A Protocol Using An Alkali Blotting Procedure For The Analysis Of Restriction Length Fragments Of Human DNA.

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ABSTRACT

The sensitivity and practicability of a combination of techniques have been investigated in a study of human tissues using restriction length fragments of DNA. In the protocol established the DNA was restricted using either EcoRl, HaeIII or Taql restriction enzymes. Studies of the capillary transfer step ("Southern blotting") of the DNA fragments from agarose gels to nylon membranes showed that the use of very dilute alkali gave improved yields. The DNA was probed with a Satellite III sequence related probe (228S) labelled with [32P]-ATP. The HaeIII/228S and the EcoRl/228S restriction patterns both show discrimination between male DNA and female DNA. The Taq 1/228S patterns are complex and have been shown to be highly discriminating of individual genomes. They have been able to distinguish between four members of a three-generation family. The protocol is satisfactory for the analysis of very small quantities of DNA.

INTRODUCTION

Capillary transfer of DNA fragments ("Southern Blotting") from electrophoresis gels to receptor membranes followed by hybridisation of the bound DNA with suitably labelled oligonucleotide probes is well established methodology (Mathew, 1984). Recently nylon membranes have been shown to bind (apparently covalently) single stranded DNA which is both denatured and transferred in dilute alkali (Reed and Mann, 1985). An advantage in forensic science of this method is that multiple probing of membranes is possible.

We have investigated the optimal conditions of alkali transfer. Such conditions should maximise the quantity of DNA transferred from the gel to the membrane and maximise the proportion which is single stranded and therefore available for hybridisation with single stranded probes.

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MATERIALS AND METHODS

Isolation of DNA

Bloodstains on cloth (or single (anagen) roots for hair samples) were digested at 37°C with continuous agitation for 14 hours in an aqueous buffer (10 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 8.0) containing 1% SDS and proteinase K (50 ug/ml). Digest volumes were a minimum of ten times the volume of the estimated volume of any stain. Hair root tissue was digested in 50 ul of digest buffer. The aqueous digests were extracted with 0.5 volumes phenol (redistilled, aqueous saturated pH 7.6) and once with 0.5 volumes chloroform. The DNA was precipitated by addition of 2.5 volumes redistilled ethanol and 0.1 volume sodium acetate (3 M, pH 6.0). This was stored on ice for 30 minutes and then centrifuged at 12000 rpm for 15 minutes at 4°C. The resultant pellet was washed once with 70% ethanol and dried at 37°C. Placental DNA was isolated according to Wolf et al (1980). Sperm DNA was isolated from whole human ejaculates which were allowed to liquify after collection and then the cellular component centrifuged and washed three times in an aqueous buffer (10 mM Tris, 10 mM EDTA, 100 mM NaCl; pH 8.0). The sperm pellet was demembranised by vortexing the sperm pellet in the same buffer containing 2% SDS. The resultant sperm head preparation was digested with proteinase K in SDS buffer as for bloodstains, but with the addition of 2-mercaptoethanol (0.04 M). The DNA was extracted, precipitated and washed as described above.

Restriction of DNA

The DNA recovered was restricted in minimal volumes using either EcoRl, HaeIII or Taq 1 (Boehringer Mannheim) following the manufacturers' instructions. The restriction fragments were precipitated with ethanol/sodium acetate, cooled, centrifuged, washed and dried as described by Zengin and Hartley (1985), then dissolved in 8 ul of buffer (10 mM tris, 1 mM EDTA, pH 8.0). Direct-loading of the digest into the electrophoresis gel is also possible, thus eliminating the precipitation step. This usually had no discernable effect upon the clarity of the DNA separation. One ul of a loading solution (5% Ficoll 400, 0.1% bromophenol blue (BPB), in electrophoresis buffer) was added to each 8 ul sample prior to injection into the gel.

Electrophoresis

Electrophoresis was in a 1% agarose gel (50 ml Calbiochem Type C agarose, Pharmacia GNA 100 minigel apparatus). The gel and tank buffer were identical (10 mM Tris, 5 mM sodium acetate, 0.5 mM EDTA, pH 7.8 with acetic acid). Electrophoresis (approximately 4 V/cm) was conducted for a minimum of 2.25 hours at ambient temperature. DNA molecular size markers were included in each gel (nick translated DNA-EcoRl + HindIII, MW marker III, Boehringer Mannheim).

Southern Blotting

Gels were soaked in dilute HCl (0.25 M) for approximately 10 minutes (until the Bromophenol blue (BPB) turned from blue to yellow) and then blotted by Southern transfer to nylon membranes (Bio-Rad Zeta Probe) in dilute alkali (0.4 M NaOH) (Reed and Mann, 1985). Alternatively, after acid treatment, the gels were soaked for approximately 10 minutes in dilute alkali (0.4 M, NaOH, BPB turns from yellow to blue) and then blotted to nylon membranes using lower concentrations of alkali (0.02 M, 0.004 M NaOH). Blotting was conducted for approximately 14 hours. The membrane was then recovered and washed for 10 minutes in a neutralising solution (0.3 M NaCl, 0.03 M trisodium citrate = 2 x SSC; pH 7.0) and either stored moist in plastic bags at 4°C, or probed directly.

Probe preparation

The sequence 228S was obtained from a human sperm DNA library enriched in repetitive sequences. It has been subcloned in M-13. The sequence 142F was obtained from human fibroblast DNA.

Probes were prepared by hybridisation of an oligonucleotide primer (Biotechnology Research Enterprises, S. Australia, BRESA) to the 5' side of the insert in the M-13 vector (primer 15 ug/ul, 4 ul; M-13 with insert, 20 ul; primed in a cooling temperature gradient; 80° to 37°C for 90 minutes). Synthesis of the complementary strand using [32P]-ATP (2 ul, 1800 Ci/mmole) or [35S]-ATP, (> 1000 Ci/mmole specific activity) and CTP, GTP, TTP (2 mM each) was with the Klenow fragment of DNAase I (Klenow-BRESA 4 ul; 3 units/ul) at 37°C for 30 minutes. The whole construct was precipitated by ethanol/sodium acetate, cooled on ice, centrifuged and washed twice with 70% ethanol to removed unicorporated nucleotides. The dry residue was dissolved in approximately 3 ml of hybridisation buffer (0.7 M, NaCl, 50 mM NaH_2PO_4, 5 mM EDTA containing 1% SDS and 0.5% non-fat milk powder).

Hybridisation, washing and exposure

The membrane was routinely prehybridised for 3 hours at 68° in hybridisation buffer and then hybridised with the probe for 14 hours at 68°C using a perspex cassette. The membrane was then washed within the cassette with solutions of increasing stringency at 37°C (Wash A, 2 x SSC with 0.1% SDS, 15 minutes; Wash B, 0.5 x SSC with 0.1% SDS and trace quantities of proteinase K, 2 hours; Wash C, 0.1 x SSC with 0.1% SDS, 15 minutes; Wash D, 0.1 x SSC with 1% SDS, 15 minutes at 48°). The membrane was dried thoroughly at 38°C and exposed at ambient temperature to x-ray film (Kodak X OMat RP Film) normally with an intensifier screen (when using [32P]).

Membrane Stripping

Membranes were stripped of probes by washing twice, with agitation, in alkali (0.4 M NaOH) for 15 minutes at 48°C and

tris, pH 7.5) for 30 minutes at 48°C. The membrane could then be reprobed. Probe removal was never absolute, as shown by long exposure of "blank" membranes on X-ray film, but was highly efficient.

RESULTS

The efficiency of various alkali "Southern" transfer procedures were initially compared by comparing the amount of "nick-translated" DNA standard molecular size fragments transferred to the membrane with that remaining in the gel. The procedures used were only semi-quantitative, but best transfer of DNA occurred when the gel was firstly acid treated, and then either blotted directly with alkali (0.4 M NaOH) (similar to Reed and Mann, 1985) or where the gel was treated with alkali (0.4 M NaOH) before blotting with substantially lower concentrations of alkali (0.02 M NaOH or less). Recovery was not as good when the gel was both treated and blotted in the higher concentration of alkali (0.4 M NaOH). Transfers at best were never complete and did not exceed approximately 70% efficiency for the range 20 kb to 0.5 kb DNA.

The same conditions were applied to the transfer of human DNA restriction fragments. Equal concentrations of either EcoRl or HaeIII restricted placental or sperm DNA were blotted to membranes and hybridised under identical conditions with probe 228S. The procedure of acid treatment, in situ alkali denaturation and then transfer using very dilute alkali (0.02M NaOH or less) provided small but consistent improvement in autoradiograph band intensity over other methods.

Comparison of the HaeIII/228S restriction patterns obtained from male DNA with female DNA (either placental or hair root), showed a 3.4 kb restriction fragment to be present in the male but absent in the female (Fig. 1). Additionally high molecular weight material unresolved in the gel and resistant to HaeIII digestion was present in both. This appeared largely to be Satellite III sequence related, there being little or no hybridisation of the alphoid satellite probe 142F in this region. Such probing showed the typical HaeIII/alphoid ladder-like banding (Fig. 1).

The EcoRl/228S restriction patterns obtained from male and female DNA again showed a means to distinguish them, though the patterns are more complex. The male tissue has an intense band at 3.4 kb and also has common banding with female DNA at 1.3, 1.7 and 2.0 kb (Fig. 1). However female DNA showed very weak banding at 3.4 and 3.5 kb, the intensity of which was found to be restriction dependent. Such results were reproducible, provided restriction was thorough, as could be monitored by reprobing with alphoid satellite probe 142F.

Either method, restriction with EcoRl or HaeIII and probing with 228S, is sufficiently sensitive to identify the male

3.4 kb fragment in small amounts of DNA, such as that recovered from human blood stains of 5 ul volume or human hair root tissue from individual hairs. HaeIII rather than EcoRl restriction is perhaps preferred for such samples, it giving clearer identification of the 3.4 kb fragment. Amounts as low as 5 ng of DNA have been detected using both [32S] and biotin labelled 228S probe.

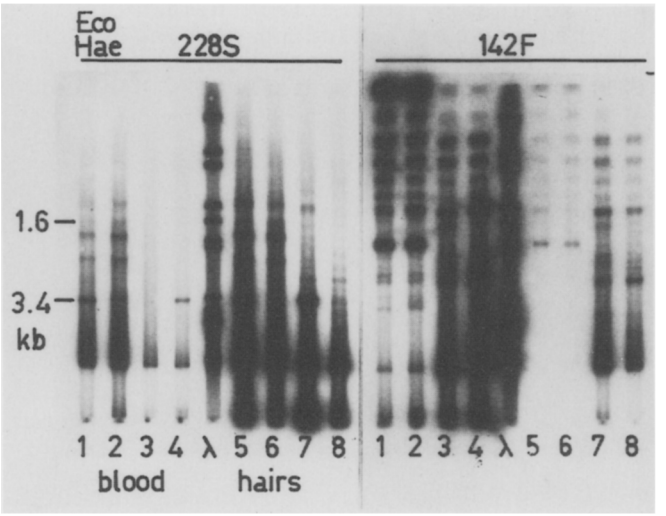

Fig. 1 EcoRl and Hae III analysis with probes 228S and 142F of blood and single hair root DNA. Samples 1,4 and 5,7 are male blood and hair DNA cleaved with Eco and Hae respectively. Samples 2,3 and 6,8 are female blood and hair DNA cleaved with Eco and Hae respectively. λ is DNA markers.

Restriction with Taq 1 and probing with 228S produced complex fragment patterns (Fig. 2). These are highly discriminating of both unrelated and related individual genomes (fig. 2). The equality of a Taq 1 restriction for each sample was also checked by stripping each membrane of sequence 228S and reprobing with 142F. As long as digestion is complete these probes provide "invariate" banding patterns for each genome.

DISCUSSION

The very dilute alkali "Southern blot" method described gives good recovery and excellent binding of single stranded DNA to nylon membranes. The membranes do not appear to physically degrade under any of the treatments used, band resolution is good and is maintained through multiple probings. The results of Reed and Mann (1985) which demonstrated quantitative

recovery of DNA agarose gels could not be reproduced by us, the best efficiency being of the order of 70% for material in 20 kb to 0.5 kb range. It is possible that a more prolonged acid treatment of the gel would provide greater depurination of double stranded DNA, hence greater fragmentation and better transfer. However an anticipated limitation might be lower hybridisation efficiencies. This may explain the improved results obtained by our method of brief acid treatment, denaturation in situ (0.4 M NaOH) and Southern blotting in dilute alkali (0.02M NaOH or less). Too dilute alkali transfer might decrease the (covalent) binding of single stranded DNA to the membrane, although transfer solutions as dilute as 4 mM NaOH still provided very satisfactory binding.

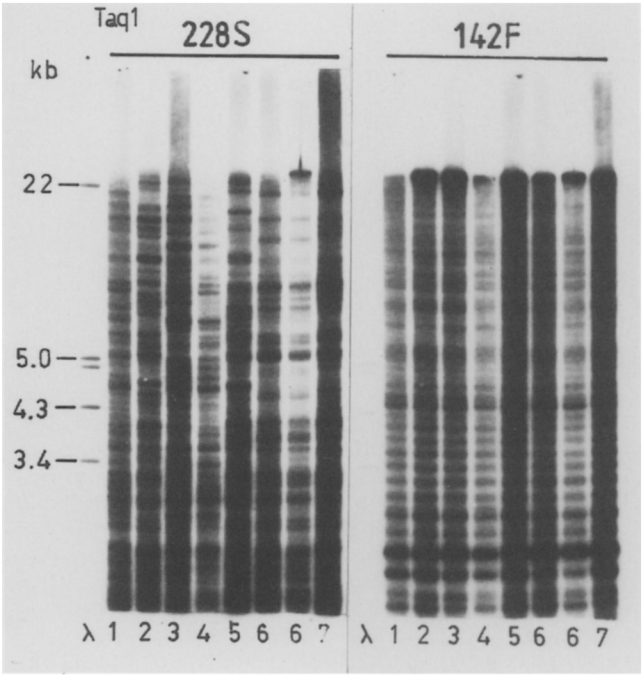

Fig. 2 Taq 1 analysis with probes 228S and 142F of individuals 1 - 7. Persons 1 - 4 are related, 5 - 7 are unrelated and duplicate 6 is sperm/hair tissue compared (3 ug DNA per sample).

The preparation of the hybridisation probe is particularly simple. The large size of the M-13/insert probe (approximately 7.5 kb including the insert 228S of 332 bp) does not seem to be inhibitory to hybridisation with either large or small DNA fragments attached to the membrane. Preliminary experimentation has shown that this probe design gave a ten-fold better autoradiographic signal when compared with a probe made by replication of the insert, restriction of the insert from the vector and using the insert in its denatured form. This improvement was consistent whether testing serial dilutions of either whole genomic DNA or size

selected DNA (approximately 500 bp). Inclusion of proteinase K in the wash procedure (Wash B) was found empirically to reduce background autoradiagraphic signals.

The probe 228S has been sequenced and classified by major sequence data banks as closely related to human Satellite III DNA. It is characterised by its high proportion of the tandem repeat TTCCA. The sequence 142F has been similarly examined and classified as homologous to human alphoid (centromeric) DNA.

The 3.4 kb Y specific fragment is believed to be composed of tandem repeats, with common and clustered EcoRl and HaeIII restriction sites, but where the intervening sequences are relatively diverged (Cooke et al, 1983). One of the sequences common to these repeats is Satellite III related (Beauchamp et al, 1979). It should be noted that it is the size of the repeat fragments which is considered to be the Y specific feature, though some short base sequences within any repeat may be male specific. The presence of the other Satellite III sequence related fragments detected in both male and female DNA provides an internal reference as to the quantity and quality of DNA under examination. This is of particular advantage in the analysis of forensic specimens. In such samples, the absence of a male specific response using a "sequence specific" probe (Tyler et al, 1986) could be explained by there either being insufficient DNA recovered or the sample being female DNA. The type of patterns produced here provide a means of resolving this ambiguity, paticularly if this evidence is supported by reprobing with other repetitive sequence probes. This advantage is especially important given that although the 3.4 kb fragment occurs in approximately 5000 copies on the Y chromosome of most males, the quantity of heterochromatin from which it is derived in the DYZl region is variable, and may even be absent in some "normal" males (Goodfellow et al, 1985). Other Y chromosome fragments have therefore been argued as more reliable indicators of sex (Goodfellow et al, 1983). However these are generally of far lower copy number, reducing their forensic usefulness. Provided results are presented with the qualifications outlined, the method here provides a very sensitive means of sexing human tissue in forensic samples.

The Taq 1 fragment patterns have the potential for forensic use in the discrimination of individuals, either from somatic or germ tissue. Again, the ability to reprobe the same samples with the centromeric probe 142F to establish the equality of the restriction is a particular advantage on forensic samples.

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