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ORIGINAL ARTICLE

Comparison of different methods for repairing damaged DNA from buffered and unbuffered formalin-fixed tissues

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Abstract Formalin fixation is considered an important process for preservation of human tissue samples for long periods. However, this process not only results in cross-linking complicating isolation of nucleic acid but also introduces polymerase "blocks" during polymerase chain reaction (PCR). At present, many protocols have already been developed aiming at extracting high amounts of amplifiable DNA from formalin-fixed tissues (FFTs). However, there are few methods for repairing formalin-damaged DNA. In this study, we compared the effectiveness of several post-extraction enzymatic repair techniques, including *Taq* DNA polymerase, DNA polymerase I and T4 DNA ligase, the PreCR™ Repair Mix and Restorase® DNA Polymerase, in restoring STR profiles from formalin-damaged DNA. Our results indicated that formalin-damaged DNA may be repaired partly with *Taq* DNA polymerase and the Restorase® DNA Polymerase, and lost alleles may be restored and STR peak heights may increase upon repair with them. Moreover, the repair ability of the protocol 2 with *Taq* DNA polymerase surpasses the Restorase® DNA Polymerase.

Keywords Formalin-fixed tissues · DNA damage · DNA repair · Short tandem repeat (STR) analysis

Introduction

Formalin fixation is most commonly used to preserve biological tissue sections for histopathology testing (formalin-fixed paraffin embedded (FFPE)) and embalming cadavers for medical study or in preparation for burial. With the advent of the polymerase chain reaction (PCR), this type of samples has become an increasingly important source of DNA for medical diagnosis [1–3] and forensic studies [4–7]. However, DNA extraction from formalin-fixed tissues (FFTs) or formalin-fixed paraffin-embedded tissues (FFPETs) and followed genetic profile analysis are known to be a challenging task. There are several reasons for the failure of PCR using DNA isolated from FFT or FFPET, for example, the generation of cross-links between nucleic acids and proteins resulting in nucleic acid fragmentation [8, 9] and the presence of remnants of substances that inhibit the amplification reaction such as formalin [10] or inhibit the proteinase K used in the extraction procedure such as xylene [11].

To cope with these difficulty, not only various DNA extraction protocols have been developed aiming at extracting high amounts of amplifiable DNA from FFT or FFPET, such as modified phenol-chloroform protocol [10], salting-out method [12, 13], CTAB method [14], hot-alkali treatment method [15, 16], and commercially available kits [14, 17, 18] but also shorter amplicon strategy have been performed, like with mini-STRs [17, 19] or SNPs [20, 21]. Another possibility is to repair the damaged DNA from FFT or FFPET. One of the most interesting developments with regard to genetic analyses of FFT or FFPET is to repair the nicked single-strand DNA using the *Taq* DNA polymerase [18, 22, 23]. In fact, in recent

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years, more and more researchers have tried to repair damaged DNA before amplification by means of DNA repair enzymes [24–34].

Without considering the influence of different DNA extraction methods from FFT, the purpose of this study is only to systematically compare the effectiveness of several post-extraction enzymatic repair techniques, including *Taq* DNA polymerase, DNA polymerase I and T4 DNA ligase, and the PreCR™ Repair Mix and Restorase® DNA Polymerase, in restoring STR profiles from formalin-damaged DNA.

Materials and methods

Sample preparation

Because it is texture homogenous and easily damaged by formalin fixation, human liver tissues were selected for preparing the artificially FFT. The fresh autopsy liver tissues were cut into the same size of blocks (approximately 2 cm of length, 1 cm of width, and 0.1 cm of thickness), and all of them were fixed respectively in 10% unbuffered formalin for 0.5, 1, 2, 3, 5, 7, 9, 11, and 15 days, and in 10% buffered formalin for 1, 3, 5, 7, 9, 11, 15, 17, 20, 25, 30, and 35 days under a constant 25 °C condition ($n = 4$ for each time-point). Approval for use of these autopsy human tissues in our research was obtained from the medical ethics committee of Tongji Medical College of Huazhong University of Science and Technology.

DNA extraction, quantity, and quality assessment

In order to get high-purity DNA samples for the subsequent repair reaction, the phenol-chloroform method was selected to extract DNA from FFT. For removal of formalin, FFT blocks were soaked in distilled water 48 h and water was changed every 12 h before DNA extraction. Twenty milligrams of tissue pellets were digested 48 h at 56 °C in a total reaction volume of 500 μL containing 360 μL of STE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 100 mM NaCl), 100 μL of 10% SDS, 20 μL of 1 M DTT, 0.12 g of urea, and 20 μL of proteinase K (20 mg/mL). Then, DNA was extracted using the standard phenol-chloroform protocol, followed by ethanol precipitation. Finally, the DNA was resuspended in 50 μL of distilled water and stored at − 20 °C.

The concentration and quality of genomic DNA were measured by using NanoDrop spectrophotometer (ND-2000, Nanodrop Technologies) at 260 and 280 nm (OD_{260}/OD_{280}). The amount of amplifiable human DNA present in each sample was quantified using the Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific, MA, USA) and the 7500 real-time PCR system (Thermo Fisher Scientific, MA, USA) following the manufacturer's instruction. In addition, DNA integrity was also determined by 1.0% agarose gel electrophoresis and STR genotyping with the AmpFlSTR® Identifiler® PCR amplification kits (Thermo Fisher Scientific, MA, USA).

Repair reaction

In order to compare the effects of different repair methods on formalin-damaged DNA, the samples fixed in unbuffered formalin for 1, 2, 5, and 11 days and in buffered formalin for 3, 7, 17, and 35 days were selected to perform repair reaction. After repair reaction, the amount of amplifiable human DNA present in each sample was also quantified using the Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific, MA, USA).

Repair with Taq DNA polymerase

Two DNA repair protocols with *Taq* DNA polymerase were performed respectively. For protocol 1, 200 ng of the DNA samples was briefly incubated for 1 h at 55 °C in 20 μL of solution containing 8 μL AmpFlSTR® PCR Reaction Mix (Thermo Fisher Scientific, MA, USA). After this step, 2 U of AmpliTaq Gold® DNA Polymerase (Thermo Fisher Scientific, MA, USA) was added and DNA polymerization was performed at 72 °C for 20 min.

For protocol 2, a total reaction volume of 20 μL containing 200 ng of the DNA samples, 8 μL of AmpFlSTR® PCR Reaction Mix (Thermo Fisher Scientific, MA, USA), and 2 U of AmpliTaq Gold® DNA Polymerase (Thermo Fisher Scientific, MA, USA) was established, and then incubated with the following conditions: 95 °C for 11 min; 30 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min; and a final hold at 72 °C for 20 min.

In addition, a control test was also set up. Briefly, a total reaction volume of 20 μL containing 200 ng of the DNA samples, 8 μL of AmpFlSTR® PCR Reaction Mix (Thermo Fisher Scientific, MA, USA), and 2 U of AmpliTaq Gold® DNA Polymerase (Thermo Fisher Scientific, MA, USA) was established, then 10 μL of the mixture (approximately 100 ng of DNA) without any subsequent treatment was directly used to establish the Identifiler® reaction.

Repair with DNA polymerase I and T4 DNA ligase

This repair method followed the report by Pusch et al. [24] and Kovatsi et al. [28]. Briefly, the repair reaction contained 5 U *Escherichia coli* DNA polymerase I (NEB, Ipswich, MA), 2.5 μL 10 × NEB buffer, 400 ng of the DNA samples, 0.2 mM each dNTP, and distilled water to a total volume of 25 μL. The reaction was carried out for 90 min at 37 °C and terminated by incubating at 75 °C for 20 min. Subsequently, all of the polymerase-treated DNA were mixed with 3 μL of

10× ligase buffer and 300 U T4 DNA ligase (NEB, Ipswich, MA). The ligation reaction was performed overnight at 16 °C. After this step, the repaired samples were purified with the TaKaRa MiniBEST DNA Fragment Purification Kit (TaKaRa, Dalian, CA).

Repair with the PreCR™ repair mix

DNA repair was performed according to the manufacturer's instructions of the PreCR™ Repair Mix (NEB, Ipswich, MA). Briefly, 20 μL of repair reaction volume consisting of 1× ThermoPol Reaction Buffer, 200 μM dNTPs (Sigma, USA), 1× NAD^+, 100 ng of DNA, and 0.5 μL of PreCR™ Repair Mix was prepared and incubated at 37 °C for 20 min.

Repair with the Restorase® DNA Polymerase

DNA repair was performed according to the manufacturer's instructions of the Restorase® DNA Polymerase (Sigma, USA). Specifically, a 20-μL repair reaction consisting of 1× Reaction Buffer, 200 μM dNTPs, 1.25 U Restorase® DNA Polymerase, and 100 ng of DNA was prepared. The mixture was incubated at 37 °C for 20 min and 72 °C for 5 min, followed by denaturation at 94 °C for 30 s.

PCR amplification and STR genotyping

All samples were amplified with a standard 25 μL reaction volume using the AmpFLSTR® Identifiler® PCR amplification kit (Thermo Fisher Scientific, MA, USA) on a GeneAmp® 9700 thermal cycler (Thermo Fisher Scientific, MA, USA). Specifically, for the repaired DNA with *Taq* DNA polymerase and DNA polymerase I and T4 DNA ligase, 10 μL of the enzyme-treated samples (approximately 100 ng of DNA) was used as DNA template, and for the repaired DNA with the PreCR™ Repair Mix and Restorase® DNA Polymerase, 5 μL of the Identifiler® Primer Set and 2.5 U AmpliTaq Gold® DNA polymerase were added directly to the repair reaction mix. PCR amplification was performed according to the manufacturer's protocol of the AmpFLSTR® Identifiler® PCR amplification kit (Thermo Fisher Scientific, MA, USA).

PCR products were electrophoresed on AB 3130 Genetic Analyzer (Thermo Fisher Scientific, MA, USA) following manufacturer's protocols. Samples were prepared as a mixture of 0.3 μL GeneScan™ LIZ-500 size standard (Thermo Fisher Scientific, MA, USA) with 8.7 μL Hi-Di™ Formamide (Thermo Fisher Scientific, MA, USA) and 1 μL PCR products. Samples were analyzed using GeneMapper ID v3.2 software (Thermo Fisher Scientific, MA, USA) after data collection.

Data analysis

The degree of DNA damage resulted from formalin fixation was evaluated using the Degradation Index (DI) value which is the ratio between DNA quantity of the short target divided by DNA quantity of the long target. For analysis of the STR profile, a detection threshold of 50 RFU was applied. A threshold of 75 RFU for heterozygote loci and 150 RFU for homozygote loci was used to determine reportable alleles. The average percentage of detectable and reportable alleles for all alleles was count respectively. Comparison of the paired data was performed by the Student's *t* test.

Results

Quantity and quality of DNA from FFT

DNA damage was confirmed by agarose gel electrophoresis, Quantifiler® Trio DNA Quantification, and STR genotyping. In general, whether fixation with unbuffered or buffered formalin, the longer the fixation time, the more serious the DNA damage, and the proportion of intact DNA decreased quickly when tissues were fixed in unbuffered formalin, while the DNA degradation of buffered FFT was significantly slower (see Electronic Supplementary Materials Fig. S1 and Table S1). STR typing results of FFT showed typical degraded DNA pattern: a left to right decrease in allelic peak heights and drop-out of the larger alleles (see Electronic Supplementary Materials Fig. S2). After being fixed in unbuffered formalin for 0.5 days or in buffered formalin for 1 day, mild DNA degradation ($1.5 < DI < 4$) and allelic drop-out at D2S1338 locus was observed in some of the DNA samples, followed by the alleles at CSF1PO after 1 day fixation in unbuffered formalin or 3 days in buffered formalin. After being fixed in unbuffered formalin for 9 days or in buffered formalin for 25 days, severe DNA degradation ($DI > 10$) and allelic drop-out at amelogenin was observed in some of the DNA samples, and after being fixed in unbuffered formalin for 15 days or in buffered formalin for 35 days, allelic drop-out was observed at almost all loci (see Electronic Supplementary Materials Fig. S3 and S4).

Spectrophotometric determination of the yield and purity of DNA was conducted (see Electronic Supplementary Materials Table S1). Based on DNA extracted from FFT of different fixation time, the mean concentration and ranges of OD ratios (OD_{260}/OD_{280}) were 2227.3 ng/μL and 1.89 to1.95 (mean = 1.92) for unbuffered FFT and 2123.9 ng/μL and 1.87 and 1.95 (mean = 1.91) for buffered FFT, respectively, which indicates the extracts are sufficiently pure and suitable for repair reaction.

Repair of formalin-damaged DNA

As shown in Tables 1 and 2, after repair with protocol 2 using *Taq* DNA polymerase and the Restorase® DNA Polymerase, the average percentage of detectable and reportable alleles in all samples fixed in unbuffered formalin for 1, 2, and 5 days and in buffered formalin for 3, 7, and 17 days was significantly higher than that of the unrepaired and control test samples ($p < 0.05$) (Fig. 1), and in samples fixed in unbuffered formalin for 11 days and in buffered formalin for 35 days, there was no significant difference ($p > 0.05$) between the repaired and unrepaired samples except for the average percentage of detectable alleles with the former method ($p < 0.05$).

After repair with the other three methods, protocol 1 with *Taq* DNA polymerase, DNA polymerase I, and T4 DNA ligase, and the PreCR™ Repair Mix, no obvious improvement in allele recovery was observed compared to the unrepaired samples (Tables 1 and 2). These results had also been supported by the similar changes in DI values after repair reaction (see Electronic Supplementary Materials Table S2).

Discussion

Ten percent formalin solution is a kind of common tissue fixative, and it is well known that formalin can cause DNA damage. In this study, DNA damage was observed from the samples fixed in unbuffered formalin for 0.5 days and in buffered formalin for 1 day, and after being fixed in unbuffered formalin for 15 days and in buffered formalin for 35 days, allelic drop-out was observed at almost all loci detected. Obviously, the duration of fixation plays an important role to DNA degradation, and the DNA damage in buffered FFT is significantly slower than that in unbuffered FFT. The results indicate that different characteristics of formalin solution have a different capacity to degrade DNA.

For many years, *Taq* DNA polymerase has served as the stalwart enzyme in the PCR amplification of DNA. However, a major limitation of *Taq* DNA polymerase is its inability to amplify damaged DNA, thereby restricting its usefulness in forensic applications [35]. Based on the fact that DNA degradation is connected to random single-strand breaks, after pre-PCR DNA restoration treatment using *Taq* DNA polymerase, DNA samples extracted from archival postmortem tissues were successfully amplified by Bonin et al. [22]. In this study, however, there was no improvement in allele recovery from FFT after being repaired with protocol 1 using *Taq* DNA polymerase. Based on a consideration that there are so many short strands in damaged DNA samples and the polymerase reaction restores the nicks after DNA rehybridization using the other strand as the template, we imitated the PCR process in protocol 2 using *Taq* DNA polymerase. After being repaired with the protocol followed by STR amplification, an obvious increase in allelic peak heights and recovery of previously undetected alleles were observed in all samples detected. Moreover, the lost amplification products of amelogenin locus could even be restored from the samples fixed in unbuffered formalin for 15 days and in buffered formalin for 35 days with the protocol but not in the control test (see Electronic Supplementary

Table 1 Comparison of several different protocols for repairing damaged DNA from unbuffered FFT ($n = 4$)

	No repair		Repair with *Taq* DNA polymerase				Repair with DNA polymerase I and T4 DNA ligase		Repair with the PreCR™ Repair Mix		Repair with the Restorase® DNA Polymerase	
			Protocol 1		Protocol 2							
	Avg.[a]	S. E.[b]	Avg.	S. E.	Avg.	S. E.	Avg.	S. E.	Avg.	S. E.	Avg.	S. E.
1 day of fixation												
All detectable alleles	88.9%	1.2%	89.3%	2.6%	96.3%*	3.0%	90.2%	2.5%	86.4%	2.1%	92.6%*	5.2%
All reportable alleles	86.1%	1.8%	86.1%	3.0%	94.4%*	2.1%	86.3%	3.0%	84.0%	2.1%	89.8%*	3.5%
2 days of fixation												
All detectable alleles	55.6%	5.3%	54.6%	3.7%	84.3%*	5.6%	55.6%	3.5%	56.5%	3.0%	77.8%*	1.3%
All reportable alleles	50.9%	3.9%	48.1%	3.1%	78.7%*	3.5%	49.1%	2.6%	49.1%	2.6%	70.4%*	2.2%
5 days of fixation												
All detectable alleles	38.9%	5.6%	36.1%	3.7%	59.3%*	8.0%	38.0%	3.3%	39.8%	2.5%	45.6%*	5.8%
All reportable alleles	31.5%	3.7%	31.5%	3.0%	50.9%*	3.6%	32.4%	3.0%	32.4%	2.3%	40.7%*	4.3%
11 days of fixation												
All detectable alleles	13.0%	2.1%	12.1%	2.2%	18.5%*	3.0%	13.9%	2.2%	11.1%	2.5%	14.8%	6.0%
All reportable alleles	10.2%	1.9%	9.2%	1.2%	13.9%	1.9%	10.2%	1.4%	8.3%	3.4%	8.3%	1.9%

*$p < 0.05$

[a] Average percentage of detectable or reportable alleles

[b] Standard error

Table 2 Comparison of several different protocols for repairing damaged DNA from buffered FFT ($n = 4$)

	No repair		Repair with *Taq* DNA polymerase				Repair with DNA polymerase I and T4 DNA ligase		Repair with the PreCR™ Repair Mix		Repair with the Restorase® DNA Polymerase	
			Protocol 1		Protocol 2							
	Avg.[a]	S. E.[b]	Avg.	S. E.	Avg.	S. E.	Avg.	S. E.	Avg.	S. E.	Avg.	S. E.
3 days of fixation												
All detectable alleles	88.0%	5.6%	88.0%	1.9%	96.3%*	4.3%	91.7%	3.5%	90.7%	3.7%	94.4%*	2.1%
All reportable alleles	83.3%	6.4%	86.1%	1.9%	95.4%*	3.5%	84.3%	6.3%	86.1%	1.9%	91.7%*	1.8%
7 days of fixation												
All detectable alleles	56.5%	9.7%	57.4%	3.7%	88.9%*	3.0%	57.4%	3.2%	57.9%	3.3%	75.5%*	5.1%
All reportable alleles	52.6%	4.7%	52.8%	3.6%	87.0%*	2.1%	52.3%	3.6%	52.8%	3.4%	70.2%*	4.3%
17 days of fixation												
All detectable alleles	36.1%	8.2%	34.3%	3.1%	64.8%*	4.8%	35.1%	3.3%	37.0%	4.1%	63.9%*	1.9%
All reportable alleles	27.8%	6.4%	25.9%	3.4%	59.3%*	5.2%	26.9%	2.9%	25.9%	4.2%	55.6%*	5.2%
35 days of fixation												
All detectable alleles	7.4%	3.5%	6.5%	2.8%	13.9%*	3.6%	8.3%	3.5%	7.4%	2.6%	9.3%	4.1%
All reportable alleles	7.4%	3.5%	6.5%	2.8%	9.3%	2.9%	6.5%	3.6%	6.5%	3.7%	7.4%	2.3%

*$p < 0.05$

[a] Average percentage of detectable or reportable alleles

[b] Standard error

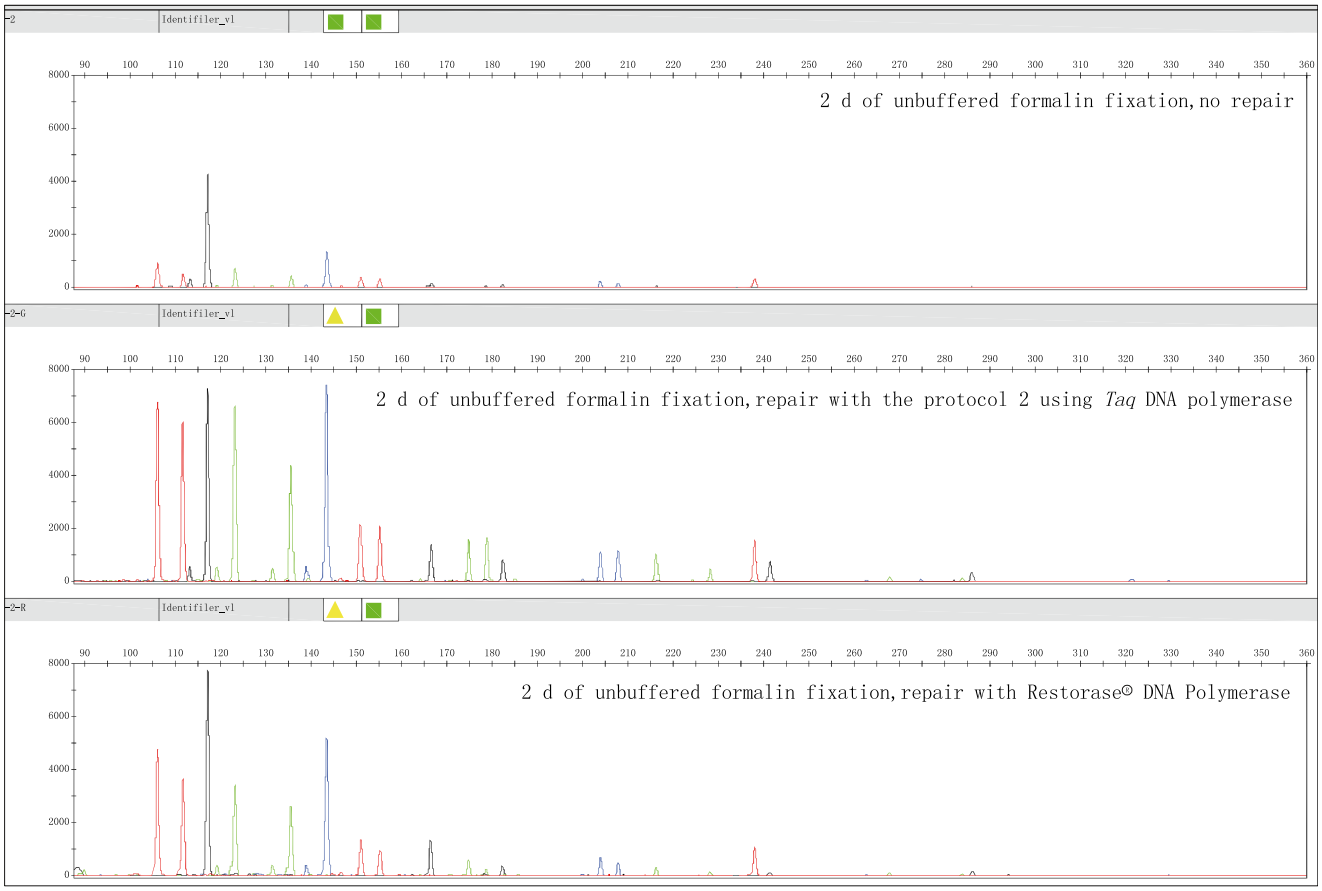

Fig. 1 Comparison of the ability to restore STR alleles from formalin-damaged DNA between protocol 2 with *Taq* DNA polymerase and the Restorase® DNA Polymerase

Materials Fig. S5). The results indicated that the repair capacity of the protocol to restore alleles was not due to the increase of *Taq* DNA polymerase amount.

A simple repair method using DNA polymerase I and T4 DNA ligase has recently been applied in ancient DNA studies [24, 28]. This approach focused on the state of preservation of the chemically altered DNA, consisting of nicked double strands due to hydrolysis, oxidation, or enzymatic destruction. Damaged DNA could be terminally elongated by DNA polymerase I, sealed by T4 DNA ligase, or filled in and then sealed by the concerted action of these two enzymes [28]. In this study, however, this approach was proven unable to repair formalin-damaged DNA.

The PreCR™ Repair Mix is an enzyme cocktail formulated to repair damaged template DNA prior to its use in PCR, microarrays, or other DNA technologies, and it is active on a broad range of DNA damages including those that block PCR (e.g., apurinic/apyrimidinic sites, thymine dimers, nicks, and gaps) and those that are mutagenic (e.g., deaminated cytosine and 8-oxo-guanine), but not for those that inhibit/interfere with PCR [36]. The results in the present study also showed that the PreCR™ Repair Mix was unable to repair formalin-damaged DNA. Combined with the results obtained from DNA polymerase I and T4 DNA ligase, our study demonstrated that nicks and gaps were not the main types of DNA damage caused by formalin fixation.

The Restorase® DNA Polymerase combines Sigma's Long and Accurate enzyme technology with a DNA repair enzyme resulting in a blend that facilitates repair and amplification of damaged DNA. Restorase functions by modifying the damaged sites allowing subsequent template copying [37]. Skage et al. reported that the amplification success of damaged DNA extracted from FFT was greater using Restorase than with the regular PCR assay [38]. Similar results were obtained in this study, and compared to the unrepaired samples, significant improvements were observed in allele recovery and peak height after repair with the Restorase® DNA Polymerase (Fig. 1). But for the seriously damaged DNA samples, such as fixed in unbuffered formalin for 15 days or in buffered formalin for 35 days, the Restorase has no ability to restore the alleles dropped out. In addition, although the Restorase® DNA Polymerase is significantly effective in repairing formalin-damaged DNA, their repair capacity is generally not as good as protocol 2 with *Taq* DNA polymerase, and the differences are more obvious in the unbuffered samples compared to the buffered ones (Fig. 1 and Tables 1 and 2).

Conclusion

Formalin fixation process can lead to DNA damage, and the damage resulted from unbuffered formalin is more serious and rapid than that of buffered formalin. The results of this study indicate that formalin-damaged DNA may be repaired with *Taq* DNA polymerase and the Restorase® DNA Polymerase. Lost alleles may be restored and STR peak heights may increase upon repair with them. Moreover, the repair ability of protocol 2 with *Taq* DNA polymerase to FFT surpasses the Restorase® DNA Polymerase.

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