Title page:

A heteroduplex-preferential T_m depressor for the specificity-enhanced DNA polymerase chain reactions

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Abstract

A macrocyclic tetraamine zinc(II) complex appended with two quinoline groups, Zn^{2+}–1,7-bis(4-quinolylmethyl)-1,4,7,10-tetraazacyclododecane (Zn^{2+}–Q_2-cyclen), was successfully used as a novel additive to suppress non-specific products in DNA polymerase chain reaction (PCR). In the presence of Zn^{2+}–Q_2-cyclen, the T_m drop of 20-bp heteroduplexes containing a non-complementary base pair was greater than that of the corresponding homoduplex (*i.e.*, primer DNA). Here, we applied such preferential DNA melting to a specificity-enhanced PCR using micromolar concentrations of Zn^{2+}–Q_2-cyclen. We demonstrated the selective amplification of target DNA fragments (*i.e.*, the human heart sodium channel $Na_v1.5$ gene) from genomic DNA or a cDNA library. The optimum condition for the specificity-enhanced PCR could be determined in the concentration range of 1–50 μM of Zn^{2+}–Q_2-cyclen.

Keywords: Macrocyclic tetraamine; Zinc(II) complex; T_m depressor; Specificity-enhanced PCR

Introductory statement

The polymerase chain reaction (PCR) [1] is the most accepted and versatile procedure for the rapid amplification of target DNA fragments. When genomic DNA or a complex cDNA library is used as a template, undesirable multiple satellite band DNAs often appear with the target DNA on the electrophoretic gel. A large variety of parameters can influence the outcome of PCR amplification. These variables include cycling parameters, such as the annealing temperature, as well as the concentrations of Mg^{2+}, H^+, DNA polymerase, dNTP, primer, and template. In general, each optimization of the PCR condition is empirical and time-consuming. Even when all the parameters are optimized, the multiple fragments, frequently attributed to non-specific priming (mispriming), sometimes remain. Some reagents, such as tetramethylammonium (TMA) salts, formamide, dimethyl sulfoxide (DMSO), hexadecyltrimethylammonium bromide, betaine monohydrate, and benzyldimethylhexadecylammonium chloride have been reported to improve the specificity of PCR [2]. TMA derivatives increase the specificity and efficiency of low-performance PCR by presumably enhancing the thermal stability of DNA double-strands [2–4]. In contrast, the reagents, such as formamide [5], DMSO [6–9], and betaine [9–13], are capable of improving the performance by lowering the DNA melting temperature (T_m), where DNA double-strands are destabilized. The latter are often used as components of the commercial optimization and enhancer kit for PCR in the amplification of GC-rich DNA sequences.

Previously, Shionoya *et al.* [14] reported that a macrocyclic tetraamine zinc(II) complex (Zn^{2+}–cyclen, cyclen = 1,4,7,10-tetraazacyclododecane) selectively binds to deoxythymidine (dT) in aqueous solution with a dissociation constant K_d = [free dT][free Zn^{2+}–cyclen]/[dT–Zn^{2+}–cyclen] = 0.3 mM at pH 8. We have also found that the derivative of Zn^{2+}–cyclen-attached aromatic rings, Zn^{2+}–1,7-bis(4-quinolylmethyl)-1,4,7,10-tetraazacyclododecane (Zn^{2+}–Q_2-cyclen), binds to dT (K_d = 10 μM at pH 8.0) much more strongly due to additional double π-π stacking interactions between the two quinoline rings and the nucleobase [15, 16]. The macrocyclic tetraamine zinc(II) complexes preferentially bind to dT in double-stranded DNA to dissociate

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the A–T hydrogen bonds, lowering the melting point, T_m [17–19]. Furthermore, the disrupture of the DNA double-strands in the A–T rich region by the zinc(II) complexes was revealed by nuclease footprinting experiments [15, 19, 20]. Recently, such T-recognizing and local conformation-changing properties of Zn^{2+}–cyclen were applied to the detection of DNA fragments containing single nucleotide polymorphisms (SNPs) using polyacrylamide gel electrophoresis (Zn^{2+}–cyclen–PAGE) [21]. In this study, we have extended the T-recognizing and T_m-depressing properties of the Zn^{2+}–Q_2-cyclen in PCR. We here describe a simple and reliable specificity-enhanced PCR using micromolar concentrations of Zn^{2+}–Q_2-cyclen.

Materials and methods

Reagents

Zn^{2+}–1,7-bis(4-quinolylmethyl)-cyclen was prepared as dinitrate salt (Zn^{2+}–Q_2-cyclen·2NO$_3^-$), as described previously [15]. DMSO and formamide were purchased from Sigma-Aldrich (St. Louis, MO). Betaine monohydrate and ethidium bromide were obtained from Nacalai Tesque (Kyoto, Japan). KOD-plus- DNA polymerase and reverTra Ace were supplied by Toyobo (Osaka, Japan). A fluorescence dye, LCGreen I, was purchased from Idaho Technology (Salt Lake City, UT). λ-*Hin*d III and a 100-base pair (bp) DNA ladder as a DNA size marker were supplied by Takara (Kyoto, Japan). Human genomic DNA and human heart total RNA were purchased from BD Biosciences Clontech (Franklin Lakes, NJ). A pGEM-T Easy Vector System was obtained from Promega (Madison, WI). All oligonucleotides and PCR primers were purchased from Espec Oligo Service (Tsukuba, Japan). The other reagents and solvents used were of analytical quality and were used without further purification. All aqueous solutions were prepared using deionized and distilled water.

Apparatus

The pH measurement was conducted with a Horiba F-23 pH meter (Kyoto, Japan)

and a combination pH electrode Horiba-6378, which was calibrated using pH standard buffers (pH 4.01 and 6.86) at 25 ˚C. All PCR reactions were performed using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The DNA sequence cloned as a template was confirmed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The T_m values were measured using a high-resolution melting instrument, HR-1 (Idaho Technology). Polyacrylamide gel electrophoresis (PAGE) was carried out using a mini slab electrophoresis system, AE-6500 (Atto, Tokyo, Japan). Agarose gel electrophoresis was performed using a mini gel electrophoresis apparatus, Mupid (Advance, Tokyo, Japan).

Polymerase chain reactions and polyacrylamide gel electrophoresis

When the pGEM-T Easy Vector plasmid into which a target DNA sequence was inserted was used as a template, each PCR reaction was performed with the following mixture: 0.15 ng of the plasmid as a template, 0.20 µM forward and reverse primers, 1.0 mM $MgSO_4$, 0.1 unit of KOD-plus- DNA polymerase and its special buffer, dNTPs (each at 0.20 mM), and various concentrations of Zn^{2+}–Q_2-cyclen in a volume of 5.0 µL. After initial denaturation at 95 ˚C for 3 min, the PCR amplification was performed for 30 cycles of 30-sec denaturation at 95 ˚C, 30-sec annealing at 55–68 ˚C, and 30-sec extension at 68 ˚C. When the human genomic DNA was used as a template, each PCR reaction was performed using a mixture of 2.5 ng of human genomic DNA, 0.20 µM forward and reverse primers, 1.0 mM $MgSO_4$, 0.1 unit of KOD-plus- DNA polymerase and its special buffer, dNTPs (each at 0.20 mM), and various concentrations of Zn^{2+}–Q_2-cyclen in a volume of 5.0 µL. The PCR was performed with the same cycle described above. PAGE was performed at 200 V for 60 min at room temperature in a 1 mm-thick, 9 cm-wide, and 9 cm-long gel prepared with 8% (w/v) polyacrylamide (30:1 ratio of acrylamide to *N,N'*-methylenebisacrylamide), 90 mM Tris, 90 mM borate, and 2.0 mM EDTA (pH 8.0) on an Atto mini slab PAGE apparatus. The PCR products were dissolved in 0.50 µL of a loading buffer containing 60% (v/v) glycerol, 18 mM Tris/18 mM borate, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol and then applied to the well of the gel. The running buffer was a solution containing 90 mM Tris, 90

mM borate, and 2.0 mM EDTA (pH 8.0). At the end of the run, the gels were stained in an aqueous solution of ethidium bromide (10 µg/mL).

Reverse transcription-polymerase chain reaction and agarose gel electrophoresis

A mixture of 1.0 µg of human heart total RNA and 50 pmol oligo(dT)$_{20}$ in volume 12 µL was heated at 65 ˚C for 5 min. The mixture was then cooled on ice, and 1.0 µL RNase inhibitor, 0.20 µmol dNTPs, and 100 units reverTra Ace and its special buffer were added to a final volume of 20 µL. The reverse transcription was started by 42 ˚C incubation for 60 min followed by inactivation at 85 ˚C for 5 min. Subsequent PCR was performed using the following mixture: 1.0 µL of the above mixture, 0.20 µM forward and reverse primers, 1.0 mM MgSO$_4$, 0.1 unit of KOD-plus- DNA polymerase and its special buffer, dNTPs (each at 0.20 mM), and various concentrations of Zn^{2+}–Q$_2$-cyclen in a volume of 5.0 µL. PCR was started by 3-min denaturation at 95 ˚C followed by 30 cycles with 10-sec denaturation at 98 ˚C, 30-sec annealing at 60 or 63 ˚C, and 3-min extension at 68 ˚C. The PCR products were dissolved in 0.50 µL of the above loading buffer, and 1.0 µL of the products was then used for the following agarose gel electrophoresis. Electrophoresis was performed at 100 V for 30 min at room temperature in a 0.5 cm-thick, 10.5 cm-wide, and 5.8 cm-long gel prepared with 0.8% (w/v) agarose containing 10 µg/mL ethidium bromide on a Mupid mini gel maker. The running buffer was a solution of 40 mM Tris and 1.0 mM EDTA adjusted to pH 8.0 with acetic acid.

Measurement of DNA melting temperature

Before measuring the practical melting temperature (T_m) of double-stranded DNA, each pair of single-stranded 20-mer oligonucleotides (each at 1.0 µM) was mixed in the same buffer as described in the following experiment. After the mixture was heated at 95 ˚C for 3 min, it was returned to room temperature to form double-stranded DNA. The T_m was measured in a mixture containing 0.20 µM double-stranded DNA (20 bp), 1.0 mM MgSO$_4$, a

special buffer for KOD-plus- DNA polymerase, 1.0 μL of LCGreen I dye (a commercially available solution), and 0–10 μM Zn^{2+}–Q_2-cyclen, 0–10% (v/v) formamide, 0–10% (v/v) DMSO, or 0–1.0 M betaine monohydrate in a volume of 10 μL. The mixture was placed in a glass capillary tube, set in a high-resolution melter, HR-1, and heated at 0.3 ˚C/sec. During heating, the fluorescence (F) of the LCGreen I dye (*i.e.*, a double-strand binder) was monitored and plotted versus temperature (T) to obtain a melting curve, which was converted to a differential plot ($-dF/dT$ vs. temperature) to determine the T_m value. The temperature at the peak of the plot is the practical T_m value (see Fig. 2A). The theoretical melting temperature (T_m') of each PCR primer was calculated using the following formula of Baldino *et al.* [22] modified by Hecker and Roux [23]: T_m' = 81.5 + 16.6 log [Na^+] + 0.41 (%GC) – 675/(number of base), where [Na^+] is assumed to be 1.0 M.

Results and discussion

Preliminary PCRs were performed using the partial rat $Na_v1.4$ gene [24] (a 621-bp sequence of 1162–1782 entered as GenBank accession number M26643) cloned as a template and a combination of the 20-mer primer 1 (5'-TGGTACATCAATGACACTTG, T_m' = 64.1 ˚C) and the 20-mer primer 2 (5'-CGTACGCCATGGCCACCACG, T_m' = 76.4 ˚C). The target DNA was a 400-bp fragment corresponding to the yellow region, as shown in Fig. 1A. The PCR always gave a few non-specific products together with the target DNA at annealing temperatures of 55 to 64 ˚C set in consideration of the T_m' of primer 1 (64.1 ˚C). At over 64 ˚C, almost all PCR products disappeared. The template used had three homologous repeated sequences to primer 1 (see the green, blue, and pink regions in Fig. 1A). Thus, spurious products were observed at an annealing temperature of 60 ˚C, a phenomenon that is due to the mispriming of primer 1 on the homologous sequences (*i.e.*, the formation of 20-bp heteroduplexes). The major non-specific products are a 436-bp fragment (from the blue region containing a non-complementary base pair) and a 454-bp fragment (from the green region containing three non-complementary base pairs). A 418-bp fragment (from the pink region) was not generated under the experimental conditions. The non-specific products were not

eliminated even when a duplex-stabilizer, Mg^{2+} (2.0 mM), was added at the same annealing temperature of 60 ˚C. To determine whether the specificity could be improved, Zn^{2+}–Q_2-cyclen was added to the PCR mixture, and the products were analyzed after electrophoretic fractionation on polyacrylamide gel. A typical effect of Zn^{2+}–Q_2-cyclen on the PCR amplification at an annealing temperature of 60 ˚C is shown in Fig. 1B. With increasing the concentrations of Zn^{2+}–Q_2-cyclen (up to 4.0 μM), there was an increase in the quantity of the specific product (400 bp) and a decrease in that of non-specific ones (436 bp and 456 bp) (see Fig. 1B). At 5.0 μM, only the specific product was observed with a slight decrease in quantity. The quantity of the specific product at [Zn^{2+}–Q_2-cyclen] = 5.0 μM was almost half of that of the specific product generated at [Zn^{2+}–Q_2-cyclen] = 0 M (see the second lane and lane *a* in Fig. 1B). Lane *b* of Fig. 1B, in which 3-fold amounts of the PCR product at [Zn^{2+}–Q_2-cyclen] = 5.0 μM were applied, clearly shows that Zn^{2+}–Q_2-cyclen can eliminate the non-specific products. At a lower annealing temperature of 55 ˚C, a higher concentration of 8.0 μM (= [Zn^{2+}–Q_2-cyclen]) was required to eliminate the non-specific products. Thus, the dose-dependent effect of Zn^{2+}–Q_2-cyclen should be applicable to the specificity-enhanced PCR.

Insert Figure 1.

Next, to evaluate whether the T_m-depressing property of Zn^{2+}–Q_2-cyclen improves the specificity of PCR, we investigated the effect of the zinc(II) complex on the melting temperature (T_m) of double-stranded oligonucleotides designed on the basis of the above experiments. A homoduplex and a T–C mismatching-heteroduplex tested are shown in Fig. 2A. Those double-stranded 20-bp oligonucleotides are annealing models of primer 1 with a complementary sequence (for the specific PCR product) and a non-complementary blue region (for the main spurious one) in the template of Fig. 1A. The T_m of 61.9 ± 0.2 ˚C for the homoduplex (*cf.,* theoretical melting temperature = 64.1 ˚C) was higher than that of 55.5 ± 0.3 ˚C for the heteroduplex. The zinc(II) complex dose-dependently lowered the T_m values for both duplexes (see Table 1). Differential curves of –dF/dT vs. temperature and plots of ΔT_m

($= T_m - T_m([Zn^{2+}\text{–}Q_2\text{-cyclen}] = 0\ \mu M)$) are shown in Fig. 2A and Fig. 2B, respectively. The T_m drop of the heteroduplex was greater than that of the homoduplex (see ΔT_m values in Fig. 2B and Table 1). In the presence of over 5 μM, the peak of the differential curve for the heteroduplex became still broader, and the melting temperature was not estimated. This fact shows that the double-stranded structure of the heteroduplex is preferentially disrupted by $Zn^{2+}\text{–}Q_2$-cyclen compared to the homoduplex. In contrast, such a preferential T_m-depressing effect was not observed with the conventional T_m depressors, such as formamide, DMSO, and betaine. Although those reagents dose-dependently lowered the T_m values of the homoduplex and the heteroduplex, there was almost no difference in the ΔT_m values at the same concentration of the conventional T_m depressor (Table 1). From the results, therefore, the preferential T_m-depressing effect by $Zn^{2+}\text{–}Q_2$-cyclen just might become advantageous to prevent the mismatched annealing of a primer and enhance the specificity of low-performance PCR.

Insert Figure 2 and Table 1.

Finally, we applied $Zn^{2+}\text{–}Q_2$-cyclen to PCR using genomic DNA or a complex cDNA library as a template. Preliminary PCR experiments for the amplification of each of exon 1 to exon 28 in the $Na_v1.5$ gene (*SCN5A*) [25] from the human genome at a constant annealing temperature of 60 °C showed that *ca.* 50% PCR reactions were accompanied by the generation of non-specific products. Typical results with non-specific products are shown in Fig. 3, where a lot of larger DNA bands are observed at $[Zn^{2+}\text{–}Q_2\text{-cyclen}] = 0\ \mu M$ at 60 °C. In the case of exon 15, the target DNA (247 bp) was not amplified at 60 °C, and, thus, the PCR was performed at an annealing temperature of 57 °C. The primer sets for the 151-bp DNA of exon 8 and the 247-bp DNA of exon 15 are 5'-CGAGTGCCCCTCACCAGCATG (theoretical melting temperature, $T_m' = 76.7$ °C) and 5'-GGAGACTCCCCTGGCAGGACAA ($T_m' = 76.9$ °C), and 5'-CTTTCCTATCCCAAACAATACCT ($T_m' = 68.2$ °C) and 5'-CCCCACCATCCCCCATGCAGT ($T_m' = 76.7$ °C), respectively. In both exons, the non-specific products decreased with an increase in the concentration of $Zn^{2+}\text{–}Q_2$-cyclen. In

the amplification of exon 8, the optimal annealing temperature was estimated as 68 ˚C from the theoretical T_m values (76.7 and 76.9 ˚C) of the primers. The quality and quantity of PCR products at the optimal annealing temperature of 68 ˚C were almost equal to those at 60 ˚C in the presence of 40 μM Zn^{2+}–Q_2-cyclen (see Fig. 3A). On the other hand, the annealing temperature for the amplification of exon 15 could not be optimized because the specificity of the PCR was not high enough to eliminate the non-specific products without losing the specific one at annealing temperatures below 60 ˚C. Such a situation may often occur in general PCR experiments and requires the use of other protocols (*e.g.*, a redesign of primers). In the presence of 20 μM Zn^{2+}–Q_2-cyclen, however, the target DNA (247 bp) was selectively amplified at 57 ˚C (see Fig. 3B). These results indicate that a novel optimization for specificity-enhanced PCR could be available by using Zn^{2+}–Q_2-cyclen.

Insert Figure 3.

Similar PCR experiments were performed using a cDNA library. A typical result, as shown in Fig. 4, demonstrated that Zn^{2+}–Q_2-cyclen enhances the specificity at an annealing temperature of 60 ˚C. Primers 5'-TTCACCGCCATTTACACCTT (T_m' = 66.2 ˚C) and 5'-TGTGTTCAGGGTGGGCCAGT (T_m' = 72.4 ˚C) were used to define a 1993-bp DNA from the *SCN5A* gene (the sequence from 639 to 2631 entered as GenBank accession number M77235). The template was the cDNA library synthesized from human heart total RNA by reverse transcription. In this case, two bands of smaller DNA were observed at [Zn^{2+}–Q_2-cyclen] = 0 μM at 60 ˚C. The non-specific products were suppressed at a higher annealing temperature of 63 ˚C. With an increase in the concentration of Zn^{2+}–Q_2-cyclen (up to 34 μM), the non-specific products were completely suppressed even at a lower annealing temperature of 60 ˚C. The specificity-enhanced PCR using genomic DNA or a complex cDNA library required higher concentrations of Zn^{2+}–Q_2-cyclen than that using cloned DNA, as shown in Fig. 1B, which might be due to the binding of Zn^{2+}–Q_2-cyclen to excess amounts of thymidine groups in the DNA template and the oligo(dT)$_{20}$ carried over from the reverse transcription protocol.

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Insert Figure 4.

Conclusions

The macrocyclic tetraamine zinc(II) complex, Zn^{2+}–Q_2-cyclen, is a very unique and useful T_m-depressing reagent that greatly facilitates the specificity of PCR by preferentially lowering the T_m of the heteroduplex. The optimum concentration of Zn^{2+}–Q_2-cyclen for the specificity-enhanced PCR depends on the amount of the template DNA and primers (*e.g.*, PCR primers and oligo(dT)$_{20}$). The T_m depressor could preferentially bind to a primer having a mismatched binding site and then suppresses non-specific products. Such a duplex-selective characteristic has not been observed in other reagents with a T_m-depressing property, such as formamide, DMSO, and betaine. Taking advantage of this characteristic is likely to enhance the performance of various molecular strategies aimed at DNA amplification.

Acknowledgements

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Table 1. T_m and ΔT_m of homo- and heteroduplexes in the absence and presence of Zn^{2+}–Q_2-cyclen and conventional T_m depressors

Homoduplex

Zn^{2+}–Q_2-cyclen			Formamide			DMSO			Betaine		
Conc. (µM)	T_m (S.D.) (˚C)	ΔT_m (S.D.) (˚C)	Conc. (v/v%)	T_m (S.D.) (˚C)	ΔT_m (S.D.) (˚C)	Conc. (v/v%)	T_m (S.D.) (˚C)	ΔT_m (S.D.) (˚C)	Conc. (M)	T_m (S.D.) (˚C)	ΔT_m (S.D.) (˚C)
0	61.9 (± 0.2)	–	0	61.5 (± 0.3)	–	0	61.7 (± 0.2)	–	0	61.4 (± 0.5)	–
2.0	59.8 (± 0.1)	-1.6 (± 0.4)	2.5	59.3 (± 0.6)	-2.3 (± 0.3)	2.5	60.3 (± 0.5)	-1.2 (± 0.3)	0.2	60.7 (± 0.7)	-0.8 (± 0.2)
4.0	57.2 (± 0.2)	-4.5 (± 0.2)	5.0	56.4 (± 0.4)	-5.1 (± 0.2)	5.0	58.5 (± 0.5)	-3.2 (± 0.3)	0.4	60.2 (± 0.7)	-1.2 (± 0.2)
6.0	54.5 (± 0.7)	-6.6 (± 0.2)	7.5	54.4 (± 0.4)	-7.1 (± 0.2)	7.5	56.9 (± 0.6)	-4.8 (± 0.4)	0.6	59.9 (± 0.6)	-1.5 (± 0.2)
8.0	52.4 (± 0.6)	-9.3 (± 0.6)	10.0	52.5 (± 0.5)	-9.1 (± 0.2)	10.0	55.3 (± 0.4)	-6.4 (± 0.2)	0.8	59.6 (± 0.5)	-1.8 (± 0.1)
10.0	50.2 (± 0.8)	-11.5 (± 0.7)							1.0	59.4 (± 0.6)	-2.0 (± 0.2)

Heteroduplex

Zn^{2+}–Q_2-cyclen			Formamide			DMSO			Betaine		
Conc. (µM)	T_m (S.D.) (˚C)	ΔT_m (S.D.) (˚C)	Conc. (v/v%)	T_m (S.D.) (˚C)	ΔT_m (S.D.) (˚C)	Conc. (v/v%)	T_m (S.D.) (˚C)	ΔT_m (S.D.) (˚C)	Conc. (M)	T_m (S.D.) (˚C)	ΔT_m (S.D.) (˚C)
0	55.5 (± 0.3)	–	0	54.9 (± 0.3)	–	0	55.1 (± 0.4)	–	0	55.1 (± 0.4)	–
2.0	51.6 (± 0.4)	-3.5 (± 0.1)	2.5	52.5 (± 0.5)	-2.5 (± 0.2)	2.5	53.2 (± 0.4)	-1.8 (± 0.1)	0.2	54.7 (± 0.3)	-0.4 (± 0.1)
4.0	49.0 (± 0.5)	-6.1 (± 0.2)	5.0	49.9 (± 0.5)	-5.1 (± 0.2)	5.0	51.4 (± 0.5)	-3.7 (± 0.3)	0.4	54.3 (± 0.4)	-0.8 (± 0.3)
6.0	ND	ND	7.5	47.6 (± 0.2)	-7.3 (± 0.1)	7.5	50.2 (± 0.3)	-4.8 (± 0.2)	0.6	54.0 (± 0.3)	-1.1 (± 0.3)
8.0	ND	ND	10.0	45.0 (± 0.3)	-10.0 (± 0.1)	10.0	48.9 (± 0.6)	-6.2 (± 0.3)	0.8	53.7 (± 0.2)	-1.4 (± 0.3)
10.0	ND	ND							1.0	53.5 (± 0.2)	-1.6 (± 0.3)

Data are presented as mean ± S.D. by experiments performed in triplicate.

ND = Not Detectable

Figure Legends

Fig. 1. (**A**) A partial DNA sequence of the rat $Na_v1.4$ gene used as a template. Two arrows show the 20-mer primers 1 and 2 used for the amplification of a 400-bp fragment (yellow region). The green, blue, and pink regions are highly homologous regions to primer 1. (**B**) Electrophoretic analysis of PCR products (8% PAGE followed by ethidium bromide staining) in the absence and presence of Zn^{2+}–Q_2-cyclen (0–5 µM). The volume for each of the PCR products applied is 1.0 µL except for lanes *a* and *b*. A half-volume (0.5 µL) of the sample at [Zn^{2+}–Q_2-cyclen] = 0 and a 3-fold volume (3 µL) of the sample at [Zn^{2+}–Q_2-cyclen] = 5 µM were applied in lanes *a* and *b*, respectively. Lane *M* represents the 400-bp and 500-bp DNA of a 100-bp ladder size marker.

Fig. 2. (**A**) Differential melting curves ($-dF/dT$ vs. temperature) for two kinds of 20-bp DNA. Each peak temperature is the melting temperature (T_m). (**B**) Plots of ΔT_m (= T_m − T_m([Zn^{2+}–Q_2-cyclen] = 0 µM) against [Zn^{2+}–Q_2-cyclen]. Each symbol is the mean value of experiments performed in triplicate.

Fig. 3. (**A**) Electrophoretic analysis of PCR products containing the sequences of exon 8 (151 bp) of the *SCN5A* gene from the human genome (8% PAGE followed by ethidium bromide staining). (**B**) Electrophoretic analysis of the PCR products containing the sequences of exon 15 (247 bp) of the *SCN5A* gene from the human genome (8% PAGE followed by ethidium bromide staining).

Fig. 4. Electrophoretic analysis of the reverse transcription-PCR product (1993 bp) amplified in a partial *SCN5A* gene (0.8% agarose gel containing ethidium bromide).

Figure 1

(A)

CAATTGTGGG AGCCCTGATC CAGTCTGTGA AAAAGCTGTC GGATGTGATG
ATCCTCACTG TCTTCTGCCT GAGTGTCTTT GCCCTGGTGG GGCTGCAGCT
TTTCATGGGA AACCTGCGTC AGAAGTGCGT GCGTTGGCCC CCGCCCATGA

ATGACACCAA CACCACGTGG TATGGCAATG ACACTTGGTA CAGCAATGAC

ACTTGGTACG GCAATGACAC TTGGTACATC AATGACACTT GGAACAGCCA
Primer 1
GGAGAGCTGG GCCGGCAACT CTACCTTTGA CTGGGAGGCC TACATCAATG
ACGAAGGGAA CTTCTATTTC TTGGAGGGCT CCAATGATGC TCTGCTCTGT
GGGAATAGCA GTGATGCTGG GCACTGCCCT GAGGGCTACG AATGCATAAA
GGCTGGGCGG AACCCCAACT ATGGCTACAC CAGCTATGAC ACCTTCAGCT
GGGCTTTCCT GGCTCTCTTC CGGCTCATGA CGCAGGACTA CTGGGAGAAC
CTTTTCCAGC TGACCCTACG AGCTGCTGGC AAGACCTACA TGATCTTCTT
CGTGGTCATC ATCTTCCTGG GCTCCTTCTA CCTCATCAAT CTGATCCTGG
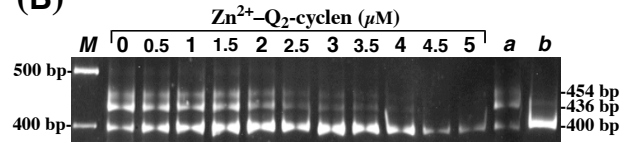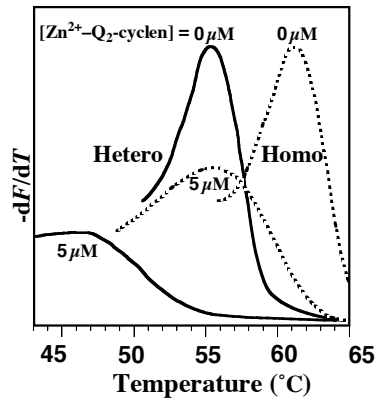CCGTGGTGGC CATGGCGTAC G
Primer 2

(B)

Figure 2

(A)   **Homoduplex**

5'-TGGTACATCAATGACACTTG
3'-ACCATGTAGTTACTGTGAAC

Heteroduplex

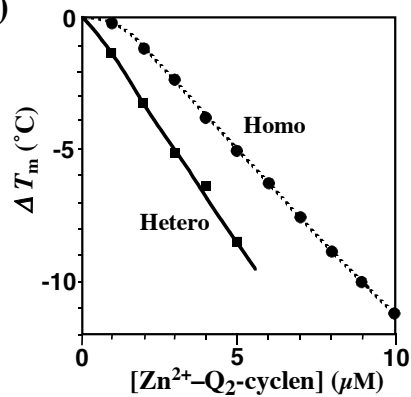5'-TGGTACA **T** CAATGACACTTG
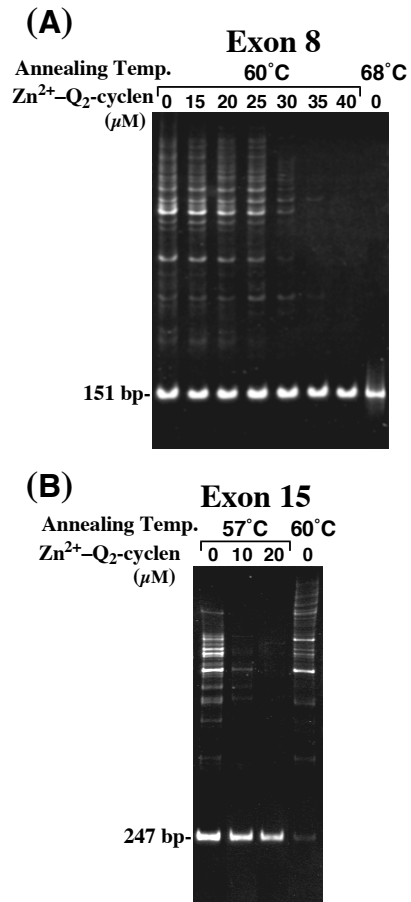3'-ACCATGT **C** GTTACTGTGAAC

(B)

Figure 3

(A)

Exon 8

(B)

Exon 15

Figure 4