

used in PCR, the secondary structure is eliminated. Once one end of the CLAM oligonucleotide is incorporated into a PCR amplicon it no longer functions with the secondary structure and provides a single stranded region **82**. In step **83**, the temperature is below that required to generate secondary structure of unincorporated CLAM primer sequences. As a result, CLAM primers that have been incorporated into a PCR amplicon will have single stranded regions capable of binding to the capture oligonucleotide.

Enzymatic Removal

[0156] Two enzymatic approaches have been devised for removal of primers, these relate to TdT-tails on unincorporated oligonucleotides and degradation of unincorporated oligonucleotides. Within a PCR reaction mixture there exist two types of structures, amplicons with single stranded regions, in the example above having an iSp 18 primer and unincorporated synthetic oligonucleotides. The primers on the amplicons only have extending 5' regions, whereas the unincorporated primers have free 5' and 3' single stranded ends. Using enzymes specific to these differences at the 3' end, strategies to differentially remove these molecules was developed.

[0157] Calf Thymus Terminal deoxynucleotidyl transferase (TdT) enzymatic treatment of the PCR reaction product is specific to single stranded 3' extensions, thus only the unincorporated primer will generate a newly incorporated tail. By contrast, the amplicon only has single stranded regions with 5' tails, which are unreactive with TdT.

[0158] While it is inefficient and not unique for a universal capture system, one could use a single nucleotide (dNTP) such as 'T' to create an extended T tail at the 3' end of the PCR primer. Any nucleotide, including modified nucleotides, including ribonucleotides could be used for this application and which function with TdT or poly(A) polymerase. The modified PCR reaction mix with T tailed unincorporated primer sequences can then be exposed to a capture oligonucleotide with a poly(A) sequence. Only unincorporated PCR primers with T tails will be bound to the capture poly(A) sequence. This enriches the reaction mixture for PCR amplicons with associated poly(T) sequences. The poly(A) capture oligonucleotide can be bound to solid surfaces, beads, in a matrix like agarose, acrylamide, poly vinyl alcohol or other appropriate hydrocolloids.

[0159] An alternative method is based on the use of an endonuclease. As the unincorporated oligonucleotide primer has a free 3'-hydroxyl group and the amplicon does not, a 3'-5' exonuclease is employed to remove unincorporated oligonucleotide primer. Enzymes including ExoI and ExoT have specific 3'-5' exonuclease activity with single stranded DNA with free 3'-hydroxyl groups. In this embodiment it is preferable to use primers with 5' phosphate groups.

Post-PCR Hybridization

[0160] In the PCR reaction described above, amplicons are generated containing two primers which generate two different single stranded regions. In order to generate a signal, both single stranded regions are necessary, as well as the newly amplified region, which is a bridge between the two single stranded regions.

[0161] In this example, single stranded A region binds to the complementary A prime capture oligonucleotide at the biosensor. The single stranded B region binds to a synthetic

oligonucleotide B-prime which has a moiety for the enzymatic conjugate. Alternatively, the enzymatic conjugate binds directly to the B region.

[0162] By first creating a solid substrate with B prime capture oligonucleotides bound to a solid substrate, and in this example in a channel leading to the detection region, and allowing the PCR reaction material to hybridize under the appropriate conditions, any B region oligonucleotides that were not incorporated into amplicons are lost from the channel, enriching the channel for B region oligonucleotides and B region oligonucleotides incorporated into amplicons. Unbound material is washed away.

[0163] The enriched bound B region oligonucleotides and amplicons are then released from the solid support by heat or alkaline conditions. The material is allowed to move towards the detection region of the device. Oligonucleotides with A regions or oligonucleotides incorporated into amplicons will be bound to A prime capture oligonucleotides at the biosensor. The biosensor can be washed, removing any unincorporated B primers, leaving only fully incorporated amplicons. This effectively removes background from unincorporated oligonucleotides.

Detailed Description of Nucleic Acid Testing Cartridges

[0164] An integrated single-use device for performing a nucleic acid analysis and its interaction with the reading instrument is shown topologically in FIG. 6. It comprises a housing **100** with an entry port **101** for accepting a sample suspected of containing a target nucleic acid. The entry port leads to a chamber **102** which has a reagent for extracting said target nucleic acid. The reagent **103** can be coated on to the wall of the chamber. The chamber may contain beads **104**, e.g. magnetic beads with a coating suitable for binding nucleic acid. The chamber also preferably contains a wax, which can melt to form a contiguous wax layer **105** in the region of egress to a conduit **106**. Once the preferred magnetic beads have associated with said target nucleic acid a magnetic field is applied to draw them through the wax layer and into the conduit. Note that this applied magnetic field may also be oscillated in the chamber to promote extraction of nucleic acid from the sample. Optionally a wash fluid may be applied to the beads prior to leaving the extraction chamber. A wash fluid chamber **122** is connected between the entry port and the extraction chamber. In addition, a sample and wash fluid waste chamber **123** is connected at the distal end of the extraction chamber, with respect to the entry port. In operation, after the extraction step the beads are held on the wall of the chamber by magnetic means and the wash fluid is then passed from chamber **122** through chamber **102** and into chamber **123**. This displaces unwanted sample material and leaves chamber **102** containing the beads and predominantly wash fluid. The instrument **200** contains an actuating means **211** which is aligned to chamber **122** and provides a force to a flexible diaphragm **124** to expel the wash fluid out of the chamber.

[0165] After washing, the beads then pass through the wax layer and into conduit **106** and then into the amplification chamber **107**. Movement of the beads in the conduit is preferably by the same magnetic means, or can be pneumatic. The amplification chamber is also attached to an amplification reagent holding chamber **108**, which can deliver these reagents to the amplification chamber with the beads, as in the preferred embodiment, or in a separated step before or after the beads enter this chamber. Alternatively, these reagents