

PCR Amplification of DNA from Aged Blood Stains:
Quantitative Evaluation of the “Suitability for Purpose” of Four Filter-Papers
as Archival Media

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ABSTRACT

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 (STR) multiplex with dried whole blood stains on four different commercially available identification card matrices. 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 typable (qualitatively identical) samples. After standardization, the average among-locus fluorescence intensity (electropherographic peak height and/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.

INDEXING TERMS

Forensic Science
DNA Amplification, Extraction, Fingerprinting, Quantification, Storage, Typing
Blood Stains, Short Tandem Repeat (STR)

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 (1–5). Significant ethical and procedural issues have been raised concerning access to such archived samples (6–11). 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 (12–15).

Whole blood, plasma, hair roots, and buccal epithelium are convenient and can be minimally intrusive sources of DNA for current analysis technologies (16, 17). 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 (12, 18–25). 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 (12, 26–28). 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 (15, 29, 30). A variety of specialized coatings have been developed, designed to further improve handling safety, sample longevity, and ease of use (31–35).

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 matrices. 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) (26, 36, 37). The multiplex kit used detects PCR amplification products of size from 100 through 320 nucleotide basepairs (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 polymerase chain reaction (PCR) inhibitors leaving the DNA bound to the storage matrix.

Samples

Eighty cards of each prototype were prepared 20-Jan-1999 from residual "purple-top" (EDTA stabilized) vacutainer blood drawn that morning from US 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 25-Jan-1999, the 160 bloodstain cards were individually vacuum sealed with a desiccant in pouches, again labeled only with the sequential number and type of the 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 1-Sep-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 12-July-2000. On 31-July-2000, 70 pouches containing stains on media A and B were opened in a laminar flow hood, card images recorded, and one 3 mm punch each taken

from each medium. 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, USA) and were extracted 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 15-August-2000, 70 pouches containing stains on media C (untreated) and D (DNA-binding) were opened in a laminar flow hood, card images recorded, and one 3 mm punch each taken from each medium. 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 18-Sep-2000, all 70 pouches containing the stains on media C and D were re-opened 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 (36). One mL of Milli-Q® Plus Water System (Millipore Corp., Bedford, MA, USA) 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 m, then centrifuged for 3 min at 12,500 g_n . One mL liquid was removed from each tube. One mL DI water was added to each tube and the tubes were

vortexed, shaken, and centrifuged as above. One mL liquid was removed from each tube. Four hundred μL of a freshly prepared, continuously stirred 5 % (mass fraction) suspension of Chelex[®]-100 (Bio Rad Laboratories, Hercules, CA, USA) in DI water was added. The tubes were vortexed for a few s, centrifuged for 10 s at 12,500 g_n , stored in a heat block at 56 °C for 2 h, vortexed for 10 s, boiled for 8 m, and centrifuged for 3 min at 12,500 g_n . Three hundred μL 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 B punches . Five hundred μL 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. Three hundred μL of DI water 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 D punches. Two hundred fifty μL 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, vortexed for a few s, 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 μL of DI water. As much as possible of this water was removed and 250 μL 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 approximately 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 µL 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 AmpfℓSTR® COfiler™ PCR Amplification Kit (Applied Biosystems, Inc., Foster City, CA, USA) 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, and D7S820) and the amelogenin sex marker locus. The manufacturer’s recommended amplification protocol was used for all samples, with volumes adjusted proportionally for reaction volume of 25 µL. Four µL of DNA extract were used per 25 µL 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 approximately 30 min per 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 to 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 and/or analyzer failures) were re-amplified 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 (38). The DNA concentration in these materials was evaluated with several techniques, including UV spectrophotometry, yield gel, and several commercial slot-blot methods (39, 40). 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 (41). The interlaboratory median and quartile-estimated standard deviation are used to estimate the concentration of DNA in these materials.

All linearity samples were amplified with the AmpfℓSTR 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, and 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 or “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 evaluating the genetic type of a single-source sample, allelic signal intensities (and peak or band shapes) are mostly used to help differentiate signals of “true” alleles from system noise and/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 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 and/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 (42).

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 one-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 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 area for several of the controls are up to five-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 d 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 mm vs. 3 mm), fewer thermocycler cycles were required to obtain an adequately strong signal. For convenience, treatment d signal intensities have been normalized to have the same average magnitude as the other treatments: $\text{normalized height} = (\text{observed height})(\text{average of d heights})/(\text{average of A, B, C, and D heights})$

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 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 differs 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 (43).

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 about 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 (4.5×10^9 leukocyte/L to 10.5×10^9 leukocyte/L) of the "normal" white blood cell count reference range (44). The residual variance then arises from stain heterogeneity and various 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 C. Likewise, the majority of the Chelex extracted D punches 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 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 direct amplified d, the few outlier signals of the B extracts are unusually large.

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DISCLAIMER

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.

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FIGURE CAPTIONS

Figure 1 Example AmpfℓSTR 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.

Figure 2 AmpfℓSTR 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.

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 AmpfℓSTR COfiler™ kit control sample was used with all DNA extracts (8/14 through 8/29); a cell line K562 sample was used for both batches of directly amplified punches.

Figure 4 Area Vs height of AmpfℓSTR 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 ten 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.

Figure 5 AmpfℓSTR 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 µL, 2 µL, 3 µL, and 4 µL of a single sample of “known” concentration. The dark vertical bars denote ±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” (41). The light vertical bars denote ±1 SD about the average area from two to four replicate injections of these samples and the horizontal bars denote ±1 SD about the median interlaboratory result. The light dashed lines connect results for 1 µL and 2 µL amplifications of given samples.

Figure 6 Pairwise comparisons of standardized AmpfℓSTR 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.

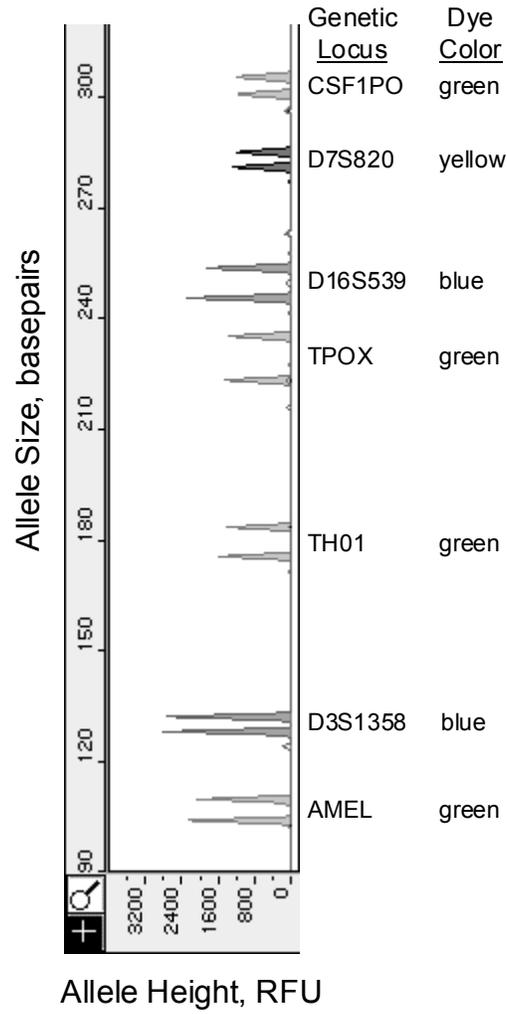


Figure 1

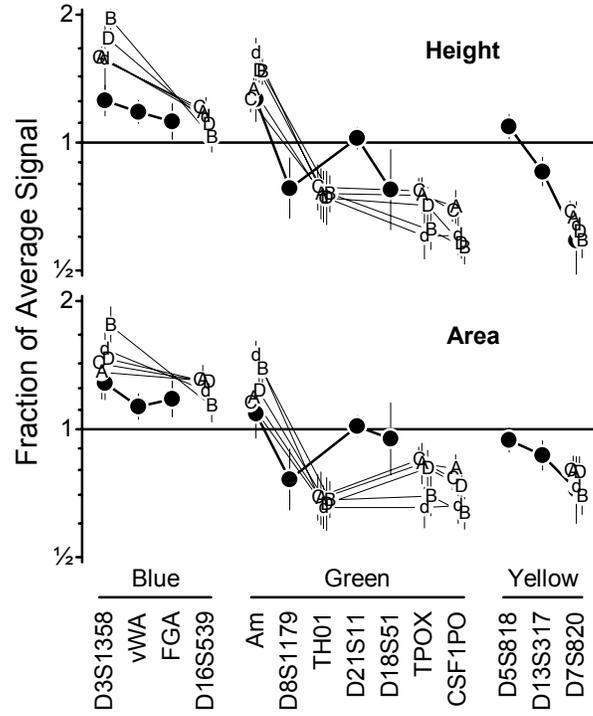


Figure 2

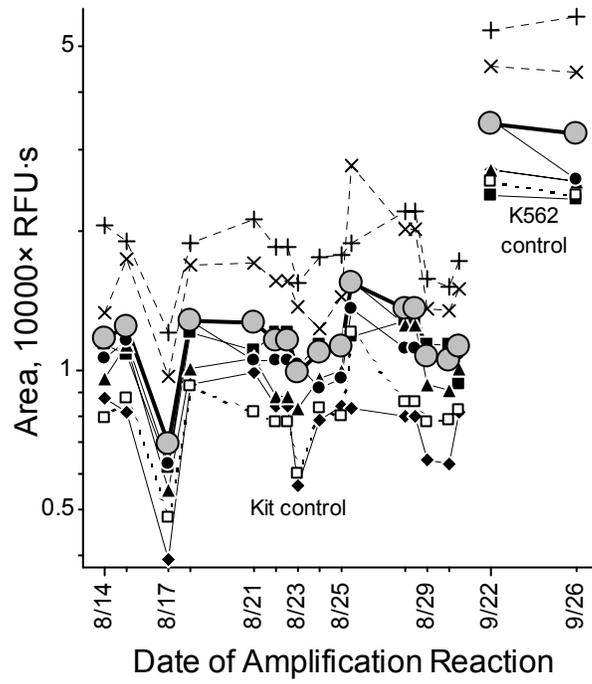


Figure 3

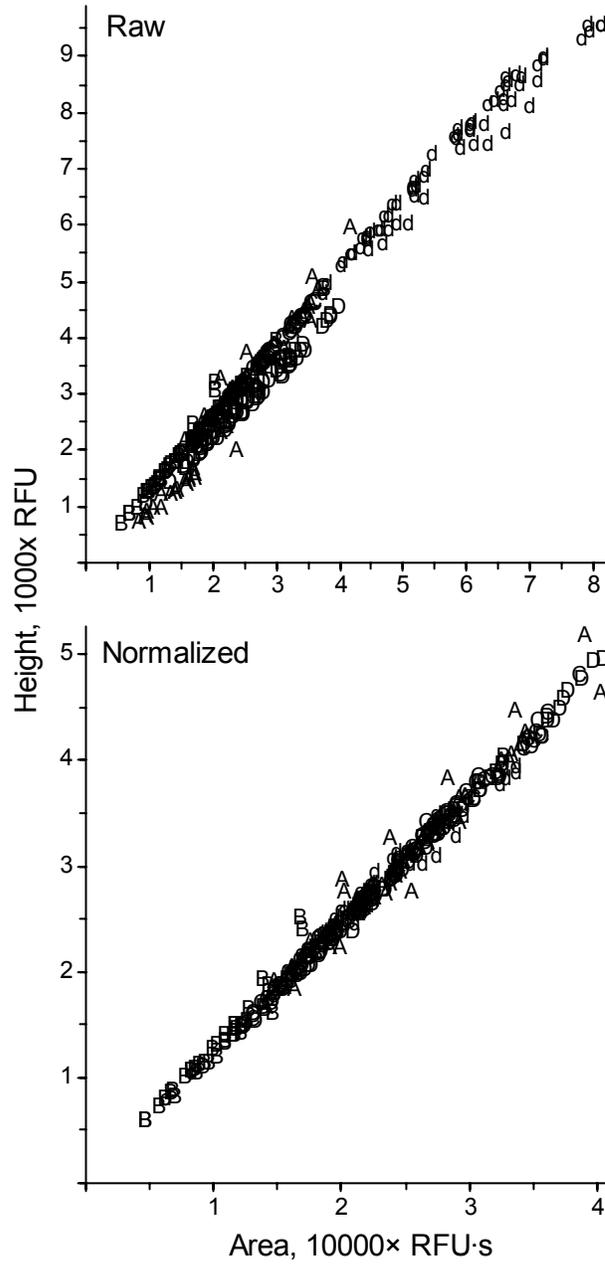


Figure 4

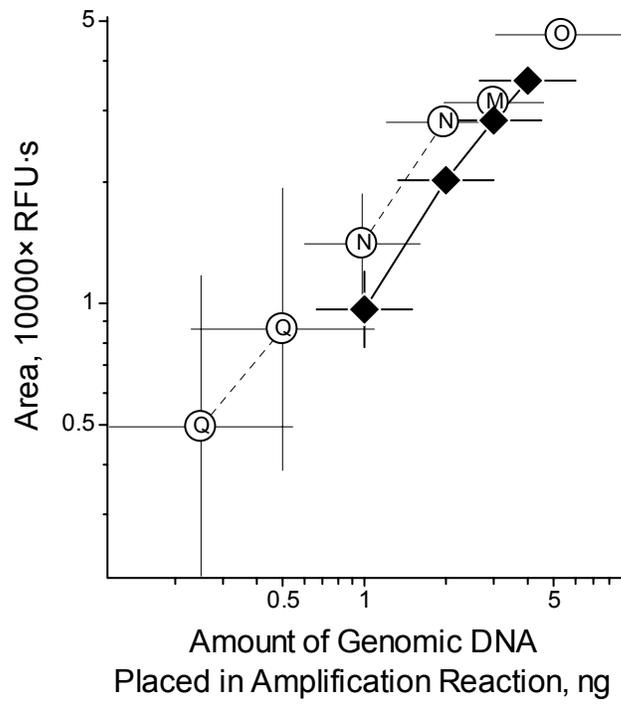


Figure 5

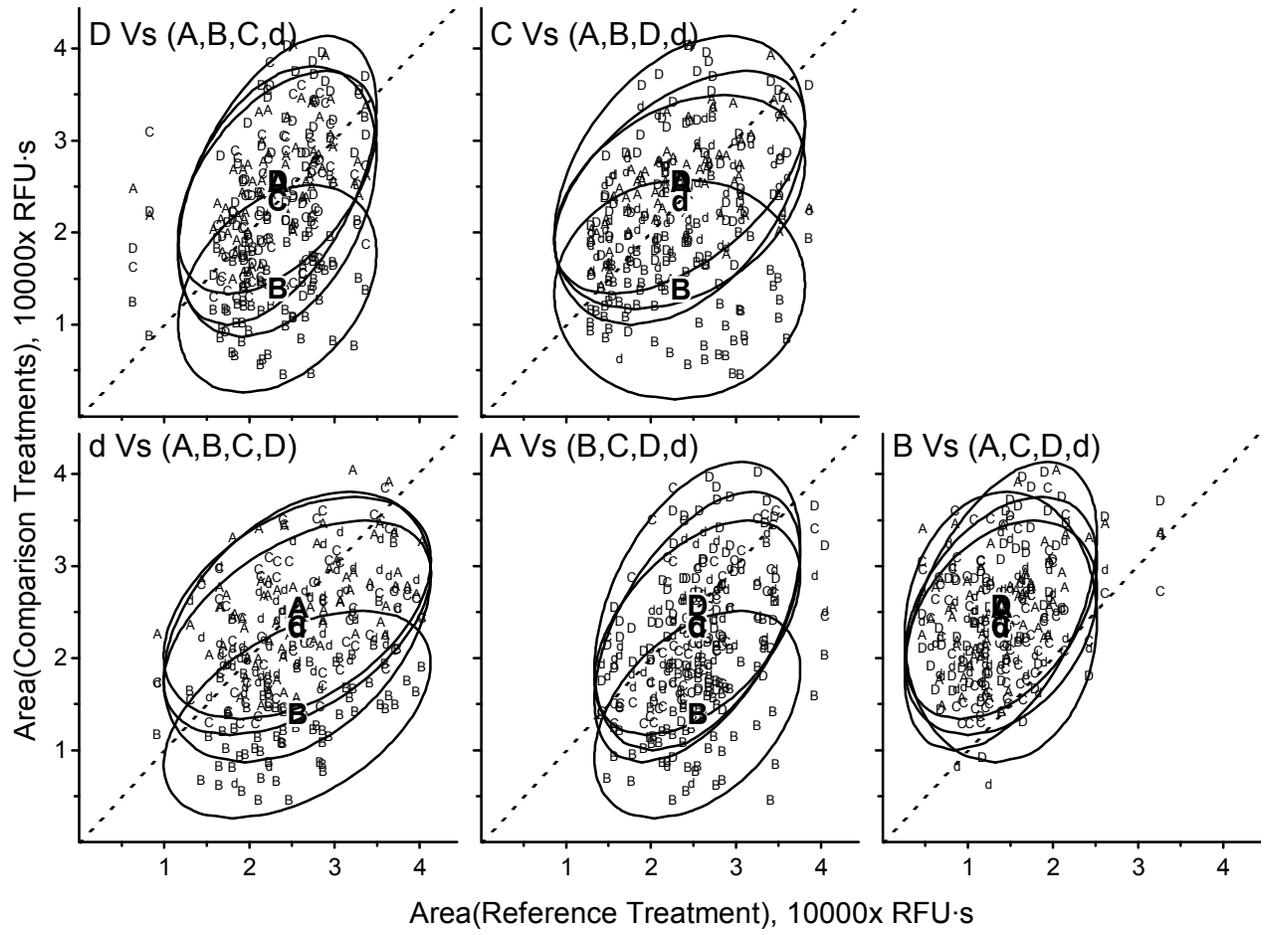


Figure 6