below and the clusterin isoforms noted above are determined in the assay and are thus considered to be reference materials.

b. Calibrators/calibration.

A calibration standard is supplied with kits. The proteins and their concentration are as follows; transferrin (4 x 10^{-3} g/ml), haptoglobin (1.25 x 10^{-3} g/ml), α_1-antitrypsin (1.4 x 10^{-3} g/ml), α_2-macroglobulin (1.8 10^{-3} g/ml), clusterin (1.08 x 10^{-4} g/ml), fibrinogen (2.72 x 10^{-3} g/ml), and plasminogen (1.08 x 10^{-4} g/ml). The clusterin, fibrinogen, and plasminogen contain all the glycoforms found in normal subjects who have no diagnosed form of cancer.

All these protein standards were obtained from commercial suppliers. The concentration of each standard in the mixture was quantified by mass spectrometry using ^{13}C-labeled peptides in the multiple reaction monitoring (MRM) mode of quantification. At least three peptides were used to quantify each protein. This approach both confirms the identity of the protein and its concentration. During the course of the assay any impurities above 1% are generally identified as well.

Commented [A88]: Provide more details about calibration process such as: the mathematical form of calibration curve, how many parameters, etc.

K. VALIDATION.

1. Repeatability/precision (runs, days, and operators) and acceptable variation limits.

Variations in the relative standard deviation of measurements ranging from 50% are acceptable. This is based on the substantial differences in marker concentration between control and cancer subject as seen in Table 3. Differences between runs, on different days, with different operator, and at multiple sites were seen to exceed 20%.

Commented [A89]: These seem to be very large variations, perhaps unacceptable in many cases. Analytical and clinical studies would have to show that this is acceptable for this assay and intended use.

2. Reproducibility/portability (all sources of variation across sites)

See Figures 3-6 and 23-26.

Commented [A90]: See comments related to these studies.

3. Linearity, quantitativeness, range of measurement.

See Figure 3-6.

Commented [A91]: This is describing precision. Our understanding is that section 1 is precision at 1 internal site. Please describe detailed study design and concentrations. Section 2 is related to the precision at 3 sites (at least 2 external sites). Please describe detailed study design, such as how many days, how many operators per site, how many runs per day, how many replicates per run, etc.
From what you provided, it is not clear what were exact studies performed and which additional studies, specimens, and sources or variability would need to be evaluated in your precision/reproducibility studies.

2. **Limits of detection, signal/noise (sensitivity)**

See Figures 3-6.

Commented [A92]: We recommend that you follow CLSI EP6-A.

3. **Accuracy/bias; is identity important, comparison method.**

A biased population was used in the analytical validation of the platform. These measurements were carried out using BI-RADS 1 and 2 patients as breast cancer free controls while individuals with a breast cancer positive biopsy were used as the other extreme.

Commented [A93]: How was this study performed? From these figures, it seems there would be some additional studies needed.

Identity of antigens captured by antibodies at array elements is important in addition to other proteins that are captured. It is for this reason that antibodies used to prepare arrays were first immobilize in POROS affinity chromatography columns and used to isolate glycoproteins that were then identified and quantified by modern proteomics methods as described above.

Commented [A94]: This section seem to mainly address other issues, such as analytical specificity (i.e. cross-reactivity, interference).

Types of interferences are illustrated in Figure 28. One type is from cross-reacting species that share an epitope with the antigen. It is seen in this illustration that when immunoaffinity chromatography (IAC) selected proteins are chromatographed on a

Commented [A95]: This is not an appropriate study design for analytical accuracy. It is possible that the BI-RADS 1 and 2 have very small amounts of the proteins.
Among other means, analytical accuracy may be assessed using recovery study and using international standards.

reversed phase chromatography (RPC) column they are separated. The same is true of epitope bearing degradation products of antigens. RPC columns easily differentiate the intact antigen from degraded forms. In both of these cases the relative concentration of the antigen and the interfering species is related to the area under the curve. When an IAC fraction from one antibody column is chromatographed on a second IAC column and examined by RPC again it is generally the case that no other protein is found. Selection through two dimensions of antibody select eliminates everything but the antigen being targeted. If a huge excess of sample of double IAC selected protein is applied to an RPC column it is sometimes possible to see a small fraction of non-specifically bound protein. The concentration of these proteins is generally so low that they cause less than 1% error in antibody assessment.

Commented [A96]: What is described in this paragraph would need to be experimentally shown in appropriately designed analytical studies, not just using an example. You may already have these studies judging from your conclusions, but they would need to be provided so that we can evaluate.

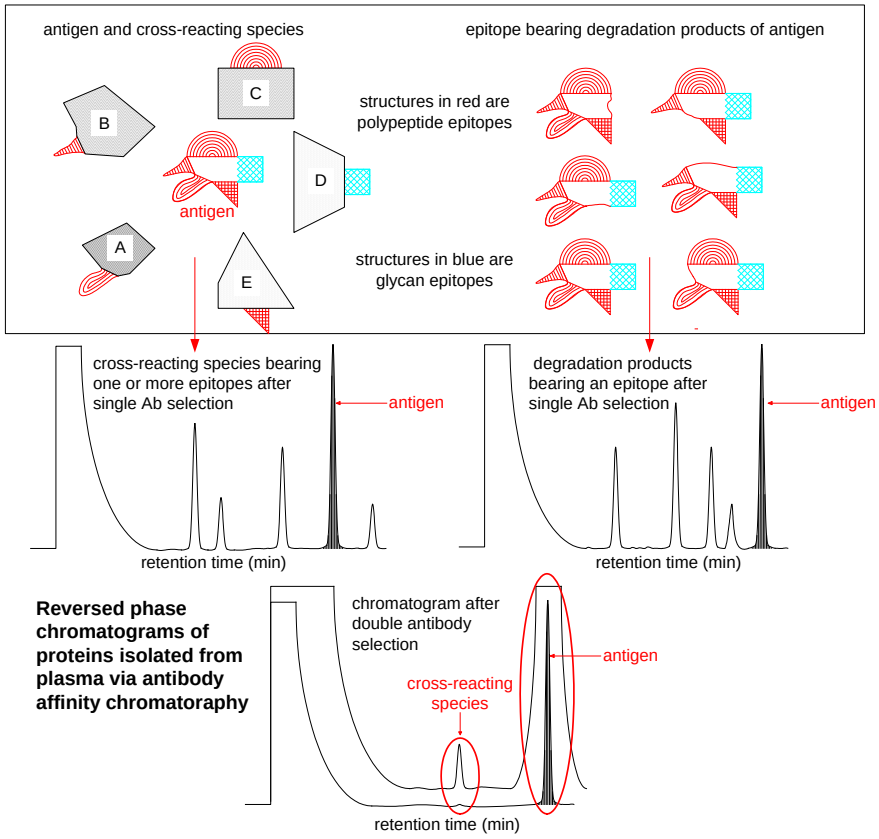

Figure 28. An illustration of the types of interference encountered in immunosorbent selection. The reversed phase chromatograms under the illustration show that cross-reacting species and antigen degradation products are easily differentiated from the antigen.

Having noted that epitope bearing degradation products are not the antigen, it is possible that they will be sample by the capture antibody at an array element and then detected by the second antibody. Degradation products can containing both antigens. Although they are recognizable and separable by double IAC, they can not be differentiated in a sandwich assay. The question is whether this is bad or good. Actually it is a good thing, because degradation products used to be the targeted antigen. They are not interferents. What is worse is to have an epitope eliminated in the course of degradation and miss the former antigen. It is missed in the assay. One would get a partial false negative. There is no way in this assay platform to eliminate that prospect. It is detectable in the double IAC approach with proteomics identification by not in a simple sandwich assay system.

Commented [A97]: This would be visible from data and would probably end up as one of the assay limitations.

4. **Specificity:false positives, interference, cross-reactivity.**

In addition to interferences from specific binding noted above, there is concern about non-specific binding (NSB). NSB is defined here as the binding of non-antigen proteins to 1) either the antibody or support matrix at a site other than the paratope and/or 2) antigens biospecifically bound to array elements.

The degree to which NSB is a problem is assessed in several ways. One is by noting the change in SDI and LIF signal intensity when discs are read before and after sample application. Another is by running tests on samples after abundant protein removal from plasma with a MARS-20 column from Agilent. This column removes the 20 most abundant proteins, including the internal standard proteins in plasma. NSB was assessed at the array elements where these proteins would bind.

Commented [A98]: This data would need to be provided.

False positives occur in an immunological assay when signal reporting species binds to something other than the antigen. In the case of SDI detection, this would be any protein that binds to an array element by any mechanism. All proteins that bind to he surface of an array element will give a signal. The human IgG array elements in sample wells determine non-specific binding (NSB). Because human IgG is of very similar structure to the antibodies bound at other array elements, NSB at all the other array elements will be very similar. Another form of NSB that can occur in SDI detection is the binding of protein(s) to captured antigens, referred to as a protein interaction partner (PIP). This would occur if the level of a PIP were elevated in the plasma of a subject for some reason. If the level of the PIP were high and stoichiometric saturation of the antigen occurred an Ag:PIP or perhaps an $Ag:(PIP)_2$ complex could be formed. Depending on the mass of the PIP relative to Ag mass, the signal for one of the reference proteins could increase many fold. Such an event would be easily recognized as a false positive because the concentration of all the internal standards and reference proteins measured by SDI are relatively constant in plasma and measurement variation is small (Tables I and II).

False positives in the sandwich assay are harder to imagine. Again binding to the IgG array elements is used to assess NSB of the fluorescent labeled reporter antibody to capture antibodies. This is automatically assessed with the IgG reference array elements. The only other way a false positive could occur is through the binding of CHO-121 or TG-1 to a protein captured on an array element that is not an antigen. The literature and our experience indicate that these two antibodies are very specific for sLe^x and Le^x, respecitvley. In fact, they are so specific they only bind to ~30 other proteins

in plasma and 40% of those proteins are associated with cancer. Because these antibodies were used to discover the breast cancer markers being targeted here, all the proteins they capture from normal plasma are known. Irrespective of the number of glycosylation sites in a protein, the only way these fluorescent labeled antibodies will be captured from solution is by the antigen they target or another protein carrying Le^x or sLe^x. A false positive could only arise from i) a new protein associated with some other disease, ii) that happens to also have a Le^x or sLe^x antigen, and 3) an epitope targeted by one of the array elements or a high propensity to non-specifically bind to only one type of array element. There is no way in this system to automatically recognize such an event.

The good news about false positives, cross-reactivity, and non-specific binding is that it will either impact all the array elements in a well or only those for a particular antigen. The probability of two false positives in a single 8-plex assay is very low. If it affects them all, the background will move up on all array elements including the IgG reference array elements. If there is a single false positive, it is one out of eight. It is unlikely that only one in eight biomarkers would be elevated in a BI-RADS 3 classified subject.

5. **Signal saturation, max/min sample volume/mass.**

This topic has been discussed above.

6. **Quantitative vs qualitative?**

As discussed above, this is a semiquantitative assay.

7. **Establishment of cutoff/normal values across disease population.**

All of the breast cancer markers identified in the discovery phase of this work were at 3 times or high concentration that the same proteins in a BI-RADS category 1 and 2 population. Based on the discovery phase finding the cutoff was set at 3 times the average value for this population.

Additional FDA comments on analytical validation studies can be found in the review memorandum.

L. CLINICAL PERFORMANCE

1. Study design

The criteria for an FDA OIVD is that it has to be safe and effective. It is assumed here that a plasma biomarker test requiring a venipuncture is regarded as safe.

Then there is the crucial issue of interpretation of "clinical effectiveness". Two interpretations of "clinical effectiveness" are possible and guidance from the FDA as to which is the most appropriate interpretation for the intended use would be very helpful:

1. The test is effective if it achieves an objective measure such as a pre-specified level of sensitivity at a given level of specificity.
2. The test is effective if it significantly improves on the medical judgment made without the use of the test. (As indicated by the FDA in discussions

Commented [A99]: Probably would be a limitation, unless this effect leads to unacceptable performance.

Commented [A100]: This should be set upfront and included in assay interpretation – how many biomarkers should be elevated.

Commented [A101]: See comments above.

Commented [A102]: See comments above – clinical study and performance derived from it would need to prove this cutoff.

Commented [A103]: Method Comparison studies, as applicable may need to be provided, and would be a part of analytical performance but performed with clinical specimens.

with the NCI in the context of another 510k application for a differential diagnostic test)

A study is being conducted to determine the effectiveness of a test for differential diagnosis of breast lesions with BI-RADS 4 results using the first criterion and then discuss requirements for the second criterion.

First Criterion

A study is being conducted in which subjects at multiple sites are enrolled if found to be in the BI-RAD 4 breast lesion category by mammography. Among these sites are the four NCI Clinical Proteomics Technology Assessment for Cancer (CPTAC) sites:

1. Fred Hutchinson Cancer Research Center – via UW SSCA
2. Vanderbilt University through the Breast Clinic
3. UC San Francisco
4. Memorial Sloan Kettering Cancer Center

The accrual goal at each site is 500 patients with a BI-RADS 4 breast lesion, for a total of 2,000 patients satisfying the inclusion/exclusion criteria listed below. This sample size will provide the following expected number of patients with conditions as determined by subsequent breast biopsy diagnosis:

1. 25% with breast cancer (500) (Cases)
2. 75% with benign disease (1500) (Controls)

Of the patients with breast cancer, half (250) are expected to have ductal carcinoma in situ (DCIS) and half (250) are expected to have invasive breast cancer. Within each of these three groups, patients will be randomly split between a training set and a validation set. Biomarker based diagnostics will be developed and optimized on the training set. It is important to note that the cutoffs are being developed by applying the tests to women in the intended use population (BI-RADS 4) and **not** in a population of *healthy women.* If any of the tests are above their respective cut-offs then the overall test is considered positive. When the final algorithm and cut-points are "written in stone" the test will be applied in a blinded manner to the validation set. The proportion of patients in the training set is set at 50%.

2. Sample Size

To determine a realistic combination of sensitivity and specificity with a plasma biomarker test, the separation between cases and controls observed in another plasma biomarker test was used for guidance. The measure of separation is the number of standard deviations the tests results are apart between cancer cases and controls, and is termed the *effect size.* This measure is *only* being used to determine a clinically realistic difference that might be expected for plasma biomarker results between cancers in breast cancer patients and subjects with benign breast disease. From this measure of separation between the two distributions, the sensitivity can be set, and the expected performance of specificity can be estimated. The effect size is used for no other purpose.

It is expected that differentiating between a benign and malignant breast lesion with a plasma marker will be more difficult than using CA125 to differentiate between a benign and malignant pelvic mass. However, with 8 biomarkers together anyone of which can be positive for the overall test to be positive, the effect size to differentiate breast lesions may achieve similar separation as CA125 does between benign and malignant pelvic masses. This analogy is purely for illustration of a realistic effect size for oncology diagnostics based on a test in

Commented [A104]: is expected to

Commented [A105]: We recommend that you use terminology recommended by STARD initiative (target condition present / target condition absent).

Commented [A106]: Please note that you did not address the problem of verification bias. Not all women with BI-RADS of 4 will have biopsy results. Women who did not have a biopsy can have different characteristics compared to the women with BI-RADS of 4 who did have a biopsy.

Commented [A107]: This is a way to consider doing the study, however any bias in the particular center would be randomized across both sets, and results might be different when introducing the test in clinical use in some other center. Perhaps less bias would be introduced if data from 1-2 centers were used for training, and other 2 or 3 centers for validation.

The training set should be data from one or more sites and the validation set is data from another independent sites.
If the clinical studies at the training data sites and at the validation data sites are going in parallel, we should discuss to assure that the data from validation set is not available during the establishing the linear combination.

current clinical use. In an actual breast cancer diagnostic study, there would be pilot data to estimate effect size. Here we proceed assuming the effect size is the same as for CA125 in differentiating benign from malignant disease. In patients with ovarian cancer, CA125 has a median value of 120 U/mL with a between patient CV of 50%, while in patients with a benign pelvic mass, CA125 has a median value of 20 with a between patient CV of 50%. On the natural logarithm scale, the effect size or the number of standard deviations by which the two means differ, is $\log(120/20)/\sqrt{(0.50^2 + 0.50^2)} = 2.53$. Since pilot data are absent in this hypothetical example, we proceed assuming the effect size of the test based on glycoprotein biomarkers is the same size as for the glycoprotein CA125 in differentiating malignant from benign pelvic masses. Then glycoprotein biomarkers could achieve the following operating characteristics.

The intended use of the glycoprotein biomarker test is to differentiate patients with BI-RADS 4 results into two groups, namely patients with a low probability of having cancer for whom the physician may recommend waiting a few months for subsequent testing, and thus avoid the morbidity associated with a biopsy, and all other patients for whom a biopsy would be recommended as currently occurs under standard of care. The definition of low probability for having cancer is for this group of patients to have the same probability as patients with a BI-RADS 3 result, namely 2% or lower, since BI-RADS 3 patients are usually recommended not to have a biopsy and wait a few months for further tests.

A probability for malignancy of 2% for women with a negative test means the negative predictive value is 98% (in other words, among 100 women with negative test results, two women have malignancies). With a prevalence of 25% of patients with a BI-RADS 4 result with malignancy (π=25%),, 75% of patients will have benign breast disease (1-π=75%). If PepCa10 has a specificity of 50%, half of the patients with benign breast disease will avoid having an unnecessary biopsy. This goal would seem to be clinically significant and therefore the sensitivity required to achieve it is now calculated. The sensitivity required to achieve an NPV of 98% with a specificity of 50% and prevalence of 25% is 96.9% (for Se=96.9%, Sp=50%, π=25%; the NPV is 98.0% and PPV is 39.3%; percent of subjects with negative test results is 38.3%).

With an effect size of 2.53 as hypothesized above, a plot of the NPV versus specificity is given in the Figure below, showing that an NPV exceeding 98% is achieved for all specificities of 80% or less. The clinical benefit will mainly be on the patients with benign disease for whom a biopsy is not recommended (specificity) based on the test results, so that they will not have to undergo the morbidity of an unnecessary biopsy.

As a specific example, if the specificity is 75% (the proportion of benign patients for whom a unnecessary biopsy will be avoided is 75%), the sensitivity is 97% (the proportion of malignant patients for whom necessary biopsy will be missed is 3%), and the prevalence is 25% (pre-test probability of malignancy is 25%); then NPV is 98.7%, PPV is 56.4%, and percent of patients with negative test results is 57.0%. It means that

i) 57% of women with BI-RADS of 4 can avoid a biopsy because of the negative test results; among them, probability of malignancy is 1.3% (1-NPV);
ii) 43% of women with BI-RADS of 4 will be recommended for biopsy because of the positive test results; among them, probability of malignancy is 56.4% (pre-test probability of malignancy was 25%).

Commented [A108]: We understand there is no basis to expect this test would have the same size effect and this in only used for illustration of a realistic effect size before pilot data become available (at which time pilot data will be used to estimate effect size or other parameters, in place of this assumption).

Commented [A109]: This can be one way to define this.

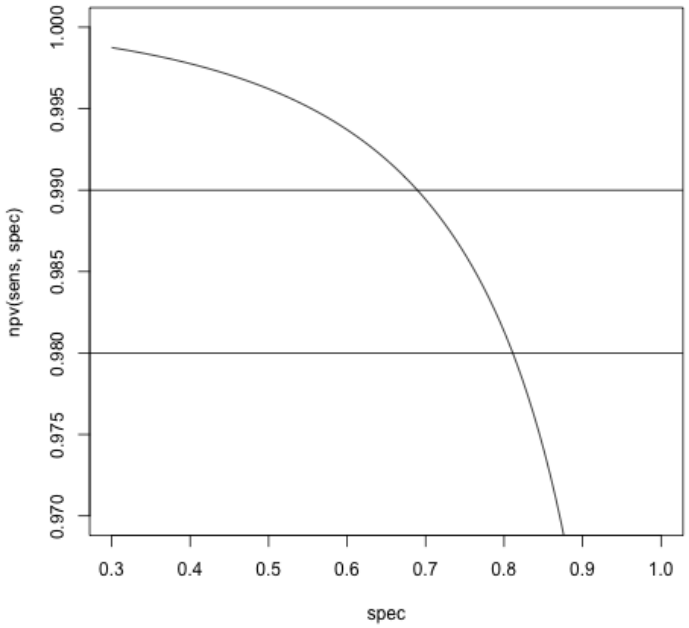

Figure 29: Negative predictive value of PepCa10 if the mean plasma test in patients with the target condition is 2.53 standard deviations above mean plasma test in patients without the target condition (on logarithmic scale) and the prevalence of the target condition is 25%.

To achieve an estimate of the sensitivity and specificity with small uncertainty, narrow confidence intervals are required. With a sample size of n=144 cancer cases and n=288 patients with benign disease in the validation set, the 95% confidence interval width is expected to be ±5% assuming a specificity of 75% and for sensitivities exceeding 90%. The 95% confidence intervals for NPV of 98% having a 95% CI as (96.4%, 99.4%), for an NPV of 99% it is (97.8%, 99.9%), and for an NPV of 99.5% it is (98.4%,99.95%).

To determine whether glycoprotein biomarker provide a contribution beyond available clinical information, we include the glycoprotein biomarker test results (continuous) in a logistic regression and assess whether the glycoprotein biomarker coefficient is significantly different from zero (which would imply no additional contribution). The clinical information would include age, menopausal status, family history, and other known risk factors for breast cancer.

Second Criterion for Effectiveness

To address the second criterion, we collected the judgment of the physician (usually radiologist) following mammography and identification of the lesion as to whether the lesion is benign or malignant. The medical action will be altered if the test indicates a difference from the physician's judgment, and will be clinically relevant if 5% or more of patients have a better

Commented [A110]: What level of sensitivity and specificity did you consider in these calculation?
Did you use exact confidence intervals for the negative likelihood ratios when you calculated the 95% CI for the NPV?

Commented [A111]: Please provide a list of these risk factors and how they will be included in the logistic regression analysis.

Commented [A112]: The "clinical effectiveness" should be demonstrated. This section seems to be a continuation of "clinical effectiveness", dealing with the available clinical information for a patient with BI-RADS 4 and the score result. See FDA comments (below) for a continuation of this comment.

Commented [A113]: By a pathologist reading biopsy results.

Commented [A114]: Please clarify how you propose to use the test in a real life setting, considering a radiologist would normally read a mammogram and may provide results according to BI-RADS assessment categories.

See FDA comments (below) for a continuation of this comment.

judgment with the test, where biopsy results will be considered the "gold standard". The validation sample will have an equal number of cancer cases and benign lesions. The test will be effective if the 95% confidence interval of the improved proportion rules out 1% or less. The confidence interval for a proportion is derived from the standard error for a binomial proportion which is √(p(1-p)/n). With a total sample size of n=232, or 116 cases and 116 controls, and 5% of the patients have an improved judgment by the test, then we have 80% power to rule out 1% improvement or less at 95% two-sided confidence.

Commented [A115]: The design of this study should be clarified.

Continuation of comments regarding "Second Criterion for Effectiveness"

- *The "clinical effectiveness" should be demonstrated. This section seem to be a continuation of "clinical effectiveness", dealing with the available clinical information for a patient with BI-RADS 4 and available SDIA result.*

  For example, consider a hypothetical case where "additional clinical information" such as whether a woman is pre-menopausal, no family history, comparison to previous mammogram, no risk factors, etc (we would need to consult expert physicians/ radiologists on what information they use and how they actually account for it). Then all patients with BI-RADS of 4 may be divided into 4 groups: clinical information positive, SDIA positive; clinical information positive, SDIA negative; clinical information negative, SDIA positive and clinical information negative, SDIA negative. You would need to investigate the percent of referral to biopsy in each group separately.

- *Please clarify how you propose to use SDIA test in a real life setting, considering a radiologist would normally read a mammogram and may provide results according to BI-RADS assessment categories*

  In the hypothetical case that this type of test were available, and if the BI-RADS results are 4, when making a decision about referral to biopsy the physician would be able to decide whether to take this test into account. Depending on how the clinical study and intended use population is defined, the decision may not necessarily be about "malignant/benign" but about "refer to biopsy/not to refer to biopsy."

3. Patient samples or specimen.

a. Inclusion Criteria

Women with no prior cancer history underwent image-guided breast biopsy for lesions of unknown diagnosis in the breast imaging clinics at UW and SCCA, the outpatient clinical site for the FHCRC/UW Cancer Consortium, and the other CPTAC sites, where all patients have a BI-RADS 4 result.

Approximately 800 image-guided core needle biopsy procedures are performed annually in the breast imaging clinics at the University of Washington (UW) and Seattle Cancer Care Alliance (SCCA) (ca. 450 US-guided procedures, 250 stereotactic (mammography)-guided

procedures, 100 MR-guided procedures). It is anticipated that at least 50% of these patients will meet criteria for study inclusion. Thus, we expect to have the opportunity to approach approximately 1000 patients and enroll approximately 500 of those individuals during the 2–2.5 years of clinical sample collection. The expected distribution of diagnoses in those 500 study participants is approximately 375 with benign breast disease and 125 with cancer. Of the 125 with cancer, it is expected that 50% (approximately 62) will have invasive disease.

b. Exclusion Criteria

- Known (biopsy-proven) current breast cancer
- Any other *in situ* or invasive cancer
- Prior chemo, radiation or hormonal (e.g., tamoxifen, AIs) therapy; HRT OK
- Current pregnancy
- Blood transfusion within the last 6 mos.
- Those not competent to provide informed consent

c. Constraints on Eligibility

- Patients must have an undiagnosed breast lesion prior to blood draw
- Blood must be drawn prior to breast biopsy diagnosis
- Patients must sit quietly for five minutes prior to draw
- Blood must be processed, aliquoted, labeled and stored within 75 min. of collection

NOTE: A prior benign breast biopsy is not an exclusion criteria.

d. Speciment collection

Specimen collection was done as a collaborative effort between the UW/SCCA Breast Imaging group and the FHCRC/UW Breast Specimen Repository and Registry (BSRR), and performed similarly in the other CPTAC sites. Patients were identified, approached and consented to the BSRR by a Clinical Research Coordinator or Nurse (RC). At the time of blood draw, the RC paged the BSRR Tissue Collection Specialist who retrieveed, processed, aliquoted and stored the blood samples and entered specimen-related information into the BSRR database. Prospective specimen and data collection protocols are well established at both sites.

Additional FDA comments on specimen:
You have provided specific instructions for blood collection tube handling and specimen processing. You will need to demonstrate the stability of the specimens across the extremes of these parameters (e.g., temperature, time to freezing, freeze-thaw, and shipping).
Sample amount: You have not provided information on how the blood sample volume was determined. What happens if user obtains a short-draw sample?

Shipment study
Samples are processed at collection site but shipped to test site. You will need to perform a shipment study to validate recommended shipping conditions and

also test extreme shipping conditions. Number of days between collection and testing was not evaluated.

Stability data: Stability data should include different peptide analyte concentrations.

Additional general FDA comments:

The description of the proposed studies lacks sufficient detail to determine how the studies will support the proposed intended use. The following are some general comments:

- *Sponsor should be prepared to provide information related to the patient (in addition to age, menopausal status, smoking habits and BMI) co-existing or previous medical conditions, mammography method (e.g., digital, x-ray, w/ or w/o CAD), size of the lump (if there is one). How will other variables be accounted for in the enrollment to avoid bias?*
- *Please provide more information about how patient samples will be chosen for your validation set.*
- *Indicate whether sub-analyses based on test performance by stage is intended.*

Expected Values in benign and malignant conditions

The target population may have a wide variety of conditions unrelated to cancer but present at the time a breast mass has been identified. These other conditions could dramatically affect the analytes in question (especially considering that you plan to test plasma specimens) and confound interpretation of results. Please demonstrate the results of your assay results in patients with other benign and malignant conditions that may be occurring concurrently. Include ovarian cancer, cervical cancer, GI cancers and disorders, lung, leukemia/lymphoma, liver, renal, endometriosis, diabetes, cardiac disorders, autoimmune disorders such as SLE, rheumatoid arthritis, infection, and anemia of chronic disease.

Reference Interval

Provide the values in non-diseased women. You should include women that span the age range of the intended use population and evaluate a minimum of 120 premenopausal women and 120 postmenopausal women unless you are able to demonstrate that there is no difference between the two populations. We recommend you include other ethnicities representing US population in addition to Caucasian and African American, if possible.

Software and Risk Hazard Analysis: *Software information and a risk hazard analysis for the assay should be submitted.*

FDA comments related to software/instrument:

- *Your submission implies the use of all specified components (instruments) in the system, although it has not been specified whether all components or just some of the components be provided to the end user. Even if you do not market all components is appears likely that you will recommend them as validated for use*

with your assay, therefore evaluation of all will be required as a part of the review. Alternatively, you could make generic recommendations if there are similar components out there for use.

- *Once the issues above are at a more defined stage for your system/assay, we can provide more specific regulatory requirements needed to support the test system's claims. Overall, you would need to ensure that all components of the test system (other than perhaps the centrifuge) are controlled under FDA's Quality System, which includes the need for design and purchasing controls for the components of the system. Regarding the submitted material, the recommended software documentation is summarized below and should be documented at a moderate level of concern. Software validation requirements can be found in the "Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm089543.htm) and the "Guidance for Industry, FDA Reviewers and Compliance on Off-The-Shelf Software Use in Medical Devices" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/uc073778.htm).*
- *The computer workstation that will be used with this device and the software used on it should be considered off-the-shelf software which the guidance above describes what analysis and documentation should be kept on file for these software components.*
- *The Sample Processor, Quadraspec Inspira Reader, and BioCD software are key components of the test system in which a failure could produce incorrect results. These components should have complete software documentation submitted in the 510(k) or PMA based on the level of concern for these devices (as described in premarket software guidance above). Additionally, if this device is to be used for a regulated assay, then the instrument (Sample Processor, Quadraspec Inspira Reader and BioCD software) would need to be produced under GMP.*

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