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Study on rapid detection of seven common foodborne pathogens by gene chip

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To develop a rapid, effective, specific, and sensitive method to detect foodborne pathogens, 13 sets of primers were designed to amplify the conservative and specific genes of *rfbE, fliC, invA, hilA, ipaH, femA, nuc, hlyA, prfA, tuf, speB, tlh* and *tdh*, respectively. Establishment of foodborne pathogens detection chips was conducted by spotting the target genes on the chips by Nano-PlotterTM NP 1.2 printing system. The DNA of 7 standard pathogenic strains and 147 strains extracts from food samples was amplified and labeled for hybridization. The results demonstrated that *enterhemorrhagic Escherichia coli O157:H7, Salmonella enteritidis, Shigella flexner, Staphylococcus aureus, Listeria monocytogenes, β-hemolytic streptococcus,* and *Vibrio parahaemolyticus* could correctly be identified by the designed gene chip at an optimal temperature of 58°C and were proved as a potential method with good stability and sensitivity (5 pg/µl of template DNA).

Key words: Gene chip, food-borne pathogen, virulence gene, detection.

INTRODUCTION

It is well known that food is the basis of human beings. In recent years, hundreds of outbreaks of foodborne infection cases occur in the world (Keener et al., 2014; Scallan et al., 2015; Centers for Disease Control and Prevention, 2013) and the species of bacteria causing foodborne infections have continuously become more diversified (Van Doren et al., 2013; Crim et al., 2015; Korsak et al., 2015), resulting in serious harmfulness to human's health. Generally, the food-borne pathogens are some micro-organism from food-processing and transporting, which usually are the main murderers making human's diseases. Therefore, analyzing pathogenic bacteria in food is a standard practice to ensure safety and quality of the food. Presently, there are

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many methods, such as culture-medium enriching, culturing, isolating, and biochemical identification available to detect foodborne pathogens (Feng et al., 2013; Wang et al., 2014, 2012). However, these methods are as long as 3-to-7-day duration, laborious, and fewer species detected. Compared with the above methods, though polymerase chain reaction (PCR) is rather rapid, its disadvantages are that only one strain and gene can be detected at a batch sample, which greatly lowers the working effectiveness and prevents the feasibility of detecting high-flux samples. Further studies are needed to develop rapid and objective methods for foodborne pathogen detection. In this study, the multi-gene methods have been successfully applied for the simultaneous detection of common foodborne pathogens in real food samples. The design of primers, the amplification of PCR, and the formation of gene chip matrix of probes are based on the steady virulence gene or non-virulence gene in the *enterohemorrhagic Escherichia coli (EHEC) O157:H7, Salmonella enteritis, Shigella, Staphylococcus aureus, Listeria monocytogenes, β-hemolytic streptococcus*, and *Vibrio parahaemolyticus.* Our methods are highly specific, sensitive, time-saving, and effective in the simultaneous detection of foodborne pathogens. The established methods have shown satisfactory results applied to detect the 7 standard strains of foodborne pathogens and 147 isolation strains from food samples.

MATERIALS AND METHODS

Standard strains and food isolation strains

Seven standard strains

EHEC O157:H7 (CCTCCAB200051), *S. enteritis* (CCTCCAB94018), *S. aureus* (CCTCCAB94006), and *L. monocytogenes* (CCTCCAB97021) were purchased from Chinese Presentative Culture Preservation Center (Beijing, China). *Shigella* (51571-10) and *β-hemolytic streptococcus* (32210-18) were from Chinese Medicine Bacteria Center of Microscobial Preservation Committee (Beijing, China). *V. parahaemolyticus* (VPL4-90) was provided by Guangzhou Microbiological Institute, Chinese Academy of Sciences (Guangzhou, China).

Seven food isolation strains

Each isolation strain is composed of 21 strains, including 5 negative and 16 positive ones, and all were granted by the Food Safety Laboratory of Technology Center, Zhuhai Entry-Exit Inspection, and Quarantine Bureau (Zhuhai, China).

Reagents

TaKaRa *Ex* Taq (5 U/μl), DL2000 Ladder Marker, λDNA, dATP, dGTP, dCTP, dTTP, and TaKaRa Spaced Cover Glass, Code No.: TX702, TX703, Lot: TAGA0508, GB010920 were from Baosheng Biological Engineering Co., Ltd. (Dalian, China). TIANamp Bacteria DNA Kit (Lot: DP302) was provided by Tiangen Biotech (Beijing) Co., Ltd. Omega Bio-tek E.Z.N.A^{TM} Gel Extraction Kit, Lot: D050520 was purchased from Qikete Company (Guangzhou, China) and stored at room temperature. Baio® amido-slide, 2×spotting buffer solution, pre-hybridization and hybridization buffer solution were from Shanghai Baiao High-tech Co. Ltd. (Shanghai, China). Cy3-dCTP: 25 nmol, PA53021, Lot: 334872 was purchased from Amersham Biosciences (UK). Cleaning solution 1: 0.1% SDS 2×SSC; cleaning solution 2: 0.1% SDS 0.2×SSC; cleaning solution 3: 0.2×SSC.

Design of the primers

The good conservative domains were picked by DNAMAN software from the conservative and specific genes of seven types of foodborne bacteria. The various primers (Shanghai Yingjun Co. Ltd., China) were also designed by Array Designer 2.0 software (Primer Biosoft International, CA).

Design of aligned genes (positive references)

The aligned gene was picked up from one specific DNA gene existing in the lambda bacteriophage, a positive reference, used as quality control (QC) in the experimental program. The results will not be reliable unless the aligned gene was found. The designs of prime and the composition of aligned genes are identical to the aforementioned workflow.

Extraction and preparation of the target and the aligned genes in samples

The extraction and preparation of DNA sample template was processed based on the User's Manual from TIANamp Bacteria DNA Kit. The amplification was processed by using PCR gradient instrument and then the production was recovered by the gel-cut after electrophoresis (the annealing temperature of the primers shown in Table 1. The cut gel was amplified as a template after ten-fold dilution and the purification was processed with isopropyl alcohol precipitation method. The amplification gene was sequenced by Shanghai Yingjun Company and Shanghai Biological Engineering Company (Shanghai, China), from which the obtained data was analyzed by Blast search and DNAMAN software.

Preparation of the gene chip of foodborne pathogens

The concentrations of target gene and aligned gene were diluted to 250 to 300 ng/μl prior to spotting on the amido slide by spotting instrument. Three sets of comparative experiments were designed as follows: positive group (aligned gene), negative group (3×SSC), and blank group (Milli-Q water), among which each sample had ten same positions and each slide repeated two arrays. Prior to being fixed at 65°C for an hour in an oven, the prepared samples should first be dried overnight; next hydrated and crosslinked by UV; finally, centrifugated after washing by 0.2% SDS solution and stored at room temperature.

Preparation of the probes and detection of concentrations

Probes were prepared by enzymatic reaction notation and the notated dCTP was taken to detect the target gene and the aligned gene. In total 50 μl solution, where DNA template shared 1 μl, 10×*Ex* Taq Buffer 5 μl, dATP, dGTP, dTTP Mixture (2.5 mmol/L, respectively) 4 μl, 10 mmol/L dCTP 0.5 μl, 1 mmol/L Cy3-dCTP 0.5 μl, each primer 1 μl, and *Ex* Taq (5 U/μl) 0.5 μl. The optimized

Table 1. Related information on the primers of target gene.

Target gene	Primer sequence(5'->3')	Fragment sizes of the target gene (bp)	Tm (°C)
O157:H7 rfbE gene	AAGATTGCGCTGAAGCCTTTG CATTGGCATCGTGTGGACAG	497	52
O157:H7 fliC gene	ATTCAGCAGGTAATATCAC TATCATCCACATAAGACTTC	390	52
Salmonella enteritis invA gene	TCCATTACCTACCTATCTG GGCATCAATACTCATCTG	382	50
Salmonella enteritis hilA gene	TAATCCTGTTCCTGTATCG GAAGTATCGCCAATGTATG	371	50
Shigella ipaH gene	AATTCTGGAGGACATTGC TCATACTTCTGCTCTTCTG	303	50
Staphylococcus aureus femA gene	AGCACATAACAAGCGAGATAAC CCAGCATCTTCAGCATCTTC	300	50
Staphylococcus aureus nuc gene	TGGCGTAAATAGAAGTGGTT GCTTGTGCTTCACTTTTTCT	438	50
Listeria monocytogenes hlyA gene	AACCTACAAGACCTTCCAG CGTATCCTCCAGAGTGATC	498	50
Listeria monocytogenes prfA gene	ATACACGATAACTTTCTCTTGC GAACAGGCTACCGCATAC	336	50
β-hemolytic streptococcus tuf gene	TTCCAGTTATCCAAGGTTC CGGTAGTTGTTGAAGAATG	484	50
β-hemolytic streptococcus speB gene	TAGACAATACAACTGGAACAAC GTCAAGACGGAAGAAGCC	400	50
Vibrio parahaemolyticus tlh gene	TACGCTTGAGTTTGGTTTG GGTGAGTTGCTGTTGTTG	476	50
Vibrio parahaemolyticus tdh gene	CCATCTGTCCCTTTTCCTGC CCACTACCACTCTCATATGC	426	50
λbacteriophage DNA gene as aligned gene	AAAGCGACGCAATGAGGCACT GTTCCACGACCGCAACTGC	500	54

concentration of probes was 3000 pg/μl (Takara Biotechnology (Dalian, China) Co. Ltd., 2014-2015).

Hybridization, scanning and data analysis for gene chip

Gene chip was hybridized with the introduced probes at 58°C for 10n to 18 h after pre-hybridization at 50°C for an hour. After hybridization, the chip was in turn washed by cleaning solution 1, 2, 3, and Milli-Q water, prior to being centrifuged and dried, respectively. The gene chip signal was obtained by a 532-nm excitation light source with 100% laser power, 600 Photo Multiplier Tube (PMT) Gain, and 10-μm resolution. The final information was taken using GenePix Pro Ver. 4.1 software, both mean values of ten-point signals from same target gene and ten-sample signal-to-noise ratio (SNR) were used to characterize the signal value of hybridization for each target gene.

Optimized temperature of hybridization of gene chip

Different tagged probes and prepared chip were hybridized at 52, 56, 58, and 60°C, respectively. The optimized temperature was obtained by analyzing specificity of hybridization of gene chip at different temperature.

Testing of specificity and sensitivity

The testing of specificity has been done based on seven probes of standard foodborned pathogens and detection chip hybridized at an optimized temperature, respectively. The results have shown that there are not distinct interferences in difficult matrix samples. Additionally, DNA template purified by *Shigella* was diluted to 3000, 300, 30, 10, 5, and 1 pg/μl and then hybridized with relevant detection chip before getting data and verifying sensitivity of chip by fluorescence scanner.

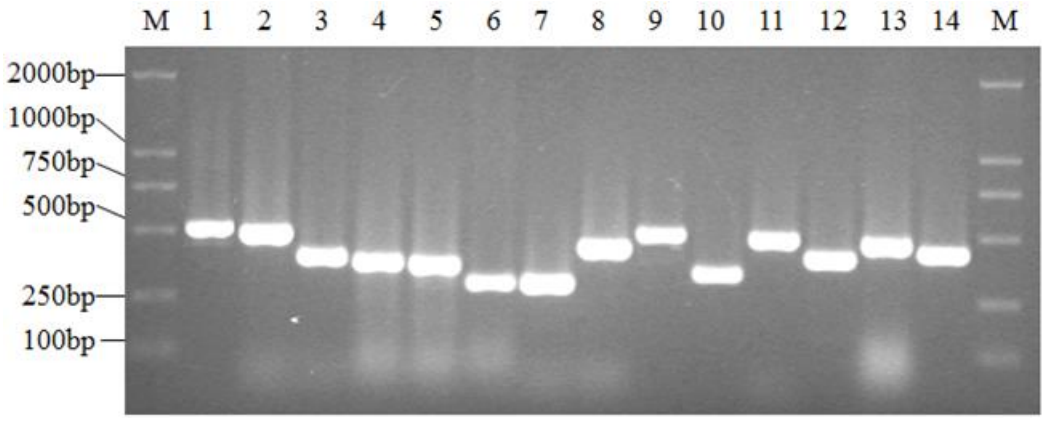

Figure 1. PCR amplification electrophoresis of target genes and aligned genes of seven foodborne pathogens. M: DL2000 Ladder Marker; 1: TλDNA; 2: TrfbE; 3: TfliC; 4: TinvA; 5: ThilA; 6: TipaH; 7: TfemA; 8: Tnuc; 9: ThlyA; 10: TprfA; 11:Ttuf; 12: TspeB; 13: Ttlh; 14: Ttdh.

Testing of isolated food strains

The data was obtained by scanning after the isolated food strains were amplified to notate by relevant primers and hybridized with the detection chips, successively.

RESULTS

Design of primers, sequence comparison of fragment and analysis of homology

Fourteen pairs of primers were made among which *rfbE, tdh,* and *λDNA* were candidates and the remaining was designed by array designer (design software of gene-chip primer). As shown in Table 1, the target gene fragments with other genus or species strains have more than 88% sequence homologies except *tdh* gene. It approved that the designed target gene fragments have shown good conservation and are irrelative with homology with nucleoside sequence of other genus and species. Moreover, Less than 43% ratio of homologies was produced between 13 sets of target genes fragments and aligned genes, greatly preventing gene-gene from being hybridized, which theoretically guaranteed the good specificity of detecting chip. Moreover, double gene combined detection supplied to each foodborne pathogen will greatly lower the probability of false positive and improve the specificity in the difficult matrix samples.

Testing of amplification effect and specificity of target gene and aligned gene

In this research, thirteen target genes and one aligned gene were successfully amplified from seven types of foodborne pathogens and *λbacteriophage* DNA gene, respectively. According to the data obtained by nucleic acid protein spectrophotometer, the concentrations and the purities (A260:A280) of genes were 449.3 to 1917.0 ng/μL and 1.70 to 1.87, respectively. Figure 1 shows that the strips of targeted genes and aligned genes are not distinct interferences and tailing found, meaning that the methods may be reasonable for the successful application of the detection.

Preparation of probes and detection of concentration

Probes were prepared by using enzymatic reaction notation. The experimental data shows that the values of A260:A280 were 1.70 to 1.96 and the concentrations were 307.2 to 846.3 ng/μl, consistent with the experimental requirements (Figure 2).

Experimental results of target gene at different temperature

The gene chip was hybridized at 52, 56, 58 and 60°C, respectively. The experimental data showed that better results can be obtained at 56, and 58°C, where each target gene had a strong signal of hybridization with relevant one on the detection chip and visible interference signals were ‘hidden’. Taking into account the signal intensity and stability, 58°C was employed as the optimized temperature.

Experimental results of hybridization of seven pathogens

The tagging probes can well hybridize with the detection chips (Figure 3a to g). The results showed that more than both 500 strong signals and 4.0 SNR were obtained and invisible interference signal appeared. These proved that the prepared chips had good specificity and did not hybridize with irrelevant genes.

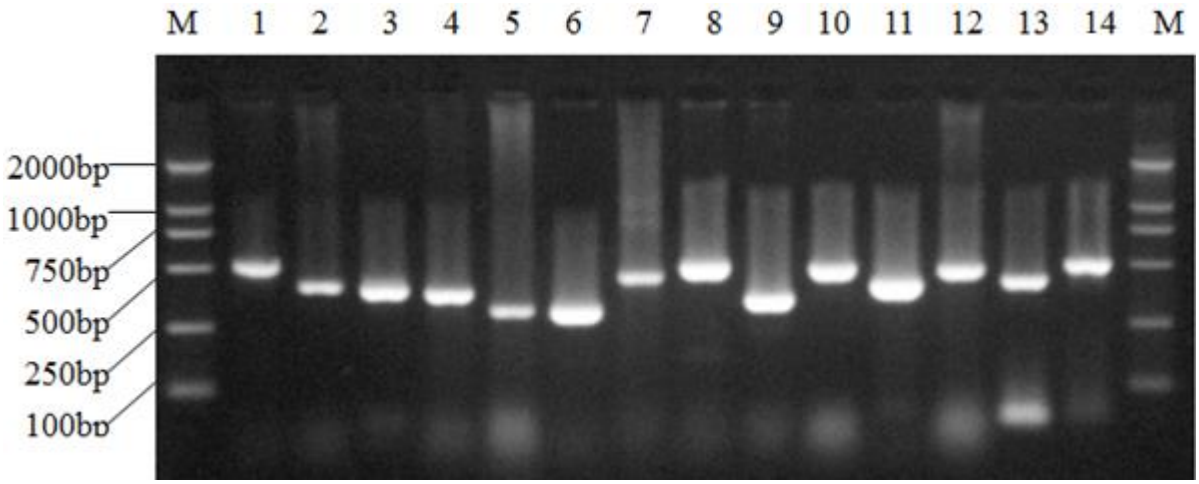

Figure 2. PCR amplification electrophoresis of the probes. M: DL2000 Ladder Marker; 1: PbrfbE; 2: PbfliC; 3: PbinvA; 4: PbhilA; 5: PbipaH; 6: PbfemA; 7: Pbnuc; 8: PbhlyA; 9: PbprfA; 10: Pbtuf; 11: PbspeB; 12: Pbtlh; 13: Pbtdh; 14: PbλDNA.

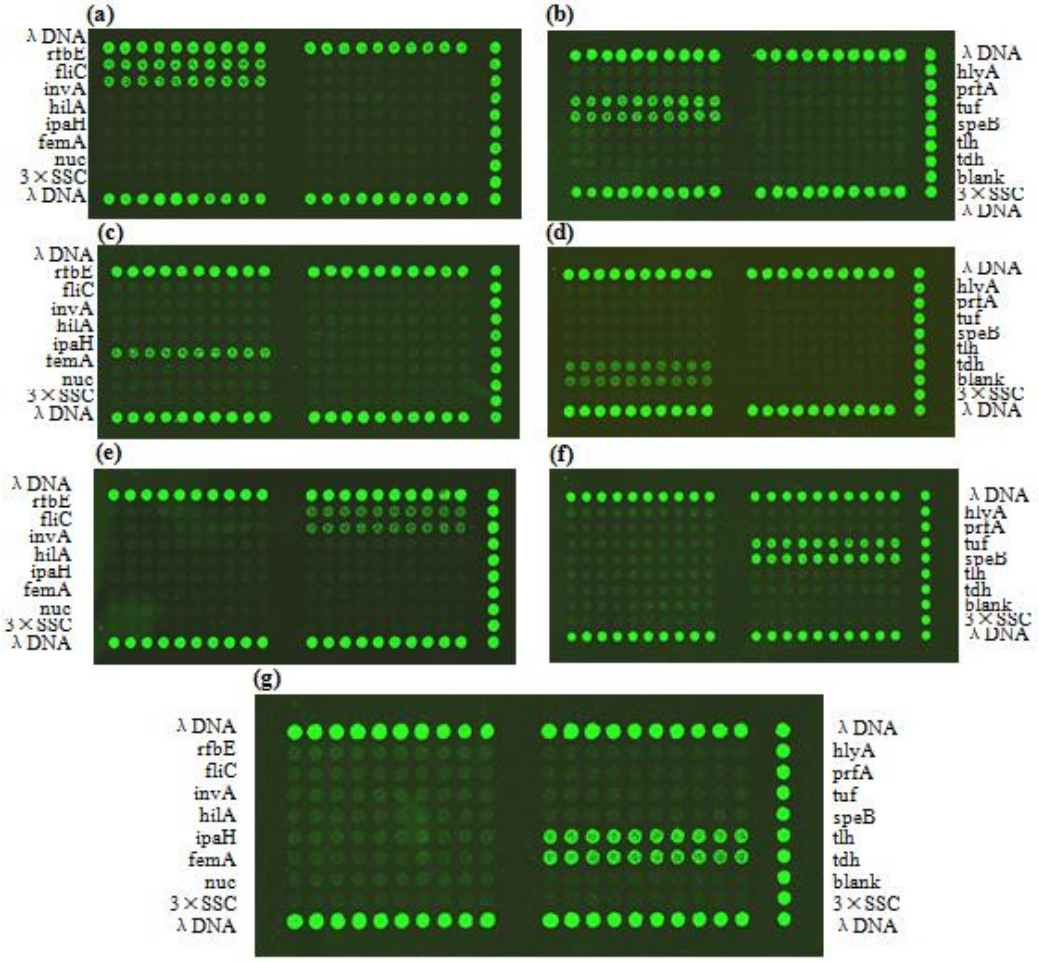

Figure 3. Fluorescence images of hybridization of seven food-borne pathogens taken by nucleic acid protein spectrophotometer: **(a)** *O157:H7* hybridization; **(b)** *Salmonella enteritis* hybridization; **(c)** *Shigella* hybridization; **(d)** *Staphylococcus aureus* hybridization; **(e)** *Listeria monocytogene* hybridization; **(f)** *β-hemolytic streptococcus* hybridization; **(g)** *Vibrio parahaemolyticus* hybridization.

Sensitivity of gene-chip detection for *Shigella*

When the concentrations of template DNA varied from 5 to 3000 pg/µl, SNR was 1.5 above and all signal intensities of hybridization were more than 150, with distinct hybridization spots. Notably, when the concentration was down to 1 pg/µl, SNR was 0.40 and the signal intensities of hybridization were less than 100, without visible signals that appeared. These indicated that 5 pg/µl was the sensitivity of detection in this study.

Specificity, repeatability and sensitivity of the isolation strains for food samples

Using the detecting chips of gene, the detecting results of 147 food isolation strains of seven foodborne pathogens have shown that the probes from 16 positive strains and the targeted genes on the chips had strong hybridization signal, meaning good repeatability; at the same time, there are no distinct interferences between positive strains and targeted genes of other strains, also five negative strains did not show any hybridization signal, bringing high specificity.

In difficult matrix samples, the detecting results of 147 food isolation strains displayed that the limit of detection can be 5 pg/µl.

Analytical applications

Each probe appeared strong signal of hybridization with relevant target gene but did not display similar phenomenon with irrelevant ones. High sensitivity of detection and good specificity had proved that our methods can well be applied to rapid and simultaneous detection of foodborne pathogens in real food samples.

DISCUSSION

Design of the target gene chip

The selection of the target genes will greatly affect the detection chip, so the target genes must be designed in accordance with conservative and specificity in its genus or species. Using the homology analysis of the detection genes, it can be clearly know whether target gene may be detected. Girke et al. (2000) have reported that the hybridization of crossing can be processed in the case of more than 70 to 80% homologies of gene sequences, whereas the hybridization will least or even do not appear under 75% homologies of sequences (Schena, 2003). In this study, the homology between 13 target genes and 1 aligned gene is less than 43%, indicating a good specificity. The homologies of the fragments are more than 88% in the remaining target genes and different genus or species except 70% homology in *tdh*, displaying a satisfactory conservative. The remaining bacteria were simultaneously detected by bi-genes except *Shigella*, thus greatly lowering the ratio of false positive and improving the specificity of detection.

UV-crosslinked and hydration time

To obtain a uniform distribution after inoculation, DNA must be re-hydrated and dried quickly. It is worthy of mentioning that excessively low water temperature and insufficient time will greatly suffer irregular sampling sites which affect the following hybridization and data analysis. By contrast, the sampling sites may be rapidly enlarged under excessively high water temperature and time and thus lead to the pollution of blending of the sampling sites. Generally, the optimized temperature and time is 70 to 80°C and 10 s, respectively. In addition, the chip should be crosslinked from a 10-cm vertical distance by a 15-mJ UV irradiation, which will successfully form a crosslink bond between a fraction of thymine residues in DNA and amino groups on the surface of slide, thus greatly improving the fixed effect. Noticeably, DNA will severely be destroyed for excessive crosslinking time, 20 min were taken as the optimization time.

Treatment parameters of images and choice of signal output mode

The images were scanned to produce the exactly aligned sites of gridding by GenePix Pro Ver.4.1 chip data analysis software and then automatically analyze signal median value, mean value, SNR and standard deviation in each sampling site. Among the mentioned data, the median value is widely used (He and Zhou, 2008; Sarder et al., 2008; Wentzell and Karakach, 2005) owing to its less sensitivity with fragments and dust pollution (Wentzell and Karakach, 2005). In this work, compared with mean value, one is that the median value can better embody hybridization signal in the case of pollution or high background signal; the other is that SNR is relative to signal and background and may well evaluate reliability of the obtained data, bigger SNR value with stronger sampling site signal and weaker background signal. Based on the discussion, median value and SNR were used to characterize the hybridization results.

Detection standard of positive signal

So far, there has still not been a standard model for gene chip adjustment. Al-Khaldi et al. (2004) have reported that the adjustment standard is to contrast the fluorescence signals in the sampling sites and the probe sites. Murray et al. (2001) have suggested that 55% pixels in the

sampling sites are above 1.5-fold local background and the intensities of signals are more than the standard deviation of 2-fold mean value of background signal (Murray et al., 2001). Until now, it is an accepted-widely method to find a proper threshold such as background mean value plus 2-fold variance or negative contrast mean signal plus 2-fold variance, in which the value of less than threshold should directly be ignored and the remaining should be kept for further data analysis. It was considered that the reliable adjustment standard of positive signals is based on more than 1.5 SNR, visible hybridization sampling sites in the scanned images and more than 150 median value of fluorescence intensity in the 10 repeated sampling sites of target genes.

Conflict of interests

The authors have not declared any conflict of interests.

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