METHODS

A Gel-Free SNP Genotyping Method: Bioluminometric Assay Coupled With Modified Primer Extension Reactions (BAMPER) Directly From Double-Stranded PCR Products

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Communicated by Paolo M. Fortina

Inexpensive, high-throughput genotyping methods are needed for analyzing human genetic variations. We have successfully applied the regular bioluminometric assay coupled with modified primer extension reactions (BAMPER) method to single-nucleotide polymorphism (SNP) typing as well as the allele frequency determination for various SNPs. This method includes the production of single-strand target DNA from a genome and a primer extension reaction coupled with inorganic pyrophosphate (PPi) detection by a bioluminometric assay. It is an efficient way to get accurate allele frequencies for various SNPs, while single-strand DNA preparation is labor intensive. The procedure can be simplified in the typing of SNPs. We demonstrate that a modified BAMPER method in which we need not prepare a single-strand DNA can be carried out in one tube. A PCR product is directly used as a template for SNP typing in the new BAMPER method. Generally, tremendous amounts of PPI are produced in a PCR process, as well as many residual dNTPs, and residual PCR primers remain in the PCR products, which cause a large background signal in a bioluminometric assay. Here, shrimp alkaline phosphatase (SAP) and E. coli exonuclease I were used to degrade these components prior to BAMPER detection. The specific primer extension reactions in BAMPER were carried out under thermocycle conditions. The primers were extended to produce large amounts of PPi only when their bases at 3'-termini were complementary to the target. The extension products, PPis, were converted to ATP to be analyzed using the luciferin–luciferase detection system. We successfully demonstrated that PCR products can be directly genotyped by BAMPER in one tube for SNPs with various GC contents. As all reactions can be carried out in a single tube, the method will be useful for realizing a fully automated genotyping system. Hum Mutat 24:155–163, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: SNP; genotyping; BAMPER; bioluminometric assay; mutation detection

INTRODUCTION

The completion of the human genome sequence [McPherson et al., 2001; Venter et al., 2001], as well as an initial map of human genome sequence variation [Sachidanandam et al., 2001], will help enable the provision of medical care to patients based on each individual’s specific genotype. Although the map identifies and localizes 1.42 million single-nucleotide polymorphisms (SNPs) in the genome, the relationships between diseases and SNPs have not being clarified. To associate genetic variations with a variety of diseases, a large amount of SNPs need to be analyzed. Consequently, an automated and high-throughput SNP typing method is required. Previously, various SNP genotyping approaches have been developed to identify genetic alterations, including electrophoresis [Medintz et al., 2001], denaturing liquid chromatography [Xiao and Oefner, 2001], mass spectrometry [Bray et al., 2001], flow-cytometry with color beads containing DNA probes [Chen et al., 2000], DNA chips coupled with electric field–controlled DNA hybridization [Sosnowski et al., 1997], minisequencing [Cai et al., 2000; Syvanen, 1999], dynamic allele specific hybridization (DASH) [Prince and Brookes, 2001], enzymatic cleavage methods such as Invader [Hsu et al., 2001] and 5'–nuclease TaqMan

Received 21 October 2003; accepted revised manuscript 20 February 2004.

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Grant sponsor: New Energy and Industrial Technology Development Organization in Japan (NEDO).

DOI 10.1002/humu.20052
Published online in Wiley InterScience (www.interscience.wiley.com).
Sodium 2

All solutions were prepared in deionized and sterilized water. The diameter (ID) was purchased from Dynal A.C. (Oslo, Norway). To remove PPi in the PPi filter-tube having the nominal molecular weight cutoff of 10,000, the residual PPase was removed by ultrafiltration with a centrifugal membrane. The mixture was thoroughly mixed and incubated at 37°C for 40 min, and then heated at 80°C for 10 min to inactivate the enzymes. Generally, 3 μL of PCR products were used for each SNP typing: three BAMPER reactions (for wild-type, mutant, and control) were carried out, and each reaction used only 1 μL of PCR products.

**Materials and Methods**

**Chemicals**

Platinum® Taq DNA polymerase was purchased from Gibco-BRL Life Technologies (Grand Island, NY). Polynucleopyrrolidone (PVP), deoxynucleotide (dNTPs), and QuantiLum™ recombinant luciferase (95%) were purchased from Promega (Madison, WI; www.promega.com). Sodium pyrophosphate decahydrate (PPi), inorganic pyrophosphatase (PPase), and adenosine 3′-phosphosulfate (APS) were purchased from Sigma (St. Louis, MO).

**Degradation of Endogenous PPi Contaminated in Reagents**

A total of 50 μL of 10 mM dNTPs containing 25 mM magnesium acetate tetrahydrate (Mg(Ac)2) and 5 mM Tris-HAc, pH7.7, were incubated with 0.4 U PPase for 30 min at room temperature. The residual PPase was removed by ultrafiltration with a centrifugal filter-tube having the nominal molecular weight cutoff of 10,000 (Millipore, Japan; www.millipore.com). To remove PPi in the PPi detection mixture, 0.1 U PPase was directly added to 50 mL PPi detection solution before adding APS, and the solution was incubated for 30 min at room temperature.

**Primer and Target Sequences**

All the oligonucleotides were synthesized and purified by Amersham Pharmacia Biotech (Hokkaido, Japan). The sequences of the PCR primers are listed in Table 1. Pairs of genotyping probes (primers) were designed so as to hybridize in the target-specific regions. As there were two choices for selecting the target DNA strand, two sets of genotyping primers could be designed. The sequences of the genotyping primers (specific primers used for typing SNPs) in this article are listed in Table 1.

**PCR Amplification and Enzymatic Cleanup**

Genome DNA for method development was extracted from the blood of volunteers in our laboratory with DNAZOL™ reagent (Life Technologies, Gibco BRL; www.lifetech.com). Various genome samples for method validation were obtained from the Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan. Written informed consent was obtained from each subject after a full explanation of the study, which was approved by the institutional ethics committee. DNA fragments used for SNP testing were amplified from the genome DNA with a PTC-225 thermocycler PCR System (MJ Research, Inc.; www.mjr.com) according to the following protocol: denatured at 94°C for 2 min, followed by 35 thermal reaction cycles (94°C for 30 s; 57°C for 60 s; 72°C for 1 min). After the cycle reaction, the product was incubated at 72°C for 5 min and held at 4°C. The annealing temperature was changed according to the Tm of each PCR primer.

In the case of multiplex PCR, 10 μL of the amplification was carried out in a 384-well plate. The concentration of each primer used in single-PCR or multiplex-PCR was identical, 0.2 pmol/μL. The cycling condition was the same as that described above except for the annealing temperature, which was the average of each primer’s Tm.

Residual components, such as PCR primers, dNTPs, and PPis produced in the PCR step, produce a large background signal. These components were degraded by adding 1 U of shrimp alkaline phosphatase (SAP) and 2 U of exonuclease I to 5 μL of the PCR product. The mixture was thoroughly mixed and incubated at 37°C for 40 min, and then heated at 80°C for 10 min to inactivate the enzymes.

Generally, 3 μL of PCR products were used for each SNP typing: three BAMPER reactions (for wild-type, mutant, and control) were carried out, and each reaction used only 1 μL of PCR products.

**Allele-Specific Extension Reactions**

Thermostable DNA polymerase without exonuclease activity was used for allele-specific primer extension reactions. A pair of extension reactions for one template species with two typing primers, corresponding to wild-type and mutant, respectively, was carried out under the same conditions. The reaction mixtures contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM magnesium chloride, 125 μM of deoxy-CTP (dCTP), 125 μM of deoxy-TPP (dTTP), 125 μM of deoxy-GTP (dGTP), 125 μM of deoxy-adenosine triphosphate-25S (dATP25S), 1.25 μM of genotyping primers, 0.05 U/μL of Platinum Taq DNA polymerase, and 1 μL of PCR products. Each 4-μL reaction mixture was incubated at 94°C for 30 s. This was followed by five thermal cycles (94°C for 10 s; 55°C for 10 s; 72°C for 20 s) in a PTC-225 thermocycler. Finally, the product was kept on ice before the PPi assay. Because of the small volume of extension solution, mineral oil was added to prevent the evaporation of water.
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<th>SNP code</th>
<th>Gene symbol</th>
<th>Accession number</th>
<th>SNP identifier*</th>
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<th>Specific primer sequencec</th>
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</table>

*aThe nucleotide residue 1 is located at the start of the reference sequence for 'g.' numbers, and at the A of the ATG translation initiation start site for 'c.' numbers.
*bFW and RV represent forward and reverse primer, respectively.
*cThe two capital letters in the 3-terminus of the primer represent the specific base to the SNP type, and the third capital letter represents the artificially mismatched base. The number in the brackets represents the GC content in a probe.
*dGE means SNP typing by gel-based electrophoresis.
PPI Detection

The detection of PPI produced in the genotyping primer extension reactions was performed by a method similar to one previously reported [Zhou et al., 2001]. The PPI detection solution contained the following components: 0.1 M Tris-acetate (pH 7.7), 2 mM EDTA, 10 mM magnesium acetate, 0.1% BSA, 1 mM dithiothreitol, 2 μM APS, 0.4 mg/ml PVP, 0.4 mM D-luciferin, 200 mU/ml ATP sulfurylase, and 3 μg/ml luciferase. We also added 40 μl of PPI detection solution to each well containing extension solution.

Experimental Apparatus

The bioluminescence was measured with a LUMINOUS CT-9000D luminometer (DIA-IATRON, Tokyo, Japan). The detection solution was supplied in a reaction well of the titer plate by hand or by a dispenser installed in the luminometer. Signals from a 96-well plate containing target DNA from 48 different genome samples were obtained to determine the allele species.

THE PRINCIPLE OF SIMPLIFIED BAMPER

The BAMPER process can be simplified by using double-strand DNAs as the template DNAs instead of single-strand DNAs. A PCR product can be directly used for the new BAMPER assay after degrading the residual PPI, dNTPs, and PCR primers. The process consists of four steps (Fig. 1): 1) PCR amplification to produce double-strand DNAs containing the target SNP sites; 2) degradation of residual components such as dNTPs, PPI, and PCR primers in the PCR products with SAP and *E. coli* exonuclease I, followed by dividing the products into two equal aliquots; 3) allele-specific cycle primer-extension reactions in the two aliquots produced inorganic pyrophosphate; and 4) bioluminometric detection using a luciferin-luciferase and an APS-ATP sulfurylase reaction system. As the two genotyping primers are designed so that the 3'-termini come to the SNP position, the allele type can be determined by comparing the photosignal produced by the primer extension reaction products, PPI, in each aliquot.

As the primer extension reactions cannot be controlled perfectly through primer terminus matching or mismatching, a mismatched primer extension frequently occurs when a regular genotyping primer is used, as shown on the right side of Figure 1. The introduction of an artificially mismatched nucleotide in the 3'-terminal...
region of the specific primers is used to minimize the false positive signals. For simplicity, the artificial mismatch base species were designed to be the same as that of the template. If the 3'-terminus of the genotyping primer is mismatched to the template, there are two mismatched bases in the 3'-terminal region, so the mismatch extension is greatly reduced. However, if the 3'-terminus of the genotyping primer is matched to the template, there is only one mismatched base at the third position from the 3'-terminus, which will not affect the extension reaction. Because the template DNAs are double-stranded, the primer extension reactions brought about by thermal-cycling are suitable for obtaining sufficient signal intensities. As dNTPs are decomposed at high temperature, a thermal cycle of less than 10 is recommended. The cycle strand extension reaction gives bigger BAMPER signals than those obtained by a single strand-extension reaction with single-strand DNAs so that accurate and reliable SNP typing can be carried out.

**RESULTS AND DISCUSSION**

*Control of Primer Extension Reactions by Artificially Mismatched Base in Each SNP-Specific Primer*

BAMPER utilizes the switching characteristics of primer extension reactions for typing an SNP, in which mismatched primer extension should be avoided. To evaluate the primer extension characteristics, we used an SNP, I823M in the ABCA1 gene, as a test target. Three samples including two homogeneous samples (AA and GG) and a heterogeneous sample (A/G) were analyzed. A pair of primers containing the artificial mismatch bases in the third positions from the 3' ends was designed to distinguish the wild-type and mutant type. Also, a pair of genotyping primers without an artificial mismatch base at the 3'-terminus region was used for the control experiment. The results (Fig. 2) indicate that the introduction of the artificial mismatch base was an effective way to decrease the false-positive signals. For example, the false-positive signal for the homogenous sample (GG) was decreased from 36% to several percent.

**Strategy for Decreasing Background Signals**

The key point to simplifying the process in the new BAMPER method is how to skip the cleanup step for the PCR products. In PCR products, various components, including residual PCR primers, dATP, and PPi will affect the subsequent bioluminometric assay for SNP typing. PCR primers produce unwanted PPi in the extension step of SNP detection and give a very high background signal. dATP is an analog of ATP which reacts with luciferin to emit luminescence. The large amount of PPi produced in PCR amplification makes accurate SNP detection impossible. Consequently, such components must be degraded before the specific-primer extension reaction. The process of degrading these components should not affect the following SNP detection process. Therefore, we used thermosensitive enzymes for the degradation at first, and followed this with heating-inactivation of the enzymes. Here, exonuclease I was used for degrading PCR primers and SAP was used for degrading dATP and PPi. Both of these were inactivated by high-temperature incubation. This treatment had no observed effect on the subsequent SNP detection. As a result, one-tube cleanup of the PCR products was realized by simply adding a small amount of SAP and exonuclease I, as has also been used in pyrosequencing [Nordstrom et al., 2000a, b] and another method [Taylor et al., 2001]. The optimum concentrations of SAP and exonuclease I were 0.5–2.0 U and 1.0–4.0 U, respectively, for 10 μl of PCR products. If a large background signal is still observed after the enzyme treatment, a single-strand DNA binding protein (SSB) can be added to prevent primer dimer production [Ronaghi, 2000].

Although the components which affect the detection of SNPs were eliminated from the PCR products, there were several reagents that produced high luminescence in the BAMPER process using thermal cycling. The background signal due to the dATP used for the extension reactions was very strong. We reduced this signal to a level corresponding to about one-fiftieth of the normal positive signal observed in BAMPER by using the analog, dATPαS. The thermal decomposition of dNTPs was the main background source in BAMPER using the thermalcycling procedure, and this was minimized by decreasing the dNTP concentration.

![FIGURE 2. SNP typing of I823M in the ABCA1 gene using artificial mismatch primers (A) and regular primers (B) in cycle extension reactions for BAMPER. Three samples with different SNP types were analyzed: homo-AA, hetero-AG, and homo-GG. Primer-A and primer-B represent the specific primer for typing allele-A and allele-G, respectively. Homo-AA and homo-GG represent homogeneous samples with the allele of AA and GG, respectively, and hetero-AG represents a heterogeneous sample with the allele of A and G.](image-url)
and shortening the high-temperature incubation time. However, the signal intensity from the cycle extension reaction is affected by the amount of dNTPs. Therefore a balance must be struck between the background and the positive signal.

As shown in Figure 3A, the positive signal intensities became stronger with an increasing dNTP concentration, as well as with an increased cycling number applied for the extension reaction. However, the background, mainly produced by the decomposition of dNTPs, also increased in proportion to the cycles, as shown in Figure 3B. The optimum dNTP concentration for genotyping was obtained from Figure 3C. When dNTP concentration was more than 0.1 mM, one cycle was enough to get a good signal-to-background ratio. Multiple cycle reactions were needed when the dNTP concentration was less than 0.01 mM. From the viewpoint of rapid analysis with adequate sensitivity, we selected a concentration of 0.125 mM for each dNTP and five cycles for the extension reaction in the simplified BAMPER experiment.

**Genotyping of Various SNPs for Different Genome Samples**

Genotyping of 27 individual SNPs in different heart-related genes was carried out with the modified BAMPER. The details of the SNPs are listed in Table 1. To remove the background signals due to primer dimer extension and decomposition of dNTPs, a control reaction without a genotyping primer was carried out to eliminate the contribution of the background in the observed signal intensities. The result was sufficient for genotyping of the 27 different SNPs (Fig. 4a). The BAMPER genotyping results coincided with results obtained through DNA sequencing, indicating that BAMPER worked well for genotyping primers containing GC rich sequences, such as eNOS-786T/C (Table 1).

To investigate the sample-to-sample instability of typing results in BAMPER, we analyzed 10 different SNPs for various genomic samples. All the results coincided with those obtained by DNA sequencing. Part of the genotyping results for 23 genomic samples are shown in Figure 4b. These results show that the simplified BAMPER method is applicable to different kinds of genome samples, and is accurate enough to type either allele of an SNP.

**Required Sample Amounts**

To evaluate the minimum required sample amount in BAMPER, we used various amounts of genome sample as PCR templates. For each specific primer-extension reaction, we used 2 μl of the PCR products. As shown in Figure 5A, 2 ng of genome sample was enough for a BAMPER assay. As 2 μl PCR products is enough for typing a SNP, as shown in Figure 5B, we used 5 μl PCR amplification with a 384-well plate for the BAMPER assay.

To enable low-cost, high-throughput SNP typing, a small reaction volume should be used. We tried using a total of 1 μl of extension solution containing 0.5 μl of PCR products for typing SNP-12 in Table 1. The signal intensity, subtracting the control value, was 6,165 arbitrary units for the matched primer and 261 for the mismatched primer. An intensity ratio of 23.6 arbitrary units was obtained from the two aliquots, which is sufficient for reliable judgment for the type of an SNP.

**Multiplex PCR for SNP Typing by BAMPER**

The most expensive process in BAMPER is the PCR amplification process. If multiplex PCR can be used for BAMPER, the testing cost will be lowered. In our BAMPER detection, only 1 μl of PCR product was used. If, for example, 10 μl of PCR product was prepared, we would discard most of the PCR product. As BAMPER is based on a specific primer-extension reaction, the specific primer only hybridizes the complementary target in the mixture, even if many amplified DNA fragments are present in the same solution. We attempted two-plex,
BAMPER SNP GENOTYPING

for typing SNPs can thus be realized. 3) Multiplex
A high-throughput device using 0.5
384-well plate was enough for a regular BAMPER assay.
one SNP typing. We found that 5

PCR products directly: 1) The detection procedure is
benefits are gained from BAMPER using double-strand
primer-extension reaction, and PPi detection. Because of

strand binding protein, as indicated by Ronaghi [2000],
was due to incomplete degrada-
three-plex, four-plex and five-plex PCR for SNP typing
by BAMPER. For comparison, we also carried out regular
PCRs for all five SNPs. As shown in Figure 6, BAMPER
could be applied with multiplex PCR products. Although
a large background appeared frequently in the multiplex
mode, we overcame this problem by adding a single-
strand binding protein, as indicated by Ronaghi [2000],
because the background was due to incomplete degrada-
tion of the PCR primer dimers. The running cost of
BAMPER with multiplex PCR was much lower than that
with a single PCR.

CONCLUSION

The BAMPER system described here comprises four
steps: PCR amplification, enzymatic treatment, specific
primer-extension reaction, and PPi detection. Because of
the introduction of an artificial mismatch base into the
specific extension primer, BAMPER is highly accurate
for 3’ end-base controlled extension reactions. Several
benefits are gained from BAMPER using double-strand
PCR products directly: 1) The detection procedure is
very simple because it is not necessary to prepare single-
stranded DNA as a template. All of the reactions can be
carried out in one tube. 2) The high sensitivity of
BAMPER allows use of a small amount of genome for
one SNP typing. We found that 5 µl of PCR products in a
384-well plate was enough for a regular BAMPER assay.
A high-throughput device using 0.5 µl of PCR products
for typing SNPs can thus be realized. 3) Multiplex

three-plex, four-plex and five-plex PCR for SNP typing
by BAMPER. For comparison, we also carried out regular
PCRs for all five SNPs. As shown in Figure 6, BAMPER
could be applied with multiplex PCR products. Although
a large background appeared frequently in the multiplex
mode, we overcame this problem by adding a single-
strand binding protein, as indicated by Ronaghi [2000],
because the background was due to incomplete degrada-
tion of the PCR primer dimers. The running cost of
BAMPER with multiplex PCR was much lower than that
with a single PCR.

FIGURE 4. Genotyping results from the simplified BAMPER using double-stranded DNAs directly. a: Typing various SNPs in a genome sample. The information on SNP-1 to SNP-27 is listed in Table 1. Primer-A and prime-B correspond to a pair of genotyping primers for strand extension after target hybridization. b: Typing an SNP, G664A in the ANP gene, for 23 different genome samples. Primer-A and prime-B denote the genotyping primers for distinguishing allele C and allele T, respectively. In both figures, the relative intensities were obtained after subtracting the control background (no specific primer) from the observed signals. The intensities with primer-A and prime-B are shown as gray bars and black bars, respectively.


PCR amplification can be used in BAMPER to reduce
the reagent cost. Although only a small amount of PCR
product is needed for BAMPER, it is difficult to carry out
PCR with such a small volume in a commercialized PCR
device. Use of a 10 μl reaction volume coupled with multiplex PCR overcomes this difficulty and provides an efficient and cost-effective means of genotyping. 4) The SNP typing cost in BAMPER is low because expensive reagents are not used and an inexpensive luminometer can be used for the assay.

ACKNOWLEDGMENTS

This work was performed as a part of the research and development project of the Industrial Science and Technology Program supported by NEDO (New Energy and Industrial Technology Development Organization in Japan). We thank Dr. Yashushi Imai (Department of Cardiovascular Medicine, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan) for generously supplying the genome sample and SNP information listed in Table 1.

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