

A Method for HLA Genotyping Using the Specific Cleavage of DNA-rN₁-DNA/DNA with RNase HII from *Chlamydia pneumoniae*

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ABSTRACT

Single nucleotide polymorphisms (SNPs) provide a great opportunity for the study of human disease and bacterial drug resistance. However, many SNP typing techniques require dedicated instruments and high cost. Here, we develop a novel method for SNP genotyping based on specific cleavage properties of RNase HII from *Chlamydia pneumoniae* (CpRNase HII), termed the “CpRNase HII-based method.” CpRNase HII cleaves the DNA-rN₁-DNA/DNA duplex at the 5'-side of the ribonucleotide (rN₁ = one ribonucleotide). Moreover, the cleavage efficiencies of the perfectly matched DNA-rN₁-DNA/DNA duplexes are higher than those carrying a mismatched ribonucleotide. DNA-rN₁-DNA fragments are modified with a fluorophore at the 5'-end and a quencher at the 3'-end to generate molecular beacons (MBs), which hybridize with single-stranded DNA (analyte) to be cleaved by CpRNase HII. As perfectly matched duplexes can be cleaved efficiently and mismatched duplexes cannot, CpRNase HII-catalyzed reactions can differentiate between one-nucleotide variations on the DNA-rN₁-DNA/DNA duplexes. We have validated this method with nine SNPs of the HLA gene, which were successfully determined by endpoint measurements of fluorescence intensity. The new method is simple and effective, because the design of MBs is easy, and all steps of the genotyping consist of simple additions of solutions and incubation. This method will be suitable for large-scale genotyping.

INTRODUCTION

MANY SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) in genomes are usually related with human disease and bacterial drug resistance (Brookes, 1999). Up to date, various approaches of SNP genotyping have been reported including allele-specific hybridization (Prince et al., 2001), allele-specific extension (Ahmadian et al., 2001), allele-specific ligation (Khanna et al., 1999), and enzyme cleavage-based methods (Myers et al., 1985; Oleykowski et al., 1998; Krebs et al., 2003; Till et al., 2004; Ichinose et al., 2005; Yeung et al., 2005).

Nucleases are important and useful analytical enzymes, which have been used in a series of application studies of SNP discovery and genotyping (Myers et al.,

1985; Oleykowski et al., 1998; Krebs et al., 2003; Till et al., 2004; Ichinose et al., 2005; Yeung et al., 2005). Among a number of nuclease-based techniques, the ribonuclease-based technique is one of the most attractive approaches in which ribonucleases can recognize and cleave single base mismatch in an RNA/DNA and RNA/RNA target but not cleave the perfect matched RNA/DNA and RNA/RNA target (Myers et al., 1985; Krebs et al., 2003; Ichinose et al., 2005). However, only a part of mismatches are cleaved by RNaseA, RNase1, or RNaseT, respectively, in the standard digestion buffer (Goldrick, 2001), which is not beneficial for them to genotype SNP.

Ribonuclease H (RNase H) is a ribonucleotide-specific endonuclease that specifically degrades the RNA strand

of the RNA/DNA heteroduplex (Ohtani et al., 1999a). Based on the difference in the amino acid sequence and biochemical properties, RNase H is classified into two major families: type 1 and type 2 RNase H (Ohtani et al., 1999a, 1999b). It is believed that type 2 RNase H, which includes bacterial RNase HII and HIII, archaeal RNase HII, and eukaryotic RNase H2, is required for the removal of RNA primers from Okazaki fragments (Kanaya and Ikehara, 1995; Sato et al., 2003).

It has been reported that *Bacillus subtilis* RNase HII and *Thermococcus kodakaraensis* RNase HII can cleave DNA-rN₁-DNA/DNA duplexes at the 5'-side of the single ribonucleotide. In contrast, *Escherichia coli* RNase HI and *B. subtilis* RNase HIII cannot cleave these duplexes (Hogrefe et al., 1990; Haruki et al., 2002). Two type 2 RNase Hs, RNase HII, and HIII from *Chlamydia pneumoniae* (CpRNase HII and CpRNase HIII), were successfully expressed and characterized previously (Pei et al., 2005). Recently, it was demonstrated that CpRNase HII, like those from *B. subtilis* and *Thermococcus kodakaraensis*, could cleave the DNA-rN₁-DNA/DNA duplex efficiently. Moreover, the cleavage rates of CpRNase HII were negatively affected by mismatches carried by DNA-rN₁-DNA/DNA duplex (Hou et al., 2007). Here we present a novel strategy for SNP genotyping based on CpRNase HII-catalyzed reactions. We propose that the cleavage properties of CpRNase HII would facilitate the development of SNP genotyping.

MATERIALS AND METHODS

Materials

Molecular beacons (MBs) were designed using the mfold DNA folding program (Zuker, 2003). MBs,

oligonucleotides, T4-polynucleotide kinase, and ribonuclease inhibitor were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). CpRNase HII was prepared as Pei et al. (2005). λ -Exonuclease and *Taq* DNA polymerase were purchased from Gene Co., Ltd. (Shanghai, China). Human genomic DNA samples were independently extracted from blood of healthy Chinese individuals who were enrolled in our study after informed consent, following the Helsinki declaration.

Preparation of MB/oligonucleotide duplexes for CpRNase HII cleavage

MBs, which were labeled with FAM at the 5'-end and Dabcyl at the 3'-end, were the oligonucleotides containing one ribonucleotide at the middle of the sequences (Table 1). Oligo-T(21), Oligo-T(19), Oligo-T(17), Oligo-T(15), and Oligo-T(13) were incubated with MB-ra, respectively, at 37°C for 5 minutes to generate the substrates of CpRNase HII, which contained different lengths of base pairs. Sixteen kinds of cleavage substrates, containing different kinds of base pairs at the position of ribonucleotide, were prepared by incubating one kind of MB-ra, rc, rg, or ru with different amounts of one kind of oligo-A, C, G, or T(15), respectively, at 37°C for 5 minutes to generate the duplexes being cleaved by CpRNase HII.

Real samples for SNP genotyping preparation by PCR and λ -exonuclease digestion

Human genomic DNA extracted from blood of 10 unrelated individuals was used in the analysis. PCR was used to obtain abundant amount of target DNA. Primers of PCR were listed in Table 2, in which the forward primers were phosphorylated at the 5'-end by T4-polynu-

TABLE 1. OLIGONUCLEOTIDES USED FOR CpRNASE HII CLEAVAGE REACTION

Oligonucleotides ^a	Nucleotide sequences (5'-3')
MB-ra	(FAM)-CGCGATGCTGCAGGAA a TCGATATCAAATCGCG-(Dabcyl)
Oligo-T(21)	TTGATATCGATTTCTCCTGCAGC
Oligo-T(19)	TGATATCGATTTCTCCTGCAG
Oligo-T(17)	GATATCGATTTCTCCTGCA
Oligo-T(15)	ATATCGATTTCTCCTGC
Oligo-T(13)	TATCGATTTCTCCTG
MB-rc	(FAM)-CGCGATGCTGCAGGAA c TCGATATCAAATCGCG-(Dabcyl)
Oligo-G	ATATCGAGTTCTCCTGC
MB-rg	(FAM)-CGCGATGCTGCAGGAA g TCGATATCAAATCGCG-(Dabcyl)
Oligo-C	ATATCGACTTCTCCTGC
MB-ru	(FAM)-CGCGATGCTGCAGGAA u TCGATATCAAATCGCG-(Dabcyl)
Oligo-A	ATATCGAATTTCTCCTGC

^aDeoxyribonucleotides are shown by capital letters, and ribonucleotides are in bold and shown by small letters.

TABLE 2. PRIMERS FOR PCR AMPLIFICATION OF HUMAN DNA

Primers ^a	Nucleotide sequences (5'–3')	Position in HLA gene ^b (length)
F-A	GGCTACGTGGACGACACGCAG	278–978 (701 bp)
R-A	GCGTCTCCTTCCCGTTCTCCAG	
F-B	GCTACTACAACCAGAGCGAGGC	
R-B(1)	CGGGGATTTTGGCCTCAACTG	448–656 (209 bp)
R-B(2)	GGAAACTCATGCCATTCTCCAT	448–1125 (678 bp)
R-B(3)	GAACACTTCTACCTGGGGCTTG	448–2127 (1680 bp)

^aF indicates forward primer, and R indicates reverse primer. A or B represents HLA-A or B, respectively.

^bIndicates the amplified regions of HLA-A or B by PCR, respectively.

cleotide kinase. PCR was performed in a 25- μ L volume containing 100 ng human DNA, 10 mM Tris–HCl, 50 mM KCl, 2 mM MgCl₂, 200 μ M each of dNTP, and 2.5 U of *Taq* DNA polymerase. The PCR protocol consisted of a 5-minute heat step at 95°C, followed by 30 cycles of denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 1 to 2 minutes, with a final 5-minute extension step at 72°C. The PCR fragment was purified and incubated with λ -exonuclease at 37°C to obtain target ssDNA.

Substrates for HLA typing

For each selected SNP site, a group of allele-discriminating DNA-rN₁-DNA MBs was designed as described below (Table 3). These MBs contained 15 nucleotides

complementary to the corresponding SNP target sequence. One single ribonucleotide at the middle of the MBs was designed as the polymorphic sequence. The substrates for HLA typing were generated by incubating MBs (Table 3) with target ssDNAs at 37°C for 5 minutes.

CpRNase HII-catalyzed reactions

CpRNase HII reaction solution (100 μ L) contained 10 mM Tris–HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl₂, 1 mM β -ME, 10 μ g/mL bovine serum albumin, 2 U ribonuclease inhibitor, a special amount of substrates, and a special amount of CpRNase HII (100 ng \sim 1.8 \times 10⁻³ units) (Pei et al., 2005). The reaction mixture was incubated at 37°C in a Fluoroskan Ascent FL & Fluoroskan Ascent Type 374 (Thermo LabSystem, Finland) and fluo-

TABLE 5. COMPARISON BETWEEN CpRNase HII-BASED METHODS AND OTHER ENZYME CLEAVAGE-BASED METHODS

	RNase ^e	DNA endonuclease ^f	Muts-exonuclease ^g	CpRNase HII
Accuracy ^a	high	moderate	high	high
Throughput ^b	high	high	—	—
Simplicity of workflow ^c	moderate	simple	moderate	simple
Cost-effectiveness ^d	moderate	high	moderate	moderate

^aRefers to the specificity of the method for SNP genotyping.

^bRefers to the commercial support available.

^cRefers to the complexity of manipulation and the stress level of day operation required in a SNP genotyping assay. For example, a “simple” assay is one in which all steps of the assay consist of simple additions of solutions and incubation, and the detection steps have a short learning curve.

^dRefers to the preparation of all of the solutions and components in the assay. For example, a “high” assay is one in which all of the solutions and components can be produced easily and reliably.

^eRefers to the articles from Myers et al. (1985), Prescott et al. (1999), Taylor et al. (1999), Faudoa et al. (2000), Goldrick (2001), Krebs et al. (2003), and Ichinose et al. (2005).

^fRefers to the articles from Oleykowski et al. (1998), Howard et al. (1999), Taylor et al. (1999), Till et al. (2004), and Yeung et al. (2005).

^gRefers to the articles from Ellis et al. (1994), Smith and Modrich (1996), and Taylor (1999).

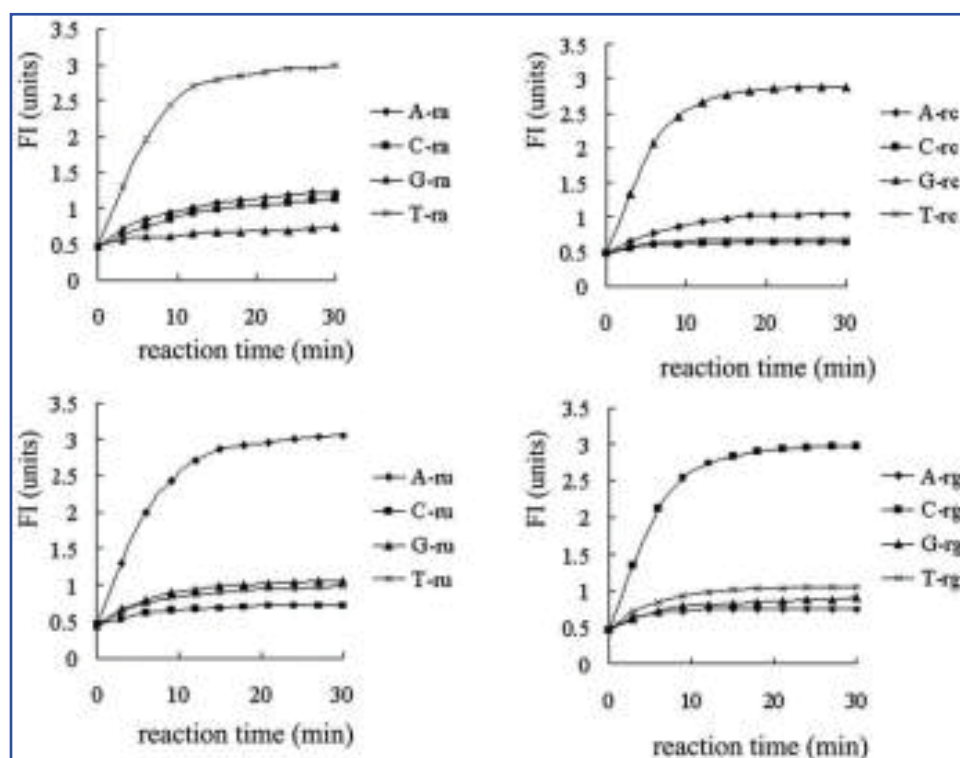


FIG. 1. Real-time fluorescence intensity of CpRNase HIII-catalyzed cleavage of DNA-rN₁-DNA/DNA duplexes. The substrates, which were shown with the base pair of deoxyribonucleotide to ribonucleotide in the diagram, respectively, were the mixture of all possible combinations of oligo-A, C, G, or T(15) and equimolar MB-ra, rc, rg, or ru. FI (units) represents the fluorescence intensity of the reaction mixture. One hundred microliters of reaction solutions, containing 0.1 μ M substrates and 100 ng CpRNase HIII, were incubated at 37°C in Fluoroskan Ascent FL & Fluoroskan Ascent Type374 for real-time assay.

rescence was monitored at time points ranging from 3 to 30 minutes. The excitation was set at 485 nm and emission was set at 527 nm.

Match/mismatch signal ratio means the ratio of fluorescence intensity change of matched duplex to the mismatched duplex. The fluorescence intensity changes were obtained according to the formula: $\Delta F = F_t - F_0$, where F_t is the fluorescence intensity of reaction mixture after CpRNase HIII cleavage and F_0 represents fluorescence intensity at the start of the reaction. The signal-to-background represents the ratio of F_t of matched duplex to F_0 .

RESULTS

The effect of oligonucleotides size on the structure of MBs in the reaction mixture for CpRNase HIII cleavage

In order to measure the effect of ssDNAs, which hybridize with the loop of MBs, on the stem-loop structure of MBs, 0.1 μ M of five oligonucleotides, oligo-T(13), oligo-T(15), oligo-T(17), oligo-T(19), and oligo-T(21), were mixed with 0.1 μ M MB-ra, respectively (Table 1). After incubation at 37°C for 30 minutes, the fluorescence

intensity of the mixture increased 0.015, 0.068, 0.1917, 0.3722, and 0.4256 units, respectively. In general, an MB is a single-stranded nucleic acid chain that forms a stem-loop structure. In this configuration, the fluorescence is inhibited by the proximity of the fluorophore to the quencher (Tyagi and Kramer, 1996). At the presence of the target sequence, the MB undergoes a structural reorganization by hybridization of the loop with the target sequence leading to the formation of a double-strand helix and the spatial separation of the fluorophore and the quencher (Tyagi and Kramer, 1996). That is why the fluorescence intensity increases evidently when oligo-T(21) is incubated with MB-ra. However, the fluorescence intensity of mixture generated by MB-ra with oligo-T(13) or oligo-T(15) increases a little, indicating that oligo-T(13) or oligo-T(15) cannot destroy the stem-loop structure of MBs by hybridization compared with the other measured oligonucleotides.

The MB-ra was cleaved by CpRNase HIII at 37°C for 30 minutes in the presence of oligo-T(13) or oligo-T(15). 0.575 units, and 2.421 units of fluorescence intensity were generated, respectively. These results indicate that MB-ra/oligo-T(13) and MB-ra/oligo-T(15) can be cleaved by CpRNase HIII. Furthermore, MB-ra/oligo-T(15) is the better substrate of CpRNase HIII, compared

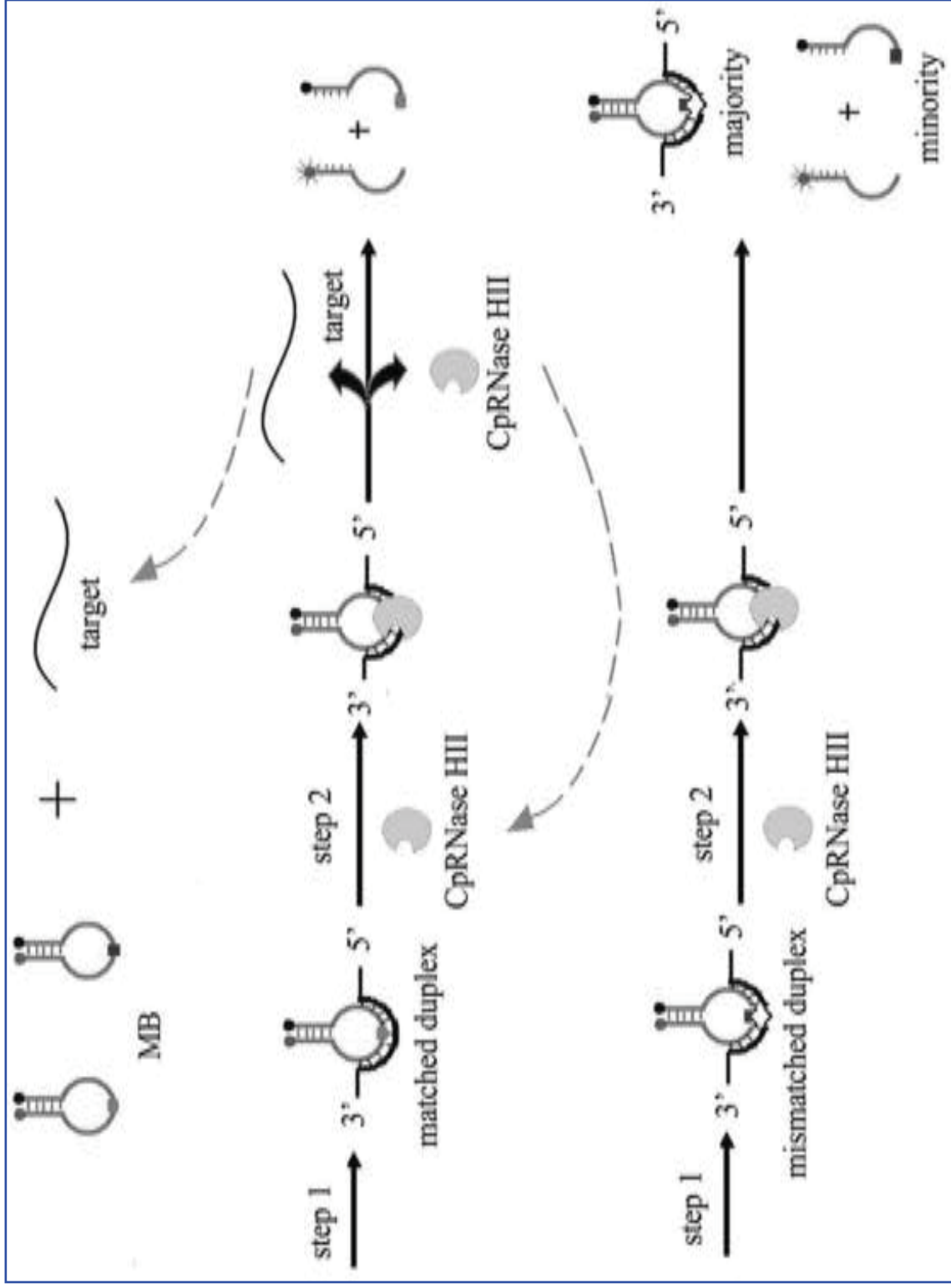


FIG. 2. Schematic diagram of CpRNase HIII-based method in genotyping. MBs are incubated with target ssDNA to generate the matched or mismatched duplexes (step 1). CpRNase HIII binds to the duplexes and cleaves the perfectly matched ones to produce two short fragments, while mismatched duplexes are not or little cleaved by CpRNase HIII (step 2). After the first cycle of cleavage, the target ssDNAs, which are released from the cleavage complex, hybridizes with other MBs to generate the new duplexes to be cleaved. The dots in the middle of the MBs represent the ribonucleotide.

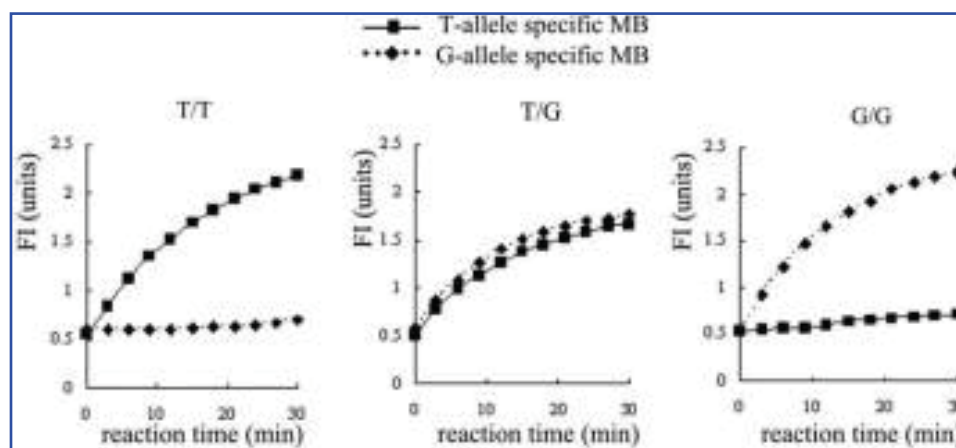


FIG. 3. Validation of the method on genotyping. Real-time fluorescence intensity profiles for the three known genotypes (T/T, G/G, and T/G) of the HLA-B gene (678 nt). FI (units) represents the fluorescence intensity of the reaction mixture. One hundred microliters of reaction solutions, containing 0.1 μ M substrate (equal mols of MB and ssDNA) and 100 ng CpRNase HII, were incubated at 37°C in Fluoroskan Ascent FL & Fluoroskan Ascent Type374 for real-time assay.

with MB-ra/oligo-T(13). Thus, the 15-nt oligonucleotides or ssDNAs that have 15-nt complementary with the loop of MBs were chosen to hybridize with MBs to form substrates of CpRNase HII. The reason why MB-ra/oligo-T(13) cannot be cleaved effectively may be that the oligo-T(13) could not effectively hybridize with MB-ra to form the stable duplex under the assay conditions.

Only perfectly matched duplexes were cleaved efficiently by CpRNase HII

Four DNA-rN₁-DNA MBs were incubated with the equal mole of corresponding oligonucleotides, respectively, to generate 16 kinds of MB/oligo duplexes (Table 1). Only perfectly matched duplexes, MB-ra/oligo-T(15), MB-rc/oligo-G, MB-rg/oligo-C, and MB-ru/oligo-A, could be cleaved by CpRNase HII efficiently (Fig. 1). All duplexes carrying a mismatched ribonucleotide were cleaved poorly (Fig. 1).

From real-time fluorescence intensity shown in Figure 1, two conclusions could be deduced: (1) the cleavage kinetics was almost same for all perfectly matched duplexes, indicating that the cleavage of CpRNase HII on DNA-rN₁-DNA/DNA duplexes had no base preference; (2) fluorescent intensity changes of matched duplexes were at least 3.6 times compared with those mismatched duplexes after the reaction solutions were incubated at 37°C for 30 minutes. These observations offer us the possibility to apply CpRNase HII-catalyzed reactions for SNP genotyping.

SNP genotyping strategy by CpRNase HII-based method

CpRNase HII-based method is designed to genotype SNP by specific cleavage of CpRNase HII on the DNA-rN₁-DNA/DNA duplexes (Fig. 2). The target ssDNAs

surrounding the target SNP are prepared by PCR amplification and λ -exonuclease digestion. DNA-rN₁-DNA MBs for each SNP are mixed with the target ssDNA individually to generate the substrates of CpRNase HII. The ribonucleotide of each MB is aligned with the polymorphic base. Therefore, if MBs are matched with the target DNA, MBs are cleaved by CpRNase HII to generate fluorescence. After the first cycle of cleavage, the excessive MBs would hybridize with the released target ssDNA

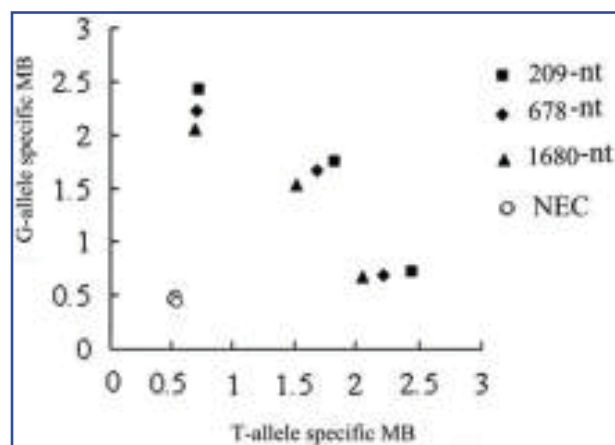


FIG. 4. Effect of template size on genotyping. Scatter plot of the endpoint fluorescence intensity for the three known genotypes (T/T, G/G, and T/G) of the HLA-B gene obtained with 209-, 678-, and 1680-nt ssDNA as a template. The x and y axis denote the fluorescence intensity (units) of the two artificial MBs in the reaction, respectively. NEC represents no enzyme (CpRNase HII) control. One hundred microliters of reaction solutions, containing 0.1 μ M substrates (equal mols of MBs and ssDNAs) and 100 ng CpRNase HII, were incubated at 37°C for 30 minutes and measured with Fluoroskan Ascent FL & Fluoroskan Ascent Type374.

TABLE 3. MBs USED FOR HLA TYPING

MBs ^a	Nucleotide sequences (5'-3') ^b	Predicated ΔG^c
A370g	(FAM)-CGCGATAATTAGGAGG g CCGGAGTTCAATCGCG-(Dabcyl)	-3.61
A370u	(FAM)-CGCGATAATTAGGAGG u CCGGAGTTCAATCGCG-(Dabcyl)	
A824c	(FAM)-CGCGATAATCCCTGAA c GAGGACCTCAATCGCG-(Dabcyl)	-3.75
A824a	(FAM)-CGCGATAATCCCTGAA a GAGGACCTCAATCGCG-(Dabcyl)	
A860a	(FAM)-CGCGATAATTACATGGC a GCTCAGACTCATCGCG-(Dabcyl)	-3.41
A860g	(FAM)-CGCGATAATTACATGGC g GCTCAGACTCATCGCG-(Dabcyl)	
B521u	(FAM)-CGCGATTCTCCCCACG u ACGGCCCCCTCATCGCG(Dabcyl)	-3.31
B521g	(FAM)-CGCGATTCTCCCCACG g ACGGCCCCCTCATCGCG(Dabcyl)	
B836a	(FAM)-CGCGATAATGGACCTG a GCTCCTGCTAATCGCG-(Dabcyl)	-3.61
B836c	(FAM)-CGCGATAATGGACCTG c GCTCCTGCTAATCGCG-(Dabcyl)	
B900a	(FAM)-CGCGATGTAGCCCGT a GGCGGAGATTATCGCG-(Dabcyl)	-5.24
B900u	(FAM)-CGCGATGTAGCCCGT u GGCGGAGATTATCGCG-(Dabcyl)	
B1583c	(FAM)-CGCGATAACAGACACA c GTGACCCATAATCGCG-(Dabcyl)	-3.61
B1583u	(FAM)-CGCGATAACAGACACA u GTGACCCATAATCGCG-(Dabcyl)	
B1871c	(FAM)-CGCGATAACCCATATCT c TTCTCAGTAAATCGCG-(Dabcyl)	-3.61
B1871g	(FAM)-CGCGATAACCCATATCT g TTCTCAGTAAATCGCG-(Dabcyl)	
B1940a	(FAM)-CGCGATAATCAGAGCC a TCTTCCCCTAATCGCG-(Dabcyl)	-3.61
B1940g	(FAM)-CGCGATAATCAGAGCC g TCTTCCCCTAATCGCG-(Dabcyl)	

^aThe first capital letter represents the type of HLA. The last small letter represents the ribonucleotide carried by the MB. The number between two letters represents the position of nucleotide typed in HLA-A or -B, respectively.

^bThe italic portions of the sequences indicate the random sequences that are not complementary with the target DNA. The polymorphic sites in each probe were in bold and underlined.

^cGenerated by mfold for most stable secondary structure in 50 mM NaCl and 10 mM MgCl₂ at 37°C.

from the cleavage complex and initiate the second cycle cleavage by CpRNase HII. Thus, further fluorescence is released from the reaction mixture. On the other hand, if MBs are mismatched with the target, MBs are not cleaved by CpRNase HII effectively, leading to the invalid release of target ssDNA and preventing further cleavage of excessive MBs in subsequent cycles of reaction as well as the accumulation of the fluorescent signal.

Validation of the method

The new method was examined with the 678-nt ssDNA of the HLA-B gene, which contained a T521G polymorphism (T/T, T/G, and G/G). The DNA fragment was prepared from genomic DNA by PCR and digestion of λ -exonuclease, and then subjected to the CpRNase HII-cleavage reaction with the T or G allele-specific MBs (Table 3). Figure 3 shows the real-time fluorescence intensity profiles of the three known genotypes in the 678-nt fragment. The three genotypes gave clearly different profiles, with the two specific MBs recognizing the T and G alleles. For example, the G/G homozygous sample showed a progressive rise in fluorescence with the G allele-specific MB, whereas no or little fluorescence change was observed with the T allele-specific MB. This indicates that the three genotypes can be clearly distinguished from one another by measuring the

intensity of the fluorescence at the endpoint of the reaction.

Effect of template size

The effect of template size on SNP genotyping was examined with 209-, 678-, and 1680-nt fragments of the HLA-B gene, which contained a T521G polymorphism. The fluorescence intensities from CpRNase HII-cleavage reaction with the G and T allele-specific MBs were plotted on the x-axis and the y-axis, respectively (Fig. 4). The three genotypes were discriminated as clusters on the graph, and the two negative controls were separated from them. From these data, the genotypes were easily distinguishable with 209-, 678-, and 1680-nt fragments. In subsequent experiments, we used target ssDNAs that are from 701 to 1680 nt for SNP genotyping.

Effect of excessive MBs

We used equal or 5-, 10-, 15-, 20-, 30-, and 50-fold mole MBs of B521u/g, respectively, to discriminate a T521G polymorphism of 678-nt ssDNA (1 pmol) of the HLA-B gene at 100 μ L reaction solutions. The fluorescence intensity changes increased with the increased MBs (Table 4). Furthermore, the match/mismatch signal ratios were greater than 12 when more than 10-fold MBs

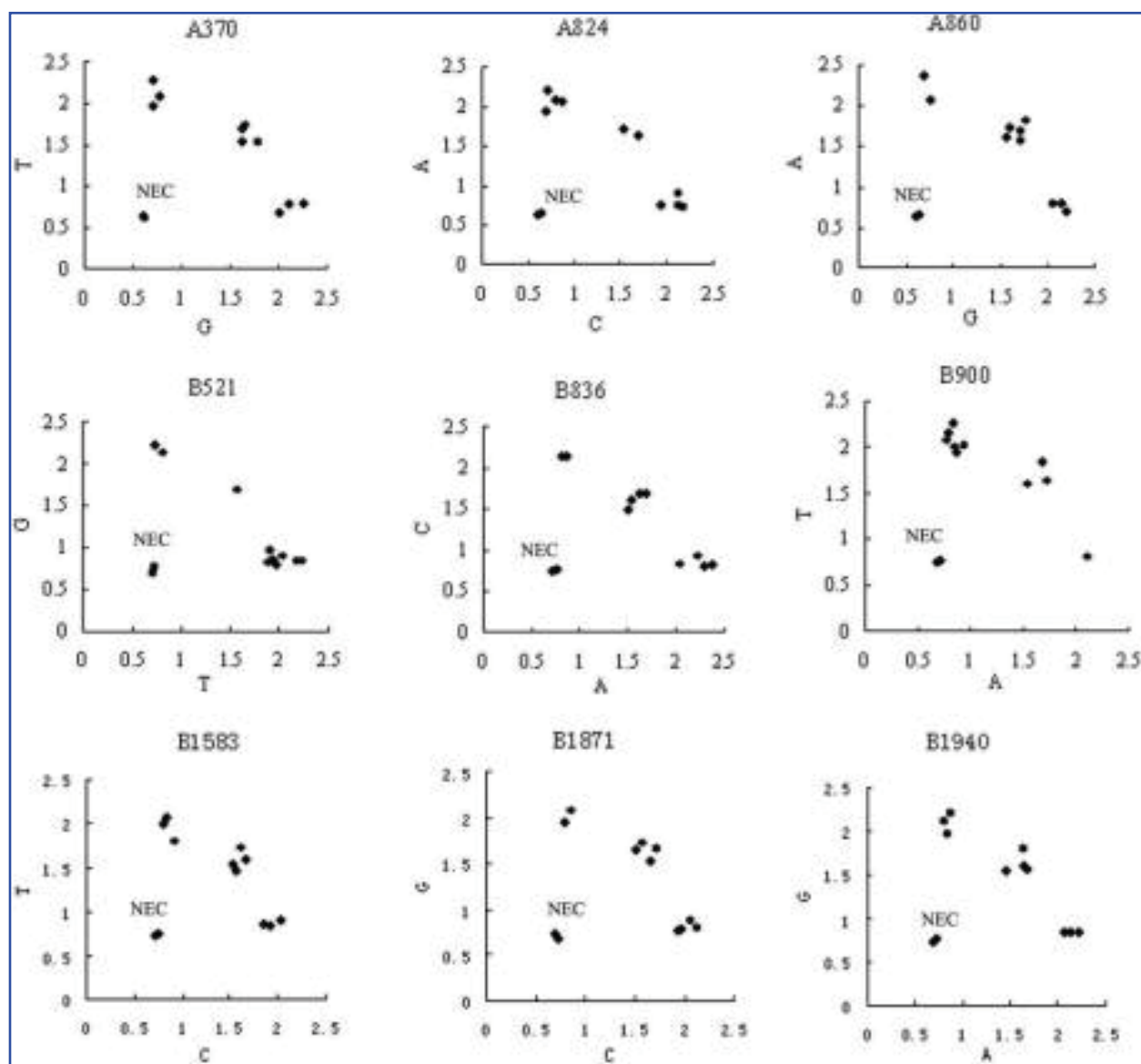


FIG. 5. Results from HLA genotyping using the CpRNase HII-based method. Scatter plots showing the genotype assignment at nine SNP sites of HLA gene from known SNP carriers. The x and y axis denote the fluorescence intensity (units) of the two artificial MBs in the reaction, respectively. NEC represents no enzyme (CpRNase HII) control.

were added in the experiments. These data indicate that both fluorescence intensity changes and match/mismatch signal ratios are amplified with increased MBs at the presence of the same amount of target ssDNA, which make SNP genotyping more simple and reliable.

SNP genotyping

The new method was used to type nine different SNPs (Table 3), which were selected to represent various types of single nucleotide substitutions, that is, A/G, C/T, G/C, G/T, and A/C. The genotypes were determined by our method with the two allele-specific MBs shown in Table 3. A scatter plot was shown in Figure 5. In this figure, the

fluorescence intensities generated by two allele-specific MBs were plotted on the x - and y -axis, respectively. For each SNP, homozygous and heterozygous SNP genotypes were discriminated as clusters on the graph, and the two negative controls were separated from them. Thus, the three genotypes of all SNPs were clearly distinguishable. All genotypes were determined independently by sequencing to verify the results of our method.

DISCUSSION

CpRNase HII-based method was designed to type SNP. CpRNase HII does not show preference to the types

TABLE 4. THE FLUORESCENCE INTENSITY CHANGES IN THE PRESENCE OF DIFFERENT FOLDS OF MBs

	Folds ^b	ΔF^a						
		1	5	10	15	20	30	50
MBs	B521u	0.124	0.403	0.846	1.325	1.773	2.349	2.716
	B521g	0.021	0.048	0.070	0.098	0.127	0.164	0.187
Match/mismatch signal ratio ^c		5.9	8.4	12.1	13.5	14.0	14.3	14.5

0.01 μM –0.5 μM B521u or B521g were mixed with 678-nt ssDNA (0.01 μM), obtained from one sample of human genomic DNA, respectively, to be cleaved by 100 ng CpRNase HII at 37°C for 30 minutes and measured with Fluoroskan Ascent FL & Fluoroskan Ascent Type 374.

^aIndicates the fluorescence intensity change of the reaction mixture formed by MB with target DNA in the presence of CpRNase HII.

^bIndicates the ratio of MB to target ssDNA.

^cIndicates the ratio of ΔF of B521u/ssDNA to ΔF of B521g/ssDNA.

of the single ribonucleotide in the DNA-rN₁-DNA/DNA duplexes. Moreover, the cleavage efficiencies of CpRNase HII against perfectly matched DNA duplexes exceed those against duplexes containing a mismatched ribonucleotide. Those are the strong molecular basis for SNP genotyping with CpRNase HII-catalyzed reactions.

The excessive MBs are benefit to amplify both the fluorescence intensity changes and the match/mismatch signal ratios, suggesting the practicality of CpRNase HII-based method in SNP genotyping. As shown in Figure 2, the matched MB/ssDNA can be cleaved by CpRNase HII to generate halves of MB, which leave the target free for hybridization to another MB. In this way, further fluorescence is released from the reaction mixture. However, the mismatched MB/ssDNA cannot be cleaved by CpRNase HII effectively, leading to the invalid release of target and preventing further cleavage of excessive MBs in subsequent cycles of reaction. In this manner, the accumulation of the fluorescent signal is acquired after several cycles of cleavage for the matched duplexes, but not for the mismatched duplexes. From the results in genotyping HLA gene, flanking sequence context apparently does not adversely affect the ability of CpRNase HII to type SNP.

In our experience, ssDNAs generated by either heat-denaturing or alkaline-denaturing/neutralization treatment seemed to be poor templates in supporting CpRNase HII-catalyzed reactions. Considering optimization was laborious, we tried to prepare ssDNA with λ -exonuclease digestion instead of those common denaturing strategies. We found that PCR products digested with λ -exonuclease were the excellent target guiding the cleavage of MB by CpRNase HII. The reason for this phenomenon may be that only partial DNA are transformed to ssDNA with those common denaturing strategies but most are ssDNA through the digestion of λ -exonuclease.

TaqMan assay and the common molecular beacon assay based on hybridization are commonly used for SNP

genotyping. These assays are based on the T_m difference between the perfectly matched and mismatched duplexes (Gibson, 2006). The perfectly matched duplex is more stable than mismatched one. However, probes for different SNPs have different T_m s, which is suitable for the differentiation. When performing multiple assays, such as 96-well plates, the temperature suitable for the differentiation of all the SNPs needs to be compatible from one another. It is a challenge to optimize all probes.

In our HLA typing assay, we use CpRNase HII-catalyzed reactions. CpRNase HII cleaves ribonucleotide more efficiently on perfectly matched DNA-rN₁-DNA/DNA duplexes than mismatched ones. Therefore, all samples or sequences could be assayed under same conditions. In other words, multiple assays can be performed under same conditions.

The other advantage of our method over TaqMan or common molecular beacon is the amplification at cleavage level. CpRNase HII-catalyzed cleavage generates halves of the MB, which leaves the target free for hybridization to another MB. In this way, a copy of the DNA target supports multiple copies of MBs cleavage by CpRNase HII.

It is true that the DNA-rN₁-DNA MBs are more expensive than TaqMan probes and common DNA MBs. In China, the price difference is under 10 USD per probe. Considering the novel properties of CpRNase HII-catalyzed reactions, including easy design of probes, uniform conditions for reaction, and signal amplification at detection level (not at target level), it would be worthwhile to spend a little more on probe synthesis.

The CpRNase HII-based method has the advantage over other enzyme cleavage-based methods. Table 5 compares several enzyme cleavage-based methods with the CpRNase HII-based method in terms of genotyping accuracy, throughput, simplicity of workflow, and the cost-effectiveness. CpRNase HII has the ability to detect all kinds of mismatches with high specificity. However,

RNase and muts-exonuclease cleavage-based methods cannot detect all kinds of mismatches (Taylor, 1999; Yeung et al., 2005). Although the DNA endonuclease cleavage-based method can detect all kinds of mismatches, the background of the assay is high (Taylor, 1999; Yeung et al., 2005). The manipulation of the CpRNase HIII-based method is simple and robust because there is no separation, purification, or centrifugation steps. Currently, the assay takes only about 35 minutes after ssDNA are obtained. Multiple assays can be carried out in one 96-well plate, where it is beneficial to increase the throughput of the assay.

The signal-to-background was 5.8–6.5 for four kinds of matched duplexes after 30 minutes of cleavage of CpRNase HIII (Fig. 1). The quenching efficiency of MBs in our experience exceeded 80%, which could meet the requirements for SNP genotyping.

In conclusion, we have developed a new method based on the specific cleavage of CpRNase HIII to type SNP in the HLA gene. The CpRNase HIII-based method offers a platform for easy and accurate SNP analysis, and could potentially be used to develop high-throughput genotyping.

ACKNOWLEDGMENTS

This work was partially supported by the National High Technology Research and Development Program of China (Grant No. 2006AA02Z108), the National Basic Research Program of China (Grant No. 2005CB724301), and the National Science Foundation of China (Grant No. 30571012/C011003).

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Received May 13, 2006; accepted in revised form July 6, 2007.

