Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

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1. Materials and Methods

All chemicals were purchased from Sigma-Aldrich Chemicals (Yongin-si, South Korea). All the oligonucleotides were purchased from Bioneer (Daejeon, South Korea). The monoclonal mouse anti-human CYFRA 21-1 capture antibody (Catalog #. 4CY1-XC42) was purchased from HyTest (Turku, Finland), mouse anti-human CYFRA 21-1 detection antibody (Catalog #. 1605 SPTN-5) was from Medix Biochemica (Joensuu, Finland) and goat anti-human IgG (Catalog# ABIGG-0500) and goat anti-mouse IgG (Catalog# ABGAM-0500) were purchased from Arista Biologicals Inc. (Allentown, PA, USA), CYFRA 21-1 antigen (Catalog #. 30-AC69) was purchased from Fitzgerald (Anaheim, CA, USA), Hemoglobin (Catalog #. H7379) and biotin (Catalog #. B4501) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carboxyl group-modified fluorescent beads (FB) of 0.2 um size (excitation wavelength 622 nm and emission wavelength 645 nm, Catalog #. F8806) were purchased from Life Technologies Corporation (Eugene, OR, USA).

2. Preparation of bio-conjuates

2.1 Synthesis of CYFRA 21-1-cAb-DNA conjugate

For the synthesis of CYFRA21-1-cAb-DNA, the cAb was first activated by reacting them with 2-iminothiolane in bicarbonate buffer. The amine modified DNAs were activated with sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate) linker in 1X PBS buffer to obtain the DNA-sulfo-SMCC. Then the iminothiolane-activated CYFRA21-1-cAb was reacted with the DNA-sulfo-SMCC in 1X PBS buffer solution to obtain CYFRA21-1-cAb-DNA.[1]

2.2 Synthesis of anti-mouse IgG-Cy5

Cy5 labeled goat anti-mouse IgG, were obtained by the reaction of the amine functions in the antibodies with the Cy5 dye mono-reactive NHS ester, respectively, by following the standard protocol provided by the manufacture with the mono-reactive Cy5Dye^{TM} (GE Healthcare UK Limited, Buckinghamshire, UK).

2.3 Synthesis of anti-human IgG-Cy5

Cy5 labeled goat anti-human IgG, were obtained by the reaction of the amine functions in the antibodies with the Cy5 dye mono-reactive NHS ester, respectively, by following the standard protocol provided by the manufacture with the mono-reactive Cy5Dye^{TM} (GE Healthcare UK Limited, Buckinghamshire, UK).

2.4 Synthesis of CYFRA 21-1-dAb-FB

The labeling of CYFRA 21-1-dAb with FB were done by following the reported method. In brief, the carboxylic acid functional groups on the surface of FB were first activated by reacting them with the EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) cross-linker. The activated FB were then allowed to react with the amine functions in dAb to produce FB-dAb conjugates.[2]

2.5 Synthesis of anti-human-IgG-FB

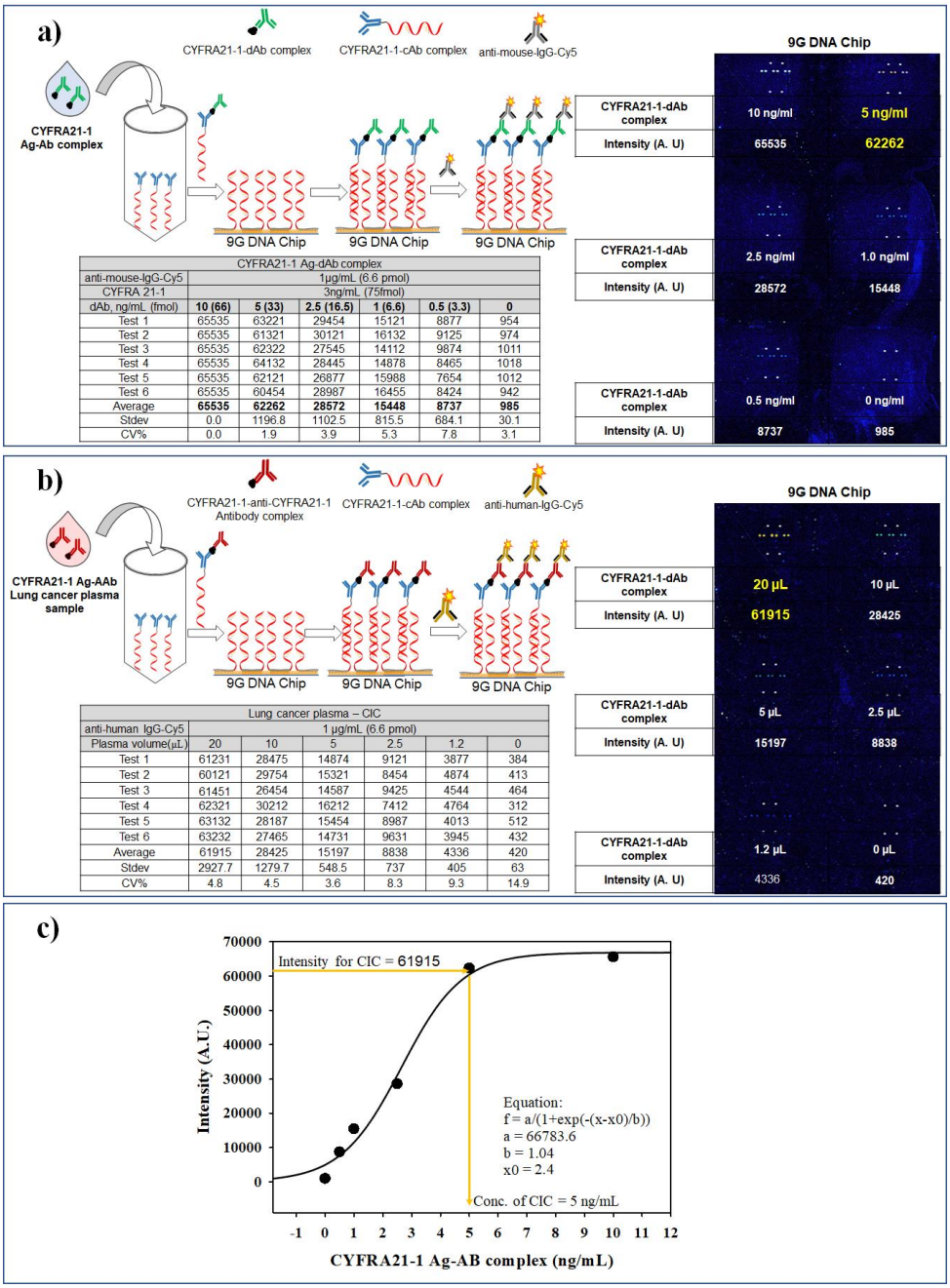
The labeling of anti-human-IgG-FB with FB were done by same above method.The carboxylic acid functional groups on the surface of FB were first activated by reacting them with the EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) cross-linker. The activated FB were then allowed to react with the amine functions in dAb to produce anti-human-IgG-FB conjugates.

2.6 Synthesis of Cy5-DNA

Cy5-DNA was obtained by the reaction of the amine functions in the amine modified oligonucleotide with the Cy5Dye mono-reactive NHS ester, respectively, by following the standard protocol provided by the manufacture with the mono-reactive Cy5DyeTM (GE Healthcare UK Limited, Buckinghamshire, UK).

3. Confirmation and quantification of CIC in the lung cancer sample using 9G DNAChip

The presence of CIC in a lung cancer sample was confirmed by using the sandwich immunoassay based on 9G DNAChip. Similarly, the amount of CIC in the lung cancer sample was quantified by using the 9G DNAChip. The lung cancer sample was found to contain 5.0 ng/mL of CIC, and this sample was used as a standard for the development of CIC detection method presented in this article. The confirmation and quantification of CIC in the lung cancer sample is explained here in brief as follows.



CYFRA21-1 Ag-dAb complex

anti-mouse-IgG-Cy5	1µg/mL (6.6 pmol)					
CYFRA 21-1	3ng/mL (75fmol)					
dAb, ng/mL (fmol)	**10 (66)**	**5 (33)**	**2.5 (16.5)**	**1 (6.6)**	**0.5 (3.3)**	**0**
Test 1	65535	63221	29454	15121	8877	954
Test 2	65535	61321	30121	16132	9125	974
Test 3	65535	62322	27545	14112	9874	1011
Test 4	65535	64132	28445	14878	8465	1018
Test 5	65535	62121	26877	15988	7654	1012
Test 6	65535	60454	28987	16455	8424	942
Average	**65535**	**62262**	**28572**	**15448**	**8737**	**985**
Stdev	0.0	1196.8	1102.5	815.5	684.1	30.1
CV%	0.0	1.9	3.9	5.3	7.8	3.1

Lung cancer plasma – CIC

anti-human IgG-Cy5	1 µg/mL (6.6 pmol)					
Plasma volume(µL)	20	10	5	2.5	1.2	0
Test 1	61231	28475	14874	9121	3877	384
Test 2	60121	29754	15321	8454	4874	413
Test 3	61451	26454	14587	9425	4544	464
Test 4	62321	30212	16212	7412	4764	312
Test 5	63132	28187	15454	8987	4013	512
Test 6	63232	27465	14731	9631	3945	432
Average	61915	28425	15197	8838	4336	420
Stdev	2927.7	1279.7	548.5	737	405	63
CV%	4.8	4.5	3.6	8.3	9.3	14.9

Figure S1: (a) CYFRA 21-1 and mouse origin anti-CYFRA 21-1 detection antibody complex detection, (b) Plasma CIC detection, (c) Standard curve using CYFRA 21-1 and mouse origin anti-CYFRA 21-1 detection antibody complex detection.

3.1. Obtaining a standard curve for CIC quantification

As shown in **Fig. S1 a)**, a standard curve was obtained by mixing the 3.0 ng/mL of CYFRA 21-1 with various concentrations of mouse origin detection antibody (dAb, 10.0, 5.0, 1.0, 0.5, 0.1 ng/mL) to obtain the CYFRA 21-1-dAb complexes, which resembles the CIC found in lung cancer samples. The obtained complexes were mixed with the hybridization solution containing CYFRA 21-1-cAB-DNA conjugate (see section 2.1 for synthesis). The 60 µL of these solutions were loaded in each chamber of 9G DNAChip and then incubated at 25 ^{0}C for 30 min. Then 9G DNAChip was rinsed with washing buffer solutions A and B successively for 2 min each and dried with commercial centrifuge (1000 rpm). Then 60 µL of solution containing anti-mouse-IgG-Cy5 (see section 2.2 for synthesis) was loaded in each chamber of 9G DNAChip and then incubated at 25 ^{0}C for 30 min. Then 9G DNAChip was rinsed with washing buffer solutions A and B successively for 2 min each and dried with commercial centrifuge (1000 rpm). The fluorescence signal on the 9G DNAChips were measured on ScanArrayLite, and the images were analyzed by Quant Array software. Each test was repeated for six times and the average values were used for the construction of a standard curve as shown in **Fig. S1 c)**.

3.2. Confirmation of presence of CIC in lung cancer sample and its quantification

As shown in **Fig. S1 b)**, lung cancer plasma sample (20.0, 10.0, 5.0, 2.5, 1.2, and 0 µL) was added to a hybridization solution containing CYFRA 21-1-cAb-DNA conjugate (see section 2.1 for synthesis). The 60 µL of these solutions were loaded in each chamber of 9G DNAChip and then incubated at 25 ^{0}C for 30 min. Then 9G DNAChip was rinsed with washing buffer solutions A and B successively for 2 min each and dried with commercial centrifuge (1000 rpm). Then 60 µL of solution containing Cy5-anti-human-IgG-Cy5 (see section 2.3 for synthesis) was loaded in each chamber of 9G DNAChip and then incubated at 25 ^{0}C for 30 min. Then 9G DNAChip was rinsed with washing buffer solutions A and B successively for 2 min each and dried with commercial centrifuge (1000 rpm). The fluorescence signal on the 9G DNAChips were measured on ScanArrayLite, and the images were analyzed by Quant Array software. Each test was repeated for six times and the average values were used for the construction of a standard curve as shown in **Fig. S1 b)**. As shown in **Fig. S1 b**, the fluorescence intensity of 61915 was observed for the 20 µL lung cancer plasma sample. By extrapolating this fluorescence intensity in a standard curve presented in the **Fig. S1 c)**, the lung cancer sample was found to contain 5.0 ng/mL of CIC.

Hence, these experiments proved the presence of CIC in lung cancer sample and this lung cancer sample containing 5.0 ng/mL of CIC was used as the standard for further experiments.

4. Optimization of Incubation time, Hybridization time, and Washing time

4.1 Incubation time

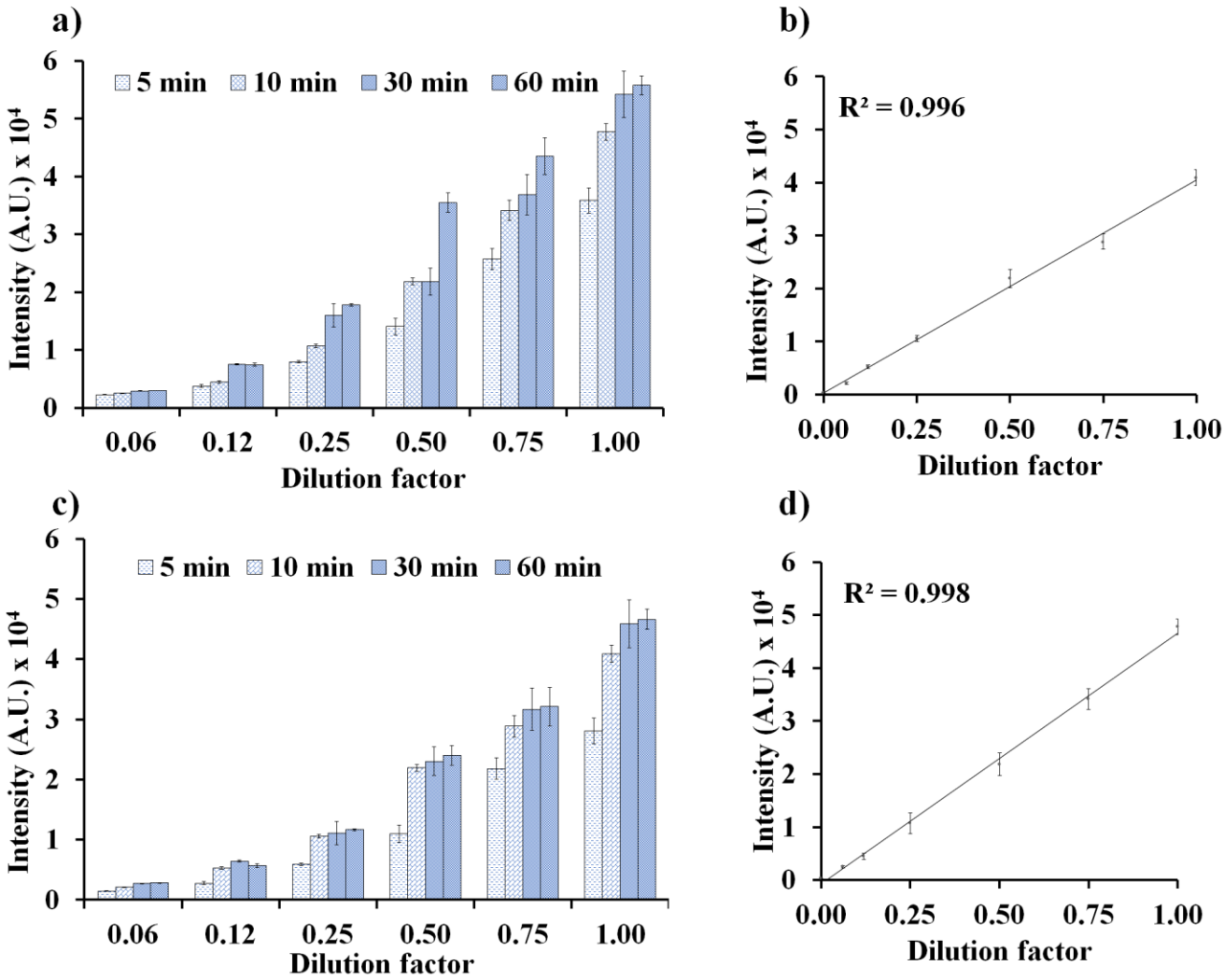


Figure S2: Optimization of the incubation time required for the detection of (a), (b) CIC and (c), (d) CYFRA 21-1. The optimum incubation time was considered as 10 min for both CIC and CYFRA 21-1 detection because the linearity coefficient (R^2) for serial dilution study (dilution factors: 1.0, 0.5. 0.25, 0.12, and 0.06) were 0.996 and 0.998, respectively. The original concentrations of CIC and CYFRA 21-1 were 2.7 ng/mL and 2.5 ng/mL, respectively.

4.2 Hybridization time

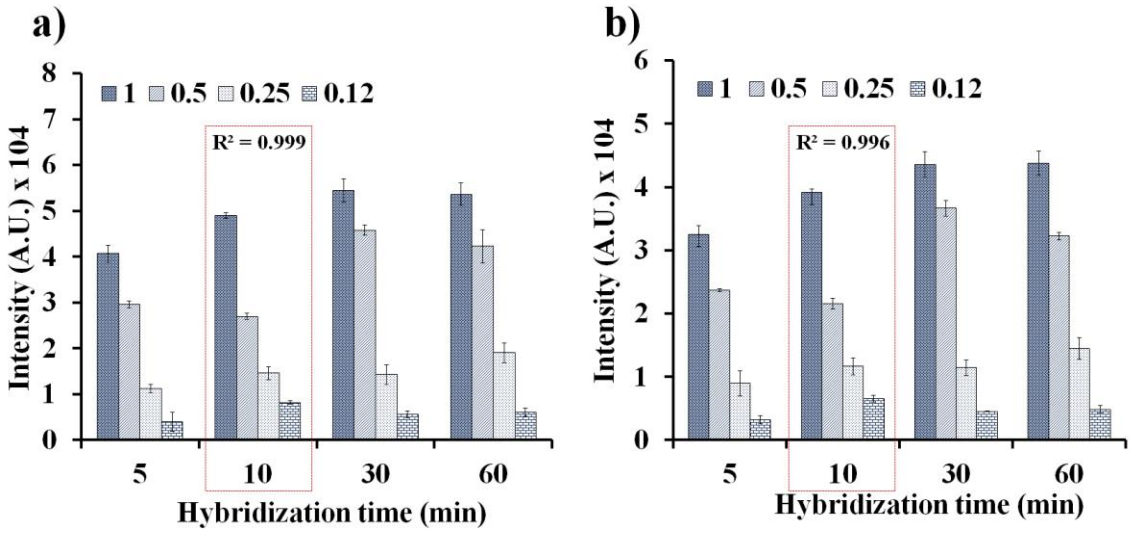


Figure S3: Optimization of the hybridization time required for the detection of (a) CIC and (b) CYFRA 21-1. The optimum hybridization time was considered as 10 min for both CIC and CYFRA 21-1 detection because the linearity coefficient (R^2) for serial dilution study (dilution factors: 1.0, 0.5. 0.25, and 0.12) were 0.999 and 0.996, respectively. The original concentrations of CIC and CYFRA 21-1 were 2.7 ng/mL and 2.5 ng/mL, respectively.

4.3 Washing time

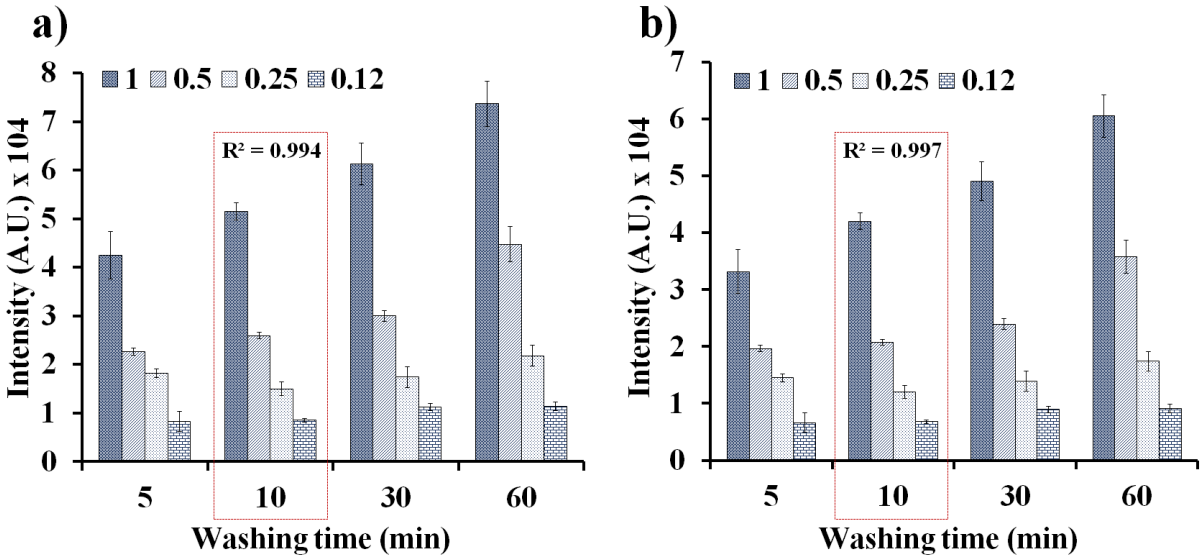


Figure S4: Optimization of the washing time required for the detection of (a) CIC and (b) CYFRA 21-1. The optimum washing time was considered as 10 min for both CIC and CYFRA 21-1 detection because the linearity coefficient (R^2) for serial dilution study (dilution factors: 1.0, 0.5. 0.25, and 0.12) were 0.994 and 0.997, respectively. The original concentrations of CIC and CYFRA 21-1 were 2.7 ng/mL and 2.5 ng/mL, respectively.

5. Linearity Study

The dilution linearity of the test was determined by diluting healthy control plasma samples (n=3) and lung cancer plasma samples (n=3). Each sample was serially diluted. Each experiment was performed in triplicate.

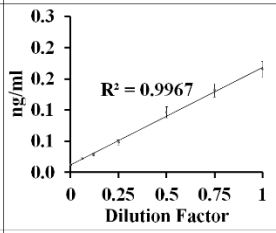
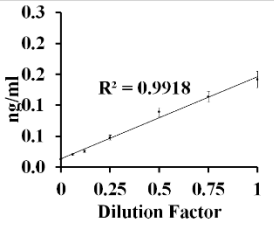
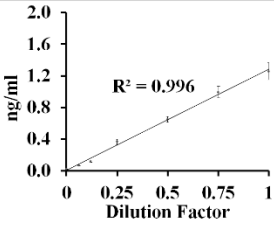
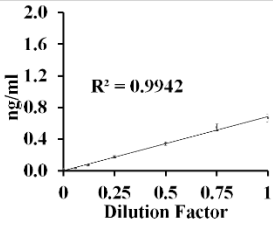
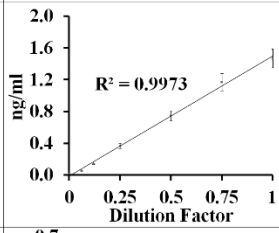
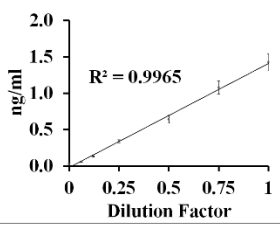
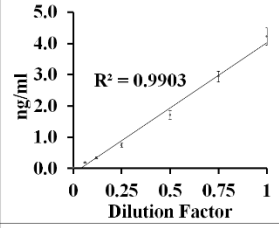
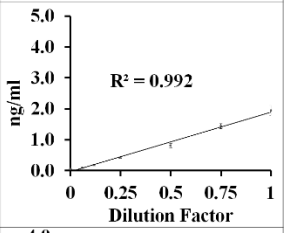
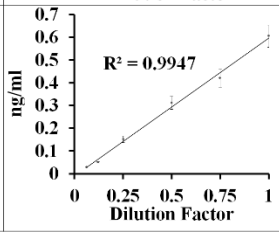
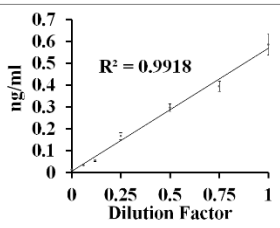
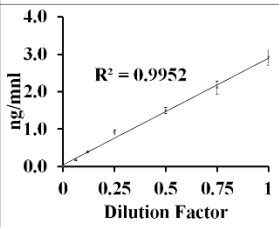
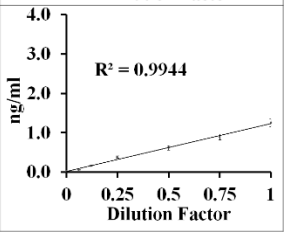
Healthy Control			Lung Cancer		
Sample	CIC	CYFRA 21-1	Sample	CIC	CYFRA 21-1
1			1		
2			2		
3			3		

Figure S5: Linearity in the serial dilution test for the detection of CIC and CYFRA 21-1 in three healthy control samples and three stage I lung cancer samples.

6. Interference study by spiking biotin, bilirubin, lipid and hemoglobin

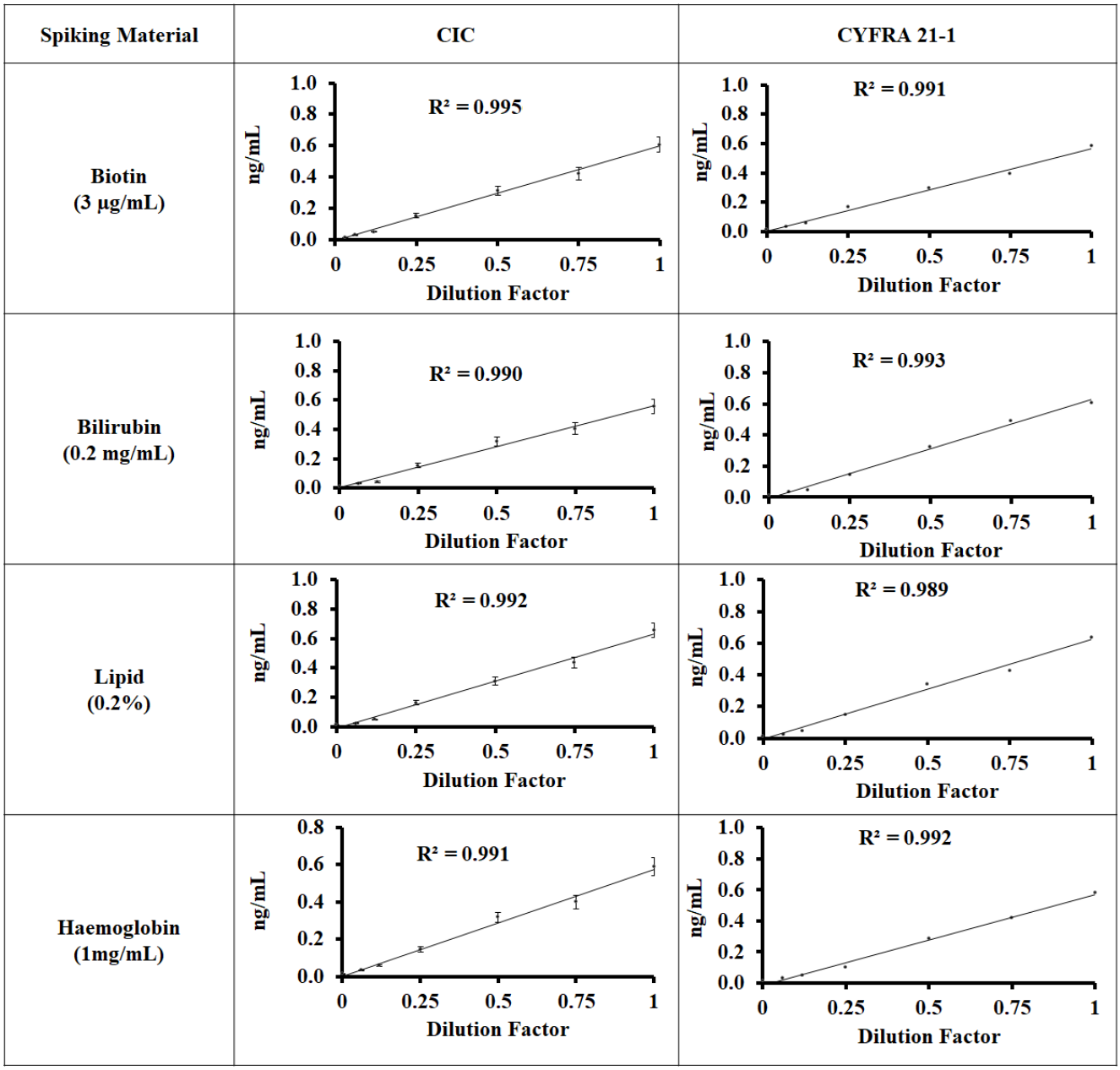


Figure S6: Determination of interference of biotin (3 µg/mL), Bilirubin (0.2 mg/mL), intra lipid (0.2%), hemoglobin (1 mg/mL).

7. Clinical Samples

During May 22, 2017 through December 31, 2018 about 120 individuals in general population and 50 cancer patients were enrolled in the study. The cancer patients with biopsy-proven primary lung cancer at Korea Cancer Central Hospital, Korea Institute of Radiological & Medical Sciences, Seoul, Korea were chosen for the study. The population included patients who had positive findings via CT imaging and who were diagnosed with pathologic or clinical stage I to IV lung cancer. Demographic data, including gender, age at diagnosis, and other clinical information, were provided by medical record. Blood samples were collected before treatment or removal of the tumor by standard surgical procedures. Written

informed consent was obtained from all participants. Ethical Clearance Committee on Human Rights Related to Research Involving Human Subjects of Korea Cancer Central Hospital, Korea Institute of Radiological & Medical Sciences, Nowon-Gu, Seoul, South Korea, approved this study (KIRAMS 2018-10-006).

8. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) or median with interquartile range (IQR) as required. Categorical data are presented as counts and percentages. Difference between healthy individuals and cancer patients of different cancer types was calculated using the Mann–Whitney U test. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) at a 95% confidence interval (CI) were calculated. Statistical analyses were performed using Medcalc for Windows version 17.4.4 (Medcalc Software, Mariakerke, Belgium).

Table S1. Characteristics of the Study Participants (n=170).

Characteristic	Healthy population (n=120)	Lung Cancer patients (n=50)
Age, years (SD)	43(35.2 - 49.2)	64.5 (59.25 - 69.75)
Male gender, n (%)	55(45.8 %)	49(98%)
CIC, ng/mL (IQR)	1.09 (0.6 – 1.8)	2.09 (1.08 – 3.77)
CYFRA 21-1, ng/mL (IQR)	0.99 (0.4 – 1.8)	1.10 (0.66 – 1.3)
CIC/ CYFRA ratio (IQR)	1.15 (0.9 – 1.4)	2 (1.60 – 2.61)

Continuous variables are given as means and standard deviation. Counts are given as numbers and percentages. Tumor marker levels are given as median and IQR. Counts are given as numbers and percentages.

Table S2: Sensitivity, specificity, PPV, and NPV of 0 ~ IV lung cancer detection by using CIC/CYFRA 21-1 ratio (n=170).

Clinical samples (n=170)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Stage I (n=25)	76 (54.8–90.6)	87.5 (80.2–92.8)	55.8 (42.9-68.1)	94.5 (89.6-97.2)
Stage II (n=10)	80 (43.3–97.5)	87.5 (80.2–92.8)	81.8 (48.2-97.7)	88.9 (51.8-99.7)
Stage III (n=13)	76.9 (40.1-94.9)	87.5 (80.2–92.8)	40 (27.5-53.8)	97.2 (92.8-98.9)
Stage IV (n=2)	50 (1.2-98.7)	87.5 (80.2–92.8)	6.2 (1.5-22.3)	99 (96.3-99.7)
Overall lung cancer (n=50)	76.0 (61.8 - 86.9)	87.5 (80.2-92.8)	66.7 (56.3-75.7)	89.4 (83.6 - 93.3)

References:

1. Y. Jung, J. M. Lee, H. Jung, B. H. Chung, *Anal. Chem*., 2007, **79**, 6534-6541.

2. S. Y. Song, Y. D. Han, K. Kim, S. S. Yang, H. C. Yoon, Biosens. Bioelectron., 2011, 26, 3818–3824.