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Research Article

Detection of *Cronobacter sakazakii* in Powdered Infant Formula using Real-time Fluorescence Single Primer Isothermal Amplification

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Abstract: Specific primers and blockers were designed based on *Cronobacter sakazakii* gene (16SrRNA) and Real-time Fluorescence Single Primer Isothermal Amplification (RF-SPIA) was established to detect *C. sakazakii*. This method identified that *C. sakazakii* genes were positive, whereas other non-*C. sakazakii* genes were negative. The sensitivity of this test for *C. sakazakii* pure culture reached 1.4×10^{0}CFU/mL and that for *C. sakazakii* genome reached 7.5×10^{1}fg/mL. The detection limit of RF-SPIA in milk powder artificially contaminated with *C. sakazakii* could reach 8.1×10^{1}CFU/mL. These results demonstrate that the RF-SPIA detection method for *C. Sakazakii* is highly sensitive, strong, specific and convenient.

Keywords: *Cronobacter sakazakii*, 16SrRNA, detection, Real-time fluorescence single primer isothermal amplification

INTRODUCTION

In 2002, the International Commission on Microbiological Specifications for Foods has listed *Cronobacter sakazakii* as a serious hazard, life threatening, or substantial chronic sequelae for specific populations (Lu *et al*., 2011). *C. sakazakii* is the wide spread bacteria that exists in contaminated foods and food factory, as well as in almost all environments (Leclercq *et al*., 2002). *C. sakazakii* can cause infection in all age groups; however, infants are mainly at high risk. Infant mortality rate from *C. sakazakii* infection is as high as 20 to 50%. Infant survivors of *C. sakazakii* infection may have serious neurological sequelae (FAO/WHO, 2004; Arvanitoyannis, 2010; Liu, 2004). *C. sakazakii* infects this population mainly through infant formula. Therefore, establishing a reliable, rapid, efficient and sensitive method for detection of *C. sakazakii* in infant formula is crucial.

The traditional detection methods of *C. Sakazakii* require a long period of time to complete and the operation is cumbersome. Serum detection is simple and affordable, but this method easily cross-reacts. Thus, this method cannot be independently used (Wang, 2014). Real-time quantitative PCR can be simultaneously performed and the reaction system is obtained by detecting the fluorescence signal strength. Its detection sensitivity is much higher than the conventional PCR; however, the method is entirely dependent on specific primers and probes are expensive. Thus, the method is not conducive to the promotion at the grassroots level. Furthermore, these methods fail to meet the demand for rapid detection (XiuJuan *et al*., 2010).

Single Primer Isothermal Amplification (SPIA) is a new type of linear isothermal nucleic acid amplification technique (Kurn *et al*., 2005). This technology uses a mixed primer with 3′ end of DNA and 5′ end of RNA, as well asendoribo nuclease (RNaseH) and strong strand displacement activity of DNA polymerase, to achieve a linear isothermal DNA amplification in vitro. In the amplification reaction, RNaseH constantly degrades the RNA portion of the DNA/RNA hybrid chain formed from primer and template DNA for the unbound primer to continue to obtain the binding site. Furthermore, RNaseHbinds with the template, undergoes strand-displacement synthesis and then terminates at the end of the template chain or the junction of polynucleotide chain termination (blocker), eventually amplifying numerous single-stranded cDNA with high fidelity (Kurn *et al*., 2005). RF-SPIA adds a corresponding proportion of the fluorescent dye (SYBR Green II) in the reaction system of SPIA. The cDNA products increase with trials and the fluorescence signal constantly enhances. The real-time fluorescence monitoring device can be used to monitor throughout

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Table 1: Strains used in this study

Serial number	Strain number	Strains of Latin name	Source
1	ATCC 51329	*Cronobactersakazakii*	China's general microbiology culture collection center
2	ATCC 51024	*C.sakazakiii*	China's general microbiology culture collection center
3	N1	*C.sakazakiii*	stored in the laboratory
4	N2	*C.sakazakiii*	stored in the laboratory
5	N3	*C.sakazakiii*	stored in the laboratory
6	N4	*C.sakazakiii*	stored in the laboratory
7	N5	*C.sakazakiii*	stored in the laboratory
8	N6	*C.sakazakiii*	stored in the laboratory
9	N7	*C.sakazakiii*	stored in the laboratory
10	R2	*C.sakazakiii*	stored in the laboratory
11	R3	*C.sakazakiii*	stored in the laboratory
12	R4	*C.sakazakiii*	stored in the laboratory
13	R5	*C.sakazakiii*	stored in the laboratory
14	R6	*C.sakazakiii*	stored in the laboratory
15	R7	*C.sakazakiii*	stored in the laboratory
16	O157	*Escherichia coli EHEC O157:Hund*	stored in the laboratory
17	CICC 10411	*Diagrrheagenic Escherichia coli*	China's industrial microbial preservation management center
18	ATCC 25922	*Escherichia coli*	China's general microbiology culture collection center
19	CMCC 44103	*Escherichia coli*	The Chinese medicine bacteria preservation management center
20	CICC 21495	*Salmonella paratyphi-B*	China's industrial microbial preservation management center
21	CMCC 50115	*Salmonella typhimurium*	The Chinese medicine bacteria preservation management center
22	CMCC 50041	*Salmonella enteritis*	The Chinese medicine bacteria preservation management center
23	Jia	*Salmonella*	stored in the laboratory
24	CMCC 50001	*Salmonella schottmuelleri*	The Chinese medicine bacteria preservation management center
25	CMCC 51522	*Shigellaboydii*	The Chinese medicine bacteria preservation management center
26	CMCC 51334	*Shigellasonnei*	The Chinese medicine bacteria preservation management center
27	CICC 21519	*Klebsiellapneumoniae*	China's industrial microbial preservation management center
28	CICC 21669	*Yersiniaenterocolitica*	China's industrial microbial preservation management center
29	ATCC 17802	*Vibrio parahaemolyticus*	China's general microbiology culture collection center
30	CMCC 49027	*Proteusbacillus vulgaris*	The Chinese medicine bacteria preservation management center
31	CICC 21636	*Pseudomonas aeruginosa*	China's industrial microbial preservation management center
32	CICC 22945	*Bacillus thuringiensis*	China's industrial microbial preservation management center
33	CMCC 63302	*Bacillus cereus*	The Chinese medicine bacteria preservation management center
34	CMCC 54001	*Listeria monocytogenes*	The Chinese medicine bacteria preservation management center
35	CICC 21670	*Listeria grayi*	China's industrial microbial preservation management center
36	CICC 21663	*Listeria ivanovii*	China's industrial microbial preservation management center
37	CICC 21672	*Listeria welshimeri*	China's industrial microbial preservation management center
38	CMCC 26073	*Staphylococcus aureus*	The Chinese medicine bacteria preservation management center
39	CMCC 26003	*Staphylococcus aureus*	The Chinese medicine bacteria preservation management center
40	ATCC 43300	*Staphylococcus aureus*	China's general microbiology culture collection center
41	Ji Kong	*Staphylococcus aureus*	stored in the laboratory
42	CICC 23664	*Staphylococcus epidermidis*	China's industrial microbial preservation management center
43	CICC 20237	*Staphylococcus xylosus*	China's industrial microbial preservation management center
44	CICC 22943	*Staphylococcus xylosus*	China's industrial microbial preservation management center

the SPIA and the obtained results are converted into data or charts to reflect in the computer. The basic principle is similar to real-time fluorescent ring-mediated isothermal amplification (Lucchi *et al*., 2010). The experimental results can be visually observed.

This study uses the RF-SPIA technology principle, in which specific primers and blockers were designed based on 16SrRNA gene of *C. sakazakii*, through optimization of various reaction conditions and systems. RF-SPIA method was established to detect *C. sakazakii* in infant formula milk powder. This method is rapid and sensitive for *C. sakazakii* detection.

MATERIALS AND METHODS

Materials:

Bacterial strains: The bacterial strains used in this study are listed in Table 1.

Instrument and equipment: The instruments and equipment used were ESE Quant tube scanner (ESEGmbh, Stockach, Germany), PCR instrument (Biometra, Germany) and 752 UV-vis spectrophotometer (Shanghai Instrument Co., Ltd.).

Samples and biochemical reagents: The following biochemical reagents were employed:

- Infant formula (supermarket in the South of Baoding, Hebei Province)
- RF-SPIA reagents: 25mmol/L $MgSO_4$,10mmol/L dNTPs, 2× Bca DNA polymerase buffer, 5× RNaseH buffer, 40 U/μL recombinant RNase inhibitor, 20 mg/mL bovine serum albumin, 20U/μLBca DNA polymerase, 60U/μLRNaseH, RNase-free water (Dalian Takara Biotechnology) and SYBR Green II(Beijing Trans Gen Biotech)

Table 2: Primer and blocker of RF-SPIA

Primer name	Sequence(5′→3′)
Primer Y1	CGCCACCUGCTGGTAATG
Blocker B1	AAACTCGCAGCACGAAGA

- Synthesized primers and blockers (Takara Biotechnology)
- Bacterial genome DNA extraction kit (Takara MiniBEST Bacteria Genomic DNA Extraction Kit Ver.3.0; Takara Biotechnology)
- Sodium chloride, anhydrous ethanol and other domestic analyses of pure products (Beijing Bridge Company).

Method:

Extraction of bacterial DNA template: One ring from *C. sakazakii* was selected by using a vaccination ring and inoculated in fresh sterile nutrient broth medium at 37°C overnight. Common thermal cracking and Kitmethods were respectively used to extract *C. sakazakii* genome DNA as the template.

RF-SPIA reaction primer design: According to related sequences in GenBank, the highly conserved sequence of *C. sakazakii* 16SrRNA gene was analyzed using the DNAMAN software to determine the target sequence. The specificity of the primers and RF-SPIA blockers was evaluated based on *C. sakazakii* in GenBank 16SrRNA gene sequence (HQ880288.1) through Primer Premier 5.0 and DNAMAN. Table 2 shows the detailed sequences.

RF-SPIA response system and major procedures:

RF-SPIA reaction system: The real-time fluorescence RF-SPIA reaction system was consisted of 25mmol/L $MgSO_4$, 0.5μL; 10mmol/L dNTPs,1.5μL; 10μmol/L primer, 1μL; 2× Bca DNA polymerase buffer, 2.5μL; 5× RNaseH buffer, 2.5μL; 40U/μL recombinant RNase inhibitor, 0.5μL; 20mg/mLbovine serum albumin, 0.5μL; 10μmol/L blocker, 1.5μL; 20U/μLBca DNA polymerase, 0.5μL; 60U/μLRNaseH, 0.5μL; Template, 1.0 μL; SYBR Green II, 0.5μL; and RNase-free water.

Main operating procedures of RF-SPIA reactions: The initial denaturation of RF-SPIA was at 95°C for 1 min 20s. RF-SPIA was then reacted at 61°C for 120 min. The reaction can be stopped at any time point according to the fluorescence curve.

Optimization condition of RF-SPIA reactions: Optimal reaction conditions of RF-SPIA for *C. sakazakii* detection were established based on the experimental design of primers and blockers through a series of component concentrations of optimized reaction system and amplification temperature.

Detection of specific primers: The detection consisted of 15strains of *C. sakazakii* (ATCC 51329, ATCC51024 and other 13 strains of *C. sakazakii* isolated in the laboratory) and 29 strains of non-*C. sakazakii*, they were cultured under suitable conditions after 7–12h, approximately 1mL of the test bacterial suspension was removed to the corresponding sterile centrifuge tube and its genome was extracted by using the conventional thermal cracking method and stored in a refrigerator at -20 °C.

Sensitive detection of *C. sakazakii*:

RF-SPIA detection sensitivity of pure *C. sakazakii* culture: The standard strain of *C. sakazakii* (ATCC 51024) was inoculated in fresh sterile nutrient broth medium at 37°C and cultured for 12 h. A pure culture of living bacteria was determined by series of 10-fold dilution with physiological saline through dilution plate method. Simultaneously, 1 mL from each dilution broth was obtained and transferred to a 1.5 mL centrifuge tube, as the initial fluid of *C. sakazakii*, an ordinary thermal cracking method was used to extract *C. sakazakii* genome DNA as the template. The sensitivity of the RF-SPIA was verified according to the above optimization steps to select the best concentration and reaction conditions of RF-SPIA.

RF-SPIA detection sensitivity of *C. sakazakii* genome: Kit method was used to extract the genomic DNA of the overnight pure culture bacteria liquid of *C. sakazakii* standard strain (ATCC 51024). DNA purity was verified by using the ratio of OD260/OD280 and the DNA concentration was determined through a spectrophotometer OD260. The DNA was determined by series of 10-fold dilution and 1 μL of per dilution as to the template. The sensitivity of the RF-SPIA was verified according to the above optimization steps to select the best concentration and reaction conditions of RF-SPIA.

Detection limit of RF-SPIA in milk powder artificially contaminated with *C. sakazakii:* Before using, the national standard (GB 478940-2010) was used to detect the infant milk powder bought from the supermarket didn't contain *C. sakazakii*, then did the artificial pollution. Afterward, 25g of milk powder was dissolved in 225mL of sterile saline and then the milk solution was artificially contaminated with *C. sakazakii*. A 1:10 series of dilute solution was prepared for the sterilized milk powder solution. Hu (2009) and Chen (2014) compared the methods for the extraction of *C. sakazakii* DNA in infant formula milk powder, such as ordinary heat crack hydrolysis, protease K method, chemical reagent and reagent kit method, their results were not much difference. Thus, this experiment extracted the genomic DNA template by using ordinary heat analysis from infant formula milk powder. Through the RF-SPIA reaction system and detection

procedure, the instrument determined positive results through if the typical fluorescence curve appeared, thus identify the minimum detection limit.

Analysis of the results of RF-SPIA amplified products:

Real-time fluorescence curve analysis of RF-SPIA amplified products: RF-SPIA results could be directly observed by using an ESE Quant tube scanner. This scanner could effectively monitor the change in fluorescence intensity of the reaction system during the RF-SPIA amplification reaction. The scanner was connected to a computer, where the amplification curve was displayed. As the reaction proceeded, there would be a significant peak pattern. The earlier and the higher the peak appeared, indicated the higher efficiency of RF-SPIA amplification reaction.

Visual analysis of RF-SPIA amplified products: Pyrophosphate and magnesium ions formed white magnesium pyrophosphate precipitate after the RF-SPIA reaction. White turbidity phenomenon was observed through visual observation in the reaction tube; the reaction tube was centrifuged at 5000rpm for 40s. The white precipitate could be observed with the naked eye at the bottom of the tube. The test results were then evaluated.

After the RF-SPIA reaction, if amplification reaction is caused by the fluorescent dye SYBR Green II in the reaction system, the dye will bind with the product. Under the irradiation of the UV lamp system, the product was illuminated through UV light fluorescent to verify whether RF-SPIA amplification reaction occurred.

RESULTS AND DISCUSSION

RF-SPIA reaction condition optimization: The optimum reaction temperature was 61°C and the reaction system was optimized for each additive. The 25μL reaction system used to determine the RF-SPIA consisted of 25mmol/L $MgSO_4$,0.8μL; 10mmol/L dNTPs, 2.0μL; 10μmol/L primer, 2.0μL; 2× Bca DNA polymerase buffer, 2.0μL; 5× RNaseH buffer, 1.5μL; 40U/μL recombinant RNase inhibitor, 0.6μL; 20mg/mL bovine serum albumin, 0.9μL; 10μmol/L blocker, 1.0μL; 20U/μLBca DNA polymerase, 1.0μL; 60U/μL RNaseH,0.6μL; Template, 1.0 μL; fluorescent dye SYBR Green II (1:400), 1.0μL; and RNase-free water.

Analysis of the results of RF-SPIA amplified products:

Real-time fluorescence curve analysis of RF-SPIA amplified products: By using the established RF-SPIA method to amplify reaction, Tube 1 was the negative control and Tube 2 was the standard strain of *C. sakazakii* (ATCC 51024). The results were shown in Fig. 1. The ESE Quant tube scanner effectively monitored the change in fluorescence intensity of the reaction system during the RF-SPIA amplification reaction. When the scanner was connected to a computer, the amplification curves were set up in real-time as the reaction proceeded. A negative reaction resulted in the absence of fluorescence curve peak (Tube 1), where as a positive reaction resulted in apparent peak (Tube 2). The earlier and the higher the peak appeared, indicated the higher efficiency of RF-SPIA amplification reaction.

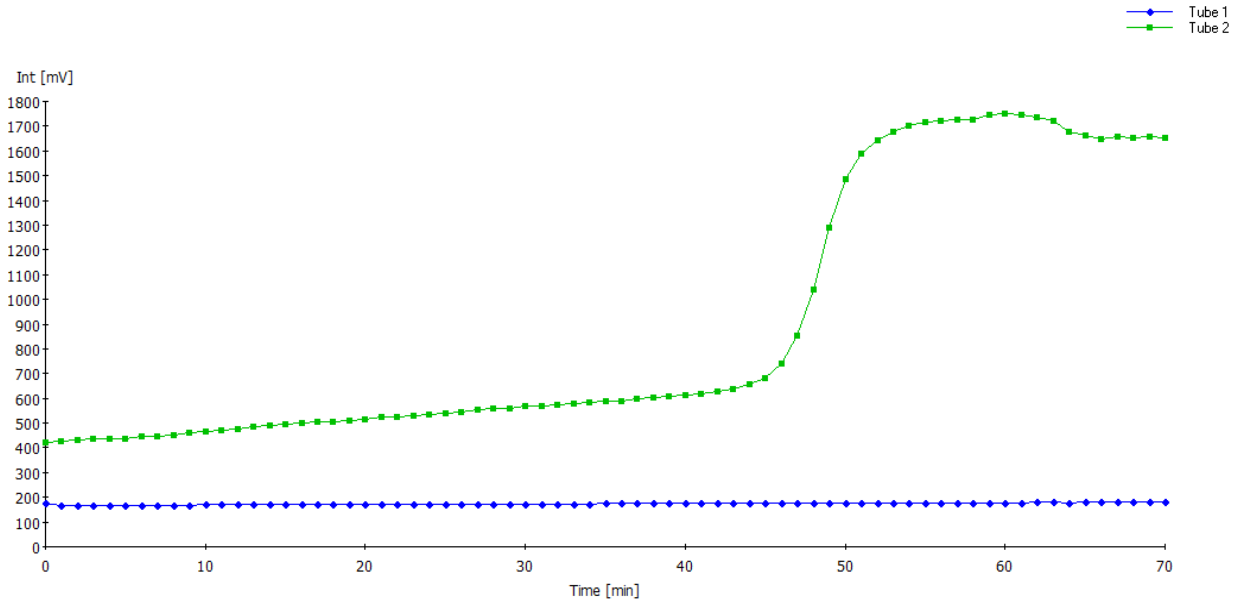
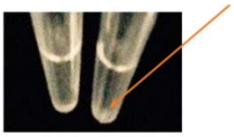


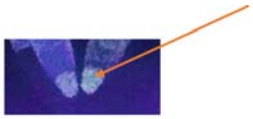
Fig. 1: Real-time fluorescence curve; Tube1: Negative control; Tube2: Standard strain of *C. sakazakii* (ATCC 51024)



A B

Fig. 2: Visual results of the RF-SPIA reaction; A: Negative control; B: Positive result

Visual analysis of RF-SPIA products: By the naked eye to observe whether the white magnesium phosphate precipitation, to determine whether the occurrence of RF-SPIA reaction. Figure 2 showed the visual result of the reaction. Tube A was the negative control, there was no white precipitate and was no RF-SPIA reaction.

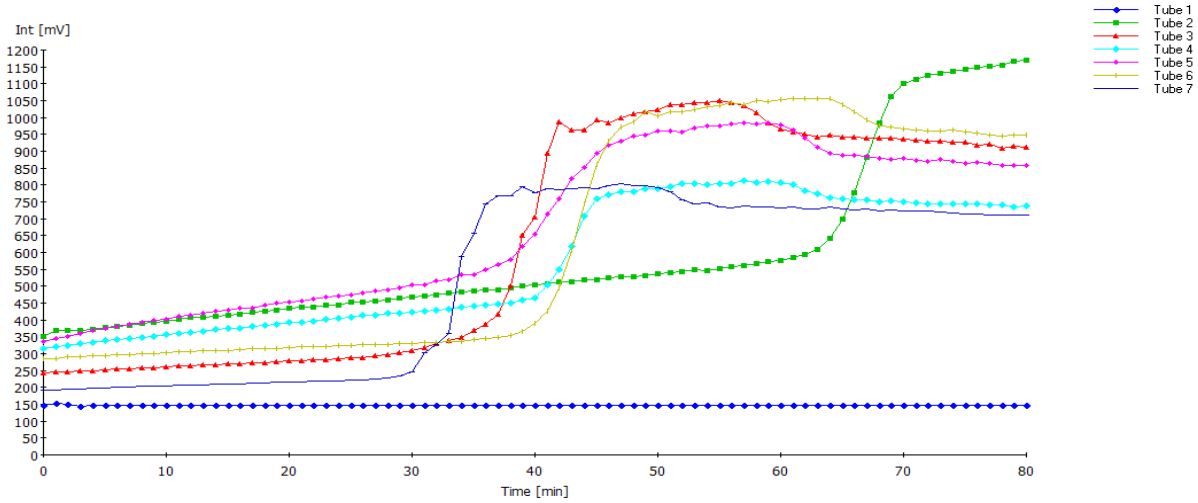


A B

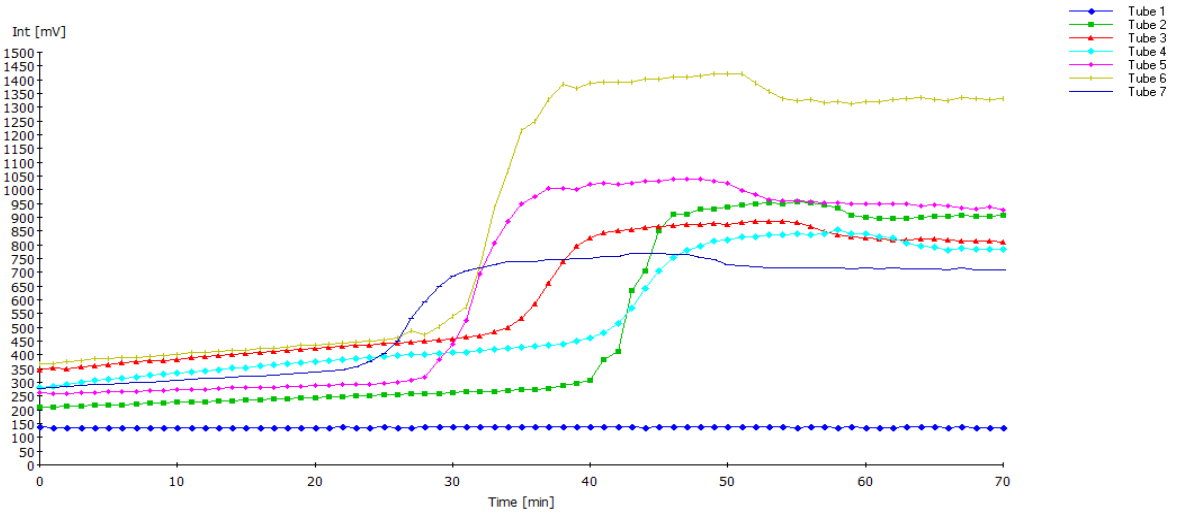
Fig. 3: Fluorescent analysis of RF-SPIA; A: Negative control; B: Positive result

Tube B has white precipitate and RF-SPIA reaction occurred.

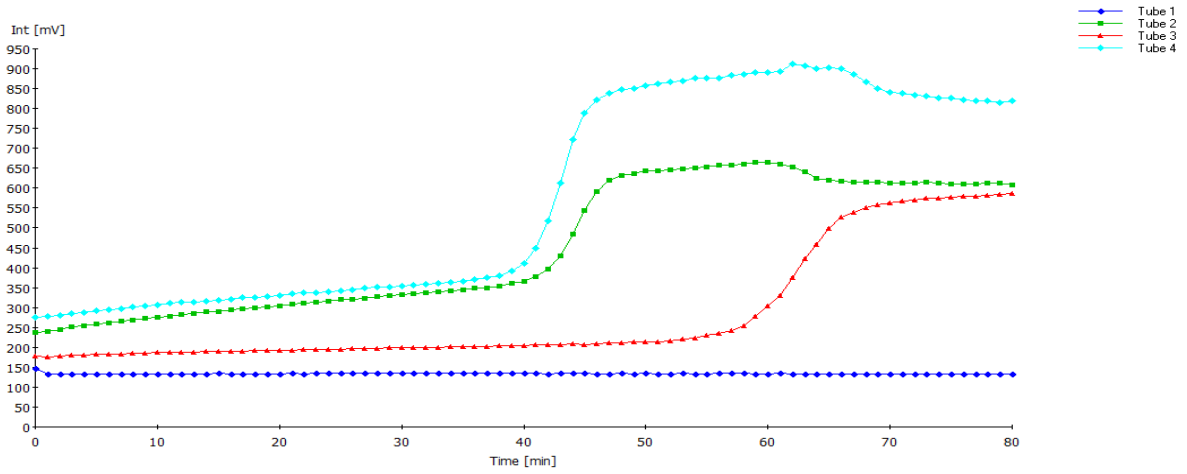
Figure 3 showed the reaction product of UV light irradiation after the RF-SPIA reaction. Tube A was the negative control, there was no fluorescence and was no RF-SPIA reaction. By contrast, Tube B has light green fluorescence and RF-SPIA reaction occurred.



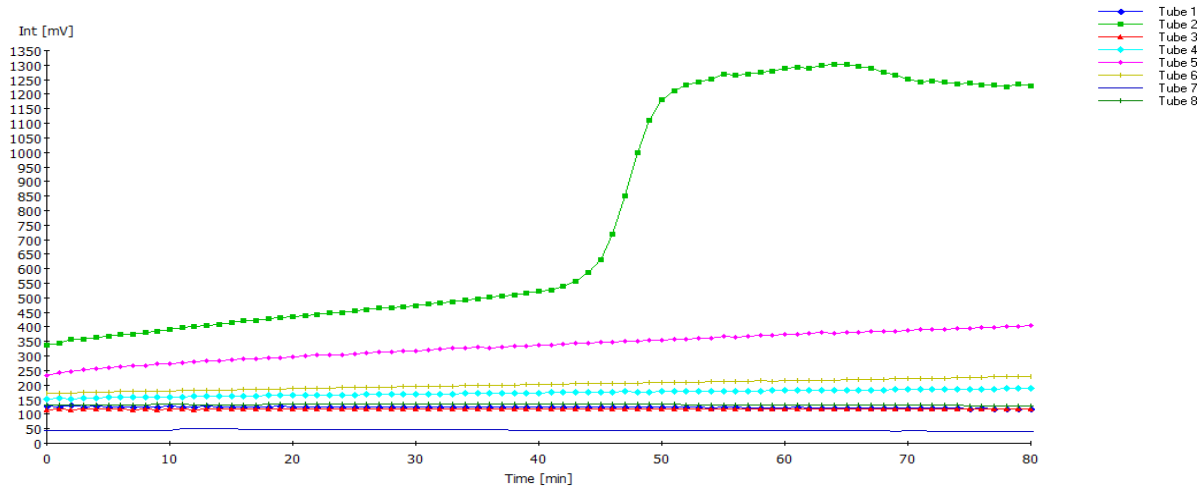
(A): Tube 1: Negative control; Tube 2: *Cronobacter sakazakii* (ATCC51329); Tube 3: *Cronobacter sakazakii* (ATCC 51024); Tube 4: *Cronobacter sakazakii* (N1); Tube 5: *Cronobacter sakazakii* (N2); Tube 6: *Cronobacter sakazakii* (N3); Tube 7: *Cronobacter sakazakii* (N4)



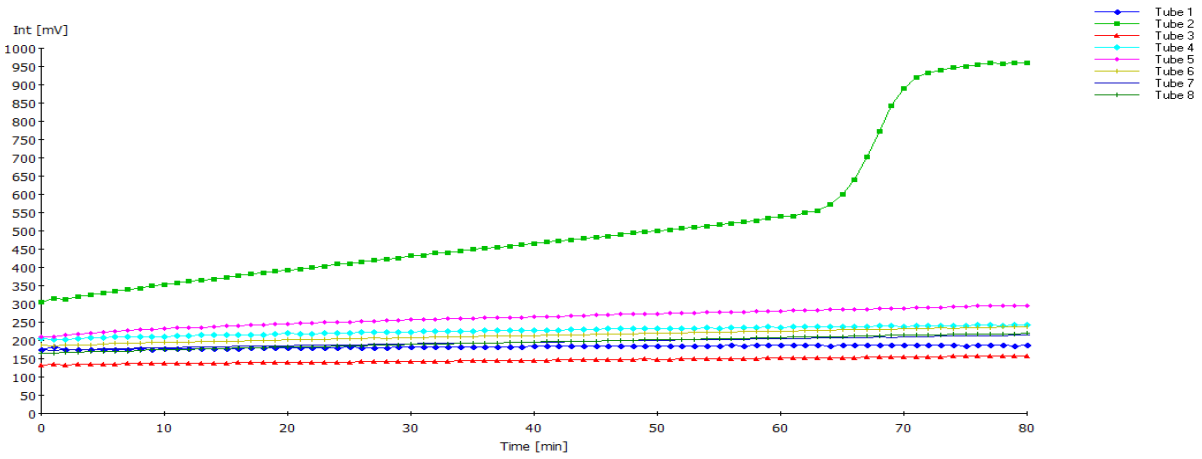
(B): Tube 1: Negative control; Tube 2: *Cronobacter sakazakii* (N5); Tube 3: *Cronobacter sakazakii* (N6); Tube 4: *Cronobacter sakazakii* (N7); Tube 5: *Cronobacter sakazakii* (R2); Tube 6: *Cronobacter sakazakii* (R3); Tube 7: *Cronobacter sakazakii* (R4)



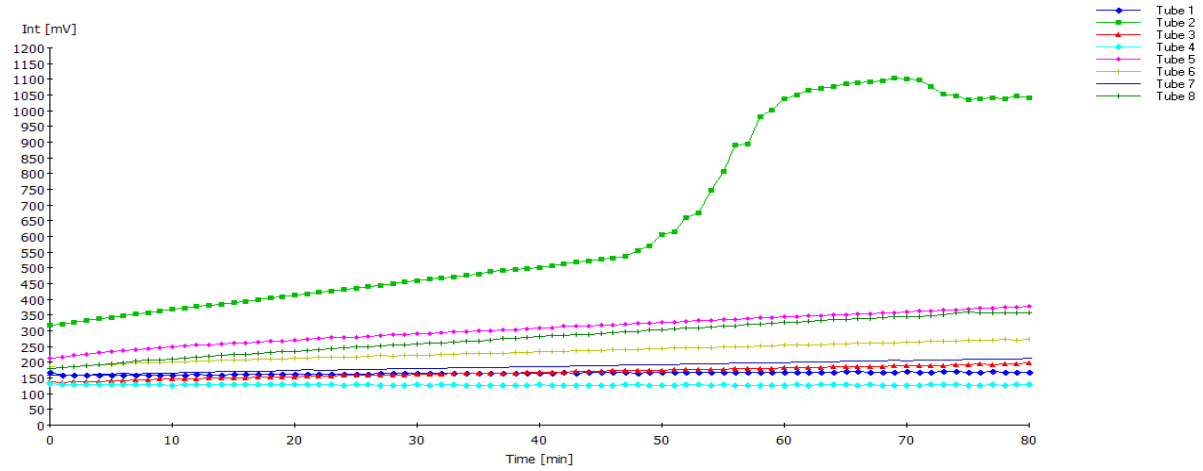
(C): Tube 1: Negative control; Tube 2: *Cronobacter sakazakii* (R5); Tube 3: *Cronobacter sakazakii* (R6); Tube 4: *Cronobacter sakazakii* (R7)



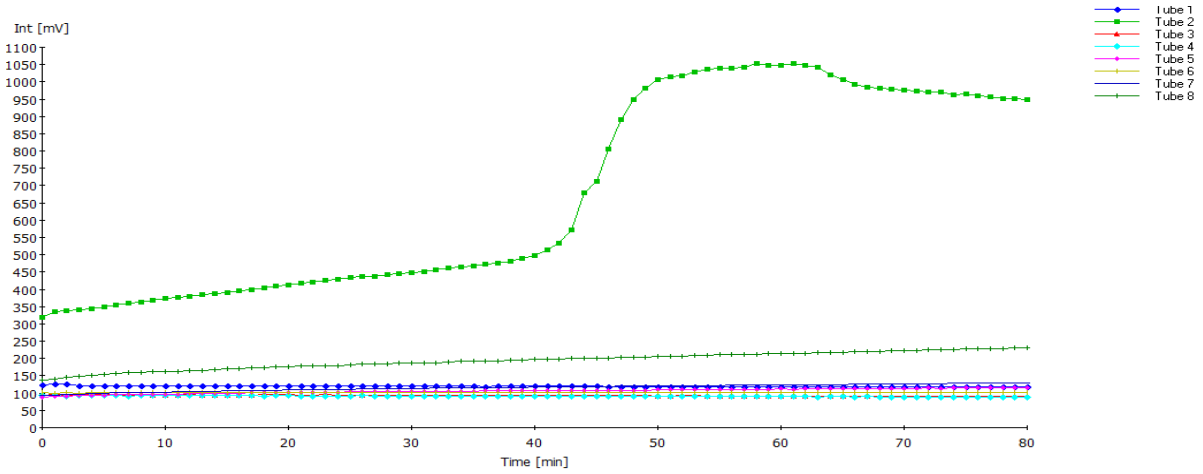
(D): Tube 1: Negative control; Tube 2: *Cronobacter sakazakii* (N5); Tube 3: *Escherichia coli EHEC* O157: *Hun* (O157); Tube 4: *Diagrrheagenic Escherichia coli* (CICC 10411); Tube 5: *Escherichia coli* (ATCC 25922); Tube 6: *Escherichia coli* (CMCC 44103); Tube 7: *Salmonella paratyphi-B* (CICC 21495); Tube 8: *Salmonella typhimurium* (CMCC 50115)



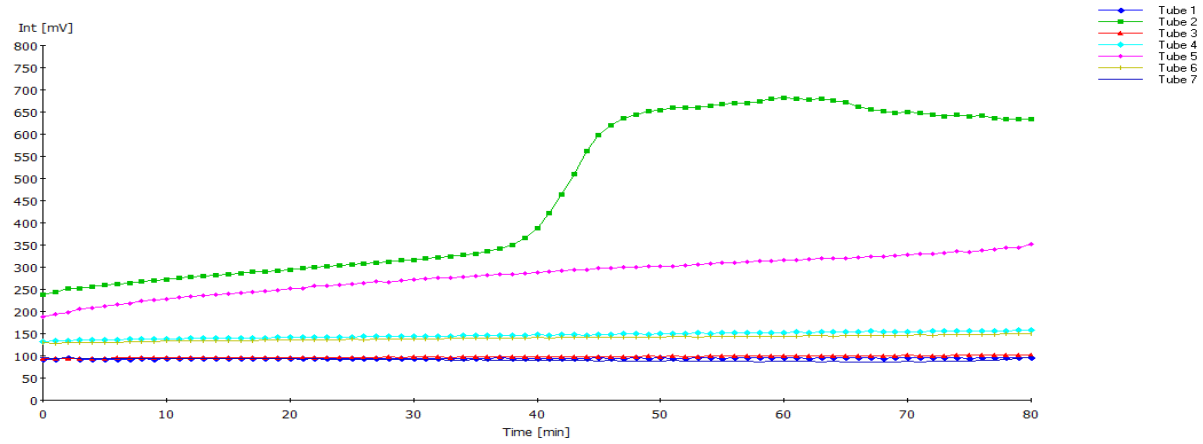
(E): Tube 1: Negative control; Tube 2: *Cronobacter sakazakii* (ATCC51329); Tube 3: *Salmonella enteritis* (CMCC 50041); Tube 4: *Salmonella* (Jia); Tube 5: *Salmonella schottmuelleri* (CMCC 50001); Tube 6: *Shigella boydii* (CMCC 51522); Tube 7: *Shigella sonnei* (CMCC 51334); Tube 8: *Klebsiella pneumoniae* (CICC 21519)



(F): Tube 1: Negative control; Tube 2: *Cronobacter sakazakii* (N7); Tube 3: *Yersinia enterocolitica* (CICC 21669); Tube 4: *Vibrio parahaemolyticus* (ATCC 17802); Tube 5: *Proteusbacillus vulgaris* (CMCC 49027); Tube 6: *Pseudomonas aeruginosa* (CICC 21636); Tube 7: *Bacillus thuringiensis* (CICC 22945); Tube 8: *Bacillus cereus* (CMCC 63302)



(G): Tube 1: Negative control; Tube 2:*Cronobacter sakazakii* (N3); Tube 3: *Listeria monocytogenes* (CMCC 54001); Tube 4: *Listeria grayi* (CICC 21670); Tube 5: *Listeria ivanovii* (CICC 21663); Tube 6: *Listeria welshimeri* (CICC 21672); Tube 7: *Staphylococcus aureus* (CMCC 26073); Tube 8: *Staphylococcus aureus* (CMCC 26003)



(H): Tube 1: Negative control; Tube 2: *Cronobacter sakazakii* (R5); Tube 3: *Staphylococcus aureus* (ATCC 43300); Tube 4: *Staphylococcus aureus* (Ji Kong); Tube 5: *Staphylococcus epidermidis* (CICC 23664); Tube 6: *Staphylococcus xylosus* (CICC 20237); Tube 7: *Staphylococcus xylosus* (CICC 22943)

Fig. 4: Specificity test by RF-SPIA

Study on primer specificity: The genomic DNA of 44 tested strains listed in Table 1 was extracted as a template and RNase-free water as a negative control. RF-SPIA amplification was conducted to verify the specificity of this method. Figure 4 showed that only 15 strains of *C. sakazakii* fluorescence curves had evident peak and the ESE Quant tube scanner automatically determined positive (+). And other 29 bacterial strains without peak, the ESE Quant tube scanner automatically determined negative (−). It can proved that the specificity of this method established in the study is strong, it can be used for the detection of *C. sakazakii*.

Sensitivity detection of *C. sakazakii*:

RF-SPIA detection sensitivity of *C. sakazakii* pure culture: The original bacterium liquid concentration of the standard strain of *C. sakazakii* (ATCC 51024) determined through dilution plate colony counting method was1.4×10^{9}CFU/mL. The pure culture was 10-fold serially diluted and by using an ordinary thermal cracking method to extract the genomic DNA of *C. sakazakii* as a template. Then they were detected according to the above optimization steps to select the best concentration and reaction conditions of RF-SPIA. The results were shown in Fig. 5.

Tube 1 was the negative control. Tubes 2 to 8 were the templates of pure bacteria culture from which liquid with 10-fold serial dilutions. When pure culture concentration was diluted to 1.4×10^{-1} CFU/mL (Tube8), the ESE Quant tube scanner exhibited no peak. Thus, the sensitivity of RF-SPIA of *C. sakazakii* pure culture could reach 1.4 × 10°CFU/mL.

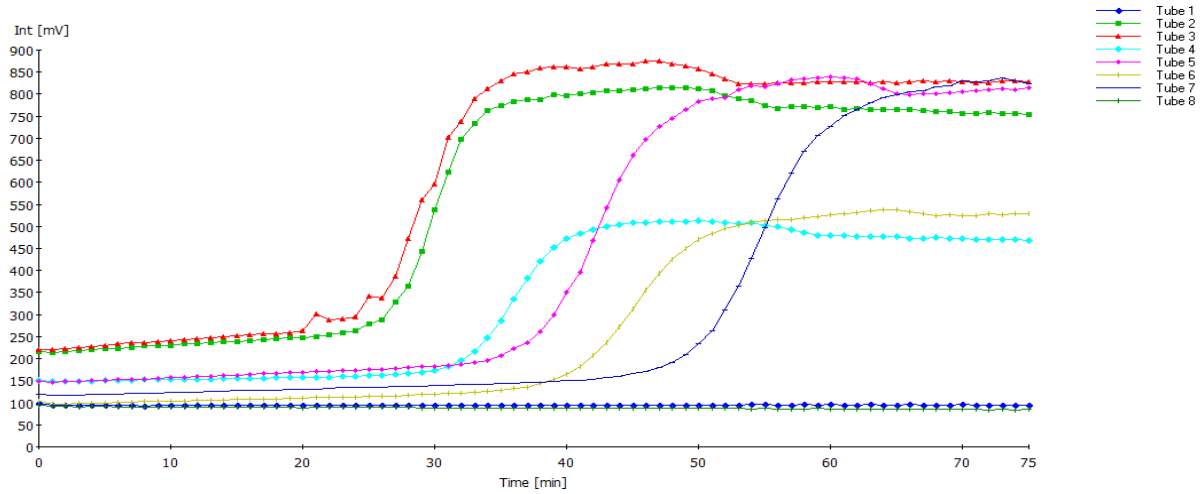


Fig. 5: Sensitivity of detection of a pure culture of *C. sakazakii* by RF-SPIA; Tube 1: Negative control; Tube 2:1.4×10^{9}CFU/mL; Tube 3: 1.4×10^{7}CFU/mL; Tube 4: 1.4×10^{5}CFU/mL; Tube 5: 1.4×10^{3}CFU/mL; Tube 6: 1.4×10^{1}CFU/mL; Tube 7: 1.4×10^{0}CFU/mL; Tube 8: 1.4×10^{-1}CFU/mL

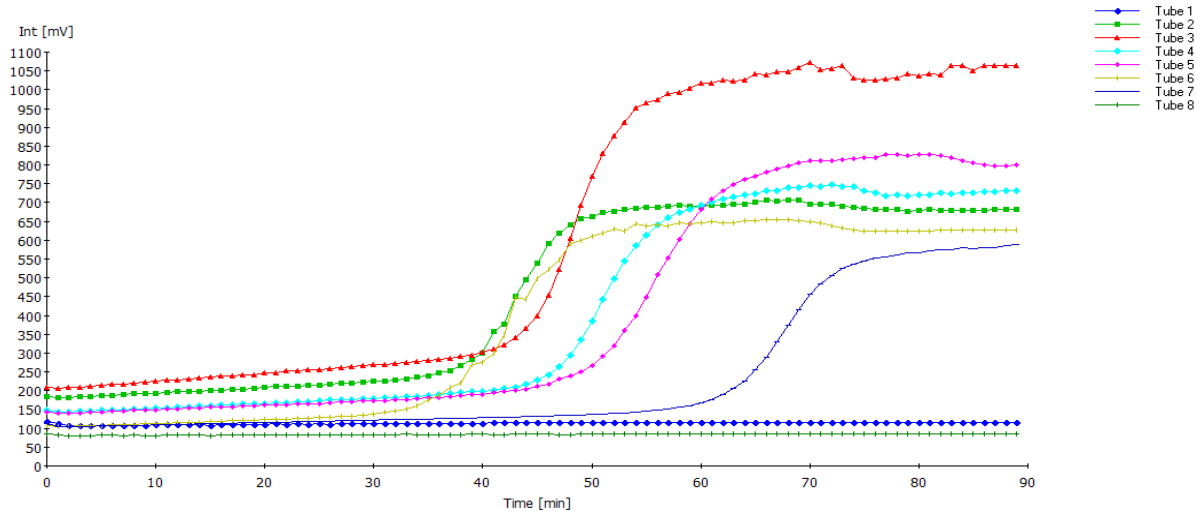


Fig. 6: Sensitivity of detection of *C. sakazakii* geneome by RF-SPIA; Tube 1: Negativecontrol; Tube 2: 7.5×10^{10}fg/mL; Tube 3: 7.5×10^{8}fg/mL; Tube 4: 7.5×10^{7}fg/mL; Tube 5: 7.5×10^{5}fg/mL; Tube 6: 7.5×10^{3}fg/mL; Tube 7: 7.5×10^{1}fg/mL; Tube 8: 7.5×10^{0}fg/mL

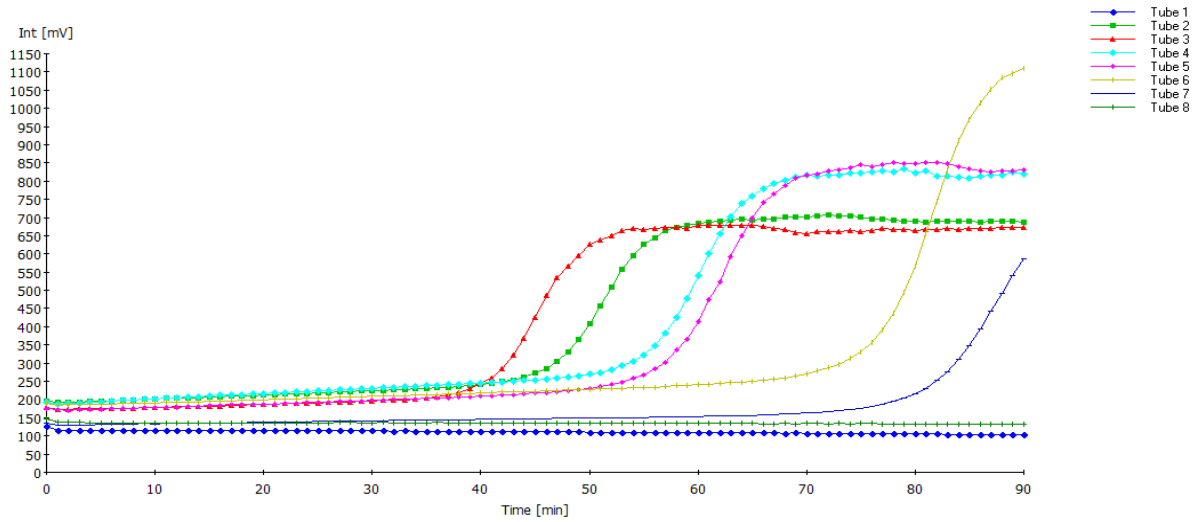


Fig. 7: Detection limit of RF-SPIA in milk powder artificially contaminated with *C.sakazakii*; Tube 1: Negative control; Tube 2: 8.1×10^{8}CFU/mL; Tube 3: 8.1×10^{6}CFU/mL; Tube 4: 8.1×10^{5}CFU/mL; Tube 5: 8.1×10^{4}CFU/mL; Tube 6: 8.1×10^{2}CFU/mL; Tube 7: 8.1×10^{1}CFU/mL; Tube 8: 8.1×10^{0}CFU/mL

RF-SPIA detection sensitivity of *C. sakazakii* genome: The genomic DNA of the overnight culture of the standard strain of *C. sakazakii* (ATCC 51024) was extracted by using the kit. The genomic stock solution diluted 50-fold was 1.78 as measured at OD260/OD280 in a spectrophotometer. Thus, the genome had a high purityof750 μg/mL as calculated from the measured OD260 dope genomic concentration. The DNA stock solution was10-fold serially diluted, which depicted 1μL of per dilution as to the template. Then they were detected according to the above optimization steps to select the best concentration and reaction conditions of RF-SPIA. Figure 6 showed the results.

Tube 1 was the negative control. Tube 2 to 8 were the DNA solution with 10-fold serial dilutions. When the genomic DNA was diluted to 7.5×10^{0}fg/mL (Tube 8), the ESE Quant tube scanner exhibited no peak; thus, the sensitivity of RF-SPIA of *C. sakazakii* genome could reach 7.5×10^{1}fg/mL.

Detection limit of RF-SPIAin milk powder artificially contaminated with *C. sakazakii*: The milk powder solution was contaminated with 8.1×10^{8} CFU/mL *C. sakazakii* bacteria through the dilution plate method colonycount. The contaminated milk powder solution was 10-fold serially diluted, by using a conventional pyrolysis method to directly extract the genomic DNA of *C. sakazakii* as a template, according to the above mentioned optimization step. Then they were detected according to the above optimization steps to select the best concentration and reaction conditions of RF-SPIA. The results were shown in Fig. 7.

Tube 1 was the negative control. Tube 2 to 8 was 10-fold serial dilutions of template extraction. When these extracts are diluted to 8.1×10^{0} CFU/mL (Tube 8), the ESE Quant tube scanner exhibited no peak. Thus, the detection of RF-SPIA artificial pollution *C. sakazakii* in milk powder detection limit can reach 8.1×10CFU/mL.

CONCLUSION

With the development of biological technology, in-depth studies on *C. sakazakii* have been conducted. Consequently, numerous approaches to the detection of *C. sakazakii* emerged. The traditional detection methods are time-consuming, inefficient, slow and complicated. Several molecular biological techniques, such as PCR, nucleic acid hybridization and gene chip, have been successfully established to detect *C. sakazakii*. Nevertheless, these methods require complex operation and expensive equipment. Therefore, they are not conducive to the promotion at the grassroots level.

The RF-SPIA is established in this experiment, adding fluorescent dye in to the reaction system. With the reaction, the amplified product is increased and the fluorescence signal is strengthened, the ESE Quant tube scanner monitors the RF-SPIA reaction through fluorescence signal accumulation. The results can be visually analyzed and automatically displayed without complex agarose gel electrophoresis. Meanwhile, the risk of injury or damage to the test operator is reduced and contact with ethidium bromide, which is a strong carcinogen, can be avoided. The product can directly irradiate under ultraviolet light and color change can be observed. A blocker is added to the reaction system and its own 3′-OH is replaced or modified. Thus, a primer cannot be used for non-specific amplification, avoiding the amplification primer itself. The entire reaction process uses closed tube amplification, which prevents false-positive results caused by pollution and controls the negative pollution at the source. Moreover, the used

ESE Quant tube scanner is simple, portable and convenient for promotion in the primary.

The RF-SPIA for the detection of *C. sakazakii* in infant formula milk powder is highly sensitive, specific, simple and swift. Thus, the method has broad prospect of application. Nevertheless, the methods have several limitations as follows:

- The primer synthesis is relatively complex. The reaction requires DNA primer and RNA hybrid primer. The synthesis of DNA or RNA primer is relatively complex compared with the conventional method.
- The blocker needs to modify the base to enhance its binding force with the template.
- No real-time quantitative analysis can be conducted and the SPIA cannot quantitatively analyze the amplification reaction.

Although the RF-SPIA technology has incomparable advantages, improvement is still required. RF-SPIA technology to the final analysis of the template can be real-time fluorescence quantitative analysis of the direction of development.

In summary, the RF-SPIA method with high sensitivity, strong specificity and simple operation for detecting *C. Sakazakii* in infant formula milk powder within a short time, reduces detection time and provides intuitive reaction results, this method has broad prospect of application.

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