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(54) **Polynucleotide probes**

(57) A method for the assessment of sample DNA fragmentation which comprises detecting the presence or absence of a control fragment formed, during sample DNA cleavage, after one or more of the DNA fragments to be characterised. Polynucleotides and polynucleotide probes for use in the method as well as control kits comprising these. In particular the method of the invention is useful as a digestion control in methods of genetic characterisation.

Probes binding to sequence 5'-ACATGGCAGG (AGGGCAGG)$_n$ TGGAGGG-3' where n= 1 or 2 are claimed.

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This print takes account of replacement documents submitted after the date of filing to enable the application to comply with the formal requirements of the Patents Rules 1982.

FIG.1

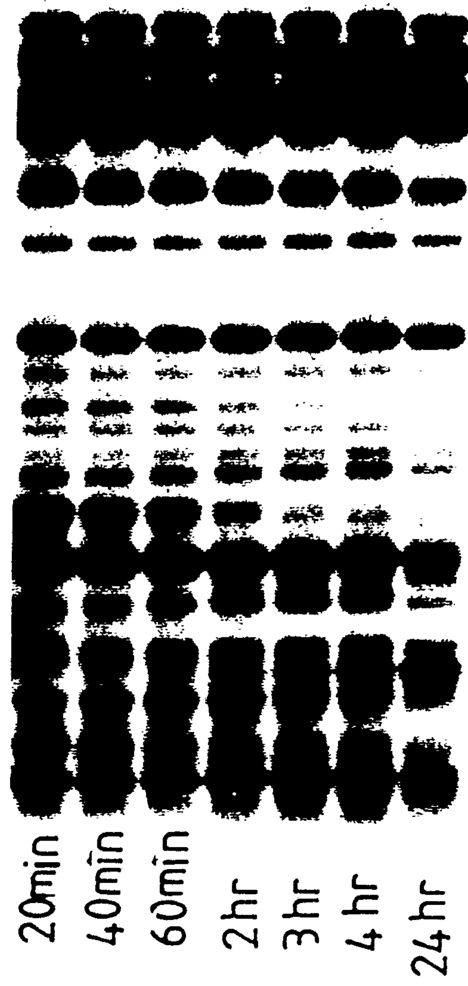


Fig.2

MS 51

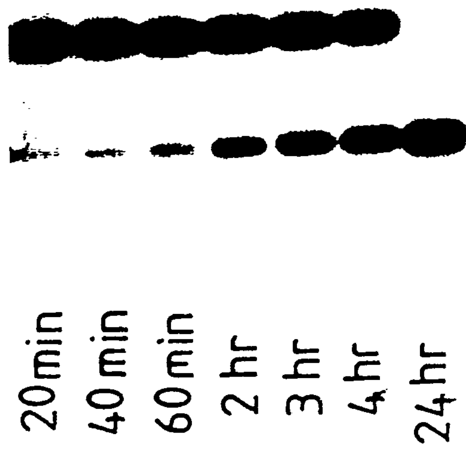
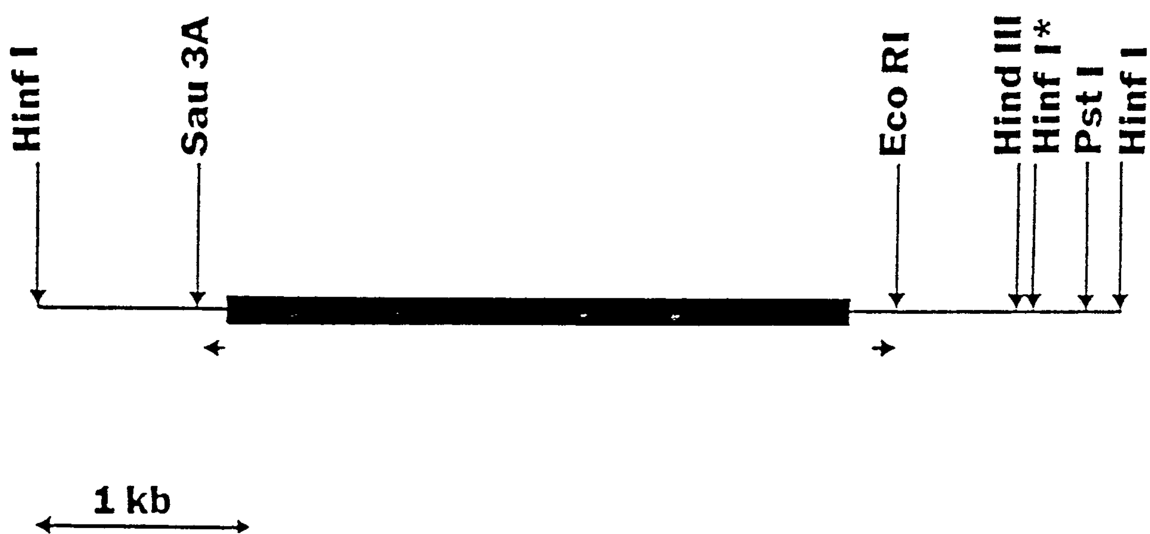


Fig.3

G3

20 min
40 min
60 min
2 hr
3 hr
4 hr
24 hr

Fig 4



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POLYNUCLEOTIDE PROBES

This invention relates to a method for the assessment of sample DNA fragmentation as well as to polynucleotides and polynucleotide probes for use in the method. In particular the method of the invention is useful as a digestion control in methods of genetic characterisation.

Analytical methods have been developed to investigate the size and/or composition of DNA fragments. In particular methods have been developed where the DNA fragments are rendered single stranded for hybridisation to probes specific for one or more genetic loci. The probes are conveniently labelled for identification purposes. The probe-fragment hybrids thus obtained may be detected and characterised, for example according to size, using known techniques such as gel electrophoresis. Autoradiography is a convenient method for the identification of radiolabelled probe-fragment hybrids.

Sample DNA fragmentation is achieved by various methods, including sonication and chemical means. Commonly however the sample DNA is cleaved enzymatically, for example by restriction enzymes or endonucleases. Restriction endonucleases are capable of recognising target nucleotide sequences in DNA and are capable of cleaving the DNA in a predictable manner in relation to the target sequence. Such endonucleases may therefore serve as useful analytical tools. Enzymic fragmentation of DNA is often termed digestion.

The human genome has already been investigated to identify genetic loci which are informative. Particular interest has been shown in

polymorphic or hypervariable loci. The total number of such loci is unknown but is likely to be large. Indeed the human genome might contain at least 1500 such loci. It has been shown that certain of these loci are useful in methods of genetic characterisation. In UK Patent No. 2166445 (Lister Institute of Preventive Medicine) there are disclosed probes capable of characterising genomic DNA by reference to more than one informative genetic locus. Probes which are capable of characterising genomic DNA by reference to a single informative locus have also been developed and are disclosed in our European Patent Application, Publication No. 238329.

A problem often associated with analytical methods outlined above and involving the preparation of DNA fragments is that the extent of sample DNA fragmentation cannot be readily assessed or estimated without full knowledge of all the relevant parameters. These may include the power of the fragmentation means, for example enzyme concentration, DNA sample concentration, buffer concentration and temperature. It is therefore often necessary to allow a long period of time to elapse before complete or sufficient DNA fragmentation can be assumed. Such delays introduce a degree of inefficiency, especially where many analytical tests are being performed. Moreover it is desirable that all informative DNA fragments, for example all DNA fragments resulting from complete digestion of the sample DNA with one or more restriction enzymes, are produced prior to characterisation so that the maximum information which may be gained from the fragments, for example their size and/or composition can be obtained.

The present invention is based on the discovery that certain informative loci in genomic DNA, useful for example in genetic characterisation methods, are also associated with regions of DNA resistant to fragmentation. The identification of fragments containing such loci may therefore be used to provide a DNA fragmentation control in analytical methods.

Therefore according to one feature of the present invention we provide a method for the assessment of sample DNA fragmentation which comprises detecting the presence of a control fragment formed, during sample DNA cleavage, after the formation of one or more of the DNA fragments to be characterised.

Sample DNA fragmentation is effected by any convenient cleavage means, such as for example, sonication or chemical means but is preferably effected using one or more enzymes. Thus the expression "sample DNA fragmentation" and "sample DNA cleavage" are used synonymously. Examples of convenient enzymes for use in the present invention include restriction enzymes such as restriction endonucleases. The method of the present invention has been found to be particularly useful as a digestion control where the restriction enzyme Hinf I is used.

Complete DNA fragmentation is not essential for the performance of the invention. It is only necessary that the DNA control fragment is formed after the fragments to be characterised. The control fragment can therefore be selected to detect any desired degree of fragmentation, such as for example up to 50%, up to 60%, up to 70%, up to 80% or up to 90% fragmentation. However, conveniently, the control fragment is chosen so that the control fragment indicates complete sample DNA fragmentation. The sample DNA is conveniently genomic DNA, such as for example human DNA.

A control fragment may be of any convenient size or composition provided that it is present within a fragmentation resistant locus. It will be understood that the expression "fragmentation resistant locus" refers to a locus more resistant to fragmentation than one or more loci containing the DNA fragments to be characterised. A control fragment may, for example, be identified by cleaving DNA, for example using restriction enzymes and monitoring the formation of DNA

fragments. Fragments comprising a fragmentation resistant locus will only disappear later in the fragmentation process whereby convenient control fragment(s) will be formed. The appearance of a control fragment may be detected by any convenient means for the detection of DNA fragments. Once a control fragment has been identified, it is not necessary to constantly monitor the DNA fragmentation process. Conveniently a sufficient degree of fragmentation is estimated based on empirical observations and the control fragment is then used to confirm the validity of the estimation.

The method of the present invention is believed to be useful in any situation where the extent of sample DNA fragmentation requires assessment, for example where sample DNA fragments are characterised according to their size and/or composition. The method is particularly useful in methods of genetic characterisation.

It has already been demonstrated that minisatellite regions or hypervariable loci in genomic DNA may be used in methods of genetic characterisation. In UK Patent No. 2166445 (Lister Institute of Preventive Medicine) there are disclosed methods and probes for characterising genomic DNA by reference to more than one minisatellite region or hypervariable locus. In addition our European Patent Application, Publication No. 238329 discloses methods and probes for characterising genomic DNA by reference to individual minisatellite regions or hypervariable loci.

The method of the present invention is particularly useful where sample genomic DNA is characterised using probes for minisatellite regions or hypervariable loci. The provision of a fragmentation control allows an even greater degree of certainty when interpreting results, for example in paternity and forensic cases.

Convenient minisatellite probes include the multilocus probes 33.6 and 33.15 claimed in UK Patent No. 2166445 (Lister Institute of

Preventive Medicine) and the single locus probes p lambda g3, MS1, MS8, MS31, MS 32 and MS43 claimed in our European Patent Application, Publication No. 238329.

The detection of a control fragment is conveniently effected using polynucleotides or polynucleotide probes capable of hybridisation to the control fragment. Where the control fragment includes a minisatellite region or hypervariable locus the polynucleotide or polynucleotide probe conveniently hybridises to the said region or locus.

The control fragment is preferably identified by probing sample DNA fragments with a minisatellite probe to detect a single minisatellite region or hypervariable locus. The terms "minisatellite region" and "hypervariable locus" are used synonymously. The minisatellite probe can be considered as a control probe. This allows the formation of a control fragment to be more easily monitored since for example hybridisation filters are readily reprobed with the control probe.

Polynucleotides of DNA, RNA and of any other kind hybridisable to DNA can be used. The polynucleotides are unlabelled and can be in double stranded (ds) or in single stranded (ss) form. Generally at least the nucleotide sequence of the polynucleotide will be in single stranded form, but preferably the probe will comprise the polynucleotide wholly in single stranded form. Polynucleotides for use in the method of the present invention are conveniently prepared by microbiological reproduction of cloned material or by direct synthesis.

Polynucleotide probes may be obtained by labelling or marking polynucleotides. Labelled polynucleotides in ss-form can be used as probes as well as their labelled precursors, from which ss-probes can be produced. The polynucleotide probes used in the present invention are preferably radiolabelled, for example with ^{32}P or with ^{35}S, for

example in any conventional way. The probes may alternatively be labelled by non-radioactive means well known in the hybridisation art, for example with fluorescent groups or they may be labelled with biotin or derivatives thereof.

It will be appreciated that where polynucleotide or polynucleotide probes capable of hybridisation to polymorphic or hypervariable loci are used and restriction enzymes are used to cleave the sample DNA then it is desirable that the probe/enzyme combination selected is such that enzymic cleavage of the DNA does not interfere with probe/fragment hybridisation.

Digestion resistance has been observed adjacent to the minisatellite region termed MS51. This primarily affects Hinf I restriction endonuclease cleavage adjacent this minisatellite. Whilst we do not wish to be bound by theoretical considerations it is believed that fragmentation resistance, for example digestion resistance, may result from changes in the tertiary structure of the DNA. These alternative structures can be caused for example by a particular sequence of nucleotides, by environmental conditions (pH, ionic strength) or long range interactions. The properties of such alternative structures have been reviewed by R.D. Wells and S.C. Harvey in "Unusual DNA Structures" (1988, Springer Verlag). Changes to the tertiary structure may affect the ability of, for example a restriction enzyme to recognise a nucleic acid binding site and/or its ability to cleave nucleic acid. Nucleotide sequences of interest include those which are involved in, for example intra-strand hybridisation and in particular non Watson Crick pairing. Such nucleotide sequences may for example be G-rich. The term "G-rich" can for example refer to nucleotide sequences which comprise at least 50%, at least 60%, at least 70%, at least 80% or at least 90% guanine residues. Such changes in tertiary structure are conveniently illustrated in the paper of V.I. Lyamichev et al in Nature, 339, (1989), 634-637. A further possible structure change may involve the

(1989), 634-637. A further possible structure change may involve the formation of triple strands, for example homopyrimidine nucleic acid. This is conveniently illustrated in the paper of P. Rajagopal et al in Nature, 339, (1989), 637-640.

Fragments which comprise the MS51 minisatellite, and in particular Hinf I fragments may therefore be useful as control fragments in the method of the present invention. It will of course be appreciated that the MS51 minisatellite is not only useful in the assessment of sample DNA fragmentation but also for example as a single locus probe in methods of genetic characterisation for example as disclosed in our European Patent Application, Publication No. 238329.

The MS51 minisatellite is specifically identifiable by the nucleotide sequence referred to hereinafter as pMS51 namely 5'-ACATGGCAGG$(AGGGCAGG)_n$TGGAGGG-3' where n=1 or 2, and tandem repeats thereof. Any convenient number of repeats may be present.

Therefore in a further aspect of the present invention we provide a polynucleotide which selectively hybridises to a sample DNA fragment specifically identifiable by the nucleotide sequence 5'-ACATGGCAGG$(AGGGCAGG)_n$TGGAGGG-3' where n=1 or 2 and tandem repeats thereof. The sample DNA fragment conveniently results from restriction enzyme cleavage, for example Hinf I cleavage, of sample DNA. It will be appreciated that the tandem repeats are not necessarily perfect repeats of the given sequence provided that a reasonable degree of sequence homology is present.

In a preferred aspect of the present invention there is provided a polynucleotide which comprises the nucleotide sequence given directly above and tandem repeats thereof and sequences complementary thereto or any convenient part thereof for example of at least 6, at least 8 or preferably at least 10 nucleotides. The polynucleotide more preferably comprises the nucleotide sequence given above and any

convenient number of repeats thereof.

The invention also relates to probes which conveniently comprise any one of the above polynucleotides together with a label or marker component to facilitate the detection thereof.

The present invention also relates to a fragmentation control kit for the detection of a control fragment. The kit conveniently includes a minisatellite probe, which selectively hybridises to a control fragment formed during sample DNA digestion with Hinf I after the formation of one or more of the DNA fragments to be characterised. At least one of the following items is also present in the kit: appropriate buffer, packaging and instructions for use. If required, the kit also includes the restriction enzyme Hinf I.

In a preferred aspect of the present invention the control kit comprises a polynucleotide probe which selectively hybridises to a sample DNA fragment specifically identifiable by the nucleotide sequence 5′-ACATGGCAGG(AGGGCAGG)$_n$TGGAGGG-3′ where n=1 or 2 and tandem repeats thereof. The sample DNA fragment conveniently results from restriction enzyme cleavage, for example Hinf I cleavage, of sample DNA.

In a more preferred aspect of the present invention the control kit includes a polynucleotide probe which comprises the nucleotide sequence given directly above and tandem repeats thereof and sequences complementary thereto. The means for sample DNA fragmentation are preferably a restriction enzyme and more preferably Hinf I.

The invention will now be illustrated but not limited by reference to the accompanying Figures and Example and wherein:

Figure 1 shows the results of partial digestion of a genomic DNA

sample. The sample was digested for 20 mins, 40 mins, 60 mins, 1hr, 2hrs, 4hrs, and 24hrs respectively.
The bands clearly show how the DNA fingerprint changes with time.

Figure 2 shows the results of probing the same DNA sample with pMS51. The bands clearly show the resistance of MS51 alleles to digestion and the changing pattern of the bands.

Figure 3 shows the results of probing the same DNA sample with the probe p lambda g3. It can be seen that the g3 alleles do not show any appreciable degree of fragmentation resistance and that the bands rapidly stabilise. It should be noted that only one allele is visible using this sample.

Figure 4 shows the location of the Eco RI, Hind III, Hinf I, Pst I and Sau 3A restriction sites adjacent the MS51 minisatellite locus.

In the following Examples DNA isolation, restriction enzyme digestion, electrophoresis and Southern blotting were performed as follows:

DNA isolation

DNA was isolated from whole blood of a human donor (AFM16). White blood cells were separated from 20 mls of blood and washed once in 1xSSC (20xSSC is 3.0M NaCl/0.3M tri-sodium citrate).
The cells were then lysed in 3.75 ml of 0.2M sodium acetate pH 7.0, 100µl 10mg/ml proteinase-K (Boehringer-from Tritirachium album), 250ul 10% SDS at 56°C for 3 hours. The lysate was then extracted with phenol/chloroform (25 parts phenol: 24 parts chloroform: 1 part isoamyl alcohol) and once with chloroform (24 parts chloroform: 1 part isoamyl alcohol).
The DNA was spooled out after addition of 2 volumes of 100% ethanol and resolvated in 0.2M sodium acetate. It was then precipitated with 100% ethanol and rinsed in 80% ethanol, then dried. Finally the DNA

was fully resolvated in 10mM Tris pH8/ 1mM EDTA buffer.

Restriction Enzyme Digestion

The concentration of DNA was estimated by fluorimetry on an aliquot of HinfI digested stock DNA using a Hoeffer T120-100 DNA fluorimeter. 30ug of high molecular weight DNA extracted from AFM16 blood was digested in a volume of 140µl with HinfI enzyme (supplied by BCL at 20 units/µl). 140 units of HinfI enzyme were used and incubated at 37°C, mixing at 10 minute intervals for the first hour of incubation.

20µl aliquots were sampled into 1µl of 100mM EDTA pH8 and frozen at -20°C after 20, 40 and 60 minutes as well as after 2, 3, 4, and 24 hours.

Electrophoresis

A 0.7% 20cm x 25cm agarose gel (Sigma-Low EEO Type I) was prepared in 1xTBE (134mM Tris, 74.9mM H_3BO_3, 2.5mM Na_2EDTA pH8) electrophoresis buffer. The HinfI restricted DNA samples were loaded onto the gel together with molecular weight markers and 0.5µg of pBR322 plasmid DNA (Anglian Biotechnology, 2mg/ml) digested with BglI restriction enzyme (supplied by Anglian Biotechnology at 10 units/µl). A constant voltage of 75 volts is applied across the agarose gel and the 2.3 kilobase molecular weight marker from the pBR322 BglI DNA is allowed to run to 18.5 cms from the origin.

Southern Blotting

The gel was depurinated in 0.25M HCl for 15 minutes, denatured in 0.5M NaOH/1.5M NaCl for 30 minutes and neutralised in 0.5M Tris pH7.5, 3.0M NaCl for 30 minutes. The DNA was transferred to hybond-N (Amersham) by Southern blotting and fixed by uv. irradiation.

Example 1

Probe Labelling and Hybridisation

80ng 33.15 probe (prepared according to the methods described in UK patent 2166445) was labelled with alpha ^{32}P dGTP (DuPont-3000 Ci/mMol in 10mM Tricine) by primer extension.

36µl of buffer B is added to 24µl of 33.15 buffer and boiled for 5 minutes, then cooled on ice for 10 minutes. 16µl of buffer C was then added together with 20µl alpha ^{32}P dGTP and 4µl of Klenow enzyme (BCL sequencing grade-

5 units/µl). The reaction is incubated at room temperature for three hours, this is enough probe for one hybridisation chamber (a 21cm x 21cm perspex sealable box).

A NICK column (Parmacia-Sephadex G-50 column) is equilibrated with 10 ml of column buffer (20mM Tris pH 7.5, 20mM NaCl, 2mM EDTA, 0.25% SDS). The probe reaction mix is passed through the column, 0.4 ml of column buffer is then added and allowed to run to waste, a second 0.4 ml column buffer is added and this is collected and used as the purified probe.

The hybond-N filter is wetted in 1xSSC and prehybridised in 1x Denhardts (0.2% BSA (Sigma fraction V), 0.2% Ficoll 400, 0.2% polyvinyl pyrolidone (average molecular weight of 40000, 1xSSC) for 60 minutes at 62°C.

0.5 ml of 1mg/ml sheared human placental DNA (sheared in 0.3M NaOH, 20mM EDTA at 100°C for 5 minutes then neutralised with HCl) is added to the probe to be used per hybridisation chamber and boiled for 7 minutes, then cooled on ice for 10 minutes.

The probe is added to 160 ml CHFM (6% polyethylene glycol 6000 (1x Denhardts, 0.1% SDS, 6% polyethylene glycol 6000) in a prehybridisation chamber. The filter is transferred from the prehybridisation solution and incubated at 62°C for 16 hours, gently shaking.

The filter is washed in 400 ml of 1xSSC, 25µg/ml herring sperm DNA (Sigma), 0.1% SDS at 62°C for 30 minutes, repeated four times. It is then rinsed in 3xSSC and air dried briefly before wrapping in saran wrap. It is then placed in a light proof cassette with a Curix MR 800 intensifying screen (Agfa) and exposed to X-ray sensitive film

for 10 days at -70°C.

Buffer B: 44µl buffer A (1.21M Tris pH8.0, 0.12M $MgCl_2$, 0.25M 2-mercaptoethanol), 110µl 2M Hepes pH 6.6, 242µl MilliQ H_2O (Millipore-reagent grade water system), aliquoted and stored at -20oC.

33.15 buffer: 20µl Diag 25 7.7µm (custom prepared oligonucleotides), 4ul 20ng/ul 33.15 insert.

Buffer C: 44ul 1mM dATP, 44ul 1mM dCTP, 44ul 1mM TTP and 44ul 10mg/ml BSA, aliquoted and stored at -20°C (dATP, dCTP, TTP -Pharmacia 100mM solutions).

Example 2.

Probe Labelling and Hybridisation

20ng of the probe MS51 (the insert sequence of which is outlined in this specification) were labelled by random oligonucleotide priming (Feinberg and Vogelstein, 1984, Anal. Biochem, 137, pages 266-267). The probe was boiled for 5 minutes and then cooled on ice for 10 minutes. 10µl of oligolabelling buffer was added together with 4µl 5mg/ml bovine serum albumin (BRL), 7µl alpha ^{32}P dGTP (DuPont) and 2ul Klenow enzyme (Boehringer-sequencing grade at 5units/µl). The reaction volume is made up to 50µl by adding MilliQ H_2O, mixed and incubated at room temperature for three hours. The probe is purified through a NICK column as outline in Example 1 above. The hybond-N filter was striped of the 33.15 probe of Example 1 by washing in 0.4m NaOH at 45°C for 30 minutes, then neutralised in 0.1xSSC, 0.1xSDS, 0.2M Tris pH 7.5 for 30 minutes at 45°C.
The filter was then rinsed in 1xSSC and prehybridised at 65°C for 20 minutes in 0.1% bovine serum albumin (Bethesda Research

Laboratories-nucleic acid enzyme grade), 1mM EDTA, 7% SDS, 0.5M sodium pyrophosphate pH 7.2 [Church and Gilbert, 1984, P.N.A.S., USA, 81, pages 1991-1995].
The purified probe was boiled for 5 minutes with 0.5 ml 1mg/ml sheared human placental DNA, then cooled on ice for 10 minutes, and added to 160 ml of 1xSSC, 6% polyethylene glycol 6000. The filter was transferred from hybridisation solution and incubated at 65°C for 16 hours. After hybridisation, filters were washed at 65°C in 40mM sodium phosphate, 0.1% SDS (pH7.2) for 15 minutes, followed by two 15 minute washes at 65°C in 0.5x SSC, 0.01% SDS and finally two 15 minute washes at 65°C in 0.2x SSC, 0.01 SDS. The filter was then rinsed in 3xSSC, briefly air dried and wrapped in saran wrap for autoradiograpy as described in Example 1, but with 1-2 days exposure.

Example 3

The materials and methods were exactly as described for Example 2 except that the probe p lambda g3 (prepared according to the methods disclosed in European Patent Application, Publication No. 238329) was used in place of pMS51. The sequence of p lambda g3 is AGGAATAGAAAGGCGGGYGGTGTGGGCAGGGAGRGGC (wherein Y is C, T or U, R is A or G and T is T or U) and tandem repeats thereof.

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CLAIMS FOR USE IN: GB

1. A method for the assessment of sample DNA fragmentation which comprises detecting the presence of a control fragment formed, during sample DNA cleavage, after the formation of one or more of the DNA fragments to be characterised.

2. A method as claimed in claim 1 wherein sample DNA is fragmented by enzymic digestion.

3. A method as claimed in claim 1 or claim 2 wherein the control fragment indicates complete sample DNA fragmentation.

4. A method as claimed in any one of claims 1-3 wherein the control fragment is detected using a minisatellite probe.

5. A method as claimed in claim 4 wherein the minisatellite probe is used to detect a single minisatellite region or hypervariable locus.

6. A method as claimed in claim 5 wherein the minisatellite probe selectively hybridises to a sample DNA fragment specifically identifiable by the nucleotide sequence 5'-ACATGGCAGG(AGGGCAGG)$_n$TGGAGGG-3' where n=1 or 2 and tandem repeats thereof.

7. A method as claimed in any one of claims 1-6 wherein the restriction enzyme Hinf I is used to cleave the sample DNA.

8. A polynucleotide which selectively hybridises to a sample DNA fragment specifically identifiable by the nucleotide sequence 5'-ACATGGCAGG(AGGGCAGG)$_n$TGGAGGG-3' where n=1 or 2 and tandem repeats thereof.

9. A polynucleotide as claimed in claim 8 together with a label or marker component.

10. A polynucleotide probe which comprises the nucleotide sequence 5′-ACATGGCAGG(AGGGCAGG)$_n$TGGAGGG-3′ where n=1 or 2 and tandem repeats thereof.

11. A control kit which comprises a minisatellite probe which selectively hybridises to a control fragment formed during sample DNA digestion with Hinf I after the formation of one or more of the DNA fragments to be characterised and at least one of buffer, packaging and instructions for use.

12. A control kit as claimed in claim 11 wherein the minisatellite probe comprises the nucleotide sequences.
5′-ACATGGCAGG(AGGGCAGG)$_n$TGGAGGG-3′ where n=1 or 2 and tandem repeats thereof.

13. A method for the assessment of sample DNA fragmentation substantially as hereinbefore described, with reference to the accompanying figures and example.

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