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 free of any bridge DNA sequence;

CLAIMS

1. 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, tetramethylammonium chloride (TMAC) or another Hofmeister salt, 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 for each intended rare mutant target sequence, a limiting concentration of a unique multi-part primer possessing at least one unique DNA sequence that is not complementary to any target DNA sequence, and the complement of one of said unique sequences 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, wherein the sequence of each multi-part primer comprises, in the 5’ to 3’ direction, the following three contiguous DNA sequences that are copied by extension of the reverse primer:

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 24 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 primer's 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 (C_T) as compared to the C_T that would occur using a conventional primer that is free of any bridge DNA sequence;

(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 (ΔC_T) 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 (C_T) 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 C_T 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.

2. The method according to claim 1 wherein cycling is temperature cycling in a polymerase chain reaction (PCR) method.

3. The method according to claim 1 or claim 2, wherein for at least one intended rare target sequence the multi-part primer includes a 5’ tag sequence that is not complementary to any target sequence, that is unique for each target sequence or target-sequence group that is to be separately identified, and whose complement in the amplicon strand initiated by the reverse primer is the target of the probe.4. The method according to claim 1 or claim 2, wherein for at least one intended rare target sequence the target of the probe is the complement of the bridge sequence in the amplicon strand initiated by the reverse primer.

5. The method of any one of claims 1 to 4 wherein 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.

6. The method of any of claims 1 to 5, wherein the foot sequence is 8 to 10 nucleotides long.

7. The method of any of claims 1 to 6, wherein the lengths of the bridge and intervening sequences are unequal, and the bubble is asymmetric.

8. The method according to claim 2 wherein a C_T value for one target DNA sequence represents the same number of starting templates as it does for any other target DNA sequence.

9. The method according to claim 1 that 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 claim 1, 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 C_T value obtained for each mutant target DNA sequence and the

C_T 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.

10. The method according to any of claims 1-9, wherein 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.

11. A multiplexed assay method according to any of claims 1-10, wherein amplification and detection are a digital PCR method.

12. A highly multiplexed assay method according to claim 11, wherein the probes are color-coded molecular beacon probes.

13. The method according to any of claims 1 to 12 that 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 hybrids formed by 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.

14. A reagent kit that includes reagents sufficient for performing amplification and detection according to any of claims 1-13, comprising said DNA polymerase, said deoxyribonucleoside triphosphates, said other reagents required for amplification, TMAC or said another Hofmeister salt, said distinguishably labeled homogeneous fluorescence detection probe, said reverse primer, and said unique multi-part primer.