

Multiplexed Detection of Protein Cancer Markers with Biobarcode Nanoparticle Probes

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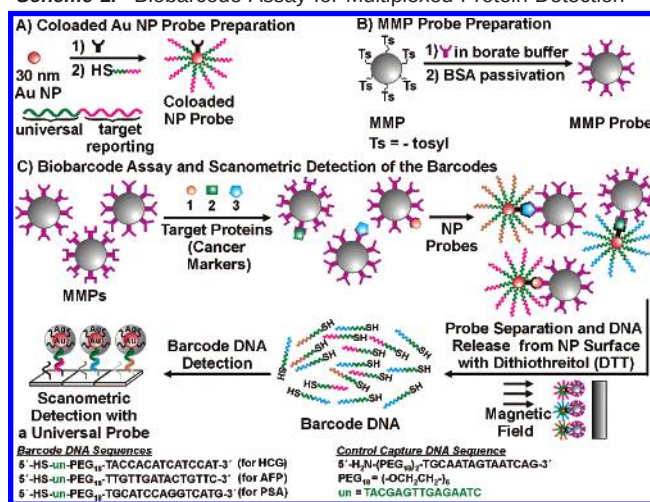
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Highly sensitive and selective immunoassays capable of detecting proteins in a multiplexed fashion are essential for disease diagnostics, drug screening, and biodefense applications.¹ As our understanding of disease increases, multiplexed protein assays will become increasingly important. For many types of cancers, it will not be sufficient to study a single protein marker, but rather a suite of markers that allow one to diagnose and follow the disease as a function of treatment.^{1–3} Heterogeneous enzyme-linked immunosorbent assays (ELISAs) coupled with optical probes (chemiluminescent or fluorophore) (1) lack the required sensitivity to diagnose and study many types of diseases, including many forms of cancer, (2) provide slow antibody–antigen binding kinetics, leading to long assay times at low target levels, and (3) in the case of multiplexed analysis, suffer from overlapping spectral features, nonuniform photobleaching rates, false positive signals, the need for multiple laser excitation sources, and complex instrumentation for assay readout.⁴ The sensitivity of ELISA is significantly improved by the immuno-PCR method, whose major drawbacks are the necessity of cumbersome antibody–DNA conjugation protocols and PCR requirements, which ultimately have hampered its widespread use for multiplexed protein analysis.⁵ To circumvent the aforementioned limitations, other alternative approaches for labeling and detection of biomolecules have been suggested, such as DNA-directed immobilization of proteins on supports,⁶ fluorophore-encoded microspheres,⁷ nanowires,⁸ microcantilevers,⁹ electrochemical coding,¹⁰ multimetal microrods,¹¹ and metal and semiconductor nanoparticle probes.¹²

Oligonucleotide-functionalized gold nanoparticles (Au NPs) have a demonstrated advantage over conventional probes in a variety of biodetection schemes as a result of their unique chemical and physical properties.¹³ The biobarcode assay, based on Au NPs functionalized both with oligonucleotides (the barcodes as a surrogate target) and a target recognition element (an antibody for proteins and a portion of the barcode for nucleic acids), is a powerful amplification and detection system for proteins¹⁴ and nucleic acids.¹⁵ It allows one to rapidly detect nucleic acid targets with sensitivity of PCR and protein targets at limits of detection that are 4–6 orders of magnitude lower than ELISAs, depending upon capture antibody and background signal. In the research and clinical diagnostic arenas, the biobarcode assay offers the following opportunities: (1) the potential use of new protein markers that could not be utilized for disease diagnosis with current technology because of sensitivity limitations;^{14b} (2) the assessment and study of disease recurrence using existing markers; and (3) the availability of a large library of discernible barcode oligonucleotides allowing the simultaneous detection of a panel of markers with high selectivity enabling an accurate diagnosis of a complex disease. Until now, however, it has remained unclear whether the biobarcode assay could be utilized for the multiplexed detection of proteins. The realization of a multiplexing system faces significant challenges, including the elimina-

Scheme 1. Biobarcode Assay for Multiplexed Protein Detection



tion of probe set/target set cross reactivity, selection of well-characterized high-affinity antibodies, minimization of nonspecific binding, synthesis of stable probes, and design of noncrossreacting reporters.

Herein, we present the first example of multiplexed protein detection with the biobarcode assay in the context of three established cancer markers at low-femtomolar concentration: prostate specific antigen (PSA, prostate cancer marker), human chorionic gonadotropin (HCG, testicular cancer marker), and α -fetoprotein (AFP, hepatocellular carcinoma marker).³ In addition, HCG and AFP in combination are suggested as the most effective markers for the diagnosis and monitoring of patients with testicular germ-cell tumors.³ The assay can be performed in a 96-well plate format in a high-throughput manner in buffer or serum.

The multiplexed barcode assay utilizes three 30 nm Au NP probes, each cofunctionalized with an antibody (monoclonal or polyclonal) that recognizes the target protein and barcode oligonucleotides as surrogates for the target antigen (Scheme 1A). Note that half of the barcode for each target is the target-reporting oligonucleotide probe, while the other half is identical for all the barcodes and is in effect a universal sequence (Scheme 1A). Such a barcode design enables one to use only one type of 13 nm Au NP probe for the scanometric identification of the barcodes (Scheme 1), which significantly simplifies the system and reduces assay time and effort. The target antigens are captured in solution by three magnetic microparticle (MMP) probes, each conjugated with a monoclonal antibody specific for an epitope of the target antigen different from the one recognized by the Au NP probe. The target-MMP complexes are then sandwiched by a Au NP probe with an antibody that can bind to the target in a different region than the MMP (Scheme 1B,C). These complexes are isolated with a magnetic field and washed, and the barcode strands are released by a ligand exchange process induced by the addition of dithiothreitol (DTT, Pierce).^{15b} The barcode strands are then identified by the chip-based

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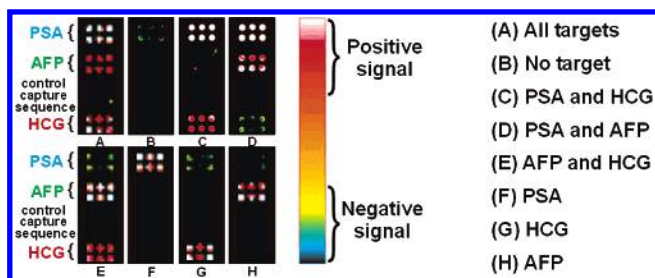


Figure 1. Scanometric detection of the barcodes. The gray scale images from the Verigene ID system are converted into a color scale using GenePix Pro 6 software (Molecular Devices).

scanometric method,^{13b} developed in our laboratory and commercialized by Nanosphere, Inc.

Each Au NP probe (30 nm diameter, Ted Pella, Inc.) is cofunctionalized with a detection antibody and barcode DNA strands (~180 barcodes/particle),¹⁶ following a new chemical procedure (Supporting Information). The Au NP probes are stored at a concentration of 6 nM, with the excess barcode DNA removed in 0.15 M NaCl, 0.025% Tween 20, 0.1% BSA, and 10 mM phosphate buffer, pH = 7.2 (assay buffer). The NP probe solution, composed of all three NP probes (NP multiplexing solution), is prepared by diluting equal volumes of each NP probe (6 nM) in assay buffer to a final NP concentration of 240 pM in the presence of 6.6 mg/mL tRNA to reduce nonspecific binding (Sigma-Aldrich).^{14d} Each MMP probe is prepared by coupling tosyl-functionalized MMPs (1 μ m diameter, Invitrogen) to the primary amino groups of the corresponding antibodies in borate buffer at pH = 9.5 at 37 °C. The surfaces of the MMP probes are passivated with bovine serum albumin (BSA, R&D Systems). The MMP probes are stored in assay buffer at a concentration of 10 mg/mL. The MMP probe solution, composed of all three MMP probes (MMP multiplexing solution), is prepared by diluting equal volumes of each MMP probe (10 mg/mL) in assay buffer to a final total MMP concentration of 0.0125 mg/mL.

To evaluate the selectivity of the system, eight tests are run with different combinations of the target antigens (Figure 1). In the first combination, all three targets are present. In the next seven tests, we systematically combine targets to evaluate the multiplexing capability of the barcode method. Significantly, the operator does not know the contents of each sample. The concentrations of the target antigens are kept constant for all experiments (each 170 fM final concentration in the assay). First, the antigens are captured by the MMP probes at 25 °C for 1.5 h, the MMP/target complexes are washed, and the NP probes are allowed to bind at 25 °C for 1 h. Next, the sandwiched structures are washed seven times with assay buffer, and the barcode DNA strands are released by the addition of 0.5 M DTT in 0.5 M NaCl, 0.01% Tween 20, 10 mM phosphate buffer, pH = 7.2 (scanometric buffer). The barcodes are detected with the chip-based scanometric method, and the signal is identified with a Verigene ID system (Nanosphere, Inc.) that measures the scattered light from the developed spots after silver amplification.

The scanometric identification of the barcode oligonucleotides is presented in Figure 1. In each combination, the presence of target proteins is accurately correlated to a high-intensity positive signal (white/red color), clearly distinguishable from the low intensity background negative signal (yellow/black color). The quantitation of the net signal intensities is given in the Supporting Information (SI, Figure 1S). The difference in the signal intensities of the positive signals are likely related to the different binding constants of the antigens with the corresponding antibodies.¹⁷ No hybridization is observed between the released barcode strands or the universal probe and the negative control capture DNA sequence printed on

the chip. Significantly, one also can utilize the developed multiplexed barcode assay in a complex medium, such as goat serum, and selectively detect the three antigens at low-pM concentration in an undiluted serum or 170 fM in diluted serum (1:200, see SI).

In conclusion, we have demonstrated the highly selective multiplexed detection of three protein cancer markers in buffer and serum media utilizing a new multiplexed version of the biobarcode amplification method. The results presented here validate the multiplexing capabilities of the barcode method and open new avenues for developing highly selective panel assays for the early detection of a wide range of diseases and potentially the evaluation of residual disease and disease recurrence, where high sensitivity is essential for early identification. In addition to the uses of the high sensitivity of the barcode assay and these markers to diagnose cancer, two of these markers, AFP and HCG, are important for screening fetal Down's syndrome. Since AFP is at a low concentration in Down's syndrome pregnancies, high sensitivity and multiplexed panels could contribute to an earlier diagnosis of this fetal abnormality.¹⁸

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Supporting Information Available: Detailed procedures for Au and MMP probes preparation, multiplexed barcode assay in buffer and goat serum, and scanometric barcode DNA detection. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Supporting Information

Multiplexed Detection of Protein Cancer Markers with Biobarcode Nanoparticle Probes

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Antigens and Antibodies

a) PSA

Prostate specific antigen (P3235, Sigma-Aldrich), MMP-monoclonal PSA antibody (ab403, Abcam), Au NP-polyclonal PSA antibody (AF1344, R&D Systems)

b) AFP

α -Fetoprotein antigen (A32260H, Biodesign International), MMP-monoclonal AFP antibody (10-A05, clone M19301, Fitzgerald Industries International, Inc.), Au NP-polyclonal AFP antibody (70-XG05, Fitzgerald Industries International, Inc.)

c) HCG

Human Chorionic Gonadotropin (A81355M, Biodesign International), MMP-monoclonal HCG antibody (E20579, Biodesign International), Au NP-monoclonal HCG antibody (E20106, Biodesign International)

Preparation of Au NP Probes

a) Preparation of 30 nm coloaded Au NP probes

Gold nanoparticles with an average diameter of 30 nm were purchased from Ted Pella, Inc. and used as received at a concentration of approximately 330 pM ($\sim 2 \times 10^{11}$ particles/mL). Prior to functionalization, the pH of the Au colloid was adjusted to 9.2 using 0.2 M NaOH. First, the 30 nm Au particles were functionalized with antibodies by adding each antibody at 0.1 $\mu\text{g}/\mu\text{L}$ starting concentration to each of the Au colloids and incubating the solutions for 30 min at 10 °C under slow vortex. The final concentrations of the antibodies in the Au colloids are the following: 2 $\mu\text{g}/\text{mL}$ for PSA, 4 $\mu\text{g}/\text{mL}$ for AFP, and 4 $\mu\text{g}/\text{mL}$ for HCG. Next, freshly deprotected thiol-functionalized oligonucleotides (Integrated DNA Technologies, Inc.) were added to the Au colloids (final concentration of oligonucleotides 3-4 μM) and the solutions were shaken gently for 5 min at 10 °C. The disulfide bonds in all oligonucleotides were reduced prior to the addition to the Au colloids by soaking in 0.1 M dithiothreitol (DTT, Pierce) in 0.17 M phosphate buffer, pH=8 for 2 h (10 OD of lyophilized DNA is typically reduced with 150 μL 0.1 M DTT solution). The deprotected DNA solutions were purified through desalting NAP-5 columns (GE Healthcare), and the amount of DNA from each column was determined by reading the absorbance of the solutions at 260 nm.

The colloids were first buffered to 10 mM phosphate concentration, pH=7.2 including 0.02 % Tween 20 (Sigma-Aldrich), and the NaCl concentration was brought to 0.15 M in one step under vortex. The solutions were incubated for 30 min at 10 °C, purified from excess DNA by repeated centrifugation at 10,000 rpm at 10 °C for 10 min, and washed with 0.15 M NaCl, 0.025 % Tween 20, 0.1 % BSA, 10 mM phosphate buffer, pH=7.2 (assay buffer). As a final step, all Au NP probes were re-dispersed in assay buffer at a concentration of ~ 6 nM. The exact Au nanoparticle concentration was determined by reading the absorbance of the colloid at 530 nm. The molar extinction coefficient for 30 nm particles at 530 nm is 3×10^9

$\text{M}^{-1}\text{cm}^{-1}$ (calculated from the measured UV-Vis absorbance of a colloid with a known particle concentration). The Au NP probes were stored at 4 °C prior to use.

b) Preparation of 13 nm Au NP chip-probes

Gold nanoparticles with a 13 nm average diameter were synthesized by citrate reduction of HAuCl_4 . The 13 nm Au particles were functionalized with freshly deprotected thiol-functionalized oligonucleotides and salted in a step-wise fashion to 0.3 M NaCl, 10 mM phosphate buffer, 0.01 % SDS, pH=7.2 over one day. Each 4 hour, the salt concentration was increased by 0.1 M. The 13 nm Au probe was purified from excess DNA by centrifugation at 14,000 rpm at 20 °C for 25 min, which was repeated three more times. The particles were washed and finally resuspended in 0.5 M NaCl, 10 mM phosphate buffer, 0.01 % Tween 20, pH=7.2 (scanometric buffer) at a concentration of ~ 10 nM, and stored at 4 °C prior to use. The exact Au nanoparticle concentration was determined by reading the absorbance of the colloid at 525 nm. The molar extinction coefficient for 13 nm particles at 525 nm is 2.4×10^8 $\text{M}^{-1}\text{cm}^{-1}$ (calculated from the measured UV-Vis absorbance of a colloid with a known particle concentration).

Preparation of MMP Probes

Tosyl-functionalized MMPs (1 μm diameter, Cat. No. 655-01, Invitrogen) were covalently linked to the primary amino groups of the corresponding antibodies (*see Antigens and Antibodies Section*) at 37 °C in borate buffer solution at pH=9.5. First, 100 μL of tosyl-functionalized MMPs (100 mg/mL) was washed twice with 1 mL of borate buffer while physically retaining the particles with a magnetic field. They were then re-suspended in 100 μL of borate buffer. The conjugation solution in each case was prepared by mixing 66 μL of borate buffer, 84 μL of 3 M $(\text{NH}_4)_2\text{SO}_4$ in borate buffer, 20 μL of the pre-washed magnetic beads (*vide supra*) and 80 μL of each MMP-monoclonal antibody solution (1.0 $\mu\text{g}/\mu\text{L}$ in borate buffer). The conjugation was carried out in 200 μL PCR tubes for 24 hours under vortex at 1,400 rpm at 37 °C. Next, the MMP solutions were placed on a magnet, the

supernatant aspirated, and the MMPs were passivated by adding 250 μ L of blocking buffer (0.15 M NaCl, 10 mM phosphate, 0.025 % Tween 20, 0.5 % BSA, pH=7.4). The passivation step proceeded for 24 hours under vortex at 1,400 rpm at 37 °C. The MMP probes were washed three times, each with 1 mL of storage buffer (0.15 M NaCl, 10 mM phosphate, 0.025 % Tween 20, 0.1 % BSA, pH=7.4), resuspended in 200 μ L of storage buffer (10 mg/mL final concentration), and stored at 4 °C. The loading of antibodies on the MMP probes is $\sim 1 \times 10^4$ mAbs/bead from UV/Vis measurement at 288 nm before and after the coupling reaction.

Multiplexed Biobarcode Assay

a) In buffer

Prior to the experiment, each MMP probe was washed twice with assay buffer and MMP probe solution composed of all three MMP probes (MMP multiplexing solution) was prepared by diluting equal volumes of each MMP probe (10 mg/mL) in assay buffer to a final total MMP concentration of 0.0125 mg/mL. The assay was initiated by mixing 150 μ L of assay buffer, 40 μ L of MMP multiplexing solution and 10 μ L of the appropriately mixed target solution in a PCR tube. The concentrations of the target antigens were kept constant for all experiments (each 170 fM final concentration in the assay). The system was incubated at 25 °C for 1.5 hours under constant vortex to allow binding between the MMP probes and the target antigens. The antigen capturing time can be reduced in the future. For example, we have found that a 30 min time period results in efficient capturing for a single PSA detection. In the case of multiplexed detection, one has to consider the diversity of target antigens and the different binding constants of the capturing antibodies. Next, the reaction tube was placed on a 96-well plate magnet (Invitrogen), and the MMP/target complexes were washed twice with 200 μ L of assay buffer. A NP probe solution, composed of all three NP probes (NP multiplexing solution), was prepared by diluting equal volumes of each NP probe (6 nM) in assay buffer to a final total NP concentration of 240 pM in the presence of 6.6 mg/mL tRNA

(R9001, Sigma-Aldrich). The NP multiplexing solution (50 μ L) was added to the MMP/target complexes, and the solution was incubated under constant vortex for 1 hour at 25°C. The system was washed seven times with 200 μ L of assay buffer each time to remove non-specifically bound NPs and any free barcode DNA. The barcode DNA strands were released from the NP probes by the addition of 75 μ L scanometric buffer (*vide supra*). The DTT-induced release of the barcodes was allowed to proceed for 10 min at 50 °C and 50 min at 25 °C under vortex. Magnetic field introduction removed all MMP probes from the solution, leaving barcode DNA for detection with the chip-based scanometric method. The signal was identified with a Verigene ID system (Nanosphere, Inc.) that measured the scattered light from the developed spots after silver amplification.

b) In goat serum

The barcode assay was initiated by mixing 140 μ L of assay buffer, 10 μ L of 10 % goat serum (G9023, Sigma-Aldrich) in assay buffer, 40 μ L of MMP multiplexing solution and 10 μ L of the appropriate mixed target solution in a PCR tube. The concentration of each target in undiluted goat serum corresponded to 33 pM. Further, the multiplexed barcode assay was carried out under conditions similar to the ones used in buffer solution (*vide supra*). The scanometric results are presented in Figure 1S.

Scanometric Detection of the DTT-Released Barcodes

For scanometric barcode DNA detection, N-hydroxysuccinimide-activated Codelink glass microarray slides (GE Healthcare) were spotted at Nanosphere, Inc. with amine-terminated barcode capture and control capture DNA sequences of the type 5'-H₂N-(PEG₁₈)₂-DNA (Integrated DNA Technologies) following the manufacturer's protocol. Each DNA was spotted six times. DTT-released barcodes in scanometric buffer (20 μ L) were injected into hybridization chambers (Nanosphere, Inc.) attached to a microarray slide. The barcodes were hybridized to the slide for 15 min at 60 °C, 30 min at 37 °C, and 15 min at 25 °C under mild shaking in an incubator. The gaskets were disassembled, the slides were copiously rinsed

with scanometric buffer, spin-dried and reassembled with new hybridization gaskets. The universal Au NP chip-probe solution (20 μ l, 500 pM Au NP, 10 % formamide in scanometric buffer) was introduced into the hybridization chambers and allowed to hybridize for 45 min at 37 °C. The gaskets were disassembled, washed three times in 0.5 M NaNO₃ solution, quickly dipped in cold 0.1 M NaNO₃, and spin-dried. Silver enhancement was performed by applying 2 mL of silver staining solution (Nanosphere, Inc.) on the dried chips for 5.5 min. The reaction was terminated by washing the slides with Nanopure water (18 M Ω , Barnstead International). The slides were spin-dried and imaged with the Verigene ID system (Nanosphere, Inc.) that measures the scattered light from the developed spots.

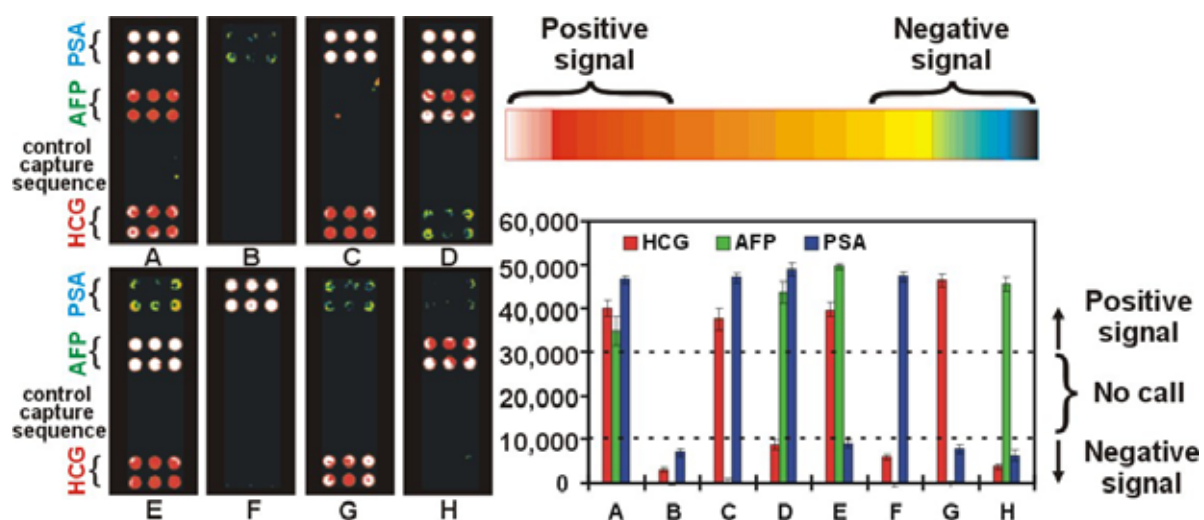


Figure 1S. Scanometric identification of the barcodes released from the 30 nm Au NPs in buffer and the corresponding quantitation of the net signal intensities (determined with a GenePix Pro 6 software, Molecular Devices). The minimum signal-to-noise ratio is 3 based on the absolute signal net intensity. If we have a net signal intensity of more than 30,000, we consider it a positive signal, and in case of less than 10,000, it is considered a negative signal. Any net signal intensity between 10,000 and 30,000 is considered a “no call”, and it indicates that we need to repeat the assay.

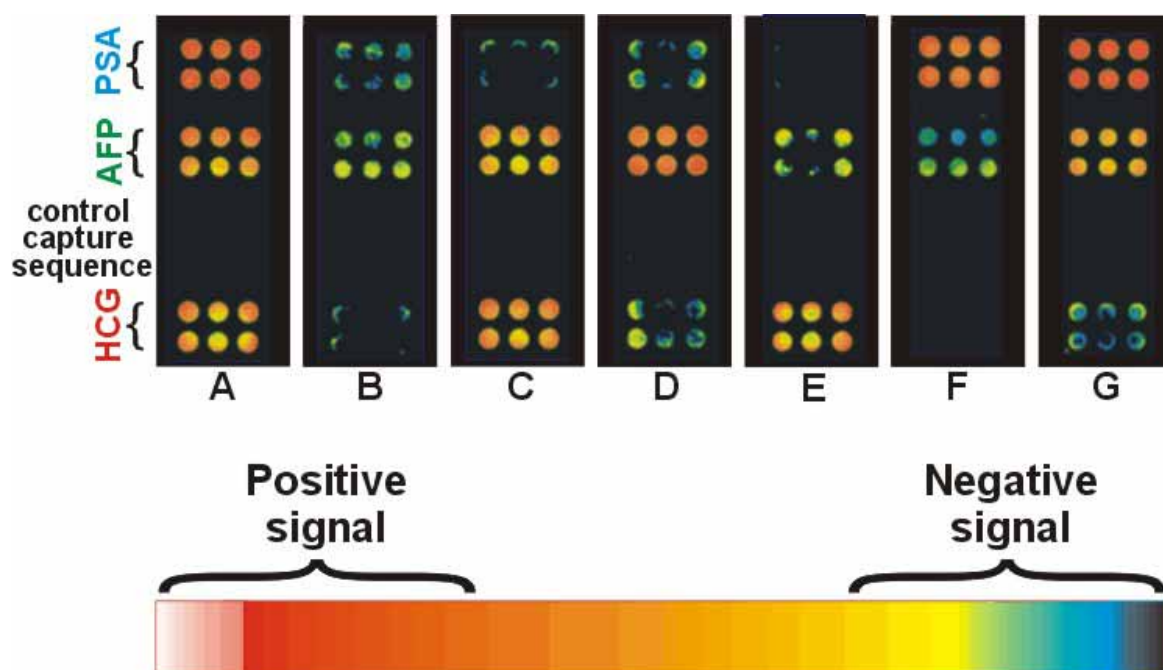


Figure 2S. Scanometric detection of the barcode DNA strands from an assay carried out with goat serum. (A) All targets are present. (B) No target. (C) AFP and HCG. (D) AFP. (E) HCG. (F) PSA. (G) PSA and AFP. The gray scale images from Verigene ID system are converted into colored ones using GenePix Pro 6 software (Molecular Devices).