

# Polymerase Chain Reaction Amplification of DNA from Aged Blood Stains: Quantitative Evaluation of the “Suitability for Purpose” of Four Filter Papers as Archival Media

Margaret C. Kline,\* David L. Duewer, Janette W. Redman, and John M. Butler

Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899

David A. Boyer

Department of Defense DNA Registry, Armed Forces Institute of Pathology, Suite 100, 16050 Industrial Drive, Gaithersburg, Maryland 20877

**In collaboration with the Armed Forces Institute of Pathology’s Department of Defense DNA Registry, the National Institute of Standards and Technology recently evaluated the performance of a short tandem repeat multiplex with dried whole blood stains on four different commercially available identification card matrixes. DNA from 70 stains that had been stored for 19 months at ambient temperature was extracted or directly amplified and then processed using routine methods. All four storage media provided fully typeable (qualitatively identical) samples. After standardization, the average among-locus fluorescence intensity (electropherographic peak height or area) provided a suitable metric for quantitative analysis of the relative amounts of amplifiable DNA in an archived sample. The amounts of DNA in Chelex extracts from stains on two untreated high-purity cotton linter pulp papers and a paper treated with a DNA-binding coating were essentially identical. Average intensities for the aqueous extracts from a paper treated with a DNA-releasing coating were somewhat lower but also somewhat less variable than for the Chelex extracts. Average intensities of directly amplified punches of the DNA-binding paper were much larger but somewhat more variable than the Chelex extracts. Approximately 25% of the observed variation among the intensity measurements is shared among the four media and thus can be attributed to intrinsic variation in white blood count among the donors. All of the evaluated media adequately “bank” forensically useful DNA in well-dried whole blood stains for at least 19 months at ambient temperature.**

Many reference DNA sample repositories or “DNA banks” are now in existence, primarily for support of epidemiological and genetic research, definitive diagnosis, and recurrence risk counseling or to enable identification of forensic evidence or human

remains.<sup>1–5</sup> Significant ethical and procedural issues have been raised concerning access to such archived samples.<sup>6–11</sup> However, from a practical scientific standpoint, no DNA bank can satisfy its intended goals unless the desired genetic information latent in the original sample can indeed be accessed analytically. The nature of the sample, how it is collected, and how it is stored are critical issues for the ultimate utility of any DNA banking effort.<sup>12–15</sup>

Whole blood, plasma, hair roots, and buccal epithelium are convenient and can be minimally intrusive sources of DNA for

- (1) Muti, P.; Trevisan, M.; Modlich, F.; Krogh, V. *Nutr. Metab. Cardiovasc. Dis.* **1998**, *8*, 200–204.
- (2) de Montgolfier, S.; Moutel, G.; Herve, C. *Presse Med.* **2000**, *29*, 1752–1758.
- (3) Farkas, D. H.; Kaul, K. L.; Wiedbrauk, D. L.; Kiechle, F. L. *Arch. Pathol. Lab. Med.* **1996**, *120*, 591–596.
- (4) Weedn, V. W. *Atlanta Med.* **1993**, *67* (3), 55–57. See also: Armed Forces Institute of Pathology. Repository History. <http://www.afip.org/Departments/oa/me/dna/history.htm>. Gillert, D. J. American Forces Information Service News Articles: Who Are You? DNA Registry Knows. [http://www.defenselink.mil/news/Jul1998/n07131998\\_9807131.html](http://www.defenselink.mil/news/Jul1998/n07131998_9807131.html).
- (5) Parson, W.; Steinlechner, M.; Scheithauer, R.; Schneider, P. M. In *Proceedings from the Ninth International Symposium on Human Identification, Orlando, Florida, October 8–10, 1998*; Promega Corp.: Madison WI, 1998; pp 52–54. <http://www.promega.com/geneticidproc/ussymp9proc/content/11.pdf>.
- (6) Annas, G. J. *JAMA, J. Am. Med. Assoc.* **1993**, *270*, 2346–2350.
- (7) Proceedings of the European Symposium “Ethical and Legal Issues of DNA Typing in Forensic Medicine”; Schneider, P. M., Rittner, C., Martin, P. D., Eds. *Forensic Sci. Int.* **1997**, *88*, 1–110.
- (8) McQueen, M. J. *Clin. Chem. Lab. Med.* **1998**, *36*, 545–549.
- (9) Guillén, M.; Lareu, M. V.; Pestoni, C.; Salas, A.; Carracedo, A. *J. Med. Ethics* **2000**, *26*, 266–271.
- (10) American Civil Liberties Union of Massachusetts. DNA Testing ACLU Q&A. <http://www.aclu-mass.org/privacy/dnaqa.html>.
- (11) McEwen, J. E.; Reilly, P. R. *Am. J. Hum. Genet.* **1994**, *55*, 196–200.
- (12) Madisen, L.; Hoar, D. I.; Holroyd, C. D.; Crisp, M.; Hodes, M. E. *Am. J. Med. Genet.* **1987**, *27*, 379–390.
- (13) Cann, R. L.; Feldman, R. A.; Freed, L. A.; Lum, J. K.; Reeb, C. A. *Methods Enzymol.* **1993**, *224*, 38–51.
- (14) Austin, M. A.; Ordovas, J. M.; Eckfeldt, J. H.; Tracy, R.; Boerwinkle, E.; Lalouel, J. M.; Printz, M. *Am. J. Epidemiol.* **1996**, *144*, 437–441.
- (15) Therrell, B. L.; Hannon, W. H.; Pass, K. A.; Lorey, F.; Brokopp, C.; Eckman, J.; Glass, M.; Heidenreich, R.; Kinney, S.; Kling, S.; Landenburger, G.; Meaney, F. J.; McCabe, E. R. B.; Panny, S.; Schwartz, M.; Shapira, E. *Biochem. Mol. Med.* **1996**, *57*, 116–124.

\* Corresponding author. Tel: 301-975-3134. Fax: 301-975-8505. E-mail: Margaret.Kline@nist.gov.

current analysis technologies.<sup>16,17</sup> Whole blood is often the least expensive source and has the great advantage of providing immediate visual evidence that a sample of adequate size has been obtained. A number of storage media have been investigated: liquid, liquid frozen, lyophilized, and dried on glass slides, cotton swabs, filter papers, and other solid media.<sup>12,18–25</sup> DNA of sufficient quantity and quality for successful amplification via the polymerase chain reaction (PCR) has been isolated from a variety of sources after many years of storage.<sup>12,26–28</sup> High-purity cotton linter pulp paper “cards” are by far the most widely used media, providing a relatively safe sequestering of a sample, and are easy to label, inexpensive to transport, and compact to store.<sup>15,29,30</sup> A variety of specialized coatings have been developed, designed to further improve handling safety, sample longevity, and ease of use.<sup>31–35</sup>

As one component of its commitment to the support of the United States human identity communities, the National Institute Standards and Technology (NIST) studies the effects of DNA sample storage media, time, and conditions on DNA typing technologies. In collaboration with the Department of Defense DNA Registry of the Armed Forces Institute of Pathology's Office of the Armed Forces Medical Examiner, NIST recently evaluated the performance of a short tandem repeat (STR) multiplex with dried whole blood stains stored for 19 months on four different commercially available identification card matrixes. Evaluation of the stains using a commercial short tandem repeat (STR) multiplex kit ensured the relevance of our results for many current DNA analysis applications. Successful STR typing requires that a sample contain an adequate quantity of relatively intact DNA and

that this DNA can be isolated from all PCR inhibitors (heme, proteins, and many other whole blood components).<sup>26,36,37</sup> The multiplex kit used detects PCR amplification products of size from 100 to 320 nucleotide base pairs (bp).

We here describe our evaluation of the basic fitness of these four media for storage of DNA in whole blood. We elsewhere describe results from separate but related studies of DNA sample storage environment and duration. We base our analysis on qualitative and quantitative results provided by commercial STR multiplex systems. Although the analytical needs of future DNA typing technologies cannot be fully anticipated, the evaluation considerations and quantitative assessment methods developed for this study should be applicable for most typing systems based upon amplification of extracted DNA.

## METHODS AND MATERIALS

**Storage Media.** Two high-purity cotton linter pulp specimen collection papers were evaluated, using prototype cards that were half-untreated base paper and half-treated with their manufacturers' specialized coating. These two untreated papers are coded as media A and C. Medium B is paper A treated with a coating designed to tightly bind non-DNA blood components, enabling fast selective extraction of DNA from the matrix. Medium D is paper C treated with a coating designed to tightly bind DNA and RNA, enabling selective removal of PCR inhibitors leaving the DNA bound to the storage matrix.

**Samples.** Eighty cards of each prototype were prepared on 20 January 1999 from residual “purple-top” (EDTA stabilized) Vacutainer blood drawn that morning from U.S. Army recruits at the Fort Benning Reception Station, Ft. Benning, GA. The two prototype cards for each donor were labeled with the same sequence number, but no other information was recorded. After air-drying overnight, the stained cards were sealed in individual transfer pouches and hand carried to the Department of Defense DNA Registry, Rockville, MD. On January 25, 1999, the 160 bloodstain cards were individually vacuum-sealed with a desiccant in pouches, again labeled only with the sequential number and type of card it contained. Specimen collection approval was obtained from the Director of the Armed Forces Institute of Pathology.

Seventy sets of the prototype card pairs were relinquished to NIST on September 1, 1999, for evaluation and testing. Samples were stored at room temperature.

**Sample Analysis.** Proposed sample analysis protocols were approved by the Department of Defense DNA Registry on July 12, 2000. On July 31 2000, 70 pouches containing stains on media A and B were opened in a laminar flow hood, card images were recorded, and one 3-mm punch was taken from both stains of each card. The stains were of very different color and shape. Three punches of a clean paper towel were taken between each sample punch to ensure no cross-contamination between samples. Each pouch was resealed under vacuum prior to opening the next in turn. The punches from the stains stored on medium A (untreated) were stored in labeled HI-YIELD 1.5-mL Nucleic Acid Recovery tubes (Robbins Scientific Corp., Sunnyvale, CA) and were ex-

(16) Visvikis, S.; Schlenck, A.; Maurice, M. *Clin. Chem. Lab. Med.* **1998**, *36*, 551–555.

(17) Thomson, D. M.; Brown, N. N.; Clague, A. E. *Clin. Chim. Acta* **1992**, *207*, 169–174.

(18) Towne, B.; Devor, E. *J. Hum. Biol.* **1990**, *62*, 301–306.

(19) Polakova, H.; Kadasi, L.; Zelinkova, M. *Bratisl. Lek. Listy* **1989**, *90*, 844–7.

(20) Torrance, H.; MacLeod, H. L.; Haché, R. J. G. *BioTechniques* **1993**, *15*, 64–67.

(21) Huckenbeck, W.; Bonte, W. *Int. J. Legal Med.* **1992**, *105*, 39–41.

(22) Aggarwal, R. K.; Lang, J. W.; Singh, L. *Genet. Anal. Tech. Appl.* **1992**, *9*, 54–57.

(23) McCabe, E. R. B.; Huang, S. Z.; Seltzer, W. K.; Law, M. L. *Hum. Genet.* **1987**, *75*, 213–216.

(24) Zhong, K. J. Y.; Salas, C. J.; Shafer, R.; Gubanov, A.; Gasser, R. A.; Magill, A. J.; Forney, J. R.; Kain, K. C. *J. Clin. Microbiol.* **2001**, *39*, 1195–1196.

(25) Nurgalieva, Z. Z.; Almuchambetova, R.; Machmudova, A.; Kapsultanova, D.; Osato, M. S.; Peacock, J.; Zoltek, R. P.; Marchildon, P. A.; Graham, D. Y.; Zhangabylov, A. *Clin. Diagn. Lab. Immunol.* **2000**, *7*, 882–884.

(26) Makowski, G. S.; Davis, E. L.; Hopfer, S. M. *Ann. Clin. Lab. Sci.* **1996**, *26*, 458–469.

(27) Gino, S.; Robino, C.; Torre, C. In *Progress in Forensic Genetics 8*; Sensabaugh, G. F., Lincoln, P. J., Olaisen, B., Eds.; Elsevier Science BV: Amsterdam, 2000; pp 476–478.

(28) Johansson, P. J. H.; Jonsson, M.; Ahlfors, K.; Ivarsson, S. A.; Svanberg, L.; Guthenberg, C. *Scand. J. Infect. Dis.* **1997**, *29*, 465–468.

(29) Steinberg, K. K.; Sanderlin, K. C.; Ou, C. Y.; Hannon, W. H.; McQuillan, G. M.; Sampson, E. *J. Epidemiol. Rev.* **1997**, *19*, 156–162.

(30) Parker, S. P.; Cubitt, W. D. *J. Clin. Pathol.* **1999**, *52*, 633–639.

(31) Burgoyne, L. A. U.S. Patent 5496562, 1996. See also: Whatman BioScience. FTA Products and Accessories. [http://www.whatman.plc.uk/products/biosciences/bs\\_fta\\_prod.html](http://www.whatman.plc.uk/products/biosciences/bs_fta_prod.html).

(32) Schleicher and Schuell, Inc. Brochure 658 1/898, 1998. <http://www.s-und-s.de/Pages-NEU-eng/UB3/Diagnostic%20Components/UB3-DC-Literature.html>.

(33) Natarajan, P.; Trinh, T.; Mertz, L.; Goldsborough, M.; Fox, D. K. *BioTechniques* **2000**, *29*, 1328–1333.

(34) Harty, L. C.; Garcia-Closas, M.; Rothman, N.; Reid, Y. A.; Tucker, M. A.; Hartge, P. *Cancer Epidemiol., Biomarkers Prev.* **2000**, *9*, 501–506.

(35) Makowski, G. S.; Davis, E. L.; Hopfer, S. M. *J. Clin. Lab. Anal.* **1997**, *11*, 87–93.

(36) Walsh, P. S.; Metzger, D. A.; Higuchi, R. *BioTechniques* **1991**, *10*, 506–513.

(37) Makowski, G. S.; Davis, E. L.; Nadeau, F.; Hopfer, S. M. *Ann. Clin. Lab. Sci.* **1998**, *28*, 254–259.

tracted with the modified Chelex procedure described below. The punches from the stain stored on medium B (DNA-releasing) stain were stored in Microcentrifuge 0.6-mL tubes (Robbins Scientific Corp.) and were extracted with the modified aqueous procedure described below.

On August 15, 2000, 70 pouches containing stains on media C (untreated) and D (DNA-binding) were opened in a laminar flow hood, card images were recorded, and one 3-mm punch was taken from both stains of each card. Three punches of a paper towel were taken between each sample punch. Each pouch was resealed under vacuum prior to opening the next in turn. The punches from both stains were stored in labeled HI-YIELD 1.5-mL Nucleic Acid Recovery tubes and extracted with the modified Chelex procedure described below.

On September 18, 2000, all 70 pouches containing the stains on media C and D were reopened in a laminar flow hood and three 1.2-mm punches taken from stains stored on the D (DNA-binding) medium. Three punches of a paper towel were made between each set of sample punches. Each pouch was again resealed under vacuum prior to opening the next in turn. The sample punches were stored in labeled Microcentrifuge 0.6-mL tubes and prepared for amplification with the modified direct procedure described below.

**Chelex Extraction.** The following version of the standard Chelex extraction procedure was used.<sup>36</sup> One milliliter of Milli-Q Plus Water System (Millipore Corp., Bedford, MA) deionized (DI) water was added to each 1.5-mL tube containing a single 3-mm punch. The tubes were vortexed for 10 s, shaken for 15 min, and then centrifuged for 3 min at 12500 $g_n$ . One milliliter of liquid was removed from each tube. One milliliter of DI water was added to each tube, and the tubes were vortexed, shaken, and centrifuged as above. One milliliter of liquid was removed from each tube. Four hundred microliters of a freshly prepared, continuously stirred 5% (mass fraction) suspension of Chelex-100 (Bio-Rad Laboratories, Hercules, CA) in DI water was added. The tubes were vortexed for a few seconds, centrifuged for 10 s at 12500 $g_n$ , stored in a heat block at 56 °C for 2 h, vortexed for 10 s, boiled for 8 min, and centrifuged for 3 min at 12500 $g_n$ . Three hundred microliters of liquid DNA extract was removed from each tube, placed into an appropriately labeled 0.6-mL tube, and stored at 4 °C prior to amplification.

**Aqueous Extraction.** The following version of the manufacturer's DNA isolation procedure was used for all media B punches. Five hundred microliters of DI water was added to each 0.6-mL tube containing a single punch. The tubes were pulse vortexed three times for a total of 5 s. The punch was removed from the original tube and placed in an appropriately labeled second tube. A 300- $\mu$ L aliquot of DI water was added. The tubes were heated at 95 °C for 30 min and pulse vortexed for 15 s. The punches were removed from the tubes. The tubes containing the extracted DNA were stored at 4 °C prior to amplification.

**Direct Amplification.** The following version of the manufacturer's recommended procedure for PCR analysis was used for the 1.2-mm media D punches. Two hundred fifty microliters of the manufacturer's proprietary reagent was added to each 1.5-mL tube containing three 1.2-mm paper punches from the same stain. The tubes were vortexed for 2 s at low speed, sat for 5 min at room temperature, were vortexed for a few seconds, and as much

reagent as possible was removed. Two additional reagent washes were performed in an identical manner. Each set of three punches was washed with 500  $\mu$ L of DI water. As much as possible of this water was removed and 250  $\mu$ L of tris-EDTA buffer (TE, 10 mmol/L Tris-HCl at pH 8.0, 0.1 mmol/L EDTA) added. The tubes were vortexed for 2 s at low speed and allowed to set for 5 min at room temperature. As much of the TE buffer was removed as possible, and the tubes were dried under laminar flow at room temperature for  $\sim$ 1 h. One punch of each set of three was added to an appropriately labeled 0.2-mL Strip-Ease tube (Robbins Scientific Corp.) containing 10  $\mu$ L of PCR reaction mix. The tubes were centrifuged briefly to ensure that the punches were at the bottom of the tubes and then amplified. The results for these directly amplified medium D punches are coded as "d".

**PCR Amplification and STR Detection.** All samples were amplified for the AmpfSTR COfiler PCR Amplification Kit (Applied Biosystems, Inc., Foster City, CA) using a GeneAmp-PCR System 9600 (Applied Biosystems, Inc.). The COfiler heptaplex enables amplification of products at six STR loci (D3S1358, D16S539, TH01, TPOX, CSF1PO, D7S820) and the amelogenin sex marker locus. The manufacturer's recommended amplification protocol was used for all samples, with volumes adjusted proportionally for a reaction volume of 25  $\mu$ L. Four microliters of DNA extract were used per 25- $\mu$ L reaction. The manufacturer's recommended protocol was used for all directly amplified D punches except that the number of PCR cycles was reduced from 28 to 25. The amplified products were processed with a ABI PRISM 310 genetic analyzer using the manufacturer's recommended conditions. This is a single-capillary electrophoretic system that requires  $\sim$ 30 min/sample and can analyze batches of up to 96 samples. Figure 1 presents amplification and analysis results for one sample in the form of an electropherogram.

Samples from a given storage medium were processed in batches of 20–40. One or two control samples were amplified and processed with each batch. The COfiler STR multiplex kit control sample was used with all extracted samples; a cell line K562 solution was used with the directly amplified d punches. The few samples that were not successfully characterized by their initial analysis (amplification or analyzer failures) were reamplified and reprocessed with appropriate blank and control samples.

**Linearity Study.** Five DNA extracts were used to calibrate the relationships between the STR signal intensity metrics and amount of DNA in the reaction mixture; these extracts were prepared for distribution in the NIST-sponsored Mixed Stain Study #3.<sup>38</sup> The DNA concentration in these materials was evaluated with several techniques, including UV spectrophotometry, yield gel, and several commercial slot-blot methods.<sup>39,40</sup> Four DNA extracts were used to evaluate the predictive utility of the calibrations; these extracts were distributed as unknowns in the NIST-sponsored Mixed Stain Study #2.<sup>41</sup> The interlaboratory median

(38) Kline, M. C.; Redman, J. W.; Duewer, D. L.; Butler, J. M. Presented at the Twelfth International Symposium on Human Identification, Biloxi, MI, October 9–12, 2001; Poster 4.

(39) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1982; Appendix A, pp 468–469.

(40) Waye, J. S.; Presley, L. A.; Budowle, B.; Shutler, G. G.; Fourney, R. M. *BioTechniques* **1989**, 7, 852–855.

(41) Duewer, D. L.; Kline, M. C.; Redman, J. W.; Newall, P. J.; Reeder, D. J. *J. Forensic Sci.* **2001**, 46, 1199–1210.

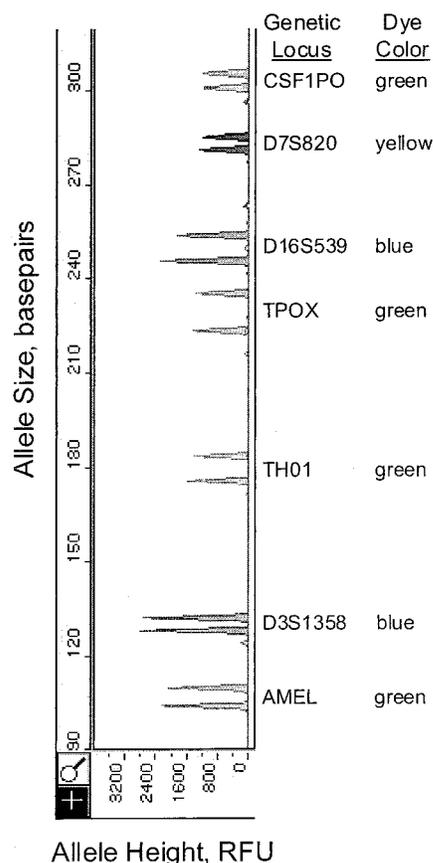


Figure 1. Example of an Ampf/STR COfiler electropherogram. The loci are identified by bp size and fluorescent dye color. This sample has an unusual DNA profile in that there are two alleles (i.e., heterozygous) at all seven COfiler loci and there is no intermixing of alleles among neighboring loci.

and quartile-estimated standard deviation are used to estimate the concentration of DNA in these materials.

All linearity samples were amplified with the Ampf/STR PROFILERPlus PCR Amplification Kit (Applied Biosystems, Inc.) but were otherwise analyzed as described above. The PROFILERPlus decaplex enables amplification of products at nine STR loci (D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820) and the amelogenin sex marker locus.

**Data Analysis.** The genetic type and signal intensity metrics (peak height and area, expressed in relative fluorescence units, RFUs) for all alleles for all amplification products were evaluated with GeneScan 2.1 and Genotyper 2.0 software (Applied Biosystems, Inc.) following typical genotyping practice. The types, heights, and areas were exported for statistical analysis as spreadsheets.

## RESULTS AND DISCUSSION

The analytical signal characteristic of an STR multiplex is an ordered set of "peak" (in electropherograms) or "band" (in gel images) locations and intensities, almost always with one or two of these signal elements per genetic locus evaluated. The STR multiplexes used in the current study differentiate signals from the different loci on the basis of fluorescent dye type (color; see Figure 1) and electrophoretic migration differences (size) of the amplification products. Each element at each locus represents a

particular DNA sequence (allele). Allele locations are defined (typed) relative to a set of reference alleles (typing ladder) for each locus.

Forensically useful polymorphic loci typically provide two signals of approximately equal intensity, one each from analytically distinguishable maternal and paternal alleles (heterozygosity). When the maternal and paternal alleles are analytically indistinguishable (homozygosity), one signal of approximately twice the expected single allele intensity is observed. The signal intensity for a given allele is evaluated as a peak height and area or band optical density.

**Qualitative Observations.** All amplification products from all storage media were typed successfully. The only differences among the sets of amplification products for each of the 70 DNA sources evaluated were the magnitudes of the allelic peak height and area signals.

**Quantitative Observations. Metric for Comparing Allelic Intensities.** When the genetic type of a single-source sample is evaluated, allelic signal intensities (and peak or band shapes) are mostly used to help differentiate signals of "true" alleles from system noise or amplification artifacts. As long as the intensities are above the chosen threshold value, the allelic signals are compared (generally "by eye"; see Figure 1) relative to other signals for the same sample. Quantitative comparison among samples is seldom necessary; therefore, there has been little attention given to parsimoniously describing the entire set of STR multiplex signal intensities for a given amplification. Since we are interested in evaluating the suitability of different storage media for STR multiplex typing, the initial task is to define a suitable comparison metric.

Since there are, by explicit analytical design, a large number of different genetic types possible at STR loci, it is not practical to quantitatively compare signal intensities among different samples per allelic type. While there are systematic differences in signal intensity (particularly peak height; see below) as a function of allele size, within each locus, the allelic peaks are typically similar in size and shape. Little information is lost by characterizing the signal intensity for a given locus as the sum of its valid allelic signals regardless of genetic type. Since the final typing document produced by some commercial typing software reports homozygous alleles as two replicates, both having the full signal intensity of the single observed allele, some care must be exercised to avoid "double counting" such signals.

Comparing signal intensities for each locus independently is inconvenient and, when comparing results for STR multiplexes that examine different loci, may not be possible. Since the same amount of target DNA is available at each genetic locus for a given amplification, the signal intensities among the different loci of a multiplex should in principle be strongly correlated. However, the intensity of the final signal is a complex function of the amount and character of the amplification reagents and the fluorescent dyes employed and the state of degradation of the sample DNA. Commercial STR multiplexes are carefully engineered to provide fairly well "balanced" signal intensities across all loci they examine. Figure 2 displays the ratios of individual locus signals relative to the average signal across all loci for the COfiler and PROFILERPlus systems. Two trends are evident in both systems: (1) "Blue" loci signals are somewhat larger than those from "yellow" loci and

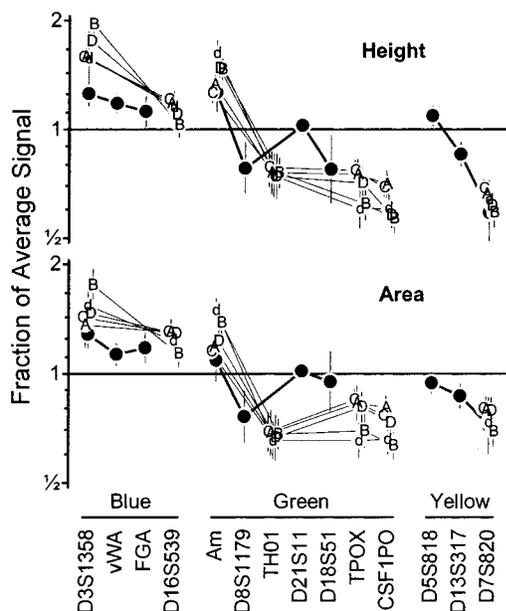


Figure 2. Ampf/STR COfiler and PROfiler Plus signal magnitude at each locus as fractions of the average signal. The loci are grouped by their fluorescent dye color; within each color group the loci are arranged in order of increasing bp size. The upper segment displays the observed distributions of peak heights relative to the all-locus average height. The letter symbols and their accompanying vertical bars denote the average  $\pm$  SD of each of the five sets of 70 COfiler amplification products, where "A", "C", and "D" represent the Chelex extracts of media A, C and D punches; "B" represents the aqueous extracts of medium B punches, and "d" represents the directly amplified medium D punches. The solid circles and their accompanying vertical bars denote the average  $\pm$  SD of the 32 PROfiler Plus amplification products of the linearity study. The lower segment displays the same results for peak areas.

(2) within a given color group, peak height and (to a lesser extent) peak area decline with increasing bp size of the amplified products.

The differences among the color groups arise in intrinsic characteristics of the fluorescence dyes or the analytical sensor system used to detect them. The decline in peak height and area with increasing bp size is compatible with both increased impact of DNA degradation and decreased amplification efficiency with increasing molecular weight.<sup>42</sup>

The distributions of the individual locus intensities are very similar among the five sets of COfiler results displayed in Figure 2. Although the average signal intensities for the different loci do differ, they are in the same relative proportion throughout the study. Some linear combination(s) of the individual locus-specific intensities thus should adequately summarize the overall STR multiplex signal intensity results for each given sample. Since none of the single locus signal heights are more than twice nor less than half of the average height, we use the simple average intensity over all loci as our comparison metric. Since areas are somewhat more similar than heights, we expect that the area-based average should have better statistical properties than that based upon heights.

**Batch Standardization.** Being kinetically limited processes, the quantitative reproducibility of both the PCR amplification and

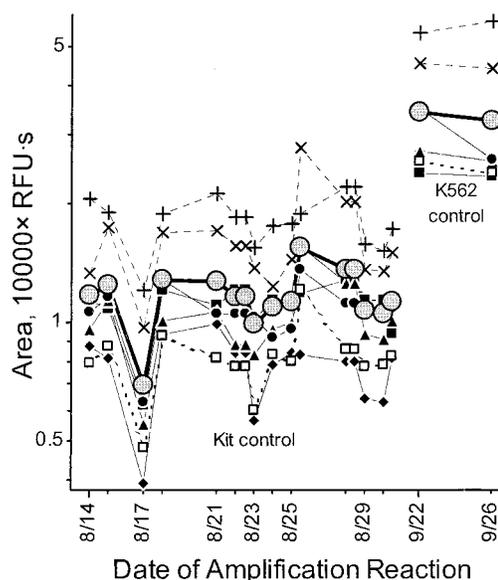


Figure 3. Control sample areas. The large gray circles connected with a thick solid line denote the all-locus average peak areas for the control samples amplified with each batch of samples. The areas of the seven individual loci are denoted: color group blue D3S1358 (+) and D16S539 (x) connected by thin dashed lines, color group green amelogenin (solid square), TH01 (solid diamond), TPOX (solid triangle), and CSF1PO (solid circle) connected by thin solid lines, and color group yellow D7S820 (open square) connected by thick dashed line. The Ampf/STR COfiler kit control sample was used with all DNA extracts (8/14–8/29); a cell line K562 sample was used for both batches of directly amplified punches.

capillary electrophoretic separation stages of analysis is very sensitive to small environmental differences. When identical samples in different batches are analyzed under nominally identical conditions, relatively large signal intensity differences are sometimes observed. Figure 3 displays the peak areas for the control samples analyzed with each batch of samples in this study; the peak heights (data not shown) follow identical patterns. While generally of similar magnitude for a given control sample, the average areas for several of the controls are up to 5-fold different from the norm.

The signal intensities of the unknown samples in the batches with unusually intense or weak controls tended to be similarly unusual. We thus divide the signal intensities of the unknowns of a given batch by the same signals of the control samples. To maintain the native units and typical magnitude of the intensity signals, these fractions are multiplied by the average value of the signal for the control samples. The upper segment of Figure 4 displays the "raw" average areas versus average heights for all five sets of samples; the lower segment displays the identical data after standardization to the control standards. We attribute the several different height/area ratios expressed in the raw data to degradation of the capillary, resulting in decreased resolution. In addition to bringing the signal intensities of the various sample batches into better accord, standardization removes these height/area artifacts.

The absolute signal intensities of the directly amplified punches are not directly comparable to those from the Chelex (media A, C, and D) and aqueous (medium B) extractions. Not only was the size of the punch different (1.2 vs 3 mm), but fewer thermocycler cycles were required to obtain an adequately strong

(42) Walsh, P. S.; Erlich, H. A.; Higuchi, R. *PCR Methods Appl.* **1992**, *1*, 241–250.

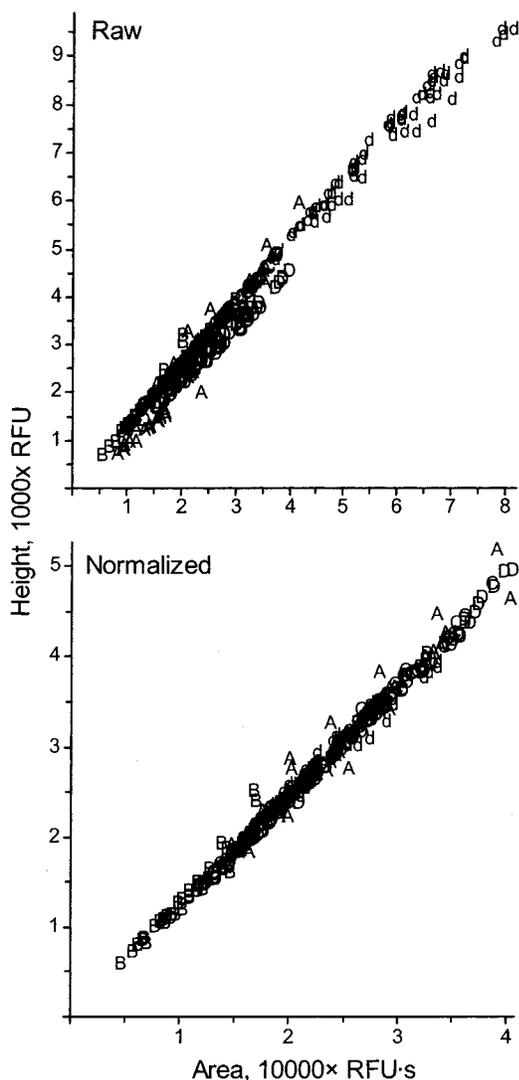


Figure 4. Area vs height of Ampf/STR COfiler signals for a series of blood stains stored on four different storage media. The upper graphical segment displays the average height and areas of the unique allelic signals for each unique DNA over 10 genetic loci. The lower segment displays the height and areas after standardization to the signals from the control DNA amplified at the same time as the sample extracts. Results are coded as in Figure 2.

signal. For convenience, treatment d signal intensities have been normalized to have the same average magnitude as the other treatments:  $\text{normalized height} = (\text{observed height}) / (\text{average of d heights}) \times (\text{average of A, B, C, and D heights})$

**Proportionality to Amount of Template DNA.** Quantitative comparison of different DNA storage media and extraction techniques requires that there be a predictive relationship between the quantity of DNA in the amplification reaction and the summary metric. Figure 5 displays the monotonically proportional relationships of the area-based averages; the height-based averages (data not shown) are nearly identical within graphical resolution. For a given sample, both height- and area-based signal intensity metrics are essentially linear with quantity of DNA in the amplification reaction. Most of the among-sample differences can be attributed to uncertainty in their "true" DNA concentrations.

Due to resource availability, the relationships shown in Figure 5 were evaluated using the PROfiler Plus decaplex. COfiler and

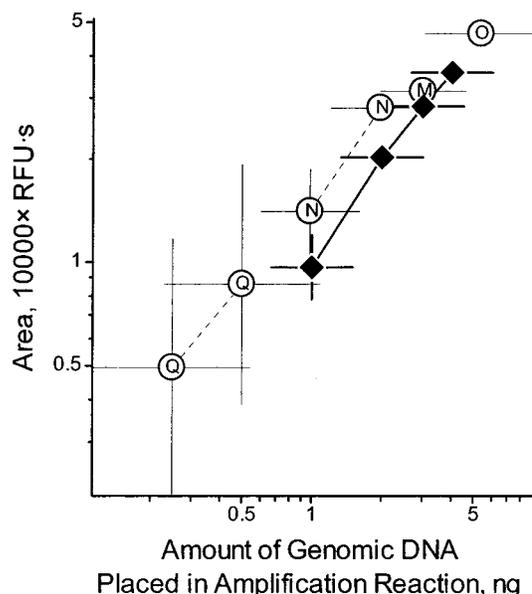


Figure 5. Ampf/STR PROfiler Plus average peak area as a function of amount of genomic DNA in the PCR reaction. The solid diamonds denote results for the amplification of 1, 2, 3, and 4  $\mu\text{L}$  of a single sample of "known" concentration. The dark vertical bars denote  $\pm 1$  SD about the average area from three to six replicate injections of these samples, the horizontal bars represent an approximate 70% confidence interval on the total amount of DNA amplified, and the dark line connects the average values. The open circles denote interlaboratory comparison results for the four Mixed Stain Study #2 reference samples "M", "N", "O", and "Q".<sup>41</sup> The light vertical bars denote  $\pm 1$  SD about the average area from two to four replicate injections of these samples, and the horizontal bars denote  $\pm 1$  SD about the median interlaboratory result. The light dashed lines connect results for 1- and 2- $\mu\text{L}$  amplifications of given samples.

PROfiler Plus multiplexes share three loci, one in each color group; as shown in Figure 2, the locus-specific signal ratios for these loci are quite similar between these two multiplexes. While the relationships between DNA quantity amplified and locus-average signal intensity differ in detail among multiplexes (and among different batch lots of the same multiplex), our experience is that their signal intensities also increase roughly in proportion to the amount of DNA amplified over the manufacturer's recommended range.

**Comparison among Treatments.** Figure 6 displays all pairwise comparisons of standardized average peak areas among the five treatments; the standardized heights (data not shown) show very similar patterns. All of the comparisons are well described as bivariate normal distributions using the 70-sample average and standard deviations of each of the involved treatments and the bivariate correlation coefficient between them.<sup>43</sup>

The strongest correlations among the treatment sets occur with the most similar treatments: 0.54 between the two untreated filter papers (A and C) and 0.51 between the direct amplification and Chelex-extracted medium D treatments (d and D). This suggests that  $\sim 25\%$  of the observed variance among the measurements is attributable to differences among the 70 stain donors, a result that is compatible with the 2-fold span ( $4.5 \times 10^9 - 10.5 \times 10^9$  leukocyte/L) of the "normal" white blood cell count reference range.<sup>44</sup> The residual variance then arises from stain heterogeneity and various

(43) Duewer, D. L.; Liu, H.-K.; Reeder, D. J. *J. Forensic Sci.* **1999**, *44*, 969–977.

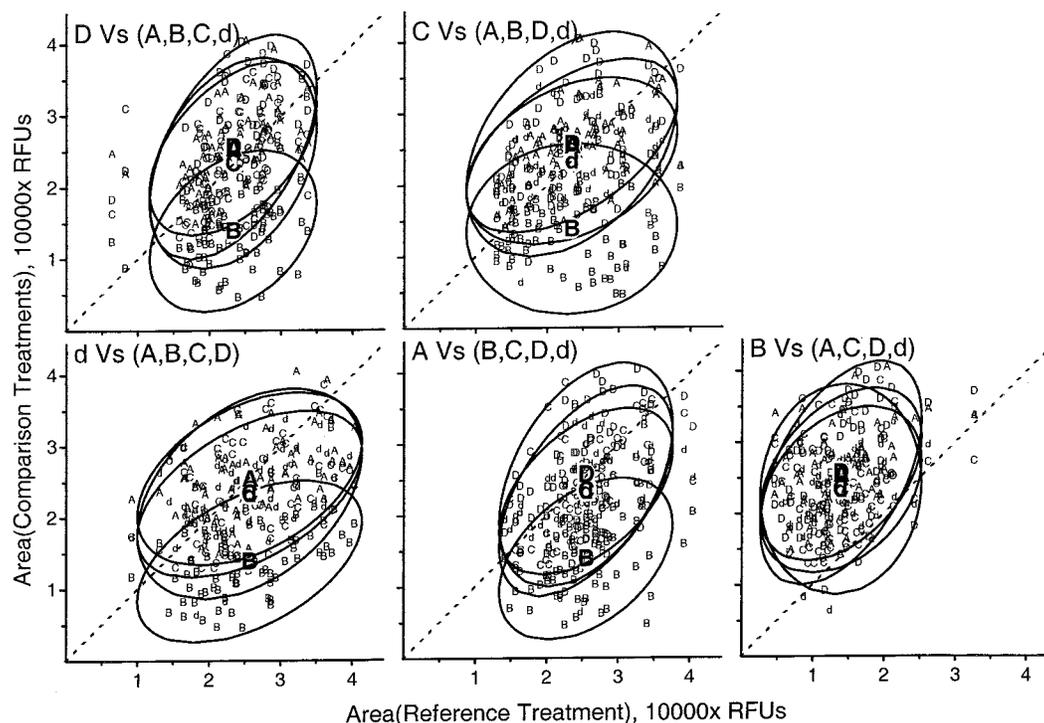


Figure 6. Pairwise comparisons of standardized Ampf/STR COfiler areas. Results are coded as in Figure 2. Each small symbol denotes the average area for one of the 70 samples of the given treatment; the large symbols denote the average area over the 70 samples. The ellipses represent the 90% confidence interval on each bivariate distribution.

aspects of the measurement process: sample processing, amplification, separation, and detection.

While highly correlated and on average of the same magnitude, signal intensities for untreated paper A are somewhat more uniform than are those for paper C. Likewise, the majority of the Chelex-extracted D punch intensity signals are more uniform than are those directly amplified (d). However, two of the D punch extracts did not amplify well—suggesting the presence of more than usual quantities of PCR inhibitors.

The signals from aqueous extracts of medium B punches are generally of lower intensity than the other treatments; however, they are as uniform as those of media A and D. While modestly well correlated to d (0.35), D (0.49) and A (0.42), the values for the aqueous B extracts are anomalously uncorrelated (0.06) with Chelex C extracts. In contrast to the few unusually low signals from the directly amplified d, the few outlier signals of the B extracts are unusually large.

#### ACKNOWLEDGMENT

We thank Whatman Bioscience and Schleicher and Schuell for providing the prototype identity cards evaluated in this study;

(44) Ravel, R. *Clinical Laboratory Medicine: Clinical Applications of Laboratory Data*, 6th ed.; C. V. Mosby: St. Louis, MO, 1995.

Col. Brion C. Smith, DDS, who assisted with the donor collection effort; Martin Army Community Hospital, Ft. Benning, GA, for the use of their facilities, and the 80 anonymous Army recruits who provided the blood samples. This work was funded in part by the Department of Defense under contract agreement W74MYH99144-014 and by the National Institute of Justice through the National Institute of Standards and Technology Office of Law Enforcement Standards. Certain commercial materials, instruments, software, and equipment are identified in this paper to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by Armed Forces Institute of Pathology or the National Institute of Standards and Technology, nor does it imply that the material, instrument, software, or equipment is necessarily the best available for the purpose. The opinions and assertions expressed herein are solely those of the authors and are not to be construed as official or as the views of the United States Department of Defense or the United States Department of the Army.

Received for review December 3, 2001. Accepted February 7, 2002.

AC015715E