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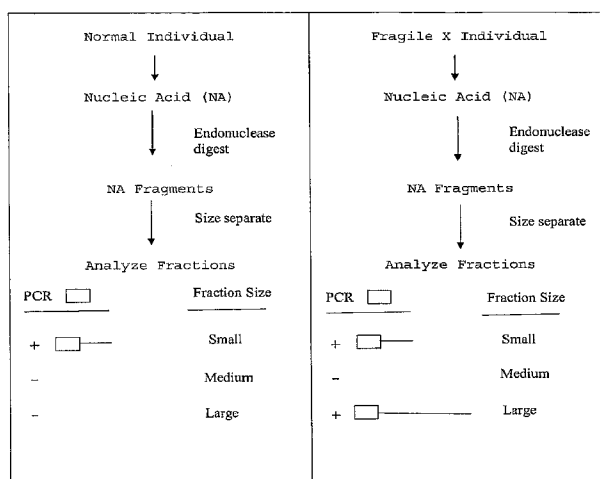
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(54) Title: NUCLEIC ACID SIZE DETECTION METHOD



□ = flanking marker segment

—] = Tandem Repeat Segment

(57) Abstract: The present invention provides methods of determining the size of a particular nucleic acid segment of interest in a sample of nucleic acids through fragmentation of DNA, size fractionation, an optional second fragmentation, and identification using a marker sequence. In particular aspects, an expansion or reduction of tandem repeat sequences can be detected. In further aspects, carriers and individuals afflicted with fragile X syndrome or other diseases associated with tandem repeats can be distinguished from normal individuals.

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NUCLEIC ACID SIZE DETECTION METHOD

FIELD OF THE INVENTION

[0001] The present invention relates generally to the field of medical diagnostics. In particular, the present invention relates to methods of detecting genetic mutations characterized by an expansion of tandem repeats.

BACKGROUND

[0002] A tandem repeat in DNA represents two or more contiguous approximate copies of a pattern of nucleotides. Tandem repeats have been shown to be associated cause a variety of human diseases. Dramatic expansion of trinucleotide repeats has been associated with such diseases as fragile-X mental retardation (see Verkerk, et al., (1991) Cell, 65, 905-914), Huntington's disease (see Huntington's Disease Collaborative Research Group. (1993) Cell, 72, 971-983), myotonic dystrophy (see Fu, et al., (1992) Science, 255, 1256-1258), spinal and bulbar muscular atrophy (see La Spada, et al., (1991) Nature, 352, 77-79) and Friedreich's ataxia (see Campuzano, et al., (1996) Science, 271, 1423-1427).

[0003] Fragile X syndrome is one of the most common causes of inherited mental retardation, occurring in approximately one in 1,250 males and approximately one in 2,500 females. Males with fragile X syndrome typically exhibit some degree of mental impairment, ranging from learning disabilities to mental retardation to autism. Characteristic physical features (e.g., enlarged ears, elongated face with prominent chin), connective tissue problems (e.g., mitral valve prolapse, and double-jointed fingers), and characteristic behaviors (e.g., attention deficit disorders, speech disturbances, and unusual responses to various touch, auditory, or visual stimuli) may also be exhibited. Affected females present with similar but milder mental impairment, physical characteristics, and behavioral characteristics as those of affected males.

[0004] The mutation responsible for fragile X syndrome involves expansion of a trinucleotide (CGG) tandem repeat sequence located in the 5' untranslated region of the FMR1 gene on the X chromosome. The number of CGG repeats in the FMR1 gene determines whether an individual is normal or has one of the two categories of mutation:

premutation and full mutation. The number of repeats ranges from less than 55 repeats in normal, non-carrier individuals, whereas a premutation consists of 55 to 200 repeats and full mutation consists of more than 200 repeats (Chen et al. Hum. Mol. Genetics 12(23):3067-74, 2003).

[0005] Both males having a premutation and females having a premutation in one FMR1 gene are carriers but are unaffected. Male carriers are referred to as “normal transmitting” males, and pass on the mutation, relatively unchanged in size to each daughter. Although such daughters are unaffected, they are at risk of having affected offspring because a premutation is susceptible to expansion after passage through a female meiosis. Furthermore, the larger the premutation, the higher the risk of expansion to a full mutation in any offspring.

[0006] Most males with a full mutation exhibit mental retardation and stereotypical physical and behavioral characteristics. For females with a full mutation in one FMR1 gene, about one-third exhibit normal intelligence, about one-third exhibit borderline intelligence, and about one-third exhibit mental retardation.

[0007] Currently, the industry standard for screening for carriers or affected individuals with expansion of tandem repeat regions such as Fragile X is a combination of PCR amplification of the tandem repeat region and analysis by Southern blotting.

SUMMARY OF THE INVENTION

[0008] In one aspect of the invention, there are provided methods of determining the size of a particular nucleic acid segment of interest in a sample of nucleic acids. This method is accomplished by separating fragments of a nucleic acid, wherein the fragments are prepared from a nucleic acid-containing sample, wherein the fragments include some which contain the segment and a marker sequence, wherein separating is into fractions according to size under conditions in which a fragment containing the segment will be located in the fractions according to the size of the segment, and identifying those fraction(s) containing the segment by detecting the marker sequence, wherein the size of the segment is determined by the fraction in which it is identified.

[0009] This method is applicable to essentially any nucleic acid segment of interest, however the method is particularly amenable to determining the size of nucleic acid segments

which are, for example, difficult to size by methods utilizing amplification (e.g. PCR) across the nucleic acid segment. Examples of nucleic acid segments which are difficult to size by methods utilizing amplification across the nucleic acid segment include nucleic acid segments which have high content of the bases guanine and cytosine, large nucleic acid segments, and/or segments having large numbers of tandem repeats. In a preferred embodiment, the particular nucleic acid segment of interest is a tandem repeat nucleic acid sequence. A length of nucleic acid which is difficult to amplify by PCR is generally greater than 50,000 bases, more typically greater than 100,000 bases, more typically greater than 150,000 bases, or more than 200,000 bases, or even more than 250,000 bases.

[0010] In another aspects, invention methods are used to determine the size of a particular nucleic acid segment in a sample from an individual, thereby determining if that individual has an abnormality in size of that particular nucleic acid segment, wherein the abnormality is due to a duplication, addition, or deletion in the particular nucleic acid segment.

[0011] In still another aspect, the invention provides a method of detecting a mutation in a tandem repeat segment of a gene in a nucleic acid sample, wherein the mutation is characterized by an increase in the number of repeats compared to the number of repeats in the wild type allele. The method is accomplished separating fragments of a nucleic acid, wherein the fragments are prepared from a nucleic acid-containing sample, wherein the fragments include some which contain the tandem repeat segment and a marker sequence, wherein the separating is into fractions according to size under conditions in which a fragment containing the tandem repeat segment will be located in the fractions according to the number of repeats in the tandem repeat segment; and identifying those fraction(s) containing the segment by detecting the marker sequence. The number of repeats in the tandem repeat segment is determined by the fraction in which it is identified. The number of repeats is compared to the number in the corresponding wild type allele, wherein a number of repeats greater than the number in wild type allele is indicative of a mutation.

[0012] In certain embodiments, the above aspect of the invention further includes determining if a mutation is a premutation or a full mutation. This determination is accomplished by comparing the number of repeats in the tandem repeat segment from the nucleic acid sample to the number in the corresponding full mutation allele, wherein a number of repeats greater than the wild type allele but less than the full mutation is indicative

of a premutation allele, and a number of repeats greater than or equal to the full mutation is indicative of a full mutation allele. In other embodiments the number of repeats in the tandem repeat region of the nucleic acid sample can be compared to the number of repeats found in each of a wild type allele, a premutation allele, and a full mutation allele.

[0013] In particular embodiments of the above aspect of the invention there are provided methods of identifying FMR1 alleles having a normal number of tandem repeats, a premutation, or a full mutation in the nucleic acid of an individual, in which the method includes,

fragmenting the nucleic acid in the sample from the individual into fragments, wherein the tandem repeat segment of the FMR1 gene is associated with a marker sequence in the fragment,

separating fragments of a nucleic acid, wherein the fragments are prepared from a nucleic acid containing sample of the individual, wherein the fragments include some which contain a tandem repeat segment of the FMR1 gene and a marker sequence, the separating into fractions according to size under conditions in which a fragment containing the tandem repeat segment having a normal number of repeats will be located in a first fraction; and a fragment containing a tandem repeat segment having a premutation will be located in a second fraction; and a fragment having a tandem repeat region having a full mutation will be located in a third fraction,

identifying those fraction(s) containing the segment by detecting the marker sequence, wherein the number of repeats in the tandem repeat segment is determined by the fraction in which it is identified, wherein

a positive result in the first fraction indicates the individual has an FMR1 allele with a normal number of tandem repeats;

a positive result in the second fraction indicates the individual has a premutation FMR1 allele; and

a positive result in the third fraction indicates the individual has a full mutation FMR1 allele.

[0014] In other aspects, there are provided methods for detecting carriers of genetic mutations characterized by the expansion or reduction of a tandem repeat segment of a gene and diagnosing individuals afflicted with diseases caused by such an expansion. The method

involves the detection of wild type alleles, premutation alleles and/or full mutation alleles for a particular gene as described above. A genotype may then be determined based on the allele(s) present in an individual, allowing the designation of normal, carrier, or affected status.

[0015] As used herein a “carrier” is an individual who carries an mutated or altered allele of a gene but is not affected by the disorder or disease associated with mutation. Carriers can pass the mutation to a child or offspring in future generations, who may be affected with the disease or disorder. With respect to Fragile X syndrome, both males and females may be carriers. As used herein in reference to males, the term “carrier” is used interchangeably with “premutation carrier” and refers to males having a premutation allele. As used herein in reference to females, the term carrier encompasses females having a premutation allele or a full mutation allele. Such female carriers may also be referred to herein as “premutation carrier” (i.e., having a premutation FMR1 allele) or a “full mutation carrier” (i.e., having a full mutation FMR1 allele).

[0016] As used herein “affected” refers to individuals who possess one or more mutated alleles of a particular gene and exhibit the disease or disorder (i.e., phenotype) associated that mutation. With respect to Fragile X syndrome, males having a full mutation FMR1 allele are affected, whereas females having a single full mutation allele may be affected or may be a full mutation carrier.

[0017] In some embodiments of the above aspect of the invention, male individuals afflicted with Fragile X syndrome (full mutation) can be distinguished from individuals that are carriers (premutation) or from those that are normal. A nucleic acid sample from the individual is fragmented to produce nucleic acid fragments in which the tandem repeat segment of the FMR1 gene is associated with a marker sequence in a fragment. The fragments are separated into fractions according to size under conditions in which the fragment(s) containing the tandem repeat segment will be located in the fractions according to the number of repeats in the tandem repeat segment, and identifying those fraction(s) containing the segment by detecting the marker sequence. In some embodiments, the fractions are chosen so that the first fraction captures fragments having a number of repeats within the range of repeats for a normal allele; the second fraction captures fragments having a number of repeats within the range of repeats for a premutation allele; and the third fraction

captures fragments having a number of repeats within the range of repeats for a full mutation allele. In accordance with current research in the field of fragile X, the range of repeats for a normal normal allele of the FMR1 gene is less than 55 repeats; the range of repeats for a premutation allele is 55-200; and the range of repeats for a full mutation allele is greater than 200 repeats. These ranges may be +/- 10%. These ranges may change over time as new studies are conducted and more is learned about the correlation of number of repeats and disease status. Since males generally have a single X-chromosome (which is where the FMR1 gene resides), only one fraction should be positive for the marker sequence. Therefore, males can be assigned a phenotype, based on the genotype according to the following: if the first fraction is positive for the marker sequence, the individual is normal; if the second fraction is positive for the marker sequence, the individual is a carrier; if only the third fraction is positive for the marker sequence, the individual is affected with fragile X.

[0018] In another aspect, there are provided methods for screening male and female individuals for carrier status of mutations in the tandem repeat region of the FMR1 gene, the method includes

assaying nucleic acids from an individual to determine gender; and

assaying the nucleic acid to determine the length of the tandem repeat region of the FMR1 gene wherein the determining comprises

amplifying tandem repeat region,

detecting an amplification product, and

determining the number of tandem repeats in the amplification product,

wherein,

in male individuals:

the presence of an amplification product having less than 55 tandem repeats indicates the individual is not a carrier,

the presence of an amplification product having 55 or more tandem repeats indicates the individual is a carrier, or

in the absence of an amplification product, the carrier status is undetermined;

and

in female individuals:

the presence of an amplification product having more than 55 tandem repeats indicates the individual is a carrier; or

the presence of a single amplification product having less than 55 tandem repeats, the carrier status is undetermined.

[0019] In certain embodiments, the above method further includes, analyzing undetermined individuals to determine carrier status, wherein the analyzing includes, separating fragments of a nucleic acid, wherein the fragments are prepared from a nucleic acid containing sample of the individual, wherein the fragments include some which contain a tandem repeat segment of the FMR1 gene and a marker sequence, the separating into fractions according to size under conditions in which a fragment containing the tandem repeat segment having a normal number of repeats will be located in a first fraction; and a fragment containing a tandem repeat segment having a premutation will be located in a second fraction; and a fragment having a tandem repeat region having a full mutation will be located in a third fraction,

identifying those fraction(s) containing the segment by detecting the marker sequence, wherein the number of repeats in the tandem repeat segment is determined by the fraction in which it is identified, wherein

in male individuals:

a positive result in the first fraction indicates the individual is not a carrier,

a positive result in the second fraction indicates the individual is a premutation carrier,

a positive result in the third fraction indicates the individual is affected; and

in female individuals:

a positive result in only the first fraction indicates the individual is homozygous for the a normal allele;

a positive result in the second fraction indicates the individual is a premutation carrier; and

a positive result in the third fraction indicates the individual is a full mutation carrier.

[0020] In preferred embodiments of the above method, the assaying of nucleic acids to determine gender includes amplification of a region of the nucleic acid, preferably by PCR. For example, sequences specific to the Y chromosome, such as the SRY locus may be targeted for amplification. In this case, amplification only occurs in the presence of a Y

chromosome. (See Sinclair A. H., et al., Nature 346:240 244 (1990)). In other examples, certain genes which occur on both the X chromosome and the Y chromosome may be detected for gender determination, if the lengths of the corresponding genes are different on each chromosome. Thus, amplification results in different sized amplicons having lengths specific to either the X or the Y chromosome. In this case, amplification of nucleic acids from males would result in both amplicons, whereas samples from females would have only one amplicon. Examples of such genes include DXZ1 and DYZ1 and the amelogenin gene. In more preferred embodiments, the assaying to determine gender includes

amplifying a region of the amelogenin gene which produces different sizes of amplification products from the amelogenin gene on the X chromosome and the amelogenin gene on the Y chromosome,

determining the size of the amplification product or products, wherein the presence of one product of a single size indicates the gender is female and the presence of two products of different sizes indicates the gender is male.

[0021] In still further embodiments, the assay to determine gender is performed in multiplex with the amplification of the tandem repeat region; preferably in multiplex PCR; preferably one or more internal controls are included in the multiplex reaction. In preferred embodiments, a region of the androgen insensitivity gene is amplified as an internal control.

[0022] In a preferred embodiment of the above aspect of the invention, a sample containing genomic DNA is assayed for an expansion in the tandem repeat region of the FMR1 gene. Thus, genomic samples are subjected to nucleic acid fragmentation and the resulting nucleic acid fragments are separated by size into fractions. A marker sequence upstream or downstream of the tandem repeat region of the FMR1 gene, which is associated with the tandem repeat region in the fragmented nucleic acid, is amplified by polymerase chain reaction. In some embodiments the amplification of the marker sequence and detection of the amplicon is done using the TaqMan system. In other embodiments the marker sequence is amplified and using a labeled primer and the resulting labeled amplicon is detected using capillary electrophoresis.

[0023] One of skill in the art would readily recognize that the separation of fragments into fractions by size can be modified so that the fractions correspond to either a normal number of tandem repeats or an abnormal number of tandem repeats. In some embodiments,

the fragmented nucleic acid may be separated by size into two fractions, an upper fraction of larger size fragments and a lower fraction of smaller size fragments. In this approach, the fractions are designed such that fragments from nucleic acid containing a normal number of tandem repeats will be found in the lower fraction while fragments from nucleic acid containing an abnormally increased number of tandem repeats will be found in the upper fraction. In other embodiments, the fragmented DNA may be separated into any number of fractions. In some embodiment the fragmented DNA may be separated into a number of fractions selected from the group consisting of 2-16, preferably 3 fractions, or 4 fractions, or 5 fractions, or 6 fractions, or 8 fractions, or even 16 fractions.

[0024] In a preferred embodiment of the above method, the fragmented DNA is separated into lower and upper fractions, wherein the lower fraction corresponds to a tandem repeat region containing less than 55 repeats (normal number of repeats) and an upper fraction containing 55 or more tandem repeats (premutation and full mutation). A normal allele can therefore be distinguished from a premutation or a full mutation.

[0025] In another preferred embodiment of the above method, the fragmented DNA is separated into three fractions, wherein a first fraction corresponds to a tandem repeat region of a normal allele (i.e., less than 55 repeats), a second fraction corresponds to a tandem repeat region of a premutation allele (i.e. 55-200 repeats), a third fraction corresponds to a tandem repeat region of a full mutation allele (i.e., greater than 200 repeats).

[0026] In yet another preferred embodiment of the above method, the fragmented DNA is separated into four fractions, wherein a first fraction corresponds to a tandem repeat region of a normal allele, a second fraction corresponds to a tandem repeat region of a small premutation allele, a third fraction corresponds to a tandem repeat region of a large premutation allele, and a fourth fraction corresponds to a tandem repeat region of a full mutation allele. In particular embodiments, the first fraction corresponds to 0-60 repeats; preferably the second fraction corresponds to 60-200 repeats; preferably the third fraction corresponds to 200-2000 repeats; and preferably the fourth fraction corresponds to 2000+ repeats. In another embodiment, the DNA is fragmented with *BlpI* and *MlyI* and fractionated such that the first fraction corresponds to 6-62 repeats; preferably the second fraction corresponds to 63-140 repeats; preferably the third fraction corresponds to 141-220 repeats; and preferably the fourth fraction corresponds to 221-2000+ repeats. In another embodiment,

the DNA is fragmented with AluI and fractionated such that the first fraction corresponds to 6-68 repeats; preferably the second fraction corresponds to 69-102 repeats; preferably the third fraction corresponds to 102-202 repeats; and preferably the fourth fraction corresponds to 203+ repeats. In yet another embodiment, the DNA is fragmented with SphI and BmtI and fractionated such that the first fraction corresponds to 6-62 repeats; preferably the second fraction corresponds to 63-163 repeats; preferably the third fraction corresponds to 164-196 repeats; and preferably the fourth fraction corresponds to 197+ repeats.

[0027] Also provided are methods of estimating the number of tandem repeats in a sample of genomic DNA. In this method, test samples containing genomic DNA are subjected to nucleic acid fragmentation. The resulting nucleic acid fragments are separated by size into three or more size range fractions, and fragments containing tandem repeat segments in the various fractions are identified by detecting a marker sequence flanking the tandem repeat segment, which is associated with the tandem repeat region in the fragmented nucleic acid. The size of the tandem repeat region detected is then determined by relating the fraction size containing the repeat to the size of a tandem repeat segment present in such nucleic acid fragments. Separation into three or more size range fractions allows a finer estimation of the number of tandem repeats. In the case of fragile X, the extent of the expansion of a premutation or full mutation can be assessed.

[0028] In preferred embodiments of the above aspects of the invention, the method includes a second nucleic acid fragmentation. Preferably the second fragmentation occurs after the size separation, which follows the first nucleic acid fragmentation; preferably the second fragmentation is by restriction enzyme digestion. In more preferred embodiments, the second fragmentation cleaves the particular nucleic acid segment of interest (e.g., a tandem repeat segment) from a marker sequence flanking the particular nucleic acid segment. Preferably the second nucleic acid fragmentation does not cleave within the marker sequence.

[0029] In some embodiments of the above aspects of invention, the marker sequence is detected by amplification of all or a portion of the marker sequence and detection of the amplicon. In preferred embodiments, the marker sequence is amplified by PCR and the amplicon is detected by electrophoresis. In more preferred embodiments, a primer used in the PCR amplification reaction comprises a label, thereby labeling the resulting amplicon. The so-labeled amplicon can then be detected by methods such as capillary electrophoresis.

[0030] In other embodiments of the above aspects of the invention, the marker sequence is detected using real time PCR methods such as the TaqMan system. In this approach a probe is used to detect the amplified region of the marker sequence.

[0031] In still other embodiments of the above aspects of the invention, the marker sequence need not be amplified and can be detected directly by hybridization to two differentially labeled oligonucleotide probes. The two probes, which hybridize to distinct segments of a marker sequence, such that both probes can bind simultaneously, are contacted with the fragmented nucleic acids under hybridization conditions. The simultaneous detection of differentially labeled probes hybridized to a single nucleic acid fragment in the fractions indicates the presence of a tandem repeat region in a fragment contained in that fraction. The two oligonucleotide probes of this embodiment may be designed to hybridize to segments of a marker sequence upstream or downstream of the tandem repeat. These segments of the marker sequence may be adjoining the tandem repeat region or may be a distance upstream or downstream. In preferred embodiments, the segments of the marker sequence are within 500 bases upstream or downstream of the tandem repeat region; in more preferred embodiments the segments of the marker sequence are within 250 bases upstream or downstream of the tandem repeat region; in most preferred embodiments the segments of the marker sequence are within 100 bases upstream or downstream of the tandem repeat region. The probes may be designed to hybridize to the same or to opposite strands of a double-stranded marker sequence. The probes may both hybridize upstream or both downstream of the tandem repeat. Alternatively, one probe may hybridize upstream of the tandem repeats whereas the other probe hybridizes downstream. The probes may hybridize to segments of the marker sequence that are separated by zero bases to several hundred thousand bases provided both segments are located on the same contiguous nucleic acid molecule after the fragmentation step or steps. Preferably the probes are separated by less than 1 kb, or preferably less than 500 bases, or less than 300 bases, or less than 200 bases, or less than 100 bases, or less than 50 bases, or less than 20 bases, or less than 10 bases, or less than 5 bases, or 1 base, or 0 bases.

[0032] In another aspect of the invention, there are provided methods of determining the size of a particular nucleic acid segment in a sample of nucleic acids, wherein size is determined using information obtained using a first method and a second method. Thus, the

method comprises measuring the size of a tandem repeat segment by a first method, measuring the size of the tandem repeat segment by a second method, and using the information obtained by the first and second methods to determine the size of the tandem repeat region. In some embodiments, the first method includes an amplification of the tandem repeat region, preferably the amplification is by PCR. In preferred embodiments, the PCR amplification includes a labeled primer. In other preferred embodiments the amplicon is subjected to electrophoresis, preferably capillary electrophoresis, and the size of the amplicon is determined by comparison to a standard run in parallel. In other embodiments the first method includes Southern blotting. In preferred embodiments the second method comprises, fragmenting the nucleic acids of the sample, separating the fragmented nucleic acids into fractions according to size, and detecting a marker sequence upstream or downstream of the particular nucleic acid segment of interest, wherein the marker sequence is associated with the particular nucleic acid segment of interest in the fragmented nucleic acid. The size of the particular nucleic acid segment of interest is then determined by relating the fraction size containing the particular nucleic acid segment to the size of the particular nucleic acid segment in the sample of nucleic acids. In certain embodiments, the first method is used as an initial screen and samples for which the size of the particular nucleic acid segment is unable to be determined by this method are further analyzed by the second method. In other embodiments, sizing by one of the above two methods is used to confirm the results of the sizing by the other of the above two methods. In still other embodiments, sizing by the first method is used for finely determining the size of the particular nucleic acid fragment.

[0033] In another aspect of the invention there are provided primers for amplification of marker sequences flanking the tandem repeat region of the FMR1 gene. In particular embodiments, the primers are selected from the group consisting of SEQ ID NOs:4-9

[0034] In yet another aspect of the invention, there are provided kits for detecting the size of a particular nucleic acid segment in a sample comprising a primer pair for amplifying a marker nucleotide sequence upstream or downstream of the particular nucleic acid segment, and one or more restriction endonucleases for cleaving the nucleic acid sample to generate a fragment of the nucleic acid sample which contains the particular nucleic acid segment and the upstream or downstream marker sequence. In certain embodiments the kit further comprises one or more restriction endonucleases for cleaving the particular nucleic acid

segment from the marker nucleotide sequence. In still other embodiments, the kit may further contain one or more controls for verifying proper size separation of fragments; preferably the control consists of one or more primer pairs that are used to amplify one or more control fragments from the size-separated nucleic acid sample. In further embodiments, the kit may further contain one or more controls for verifying the completion of the one or more enzyme digests; preferably the control consists of one or more primer pairs designed to amplify a control fragment that includes a recognition site for the enzyme used. The kit may further contain any necessary buffers or other reagents.

[0035] In preferred embodiments of the above aspect of the invention, the particular nucleic acid segment contains a tandem repeat segment. In more preferred embodiments, the kit is for the detection of the tandem repeat segment of the FMR1 gene; preferably the enzyme for generating fragments of the nucleic acid sample is *AluI*; preferably the enzyme for cleaving the marker sequence from the tandem repeat is *BstNI*; preferably the kit contains one or more control primers pairs to determine if enzyme digestions are completed; preferably the kit contains one or more primer pairs to detect the presence of one or more control fragments in the size-separated nucleic acid sample. In other preferred embodiments, the kit is for the detection of the tandem repeat segment of the FMR1 gene; preferably the enzymes for generating fragments of the nucleic acid sample are *BlnI* and *MlyI*; preferably the enzyme for cleaving the marker sequence from the tandem repeat is *BmtI*; preferably the kit contains one or more control primers pairs to determine if enzyme digestions are completed; preferably the kit contains one or more primer pairs to detect the presence of one or more control fragments in the size-separated nucleic acid sample. In still other preferred embodiments, the kit is for the detection of the tandem repeat segment of the FMR1 gene; preferably the enzymes for generating fragments of the nucleic acid sample are *SphI* and *BmtI*; preferably the enzyme for cleaving the marker sequence from the tandem repeat is *BstNI*; preferably the kit contains one or more control primers pairs to determine if enzyme digestions are completed; preferably the kit contains one or more primer pairs to detect the presence of one or more control fragments in the size-separated nucleic acid sample.

[0036] "Segment" as used herein in reference to nucleic acid, refers to a piece of contiguous nucleic acid.

[0037] “Particular nucleic acid segment of interest” as used herein refers to a specific “segment” or piece of nucleic acid having a known sequence, preferred segments are those segments that are difficult to amplify by PCR. Examples include nucleic acid segments having high content of the bases guanine and cytidine, large segments of nucleic acid or segments having large numbers of tandem repeats, generally more than 100 tri-nucleotide repeats. In some embodiments the segment comprises a deletion, a duplication or an insertion. In a preferred embodiment, the particular nucleic acid segment of interest comprises a tandem repeat region.

[0038] “Nucleic acid segments which have high content of the bases guanine and cytosine” or “GC-rich” refer to those nucleic acid segments of a genome are more than the average for that genome. Generally, GC-rich is more than 40% guanine and cytosine bases, or more than 50%, or more than 60%, or more than 75%.

[0039] “Size” as used in reference to a particular nucleic acid segment of interest refers to quantity or amount that describes the magnitude of that segment and can be represented by, for example, molecular weight, number of base pairs, or number of copies of a tandem repeat.

[0040] “Fragment” as used herein refers to a portion of nucleic acid resulting from a process in which longer lengths of nucleic acid are broken up into shorter lengths of nucleic acid. Nucleic acids may be broken up or fragmented by chemical or biochemical means, preferably nucleic acids are fragmented in a manner that is reproducible, preferably nucleic acids are fragmented by one or more restriction endonucleases. Preferably nucleic acids are fragmented so that the particular nucleic acid segment of interest and its associated marker sequence are located on the same fragment. The length of a fragment containing the nucleic acid segment of interest will depend on the length of the nucleic acid segment of interest as well as the restriction enzyme chosen to fragment the DNA. Thus, the length of the of the fragment includes the nucleic acid segment of interest plus the region upstream of the segment to the 5' restriction enzyme recognition site (i.e., the 5' end of the fragment) and the region downstream of the segment region to the 3' restriction enzyme recognition site (i.e., the 3' end of the fragment).

[0041] “Separating fragments” as used herein refers to the process whereby the fragments contained in a mixture of different fragments are physically separated from one another.

[0042] “Fractionation” as used herein refers to a process whereby a single mixture of individual components is processed so that at least some of the individual components in the mixture become separated from each other. For example, chromatography is a fractionation method that separates a mixture of components based on some physical/chemical principle. The components may be separated in a gel or on a membrane so that the individual components may be separately identified. The individual components of a mixture may be fractionated by separating the mixture into the different components which are captured in separate aliquots of liquid (i.e. fractions). As used herein “fraction” in the context of the invention refers to a collection of fragments having a certain size or range of sizes that differs from the size or range of sizes of the starting non-fractionated mixture of fragments.

[0043] “Identifying those fractions containing the segment” as used herein means that the fraction of size-separated fragments that contains the segment of interest, is determined by the detection of a marker sequence associated with that segment on a fragment of nucleic acid.

[0044] The phrase “tandem repeat region” or “tandem repeat segment” as used herein refers to a region of DNA that contains a multiple copies of a short sequence of DNA. “Tandem repeat sequences” or “tandem repeats” or simply “repeats” are used interchangeably herein and refers to the short sequence of DNA that is repeated in the tandem repeat region. Such tandem repeats can lie adjacent to each other in the same orientation (i.e., direct tandem repeats) or in the opposite direction to each other (i.e., inverted tandem repeats). The repeated sequences may be di-, tri-, tetra-, or more nucleotides in length. Expansion the number of copies of the tandem repeat sequences within the coding or noncoding regions of some human genes is associated with repeat expansion disease.

[0045] As used herein, the term “sample” or “test sample” refers to any liquid or solid material containing genomic DNA. In preferred embodiments, a test sample is obtained from a biological source (i.e., a “biological sample”), such as cells in culture or a tissue sample from an animal, most preferably, a human. Preferred sample tissues include, but are not limited to, blood, bone marrow, body fluids, cerebrospinal fluid, plasma, serum, or tissue (e.g. biopsy material).

[0046] As used herein, “nucleic acid” refers broadly to segments of a chromosome, segments or portions of DNA, cDNA, and/or RNA. Nucleic acid may be derived or obtained from an originally isolated nucleic acid sample from any source (*e.g.*, isolated from, purified from, amplified from, cloned from, reverse transcribed from sample DNA or RNA).

[0047] “Target nucleic acid” as used herein refers to segments of a chromosome, a complete gene with or without intergenic sequence, segments or portions a gene with or without intergenic sequence, or sequence of nucleic acids to which probes or primers are designed. Target nucleic acids may include wild type sequences, nucleic acid sequences containing mutations, deletions or duplications, tandem repeat regions, a gene of interest, a region of a gene of interest or any upstream or downstream region thereof. Target nucleic acids may represent alternative sequences or alleles of a particular gene. Target nucleic acids may be derived from genomic DNA, cDNA, or RNA. As used herein target nucleic acid may be native DNA or a PCR amplified product.

[0048] The term “marker sequence” as used herein refers to a segment of nucleic acid which is associated with a nucleic acid segment of interest so that detection of the marker sequence in a sample is indicative of the presence of the nucleic acid segment of interest. The marker sequence for detecting a particular nucleic acid segment of interest should be selected on the basis that the marker is uniquely or substantially associated with the nucleic acid segment of interest in fragments present in a particular size fraction. Marker sequences can be detected by nucleic acid amplification using primer based hybridization methods. Marker sequences can also be detected by hybridization to one or more nucleic acid probe(s). In accordance with the methods disclosed herein, a fragment containing a tandem repeat segment is identified in size fractioned nucleic acid fragments by detecting a marker sequence that is either upstream or downstream of the tandem repeat.

[0049] Marker sequences may be within the nucleic acid segment of interest or may be flanking the nucleic acid of interest. The term “flanking” as used herein refers to a region of DNA either adjoining or a distance from a region of interest. The flanking region may be “upstream” (*i.e.*, 5') or “downstream” (*i.e.*, 3') of the region of interest. The marker sequence may be adjoining the tandem repeat region or may be located a distance upstream or downstream. In preferred embodiments, the marker sequence is within 500 bases upstream

or downstream of the tandem repeat region; in more preferred embodiments the marker sequence is within 250 bases upstream or downstream of the tandem repeat region; in most preferred embodiments the marker sequence is within 100 bases upstream or downstream of the tandem repeat region. The flanking region may be coding or non-coding sequence and may be the same or a different gene as the gene comprising the region of interest. In preferred embodiments, the marker sequence is flanking the nucleic acid segment of interest.

[0050] In certain embodiments, the size of the particular nucleic acid segment of interest is determined by relating the fraction size containing the particular nucleic acid segment to the size of the particular nucleic acid segment in the sample of nucleic acids. This step of relating the fraction size containing the particular nucleic acid segment to the size of the particular nucleic acid segment in the sample of nucleic acids can be accomplished using a look-up table as disclosed herein. In other embodiments this step is accomplished with a computer program.

[0051] The phrase “relating the fraction size containing the particular nucleic acid segment of interest to the size of the particular nucleic acid segment of interest that would be present in that fraction under the conditions which generated the fragment” as used herein refers to the means by which the size of the particular nucleic acid segment of interest is determined from its location in a particular fraction size. In one approach, a look-up table is established for each combination of particular nucleic acid segment of interest and fragmentation approach (e.g. particular restriction endonuclease(s) used). The look-up table links each fraction (containing a range of fragment sizes) to the length of the segment of interest that is present in such fragments. In any particular fraction, there are fragments that contain the segment of interest and other sequence. Thus, in any fraction, one can calculate from sequence data the number of bases in the fragments that represent the segment of interest. This correlation may be established experimentally or by using known DNA sequence for the fragments generated. For any particular unknown sample, the size of a segment of interest can be determined by relating the fragment size that contains the segment of interest to the appropriate look-up table reflecting the same conditions for fragment generation. By using this process one relates the fraction size containing the particular nucleic acid segment of interest to the size of the particular nucleic acid segment of interest that would be present in the fraction under the conditions which generated the fragment. It is

not essential that one prepare a look-up table to perform the method. For example, one could generate a computer program to perform the relating step.

[0052] “Genomic nucleic acid” or “genomic DNA” refers to some or all of the DNA from the nucleus of a cell. Genomic DNA may be intact or fragmented (e.g., digested with restriction endonucleases by methods known in the art). In some embodiments, genomic DNA may include sequence from all or a portion of a single gene or from multiple genes, sequence from one or more chromosomes, or sequence from all chromosomes of a cell. In contrast, the term “total genomic nucleic acid” is used herein to refer to the full complement of DNA contained in the genome of a cell. As is well known, genomic nucleic acid includes gene coding regions, introns, 5' and 3' untranslated regions, 5' and 3' flanking DNA and structural segments such as telomeric and centromeric DNA, replication origins, and intergenic DNA. Genomic nucleic acid may be obtained from the nucleus of a cell, or recombinantly produced. Genomic DNA also may be transcribed from DNA or RNA isolated directly from a cell nucleus. PCR amplification also may be used. Methods of purifying DNA and/or RNA from a variety of samples are well-known in the art.

[0053] The terms “allele” and “allelic variant” are used interchangeably herein. An allele is any one of a number of alternative forms or sequences of the same gene occupying a given locus or position on a chromosome. A single allele for each locus is inherited separately from each parent, resulting in two alleles for each gene. An individual having two copies of the same allele of a particular gene is homozygous at that locus whereas an individual having two different alleles of a particular gene is heterozygous.

[0054] “Repeat expansion disease” refers to any of about two dozen human diseases displaying Mendelian inheritance patterns shown to be caused by expansions of intrinsically polymorphic tandem repeats, mainly involving different trinucleotide motifs but also longer repetitive sequences up to 12-mers (Table 1). A characteristic of an allele containing an expanded tandem repeat is an excessive instability in successive generations (dynamic mutations). Furthermore, these alleles can differ in lengths among cell populations of the same organism (mosaicism). One type of repeat expansion disease is the trinucleotide repeat disorders (e.g., fragile X syndrome, myotonic dystrophy 1, *etc.*), the most abundant form of repeat expansion diseases. These diseases exhibit intergenerational repeat instability with a tendency towards further expansion of the tandem repeat. Increased repeat lengths in

successive generations can lead to an earlier age of onset in affected individuals and/or an accentuation of clinical symptoms. The methods of measuring tandem repeat length as described herein can be applied to measures tandem repeat length for any of the diseases/genes in Table 3.

Table 1. Exemplary tandem repeat expansion diseases

Disease (gene designation)	Protein	Tandem repeat sequence	Repeat Localization	Normal repeat range	Expanded repeat
Dentatorubral-pallidoluysian atrophy (DRPLA)	atrophin-1	CAG	coding	6-35	49-88
Progressive myoclonus epilepsy (EPM1)#	CSTB	CCCCGCCC CGCG (SEQ ID NO:39)	5' UTR	2-3	50-80
Fragile XA-syndrome (FRAXA)*	FMR1	CGG	5' UTR	6-53	>230
Fragile XE-syndrome (FMR1)*	FMR-2	GCC	5' UTR	6-35	>200
Friedreich ataxia (FRDA)#	Frataxin	GAA	intronic	7-34	>100
Huntington disease (huntingtin)	huntingtin	CAG	coding	4-35	36-250
Huntington disease-like 2 (JPH-3)	junctophilin-3	CTG	coding	6-27	>40-60
Spinobulbar musc. atrophy, Kennedy disease (AR)*	androgen rec.	CAG	coding	9-36	38-62
Myotonic dystrophy 1 (DM1)	DMPK/SIX5	CTG	3' UTR	5-37	>50
Myotonic dystrophy 2 (DM2)	ZNF9	CCTG	intronic	10-26	75- >11000
Spinocerebellar ataxia 1 (SCA1)	ataxin-1	CAG	coding	6-44	39-82
Spinocerebellar ataxia 2 (SCA2)	ataxin-2	CAG	coding	15-31	36-63

Spinocerebellar ataxia 3, Machado-Joseph disease (SCA3)	Ataxin-3	CAG	coding	12-40	55-84
Spinocerebellar ataxia 6 (SCA6)	CACNA1A	CAG	coding	4-18	21-33
Spinocerebellar ataxia 7 (SCA7)	Ataxin-7	CAG	coding	4-35	37-306
Spinocerebellar ataxia 8 (SCA8)	not known	CTG	3' UTR	16-37	110-250
Spinocerebellar ataxia 10 (SCA10)	Ataxin-10	ATTCT	intronic	10-22	800-4600
Spinocerebellar ataxia 12 (SCA12)	PP2A-PR55B	CAG	promotor	7-28	66-78
Spinocerebellar ataxia 17 (SCA17)	TBP	CAG	coding	25-42	47-63
Oculopharyngeal muscular dystrophy (PABPN1)	PABPN1	GCG	coding	6	7-13
Synpolydactyly, type II (HOXD13)	HOXD13	GCN	coding	15	22-24

* = X chromosome; # = autosomal recessive; undesignated = autosomal dominant

[0055] As used herein, the term “oligonucleotide” refers to a short polymer composed of deoxyribonucleotides, ribonucleotides or any combination thereof. Oligonucleotides of the invention are generally between about 10 and about 100 nucleotides in length.

Oligonucleotides are preferably 15 to 70 nucleotides long, with 20 to 26 nucleotides being the most common. The single letter code for nucleotides is as described in the U.S. Patent Office Manual of Patent Examining Procedure, section 2422, table 1. In this regard, the nucleotide designation “R” means guanine or adenine, “Y” means thymine (uracil if RNA) or cytosine; and “M” means adenine or cytosine. An oligonucleotide may be used as a primer or as a probe.

[0056] As used herein, the term “substantially purified” in reference to oligonucleotides does not require absolute purity. Instead, it represents an indication that the sequence is relatively more pure than in the natural environment. Such oligonucleotides may be obtained by a number of methods including, for example, laboratory synthesis, restriction enzyme

digestion or PCR. A “substantially purified” oligonucleotide is preferably greater than 50% pure, more preferably at least 75% pure, and most preferably at least 95% pure.

[0057] As used herein, an oligonucleotide is “specific” for a nucleic acid if the oligonucleotide has at least 50% sequence identity with a portion of the nucleic acid when the oligonucleotide and the nucleic acid are aligned. An oligonucleotide that is specific for a nucleic acid is one that, under the appropriate hybridization or washing conditions, is capable of hybridizing to the target of interest and not substantially hybridizing to nucleic acids which are not of interest. Higher levels of sequence identity are preferred and include at least 75%, at least 80%, at least 85%, at least 90%, at least 95% and more preferably at least 98% sequence identity.

[0058] As used herein, the term “hybridize” or “specifically hybridize” refers to a process where two complementary nucleic acid strands anneal to each other under appropriately stringent conditions. Hybridizations are typically and preferably conducted with probe-length nucleic acid molecules, preferably 20-100 nucleotides in length. Nucleic acid hybridization techniques are well known in the art. See, e.g., Sambrook, et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Plainview, N.Y. Those skilled in the art understand how to estimate and adjust the stringency of hybridization conditions such that sequences having at least a desired level of complementarity will stably hybridize, while those having lower complementarity will not. For examples of hybridization conditions and parameters, see, e.g., Sambrook, et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Plainview, N.Y.; Ausubel, F. M. et al. 1994, *Current Protocols in Molecular Biology*. John Wiley & Sons, Secaucus, N.J.

[0059] The term “substantially complementary” as used herein means that two sequences hybridize under stringent hybridization conditions. The skilled artisan will understand that substantially complementary sequences need not hybridize along their entire length. In particular, substantially complementary sequences comprise a contiguous sequence of bases that do not hybridize to a target or marker sequence, positioned 3' or 5' to a contiguous sequence of bases that hybridize under stringent hybridization conditions to a target or marker sequence.

[0060] The term “complement” as used herein means the complementary sequence to a nucleic acid according to standard Watson/Crick pairing rules. A complement sequence can also be a sequence of RNA complementary to the DNA sequence or its complement sequence, and can also be a cDNA.

[0061] The term “coding sequence” as used herein means a sequence of a nucleic acid or its complement, or a part thereof, that can be transcribed and/or translated to produce the mRNA for and/or the polypeptide or a fragment thereof. Coding sequences include exons in a genomic DNA or immature primary RNA transcripts, which are joined together by the cell's biochemical machinery to provide a mature mRNA. The anti-sense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

[0062] The term “non-coding sequence” as used herein means a sequence of a nucleic acid or its complement, or a part thereof, that is not transcribed into amino acid in vivo, or where tRNA does not interact to place or attempt to place an amino acid. Non-coding sequences include both intron sequences in genomic DNA or immature primary RNA transcripts, and gene-associated sequences such as promoters, enhancers, silencers, etc.

[0063] The term “amplification” or “amplify” as used herein means one or more methods known in the art for copying a target nucleic acid, thereby increasing the number of copies of a selected nucleic acid sequence. Amplification may be exponential or linear. A target nucleic acid may be either DNA or RNA. The sequences amplified in this manner form an “amplicon.” While the exemplary methods described hereinafter relate to amplification using the polymerase chain reaction (“PCR”), numerous other methods are known in the art for amplification of nucleic acids (*e.g.*, isothermal methods, rolling circle methods, *etc.*). The skilled artisan will understand that these other methods may be used either in place of, or together with, PCR methods. See, *e.g.*, Saiki, “Amplification of Genomic DNA” in PCR Protocols, Innis et al., Eds., Academic Press, San Diego, CA 1990, pp 13-20; Wharam et al., Nucleic Acids Res. 2001 Jun 1;29(11):E54-E54; Hafner et al., Biotechniques 2001 Apr;30(4):852-6, 858, 860 *passim*; Zhong et al., Biotechniques 2001 Apr;30(4):852-6, 858, 860 *passim*.

[0064] As used herein, a “primer” for amplification is an oligonucleotide that specifically anneals to a target or marker nucleotide sequence. The 3' nucleotide of the primer should be

identical to the target or marker sequence at a corresponding nucleotide position for optimal amplification.

[0065] “Sense strand” means the strand of double-stranded DNA (dsDNA) that includes at least a portion of a coding sequence of a functional protein. “Anti-sense strand” means the strand of dsDNA that is the reverse complement of the sense strand.

[0066] As used herein, a “forward primer” is a primer that anneals to the anti-sense strand of dsDNA. A “reverse primer” anneals to the sense-strand of dsDNA.

[0067] As used herein, sequences that have “high sequence identity” have identical nucleotides at least at about 50% of aligned nucleotide positions, preferably at least at about 75% of aligned nucleotide positions, more preferably at least at about 90% of aligned nucleotide positions, and most preferably at least at about 95% of aligned nucleotide positions.

[0068] As used herein “TaqMan PCR detection system” refers to a method for real time PCR. In this method, a TaqMan probe which hybridizes to the nucleic acid segment amplified is included in the PCR reaction mix. The TaqMan probe comprises a donor and a quencher fluorophore on either end of the probe and in close enough proximity to each other so that the fluorescence of the donor is taken up by the quencher. However, when the probe hybridizes to the amplified segment, the 5'-exonuclease activity of the Taq polymerase cleaves the probe thereby allowing the donor fluorophore to emit fluorescence which can be detected.

BRIEF DESCRIPTION OF THE FIGURES

[0069] Figure 1. A schematic showing one embodiment of the method of detecting the tandem repeats. The presence of FMR1 fragments with a particular length of tandem repeat is shown schematically at the bottom for each size fraction indicated (i.e. small, medium and large). The designation of + or – is shown below PCR to indicate whether PCR amplification of the flanking marker sequence occurs when the particular fragment is present in the fraction.

[0070] Figure 2. Exemplary sequence (SEQ ID NO:1) of a region of the FMR1 gene showing the CGG tandem repeat region (single underlining), preferred locations for hybridizing PCR primers (shaded regions), and a preferred location for a hybridizing probe (double-underlining).

[0071] Figure 3. Exemplary sequence (SEQ ID NO:2) of the downstream 3' untranslated region of the DM-1 gene showing the CTG tandem repeat region (single underlining), preferred locations for hybridizing PCR primers (shaded regions), and a preferred location for a hybridizing probe (double-underlining).

[0072] Figure 4. Exemplary sequence (SEQ ID NO:3) of the first intronic region of the FRDA gene showing the CAA tandem repeat region (single underlining), preferred locations for hybridizing PCR primers (shaded regions), and a preferred location for a hybridizing probe (double-underlining).

[0073] Figure 5. Restriction enzyme map of a region of the FMR1 gene. FXCEF3, FXCER3, FMR1F4, FMR1R4, FXCEF2, and FXCER2 show the location of hybridization of preferred oligonucleotide primers. FXCEF3/FXCER3, FMR1F4/FMR1R4, and FXCEF2/FXCER2 are preferred primer pairs for amplification of marker sequences when the nucleic acid is fragmented with SphI/BmtI, AluI, and BlnI/MlyI, respectively.

DETAILED DESCRIPTION OF THE INVENTION

[0074] In accordance with the present invention, there are provided methods of detecting a particular nucleic acid segment of interest in a sample of nucleic acids. In particular embodiments, the particular nucleic acid segment of interest is a tandem repeat and the method is used to determine information about the size of such tandem repeat. This information may be used to determine if an individual carries a genetic mutation characterized by an increase (i.e., expansion) or a decrease (i.e., reduction) in the number of tandem repeats associated with a particular gene. Thus, described is a method of measuring the size of a tandem repeat segment in a sample of nucleic acids, the method comprising identifying the tandem repeat in fractions of size-separated nucleic acid fragments by detecting a marker sequence flanking the tandem repeat segment and then relating the fraction size containing the repeat to the size of a tandem repeat segment present in such nucleic acid fragments. Figure 1 shows one embodiment of this method in schematic form.

As will be discussed, a variation of this method is to perform a second fragmentation after the size separation and prior to the "analysis" step.

Sample Preparation

[0075] The methods of the present invention can be used to detect mutations characterized by an expansion or reduction of tandem repeat region of a gene in the genomic DNA of a test sample. Therefore, the method may be performed using any biological sample containing genomic DNA. Examples include tissue samples or any cell-containing bodily fluid. Blood is the preferred biological sample. Biological samples may be obtained by standard procedures and may be used immediately or stored, under conditions appropriate for the type of biological sample, for later use.

[0076] Methods of obtaining test samples are well known to those of skill in the art and include, but are not limited to, aspirations, tissue sections, drawing of blood or other fluids, surgical or needle biopsies, and the like. The test sample may be obtained from individual or patient. The test sample may contain cells, tissues or fluid obtained from a patient suspected being afflicted with or a carrier for a disorder caused by an expansion of tandem repeat sequences. The test sample may be a cell-containing liquid or a tissue. Samples may include, but are not limited to, amniotic fluid, biopsies, blood, blood cells, bone marrow, fine needle biopsy samples, peritoneal fluid, plasma, pleural fluid, saliva, semen, serum, tissue or tissue homogenates, frozen or paraffin sections of tissue. Samples may also be processed, such as sectioning of tissues, fractionation, purification, or cellular organelle separation.

[0077] The invention methods can be used to perform prenatal diagnosis using any type of embryonic or fetal cell or nucleic acid containing body fluid. Fetal cells can be obtained through the pregnant female, or from a sample of an embryo. Thus, fetal cells are present in amniotic fluid obtained by amniocentesis, chorionic villi aspirated by syringe, percutaneous umbilical blood, a fetal skin biopsy, a blastomere from a four-cell to eight-cell stage embryo (pre-implantation), or a trophoctoderm sample from a blastocyst (pre-implantation or by uterine lavage).

[0078] In particular embodiments, genomic DNA may be used. Genomic DNA may be isolated from cells or tissues using standard methods, see, e.g., Sambrook, et al., 1989,

Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, Plainview, NY.

Fragmentation of Genomic DNA

[0079] Genomic DNA may be fragmented by various methods well-known in the art. Preferably, a restriction endonuclease digestion is used to fragment the DNA.

[0080] A “restriction endonuclease” or “restriction enzyme” as used herein refers to an enzyme that cuts double-stranded DNA at a specific sequence (i.e., the recognition sequence or site). The frequency with which a given restriction endonuclease cuts DNA depends on the length of the recognition site of the enzyme. For example, some enzymes recognize sites that are four nucleotides long (referred to as “four cutters”). In general one can estimate how frequently an enzyme should cut a piece of DNA based the length of the recognition site and the assumption that the probability of any one nucleotide occurring at a given location is $\frac{1}{4}$. In the case of a “four cutter” a specific sequence of four nucleotides must be present. Assuming that each nucleotide has an equal chance (i.e., $\frac{1}{4}$) of occurring at any particular site within the four nucleotide sequence, then a four-cutter should on average cut once every 256 base pairs (i.e., $\frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} = 1/256$). A similar calculation can be applied to any restriction enzyme as long as the length of its recognition site is known, making it possible to predict the size and number of a DNA fragments that would be obtained by cutting a DNA molecule of known size. This allows one of skill in the art to produce DNA fragments of known size. Restriction endonucleases are obtained from bacteria or are produced through recombinant technology and are readily available through numerous commercial sources.

[0081] In the restriction endonuclease fragmentation method, a restriction endonuclease is combined with a sample of genomic DNA and buffer appropriate for optimal activity of the endonuclease. In general, 1 unit of endonuclease will digest $1\mu\text{g}$ of DNA in 1 hour at 37°C .

[0082] One of skill in the art would recognize that this fragmentation method can be modified by using a restriction enzyme that cuts at a particular frequency or a particular site, or by using multiple restriction enzymes. The choice of enzyme or enzyme combinations is chosen to suit the gene of interest in an assay. In general, one would choose an enzyme or enzyme combination to generate a fragment containing the entire tandem repeat region and the upstream or downstream marker sequence. Enzymes for fragmentation can be chosen by

using a restriction enzyme map of the region surrounding the tandem repeat. Such maps can be readily generated by software programs well-known to those of skill in the art.

[0083] In particular, one would choose an enzyme or a combination of enzymes to obtain an appropriately sized fragment to distinguish a normal length tandem repeat region from an abnormal length tandem repeat region. In determining an appropriate size for a fragment, one would consider the difference in the range of lengths of a normal tandem repeat region as compared to that for an abnormal tandem repeat region. For example, if the difference between a normal tandem repeat region and an abnormal tandem repeat region is small, one would choose a shorter length fragment, whereas if the difference is large one would choose a longer length fragment.

[0084] In preferred embodiments, *AluI* is used to fragment the nucleic acids. *AluI* is a restriction enzyme that recognizes a 4-nucleotide sequence of double-stranded DNA (*i.e.*, -AGCT-). One of skill in the art would recognize that isoschizomers (*i.e.*, restriction enzymes with the same recognition sequence and cut site) of *AluI* can be readily substituted for *AluI*. Examples of *AluI* isoschizomers include, but are not limited to, *BsaLI*, *MarI*, *MltI*, and *OxaI*. One of skill in the art would further recognize that a neoschizomer (*i.e.*, a restriction enzyme with the same recognition sequence as another enzyme but with a different cut site) of *AluI* could also be substituted for *AluI*.

[0085] One of skill in the art would recognize that other restriction enzymes with the same cutting frequency as *AluI* could be substituted for *AluI* in this method. For example, *AluI*, which recognizes a 4-nucleotide sequence, cuts DNA at approximately every 256 bases. Other enzymes with different 4-nucleotide recognition sequences (*e.g.*, *DpnI*, *RsaI*, *MboI*, and *NlaI*) would be expected to cut at a similar frequency to *AluI* and would therefore produce fragments of a size similar to those of *AluI*.

[0086] In other preferred embodiments, *BlpI* and *MlyI* are used in combination to fragment the nucleic acids. In still other preferred embodiments, *SphI* and *BmtI* are used in combination to fragment the nucleic acids.

Size Separation of DNA Fragments

[0087] Separation of DNA fragments according to size may be accomplished by various methods known to those of skill in the art. For example, various methods of gel electrophoresis or column chromatography (*e.g.*, size-exclusion high performance liquid chromatography (SEC-HPLC) and denaturing HPLC (DHPLC)) may be used.

[0088] In gel electrophoresis, a gel matrix, to which an electric field is applied, is utilized to separate nucleic acid molecules or fragments thereof based on size. In general, smaller nucleic acid fragments will move faster through the gel matrix than larger fragments. Preferred gel matrices include agarose and polyacrylamide.

[0089] In preferred embodiments, capillary electrophoresis is used to separate the fragmented nucleic acids. Capillary electrophoresis is a separation method based on the differential electrophoretic migration rate of sample components in a capillary when a voltage is applied. Separated fragments or molecules are generally detected "on-column" using UV spectrometric or fluorescence analysis through a window in the capillary. In general, one or more standards (*i.e.*, a segment of nucleic acid having known length) are first injected into the capillary to determine the elution time for each standard using on-column detection. Then, the elution times of the standards are used to determine the length of time over which a fraction will be collected in order to achieve a desired size range for that fraction.

[0090] In some embodiments, the size ranges for the fractions used in an assay may be chosen based on the length in base pairs of commercially-available standards. A number of standards are available containing mixtures of lengths of nucleic acids. In one example, a standard containing nucleic acid fragments having the following lengths is used: 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, and 1000 bp. Thus, fractions would then be chosen based on one or more of the sizes present in the standard. For example, one might choose the following three fractions: 1) less than or equal to 300 bp, 2) 301-500 bp, and 3) greater than or equal to 501. One would then collect fractions of the fragmented nucleic acid sample based on the elution times of the 300 bp and 500 bp standards. The range in number of tandem repeats present in each fraction can be calculated.

[0091] Alternatively, fractions can be chosen based on a desired number of tandem repeats for each fraction. In this case, standards representing the upper and lower limits in size of each fraction can be synthesized if not commercially available.

[0092] In some embodiments, the fragments are separated into the lowest number of fractions in order to distinguish normal from abnormal length tandem repeat regions. In particular embodiments, the fragments may be separated into two fractions, one corresponding to a normal tandem repeat region and one corresponding to an abnormal tandem repeat region.

[0093] In other embodiments, the fragments are separated into a larger number of fractions in order to determine the size of the tandem repeat region. In general, more fractions will allow a more precise determination of the length of the tandem repeat region. The number of fractions may be chosen in order to achieve a desired level of precision in determining the length of the tandem repeat region.

[0094] In a preferred embodiment of the assay to determine the number of tandem repeats of the FMR1 gene, AluI fragmented DNA is separated into two fractions corresponding to sizes of approximately 211 – 358 bp (lower fraction) and 359 bp – 9 kb (upper fraction). Fragments from samples containing a normal FMR1 gene (*i.e.*, less than 55 tandem repeats), will separate into the lower fraction, whereas fragments from FMR1 genes containing premutations (*i.e.*, 55-200 tandem repeats) or full mutations (*i.e.*, greater than 200 tandem repeats) will separate into the upper fraction.

[0095] In another preferred embodiment of the assay to determine the number of tandem repeats of the FMR1 gene, AluI fragmented DNA is separated into two fractions corresponding to sizes of approximately 211 – 400 bp (lower fraction) and 401 bp – 9 kb (upper fraction). Fragments from samples containing a normal FMR1 gene (*i.e.*, less than 55 tandem repeats) and premutations having 56-69 tandem repeats, will separate into the lower fraction, whereas fragments from FMR1 genes containing premutations having 70-200 tandem repeats or full mutations (*i.e.*, greater than 200 tandem repeats) will separate into the upper fraction.

[0096] In another preferred embodiment of the assay to determine the number of tandem repeats of the FMR1 gene, AluI fragmented DNA is separated into four fractions corresponding to sizes of approximately less than or equal to 400 bp (first/lowest fraction), 401-500 bp (second fraction), 501-800 bp (third fraction) and 800 bp – 9 kb (fourth/highest fraction). Fragments from samples containing a normal FMR1 gene (*i.e.*, less than 55 tandem

repeats) and those containing premutations having 56-68 tandem repeats will separate into the first/lowest fraction, whereas fragments from FMR1 genes containing small premutations (*i.e.*, 69-102 tandem repeats) will separate into the second fraction, whereas fragments from FMR1 genes containing large premutations (*i.e.*, 103-200 tandem repeats) and full mutations having 201-202 tandem repeats will separate into the third fraction, and whereas full mutations having greater than 202 tandem repeats will separate into the fourth/highest fraction.

[0097] In yet another preferred embodiment of the assay to determine the number of tandem repeats of the FMR1 gene, DNA fragmented with a combination of SphI and BmtI is separated into four fractions corresponding to sizes of approximately less than or equal to 500 bp (first/lowest fraction), 501-800 bp (second fraction), 801-900 bp (third fraction) and 901 bp – 9 kb (fourth/highest fraction). Fragments from samples containing a normal FMR1 gene (*i.e.*, less than 55 tandem repeats) and those containing premutations having 56-62 tandem repeats will separate into the first/lowest fraction, whereas fragments from FMR1 genes containing small premutations (*i.e.*, 63-163 tandem repeats) will separate into the second fraction, whereas fragments from FMR1 genes containing large premutations (*i.e.*, 164-196 tandem repeats) will separate into the third fraction, and whereas large premutations having 197-200 tandem repeats and full mutations (*i.e.*, greater than 200 tandem repeats) will separate into the fourth/highest fraction.

[0098] In still another preferred embodiment of the assay to determine the number of tandem repeats of the FMR1 gene, DNA fragmented with a combination of BlnI and dMlyI is separated into four fractions corresponding to sizes of approximately less than or equal to 603 bp (first/lowest fraction), 604-840 bp (second fraction), 841-1078 bp (third fraction) and 1079 bp – 9 kb (fourth/highest fraction). Fragments from samples containing a normal FMR1 gene (*i.e.*, less than 55 tandem repeats) and those containing premutations having 56-62 tandem repeats will separate into the first/lowest fraction, whereas fragments from FMR1 genes containing small premutations (*i.e.*, 63-140 tandem repeats) will separate into the second fraction, whereas fragments from FMR1 genes containing large premutations (*i.e.*, 141-200 tandem repeats) and full mutations having 201-220 tandem repeats will separate into the third fraction, and whereas large premutations having greater than 220 tandem repeats will separate into the fourth/highest fraction.

[0099] In another preferred embodiment, fragmented DNA is separated into a multiplicity of fractions, according to size. Automated fraction collection is accomplished using, for example, a preset fraction time window, beginning at approximately 200 bp and ending at 9 kb. This method allows for a finer estimation of fragment size and thereby, an estimation of the number of repeats.

Second Fragmentation of DNA

[00100] In yet other preferred embodiments, the methods of the invention include a second nucleic acid fragmentation following the size separation of the first nucleic acid fragmentation. Preferably, a restriction endonuclease digestion is used to further fragment the DNA. Preferably, the second fragmentation separates the tandem repeat segment from an associated marker sequence.

[00101] In preferred embodiments, restriction enzyme *BsaWI*, *HpyI88I*, *HphI* or *BstNI* is used for the second nucleic acid fragmentation when the marker sequence is upstream of the tandem repeat segment. In other preferred embodiments, restriction enzyme *SmlI*, *BbvI*, or *BmtI* is used for the second nucleic acid fragmentation when the marker sequence is downstream of the tandem repeat segment. One of skill in the art would recognize that isoschizomers and neoschizomers of the listed restriction enzymes could also be used. One of skill in the art would be able to identify a suitable restriction enzyme for the second nucleic acid fragmentation by analyzing factors which include, but are not limited to, the location of the marker, the sequence of the marker and the sequence between the marker sequence and the tandem repeat segment.

Amplification of Size-separated DNA Fragments or DNA Fragments following Second Fragmentation

[00102] Size-separated DNA may be amplified by various methods known to the skilled artisan. Amplification methods suitable for use with the present methods include, for example, polymerase chain reaction (PCR), ligase chain reaction (LCR), transcription-based amplification system (TAS), nucleic acid sequence-based amplification (NASBA) reaction, self-sustained sequence replication (3SR), strand displacement amplification (SDA) reaction, boomerang DNA amplification (BDA), Q-beta replication, or isothermal nucleic acid

sequence based amplification. These methods of amplification each described briefly below and are well-known in the art.

[00103] PCR is a technique for making many copies of a specific template DNA sequence. The reaction consists of multiple amplification cycles and is initiated using a pair of primer sequences that hybridize to the 5' and 3' ends of the sequence to be copied. The amplification cycle includes an initial denaturation, and up to 50 cycles of annealing, strand elongation and strand separation (denaturation). In each cycle of the reaction, the DNA sequence between the primers is copied. Primers can bind to the copied DNA as well as the original template sequence, so the total number of copies increases exponentially with time. PCR can be performed as according to Whelan, *et al*, *Journal of Clinical Microbiology*, 33(3):556-561(1995). Briefly, a PCR reaction mixture includes two specific primers, dNTPs, approximately 0.25 U of Taq polymerase, and 1x PCR Buffer. For every 25 µl PCR reaction, 2 µl sample (e.g., isolated DNA from target organism) is added and amplified using a thermal cycler.

[00104] LCR is a method of DNA amplification similar to PCR, except that it uses four primers instead of two and uses the enzyme ligase to ligate or join two segments of DNA. LCR can be performed as according to Moore *et al.*, *Journal of Clinical Microbiology* 36(4):1028-1031 (1998). Briefly, an LCR reaction mixture contains two pair of primers, dNTP, DNA ligase and DNA polymerase representing about 90 µl, to which is added 100 µl of isolated nucleic acid from the target organism. Amplification is performed in a thermal cycler (e.g., LCx of Abbott Labs, North Chicago, IL).

[00105] TAS is a system of nucleic acid amplification in which each cycle is comprised of a cDNA synthesis step and an RNA transcription step. In the cDNA synthesis step, a sequence recognized by a DNA-dependent RNA polymerase (i.e., a polymerase-binding sequence or PBS) is inserted into the cDNA copy downstream of the target or marker sequence to be amplified using a two-domain oligonucleotide primer. In the second step, an RNA polymerase is used to synthesize multiple copies of RNA from the cDNA template. Amplification using TAS requires only a few cycles because DNA-dependent RNA transcription can result in 10-1000 copies for each copy of cDNA template. TAS can be performed according to Kwoh *et al.*, *PNAS* 86:1173-7 (1989). Briefly, extracted RNA is combined with TAS amplification buffer and bovine serum albumin, dNTPs, NTPs, and two

oligonucleotide primers, one of which contains a PBS. The sample is heated to denature the RNA template and cooled to the primer annealing temperature. Reverse transcriptase (RT) is added the sample incubated at the appropriate temperature to allow cDNA elongation. Subsequently T7 RNA polymerase is added and the sample is incubated at 37°C for approximately 25 minutes for the synthesis of RNA. The above steps are then repeated. Alternatively, after the initial cDNA synthesis, both RT and RNA polymerase are added following a 1 minute 100°C denaturation followed by an RNA elongation of approximately 30 minutes at 37°C. TAS can be also be performed on solid phase as according to Wylie *et al.*, *Journal of Clinical Microbiology*, 36(12):3488-3491 (1998). In this method, nucleic acid targets are captured with magnetic beads containing specific capture primers. The beads with captured targets are washed and pelleted before adding amplification reagents which contains amplification primers, dNTP, NTP, 2500 U of reverse transcriptase and 2500 U of T7 RNA polymerase. A 100 µl TMA reaction mixture is placed in a tube, 200 µl oil reagent is added and amplification is accomplished by incubation at 42°C in a waterbath for one hour.

[00106] NASBA is a transcription-based amplification method which amplifies RNA from either an RNA or DNA target. NASBA is a method used for the continuous amplification of nucleic acids in a single mixture at one temperature. For example, for RNA amplification, avian myeloblastosis virus (AMV) reverse transcriptase, RNase H and T7 RNA polymerase are used. This method can be performed as according to Heim, *et al.*, *Nucleic Acids Res.*, 26(9):2250-2251 (1998). Briefly, an NASBA reaction mixture contains two specific primers, dNTP, NTP, 6.4 U of AMV reverse transcriptase, 0.08 U of Escherichia coli Rnase H, and 32 U of T7 RNA polymerase. The amplification is carried out for 120 min at 41°C in a total volume of 20 µl.

[00107] In a related method, self-sustained sequence-replication (3SR) reaction, isothermal amplification of target DNA or RNA sequences in vitro using three enzymatic activities: reverse transcriptase, DNA-dependent RNA polymerase and Escherichia coli ribonuclease H. This method may be modified from a 3-enzyme system to a 2-enzyme system by using human immunodeficiency virus (HIV)-1 reverse transcriptase instead of avian myeloblastosis virus (AMV) reverse transcriptase to allow amplification with T7 RNA polymerase but without E. coli ribonuclease H. In the 2-enzyme 3SR, the amplified RNA is obtained in a

purified form compared with the 3-enzyme 3SR (Gebinoga & Oehlenschläger *European Journal of Biochemistry*, 235:256-261, 1996).

[0100] SDA is an isothermal nucleic acid amplification method. A primer containing a restriction site is annealed to the template. Amplification primers are then annealed to 5' adjacent sequences (forming a nick) and amplification is started at a fixed temperature. Newly synthesized DNA strands are nicked by a restriction enzyme and the polymerase amplification begins again, displacing the newly synthesized strands. SDA can be performed as according to Walker, *et al.*, *PNAS*, 89:392-6 (1992). Briefly, an SDA reaction mixture contains four SDA primers, dGTP, dCTP, TTP, dATP, 150 U of Hinc II, and 5 U of exonuclease-deficient of the large fragment of *E. coli* DNA polymerase I (exo⁻ Klenow polymerase). The sample mixture is heated 95°C for 4 minutes to denature target DNA prior to addition of the enzymes. After addition of the two enzymes, amplification is carried out for 120 min. at 37°C in a total volume of 50 µl. Then, the reaction is terminated by heating for 2 minutes at 95°C.

[0101] Boomerang DNA amplification (BDA) is a method in which the polymerase begins extension from a single primer-binding site and then makes a loop around to the other strand, eventually returning to the original priming site on the DNA. BDA differs from PCR through its use of a single primer. This method involves an endonuclease digestion of a sample DNA, producing discrete DNA fragments with sticky ends, ligating the fragments to "adapter" polynucleotides (comprised of a ligatable end and first and second self-complementary sequences separated by a spacer sequence) thereby forming ligated duplexes. The ligated duplexes are denatured to form templates to which an oligonucleotide primer anneals at a specific sequence within the target or marker sequence of interest. The primer is extended with a DNA polymerase to form duplex products followed by denaturation of the duplex products. Subsequent multiple cycles of annealing, extending, and denaturing are performed to achieve the desired degree of amplification (U.S. Patent No. 5,470,724).

[0102] The Q-beta replication system uses RNA as a template. Q-beta replicase synthesizes the single-stranded RNA genome of the coliphage Q β . Cleaving the RNA and ligating in a nucleic acid of interest allows the replication of that sequence when the RNA is replicated by Q-beta replicase (Kramer & Lizardi *Trends Biotechnol.* 1991 9(2):53-8, 1991).

[0103] A variety of amplification enzymes are well known in the art and include, for example, DNA polymerase, RNA polymerase, reverse transcriptase, Q-beta replicase, thermostable DNA and RNA polymerases. Because these and other amplification reactions are catalyzed by enzymes, in a single step assay the nucleic acid releasing reagents and the detection reagents should not be potential inhibitors of amplification enzymes if the ultimate detection is to be amplification based. Amplification methods suitable for use with the present methods include, for example, strand displacement amplification, rolling circle amplification, primer extension preamplification, or degenerate oligonucleotide PCR (DOP). These methods of amplification are well known in the art and each described briefly below.

[0104] Preferably, PCR is used to amplify a target or marker sequence flanking the tandem repeat segment of interest. In this method, two or more oligonucleotide primers that anneal to opposite strands of a target or marker sequence are repetitively annealed to their complementary sequences, extended by a DNA polymerase (*e.g.*, AmpliTaq Gold polymerase), and heat denatured, resulting in exponential amplification of the target nucleic acid sequences. Cycling parameters can be varied, depending on the length of nucleic acids to be extended. The skilled artisan is capable of designing and preparing primers that are appropriate for amplifying a target or marker sequence. The length of the amplification primers for use in the present invention depends on several factors including the nucleotide sequence identity and the temperature at which these nucleic acids are hybridized or used during in vitro nucleic acid amplification. The considerations necessary to determine a preferred length for an amplification primer of a particular sequence identity are well-known to a person of ordinary skill and include considerations described herein. For example, the length of a short nucleic acid or oligonucleotide can relate to its hybridization specificity or selectivity.

[0105] In some embodiments, the amplification may include a labeled primer, thereby allowing detection of the amplification product of that primer. In particular embodiments the amplification may include a multiplicity of labeled primers, preferably such primers are distinguishably labeled, allowing the simultaneous detection of multiple amplification products.

[0106] Oligonucleotide primers can be designed which are between about 10 and about 100 nucleotides in length and hybridize to the marker sequence. Oligonucleotide primers are

preferably 12 to 70 nucleotides; more preferably 15-60 nucleotides in length; and most preferably 15-25 nucleotides in length.

[0107] In one embodiment, a primer pair is designed to amplify a marker sequence upstream of the tandem repeat region of the FMR1 gene following size separation of nucleic acids fragmented by AluI. An exemplary marker sequence upstream of the FMR1 tandem repeat region for designing hybridization primers is depicted in Figure 2 (SEQ ID NO:1). A forward primer can hybridize to SEQ ID NO:1 between nucleotides 1 and 45, more preferably between positions 22 and 39 while a reverse primer can hybridize to SEQ ID NO:1 between positions 70 and 115, more preferably between 97 and 113. One example is to use a primer pair to amplify a region of the flanking sequence corresponding to approximately 95 bp upstream of the tandem repeat region; more specifically using a forward primer, SEQ ID NO:4 and a reverse primer, SEQ ID NO:5 to amplify a 93 bp region of the marker sequence. Thus, preferred oligonucleotides which may be used as amplification primers include SEQ ID NO:4 (5'-GGTGGAGGGCCGCCTCTG-3') and SEQ ID NO:5 (5'-AGCGGCGCCTCCGTCACC-3'). Other preferred oligonucleotide primers are approximately 15-100 nucleotides in length and comprise SEQ ID NO:4 or SEQ ID NO:5. Still other preferred oligonucleotide primers include an oligonucleotide sequence that hybridizes to the complement of a 15-100 nucleotide sequence including SEQ ID NO:4 or SEQ ID NO:5. Such oligonucleotides may be substantially purified.

Table 2. Primers for amplifying marker sequences flanking FMR1

Primer Name	SEQ ID NO:	Primer Sequence
FMR1F4	4	5'-GGTGGAGGGCCGCCTCTG-3'
FMR1R4	5	5'-AGCGGCGCCTCCGTCACC-3'
FXCEF2	6	5'- GATGGAGGAGCTGGTGGTGG -3'
FXCER2	7	5'- GGAAGGGCGAAGATGGGG -3'
FXCEF3	8	5'- CGTGACGTGGTTTCAGTGTTTACA -3'
FXCER3	9	5' GGAAGTGAAACCGAAACGGAG -3'

[0108] In another embodiment, a primer pair is designed to amplify a marker sequence flanking the tandem repeat region of the FMR1 gene following size separation of nucleic acids fragmented by BlnI and MlyI. In one example, a primer pair is used to amplify a region

of the flanking sequence downstream of the tandem repeat region; more specifically using a forward primer, FXCEF2 (SEQ ID NO:6), and a reverse primer, FXCER2 (SEQ ID NO:7), to amplify an 86 bp region of the marker sequence. Thus, preferred oligonucleotides which may be used as amplification primers include SEQ ID NO:6 (5'-GATGGAGGAGCTGGTGGTGG -3') and SEQ ID NO:7 (5'-GGAAGGGCGAAGATGGGG -3'). Other preferred oligonucleotide primers are approximately 15-100 nucleotides in length and comprise SEQ ID NO:6 or SEQ ID NO:7. Still other preferred oligonucleotide primers include an oligonucleotide sequence that hybridizes to the complement of a 15-100 nucleotide sequence including SEQ ID NO:6 or SEQ ID NO:7. Such oligonucleotides may be substantially purified.

[0109] In another embodiment, a primer pair is designed to amplify a marker sequence flanking the tandem repeat region of the FMR1 gene following size separation of nucleic acids fragmented by SphI and BmtI. In one example, a primer pair is used to amplify a region of the flanking sequence downstream of the tandem repeat region; more specifically using a forward primer, FXCEF3 (SEQ ID NO:8), and a reverse primer, FXCER3 (SEQ ID NO:9), to amplify an 86 bp region of the marker sequence. Thus, preferred oligonucleotides which may be used as amplification primers include SEQ ID NO:8 (5'-CGTGACGTGGTTTCAGTGTTCACA -3') and SEQ ID NO:9 (5'-GGAAGTGAAACCGAAACGGAG -3'). Other preferred oligonucleotide primers are approximately 15-100 nucleotides in length and comprise SEQ ID NO:8 or SEQ ID NO:9. Still other preferred oligonucleotide primers include an oligonucleotide sequence that hybridizes to the complement of a 15-100 nucleotide sequence including SEQ ID NO:8 or SEQ ID NO:9. Such oligonucleotides may be substantially purified.

[0110] Assay controls may be used in the assay for detecting carriers and individuals afflicted with fragile X syndrome. Positive controls for normal or wild type FMR1 gene (*i.e.*, less than 55 tandem repeats), the premutation (55-200 tandem repeats), and the full mutation (greater than 200 tandem repeats) may be used.

[0111] Additional controls may be included in the assay to determine if the restriction enzyme digestion of the genomic DNA is complete. One approach to evaluate the completeness of digestion by a particular restriction enzyme is to determine if the digested DNA can support a PCR amplification using a test primer pair that spans the restriction

enzyme site used for digestion. Thus, if the nucleic acid has been fully digested by the restriction enzyme, there should be no amplification from the test primer pair, however, if digestion is incomplete, leaving some intact nucleic acid, the test primer pair should amplify the target. This test digestion PCR amplification can be conducted anytime after digestion including during amplification of the marker sequence.

Table 3. Exemplary control primers

Primer Name	SEQ ID NO:	Primer Sequence
AluIF	10	5'-CTCCAATGCCTCCTGCGTCC-3'
AluIR	11	5'-GGGGGTAGGGAGTGTCTGAGAGTCT-3'
BlpIF	12	5'- AGTGTTTAGAAGGAAAAGGCTGAGC-3'
BlpIR	13	5'- GCCCAAAGTTTCATAGGTAGCAAA -3'
LargeF	14	5'-CACCCCTACAAGCCGTCGCTAACA-3'
LargeR	15	5'-CGTGCCTTGTCGGTATCATTAGCAA-3'
FIIctrl1F	16	5'- CTGAATTTGTTTGGTTTGATGATGC-3'
FIIctrl1R	17	5'- CCTGTGTTATCTGTGCCCATTTTAA-3'
FIIctrl3F	18	5'- /6-FAM/ATCTGGGTCTGAATAATGTGAGGAG -3'
FIIctrl3R	19	5'- CCTAACTTTTCATTCTTGTCACCCTT-3'
LgctrlF	20	5'- /6-FAM/AGGTTTGAGTGTATCGCCTGATAGA -3'
LgctrlR	21	5'- TGAGTTTCATGTTTGCTCTTGCTC -3'
AluIFtaq	22	5'-GCCTCCTGCGTCCTTGTAAGA-3'
AluIRtaq	23	5'-TGAGAGTCTTGTTTCAGCAGTGTTAA-3'
LargeFtaq	24	5'-CAAGCCGTCGCTAACAAGGA-3'
LargeRtaq	25	5'-GATGTCTTGATGGTGCCCTCAT-3'

[0112] In a particular embodiment of an assay to distinguish normal individuals from carriers and affected individuals of fragile X syndrome, the genomic DNA is digested with *AluI*. Thus, the completion of the digestion of genomic DNA by *AluI* can be determined by amplifying a region containing an *AluI* recognition site. In one example of such a control, a pair of primers, AluIF and AluIR (SEQ ID NO:10 and SEQ ID NO:11, respectively) are used to amplify a 103 bp target segment of a nucleic acid fragment containing an *AluI* recognition site. Amplification of this segment will only occur when *AluI* digestion is incomplete. In another embodiment of this assay, the genomic DNA is digested with *BlpI* and *MlyI*. Thus, the completion of the digestion of genomic DNA by *BlpI* and *MlyI* can be determined by amplifying a region containing a *BlpI* or an *MlyI* recognition site. In one example of such a control, a pair of primers, BlpIF and BlpIR (SEQ ID NO:12 and SEQ ID NO:13, respectively) are used to amplify a 138 bp segment of target segment of nucleic acid

containing a *BlpI* recognition site. Amplification of this segment will only occur when *BlpI* digestion is incomplete.

[0113] Additional controls may be included in the assay to verify proper size-separation of fragmented DNA. Using sequence analysis tools known to those of skill in the art, one could determine the size distribution of fragments obtained using a particular restriction enzyme. One could then identify a specific control fragment having a size that corresponds to the size range of a particular fraction. Thus one could verify proper size separation of the fragmented DNA by detecting the control fragment in the appropriate fraction for its size. One could use a single control fragment in one fraction, a control in each fraction relevant to the determination of a normal versus abnormal tandem repeat, or a control fraction in all fractions.

[0114] In a particular embodiment of an assay to distinguish individuals having a normal FMR1 allele from those having a premutation FMR1 allele and those having a full mutation FMR1 allele, a control amplification is also included to determine if the largest tandem repeat-containing fragments (obtained through digestion with *AluI*) are collected into the size-appropriate fraction. In one example of such a control, a pair of primers, LargeF and LargeR (SEQ ID NO:14 and SEQ ID NO:15, respectively) will amplify a 121 bp segment of an 8,479 bp *AluI* fragment of the USP41 gene when present in a fraction. In another embodiment of the assay, in which the genomic DNA is digested with *BlpI* and *MlyI*, controls are included to detect proper size separation of fragments of 675 bp, 905 bp, and 7,031 bp. In one example, a pair of primers, FIIctrl1F and FIIctrl1R (SEQ ID NO:16 and SEQ ID NO:17, respectively) will amplify a 102 bp segment of a 675 bp *BlpI* / *MlyI* fragment of the CFTR gene when present in a fraction. In another example, a pair of primers, FIIIctrl3F and FIIIctrl3R (SEQ ID NO:18 and SEQ ID NO:19, respectively) will amplify a 113 bp segment of a 905 bp *BlpI* / *MlyI* fragment of the CFTR gene when present in a fraction. In another example, a pair of primers, LgctrlF and LgctrlR (SEQ ID NO:20 and SEQ ID NO:21, respectively) will amplify a 156 bp segment of a 7,031 bp *BlpI* / *MlyI* fragment from chromosome 21 (21:20912766-20919795) when present in a fraction.

Detection of Marker Sequences

[0115] Marker sequences may be amplified prior to detection or may be detected directly after size separation without an amplification step. In some embodiments, the marker sequence is amplified and the resulting amplicon is detected by electrophoresis, preferably capillary electrophoresis. In preferred embodiments, the marker sequence is amplified using a labeled primer such that the resulting amplicon is detectably labeled. In preferred embodiments, the primer is fluorescently labeled, however, the primers may be labeled according to the methods described below for oligonucleotide probes.

[0116] In preferred embodiments, the fragmented DNA is detected directly, without an amplification step, using two distinguishably-labeled nucleic acid probes which hybridize to two separate segments of a marker sequence upstream or downstream of the tandem repeats. The simultaneous detection of both labels in one hybridization complex indicates the presence of the marker sequence (and thus the associated tandem repeat). In one embodiment, detection is accomplished using a Trilogy 2020 Analyzer (US Genomics Woburn, MA). In this embodiment, two probes with distinguishable fluorescent labels are contacted with the size-separated DNA fragments. The resulting mixture of hybridization complexes is directed into a capillary tube where it is exposed to multiple lasers of differing wavelengths. The fluorescent labels of the probes are excited such that photons are emitted and detected. The simultaneous detection of fluorescent labels of different colors indicates the presence of the target region.

[0117] Probe oligonucleotides may be detectably labeled by methods known in the art. Useful labels include, e.g., fluorescent dyes (e.g., Cy5®, Cy3®, FITC, rhodamine, lanthamide phosphors, Texas red), ^{32}P , ^{35}S , ^3H , ^{14}C , ^{125}I , ^{131}I , electron-dense reagents (e.g., gold), enzymes, e.g., as commonly used in an ELISA (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase), colorimetric labels (e.g., colloidal gold), magnetic labels (e.g., Dynabeads™), biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available. Other labels include ligands or oligonucleotides capable of forming a complex with the corresponding receptor or oligonucleotide complement, respectively. The label can be directly incorporated into the nucleic acid to be detected, or it can be attached to a probe (e.g., an oligonucleotide) or antibody that hybridizes or binds to the nucleic acid to be detected.

[0118] In preferred embodiment the detectable label is a fluorophore. The term “fluorophore” as used herein refers to a molecule that absorbs light at a particular wavelength (excitation frequency), and subsequently emits light of a different, typically longer, wavelength (emission frequency) in response. Suitable fluorescent moieties include the following fluorophores known in the art:

4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid
acridine and derivatives:

acridine

acridine isothiocyanate

Alexa Fluor® 350, Alexa Fluor® 488, Alexa Fluor® 546, Alexa Fluor® 555, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 647 (Molecular Probes)

5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS)

4-amino-N-[3-vinylsulfonyl]phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS)

N-(4-anilino-1-naphthyl)maleimide

anthranilamide

Black Hole Quencher™ (BHQT™) dyes (biosearch Technologies)

BODIPY® R-6G, BOIPY® 530/550, BODIPY® FL

Brilliant Yellow

coumarin and derivatives:

coumarin

7-amino-4-methylcoumarin (AMC, Coumarin 120)

7-amino-4-trifluoromethylcoumarin (Coumarin 151)

Cy2®, Cy3®, Cy3.5®, Cy5®, Cy5.5®

cyanosine

4',6-diaminidino-2-phenylindole (DAPI)

5', 5''-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red)

7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin

diethylenetriamine pentaacetate

4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid

4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid

5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansyl chloride)

4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL)

4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC)

Eclipse™ (Epoch Biosciences Inc.)

eosin and derivatives:

eosin

eosin isothiocyanate

erythrosin and derivatives:

erythrosin B

erythrosin isothiocyanate

ethidium

fluorescein and derivatives:

5-carboxyfluorescein (FAM)

5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF)

2',7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE)

fluorescein

fluorescein isothiocyanate (FITC)

hexachloro-6-carboxyfluorescein (HEX)

QFITC (XRITC)

tetrachlorofluorescein (TET)

fluorescamine

IR144

IR1446

Malachite Green isothiocyanate

4-methylumbelliferone

ortho cresolphthalein

nitrotyrosine

pararosaniline

Phenol Red

B-phycoerythrin, R-phycoerythrin

o-phthaldialdehyde

Oregon Green®

propidium iodide

pyrene and derivatives:

pyrene
pyrene butyrate
succinimidyl 1-pyrene butyrate
QSY® 7, QSY® 9, QSY® 21, QSY® 35 (Molecular Probes)
Reactive Red 4 (Cibacron® Brilliant Red 3B-A)
rhodamine and derivatives:
6-carboxy-X-rhodamine (ROX)
6-carboxyrhodamine (R6G)
lissamine rhodamine B sulfonyl chloride
rhodamine (Rhod)
rhodamine B
rhodamine 123
rhodamine green
rhodamine X isothiocyanate
sulforhodamine B
sulforhodamine 101
sulfonyl chloride derivative of sulforhodamine 101 (Texas Red)
N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA)
tetramethyl rhodamine
tetramethyl rhodamine isothiocyanate (TRITC)
riboflavin
rosolic acid
terbium chelate derivatives

[0119] Other fluorescent nucleotide analogs can be used, see, e.g., Jameson, Meth. Enzymol. 278:363-390, 1997; Zhu, Nucl. Acids Res. 22:3418-3422, 1994. U.S. Patent Nos. 5,652,099 and 6,268,132 also describe nucleoside analogs for incorporation into nucleic acids, e.g., DNA and/or RNA, or oligonucleotides, via either enzymatic or chemical synthesis to produce fluorescent oligonucleotides. U.S. Patent No. 5,135,717 describes phthalocyanine and tetrabenztriazaporphyrin reagents for use as fluorescent labels.

[0120] The detectable label can be incorporated into, associated with or conjugated to a nucleic acid. Label can be attached by spacer arms of various lengths to reduce potential

steric hindrance or impact on other useful or desired properties. See, *e.g.*, Mansfield, *Mol. Cell. Probes* 9:145-156, 1995.

[0121] Detectable labels can be incorporated into nucleic acids by covalent or non-covalent means, *e.g.*, by transcription, such as by random-primer labeling using Klenow polymerase, or nick translation, or, amplification, or equivalent as is known in the art. For example, a nucleotide base is conjugated to a detectable moiety, such as a fluorescent dye, *e.g.*, Cy3® or Cy5® and then incorporated into genomic nucleic acids during nucleic acid synthesis or amplification. Nucleic acids can thereby be labeled when synthesized using Cy3®- or Cy5®-dCTP conjugates mixed with unlabeled dCTP.

[0122] Nucleic acid probes can be labeled by using PCR or nick translation in the presence of labeled precursor nucleotides, for example, modified nucleotides synthesized by coupling allylamine-dUTP to the succinimidyl-ester derivatives of the fluorescent dyes or haptens (such as biotin or digoxigenin) can be used; this method allows custom preparation of most common fluorescent nucleotides, see, *e.g.*, Henegariu, *Nat. Biotechnol.* 18:345-348, 2000.

[0123] Nucleic acid probes may be labeled by non-covalent means known in the art. For example, Kreatech Biotechnology's Universal Linkage System® (ULS®) provides a non-enzymatic labeling technology, wherein a platinum group forms a co-ordinative bond with DNA, RNA or nucleotides by binding to the N7 position of guanosine. This technology may also be used to label proteins by binding to nitrogen and sulphur containing side chains of amino acids. See, *e.g.*, U.S. Patent Nos. 5,580,990; 5,714,327; and 5,985,566; and European Patent No. 0539466.

[0124] The binding of a probe to the marker sequence flanking the tandem repeat region may be determined by hybridization as is well known in the art. Hybridization may be detected in real time or in non-real time.

[0125] One general method for real time PCR uses fluorescent probes such as the TaqMan® probes, molecular beacons and scorpions. The probes employed in TaqMan® and molecular beacon technologies are based on the principle of fluorescence quenching and involve a donor fluorophore and a quenching moiety.

[0126] The term “donor fluorophore” as used herein means a fluorophore that, when in close proximity to a quencher moiety, donates or transfers emission energy to the quencher. As a result of donating energy to the quencher moiety, the donor fluorophore will itself emit less light at a particular emission frequency that it would have in the absence of a closely positioned quencher moiety.

[0127] The term “quencher moiety” as used herein means a molecule that, in close proximity to a donor fluorophore, takes up emission energy generated by the donor and either dissipates the energy as heat or emits light of a longer wavelength than the emission wavelength of the donor. In the latter case, the quencher is considered to be an acceptor fluorophore. The quenching moiety can act via proximal (i.e. collisional) quenching or by Förster or fluorescence resonance energy transfer (“FRET”). Quenching by FRET is generally used in TaqMan® probes while proximal quenching is used in molecular beacon and scorpion type probes.

[0128] In proximal quenching (a.k.a. “contact” or “collisional” quenching), the donor is in close proximity to the quencher moiety such that energy of the donor is transferred to the quencher, which dissipates the energy as heat as opposed to a fluorescence emission. In FRET quenching, the donor fluorophore transfers its energy to a quencher which releases the energy as fluorescence at a higher wavelength. Proximal quenching requires very close positioning of the donor and quencher moiety, while FRET quenching, also distance related, occurs over a greater distance (generally 1 –10 nm, the energy transfer depending on R^{-6} , where R is the distance between the donor and the acceptor). Thus, when FRET quenching is involved, the quenching moiety is an acceptor fluorophore that has an excitation frequency spectrum that overlaps with the donor emission frequency spectrum. When quenching by FRET is employed, the assay may detect an increase in donor fluorophore fluorescence resulting from increased distance between the donor and the quencher (acceptor fluorophore) or a decrease in acceptor fluorophore emission resulting from increased distance between the donor and the quencher (acceptor fluorophore).

[0129] TaqMan® probes (Heid et al., 1996) use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target or marker sequences in DNA samples. TaqMan® probes are oligonucleotides that contain a donor fluorophore usually at or near the 5' base, and a quenching moiety typically at or near the 3' base. The quencher moiety may be

a dye such as TAMRA or may be a non-fluorescent molecule such as 4-(4 - dimethylaminophenylazo)benzoic acid (DABCYL). See Tyagi et al., *Nature Biotechnology* 16:49-53 (1998). When irradiated, the excited fluorescent donor transfers energy to the nearby quenching moiety by FRET rather than fluorescing. Thus, the close proximity of the donor and quencher prevents emission of donor fluorescence while the probe is intact.

[0130] TaqMan® probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan® probe is bound, its 5' exonuclease activity cleaves the probe. This ends the activity of quencher (no FRET) and the donor fluorophore starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR product is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). If the quencher is an acceptor fluorophore, then accumulation of PCR product can be detected by monitoring the decrease in fluorescence of the acceptor fluorophore.

[0131] TaqMan® assay uses universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridizes to the target, the fluorescence detected originates from specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of the product. One specific requirement for fluorogenic probes is that there be no G at the 5' end. A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage.

[0132] Other methods of probe hybridization detected in real time can be used for detecting amplification a target or marker sequence flanking a tandem repeat region. For example, the commercially available MGB Eclipse™ probes (Epoch Biosciences), which do not rely on a probe degradation can be used. MGB Eclipse™ probes work by a hybridization-triggered fluorescence mechanism. MGB Eclipse™ probes have the Eclipse™ Dark Quencher and the MGB positioned at the 5'-end of the probe. The fluorophore is located on the 3'-end of the probe. When the probe is in solution and not hybridized, the three dimensional conformation brings the quencher into close proximity of the fluorophore, and the fluorescence is quenched. However, when the probe anneals to a target or marker sequence, the probe is unfolded, the quencher is moved from the fluorophore, and the resultant fluorescence can be detected.

[0133] Suitable donor fluorophores include 6-carboxyfluorescein (FAM), tetrachloro-6-carboxyfluorescein (TET), 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC), and the like. Suitable quenchers include tetra-methylcarboxyrhodamine (TAMRA) 4-(4-dimethylaminophenylazo)benzoic acid ("DABCYL" or a DABCYL analog) and the like. Tetramethylrhodamine (TMR) or 5-carboxyrhodamine 6G (RHD) may be combined as donor fluorophores with DABCYL as quencher. Multiplex TaqMan assays can be performed using multiple detectable labels each comprising a different donor and quencher combination. Probes for detecting amplified sequence in real time may be stored frozen (-10° to -30°C) as 100 µM stocks. TaqMan probes are available from Applied BioSystems (4316032).

[0134] In a preferred embodiment, real time PCR is performed using TaqMan® probes in combination with a suitable amplification/analyzer such as the ABI Prism 7900HT Sequence Detection System. The ABI PRISM® 7900HT Sequence Detection System is a high-throughput real-time PCR system that detects and quantitates nucleic acid sequences. Briefly, TaqMan™ probes specific for the amplified target or marker sequence are included in the PCR amplification reaction. These probes contain a reporter dye at the 5' end and a quencher dye at the 3' end. Probes hybridizing to different target or marker sequences are conjugated with a different fluorescent reporter dye. During PCR, the fluorescently labeled probes bind specifically to their respective target or marker sequences; the 5' nuclease activity of Taq polymerase cleaves the reporter dye from the probe and a fluorescent signal is generated. The increase in fluorescence signal is detected only if the target or marker sequence is complementary to the probe and is amplified during PCR. A mismatch between probe and target greatly reduces the efficiency of probe hybridization and cleavage. The ABI Prism 7700HT or 7900HT Sequence detection System measures the increase in fluorescence during PCR thermal cycling, providing "real time" detection of PCR product accumulation.

[0135] Real Time detection on the ABI Prism 7900HT or 7900HT Sequence Detector monitors fluorescence and calculates Rn during each PCR cycle. The threshold cycle, or Ct value, is the cycle at which fluorescence intersects the threshold value. The threshold value is determined by the sequence detection system software or manually.

[0136] Oligonucleotide probes can be designed which are between about 10 and about 100 nucleotides in length and hybridize to the amplified region. Oligonucleotide probes are preferably 12 to 70 nucleotides; more preferably 15-60 nucleotides in length; and most

preferably 15-25 nucleotides in length. The probe may be labeled. In one example, SEQ ID NO:26 can be used as an oligonucleotide probe to detect a marker sequence associated with the tandem repeat region of the FMR1 gene (following genomic fragmentation by *AluI*), when the marker sequence is amplified by forward and reverse primers as set forth in SEQ ID NO:4 and SEQ ID NO:5, respectively. SEQ ID NO:27 can be used to detect an *AluI* control fragment amplicons amplified by *AluIFtaq* and *AluIRtaq* (SEQ ID NOs:22 and 23, respectively) and . SEQ ID NO:28 can be used to detect the 8,479 bp *AluI* control fragment amplicon, as amplified by *LargeFtaq* and *LargeRtaq* (SEQ ID NOs: 24 and 25, respectively).

Table 4. TaqMan Probes

Probe Name	SEQ ID NO:	Probe Sequence
CGG flanking	26	5'-6-FAM-CTCTGAGCGGGCG-3'
<i>AluI</i> control	27	5'-VIC-AGGAAGCTAAGCAGTTG-3'
Large control	28	5'-NED-ACTGTACACAACATCG-3'

[0137] Amplified fragments may be detected using standard gel electrophoresis methods. For example, in preferred embodiments, amplified fractions are separated on an agarose gel and stained with ethidium bromide by methods known in the art to detect amplified fragments.

Sizing by PCR Amplification and Electrophoresis

[0138] In certain embodiments, methods involving amplification of the tandem repeat region are used to measure the size of that region. In some embodiments, such methods are used as a screen prior to the use of a second method for sizing the tandem repeat region. In preferred embodiments, the amplification is preferably done by PCR. In this method, the entire tandem repeat region is amplified. The resulting amplicons are sized using electrophoresis, preferably capillary electrophoresis.

[0139] In one example, forward primer FX-5F (SEQ ID NO:29; 5'-GCT CAG CTC CGT TTC GGT TTC ACT TCC GGT 3') is used in an amplification reaction with reverse primer FX-3F(SEQ ID NO:30; 5'-AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA-3') to amplify the tandem repeat region of the FMR1 gene. Preferably, one of the primers of this primer pair is labeled, preferably the label is a fluorescent label. Amplification product may

be detected and sized by electrophoresis, preferably capillary electrophoresis. Alternatively, amplification products can be detected and sized using Southern blot.

Correlation of Fragment Size to Normal or Carrier/Affected Status

[0140] The fraction in which the marker sequence upstream or downstream of the tandem repeat region is detected corresponds to the size of the fragment containing the tandem repeat. This correlation enables an estimation of the number of tandem repeats and thus, whether an individual is normal or carries an allele having an expansion in the tandem repeat region.

[0141] In some embodiments, individuals that are afflicted with a disease associated with a mutation in the tandem repeat region of a gene can be distinguished from those that are normal. A nucleic acid sample from the individual is fragmented to produce nucleic acid fragments in which the tandem repeat segment of the gene is associated with a marker sequence in a fragment. The fragments are separated into fractions according to size under conditions in which the fragment(s) containing the tandem repeat segment will be located in the fractions according to the number of repeats in the tandem repeat segment, and identifying those fraction(s) containing the segment by detecting the marker sequence. The fractions are chosen so that one fraction corresponds to tandem repeat regions having a normal number of repeats (i.e., a normal allele) and another fraction that corresponds an abnormal number of repeats (i.e., a mutated allele). If only the former fraction is positive, the individual is normal; if only the latter fraction is positive, the individual may be a carrier or may be afflicted with the disease. A positive result in both fraction indicates a heterozygote and the individual may or may not be affected, depending on whether the disease is dominant or recessive. If the disease is dominant, heterozygotes will be affected; if the disease is recessive, the heterozygote will not be affected but will be a carrier of the disease.

[0142] In other embodiments, individuals that have a normal allele can be distinguished from those that have a premutation or a full mutation in the tandem repeat region of a gene. A nucleic acid sample from the individual is fragmented to produce nucleic acid fragments in which the tandem repeat segment of the gene is associated with a marker sequence in a fragment. The fragments are separated into fractions according to size under conditions in which the fragment(s) containing the tandem repeat segment will be located in the fractions

according to the number of repeats in the tandem repeat segment, and identifying those fraction(s) containing the segment by detecting the marker sequence. The fractions are chosen so that a first fraction corresponds to tandem repeat regions having a normal number of repeats (i.e., a normal allele), a second fraction corresponds to tandem repeat regions having a number of repeats in a premutation (i.e., a premutation allele), and a third fraction that corresponds to tandem repeat regions having a number of repeats in a full mutation (i.e., a full mutation allele). Generally, if only the first fraction is positive, the individual is normal; if only the second fraction is positive, the individual carries the premutation, and if only the third fraction is positive, the individual is affected with the disease. A positive result in more than one fraction indicates a heterozygote. A heterozygote may be a carrier or an affected individual depending on the gene involved and the dominance of the disease.

[0143] In some embodiments, individuals having a mutation associated with fragile X syndrome (i.e., a full mutation in the FMR1 gene) can be distinguished from individuals having a premutation or a normal allele. A nucleic acid sample from the individual is fragmented to produce nucleic acid fragments in which the tandem repeat segment of the FMR1 gene is associated with a marker sequence in a fragment. The fragments are separated into fractions according to size under conditions in which the fragment(s) containing the tandem repeat segment will be located in the fractions according to the number of repeats in the tandem repeat segment, and identifying those fraction(s) containing the segment by detecting the marker sequence. The fractions are chosen so that a first fraction corresponds to tandem repeat regions having a normal number of repeats (i.e., a normal allele), a second fraction corresponds to tandem repeat regions having a number of repeats in a premutation (i.e., a premutation allele), and a third fraction that corresponds to tandem repeat regions having a number of repeats in a full mutation (i.e., a full mutation allele). In preferred embodiments, the fractions are chosen so that the first fraction corresponds to tandem repeat regions having less than 55 repeats, the second corresponds to tandem repeat regions having 55-200 repeats, and the third corresponds to tandem repeat regions having greater than 200 repeats. Therefore, if the first fraction is positive (i.e., the marker sequence was detected in this fraction), it indicates that the tandem repeat region contains less than 55 repeats (i.e., a normal allele), if the second fraction is positive, it indicates that the tandem repeat region contains 55-200 repeats (i.e., a premutation allele), and if the third fraction is positive, it indicates that the tandem repeat contains greater than 200 repeats (i.e., a full mutation allele).

In male individuals, a phenotype or disease status may be assigned based on these results. Males generally possess only one X chromosome (the chromosome which contains the FMR1 gene), therefore, if the first fraction is positive, the individual is normal; if the second fraction is positive, the individual is a carrier of the premutation; if the third fraction is positive, the individual is afflicted with fragile X. Female individuals possess two X chromosomes, therefore, females possessing two normal alleles are normal and females possessing a premutation allele or a full mutation allele are carriers. Females heterozygous for a normal allele and a full mutation allele may or may not be affected, depending on other factors such as methylation status of the gene.

[0144] In certain embodiments of an assay to distinguish individuals having a normal tandem repeat region of the FMR1 gene from those having a premutation or full mutation, two fractions of *AluI* fragmented genomic DNA are separated by capillary electrophoresis and collected by automatic fraction collector, one between 211 – 400 bp, one between 401 bp – 9 kb. *AluI* fragments having 6-68 repeats will be present in the lower fraction, thus normal alleles and those with small premutations (i.e., 55-68 repeats) will be separated into this fraction. Normal alleles and small premutations can be further distinguished by amplification of the tandem repeat region and sizing with electrophoresis. The premutation, encompassing a range of 69-200 repeats, and the full mutation, encompassing 201-2000+ repeats will be present in the upper fraction. Therefore, if the lower fraction is positive (i.e., the marker sequence was detected in this fraction), it indicates that there is a CGG tandem repeat region that contains 6-68 repeats; if the upper fraction is positive, it indicates that there is CGG tandem repeat region that contains 68-2000+ repeats. A positive result in both fractions would indicate a heterozygote in which one allele is normal and the other allele contains the premutation or the full mutation.

[0145] In other embodiments of the assay to determine size of the tandem repeat region of the FMR1 gene, DNA fragmented with a combination of *BlnI* and *dMlyI* is separated into four fractions corresponding to sizes of approximately less than or equal to 603 bp (first/lowest fraction), 604-840 bp (second fraction), 841-1078 bp (third fraction) and 1079 bp – 9 kb (fourth/highest fraction). Fragments from samples containing a normal FMR1 gene (i.e., less than 55 tandem repeats) and those containing premutations having 56-62 tandem repeats will separate into the first/lowest fraction. Normal alleles and small premutations can be further

distinguished by amplification of the tandem repeat region and sizing with electrophoresis. Fragments from FMR1 genes containing small premutations (*i.e.*, 63-140 tandem repeats) will separate into the second fraction, whereas fragments from FMR1 genes containing large premutations (*i.e.*, 141-200 tandem repeats) and full mutations having 201-220 tandem repeats will separate into the third fraction, and whereas large premutations having greater than 220 tandem repeats will separate into the fourth/highest fraction. Large premutations 141-200 repeats can be distinguished from full mutations of 201-220 using, for example, standard Southern blot methods.

[0146] In another embodiment, the *AluI* digested genomic DNA is size fractionated using capillary electrophoresis into a multiplicity of fractions. Automated fraction collection is accomplished using a preset fraction time window of approximately 30 seconds per fraction, beginning at 200 bp and ending at 9 Kb. Approximately 16 fractions are collected. The fraction or fractions that are positive for detection of the marker sequence upstream or downstream of the tandem repeat region correspond to a size range and thus, the number tandem repeats can be estimated.

Gender Determination

[0147] In some embodiments, a nucleic acid assay to determine gender is combined with an assay to determine the length of the tandem repeat region of the FMR1 gene. In preferred embodiments, the nucleic acid assay includes DNA amplification. Such DNA amplification assays may target amplification of sequences specific to the Y chromosome (e.g., the SRY locus (Sinclair, et al., Nature 346:240 244, 1990)). In this case, amplification only occurs in the presence of a Y chromosome, indicating the nucleic acids are from a male. The absence of amplification suggests the nucleic acids are from a female. However, in these assays, a positive control is preferably included to detect false negatives.

[0148] In other examples, certain genes which occur on both the X chromosome and the Y chromosome but having different lengths depending on whether the gene occurs on the X chromosome or the Y chromosome, may be targeted for amplification. In this example, a region encompassing the segment of the gene which differs between the X and Y chromosomes would be amplified. This results in amplification products having different sizes, corresponding to the template nucleic acid (*i.e.*, the X chromosome or the Y

chromosome). Thus, amplification of nucleic acids from males would result in amplicons of both sizes, whereas samples from females would result in amplicons having only one size.

[0149] In a preferred embodiment, the amelogenin gene is targeted for gender determination. Sequence differences between the X and Y homologs of the amelogenin gene have been used to differentiate males from females. For example, two primer sets spanning a 6 base pair (bp) deletion of the amelogenin gene on the X chromosome have been used to generate fragments of 106/112 bp or 212/218 bp for X/Y products, respectively (Sullivan et al., BioTechniques 15:636-9, 1993). In preferred embodiments, the following primers are used to amplify a region of the amelogenin gene:

AMLF2 primer, 5'-AGTACTTGACCACCTCCTGATCTACAAGG 3' (SEQ ID NO:40) and
AMLR2 primer, 5'-TTTTTAACAGTTTACTTGCTGATAAACTCAYCCC 3' (SEQ ID NO:41).

This primer pair results in a 134 bp amplicon corresponding to the X chromosome homolog and a 140 bp amplicon corresponding to the Y chromosome. Thus, both amplicons would be generated by amplification of nucleic acids from males, whereas only one amplicon would be generated by amplification of nucleic acids from females.

[0150] The following examples serve to illustrate the present invention. These examples are in no way intended to limit the scope of the invention.

EXAMPLE 1

Restriction Enzyme Digestion

[0151] This example describes methods to detect expansion of the tandem repeat region of the FMR1 gene. Genomic DNA test samples and control samples of DNA were restriction endonuclease digested with *AluI*. 1.0 µg of test or control DNA was used for each digest. Genomic DNA was purified and diluted to a concentration of 50 ng/µL. The reaction mix for the digest was prepared according to the following table.

Table 5. *AluI* reaction mix

Reagent	(μ L/sample)
10X NEBuffer 2	3.0
<i>AluI</i> (10 U/ μ L)	1.0
Sterile H ₂ O	7.0
Total	10.0

[0152] 10 μ L of the *AluI* reaction mix was added to each sample of DNA. The samples were mixed by vortexing and spinning in a microfuge and are then incubated overnight at 37°C.

EXAMPLE 2

Size Separation of Digested DNA

[0153] A. Two fraction approach

[0154] Restriction enzyme digested DNA as described in Example 1 was separated into fractions according to size. 1 kb DNA ladder and the digested test genomic DNA samples were placed into a 96-well plate. The plate was subjected to auto-sampling and automatic fractionation using a Beckman Coulter P/ACE MDQ Series Capillary Electrophoresis System in reversed polarity separation mode. The ladder was first injected into the capillary with 6kv/60sec, then run with 200V/cm to determine the correct sizing cutoff time. Automated fraction collection was accomplished using a preset fractionation time window corresponding to 211-400 bp (lower fraction) and 401 bp – 9 kb (upper fraction). The separations are monitored on-column by UV detection. The data were acquired and evaluated by the P/ACE MDQ 32 Karat software package.

[0155] The digested samples were fractionated using the same conditions as the 1 kb DNA ladder. The lower fraction (211-396 bp) and the upper fraction (396 bp -9 kb) were collected based on the sizing cutoff times as determined using the 1 kb ladder for each size range. Fractions were collected using the P/ACE MDQ auto-collector and stored in a 96-well plate in 30 μ L 0.1X TBE buffer per well.

[0156] A. 16 fraction approach

[0157] Automated fraction collection was accomplished using a preset fractionation time window 30 seconds per fraction, starting roughly from 200bps and ending at 9Kbs. The separations were monitored on-column by UV detection. The data were acquired and evaluated by the P/ACE MDQ 32 Karat software package.

[0158] 1-kb DNA ladder and the digested genomic DNA samples were placed into a 96 well plate. The plate was subjected to autosampling, automatic fractionation on the Beckman Coulter P/ACE MDQ machine with cooling control. The ladder was first electrophoretically injected into the capillary with 6kV/60sec, then run with 200V/cm to determine the correct sizing window (preferably 200bps-9Kbs).

[0159] The digested samples were fractionated based on the same condition as the 1-kb DNA ladder running condition. A total 16 fractions were collected using P/ACE MDQ's auto collector and stored in a 96-well plate in 5 μ L dH₂O per well.

EXAMPLE 3

PCR Amplification and Detection of Fragments with Tandem Repeats

[0160] In an assay to detect mutations characterized by an expansion of the tandem repeat region of the FMR1 gene, working PCR master mixes were made as described.

[0161] A. PCR amplification (non-TaqMan)

[0162] A PCR Master Mix for amplifying fragments and for size separation analysis of PCR product was prepared as shown in Table 6.

Table 6. Preparation of PCR primer (non-Taqman) master mix for CCG 5' flanking sequence

Stock Solution	One Reaction (μ L)	500 Reactions (μ L)	Final Concentration
10X PCR Buffer (with 15 mM $MgCl_2$)	2.5	1250	1X (1.5 mM)
5X Q-Solution	5.00	2500	1X
25 mM $MgCl_2$	0.5	250	0.5 mM
25 mM dNTPs	0.2	100	0.2 mM
100 μ M FMR1F4 Primer (SEQ ID NO:4)	0.5	250	2.0 μ M
100 μ M FMR1R4 Primer (SEQ ID NO:5)	0.5	250	2.0 μ M
100 μ M AluIF Primer (SEQ ID NO:10)	0.1	50	0.4 μ M
100 μ M AluIR Primer (SEQ ID NO:11)	0.1	50	0.4 μ M
100 μ M LargeF Primer (SEQ ID NO:14)	0.10	50	0.4 μ M
100 μ M LargeR Primer (SEQ ID NO:15)	0.10	250	0.4 μ M
DMSO	1.25	625	5%
1M KCl_2	1.25	625	50mM
dH ₂ O	7.5	3750	
Total	19.6	9800	

[0163] The PCR primer master mix was stored in 1.5 mL aliquots at -20 °C prior to use. When ready for use, PCR reactions were prepared as shown in Table 7.

Table 7. Final. PCR amplification mixture for CCG 5' flanking sequence

Reagent	Per Rxn (μ L)	1/2 plate (μ L)	full plate (μ L)
Master Mix	19.6	940.8	1881.6
HotStar Taq	0.4	19.2	38.4
DNA fraction	5.0	-	-
Total	25.0	1200	2400

[0164] The final amplification mixtures were sealed tightly in plates with Microseal A film. The plates were vortexed briefly (approximately 5 sec) and spun down for approximately 30 sec in a plate centrifuge at 2,000-6,000g (1,600 rpm in a Sorvall T6000D centrifuge). The plate was transferred to the ABI 9700 thermal cycler.

[0165] The thermal cycler conditions for amplification were as follows:

Step 1	95°C for 15 minutes.
Step 2	95°C for 60 seconds
Step 3	64°C for 60 seconds.
Step 4	72°C for 30 seconds
Step 5	Steps 2-4 repeated, 34 times.
Step 6	72°C for 5 minutes.
Step 7	4°C indefinitely.

[0166] PCR products were then loaded onto the ABI 3100 genetic analyzer for detection.

[0167] B. TaqMan PCR amplification

[0168] A Taqman PCR master mix for detecting CCG 5' flanking sequence was prepared as shown in Table 8.

Table 8. Preparation of Taqman PCR master mix for CCG 5' flanking sequence

Stock Solution	One Reaction (μ L)	500 Reactions (μ L)	Final Concentration
5X Q-Solution	10.00	5000	1X
25 mM MgCl ₂	1.0	500	0.5 mM
100 μ M FMR1F4 Primer (SEQ ID NO:4)	1.0	500	2.0 μ M
100 μ M FMR1R4 Primer (SEQ ID NO:5)	1.0	500	2.0 μ M
100 μ M AluIFtaq Primer (SEQ ID NO:22)	0.40	200	0.8 μ M
100 μ M AluIRtaq Primer (SEQ ID NO:23)	0.40	200	0.8 μ M
100 μ M LargeFtaq Primer (SEQ ID NO:24)	0.40	200	0.8 μ M
100 μ M LargeRtaq Primer (SEQ ID NO:25)	0.40	200	0.8 μ M
DMSO	2.50	1250	5%
1M KCl ₂	2.50	1250	50mM
CGG Flanking Probe (SEQ ID NO:26)	0.10	100	0.2 μ M
AluI Control Probe (SEQ ID NO:27)	0.10	100	0.2 μ M
Large Control Probe (SEQ ID NO:28)	0.10	100	0.2 μ M
dH ₂ O	0.10	100	
Total	20	10000	

[0169] The Taqman PCR master mix was stored in 1.5 mL aliquots at -20 °C prior to use. When ready for use, Taqman PCR reactions were prepared as shown in Table 9.

Table 9. TaqMan PCR Master Mix

Reagents	1 reaction (μ L)	¼ plate (μ L)	½ plate (μ L)	¾ plate (μ L)	full plate (μ L)
TaqMan 2X Universal Master Mix	25.0	750	1600	2150	2750
Primer/Probe Mix	20.0	600	1280	1720	2200
Total	45.0	1350	2880	3870	4950

[0170] 45 μ L of the TaqMan PCR master mix was added to each well of the 96-well plate containing the fractions of size separated DNA. The wells are sealed tightly with Microseal A film. The plates were vortexed briefly (approximately 5 sec) and spun down for approximately 30 sec in a plate centrifuge at 2,000-6,000g (1,600 rpm in a Sorvall T6000D centrifuge). The plate was transferred to the ABI 7700 (or 7900HT) sequence detector.

[0171] The thermocycler conditions for TaqMan were as follows:

Step 1	95°C for 15 minutes.
Step 2	95°C for 60 seconds
Step 3	64°C for 60 seconds.
Step 4	72°C for 30 seconds
Step 5	Steps 2-4 repeated, 40 times.
Step 6	72°C for 5 minutes.
Step 7	4°C indefinitely.

EXAMPLE 4

Detection of Amplified Fractions by Gel Electrophoresis

[0172] Gel electrophoresis was used to identify the size of PCR amplified fragments prepared as described in Example 3A. 6 μ L of 6X FEB (ficoll, EDTA bromphenol blue loading dye) was added to 6 μ L PCR products and the resulting mixture was loaded into the gel. 50 bp DNA ladder was loaded into the first and last well of the gel. Samples were electrophoresed in 0.8% agarose at 200V for 1.5 hours. Completed gel was photographed with UV photodocumentation apparatus (Alpha Innotech Image Analysis System).

EXAMPLE 5

Detection of Expansion Mutations of the Tandem Repeat Region of the DM-1 Gene

[0173] In an assay to detect mutations characterized by an expansion of the tandem repeat region of the DM-1 gene, genomic DNA test samples are restriction endonuclease digested with *AluI*. Approximately 1.0 μ g of test genomic DNA is used for each digest. The reaction mix for the digest is prepared according to the enzyme supplier's protocol. The samples are mixed and incubated at 37°C to complete digestion.

[0174] The restriction enzyme digested DNA is separated according to size using capillary electrophoresis and two fractions are collected, such that the first fraction (250-360 bp) corresponds to the normal repeat range (*e.g.*, 5-37 repeats) and the second fraction (400 bp – 9 kb) corresponds to an expanded repeat mutation (*e.g.*, greater than 50 repeats). 1 kb DNA ladder is first injected into the capillary to determine the correct sizing cutoff time. Automated fraction collection is accomplished using a preset fractionation time window corresponding to a lower fraction and an upper fraction. The separations are monitored on-column by UV detection. The digested samples are then fractionated using the same conditions as the 1 kb DNA ladder. The lower fraction and the upper fraction are collected based on the sizing cutoff times as determined using the 1 kb ladder for each size range.

[0175] Each fraction is analyzed using the TaqMan real time PCR method for the presence of a fragment containing the DM-1 tandem repeat region. In this method, a segment of the 3'-untranslated region of DM-1 gene is amplified using a forward primer (*e.g.*, 5'-CCATTTCCTTTCTTTTCGGCCA-3'; SEQ ID NO:31) and a reverse primer (*e.g.*, 5'-AGGCCTGCAGTTTGCCC-3'; SEQ ID NO:32). The amplified fragment is detected with a TaqMan labeled probe, 5'-TGAGGCCCTGACGTGG-3' (SEQ ID NO:33).

[0176] The presence of the amplified segment in only the lower fraction is indicative of an individual homozygous for the normal DM-1 allele. The presence of the amplified segment in only the upper fraction is indicative of an individual homozygous for a mutant allele(s). The presence of the amplified segment in both fractions is indicative of a heterozygote.

EXAMPLE 6

Detection of Expansion Mutations of the Tandem Repeat Region of the FRDA Gene

[0177] In an assay to detect mutations characterized by an expansion of the tandem repeat region of the FRDA gene, genomic DNA test samples are restriction endonuclease digested with *AluI* and *RsaI*. Approximately 1.0 µg of test genomic DNA is used for each digest. The reaction mix for the digest is prepared according to the enzyme supplier's protocol. The samples are mixed and incubated at 37°C to complete digestion.

[0178] The restriction enzyme digested DNA is separated according to size using capillary electrophoresis and two fractions are collected, such that the first fraction (300-405 bp)

corresponds to the normal repeat range (*e.g.*, 7-34 repeats) and the second fraction (600 bp – 9 kb) corresponds to an expanded repeat mutation (*e.g.*, greater than 100 repeats). 1 kb DNA ladder is first injected into the capillary to determine the correct sizing cutoff time. Automated fraction collection is accomplished using a preset fractionation time window corresponding to a lower fraction and an upper fraction. The separations are monitored on-column by UV detection. The digested samples are then fractionated using the same conditions as the 1 kb DNA ladder. The lower fraction and the upper fraction are collected based on the sizing cutoff times as determined using the 1 kb ladder for each size range.

[0179] Each fraction is analyzed using the TaqMan real time PCR method for the presence of a fragment containing the FRDA tandem repeat region. In this method, a segment of the first intronic region of the FRDA gene is amplified using a forward primer (*e.g.*, 5'-AGGCCTAGGAAGGTGGATCAC-3'; SEQ ID NO:34) and a reverse primer (*e.g.*, 5'-ACCATGTTGGCCAGGTTAGTCT-3'; SEQ ID NO:35). The amplified fragment is detected with a TaqMan labeled probe, 5'-TGAGGTCCGGAGTTC-3' (SEQ ID NO:36).

[0180] The presence of the amplified segment in only the lower fraction is indicative of an individual homozygous for the normal FRDA allele. The presence of the amplified segment in only the upper fraction is indicative of an individual homozygous for a mutant allele(s). The presence of the amplified segment in both fractions is indicative of a heterozygote.

EXAMPLE 7

Detection of Expansion Mutations of the Tandem Repeat Region of the FMR1 Gene

[0181] In this example, expansion mutations of the tandem repeat region of the FMR1 gene were detected by fragmentation with *AluI*, size fractionation, followed by a second restriction enzyme digestion with *BstNI*. Thus, genomic DNA test samples were restriction endonuclease digested with *AluI* as described above in Example 1. The digested DNA was then fractionated into four fractions using capillary electrophoresis. Fraction 1 contains nucleic acids with 0-60 CCG tandem repeats, fraction 2 contains 60-200 CCG tandem repeats, fraction 3 contains 200-2000 CCG tandem repeats and fraction 4 contains 2000+ CCG tandem repeats. Following fractionation, an aliquot of each of the four fractions was digested with a second restriction endonuclease, *BstNI*, that cleaved the marker from the CCG tandem repeat region. Following the second nucleic acid fractionation, each of the four

fractions were subjected to PCR as described in Example 3B (*i.e.*, TaqMan PCR Amplification).

[0182] A. Second Restriction Enzyme Digestion of Samples from Affected Individuals

[0183] Affected samples were tested with and without a second restriction enzyme digestion in which the marker sequence was cleaved from the tandem repeat region. Samples having the second digestion had much stronger signals (*i.e.*, higher relative fluorescence unit (RFU) signals) as compared to samples that did not undergo a second enzyme digestion. Each sample was run in duplicate; data are shown below in table 9. These data suggest that there is increased amplification of the marker sequence when the marker sequence is cleaved from the tandem repeat region.

Table 10. Relative Fluorescence Unit (RFU) Signals of Affected Samples With (“with 2°”) and Without a Second Restriction Enzyme Digestion (“without 2°”).

Sample No.	Fraction 1	Fraction 2	Fraction 3	Fraction 4
15251 without 2°	75	0	445	0
<i>Duplicate</i>	0	0	0	0
15251 with 2°	1752	781	787	0
	2566	931	695	0
15050 without 2°	980	0	210	0
	259	0	318	0
15050 with 2°	3571	0	873	0
	2270	586	1042	0
14792 without 2°	575	0	328	0
	662	280	246	0
14792 with 2°	1223	243	658	0
	1308	450	967	0

[0184] B. Second Restriction Enzyme Digestion of Samples from Normal Individuals

[0185] Normal samples were tested with and without a second restriction enzyme digestion in which the marker sequence was cleaved from the tandem repeat region. Samples having less starting material that underwent the second digestion, had comparable or higher signals (i.e., higher relative fluorescence unit (RFU) signals) as compared to samples having more starting material that did not undergo a second enzyme digestion. Data are shown below in table 10. These data suggest that there is increased amplification of the marker sequence when the marker sequence is cleaved from the tandem repeat region.

Table 11. Relative Fluorescence Unit (RFU) Signals of Normal Samples With (“with 2°”) and Without a Second Restriction Enzyme Digestion (“without 2°”).

Sample No.	Fraction 1	Fraction 2	Fraction 3	Fraction 4
14028 without 2°	3044	0	0	0
14028 with 2°	4356	0	0	0
13616 without 2°	2701	0	0	0
13616 with 2°	2396	0	0	0
13488 without 2°	1399	0	0	0
13488 with 2°	2258	0	0	0
13524 without 2°	1051	0	0	0
13524 with 2°	1074	0	0	0

EXAMPLE 8

Detection of Expansion Mutations of the Tandem Repeat Region of the FMR1 Gene

[0186] In this example, expansion mutations of the tandem repeat region of the FMR1 gene are detected by fragmentation with *BlpI* and *MlyI*, size fractionation, followed by a second restriction enzyme digestion with *BmtI*.

[0187] Genomic DNA test samples are restriction endonuclease digested with *BlpI* and *MlyI* using approximately 1.5 µg of test or control DNA (purified and diluted to a concentration of 50 ng/µL) for each digest. The reaction mix for the digest is prepared according to the following table.

Table 12. *BlpI* / *MlyI* reaction mix

Reagent	(µL/sample)
Genomic DNA 50 ng/µL	30 µL
10X NEBuffer 4 (New England BioLabs)	3.5
<i>BlpI</i> (10 U/µL)	0.75
<i>MlyI</i> (10 U/µL)	0.75
Total	35.0

[0188] The samples are mixed by vortexing and spinning in a microfuge and are then incubated at 37°C for 16 hours and stored at 4°C. The digested DNA is then fractionated into four fractions using capillary electrophoresis using P/ACE MDQ Capillary Electrophoresis System. Fraction 1 (less than 603 bp) contains nucleic acids with 6-62 CCG tandem repeats, fraction 2 (603-840 bp) contains 63-140 CCG tandem repeats, fraction 3 (841-1078bp) contains 141-220 CCG tandem repeats and fraction 4 (1079 bp – 9 kb) contains 221-2000+ CCG tandem repeats. Following fractionation, an aliquot of each of the four fractions is digested with the restriction endonuclease, *BmtI*, which cleaves the marker from the CCG tandem repeat region. The *BmtI* reaction mix is prepared according to the following table.

Table 13. *BmtI* reaction mix

Reagent	(µL/sample)
Fractionated genomic DNA	30 µL
10X NEBuffer 2 (New England BioLabs)	3.0
<i>BmtI</i> (10 U/µL)	0.75
Total	33.75.0

[0189] The digestion reaction mixes are incubated at 37°C for 16 hours and stored at 4°C.

[0190] Following the second nucleic acid digestion, each of the four fractions are subjected to PCR using the following primers:

Table 14. Primers for PCR amplification of *BlnI* / *MlyI* fragments

Primer Name	SEQ ID NO:	Primer Sequence
FXCEF2	6	5'-/6-FAM/GATGGAGGAGCTGGTGGTGG-3'
FXCER2	7	5'-GGAAGGGCGAAGATGGGG-3'
BlnIF	12	5'-/HEX/AGTGTTTAGAAGGAAAAGGCTGAGC-3'
BlnIR	13	5'-GCCCAAAGTTTCATAGGTAGCAA-3'
FIIctrl1F	16	5'-/HEX/CTGAATTTGTTTGGTTTGATGATGC-3'
FIIctrl1R	17	5'-CCTGTGTTATCTGTGCCCATTTTAA-3'
FIHctrl3F	18	5'-/6-FAM/ATCTGGGTCTGAATAATGTGAGGAG-3'
FIHctrl3R	19	5'-CCTAACTTTCATTCTTGTCACCCTT-3'
LgctrlF	20	5'-/6-FAM/AGGTTTGAGTGTATCGCCTGATAGA-3'
LgctrlR	21	5'-TGAGTTTCATGTTTGCTCTTGCTC-3'

[0191] A PCR master mix for amplifying fragments for size analysis is prepared according to Table 15.

Table 15. PCR Master Mix

Reagent	Vol. for 1 reaction (μL)	Final Conc in PCR Reaction
Qiagen 10X Buffer	2.5	1X
Qiagen 25 mM MgCl ₂ *	0.75	2.25 mM
DMSO	1.25	5%(v/v)
1 M KCl**	1.25	105 mM
25 mM dNTP	0.2	0.2 mM
100 μM FXCEF2	0.25	1 μM
100 μM FXCER2	0.25	1 μM
100 μM BlnIF	0.015	0.06μM
100 μM BlnIR	0.015	0.06μM
100 μM lgctrlF	0.06	0.24μM
100 μM lgctrlR	0.06	0.24μM
100 μM FIIctrl1F	0.015	0.06μM
100 μM FIIctrl1R	0.015	0.06μM
100 μM FIHctrl3F	0.02	0.08μM
100 μM FIHctrl3R	0.02	0.08μM
Water	12.83	
Total	19.5	

*, ** 2.5 μL 10X PCR buffer contains 15 mM MgCl₂, 50 mM KCl. Total reaction MgCl₂ is 2.25 mM, KCl is 105mM.

[0192] A final PCR amplification mixture is made by adding 0.5 μ L HotStarTaq (Qiagen) is added to each individual PCR reaction followed by 5 μ L digested fractionated DNA. The final amplification mixtures are sealed tightly in plates with Microseal A film. The plates are vortexed briefly (approximately 5 sec) and spun down for approximately 30 sec in a plate centrifuge at 2,000-6,000g (1,600 rpm in a Sorvall T6000D centrifuge). The plate is transferred to the ABI 9700 thermal cycler.

[0193] The thermal cycler conditions for amplification are as follows:

Step 1	95°C for 15 minutes.
Step 2	95°C for 30 seconds
Step 3	55°C for 30 seconds.
Step 4	72°C for 60 seconds
Step 5	Steps 2-4 repeated, 33 times.
Step 6	72°C for 10 minutes.
Step 7	4°C indefinitely.

[0194] 2 μ L PCR product is combined with 10.5 μ L Hi-Di formamide (Applied Biosystems) with ROX 350 size standard (Applied Biosystems), heated at 95 °C for 5 minutes followed by 5 minutes on ice. Samples are then loaded onto the ABI 3100 genetic analyzer for detection.

EXAMPLE 9

Detection of Expansion Mutations of the Tandem Repeat Region of the FMR1 Gene

[0195] In this example, expansion mutations of the tandem repeat region of the FMR1 gene are detected by fragmentation with *SphI* and *BmtI*, size fractionation, followed by a second restriction enzyme digestion with *BstNI*. Thus, genomic DNA test samples are restriction endonuclease digested with *SphI* and *BmtI* using approximately 1.5 μ g of test or control DNA (purified and diluted to a concentration of 50 ng/ μ L) for each digest. The reaction mix for the digest is prepared according to the following table.

Table 16. *SphI* / *BmtI* reaction mix

Reagent	(μ L/sample)
Genomic DNA (50ng/ μ L)	30
10X NEBuffer 2 (New England BioLabs)	3.5
<i>SphI</i> (10 U/ μ L)	0.75
<i>BmtI</i> (10 U/ μ L)	0.75
Total	35.0

[0196] The samples are mixed by vortexing and spinning in a microfuge and are then incubated overnight at 37°C. The digested DNA is then fractionated into four fractions using capillary electrophoresis. Fraction 1 contains nucleic acids with 6-62 tandem repeats, fraction 2 contains 63-163 tandem repeats, fraction 3 contains 164-196 tandem repeats and fraction 4 contains greater than tandem repeats. Following fractionation, an aliquot of each of the four fractions is digested with a second restriction endonuclease, *BmtI*, that cleaved the marker from the CCG tandem repeat region. Following the second nucleic acid fractionation, each of the four fractions are subjected to PCR using the following primers:

Table 17. Primers for PCR amplification of *SphI* / *BmtI* fragments

Primer Name	SEQ ID NO:	Primer Sequence
FXCEF3	8	5'- CGTGACGTGGTTTCAGTGTTTACA -3'
FXCER3	9	5'- GGAAGTGAAACCGAAACGGAG - 3'

[0197] PCR is conducted using the following conditions:

- Step 1 95°C for 15 minutes.
- Step 2 95°C for 30 seconds
- Step 3 55°C for 30 seconds.
- Step 4 72°C for 60 seconds
- Step 5 Steps 2-4 repeated, 33 times.
- Step 6 72°C for 10 minutes.
- Step 7 4°C indefinitely.

[0198] PCR products are then loaded onto the ABI 3100 genetic analyzer for detection.

EXAMPLE 10

Identification of a Control Fragment for Use in the FMR1 Assay

[0199] An *AluI* fragment from a region of the genome distinct from FMR1, that was larger than 6000 bases, containing trinucleotide repeats, and having a high GC content and/or CpG islands was identified for use as a control fragment in the FMR1 assay. This fragment was identified as follows. All *AluI* sites in the human genome were identified using the EMBOSS Restrict program (Rice et al., "EMBOSS: The European Molecular Biology Open Software Suite." *Trends in Genetics* 16(6):276-7 (2000)), resulting in a predicted 11.5 million fragments produced by a digestion with *AluI*. From these fragments, 20 fragments having a length longer than 6000 bases were identified using the TACG program (Mangalam, HJ. "tacg - a grep for DNA." *BMC Bioinformatics* 3:8 (2002)). The sequences of these 20 fragments were obtained using EMBOSS ExtractSeq. Of these 20 fragments, one fragment corresponding to a region of the USP41 gene on chromosome 22 (i.e., chromosome 22:19033185-19041663) was found to have a large trinucleotide repeat region. The GC content was analyzed using EMBOSS geecee and the CpG island analysis was performed using EMBOSS cpgseek and newcpgreport.

EXAMPLE 11

Determination of the Size of the Tandem Repeat Region of FMR1 using PCR

[0200] To determine the size of the tandem repeat region of the FMR1 region, the region is amplified by the polymerase chain reaction in the presence of a fluorescently-labeled primer (e.g., 6-FAM) and the sizes of the resulting labeled amplicons are determined by capillary electrophoresis. A second trinucleotide repeat (CAG in the X-linked androgen receptor gene) is co-amplified using a primer pair in which one of the primers is fluorescently-labeled and co-analyzed to provide an internal amplification control.

[0201] Thus, the tandem repeat region of the FMR1 gene is amplified using FX-5F (SEQ ID NO:29) as the forward primer and FX-3F (SEQ ID NO:30) as the reverse primer, and trinucleotide repeat of the X-linked androgen receptor gene using AR-5F (SEQ ID NO:37) as

the forward primer and AR-R2 (SEQ ID NO:38) as the reverse primer, as set forth in the table below.

Table 18. Primers for PCR amplification

Primer Name	SEQ ID NO:	Primer Sequence
FX-5F	29	5'-(6-FAM) GCT CAG CTC CGT TTC GGT TTC ACT TCC GGT 3'
FX-3F	30	5'-AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA-3'
AR-5F	37	5'-(HEX) ACC AGG TAG CCT GTG GGG CCT CTA CGA TGG GC-3'
AR-R2	38	5'-GCT TTC CAG AAT CTG TTC CAG AGC GTG CGC GA-3'

[0202] A PCR master mix for amplifying fragments for size analysis is prepared according to Table 19.

Table 19. PCR Master Mix

Reagent	Vol. for 1 reaction (μL)	Vol. for 1000 reactions (μL)
10X Buffer (Qiagen)	1.0	1000
Qiagen 25 mM MgCl ₂ (Qiagen)	0.2	200
25 mM dNTP	0.08	80
100 μM FX-5F primer	0.04	40
100 μM FX-3F primer	0.04	40
100 μM AR-5F primer	0.04	40
100 μM AR-R2 primer	0.04	40
DMSO	0.2	200
5X Q (Betaine) solution	5.0	5,000
Water	1.16	1,160
Total	10.0	

[0203] The PCR master mix is aliquoted into 1,100 μL aliquots to which 5.5 μL of Taq polymerase (5 U/μL) from Qiagen and 22 μL of Pfu DNA polymerase (2.5 U/μL) from Stratagene are added to make the polymerase/master mix solution.

[0204] Genomic DNA is diluted to 20 ng/μL in TE buffer. Samples are heated to 93-97°C for 4-6 minutes and cooled on ice. 10 μL of polymerase/master mix solution is added to 2 μL (40 ng) diluted genomic DNA.

[0205] The samples are transferred to the ABI 9700 thermal cycler once the thermal cycler has reached 85°C +/- 2°C. The PCR conditions for amplification are as follows:

Step 1	95°C for 6 minutes
Step 2	95°C for 1 minute
Step 3	60°C for 2 minutes
Step 4	75°C for 5 minutes
Step 5	Steps 2-4 repeated, 31 times
Step 6	75°C for 13 minutes
Step 7	4°C indefinitely.

[0206] PCR products are then loaded onto the ABI 3100 genetic analyzer for detection.

EXAMPLE 12

Carrier screening for Fragile X Syndrome

[0207] In this example, samples from male and female individuals were screened for carrier status of fragile X syndrome by a two-step method, in which samples were initially screened with multiplex PCR to establish gender and size of the FMR1 region and were subjected to further sizing analysis based on the results of the multiplex PCR. All samples that were determined to be female and heterozygous for two normal alleles were not subjected to further analysis. All samples determined to be female and apparently homozygous at the FMR1 locus (24% of all analysis) were subjected to further analysis to determine the size of the FMR1 tandem repeat region. Samples identified as male and hemizygous for a normal FMR1 allele were not subjected to further analysis, whereas samples identified as male but exhibiting no FMR1 amplification are subjected to further analysis to determine the size of the FMR1 tandem repeat region.

[0208] Genomic DNA was extracted from 150 µL whole blood collected in EDTA anticoagulated blood collection vacuum tubes using an Xtractor Gene™ (Corbett Life Science, Mortlake, NSW, Australia) according to manufacturer's Whole Blood DNA Extraction Protocol. The final elution was carried out in 100 µL buffer to consistently yield concentrations of 50 – 100 ng/µL.

[0209] Genomic DNA samples were then analyzed by a multiplex PCR consisting of an amplification of the FMR1 tandem repeat region using FX-5F primer (FAM-labeled; SEQ ID NO:29) and FX-3F primer (SEQ ID NO:30); amplification of a region of the amelogenin

gene to establish gender using AMLF2 primer, 5'-AGTACTTGACCACCTCCTGATCTACAAGG 3' (FAM-labeled; SEQ ID NO:40) and AMLR2 primer, 5'-TTTTTAACAGTTTACTTGCTGATAAACTCAYCCC 3' (SEQ ID NO:41), and amplification the trinucleotide repeat of the X-linked androgen receptor using AR-5F (HEX-labeled SEQ ID NO:37) and AR-R2 primer (SEQ ID NO:38) as a positive internal control.

[0210] A PCR mastermix for the multiplex PCR was prepared consisting of 3.3 μ M of each of the above primers, 1X Qiagen Standard PCR buffer, 0.4 mM $MgCl_2$, 2% DMSO, 1 X Qiagen Q Solution, 0.2 mM dNTP, and 0.25 unit Qiagen Taq DNA polymerase (Qiagen, Valencia, CA), 0.5 unit Pfu DNA Polymerase (Stratagene, La Jolla, CA). One μ L of isolated DNA solution was added to 10 μ L of the multiplex primer mix to a final volume of 11 μ L. The PCR conditions were as follows: 95 °C for 6 min followed by 32 cycles of 95 °C for 1 min, 60 °C for 2 min, 75 °C for 5 min, and finally the amplified products were extended at 75 °C for 15 min. The PCR fragments were analyzed on an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) and fragment analysis accomplished with ABI GeneScan™ V3.7 and Genotyper™ V3.7 software (Applied Biosystems). The amelogenin primer pair results in a 134 bp amplicon corresponding to the X chromosome homolog and a 140 bp amplicon corresponding to the Y chromosome.

[0211] For the further analysis to determine size of the tandem repeat region of the FMR1 gene, genomic DNA was digested for 16 hours at 37°C with restriction enzymes BlnI and MlyI (New England BioLabs, Ipswich, MA, USA). Following incubation the restriction fragments were either pressure injected or vacuum injected onto a P/ACE™ MDQ capillary electrophoresis system with an UV/Vis Detector (Beckman Coulter, Fullerton, CA, USA). Undenatured double stranded DNA was separated at an electric field strength of 100 V/cm, in 1X TBE buffer (90 mM Tris-Borate, 2 mM EDTA, pH 8.3). Capillary temperature was maintained at 25 °C. Four fractions were collected into 0.1X TBE buffer (9 mM Tris-borate, 0.2 mM EDTA, pH 10) The initial fraction consisted of molecular weights between 400 bps to 600 bps; the second fraction included molecular weights of between 600 bps to 800 bps; the third fraction included molecular weights of 800 bps to 1,000 bps; and the fourth fraction included molecular weights of 1,000 bps to 8,000 bps. No internal control for incomplete

restriction digestion was used because failure of either enzyme to cleave in FMR1 would result in a fragment too large to be collected in any fraction.

[0212] All collected fractions were then subjected to restriction enzyme digestion with BmtI (New England BioLabs) according to manufacturer's procedure in order to cleave the marker sequence from the tandem repeat region. Five μ L of each digested fraction was transferred to 96-well plates containing 20 μ L PCR mix in each well. This PCR mix consists of 1X Qiagen Standard PCR buffer, 1.5 mM $MgCl_2$, 5% DMSO, 100 mM KCl, 0.2 mM dNTP, 2.5 units HotStart Taq DNA polymerase (Qiagen Inc), and 1 μ M each of following primers: FXCEF2 primer (FAM-labeled; SEQ ID NO:6), FXCER2 primer (SEQ ID NO:7), and 0.01 μ M each of following primers: BIpIF primer (HEX-labeled, SEQ ID NO:12), BIpIR primer (SEQ ID NO:13), IgctrlF primer (FAM-labeled, SEQ ID NO:20), IgctrlR primer (SEQ ID NO:21), F2ctrl1F primer (HEX-labeled, SEQ ID NO:16), F2ctrl1R primer (SEQ ID NO:17), F3ctrl3F primer (FAM-labeled SEQ ID NO:18), F3ctrl3R primer (SEQ ID NO:19). The PCR conditions were as follows: 95 °C for 15 min following by 33 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min, and finally the amplified products were extended at 72 °C for 10 min. The final PCR products were then analyzed on a 3100 Prism Genetics Analyzer (Applied Biosystems) using Genescan™-350 ROX size standard (Applied Biosystems).

[0213] 1,662 blood samples, having been stripped of identifying data were submitted to analysis by the above described method. In this population of samples, there were 995 females and 557 males. Of the female individuals, 6 were determined to be premutation carriers (0.6%) and 7 were determined to be full mutation carriers (0.7%). All 13 carriers were correctly identified as verified by standard PCR/Southern blot analysis, leading to a sensitivity of 100%. A single patient interpreted as a noncarrier by the standard PCR / Southern blot assay, appeared to be a premutation carrier by the above method. This patient may be mosaic for a premutation allele or this represents a false positive result. Due to the anonymous nature of the samples, it was not possible to review the Southern blot data or to retest the sample. Assuming this result is a false positive, the specificity of the above method is 99.5%. Of the 557 males, there was one premutation carrier and 5 affected individuals. These determinations were confirmed by Southern blot analysis. Thus, the above method

detected all premutation carrier males and affected males with no false positive results on the 551 unaffected males.

[0214] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All nucleotide sequences provided herein are presented in the 5' to 3' direction.

[0215] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including," containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed.

[0216] Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification, improvement and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications, improvements and variations are considered to be within the scope of this invention. The materials, methods, and examples provided here are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention.

[0217] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0218] In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0219] All publications, patent applications, patents, and other references mentioned herein are expressly incorporated by reference in their entirety, to the same extent as if each were incorporated by reference individually. In case of conflict, the present specification, including definitions, will control.

[0220] Other embodiments are set forth within the following claims.

That which is claimed is:

1. A method for determining the size of a nucleic acid segment in a nucleic acid sample, comprising,
separating fragments of a nucleic acid, said fragments prepared from a nucleic acid-containing sample, wherein said fragments include some which contain said segment and a marker sequence, said separating into fractions according to size under conditions in which a fragment containing said segment will be located in said fractions according to the size of the segment, and
identifying those fraction(s) containing the segment by detecting the marker sequence, wherein the size of the segment is determined by the fraction in which it is identified.
2. The method of claim 1 wherein the nucleic acid segment is difficult to amplify by polymerase chain reaction.
3. The method of claim 2 wherein said nucleic acid segment is rich in guanine and cytosine bases.
4. A method according to claim 1, wherein said fragments are prepared using one or more restriction endonucleases.
5. A method according to claim 1, wherein said fractions are chosen to separate fragments containing segments having a normal size from fragments containing segments having an abnormal size.
6. A method according to claim 1, wherein said separating is into a number of fractions selected from the group consisting of 2-16.
7. A method according to claim 1, wherein said separating is by electrophoresis.
8. A method according to claim 7, wherein said electrophoresis is capillary electrophoresis.
9. A method according to claim 1, wherein said separating is by chromatography.
10. A method according to claim 1, further comprising after said separating step, cleaving all or a portion of the segment from said marker sequence in fragments which contain said segment and said marker sequence.

11. A method according to claim 10, wherein said cleaving is accomplished using a restriction endonuclease.
12. A method according to claim 1, wherein said detecting further comprises amplifying said marker sequence.
13. A method according to claim 12, wherein said amplifying is accomplished with polymerase chain reaction (PCR).
14. A method according to claim 13, wherein said PCR includes a labeled primer.
15. The method of claim 1, wherein said detecting is accomplished using the Taqman PCR detection system.
16. A method according to claim 1, wherein said marker sequence is detected by hybridizing to two probes that hybridize simultaneously to the marker sequence.
17. A method according to claim 1, wherein said segment comprises a tandem repeat region.
18. A method according to claim 17, wherein the size determined is the number of repeats in said tandem repeat region.
19. A method according to claim 19, wherein said fractions are chosen to separate fragments with a normal number of tandem repeats from fragments containing an abnormal number of tandem repeats.
20. A method according to claim 17, wherein said tandem repeats are trinucleotide tandem repeats.
21. A method according to claim 17, wherein said tandem repeat is associated with a gene selected from the group consisting of DRPLA, EPM1, FRAXA, FMR1, FRDA, huntingtin, JPH-3, AR, DM1, DM2, SCA1, SCA2, SCA3, SCA6, SCA7, SCA8, SCA10, SCA12, SCA17, PABPN1 and HOXD13.
22. A method according to claim 21 wherein said tandem repeat is associated with a gene selected from the group consisting of FMR1, FRDA, and DM1.
23. A method according to claim 22, wherein said gene is FMR1.
24. A method of detecting a mutation in a tandem repeat segment of a gene in a nucleic acid sample, wherein said mutation is characterized by a change in the number of

repeats compared to the number of repeats in the wild type allele, said method comprising separating fragments of a nucleic acid, said fragments prepared from a nucleic acid-containing sample, wherein said fragments include some which contain said tandem repeat segment and a marker sequence, said separating into fractions according to size under conditions in which a fragment containing the tandem repeat segment will be located in said fractions according to the number of repeats in the tandem repeat segment;

identifying those fraction(s) containing the segment by detecting the marker sequence, wherein the number of repeats in the tandem repeat segment is determined by the fraction in which it is identified, and

comparing the number of repeats in the tandem repeat segment from the nucleic acid sample to the number in the corresponding wild type allele, wherein a number of repeats differing from the number in the wild type allele is indicative of a mutation.

25. A method according to claim 24, further comprising,

determining if a mutation is a premutation or a full mutation by comparing the number of repeats in the tandem repeat segment from the nucleic acid sample to the number in the corresponding full mutation allele, wherein a number of repeats greater than the wild type allele but less than the full mutation is indicative of a premutation allele, whereas a number of repeats greater than or equal to the full mutation is indicative of a full mutation allele.

26. A method according to claim 24, wherein said fragments are obtained using one or more restriction endonucleases.

27. A method according to claim 26, wherein said one or more restriction endonucleases are selected from the group consisting of AluI, SphI, BmtI, BlnI, MlyI, and BstNI.

28. A method according to claim 27, wherein said one or more restriction endonuclease is AluI.

29. A method according to claim 27, wherein said one or more restriction endonucleases are SphI and BmtI.

30. A method according to claim 27, wherein said one or more restriction endonucleases are BlnI and MlyI.

31. A method according to claim 24, wherein said separating is by electrophoresis.
32. A method according to claim 33, wherein said electrophoresis is capillary electrophoresis.
33. A method according to claim 24, wherein said separating is by chromatography.
34. A method according to claim 24, wherein said fractions are chosen to separate fragments containing tandem repeat segments having a normal number of tandem repeats from fragments containing tandem repeat segments having an abnormal number of repeats.
35. A method according to claim 24, wherein said separating is into a number of fractions selected from the group consisting of 2-16.
36. A method according to claim 24, further comprising after said separating step, cleaving all or a portion of the tandem repeat segment from said marker sequence in fragments which contain said tandem repeat segment and said marker sequence.
37. A method according to claim 36, wherein said cleaving is accomplished using a restriction endonuclease.
38. A method according to claim 37, wherein said restriction endonuclease is selected from the group consisting of BsaWI, HphI, BbvI, BstNI, Hpy1881, SmlI, and BmtI.
39. A method according to claim 24, wherein said detecting further comprises amplifying said marker.
40. A method according to claim 39, wherein said amplifying is accomplished with polymerase chain reaction (PCR).
41. A method according to claim 40 wherein said amplifying employs a detectably labeled primer.
42. A method according to claim 41 said detecting is accomplished with electrophoresis.
43. The method of claim 24, wherein said detecting is accomplished using the Taqman PCR detection system.
44. A method according to claim 24, wherein said marker sequence is detected by hybridizing to two probes that hybridize simultaneously to the marker sequence.

45. A method according to claim 24, wherein said tandem repeats are trinucleotide tandem repeats.

46. A method of identifying FMR1 alleles having a normal number of tandem repeats, a premutation, or a full mutation in the nucleic acid of an individual, said method comprising,

separating fragments of a nucleic acid, said fragments prepared from a nucleic acid containing sample of the individual, wherein said fragments include some which contain a tandem repeat segment of the FMR1 gene and a marker sequence, said separating into fractions according to size under conditions in which a fragment containing the tandem repeat segment having a normal number of repeats will be located in a first fraction; and a fragment containing a tandem repeat segment having a premutation will be located in a second fraction; and a fragment having a tandem repeat region having a full mutation will be located in a third fraction,

identifying those fraction(s) containing the tandem repeat segment by detecting the marker sequence, wherein the number of repeats in the tandem repeat segment is determined by the fraction in which it is identified, wherein

a positive result in the first fraction indicates the individual has an FMR1 allele with a normal number of tandem repeats;

a positive result in the second fraction indicates the individual has a premutation FMR1 allele; and

a positive result in the third fraction indicates the individual has a full mutation FMR1 allele.

47. A method according to claim 46, wherein said fragments are obtained using one or more restriction endonucleases.

48. A method according to claim 47, wherein said one or more restriction endonucleases are selected from the group consisting of AluI, SphI, BmtI, BlnI, MlyI, and BstNI.

49. A method according to claim 48, wherein said one or more restriction endonuclease is AluI.

50. A method according to claim 48, wherein said one or more restriction endonucleases are SphI and BmtI.

51. A method according to claim 48, wherein said one or more restriction endonucleases are BlnI and MspI.

52. A method according to claim 46, wherein said separating is by electrophoresis.

53. A method according to claim 46, wherein said separating is by chromatography.

54. A method according to claim 46, further comprising after said separating step cleaving all or a portion of the tandem repeat segment from said marker sequence fragments which contain said tandem repeat segment and said marker sequence.

55. A method according to claim 54, wherein said cleaving is accomplished using a restriction endonuclease.

56. A method according to claim 55, wherein said restriction endonuclease is selected from the group consisting of BsaWI, HphI, BbvI, BstNI, Hpy188I, SmaI, and BmlI.

57. A method according to claim 46, wherein said detecting further comprises amplifying said marker.

58. A method according to claim 57, wherein said amplifying is accomplished with polymerase chain reaction (PCR).

59. A method according to claim 58 wherein said amplifying employs a detectably labeled primer.

60. A method according to claim 59 said detecting is accomplished with electrophoresis.

61. The method of claim 46, wherein said detecting is accomplished using the Taqman PCR detection system.

62. A method of determining the size of a tandem repeat segment in a sample of nucleic acids, said method comprising

- a) measuring the size of said tandem repeat segment by a first method which comprises amplification of the tandem repeat segment,
- b) measuring the size of said tandem repeat segment by a second method according to claim 17, and

c) using the information obtained in steps a) and b) to determine the size of the tandem repeat segment.

63. A method according to claim 62, wherein the amplification of step a) is accomplished with polymerase chain reaction (PCR).

64. A method according to claim 63, further comprising sizing of the amplified tandem repeat region using electrophoresis.

65. A method for screening male and female individuals for carrier status of mutations in the tandem repeat region of the FMR1 gene, said method comprising
assaying nucleic acids from an individual to determine gender; and
assaying said nucleic acid to determine the length of the tandem repeat region of the FMR1 gene wherein said determining comprises

amplifying tandem repeat region,

detecting an amplification product, and

determining the number of tandem repeats in the amplification product,

wherein

in male individuals:

the presence of an amplification product having less than 55 tandem repeats indicates the individual is not a carrier,

the presence of an amplification product having 55 or more tandem repeats indicates the individual is a carrier, or

in the absence of an amplification product, the carrier status is undetermined; and

in female individuals:

the presence of an amplification product having more than 55 tandem repeats indicates the individual is a carrier; or

the presence of a single amplification product having less than 55 tandem repeats, the carrier status is undetermined.

66. A method according to claim 65, further comprising, analyzing undetermined individuals to determine carrier status, wherein said analyzing comprises,

separating fragments of a nucleic acid, said fragments prepared from a nucleic acid containing sample of the individual, wherein said fragments include some which contain

a tandem repeat segment of the FMR1 gene and a marker sequence, said separating into fractions according to size under conditions in which a fragment containing the tandem repeat segment having a normal number of repeats will be located in a first fraction; and a fragment containing a tandem repeat segment having a premutation will be located in a second fraction; and a fragment having a tandem repeat region having a full mutation will be located in a third fraction,

identifying those fraction(s) containing the segment by detecting the marker sequence, wherein the number of repeats in the tandem repeat segment is determined by the fraction in which it is identified, wherein

in male individuals:

a positive result in the first fraction indicates the individual is not a carrier,

a positive result in the second fraction indicates the individual is a premutation carrier,

a positive result in the third fraction indicates the individual is affected; and

in female individuals:

a positive result in only the first fraction indicates the individual is homozygous for the a normal allele;

a positive result in the second fraction indicates the individual is a premutation carrier; and

a positive result in the third fraction indicates the individual is a full mutation carrier.

67. A method according to claim 65, wherein said assaying nucleic acids to determine gender comprises nucleic acid amplification.

68. A method according to claim 67, wherein said nucleic acid amplification comprises

amplifying a region of the amelogenin gene which produces different sizes of amplification products from the amelogenin gene on the X chromosome and the amelogenin gene on the Y chromosome,

determining the size of the amplification product or products, wherein the

presence of one product of a single size indicates the gender is female and the presence of two products of different sizes indicates the gender is male.

69. A method according to claim 67, wherein said amplifying of a region of the amelogenin gene is performed in multiplex with the amplifying of the tandem repeat region of the FMR1 gene.

70. A method according to claim 69, wherein said multiplex amplification further comprises amplifying one or more control sequences.

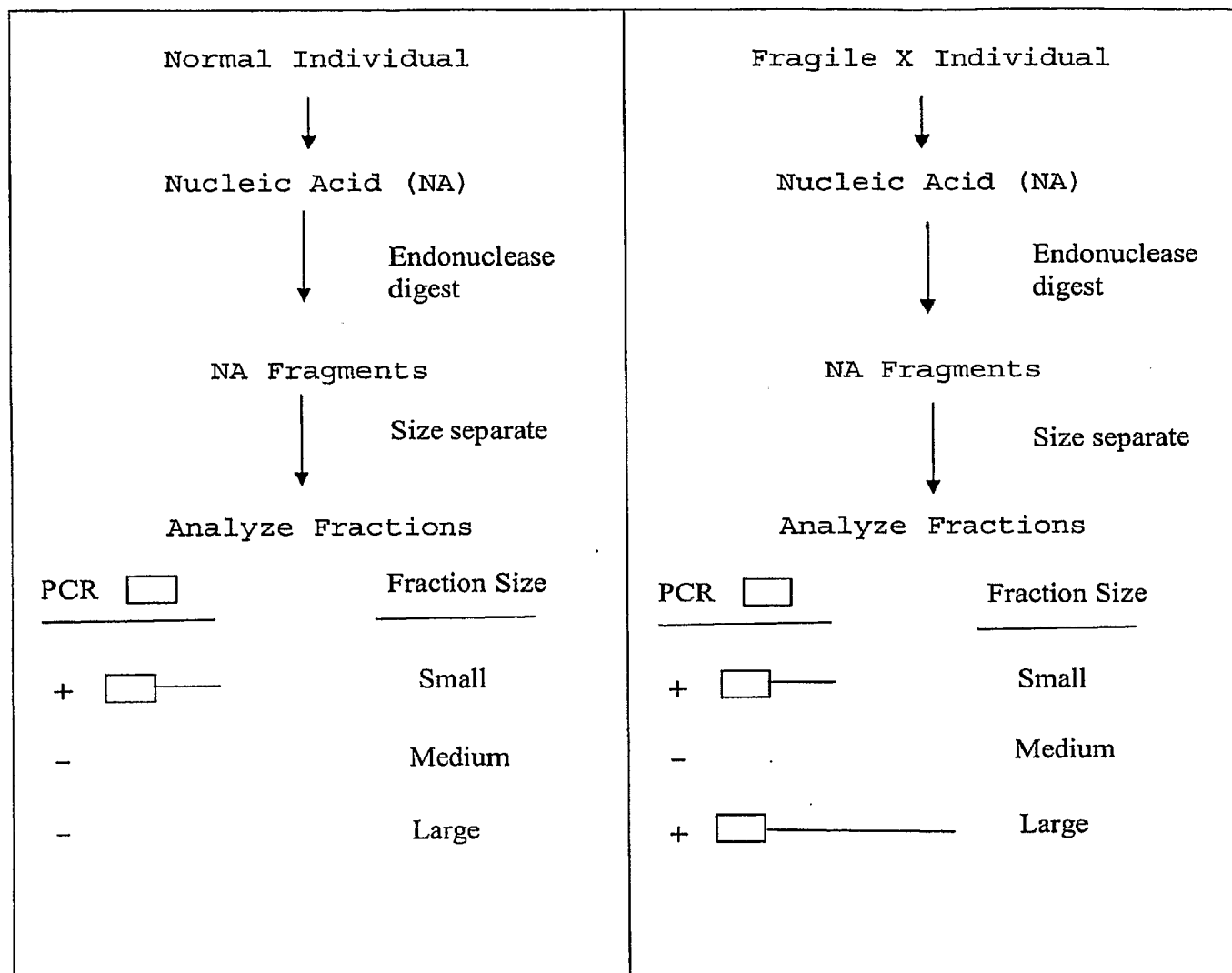
71. A method according to claim 66, further comprising after said separating step cleaving all or a portion tandem repeat segment from said marker sequence fragments in which contain said tandem repeat segment and said marker sequence.

72. A kit for detecting the size of a particular nucleic acid segment in a sample comprising a primer pair for amplifying a marker nucleotide sequence upstream or downstream of the particular nucleic acid segment, and one or more restriction endonucleases for cleaving the nucleic acid sample to generate a fragment of the nucleic acid sample which contains the particular nucleic acid segment and the upstream or downstream marker sequence, wherein said particular nucleic acid segment is a tandem repeat sequence.

73. A kit according to claim 72, further comprising one or more restriction enzymes for separating the particular nucleic acid segment from the marker sequence.

74. A kit according to claim 72, further comprising one or more controls that verify proper size separation of nucleic acids digested with the restriction endonuclease.

75. A kit according to claim 72 wherein the tandem repeat segment is from the FMR1 gene.



= flanking marker segment

—————] = Tandem Repeat Segment

FIGURE 1

CTCCGTTTTCGGTTTCACTTCCGGTGGAGGGCCGGCTGTGAGCGGGCGGCGGGCCGACGGCGA
GCGCGGGCGGCGGCCTGACGGAGCGGGCGGTGCCAGGGGGCGTGCGGCAGCGCGGCGGCGG
CGGCGGCGGCGGCGGCGGCGGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCTGGGCCTCGA
GCGCCCGCAGCCACCTCTCGGGGGCGGGCTCCCGGCGCTAGCAGGGCTGAAGAGAAGATGG
AGGAG

FIGURE 2

CTCCAGTCCTGTGATCCGGGCCCCGCCCCCTAGCGGCCGGGGAGGGAGGGGCCGGGTCCGCGG
CCGGCGAACGGGGCTCGAAGGGTCCTTGTAGCCGGGAATGCTGCTGCTGCTGCTGCTGCTGC
TGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGGGGGATCACAGACCATTCTTTC
TTTCGGCCAGGCTGAGGCCCTGACGTGGATGGGCAAACCTGCAGGCCTGGGAAGGCAGCAAGC
CGGGCCGTCCGTGTTCCATCCTCCACGCACCCCCACCTATCGTTGGTTCGCAAAGTGCAAAG

FIGURE 3

AGGCCTAGGAAGGTGGATCACCTGAGGTCCGGAGTTCAAGACTAACCTGGCCAACATGGTGA
AACCCAGTATCTACTAAAAAATACAAAAAAAAAAAAAAAAAAGAAGAAGAAGAAGAAAATA
AAGAAAAGTTAGCCGGGCGTGGTGTCGCGCGCCTGTAATCCCAG

FIGURE 4

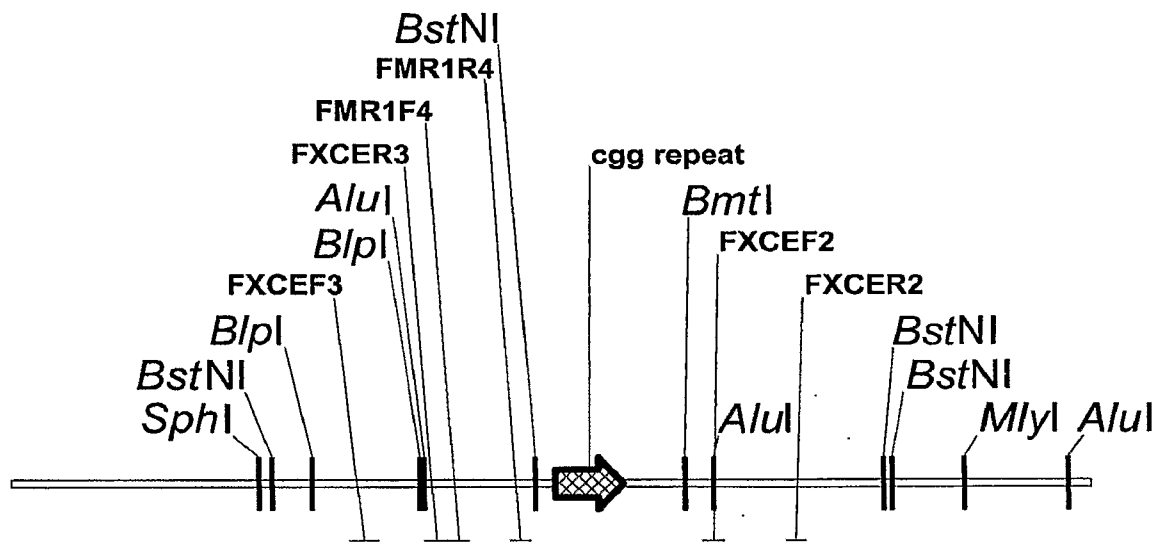


FIGURE 5