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Universal Quenching Probe System: Flexible, Specific, and Cost-Effective Real-Time Polymerase Chain Reaction Method

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We have developed a flexible, specific, and cost-effective real-time polymerase chain reaction (PCR) method. In this technique, a quenching probe (QProbe) and a nonfluorescent 3′-tailed probe are used. The QProbe is a singly labeled oligonucleotide bearing a fluorescent dye that is quenched via electron transfer between the dye and a guanine base at a particular position. The nonfluorescent 3′-tailed probe consists of two parts: one is the target-specific sequence on the 5′ side, and the other is complementary to the QProbe on the 3′ side. When the QProbe/nonfluorescent 3′-tailed probe complex hybridizes with the target in PCR, the fluorescence of the dye is quenched. Fluorescence quenching efficiency is proportional to the amount of the target. We called this method the universal QProbe system. This method substantially reduces the cost of real-time PCR setup because the same QProbe can be used for different target sequences. Moreover, this method allows accurate quantification even in the presence of nonspecific PCR products because the use of nonfluorescent 3′-tailed probe significantly increases specificity. Our results demonstrate that this method can accurately and reproducibly quantify specific nucleic acid sequences in crude samples, comparable with conventional TaqMan chemistry. Furthermore, this method is also applicable to single-nucleotide polymorphism (SNP) genotyping.

Nucleic acids (DNA and RNA) are among the most fundamental molecules in all life forms. Specific nucleic acid sequence quantification is essential in biological and biomedical studies, such as those of medical diagnostics, gene expression analysis, genotyping of individuals, detection of infectious diseases, and examination of genetically modified organisms.[1−4] Many analytical methods for specific nucleic acid sequence quantification have already been developed. In particular, real-time polymerase chain reaction (PCR)[5−11] has been most widely used because of its rapidity, high sensitivity, high reproducibility, and low risk of carryover contamination.

Real-time PCR is a method that combines the amplification of the gene of interest (target) and the detection of amplified products at each reaction cycle. The cycle at which the amount of amplified products attains a certain preset value is inversely related to the starting quantity of the target. The accumulation of the amplified products is monitored in real time using DNA-binding fluorophores such as SYBR Green I,[6] or sequence-specific fluorescent probes such as TaqMan probes,[7] molecular beacons,[8] and quenching probe (QProbe).[9−11]

A DNA-binding fluorophore is incorporated into double-stranded DNA, causing fluorescence to increase. An increase in the amount of amplified products during PCR leads to an increase in fluorescence intensity. DNA-binding fluorophores can be used in conjunction with any primers for any target without an extra probe design step, which is complicated. However, if nonspecific PCR products such as the primer−dimer complexes are present, the fluorophores will bind to these products and generate fluorescent signals. Therefore, methods using DNA-binding fluorophores are less specific than probe-based detection methods.

Sequence-specific fluorescent probes only bind to the amplified products containing sequences that are complementary to the probe. The use of sequence-specific fluorescent probes significantly increases specificity and allows quantification even in the presence of nonspecific PCR products; however, more than one sequence-specific fluorescent probe is required to be designed, synthesized, and tested for each target sequence of interest in general, which is expensive and troublesome in practical use.

Recently, simple and cost-effective methods using a universal-tailed primer have been developed.[12−14] The universal-tailed

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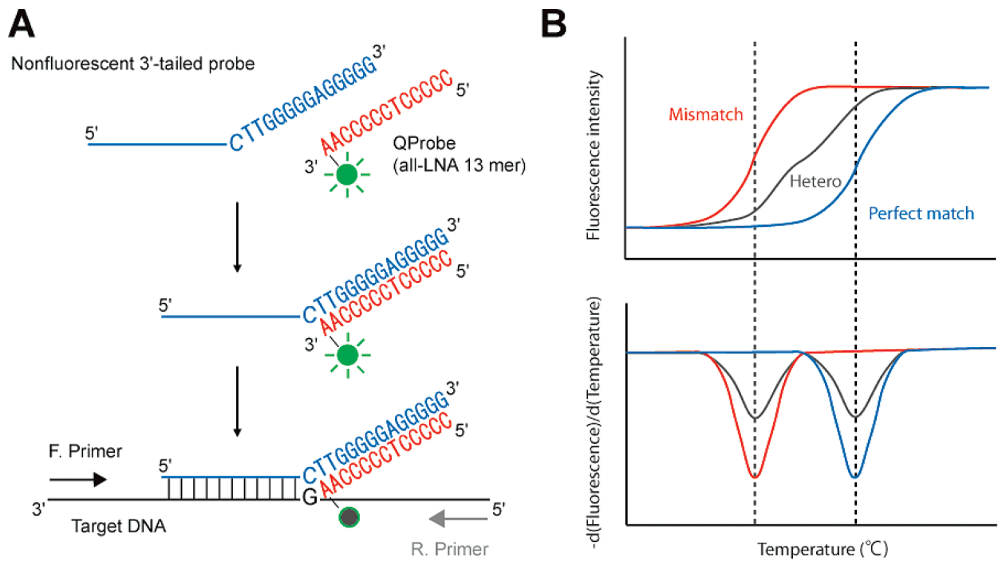

Figure 1. (A) Schematic representation of the universal QProbe system for real-time PCR. The QProbe is an all-locked nucleic acid (LNA) 13 mer oligonucleotide. The nonfluorescent 3′-tailed probe is designed to possess a C base at the 3′-end in the target-specific part. The target should possess a G base complementary to the C base at the 3′-end in the target-specific part of the nonfluorescent 3′-tailed probe to quench fluorescence. When the QProbe/nonfluorescent 3′-tailed probe complex hybridizes with the amplified target at the annealing temperature, the fluorescence of the dye is quenched by the guanine base in the target. The presence of other guanine bases at the 5′ side in the target also slightly quenches fluorescence.[9] This is preferable, but not necessary. The fluorescence quenching efficiency is proportional to the amount of the target DNA. Then, the QProbe/nonfluorescent 3′-tailed probe complex is not degraded during the extension step because the DNA polymerase used in this method has no 5′-to-3′ exonuclease activity. (B) Schematic representation of universal QProbe system for SNP genotyping. Homozygotes that are complementary to the probe dissociate at a relatively high temperature, homozygotes that are mismatched with the probe dissociate at a relatively low temperature, and heterozygotes dissociate between the two transitions.

primer (forward primer) consists of two parts: one is the 5′-universal tail that specifically hybridizes with fluorescent probes such as a TaqMan probe[12] and molecular beacon,[13] and the other part specifically hybridizes with the target sequence. During amplification, the fluorescent probe that hybridizes with the universal-tailed primer is degraded or displaced by the extension of the reverse primer, and the fluorescent signal corresponds to the amount of the target. The different target sequences can be detected by employing the same fluorescent probes; thus, these methods reduce the cost of real-time PCR setup. However, the methods still have the problem of detecting nonspecific PCR products because the primer−dimer complex generates fluorescent signals in these methods.

Here, we have developed a novel real-time PCR method to overcome these limitations. In this technique, a QProbe[9−11] and a nonfluorescent 3′-tailed probe are used (Figure 1A). The QProbe is a singly labeled oligonucleotide bearing a fluorescent dye that is quenched via electron transfer between the dye and a guanine base at a particular position. The nonfluorescent 3′-tailed probe consists of two parts: one is the target-specific sequence on the 5′ side, and the other is complementary to the QProbe on the 3′ side. In this method, the same QProbe can be used for different target sequences, and the use of nonfluorescent 3′-tailed probe ensures high specificity for detecting the target amplicon. Moreover, the evaluation of the performance of the designed probe is rapid and cost-effective because the quenching efficiency of the QProbe/nonfluorescent 3′-tailed probe complex can be measured using a complementary oligonucleotide without performing real-time PCR. In other methods using fluorescent probes except QProbe, several examinations of real-time PCR are usually required for the evaluation of the performance of the designed probes, and this step is time-consuming. Therefore, this method significantly reduces the time and cost of real-time PCR setup and allows the accurate quantification even in the presence of nonspecific PCR products. We called this method the universal QProbe system. In addition, we also demonstrate the applicability of the method to single-nucleotide polymorphism (SNP) genotyping using melting curve analysis (Figure 1B). This technique will provide academic and industrial researchers with the opportunity to use flexible, specific, and cost-effective genetic tests in broad applications.

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EXPERIMENTAL SECTION

Oligonucleotides. The sequences of the primers, probes, and complementary oligonucleotides used in this study are listed in Tables 1 and 2. All melting temperatures (T_m's) of the primers and probes were predicted using Genetyx-win version 5.1 (Genetyx Corporation) except for the QProbe. The T_m of the QProbe was predicted using the Exiqon T_m prediction tool (http://lna-tm.com). The primers and complementary oligonucleotides were purchased from Hokkaido System Science

Table 1. Primers, Probes, and Complementary Oligonucleotides Used for Real-Time PCR[a]

target	orientation	sequence (5′ to 3′)	predicted T_m (°C)	ref
β-actin	forward primer	CATGTACGTTGCTATCCAGGC	62.5	15 (PrimerBank ID: 4501885a1)
	reverse primer	CTCCTTAATGTCACGCACGAT	61.9	15 (PrimerBank ID: 4501885a1)
	f. primer for DNA template	CCAGCCATGTACGTTGCTATC	62.5	this work
	r. primer for DNA template	AGCTTCTCCTTAATGTCACG	58.9	this work
	nonfluorescent 3′-tailed probe	CTGACTGACTACCTCATGAAGATCCTCACttgggggaggggg	66.7[b]	this work
	complementary oligonucleotide	GCTCGGTGAGGATCTTCATGAGGTAGTCAGTCAGGTCCC		this work
albumin	forward primer	TTTATGCCCCGGAACTCCTTT	63.8	15 (PrimerBank ID: 4502027a1)
	reverse primer	ACAGGCAGGCAGCTTTATCAG	64.5	15 (PrimerBank ID: 4502027a1)
	f. primer for DNA template	TTACTTTTATGCCCCGGAACT	61.2	this work
	r. primer for DNA template	TGGCAACAGGCAGGCAG	65.0	this work
	nonfluorescent 3′-tailed probe	CTTGGCAACATTCTGTAAAAGCAGCTTTATACttgggggaggggg	66.2[b]	this work
	complementary oligonucleotide	AAAAGGTATAAAGCTGCTTTTACAGAATGTTGCCAAGCTGCT		this work
β-globin	forward primer	GGTTGGCCAATCTACTCCCAGG	66.5	16
	reverse primer	TGGTCTCCTTAAACCTGTCTTG	61.3	16
	f. primer for DNA template	CCTAGGGTTGGCCAATCTACTCCCAGG	71.0	this work
	r. primer for DNA template	TCTATTGGTCTCCTTAAACCTGTCTTG	63.5	this work
	nonfluorescent 3′-tailed probe	GGTGTCTGTTTGAGGTTGCTAGTGAACttgggggaggggg	66.8[b]	this work
	complementary oligonucleotide	ACTGTGTTCACTAGCAACCTCAAACAGACACCATGGT		this work
	TaqMan probe	(FAM)−GGTGTCTGTTTGAGGTTGCTAGTGAAC−(TAMRA)	66.8	this work
QProbe	QProbe	*CCCCCTCCCCCAA*−(BODIPY FL)	102	this work

[a] The lowercase sequences are complementary to the QProbe. The italicized sequence is locked nucleic acid (LNA). [b] The temperatures indicate that the predicted T_m's of the target-specific sequence (uppercase) are on the 5′ side.

Table 2. Primers and Probes Used for SNP Genotyping[a]

target	orientation	sequence (5′ to 3′)	predicted T_m (°C)	ref
ADRB2	forward primer	CATGTACGTTGCTATCCAGGC	62.5	this work
(dbSNP ID; rs1042713)	reverse primer	CTCCTTAATGTCACGCACGAT	61.9	this work
	f. primer for DNA template	CGCTGAATGAGGCTTCC	59.5	this work
	r. primer for DNA template	CAGCACATTGCCAAACAC	59.2	this work
	nonfluorescent 3′-tailed probe	CTTCCATTGGGTGCCAGCttgggggaggggg	63.0[b]	this work
ADRB3	forward primer	AGCTCTCTTGCCCCATG	60.9	this work
(dbSNP ID; rs4994)	reverse primer	GCCAGCGAAGTCACGAA	61.7	this work
	f. primer for DNA template	TGGCCTCACGAGAACAG	60.5	this work
	r. primer for DNA template	GAGTCCCATCACCAGGTC	60.7	this work
	nonfluorescent 3′-tailed probe	CCATCGCCCGGACTCCGAGACTCttgggggaggggg	71.8[b]	this work
UCP1	forward primer	AGTGGTGGCTAATGAGAGAA	60.0	this work
(dbSNP ID; rs1800592)	reverse primer	AAGGAGTGGCAGCAAGT	60.7	this work
	f. primer for DNA template	TTCTTCTGTCATTTGCACATTTATCT	60.8	this work
	r. primer for DNA template	AACTGACCCTTTATGACGTAG	58.6	this work
	nonfluorescent 3′-tailed probe	CACTCGATCAAACTGTGGTCttgggggaggggg	59.9[b]	this work
QProbe	QProbe	*CCCCCTCCCCCAA*−(BODIPY FL)	102	this work

[a] The underlined sequences correspond to SNP site. The lowercase sequences are complementary to the QProbe. The italicized sequence is locked nucleic acid (LNA). [b] The temperatures indicate that the predicted T_m's of the target-specific sequence (uppercase) are on the 5′ side.

Co., Ltd., Tsukuba Oligo Service Co., Ltd., and Nihon Gene Research Laboratories Inc. The nonfluorescent 3′-tailed probes were purchased from Tsukuba Oligo Service Co., Ltd. The primers, complementary oligonucleotides, and nonfluorescent 3′-tailed probes were purified by gel filtration. The TaqMan probe was purchased from Tsukuba Oligo Service Co., Ltd. The QProbe was purchased from J-Bio 21 Corporation. The QProbe was labeled at the 3′ end with BODIPY FL via an aminohexyl phosphate linker having a seven-carbon spacer. The TaqMan probe and QProbe were purified by high-performance liquid chromatography (HPLC).

DNA Templates. First-strand cDNAs were synthesized from β-actin mRNA, human (Nippon Gene) and albumin mRNA, human (Nippon Gene) by RT using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) with Oligo(dT)$_{20}$ as described by the manufacturer. DNA fragments were amplified from the cDNAs (for the β-actin and albumin genes) or human genomic DNA (Novagen; for the β-globin gene) by PCR using each gene-specific primer set for the DNA template (as described in Table 1). The lengths of the amplified products for the β-actin, albumin, and β-globin genes were 260, 100, and 272 bp, respectively, which are longer than regions that are amplified by gene-specific primers for real-time PCR. Then, the PCR products were purified using Microcon YM-30 (Millipore) or a QIAquick PCR purification kit (Qiagen). The fragment length and

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concentration of the purified products were determined using an Agilent 2100 bioanalyzer (Agilent Technologies). The products were used as references for real-time PCR.

Genomic DNA was extracted from human oral swab samples of volunteers using a preserved solution of a DNA test sample for oral swab (J-Bio 21 Corporation). SNP types were determined using the QP system (J-Bio 21 Corporation).[10,17,18] DNA fragments were amplified from genomic DNA (for the ADRB2, ADRB3, and UCP1 genes) by PCR using each gene-specific primer for the DNA template (as described in Table 2). The lengths of the amplified products for the ADRB2, ADRB3, and UCP1 genes were 266, 255, and 277 bp, respectively, which are longer than regions that are amplified by gene-specific primers for SNP genotyping. Then, the PCR products were purified using Montage PCR centrifugal filter devices (Millipore). The fragment length and concentration of the purified products were determined using an Agilent 2100 bioanalyzer (Agilent Technologies). The products were used as references for SNP genotyping.

Measurement of the Fluorescence Quenching Efficiency of the QProbe/Nonfluorescent 3′-Tailed Probe Complex. The reaction mixture (20 μL) contained 1× titanium buffer (Clontech), 200 μM each of dATP, dCTP, and dGTP, 600 μM dUTP (Roche Diagnostics), 0.25 mg/mL BSA, 0.3 (for the β-actin gene) or 1.0 μM (for the albumin and β-globin genes) forward primer, 1.0 (for the β-actin gene) or 0.3 μM (for the albumin and β-globin genes) reverse primer, 0.05 μM QProbe, 0.1 μM nonfluorescent 3′-tailed probe, 1× titanium Taq (Clontech), 0.2 unit of uracil−DNA glycosylase (heat-labile; Roche Diagnostics), and 0.4 μM complementary oligonucleotide. For preparation of negative controls, the complementary oligonucleotide was eliminated from the mixture. The reaction mixture was heated to 95 °C for 15 s, cooled to 37 °C, kept at 37 °C for 60 s, and then slowly heated back to 95 °C at a ramp rate of 0.1 °C/s with continuous fluorescence acquisition using a LightCycler 1.5 (Roche Diagnostics). To normalize fluorescence fluctuations from tube to tube, the fluorescence intensity at 95 °C of the reaction mixture without complementary oligonucleotide ($F_{1,95}$) is divided by that with the complementary oligonucleotide ($F_{2,95}$), and $F_{1,95}/F_{2,95}$ was multiplied by the fluorescence intensity at each temperature with the complementary oligonucleotide (F_2). Normalized F_2 is expressed as $F_{2,\text{normalized}}$. Fluorescence quenching efficiency at each temperature was calculated using eq 1:

$$\text{fluorescence quenching efficiency (\%)} = [(F_1 - F_{2,\text{normalized}})/F_1] \times 100 \quad (1)$$

where F_1 is the fluorescence intensity at each temperature of the reaction mixture without the complementary oligonucleotide.

Real-Time PCR of the Universal QProbe System. Real-time PCR was carried out using a LightCycler 1.5 (Roche Diagnostics). The reaction mixture (20 μL) contained 10^1−10^8 copies of the DNA templates as references or 0.2−200 ng of the human genomic DNA, 1× titanium buffer (Clontech), 200 μM each of dATP, dCTP, and dGTP, 600 μM dUTP (Roche Diagnostics), 0.25 mg/mL BSA, 0.3 (for the β-actin gene) or 1.0 μM (for the albumin and β-globin genes) forward primer, 1.0 (for the β-actin gene) or 0.3 μM (for the albumin and β-globin genes) reverse primer, 0.05 μM QProbe, 0.1 μM nonfluorescent 3′-tailed probe, 1× titanium Taq (Clontech), and 0.2 unit of uracil−DNA glycosylase (heat-labile; Roche Diagnostics). The lengths of the amplified products for the β-actin, albumin, and β-globin genes were 250, 90, and 262 bp, respectively. To produce the single-stranded DNAs that possess the probe binding site, one of the primers was added to the reaction mixture at 3- or 4-fold the amount of the other primer. Titanium Taq is an N-terminal deletion mutant of thermostable recombinant Taq DNA polymerase that lacks 5′-to-3′ exonuclease activity. Uracil−DNA glycosylase was added to the reaction mixture to prevent the carryover contamination of the amplified products. The glycosidase reaction was performed during the preparation of the reaction mixture at room temperature before PCR. The PCR conditions were as follows: initial denaturation at 95 °C for 2 min, 50 cycles of denaturation at 95 °C for 30 s, annealing at 55 (for the β-actin and albumin genes) or 62 °C (for the β-globin gene) for 30 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 2 min. Fluorescence intensity was measured at the end of denaturation and annealing steps in each cycle, and fluorescence quenching efficiency at each cycle was calculated in accordance with a previous report.[19] The cycle at which the fluorescence quenching efficiency plot crosses the threshold is defined as Ct (cycle of threshold), and the standard curve was constructed from the mean Ct values of triplicate determinations. The amplified products were electrophoresed in a 2% agarose gel.

Real-Time PCR of the TaqMan Chemistry. The TaqMan assay was carried out using an ABI PRISM 7900 HT (Applied Biosystems). The reaction mixture (50 μL) contained 1× TaqMan universal master mix (Applied Biosystems), 0.1 μM TaqMan Probe, 0.3 μM forward primer, and 0.3 μM reverse primer. The PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Results were analyzed using ABI PRISM 7900 HT Sequence Detection System software 2.1 (Applied Biosystems).

SNP Genotyping. PCR and melting curve analyses were carried out using a LightCycler 480 (Roche Diagnostics). The reaction mixture (20 μL) contained 10^4 copies of the DNA templates as references, 1× LC480 genotyping master (Roche Diagnostics), 0.25 mg/mL BSA, 0.5 (for the ADRB2 and UCP1 genes) or 0.15 μM (for the ADRB3 gene) forward primer, 0.15 (for the ADRB2 and UCP1 genes) or 0.5 μM (for the ADRB3 gene) reverse primer, 0.15 μM QProbe, 0.5 μM nonfluorescent 3′-tailed probe, and 0.1 unit of uracil−DNA glycosylase (heat-labile; Roche Diagnostics). The lengths of the amplified products for the ADRB2, ADRB3, and UCP1 genes were 150, 215, and 203 bp, respectively. The polymerase in the LC480 genotyping master is an N-terminal deletion mutant of thermostable recombinant Taq DNA polymerase that lacks 5′-to-3′ exonuclease activity and is chemically modified. The PCR conditions were as follows: initial denaturation at 95 °C for 5

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min, 50 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 20 s, and extension at 72 °C for 10 s, and a final extension at 72 °C for 5 min. After the PCR, the reaction mixture was heated to 95 °C for 15 s, cooled to 37 °C, kept at 37 °C for 60 s, and then slowly heated back to 95 °C at a ramp rate 0.1 °C/s with continuous fluorescence acquisition. T_m was calculated from peaks generated by plotting the negative derivative of fluorescence intensity over temperature versus temperature.

RESULTS AND DISCUSSION

Measurement of the Fluorescence Quenching Efficiency of the QProbe/Nonfluorescent 3′-Tailed Probe Complex. We measured the fluorescence quenching efficiency of the QProbe/nonfluorescent 3′-tailed probe complex using a complementary oligonucleotide without performing real-time PCR. As model targets, we chose three genes: the β-actin, albumin, and β-globin genes. The QProbe was designed to not contain guanine bases to prevent self-quenching due to secondary structure formation. The nonfluorescent 3′-tailed probes were designed specifically for the β-actin, albumin, and β-globin genes (Table 1). The T_m of the target-specific portion of the nonfluorescent 3′-tailed probe was approximately 5−10 °C higher than that of the primer. The 3′-tail sequence of the probe was designed to be as short as possible to minimize hybridization of the 3′-tail with other locations in the genome. The predicted T_m of the designed 3′-tail sequence is 57.9 °C. Moreover, we examined the similarity between the designed 3′-tail sequence and any known genomic sequences on the DDBJ BLAST search program (http://www.ddbj.nig.ac.jp/search/top-e.html). No hits were found. Furthermore, to stabilize the QProbe/nonfluorescent 3′-tailed probe complex, we used the locked nucleic acid (LNA) in the QProbe sequence (Figure 1A). LNA is a DNA analogue in which the furanose ring in the sugar−phosphate backbone is chemically locked. LNA obeys the Watson−Crick pairing rules but has an increased specificity and a high affinity to its complementary DNA sequence.[20,21] The theoretical T_m of the designed QProbe that is all-LNA oligonucleotides was calculated to be 102 °C using the Exiqon T_m prediction tool, which is available online (http://lna-tm.com). Because the theoretical T_m of the QProbe was over 100 °C, we considered that the QProbe/nonfluorescent 3′-tailed probe complex was prevented from dissociation during the PCR cycle. As shown in Figure 2, the fluorescence quenching efficiencies of the QProbe/nonfluorescent 3′-tailed probe complex for the β-actin, albumin, and β-globin genes were 37.4%, 24.1%, and 17.9%, respectively, at the annealing temperature in the PCR cycle. The differences in fluorescence quenching efficiency among the three genes are probably due to the difference in the target-specific sequence of the nonfluorescent 3′-tailed probes and/or the other guanine bases at the 5′ side in the target that quenches the fluorescence.

Real-Time PCR. We tested the feasibility of the universal QProbe system for real-time PCR using the three different genes as model targets as described above. The DNA templates were prepared from commercially produced mRNA or human genomic DNA as described in Experimental Section and diluted to serial 10-fold solutions, ranging from 10^1 to 10^8 copies. Then, the DNA mixtures were amplified by PCR with real-time monitoring of fluorescence intensity. To make the universal QProbe system reliable, two points were considered to be particularly important. First, the asymmetric PCR should be used to minimize the hook effect.[22] The hook effect is the phenomenon of a decrease in fluorescence intensity (or fluorescence quenching efficiency) observed in later cycles in symmetric PCR, because the amplified strands reanneal before the probes can bind to generate (or quench) fluorescence. This may confuse the results. Second, the DNA polymerase that has no 5′-to-3′ exonuclease activity should be used to prevent the degradation of QProbe/nonfluorescent 3′-tailed probe. Figure 3 shows amplification plots and standard curves for the β-actin, albumin, and β-globin genes obtained using the universal QProbe system. The maximum fluorescence quenching efficiencies of the amplification plots for the β-actin, albumin, and β-globin genes were 38.5%, 32.2%, and 21.5%, respectively. These results are slightly lower than those obtained using a complementary oligonucleotide as the DNA template (without performing real-time PCR) as described above, probably owing to the difference in the length of the target DNA. The calculated R^2 values of the standard curves for the β-actin, albumin, and β-globin genes were 0.9993, 0.9997, and 0.9989, respectively. Thus, the standard curves in the universal QProbe system were linear, and the dynamic range was at least 7 orders of magnitude ranging from 10^1 and 10^8 target copy numbers. Then, the reproducibility of the universal QProbe system was evaluated using the Ct values for constructing the standard curves. The relative standard deviations (RSDs) ($N = 3$) of the β-actin, albumin, and β-globin genes were all less than 2.7% in a test range of 10^1−10^8 copies. To compare the results of our method with those of a traditional quantitative method, we also performed the real-time PCR of TaqMan chemistry for constructing the standard curves for the β-globin gene. The results show that the calculated R^2 value of the standard curve was 0.9976 in the test range of 10^1−10^8 copies, and the *y*-intercept was 43.5 (data not shown). This *y*-intercept obtained by TaqMan chemistry was slightly lower than that obtained using the universal QProbe system (44.2), as shown in Figure 3F. The RSDs ($N = 3$) obtained by TaqMan chemistry were all less than 2.2% in the test range of 10^1−10^8 copies. The RSDs obtained by TaqMan chemistry were slightly lower than those obtained using the universal QProbe system. These results indicate that the universal QProbe system has a slightly lower sensitivity and reproducibility for constructing standard curves than the conventional TaqMan chemistry. However, we considered that the universal QProbe system has acceptable sensitivity and reproducibility in practical use. Moreover, when the amplified products were electrophoresed using a 2% agarose gel, some bands of less than 100 bp, considered to be the primer−dimer complex, were observed in all samples including the no-template controls for the three genes (Figure S-1 in the Supporting Information). However, no decreases in fluorescence intensity were observed from the amplification plots of the no-template controls (Figure 3). These results strongly suggest that this method allows quantification

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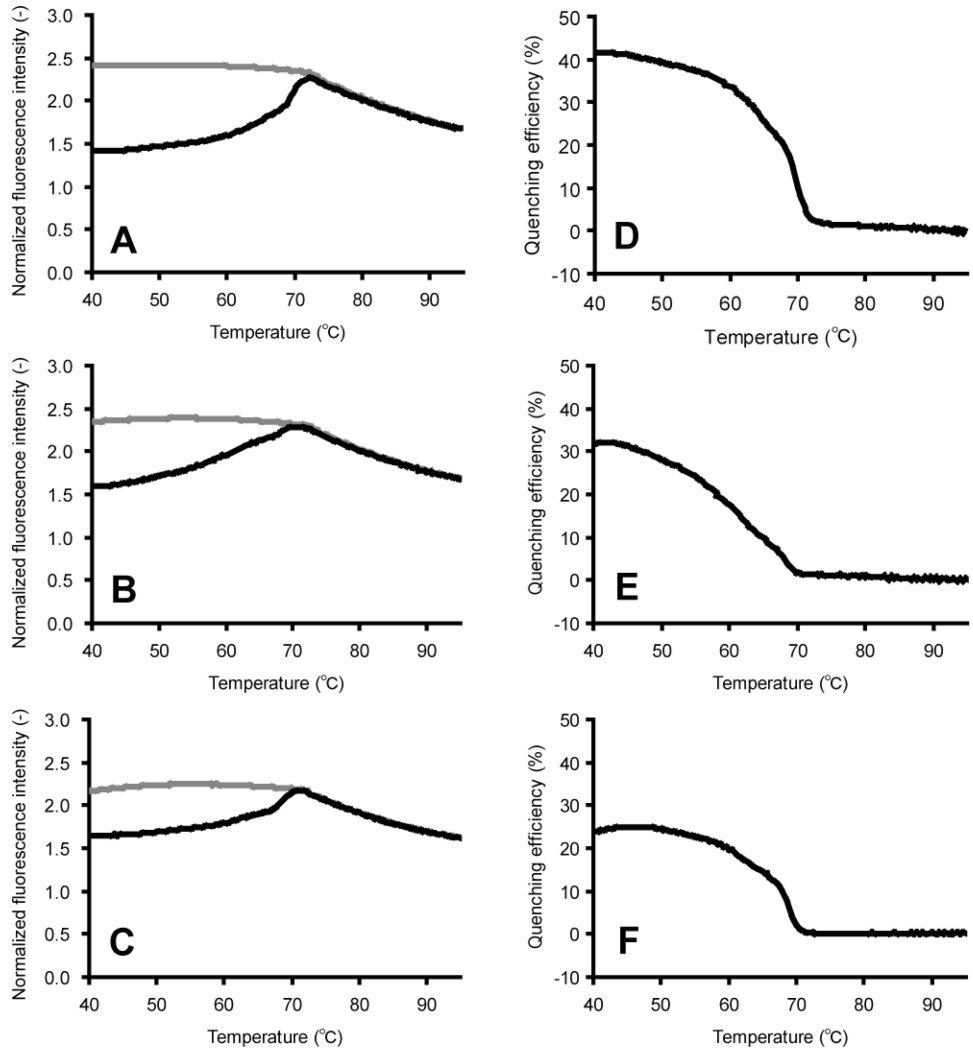

Figure 2. Fluorescence quenching efficiency of QProbe/nonfluorescent 3′-tailed probe complex using a complementary oligonucleotide. Fluorescence intensity was measured for the β-actin (A), albumin (B), and β-globin (C) genes with complementary oligonucleotides (black line), and without complementary oligonucleotides (gray line). Fluorescence quenching efficiency was calculated for the β-actin (D), albumin (E), and β-globin (F) genes.

even in the presence of nonspecific PCR products. We also confirmed that no misamplifications that cause the decrease in fluorescence intensities were observed in the presence of the DNA templates for nontarget genes (Figure S-2 in the Supporting Information) and no decreases in fluorescence intensity were observed in the presence of the amplified products of nontarget genes (Figure S-3 in the Supporting Information).

To determine the accuracy and precision of the universal QProbe system for real-time PCR, we quantified the gene of β-globin of the human genomic DNA, ranging from 0.2 to 200 ng. TaqMan chemistry was also applied to the same samples, and the results were compared. The experiments were repeated three times, and the mean β-globin copy, SD, and RSD at each amount of human genomic DNA were calculated. Because one human cell contains ~6 pg of genomic DNA and two copies of the β-globin gene,[9] 0.2−200 ng/μL solution was calculated to contain about 7×10^1 to 7×10^4 copies/μL of the human β-globin gene. The numbers of copies of β-globin gene measured using the universal QProbe system were 29 694 ± 2339, 4148 ± 482, 548 ± 69, and 55 ± 5 in 200, 20, 2, and 0.2 ng of human genomic DNA, respectively. The results obtained using the universal QProbe system correlated with true values. The RSDs (N = 3) of the universal QProbe system were less than 13%. The numbers of copies of β-globin genes measured by TaqMan chemistry were 133 995 ± 9048, 18 666 ± 2652, 1611 ± 133, and 160 ± 17 in 200, 20, 2, and 0.2 ng of human genomic DNA, respectively. The RSDs (N = 3) obtained by TaqMan chemistry were less than 15%. These results indicate that the universal QProbe system can accurately and reproducibly quantify specific nucleic acid sequences in crude samples, comparable with TaqMan chemistry. The differences in the number of copies between the two methods are probably due to the difference in detection chemistry.

SNP Genotyping. QProbe has already been used for SNP genotyping in the QP system.[10,17,18] However, this system requires more than one specific fluorescent probe for one SNP genotype; therefore, this system is relatively expensive and troublesome in practical use. On the other hand, the universal QProbe system can reduce the cost of a SNP genotyping setup because different SNPs can be genotyped by employing the same QProbe. Here, we tested the feasibility of the universal QProbe system for SNP genotyping using the three different SNPs, namely, the ADRB2

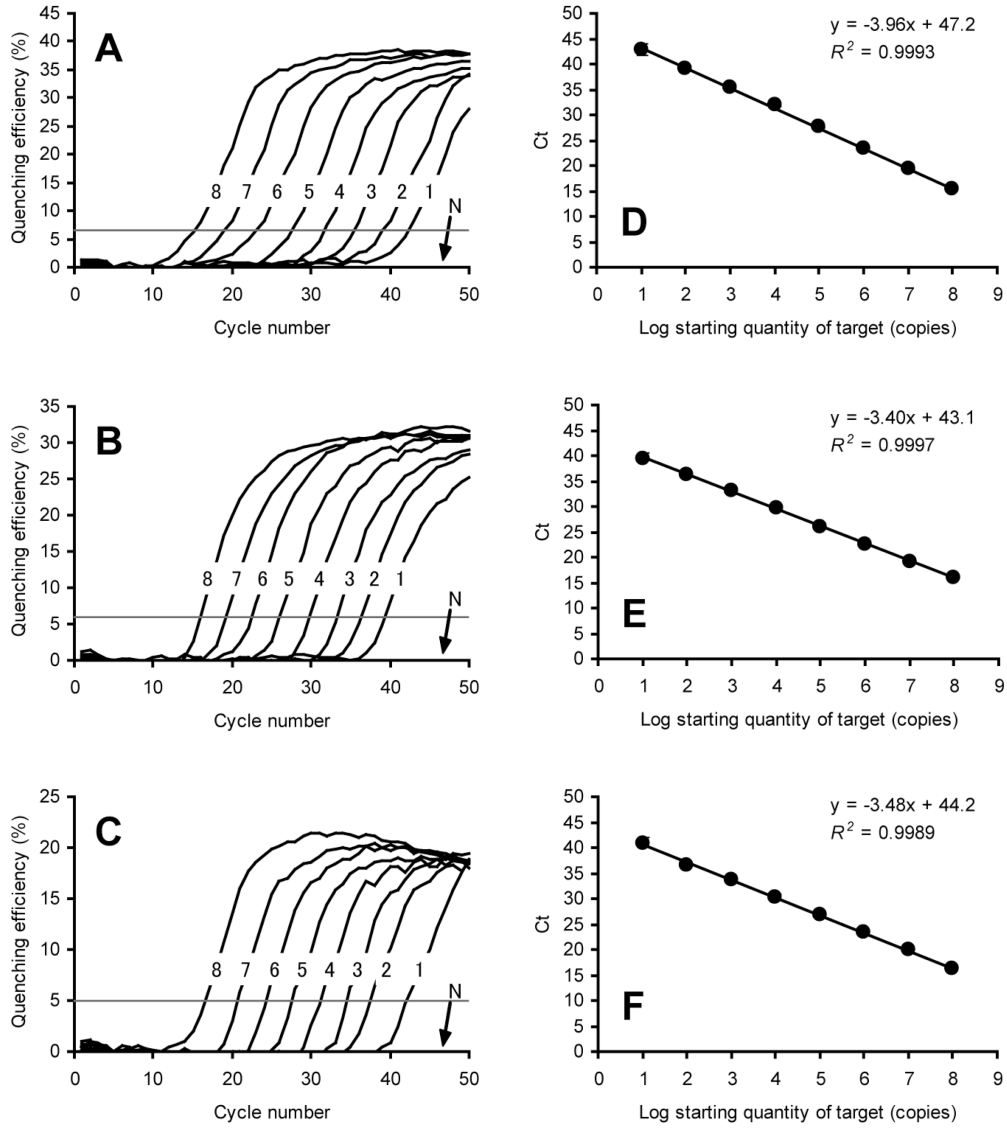

Figure 3. Real-time PCR. Amplification plots for β-actin (A), albumin (B), and β-globin (C) genes with serial dilutions (10-fold) of each DNA template per dilution: (1) 10^1, (2) 10^2, (3) 10^3, (4) 10^4, (5) 10^5, (6) 10^6, (7) 10^7, (8) 10^8, (N) no-template control. These graphs show representative traces for all the dilutions and no-template control in triplicate determinations. The gray lines represent the threshold lines. Standard curves for β-actin (D), albumin (E), and β-globin (F) genes. The error bars represent the standard deviation. Standard error bars that are not visible are within the symbols.

gene (A/G), ADRB3 gene (C/T), and UCP1 gene (A/G) as model targets. The nonfluorescent 3′-tailed probes were designed specifically for the ADRB2, ADRB3, and UCP1 genes (Table 2). The DNA templates were prepared from genomic DNA as described in Experimental Section and diluted to 10^4 copies of each allele. Then, the DNA mixtures were amplified by PCR, and fluorescence intensity was monitored continuously during the increase in temperature to determine the melting temperature at which the QProbe/nonfluorescent 3′-tailed probe complex dissociates from the amplified products. The position of the melting peak provides T_m, which indicates the genotypes. Figure 4 shows the melting peaks for the ADRB2, ADRB3, and UCP1 genes obtained using the universal QProbe system. The results show that homozygous and heterozygous SNP genotypes were discriminated by the differences in the position of the melting peaks in the three SNP genotypings; i.e., the T_m of the homozygous mutant type is higher (61.6 °C) than that of the homozygous wild type (53.0 °C) for the ADRB2 gene, the T_m of the mutant type is higher (70.2 °C) than that of the wild type (63.9 °C) for the ADRB3 gene, and the T_m of the mutant type is higher (60.2 °C) than that of the wild type (52.5 °C) for the UCP1 gene. Heterozygous types exhibited the presence of both the two melting peaks (peaks at T_m of homozygous wild and mutant types). These results indicate that the three genotypes can be clearly distinguished from one another by melting curve analysis. Therefore, the universal QProbe system is applicable to SNP genotyping.

Advantages of the Universal QProbe System. In this study, we have developed a flexible, specific, and cost-effective real-time PCR method, called the universal QProbe system. The three different genes (β-actin, albumin, and β-globin genes) have been quantified with the universal QProbe system using the same QProbe. This method can accurately and reproducibly quantify specific nucleic acid sequences in crude samples, comparable with conventional TaqMan chemistry. This method is comparable in

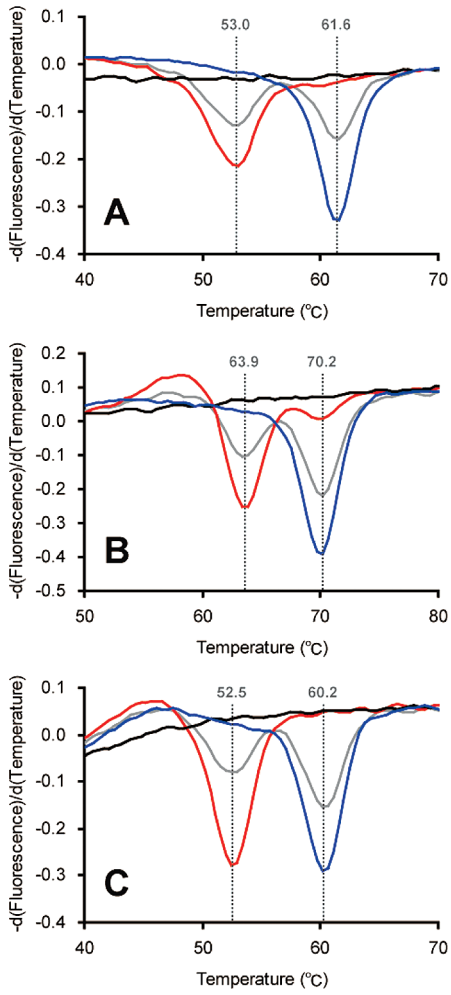

Figure 4. SNP genotyping using melting curve analysis. Melting peaks of the ADRB2 (A), ADRB3 (B), and UCP1 (C) genes with the homozygous wild type, which has a mismatched nucleotide to the probe (red line), homozygous mutant type, which is complementary to the probe (blue line), heterozygous (gray line), and no-template control (black line) are shown. All the graphs show representative traces for all the genotypes and no-template control in triplicates.

quality to other published methods.[14,23] This method was also shown to be applicable to SNP genotyping, in which we have established the genotyping system for three different SNPs (ADRB2, ADRB3, and UCP1) using the same QProbe. The present method has several major advantages as follows:

(i) In this method, the same QProbe can be used for any targets. Thus, this method significantly reduces the cost of real-time PCR setup in comparison with other sequence-specific fluorescent PCR techniques.[7−10] Moreover, the evaluation of fluorescence quenching efficiency of the designed probe is rapid and cost-effective because this can be carried out without performing the real-time PCR.

(ii) Unlike the universal-tailed primer techniques,[12−14] the universal QProbe system uses the nonfluorescent 3′-tailed probe that is specific for target sequences. Thus, this new the method allows the accurate quantification even in the presence of nonspecific PCR products.

(iii) This method can significantly reduce the cost of a SNP genotyping setup because the different SNPs can be genotyped by employing the same QProbe. All steps of the preparation and measurement can easily be performed in a single vessel, and then genotypes are discriminated by melting curve analysis. Thus, this method is suitable for automation.

(iv) In principle, this method can be applied not only to PCR systems but also to other amplification systems such as loop-mediated isothermal amplification (LAMP),[24] rolling-circle amplification (RCA),[25] nucleic acid sequence-based amplification (NASBA),[26] and helicase-dependent amplification (HDA).[27] These systems can amplify nucleic acids under isothermal conditions, thereby allowing the use of simple and cost-effective reaction equipment.

There may be some difficulties with regard to the design of the sequence-specific probes in this method because the nonfluorescent 3′-tailed probe should possess a C base at the 3′-end in the target-specific part and the target should possess a G base complementary position. However, G bases are ubiquitous in the target; therefore, this limitation can be negligible.

This novel concept can be applied to other quantitative systems such as alternately binding probe competitive PCR (ABC-PCR), which we previously reported.[28,29] This method combines the use of competitive PCR and a sequence-specific fluorescent probe that binds to either the gene of interest or the internal standard. Unlike the real-time PCR techniques including the universal QProbe system, ABC-PCR enables a reliable quantification of DNA in biological samples that contain high concentrations of PCR-inhibiting compounds and can quantify specific nucleic acid sequences by end point fluorescence measurement using a simple fluorometer. By combining the present universal system with ABC-PCR, we believe that it may lead to the widespread use of quantitative genetic tests in many fields.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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