

Colorimetric Bio-Barcode Amplification Assay for Cytokines

Jwa-Min Nam, Amber R. Wise, and Jay T. Groves*

Department of Chemistry, University of California, Berkeley, California 94720 and Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720

The bio-barcode amplification assay has become a powerful tool in detecting tens to hundreds of biological targets such as proteins and nucleic acids in the entire sample. However, current bio-barcode detection schemes still require many experimental steps including microarrayer-based immobilization of oligonucleotides on a glass chip, silver enhancement of immobilized gold nanoparticles on a chip, and light-scattering measurement. Here, we report a colorimetric bio-barcode method that minimizes the above requirements while detecting 30 aM concentrations of cytokines (~3 orders of magnitude more sensitive than conventional nonenzymatic cytokine detection assays). The assay is based on porous microparticles, which enable loading of a large number of barcode DNA per particle, and gold nanoparticle-based colorimetric barcode detection method.

Numerous high-sensitivity biomolecule detection methods have been developed,^{1–12} but few have achieved the sensitivity of the polymerase chain reaction (PCR).^{13,14} The bio-barcode amplification assay is the only biodetection method that has the PCR-like sensitivity for both protein and nucleic acid targets without a need for enzymatic amplification.^{12,15–18} However, current bio-barcode detection schemes still require microarrayer-based immobilization

of oligonucleotide on a glass chip, surface passivation chemistry to minimize nonspecific binding, silver enhancement of immobilized gold nanoparticles on a chip, light-scattering measurement, and a quantification step. Importantly, sophisticated instruments such as microarrayers and chip-imaging tools limit portability, and the assay cost is bound to be expensive. It would be beneficial if one can obviate or minimize the above requirements without sacrificing attomolar sensitivity of the bio-barcode assay.

Herein, we report an ultrasensitive colorimetric bio-barcode amplification method that relies on porous silica beads and the gold nanoparticle-based colorimetric DNA detection scheme^{19,20} for straightforward readout (Figure 1). This ultrasensitive colorimetric bio-barcode assay is possible because the porous microparticle accommodates millions of barcode DNA per particle, thus allowing the use of a colorimetric barcode DNA detection scheme (typical detection limit of gold nanoparticle-based colorimetric detection method is approximately nanomolar). This is an important advance because this scheme has the attomolar (10^{-18} M) sensitivity of the bio-barcode amplification method as well as the simplicity, portability, and low cost of gold nanoparticle-based colorimetric detection methods. Moreover, a graphic processing method to quantify the spotted gold nanoparticle aggregates on a thin-layer chromatography (TLC) plate has been developed herein (Figure 2).

In this work, our assay target is interleukin-2 (IL-2). IL-2 is a secreted human cytokine protein that mediates local interactions between white blood cells during inflammation and immune responses. Cytokines play a central role in the regulation of hematopoiesis, mediating the differentiation, migration, activation, and proliferation of phenotypically diverse cells.^{21,22} Improved detection limits of cytokines will allow for earlier and more accurate diagnosis and treatments of cancers and immunodeficiency-related diseases and lead to an increased understanding of cytokine-related diseases and biology,²³ because cytokines are signature biomarkers when humans are infected by foreign

* Corresponding author. E-mail: JTGroves@lbl.gov.

- (1) Nicewarner-Pena, S. R.; Freeman, R. G.; Reiss, B. D.; He, L.; Peña, D. J.; Walton, I. D.; Cromer, R.; Keating, C. D.; Natan, M. J. *Science* **2001**, *294*, 137–141.
- (2) Han, M. Y.; Gao, X.; Nie, S. *Nat. Biotechnol.* **2001**, *19*, 631–635.
- (3) Taton, T. A.; Mirkin, C. A.; Letsinger, R. L. *Science* **2000**, *289*, 1757–1760.
- (4) Cao, Y. W. C.; Jin, R.; Mirkin, C. A. *Science* **2002**, *297*, 1536–1540.
- (5) Niemeyer, C. M.; Ceyhan, B. *Angew. Chem., Int. Ed.* **2001**, *40*, 3685–3688.
- (6) Cui, Y.; Wei, Q.; Park, H.; Lieber, C. M. *Science* **2001**, *293*, 1289–1292.
- (7) Gearheart, L.; Ploehn, H. J.; Murphy, C. J. *J. Phys. Chem. B* **2001**, *105*, 12609–12615.
- (8) Bayley, H.; Martin, C. R. *Chem. Rev.* **2000**, *100*, 2575–2594.
- (9) Marinakos, S. M.; Anderson, M. F.; Ryan, J. A.; Martin, L. D.; Feldheim, D. L. *J. Phys. Chem. B* **2001**, *105*, 8872–8876.
- (10) Zhao, X.; Tapecc-Dytioco, R.; Tan, W. *J. Am. Chem. Soc.* **2003**, *125*, 11474–11475.
- (11) Wang, J.; Liu, G.; Munge, B.; Lin, L.; Zhu, Q. *Angew. Chem., Int. Ed.* **2004**, *43*, 2158–2161.
- (12) Rosi, N.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547–1562.
- (13) Saiki, R. K.; Scharf, S.; Faloona, F.; Mullis, K. B.; Horn, G. T.; Erlich, H. A.; Arnheim, N. *Science* **1985**, *230*, 1350–1354.
- (14) Sano, T.; Smith, C. L.; Cantor, C. R. *Science* **1992**, *258*, 120–122.
- (15) Nam, J.-M.; Park, S.-J.; Mirkin, C. A. *J. Am. Chem. Soc.* **2002**, *124*, 3820–3821.
- (16) Nam, J.-M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301*, 1884–1886.
- (17) Nam, J.-M.; Stoeva, S. I.; Mirkin, C. A. *J. Am. Chem. Soc.* **2004**, *126*, 5932–5933.

- (18) Georganopoulou, D.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 2273–2276.
- (19) Elghanian, R.; Storhoff, J. J.; Mucic, R. C.; Letsinger, R. L.; Mirkin, C. A. *Science* **1997**, *277*, 1078–1081.
- (20) Storhoff, J. J.; Elghanian, R.; Mucic, R. C.; Mirkin, C. A.; Letsinger, R. L. *J. Am. Chem. Soc.* **1998**, *120*, 1959–1964.
- (21) Ferrua, B.; Aussel, C.; Fehlmann, M. *J. Immunol. Methods* **1987**, *97*, 215–220.
- (22) Carson, R. T.; Vignali, D. A. *J. Immunol. Methods* **1999**, *227*, 41–52.
- (23) Janeway, C. A.; Travers, P.; Walport, M.; Capra, J. D. *Immunobiology: the immune system in health and disease*, 6th ed.; Garland Science Publishing: New York, 2005.

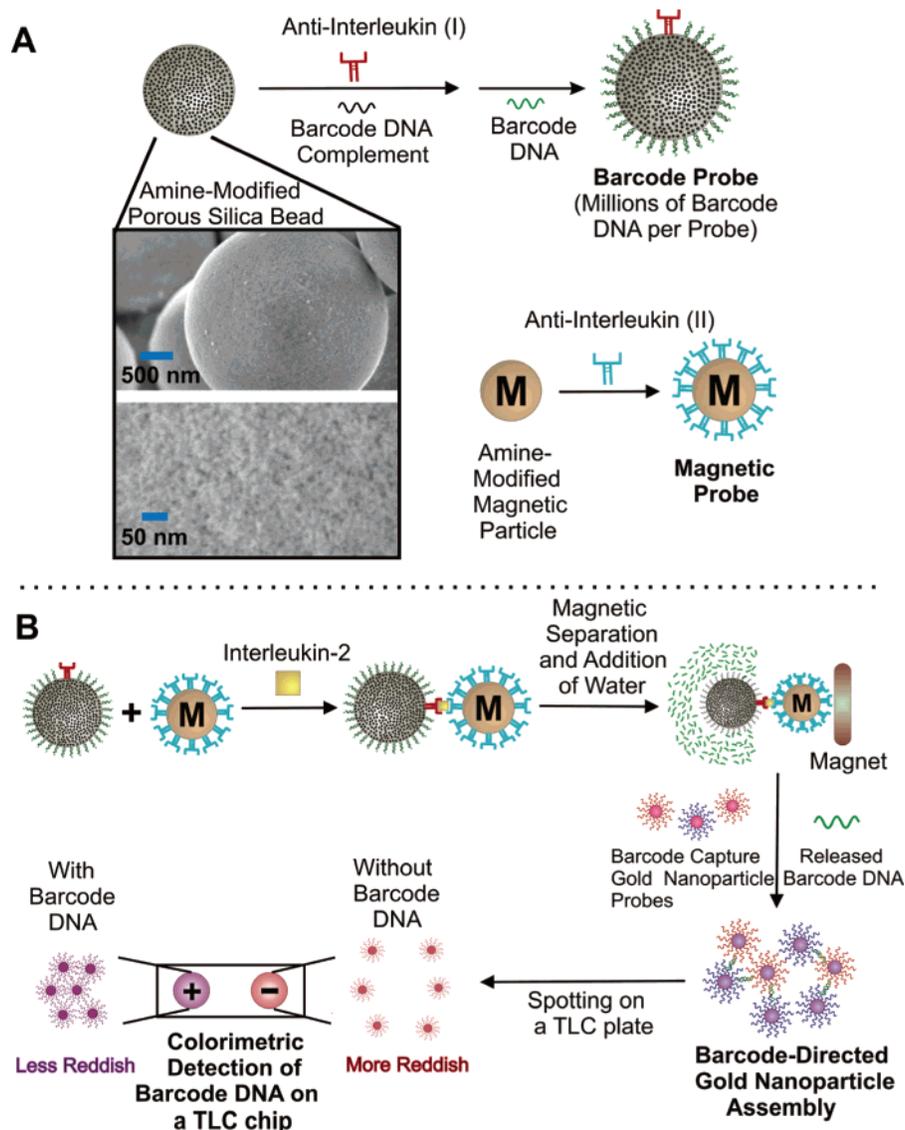


Figure 1. Colorimetric bio-barcode assay. (A) Probe preparation and electron micrograph images of amine-modified porous silica beads (inset). (B) Interleukin-2 detection scheme.

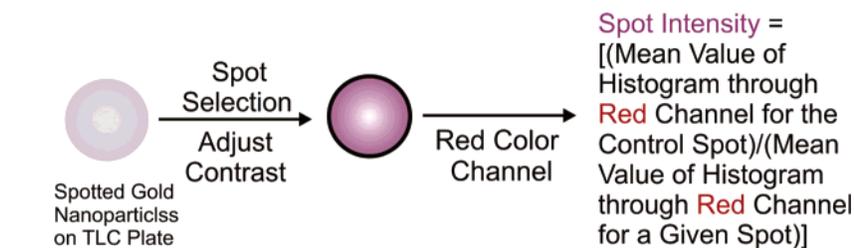


Figure 2. Quantification method for gold nanoparticle aggregates spotted on a TLC plate. Spot intensity value is proportional to the number of barcode DNA (the more gold nanoparticles aggregated, the less red color appeared) and the number of barcode DNA is proportional to the amount of target proteins present.

antigens. Conventional cytokine detection assays have a detection limit of ~ 50 fM, and the detection limit of enzyme-based rolling-circle amplification method is ~ 500 aM.^{21,22,24–26}

(24) Multenix, M. C.; et al. *Clin. Chem.* **2002**, *48*, 1855–1858.
 (25) Schweitzer, B.; Roberts, S.; Grimwade, B.; Shao, W.; Wang, M.; Fu, Q.; Shu, Q.; Laroche, I.; Zhou, Z.; Tchernev, V. Y.; Christiansen, J.; Velleca, M.; Kingsmore, S. F. *Nat. Biotechnol.* **2002**, *20*, 359–365.
 (26) Kingsmore, S.; Patel, D. D., *Curr. Opin. Biotechnol.* **2003**, *14*, 74–81.

EXPERIMENTAL SECTION

Electron Micrographs. The LEO 1550 scanning electron microscope at UC Berkeley Microlab facility was used. The images were taken using 3-kV acceleration voltage at a working distance of 3 mm after vapor deposition of ~ 3 -nm chromium onto the sample.

Barcode Probe Preparation. To prepare the barcode probes, 1 mL of an aqueous suspension of the amino-functionalized porous

silica microparticles (1.57×10^9 mL⁻¹; diameter 3.53 ± 0.49 μ m; obtained from Phenomenex, Torrance, CA) was centrifuged for 5 min at 10 000 rpm, and the supernatant was removed. The particles were resuspended in PBS solution, and the centrifugation step was repeated once more. The resulting polystyrene particle pellet was resuspended in 1 mL of 8% glutaraldehyde in PBS solution at pH 7.4. The solution was mixed for 5 h on a rocking shaker. Centrifugation followed for 5 min at 10 000 rpm, and the supernatant was discarded (this step was repeated two more times). The resulting pellet was resuspended in PBS, and 5 μ g of monoclonal antibody for IL-2 was added to the solution. The amount of antibody (5 μ g) is much less than the amount of antibody recommended by Polysciences, Inc. to fully modify the particle surface (antibodies were purchased from Abcam, Inc., Cambridge, MA). The solution was left on a shaker overnight to link the anti-IL-2 to the activated polystyrene particles. Analogous glutaraldehyde linker chemistry has been extensively used by others to effect protein linking to amino-functionalized particles. 3'-Amino-functionalized barcode DNA complements (1 mL at 100 μ M; 5' CGTCGCATTCAGGATTCTCAACTCGTAGCT-A₁₀-C6-amine 3') were then added to the monoclonal antibody-modified silica particles, and the centrifugation step was repeated twice. The resulting pellet was resuspended in 1 mL of 0.2 M ethanol-amine to passivate all unreacted glutaraldehyde sites on the microparticles for 30 min at room temperature. Centrifugation was performed to remove supernatant. A 10% bovine serum albumin (BSA) solution was subsequently added to further passivate the protein-inactive regions of the particle surface. The centrifugation step was repeated twice, and the supernatant was removed. The resulting pellet was resuspended in 1 mL of 0.15 M PBS solution.

Magnetic Probe Preparation. Amino-functionalized magnetic particles (DynaL Biotech, Brown Deer, WI) were linked to monoclonal antibodies for IL-2. The epitopes of these antibodies are different from those of the antibodies used to prepare the barcode probes (Abcam, Cambridge, MA) using glutaraldehyde-amine coupling chemistry. Amino-functionalized magnetic particles in 0.05 mM EDTA solution (5 mL of solution at 1 mg/mL) were washed with 10 mL of pyridine wash buffer. The resulting solution was magnetically separated, and the supernatant was removed (repeated two more times). The magnetic particles were then activated with 5 mL of 5% glutaraldehyde in pyridine wash buffer for 3 h at room temperature. The activated magnetic particles were then magnetically separated, and the supernatant was removed. This magnetic separation step was repeated twice, and the magnetic particles were resuspended in 10 mL of pyridine wash buffer. The monoclonal anti-IL-2 in pyridine wash buffer (1 mL at 750 μ g/mL) was then added to magnetic particles, and the solution was mixed for 10 h at room temperature. Then, 1 mg of BSA was added to the magnetic particle solution, and the solution was mixed for an additional 10 h at room temperature. The magnetic separation step was repeated twice, and the magnetic particles were resuspended in 5 mL of pyridine wash buffer. Then 3 mL of glycine solution (1 M at pH 8.0) was added to the resulting solution to quench all of the unreacted aldehyde sites, and the resulting solution was stirred for 30 min. After the magnetic separation step, 5 mL of wash buffer was added to the monoclonal antibody-functionalized magnetic particles and mixed vigorously (this step was repeated two more times). The magnetic particles

were then magnetically separated, and the supernatant was removed. This washing step was repeated three more times. Finally, the magnetic probes were resuspended in 0.15 M PBS solution.

Barcode DNA Quantification. After adding barcode DNA to gold nanoparticle probes, the solution was spotted and dried on a TLC plate. The plate was scanned using a flatbed scanner, and the scanned image was adjusted using Adobe Photoshop software (all the spots were adjusted together). Each nanoparticle spot was then selected, and the selected area was quantified using the histogram function with red channel option of the Adobe Photoshop (Adobe Systems Inc., San Jose, CA). The mean value from the histogram window was used to calculate the spot intensity of each spot (Figure 2).

RESULTS AND DISCUSSION

In a typical experiment, two types of probes were prepared (Figure 1A). The first is the barcode probe, a 3- μ m porous silica particle modified with anti-IL-2 and the oligonucleotide, which is complementary to a barcode sequence (5' AGCTACGAGT-TGAGAATCCTGAATGCGACG 3') that is a unique identification tag for the target molecule. In the bio-barcode approach, the number of barcode DNA per probe is important because the final detection signal is proportional to the amount of captured barcode DNA. The large size (a few micrometers) and porosity of probe result in significantly increased barcode DNA loading relative to past approaches (tens-of-nanometer particle without pores). Using UV-visible spectroscopy (the UV absorption peak for single-stranded DNA is at 260 nm), we determined the average total number of barcode DNA per bead to be $\sim 3.6 \times 10^6$. Compared with nanoparticle-based barcode probes (hundreds of barcode DNA per nanoparticle probe), this is several orders of magnitude more amplification in terms of the number of barcode DNA per barcode probe. The second probe is a 2.8- μ m iron oxide magnetic probe particle, which has a magnetic iron oxide core with an amine-modified silane coating (DynaL Biotech). These particles were functionalized with anti-IL-2 molecules that can capture IL2 targets.

In the IL-2 detection assay (Figure 1B), 15 μ L of magnetic probe solution (1.5×10^9 beads/mL) was added to 20 μ L of IL-2 solution, followed by the addition of 15 μ L of barcode probe solution (1×10^9 beads/mL). The resulting solution was incubated at 37 $^\circ$ C for 50 min on an orbital shaker. Next, the solution was placed in a magnetic separator (DynaL Biotech), and the supernatant was removed. Then the probe complex solution was washed with 0.15 M PBS solution three more times. Finally, 50 μ L of NANOpure water (18 M Ω) was added to the magnetically separated complexes to release the barcode DNA, and the complexes were kept on a rocking shaker at 70 $^\circ$ C for 10 min. After magnetic separation, the supernatant including free barcode DNA strands was collected for barcode DNA detection. To detect the barcode DNA, 30-nm gold nanoparticle probes (25 μ L at 1 nM for both probes 1 and 2)²⁰ functionalized for barcode DNA capture (barcode capture probe 1, 5' TCTCAACTCGTAGCTA-AAAAAAAA-triethylene glycol-SH 3'; barcode capture probe 2, 5' SH-triethylene glycol-AAAAAAAAACGTCGCATTCAGGAT 3') were added to the barcode DNA in 0.15 M PBS solution. The resulting solution was kept at room temperature for 1.5 h. The

solution was then centrifuged to increase the concentration of probe complexes and to collect small nanoparticle aggregates (10 000 rpm for 5 min), and the supernatant was discarded. Although a centrifugation step is used here, this step may not be essential for actual implementation of the assay after further optimization. Finally, 5 μ L of nanoparticle probe solution from the concentrated nanoparticle solution was spotted on a reversed-phase silica TLC plate (EMD Chemicals, Inc., Gibbstown, NJ) for target verification and quantification (Figure 2A). The spot test was ranged from 30 aM to 300 fM and included a control sample where no IL-2 is present. This assay can detect as low as 30 aM IL-2 targets in the presence of background proteins (1 μ L of 5 μ M anti-biotin and 1 μ L of 5 μ M anti-fibronectin per sample). Spotted dots show not only different colors but also different intensities. Each spot intensity was quantified using image analysis software based on the red color intensity that reflects the aggregation of gold nanoparticles (Adobe Photoshop). Because this colorimetric assay is based on the color change from red (without barcode DNA) to purple (with barcode DNA), a lower mean red color channel value is indicative of more barcode DNA present in solution (Figure 2). Spot intensity herein is defined by the mean red channel value of a control spot divided by the mean red channel value of a given sample spot. These spot intensity values are plotted in Figure 3A (experiments were repeated five times, and the highest and the lowest values were not used for the final spot intensity calculation). The spot intensity of a 30 aM target solution is higher than that of the control spot, and the dynamic range of this assay ranges from 30 aM to 300 fM (Figure 3A).

To validate this colorimetric bio-barcode system for real samples, IL-2 molecules in human serum samples (Cambrex Corp., East Rutherford, NJ) were tested with the same protocol that was used for IL-2 detection in PBS buffer solution. Nanoparticle-based barcode detection spots for 300 aM and 3, 30, and 300 fM IL-2 samples were distinctively different from the control spot (Figure 3B). The spot intensity rapidly saturates after 30 fM.

CONCLUSION

A simple, ultrasensitive colorimetric bio-barcode assay has been developed. This bio-barcode approach to IL-2 detection is important for the following reasons. First, this new method has shown that one can dramatically increase the number of barcode DNA per probe by adjusting surface and size of the barcode probe. This allows for various other ways (in this paper, we used a colorimetric assay) to detect barcode DNA. Second, the detection limit for this assay was orders of magnitude better than other conventional immunoassays in detecting IL-2 (30 aM IL-2 in PBS buffer solution).^{24–26} Significantly, this detection limit is \sim 15 times more sensitive than an enzyme-based amplification method in detecting IL-2. Third, this bio-barcode method does not require complicated instrumentation or experiment steps. Simple mixing and separation of probe solutions would result in attomolar sensitivity without using a microarrayer, complicated signal amplification steps such as enzymatic amplification and silver enhancement, or sophisticated signal measurement tools. Since the readout is based on color change, minimal expertise is

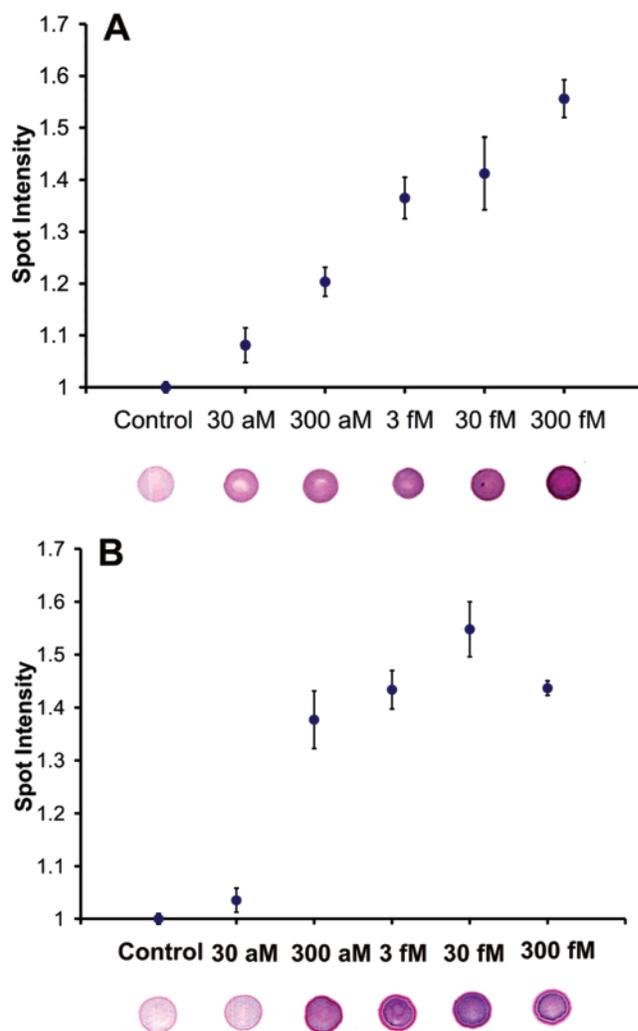


Figure 3. Gold nanoparticle-based colorimetric barcode DNA IL-2 detection (top, quantification data; bottom, gold nanoparticle spots on a TLC plate). (A) In buffer. (B) In human serum samples.

required to perform the assay. Fourth, a quantification method using graphic software was developed for quantitative colorimetric barcode DNA detection assay, which was not possible with previous gold nanoparticle-based colorimetric DNA detection schemes. Finally, this assay should be suitable for point-of-care applications with the requirement only for probe solutions and TLC plates. Efforts to synthesize monodispersed porous microbeads, optimize the detection system for better quantification, and multiplex the system with other cytokines are currently ongoing.

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