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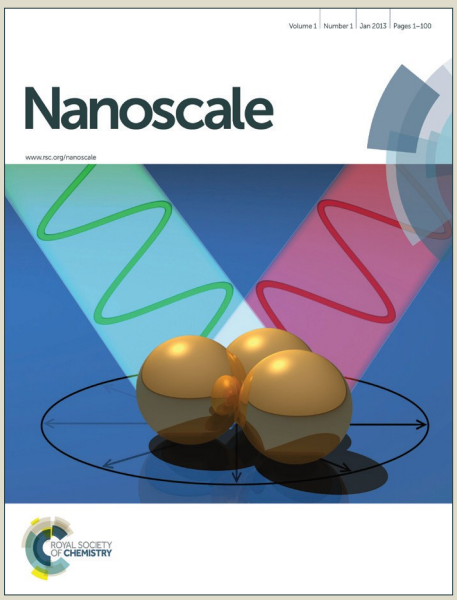





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# Visual and efficient immunosensor technique for advancing biomedical applications of quantum dots on *Salmonella* detection and isolation

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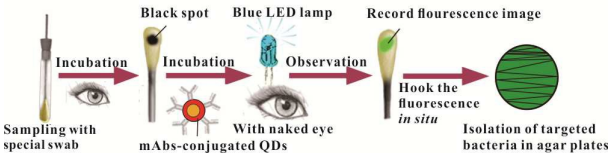


A visual immunosensor technique for efficient detection and isolation of *Salmonella* was established by applying fluorescent nanobioprobes on cellulose-based swab

## Abstract

It is a great challenge in nanotechnology for fluorescent nanobioprobes to be applied to visually detect and directly isolate pathogens *in situ*. A novel and visual immunosensor technique for efficient detection and isolation of *Salmonella* was established here by applying fluorescent nanobioprobes on a specially-designed cellulose-based swab (a solid-phase enrichment system). The selective and chromogenic medium used on this swab can achieve the ultrasensitive amplification of

target bacteria and form chromogenic colonies *in situ* based on a simply biochemical reaction. More importantly, due to this swab can be served as attachment sites for the targeted pathogens to immobilize and immunologically capture nanobioprobes, our mAbs-conjugated QDs bioprobes were successfully applied on the solid-phase enrichment system to capture the fluorescence of targeted colonies under a designed excitation light instrument based on blue light-emitting diodes combined with stereomicroscopy or laser scanning confocal microscopy. Compared with the traditional methods using 4-7 days to isolate *Salmonella* from the bacterial mixture, this method took only 2 days to do this, and the process of initial screening and preliminary diagnosis can be completed in only one and half days. Furthermore, the limit of detection can reach as low as $10^1$ cells $mL^{-1}$ *Salmonella* on the background of $10^5$ cells $mL^{-1}$ non-*Salmonella* (*Escherichia coli, Proteus mirabilis or Citrobacter freundii, respectively*) in experimental samples, and even in human anal samples. The visual and efficient immunosensor technique may be proved to be a favorable alternative for screening and isolating *Salmonella* in large number of samples related to public health surveillance.



## Introduction

Foodborne pathogen bacteria have always been a serious worldwide health threat.[1] In 2013, the Foodborne Diseases Active Surveillance Network (FoodNet) identified 19,056 cases of infection, 4,200 hospitalizations and 80 deaths in the United States.

*Salmonella* accounted for the highest incidence of 7, 277 per 100, 000 individuals among all the pathogens.[2] Worldwide, there are an estimated 94 million cases of nontyphoidal *Salmonella* gastroenteritis and about 155, 000 deaths.[3] However, because of the insufficient sensitivity of current detection and isolation techniques, the wild strains of this bacterium are not fully detected. The real number of individual with *Salmonella* in public may be many times greater than the current reports.[4] Since the minimum infectious dose of food-borne pathogens such as *Salmonella* is very low,[5, 6] there is an urgent need for developing a reasonable technique to improve the detection rates and isolation rates of this bacterium, especially when screening large number of samples related to public health surveillance.[7, 8]

The commonly used *Salmonella* detection methods such as the traditional culture method[9] and the enzyme-linked immunosorbent assay (ELISA)[10] suffer from low sensitivity, complicated procedures and poor efficiency. As a very simple and highly specific technique, polymerase chain reaction (PCR) can not be helpful for obtain pure strains. Reasonable detection methods may depend upon an adequate sampling procedure combined with a sensitive culture method,[10] and the application of novel technologies, *i.e.* immunosensors and nanotechnology.[11-13] Recently, fluorescent semiconductor nanocrystals, known as "quantum dots" (QDs), have been demonstrated to have many remarkable optoelectronic properties, for instance, they have tunable emission from the ultra-violet (UV) to the near infra-red (NIR) region when excited by a given wavelength due to the fact that their size can be easily modulated,[14, 15, 16] which are particularly appropriate as sensitive fluorescent labels in

biochemical optical sensing systems.[17] Some studies have tried to apply fluorescent nanoprobe on biological systems and developed nanoparticle substrates for ultra-sensitive sensors and trace chemical analysis,[14, 18] other investigations tried to use immunological methods based on gold nanoparticles to detect *Salmonella typhi (S. typhi)*,[19, 20] however, these methods did not involve in the process of isolation and purification of this bacterium, which is of great importance for epidemiological analysis or even for further drug sensitivity tests in clinical practice.

In addition, cellulose-based materials have been widely used in analytical chemistry as platforms for chromatographic and immunodiagnostic techniques.[21, 22] Biocompatible polymeric materials, such as cellulose,[23] have high protein capture ability, resulting in significantly increased detection signal and improved sensitivity. The crosslinking cellulose-based materials may be used to prepare fibrous forms of engineered elastins and allow attachment and spreading of cultured cells on it. Currently, this swab-based method has just been applied as a sampling and transport system for bacteria detection,[24] however, to the best of our knowledge, few previous investigations have tried to use the traditional cellulose-based swab for culture and immunodetection of *Salmonella*.

In the present study, a specially-designed cellulose-based swab was directly submersed in a sample solution and cultured for early screening. As a sulfate-reducing bacterium, *Salmonella* can reduce thiosulfate and produce hydrogen sulfide ($H_2S$), which reacts with ammonium ferric citrate to produce ferrous sulfide, generating a

black precipitate. These developing black spots can be easily observed by naked eyes. The swabs with black spots were incubated with amino-functionalized water-soluble *CdSe/ZnS* QDs[25] and fluorescence emission was recognized *in situ* on the black spots by the naked eyes under a designed excitation light source instrument using on blue light-emitting diodes (LED) ring light combined with stereomicroscopy or using confocal microscopy. The strains showing strong fluorescent signals were then transferred onto Xylose lysine deoxycholate (XLD) and Rappaport–Vassiliadis (RV) agar plates, followed by isolation and identification procedures consistent with current international standards **(Figure 1)**.[5] In this way, we can successfully, timely and visually detect, isolate the targeted bacteria on Solid Phase Support.

## Experimental section

Information about reagents, instruments and bacterial strains is available in the Supporting Information (SI).

### Ethical standards

The protocol of this study was approved by the human research advisory committees of the Wuhan Centers for Disease Prevention and Control (CDC), the Wuhan Children's Hospital and the Huazhong University of Science and Technology for Medical Sciences. All participants in this investigation were informed about the aim and procedures included in the protocol and were needed to sign a written

informed consent. They could withdraw from this study at any time. Written informed consent was obtained from all participants according to the guidelines of the Chinese National Ethics Regulation Committee before enrollment in the study, and all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, revised in 2008.

**Purification and preparation of bacteria**

The following bacterial strains were prepared: nine types of *Salmonella* strains of serotype groups A - H, forty local serotypes of *Salmonella*, and three type non-*Salmonella strains*, including *E. coli, Proteus mirabilis* and *Citrobacter freundii*. These bacteria were purified on XLD plates, cultured on Tryptone Soy Agar, and then diluted with sterile water to obtain serial dilutions using a turbidity meter. Bacterial counts were performed in duplicate by plating bacteria at densities of $10^2$ cells $mL^{-1}$ and $10^3$ cells $mL^{-1}$ in 100 μL of bacterial solution on agar plates.

**Production of swabs containing medium and optimization of the medium**

Each swab, 25 - 30 mm long and 9 - 10 mm wide with a 14.5 cm bamboo handle, contained 0.11 g of 100% medical-grade degreasing cotton. The swabs were capable of absorbing approximately 600 μL medium or sample solution **(Figure 1)**. None of the specially-designed swabs was treated with any reagents such as formaldehyde, decolorizer or fluorescent agents. The swabs were inserted into wells containing 600

μL of designed medium for 30 min at room temperature, dried at 48 $^{o}$C for 6 h, and finally stored at 20 $^{o}$C.

Design-Expert® software (Version 8.0.4, StatEase, Minneapolis, USA) for response surface methodology (RSM) was used to optimize the composition of the medium.[26] Most subspecies of *Salmonella* produce hydrogen sulfide, which can be easily visualized as black spots on the swab due to the formation of ferrous sulfide. This property is useful for early screening of *Salmonella* on the swab. Three specific compounds were screened, which were directly related to the generation of hydrogen sulfide, including ferric citrate amine, sodium hyposulfite and cystine. Super-speed Super Absorbing Polymer (SSAP) was added to the medium to increase its water retention capacity.

**Synthesis of mAbs-conjugated QDs**

The mAbs-conjugated QDs bioprobes were prepared using water-soluble amino-functionalized (NH2-PEG-NH2) *CdSe/ZnS* QDs (Product number: Q4525, Wuhan Jiayuan Quantum Dots Co., Ltd., China.) with a maximum emission wavelength of 525 nm and a mouse monoclonal antibody to *Salmonella* group O (Product number: B343M, Abcam, England).

The successful synthesis required five steps. (i) The QDs (5~8 nm) were activated with hetero-bifunctional crosslinker agent succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1- carboxylate (SMCC) (10 mM) at 20$^{o}$C for 1 h. Excess SMCC was removed *via* a NAP-5 desalting column with

exchange buffer. Approximately 500 μL of QDs were collected. (ii) 300 μL of 1 mg $mL^{-1}$ monoclonal antibody IgG to *Salmonella* was stirred and reduced with dithiothreitol (DTT) (20 mM) at 20°C for 0.5 h to expose free sulfhydryl (-SH) groups, and then purified with the NAP-5 desalting column. (iii) The activated QDs and antibodies were conjugated at 20°C for 1 h. (iv) The reaction was quenched by β-mercaptoethanol and reactants were concentrated to a final volume of 20 μL *via* ultrafiltration (7000 rpm, 10–15 min). (v) Finally, the mAbs-conjugated QDs conjugates were purified by superdex 200 gel chromatography, and the unconjugated antibody was removed with phosphate buffered saline solution (PBS) (10 mM, pH 7.4). The initial green eluent in the dead space of the column (~200 μL) was collected and stored at 4°C for the following experiments. The successful conjugation of QDs with the antibodies was detected using agarose gel electrophoresis.[27]

**Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) sample preparation**

After treatment with mAbs-conjugated QDs, the presence of QDs on the surface of *Salmonella* was verified by TEM (**Figure S1C**).[28] 50 μL of *Salmonella manhattan* ($10^5$ cells $mL^{-1}$) were incubated with 10 μL mAbs-conjugated QDs ($98.6\times10^{-2}$ μg/μL) in 1.0 mL tris buffered saline (TBS) (0.01 M, pH 7.4) for 30 min. Twenty μL coupling solution of mAbs-conjugated QDs and *Salmonella manhattan* were deposited on the surface of the carbonate support film and then dried out.

The conformation and interrelation between *Salmonella* and mAbs-conjugated QDs on the surface of fibers was further evaluated and characterized by field emission scanning electron microscopy (FESEM) **(Figure 5)**.[29] All samples were treated as: positive swabs containing *Salmonella* before and after incubation of mAbs-conjugated QDs, negative samples containing *Proteus mirabilis* and blank fibers containing purified water without bacteria. After incubation with mAbs-conjugated QDs bioprobes, samples were fixed with 2.5% glutaraldehyde solution followed by dehydration in a gradient of ethanol solutions. The samples were then dried in vacuum and coated with platinum particles.

**Preparation of artificial light based on a blue LEDs and stereomicroscope**

After verifying the emission characteristics of quantum dots with a dark field microscope,[30] a novel and simple apparatus was specially designed and manufactured to directly observe the fluorescent spots on positive swabs. The apparatus provided an artificial excitation light ($\lambda_{ex}$ = 405 nm) and adjustable zoom lenses for the visualization with the naked eyes. An artificial excitation light source based on a blue LEDs **(Figure 2C)** and a matched optical cut-off filter (Standard serial number ZWB2) **(Figure 2D)** were designed, whose physical characteristics can be found in the SI section. The blue LED was made of a mix of gallium nitride (GaN) and indium nitride (InN).[31, 32] The inner diameter of annular light was 44 mm and the outer one was 100 mm, containing 156 LED lamp beads. This system provided adjustable excitation light ($\lambda_{ex}$ = 405 nm) and a total power of 10 watts (input voltage: 90 V-264

V, output voltage: DC 12 V). The transmittance and wavelength range of the cut-off filters were detected with fluorescence spectrophotometers **(Figure 2E)**. The cut-off filter that covers the annular fluorescent light was 9.8 cm in inner diameter, 4.6 cm in outer diameter and 1.0 mm in thickness. Since the excited light occurred in the wavelength range of 400-680 nm was blocked by the cut-off filter, the emitted fluorescence in the same range could be clearly visualized and distinguished by the naked eyes in the darkroom (**Figure 2F**).[33] The artificial LEDs light ring and cut-off filter were assembled and fixed on the outer layer of the objective lens **(Figure 2A)** of a stereomicroscope (14- 90×, trinocular continuous zoom microscope). By hand-held or inserted in a fixed support, the swab can be directly observed under the light ring **(Figure 2B)**.

**Detection and observation procedures**

Swabs were inserted into sample tubes to absorb sufficient solution at room temperature, and then cultured for 18-24 h at 37 $^{o}$C. The swabs without black spots were directly confirmed as non-*Salmonella* samples and discarded. The swabs with black spots were put into the mAbs-conjugated QDs bioprobes solution (0.01 mg/mL) in TBS (0.01 M, pH 7.4), and then incubated for 0.5 h at 37 $^{o}$C. When developed with artificial light ($\lambda_{ex}$ = 405 nm), samples showing fluorescent spots were considered positive which would suffered from further observation under the stereomicroscope. The intensely fluorescent fibers on the swabs were finally picked up *in situ* with a small iron hook (15 cm length handle and 2 mm elbow) or ophthalmic tweezers and

plated onto the XLD and RV plates for 24 h at 37 °C for isolation of positive colonies. For the remaining samples without visible fluorescent spots, a few cotton fibers were clipped from the light-sensitive part and rinsed five times with 1 mL TBS and Tween 20 (TBST, 0.01 M). Afterwards, the fibers were completely stretched and placed on a glass slide, fixed with 5% glycerol and observed under laser scanning confocal microscopy (LSCM). Aggregates or fragments with intense fluorescence observed with the LSCM ($\lambda_{ex}$ = 405 nm) were also identified as positive. The positive fibers fixed on the glass slide were transferred onto the XLD and RV plates for bacterial isolation and identification.

During the entire process of detection and observation, *Proteus mirabilis* ATCC 12453 (hydrogen sulfide-positive) and *E.coli* ATCC 25922 (hydrogen sulfide-negative) were used as negative controls, and the sample containing purified water without bacteria was used as a blank control.

## Results and discussion

### Preparation and characterization of mAbs-conjugated QDs bioprobes

The mouse monoclonal *Salmonella* antibody (1 mg/mL) is specific for the core regions of lipopolysaccharides (LPS). It reacts with all common *Salmonella* O-serogroups A, B, C1, C2, D, E1, E3, E4, F, G1, G2 and H, but does not react with *E.coli, Klebsiella, Citrobacter, Pseudomonas, Yersinia, Shigella, Proteus and*

*Legionella*. Gel electrophoresis images of mAbs-conjugated QDs are shown in the SI section (Figure S1A). The presence of QDs on the surface of *Salmonella* was verified by TEM as shown in the SI section (Figure S1C). TEM demonstrated that mAbs-conjugated QDs bioprobes attached to the surface of *Salmonella*. The fluorescence absorption and emission spectra of these mAbs-conjugated QDs are shown in the SI section (Figure S1B). The fluorescence emission spectra of water-soluble QDs before and after the conjugation are almost entirely consistent. The fluorescence emission spectrum of the mAbs-conjugated QDs is 526 nm, however the human eyes is most sensitive to light at wavelength around 550 nm. The conformation between *Salmonella* and QDs probes on the surface of fibers was further characterized by FESEM (**Figure 5**).

**Preparation of cellulose-based developed swab**

Selection of the appropriate material for SPS is critical for characterizing the optimal medium to be used on the swabs. The material needs to be strong and highly water-absorptive. After a few trials, 100% medical-grade degreasing cotton was optimal among natural celluloses and synthetic fibers. This cellulose-based developed swab was proved to be not only a very good carrier with netting structure to reduce the interference of peripheral bacteria and enhance the proliferation of local dominant bacteria, but also the “docking sites” for the selective immobilization of pathogens and the immuno-capture of antibodies.

**Optimization of swab medium**

Since the medium components can seriously effect bacterial proliferation and inhibition on the specially-designed cellulose-based swab, optimal medium formula for *Salmonella* is of great importance. In the present study, we used a statistical technique known as "response surface methodology" (RSM) to identify and quantify changes in a variety of important chemical components that are directly related to the enrichment effect and the chromogenic reaction originated from the production of hydrogen sulfide during the early screening.[26, 34] Observation of the result of this biochemical reaction on a novel solid-phase enrichment system such as the cellulose-based developed swab may be helpful for visual detection of bacteria by the naked eye.

As an empirical statistical technique, the mathematical model of RSM was used to predict the response of a system to any new conditions, as well as to evaluate the relationship between a set of controllable experimental factors and observed results. Study of the interactions among key ingredients can be done with contour plots.[26] We finally determined an optimal medium formula for *Salmonella,* which contained 40 μL of 10% polyvalent polypeptone, 40 μL of 10% buffered peptone water, 6 μL of 10% ferric citrate amine, 6 μL of 20% sodium hyposulfite, 6 μL of 5% cystine, 6.4 μL of 20% sodium carbonate, 8 μL of 1% sodium deoxycholate and 0.01 μg of SSAP in 487.6 μL of distilled water.

Among ingredients of our optimal medium, as a nontoxic polymer material with polyvinyl alcohol and acrylic acid as raw material,[35] SSAP has a special structure with

hydrophilic groups for water absorption. Right amount of SSAP can maintain moisture for the prolonged growth of bacteria on the swab, which is very favorable for the full recovery of few or inactive *Salmonella* cells and can improve the detection sensitivity.

**Visual observation with the naked eyes and artificial blue LEDs**

As described by Edwards and Ewing, hydrogen sulfide is produced by 97.7% of *Salmonella spp.*, whereas the most common interfering bacteria in the intestinal microflora, *E. coli,* cannot produce hydrogen sulfide.[36] This biochemical reaction has only been applied for the identification of colonies on agar medium in the bacterial isolation stage.[37] In our system, the reaction was innovatively used for early screening on swabs in the stage of bacterial culture. All samples containing *Salmonella* strains (including *S. typhi*) produced clear and obvious black spots on the swab with concentrations above $10^1$ cells $mL^{-1}$ within 18 - 24 h, while no black spots were observed in the samples containing *E. coli*. The swabs containing black sediments or spots were preliminarily considered as suspicious positive samples (**Figure 3A)**, which can be visually observed by the naked eye. The swabs without black sediments were directly confirmed as negative samples and discarded. The suspicious positive samples were further incubated with mAbs-conjugated QDs bioprobes. Since this feature of production of hydrogen sulfide is not unique to *Salmonella*, mAbs-conjugated QDs bioprobes based on antibody specificity were applied for further detection and visual isolation.

The swabs with black spots can absorb a large amount of bioprobes.[38] Using a cut-off filter, the fluorescence signal of the bioprobes conjugated with the targeted strains on swab fibers was directly visualized under annular artificial blue LEDs ($\lambda_{ex}$= 405 nm) **(Figure 3 (C - F))**. The fluorescence image of suspicious fibers could be moderately enlarged and clearly distinguished using the magnification of the stereomicroscope **(Figure 3 (F - G))**. These *Salmonella* strains on fibers were collected using a small hook **(Figure 3B)** or ophthalmic tweezers under the stereomicroscope and transferred onto the XLD and RV plates for further isolation. Swabs showing visible fluorescence with the naked eye were considered as positive samples.

LED has become a potentially suitable material for excitation light source.[39] Among various fluorescent light sources such as laser, tungsten lamp, high pressure mercury lamp, xenon lamp and metal halide lamp, we used blue-LED made of indium gallium nitride (InGaN) because of its high heat capacity, small size, low power consumption, sturdiness, energy conservation and environmental protection.[40, 41] Based on these features of blue-LED, in the present study, the annular fluorescent light was successfully manufactured for the final three-dimensional observation.

**Observation with confocal microscopy**

For samples whose fluorescence could not be easily observed by the naked eye, we used LSCM ($\lambda_{ex}$ = 405 nm)[42] to identify the fluorescence emission of immuno-agglutination complexes on positive samples. In order to avoid subjective

differences, all swabs with black spots on which explicit fluorescent spots or fibers were not found by the naked eye were analyzed with LSCM **(Figure 4 (A - C))**. Negative controls on which a few *Proteus strains* were growing and blank controls containing purified water without bacteria are respectively shown in **Figure 4 (D - F)** and **Figure 4 (G - I).** The samples with fluorescence three times more intensive than the standard deviation of the negative controls were considered to be positive.[43] All positive fibers were directly transferred to the selective agar plates for efficient isolation. Image J software was applied for the analysis (v2.1.4.7, USA). The screening step with LSCM represents a semi-quantitative analysis of the fluorescence signal.

**SEM imaging of *Salmonella* and mAbs-conjugated QDs on the surface of the swab**

To confirm the specificity and reliability of the visual detection technique, we used FESEM to image mAbs-conjugated QDs bioprobes and *Salmonella* presenting on fibers from the swabs.[29] FESEM images in **Figure 5 (C-D)** clearly show that mAbs-conjugated QDs bioprobes adhered to positive samples and that the clusters of bacteria were growing on the surface of the swabs**.** No deposition of mAbs-conjugated QDs was observed on the surfaces of the positive samples **(Figure 5 (A - B))** before the incubation of bioprobes, **or** on the negative strains (*Proteus mirabilis*) **(Figure 5 (E - F))** and blank swab fibers containing purified water without

bacteria **(Figure 5 (G - H))** after incubation. The SEM analysis proved that the bioprobe has specificity for the targeted bacteria on the swabs.

**Accuracy and analytic sensitivity of the assay**

Based on the Cumitechs (Cumulative Techniques and Procedures in Clinical Microbiology) from the American Society for Microbiology (ASM), analytic sensitivity means the smallest quantity of an analyte that can be reproducibly distinguished from background levels, which is also known as the limit of detection (LOD).[44] To evaluate the accuracy and analytic sensitivity of the new assay, mixed samples containing $10^1$ cells $mL^{-1}$ *Salmonella Typhimurium* and $10^5$ cells $mL^{-1}$, $10^6$ cells $mL^{-1}$ or $10^7$ cells $mL^{-1}$non-*Salmonella* strains, including *E.Coli*, *Proteus mirabilis* or *Citrobacter freundii,* were analyzed in groups using serial dilutions with purified water (40 samples in each group). Purified water without bacteria was the blank sample.[43] Evaluation criteria were based on the production rate of black and fluorescent spots observed by the naked eye or confocal microscopy, respectively. All strains isolated from the swabs were confirmed by traditional standard identification procedures,[5, 33] including conventional biochemical and serological identification.

Among the sample groups containing $10^1$ cells $mL^{-1}$ *Salmonella* and $10^5$ cells $mL^{-1}$ non-*Salmonella (E. coli*, *Proteus mirabilis* or *Citrobacter freundii)*, our new assay detected 97.50%，97.50% and 100% of *Salmonella* respectively, and visible fluorescence with the naked eye was observed correspondingly in 52.50%, 57.50% and 62.50% of positive swabs as shown in **Figure 6A**. In particular, the present

screening system identified and excluded all of the negative samples containing non-*Salmonella* because they neither exhibited black spots nor emitted fluorescence. This screening prevents a substantial amount of subsequent processes. Parallel experiments also showed that monoclonal antibodies conjugated with QDs maintained their bioactivity and biospecificity for *Salmonella*.[45] Among the groups containing $10^1$ cells $mL^{-1}$ *Salmonella Typhimurium* plus $10^6$ cells $mL^{-1}$ and $10^7$ cells $mL^{-1}$ non-*Salmonella* strains respectively, all of the detection rates within 24h were less than 60% (data not shown). In fact, we used various *Salmonella* strains (Supplementary Note 2) in this study to verify our results (data not shown).

Numerous studies have reported CdSe/ZnS as a fluorescence marker for detecting bacterial counts.[46] After high-quality CdSe/ZnS nanocrystals are synthesized and successfully conjugated with bacteria, the fluorescence intensity is proportional to bacterial count in the range of $10^2$-$10^8$ cells $mL^{-1}$, and the low detection limit has been reported to be $10^2$ cells $mL^{-1}$.[47] Some immunoassays using gold nanoparticles were developed for the rapid detection of *Salmonella* in human serum or food samples,[19, 48] whose LOD was higher than $10^3$ cells $mL^{-1}$. Our newly developed method has improved analytic sensitivity, showing that $10^1$ cells $mL^{-1}$ *Salmonella* can fully proliferate and be screened under the interference of $10^5$ cells $mL^{-1}$ non-*Salmonella* strains.

In our study, a few positive samples were not confirmed to be positive by only naked eye, which may be due to the fact that *Salmonella* does not reproduce fast enough to form high-density colonies on the swab.

**Detection analysis of *Salmonella* in human anal samples**

In the department of public health surveillance, *i.e.* the Center for Disease Control and Prevention (CDC), monitoring *Salmonella* in employees in the food and catering industry is very important. In order to determine the detection rate and practicality of the immunosensor technology, 120 fresh anal samples uncontaminated with *Salmonella* were collected and prepared for analysis. All individuals who passed the relevant medical examination signed an informed consent. To observe the growth of certain concentrations of *Salmonella* in anal samples from healthy people and measure the differences between our method and conventional culture-based methods for bacterial identification and isolation, $10^1$ cells $mL^{-1}$ *Salmonella Typhimurium* were added to the related test tubes of each sample for further evaluation with the newly developed assay and the traditional standard method.

A disposable sampling stick was gently inserted into the anus about 4.0 - 5.0 cm and repeatedly rotated to obtain fingernail-sized feces. After the collection of the anal samples, the disposable sampling stick was submerged in 2.40 mL of purified water and mixed well.[49] Each sample was divided into four tubes (600 μL per tube), which were distributed into four groups (each n = 120): group A contained *Salmonella* ($10^1$ cells $mL^{-1}$) and group B contained no *Salmonella*, which were subjected to the new method; group C contained *Salmonella* ($10^1$ cells $mL^{-1}$) and group D contained no *Salmonella*, which were subjected to the conventional culture-based method. Purified

water without bacteria was used as a blank control.[43] All isolates were identified by the current standard procedures.[36, 49]

Using the enrichment assay with a specially-designed swab and mAbs-conjugated QDs bioprobes, the newly developed technique achieved better visualization and isolation of targeted bacteria compared to the international standard culture-based method. The results are shown in **Figure 6**. The detection rate was 96.67% with LSCM and 91.67% with traditional culture-based method, suggesting that the prognosis of *Salmonella* infection was improved by the new method **(Figure 6 (B - C))**. Based on the Cumitechs from ASM,[44] clinical sensitivity is a measure of a test's ability to accurately detect a specific disease in patients. In our study, the sensitivity was 96.67% (116/120) in simulated human anal samples containing *Salmonella*, and 91.67% (110/120) with a traditional culture-based method. More importantly, the new method had a higher detection rate compared to the traditional method in the low dose of $10^1$ cells $mL^{-1}$ *Salmonella* in human anal samples. Compared with previous similar studies using immunosensor technique,[18, 19] the whole amount of sample collected was subjected to the bacterial detection and isolation, which can help us to identify and isolate the amount as low as $10^1$ cells $mL^{-1}$ *Salmonella* from a complex matrix of human anal sample.

In the samples without *Salmonella*, the production rate of black spots was 25.00% (30/120), and 75.00 % (90/120) of samples could be directly judged as negative and excluded (**see step 2 of Figure 1**). Compared with the conventional method **(Figure 6 (B - C))** which need to keep the whole samples in the process of

bacterial detection and isolation, 75.00 % excluded samples in this study will result in a significant decrease of workload including bacterial detection and isolation (**see step 3 of Figure 1**) .

In addition, all samples in this study containing *Salmonella* produced black spots of different sizes within 24 h, which make it possible to detect the bacteria in one and a half days and isolate the bacteria in two days, while the conventional culture-based method needs four to seven days to fulfill this procedure.[36, 49]

In the human anal samples where *Salmonella* was not added, there were two false-positive samples (1.67%) using the present method (**see step 4 of Figure 1**), and these two samples were identified as *Citrobacter freundii* by further biomedical identification methods. These strains were also proved to have the agglutination ability of the *Salmonella* O antibody (A-F) which may lead to false positives. Many studies have previously reported that some *Citrobacter* and *Salmonella* strains may share a similar O antigen gene originated from a common ancestor.[50, 51] Based on the Cumitechs from ASM,[44] the specificity is a measure of a test's ability to accurately identify all non-infected patients. Therefore, the specificity of the present method was 98.33 % (118/120) in anal samples that did not contain *Salmonella*. Actually, 4 in 120 anal samples (3.33%) containing *Salmonella* were identified as negative (false-negative samples) may due to insufficiently proliferation and shedding of the immune aggregates (antigen of bacteria with mAbs-conjugated QDs).

## Conclusions

In summary, a specific, efficient, ultrasensitive, simple and visual immunosensor technique for *Salmonella* has been established, based on fluorescent nanobioprobes using mAbs-conjugated QDs and an improved microbiological enrichment method using a specially designed swab made of cotton fiber. It may become a useful tool for screening and isolating *Salmonella* in large number of samples from public health screening. Technically, under excitation light with the same wavelength, multi-color QDs without spectral overlap can be applied to simultaneously detect and isolate multiple pathogens in future applications. The efficient isolation of *Salmonella* will help to do further molecular epidemiological analysis of public health surveillance such as food poisoning and water pollution or even have the drug sensitivity tests and rationally selection of antibiotics in clinic practice.

**Conflict of Interest:** The authors declare no competing financial interest.

**Supporting Information Available:** Two additional figures (**Figures S1**), five additional tables (**Table S1-S2**) and additional information. Refer to Web version on PubMed Central for supplementary material.

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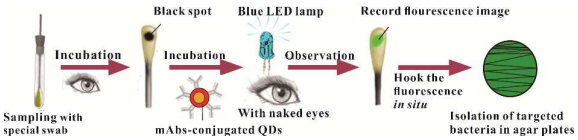
## TOC



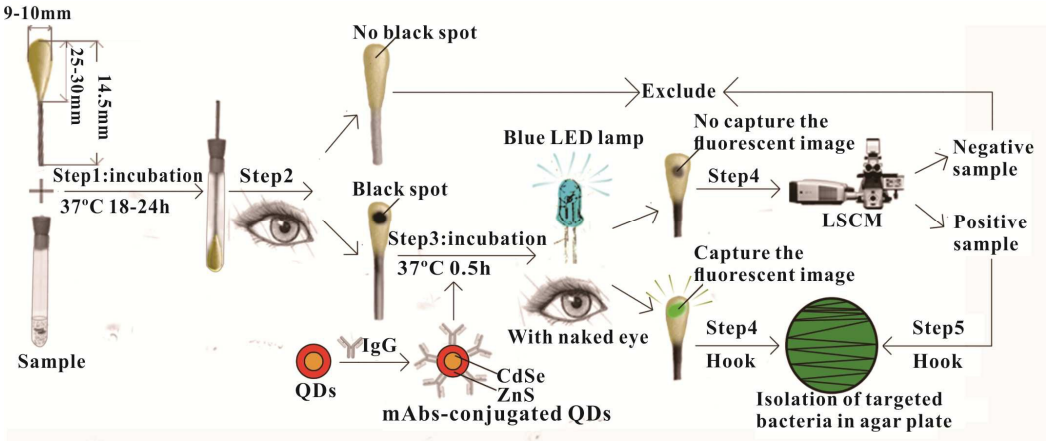


**Figure 1. Summary of technical procedure.** The developed swabs are inserted into sample tubes to absorb sample solution, then cultured for 18 - 24 h at 37 $^{o}$C. The swabs without black spots are directly confirmed as negative samples and then discarded. The swabs with black spots are put into the mAbs-conjugated QDs bioprobes solution, and then incubated at 37 $^{o}$C for 0.5 h. The samples showing fluorescent spots under artificial LEDs light source are considered as positive samples. The fluorescent fibers on the swabs are picked-up *in situ* with a small iron hook and plated onto the XLD and RV plates to isolate the positive colonies. For the remaining samples without visible fluorescent spots, a few cotton fibers are clipped from the light-sensitive part. The fibers are observed under LSCM. Once aggregates or fragments with intense fluorescence are found, they are also identified as positive. The remaining samples are confirmed as negative and discarded. The positive fibers are transferred onto the XLD and RV plates for bacterial isolation and identification.

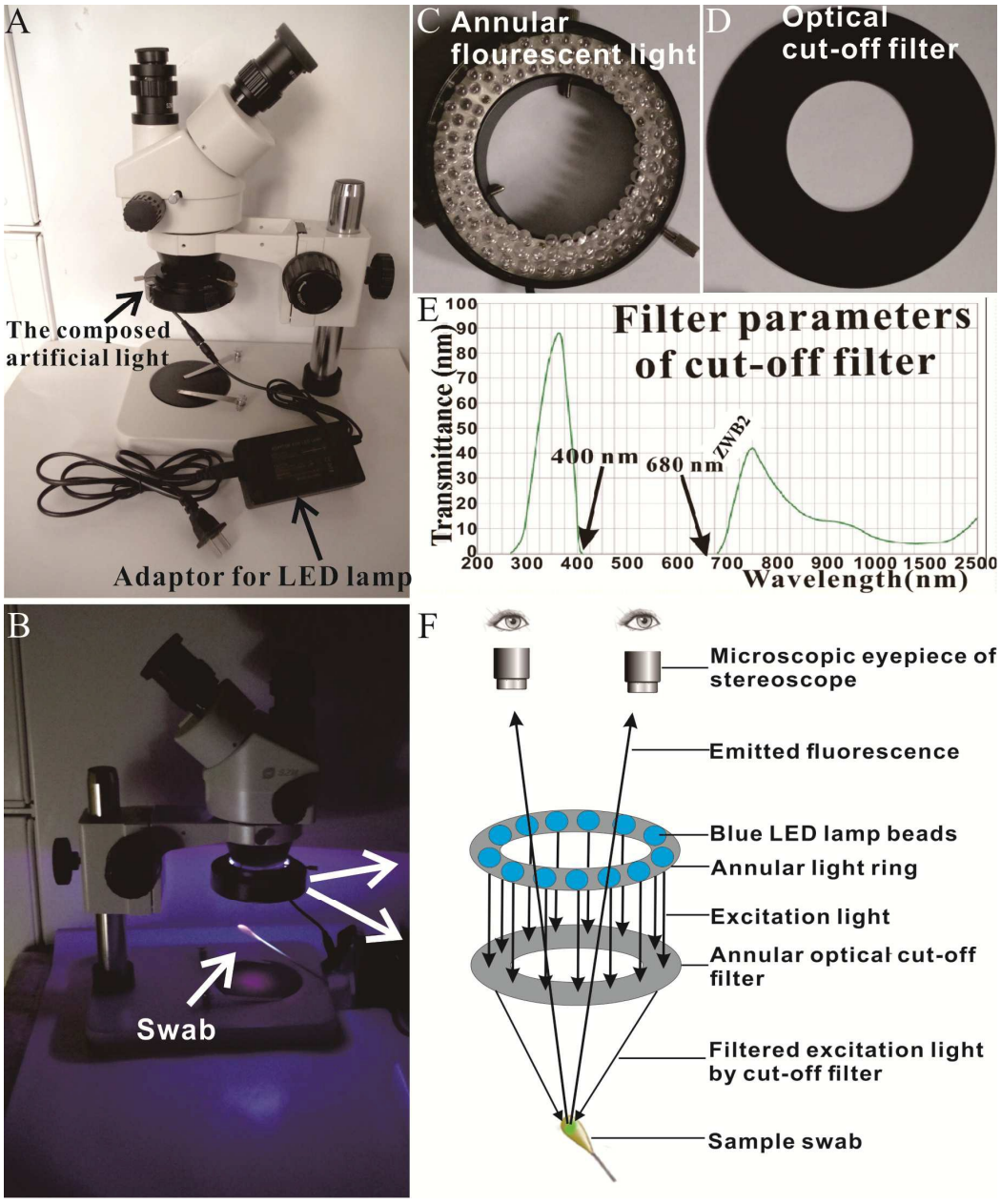



**Figure 2. Components of the optical observation device.** The complete system is composed of a power adaptor, a stereomicroscope (A), the annular fluorescent light (C) and the annular optical cut-off filter (ZWB2) (D). The transmittance and wavelength range of optional cut-off filters is shown in (E). The combined fluorescent light ring and cut-off filter is assembled on the objective lens of the stereomicroscope and the swab is observed with the optical device in darkroom (B). The complete optical path diagram of the observation device

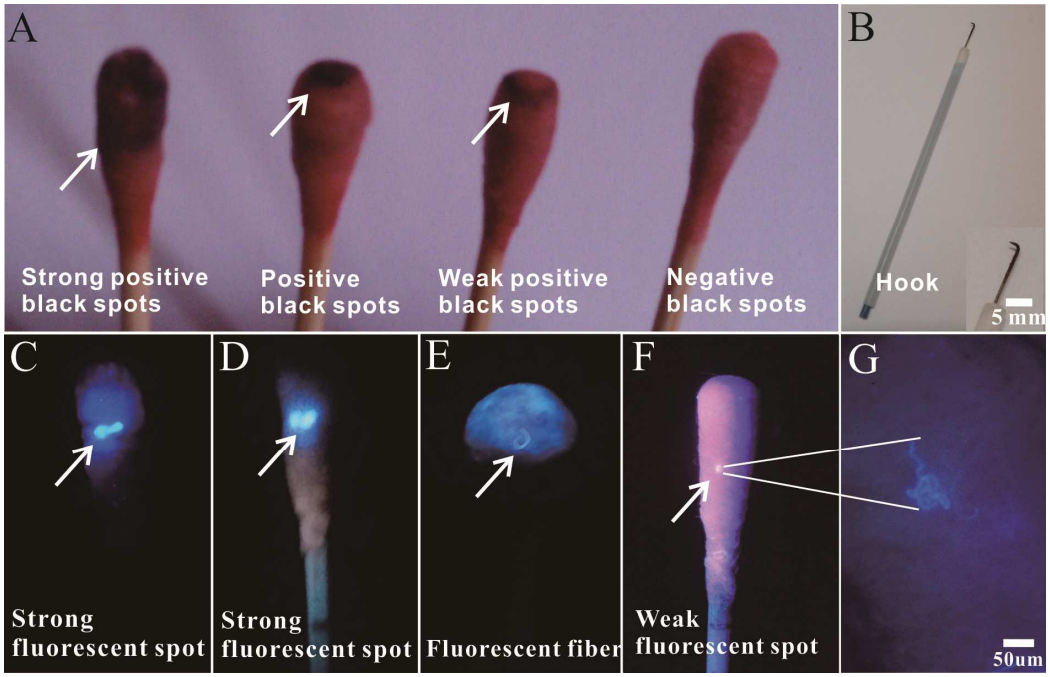



**Figure 3. Visual observation of mAbs-conjugated QDs bound to *Salmonella* with the naked eye and artificial blue LEDs light source.** (A) Black spots on swabs are arranged as strongly positive, positive, weakly positive and negative from left to right. (B) Engineered iron hook for picking up fibers from the swab. The handle length is 15 cm and the elbow is 2 mm. (C and D) Strong fluorescent spot on swab. (E) Fluorescent fiber on the swab. (F) Weak fluorescent spot on the swab. (G) Weak fluorescent fibers are from (F) under stereomicroscope.

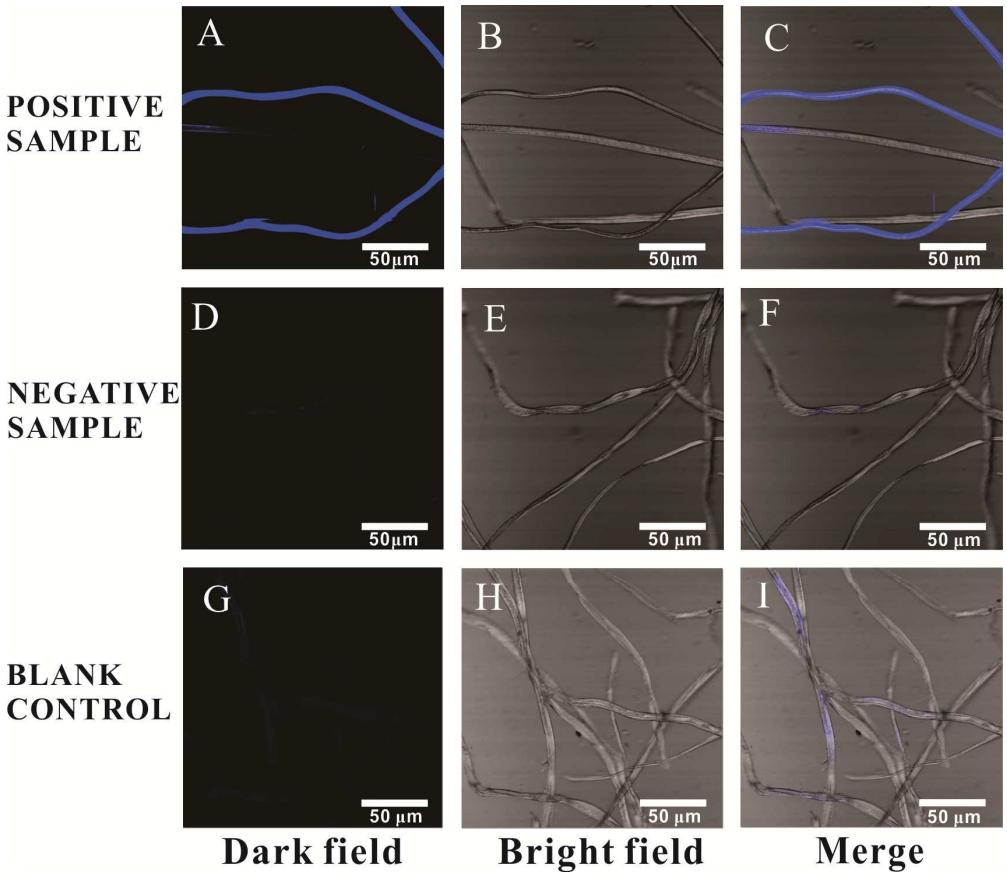



**Figure 4. Fluorescence imaging with confocal microscope.** (A - C) positive sample, (D - F) negative sample and (G, H and I) blank control. The mean values of fluorescence intensity are calculated using image J software (A = 164.729, D = 0.382, G = 0.341).

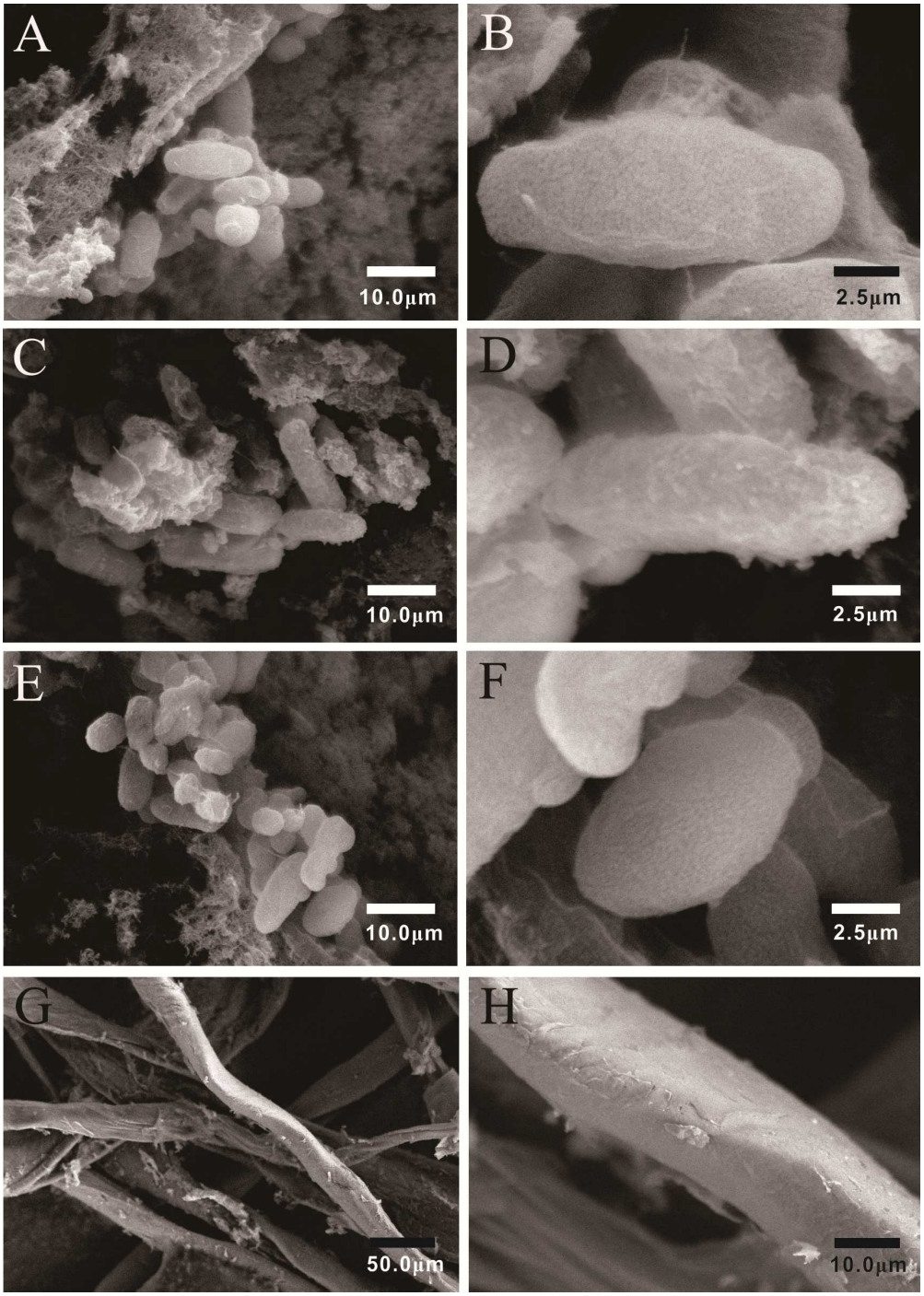



**Figure 5. SEM images of the surface conformation.** Surface conformation of a positive sample before (A - B) and after (C - D) incubation with mAbs-conjugated QDs bioprobes, negative sample (E - F) and blank fibers (G - H) after incubation with mAbs-conjugated QDs bioprobes. Clusters of bacteria are seen growing on the fibers (A, C, E). Right column pictures are magnifications of the pictures on the left.

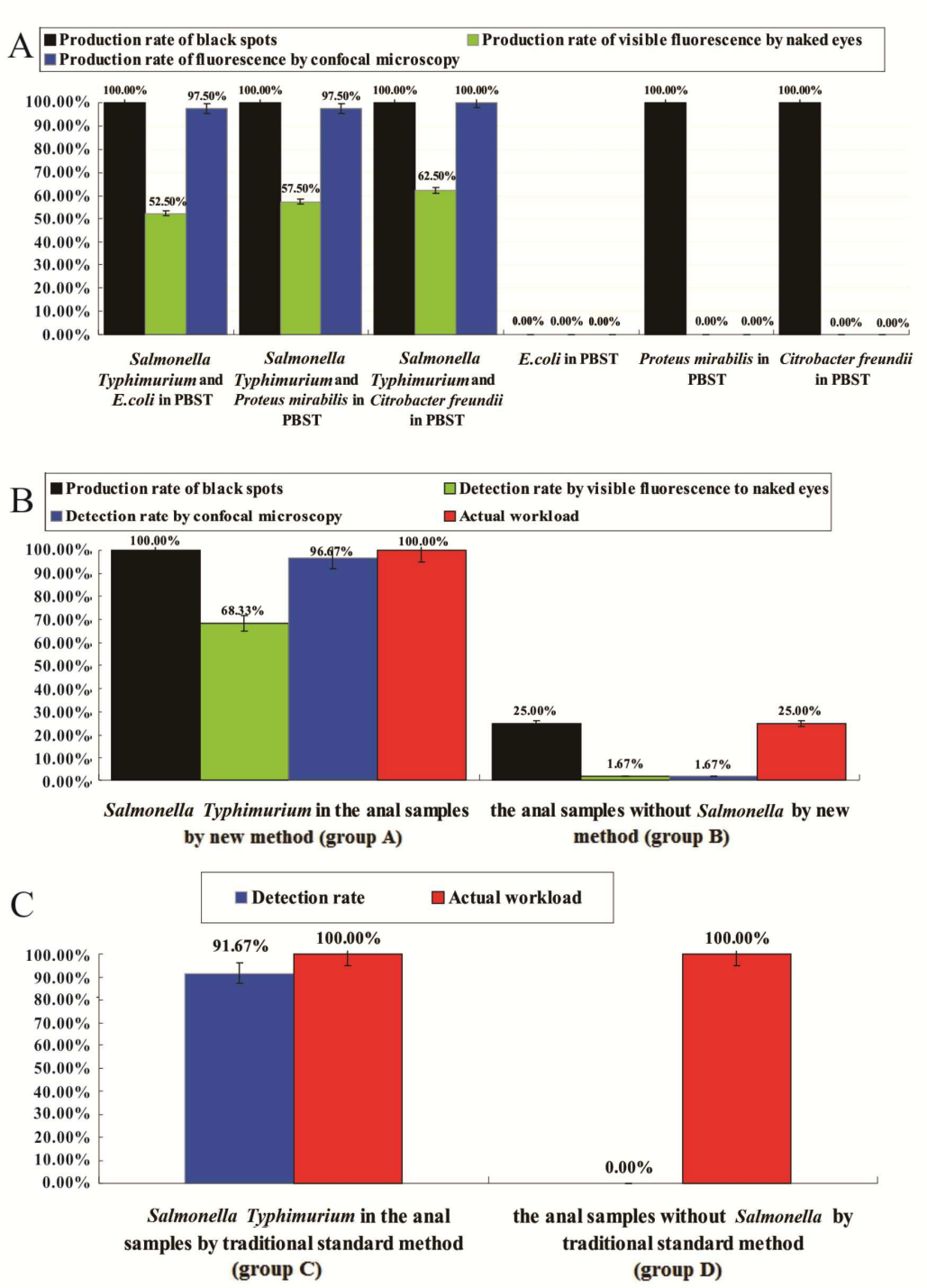



**Figure 6. Evaluation of the new method in mixed samples and human anal samples.** (A) Accuracy and analytic sensitivity of the new assay in mixed samples containing $10^1$ cells $mL^{-1}$ *Salmonella* and $10^5$ cells $mL^{-1}$ non-*Salmonella*. The evaluation in human anal samples and corresponding workload including follow-up

separation steps applying the new assay are shown in (B) and the traditional culture-based method is shown in (C).