**[0067]** As noted above, typically the invention is used to simultaneously assay for multiple different target sequences in the same sample (e.g., sequences of multiple different genes or gene segments, or alternative sequences of a single gene). For example, provided with a patient blood sample, it may be useful to assay the sample for the presence of several (e.g., 10, 20 or 100) different sequences each characteristic of a different pathogen. In one embodiment, the different sequences are amplified in different reaction chambers. For example, an assay to detect the presence of multiple mutations in different genes from an individual sample will result in analysis of products in different reaction chambers if the genes are on fragments distributed to different sub-samples. In other embodiments, multiple different target sequences are assayed for and/or detected in a single reaction chamber, such as, for example, when the reaction chamber contains a single rare cell and the assay is designed to analyse several genetic loci in the cell genome. For example, an assay to determine which of several possible polymorphisms (e.g. defining a haplotype) are represented at a specific genomic site, multiple primers or probes may be used to determine which of several possible target sequences are present in a single reaction chamber. Very often both types of amplification are used.

**[0068]** A variety of methods are known for "multiplex" analysis (amplification of multiple sequences from one reaction) and can be adapted for use in a single reaction chamber MPDs. In one embodiment amplification is conducted using a universal primer strategy in which each target sequence is initially amplified by a pair of target sequence specific primers that include a 3-prime domain with a gene-specific sequence and a 5-prime domain with a universal (not target specific) sequence. For example, N different target sequences can be detected using primers 5'-$U_1$-$F_N$-3' and 5'-$U_2$-$R_N$-3' where $F_N$ and $R_N$ are forward and reverse PCR primers for each gene N, and $U_1$ and $U_2$ are sequences common to all of the primer pairs and $U_i$ and $U_2$ may be the same or different. Subsequent rounds of amplification can be conducted using primers 5'-$U_1$-3' and 5'-$U_2$-3'. See, e.g.,. Zhenwu Lin et al., 1996, "Multiplex genotype determination at a large number of gene loci" *Proc. Nat'l Acad. Sci USA* 93: 2582-87. When used in an MPD this strategy allows use of a low concentration of target specific primers (thereby reducing expense and the chances of primer dimer and other unintended side reactions) and a higher concentration of universal primers.

**[0069]** In one embodiment, the multiplex amplification is carried out in a MPD using the primers and strategy described below in Section C(v).

**[0070]** Amplification reagents, including primers, can be provided by prepositioning reagents in reaction chambers, by combining reagents with the sample before partitioning, by a combination of prepositioning and combining, or by any other suitable method. Specific amplification reagents will depend on the amplification method and sample, but can include primers, polymerase, reverse transcriptase, nucleotides, cofactors, metal ions, buffers, and the like. Methods for prepositioning reagents in a microfluidic device have been described (see McBride et al., supra). In addition, detection reagents such as labeled probes can be provided by prepositioning and/or combining.

**[0071]** To produce amplicons the environment of the MPD reaction chambers is manipulated as required to accomplish amplification by the amplification method selected. For example, thermocycling necessary for a PCR-type amplification reaction can be accomplished by placing the device on a thermocycling plate and cycling the device between the various required temperatures for melting of the DNA duplex (either target or amplicon), annealing of primers, and DNA synthesis. For example a protocol with an initial ramp to 95° C. and maintain for 1 m; three step thermocycling for 40 cycles [92° C. for 30 s, 54° C. for 30 s, and 72° C. for 1 m] or two step thermocycling for 40 cycles [92° C. for 30 s and 60° C. for 60 s] can be used. A variety of thermocycling plates are available from commercial sources, including for example the ThermoHybaid Px2 (Franklin, Mass.), MJ Research PTC-200 (South San Francisco, Calif.), Eppendorf Part# E5331 (Westbury, N.Y.), Techne Part# 205330 (Princeton, N.J.).

**[0072]** Although in some cases a single target sequence in the sample is amplified, more often at least 2, at least 3, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, or at least 100 different target sequences are amplified. Thus, in some embodiments, sufficient reagents are provided to amplify more than one target sequence.

**[0073]** Thus, the amplification procedure can produce from zero (if no target sequence is present) to 100 or more different amplicons. Any particular chamber may have zero, one or more than one amplicons species (where amplicons corresponding to the same target sequence are of the same "species") depending on the nature of the assay and sample.

**[0074]** Usually, at least about $10^3$, at least about $10^4$, at least about $10^5$, at least about $10^6$, at least about $10^7$, or at least about $10^8$ amplicon molecules are produced corresponding to some or all of the target sequences present in the sample. Most typically, from about $10^7$ to about $10^9$ amplicon molecules are produced for each target sequence, although the number may be lower when multiple reactions are conducted in a single reaction chamber.

**[0075]** iv) Detection of Amplicons

**[0076]** The amplicon products can be detected in individual chambers and/or they can be pooled for subsequent detection and analysis (as described below in Section C(vi)). That is, amplicons can be detected and then pooled, pooled without previously being detected, or detected and not subsequently pooled.

**[0077]** Amplicons can be detected and distinguished (whether isolated in a reaction chamber or at any subsequent time) using routine methods for detecting nucleic acids. Amplicons comprising double-stranded DNA can be detected using intercalation dyes such as SYBR™, Pico Green (Molecular Probes, Inc., Eugene, Oreg.), ethidium bromide and the like (see Zhu et al., 1994, *Anal. Chem.* 66:1941-48) and/or gel electrophoresis. More often, sequence-specific detection methods are used (i.e., amplicons are detected based on their nucleotide sequence). Examples of detection methods include hybridization to arrays of immobilized oligo or polynucleotides, and use of differentially labeled molecular beacons or other "fluorescence resonance energy transfer" (FRET)-based detection systems. FRET-based detection is a preferred method for detection. In FRET-based assays a change in fluorescence from a donor (reporter) and/or acceptor (quencher) fluorophore in a donor/acceptor fluorophore pair is detected. The donor and acceptor fluorophore pair are selected such that the emission spectrum of the donor overlaps the excitation spectrum of the acceptor. Thus, when the pair of fluorophores are brought within sufficiently close proximity to one another, energy transfer from the donor to the acceptor can occur and can be detected. A variety of assays are known including, for example and not limitation,