

# Enhancing the immunofluorescent sensitivity for detection of *Acidovorax citrulli* using fluorescein isothiocyanate labeled antigen and antibody

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**Abstract** A rapid lateral flow immunochromatographic strip (ICS) using fluorescein isothiocyanate (FITC) labeled antigen and antibody was developed for the detection of *Acidovorax citrulli* (Ac) in melons and vegetable samples. In the ICS, signal amplification was realized based on antigen Ac and anti-Ac monoclonal antibody (McAb) 4F conjugated with FITC, respectively, which were forming two probes. The control line and the test line were obtained by immobilizing the goat anti-mouse IgG antibody and anti-Ac McAb 6D on both sides of the nitrocellulose membrane. The visual detection limit of the strip was  $10^5$  CFU/mL, which was 10-fold sensitive compared to the strip of FITC only labeling antigen or antibody. Signal amplification ICS was successfully applied to the detection of Ac in melon and vegetable samples with less detection time and operation procedures compared to the traditional enzyme-linked immunosorbent assay (ELISA) and PCR methods. This is the first report of using FITC labeled antigen and McAb as dual fluorescent probes to develop a direct-type immunofluorescence strip for the rapid and sensitive detection of Ac, which demonstrates a powerful tool for rapidly screening Ac in plant materials and other samples.

**Keywords** FITC · Antigen and antibody dual labeled · Immunoassay · *Acidovorax citrulli*

## Introduction

Bacterial fruit blotch (BFB) caused by gram-negative bacterium *Acidovorax citrulli* (Ac) is a devastating bacterial disease to many cucurbitaceous plant species, e.g., watermelon, melon, cucumber, pumpkin, and other cucurbits [1]. Ac, as a seed-borne pathogen, can infect seeds, seedlings, and fruits, and the infested seeds are usually the most important source for BFB outbreaks [2]. Since the first report in commercial products in 1965 [3], BFB has caused millions of dollars of loss around the world, such as United States, Australia, Brazil, Turkey, Japan, Thailand, Israel, Iran, Hungary, Greece, and China. So far, there are still no commercial species that could completely resist BFB [4]. Hence, relatively effective BFB management is early detection, diagnosis, and clearance of Ac from all commercial seeds and seedlings.

As we all know, membrane-based lateral flow immunochromatographic strip (ICS) has been a powerful tool for simple, rapid, and low-cost detection. The assay procedure could be significantly simplified to avoid the requirements for a long incubation time and complex operation steps, which is especially suitable for personal detection or in places where complicated equipment is not available. Lately, ICS tests based on colloidal gold as a tracer have been widely used for the detection of foodborne pathogens [5], mycotoxins [6], pesticide residues [7], heavy metal ions [8], prohibited additives [9], antibiotics residues [10] etc. However, ICS based on colloidal gold shows a serious limit when high sensitivity is needed. Many strategies, such as selecting new labels, e.g., quantum dot [11] and

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upconverting nanophosphors [12], combining with other reagent or other techniques [13], e.g. magnetic capture [14] and PCR-based methods [15], designing new patterns [16], and developing signal amplification system [17], were applied to enhance the sensitivity of lateral flow immunoassay.

Fluorescein isothiocyanate (FITC), a fluorochrome dye, which could emit a visible yellow-green light after absorbing blue or ultraviolet light. As a fluorescent probe, FITC has been widely used to show the intracellular localization of pathogens [18], visualize live cells [19], trace drugs in vivo delivery [20, 21], estimate in vivo counts during passage of probiotics [22], and other fields. FITC was also regarded as a promising tracer for immunochromatographic assay due to the unique properties, such as strong luminescence, photo-stability, satisfactory labeling effect, and low cost. These properties make it possible to be a new label material for developing highly sensitive ICS assays.

Recently, monoclonal antibody (McAb) 4F and 6D against Ac have been prepared in our lab, which possessed sufficient sensitivity and specificity for the analysis of Ac. In this study, a novel signal amplification fluorescent immunoassay method based on a common fluorochrome dye, FITC, was developed. ICS was successfully applied with the target bacteria as a novel fluorescent probe after incubation with FITC and the FITC labeled McAb 4F as the second fluorescent probe. The results indicated that the dual labeled method could enhance the sensitivity for the detection of Ac, and the assay would be a useful tool for the early diagnosis, prevention, and control of BFB in the future.

## Materials and methods

### Bacterial strains and materials

The McAbs (6D and 4F) against Ac were produced in our lab as previously reported [23]. Strains used for testing the cross-reaction of the strip are listed in Table 1. All the strains were obtained from the Chinese Academy of Inspection and Quarantine (Beijing, China). Brain heart infusion (BHI) medium was purchased from Land Bridge (Beijing, China). The nitrocellulose (NC) membrane, sample pad, conjugate pad, and absorbent pad were purchased from Millipore (Bedford, MA, USA). FITC  $\geq$  90% (HPLC) and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). All solvents and other chemicals were of analytical reagent grade.

### Conjugate of antibodies to FITC

Bicarbonate/carbonate buffer at pH 9.0 was used to dilute the purified monoclonal antibody (McAb) 4F from 10 mg/mL to 4 mg/mL. Freshly prepared FITC solution dissolved in DMSO was added slowly dropwise to the antibody solution until a final concentration of about 150  $\mu$ g FITC per mg

McAb. The mixture was kept in the dark and reacted at room temperature for 2 h. Then the reaction mixture was dialyzed to separate the conjugated antibody from the free FITC. The labeled antibody was stored at 4 °C before use.

### Bacteria culture and sample pretreatment

Freshly prepared FITC solution dissolved in DMSO was added to BHI medium, which was used for bacteria culture. A single colony was added to the modified BHI (45 mL) containing plant juice sample (5 mL) and incubated at 37 °C overnight. Bacterial concentration was diluted by sterile saline and determined by plate count or by microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA). The fluorescence intensity of bacteria was determined by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

### The optimum concentration of FITC

The concentration of FITC solution for sample incubation could influence the background and final fluorescence intensity of the test line. Freshly prepared FITC solution was diluted to different concentrations of 10  $\mu$ g/mL, 25  $\mu$ g/mL, 50  $\mu$ g/mL, 100  $\mu$ g/mL, 200  $\mu$ g/mL, 300  $\mu$ g/mL, 400  $\mu$ g/mL to culture Ac. After incubating at 37 °C for 12 h, fluorescence intensity was detected by flow cytometry.

### Fabrication of the immunoassay strip and results judgment

The immunofluorescence strip is usually composed of the following five parts: a sample pad, a conjugate pad, a NC membrane, an absorbent pad, and a plastic backing board. A schematic presentation of the test strip is shown in Fig. 1a. The sample pad (2.5  $\times$  30 cm) and conjugate pad (0.5  $\times$  30 cm) were made of the same type of glass fiber, and the adsorbent pad (3  $\times$  30 cm) was made of cellulose fiber. The anti-Ac McAb 6D and goat anti-mouse IgG were transferred onto both sides of the NC membrane (2.5  $\times$  30 cm) using an XYZ arrayer (Bio-Dot, Irvine, CA, USA) with 1  $\mu$ L/cm volume at 4 cm/s dispensed rate to form test line and control line, respectively. FITC labeled anti-Ac McAb 4F was dispensed onto the conjugate pad followed by drying at room temperature. The spotted NC membrane, absorbent pad, conjugate pad, and sample pad were assembled sequentially on the backing board (8  $\times$  30 cm) with approximately 2 mm overlap. The assembly was cut into 3 mm wide strips using a guillotine cutter (Bio-Dot). All strips were sealed with desiccant and stored at room temperature.

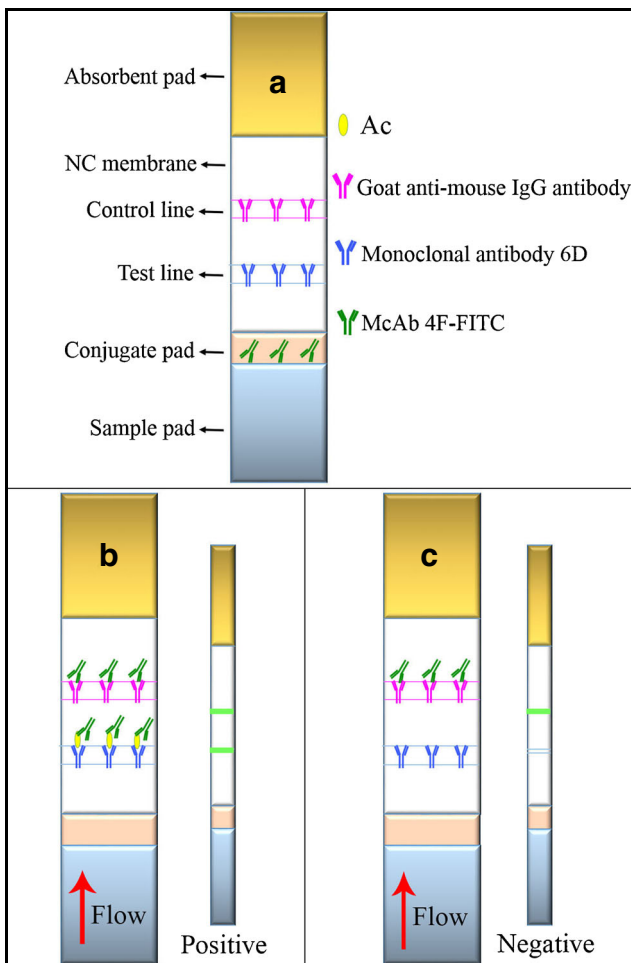
The preparation of the strip is based on the classic double antibodies sandwich theory. The complex FITC Ac from sample was first combined with FITC-4F coated on conjugate pad, and then the complex FITC Ac-4F FITC flowed through the NC membrane via capillary action. Subsequently, the FITC

**Table 1** Strains used in this study

No.	Species/strains	Host	Origin	PCR
1	<i>Acidovorax citrulli</i> SD01	Watermelon	Shandong, China	+
2	<i>Acidovorax citrulli</i> ATCC 29625	Watermelon	Georgia, USA	+
3	<i>Acidovorax citrulli</i> tw31	Watermelon	Taiwan, China	+
4	<i>Acidovorax citrulli</i> plsb91	melon	Inner Mongolia, China	+
5	<i>Acidovorax citrulli</i> xj112	Cantaloupe	Xinjiang, China	+
6	<i>Acidovorax citrulli</i> 99-5	Cantaloupe	Georgia, USA	+
7	<i>Acidovorax citrulli</i> PLSB1	melon	Inner Mongolia, China	+
8	<i>Acidovorax citrulli</i> 00-1	Watermelon	Georgia, USA	+
9	<i>Acidovorax konjaci</i> ATCC 33996	Konjac	Japan	+
10	<i>Acidovorax cattlesiae</i> NCPPB 961	Unknown	Unknown	+
11	<i>Acidovorax avenae</i> ATCC 19307	Sugarcane	Reunion	+
12	<i>Xanthomonas Cucurbitae</i> NCPPB 2597	Curcubita maxima	New Zealand	+
13	<i>Pseudomonas Lachrymans</i> NCPPB 540	Cucumber	Denmark	+
14	<i>Erwinia tracheiphila</i> NCPPB 2975	Cucumis sativus	USA	+

Ac-4F FITC bound to McAb 6D was immobilized on test line, which formed a tripolymer FITC 4F-FITC Ac-6D in test line area. Accumulative FITC displayed yellow-green

fluorescence under ultraviolet light as shown in Fig. 1b. Conversely, if there was no Ac in the samples, the FITC did not accumulate on the test line (Fig. 1c).



**Fig. 1** (a) Schematic presentation of the test strip; (b) positive result of the test strip; (c) negative result of the test strip

**Sensitivity and specificity of the test strip**

To evaluate the sensitivity, the SD01 strain diluted to  $10^4$ – $10^8$  CFU/mL concentrations were analyzed with the strip. The Ac detection was carried out by applying 75  $\mu$ L sample solution to the sample pad. Modified BHI medium containing FITC was used as negative control. After 10 min, the results of test line and control line were visualized in a black box equipped with an ultraviolet light source and a fluorescent filter of 525 nm (Semrock, Rochester, NY, USA), and then recorded with a digital camera.

The strip was stored at room temperature for 1, 2, and 3 mo to assess the stability by evaluating the sensitivity in  $10^5$  CFU/mL. The same test was conducted as described above.

Eight strains of Ac (SD01, ATCC 29625, 99-5, xj112, PLSB1, 00-1, plsb91, tw31) and six strains of Ac genetic nearly plant pathogens (ATCC 33996, NCPPB 961, ATCC 19307, NCPPB 2597, NCPPB 540, NCPPB 2975) were cultured in modified BHI medium and tested to evaluate the specificity of the strip. All bacterial suspensions were adjusted to the same concentrations using a microplate reader.

**Application to real samples and comparison with PCR methods**

The Ac free plant samples collected from the field, including watermelon, muskmelon, cantaloupe, luffa, wax gourd, pumpkin, cucumber, and summer squash, were verified by PCR methods. The modified BHI (45 mL) containing juice (5 mL) from plant samples was used to cultivate SD01 strain.

Then, these inoculums were diluted to  $10^5$  CFU/mL for strip detection. The purpose was to determine whether the impurities in the juice from the plants could impact the smallest detectable concentration.

## Results

### Optimization of parameters

To develop the strip, many factors should be optimized to increase the sensitivity. After incubation at different FITC concentrations of 10  $\mu\text{g/mL}$ , 25  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$ , 200  $\mu\text{g/mL}$ , 300  $\mu\text{g/mL}$ , 400  $\mu\text{g/mL}$ , the Ac was with different fluorescence intensity. As shown in Fig. 2, the Ac without fluorescence was distributed in M1 region (Control), and the fluorescent bacteria were distributed in M2, M3, or M4 areas according to the fluorescence intensity from  $10^1$  to  $10^4$ . The percentage of fluorescent bacteria (in M2+M3+M4 area) and the distribution in M2, M3, or M4 areas were different. Since there were almost no bacteria in the M4 region, the proportion of bacteria distributed in M3 region was the basis for determining the optimum FITC concentration.

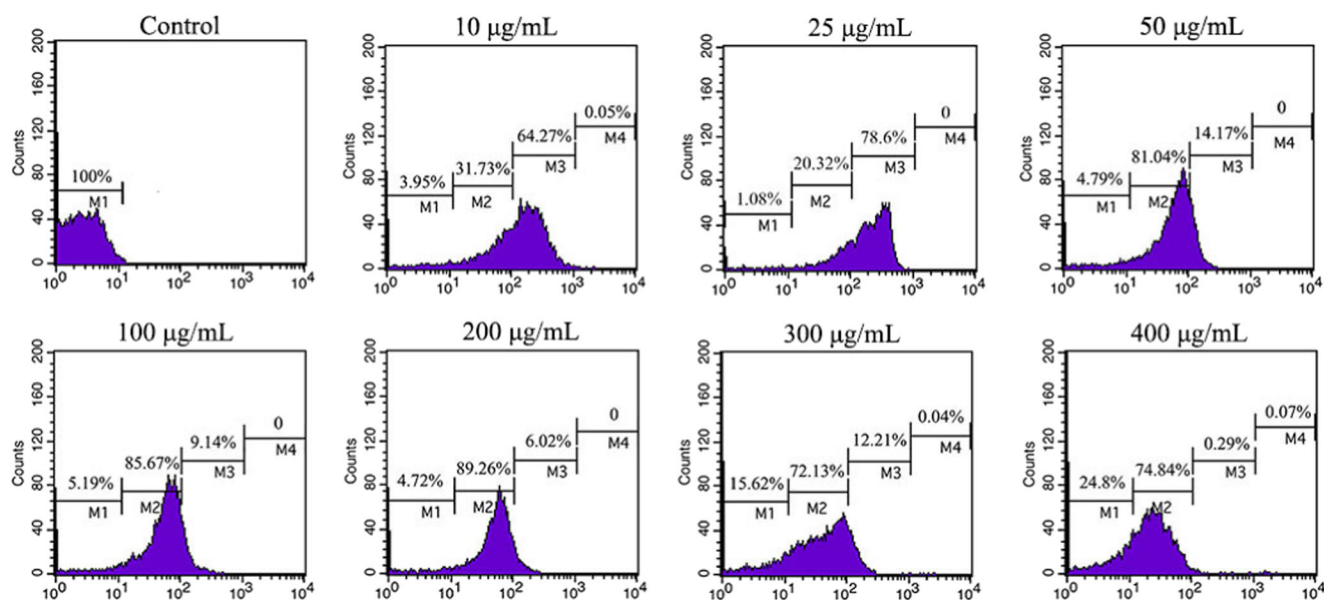
Comparison with the proportion of the M3 area, 25  $\mu\text{g/mL}$  was considered to be the optimal FITC concentration for detection. In addition, when the FITC concentration was at 25  $\mu\text{g/mL}$ , the proportion of fluorescent bacteria (M2+M3+M4) was up to 98.9%, which was higher than others. Figure 3 was the morphological picture of Ac cultured with FITC viewed under fluorescent microscope with blue light. After incubating with FITC, the bacteria became fluorescent probe.

The concentration of McAb 6D is also important for the performance of the strip since the color of the test line deepened with the increase of 6D concentration. However, the blank control had a false-positive result when the 6D concentration was higher than 2 mg/mL. Thus, the optimal concentration of 6D was determined to be 2 mg/mL. The concentration of FITC-4F measured by NanoDrop (Thermo, Waltham, MA, USA) was 2 mg/mL.

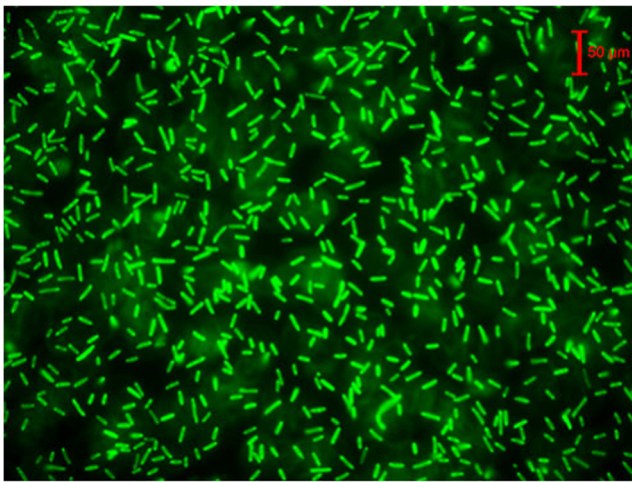
### Sensitivity and stability of the ICS

To identify the sensitivity, a diluted gradient of SD01 ( $10^8$ – $10^4$  CFU/mL) was added to the sample pad of the dual FITC labeled ICS. As shown in Fig. 4c, the yellow-green light of the test line was evident for concentrations ranging from  $10^8$  to  $10^5$  CFU/mL. When the concentration was below  $10^4$  CFU/mL, there were no lines observed, which indicated that the detection limit was  $10^5$  CFU/mL. However the immunoassay strip based on FITC just labeling antigen (Fig. 4a) or antibody (Fig. 4b) displayed the sensitivity of  $10^6$  CFU/mL. All the results indicated that the method of FITC labeling antigen and antibody successfully achieved the effect of signal amplification, which realized a 10-fold increase in sensitivity compared with FITC just labeling antigen or antibody.

The stability of the strip was assessed through the sensitivity test. After storing at room temperature for 1, 2, and 3 the sensitivity test was performed and no exceptional results appeared. Therefore, we concluded that the strip could be stored for at least 3 mo without losing stability.



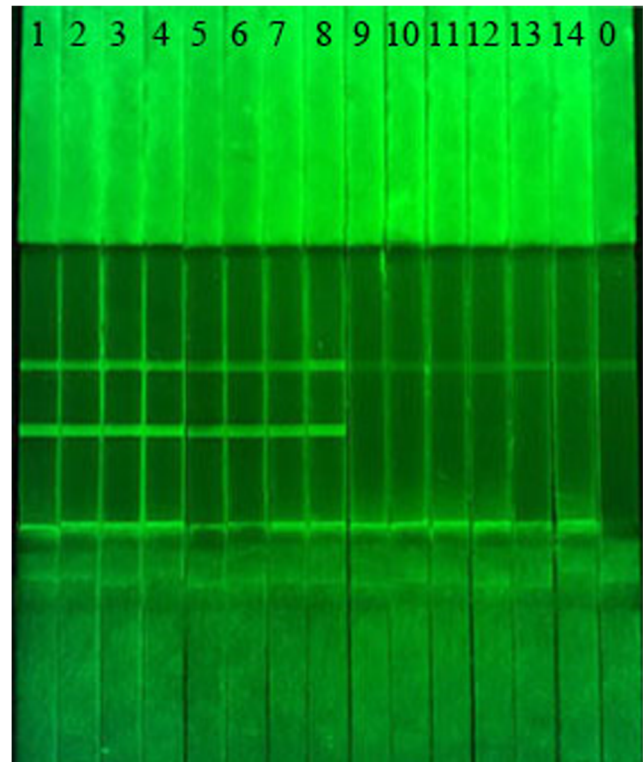
**Fig. 2** The fluorescence intensity detected by flow cytometry. **M1**: bacterial with fluorescence intensity of 1–10; **M2**: bacterial with fluorescence intensity of  $10$ – $10^2$ ; **M3**: bacterial with fluorescence intensity of  $10^2$ – $10^3$ ; **M4**: bacterial with fluorescence intensity of  $10^3$ – $10^4$



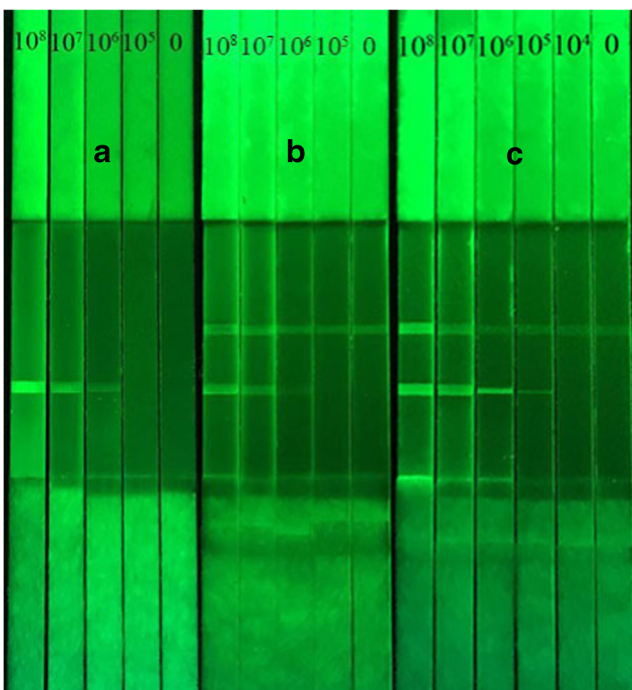
**Fig. 3** The morphological picture of Ac cultured with FITC viewed under fluorescent microscope with blue light

**Specificity of the ICS**

Since the common antigen of most bacteria, including Ac, is still unknown, the detection of different serotypes to evaluate the specificity of the strip are of critical importance. The specificity of the ICS was evaluated using eight strains of Ac and six genetically similar species to Ac. As shown in Fig. 5, eight strains of Ac (1 to 8) demonstrated positive results, and the other strains similar to Ac (9 to 14) produced negative results,



**Fig. 5** The cross-reaction results of the strip. 0: Blank control; 1: SD01; 2: PLSB1; 3: xjl112; 4: 00-1; 5: plsb91; 6: 99-5; 7: tw31; 8: ATCC 29625; 9: ATCC 19307; 10: NCPPB 961; 11: ATCC 33996; 12: NCPPB 2975; 13: NCPPB 540; NCPPB 2597



**Fig. 4** The sensitivity of the ICS (CFU/mL). 0: blank control; a: immunoassay strip based on FITC labeling antigen; b: immunoassay strip based on FITC labeling antibody coated on conjugate pad; c: immunoassay strip based on FITC labeling antigen and antibody

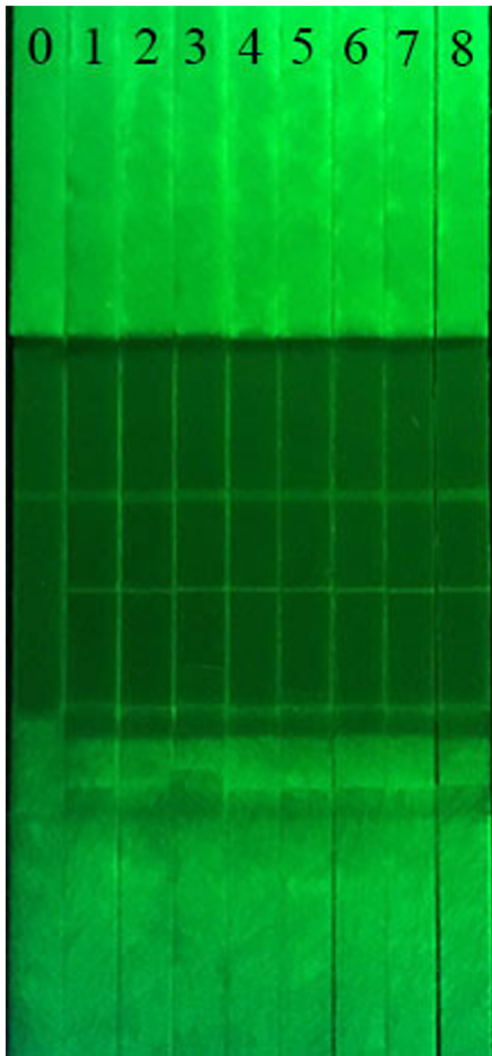
which indicated that the strip possessed high specificity for the detection of Ac.

The fluorescence intensity of the control line for the positive results (1 to 8) was visibly higher than the fluorescence intensity of the negative results (9 to 14). The fluorescence of control line for positive results was from fluorescent bacteria and 4F-FITC, and the fluorescence was just from 4F-FITC after 4F-FITC combining with goat anti-mouse IgG antibody for negative results.

**Sample analysis**

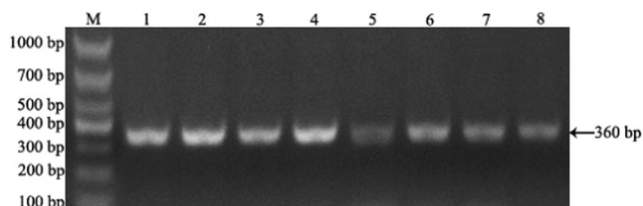
To ensure the applicability and accuracy of the strip in real samples, eight species of cucurbitaceous plant, including watermelon, cantaloupe, muskmelon, pumpkin, cucumber, luffa, wax gourd, and summer squash were used to simulate Ac infection. As shown in Fig. 6, all Ac samples presented positive results, indicating the impurities in plant juice would not impact the accuracy of detection results, and the smallest detectable concentration in real samples was  $10^5$  CFU/mL, which was consistent with the PCR results (Fig. 7).

To further confirm the specificity of the strip and the detection performance for real samples, 14 strains of cucurbitaceous common pathogen, including eight strains of



**Fig. 6** The detection results of artificial contaminated samples in  $10^5$  CFU/mL. **0**: blank control; **1**: Watermelon; **2**: Cantaloupe; **3**: Muskmelon; **4**: Pumpkin; **5**: Cucumber; **6**: Luffa; **7**: Wax gourd; **8**: Summer squash

Ac and six stains of plant pathogens, were mixed with watermelon juice for strip test. As shown in Table 2, the detection results were not affected by the juice, indicating the strip possessed high specificity and was suitable for the detection of Ac in real samples.



**Fig. 7** The PCR results of artificial contaminated samples in  $10^5$  CFU/mL. **M**: marker; **1**: Watermelon; **2**: Cantaloupe; **3**: Muskmelon; **4**: Pumpkin; **5**: Cucumber; **6**: Luffa; **7**: Wax gourd; **8**: Summer squash

**Table 2** The specificity of the strip for real samples

No.	Species/strains	Detection by ICS
1	SD01	+
2	ATCC 29625	+
3	tw31	+
4	plsb91	+
5	xj112	+
6	99-5	+
7	PLSB1	+
8	00-1	+
9	ATCC 33996	-
10	NCPPB 961	-
11	ATCC 19307	-
12	NCPPB 2597	-
13	NCPPB 540	-
14	NCPPB 2975	-

## Discussion

In this study, a new antigen and antibody dual labeled method using FITC was developed. FITC was used to make the bacteria fluoresce during bacterial enrichment process; the fluorescence stability of the labeling method has been proven before. The assay of FITC-labeled probiotic during gastrointestinal transit in broilers [22] and FITC-BSA-loaded chondroitin sulfate-chitosan nanoparticles upon Caco-2 cell uptake [24] verified that the bacterial fluorescence was very stable and was not quenched after entering the cell. In summary, the labeling method *in vivo* could make the bacteria emit stable and bright fluorescence.

Many methods for Ac detection were reported in recent years. The sensitivity of ELISA [25] and microsphere-based assay [26] were  $10^4$  CFU/mL with long time incubation and complex steps. PCR-based methods [27, 28] with the sensitivity of 32 and 10 CFU/mL, required trained technicians and precise instruments. Recently, DNA strip [29] has gained much attention owing to the high sensitivity of 4 nM. However, the process of pretreating sample to obtain DNA before detection was complex and time-consuming. The colloidal gold-based strip for Ac detection has been reported with the detection limit of  $10^5$  CFU/mL, while the chemical and colloidal instability in biological environment may lead to inaccurate result [30].

Compared with the methods mentioned above, antigen and antibody dual labeled immunofluorescence strip developed in this study is simple, fast, convenient, and easy to operate. The detection limit was successfully realized to  $10^5$  CFU/mL, which was 10-fold more sensitive compared with the strip of FITC only labeling antigen or antibody. In addition, there was a positive correlation between the Ac concentration and the fluorescence intensity of test line. The developed strip could detect the majority of Ac and had no cross-reactions with other

plant pathogens. This antigen and antibody dual labeled, one-step Ac detection method have many advantages, such as reducing the cost and possessing high stability, which will be a more popular and useful tool for the early diagnosis, prevention, and control of BFB in the future.

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#### Compliance with Ethical Standards

**Conflict of Interest** The authors confirm that no conflict of interest exists for this manuscript and all of the authors agree to the submission of this paper.

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