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Rapid, highly sensitive and highly specific gene detection by combining enzymatic amplification and DNA chip detection simultaneously

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ABSTRACT

We have developed a novel gene detection method based on the loop-mediated isothermal amplification (LAMP) reaction and the DNA dissociation reaction on the same DNA chip surface to achieve a lower detection limit, broader dynamic range and faster detection time than are attainable with a conventional DNA chip. Both FAM- and thiol-labeled DNA probe bound to the complementary sequence accompanying Dabcyl was immobilized on the gold surface via Au/thiol bond. The LAMP reaction was carried out on the DNA probe fixed gold surface. At first, Dabcyl molecules quenched the FAM fluorescence. According to the LAMP reaction, the complementary sequence with Dabcyl was competitively reacted with the amplified targeted sequence. As a result, the FAM fluorescence increased owing to dissociation of the complementary sequence from the DNA probe. The simultaneous reaction of LAMP and DNA chip detection was achieved, and 10^3 copies of the targeted gene were detected within an hour by measuring fluorescence intensity of the DNA probe.

1. Introduction

The DNA chip (microarray) is widely used in genotyping and gene expression analysis as a tool capable of detecting two or more genes simultaneously [2,16,19,20]. However, it is suitable for use only in research since the detection limit, dynamic range and detection time are insufficient compared with the enzymatic amplification reaction of nucleic acid sequences such as the real-time polymerase chain reaction (PCR) [1,8] and loop-mediated isothermal amplification (LAMP) [10,21]. One possible way of making a DNA chip with a lower detection limit, broader dynamic range and faster detection time is to combine enzymatic amplification and a DNA chip. But there is serious problem concerning composition of the reaction solution. A DNA chip is based on the hybridization reaction with the DNA probe and targeted gene on the solid surface. This solid phase reaction is highly salt-dependent. The commonly used hybridization solution 2xSSC contains 300 mM sodium chloride [11]. On the other hand, DNA polymerase using enzymatic amplification reaction is not resistant to high salt concentration since it usually uses 50 mM sodium chloride for PCR and 10 mM potassium chloride for LAMP [6]. The rate of hybridization on 300 mM monovalent cation concentration is a thousand times higher than that of 30 mM [3,4,24]. Consequently, hybridization and amplification reaction are incompatible, making it necessary to carry out a divided solution [7,14,22].

Combining the enzymatic amplification and DNA chip detection, we have developed a novel gene detection method based on the LAMP reaction and DNA dissociation reaction on the same DNA chip surface simultaneously. LAMP is isothermal gene amplification method conducting a constant temperature (60–65 °C). The targeted sequence is exponentially amplified over 10^9 fold within an hour [15] and a large quantity of amplicon that has a sequence specific to the complementary sequence is produced. So it is suitable for competitive dissociation reaction on the DNA chip surface. The novel gene detection method is expected to enhance the applicability and utility of conventional DNA chips.

2. Materials and methods

2.1. Materials

A plasmid DNA (4 kbp) having parvovirus VP2 gene was used as a model for verification of a novel gene detection method. Concentration of the plasmid DNA was estimated by measuring the absorbance at 260 nm (A260 of 1.0 = 50 μg/mL) then it was diluted using TE buffer (10 mM Tris–HCl (pH 8), 1 mM EDTA) to the appropriate concentration. The oligonucleotides were purchased from Fasmac Co. Ltd. and Life Technologies Japan. The primers for LAMP reaction were 5′-GAACATCATCTGGATCTGTACCAACCATCTCATACTGGAACT AGTGGC-3′ (FIP), 5′-CTGTGCCAGTACACTTACTAAGAGTGTTAGTCTA CATGGTTTACAATC-3′ (BIP), 5′-GAGATATTATTTTCAATGGGATAG AAC-3′(F3), 5′-CAATGCTCTATTTGTTTGCCATG-3′ (B3), and 5′-TGGT

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ATATATTTGTTGGTGTGCC-3′ (Lpf). *Bst* polymerase used for LAMP reaction was obtained from New England Biolabs. All other chemicals used were of analytical grade. All buffers were prepared with a Milli-Q system (Millipore). The Pyrex glass substrate (20 mm × 30 mm × 8 mm) sputtered with titanium (500 nm) and gold (2000 nm) was purchased from the Toshiba Hokuto Electronics.

2.2. Methods

2.2.1. The principle of novel gene detection method

Fig. 1 shows the principle of the simultaneous reaction both enzymatic amplification reaction (LAMP) and DNA dissociation reaction on the same DNA chip surface.

The DNA probe is labeled with thiol at 3′ end and carboxyfluorescein (FAM) as a fluorophore at 5′ end. The probe bound to a complementary sequence is immobilized on the gold substrate via Au/thiol bond. The complementary sequence accompanying Dabcyl quencher molecule at 3′ end is specific to a targeted gene. Dabcyl molecules quench the FAM when the DNA probe formed double-stranded structure with complementary sequence [18].

Firstly, the DNA probe is bound to complimentary sequence accompanying Dabcyl molecules so fluorescence derived from the DNA probe is quenched and only weak fluorescence is observed on the gold surface. Then LAMP reaction is carried out on the DNA probe fixed gold surface. The targeted DNA is exponentially amplified and large quantity of amplicon which has specific sequence of the targeted gene is produced. The complementary sequence bound to the DNA probe is competitively reacted with the amplified sequence of the targeted gene during LAMP reaction and it dissociates from the DNA probe. The FAM molecule is released from Dabcyl molecule then the fluorescence of FAM is unquenched. The fluorescence signal of FAM increases according to the dissociation of the complementary sequence with Dabcyl. We can quantify the amount of targeted DNA by the event of fluorescence increase during LAMP reaction.

2.2.2. Immobilization of DNA probe on gold surface

The gold surface of the Pyrex substrate was cleaned by UV-ozone cleaner UV-208 (UVFAB Systems) for 30 min. Then the substrate was immersed in ultrapure water for 15 min and dried by blowing air.

3 μM of oligonucleotides labeled with FAM and Dabcyl was mixed in 20 mM PIPES buffer (pH 7) and then incubated at 37 °C for 30 min in order to form double-stranded structure. After incubation, 1 μL of DNA probe solution was spotted on the gold surface and left at room temperature until dry (few minutes). The spot size of dried DNA probe was about 2 mm. Then, the substrate was washed with 10 mM phosphate buffer (pH 7) and dried by blowing air. The DNA probe fixed substrate was stored at −20 °C until use.

2.2.3. LAMP reaction on the gold surface

LAMP reagent containing primers, *Bst* polymerase, dNTPs and buffers was described previously [13]. 25 μL of LAMP reagent was added to the cover well PC20 (0.2 mm × 13 mm), Grace Bio-Labs), and then gold substrate with DNA probe was closely attached to the cover well. LAMP reaction was carried out at 63 °C using a hot plate. After LAMP reaction, the gold substrate was washed with 10 mM phosphate buffer and then dried by blowing air.

2.2.4. Detection of fluorescence image

Fluorescence image of gold surface was captured by Typhoon8620 (GE Healthcare) with 532 nm laser (excitation wavelength) and 526 nm short-pass filter (emission wavelength) for FAM fluorophore. The substrates were set on the scanner plane with 3 mm spacer and scanned at 200 μm pixel size. The fluorescence intensity of the DNA

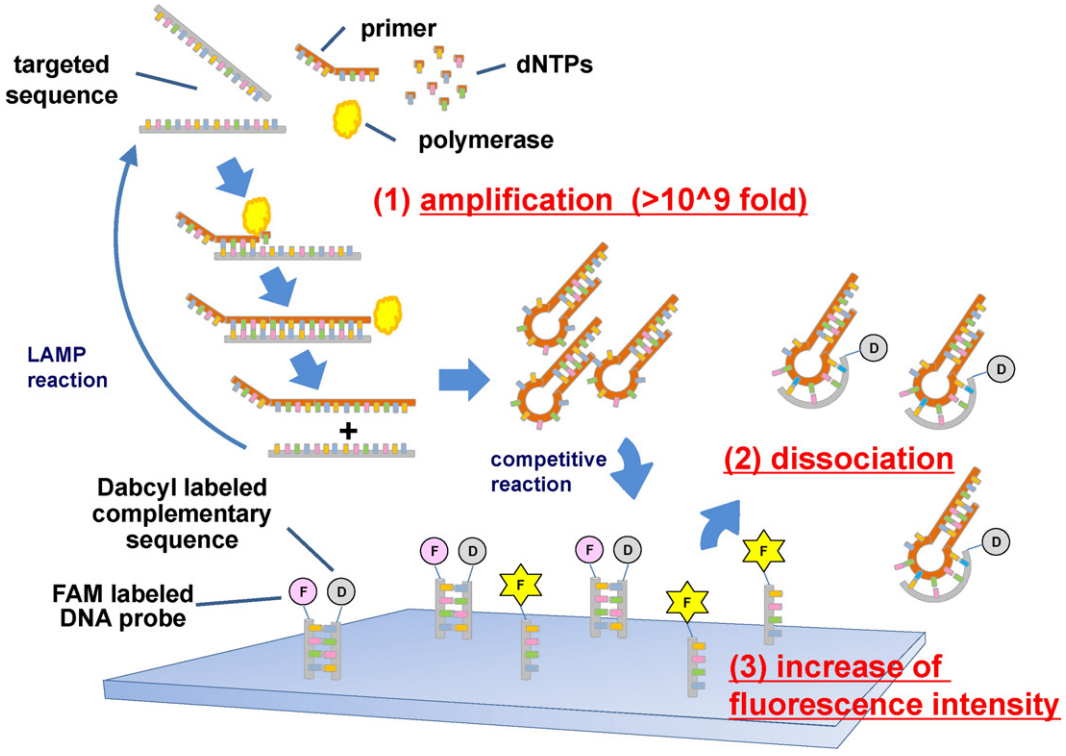

Fig. 1. The principle of a novel gene detection method based on the enzymatic amplification reaction and DNA dissociation reaction on the same DNA chip surface.