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Multiple Labeling of Antibodies with Dye/DNA Conjugate for Sensitivity Improvement in Fluorescence Immunoassay

Qin Zhang and Liang-Hong Guo*

State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-environmental Sciences, Chinese Academy of Sciences, P.O. Box 2871, 18 Shuangqing Road, Beijing 100085, China. Received September 17, 2006; Revised Manuscript Received June 27, 2007

Fluorescence immunoassays are widely used in life science research, medical diagnostics, and environmental monitoring due to the intrinsically high specificity, simplicity, and versatility of immunoassays, as well as the availability of a large variety of fluorescent labeling molecules. However, the sensitivity needs to be improved to meet the ever-increasing demand in the new proteomics era. Here, we report a simple method of attaching multiple fluorescent labels on an antibody with a dye/DNA conjugate to increase the immunoassay sensitivity. In the work, mouse IgG adsorbed on the surface of a 96-well plate was detected by its immunoreaction with biotinylated goat anti-mouse antibody. A 30 base pair double-stranded oligonucleotide terminated with biotin was attached to the antibody through the biotin/streptavidin/biotin interaction. Multiple labeling of the antibody was achieved after a fluorescent DNA probe was added into the solution and bound to the oligonucleotide at high ratios. By comparison with fluorescein-labeled streptavidin, the assay with the dye/DNA label produced up to 10-fold increase in fluorescence intensity, and consequently about 10-fold lower detection limit. The multiple labeling method uses readily available reagents, and is simple to implement. Further sensitivity improvement can be obtained by using longer DNAs for antibody labeling, which can incorporate more fluorescent dyes on each DNA.

INTRODUCTION

Immunoassays are widely used in life science research, drug discovery, clinical diagnostics, and environmental monitoring due to their high specificity, simplicity, and versatility. In general, in order to detect and quantify the formed antibody−antigen complex in the immunoassay, a label is attached to the antibody to generate a detectable signal. Since Berson and Yalow (*1*) first introduced radioisotope-labeled immunoassays in 1959, a variety of labels have been developed (*2*), including fluorescent dyes and metal complexes (*3*), enzymes for colorimetric (*4*), chemiluminescent (*5*), and amperometric detection (*6*), and metal chelates for electrochemiluminescent assays (*7*). Among them, fluorescence immunoassay perhaps offers one of the best combinations in terms of label stability, assay robustness, detection sensitivity, and multiplexity. As such, it has become the standard detection platform for protein microarrays (*8*).

However, the sensitivity of the current fluorescence immunoassay technology needs to be improved substantially to meet the demand in the proteomics study. Unlike gene detection in which the nucleic acid target can be amplified by PCR by orders of magnitude, currently there is no amplification technique for proteins. Therefore, sensitivity improvement for protein detection relies on signal amplification. In 1992, Sano et al. established the immuno-PCR method by using nucleic acids as immunoassay labels and amplifying them by PCR after the immunoreaction (*9*). As a result of nucleic acid amplification, the detection limit is improved by 100- to 10 000-fold (*10*). The limitation of immuno-PCR is that it employs gel electrophoresis, PCR-ELISA, or real-time PCR for assay readout, which adds a substantial amount of time and reagent cost. In addition, it is difficult to implement the method in the protein microarray technology.

An alternative approach for signal amplification in fluorescence immunoassay is to increase the number of labels conjugated to each antibody. This can be done by either attaching multiple labels at multiple sites of the antibody with only one label at each site or attaching multiple labels at one site with a macromolecular carrier. Due to the finite size and hydrophobic nature of most fluorescent dyes, the multiple-site approach suffers from reduced immunoreactivity and increased nonspecific binding of the labeled antibody. In the single-site approach, an organic dendrimer (*11*, *12*) or protein loaded with a large number of fluorescent dyes (*13*) is linked covalently or noncovalently (e.g., through biotin−streptavidin interaction) to the antibody at a single site. The single-site linkage minimizes structural alteration of the antibody, thus maintaining its native immunoreactivity. The challenge of this approach is the synthesis and purification of dye-conjugated macromolecules.

More recently, fluorescent micro- and nanometer-sized particles have drawn much attention as a new type of nonisotopic immunoassay labels. Because each particle contains hundreds or even thousands of fluorescent entities, its emission is substantially brighter than the most efficient organic dyes. In 1998, Alivisatos (*14*) and Nie's group (*15*) described separately the application of semiconductor nanocrystals (quantum dots) in biological detections. The new label exhibited such advantageous features as single excitation wavelength, tunable emission wavelength, and improved photostability. Immunoassay sensitivity can also be improved by using fluorophore-doped silica (*16*) and latex beads (*17*). As with the quantum dots, the

* Corresponding author. Phone & Fax: (86)-10-62849685. E-mail: LHGuo@rcees.ac.cn.

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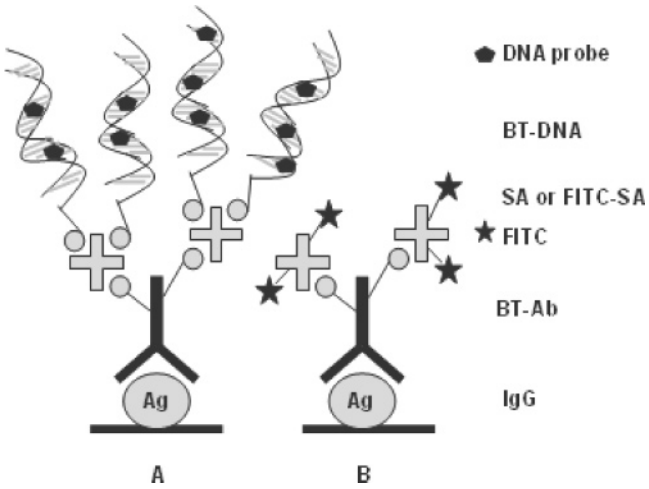
Scheme 1. Illustration of the Two Immunoassay Labeling Formats for the Detection of Goat Anti-Mouse Antibody Using the Conjugate Label (A) and Dye Label (B)[a]



[a] Mouse IgG of various concentrations is coated on a 96-well plate. Biotinylated goat anti-mouse antibody is reacted with mouse IgG. (A) The dye/DNA conjugate with multiple DNA probes is attached to the antibody through the biotin-streptavidin-biotin linkage. (B) The molecular label fluorescein is attached to the antibody by the biotin−streptavidin interaction.

difficulties include the uniformity of the particle, conjugation of biomolecules, dispersion in aqueous solution, and reaction kinetic.

In the work described below, we employed a 30 base pair biotinylated double-stranded oligonucleotide as a carrier for the conjugation of multiple fluorescent dyes. It was attached to a biotin-modified goat anti-mouse antibody through the high-specificity, high-affinity biotin−streptavidin interaction. A highly fluorescent DNA probe, SYBR Green I, binds in situ to the oligonucleotide at high ratios by the specific binding interaction with DNA. Because the free (unbound) probe does not emit fluorescence, measurement can be performed without a wash step. A direct comparison was made with fluorescein-labeled streptavidin in the immunological detection of goat anti-mouse antibody on a mouse IgG-coated 96-well plate (Scheme 1). The dye/DNA conjugate labeled immunocomplex produced up to 10-fold increase in the fluorescence intensity.

EXPERIMENTAL PROCEDURES

Fluorescein isothiocyanate (FITC) was purchased from Amresco (Solon, OH), and SYBR Green I from Invitrogen (Carlsbad, CA). Biotinyl-*N*-hydroxysuccinimide (BT-NHS) was from Sigma (St. Louis, MO). Streptavidin was obtained from Promega (Madison, WI). Bovine serum albumin fraction V (BSA), mouse IgG, and goat anti-mouse IgG were from Xinjingke Corp. (Beijing, China). All buffers were prepared in high-purity water from a Millipore Milli-Q (Biocel) purification system. Unless specified, all antibodies were dissolved in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 0.1% BSA and 0.1% NaN_3.

Biotinylated goat anti-mouse antibody (BT-Ab) was obtained by mixing the antibody with BT-NHS at a molar ratio of 5.6 BT-NHS: 1 antibody in 0.1 M sodium bicarbonate, pH 9.15, for 4 h at room temperature with stirring. After the reaction, the remaining free BT-NHS was separated from the antibody by gel filtration on a D-Salt polyacrylamide desalting column from Pierce (Rockford, IL). The antibody concentration was determined by its absorbance at 280 nm on a UNICO UV-2800 visible spectrophotometer (Shanghai, China). The biotin content was determined with the HABA/avidin reagent from Sigma (St. Louis, MO), and was found to be 1.9 biotins per antibody. Mouse IgG, goat anti-mouse IgG, and streptavidin were labeled with FITC by following the same procedure. The protein and FITC concentration of the labeled biomolecules were determined by absorbance at 280 nm and 495 nm, respectively, and the label ratios were 2.0, 4.0, and 3.5 per protein, respectively. They were stored at 4 °C in the dark until use.

A 30 base pair biotinylated double-stranded oligonucleotide (BT-DNA) was obtained by hybridizing two complementary single-stranded oligonucleotides purchased from Invitrogen (Shanghai, China): strand A, biotin-5′-TTT TTT TTT GCG GGT AAC GTC AAT ATT AAC TTT ACT CCC-3′; strand B, 5′-GGG AGT AAA GTT AAT ATT GAC GTT ACC CGC-3′. The nine thymine nucleotides on strand A are designed to minimize possible spatial hindrance to binding with streptavidin. The two oligonucleotides were mixed in 2 × SSC buffer (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), denatured at 95 °C for 5 min on a Biometra T-Gradient thermocycler (Goettingen, Germany), and then naturally cooled down to room temperature. The concentration of the oligonucleotide was determined by absorbance at 260 nm.

The following is an optimized immunoassay protocol for the detection of mouse IgG using the dye/DNA conjugate labels. First, a white 96-well plate was coated with 100 μL mouse IgG of various concentrations (in 50 mM $NaHCO_3$, pH 9.6, with 0.01% rabbit IgG) by incubating overnight at 4 °C. The plate was washed three times with 300 μL of washing buffer (50 mM Tris, 50 mM NaCl, 0.1% Tween 20, pH 8.0). The plate was then blocked overnight at 4 °C with 300 μL of 1% BSA in PBS, pH 7.4. The plate was washed as above. In the second step, 100 μL of 784 μg/mL BT-Ab in dilution buffer (0.1% BSA, 50 mM Tris, 50 mM NaCl, pH 8.0) was added into the coated well and incubated at 37 °C for 2 h with shaking. The plate was again washed. In the third step, 100 μL of 7.4 μg/mL streptavidin was added into the well and reacted at 37 °C for 2 h with shaking, then washed. In the fourth step, BT-DNA was added and reacted at 37 °C for 1 h with shaking, and washed again. Finally, SYBR Green I was added into the plate and incubated for 10 min at room temperature with shaking. The fluorescence intensity was measured on a Thermo Electron Varioskan Flash luminescence spectrometer (Waltham, MA) with 495 nm excitation, 520 nm emission, 5 nm slit width, and 515 nm cutoff filter. In the dye-labeled immunoassay, fluorescence of fluorescein was measured with 495 nm excitation, 525 nm emission, 5 nm slit width, and 515 nm cutoff filter.

RESULTS AND DISCUSSION

As described above, our strategy is to use a strand of DNA as a carrier to attach multiple fluorescent dyes to an antibody at a single site. The dye/DNA conjugate is not presynthesized, but rather formed in situ as part of the immunoassay. For this strategy to work, there are a few requirements for the fluorescent label. First of all, it must bind to DNA only, not to proteins or immunoglobulins. And the binding stoichiometry is preferably high to ensure a high labeling ratio. Second, the quantum yield of the DNA-bound probe should be comparable to that of the commonly used fluorescent dyes such as fluorescein, Cy-3, Cy-5, and rhodamine, which is in the range 0.1−0.9 (*18*). Third, fluorescence of the unbound probe should be minimal so that the immunoassay can be quantified without wash.

Among the commercially available fluorescent DNA probes, PicoGreen, SYBR Green I, SYBR Gold, and YOYO-1 fulfill the above requirements (*19*). SYBR Green I was selected in our work because its fluorescence excitation and emission maximum wavelengths are close to that of fluorescein, to which the SYBR Green/DNA conjugate is going to be compared. To characterize the binding interaction between SYBR Green and

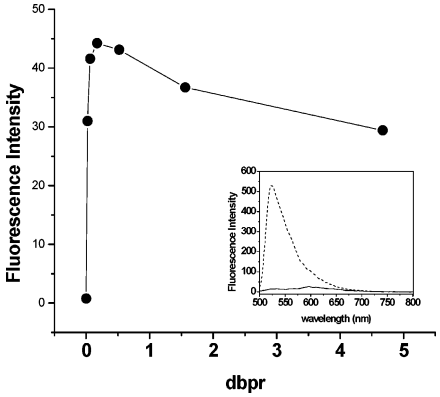


Figure 1. Fluorescence intensity of SYBR Green as a function of the dye to base pair ratio (dbpr). The DNA probe is titrated from 0 to 2.1 μM into a 0.15 μM (base pair) solution of the biotinylated oligonucleotide in 50 mM Tris/50 mM NaCl, pH 8.0. Inset shows the fluorescence emission spectra of the free (solid line) and oligonucleotide-bound dye at 0.02 dbpr (dotted line).

BT-DNA, the probe was titrated into the BT-DNA solution at increasing dye to base pair ratios (dbpr). As illustrated in Figure 1, the fluorescence intensity increased rapidly from 0 to 0.17 dbpr, reached a plateau between 0.17 and 0.52 dbpr, and started to drop at higher ratios. Vitzthum and co-workers have investigated the fluorescent response of SYBR Green I after binding with double-stranded (ds-) and single-stranded (ss-) plasmid DNA (heat-denatured) (*20*). In ds-DNA, the fluorescence increased gradually with the dye concentration until 2 dbpr, after which it became constant. In ss-DNA, however, the response increased more rapidly with the dye concentration, reached the maximum at 0.2 dbpr, and then dropped with further increase of dbpr. The reduction in fluorescence at high dbpr was attributed to self-quenching. The dependence of fluorescence on dbpr with BT-DNA in our work resembles more closely that of ss-DNA in Vitzhum's investigation. This can be explained by the fact there are nine unpaired thymine bases in BT-DNA that bear the characteristics of ss-DNA. Due to the quenching effect, binding constant and stoichiometry between the probe and BT-DNA cannot be determined from the fluorescence titration curve. Cosa et al. revealed (*19*) that the quantum yield of SYBR Green I was 0.69 when bound to double-stranded calf thymus DNA, an increase of more than 1500-fold over the free dye. The drastic enhancement was also observed for the dye/BT-DNA conjugate, as shown in the inset of Figure 1. Furthermore, at the same dye concentration of 20 nM, the emission intensity of SYBR Green/BT-DNA conjugate with 0.22 dbpr is 88% of that of fluorescein-labeled streptavidin (FITC-SA), indicating that the quantum yields of the two fluorescent dyes are similar. All the experimental data obtained above support our notion that the SYBR Green/BT-DNA conjugate can be used effectively to attach multiple fluorescent labels in immunoassays.

After the fluorescence property of the SYBR Green/BT-DNA conjugate was investigated, a 96-well plate based immunoassay for the detection of mouse IgG was carried out. The assay protocol described in the Experimental Section was established after a series of optimization experiments were performed to determine the optimal concentration, reaction time, and temperature for IgG/BT-Ab immunoreaction and biotin/streptavidin binding. The results of the optimization experiments are described in the Supporting Information.

The optimal condition for the binding of BT-DNA/SYBR Green conjugate to the surface-attached IgG/BT-Ab/SA immunocomplex was investigated as follows. The concentrations of the reagents for the formation of the immunocomplex were 30

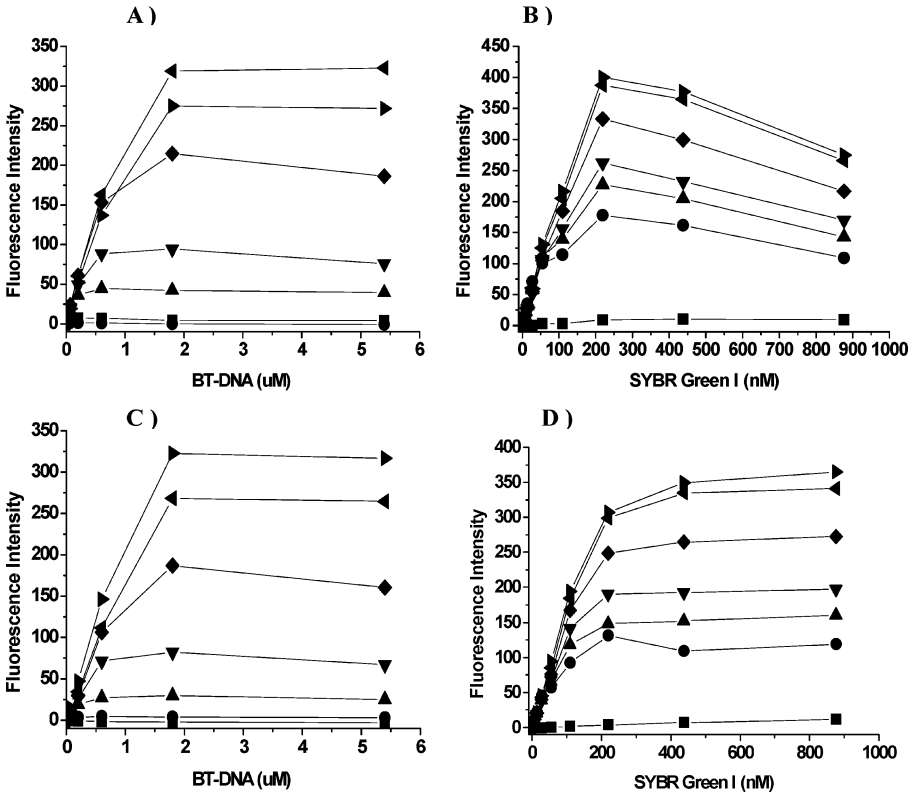


Figure 2. Fluorescence intensity of the SYBR Green/BT-DNA conjugate attached to the IgG/BT-Ab/SA complex formed on a 96-well plate as a function of BT-DNA (A,C) and SYBR Green (B,D) concentration. Different curves in (A) and (C) correspond to 0 nM (●), 14 nM (■), 27 nM (▲), 55 nM (▼), 110 nM (◆), 219 nM (right-facing solid triangle), and 438 nM (left-facing solid triangle) SYBR Green. Different curves in (B) and (D) correspond to 0 μM (■), 0.04 μM (●), 0.12 μM (▲), 0.37 μM (▼), 1.11 μM (◆), 3.33 μM (left-facing solid triangle), and 10 μM (right-facing solid triangle) BT-DNA. Fluorescence was measured before (A,B) and after (C,D) plate wash to remove unbound dyes.

μg/mL IgG for plate coating, 784 μg/mL BT-Ab for immunoreaction, and 7.4 μg/mL (114 nM) SA for BT-Ab binding. BT-DNA of various concentrations was then added to the plate. After binding of BT-DNA to SA on the surface, SYBR Green I was added into the plate from 0 to 876 μM at approximately 2-fold increments for each BT-DNA concentration. Fluorescence intensity was measured, first without wash (in the presence of excessive SYBR Green), then after wash to remove unbound dye molecules. From Figure 2A,C, it is apparent that the binding site for BT-DNA on the plate surface is saturated when the DNA concentration exceeds 1.8 μM. An increase in the dye concentration only enhanced the detection signal, and had no effect on the saturation concentration of BT-DNA. In the unwashed measurement, the light intensity reached its maximum with 219 nM SYBR Green in solution, and decreased at higher concentrations (Figure 2B). This is similar to the trend observed in the solution study of the dye/BT-DNA binding reaction illustrated in Figure 1. The fact that the signal starts to decrease at the same dye concentration but is independent of BT-DNA concentration suggests the reduction is due to quenching by the free dye molecules in solution. This notion is further supported by the results displayed in Figure 2D for the washed measurement. Once excessive SYBR Green molecules were removed from the plate, there was no loss of fluorescence at high dye concentrations. Therefore, a combination of 1.8 μM BT-DNA and 219 nM dye produced the most intense signal in the unwashed measurement, whereas the intensity was slightly lower for the washed assay.

After all the reaction parameters were optimized, a direct comparison was made between the dye label and the dye/DNA conjugate label format in the immunoassay for mouse IgG. The experiment is illustrated in Scheme 1. IgG coating and BT-Ab reaction were performed in the same way for the two labeling formats, with the IgG concentration varied from 0 to 30 μg/mL. In the conjugate-labeled assay, 120 nM unlabeled SA was reacted with BT-Ab on the plate. BT-DNA (1.8 μM) and SYBR Green (219 nM for the nonwashed assay and 438 nM for the washed assay) were then added sequentially to the plate and reacted. The fluorescence was measured first without wash, then with wash. In the dye-labeled assay, 120 nM FITC-SA was reacted with the biotin group on the antibody and its fluorescence measured after wash. A more preferred way for carrying out the single-label assay would be to use a biotin−dye conjugate to bind to the unlabeled SA. Unfortunately, most of the conjugates lose their fluorescence upon binding to streptavidin (*17*), and cannot be used. The results obtained in the comparison experiment are shown in Figure 3. The signal is subtracted by the background, which was found to be caused by the scattered light, not by nonspecific binding of the DNA probe. In both assays, the fluorescence signal is progressively higher with increasing IgG concentration, suggesting that both methods can be used for quantitative detection of immunoassays. However, in the whole range of the IgG concentration examined, the intensity of the SYBR Green/DNA labeled assay is persistently higher than the dye-labeled assay by about 8-fold. The calculated limit of lower detection (3 times the standard deviation above the blank) is 1.7 μg/mL for the dye-labeled assay and 0.14 μg/mL for the dye/DNA conjugate-labeled assay, respectively. Obviously, the dye/DNA multiple labeling strategy improved the detection sensitivity for IgG by 10-fold.

To evaluate the specificity of the assay for mouse IgG, an experiment was carried out in which the surface-adsorbed protein was replaced with either rabbit IgG or BSA, while keeping the protein concentration the same as mouse IgG. The subsequent assay procedure was also the same as for mouse IgG. As illustrated in Figure 4, at a protein concentration of 30 μg/mL, the fluorescence intensity of either rabbit IgG or BSA

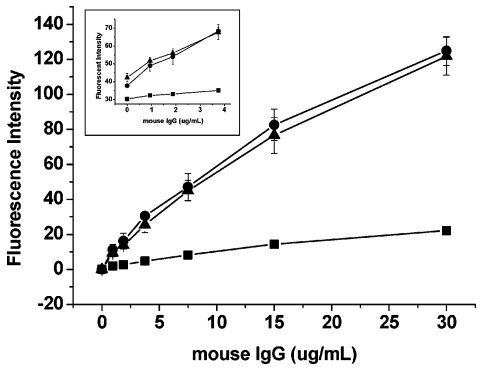


Figure 3. Fluorescence immunoassay for the detection of mouse-IgG on a 96-well plate using the SYBR Green/BT-DNA conjugate label and fluorescein molecular label. Mouse-IgG was dissolved in 50 mM $NaHCO_3$, pH 9.6, with 0.01% rabbit IgG. The concentrations of the reagents used in the assay were 4.9 μM BT-Ab for the immunoreaction, 120 nM FITC-SA (filled square) in the dye-labeled assay, or 120 nM unlabeled SA/1.8 μM BT-DNA/438 nM SYBR Green (filled triangle) in the washed conjugate labeled assay, or 120 nM unlabeled SA/1.8 μM BT-DNA/219 nM SYBR Green (filled circle) in the unwashed conjugate-labeled assay. Inset is an expanded view.

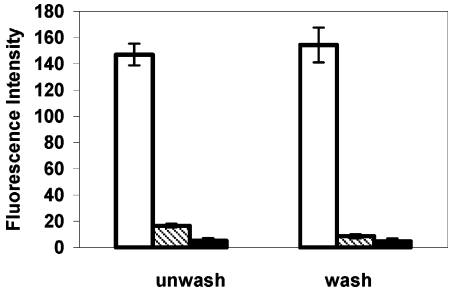


Figure 4. Comparison of fluorescence immunoassay for mouse-IgG (open column), rabbit IgG (hatched column), and BSA (filled column) on a 96-well plate using the SYBR Green/BT-DNA conjugate label. The concentrations of the reagents used in the assay are the same as in Figure 3 for the unwashed (left) and washed (right) assay.

sample is very low by comparison with that of mouse IgG. The result indicates that the immunoassay is highly specific for mouse IgG.

As discussed in the Introduction, although there are a few approaches reported in the literature of employing the multiple-label strategy for signal amplification in immunoassays, none of them can be readily adopted for the sensitive detection of protein microarrays, which is highly sought after due to the low concentration in biological samples and lack of target amplification method. While chemiluminescence has been proven to be a very sensitive detection method, it is very difficult to implement the method on microarrays, since the light-emitting species are not confined to the surface (*22*). Immuno-PCR has been reported to improve the immunoassay sensitivity by 100- to 10 000-fold (*11*), but requires a separate set of assay for DNA quantitation. The covalent labeling approach using organic polymers or proteins as the label carrier faces a severe challenge in chemical synthesis and post-labeling purification. By comparison, the dye/DNA conjugate approach reported in this paper uses all the common reagents such as biotin, streptavidin, oligonucleotides, and fluorescent DNA probes. Multiple labeling of antibodies is achieved in situ by reacting with the oligonucleotide and DNA probe on a 96-well plate. There is even no need to remove the unbound dyes before measurement. If one is concerned about the assay time, the BT-Ab/SA/BT-DNA can be premixed at a controlled ratio to save time. Furthermore, the signal amplification efficiency can potentially be enhanced substantially by using longer DNAs to carry more fluorescent

dyes. DNA markers of 150 to 300 base pairs are commonly used in immuno-PCR (*23*). By simple extrapolation, such a long DNA sequence would increase the fluorescent signal by 50−100 fold over the dye-labeled assays, and probably with similar improvement in detection sensitivity. The improved sensitivity would be very attractive for protein microarray detection.

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Supporting Information Available: Details of the experiments in determining the optimal concentration, reaction time, and temperature for IgG/BT-Ab immunoreaction and biotin/streptavidin binding. This material is available free of charge via the Internet at http://pubs.acs.org.

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