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Antibody Array in a Multiwell Plate Format for the Sensitive and Multiplexed Detection of Important Plant Pathogens

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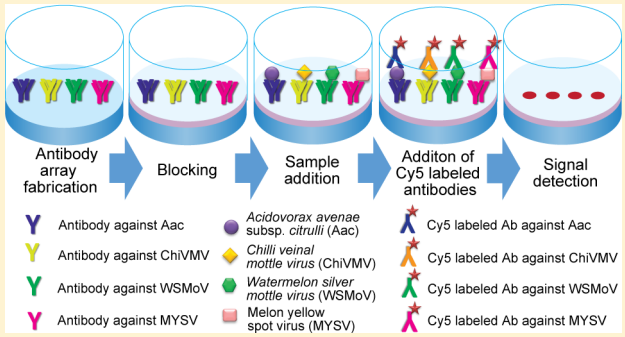
Supporting Information

ABSTRACT: The global seed market is considered to be an important industry with a total value of $10,543 million US dollars in 2012. Because plant pathogens such as bacteria and viruses cause a significant economic loss to both producers and exporters, the seed export industry urgently requires rapid, sensitive, and inexpensive testing for the pathogens to prevent disease spreading worldwide. This study developed an antibody array in a multiwell plate format to simultaneously detect four crucial plant pathogens, namely, a bacterial fruit blotch bacterium *Acidovorax avenae* subsp. *citrulli* (Aac), *Chilli veinal mottle virus* (ChiVMV, potyvirus), *Watermelon silver mottle virus* (WSMoV, tospovirus serogroup IV), and Melon yellow spot virus (MYSV, tospovirus). The capture antibodies specific to the pathogens were immobilized on each well at preassigned positions by an automatic microarrayer. The antibodies on the arrays specifically captured the corresponding pathogens present in the sample extracts. The presence of pathogens bound on the capture antibodies was subsequently detected by a cocktail of fluorescently conjugated secondary antibodies. The limits of detection of the developed antibody array for the detection of Aac, ChiVMV, WSMoV, and MYSV were 5×10^5 CFU/mL, 30 ng/mL, 1000 ng/mL, and 160 ng/mL, respectively, which were very similar to those of the conventional ELISA method. The antibody array in a multiwell plate format accurately detected plant pathogens in single and multiple detections. Moreover, this format enables easy handling of the assay at a higher speed of operation.



Seed export is one of the important agricultural industries accounting for 3 million metric tons in 2012 (International Seed Federation (ISF) compilation based on official statistics and international seed trade reports). Europe, America, and Asia were the world's leading seed exporters. Plant pathogens such as viruses, bacteria, fungi, and parasitic plants are serious threats to the seed export businesses worldwide and global food supply.[1] These pathogens reduce quantity and quality of products as well as damage the creditability of exporting countries. For instance, the bacterium *Acidovorax avenae* subsp. *citrulli* (Aac) causes fruit blotch in watermelon and cucurbits. Potyvirus infection reduces fruit set and fruit distortion in all melon types, and tospovirus causes loss in agricultural, horticultural, and ornamental values.[2] Therefore, seed and plant export businesses urgently require a rapid, low-cost, and high-throughput screening method to ensure disease-free seeds and plants.

Current methods for plant disease detection are based mainly on polymerase chain reaction (PCR) and immunochemical techniques. For instance, a PCR method was developed to detect a single type of plant pathogen, such as *Acidovorax avenae* subsp. *avenae*,[3] *Xanthomonas oryzae* pv. *oryzae*,[4] and *Pseudomonas syringae* pv. *phaseolicola*.[5] Several multiplex PCR assays were developed to simultaneously detect multiple pathogens such as two clades of *Tomato leaf curl virus* (TYLCV) in tomato[6] and multiplex *Cucumber vein yellowing virus* (CVYV) and *Cucurbit yellow stunting disorder virus* (CYSDV) in the whitefly vector *Bemisia tabaci*.[7] These molecular techniques are highly sensitive but require highly-skilled workers for tedious DNA extraction and purification which also incur an additional cost for sample preparation. Immunochemical based techniques, such as enzyme-linked immunosorbent assays (ELISAs), are widely used as screening tools; however, they are not suitable for detecting multiple pathogens in a single well.

This paper describes a development of an alternative immuno based method using a high throughput antibody array technology that allows multiplexing to be employed. With its high-throughput and miniaturized format, the antibody array can be considered as an alternative screening method with shorter assay time and reduced associated costs of reagents and labor. The antibody array has been used for diagnostic purposes to detect

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Table 1. Antibodies Used in the Study

pathogen	capture antibody		secondary antibody	
	clone	type of antibody	clone	type of antibody
Acidovorax avenae subsp. *citrulli* (Aac)	11E5	mouse monoclonal antibody	MPC	rabbit polyclonal antibody
Chilli veinal mottle virus (ChiVMV)	1B4	mouse monoclonal antibody	1G8	mouse monoclonal antibody
Watermelon silver mottle virus (WSMoV)	2D6	mouse monoclonal antibody	MYSV6	rabbit polyclonal antibody
Melon yellow spot virus (MYSV)	5E7	mouse monoclonal antibody	MYSV6	rabbit polyclonal antibody

diseases[8−13] and foodborne pathogens[14−16] and for many other applications.[17] This study describes the first application of an antibody array in a multiwell plate format to detect the widespread plant pathogens: *Acidovorax avenae* subsp. *citruli* (Aac), *Chilli veinal mottle virus* (ChiVMV), *Watermelon silver mottle virus* (WSMoV), and Melon yellow spot virus (MYSV). The assay was optimized to achieve high accuracy and sensitivity required by the seed and plant industry sector.

MATERIALS AND METHODS

Conjugation of the Antibodies with the Fluorescent Dye and Alkaline Phosphatase. All antibodies used in this study were obtained from the Monoclonal Antibody Production Laboratory, BIOTEC, Thailand, except for MPC which was purchased from the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus, Thailand (Table 1). The secondary antibodies (MPC, 1G8, and MYSV6) were conjugated with a fluorescent dye (Cy5) using an antibody conjugation kit (LNK101CY5, AbD Serotec, USA.). Briefly, 10 μL of Modifier was added into 100 μL of 1 mg/mL antibody and incubated with LYNX lyophilized Cy5 at 25 °C overnight before 10 μL of Quencher was added to the solution for 30 min. The fluorescently labeled antibodies were kept at 4 °C until use.

The conjugation of the antibodies with alkaline phosphatase (AP) was performed using the SureLINK modified AP kit (Kirkegaard & Perry Laboratories, #85−01−01). Briefly, antibody was diluted in AP modification buffer (200 μL, 2 mg/mL), and then, 3.8 μL of succinimidyl-P-formyl benzoate (SFB) was added and incubated at room temperature (RT) for 1 h. The Ab-SFB solution was added to 0.2 mg of modified AP before adding 80 μL of AP conjugation buffer. Distilled water was added to make up a final volume of 400 μL, and the reaction mixture was incubated at RT for 2 h. The antibodies labeled alkaline phosphatase were kept at 4 °C until use.

Antigen Preparation. *Acidovorax avenae* subsp. *citrulli* (Aac) was inoculated in Nutrient broth (Difco laboratory, #234000) and shaken at 200 rpm and 30 °C for 16 h. The bacterial cells were harvested by centrifugation (5000 rpm, 10 min) and resuspended in phosphate buffered saline (PBS), pH 7.4, containing 1 mM KH_2PO_4, 0.15 mM Na_2HPO_4, and 3 mM NaCl. The optical density (OD) values were measured at 600 nm (Spectrophotometer Cintra 404), and the corresponding colony forming unit (CFU) numbers were calculated from a conversion factor of 1 OD equivalent to 3×10^9 CFU/mL by the plate count method.

Recombinant capsid coat protein (CP) of ChiVMV and nucleocapsid proteins (NPs) of WSMoV and MYSV were produced to represent the plant virus during the assay optimization. PCR products of the coat proteins were amplified using gene specific primers from the previously reported nucleic acid sequences (http://www.ncbi.nlm.nih.gov; GenBank accession numbers U72193, AY514625, and AY574574 for ChiVMV, WSMoV, and MYSV, respectively). Each purified PCR product was cloned into an expression vector, pQE80L (QIAGEN), with 6xHis tag at the N-terminus of virus protein. The resulting plasmid was transformed into designated *E. coli* host (DH5α), and the expression was induced using isopropyl-1-thio-β-D-galactoside (IPTG, final concentration of 1 mM). The 6xHis-protein was purified with Ni-NTA agarose resin column under a denaturing condition. The CP and NPs of the viruses were around 30−34 kDa in weight.[18−21]

To obtain pure virus, the ChiVMV was inoculated into healthy *Datura metel*. The virus infected leaves (100 g) were ground and extracted in 150 mL of 0.5 M potassium phosphate buffer (KPB), pH 7.5, containing 1% (w/v) Na_2SO_3 and then clarified by adding 8% (v/v) butanol and centrifuged at 4000 rpm for 10 min. The supernatant was precipitated with 8% polyethylene glycol (PEG-6000) at RT for 1 h and kept at 4 °C for 1 h before centrifugation at 7000 rpm for 20 min. The virion pellet was resuspended in 20 mL of 0.25 M KPB, pH 7.5, containing 1% (w/v) Na_2SO_3, 0.1% (v/v) tritonX-100, and 0.01 M $MgCl_2$. The centrifugation steps were repeated at 4000 rpm for 10 min and 28 000 rpm for 90 min, respectively. The pellet was collected and resuspended in 1.5 mL of 0.25 M KPB, pH 7.5, containing 0.01 M $MgCl_2$ and then centrifuged at 4000 rpm for 10 min. The virion was purified by using a sucrose density gradient (10%, 20%, 30%, and 40%) at 20 000 rpm for 90 min. The absorbance at 260 nm of the obtained virion solution was measured and used for calculation of the concentration of the virion by using an extinction coefficient of 2.4.[22]

Optimization of the Antibody Array Fabrication in a Multiwell Plate Format. To optimize antibody array fabrication, Aac was used as a model target. Four different multiwell plates were purchased from Nunc brand (black-rim coverglass base: #265300; and black-rim polystyrene: #265301) and Costar brand (clear-rim with bottom polystyrene: #3590; and black-rim with clear bottom polystyrene: #3601). The plates were compared for antibody array fabrication performance. The capture antibody to Aac (11E5) at concentrations of 31, 62, 125, 250, and 500 μg/mL was diluted in a spotting buffer. Two spotting solutions: phosphate buffered saline, pH 7.4, containing 1 mM KH_2PO_4, 0.15 mM Na_2HPO_4, and 3 mM NaCl (PBS) and 50 mM sodium carbonate−bicarbonate buffer (CB), pH 9.6, containing various combinations of 0.05% Tween 20, 1% sucrose, and/or 0.5% glycerol were compared for spot morphology and signal enhancement. Fabrication on a 96-well microtiter plate was performed by a robotic arrayer with a 946MP10 pin (NanoPrint 210, TeleChem USA) and 30−35% humidity at 23−25 °C to create an array (6 × 6 arrangement) of spots with 500 μm center-to-center spacing between spots. Spotting buffer was used as a negative control. After antibody fabrication, the plate was kept at 4 °C overnight before being blocked with the designated blocking buffer. Three blocking buffers, 1% gelatin, 2% BSA (Sigma, #A9647), and 3% skim milk (Difco laboratory, #232100), in PBS containing 0.05% Tween 20 (v/v, PBST) were compared. The blocking buffer (100 μL/well) was used to block each well for 1 h at RT.

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The plates were washed with 300 μL of PBST 4 times before Aac at 1×10^9 CFU/mL in 0.5% gelatin, 0.5% BSA, or 1% skim milk in PBST or in plant extract was added to each well (100 μL) for 1 h at RT. The washing step was repeated before the fluorescence labeled secondary antibody (MPC) (25 μL/well, diluted in 0.5% gelatin, 0.5% BSA, or 1% skim milk in PBST) was added and incubated for 1 h at RT. Another washing step was performed before the microtiter plate was dried by centrifugation (1000 rpm, 5 min). The fluorescent signals were scanned using a fluorescence scanner (Alpha Innotec NovaRay USA) before signals were acquired using ArrayVision software version 8.0.

To examine reproducibility and stability of the selected spotting and blocking buffers, different concentrations (0, 31, 63, 125, and 500 μg/mL) of capture antibody 11E5 in a carbonate buffer with 0.05% Tween 20 and 1% sucrose were spotted in each well before 3% skim milk was used as a blocking buffer. The fluorescent (Cy5) labeled goat-antimouse was added and used for surface intensity plot analysis by using Array Pro Analyzer software (version 4.5.1.73). For intra-assay analysis, six repeats of each concentration of capture antibody in a carbonate buffer with 0.05% Tween 20 and 1% sucrose were spotted per well and 24 different wells were tested within a day. For interanalysis, the same experimental scheme was repeated in two different days. The variation of the signals was examined using the following equation.

$$\%\ \text{coefficient of variation (CV)} = \text{mean of the standard deviation (SD)} \times 100/\text{mean} \quad (1)$$

Optimization of Concentrations for Capture Antibodies and Secondary Antibodies. To find the optimal concentration of antibodies, five concentrations of each antibody (0, 31, 63, 125, and 250 μg/mL for Aac-antibody 11E5; 0, 125, 250, 500, and 1000 μg/mL for ChiVMV-antibody 1B4; and 0, 250, 500, 1000, and 1500 μg/mL for WSMoV-antibody 2D6 and MYSV-antibody 5E7) and four concentrations (5, 10, 20, 40 μg/mL) of each fluorescently labeled secondary antibody (MPC for Aac detection, Cy5 labeled 1G8 for ChiVMV detection, Cy5 labeled MYSV6 for WSMoV and MYSV detection) were tested (Figure S-3, Supporting Information). Each concentration of capture antibodies was spotted in a well (six replicates for each concentration). Then, a single antigen (Aac, 10^9 CFU/mL for Aac detection; ChiVMV, 0.5 μg/mL for ChiVMV detection; WSMoV, 50 μg/mL for WSMoV detection; and MYSV, 10 μg/mL for MYSV detection) was added after the blocking step. A corresponding fluorescently labeled secondary antibody (Cy5 labeled MPC for Aac detection, Cy5 labeled 1G8 for ChiVMV detection, Cy5 labeled MYSV6 for WSMoV and MYSV detection) was added before the washing step. The fluorescent signals were obtained and reported in terms of mean fluorescent intensity (MFI).

Sandwich ELISA. 96-well microtiter plates (Costar, #3590) were coated overnight at 4 °C with 100 μL per well of the capture antibodies serially diluted in carbonate–bicarbonate buffer (50 mM, pH 9.6). The plates were washed 4 times with 400 μL of PBS containing Tween 20 (0.05% v/v) before blocking with 100 μL of 3% skim milk in PBST for 1 h at RT. The same washing steps were repeated afterward.

Test samples diluted in 100 μL of 1% skim milk in PBST were added and incubated for 1 h at RT. Each plate was washed before alkaline phosphatase conjugated antibodies specific to each antigen were added and incubated for 1 h at RT. The same washing steps were repeated. After washing, 100 μL of substrate for alkaline phosphatase (*p*-nitrophenyl phosphate, pNPP, Zymed, Invitrogen, #00-2201) was added and incubated for 60 min in the dark at RT before signals were measured at 405 nm.

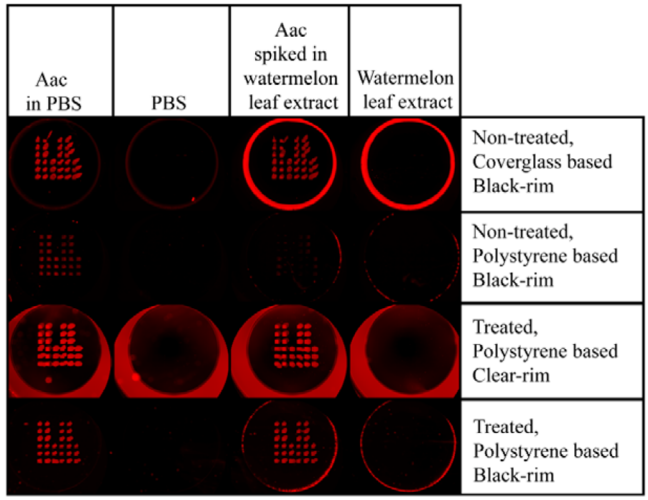

Figure 1. 96-well plate selection for antibody array construction. Two surface chemistries (nontreated and treated), two kinds of material (coverglass and polystyrene based), and two types of rim of the well (black and clear-rim) were tested using *Acidovorax avenae* subsp. *citrulli* (Aac) in PBS and spiked in watermelon leaf extract as a model.

Limit of Detection. The capture antibodies (11E5, 1B4, 2D6, and 5E7) were diluted in 50 mM carbonate–bicarbonate buffer containing 0.05% Tween 20 and 1% sucrose at the optimal concentrations of 62.5, 500, 1000, and 1500 μg/mL, respectively (Figure S-3, Supporting Information), and spotted in each well of 96-well plate. The subsequent assay steps were the same as those described in the Optimization of the Antibody Array Fabrication in a Multiwell Plate Format section, and a total of 25 μL of the cocktail of Cy5 labeled antibodies (20, 10, and 40 μg/mL for MPC, 1G8, and MYSV6, respectively) was used to report signals. Different concentrations of Aac (0–10^8 CFU/mL), ChiVMV or purified ChiVMV (0–1000 ng/mL), and WSMoV and MYSV (0–10 000 ng/mL) were tested with optimized conditions. The signals from the microarray fluorescent scanner of each spot were used to prepare standard curves. The following dose–response curve fitting equation was used.[23]

$$Y = A + B/(1 + 10^{C-X}) \quad (2)$$

Y was the ratio between signals of spot and background when detecting bacteria or coat protein of virus concentration (*X*), while *A*, *B*, and *C* were constants from curve fitting. The limit of detection (LOD) values were calculated using the intensity values greater than twice of the background or negative values.[24]

Multiplex Detection Using Antibody Array in 96-Well Plate Format. To test accuracy and specificity of the assay, different combinations (single, two, three, and four) of plant pathogens were tested. Aac (1×10^7 CFU/mL), recombinant coat proteins for ChiVMV (250 ng/mL), WSMoV (5000 ng/mL), and MYSV (1000 ng/mL) spiked in watermelon leaf extract were used for testing accuracy of the detections. A total volume of 25 μL of the cocktail of Cy5 labeled antibodies (20, 10, and 40 μg/mL for MPC, 1G8, and MYSV6, respectively) was added for signal detection.

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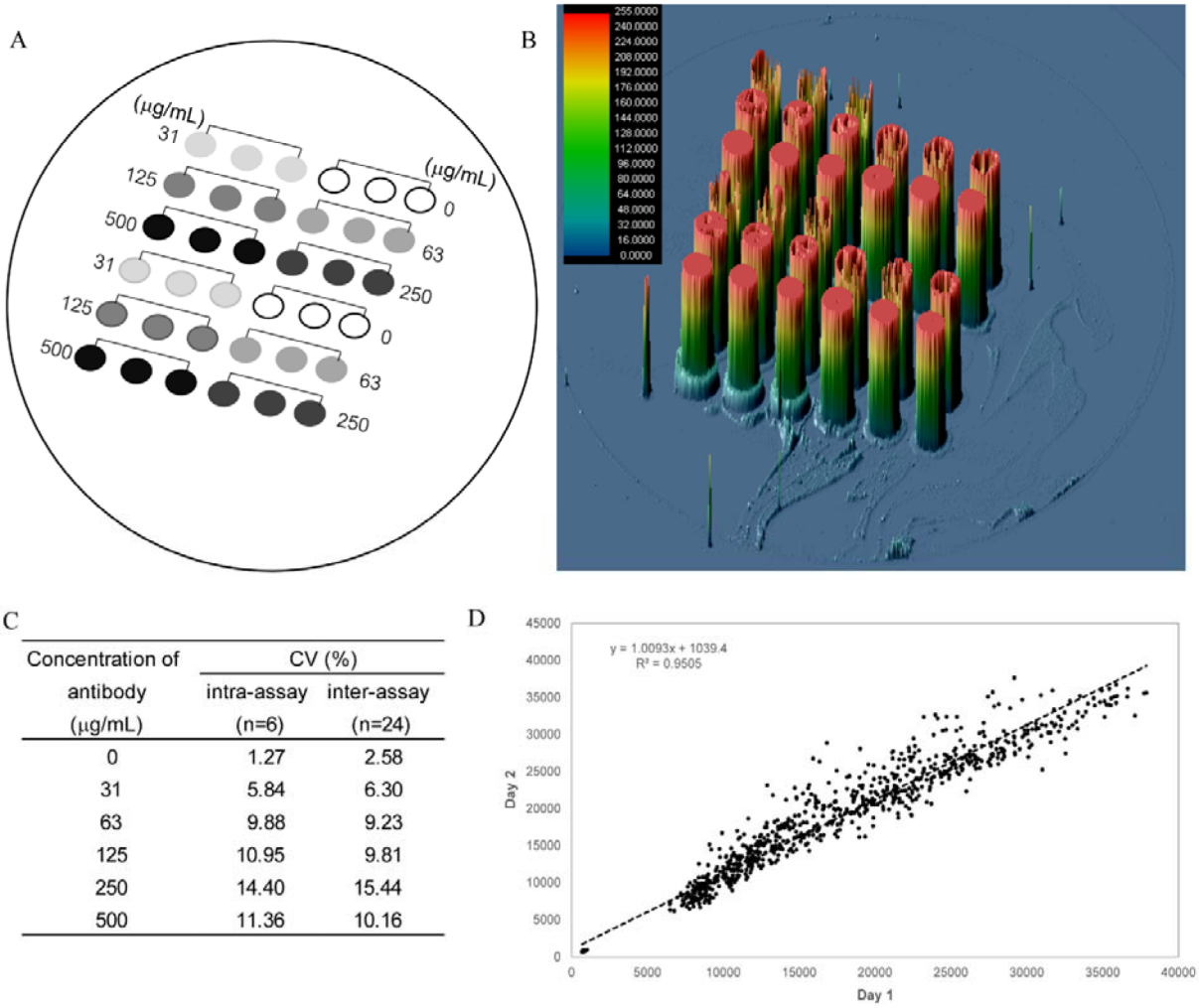

Concentration of antibody (μg/mL)	CV (%) intra-assay (n=6)	CV (%) inter-assay (n=24)
0	1.27	2.58
31	5.84	6.30
63	9.88	9.23
125	10.95	9.81
250	14.40	15.44
500	11.36	10.16

Figure 2. Reproducibility and stability of spotting and blocking buffers. Five concentrations (0, 31, 63, 125, 250, and 500 μg/mL) of capture antibody specific to *Acidovorax avenae* subsp. *citrulli* (Aac) (11E5 Ab) were spotted in three replicates for two sets as in diagram (A). The Cy5 labeled goat antimouse IgG (2 μg/mL) was used to detect the signal from the spotted capture antibody. Each spot was intensity plotted (0−255) and analyzed by Array Pro Analyzer software (B). The percentages of coefficient of variation (CV) were calculated for each concentration of capture antibody for intra-assay (n = 6, 6 spots) and interassay (n = 24 well, 144 spots) (C). The correlation of results from interassay (2 days, 6 concentrations of capture antibody, 864 spots for each day) was compared (D).

RESULTS AND DISCUSSION

To construct a multiplex detection of important plant pathogens using antibody array technology in a 96-well plate format, assay conditions were systematically optimized by considering several important factors to obtain high sensitivity and specificity of the multiplex detection.

Assay Optimization. *Types of 96-Well Plate.* In order to select the most suitable 96-well plate for antibody array fabrication, four types of plates, black-rim with nontreated coverglass based, black-rim with nontreated polystyrene based, clear-rim with treated polystyrene based, and treated polystyrene based, were tested by using *Acidovorax avenae* subsp. *citrulli* (Aac) as a model for detection. In Figure 1, it can be observed that all types of the plate gave a strong signal for both Aac in PBS and spiked in healthy watermelon leaf extract, low background of fluorescence, and low nonspecific binding with healthy watermelon leaf extract except the black-rim nontreated polystyrene based plate. The assay on the black-rim nontreated polystyrene based plate not only gave the lowest signal for Aac in PBS but also was not able to detect Aac in healthy watermelon leaf extract. The polystyrene based treated plates were exposed to γ radiation that assures sterility and enhances protein binding;[25,26] therefore, they provided a higher signal than nontreated plates. The coverglass based plate also showed good performance; however, these were not exposed to γ radiation but had a modified surface with CC^2 treatment which mimics the surface in a similar way to poly-D-lysine. In addition, the clear-rim treated polystyrene based plate gave a higher background than the black-rim type (PBS wells in Figure 1) due to the wall-to-wall interference. Finally, the black-rim treated polystyrene based plate was chosen for further work as these gave a high signal and low background and was cheaper than the similarly performing coverglass plates.

Spotting Buffers. To select a spotting buffer for acquiring high intensities of spots with minimal nonspecific binding and with good morphology, 14 buffers based on phosphate buffered saline (PBS), pH 7.4, and carbonate−bicarbonate buffer, pH 9.6, with the addition of various combinations of Tween 20, sucrose, and/or glycerol were tested. Figure S-1, Supporting Information, shows that all spotting buffers did not give rise to nonspecific binding. PBS without any supplements gave higher signal intensities than carbonate−bicarbonate buffer, and it also

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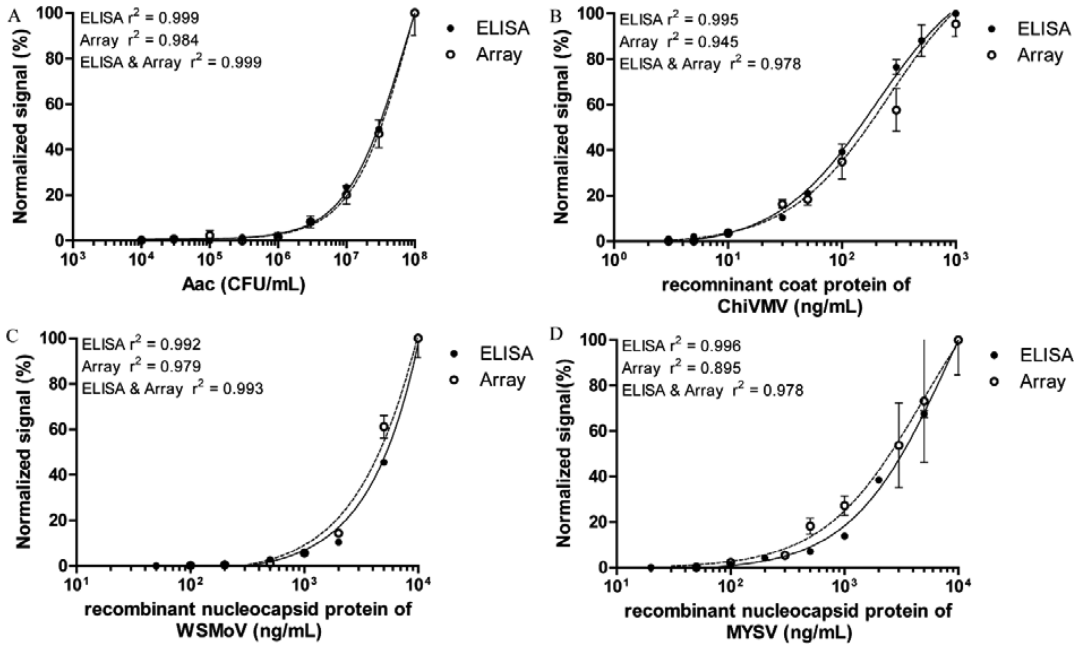

Figure 3. Comparison of the sensitivity of detection between the antibody array (circle and dashed line) and the ELISA method (black circle and solid line). The fluorescent signals obtained from detecting different concentrations of (A) *Acidovorax avenae* subsp. *citrulli* (Aac), (B) *Chilli veinal mottle virus* (ChiVMV), (C) *Watermelon silver mottle virus* (WSMoV), and (D) Melon yellow spot virus (MYSV). Data were normalized by taking minimum and maximum values as 0% and 100%, respectively. Each data point was plotted as a mean of six replicates for the antibody array and duplicates for ELISA ± standard deviation.

provided good spot morphology with a full circle spot when Tween 20 was added. The percentages of coefficient of variation (%CV) of signals from the examined spotting buffers showed different degrees of variation. In the case when Tween 20 was included, the reduced %CVs of 8.5−13.2% and 3.0−20.1% were obtained for phosphate buffer based and carbonate−bicarbonate buffer based, respectively (Figure S-1D, Supporting Information). The nonionic surfactants such as Tween help reduce surface tension and promote formation of a well-shaped microarray spot from a small volume of spotting solution which helps minimize intracoefficient of variation (CV) of spots. The use of surfactant was also advantageous for stealth pins or quill pins because it reduced the viscosity of the antibody solution. A spotting buffer with surfactant has previously been applied in cancer cell line microarrays for proteomic profiling purposes.[27]

Carbonate−bicarbonate buffers containing sucrose and glycerol gave higher intensities of spots than carbonate buffer without sucrose and glycerol at 125, 250, and 500 μg/mL of capture antibody. It has previously been demonstrated that sucrose helps in pocketing the binding site of antibodies after immobilization, resulting in the improvement of the antibody−antigen interaction[28] while glycerol should prevent the evaporation of a small volume of spots and help preserve the conformation of antibodies. However, when buffer containing glycerol was used for spotting in this study, the spot morphology was poor and high intra-CV was obtained (13.2−98.6%). The higher concentrations of glycerol (0.5%, 2.5%, 5%, and 25%) in both PBS and carbonate−bicarbonate buffer were also tested but no improvement was observed (data not shown). In contrast, increasing percentages of glycerol were shown to improve density, decrease intracoefficient of variation (CV), and protect drying artifacts of antibody spots on the epoxy-silane slide by using a noncontact printing system.[29]

The results for the combination of glycerol, Tween 20, and sucrose in both of the spotting buffers confirmed that glycerol caused an increase in intra-CV (13.2−81.7%) while Tween 20 gave the best spot morphology. Carbonate−bicarbonate buffer containing Tween 20 (0.05% v/v) and sucrose (1% w/v) gave the highest signal for all the concentrations of capture antibodies with low intra-CV and good spots morphology; therefore, it was selected and used for subsequent experiments.

Blocking Buffers. Three blocking buffers (1% gelatin, 2% BSA, and 3% skim milk in PBST) were compared for the ability to reduce nonspecific binding and background for the detection of pathogens in real plant extracts. A 1% gelatin solution gave the highest background for Aac detection in PBST and also did not provide a good distinguishing signal between the spots and background for the detection of Aac spiked in healthy watermelon extracts. Conversely, 2% BSA and 3% skim milk gave good results for Aac detection and provided distinct separation between the signal of spots and background in both PBST and healthy watermelon leaf extracts (Figure S-2A, Supporting Information). The intensities of spots were similar for both blocking buffers when comparing results for Aac spiked in healthy watermelon leaf extract (Figure S-2B, Supporting Information). To reduce costs of the final assay, 3% skim milk was selected for further work.

Reproducibility and Stability of Spotting and Blocking Buffers. To examine reproducibility and stability of the selected spotting and blocking buffers, different concentrations of capture antibody (11E5) were resuspended in a carbonate buffer with 0.05% Tween 20 and 1% sucrose as diagrammed in Figure 2A before a blocking buffer (3% skim milk in PBST) was added. The fluorescent (Cy5) labeled goat antimouse was subsequently added, and the obtained fluorescent signal was used for surface and spot florescent intensity analysis. The 3% skim milk in PBST seemed to be able to block nonspecific binding effectively as it gave a low background. The fluctuation of intensity was observed when 31 and 125 μg/mL of capture antibody were used whereas the higher concentrations of capture

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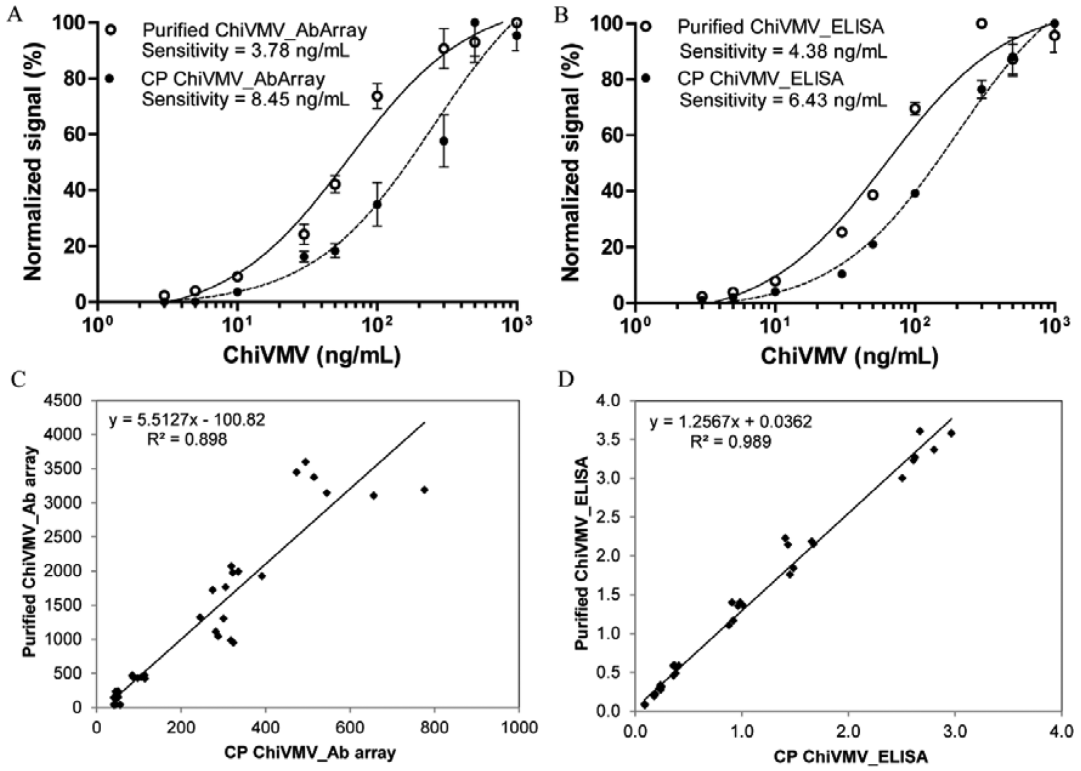

Figure 4. Comparison of coat protein (CP) and purified *Chilli veinal mottle virus* (ChiVMV) detection in the antibody array and ELISA. Normalized signals obtained from the detection of CP and purified ChiVMV in antibody array (A) and ELISA (B) were obtained by taking minimum and maximum signal values as 0% and 100%, respectively. Each data point was plotted as a mean of six replicates. The correlations between the signals from the detection of CP and ChiVMV (0−100 ng/mL) by antibody array (C) and ELISA (D) were scatter plotted and calculated by linear regression.

antibody between 250 and 500 μg/mL resulted in steady signals (Figure 2B). Although the lower concentrations of capture antibody (31−125 μg/mL) exhibited poorer unity of spot intensities, % CV of intensities was less than 10% in both intra-assay and interassay (Figure 2C). Furthermore, a good correlation of >95% was obtained between the results from assays performed on different days (Figure 2D). This quantitative analysis confirmed our results from spot morphology analysis that the carbonate buffer containing Tween and sucrose and the 3% skim milk were appropriate spotting buffer and blocking buffer, respectively, as they gave the most consistent results for the antibody array.

Comparison of the Sensitivity of Antibody Array and ELISA Method. The sensitivity of the antibody array was compared to a conventional ELISA method for the detection of the plant pathogens (Sandwich ELISA section). For the antibody array method, the LODs for the detection of Aac, ChiVMV, WSMoV, and MYSV were 5×10^5 CFU/mL and 30, 1000, and 160 ng/mL, respectively, whereas for an ELISA method they were 5×10^5 CFU/mL and 10, 800, and 230 ng/mL, respectively (Figure 3). The sensitivities of these two methods were of the same order of magnitude. The correlations between the two methods were calculated using Pearson analysis, and the results between 0.938 and 0.999 were obtained for all comparisons. Usually, the viral coat or nucleocapsid proteins have been used to represent actual virus in infected plant samples in the immunoassay development[30−33] because the plant virus can only survive when it resides in the host system which is unstable to use during the assay optimization. Therefore, the recombinant coat proteins of viruses have always been used in such studies rather than the actual viral particles. In our study, coat protein (CP) of ChiVMV, nucleocapsid protein (NP) of WSMoV, and NP of MYSV were used to represent ChiVMV, WSMoV, and MYSV, respectively. In order to correlate the amount of these coat proteins to the amount of the virus, it is important to know how many coat protein copies are in each virus. ChiVMV is member of the *Potyviridae* family which has 2000 copies of CP[34] with a size of 33 kDa.[18] WSMoV and MYSV are members of the *Bunyaviridae* family which have tripartite genomes (L, M, and S RNA segments). The L, M, and S RNA segments encode L proteins (transcriptase component), glycoprotein, and nucleocapsid protein (NP), respectively. In general, the NP of *Bunyaviridae* has 2100 copies per particle of virus[35] and the NPs of WSMoV and MYSV are about 31 kDa.[21,36] From the protein copy numbers and molecular weights of the coat proteins, the sensitivity obtained from our results can then be converted to 3×10^8, 8.3×10^9, and 1×10^9 viral particles/mL for ChiVMV, WSMoV, and MYSV, respectively. Although there has never been any immunological assay successfully developed for these three important plant viruses, the sensitivities obtained in our study are similar to the detection of Potato Y potyvirus (*Potyviridae* family) using the double antibody sandwich (DAS) ELISA whose sensitivity was 10 ng/mL of purified virus or 10^8 particles/mL.[37] In order to benchmark the antibody array's capability to the gold standard, the ELISA method was performed and the same sensitivity was obtained.

In our study, in order to demonstrate that the coat protein (CP) of virus can indeed be used as a representative of the real whole virus, CP and purified of ChiVMV were tested with the

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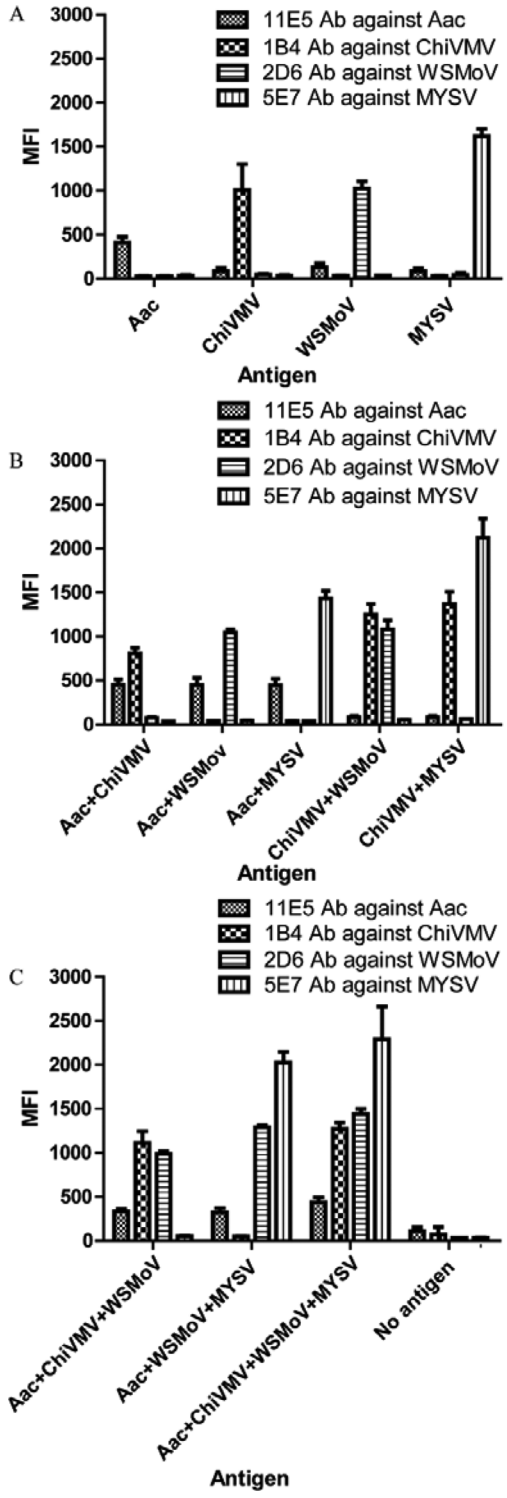

Figure 5. Multiplex detection by the antibody array in a 96-well plate using antibodies 11E5, 1B4, 2D6, and 5E7 for Aac, ChiVMV, WSMoV, and MYSV detection, respectively. The fluorescent signals obtained from detecting molecules are reported in terms of mean fluorescent intensity (MFI). The system shows (A) single detection, (B) duplex detection, and (C) multiplex detection and no added antigens. The *X*-axis is different combinations of antigens tested, and the *Y*-axis is the fluorescent signals. Each data point was plotted as a mean of six replicates ± standard deviation.

antibody array and ELISA methods (Figure 4). Figure 4A,B showed that the LODs were achieved at the same order of magnitude (in antibody array, 3.78 and 8.45 ng/mL for CP and purified virus, respectively; in ELISA, 4.38 and 6.43 ng/mL for CP and purified virus, respectively). Figure 4C,D showed good correlations of 90% and 99% between the CP and purified virus detection by the antibody array and ELISA, respectively. These results therefore confirm that the coat protein of virus (CP) can be used as a representative of the purified virus for the antibody array development.

Multiplex Detection. To investigate the possibility of multiplex detection for four plant pathogens using the antibody array, different combinations of single, duplex, triplex, and multiplex analytes were tested. In each well, four different antibodies were spotted in 6 replicates (11E5 for Aac, 1B4 for ChiVMV, 2D6 for WSMoV, and 5E7 for MYSV). For a single detection, the signal intensities at the expected positions of antibody against the tested antigens were obtained. For example, the positive signals from the 11E5 spots were obtained when only Aac was added into the system (Figure 5A). Moreover, the detection results of different combinations of two pathogens were accurate and similar to those obtained from the single detection. On the other hand, the signals for the virus protein spots were slightly higher in the duplex detections than in the single detection (Figure 5B). Furthermore, combinations of three and four pathogens were tested, and accurate results were obtained (Figure 5C). From these results, we have demonstrated that the developed antibody array was able to detect Aac, ChiVMV, WSMoV, and MYSY simultaneously in one single well.

■ CONCLUSION

The optimization of numerous factors such as types of plates, spotting buffers, and blocking buffers for antibody array fabrication in the 96-well plate was successfully completed. The potential of the antibody array in a 96-well plate format was clearly demonstrated by detecting the four important plant pathogens selected for inclusion in the study. This work will help increase the screening capacity especially for seed and plant industries that requires large testing volumes for export and cultivation purposes. Not only can the antibody array in a plate format reduce the cost of testing, but also the nature of the 96-well plate will allow easy automatization of the assay for high-throughput screening.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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