

484 describes an apparatus for performing the polymerase chain reaction and subsequent capillary electrophoretic separation and detection in an integrated device.

[0016] There are several nucleic acid amplification technologies that differ from the PCR reaction in that the reaction is run at a single temperature. These isothermal methods include the cycling probe reaction, strand displacement, Invader™, SNPase, rolling circle reaction and NASBA. U.S. Pat. No. 6,379,929 describes a device for performing an isothermal nucleic acid amplification reaction.

[0017] More recently, a strategy for performing the polymerase chain reaction isothermally has been described by Vincent et al., 2004, EMBO Reports, vol 5(8), see also US Application 20040058378. A DNA helicase enzyme is used to overcome the limitations of heating a sample to perform PCR DNA amplification.

Enzymes Used for the Polymerase Chain Reaction (PCR)

[0018] The polymerase chain reaction (PCR) is based on the ability of a DNA polymerase enzyme to exhibit several core features, which include its ability to use a primer sequence with a 3'-hydroxyl group and a DNA template sequence and to extend a newly synthesized strand of DNA using the template strand, all well known to those skilled in the art. In addition, DNA polymerases used in the PCR reaction must be able to withstand high temperatures (e.g. 90 to 99° C.) used to denature double stranded DNA templates, as well as be inactive at lower temperatures (e.g. 40 to 60° C.) at which DNA primers hybridize to the DNA template. Further, to have optimal DNA synthesis at a temperature near to the hybridization temperature (e.g. 60 to 80° C.).

[0019] In addition to these core characteristics, DNA polymerases also exhibit proofreading capabilities, which are due to the 3'-5' exonuclease activity inherent in most DNA polymerases. For the purpose of single nucleotide polymorphism (SNP) detection based on differential primer extension using PCR (also called 3'-allele specific primer extension), it is a disadvantage to use an enzyme that exhibits a 3'-5' exonuclease activity, as the terminal 3' nucleotide can be excised from a standard nucleic acid primer, permitting synthesis of both alleles.

[0020] Zhang et al., (2003, Laboratory Investigation, vol 83(8):1147) describe the use of a terminal phosphorothioate bond to overcome the limitations of DNA polymerases used for 3'-5' exonuclease activity. The phosphorothioate bond is not cleaved by 3'-5' exonucleases. This prevents DNA polymerases with 3'-5' exonuclease activities from removing the terminal mismatch and proceeding with DNA elongation, alleviating the lack of discrimination observed with normal DNA.

[0021] Another characteristic of DNA polymerases is their elongation rate. Takagi et al., (1997, Applied and Environmental Microbiology, vol 63(11): 4504) teach that *Pyrococcus* sp. Strain KOD1 (now *Thermococcus kodakaraensis* KOD1), *Pyrococcus furiosus*, Deep Vent (New England Biolabs, Beverly, Mass.), and *Thermus aquaticus* have elongation rates of 106 to 138,25,23 and 61 bases/second, respectively. The processivity rates of these enzymes are also described, and behave similarly to the elongation rates. Clearly, *Thermococcus kodakaraensis* KOD1 has much higher elongation and processivity rates compared to the other well-known enzymes, which would make this enzyme beneficial in applications where sensitivity and speed are an issue. Further, *Thermococcus kodakaraensis* KOD1 pos-

sesses an exonuclease activity which would be detrimental for use in a 3'-allele specific primer extension assay used for SNP analysis.

Design of Synthetic Oligonucleotides

[0022] Regarding the design of synthetic oligonucleotides for use in amplification reactions, Rychlik et al., (1989, Nucleic Acids Research, vol 17(21):8543-8551) and Rychlik (1995, Molecular Biotechnology, vol 3: 129-134), describe selection criteria and computer programs to design probes and primers, including primers for in vitro amplification of DNA. Both teach that primers should not generate secondary structure or exhibit self-hybridization.

[0023] PCR primers designed as molecular beacons (Bonnet et al., 1999, Proc. Natl. Acad. Sci. USA, vol 96: 6171-6176) have a short region at both the 5' and 3' ends which are complementary generating what is known as hairpin loop structures, to quench the fluorescent signal by placing the donor and quencher molecules in close physical proximity to each other. After polymerization and incorporation into a newly synthesized double stranded molecule, the donor and quencher molecules are physically distant to each other, permitting the generation of a fluorescent signal. The region of complementarity is short and typically has only about 5 nucleotides which are complementary, preferably generating a hairpin stem. Tsourkas et al., 2003, Nucleic Acids Research, vol 31(4):1319-1330, teaches that molecular beacons with longer stem lengths have an improved ability to discriminate between targets over a broader range of temperatures. However, this is accompanied by a decrease in the rate of molecular beacon-target hybridization. Molecular beacons with longer probe lengths tend to have lower dissociation constants, increased kinetic rate constants and decreased specificity. Therefore, having longer stem loops will have an impact on reducing the efficiency of hybridization kinetics, which in turn will reduce the levels of PCR amplification. Therefore, PCR using a stem loop structure is generally undesirable in the art. Kaboev et al., (2000, Nucleic Acids Research, vol 28(21):e94) teaches that designing a PCR primer with a stem loop structure by adding additional sequences to the 5'-end of the primer, which are complementary to the 3'-end. This reference also teaches that adding this secondary structure increases the specificity of the PCR reaction, though it does use a PCR primer that permits the generation of single stranded tails. Further, Kaboev does not teach that the generation of the secondary structure prevents the hybridization of the single stranded regions to a capture moiety.

Detection Methods

[0024] Conventional detection methods for the final step in a nucleic acid analysis are well known in the art and include sandwich-type capture methods based on radioactivity, colorimetry, fluorescence, fluorescence resonance energy transfer (FRET) and electrochemistry. For example, jointly owned U.S. Pat. No. 5,063,081 covers a sensor for nucleic acid detection. The sensor has a permselective layer over an electrode and a proteinaceous patterned layer with an immobilized capture oligonucleotide. The oligonucleotide can be a polynucleotide, DNA, RNA, active fragments or subunits or single strands thereof. Coupling means for immobilizing nucleic acids are described along with methods where an immobilized nucleic acid probe binds to a complimentary