

al., 1994, Padlock probes: circularizing oligonucleotides for localized DNA detection, *Science* 265:2085-8.

**[0085]** v) Detection of Multiple Different Target Nucleic Acid Sequences Using the “Universal Amplification” Method

**[0086]** As described above, a variety of multiplex amplification systems can be used in conjunction with the present invention. In one type, several different targets can be detected simultaneously by using multiple differently labeled probes each of which is designed to hybridize only to a particular target. Since each probe has a different label, binding to each target to be detected based on the fluorescence signals. By judicious choice of the different labels that are utilized, analyses can be conducted in which the different labels are excited and/or detected at different wavelengths in a single reaction. See, e.g., Fluorescence Spectroscopy (Pesce et al., Eds.) Marcel Dekker, New York, (1971); White et al., Fluorescence Analysis: A Practical Approach, Marcel Dekker, New York, (1970); Berlmann, Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd ed., Academic Press, New York, (1971); Griffiths, Colour and Constitution of Organic Molecules, Academic Press, New York, (1976); Indicators (Bishop, Ed.), Pergamon Press, Oxford, 19723; and Haugland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Eugene (1992).

**[0087]** Conventional multiplex fluorescence detection using many different probes is limited, however, by fluorescence background because probe concentration must be high enough to allow detection of many different probes (i.e., one for each sequence to be detected). Combining many probes results in fluorescence that is the sum of all the probes. This also results in a fluorescence background that is the sum of the background from all of the probes. This background may be so high as to interfere with detection of the reaction product (s). An alternative approach, referred to as the Universal Amplification (“UA”) method uses multiple sets of primer pairs, referred to here as “UA primer” amplification in a PCR-type reaction. Universal amplification allows many different sequences in a sample to be amplified using a single reaction mixture, with lower background and cost than conventional systems, and is particularly well suited for use with an MPD.

**[0088]** UA primers can be used to determine whether or not any one or more of a number of different target nucleic acid sequences are present in a sample (without necessarily identifying which of the several target sequences is present). If the assay indicates that at least one of the different nucleic acid sequences is present in the sample, subsequent analysis can be conducted to determine which of the different sequences is present. Such two-step analysis is advantageous in many applications. For example, using the present invention, in a first step a sample can be assayed to determine whether any of 100 (for example) different pathogenic agents is present in the sample. If it is determined in the first step that at least one pathogenic agent is present, the sample can be subjected to further analysis to identify and characterize the particular pathogen.

**[0089]** In one aspect the invention provides a method for detecting multiple amplification products (i.e., amplicons) using the Universal Amplification method. The total number of target sequences detected is usually at least two, and is sometimes at least 5, more often at least 10, at least 20 or at least 30. In some embodiments the total number of target sequences is between 2 and 100, between 5 and 100, between

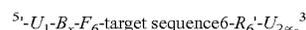
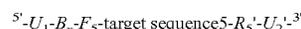
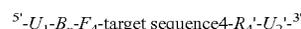
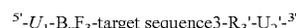
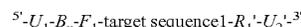
10 and 100, between 20 and 100, or between 30 and 100. In some embodiments the total number of target sequences is between 2 and 50, between 5 and 50, between 10 and 50, between 20 and 50, or between 30 and 50. In some embodiments the total number of target sequences is more than 100. The UA method makes use of three or more types of primers: **[0090]** The Type 1 primer has the structure 5'-U<sub>F</sub>-B<sub>X</sub>-F<sub>N</sub>-3' where U<sub>F</sub> is a universal forward primer sequence, B<sub>X</sub> is a sequence recognized by a detectable (e.g., detectably labeled) labeled probe, Probe X, and F is a forward primer sequence specific to a target sequence N so that F<sub>1</sub> is primer for a first target sequence, F<sub>2</sub> is a primer sequence for a second target sequence, and so on. Probe X can be a molecular beacon, Taqman-type probe, or other probe (such as, but not limited to, those described above) that specifically binds or hybridizes to sequence B.

**[0091]** The Type 2 primer has the structure 5'-U<sub>R</sub>-R<sub>N</sub>-3' where U<sub>R</sub> is a universal reverse primer sequence and R<sub>N</sub> is a primer specific to a target sequence N so that R<sub>1</sub> is primer for a first target sequence, R<sub>2</sub> is a primer for a second target sequence and so on. U<sub>R</sub> may or may not be the same sequence as U<sub>F</sub>. In one embodiment, 5'-U<sub>R</sub>-3' has the same sequence as 5'-U<sub>F</sub>-3'.

**[0092]** The Type 3 primer comprises the sequence 5'-U<sub>F</sub>-3' (or 5'-U<sub>1</sub>-3').

**[0093]** The Type 4 primer comprises the sequence 5'-U<sub>R</sub>-3' (or 5'-U<sub>2</sub>-3').

**[0094]** Each pair of Type 1 and Type 2 primers is specific to a particular target. The cognate pair of primers that amplify the same target is a “UA primer pair.” Thus, if there are 20 target sequences to be detected (for example, sequences corresponding to 20 different pathogens) twenty different primer pairs can be prepared, i.e., 5'-U<sub>1</sub>-B<sub>X</sub>-F<sub>[1→20]</sub>-3' and 5'-U<sub>2</sub>-R<sub>[1→20]</sub>-3'. The various pairs of Type 1 and Type 2 primers are combined at low concentrations with the sample, and Type 3 and Type 4 primers are added at a higher concentration. During the initial rounds of amplification, the Type 1 and Type 2 primers will amplify any target sequences present in a sample or sub-sample. It will be appreciated that U<sub>1</sub> and U<sub>2</sub> sequences can be designed with sequences not present (or unlikely to be present) in the initial sample nucleic acid, to avoid amplification of non-target sequences in the sample. For example, for analysis of human DNA, U<sub>1</sub> and U<sub>2</sub> can be selected to have sequences not found on the human genome. During subsequent rounds of amplification, the amplification products generated in the first rounds of amplification are themselves amplified by the Type 3 and Type 4 primers. The resulting double stranded amplification products will have the structure (showing one strand):



etc.

where U<sub>1</sub>' is the complement of U<sub>1</sub> and U<sub>2</sub>' is the complement of U<sub>2</sub>. Each of the amplification products shown above can be detected by a probe (e.g., molecular beacon, Invader probe,