TECHNICAL NOTE

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Effect of Storage Conditions on Restriction Fragment Length Polymorphism (RFLP) Analysis of Deoxyribonucleic Acid (DNA) Bound to Positively Charged Nylon Membranes

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ABSTRACT: The ability to generate an autoradiogram from deoxyribonucleic acid (DNA) immobilized on a positively charged nylon membrane could be compromised by the storage conditions of the membrane. *Hae*III-digested human DNA was size fractionated and transferred to two types of positively charged nylon membranes. The membranes were stored at −20°C, 4°C, and ambient temperature and humidity for times ranging from 1 day to 13 weeks, then hybridized to variable number of tandem repeat (VNTR) probes to examine the effect of the storage conditions on the membrane-bound DNAs. It was shown that such membranes could be successfully hybridized and rehybridized if they were stored at −20 or 4°C, but storage under ambient conditions reduced or eliminated the likelihood of successful hybridization.

KEYWORDS: pathology and biology, deoxyribonucleic acid (DNA), hybridization, restriction fragment length polymorphisms, storage conditions, nylon membranes, variable number of tandem repeats

The ability to extract and analyze deoxyribonucleic acid (DNA) from biological specimens has had a profound impact on the field of forensic serology [*1–4*]. The prevailing method of forensic DNA analysis involves the typing of variable number of tandem repeat (VNTR) loci by restriction fragment length polymorphism (RFLP) analysis. The method of RFLP analysis employed by the authors' laboratory involves the transferring of DNA out of an electrophoretic gel via Southern blotting to a positively charged nylon membrane [*5*]. The generation of a permanent record of the fractionated DNA in the form of the Southern blot is an important aspect of the process. This format of immobilized

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DNA allows sequential examination of the DNA for single-locus markers by denaturing the currently hybridized probe from the membrane (a process generally referred to as "stripping") and hybridizing with a probe for another VNTR locus.

It has been observed by the authors of this paper that at times the storage conditions of positively charged nylon membranes with immobilized DNA can have an effect on their ability to yield results. In fact, under certain storage conditions, the ability to generate an autoradiogram from a given membrane can be compromised. This paper describes the storage conditions for DNA bound to positively charged nylon membranes that impact positively and negatively on the ability to obtain RFLP results.

Materials and Methods

Membrane Generation

Human DNA from whole blood samples was isolated and analyzed according to procedures described by Budowle and Baechtel [*5*]. DNA from an immortalized human cell line (CL-100) was obtained from Collaborative Research (Bedford, Massachusetts). The human DNA was applied to the gels in amounts from 10 to 500 ng. DNA size markers, consisting of a mixture of defined fragment lengths from enzymatically digested bacteriophage (lambda and phiX 174) DNAs were obtained from Lifecodes Corp. (Valhalla, New York). Two sources of positively charged nylon membranes were used: Zetaprobe (Cuno\Bio-Rad) and Biodyne B (Pall Corp.). Immediately after the DNA was fixed on the membranes by heat baking at 80°C for 30 min, the membranes were analyzed by hybridization with VNTR-specific probes or stored under various conditions.

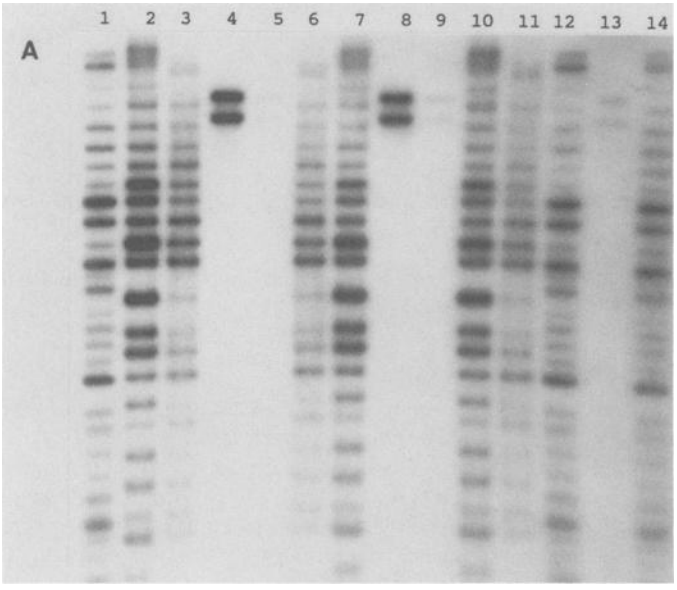

FIG. 1—*Effect of successive hybridizations on the detectability of VNTR markers. Lanes 1, 12, and 14 contain 0.6 to 23-kb markers (Lifecodes, Valhalla, New York). Lanes 2, 7, and 10 contain 0.7 to 30-kb markers (Promega, Madison, Wisconsin). Lanes 3, 6, and 11 contain 0.5 to 14-kb markers (Promega, Madison, Wisconsin). Lanes 4 and 8 contain 500 ng of* Hae*III-digested human DNA; Lanes 5 and 9 contain 50 ng of* Hae*III-digested human DNA; and Lane 13 contains 100 ng of* Hae*III-digested human DNA.* (a) *Result of the second hybridization of a membrane to the probe for the VNTR locus D4S139 (Genelex, Seattle, Washington)* [6].

Storage Conditions

The membranes were stored under three different conditions: −20°C, 4°C, and ambient temperature and humidity. Following autoradiography, the probes were stripped from the membranes using a solution of 55% formamide, 2× standard saline citrate (SSC), and 1% sodium dodecyl sulfate (SDS) (1× SSC = 0.15*M* sodium chloride and 15m*M* sodium citrate at pH 7.0) in a plastic box (Rubbermaid) for 1 h at 65°C with gentle agitation. All the membranes were blotted between two sheets of Whatman 3MM paper to remove excess moisture; then some were wrapped in plastic wrap (or placed in sealable plastic bags) and stored at −20 or 4°C, while others were placed exposed on bench tops at ambient temperature and humidity. After varying times, the membranes were rehybridized to examine the effect of the different storage conditions.

Results and Discussion

It had been our experience that some membranes generated for RFLP analysis yielded no results, even on the first hybridization. The hypothesis is that the ability to hybridize the membranes successfully was reduced or eliminated by the storage conditions of the membranes, a phenomenon we termed "hybridization inactivation." A suitable means of membrane storage could overcome the phenomenon of hybridization inactivation without a decrease in the sensitivity of detection. To test this hypothesis, 23 positively charged nylon membranes with immobilized DNA were used. Of the 23 membranes, 20

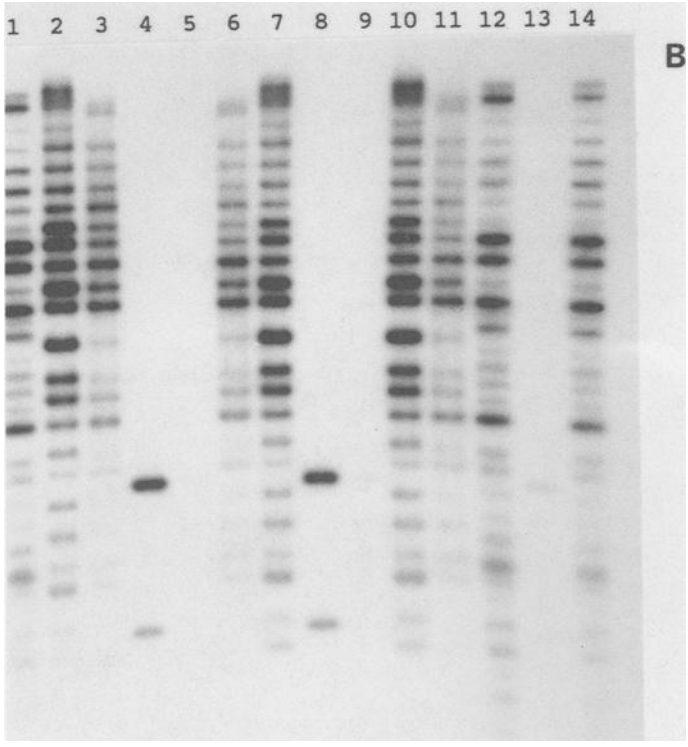

FIG. 1b—*Result of the sixth hybridization of a membrane to the probe for the VNTR locus D10S28 (Promega, Madison, Wisconsin)* [7]. *The autoradiography time was five days.*

were hybridized immediately after heat baking; 2 membranes were stored at −20°C for four weeks prior to the initial hybridization; and 1 membrane was stored for three weeks at −20°C, then placed at ambient temperature and humidity for one week prior to its initial hybridization. No significant difference was observed between the membranes hybridized immediately after DNA fixation and those stored frozen for one month, while the membrane stored for one week at ambient conditions failed to hybridize with the radioactive probes. This membrane was not subjected to further analyses.

Six of the 20 membranes immediately hybridized after DNA fixation were stripped and hybridized five times in immediate succession, while 1 membrane of the 20 was stripped and hybridized two more times in immediate succession (that is, with no storage time). There was only a slight decrease in signal intensity between the initial and final hybridizations (Fig. 1). Five of the above 7 membranes then were stored at −20°C, and 2 were stored under ambient conditions for two weeks. The results indicate that the processes of hybridization and probe stripping do not affect the ability to obtain an RFLP profile.

The remaining 15 membranes were subjected to probe stripping conditions, and 14 of those then were stored at −20°C and 1 was stored under ambient conditions. Including those membranes mentioned in the previous paragraph, a total of 19 membranes were stored at −20°C for periods ranging from 1 to 13 weeks. Upon rehybridization, all resulting autoradiograms from the 19 membranes stored at −20°C displayed bands with signal intensities consistent with the expected level of sensitivity of the procedure [5], while those membranes that were stored under ambient conditions yielded no results (Figs. 2*a* and *b*). After storage at −20°C, 15 of the 19 membranes were stored for two

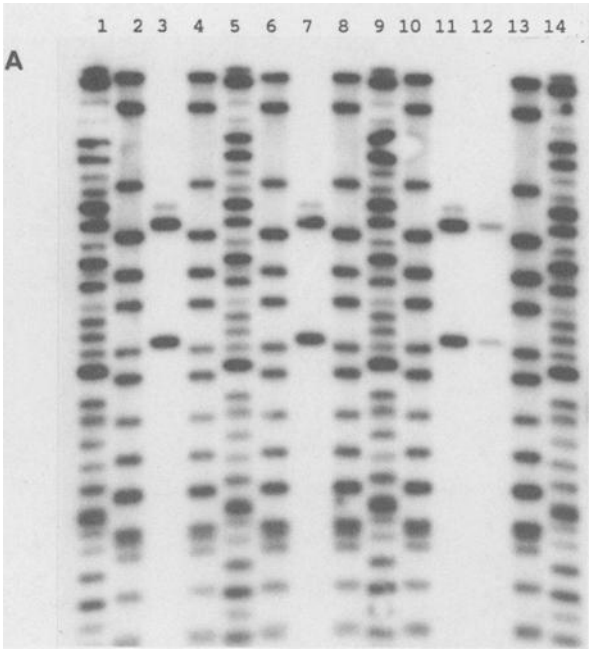

FIG. 2—*Effect of storage conditions on hybridization results. Lanes 1, 5, 9, and 14 contain 0.6 to 23-kb markers, while Lanes 2, 4, 6, 8, 10, and 13 contain 0.7 to 16-kb markers. Lanes 3, 7, and 11 contain 500 ng of* Hae*III-digested human DNA, and Lane 12 contains 50 ng of* Hae*III-digested human DNA.* (a) *Membrane stored for nine days at −20°C. The probe was CMM101 for the locus D14S13* [8].

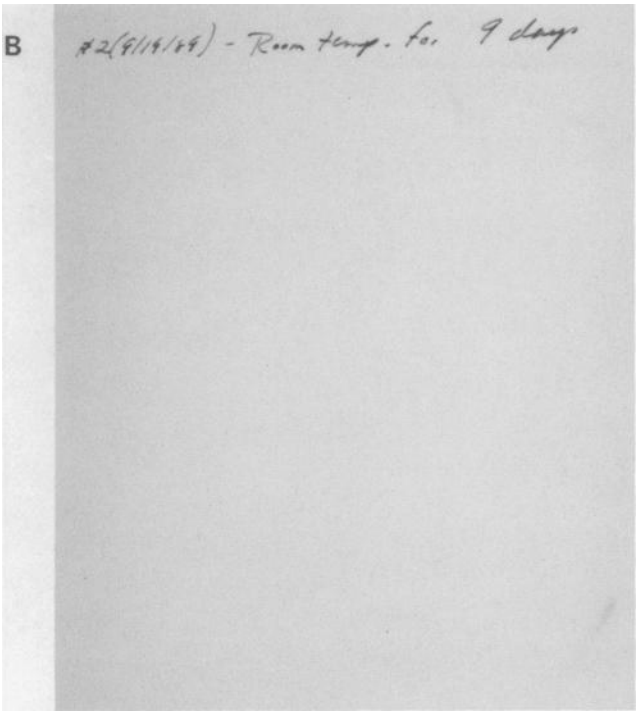

FIG. 2b—*Membrane stored for nine days at ambient conditions. The probe was CMM101 for the D14S13 locus.*

weeks at 4°C and then hybridized. Again, all the stored membranes generated autoradiograms with the expected signal intensity (Fig. 2*c*).

This study shows that membranes can be probed at least eight times, and that membranes can be stored under the proper conditions for a reasonable period of time prior to hybridization. In our hands, however, the storage of nylon membranes at ambient temperature prior to hybridization or after hybridization and stripping can have a deleterious effect on subsequent hybridizations. Thus, the proper storage conditions for the membrane can be critical to successful reprobing. Hybridization inactivation did not occur every time, as there were instances when it was possible to generate an autoradiogram or partial autoradiogram from membranes stored under ambient conditions, although the efficiency of obtaining a RFLP profile was compromised (results not shown). This phenomenon is not restricted to the Zetaprobe membrane, as two of the membranes stored under ambient conditions in this study were Biodyne B membranes. Currently, there is no explanation for hybridization inactivation. Perhaps the membrane or the membrane-bound DNA requires a certain degree of hydration that is not eliminated by heat-baking but is eliminated if the membrane is allowed to air-dry for an extended period of time. In fact, membranes that have been securely wrapped and stored at room temperature are able to generate autoradiograms upon rehybridization, even after several months (L. Presley, personal communication, DNA Unit, FBI, Washington, DC, 1991). This suggests that membrane hydration may be the critical parameter of storage conditions. However, attempts to rehydrate membranes stored under ambient conditions by incubation in a moisture chamber were unsuccessful. Therefore, the change affecting the membrane may be irreversible or the DNA may be no longer intact on the membrane.

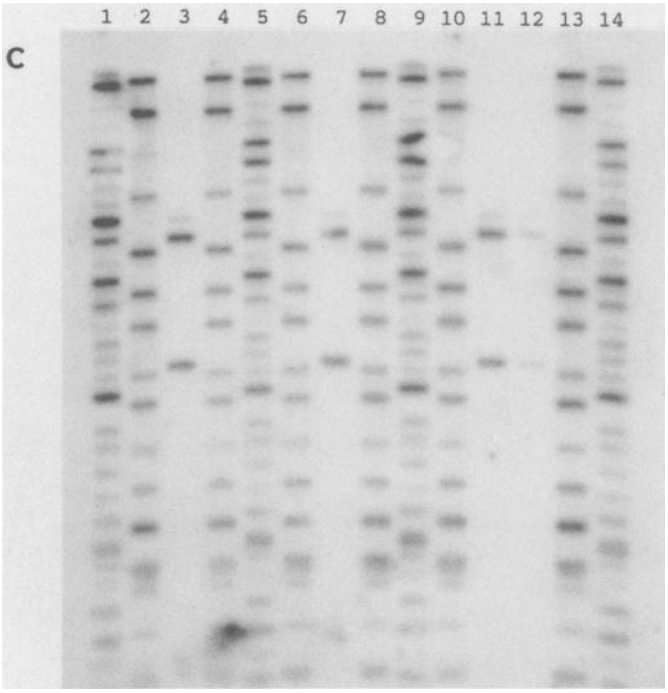

FIG. 2c—*Hybridization of a membrane stored for 15 days at 4°C. The probe was CMM101 for the D14S13 locus. The autoradiography time was five days.*

Nevertheless, the immediate concern is proper storage conditions for the membranes so that RFLP profiles can be derived from the immobilized DNA over an extended period of time. Based upon this study, it is clear that the two brands of positively charged membranes examined can be stored at −20°C or at 4°C without any significant loss of detection sensitivity. However, we recommend storage in sealed plastic bags or by thorough wrapping in plastic wrap at −20°C to reduce the possibility of bacterial or fungal growth, which might affect the integrity of the membrane or human DNA.

References

[1] Gill, P., Jeffreys, A. J., and Werrett, D. J., "Forensic Application of DNA 'Fingerprints'," *Nature*, Vol. 318, Dec. 1985, pp. 577–579.

[2] Kanter, E., Baird, M., Shaler, R., and Balazs, I., "Analysis of Restriction Fragment Length Polymorphisms on Deoxyribonucleic Acid (DNA) Recovered from Dried Bloodstains," *Journal of Forensic Sciences*, Vol. 31, No. 2, April 1986, pp. 402–408.

[3] Giusti, A., Baird, M., Pasquale, S., Balazs, I., and Glassberg, J., "Application of Deoxyribonucleic Acid (DNA) Polymorphisms to the Analysis of DNA Recovered from Sperm," *Journal of Forensic Sciences*, Vol. 31, No. 2, April 1986, pp. 409–417.

[4] Gill, P., Lygo, J., Fowler, S., and Werrett, D. J., "An Evaluation of DNA Fingerprinting for Forensic Purposes," *Electrophoresis*, Vol. 8, 1987, pp. 38–44.

[5] Budowle, B. and Baechtel, F. S., "Modifications to Improve the Effectiveness of Restriction Fragment Length Polymorphism Typing," *Applied and Theoretical Electrophoresis*, Vol. 1, 1990, pp. 181–187.

[6] Milner, E. C. B., Lotshaw, C. L., Willems van Dijk, K., Charmley, P., and Schroeder, H. W., Jr., "Isolation and Mapping of a Polymorphic DNA Sequence pH30 on Chromosome 4 (HGM Provisional No. D4S139)," *Nucleic Acids Research*, Vol. 17, p. 4002.

[7] Bragg, T., Nakamura, Y., Jones, C., and White, R, "Isolation and Mapping of a Polymorphic DNA Sequence (cTBQ7) on Chromosome 10 (D10S28)," *Nucleic Acids Research*, Vol. 16, p. 11395.

[8] Nakamura, Y., Culver, J. G., O'Connell, P., Leppert, M., Lathrop, G. M., Lalouel, J.-M., and White, R., "Isolation and Mapping of a Polymorphic DNA Sequence pMLJ14 on Chromosome 14 (D14S13)," *Nucleic Acids Research*, Vol. 16, 1988, p. 381.

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