Notes & Tips

Zinc(II)–cyclen polyacrylamide gel electrophoresis for detection of mutations in short Ade/Thy-rich DNA fragments

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Short title: Improved Zn^{2+}-cyclen–PAGE procedure

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[1] *Abbreviations used:* SNPs, single-nucleotide polymorphisms; Zn^{2+}-cyclen, zinc(II) complex of 1,4,7,10-tetraazacyclododecane; bp, base pairs; T_m, melting point; FFPE, formalin-fixed paraffin-embedded.

Abstract

We describe an improved gel-based method with an additive Zn^{2+}-cyclen complex (cyclen = 1,4,7,10-tetraazacyclododecane), Zn^{2+}-cyclen–PAGE, for mutation detection in DNA fragments by PCR that contain more than 65% Ade/Thy bases and fewer than 100 base pairs (bp). Existing techniques have a problem in analyzing such short Ade/Thy-rich fragments because the duplexes are disrupted and are not detectable due to binding of Zn^{2+}-cyclen to Thy bases. In this strategy using a PCR primer with a Gua/Cyt-lined sequence attached at its 5'-end, we successfully detected a mutation in an 86-bp Ade/Thy-rich region of the *BRCA1* gene from formalin-fixed paraffin-embedded breast cancer-tissue sections.

Key words: Zn^{2+}-cyclen; PAGE; SNP; Mutation; FFPE; *BRCA1*

We have previously reported a method for the detection of single-nucleotide polymorphisms (SNPs)[1] by means of PAGE in the presence of the zinc(II) complex of 1,4,7,10-tetraazacyclododecane (Zn^{2+}-cyclen) as an additive [1]. The method is based on the ability of Zn^{2+}-cyclen to recognize Thy moieties in a double-stranded DNA [2–4]. In combination with a PCR-based heteroduplexing method, Zn^{2+}-cyclen–PAGE permits the separation of heteroduplex bands from the corresponding homoduplex bands. The appearance of slow or differentially migrating bands indicates the presence of heteroduplex bands that imply the presence of mutations or SNPs. With this gel-based method, up to four distinct migration bands per DNA sample can be detected. Zn^{2+}-cyclen–PAGE has been used for screening of mutations in the *SCN5A* gene, which is related to inherited arrhythmia syndrome [5], or for genomapping of SNPs in the *CYP3A5* gene, which is associated with enzymatic activity for drug metabolism [6]. In addition, we have demonstrated that single mutations in Gua/Cyt-lined sequences with a length of up to 20 base pairs (bp) were detectable through binding of Zn^{2+}-cyclen to Thy bases near the sites of mutations [7]. However, the existing procedure has a limitation in the analysis of Ade/Thy-rich PCR products of fewer than 100 bp that contain more than 65% Ade/Thy bases. Because the Zn^{2+}-cyclen in the gel binds preferentially to Thy bases in a double-stranded DNA and thereby dissociates the double strand, it does not permit the visualization of such short Ade/Thy-rich products as double-stranded DNA bands. To overcome this limitation, we have developed an improved Zn^{2+}-cyclen–PAGE procedure that uses a PCR primer with a Gua/Cyt-lined sequence attached at its 5'-end to increase the melting point (T_m) of the resultant PCR amplicon.

We selected the Ade/Thy-rich DNA region of the breast cancer *BRCA1* gene (GenBank accession No. AY273801, 19772 to 19892) as a target sequence for this study (see Supplementary Fig. S1). The sequence was cloned and then PCR-based mutagenesis was performed in the substitution from Ade to Thy at nucleotide position 19861 (19861A>T), which has been identified as a mutation that leads to a codon transversion from Leu-63 to the terminus [8]. The Ade/Thy-rich DNA fragments of 51 bp (containing 73% Ade/Thy bases) were amplified as Wild-type (W) and Mutated (M) sequences. The heterozygosity mimic (H)

was obtained as follows: equal amounts of W and M fragments were mixed, and then ten cycles of denaturing (95 °C, 15 s) and annealing (60 °C, 15 s) were performed. Three fragments of W, H, and M were considered as a one-set sample, and they were subjected to analysis by Zn^{2+}-cyclen–PAGE. Details for DNA sample preparation and sequence of primers used were described in Supplementary Fig. S1.

We subjected the set of 51-bp DNA fragments to Zn^{2+}-cyclen–PAGE (with 3 mM Zn^{2+}-cyclen) (Fig. 1, left panel). Electrophoresis was performed as previously described [5–7]. All the DNA fragments were detected as complicated multiple bands that were caused by complete or partial dissociation of the short Ade/Thy-rich DNA fragments through binding of Zn^{2+}-cyclen to the Thy bases. Even in the analysis with 1 mM Zn^{2+}-cyclen (see Supplementary Fig. S2, rightmost panel of b), we were unable to observe typical heteroduplex bands in the hetero sample (lane H). These results show that the use of lower concentrations of Zn^{2+}-cyclen has limitations for the analysis of mutation in short Ade/Thy-rich DNA fragments. In an attempt to overcome this limitation, we attached a 10-bp Gua/Cyt-lined sequence (CCCGCGCGCC) to the 51-bp Ade/Thy-rich DNA fragment. The 51-bp region was re-amplified, and the resultant PCR product consisted of a 61-bp fragment containing 61% Ade/Thy bases (see Supplementary Fig. S1). The Gua/Cyt-attached DNA fragment sample set was subjected to analysis using 3 mM Zn^{2+}-cyclen–PAGE (Fig. 1, right panel). The separation of heteroduplexes and the corresponding homoduplexes of the hetero sample was dramatically improved by the attachment of the 10-bp Gua/Cyt-lined sequence. The presence of slower-migrating bands corresponding to the heteroduplexes was clearly detected. This result demonstrates that the attachment of Gua/Cyt is effective in preventing dissociation of the short Ade/Thy-rich DNA fragment in the Zn^{2+}-cyclen–PAGE gel. Furthermore, we attached other Gua/Cyt-lined sequences (10 or 20 bp) to the same 51-bp *BRCA1* region, and the resultant five types of sample sets were examined (see Supplementary Fig. S3). The artificial single-nucleotide substitution was detected in only two sample sets. These results indicate that the Gua/Cyt-attached PCR primer containing a 10- or 20-bp Gua/Cyt-lined sequence should be designed in the present method. In addition, it is desirable to test several

kinds of Gua/Cyt-lined sequences form the viewpoints of the order and attached sides (forward or reverse side for PCR).

As the first practical example of an application of the improved procedure, we analyzed short Ade/Thy-rich PCR products amplified from the genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) breast-cancer tissue sections. A human tissue array of ninety-six FFPE sections derived from breast-cancer tissues and adjacent normal tissues was purchased from US Biomax (Rockville, MD, USA). *BRCA1* is a tumor-suppressor gene, and a relationship between mutations in this gene and breast cancer has been reported [8–10]. It would be useful to have prognostic and predictive markers of breast cancer in FFPE tissues collected from clinical trials. However, it is generally difficult to amplify long DNA regions from FFPE tissues by PCR because the DNA is frequently damaged during the process of fixation and long-term storage. Genomic DNA was extracted from each of ninety-six FFPE sections arrayed on a slide glass, according to the standard protocol, by using TaKaRa DEXPAT (Takara Bio, Ootsu, Japan). We performed PCR of the *BRCA1* region (GenBank accession No. AY273801, 19772 to 19902) from the FFPE subjects by using pairs consisting of primers p1 and p7, p4 and p7, p5 and p7, p6 and p7, and p2 and p7 (see Supplementary Figs. S1 and S2, and Table S1) to give 131-bp, 109-bp, 86-bp, 71-bp, and 61-bp DNA fragments, respectively. DNA fragments of three sizes (86, 71, and 61 bp) were sufficiently amplified from all the ninety-six FFPE subjects under the experimental conditions. The longest (86-bp) fragments, containing 66% Ade/Thy bases (GenBank accession no. AY273801, 19817 to 19902), were subjected to 3 mM Zn^{2+}-cyclen–PAGE for screening of mutations. Typical results from Zn^{2+}-cyclen–PAGE of the three DNA-fragment samples are shown in the left-hand panel of Fig. 2. Multiple bands produced by partial dissociation of the double strand were observed similarly in all the ninety-six samples. We were unable to confirm the presence of heteroduplex bands. The same 86-bp region was re-amplified by using a pair consisting primers p5+GC and p7 (see Supplementary Table S1), and the resultant 96-bp fragments, containing 59% Ade/Thy bases, were subjected to 3 mM Zn^{2+}-cyclen–PAGE. The attachment of the Gua/Cyt-lined sequence suppressed the

dissociation of the double strands in all the ninety-six samples. As shown in right-hand panel of Fig. 2, we detected typical heteroduplex bands (two slower-migrating bands in sample No. 2), indicating the existence of a mutation in the sequence that was amplified from the genomic DNA extracted from the breast-cancer tissue section. Samples No. 1 and No. 3 were derived from the adjacent normal tissue sections. To determine the position of the mutation in sample No. 2, we performed direct sequencing using the same samples Nos. 1, 2, and 3. However, direct sequencing did not permit the detection of any mutations in any of the three sequences (data not shown). This result suggests that in the cancer-tissue specimen the content of mutated DNA may be much lower than that of wild-type DNA. Consequently, it would be very difficult to detect a mutated-nucleotide base from the direct-sequencing charts in such a case, as previously described [11]. Next, we cloned the 86-bp region from the same three samples, and then we examined the sequences of 32 clones randomly selected from each sample. Finally, we detected a mutation of 19861A>T in five clones from Sample No. 2. Other clones from the three samples showed no variations in the 86-bp region of *BRCA1*. We have therefore demonstrated that screening by using the improved Zn^{2+}-cyclen-PAGE method is much more sensitive and accurate than is direct sequencing in this case. It is therefore worthwhile considering our current strategy for highly sensitive screening of mutation/polymorphism in relatively short DNA sequences from FFPE tissue samples.

Acknowledgments

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Figure legends

Fig. 1. Analysis of mutations in the native and Gua/Cyt-attached DNA fragment sample sets. Each sample set (W, H, and M) of the native (51 bp) and 10-bp Gua/Cyt-attached (51 + 10GC: 61 bp) DNA fragments was applied to a 15% (w/v) polyacrylamide gel containing 3.0 mM Zn^{2+}-cyclen. The DNA size (bp), the contents of Ade/Thy bases (%), and the results of mutation detection are shown above each panel. ND = not detected. The R_f value was defined as the ratio of the migration distance to the length of the gel slab.

Fig. 2. Detection of mutations of the *BRCA1* gene in genomic DNA extracted from FFPE tissue sections. The native (86 bp) and 10-bp Gua/Cyt-attached (86 + 10GC; 96 bp) DNA fragments were applied to a 15% (w/v) polyacrylamide gel containing 3.0 mM Zn^{2+}-cyclen. Sample No. 2 was amplified from the genomic DNA extracted from the breast-cancer tissue section, whereas samples Nos. 1 and 3 were derived from sections of adjacent normal tissue. The DNA size (bp), the contents of Ade/Thy bases (%), and the results of mutation detection are shown above each panel. ND = not detected. The R_f value was defined as the ratio of the migration distance to the length of the gel slab.

Figure 1

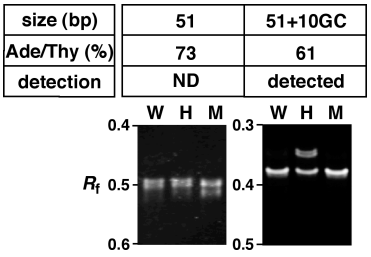
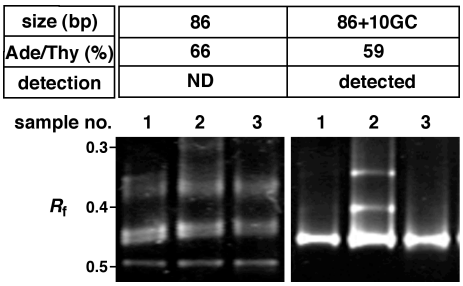


Figure 2



Zinc(II)–cyclen polyacrylamide gel electrophoresis for detection of mutations in short Ade/Thy-rich DNA fragments

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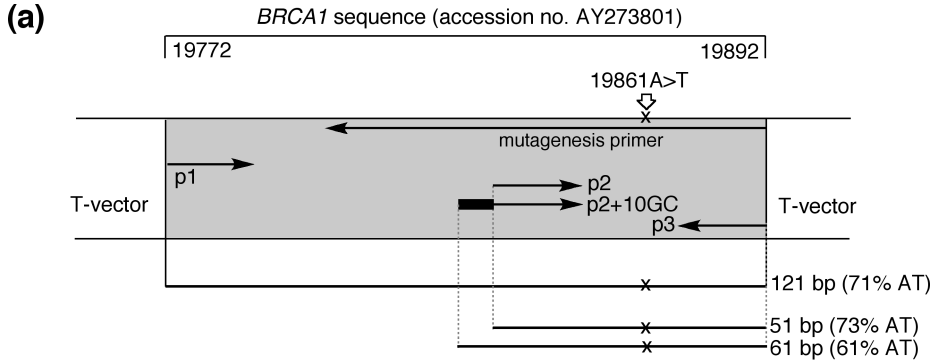
Supplementary Materials

Supplementary Figure S1



(b)

p1:
5'-TAAATTGTTCTTTCTTTCTT

p2:
5'-CTTCACAGTGTCCTTTAT

p2+10GC:
5'-CCCGCGCGCCCTTCACAGTGTCCTTTAT

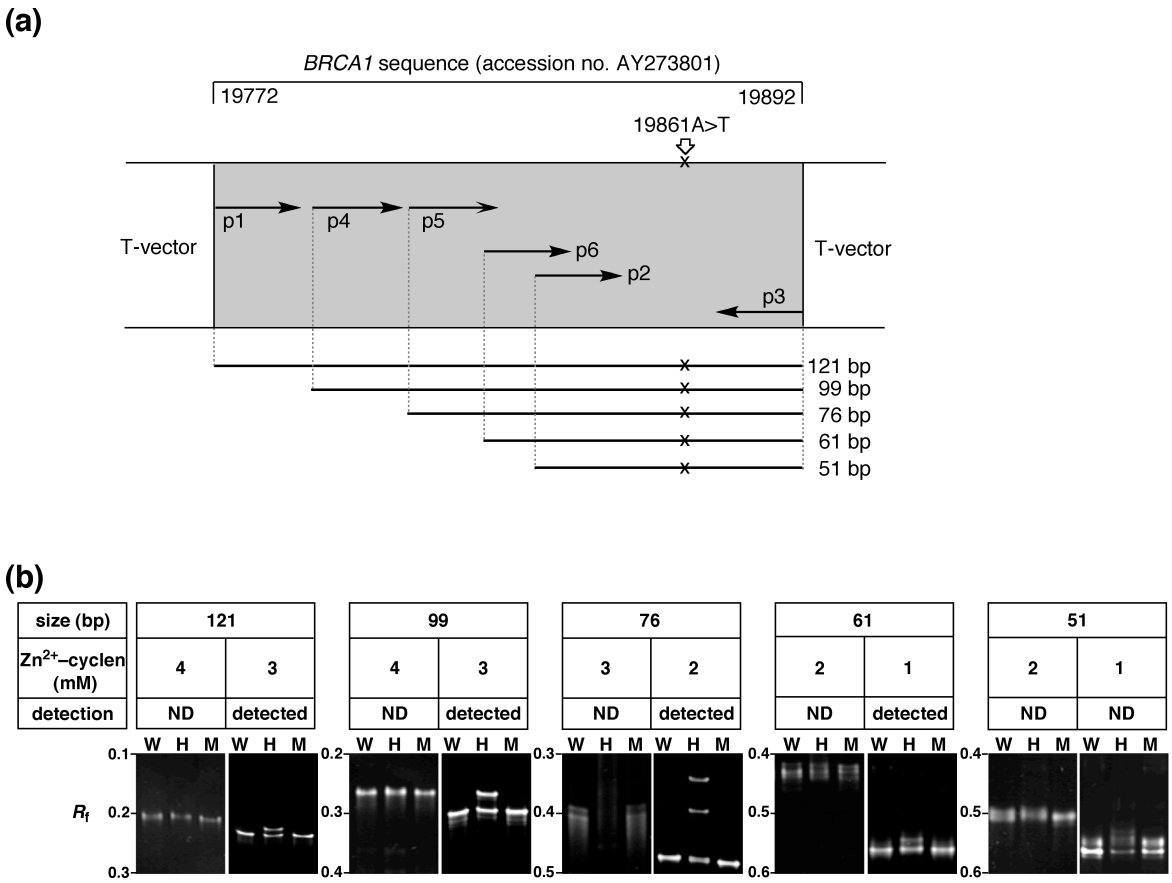
p3:
5'-AAGAAAGGGCCTTCACAGTG

mutagenesis primer:
5'-AAATTATATACCTTTTGGTTATATCATTC
TT*A*CATAAAGGACACTGTGAAGGCCCTT

Supplementary Fig. S1. (a) Schematic representation of the *BRCA1* sequence (GenBank accession no. AY273801, 19772 to 19892, 121 bp) cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). Each arrow shows the location of the PCR primers used. The lengths of DNA fragments amplified by each PCR were shown together with the contents of Ade/Thy bases (%, AT). The 121-bp sequence was amplified from human genome DNA (BD Biosciences, Franklin Lakes, NJ, USA) by using a pair of primers p1 and P3. The PCR product was cloned into a pGEM-T Easy Vector, and the resultant plasmid was named 'wild-type BRCA1'. PCR-based site-directed mutagenesis at nucleotide position 19861 (19861A>T) was performed using mutagenesis primer, p1, and the wild-type BRCA1 plasmid as a template DNA. The resultant PCR product was cloned into pGEM-T Easy Vector to give a plasmid that we named 'mutant BRCA1'. The sequences that were cloned were checked by using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The short Ade/Thy-rich DNA fragments targeted for analysis by Zn^{2+}-cyclen–PAGE were prepared by PCR using the wild-type and mutant BRCA1 plasmids as templates. The 51-bp region in the cloned sequence (19842–19892) was amplified by using a pair of primers p2 and p3. The Gua/Cyt-attached DNA fragment (61 bp) was amplified by using a pair of primers p2+10GC and p3. The concentration of each PCR product was adjusted to 20 ng/μl. Bold line indicates a Gua/Cyt-lined sequence (10 bases) attached at the 5'-end of the p2 primer (p2+10GC). (b) The sequences of p1, p2, p2+10GC, p3, and mutagenesis primer are shown. The underlined Gua/Cyt-lined sequence (10 bases) was attached. The nucleotide base (arrowed) written as an italic small capital in the mutagenesis primer indicates the single-substituted position 19861 in the *BRCA1* gene.

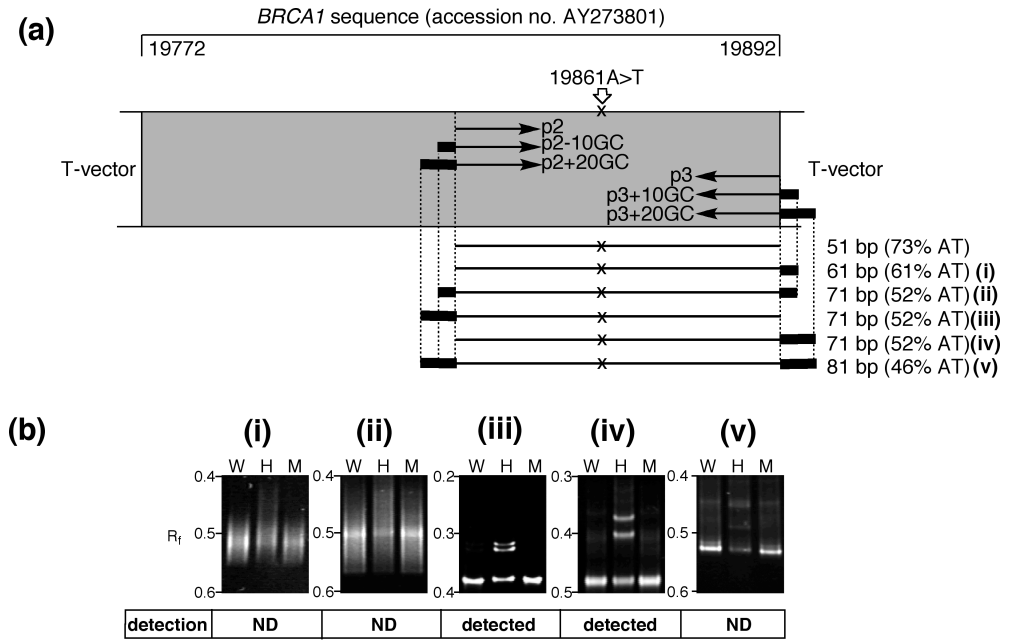
Supplementary Figure S2



Supplementary Fig. S2. (a) Schematic representation of the *BRCA1* (GenBank accession no. AY273801, 19772 to 19892) sequence cloned into the pGEM-T Easy Vector, showing the location of the PCR primers used in this experiment and the lengths of DNA fragments amplified by PCR. For the analysis of the mutation of 19861A>T by the Zn^{2+}-cyclen–PAGE procedure, five Ade/Thy-rich DNA fragments of 121 bp (containing 71% Ade/Thy bases), 99 bp (69%), 76 bp (67%), 61 bp (69%), and 51 bp (73%), respectively, were amplified separately from the wild-type BRCA1 and mutant BRCA1 plasmids described above by using pairs consisting of primers p1 and p3, p4 and p3, p5 and p3, p6 and p3, and p2 and p3, respectively. And then, we prepared sets (W, H, and M) of the five variously sized DNA fragments. The sequences of p1, p2, and p3 are shown in Supplementary Fig. S1b. The sequences of p4, p5, and p6 are shown in Supplementary Table S1. (b) Analyses with lower concentrations of Zn^{2+}-cyclen. Each sample set (W, H, and M) of the five variously sized

DNA fragments (121, 99, 76, 61, and 51 bp) was applied to a 15% (w/v) polyacrylamide gel containing 4.0–1.0 mM Zn^{2+}-cyclen. The DNA size (bp), the concentration of Zn^{2+}-cyclen (mM), and the results of mutation detection are shown above each panel. ND = not detected. Although we first subjected each set of the five variously sized DNA fragments to 5 mM Zn^{2+}-cyclen–PAGE that is a standard concentration procedure described previously [5–7], we were unable to observe typical heteroduplex bands in the hetero samples (data not shown); all the DNA fragments were detected as smeared and complicated multiple bands. The resultant migration images were caused by complete or partial dissociation of the short Ade/Thy-rich DNA fragments through binding of Zn^{2+}-cyclen to the Thy bases. Subsequently, we examined the effects of reducing the concentration of Zn^{2+}-cyclen (4, 3, 2, or 1 mM) on the analysis of the mutation by using Zn^{2+}-cyclen–PAGE with the same sample sets. In the 3 mM Zn^{2+}-cyclen–PAGE procedure for the 121-bp and 99-bp DNA samples, separation of heteroduplexes (slower-migrating bands) and the corresponding homoduplexes (faster-migrating bands) of the hetero samples (see lanes H) was clearly observed. We also detected the presence of heteroduplex bands with 2 mM Zn^{2+}-cyclen for the 76-bp DNA sample, and with 1 mM Zn^{2+}-cyclen for the 61-bp sample. For the 51-bp DNA fragment sample set, however, multiple bands were observed in all lanes, even with 1 mM Zn^{2+}-cyclen. These results show that the use of lower concentrations of Zn^{2+}-cyclen has limitations for the analysis of mutation in short Ade/Thy-rich DNA fragments. The R_f value was defined as the ratio of the migration distance to the length of the gel slab.

Supplementary Figure S3



(c) p2+20GC:
5'-CCCGCGCGCCCGCCCCCGCCCTTCACAGTGTCCTTTAT

p3+10GC:
5'-CGCCCGCCGCAAATTATATACCTTTTGGTT

p3+20GC:
5'-CGCCCGCCGCGCCCCGCGCCAAATTATATACCTTTTGGTT

Supplementary Fig. S3. (a) Schematic representation of the *BRCA1* (GenBank accession no. AY273801, 19772 to 19892) sequence cloned into the pGEM-T Easy Vector, showing the location of the PCR primers used in this experiment and the lengths of DNA fragments amplified by PCR (i-v), together with the contents of Ade/Thy bases (%, AT). Bold lines indicate the 10-bp and 20-bp Gua/Cyt-lined sequences attached to the 5'-end of each primer. (b) Analysis of mutations in five kinds of DNA fragment sample sets (W, H, and M) by using the Zn^{2+}-cyclen–PAGE procedure on 15% (w/v) polyacrylamide gel containing 3 mM Zn^{2+}-cyclen (i–v). The results for mutation detection are shown above each panel. ND = not detected. The R_f value was defined as the ratio of the migration distance to the length of the gel slab. (c) The sequences of p2+10GC, p3+10GC, and p3+20GC are shown. The underlined Gua/Cyt-lined sequence was attached.

Supplementary Table S1. Sequences of PCR primers used in this practical study

Primer	Sequence (5'–3')
p4	TAATTTATAGATTTTGCATG
p5	AAACTTCTCAACCAGAA
p5+10GC	CCCGCGCGCCAAACTTCTCAACCAGAA
p6	AAGAAAGGGCCTTCACAGTG
p7	CATCATTACCAAATTATATA

The underlined Gua/Cyt-lined sequence (10 bases) was attached to the 5'-end of primers p5 to increase the T_m of the resultant PCR amplicon.