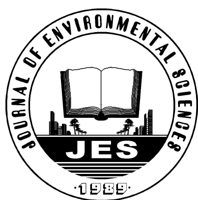

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Real-time fluorescent quantitative immuno-PCR method for determination of fluoranthene in water samples with a molecular beacon

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Abstract

A reliable and sensitive competitive real-time fluorescent quantitative immuno-PCR (RTFQ-IPCR) assay using a molecular beacon was developed for the determination of trace fluoranthene (FL) in the environment. Under optimized assay conditions, FL can be determined in the concentration range from 1 fg/mL to 100 ng/mL, with $y = 0.194x + 7.859$, and a correlation coefficient of 0.967 was identified, with a detection limit of 0.6 fg/mL. Environmental water samples were successfully analyzed, recovery was between 90% and 116%, with intra-day relative standard deviation (RSD) of 6.7%–12.8% and inter-day RSD of 8.4%–15.2%. The results obtained from RTFQ-IPCR were confirmed by ELISA, showing good accuracy and suitability to analyze FL in field samples. As a highly sensitive method, the molecular beacon-based RTFQ-IPCR is acceptable and promising for providing reliable test results to make environmental decisions.

Key words: fluoranthene; real-time fluorescent quantitative immuno-PCR; molecular beacon

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that are formed through the combustion of fossil fuel and the burning of various substances (Tsapakis and Stephanou, 2007; Zedeck, 1980). Because combustion of organic materials is involved in countless natural processes and human activities, PAHs are omnipresent and abundant pollutants in the environment (Perez et al., 2008). Therefore, they pose serious threats to the health of aquatic and human life through bioaccumulation.

The International Agency for Research on Cancer has classified several of these compounds as probable human carcinogens. In fact, the US Environmental Protection Agency has identified 16 unsubstituted PAHs as priority pollutants. One of these is fluoranthene (FL), which is currently being monitored routinely for regulatory purposes.

At present, the most common methods used for analysis of FL are gas chromatography (GC) and high-performance liquid chromatography (HPLC), while HPLC is the standard method for determination of FL in China. However, these methods are known to manifest underlying disadvantages such as complicated pretreatments, high costs, and time-consuming processes. Moreover, they are deemed unsuitable for detecting very low quantities of PAHs in the environment. Sensitive and reliable analytical methods are therefore required to evaluate the presence of PAHs at very low concentrations in the environment. At present, a highly sensitive and specialized method, enzyme-linked immuno sorbent assay (ELISA), is being studied and utilized to monitor endocrine disruptors in the environment. Based on ELISA, another highly sensitive antigen detection system, immuno polymerase chain reaction (IPCR) is being developed for the above-mentioned purposes (Sano et al., 1992; Fránek et al., 2001; Hinfray et al., 2006; Wang et al., 2006). A number of research applications describe the advantages of the method (Niemeyer et al., 1997, 1999; Loge et al., 2002), that is, in particular, its high sensitivity and good quantification capabilities due to the great linearity and compatibility with established ELISA protocols.

Further development in the technology and instrumentation used for the signal detection of IPCR has resulted in the development of real-time IPCR (RT-IPCR). RT-IPCR is still relatively undeveloped in comparison to the use of both real-time PCR and IPCR.

The primary advantage of using RT-IPCR in place of IPCR is the immediate interpretation of positive data (quantification of proteins) as the PCR reaction proceeds. To date, only a few mono- or polyclonal antibody-based immunoassays for PAHs have been reported (Ye et al., 2009; Zhou et al., 2008; Zhuang and Zhou, 2009). However, the detection of FL using RT-IPCR has not been

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reported. In this article, a new method was developed to establish a selective RT-IPCR assay for the determination of trace FL in the environment. Molecular beacon (MB) probe, which is widely used in biology and clinical medicine (Orru et al., 2006; Zuo et al., 2007; Ma et al., 2007; McKillen et al., 2007), was selected as the DNA marker for the determination of FL in water samples.

1 Materials and methods

1.1 Reagents and chemicals

Phosphate-buffered Saline-Tween (PBST) solutions were prepared from 0.01 mol/L buffer salts, pH 7.4, 0.15 mol/L NaCl, and 0.05% (*V/V*) Tween 20. The carbonate buffer solution (CBS) was 0.2 mol/L at pH 9.6. Hot start fluorescent PCR core reagent kit, DNA PCR kit, UNIQ-10 PCR DNA extraction kit, and avidin were procured from Shanghai Sangon Biological Engineering Technology and Service (China). Ovalbumin (OVA) was purchased from Sino-American Biotechnology (USA). The standard samples of anthracene, naphthalene, fluorescence, and phenanthrene were bought from AccuStandard, Inc. (USA), while the article antigens of FL, Anti-FL polyclonal antibodies and biotinylated polyclonal antibodies of FL were developed in our laboratory, freeze-dried, and stored at –20°C for subsequent use.

All chemicals for the preparation of buffers and reagents were of analytical reagent grade, unless specified otherwise.

1.2 Preparation of reporter DNA

The reporter DNA was produced through PCR amplification of the pUC18 plasmid purchased from Shine Gene Bio, Inc. (Shanghai, China) using the biotinylated forward and reverse primers 5′-CTGACTCCCCGTCGTGTAGA-3′ and 5′-GCTGGCTGGTTTATTGCTGAT-3′, respectively. The PCR products were analyzed on 1.5% agarose gel stained with 0.5 g/mL ethidium bromide. Agarose gels were then examined in a UV light vision system (Beijing Purkinje General Instrument Co., China) after electrophoresis. The target DNA samples (121 bp) were recovered and purified using a UNIQ-10 PCR DNA extraction kit.

1.3 Preparation of molecular beacon probe

Based on the sequence of the pUC18 plasmid, we designed the molecular beacon probe using the Primer Premier v5.0. The probe is represented as 5′-FAM-CAGCGATCTGGCCCCAGTGCTGCAATCGCTG-DABCYL-3, with the underlined portions representing the five base stems. The probe was labeled with fluorescein (6-FAM) at the 5′ end and quencher 4-(4-dimethylaminophenylazo) benzoic acid (DABCYL) at the 3′end. It was synthesized by Shine Gene Bio., Inc. (Shanghai, China) before storage in the dark at –20°C.

1.4 RTFQ-IPCR

The processes of the direct competitive RTFQ-IPCR are shown in Fig. 1. First, a 20-μL volume FL-OVA coated antigen (20 μg/mL) solution was injected into a polypropylene PCR-tube, which was treated with 0.8% glutaraldehyde solution to improve absorbability. The tubes were sealed and incubated at 4°C overnight to allow sufficient adsorption of the coated antigen onto the tube wall. The tubes were washed three times with PBST (PBS and 0.05% Tween 20) to remove unbound and other dissociative compounds. The residual adsorption sites were blocked by a 200-μL blocking buffer (PBS with 1% OVA) at 37°C for 30 min. Upon washing the tubes as described in the first step detailed above, 10 μL FL molecular dilutions of different concentrations (from 1 fg/mL to 100 ng/mL) and biotinylated capture antibody were added to each tube, with each set at 37°C for 1 hr. During this step, the FL-OVA antigen and FL molecules compete to combine with the biotinylated antibodies. Three washing steps were performed, after which the unbound and other dissociative compounds were removed, while those present in the FL-OVA-biotinylated antibodies remaining. Next, 20 μL avidin was added to link with the attached biotinylated antibodies before incubation at room temperature for 30 min. Avidin was used as bridge to link the biotinylated reporter DNA to the biotinylated FL antibody. After undergoing the same washing process as above described, biotinylated DNA was added, after which the mixtures were incubated at room temperature for 30 min. Finally, the wells were washed five times with PBST and Milli-Q water, respectively. This was done to fully remove the unbound biotinylated DNA and other dissociative leftover compounds. All the processes mentioned were completed for the preparation of the real-time PCR assay.

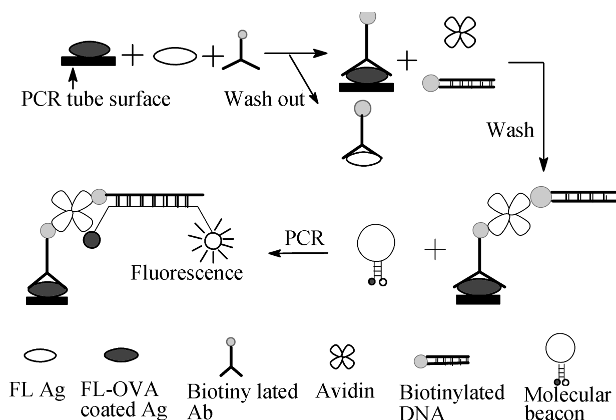

Fig. 1 MB-based direct competitive real-time immune-PCR assay process. The PCR tube surface was used as the coated antigen carrier. FL-OVA and FL Ag competed to combine the biotinylated Ab and the biotinylated DNA linked the remaining biotinylated Ab by avidin. When the PCR assay was carried out, the molecular beacon opened the stem-loop, and combined with the biotinylated ssDNA; the fluorescence was recorded at the annealing stage every cycle.

PCR was performed directly in PCR tubes using the Rotor-gene 3000 real-time rotary analyzer (Corbett Life Science Corbett Robotics Inc., Australia). The reaction mixture, in a final volume of 50 μL, contained Hot Start Fluo-PCR mix (1×), 0.5 μmol/L of each primer, 0.5 μmol/L of MB probe, and the rest was Milli-Q water. The

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PCR parameters were: 2 min at 55°C; 4 min at 94°C; then 30 cycles consisting of 15 sec at 94°C, 30 sec at 55°C, and 20 sec at 72°C. Fluorescence was measured at an annealing temperature of 55°C. Thus, in the assay of this study, a complete test from the immunoassay to completing the PCR assay, it may cost about four hours and 10 μL volume of sample.

1.5 Determination of water samples

In order to assess the validity of the proposed method, FL in double-distilled water, tap water, and water samples from Suzhou River (Shanghai, China) were determined. Tap water and river water samples were collected in bottles, filtered and adjusted to pH 7.5 with 1 mol/L HCl or 1 mol/L NaOH, and then stored at 4°C until required.

The same water samples were performed by ELISA to assess the results from RTFQ-IPCR.

2 Results and disscusion

2.1 Optimized conditions in RTFQ-IPCR

In the immunoassay, the solution used as a blocking reagent could provide good cover over the antigen-OVA on the tube surface. Three sets of blocking buffers were tested, and PBST with 1% OVA produced the best results.

To select the optimal recording temperature for the real-time PCR assay which could decrease the background signal effectively, we investigated the relation between MB probe concentration and annealing temperature. According to the Tm value of the primers, we set the annealing temperatures at 53, 55, and 60°C, and analyzed three concentration series of the probe (0.4, 0.5, and 0.6 μmol/L) at these temperatures. We then recorded the fluorescence intensities of the molecular beacon and the background signals at each temperature and concentration combination. It was observed that at 55°C and 0.5 μmol/L the highest intensity of fluorescence and signal-to-background ratio were obtained, which could be attributed to the primers. Hence, the optimal annealing temperature is 55°C and the concentration of MB is 0.5 μmol/L.

Different concentrations of avidin (25, 12.5, and 6.5 ng/mL) in the PBS solution were likewise tested. The 12.5 ng/mL-avidin solution was selected as the best concentration.

To assess the influence of reporter DNA concentration (original concentration of 37.5 ng/mL) on the PCR, we took the diluted DNA at 1:100, 1:500, and 1:700 ratios for the experiment. Based on the high specificity of the MB probe and the amplifying efficiency of PCR, the results proved that 1:500 DNA was the most appropriate for the assay.

2.2 Cross-reactivity

To define the specificity of our method, we selected the samples (1-naphthoxy acetic acid, 2-naphthoxy acetic acid, anthracene, and phenanthrene) in similar molecular structures with FL acting as FL substitutes, and performed the same immuno-PCR assay five times. In addition, three parallel collators from each concentration were tested. The cross-reactivity of the immunoassay toward the compounds applied was obtained using 50% inhibition value (IC_{50}) as the evaluated criterion. All of these assays showed negative results, and the cross-reaction ratios were below 11.6%. It indicated that there was low cross-reaction ratio and non-specific binding in our assay.

2.3 Amplifying curves and standard curves

Since the threshold cycle Ct value represented the PCR cycle at which the fluorescent intensity rose above the threshold, it could be used to quantify the input target concentration (Sandhya et al., 2008). The sensitivity of the system was assessed after using standard samples with known, isolated concentrations of tenfold dilution series (Fig. 2). To cover a wide detection range with the RTFQ-IPCR, FL concentrations were formulated in a tenfold dilution series from 1 fg/mL to 10^8 fg/mL. The standard curves are shown in Fig. 3. The fluorescent threshold was automatically set by the cycler instrument, and was defined as the mean standard deviation of fluorescence in the sample tube over baseline cycles. In Fig. 2, the fluorescence signal of the curve (100 ng/mL) reaching the threshold was at around cycle 9.6, and there was a fall in Ct

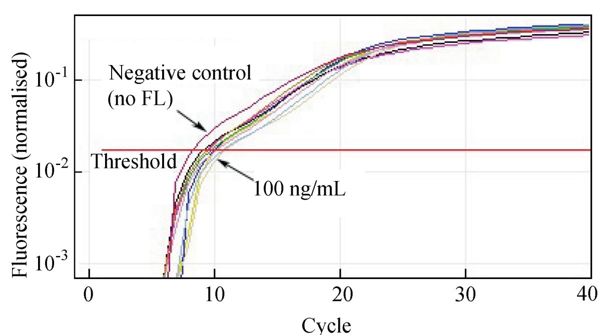

Fig. 2 Fluorescence signal data for each cycle of FL amplification. The curves delegated the amplifying efficiency of the dilution series of FL ranging from 1 fg/mL to 100 ng/mL, including negative control (no FL) on the far left.

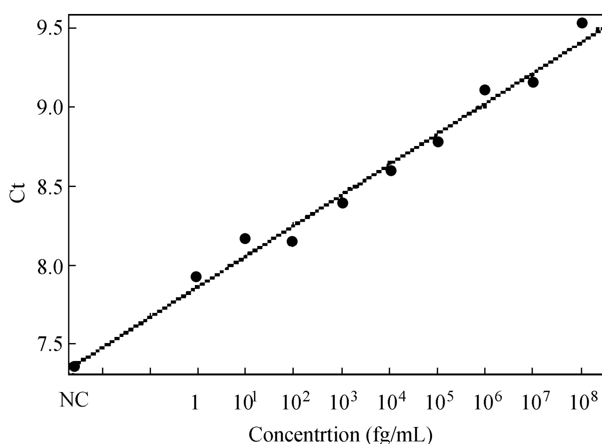

Fig. 3 The FL standard curves. RTFQ-IPCR was performed on serial dilutions of known concentrations of NA, equivalent to ten-fold dilutions from 1 fg/mL to 10^8 fg/mL. Correlation coefficient 0.967; $y = 0.194x + 7.859$. The point at the bottom-left delegated the negative control (NC) sample (no FL).

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Table 1 Recovery and precision of spiked samples tested by RTFQ-IPCR

Samples	FL levels (pg/mL)	FL added (pg/mL)	Total found (pg/mL)	Recovery (%)	RSD (%) (n = 9)	
					Intra-day	Inter-day
Double-distilled water	No detected	0.005	0.005	100	12.8	15.2
		0.01	0.011	110	11.7	14.8
		0.050	0.053	106	9.9	14.0
Tap water	0.2	0.05	0.258	116	9.4	13.7
		0.1	0.314	114	9.2	12.6
		0.5	0.721	104	8.8	12.2
River water	75	10	86	110	8.2	10.1
		50	120	90	7.5	9.6
		100	182	107	6.7	8.4

RSD: relative standard deviation.

value from 100 ng/mL to 1 fg/mL. It implied that the time expended to reach threshold for the higher concentration of FL molecules was much longer compared to other lower concentrations. This can be explained as follows: if there were more FL molecules added to compete and combine with the biotinylated antibodies, there would be fewer biotinylated antibodies combined with the coated antigen, which adsorbed on the tube surface; therefore, there were fewer compounds after washing. Similarly, the biotinylated DNA that integrated with the MB probe were fewer, so more time was needed to reach the threshold. The curve of negative control (no FL) was a good example.

The standard curves (Fig. 3) displayed a correlation coefficient of 0.967, with y = 0.194x +7.859, and this quantification proved to be linear over a wide range of initial target concentrations (from 1 fg/mL to 100 ng/mL). The good correlation coefficient of the standard curves guaranteed the correctness of the quantification.

2.4 Analysis of real samples

Standards and samples were run nine times and the mean Ct values were recorded. The results showed that the presence of FL in these water samples was trace due to its insolubility in water, and demonstrated that these water samples were away from FL pollutants. The recovery rates were between 96% and 118.3%, and the results for intra-day and inter-day precision were obtained and showed in Table 1. For intra-day assay, the relative standard deviation (RSD) within a batch was below 12.8%, and for inter-day assay, the RSD were higher and below 15.2%. The results show that the recovery and reproducibility of the proposed method are satisfactory.

To validate the results obtained from RTFQ-IPCR, the same samples were tested by enzyme-linked immuno sorbent assay (ELISA). We obtained the standard curve of inhibition from ELISA, y = 8.21log$x$ + 47.51, and a correlation coefficient of 0.9987 was identified, with a detection limit (IC_{20}) of 0.45 pg/mL. The results from ELISA showed that there was no FL found in either the double-distilled water or tap water. The probable reason is that the amount of FL is too low in these two samples to be detected with ELISA for its higher limit of detection of 0.45 pg/mL. However, there was about 68 pg/mL of FL tested by ELISA in river water, and the recovery tested by ELISA was about 89%–106%. The results from two methods were close within margin of error, showing a good accuracy and suitability to analyze FL in field samples by MB-based RTFQ-IPCR.

2.5 MB-based RTFQ-IPCR

We introduced this method to detect the environmental pollution, and found a novel way in detecting method research, especially in the microanalysis of samples, and the increased sensitivity should improve the efficiency and reduce costs. The large amount of statistical data collected during this work and the sensitivity would underline the enormous potential of the RTFQ-IPCR as an ELISA-enhancing detection tool.

The standard curve displayed a correlation coefficient of 0.967, which may be not as precise as that of an optimized RTFQ-PCR amplification of a DNA template alone. Unlike the RTFQ-PCR protocol, the RTFQ-IPCR protocol involves the primary step of antibodies' recognition of FL. This variable in the RTFQ-IPCR test may account for a loss of precision when compared to a standard curve generated by RTFQ-PCR of a DNA template alone. However, a semi-quantitative dose response was evidenced by an increasing Ct value with increasing concentrations of FL.

On the other hand, as a marker for the reporter DNA, the MB probe made the RTFQ-PCR assay more efficient and precise. The MB-based RTFQ-PCR assay made real-time quantitative detection of specific targets directly in the PCR tubes possible. Reagents were mixed in one step, and reactions were carried out in closed tubes to prevent contamination. Data were recorded during each cycle and results were analyzed immediately after the completion of the reaction, usually within 2 hr. The most powerful aspect of MB was its capability to distinguish false positive results from PCR amplification. The critical cycle (Ct) is inversely proportional to the logarithm of the initial number of target molecules. Perhaps this would be the most popular tool in overcoming false positive results. Owing to their high sensitivity and excellent specificity, MBs were widely used in chemistry, biology, biotechnology, and medical sciences for biomolecular recognition. MB is a useful and promising tool not only in DNA/RNA studies or DNA-protein interactions (Wang et al., 2005; Patel et al., 2006; Churruca et al., 2007; Yeo et al., 2005), but also in monitoring organic pollutants in the environment.

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3 Conclusions

Greatly enhanced detection sensitivity is the major advantage of RTFQ-IPCR. Using a molecular beacon, FL can be detected by RTFQ-IPCR in both pure cultures and environmental samples at concentrations ranging from 1 fg/mL to 100 ng/mL with a detection limit calculated from ten times the standard deviation of the blank is 0.6 fg/mL. Although the RTFQ-IPCR method is not yet reproducible to the standards required for environmental monitoring, there is little doubt that it has the potential with continued refinement to become the most sensitive analytical method for the detection of environmental pollutants.

Acknowledgments

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