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TECHNICAL NOTE

PATHOLOGY/BIOLOGY; CRIMINALISTICS

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Effects of Histological Staining on the Analysis of Human DNA from Archived Slides*

ABSTRACT: Archived slides of cell smears treated with histological stains for sperm detection are often the only source of DNA available when cold cases are reopened. There have been conflicting reports as to the negative effects of particular histological stains on DNA recovery and quality from human cells, making stain selection an important consideration for forensic laboratories. This study investigates the effect of several staining systems on DNA recovery from histological slide samples stored from 0 to 10 weeks. DNA profiles obtained after analysis of these samples with AmpF*l*STR® Identifiler™ and increased cycle AmpF*l*STR® SGM Plus™ short tandem repeat (STR) profiling systems and the effects that these stains have on DNA quantity and quality over time are described. Results indicate that Christmas Tree and Hematoxylin and Eosin stains do not have significantly different effects on DNA quality after 10-week storage of slides. This research will assist scientists to select staining systems that have minimal deleterious effects on the DNA recovered.

KEYWORDS: forensic science, histochemistry, epithelial cells, archived slides, STR profiling, DNA quality, laser microdissection

The advent of improved cell selection methods using laser microdissection (LMD) technology has given forensic scientists the ability to isolate cells that are differentially stained to indicate cell type, and the increased sensitivity offered by sensitive multiplex short tandem repeat (STR) systems allows DNA profiling from very few selected cells. Although LMD is becoming more commonplace in forensic laboratories, glass slide preparations for the detection of sperm are still standard practice and may be the only available DNA source in the reopening of cold cases. Because of the sensitivity of the DNA profiling systems to any co-extracted inhibitors, and the possibility that only small numbers of cells are available for testing from the slides, it is important that the staining methods utilized for cell identification have minimal impact on downstream DNA analysis.

There are a range of histological stains used to detect the presence of sperm in forensically important biological samples. The most commonly used histological stains in forensic laboratories are the Hematoxylin and Eosin (H&E), Papanicolaou, and Christmas Tree stains (1,2) but Giemsa, Crystal Violet, and Methylene Blue stains are also used. Recently, there have been conflicting reports as to the negative effects of histological stains on the quality and quantity of DNA extracted from slides, although the majority of these focus on the use of LMD slides. In particular, studies by Di Martino et al. (3) and Sanders et al. (4) have indicated that the Picric acid in Christmas Tree staining degrades DNA and inhibits the PCR, while Pritchard et al. (5) reported no adverse effects from using this stain. Because of its ease of interpretation, the Christmas Tree stain is one of the most widely used histological tests for the identification of sperm in sexual assault cases, so its effects on DNA recovery have far-reaching implications. H&E staining is one of the most common stains used in human histology, including forensic laboratories, and can be used for identification of sperm by cell shape and color variation. Amplification of DNA from H&E- and Papanicolaou-stained cytological slides has been previously investigated using individual STR PCR (6), but not with the multiplex systems currently in use.

This study focuses on the analysis of DNA from stained vaginal epithelial cells permanently mounted on glass slides stored for varying lengths of time. Epithelial cells recovered from an archived slide are generally considered to be more problematic for DNA profiling. The H&E and Christmas Tree staining methods were selected for investigation because of their widespread use in forensic laboratories. Two further stains were selected because of our laboratory's interest in identification of epithelial cell types. A modified Danes stain was developed to distinguish vaginal from buccal and skin epithelial cells (7), and we have been investigating the use of 3.3′-diaminobenzidine tetrahydrochloride–peroxidase immunohistochemistry (immuno-DAB) and specific protein markers as an alternate method to identify specific epithelial cell types in forensic samples (8).

Materials and Methods

Epithelial Cell Slide Preparation

Vaginal cells from a single donor were collected onto cotton swabs (Medical Wire & Equipment Co., Bath, UK), stored at room temperature for up to 24 h, and then smeared directly onto glass slides (Esco, Oakridge, NJ). The majority of cells were fixed in 50:50 acetone:methanol for 90 sec. Because of the particular requirements of the modified Danes staining procedure, samples to be stained using this method were smeared onto Poly-L-lysine-coated slides and fixed in methanol for 10 sec. A number of unstained cell slides were also prepared using the Poly-L slides and methanol fixation to confirm that these changes had no additional

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effect on the DNA extraction (data not shown). Six slides were prepared for each treatment at each time point.

Slide Staining

Four different staining methods were examined during this study. H&E staining was carried out by pipetting the stains directly onto slides to avoid cross contamination by use of stain baths. Slides were stained with Hematoxylin (BDH Chemicals, Poole, UK) for 5 min and washed with tap water, then stained with Eosin (Merck KGaA, Darmstadt, Germany) for 1 min and washed with tap water. Christmas Tree staining was also carried out directly onto slides that were incubated at room temperature with Nuclear Fast Red (BDH Chemicals) for 15 min, washed with distilled water, stained with Picroindigocarmine for 15 sec and then rinsed with absolute ethanol. Treatment of methanol-fixed cells with the modified Danes staining process was performed as described in French et al. (7) and included use of Hematoxylin, Lithium Chloride (BDH), Phloxine (Harleco, Philadelphia, PA), Alcian Blue (Serva, Heidelberg, Germany), and Orange G (BDH) stains. DAB immunohistochemistry was carried out as described previously (8), but using mouse anti-cytokeratin 13 (anti-CK13) (Novocastra, Newcastle, UK) as the primary antibody. Slides containing fixed, unstained cells were used as the positive control in this experiment.

After the staining treatment, all slides were air-dried, then mounted with a coverslip using DPX permanent histology mountant (Fluka, Sigma Aldrich, Germany), and left to set overnight in a fume hood.

Slide Storage

Mounted slides were stored at room temperature for a range of time points (0, 2, 4, 10 weeks). For the 0-week storage time point, the slides were dried overnight to allow the mountant to set and then the next day transferred to the xylene soak for coverslip removal. Stained and mounted slides were visualized with a Leica DM 1000 LED compound light microscope and the Leica LAS imaging software (Leica Microsystems, Wetzlar, Germany). Representative images of the samples were captured for each slide. The numbers of cells present in each of five randomly selected fields of view within the stained area of the slide were counted, and the total number of cells present per slide calculated using the area of the fields of view and the total area covered by the stain.

DNA Extraction

The coverslips were removed from the sealed slides by soaking overnight in xylene. Cells were scraped from the surface of the glass slide using a sterile scalpel blade and washed into a microfuge tube with small aliquots of xylene (not exceeding 100 μL in total). Cells were collected by centrifugation at 15,700 × *g* for 10 min, washed with ethanol to remove xylene from the samples, and recentrifuged. DNA was extracted following an organic extraction method adapted from Bright and Petricevic (9). The cell pellet was resuspended in 300 μL of TBE extraction buffer (1.2 g/L Tris, 3.72 g/L EDTA, 5.84 g/L NaCl, pH 8.0), 2% SDS and 10% proteinase K (Boehringer Mannheim, Mannheim, Germany) and incubated at 55°C overnight. Following a phenol : chloroform clean-up, DNA was then recovered by ethanol precipitation (1/10th volume of 2 M Na Ac, 2.5 volume absolute ethanol, at −20°C for 1 h), centrifugation at 15,700 × *g* for 15 min, air-dried, and resuspended into 45 μL of distilled water.

DNA Analysis

The concentration of the DNA sample extracted from each slide was determined using the Quantifiler™ (QF) Human DNA Quantification kit (Applied Biosystems Incorporated, Foster City, CA) in accordance with the manufacturer's instructions and using an ABI PRISM® 7500 Real-time PCR System. If inhibition was indicated by results, then samples were diluted by half and requantified. DNA amplification was performed using the AmpF*l*STR® Identifiler™ (Applied Biosystems Incorporated) STR profiling system at 28 cycles and according to the manufacturer's recommendations. Approximately 1.5 ng of DNA was added per reaction. Increased amplification cycle reactions were carried out using the AmpF*l*STR® SGM Plus™ (Applied Biosystems Incorporated) STR profiling system. This was performed following the manufacturer's recommendations except for the PCR cycle number that was increased to 34. Approximately 0.1 ng of DNA per reaction was used in the increased cycle reactions for samples at low template DNA levels. All amplified products were analyzed on an Applied Biosystems™ 3130 capillary electrophoresis instrument using a 10 sec injection at 3 kV. Allele peak height and peak area data of the resultant DNA profiles were analyzed using FaSTR, an in-house program developed by ESR (10).

Results and Discussion

Four different treatments were used to stain the cells for this experiment. The positive controls were slides of unstained cells, fixed and mounted in the same way as the stained samples, and compared with cells treated with H&E, Christmas Tree, Modified Danes, and immuno-DAB stains. Representative images from the four cell staining methods used are shown in Fig. 1. Once mounted with DPX and a coverslip, the unstained cells could not be easily visualized with the microscope, and so the number of cells present on these slides could not be calculated.

The stained slides were stored at room temperature for a range of time periods from 0 to 10 weeks, and then the DNA was extracted from the samples using an organic extraction method. Although stained slides from forensic casework may be stored for years, the 10-week period was selected for the initial study on the effect of staining on DNA profiling. The amount of DNA extracted from the various histologically stained slides was calculated using the Quantifiler™ kit and is presented in Fig. 2. The unstained cells produced the largest amounts of DNA, followed by the slides containing Christmas Tree- and H&E-stained cells. The other staining methods produced much smaller amounts of DNA, in particular the immuno-DAB-stained slides where the quantities were very low.

As the amount of DNA extracted from any particular slide is affected by the number of cells on the slide in question, the amount of DNA extracted from the slides was normalized against the number of cells present. The normalized data are presented in Fig. 3 but do not include the positive control slides, where the unstained cells could not be counted. Once adjusted for number of cells, the amount of DNA extracted from H&E- and Christmas Tree-stained slides at each time point was more similar.

The Quantifiler™ kit also contains an internal control amplification reaction that enables the investigator to determine whether the quantification is being inhibited by compounds present in the extracted DNA. This is judged by the number of PCR cycles it takes the internal control reaction to reach a predetermined threshold. None of the reactions with DNA from unstained, H&E-, or Christmas Tree-stained cells showed any inhibition, but both the

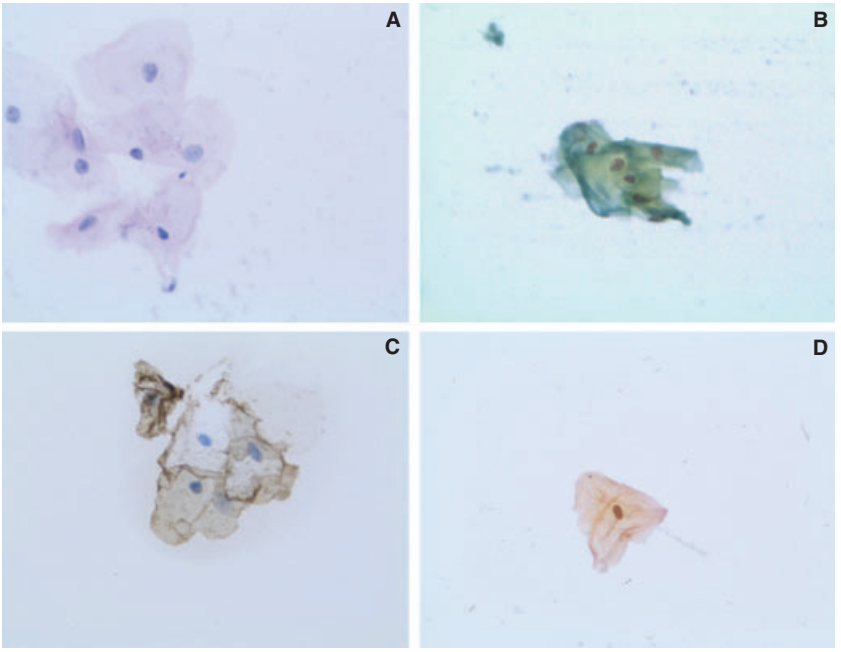

FIG. 1—*Representative examples of vaginal epithelial cells treated with the range of stains used in this study and permanently mounted with DPX and a coverslip. (A) Hematoxylin and Eosin stain. (B) Christmas Tree stain. (C) Immuno-DAB staining, using mouse anti-CK13 as the primary antibody. (D) Modified Danes stain.*

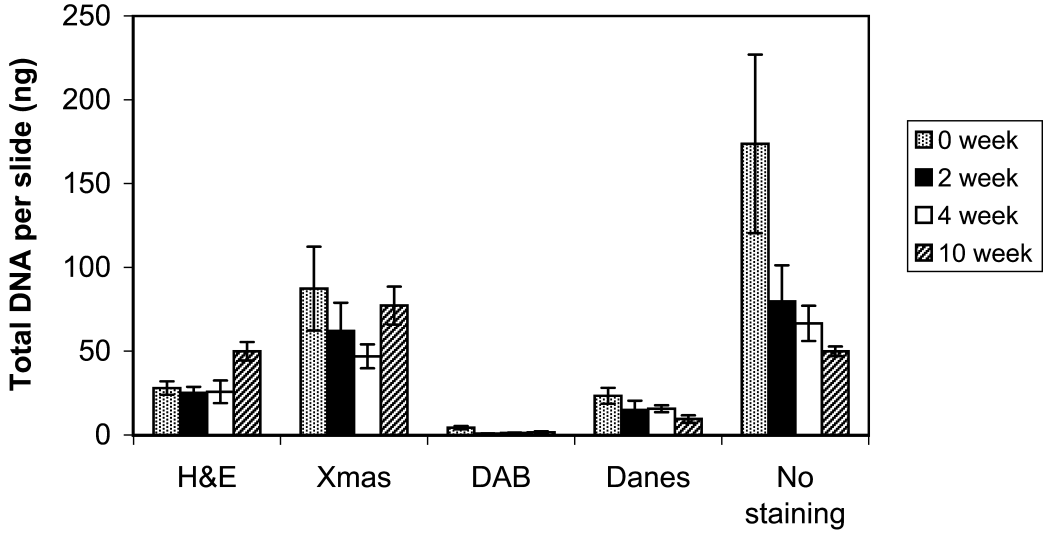

FIG. 2—*Total amounts of DNA (ng) extracted per slide for samples stored from 0 to 10 weeks. Data presented as mean total DNA extracted per slide, error bars indicate standard errors of the mean* ($n \geq 6$). *H&E, Hematoxylin and Eosin staining; CT, Christmas Tree staining; DAB, immuno-DAB staining; Danes, modified Danes stain.*

immuno-DAB- and modified Danes-stained cells were affected (Table 1).

Statistical Analysis of Effects on DNA Recovery by Staining Treatments

Examination of the data suggested that there was likely to be an effect of staining treatment on the mean amounts of DNA extracted per cell. In particular, it was noted that the amounts of DNA extracted from immuno-DAB- and modified Danes-treated slides were lower than that from Christmas Tree- and H&E-treated slides (Fig. 3). The situation regarding the effect of storage time was more complex. It would seem that there is an initial rise followed by decay in amounts of DNA recovery over time; however, this early rise in DNA extracted per cell is strongly counter-intuitive. To examine this in a more objective fashion, two-factor analysis of variance (ANOVA) was undertaken in EXCEL™. When two factors are examined, the analysis gives us information on each factor separately and on any potential interaction between the factors. The analysis confirms a significant effect of staining treatment ($F = 14.5$, p-value = 1.25E-07, d.f. = 3). The ambiguous situation regarding storage time is reinforced by the ANOVA statistic, which

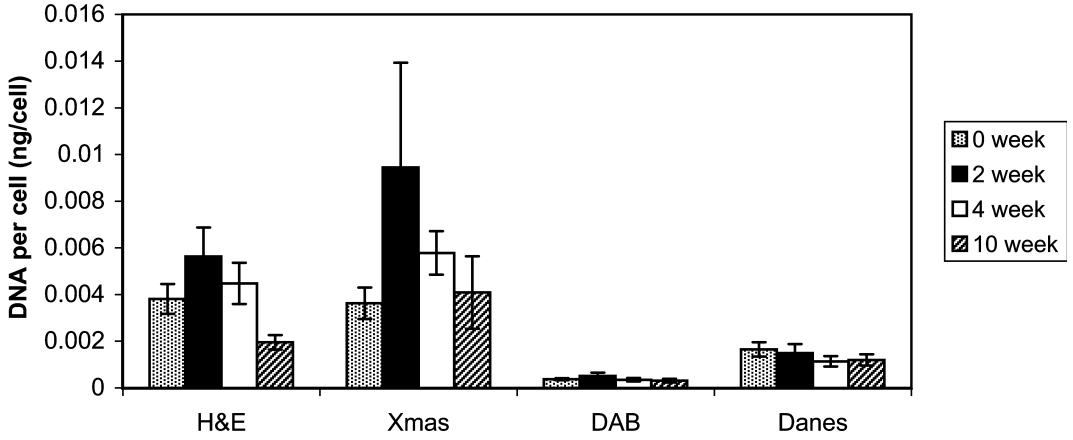

FIG. 3—*Average quantity of DNA extracted per cell for slides stored from 0 to 10 weeks. Data presented as mean total DNA extracted (ng) per cell, across slides in a single treatment, error bars indicate standard errors of the mean* ($n \geq 6$). *H&E, Hematoxylin and Eosin Staining; CT, Christmas Tree staining; DAB, immuno-DAB staining; Danes, modified Danes stain.*

TABLE 1—*Percentage of inhibited samples for each staining treatment.**

Stain Treatment Used	Number of Samples Quantified	% Showing Inhibition	% Showing Inhibition After 1/2 Dilution
H&E	24	0	0
Christmas Tree	24	0	0
Immuno-DAB	23†	50	9
Modified Danes	24	37.5	4
No stain	30‡	0	0

*Determined by Quantifiler™ internal control results. Samples were quantified at both full concentration and when diluted by half with sterile filtered water.

†One slide deemed unacceptable for analysis.

‡Includes 6 poly-L lysine slides with unstained cells.

gives indications of some effect ($F = 2.5$, p-value = 0.064, d.f. = 3). No indication of an interaction between staining treatment and storage time was found ($F = 1.04$, p-value = 0.42, d.f. = 9).

The histograms of total DNA extracted from the slides suggested that there is some difference in the total amount of DNA extracted from slides stained with Christmas Tree or H&E (Fig. 2), although there was less evidence of this difference when the data were adjusted for the number of cells on the slide (Fig. 3). One-factor ANOVA was carried out in EXCEL™ to determine whether there were any significant differences caused by treatment of slides with Christmas Tree stain compared to H&E staining. The analysis showed that there was no indication of a significant effect of treatment on the amount of DNA extracted per cell when comparing these two stains ($F = 1.79$, p-value = 0.186, d.f. = 1).

STR Profiling of DNA Extracts

As shown in Fig. 3, the lowest amount of DNA per cell was extracted from the samples that had been stored for 10 weeks at room temperature. It was reasonable to assume that these samples would also display the greatest negative effects on DNA profiling, and the AmpF*l*STR® Identifiler™ STR profiling system was first used to analyze the DNA from these extracted samples.

Full Identifiler™ profiles were generated from the DNA extracted from both the H&E-stained slides (see Fig. 4) and the positive control slides where the cells were not stained. The profiles from the Christmas Tree-stained slides showed drop-out of one or two alleles in some cases (at the FGA, D16S539, and CSF1PO loci), but samples also produced complete profiles. The decreased concentrations of DNA extracted from the immuno-DAB- and modified Danes-stained slides had an effect on the quality of the DNA profiles produced by these samples. Under standard amplification conditions (i.e., 28 cycles), DNA from these slides produced partial profiles, with between 14 and 19 alleles present of a possible 32 for the immuno-DAB DNA, and 11–13 alleles present of 32 for the modified Danes-stained DNA. To assess the effects of inhibitors from the immuno-DAB and modified Danes stains, DNA from these extractions was also diluted 1/10 before standard amplification, but this did not improve the profiling results (data not shown).

The greatest inhibition of the Quantifiler™ PCR reaction was seen in samples extracted from immuno-DAB slides, and some inhibition was seen in the DNA from the modified Danes-stained slides, although this effect could be significantly ameliorated by dilution of the extracted sample. Both of these treatments resulted in significantly lower levels of DNA being extracted from the stored slides. Despite the fact that modified Danes-stained slides produced greater concentrations of DNA than the immuno-DAB slides, profiles from these stained cells tended to show a greater level of allelic drop-out. Increased cycle amplification reactions (34 cycles) were carried out on the DNA extracted from the immuno-DAB- and modified Danes-treated slides in an attempt to improve the DNA profiles. This procedure resulted in profiles with a higher proportion of alleles present in all cases with 12–17 of a possible 22 alleles present from the immuno-DAB-treated slides, and 12–13 of 22 alleles present from the modified Danes-treated slides. The analysis of these samples led to an overall increase in informative alleles when combined with the Identifiler™ profiling results (see Table 2). The immuno-DAB and modified Danes staining treatments are less likely to have been used on archived casework slides, but if used in current analysis, then the profiling results obtained from standard analysis when combined with those from increased cycle amplification should provide sufficient DNA profiling information to be of evidential use in a case.

We selected the organic extraction method for this study, as it is the method our forensic casework laboratory currently uses for retrieval of DNA from archived slides, and because of the necessity of removing all traces of contaminants such as xylene from the extracted sample before PCR amplification. It is important to realize that a combination of alternative extraction methods with

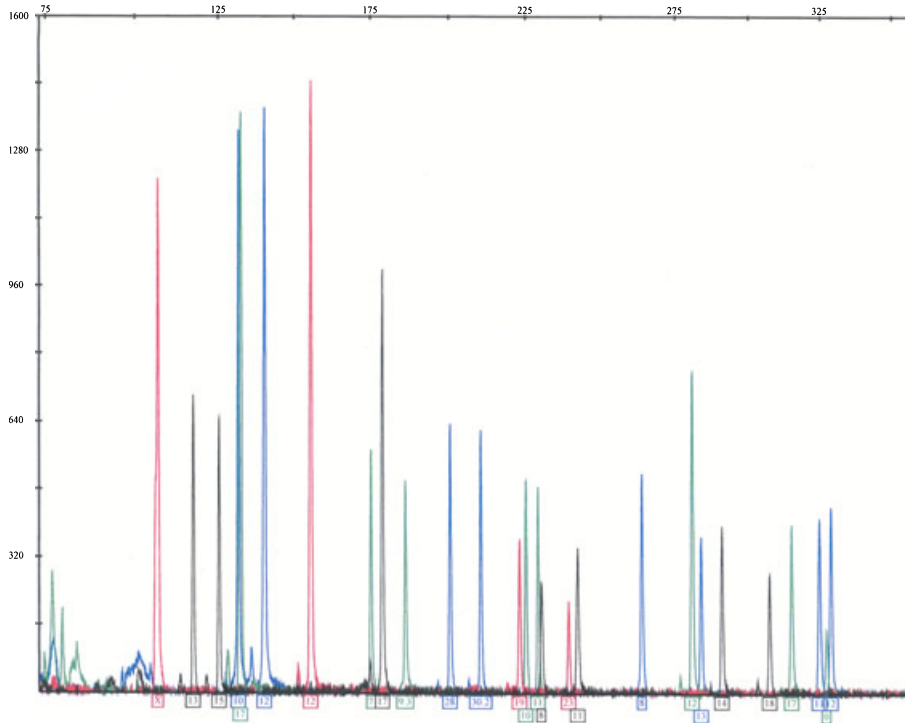

FIG. 4—*Example of electropherogram of Identifiler™ profile of human DNA produced by the FaSTR program. This profile was produced from a slide of Hematoxylin and Eosin (H&E)-stained epithelial cells that had been stored for 10 weeks. A full profile was produced with no allelic drop-out.*

TABLE 2—*Percentage of total possible alleles present detected during profile analysis.*

	Percentage of Total Available Alleles Detected*	
Stain Treatment Used	Identifiler Analysis	Combined Identifiler and LCN SGM Plus Analysis
Immuno-DAB	51.56 ± 6.05%	65.63 ± 4.42%
Modified Danes	37.5 ± 4.41%	44.79 ± 1.80%

*Data presented for Modified Danes- and Immuno-DAB-stained samples, with standard Identifiler profiling, and with the combined results of Identifiler and LCN SGM Plus profiling. Data are presented as mean result ± standard deviation of the mean.

particular stains and amplification systems may interact differently and produce different results from those obtained in this study.

Apart from the quantity and quality of the DNA produced after staining with the methods described, laboratories may have other considerations when choosing histological stains, such as for health and safety reasons. In particular, the use of Picric Acid to make Picroindigocarmine may be of concern in Christmas Tree staining. Because of safety and other concerns, some laboratories have converted from Christmas Tree staining to the sole use of the Nuclear Fast Red part of the stain to label cell nuclei in cell smears (11).

The type of slide used and the method of cell recovery from the slide may also affect the quality of the DNA recovered. In particular, the development of LMD systems has provided alternate methods of retrieving tissue from archived cytological slides, and many LMD systems use alternative slide formats to glass. Elliott et al. (12) described sperm cell recovery from archived slides using the Arcturus LMD system, which seals target cells to a membrane coated cap. LMD and pressure catapulting from archived slides can be performed using the PALM microlaser system, but because of the sample damage that may occur it is of more use with robust samples such as cryptosporidium oocysts (13) or paraffin-embedded tissue sections (e.g., [14]). Other LMD systems describe transfer of cells from archived slides to LMD membrane slides using a resin peel, but this method may involve some loss of the original sample.

The use of fluorescent-labeling techniques for cell differentiation, such as sex-chromosome *in situ* hybridization (15) and Sperm Hy-liter (16), is being considered for use in some forensic laboratories, and some of these techniques may also have effects during storage on the DNA that may be later extracted from the cells. Analysis of the effects over time of these stains, particularly when used in collaboration with the LMD membrane slides would be a useful next step in determining best practices in forensic histology and DNA profiling with long-term storage in mind.

The adverse effects of some histological stains on the quality of extracted DNA do not necessarily preclude their use in forensic situations but rather require, where able, adaption of other parts of the DNA profiling methodology. For example, stains that produce low levels of good quality DNA can be complemented by the use of more sensitive DNA profiling systems such as increased amplification cycles or enhanced injection parameters, whereas a staining system that results in co-extraction of inhibitors may benefit from dilution of the extracted DNA prior to amplification provided sufficient DNA is recovered. DNA profiling results from DNA that has been degraded by a stain may improve with the use of a mini-STR amplification system, such as the AmpF*l*STR® Minifiler™ profiling system. These systems have been specifically designed for the analysis of degraded DNA.

Conclusion

Our statistical analysis showed that there was no significant difference in the amount of DNA recovered from stained slides treated with the H&E and Christmas Tree stains. None of the samples

treated with either of these stains showed inhibition during the Quantifiler™ PCR reaction used to determine DNA concentrations. Full Identifiler™ profiles were produced from the DNA extracted from the unstained positive control and H&E-stained slides. Some complete profiles were produced using the DNA recovered from Christmas Tree-treated slides, but occasional drop-out of a few alleles was also observed. These results indicate that it is unlikely that either stain will adversely affect downstream uses of archived slides treated with these histological stains and both could be used with confidence in the forensic laboratory.

Our data indicate that there is some effect of storage time on the quantity of DNA extracted from stained slides, but statistical analysis indicates that this is not a significant effect. We have presented data that is derived from examination of a single type of forensically relevant stained cell. Further study including a larger range of storage times and different cell types would be beneficial in determining the true effect of these variables. Additional research on the particular effects of various parts of the immuno-DAB and modified Danes staining systems could also elucidate the causes of the deleterious effects observed in this study.

Conflict of interest: The authors have no relevant conflicts of interest to declare.

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