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Tag/hybridization-based sensitive detection of polymerase chain reaction products

Kousuke Niwa [a,*], Akinobu Oribe [a], Hidemasa Okumura [a], Masahiro Shimono [a], Kenkichi Nagai [a], Toshikazu Hirota [a], Hiroshi Yasue [b], Mitsuo Kawase [a,c]

[a] Future Technology Management Center, Corporate R&D, NGK Insulators, Mizuho, Nagoya 467-8530, Japan
[b] Tsukuba Gene Technology Laboratories, Tsuchiura, Ibaraki 300-0873, Japan
[c] Graduate School of Biomedical Engineering, Tohoku University, Aoba, Sendai 980-8579, Japan

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ABSTRACT

The polymerase chain reaction (PCR) is an important technology to amplify a single copy or a few copies of DNA segment in genomic DNAs, visualizing the segment as DNA fragment. Thus, PCR is frequently used in various examinations such as detection of bacteria and fungi in the food industry. Here, we report a simple and sensitive method for detection of PCR products using single-strand tag sequence and hybridization of the tag sequence to the complementary tag sequence immobilized on solid material (STH). The detection sensitivity was found to be at least 50 times higher than electrophoresis/ethidium bromide (EtBr) visualization for approximately a 500-bp fragment and higher than the ordinary hybridization, that is, hybridization of denatured PCR product to probe sequence immobilized on solid material.

The polymerase chain reaction (PCR)[1] was developed by Mullis and Falloona in 1985 to detect a single copy or a few copies of DNA segment in DNA sequences [1–4]. Since then, PCR has been increasingly used in various fields, for example, the detection of pathogenic agents in medicine [5,6] and the food industry [7]. Simplification of the PCR product detection system and more sensitive system has been demanded with growing commitment of PCR in biological research and industries. Currently, ethidium bromide (EtBr) staining of PCR products after gel electrophoresis [8], hybridization with labeled probe [9], incorporation of label into PCR product [10], and silver staining of PCR products after gel electrophoresis [11] have been used for the detection of PCR products. Of these detection methods, EtBr staining is most frequently used because of its simplicity and low cost.

However, the EtBr staining method presents some difficulties such as time-consuming electrophoresis and the fact that EtBr is a carcinogenic agent [12,13]. In the current study, we devised a simple and sensitive method using single-strand tag/hybridization (STH) for the detection of PCR product.

Materials and methods

Primer design

The detection method devised in the current study, STH, is schematically described in Fig. 1. The essence of the current method is as follows. One of the primer pairs (designated STH primer) consists of two sequences; the 3′ part of the sequence was the primer sequence that was designed to amplify the specific DNA sequence, and the 5′ part was the 23-mer tag sequence [14]. The two parts were joined through three-carbon spacer, $(CH_2)_3$, using Phosphoramidite C3 spacer (Glen Research, Sterling, VA, USA; http://www.glenresearch.com/ProductFiles/10-1913.html). The insertion of three-carbon spacer terminates DNA synthesis of Taq DNA polymerase at the insertion site, so that the primer sequence from the site to 3′ terminus is left as a single strand in the PCR [15].

The other of the primer pair was designed to amplify the specific DNA sequence together with the primer described above and labeled with Cy3 at its 5′ end (Nihon Gene Research Laboratories, Sendai, Japan). In the current study, the primer sequences were designed to amplify a DNA segment encompassing rs6720173 (SNP) using human genomic DNA (Japan Health Sciences Foundation, Tokyo, Japan) (Table 1).

As a control of STH primer, a primer was designed to connect the 3′ part of the STH primer sequence with a complementary

* Corresponding author. Fax: +81 52 872 7537.
E-mail address: kniwa@ngk.co.jp (K. Niwa).

[1] *Abbreviations used:* PCR, polymerase chain reaction; EtBr, ethidium bromide; STH, single-strand tag/hybridization; cTag, complementary tag; NHS, *N*-hydroxysuccinimide; SSC, saline sodium citrate; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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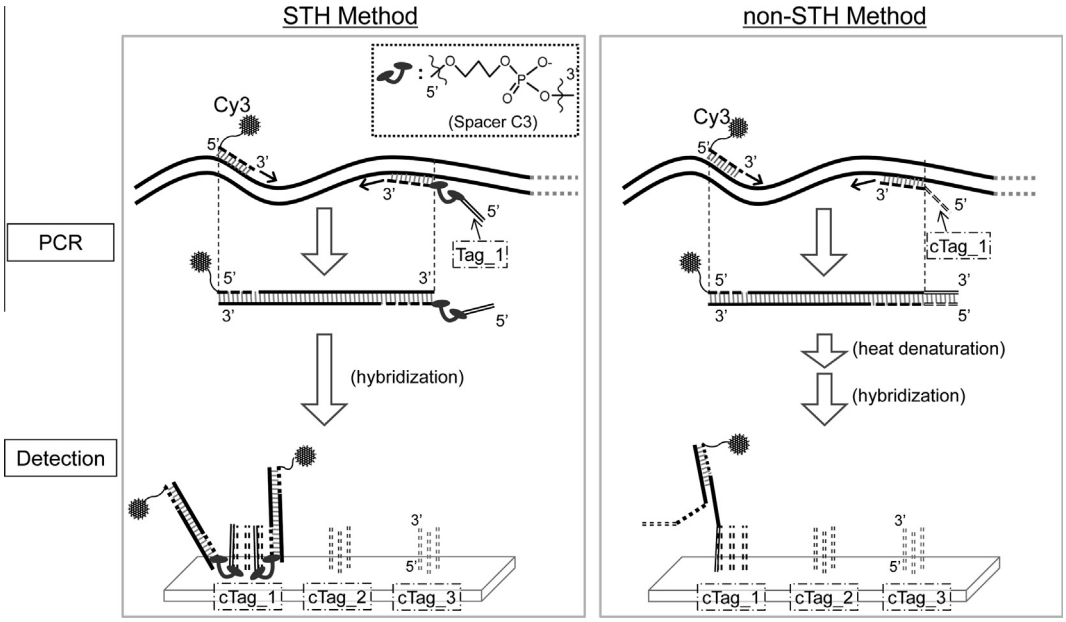

Fig.1. Schematic presentation of STH and non-STH PCR detection systems.

Table 1
Primer sequences of STH and non-STH systems for amplification of a DNA segment for human genomic DNA.

Primer		Sequence (5′ to 3′)
Forward	Common	[Cy3]ACCAAAGAATATGGCTGAATTTAGTAGTGTTTTAAATAATTTTAA
Reverse	STH/Tag_1	gcagattcattggtcagagaacaXACCTGCTAATGAGATGATCCCTTATTTTGAAAACAACTATTCCTA
	STH/Tag_t (control)	ttttttttttttttttttttXACCTGCTAATGAGATGATCCCTTATTTTGAAAACAACTATTCCTA
	Non-STH/ cTag_1 (control)	tgttctctgaccaatgaatctgcACCTGCTAATGAGATGATCCCTTATTTTGAAAACAACTATTCCTA
	Non-STH/ cTag_t (control)	aaaaaaaaaaaaaaaaaaaaaaaACCTGCTAATGAGATGATCCCTTATTTTGAAAACAACTATTCCTA

Note. Uppercase letters of primer sequences represent sequences necessary for amplification of human genomic DNA segment, and lowercase letters represent tag sequences. "**X**" represents "spacer C3".

tag (cTag) sequence without spacer (non-STH primer), which enables PCR to proceed to the 3′ end of the primer (Table 1).

Detection of PCR product

The single-strand cTag sequences joined with EC amino linker (Sigma–Aldrich Japan, Hokkaido, Japan) in polyethylene glycol aqueous solution (10%, w/v) were spotted on an *N*-hydroxysuccinimide (NHS) ester-activated glass slide, GeneSlide (10 fmol/spot; Toyo Kohan, Tokyo, Japan), with a diameter of approximately 100 μm, and immobilized by amidation of NHS ester on the glass surface. The glass slides were incubated at 80 °C for 60 min and then washed successively with 2× saline sodium citrate (SSC) containing 0.2% sodium dodecyl sulfate (SDS) for 15 min at room temperature, with 2× SSC containing 0.2% SDS for 5 min at 90 °C, and with water for 1 min, followed by drying at room temperature. The DNA array layout on the glass slide and its signal image are shown in Fig. 2.

PCR was performed using primer sets and template DNA described above following the procedure described by Nishida and coworkers [14]. The PCR product generated by non-STH primer pair was first subjected to electrophoresis in a 4% NuSieve 3:1 agarose gel and subsequent staining with EtBr to confirm that the PCR product was the expected size.

Then, the PCR product generated by STH primer pair was purified with a MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA). The amount of the purified PCR product was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The PCR product obtained in this manner was serially diluted with a buffer consisting of 10 mM Tris–HCl (pH 8.0) and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The diluted PCR products were electrophoresed in a 4% NuSieve 3:1 agarose gel (Fig. 3) to examine their integrity and visibility with EtBr staining.

After the examination, the diluted PCR products were subjected to hybridization detection on the glass slide, to which cTag sequence had been fixed as a spot. Prior to the hybridization, non-STH PCR products were denatured at 90 °C for 1 min and chilled quickly to make single-strand DNA. Then, the STH and non-STH PCR products were hybridized with cTag sequence on the slide according to the following procedure. Hybridization was performed at 37 °C for 30 min in 10 μl of hybridization mixture consisting of 0.5× SSC (0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS, 15% formamide, 1 mM EDTA, and an appropriate amount of PCR product. After the hybridization, the slide was washed once with a solution consisting of 1× SSC and 0.1% SDS for 3 min at 25 °C and then twice with 1× SSC for 1 min at 25 °C. The resulting glass slide was dried by spinning and subjected to measurement of hybridization signal (i.e., Cy3 signal) using a laser fluorescent scanner (GenePix 4000B and GenePix Pro 6.1 software package; Molecular Devices, Sunnyvale, CA, USA).

Results and discussion

As described in Materials and methods, primer pairs for STH and non-STH PCRs were used for amplification of DNA segment