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A Fully Integrated Lab-on-a-Disc for Multiplex Immunoassay from Whole Saliva

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2011

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A thesis
submitted to the School of Nano-
Bioscience and Chemical Engineering
and the Graduate School of UNIST in
partial fulfillment of the requirements for
the degree of Master of Science

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05. 31. 2011

Approved by

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A Fully Integrated Lab-on-a-Disc for Multiplex Immunoassay from Whole Saliva

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This certifies that the thesis of Jiwoon Park is approved.

05. 31. 2011

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Thesis supervisor: Yoon-Kyoung Cho

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Abstract

This research presents a cost-effective, rapid, and fully automated lab-on-a-disc for simultaneous detection of multiple protein biomarkers in raw samples such as whole blood or whole saliva. For the diagnosis of cardiovascular disease, here a novel centrifugal microfluidic layout was designed to conduct the simultaneous detection of high sensitivity C-reactive protein (hsCRP), cardiac troponin I (cTnI), and N-terminal pro-B-type natriuretic peptide (NT-proBNP) based on a bead-based sandwich type enzyme-linked immunosorbent assay (ELISA). Three reaction chambers are initially interconnected for the common processes such as sample injection, incubation, and washing and isolated on-demand for the independent processes such as substrate incubation and final detection. The assay performances such as the limit of detection and the dynamic range were comparable with those of the conventional ELISA despite the significant reduction of the minimum sample volume (200 μL), the amount of washing buffer (700 μL), and the total process time (20 min). In addition, two multiplex assays of three cardiovascular disease markers could be simultaneously conducted on a simple disc-shaped device in a fully automated manner.

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1. Introduction

1.1 Lab-on-a-disc

1.1.1 Lab-on-a-chip

There have been significant advances in microchip-based diagnostics. The miniaturized bioanalytical devices have many advantages compared to the conventional systems used in hospital laboratories; e.g. less consumption of sample reagents, smaller sample volume required and short assay time as well as smaller device size to make it portable. In “lab on a chip” or “Micro TAS” type of devices, all the necessary process such as sample preparation, reaction, and detection are integrated in a single chip. The Lab-on-a-chip can be useful for point-of-care test, and there are a few already commercialized examples; e.g. Cardiac reader of Roche[6] and Triage system of Biosite[7] (Figure 1). These are desktop size analyzers that can determine the concentration of important biomarkers, e.g. Troponin I, Myoglobin, D-dimer, proBNP, CK-MB to test cardiovascular disease from whole blood. The fluid is driven by capillary action inside the test-strip, and the assay is based on the lateral flow immunoassay. These kinds of devices could be useful in private hospitals or emergency rooms because they require little space, and the total analysis time is faster compared to the conventional large-sized instruments.

1.1.2 Lab-on-a-disc

The lab-on-a-disc or “LabCD” is the device that uses the centrifugal force to pump liquids. In **centrifugal pumping**, the flow rates depend on the rotational speed, radial location of the fluid reservoirs/channels, channel geometry, and fluidic properties (e.g., viscosity, density, etc.) of the sample.[15] The average velocity, U, of centrifugally-pumped liquid in a microchannel can be derived from centrifugal theory as :

$$U = \frac{D_h^2 \rho \omega^2 \bar{r} \Delta r}{32 \mu L} \tag{1}$$

Where D_h is the hydraulic diameter of the channel (defined as $4A/P$, A is the cross-sectional area and P is the wetted perimeter of the channel), ρ is the density of the liquid, ω is the angular velocity of the disc, $\bar{r}$ is the average distance of the liquid in the channels from the center of the disc, Δr is the radial extent of the fluid, μ is the viscosity of the fluid, and L is the length of the liquid in the microchannel.[15] $\bar{r}$ and Δr are defined in terms of r_0, r_1, and H, such that, $\bar{r} = (r_1 + (r_0 - H))/2$ and $\Delta r = r_1 - (r_0 - H)$ (Figure 2).[16] The volumetric flow rate, Q, is then

defined as $U \cdot A$, where U is from eqn (1). As shown in eqn (1), both the channel geometries and the fluidic properties play a large role in centrifugal pumping, in addition to the spin speed.

Valve is an essential component to control fluid flow on a disc. The chambers including several reagents required for the assay are normally closed by valve, and then the valve should be open sequentially according to the reaction protocols. In the passive type of valves such as capillary or hydrophobic or siphoning valves as shown in Figure 3, the valve opening is simply controlled by the spin speed of the disc with motor. For example, the valve opens when the centrifugal force overcomes the capillary force or surface force. However, it has disadvantages that it cannot prevent liquid from vaporizing. And the operation frequency of the valve is strongly dependent of the channel dimension. Therefore, the operation condition is very sensitive to the manufacturing error and the operation is not robust enough. The valves located in the same radial position have the similar operation condition. Therefore, it could be advantageous for high-throughput applications. However, it is not possible to integrate complicated multi-step reaction protocols. Furthermore, the spin speed should be sequentially increased. Otherwise, if the spin speed of the initial operation is higher than the following steps, all the valves could be open at once. In addition, only normally-closed valves were available for the passive type of valves.

An active type of valve, Laser irradiated ferrowax microvalve (LIFM), has been developed by Park et al.[17] The valves are made of nanocomposite materials in which 10 nm-sized iron oxide nanoparticles are dispersed in paraffin wax. The LIFM tightly seals the channel preventing the evaporation of the liquid samples and furthermore, the normally opened valves are also possible.

One of the unique capability of the lab-on-a-disc is **metering.** It is required to use precise volume of sample to achieve not only good reproducibility of the biological assay but also better quantitative analysis. For example, Gyrolab MALDI SP1 CD developed for matrix assisted laser desorption ionization (MALDI) sample preparation, a common distribution channel feeds several parallel individual sample preparation fluidic structures.[18] Reagents are introduced by the capillary force exerted by the hydrophilic surfaces into the common channel and defined volume (200 nL) chambers until a hydrophobic valve stops the flow. When all of the defined-volume-chambers are filled, the CD is spun at a velocity large enough to move the excess liquid from the common channel into the waste. The velocity is not so high as to allow liquid to move past the hydrophobic valve and the well-defined volume chambers remain filled.

Mixing is also important step in most of biological assays. However, mixing is a difficult task in microfluidic systems due to laminar flow because there is no convection.[19-21] To overcome this challenge, a number of approaches have been demonstrated on centrifugal microfluidic platforms.[22-29] One method is to utilize the rapid oscillations of disc between clockwise and counter clockwise rotation.[25,27] In addition, Brenner et al. used magnetic particles in the chamber and an external magnet to achieve effective mixing.[25,30]

Lab-on-a-disc can be applied to various biomedical diagnostic applications. It has been applied to bioanalysis such as sample preparation,[8,12,31-36] immunoassay,[12,14,37-40] DNA microarray,[41-45] PCR,[46,47] Cell lysis,[23,48-51] etc. Additionally, it has been successfully commercialized as a diagnostic device for point of care test. For example, Piccolo clinical blood analyzer which is for blood-based clinical biochemistry analysis has been developed by Abaxis (Figure 4A).[8] The GyroLab workstation handles all the operating steps of immunoassay automatically (Figure 4B).[10] More recently, Samsung launched the blood analyzer in the market and they also demonstrated the capability of analysis of immunoassay (Figure 4C).[12,11]

Lab on a disc for immunoassay developed from Samsung has advantages comparing with Gyros. It fully integrates all procedure of immunoassay from blood separation to detection. And it does not require robotic system for loading reagent because all reagents already are stored in chamber of disc.

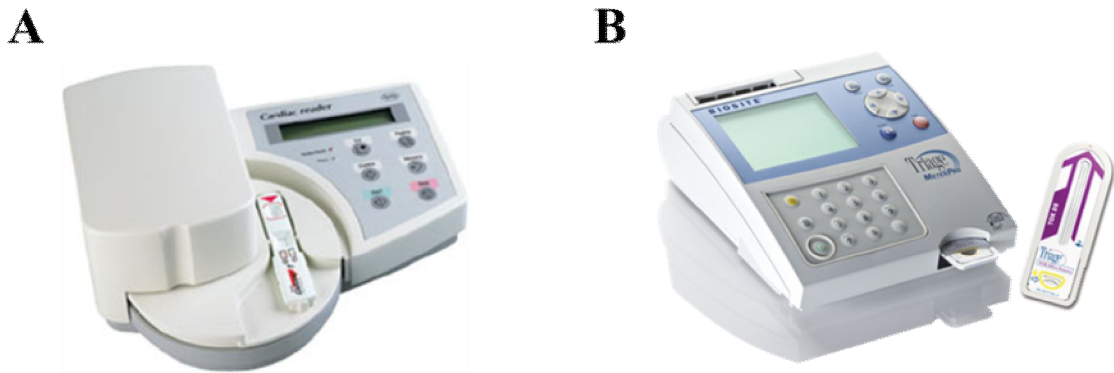


Fig 1 Examples of commercially available diagnostic devices for point-of-care. (A) Cardiac reader of Roche.[6] (B) Triage system of Biosite.[7]

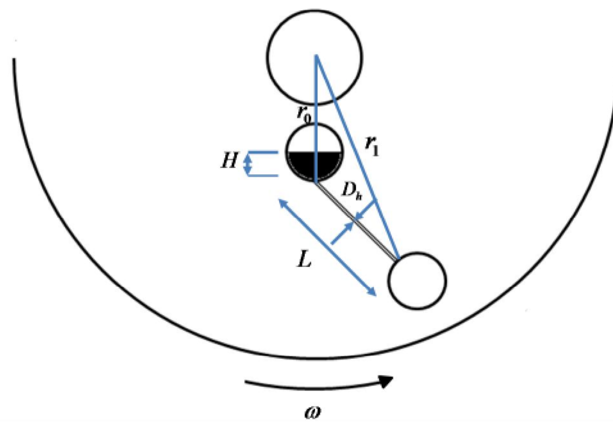


Fig 2 Schematic diagram of a liquid being pumped through a simple fluidic network in a centrifugal system. r_0 and r_1 are inner and outer radii of the flowing fluid, respectively; H is the "head" of liquid in the feed reservoir, D_h is the hydraulic diameter of the channel; L is the length of the channel; ω is the rate of rotation of the spinning disc.

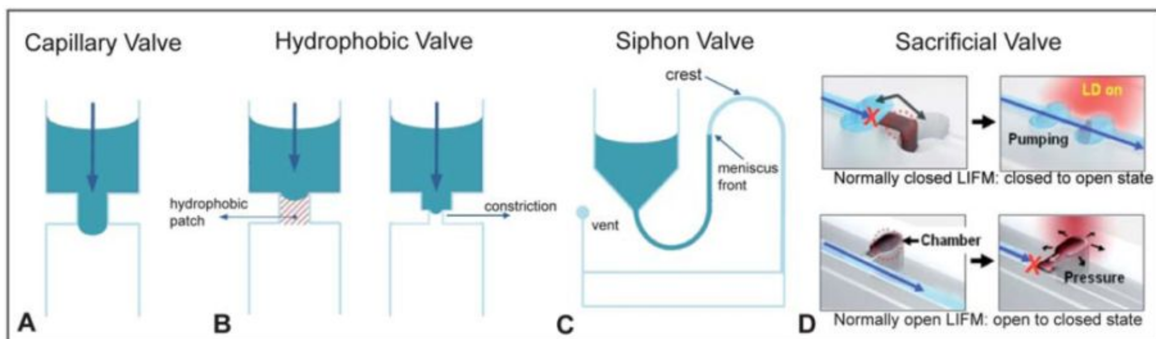


Fig 3 Centrifugal microfluidics-based valving methods.[11] (A) Capillary valve using a hydrophilic microchannel. (B) Implementation of hydrophobic valving on a disc. (C) Siphon valve. (D) Sacrificial valve made from wax utilizing laser assisted heating for operation.

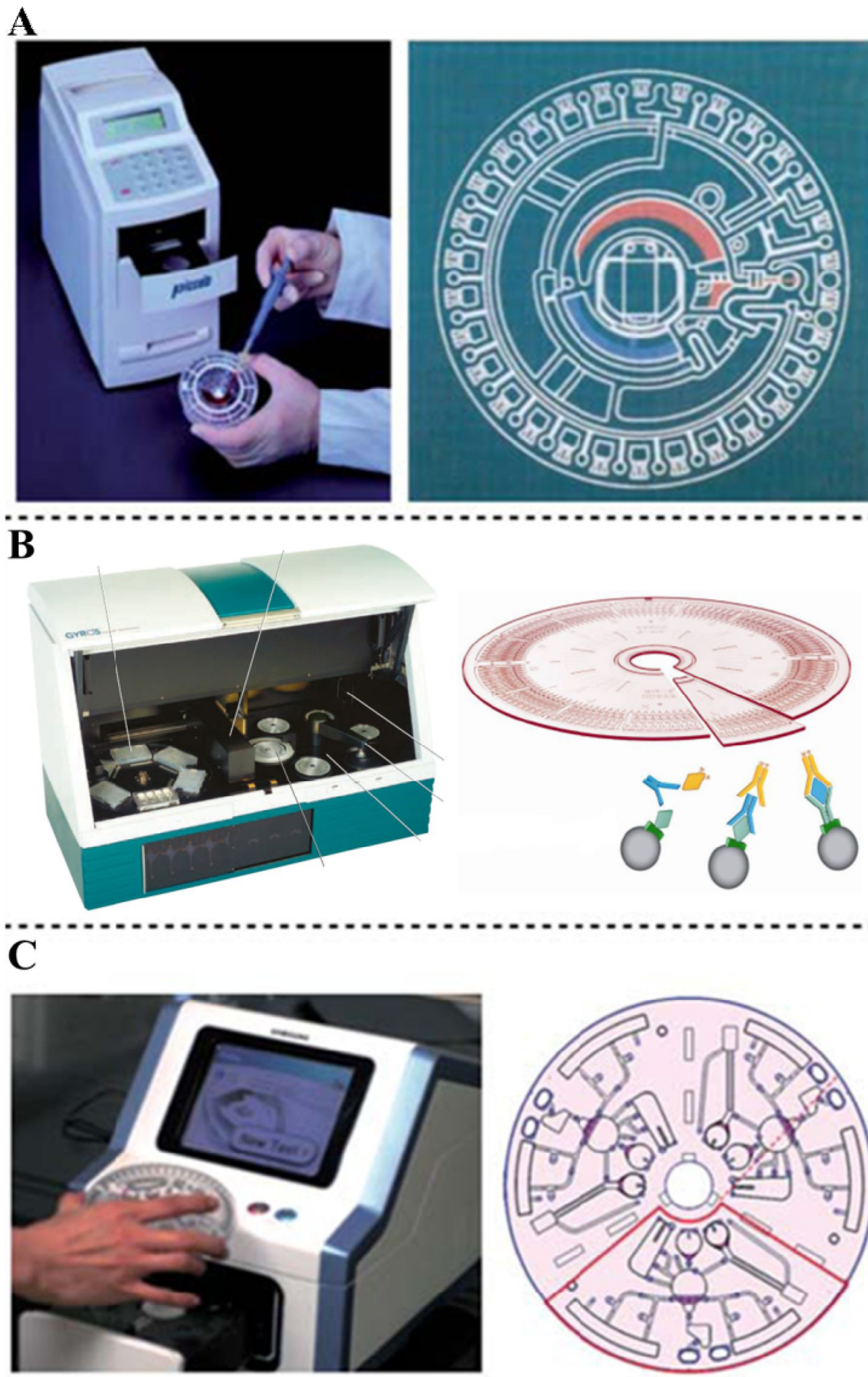


Fig 4 Example of commercial disc-based device. (A) The Piccolo clinical blood analyzer system from Abaxis.[8] (B) The immunoassay system from Gyros.[10] (C) The integrated ELISA assay system from Samsung.[12,]

1.2 Immunoassay

1.2.1 Immunoassay

Immunoassay is used to determine the concentration of a target protein in biological samples such as serum, saliva, and urine. Immunoassay is based on the specific binding capability of an antibody to the target antigen. The antibody binding affinity for the antigen is a critical factor to determine the detection sensitivity of the immunoassay.

The ELISA (Enzyme-linked immunosorvent assay) has been used as a diagnostic tool (Figure 5). Two types of ELISA which are sandwich immunoassay and competitive immunoassay are used generally to quantify unknown amounts of target analytes.

A sandwich immunoassay is a method using two kinds of antibodies, which bind to different sites on the antigen (Figure 6A). The capture antibody is attached to a solid surface. The antigen is added and then followed by addition of a detection antibody. The detection antibody binds the antigen to a different epitope than the capture antibody. The substrate for the enzyme is added to the reaction that forms a colorimetric readout as the detection signal. The signal generated is proportional to the amount of antigen present in the sample.

A competitive immunoassay is based on the competition of labeled and unlabeled antigen for a limited number of binding sites (Figure 6B). Competitive immunoassays are often used to measure small amount analytes. These assays are also used when a matched pair of antibodies to the analyte does not exist. Only one antibody is used in competitive ELISA. As the concentration of unlabeled analyte is increased, less labeled analyte can bind to the antibody and the measured response decrease. Thus the lower the signal, the more unlabeled analyte there is in the sample. The standard curve of a competitive binding assay has a negative slope.

Technically, newer assays are not strictly ELISAs as they are not “enzyme-linked” but are instead linked to some non-enzymatic reporter such as fluorescent, chemiluminescent dye, gold, silver particle, magnetic particle etc. However, given that the general principles in these assays are largely similar, they are often grouped in the same category as ELISAs.

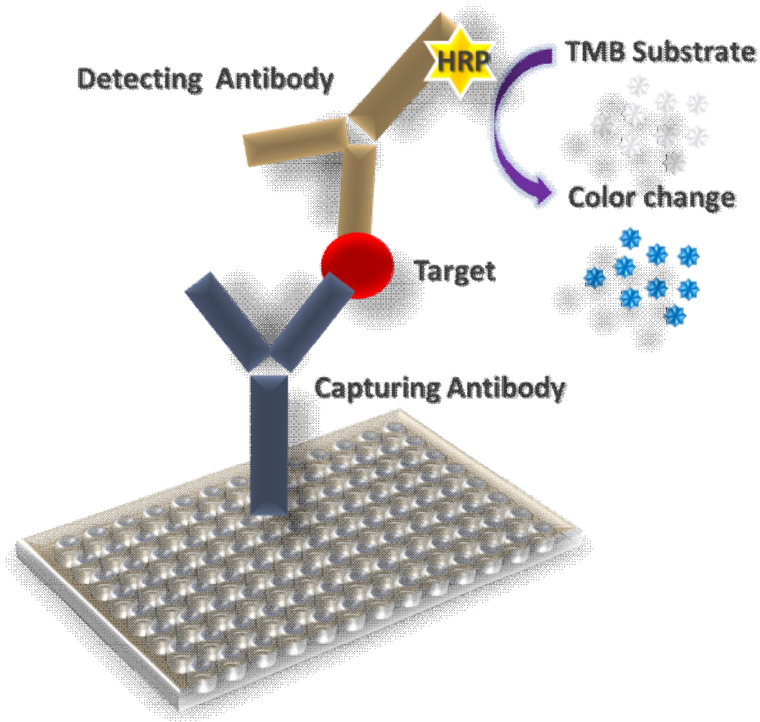
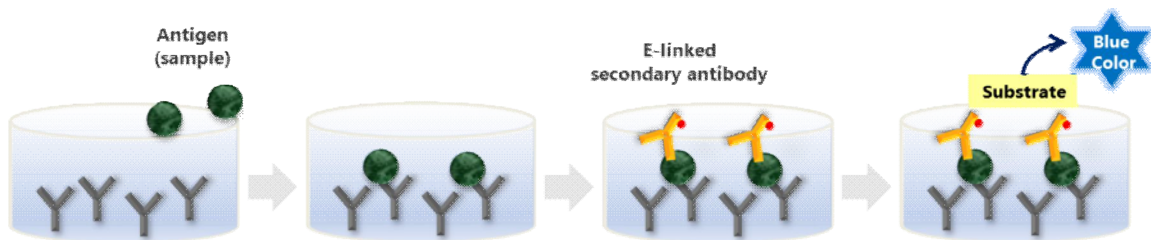


Fig 5 Schematic diagram of ELISA.



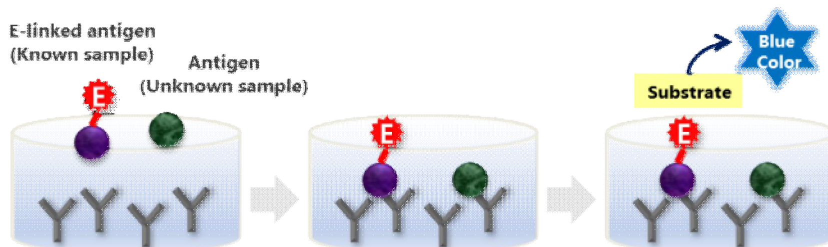


Fig 6 Schematic diagram of the principle of the ELISA. (A) Sandwich immunoassay. (B) Competitive Immunoassay.

1.2.2 Microfluidic chip-based immunoassays

Commercial ELISA kit is routinely used in medical diagnostics. However, it is labor-intensive because the assay protocol requires multiple manual operation steps, it takes long reaction time, typically more than 3 hours, and it is expensive, the price of the typical 96 well plate is more than $50, and the detection sensitivity needs to be improved.

To compensate these drawbacks, many kinds of microfluidic systems have been developed for immunoassay. Using microchip-based immunoassays, the reaction between antigen and antibody could be faster because of high surface to volume ratio. Therefore, it could reduce the reagents consumption as well as the assay time.

Previous studies about the immunoassays on a microfluidic chips could be grouped depending upon the method of antibody immobilization on the substrates, driving force to transfer liquids, and the strategies to incorporate the multiplex detection capabilities.

Several technologies were demonstrated to immobilize antibody on the surface of chip. Solid supports may consist of microchip channel or beads made form plastic, silica or glass, and magnetic materials. One of the immobilization methods is physical adsorption of antibodies onto the surface.[52,53] This method results in a random orientation and potentially lowered binding activity. To overcome these issues, covalent bonding and bioaffinity binding methods have been developed by using surface modification technology.[54-62] For examples, long flexible linkers such as polyethyleimine (PEI)[57] and dextran (DEX)[58] are used for covalent attachment. Other strategies of immobilization include the use of lipid,[54] DNA,[61] poly (ethylene glycol) (PEG),[60] Protein G,[62] etc.

Microbeads as solid supports offer higher surface to volume ratio than 2D surface. Therefore the reaction efficiency is better due to the reduced diffusion distance. Sato *et al.* has demonstrated to perform immunoassay using 45μm diameter polystyrene beads, and they made dam structure in microchannel to trap the beads (Figure 7A).[1] However, the reaction and washing efficiency were decreased because the efficient mixing was difficult. Magnetic beads were often used as well because the surface modification was relatively simple and the handling for particle concentration, separation and resuspension were relatively easy.[63]

The most popular method of for the fluid transfer on the chip is pressure-driven force using syringe pumps. However, this could be troublesome to miniaturize because each reagents need to be connected to individual pumps.

Capillary force is easy to operate and require minimum power consumption,[64] and it can be implemented to the portable device. As an interesting example, Gervais *et al.* developed portable microfluidic system which enables one step immunoassay by using capillary force to drive fluid (Figure 7B).[9] According to this report, within a minute after the addition of the sample onto the loading pad, the sample fills the chip up to the capillary pump. The capillary pump has the largest capillary pressure on the chip and it determines the flow rate together with the flow resistor, and the maximum volume of sample used in the immunoassay. Using this chip, they detected CRP at a concentration below 1 ng/mL in 5 minutes using 5 μL of human serum. It is advantage compared to commercially available lateral flow chip for immunoassay that requires large volume of sample, e.g, Triage system from Biosite uses 150 μL serum, Cardiac reader from Roche uses 150 ± 15 μL.

Lab-on-a-disc type devices use centrifugal force to transfer liquids. It is possible to perform sequential procedures of the immunoassay by designing the location of the chamber and controlling the spin frequency properly. The centrifugal microfluidic devices are easily implemented to the bioanalysis system because it is not very sensitive to physiochemical properties of fluids compared to electrokinetic transport mechanisms. In addition, it is capable to integrate several types of detection method; e.g. colorimetric or fluorescence detection.[12,14,39,40]

In the most innovative approach so far, Lee *et al.* has developed bead-based immunoassay on a lab-on-a-disc to detect antigen or antibody of hepatitis B virus (Figure 7C).[12] The device is fully integrated with the following steps; e.g. plasma separation from whole blood by using centrifugal force, binding of target sample on the surface of microbeads previously modified with antibodies, several steps of washing, reaction with enzymes, and the detection.

Multiplex detection is important in many biomedical applications because the diagnostics based upon multiple biomarkers could be more accurate. There have been many approaches to achieve the multiplex immunoassay on the microfluidic system.[13,65-67] First, a microarray type platform was developed by patterning various antibodies or antigens on the surface. For examples, mosaic format immunoassays based on patterning lines of antigens onto a surface by means of a microfluidic network.[68] Solutions are delivered by the channels of a second microfluidic network across the pattern of antigens. Another pattering method is by using single strand DNA for immobilizing capture antibody. The ssDNA is immobilized on the surface and then complementary ssDNA labeled antibodies are immobilized by binding of two complementary ssDNA on the surface. Fan et al. is demonstrated multiplex immunoassay by using this kind of patterning technology using ssDNA (Figure 7D).[13] This system is capable of high throughput multiplex immunoassay. But it requires several complex procedures such as synthesis of ssDNA, surface modification, and so on.

Second, multiple kinds of microbeads were utilized as a surface for the immunoassay.[14,69,70] For example, Riegger et al. developed centrifugal microfluidic device which use three different quantum dot labeled beads to do multiplex immunoassay (Figure 7E).[14] Three different antibodies coated on the quantum dot bead, it was capable to identify each single bead by using specific wavelength characteristic of quantum dot beads. This system has a single reaction chamber. Though different kinds of beads are mixed in single chamber, it could be identified each single bead by using CCD camera, and then measured fluorescence signal. Using quantum dot for multiplex immunoassay is limited because several kinds of quantum dots should be used without overlapping the wavelength characteristic. And fluorescent labels may increase the sensitivity, but will increase the cost of the assay, because more sophisticated hardware and software are needed to read the signal by scanning each bead.

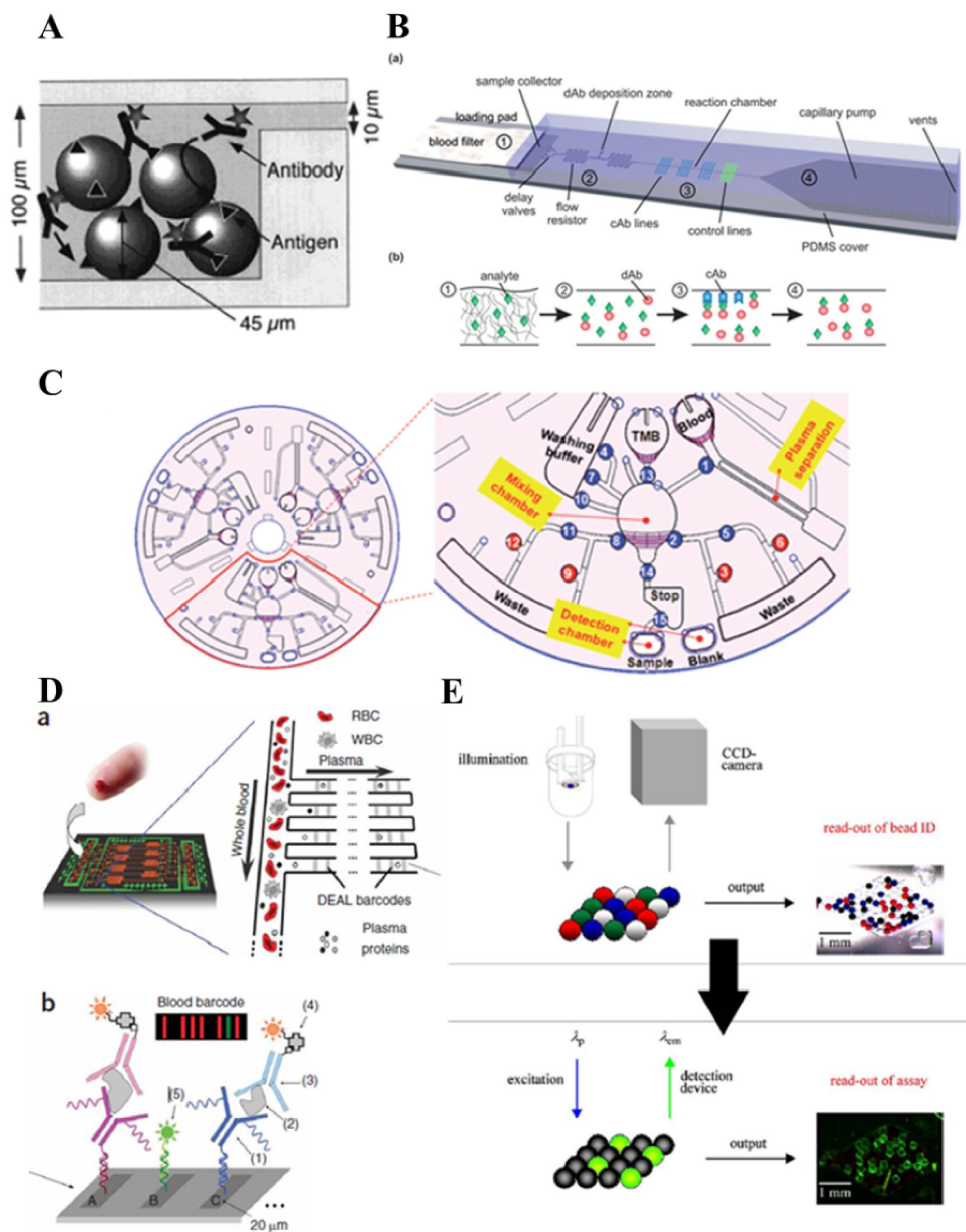


Fig 7 Examples of immunoassay in microfluidic systems. (A) Immunoassay on microbeads. This figure shows the dam structure for trapping of microbeads.[1] (B) Lateral flow chip for immunoassay. This chip uses capillary force.[9] (C) Centrifugal-based microfluidic system for immunoassay.[12] (D) Blood barcode chip for immunoassay. The antibody is immobilized by using ssDNA on the surface. [13] (E) Multiplex immunoassay using quantum dot beads.[14]

1.3 Oral Fluid based Diagnostics

1.3.1 Properties of oral fluids

Saliva is a hypotonic fluid composed mostly of water, electrolytes and organic molecules (i.e., amino acids, proteins and lipids). The concentration of components in saliva is mostly smaller than blood (Table 1). For example, the steroid hormone levels in saliva/plasma ratio are at minimum about 1:10 (e.g. cortisol) up to more than 1:100 (e.g. testosterone) (Table 2). In the following tables many analytes present very small concentration in saliva and this requires the development of highly sensitive method for assessment of analyte levels in saliva.

In addition, saliva consists of other compounds such as mucus, bacteria cells. Mucus has unique rheological properties (e.g., high elasticity, adhesiveness, and low solubility). Such properties can pose problems in microfluidic system for salivary diagnostic. The viscosity can be decreased by removing mucus by ultra filtration,[71] or changing of the pH,[72] or freezing and thawing.[3]

1.3.2 Biomarkers for salivary diagnostics

The most common sample for the biomedical diagnostics is cellular and chemical constituents of blood. However, other biological fluids are also utilized for the diagnosis of disease, and saliva offers unique advantage in sample collection because it is less invasive, painless and convenient. Whole saliva contains locally produced as well as serum-derived biomarkers that have been found to be useful in the diagnosis of variety of systemic diseases (Figure 8).

For example, periodontal disease can be determined by detecting many biomarkers in saliva.[73] Periodontal disease is a chronic infection involving biofilms of Gram-negative and Gram-positive bacteria characterized by persistent inflammation, breakdown of the connective tissue and destruction of alveolar bone.[74] It affects approximately 45 % of adults in the USA over 50 years of age, and is a major cause of tooth mobility and tooth loss worldwide.[75] Systemic markers related to periodontal disease are C-reactive protein (CRP)[76] and matrix metalloproteinase-8 (MMP-8).[77] Interleukin-1 beta released during the initiation of an inflammatory response and pyridinoline cross-linked carboxyterminal telopeptide of type Ⅰ collagen (ICPT) are also used as potential biomarkers for periodontal disease.[78,79]

Some preliminary studies have suggested that salivary proteins can serve as biomarkers for human cancer detection.[80-85] Testing of human epidermal growth factor receptor-2 (HER2) and cancer antigen CA 15-3 in saliva may be promising way for monitoring progression and recurrence of breast cancer.[84] And cancer antigen CA125 is used as biomarker to determine the ovarian cancer. Oral cancer is high-

effect local disease in the oral cavity affecting over 300,000 people worldwide annually.[86] Delayed detection increase mortality rate of oral cancer patients because patients with oral cancer often present symptoms at a late stage. This supports the need for sensitive biomarkers to improve early detection of oral cancer. Recently, protein biomarkers for oral cancer is identified, including Mac-2 binding protein, CD59, Calgranulin B, Catalase, Profilin.[87]

Cardiovascular diseases (CVD) are the most murderous disease in the United States. According to the report from American Heart Association, nearly 2,300 Americans die of cardiovascular disease each day on the basis of 2006 mortality rate data. Symptoms of cardiovascular disease vary depending on the type of cardiovascular disease. The general symptom is chest pain. However, some people even have a cardiovascular disease without chest pain. Such disease increases the risk of developing life-threatening complications. Thus, the early diagnosis is required to detect cardiovascular disease in its early stage before serious complications occur.[88] In addition, the people who have experience cardiovascular disease need to monitor the possibilities of other outcome.

Generally, the diagnosis of cardiovascular disease is to measure protein level in blood. The typical biomarkers to determine cardiovascular disease are cardiac enzymes; Troponin I, Troponin T, CK-MB, Myoglobin.[89] The temporal pattern of appearance of these markers is important for diagnosis. Recently, high-sensitivity C-reactive protein (hsCRP), and proBNP are used as promising biomarkers for diagnosis and prognosis for cardiovascular disease.[90] Especially, salivary levels of hsCRP are significantly high in patients who have cardiovascular disease.[91] According to report, hsCRP showed the highest ration in median concentrations of acute myocardial infarction (AMI) /control; ratio value is 72.

Table 1 Analyte levels in saliva and in plasma[4]

Analyte	Mixed Saliva	Plasma	Unit
In General			
Water	97 – 99.5	90 - 93	%
pH	(5.6)6.4 – 7.4 (7.9)	7.4	
Substrates			
Albumin	246 – 344	34 000 – 48 000	mg/l
Cholesterol	3 - 15	150 – 300	mg/dl
Creatinine	0.07 – 0.2	< 1.1	mg/dl
Glucose	< 2	55 – 115	mg/dl
Protein	1.1 – 1.8 (6.4)	66 – 87	g/l
Urea	17 – 41	< 50	mg/dl
Uric Acid	0.7 – 6.0	< 7.0	mg/dl
Enzymes (37 °C)			
α-Amylase	11 900 – 305 000	< 220	U/l
AP	< 11	< 270	U/l
SGOT (ASAT)	< 43	< 38	U/l
SGPT (ALAT)	< 11	< 41	U/l
LDH	113 - 609	< 480	U/l
Lysozyme	6 - 12	3 – 9	mg/l
Electrolytes/Minerals			
Calcium	0.88 – 2.05	2.20 – 2.55	mmol/l
Chloride	5 – 40	96 – 108	mmol/l
Magnesium	0.08 – 0.56	0.70 – 1.05	mmol/l
Phosphate	1.4 – 13.2	0.87 – 1.45	mmol/l
Potassium	6.4 – 37	3.3 – 5.1	mmol/l
Sodium	2 – 21	133 – 145	mmol/l
Other			
IgA	42 – 174	850 – 4 000	mg/l
CBG, male	38 ± 18	39 700 ± 6 300	µg/l
CBG, female	72 ± 71	42 200 ± 5 600	µg/l
SHBG, male	19 ± 10 ??	15 – 100	nmol/l
SHBG, female	63 ± 60 ??	15 - 120	nmol/l
Transferrin	< 0.5	250 - 350	mg/dl

Table 2 Steroid hormone levels in saliva and in plasma[4]

Analyte	Remark	Mixed Saliva	Plasma	Unit
Aldosterone	non pregnant female	29 – 118	80 – 790	pmol/l
Androstenedione	adult men	140 – 630	1200 – 11000	pmol/l
	adult women	62 – 482	1400 – 11900	pmol/l
Cortisol	Cortisol peak in adults	13.8 – 48.9	190 – 690	nmol/l
	8 hrs. after peak	1.4 – 8.6	55 – 250	nmol/l
DHEA	premenop. women	0.3 – 1.0	4.5 – 34.5	nmol/l
	adult men	0.3 – 1.7	6.2 – 43.3	nmol/l
Estradiol	follicular phase	2 – 18	26 – 650	pmol/l
	midcycle „peak"	9 – 29	180 – 1420	pmol/l
Estriol	40 weeks gestation	4.5 – 9.8	330 – 1596	mmol/l
Estrone	adult women	10 – 21	92 – 1294	pmol/l
	adult men	10 – 21	92 – 555	pmol/l
17- OH Progesterone	adult men	50 – 360	150 – 4900	pmol/l
	luteal phase	140 – 320	600 – 8800	pmol/l
Progesterone	follicular phase	< 160	500 – 3500	pmol/l
	luteal phase	200 – 1600	4900 – 72000	pmol/l
Testosterone	premenop. women	10 – 52	200 – 2860	pmol/l
	adult men	95 – 205	9900 – 27800	pmol/l
5α - Dihydrotestosterone	premenop. women	10 – 26	80 – 1270	pmol/l
	adult men	34 – 172	860 - 3410	pmol/l

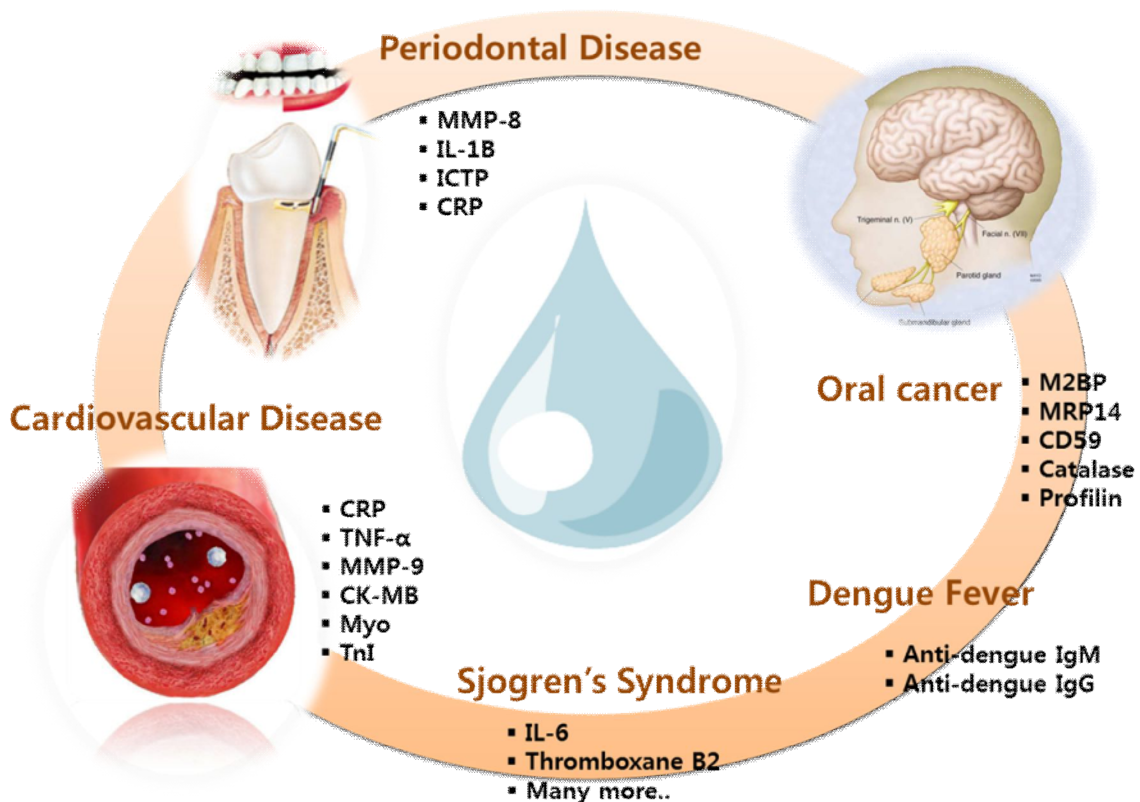


Fig 8 The potential diseases and biomarkers for salivary diagnostics.

1.3.3 The biomarker of cardiovascular disease: hsCRP

The American Heart Association (AHA) and the Centers for Disease Control and Prevention (CDC) group have established guidelines for hsCRP test using whole blood: Levels less than 1 μg/mL indicate a low cardiovascular risk levels between 1 μg/mL and 3 μg/mL correspond to moderate cardiovascular risk, and those greater than 3 μg/mL correspond to high risk (Table 3).

The studies on measuring hsCRP in microfluidic chip have been advanced for highly sensitive detection (Table 4).[2,92-98] Tsai *et al*. measured hsCRP using antibody-coated magnetic nanoparticle.[92] In this study, the limit of detection is 120 ng/mL which is not better than traditional ELISA. Yang *et al.* used DNA aptamer immobilized magnetic bead for detection hsCRP.[93] Aptamer is used instead of antibody because it is easy to synthesize and cheaper than antibody. In this study, the LOD is 12.5 ng/mL in PBS buffer. Additionally, Yamada *et al.* developed anisotropically patterned nanosieve array (ANA) chip that can measure hsCRP by separating bound target antibody from unbound antibody.[94] The LOD is 50 ng/mL. And another study is using SPR-based detection as label-free method. Meyer et al. developed immunosensor for monitoring complex of antigen-antibody by immobilizing antibody on the gold surface by using biotin-streptavidin complex.[96] SPR-based immunosensor is advantageous because it enables monitoring in real-time and detecting for multi-targets. However, in this study, the LOD is 1 μg/mL that is not better than other studies. In addition, it requires longer time for the measurement.

Table 3 Clinical cut-off level of hsCRP in blood

Disease	*Risk classification*	*hsCRP level(μg/mL)*
Cardiovascular disease(CVD)	Low risk	< 1.0
	Average risk	1.0-3.0
	High risk	>3.0

Table 4 Representative examples of microfluidic chip for detection of hsCRP

Method	*LOD*	*Assay Time*	*Dynamic Range*	*Sample Type*	*Reference*
Lateral flow polymer chips	2.6ng/mL	Not mentioned	2.6ng/mL-0.3μg/mL	CRP depleted serum	Lab chip, 2008, 8, 1191.
Anisotropic nanosieve array(ANA) structures	50ng/mL	Not mentioned	0.02μg/mL-11.5μg/mL	CRP free human serum	Anal.Chem., 2009, 81, 7067.
Power free Microchip dendritic amplification	0.15pM (0.017ng/mL)	<30min	0-2.3μg/mL	CRP free human serum	Anal.Chem., 2007, 79, 6000.
Magnetic Biosensor	10-25ng/mL	<30min	25ng/mL-2.5μg/mL	Serum, Saliva, Urine	Biosensors & Bioelectronics, 2007, 22, 973.
Magnetic Nanoparticles	0.2μg/mL	11.5min	0-20μg/mL	Whole blood	Anal.Chem., 2005, 77, 5920.
Magnetic Nanoparticles	0.12μg/mL	<10min	1.2-310μg/mL	Serum	Anal.Chem., 2007, 79, 8416.
Magnetic beads with aptamer	12.5ng/mL	<25min	0.01-10μg/mL	Buffer (1%BSA)	Biosensors & Bioelectronics, 2009, 24, 3091.
SPR based immunosensor	1μg/mL	<1h	2-5μg/mL	Buffer	Biosensors & Bioelectronics, 2006, 21, 1987.
Electronic taste chip	5fg/mL	12min	10fg/mL-10pg/mL	Saliva	Lab chip, 2005, 5, 261.

1.3.4 Examples of oral fluids based diagnostics in microfluidic chip

The salivary diagnostics using POCT have been an interesting topic. However, the salivary diagnostics is much more challenging because the target concentration in saliva is much lower, e.g. 10 ~ 100 times lower, compared to the one in blood.

For example, Christodoulides *et al.* in University of Texas at Austin group have developed electronic taste chip for the measurement of hsCRP in human saliva.[2] They used bead array as an assay platform and fluorescence modified antibody (Figure 9A). They reported much higher sensitivity compared to the traditional ELISA, 5 fg/mL vs. 1 ng/mL. However, the whole saliva could not be used directly and the sample was required to be diluted at least 1000-fold because of the viscosity of whole saliva. Therefore, the sensitivity starting with real sample is 5 pg/mL and has a useful detection range between 10 fg/mL to 10 pg/mL.

Herr *et al.* in Sandia National Laboratories developed a microchip-based immunoassay, which could do sample enrichment using electrophoresis technique (Figure 9B).[3] They detected the collagen-cleaving enzyme matrix metalloproteinase-8 (MMP-8) as a biomarker of periodontal diseases in less than 10 min. The MMP-8 values measured by the proposed method was comparable to the one measured by conventional ELISA. But, they also used diluted saliva sample for the assay in the microchips.

Fang Wei *et al.* in UCLA group demonstrated the detection of the multiplex biomarkers to determine the oral cancer related biomarkers by using electrochemical sensors.[5] They detected two biomarkers; IL-8 mRNA and IL-8 protein (Figure 9C). The results are very close to the data measured by ELISA and PCR method. But, this system was not fully integrated. The table 5 is summarized about these salivary diagnostic systems.

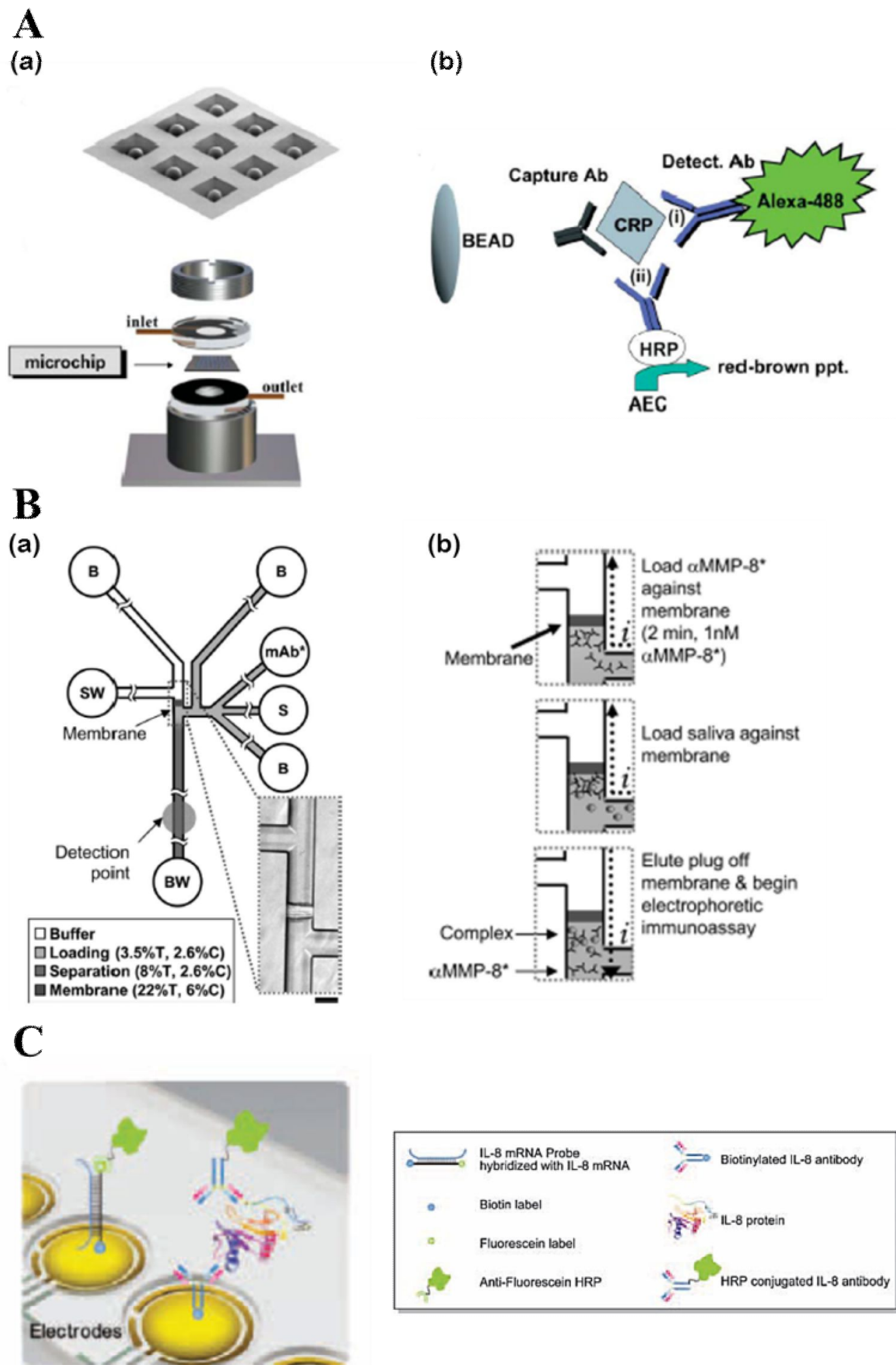


Fig 9 Examples of oral fluids based diagnostics. (A) This figure shows the position of bead-loaded microchip (a), The immunocomplex of bead-based CRP assay (b).[2] (B) The microchip electrophoretic immunoassay device layout (a), The procedure of sample enrichment on the device (b).[3] and (C) Illustration of the EC sensor with mRNA and protein detection.[5]

Table 5 Comparison with various micrfluidic system for salivary diagnostics

Target	***Platform***	***Assay Time***	***Sample volume***	***Sample Type***	***Detection method***	***Integr -ation***	***Reference***
hsCRP	Bead-based array microchip	12min	Not mentioned	Diluted saliva	Fluorescence	X	Christodoulides et al., Lab chip, 2005
MMP-8	Electrophoretic microchip	<10min	20μL	Diluted saliva	Fluorescence	Δ	Herr et al., PNAS, 2007
IL-8 mRNA, IL-8 protein	Electrochemical sensor	<10min	100μL	Whole saliva	Electrochemical	X	Fang Wei et al., Clin.Chem, 2009

1.4 Research Outline

1.4.1 Objective of the thesis

The aim of the proposed research is to develop a fully integrated lab-on-disc for multiplex immunoassay from whole saliva. The lab-on-a-disc can finish the full procedures of the multiplex immunoassay (up to 3 target analytes) starting from whole saliva to the final detection within 20 minutes.

1.4.2 Novelty of the thesis

✧ **Saliva sample preparation on a disc**

In this work, centrifugal separation was used to remove large molecular weight compounds, including mucus. And then, the supernatant was used for further assay.

✧ **Fully integrated lab-on-a-disc for multiplex immunoassay**

The lab-on-a-disc is designed for the simultaneous detection of multiple analytes, e.g. high sensitive C-reactive protein (hsCRP), cardiac troponin I (cTnI), and N-terminal B-type natriuretic peptide (NT-proBNP), for the diagnosis of cardiovascular disease starting from whole saliva.

hsCRP has been used primarily as a marker of systemic inflammation. And it is useful prognostic indicator in patients with acute coronary syndrome (ACS), as elevated CRP levels are independent predictors of cardiac death, acute myocardial infarction (AMI), and congestive heart failure (CHF). cTnI is one of sensitive and specific markers of AMI and cTnI levels indicates to predict the risk of mortality in patients with ACS.[99] NT-proBNP levels have been shown to be elevated in ACS.[100] Like this, hsCRP, cTnI and NT-proBNP each are associated with higher rates of death and predict ACS. Recently, the utility of these biomarkers in combination has been reported.[101] According to that report, simultaneous assessment of hsCRP, cTnI and NT-proBNP in acute coronary syndromes offer complementary information and provide powerful prognostic ability for a clinically meaningful composite endpoint.

Based on the bead-based immunoassay, here the novel centrifugal microfluidic layout was designed and tested to accomplish the multiplex immunoassay on a disc. The assay protocol for each target analyte such as hsCRP, cTnI, and NT-proBNP was optimized to be employed on a disc. The total saliva volume, washing buffer volume, and total reaction time were significantly reduced compared to the conventional ELISA, 250 μL vs. 300 μL, 700 μL vs. 3 mL, 20 min. vs. 3 hrs, respectively. The assay protocol was optimized to achieve similar performance compared to the conventional ELISA kit

e.g the LOD of hsCRP, cTnI and NT-proBNP was 0.05 ng/mL, 0.19 ng/mL and 0.26 ng/mL, respectively. To the best of our knowledge, this is the first example of chip-based fully integrated multiplex ELISA starting from real samples to the final detection.

2 Experimental Method & Materials

2.1 Reagents & Materials

In this study, 10 polystyrene beads (diameter: 2 mm, Hoover, USA) were used per one batch of reaction. Its total surface area is approximately 500 mm^2. The surface area of 10 PS beads is about 3 times larger than 96 well-plate (96 Well Clear Flat Bottom Polystyrene High Bind Microplate, Corning Inc., USA) which is used for conventional ELISA.

As capture antibodies, monoclonal mouse anti-human C-reactive protein (clone # C5 in PBS) and monoclonal mouse anti-cardiac troponin I (clone # 19C7 in PBS) and monoclonal mouse anti-human N-terminal proBNP (clone # 15F11 in PBS) was coated on the PS beads by using coating buffer (100 mM Bicarbonate/carbonate, pH 9.6). The capture antibodies were purchased from Hytest. Horseradish peroxidase (HRP) conjugated goat polyclonal anti-CRP and mouse monoclonal anti-cTnI (clone # 16A11 in PBS) and mouse monoclonal anti-proBNP (clone # 24E11 in PBS) were used as detection proteins to detect CRP (Abcam, USA), cTnI (Fitzgerald, USA) and proBNP (Abcam, USA), respectively. As blocking solution, 1 % BSA/1X PBS was used (99 % purified Bovine Albumin, Bio Basic Inc., Canada). The washing solution was purchased from AbD serotec (UK) which was 0.05 % Tween 20 in PBS at pH 7.4. The TMB (3,3',5,5'-tetramethylbenzidine) solution (AbD serotec, UK) was used as substrate for react with HRP. The stop solution was composed 0.2 M sulphuric acid (Matsunoen Chemicals Ltd., Japan) prepared in the lab.

Target-free serum (Fetal Bovine Serum-Charcoal Stripped, GeneTex, USA) was used to achieve the calibration curves for the serum-based immunoassays. QuantaRed Enhanced Chemifluorescent HRP Substrate was used as the substrate for the fluorescence signal measurements (Thermo Fisher Scientific Inc., USA).

The ferrowax valve is made of a nanocomposite materials composed of 50 % of paraffin wax (T_m: 50 - 52 ℃, Fluka Chemie GmbH) and 50 % ferrofluids (10 nm sixed iron oxide nanoparticles dispersed in oil; APC 314, Ferrotec Inc., CA, USA).

2.2 Immunoassay protocols

In this study, immunoassay was performed according to general sandwich immunoassay. The capture antibody is coated the PS beads by using carbonate/bicarbonate buffer (pH 9.6) in the tube, and then incubated at 37℃. After that, the coating solution is removed and then filled with 1 mL blocking

solution, and it is incubated at 37℃. After incubation, the blocking solution is removed. The 50 μL of diluted samples in PBS and 50 μL of diluted detection antibody in conjugate diluents are added together and mixed well, and then incubate at 37℃. The solution is removed and added washing buffer which is 0.05 % (v/v) Tween #20/PBS. This step is repeated one more time. 100 μL of TMB solution is added and then incubated at 37℃. After that, the stop solution 50 μL is added to the tubes. The solutions is collected by pipette and then dispensed to the well plate to read absorbance. The absorbance of solution was measured by spectrophotometer at 450 nm.

2.3 Fabrication of disc

As shown in Figure 10, both the top and bottom plates of the disc are made of polycarbonate (PC). The first fabrication step is the microstructuring of the geometric design of disc by using a conventional Computer Numerical Control (CNC) micromachining (3D modeling machine; M&I CNC Lab, Osan, Korea) in Figure 10A. The bottom plate is composed storage chambers and channels.

The top plate has inlet hole and dispensing hole of ferrowax. Ferrowax consists of paraffin wax (T_m 50 - 52 ℃, Fluka Chemie, GmbH) and ferrofluid (APG 314, Ferrotec Inc., CA, USA). Molten ferrowax is much less viscous than molten paraffin wax and thus injection of the wax material to the desired position is much easier. The compositions of the ferrofluid are hydrocarbon based carrier oil (77 - 92 %), nanosized iron oxide molecules (1 - 5 %), oil soluble dispersant (6 - 16 %), and oil soluble additives (1 - 2 %).[17] Molten paraffin wax and ferrofluid solution are vortexed for 3 sec above the melting temperature of the paraffin wax. After heating the plastic test chip or disc using a hot plate at 80 ℃, the molten ferrowax was placed into each inlet holes located on the top plate of the chip using a wax dispensing machine (Hanra Precision Eng.Co.LTD., Incheon, Korea) in figure 10B. The dispensing volume of the ferrowax was automatically controlled.

Before bonding two plates, PS beads were added in the reaction chamber. The top and bottom plates were bonded by using a double-sided adhesive tape (Flexmount DFM 200 Clear V-95 150 POLY H-9 V-95 4, FLEXcon Inc., MA, USA) which was printed by using tape-cutting plotter (Graphtec CE3000-60 MK2, Graphtec, USA) in Figure 10C. And then it was compressed by using press machine (Mechanical Power Vise AVQ-160GHV, Auto Well Enter Co., LTD., Taiwan) for a few minute in Figure 10D.

2.4 Operation of multiple LIFM on disc

The molten ferrowax is oily and has very low surface tension because its major component is the

hydrocarbon based oil. Therefore, the molten ferrowax flows into the microchannels by capillary action. In order to open the NC-LIFM, a polar coordinate was used for the control of the ferrowax valves on a disc. For example, in order to open the valve positioned at (r, h) with respect to the laser home position (r_0, 0), the disc is rotated with an angle of θ and the laser diode is moved with the distance of $r–r_0$ from the laser home to the center of the disc. For example, in order to transfer 100 µL of solution in reservoir A to reservoir B, as soon as the ferrowax valve is melted by laser irradiation for 1 sec, the disc is spinning up to 3600 rpm. These basic valve operation steps are repeated many times to control multiple valves on the disc.

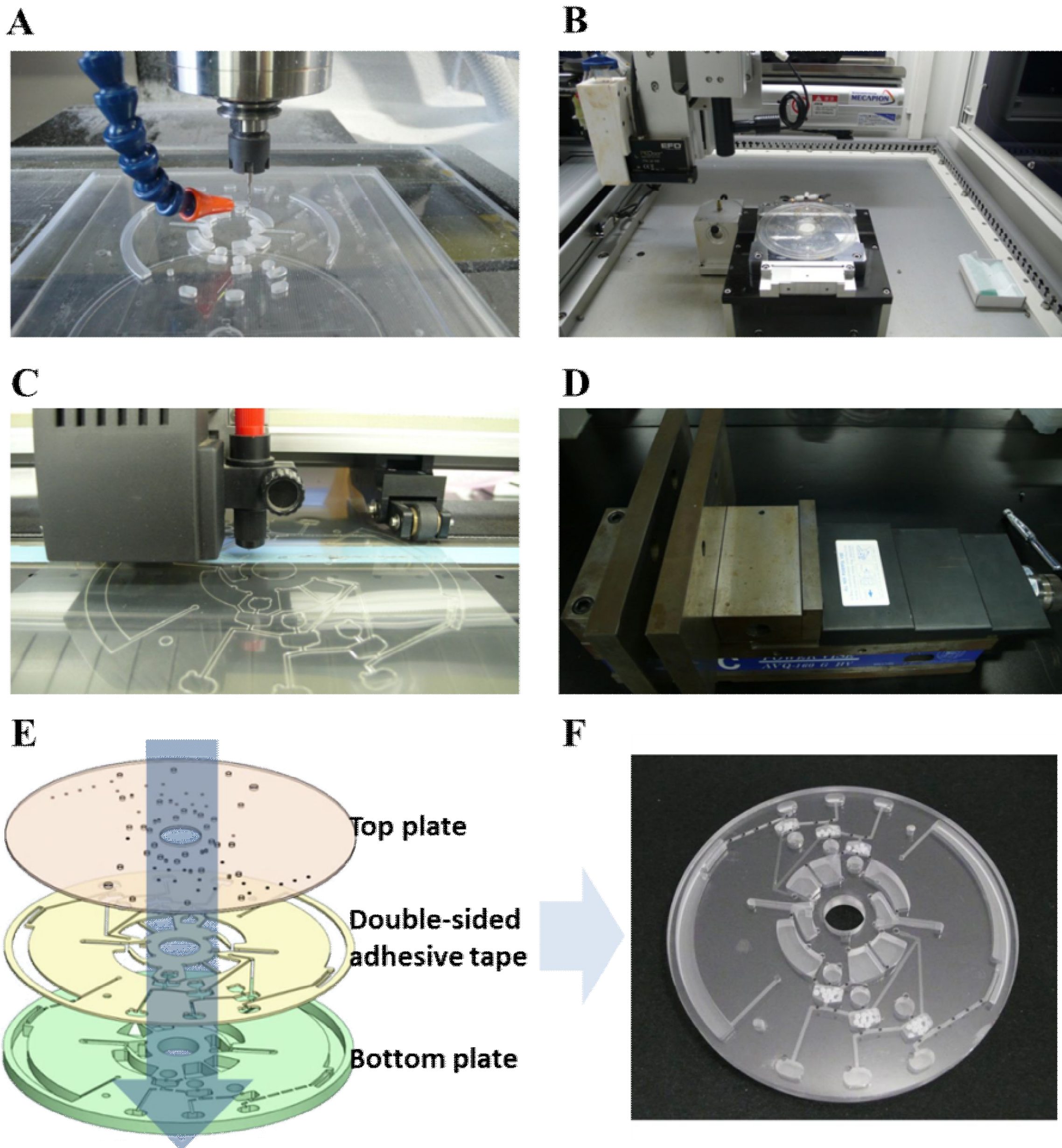


Fig 10 Fabrication procedures of disc. (A) The microstructuring of the geometric design of disc by using a conventional Computer Numerical Control (CNC) micromachining (B) Injecting of wax in the hole of the top plate by using wax dispenser machine (C) Printing of the double-side adhesive tape by using tape-cutting plotter (D) Compressing of top plate and bottom plate by using press machine (E) Schematic image presented bonding process of top and bottom plate. (F) Photo image of completed disc.

2.5 Motion Set-up

The visualization system is used for lab-on-a-disc operation that supports to rotate a disc up to 6000 rpm by main motor. In addition, this system is possible to record disc operation such as fluid flow by using CCD camera and strobe light (Figure 11).

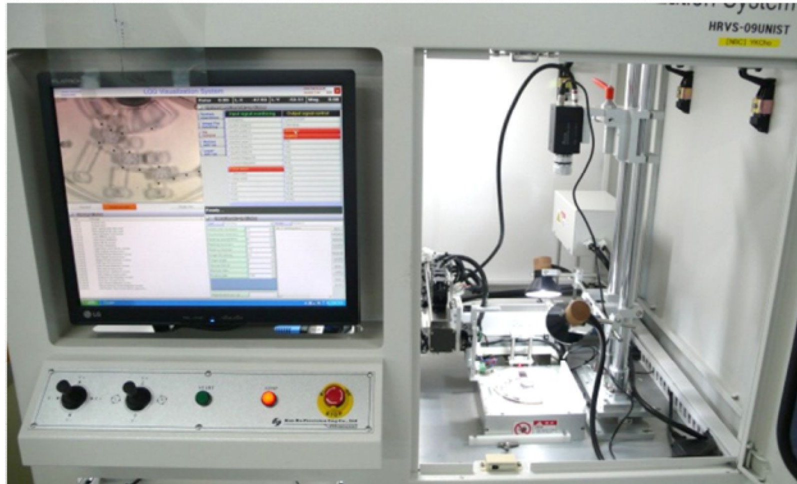
2.5.1 System composition

This system is composed of modules for imaging, motion control, laser control and a PC. The imaging device is used to record the fluidic movement on a rotating disc. This device contains CCD camera, lens, strobe light and manual stage. The camera is a CCD digital color camera (IK-TF5C, Toshiba America, Inc., USA) that video signal can save the 60 frame per 1 sec. The motion device contains rotor, laser stage, magnet stage and controller. Rotor (SGMJV-02A 200W servomotor, Yaskawa Electric Co., Japan) is possible to rotate up to 6000 rpm. Laser (808 nm High-Power Laser Diode AL808T3000, Associated Opto-Electronics (Chongqing Co., China) can move by X-Y axis stage. Motor controller control the rotor servo pack, stepped motor for operating laser and stepped motor located on magnet stage. Laser device is used to open/close the valve which is wax pre-loaded in the hole of top plate. The laser has 3W power that can be controlled.

2.5.2 Usage of Software

Control PC has software which executes operation according to the pre-designed operating program. Using this software, we can make an operating program of disc to control the operation variables such as rotating speed and duration, laser irradiation time and position as well as image file saving conditions. In the spin program control window, we set up the acceleration time, deceleration time, rotating speed, rotating time and rotating direction, e.g., clockwise or counterclockwise direction. And in the laser set-up window, we set up the parameter such as laser power, irradiation time and laser position. The operating program can be added several working items. For example, to operate fluid transferring step, the laser working is executed and then followed by rotating working. For this study, the operating program was created as shown Table 6. In addition, using this software, we can edit the video files.

A



B

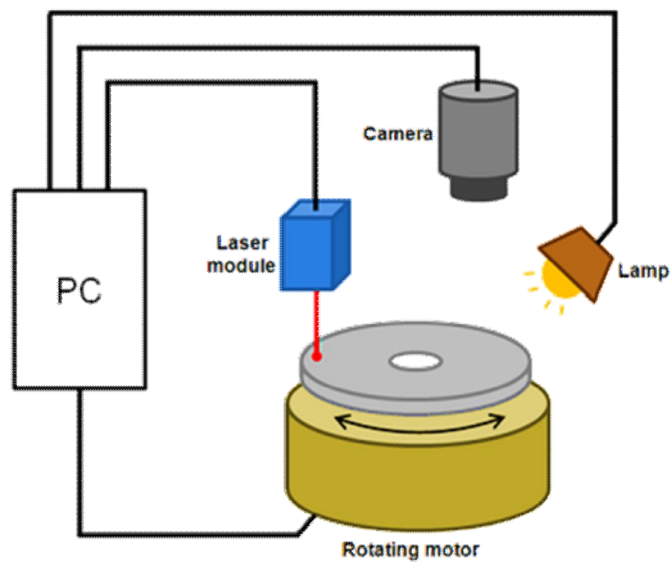


Fig 11 Motion system to operate disc. (A) Photo image of the visualization system. (B) Schematic diagram of composition of visualization system.

Table 6 Spin program

Spin No.	*Speed (rpm)*	*Time (sec.)*	*operation*
1	3600	120	Saliva preparation
2	2400	5	Transfer saliva into detecting Ab reservoir
3	+1200~-1200	4	Mix saliva and detecting Ab
4	2400	8	Transfer mixture into reaction chambers
5	+1200~-1200	600	Mix beads, saliva and detecting Ab
6	2400	5	Remove reaction residues into waste chamber
7	3600	8	Spin to dry the channel
8	-	2	Close the 1st valve of waste channel
9	2400	8	Transfer 1st washing buffer into reaction chambers
10	+1200~-1200	20	Mix beads and washing buffer
11	2400	5	Remove 1st washing buffer into waste chamber
12	3600	8	Spin to dry the channel
13	-	2	Close the 2nd valve of waste channel
14	2400	8	Transfer 2nd washing buffer into reaction chamber
15	+1200~-1200	20	Mix beads and washing buffer
16	2400	8	Remove 2nd washing buffer into waste chamber
17	3600	8	Spin to dry the channel
18	-	1	Close the 3rd reaction chamber channel
19	-	1	Close the 2nd reaction chamber channel
20	-	1	Close the 1st reaction chamber channel
21	2400	3	Transfer 1st TMB into 1st reaction chamber
22	2400	3	Transfer 2nd TMB into 2nd reaction chamber
23	2400	3	Transfer 3rd TMB into 3rd reaction chamber
24	+1200~-1200	300	Mix beads and TMB
25	2400	3	Transfer into 1st detection chamber
26	2400	3	Transfer into 2nd detection chamber
27	2400	3	Transfer into 3rd detection chamber
Total		19.3 min	

3 Results and discussion

3.1 Optimization of immunoassay

3.1.1 Capture antibody concentration

The concentration of capture antibody is first variable factor to optimize immunoassay on beads. In the case of hsCRP, the capture antibody was coated at different concentrations, ranging between 10 - 40 μg/ml, onto the surface of beads. For the binding assay, 25 ng/mL concentration of antigen was used in order to expose the capture antibodies to a sufficient amount of target molecules. Figure 12A shows that the signal increased slightly from 10 μg/ml and became saturated at 20 μg/ml concentration. The negative control was used to check for non-specific binding and false positive results. Overall, the optimal concentration for the capture antibody was determined to be 20 μg/ml.

For other targets, cTnI and NT-proBNP, the concentration of capture antibody was also optimized as the aforementioned way. Each capture antibody concentration was optimized at ranges between 10 - 40 μg/ml. As the result, the optimum concentration of both was 20 μg/ml (Figure 12B - C).

3.1.2 Detection antibody concentration

The next parameter that was optimized in the sandwich immunoassay was the concentration of the detection antibody as its concentration is critical to the sensitivity of the system. The performance of the immunoassay was evaluated using different concentrations of the detection antibody (10 - 1000 ng/ml) for hsCRP. As shown in Figure 12D, the signal increased with the amount of detection antibody and the optimum concentration was found to be 200 ng/mL.

For the concentration of cTnI and NT-proBNP detection antibody, the evaluated range of the concentration of detection antibody was between 100 - 2000 ng/mL. As shown in Figure 12E & F, the optimum concentration of both was found to be 500 ng/mL which has the highest signal to noise ratio.

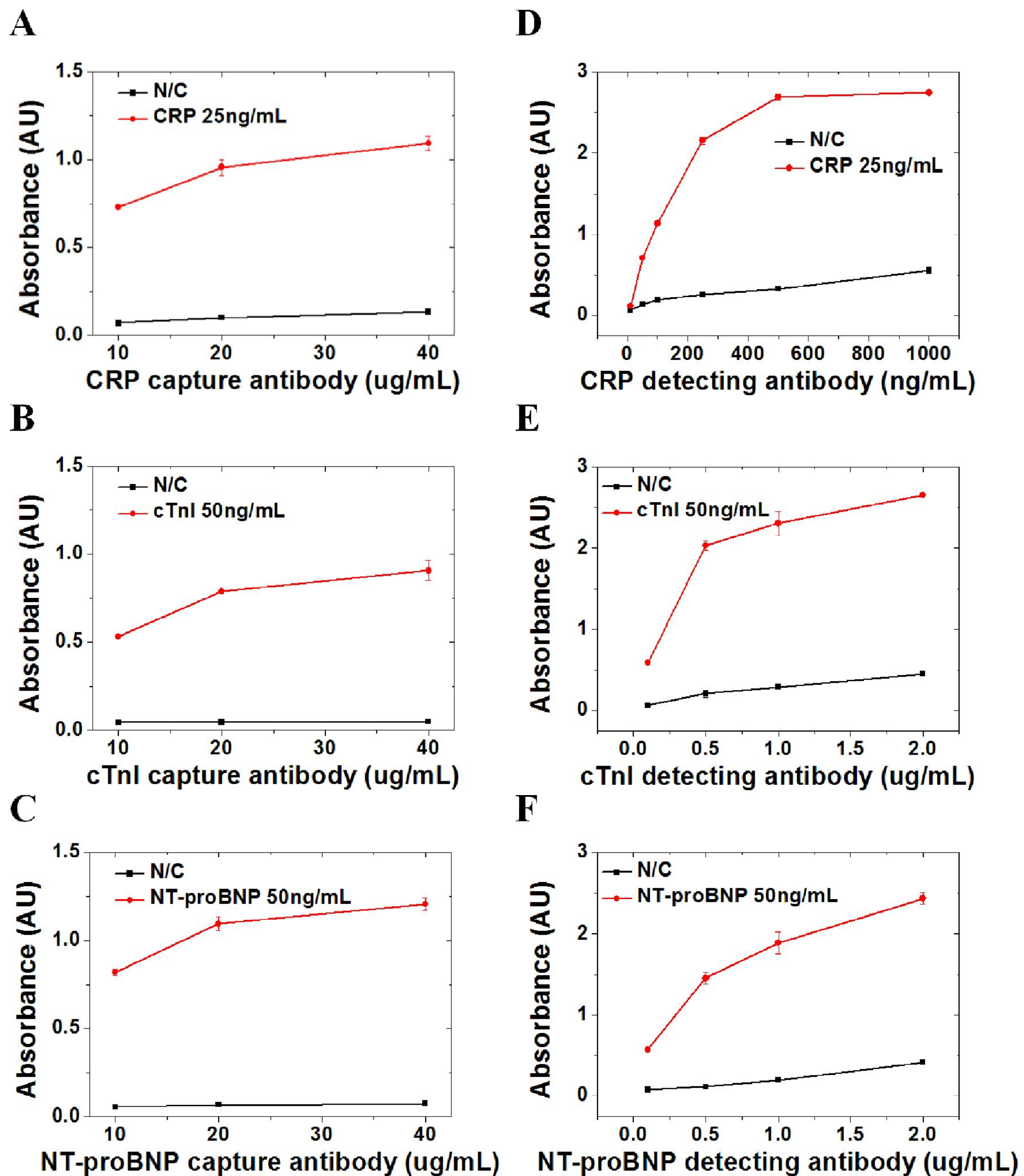


Fig 12 Optimization of variable factors which are concentration of capture antibody and detection antibody. (A) Optimization of concentration of hsCRP capture antibody, (B) Optimization of concentration of cTnI capture antibody, (C) Optimization of concentration of NT-proBNP capture antibody, (D) Optimization of concentration of hsCRP detection antibody, (E) Optimization of concentration of cTnI detection antibody, (F) Optimization of concentration of NT-proBNP detection antibody.

3.1.3 Reaction time

The immunoassay for detection of hsCRP performed under various incubation times, ranging from 5 min to 60 min. As shown in Figure 13A, the signal reached a plateau at 30 min of incubation time. However, as the difference between the signals at 10 min and 30 min was low, 10 min of incubation time was chosen to minimize incubation time. Another variable that was evaluated was the "detection time". We remind the reader that the HRP enzyme oxidizes TMB and generates a blue color and the intensity of the generated signal depends on the concentration of antigen. Figure 13B shows that the signal increased gradually with time and the colorimetric signal at 5 min was adequate to take a reading of the assay. Therefore, the total immunoassay time was reduced to 20 min including reaction and detection time, significantly shorter than conventionally ELISA (3 hours).

3.1.4 Comparison with 3D substrate and 2D substrate

The immunoassay performed on the PS beads was compared with performed on the 96 well-plate. In this study, as solid support, 10 PS beads were used for the sandwich immunoassay. As shown in Figure 14A, in case of hsCRP, the absorbance signal of immunoassay performed on PS beads was higher than performed on 96 well-plate. The surface area of 10 PS beads was about 2 times larger than 96 well-plate. Thus, since it has an effect that a large number of capture antibody could coat the PS beads, it was capable to capture antigen ranging from low to high concentration. Additionally, it resolves the diffusion limitation for reacting between antibody and antigen because the beads could move in limited chamber, and therefore, the antibody and antigen have more chance to meet each other in short time.

As shown in Figure 14B and 14C, for cTnI and NT-proBNP, respectively, it also shows the results of immunoassay performed on PS beads compared with performed on 96 well-plate. As the same reasons, the obtained signal on PS beads was higher than the obtained signal on 96 well-plate.

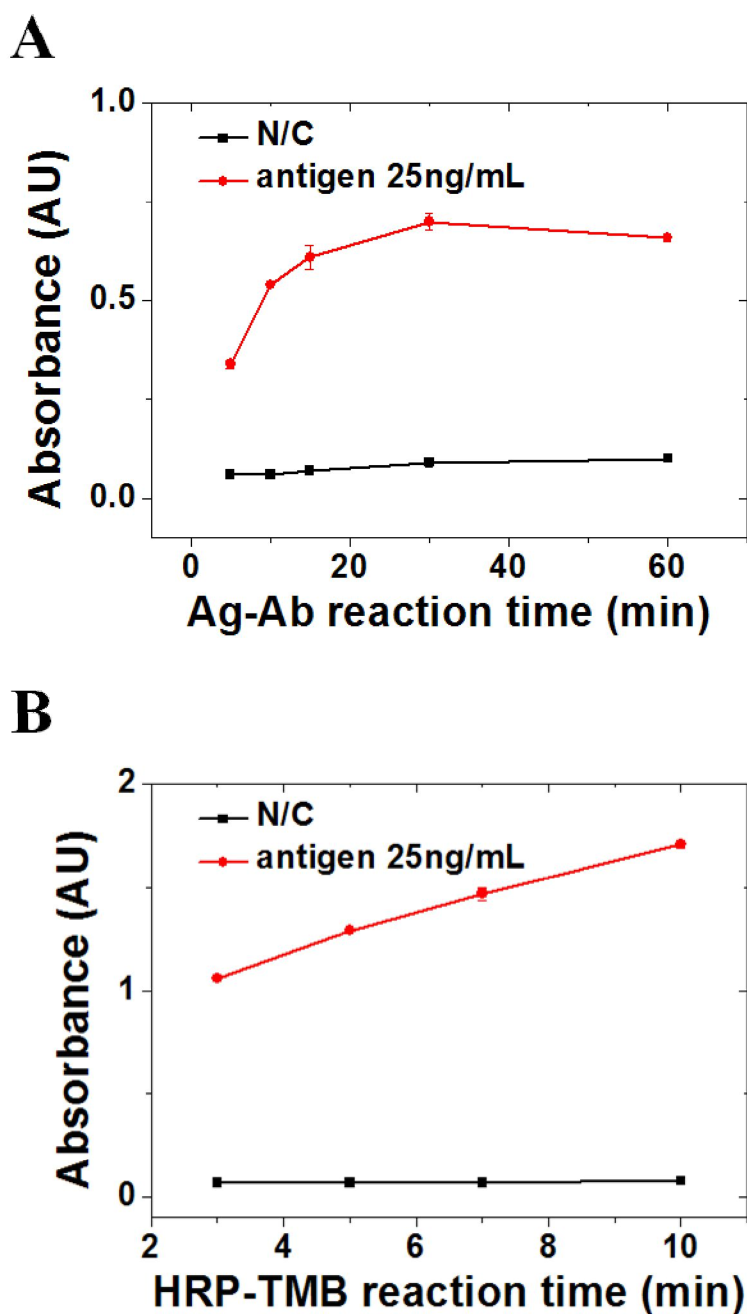


Fig 13 Optimization of reaction time and detection time. (A) Optimization of reaction time that the antigen and detection antibody complex was bound to capture antibody coated on PS beads. (B) Optimization of detection time that the HRP labeled on a detection antibody reacts with substrate, and then detectable signal was released as blue color.

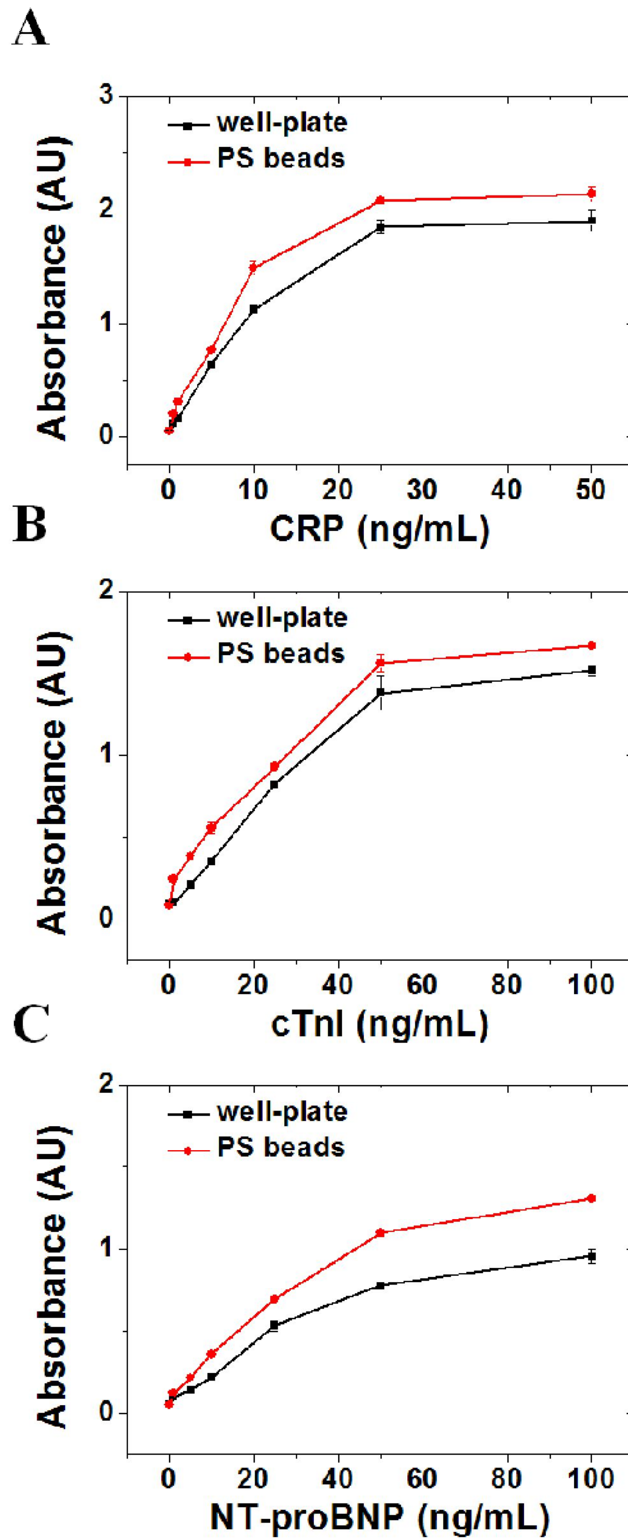


Fig 14 Comparison of immunoassay performed on PS beads and performed on 96-well plate. (A) hsCRP, (B) cTnI, (C) NT-proBNP.

3.2 Saliva sample detection

3.2.1 Saliva sample preparation

Saliva is composed mostly of water, electrolytes and proteins, RNAs, lipids. Moreover, it consists of other compounds such as mucus, bacterial cells, etc. Mucus has rheological properties, e.g., high elasticity, adhesiveness and low solubility. This thick and sticky substance can cause problems in transferring small volumes. Conventional salivary ELISA kit advises freezing all saliva samples once before performing the assay, followed by vortexing after the sample is thawed. This procedure helps break up the mucus, and it can then be centrifuged into the bottom of the tube.[102]

In this study, whole saliva was collected in the tube directly without stimulation. And centrifugation with 3600 rpm for 2 minutes was used to remove mucus, bacterial cell which is large molecule. Any other particles that were present are also removed in this step. After centrifuging, the supernatant was transferred into next chamber for further assay step.

3.2.2 Immunoassay using saliva sample

To verify whether saliva affects assay performance or not, we attempted to do immunoassay in saliva sample. Target analyte was spiked in saliva, and then we measured the absorbance signal of each concentration of analyte. As shown in Figure 15A - C, when we performed immunoassay in target protein-spiked whole saliva, there is a little difference compared with result performed in target protein-spiked PBS buffer. In case of performed in whole saliva, assay efficiency was reduced at high concentration of target protein. However, at low concentration of target protein, the result measured absorbance signal was similar with the result performed in PBS. In addition, separated saliva was mixed with the solution of the detection antibodies with 1:1 ratio. Thus, the whole saliva was diluted in half, and it reduced viscosity of saliva. In this result, we confirmed that the constituents of whole saliva do not have a great effect on the assay performance.

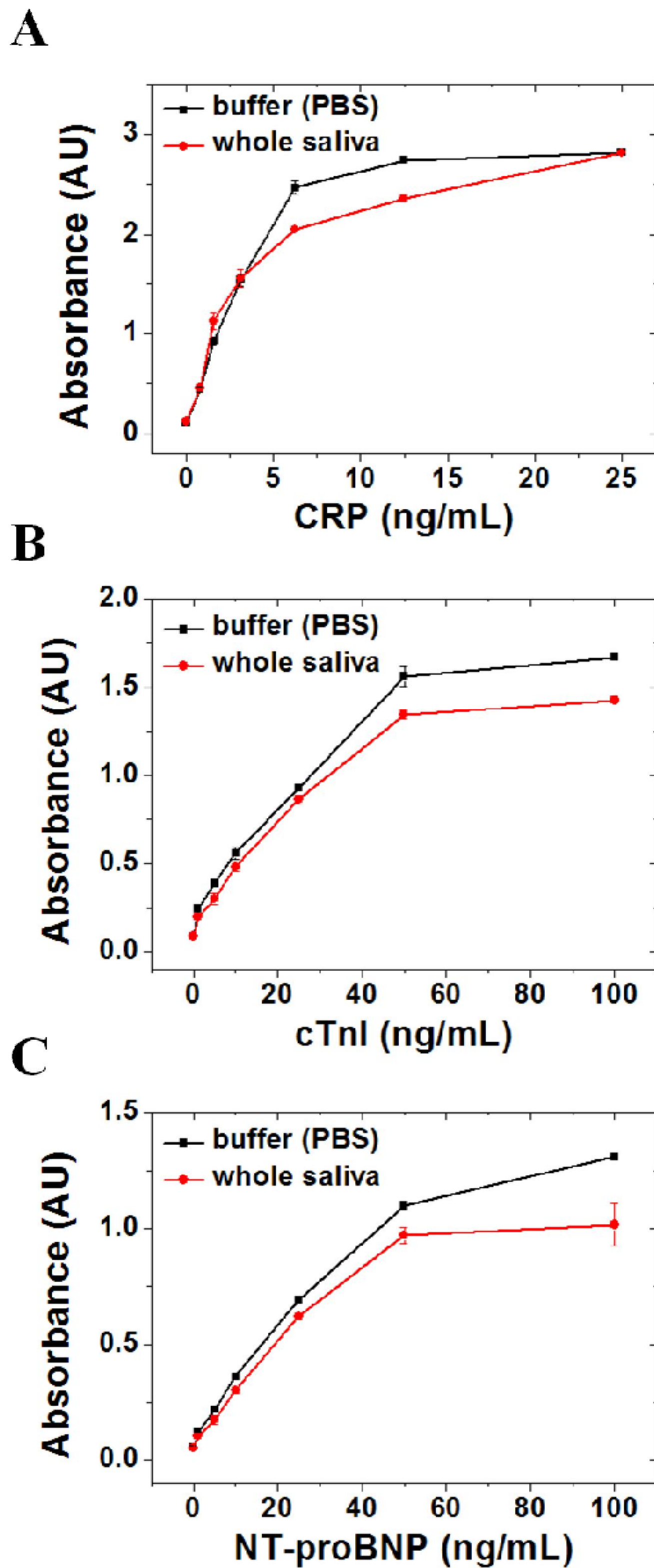


Fig 15 Immunoassay in whole saliva sample. (A) Comparison with calibration curve with hsCRP in whole saliva and in PBS. (B) Calibration curve of cTnI in whole saliva, (C) Calibration curve of NT-proBNP in whole saliva.

3.3 Multiplex immunoassay on a disc

3.3.1 Cross reactivity

Cross reactivity refers to the ability of an individual combining site to react with more than one antigen. For multiplex immunoassay in this system, there should be no cross reactivity between multiple antigens and multiple antibodies. To confirm cross reactivity between hsCRP capture antibody and other antigens; cTnI and NT-proBNP, we added each cTnI and NT-proBNP on the hsCRP capture antibody-coated beads. And then each cTnI detecting antibody and NT-proBNP detecting antibody was added on the beads. Additionally, we tested cross reactivity between hsCRP and other detecting antibodies; cTnI detecting antibody, NT-proBNP detecting antibody. In this case, hsCRP bound to hsCRP capture antibody and then each cTnI detecting antibody and NT-proBNP detecting antibody was added on the beads. As show in Figure 16A, there is no cross reactivity between capture antibody and other antigens as well as cross reactivity between antigen and other detecting antibodies. Likewise, for other proteins, the results indicate that there is no cross reactivity between antigens and different kinds of antibodies (Figure 16B - C). This result demonstrated the multiple antigen and antibodies mixture does not affect to each other. Thus, this target set is usable for multiplex immunoassay system.

3.3.2 Disc design

The disc scheme is shown in Figure 17A. Disc is designed to accommodate immunoassays using two different samples. Disc is composed of a sample preparation chamber, a detecting antibody storage chamber, two washing buffer storage chamber, a waste chamber, and three reaction chambers. Also, there are three TMB solution storage chambers, and three detection chambers. The channel is connected to each chamber. In order to finish multiplexed-immunoassay to measure 3 independent biomarkers in a fully automatic fashion, there are total 11 normally closed LIFM valves and 5 normally opened LIFM. The normally closed valves sequentially open based upon the pre-designed spin program by laser irradiation.

The three reaction chambers are connected by connection channels so that each fluid (e.g. sample & detection antibody, washing buffer) can be filled in three reaction chambers at a single step of fluidic operation. This unique design of the centrifugal microfluidic layout offers reducing the complexity of microfluidic operation procedures and required chip space. Furthermore, it was the design that we came up with in order to have a minimized number of valves.

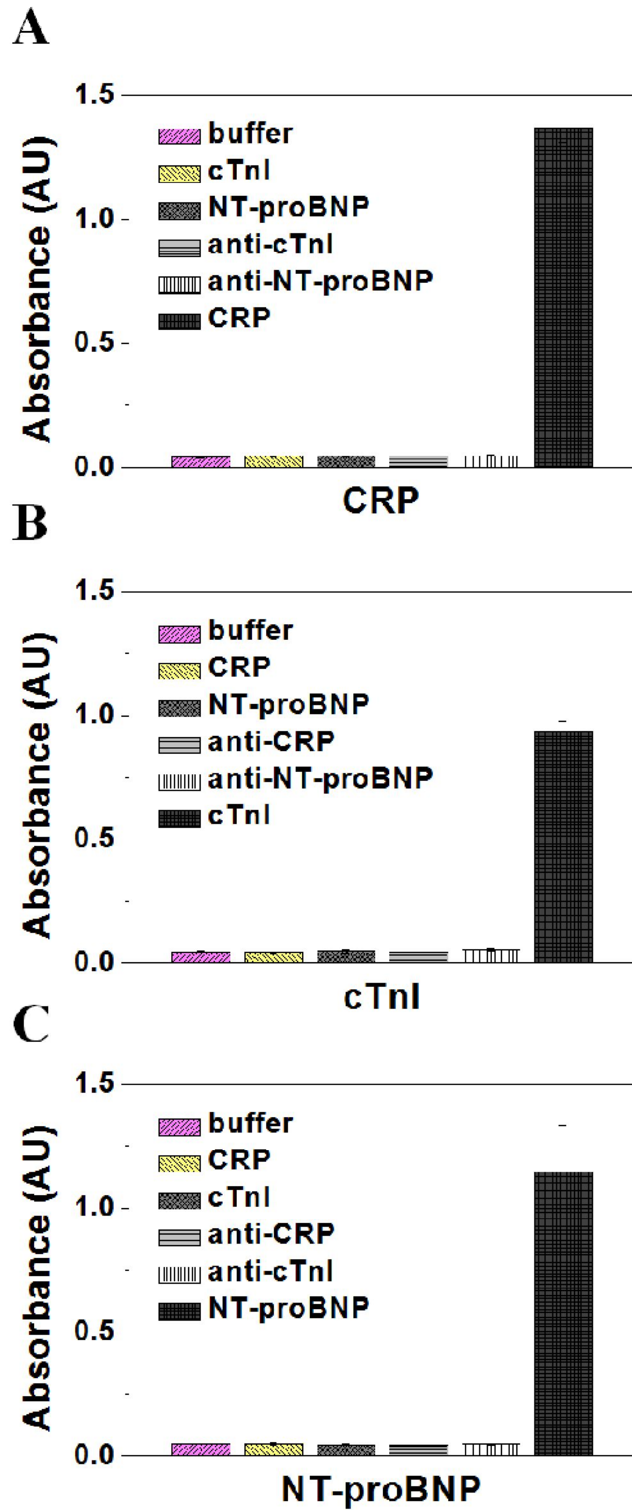


Fig 16 Evaluation of cross-reactivity between target antigen and antibody for (A) hsCRP, (B) cTnI, (C) NT-proBNP, respectively.

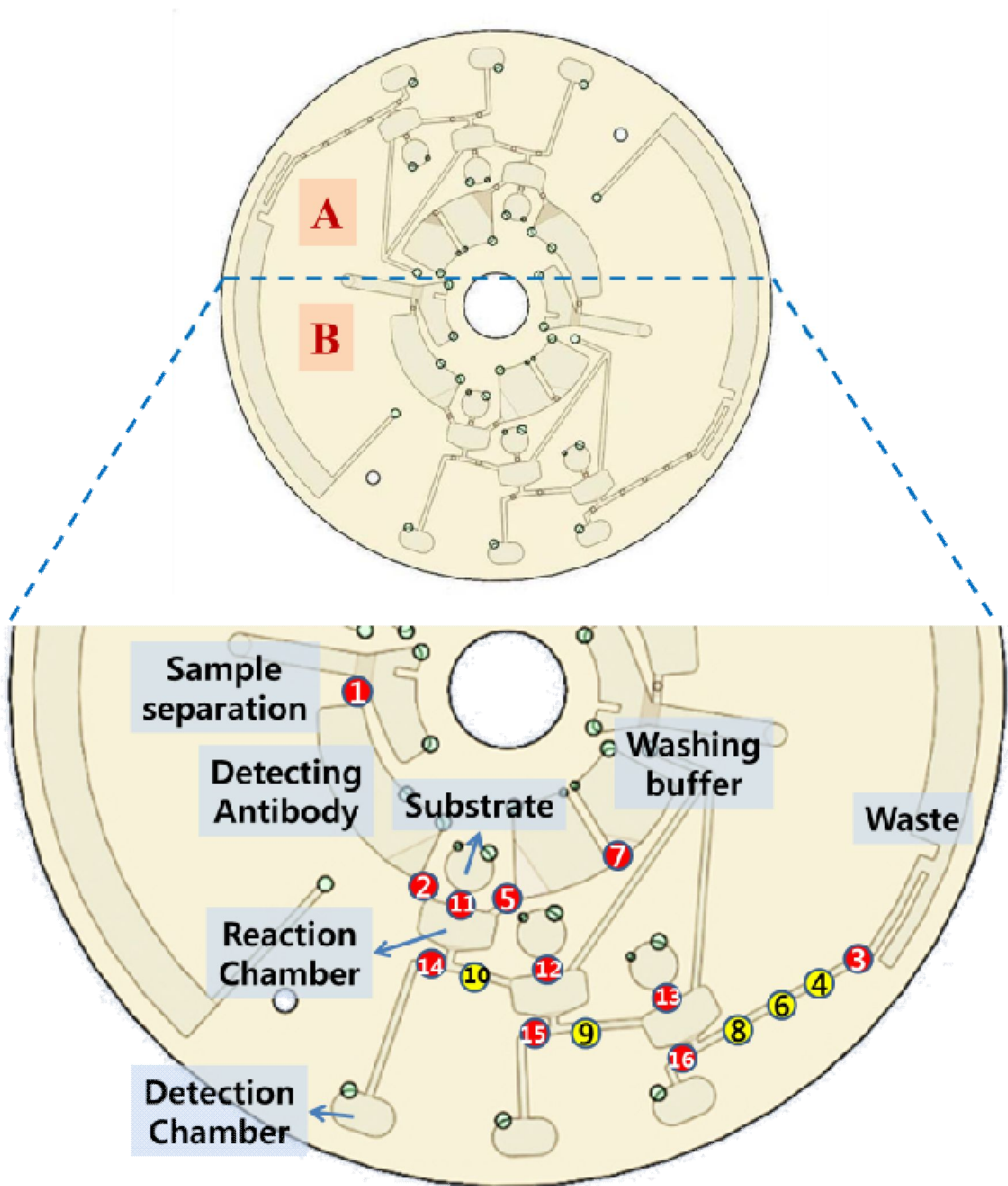


Fig 17 Disc design for fully integrated multiplex immunoassay. Red dots indicate the normally closed valves and the yellow dots are for the normally open valves. The main three reaction chambers are connected during the reaction and washing steps but isolated for the final reaction with substrate solution and the detection.

3.3.3 Fluids control on a disc

After introducing 250 μL of whole saliva, the disc spins with the spin speed of 3600 rpm for 2 minutes to deposit the large molecules and bacterial cells from whole saliva. Upon laser irradiation at the valve #1, 200 μL of the supernatant is transferred into the detecting antibody storage chamber. The 200 μL of supernatant and 200 μL of detecting antibody is mixed by spinning in clockwise and counter-clockwise direction.

After opening the valve #2, the mixture is transferred into the three reaction chambers. The reaction occurs for 10 min in mixing mode, and then the reaction residue is removed into waste chamber by opening the valve #3. After closing the valve #4, the washing buffer is transferred into reaction chambers by opening the valve #5. After washing the beads, the waste solution is removed to the waste chamber by opening the valve #4 and the chamber is sealed by closing the valve #6. After repeating the washing step for one more time by operation of the valve #7 and the valve #6. After each reaction chambers are sealed by closing the valve #8, #9, #10, the washed beads are reacted with the TMB solution by opening the valve #11, #12, #13.

After 5 minutes, the solution is transferred into each detection chamber preloaded with the stopping solution by opening the valve #14, #15, #16. Finally, the optical absorbance is measured at the detection chamber and the concentration is calculated by comparing the absorbance measured at the control chamber.

In Figure 18, the sequence of flow control on a disc to fully integrate the multiplex ELISA was demonstrated using color dye solution for clear visualization. As shown in Figure 18, each reaction chamber is preloaded with the PS beads modified with capture antibodies. Saliva is prepared by spinning the disc at 3600 rpm for two minutes in Figure 18A and the 200 μL of saliva is transferred to the chamber preloaded with detecting antibodies as shown in Figure 18B. Then, the solution is introduced in reaction chamber for target binding in Figure 18C. After reaction, the solution is transferred into waste chamber (Figure 18D). Then, the washing buffer is introduced to remove the serum residues (Figure 18E - G). After the washing step, each reaction chamber is isolated by closing the valves between. The substrate solution is now transferred to each reaction chamber (Figure 18H) and the final solution is moved to the detection chamber for the concentration measurements (Figure 18I).

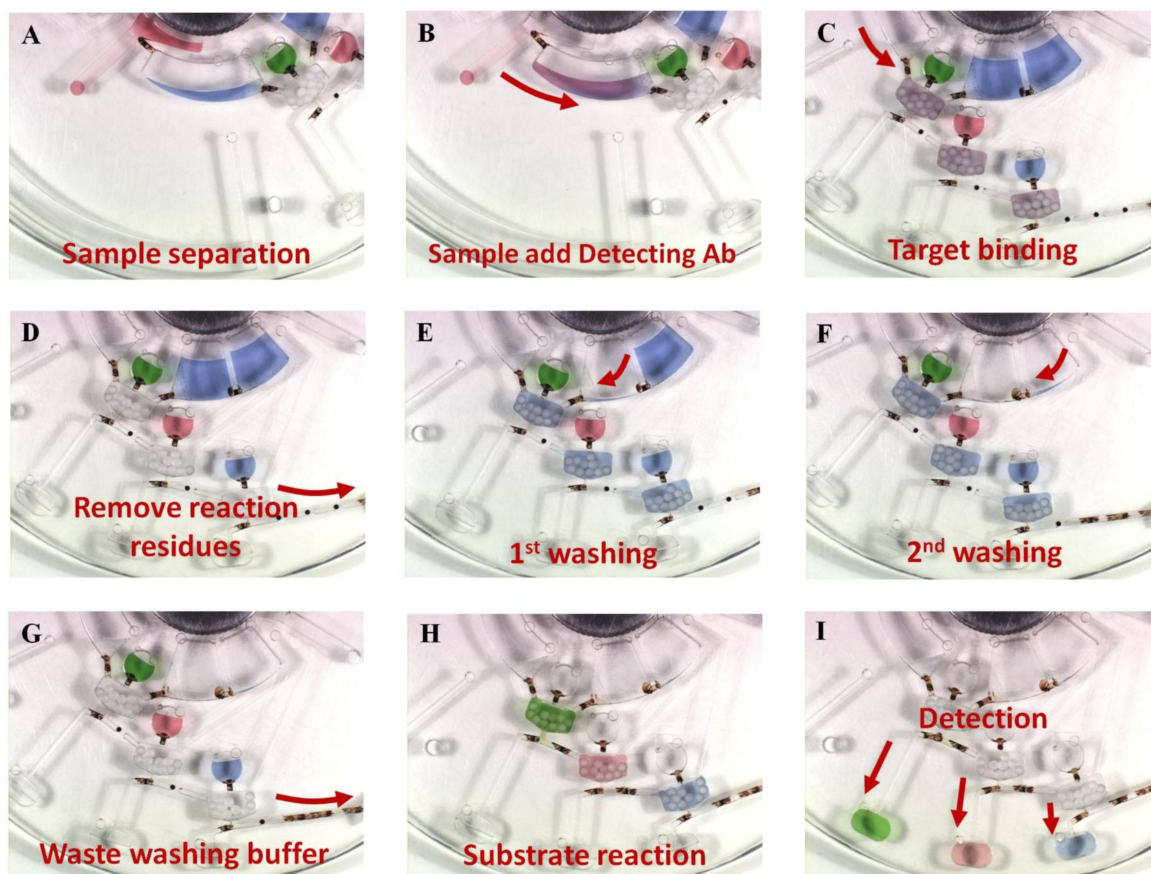


Fig 18 Visualization of the centrifugal microfluidic control on the disc with color dye solution. (A) Sample is separated by high speed spinning. (B) Separated sample is transferred into the chamber preloaded with detecting antibody. (C) Sample mixed with detecting antibody solution is transferred into the reaction chambers for the target binding reaction. (D) After reaction, the reaction residue is removed into the waste chamber. (E-F) Washing buffer is transferred through the reaction chambers. (G) After washing, the waste solution is removed into waste chamber. (H) The substrate solution is transferred to the reaction chamber. Note each reaction chamber is now isolated. (F) The final solution is transferred into the detection chamber and mixed with the preloaded stopping solution, in which signal is measured by spectrophotometer.

3.3.4 Evaluation of multiplex immunoassay on a disc

Figure 19A – C show the calibration curves for the detection of hsCRP, cTnI, and NT-proBNP spiked on whole saliva. The result that immunoassay performed on a disc with various concentrations of the target protein-spiked sample indicates similar with the result that measured by the manual assay on a tube. In addition, as shown in Table 7, the CV(%) between each reaction chamber is below 10% regardless the location of the chamber when we used the beads coated with the same antibody for all three chambers. This result demonstrates that the binding reaction occurs uniformly in each reaction chambers.

For multiplex immunoassay, a sample solution prepared as mixture of hsCRP, cTnI and NT-proBNP are all pre-mixed with the corresponding three kinds of detecting antibodies that are pre-loaded in the storage chamber. In each reaction chambers, hsCRP, cTnI and NT-proBNP capture antibody modified beads were loaded, respectively. After multiplex immunoassay, we compared the result in each reaction chamber with the result that was obtained when we used single target protein for all three chambers. As shown in Figure 20A - C, for all of the target protein, the results obtained when we used multiple target proteins was similar with the results of immunoassay performed by using single target protein on a disc. As we confirmed before in the off-disc (tube-based) experiments, there was no significant cross reactivity between different kinds of antibody and antigen. Therefore, we confirmed that the antibodies that we chose for the multiplex immunoassay do not have significant cross-reactivity and therefore provide good specificity for the diagnostics.

Table 7 Raw data of immunoassay result in each reaction chamber for hsCRP

ng/mL	*Chamber1*	*Chamber2*	*Chamber3*	*AVERAGE*	*STDEV*	*%CV*
0	0.175	0.196	0.188	0.186	0.015	7.97
0.5	0.302	0.334	0.337	0.324	0.023	6.98
1	0.428	0.457	0.423	0.436	0.021	4.70
5	0.844	0.814	0.863	0.840	0.021	2.52
10	1.262	1.309	1.21	1.260	0.033	2.64
25	2.725	2.726	2.644	2.698	0.001	0.03
50	2.802	2.763	2.76	2.775	0.028	0.99

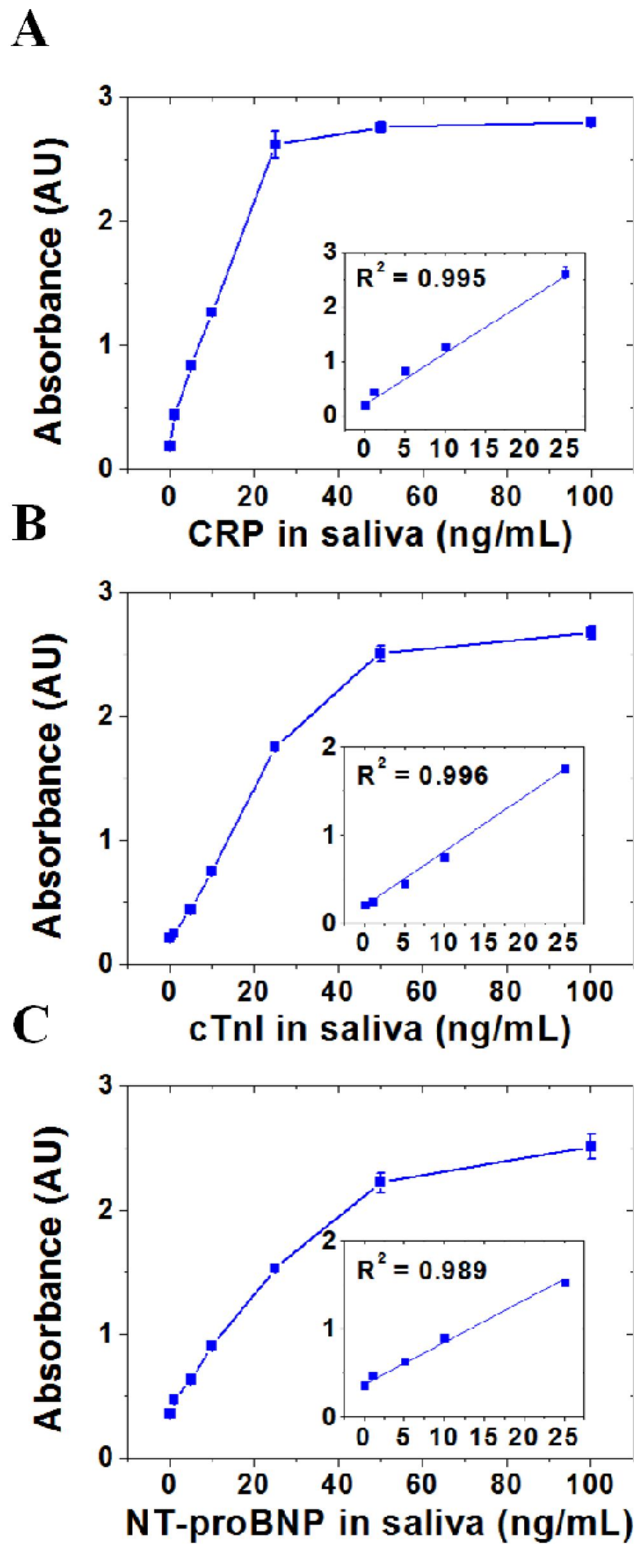


Fig 19 Calibration curve of (A) hsCRP, (B) cTnI, (C) NT-proBNP on a disc. The immunoassay performed with single target protein on a disc. It is compared with manually performed immunoassay on a tube.

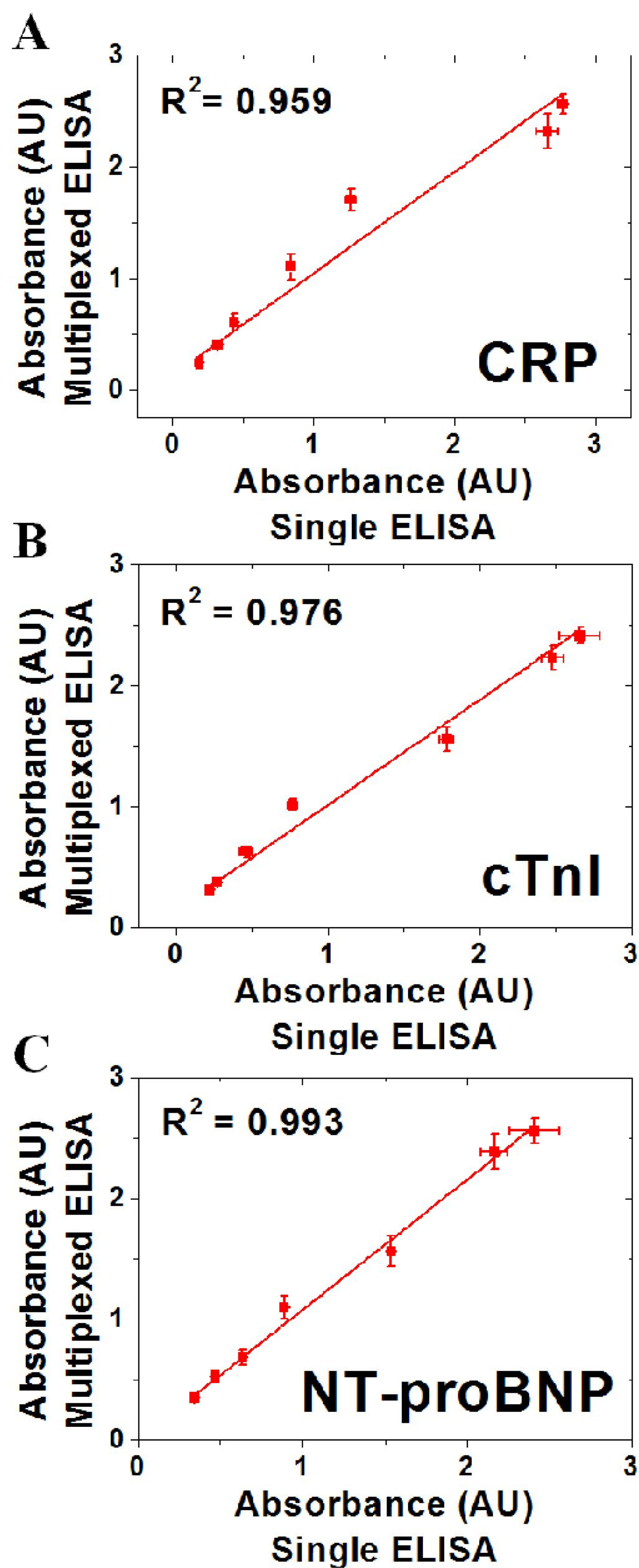


Fig 20 Correlation curve of between used single target immunoassay and used multiple target immunoassay on a disc for (A) hsCRP, (B) cTnI, (C) NT-proBNP.

3.3.5 Fluorescent detection for immunoassay in saliva

In addition, the detection sensitivity could be further enhanced by using the same disc but simply replacing the substrate for HRP from colorimetric to a chemifluorescent signal generating reagent. The signal was measured at excitation and emission wave numbers 570 nm and 585 nm, respectively. As shown in Figure 21, the calibration curve was obtained in saliva sample for each target protein and the sensitivity is enhanced significantly.

3.3.6 ELISA in serum

In this study, the disc was designed to use whole blood as well as whole saliva. We tested immunoassay performance to confirm antibodies activity in serum sample. We used various concentration of target spiked in target-free serum, and Figure 22 shows calibration curves for the detection of CRP, cTnI, and NT-proBNP. In results, all antibodies were functioning and immunoassay performance was not affected by serum condition.

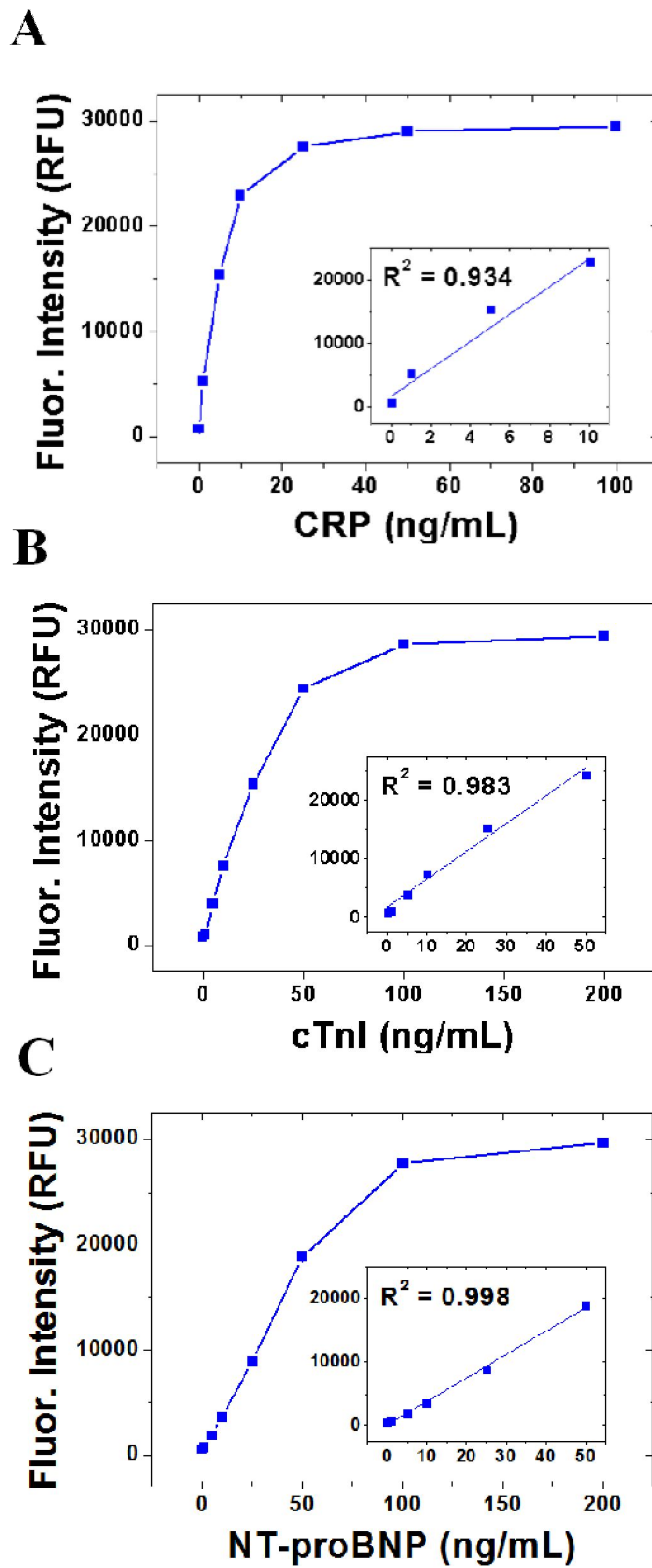


Fig 21 Fluorescent detection of the multiplex immunoassay on a disc to detect hsCRP (A), cTnI (B), and NT-proBNP (C)

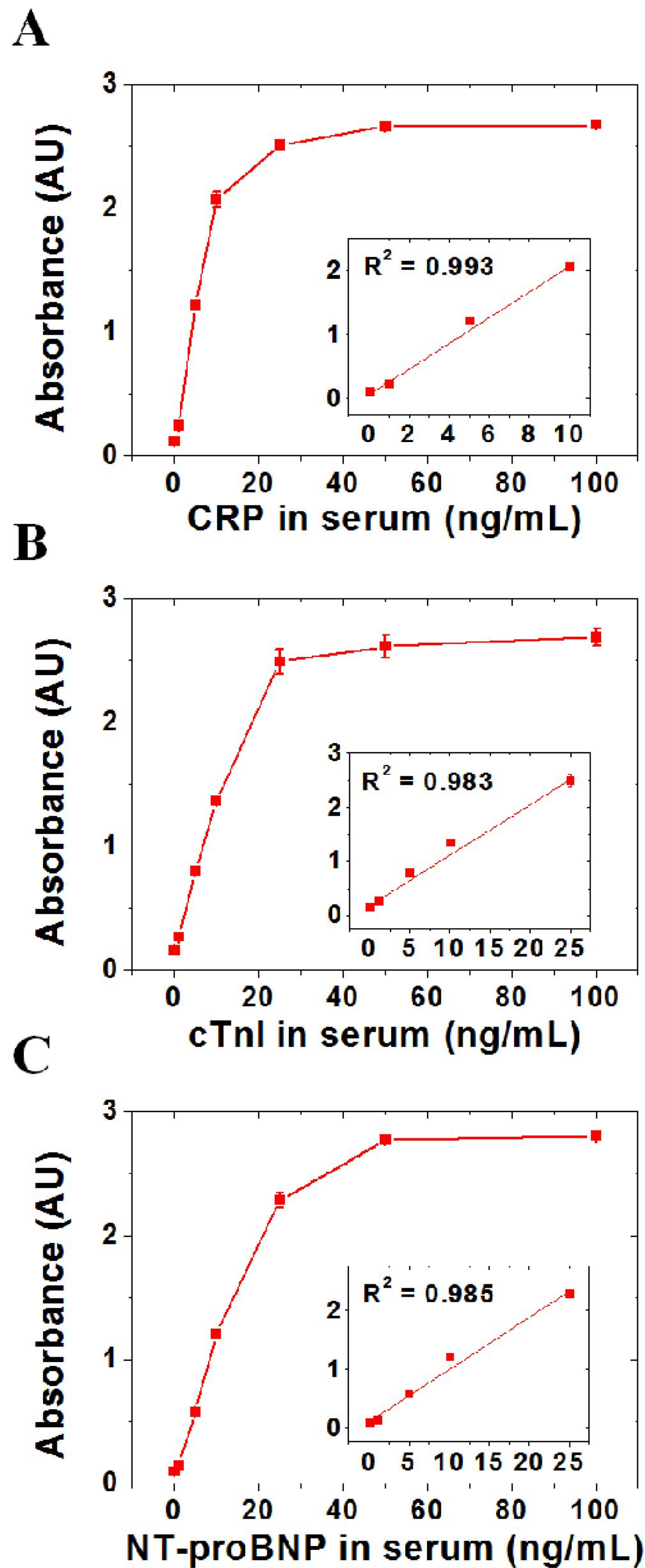


Fig 22 Immunoassay results for detection of CRP, cTnI and NT-proBNP (A – C) performed on a disc. The target proteins were spiked in target-free serum.

3.3.7 Assay performance on a lab-on-disc

To evaluate immunoassay, we compared assay performance specifications such as LOD, dynamic range, assay time, etc. between different lab-on-a-disc and conventional ELISA kit as shown in Table 8. First, we used 250 μL of the sample volume for the multiplex immunoassay on a disc to detect three different target proteins. It was similar to the conventional ELISA kits; 300 μL. We have not tried to reduce the sample volume further not only because the current design of the chip require 200 μL sample volume to fill the reaction chambers but also small reduction of the target sample volume does not mean much especially for saliva sample (or even for whole blood) considering the hospital environments to have the actual tests.

Second, the operation time was significantly reduced compared to the conventional ELISA; 20 min vs. 3 hrs. Because this system was fully integrated and automated, not only it can reduce the total operation time but also the manual errors. Previously, it was reported that one immunoassay could be finished within 30 minutes using a lab-on-a-disc.[12] It was the world first report for a fully automated ELISA type immunoassay using real samples such as whole blood. Here, we have demonstrated a novel disc design that can finish multiplexed immunoassay to test three independent target biomarkers within 20 minutes, again in a fully automated fashion and starting from raw samples such as whole blood, saliva and urine.

Third, the dynamic range was optimized to achieve similar result with the conventional ELISA kits despite that we use smaller sample volume and reduced binding reaction time. In case of conventional ELISA, the dynamic range of hsCRP, cTnI and NT-proBNP is 1 - 25 ng/mL, 2 - 75 ng/mL, 0.3 - 20 ng/mL, respectively. The dynamic range, in this system using blood sample, of hsCRP, cTnI and NT-proBNP is 1 - 10 ng/mL, 1 - 25 ng/mL and 1 - 25 ng/mL, respectively. In addition, the dynamic range using fluorescent detection in saliva of hsCRP, cTnI and NT-proBNP is 1 – 10 ng/mL, 1 – 50 ng/mL, 1 – 50 ng/mL, respectively.

Finally, the limit of detection (LOD), defined as 2 times the standard deviation of the signal measured with the blank sample of hsCRP, cTnI and NT-proBNP are 0.03 ng/mL, 0.08 ng/mL and 0.08 ng/mL, respectively. These are as good as or more sensitive them the values obtained by conventional ELISA kits. In addition, using fluorescent detection, the LOD of hsCRP, cTnI and NT-proBNP is 0.007 ng/mL, 0.02 ng/mL, 0.02 ng/mL, respectively. It could be improved detection sensitivity in saliva.

For the evaluation of reproducibility of disc performance, we investigated CV(%) of inter-discs and intra-disc. In this case, each sample at 7 different concentrations was repeated minimum 4 times using

different discs and both of the within CV(%) and Run-to-run CV(%) were below 10%, which demonstrate the good reproducibility of the analysis.

Table 8 Comparison of the immunoassay performance measured by lab-on-a-disc and conventional ELISA kit

		ELISA Kits	***Lan-on-a-disc (Absorbance)***	***Lab-on-a-disc (Fluorescence)***
Sample type		Serum	Whole blood	Whole saliva
Sample volume (μL)		300	200(serum)	200
Dynamic range (ng/mL)	hsCRP	1 - 25	1 - 10	1 - 10
	cTnI	2 - 75	1 - 25	1 - 50
	NT-proBNP	0.3 - 20	1 - 25	1 - 50
Limit of detection (ng/mL)	hsCRP	0.124	0.03	0.007
	cTnI	0.48	0.08	0.02
	NT-proBNP	0.08	0.08	0.02
Assay time		> 3 h	< 20 min	< 20 min
Operation		Manual	Automatic	Automatic

4 Conclusions

We have developed a fully integrated and automated lab-on-a-disc for multiplex immunoassay starting from raw samples such as whole blood, urine, saliva etc. to the final detection. To the best of our knowledge, it is the world first report that is capable of fully integrated ELISA type multiplexed immunoassay starting from real samples to the final detection. The disc is designed to have several chambers to have preloaded necessary reagents such as PS beads immobilized with capture antibody, washing buffers, detection antibody, substrate solution etc. In order to have diversity in terms of applications, simply the beads could be replaced with different kinds of beads modified with the corresponding biomarkers.

A novelty of the proposed disc design is that the three different reaction chambers are fluidically connected during the reaction and washing steps but isolated during the detection steps. Using this concept, we could reduce the number of valves and the space to accommodate multiplex immunoassay. To enhance the binding reaction, we used PS beads coated with antibodies and it can freely move within the reaction chambers when the disc spin direction is changed, which results improved binding efficiency.

Assay performance values of the lab-on-a-disc such as LOD or sensitivity were as good as the conventional ELISA kits even though the total assay time was significantly reduced, 20 min. vs. over 3 hours. We believe the current design is good enough for the application with whole blood. In addition, sensitivity was increased by employing fluorescent detection technologies to use saliva sample.

5 Future work

For the future work, it might be necessary to increase sensitivity further in order to be used for saliva diagnostics because the target concentration is 10 ~ 100 times lower for the protein concentration in saliva compared to whole blood. This could be achieved by developing functions such as sample concentration and more sensitive detection technologies such as fluorescence, chemiluminescence, electrochemical based detection.

Furthermore, it is noteworthy that the total reaction time can be further reduced, for example, if we use fluorescence tagged detection antibodies or if we reduce the reaction time by optimizing the binding reaction. In this study, we have used the binding reaction time, 10 minutes, that was optimum condition that we achieved in tube-based experiments. Therefore, it could be further decreased by applying much more efficient mixing condition on disc.

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RESEARCH INTERESTS

- Biomedical application in centrifugal microfluidic system, Lab-on-a-disc
- Immunoassay
- Cell biology

RESEARCH PUBRICATIONS

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