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Enhanced Conditions for DNA Fingerprinting with Biotinylated M13 Bacteriophage

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ABSTRACT: Deoxyribonucleic acid (DNA) fingerprints are Southern blots which have a pattern resembling bar codes. The pattern is created by DNA probes that bind to variable-length repeated sequences of human genomic DNA digested with restriction endonucleases. To improve DNA fingerprints obtained with biotin-labeled M13mp8 replicative form (RF) bacteriophage as the gene probe, the conditions for hybridization and the subsequent washing steps of the filter were refined. Experiments were conducted varying the electrophoresis time, blotting membranes, hybridization solution, and posthybridization washes. The simplicity, sensitivity, and reliability of this nonisotopic technique make possible its application for identification of individuals within a species, for parentage testing, and for monitoring bone marrow transplantation.

KEYWORDS: pathology and biology, deoxyribonucleic acid (DNA), bacteriophage

The use of wild Type M13 phage deoxyribonucleic acid (DNA) to detect highly variable minisatellite sequences (DNA fingerprints) was reported initially by Vassart et al. [*1*]. This ability to detect the hypervariable sequences can be attributed to a sequence present in the protein III gene of the M13 bacteriophage, in a region between coordinates 1013 and 2528 on the M13 map [*2*]. Devor et al. [*3*] modified Vassart's technique, using the 849 and 309 base pair fragments of M13 digested with Hae III as the gene probe to identify cultured cell lines. Improvement in the fingerprint has been claimed [*4*], obtained by using bovine serum albumin in place of "blotto" during hybridization. A comparative study using M13 and other minisatellite probes for DNA fingerprinting in domestic animals has been reported [*5*]. Although the banding patterns are different in different species, care needs to be taken to identify the origin of the specimen in forensic science samples.

All the studies just described used radioactively labeled M13 as the probe. We recently described [*6*] the basic procedure for performing DNA fingerprinting with biotinylated M13 instead of the bacteriophage labeled with phosphorus-32 [^{32}P]. In this paper, we have expanded and refined the system of DNA fingerprinting by using nonradioactive M13 probes. At this time, the best DNA fingerprints were obtained under the following

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conditions: approximately 41 h of electrophoresis at 1.5 V/cm, alkaline transfer by Southern blot to Oncor nylon, low-stringency hybridization conditions, and low-stringency posthybridization washes. We have also investigated the lower limits of detection of genomic DNA. A DNA fingerprint can easily be made visible with as little as 2 μg of human genomic DNA. Since M13 is so universally available, the improved method described in this paper for DNA fingerprinting with nonradioactively labeled M13 becomes more functional than before [7].

Material and Methods

DNA extraction was performed in silica gel polymer tubes as previously described [8]. The M13 probes were labeled with biotin by the nick translation method. Varying concentrations of human genomic DNA were digested with Hae III restriction endonuclease (Promega, Madison, WI) and loaded onto 20 by 20.5 by 0.5-cm, 0.8% (w/v) agarose gels containing 0.089*M* tris(hydroxymethyl)aminomethane (Tris)-borate and 2.5m*M* disodium ethylenediaminetetraacetic acid (Na_2 EDTA), pH 8.3. The electrophoresis parameters varied from 1.5 to 2.0 V/cm performed for 16 to 41 h.

Southern Blots

After electrophoresis, the gels were depurinated for 10 min in 0.25*M* hydrochloric acid (HCl) and denatured for 30 min in 0.5*M* sodium hydroxide (NaOH) containing 0.6*M* sodium chloride (NaCl). With nitrocellulose (BRL, Gaithersburg, MD) and Nytran nylon (Schleicher & Schuell, Keene, NH) the membranes were neutralized with 0.5*M* Tris-HCl, pH 7.4, and 3*M* NaCl before transfer. The DNA was transferred according to Southern [9] to nitrocellulose and different brands of nylon. The DNA was blotted to nitrocellulose with ×20 standard saline citrate (SSC) (×1 SSC contains 0.15 mol/L NaCl and 0.015 mol/L sodium citrate, pH 7.0). The blotting to Nytran nylon was performed with ×10 SSC. Transfer to Zeta-probe nylon (Bio-Rad, Richmond, CA) and Sure-blot nylon (Oncor, Gaithersburg, MD) was accomplished using denaturing solution, rinsing the membranes with ×2 SSC before they were baked. All the membranes were baked 30 min at 80°C.

Hybridization

The prehybridization solution was comprised of 1% Hammersten casein, Tris-buffered saline (pH 7.5) (0.1*M* Tris-HCl and 0.5*M* NaCl), 3% liquid Hipure gelatin, and 0.05% Tween-20. Prehybridization was carried out at 42°C for 30 min using 0.3 mL/cm^2 of membrane. The hybridization was performed by diluting 1:1 the hybridization solution [×1 Denhart's solution, ×3 SSC, 45% formamide, 0.02 mol/L monobasic sodium phosphate (NaH_2PO_4) (pH 6.5), 0.5% sodium dodecyl sulfate (SDS), and 10% dextran sulfate] with either the prehybridization solution or sterile distilled water. Hybridization was performed at 42°C overnight using 0.065 mL/cm^2 of membrane. The same amount of probe was used in all hybridizations (20 ng/mL). The posthybridization washes consisted of two 5-min washes at room temperature, followed by one wash at 42°C for 30 min, varying the SSC concentration from ×0.1 to ×2.0 SSC with 0.1% SDS. Finally, two washes of 3 min each were performed in ×2 SSC.

Color Detection

After the posthybridization washes, the membranes were blocked for 30 min at 42°C with prehybridization solution. The blocking solution was replaced by streptavidin diluted

1:1000 in 0.065 mL of prehybridization solution per square centimetre of membrane. The membranes were incubated at room temperature for 10 min, then washed three times for 5 min each in × 1 SSC. This was followed by a washing with biotinylated alkaline phospatase diluted 1:1000 with 0.065 mL of prehybridization solution per square centimetre of membrane. The washing was carried out using 0.1*M* Tris-HCl (pH 7.5) containing 0.15*M* NaCl, four times at 5 min each. After 2 min in 0.1*M* Tris-HCl (pH 9.5), 0.1*M* NaCl, and 50 m*M* magnesium chloride ($MgCl_2$), the membranes were incubated at 37°C for 2 to 20 h in nitroblue tetrazolium (165 mg/mL in 70% dimethylformamide) and 5-bromo-4-chloro-3-indolylphosphate (83.33 mg/mL in dimethylformamide), diluted 2:1000 in 0.35 mL of 0.1*M* Tris-HCl (pH 9.5), 0.1*M* NaCl, and 50m*M* $MgCl_2$ per square centimetre of membrane. After color detection, the membranes were rinsed in distilled water and dried in the dark on filter paper at room temperature.

Results

Membranes and Southern Blot

Various blotting media were evaluated for their ability to produce clear and distinct DNA fingerprints. Nitrocellulose (Fig. 1, *A*) yielded the poorest results. The DNA fingerprints were much improved when blotted to nylon membranes. A better pattern than that with nitrocellulose was produced by using Nytran nylon (Fig. 1, *C*). Bio-Rad's Zeta-probe nylon yielded a better pattern still, in comparison with Nytran nylon (Fig.

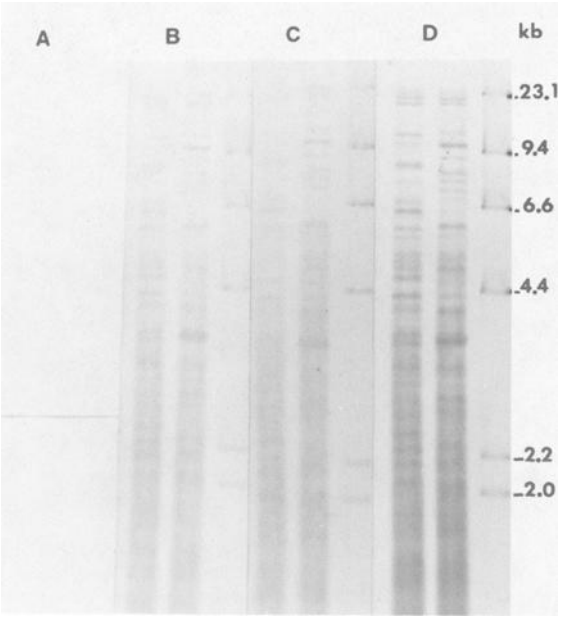

FIG. 1—*Southern blots obtained with various blotting membranes. The membranes used include* (A) *nitrocellulose,* (B) *Zeta-probe nylon,* (C) *Nytran nylon, and* (D) *Sure-blot nylon. The three different lanes on each membrane were 10* μg *of Hae III digested human genomic DNA (Lanes 1 and 2), and 500 pg of Hind III digested lambda virus (Lane 3). The duration of electrophoresis was 16 h; hybridization was accomplished with hybridization solution containing prehybridization solution (1:1) and* ×*0.7 SSC posthybridization washes.*

1, *B*). The strongest, clearest pattern was obtained using Oncor's Sure-blot nylon (Fig. 1, *D*). The bands were darkest and best resolved between 4 and 23 kilobase (kb) when using the Oncor nylon. Of course, other media not tested, or even these membranes used with other protocols, may provide as good, or even better, patterns than the Sure-blot nylon.

Electrophoresis Time and Sensitivity Tests

Changes in the duration and voltage of electrophoresis made a difference in the quality of DNA fingerprints. Increasing the electrophoresis time from 16 to 41 h enhanced the resolution of the bands (compare *C* and *B* in Fig. 2). For longer electrophoresis durations the voltage was reduced to 1.5 V/cm of gel. The separation of bands from 4 to 23 kb was better with the longer electrophoresis, and the smear that appeared in the region less than 2.3 kb was electrophoresed off the gel. The bands were sharper when a lower voltage per centimetre ratio was used. The longer electrophoresis time allowed the more informative bands to be resolved, improving the value of the pattern.

Decreasing amounts of genomic DNA were digested, electrophoresed, blotted, and

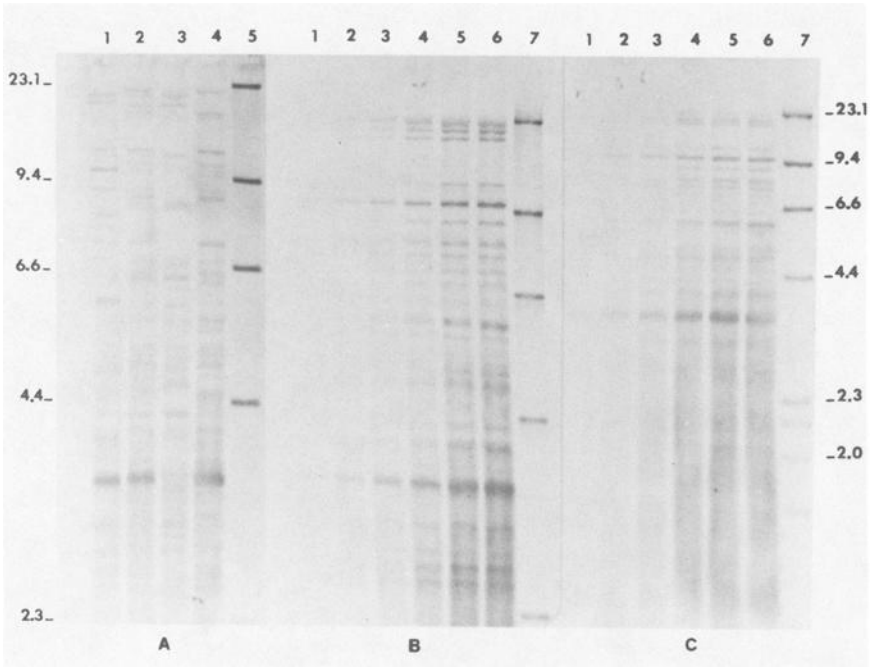

FIG. 2—(A) *Southern blots obtained using the following reagents and conditions: Sure-blot nylon membrane, hybridization solution/prehybridization solution (1:1), 41-h electrophoresis, and post-hybridization washes using ×0.7 SSC. Lanes 1 through 4 were 10 μg of genomic DNA digested with Hae III from four different samples. Lane 5 is marker lambda DNA. The size ranges in kilobase units (kb) are on the left.* (B) *The reagent and conditions are the same as in* (A) *except that the posthybridization washes were with ×2 SSC. Increasing quantities of Hae III digested DNA from the same patient were applied to Lanes 1 through 6. The quantities were 0.5, 1.0, 2.0, 4.0, 8.0, and 10 μg of DNA. Lane 7 is marker lambda DNA.* (C) *Southern blots obtained using the following reagents and conditions: Sure-blot nylon, hybridization solution/prehybridization solution (1:1), 16-h electrophoresis, and ×0.7 SSC posthybridization washes. Lanes 1 through 6 contain increasing quantities of DNA similar to those in* B. *Lane 7 is marker lambda DNA.*

hybridized, revealing an excellent pattern for from 10 to 4 μg of sample applied (Fig. 2, *B* and *C*). Even 2 μg of genomic DNA produced a good, discernible pattern. With 1 μg of DNA one could still observe the basic pattern of the fingerprint (Fig. 2, *B* and *C*, Lane 2). The longer electrophoresis time and less stringent posthybridization washes improved the sensitivity, even at the lower quantities of DNA. The ½ μg sample results were difficult to see, no matter what electrophoresis time or posthybridization washes were used.

Stringency Conditions

The effect of stringency, during the hybridization and posthybridization washes, is shown in Fig. 3. It has been previously demonstrated [*6*] that diluting the standard hybridization solution with prehybridization solution 1:1 improved the signal of the DNA fingerprint. This was further investigated by diluting the hybridization solution with sterile distilled water. The quality of the DNA fingerprint obtained (Fig. 3, Lane 8) was not as impressive as that obtained when prehybridization solution was used as the diluent (Fig. 3, Lane 3). Posthybridization washes revealed usable DNA patterns from ×0.4 to ×2.0 SSC. Greater signal intensity for the upper bands was noted with the less stringent washes. The best signal-to-noise ratio was found with ×0.6 to ×0.7 SSC. When the washing was at a high stringency, ×0.1 SSC, almost no bands were observed.

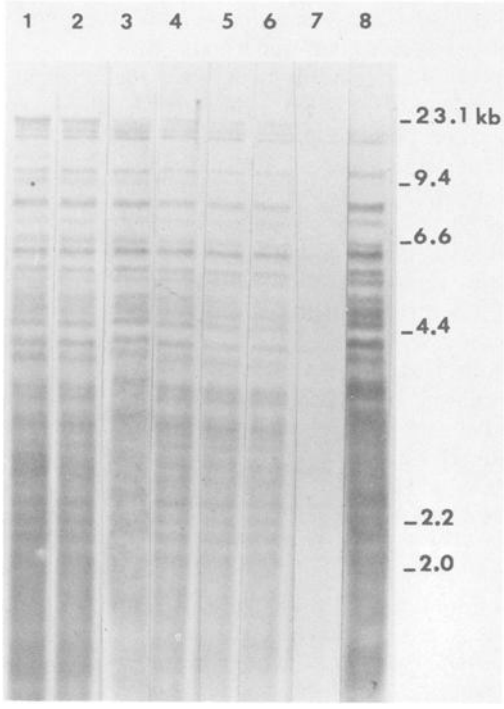

FIG. 3—*Southern blots obtained using "Sure-blot" after an 18-h electrophoresis. Lanes 1 through 7 were hybridized with hybridization and prehybridization solution (1:1), and Lane 8 with hybridization solution and water (1:1). Lanes 1 through 7 were washed after hybridization with ×2.0, ×1.0, ×0.7, ×0.6, ×0.5, ×0.4, and ×0.1 SSC respectively. Lane 8 was washed with ×0.7 SSC before color detection.*

Discussion

We recently demonstrated that DNA fingerprinting could be done without radioactive probes [*6*]. We described a system that produced a good DNA fingerprint using biotin-labeled M13 as a probe. This paper describes the procedures used to improve the banding patterns.

We have examined the conditions of electrophoresis, the type of blotting medium, the hybridization solutions, and the subsequent washes used in this method. We have observed that increasing the electrophoresis time at a lower voltage produces a superior banding pattern in comparison with overnight (16-h) electrophoresis. With the longer electrophoresis period, more informative and sharper bands were observed, with a reduction of background. These results could probably be improved further by using gels longer than 20 cm. A wide range of genomic DNA quantities may be used, and the patterns are readable for amounts as small as 2 μg of DNA. Recent experiments using digoxigenin-labeled M13 probes have shown sensitivity for undegraded DNA in amounts under 1 μg. This should allow forensic science application of this technique in rape cases, since 30 μg of an average ejaculate contains 2 μg of DNA. However, recovery and degradation factors encountered in certain forensic science cases also affect the results.

Our previous study [*6*], which used nonradioactive probes for DNA fingerprinting, showed that diluting the hybridization solution produced a better signal. We investigated in this paper whether dilution with the prehybridization solution provided a special protection to the membrane during hybridization to increase the signal-to-noise ratio or whether it simply changed the stringency of the hybridization solution (lowering the salt and formamide concentration). As shown in Fig. 3, Lane 8, simply diluting the hybridization solution with water produced an insignificant background and a DNA fingerprint with bands almost as dark as those obtained with the prehybridization solution as diluent. Thus, we concluded that the important enhancer is the adjusted stringency of the hybridization solution. It should be noted, however, that the banding pattern produced when using the prehybridization solution as diluent provided uniform intensity of the bands (Fig. 2, *A*). The improved pattern occurs partly because the casein, nonionic detergent, and fish gelatin help to reduce nonspecific binding while stabilizing the target-probe hybrids. These three ingredients were individually analyzed in experiments (the results are not shown) to support this conclusion.

For the electrophoresis conditions used initially in our study (16 to 18 h at 1.5 to 2 V/cm), the optimal salt concentration in the posthybridization washes was found to be ×0.6 to ×0.7 SSC. The ×2 SSC washing produced a strong background in the region of fragments less than 4 kb. However, when the ×2 SSC wash was used, the bands between 4 and 23 kb were stronger and required less time to develop. Since longer periods of electrophoresis give better resolution in this range, ×2 SSC becomes the washing condition of choice, yielding a clean background and more sensitivity, adequate for detection of at least 2 μg of genomic DNA.

A recent paper by Medeiros et al. [*10*] demonstrated a system of DNA fingerprinting using nonradioactive M13. The authors claim their procedure did not work with the common labeling technique of nick translation. Our method easily provided sensitivity below 2 μg with nick-translated M13. Conditions affecting the stringency during hybridization seem to be the dominant factor in obtaining the results.

For laboratories installing DNA technology in their case loads, the fingerprints rival those produced by radioactively labeled M13. The advantages of this method over radioactivity are its low cost, speed, and safety. Despite the restrictions on sensitivity, this method can be used for rapid accumulation of a population database, training of new personnel, and application to some forensic science cases. Other attributes of the method allow it to be applied in parentage testing, bone marrow transplantation, and detection of somatic changes in tumors [*11*].

References

[1] Vassart, G., Georges, M., Monsieur, R., Brocas, H., Lequarre, A. S., and Christophe, D., "A Sequence in M13 Phage Detects Hypervariable Minisatellites in Human and Animal DNA," *Science*, Vol. 235, No. 4789, Feb. 6, 1987, pp. 683–684.

[2] Messing, J., Gronenborn, B., Müller-Hill, B., and Hofschneider, P. H., "Filamentous Coliphage M13 as a Cloning Vehicle: Insertion of a Hind II Fragment of the Lac Regulatory Region in M13 Replicative Form in Vitro," *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 74, No. 9, Sept. 1977, pp. 3642–3646.

[3] Devor, E. J., Ivanovich, A. K., Hickok, J. M., and Todd, R. D., "A Rapid Method for Confirming Cell Line Identity: DNA "Fingerprinting" with a Minisatellite Probe from M13 Bacteriophage," *Biotechniques*, Vol. 6, No. 3, 1988, pp. 200–201.

[4] Westneat, D. F., Noon, W. A., Reeve, H. R., and Aquadro, C. F., "Improved Hybridization Conditions for DNA "Fingerprints" probed with M13," *Nucleic Acids Research*, Vol. 16, No. 9, 1988, p. 4161.

[5] Georges, M., Lequarre, A. S., Castelli, M., Hanset, R., and Vassart, G., "DNA Fingerprinting in Domestic Animals Using Four Different Minisatellite Probes." *Cytogenetics and Cell Genetics*, Vol. 47, No. 3, 1988, pp. 127–131.

[6] Tilzer, L., Moreno, R. F., Booth, F., Wilbur, S., and Thomas S., "DNA Fingerprinting with Nonradioactive Gene Probes," *Clinical Chemistry*, Vol. 35, No. 10, 1989, p. 2147.

[7] Jeffreys, A. J., Wilson, V., Thein, S. L., Weatherall, D. J., Ponder, B. A. J., *American Journal of Human Genetics*, Vol. 39, No. 1, July 1986, pp. 11–24.

[8] Thomas, S. M., Moreno, R. F., and Tilzer, L. L., "DNA Extraction with Organic Solvents in Gel Barrier Tubes," *Nucleic Acid Research*, Vol. 17, No. 13, 1989, p. 5411.

[9] Southern, E. M. "Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis," *Journal of Molecular Biology*, Vol. 98, No. 3, pp. 503–517.

[10] Medeiros, A. C., Macedo, A. M., and Pena, S. D. J., "A Simple Nonisotopic Method for DNA Fingerprinting with M13 Phage," *Nucleic Acids Research*, Vol. 16, No. 21, Nov. 1988, p. 10394.

[11] de Jong, D., Voetdijk, B. M. H., Kluin-Nelemans, J. C., van Ommen, G. J. B., and Kluin, P. M., "Somatic Changes in B-Lymphoproliferative Disorders (B-LPD) Detected by DNA-Fingerprinting," *British Journal of Cancer*, Vol. 58, 1988, pp. 773–775.

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