Detection of the Gua/Cyt-to-Cyt/Gua mutation in a Gua/Cyt-lined sequence using Zn^{2+}–cyclen polyacrylamide gel electrophoresis

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Short title: SNP detection using Zn^{2+}–cyclen–PAGE

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[1] *Abbreviations used:* PAGE, polyacrylamide gel electrophoresis; SNP, single-nucleotide polymorphism; PCR, polymerase chain reaction.

Abstract

We have previously reported a method for the detection of single-nucleotide polymorphisms by polyacrylamide gel electrophoresis (PAGE) with an additive Zn^{2+}–cyclen complex (cyclen = 1,4,7,10-tetraazacyclododecan), called Zn^{2+}–cyclen–PAGE. The method is based on the difference in mobility of mutant DNA (in the same length) in PAGE, which is due to Zn^{2+}–cyclen binding to thymine bases accompanying a total charge decrease and a local conformation change of target DNA. In combination with a heteroduplexing technique, the method is more accurate, as shown by clear gel-shifting bands. However, the question remains of whether the Gua/Cyt-to-Cyt/Gua mutation, which is far apart from the Thy/Ade base (i.e., in a Gua/Cyt-lined sequence), can be detected by this Thy-dependent method. In this study, we determined the potency of Zn^{2+}–cyclen–PAGE for the detection of the Gua/Cyt-to-Cyt/Gua single substitutions in some artificial Gua/Cyt-lined sequences derived from a human cardiac sodium channel gene, *SCN5A*. All Gua/Cyt-to-Cyt/Gua substitutions in the 28-set samples tested, which are 1 to 10 bases away from the nearest Thy/Ade, were successfully detected by designing DNA fragments of the appropriate length.

Keywords: Guanine; Cytosine; SNP; Mutation; Heteroduplex; Zn^{2+}–cyclen; Electrophoresis

Introduction

Recently, we have reported a polyacrylamide gel electrophoresis (PAGE)[1]-based single-nucleotide polymorphism (SNP) detection method, Zn^{2+}–cyclen–PAGE, for the small-scale screening of various disease-causing mutations [1, 2]. The combination of a polymerase chain reaction (PCR)-based heteroduplex method and Zn^{2+}–cyclen–PAGE enabled a more accurate detection of single mutations introduced artificially even for less detectable substitutions, such as Ade/Thy-to-Thy/Ade and Gua/Cyt-to-Cyt/Gua. This combination procedure does not require any radioisotopes or fluorescent probes and basically detects heteroduplex bands on the electrophoresis gel, which arise due to the annealing of complementary strands, one from mutant and one from wild-type DNA (heterozygosity), during PCR. Our approach is based on three principles: i) A single-base mismatch can produce a local conformational change in the double-stranded DNA, leading to differential migration of heteroduplex and homoduplex bands. ii) The addition of Zn^{2+}–cyclen in the gel, which selectively binds to the thymine bases and disrupts the double strands, can intensify the local conformational change, resulting in increased differential migration of both duplexes. iii) Binding of the Zn^{2+}–cyclen to the thymine bases decreases the total charge of the target DNA, helping the detection further. The appearance of slow or differentially migrating bands on the gel indicates the presence of heteroduplex bands, which are suggestive of the existence of mutations or polymorphisms. Furthermore, a homoduplex of a certain mutant allele is separated from a homoduplex of the homologous wild-type allele by using Zn^{2+}–cyclen–PAGE. In this case, four distinct migration bands per DNA sample are detectable at maximum [1, 2].

The Zn^{2+}–cyclen–PAGE has been applied to the comprehensive screening of heterozygous mutations scattered throughout a human cardiac sodium channel gene, *SCN5A*, that is related to inherited arrhythmia syndromes [2, 3]. In addition, we have demonstrated that the method enables the detection of the Gua/Cyt-to-Cyt/Gua single mutations by binding the Zn^{2+}–cyclen to the thymine base in the vicinity of the mutation sites [1]. However, the

question still remains whether the Gua/Cyt-to-Cyt/Gua mutation, which is far apart from the Thy/Ade base, is detected by the Thy-dependent method. Namely, the detection limit of the method for a Gua/Cyt-rich region is ambiguous. A Gua/Cyt-lined DNA sequence, $(Gua/Cyt)_n$ ($20 \geq n \geq 10$), which can influence the phenotype by altering the encoded proteins, often appears in the genetic coding regions of the human genome. In this study, from the viewpoint of SNP screening for the coding regions, we determined the potency of Zn^{2+}–cyclen–PAGE for the detection of the Gua/Cyt-to-Cyt/Gua single mutations using artificial Gua/Cyt-lined sequences with a length of up to 20 bp.

Materials and methods

Cloning of the SCN5A sequence and mutagenesis

The *SCN5A* sequence (GenBank accession no. EF179185, 17042 to 17329, 288 bp) was amplified from the human genome DNA (BD Biosciences, Franklin Lakes, NJ, USA) using a pair made of primers O_{10} and P5 (see Fig. 1 and Table 1). The PCR product was cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA), and the sequence was checked using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The plasmid carrying the *SCN5A* sequence was used as a template for the subsequent PCR-based mutagenesis. Forty-one other sequences were prepared by PCR using a pair made of P5 and each GC-rich primer listed in Table 1. All PCR products (288 to 298 bp) were cloned into the same T-vector, and the sequences were confirmed using the CUGA DNA sequencing kit (Nippon Gene, Tokyo, Japan). All oligonucleotides for PCR were obtained from Invitrogen Japan (Tokyo, Japan).

Insert Figure 1 and Table 1

Preparation of Gua/Cyt-lined sequences for the analysis of Zn^{2+}–cyclen–PAGE

The target Gua/Cyt-lined sequences for the analysis of Zn^{2+}–cyclen–PAGE were prepared by PCR using the 42 plasmids described above. Various-sized PCR products were amplified from each template using a pair made of primers P1 and P2 (166 to 176 bp), P1 and P3 (231 to 241 bp), P1 and P4 (296 to 306 bp), and P1 and P5 (335 to 345 bp) (see Fig. 1 and Table 1). The abbreviated names of the resulting PCR products were the same as those of the corresponding GC-rich primers (e.g., O_{10} PCR product [166 bp]). The concentration of each PCR product was adjusted to be 20 ng/μL. For heteroduplexing, an equal amount of two PCR products from the non-substituted sequence (see the O and I series primers in Table 1) and the

substituted counterpart (see S series primers in Table 1) were mixed, and then 10 cycles of denaturing (95 ˚C, 15 sec) and annealing (60 ˚C, 15 sec) were performed.

Zn^{2+}–cyclen–PAGE

Electrophoresis was performed in a 1-mm-thick, 9-cm-wide, and 9-cm-long gel on a standard mini-slab PAGE apparatus (model AE6500; ATTO, Tokyo, Japan) at 25 mA for 100 min at room temperature. The gel consisted of 8.5 mL of a polyacrylamide gel (5.0 mM Zn^{2+}–cyclen, 20 to 18% [w/v] polyacrylamide, and 375 mM Tris-HCl at pH 8.8). An acrylamide stock solution was prepared as a mixture of a 99:1 ratio of acrylamide to *N,N'*-methylenebisacrylamide. The cathode buffer was 25 mM Tris and 192 mM glycine, and the anode buffer was 25 mM Tris and 192 mM glycine containing 5.0 mM $Zn(NO_3)_2$. The DNA samples were added to one-half the amount of a loading dye containing 50 mM EDTA, 0.05% (w/v) bromophenol blue, and 30% (v/v) glycerol and then applied (10 ng of DNA per well). The DNA bands were visualized with 10,000-fold-diluted SYBR green I (Cambrex Bio Science Rockland, Rockland, ME, USA) staining (15 mL per gel) after electrophoresis.

Elution of DNA from the Zn^{2+}–cyclen–PAGE gel

The target DNA band stained with ethidium bromide on the Zn^{2+}–cyclen–PAGE gel was cut out. The gel slice was then transferred to a microtube and minutely crushed using a disposable pipette tip against the wall of the tube. After calculating the approximate volume of the gel pieces, an elution buffer (*ca*. 2-fold volume of the gel pieces) consisting of 0.50 M ammonium acetate, 10 mM magnesium acetate, 1.0 mM EDTA (pH 8.0), and 0.10% (w/v) SDS was added to the tube, and the gel pieces were shaken in the buffer for 12 h. The supernatant was filtrated through a 0.22-μm-pore filter. The resultant filtrate was mixed with a 2-fold amount of ethanol, and DNA was precipitated. The recovered DNA was dissolved in distilled water.

Results and discussion

Preparation of DNA samples

We selected the DNA sequence of 5'-CCCCGGCCCC located in the exon 2 of the human cardiac sodium channel gene, *SCN5A* (GenBank accession no. EF179185, 17310 to 17319), as the original sequence (see O_{10} primer in Table 1). The GC-rich region was cloned as a 288-bp DNA fragment for the analysis of Zn^{2+}–cyclen–PAGE. Next, the original sequence was modified by inserting 1 to 10 Gua/Cyt bases (see the I series primers in Table 1) and/or by substituting Gua/Cyt to Cyt/Gua (see the S series primers in Table 1). By using a PCR-based mutagenesis, we produced 42 plasmids containing the target Gua/Cyt-lined sequences to prepare 28-set samples by coupling the substituted sequences ($S_{10–20}$) and their counterparts (O_{10} and $I_{11–20}$) (see Table 1). For the detection of the single-base substitutions by Zn^{2+}–cyclen–PAGE, various-sized DNA fragments containing the target sequences were amplified by PCR. In previous studies, the length of each PCR product was not supposed to exceed *ca.* 200 bp for the accurate detection of mutations [1, 2]. In this study, to determine the optimum length for the detection of the Gua/Cyt-to-Cyt/Gua single mutations in a Gua/Cyt-lined sequence, the fragments were designed to be 166 to 345 bp using four pairs of primers (see Fig. 1). Because Zn^{2+}–cyclen–PAGE enables the detection of heterozygosity more accurately than that of homozygosity [1, 2], we performed heteroduplexing by mixing two PCR products of the substituted sequence and its counterpart to produce heterozygosity mimics. The DNA fragments from the heterozygosity mimic, substituted, and non-substituted sequences were considered as a 1-set sample and were subjected to Zn^{2+}–cyclen–PAGE.

Detection of the Gua/Cyt-to-Cyt/Gua substitution in a Gua/Cyt-lined sequence

First, we examined the $(Gua/Cyt)_{10}$-lined original sequence (5'-CCCCGGCCCC) and the substituted counterparts amplified from 7 plasmids containing the O_{10} and $S_{10}1–6$ sequences

(see Table 1). The Gua/Cyt-to-Cyt/Gua single substitutions in the 161- and 231-bp PCR products, which are 1 to 5 bases away from the nearest Thy/Ade, were detected as multiple separated DNA bands from all heterozygosity mimics of the 6-set samples (Fig. 2). In the previous report on Zn^{2+}–cyclen–PAGE [1], some single mutations on DNA fragments with lengths of over 300 bp were not detected. Similarly, the heterozygosity mimics of 296- and 335-bp PCR products containing O_{10} and $S_{10}5$ sequences were not separated (Fig. 2). Thus, we adopted the appropriate length of DNA fragments within 241 bp for the subsequent analysis. Next, we examined the $(Gua/Cyt)_{11–17}$-lined sequences using the PCR products of 232 to 238 bp. In the 12-set samples tested, all Gua-to-Cyt substitutions, which are 6 to 8 bases away from the nearest Thy/Ade, were detected (see lanes of heterozygosity mimics in Fig. 3). Finally, we analyzed the $(Gua/Cyt)_{20}$-lined sequences using the PCR products (I_{20} and $S_{20}1–10$) of 241 bp. The results of Zn^{2+}–cyclen PAGE for the 10-set sample combinations of I_{20} and $S_{20}1–10$ are shown in Fig. 4A. The Gua/Cyt-to-Cyt/Gua single substitutions, which are 1 to 10 bases away from the nearest Thy/Ade, were detected as multiple separated bands in the heterozygosity mimics. As for the less detectable substitution $S_{20}2$, the migration bands were more clearly separated for the PCR product of 176 bp amplified using a pair made of primers P1 and P2 (Fig. 4B). Remarkable separation bands were observed in the heterozygosity mimics of $S_{20}4 + I_{20}$ and $S_{20}5 + I_{20}$, whose substituted sites were located four and five bases away from the nearest Thy/Ade, respectively. In both samples, two bands of the heteroduplexes were clearly separated from the homoduplex bands. As for the heterozygosity mimics of $S_{20}1 + I_{20}$, $S_{20}2 + I_{20}$, and $S_{20}3 + I_{20}$, on the other hand, a relatively small degree of separation was observed. By comparing the density between the upper and lower bands in each lane of the heterozygosity mimics, we estimated that a heteroduplex had shifted from three other duplexes (the other heteroduplex and two homoduplexes). These results suggested that the separation degree of a duplex is not correlated with the distance between the substitution site and the nearest Thy/Ade in Zn^{2+}–cyclen–PAGE. Furthermore, a small degree of separation was also observed in the heterozygosity mimics of $S_{20}6 + I_{20}$, $S_{20}7 + I_{20}$, $S_{20}8 + I_{20}$, $S_{20}9 + I_{20}$, and $S_{20}10 + I_{20}$. These results suggest that the influence of the

binding of the Zn^{2+}–cyclen on a local conformation change of a heteroduplex might reach a mismatched site that is even 10 bases away from the nearest Thy/Ade.

Insert Figures 2 to 4

Sequencing analyses of the duplexes eluted from the Zn^{2+}–cyclen–PAGE gel

To determine which heteroduplex containing a mismatched Gua:Gua or Cyt:Cyt base pair migrates more slowly in Zn^{2+}–cyclen PAGE, we performed sequencing analyses of the slower migrated DNA band (upper) and faster migrated ones (lower) in all heterozygosity mimics of S_{20} + I_{20} series (10-set samples). Typical results of the mimics showing a relatively small degree of separation ($S_{20}2$ + I_{20} and $S_{20}3$ + I_{20}) and a remarkable one ($S_{20}4$ + I_{20} and $S_{20}5$ + I_{20}) are shown in Fig. 5. The DNA samples eluted from each area surrounded in the electrophoresis images were sequenced using the primers P1 and P2 (see Fig. 1). The sequencing data of the upper bands from all heterozygosity mimics showed that the substitution site in both strands is a guanine base, i.e., each upper band corresponds to a heteroduplex with a mismatched Cyt:Cyt base pair. Consequently, we confirmed that all faster migrated bands contain a Gua:Gua heteroduplex and two homoduplexes (Gua:Cyt and Cyt:Gua). These facts indicate that the Cyt:Cyt region of the heteroduplex is more significantly untangled than the Gua:Gua one in the presence of Zn^{2+}–cyclen. This is possibly due to the Hoogsteen base pairing of the Gua:Gua mismatched site in a DNA duplex [4]. Thus, the conformation change in the Gua:Gua heteroduplex would be similar to that of the corresponding homoduplex. Such Gua:Gua base pairing is well known to play a major role in stabilizing the telomere structure [5] and to be important in purine:purine DNA triplexes [6] and in various RNA stem bulges [7].

Insert Figure 5

Conclusions

Zn^{2+}–cyclen–PAGE is based on the principle that the binding of Zn^{2+}–cyclen (i.e., a mononuclear zinc(II) complex) to the thymine bases changes the local DNA conformation, resulting in differences in the electrophoresis migration of a mutant DNA. In combination with a heteroduplexing technique, the method has enabled a more accurate detection of single-nucleotide mutations for SNP mapping and heterozygosity screening [1–3]. In this article, we demonstrated that single substitutions of Gua/Cyt to Cyt/Gua in the artificial Gua/Cyt-lined sequences were derived from a human cardiac sodium channel gene, *SCN5A*. All Gua/Cyt-to-Cyt/Gua substitutions in the 28-set samples tested, which are 1 to 10 bases away from the nearest Thy/Ade, were successfully detected by determining the appropriate DNA length (176 to 241 bp). In addition, we disclosed the DNA binding properties of Zn^{2+}–cyclen moiety by sequencing analyses of the DNA bands eluted from the Zn^{2+}–cyclen–PAGE gel. We found that the slowest migration of a Cyt:Cyt heteroduplex in the presence of Zn^{2+}–cyclen contributes to accurate detection when analyzing the Gua/Cyt-to-Cyt/Gua single substitutions in the Gua/Cyt-lined sequences. The disruption of Ade:Thy base pairing due to the binding of Zn^{2+}–cyclen affects even a mismatched site that is 10 bases away, causing a detectable local conformation change of target DNA in the Zn^{2+}–cyclen–PAGE gel. Therefore, it is worthwhile to consider using Zn^{2+}–cyclen–PAGE for the detection of various disease-causing mutations in the genomic coding region from a small number of samples.

Acknowledgements

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References

1. E. Kinoshita-Kikuta, E. Kinoshita, T. Koike, A novel procedure for simple and efficient genotyping of single nucleotide polymorphisms by using the Zn^{2+}–cyclen complex. Nucleic Acids Res. 30 (2002) e126.
2. E. Kinoshita, E. Kinoshita-Kikuta, H. Kojima, Y. Nakano, K. Chayama, T. Koike, Reliable and cost-effective screening of inherited heterozygosity by Zn^{2+}–cyclen polyacrylamide gel electrophoresis. Clin. Chem. 51 (2005) 2195–2197.
3. Y. Nakano, S. Tashiro, E. Kinoshita, E. Kinoshita-Kikuta, S. Takenaka, M. Miyoshi, H. Ogi, E. Sakoda, N. Oda, K. Suenari, Y. Tonouchi, T. Okimoto, Y. Hirai, F. Miura, K. Yamaoka, T. Koike, K. Chayama, Non-SCN5A related Brugada syndromes: Verification of normal splicing and trafficking of SCN5A without exonic mutations. Ann. Hum. Genet. 71 (2007) 8–17.
4. J.V. Skelly, K.J. Edwards, T.C. Jenkins, S. Neidle, Crystal structure of an oligonucleotide duplex containing G.G base pairs: influence of mispairing on DNA backbone conformation. Proc. Natl. Acad. Sci. USA 90 (1993) 804–808.
5. E.H. Blackburn, Structure and function of telomeres. Nature 350 (1991) 569–573.
6. P.A. Beal, P.B. Dervan, Second structural motif for recognition of DNA by oligonucleotide-directed triple-helix formation. Science 251 (1991) 1360–1363.
7. D.P. Bartel, M.L. Zapp, M.R. Green, J.W. Szostak, HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. Cell 67 (1991) 529–536.

Figure legends

Fig. 1. Schematic representation of the *SCN5A* (GenBank accession no. EF179185) sequence cloned into the pGEM-T Easy Vector and the location of the PCR primers used in this study. Each primer sequence is shown in Table 1.

Fig. 2. Detection of the Gua/Cyt-to-Cyt/Gua mutation in the $(Gua/Cyt)_{10}$-lined sequence. The PCR products from the original (O_{10}) and corresponding substituted sequences ($S_{10}1–6$) were amplified to be 166, 231, 296, and 335 bp in DNA length. By heteroduplexing the mixture of the amplified original and substituted sequences, six heterozygosity mimics ($S_{10}1–6 + O_{10}$) were produced. The resulting samples were applied to a 20% (w/v) polyacrylamide gel containing 5.0 mM Zn^{2+}–cyclen.

Fig. 3. Detection of the Gua/Cyt-to-Cyt/Gua mutation in the $(Gua/Cyt)_{11–17}$-lined sequence. The PCR products from the inserted ($I_{11–17}1$, 2) and corresponding substituted sequences ($S_{11–17}1$, 2) were amplified. By heteroduplexing the mixture of the amplified inserted and substituted sequences, 12 heterozygosity mimics ($S_{11–17}1$, 2 + $I_{11–17}1$, 2) were produced. The resulting samples were applied to an 18% (w/v) polyacrylamide gel containing 5.0 mM Zn^{2+}–cyclen. The lengths of the analyzed DNA were 232 bp (I_{11} and S_{11}), 233 bp (I_{12} and S_{12}), 235 bp (I_{14} and S_{14}), 236 bp (I_{15} and S_{15}), 237 bp (I_{16} and S_{16}), and 238 bp (I_{17} and S17).

Fig. 4. Detection of the Gua/Cyt-to-Cyt/Gua mutation in the $(Gua/Cyt)_{20}$-lined sequence. The PCR products from the inserted (O_{20}) and corresponding substituted sequences ($S_{20}1–10$) were amplified to be 241 (A) and 176 bp (B) in DNA length. By heteroduplexing the mixture of the amplified inserted and substituted sequences, 10 heterozygosity mimics ($S_{20}1–10 + I_{20}$) were produced. The resulting samples were applied to 18 and 20% (w/v) polyacrylamide gels containing 5.0 mM Zn^{2+}–cyclen. The 18 and 20% (w/v) polyacrylamide gels were used for 241 and 176 bp of DNA, respectively.

Fig. 5. Sequencing analyses of the duplexes eluted from the Zn^{2+}–cyclen–PAGE gel. The slower migrated DNA band (upper) and faster migrated ones (lower) in four heterozygosity mimics of $S_{20}2 + I_{20}$, $S_{20}3 + I_{20}$, $S_{20}4 + I_{20}$, and $S_{20}5 + I_{20}$ (each area surrounded with dotted lines in the electrophoresis images) were analyzed after electrophoresis using an 18% (w/v) polyacrylamide gel containing 5.0 mM Zn^{2+}–cyclen. Each DNA eluted from the gel was sequenced using the primers P1 and P2 (see Fig. 1). The Gua/Cyt-to-Cyt/Gua mutation sites are indicated with arrows in the sequence charts.

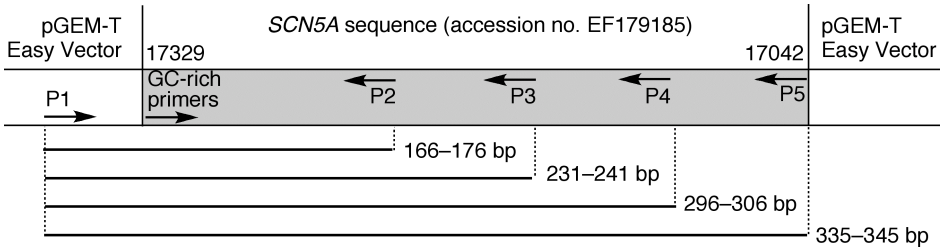
Table 1 Sequence of PCR primers used in this study

Primers	Sequence (5'-3')
P1	CTCTCCCATATGGTCGACCTGC
P2	AGTCCCTGGCAGCCATCGAG
P3	GGATGAGAAGATGGCAAACT
P4	GGCCCATTGTCTGTGTCTTC
P5	GGTCTGCCCACCCTGCTCTC
GC-rich primers	
O_{10}	AGGTCCAGCTGGGGCCGGGGAGCCTCCTCC
$S_{10}1$	AGGTCCAGCTGGGGCCGGG**C**AGCCTCCTCC
$S_{10}2$	AGGTCCAGCTGGGGCCGG**C**GAGCCTCCTCC
$S_{10}3$	AGGTCCAGCTGGGGCCG**C**GGAGCCTCCTCC
$S_{10}4$	AGGTCCAGCTGGGGCC**C**GGGAGCCTCCTCC
$S_{10}5$	AGGTCCAGCTGGGGC**G**GGGGAGCCTCCTCC
$S_{10}6$	AGGTCCAGCTGGGG**G**CGGGGAGCCTCCTCC
$I_{11}1$	AGGTCCAGCTGGGG*G*CCGGGGAGCCTCCTCC
$S_{11}1$	AGGTCCAGCTGGGG*G***G**CGGGGAGCCTCCTCC
$I_{11}2$	AGGTCCAGCTGGGG*C*CCGGGGAGCCTCCTCC
$S_{11}2$	AGGTCCAGCTGGGG*C***G**CGGGGAGCCTCCTCC
$I_{12}1$	AGGTCCAGCTGGGG*GG*CCGGGGAGCCTCCTCC
$S_{12}1$	AGGTCCAGCTGGGG*G****C***CCGGGGAGCCTCCTCC
$I_{12}2$	AGGTCCAGCTGGGG*CC*CCGGGGAGCCTCCTCC
$S_{12}2$	AGGTCCAGCTGGGG*C****G***CCGGGGAGCCTCCTCC
$I_{14}1$	AGGTCCAGCTGGGG*GGGG*CCGGGGAGCCTCCTCC
$S_{14}1$	AGGTCCAGCTGGGG*GG****C****G*CCGGGGAGCCTCCTCC
$I_{14}2$	AGGTCCAGCTGGGG*CCCC*CCGGGGAGCCTCCTCC
$S_{14}2$	AGGTCCAGCTGGGG*CC****G****C*CCGGGGAGCCTCCTCC
$I_{15}1$	AGGTCCAGCTGGGG*GGGGG*CCGGGGAGCCTCCTCC

$S_{15}1$	AGGTCCAGCTGGGG*GG**<u>C</u>**GG*CCGGGGAGCCTCCTCC
$I_{15}2$	AGGTCCAGCTGGGG*CCCCC*CCGGGGAGCCTCCTCC
$S_{15}2$	AGGTCCAGCTGGGG*CC**<u>G</u>**CC*CCGGGGAGCCTCCTCC
$I_{16}1$	AGGTCCAGCTGGGG*GGGGGG*CCGGGGAGCCTCCTCC
$S_{16}1$	AGGTCCAGCTGGGG*GGG**<u>C</u>**GG*CCGGGGAGCCTCCTCC
$I_{16}2$	AGGTCCAGCTGGGG*CCCCCC*CCGGGGAGCCTCCTCC
$S_{16}2$	AGGTCCAGCTGGGG*CCC**<u>G</u>**CC*CCGGGGAGCCTCCTCC
$I_{17}1$	AGGTCCAGCTGGGG*GGGGGGG*CCGGGGAGCCTCCTCC
$S_{17}1$	AGGTCCAGCTGGGG*GGG**<u>C</u>**GGG*CCGGGGAGCCTCCTCC
$I_{17}2$	AGGTCCAGCTGGGG*CCCCCCC*CCGGGGAGCCTCCTCC
$S_{17}2$	AGGTCCAGCTGGGG*CCC**<u>G</u>**CCC*CCGGGGAGCCTCCTCC
I_{20}	AGGTCCAGCTGGGG*CCCGGGGCCC*CCGGGGAGCCTCCTCC
$S_{20}1$	AGGTCCAGCTGGGG*CCCGGGGCCC*CCGGG**<u>C</u>**AGCCTCCTCC
$S_{20}2$	AGGTCCAGCTGGGG*CCCGGGGCCC*CCGG**<u>C</u>**GAGCCTCCTCC
$S_{20}3$	AGGTCCAGCTGGGG*CCCGGGGCCC*CCG**<u>C</u>**GGAGCCTCCTCC
$S_{20}4$	AGGTCCAGCTGGGG*CCCGGGGCCC*CC**<u>C</u>**GGGAGCCTCCTCC
$S_{20}5$	AGGTCCAGCTGGGG*CCCGGGGCCC*C**<u>G</u>**GGGGAGCCTCCTCC
$S_{20}6$	AGGTCCAGCTGGGG*CCCGGGGCCC**<u>G</u>**CGGGGAGCCTCCTCC
$S_{20}7$	AGGTCCAGCTGGGG*CCCGGGGCC**<u>G</u>**CCGGGGAGCCTCCTCC
$S_{20}8$	AGGTCCAGCTGGGG*CCCGGGGC**<u>G</u>**C*CCGGGGAGCCTCCTCC
$S_{20}9$	AGGTCCAGCTGGGG*CCCGGGG**<u>G</u>**CC*CCGGGGAGCCTCCTCC
$S_{20}10$	AGGTCCAGCTGGGG*CCCGGG**<u>C</u>**CCC*CCGGGGAGCCTCCTCC

The O_{10} primer is the original sequence located in exon 2 of *SCN5A*. The original sequence was modified by inserting 1 to 10 Gua/Cyt bases ($I_{11–20}$ primers) and/or by substituting Gua/Cyt to Cyt/Gua ($S_{11–20}$ primers). The bases in italics indicate inserted bases to the sequence of O_{10}. The underlined bold G and C in the $S_{11–20}$ primers indicate substitution sites for the corresponding non-substituted primers (O_{10} and $I_{11–20}$).

Figure 1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GC-rich
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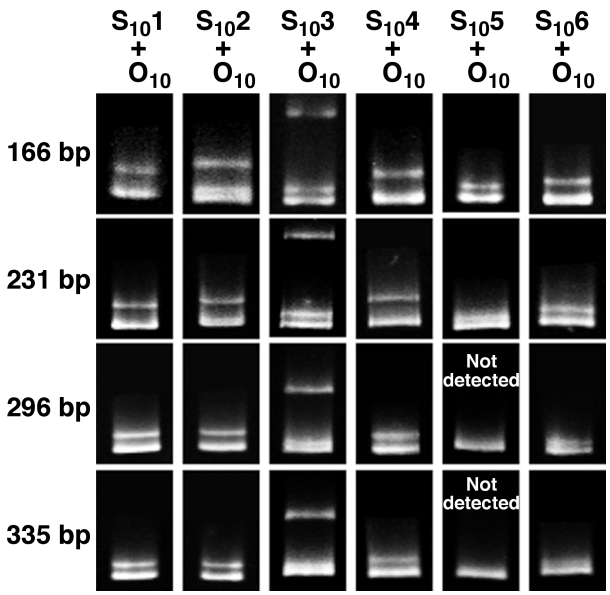
P2
P3
P4
P5
166–176 bp
231–241 bp
296–306 bp
335–345 bp

Figure 2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335 bp
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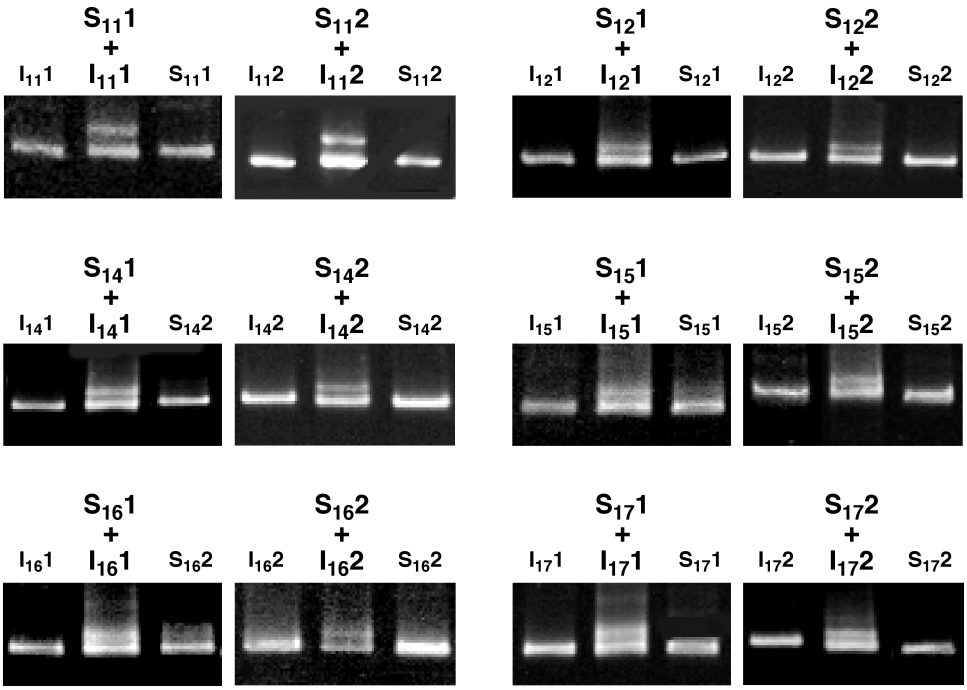
Not detected

Figure 3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I16 2
S16 2
S17 1 + I17 1
I17 1
S17 1
S17 2 + I17 2
I17 2
S17 2

Figure 4

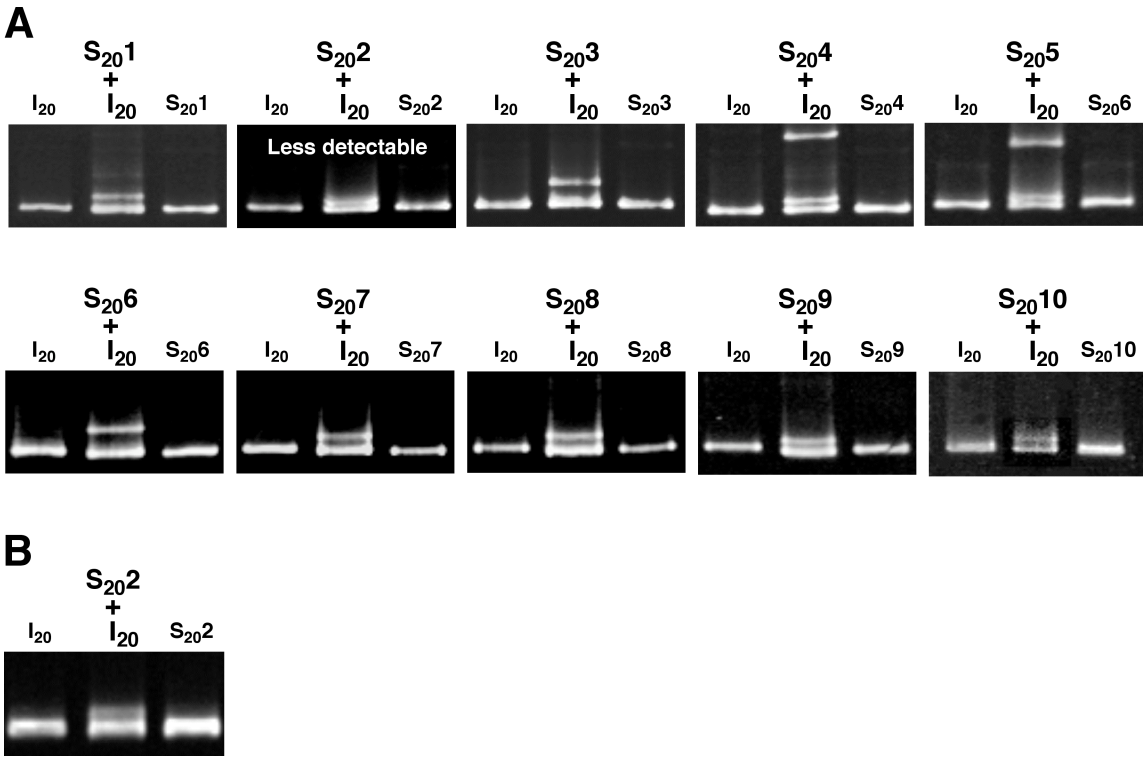
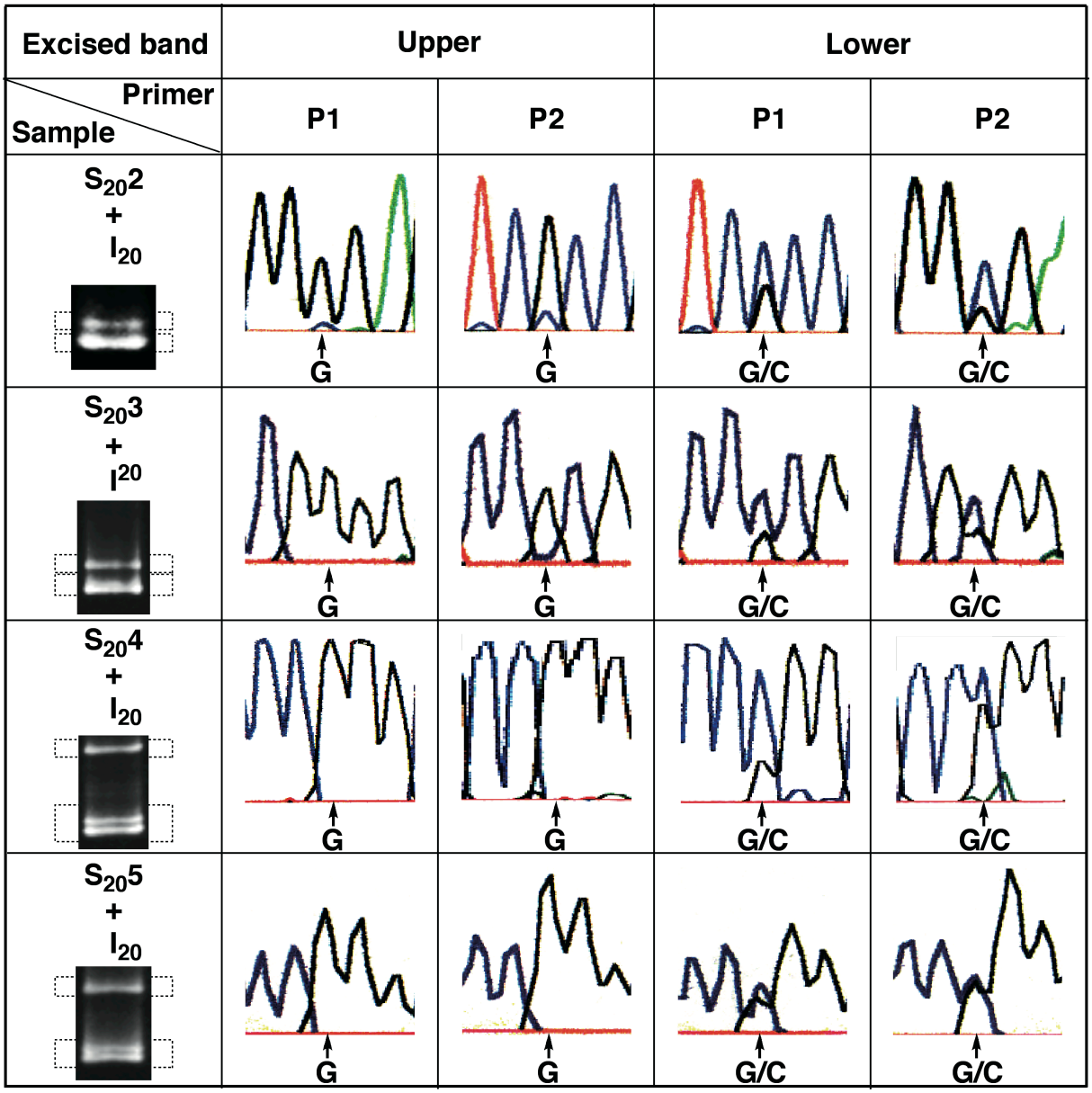

A
S20 1 + I20
I20 I20 S20 1
S20 2 + I20
I20 I20 S20 2
Less detectable
S20 3 + I20
I20 I20 S20 3
S20 4 + I20
I20 I20 S20 4
S20 5 + I20
I20 I20 S20 6
S20 6 + I20
I20 I20 S20 6
S20 7 + I20
I20 I20 S20 7
S20 8 + I20
I20 I20 S20 8
S20 9 + I20
I20 I20 S20 9
S20 10 + I20
I20 I20 S20 10
B
S20 2 + I20
I20 I20 S20 2

Figure 5


Excised band
Upper
Lower
Primer
Sample
P1
P2
P1
P2
S20 2 + I20
S20 3 + I20
S20 4 + I20
S20 5 + I20
G
G
G/C
G/C
G
G
G/C
G/C
G
G
G/C
G/C
G
G
G/C
G/C