

## Detection of cariogenic bacteria genes by a combination of allele-specific polymerase chain reactions and a novel bioluminescent pyrophosphate assay

Hidetoshi Arakawa<sup>a,\*</sup>, Koji Karasawa<sup>a</sup>, Takeshi Igarashi<sup>b</sup>, Shigeya Suzuki<sup>c</sup>,  
Nobuichi Goto<sup>b</sup>, Masako Maeda<sup>a</sup>

<sup>a</sup> School of Pharmaceutical Sciences, Department of Analytical Chemistry, Showa University, Tokyo 142-8555, Japan

<sup>b</sup> School of Dentistry, Department of Oral Microbiology, School of Dentistry, Showa University, Tokyo 142-8555, Japan

<sup>c</sup> Research & Development Division, Kikkoman Corporation, Chiba 278-0037, Japan

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### Abstract

We developed a novel bioluminescent assay for detection of pyrophosphate in polymerase chain reaction (PCR) product. The principle of this method is as follows: pyrophosphate released by PCR is converted to adenosine 5'-triphosphate (ATP) by pyruvate phosphate dikinase in the presence of the substrate pyruvate phosphate and the coenzyme adenosine 5'-monophosphate; subsequently, ATP concentration is determined by firefly luciferase reaction. The detection limit of pyrophosphate is  $1.56 \times 10^{-15}$  mol/assay. Additionally, luminescent intensity reached a maximum at ~100s and remained elevated beyond 10min. This approach is applicable to the detection of cariogenic bacteria in dental plaque. Thus, the allele-specific PCR products of *Streptococcus mutans* and *Streptococcus sobrinus* developed in this study were measured via the proposed bioluminescent assay. This protocol, which does not require expensive equipment, can be utilized to rapidly monitor cariogenic bacteria in dental plaque.

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Maintenance of tooth health, which is important to the health of the entire body, enhances the quality of life. Removal of dental plaque by tooth brushing following meals is effective in the prevention of caries. Bacteria comprise 75% of dental plaque. One gram of dental plaque contains  $2 \times 10^{11}$  bacterial cells [1]. *Streptococcus mutans* and *Streptococcus sobrinus*, which are the primary cariogenic species, play a role in the generation of caries; consequently, these bacteria have been vigorously examined [2–6]. Previous data clearly demonstrated that cariogenic ability is promoted when two species coexist [7]. Therefore, identification and determination of the habitation ratio of

these species as a diagnostic method for caries prevention in a clinical setting are essential.

Currently, identification of *S. mutans* and *S. sobrinus* is conducted with biochemical and immunological techniques or via analysis of a bacillus gene involving allele-specific PCR, restriction enzyme fragment length polymorphism, and hybridization methodologies, etc. However, these approaches generally employ slab gel electrophoresis; as a result, considerable time and skill are required for proper utility of these distinct procedures. In addition, results are occasionally unsatisfactory. Furthermore, application of these techniques in a clinical setting presents problems.

Previously, we developed a bioluminescent detection method for the O157 VT gene, which involved the

\* Corresponding author. Fax: +011-03-3784-8194.

E-mail address: [arakawa@pharm.showa-u.ac.jp](mailto:arakawa@pharm.showa-u.ac.jp) (H. Arakawa).

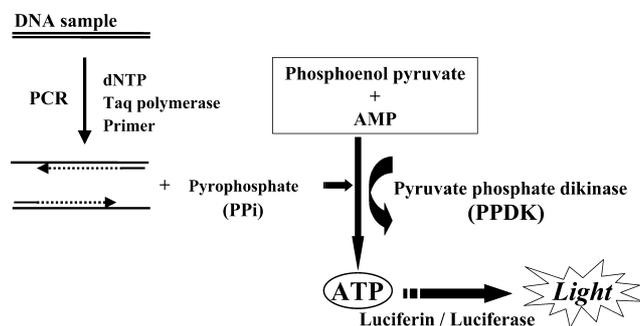


Fig. 1. Principle of bioluminescent assay for DNA-amplified product by PPKD system.

luciferin–luciferase reaction following transformation of pyrophosphate produced during PCR to ATP by adenosine 5'-phosphosulfate (APS)<sup>1</sup> and ATP sulfurylase [8]. However, the sensitivity of this technique was insufficient due to the slight light emission of APS during the luciferin–luciferase reaction, leading to elevation of the blank value. Furthermore, APS and ATP sulfurylase, which are expensive, display poor stability for utility in routine analysis.

Pyruvate phosphate dikinase (PPDK) catalyzes the conversion of phosphoenolpyruvate (PEP) to ATP, phosphate, and pyruvate in the presence of AMP and pyrophosphate [9]. PPKD catalyzes ATP synthesis in microorganisms lacking pyruvate kinase such as *Entamoeba histolytica* and *Clostridium symbiosum*. Furthermore, significant work on this enzyme has been conducted in plants, including *Propionibacterium shermanii*, *Acetobacter xylinum*, photosynthetic bacteria, rice, and corn with respect to the glycolytic pathway, i.e., PEP synthesis [10,11]. Generally, PPKD occurs in low concentrations; moreover, this protein is unstable upon freezing and under alkali conditions [9].

Eisaki et al. [10] isolated PPKD in the heat-resistant microorganism *Microbispora rosea aerata* (IFO 14047); subsequently, the PPKD gene was transfected into *Escherichia coli* [10]. This PPKD displayed molecular weight of 230,000. In addition, PPKD is stable in a wide pH range; it also exhibits thermal stability and stability with regard to chemical modification. PPKD produced by the recombinant technique was also applied to the high-sensitivity measurement of ATP, AMP, and RNA [12,13]. During elongation of template DNA by DNA polymerase in PCR, one pyrophosphate molecule was released consequent to incorporation of deoxynucleotide (dNTP) as described above. At this point, the amount of released pyrophosphate increased with amplification of PCR. Therefore,

<sup>1</sup> Abbreviations used: APS, adenosine 5'-phosphosulfate; PPKD, pyruvate phosphate dikinase; PEP, phosphoenolpyruvate.

detection of PCR amplification is possible with the luciferin–luciferase reaction following conversion of pyrophosphate to ATP by PPKD. The reaction schema is presented in Fig. 1.

In this study, the dextranase gene (*dex*) in *S. mutans* was selected as a marker gene for preventive diagnosis of cariogenicity. Allele-specific PCRs, which consisted of *mutans* PCR and *sobrinus* PCR, were developed relative to slightly distinct *dex* genes in *S. mutans* and *S. sobrinus* [14]. Furthermore, a bioluminescent assay for pyrophosphate functioned as the detection methodology of PCR amplification.

## Materials and methods

### Reagents

PPDK from *M. rosea aerata* (EC2.7.9.1) and thermostable *Luciola cruciata* firefly luciferase (EC 1.13.12.7) were obtained from Kikkoman (Chiba, Japan). Pyrophosphate, lysozyme, and proteinase K were purchased from Wako Pure Chemical Industries (Osaka, Japan). dNTP mixture, *Taq* DNA polymerase, and 10× PCR buffer were acquired from Takara Shuzo (Osaka, Japan). Primers were synthesized by Takara Shuzo (Osaka, Japan). Luciferin and mutanolysin were obtained from Sigma Chemical (St. Louis, MO). Perfect match (PCR Enhancer) was manufactured by Toyobo. Other reagents were of analytical grade.

### Apparatus

The Luminescence Reader BLR-301 (Aloka, Tokyo, Japan), Wallac 1420 ARVosx multilabel counter, and Gene Amp PCR System 9600 (Perkin–Elmer, Norwalk, CT) were employed.

### Extraction of DNA from *S. mutans* and *S. sobrinus* in dental plaque

The *dex* genes of *S. mutans* and *S. sobrinus* consist of 2550 and 4011 bp, respectively. DNA extraction of bacterial plaque samples (dental plaque) was conducted following protein degradation with lysozyme [4]. Collected dental plaque was suspended in 500 μL of 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA (TE buffer). The suspension was heated at 100 °C for 10 min. After cooling, DNA extraction was effected via addition of 40 μL of lysozyme (50 mg/mL), 1000 U of mutanolysin, and 3 μL of proteinase K (20 mg/mL) to *S. mutans* and *S. sobrinus* cells in dental plaque. All reagents were dissolved in a final concentration of 1.3% SDS. Following phenol–chloroform extraction, DNA was refined by ethanol precipitation.

Isolated DNA samples were dried, followed by dissolution in TE buffer. Samples were stored at  $-40^{\circ}\text{C}$  until use.

#### Allele-specific PCR and common PCR

Strains of *S. mutans* and *S. sobrinus* were derived from dental plaque samples following clinical isolation. PCR conditions with regard to dNTP concentration and improvement of PCR specificity were assessed to obtain specific strong signals with respect to bioluminescence detection. Two allele-specific PCR methods (*mutans* and *sobrinus* PCR), involving individual primers specific to *mutans* and *sobrinus*, and common PCR, involving a primer common to *mutans* and *sobrinus*, were developed. In allele-specific PCR, sequences of the sense (MF) and antisense (MR) primers for amplification of *S. mutans dex* (*mutans* PCR) were 5'-GACCTTAAGGTGCAAGAGAA-3' and 5'-GGTTCTGTGCAGTTTCTAGC-3', respectively, and sequences of the sense (SF) and antisense (SR) primers for amplification of *S. sobrinus dex* (*sobrinus* PCR) were 5'-GGGTTATCCGAGCAACAAGT-3' and 5'-GGTTGACATCTTCACCCTGA-3', respectively. In common PCR, sequence of the sense (CF) and antisense (CR) primers for amplification of *S. mutans dex* and *S. sobrinus dex* were 5'-CTGTTTTGCCAGAGACGGAATAC-3' and 5'-GTATCACCTGCCAACCATCAAAA-3', respectively. These PCR protocols were performed as follows. Template DNA (1  $\mu\text{L}$ ), which corresponded to 20 ng as bacterial DNA and 1  $\mu\text{L}$  of *Taq* polymerase (2.5 U), was introduced to 48  $\mu\text{L}$  of mixed solution containing 5  $\mu\text{L}$  of 10 $\times$  buffer, dNTP (dATP, dCTP, dGTP, and dTTP), 1  $\mu\text{L}$  of each primer (20 pmol), and  $\text{H}_2\text{O}$ . The reaction was conducted at  $94^{\circ}\text{C}$  after a 5-min heating process ( $94^{\circ}\text{C}$  for 1 min) at  $45^{\circ}\text{C}$  for common PCR or at  $55^{\circ}\text{C}$  for allele-specific PCR for 1 min and at  $72^{\circ}\text{C}$  for 1 min. The number of PCR cycles was 25. Following the final PCR cycle, the reaction was completed at  $72^{\circ}\text{C}$  for 7 min. Allele-specific and common PCR products were determined with the bioluminescent assay.

#### Bioluminescent detection of PCR products and pyrophosphate

Pyrophosphate solution or PCR product (10  $\mu\text{L}$ ), which was diluted 10 times with  $\text{H}_2\text{O}$ , was introduced to a test tube; subsequently, 100  $\mu\text{L}$  of PPDK luciferin–luciferase solution (PPDK reagent) (2.34 U/mL PPDK, 0.2 mM luciferin, 5.5 U/mL luciferase, 0.0125 mM AMP, 0.04 mM PEP, 0.005 U/mL apyrase, 0.05 mM dithiothreitol, 5% trehalose, 1 mM EDTA, 7.5 mM  $\text{MgSO}_4$ , 30 mM 2[bis(2-hydroxyethyl)amino]ethanesulfonic acid, pH 8.0) was added. After 150 s, emission intensity was measured for 10 s in the luminescence reader (Aloka).

## Results and discussion

#### Measurement of pyrophosphate via bioluminescence detection

PPDK–luciferin/luciferase reaction has been employed for measurement of AMP and RNA. To apply the PPDK–luciferin–luciferase reaction to measurement of pyrophosphate in the present investigation, conditions of the AMP measurement system involving this reaction were improved and the optimal conditions for measurement of pyrophosphate based on the presence of excess AMP were determined as PPDK–luciferin–luciferase solution described under Materials and methods.

#### Kinetics of light emission for measurement of pyrophosphate

To obtain optimum reaction time for the measurement of pyrophosphate, PPDK–luciferin–luciferase reagent (100  $\mu\text{L}$ ) was introduced to 10  $\mu\text{L}$  of pyrophosphate; subsequently, light emission intensity was measured from the initiation of the reaction. As a result, light emission intensity reached a plateau after 100 s; the reading remained elevated beyond a 10-min period. Kinetics of light emission is presented in Fig. 2.

#### Calibration curve of pyrophosphate

The calibration curve of pyrophosphate was obtained via this method. Pyrophosphate from  $1 \times 10^{-12}$  to  $1 \times 10^{-3}$  M was examined. The calibration curve of pyrophosphate is illustrated in Fig. 3. The detection limit of pyrophosphate was  $1.56 \times 10^{-15}$  mol/assay (as blank + 2 SD).

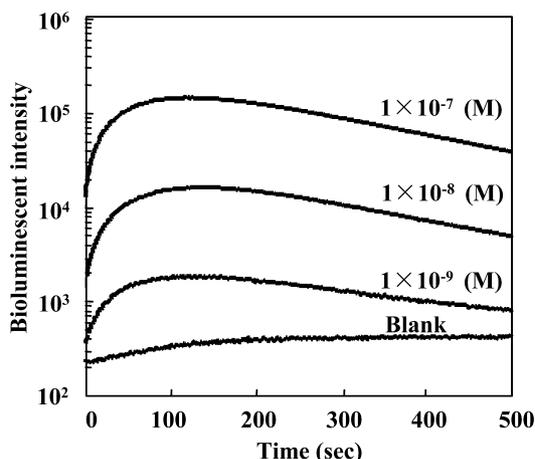


Fig. 2. Time course of bioluminescent intensity. Pyrophosphate concentrations of  $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$ , and 0 M ( $\text{H}_2\text{O}$ ) were measured. The 10- $\mu\text{L}$  samples were measured according to the procedure under Materials and methods.

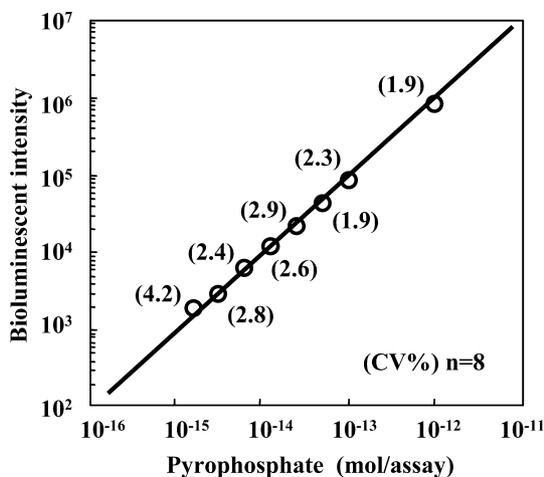


Fig. 3. Standard curve for pyrophosphate.

### PCR conditions

The underlying principle of this approach involves the determination of pyrophosphate released during allele-specific amplification of genes of cariogenic species via the bioluminescent PPKK–luciferin–luciferase reaction. Previously, we developed allele-specific PCRs for *S. mutans* and *S. sobrinus* with regard to detection of cariogenic bacterial genes, which are analyzed by microchip electrophoresis. However, excess dATP used in PCR and nonspecific PCR amplification observed in electrophoresis may raise the blank value during light emission detection based on this principle, which influences the sensitivity and specificity. Therefore, dNTP concentration, *Taq* DNA polymerase amount, PCR cycle number, and annealing temperature were reexamined.

**Effect of dNTP concentration.** Pyrophosphate is formed in the PCR extension reaction; pyrophosphate was released during thermal decomposition of dNTP during PCR. In addition, use of high concentrations of dATP leads to elevation of the blank value of the bioluminescence reaction following PCR (data not shown). Luciferin/luciferase utilized in this study reacts with dATP; consequently, it corresponds to approximately 10% of ATP. Thus, reduction of dNTP quantity to as large an extent as possible for PCR is desirable. In this experiment, the optimum dNTP concentration was examined under conditions in which PCR product quantity was not diminished. As a result, *S/N* ratio (*S*, emission intensity at template DNA; *N*, emission intensity with no template DNA in PCR) of light emission exceeded 10 when 1/5 (dNTP 0.8 μL/50 μL) of the usual dNTP quantity was employed. In the case of 1/20 (dNTP 0.2 μL/50 μL) of the usual dNTP quantity, PCR product decreased significantly. These findings demonstrated that the efficiency of amplification decreases when the concentration of dNTP in PCR is reduced

sharply. Therefore, 0.8 μL of dNTP was selected for PCR conditions characterized by a *S/N* ratio at emission intensity in excess of 10 and a smaller reduction of the PCR product.

**Effect of *Taq* DNA polymerase quantity.** Optimum conditions for *Taq* polymerase consist of pH 8.3–8.8 and temperature of 70–75°C; moreover, unit amount in PCR is the usual 1–2.5 U. In this study, *Taq* polymerase quantity of 0.25–5 U was examined. As a result, the steady state was present upon addition of 1 μL of *Taq* polymerase (data not shown). Therefore, polymerase quantity of 1 μL (5 U) was utilized in the following experiment.

**Effect of cycle number.** dNTP decomposes upon heating above 90°C; slight pyrophosphate release occurs. Therefore, limiting the number of PCR cycles associated with heating is highly desirable. In addition, PCR characterized by increased cycle number generally produces nonspecific PCR product. Consequently, reduced cycle number is important to enhance specificity. As a result, cycle number was examined. Light emission intensity increased with cycle number. However, increases in the number of PCR cycles exceeding 30 diminished light emission intensity of the PCR product; in contrast, the blank value increased due to decomposition consequent to extended heating and nonspecific amplification of dNTP. Therefore, to obtain optimum light emission intensity, high *S/N* ratio and rapid completion are required; 25 cycles appear optimum.

**Effect of annealing temperature.** This investigation was conducted employing optimum conditions (dNTP 0.8 μL, *Taq* polymerase 1 μL, PCR, 25 cycles). Annealing temperature was examined subsequently. The experiment was performed at temperatures of 45, 50, and 55°C. However, changes in the *S/N* ratio could not be observed at these temperatures. 55°C was selected for specific duplex allele-specific PCR; on the other hand, 45°C was selected for common PCR to achieve equal amplification of *S. mutans* and *S. sobrinus*.

### Measurement of PCR product

Allele-specific PCRs (*mutans* and *sobrinus* PCRs) employing a set of MF and MR primers for the *dex* gene in *S. mutans* and a set of SF and SR primers for the *dex* gene in *S. sobrinus* can be amplified specifically; consequently, *S. sobrinus* and *S. mutans* can be identified readily. Common PCR is not specific for *S. sobrinus* and *S. mutans*; therefore, identification of these bacteria was conducted via allele-specific PCR (*mutans* and *sobrinus* PCRs). Results of bioluminescent detection for the two allele-specific PCR techniques are exhibited in Fig. 4A. Light emission intensity was presented as the *S/N* ratio (*S*, signal of PCR product using template DNA; *N*, noise of PCR product without DNA). When

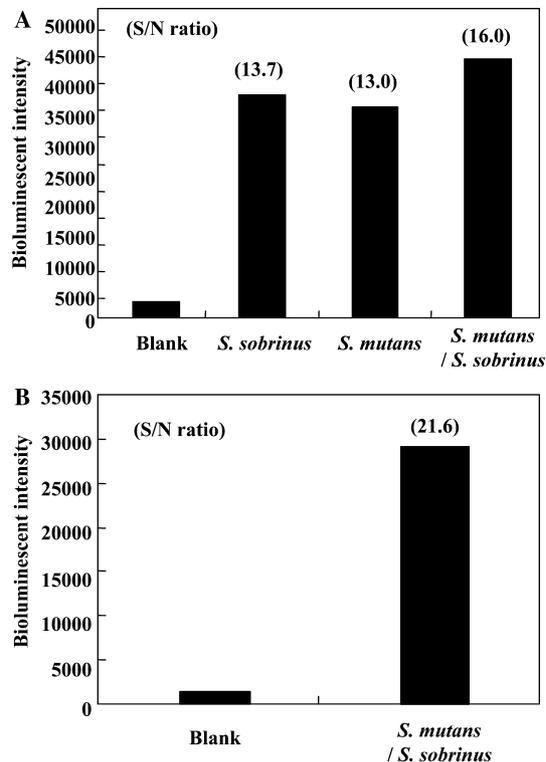


Fig. 4. Measurement of PCR product. Bioluminescent intensity obtained by specific PCR products (A) and common PCR products (B). Cell DNA (20 ng) extracted from cultured *S. mutans*, *S. sobrinus*, and a mixture of both bacteria were detected by allele-specific PCRs (A) and common PCR (B).

*S. mutans* and *S. sobrinus* DNA which corresponded to 20 ng as bacterial DNA were used, the *S/N* ratio of *S. mutans* was 13.7 and that of *S. sobrinus* was 13.0. This finding indicates that *S. mutans* and *S. sobrinus* were clearly identified by the respective bioluminescent allele-specific PCR approaches.

#### Bioluminescence detection of PCR product obtained by common PCR

As described above, identification of *S. mutans* and *S. sobrinus* is challenging due to nonspecific amplification of both bacteria by common PCR. However, types of fungi, which inhabit the human mouth, approximate *S. mutans* and *S. sobrinus* with regard to the *dex* gene; consequently, detection of one or the other bacterium is sufficient for cariogenic prevention. Therefore, to identify *S. mutans* and *S. sobrinus*, common PCR with CF and CR primers was conducted; subsequently, PCR products were determined by bioluminescent PPDK–luciferin–luciferase reaction. As a result, super-sensitive detection of both genes of *S. mutans* and *S. sobrinus* was possible; *S/N* ratios were 21.6. This finding indicates that common PCR is suitable for sensitive determination of cariogenic species. The result is presented in Fig. 4B.

Table 1  
Types and grouping of oral bacteria

Oral bacteria	<i>dex</i> gene/inhabitant
Group 1	
1. <i>S. mutans</i>	(+)/human
2. <i>S. sobrinus</i>	(+)/human
Group 2	
3. <i>S. rattus</i>	(+)/rat
4. <i>S. cricetus</i>	(+)/mouse
5. <i>S. downei</i>	(+)/monkey
6. <i>S. macacae</i>	(+)/monkey
7. <i>S. salivarius</i>	(+)/human
Group 3	
8. <i>S. bovis</i>	(-)
9. <i>S. oralis</i>	(-)
10. <i>S. mitis</i>	(-)
11. <i>S. gordonii</i>	(-)
12. <i>S. faecalis</i>	(-)
13. <i>S. sanguis</i>	(-)

#### Specificity of allele-specific PCRs and a common PCR

The specificity of allele-specific PCR (*mutans* and *sobrinus* PCR) was determined with DNA extracted from bacteria. These bacteria were divided into three groups (Table 1): group 1, mixed sample of *S. mutans* and *S. sobrinus*; group 2, mixed sample of *dex*(+) bacteria (3–7) in Table 1; and, group 3, mixed sample of *dex*(-) bacteria (8–13) in Table 1. DNA extracted from these groups was mixed and amplified by duplex PCR, which consisted of *mutans* PCR and *sobrinus* PCR, and by common PCR following bioluminescent pyrophosphate assay.

As a result, bioluminescent assay of duplex PCR composed of *mutans* PCR and *sobrinus* PCR demonstrates bioluminescent intensity exclusively for a mixture of *S. mutans* and *S. sobrinus* in group 1. In contrast, bioluminescent assay involving common PCR reveals bioluminescent intensity with respect to DNA of groups 1 and 2 (*dex*(+)); however, group 3 (*dex*(-)) exhibits no bioluminescent intensity. Therefore, the two allele-specific PCR techniques (*mutans* and *sobrinus* PCRs) were specific for *S. mutans* and *S. sobrinus*; moreover, common PCR, which amplified DNA involving *dex*(+), did not amplify specific DNA with *dex*(-). Consequently, allele-specific PCR can function as a specific detection method for *S. mutans* and *S. sobrinus*, whereas common PCR is applicable to fungi characterized by *dex*(+). These results are depicted in Fig. 5.

#### Detection limit of *dex* gene of *S. mutans* and *S. sobrinus*

The detection limit of the bioluminescent pyrophosphate assay of *dex* genes of *S. mutans* and *S. sobrinus* was examined; 20, 2, 0.2, 0.02, 0.002, and 0.0002 ng/ $\mu$ L DNA of *S. mutans* and *S. sobrinus* served as a template. As a result, 20 pg of DNA of both *S. mutans* and

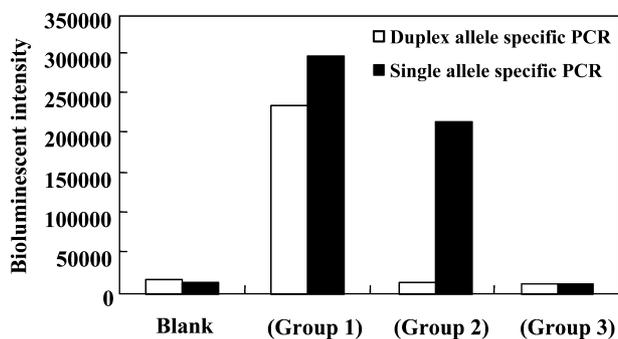


Fig. 5. Specificities of allele-specific and common PCRs. DNA (20 ng) extracted from groups 1–3 was mixed and amplified by duplex PCR, which consisted of *mutans* PCR and *sobrinus* PCR, and by common PCR following bioluminescent pyrophosphate assay.

*S. sobrinus* was detectable by both allele-specific PCR protocols consisting of 25 cycles. The detection limit was expressed as an *S/N* ratio exceeding 1.5 (data not shown). This sensitivity corresponded to that of agarose gel electrophoresis involving nondiluted PCR product (5  $\mu$ L) and ethidium bromide as an intercalating agent.

#### Reproducibility of bioluminescence detection method

The reproducibility of the proposed method was examined using allele-specific PCR products of *S. mutans* and *S. sobrinus*. Reproducibility (CV%,  $n = 6$ ) demonstrated high accuracy, 4.65 and 3.28%, respectively.

#### Application to dental plaque samples

Bioluminescent detection of allele-specific PCR (*mutans* and *sobrinus* PCRs) was applied to the detection of *S. mutans* and *S. sobrinus* in dental plaque with regard to a preventive diagnosis of cariogenicity. DNA was extracted from dental plaque samples (YBK1~YBK11); subsequently, *mutans* and *sobrinus* PCRs were

performed followed by bioluminescent pyrophosphate assay.

The specificity of this method was examined previously using the gene of 13 types of *dex*(+) and *dex*(-) bacteria. Those findings demonstrated that these PCR approaches afforded great specificity for *S. mutans* and *S. sobrinus* (Fig. 5). However, specificity of PCR with respect to dental plaque samples declined consequent to the large number of bacteria, usually 300–400 types, that exist in dental plaque; that is, allele-specific PCR non-specifically amplified other DNA. Subsequently, PCR conditions (annealing temperature, PCR cycle number) for specific amplification of *S. mutans* and *S. sobrinus* genes in DNA extracted from dental plaque specimens were reexamined.

As a result, PCR conditions were modified as follows: addition of 5  $\mu$ L of dimethyl sulfoxide, annealing temperature of 57°C, and PCR cycle number of 30. Under these conditions, nonspecific PCR amplification of dental plaque samples was not observed.

#### Measurement of *S. mutans* and *S. sobrinus* in dental plaque samples by bioluminescent detection of allele-specific PCR

*S. mutans* and *S. sobrinus* in dental plaque samples (YBK1~YBK11) were identified via allele-specific PCR (*mutans* and *sobrinus* PCRs). In this experiment, 96-well microtiter plates and a light emission measurement instrument (Wallac 1420 ARVOsx multilabel counter) were utilized. As shown in Fig. 6, DNA of *S. mutans* and *S. sobrinus* from all 11 specimens was detected. In each sample, the abundance ratio of *S. mutans* and *S. sobrinus* is distinct (Fig. 6); furthermore, fungus quantities may vary also. The size of dental plaque samples is different; therefore, accurate quantitative determination of *S. mutans* and *S. sobrinus* is not possible. Consequently, several methods for correct dental plaque

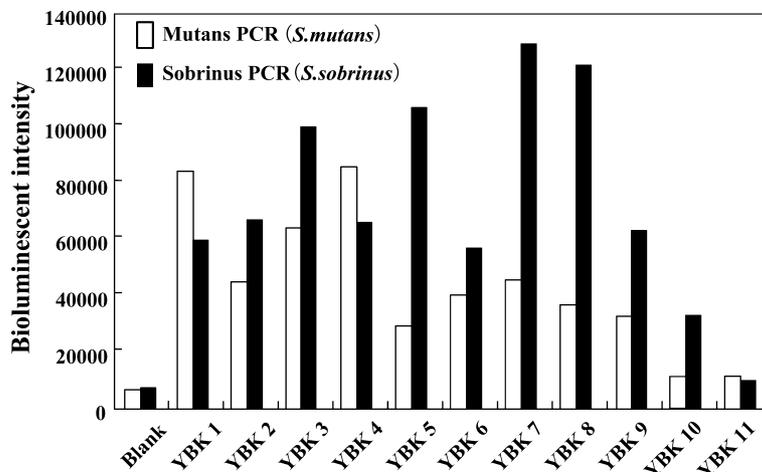


Fig. 6. Application to dental plaque samples.

sampling are necessary. At present, a technique for amplification of *mutans* streptococcus DNA employing PCR, which simultaneously detects normal oral cavity bacteria in dental plaque as a control, and a correction method related to sampling amount utilizing pigment to dye dental plaque are under development. In addition, the improvement in sensitivity of allele-specific PCR employing the longer PCR product, which generates larger quantities of pyrophosphate, was examined.

### Conclusion

A novel bioluminescent pyrophosphate assay utilizing the PPDK–luciferin–luciferase reaction was established to quantitatively measure PCR product. Detection of pyrophosphate ( $1.56 \times 10^{-15}$  mol/assay) characterized by enhanced sensitivity was possible with the proposed method. Furthermore, this bioluminescent assay in association with allele-specific PCR was applied to the analysis of the *dex* gene of *mutans* streptococcus. Allele-specific PCR for *S. mutans* and *S. sobrinus* involving *mutans* and *sobrinus* primers and common PCR employing the primer common to both *S. mutans* and *S. sobrinus* were developed for this analysis. Consequently, detection and identification of *S. mutans* and *S. sobrinus* were possible; moreover, combination with common PCR afforded supersensitive detection of *S. mutans* and *S. sobrinus*. Based on the aforementioned data,

analysis of *mutans* streptococcus via the novel bioluminescent assay for PCR product appears to afford a technique suitable for diagnosis and prevention of cariogenic bacteria.

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