A single nucleotide polymorphism genotyping method using phosphate-affinity polyacrylamide gel electrophoresis

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Short title: SNP detection using phosphate-affinity PAGE

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Abstract

To date, various methods have been developed to facilitate the genotyping of a single nucleotide polymorphism (SNP) for aiding in the diagnosis and treatment of inherited diseases. The most commonly used method for SNP genotyping is an allele-specific hybridization procedure using an expensive fluorochrome-labeled oligonucleotide probe and a specialized fluorescence analyzer. Here, we introduce a simple and reliable genotyping method using a 1:1 mixture of 5'-phosphate-labeled and non-labeled allele-specific PCR primers. The method is based on the difference in mobility of the phosphorylated and nonphosphorylated PCR products (in the same number of base pairs) on phosphate-affinity polyacrylamide gel electrophoresis. The phosphate-affinity site is a polyacrylamide-bound dinuclear zinc(II) complex, which preferentially captures the 5'-phosphate-labeled allele-specific product compared with the corresponding non-labeled product. The obtained DNA migration bands can be visualized by ethidium bromide staining. We demonstrate the genotyping of a SNP reported in a human cardiac sodium channel gene, *SCN5A*, using this novel procedure.

Key words: SNP genotyping; Allele-specific PCR; Phosphate-affinity PAGE; Zinc

Introduction

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide in the genome sequence is altered. The use of SNPs is expected to lead to a better understanding of the genetic basis for diseases and to realize the potential for clinical diagnostics and pharmacogenetics [1]. Now, a number of methods are available for SNP genotyping, and the choice of the method depends on the assay scale. In a large-scale assay, an allele-specific hybridization method using a TaqMan probe [2], fluorescence resonance energy transfer probe [3], or molecular beacon [4] is most commonly used for SNP genotyping. The method allows accurate allele discrimination in a one-step procedure without separation of the analytes or removal of the surplus fluorescent contaminants. However, expensive fluorochrome-labeled oligonucleotides are required, in addition to a special apparatus for the fluorescence measurement. On the other hand, the genotyping methods for a small-scale assay require complicated processes and a skillful analyst; many of these methods are gel electrophoresis-based ones, such as single-strand conformation polymorphism [5], denaturing gradient gel electrophoresis [6], and conformation-sensitive gel electrophoresis [7].

In 2002, we reported a gel electrophoresis-based SNP detection method, Zn^{2+}–cyclen PAGE, for the small-scale screening of various disease-causing mutations [8]. The method is based on the principle that the binding of Zn^{2+}–cyclen (*i.e.*, a mononuclear zinc(II) complex) to the thymine base changes the local DNA conformation, resulting in differences in the electrophoretic mobility of a mutant DNA. The Zn^{2+}–cyclen PAGE was applied for the comprehensive screening of heterozygous mutations scattered throughout a human cardiac sodium channel gene, *SCN5A*, that is related to inherited arrhythmia syndromes [9]. In this report, we introduce a newly developed gel electrophoresis-based method for SNP genotyping. The principle of the method is based on our recent findings on a phosphate-binding tag molecule, Phos-tag (*i.e.*, a dinuclear metal (Mn^{2+} or Zn^{2+}) complex of 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olate) [10–16]. The Phos-tag molecule has a vacancy on two metal ions that is suitable for the access of a phosphomonoester dianion

(R-OPO_3^{2-}) as a bridging ligand. This fact has resulted in the development of phosphate-affinity gel electrophoresis (Mn^{2+}–Phos-tag SDS-PAGE) for the mobility shift detection of phosphoproteins from their nonphosphorylated counterparts (13–15). We have adapted the electrophoretic separation method for the SNP genotyping in the present study; the separation of phosphate-labeled and non-labeled PCR products (*i.e.*, allele-specific DNA in the same number of base pairs) was conducted by a similar phosphate-affinity PAGE but with a Phos-tag complex with two zinc(II) ions (Zn^{2+}–Phos-tag PAGE). As the first practical example, we demonstrated the SNP genotyping of a silent mutation reported in the *SCN5A* gene [9] using Zn^{2+}–Phos-tag PAGE.

Materials and methods

Materials

The acrylamide-pendant Phos-tag ligand (*N*-(5-(2-acryloylaminoethylcarbamoyl)pyridin-2-ylmetyl)-*N,N',N'*-tris(pyridin-2-yl-methyl)-1,3-diaminopropan-2-ol) was obtained from the Phos-tag consortium (http://www.phos-tag.com, Japan). The 100-base pair (bp) DNA ladder as a DNA size marker was purchased from Takara Bio (Otsu, Japan). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). KOD-plus- DNA polymerase was purchased from Toyobo (Osaka, Japan). All oligonucleotide PCR primers were obtained from Invitrogen Japan (Tokyo, Japan). Ethidium bromide was purchased from Nacalai Tesque (Kyoto, Japan). All reagents and solvents used were of the highest commercial quality and were used without further purification.

Preparation of genomic DNA

Peripheral blood (10 mL) was obtained from seven healthy blood donors, and genomic DNA was extracted from the leukocytes according to the standard protocol using the QIAamp DNA Blood Maxi Kit (QIAGEN, Hilden, Germany). Written informed consent for participation was obtained from all the donors. The DNA concentration was determined by using an ethidium bromide fluorescent quantitative method [17].

Design of the allele-specific primers and procedure of PCR for genotyping

Allele-specific PCR primers containing a single nucleotide variation of G and A in the exon 2 of the *SCN5A* gene (a G-allele-specific primer, 5'-GCCGCGGGCTTGCTTCTCCG-3', and an A-allele-specific primer, 5'-GCCGCGGGCTTGCTTCTCTG-3', underlined at the variation site) and a reverse PCR primer, 5'-GGTCTGCCCACCCTGCTCTC-3', were

designed by referring to the sequence of GenBank Accession No. M77235 (cDNA of *SCN5A*). The allele-specific PCR primers for the SNP genotyping are illustrated in Figure 1. The G-allele-specific primer (50 μM) was phosphorylated with T4 polynucleotide kinase (5 U) in a reaction mixture (final volume, 20 μL) consisting of 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 1 mM ATP at 37 ˚C for 30 min. The reaction mixture was incubated at 75 ˚C for 5 min to stop the kinase reaction. The A-allele-specific primer and the reverse primer were used without the kinase treatment. PCR for amplification of the target region (220 bp) was performed with the following reaction mixture: a 1:1 mixture of allele-specific primers (each at 0.10 μM), a 0.20 μM reverse primer, various concentrations of the genomic DNA template (500, 250, 125, 63, or 32 pg), dNTPs (each at 0.20 mM), 0.80 mM $MgSO_4$, 0.10 U KOD-plus- DNA polymerase, and its special buffer in a volume of 5 μL. After initial denaturation at 95 ˚C for 3 min, the PCR amplification was carried out for 30 cycles of 15-sec denaturation at 95 ˚C and 30-sec annealing/extension at 68 ˚C to obtain 5'-OPO_3^{2-}-labeled and/or non-labeled DNA fragments.

Zn^{2+}–Phos-tag PAGE

PAGE was performed using a 1-mm-thick, 9-cm-wide, and 9-cm-long gel prepared with 18% (w/v) polyacrylamide (99:1 ratio of acrylamide to *N,N'*-methylenebisacrylamide), an acrylamide-pendant Phos-tag ligand (5 – 20 μM), 2 equivalents of $Zn(NO_3)_2$, and 375 mM Tris-HCl (pH 8.8) on a standard mini-slab PAGE apparatus (model AE-6500; ATTO, Tokyo, Japan) at 15 mA/gel for 70 min at room temperature. The electrophoresis running buffer (pH 8.4) was 25 mM Tris and 192 mM glycine. Each PCR reaction mixture was dissolved in 5.0 μL of a loading buffer (50 mM EDTA (pH 8.0), 30% (v/v) glycerol, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol). Then, 2.0 μL of each sample solution was applied to the polyacrylamide gel containing gel-bound Zn^{2+}–Phos-tag. The DNA bands were stained with an aqueous solution (20 mL/gel) of ethidium bromide (10 μg/mL) after electrophoresis. The procedure for SNP genotyping using the 5'-OPO_3^{2-}-labeled and

non-labeled PCR product is shown in Fig. 1.

Insert Figure 1

Results and discussion

Separation of 5'-OPO_3^{2-} and 5'-OH DNA fragments in a polyacrylamide gel

Recently, we found that a dinuclear manganese(II) complex of Phos-tag ligand can capture *R*-OPO_3^{2-} anions preferentially, such as phosphoserine and phosphotyrosine, at alkaline pH *ca*. 9. This finding has contributed to the development of a novel type of phosphate-affinity electrophoresis for the mobility shift detection of phosphorylated proteins from their nonphosphorylated counterparts [13–16]. We were able to utilize successfully an acrylamide-pendant Mn^{2+}–Phos-tag as a novel additive of the separating gel in SDS-PAGE (Mn^{2+}–Phos-tag SDS-PAGE). However, a similar PAGE, but without SDS, caused remarkable waving and tailing of the migration of 5'-phosphorylated DNA bands on the gel (data not shown). Therefore, we investigated the potency of a zinc(II) homologue instead of the manganese(II) one in the PAGE system. To evaluate the effect of the polyacrylamide-bound Zn^{2+}–Phos-tag on the migration of 5'-phosphorylated DNA fragments, we conducted Zn^{2+}–Phos-tag PAGE using DNA fragments treated with and without T4 polynucleotide kinase (Fig. 2). The commercially available 100-bp DNA ladder marker is a mixture of eleven DNA fragments having the 5'-OH group (nonphosphorylated DNA fragments) in the size range of 100 – 1500 bp (see Lane 1). The migration distance of each fragment was almost the same in the absence (Fig. 2a) and presence of 5, 10, or 20 μM polyacrylamide-bound Zn^{2+}–Phos-tag (Figs. 2b–d). When the ladder marker was treated with T4 polynucleotide kinase, the migration of the 5'-phosphorylated all fragments was retarded (see Lane 2) in comparison with that of the corresponding nonphosphorylated counterparts (see Lane 1). Although remarkable retardation was observed as the Zn^{2+}–Phos-tag concentration was increased, the migration patterns were disordered at more than 10 μM of Zn^{2+}–Phos-tag, and the discrimination of each band became difficult (see Lane 2 of Figs. 2c and d). In contrast with the phosphorylated fragments, the migration images of the nonphosphorylated counterparts did not change even in the presence of 20 μM

polyacrylamide-bound Zn^{2+}–Phos-tag (see Lane 1 of Fig. 2d). These facts show that the Zn^{2+}–Phos-tag molecules selectively interact with the 5'-OPO_3^{2-} groups of DNA fragments in a polyacrylamide gel. The behavior of 5'-phosphorylated DNA on the electrophoresis gels is consistent with that of phosphorylated proteins in a previous report [13–16].

Next, we determined single PCR products by using Zn^{2+}–Phos-tag PAGE. The PCR products used were amplified from a human cardiac sodium channel gene, *SCN5A*. We have reported a single nucleotide variation of G or A at the 87 position of the *SCN5A* in healthy individuals, which causes no change in the amino acid sequence [9]. PCR was performed to amplify the 220-bp DNA fragment containing this SNP position using the G-allele-specific and reverse primers (see the section of Materials and methods) and the genomic template of the G/G homozygote from a selected individual. In order to compare the electrophoresis migration of the PCR products attached with a different group at the 5'-end, the following three kinds of PCR products were prepared by amplification using three different pairs of primers: i) the product using a pair of the G-allele-specific and reverse primers having the 5'-OH group (applied to Lane 3 in Figure 2), ii) the product using a pair of the G-allele-specific primer having the 5'-OPO_3^{2-} group and the reverse primer having the 5'-OH group (Lane 4), and iii) the product using a pair of the G-allele-specific and reverse primers having the 5'-OPO_3^{2-} group (Lane 5). The migration of the products with the 5'-OPO_3^{2-} group was retarded as the Zn^{2+}–Phos-tag concentration was increased (see Lanes 4 and 5 of Figs. 2b–d). The mobility of the two-sided 5'-OPO_3^{2-} DNA fragment (Lane 5) was smaller than that of the one-sided one (Lane 4). On the other hand, the migration distance of the product without the phosphate group (see Lane 3) was almost the same in the absence (Fig. 2a) and presence of polyacrylamide-bound Zn^{2+}–Phos-tag (Figs. 2 b–d). In the presence of 20 μM (Fig. 2d), the one-sided 5'-OPO_3^{2-} DNA fragment (see Lane 4) showed a significant separation from the corresponding non-labeled counterpart (see Lane 3) without disordering of the migration image. Thus, we adopted the suitable concentration (20 μM) of polyacrylamide-bound Zn^{2+}–Phos-tag for the following SNP genotyping.

Insert Figure 2

SNP genotyping of SCN5A using Zn^{2+}–Phos-tag PAGE

We performed allele-specific amplifications of the same region of *SCN5A* (220 bp) described above using a 1:1 mixture of 5'-OPO_3^{2-}-labeled and non-labeled allele-specific primers, the reverse non-labeled primer, and each genomic template from seven healthy individuals with the following three allele genotypes, *i.e.*, the G/A heterozygote in two individuals, the G/G homozygote in three individuals, and the A/A homozygote in two individuals. Subsequently, the resultant PCR products were analyzed using 20 μM Zn^{2+}–Phos-tag PAGE (Fig. 3). It is important for genotyping to obtain a significant difference in quantity between the allele-specific PCR products in the simultaneous amplification of all individuals. However, it is difficult to distinguish the kinetics of allele-specific amplification from the individual genomic templates different in the qualities, such as the concentration and/or the level of purification, under the fixed thermal cycle protocol (*i.e.*, fixed annealing temperature and amplification cycle numbers). To avoid this problem, we performed five kinds of PCR reactions using 500, 250, 125, 63, and 32 pg of the genomic template in each individual genotyping. Figure 3 shows that the allele-specific PCR products were clearly observed as a slower migration band (the 5'-OPO_3^{2-}-labeled G-allele product) and a faster one (a non-labeled A-allele product). In the genotype of the G/A heterozygote, both allele amplifications were observed almost equally in all lanes. As for the G/G or A/A homozygote sample, a small amount of the non-specific product was observed together with the specific product. However, there was a significant difference in quantity between both products amplified. The genome in the range of 125 – 500 pg was sufficient to identify the allele genotype when used as template for allele-specific amplification.

Insert Figure 3

Conclusions

We introduced a novel type of phosphate-affinity gel electrophoresis, Zn^{2+}–Phos-tag PAGE, for a simple and accurate analysis of DNA mutations. The Zn^{2+}–Phos-tag PAGE is based on the principle that the binding of Zn^{2+}–Phos-tag immobilized in the gel to the phosphate group at the 5'-end of DNA fragment results in slower electrophoresis mobility of the 5'-OPO_3^{2-}-labeled DNA fragment compared with the corresponding non-labeled one. The combination of the allele-specific PCR technique and Zn^{2+}–Phos-tag PAGE enables simple and accurate typing of SNPs without using expensive fluorescent primers or a special apparatus for real-time PCR. Since this procedure requires a general PCR apparatus, a mini-slab PAGE system, and a gel-bound Zn^{2+}–Phos-tag molecule, it would be a very convenient tool for clinical researchers and physicians to obtain the useful SNP data from their small number of patients. It is worthwhile to consider using Zn^{2+}–Phos-tag PAGE in the medical field for the genotyping of various disease-causing mutations.

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Figure Legends

Fig. 1. Novel SNP genotyping by using a two-step procedure of allele-specific PCR and Zn^{2+}–Phos-tag PAGE.

Fig. 2. Comparison of the migration patterns between 5'-OPO_3^{2-} and 5'-OH DNA fragments in electrophoresis with 18% (w/v) polyacrylamide gel in the absence (**a**) and presence of 5 (**b**), 10 (**c**), and 20 μM (**d**) of polyacrylamide-bound Zn^{2+}–Phos-tag. Lanes 1 and 2: 100-bp ladder markers untreated with T4 polynucleotide kinase (Lane 1, 5'-unphosphorylated markers) and treated with T4 polynucleotide kinase (Lane 2, 5'-phosphorylated markers at the both ends). Lanes 3–5: the 220-bp PCR products of *SCN5A* amplified from the homozygote of the G-allele using a pair of the allele-specific and reverse primers having the 5'-OH group (Lane 3, non-labeled DNA fragment), a pair of the allele-specific primer having the 5'-OPO_3^{2-} group and the reverse primer having the 5'-OH group (Lane 4, one-sided 5'-OPO_3^{2-} DNA fragment), and a pair of the allele-specific and the reverse primers having the 5'-OPO_3^{2-} group (Lane 5, both-sided 5'-OPO_3^{2-} DNA fragment). The R_f value was estimated as the relative ratio against bromophenol blue dye. All PAGE gels were stained with ethidium bromide.

Fig. 3. Zn^{2+}–Phos-tag PAGE of the allele-specific PCR products (220 bp) amplified from three *SCN5A* genotypes in seven healthy individuals. Each panel shows the result of genotyping in each individual. The allele genotype (G/A heterozygote, G/G homozygote, and A/A homozygote) of each individual is represented on the left side of each panel. The amounts of genomic template DNA used (500, 250, 125, 63, and 32 pg) are shown above each lane. The Zn^{2+}–Phos-tag PAGE gels (20 μM polyacrylamide-bound Zn^{2+}–Phos-tag and 18% (w/v) polyacrylamide) were subjected to ethidium bromide staining.

Fig. 1

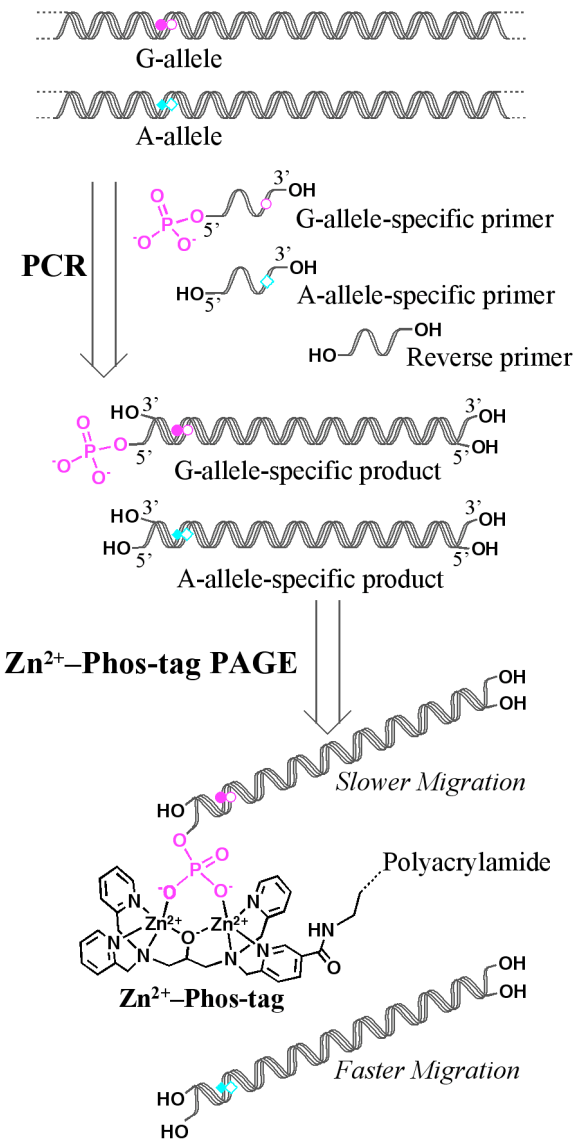
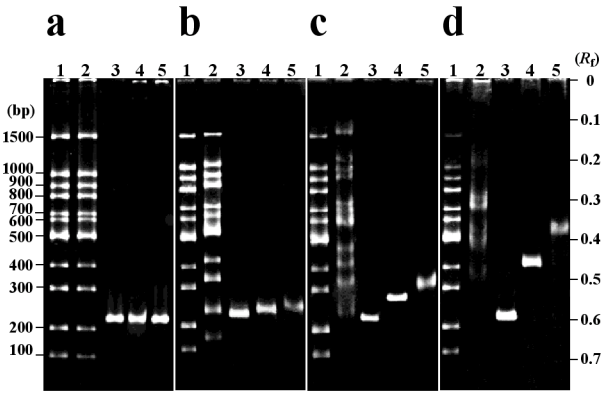

G-allele
A-allele
PCR
G-allele-specific primer
A-allele-specific primer
Reverse primer
G-allele-specific product
A-allele-specific product
Zn2+–Phos-tag PAGE
Slower Migration
Polyacrylamide
Zn2+–Phos-tag
Faster Migration

Fig. 2


a
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(bp)
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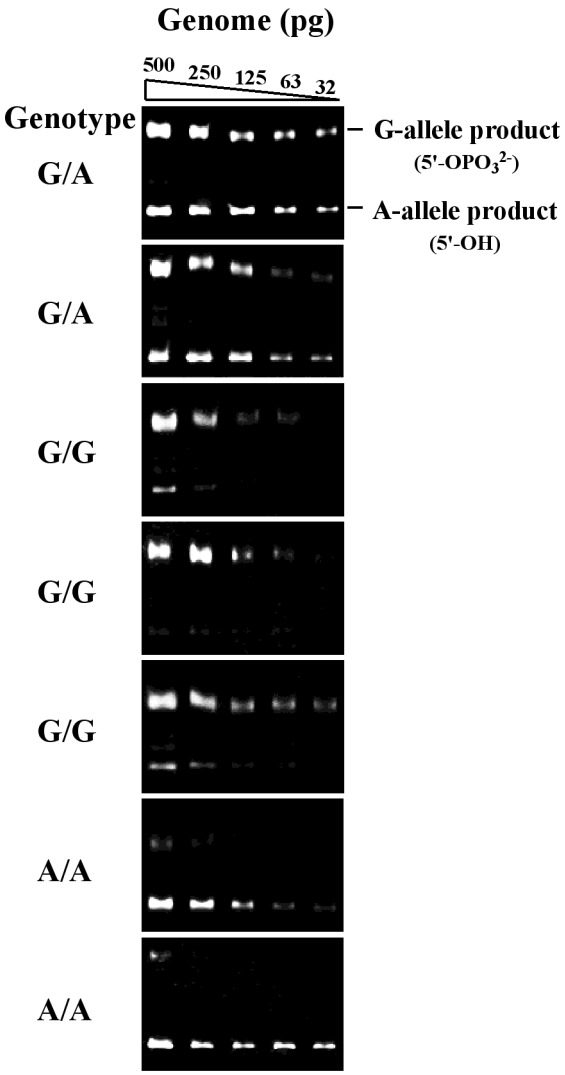
Fig. 3


Genome (pg)
500
250
125
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32
Genotype
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G-allele product
(5'-OPO3 2-)
A-allele product
(5'-OH)