

TECHNICAL NOTE

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A LDR-PCR Approach for Multiplex Polymorphisms Genotyping of Severely Degraded DNA with Fragment Sizes <100 bp*

ABSTRACT: Reducing amplicon sizes has become a major strategy for analyzing degraded DNA typical of forensic samples. However, amplicon sizes in current mini-short tandem repeat-polymerase chain reaction (PCR) and mini-sequencing assays are still not suitable for analysis of severely degraded DNA. In this study, we present a multiplex typing method that couples ligase detection reaction with PCR that can be used to identify single nucleotide polymorphisms and small-scale insertion/deletions in a sample of severely fragmented DNA. This method adopts thermostable ligation for allele discrimination and subsequent PCR for signal enhancement. In this study, four polymorphic loci were used to assess the ability of this technique to discriminate alleles in an artificially degraded sample of DNA with fragment sizes <100 bp. Our results showed clear allelic discrimination of single or multiple loci, suggesting that this method might aid in the analysis of extremely degraded samples in which allelic drop out of larger fragments is observed.

KEYWORDS: forensic science, DNA typing, DNA degradation, rs17750303, rs2307647, rs2307557, rs17250992, ligase detection reaction, polymerase chain reaction

The breakdown of genomic DNA into small fragments by chemical or physical factors is always a special challenge in the genetic identification of human remains or crime stains (1). One strategy that addresses some of the problems encountered in analyzing degraded DNA is the reduction of polymerase chain reaction (PCR) amplicon size (2). Mini-short tandem repeats (STRs) and single nucleotide polymorphisms (SNPs) are two types of genetic markers that can be analyzed in an amplicon smaller than 150 bp (3). A number of validated mini-STR and SNP assays have been used in casework in which current STR kits failed to achieve complete DNA profiles (4–9). However, these two techniques require an average DNA fragment length of 100–200 bp and may not be applicable to casework with extremely degraded DNA consisting of fragments <100 bp in length.

Ligase detection reaction (LDR) has been used to detect mutations in disease genes in clinical samples (10–13) and provides an elegant technique for polymorphism typing that could be applied to severely degraded DNA. Here we present a multiplex polymorphism typing method that couples LDR with PCR (LDR-PCR). In the LDR portion of the technique, probes that are designed to be complementary to the polymorphism and to the region directly adjacent are ligated when brought into juxtaposition through hybridization with the DNA fragment. In the subsequent PCR step, the ligation products are

amplified using primers that are complementary to the generic priming sites built into the hybridization probes (Fig. 1). In this study, the LDR probes were designed such that only 20–40 bp of intact DNA was required for their hybridization. These techniques were applied to samples of artificially degraded DNA consisting of fragments <100 bp to determine whether each allele of four different polymorphisms could be discerned.

Materials and Methods

Selection of Polymorphic Markers

The polymorphic markers used in this study were selected from the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) and included two single-base nucleotide substitutions and two three-base deletions/insertions (Table 1). These markers were chosen because they met certain empirical criteria including randomness of nucleotides in the target region, low secondary structure of the target sequence, and uniqueness of the target sequence within the whole genome. The exact chromosomal locations were determined using BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

Oligonucleotide Primers and Probes

All oligonucleotide primers and probes were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China) and Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). Primer and probe sequences were given in Tables 2 and 3. The DNA sequences for design work were downloaded from the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>).

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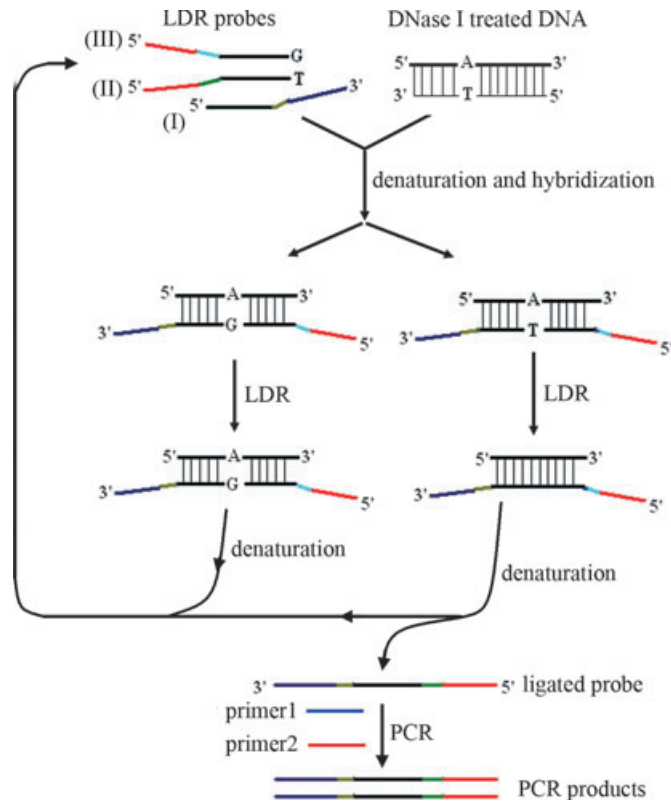


FIG. 1—Schematic representation of LDR-PCR assay. Genotyping of each polymorphism requires three LDR probes, one common (I), and two discriminating (II and III, one for each allele of the polymorphism). The common probe is composed of target-specific sequence complementary to the region adjacent to the polymorphism (shown in black) with 3' downstream flanking PCR primer-specific sequence (shown in blue, marked as primer1). The discriminating probes include target-specific sequence complementary to the allele of the polymorphism (shown in black) and 5' upstream flanking PCR primer-specific sequence (shown in red, marked as primer2). Some nucleotides (shown in green, light blue or brown) are added between the target-specific and the primer-specific regions of each probe so that the ligated products corresponding to each allele are all different in length. In this example, the individual is homozygous for the A-allele, thus only the discriminating probe (II) hybridizes to the template and becomes ligated to the common probe, generating a single type of ligation product. Several cycles of hybridization and ligation are required to generate sufficient ligation product to serve as template in the subsequent PCR step.

TABLE 1—Polymorphic markers studied.

Locus	Chromosome	Chromosome Position	Alleles
rs17750303	16	10011190	A/C
rs2307647	3	192569108...192569110	-/GAA
rs2307557	X	118632543...118632544	-/ACA
rs17250992	Y	8905380	C/T

Preparation of Genomic DNA Samples and Genotyping of the Polymorphic Loci

Twelve anonymous liquid blood samples were provided by the Department of Forensic Serology, China Medical University (Shenyang, China). Genomic DNA was extracted by the standard phenol–chloroform procedure and was quantitated based on UV spectrophotometry (A260). To avoid any possible contamination, all procedures for DNA isolation were carried out in separate rooms using dedicated equipment.

TABLE 2—Sequencing primers of each SNP locus.

Primer	Sequence (5' → 3')
rs17750303	
F	CTAAGCCTTCCTTTATCACC
R	TTACAGTCGTGCTTGTTCCT
rs2307647	
F	ATCGTTCATAAGGAATACAAG
R	TACCTAGAATGAGCATCCAG
rs2307557	
F	GGGAGCCCACTGACTGAAC
R	GTCTCCAGAAAAGGAGGACATC
rs17250992	
F	GATGTAAAGCCAACAAAATCTC
R	TAGGACTGTAAAAGACGAAAG

Prior to degradation, all 12 genomic DNA samples were genotyped at each of the four polymorphisms studied. DNA was amplified with each set of sequencing primers (Table 2). The PCR products were purified and sequenced by Shanghai GeneCore Bio-Technologies Co., Ltd. (Shanghai, China). The genotypes of each of the 12 samples for the four polymorphic loci were identified according to the sequencing results.

Preparation of the Reference Sample of Artificially Degraded DNA

We subjected the genomic DNA samples to DNase I digestion, then assessed the severity of degradation by PCR using primers 100 bpF/R (size of PCR products: 102 and/or 105 bp). Each 90 µL digestion reaction consisted of 9 µL of DNase I (1 U/µL) (Fermentas, Vilnius, Lithuania), 9 µL of 10 × DNase I reaction buffer without MnCl₂ (100 mM Tris–HCl [pH 7.5 at 25°C], 1 mM CaCl₂), 9 µL of 100 mM MnCl₂, and 10 µg genomic DNA, incubated at 37°C. Nine µL aliquots were removed at 0, 10, 30, 60, 90, 120, 150, 180, 210 min time points, and DNase I activity was quenched by adding 1 µL of 25 mM EDTA with heating at 65°C for 15 min.

The extent of DNA fragmentation was assessed by PCR amplification using primers 100 bpF (5'-GGGAGCCCACTGACTGAAC-3') and 100 bpR (5'-GTCTCCAGAAAAGGAGGACATC-3') performed in a Tgradient thermocycler (Whatman Biometra, Goettingen, Germany). Each 20 µL PCR reaction contained 0.1 µg of DNase I digested DNA, 0.2 µM 100 bpF, 0.2 µM 100 bpR, 200 µM dNTPs, 1 U of *rTaq* DNA polymerase (Takara Biotechnology Co., Ltd.), and 1 × PCR buffer. Reaction parameters consisted of an initial denaturation step at 94°C for 2 min, followed by 34 cycles of 94°C for 30 s, 60°C for 20 s, and 72°C for 20 s, with a final extension at 72°C for 3 min. PCR products were separated on a 3% polyacrylamide gel and visualized by ethidium bromide staining. Samples too degraded to be amplified were used as the reference sample for the subsequent LDR experiments.

Ligase Detection Reaction Coupled with PCR

Each 20 µL ligation reaction contained 4 U of Ampligase[®] Thermostable DNA Ligase (Epicentre Technologies), 1 × Ampligase[®] Reaction Buffer, 50 ng of DNase I digested DNA, and 1 or 2 pmol of the LDR probes (see legends for Figs. 3–5). Following an initial denaturation at 94°C for 3 min, the ligation parameters consisted of 20 cycles of denaturation at 94°C for 30 s, and ligation at 37, 42, or 45°C for 8 min (see legends for Figs. 3–5). Different probe concentrations and ligation temperatures were used depending on whether a polymorphism was being determined individually (Fig. 3) or in a multiplex reaction (Figs. 4 and 5).

TABLE 3—Probes and primers for LDR-PCR assay.

LDR Probe*	Total Sequence (5' → 3') [†]	Length of Target-Specific Region (nt)	Probe Length (nt)	LDR-PCR Amplicon Size (I+II/III, bp)
rs17750303				
I	p-GACTCTTCTGT <u>agttgctcattagctctaataacc</u>	11	34	
II	<u>gtcctaataaccagttcgtctt</u> <i>cc</i> TAGCTGCCTG	10	34	68
III	<u>gtcctaataaccagttcgtctt</u> TAGCTGCCTT	10	31	65
rs2307647				
I	p-GAATACCCTCAG <u>agttgctcattagctctaataacc</u>	12	36	
II	<u>gtcctaataaccagttcgtctt</u> <i>caca</i> AGGTGCGAATGAA	13	38	74
III	<u>gtcctaataaccagttcgtctt</u> <i>caca</i> AGGTGCGAAT	10	35	71
rs2307557				
I	p-TTAGCTCAGGTAGCCTTCA <u>agttgctcattagctctaataacc</u>	19	42	
II	<u>gtcctaataaccagttcgtctt</u> GCCAGGTCTTTAACTTACA	20	41	83
III	<u>gtcctaataaccagttcgtctt</u> GCCAGGTCTTTAACTT	17	38	80
rs17250992				
I	p-GTCCATAAGAGACTAGCACCC <u>agttgctcattagctctaataacc</u>	20	46	
II	<u>gtcctaataaccagttcgtctt</u> <i>tc</i> TCAGTGCTCAGTTTGCC	17	41	87
III	<u>gtcctaataaccagttcgtctt</u> <i>tttc</i> TCAGTGCTCAGTTTGCT	17	44	90
Generic primers for PCR of ligation products				
Primer 1	GGTATTAGGACTAATGAGCAACT			
Primer 2	GTCCTAATACCAGTTCGTCTT			

*Each set of probes includes a common probe (I) and two allele-specific probes (II and III).

[†]p = 5' phosphorylation. Underlined nucleotides are complementary to the generic PCR primers and those in rectangle are target-specific sequences of LDR probes. Nucleotides in italics were added to change the length of LDR probes to allow allele discrimination in the PCR step.

PCR reactions were performed using the ligation product of the LDR as amplification template. Each 30 μ L reaction contained 3 μ L of the LDR product, 200 μ M each dNTP, 0.2 μ M each generic primer (Table 3), 1 U *r*Taq polymerase (Takara Biotechnology Co., Ltd.), and 1 \times PCR buffer. The PCR parameters consisted of an initial denaturation at 94°C for 3 min, followed by six cycles of 94°C for 30 s, 54°C for 20 s, and 72°C for 15 s, followed by 28 cycles of 94°C for 30 s, 50°C for 20 s, and 72°C for 15 s, and a final extension at 72°C for 5 min. The products were separated on a 10% polyacrylamide gel and visualized by ethidium bromide staining.

Results and Discussion

Preparation of the Reference Sample of Degraded DNA with Fragment Sizes <100 bp

In crime case investigations, DNA samples are often qualitatively inadequate since the degradation of human DNA varies with light, humidity, elevated temperatures as well as bacterial and fungal contaminations followed by the growth of these microorganisms (1). To systematically assess the performance of different typing techniques, it would be desirable to have a standardized reference sample of degraded DNA in sufficient amount and specific length (14). Artificially degraded DNA can be obtained by sonication, enzyme digestion, or physical methods such as elevated temperature or humidity (3,15,16). DNase I digestion for controlled production of degraded DNA has been described in several studies (15–18).

In the presence of Mn²⁺, DNase I enzyme cleaves double-stranded DNA and completely digested products are tetranucleotides (19). Increasing incubation times with DNase I resulted in increasing severity of degradation. Analysis by agarose gel electrophoresis (data not shown) showed that the sample incubated for 0 min consisted of high molecular weight DNA, and samples incubated for 10, 30, and 90 min consisted of fragments that were approximately 600, 200, and 100 bp, respectively. However, since

the sizes of the degraded DNA fragments could not be reliably assessed by agarose gel, assessment by PCR amplification was used as described previously (16,20). We designed a pair of primers (100 bpF/R) and attempted to amplify a 102/105 bp amplicon using the degraded DNA as template. In order to ensure that there were no detectable large DNA fragments remaining, we increased the quantity of DNA template (0.1 μ g of DNase I digested DNA) and the cycle numbers of PCR (34 cycles). With increasing digestion time, we observed decreasing amounts of PCR product, with no amplification product from the DNA samples digested for 150 min or more (Fig. 2). As an additional check, we attempted to amplify 8 ng genomic DNA digested for 150 min using the

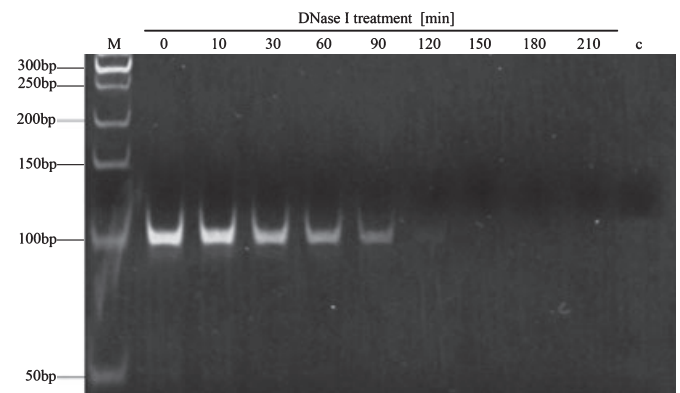


FIG. 2—Verification of DNA degradation by PCR. Ethidium bromide stained 3% polyacrylamide gel showing PCR amplification products of 1 microliter samples of degraded DNA digested with DNase I for 0, 10, 30, 60, 90, 120, 150, 180, and 210 min using the 100 bpF/R primer pair, which gives a 102/105 bp amplicon. The amount of PCR product visualized decreased with increasing DNase I digestion time such that after 150 min, no amplification is detected. Lane M, the 50 bp DNA Ladder Marker (Takara Biotechnology Co., Ltd.). Lane c, the negative control lacking template DNA.

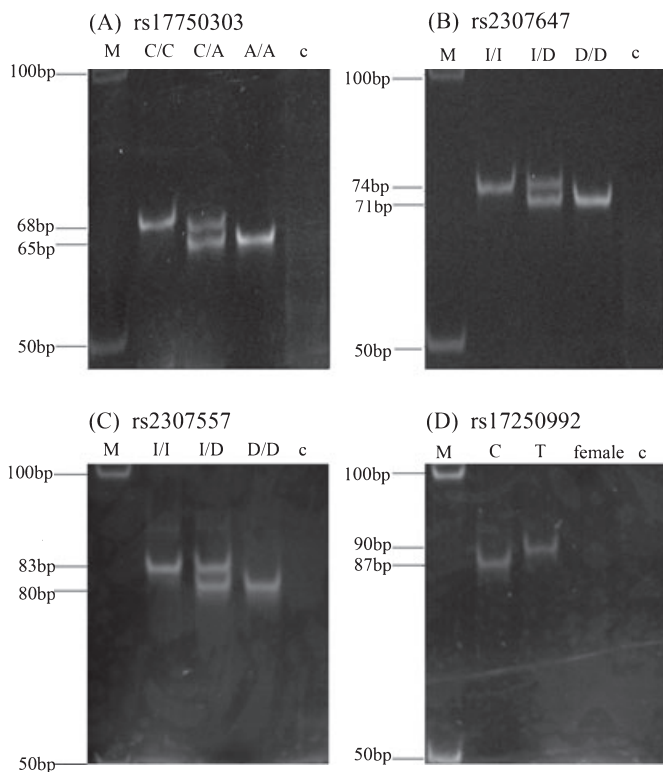


FIG. 3—LDR-PCR and signal detection of each polymorphism. I = the allele of insertion mutation. D = the allele of deletion mutation. PCR products of ligated probes were separated on 10% nondenaturing polyacrylamide gels. DNA was visualized by ethidium bromide. The nucleotides in capitals represent the genotype of the target polymorphism locus. Lane M, the 50 bp DNA Ladder Marker (Takara Biotechnology Co., Ltd.). Lane c, the negative control lacking target DNA. (A) Analysis of rs17750303 locus. LDR was carried out with 2 pmol of each of the rs17750303 probes (rs17750303 I+II+III) at 37°C. (B) Analysis of rs2307647 locus. LDR was carried out with 2 pmol of each of the rs2307647 probes (rs2307647 I+II+III) at 37°C. (C) Analysis of rs2307557 locus. LDR was carried out with 2 pmol of each of the rs2307557 probes (rs2307557 I+II+III) at 45°C. (D) Analysis of rs17250992 locus located in Y chromosome. DNA samples included two males and one female. LDR was carried out with 2 pmol of each of the rs17250992 probes (rs17250992 I+II+III) at 45°C.

AmpFISTR® Identifiler® PCR Amplification Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Analysis of this reaction gave no results (data not shown). Therefore, we were confident that the length, and not the quantity, of DNA fragments caused the PCR reactions to fail, and that the 150 min digestion sample could be used as the reference sample of degraded DNA with fragment sizes <100 bp. In our experiments, every DNA digestion sample was verified to contain fragments under 100 bp by failure of PCR using primers 100 bpF/R before use in subsequent LDR experiments.

Rationale for Using LDR-PCR and Selection of Polymorphic Markers

A forensic DNA sample frequently is too degraded to give a full profile of STRs. Genotyping methods that can be successfully performed on smaller fragments of genomic DNA are of great interest to forensic science. Reducing the amplicon sizes of target DNA fragments has become a major strategy of analyzing the degraded DNA, and mini-STR-PCR and mini-sequencing techniques have been used in casework (5,6,8). However, due to the length limitation of PCR amplicons (21,22), these PCR-based assays would not provide

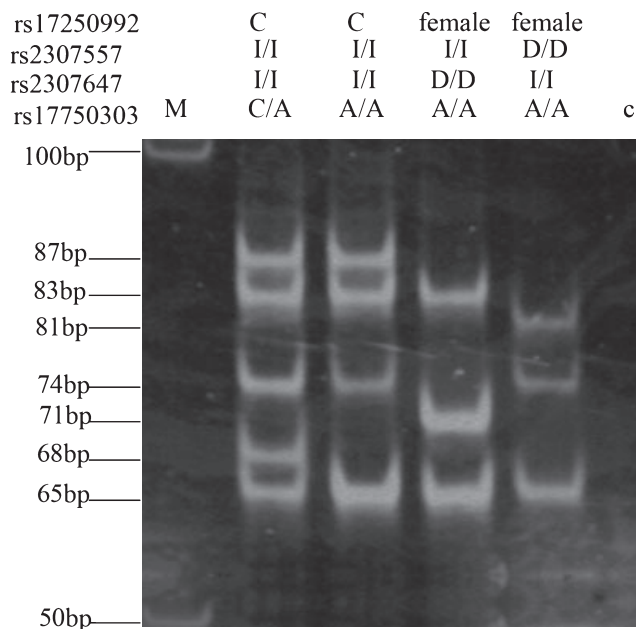


FIG. 4—Genotyping of four samples of degraded DNA by a multiplex LDR-PCR reaction characterizing alleles of all four polymorphism loci simultaneously. I = the allele of insertion mutation. D = the allele of deletion mutation. Four samples of degraded DNA (two male and two female) were genotyped using a multiplex LDRPCR. LDR was carried out with 2 pmol of each probe of rs17750303 and rs2307647, and 1 pmol of each probe of rs2307557 and rs17250992 at 42°C. PCR products of ligated probes were separated on 10% nondenaturing polyacrylamide gels and visualized by ethidium bromide. The nucleotides in capitals represent the genotypes of the target polymorphism. Lane M, the 50 bp DNA Ladder Marker (Takara Biotechnology Co., Ltd.). Lane c, the negative control lacking target DNA. Genotype findings for the four samples using the multiplex reaction were identical to prior findings for these samples.

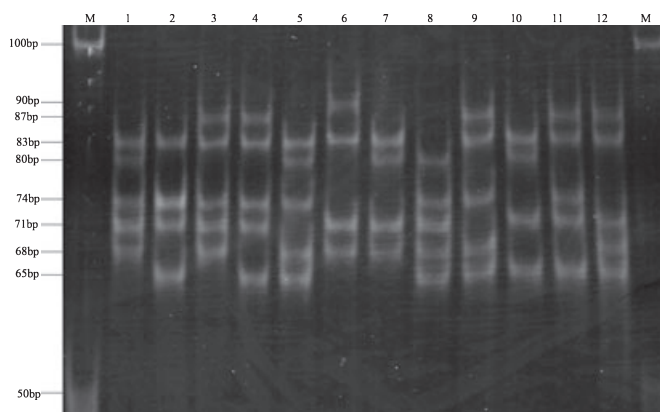


FIG. 5—Verification of the multiplex LDR-PCR genotyping method using coded DNA samples. DNA from 12 anonymous blood samples (six male and six female) were PCR amplified surrounding the four polymorphic loci and sequenced to obtain the genotype profile. The samples were then degraded with DNase I until the largest fragments were <100 bp and randomly coded by a researcher blinded to the genotype results. The coded samples were then genotyped for the four polymorphic markers using the LDR-PCR method. LDR was carried out with 2 pmol of each probe for 17750303 and rs2307647, and 1 mol of each probe for rs2307557 and rs17250992 at 42°C. Lanes 1–12, PCR products of ligated probes were separated on 8% nondenaturing polyacrylamide gels and stained with ethidium bromide. Lane M, the 50 bp DNA Ladder Marker (Takara Biotechnology Co., Ltd.). Lane c, the negative control lacking target DNA. After completing the analysis, the code was broken. All 12 samples were correctly genotyped using the LDR-PCR method.

reliable DNA profiles from a sample of severely degraded DNA such as our reference sample (DNA digested with DNase I for 150 min).

To characterize a bi-allelic polymorphism by LDR, three probes (one common and one specific to each allele) must be designed (Fig. 1). When allowed to hybridize to the target DNA, the probes will be brought into juxtaposition and ligated by DNA ligase. The probes that discriminate the two alleles (probes II and III) have, as their 3' terminus, the nucleotide(s) corresponding to their target allele. This strategy capitalizes on the sensitivity of DNA ligase to all base mismatches on the 3'-side of the nick (23). Only probes that hybridize completely become ligated by the thermostable ligase used in this study. The two probes together only hybridize to approximately 20–40 bp of target sequence, making a degraded DNA sample an adequate template. After several cycles of hybridization and ligation, the ligation products would be sufficient for the subsequent PCR amplification.

Each LDR probe was designed to contain a generic primer-specific sequence (Table 3) so that every set of LDR-ligated product could be simultaneously amplified in the PCR step (Fig. 1). In order to avoid nonspecific priming, the sequences added to the LDR probes used for priming the PCR reactions were derived from *E. coli* plasmid replication origin and adjusted by ourselves. In addition, several nucleotides were added between the primer-specific and target-specific regions of the probes so that all PCR products of ligated LDR probes varied in length by 3 bp and could be discerned by polyacrylamide gel electrophoresis (Table 3 and Fig. 1). Together, these measures reduce the risk of amplification artifacts and provide for low cost, high throughput analysis of multiple polymorphic loci.

Our next challenge was to identify polymorphic markers that could be accurately distinguished by the LDR-PCR method in a degraded DNA sample. In the interest of sample conservation, it was also important that the design accommodate multiplexing of the reactions. Using the dbSNP database, we identified two SNPs (rs17750303 and rs17250992) and two three-base deletion/insertion polymorphisms (rs2307647 and rs2307557) that met our criteria and were selected for investigation (Table 1).

Genotyping of the Reference Sample of Degraded DNA by LDR-PCR

After genomic DNA was digested with DNase I for 150 min, DNA fragments were less than 100 bp in length (Fig. 2). Despite the severe degradation of the DNA samples, each of the four polymorphic loci were successfully discriminated using our LDR-PCR method (Fig. 3). LDR capitalizes on the ability of DNA ligase to covalently join two adjacent oligonucleotides hybridized to target DNA in which there is perfect complementarity at the nick junction (13). Without pre-amplification of the target sequences, our LDR probes could directly recognize and hybridize their targets within the degraded reference sample. Therefore, only a very short target sequence (20–40 bp surrounding the polymorphism) within the DNA sample was required for the ligation reaction. LDR-PCR is therefore a genotyping technique suitable for use in extremely degraded DNA samples.

Studies of the recognition process of DNA ligases show that when binding to the DNA substrate, their footprints extend approximately 5–9 nucleotides on the 3'-OH side of the nick, and 9–12 nucleotides on the 5'-phosphate side of the nick (24,25). Thus, DNA ligases require a certain length of double stranded DNA template in order to stably bind to the nick and catalyze the repair reaction. The design of LDR probes rs2307557 and rs17250992 included 17–20 bp complementary to the target DNA (Table 3),

which would provide more than sufficient double stranded DNA for ligase recognition and binding. However, the primary goal of this LDR technique was to detect polymorphisms in severely degraded DNA samples containing very small fragments. Therefore, for the other two polymorphisms, the LDR probes were designed with shorter target-specific sequences (rs17750303 and rs2307647, Table 3), containing only 10–13 bases complementary to the target region. The results showed that Ampligase[®] allowed for successful ligation reaction and polymorphism discrimination when the target-specific sequence of LDR probe was as short as 10 nt (Fig. 3A, B). This characteristic of Ampligase[®] is valuable in this type of analysis.

While most DNA ligases have very stringent requirements for perfect complementarity on the 3'-side of the nick, it has been reported that T–G and G–T mismatches at the 3'-end of the nick can generate a quantifiable level of ligation product (23). A ligation product formed despite a sequence mismatch would result in unreliable typing because erroneous ligation products would also be amplified in the subsequent PCR step. In this study, the rs17250992 polymorphism is a C/T SNP and therefore there was the possibility that the probe used to discriminate the “C” allele would erroneously bind to the DNA template having the “T” allele, producing a G–T mismatch at the 3'-end of the nick. Despite this situation, this polymorphism was accurately discriminated (Fig. 3D). For each polymorphism studied, two allele-discriminating probes competed to anneal to DNA template adjacent to the common probe, yet we observed that only the allelic probe with perfect complementarity to the template became ligated to the common probe by Ampligase[®]. This high degree of allele discrimination might result from the use of short LDR probes (23), intermolecular recognition and fidelity of Ampligase[®].

Multiplex LDR-PCR Reaction

Design of a multiplex LDR-PCR reaction requires similar annealing temperatures for all probes and an avoidance of interaction effect between them. When designing the probes for the LDR-PCR assays, T_m of each LDR probe was calculated using the online HyTher software (26). We then tested the specificity of each pair of probes (probes I+II or probes I+III) by performing the hybridization step at different temperatures (the T_m, and 5°C above and below the T_m). PCR amplicons of the ligation products from each hybridization were similar (data not shown), therefore we were confident that the specificity of the reaction would remain even in the presence of slight fluctuations in hybridization temperature. Additionally, Primer Premier 5.0 software was used to analyze any interaction between the LDR probes, which found no significant risk of mis-hybridization between probes and target loci.

We then mixed all four sets of probes and carried out the LDR-PCR procedure in a single tube. Based on the fact that the target-specific oligos of LDR probes were different in length and sequence, the ligation temperatures and concentrations of probes were modified experimentally. Using 2 pmol of each probe for rs17750303 and rs2307647, and 1 pmol of each probe for rs2307557 and rs17250992 at a hybridization temperature of 42°C, we correctly genotyped four samples of DNase I digested DNA in a multiplex LDR-PCR reaction (Fig. 4). All alleles of the four different loci were readily distinguished, confirming a lack of interference between probes.

Finally, we confirmed the genotypes of all 12 reference samples of digested DNA at the four polymorphic loci using our multiplex LDR-PCR method. The 12 samples had been genotyped by direct DNA sequencing of the polymorphic regions before digestion with

DNase I. The digested samples were then randomly coded by a researcher who was blind to the original genotyping results. The coded samples were then genotyped using the LDR-PCR method. After analysis, the code was broken, revealing that the genotypes of each sample for each of the four polymorphic loci had been correctly identified (Fig. 5). Therefore, this LDR-PCR method can be used to perform multiplex genotyping of polymorphic loci in samples of severely degraded DNA with fragment sizes less than 100 bp.

Indeed, SNPs and small-scale multi-base deletions/insertions are not as powerful as STRs for identification purposes, but they can provide valuable information in cases of highly degraded samples that yield incomplete STR profiles or no profile at all (3,27,28). This pilot study confirmed the potential of LDR-PCR methods in genotyping polymorphic loci in severely degraded DNA samples. Future work should focus on selection of target polymorphic loci, design of LDR probes, and optimization of reaction conditions such that dozens of loci could be discriminated simultaneously in a single tube. Additional modifications to the protocol, such as using fluorescent dyes for allele detection rather than ethidium bromide, will only improve the assay, making it sensitive enough for forensic casework.

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