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Chemiluminescent Detection of RFLP Patterns in Forensic DNA Analysis

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ABSTRACT: DNA testing by restriction fragment length polymorphism (RFLP) analysis is an extremely important technique used in forensic science laboratories. While RFLP testing is a highly informative method, it traditionally has had several disadvantages. It is time consuming and involves work with radioactive phosphorous. A detection method that is faster and safer than isotopic detection is presented. Various membranes, fixation methods and transfer procedures were evaluated for DNA retention and sensitivity using alkaline phosphatase conjugated oligonucleotide probes and a chemiluminescent substrate. Blood samples and evidentiary material from forensic casework were analyzed by both chemiluminescent and isotopic detection. Results of each method were compared for pattern appearance, band size, and composite profile frequency. The chemiluminescent system had very good sensitivity, detecting 3–25 ng K562 DNA. Most patterns developed by both methods appeared the same. The variation observed between band sizes and frequency estimates generated by each method was as expected for an inter-gel comparison. The chemiluminescent detection procedure described here is suitable for use in forensic casework.

KEYWORDS: forensic science, DNA typing, chemiluminescence, restriction fragment length polymorphism, detection

The adoption of restriction fragment length polymorphism (RFLP) testing into routine use by the forensic science community in the late 1980s and early 1990s dramatically changed the quality of the information laboratories were able to provide to courts regarding the source of biological evidence. Although the technique is highly valuable it has a number of drawbacks. Some of the major disadvantages of current RFLP methods are due to the use of radioisotope labeled insert probes for detection. The use of ^{32}P is a safety hazard for personnel and requires a significant administrative commitment. The process of creating a film image by autoradiography is slow, routinely taking several days to obtain an acceptable image for each probe. Because of this time consuming exposure process, many RFLP cases take six to eight weeks to complete.

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Non-isotopic detection is an attractive alternative and has been of interest to researchers for years. Early methods included probes conjugated to biotin and digoxigenin and colorimetric detection (1–4). Many of these procedures did not give the sensitivity or image quality of isotopic detection and thus were not suited to forensic casework. The development of direct alkaline phosphatase (AP) conjugated probes and improved chemiluminescent substrates have provided a non-isotopic detection method that equals or surpasses ^{32}P detection systems (4–6).

Both analysis time and safety can be improved by the use of AP conjugated oligonucleotide probes and a chemiluminescent substrate for detection. Images can be generated in a matter of hours, not days. In this study the parameters of membrane, Southern transfer method and DNA fixation were evaluated with one hybridization protocol and a variety of variable number of tandem repeat (VNTR) probes for sensitivity and DNA retention. Subsequently blood samples from eighty different individuals and evidentiary material from forensic cases were examined to assess the performance of the system for forensic casework.

Materials and Methods

Membrane Preparation, Hybridization and Detection

Bloodstains and casework samples were prepared by the process described by Budowle and Baechtel (7) with some modifications (8) through the analytical gel. All analytical gels were made with GenePrint agarose (Promega Corp., Madison, WI), EEO 0.20–0.26. Ethidium bromide (500 ng/mL) was incorporated in the tank and gel buffers. Sensitivity dilution series were made with commercially quantitated HaeIII digested K562 DNA (Life Technologies Inc., Gaithersburg, MD).

Analytical gels intended for chemiluminescent detection included the ACES Chemiluminescent Marker DNA (Life Technologies Inc.) and were transferred to neutral nylon membrane, either Biodyne A or Nylon-1 (Life Technologies Inc.) by an alkali transfer or a neutral high salt transfer. The alkali transfer method consisted of a 30 minute denaturation in 500 mM NaOH, 500 mM NaCl and a 6 hour transfer in the same solution (9). For the neutral transfer, gels were denatured for 20 minutes in 500 mM NaOH, 1500 mM NaCl, briefly rinsed in water, neutralized for 20 minutes in 1500 mM NaCl, 1000 mM Tris, pH 8.0 and then transferred for 6 hours in 10x SSC (6) (20x SSC consists of 3000 mM sodium chloride and 300 mM sodium citrate, pH 7.0.) After transfer all membranes were washed for 10–15 minutes in 2x SSC, 200 mM Tris, pH 7.5. DNA was fixed to the membranes through a combination of baking at 80°C and UV crosslinking at 254 nm with a Stratalinker 2400 (Stratagene, La Jolla, CA).

Hybridization to AP conjugated oligonucleotide probes was performed by the procedure described by Klevan et al. (10). Membranes were prehybridized for 20 minutes at 55°C in 500 mM $NaPO_4$, pH 7.2, 1% Hammerstein casein, 0.5% Tween 20 (ACES 2.0 Hybridization Solution, Life Technologies, Inc.). Membranes were hybridized in the same solution at 55°C with hybridization time and probe concentrations varying according to manufacturer's recommendations. Probes to the loci D1S7, D4S139 and D5S110 were obtained from Life Technologies, Inc. (catalog numbers 14231-013, 24230-013 and 14232-011). Probes to the loci D2S44, D10S28 and D17S79 were obtained from Promega Corp. (catalog numbers DK5411, DK5481 and DK5431). Subsequent to hybridization, membranes were washed twice for 10 minutes at 55°C in 50 mM $NaPO_4$, pH 7.2, 0.5% Tween 20 (ACES 2.0 10x Wash Buffer 1, Life Technologies, Inc.) and twice for 5 minutes at room temperature in 10 mM Tris, pH 8.6, 150 mM NaCl (ACES 2.0 10x Final Wash Buffer, Life Technologies, Inc.). Membranes were removed from the final wash, touched briefly to Whatman 3 MM CHR paper (Whatman Lab Sales, Hillsboro, OR) and incubated for five minutes with agitation in LumiPhos Plus, 3.75 mL/membrane (Lumigen, Inc., Detroit, MI). After sealing in plastic folders, membranes were allowed to ramp overnight and were then exposed to Kodak XRP film. All ramps and exposures were done at ambient temperature.

After film exposure the probe was stripped in 10 mM Tris, pH 7.5, 1 mM EDTA, 0.5% Tween 20. The solution was heated to 95°C and added to the membranes. The membranes were agitated at 65°C for one hour then rinsed in 2x SSC for 5 minutes.

Analytical gels intended for radioactive detection included the LifeCodes 23kb Size Marker (LifeCodes Corp., Stanford, CT) and were transferred to the charged nylon membrane Biodyne B (Life Technologies, Inc.) by an alkali transfer procedure. Gels were denatured for 30 minutes in 400 mM NaOH and transferred for 6 hours in the same solution. Membranes were washed for 15 minutes in 2x SSC, 200 mM Tris, pH 7.5 and baked at 80°C for 30 minutes (7).

Hybridization to ^{32}P labeled insert probes was performed as described by Budowle and Baechtel (7). Autoradiographs were made on Kodak XRP with intensifying screens. Most exposures took three to five days. After autoradiography probe was stripped with a hot SDS solution before rehybridization (11).

UV Crosslinking

The optimum energy for UV crosslinking was determined. Analytical gels were loaded with one lane of size marker and one lane of 200 ng Hae III digested K562 DNA followed by an empty lane. This pattern was repeated five times across the gel. Four gels were transferred to Nylon-1 by the neutral high salt method. After the membranes were rinsed in 2x SSC/200 mM Tris, pH 7.5 they were blotted on Whatman 3 MM CHR paper. Two of the membranes were then crosslinked while still damp by irradiating each set of lanes with a different amount of energy: 15×10^4, 12×10^4, 8×10^4, 4×10^4 and 0 $\mu J/cm^2$. Heavy black paper and glass were used to mask the lanes that were not being exposed. The membranes were then baked at 80°C for 30 minutes. The other two membranes were baked first and then irradiated in the same manner.

Four additional gels were transferred to Biodyne A: two by the neutral transfer method and two by the alkali transfer method. After the membranes were rinsed and blotted, all four were baked at 80°C for 30 minutes and then UV crosslinked by irradiating each set of lanes with a different amount of energy: 12×10^4, 8×10^4, 4×10^4, 2×10^4 and 0 $\mu J/cm^2$.

All membranes were hybridized, stripped and rehybridized. The images produced were evaluated for sensitivity and signal to noise ratio.

DNA Retention and Sensitivity

To evaluate the retention of DNA through multiple hybridizations and to determine the sensitivities of the different probes, analytical gels were run containing a dilution series of Hae III digested K562 DNA ranging from 400 ng to 3 ng. Gels were transferred by the neutral transfer method to Nylon-1, baked at 80°C for 30 minutes and UV crosslinked with 4×10^4 $\mu J/cm^2$. Gels were transferred to Biodyne A by both transfer methods, baked at 80°C for 30 minutes and UV crosslinked with 2×10^4 $\mu J/cm^2$. Membranes produced with the neutral transfer method were hybridized and stripped twelve times as described above. Membranes produced by the alkali transfer method were hybridized and stripped six times.

Blood Sample Study

DNA from eighty individuals was analyzed to evaluate the chemiluminescent detection system on a range of band sizes at each locus. Anonymous blood samples (40 Caucasian, 40 African-American) were obtained from an Atlanta area blood drive, made into stains and processed according to standard laboratory protocol (8). Identical aliquots of approximately 400 ng were electrophoresed in two analytical gels. One gel was transferred to Biodyne B as described above and hybridized to ^{32}P labeled insert probes. The second gel was transferred to Biodyne A by the alkali transfer method, baked at 80°C for 30 minutes, UV crosslinked with 2×10^4 $\mu J/cm^2$ and subsequently hybridized to AP conjugated oligonucleotide probes as described.

The images generated by each method were evaluated for band appearance and were sized with the BioImage sizing software Visage 4.60 (Millipore Corp. Imaging Systems, Ann Arbor, MI, 1993). The band sizes obtained from the lumigraphs were compared with those from the autoradiographs. While the frequency of a profile is obviously wholly dependent upon the band sizes, we chose to evaluate the frequencies as well. The frequency of occurrence for each profile was calculated across all loci using the band sizes produced by each method. The two frequencies for each sample were compared. The comparison was done in three different databases.

Casework Sample Study

Nonprobative evidentiary material was examined to evaluate the performance of the chemiluminescent detection system on casework samples. Aliquots identical to those loaded on casework gels were obtained from approximately 50 casework samples where excess DNA was available. These samples were electrophoresed in an analytical gel and transferred by the alkali method to Biodyne A. The membranes were baked for 30 minutes at 80°C, UV crosslinked at 2×10^4 $\mu J/cm^2$ and hybridized to AP conjugated oligonucleotide probes as described. The images generated by standard exposure were compared to the casework autoradiographs for band appearance and band size.

Low Molecular Weight Alleles

Eight samples from the blood sample study that were found to have alleles below 1 kb at the locus D2S44 were processed according to standard laboratory protocol (8). Identical aliquots of approximately 200 ng were electrophoresed in two analytical gels. One gel was transferred to Biodyne A by the alkali method; the other gel was transferred to Biodyne A by the neutral high salt method. Both membranes were baked at 80°C, UV crosslinked at 2×10^4 μJ/cm^2 and hybridized to AP conjugated oligonucleotide YNH24. The images were evaluated based on the appearance of the low bands.

Results and Discussion

UV Crosslinking

Optimized UV crosslinking is critical to achieve maximum DNA retention and sensitivity on neutral nylon membrane. Different amounts of energy were applied to both damp and dry membranes in an effort to find conditions that give optimum results. From Nylon-1 the strongest signal over the range of band sizes was obtained with the neutral transfer when the membrane was baked first and then crosslinked with 4×10^4 μJ/cm^2. See Fig. 1. From Biodyne A the strongest signal over the range of band sizes was obtained with both transfer methods when the membrane was baked first and then crosslinked with 2×10^4 μJ/cm^2 (data not shown). Baking alone did very little to fix the DNA. Signal strength in the lanes that were baked but not crosslinked is significantly less than in any other lane. In subsequent studies membranes were baked first and crosslinked using these conditions.

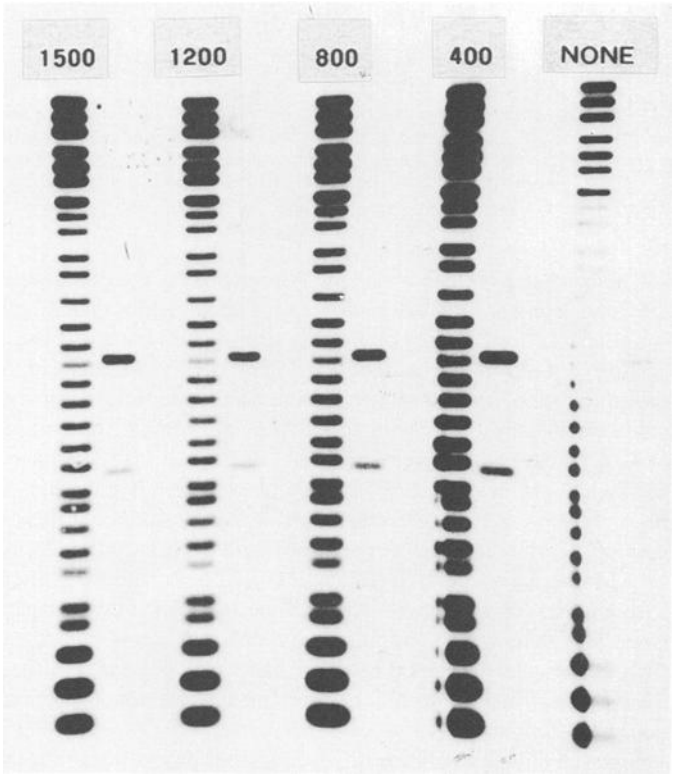

FIG. 1—*UV crosslinking energy determination. Nylon-1 membrane generated by the neutral transfer and hybridized to YNH24. Each pair of lanes contains the BRL ladder followed by 200 ng K562 DNA. The membrane was baked for 30 minutes at 80°C before each set of lanes was crosslinked with a different amount of energy. The figure shows the Stratalinker setting used. A setting of 1500 gives 1.5 × 10^4 μJ/cm^2.*

DNA Retention and Sensitivity

Alkaline phosphatase catalyzes the breakdown of Lumigen®-PPD (4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane]) in the LumiPhos Plus, releasing photons in the process. The rate of light emission increases with time until a maximum light output is reached. Approximately 75% of the maximum output is reached in 10 hours (12). By allowing membranes to ramp overnight before making exposures the time required to make the desired exposure can be more easily determined.

The sensitivities (nanograms K562 DNA) observed with two hour exposures on Kodak XRP following an overnight ramp are given in Table 1. All lumigraphs had acceptable to very good background and were clearly suitable for interpretation.

Under the fixation conditions described, the retention of DNA was good on both membranes. No significant change in sensitivity was observed after membranes were hybridized and stripped 12 times. Lumigraphs made from Nylon-1 membranes showed less sensitivity and less background than those made from Biodyne A membranes. On longer exposures, the sensitivity of Nylon-1 as well as the background was similar to that of Biodyne A. For Biodyne A, the neutral transfer gives slightly better sensitivity than the alkali transfer although the sensitivity of both methods is very good. Minor variation between repeated hybridizations of the same probe is not significant because factors such as ramp time and amount of substrate absorbed, which can be difficult to control, can cause small differences in the exposure strength. Ramp temperature may also be a factor. Film exposure time can be adjusted to compensate for this variation if needed.

In addition to ramp condition and substrate volume other parameters can be used to manipulate exposures. The use of a white surface behind the membrane (for example, Whatman 3 MM CHR or an intensifying screen) will give a stronger image than the use of a black surface. See Fig. 2. Film speed can dramatically effect

TABLE 1—*Sensitivity and DNA retention.*

Hyb number	Probe	Nylon-1 neutral transfer	Biodyne A neutral transfer	Biodyne A alkali transfer
1	TBQ7	100 ng	25 ng	12–25 ng
2	LH1	25 ng	3 ng	6–12 ng
3	YNH24	25 ng	3 ng	25 ng
4	pH30	100 ng	12 ng	25 ng
5	MS1	200 ng	6 ng	6 ng
6	V1	50 ng	6 ng	12 ng
7	TBQ7	200 ng	25 ng	...
8	LH1	25 ng	6 ng	...
9	YNH24	50 ng	6 ng	...
10	pH30	200 ng	25 ng	...
11	MS1*	25 ng	3 ng	...
12	V1	50 ng	6 ng	...

NOTE: ... = not tested.

*Probe used in this hybridization was a different lot from that used in No. 5. Determinations (nanograms K562 DNA detected) were based on the results of 2 membranes, all exposed for 2 hours on Kodak XRP following an overnight ramp. All lumigraphs had acceptable to very good background and were suitable for interpretation.

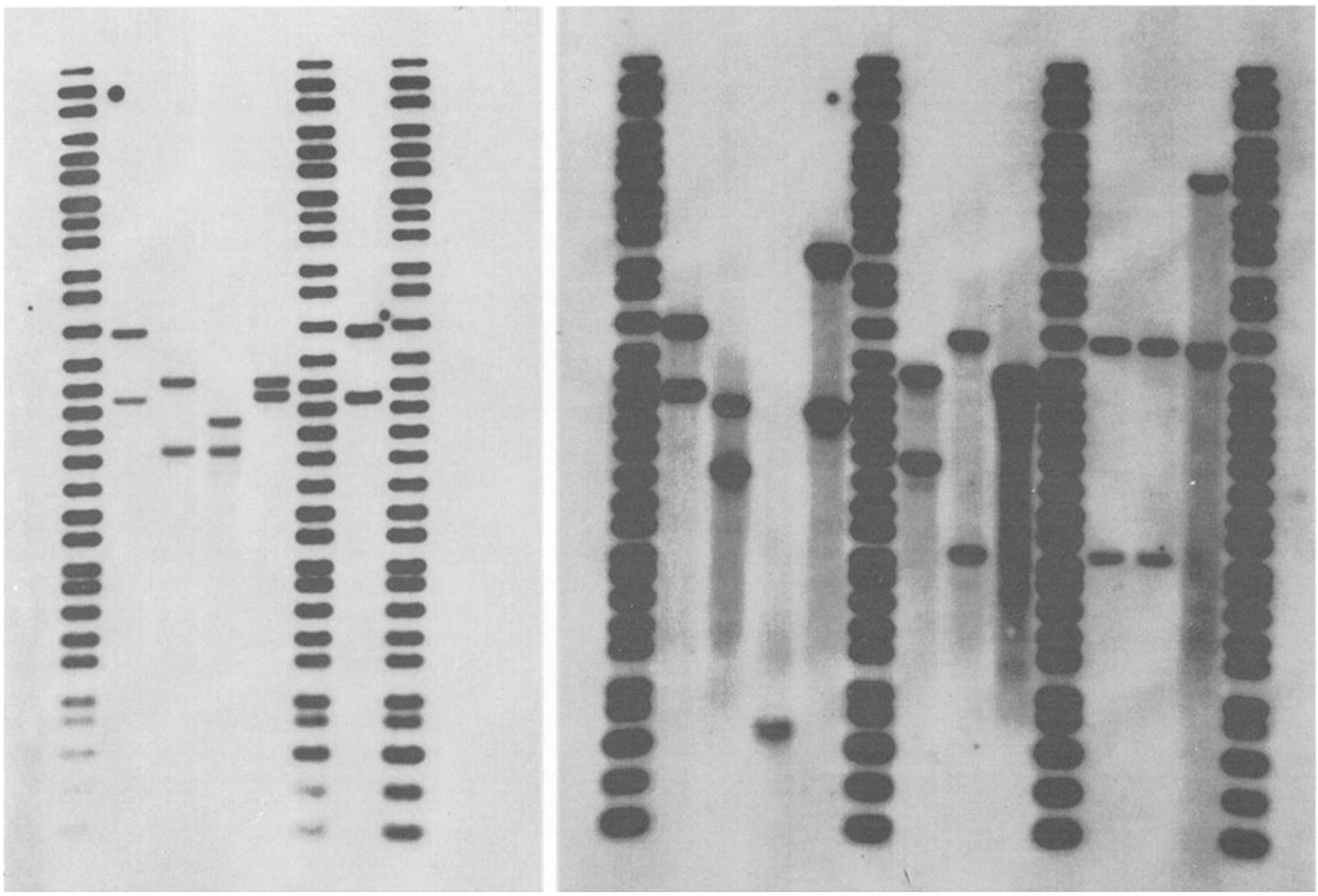

FIG. 2—*Effect of light or dark surface. Both membranes are Biodyne A and were generated by the alkali transfer, hybridized to LH1 and exposed together to Kodak XRP for 80 minutes following an overnight ramp. The membrane on the left had a black surface behind the membrane and the film. The membrane on the right had a white surface behind the membrane and the film. Both membranes contain the same amount of ladder and 400 ng of K562 DNA (in lane 7 on the left and lane 2 on the right).*

exposure time. Reflection® film (DuPont) gives a result equivalent to Kodak XRP in roughly one-fourth the time. See Fig. 3.

Blood Sample Study

Eighty blood samples were typed by both chemiluminescent and isotopic detection methods at five loci for a potential 800 bands. Only one band was detected by either method in 15 samples. These were determined to be single band patterns, making the total number of bands available for detection 785. The isotopic method detected 783 bands (99.7%) while the chemiluminescent method detected 780 bands (99.4%). The images generated by chemiluminescence were in general stronger and sharper than the images generated by radioactivity. See Fig. 4. The two bands not detected by the isotopic method, a band near 1 kb at the locus D10S28 and a band near 2.2 kb at D4S139, were most likely missed due to lesser sensitivity. Of the five bands not detected by the chemiluminescent method, four were near or below 1 kb at the locus D2S44 and the remaining band was near 1 kb at D10S28. Six other small alleles (below 1 kb) at the locus D2S44 were visible but much weaker than their isotopic counterparts. While bands were missed by each method, the apparent pattern of chemiluminescent detection missing low bands at D2S44 prompted additional investigation of the transfer method. See the section below on low molecular weight alleles.

The band size obtained from the lumigraph was compared with the band size from the autoradiograph. Bands greater than 10 kb were not included in the comparison leaving 742 pairs of bands. The percent difference between bands in a pair was calculated as the difference between the chemiluminescent value and the isotopic value divided by their average. For the 742 comparisons made, 58% of these values were between −1.0 and 1.0, 88% were between −2.0 and 2.0 and 97% were between −3.0 and 3.0. See Fig. 5. Some variation between duplicate samples run on different gels with different molecular weight markers (LifeCodes' 23 kb Size Marker versus Life Technologies' ACES Chemiluminescent Size Marker) was expected (13,14). Of the 742 pairs in this comparison, 741 were within the laboratory match window at ±2.5% (that is, percent difference between bands equal to or less than 5%). One pair near 9 kb at D1S7 was outside the window with a percent difference of 5.25%.

Figure 5 shows an apparent trend for band sizes obtained from lumigraphs to be slightly larger than those obtained from the autoradiographs. The mean size measurement differences (mean of percent differences) by locus were 0.82% for D1S7, 0.80% for D2S44, 0.90% for D4S139, 0.69% for D5S110 and 0.58% for D10S28. Sequential probing with MS1 was performed on half of the blood samples. The Biodyne A membranes, containing 40 samples, that were first hybridized to the alkaline phosphatase

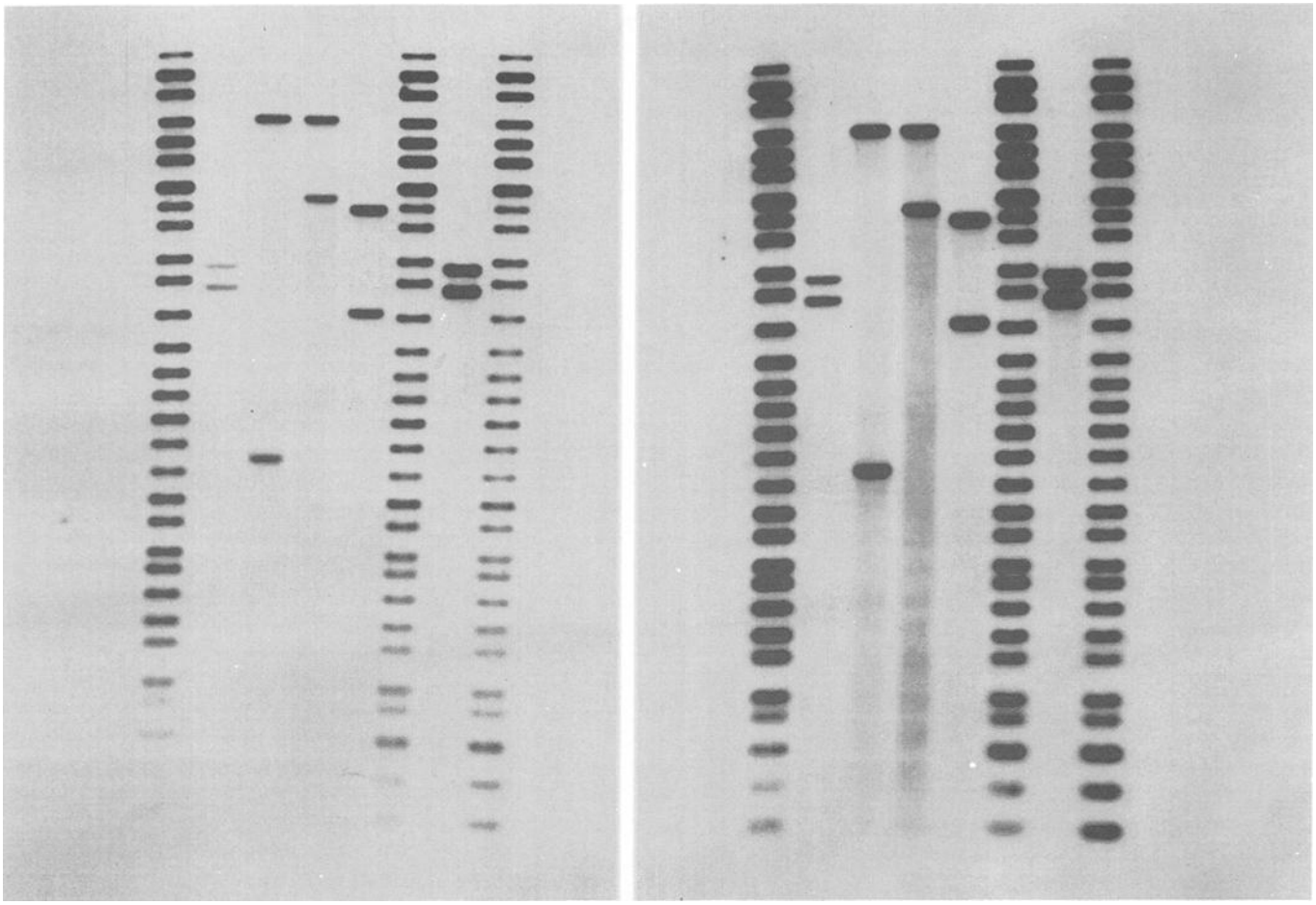

FIG. 3—*Film speed. This Biodyne A membrane was made by the alkali transfer, hybridized to MS1 and exposed to film following an overnight ramp. The image on the left results from a 30 minute exposure to Kodak XRP and the image on the right from a 30 minute exposure to DuPont Reflection®.*

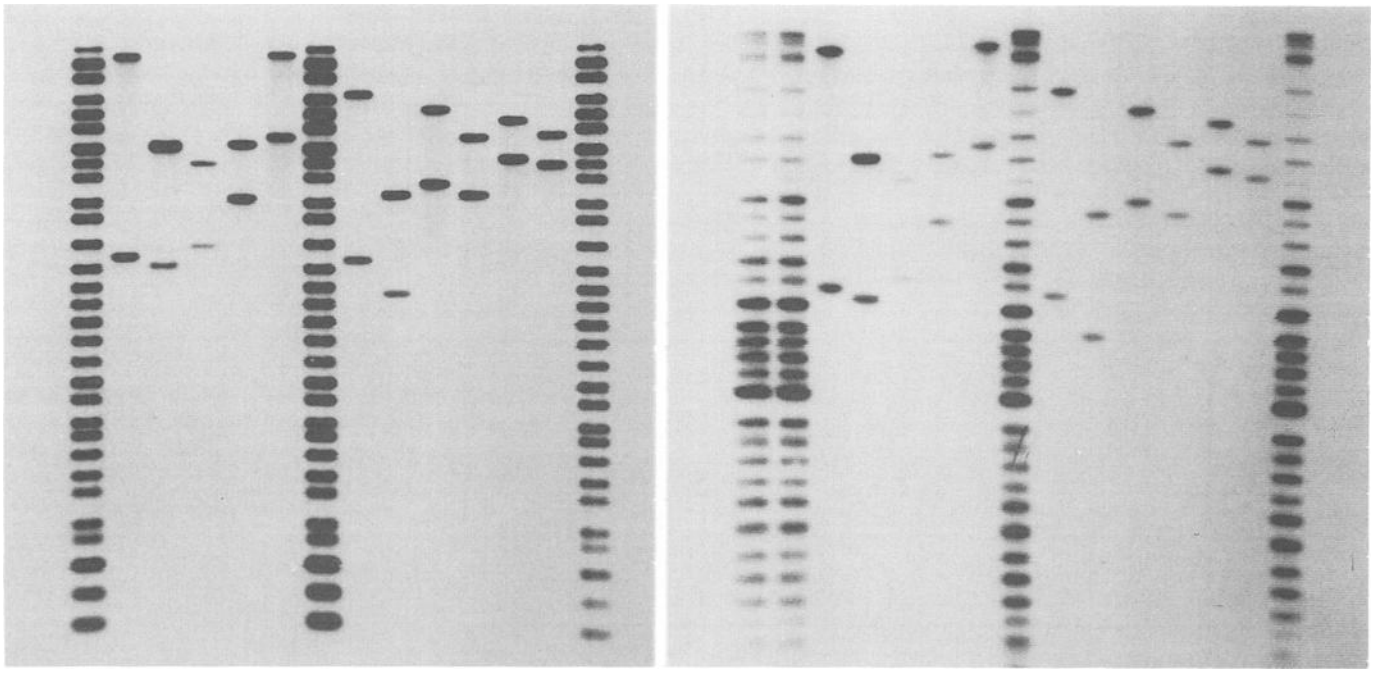

FIG. 4—*Blood sample study—chemiluminescence versus ^{32}P. Samples contain 400 ng DNA from 10 individuals plus K562 (second sample lane). The membrane on the left was hybridized to AP conjugated pH30 and exposed to Kodak XRP for 1 hour following and overnight ramp. The membrane on the right was hybridized to ^{32}P labeled pH30 and exposed to Kodak XRP at −80°C for 5 days.*

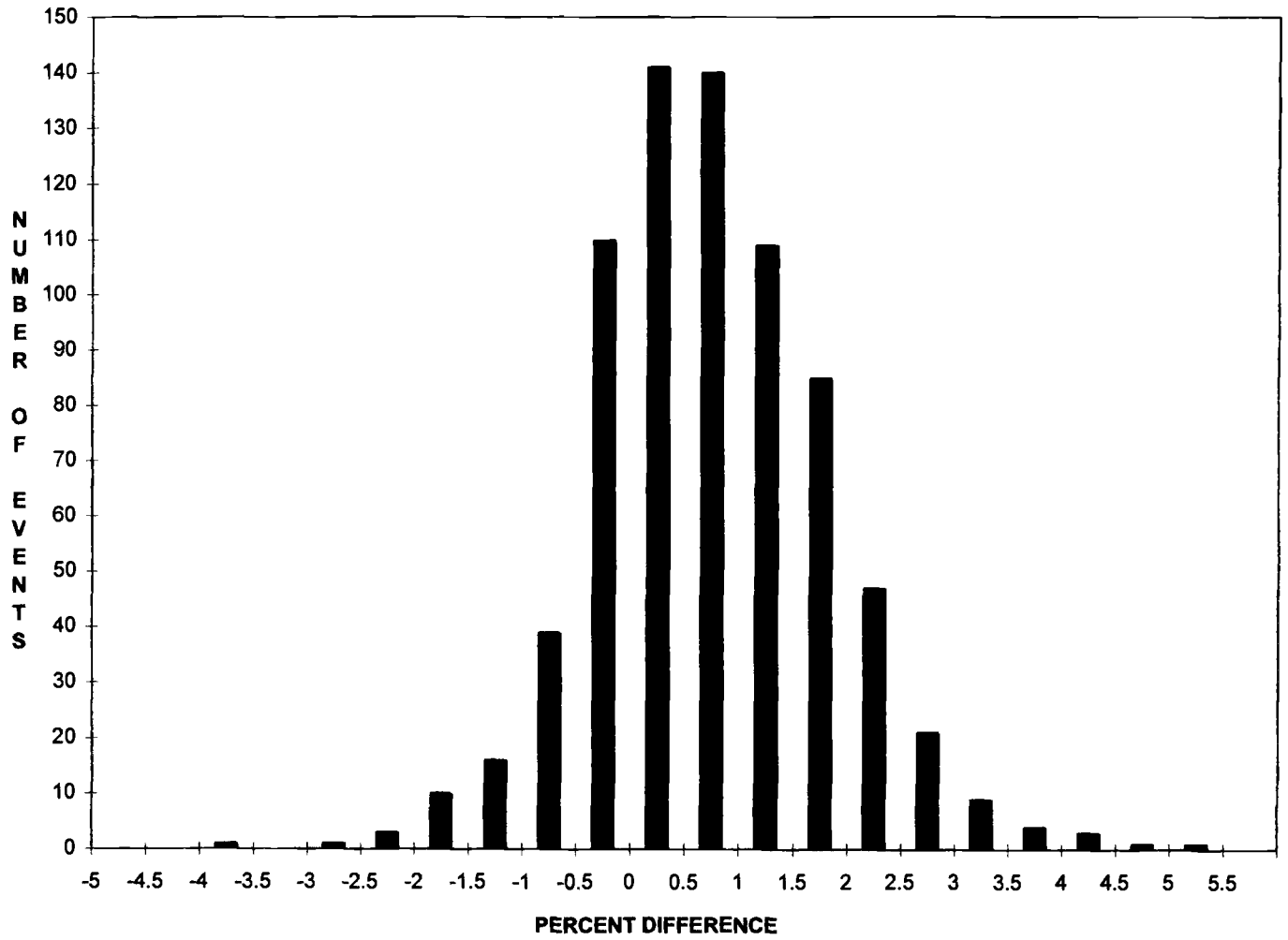

FIG. 5—*Distribution of percent differences between sizes from lumigraphs and sizes from autoradiographs in the blood sample study.*

conjugated oligonucleotide probes were rehybridized to ^{32}P labeled insert probe MS1 as described above for Biodyne B membranes with the only change being the addition of probe for the Life Technologies size marker rather than the LifeCodes marker. For these 40 samples at D1S7 the mean size measurement difference from the parallel comparison was 0.90%. When the chemiluminescent detection sizes for the same group were compared to the sizes generated by sequential isotopic probing the mean size measurement difference was −0.02%, with 96% of the values falling between −1.0 and 1.0%. For this limited test group, when the same membranes were probed sequentially, eliminating variation due to different gels and molecular weight markers, the bias was not observed. This finding suggests the apparent trend is not due to detection method. Benzinger et al. found no variation in molecular weight due to detection method (15).

Frequency estimates were calculated for the composite profiles of all 80 individuals using the method described by Budowle et al. (13). One estimate was calculated with the band sizes obtained from the lumigraphs. A second estimate was calculated using the sizes from the autoradiographs. Bands greater than 10 kb were not included. The two frequencies for each sample were estimated in the Caucasian, African-American and Combined Hispanic databases published by the Federal Bureau of Investigation for a total of 240 comparison pairs (16). All samples had results at five loci. The largest frequency estimate obtained was 5.2×10^{-7}; the smallest was 1.5×10^{-15}.

Figures 6 through 8 show the log of the inverse frequency based on chemiluminescent detection plotted against the log of the inverse frequency based on isotopic detection. Where the estimates are the same the point falls on the line $x = y$. Points for estimates that are not the same are distributed above and below the line. In some cases the more common estimate was generated by the isotopic profile, in other cases by the chemiluminescent profile. Some variation around the $x = y$ line is expected as even multiple sizings of the same lane can generate different frequencies due to changes in bin assignments (13).

In all three databases the majority of the profile pairs had the same frequency estimate or estimates that differed by a factor of two or less. In the Caucasian database 87% of the pairs fell into one of these two categories, in the African-American and Combined Hispanic databases 90% did. See Fig. 9. In no pair did the estimates differ by an order of magnitude. Only three of the 240 pairs had a ratio between the estimates in the 5 to 7 range. In these three pairs the estimates, chemiluminescent versus isotopic, were 7.6×10^{-11} versus 1.2×10^{-11}, 1.1×10^{-11} versus 1.8×10^{-12}, and 1.5×10^{-15} versus 7.9×10^{-15}; all of these profiles are very rare. This variation is not meaningful in the forensic context in which these estimates are used (17).

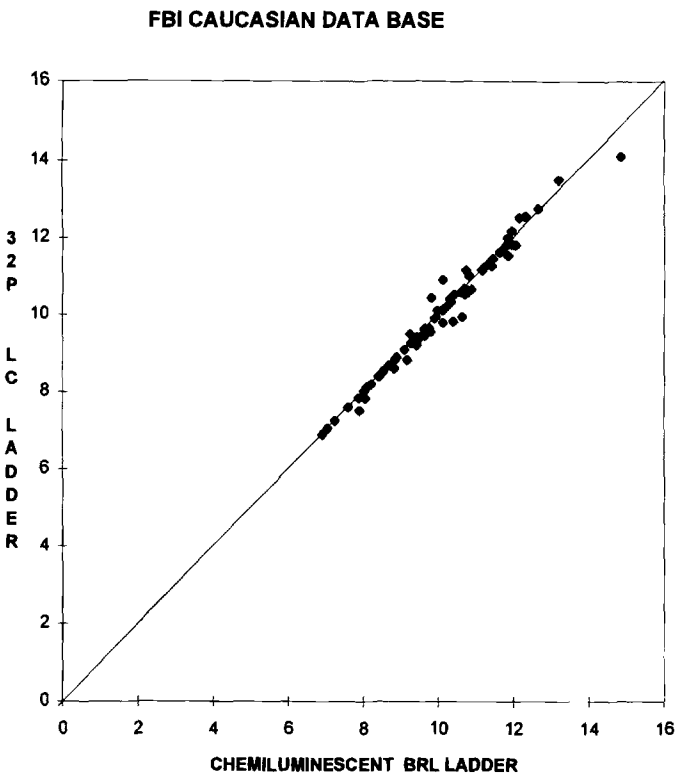

FIG. 6—*Frequency estimates for the 80 individuals in the blood sample study determined in the FBI Caucasian database. Estimates generated by chemiluminescent detection (as logs) are plotted against estimates generated by isotopic detection (as logs).*

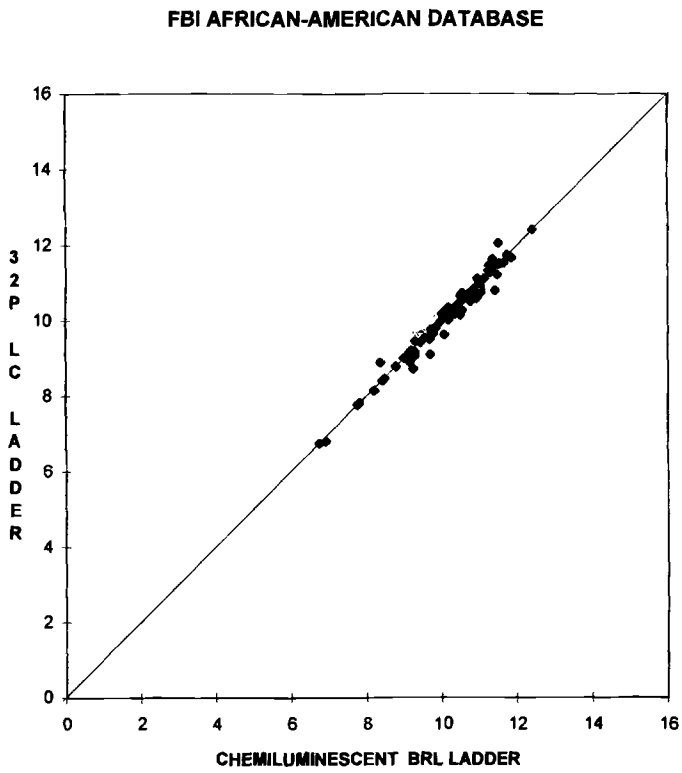

FIG. 7—*Frequency estimates for the 80 individuals in the blood sample study determined in the FBI African-American database. Estimates generated by chemiluminescent detection (as logs) are plotted against estimates generated by isotopic detection (as logs).*

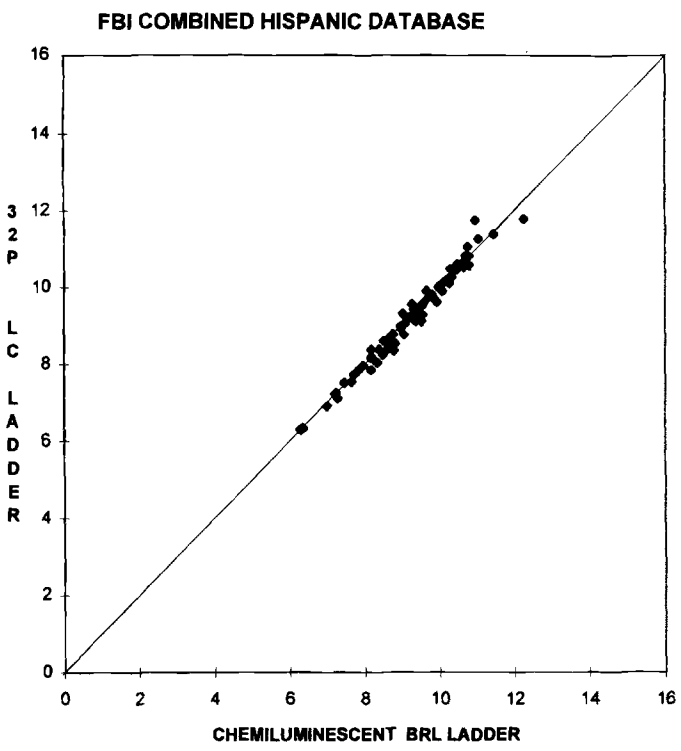

FIG. 8—*Frequency estimates for the 80 individuals in the blood sample study determined in the FBI Combined Hispanic database. Estimates generated by chemiluminescent detection (as logs) are plotted against estimates generated by isotopic detection (as logs).*

Casework Sample Study

Non-probative evidentiary material was tested to evaluate the chemiluminescent detection system on the type of insulted samples encountered in forensic casework. The band and degradation patterns detected by each method were visually compared for each probe. Forty-seven samples were typed at up to six loci for a total of 249 probe patterns that could be compared to casework autoradiographs. The majority of the patterns appeared the same by both methods. In some patterns bands were detected by the isotopic method that were missed by chemiluminescence and in other patterns vice versa. However, for the samples examined here in no instance did the different detection methods yield a different overall interpretation of the evidence.

For 223 of the 249 patterns (89.5%) the appearance of the patterns was the same: the primary bands appeared the same and in-lane background and additional bands, if any, were the same.

Eight patterns (3.2%) from eight different samples showed primary bands on the autoradiograph that were not detected by chemiluminescence. Six of these patterns contained bands below 1 kb at D2S44 or D10S28. In the remaining two patterns bands were missed on weak samples on a standard exposure from D1S7. These bands were visible on a longer exposure. The same trend of not detecting low bands at D2S44 that was seen with the blood sample study was observed here. See the section below on low molecular weight alleles.

Four patterns (1.6%) from four different samples showed primary bands on the lumigraphs that were not seen with isotopic detection. Seven bands ranging from 1317 bp to 5762 bp at the loci D2S44, D4S139 and D10S28 were missed. All four samples were weak and these bands were most likely missed by isotopic detection due to its lesser sensitivity.

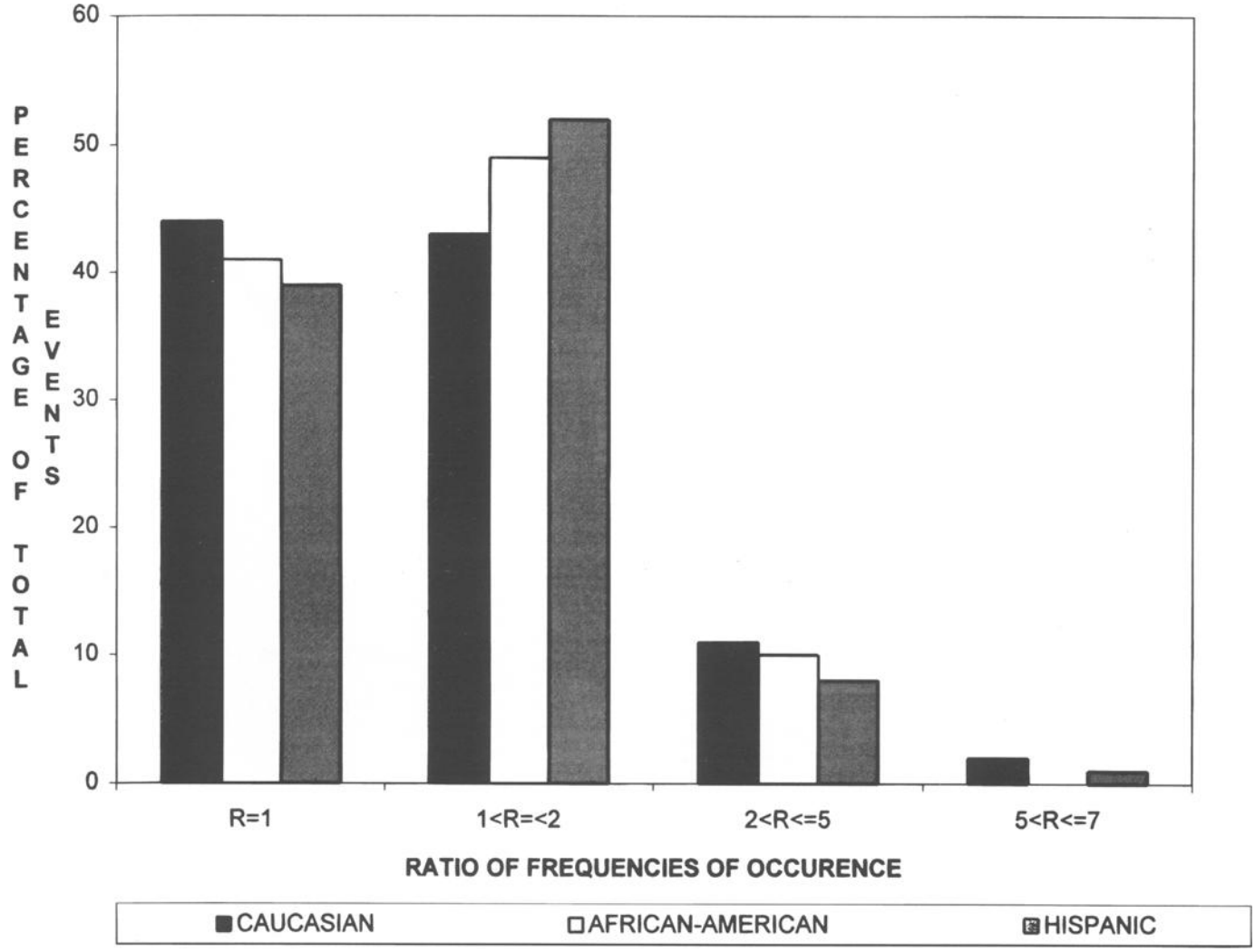

FIG. 9—*Distribution of ratios of frequencies of occurrence. The ratio was calculated as the larger value divided by the smaller value.*

Fourteen patterns (5.5%) showed the same primary bands by both methods but showed additional bands or background on the lumigraphs. The additional bands in five of the 14 samples appear to be consistent with partial digestion and were most likely missed by isotopic detection due to its lesser sensitivity. The additional bands seen in eight of the remaining patterns are weak upper bands on strong samples at the locus D17S79 that do not appear to be consistent with partial digestion.[2] The remaining sample showed some in-lane degradation and weak additional bands at the locus D1S7.

All primary bands below 10 kb were sized and the sizes compared to the casework results. All comparisons were within the laboratory match window of ±2.5%. For the 481 pairs of bands in this inter-gel comparison 56% had percent differences between −1.0 and 1.0, 90% between −2.0 and 2.0 and 96% between −3.0 and 3.0. Percent difference was calculated as in the blood sample study.

[2]These bands were also observed in about 75% of the samples in the blood sample study on long exposures. They range in size from about 15 kb down into the V1 range and do not fit the pattern of partial digestion bands described by Deadman for D17S79 (Hal Deadman, personal communication). The probe used in both studies was Promega lot number 4105801.

The same trend for the lumigraph size to be greater than the autoradiograph size that was observed in the blood sample study was also seen with these samples. The mean size measurement differences by locus were 0.50% (D1S7), 0.56% (D2S44), 0.83% (D4S139), 0.43% (D10S28) and 0.53% (D17S79). Again, these duplicate samples were run in different gels with different molecular weight markers and some variation was expected (13,14). Both the distribution of percent differences and the mean size measurement differences for this group of samples are similar to those observed in the blood sample study.

Low Molecular Weight Alleles

DNA samples with small alleles (near or below 1 kb) can display a great difference in intensity between the upper and lower bands. This difference in intensity is a property of the sample and occurs regardless of the detection method used. The lower band is more difficult to detect and can fail to be detected well before the upper band in a titration series, again with either detection method (data not shown). In both the blood sample and casework sample studies some small alleles were not detected by the chemiluminescent method that were detected by the isotopic method. This occurrence was most commonly observed at D2S44. It has been demonstrated

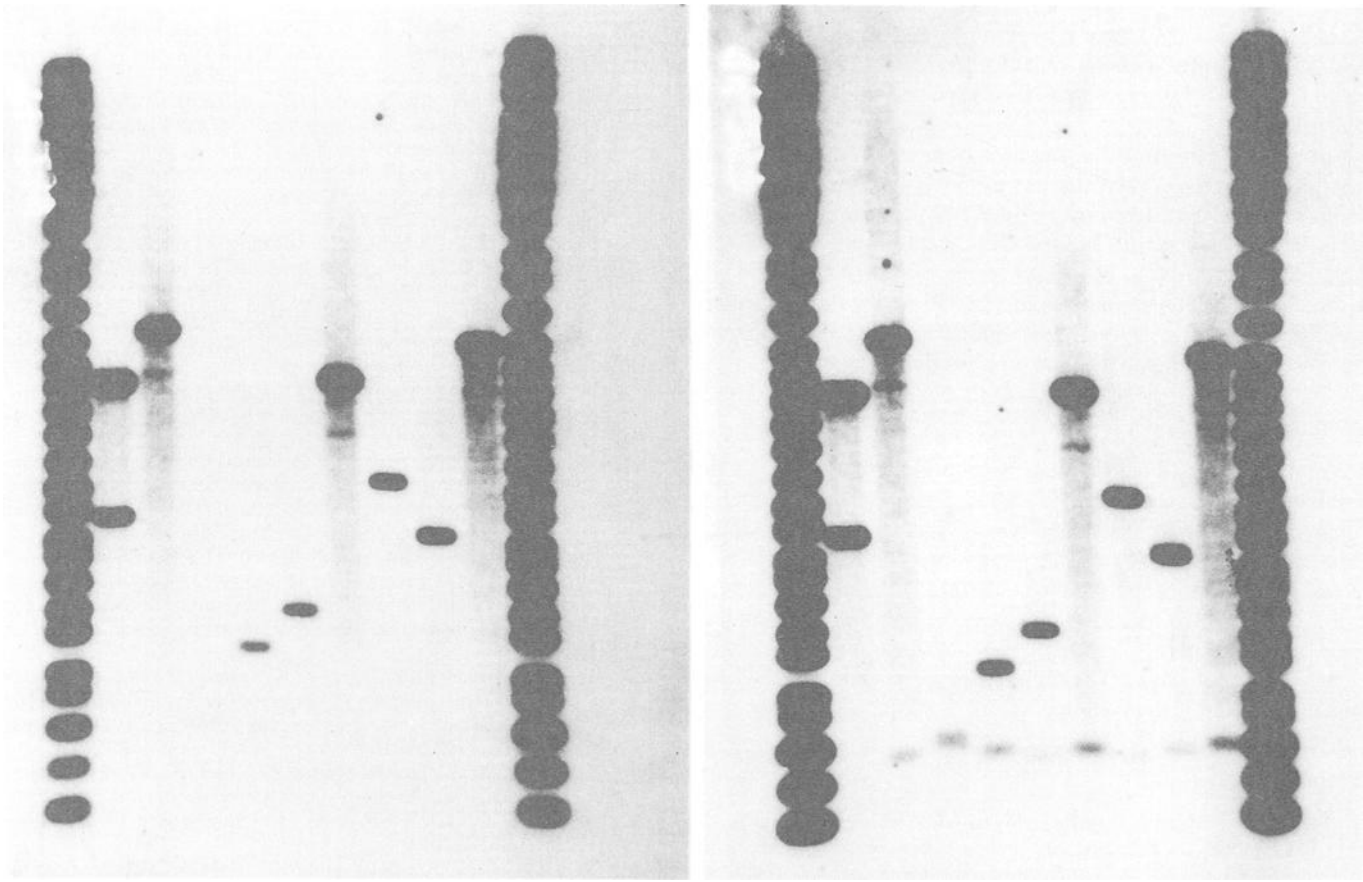

FIG. 10—*Low bands. Both membranes are Biodyne A, were hybridized to YNH24 and exposed together to Kodak XRP for 2 hours following an overnight ramp. The membrane on the left was made with the alkali transfer method while the membrane on the right was made with the high salt neutral transfer method. Both membranes contain 200 ng K562 DNA in lane 2 and eight samples, also 200 ng, known to have a small allele at D2S44.*

that this is a result of decreased sensitivity in this region and is not due to a difference in probe specificity (15). While the overall detection capability and sensitivity of the chemiluminescent system are very good, usually better than that of our ^{32}P system, improvement in this area was desirable to reduce instances of the lower band being missed.

The alkali transfer was used for the blood sample and casework sample studies because of its simplicity and economy. In an effort to improve detection of low bands the alkali transfer and the neutral transfer were compared with respect to small alleles. Eight blood samples with alleles below 1 kb at D2S44 were electrophoresed in duplicate on identical gels and one transferred by each method to Biodyne A. The membranes were hybridized together to YNH24. See Fig. 10. In all eight samples the low bands were visible when transferred by the neutral method. When the samples were transferred by the alkali method the low bands are very weak or not visible at all. The substantial difference in intensity between the larger and smaller alleles in these samples is also observed with isotopic detection (data not shown); the low bands are weak with both detection methods.

The alkali transfer is more economical and requires less labor than the neutral transfer and may be the method of choice when the quantity of DNA is not limited and the detection of small alleles can be enhanced by increasing the amount of DNA loaded on the gel. When the quantity of DNA is limited, as it often is in forensic samples, the neutral transfer is preferable.

Conclusions

Detection of RFLP patterns with alkaline phosphatase conjugated oligonucleotide probes and a chemiluminescent substrate provides an excellent alternative to isotopic detection. Relative to methods requiring radiation, the method described here is easy to implement, easy to use and provides results in a fraction of the time. With this system, results from five probes can be obtained in a week.

The system is very sensitive with the six probes tested, more sensitive than the isotopic system in our laboratory. On standard two hour exposures 3–25 ng of K562 DNA were detected. The combination of membrane, Southern transfer and UV fixation that was selected gives excellent DNA retention. Membranes that went through the hybridization/strip cycle 12 times had very good sensitivity, showing 6 ng of K562 DNA on a two hour exposure.

The hybridization protocol was tested on membranes made by an alkaline transfer method and a neutral high salt transfer method. Both methods worked well. The neutral method was slightly more sensitive and gave better results in detection of low molecular weight alleles at D2S44 and is therefore a better choice for samples with limited DNA as are often encountered in forensic casework.

In a study of 80 blood samples chemiluminescent detection was very successful, finding 99.4% of bands compared to 99.7% for isotopic detection. Samples in this study were transferred by the alkali method. Had the neutral transfer been used the percentage

of bands detected may have been even higher. A comparison of band sizes and profile frequencies generated by each detection method showed variation that was expected for an inter-gel comparison.

Forty-seven non-probative samples from evidentiary material were tested and the probe patterns visually compared to the casework results. The vast majority of the 249 patterns appeared the same. Where the patterns differed there were no differences that altered the interpretation of the evidence.

The chemiluminescent detection system described in this paper provides an attractive alternative to isotopic methods. It is sensitive and robust and well suited to use in forensic casework.

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