

Biomarkers Quantification with Antibody Arrays in Cancer Early Detection

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KEYWORDS

- Cancer biomarkers • Antibody arrays • Biochip
- Cancer screening

Cancer is the second most common cause of death in the United States, accounting for nearly one of every four deaths, exceeded only by heart disease. Cancer is more treatable when diagnosed at the early stage, and this is particularly relevant for cancers of the breast, cervix, mouth, larynx, colon and rectum, and skin. Moreover, cancers of the cervix, colon, and rectum can be prevented by removal of precancerous tissue. There are two major components of early detection of cancer: education to promote early diagnosis and screening.¹

Recognizing possible warning signs of cancer and taking prompt action leads to early diagnosis and can have a great impact on the disease. Some early signs of cancer include lumps, sores that fail to heal, abnormal bleeding, persistent indigestion, and chronic hoarseness.

Screening refers to the use of simple tests across a healthy population in order to identify individuals who have disease but do not yet have symptoms. Examples include breast cancer screening using mammography and cervical cancer screening using cytology screening methods including Pap smears. Screening programs should be undertaken only when their effectiveness has been demonstrated, when resources (personnel, equipment, and so forth) are sufficient to cover nearly all of the target group, when facilities exist for confirming diagnoses and for treatment and follow-up of those with abnormal results, and when prevalence of the disease is high enough to justify the effort and costs of screening.¹

BIOMARKERS AND THEIR USER IN COMBINATION

Circulating biomarkers represent a simple, noninvasive, promising approach for improving detection, diagnosis, and management of some types of cancer. The screening tool must be sufficiently noninvasive and inexpensive to allow widespread applicability. A

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substance secreted by tumor tissues easily and cheaply detectable in biological fluids is, therefore, an ideal biomarker because the cancer is detected specifically and noninvasively. Biomarkers measurement and interpretation could be indirect; for example, measures of immune response or hormonal changes induced by tumors.²

Cancer is a diverse disease, and it is unlikely that a single biomarker could detect all patients affected by a particular cancer with high specificity and sensitivity. Indeed, biomarkers, such as prostate-specific antigen (PSA) that purport to have high sensitivity tend to have low specificity. Maintaining high specificity (low false-positive rates) is a high priority for population screening. Even a small false-positive rate translates into a large number of people subjected to unnecessary costly diagnostic procedures and psychological stress. Thus, biomarkers need to be highly specific for cancer, and the use of many different biomarkers will likely be necessary for an overall screening program that is both sensitive and specific. Measurement with equivalent accuracy of elevated levels of more than two biomarkers for a given cancer should provide a much better statistical basis for successful prediction than measurement of a single biomarker. Some biomarkers may be useful for the classification, but when combined with others they may become ineffective if they are highly correlated (overlapping). In a stepwise backward selection method, biomarkers that contribute redundant information should be omitted in order to retain a model that contains only biomarkers that are able to improve the diagnostic accuracy. For example, in hepatocellular carcinoma, biomarker-immunoglobulin M (IgM) complexes (squamous cell carcinoma antigen-IgM [SCCA-IgM] and alpha fetoprotein-IgM [AFP-IgM]) may be combined to improve diagnostic accuracy (Fig. 1).³

A single biomarker expected for a given cancer may for some biochemical reasons be poorly expressed in a particular patient, but it would be unlikely that an entire panel

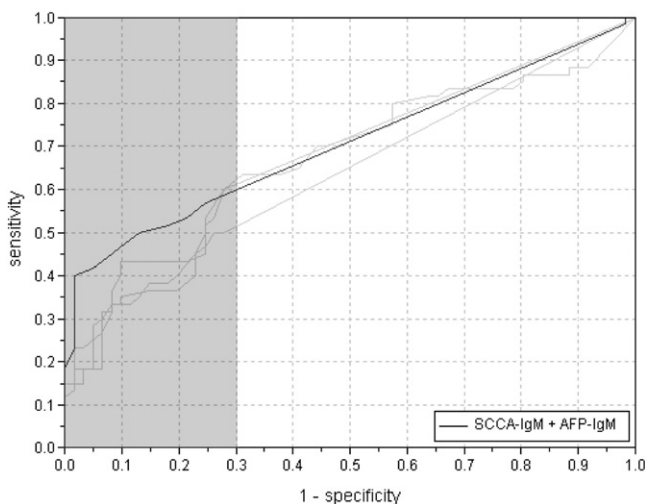


Fig. 1. Receiver operating characteristic curves from 60 patients with hepatocellular carcinoma versus 66 cirrhosis donors. The gray area shows the range of specificity between 70% and 100% where partial AUC was calculated ($\text{pAUC}_{0.3}$). SCCA-IgM ($\text{AUC} = 0.689$, $\text{pAUC}_{0.3} = 0.121$), AFP-IgM ($\text{AUC} = 0.632$, $\text{pAUC}_{0.3} = 0.105$) and their combination by linear logistic regression (SCCA-IgM + AFP-IgM; $\text{AUC} = 0.722$, $\text{pAUC}_{0.3} = 0.146$). AFP, alpha fetoprotein; AUC, area under curve; IgM, immunoglobulin M; SCCA, squamous cell carcinoma antigen.

of protein biomarkers indicative of that cancer would fail to be expressed. In addition, false-positives and false-negatives may appear frequently with a single biomarker but would be minimized by using a biomarker panel.

Another issue to be considered is that many protein biomarkers are indicative of more than one disease; for example, serum PSA is elevated in some benign prostate diseases as well as prostate cancer. Single cancer biomarkers are often not unique to a specific cancer, but it should be possible to define collections of biomarkers whose elevated individual levels taken together would be reliable predictors for specific cancers.⁴

Detection of panels of biomarkers is further complicated by the fact that ideally both normal and elevated serum levels of biomarkers need to be accurately measured. Ideally, measurements need to be done at high accuracy and cheaply at point of care (POC) such as in a physician's office or clinic to reduce costs, minimize sample decomposition, facilitate on-the-spot diagnosis, and alleviate patient stress. Such POC can also help to guide therapy, especially when timely adjustments in treatment become critical. Given these realities, development of POC bioanalytical devices to measure multiple cancer biomarkers presents a discouraging challenge, but one that nevertheless should be realizable.

In this article the focus is on planar antibody arrays, a possible answer for these POC devices. Antibody arrays are a specific form of protein microarrays in which captured antibodies are spotted and fixed on a solid surface for the purpose of detecting antigens in cancer early detection. The theoretical background for protein microarray-based ligand binding assays was initially developed by Ekins⁵ in the late 1980s. According to the model, antibody microarrays not only would permit simultaneous screening of an analyte panel but would also be more sensitive and rapid than conventional screening methods.⁵

ANTIBODY ARRAYS

Antibody arrays consist of miniaturized devices in which antibodies can be immobilized to capture and quantify specific proteins. One of the main advantages of this technology is the ability to measure hundreds of proteins (biomarkers) using low sample volumes of both precious clinical or biological material and expensive antibodies.^{6–8} Understanding the biological events that occur during progression from a normal tissue to cancer and the evidence to use many biomarkers to improve diagnostic accuracy regarding tumors has provided the stimulus to sustain biomarker discovery activities and efforts to evaluate their combination.

As shown in **Fig. 2** and summarized in **Table 1**, several biomarker combinations are suggested for many different tumors. Breast, prostate, and colorectal cancers are the main cancers studied.

TECHNOLOGICAL OVERVIEW

The high versatility of the design and format of planar antibody arrays has led to multiple versions, and their classification is related to antibody surface, printing methods, labeling, and detection methods.

Antibody Surface

Immobilization of the antibodies can be performed on planar surfaces such as filter supports, microtiter plates, or glass slides, leading to planar arrays.^{5–8} As illustrated in **Box 1**, the two main surfaces used to develop antibody arrays are amorphous material (glass and plastic) and nitrocellulose.

Antibody Array according to Type of Cancer

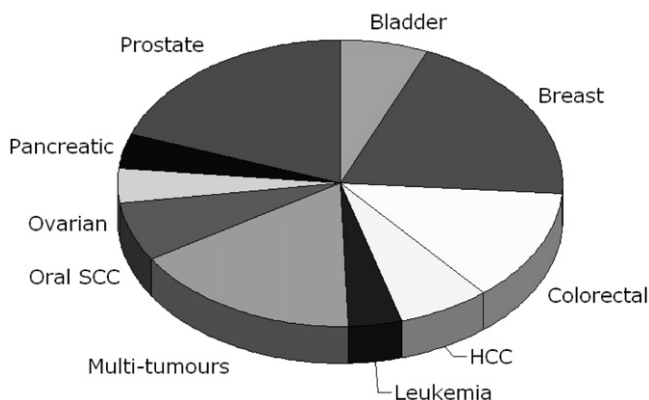


Fig. 2. Antibody array according to type of cancer. HCC, hepatocellular carcinoma; SCC, squamous cell carcinoma.

The surface properties of the substrate play an important role in the orientation and conformation of the biomolecule on printed microarray. This orientation is of crucial importance for the functional properties of the biomolecule, the sensitivity of the device, and, hence, on the quality of the applications developed.

Printing Methods

The antibody must be deposited as microspots in specified positions on the chip. Antibody matrix technology is based mainly on microcontact printing or ink-jet technology (**Box 2**).

Microcontact printing is a form of soft lithography that uses the relief patterns on a master polydimethylsiloxane stamp to form patterns. Advantages of this technique are the simplicity and ease of creating patterns with microscale features, the ability for the array to be created from a single master, the fact that individual stamps can be used several times with minimal degradation of performance, and the fact that this method is inexpensive to fabricate.⁴⁷

Ink-jet technology is a noncontact microdispensing system that allows printing of antibodies on various substrates (nitrocellulose, polystyrene, glass, epoxy, carboxyl, amine, hydrogel). Not all of these surfaces may be treated with contact methods. With ink-jet technology, it is easier to control the antibody amount deposited on each spot, a very important characteristic for developing in vitro diagnosis with high reproducibility. Moreover, with ink-jet technology the minimum amount of antibody deposited for each spot is much less than contact methods (nanoliters vs microliters).⁴⁸

Labeling and Detection Methods

Fig. 3 summarizes capture methods and label types of planar antibody arrays.^{6–8,49}

Capture methods of antibody arrays comprise the following:

1. *Label-based (one-antibody assays)*. The targeted proteins are captured by an immobilized antibody and detected through labeling with a tag.
2. *Sandwich (two-antibody assays)*. Immobilized antibodies capture unlabeled proteins, which are detected by another antibody using several methods to generate the signal for detection.

Table 1
Tumors and most often-used markers for screening

Type of Cancer	Marker
Colorectal Cancer	Apoptotic factors ⁹ Various antigens ^{10–12}
Breast Cancer	CA15-3, CEA, HER2, MMP9 and uPA ¹³ CA 15-3, EGFR ¹⁴ IL-8 ¹⁵ Cytokines and chemochines ¹⁶ HGF ¹⁷ Various antigens ¹⁸
Prostate Cancer	AMACR ¹⁹ IL-6, PSA ²⁰ anti-huTSP1 ²¹ PSA, PSMA, IL-6, PF-4 ²² PSA, IL-6 ²³ Various antibodies ²⁴
Oral Squamous Cell Carcinoma	EGFR ²⁵ Various antibodies ²⁶
Hepatocarcinoma	AFP, ANG ²⁷ Apoptotic factors ²⁸
Leukemia	3D9, 6A5, 4D9, 2D5 ²⁹
Ovarian Cancer	Various antigens ³⁰
Pancreatic Cancer	Various antigens ³¹
Bladder Cancer	Cytokines ³²
Multitumors	CA125, CA15-3, CA19-9, CA242, CEA, AFP, PSA, fPSA, HGH, beta-HCG, NSE and ferritin ³³ p-Tyr antibodies ³⁴ AFP, CEA, CA19-9, CA15.3, CA125, b-HCG ³⁵ AFP, PSA, CRP ³⁶ Various antigens ³⁷

(continued on next page)

Table 1 (continued)	
Type of Cancer	Marker
Other ^a	CRP, CEA ³⁸
	CA19-9, CA125 ³⁹
	CEA, CA125, Her-2/Neu (C-erbB-2) ⁴⁰
	CEA, AFP ⁴¹
	CEA ⁴²
	PSA, PSMA, PF-4 and IL-6 ⁴³
	Various antigens ⁴⁴

Abbreviations: AMACR, Alpha-methylacyl-CoA racemase; ANG, angiogenin; CA, cancer antigen; CEA, anticarcinoembryonic antigen; CRP, C-reactive protein; EGFR, epidermal growth factor receptor; fPSA, free prostate-specific protein; HCG, human chorionic gonadotropin, HER, human epidermal growth factor; HGF, hepatocyte growth factor; HGH, human growth hormone; hu-TSP, human testis-specific protein; IL, interleukin; MMP, matrix metalloproteinase; NSE, neuron-specific enolase; PF, platelet factor; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; p-TYR, phosphotyrosine; uPA, urokinase plasminogen activator.

^a Tumor not defined.

Label types comprise the following:

1. *Direct labeling.* Proteins/biomarkers (for label-based assays) or secondary antibody (for sandwich assays) are labeled with a fluorophore such as cyanine family (Cy3 or Cy5), quantum dots (QDs) or with an enzyme such as horseradish peroxidase (HRP) in sandwich labeling.

Box 1 Types of surfaces used for antibody arrays
Amorphous material – glass and plastic slides Superaldehyde slides ^{9,18} Poly(methyl methacrylate) substrate ⁴⁴ Aminosilanated slides ^{17,36,38} Polydimethylsiloxane chip ^{14,26} Silicon chip ^{21,41}
Nitrocellulose slides Nitrocellulose ^{15,18,25,32,33} PATH slides (Gentel) ^{22,45} FAST slides (Whatman) ^{12,26,28}
Hydrogels Hydrogels pads on slide ^{16,30,34}
Other Gold-patterned microarray chip ^{23,27}

Box 2**Biomarker printing methods****Ink-Jet**

Packard BioChip Arrayer (PerkinElmer)^{16,40}

ScanArray Lite microarray scanner (Packard Bioscience)¹⁹

PixSys 5000 robot (Cartesian)¹⁷

Nanoarrayer²¹

GT5000 Gantry System (Cartesian)³³

PROSYS 7500° (Cartesian)³⁵

Microgrid microarraying robot printed (BioRobotics)³⁷

Contact

GMS 417 microarray printer (Genetic Microsystems Affymetrix)^{13,20,26}

VersArray chip writerTM (Bio-Rad)⁴²

Proteogen CM-1000 (Proteogen)³⁸

Biochip Arrayer (PerkinElmer Life & Analytical Sciences)^{22,31,44,45}

M365 syringe pump (Sage)²⁹

Polybutadiene PAP pens (Sigma-Aldrich)⁴⁶

Other

Hand spotted^{9,36}

Photoresist coating²⁷

2. *Indirect labeling.* Proteins/biomarkers (for label-based assays) or secondary antibody (for sandwich assays) are conjugated with a tag (such as biotin) that is later detected by a second labeled tag (such as streptavidin-HRP, streptavidin-fluorophore, or rolling circle). This approach is an amplification protocol and improves the sensitivity.

Direct label-based assays allow the incubation of two different samples, each labeled with a different tag on the arrays. This type of assay allows the use of a reference sample to be coincubated with a test sample and facilitates normalization. Another benefit is that these assays are competitive, because the analytes in the test and reference solutions compete for binding with the antibodies. This attribute leads to improvement in linearity of response and dynamic range compared with noncompetitive assays. The main disadvantage is related to the disruption of the analyte-antigen interaction by the label, which may also limit the detection as well as the sensitivity and specificity. Moreover, the sample must be treated before introduction into the array, which limits the capability to develop a useful POC device.

The sandwich format increases the specificity compared with label-based assays and reduced background with increasing the sensitivity. This method only allows noncompetitive assays, because only one sample can be incubated on each array. This technique results in a sigmoidal binding response compared with the linear one in the competitive format and requires standard curves of known concentrations of analytes to achieve accurate calibration of concentrations. Compared with label-based assays, the weakness of sandwich assays is the availability of pairs of antibodies for

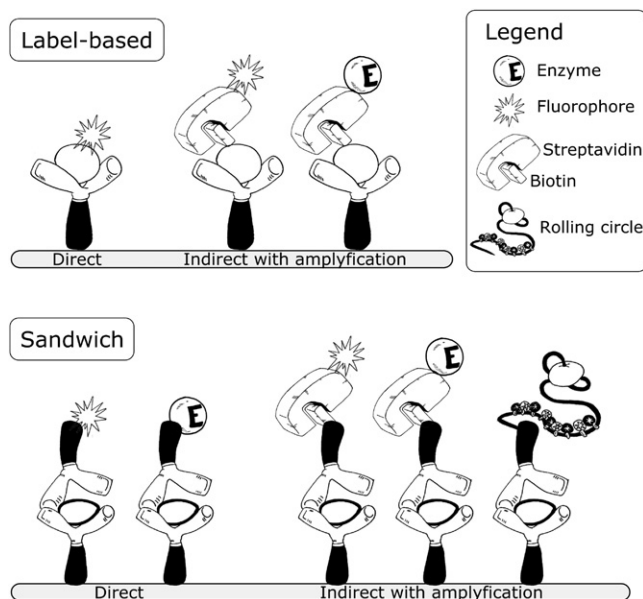


Fig. 3. Label-based and sandwich capture methods of planar antibody arrays with different label types.

each antigens and the potential cross-reactivity among detection antibodies increasing with additional analytes. This weakness contrasts with one-antibody assays in which only the availability of antibodies and space on the substrate limit the number of targets analyzed.

Commonly used labels for the visualization/quantification of biomarkers include mainly fluorophores (Cy3, Cy5, QDs, and so forth) and enzymes (alkaline phosphatase, HRP, and so forth).

Detection is by fluorescence or chemiluminescence (**Box 3**). Fluorescence detection is based on the use of fluorochromes that emit light when excited by light of a shorter wavelength. In chemiluminescence, when a soluble organic substrate is applied, the enzyme reacts with the substrate to generate light localized to the sites of enzyme. Chemiluminescence detection is more sensitive than fluorescence, but it requires more incubation and blocking steps.

Sensitivity and Specificity

Sensitivity and specificity are key parameters for quality and critical steps for the development of antibody array devices. Typical detection range of antibody array has been reported from some picograms to nanograms and usually is better than established enzyme-linked immunoabsorbent assay.^{24,49}

Ideally, only the protein of interest reacts with its corresponding binding partner on the microchip. Nonspecific binding may lead to false-positives, rendering protein microchip measurements inaccurate. The potential problem of specificity in the case of antibody microchips is antibodies that interact with multiple proteins. The main causes of this problem are cross-reactivity and multispecificity. Cross-reactivity is the tendency of an antibody to react with an antigen that did not stimulate its production.

Box 3**Biomarker detection systems****Chemiluminescence**HRP^{15,23,33,46}BCIP/NBT^{18,26}Electrochemiluminescence^{14,32}**Fluorescence**Cyanine (Cy3,Cy5)^{9,10,17,19,20,22,24,28,30,31,34,36,44,45}Alexa Fluors^{12,21,24,41,43}Texas Red¹⁶Fluorescein isothiocyanate^{29,35}Dyomics (Dy-549, Dy-649)³⁷*Abbreviation:* BCIP/NBT, 5-bromo-4-chloro-3-isdolyphosphate/nitro blue tetrazolium.

Multispecificity is a phenomenon when the same antibody reacts with two very different antigens. Both may lead to false-positives.

In label-based direct array, the main disadvantage of antibody array is the disruption of the analyte-antigen interaction by the label, which may limit detection as well as sensitivity and specificity. Moreover, because antibodies cannot be manufactured with preselected affinity and specificity, array sensitivity is antibody-dependent. It is advisable to validate the specificity and sensitivity of each antibody before using it as a probe for antibody arrays.^{6,24}

Sensitivity enhancement could be achieved by signal amplification using rolling circle amplification (RCA). In fact, with RCA it is possible to detect 0.1 pg/mL of protein, but this technology requires extensive chemical labeling of the detection antibody and is not easily adaptable in all laboratories.^{17,45} An alternative is to use an enzymatic signal enhancement method known as tyramide signal amplification, with which sub-pg/mL levels of protein may be detected.¹⁷

Reducing background is important to increase the sensitivity. The choices of antibodies and substrates used to develop the device and the step solution applied to treat the array play a key role.

Sensitivity of the microarray could be improved just by minimizing nonspecific binding using washing steps.²³ Another method to reduce background is to develop an alternative microarray using monoclonal antibodies or F(ab')₂ fragments as capture probes.³⁵

Wang and colleagues¹⁶ show the importance of substrate; in fact, while the nitrocellulose-coated slide performs similar to HydroGel-coated slides at the higher target concentrations, its sensitivity is much lower because of high levels of inherent fluorescent background. Moreover, Lee and colleagues³⁸ show that the high detection sensitivity can be attributed to the improved surface chemistry of protein immobilization by the bifunctional affinity linker. It is thus proposed that most antibody molecules immobilized are in the correct orientation resulting in increased detection sensitivity.³⁸

Reproducibility

Reproducibility (or precision) is the degree to which repeated measurements of the same sample will show the same or similar results. High reproducibility is fundamental

to correctly assess the protein quantity and is essential to develop devices for clinical application. Each step in the preparation of an antibody array contributes to generating noise that can affect its reproducibility. Usually, measurements are affected by an error that makes repeated measurements differ from each other. Given a set of measurements, the precision is usually measured by the coefficient of variation (CV; the standard deviation divided by the mean). The precision determined at each concentration level should not exceed 15% of CV except for the lower limit of quantification, where it should not exceed 20%. For high levels of reproducibility, a CV less than 10% on a minimum of three concentrations in the range of expected concentrations is usually considered. Moreover, reproducibility of the system should be evaluated both from an intraassay and interassay approach.⁵⁰

Stability

Another important aspect for the development of antibody arrays is the stability of the device. Thus, the storage stability of the immunosensor array should be adequate for practical use in order to propose biochips as suitable for clinical diagnostics.

All components of the device should be tested for stability in order to define the shelf life, but the conformational stability of antibody is the most important issue for the reliable detection of biomarkers with microchip-based approaches.

Antibody recognition against protein site (epitope) is dependent on hydrogen bonds, hydrophobic interactions, electrostatic forces, and van der Waals forces. The two- and three-dimensional antibody structure is necessary for binding. Printing methods and arrays surface are the most critical aspects affecting antibody stability. In addition, changes of structure that influence epitope recognition can be caused by an altered reaction environment such as pH, salt concentration, hydrophobicity, or by analysis-related artifacts that are generated during sample processing. Even partially degraded spotted antibodies may bind their corresponding proteins, resulting in decreased signal intensity with consequent quantification errors and/or increases in the coefficients of variation. Manufacturing, storage, and shipping are critical points to maintain the declared stability parameters.

The antibody array stability may vary considerably, depending on different storage times and conditions.²⁹ Stability may be increased by storing devices in dry air at 4°C.⁴⁰

In stability tests of oriented immobilized antibody, it was found that the antibody array was stable without loss of activity for more than 3 months at 47°C.³⁸ The advantages of the oriented immobilization of proteins are the good steric accessibility of the active binding site and the increased stability.³⁶ In addition, stability tests could be used to select the surface and to define the best conditions for the oriented immobilization of antibodies.

SUMMARY

The aging process in our society will become one of the key driving forces of change over the next decades. The specific demands of older generations constitute a key market of the future, and the pressure to improve and expand health services increases, especially as far as chronic diseases such as cancer are concerned. Availability of efficient and cost-effective screening devices for early detection of cancer are urgently needed to improve quality of life. Mass screening for early detection of cancer will reduce the ever-increasing social costs associated with treating patients with advanced-stage disease where existing diagnostic procedures have failed or screening has been inadequate for broad use. Nanosized technological platforms are the answer for mass screening, especially in those cases in which many

different biomarkers need to be determined simultaneously to provide clinical significance even in a decentralized fashion (POC testing). Because biochips automate highly repetitive laboratory tasks by replacing cumbersome equipment with miniaturized assay chemistries, they are able to provide ultrasensitive detection methodologies at significantly lower cost per assay than traditional methods in a significantly smaller amount of space. Biomarkers selection is a key factor for biochip design, in addition to managing the many variables that afflict an array experiment such as instrumental and biological error, reproducibility, array data processing, and normalization.

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