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Cotton-based Diagnostic Devices

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A good diagnostic procedure avoids wasting medical resources, is easy to use, resists contamination, and provides accurate information quickly to allow for rapid follow-up therapies. We developed a novel diagnostic procedure using a “cotton-based diagnostic device” capable of real-time detection, i.e., *in vitro* diagnostics (IVD), which avoids reagent contamination problems common to existing biomedical devices and achieves the abovementioned goals of economy, efficiency, ease of use, and speed. Our research reinforces the advantages of an easy-to-use, highly accurate diagnostic device created from an inexpensive and readily available U.S. FDA-approved material (i.e., cotton as flow channel and chromatography paper as reaction zone) that adopts a standard calibration curve method in a buffer system (i.e., nitrite, BSA, urobilinogen and uric acid assays) to accurately obtain semi-quantitative information and limit the cross-contamination common to multiple-use tools. Our system, which specifically targets urinalysis diagnostics and employs a multiple biomarker approach, requires no electricity, no professional training, and is exceptionally portable for use in remote or home settings. This could be particularly useful in less industrialized areas.

Healthcare in the 21st century is remarkably complex, but can be generally conceptualized as consisting of two major facets, diagnosis and therapy. Widely-used experimental techniques in diagnosis are economically frugal, they do not waste costly or scarce medical resources, and they do not defer treatment as a result of poor time management, i.e., delayed diagnosis resulting from the need to schedule clinical sampling and then wait for the sample to be analyzed before successive therapy can be prescribed. Real-time laboratory results in healthcare demand real-time diagnostics, and *in vitro* diagnostic (IVD) solutions are becoming necessary to provide effective treatments at the population level, especially in the developing world. According to the World Health Organization (WHO), IVD devices for use in developing nations (resource-limited areas and others) should be “ASSURED” (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end users)^{1,2}. Present traditional IVD systems require significant manpower, materials, effort, instruments, time, and expenditure to determine the occurrence or state of a disease in a timely manner. Timely and proper diagnosis currently requires institutional facilities, e.g., large hospitals, which are scarce or unobtainable in developing areas^{3–7}. Ultimately, however, untimely procedures may run the risk of obtaining diagnoses so late that subsequent emergency healthcare costs become escalated. An IVD approach for diagnosis is a step toward the ultimate goal of more effective therapy. While clearly relevant to healthcare demands, IVD can fall short in providing sufficient information if it is used from a one-step perspective, as healthcare professionals must also be able to monitor disease state. This is accomplishable with an IVD platform, but more efforts need to be taken to develop “all-in-one” IVD devices as the science of healthcare and growing real-time demands evolve.

All-in-one detection devices do currently exist, e.g., dipstick-related products (i.e., urine screening) and lateral flow systems products (i.e., pregnancy tests). These served as an inspirational foundation for our work. Clearly, dipstick devices can be layered with multiple detection zones, leveraging the superior all-in-one conceptual approach, and lateral flow systems possess valuable functions of filtration and chromatography. We sought to establish an IVD device with a lateral-based flow format that also used a U.S. FDA-approved material (cotton) because of advantages in cost (~\$ 0.38/per piece), operating time (<10 minutes), sample saving, and the capacity for detecting multiple clinical targets through one step using one device. Our next-generation IVD device leverages the very nature of the material (cotton) used. Cotton is characterized by intertwined surface fibers that are compacted to a high enough degree to provide considerable hydrophobicity. When combined with the highly absorptive qualities of chromatography paper, as in our device, contamination between adjacent reaction areas is

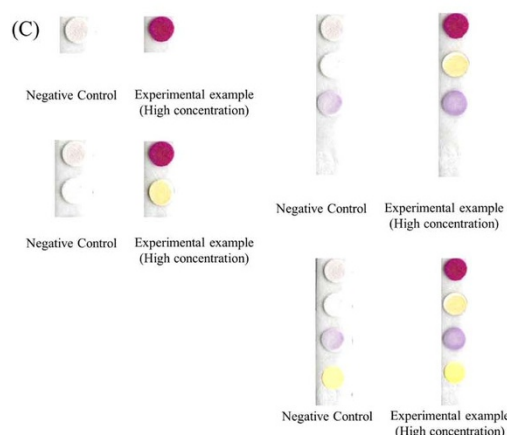
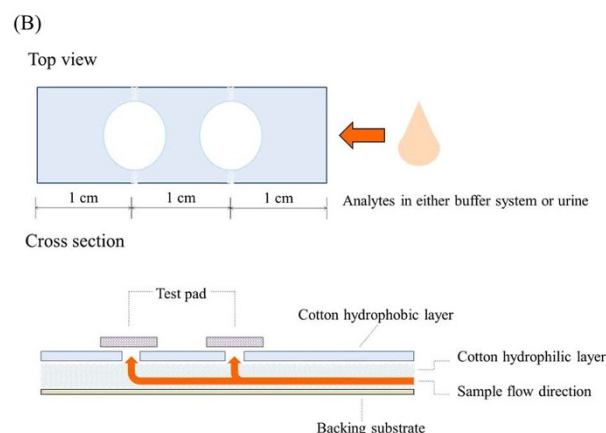
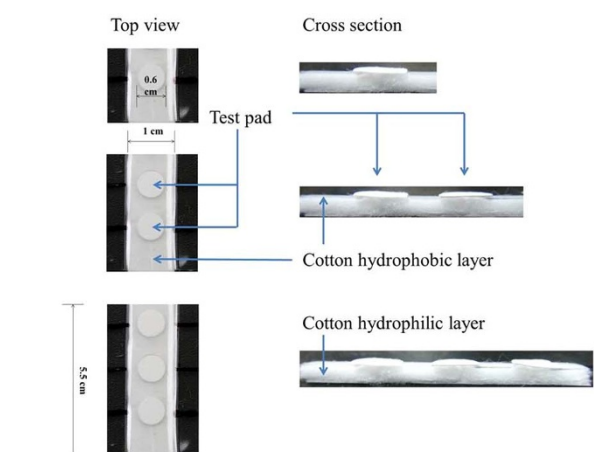
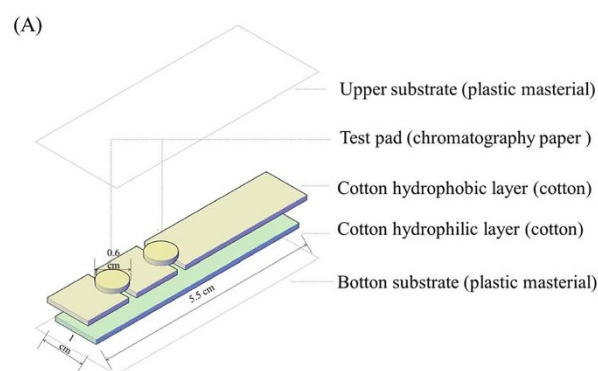


Figure 1 | Schematic diagram of our novel lateral flow cotton-based diagnostic device. This cotton-based device was designed for the diagnosis in a buffer system or in a clinical system. (A) Our cotton-based diagnostic device contains three parts: sample/reaction zone test pad (a single-layer membrane, Whatman chromatography paper), flow channel of cotton hydrophobic and hydrophilic layer (a cotton membrane, Shiseido cleansing cotton), and a background packaging substrate (a thick plastic material lamination film). (B) Top view and cross section show the distance of each test pad (1 cm) and sample flow direction (orange line). (C) This lateral flow-based device has been shown for the diagnosis of BSA, nitrite, and uric acid assays in a buffer system. Photographic evidence of our device test pads (top view) showing negative control and experimental example after we immobilized BSA, nitrite, and uric acid assays on the test pad.

limited between reaction areas, and multiple biomarker-based tests can be performed with greater accuracy (standard curve) and sensitivity (limit of detection).

A number of items encountered in daily life use cotton in their manufacture, including cleansing cotton, panty liners, and diapers. Further, cotton is U.S. FDA-approved for healthcare uses, in part because it does not cause inflammatory skin responses (e.g., as with tampons or diapers). The function of cotton is often to absorb liquid matter, typically from the skin, making it a natural choice for a biomedical device requiring an absorptive function. However, in the design of our fluid channel, we used a specific kind of cotton consisting of hydrophobic (cotton surface layers) and hydrophilic (cotton interior) properties that are jointly leveraged to create effective channels for sample delivery. For reaction areas and results display, we embedded chromatography paper, our test pad, onto the exterior surface of the cotton via stacking. As liquid sample is added, it flows along hydrophilic cotton layers, and a portion of the liquid sample subsequently flows to the first assay reagent pad then proceeds through that to the second assay pad as shown in Fig. 1. Cross-contamination is avoided, and an accurate multiple-biomarker standard calibration curve is performed immediately. We strongly believe that cotton-based analysis devices have more potential and offer greater plasticity for advanced IVD system development, especially for various point-of-care diagnostic implementations. In summary, this cotton-based diagnostic device supplies a number of benefits including rapid detection time (less than 5 minutes), high sensitivity via the dilution effect provided by cotton channels and the chromatographic effect of paper (chromatography paper) at the test pad point, and avoidance of common contamination issues between reaction areas during fluid delivery while allowing for the adoption of standard calibration curve analyses to accurately obtain semi-quantitative information.

Results

Cellulose-based materials (e.g., paper and nitrocellulose) have been widely used as a support material/platform for the implementation of both qualitative and quantitative testing including clinical diagnostics (e.g., dengue fever assay, VEGF level), organic/inorganic chemical analysis, environmental/geochemical analysis, pharmaceutical and food chemistry^{8–17}. Cotton, a U.S. FDA-approved material, is also categorized as a fiber-based material with good flexibility and biocompatibility that is suitable for use in creating a lateral flow-based diagnostic device. Interestingly, we found that a cleansing cotton produced by Shiseido® possessed both hydrophilic (inner part of cleansing cotton) and hydrophobic (exterior part of cleansing cotton) characteristics (Fig. 1A) as confirmed by contact angle (Fig. 2 and Supplementary Fig. 1) and maintained time test experiments (Fig. 2). The mean contact angle of the hydrophobic cotton was $124.41 \pm 6.53^\circ$ (average \pm standard deviation, $N = 4$) and the limit of maintained droplet of time is as long as possible for the hydrophobic cotton. Fig. 2 illustrates the mean time of water main-

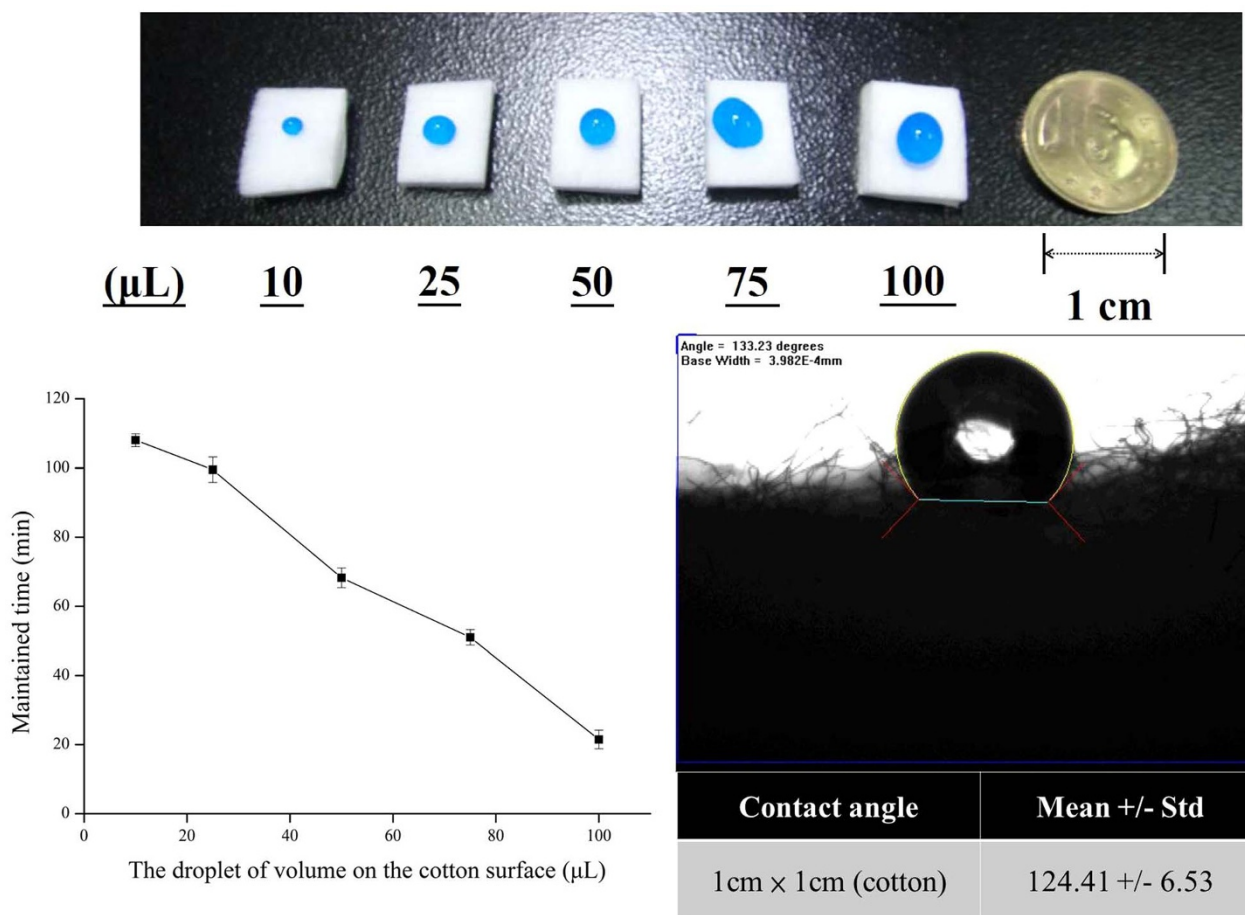


Figure 2 | Shiseido cleansing cotton features illustrated by water maintained time and contact angle on cotton hydrophobic layer. We dropped different volumes of blue dye (10, 25, 50, 75, and 100 μL) onto the hydrophobic layer to determine water maintained time (samples number $N = 4$). We examined contact angle effects using 10 μL of blue dye onto the cotton hydrophobic layer (1 cm \times 1 cm).

tained at various water volumes for the cotton used in our device and demonstrates the excellent linear relationship at maximized water volume (100 μL) in a sheet of said hydrophobic cotton (1 cm \times 1 cm) and hydrophobic layer of cotton is one of important sections in the cotton-based device that could be affected with greater accuracy (standard curve) and cross contamination or not (Supplementary Fig. 2). We made excellent use of this particular feature, as we attempted to develop a novel diagnostic platform while following the developmental standards for a postamendment medical device, in regard to FDA regulations, that exploits the dual nature of this particular cotton. Colorimetric assays, commonly used, are well suited for paper-based (test pad) assays of the type employed in our cotton-based diagnostic device (Fig. 1C) and the difference of diameter in test pads (0.6 cm and 0.3 cm) could be performed with different variation and sensitivity in the cotton-based device (Supplementary Fig. 3). In this assay, color intensity was distinguishable both by the naked eye and by statistical/semi-quantitative analysis using imaging software (ImageJ).

Cotton-based diagnosis devices for colorimetric assays. Fig. 3A~D illustrates the use of cotton-based diagnostic devices for the diagnosis of different analytes (nitrite, BSA, urobilinogen and uric acid) in a buffer system. Using our colorimetric assays, we analyzed artificial samples of urine protein, nitrite, urobilinogen and uric acid in clinically relevant ranges of approximately 0.38 ~ 30 μM for urine protein, 0.156 ~ 2.5 mM for nitrite, 7.8 ~ 125 μM for urobilinogen and 100 ~ 1600 μM for uric acid (Fig. 3A~D)^{18–20}. In our assays, we dipped the bottom of each cotton-based device into a premade test

solution (~2 mL). The fluid channel (both single and multiple test pads) filled the entire cotton-based device within approximately 10 seconds, and assays required approximately 10 minutes for color development of colorimetric analysis (Supplementary Fig. 4).

BSA is also commonly used as a quantitative standard proteins, by analyzing an unknown quantity of protein to known amounts of BSA (e.g., Bradford protein assay)^{21–23}, so that we have adopted BSA to replace total urine protein in the clinical urine. In healthy adults, the daily urinary excretion of total protein should be less than 150 mg and the approximate concentration of total protein should be less than 4 μM ²⁴. The presence of nitrite ions is a clinical indication of abnormality in human urine, i.e., urinary tract infections or bacterial infections. Therefore, the limit of detection of nitrite ions is as low as possible for *in vitro* urinary diagnostic devices. Uric acids are general components in human serum. They act as an important biomarker for the detection of gout. The concentration of uric acid should be less than 400 μM ¹⁹. Most of the urobilinogen is excreted by the feces and recovered by the enterohepatic circulation, some loss of urine a day by about 1 to 4 mg. When hemolytic disease or liver disease existed, a significant increase in value of urine urobilinogen will be detected²⁰. Fig. 3A~D illustrate the mean intensity of four assays (total protein, nitrite ions, urobilinogen and uric acid) in a buffer system at various concentrations. The results indicate that mean intensity corresponds to concentrations of BSA, nitrite, urobilinogen and uric acid in our cotton-based diagnostic device, allowing for the establishment of a clear calibration curve that defines the lower limits of the limit of detection (LOD) for which these assays can be used (Fig. 3A~D). The LOD was calculated to be the concentration that

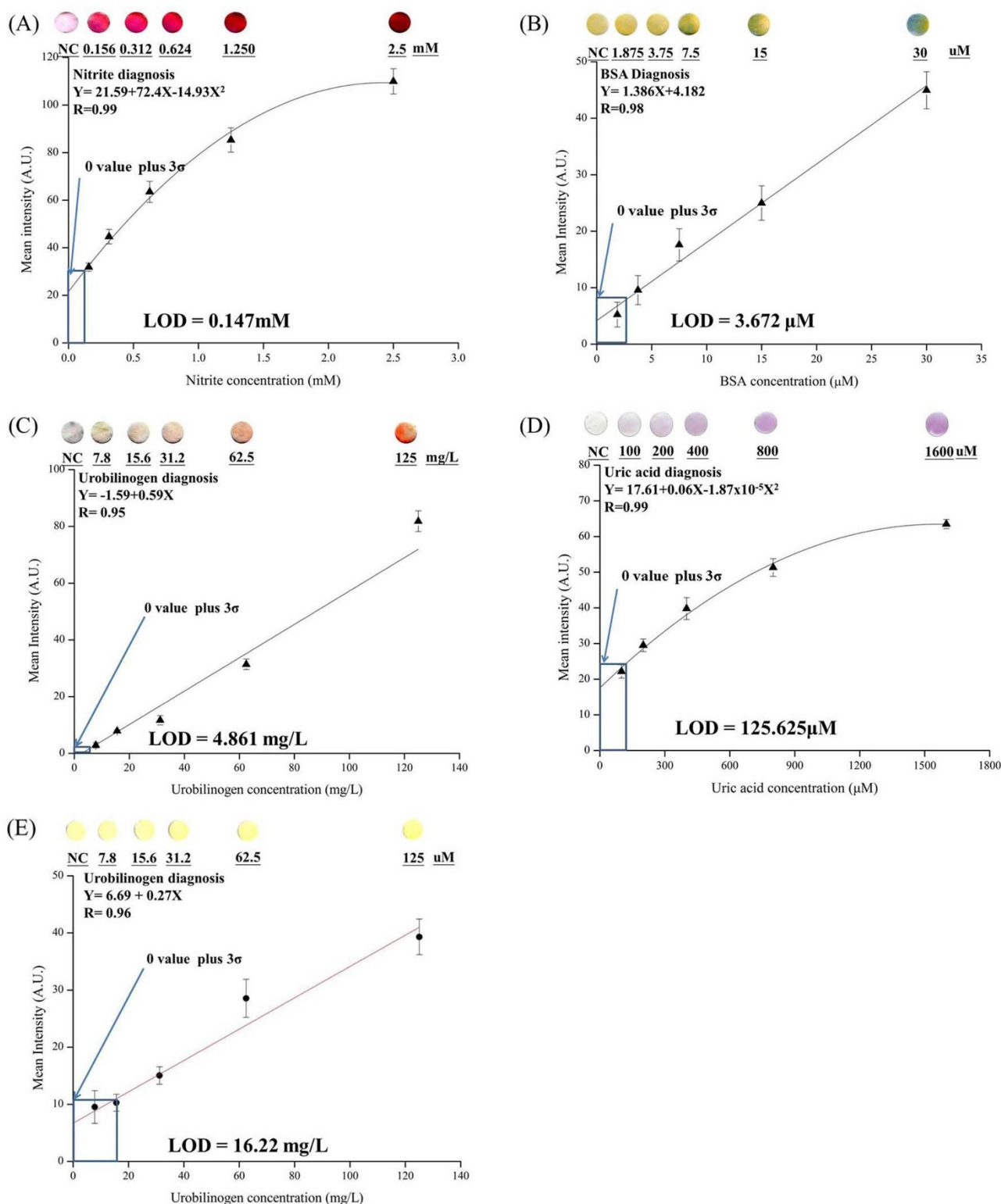


Figure 3 | The results of cotton-based diagnostic device analysis of nitrite, urobilinogen, BSA, and uric acid in a buffer system and clinical sample. We diluted purified nitrite (0.156 ~ 2.5 mM, 2-fold serially dilution) for A, BSA (1.875 ~ 30 μ M, 2-fold serially dilution) for B, urobilinogen (7.8 ~ 125 μ M, 2-fold serially dilution) for C, uric acid (100 ~ 1600 μ M, 2-fold serially dilution) for D with PBS buffer and urobilinogen (7.8 ~ 125 μ M, 2-fold serially dilution) for E with urine sample. After data readout (via desktop scanner to scan the reacting test pad and turn the colorimetric output signal into a grayscale value to analyze the color intensity via the graphics processing software, ImageJ), we obtained sensitivity by parabola (nitrite and uric acid assays) and a linear (BSA and urobilinogen assays) equation to generate an Originpro 8 curve fitting. This figure shows the calibration plot for the mean intensity (I) of the colorimetric results for reagent compound reaction in our cotton-based diagnostic device versus the amount of the substrate absorbed by each test pad. Each value is the mean of ten replicates (samples number $N = 10$), and the error bars represent the standard deviations of the measurements. The R^2 values of the curve fit to the data using the equation are nitrite: 0.99, BSA: 0.98, urobilinogen: 0.95, uric acid: 0.99 and urobilinogen in clinical sample: 0.95 in this study.

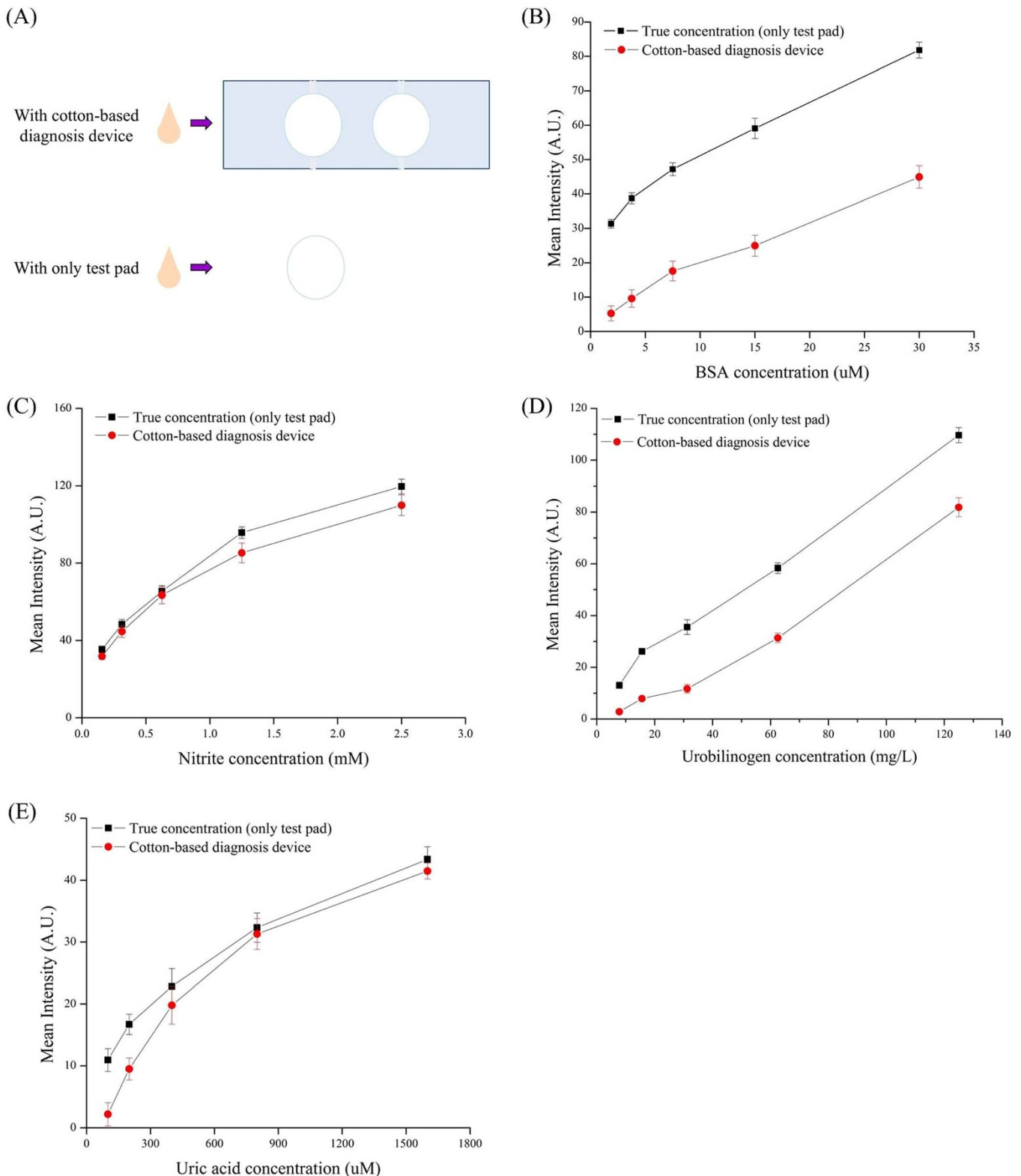


Figure 4 | The results between test pad and cotton-based diagnostic device analysis of nitrite, BSA, urobilinogen and uric acid in a buffer system. A comparison of diagnosis data in our buffer system: sample detection using our cotton-based diagnostic device and test pad assays only (samples number $N = 3$).

generated an intensity level three times the standard deviation of intensities measured in zero concentration A.U. (all calculations were conducted at linear regions). Here, the LODs of BSA, nitrite, urobilinogen and uric acid in a buffer system were 3.672 μM , 0.147 mM, 4.861 μM and 125.625 μM , respectively.

Dilution effect of cotton-based fluidic channels. Commercial products “strip tests” (i.e., lateral flow assays) have already found a place in the medical market (e.g., pregnancy test strips). Enclosed flow channel strip tests have advantages in that they employ the dilution effect to create clearer and amplified ELISA signals. We


Table 1 | A comparison of detection values using our cotton-based device to detect nitrite, BSA, and uric acid in a buffer system

Nitrite concentration [mM]	Paper (only test pad)	Cotton device
0.156	35.46	31.86
0.312	48.4	44.67
0.624	65.43	63.5
1.25	95.76	85.29
2.5	119.67	109.94
BSA concentration [μ M]	Paper (only test pad)	Cotton device
1.875	31.36	5.24
3.75	38.76	9.57
7.5	47.21	17.59
15	59.08	24.98
30	81.83	44.95
Urobilinogen concentration [mg/L]	Paper (only test pad)	Cotton device
7.8	13.09	2.87
15.6	26.19	7.90
31.2	35.60	11.72
62.5	58.31	31.41
125	109.69	81.83
Uric acid concentration [μ M]	Paper (only test pad)	Cotton device
100	10.94	2.18
200	16.71	9.49
400	22.84	19.79
800	32.34	31.31
1600	43.37	41.5

have compared the experimental data (output signal value) of our cotton-based device and chromatograph paper only (i.e., the test pad of our diagnostic device). We determined standard detection curves for our assays (BSA, nitrite, urobilinogen and uric acid) via our cotton-based device (Fig. 3A~D), and then measured the same samples by dipping them onto test pad paper (Supplementary Fig. 4). Fig. 4B~E show that the output value of the standard curve from our test pad (only) was significantly higher than the curve found using our cotton-based device. These results indicate that our cotton-based device was strongly affected by the dilution effect, which would adversely affect the concentration readout. To overcome this issue, we attempted to establish a compensation curve to calibrate data accuracy (Fig. 3A~D and Table 1). We noted that the difference in output signal value for nitrite analysis between cotton and paper (only) in our study was less severe than in other sample fluids, thus using a cotton-based device is more suitable for nitrite assays than for analysis of BSA, uric acid and urobilinogen in a buffer system. We found that the dilution effect of cotton produced a normal siphon effect in our experiment that lowered our detected concentration. To resolve this, we used a standard calibration curve for our cotton-based diagnostic device in order to more accurately determine results for BSA and uric acid assays (Fig. 4B~E). In addition to examine impact of dilution, we assessed the association of the distance from the device's terminal to the vertical test pad on our cotton-based diagnostic device (Supplementary Fig. 5). Finally, we found proper sequence of the test-pad on our cotton-based device: urobilinogen (1st), urine protein (2nd), uric acid (3rd) and nitrite (4th) and this order optimize our device.

Cotton-based diagnostic devices for urinalysis. Chronic kidney disease affects between 8 and 10% of the adult population around the world, and every year millions of people die from complications related to chronic kidney diseases²⁵. In the United States, it is estimated that more than 90,000 people are waiting for kidney transplants, and each year as many as 4,500 people may die while waiting²⁶. In Taiwan, the number of regular dialysis treatment

patients has exceeded 70,000²⁷. The prevalence and incidence of chronic kidney disease is increasing around the world. Albuminuria not only reveals impairment of kidney function, it has been independently associated with greater risk of coronary artery disease²⁸. Patients with levels of proteinuria in excess of 3.5 g/day would show evident clinical manifestations of nephrotic syndrome. Further immunochemical assays have shown that urine albumin accounts for more than 80% of the excreted proteins within urine²⁹. Thus, early screening of urine albumin is a crucial and proper treatment that can slow the progression of chronic kidney disease. The end-stage of kidney disease requires regular hemodialysis and is a tremendous global healthcare concern with demands that increase the cost of available medical resources worldwide. Beyond urinalysis for kidney disease, examination of urine for the presence of nitrate can provide guidance for determining types of urinary tract infections, and analysis for uric acid may be indicative of kidney stone presence. Clearly, there is a need worldwide for the development of IVD devices that are specific to detection of kidney-related diseases. Urinalysis dipsticks and litmus paper are commonly used paper-based IVDs. Confirming qualitative analysis as a clinical assay is an important initial test or stepping stone in designing new diagnostic devices with minimal errors and high readability.

For multiple target detection in clinical samples, the most difficult problem for IVD devices is cross-contamination of testing specimens. To find out whether our cotton-based device was affected by this issue, we examined clinical samples of urine. According to commercially available dipsticks, the clinical mean for critical urine protein concentration is approximately 1.4 μ M, nitrite concentration is approximately 0.3 mM, urobilinogen concentration is approximately 25 μ M and pH is approximately 4.6 ~ 8)^{19,20,30,31}. To simulate real clinical urine specimens, we spiked BSA (replaced urine protein), nitrite, urobilinogen and hydrochloric acid to create samples that mimicked human physiological conditions. Our results indicate that as the distinguished results on device can be read out by naked eye on different numbers of test pads (2, 3, 4 separately) and different dia-

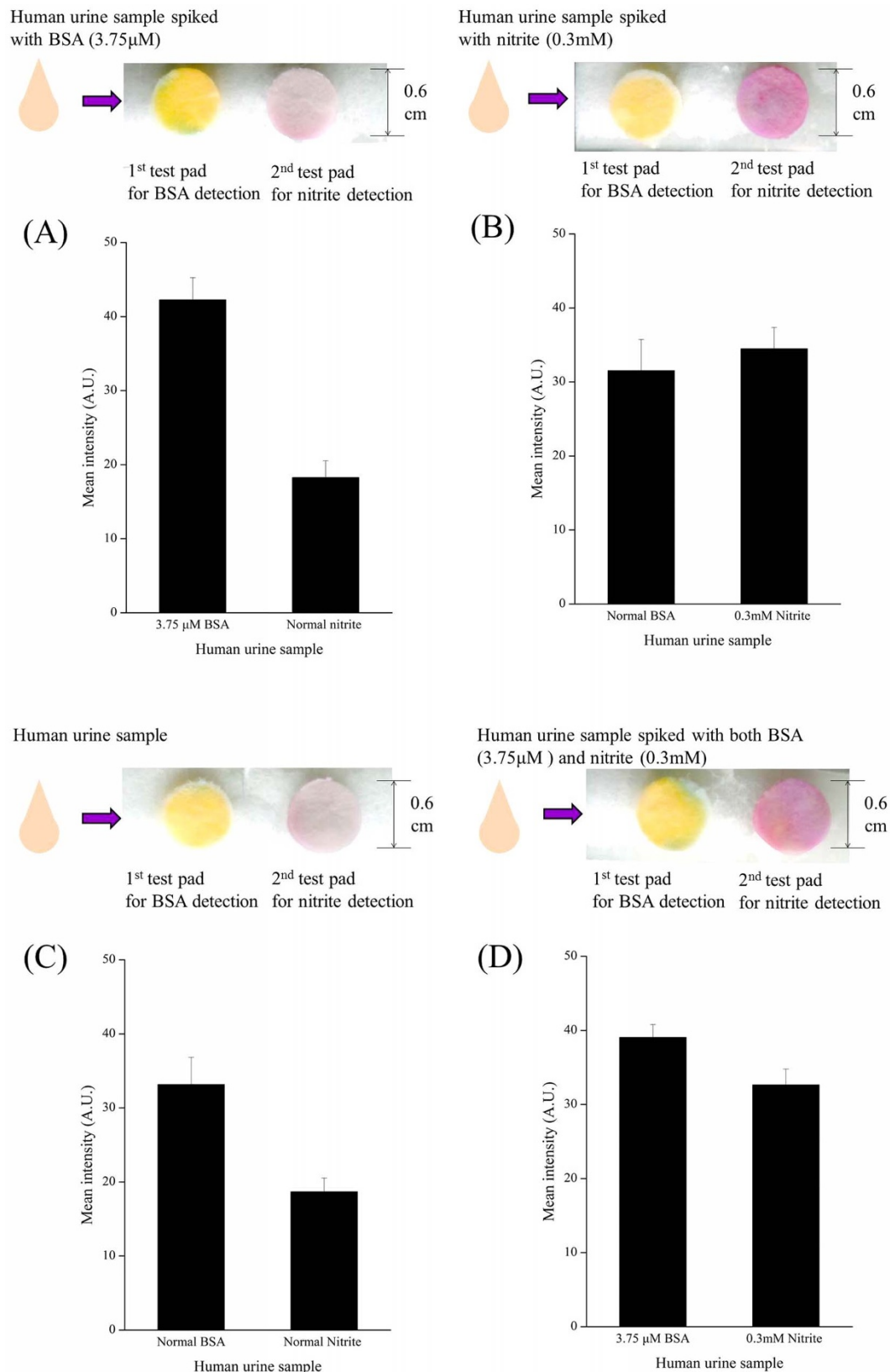


Figure 5 | Examination for cross-contamination in 3.5 cm cotton-based diagnosis with 0.6 cm test pad for BSA and nitrite assays in a clinical urine system. The total diagnosis procedure was almost the same as the protocol shown in Figure 3. Briefly, we diluted the purified nitrite (0.3 mM) and BSA ($3.75\text{ }\mu\text{M}$) using PBS buffer. After data readout (using a desktop scanner to scan the reacting test pad and turning the colorimetric output signal into a grayscale value to analyze the color intensity via the graphics processing software, ImageJ), we obtained the sensitivity to generate an Originpro 8 histogram. The above histogram illustrates the calibration plot for the mean intensity (I) of the colorimetric result by reagent compound (nitrite and BSA assays) reaction in the cotton-based diagnostic assay versus the amount of the substrate (nitrite, BSA) absorbed to each test pad. Each value is the mean of five replicates (samples number $N = 5$), and the error bars represent the standard deviations of the measurements.

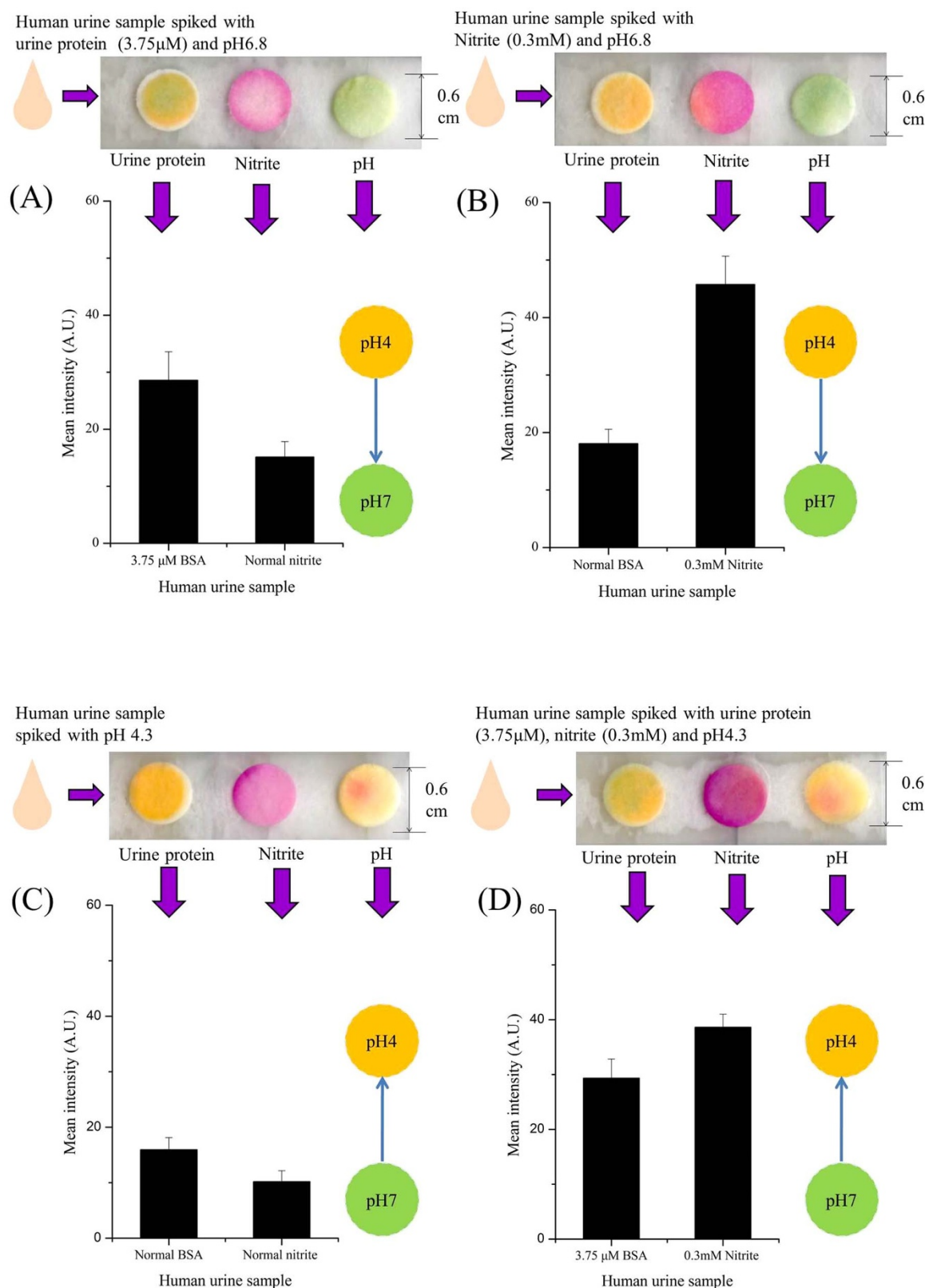


Figure 6 | Examination for cross-contamination in 5.5 cm cotton-based diagnosis with 0.6 cm test pad for BSA, nitrite and pH assays in a clinical urine system. The total diagnosis procedure was almost the same as the protocol shown in Figure 3. Briefly, we diluted the purified nitrite (0.3 mM), BSA (3.75 μ M) and hydrochloric acid (pH 6.8 to 4.3) using clinical urine. After data readout (using a desktop scanner to scan the reacting test pad and turning the colorimetric output signal into a grayscale value to analyze the color intensity via the graphics processing software, ImageJ), we obtained the sensitivity to generate an Originpro 8 histogram. The above histogram illustrates the calibration plot for the mean intensity (I) of the colorimetric result by reagent compound (nitrite and BSA assays) reaction in the cotton-based diagnostic assay versus the amount of the substrate (nitrite and BSA) absorbed to each test pad. And pH assays results could be directly observed by the naked eye. Each value is the mean of five replicates (samples number $N = 5$), and the error bars represent the deviations of the measurements.



Table 2 | The results of detecting nitrite, BSA, and urobilinogen of different concentrations via our cotton-based device to examine clinical urine samples

Urinalysis (size of cotton: 3.5 cm × 1 cm)	1 st test pad (0.6 cm) for BSA assay	2 nd test pad (0.6 cm) for nitrite assay	
Human urine sample	33.15 +/− 3.67	18.68 +/− 1.86	
Human urine sample spiked with nitrite (0.3 mM)	31.52 +/− 4.23	34.46 +/− 2.88	
Human urine sample spiked with BSA (3.75 μM)	42.25 +/− 3.0	18.26 +/− 2.26	
Human urine sample spiked with BSA (3.75 μM) and nitrite (0.3 mM)	39.06 +/− 1.74	32.64 +/− 2.15	
Urinalysis (size of cotton: 5.5 cm × 1 cm)	1 st test pad (0.6 cm) for BSA assay	2 nd test pad (0.6 cm) for nitrite assay	
Human urine sample	15.95 +/− 2.18	10.19 +/− 1.98	
Human urine sample spiked with nitrite (0.3 mM)	18.05+/− 2.49	45.75 +/− 4.93	
Human urine sample spiked with BSA (3.75 μM)	28.58 +/− 5.00	15.13 +/− 2.71	
Human urine sample spiked with BSA (3.75 μM) and nitrite (0.3 mM)	29.33 +/− 3.49	38.6 +/− 2.42	
Urinalysis (size of cotton: 5.5 cm × 1 cm)	1 st test pad (0.3 cm) for urobilinogen assay	2 nd test pad (0.3 cm) for BSA assay	3 rd test pad (0.3 cm) for nitrite assay
Human urine sample spiked with BSA (3.75 μM) , nitrite (0.3 mM) and urobilinogen (25 mg/L)	87.66 +/− 4.11	75.41 +/− 4.23	83.72 +/− 5.67

meter of test pads (0.3 cm and 0.6 cm). We could clearly observe that the mean intensity increased approximately 1.2 ~ 1.5 times for urine protein and 2.7 ~ 3 times for nitrite at least and each assay's variation of mean intensity was approximately 4 ~ 9% (Fig. 5, 6 and Table 2). Fig. 7 illustrates the use of 4 separate test pads on cotton-based

diagnostic devices and each pad with 0.3 cm diameter for the diagnosis of different analytes (nitrite, BSA, urobilinogen and pH) in a clinical urine, and than different analytes could be directly observed that mean intensity significant increased (nitrite, BSA, urobilinogen) and different color (pH), but urobilinogen assays has different color

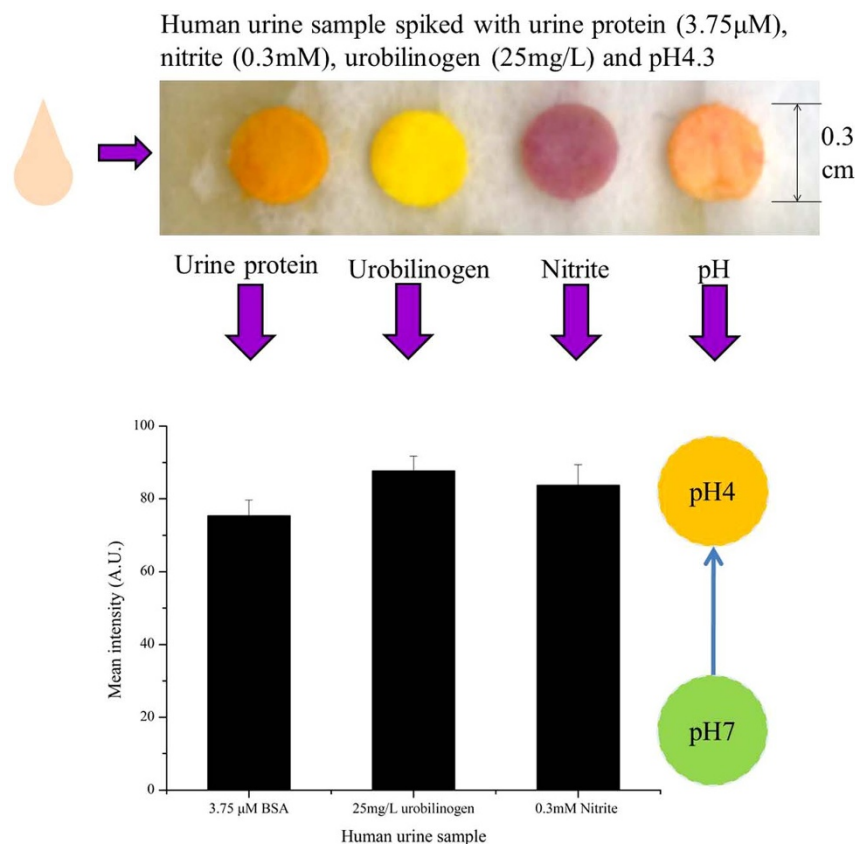


Figure 7 | Examination for cross-contamination in 5.5 cm cotton-based diagnosis with 0.3 cm test pad for BSA, nitrite, pH and urobilinogen assays in a clinical urine system. The total diagnosis procedure was almost the same as the protocol shown in Figure 3. Briefly, we diluted the purified nitrite (0.3 mM), BSA (3.75 μM), hydrochloric acid (pH 6.8 to 4.3) and urobilinogen (25 mg/L) using clinical urine. After data readout (using a desktop scanner to scan the reacting test pad and turning the colorimetric output signal into a grayscale value to analyze the color intensity via the graphics processing software, ImageJ), we obtained the sensitivity to generate an Originpro 8 histogram. The above histogram illustrates the calibration plot for the mean intensity (I) of the colorimetric result by reagent compound (BSA, urobilinogen and nitrite assays) reaction in the cotton-based diagnostic assay versus the amount of the substrate (BSA, urobilinogen and nitrite) absorbed to each test pad. And pH assays results could be directly observed by the naked eye. Each value is the mean of five replicates (samples number $N = 5$), and the error bars represent the deviations of the measurements.

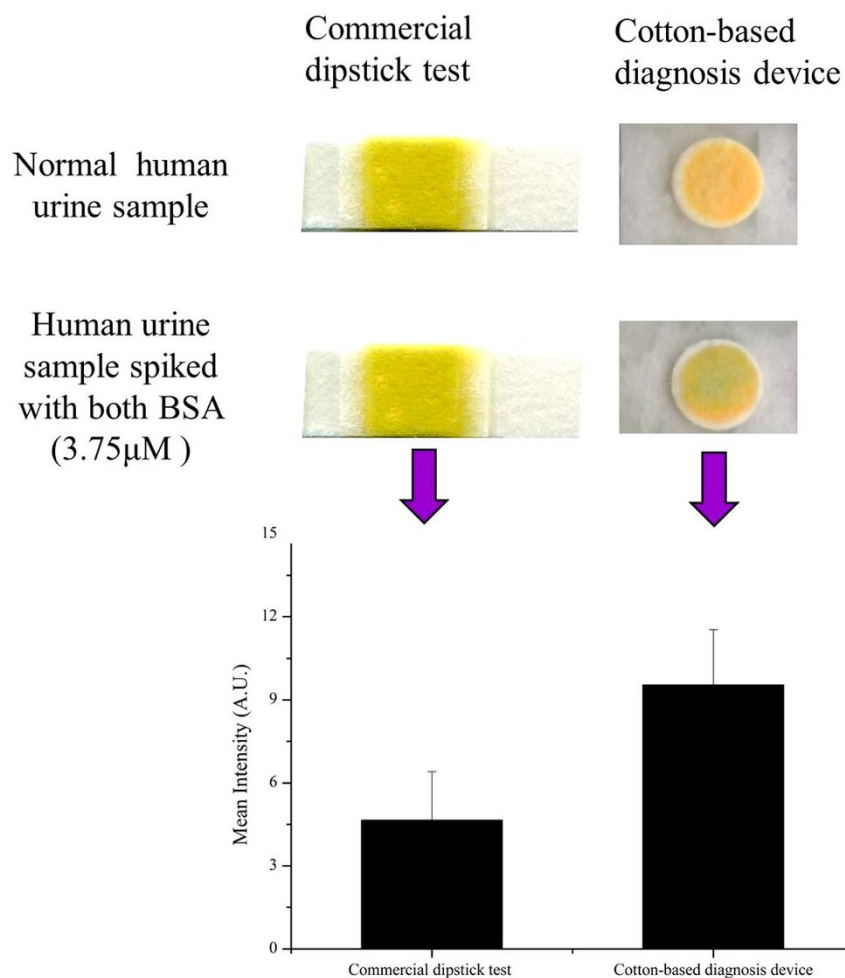


Figure 8 | The comparisons commercial dipstick and cotton-based diagnostic devices on the urinalysis assays by visual observation and quantitative. Photographic evidence comparing commercial strip test and cotton-based diagnosis of a urine sample shows that the cotton-based diagnostic device changed color (yellow to green) as visible by the naked-eye in spiked BSA (3.75 μM) sample. The total quantitative procedure was almost the same as the protocol shown in Figure 3. After data readout (using a desktop scanner to scan the reacting test pad and turning the colorimetric output signal into a grayscale value to analyze the color intensity via the graphics processing software, ImageJ), we obtained the sensitivity to generate an Originpro 8 histogram. Each value is the mean of three replicates (samples number $N = 3$), and the error bars represent the deviations of the measurements. The histogram illustrates the comparisons commercial dipstick and cotton-based diagnostic devices calibration plot for the mean intensity ($I = \text{spiked BSA } 3.75 \mu\text{M} - \text{normal}$) of the colorimetric results.

in the test pad between clinical urine (yellow) and buffer (orange) system (Fig. 3C and E) and pH. The results support the effectiveness of our device in limiting cross-contamination during multiple target detection analyses and suggest it may have potential for use in an array of clinical diagnostic and bio-detection assays. We considered the dilution effect within our cotton-based fluid channel and determined that a calibration could accommodate this effect, because nitrite in normal urine situation is almost non-existent, for example nitrite mean intensity is 20 ~ 40 A.U. can useful range in real urine composition nitrite concentration 0 mM ~ 0.3 mM from Fig. 3A. Because urine protein of trace is in normal urine, so our analysis method only displays relative concentrations, for example Table 2 subtracted BSA (urine protein) mean intensity ~ 9 A.U. transform to

BSA mean intensity curve ~3.75 μM from Fig. 3B). Visual observation of colorimetric values for BSA in urine samples applied to our cotton-based device achieved the same effect values found when using a commercial dipstick test assay (TERUMO, Japan; No. UA-C03K1), indicating that about 2 times more sensitive than commercial dipstick test assay and thus cotton-based device is more suitable for urine protein detection. (Fig. 8 and Table 3).

Discussion

At present, the same set of clinical samples can be detected for one fluid channel using multiple-biomarker assays in paper-based material, which allows for practical assays of urine, glucose, nitrite, kidney protein, urinary protein, ketone body, bilirubin, uric acid, and

Table 3 | Comparisons of sensitivity, cross-contamination, and market share between commercial dipstick devices and cotton-based diagnostic devices during urinalysis assays

Urinalysis	Commercial dipstick for BSA assay (dipstick system)	Cotton-based for BSA assay (lateral flow system)
Sensitivity	*	**
No cross-contamination	*	**
Market share	**	*



more^{19,32}. The objective of our novel design is to provide a biomedical diagnostic device for detecting the concentration of minute clinical samples in fluid. Various commercial products already exist that can perform a single assay, and dipstick tests can perform multiple-biomarker assays, but they carry a high possibility of clinical sample contamination. We were able to create a cotton-based diagnostic platform that can be used for multiple assays in a single fluid channel (in one step/in one device) that employs a combination of hydrophobic and hydrophilic layers to build a microfluidic boundary and an active delivery channel to isolate and apply clinical samples for analysis. The dilution effect was calibrated with the flow distance for cotton—both parameters are important considerations when designing any such device as evidenced by the discovered slopes of standard curves. This allowed us to determine optimal sequence for our multiple-biomarker assays on the cotton-based diagnostic devices in the buffer system as follows: urobilinogen, BSA, nitrite and uric acid, respectively. In medical assays, sensitivity and precision of data are often affected by the unpurified state of samples. The design of our device uses chromatography paper test pads and cotton material to create a fluid channel that dilutes concentration and filters unpurified samples before they are picked up by the chromatography paper. This allows for highly sensitive assays. We employed a material with different characteristics to enable our biomedical essays to provide the best performance, and our evidence indicates an impressive capacity for multiple high-quality diagnoses. It is our hope to improve the sensitivity and accuracy of our device platform to allow for the detection of biomarkers (biomolecules), such as uric acid do not work in the urine clinical samples. The design of a cotton-based diagnostic device comprises hydrophilic and hydrophobic layers of cotton that act as a lateral flow channel and a test pad of chromatography paper that could act as a multi-assay reaction zone. We further discovered that the reagent contamination often found with other biomedical assay devices could be limited to negligible levels using our cotton array. These experimental results indicate that our novel diagnostic tool is simple, inexpensive, eliminates cross-contamination, does not require electricity consumption, and is portable (flexible). This device has great potential for use in remote or home settings, and could be highly impactful in less industrialized areas. We hope that this research stimulates further study with a view to developing *in vitro* diagnostic devices for the clinical diagnosis of modern diseases, particularly in economically developed or developing nations.

Methods

Reagents of urine-based assays. The patterned paper for our cotton-based diagnostics device can be derivatized for biological assays by adding appropriate reagents to the test pads (reaction areas). We demonstrated this concept by detecting urine total protein (using bovine serum albumin as a demonstration), nitrite, and uric acid. The bovine serum BSA stock solution (60 μ M) was prepared by dissolving 415 mg BSA ($\geq 99\%$, Sigma-Aldrich, US, No. 9048468) in 100 mL PBS solution (pH = 7.0). Then, this stock solution was diluted with water to obtain serially diluted nitrite standard solutions with concentrations of 30, 15, 7.5, 3.75, and 1.875 μ M. The nitrite stock solution (10.0 mM) was prepared by dissolving 69.0 mg sodium nitrite ($\geq 99\%$, Sigma-Aldrich, US, No. 7632000) in 100 mL water. This stock solution was then diluted with water to create serially diluted nitrite standard solutions with concentrations of 2,500, 1,250, 625, 312, 156, and 78 μ M. The urobilinogen stock solution 25 g/L was prepared by dissolving 2.5 g urobilinogen (Santa Cruz Bio, US, No. SC-296690) in 100 mL. This stock solution was then diluted with water to create serially diluted urobilinogen standard solutions with concentrations of 7.8, 15.6, 31.2, 62.5, and 125 mg/L. The uric acid stock solution 12.8 mM was prepared by dissolving 215.0 mg uric acid ($\geq 99\%$, Sigma-Aldrich, US, No. 69932) in 100 mL sodium hydroxide solution (0.2 M). The serially diluted uric acid standard solutions were prepared by diluting the stock solution with 0.2 M NaOH to provide concentrations of 1,600, 800, 400, 200, and 100 μ M. The indicator solution for BSA contains 250 mM citrate ($\geq 99\%$, Sigma-Aldrich U.S., No. 6132043) buffer solution (pH = 1.8) in a separate well and then a 3.3 mM solution of tetrabromophenol blue (TBPB $\geq 99\%$, Sigma-Aldrich U.S., No. 4430255) in 95% ethanol was layered over the citrate buffer solution. The indicator solution for nitrite contains 50 mM sulfanilamide ($\geq 99\%$, Sigma-Aldrich U.S., No. 63741), 330 mM citric acid ($\geq 99.5\%$, Sigma-Aldrich U.S., No. 77929), and 10 mM N-(1-naphthyl) ethylenediamine dihydrochloride ($\geq 98\%$, Sigma-Aldrich U.S., No. 1465254). The indicator solution for urobilinogen contains 0.1 M 4-(Dimethylamine)benzaldehyde (98%, AlfaAesar, U.S., No. A11712). The indicator solution for uric acid consisted of a 1 : 1 mixture of solution A (2.56% (w/v),

2'-biquinoline-4,4'-dicarboxylic acid disodium salt hydrate, $\geq 98\%$, Sigma-Aldrich U.S., No. 979884) and solution B (20 mM sodium citrate, Sigma-Aldrich U.S., No. 6132043 and 0.08% (w/v) copper (II) sulfate, $\geq 99\%$, Sigma-Aldrich U.S., No. 7758987)^{18,33,34}. The indicator solution for pH contains 0.02% Methyl Red sodium salt (95%, sigma, U.S., No. 114502) and 0.25% mM Bromothyle blue (95%, sigma, U.S., No. 114413).

Materials of cotton-based diagnostic devices. We used a cotton pad (Shiseido Cleansing Cotton, Shiseido, Japan; No. 79014) uniquely equipped with hydrophilic and hydrophobic layers in an intact sheet to establish a lateral flow channel in our cotton-based diagnostic device. Commercial chromatography paper (Whatman chromatography paper, GE Healthcare Whatman, Springfield Mill, UK; No. 30306132) was used as a test pad for coating reagents and establishing a reaction zone for colorimetric assays. We introduced a plastic substrate (lamination film, MAS, A4, 216 mm \times 303 mm), using a laminator, to reinforce our tightly compacted cotton channel and act as the top and bottom substrate for our cotton-based diagnostic device.

Fabrication and process of cotton-based diagnostic devices. In our search for simple fabrication that would meet the demand for multiple assays, we created a fluid channel system composed of cotton material as a base to explore multiple detection settings and provide an alternative solution to address the contamination issue which commonly affects lateral flow-based biomedical devices. The design of this study was as follows (Fig. 1A) : 1) we tailored the cleansing cotton into a rectangular-shaped piece (5.5 cm \times 1 cm) (fitting our temporal processing skill), identified and rearranged the hydrophobic and hydrophilic (as a fluidic channel) parts toward the upper and bottom sides, respectively, and subsequently cut access holes on the upper sides of the hydrophobic zone for test pad insertion; 2) we placed a test pad (chromatography paper, ϕ = 5 mm) that had already been coated with a detection reagent (i.e., BSA, nitrite, and uric acid detection reagents) on the top of the hydrophobic structure so that the individual test pads corresponded to each access hole at a distance of 1 cm, which allowed for direct and convenient observation of the reaction intensity (Fig. 1B), and; 3) we used a laminator to solidify the multilayered device and provide natural, impervious top and bottom limits.

The operational procedure and reactive mechanism was multiphased. First, we immersed the absorptive terminal of our cotton-based diagnostic device into our liquid detection sample so that the sample solution would flow along the fluidic channel and pass through the hole to the test pad. Fluid flowed from the front of the hydrophilic layer to the test pads and then to the hydrophilic layer followed by the hydrophobic layer and finally to the plastic material. Because the liquid absorptive strength of our test pads (chromatography paper) was greater than that of cotton, one part of the sample contacting paper did not release reagent or reacted reagent flowing into the channel (Fig. 1B and the Supporting Movie). Second, we acquired colorimetric results using a desktop scanner and by analyzing the correlation between color intensity (converted to grayscale) and analyte concentration using image processing software (ImageJ). The lamination film (a transparent shell) not only provided the backing substrate for our cotton-based diagnostic device to facilitate packaging and promote internal hydrophilicity/hydrophobicity, it protected the overall function of the device and allowed for easy observation of flow channel dynamics.

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Author contributions

C.M.C. and F.G.T. conceived the idea of the project and wrote the manuscript. S.C.L., C.M.K., and H.K.W. designed the experiments. S.C.L., C.M.K., and H.K.W. performed the experiments and analyzed the data. M.Y.H. and C.L.C. provided recommendations for the clinical experiment and wrote the manuscript. All authors discussed the results and commented on the paper. C.M.C. and F.G.T. revised the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: S.C.L., C.M.K., F.G.T. and C.M.C. are inventors of a patent (patent No.:US 8691162 B1) owned by National Tsing Hua University on the basis of the work reported in this paper. S.C.L., C.M.K., F.G.T. and C.M.C. have a patent pending in Taiwan and China based on the work reported in this paper. H.K.W., M.Y.H. and C.L.C. declare no potential conflict of interest.

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