Identifying DNA RFLP'S in Routine Paternity Cases: Non-Isotopic Methods of Detection.

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An ever increasing number of polymorphic DNA probes have been reported in the literature. Thus it should be theoretically possible to select from a broad range of probes to be used in cases of disputed parentage. However, the lack of availability of many probes from the commercial sector, the general view that probes must be labeled with isotopes and various technical problems inherent with the techniques have hindered the widespread use of RFLP's for this application.

For this study we will describe data on the use of non-isotopically labeled probes for detecting RFLP's in paternity cases. Probes were labeled with biotin and subsequently detected with alkaline phosphatase conjugates using modifications of the techniques of Leary et al. (1983) and Dykes et al (1986). The probes were obtained either in plasmid vectors, bacteriophage lambda or as inserts from three commercial companies and labeled in our laboratory. The technique permitted the use of probes labeled up to 18 months prior to hybridization. Rehybridization of membranes a minimum of 4-5 times with non-isotopically labeled probes was possible. Speed, cost effectiveness and reproduceability are all important factors which make this technique well suited for general use in the area of parentage testing.

MATERIALS AND METHODS

DNA extracts used in this study were obtained from paternity cases routinely tested in our laboratory for 14-16 genetic marker systems (RBC antigens, enzymes and serum proteins). Single copy human probes used in this study were obtained from Integrated Genetics, Framingham, MA. Collaborative Research Inc, Bedford, MA. and Oncor Inc. Gathersburg, MD. Probes were used only if they had extensive family data to document inheritance, were characterized for chromosomal location and in most cases documented in the literature.

Probes were labeled with a biotin-11-dUTP nucleotide by nick translation as described by Dykes et al. (1986). Labeled probes were stored at 4°C for up to 18 months prior to use. Length of electrophoresis depended upon the probe being used. Southern blotting generally was accomplished in 2-3.5 hours for gels 6-8 mm thick. For this study we tried many membranes and finally settled on a nylon membrane from Oncor, Inc. called Sure Blot® which provided the best signal to noise ratio of any membranes tested. The membrane worked well in

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both an alkaline and high salt transfer buffer. Membranes were baked at 80°C for 20-30 minutes after transfer. Listed below, with minor modifications, is the hybridization and staining procedure we described in 1986.

1. Block membrane 30-45 minutes at RT using pH 7.5 TBS (0.1M Tris-HCl, 0.5M NaCl), 1% Hammarsten casein, 3% Liquid Hipure Geletin and 0.05% Tween-20.
2. Hybridize at 42°C overnight using 20-40 ng probe/ml hybridization buffer.
3. Wash 2-3 minutes at RT in 0.16 X SSC, 0.1% SDS.
4. Stringency wash at 50-60°C in 0.16 X SSC, 0.1% SDS.
5. Wash 2 X 3 minutes in 2 X SSC.
6. Block membrane for 15 minutes (#1)
7. Prewash in complex dilution buffer of pH 7.5 TBS, 5% Tween-20.
8. Treat with streptavidin-biotin-alkaline phosphatase (Enzo Biochem or Oncor, Inc.) in complex dilution buffer for 20 minutes at RT.
9. Blot membrane and wash 3 X 5 minutes in pH 7.5 TBS buffer.
10. Wash 5 minutes in 0.1 M TBS pH 9.1 stain buffer.
11. Stain membrane on agarose stain plates.
12. Wash briefly in tap water and thoroughly dry in dark.

Membranes were rehybridized by first removing the labeled probe followed by two brief washes in dimethlyformamide to remove the bands and two washes of 2 X SSC for 4-5 minutes. Membranes were then reblocked as above and rehybridized.

RESULTS AND DISCUSSION

Purified probe inserts and probes in plasmid or lambda bacteriophage were all capable of providing good signal after nick translation with biotin. Although a large number of probes were analyzed we concentrated on the probes shown in Table 1. There appeared to be a consistent difference in signal obtained from some of the probes which did not seem to be related to the size of the probe insert. This phenomenon also holds true for probes labeled with p32 (personnal communication with labs providing probes). For example probe pPW228C at 1500 bp provided a better signal than pPW513 at 3200 bp when applied at equal concentrations to the membrane.

TABLE 1 PROBES USED IN STUDY

Probe	Size	Vector	Enzyme	Source*
pPW228C	1.5	pBR328	MspI	IG
pPW513-5H	3.2	pBR322	MspI	IG
pPW523-10B	6.5	pBR322	TaqI	IG
pPW518-1R	3.2	pBR322	XbaI	IG
cMetH	1.6	pBR322	TaqI	IG
cMetD	1.1	pBR322	TaqI	IG

TABLE 1 (CONTINUED)

Probe	Size	Vector	Enzyme	Source*
L-45	3.2	Lambda	MspI	CR
L-892	12.6	Lambda	TaqI	CR
pS-194	1.4	pUC-8	TaqI	CR
cMetH	1.6	insert	TaqI	OI
cMetD	1.1	insert	TaqI	OI

*IG = Integrated Genetics
CR = Collaborative Research
OI = Oncor Inc.

The type of membrane is vital to the use of non-isotopic probes. Nylon worked best, with Genetran-45® and Sure blot® showing the most reproducable results. Sure Blot, however, retained the most DNA after transfer and demonstrated the clearest background. Excessive blocking and the use of carrier DNA was found to be unnecessary. Carrier DNA non-specifically reacted with some enzyme conjugates and caused unwanted back-ground.

Alkaline phosphatase conjugates provided greater sensitivity when compared to streptavidin-horseradish peroxidase conjugates. Although the latter is faster it often produced excessive background if not carefully watched. It was very light sensitive and losses approximately 20-25% of band intensity when dried.

When such parameters as membrane type and source, form of enzyme conjugate and probe size are optimized the biotinylated probes easily detect single copy RFLP's when 3-5 ug of total human genomic DNA are applied to the gel. Dot blots are capable of detecting 0.1-0.5 pg of target DNA. The stability of these non-isotopically labeled probes was at least 18 months. This enabled us to use the same quality controlled probe over extended periods of time. Isotopic probes using p32 have a short half-life and require labeling probes every 10-14 days.

Table 2 lists the observed gene frequencies of the RFLP's observed in this study using the various single copy human DNA probes. Examples of single probing are shown in figures 1a and b. For two allele probes with exclusion probabilities of 0.15 - 0.19 we found it more practical to do multiple probings if overlapping of bands was not a problem, figure 2. For more polymorphic probes such as 518-1R and PS-194 we found that the non-isotopic approach demonstrated additional alleles. The better resolution obtained with this technique and hence the detection of more variants is a result of confining the signal in a narrow region versus the more diffuse signal generated by the isotopic approaches.

TABLE 2

GENE FREQUENCIES AND EXCLUSION PROBABILITIES DETECTED WITH PROBES

Probe	No. Tested	Alleles	Size	Gene Freq.	Exclusion Prob (P)
228C	474	A1	4.6	0.62	0.18
		A2	7.8	0.38	
513-5H	407	A1	3.4	0.67	0.17
		A2	2.1	0.32	
523-10B	277	A1	7.9	0.51	0.19
		A2	5.5	0.49	
518-1R	208	A1	2.45	0.224	0.43
		A2	2.41	0.293	
		A3	2.33	0.415	
		A4	2.25	0.050	
		A5	2.33	0.012	
		A6	2.35	0.005	
		A7	2.60	0.001	
cMetH	323	1	7.7	0.57	0.18
		2	4.0	0.43	
cMetD	135	1	6.3	0.83	0.12
		2	4.7	0.17	
L-45	171	1	7.4	0.336	
		2	4.0+3.0	0.342	
		3	3.8+3.2	0.281	0.41
		4	3.95+2.8	0.041	
L-892	154	1	9.7	0.045	
		2	9.2	0.341	
		3	8.8	0.247	0.48
		4	8.2	0.308	
		5	9.2+4.5	0.042	
		6	8.6	0.017	
PS 194	154	1	14	0.023	0.67
		2	12.3	0.003	
		3	11.1	0.006	
		4	10.7	0.039	
		5	10.5	0.042	
		6	10.3	0.049	
		7	10.2	0.032	
		8	10.1	0.078	
		9	10.0	0.019	
		10	9.9	0.206	
		11	9.6	0.081	
		12	9.4	0.062	
		13	9.2	0.019	
		14	9.1	0.006	
		15	8.9	0.003	
		16	8.6	0.332	

Membranes were also rehybridized up to 4-5 times without a noticeable loss of signal. This permitted us to return to earlier hybridized membranes and study older paternity cases and population studies, figure 3.

As expected the observed rate of exclusion varied with the "P" value of the individual probes. Multiple simultaneous probings, such as 228C and 513-5H after MspI restriction or cMetH and 523-10B, sometimes included with pS-194 after TaqI restriction provided the anticipated number of combined exclusions, table 3. Some of the variation from expected values was due to the small sample size. No additional exclusions were observed in cases with PI $\geq$ 100. In cases with multiple exclusions by our normal test battery we observed that the putitive father was excluded 7/8 times when using L-45 and L-892 and pS 194.

In conclusion the use of biotinylated probes permits easy and inexpensive analysis of RFLP's in parentage testing. The substrates are not a biohazard, are stable upon storage, give clearly resolved bands and are easily adapted to the normal laboratory setting.

TABLE 3
RESULTS OF BIOTINYLATED PROBES ON PATERNITY CASES

Probe	# cases Tested	# Exclusion Other Systems	# Exclusion with Probe	Observed Excl. Rate	Expected* Excl. Rate(P)
L-45	43	8	3	0.43	0.38
L-892	42	13	8	0.62	0.48
PS-194	46	16	12	0.75	0.67
pPW228C	108	29	6	0.20	0.18
513-5H	89	25	8	0.32	0.17
523-10B	47	11	2	0.18	0.19
518-1R	23	7	2	0.29	0.43
cMetH	71	19	5	0.26	0.18
cMetD	15	6	0	-	0.12

* Initial battery of tests included 14-16 markers with P $\cong$ 0.98. This value is close enough to 1.00 that "P" for each marker system should approximate observed rate of exclusion if sampe size is large enough.

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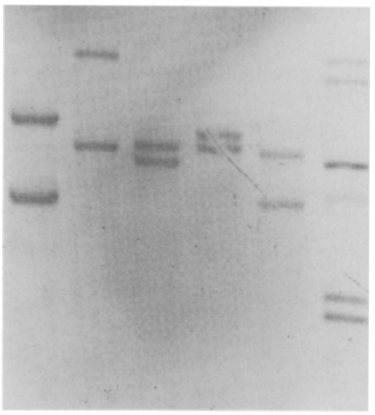

Fig. 1a pS 194 probing with size markers on far right.

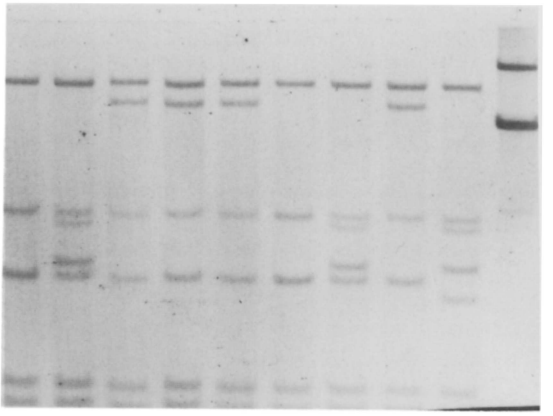

Fig. 1b L-45 probing with size markers on far right.

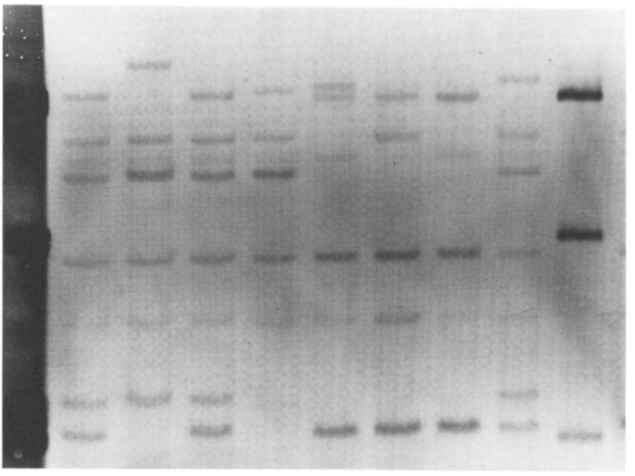

Fig. 2 Multiple probing with cMetH, cMetD, 523-10B and pS194. Size markers on far right and left.

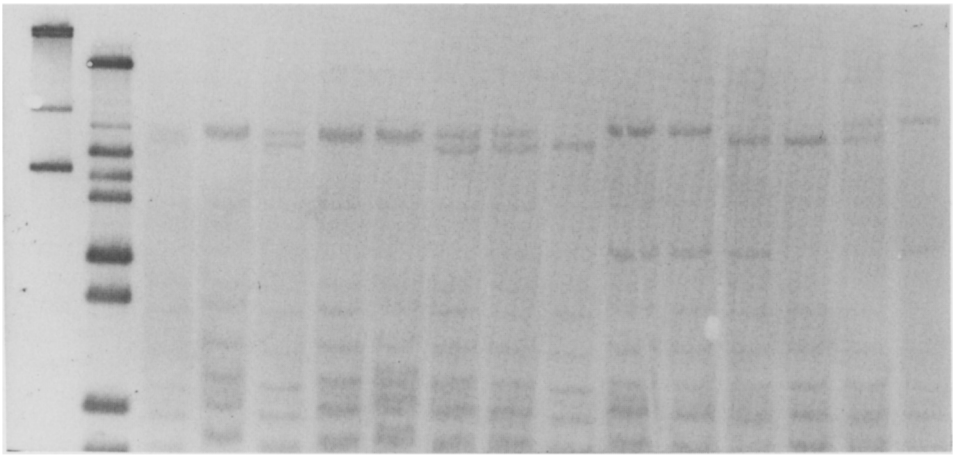

Fig. 3a Probing with L-892. Size marker controls on far left and right.

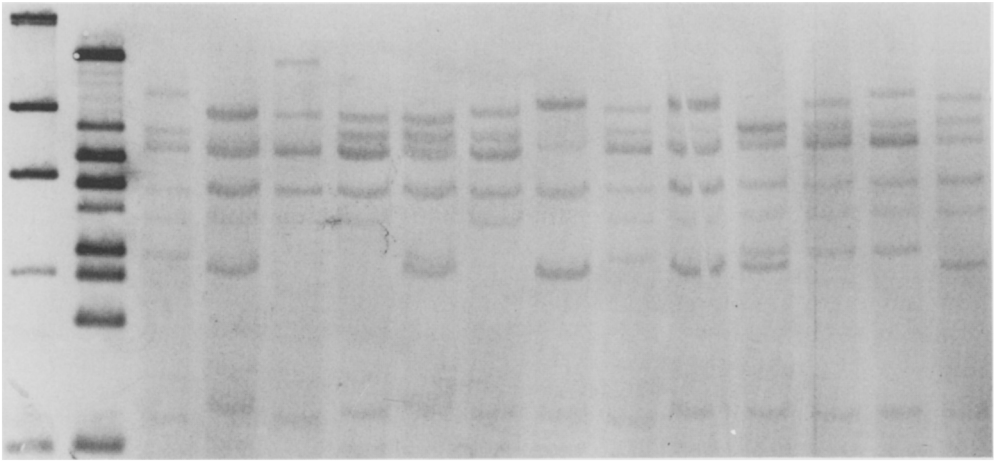

Fig. 3b Reprobing of membrane from Fig. 3b using probes cMetH, cMetD, 523-10B and pS194. Reason for darker size markers is a different source of biotinylated - λ .