Analytica Chimica Acta 664 (2010) 95–99

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Sensitive and simple detection of *Escherichia coli* strain based on time-resolved fluorescence DNA hybridization assay

Min Ruan[a,b], Cheng-Gang Niu[a,b,*], Pin-Zhu Qin[a,b], Guang-Ming Zeng[a,b], Zhao-Hui Yang[a,b], Hui He[a,b], Jing Huang[a,b]

[a] College of Environmental Science and Engineering, Key Laboratory of Environmental Biology and Pollution Control, Ministry of Education, Hunan University, Changsha 410082, China
[b] State Key Laboratory of Powder Metallurgy, Central South University, Changsha 410083, China

ARTICLE INFO

Article history:
Received 1 December 2009
Received in revised form 27 January 2010
Accepted 31 January 2010
Available online 6 February 2010

Keywords:
Escherichia coli
Two-probe tandem DNA hybridization assay
Time-resolved fluorescence
Europium complex

ABSTRACT

A two-probe tandem DNA hybridization assay based on time-resolved fluorescence was employed to detect *Escherichia coli* strain. The amino modified capture probe was covalently immobilized on the common glass slide surface. The Eu(TTA)$_3$(5-NH$_2$-phen) with the characteristics of long lifetime and intense luminescence was labeled with reporter probe. The original extracted DNA samples without the purification and amplification process were directly used in the hybridization assay. The concentration of capture probe, hybridization temperature, hybridization and washing time were optimized. The detection limit is about 1.49×10^3 CFU mL^{-1} *E. coli* cells, which is comparable to the value of most microbiology methods. The proposed method has the advantages of easy operation, satisfactory sensitivity and specificity, which can provide a promising technique for monitoring the microorganisms.

1. Introduction

The presence of *Escherichia coli* in water and food mainly originated from warm-blooded animals as a result of fecal contamination. The organism is used as an index of water quality as well as indicator microorganism for fecal source tracking owing to its easy culture [1–4]. Current regulations require drinking water to contain less than one *E. coli* cell per 100 mL and certain foods to contain less than 10 *E. coli* cells per gram. Therefore, enumeration of *E. coli* cells is essential to access the level of contamination. Traditionally, enumeration of *E. coli* cells is done by counting colonies depended on a selective or indicator medium according to their morphological, biochemical, and/or immunological characteristics. Theoretically, each of the living cells should give a colony. However, the physiological state of the cells has a significant influence on their efficiency of plating, and the testing results in this method require many days from initiation to readout [5].

As a result of increasing availability of 16S and 23S ribosomal ribonucleic acid (rRNA) sequences in databases and the accessibility of sequencing technology, molecular methods are extensively used for the detection and identification of microorganisms [6–10]. PCR-based diagnostic methods are now routinely used but the confidence of these approaches is highly depended on the preparation of adequate positive and negative controls [11,12]. Minor contamination and the presence of PCR inhibitors in testing samples may lead to false negative results. Fluorescent in situ hybridization (FISH) is a hybridization-based technique to detect microorganisms directly in complex samples without the necessity of an initial enrichment step. FISH uses fluorescence-labeled nucleic acid probes that bind specifically to the target organism, and are later detected by fluorescence microscopy or flow cytometry [13–15]. However, the existence of autofluorescence in some bacteria, the decay of probe fluorescent signal and the insufficient bacterial fixation may all lead to misleading results [16]. Nucleic acid-based microarrays offer a fast and convenient alternative means to time-consuming and conventional microbiological methods. These high-density chips combine the detection of target DNA signatures with the advantages of DNA sequencing, which allows the ultimate resolution for microorganism identification to be achieved [17–20]. The requirements of technological resources and the high costs are critical problems, which will determine whether the microarrays could take a leading role as microbial diagnostic tools or not.

In this work, the authors developed a method based on a novel europium complex for the specific detection of original extracted DNA from *E. coli* strain. A bifunctional europium complex of Eu(TTA)$_3$(5-NH$_2$-phen) using 2-thenoyltrifluoroacetonate (TTA) and 5-amino-1,10-phenanthrolin (5-NH$_2$-phen) as ligand

* Corresponding author at: College of Environmental Science and Engineering, Key Laboratory of Environmental Biology and Pollution Control, Ministry of Education, Hunan University, Changsha 410082, Hunan, China. Tel.: +86 731 88823820; fax: +86 731 88822829.
E-mail address: cgniu@hotmail.com (C.-G. Niu).

0003-2670/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.aca.2010.01.064

reagents was applied to the microorganisms detection. Compared to the other fluorescent dyes, the complex has the advantages of long fluorescence lifetime, intense luminescence, high fluorescence quantum yield and easy label. Therefore, when the time-resolved fluorescence detection method and the complex were employed to the hybridization assay, it could effectively eliminate the background noises and improve the detection sensitivity. The method could be operated without the purification and amplification procedures of nucleic acid and simplified the detection process. It could be an alternative means to monitoring the microorganism.

2. Experimental

2.1. Reagents

The complex of Eu(TTA)$_3$(5-NH$_2$-phen) was synthesized by our group and has the lifetime of 0.688 ms and a very high fluorescence quantum yield of 0.62 [21]. 3-Aminopropyltrimethoxysilane, a silanization reagent, was purchased from Acros organics. Ammonium hydroxide (25–28 wt.%), ethanol, glutaraldehyde (50 wt.%), acetic acid and hydrogen chloride (HCl) were purchased from Hengxing Reagent Co. (Tianjin, China). Sodium phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium citrate and sodium dodecyl sulfate were from Kermel (Tianjin, China). Unless otherwise stated, all other reagents were of analytical reagent grade and used without purification or treatment.

2.2. Media and buffers

LB (Luria-Bertani) medium contained 10.0 g peptone, 5.0 g NaCl, 5.0 g yeast extract and 15.0 g agar (if necessary) per litre (pH 7.4). Nutrient medium contained 10.0 g peptone, 5.0 g nutrient broth, 5.0 g NaCl and 15.0 g agar (if necessary) per litre (pH 7.0–7.2). EMB (eosin methylene blue) medium was purchased from Hangzhou Microbial Reagent Corporation (Hangzhou, China).

Phosphate buffered saline (PBS) consisted of 10 mM NaH_2PO_4, 137 mM NaCl, 2 mM KH_2PO_4 and 2.7 mM KCl (pH 7.4). TE buffer consisted of 10 mM Tris–HCl and 1 mM EDTA (pH 8.0). All of the culture media and buffers were sterilized by autoclave treatment before being used.

2.3. Bacterial strains and enumeration

All the *E. coli* strains (CCTCC 200068, CCTCC 94092 and CCTCC 91117) were purchased from China Center For Type Culture Collection (Wuhan, China), and were routinely grown on LB media at 37 °C. The *E. coli* strain CCTCC 200068 was used as the reference strain in all optimization and sensitivity experiments. The *Paenibacillus polymyxa* strain CCTCC M206017 was supplied by other laboratory. The *Staphylococcus aureus* and *Enterobacter cloacae* strains were preserved by our lab.

Numbers of cultivable cells were determined by conventional agar plate counting. One milliliter of 10-fold serial dilutions of freshly grown cells was spread on an EMB agar plate and incubated overnight at 37 °C and the melt brilliant bacterial colony was counted.

2.4. Selection of sequences (probes)

In this research, a two-probe tandem DNA hybridization assay was employed. Sequences of the oligonucleotide are outlined in Table 1 and all the sequences were designed by Primer Premier 5.0 software. The specificity of those sequences was checked by the program 'Probe Match' provided by the Ribosomal Database Project II (RDP II, http://rpd.cme.msu.edu/) and the NCBI Blast 2 alignment tool in GenBank database. The 16S rDNA oligonucleotide sequences of the capture and reporter probe were complemented with the specified target DNA sequences. Meanwhile, the melting temperature of the two probes was selected with the approximate value to ensure the uniform condition of renaturation during hybridization. Ten bases of 'T' were added on the 3′ end of capture probe and 5′ end of reporter probe, which could avoid the sterically hindered immobilization substrate. For the purpose of coupling, an amine group was introduced to the end of two probes. And all the oligonucleotide sequences were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China).

2.5. Nucleic acid extraction

Total genomic DNA was extracted by a Generay™ Genomic DNA Extraction Kits following the instructions of the manufacturer. An overnight culture of the bacterial cells in fresh LB media was grown to 0.6–1.0 OD_{600}. For each DNA extracted preparation, a pellet contained approximately 1×10^8 CFU (colony forming unit) mL^{-1} of bacteria. The purity of the collected DNA was verified by a spectrophotometer (DU®640Bechman, US) using the A_{260}/A_{280} ratio.

2.6. Capture probe coupling onto the glass slides surface

Common glass slides were modified with aldehyde groups on their surfaces according to the literature [22]. The clean glass slides were immersed in 25% ammonium hydroxide for 2 h and rinsed with double-distilled water. Then they were soaked in the solution of 2% 3-aminopropyltrimethoxysilane at pH 4.5 adjusted by glacial acetic acid. And they were ultrasonically washed with double-distilled water after 30 min. Finally, they were immersed in 2.5% glutaraldehyde for 3 h and washed with PBS buffer and double-distilled water in turn and dried at room temperature.

The capture probe diluted in TE buffer with the volume of 40 μL was activated and pipetted onto the aldehyde glass slides surface. After incubated at room temperature for 5 h, the glass slides were washed with 0.2% sodium dodecyl sulfate and double-distilled water twice to remove unbound DNA. Subsequently, the glass slides were dried under a filtered air-stream. To block the surplus aldehyde groups on the surface, the glass slides were immersed in a glycin solution for 1 h. The final glass slides coupling with capture probe were dried after washing with 0.2% sodium dodecyl sulfate and double-distilled water twice, respectively.

2.7. Reporter probe coupling with the ternary europium complex

The synthesized europium ternary complex of Eu(TTA)$_3$(5-NH$_2$-phen) was applied as the biomarker. The complex was dispersed in a 2.5% glutaraldehyde solution and stirred for 3 h at room tem-

Table 1
Sequences of all probes.

Probe	Sequence (5′–3′)	mer
Capture probe	CAT TAC ATT GAC GCA GGT GAT CGG ACG $(T)_{10}NH_2$	47
Reporter probe	$H_2N(T)_{10}$ GTA TCG GTG TGA GCG TCG CAG AA	43
Target DNA sequence	CGT CCG ATC ACC TGC GTC AAT GTA ATG TTC TGC GAC GCT CAC ACC GAT AC	50

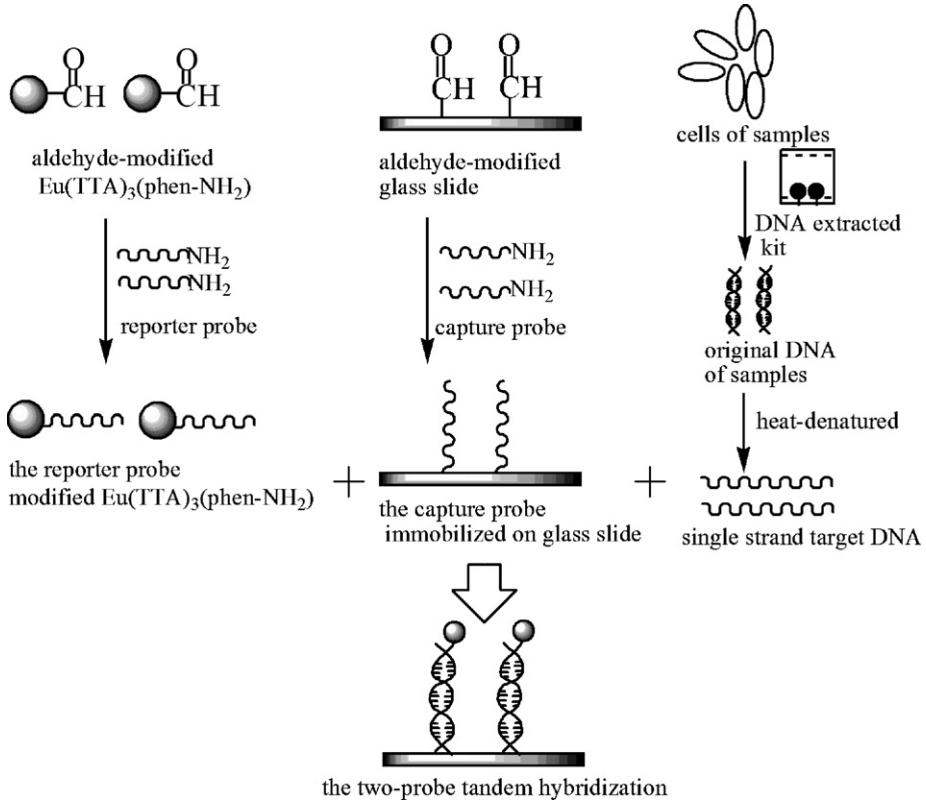

Fig. 1. The schematic diagram of this detection method.

perature. Then, the mixture was centrifuged and washed with double-distilled water twice, and finally suspended in PBS buffer. A certain concentration of reporter probe was added to the complex suspended solution with continuous stirring at room temperature for 5 h and then centrifuged and washed with PBS buffer to remove the unbound oligonucleotides. Finally, the mixture was treated with glycin solution in order to inactivate the unreacted aldehyde groups.

2.8. Hybridization assay format

The primitive nucleic acid stored at 4 °C was thawed and heat-denatured at 95 °C for 5 min and immediately immersed in mixture of ice and water for 5 min to obtain the single strand of target DNA. A hybridization box was prepared and filled with cotton soaked in 6× SSC solution to guarantee a uniform moistening of the glass slide surface. Target DNA of single strand 10 μL, hybridization buffers (6× SSC) 10 μL and dye modified reporter probe 10 μL were pipetted onto the glass slides and mixed thoroughly. Then the box was incubated at a designed temperature in a thermostat. After hybridization reaction is completed, the glass slides were washed with three types of washing buffers in turn. A two-stage stringent wash was performed to disrupt undesired hybrids and then the glass slides were dried at room temperature. Unless otherwise stated, blank control was used of double-distilled water instead of the DNA sample and all the assays were performed in triplicates. A schematic diagram for illustrating the sensing strategy was shown in Fig. 1.

The time-resolved fluorescent intensity of the glass slide was monitored by a detection system, which consisted of a PerkinElmer LS-55 spectrofluorimeter, a personal computer, two arms of the bifurcated optical fibers and a home-made poly(tetrafluoroethylene) detection cell. The optical fibers were fixed in the detection chamber of the spectrofluorimeter to carry the excitation and emission light. The detection cell acted as an immobilized platform for the glass slide and optical fibers.

3. Results and discussion

3.1. Optimal concentration of capture probe

In preliminary experiments, the concentration of capture probe was found to influence the hybridization efficiency. Capture probe of six different concentrations, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9} and 1×10^{-10} mol L^{-1} were examined. As shown in Fig. 2, the concentration at 1×10^{-7} mol L^{-1} presents a stable and strong signal than the others. It should be the appropriate concen-

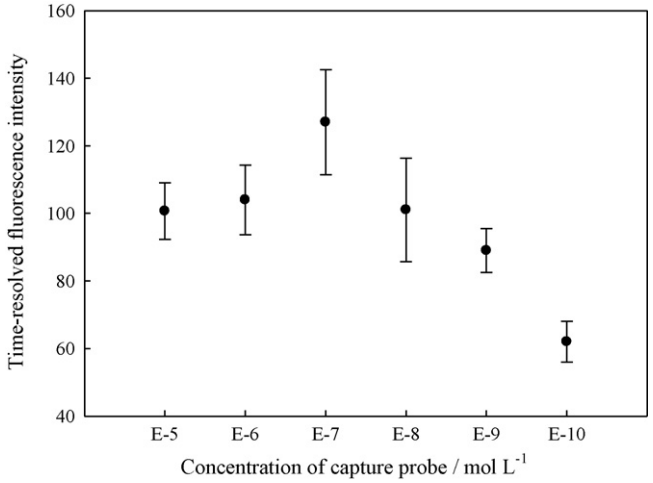

Fig. 2. Optimization of the concentration of capture probe coupling on glass slides. Background signals from blank controls are subtracted.

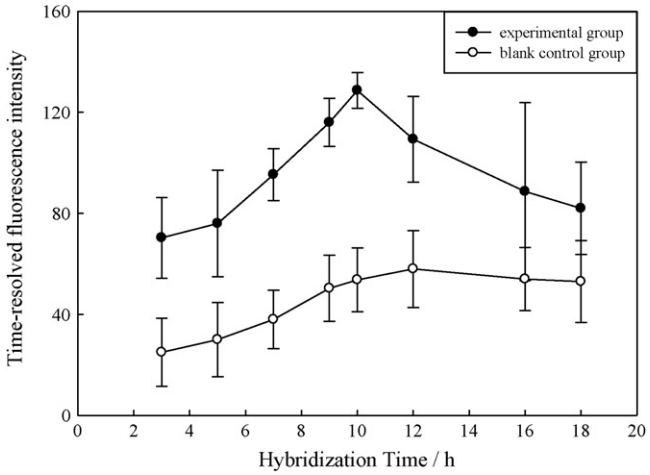

Fig. 3. Optimization of the hybridization time.

tration for coupling with the aldehyde group on the glass slides. The capture probe concentration of 1×10^{-7} mol L^{-1} is chosen in the following experiments.

3.2. Optimal hybridization temperature

Generally, there are three types of hybridization temperature, the optimum renaturation temperature (TOR, equal to T_m (melting temperature) minus 25 °C), stringent renaturation temperature (T_s, equal to T_m minus 10 or 15 °C) and non-stringent renaturation temperature (T_{ns}, equal to T_m minus 30 or 35 °C). Here the value of T_m was 53 °C. The results demonstrated that the signals were relatively low at the temperature of TOR and T_{ns} while it had a satisfactory signal at the temperature of T_s. It might be due to the reason that high molecular weight of the specimen DNA was too difficult to diffuse at low temperature and led to the incomplete reaction of complementary base pairing. Thus, the optimal hybridization temperature is T_s (43 °C) which is similar to the temperature reported in those literatures [9,13,23].

3.3. Influence of hybridization time

The desirable hybridization time depended on many factors such as the length and sequence of the coupled probe. For a given assay, it is necessary to examine the best hybridization time in performing a hybrid course. As shown in Fig. 3, the fluorescent intensity obviously increases from 3 h to 10 h and gets to the summit at 10 h. Meanwhile, the blank control signal is almost maintained in a certain range after 10 h. According to the literature, hybrids formed between short oligonucleotides and target DNA sequence at 5–10 °C minus the T_m could unwind, which also could be considered reversible [24]. Consequently, lengthening the hybridization time might not be a better option. Furthermore, the specimen nucleic acid was not purified and many unknown factors might interfere with the process of hybridization. These interference units could compete for the adsorption sites. In the surplus hybridization time, they dominated the adsorption sites that were released by target DNA base. Therefore, the curve appears a downtrend after 10 h.

3.4. Optimal washing time

A two-stage stringent wash was performed to disrupt undesired hybrids after hybridization. Low stringent wash (high salt concentrations and low temperatures) could remove nonspecifically bound probe but the low homology hybrids would not be disrupted in this procedure. And high stringent wash (low salt concentration and high temperatures) could remove undesired hybrids of low homology. Protocol of low and high stringent washing is shown in Table 2. The results demonstrated that it could obtain the best washing efficiency when washing time was 6 min. And the signals sharply decreased when it exceeded 6 min. One possible explanation for the phenomenon may be that some of the target bases were broken during the overlong wash, which was a non-reversible destruction procedure.

3.5. Analysis of specificity for E. coli

The specificity of this hybridization method was determined by the sequence of probe designed. The *E. cloacae* strain was chosen, which was known to be closely related to *E. coli*. The *S. aureus* strain was usually presented with the *E. coli* in contaminations. And the *P. polymyxa* strain was chosen to serve as a random interfered strain in this experiment. All the samples were monitored under the optimal conditions.

The results are obtained from the hybridization reaction (see Fig. 4). The *E. coli* samples and positive controls give signals between 139 and 154, while all the other organisms have values close to that of the blank control. No false positive signals are appeared, which indicates that it is a good specific hybridization assay for the detection of *E. coli* strains. For the DNA hybridization method without purification and amplification procedure, its specificity is a significant characteristic for its further exploration and application.

3.6. Sensitivity of the hybridization method

The cultivable *E. coli* concentration was determined on 10-fold serial dilutions, which were prepared by sterile PBS buffer. Then, 1 mL of each dilution was plated on EMB agar plates to obtain an accurate estimation of the cultivable *E. coli* concentration. Simultaneously, the cells of each dilution were extracted nucleic acid and investigated under the optimal conditions. The results are shown in Fig. 5. The threshold for the positive detection is set as background (blank) signal plus three times the standard deviation of the blank [25]. According to this criterion, the detection limit of this method is determined as 1.49×10^3 colony forming unit (CFU) per milliliter. This concentration is comparable to that reported literature of detection for *E. coli* with PCR procedure [11]. And it is a

Table 2
Formulation of washing solution.

Step	Type	Final concentration	Volume	Temperature
Low stringency washes	Washing I	1× SSC, 0.03%SDS	10 mL 20× SSC 600 μL 10% SDS 189.4 mL double-distilled water	RT
High stringency washes	Washing II	0.2× SSC	2 mL 20× SSC 198 mL double-distilled water	43 °C
	Washing III	0.05× SSC	500 μL 20× SSC 199.5 mL double-distilled water	43 °C

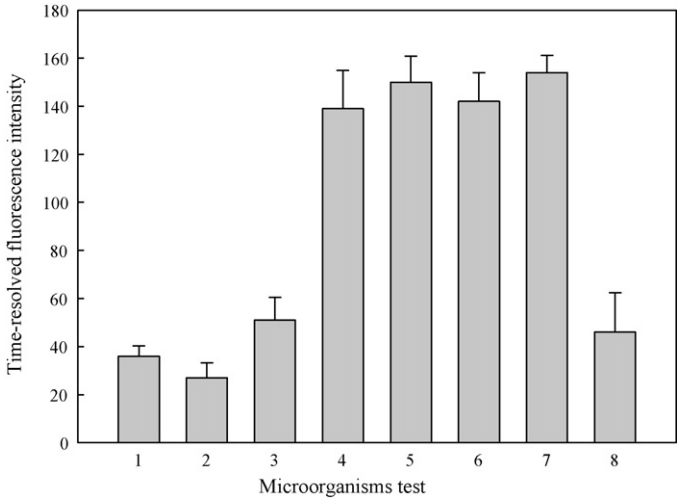

Fig. 4. Investigation of the specificity in this method. The numbers correspond to the following samples: (1) *S. aureus*; (2) *E. cloacae*; (3) *P. polymyxa*; (4) *E. coli* CCTCC 94092; (5) *E. coli* CCTCC 200068; (6) *E. coli* CCTCC 91117; (7) positive control (target DNA sequence) and (8) blank control (H_2O).

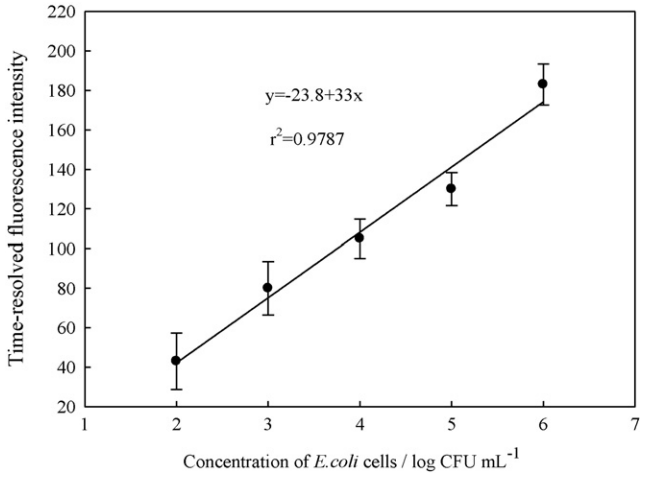

Fig. 5. Correlation between fluorescence intensity and the concentration of the *E. coli* cells.

major improvement for the method of DNA hybridization assay in microorganism detection without nucleic acid amplification.

4. Conclusions

A sensitive and simple method for the detection of the *E. coli* strain is presented. The $Eu(TTA)_3(5\text{-}NH_2\text{-}phen)$ is an appropriate biomarker with the characteristic of long lifetime and intense luminescence. In the experimental procedure, the concentration of capture probe, hybridization temperature, hybridization and washing time were investigated and optimized. The detection limit of this method is as few as 1.49×10^3 CFU mL^{-1} *E. coli* cells and no false positive signals were obtained from the other contrasted microorganisms. Considering the satisfactory sensitivity and specificity, this proposed assay could be an alternative method for microorganisms monitoring.

Acknowledgements

This work was financially supported by the National 863 High Technology Research Foundation of China (2006AA06Z407), the National Natural Science Foundation of China (20977026), the Research Fund for the Doctoral Program of Higher Education of China (20090161110009), the Hunan Provincial Natural Science Foundation of China (06JJ20062), and the Changjiang Scholars and Innovative Research Team in University (PCSIRT).

References

[1] T.M. Straub, D.P. Chandler, J. Microbiol. Methods 53 (2003) 185–197.
[2] S. Ram, P. Vajpayee, R. Shanker, Environ. Sci. Technol. 42 (2008) 4577–4582.
[3] C. Pöhlmann, Y.R. Wang, M. Humenik, B. Heidenreich, M. Gareis, M. Sprinzl, Biosens. Bioelectron. 24 (2009) 2766–2771.
[4] L. Heijen, G. Medema, Water Res. 43 (2009) 3124–3132.
[5] T.M. Scott, J.B. Rose, T.M. Jerkins, S.R. Farrah, J. Lukasik, Appl. Environ. Microbiol. 68 (2002) 5796–5803.
[6] H. Stender, K. Oliveira, S. Rigby, F. Bargoot, J. Coull, J. Microbiol. Methods 45 (2001) 31–39.
[7] R.Y.C. Kong, S.K.Y. Lee, T.W.F. Law, S.H.W. Law, R.S.S. Wu, Water Res. 36 (2002) 2802–2812.
[8] T. Kostić, A. Weilharter, S. Rubino, G. Delogu, S. Uzzau, K. Rudi, A. Sessitsch, L. Bodrossy, Anal. Biochem. 360 (2007) 244–254.
[9] A.P. Bauer, W. Ludwig, K.H. Schleifer, Syst. Appl. Microbiol. 31 (2008) 50–61.
[10] F.R.R. Teles, L.P. Fonseca, Talanta 77 (2008) 606–623.
[11] S. Sandhya, W. Chen, A. Mulchandani, Anal. Chim. Acta 614 (2008) 208–212.
[12] L.R. Fogarty, S.K. Haack, M.J. Wolcott, R.L. Whittman, J. Appl. Microbiol. 94 (2003) 865–878.
[13] M.W. Mittelman, M. Habash, J.M. Lacroix, A.E. Khoury, M. Krajden, J. Microbiol. Methods 30 (1997) 153–160.
[14] B. Regnault, M.D. Sylvie, L.C. Monique, M. Lefèvre, A.D.G. Patrick, Res. Microbiol. 151 (2000) 521–533.
[15] J.R. Epstein, I. Biran, D.R. Walt, Anal. Chim. Acta 469 (2002) 3–36.
[16] O. Stabnikova, X.Y. Liu, J.Y. Wang, V. Ivanov, Appl. Microbiol. Biotechnol. 73 (2006) 696–702.
[17] R. Naveen, R. Zhang, H.B. Liu, C.C. Dai, K. Rajni, R. Bandi, H.Q. Gong, Sensor Actuat. B, doi:10.1016/j.snb.2009.11.025, in press.
[18] J.C. Cho, J.M. Tiedje, Appl. Environ. Microbiol. 68 (2002) 1425–1430.
[19] C. Maynard, B. Frédéric, K. Lemarchand, H. Josée, P. Payment, P. Bayardelle, L. Masson, R. Brousseau, Appl. Environ. Microbiol. 12 (2005) 8548–8557.
[20] B.C. Lin, K.M. Blaney, A.P. Malanoski, A.G. Ligler, J.M. Schnur, D. Metzgar, K.L. Russell, D.A. Stenger, J. Clin. Microbiol. 45 (2007) 443–452.
[21] P.Z. Qin, C.G. Niu, G.M. Zeng, M. Ruan, L. Tang, J.L. Gong, Talanta 80 (2009) 991–995.
[22] J.R. Falsey, M. Renil, S. Park, S.J. Li, K.S. Lam, Bioconjug. Chem. 12 (2001) 346–353.
[23] J.B. Antje, N.C. Richard, M. Vonya, H.M. Jun, Biosens. Bioelectron. 18 (2003) 405–413.
[24] C.G. Miyada, R.B. Wallace, Methods Enzymol. 154 (1987) 97–107.
[25] X.L. Mao, L.J. Yang, X.L. Su, Y.B. Li, Biosens. Bioelectron. 21 (2006) 1178–1185.