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(54) Title: SOLID PHASE BASED NUCLEIC ACID ASSAYS COMBINING HIGH AFFINITY CAPTURING AND DETECTION BY SPECIFIC HYBRIDIZATION

(57) Abstract: This invention relates to methods for detection of nucleic acids on a solid phase with high affinity and high specificity. More particularly, the invention relates to methods combining high-affinity hybridization-based capturing with highly specific hybridization-based discrimination in solid phase based nucleic acid assays. This invention further relates to kits containing the reagents necessary for carrying out the disclosed assays. The detection of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) is of importance in human or veterinary diagnostics, food control, environmental analysis, crop protection, biochemical/pharmacological research, or forensic medicine.



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SOLID PHASE BASED NUCLEIC ACID ASSAYS
COMBINING HIGH AFFINITY CAPTURING AND DETECTION
BY SPECIFIC HYBRIDIZATION

5 Field of the Invention

This invention relates to methods for detection of nucleic acids on a solid phase with high affinity and high specificity. More particularly, the invention relates to methods combining high-affinity hybridization-based capturing with highly specific hybridization-based discrimination in solid phase based nucleic acid assays.

10 This invention further relates to kits containing the reagents necessary for carrying out the disclosed assays. The detection of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) is of importance in human or veterinary diagnostics, food control, environmental analysis, crop protection, biochemical/pharmacological research, or forensic medicine.

15 Background of the Invention

In a typical solid phase based nucleic acid assay, capture oligonucleotides are immobilized on a solid support. The labeled or unlabeled nucleic acid target is specifically hybridized to the capture probes. After hybridization and, if necessary, labeling, the hybridization event can be detected using e.g. optical, electrical, mechanical, magnetic etc. readout technology. Generally, the high specificity of base pairing interactions between strands of nucleic acids is used in these methods to differentiate between different targets. Using a solid phase enables facile multiplexing of nucleic acid hybridization assays by spatially separating different capture oligonucleotides having different sequences. A huge number of different supports e.g. planar surfaces ("chips"), beads or gel matrices can be used as solid phases. Methods for preparation of DNA oligonucleotide arrays are summarized e.g. in S.L. Beaucage, Curr. Med. Chem. 2001, 8, 1213-1244 or M. C. Pirrung, Angew. Chem. 2002, 114, 1326-1341). Solid phase based nucleic acid hybridization assays are widely used e.g. for analysis of single nucleotide polymorphisms (SNPs),

expression profiling or viral detection (for a summary see e.g. J. Wang, Nucl. Acids Res. 2000, 28, 3011-3016.

Methods for genotyping single nucleotide polymorphisms are described e.g. in P.-Y. Kwok, Annu. Rev. Genomics Hum. Gen. 2001, 2, 235-258. One current
5 general method for detection of SNPs relies on a three step procedure: purification of genomic DNA from biological material, amplification of the desired gene fragment e.g. by PCR and subsequent detection by allele specific hybridization, enzymatic reactions etc. Due to the current lack of highly sensitive nucleic acid detection
10 methods, the amplification step is unavoidable. However, this step is very laborious, time consuming, expensive, difficult to multiplex and difficult to integrate into fully automated devices as they are needed for nucleic acid analysis at the point of care.

In summary, there is a need for fast, simple and inexpensive assays that allow for highly sensitive, highly selective detection of nucleic acids, e.g. comprising SNPs, directly from genomic DNA, without prior amplification. These assays are
15 especially needed for genotyping in a point-of-care environment.

The present invention is directed to a method for combining high specificity with high sensitivity in order to enable nucleic acid analysis on a solid surface from biological sources without prior amplification.

An example of a nucleic acid assay which employs multiple hybridization
20 reactions for combination of affinity and specificity is given in WO95/16055. In this approach, capture probes are bound to a surface. One or more capture extender molecules are employed, each containing a target specific binding sequence and a support binding sequence able to hybridize to the surface bound capture probes. The capture extender sequences are used to bind the target to the support with high
25 affinity. For detection, e.g. amplification multimers are hybridized to the target in order to amplify signals. Different sequences can be discriminated by specific hybridization of capture extenders containing sequences specific to different target regions. In case of targets with closely related sequences (e.g. targets containing mutations like single nucleotide polymorphisms) this approach does not work for

more than one capture extender, because the differences in thermodynamic stabilities and thus melting temperatures are too small for effective discrimination.

5 A nucleic acid hybridization assay combining affinity and specificity has been described by Wanda L.B. White (Poster-title "SNP determination by dual hybridization with DNA and PNA probes" at the Cambridge Healthtech Institute Conference on Nucleic Acid Based Technologies, Washington D.C., 2002). An immobilized 40mer DNA is used to capture the target with high affinity, while a short PNA probe is used for allele-specific hybridization. Drawbacks of this assay principle are that long capture sequences are expensive in synthesis, not easily
10 accessible in sufficient purity and often not specific enough for multiplexed assays.

US 2004/0009506 A1 describes a nucleic acid hybridization assay that enables highly sensitive target capturing by using an immobilized capture polymer. The capture polymer consists of target-specific capture sequences that are either
15 directly or indirectly bound to a solid support. The captured nucleic acid sequences are detected by hybridization of label probes containing two or more units of label bound to the label probe. The disclosed assay type does not provide methods for high sensitivity detection of closely related sequences, e.g. sequences differing by a single base. A similar assay type, combining high sensitivity capturing with highly specific
20 enzymatic discrimination is disclosed in WO 2004/020654 A2. Here, target-specific capture sequences that are bound to a solid support either directly or indirectly capture a target from solution with high affinity. Closely related sequences, e.g. sequences differing by a single base, are discriminated by using enzymes that incorporate the label during e.g. ligation or extension reactions. Although this highly
25 sensitive assay type enables qualitative and quantitative detection of closely related sequences from genomic samples without target amplification, it relies on the discriminative power of enzymes. The incorporation of enzymes into the assay workflow adds a process step, increases assay cost and makes integration into fully-automated devices complicated.

30

In summary, many nucleic acid assay formats that make use of a hybridization reaction of a target probe to a capture probe immobilized on a solid

phase suffer from either sensitivity or selectivity or require enzymatic steps. Therefore, problems occur if e.g. single nucleotide polymorphisms must be detected in samples without prior target amplification in a fast, simple and inexpensive way.

5 Summary of the Invention

Methods and kits are provided for detecting nucleic acids with high sensitivity and high specificity on a solid support. In general, the methods combine high affinity capture using one or more target specific oligonucleotides with highly
10 specific hybridization-based discrimination methods. Preferred methods include the use of one or more capture extender molecules for capturing the target with high affinity, in combination with a set of two or more hybridization probes that are specific for a particular variant of the target (e.g. wild type or single base mutated target). The different hybridization probes are encoded by a particular type (e.g. a
15 specific color) of label.

Brief Description of the Drawings

Figure 1: Summary of the assay in a preferred embodiment. A target (5)
20 containing a single base mutation (8) is hybridized to mixtures of target-specific capture probes (1) that are immobilized on a solid support (6) with or without a spacer (7) and are complementary to sequences of the target (2). Allelic discrimination is achieved by hybridization of probes that contain an allele-specific site (4) and are labeled with different labeling entities (3).

25 **Figure 2:** Summary of the assay in a preferred embodiment. A target (5) containing a single base mutation (8) is hybridized to a mixture of immobilized capture probes (1) and an allele-specific discrimination probe containing an allele-specific site (4) on a solid support (6) with or without a spacer (7). The capture
30 probes are hybridized to sequences on the target (2) outside the single base mutation. The target (6) is labeled with labeling entities (3) that are directly or indirectly attached to the target.

Detailed Description of the Invention

The invention combines high-affinity oligonucleotide capture with highly specific hybridization-based discrimination on a solid support, preferably for the detection of single nucleotide polymorphisms in multiplex assays without prior amplification of genomic DNA. The invention makes use of two or more target-specific capture probes that are directly or indirectly attached to a solid support. The capture probes can be spotted as mixtures of different oligonucleotides and covalently or noncovalently attached to a support. Alternatively, they can be hybridized to universal capture sequences immobilized as arrays on planar substrates. By spotting or hybridizing mixtures of capture sequences each target can be separately captured in defined spots on the surface. This enables a multiplex detection of e.g. different gene fragments of a gene of interest in different spots on the surface. If more than one capture sequence hybridizes to the same target, cooperative binding leads to a significant increase in sensitivity thereby enabling detection of targets directly from genomic DNA without prior amplification.

In a preferred embodiment of the disclosed invention the capture probes are immobilized on a solid support. In order to ensure effective hybridization of the capture probes to the target, a spacer can be introduced between the surface and the hybridizing sequence. This spacer can be composed of units that are capable of hybridization such as nucleotides and nucleotide analogs. Alternatively, the spacer can be composed of units that are not capable of hybridization such as a hydrocarbon chain. The hydrocarbon chain can also contain atoms taken from the 5th or 6th group of the periodic system, preferably nitrogen, phosphorous, oxygen or sulfur. The spacer can also be composed of both nucleotide and non nucleotide units. In one particularly preferred embodiment the capture probes are covalently immobilized on the planar surface via their 3'- or 5'-termini without a spacer on a planar substrate that is coated with a polymeric layer. In this embodiment, the polymeric layer ensures effective hybridization of the capture probes to the target. In another particularly preferred embodiment, the capture probes are covalently immobilized on a planar surface via their 3'- or 5'-termini with a short spacer containing 2-20 units capable of hybridization, particularly preferred 2-10 units capable of hybridization on a planar

substrate that is coated with a polymeric layer. A variant in which the capture probes are covalently immobilized on a planar surface via their 3'- or 5'-termini with a spacer containing 3-30 ethylene glycol units is particularly preferred. Examples for spacer molecules that can be attached to the 5'-terminus of capture probes include the
5 commercially available Spacer Phosphoramidite 9 (3 ethylene glycol units and one phosphate unit), 18 (6 ethylene glycol units and one phosphate unit) and C3 (propanol unit and phosphate unit) as well as the Spacer C12 CE Phosphoramidite (dodecanol unit and phosphate unit) from Glen Research, Sterling, Virginia, USA. One example for a spacer molecule that can be attached to the 3'-terminus of the
10 capture probes is the 3' Spacer 9 CPG (3 ethylene glycol units) which is also commercially available from Glen Research, Sterling, Virginia, USA. In a further preferred embodiment of the disclosed invention the capture probes are covalently immobilized on a solid support via a 5'-terminal spacer that contains 3-10 nucleotide units and 3-30 ethylene glycol units).

15

In order to capture the targets with high sensitivity and high specificity, it is necessary to provide two or more capture probes that can hybridize to one target. In a preferred embodiment of the disclosed invention 2-10 capture extenders are provided for each target and are covalently or non-covalently immobilized on a planar
20 substrate as mixtures resulting in an array of mixtures of immobilized capture probes wherein a particular target can be captured within a particular spot. The capture probes comprise a sequence of 5-100 nucleotides, preferably 10-50 nucleotides that is complementary to a particular target.

25

The targets, captured by mixtures of capture probes that are arrayed on a solid substrate, are labeled during hybridization of label probes. These label probes can be chosen from the group of molecules capable of hybridization that are known to persons skilled in the art e.g. DNA, DNA analogs, LNA and PNA. The length and sequence of the label probes depend on the target to be detected. In a preferred
30 embodiment of the disclosed invention mutations (e.g. single nucleotide polymorphisms and deletions) within targets are detected by differential hybridization of allele-specific hybridization probes. In a preferred embodiment of the disclosed invention targets are captured on an array of immobilized mixtures of

capture probes, whereupon both alleles of a gene fragment containing a single nucleotide polymorphism are captured within the same spot. Before, during or after hybridization of the targets to the array, allele-specific label probes are hybridized to the targets. To discriminate between alleles, the hybridization probes are encoded
5 with different labels such that each allele corresponds to a particular type of label. In a particularly preferred embodiment of the present invention two allele-specific label probes per target each encoded by a specific label compete during hybridization to a particular sequence containing a mutation. This competing hybridization (also termed differential hybridization) increases the specificity and is especially useful if
10 single nucleotide polymorphisms are going to be detected. Thus in one preferred embodiment the disclosed invention combines two levels of multiplexing: spatially resolved immobilization of mixtures of capture probes that are specific for particular gene fragments and specific hybridization of label-encoded probes that can distinguish between different variants of a target.

15 In one preferred embodiment of the invention, different hybridization probes are encoded by different labels. Therefore, two or more types of labels are needed for one preferred embodiment. Labels or groups enabling labeling reactions can be e.g. fluorophors, nanoparticles, redox active moieties, antibodies, antibody fragments,
20 biotin, aptamers, peptides, proteins, mono- or polysaccharides, nucleic acids, nucleic acid analogs, complexing agents, cyclodextrins, crown ethers, anticalins, receptors etc. In one particularly preferred embodiment two or more hybridization probes are encoded by two or more different fluorescent dyes that emit fluorescence of different wavelengths. In a second particularly preferred embodiment two or more
25 hybridization probes are encoded by two or more different fluorescent nanoparticles that emit fluorescence of different wavelengths. If the invention is carried out with one type of label, the sample to be analyzed must be split into two separate hybridization reactions, carried out on two different arrays. In a particular
30 embodiment of this invention, two different alleles of a set of targets are detected on two different arrays, whereupon the labeled probe specific for one allele competes with the unlabeled probe of the other allele for hybridization to the target.

An alternative embodiment of the invention that also uses one type of label makes use of immobilized mixtures of probes, each of the hybridizing to a specific target. In addition, capture probes are immobilized within the spots, that are specific to a particular variant of a target, e.g. a particular allele. In this particularly preferred
5 embodiment each allele of a target binds to a specific spot only if both the target-specific capture probes and the allele-specific capture probes become hybridized to the target. In this assay configuration the target can be labeled prior, during or after hybridization to the array using a method known to a person skilled in the art. Examples for labeling reactions include, but are not limited to, incorporation of dyes
10 during primer extension, hybridization of labeling probes or chemical attachment of dyes.

If the disclosed assays are used to detect DNA- or RNA-Sequences without prior amplification of the respective target it may be beneficial to fragment the
15 nucleic acids prior to hybridization to the planar surface. Fragmentation can be performed using different methods known to a person skilled in the art. Examples for fragmentation methods include, but are not limited to, sonication, enzymatic digestion, chemical fragmentation e.g. using radicals (as described e.g. in Y. Zhang et al., Nucleic Acids Res. 2001, 29, 13, e66). Fragmentation allows for the detection
20 of a small (i.e. 50-1000 bp) sequence stretch within a target gene on one spot of the array. Thus, close-by mutations can be detected on different gene fragments in different spots of an array.

Depending on the type of label that has been introduced during the
25 hybridization-based detection step, different readout methods can be used to assess the result of the assay. Examples for readout methods include optical, electrical, mechanical or magnetic detection. More specifically, fluorophores can be detected using e.g. planar optical waveguides as disclosed in US 5959292, total reflection on interfaces as disclosed in DE 196 28 002 or using optical fibers as disclosed in US
30 4815843. Nanoparticle labels can be detected e.g. via optical methods or e.g. by direct electrical detection after autometallographic enhancement as disclosed in US patents US 4794089, US 5137827 and US 5284748.

The disclosed invention requires a solid phase which is used to immobilize the capture probes. Examples for solid phases include, but are not limited to, beads, planar surfaces, metallic particles and gel matrices.

5 Examples

Example 1: Shearing of human genomic DNA samples

Four different samples of genomic DNA, representing wild type (WT), variant (VAR) and heterozygous (HET) samples for two different SNP sites were
10 acquired. As WT sample human genomic DNA (Roche, Switzerland) was used (sample 1). HET samples were obtained from the Coriell Cell Repository (Coriell Institute, Camden, NJ, USA): NA11497 (containing the SNP G542X, heterozygote, sample 2) and NA12585 (containing the SNP R1162X, heterozygote, sample 3). VAR sample was also obtained from the Coriell Institute: NA11496 (G542X,
15 variant, sample 4). 60 µg genomic DNA from each sample was dissolved in Tris-EDTA buffer (10 mM Tris base, 1mM EDTA, pH=8.0) to give a volume of 400µl. The samples were cooled on ice and sonified using a Branson Sonifier B12 (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a microtip (3mm diameter) under the conditions of power 3 and 50% duty cycle for a total of 10 min.
20 The sonicated samples were precipitated by addition of 20 µl aqueous 5M NaCl and 2 µl glycogen solution (20mg/ml in water) and 1ml of ice cold ethanol. Samples were incubated for 30 min. at -20°C. Subsequently, the solutions were centrifuged 15 min at 13000 g. The supernatant was removed and 400 µl of 70% ethanol were added to the precipitate. Subsequently the samples were centrifuged for 5 min. at 13000 g.
25 The supernatant was removed, the pellets were dried under vacuum and redissolved in 40 µl of distilled water.

Example 2: Preparation of DNA arrays with mixed capture probes

Four biochips (Unaxis AG, Balzers, Liechtenstein) with outer dimensions of 2x1
30 cm were obtained. The chips consisted of glass and an optical grating with 18 nm deepness etched into the glass. They were coated with a 155nm thick layer of Ta₂O₅. The chips were cleaned by the following protocol: 10 min sonication in 65% HNO₃, rinsing by pure distilled water, 10 min sonication in 30% hydrogen peroxide, rinsing

by pure distilled water, 10 min sonication in pure distilled water. Then, the PWG-chips were coated with aminopropyltriethoxysilane (APTES, Sigma, Deisenhofen, Germany) using the following protocol: drying of the cleaned chips @80°C for 5min, preparation of 100 ml silanization solution (1% APTES in acetone/water (95:5 v/v)),
5 submerging the warm chips in the silanization solution, incubation for 15 min. at room temperature, rinsing of the chips 5 times by acetone, drying of the chips at 110°C for 45 min.

For immobilization of capture probes, the silanized chips were incubated in a 100ml solution of 10mM suberic acid bis(N-hydroxysuccinimideester) in
10 dimethylsulfoxide (DMSO) and 50 µL triethylamine for one hour. Thereafter, the chips were rinsed two times by DMSO and once by distilled water and blown dry in a stream of nitrogen. Two different mixtures of capture probes were prepared: mix 1 consisted of the following capture probe sequences:

CE001: TCAGATTGAGCATACTAAAAGTGACTCTTTTTT-NH2

15 CE002: CTAATTTTCTATTTTGGTAATAGGACATTTT-NH2

CE003: TCTCCAAGTTTGCAGAGAAAGACATTTT-NH2

CE004: CAAGAATTTCTTTAGCAAGGTGAATAATTTT-NH2

CE005: CTAATTATTGGTCTAGCAAGCATTTT-NH2

Mix 2 consisted of the following five sequences:

20 CE01: GGCAGACAATTTTACAATTAGTTTGTTTTT-NH2

CE02: AAATAACAACATTTTGTTTTAAGAATTTT-NH2

CE03: GACTTGGTAGGTTTACCTTCTGTTGTTTTT-NH2

CE04: GGCCATTCTTGTATGGTTTGGTTTTTT-NH2

CE05: TCTCAATAATCATAACTTTTCGAGAGTTTTTT-NH2

All ten sequences were obtained from Thermo Hybaid Corporation, Ulm, Germany and were synthesized with a 3'-terminal amine (3'-amino modifier by Glen Research, Sterlington, VA, USA). The oligonucleotides were mixed in equal amounts to give solutions of mix 1 and mix 2 with a final DNA concentration of 5x10⁻⁵M in potassium phosphate buffer, pH 7.2. These solutions were placed in small droplets (0.4 nl) onto the chip surface using a Biochip Arrayer (Perkin Elmer, Germany), creating two arrays of 4x4 spots on each chip and incubated overnight in a humid chamber. Thereafter, the chips were rinsed once by 1 M NaOH solution and six times by distilled water. Capping of unreacted amino groups on the surface was performed by reaction with a solution of 0.4 mg/ml bis-sulfo-succinimidyl-suberate (BS3, Pierce, Rockford, IL, USA) in 0.1M potassium phosphate buffer, pH 7.2 overnight. Chips were washed three times with distilled water, dried in a stream of nitrogen and used thereafter.

Example 3: Hybridization of genomic DNA to arrays of mixed capture probes

Hybridization chambers were mounted onto the four biochips, thereby creating eight discrete chambers with a volume of approximately 100 µl each. Eight different Allele Specific Hybridization probes (ASH-probes) were obtained from Thermo Hybaid Corporation, Ulm, Germany. The ASH-probes comprised the following oligonucleotides:

fA: CY5-ttatatcaagaaccttccacctt

A: ttatatcaagaaccttccacctt

fB: CY5-ttatatcaagaaacttccacctt

B: ttatatcaagaaacttccacctt

fC: CY5- gatctgtgagccgagtctttaag

C: gatctgtgagccgagtctttaag

fD: CY5- gatctgtgagctgagtctttaag

D: gatctgtgagctgagtctttaag

As indicated, four oligonucleotides were modified at their 5'end using a CY5-modifier (Glen Research, Sterling, VA, USA).

20 µl each of the sonicated DNA samples (see example 1) was mixed with an equal volume of denaturing diluent (taken from the Versant 3.0 bDNA assay kit, Bayer HealthCare LLC, Tarrytown, NY, USA) and incubated for 1h at 65°C. Two different mixtures of ASH probes were prepared: A wild type probe mix containing fA, B, fC and D at 5x10⁻⁵M concentration each in a 1:1 solution of neutralization solution (taken from the Versant 3.0 bDNA assay kit, Bayer HealthCare LLC, Tarrytown, NY, USA) and HIV 3.0 lysis solution (taken from the Versant 3.0 bDNA assay kit, Bayer HealthCare LLC, Tarrytown, NY, USA) supplemented with 5x Denhardt's solution (Sigma, Deisenhofen, Germany). The variant probe mix consisted of the ASH probes fB, A, fD and C with the same buffers and additives as above. After incubation, the DNA samples (40µl each) were mixed with 40µl of the ASH probe mixes to give a total volume of 80 µl. Each DNA sample was prepared in two different reaction mixtures containing the two different sets of ASH probes. The resulting solutions were pipetted into the eight different hybridization chambers, sealed tightly and were incubated overnight at 54°C. Following incubation, the chips were read out on a fluorescence reader, type Minifluo IV (Bayer Technology Services, Leverkusen Germany). The reader enabled to couple laser light into the optical grating and image the fluorescence with a CCD camera. Fluorescent intensity of each spot on the array was quantified and the mean of fluorescence for all mix 1 and mix 2 spots was calculated. The results are summarized in the table below:

Sample	1	1	2	2	3	3	4	4
Probes	fA+B fC+D	fB+A fD+C	fA+B fC+D	fB+A fD+C	fA+B fC+D	fB+A fD+C	fA+B fC+D	fB+A fD+C
RFU Mix 1	2,2	1,2	2,7	0,9	1,1	2,5	1,5	1,6
RFU Mix 2	5,0	1,1	1,6	1,6	6,7	1,2	3,0	1,1

For genotype determination, the ratio of mean fluorescence intensities for each sample was calculated by dividing the signal for the wild type probe mix by the signal for the variant probe mix for the respective type of spots (spots mix 1 and

spots mix 2). The results are given in the table below, expressed as log (wild type RFU / variant RFU):

Sample	1	2	3	4
Log (WT/VAR) Mix 1	0,3	0,5	-0,4	0,0
Log (WT/VAR) Mix 2	0,7	0,0	0,7	0,4

- 5 The results are consistent with the assumed genotypes of WT/WT (sample 1), WT/HET (sample 2), VAR/WT (sample 3) and HET/WT (sample 4). In this example the DNA samples analyzed were split into two separate mixtures in order to perform the experiment with only one type of label. In case of two types of fluorescent labels ("two colors"), the wild type and variant probes can be labeled differently and the
- 10 analysis of one sample can be performed on one array.

Claims

We claim:

- 5 1. A solid phase based nucleic acid assay combining high sensitivity and high specificity, comprising (a) capturing one or more targets by high-affinity hybridization to mixtures of capture probes immobilized on a solid support, (b) discriminating different variants of the targets by hybridization of labeled probes and (c) detecting the result exploiting the physical properties of the labels.
- 10 2. A solid phase based nucleic acid assay combining high sensitivity and high specificity, comprising capturing the target by high-affinity hybridization to mixtures of target-specific capture probes and capture probes specific for a variant of the respective target immobilized on a solid support.
- 15 3. The assay of claim 2 wherein the targets to be detected are labeled directly or indirectly prior, during or after hybridization of the targets to the array.
4. The assay of claims 1 and 2 wherein mixtures of two or more target-specific capture probes per target are immobilized directly or indirectly on a solid support, forming an array.
- 20 5. The assay of claim 1 wherein the hybridization probes that are specific for certain variants of a target are encoded by a specific label so that each allele of a target can be identified by an allele-specific hybridization probe.
- 25 6. The assay of claims 1-5 wherein fluorescent molecules or particles are used for labeling.
- 30 7. The assay of claim 5 wherein differential hybridization of differently labeled hybridization probes that compete for hybridization is used to enhance discrimination between different variants of a target.

8. The assay of claim 5 wherein the sample to be analyzed is separated in two parts and one part is hybridized to labeled probes that are specific for a first variant of the target in the presence of non labeled probes that are specific for a second variant of the target and that compete with the labeled probes for hybridization and wherein the second part of the sample is hybridized to labeled probes that are specific for a second variant of the target in the presence of non labeled probes that are specific for a first variant of the target and that compete with the labeled probes for hybridization.
9. The assay of claim 4 wherein the mixtures of capture probes are immobilized on a solid support via a short spacer of 1-20 nucleotides between the attaching unit and the hybridizing sequence.
10. The assay of claim 4 wherein the mixtures of capture probes are immobilized on a solid support that is coated with a polymeric layer with a spacer of 0-20 atoms between the hybridizing sequence and the group that is used for attachment to the support.
11. The assays of claims 1-10 wherein planar optical waveguides are used for reading out the labels during or after hybridization to the array.
12. The assays of claims 1-11 wherein the target is fragmented prior to hybridization to the array.
13. The assays of claims 1-12 wherein the targets contain mutations selected from the group of single base substitutions, single nucleotide polymorphisms, insertions, deletions or duplications.
14. The assays of claims 1-13 wherein the solid support is selected from the group consisting of: beads, planar surfaces, metallic particles and gel matrices as supports for multiplexing.

15. The assays of claims 1-14 wherein optical, electrical, mechanical, magnetic methods are used for discriminating sequences.

16. Kits for performing the assays of claims 1-15.

5

17. An array according to claims 1-16.

Figure 1

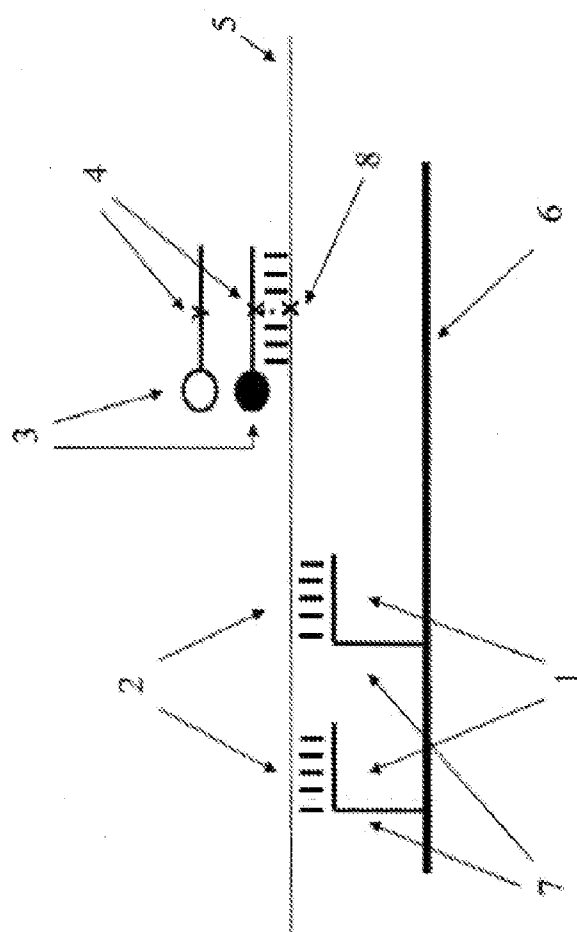


Figure 2

