

## Blot hybridisation analysis of genomic DNA

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**SUMMARY** Restriction endonuclease analysis of specific gene sequences is proving to be a valuable technique for characterisation and diagnosis of inherited disorders. This paper describes detailed protocols for isolation, restriction, and blot hybridisation of genomic DNA. Problems and alternatives in the procedure are discussed and a troubleshooting guide has been provided to help rectify faults.

The development of techniques for the cloning<sup>1</sup> and analysis<sup>2</sup> of genes from complex organisms laid the foundation for the study of mutant genes associated with human inherited disorders. DNA from a person can now be cleaved into fragments of defined length by restriction endonucleases. The fragments are then separated by gel electrophoresis, blotted onto filters,<sup>2</sup> and incubated with radioactively labelled gene specific probes. These probes, obtained by molecular cloning techniques, are isolated and characterised DNA sequences which will associate specifically with homologous genomic DNA sequences on the filter. Thus, only fragments containing part or all of the gene of interest will be detected. This new recombinant DNA technology was rapidly applied to the molecular characterisation and antenatal diagnosis of the haemoglobinopathies and thalassaemia.<sup>3-7</sup> As cloning techniques have become more sophisticated, the number of purified, cloned human genes has proliferated to the extent that a recently published list<sup>8</sup> is already out of date. A considerable number of genetic diseases are therefore amenable to DNA analysis, and the use of linked restriction fragment length polymorphisms<sup>9,10</sup> has further extended the applicability of the technique.

These advances have brought DNA analysis within the scope of the clinical geneticist, and the techniques will ultimately become part of the routine service provided by human genetics departments. DNA blotting and molecular hybridisation

do, however, present a considerable technical challenge to workers new to the field. Furthermore, although standard protocols have been described,<sup>11,12</sup> they do not provide details of all aspects of genomic DNA analysis or a troubleshooting guide for the non-specialist.

In this paper, we present details of a protocol which works reproducibly in our hands and discuss some of the difficulties and alternatives with which the researcher may be confronted.

### Materials

#### CHEMICALS AND EQUIPMENT

##### *Chemicals*

Restriction enzymes were obtained from Bethesda Research Laboratories, Boehringer-Mannheim, and New England Biolabs. Bovine serum albumin, fraction V, and pancreatic ribonuclease A were purchased from Bayer-Miles. Sigma provided herring sperm DNA, polyadenylic acid, polyvinylpyrrolidone (type 360), and ficoll (type 400). Nitrocellulose (BA 85, 0.45  $\mu\text{m}$ ) was obtained from Schleicher and Schuell. Nuclease free bovine serum albumin, proteinase K, T4 DNA polymerase, crystalline redistilled phenol,  $\lambda$  DNA, and the nick translation kits were purchased from Bethesda Research Laboratories. Agarose (Seakem) was obtained from Marine Colloids. [ $\alpha^{32}\text{P}$ ]-deoxycytidine triphosphate (3000 Ci/mmol, 10 mCi/ml in a stabilised aqueous solution) was purchased from Amersham.

All chemicals not further described were of Analar (or equivalent) grade.

**Equipment**

A horizontal gel electrophoresis apparatus based on the design described by Southern<sup>2</sup> was used. The dimensions of the gel mould were 183 mm (width) × 170 mm (length). The teeth of the 'comb' or well former were 10 mm in width and 1 mm thick. The gel chamber and 'comb' were constructed of Perspex with platinum wire electrodes.

The hybridisation chamber was made of Perspex and designed by Alec Jeffreys (Leicester University) (see fig 1 for details).

Kodak X-Omatic x-ray cassettes with Fuji Mach 2 calcium tungstate intensifying screens were used. The films used were 'Cronex Safety' and Kodak X-Omat AR.

The transilluminator (model C62) was obtained from Ultra-violet Products Inc, San Gabriel, California.

**SOLUTIONS****(1) Cell lysis buffer**

320 mmol/l sucrose.

1% (v/v) triton X-100.

5 mmol/l MgCl<sub>2</sub>.

10 mmol/l Tris-HCl, pH 7.6.

**(2) Saline-EDTA (pH 8.0)**

25 mmol/l EDTA.

75 mmol/l NaCl.

**(3) Phenol:chloroform**

To a phenol:chloroform (1:1 v/v) mixture add 0.5 volume 1 × TE (solution 16). Store at 4°C in a light-tight bottle.

**(4) 10 × gel electrophoresis buffer**

0.89 mol/l Tris-borate.

0.89 mol/l boric acid.

0.02 mol/l EDTA.

(108 g Tris base, 55 g boric acid, and 40 ml 0.5 mol/l EDTA, pH 8.0, per litre H<sub>2</sub>O).

**(5) Ficoll-Orange G**

0.1% (w/v) Orange G

in 20% (w/v) ficoll.

10 mmol/l EDTA (pH 7.0).

**(6) Chloroform:octanol**

(24:1 v/v).

**(7) Restriction endonuclease buffer**

Prepare as a 10 × stock according to manufacturer's instructions.

**(8) Denaturation solution**

0.5 mol/l NaOH.

1.5 mol/l NaCl.

**(9) Neutralisation buffer**

0.5 mol/l Tris-HCl, pH 5.5.

3.0 mol/l NaCl.

0.3 mol/l sodium citrate.

**(10) 20 × SSC**

3.0 mol/l NaCl.

0.3 mol/l sodium citrate, pH 7.0.

**(11) 100 × Denhardt's**

2% (w/v) bovine serum albumin fraction V.

2% (w/v) polyvinylpyrrolidone type 360.

2% (w/v) Ficoll type 400.

Heat to 40°C while stirring, followed by gentle stirring at 4°C overnight.

**(12) Hybridisation solution**

3 × SSC.

10 × Denhardt's.

0.1% SDS (sodium dodecyl sulphate).

10 µg/ml polyadenylic acid.

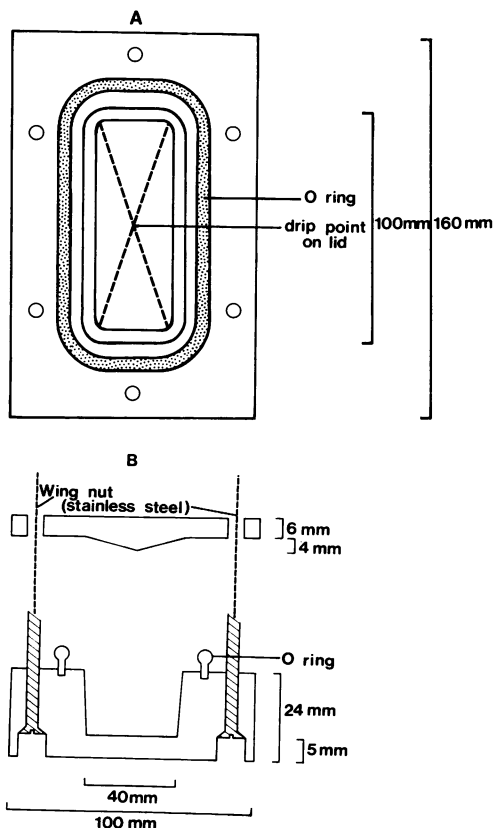


FIG 1 Diagram of the hybridisation chamber designed by Alec Jeffreys showing the plane (A) and elevation (B).

50 µg/ml heat denatured sonicated herring sperm DNA (see solution 14).

(13) *Post-hybridisation wash solution*

3 × SSC.

10 × Denhardt's.

0.1% sodium dodecyl sulphate.

(14) *Heat denatured sonicated herring sperm DNA*

2 mg/ml stock solution.

Dissolve and sonicate to an average length of approximately 600 base pairs. (Determine by agarose gel electrophoresis. See Method section on agarose gel electrophoresis of DNA restriction fragments.) Denature in boiling waterbath for 10 minutes. Cool rapidly on ice.

(15) *Stringent wash solution*

0.1% (w/v) sodium dodecyl sulphate.

0.1 × SSC (see discussion).

(16) *1 × TE*

10 mm Tris-HCl, pH 7.5.

1 mmol/l EDTA.

(17) *10 × T4 polymerase buffer*

330 mmol/l Tris-acetate, pH 7.9.

660 mmol/l potassium acetate.

100 mmol/l magnesium acetate.

1 mg/ml nuclease free bovine serum albumin.

5 mmol/l dithiothreitol.

The following stock solutions are also recommended:

- (a) 10% (w/v) sodium dodecyl sulphate.
- (b) 10 mg/ml proteinase K.
- (c) 10 mg/ml ribonuclease, heat treated at 80°C for 10 minutes.
- (d) 10 mg/ml ethidium bromide.
- (e) 0.1 mol/l EDTA, pH 7.0. Adjust pH in order to dissolve EDTA.
- (f) 5 mol/l sodium perchlorate.

## Methods

### PREPARATION OF GENOMIC DNA

Human DNA was isolated from lymphocytes using a procedure modified from Kunkel *et al.*<sup>13</sup>

A total of 10 ml of whole blood was collected in vacutainer tubes containing EDTA or citrate as anticoagulant and added to 60 ml of lysis buffer. This suspension was then gently homogenised in a Dounce homogeniser (5 strokes up and down). The nuclei were pelleted by centrifugation at 2500 *g* for 20 minutes at 4°C. The nuclear pellet was suspended in 8 ml of 25 mmol/l EDTA, 75 mmol/l NaCl, pH 8.0, using a sterile pipette. After the addition of 800 µl of 10% (w/v) sodium dodecyl sulphate and 0.1 ml of the 10 mg/ml proteinase K solution, the mixture was incubated for 2 hours at 37°C. A total

of 500 µl of a 5 mol/l sodium perchlorate solution was added. The digest was gently mixed with 8 ml of phenol:chloroform until homogeneous. The phases were separated by centrifugation for 10 minutes at 12 000 *g* at 10°C. The upper, aqueous, phase was removed and further extracted with an equal volume of chloroform:octanol (24:1). The phases were again separated after gentle mixing. DNA was precipitated from the aqueous phase by adding 2 volumes of cold absolute ethanol. The precipitate was lifted out with the sealed end of a Pasteur pipette and shaken into 1 × TE. The DNA was allowed to dissolve overnight at 4°C. A total of 0.1 volumes 20 × SSC and 0.01 volumes 5 mg/ml ribonuclease were added and the mixture incubated for 1 hour at 37°C. Then, 2 ml of sterile water was added and the solution was extracted twice with chloroform:octanol (24:1). The DNA was precipitated by adding 2 volumes of absolute ethanol and washed twice with 70% ethanol. The DNA pellet was dried under a vacuum for 15 minutes and finally dissolved in 0.5 ml of sterile double distilled water.

The DNA concentration is estimated by the determination of its absorbance at 260 nm assuming that its  $A_{1\text{cm}, 260}^{1\%}$  is 200 (that is, a 1 g/100 ml solution in a 1 cm lightpath has an absorbance at 260 nm of 200).<sup>14</sup>

### PREPARATION OF PROBE DNA

Details of the isolation of recombinant plasmids containing specific sequence probes are given by Maniatis *et al.*<sup>12</sup> Approval for these experiments by the Genetic Manipulation Advisory Group (GMAG) is required.

### RESTRICTION ENDONUCLEASE DIGESTION OF GENOMIC DNA

An incubation mixture with a final volume of 50 µl was prepared containing 10 µg DNA, 0.1 volume of 10 × restriction endonuclease buffer, 30 units restriction enzymes (3 U/µg DNA), and 100 µg/ml nuclease-free bovine serum albumin. This was then incubated for 16 hours at the temperature required for the enzyme used. The condensate was collected by centrifugation for 10 seconds in an Eppendorf microcentrifuge. Digestion was terminated by the addition of 0.1 volumes of a 100 mmol/l EDTA stock (pH 7.0).

A 5 µl aliquot was removed from each incubation mixture, mixed with 1 µl of Orange G-ficoll, and electrophoresed in order to determine whether digestion was complete (see Discussion).

### AGAROSE GEL ELECTROPHORESIS OF DNA RESTRICTION FRAGMENTS

Firstly, 0.6% agarose horizontal slab gels were

prepared by adding 1.2 g of agarose powder to 200 ml of  $1 \times$  electrophoresis buffer and boiling until completely dissolved. The solution was then cooled to  $65^{\circ}\text{C}$  and ethidium bromide added to a concentration of  $1 \mu\text{g}/\text{ml}$ . The molten agarose was poured into the gel mould with comb in place (0.5 mm off gel bed) and allowed to set at room temperature for 1 hour.

A total of  $5 \mu\text{l}$  ficoll-Orange G solution was added to each DNA digest. The samples were then applied to the gel and electrophoresed (in  $1 \times$  electrophoresis buffer, containing  $1 \mu\text{g}/\text{ml}$  ethidium bromide) at 30 V for 30 minutes at constant voltage. After the samples had entered the gel, it was submerged in electrophoresis buffer and the electrophoresis continued overnight. About 10 000 cpm of a radiolabelled DNA molecular weight marker (see T4 DNA labelling, Methods) was applied to one lane of the gel before electrophoresis.

After electrophoresis was completed (Orange G had migrated to end of gel), the gel was placed on a UV transilluminator and photographed, using an orange filter.

#### TRANSFER OF DNA FROM AGAROSE GEL ONTO NITROCELLULOSE

The original method as described by Southern<sup>2</sup> was used with a few modifications.

#### Pre-treatment of gel

The DNA in the gel was denatured by submersion in 300 ml denaturation buffer for 2 hours with gentle shaking. The gel was briefly rinsed with distilled

water and then neutralised by submerging in 300 ml neutralising buffer for 1 hour with gentle shaking.

#### DNA transfer

Details of the DNA transfer system are shown in fig 2.

A tray was filled with  $20 \times \text{SSC}$  and a glass plate was supported in the tray. A piece of Whatman 3M filter paper was draped over the glass plate with the sides in contact with the SSC solution. The top of the filter paper was also soaked with  $20 \times \text{SSC}$ . A piece of nitrocellulose was then cut to the size of the gel using a sterile blade. (Nitrocellulose must be handled with forceps or washed gloves.) The nitrocellulose was wet by flotation on  $2 \times \text{SSC}$  solution for 5 minutes. The pre-treated gel was then carefully slid onto the wet filter paper on the glass plate, taking care to avoid trapping air beneath it. The paper around the gel was then covered with a layer of waterproof film (for example, Saran wrap, cling film, etc). Excess liquid was removed from the gel surface and the soaked nitrocellulose was placed on the gel, taking care not to trap air beneath it.

Another two pieces of Whatman 3M paper were cut according to gel size and soaked in  $2 \times \text{SSC}$ . These were then placed on top of the nitrocellulose (making sure no air was trapped beneath). Dry absorbant paper was placed on top of the filter paper and compressed with weights ( $\pm 1 \text{ kg}$ ).

The time allowed for transfer was about 40 hours at  $4^{\circ}\text{C}$ . During this time the level of SSC in the tray was checked and the wet absorbant paper replaced by dry paper.

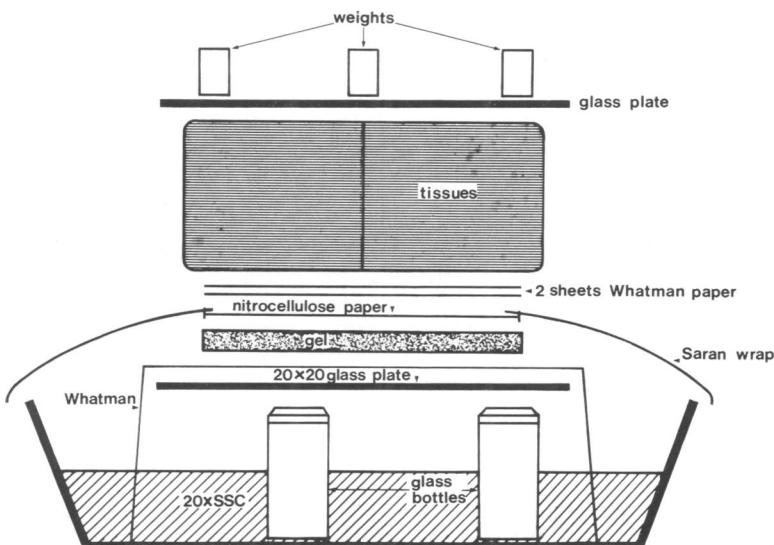


FIG 2 DNA transfer system. Strips of Saran wrap are suspended from the outer edges of the gel to the sides of the tray so that SSC is forced to move through the gel, and to prevent evaporation of the SSC.

After transfer was completed, the position and orientation of the lanes of DNA in the gel were marked on the cellulose nitrate sheet with a black waterproof marker pen. The sheet was then cut into rectangular strips (two lanes per strip) to fit into the hybridisation chamber. The filters were then soaked in  $2 \times \text{SSC}$  for 10 minutes and baked at  $80^\circ\text{C}$  for 2 hours. (The filters should not be allowed to adhere to each other during soaking.)

#### HYBRIDISATION

The protocol described by Jeffreys and Flavell<sup>15</sup> was used for washing and hybridisation of the filters.

#### *Pre-hybridisation washes*

The baked nitrocellulose strips were wet by flotation on a  $3 \times \text{SSC}$  solution at room temperature. The filters were then incubated with gentle shaking at  $65^\circ\text{C}$  for 30 minutes in 50 ml preheated  $3 \times \text{SSC}$ . The filters were then washed in 50 ml of a solution containing  $3 \times \text{SSC}$ ,  $10 \times \text{Denhardt's}$  (preheated to  $65^\circ\text{C}$ ) for 60 minutes. Finally, the filters were washed for 30 minutes at  $65^\circ\text{C}$  in 50 ml of preheated hybridisation solution.

#### *Hybridisation*

Hybridisation was carried out in a hybridisation chamber as described in the Materials section. A total of 10 ml of the hybridisation buffer was placed in the chamber together with the heat denatured radioactively labelled probe (see next section). The washed filters were carefully placed into this mixture and the unit sealed and incubated at  $65^\circ\text{C}$  with gentle shaking for 40 hours.

#### RADIOLABELLING OF DNA

##### *Nick translation of the DNA probe*

Nick translation<sup>16</sup> was performed with 100  $\mu\text{Ci}$  of  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  (specific activity of 3000 Ci/mmol, concentration 10 mCi/ml). The standard nick translation labelling protocol, supplied with the kit obtained from Bethesda Research Laboratories, was followed.

A total of 5  $\mu\text{l}$  of a solution containing 0.2 mmol/l dNTPs (dATP, dTTP, dGTP) was pipetted into a 1.5 ml microcentrifuge tube on ice. Then, 0.5  $\mu\text{g}$  of probe DNA and 100  $\mu\text{Ci}$  of radioactive nucleotide was added, the mixture was made up to a volume of 45  $\mu\text{l}$  with sterile distilled water, and 5  $\mu\text{l}$  of a mixture of the DNA polymerase I (0.4 U/ $\mu\text{l}$ ) and DNase I (40 pg/ $\mu\text{l}$ ) was added. The solution was mixed gently but thoroughly. It was centrifuged briefly (microfuge 15 000  $g$  for 5 seconds) and incubated at  $15^\circ\text{C}$  for 60 minutes. The reaction was stopped by the addition of 5  $\mu\text{l}$  Stop Buffer (300 mmol/l  $\text{Na}_2\text{EDTA}$ , pH 8.0) and extracted with an

equal volume of phenol:chloroform. The phenol phase was re-extracted with 100  $\mu\text{l}$   $1 \times \text{TE}$ . The labelled DNA in the pooled aqueous phases was then separated from the free nucleotides by chromatography on a small column of Sephadex G-50 (medium), poured in a Pasteur pipette, and equilibrated with  $3 \times \text{SSC}$ . The DNA was eluted with  $3 \times \text{SSC}$ . Twenty fractions (three drops) were collected and 1.0  $\mu\text{l}$  of each fraction was counted. The excluded peak containing the labelled DNA was pooled, an aliquot was counted, and the specific activity of the probe calculated.

##### *T4 DNA polymerase labelling of $\lambda\text{DNA}$*

$\lambda\text{DNA}$  was labelled with  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  for use as a DNA molecular weight marker. A total of 6  $\mu\text{g}$  of  $\lambda\text{DNA}$  was digested with 18 units of *Hind* III for 90 minutes at  $37^\circ\text{C}$  in an incubation volume of 60  $\mu\text{l}$  which contained 6  $\mu\text{l}$   $10 \times \text{T4}$  polymerase buffer. After incubation, 20  $\mu\text{l}$  was run on a 0.6% agarose gel to determine if digestion was complete.

Then, 2  $\mu\text{g}$  of the digested  $\lambda\text{DNA}$  (20  $\mu\text{l}$  of the *Hind* III digest) was added to 2.5 units of T4-DNA polymerase and incubated for 5 minutes at  $37^\circ\text{C}$ . After this, 1  $\mu\text{l}$  of a solution containing 2 mmol/l dGTP, 2 mmol/l dATP, 2 mmol/l dTTP, and 1  $\mu\text{Ci}$   $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  was added and incubated for 1 minute at  $37^\circ\text{C}$ . Then, 5  $\mu\text{l}$  cold 1 mM dCTP was added and incubated for 10 minutes at  $37^\circ\text{C}$ . The reaction was terminated by incubating for 5 minutes at  $70^\circ\text{C}$ . This mixture was then phenol extracted and the DNA isolated on a Sephadex G-50 (medium) column as described in the previous section.

A total of  $1 \times 10^4$  cpm of  $^{32}\text{P}$ -labelled *Hind* III digested  $\lambda\text{DNA}$  was used as a DNA molecular weight marker (see fig 3).

#### POST-HYBRIDISATION WASHES

After hybridisation, the filters were washed in post-hybridisation buffer; 300 ml of post-hybridisation buffer preheated to  $65^\circ\text{C}$  was divided equally among six containers. The filters were then washed for 1 minute at  $65^\circ\text{C}$  in each of four containers, then washed for 30 minutes each in the remaining two containers. The filters were finally given  $2 \times 30$  minutes stringency washes at  $65^\circ\text{C}$  in 50 ml of stringency buffer. The wet filters were then aligned and heat sealed within a plastic bag.

The filters and x-ray film were placed in a cassette between two intensifying screens and the film was exposed at  $-70^\circ\text{C}$  for 1 to 14 days.

#### RE-USE OF FILTERS

Used filters can be rehybridised to a second probe after removal of the original probe with NaOH. Filters were soaked in denaturation solution

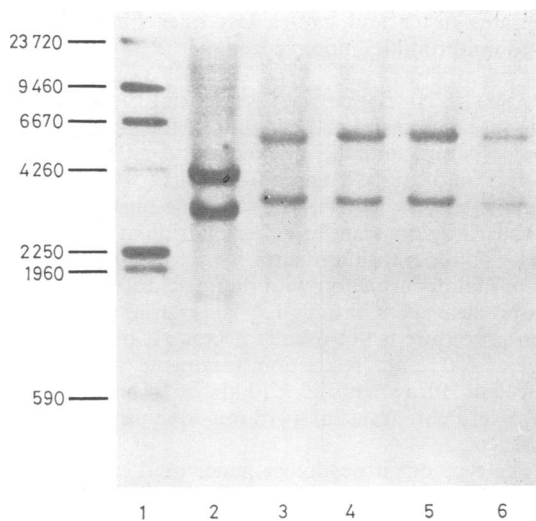


FIG 3 Detection of specific genomic sequences by blot hybridisation. DNA was digested with restriction enzymes, electrophoresed on 0.6% agarose gel, blotted, and hybridised with a  $\text{pro } \alpha_2(I)$  collagen cDNA probe.<sup>17</sup> Lane 1. <sup>32</sup>P-labelled  $\lambda$  molecular weight marker. Lanes 2-6.  $\text{Pro } \alpha_2(I)$  collagen sequences. (This result was obtained from an overnight exposure.)

(solution 8) for 5 minutes, neutralisation buffer (solution 9) for 2 hours, and finally  $3 \times \text{SSC}$  for 15 minutes. They were then baked at 80°C for 2 hours before being prehybridised in the usual way.

### Discussion

We will discuss each step in the procedure with regard to possible problems and alternatives.

### CHEMICALS

It is probable that chemicals from suppliers other than those mentioned can be used. However, this should be established in controlled experiments before a chemical from a different supplier is used routinely.

### PREPARATION OF GENOMIC DNA

Whole blood is a convenient source of DNA. If the blood is collected in EDTA, which inactivates endogenous nucleases, it can be stored at 4°C for at least 7 days before isolation of the DNA. White blood cells prepared from buffy coats or ficoll gradients can be stored in physiological saline at -70°C for several years.

Permanent cell lines of patients can be obtained by transformation of lymphocytes with Epstein-Barr

virus.<sup>18</sup> Transformed cells can be stored in liquid nitrogen, thus providing a constantly renewable source of DNA.

The yield of DNA obtained will depend on the white cell count of the patient, but is generally 150 to 500  $\mu\text{g}$  per 10 ml of whole blood. An absorbance scan of the DNA (220 to 300 nm) should be run to determine whether impurities such as phenol or proteins are present (see fig 4). DNA samples contaminated with phenol should be re-extracted with  $\text{CHCl}_3$ . Protein can be removed by repeating the phenol and chloroform extractions. The DNA solution can be stored at -70°C or -20°C.

### RESTRICTION ENDONUCLEASE DIGESTION

It is important to obtain complete digestion of the DNA by the restriction enzyme. Partial digests will produce spurious high molecular weight DNA fragments. The completeness of digestion should be checked by electrophoresis of an aliquot of the digest. Examples of complete and partial digests are shown in fig 5. Partial digests can also be obtained if enzyme action is affected by methylation of the DNA (fig 5). In this case an enzyme which recognises the same sequence, but which is not affected by methylation, should be used.

### AGAROSE GEL ELECTROPHORESIS

A variety of designs of apparatus are suitable for electrophoresis of the DNA. Horizontal slab gels are convenient for gels of low agarose concentration. A number of such apparatuses are commercially available (for example, BRL, Bio-Rad) but they can easily be constructed by hospital or university

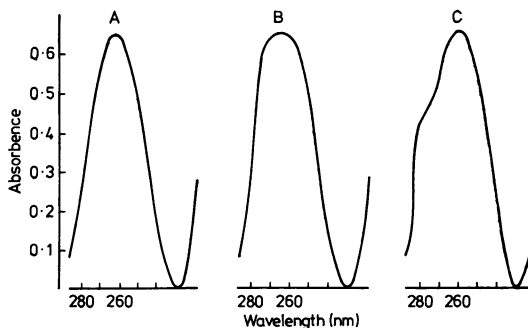


FIG 4 Absorbance spectrum of human DNA. A. Spectrum of uncontaminated DNA showing absorbance maximum at 260 nm. B. Phenol contaminated DNA showing broadening of the peak as a result of absorption by phenol at 270 nm. C. Protein contaminated DNA showing protein absorbance shoulder at 280 nm.

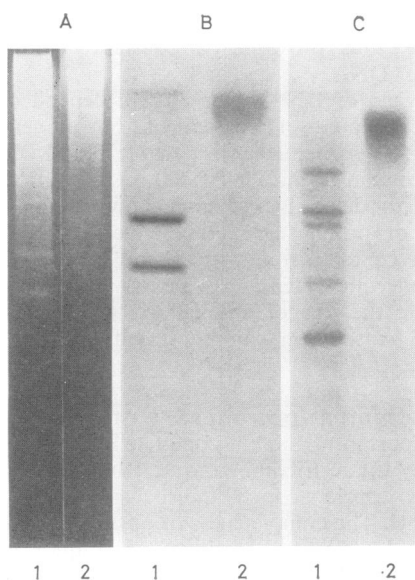


FIG 5 Restriction endonuclease digestion of human genomic DNA. A. Ethidium bromide staining of *EcoRI* digests. Lane 1 shows discrete bands of highly repeated DNA sequences indicative of complete digestion. Lane 2 shows DNA partially digested by *EcoRI*. B. Hybridisation of the *EcoRI* digested DNA in A with a collagen  $\text{pro } \alpha_2(\text{I})$  cDNA probe.<sup>17</sup> C. Partial digestion of genomic DNA as a result of methylation. Lane 1. DNA digested with *Msp I* showing complete digestion and the correct fragment pattern. Lane 2. DNA from the same person digested with *Hpa II* which recognises the same base sequence as *Msp I*, but will not cut if the internal cytosine of this sequence is methylated.

workshops. Gels should be at least 150 mm in length to ensure accurate measurements of DNA fragment sizes and high resolution of the bands. Resolution will also be improved by electrophoresis at a low voltage for 12 to 16 hours rather than at a high voltage for a shorter period. Changes of pH which occur as a result of electrophoresis can be minimised by using a gel tank which holds a larger volume of buffer (2 to 3 l). Alternatively, the buffer can be circulated through the cathode and anode compartments.

Glycerol or sucrose are often added to the restriction digests before loading on the gel to increase the density of the solution. However, these low molecular weight solutes cause streaming of the sample up the side of the well, which leads to the production of U shaped DNA bands. Ficoll avoids this effect. Bromophenol blue or Orange G can be used as a sample marker dye, but Orange G

migrates faster and causes less quenching of the ethidium bromide fluorescence.<sup>11</sup>

#### TRANSFER OF DNA ONTO NITROCELLULOSE

The rate of transfer of DNA out of the gel depends on DNA size and the thickness and agarose concentration of the gel. Large fragments (>10 kb) are transferred very slowly. They may be broken down in the gel before transfer either by irradiation of the DNA on the transilluminator for 5 to 10 minutes or by partial depurination with dilute acid followed by strand cleavage with alkali.<sup>19</sup> The partial depurination procedure is not usually necessary, but may be included if large restriction fragments are to be detected. If fragments of <10 kb are to be detected, an overnight transfer (without depurination) is sufficient.

The type of nitrocellulose paper used can significantly influence the sensitivity of detection of DNA fragments. We have found that Schleicher and Schuell nitrocellulose binds DNA more efficiently than several other brands.

Once the filters have been baked after transfer, they can be stored for several months at 4°C before hybridisation with the DNA probe.

#### HYBRIDISATION OF PROBE TO FILTERS

Hybridisation can be carried out in a perspex box (fig 1) or a sealed plastic bag. We have found that the slight inconvenience of cutting up the filters to fit the hybridisation box is more than adequately compensated by the lack of background signal obtained. The filters should be well covered by the probe solution during hybridisation and should not be allowed to dry out until after the final stringent washes have been done.

The time required for adequate hybridisation depends on the concentration and sequence complexity of the probe, the temperature of hybridisation, and the salt concentration of the probe solution.<sup>12 20</sup> An overnight hybridisation is sufficient to detect single copy genomic sequences using the conditions described here (see methods), provided that the specific activity of the probe is not less than  $1 \times 10^8$  cpm/ $\mu\text{g}$  DNA. However, we routinely hybridise for about 40 hours.

Sequence specific probes are generally recombinant plasmids containing complementary DNA (cDNA) or genomic DNA sequences. Before using such a probe for the first time it is important to establish that it contains the desired insert. This can be done by comparing its electrophoretic mobility with that of the parental plasmid and checking that it produces the expected fragment sizes after digestion with one or two restriction enzymes. Either the entire recombinant plasmid can be

TABLE Troubleshooting guide.

Problem	Possible causes	Remedy
Atypical DNA scan DNA appears degraded on gel	Phenol or protein contamination Blood kept too long before DNA extracted Nuclease contamination of DNA, buffers, or enzyme	Re-extract DNA with $\text{CHCl}_3$ or phenol and $\text{CHCl}_3$ Check undigested DNA for degradation If degraded, obtain fresh blood If not, use fresh buffer and enzyme
DNA not digested	Faulty buffer or restriction enzyme Impurities in DNA	Check enzyme and buffer with $\lambda$ DNA Re-extract DNA
Incomplete DNA transfer	No Saran wrap between filter paper wick and nitrocellulose Air trapped between gel and filter	Repeat transfer Increase transfer time Pre-treat gel with HCl
High background signal in DNA lanes	Post-hybridisation wash stringency too low Probe contains repeat sequences	Increase stringency of final washes Change probe
High background all over filters	Inadequate pre-hybridisation Handling filters without gloves Drying out of filters in contact with probe	Repeat stringency washes If inadequate, remove probe with NaOH and rehybridise
No bands detected on autoradiograph	DNA not binding to nitrocellulose  No insert in plasmid probe  Inadequate sensitivity of detection Incorrect hybridisation or wash solutions Specific activity of probe too low Probe too short after nick translation Probe not denatured before hybridisation	Include $^{32}\text{P}$ -labelled $\lambda$ DNA on gel If not detected, change nitrocellulose and make fresh $20 \times \text{SSC}$ Electrophorese 20–100 pg of probe on one lane of gel If signal obtained, prepare and characterise new probe Remake solutions Repeat nick translation with fresh $^{32}\text{P}$ dNTP or fresh enzyme Reduce DNase concentration in nick translation Denature probe

labelled, or the insert cut out and purified by preparative gel electrophoresis. The latter approach has the advantage that spurious restriction fragments resulting from contamination of human DNA samples with plasmid DNA would not be detected. If a particular probe consistently produces high background signals, a new stock of the recombinant plasmid should be prepared from a fresh culture.

After hybridisation the filters should be given high stringency (low salt concentration) washes in order to remove non-specifically bound probe and to reduce hybridisation to other related gene sequences.<sup>20</sup> We use  $0.1 \times \text{SSC}$  washes when hybridising with a probe containing genomic DNA and  $0.1$  to  $0.25 \times \text{SSC}$  for cDNA probes.

When a new probe is being used, a series of washes can be carried out to establish a suitable stringency.

#### AUTORADIOGRAPHY

The conditions for autoradiography and the types of x-ray film and intensifying screens available have been reviewed.<sup>21</sup> We have found Kodak X-Omat AR, Fuji RX, and Cronex Safety films to be suitable, used with Dupont Cronex Lightning Plus or Fuji Mach 2 calcium tungstate intensifying screens. Note that exposure at  $-70^\circ\text{C}$  is more efficient than at  $-20^\circ\text{C}$ .

The length of exposure required to detect specific sequences will depend on factors such as the specific activity of the probe and the amount of unique sequence DNA on the filter. Using the conditions

described here, faint bands can be detected after an overnight exposure, whereas intense bands can be seen after exposures of 3 to 7 days.

#### Conclusion

If the protocol described in this review is followed carefully, results such as that illustrated in fig 3 should be obtained routinely. However, problems do occur, especially when first setting up this technique. A troubleshooting guide is given in the table to assist in diagnosing and rectifying the problem.

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### Correction

In the Review article 'The fragile X syndrome: the patients and their chromosomes' by De Arce and Kearns published in the April 1984 issue of the journal (vol 21, pp 84-91), the sentence starting 11 lines from the bottom of p 87, column 2, should read: "From the work of Sutherland<sup>42</sup> and others,<sup>58 61 62</sup> we know that TC 199 induces the expression of certain fragile sites and non-specific gaps on C group autosomes in normal controls".