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(54) Title: METHOD FOR DETECTION OF NON-LABELED PCR PRODUCTS ON SANDWICH HYBRIDIZATION BASED ARRAY PLATFORMS

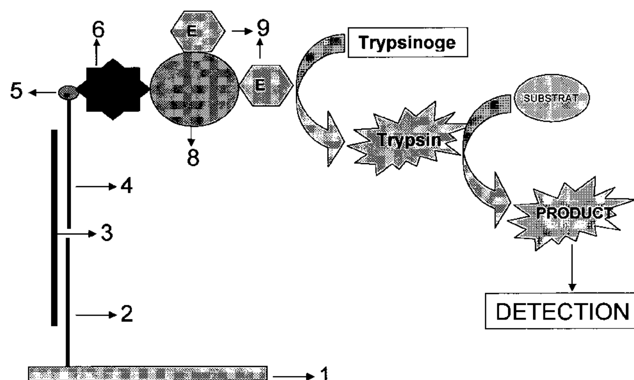

Figure 2

(57) Abstract: The present invention provides methods for selectively detecting at least one complementary oligonucleotide target species in a mixture of different oligonucleotide fragments in double or single stranded format by using a sandwich hybridization assay. The sandwich hybridization assay requires at least two different specific probes hybridize to the target nucleic acid of interest. In this format, the first probe (the capture sequence) is bound to a solid support and is allowed to bind (capture) the target nucleic acid sequence. A second probe (the signal probe) with a sequence that is adjacent or close to the capture sequence on the target DNA molecule is then allowed to hybridize to the captured target nucleic acid sequence. One preferred embodiment of the method is direct utilization of non-labeled double stranded nucleic acid species (such as PCR products or genomic DNA fragments) in the array platforms. This double stranded nucleic acid product acts as target probe and establishes a sandwich between the capture and signal probes. Once PCR reaction is over there is no need for further purification of PCR products and/or isolation of single strands. Another embodiment of the method is employment of alternative signal generating strategies such as using streptavidin conjugated quantum dots and use of CELISA (Cascade enzyme-linked immunosorbent assay) method.

DESCRIPTION

METHOD FOR DETECTION OF NON-LABELED PCR PRODUCTS ON SANDWICH HYBRIDIZATION BASED ARRAY PLATFORMS

FIELD OF THE INVENTION

The present invention provides methods for selectively detecting at least one complementary oligonucleotide target species in a mixture of different oligonucleotide fragments in double or single stranded format by using a sandwich hybridization assay. The sandwich hybridization assay requires at least two different specific probes hybridize to the target nucleic acid of interest. In this format, the first probe (the capture sequence) is bound to a solid support and is allowed to bind (capture) the target nucleic acid sequence. A second probe (the signal probe) with a sequence that is adjacent or close to the capture sequence on the target DNA molecule is then allowed to hybridize to the captured target nucleic acid sequence.

One preferred embodiment of the method is direct utilization of non-labeled double stranded nucleic acid species (such as PCR products or genomic DNA fragments) in the array platforms. This double stranded nucleic acid product acts as target probe and establishes a sandwich between the capture and signal probes. Once PCR reaction is over there is no need for further purification of PCR products and/or isolation of single strands.

Another embodiment of the method is employment of alternative signal generating strategies such as using streptavidin conjugated quantum dots and use of CELISA (Cascade enzyme-linked immunosorbent assay) method.

BACKGROUND OF INVENTION

DNA has become an important part of various analysis and delivery systems in recent years. In analysis, many of the applications are for detection, genetic desises and both of pathogens and contamination in food and the environment. Nucleic acids are perfect for detection assays because they are highly specific, being able to determine which strain of a species a bacterium belongs to. DNA is a more robust nucleic acid than RNA, and is therefore more suitable to an assay. In many of these assays, a short sequence of DNA, called a probe, is used to detect a longer sequence of DNA or RNA, called the target, which is generated by the organism in question. The probe is generally immobilized on some surface and then mixed with the target to allow them to hybridize. At this point, a signal needs to be generated and measured. This signal is usually generated by adding a second

DNA probe with a modification, such as the attachment of a fluorescent molecule that allows the detection of the probe that is bound to the target once the excess has been washed out. This combination of probe-target-probe is often called a DNA sandwich assay.

Sandwich hybridization methods have incorporated several major improvements within the last couple of years. The addition of helper probes facilitates the hybridization of capture and detection probes to target nucleic acids, resulting in greater sensitivity of the assay.

Some amplification reactions are isothermal, such as nucleic acid sequence based amplification (NASBA). Others employ thermal cycling, such as the polymerase chain reaction (PCR). Preferred amplification assays employing fluorescence detection of amplified product are homogeneous, that is, they do not require the physical separation of reagents to permit detection (for example, separation of bound probes from unbound probes) and can be performed in a single closed vessel. Such assays may be end-point, wherein product is detected after amplification, or they may be real-time, wherein product is detected, as amplification proceeds.

Nucleic acid amplification and assays employing PCR are described, for example, in U.S. Patents 4 683 202, 4 683 195 and 4 965 188, and, generally, PCR PROTOCOLS, a guide to Methods and Applications, Innis et al. eds., Academic Pres (San Diego, CA (USA) 1990). Homogeneous PCR assays, including real-time assays, in which amplified product is detected during some or all of the PCR cycles as the reaction proceeds are described, for example, in U.S. Patents 5 994 056, 5 487 972, 5 925 517 and 6 150 097.

PCR amplification reactions generally are designed to be symmetric, that is, to make double-stranded amplicons exponentially by utilizing forward primer and reverse primer in equimolar concentrations and equal melting temperatures (Tm's).

Fluorescent-labeled probes are used in homogeneous nucleic acid amplification assays, including PCR assays, to measure the accumulation of desired amplicon, either in real time or by end-point analysis. Several available types of probes are significantly allele-discriminating as compared to linear single-stranded probes. Real-time probes include dual-labeled linear probes that are cleaved by 5' -to-3' exonuclease activity of DNA polymerase during the extension step of a PCR cycle (U.S. patents 5 210 015, 5 487 972 and 5 538 848); molecular beacon probes (U.S. patents 5 925 517, 6 103 476 and 6 365 729); minor groove binding probes (*Afonina et al. "Minor Groove Binder-Conjugated DNA Probes for Quantitative DNA Detection by Hybridization-Triggered Fluorescence," Biotechniques 32, (2002), 946-949)*; linear probe pairs that FRET when hybridized adjacently on a target strand; quenched double-stranded linear probes for which a target competes to hybridize to the labeled probe

strand (*Li, Q. et al. Nucl. Acid. Res. 30, (2002), e5*); and so-called "light-up" probes, which are peptide nucleic acid (PNA) oligomers linked to an asymmetric cyanine dye that fluoresces when the probe binds to target to form a double-stranded region.

More recently however, the value of using immobilized, spatially distinguishable, hybridization probes for concurrent analyses of multiple gene sequences has been recognized and resulted in the development of miniaturized hybridization assays using solid matrix assays [*Southern, E.M., Trends in Genetics 12, (1996) 110-115*]. Thus, hybridization using said matrix arrays have been performed on glass surfaces [*Maskos, U. and E.M. Southern, Nuc. Acids. Res. 20: 1679-1684 (1992); Guo et al., Nuc. Acid. Res. 22: 5456-5465 (1994)]*; on microtiter plates *[Kalakowski et al., Anal. Chem. 68: 1197-1200 (1996); Nikiforov et al., Nuc. Acids Res. 22: 4167-4175 (1994); Rasumussen et al., Anal. Biochem. 198: 138-142 (1991)]*; on plastic sheets *[Matson et. al. Anal. Biochem. 224: 110-116 (1995)]*; on thin polymer gels *[Khrapko et al., J. DNA Seg. Map. 1: 375-388 (1991)]*; and using semiconductor devices *[Eggers et al., Bio Techniques 17: 516-524 (1994); Kreiner, T, Am. Lab.: 39-43 (1996)]*. In addition, the desire for using non-radioactive means for detection have caused a surge of interest in means for detection of hybridization on solid matrix supports which employ fluorescence *[Kumke et. al., Anal. Chem. 67: 3945-3951 (1995); Piunno et. al., Anal. Chim. Acta. 288: 205-214 (1994)]*; chemiluminescence *[Ito et. al., J. Neurosci. Methods 59: 265-271 (1995); Nguyen et. al., Biosen. Bioelectron. 7: 487-493 (1995)]*; evanescent wave technology *[Graham et al., Biosen. Bioelectron. 7: 487-493 (1992); Strachan et. al., Lett. App. Microbiol. 21: 5-9 (1995); Watts et. al., Anal. Chem. 67: 4283-4289 (1995)]*; confocal microscopy *[Fodor et. al., Nature (London) 364: 555-556 (1993*)]; light scattering *[Stimpson et. al., Proc. Natl. Acad. Sci. USA 92: 6379-6383 (1995)]*; electrochemistry *[Milland et. al., Anal. Chem. 66: 2943-2948 (1994); Pandey et. al., Anal. Chem. 66: 1236-1241 (1994); Hashimoto et. al., Anal. Chim. Acta. 286: 219-224 (1994)]*; and surface resonance phenomena *[Yamaguchi et. al., Anal. Chem. 65: 1925-1927 (1993)]*. Despite these recent innovations using probes immobilized on solid matrix arrays the major obstacles and limitations of hybridization methods generally continue to restrict and contain the currently available techniques and formats. These demands and limitations include a requirement for a large sample volume; an inability to perform multiple analyses concurrently in real time; a requirement for a relatively high concentration of target DNA (the complementary target sequence) in the fluid sample; an inability to detect multiple species concurrently; relatively slow kinetics for hybridization to occur between the target sequences and the immobilized probes within the assay format; and a dependence upon lengthy assays. Moreover, despite the use of new in-vitro amplification techniques such as

the polymerase chain reaction procedure, the problems of assay sensitivity, lengthy times for analysis, the quantum of background signal noise, and the inability to detect more than one target nucleic acid sequence at a time remain as recurring handicaps and continuing obstacles for each of these techniques. It will be recognized and appreciated by persons working in this field today, therefore, that the development of a unique platform which overcomes and eliminates most, if not all, of these major limitations and procedural hindrances would be seen as a major advance and unforeseen improvement in this art.

A conventional nucleic acid array platform employs a labeling step of the target nucleic acids prior to hybridization. Following hybridization the signal is detected according to the nature o the label.

Alternatively in certain nucleic acid array platforms, prior to signal generation PCR products are labeled with biotin and following hybridization with the capture probe signal is generated with enzyme conjugated streptavidin *(Sachse K, Hotzel H, Slickers P, Ellinger T, Ehricht R, DNA microarray-based detection and identification of Chlamydia and Chlamydophila spp. Mol Cell Probes 2005, 19, 41-50; Ralf Ehricht, Peter Slickers, Stefanie Goellner, Helmut Hotzel, Konrad Sachse, Optimized DNA microarray assay allows detection and genotyping of single PCR-amplifiable target copies, Molecular and Cellular Probes 20 (2006) 60–63 and US 20030215891).* In such platforms depending on the conjugated enzyme the signal is generated by addition of the necessary substrates. For instance in case of horse radish peroksidase TBM (3,3,5,5 Tetrametilbenzydin) is a common subtrate and forms a blue precipitate. Alkaline phosphatase is another commonly used alternative and utilizes pNPP (para-nitrophenylphosphate) as substrate.

Other nucleic acid based sandwich hybridization assay platforms on the other hand utilizes RNA molecules which can directly establishes a sandwich between the capture and detection probe *(Huhtamella, S., Leinonen, M., Nieminen, T., Myllykoski, L., Fahnert, B., Breitenstein, A., Neubauer, P., RNA based sandwich hybridisation method for detection of lactic acid bacteria in brewery samples, J. Micro-biol. Methods. 68 (3), (2007) 543-553).* As explaind in this and other methods (WO01/48244 A2) the sandwich is established on micro/nano particles with or without magnetic properties.

Some application also utilizes genomic DNA extracts to establish a sandwich between a opitical thin film immobilized capture probe and biotin labeled signal probe. Then a blue visible signal is achieved by addition of HRP conjugated streptavidin and TBM substrate *(Wesley C. Lindsey, Evelyn S. Woodruff, Diane Weed, David C. Ward, Robert D. Jenison, Development of a rapid diagnostic assay for methicillin-resistant Staphylococcus aureus and*

methicillin-resistant coagulase-negative Staphylococcus, Diagnostic Microbiology and Infectious Disease 61 (2008), 273–279)

BRIEF DESCRIPTION OF INVENTION

The present invention includes methods of detection of double stranded nucleic acid products such as PCR amplicons present in analytes. The process includes sandwich hybridization that has been achieved with unpurified master mix of dsDNA at different hybridization times. The invention also describes novel signal generating techniques which includes streptavidin conjugated quantum dots and CELISA (Cascade enzyme-linked immunosorbent assay) method. The method is applicable by using capture probes immobilized to a variety of supports including Poly-L-Lysine or epoxy coated microscope glass slides, micro/nano particles and plastic membranes.

DEFINITION OF FIGURES

Figures are given below to explain well of the invention of methods for detection of non-labeled PCR products on sandwich hybridization based array platforms

Figure 1. Sandwich hybridization platform.
Figure 2. Signal generation by CELISA method. Use of streptavidin and enterokinase conjugated micro/nanoparticles.
Figure-3 Signal generation by CELISA method. Use of biotin and enterokinase conjugated micro/nanoparticles.

DETAILED DESCRIPTION OF THE INVENTION:

DESCRIPTION OF THE ELEMENTS/PARTS/SECTIONS OF THE INVENTION:

1: Support matarial for the capture probe
2: Capture probe
3: Target nucleic acid (target probe)
4: Signal probe
5: Label of signal probe
6: Streptavidin

7: Streptavidin conjugated agent (Enzymes like HRP, AP, GOD or QD or magnetic particles)
8. Micro/Nano particles
9: Enterokinase

The present invention describes a platform and method to directly utilize double stranded amplicons (for example PCR products) as target nucleic acid in establishing a sandwich between the capture probe and signal probe. Compared to other methods, the described procedure allows direct utilization of double stranded amplicons without any labeling process.

The present invention describes a method for detecting a target nucleic acid (3) sequence in an analyte, where the sequence have at least two complementary binding sites characterized in comprising an unlabeled single stranded target nucleic acid (3) sequence which is originated from a standard PCR reaction, able to bind to capture probe (2) and signal probe (4) at the same time to establish a sandwich hybridization assay platform.

The invention also describes methods for signal generation with quantum dot conjugated streptavidin molecules. This detection method allows direct signal generation without any substrate utilization. The signal can be easily observable by subjecting the hybridization product to a conventional UV transillimunator or UV-LEDS or flourecence scanner or by illuminating by an appropriate light source like light emitting diodes (LEDs).

In another embodiment for signal amplification after capture and target probe hybridization, more than one signal probe can be attached to the remaining portion of the target probe. Together with multiple biotin labels on the signal probe this allows a very strong signal amplification to detect low concentration of target sequences.

Taken all together the embodiments described in this art allows detectable (preferably visual) signal generation even at very low concentrations of target nucleic acid which might be in single or double stranded format.

The first probe, the capture sequence (2), is bound to a solid support (1) and is allowed to bind (capture) the target nucleic acid sequence (3). A second probe, the signal probe (4), with a sequence that is adjacent or close to the capture sequence on the target DNA molecule is then allowed to hybridize to the captured target nucleic acid sequence.

To aid in understanding terms used in describing the invention, the following definitions are provided.

By "oligonucleotide" or "oligomer" is meant a nucleic acid having generally less than 1,000 residues, including polymers in a size range having a lower limit of about 2 to 5 nucleotide residues and an upper limit of about 500 to 900 nucleotide residues. Preferred

oligomers are in a size range having a lower limit of about 5 to about 15 residues and an upper limit of about 50 to 600 residues; more preferably, in a size range having a lower limit of about 10 residues and an upper limit of about 100 residues. Oligomers may be purified from naturally occurring sources, but preferably are synthesized using well known methods.

By "amplification oligonucleotide" or "amplification oligomer" is meant an oligonucleotide that hybridizes to a target nucleic acid, or its complement, and participates in an in vitro nucleic acid amplification reaction (e.g., primers and promoter primers). Preferably, an amplification oligonucleotide contains at least about 10 contiguous bases, and more preferably at least about 12 contiguous bases, which are complementary to a region of the target nucleic acid sequence (or a complementary strand thereof). The contiguous bases are preferably at least 80%, more preferably at least 90% complementary to the sequence to which the amplification oligonucleotide binds. An amplification oligonucleotide is preferably about 10 to about 60 bases long and may include modified nucleotides or base analogs (like locked nucleic acids LNA or peptide nucleic acids PNA).

By "amplification" is meant any known in vitro procedure for obtaining multiple copies of a target nucleic acid sequence or its complement or fragments thereof. In vitro amplification refers to production of an amplified nucleic acid that may contain less than the complete target region sequence or its complement. Known amplification methods include, for example, transcription-mediated amplification, replicase-mediated amplification, polymerase chain reaction (PCR) amplification, ligase chain reaction (LCR) amplification and strand-displacement amplification (SDA).

By "capture probe" (2) or "immobilized nucleic acid" is meant a nucleic acid that joins, directly or indirectly, a capture oligomer to a solid support (1). An immobilized capture probe is an oligomer joined to a solid support that facilitates separation of bound target sequence from unbound material in a sample. Any known solid support (1) may be used, such as matrices and particles free in solution, made of any known material (e.g., nitrocellulose, nylon, glass, polyacrylate, mixed polymers, polystyrene, silane polypropylene and metal particles, such as paramagnetic particles)

By "target probe" (3) (is meant at least one nucleic acid oligomer that provides means for specifically joining a signal probe and an capture probe based on base pair hybridization. Generally, a target includes two binding regions: a signal probe -specific binding region and an immobilized capture probe-specific binding region. Basicly the target probe is the nucleic acid sequence that is planned to be detected in a given analyte and it might be an oligonucleotide, a PCR product, a genomic or plamid DNA or a fragment of it, a

cDNA molecule, a cRNA molecule, a RNA molecule, ribosomal RNA molecules like 16S, a tRNA molecule or mRNA molecule.

By "signal probe" (4) is meant a nucleic acid oligomer that hybridizes specifically to a target sequence in a nucleic acid or its complement, preferably in an amplified nucleic acid, under conditions that promote hybridization, thereby allowing detection of the target sequence or amplified nucleic acid (3). Signal probe carries a label (5) such as magnetic particles, flouresence dye, biotin, quantum dots or gold nanoparticles. Detection may either be **direct** (i.e., resulting from a fluorescently, QD or magnetic particle labeled signal probe hybridizing to the target sequence) or **indirect** (i.e., resulting from binding of quantum dot, enzyme or magnetic particle conjugated streptavidin binding to the biotin label present on the signal probe (6,7)). A signal probe's "target" generally refers to a sequence within (i.e., a subset of) an amplified nucleic acid sequence which hybridizes specifically to at least a portion of a capture probe oligomer using standard hydrogen bonding (i.e., base pairing).

By "label" (5) is meant a molecular moiety or compound that can be detected or can lead to a detectable response. A label is joined, directly or indirectly, to a nucleic acid probe or to the nucleic acid to be detected (e.g., amplified product). Direct labeling can occur through bonds or interactions that link the label to the probe (e.g., covalent bonds or non-covalent interactions). Indirect labeling can occur through use of a bridging moiety or "linker", such as additional oligonucleotide(s), which is either directly or indirectly labeled. Bridging moieties can be used to amplify a detectable signal. Labels can be any known detectable moiety (e.g., a radionuclide, ligand such as biotin or streptavidin/avidin, enzyme or enzyme substrate, reactive group, or chromophore, such as a dye or colored particle, luminescent compond, including bioluminescent, phosphorescent or chemiluminescent compounds, fluorescent compound and/or nano gold particles). A labeled probe is referred to as a "signal probe" (4).

By "Quantum dots (QDs)" is meant semiconductor nanocrystals or artificial atoms which are semiconductor crystals that contain anywhere between 100 to 1,000 electrons and range from 2-10 nm. Some QDs can be between 10-20 nm in diameter. QDs have high quantum yields, which makes them particularly useful for optical applications. QDs are fluorophores that fluoresce by forming excitons, which are similar to the excited state of traditional fluorophores, but have much longer lifetimes of up to 200 nanoseconds. This property provides QDs with low photobleaching. The energy level of QDs can be controlled by changing the size and shape of the QD, and the depth of the QDs' potential. One optical feature of small excitonic QDs is coloration, which is determined by the size of the dot. The larger the dot, the redder, or more towards the red end of the spectrum the fluorescence.

The smaller the dot, the bluer or more towards the blue end it is. The bandgap energy that determines the energy and hence the color of the fluoresced light is inversely proportional to the square of the size of the QD. Larger QDs have more energy levels which are more closely spaced, thus allowing the QD to absorb photons containing less energy, i.e., those closer to the red end of the spectrum. Because the emission frequency of a dot is dependent on the bandgap, it is possible to control the output wavelength of a dot with extreme precision. QDs have broad excitation and narrow emission properties which, when used with color filtering, require only a single electromagnetic source to resolve individual signals during multiplex analysis of multiple targets in a single sample. Colloidally prepared QDs are free floating and can be attached to a variety of molecules via metal coordinating functional groups. These groups include but are not limited to thiol, amine, nitrile, phosphine, phosphine oxide, phosphonic acid, carboxylic acids or other ligands. By bonding appropriate molecules to the surface, the quantum dots can be dispersed or dissolved in nearly any solvent or incorporated into a variety of inorganic and organic films. Quantum dots (QDs) can be coupled to streptavidin directly through a maleimide ester coupling reaction or to antibodies through a meleimide-thiol coupling reaction. This yields a material with a biomolecule covalently attached on the surface, which produces conjugates with high specific activity. Thus, in some embodiments QD conjugated streptavidin is used to generate a detectable signal.

By "CELISA" is meant that Cascade enzyme-linked immunosorbent assay, which is a recently described signal generation method for sandwich hybridization assays for ELISA method *(Young-mi Lee, Yujin Jeong, Hyo Jin Kang, Sang J. Chung, Bong Hyun Chung, Cascade enzyme-linked immunosorbent assay (CELISA), Biosensors and Bioelectronics 25 (2009) 332–337).* CELISA involves incorporation of an enzyme-cascading step into an ELISA system, in place of the use of enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) used in conventional ELISA *(Porstmann, B., Porstmann, T., Nugel, E. and Evers, U. Which of the commonly used marker enzymes gives the best results in colorimetric and fluorimetric enzyme immunoassays: horseradish peroxidase, alkaline phosphatase, ß-galactosidase.* J. Immunol. Meth. ***79****, (1985), 27-37; Porstmann T. and Kiessig S. T., Enzyme immunoassay techniques J. Immunol. Meth. 150 (1–2), (1992), 5–21); REGALADO, Carlos; GARCIA-ALMENDAREZ, Blanca E. and DUARTE-VANQUEZ Miguel A. Biotechnological applications of peroxidases. Phytochemistry Reviews, vol.3, no. 1-2, (2004), 243-256)*

CELISA consists of a capture antibody immobilized on a solid support such as nano/microparticles or well plates, a detection antibody accompanied by a cascading enzyme, a proenzyme, and a substrate. The major difference between CELISA and ELISA is

an enzyme which accompanies the detection antibody. In ELISA, HRP catalyzes a substrate change, resulting in an altered substrate absorption or fluorescence emission. In CELISA, enterokinase (EK) converts catalytically inactive trypsinogen (the trypsin precursor protein) to active trypsin, which in turn catalyzes change of a chromogenic substrate. In this context, EK and trypsinogen are termed a cascading enzyme and a proenzyme, respectively. The turnover number (k_{cat}) of trypsinogen activation by EK is $414 min^{-1}$, meaning that EK can activate 414 molar equivalents of trypsinogen per minute in the presence of sufficient trypsinogen. This means that every EK molecule trapped via antigen–antibody interaction can activate 10^4 trypsinogen molecules every 25min, thereby amplifying the signal by 10^4.

An important embodiment of the present invention is application of CELISA method to detect nucleic acid hybridization events (Figure 2, 3). The first probe, the capture sequence (2), is bound to a solid support (1) and is allowed to bind (capture) the target nucleic acid sequence (3). A second probe, the signal probe (4), with a sequence that is adjacent or close to the capture sequence on the target DNA molecule is then allowed to hybridize to the captured target nucleic acid sequence. The sandwich formation thus presence of the target nucleic acid is then detected by application of micro/nanoparticles (8) (Figure 2-3).

The present invention discloses a process which includes the following steps:

1. **Capture Probe solution preparation** where capture probe (2) is 5′ oligonucleotide that is modified with a functional group like thiol or amino group. In case of thiol group the oligonucleotide firstly reduced to –SH form in the presence of a reducing agent TCEP (tris (2-carboxyethyl) phosphine).
2. **Capture Probe immobilization** where 0,5 µL probe-2 solution is applied onto corresponding spots of of poly-L lysine slides. Alternatively amino labeled probes can be immobilized to epoxy coated surfaces.with known procedures present in the art.
3. **Incubation** where slides are incubated in humidified chamber at 30°C for 1.5 hours
4. **Post-immobilization washing and Drying** where slides are washed for 15 minutes with 5X SSC of pH 7.0 + 0.1% SDS at room temperature with agitation (130 rpm), 3 times for 15 minutes with distilled water at room temperature with agitation (130 rpm) and dried by means of the spinner at maximum speed for 1min.
5. **Blocking** where slides are dipped into 2% BSA (Bovine Serum Albumin) (in 10 mM PBS (Phosphate Buffered Saline) pH 7.2) solution for 2 hr to reduce high background and increase immobilization efficiency.

6. **Washing and Drying** where slides are washed twice for 15 minutes with 0.1X SSC pH 7.0 with agitation (130 rpm), once with distilled water (squirt but not on the spots) and dried by means of the spinner at maximum speed for 1min.
7. **PCR Master Mix Preparation** where label-free PCR-amplified DNA sequence obtained after 30-45 cycles of PCR is used as a master mix form.
8. **Hybridization Solution preparation** where positive and negative control solutions are prepared using dsDNA master mix from 45 cycles of PCR, signal probe (probe1), SDS and BSA.
9. **Hybridization** where hybridization solution is applied onto spots of each slide, coverslip is situated on this spot area, slides are placed in humidified chamber and incubated at 40°C for 15, 30 and 120 minutes.
10. **Post-hybridization washing and drying** where slides are washed once for 5 minutes with 1X SSC + 0.1% SDS at room temperature with agitation (130 rpm), once for 5 minutes with 0.1X SSC at room temperature with agitation (130 rpm), 3 times for 1 minute with distilled water at room temperature with agitation (130 rpm) lastly plunge into distilled water gently and dried by means of the spinner at maximum speed for 1 minute.
11. **Signal Generation using biotin labeled signal probe** where a detectable signal is generated according to any of the following alternative components Various alternatives are possible and some of them are given below. These alternatives are not limited with the ones explained in this document. The signal probe can carry a single biotin label. Alternaitvely, for signal amplification multiple biotin labeled probes can be used.

QD application where streptavidin coated quantum dots are used to bind signal probe (biotinylated signal probe) with high affinity and applied onto spots area of slides. Coverslips are situated on spot area, slides are placed in humidified chamber and incubated at 30°C for 1 hour. Post-QD application where slides are washed once for 5 minutes with 1X SSC at room temperature with agitation (130 rpm), once for 5 minutes with 0.1X SSC at room temperature with agitation (130 rpm), 3 times for 1 minute with distilled water at room temperature with agitation (130 rpm) and lastly plunge into distilled water gently. **UV screening** where slides are placed on a standard UV-illuminating tray or by using UV-LEDs and the images were captured.

Enzyme (Horse radish peroxidase-HRP, alkaline phosphatase-AP or glucose oxidase-GOD) application, where membranes are dipped into 2% BSA in

10 mM PBS (Phosphate Buffered Saline) pH 7.2 solution for 2 hours. Then the platform is washed 2 times for 15 sec in 0.1X SSC pH 7.0 with agitation (130 rpm) and rinsed once with dH_2O. In case of membrane platforms, the membranes are dried at 40°C for 30 min. Streptavidin coated HRP is diluted as 1:20000 ratio in 1%BSA in PBS , pH 7.3, and the membranes are incubated in this solution at 37°C for 1 hour. Substrate addition and signal generation where appropriate substrates for HRP (3,3,5,5 Tetrametilbenzydin) is added onto the spot area and blue signal is generated in 15-20 minutes incubation at RT.

Cascade enzyme-linked immunosorbent assay (CELISA) application where trypsinogen–enterokinase combination as the cascading enzyme system is applied to obtain a visual signal as explained by *Lee Y. et al, 2009. (Young-mi Lee, Yujin Jeong, Hyo Jin Kang, Sang J. Chung, Bong Hyun Chung, Cascade enzyme-linked immunosorbent assay (CELISA), Biosensors and Bioelectronics 25 (2009) 332–337).* This method allows a strong signal amplification via enhancing the generation of more products (thus signals) by the help of enzymes. Micro or nanoparticles (8) carrying streptavidin (6) and enterokinase (9) are applied to the spot area of the array slides to bind signal probe (4) (biotinylated signal probe). After 1 h incubation with gentle shaking at room temperature, slides were washed 3X with TBST (150mM NaCl, 50mM Tris; pH 7.5). Then 100 μM trypsinogen in a proenzyme buffer (50mM Bis–Tris buffer; pH 5.5) is added and incubated at 37 °C for 2 hours. Finally 5mM BAPNA in 50% (v/v) DMSO was added to the spot area and trypsin activity is measured by monitoring the absorption change at 405nm upon the release of p-nitroanilide from the substrate by hydrolysis. The system is explained in Figure 2.
Alternatively, micro or nanoparticles (8) carrying biotin (5) and enterokinase (9) are applied to the spot area of the array slides to bind signal probe (4) (biotinylated signal probe). After 1 h incubation with gentle shaking at room temperature, slides were washed 3X with TBST (150mM NaCl, 50mM Tris; pH 7.5). Then streptavidin in proenzyme buffer was added and incubated for 30 minutes at RT. Then 100μM trypsinogen in a proenzyme buffer (50mM Bis–Tris buffer; pH 5.5) and incubated at 37 °C for 2 hours. Finally 5mM BAPNA in 50% (v/v) DMSO was added to the spot area and trypsin activity is measured by monitoring the absorption change at 405nm upon the release of p-nitroanilide from the substrate by hydrolysis. The system is explained in Figure 3.

Gold labeled signal probe where the signal probe is labeled with gold nanoparticles. In case the signal probe carries a gold particle, the detection is

achieved by silver deposition. The following is a typical procedure to achieve image formation on glass slides. Incubate capture probe and signal probe in TCEP at RT for 30 minutes for sulphide reduction. To immobilize the capture probe apply TCPE treated nucleic acid probes on Poly-L-Lysin surface and incubate overnight. Prepare 5% W/V Skim Milk and BSA mixture (1:1 ratio) and incubate coated Poly-L-Lysine slides in this mixture for 1 hour. Wash 3 times with ultra pure water and dry with slide spinner. Mix target nucleic acid and gold nono particle carrying signal probe and incubate at 60°C for 5 minutes, followed by incubation at 25°C for 30 minutes. Apply this mixture to capture probe immobilized slide surfaces and incubate humidity chamber at 35° C for 5 hours. Wash slides with 1X SSC + 0.1 % SDS for 5 min, 1 X SSC for 5 min and 3 X with ultra pure water and dry with slide spinner. Apply commercially available silver enhancer solution (2 ml per slide). Incubate at 24 °C until the desired stain intensity is reached (typically 5–10 minutes). Rinse slides in distilled water to remove the Silver Enhancer Mixture and fix by immersing in 2.5% Sodium Thiosulfate Solution for 2–3 minutes. Wash slides thoroughly in distilled water and dry.

CLAIMS

1. A method for detecting a target nucleic acid (3) sequence in an analyte, where the sequence have at least two complementary binding sites characterized in comprising an unlabeled single stranded target nucleic acid (3) sequence which is originated from a standard PCR reaction, able to bind to capture probe (2) and signal probe (4) at the same time to establish a sandwich hybridization assay platform.
2. A method according to claim 1, where signal probe (4) carries at least one label (5).
3. A method according to claim 2, where label (5) is selected from the group consisting of magnetic particle, fluorescence dye, biotin, quantum dot and gold nanoparticles.
4. A method according to claim 1, where detection of the signal may either be direct or indirect.
5. A method according to claim 4, where **direct** signal generation is obtained by using fluorescently, quantum dot, gold or magnetic particle labeled signal probe (4).
6. A method according to claim 4, where **indirect** signal generation is obtained by using quantum dot, enzyme, or magnetic particle conjugated streptavidine (6) bound to biotin (5) labeled signal probe (4).
7. A method according to Claim 6, wherein enzyme is selected from the group consisting of horse radish peroxidase, alkaline phosphatase, glucose oxidase.
8. A method for detecting a target nucleic acid (3) sequence in an analyte, where the sequence have at least two complementary binding sites characterize in comprising a target nucleic acid (3) establishing a sandwich with capture (2) and signal probe (4), where biotin (5) labeled signal probe interacts with streptavidin (7) conjugated quantum dot for signal generation.
9. A method according to claim 8, where target nucleic acid (3) sequence is selected from the group consisting of oligonucleotide, a PCR product, a genomic or plamid DNA or a fragment of it, a cDNA molecule, a cRNA molecule, a RNA molecule, ribosomal RNA molecule, a tRNA molecule or mRNA molecule.
10. A method for detecting a target nucleic acid (3) sequence in an analyte, where the sequence have at least two complementary binding sites characterize in comprising a target nucleic acid (3) establishing a sandwich with capture (2) and signal probe (4), where the signal is generated by CELISA method.
11. A method according to claim 10, where signal generation is obtained by using streptavidin (6) conjugated micro or nanoparticles (8) carrying multiple enterokinase(9).

12. A process for detecting a target nucleic acid (3) sequence in an analyte, where the sequence have at least two complementary binding sites according to claim 10, comprising the steps of;
    - adding micro or nanoparticles (8) carring sterptavidine (6) and enterokinase (9) to bind biotinylated signal probe (4),
    - adding trypsinogen where it is activated by enterokinase (9) to form active trypsin.
    - adding a substrate for trypsin (N_-benzoyl-l-Arg-p-nitroanilide hydrochloride (BAPNA) or Suc-AlaAlaProArg-p-nitroanilide (Suc-AAPR-pNA)) and,
    - following signal generation visually or spectrophotometrically.
13. A process for detecting a target nucleic acid (3) sequence in an analyte, where the sequence have at least two complementary binding sites according to claim 10, comprising the steps of;
    - adding free sterptavidine (6) to bind biotinylated signal probe (4),
    - adding micro or nanoparticles (8) carring enterokinase (9),
    - adding trypsinogen where it is activated by enterokinase (9) to form active trypsin.
    - adding a substrate for trypsin (N_-benzoyl-l-Arg-p-nitroanilide hydrochloride (BAPNA) or Suc-AlaAlaProArg-p-nitroanilide (Suc-AAPR-pNA)) and,
    - following signal generation visually or spectrophotometrically.
14. A method according to any of the proceeding claims, wherein signal generation is followed by using standart UV transilluminator, UV-LEDS, flourecence scanner or by illuminating by an appropriate light source like light emitting diodes.

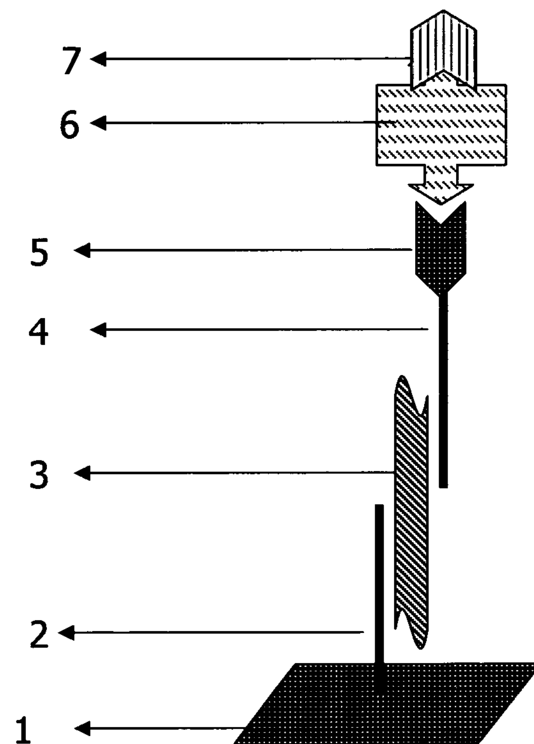

Figure 1

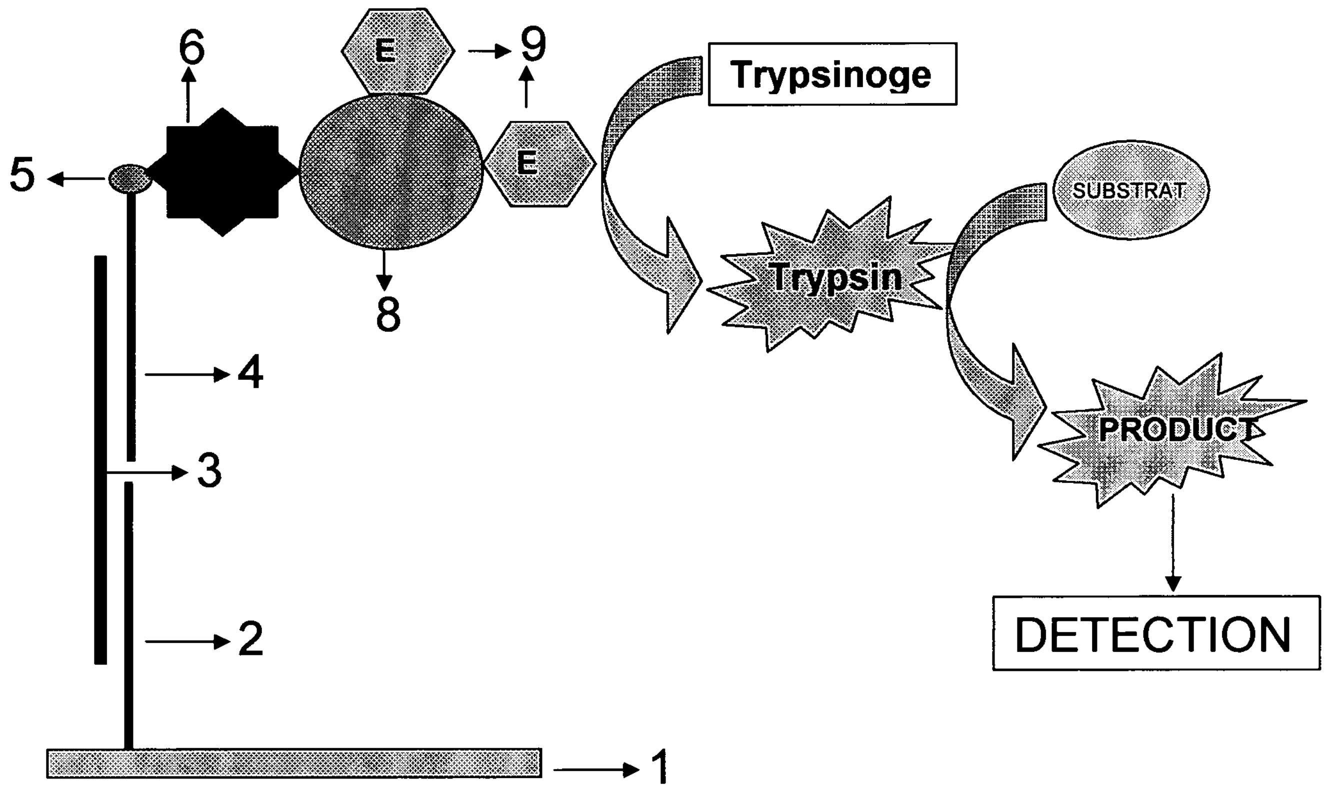

Figure 2

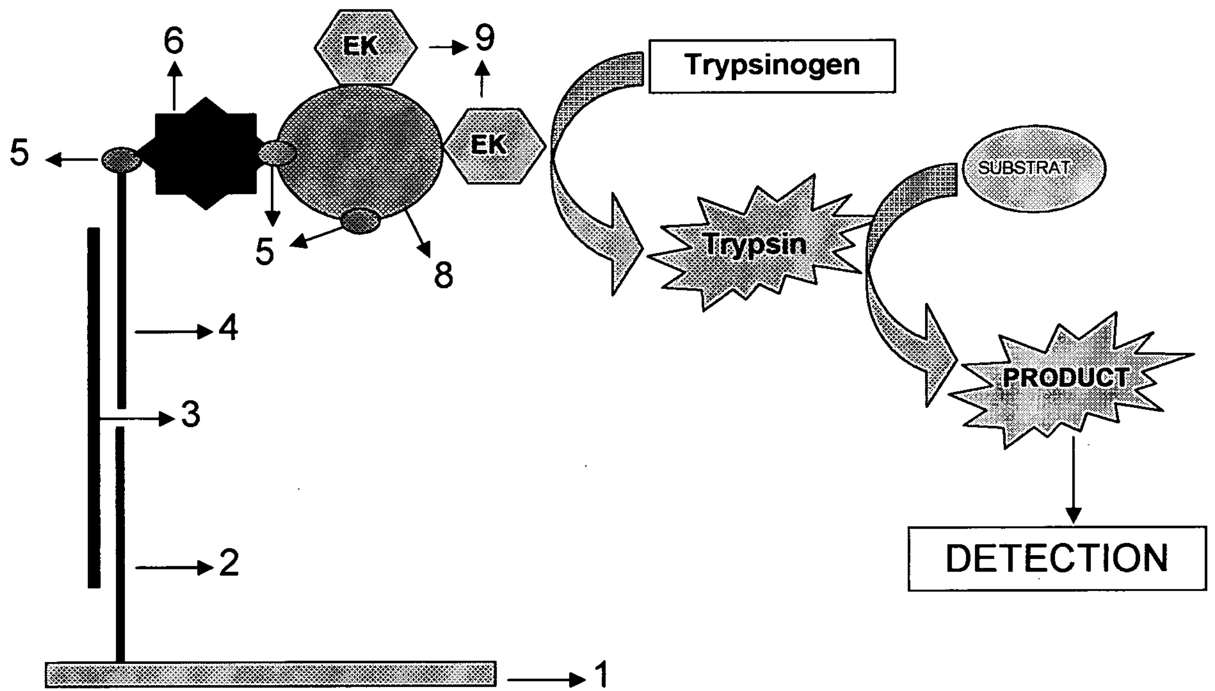

Figure 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/TR2010/000011

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 1 672 082 A2 (SYSMEX CORP [JP]) 21 June 2006 (2006-06-21) the whole document figure 9	1-4,6,7 10-13
X	US 2002/137060 A1 (BROWN-AUGSBURGER PATRICIA LEA [US] ET AL) 26 September 2002 (2002-09-26) the whole document paragraph [0114]; figure 1	1,2,4,6,7
X	US 2006/188880 A1 (HINNAH SILKE [DE]) 24 August 2006 (2006-08-24) the whole document paragraph [0012] - paragraph [0017] paragraph [0023]; figure 1	1-5

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 June 2010	30/06/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pinta, Violaine

INTERNATIONAL SEARCH REPORT

International application No
PCT/TR2010/000011

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X Y	WO 00/29617 A2 (ADVANCED RES & TECH INST [US]) 25 May 2000 (2000-05-25) the whole document figure 2; example 5	1-5 8,9,14
X	US 2004/110220 A1 (MIRKIN CHAD A [US] ET AL MIRKIN CHAD A [US] ET AL) 10 June 2004 (2004-06-10) the whole document figures 4, 25	1-5
Y	WO 2005/050171 A2 (US GENOMICS INC [US]; GILMANSHIN RUDOLF [US]; HATCH AMIE JO [US]) 2 June 2005 (2005-06-02) the whole document figure 1	8,9,14
Y	WO 2009/044985 A1 (KOREA RES INST OF BIOSCIENCE [KR]; CHUNG SANG JEON [KR]; LEE YOUNG-MI) 9 April 2009 (2009-04-09) the whole document	10-13
Y	LEE YOUNG-MI ET AL: "Cascade enzyme-linked immunosorbent assay (CELISA)." BIOSENSORS & BIOELECTRONICS 15 OCT 2009 LNKD- PUBMED:19665363, vol. 25, no. 2, 15 October 2009 (2009-10-15), pages 332-337, XP002586857 ISSN: 1873-4235 cited in the application the whole document	10-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/TR2010/000011

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