



Immuno-PCR: Very Sensitive Antigen Detection by Means of Specific Antibody- DNA Conjugates

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ing ΔSO_4^{2-} . Cloud processing is relatively less efficient in enhancing scattering for large ΔSO_4^{2-} , in part because for perturbations through gas-phase conversion only, α increases with ΔSO_4^{2-} (Table 1). In fact, the ratio $\alpha(\text{cloud})/\alpha(\text{gas phase})$ approximates unity for large values of ΔSO_4^{2-} (Fig. 3). Nevertheless, considering the calculated average sulfate pollution of about 0.5 to 2 $\mu\text{g m}^{-3}$ in the lower atmosphere in a large part of the Northern Hemisphere (Fig. 2), we conclude that the mean climate forcing by sulfate in this part of the globe may be about -0.5 to -1.0 W m^{-2} , to a large extent caused by in-cloud oxidation of anthropogenic SO_2 .

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Immuno-PCR: Very Sensitive Antigen Detection by Means of Specific Antibody-DNA Conjugates

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An antigen detection system, termed immuno-polymerase chain reaction (immuno-PCR), was developed in which a specific DNA molecule is used as the marker. A streptavidin-protein A chimera that possesses tight and specific binding affinity both for biotin and immunoglobulin G was used to attach a biotinylated DNA specifically to antigen-mono-clonal antibody complexes that had been immobilized on microtiter plate wells. Then, a segment of the attached DNA was amplified by PCR. Analysis of the PCR products by agarose gel electrophoresis after staining with ethidium bromide allowed as few as 580 antigen molecules (9.6×10^{-22} moles) to be readily and reproducibly detected. Direct comparison with enzyme-linked immunosorbent assay with the use of a chimera-alkaline phosphatase conjugate demonstrates that enhancement (approximately $\times 10^5$) in detection sensitivity was obtained with the use of immuno-PCR. Given the enormous amplification capability and specificity of PCR, this immuno-PCR technology has a sensitivity greater than any existing antigen detection system and, in principle, could be applied to the detection of single antigen molecules.

Antibody-based detection systems for specific antigens are a versatile and powerful tool for various molecular and cellular analyses and clinical diagnostics. The power of such systems originates from the considerable specificity of antibodies for their particular epitopes. A number of recent antibody technologies, including genetic engineering of antibody molecules (1) and the production of catalytic antibodies (2) and bispecific antibodies (3), are allowing a rapid expansion in the applications of antibodies. We were interested in further enhancing the sensitivity of antigen detection systems. This should facilitate the specific detection of rare antigens, which are pre-

sent only in very small numbers, and thus could expand the application of antibodies to a wider variety of biological and nonbiological systems.

Polymerase chain reaction (PCR) technology (4) has become a powerful tool in molecular biology and genetic engineering (5). The efficacy of PCR is based on its ability to amplify a specific DNA segment flanked by a set of primers. The enormous amplification capability of PCR allows the production of large amounts of specific DNA products, which can be detected by various methods. The extremely high specificity of PCR for a target sequence defined by a set of primers should avoid the generation of false signals from other nucleic acid molecules present in samples. We reasoned that the capability of antigen detection systems could be considerably

enhanced and potentially broadened by coupling to PCR. Following these ideas, we have developed an antigen detection system, termed immuno-PCR, in which a specific antibody-DNA conjugate is used to detect antigens.

In immuno-PCR, a linker molecule with bispecific binding affinity for DNA and antibodies is used to attach a DNA molecule (marker) specifically to an antigen-antibody complex, resulting in the formation of a specific antigen-antibody-DNA conjugate. The attached marker DNA can be amplified by PCR with the appropriate primers. The presence of specific PCR products demonstrates that marker DNA molecules are attached specifically to antigen-antibody complexes, which indicates the presence of antigen. A streptavidin-protein A chimera that we recently designed (6) was used as the linker. The chimera has two independent specific binding abilities; one is to biotin, derived from the streptavidin moiety, and the other is to the Fc portion of an immunoglobulin G (IgG) molecule, derived from the protein A moiety. This bifunctional specificity both for biotin and antibody allows the specific conjugation of any biotinylated DNA molecule to antigen-antibody complexes.

To test the feasibility of this concept, we immobilized various amounts of an antigen on the surface of microtiter plate wells and detected them by immuno-PCR. Bovine serum albumin (BSA) was used as the antigen because of the availability of pure protein and monoclonal antibodies against it. The detection procedure used (7) is similar to conventional enzyme-linked immunosorbent assay (ELISA). Instead of an enzyme-conjugated secondary antibody directed against the primary antibody, as in

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typical ELISA, a biotinylated linear plasmid DNA (pUC19) (8) conjugated to the streptavidin-protein A chimera (9, 10) was targeted to the antigen-antibody complexes. A segment of the attached marker DNA was amplified by PCR with appropriate primers (11), and the resulting PCR products were analyzed by agarose gel electrophoresis after staining with ethidium bromide (Fig. 1).

A specific 260-bp PCR product was observed in all the lanes that contained immobilized BSA (Fig. 1A), which indi-

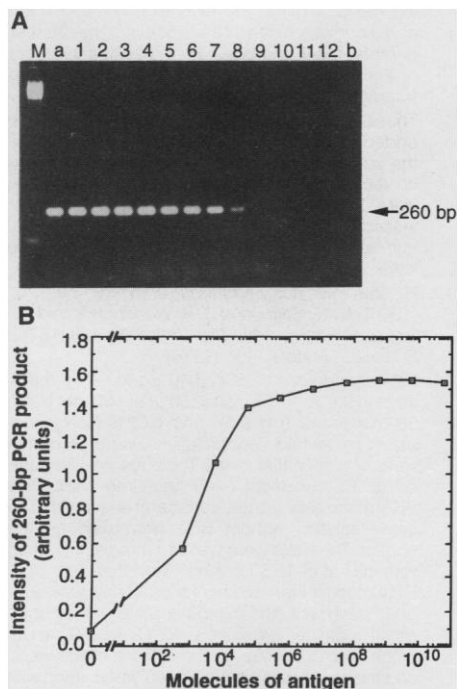


Fig. 1. Detection of BSA immobilized on a microtiter plate by immuno-PCR. **(A)** Analysis of PCR products by agarose gel electrophoresis. A PCR amplification reaction mixture (15 μ l) (7, 11) was separated on 2.0% agarose gels, and the DNA was stained with ethidium bromide. Lane M, 123-bp ladder (BRL); lane a, positive control for PCR with 2 ng of biotinylated pUC19 (8) added before PCR amplification; lane b, negative control for PCR; lanes 10 through 12, control (TBS used without BSA in the initial immobilization step). Lanes 1 through 9 contain PCR amplification reaction mixtures with immobilized antigen: lane 1, 96 fmol; lane 2, 9.6 fmol; lane 3, 960 amol; lane 4, 96 amol; lane 5, 9.6 amol; lane 6, 0.96 amol; lane 7, 9.6×10^{-20} mol; lane 8, 9.6×10^{-21} mol; and lane 9, 9.6×10^{-22} mol. Each lane contained 5.8×10^{10} , 5.8×10^9 , 5.8×10^8 , 5.8×10^7 , 5.8×10^6 , 5.8×10^5 , 5.8×10^4 , 5.8×10^3 , and 5.8×10^2 molecules, respectively (12). **(B)** Quantitation of the 260-bp PCR product. The agarose gel shown in (A) was photographed with Polaroid 665 film, and the negatives were scanned with the use of a densitometer (2202 Ultrascan laser densitometer, Pharmacia-LKB). The intensity of the 260-bp band, represented in arbitrary units, is plotted as a function of the molecules of antigen.

cates that the biotinylated pUC19 was specifically attached to the antigen-mono-clonal antibody complexes by the chimera. In contrast, almost no 260-bp fragment was observed in lanes 10 through 12, which came from wells without immobilized antigen. Quantitation of the 260-bp PCR product (Fig. 1B) demonstrates that background PCR signals generated by nonspecific binding of the antibody or the chimera-pUC19 conjugate were sufficiently small to allow clear discrimination of positive signals from background. This also indicates that the specificity of PCR amplification is high enough to avoid the generation of false signals from other DNA molecules present in the wells. Because the sequences of a marker DNA and its amplified segment are purely arbitrary, they can be changed frequently, if needed, to prevent deterioration of signal-to-noise ratios caused by contamination.

Fig. 2. Detection of BSA (14) immobilized on a microtiter plate by ELISA with the use of antibody against BSA and a chimera-biotinylated alkaline phosphatase conjugate. The amounts of immobilized BSA in wells 1 through 11 were as follows: well 1, 9.6 nmol; well 2, 960 pmol; well 3, 96 pmol; well 4, 9.6 pmol; well 5, 960 fmol; well 6, 96 fmol; well 7, 9.6 fmol; well 8, 960 amol; well 9, 96 amol; well 10, 9.6 amol; and well 11, 9.6×10^{-19} mol. Each well contained 5.8×10^{15} , 5.8×10^{14} , 5.8×10^{13} , 5.8×10^{12} , 5.8×10^{11} , 5.8×10^{10} , 5.8×10^9 , 5.8×10^8 , 5.8×10^7 , 5.8×10^6 , and 5.8×10^5 molecules, respectively (12). Well 12 is the control, where TBS without BSA was used in the initial immobilization step. When *p*-nitrophenyl phosphate was used as the substrate, color development was observed at 96 amol (5.8×10^7 molecules) (12) or more of immobilized BSA.

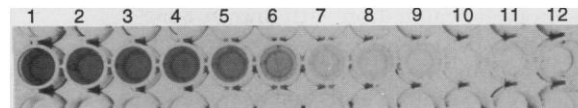
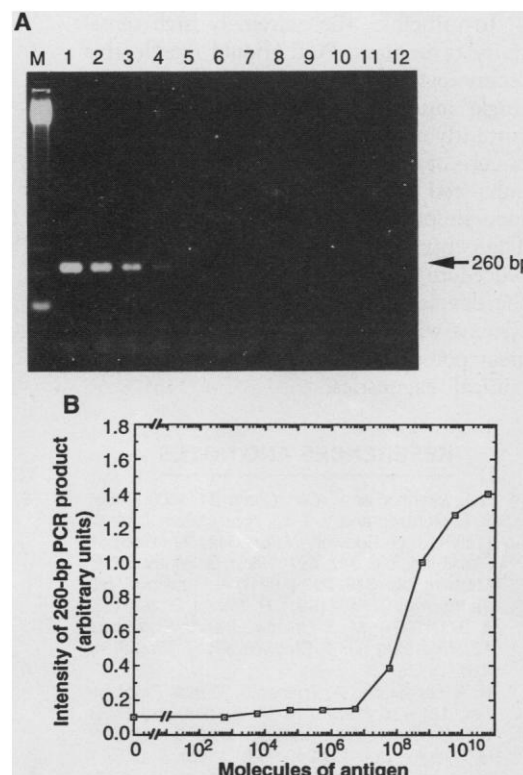


Fig. 3. Effect of a reduced concentration of the chimera-pUC19 conjugate on the sensitivity of immuno-PCR. **(A)** Analysis of PCR products by agarose gel electrophoresis. A lower concentration (1.4×10^{-17} mol/50 μ l) of pUC19 conjugated to the chimera was applied to the wells, whereas all the other conditions remained the same as those in (7, 11). Each lane contains a PCR amplification mixture derived from a well that contained the same amount of immobilized BSA as in Fig. 1A: lane 1, 96 fmol; lane 2, 9.6 fmol; lane 3, 960 amol; lane 4, 96 amol; lane 5, 9.6 amol; lane 6, 0.96 amol; lane 7, 9.6×10^{-20} mol; lane 8, 9.6×10^{-21} mol; and lane 9, 9.6×10^{-22} mol. Each lane contained 5.8×10^{10} , 5.8×10^9 , 5.8×10^8 , 5.8×10^7 , 5.8×10^6 , 5.8×10^5 , 5.8×10^4 , 5.8×10^3 , and 5.8×10^2 molecules, respectively (12). Lanes 10 through 12, control (TBS used without BSA in the initial immobilization step); M, 123-bp ladder as in Fig. 1A. **(B)** Quantitation of the 260-bp PCR product shown in (A). The procedures were the same as in Fig. 1B. The PCR amplification was saturated at around 9.6 fmol (5.8×10^9 molecules) (12) of immobilized BSA.



PCR products. The sensitivity and versatility could be enhanced considerably with the use of better detection methods for PCR products. For example, direct incorporation of a label, such as radioisotopes, fluorochromes, and enzymes, into PCR products with the use of label-conjugated primers or nucleotides allows simple analytical formats. Alternatively, gel electrophoresis could be used to detect many different antigen molecules simultaneously, each of which is labeled with a different size marker DNA.

The amount of the 260-bp fragment decreased with decreasing amounts of immobilized antigen from lanes 6 to 9 (Fig. 1A), which demonstrates that the PCR amplification was not saturated below 0.96 amol of BSA. For wells that contained more antigen (lanes 1 through 5), the PCR amplification was saturated. In principle, quantitation of PCR products below saturation should provide an estimate of the number of antigens (epitopes) after appropriate calibration with known numbers of antigen molecules. When more dilute chimera-pUC19 conjugates were used, saturation of PCR amplification occurred with larger amounts of the immobilized antigen (Fig. 3). Thus, one can control the sensitivity of the system by varying the concentration of the conjugate. Other key factors, such as the concentration of antibody, the number of PCR amplification cycles, and the detection method for PCR products, can also be used to control the overall sensitivity of the system.

In principle, the extremely high sensitivity of immuno-PCR should enable this technology to be applied to the detection of single antigen molecules; no method is currently available for this. The sensitivity of current antigen detection systems can be enhanced by at least a few orders of magnitude simply by the introduction of PCR. The controllable sensitivity and the simple procedure of immuno-PCR should allow the development of fully automated assay systems without loss in sensitivity, with a great potential promise for applications in clinical diagnostics.

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- Various amounts (6.4 ng to 6.4 ag; 9.6×10^{-14} to 9.6×10^{-22} mol) of BSA in 45 μ l of 150 mM NaCl, 20 mM tris-Cl (pH 9.5), and 0.02% Na₂S₂O₃, prepared by tenfold serial dilutions, were placed in wells of a microtiter plate (Falcon 3911; Becton Dickinson). The microtiter plate was incubated at 4°C overnight (~15 hours) to immobilize BSA molecules on the surface of the wells. The same solution without BSA was used as the control. The wells were washed three times, each for 5 min with 250 μ l of tris-buffered saline [TBS; 150 mM NaCl, 20 mM tris-Cl (pH 7.5), and 0.02% Na₂S₂O₃]. Then, 200 μ l of ETBS (TBS plus 0.1 mM EDTA) that contained 4.5% nonfat dried milk and denatured salmon sperm DNA (1 mg/ml) was added to each well. The microtiter plate was incubated at 37°C for 80 min to block reactable sites on the surface of the wells to avoid nonspecific binding in subsequent steps, and then the wells were washed seven times, each with 150 μ l of TETBS (TBS plus 0.1 mM EDTA and 0.1% Tween 20) for 5 min. To each well, 50 μ l of TETBS containing 0.45% nonfat dried milk, denatured salmon sperm DNA (0.1 mg/ml), and diluted (8000-fold) monoclonal antibody against BSA (mouse ascites fluid, IgG2a, clone BSA-33; Sigma) was added. The microtiter plate was incubated at room temperature for 45 min to allow the antibody to bind to immobilized BSA molecules. The wells were washed 15 times, each with 250 μ l of TETBS for 10 min, to remove unbound antibody molecules, and 50 μ l of TETBS containing 0.45% nonfat dried milk, denatured salmon sperm DNA (0.1 mg/ml), and 1.4×10^{-16} mol of biotinylated pUC19 conjugated to the streptavidin-protein A chimera (8-10) was added to each well. The microtiter plate was incubated at room temperature for 50 min to allow the chimera-pUC19 conjugates to bind to the antigen-antibody complexes, and then the wells were washed 15 times, each with 250 μ l of TETBS for 10 min, to remove unbound conjugates. The wells were washed three times with TBS without Na₂S₂O₃, and the microtiter plate was subjected to PCR (11). After the PCR amplification, each reaction mixture was analyzed by agarose gel electrophoresis.
- The biotinylated pUC19 used was a linear 2.67-kb Hind III-Acc I fragment, in which one biotin molecule had been incorporated at its Hind III terminus by a filling-in reaction with Sequenase version 2.0 (U.S. Biochemical) in the presence of a biotinylated nucleotide (biotin-14-deoxyadenosine triphosphate; BRL). By gel retardation, dependent on streptavidin binding, almost 100% of the 2.67-kb fragment contained biotin.
- A streptavidin-protein A chimera was expressed in *Escherichia coli* by means of the expression vector pTSA-2 and purified to homogeneity (6). The purified chimera was stored frozen at -70°C in 150 mM NaCl, 20 mM tris-Cl (pH 7.5), 0.02% Na₂S₂O₃, and 6% sucrose. No appreciable changes in the biotin- and the IgG-binding ability were observed upon frozen storage.
- We prepared the chimera-pUC19 conjugate by mixing the purified chimera (9) and the biotinylated pUC19 (8) at a molar ratio of biotin to biotin binding site of 1. The resulting conjugates contain four biotinylated pUC19 per chimera, which possesses four biotin binding sites (6).
- PCR was carried out under the following conditions: 50 mM KCl, 10 mM tris-Cl (pH 8.3 at 20°C), 1.5 mM MgCl₂, gelatin (10 μ g/ml), 0.8 mM deoxyribonucleoside triphosphates (dNTPs) (0.2 mM each), 2 μ M primers (bla-1 and bla-2, 1 μ M each), and Taq DNA polymerase (50 unit/ml) (Boehringer Mannheim). Pre-PCR mixtures sterilized by ultraviolet (UV) irradiation at 254 nm [G. Sarkar and S. S. Sommer, *Nature* **343**, 27 (1990); G. D. Cimino, K. Metchette, S. T. Isaacs, Y. S. Zhu, *ibid.* **345**, 773 (1990)] were added to the wells of a microtiter plate (40 μ l per well), and mineral oil sterilized by UV irradiation was layered (20 μ l per well) on the reaction mixture. PCR was performed with an automated thermal cycler (PTC-100-96 Thermal Cycler, MJ Research, Inc.), with the use of the following temperature profile: initial denaturation, 94°C, 5 min; 30 cycles of denaturation (94°C, 1 min), annealing (58°C, 1 min), and extension (72°C, 1 min); and final extension, 72°C, 5 min. The 30-mer primers, bla-1 and bla-2, hybridize to a segment of the *bla* gene and should generate a 261-bp fragment upon PCR amplification.
- These numbers indicate the amounts of antigen added to each well. However, it is unlikely that all the added antigen molecules were immobilized on the wells. Furthermore, some molecules that were initially immobilized may have been released in subsequent steps. Therefore, the actual number of antigens in each well is very likely to be lower than indicated.
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- Various amounts of BSA (640 μ g to 64 fg; 9.6×10^{-9} to 9.6×10^{-19} mol) in 50 μ l of 150 mM NaCl, 20 mM tris-Cl (pH 9.5), and 0.02% Na₂S₂O₃, prepared by tenfold serial dilutions, were placed in wells of a microtiter plate. The plate was incubated at 4°C overnight (~15 hours) to immobilize BSA molecules on the surface of the wells. The same solution without BSA was used as the control. The wells were washed three times, each with 100 μ l of TBS for 5 min, and then 200 μ l of ETBS containing 4.5% nonfat dried milk was added to each well. The microtiter plate was incubated at room temperature (~22°C) for 60 min to block reactable sites on the surface of the wells, and the wells were washed three times, each with 200 μ l of TETBS (TBS plus 0.1 mM EDTA and 0.04% Tween 20) for 5 min. To each well, 75 μ l of TETBS containing 0.45% nonfat dried milk and diluted (500-fold) monoclonal antibody against BSA (7) was added. The microtiter plate was incubated at room temperature for 60 min to allow the antibody to bind to immobilized BSA molecules. The wells were washed six times, each with 200 μ l of TETBS for 5 to 10 min, to remove unbound antibody, and 70 μ l of TETBS containing 0.45% nonfat dried milk and 6 pmol of biotinylated alkaline phosphatase (Boehringer Mannheim) conjugated to the streptavidin-protein A chimera (9) was added to each well. The microtiter plate was incubated at room temperature for 60 min to allow the chimera-alkaline phosphatase conjugate to bind to the antigen-antibody complexes, and then the wells were washed six times, each with 200 μ l of TETBS for 5 to 10 min. The wells were washed once with 200 μ l of TBS without Na₂S₂O₃ and then with 200 μ l of 1 M diethanolamine (pH 9.8) and 0.5 mM MgCl₂. To each well, 200 μ l of 1 M diethanolamine (pH 9.8) and 0.5 mM MgCl₂ containing 10 mM *p*-nitrophenyl phosphate was added, and a color development reaction was performed at 37°C for 60 min.
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