

Nanoparticle-Based Bio-Bar Codes for the Ultrasensitive Detection of Proteins

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Supporting Online Material

The gold nanoparticle (NP) probes were prepared by adding polyclonal antibodies (Abs) to PSA (7 μg) to an aqueous solution of 13 nm gold NPs (1 ml of 12 nM solution) at pH 9.0 (in case of 30 nm gold NPs, 0.5 μg of polyclonal anti-PSA was added to 1 ml of 400 pM NP solution). After 20 minutes, the Ab modified NPs were reacted with alkylthiol-capped bar-code DNA capture strands (0.4 OD for 13 nm gold particles and 1 OD for 30 nm gold particles; 5' CAACTTCATCCACGTTCAACGCTAGTGAACACA GTTGTGT-A₁₀-(CH₂)₃-SH 3') for 16 hours and then salt-stabilized to 0.1 M NaCl. Next, the solution was treated with 0.3 ml of a 10 % BSA solution for 30 minutes to passivate and stabilize the gold NPs. The final solution was centrifuged for 1 hour at 4 °C (20,000g), and the supernatant was removed. This centrifugation procedure was repeated for further purification. The final NP probes were re-dispersed in 0.1 M NaCl/0.01 M phosphate buffer solution at pH 7.4. The PSA-specific bar-code DNA strands (1 OD; 5'ACACA ACTGTGTTCACTAGCGTTGAACGTGGATGAAGTTG 3') were then hybridized with the bio-bar-code DNA capture strands coordinated to the NPs and purified using a similar centrifugation procedure. The oligonucleotide loading was determined by the method of Demers *et al* (SI). Amino-functionalized 1 μm diameter MMPs were obtained from Polysciences, Inc. MMPs were linked to mAbs to PSA using the commercial glutaraldehyde-amine coupling chemistry. Coupling efficiency was

determined to be 90% by UV-vis spectroscopy by comparing the absorbance at 270 nm before and after protein coupling to the MMPs. The MMPs were suspended in 40 ml of 0.1 M PBS buffer (pH 7.4) prior to use.

To assess the possibility of generating a positive signal after PCR/gel electrophoresis in the case where only primers are present to assess primer dimer formation and amplification, control experiments were done. Dimethyl sulfoxide (DMSO) was added to reduce the melting temperature of spurious hybridized primers and minimize the possibility of primer dimer formation and amplification (0 to 2 % from left to right in 0.5 % increments in Fig. S1). In addition, the number of thermal cycles was set at 25. As seen in Fig. S1, there are clear bands in the lanes with bar-code DNA amplicons (lane 1-5), while there are no observable bands when only primers are thermally cycled in the presence of Taq polymerase (lane 6-10). Therefore, 2 % DMSO was added to all bio-bar-code PCR reactions since there is no observable band trace for that concentration (lane 10 of Fig. S1) while amplification was maintained for bar-code DNA.

For PCR amplification of bar-code DNA, an aliquot of free bar-code DNA (8.9 μ l) was added to the top wax layer of an EasyStart™ Micro 20 PCR Mix-in-a-Tube (Molecular Bio-Products, San Diego, CA) along with 0.3 μ l of the appropriate primers (each at 25 μ M, Primer 1: 5' CAACTTCATCCACGTTCAAC 3', Primer 2: 5' ACACA ACTGTGTTCACTAGC 3'), 0.4 μ l DMSO (2 % final concentration), and 0.1 μ l of Taq DNA Polymerase, a polymerase shown to be compatible with the EasyStart™ system (5U/ μ l, Fisher Scientific), for a final volume of 20 μ l. The final concentrations of the PCR reaction mix were as follows: Primers 1 and 2, 0.37 μ M; dNTP mix, 0.2 mM;

PCR Buffer, 1X; and MgCl₂, 2mM. The PCR tubes were loaded into a thermal cycler (GeneAmp 9700, Applied Biosystems) and subjected to a 7 minute “hot start” at 94 °C, cycled 25 times at 94 °C for 30 seconds, 58° C for 30 seconds and 72 °C for 1 minute, with a final extension of 72 °C for seven minutes followed by a 4 °C soak.

Using gel electrophoresis, we could detect PSA concentrations as low as 3 aM (10 μl) (Fig. S2) in the presence of two background proteins (β-galactosidase and anti-dinitrophenyl). In addition, we determined that non-specific binding of the NP probe to the MMP probe was negligible, as bio-bar-code PCR generated little signal when PSA was absent from the detection assay both with the background proteins (Fig. S2A, lane 1). The relative intensity of the ethidium bromide stained bands allowed for an estimate of the relative concentration of PSA (Fig. S2B). According to graph 1B, the intensities for control bands (lane 1) are much lower than the bands with PSA present. In the graph representing low concentration (3 aM to 300 fM, lanes 2-7, respectively) PSA detection, the gel band corresponding to 3 aM (lane 2) has a relative intensity 2.5 times higher than the negative control (lane 1).

For the chip-based detection of bio-bar-code DNA, 5' alkylthiol-capped DNA capture strands (5' SH-(CH₂)₆-A₁₀-CAACTTCATCCACGTTCAAC 3') were covalently attached to a glass microscope slide via literature procedures (S2) and gold NPs (13 nm) were functionalized with 3' alkylthiol-capped oligonucleotides (5' GCTAGTGAACA CAGTTGTGT-A₁₀-(CH₂)₃-SH 3') via literature procedures (S3). The chip and NP 20-mer recognition sequences are complementary to half of the target bio-bar-code DNA sequence (40-mer), respectively. As shown in the accompanying spotting template, control DNA oligomers (5' SH-(CH₂)₆-A₁₀-GGCAGCTCGTGGTGA 3') were also

spotted to the chip surface to test the assay selectivity for bar-code DNA. After PCR, the bar-code DNA (40-mer) is in duplex form paired with its complementary 40-mer generated during amplification. Consequently, the amplified duplex must be initially denatured in order to effect hybridization between the bar-code DNA, the chip surface, and gold NP probes. Therefore, bar-code DNA amplicons were removed from the original PCR tube and added (5 μ l) to a solution of gold NP probes (5 μ l, 10 nM). This solution is diluted with 0.3 M PBS (90 μ l) to a final volume of 100 μ l in a clean 0.2 ml PCR tube. In order to hybridize bar-code DNA single strands (40-mer) and NP bound complements (20-mer), the PCR tubes were added to a thermal cycler, heated to 95 °C for 3 minutes to denature the bar-code DNA duplexes, and then cooled to a hybridization temperature of 45 °C for two minutes to bind NP probes to their complementary bar-code DNA sequences. This mixture was removed from the PCR tube and added to the microarrayed (GMS 417 Arrayer, Genetic MicroSystems) chip with immobilized capture strands (20-mer). The test solution for each experiment was confined over the active region of the array with a 100 μ l hybridization well (Grace BioLabs, Bend, OR) for 45 minutes in a humidity chamber. After hybridization, the chips are rinsed with 0.1 M NaNO₃/0.01 M phosphate buffer, pH 7.0 at 45 °C to remove excess gold NPs (repeated twice) and dried with a bench-top centrifuge. Silver enhancement solution (Ted Pella, Inc., Redding, California) is applied to the glass surface immobilized gold NPs for six minutes, rinsed with NANOpure (18 megaohm) water, and dried using a benchtop centrifuge.

The bar-code DNA can also be detected without implementing PCR (Scheme 1B) by increasing the size of NP probes in the protein detection step. The number of DNA

strands for each NP can be significantly increased by increasing size of the NP (in theory, assuming 100 DNA strands are attached to a 13 nm gold NP, there could be as many as 532 DNA strands on each 30 nm gold NP) (S1). Herein, chip-based assays were used to directly measure the amount of bar-code DNA in solution without PCR. Since PCR is not used, we also eliminate the need to denature the duplex DNA formed during PCR amplification. Therefore, after protein detection and isolation of bar-code DNA, an aliquot of the isolated bar-code sample (10 μ l) was mixed with 0.6 M PBS (85 μ l) and the 13 nm gold detection probe (5 μ l, 500 pM final concentration, same sequence as above). This mixture was added to the wells of a Verigene on-chip hybridization chamber under which the appropriate capture strands were arrayed as reported in the main text.

Incubation took place for two hours at 42 °C, whereupon the reaction mixture was removed and the chips were washed with 0.5 M NaNO₃/0.01 M phosphate buffer to remove excess gold nanoparticles. Surface immobilized gold particles were stained with silver enhancement solution (Ted Pella) for six minutes, washed with NANOpure water and imaged with the Verigene ID system, all as done previously. As shown in Fig. S3, we were able to detect as low as 30 attomolar PSA with 30 nm gold NP probes (200 pM) without doing PCR. Although one loses one log in sensitivity, one gains a significant advantage in terms of decreased cost, effort, and time by eliminating the PCR step.

To demonstrate the applicability of our method in a sample solution closer to what would be encountered clinically, we conducted the assay in goat serum with PCR and found that 30 aM PSA concentrations were clearly discernible from control experiments (Fig. S4).

To examine the theoretical lower-limit of protein detection using bio-bar-code PCR, PCR/gel electrophoresis was performed with a dilution series of bar-code DNA concentrations for PCR amplification (Fig. S5). The signal when bar-code DNA amplicons are present is quite discernible from the control band (lane 10) even when only 30 copies of bar-code DNA are added to the PCR reaction (lane 9).

Supporting Figures

Fig. S1. Control experiment to assess primer-dimer and bio-bar-code DNA amplification, and the effect of increasing DMSO concentration using 25 thermal cycles. Lanes 1 through 5 are those with bio-bar-code DNA present in the PCR reaction mixture while there is no bar-code DNA in lanes 6 through 10. Note that DMSO is increased from lane 1 to 5 and 6 to 10 (0 to 2 % in 0.5 % increments).

Fig. S2. Gel electrophoresis images and relative band intensity graph of bar-code DNA amplicons after PSA detection. **A.** Low concentration detection of PSA. Concentrations are from 3 aM to 300 fM in 10 X dilutions from lane 2 (3 aM) to lane 7 (300 fM). A negative control with only background proteins (anti-dinitrophenyl and β -galactosidase without PSA) is shown in lane 1, and the standard bio-bar-code 40-mer is shown in the first lane (lane "B"). **B.** Relative gel electrophoresis band intensity after bio-bar-code PCR. For all electrophoresis experiments, an aliquot (15 μ l) of the PCR mixture is stained with ethidium bromide (1 mg), mixed with gel electrophoresis loading dye (3 μ l, 6X, Promega, Madison, WI), and gel electrophoresis is performed (2 % agarose gel, 110 V, 35 minutes) in 1X TAE running buffer. A bio-bar-code standard (1 μ l, 6 μ M bio-bar-code duplex) is added to the gel for reference. The bio-bar-code standard (40-mer) is

made by adding the bio-bar-code DNA to its complementary strand in 0.3 M PBS. All gel images and determinations of band intensities were done using a Kodak DC-120 digital camera and Kodak ID 2.0.2 imaging software. Gel bands were also stained with ethidium bromide after electrophoresis by soaking the gel in ethidium bromide for 35 minutes (0.5 $\mu\text{g/ml}$ in 1X TAE running buffer) and qualitatively similar results to those where ethidium bromide was added to the PCR reaction prior to electrophoresis were obtained (e.g. Fig. S1C).

Fig. S3. PCR-less detection of PSA bar-code DNA with 30 nm NP probes.

Fig. S4. PSA bio-bar-code DNA detection in goat serum. Each PSA sample is prepared by diluting PSA in goat serum (ICN biomedical).

Fig. S5. Theoretical detection limit of the bio-bar-code method. A. The gel image shows bands after PCR at decreasing starting bar-code DNA concentrations. From left to right, lane 1: 3×10^9 copies, lane 2: 3×10^8 copies, lane 3: 3×10^7 copies, lane 4: 3×10^6 copies, lane 5: 3×10^5 copies, lane 6: 3×10^4 copies, lane 7: 3×10^3 copies, lane 8: 3×10^2 copies, lane 9: 3×10^1 copies, and lane 10: no bar-code DNA. B. Relative gel electrophoresis band intensity graph.

Supporting References

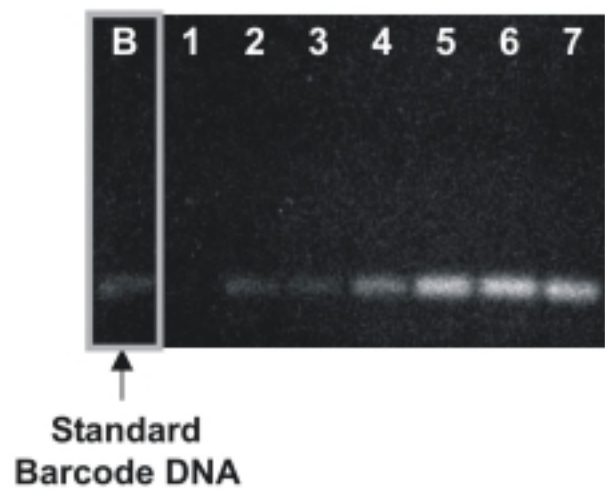
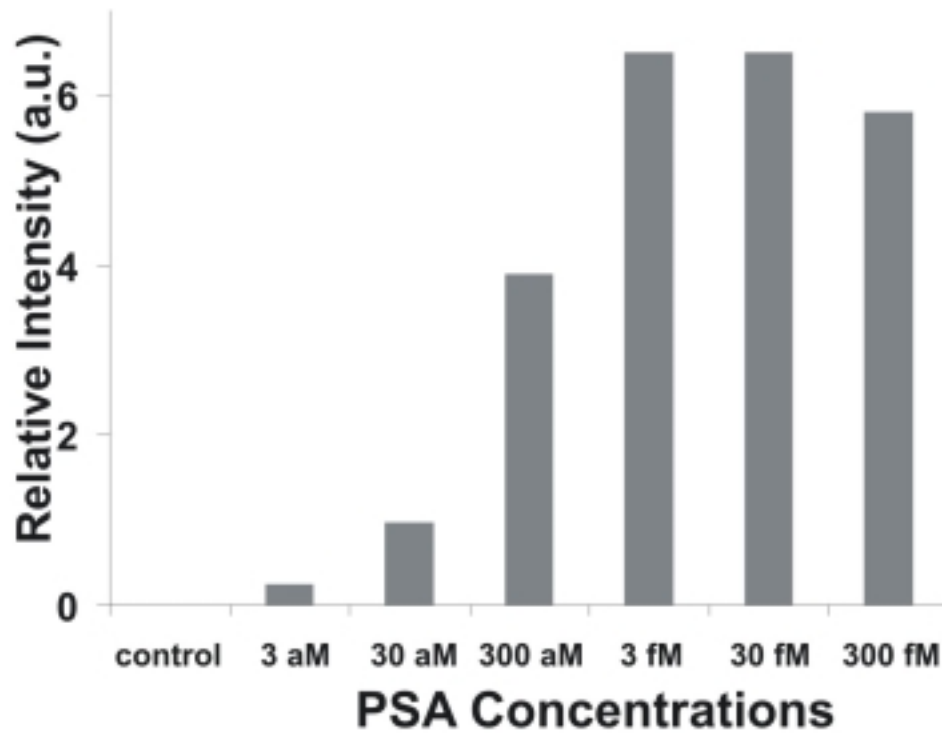
S1. L. M. Demers *et al.* *Anal. Chem.* **72**, 5535 (2000).

S2. L. A. Chrisey, G. U. Lee, C. E. Oferrall, *Nucl. Acids Res.* **24**, 3031 (1996).

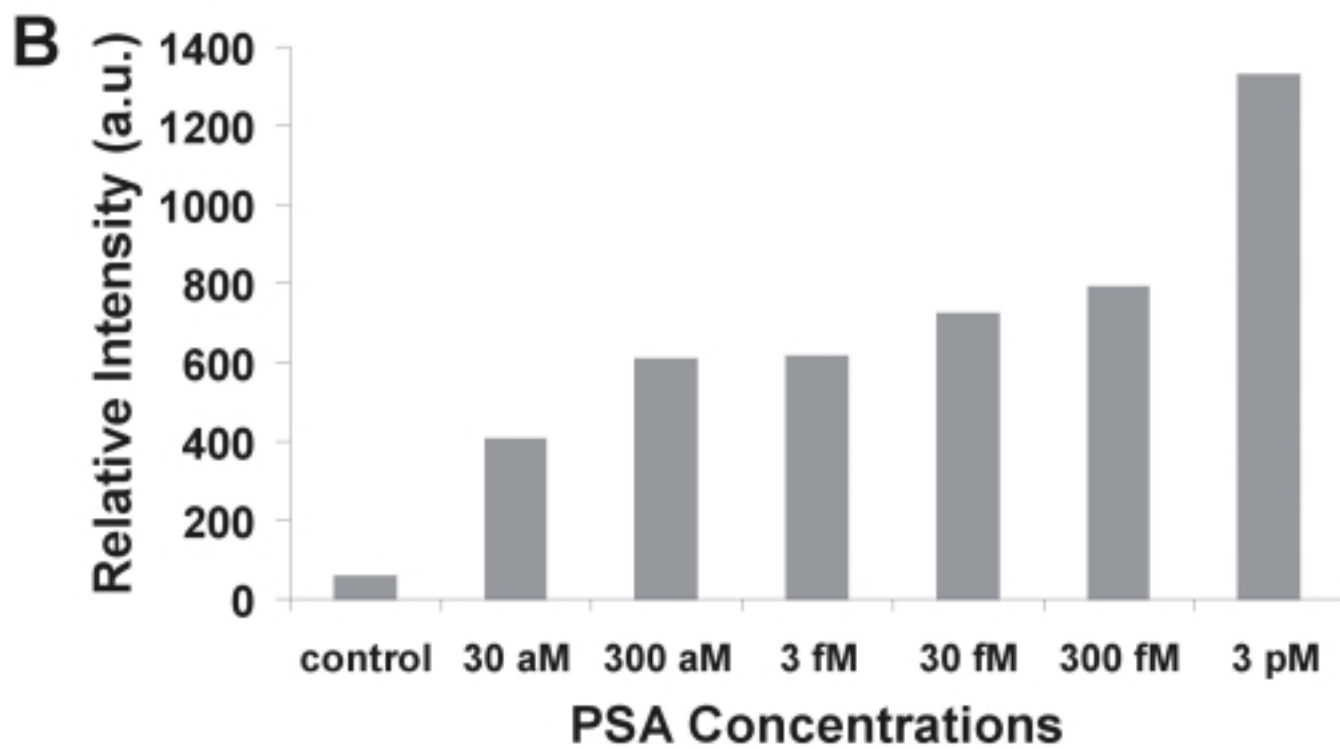
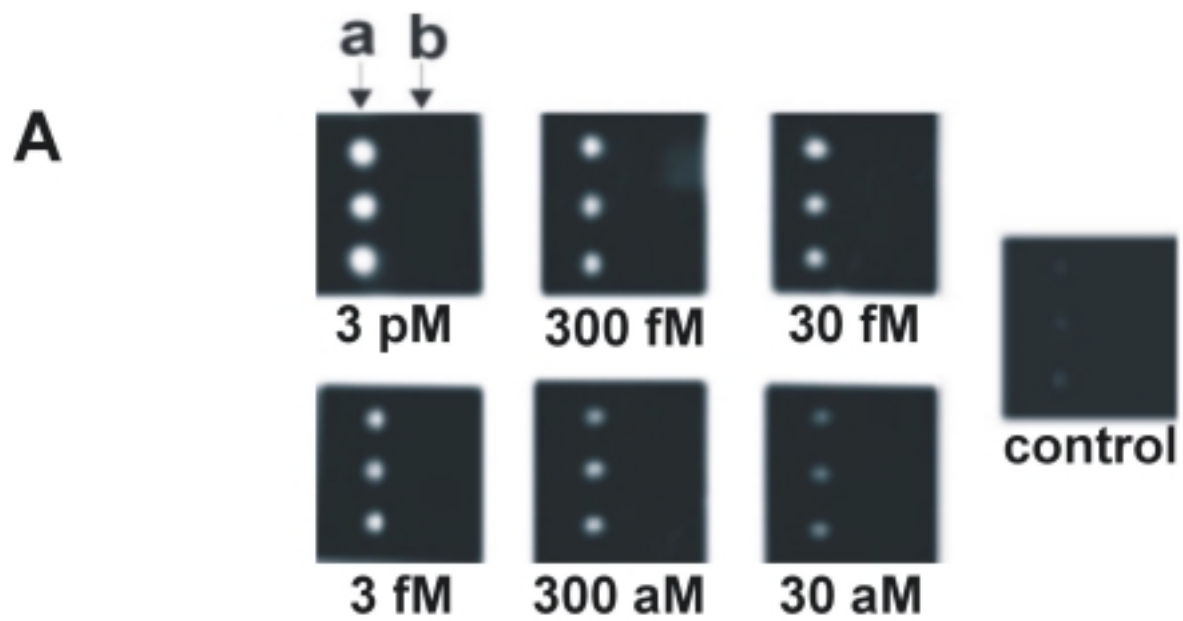
S3. J. J. Storhoff, R. Elghanian, R. C. Mucic, C. A. Mirkin, R. L. Letsinger, *J. Am. Chem. Soc.* **120**, 1959 (1998).



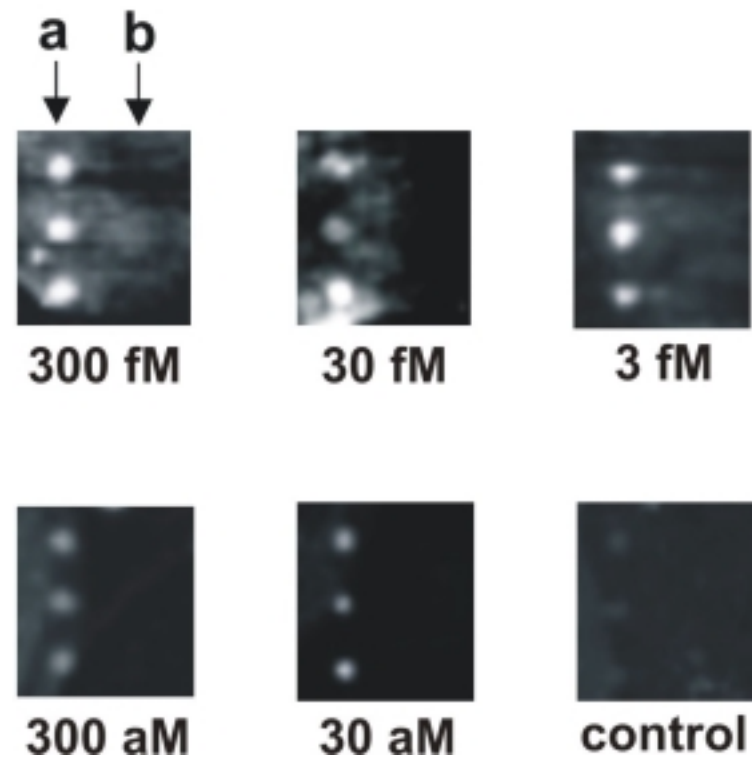
Supporting Figure 1.

A**B**

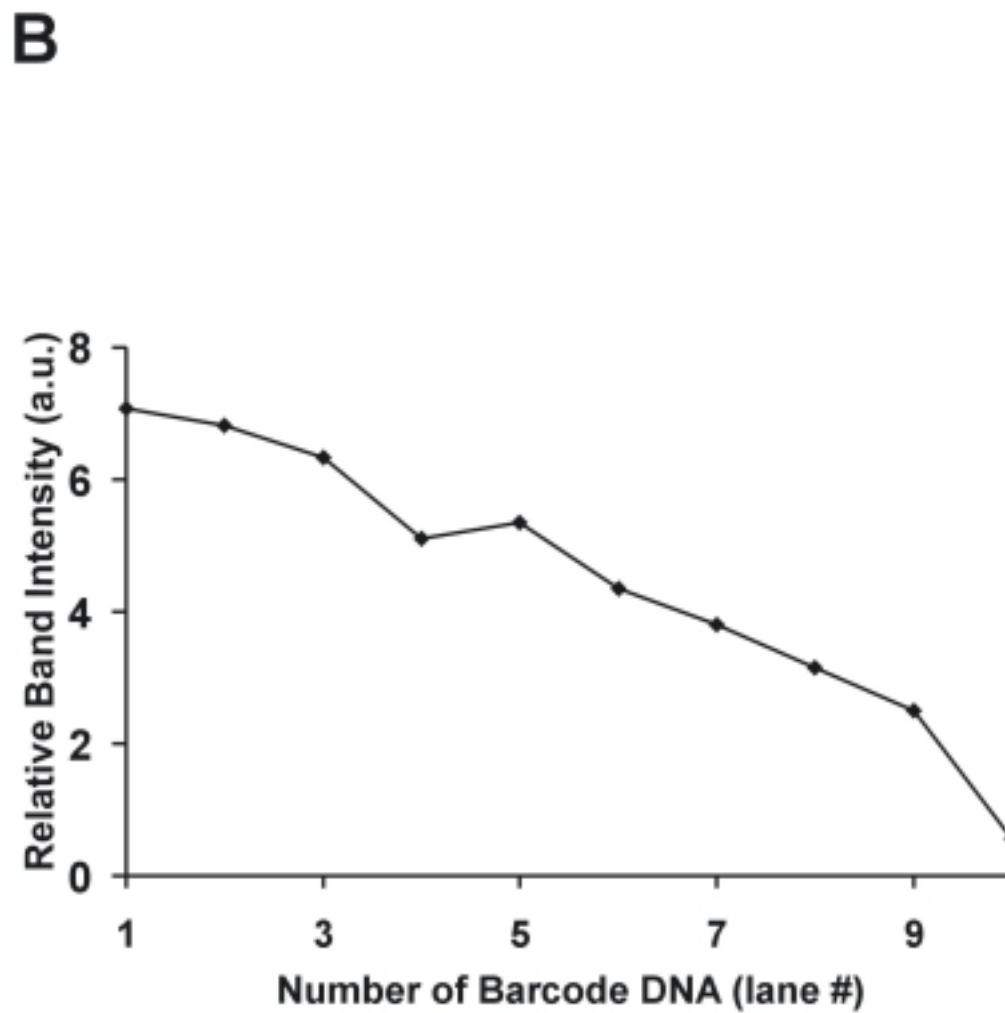
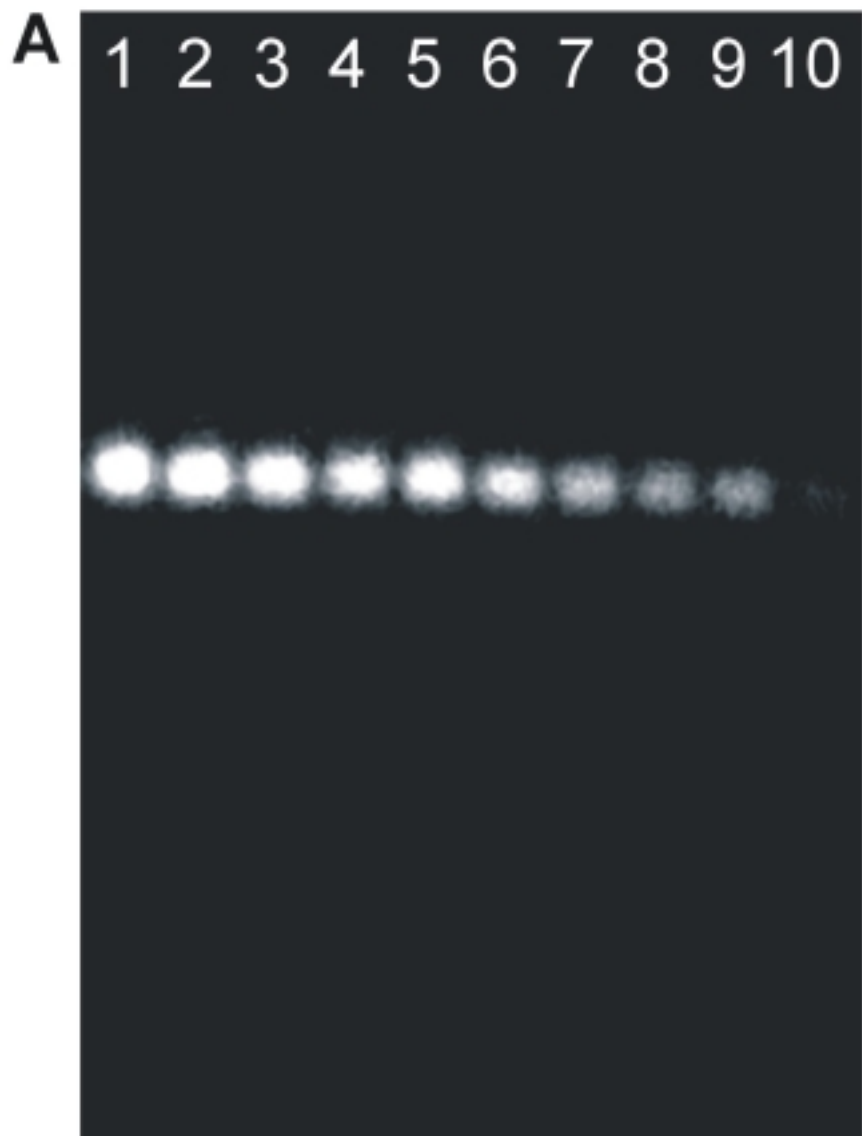
Supporting Figure 2.



Supporting Figure 3.



Supporting Figure 4.



Supporting Figure 5.