TECHNICAL NOTE

Renee L. Romero,[1] M.S.; Ann C. Juston,[2] B.S.; Jack Ballantyne,[2] M.Sc.; and Berch E. Henry,[3] Ph.D.

The Applicability of Formalin-Fixed and Formalin Fixed Paraffin Embedded Tissues in Forensic DNA Analysis

REFERENCE: Romero RL, Juston AC, Ballantyne J, Henry BE. The applicability of formalin-fixed and formalin fixed paraffin embedded tissues in forensic DNA analysis. J Forensic Sci 1997;42(4):708–714.

ABSTRACT: Historically, formalin fixed (FF) tissues could not be used as a source of DNA in forensic science due to the fact that the DNA was too degraded for DNA analysis. With the introduction of the polymerase chain reaction (PCR) technique to forensic science, the usefulness of DNA from this biological material has been re-evaluated. This study evaluates the potential use of DNA from FF and formalin fixed paraffin embedded (FFPE) tissues in 13 PCR systems; HLA DQα, LDLR, GYPA, HBGG, D7S8, GC, D1S80, vWA31, THO1, F13A1, FES/FPS, TPOX, and CSF1PO. The first six, HLA DQα, LDLR, GYPA, HBGG, D7S8, and GC are reverse dot blot systems, D1S80 is an amplified fragment length polymorphism (AmpFlp) system and the others are short tandem repeats (STRs). This study shows that FFPE tissue which has not been fixed in formalin for more than three days is a useful source of DNA for 12 of the 13 PCR systems. In contrast, FF tissue did not prove to be a reliable source of DNA for the PCR techniques examined here.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, formalin, tissue fixation, HLA-DQ, LDLR, GYPA, HBGG, D7S8, GC, short tandem repeats, VWA31, THO1, FI3A1, FES/FPS, TPOX, CSF1PO, D1S80

When DNA testing was introduced into forensic science, it was through restriction fragment length polymorphism (RFLP) analysis. This type of testing requires a relatively large amount of sample and the presence of high molecular weight DNA (1,2). A second method, the polymerase chain reaction (PCR), has had an equally significant impact on forensic science. PCR can be used to detect length polymorphisms as well as sequence polymorphisms (3,4). Forensic samples often do not fit the quality and quantity requirements of the RFLP test. Due to the fact that the initial quantity of starting material required for PCR is significantly less than that required for RFLP analysis, sensitivity is vastly improved. Because loci which are amenable to PCR analysis need only be up to several hundred base pairs in length, degraded DNA can be successfully typed.

Historically, formalin fixed (FF) tissue could not be analyzed by the RFLP technique due to the cross linking that takes place within and between DNA molecules which interferes with restriction digestion (5–8). Also, the presence in FF tissue of degraded DNA interferes with hybridization by causing a high background signal (7–10). Several studies have shown evidence for the formation of formalin-mediated methyl bridges between the amino groups of both purine and pyrimidine bases and between bases and histones (5–8). Examination of the double stranded DNA can be inhibited by this cross linking. In fact there has been little success in extracting relatively large sized DNA fragments (i.e., >1000 base pairs) from formalin fixed tissue (8,11–14).

This study was done to evaluate the potential usefulness of formalin fixed (FF) tissue and formalin fixed paraffin embedded (FFPE) tissue in 13 PCR systems. Six of these markers are reverse dot blot systems: HLA DQα (3,4), LDLR (15), GYPA (16), HBGG (17), D7S8 (18,19), and GC (20). One marker is an AmpFlp system, D1S80 (21,22). These systems have been validated for a wide variety of forensic samples and standardized protocols developed. The remaining six loci are short tandem repeats (STRs): vWA31, THO1, F13A1, FES/FPS, TPOX, and CSF1P0, which are length polymorphisms, because the variation is due to the number of tandem repeats of a short core sequence, the exact size and sequence of which is locus dependent (23). Two multiplex STR systems were used in this study. The first, which was developed by the British Home Office, utilizes the four loci vWA31, THO1, F13A1, and FES/FPS (the 'BHO QUAD') (24–26) and the second, designated 'CTT' (27), comprises CSF1PO (28), TPOX (29), and THO1 (23). The semi-automated method utilized here to type samples at the BHO QUAD and CTT loci is based upon the real-time laser-induced fluorescent detection of PCR products whose incorporated primers are labeled with appropriate fluorescent dyes. Electrophoretic separation, detection and measurement of the STR PCR products is carried out on an automated DNA sequencer with appropriate software. It is important to know if DNA extracted from these types of tissues can be examined using previously validated protocols for each of the above systems.

This study attempts to evaluate the applicability of FF and FFPE tissue fit into the field of forensic science by examining these tissues as a source of DNA and applying current methodology. The study focuses on an inorganic Chelex extraction procedure and uses DNA typing kits or primer sets which have become

[1]Criminalist, Washoe County Sheriff's Office, Forensic Science Division, Reno, NV.

[2]Forensic scientist trainee and supervisor, respectively, Suffolk County Crime Laboratory, North County Complex, Hauppauge, NY.

[3]Technical Coordinator, Alabama Department of Forensic Science, Birmingham, AL.

Received 26 Feb. 1996; and in revised form 15 May 1996, 27 Aug. 1996; accepted 11 Oct. 1996.

commercially available and are being used in a number of forensic laboratories.

Materials and Methods

Formalin Fixed Tissues—Six kidney tissues were obtained from the Washoe County Coroners Office in Reno, Nevada. The tissues were removed at autopsy, and stored in 10% buffered formalin from one to five years.

Formalin Fixed Paraffin Embedded Tissues—Nineteen kidney tissues were prepared by and received from the Memphis Tennessee Medical Examiners Office. These tissues were removed at autopsy and put in 10% buffered neutral formalin (BNF) for a period of 4 to 72 h. The tissues were then processed in an automated tissue processor as follows: 2 washes in 10% BNF, 1 wash in 80% alcohol, 2 washes in 95% alcohol, 2 washes in absolute alcohol, 2 washes in xylene, and 2 changes of paraffin. The paraffin embedded tissues with the formalin removed were then stored at room temperature. These tissues were up to two and a half years old.

Blood Standard Extraction—Known blood standards were obtained upon autopsy from each individual tissue donor. DNA was extracted from the standards (known blood dried on cotton) using a Chelex extraction method (30).

Formalin Fixed Paraffin Embedded Tissue Extraction—Approximately 30 micron thick sections of the paraffin blocks were sliced with a microtome. Small slices, approximately 5 m^2, with the paraffin cut away were placed in microcentrifuge tubes containing 200 uL 5% Chelex and 10 uL Proteinase K (10 mg/mL). The tubes were incubated at 56°C for 1 h, agitated at maximum speed using a Vortex for 10 s and boiled for 15 min then centrifuged for 3 min at maximum speed (10,950 g). This resulted in three layers: Chelex beads at the bottom layer, an aqueous middle layer containing DNA, and a top layer of paraffin.

Formalin Fixed Tissue Extraction—Two extraction procedures were used for this tissue. The first was that described above for the extraction of DNA from FFPE tissue. The second method allowed the tissue to digest in a solution of 1% sodium dodecyl sulfate (SDS) and 100 μg/mL Proteinase K in a total volume of 515 μL at 56°C overnight. The samples were then extracted with an equal volume of 25:25:1 phenol/chloroform/isoamyl alcohol. The aqueous DNA-containing layer was then passed through a Microcon 100 filter (Amicon, Beverly, MA) and washed with TE^{-4} buffer (10 mM Tris-HCl, 0.1 mM EDTA).

Quantitation of DNA—The Quantiblot Human DNA Quantitation Kit (Perkin Elmer Foster City, CA) was utilized under conditions recommended by the manufacturer. The procedure is based on the hybridization of a biotinylated oligonucleotide probe (D17Z1) to DNA samples immobilized on a nylon membrane through the use of a slot blot apparatus (31).

PCR Conditions–HLA DQα and PM—The AmpliType HLA DQα Amplification and Typing Kit and the AmpliType PM PCR Amplification and Typing Kit (Perkin Elmer, Foster City, CA) were used. Approximately 2 to 5 ng of extracted DNA were amplified in a Perkin-Elmer Model 9600 Thermal Cycler according to the protocols received with the AmpliType Kits (32,33).

PCR Conditions–D1S80—The AmpFLP D1S80 PCR Amplification Kit (Perkin Elmer, Foster City, CA) was used. Approximately 5 ng of extracted DNA were amplified in a Perkin-Elmer Model 9600 thermal cycler according to the protocol received with the amplification kit (21,22).

Determination of PCR Product—A small capacity test gel was prepared by combining 25 mL 1.0 M Tris, 0.9 M Boric Acid, 0.1 M EDTA (TBE) 0.5×, 40 mg/mL 3:1 NuSieve (FMC, Rockland, Maine) and 2.5 μL Ethidium Bromide (EtBr; 5 mg/mL). Once the gel solidified, 200 mL of 0.5× TBE containing 20 μL EtBr (5 mg/mL) was added to the electrophoresis tank. Seven microliters of amplified DNA were added to 3 μL of loading buffer (50% glycerol, 0.1% bromophenol blue, 0.1 M EDTA dissolved in TE^{-4}). The amplified DNA mixture was loaded into individual wells of the gel. Hae III digested Phi × 174 marker DNA was run in one lane of the gel. The voltage was set to 140 V for 30 min. Both the HLA DQα and PM amplified products were analyzed using this system. Additionally, the D1S80 amplified products were analyzed using the same technique as stated above without the use of the EtBr. These D1S80 gels were stained with SYBER Green (10 μL SYBER Green in 100 mL TBE) (FMC, Rockland, Maine) for one half hour.

Typing of HLA DQα and Polymarker—The typing of the amplified DNA was done according to the validated protocols received with the Amplitype kits. Briefly, the amplified DNA was added to individual wells of typing trays containing typing strips for the particular locus or loci being examined. The detection is based on a reverse dot blot system (32). The HLA DQα and PM genotypes were determined by the pattern of blue dots developed on the probe strips.

PCR Conditions–STR

Amplification was performed using 5 ng of DNA in a 50 μL reaction volume in GeneAmp Thin Walled Reaction tubes. The reaction components included 10× GeneAmp Reaction Buffer, 10 mM each dTTP, dATP, dCTP, and dGTP, 10 μM of each primer pair ((a) BHO QUAD: vWA31: labeled PCR primer [FWD]-JOE, unlabeled PCR primer [REV]; THO1: unlabeled PCR primer [FWD], labeled PCR primer [REV]-FAM; F13A1: unlabeled PCR primer [FWD], labeled PCR primer [REV]-JOE; FES/FPS: unlabeled PCR primer [FWD], labeled PCR primer [REV]-FAM, (b) CTT: CSF1PO: labeled PCR primer [FWD]-TAMRA, unlabeled PCR prime [REV]; TPOX: labeled PCR primer [FWD]-TAMRA, unlabeled PCR primer [REV]; THO1: labeled PCR primer [FWD]-TAMRA, unlabeled PCR primer [REV], and 5 U/μL AmpliTaq DNA Polymerase to give a final concentration of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM of each dNTP, 0.2 μM of each primer (except for the THO1 primers of CTT which are 0.4 μM) and 1.25 U of AmpliTaq DNA Polymerase. (All of the above reagents were obtained through Perkin Elmer, Foster City.) In order to reduce primer dimer formation in the BHO QUAD the PCR reaction was assembled in the following order: buffer, dNTP's, primers (except for FES/FPS labeled [REV]-FAM), H_2O, AmpliTaq DNA Polymerase and then FES/FPS labeled [REV]-FAM. The reaction mix was sealed with two drops of mineral oil, the DNA samples added and amplification carried out in a Perkin Elmer DNA Thermal Cycler Model 480 programmed as follows: (a) BHO QUAD: 95°C 'hold' for 4 min, 28 cycles at 95°C for 1 min, 2 min ramp to 54°C, 1 min at 54°C,

30 s ramp to 72°C, 1 min at 72°C, 30 s ramp to 95°C, final extension at 72°C for 10 min; (b) CTT: 96°C 'hold' for 2 min, 10 cycles at 94°C for 1 min, 2 min ramp to 60°C, 1 min at 60°C, 30 s ramp to 70°C, 1.5 min at 70°C, 30 s ramp to 94°C; 20 cycles at 90°C for 1 min, 2 min ramp to 60°C, 1 min at 60°C, 30 s ramp to 70°C, 1.5 min at 70°C, 30 s ramp to 94°C. PCR reactions were stored at 4°C until required for electrophoretic analysis.

Electrophoresis–STR

Electrophoresis was carried out in an Applied Biosystems 373A DNA Sequencer using 0.4 mm thick 8.3 M urea denaturing polyacrylamide gels (6%T, 5%C) in 1× TBE buffer (Gibco BRL, Grand Island, NY). 1.5 μL of the PCR product was combined with 0.5 μL (6 fmoles) of the GeneScan-2500 (ROX) size standard (Perkin Elmer, Foster City, CA) and made up to 5 μL total volume with loading buffer (41.5% formamide, 4.15 mM EDTA, 2.5 mg/mL blue dextran final concentration), heat denatured at 90°C for 2 min, snap cooled on ice and immediately loaded onto the gel. Gels with a 24-cm distance from the well to laser excitation/fluorescence detection region were utilized and were run for 9 h at a constant voltage of 1700 V.

Data Analysis–STR

Data analysis was carried out using GeneScan 672 software v1.2 (Applied Biosystems Division, Foster City, CA) for construction of the calibration curve from the internal lane standard and for size estimation of the BHO QUAD and CTT alleles. The local southern algorithm option was used to calculate fragment sizes.

Results

Formalin Fixed Paraffin Embedded Tissue

It was found that the addition of Proteinase K to the Chelex solution and incubation at 56°C for 1 h prior to boiling substantially increased the DNA yield from the FFPE tissue (data not shown). It should be noted that as expected no increase of the typing success rate was observed when non-typeable Chelex extracts were subjected to a round of organic extraction. The DNA extracted from the formalin fixed paraffin embedded tissue, where the tissue was first fixed in formalin for one to three days, yielded DNA quantities from .25 ng/μL to greater than 2 ng/μL (Table 1). The quantity determinations were based on intensity comparisons between known standards and the tissue extracts using the Quantiblot system.

TABLE 1—*Quantitation of FFPE tissue.*

Tissue	Yield (ng)	Tissue	Yield (ng)
4	>10	67	2.5
12	2.5	68	4
15	1.25	74	>10
20	8	76	>10
26	8	768	2.5
30	3	773	8
41	>10	776	>10
46	5	778	>10
55	>10	780	8
62	5		

FFPE tissue yield in nanogram quantities per 5 mL of Chelex extracted DNA.

Amplification was performed according to standard parameters previously validated. Upon amplification of the extracted DNA in the HLA DQα and the PM PCR systems a product gel was run to assess the success of the amplification. As expected, the product gels from the HLA DQα amplifications yielded one band per tissue whereas the PM product gels yielded six bands for each tissue. These bands fell in the appropriate size range as determined by comparison to the control DNA and Hae III digested Phi × 174 marker. The control blood standards from each tissue donor were assessed in the same fashion and gave the same product gel results as these tissues. An example of this banding pattern is pictured (Product Gel, Fig(s). 1 and 2). An example of the typing strip results is also pictured (Typing Strips, Fig(s). 3 and 4). The typing results obtained from all of the FFPE tissue and the blood standards at the HLA DQα and PM genetic loci are listed in Table 2. The analysis was repeated on a portion of the tissues and standards to demonstrate that the results were indeed reproducible (Results not shown).

The same extract used for the HLA DQα and PM amplifications was utilized for the D1S80 amplification. This did not produce typeable results in the D1S80 system, even though a range of input DNA (2.5–15 ng) was added, with and without the addition of

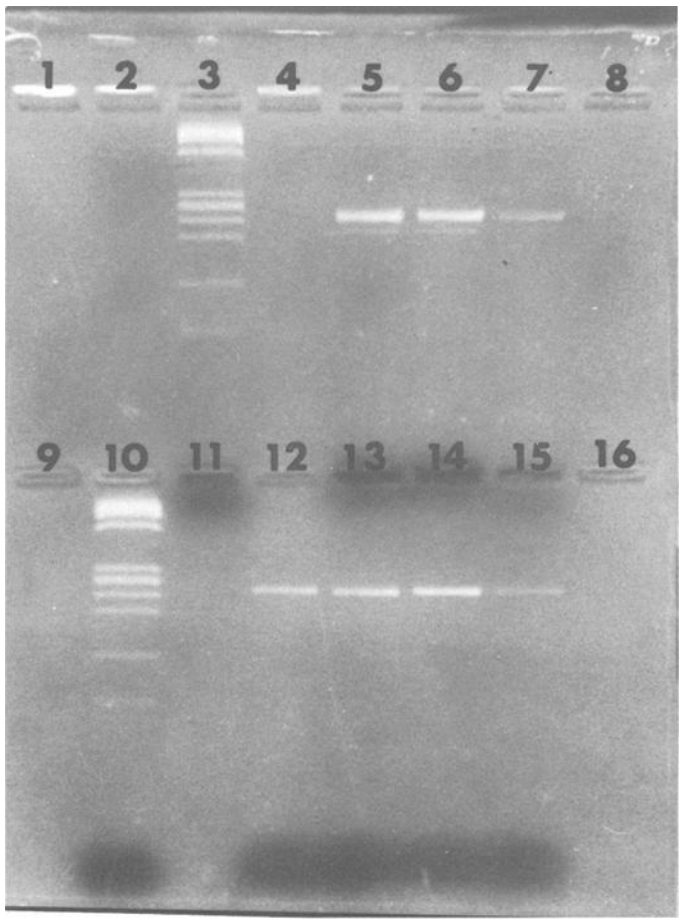

FIG. 1—*Blood standard and FFPE tissue HLA DQα product gel. HLA DQα amplimers for blood standards and formalin fixed paraffin embedded tissues. Lane 5 contains standard 776; Lane 6 contains standard 76; Lane 7 contains standard 778; Lane 13 contains tissue 776; Lane 14 contains tissue 76; Lane 15 contains tissue 778; Lane 12 contains a positive control; and Lanes 3 and 10 contain the Hae III digested Phi × marker. Lanes 4 and 11 contain extraction blanks and Lanes 1,2,8,9, and 16 are empty.*

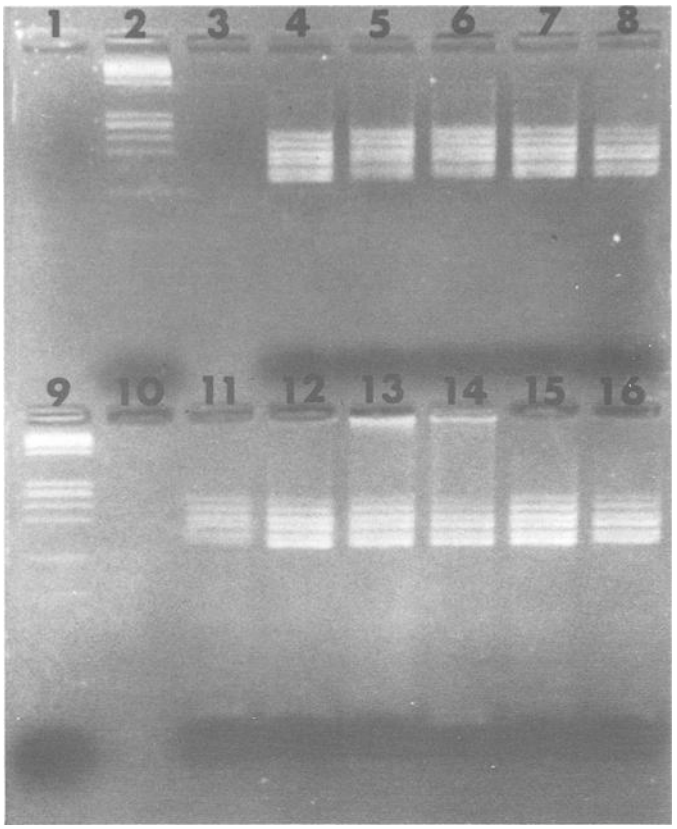

FIG. 2—*Blood standard and FFPE tissue PM product gel. PM amplimers for blood standards and formalin fixed paraffin embedded tissue. Lane 4 contains standard 776; Lane 5 contains standard 68; Lane 6 contains standard 62; Lane 7 contains standard 78; Lane 8 contains standard 778; Lane 12 contains tissue 776; Lane 13 contains tissue 68; Lane 14 contains tissue 62; Lane 15 contains tissue 76; Lane 16 contains tissue 778; Lane 11 contains a positive control sample and Lanes 2 and 9 contain the Hae III digested Phi × marker. Lanes 3 and 10 contain extraction blanks and Lane 1 is empty.*

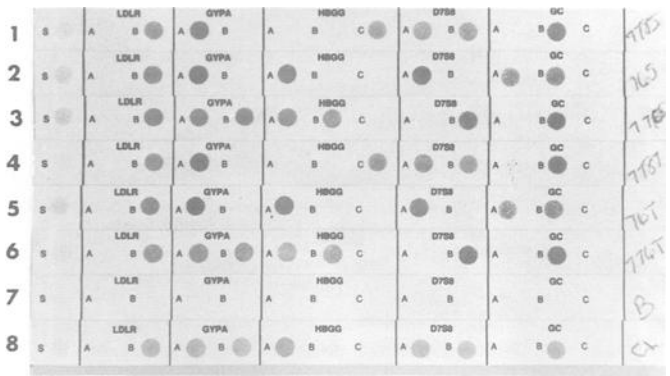

FIG. 3—*Blood standard and FFPE HLA DQα typing strips. HLA DQα typing results for blood standards and formalin fixed paraffin embedded tissues; strips 1 through 3 contain standards 778, 76, and 776, respectively, strips 4 through 6 contain tissues 778, 76, and 776, respectively. Strip 7 contains the reagent blank. Strip 8 contains the positive control DNA supplied in the AmpliType HLA DQα kit.*

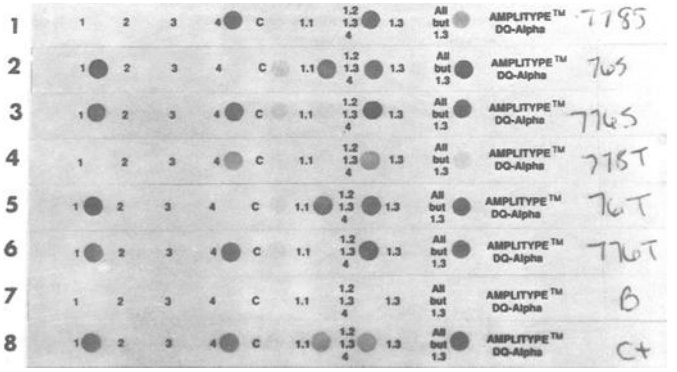

FIG. 4—*Blood standard and FFPE PM typing strips. PM typing results for blood standards and formalin fixed paraffin embedded tissues; strips 1 through 3 contain standards 778, 76, and 776, respectively, strips 4 through 6 contain tissues 778, 76, and 776, respectively. Strip 7 contains the reagent blank. Strip 8 contains the positive control DNA supplied in the AmpliType PM kit.*

TABLE 2—*FFPE and blood standard HLA DQα and PM typing results.*

STD	HLA DQα	LDLR	GYPA	HBGG	D7S8	GC
4	1.3,2	BB	AB	AB	AA	BB
12	2,4*					
15	1.2,4*					
20	1.1,1.2	BB	AB	AA	AB	BB
26	1.3,1.3	AB	BB	AB	AB	AC
30	1.2,4	BB	AB	AC	AB	AC
41	1.2,1.3	BB	AB	BC	AA	BC
46	1.1,1.1	BB	AA	AA	BB	AB
55	1.2,4	BB	BB	CC	AA	BB
62	1.2,2	BB	BB	BC	AA	BB*
67	2,4	AB	AA	AB	AA	CC†
68	3,4	BB	AA	AB	AA	BC
74	2,4	BB	BB	AA	AB	BC
76	1.1,1.2	BB	AA	AA	AA	AB
768	2,2	BB	AA	CC	AB	AB
773	1.1,1.3	AB	AB	AA	AB	BC
776	1.2,4	BB	AB	AB	BB	BB
778	4,4	BB	AA	CC	AB	BB
780	1.2,4	AB	AA	AC	AB	BB

Results of blood standards and FFPE tissues from each donor at HLA DQα and PM genetic loci.

*Tissue too degraded for analysis.

†Reproducible sample contamination in the GC region of the tissue sample.

BSA, to the amplification mix. The same extract was washed through a Microcon 100 (Amicon, Beverly, MA) filtration device to concentrate the DNA from a volume of approximately 150 μL to a volume of 20 μL and to possibly remove any inhibitory products. An attempt to amplify this concentrated volume of DNA was made and no product was detected. A second extraction technique performed on the original tissue utilizing 1% SDS and Proteinase K digestion with an organic extraction also failed to produce typeable DNA for the D1S80 system. The product gel from these tissues did not show any detectable levels of amplified DNA, whereas the blood standard which underwent the same procedure produced two discrete D1S80 bands on the product gel as expected (not pictured). Also polyacrylamide gel electrophoresis confirmed the absence of D1S80 amplified product.

Five blood and FFPE tissue sample pairs (#62, #68, #76, #776,

and #778) were subjected to STR analysis using the BHO QUAD and the CTT multiplex systems. The five pairs of blood and FFPE tissue samples were all successfully typed at the six STR loci vWA31, THO1, F13A1, FES/FPS, CSF1PO, and TPOX with the exception of one tissue sample (#62) which could not be typed at the CSF1PO and TPOX loci (Table 3). Although all five blood samples were amenable to standard multiplex analysis, some of the tissue samples initially proved refractory to typing at several of the STR loci. One of the tissue samples (#776) demonstrated loss of the loci F13A1 and FES/FPS, another (#62) lost F13A1, FES/FPS, and all CTT loci, whereas another (#68) lost all four BHO QUAD loci. However upon re-amplification of the BHO-QUAD loci using duplex analysis (F13A1 and FES/FPS (#62, #776); vWA31 and F13A1 (#68); THO1 and FES/FPS (#68)) in combination with an increased cycle number (34 cycles as opposed to the standard 28) all tissue samples were successfully typed at these loci. Re-amplification of tissue #662 by standard CTT multiplex resulted in a reportable signal for THO1 but still yielded no result for the loci CSF1PO or TPOX, which comprise the longest amplimers of the six STR loci analyzed.

Formalin Fixed Tissue

The Chelex and the organic extraction procedures used on the formalin fixed tissue obtained from the Washoe County Coroners Office did not yield a sufficient amount of intact DNA for typing in the PCR systems examined here. No results were visible in the quantitation procedure of the extracted DNA. Any DNA present from either extraction procedure was below the level of detection (0.15 ng) of the quantitation procedure used here. An attempt to amplify the DNA extracted from both procedures using the HLA DQα and PM systems failed to yield any amplified DNA product (not pictured).

Discussion

A number of studies have shown that PCR is a technique that can be used on formalin fixed paraffin embedded (FFPE) tissue when the fixation time is limited to less than three days (9,13,14,34). Several extraction techniques have been attempted and many different targets have been utilized. Successfully detected amplimers range in size from 100 to 1500 bp (10,11). Many variables need to be considered in the successful amplification of DNA extracted from FFPE; 1) length of time between sampling from body and fixation, 2) length of time of fixation, 3) fixation material, 4) age of the paraffin block, and 5) method of DNA extraction. The greatest variation in extraction procedures previously attempted was the length of time the samples were digested with Proteinase K. Although some procedures require less than a 24 h digestion (35), others require separate additions of the enzyme over time (36) and one even required a 5 day incubation (37).

TABLE 3—*STR typing results.*

Sample	VWA	THO1	F13A1	FES/FPS	TPOX	CSF1PO
62 Blood	15,17	7,7	3,5	9,12	8,11	10,12
62 Tissue	15,17	7,7	3,5	9,12	NR*	NR
68 Blood	14,17	7,9.3	5,8	11,12	10,11	10,10
68 Tissue	14,17	7,9.3	5,8	11,12	10,11	10,10
76 Blood	16,16	7,7	3,5	12,12	11,11	7,8
76 Tissue	16,16	7,7	3,5	12,12	11,11	7,8
776 Blood	17,17	7,9	3,7	10,12	9,9	10,11
776 Tissue	17,17	7,9	3,7	10,12	9,9	10,11
778 Blood	16,17	7,9.3	6,7	11,12	11,11	10,12
778 Tissue	16,17	7,9.3	6,7	11,12	11,11	10,12

*NR = No result obtained.

Formalin fixed paraffin embedded tissue appears to be a good source of DNA for the analysis of at least 12 PCR systems. The forensic value of FFPE tissues is shown throughout the entire process in obtaining PCR results. The tissues yielded greater than 2 ng/μL Chelex extracted DNA. This result indicated that the extraction procedure was successful and contained enough DNA for use in the PCR reaction of interest. The extracts were amplified according to previously validated procedures and produced typeable DNA as indicated by the product gels for HLA DQα and PM. The tissue gave bands of equal or greater intensity in comparison to the control DNA (Examples, Fig(s). 1 and 2). This result indicated that the amplification procedure was successful. The FFPE tissue gave unambiguous results for the reverse dot blot typing procedure. Referring to Table 2 and the examples in Fig(s). 3 and 4 the HLA DQα and PM types for the tissue and blood standards gave the same results.

In general STR analysis proved to be a successful means of typing FFPE tissues. However for three of the five FFPE samples modification to the standard BHO QUAD multiplex procedure had to be made in order to obtain reportable results for all specimens. Specifically a combination of increased cycle number and duplex as opposed to quadraplex analysis yielded allelic signals with acceptable signal to noise ratios. This illustrates the potential usefulness of possessing validated non-standard PCR protocols that may be required to be implemented for particularly refractory samples. In all instances the paired blood and FFPE tissue specimens demonstrated somatic stability in that the same STR alleles were detected in both specimens. Re-amplification of tissue #662 by standard CTT multiplex resulted in a reportable signal for THO1 but still yielded no result for the loci CSF1PO or TPOX which comprise the longest amplimers of the six STR loci analyzed. These results are consistent with the hypothesis that FFPE tissue consists of varying amounts of degraded and/or cross-linked DNA such that the success of amplification is dependent upon the effective number average molecular length (L_n) of the DNA molecules in the particular sample. If L_n is less than the expected amplimer size (α) at a particular locus then amplification would be unsuccessful or inefficient depending upon the extent of this difference. Conversely successful amplification would be obtained in those instances where L_n is greater than or equal to α.

The fact that the FFPE tissue and blood standards from the same individual gave the same results shows that the process of formalin fixing and paraffin embedding does not result in erroneous typing results. It is clear that DNA extracted from FFPE tissues can be used in forensic examinations. However, two FFPE tissues (#12 and #15) were not suitable for use in the systems examined here. Product gel results upon amplification at the HLA DQα and PM loci of these tissues resulted in smearing rather than banding indicating no amplifiable product. These tissues may have been at or past the three day limit of formalin fixation and were rendered too degraded for amplification at these regions. These tissues were not examined at any additional loci. As shown in Table 2, FFPE tissue sample number 67 gave reproducible contamination at the GC locus. A light B dot appeared in the tissue sample but did not appear in the blood standard for this tissue. Contamination, only observed in one of our samples, can be derived from various sources: sample handling, formalin solution, storage in close contact with other tissues, etc. Contamination is always an issue to be aware of in any PCR examination. Despite the results for the

three tissue samples mentioned above, it is still apparent that FFPE tissue can be a reliable source of DNA.

The 12 systems that did give typing results contain relatively small amplimers, the largest being 317 bp in length for the CSF1PO locus. In the D1S80 system, the targeted DNA amplimers range from 369–785 bp in length. The tissue from paraffin blocks examined in this study remained in formalin for a period of one to three days during which time the DNA is rapidly degrading and forming cross links between neighboring amino groups (5–8). Moerkerk (8) found that the amount of DNA extracted from colonic carcinomas was reduced by 90–95% after 1 h fixation. The D1S80 system contains alleles too large to be detected when the DNA sample source has been previously subjected to formalin fixation as it was in this study. It appears that the amplimer size requirement is simply too large. This is supported by the product gels and the polyacrylamide gel run on these samples as they showed no amplified product.

Formalin fixed tissue (not paraffin embedded) was examined in six PCR systems; HLA DQα and PM did not provide suitable DNA for typing for these six PCR techniques examined here. DNA extracted from the FF tissue was not detectable on the Quantiblot system used here. Upon amplification of the extracts no amplified product was obtained on the product gels. As expected no DNA types were detected on the typing strips, therefore FF tissue was not examined in additional systems. Autopsy tissue is typically stored in a container of buffered formalin. It may be feasible to use this tissue at later dates to perform histological examinations but it is not useful for currently available forensic DNA examinations. A general consensus appears to be that tissue fixed in formalin for more than three days renders the DNA undetectable by the methods used here (9,13,14,34). The tissue examined in this portion of the study was probably fixed in formalin too long to yield usable DNA for these six PCR systems.

In summary, FFPE tissue could prove to be a reliable source for victim standards in the comparison of evidence utilizing current PCR technology. However FF tissue does not appear to be quite as promising due to the detrimental cross linking effects over time. In the ideal situation the tissue extracted from the body should be fixed in buffered neutral formalin immediately upon removal. The tissue should not be left to fix for more than three days before it is prepared and stored in the paraffin block form. A paraffin block is easily stored and would be another useful resource as a standard in PCR examinations. The block could prove to be quite useful for comparison to evidence when the evidence in question is not available at the time of death and the blood sample has not been properly preserved. These types of tissues may require a DNA examination for purposes other than that of serving as a standard. Such samples may require examination to determine a common source.

Formalin was studied here because it is a very commonly used fixative and one that may be encountered more frequently than other types of fixatives. However, it should be noted that fixatives other than formalin have been examined (9,11,12,13,37). Greer (11,12) has done extensive studies on different fixatives verses the length of DNA fragments obtainable over fixation time. In fact they found that 95% ethanol was the most suitable fixative for keeping DNA in a useable condition for analysis. This fixative may prove to be of forensic value also.

Acknowledgments

We would like to acknowledge: Bobbie Stacks from the University of Tennessee Toxicology and Chemical Pathology Laboratory for supplying the formalin fixed paraffin embedded tissues, Vernon O. McCartey, Coroner of Washoe County, for supplying the formalin fixed tissue, Kristin Garvin of Perkin-Elmer/Roche Molecular Systems for supplying the AmpliType HLA DQα, PM and D1S80 Kits, Willy Stevenson for the photography and the Washoe County Sheriff's Office, Forensic Science Division for allowing the time and tools to complete this project.

References

1. Budowle B, Baechtel FS, Adams DE. Validation with regard to environmental insults of the RFLP procedure for forensic purposes. In: Forensic DNA Technology, Farley MA, Harrington JJ, editors. 1991:83–91.
2. Budowle B, Baechtel FS. Modifications to improve the effectiveness of restriction fragment length polymorphism typing. Appl Theor Electrophoresis 1990;1:181–7.
3. Blake E, Mihalovich J, Higuchi R, Walsh PS, Erlich HA. Polymerase chain reaction (PCR) amplification and human leukocyte antigen (HLA)-DQα oligonucleotide typing on biological evidence samples: Casework experience. J Forensic Sci 1992;3:700–26.
4. Saiki RK, Walsh PS, Levenson CH, Erilch HA. Genetic analysis of amplified DNA with immobilized sequence specific oligonucleotide probes. Proc Nat Acad Sci USA 1989;866:230–4.
5. Brutlag D, Schlehuber C, Bonner J. Properties of formaldehyde-treated nucleohistone. Biochemistry 1969;8:3214–8.
6. Feldman MY. Reactions of nucleic acids and nucleoproteins with formaldehyde. Progr Nucl Acid Res Mol Biol 1973;13:1–49.
7. Kiene P, Milde-Langosh K, Runkel M, Schulz K, Loning T. A simple and rapid technique to process formalin-fixed, paraffin-embedded tissues for the detection of viruses by the polymerase chain reaction. Virchows Arch A Pathol Anat 1992;420:269–73.
8. Moerkerk PT, Han KJ, Tenkate J, deGoeij A, Bosman JT. Southern and dot blot analysis of DNA from formalin-fixed paraffin-embedded tissue samples from colonic carcinaomas. Virchows Arch B Cell Pathol 1990;58:351–5.
9. Shibata D, Kurosu M, Noguchi TT. Fixed human tissue: A resource for identification of individuals. J Forensic Sci 1991;36:1204–12.
10. Goelz SE, Hamilton SR, Vogelstein B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochem Biophys Res Comm 1985;130:118–26.
11. Greer CE, Lund JK, Manos MM. PCR amplification from paraffin embedded tissues: Recommendations on fixatives for long term storage and prospective studies. PCR Meth Appl 1991;1:46–50.
12. Greer CE, Peterson SL, Kiviat NB, Manos MM. PCR amplifications from paraffin-embedded tissues: Effects of fixative and fixation time. Am J Clin Pathol 1991 Feb;117–24.
13. Koraljka G, Pavelic J, Jadro-Santel D, Poljak M, Pavelic K. DNA amplification by polymerase chain reaction form brain tissues embedded in paraffin. Int J Exp Pathol 1993;74:333–7.
14. Malmgren C, Gustavsson I, Hurte I, Saldeen T, Gyllensten U. Reliable typing of DNA amplified from formalin-fixed tissue biopsies. PCR Meth Appl 1992;2:175–6.
15. Yamamoto T, David CG, Brown MS, Schneider WJ, Casey ML, Goldstein JL, et al. The human LDL receptor a cysteine-rich protein with multiple Alu sequences in its mRNA. Cell 1984;39:27–38.
16. Siebert PD, Fukuda M. Molecular cloning of human glycophorin B cDNA: Nucleotide sequence and genomic relationship to glycophorin A. Proc Nat Acad Sci USA 1987;846:735–9.
17. Slightom JL, Blechl AE, Smithies O. Human fetal G-gamma and A-gamma glogin genes; Complete nucleotide sequences suggest that DNA can be exchanged between these duplicated genes. Cell 1980;21:627–38.
18. Bartels I, Grzeschik KH, Cooper DN, Schmidtke J. Regional mapping of six cloned DNA sequences on human chromosome 7. Am J Hum Genet 1986;38:280–7.
19. Horn GT, Richards B, Merril JJ, Klinger KW. Characterization and rapid diagnostic analysis of DNA polymorphisms closely linked to the cystic fibrosis locus. Clin Chem 1990;36:1614–9.
20. Yang F, Brune JL, Naylor SL, Apples RL, Naberhaus KH. Human group specific component (GC) is a member of the albumin family. Proc Nat Acad Sci USA 1985;82:7994–8.
21. Budowle B, Chakraborty R, Giusti AM, Eisenburg AJ, Allen RC.

Analysis of the VNTR locus D1S80 by the PCR followed by high resolution PAGE. Am J Hum Genet 1991;48:137–44.
22. Kloosterman AD, Budowle B, Daselaar P. PCR-amplification and detection of the human D1S80 VNTR locus: Amplification conditions, population genetics, and application in forensic analysis. Int J Leg Med 1993;105:257–64.
23. Edwards A, Civitello A, Hammond HA, Caskey CT. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am J Hum Genet 1991;49:746–56.
24. Kimpton CP, Gill P, Walton A, Urquart A, Millican ES, Adams M. Automated DNA profiling employing multiplex amplification of short tandem repeat loci. PCR Meth Appl 1993;3:13–22.
25. Kimpton C, Fisher D, Watson S, Adams M, Urquart A, Lygo J, et al. Evaluation of an automated DNA profiling system employing multiplex amplification of four tetrameric STR loci. Int J Leg Med 1994;106:302–11.
26. Lygo JE, Johnson PE, Holdaway DJ, Woodroffe S, Whitaker JP, Clayton TM, et al. The validation of short tandem repeat (STR) loci for use in forensic casework. Int J Leg Med 1994;107:77–89.
27. Huang NE, Schumm J, Budowle B. Chinese population on three tetrameric short tandem repeat loci-HUMTHO1, TPOX, and CSF1PO-derived using multiplex PCR and manual typing. Forensic Sci Int 1995;71:131–6.
28. Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R. Evaluation of thirteen STR loci for use in personal identification applications. Am J Hum Genet 1994;55:175–89.
29. Anker R, Steinbreuck T, Donnis-Keller H. Tetranucleotide repeat polymorphism at the human thyroid peroxidase (hTPO) locus. Hum Mol Genet 1992;1:137.
30. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for sample extraction of DNA for PCR based typing from forensic material. BioTechniques 1991;4:506–13.
31. Walsh PS, Valaro J, Reynolds R. A rapid chemiluminescent method for quantitation of human DNA. Nucl Acids 1992;20:5061–5.
32. The Perkin Elmer Corporation. AmpliType User Guide, Version 2. Norwalk, CT. 1992.
33. Herrin G Jr, Fildes N, Reynolds R. Evaluation of the AmpliType PM DNA test system on forensic case samples. J Forensic Sci 1994;39:1247–53.
34. Rogers BB, Alpert LC, Hine EA, Buffone GJ. Analysis of DNA in fresh and fixed tissue by the polymerase chain reaction. Am J Pathol 1990 Mar;136.
35. An SF, Flemming KA. Removal of inhibitors of the polymerase chain reaction from formalin fixed, paraffin wax embedded tissues. J Clin Pathol (England), 1991;44(11):924–7.
36. Grunwald K, Lyons J, Hansen-Hagge TE, Janssed JWG, Feichtinger H, Bartram CR. Molecular genetic analysis of DNA obtained from fixed, air dried of paraffin embedded sources. Hemotology 1991;62:108–14.
37. Jackson DP, Lewis FA, Taylor GR, Boylston AW, Quirke P. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. Lab Techniques 1990 Jan.;499–504.

Additional information and reprint requests:
Renee L. Romero
Criminalist
Washoe County Sheriff's Office
Forensic Science Division
911 Parr Blvd.
Reno, NV 89512