as 10 mutant alleles in a mixture containing 1,000,000 wild-type alleles, and we described a real-time monoplex PCR assay utilizing human DNA and a SuperSelective primer that successfully detected 10 mutant alleles in a sample containing 10,000 wild-type alleles although a plot of C_T values versus the log of the starting number of mutant copies (templates) departed from linearity below 100 copies. We did not, however, demonstrate multiplex assays or describe multiplex assays for closely related rare mutant target sequences in which the SuperSelective primer design and the method were capable of detecting as few as ten mutant target sequences.

SUMMARY OF THE INVENTION

This invention includes multiplex assay methods that are capable of detecting in a sample containing genomic DNA fragments as few as ten copies of each of at least two different closely related, intended rare mutant DNA target sequences in the presence of 10,000 copies of a related wild-type target sequence where the mutant target sequences differ from each other and from their related wild-type sequence by as little as a single-nucleotide polymorphism. Mutations are "closely related" if they are not amenable to PCR amplification using separate primer pairs and their amplified products (amplicons) are able to cross-hybridize and form heteroduplexes. Closely related mutations include mutations that, while they may well occur in different cells, are located in the same codon or adjacent codons, or they may be separated by up to a few codons. Closely related mutations may differ from one another and from their related wild-type sequence by as little as a single nucleotide. A single-nucleotide difference between a wild-type sequence and a mutant sequence is often referred to as a single-nucleotide polymorphism (SNP).

A first aspect of the invention is a multiplex assay method that is capable of amplifying and detecting in a sample as few as ten copies of each of at least two different closely related, intended rare mutant DNA target sequences in the presence of 10,000 copies of a related wild-type DNA target sequence, where the mutant DNA target sequences differ from each other and from the wild-type DNA target sequence by as little as a single-nucleotide polymorphism, comprising:

(a) preparing a non-symmetric primer-dependent amplification reaction mixture that includes the sample, a DNA polymerase, deoxyribonucleoside triphosphates, other reagents required for amplification, a distinguishably labeled homogeneous fluorescence detection probe, that is specific for an amplification product of each rare mutant DNA target sequence,

an excess concentration of a reverse primer for the closely related mutant target sequences, and a limiting concentration of a unique multi-part primer for each intended rare mutant target sequence, wherein the sequence of each multi-part primer comprises, in the 5' to 3' direction, the following four contiguous DNA sequences that are copied by extension of the reverse primer:

a tag DNA sequence that is not complementary to any target DNA sequence, whose complement in an amplicon strand initiated by the reverse primer is the target of the probe and that is unique for each target sequence or target-sequence group that is to be separately identified;

an anchor DNA sequence that is sufficiently long so that it is able to hybridize with the closely related mutant DNA target sequences and with the related wild-type DNA target sequence during primer annealing;

a unique bridge DNA sequence at least six nucleotides long that does not hybridize during primer annealing to the unique multi-part primer's intended DNA target sequence, to any other closely related mutant target DNA sequences, or to the related wild-type DNA target sequence during primer annealing; and

a unique foot DNA sequence that is 7 to 14 nucleotides long and that is perfectly complementary to the intended DNA target sequence but mismatches each other mutant DNA target sequence and the related wild-type DNA sequence by one or more nucleotides, at least one of which is the 3'-terminal nucleotide or the 3'-penultimate nucleotide, wherein:

(i) if the anchor DNA sequence and the foot DNA sequence of the multi-part primer are both hybridized to its intended target DNA sequences, thereby creating a primer-target hybrid, the primer-target hybrid comprises in the 5' to 3' direction of the multi-part primer: an anchor-target hybrid, a bubble, and a foot target hybrid, said bubble having a circumference of 18 to 40 nucleotides and being formed by an intervening DNA sequence in the target DNA sequence that is at least eight nucleotides long and does not hybridize to the bridge DNA sequence during primer annealing,

(ii) the bubble isolates the foot-target hybrid from the anchor-target hybrid, and the isolated foot target hybrid is a weak hybrid that makes copying the intended target DNA sequence unlikely as evidenced by a delay of at least five cycles in the threshold value (CT) as compared to the CT that would occur using a conventional primer that is 15-40 nucleotides long and forms a hybrid under primer annealing conditions;

(iii) the probability that during PCR amplification the multi-part primer for its intended target DNA sequence will initiate copying of any closely related mutant target DNA sequence or the related wild-type target DNA sequence is at least 1,000 times lower than the probability of initiating copying of its intended target sequence, as evidenced by a difference in threshold values (ΔCT) of at least ten thermal cycles;

(iv) the multi-part primer that has generated an amplicon strand has bridge and foot DNA sequences that are perfectly complementary to the amplicon strand's complementary strand; and

(v) the length and sequence of the bridge DNA sequence of each multi-part primer, together with the length of the intervening DNA sequence of its intended target DNA sequence, result in a threshold value (CT) observed for a sample containing only ten copies of its intended target DNA sequence that will occur within 60 cycles of exponential amplification and will be distinguishable from the CT observed from a sample containing no copies; and

(b) repeatedly cycling the reaction mixture to amplify the closely related rare mutant target DNA sequences present in the sample and detecting the presence of those DNA sequences by measuring an intensity of fluorescence from each distinguishably labeled probe by real-time or end-point detection.

In one embodiment of the multiplex assay method, the cycling is temperature cycling in a polymerase chain reaction (PCR) method.

In one embodiment of the multiplex assay method, the amplification reaction mixture does not contain an allele-selectivity-enhancing reagent, and the foot DNA sequence of each multipart primer is 7-9 nucleotides long.

In one embodiment of the multiplex assay method, the amplification reaction mixture contains tetramethylammonium chloride (TMAC) or another allele selectivity-enhancing reagent, and the foot DNA sequence of each multi-part primer is 8-10 nucleotides long.

In one embodiment of the multiplex assay method, the foot DNA sequence mismatches the closely related wild-type DNA sequence by one or more nucleotides, and wherein at least one of which is the 3' terminal nucleotide.

In one embodiment of the multiplex assay method, a CT value for one target DNA sequence represents the same number of starting templates as it does for any other target DNA sequence.

In one embodiment of the multiplex assay method, the multiplex assay method includes amplifying and detecting a reference wild-type DNA sequence that is not closely related to the at least two closely related mutant target DNA sequences, wherein the primer-dependent amplification reaction mixture includes a limiting multi-part primer for, an excess reverse primer for, and a homogeneous fluorescence detection probe for, the reference DNA sequence, wherein the multi-part primer for the reference DNA sequence has the structural and functional limitations described in the first aspect set forth above, and wherein the length and nucleotide sequence of the bridge DNA sequence of the multi-part primer for the reference DNA sequence are coordinated with those of the multi-part primers for the mutant target DNA sequences so that the difference between the CT value obtained for each mutant target DNA sequence and the CT value obtained for the reference wild-type DNA sequence reflects the abundance of that mutant target DNA sequence relative to the abundance of the reference wild-type DNA sequence, irrespective of the amount of DNA present in the sample.

In one embodiment of the multiplex assay method, cycling the reaction mixture is performed by an instrument, wherein the number of target DNA sequences exceeds the number of colors the instrument can separately detect, and wherein multiple different probes are thermospecific hybridization probes having the same fluorophore but having different melting temperatures.

In one embodiment of the multiplex assay method, amplification and detection are a digital PCR method, and wherein the probes are color-coded molecular beacon probes.

In one embodiment of the multiplex assay method, amplification and detection are performed in a spectrofluorometric thermal cycler, and wherein the probes are color-coded molecular beacon probes.

In one embodiment, the multiplex assay method is capable of amplifying and detecting fewer than ten copies of any of the at least two closely related rare mutant target DNA sequences, wherein the hybrids formed by the multi-part primer anchor DNA sequences and the mutant target DNA sequences have melting temperatures (Tm's) that are lower than the Tm of hybrids formed by the reverse primer and the mutant target DNA sequences, wherein before step (b) multiple cycles of linear amplification utilizing the reverse primer are

performed using a primer annealing temperature at which the reverse primer hybridizes but the multi-part primers are very unlikely to hybridize, and wherein step (b) is performed using a lower primer annealing temperature at which the multi-part primers and the reverse primer hybridize.

Assay methods according to this invention comprise selectively amplifying each closely related mutant sequence, if present in the sample, by a non-symmetric primer-dependent amplification method, such as a polymerase chain reaction (PCR) method, and separately detecting amplified products (amplicons) from each mutant target sequence by fluorescence detection that utilizes at least one fluorescently labeled probe. Methods according to this invention may be qualitative or quantitative. In some quantitative methods, the real-time PCR threshold value (or threshold cycle, C_T) reflects the amount of a mutant target sequence present in a sample. Certain embodiments further include amplifying a reference wild-type gene sequence, which may be the wild-type sequence related to the mutant sequences to be detected or a reference wild-type sequence that is present in the sample but is unrelated to those mutant sequences.

Assay methods according to this invention utilize SuperSelective primers having 5'-tag sequences that serve as targets for detection by fluorescently labeled hybridization probes. By including unique 5'-tag sequences (each different from all other 5'-tag sequences in a particular assay) in different SuperSelective primers, and including a distinguishably labeled probe targeting the complement of each different 5-'tag sequence, detecting probe hybridization identifies which tag sequence, and hence which SuperSelective primer, was amplified. Preferred hybridization probes are homogeneous detection probes whose hybridization is detectable without washing away unbound probes. Preferred fluorescent labels are fluorophores. Our most preferred homogeneous detection probes are molecular beacon probes labeled with at least one fluorophore and also labeled with a non-fluorescent quencher.

Assay methods according to this invention are multiplex assays that are capable of detecting in a sample containing genomic DNA fragments the presence of at least two closely related mutations of a selected wild-type sequence in the presence of an abundance of the wild-type sequence. Different embodiments have different objectives and features.