

age RNA polymerases requires additional reagents and generates labile RNA species for detection.

[0111] Here we describe a novel method of performing a PCR reaction by combining DNA polymerase, a target nucleic acid and amounts of two modified primers where the first modified primer has a sequence of bases to a region of the target. A polymerase blocking region is attached to this primer which is linked to a single stranded hybridization region. The second modified primer has a sequence of bases to a second region of the target and also a polymerase blocking region and a second single stranded hybridization region. A detectable moiety (e.g. biotin, fluorescein) is attached to one or both of the two modified primers. To run the PCR reaction the mixture is cycled to generate multiple copies of an amplicon incorporating the modified primers. In a second step excess unincorporated modified primers, with the detectable moiety, are substantially eliminated from the mixture. Several different methods are available and these are described below. The mixture is then added to a capture oligonucleotide which is complementary to one or both of the single stranded hybridization regions to permit hybridization with the amplicon. In the last step the moiety associated with this hybridization is detected directly, for example by optical detection of fluorescein. Alternatively, the moiety, e.g. biotin is exposed to and binds with a streptavidin-labeled enzyme, e.g. alkaline phosphatase and the enzyme activity is determined either optically or electrochemically. Again several specific methods are possible and examples of these are described below.

[0112] The reaction sequence is shown in FIG. 7(a), where **31** is the detection moiety, e.g. biotin, FAM, DNP, cholesterol, fluorescein, **32** is the first single stranded hybridization region, **33** is the polymerase blocking region, e.g. hexaPEG, **34** is the first PCR primer, **35** is the second PCR primer, **36** is the second single stranded hybridization region, **37** is a second detectable moiety, **38** is the double stranded nucleic acid target sequence, **39** is a solid substrate, e.g. bead or surface, and **40** is a hybridization region complementary to **36**.

[0113] The PCR primers, **34** and **35** are preferably synthesized using standard phosphoramidite chemistry and can include any nucleotide or modified base which is amenable to DNA polymerase, except in the polymerase blocking region **33**. An example of a polymerase blocking region sequence can consist of the spacer phosphoramidite 18-O-dimethyltritylhexaethyleneglycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (hereinafter referred to as "HPEG"). This phosphoramidite generates a hexaethyleneglycol spacer region. Other spacer molecules with similar properties can also be used for this purpose. Alternatives to phosphoramidite chemistry can be used including creating a 3'-3' or 5'-5' phosphodiester backbone, as well as modified nucleotides as described by Newton et al., (Nucleic acids research 21, 1155-62, 1993) and also U.S. Pat. No. 5,525,494.

[0114] Allowing PCR to proceed using these synthetic oligonucleotide primers in the presence of the appropriate target and DNA polymerase with associated components, generates a newly synthesized DNA molecule with incorporated single stranded regions **32** and **36**. It has been found that while the Taq DNA polymerase may be used, the preferred embodiment uses *T. kodakienensis* DNA polymerase which exhibits a significantly higher turnover number. This molecule can then be hybridized by means of **36** to a target sequence **40** on a solid support **39**. The binding moiety region can then be used for generating a signal. For example by using biotin as the

binding moiety and using streptavidin conjugated to a detection enzyme, e.g. horseradish peroxidase (HRP) and alkaline phosphatase (ALP).

[0115] The PCR primer also preferably contains a terminal phosphorothioate bond, preventing the exonuclease activity of *T. kodakienensis* KODI DNA polymerase from not discriminating allelic differences in primers used in SNP analysis based on the terminal base being different.

[0116] In the preferred embodiment using human genomic DNA isolated using the filter holder device described above, two synthetic oligonucleotides (primers **1** and **2**) were used to generate a region of the human hemochromatosis gene (hfe) of approximately 390 by in size. These were oligo 1: 5'-ACTTCATACACAACCTCCGCGTTGCATAACT-HPEG-TG-GCAAGGGTAAACAGATCC-3' and oligo 2: 5'-56-FAM-AACAATACCACCGTAGCGATCA-HPEG-AACAATACCACCGTAGCGATCA-3', where 56-FAM is a fluorescent species and HPEG is a hexa PEG sequence incorporated using an 18-0-dimethyltritylhexaethyleneglycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. In the oligo 1 sequence, the sequence located 5' to the HPEG spacer, 5'-ACTTCATACACAACCTCCGCGTTGCATAACT-3' is designated as SEQUENCE ID NO. 1 and the sequence located 3' to the HPEG spacer, 5'-TGGCAAGGGTAAACAGATCC-3' is designated as SEQUENCE ID NO. 2. In the oligo 2 sequence, the sequence located 5' to the HPEG spacer, 5'-AACAATACCACCGTAGCGATCA-3' is designated as SEQUENCE NO. 3 and the sequence located 3' to the HPEG spacer, 5'-AACAATACCACCGTAGCGATCA-3' is designated as SEQUENCE ID NO. 4.

[0117] To demonstrate the use of these primers, a buccal cell DNA sample originating from mouthwash (Scope brand) was used. A volume of 3 μ L of this bodily fluid was dispensed onto a 5 mm diameter disc punched from Whatman 4 filter-paper impregnated with 3 μ L of lytic salt and detergent solution comprising 2M guanidinium isothiocyanate, 1% Triton-X-100, 10 mM Tris buffer at pH 8.8 and 2 mM EDTA. After extraction, the filter-disc was placed immediately into a 0.5 mL M β P Easystart PCR reaction tube (Fisher Scientific, PN 21-402-49) designed to be filled to 100 μ L. The tube is supplied with 50 μ L of fluid under a waxlayer to give a final concentration of the following reagents in 100 μ L of aqueous solution; 2 mM MgCl₂, 20 mM Tris pH 8.4, 50 mM KCl and 0.2 mM dNTP. A 47 μ L upper-layer reaction mixture was added to give a final reaction concentration of primers **1** and **2** of 0.31 pM, described (Integrated DNA Technologies Inc). This aqueous solution also contained 5U Vent (exo-) polymerase (New England Biolabs) and 0.1% Triton-X-100. The amplification reaction was performed in a Techne Techgene Thermocycler. The sequence was amplified using 3 cycles of 97° C. for 3 min, 60° C. for 1 min and 72° C. for 1 min, followed by 36 cycles of 97° C. for 1 min and 62° C. for 45 s. Samples resulting from the amplification procedure were then tested in single-use cartridges using 100 μ L aliquots. A complete description of the design elements of detection cartridge containing an electrochemical sensor is found in jointly owned US 20030170881 incorporated here by reference. A general description of chronoamperometry and other electrochemical methods applicable to sensors incorporated into single-use test cartridges is found in jointly owned U.S. Pat. No. 5,112,455 incorporated here by reference.

[0118] The 100 μ L aqueous aliquots were prepared as follows; 14 μ L 1M NaCl, 1 μ L FITC-ALP conjugate 1/100 dilution, and 10 μ L amplified DNA. The FITC-ALP conju-