Development of an accurate and cost-effective quantitative detection method for specific gene sequences

— Development of a quantitative detection method for specific gene sequences using fluorescence quenching phenomenon —

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[Translation from *Synthesiology*, Vol.3, No.2, p.147-157 (2010)]

DNA and RNA quantifications are essential in various fields such as biomedicine, agriculture, fishery, environment, and food. We have developed an accurate and cost-effective method for the quantification of specific nucleic acid sequences; the method involves the use of the fluorescent quenching phenomenon via an electron transfer between the dye and a guanine base at a particular position. This paper describes the elemental key technologies and their synthesis for the development of such a gene quantification method. Furthermore, based on the findings of a collaborative research project with a private company, we report the scenario for the industrialization and the practical use of the developed method.

Keywords : Gene quantification, fluorescence quenching, life science, fluorescent probe

1 Introduction

The genetic analysis technology is used in the wide-ranging socioeconomic activities including medicine, agriculture, fisheries, environment, and foods. Its use in clinical genetic testing is particularly increasing. Specifically, the test kits for hepatitis C virus and tuberculosis bacterium are already commercially available, and the genetic analysis technology is applied to hepatitis B virus, HIV, and sepsis pathogen. The venture businesses are beginning to provide subcontract service for genetic analysis for lifestyle-related diseases. In fields other than clinical tests, genetic analysis technology is used for DNA typing in the forensic investigation, detection of the food poisoning pathogens, quantification of the contents of genetically recombined foods, breed identification, as well as in bioterrorism countermeasures and environmental measurements. It is certain that the genetic analysis technology will be applied further, and the technology to detect and quantify certain genes is one of the most basic and important genetic analysis technologies.

There are eight items of quantitative analysis including the gene quantification technology: (1) specificity, (2) trueness, (3) precision, (4) detection limit, (5) linearity, (6) range, (7) robustness, and (8) commutability. Specificity is the ability to accurately measure only the molecule to be investigated amongst the coexisting similar molecules, and the point in nucleic acid detection is whether the target nucleic acid molecule and those with other sequences can be properly identified. Trueness is the degree of match between the measurement result and the true value of the measured subject. Precision is the degree of (smallness of) variations in the results when the measurements are repeated. Detection limit is the minimum amount by which the measured molecules can be detected, and the quantification limit means the minimum amount of measured molecule that can be quantified with sufficient trueness and precision. Linearity is the degree of the ability by which the measurement result and the amount of substance of the measured molecule within a certain range can be expressed as a linear relationship. Range is the upper and lower limits of the concentration of the measured molecule that give appropriate trueness, precision, and linearity. Robustness is the degree in which the measurement value is unaffected when the measurement condition shifts, and for example, in gene quantification, the inclusion of inhibitors may affect this factor. Commutability is the equivalence of the obtained measurement value, when the obtained value is compared with the one obtained by measuring the same sample using another (standard) method. Other than these indices for quantification, convenience of use, cost performance, throughput, and speed are important factors from the perspective of realizing the measurements. Considering the development of a practical gene quantification technology, the technology must have a certain level or higher of specificity, trueness, precision, and detection limit. Furthermore, for the technology to become diffused widely, it must have high robustness (accurate quantification must be possible even in the presence of inhibitors), be easy to use, and have excellent cost performance.

In the quantification of a specific gene, it must be kept in mind that the target gene within the sample may be extremely minute in amount. Therefore, to quantify a specific gene, it

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Original manuscript received January 26, 2010, Revisions received February 15, 2010, Accepted February 19, 2010

is necessary to amplify only the gene to be quantified among the various nucleic acid mixtures. There have been various methods developed for the amplification of the target gene, and the one most frequently used is the polymerase chain reaction (PCR) method. The PCR method was developed in 1984 by Kary Mullis, an American researcher who won the Nobel Prize in Chemistry. In this method, the target gene can be amplified exponentially using a simple method where the temperature is changed cyclically using reagents such as heat-resistant polymerase or short DNA fragments (primers) that act as the originators of the reaction. However, since the amount of the final amplified product by PCR does not necessarily reflect the amount of the target gene in the initial reaction solution, the amount of the initial target gene cannot be directly quantified from the amount of the final amplified product. Therefore, to quantify the target gene using PCR (quantitative PCR), it is necessary to find a way to measure the amount of the target gene in the initial reaction solution.

In the quantitative PCR, there are several methods with different measurement principles such as the real time[1], the competitive[2], and the most probable number (MPN)[3] methods. The real time PCR (RT-PCR) method is most commonly used. In the RT-PCR, the amount of amplified product is measured at each cycle of PCR, and the number of cycles required for the reaction product to reach a certain amount (or cycle of threshold: Ct) is calculated in the region where exponential amplification reaction is occurring. The relationship between the Ct and the amount of genes in the initial reaction solution is plotted to obtain the standard curve, and the amount of the target gene in the initial reaction solution can be calculated from this standard curve based on the Ct for the unknown sample.

In the RT-PCR, it is necessary to measure the amount of the amplified product at each cycle. The method used is to label and quantify the amount of amplified product with fluorescence. The major methods are the method using intercalator such as the SYBR Green[4] or the one using fluorescent probe such as the TaqMan probe[5]. SYBR Green is a special fluorescent dye (intercalator) that emits fluorescence when incorporated into the double-stranded DNA. When the SYBR Green is added to the PCR solution, the SYBR Green intercalates into the double-stranded DNA amplified by PCR and the fluorescence increases. The amount of PCR product can be measured by measuring the intensity of the fluorescence. This method allows using the same reagent for the target genes of any sequence, and it is used widely because of its low cost and convenience. On the other hand, since fluorescence increases with nonspecific amplified product such as primer dimer, there is a disadvantage that the fluorescence intensity and the amount of PCR product may not necessarily correspond. As shown in Fig. 1, the TaqMan probe method is a method using the TaqMan probe, in which one terminal of the oligonucleotide corresponding to the base sequence of the segment of the amplified region of the target gene is labeled with a reporter (fluorescent dye), and the other end is labeled with a quencher to turn off the fluorescence of the reporter. When the TaqMan probe is added to the PCR solution, the TaqMan probe that bonded to the amplified product is broken down by the elongation reaction by the 5'→3' exonuclease activity of DNA polymerase. When the probe is broken down, the reporter fluorescent dye begins to emit its original fluorescence by separating from the quencher. The amount of PCR product can be measured by measuring the fluorescence intensity. Since the TaqMan probe bonds specifically only to the amplified product, it is not affected by any nonspecific amplified product such as the primer dimer, and allows highly specific quantification. While this method is widely used, it requires labeling by two fluorescent dyes.

RT-PCR has advantages that it can measure the amount of target gene in a short period (30 min to 2 h), and there is very little contamination of the laboratory with the PCR product since gel electrophoresis is unnecessary. It also has excellent trueness and precision. Also, the detection limit is low due to gene amplification, and the measurement range reaches 10^5~10^8 copies. However, it has the following disadvantages: 1) since it is necessary to measure the fluorescence per cycle of PCR to measure the amount of the amplified product, it is necessary to install an expensive RT-PCR device that embodies the fluorescence measurement device and the PCR thermal cycler (problem of initial cost); 2) while the specificity may increase in the fluorescent probe method, it is necessary to design and synthesize the fluorescent probe for each target gene to measure the amount of amplified product (problem of running cost performance); and 3) the amount of the target gene may be undervalued or may produce pseudo-negativeness in the case the measured sample contains a substance that inhibits PCR (problem of robustness). Considering the use of the gene quantification technology

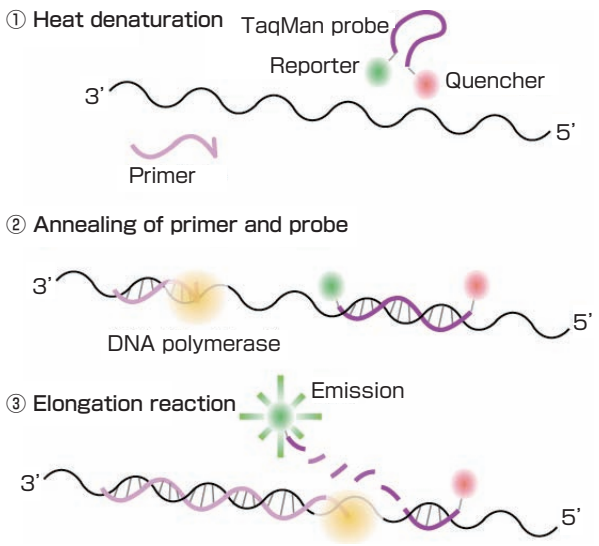

Fig. 1 TaqMan probe method

for multiple samples on site, it is necessary to concentrate on robustness, convenience, and cost performance, while maintaining an equivalent level as the current RT-PCR technology.

In this paper, the two quantitative PCR methods developed as new technologies to solve the problem inherent in the current RT-PCR are described, and the cooperation with companies for the practical application of the developed technology will be presented.

2 Scenario for the development of gene quantification technology with excellent accuracy and cost performance

2.1 Core technology for technology development: quenching phenomenon by the guanine base

To solve the problem inherent in the current RT-PCR, we developed a new quantitative PCR to overcome the two issues: 1) quantification can be done using only one type of fluorescent probe for different target genes (cost reduction by general-use fluorescent probe), and 2) accurate quantification is possible even with the presence of PCR inhibitors. The core technology in this technological development is the "quenching phenomenon by the guanine base." Fluorescence means the emission of light as the fluorescent molecule absorbs light, transforms into an excited-state molecule, and then returns to the original ground-state molecule. The difference in energy between the excited and ground states of the molecules is released as the fluorescence energy. When the molecule transforms from the excited state to the ground state, if there is another molecule with high electron density nearby, this molecule acts as an electron donor and gives away electron to the fluorescent molecule. At this moment, the electron excited by the original fluorescent molecule cannot return to the ground state, becomes unable to emit fluorescence, and the fluorescence disappears. This phenomenon is called the photo-induced electron transfer (PET), and is known to occur within and between molecules[6]. Among the bases that comprise the nucleic acid, guanine has the highest electron density, and therefore, tends to cause quenching through this PET reaction. However, not all fluorescent dyes cause quenching, and it is known that some fluorescent dyes such as BODIPY FL and TAMRA are more likely to cause quenching by the guanine base[7].

Since the quenching phenomenon by the guanine base is a reversible reaction, it can be used conveniently as a tool to detect and quantify nucleic acids. Completely complementary DNA is prepared for a fluorescent probe of about 20 bases, in which the cytosine base of the terminal is labeled with BODIPY FL. When the temperature and other conditions are adjusted to induce annealing (hybridization) in the same reaction solution, the fluorescence of the BODIPY FL is quenched. Then when the hybridization is broken by raising the temperature or other conditions, the BODIPY FL begins to emit fluorescence again. By controlling the hybridization and separation, the on/off of the fluorescence can be controlled. By measuring the degree of quenching, it becomes possible to estimate the amount of complementary strand of the fluorescent probe. The quantitative PCR method using this phenomenon was developed and put on the market as the quenching probe (QProbe) PCR method through joint research with Dr. Shinya Kurata *et al.* of the J-Bio 21 Corporation, an AIST venture that spun off from the Institute for Biological Resources and Functions[8]. The author formed the joint research structure with Dr. Kurata's group, as well as the group of Dr. Satoshi Tsuneda, School of Advanced Science and Engineering, Waseda University, to develop a new technology that extends the QProbe PCR method.

2.2 Development of the universal QProbe method that achieves cost reduction through general-use fluorescent probe

While it is necessary to label the probe with two fluorescent dyes (reporter and quencher dyes) in the TaqMan probe method, which is a method used most frequently as a RT-PCR using the fluorescent probe, only one fluorescent dye is necessary in the QProbe PCR method, which is also a RT-PCR, because it uses the guanine base as the quencher. Moreover, in the QProbe PCR, the adequacy of the amplified product can be checked by a melting curve analysis where the melting temperature of the fluorescent probe that hybridized to the amplified product is measured by raising the temperature gradually from around 40 ºC after the completion of the reaction. This cannot be done in the TaqMan probe method. Although the QProbe PCR has such advantages, it is the same as other fluorescent probe methods in that the fluorescent probes must be designed and synthesized according to the target gene. In the fluorescent probe method, although the specificity of the detection and quantification increases since a fluorescent probe specific to the amplified product is used, the cost increases since the fluorescent probe must be designed and synthesized

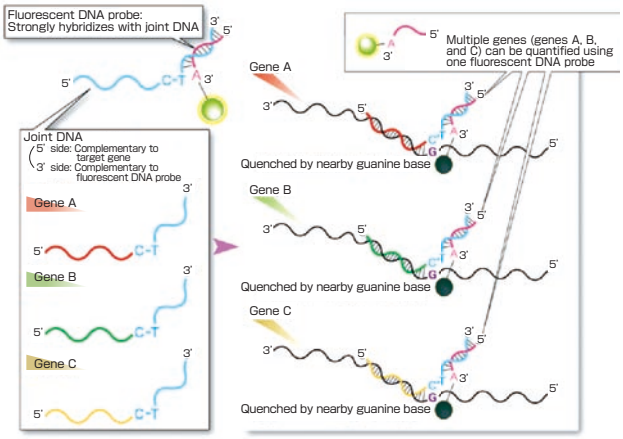

Fig. 2 Universal QProbe method

in addition to the PCR primer. While the synthetic oligonucleotide DNA without the fluorescent label can be prepared at about 2,000 yen per target gene, the probe labeled with fluorescence costs over 20,000 yen. In case there are several target genes, the fluorescent probe much be designed and synthesized for each target gene, and the cost becomes high. If it is possible to quantify any target gene with one fluorescent probe regardless of the sequence, a new gene quantification method with excellent cost performance may be established, having the advantage of mass-synthesized fluorescent probe.

The universal QProbe method was developed based on this thinking (Fig. 2)[9]. While maximizing the principle of the QProbe method that uses the quenching phenomenon by guanine base and realizing the concept of quantifying all types of target genes with one fluorescent probe (the universal QProbe) regardless of the sequence, in the universal QProbe method, we added the idea of the joint DNA that binds both the target gene and the fluorescent probe. The joint DNA is a single-stranded oligo DNA that has a complementary sequence of target gene on the 5' side and a complementary sequence of fluorescent DNA probe on the 3' side, and the two sequences are joined with cytosine and thymine. The fluorescent probe is labeled with a dye whose fluorescence is quenched by the guanine base nearby. The joint DNA bonds to both the target gene and the fluorescent probe, and the fluorescence of the fluorescent probe is quenched when the fluorescent probe approaches the guanine base in the target gene. Therefore, it is possible to measure the amount of the target gene by measuring the degree of quenching as in the QProbe method. The fluorescent probe in this method has the adenine base on the 3' terminal labeled with the fluorescent dye. It is designed so the quenching occurs when the guanine base approaches the fluorescent dye, because this adenine base is positioned across the thymine base of the cytosine-thymine sequence within the joint DNA strand, and the guanine base of the target DNA is positioned across the neighboring cytosine base.

The joint DNA must be designed and synthesized for each target gene, but the cost and time of synthesis can be reduced greatly since it is not labeled with the fluorescent dye. This method will enable the quantification using only one type of fluorescent DNA probe, even if the genes under investigation have different sequences.

2.3 Development of the alternately binding probe competitive (ABC) PCR that is resistant to PCR inhibitors

In the RT-PCR method, it is known that the quantification result may be undervalued or show pseudo-negativeness if the sample to be measured contains a substance that inhibits PCR. While such issues are negligible in samples that contain very little inhibitors or samples that are highly purified, amplification inhibitors are thought to be present in blood samples and soil samples that contain decomposed materials, and inhibition of amplification may be an issue. Although the competitive PCR method is a classical method, it solved the issue of such amplification inhibitors. In the competitive PCR, amplification is done using the same primer as the target gene, but uses an internal standard gene with different amplified base length than the target gene. Specifically, the internal standard gene that is shorter or longer than the target gene is created by removing part of the internal sequence of the target gene or by adding extra base. The internal standard gene of known concentration is added to the sample, and PCR is conducted competitively with the target gene. Since the lengths of the strands differ between the target gene and the internal standard gene, the target gene and the internal standard gene are separated by electrophoresis after PCR, the gradation of the bands of the target gene and the internal standard gene are compared quantitatively, and then the amount of the target gene can be measured from the known amount of the internal standard gene. Using this method, even if the PCR inhibitor is present in the sample, accurate quantification is possible since the inhibition equally affects both the target gene and the internal standard gene. While this method allows accurate quantification even in the presence of the PCR inhibitor, it is no longer used recently since it requires labor-intensive and time-consuming post-PCR procedures for the separation of the PCR products by gel electrophoresis.

By utilizing the advantage of the competitive PCR that can avoid the issue of PCR inhibitors, and by using the quenching phenomenon by the guanine base, we developed the alternately binding probe competitive (ABC) PCR method as a convenient gene quantification method that eliminates the electrophoresis that was a problem in competitive PCR (Fig. 3)[10]. In the ABC-PCR, internal standard gene

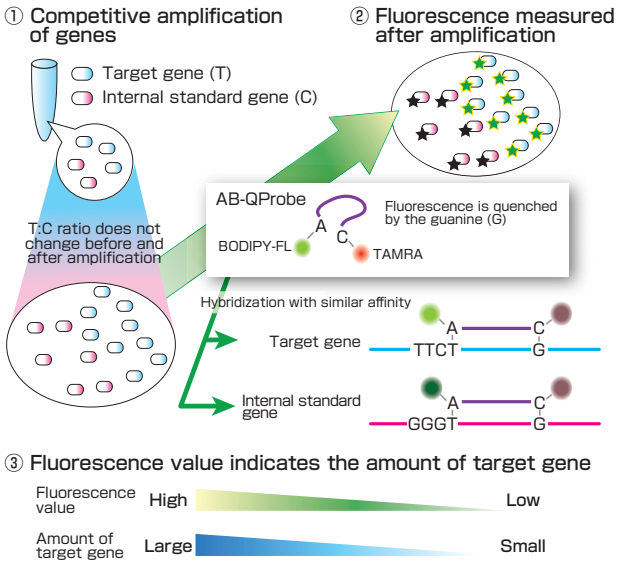

Fig. 3 ABC-PCR method

has the same strand length as the target gene, and also is amplified by the same primer and the fluorescent alternately binding probe (AB-Probe). One terminal of the AB-Probe is labeled with the green fluorescent dye (BODIPY FL) where the fluorescence is quenched with the nearby guanine base, and the other end is labeled with the red fluorescent dye (TAMRA) that is quenched by the guanine base. The sequence of the AB-Probe is designed to be complementary to the common sequence of the target gene and the internal standard gene, and it hybridizes with the same affinity to both genes. In the internal standard gene, the three exterior bases on the green fluorescent dye that hybridize with the AB-Probe are replaced with the guanine base (the bases in the target gene are those other than guanine). Therefore, the AB-Probe binds competitively to the amplified product derived from the target gene and that from the internal standard gene with the same affinity, and green fluorescence is emitted when it binds with the target gene, but does not emit fluorescence when it binds with the internal standard gene since the fluorescent dye is quenched by the guanine base. The green fluorescence becomes stronger as there are more target genes than the internal standard genes, while the green fluorescence becomes weaker as there are less target genes than the internal standard genes. The amount of the target gene can be calculated since the amount of the internal standard gene is known. TAMRA, the red fluorescent dye, is quenched in the same manner when the AB-Probe is bonded either to the target gene or the internal standard gene. The presence of amplification can be checked since the degree of quenching of TAMRA changes according to the amount of amplified product derived from the target gene and the internal standard gene.

The ABC-PCR can be considered as a method where the electrophoresis step that was mandatory in the competitive PCR method is replaced with the fluorescent probe using the quenching phenomenon by guanine. Since it is a competitive method, it not only allows accurate quantification in the presence of the PCR inhibitors, but is also an endpoint quantification method where the degree of quenching can be measured after the completion of PCR. Therefore, the expensive device needed in the RT-PCR is not necessary, and the target gene can be quantified with an inexpensive thermal cycler and a fluorescence measurement device.

3 Results of the development

3.1 Universal QProbe PCR method

We conducted an experiment to verify the principle of the universal QProbe PCR by using the β-actin, albumin, and β -globin genes as target genes. The most important point is the stability of the joint DNA and the fluorescent probe. It is desirable that the hybridization between the joint DNA and the fluorescent probe does not dissociate but remain stable during the PCR reaction. Therefore, we used a synthetic oligonucleotide where the nucleic acid of the fluorescent probe was replaced by locked nucleic acid (LNA). The LNA is an analog of the nucleic acid that has two cyclic structures in the molecule, and it is known that the oligonucleotides including LNA show dramatic heat stability against the complementary DNA and RNA[11]. A fluorescent probe labeled with BODIPY FL composed of LNA of 13 base length was synthesized, and the *Tm* of the complementary sequence of this fluorescent probe and the joint DNA was calculated using the Exiqon Tm prediction tool (http://lna-tm.com). The result was 102 ºC. Since the highest temperature encountered in PCR was 95 ºC at heat denaturation, it was thought that the complex of the fluorescent probe and joint DNA would be prevented from dissociation during the PCR cycle.

Using the designed fluorescent probe and the joint DNA, the quantification of the target gene was conducted using the universal QProbe PCR. The fluorescent quenching rate was calculated from the fluorescence value at denaturation step (state where the probe and target genes are dissociated) and the fluorescence value during annealing step (state where the probe and target genes are bonded). Figure 4 shows

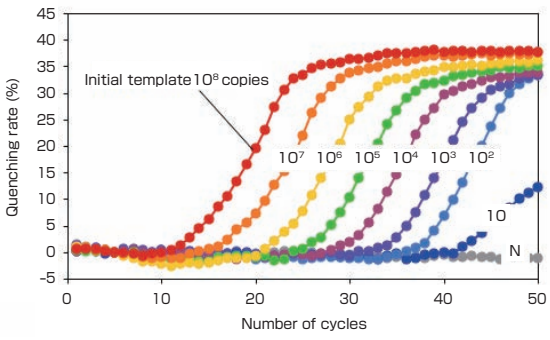

Fig. 4 Relationship between the number of cycles and quenching rate in the universal QProbe method
This shows the quenching rate when the β-actin gene is amplified from 10 to 10^8 copies. The calculation of quenching rate was done according to Reference [8].

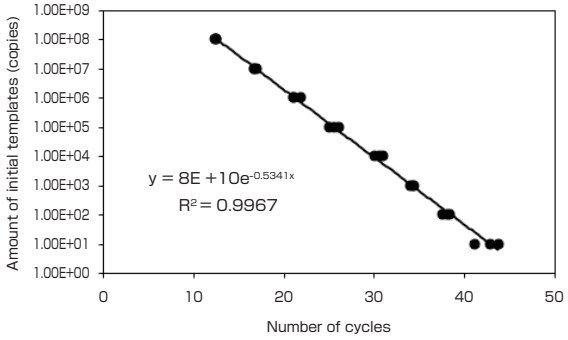

Fig. 5 Standard curve in the universal QProbe method
This shows the relationship between the number of cycles required for the reaction product to reach a certain amount and the amount of initial templates, calculated from the relationship between the number of cycles and quenching rate of Fig. 4.

the relationship of the number of cycles and fluorescent quenching rate when the β-actin gene was quantified. The fluorescent quenching rate was about 30~40 %, or about the same as the QProbe PCR. Figure 5 shows the standard curve created by calculating *Ct* from Fig. 4. The lower limit of quantification was 10 copies, and the correlation coefficient R^2 of the standard curve was 0.9967. Both the lower limit of quantification and the correlation coefficient were about the same as the QProbe PCR. The amplified product can be checked by conducting the melting curve analysis where the temperature at which the complex of the fluorescent probe and joint DNA dissociates from the amplified product is measured by gradually raising the temperature from around 40 ºC after PCR. Results with equivalent quantification precision were obtained for the albumin and β-globin genes as well as the β-actin gene. Our initial objective of developing the quantification of multiple target gene sequences using one fluorescent DNA probe while maintaining the same quantitative quality as the QProbe PCR was achieved with this universal QProbe PCR [9].

We applied the universal QProbe PCR method to the genetic analysis of single nucleotide polymorphisms (SNP) in the human gene. SNP is a single base DNA variation occurring in the genome, and is defined as a mutation seen at 1 % or higher frequency in a certain group. Recently, through the advancement in human genome and genetic analysis research, SNP is drawing attention as one of the causes of the individual differences such as susceptibility to disease or reactivity to drugs. SNP is said to occur on average in one place among 1000 bases, and there are over 3 million SNPs in the 3 billion base pairs on a human genome. We differentiated these SNP gene types through the melting curve analysis using the universal QProbe PCR. The joint DNA was designed to be completely complementary to one allele, and had one base mismatch on the other allele. After annealing the fluorescent probe and joint DNA complex with the PCR amplified product by decreasing the temperature, the SNP was analyzed by obtaining the melting curve from the fluorescence emitted by the quenched probe as the temperature was raised. The fluorescence is emitted as the dissociation occurs at low temperature if there is a mismatch, while the fluorescence is emitted at higher temperature if there is a perfect match (Fig. 6). Figure 7 shows the result of the analysis of the three gene types for SNP: wild homo, mutant homo, and hetero types. Separation was easy since the positions of the peak of emission differ for the wild and mutant types. Both peaks were observed in the hetero type where the wild and mutant types were mixed. Since the universal QProbe PCR allows quantifying multiple target genes with one fluorescent probe, it is expected to be an effective tool in genotyping the SNP, since it is said that 3 million SNPs exist in the human genome.

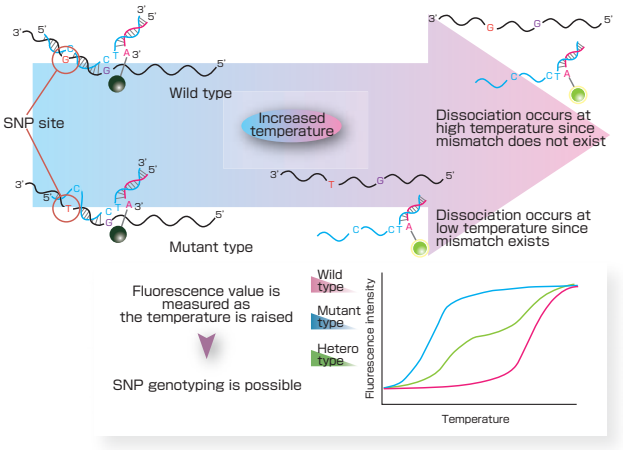

Fig. 6 Principle of SNP typing by the universal QProbe method

3.2 Alternately binding probe competitive (ABC) PCR method

We investigated the quantitative property in the ABC-PCR method using the *gfp* gene, which is the famous green fluorescent protein, as the target gene. The internal standard gene was created based on the sequence of the *gfp* gene, and the verification of ABC-PCR was conducted. The quenching rate calculated from the fluorescence values after the PCR that was corrected by several background fluorescence values was set as the relative fluorescence intensity. Figure 8 shows the graph of the relationship between this relative fluorescence intensity and the amount of the target gene in the initial template. Using this method, the standard curve could be regressed to a sigmoid curve just as in the standard curve obtained by other general competitive measurement methods such as the competitive ELISA. According to Fig. 8, the correlation coefficient of the standard curve was 0.9997. The lower limit of quantification was 10^3 copies. Since this method is competitive, the quantifiable range using one standard curve is in the order of 2~3, but the quantifiable range can be adjusted by changing the concentration of the internal standard gene. It is also possible to calculate

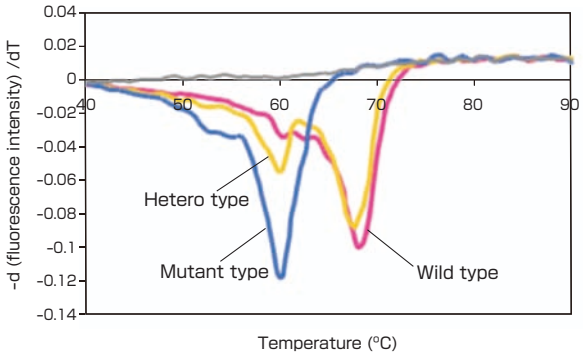

Fig. 7 Result of SNP genotyping by the universal QProbe method

SNP genotyping is conducted from the analysis of dissociation curve where the fluorescence values are measured as the temperature is gradually raised from 40 ºC to 90 ºC. The vertical axis shows the value obtained by the primary derivation of fluorescent value by time.

the amount of the standard gene from the diluted sample within the quantifiable range by conducting measurement by creating a dilution series of the unknown sample. This method can be also used as genotyping to identify the SNP as in the universal QProbe as well as for gene quantification[10].

We evaluated the effect on the quantification value in the ABC-PCR and RT-PCR methods by adding humic acid that is found in the soil and is known as a DNA amplification inhibitor. As a result, in the RT-PCR, the quantification value turned out to be lower than the true value as the concentration of the humic acid increased, while in the ABC-PCR, the quantification value was almost the same as the true value even in the presence of humic acid[10]. In the experiment using urea and Triton X-100 as the DNA amplification inhibitor, it was found that the ABC-PCR was capable of highly accurate quantification compared to the RT-PCR[12].

The ABC method not only is capable of accurate quantification in the presence of the DNA amplification inhibitor, but also is capable of quantifying the target gene by measuring the fluorescence after the gene amplification reaction. This means that the target gene can be quantified in a similar manner whether the gene amplification reaction is PCR or some other technique. Recently, methods such as the loop-mediated isothermal amplification (LAMP) and helicase-dependent amplification (HDA) have been developed as the isothermal gene amplification, as alternative to the PCR. By combining such isothermal gene amplification methods with the ABC method, similar quantification as the ABC-PCR can be done. The ABC method is not only accurate, but is also highly universal from the perspective of combining with the gene amplification methods.

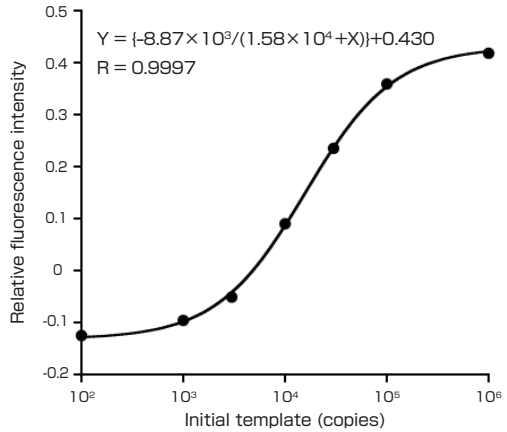

Fig. 8 Standard curve in the ABC-PCR method
This is the relationship between the amount of target genes in the initial template and the relative fluorescence intensity. The relative fluorescence intensity is the value where the fluorescence value obtained after the completion of PCR is corrected by some background fluorescence values[9]. The plot obtained is regressed by equilateral hyperbola. R is the correlation coefficient.

4 Evaluation of the development technology and scenario for realization

The advantages and the disadvantages of the two newly-developed gene quantification technologies, universal QProbe PCR and ABC-PCR methods, will be compared to the current technologies, and the scenario for their realization to maximize the advantages of each technology will be discussed (Fig. 9). Table 1 shows the comparison of the properties of the current technologies (TaqMan probe, QProbe, intercalator, competitive methods) and the universal QProbe and ABC-PCR methods. Since the technologies have their advantages and disadvantages, it is necessary to consider the ways to realize them by thoroughly understanding the properties of the technologies. The business for the realization of the universal QProbe PCR and the ABC-PCR is currently undertaken by the J-Bio 21 Corporation, the partner of the joint research.

Table 2 shows the comparison of the characteristics of the universal QProbe PCR with the conventional RT-PCR (fluorescent probe and intercalator methods). As it can be seen from Table 2, the universal QProbe PCR is a technology that takes the advantages of the fluorescent probe and the intercalator methods. Although this paper does not refer to multicolor detection, four different colors can be used as dyes where the fluorescence is quenched by guanine, and this method can be used in the multicolor detection and quantification. Since the universal QProbe PCR is a RT-PCR, the thermal cycler for RT-PCR is necessary. However, thinking alternatively, this method can be used immediately if there is a thermal cycler for RT-PCR available. Therefore, the prime strength in realizing this technology is that we can recommend it to users who are already using the RT-PCR method. In the conventional RT-PCR, the reagent kits are commercially available for the detection and quantification of specific genes (such as pathogenic bacteria, virus, or certain SNP). However, such pre-marketing method is not compatible for the universal QProbe PCR. One of the advantages of the reagent kit is the cost merit of mass synthesizing the fluorescent probe to detect a specific gene. However, since only one type of fluorescent probe is necessary for various gene sequences in the universal QProbe PCR, there is hardly any cost advantage in providing the reagent kit. Therefore, as business plans to optimize the advantage of this method, the joint DNA and fluorescent probe according to the client's target gene sequence can be provided, or the genetic analysis service can be subcontracted to detect and quantify the client's target gene sequence. In these business plans, the low cost of the fluorescent probe and the short time for the preparation of the fluorescent probe synthesis that are the characteristics of this method can be optimized fully, to provide a low-cost, quick-delivery gene analysis service. One of the clients who may benefit from the low cost and quick delivery may be a company in the field of

Table 1 Comparison of the characteristics of quantitative PCR methods

	Real time method				Internal standard method	
	TaqMan probe method	QProbe method	Intercalator method	Universal QProbe method	Competitive method	ABC method
Fluorescent probe	Needed for each target gene (label with 2 colors)	Needed for each target gene (label with 1 color)	Not necessary	Can deal with all target genes with one fluorescent probe (label with 1 color)	Not necessary	Needed for each target gene (label with 2 colors)
Internal standard gene	Not necessary	Not necessary	Not necessary	Not necessary	Necessary	Necessary
Electrophoresis	Not necessary	Not necessary	Not necessary	Not necessary	Necessary	Not necessary
Check amplified product by melting curve analysis	Impossible	Possible	Possible	Possible	Impossible	Possible
Real time PCR device	Necessary	Necessary	Necessary	Necessary	Not necessary	Not necessary
Resistance to inhibitors	No	No	No	No	Yes	Yes

Table 2 Comparison of the universal QProbe method and the conventional RT-PCR method

	Conventional method		Universal QProbe method
	Fluorescent probe method	Intercalator method	
Specificity	○ (Non specific product is not detected)	× (Non specific product is also detected)	○ (Non specific product is not detected)
Cost* (probe, primer)	× (1 gene: 20,000 yen or higher)	◎ (1 gene: about 2,000 yen)	○ (1 gene: about 6,000 yen)
Time needed for preparation*	× (1～2 weeks)	○ (Minimum next day)	○ (Minimum next day)
SNP genotyping	○	×	○
Multicolor detection	○	×	○

*Based on estimates by J-Bio 21 Corporation.

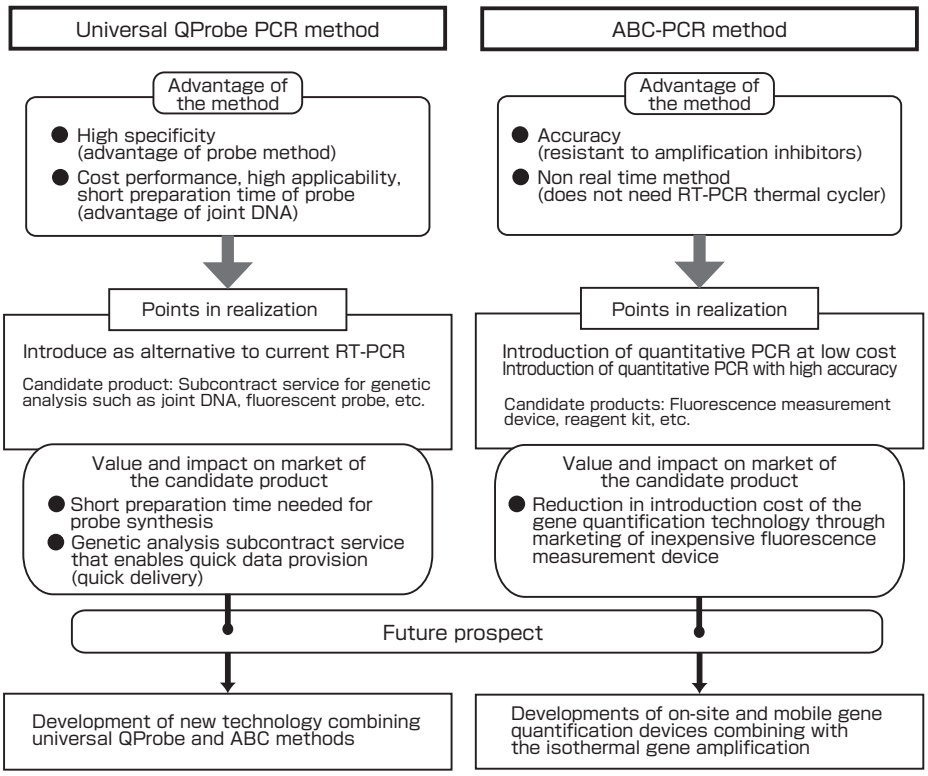

Fig. 9 Scenario for the realization of the universal QProbe PCR and ABC-PCR methods

environmental cleaning using bacteria. As covered in the newspapers and other media, the "brownfield" with soil contamination has become an issue. For cleaning such soil contamination, bioremediation using bacteria is thought to be effective in terms of cost. However, when cleaning up the contaminant by introducing the bacteria in the environment, the bioremediation guideline states that it is necessary to assess not only the introduced bacteria, but also the effect on the microbial community that originally exists in the soil. The method using the genetic information is effective for the assessment of the microbial community, and the gene quantification technology is drawing attention in this field. There is a diversity of microbes in the environment, and the microbes to be investigated change for each soil type. Therefore the universal QProbe PCR method that allows quantifying various gene sequences using one type of fluorescent probe is extremely effective in the detection and quantification of the various environmental microbes. Therefore, one of the ways of practical use for the universal QProbe PCR is the subcontracted analysis business in the field of environmental cleaning business where the various microbial community can be detected and quantified at low cost and in a short time.

Since the ABC-PCR method has different advantages and disadvantages from the universal QProbe PCR, the scenario for its realization differs. The advantages of ABC-PCR are that accurate quantification is possible without the effect of the gene amplification inhibitor, and the target gene can be quantified by simply measuring the fluorescence after the gene amplification reaction. For the former, the users who are already using the RT-PCR but are facing the problem of amplification inhibitors will probably see merit in introducing this method. Moreover, in this method, the target gene can be quantified by simply measuring the fluorescence after the gene amplification reaction, and the expensive thermal cycler for RT-PCR is not necessary. Instead, the fluorescence measurement device for measuring the fluorescence after the gene amplification reaction is necessary. The marketing of the fluorescence measurement device (named EGBox) is in progress at the joint researcher J-Bio 21 Corporation (Fig. 10). This device is specialized to measure fluorescence in the ABC method. The specifications are: one fluorescence measurement area; depth 18 cm × width 30 cm × height 15 cm; 3.5 kg; LED light source; and three excitation wavelengths. The fluorescence value can be measured simply by inserting the PCR tube into the sample port. J-Bio 21 Corporation is trying to keep the retail price below 1 million yen. By offering such inexpensive fluorescence measurement device and reagent kit, we believe we can do business with people who wish to be involved in the gene quantification technology but are hampered by the facility cost. The ABC-PCR is extremely appropriate for cases that wish to introduce the gene quantification technology at low cost such as in developing countries. To realize such a scenario, the issues are downsizing to portable size, and energy-saving configuration where the device can be powered by batteries. As the technologies to achieve downsizing and energy saving, the micro total analysis system (μ-TAS), where reaction, separation, and detection are done in microspace, by forming the flow channels and circuits on a silicon or glass substrate using the microfabrication technology, was developed and is being used in the analysis of biomolecules such as nucleic acids and proteins. Downsizing and energy saving will be achieved by fusing the μ-TAS technology and the ABC method. The ABC method can be used in combination with the gene amplification methods other than the PCR. For example, if it is combined with the isothermal gene amplification method, quantification can be done with a simple and inexpensive device consisting only of an isothermal device with low energy use instead of a thermal cycler. To develop these technologies, there are many issues that must be solved such as the selection of gene amplification technologies (development of a new isothermal gene amplification method if necessary) as well as the development of simple nucleic acid extraction technologies. However, if these issues are overcome, it is expected that a gene quantification technology that is simpler and less expensive than the one currently used widely can be achieved.

The dream of the author is the diffusion of the gene detection and quantification technologies based on the universal QProbe and the ABC methods, in pursuit of convenience and cost performance. We also aim for the development of the new technology combining the universal QProbe and the ABC methods in the future. Specifically, this involves the replacement of the fluorescent probe used in the ABC method with the universal QProbe, but there are several difficult problems because it is necessary to advance the idea on the joint DNA. However, the technology in which the universal QProbe and ABC methods are integrated will have both the accuracy of the ABC method and the flexibility of the universal QProbe method, and is expected to have great social impact due to its cost performance and the reduced preparation time for probe synthesis.

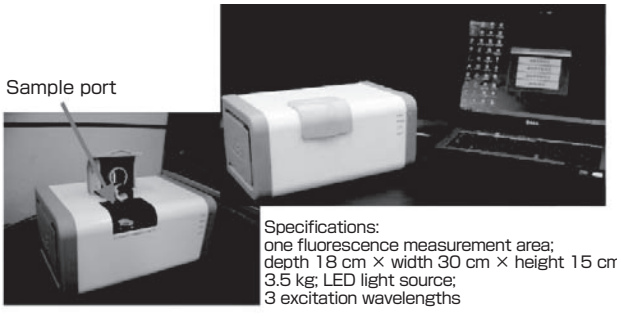

Fig. 10 Prototype of simple fluorescence measurement device (EGBox) (made by J-Bio 21 Corporation)

The Japanese market in 2009 for RT-PCR was estimated to be 6.8 billion yen for devices (0.3 billion yen increase compared to the previous year), and 4.5 billion yen for reagents (0.5 billion yen increase compared to the previous year)[13]. The demand for the quantification of gene expression such as the detailed expression analysis of human genes is increasing, and the market for RT-PCR is expected to increase further in the future. It is expected that the use will expand in the facilities and the developing countries that were reluctant to introduce gene tests due to their cost, and we believe a system that can be introduced at low cost is important. The universal QProbe PCR and ABC-PCR have excellent cost performance and universal applicability, and are expected to be the next-generation gene quantification technology in such social situations.

5 Conclusion

In this paper, for the two gene quantification technologies, universal QProbe PCR and ABC-PCR methods, the elemental technologies in the development phase and the scenario for the realization after their development were discussed from a synthesiological perspective. Although the diagram of the principle of the developed technology seems to be simple, we encountered various problems and engaged in trial-and-error in the processes from the selection of elemental technologies to their integration. Over 10 researchers combined ideas, repeated discussions, and completed the technology under the tri-party joint research of AIST, Waseda University, and J-Bio 21 Corporation. The technologies that resulted were the universal QProbe PCR and the ABC-PCR methods, and these were accomplished by repeating the work of filling each piece as if completing an extremely difficult puzzle. The core elemental technology of these technologies is the quenching phenomenon of the fluorescent dye that occurs between the guanine bases, but there were infinite quenching patterns of the fluorescent probe due to the bonding force and other factors of the probe and the amplified product as well as the positions of the fluorescent dye and guanine base. It was necessary to do trial-and-error to determine which one would quench most efficiently and stably and was appropriate for gene quantification. Since not all quenching patterns could be predicted by knowledge and experience, the work of trying out each unknown possibilities was like walking in the dark with no signs of the goal ahead. We fortunately were able to complete the technology this time, but this could not have been possible with just one or two persons. It is work accomplished by the cooperation of several researchers involved, even with them engaging in vicious discussions at times.

In the advancement of *Type 2 Basic Research*, the process of generating a practical technology by reviewing the phenomena discovered in *Type 1 Basic Research* from multiple angles is important. To advance *Type 2 Basic Research* in an effective manner, it is important not only to push forward the ideas and viewpoints of a small number of people, but the R&D must be carried out by building trusting relationships among the people of industry-academia-government, and by respecting each other's values.

6 Acknowledgements

The development of the universal QProbe PCR method was accomplished through the cooperation of: Shinya Kurata, Kohei Ichikawa, *et al.* of the J-Bio 21 Corporation; Professor Satoshi Tsuneda, Hidenori Tani (currently at the Radioisotope Center, The University of Tokyo), *et al.* of the Center for Advanced Biomedical Sciences, Waseda University; and Kazunori Nakamura, Yuji Sekiguchi, *et al.* of AIST. The development of the ABC-PCR method was accomplished through the cooperation of: Shinya Kurata *et al.* of the J-Bio 21 Corporation; Professor Satoshi Tsuneda, Hidenori Tani (currently at the Radioisotope Center, The University of Tokyo), *et al*. of the Center for Advanced Biomedical Sciences, Waseda University; Takahiro Kanagawa of Kyoto Gakuen University; and Kazunori Nakamura, Yuji Sekiguchi, *et al.* of AIST. The development of the ABC-PCR method was supported by the Grant for Industrial Technology Research, New Energy and Industrial Technology Development Organization (NEDO).

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Discussions with Reviewer

1 Specific assumed client and scenario development

Comments (Yoshifumi Jigami, Evaluation Division (current affiliation: Research Center for Medical Glycoscience), AIST)

I think you need to analyze the issues and problems that must be overcome to make this business successful. For example, you should consider the attributes of those whom you specifically assume to be your clients that can take advantage of "the low cost and quick delivery", and then present the scenario to develop the business.

Answer (Naohiro Noda)

The assumed clients that can take advantage of "the low cost and quick delivery" include the environment companies that must monitor diverse environmental microbes in bioremediation, and the genetic testing companies that must analyze innumerable gene types. We used the example of the environment companies for monitoring the environmental microbes because their market is expected to expand in the future, and revised the manuscript accordingly.

2 Issues and points for the diffusion

Comments (Yoshifumi Jigami)

The sales plan for the inexpensive fluorescence measurement device (less than 1 million yen) with accompanying reagent kit is discussed, and its diffusion as a tool for the quantification and analysis of genes at low cost in the developing countries and others is suggested as a probable business development. While this is a very interesting suggestion, you did not give the issues and points that must be overcome to realize this.

Answer (Naohiro Noda)

As the issues and problems that must be overcome to realize the diffusion to developing countries, downsizing and energy saving of the developed technology are necessary. To achieve these, I think the development of the biochip that combines the micro total analysis system (μ-TAS), which is advancing dramatically recently, is important in the future scenario. This point was described in the revised manuscript.

3 Value and social impact on the market

Comments (Yoshifumi Jigami)

Figure 9 shows the points for realization and the corresponding candidate products. I think it will be easier to understand if you describe the value and social impact of these products on the market.

Answer (Naohiro Noda)

"Value and social impact of the products on the market" was added to the product candidate in Fig. 9.

4 Problems in technological development and scenario for solution

Comments (Yoshifumi Jigami)

As future prospects, you describe "the development of the new technology combining the universal QProbe and the ABC methods" and "the development of the on-site or mobile gene quantification device combined with the isothermal gene amplification method". While these are important in looking at the "researcher's dream" or the "link between the research objective and the society (social values)", you should discuss the issues that must be overcome to achieve such technological developments, scenario to solve the issues, and the impact on the market when they are realized.

Answer (Naohiro Noda)

For "the development of the new technology combining the universal QProbe and the ABC methods", it is necessary to generate ideas about joint DNA that is compatible with the ABC method by advancing the concept of current joint DNA. (The details of the idea will be omitted here since the development is in progress.) For "the development of the on-site or mobile gene quantification device combined with the isothermal gene amplification method", selection of isothermal amplification technologies (development of new technology if necessary) and simplified nucleic acid extraction technology are needed. The issues that must be overcome for technological development and the impact on the market when they are realized are described in the revised manuscript.