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Supplementary information for

Multiplexed Cancer Biomarker Detection Using Chip-Integrated Silicon Photonic Sensor Arrays

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Table of Contents

Details on sensor calibration and calibration curve fitting 2-3

Supplementary Figure 1. Negative control experiment showing negligible non-specific binding associated with tracer and signal enhancement assay steps 4

Supplementary Figure 2. Linearity of signal enhancement steps 5

Supplementary Table 1. Calibration details and fitting parameters 6

Supplementary Table 2. Measured biomarker concentrations in patient samples 7

Supplementary Table 3. List of antigens and antibodies 8

Supplementary Table 4. List of DNA oligonucleotide sequences 9

Supplementary Table 5. Available information for patient samples from vendor 10

Supplementary Table 6. Comparison of working ranges for Luminex and microring biomarker panel described in this manuscript 11

Supplementary Table 7. Comparison of assay protocols for Luminex and microring biomarker panel described in this manuscript 12

Details on sensor calibration and calibration curve fitting

Calibration curves were made to cover a range of biomarker concentrations relevant for clinical serum specimens, as shown in Figure 3 of the manuscript. To conserve analysis time while avoiding possible interferences between antigens, calibrations were obtained simultaneously for CEA, ALCAM, AFP, and PSA. CA19-9 and CA15-3 calibrations were obtained together, but apart from the other antigens. By running a few concentration of CA15-3 with no CA19-9 and vice versa, it was possible to obtain corrections for how the stock antigen solutions interfered with each other. Similarly, osteopontin and CA-125 calibrations were run together, but apart from the other antigens. Each point on the calibration curve represents one measurement on one chip based on the net shift in resonance frequency (pm) obtained from the tertiary amplification step. Because the tertiary amplification step consisted of 6 mini-steps (anti-biotin, anti-PE, anti-biotin, anti-PE, anti-biotin, anti-PE), it was possible to use a different region of the tertiary amplification curve for each antigen. This was important because some of the antigens tended to generate higher signals and would saturate the tertiary signal faster than the antigens that generated lower signal. For example, the ALCAM calibration curve had the largest range of quantitation when the shift was measured after only one of the amplification mini-steps. At later steps of the amplification, the last two points on the calibration curve became indistinguishable from each other. In contrast, CA15-3 tended to display only small relative shifts, and thus using 6 amplification steps increased the signal that could be observed.

Further signal enhancement steps would have led to increased magnitudes of signal and lower limits of detection; however, this would consume additional reagents and time. Moreover, we empirically found that six steps of signal enhancement provided sufficient sensitivity for the biomarker screening efforts described herein. The number of amplification steps used for each antigen’s calibration curve is given in Supplementary Table 1.

The error bars shown on the calibration curves were derived using a 17% error or the standard deviation of the ring-to-ring measurements on a given chip, whichever was larger. The 17% value comes from measuring the chip-to-chip standard deviation for multiple 8-plex chips running the same assay. This same error formula was applied to the relative signal index measurements used in Figure 5.

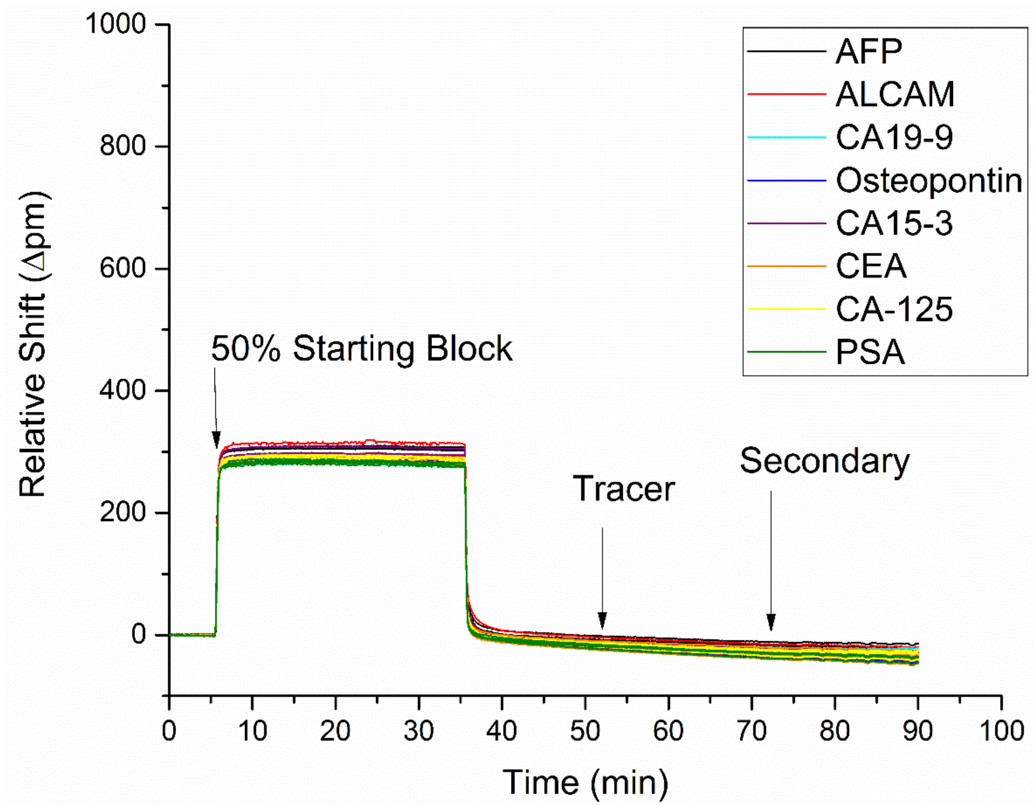
Calibration curves were based on unweighted fits with either a linear model or a dose-response model fit in OriginPro software package version 8.5. The dose-response equation used is given here:

$$y = A + \frac{(B - A)}{1 + 10^{(C-x)D}}$$

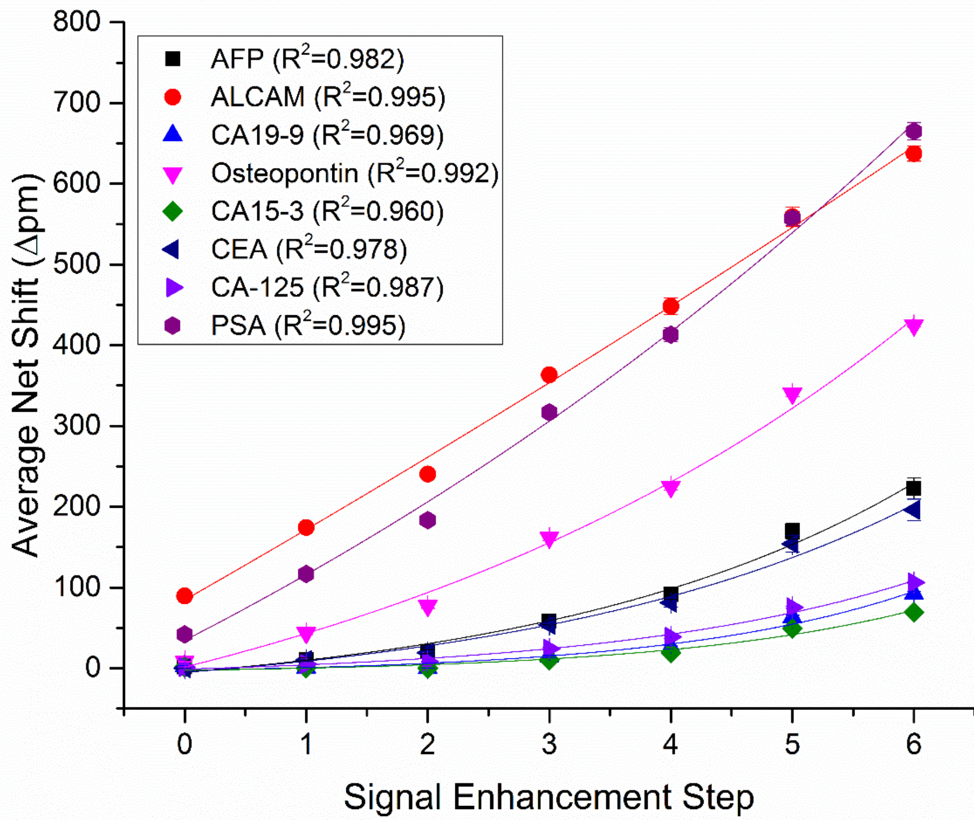
Calibration curves overlaid on data plots are shown in Supplementary Figure 1. Calibration parameters are listed in Supplementary Table 1. The units for AFP, ALCAM, Osteopontin, CEA, and PSA are given in ng/mL whereas CA19-9, CA15-3, and CA125 are given in units/mL (U/mL). These labels are based on the units given from the commercially obtained antigen solutions. Supplementary Table 2 lists the results of the calibration analysis for individual tested serum samples with reference to the upper and lower quantitation limits for the calibration curves listed in Supplementary Table 6. A "—" indicates a measurement that was below the range of quantitation used for the calibration curve. A result with a ">" indicates that the measurement fell higher than the quantitation range used for the calibration curve. CA-125 fell below the range of accurate quantitation in all samples, and so it is not listed on the table. A few of the samples still had measurable shifts even though the values fell below the range of quantitation. As a result, Figure 5 displays a relative index value for these antigens. This is a case of the limit of detection (LOD) being lower than the limit of quantitation (LOQ).

In Supplementary Table 2 the errors shows for each measurement are extrapolated from the standard deviation of the measurement signal via the calibration curve. In other words, $y \pm y_{error}$ is converted to $x \pm x_{error}$, where y_{error} was determined using relative standard deviation, as described previously.

Supplementary Figure 1. Negative control experiment where 50% Starting Block solution is flowed across the array in the primary binding step. The running buffer before primary binding is 1% starting block in PBS-Tween 20 buffer (PBST), which explains the large bulk refractive index step. The experiment demonstrates that the tracer and secondary signal enhancement steps do not lead to non-specific binding responses.



Supplementary Figure 2. A plot of the resonance shift per signal enhancement step showed a good correlation, as shown below for the antigens tested at saturating concentrations (AFP: 20 ng/ml; ALCAM: 200 ng/ml; CA19-9: 200 U/ml; Osteopontin: 50 ng/ml; CA15-3: 200 U/ml; CEA: 20 ng/ml; CA-125: 200 U/ml; PSA: 20 ng/ml). Relative responses were extracted to show the signal growth for each of the signal enhancement steps. The pre-step enhancement showed an initial exponential growth behavior, consistent previous studies of layer-by-layer protein deposition using streptavidin and multi-biotinylated antibodies, a similar system to the PE-conjugated anti-biotin antibody and multiply-biotinylated anti-PE antibody. [M.S. Luchansky, A.L. Washburn, T A. Martin, M. Iqbal, L.C. Gunn and R.C. Bailey, *Biosensors and Bioelectronics*, 2010, **26**, 1283-1291.]



Supplementary Table 1. Number of amplification steps used for the calibration curve, the upper and lower limits of quantitation used for these curves, and the fitting parameters for both the dose-response calibration curves as well as the linear calibration curves.

	Antigen	# of Enhancement Steps	A	B	C	D
Dose-Response	ALCAM	1	-45.85	66.50	2.85	0.046
	CA15-3	6	-98.22	151.91	10.09	0.019
	CEA	1	-4214.83	25.29	-109.06	0.021
	PSA	2	-37051.34	165.89	-36.65	0.064
Linear	**Antigen**	**# of Enhancement Steps**	**Intercept**	**Slope**		
	AFP	6	24.4	17.01		
	CA19-9	6	15.2	1.91		
	Osteopontin	4	-9.9	4.67		
	CA125	6	7.4	1.07		

Supplementary Table 2. Measured concentration values for each serum sample. Entries with ">" indicate values are over the limits of quantitation; entries with "—" indicate values below the limits of quantitation. Bold values indicate the concentration and non-bold entries to the right represent the error of the measurement.

	Pancreas		**Prostate**		**Liver**		**Ovarian**		**Breast**		**Lung**		**CRC**		**Healthy**	
	ng/mL	error	ng/mL	error	ng/mL	error	ng/mL	error	ng/mL	error	ng/mL	error	ng/mL	error	ng/mL	error
AFP	**1.3**	0.5	—	—	**8.8**	1.7	—	—	—	—	—	—	—	—	—	—
ALCAM	**>65**	—	**7.6**	1.5	**16.7**	4.4	**5.4**	2.8	**7.5**	5.8	**10.5**	2.2	**15.4**	3.8	**>65**	—
CA19-9	**>100**	—	—	—	—	—	—	—	—	—	—	—	**3.8**	2.0	—	—
Osteopontin	**14.6**	2.1	—	—	**21.3**	3.3	**6.1**	0.7	—	—	**6.5**	0.7	**13.3**	1.9	**5.1**	0.2
CA15-3	**12.8**	2.1	**3.6**	2.5	**5.1**	0.8	—	—	**27.2**	7.8	**4.3**	1.0	**5.1**	2.2	**3.5**	0.5
CEA	**>20**	7.1	**18.0**	5.8	**>20**	12.5	**3.9**	2.1	**12.0**	3.4	**19.9**	6.8	**>20**	—	**>20**	—
PSA	—	—	**0.14**	0.05	—	—	—	—	—	—	—	—	—	—	—	—

Supplementary Table 3. List of antigens and antibodies used for eight-plex experiments, company source, product number and antibody clone (for monoclonal antibodies)

Antibody / Antigen Type		**Source***	**Product Number**	**Antibody Clone**
AFP	**Antigen**	Meridian	A81510H	
	Capture	Meridian	MAM01-301	057-11301
	Detection	Meridian	M01254M	B491M
ALCAM	**Antigen**	R&D Systems	656-AL	
	Capture	R&D Systems	MAB656	105901
	Detection	R&D Systems	BAF656	Polyclonal
CA 125	**Antigen**	Fitzgerald	30-AC21	
	Capture	Life Span	LS-C84288/ 28658	M002201
	Detection	Meridian	10-C02F	M002203
CA 15-3	**Antigen**	Meridian	A32000H	
	Capture	Fitzgerald	10-C03E	M002204
	Detection	Fitzgerald	10-C03F	M002208
CA 19-9	**Antigen**	Fitzgerald	30AC14	
	Capture	Fitzgerald	10C04C	M8073021
	Detection	Meridian	M37301M	241
CEA	**Antigen**	Fitzgerald	30-AC32	
	Capture	Meridian	MAM02-009	057-10009
	Detection	Meridian	MAM02-008	057-10008
Osteopontin	**Antigen**	Fitzgerald	30RA0008	
	Capture	Meridian	M66102M	2C5
	Detection	Meridian	H01278M	B697M
PSA	**Antigen**	Fitzgerald	30R-AP019	
	Capture	Meridian	M66279M	B731M
	Detection	Meridian	M86506M	5A6

*Full source information:
Meridian Life Science, Saco, ME
R&D Systems, Minneapolis, MN
Fitzgerald Industries International, Concord, MA
Life Span Biosciences, Seattle WA

Supplementary Table 4. List of DNA oligonucleotide sequences used. All sequences have a 5′ terminal amino group attached via a 6-carbon chain (5AmMC6 from IDT)

Name	Sequence (5′ to 3′)
B	AAA AAA AAA AGC CTC ATT GAA TCA TGC CTA
B′	AAA AAA AAA ATA GGC ATG ATT CAA TGA GGC
C	AAA AAA AAA AGC ACT CGT CTA CTA TCG CTA
C′	AAA AAA AAA ATA GCG ATA GTA GAC GAG TGC
D	AAA AAA AAA AAT GGT CGA GAT GTC AGA GTA
D′	AAA AAA AAA ATA CTC TGA CAT CTC GAC CAT
F	AAA AAA AAA AAT CAG GTA AGG TTC ACG GTA
F′	AAA AAA AAA ATA CCG TGA ACC TTA CCT GAT
J	AAA AAA AAA ATC TTC TAG TTG TCG AGC AGG
J′	AAA AAA AAA ACC TGC TCG ACA ACT AGA AGA
K	AAA AAA AAA ATA ATC TAA TTC TGG TCG CGG
K′	AAA AAA AAA ACC GCG ACC AGA ATT AGA TTA
L	AAA AAA AAA AGT GAT TAA GTC TGC TTC GGC
L′	AAA AAA AAA AGC CGA AGC AGA CTT AAT CAC
M	AAA AAA AAA AGT CGA GGA TTC TGA ACC TGT
M′	AAA AAA AAA AAC AGG TTC AGA ATC CTC GAC

Supplementary Table 5. Available information for patient samples from vendor.

Sample	Age	Sex	Ethnicity	Clinical Diagnosis	Year Collected	Freeze Method
Pancreas	56	Female	Caucasian	High grade pancreatic intraepithelial neoplasia	2010	-20ºC
Liver	35	Female	Caucasian	—	2010	LN_2
Ovarian	62	Female	Caucasian	Clear cell adenocarcinoma of the ovary	2010	-20ºC
Breast	41	Female	—	Adenocarcinoma, invasive of the breast	2004	—
Lung	57	Male	Caucasian	Squamous cell carcinoma of the lung	2009	—
Colorectal	70	Male	Caucasian	Adenocarcinoma of the rectum	2006	—
Prostate	64	Male	Caucasian	Adenocarcinoma of the prostate gland	2011	—

Supplementary Table 6. Comparison of working ranges for the MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel and the 8-plex microring resonator cancer biomarker assay described in this manuscript.

	MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel- Cancer Multiplex Assay*	**8-plex Microring Resonator Cancer biomarker assay**
	Working ranges	
AFP (ng/mL)	0.0747-500	0.3-20.6
ALCAM	n/a	1.0-43.7
CA19-9 (U/mL)	0.3-625	2.5-96.6
Osteopontin (ng/mL)	0.2853-500	4.3-50.3
CA15-3 (U/mL)	0.03-625	2.0-91.5
CEA (ng/mL)	0.0052-500	0.16-20.2
CA-125 (U/mL)	0.2-625	2.4-95.6
PSA† (ng/mL)	0.0014-500	0.054-4.7

* Working range was assumed to span from the Minimum detectable concentration up to the upper limit of the reported dynamic range, which may be an overestimate of the upper end of the working range. Values taken from MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 Protocol.

† Reported free PSA value.

Supplementary Table 7. Comparison of assay protocols for the MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 and the 8-plex microring resonator biomarker assay described in this manuscript.

Assay protocol:

MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel- Cancer Multiplex Assay*	**8-plex Microring Resonator Cancer biomarker assay**
1. Obtain cancer multiplex assay kit with selected markers of interest 2. Analyte standards dilution and serum samples preparation 3. Wash buffer and antibody-immobilized beads preparation 4. Aliquot beads and standards/samples to a 96-well plate, then seal plate with plate sealer 5. Agitate contents of the well plate on a plate shaker at 4°C (16-18 hrs) 6. Wash plate three times manually with the aid of a hand held magnet or an automated plate washer (5-10 min) 7. Add tracer antibodies to the well plate, seal the plate and let incubate on plate shaker at room temperature (1 hr) 8. Remove well plate seal and add streptavidin-phycoerythrin to the well plate, seal the plate and let incubate on plate shaker at room temperature (30 min) 9. Remove well contents and wash plate three times manually with the aid of a hand held magnet or an automated plate washer (5-10 min) 10. Run plate on the Luminex analyzer instrument (1-2 hrs)	1. Obtain microring resonator sensor arrays chip pre-spotted with capture antibodies 2. Analyte standards diution and serum samples preparation 3. Wash buffer, tracer and secondary antibodies preparation 4. Place assay reagents into 96-well plate, and load well plate to the instrument 5. Place sensor chip into holder cartridge connected to integrated fluid handling system of the instrument 6. Load assay recipe onto instrumentation computer to control assay reagents flow from 96-well plate to the sensor chip (1.5 hrs)
Total time per run ≥19 hrs	Total time per run 1.5-2 hrs

*Adapted from MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 (Cat #: HCCBP1MAG-58K) assay protocol