TECHNICAL NOTE

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An Illustrated Guide to RFLP Troubleshooting

REFERENCE: Benzinger EA, Emerick E, Grigsby N, Lovekamp ML, Logeman R, Sallee PJ, Boster KL, Rehnstrom A, Reich AK, Small AT, Glasner JA, Coons TM, von Beroldingen C. An illustrated guide to RFLP troubleshooting. J Forensic Sci 1998; 43(3)665–679.

ABSTRACT: A large number of reagents and steps are required for restriction fragment length polymorphism (RFLP) analysis, which at times make determining the cause of any observed anomaly difficult. Troubleshooting problems in RFLP analysis is difficult and often the exact cause of a problem cannot be determined. In this paper a collection of controlled experiments detail the consequences of a number of human or materials problems. Although the focus is on forensic applications, this troubleshooting guide will be helpful to anyone employing Southern analysis.

KEYWORDS: forensic science, DNA typing, restriction fragment length polymorphism, Southern blot, troubleshooting

Restriction fragment length polymorphism (RFLP) analysis is widely employed in forensic laboratories to identify potential sources of blood, semen and other tissues. It is currently the most widely used tool available for biological analysis of evidence from sexual assault, homicide and missing persons or human remains cases. The Federal Bureau of Investigation's Combined DNA Indexing System (CODIS), a national database of genetic profiles of convicted sexual offenders and evidence from unsolved sexual assaults, employs data generated by RFLP analysis.

RFLP analysis is a complex procedure consisting of numerous steps and requiring a large number of reagents. Because of this complexity, it is susceptible to difficulties or compromise due to a wide variety of human failures or material problems. In spite of adherence to strict quality assurance regimens, laboratories using RFLP analysis occasionally encounter problems. The consequence of RFLP difficulties is partial or complete loss of data. This is particularly problematic for forensic laboratories since in many instances the limited amount of crime scene evidence precludes repetition. Forensic laboratories therefore have a high stake in identifying the causes of RFLP mishaps so that they can be prevented from occurring in the future. While the purpose or mechanism of each step in the RFLP procedure is well documented (1,2), little information is available concerning the end results of altering or eliminating these steps. As a result, RFLP troubleshooting is sometimes limited to speculation. A particular procedural error may be suspected, but often no documentation verifying the consequences of that error is available.

In the present study, a standard RFLP procedure has been systematically altered in controlled experiments to simulate procedural or materials problems. The results of these alterations have been documented for use as a troubleshooting guide. Since the most useful troubleshooting guide is one which addresses the most common problems, the procedural alterations were selected from their likelihood of occurrence, based on experience.

Methods and Results

The RFLP procedure is adapted from Budowle and Baechtel (3). The normal (control) and altered treatments are defined for each experiment. Gel formats for testing sensitivity consisted of two series of 400, 200, 100, 50, and 25 ng of *Hae* III-digested cell line K562 DNA (Life Technologies, Inc, Gaithersburg, MD) bracketed by a 30-band Molecular Weight Marker (Life Technologies).

DNA Extraction

The standard DNA extraction protocol for blood employs proteinase K and an organic extraction. Bloodstains are extracted overnight at 56°C in stain extraction buffer (SEB, 10 mM TRIS pH 7.5, 10 mM EDTA, 100 mM NaCl, 2% SDS). Just prior to use of the buffer, 5 mM dithiothreitol (DTT) and 0.5 mg mL^{-1} proteinase K are added. A single organic extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) is then performed. After addition of the phenol/chloroform/isoamyl alcohol, the extract is mixed by vigorous shaking (body fluid stains) or a 30 s vortex (liquid blood). After centrifugation to separate the aqueous and organic layers, the aqueous layer is removed and the DNA recovered by ethanol precipitation or filter centrifugation using Microcon 100 spin filters (Fisher Scientific).

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Failure to Add Proteinase K During Stain Extraction

To determine the effect of failure to add proteinase K or the addition of inactive proteinase K, bloodstains were extracted with and without the normal concentration of proteinase K in the digestion reaction. Twelve 2 cm^2 dried bloodstains from three different donors were extracted using the standard protocol. Proteinase K was left out of the extraction buffers of half of the bloodstains. Recovered DNA was quantitated by agarose gel electrophoresis against standard samples. The ability to restrict samples of DNA extracted with and without proteinase K was tested by digesting 400 ng of DNA with 40 U *Hae* III in a 40 μL volume of 1X reaction buffer at 37°C for 1 h. The digested DNA was collected by filter centrifugation and the degree of digestion was assessed following agarose gel electrophoresis of reaction products.

DNA recovery from bloodstains which had been extracted without proteinase K was approximately 1/3 that of the control bloodstain extracts. The DNA electrophoresed similarly to the controls (Fig. 1*a*). Some published DNA extraction protocols do not employ proteinase K (4). Presumably, proteinase K is effective in removing additional proteins from DNA during extraction. Any DNA still complexed with protein in extracts prepared without proteinase K would be lost to the interface during organic extraction. Absence of active proteinase K during digestion resulted in decreased yield but not decreased purity. Susceptibility to restriction digestion was not affected (not shown).

DTT Concentration

A DTT concentration of 5 mM is included in the stain extraction buffer. The effect of 0 to 100 mM DTT during extraction of bloodstains deposited on white cotton cloth and various denims (medium blue, light blue chambray, heavy dark blue cotton, heavy dark blue denim) was investigated. Eighteen 15 μL bloodstains on cotton cloth or denim were extracted in stain extraction buffer containing 0, 5, 10, 20, 50, or 100 mM DTT.

The absence of DTT from extractions of bloodstains on white cotton cloth had no effect (not shown). However, DTT increased

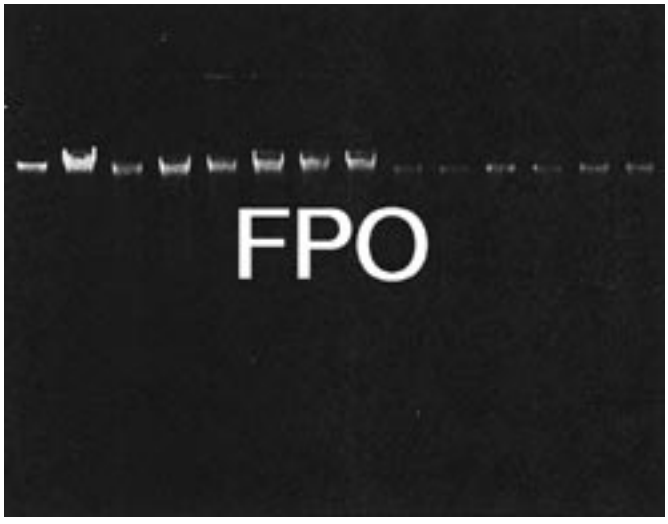

FIG. 1*a*—*Effect of omitting proteinase K from DNA extraction buffer during bloodstain extraction. Lanes 1 and 2, 30 and 125 ng lambda DNA. Lanes 3 to 8, bloodstains extracted in the presence of proteinase K. Lanes 9 to 14, bloodstains extracted without proteinase K.*

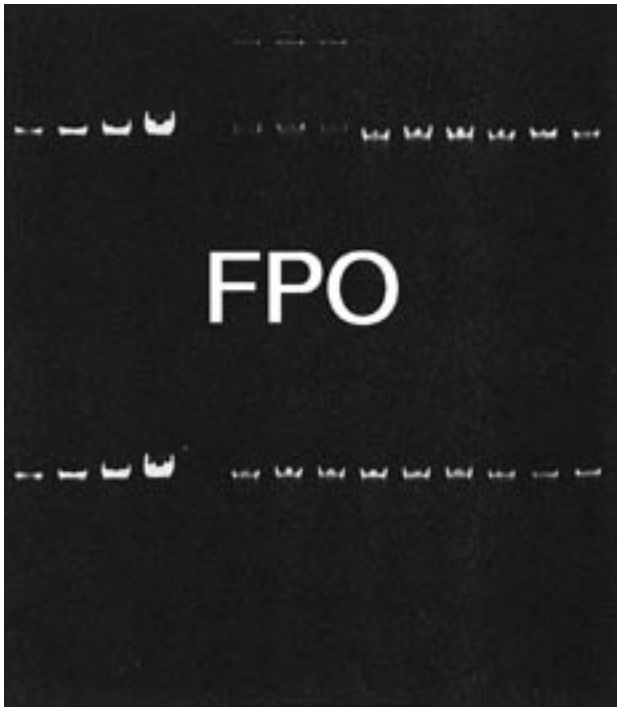

FIG. 1*b*—*Effect of omitting DTT from DNA extraction buffer during bloodstain extraction from denim.* Top: *Lanes 1 to 4, 15, 30, 60, and 120 ng lambda DNA. Lanes 6 to 14, 15 μL bloodstains extracted with no DTT (6–8), 5 mM DTT (9–11), and 10 mM DTT (12–14).* Bottom: *Lanes 1 to 4, 15, 30, 60, and 120 ng lambda DNA. Lanes 6 to 14, 15 μL bloodstains extracted with 20 mM DTT (6–8), 50 mM DTT (9–11), and 100 mM DTT (12–14).*

FIG. 1*c*—*Effect of phenol extraction vortex time during pristine bloodstain extraction.* Top: *Lanes 1 to 4, 15, 30, 60, and 120 ng lambda DNA. Lanes 5 to 7, no phenol extraction. Lanes 8 to 10, inverted four times; Lanes 11 to 14, shook vigorously 5 s.* Bottom: *Lanes 1 to 4, 15, 30, 60, and 120 ng lambda DNA. Lanes 5 to 7, vortexed maximum speed 5 s. Lanes 8 to 10, vortexed maximum speed 30 s.*

DNA yields from bloodstains on denim (Fig. 1*b*). In the denim samples that were extracted without any DTT, electrophoretic migration of the DNA was slowed. A portion of the DNA remained near the origin of the gel. Up to 50 mM DTT in the extraction buffer had no apparent deleterious effect, but 5 mM was as effective as 50 mM. A slight decrease in yield was seen at 100 mM DTT. The addition of DTT to the stain extraction buffer improves the quality and yield of DNA obtainable from bloodstains on denim. The effect of DTT on other types of forensic samples and substrates was not investigated.

Phenol Extraction Vortex Time

The consequences of shorter or absent vortexing of the phenol/chloroform/isoamyl extraction buffer mixture or failure to perform any organic extraction were determined. A single extraction was performed on a 450 μL bloodstain in 12 mL of stain extraction buffer. After the standard overnight incubation, the extract was divided into 30 equal portions. Six replicates of the following treatments were made: no organic extraction; gently inverted four times; vigorously shaken 5 s, vortexed at maximum speed 5 s, and vortexed at maximum speed 30 s. After centrifugation, DNA was recovered by ethanol precipitation.

No differences in yield were seen between any of the treatments which received a phenol extraction (Fig. 1*c*). The yield and condition of the control was the same as that of the reduced mixing treatments. Approximately 375 ng of DNA was recovered from each replicate. No increased degradation could be detected in the samples which had been vortexed the longest. Separation of the aqueous and organic phases was more difficult in the gently inverted samples.

DNA recovery was only slightly reduced by the absence of an organic extraction step. Most likely this is attributable to the pristine condition of the experimental material. Organic extraction is expected to be necessary to achieve optimum yield and purity for at least some types of forensic specimens. The susceptibility to restriction of samples which had not undergone an organic extraction was not investigated. Vortexing or shaking times limited to 5 s are sufficient for bloodstain extractions.

Substitution of Stain Extraction Buffer for Differential Extraction Reagents During the Differential Lysis of Sperm and Epithelial Cells

The most commonly used forensic protocol for separating sperm and epithelial cells during DNA extraction requires the addition of various amounts of the buffer TNE (10 mM TRIS, 100 mM sodium chloride (NaCl), 2 mM EDTA), water, sarkosyl, DTT, and proteinase K during a two-step extraction process (5). In order to simplify the differential extraction procedure, an experiment was done in which stain extraction buffer (SEB; 10 mM TRIS pH 7.5, 10 mM EDTA, 100 mM NaCl, 2% SDS) was used in place of the differential extraction cocktails. To generate uniform experimental material, 100 μL of a mixture of buccal epithelial cells spiked with semen was applied to cotton swabs. The swabs were allowed to dry at room temperature and then frozen until use. SEB was substituted for the differential extraction cocktails as shown in Table 1.

The two different protocols gave similar results in terms of total extracted DNA and efficiency of sperm/epithelial cell separation (data not shown); however the SEB method was simpler and employed a stock reagent. Note that DTT is used in the second extraction to lyse the sperm cells so it must not be included in the first extraction.

TABLE 1—*Details of substitution of SEB for differential extraction reagents.*

Reagent	1st Extraction	2nd Extraction
Standard Differential Extraction		
TNE (μL)	400	150
20% Sarkosyl (μL)	25	50
Deionized water (μL)	75	150
5 mg/mL Proteinase K (μL)	5	10
0.39 mM DTT (μL)	—	40
SEB Differential Extraction		
SEB (μL)	500	350
5 mg/mL Proteinase K (μL)	10	10
0.39 mM DTT (μL)	—	40

Restriction with *Hae* III

Digestion of DNA with *Hae* III is typically carried out at 37°C with 40 units of enzyme and up to 500 ng of DNA in a 40 μL volume of 1X reaction buffer. Reaction times vary from 1 h to overnight.

Band Shift Due to Binding of Restriction Enzyme Component

Some preparations of *Hae* III have been observed to cause marked shifting of DNA band positions in agarose gels under standard protocol conditions where the digestion reaction is loaded directly onto the analytical gel without ethanol precipitation or filter centrifugation. This effect was demonstrated by digesting DNA samples with increasing amounts of *Hae* III from a lot of enzyme previously associated with anomalous DNA migration. Identical 500 ng DNA samples were digested with 1, 2, 4, and 6 μL (10 U/μL) of the problematic enzyme lot and a different enzyme lot which had not been associated with anomalous DNA migration. After a 3 h incubation, the digestion reactions were loaded onto an analytical gel.

The severity of the anomalous migration correlated with the amount of the affected enzyme lot added to the reaction (Fig. 2*a*). The gel retardation effect was absent in the DNA samples digested with the same amount of a different lot of enzyme. *Hae* III quality control protocols that included Southern blotting of DNA samples digested with increasing amounts of enzyme preparation would test for the presence of DNA binding agents that cause anomalous migration.

Brief or Extended Incubation with Hae *III*

The causes of failure of restriction with *Hae* III were examined. To determine how long *Hae* III remains active during a digestion reaction, multiple tubes containing 40 units of *Hae* III preparations from three commercial sources were incubated for five days at 37°C in 1X restriction buffer supplied by the manufacturer. Four hundred nanograms of cell line K562 DNA was added to the *Hae* III incubations at 1, 2, 3, 4, and 5 days and restriction was allowed to proceed for 1 h. In a separate experiment, K562 DNA was incubated with *Hae* III for five days in similar reaction mixtures (Fig. 2*b*).

All pre-incubated aliquots of *Hae* III produced a limit digest of the K562 DNA within 1 h (not shown). *Hae* III maintained sufficient activity to cut the K562 DNA after five days of preincubation in the absence of DNA. The DNA samples that were incubated with *Hae* III for five days were indistinguishable from the control

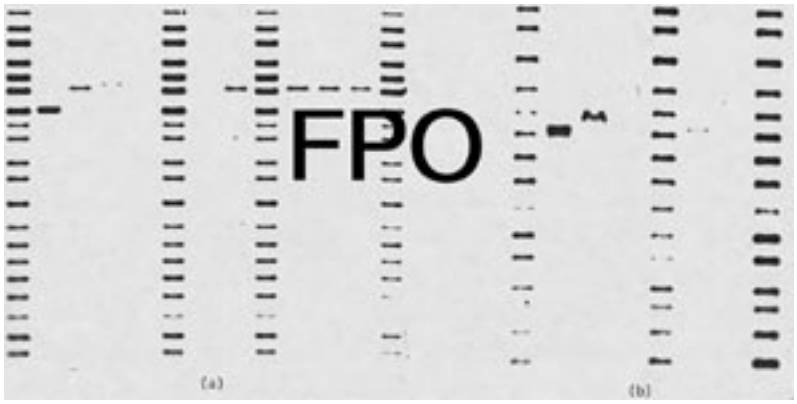

FIG. 2—*Band shift due to binding of restriction enzyme component* (a) *Lanes 1, 6, 9, and 13, molecular weight marker. Lane 2, cell line K562 control DNA. Lanes 3, 4, 5, and 7, DNA digested with 10, 20, 40, and 60 units of the affected lot of Hae* III. *Lanes 8, 10, 11, and 12, DNA digested with 10, 20, 40, and 60 units of a different lot of Hae* III. *The stronger signal of the top 7 bands of the marker is related to the relative amounts of DNA present in some lots of the product.* (b) *Lanes 1, 5 and 8, molecular weight marker. Lane 2, precut cell line K562 control DNA. Lanes 3 and 4, K562 DNA cut with 40 and 60 units of affected Hae* III *lot. Lanes 6 and 7, K562 DNA cut with 40 and 60 units of affected lot followed by proteinase K treatment, phenol extraction and filter centrifugation. The band shift is also eliminated by ethanol precipitation or filter centrifugation and resuspension of the DNA following digestion.*

samples that were digested for only 1 h. Specificity was unaffected by preincubation of *Hae* III or prolonged incubation of DNA with the enzyme. No partial digestion products or evidence of star activity was observed following Southern hybridization to various forensic probes and autoradiography. It would not be a good practice to extend digestion reactions beyond the time ordinarily required to achieve a limit digest since the absence of detectable contaminating nuclease activity in the enzyme preparations may be subject to lot-to-lot variation. Previously, it was shown that *Hae* III remains active after overnight incubation with forensic samples that fail to cut (6). It was also shown that *Hae* III can be rapidly inactivated by carryover of extraction reagents including SDS, ammonium acetate and phenol.

Incorrect Incubation Temperature During Hae *III Incubation*

The reaction temperature recommended by *Hae* III manufacturers is 37°C. However, restriction reactions at other temperatures are not harmful. Incubation temperatures of ambient (25°C) and 56°C have been shown previously to give the same results as 37°C (6).

Agarose Gel Preparation

One percent (w/v) agarose gels are prepared by heating LE agarose (either IDNA Agarose; FMC or DNA Typing Grade Agarose; Life Technologies) in 1X TAE (2) in a microwave oven for 3 to 5 min until all of the agarose particles have melted. Any volume lost due to evaporation is replaced with distilled water. The agarose solution is cooled to 50 to 60°C and gels are made by pouring 150 mL of agarose solution into leveled 11 2 20 cm trays. Well combs are inserted and the gels are allowed to set. All experiments were carried out using a Model H5 electrophoresis box (Life Technologies) modified for passive buffer recirculation (Genelex, Seattle, WA).

Gel Preparation with Water in Place of 1X TAE

Deionized water was substituted for 1X TAE in the preparation of agarose gels. After solidifying, the gels were placed in 1X TAE running buffer and loaded with DNA samples.

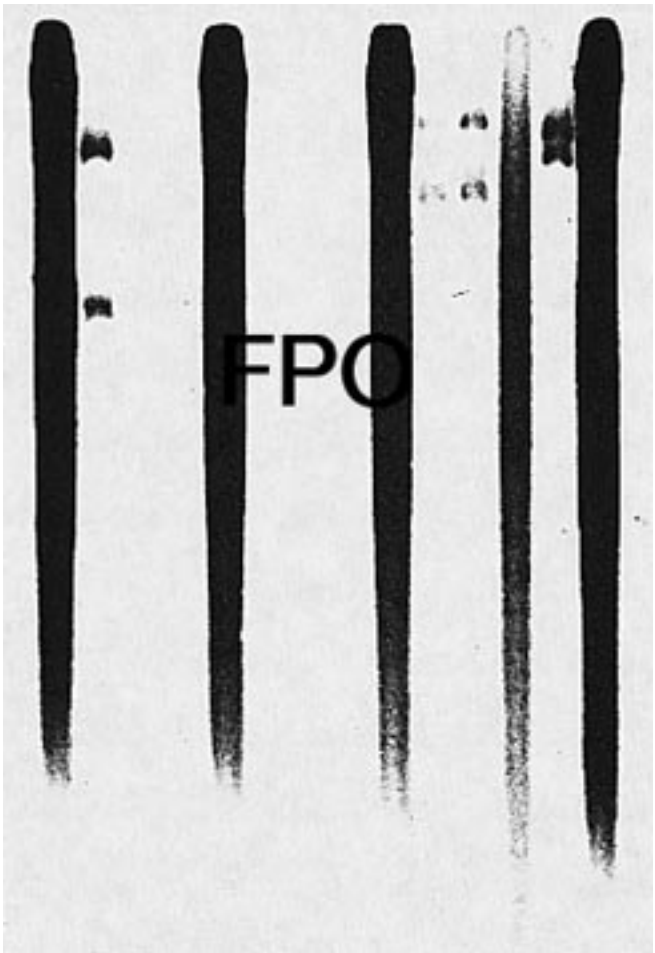

FIG. 3*a*—*One percent agarose gel prepared with water in place of 1X TAE. Electrophoresis was in 1X TAE running buffer.*

FIG. 3*b*—*Deterioration of agarose after opening of bottle. Left: gel prepared using agarose from bottle which had been in use for approximately five months. Right: gel prepared from previously unopened bottle of same lot number.*

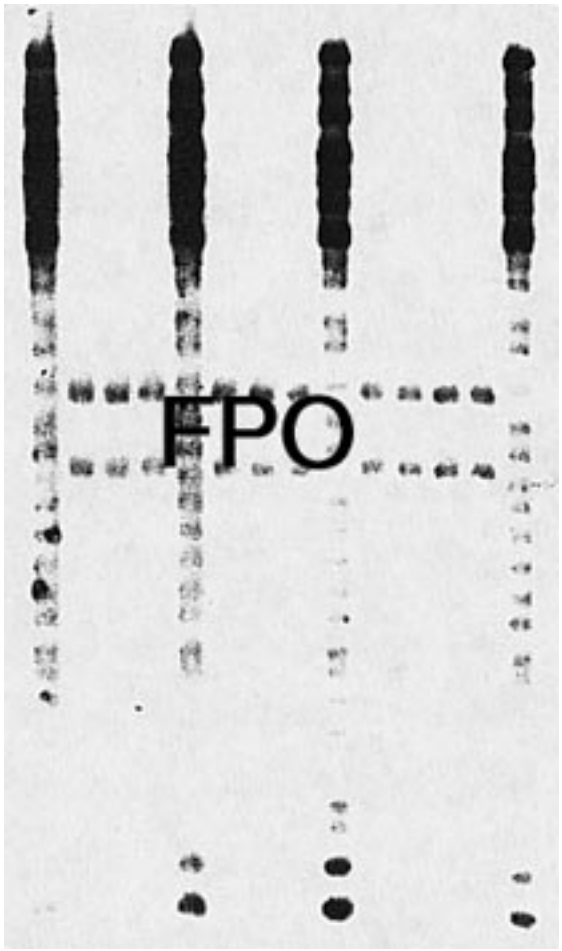

FIG. 3*c*—*Agarose particles not completely dissolved. A large amount of undissolved agarose particles are required to compromise resolution.*

The substitution of water for 1X TAE in the gel resulted in heavily smeared autoradiographs (Fig. 3*a*). This error may not be readily apparent prior to autoradiography unless expected milliamperage ranges and gel run times are established. Electrophoresis of DNA will occur as the running buffer diffuses into the gel. If

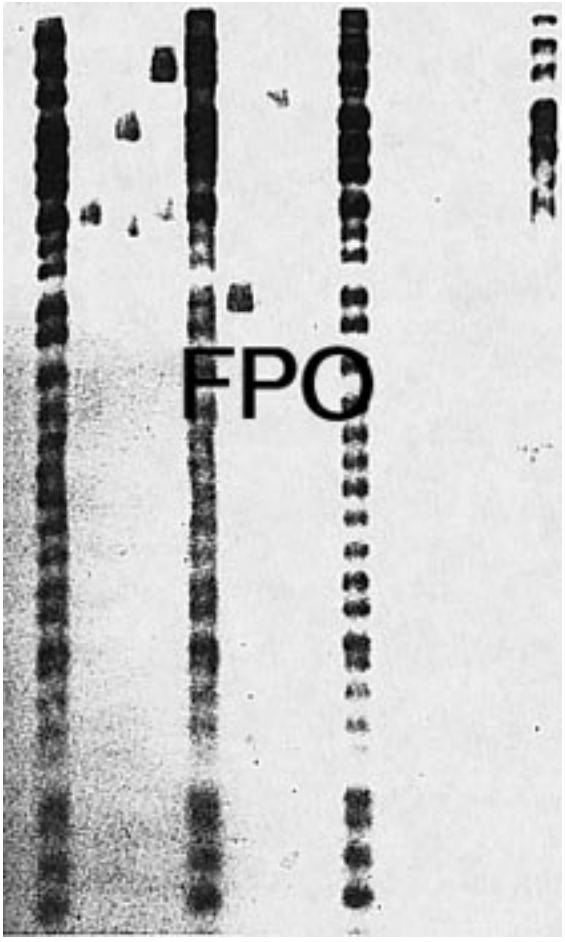

FIG. 3*d*—*Effect of prolonged heating of agarose solution. The agarose was brought to a boil in a microwave and held just below boiling for 20 min before the gel was poured.*

temperature and volume of running buffer are standardized, narrow ranges for expected source milliamperage at specific voltages can be established. A check of current draw prior to sample loading serves as an important diagnostic safeguard against errors in gel and running buffer preparations. Gels made with water will cause a decrease in milliamperage at normal voltages.

Agarose Deterioration

During the investigation of progressively worsening gel resolution, a comparison was made between agarose from a 500 g bottle which had been opened for approximately five months and from a previously unopened bottle of the same lot.

A marked difference was noted between the two containers of agarose, with agarose from the opened container giving significant loss of resolution (Fig. 3*b*). This effect was observed during consecutive years in the summer months when the indoor relative humidity is higher. A possible explanation is the absorption of moisture by the agarose. The manufacturer recommended storing the opened agarose bottle under vacuum. However, a simpler solution was to purchase agarose in smaller 100 g containers.

Agarose Not Completely Dissolved

The agarose/TAE suspension was brought to a boil, but heating was terminated with variable amounts of undissolved agarose particles remaining.

Significant loss of resolution was only observed when a very large amount of agarose particles remained undissolved (Fig. 3*c*). A small number of undissolved particles (i.e., <20) do not justify continued heating of the agarose. No electrophoretic or Southern transfer anomalies were observed in gels prepared with small amounts of undissolved agarose particles.

Agarose Overboiled

Prolonged heating of gel solutions to dissolve the agarose may result in changes in pH and ionic strength of the buffer due to evaporative loss of the acetate ion or breakdown of the agarose or both (personal communication, Carolyn Seidell, FMC Bioproducts, Inc.). Three hundred milliliters of 1% agarose in 1X TAE was brought to a boil in a microwave and held just below boiling for 20 min. The volume was readjusted to 300 mL with distilled water and gels were poured.

Gels made from agarose solution held just below boiling for 20 min exhibit a significant loss of resolution (Fig. 3*d*). Since the presence of a small number of undissolved agarose particles does not affect resolution (see preceding subsection), prolonged heating of agarose solutions should be avoided.

Comb Inserted on Angle

Well-forming combs are normally inserted so that they are perpendicular to the plane of the gel. In some gel apparatuses, it is possible to insert the comb so that it is not correctly seated in the gel box notches and is not perpendicular. To test the effect of angled comb positioning, the combs were inserted at a 45-deg angle from perpendicular pointing towards the bottom (anodal end) of the gel. No loss of resolution, smearing or detectable band broadening resulted from inserting combs at a 45-deg angle (not shown).

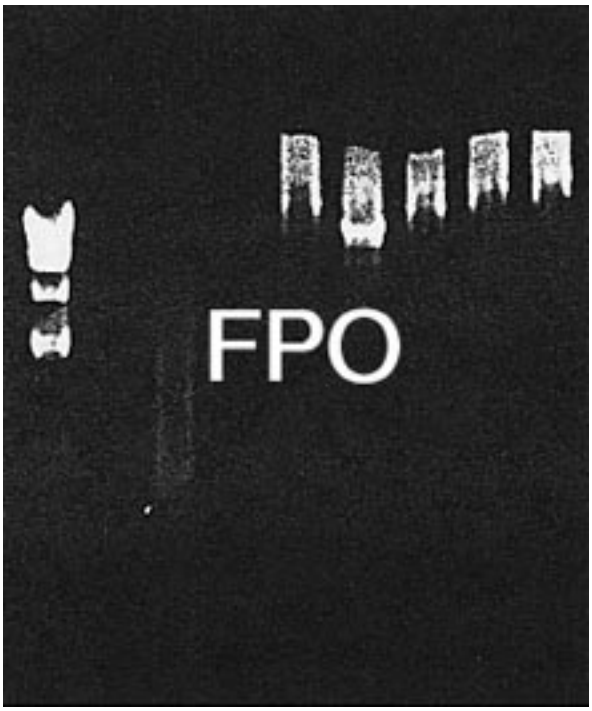

FIG. 4*a—Switching of loading buffer and ready-to-load lambda*/Hind *III size marker. Lane 1, the Lambda*/Hind *III size marker. Lanes 2 and 3, 25, and 75 ng of* Hae *III digested human DNA mixed with loading buffer. Lanes 5 to 9, approximately 75 ng of* Hae *III digested human DNA mixed with the ready-to-load lambda*/Hind *III size marker instead of loading buffer.*

FIG. 4*b—Deposition of DNA samples outside of wells during gel loading. Dropping even small amounts of sample outside of the well when loading an analytical gel may create artifacts. The anomalous bands are normally out of register with both the wells and other similarly loaded lanes.*

Gel Tray Not Level

Many laboratories routinely purchase leveling trays to provide a level surface for gel pouring. To determine the effects of failure to precisely level agarose gels, an 11 2 20 cm gel was poured on an angle. Prior to pouring the gel, the gel tray was placed on a level surface and the bottom right corner was elevated 7 mm above the surface. Normally, an 11 2 20 cm gel made with 150 mL of gel solution is 68 mm thick. The resulting gels were noticeably uneven.

No difference in autoradiographic results was seen between the leveled control gels and the uneven gels. If a gel was extremely thin and the surface angle of the gel sufficiently different from the electric field, DNA might electrophorese out of the surface of the gel. A loss of data in the thinnest part of the gel would result. Except in the most extreme cases, it is acceptable to pour submarine gels without a leveling tray.

Long/Short Gel Set Time

The effects of very short and very long gel set times were studied. To simulate a prolonged set time, agarose gels were prepared

and allowed to sit on the bench for 20 h before being placed under buffer. The short gel set time represented the earliest the comb could be removed without tearing the wells. When the agarose first sets, it is translucent and after several minutes becomes opaque. These gels were placed under buffer and had their combs removed when they were still translucent. Samples were loaded and electrophoresis begun within 2 min.

No difference was detectable between control gels and long or short set time gels (not shown).

Sample Loading

λ HinD *III Digest Ready-Load Standard Used in Place of Loading Buffer*

Some commercially available reagents are supplied in vials with labels of similar appearance to that of other reagents. These labels can be easily confused. One such set of preparations is a sample loading buffer and a λ *HinD* III digest molecular weight standard which comes mixed with loading buffer.

On a minigel used to assess the completeness of *Hae* III digestion, DNA digests mixed with 2 μL of λ *HinD* III digest instead of the standard, DNA-free loading buffer contain primarily high-molecular-weight DNA and may be confused with the products of an incomplete restriction digestion (Fig. 4*a*). Note that hybridization probes for both size markers commonly used in forensic RFLP analysis contain λ sequences; the use of ''λ loading buffer'' on analytical gels would result in autorads with high in-lane background.

Another pair of reagents whose packaging might be confused are the 30-band Molecular Weight Marker and a *KPN* I digest of Adenovirus DNA which is used as a visual marker on analytical gels. It is advisable to repackage these reagents in more distinctive tubes.

Deposition of DNA Samples Outside of Wells During Gel Loading

When a gel is being loaded, a small amount of the DNA sample is sometimes ejected from the pipette tip before or after insertion of the tip into the well. To determine if DNA samples that are deposited outside of the wells during sample loading are detectable, a drop of the molecular weight marker was deposited over the gel, on the cathodal side of the wells.

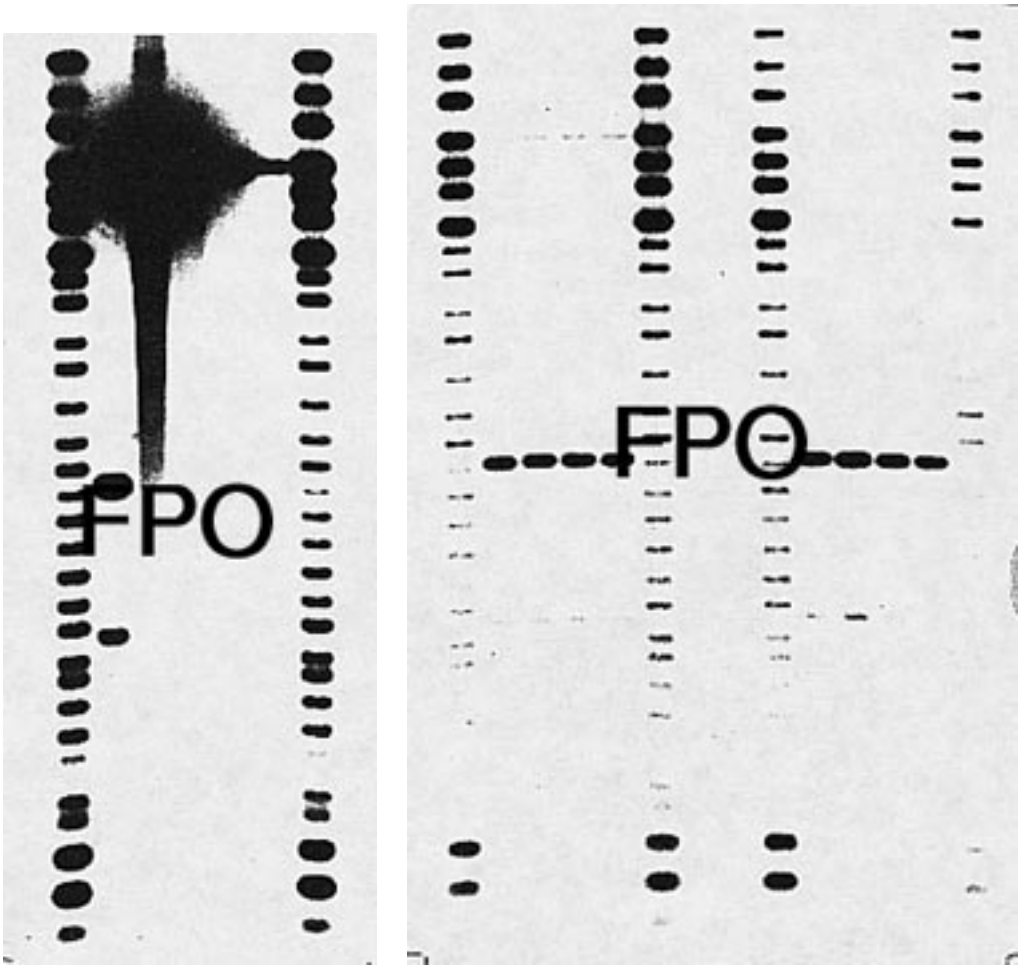

FIG. 4*c*—*Effect of Lambda*/Hind *III digest contamination of reagents.* (a) *Lanes 1 and 7, 30-band molecular weight marker; Lane 2, K562 DNA digested with* Hae *III; Lanes 3 to 6, the equivalent of* 10^{-1}, 10^{-2}, 10^{-3}, *and* 10^{-4} *μL of the ready-to-load Lambda*/*Hind III preparation. The membrane was hybridized with a mixture of probes to D2S44 and the molecular weight marker.* (b) *Autoradiograph affected by Lambda*/*Hind* III *digest contamination. Lanes 1, 6, 9, 14, 30-band Molecular Weight Marker. Lanes 2 to 5, 400 ng commercially prepared* Hae *III-digested K562 contaminated with Lambda*/*Hind III digest. Lanes 10 to 13, samples from a second tube contaminated to a lesser extent. The cause could be an unchanged pipette tip.*

A shadow of the Molecular Weight Marker was visible on some of the autoradiographs (Fig. 4*b*). The bands were out of register with the normal marker bands and fell in between two lane positions. To prevent this effect, the outside of the pipette tip should be free of sample and the tip should be inserted under the buffer directly over the well or away from the gel. Any sample dribbled over the gel will fall onto the surface and diffuse into the gel unless quickly washed away with a transfer pipette.

Contamination with λ HinD *III Digest*

A commercial preparation of a ready-to-load λ *HinD* III digest is often used as a control on post-restriction minigels. The 9416 bp fragment of the λ *HinD* III digest is detected by the probe supplied with the 30-band Molecular Weight Marker from Life Technologies, Inc. The contamination potential for this preparation is high since the 9416 bp fragment represents 19% of the 48,502 bp λ genome and thus is present in significantly higher copy number per ng of DNA than a single-copy human VNTR sequence (two copies of a 5000 bp *Hae* III fragment in a genome of 3 2 10^9 bp would constitute less than 2 2 10^{-4} % of the total DNA). Contamination of stocks of loading buffer, TE or other reagents with the λ *HinD* III digest preparation, perhaps via unchanged pipette tips, will result in an artifact band at 9416 bp. To demonstrate this effect, serial dilutions of the λ *HinD* III digest preparation were made such that the equivalent of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} μL were loaded onto an analytical gel.

After a typical exposure, even the 10^{-4} μL loading gave a strongly visible 9416 bp band (Fig. 4*c*). The viral origin of a band can be confirmed by probing with the marker and genomic probes individually.

Loading Buffer Variations

The effects of variations in a homemade loading buffer were investigated. The control loading buffer (50% glycerol, 0.1% BPB, and 1X TAE) was compared with loading buffers that contained 25% and 75% glycerol, 0.2%, 1.0%, and 2.0% BPB and no TAE. A series of 8 different loading buffer formulations was loaded onto 8 analytical gels.

When the loading buffer preparations were used to load DNA onto analytical gels, no differences between the preparations were detected in either band resolution or migration distance. However, Duewer et al. (7) have found that loading buffer formulation does influence long-term reproducibility of band sizing measurements. While none of the loading buffer formulations examined here caused apparent differences in electrophoretic properties, small sample size may have prevented our observing differences. Careful attention to loading buffer preparation is beneficial to wide-scale efforts to make interlaboratory band size comparisons such as the Combined DNA Indexing System.

Electrophoresis

Time Delay Between Sample Loading and the Beginning of Electrophoresis

To determine if a time delay between loading of an analytical gel and the beginning of electrophoresis would have a noticeable effect, *Hae* III digested K562 DNA was loaded into the wells of an analytical gel 60 min, 30 min and immediately before beginning electrophoresis.

No differences were observed between the sets of samples loaded 60 min, 30 min and immediately prior to electrophoresis. These results may also be extended to predict that interruptions of electrophoresis (for instance due to power outages) will not produce deleterious changes.

Variations in Running Buffer Concentration

To determine the effects of variations in TAE running buffer concentration that might result from sloppy solution preparation techniques, 0.5, 1.0, and 1.5 X TAE buffers were prepared. The gel and its corresponding running buffer were made from the same TAE concentration.

For similar electrophoretic run times, band migration distance was inversely proportional to TAE concentration. No differences in band sizing results were detected between the different TAE levels, although small effects could not be judged from this test. Some differences were seen in band resolution across the gel (not shown). At the same source voltage, longer electrophoretic runs are normally required for stronger buffer solutions.

Denaturation

Following electrophoresis, the DNA is denatured in the gel by gently rocking the gel in 400 mM NaOH for 30 min. Denaturation is necessary for probe binding. If DNA is double stranded when immobilized to the membrane, no signal will be detected on the autoradiographs. For transfer to positively charged nylon membrane (Biodyne B, Life Technologies or Hybond N ` , Amersham), no neutralization is performed prior to transfer. Southern transfer is carried out in the same 400 mM NaOH solution.

Shortening or Elimination of Denaturation Step

To determine whether shortening or omission of the denaturation step would adversely affect DNA detection, the denaturing step was terminated after 15 min or was completely eliminated.

No difference was detectable between gels that had been denatured for 30 min, 15 min and those that had not been denatured. Apparently, sufficient DNA denaturation takes place during the transfer step alone. However, it is not recommended to eliminate the denaturation step (see later sub-section, Wrong Transfer Solution Used).

Southern Transfer

The Southern transfer apparatus consists (from bottom to top) of two thin sponges (Lifecodes, Inc., Stamford, CT), the denatured gel, Biodyne B nylon membrane (Life Technologies), a single sheet of Whatman #3 filter paper (Fisher Scientific) and nine blot pads (Life Technologies). The sponges are wetted in 400 mM NaOH and placed in a tray containing approximately 2 to 3 cm of 400 mM NaOH prior to setup. After setup, the Southern transfer assembly is left together for 6 h, after which the nylon membrane is neutralized with a brief wash in 200 mM TRIS pH 7.5

Long/Short Transfer Period (5 min to 3 days)

Recommendations for the duration of Southern transfer vary. Transfer times of 1 h (8), 4 h (9,10), 6 h (3,11,12) and overnight (2) have been published. The optimum transfer time was determined

because rigid times may be difficult to accommodate during a normal work schedule. Southern transfers were assembled and the transfer allowed to proceed for 5, 10, 15, 30 min, 1, 2, 6 h, overnight and 3 days. A second type of experiment was performed where the nylon membrane was changed after 30, 60 and 90 min to produce membranes representing the DNA which is transferred in the first, second and third 30-min periods of the transfer, respectively. The fourth and final membrane represented the DNA that is transferred between 90 min and overnight.

Sensitivity (the smallest amount of DNA detectable) and band density (a measure of the amount of target DNA immobilized to the membrane) were affected in different ways. The smallest test sample used, 25 ng, was detectable after a 5 min transfer (Fig 5). The band intensity of the 25 ng samples did not increase after 5 min. The intensity of the molecular weight marker bands and the more concentrated genomic samples were greatly reduced in comparison with the longer transfers. Band intensity of these samples continued to increase up until 2 h of transfer time. Maximum sensitivity is achieved after approximately 15 min. Maximum band density of the higher molecular weight and the more concentrated samples continues to increase for approximately 2 h. No benefit (or harm) resulted from longer transfer times. The Southern transfers which were left assembled for three days were indistinguishable from the controls.

During one set of transfers, the nylon membrane was changed every 30 min for 2 h. The fourth membrane was left on overnight. Consistent with the transfer time course, the 25 ng samples were visible on the first membrane (Fig. 5). The bulk of the other samples had also transferred after 30 min. After 1.5 h, only DNA from the molecular weight marker bands over 4000 bp remained in the gel. The rate of transfer of DNA out of a gel is influenced not only by size, but by the amount of DNA which can sieve through the gel per unit time.

Under the conditions tested, optimum results may be obtained from Southern transfers lasting 2 h or more. In the size range tested (under 3000 bp), no loss of sensitivity in single-donor samples occurred in Southern transfers terminated after as little as 5 min. Because large fragments of DNA move through the gel more slowly than small fragments, a slightly longer time might be required to achieve maximum sensitivity for fragments over 3000 bp.

Filter Paper, Hair, Bubbles Left Under Membrane

Southern transfers were assembled where the Whatman #3 filter paper was placed below rather than on top of the nylon membrane, where 12 hairs approximately 5 cm (2 in.) in length were left between the gel and the membrane and where a large air bubble was left between the gel and the membrane.

The filter paper placed between the gel and the membrane resulted in a severe distortion of the gel pattern (Fig. 6). Bubbles under the membrane cause the transfer solution and the DNA to wick around the bubble. Bubbles can be identified by a blank space in the autoradiograph with sharp perimeters (Fig. 6). The sharp perimeter may distinguish bubbles from membrane defects or oily fingerprints. Only one defect due to hair under the membrane was noted, where a small amount of the DNA appeared to have wicked along one of the hairs (not shown).

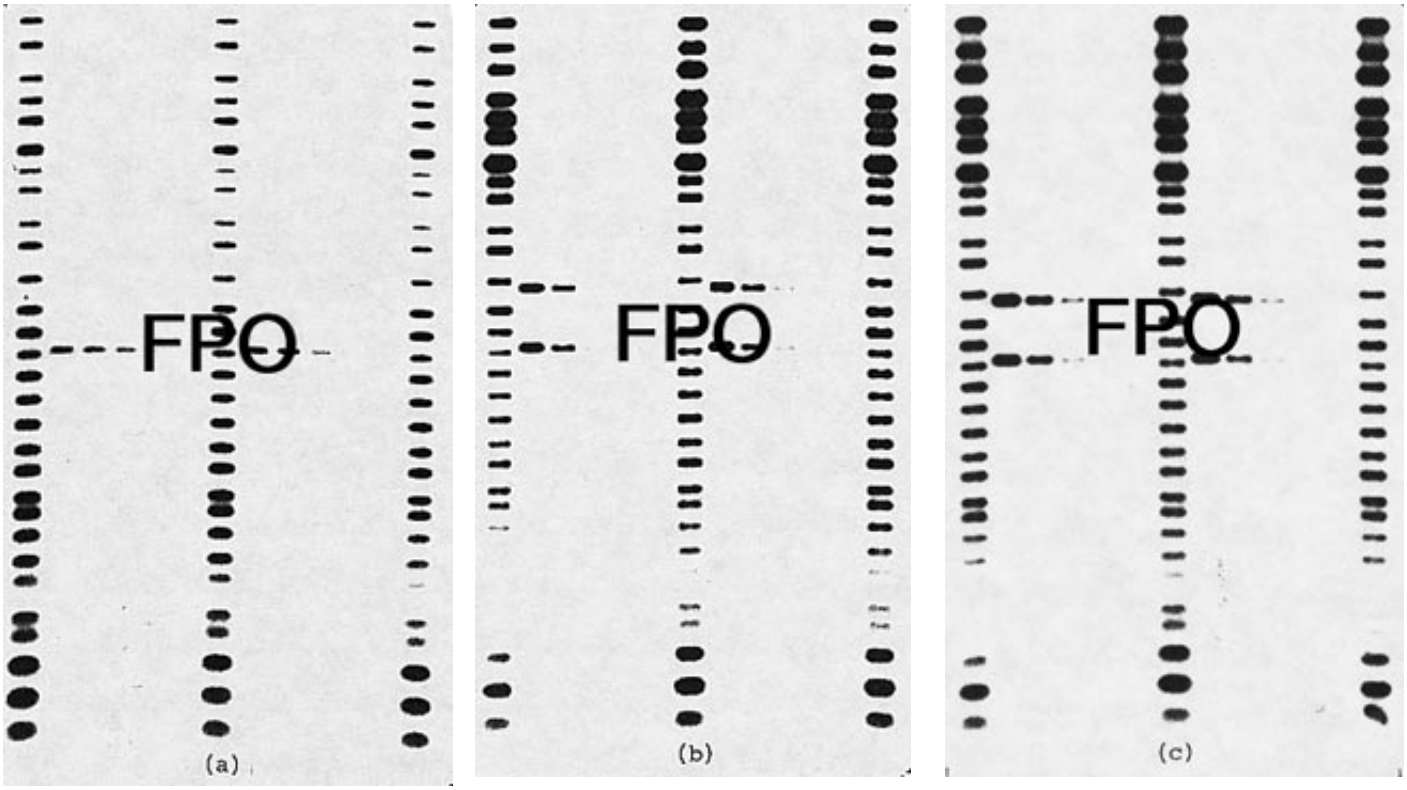

FIG. 5*a-c.—Effect of different transfer times on sensitivity and band density. a, 5 min; b, 1 h; and c, 6h.*

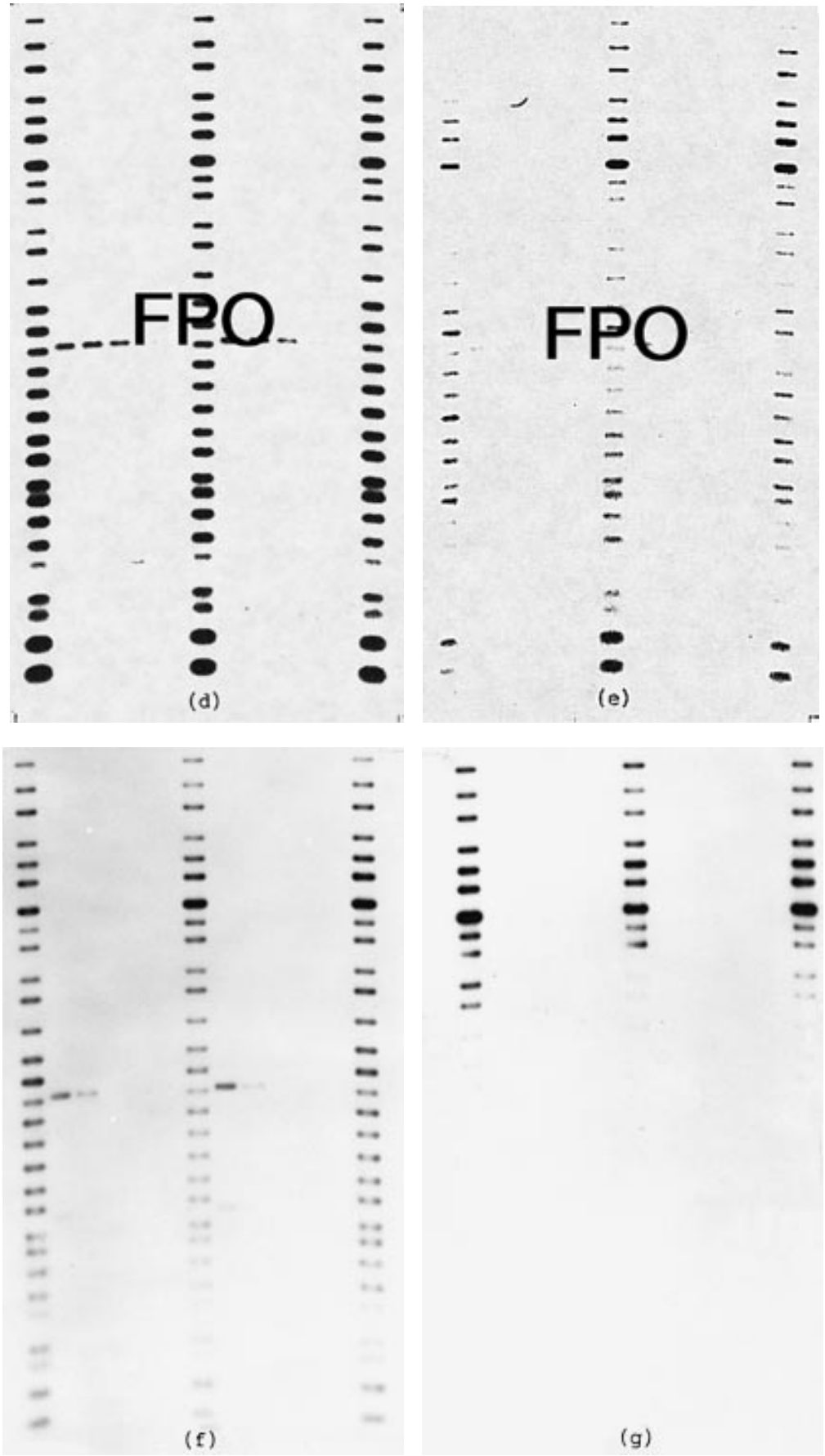

FIG. 5*d-g.—Nylon membrane changed after 30 (d), 60 (e), 90 (f) min and overnight (g).*

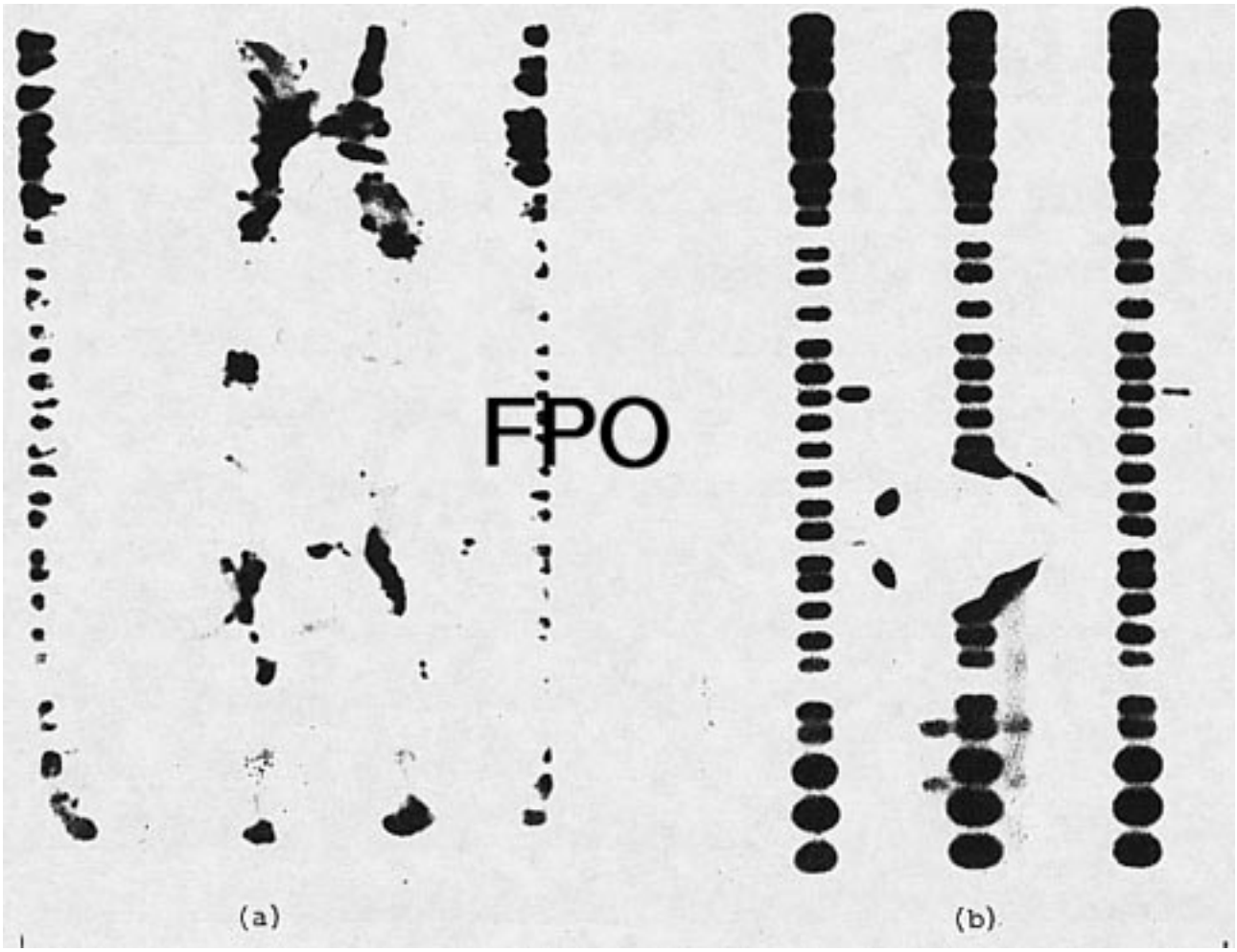

FIG. 6—*Things left under the membrane:* (a) *filter paper,* (b) *bubble.*

Wrong Transfer Solution Used

Thin sponges were placed in a tray of 1X TAE rather than 400 mM NaOH. The gels, however, had been previously denatured for 30 min in 400 mM NaOH. A second set of gels which had not been denatured were also transferred in 1X TAE.

No difference in sensitivity was detectable between denatured gels transferred in 1X TAE and 400 mM NaOH. However, total band intensity was less for the gels transferred in 1X TAE and no DNA was detectable in gels which were not denatured and were transferred in 1X TAE.

Thin Sponges Versus Blot Pads

Side-by-side comparisons were made of identically loaded gels which were blotted on top of two thin sponges or two blot pads. The blot pads were rinsed twice for 15 min in 1 L of 400 mM NaOH prior to setup. The purpose of the NaOH rinsing step is to remove a yellow manufacturing by-product from the blot pads.

No difference was detected in the use of blot pads or thin sponges. Similar results were obtained by Sprecher et al. (8).

Blot Pads Not Rinsed

Side-by-side comparisons were made of identically loaded gels which were blotted on top of rinsed or unrinsed blot pads.

A yellow stain was present in the center of the nylon membranes where unrinsed blot pads were used. On the autoradiographs, no difference was detected in the use of rinsed or unrinsed blot pads. The yellow discoloration was washed away during the first probe hybridization.

Nylon Membrane Not Baked

The practice of baking nitrocellulose or nylon membranes after transfer may enhance DNA retention (2). Positively charged nylon membrane (i.e., Biodyne B, Hybond N `) does not require baking (see product inserts) although the practice persists. To verify that baking of positively charged nylon membranes is not beneficial, Biodyne B membranes were prepared from identical gels, neutralized and either baked at 80°C for 30 min or not baked.

After several rounds of hybridization and stripping, no differences were seen between the baked and unbaked membranes (not shown).

Preneutralization Membrane Contact During Disassembly

During normal Southern transfer disassembly, the membranes are placed one at a time into a TRIS neutralization solution. This practice was compared with the practice of stacking the membranes on top of each other in a tray and then adding the 200 mM TRIS to see if close contact of membranes immediately after transfer might result in transfer of DNA from one membrane to another.

No between-membrane DNA transfer was detected (not shown).

Autoradiographic and Lumigraphic Artifacts

After hybridization with radioactively labeled probe, nylon membranes are sandwiched between two pieces of Kodak XAR film inside an X-ray cassette with two enhancing screens. The cassettes are stored at 1 75 to 1 80°C to facilitate image development. After exposure lasting from one to several days, the film is developed.

Speckled Film

Speckles that appear on autoradiograph films regardless of the presence in the cassette of a hybridized membrane are due to a defect in the intensifying screen (Fig. 7*a*).

Static Electricity Discharges

Static electricity is a familiar problem during the winter in some climates when the indoor relative humidity is very low. Light from static electricity discharges may be seen in the dark room when films are removed from the cassette for development (Fig. 7*b*). The severity of the static problem can be reduced by increasing indoor relative humidity or using a commercial anti-static spray or both. Some plastic materials which may be used to wrap the damp membranes during autoradiography may increase the static buildup. For radioactive probes which are detected using enhancing screens at 1 75 to 1 80°C, complete thawing of the cassette prior to opening is helpful.

Dark, Concentric Spots on Film

Dark, concentric spots that appear on the film may be caused by splashing of ^{32}P-labeled or alkaline phosphate conjugated probe solution onto the membrane prior to wetting in the hybridization solution. Membranes should not be left out in the area where probe is being prepared (not shown).

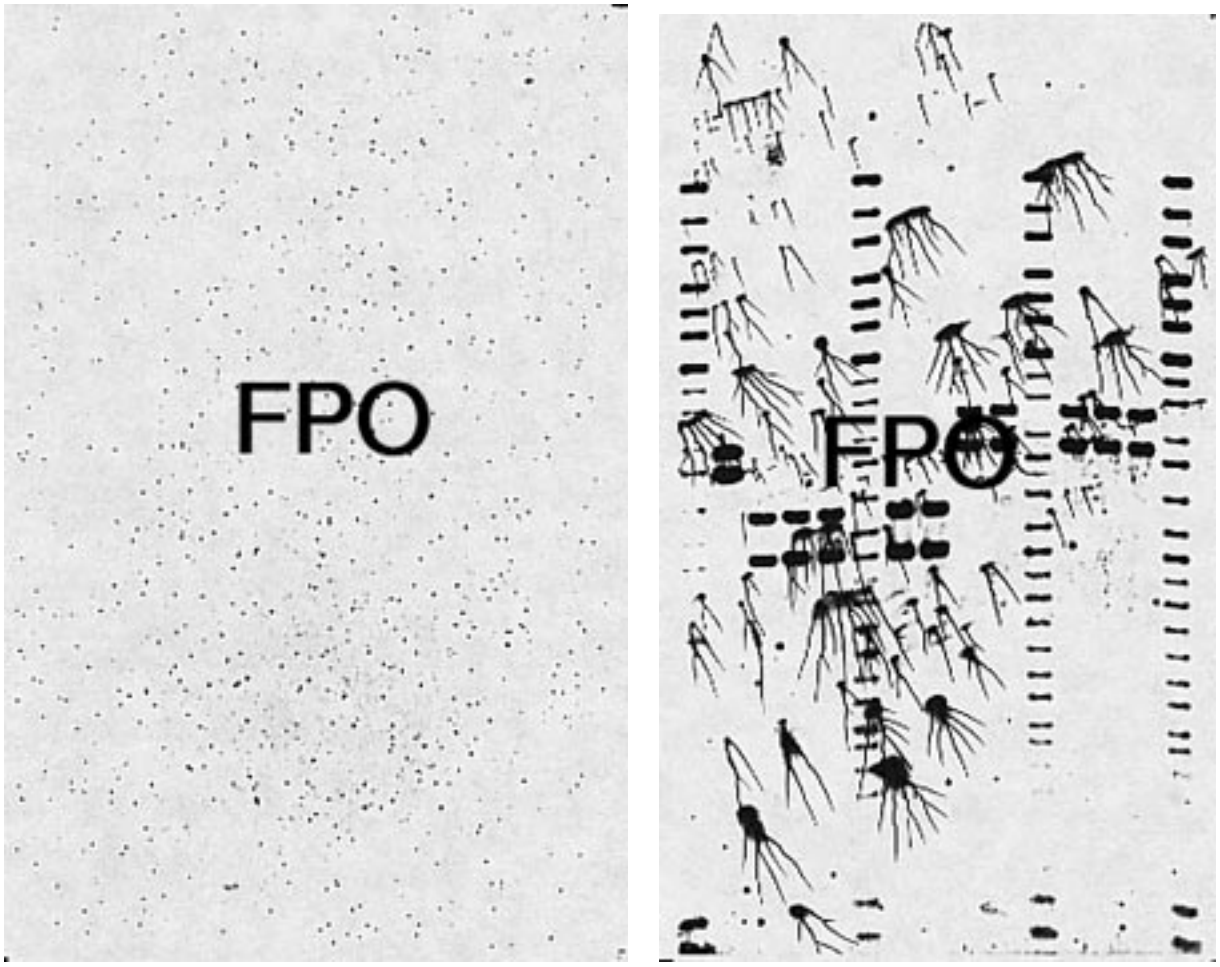

FIG. 7*a—Defective intensifying screens. The speckling seen on the autoradiograph is due to an impurity in the intensifying screens. Enlargement of film exposed in the same cassette for 7 days without hybridized membrane. The effect does not decay over time. b Lumigraph (alkaline phosphatase-conjugated probe) exposed by static electricity discharge during film removal from cassette after ambient exposure period.*

Neutralization of Membranes After Southern Transfer or Probe Stripping

After autoradiography, bound radioactive probe is removed from positively charged nylon membranes by a 30 min, ambient temperature wash in 400 mM NaOH followed by a brief neutralization in 200 mM TRIS pH 7.5. This stripping method is as effective as hot formamide, hot SDS (13) and hot NaOH methods (not shown). The membranes are then dried or damp-dried and stored frozen at 1 20°C.

Drying of Membranes Without Complete Neutralization of NaOH

While investigating the progressive loss of signal from membranes during successive hybridization, identical membranes were prepared and hybridized to radioactive probe to verify uniformity. The membranes were stripped in 400 mM NaOH, then either not neutralized or washed for 15 s or 15 min in 200 mM TRIS pH 7.5. The membranes were allowed to dry overnight on the bench top and were then reprobed.

After reprobing of the membranes, it was found that the signal was normal on the membranes which had been neutralized but was entirely absent on the membranes which had been allowed to dry with NaOH on them. Prolonged incubation of membranes in the 400 mM NaOH solution was not harmful. Membrane storage conditions have also been demonstrated to affect the quality of subsequent hybridizations (14).

Effects on DNA and Nylon Membrane of Drying in NaOH

Additional experiments were performed to investigate individually the effects of drying in the presence of NaOH on DNA and on positively charged nylon membrane. DNA (2 μg) from human cell line K562 was placed in 100 μL of either distilled water, TE, 200 mM TRIS pH 7.5, 400 mM NaCl or 400 mM NaOH, spotted onto 1 cm^2 pieces of Whatman #3 filter paper and allowed to dry overnight on the bench top. The DNA was then recovered by soaking the filter paper pieces in 400 μL TE and spinning the liquid out of the filter paper in Spin-Ease tubes (Life Technologies). The DNA was concentrated in Microcon 100 spin filters, washed with 400 μL TE and recovered in TE. The condition of the DNA was assessed by agarose gel electrophoresis. An additional experiment was performed where new pieces of nylon membrane were wetted in the same solutions and allowed to dry on the bench top overnight. They were then rinsed in 400 mM NaOH and used in Southern transfers.

Drying of DNA in deionized water, TE, 200 mM TRIS or 400 mM NaCl did not cause severe degradation, although recovery from the filter paper was low (Fig. 8*a*). The DNA which was dried in a 400 mM NaOH solution was heavily degraded.

Drying of Biodyne B membrane after wetting in deionized water, TE, 200 mM TRIS or 400 mM NaCl prior to Southern transfer assembly had no effect. However, drying after wetting in 400 mM NaOH resulted in an *enhancement* of signal (Fig. 8*b*). This effect was repeatable across membrane lot numbers. A similar effect was obtained from soaking the nylon membrane in NaOH overnight.

The neutralization step after alkaline transfer or probe stripping from positively charged nylon membranes is important. If membranes are allowed to dry without having been completely neutralized, DNA hydrolysis will result.

Discussion

The results presented here demonstrate which protocol mistakes or defective materials can and cannot be ruled out as causes of particular anomalies. Due to the controls and interpretation guidelines which are utilized in most forensic DNA laboratories, none of the situations investigated here would be expected to result in erroneous information being reported. However, a number of factors were investigated which could result in loss of data.

The results documented here are intended to be used only as an RFLP troubleshooting aid and not as a list of steps which do or do not require care and precision in their execution. Optimization of each step of the protocol is necessary to consistently achieve the best results. For instance, while neither failure to denature gels nor failure to transfer to Biodyne B in NaOH is singly detrimental, the combination of these two mistakes would result in total loss of DNA transfer to the membrane.

Finally, some of the findings may suggest desirable protocol

FIG. 8—*Effects on DNA and Biodyne B membrane of drying in NaOH.* (a) *DNA drying in NaOH. K562 DNA* (from left to right) *not dried or dried on filter paper in the presence of H_2O, TE, TRIS, NaCl, or NaOH.*

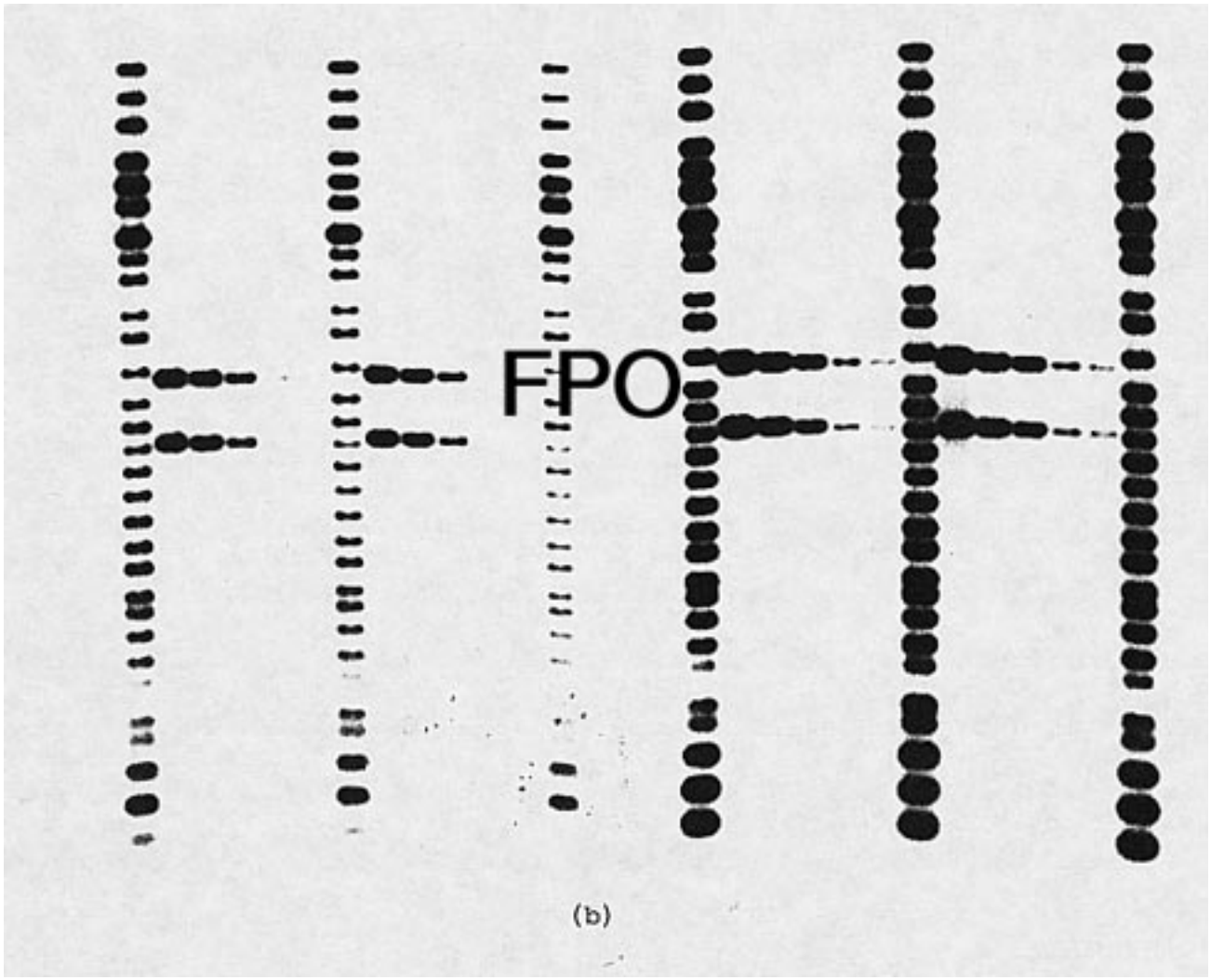

FIG. 8*b*—*Biodyne B membrane not pretreated* (left) *and wetted and dried in 400 mM NaOH prior to transfer. Pretreatment improved both sensitivity and density.*

modifications, but these should be independently validated in each laboratory using them.

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