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1 # Development of Sandwich ELISA and Immunochromatographic

2 # Strip Methods for the Detection of *Xanthomonas oryzae pv.*

3 # *Oryzae*

4 **Dezhao Kong, Shanshan Song, Liqiang Liu, Hua Kuang, and Chuanlai Xu**[*]

5 A pair of sensitive monoclonal antibodies against both *Xanthomonas oryzae pv. Oryzae* and

6 *Xanthomonas oryzae pv. Oryzicola* were generated by immunizing mice and cell fusion techniques.

7 The sandwich enzyme-linked immunosorbent assay method and immunochromatographic test

8 strip were developed based on newly generated monoclonal antibodies for detection in rice

9 samples. The lower limit of detection of the sandwich ELISA method for *X. oryzae pv. Oryzae* was

10 1.0×10^4 cfu/mL. The cut-off values for the immunochromatographic test strip for *X. Oryzae pv.*

11 *Oryzae* and *X. oryzae pv. Oryzicola* in rice samples were 3.3×10^5 cfu/mL and 1×10^6 cfu/mL,

12 respectively. The quantitative sandwich ELISA method and rapid immunochromatographic test

13 strip assay could be useful for food safety inspections.

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1　**Introduction**

2　*Xanthomonas oryzae pv. oryzae* (Xoo) and *Xanthomonas oryzae pv. oryzicola* (Xooc) are both major

3　pathogenic bacteria that infect rice within the species *Xanthomonas oryzae pathovars*. Xoo, a

4　Gram-negative bacterium that causes bacterial leaf blight (BLB) on rice, was first detected in Japan in

5　1884[1], and has now spread to many rice-growing regions worldwide[2]. This pathogen is spread mainly

6　through plant debris[3], weeds[3, 4], wild rice[5] and water[6]. Additionally, Xoo-infected rice represents a

7　serious transmission route for plant pathogens worldwide[7-10]. Because Xoo is considered to be a

8　quarantine organism and is related to several devastating strains of pathogenic bacteria found in many

9　countries, methods for the detection of Xoo in rice plants and rice are needed, especially for

10　international trade.

11　The conventional methods used to detect Xoo in rice plants and rice include field observations,

12　biochemical tests[11], serological assays[12] and fatty acid analysis[13]; however, these methods are

13　labor-intensive, time-consuming and lack sensitivity and specificity.

14　Although polymerase chain reaction (PCR) technology[14-16], the electrochemiluminescence polymerase

15　chain reaction (ECL-PCR) method[17], real-time Bio-PCR[18, 19] and loop-mediated isothermal

16　amplification[20] have been reported for the highly sensitive detection of Xoo, these methods require

17　specialized instruments and trained operators. Moreover, they have not been successful in the detection

18　of this pathogen from artificially inoculated, symptomatic and symptomless tissues or naturally

19　infected rice[14]. A padlock probe-based assay has recently been used to improve detection sensitivity

20　and reduce the cost and time required for plant disease diagnosis[21]. Additionally, MALDI-TOF MS and

21　FTIR spectra methods have be used to identify and differentiate various pathovars of *X. oryzae*[22].

22　Studies aimed at the development of a simple, sensitive, rapid and cheap method for detecting Xoo

23　remain necessary.

24　As a simple, specific, and cheap method, the enzyme-linked immunosorbent assay (ELISA) is a

25　powerful way to detect pathogenic bacteria, but is time-consuming and some plant extracts can

26　suppress the ELISA reaction, which introduces many difficulties into the detection procedure[23].

27　Moreover, ELISA test results are largely dependent on the antibody characteristics[24].

28　The lateral-flow immunochromatographic strip assay is a rapid, simple, inexpensive, and

29　instrument-free diagnostic tool. Colloidal gold nanoparticles are ideal biological tags for

1   bio-recognition and allow detection results to be obtained visually within a few minutes[25].

2   Herein, monoclonal antibodies (mAb) against Xoo and Xooc were generated by mouse immunization

3   and subsequent cell fusion. The sandwich ELISA method and immunochromatographic lateral-flow

4   strip assay for the detection of Xoo and Xooc were developed using the mAbs that we obtained.

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Materials and methods

Reagents and materials

The bacteria strains used in this study (Xoo, *Xanthomonas Oryzae pv. oryzae* NCPPB 3002; Xooc, *Xanthomonas oryzae pv. oryzicola* NCPPB 1585; *Pantoea stewartii subsp. stewartii* NCPPB 449; *Burkholderia glumae* NCPPB 3591 and NCPPB 2391; *Pseudomonas syringae pv. maculicola* NCPPB 2039; and *Pseudomonas syringae pv. syringae* NCPPB 2844) were obtained from the Hunan Entry-Exit Inspection and Quarantine Bureau (Changsha, China). Complete Freund's adjuvant, incomplete Freund's adjuvant, gelatin, casein, bovine serum albumin (BSA), horseradish peroxidase-labeled goat anti-mouse immunoglobulin, poly-vinyl pyrrolidone (PVP), polyethylene glycol 2000 (PEG 2000), and poly-vinyl alcohol (PVA) were obtained from Sigma–Aldrich (St. Louis, MO, USA), and 3,3′,5,5′-tetramethylbenzidine and horseradish peroxidase (HRP) were purchased from Aladdin Chemistry Co. (Shanghai, China). All reagents for cell fusion were obtained from Sunshine Biotechnology Co. (Nanjing, China). Other reagents and chemicals were obtained from the National Pharmaceutical Group Chemical Reagent Co. (Shanghai, China).

The nitrocellulose high-flow-plus membranes (Pura-bind RP) were obtained from Whatman-Xinhua Filter Paper Co. (Hangzhou, China). A glass fiber membrane (CB-SB08) was used as a sample pad; polyvinylchloride backing material and the absorbance pad (SX18) were supplied by Goldbio Tech Co. (Shanghai, China).

Buffers and solutions

The following solutions were used: 0.05 M sodium carbonate-bicarbonate buffer (CB, pH 9.6); 0.05 M sodium CB buffer containing 0.2% (w/v) gelatin as blocking buffer; 0.01 M phosphate-buffered saline (PBS, pH 7.4); 0.01 M PBS containing 0.05% (v/v) Tween-20 (PBST, pH 7.4) as washing buffer; 0.01 M PBS containing 0.1% (w/v) gelatin as an antibody dilution buffer; 0.1 M citrate phosphate buffer (pH 5.0) containing 180 µL 30% H_2O_2 (A solution) and ethylene glycol substrate solution containing 0.06% (w/v) 3,3',5,5'-tetramethylbenzidine (B solution), mixed at a ratio of 5:1, as substrate solution; and 2 M sulfuric acid as a stop reagent.

Preparation of monoclonal antibodies against Xanthomonas Oryzae pv. Oryzae

Microorganism preparation

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1 Xoo (*X. oryzae pv. oryzae*) NCPPB 3002 was selected as an immunogen. The cryopreserved strain was

2 cultured in Luria–Bertani medium at pH 7.0, 28°C for 2 d and inoculated on nutrient agar plates at

3 28°C for 2 d. Inoculation was performed with one colony in M210 medium (sucrose 5 g/L, casein

4 enzymatic hydrolysates 8 g/L, yeast extract 4 g/L, K_2HPO_4 3 g/L, $MgSO_4·7H_2O$ 0.3 g/L, pH 7.0) at

5 28°C for 2 d. In all cases, the microorganisms obtained were counted, treated with boiling water for 30

6 min for inactivation.

7 Immunization procedure

8 A total of five female BALB/c mice (6 weeks old) were immunized subcutaneously with 150 μL 10^8

9 cfu/mL heat-killed Xoo mixed with an equal volume of Freund's complete adjuvant at the first

10 immunization, and Freund's incomplete adjuvant for subsequent immunizations. The immunization

11 was repeated every 3 weeks until high serum antibody titers were obtained as measured by indirect

12 ELISA[26].

13 Preparation of mAbs

14 Splenocytes from mouse with the highest serum titer were fused with SP2/0 myeloma cells. Positive

15 hybridoma cell lines were obtained via indirect ELISA screening after sub-cloning three times and then

16 were expanded to inject into BABL/c mice for mAb production[27]. Monoclonal antibodies were

17 purified from ascites using the caprylic acid-ammonium sulfate precipitation method and were

18 conjugated with HRP using the sodium periodate method[28].

19 **Development of a monoclonal sandwich ELISA method**

20 To establish a sandwich ELISA method, all mAbs and HRP-labeled mAbs obtained from the previous

21 experiment were used as capture and detection antibodies, respectively[26].

22 The procedure was as follows: 96-well microtiter plates were coated with capture mAb at 37°C for 2 h

23 with 100 μL/well in CB (pH 9.6). After incubation, plates were washed three times with PBST, and

24 then were incubated with blocking buffer at 37°C for 2 h with 200 μL/well. Then, plates were washed

25 three times and incubated with heat-killed Xoo at a concentration of $1.0×10^8$ cfu/mL in 0.01 M PBS or

26 were left blank (0.01 M PBS) at 37°C for 1 h with 100 μL/well. Plates were washed three times and

27 incubated with diluted HRP-mAb at 37°C for 1 h with 100 μL/well. After washing four times, 100

28 μL/well substrate solution was added and plates were incubated at 37°C for 15 min in the dark and

29 then were stopped by the addition of 50 μL/well stop reagent. Absorbance at 450 nm was determined

1   using a microtiter plate reader (BioTek, Winooski, VT, USA).

2   The optimal antibody combination for the sandwich ELISA method was determined by pair-wise

3   interaction analysis[29]. Antibody combinations that provided the highest positive/negative value (P/N

4   value, the ratio of the optical density values of the positive test sample to the negative sample) were

5   selected as pairs for detection. Heat-killed Xoo NCPPB 3002 was diluted at the concentrations of

6   1.0×10^8, 1.0×10^7, 1.0×10^6, and 1.0×10^5 cfu/mL in 0.01 M PBS or a blank control was used (0.01 M

7   PBS) to test the selected mAb combinations. Six kinds of blocking buffers were taken in the sandwich

8   ELISA method: gelatin, PEG 2000, PVP, PVA, BSA and casein in CB (pH 9.6, 0.2% [w/v]). The

9   optimal conditions for sandwich ELISA method were obtained and the standard curve was generated

10   based on the P/N value as the ordinate, and the microorganism standard concentrations as the abscissa.

11   **Cross-reactivity of the sandwich ELISA method**

12   A series of common plant pathogens were tested using the sandwich ELISA method at a concentration

13   of 1.0×10^8 cfu/mL.

14   **Detection of naturally infested rice samples and Xoo-spiked rice samples**

15   Different samples were collected from Jiangsu province (sample numbers S1 to S20). The rice samples

16   were washed in distilled water three times and dried under aseptic conditions to avoid contamination

17   with microbes. Then, 10 g rice seed samples were immersed in 10 mL 0.002% PBS-Tween for 4–6 h at

18   5°C and then were smashed using a homogenizer and incubated at 24°C for 4–6 h. The suspension

19   solution was vortexed for 5 min and then was prepared for detection. The suspension solution was both

20   tested by the reference national standard (GB/T 28078-2011, based on the biochemical separation

21   methods) and the sandwich ELISA method. The negative sample to Xoo was selected and spiked with

22   various concentrations of Xoo and tested by sandwich ELISA method. The detected results were

23   calculated by the software OriginPro 8.5, and the recovery results were calculated with the following

24   formula,

25   Recovery (%)=(detected concentration/spiked concentration) × 100%

26   **Fabrication and characterization of the immunochromatographic strip**

27   Gold nanoparticle (GNP)-labeled mAb

28   Colloidal gold nanoparticles were synthesized in our lab, as described previously[30], with a uniform

29   particle size ~30 nm in diameter that we chose for the following studies. The GNP solution was

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1   adjusted to pH 7.0 with 0.1 M K_2CO_3 before mAb labeling (10 mL GNP solution with 30 μL 0.1 M

2   K_2CO_3). Then, 0.1 mg mAb (1 mg/mL) in 0.01 M PBS at pH 7.4 was added drop-wise into 10 mL

3   GNP solution and incubated at room temperature for 50 min for conjugation. The colloidal gold

4   nanoparticles with a negative surface charge could quickly and steadily adsorb the positively charged

5   proteins and this did not destroy its biological activity[31]. To block the gold nanoparticles, 1 mL 0.5%

6   BSA (w/v) was slowly added into the solution to stabilize the labeled mAb. After incubation for 2 h,

7   the solution was centrifuged at 8000×*g* for 30 min and the precipitate was washed three times with

8   0.01 M PBS (containing 5% sucrose, 1% BSA, and 0.5% PEG 6000, pH 7.4), dissolved in 5 mL 0.01

9   M PBS (containing 0.02% NaN_3), and finally stored at 4°C[32].

10   Preparation of the immunochromatographic strip

11   The immunochromatographic strip was assembled layer by layer with a sample pad, nitrocellulose (NC)

12   membrane, polystyrene backing card, and absorption pad. Different immunoglobulins were sprayed

13   onto the NC membrane at 1 μL/cm using a membrane dispenser machine (XinqidianGene Technology

14   Co., Beijing, China) and dried at 37°C overnight. The mAb was sprayed on the test zone to capture

15   pathogens in the samples and goat anti-mouse IgG was sprayed on the control zone for quality control.

16   The sample pad was immersed in 0.01 M PBS (containing 1% BSA and 0.2% Tween-20) for 30 min

17   and dried at 37°C for 4 h to minimize nonspecific binding and matrix interference before assembly.

18   The assembled strip was stored at 4°C, and was sealed to keep it dry[33].

19   Immunochromatographic assay

20   A biosensor system for the detection of Xoo was based on a pair of antibodies. One mAb was sprayed

21   at the test zone on an NC membrane and was used as the capture antibody; another was labeled with

22   GNP and used as a detection antibody. The intensity of the test zone signal was proportional to the

23   amount of pathogens contained in each sample.

24   For the test, sample solution was first incubated with GNP-mAb solution at room temperature for 5 min

25   and then was added onto the sample pad of the strip. This reaction solution mixture flowed to the

26   absorption pad by capillary action. After 5 min, results could be observed visually.

27   The pathogens contained in the sample solution were first bound to the GNP-mAb. When the reaction

28   solution mixture began to flow on the NC membrane, the GNP-mAb bound pathogens were caught by

29   the capture mAb immobilized on the test zone and the remaining GNP-mAb was captured by the goat

30   anti-mouse IgG immobilized on the control zone. In the positive samples, two red lines (test zone and

control zone) appear on the NC membrane because of the deposition of GNP-mAb. Additionally, in the negative samples, only one line (control zone) appears. The more pathogens that were contained in a sample, the more GNP-mAb-bound pathogens that interacted on the surface of the NC membrane at the test zone and the higher the color intensity that appeared on the test zone (Figure 1). The C-line should always appear in the test; if it did not appear, it indicated that the procedure was incorrectly performed or that the strip was invalid, and a repeat test with a new strip should be performed.

Sensitivity and specificity

The sensitivity and specificity of the immunochromatographic strip were determined by testing. Heat-killed Xoo NCPPB 3002 was diluted to 1.0×10^8, 3.3×10^7, 1.0×10^7, 3.3×10^6, 1.0×10^6, 3.3×10^5, or 1.0×10^5 cfu/mL in 0.01 M PBS or a blank control was added (0.01 M PBS). The cut-off value (a threshold Xoo concentration at which the test line appeared) was defined as the concentration of plant pathogens that was clearly visible on the T-line. Tests were repeated six times at each concentration[34].

A series of common plant pathogens were diluted by 0.01 M PBS to concentrations of 1.0×10^8 cfu/mL and were subjected to the immunochromatographic strip assay. The blank control sample consisted of 0.01 M PBS.

Detection of Xanthomonas oryzae pv. Oryzae-spiked rice samples

Rice samples were prepared as described above. The negative suspension solution (confirmed by biochemical separation methods against a reference standard, GB/T 28078-2011) was used as a negative control. The pathogens at densities of 1.0×10^7, 1.0×10^6, and 1.0×10^5 cfu/mL were spiked-in negative control samples as positive controls. Each concentration was assayed three times.

Results and Discussion

Monoclonal sandwich ELISA method

Pair-wise interaction analysis

After obtaining ten positive hybridoma cell lines from the cell fusion procedure, six combinations with high P/N values (≥2.1) were selected by pairwise interaction analysis (Table 1). Xoo NCPPB 3002 was used as a positive control at 1.0×10^8 cfu/mL in 0.01 M PBS; as a negative control, 0.01 M PBS was used. The selected combinations with high P/N values were predicted to be the most sensitive for detection.

Optimization of the sandwich ELISA method

All six combinations selected by pairwise interaction analysis were tested with different concentrations of heat-killed Xoo NCPPB 3002 as follows: 1.0×10^8, 1.0×10^7, 1.0×10^6, and 1.0×10^5 cfu/mL in 0.01 M PBS or a blank control (0.01 M PBS). As shown in Figure 2, only two combinations that showed high sensitivity for target pathogens were selected (the combinations of mAb 4D11 with 4D11-HRP and mAb 3D2 with 4D11-HRP).

Considering the high background values (negative value) in the test that can lead to a poor limit of detection (LOD) based on the P/N values, six different types of blocking buffer were tested in the optimization procedure for the two combinations. Based on our results, 0.2% (w/v) BSA in CB (pH 9.6) was considered to be the best blocking buffer for our detection method. Additionally, the combination of mAb 4D11 with 4D11-HRP was finally determined to be the optimal combination for the sandwich ELISA method. The phenomenon that the same mAb 4D11 can be used in a single ELISA assay may be due to the absolute superiority of the epitope for this clone on the surface of the microorganisms. So the Xoo could be captured and detected by just one mAb clone.

The final optimization conditions for the sandwich ELISA method were as follows: mAb 4D11 was coated on the microtiter plates as the capture mAb at a concentration of 2 μg/mL; 0.2% (w/v) BSA in CB (pH 9.6) was used as blocking buffer; HRP-labeled mAb 4D11 was used as a detection mAb at a concentration of 1 μg/mL.

The linear dynamic range of Xoo NCPPB 3002 was 1.0×10^5 to 1.0×10^8 cfu/mL and the LOD was

1   1.0×10^4 cfu/mL, based on the mean and three standard deviations of absorbance at 450 nm of the

2   negative samples (figure 3A). The linear regression equation was $y = 5.402x - 26.778$ and the linear

3   correlation coefficient (R^2) was 0.992 (figure 3 B).

4   Specificity of the sandwich ELISA method

5   A total of seven plant pathogen strains were tested by the sandwich ELISA method at a concentration

6   of 1.0×10^8 cfu/mL. Both Xoo NCPPB 3002 and Xooc NCPPB 1585 could be detected by the sandwich

7   ELISA method that we developed, and in this method little cross-reactivity with other pathogen strains

8   occurred (Table 2), indicating that the sandwich ELISA method that we developed could

9   simultaneously detect *Xoo* NCPPB 3002 and Xooc NCPPB 1585.

10   Detection of naturally infested rice samples and Xoo-spiked rice samples

11   Different samples collected from Jiangsu province (sample numbers S1 to S20) were tested by both

12   national standard GB/T 28078-2011 and the sandwich ELISA method. This experiment was repeated

13   five times. The result was shown in Table 3. The national standard method detected Xoo in 5 samples

14   of all the 20 samples in each of the five repeats with like-Xoo colonies appeared on culture plate and

15   identified as Xoo strain through biochemical identification. The sandwich ELISA method detected Xoo

16   in 4 samples of all the 20 samples in each of the five repeats. The positive results were taken based on

17   the standard which P/N values higher than 2.1 and the concentrations of detected bacteria was as

18   follows: S 3, 4.1×10^4 cfu/mL; S 7, 3.7×10^6 cfu/mL; S 12, 2.9×10^6 cfu/mL; S 18, 2.4×10^5 cfu/mL. As

19   the LOD value of the developed method was 1.0×104 cfu/mL, the samples which contained Xoo

20   lower than this LOD value could not be detected by our developed method but could be detected by

21   the national standard (S 13). By comparison, the national standard method could detecte lower

22   concentration of Xoo in the samples but consume too much time (more than 48 h - 72 h). The

23   sandwich ELISA method was quicker and simpler than the national standard method, and the limit of

24   detection was at 1.0×10^4 cfu/mL. A series of certain concentrations of Xoo were spiked into the

25   negative rice sample suspension solution at 1.0×10^7, 3.3×10^6, 1.0×10^6, or 3.3×10^5 cfu/mL, and a blank

26   control was also used. As shown in Table 4, our method could effectively detect the pathogen in rice

27   samples.

28   **Characterization of the immunochromatographic strip assay**

29   Optimization of the immunochromatographic strip assay

30   The six antibody combinations selected using the sandwich ELISA method consisted of four different

1 antibodies. All antibodies were used as capture and detection antibodies, respectively, for the

2 immunochromatographic strip assay. These combinations of antibodies were tested with Xoo at

3 1.0×10^8 cfu/mL in 0.01 M PBS (figure 4). Only when mAb 4D11 was used as a capture antibody

4 sprayed on the NC membrane, and mAb 4D11 and 3D2 were used as the GNP-labeled detection

5 antibody, did both the T- and C-lines appear on the immunochromatographic strips. These results

6 indicated that the immunochromatographic strips could successfully detect pathogen standards only for

7 two combinations: 4D11 with 4D11 and 4D11 with 3D2.

8 Then, a series of pathogen standards (1.0×10^8, 3.3×10^7, 1.0×10^7, 3.3×10^6, 1.0×10^6, 3.3×10^5, and

9 1.0×10^5 cfu/mL in 0.01 M PBS) and a blank control of 0.01 M PBS were tested using

10 immunochromatographic strips with these two antibody combinations (figure 5). The cut-off value for

11 the strips was 1×10^6 cfu/mL when the combination consisted of mAb 4D11 with 3D2, and was 1×10^5

12 cfu/mL when the combination consisted of mAb 4D11 with 4D11. Based on this comparison, mAb

13 4D11 was chosen as the capture antibody and the gold-labeled detection antibody because of the lower

14 cut-off value and the more intense color value of the strip.

15 Specificity of the strip test

16 The specificity of the immunochromatographic strip assay was determined by testing reactivity with

17 six other plant pathogens: Xooc NCPPB 1585, *Pantoea stewartii subsp. stewartii* NCPPB 449,

18 *Burkholderia glumae* NCPPB 3591, NCPPB 2391, *Pseudomonas syringae pv. maculicola* NCPPB

19 2039 and *Pseudomonas syringae pv. syringae* NCPPB 2844, at concentrations of 1×10^8 cfu/mL in 0.01

20 M PBS, pH 7.4.

21 The result indicated high cross-reactivity between the immunochromatographic strip and Xooc

22 NCPPB 1585, and no cross-reactivity with any of the other five plant pathogens that we tested (Figure

23 6A). Xooc was further detected with a series of standards as follows: 1×10^8, 3.3×10^7, 1×10^7, 3.3×10^6,

24 1×10^6, and 3.3×10^5 cfu/mL in 0.01 M PBS and a blank control of 0.01 M PBS. The cut-off value for

25 Xoo was 1×10^6 cfu/mL (Figure 6B).

26 Detection of spiked rice samples

27 Rice samples were prepared against a reference standard, GB/T 28078-2011. The negative suspension

28 solution was spiked with a series of Xoo and Xooc at the following final concentrations: 1.0×10^7,

1   3.3×10^6, 1.0×10^6, and 3.3×10^5 cfu/mL, as well as a blank control (figure 7). The cut-off values were

2   3.3×10^5 and 1×10^6 cfu/mL, respectively.

3

Conclusion

5   In this present study, sandwich ELISA and lateral-flow immunochromatography strip methods were

6   established using a pair of monoclonal antibodies obtained by immunizing mice and subsequent cell

7   fusion. Both methods could simultaneously detect the plant pathogens Xoo and Xooc in rice samples.

8   In a comparative analysis, the sandwich ELISA method showed a large detection range of 1.0×10^5 to

9   1.0×10^8 cfu/mL and a lower LOD of 1.0×10^4 cfu/mL. However, this quantitative determination method

10   was time-consuming, requiring nearly 2.5 h to complete, and required specialized equipment, such as a

11   microtiter plate reader. The colloidal gold-based immunochromatography strip assay was quicker and

12   simpler than the sandwich ELISA method. With cut-off values for Xoo and Xooc in 0.01 M PBS of

13   1.0×10^5 and 1.0×10^6 cfu/mL, and in rice samples at 3.3×10^5 and 1×10^6 cfu/mL, respectively, results

14   could be obtained by visual detection within 10 min. This method has the benefits of low fabrication

15   costs, a rapid detection process, ease of use, not requiring specialized equipment, and having the

16   potential to be used in assays involving large numbers of samples.

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3 **Acknowledgments**

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8 **Conflicts of Interest**

9 The authors declare no conflict of interest.

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References and Notes

1.   Y. Tagami and T. Mizukami, *Plant Protection Division, Ministry of Agriculture and Forestry, Tokyo, Japan*, 1962, **112**.

2.   T. Mew, *Annual review of phytopathology*, 1987, **25**, 359-382.

3.   K. Goto, R. Fuktatzu and K. Okata, *Agric. Hortic. Jpn*, 1953, **28**, 207-208.

4.   V. Valluvaparidasan and V. Mariappan, *International Rice Research Newsletter*, 1989, **14**, 27-28.

5.   S. Aldrick, I. Buddenhagen and A. Reddy, *Crop and Pasture Science*, 1973, **24**, 219-227.

6.   R. Singh, *Indian Phytopathology*, 1971, **24**, 140-144.

7.   C. Fang, C. Lin and C. Chu, *Acta Phytopath. Sin*, 1956, **2**, 173-185.

8.   D. Srivastava and Y. Rao, *Indian Phytopathology*, 1964, **17**, 77-78.

9.   S. Chattopadhyay and N. Mukherjee, *Int Rice Comm News Lett*, 1971.

10. P. Reddy, *Current Science*, 1983, **52**.

11. C. V. Cruz, F. Gossele, K. Kersters, P. Segers, M. Van den Mooter, J. Swings and J. De Ley, *Journal of general microbiology*, 1984, **130**, 2983-2999.

12. A. Benedict, A. Alvarez, J. Berestecky, W. Imanaka, C. Mizumoto, L. Pollard, T. Mew and C. Gonzalez, *Phytopathology*, 1989, **79**, 322-328.

13. A. Chase, R. Stall, N. Hodge and J. Jones, *Phytopathology*, 1992, **82**, 754-759.

14. N. Sakthivel, C. Mortensen and S. Mathur, *Applied microbiology and biotechnology*, 2001, **56**, 435-441.

15. E. S. Song, B. M. Lee, C. S. Lee and Y. J. Park, *Journal of Phytopathology*, 2012, **160**, 195-200.

16. E. S. Song, T. H. Noh and S. C. Chae, *Journal of microbiology and biotechnology*, 2014, **24**, 732-739.

17. J. Wei and B. Wu, *Sensors and Actuators B: Chemical*, 2009, **139**, 429-434.

18. W.-J. Zhao, S.-f. Zhu, X.-L. Liao, H.-y. Chen and T.-w. Tan, *Molecular biotechnology*, 2007, **35**, 119-127.

19. N. W. Schaad, S. Cheong, S. Tamaki, E. Hatziloukas and N. J. Panopoulos, *Phytopathology*, 1995, **85**, 243-246.

20. J. M. Lang, P. Langlois, M. H. R. Nguyen, L. R. Triplett, L. Purdie, T. A. Holton, A. Djikeng, C. M. V. Cruz, V. Verdier and J. E. Leach, *Applied and environmental microbiology*, 2014, **80**, 4519-4530.

21. Y. Tian, Y. Zhao, R. Xu, F. Liu, B. Hu and R. Walcott, *Phytopathology*, 2014, **104**, 1130-1137.

22. M. Ge, B. Li, L. Wang, Z. Tao, S. Mao, Y. Wang, G. Xie and G. Sun, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 2014, **133**, 730-734.

23. W. Menzel, W. Jelkmann and E. Maiss, *Journal of Virological Methods*, 2002, **99**, 81-92.

24. Dezhao Kong, Liqiang Liu, Changrui Xing, Hua Kuang, Chuanlai Xu. *Food Agric. Immunol,* 2015, 26(4), 566-576.

25. N. L. Rosi and C. A. Mirkin, *Chemical reviews*, 2005, **105**, 1547-1562.

26. M. Feng, Q. Yong, W. Wang, H. Kuang, L. Wang and C. Xu, *Food and Agricultural Immunology*, 2013, **24**, 481-487.

27. H. Kuang, C. Xing, C. Hao, L. Liu, L. Wang and C. Xu, *Sensors*, 2013, **13**, 4214-4224.

28. D. Boorsma and G. L. Kalsbeek, *Journal of Histochemistry & Cytochemistry*, 1975, **23**, 200-207.

29. J. Peng, X. Meng, X. Deng, J. Zhu, H. Kuang and C. Xu, *Food and Agricultural Immunology*, 2014, **25**, 1-8.

30. L. Liu, L. Luo, S. Suryoprabowo, J. Peng, H. Kuang and C. Xu, *Sensors*, 2014, **14**, 16785-16798.

31. W. D. Geoghegan and G. A. Ackerman, *Journal of Histochemistry & Cytochemistry*, 1977, **25**, 1187-1200.

32. F. Sun, L. Liu, W. Ma, C. Xu, L. Wang and H. Kuang, *International Journal of Food Science & Technology*, 2012, **47**, 1505-1510.

33. L. Liu, C. Xing, H. Yan, H. Kuang and C. Xu, *Sensors*, 2014, **14**, 14672-14685.

34. J. H. Cho and S. H. Paek, *Biotechnology and bioengineering*, 2001, **75**, 725-732.

14

Captions:

Fig. 1 The schematic image of the assembled strip and the principle of the detection.

Fig. 2 The sandwich ELISA method for the chosen mAb combinations.

Fig. 3 The standard curve for *Xanthomonas Oryzae pv. oryzae* NCPPB 3002 in monoclonal sandwich

ELISA (n=8).

Fig. 4 The optimization of antibodies for immunochromatographic strip.

Fig. 5 The detection of *Xanthomonas Oryzae pv. oryzae* by immunochromatographic strip.

Fig. 6 The cross-reaction and detection of the immunochromatographic strip.

Fig. 7 Detection of spiked rice samples by immunochromatographic strip.

Table 1 The sandwich ELISA for pair-wise interaction analysis (P/N value).

Table 2 The cross-reactivity by optimized Sandwich ELISA (n=8).

Table 3 The results of detecting naturally infested rice samples (n=5).

Table 4 The results of detecting *Xanthomonas Oryzae pv. oryzae* in rice samples (n=8).

Y · · · goat anti-mouse IgG

Y · · capture mAb (mouse anti-*Xanthomonas Oryzae pv. oryzae* IgG) ·

Y · gold-labeled detection mAb (mouse anti-*Xanthomonas Oryzae pv. oryzae* IgG)
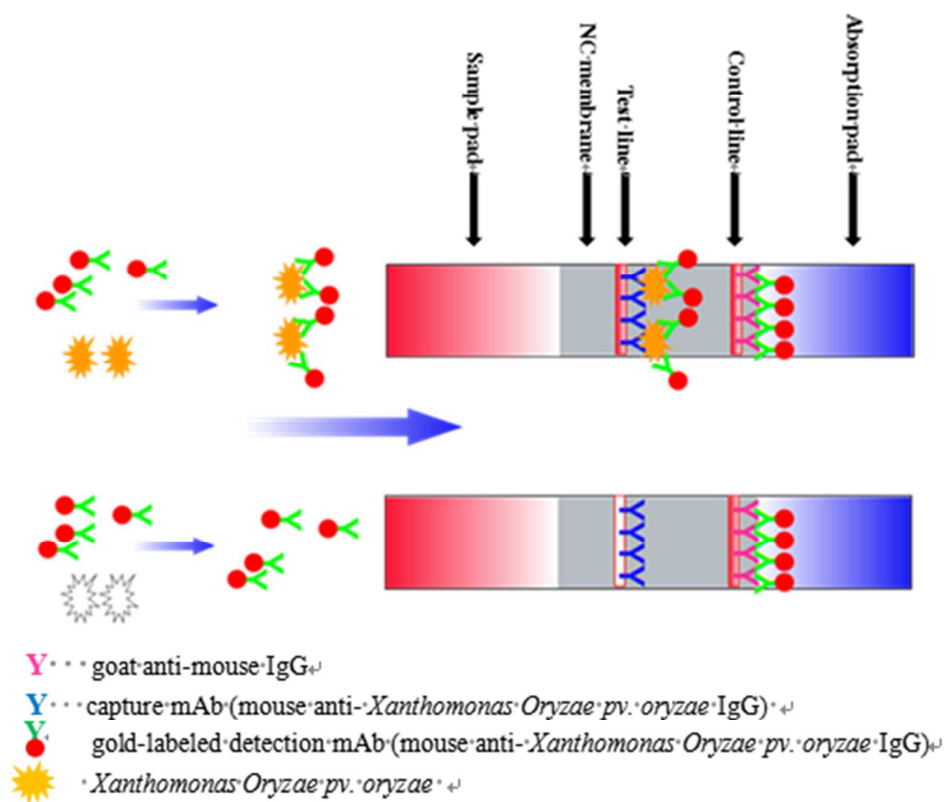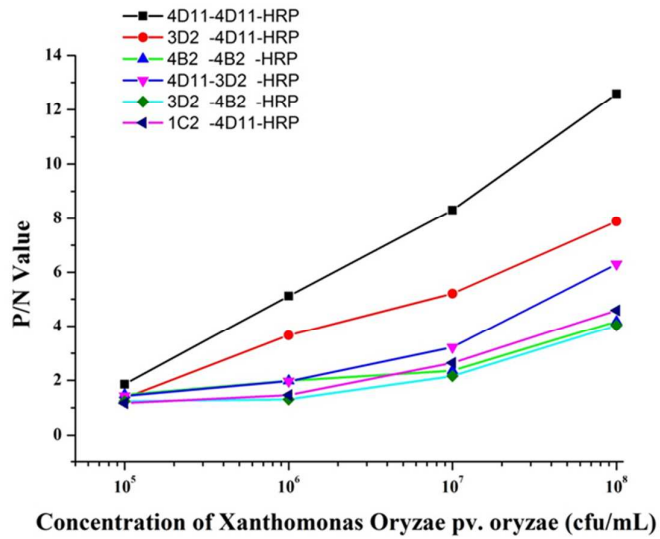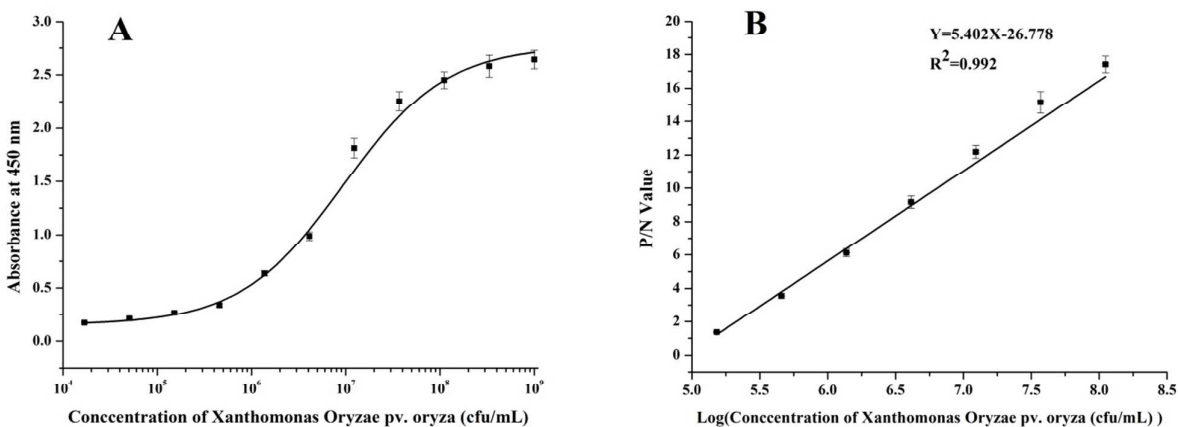
· *Xanthomonas Oryzae pv. oryzae* ·

**Fig. 1** The schematic image of the assembled strip and the principle of the detection.

**Fig. 2** The sandwich ELISA method for the chosen mAb combinations.

1 **Fig. 3** The standard curve for *Xanthomonas Oryzae pv. oryzae* NCPPB 3002 in monoclonal sandwich
2 ELISA (n=8). (A). Standard curve of the concentration ranging from 1×10^4 and 109cfu/mL; (B). The
3 linear dynamic range of *Xanthomonas Oryzae pv. oryzae* NCPPB 3002 between 1×10^5 and 10^8cfu/mL.
4 The linear regression equation was Y=5.402X-26.778. The linear correlation coefficient (R^2) was
5 0.992.

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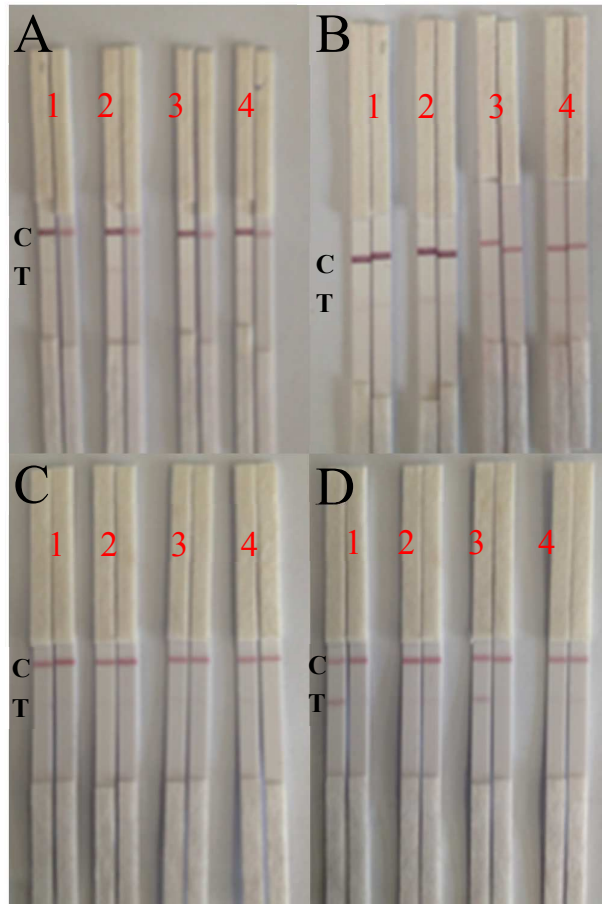
Fig. 4 The optimization of antibodies for immunochromatographic strip. The detection monoclonal antibody was: (A). 1C2; (B). 3D2; (C). 4B2; (D). 4D11; The capture monoclonal antibody was: (1) 4D11, (2) 4B2, (3) 3D2, (4) 1C2; T, test line; C, control line.
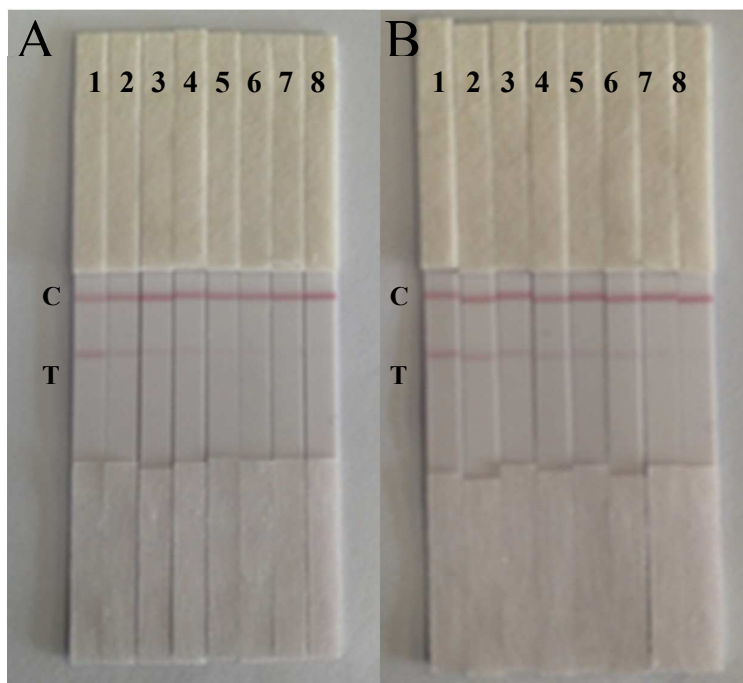
Fig. 5 The detection of *Xanthomonas Oryzae pv. oryzae* by immunochromatographic strip. (A). The capture antibody was 3D2, the detection antibody was 4D11; (B). The capture antibody was 4D11, the detection antibody was 4D11; The concentration was as follows: (1) 1×10^8, (2) 3.3×10^7, (3) 1×10^7, (4) 3.3×10^6 , (5) 1×10^6, (6) 3.3×10^5, (7) 1×10^5 cfu/mL and (8) blank. T: test line. C: control line.
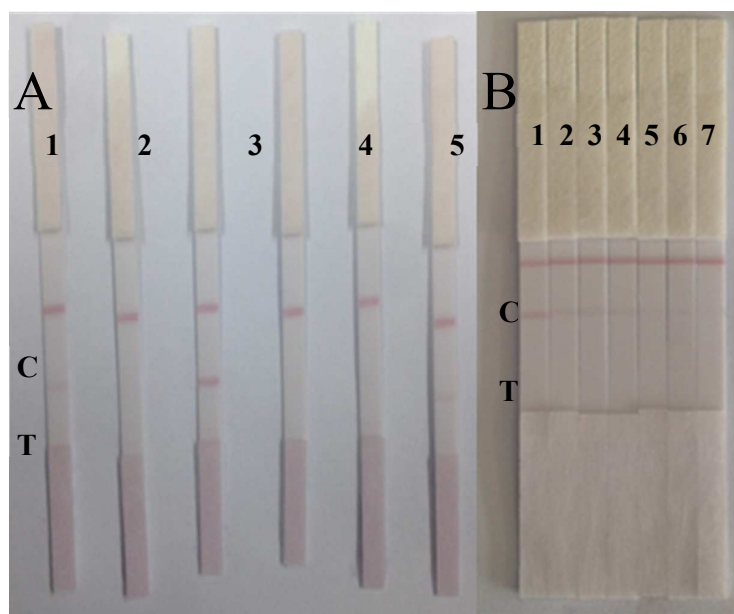
Fig. 6 The cross-reaction and detection of the immunochromatographic strip. (A). The cross-reaction of the immunochromatographic strip: (1). *Pseudomonas syringae pv. maculicola* NCPPB 2039; (2). *Pseudomonas syringae pv. syringae* NCPPB 2844; (3). *Xanthomonas oryzae pv. oryzicola* NCPPB 1585; (4). *Pantoea stewartii subsp.stewartii* NCPPB 449; (5). *Burkholderia glumae* NCPPB 3591; (6). *Burkholderia glumae* NCPPB 2391; (B). The detection of the immunochromatographic strip to *Xanthomonas oryzae pv. oryzicola* NCPPB 1585: The concentration was as follows: (1) 1×10^8, (2) 3.3×10^7, (3) 1×10^7, (4) 3.3×10^6, (5) 1×10^6, (6) 3.3×10^5 cfu/mL and (7) blank. T: test line. C: control line.
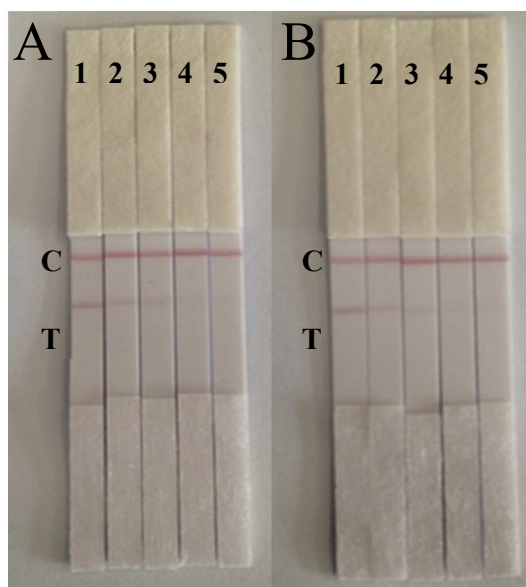
Fig. 7 Detection of spiked rice samples by immunochromatographic strip. (A): Detection of *Xanthomonas Oryzae pv. Oryzae*; (B): Detection of *Xanthomonas oryzae pv. Oryzicola*. The concentration was as follows: (1) 1×10^7, (2) 3.3×10^6, (3) 1×10^6, (4) 3.3×10^5 cfu/mL and (5) blank. T: test line. C: control line.

1   **Table 1** The sandwich ELISA for pair-wise interaction analysis (P/N value).

Detection mAb	Capture mAb									
	2H2	3G8	4B2	1B7	2H3	4D11	3D2	1C2	3G7	2D3
2H2-HRP	1.52	0.66	1.35	0.76	0.74	0.63	1.07	0.96	0.58	0.77
3G8-HRP	1.15	0.79	1.05	0.99	0.71	1.67	0.81	1.35	0.71	0.73
4B2-HRP	0.02	0.03	*4.01*	0.02	0.02	1.36	*5.25*	1.13	0.01	1.98
1B7-HRP	1.09	0.83	0.92	0.84	0.60	0.68	1.18	0.33	0.87	1.06
2H3-HRP	0.81	0.80	1.56	1.09	0.95	1.62	1.12	0.32	0.61	0.89
4D11-HRP	1.01	0.95	1.49	1.60	1.19	*12.67*	*5.00*	*4.98*	1.90	1.02
3D2-HRP	1.19	1.03	1.36	1.81	0.96	*5.34*	1.37	1.07	1.49	0.88
1C2-HRP	1.11	0.91	0.93	0.93	1.19	0.58	0.80	1.51	0.95	0.91
3G7-HRP	0.78	1.42	1.11	0.88	0.70	1.19	0.64	1.24	1.91	1.12
2D3-HRP	1.37	1.03	1.56	1.17	0.59	1.14	1.08	0.91	1.07	1.17

2   Note: P/N value, the positive/negative value, which was the ratio of optical density value of the testing

3   sample to the negative control sample.

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1 **Table 2** The cross-reactivity by optimized Sandwich ELISA (n=8).

Microorganism	Sandwich ELISA	
	OD$_{450}$ value	P/N value
Xanthomonas Oryzae pv. oryzae NCPPB 3002	2.451±0.12	19.92 (+)
Xanthomonas oryzae pv. oryzicola NCPPB 1585	2.012±0.09	13.26 (+)
Pantoea stewartii subsp.stewartii NCPPB 449	0.190±0.011	1.45 (-)
Burkholderia glumae NCPPB 3591	0.184±0.017	1.54 (-)
Burkholderia glumae, NCPPB 2391	0.212±0.011	1.72 (-)
Pseudomonas syringae pv. syringae NCPPB 2844	0.240±0.013	1.99 (-)
Pseudomonas syringae pv. maculicola NCPPB 2039	0.193±0.012	1.30 (-)

2 Note: P/N value, the positive/negative value, which was the ratio of optical density value of the testing
3 sample to the negative control sample. Values were calculated according to the formula P/N > 2.1. (+)
4 means positive, (-) means negative. NCPPB:National Collection of Plant Pathogenic Bacteria,
5 Harpenden, UK

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1   **Table 3** The results of detecting naturally infested rice samples (n=5).

Analyzed samples	National Standard method	Sandwich ELISA method	Analyzed samples	National Standard method	Sandwich ELISA method
S 1	-	-	S 11	-	-
S 2	-	-	S 12	+	+
S 3	+	+	S 13	+	-
S 4	-	-	S 14	-	-
S 5	-	-	S 15	-	-
S 6	-	-	S 16	-	-
S 7	+	+	S 17	-	-
S 8	-	-	S 18	+	+
S 9	-	-	S 19	-	-
S 10	-	-	S 20	-	-

Note: + means positive, - means negative. For the national standard method, samples with colonies appeared on culture plate and identified as Xoo strain through biochemical identification were the positive ones or to be the negative ones. For the sandwich ELISA method, samples with P/N values higher than 2.1 were the positive ones or to be the negative ones.

1   T**able 4** The results of detecting *Xanthomonas Oryzae pv. oryzae* in rice samples (n=8).

	Spiked(cfu/mL)	Recovery(%)	RSD(%)
Rice samples	0	ND	ND
	1.0×10^7	85.6	3.5
	3.3×10^6	88.4	4.1
	1.0×10^6	90.2	2.8
	3.3×10^5	88.3	3.1

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