Supplementary Material (ESI) for Lab on a Chip

Electronic Supplementary Information for:

Sensitive on-chip detection of a protein biomarker in human serum and plasma over an extended dynamic range using silicon photonic microring resonators and sub-micron beads

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Materials

3-N-((6-(N'-Isopropylidene-hydrazino))nicotinamide)propyltriethoxysilane (HyNic silane), succinimidyl 6-hydrazinonicotinate acetone hydrazone (S-HyNic), and succinimidyl 4-formyl benzoate (S-4FB) were purchased from Solulink (San Diego, CA). Capture and secondary antibodies to CRP (clones M701289 and clones M701288, respectively) were purchased from Fitzgerald Industries (Concord, MA). Capture (MAB206, clone 6708) and biotinylated secondary (BAF206) antibodies for interleukin-6 were purchased from R&D Systems, Inc (Minneapolis, MN). Recombinant human interleukin-6 and a human CRP ELISA kit (88-7502-28) were purchased from eBioscience (San Diego, CA). NHS-PEG_4-Biotin was purchased from Thermo Scientific and dissolved in DMF to make a 20 mM stock solution. Streptavidin-coated polystyrene/iron oxide beads with a mean diameter of 114 nm were purchased from Ademtech (Pessac, France). Human CRP (95% pure from human blood) was purchased from Meridian Life Science. CRP-depleted processed serum and CRP High Plasma (with a reported CRP content of 69.1 µg/mL based on a Roche Modular immunoassay) were purchased from SunnyLab (Sittingbourne, UK). Pooled normal human serum and single-donor human serum samples #1 and #2 (catalog number IPLA-SER-S, lot numbers K9207 BF 19 and 55-25114 WM 19, respectively) were purchased from Innovative Research (Novi, MI). Fresh single-donor human plasma was collected from a healthy donor under a plan approved by the University of Illinois Institutional Review Board. Zeba spin filter columns and StartingBlock (PBS) were purchased from Pierce (Rockford, IL). Vivaspin molecular weight cutoff filters (5000 and 50000 kDa) were purchased from GE Healthcare (Waukesha, WI).

The capture anti-CRP antibody was immobilized onto the microring sensors using a DNA-encoding approach.[1, 2] 30-mer DNA strands for the DNA-encoded CRP capture antibody immobilization were synthesized with a C6 spacer and 5′ amination by Integrated DNA Technologies (Coralville, IA). The surface 30-mer sequence was 5'-AAAAAAAAAAGCCTCATTGAATCATGCCTA-3', and the complement sequence conjugated to anti-CRP capture antibodies was

5'-AAAAAAAAAATAGGCATGATTCAATGAGGC-3'. The 3′- biotinylated 30-mer for the simulated human papillomavirus (HPV) DNA target, simulated HPV DNA 46-mer target, and HPV surface capture strand were also synthesized by Integrated DNA Technologies. The sequences were 5'-CTCTGGATAATAGAGAATGTAAAAAAAAAA-3', 5'-TACATTCTCTATTATCCACACCTGCATTTGCTGCATAAGCACTAGC-3', and 5'-AAAAAAAAAGCTAGTGCTTATGCAGCAAAT-3', respectively.

PBS buffer was prepared from Dulbecco's phosphate buffered saline from Sigma Aldrich (St. Louis, MO). PBST buffer consisted of PBS buffer and 0.05% Tween-20. The pH of buffers was adjusted using 1M HCl or 1M NaOH. All other chemicals were obtained from Sigma-Aldrich and used as purchased.

Experimental Details

Antibody and DNA Modification

An aldehyde moiety is added to the surface capture DNA strand and its complement by incubation with a 20-fold molar excess of 5 mg/mL S-4FB for 2-3 h at room temperature in 33% DMF in PBS pH 7.4. Excess S-4FB is removed by buffer exchange using a 5-kDa molecular weight cutoff filter. Capture antibody-HyNic conjugates are prepared by incubating the antibody with a 10-fold excess of 1 mg/mL S-HyNic at room temperature for 2 h. Excess S-HyNic is removed by buffer exchange using Zeba spin filter columns. For biotinylation, 0.5 mg/mL secondary anti-CRP M701288 in PBS pH 7.4 is incubated with a 10-fold molar excess of 20 mM NHS-PEG_4-biotin for 3 h at room temperature. Excess NHS-PEG_4-biotin is removed by buffer exchange using Zeba spin filter columns. Capture antibody- DNA conjugates are prepared by incubating the capture antibody-HyNic conjugate and the 4FB-modified surface DNA strand at a 1:20 protein:DNA ratio overnight at 4°C. The mixture is purified using a Superdex 200 10/300 GL column on an AKTA FPLC, both from GE Healthcare, and fractions containing the antibody-DNA conjugate are concentrated and exchanged into PBS buffer using a 50-kDa molecular weight cutoff filter.

Bead exchange and surface modification

Beads are exchanged into PBST buffer immediately before use to remove free streptavidin via the following procedure. First, 30 μL of 5 mg/mL streptavidin-coated beads are diluted to 150 μg/mL with PBST buffer and centrifuged at 10,000 *g* for 4 min. The bead pellet is held at the bottom of the tube using a magnet while the supernatant is removed, leaving about 30 μL of solution. The beads are then resuspended in 1 mL of PBST buffer by pipetting the solution up and down ~50 times with the tube bottom remaining in contact with the magnet. This procedure is repeated for a total of 4 exchanges. Directly before use in the assay, the final bead solution is adjusted to a concentration of 50 μg/mL using the absorbance value at 286 nm as determined by calibration based on direct dilution of non-exchanged beads. For DNA detection, beads are conjugated to a secondary complementary DNA strand. Streptavidin-coated beads are incubated with a large excess of biotinylated DNA for at least 2 h. The excess DNA is removed by buffer exchange according to the previously described method.

Chip functionalization

Chips are batch-functionalized ex-situ by a spotting method. After a 20-min sonication in ethanol, chips are immersed for 20 s in hot Piranha solution (3:1 H_2SO_4: 30% H_2O_2) [*Caution: Pirahna solution must be handled with care as it can react violently with organic compounds.*], rinsed with water, and dried under a stream of nitrogen. Each chip is then spotted with a 30-μL drop of 1 mg/mL HyNic silane in 95% ethanol and 5% DMF and incubated at room temperature in an enclosed chamber for 30 min. The chip is then rinsed with ethanol and dried. A 0.5-μL drop of 4FB-modified capture DNA strand is then manually pulled across the surface with a 2.5-μL pipette tip, avoiding those rings which are to be used as controls. The chip is then blocked by submersion in StartingBlock at 4ºC for at least 8 h.

Experimental procedure

The fluidic cell and microfluidic system have been described previously.[3] In these experiments, a Mylar gasket is used to direct flow to two microfluidic channels, each addressing 12 of the 24 active rings, allowing two experiments to be carried out on one chip. A consistent amount of capture antibody is loaded by flowing 10 µg/mL DNA-encoded anti-CRP M701289 at 2 µL/min until the antibody signal reaches ~140 Δpm. To begin the procedure for the analysis of samples, PBST buffer is flowed over the surface to establish a baseline. A 30 µL/min-flow rate is maintained for all assay segments. The sample or standard is introduced and allowed to flow over the surface for 20 min, followed by a buffer rinse. In serum and plasma samples, this rinse is longer (~20 min) than the typical 3 min rinse to allow for desorption of non-specifically bound proteins. The biotinylated secondary antibody is then flowed over the surface for 15 min followed by another short buffer rinse (3-5 min). Finally, streptavidin-coated beads (exchanged immediately before use as described above) are flowed over the surface for 16 min followed by a final buffer rinse. For DNA detection, there is not a third binding step as the secondary detection strand is directly conjugated to the bead.

Instrumental Setup

The microring resonator instrumentation and chips were acquired from Genalyte (San Diego, CA), and have been described in detail in previous publications.[3, 4] Briefly, each chip is fabricated with 32 active microring sensors covered with a perfluoropolymer cladding layer. Eight rings on each chip remain occluded by the polymer layer and serve as thermal controls. Twenty-four active sensors rings have an annular opening etched over the ring to enable exposure to the solution in the microfluidic channel. Each ring resonance is monitored via frequency attenuation in an adjacent linear waveguide. Input and output diffractive grating couplers are located at each end of the waveguide to enable independent determination of the optical cavity spectrum for each microring using a tunable, external cavity diode laser (center frequency: 1560 nm).

Data analysis

Calibration curves for primary binding are generated by determining the initial slope as a function of analyte concentration, as described previously.[3] Secondary and tertiary binding calibration curves are generated by plotting the total net relative shift of the secondary and bead binding, respectively (Fig. 2). Standard additions were prepared based on an initial estimate of the unspiked concentration in a diluted sample based on comparison to calibration plots generated in buffer. Small increments of a concentrated CRP solution are added to aliquots of diluted sample and then analyzed in the above described manner. Standard addition plots (Fig. S3) are generated based on primary, secondary, or tertiary response as a function of the concentration of spiked CRP added, and CRP concentrations are determined based on extrapolation of the linear regression fit to the x-intercept. Regression lines for calibration in buffer and standard additions were calculated using linear fitting functions in Origin Pro 8.5 software.

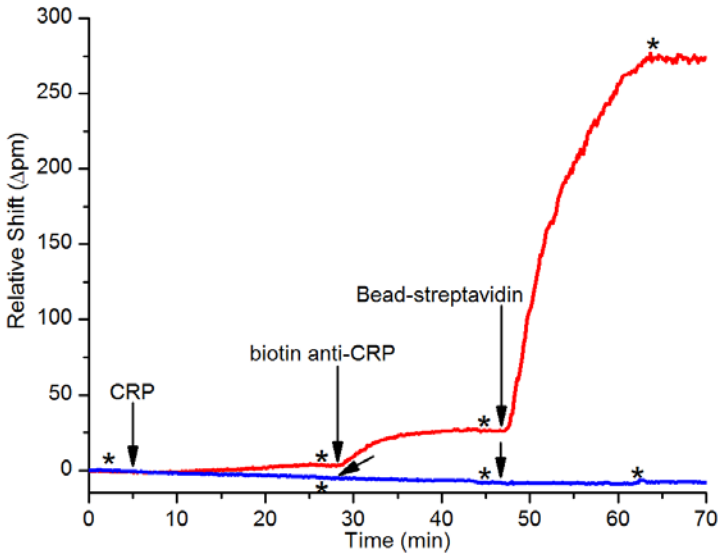

Fig. S1 Negative control experiment for 10^{-2} µg/mL CRP sandwich assay with bead amplification. The red plot represents the response to 10^{-2} µg/mL CRP (t = 5 min) followed by the introduction of 1.6 µg/mL biotin-anti-CRP M701288 (t = 28 min) and subsequent signal amplification with 50 µg/mL streptavidin-conjugated beads (t = 46 min). The blue plot represents a ring that is first exposed to buffer without any CRP followed by the same secondary antibody and bead solutions. No primary, secondary, or bead signal is observed on rings not exposed to CRP, which demonstrates no appreciable non-specific binding of secondary antibodies or beads. * indicates buffer rinse and the arrows indicate the introduction of the identified solutions at the times noted.

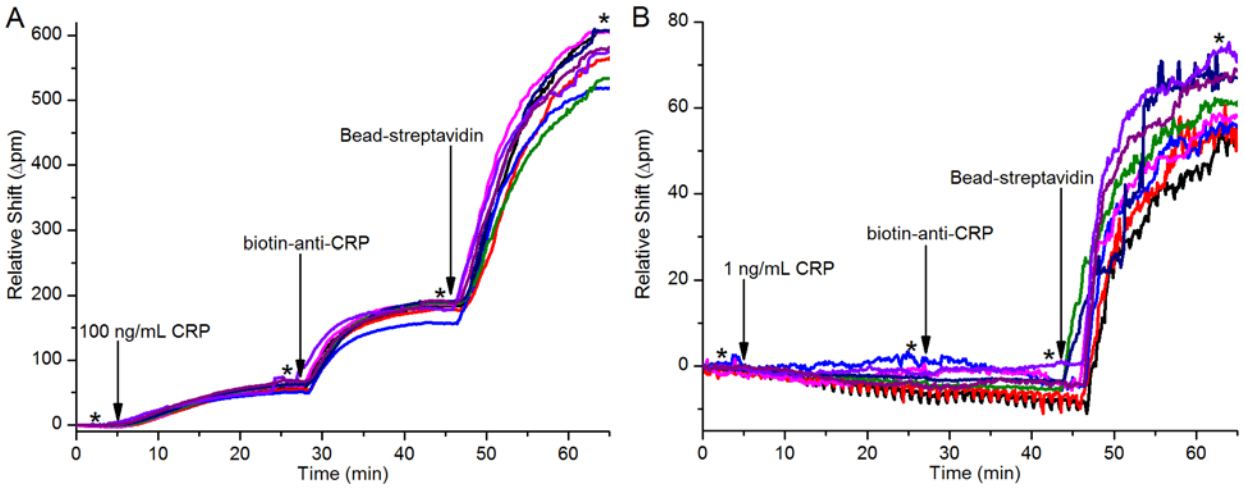

Fig. S2 Demonstration of chip-to-chip reproducibility for (**A**) 10^{-1} µg/mL and (**B**) 10^{-3} µg /mL CRP sandwich assays with subsequent bead-based amplification. Both plots display 8 representative rings, with 1 ring selected from each of 2 channels on a total of 4 chips for each CRP concentration. * indicates buffer rinse and the arrows indicate the introduction of the identified solutions at the times noted. All assays were conducted in freshly degassed 10 mM PBS pH 7.4 + 0.05% Tween-20 with 1.6 µg/mL biotin-anti-CRP M701288 and 50 µg/mL streptavidin-conjugated beads. Statistics for the 10^{-1} µg/mL and 10^{-3} µg /mL reproducibility studies are summarized in Tables S1A and S1B respectively.

A	*Initial Primary Slope*	*Secondary Shift*	*Bead Shift*
	Δpm/min	Δpm	Δpm
Average	3.7	117	369
St. Dev. (ring-to-ring)	0.7	14	47
St. Dev. (channel-to-channel)	0.3	8	33
B	*Initial Primary Slope*	*Secondary Shift*	*Bead Shift*
	Δpm/min	Δpm	Δpm
Average	-	0.9	73
St. Dev. (ring-to-ring)	-	1.5	15
St. Dev. (channel-to-channel)	-	1.0	8

Table S1 Average shifts/initial slopes and standard deviations for (**A**) 10^{-1} µg/mL and (**B**) 10^{-3} µg /mL CRP multi-chip reproducibility study. The ring-to-ring standard deviation is calculated from the standard deviation for all rings (n = 80) on all 4 chips tested, while the channel-to-channel standard deviation is calculated from the standard deviation of the average values for each of 8 channels (2 microfluidic channels/chip). In each case, the ring-to-ring variability exceeds the channel-to-channel variability, suggesting that the use of multiple chips for calibrations and quantitative analyses is satisfactory. It is also worth noting that the 0.9 ± 1.5 pm secondary shift for 10^{-3} µg /mL CRP represents a concentration on the lower boundary of what can be seen with a simple CRP sandwich assay. Although difficult to observe with secondary binding, tertiary binding creates an easily measurable response (73 ± 15 pm). Thus, in addition to enabling detection at previously impossible levels, beads also are important for amplifying small secondary signals.

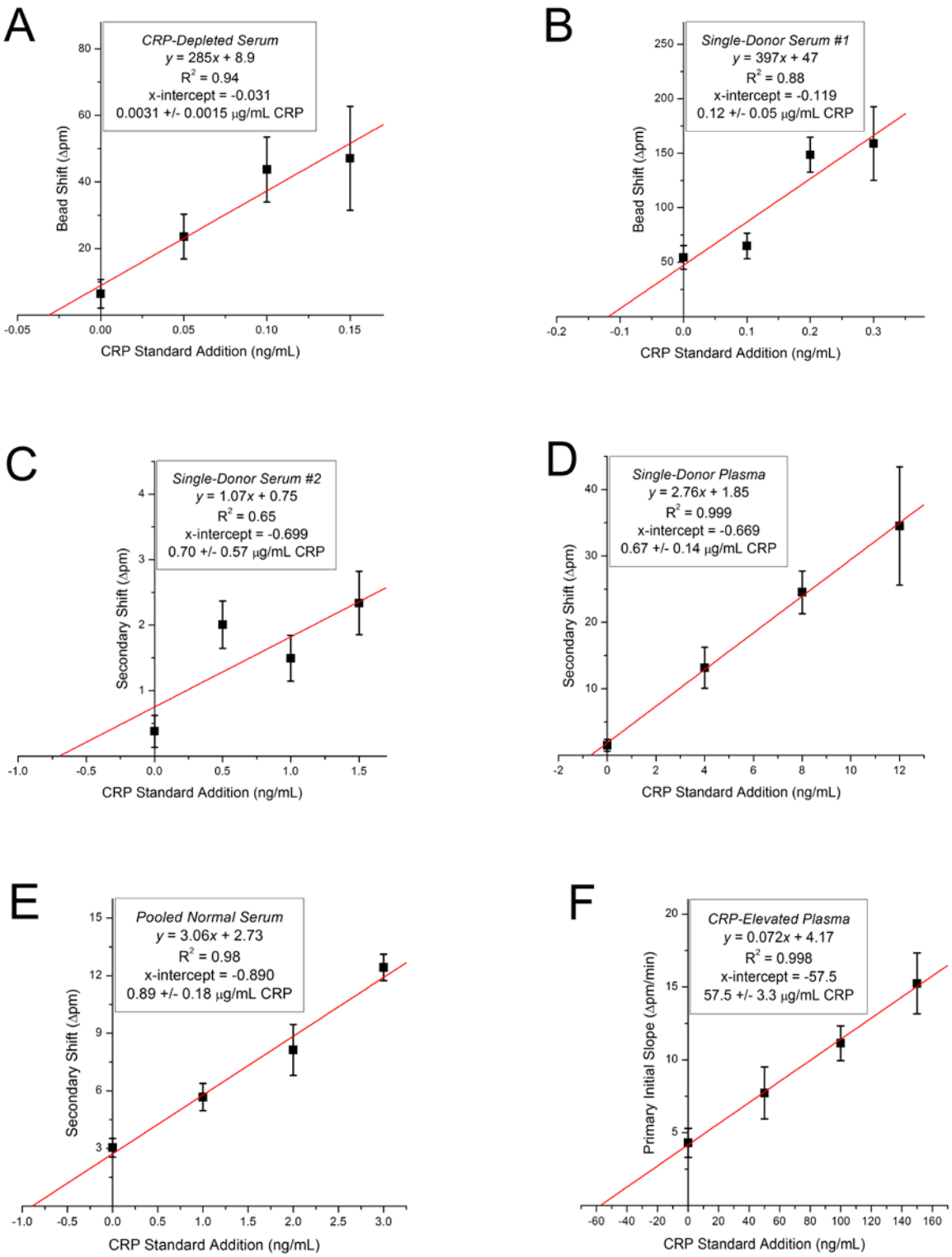

Fig. S3 Standard addition plots[5] used to determine CRP concentrations in 6 human serum and plasma samples quantified in Fig. 3. Since CRP levels vary over a wide dynamic range, quantitation was performed based on bead shift (**A**, **B**), secondary shift (**C**, **D**, **E**), or primary initial slope (**F**). Each plot displays the shift or slope data for an unspiked sample dilution plus three standard additions of CRP determined by the magnitude of the unspiked sample response. The sample identity, fitting equation, quality of fit, x-intercept, and observed CRP concentration are presented for each standard addition experiment shown above.

	CRP Concentration (µg/mL)	
	Ring Resonator Array	*CRP ELISA*
CRP-Depleted Serum	0.0031 ± 0.0015	-
Single-Donor Serum #1	0.12 ± 0.05	0.19 ± 0.08
Single-Donor Serum #2	0.70 ± 0.57	0.39 ± 0.08
Single-Donor Plasma	0.67 ± 0.14	2.05 ± 0.22
Pooled Normal Serum	0.89 ± 0.18	1.45 ± 0.10
Pooled CRP-Elevated Plasma	58 ± 3	105 ± 11

Table S2 Comparison of ring resonator and enzyme-linked immunosorbent assay (ELISA) results for six human serum and plasma samples depicted graphically in Fig. 3. Errors for the ring resonator array data represent the error in the x-intercept determination used in the standard addition assays (as described above). ELISA errors are the standard deviation of triplicate assays run according to the manufacturer's protocol. All ELISA values were measured from samples that were diluted to be within the range of the assay, with a 1:1000 dilution proving optimal for all but the CRP-elevated plasma which required a 1:40000 dilution. ELISA failed to detect CRP in the CRP-depleted serum at any dilution tested (1:100, 1:1000, 1:4000). While ring resonator analysis was in strong agreement with ELISA for most of the samples tested, some varied by as much as a factor of 2-3. This variation is typical of what is observed when comparing a variety of immunoassays, especially considering different dilution factors. Prior analyses of CRP samples by Khuseyinova *et al.* and Clarke *et al.* that each compared separate immunoassay methodologies showed a similar degree of agreement between methods, with most values in strong agreement and some varying by as much as a factor of 3-4.[6, 7] These variations also tend to be more pronounced at higher concentrations, such as the pooled CRP-elevated plasma sample, where additional dilution or use of calibration curve extremes are necessary but introduce greater error due to pipetting, regression, or signal saturation.

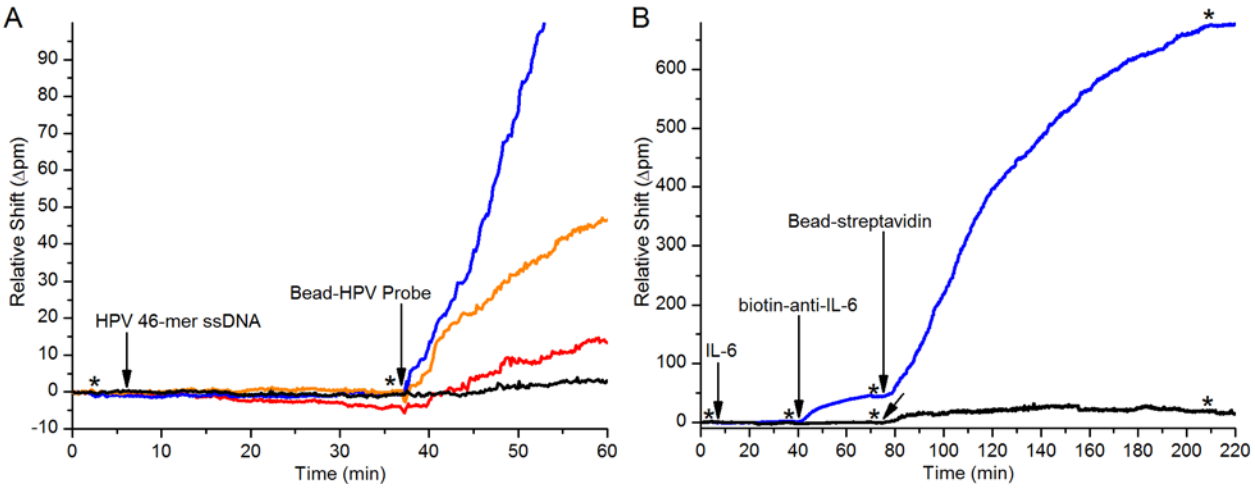

Fig. S4 Bead-based signal amplification universally augments ring resonator signals in assays designed for the detection of (**A**) a 46-mer human papillomavirus (HPV) ssDNA target and (**B**) the cytokine interleukin-6 (IL-6). In (**A**), 4 concentrations of HPV DNA prepared by serial dilution [10 nM (blue), 2 nM (orange), 0.4 nM (red), 0.16 nM (black)] are each undetectable based on primary binding (*t* = 6-36 min) to rings functionalized with a 30-mer probe complementary to the HPV target. Upon the addition of beads conjugated with a separate 30-mer DNA probe complementary to the remaining portion of the HPV 46-mer at *t* = 37 min, all 4 concentrations can be detected in a concentration-dependent manner. HPV DNA assays were conducted in freshly degassed 10 mM PBS pH 7.4 + 0.05% Tween-20 with 20 µg/mL HPV probe-conjugated beads. In (**B**), 10 ng/mL (blue) and 0.1 ng/mL (black) IL-6 are detected using bead-based amplification of a sandwich immunoassay. The secondary shift for 10 ng/mL IL-6 is amplified ~12x through the use of beads. However, the lower concentration of 0.1 ng/mL IL-6 (5 pM) is only detectable through the use of bead-based amplification to amplify the secondary signal. IL-6 assays were conducted in 10 mM PBS pH 7.4 + 0.1 mg/mL BSA with 1 µg/mL biotin anti-IL-6 BAF206 and 50 µg/mL streptavidin-conjugated beads. * indicates buffer rinse and arrows indicate the introduction of the identified solutions at the times noted.

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