

Multiplex SNP typing by bioluminometric assay coupled with terminator incorporation (BATI)

Guo-Hua Zhou^{1,2}, Mari Gotou¹, Tomoharu Kajiyama¹ and Hideki Kambara^{1,*}

¹Central Research Laboratory, Hitachi, Ltd, 1-280 Higashi-Koigakubo, Kokubunji-shi, Tokyo 185-8601, Japan and ²Huadong Research Institute for Medicine and Biotechnics, Nanjing, China

Received July 12, 2005; Revised and Accepted August 8, 2005

ABSTRACT

A multiplex single-nucleotide polymorphism (SNP) typing platform using 'bioluminometric assay coupled with terminator [2',3'-dideoxynucleoside triphosphates (ddNTPs)] incorporation' (named 'BATI' for short) was developed. All of the reactions are carried out in a single reaction chamber containing target DNAs, DNA polymerase, reagents necessary for converting PPi into ATP and reagents for luciferase reaction. Each of the four ddNTPs is dispensed into the reaction chamber in turn. PPi is released by a nucleotide incorporation reaction and is used to produce ATP when the ddNTP dispensed is complementary to the base in a template. The ATP is used in a luciferase reaction to release visible light. Only 1 nt is incorporated into a template at a time because ddNTPs do not have a 3' hydroxyl group. This feature greatly simplifies a sequencing spectrum. The luminescence is proportional to the amount of template incorporated. Only one peak appears in the spectrum of a homozygote sample, and two peaks at the same intensity appear for a heterozygote sample. In comparison with pyrosequencing using dNTP, the spectrum obtained by BATI is very simple, and it is very easy to determine SNPs accurately from it. As only one base is extended at a time and the extension signals are quantitative, the observed spectrum pattern is uniquely determined even for a sample containing multiplex SNPs. We have successfully used BATI to type various samples containing plural target sequence areas. The measurements can be carried out with an inexpensive and small luminometer using a photodiode array as the detector. It takes only a few minutes to determine multiplex SNPs. These results indicate that this novel multiplexed approach can significantly decrease the cost

of SNP typing and increase the typing throughput with an inexpensive and small luminometer.

INTRODUCTION

Although extensively characterized set of single-nucleotide polymorphisms (SNPs) covering the human genome are available (1), the role of hereditary traits on clinical consequences has still not been clarified. As there are a huge number of polymorphisms in the human genome, an accurate, high-throughput and low-cost genotyping method is required (2). At the moment, many SNP typing methods based on various principles (3), e.g. sequencing-by-synthesis (4,5), allele-specific hybridization (6–8), allele-specific extension (9,10), allele-specific ligation (11,12) and structure specific cleavage (13), have been developed. In the meantime, many detection platforms have been constructed with various readouts (14), such as fluorescence (15–18), mass spectrometry (19,20), luminescence (21–23) and electrochemical detection (24). Minisequencing is a 2',3'-dideoxynucleoside triphosphates (ddNTP)-based sequencing-by-synthesis method, and it is widely used for SNP typing detected by fluorescence and mass spectrometry. When using fluorescence for the detection, ddNTPs are labeled with different dyes, and the SNP types are based on the signal intensity at each dye-specific emission wavelength. When mass spectrometry is used for SNP detection, modified ddNTPs or a mixture of dNTPs and ddNTP are required in order to enlarge the mass differences between the extension products from each allele (20). It is expensive to employ dyes, a laser, a modified ddNTP, and a mass spectrometer for a routine SNP typing at a small scale. We have developed a METPOC method that uses chip electrophoresis with a single colored ddNTP for SNP typing as well as allele frequency analysis (25). However, it still requires two types of primers for each SNP typing and, therefore, is not so convenient. Pyrosequencing is a promising sequence-by-synthesis technology employing bioluminescence detection, which does not need dye labeling, laser excitation and electrophoresis (26). And it has been proven to be an efficient method for SNP typing.

*To whom correspondence should be addressed. Tel: +81 42 323 1111; Fax: +81 42 327 7784; Email: kambara@crl.hitachi.co.jp

© The Author 2005. Published by Oxford University Press. All rights reserved.

The online version of this article has been published under an open access model. Users are entitled to use, reproduce, disseminate, or display the open access version of this article for non-commercial purposes provided that: the original authorship is properly and fully attributed; the Journal and Oxford University Press are attributed as the original place of publication with the correct citation details given; if an article is subsequently reproduced or disseminated not in its entirety but only in part or as a derivative work this must be clearly indicated. For commercial re-use, please contact journals.permissions@oupjournals.org

Pyrosequencing uses four enzymatic reactions carried out in a single tube (27,28). They include a DNA polymerase reaction for extending DNA strands, PPi conversion for producing ATP by the catalysis of ATP sulfurylase in the presence of adenosine 5' phosphosulfate (APS), light production by a luciferase–luciferin reaction, and a dNTP degradation reaction by apyrase. A peak in a sequencing spectrum is observed when the added dNTP is complementary to the base in a template strand and incorporated into the hybridized sequencing primer. The DNA sequence is determined from the incorporated nucleotide species and the peak intensities in the spectrum. Because of the advantages of pyrosequencing in terms of the base-calling accuracy, the system flexibility and the high quantitative performance, it is widely used for SNP typing. However, it is not necessary to sequence a long DNA for SNP detections. In most cases, two-base sequencing is enough for biallelic genotyping. On the other hand, it is time consuming to sequence a long DNA for typing a known SNP by pyrosequencing, which in turn limits the sample processing speed and throughput. Moreover, the use of expensive dATP α S instead of dATP for sequencing increases the cost. Multiplex pyrosequencing for SNP typing is one way to reduce the cost (29); however, if a pyrogram contains peaks disproportional to the incorporated base number or contains a frame shift caused by incomplete extension reaction or undegraded dNTPs, the accuracy of pyrosequencing for multiplex genotyping would be reduced. To simplify the sequencing spectrum in multiplex SNP typing, we demonstrated a novel SNP-typing platform based on a 'bioluminometric assay coupled with terminator (ddNTPs) incorporation' (or 'BATI' for short). This platform does not require the use of dATP α S. There are two possible ways to increase the efficiency of SNP detection. One way is to use multiple primers to hybridize a template simultaneously and then detect the sequencing spectrum for the multiplexed SNP typing by adding ddNTPs in turn. The other way is to successively add a SNP-specific primer to a reaction mixture containing multiple templates at every cycle of four ddNTPs injection.

MATERIALS AND METHODS

Reagents

Thermostable pyruvate orthophosphate dikinase (PPDK) and thermostable luciferase were supplied by Kikkoman (Chiba, Japan). Phosphoenolpyruvate trisodium (PEP), pyrophosphate

decahydrate (PPi), apyrase-VI, bovine serum albumin (BSA) and D-luciferin were purchased from Sigma (St Louis, MO); 2'-deoxyadenosine-5'-O-(1-thiotriphosphate), Sp-isomer (Sp-dATP- α -S) and 2',3'-dideoxyadenosine-5'-O-(1-thiotriphosphate) (ddATP- α -S) were purchased from Biolog Life Science Institute (Bremen, Germany); ddNTPs were purchased from Amersham Biosciences (Piscataway, NJ); and Exo⁻ Klenow was purchased from Ambion (Austin, TX). Inorganic pyrophosphatase (40 U/ml) and Sequenase version 2.0 T7 DNA polymerase (13 U/ μ l) were obtained from USB Corporation (Cleveland, Ohio). Platinum[®] *Taq* DNA polymerase was purchased from Invitrogen (Carlsbad, CA). Sodium Dynabeads[®] M-280 Streptavidin (2.8 μ m ID) was purchased from Dynal AC (Oslo, Norway). ATP and AMP were obtained from Oriental Yeast (Osaka, Japan). All solutions were prepared in deionized and sterilized water. Other chemicals were of a commercially extra-pure grade.

Primers and target sequences

All of the oligonucleotides were purchased from Sigma Genosys (Hokkaido, Japan). The sequences of the PCR primers and SNP-typing primers are listed in Table 1. DNA fragments from TPMT and UGT1A1 genes, respectively were amplified using the PCR primers listed in Table 1 and employed as templates for SNP typing. SNP-1, SNP-2, SNP-3 and SNP-4 are on the same DNA fragment amplified by the primer pair TF and TR. SNP-5 and SNP-6 are on the same DNA fragment amplified by the primer pair GF and GR. To capture a single strand DNA with Streptavidin beads, the 5' ends of primers TR, GR and PR were modified by biotin. All the oligonucleotides were high-performance liquid chromatography purified.

Template DNA preparation

Genome DNAs were extracted from the blood of volunteers (staff from our laboratory) by using DNAZOL[™] Reagent (Invitrogen). The template DNA was obtained by means of PCRs with primer pairs of TF and TR from human genome DNA to get 181 bp fragments of TPMT gene, with primers of GF and GR to get 151 bp fragments of UGT1A1 gene, with primers of PF1 and PR1 to get 349 bp fragments of TP53 gene (defined as T-349 fragment), and with primers of PF2 and PR2 to get 454 bp fragments of TP53 gene (defined as T-454 fragment). Each PCR mixture (50 μ l) contained 1.5 mM of MgCl₂, 0.2 mM each of dNTPs, 1.25 U of Platinum *Taq*

Table 1. SNPs used for the evaluation and typing analysis

Gene symbol	PCR primer sequences	SNP code	SNP typing primer sequence	SNP identifier
TPMT	TF: 5'-tggttgaagtaccagcatgcac TR: 5'-aaaattacttaccatttgcgatca	SNP-1	5'-ttttctcttttctggtaggac	NM_000367:c.423A>C
		SNP-2	5'-tttctggtaggacaaatatt	NM_000367:c.430G>C
		SNP-3	5'-catgatttgggatagagga	NM_000367:c.460G>A
		SNP-4	5'-agaggagcattagttgccat	NM_000367:c.474C>T
UGT1A1	GF: 5'-cagcagaggggacatgaaat GR: 5'-caaaaacattatgcccgagac	SNP-5	5'-cctcgtgtgtacatcagagac	NM_000463:c.211G>A
		SNP-6	5'-aagacgtaccctgtgcca	NM_000463:c.247T>C
TP53	PF1: 5'-gttgaaagtcagggcacaagt PR1: 5'-agcctgggtaacatgatgaaa-biotin	SNP-7	5'-aacagataaagcaactggaa	dbSNPrs2856920:gG>C
		SNP-8	5'-gcagcaagaacaaacatg	dbSNPrs2909430:gC>T
		SNP-9	5'-tcttcccacctcagcctcct	dbSNPrs1794287:gA>G
TP53	PF2: 5'-agagatcacacattaagtggg PR2: 5'-ctgctcttttccaccatctac-biotin	SNP-10	5'-aaaacactgacaggaagcc	dbSNPrs1794286:gA>T
		SNP-11	5'-cagaatgcaagaagcccaga	dbSNPrs11540654:gC>A
		SNP-12	5'-tgctggtgcaggggccacg	dbSNPrs1042522:gC>G
		SNP-13	5'-tcattgcttgggacggcaa	dbSNPrs11575998:gG>T

polymerase, 20 pmol of biotinylated primer and 40 pmol of another primer. Amplification was performed on a PTC-200 thermocycler PCR system (MJ Research, Inc., MA) according to the following protocol: denatured at 94°C for 2 min and followed by 35 thermal reaction cycles (94°C for 30 s; 56°C for 1 min; and 72°C for 1–3 min). After the cycle reaction, the product was incubated at 72°C for 7 min to ensure the complete extension of the amplified DNA.

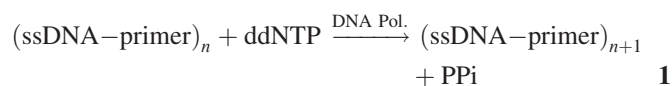
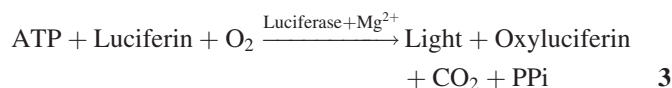
Super paramagnetic beads were used to immobilize biotinylated PCR products. The immobilization was performed by incubating the mixture of DNA and beads at room temperature for 15–40 min. Single-stranded DNAs were obtained by incubating the beaded DNAs in 0.1 M NaOH for 5–10 min. The supernatant fluid was neutralized by 1 M HCl, and the immobilized strand was washed by the buffer described in the instructions for the beads.

Eight kinds of single-stranded DNA templates, named T-1, T-2, T-3, T-4, G-1, G-2, G-3 and G-4, were prepared from the PCR products of the TPMT gene and the UGT1A1 gene amplified by the combination of an unmodified primer and a biotin-primer. T-1 and G-1 are the liquid phase of sense strands of the TPMT gene and the UGT1A1 gene, respectively. T-2 and G-2 are the liquid phase of anti-sense strands of the TPMT gene and the UGT1A1 gene, respectively. T-3 and G-3 are the beaded phase of sense strands of the TPMT gene and the UGT1A1 gene, respectively. T-4 and G-4 are the beaded phase of anti-sense strands of the TPMT gene and the UGT1A1 gene, respectively.

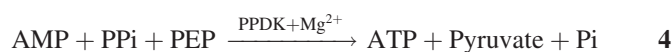
Annealing of the single-stranded template with sequencing primer was carried out in a buffer of 50 mM Tris–acetate (pH 7.8) and 20 mM magnesium acetate at 92°C for 30 s, at 65°C for 3 min and at room temperature for 5 min.

PPi conversion reaction

In conventional pyrosequencing, PPi released by the nucleotide incorporation reaction is converted into ATP by APS–ATP sulfurylase system, and luminescence is produced by ATP through a luciferase-catalyzed reaction with luciferin (30). All reactions are illustrated as the following equations:



An alternative way for converting PPi into ATP is to use PPDK-catalyzed reaction (showed in Equation 4), which was used for determining AMP by adding PPi and PEP (31). Here, we added AMP to convert PPi into ATP in the presence of PPDK.



As APS is a substrate of a luciferin–luciferase reaction, a large background signal appears when a large amount of luciferase

is added to increase the detection sensitivity. Unlike APS, AMP does not give any luminescence in the luciferin–luciferase reaction, therefore, a highly sensitive DNA detection can be carried out by adding a large amount of luciferase.

One milliliter of the AMP–PPDK-based reaction mixture contained the following components: 60 mM of Tricine (pH 7.8), 2 mM of EDTA, 20 mM of magnesium acetate, 0.2 mM of DTT, 0.4 mM of D-luciferin, 0.04 mM of PEP, 0.4 mM of AMP, 0.1% BSA, 65 U of Sequenase 2.0, 10 U of PPDK, 1 U of apyrase and an appropriate amount of luciferase.

Degradation of endogeneous PPi contained in reagents

As the proposed method is based on the bioluminescent detection of PPi released from primer extension, any contaminated PPi existing in the ddNTPs will give a spurious signal. It is necessary to degrade the endogeneous PPi in ddNTPs before the ddNTP-incorporation reactions. One milliliter of 500 μM ddNTPs containing 60 mM of Tricine (pH 7.8), 2 mM of EDTA and 20 mM of magnesium acetate was incubated with 20 mU of PPase for 10 min at room temperature. The desired concentration of ddNTP solution was diluted directly before the incorporation reaction. For a routine detection, the concentration is 50 μM for ddATP, 100 μM for ddTTP, 100 μM for ddGTP and 300 μM for ddCTP.

If PPase is used to remove the background from the detection solution instead of apyrase, PPase is added to the reaction mixture before the addition of AMP, and the solution of AMP is treated by PPase separately. The treated AMP is added just before the detection. Usually 40 mU of PPase is enough for degrading the background in 1 ml of detection mixture to a baseline.

Detection of bioluminescence signal produced by ddNTP-incorporation reaction

We used a house-made dispenser for adding a small amount of ddNTP solution to the reaction chamber. Only 250 nl of PPase-treated ddNTP was dispensed at a time. The dispenser is constructed by using a reservoir with a hole (75 μm ID) in the bottom and a pressure control device in the top. A droplet of dNTP liquid is dispensed when a pressure is added onto a dNTP reservoir. A prototype small DNA sequencer developed in our laboratory was used for the detection. It uses an array of photodiodes (S1133) (32) (made by Hamamatsu Photonics K.K) instead of PMT or CCD camera to detect photo-signals. An array of photodiodes was placed on a base plate having in-house-produced amplifiers. A resistor for converting a photo-current to a voltage was 10¹⁰ Ω and the gain of the buffer amplifier was 17.85.

RESULTS AND DISCUSSION

Principle of BATI

As ddNTPs are used instead of dNTPs for nucleotide incorporation, only one base is extended at a time. The signal intensity is proportional to the amount of DNA template in the mixture. In the case of a single SNP typing, only one peak appears in a spectrum for a homozygote and two peaks with equal height for a heterozygote. A schematic illustration for multiplex SNP typing is shown in Figure 1. There are two

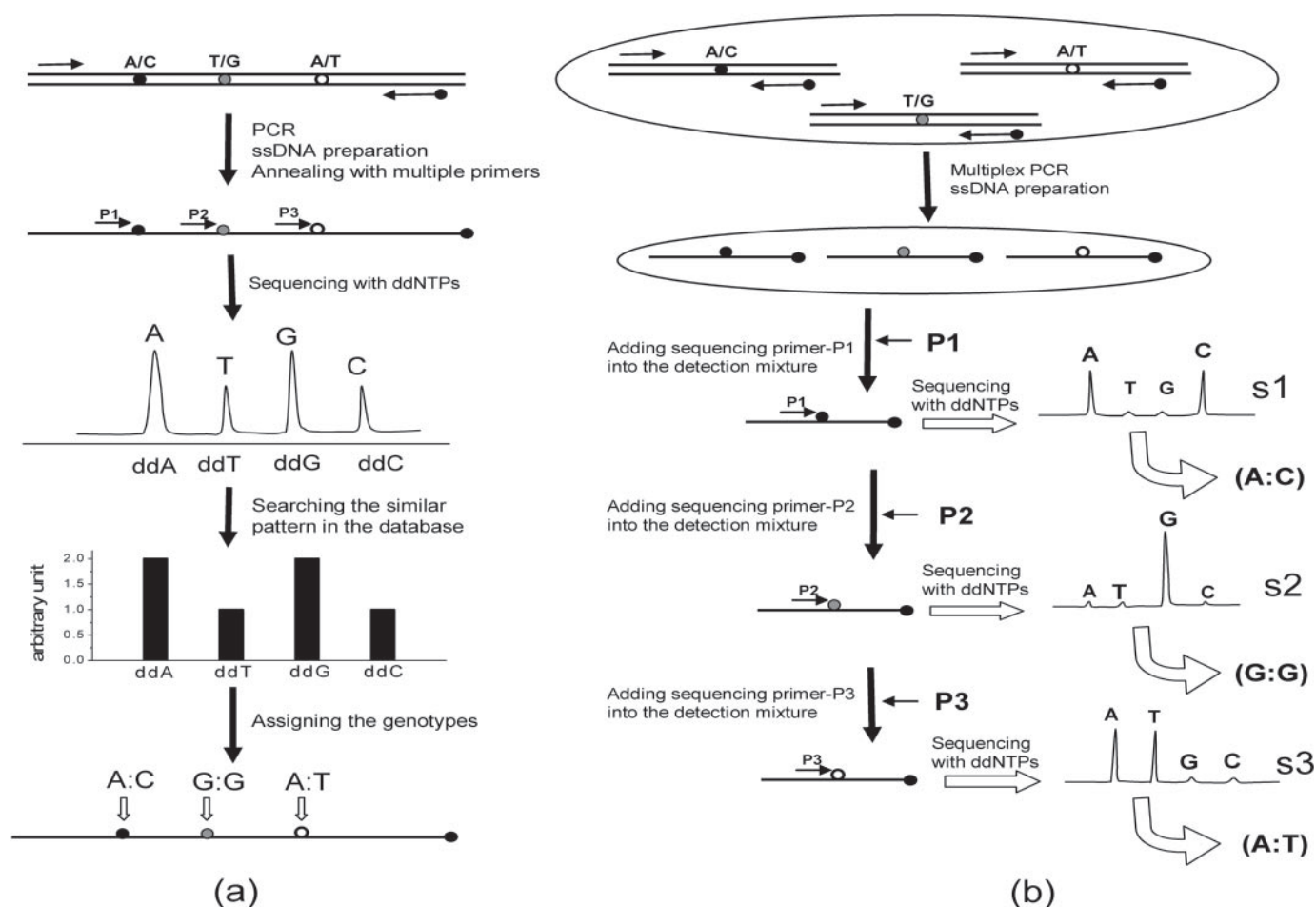


Figure 1. Schematic view of BATI for SNP typing based on (a) a single-step primer addition and (b) step-by-step primer additions.

possible ways of multiplex typing. The first way can be applied to two-plex or three-plex SNP typing, where SNPs are determined by comparing the obtained results with the estimated patterns. The second way can be applied to SNP typing with a multiplex level of more than three by adding different primers in turn. The first case is illustrated in Figure 1a for typing three SNPs with the alleles A/C, T/G and A/T on a DNA target. Firstly, a target DNA template is amplified by using a pair of primers with one biotin-primer. After preparing a single-stranded DNA, three sequencing primers with the 3' ends located just before the SNP points are hybridized with the template simultaneously. A typing spectrum is obtained by carrying out one-base extension reactions by adding ddNTP in turn, and a peak appears in a spectrum if the added ddNTP is complementary to the target at any SNP point. As signal intensities are dependent on the template amounts, the spectral patterns obtained are uniquely determined when the molar concentrations of multiple templates are equal. The result in the case that all three SNPs are on one template is shown in Figure 1a. It is easy to determine the genotypes of three SNPs by comparing the observed spectral pattern with those estimated from possible combinations of known genotypes. There are 27 different spectral patterns corresponding to three-plex SNP typing. The observed spectrum in Figure 1a matches the standard pattern for the genotypes AC, GG and AT. The multiplex typing is carried out by adding several primer species

simultaneously, but the maximum multiplexed level is three. When the peak intensity for a homozygote is defined as one, the total intensities of all four peaks from three SNPs should be three. In the example shown in Figure 1a, the sum of peak intensities from peaks A, T and G are 2.5, and the intensity for peak C is automatically determined to be 0.5. The maximum number of variables is therefore three when the observable peak number is four. The maximum number of analyzable SNPs is three in this case. This leads to the following limitation on three-plex SNP typing by ddNTP-based minisequencing: three polymorphisms must contain all four base types (A, T, G and C). However, there is no restriction on the typing of two SNPs with different base types (e.g. A/C and A/G), since these two polymorphisms contain at least three kinds of base type.

As shown in Figure 1b, multiplex SNP typing can also be performed by successive addition of sequencing primers. As the sequencing primers incorporated with ddNTPs can not extend their strands further and the added ddNTPs are degraded gradually by apyrase in the detection solution, the previously added primers and ddNTPs do not affect the next strand extension reaction with new sequencing primers added in the same tube. The detection procedure is very simple, just like a single-plex SNP typing. At first, multiple target templates containing polymorphisms are generated by multiplex PCR. Next, single-stranded DNA is prepared. A sequencing primer P1 that is specific to the allele A/C is then added into

the reaction solution containing the above template mixtures. The mixture is incubated for 3–5 min at 32°C for the primer hybridization. The one-base extension reaction is performed by adding ddNTPs in turn, and a typing spectrum is obtained as 's1' in Figure 1b. Following a cyclic addition of ddNTPs, the next sequencing primer, P2, specific to allele T/G is added again. After hybridization for 3 min, the extension reaction is carried out once more, and the spectrum is obtained as 's1' in Figure 1b. In the same way, a spectrum by adding the sequencing primer P3 is obtained as 's3'. The genotypes at all three polymorphisms are therefore determined as A:C, G:G and A:T from the spectral patterns of 's1', 's2' and 's3', respectively. Because of the simplicity of the spectral patterns (one peak or two equal peaks), the typing is carried out very accurately and rapidly. Compared with the principle described in Figure 1a, this scheme is not limited to a multiplex level or to the type of alleles at each polymorphism.

Comparison with dNTP-based pyrosequencing

Pyrosequencing technology is based on step-by-step dNTP addition (33), and the signal intensity is proportional to the number of bases incorporated by DNA polymerase at a time. Genotype is determined from a pyrogram. However, a pyrogram becomes complex when the polymorphism is located in a homogeneous region. In contrast, as for the BATI method, the spectrum is simple because ddNTPs do not have a 3' hydroxyl

group for further extension. The differences in spectra are demonstrated in Figure 2 for a sample having a sequence of -A/GAATC- (bold indicates the SNP type). As shown in Figure 2a, the typed nucleotide pattern is 1C:1T:0A:0G, and the pattern of dNTP incorporation in Figure 2b is 1C:5T:2A:2G. As the signal intensities obtained by the incorporation of >5 nt are probably not in linear proportion to the number of incorporated nucleotides in pyrosequencing, it is difficult to determine the base sequence from the observed pyrogram, even though the expected peak ratio is 1C:5T:2A:2G. On the contrary, the difficulty will not occur with the BATI method based on ddNTP incorporation because SNPs are determined by a spectral pattern: a homozygote gives a single peak and a heterozygote gives two peaks in the spectrum. This means that a typing spectrum obtained by BATI gives straightforward information on SNPs. However, the simple spectrum is less informative, so the multiplexing level in BATI is limited to three. While the multiplexing level in dNTP-based pyrosequencing may be more than three, as many informative peaks can be used for decoding the complex spectrum with the help of sophisticated software.

Blank signals from ddNTPs and their effect on the detection limit

Usually, the detection limit in the BATI method is determined by the signals from a luciferase reaction with substrates like

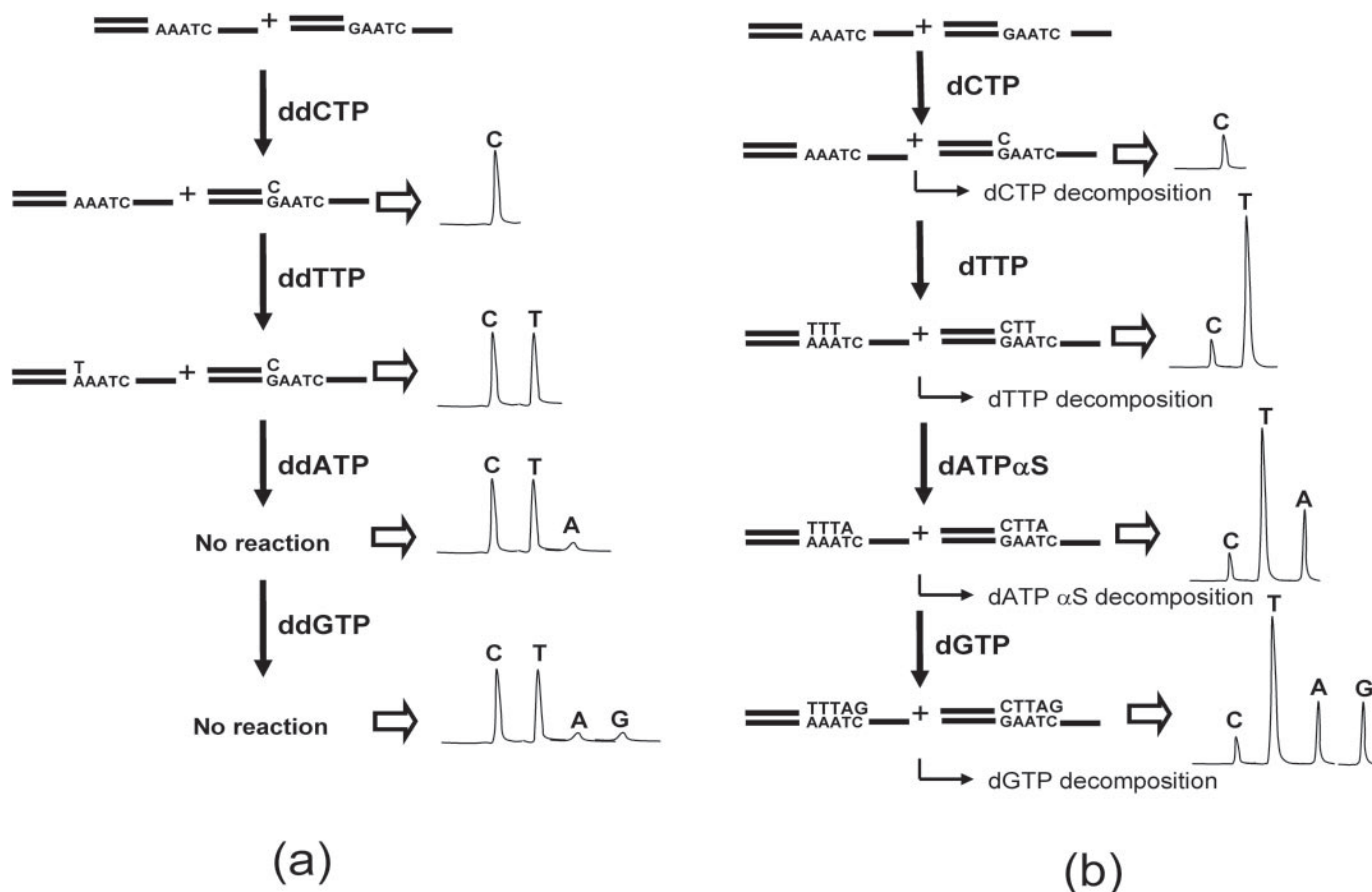


Figure 2. Comparison of ddNTP-based BATI (a) with dNTP-based pyrosequencing (b).

dNTPs, ddNTPs, APS or AMP. The amounts of these substrates are very large compared with the target DNA amount. For example, the amount of dNTPs used for the strand extension reaction in pyrosequencing is tens to hundreds of times larger than that of template DNA. If a substrate reacts with luciferase to emit luminescence, it will make a large contribution to the total signal. For example, dATP gives a relative luminescence of 8.9×10^{-2} in relation to ATP in the presence of 1 U/ml of apyrase (or 1.7×10^{-2} in relation to ATP without apyrase). Therefore, dATP α S is employed instead of dATP in pyrosequencing. On the contrary, ddATP is a poor substrate for the luciferase reaction (as shown in Figure 3). The relative intensities produced by dATP α S, ddATP and ddATP α S are 6.0×10^{-5} , 3.8×10^{-4} and 5.5×10^{-5} in relation to ATP, respectively. As the light-emission profiles for the substrates are different, the peak values are apyrase-dependent. In the

present study, 1 U/ml of apyrase was employed for all the tests.

The other component affecting the detection limit of ATP with a bioluminescent assay is the reaction of luciferase with the substrates such as APS and AMP. There are two possible ATP production reactions: APS-ATP sulfurylase reaction and AMP-PPDK reaction. Although the reaction activities of these substrates are small, a large background signal will appear if a large amount of such substances are added to the reaction mixture. A detection system using the AMP-PPDK reaction gives a smaller background; therefore, we used such a system instead of an APS-ATP sulfurylase system commonly used in pyrosequencing.

After optimizing the present AMP-PPDK-based reaction system, the minimum amount of ATP (1 fmol) was detected with a photodiode detector. The same amount of template DNA could be typed by one-base extension reaction. For stable and reliable SNP typing, the minimum amount of template DNA was 10 fmol (i.e. 10 times higher than the detection limit). Usually the amount of ddNTPs or dNTPs required for completing extension reactions is 30 times larger than that of template DNA, so 300 fmol of ddNTPs or dNTPs is dispensed. This amount of ddATP gives a small background signal, which is negligible; however, the signal produced by the same amount of dATP is three times larger than that produced by nucleotide incorporation reactions of 10 fmol of DNA templates and is not negligible. It is therefore impossible to use dATP for pyrosequencing, but it is possible to use dATP α S.

Although three ddNTPs (ddTTP, ddGTP and ddCTP) other than ddATP are not the analogs of ATP but are expected to give very low background luminescence, large blank signals were frequently observed. As all of them were treated with a small amount of PPase before typing, it is considered that the blank signals are not originated in PPi contamination. The background signals were manufacturer specific (as shown in Figure 4). It was found that the signal produced with ddCTP from Toyobo was very large. However, ddCTP from

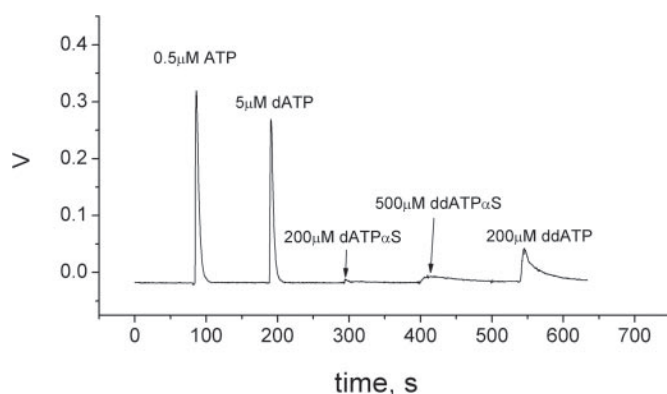


Figure 3. Emission profiles of ATP, dATP, dATP α S, ddATP and ddATP α S with luciferase in the luciferin-luciferase system. ddTTP, ddGTP and ddCTP are from Amersham Bioscience and ddATP is from TaKaRa. All of ATP analogs are treated with PPase. The peak assignments and the concentration of each ATP analog are labeled at the top of peaks directly. The dispensing volume is 0.25 μ l at a time, and the minisequencing mixture is 25 μ l. The detection sensor is a photodiode array.

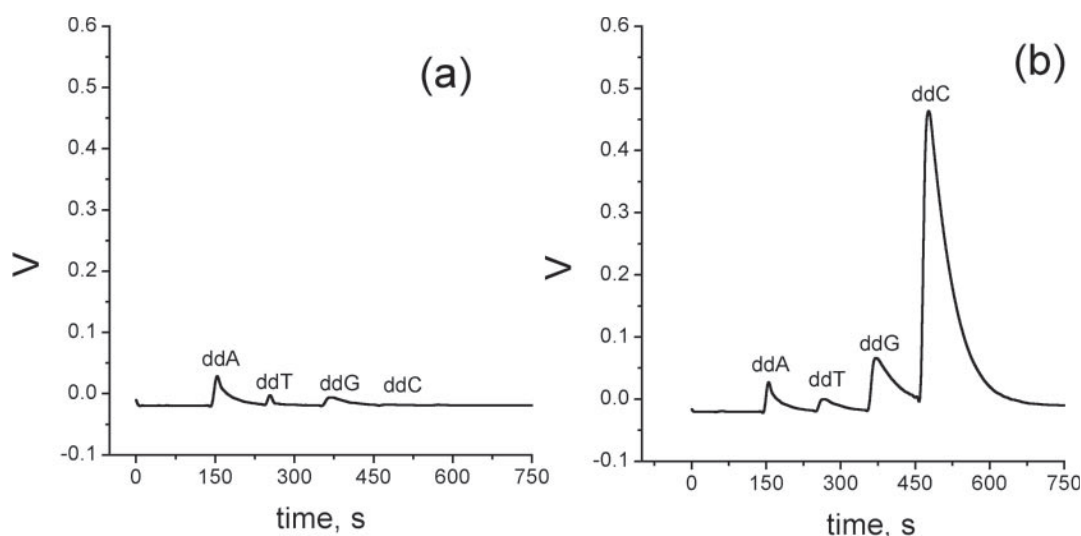


Figure 4. Reaction profiles of ddTTP, ddGTP and ddCTP from different manufacturers, (a) Amersham Bioscience and (b) Toyobo. ddATP was used as a reference and is from TaKaRa. The concentrations of all of four ddNTPs are 200 μ M. The dispensing volume is 0.25 μ l at a time, and the minisequencing mixture is 25 μ l. The detection sensor is a photodiode array. The assignment of each ddNTP is marked at the top of the peak.

Amersham Bioscience did not give a large signal. According to the manufacture's instructions, the purities of ddTTP, ddGTP and ddCTP were 98, 98 and 94% from Toyobo and 99.7, 99.9 and 99.5% from Amersham Bioscience, respectively. It is thought that the high background produced with ddCTP from Toyobo was originated in the impurities in the reagent. It is required to use highly purified ddNTPs for the present assay.

Incorporation efficiency of ddNTPs

Nucleotide incorporation reaction efficiency is dependent on the nucleotide or nucleotide analog species as well as DNA polymerase (34,35). In the present luminescence-based SNP typing, the usable nucleotide analogs are ddNTP and ddNTP α S. As exonuclease-free DNA polymerase is required for the reaction, only two kinds of DNA polymerases are available, Exo⁻ Klenow (a fragment of *Escherichia coli* DNA polymerase I) and T7 DNA polymerase (Sequenase version 2.0). Exo⁻ Klenow is frequently used in DNA sequencing using chain terminators (ddNTPs), and it incorporates a ddNTP at a rate of 1000 times slower than that for dNTP. On the contrary, the ddNTP incorporation rate by T7 DNA polymerase is only a few times lower than that for dNTP. For example, the relative incorporation rates of dATP to ddATP by T7 DNA polymerase and Exo⁻ Klenow are 3.7 and 550, respectively (36). T7 DNA polymerase seems to be more adequate for the incorporation reaction with ddNTPs. The decrease of the ddNTP incorporation rate was recovered by substituting Mg²⁺ with Mn²⁺ in DNA sequencing (36). However, Mg²⁺ is still needed for both PPi-conversion and light-production reactions. The presence of ddNTP-degrading apyrase requires rapid ddNTP incorporation before ddNTP degradation by apyrase. Consequently, it is necessary to find a good condition for efficient ddNTP incorporation.

As shown in Figure 5a, the rate of ddATP incorporation by Klenow is very low. To confirm the completion of incorporation reactions, each ddNTP was dispensed twice. The peaks marked by arrows in Figure 5a indicated that the ddATP or ddGTP incorporation reaction with Klenow was not completed before the degradation. On the contrary, the incorporation reaction of ddATP or ddGTP by Sequenase was completed,

and two equal sharp peaks with the same intensity were observed (as shown in Figure 5b). We therefore used T7 DNA polymerase with Mg²⁺.

When the amount of terminators is not sufficient, terminators are degraded before the incorporation reactions are completed. A sufficient amount of ddNTP is thus required. The optimum amount of each ddNTP should be determined because the terminator incorporation reaction efficiency is dependent on the terminator species. For example, the incorporation efficiency of ddCTP is much lower than that of ddTTP for a DNA sample immobilized on a bead. As a result, uniform peak intensities are not obtained for a heterozygote sample. However, it can be overcome by increasing the ddCTP concentration from 50 to 100 μ M. As ddCTP does not produce a large background luminescence, as shown in Figure 4a, a concentration of 300 μ M of ddCTP was employed for the routine analysis. The optimized terminator concentration was 50 μ M for ddATP, 100 μ M for ddTTP, 100 μ M for ddGTP and 300 μ M for ddCTP.

Multiplex SNP typing

The BATI method can be applied for typing multiplexed SNPs on a template. We have successfully analyzed various two-plex and three-plex SNPs. First, a total of six SNPs on two templates (T-2 and G-2) were determined by uni-plex typing, and then a combination of different SNPs for two-plex and three-plex typing were carried out. In the two-plex typing, the simultaneous addition of two primers followed by incorporation reactions with four ddNTPs was carried out for three different SNP combinations: SNP-1 + SNP-4, SNP-2 + SNP-3 and SNP-5 + SNP-6. The observed spectra are shown in Figure 6a, b and c, respectively. To decode the allele for each SNP in the spectrum, the observed spectrum was compared with standard patterns. In the case of two-plex SNP typing, there are nine possible spectral patterns. The possible nine combinations are (WW YZ), (WX YZ), (XX YZ), (WW YY), (WX YY), (XX YY), (WW ZZ), (WX ZZ) and (XX ZZ) by assuming the alleles to be W/X and Y/Z for the two SNPs. The corresponding spectral patterns were easily figured out by assigning the symbols of W, X, Y and Z as the types of interest.

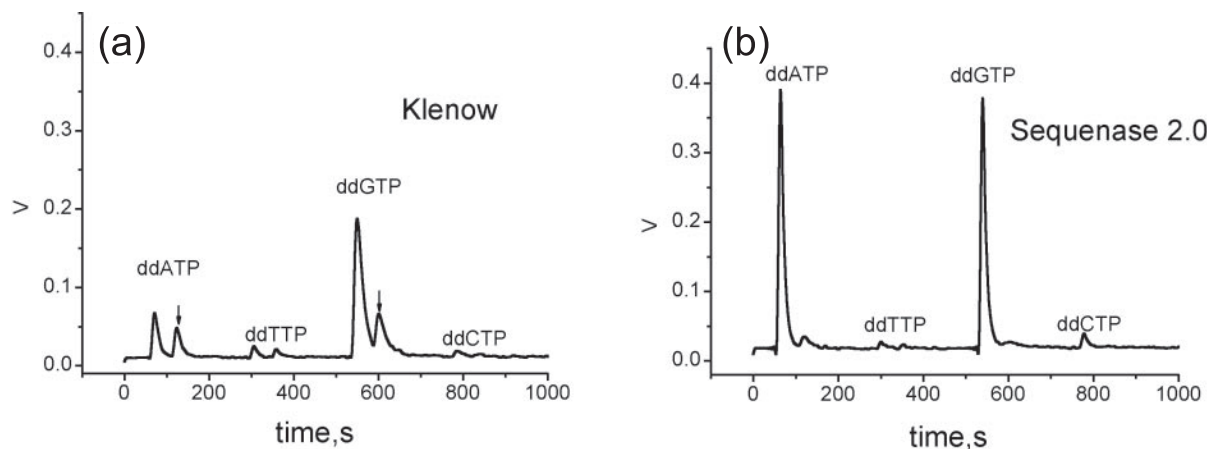


Figure 5. Incorporation efficiency of ddNTPs by Klenow (a) and Sequenase (b) with Mg²⁺. The concentration was 25 μ M for ddATP, 50 μ M for other three ddNTPs. The template was G-2 (a heterozygote of single-stranded PCR products of gene UGT1A1). The dispensing volume is 0.25 μ l at a time, and the minisequencing mixture is 25 μ l. The left arrow and the right arrow in (a) indicate the signals produced by the second addition of ddATP and ddGTP, respectively.

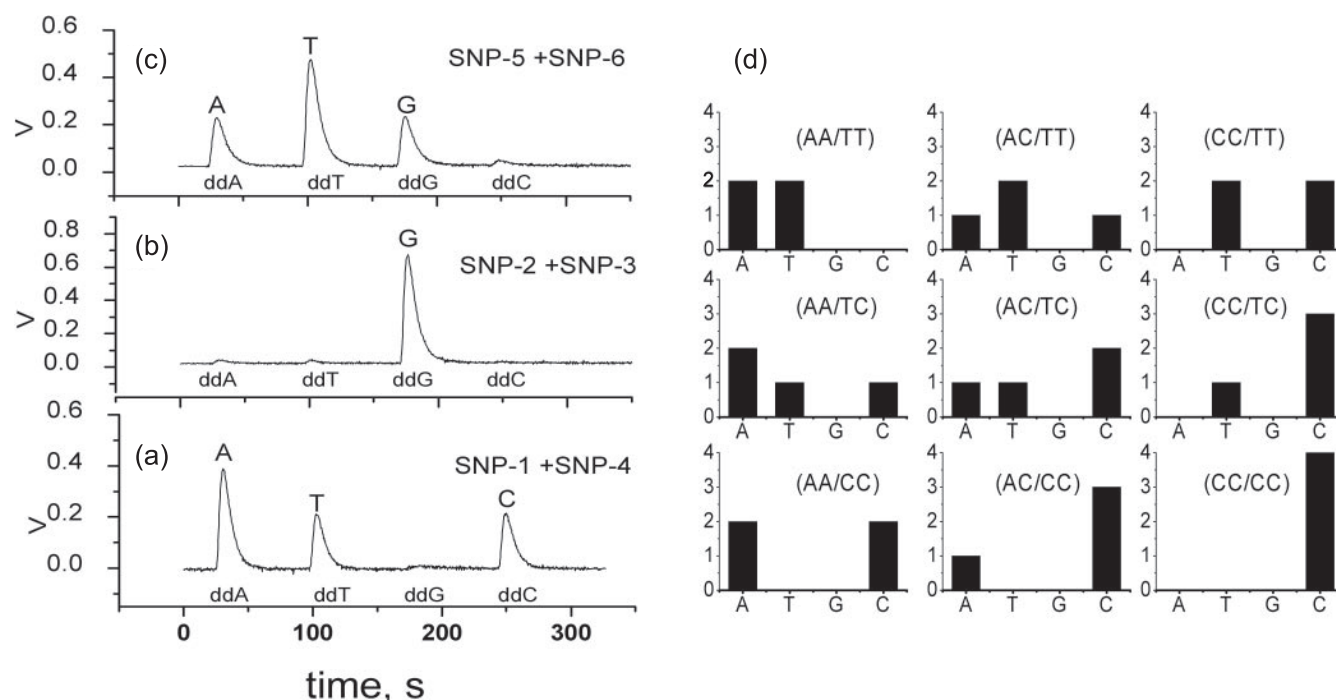


Figure 6. SNP typing spectra for different combinations of SNPs by two-plex typing (a–c) and a set of possible patterns for two-plex typing by taking the SNPs from (a) as an example (d). The combination of each allele is indicated at the top of each pattern in (d). DNA template T-2 contains SNP-1, SNP-2, SNP-3 and SNP-4, and template G-2 contains SNP-5 and SNP-6. The marked code of SNP in each spectrum is explained in the Materials and Methods section.

For example, W and X could be assigned as A and C for SNP-1, respectively, and Y and Z as T and C for SNP-4, respectively. The corresponding spectra are shown in Figure 6. The typing results of SNP-1 + SNP-4 in T-2 are indicated in Figure 6a. By comparison, the genotypes of SNP-1 and SNP-4 on T-2 were determined to be AA and TC, respectively. Similarly, the genotypes at SNP-2 and SNP-3 on T-2 (Figure 6b) were determined to be GG and GG, and those at SNP-5 and SNP-6 on G-2 (Figure 6c) were determined to be AG and TT, respectively.

For three-plex typing, there are 27 different allele combinations. It is possible to determine the genotypes by comparing the observed pattern with the standard one as far as the concentration of each template is the same. Figure 7 shows the three-plex typing profile for equal molar concentrations of template T-2 and G-2. By comparing the observed result with a set of 27 standard patterns (data not shown), the alleles of SNP-1, SNP-4 and SNP-5 in Figure 7a were determined to be AA, TC and AG, respectively, which coincided with the result by uni-plex typing. Similarly, the genotypes of SNP-2, SNP-4 and SNP-5 in Figure 7b were accurately determined to be GG, TC, and AG, respectively.

Successive SNP typing by a step-by-step addition of sequencing primers

Although multiplex typing is very efficient, the number of SNPs investigated simultaneously is limited to three. A combination of multiplex typing and successive typing by a step-by-step addition of sequencing primers improves the typing throughput and reduces the cost of SNP typing. Multiplex PCR products can be used as templates. The detection procedure is

the same as that for uni-plex typing. The results of successive multiplex typing of template mixtures, T-349 and T-454, are shown in Figure 8. The sequencing primers specific to SNP-7 + SNP-8 + SNP-9, SNP-10 + SNP-11 + SNP-12 and SNP-13, respectively, were added to get the spectra. Before adding the primers, each of four ddNTPs is dispensed in turn to remove signals due to nonspecific DNA hybridization and strand extension. In this case, no signal was observed (Figure 8a). Even if there are some DNAs that may be extended without sequencing primers, the termini are blocked by incorporating ddNTPs. The three-plex typing was carried out by adding a primer mixture for typing SNP-7, SNP-8 and SNP-9 followed by nucleotide incorporation reactions with four ddNTPs as shown in Figure 8b. Similarly, a primer mixture containing sequencing primers specific to SNP-10, SNP-11 and SNP-12 was added for three-plex typing, and the spectrum is shown in Figure 8c. Finally, a primer specific to SNP-13 was added for uni-plex typing (Figure 8d). By comparing the observed spectra with the standard patterns for three-plex typing, SNP-7, SNP-8 and SNP-9 were determined as GG, TT and GG, respectively, and SNP-10, SNP-11 and SNP-12 were determined as AA, CC and GC, respectively. All of them were coincident with the results from uni-plex typing. SNP-13 was easily identified to be TT from the spectrum directly (Figure 8d). Seven SNPs in a sample were typed easily by BATI. Generally 8 min are required to finish a typing operation from the addition of sequencing primers till the observation of a spectrum; thus, seven SNPs can be determined within half an hour (as shown in Figure 8).

The use of a terminator instead of dNTP has several advantages. In bioluminometric detection coupled with DNA-strand-extension reaction, the presence of an unspecifically

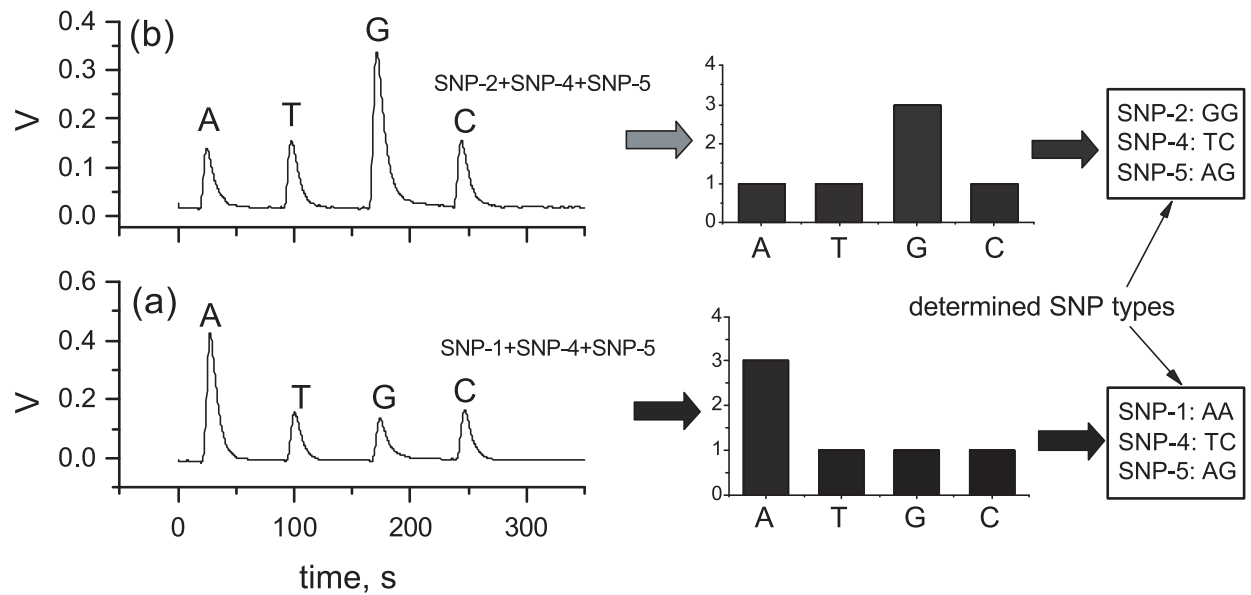


Figure 7. SNP typing spectra for different combinations of SNPs by three-plex typing. The marked code of SNP in each spectrum is explained in the Materials and Methods section.

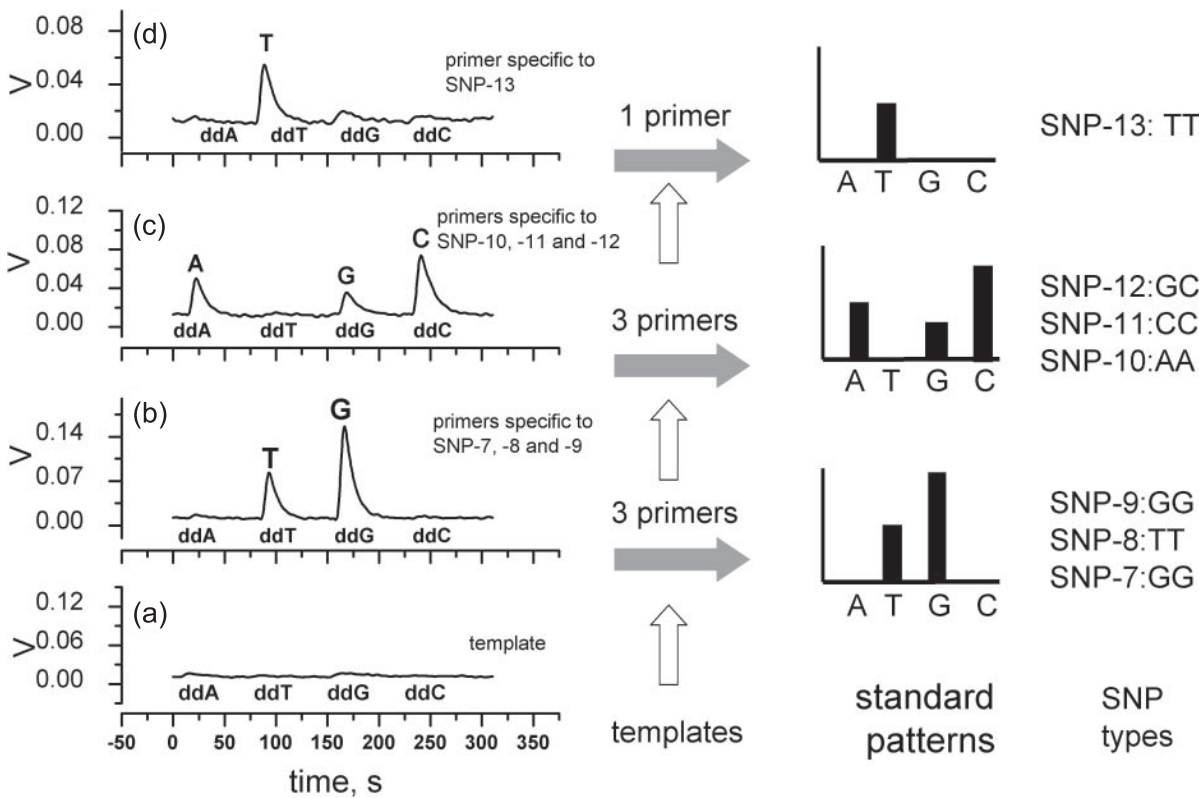


Figure 8. Successive multiplex SNP typing by step-by-step addition of sequencing primers. (a) ddNTP addition by turns without primer addition; (b) adding a primer mixture for SNP-7, SNP-8 and SNP-9, followed by terminator incorporation reactions; (c) adding a primer mixture for SNP-10, SNP-11 and SNP-12, followed by terminator incorporation reactions; (d) adding a primer specific to SNP-13. The order of sequencing-primer addition is indicated by the open arrows. The standard patterns corresponding to the observed spectra were determined as those on the right side of the solid arrows.

hybridized or a mismatched DNA duplex in a reaction mixture gives a large background, resulting in wrong typing results. However, the addition of four terminators prior to the addition of primers prevents false-signal production because all the

termini hybridized to DNAs are deactivated by the terminator incorporation. As all of the reactions are carried out in a single chamber containing multiple target DNAs, the average cost of an SNP typing (except for PCR) is decreased significantly.

Due to the high sensitivity of the present luminescence system using the new PPI-converting reagents, the amount of template required for a routine assay is 100 fmol. Consequently, 50 μ l of five-plex PCR can be used for a general template preparation. The disadvantage of the use of terminators instead of dNTPs is the slower incorporation rate of ddNTP, however, BATI works well at the optimized conditions described above.

CONCLUSION

We have successfully demonstrated a new SNP typing method based on BATI. With this method, as only one base is extended at a time, a typing spectrum is much simpler than that obtained by pyrosequencing using dNTPs. In addition, it is not necessary to use either expensive dATP α S or ddATP α S for the incorporation reactions because ddATP does not produce a large luminescence. The proposed method, BATI, reduces the analysis time to a few minutes as well as the SNP typing cost. As BATI can reduce the background signals, an inexpensive DNA-detection system consisting of a photodiode array can be used for the typing, which must be very promising for easy SNP typing at a low cost.

ACKNOWLEDGEMENTS

We are grateful to Kikkoman Corporation for kindly supplying the PPKK and thermal stable luciferase for the bioluminescent assay, and to High-technology Program of Jiangsu Province of China for the method evaluation. We also thank Teruo Togusari for designing the small pyrosequencer, Toru Habu, Kunio Harada, and Masao Kamahori for their engineering support in the design of the detection system, and Akihiko Kishimoto for his helpful comments. Funding to pay the Open Access publication charges for this article was provided by Central Research Laboratory, Hitachi Ltd.

Conflict of interest statement. None declared.

REFERENCES

- Miller, R.D., Phillips, M.S., Jo, I., Donaldson, M.A., Studebaker, J.F., Addleman, N., Alfisi, S.V., Ankener, W.M., Bhatti, H.A., Callahan, C.E. *et al.* (2005) High-density single-nucleotide polymorphism maps of the human genome. *Genomics*, **86**, 117–126.
- Syvanen, A.C. (2005) Toward genome-wide SNP genotyping. *Nature Genet.*, **37** (Suppl.), S5–S10.
- Kwok, P.Y. (2000) High-throughput genotyping assay approaches. *Pharmacogenomics*, **1**, 95–100.
- Ronaghi, M., Uhlen, M. and Nyren, P. (1998) A sequencing method based on real-time pyrophosphate. *Science*, **281**, 363–365.
- Pastinen, T., Kurg, A., Metspalu, A., Peltonen, L. and Syvanen, A.C. (1997) Minisequencing: a specific tool for DNA analysis and diagnostics on oligonucleotide arrays. *Genome Res.*, **7**, 606–614.
- Tyagi, S., Bratu, D.P. and Kramer, F.R. (1998) Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.*, **16**, 49–53.
- Shaper, M.H., Zhang, J., Loraine, A., Liu, W., Di, X., Liu, G. and Jones, K.W. (2004) MARA: a novel approach for highly multiplexed locus-specific SNP genotyping using high-density DNA oligonucleotide arrays. *Nucleic Acids Res.*, **32**, e181.
- Livak, K.J. (1999) Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet. Anal.*, **14**, 143–149.
- O'Meara, D., Ahmadian, A., Odeberg, J. and Lundberg, J. (2002) SNP typing by apyrase-mediated allele-specific primer extension on DNA microarrays. *Nucleic Acids Res.*, **30**, e75.
- Zhou, G., Kamahori, M., Okano, K., Chuan, G., Harada, K. and Kambara, H. (2001) Quantitative detection of single nucleotide polymorphisms for a pooled sample by a bioluminometric assay coupled with modified primer extension reactions (BAMPER). *Nucleic Acids Res.*, **29**, E93.
- Iannone, M.A., Taylor, J.D., Chen, J., Li, M.S., Rivers, P., Slentz, K.A. and Weiner, M.P. (2000) Multiplexed single nucleotide polymorphism genotyping by oligonucleotide ligation and flow cytometry. *Cytometry*, **39**, 131–140.
- Hardenbol, P., Baner, J., Jain, M., Nilsson, M., Namsaraev, E.A., Karlin-Neumann, G.A., Fakhrai-Rad, H., Ronaghi, M., Willis, T.D., Landegren, U. *et al.* (2003) Multiplexed genotyping with sequence-tagged molecular inversion probes. *Nat. Biotechnol.*, **21**, 673–678.
- Olivier, M. (2005) The Invader assay for SNP genotyping. *Mutat. Res.*, **573**, 103–110.
- Chen, X. and Sullivan, P.F. (2003) Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput. *Pharmacogenomics J.*, **3**, 77–96.
- Ye, F., Li, M.S., Taylor, J.D., Nguyen, Q., Colton, H.M., Casey, W.M., Wagner, M., Weiner, M.P. and Chen, J. (2001) Fluorescent microsphere-based readout technology for multiplexed human single nucleotide polymorphism analysis and bacterial identification. *Hum. Mutat.*, **17**, 305–316.
- Xiao, M. and Kwok, P.Y. (2003) DNA analysis by fluorescence quenching detection. *Genome Res.*, **13**, 932–939.
- Rockenbauer, E., Petersen, K., Vogel, U., Bolund, L., Kolvraa, S., Nielsen, K.V. and Nexø, B.A. (2005) SNP genotyping using microsphere-linked PNA and flow cytometric detection. *Cytometry A*, **64**, 80–86.
- Shen, R., Fan, J.B., Campbell, D., Chang, W., Chen, J., Doucet, D., Yeakley, J., Bibikova, M., Wickham Garcia, E., McBride, C. *et al.* (2005) High-throughput SNP genotyping on universal bead arrays. *Mutat. Res.*, **573**, 70–82.
- Griffin, T.J. and Smith, L.M. (2000) Single-nucleotide polymorphism analysis by MALDI-TOF mass spectrometry. *Trends Biotechnol.*, **18**, 77–84.
- Gut, I.G. (2004) DNA analysis by MALDI-TOF mass spectrometry. *Hum. Mutat.*, **23**, 437–441.
- Zhou, G.H., Shirakura, H., Kamahori, M., Okano, K., Nagai, K. and Kambara, H. (2004) A gel-free SNP genotyping method: bioluminometric assay coupled with modified primer extension reactions (BAMPER) directly from double-stranded PCR products. *Hum. Mutat.*, **24**, 155–163.
- Kakihara, F., Kurebayashi, Y., Tojo, Y., Tajima, H., Hasegawa, S. and Yohda, M. (2005) MagSNiPer: a new single nucleotide polymorphism typing method based on single base extension, magnetic separation, and chemiluminescence. *Anal. Biochem.*, **341**, 77–82.
- Jansson, V. and Jansson, K. (2004) An enzymatic bioluminescent assay of DNA ligation. *Anal. Biochem.*, **324**, 307–308.
- Boon, E.M., Ceres, D.M., Drummond, T.G., Hill, M.G. and Barton, J.K. (2000) Mutation detection by electrocatalysis at DNA-modified electrodes. *Nat. Biotechnol.*, **18**, 1096–1100.
- Li, Z.P., Tsunoda, H., Okano, K., Nagai, K. and Kambara, H. (2003) Microchip electrophoresis of tagged probes incorporated with one-colored ddNTP for analyzing single-nucleotide polymorphisms. *Anal. Chem.*, **75**, 3345–3351.
- Ahmadian, A., Gharizadeh, B., Gustafsson, A.C., Sterky, F., Nyren, P., Uhlen, M. and Lundberg, J. (2000) Single-nucleotide polymorphism analysis by pyrosequencing. *Anal. Biochem.*, **280**, 103–110.
- Ronaghi, M. (2003) Pyrosequencing for SNP genotyping. *Methods Mol. Biol.*, **212**, 189–195.
- Ronaghi, M. (2001) Pyrosequencing sheds light on DNA sequencing. *Genome Res.*, **11**, 3–11.
- Pourmand, N., Elahi, E., Davis, R.W. and Ronaghi, M. (2002) Multiplex pyrosequencing. *Nucleic Acids Res.*, **30**, e31.
- Zhou, G., Kamahori, M., Okano, K., Harada, K. and Kambara, H. (2001) Miniaturized pyrosequencer for DNA analysis with capillaries to deliver deoxynucleotides. *Electrophoresis*, **22**, 3497–3504.
- Sakakibara, T., Murakami, S., Eisaki, N., Nakajima, M. and Imai, K. (1999) An enzymatic method using pyruvate orthophosphate dikinase and firefly luciferase for the simultaneous determination of ATP and AMP (RNA). *Anal. Biochem.*, **268**, 94–101.

32. Kamahori, M., Harada, K. and Kambara, H. (2002) A new single nucleotide polymorphisms typing method and device by bioluminometric assay coupled with a photodiode array. *Meas. Sci. Technol.*, **13**, 1779–1785.
33. Elahi, E. and Ronaghi, M. (2004) Pyrosequencing: a tool for DNA sequencing analysis. *Methods Mol. Biol.*, **255**, 211–219.
34. Kristensen, T., Vass, H., Ansorge, W. and Prydz, H. (1990) DNA dideoxy sequencing with T7 DNA polymerase: improved sequencing data by the addition of manganese chloride. *Trends Genet.*, **6**, 2–3.
35. Tabor, S. and Richardson, C.C. (1990) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Effect of pyrophosphorolysis and metal ions. *J. Biol. Chem.*, **265**, 8322–8328.
36. Tabor, S. and Richardson, C.C. (1989) Effect of manganese ions on the incorporation of dideoxynucleotides by bacteriophage T7 DNA polymerase and *Escherichia coli* DNA polymerase I. *Proc. Natl Acad. Sci. USA*, **86**, 4076–4080.