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(54) **Title**: METHOD FOR POLYNUCLEOTIDE HYBRIDIZATION

(57) **Abstract**: There is provided a method of hybridizing a first polynucleotide to a second polynucleotide wherein the second polynucleotide is immobilized to a surface of a solid support, comprising the steps of contacting a solution containing the first polynucleotide with the surface of the solid support to which the second polynucleotide is immobilized, concentrating the first polynucleotide to the surface of the solid support to which the second polynucleotide is immobilized and allowing the first polynucleotide to hybridize to the second polynucleotide, and allowing hybridization to take place for less than 300 seconds. A method for diagnosis of Human Papillomavirus is also provided.

Title

Method for polynucleotide hybridization.

Technical field

The present invention relates to an improved method for polynucleotide hybridization where one type of nucleotide molecule is immobilized on a solid support.

Background

Hybridization of nucleotides where one type of polynucleotide molecule is immobilized (in the following referred to as simply "hybridization") is a molecular biology technology that now is a standard procedure in life science laboratories. Southern blot and northern blot are the archetypical methods for polynucleotide hybridization, but today microarray methods is an important and growing field where this approach is used. Hybridization, in particular DNA hybridization, has a multitude of uses such as in genetic analysis, expression analysis, and analysis of DNA in general. In diagnosis, DNA hybridization can be used for, for example, detecting genetic abnormalities, identification of pathogens and other types of genotyping.

DNA hybridization is carried out by allowing a single-stranded DNA molecule, the probe, to bind to the target sequence by allowing the molecules to base-pair due to complementarity between the probe and target. Base pairing occurs as hydrogen bonds are formed between complementary nucleotides, where adenine (A) always binds to thymine (T) and guanine (G) always binds to cytosine (C). Hybridization is detected by detecting a label on the polynucleotide that is not immobilized. The label can for example be of a radioactive or chemiluminescent nature.

DNA hybridization is carried out in aqueous solution under conditions that promote the forming of hydrogen bonds between base pairs. Time, ion strength and temperature are important factors for achieving efficient hybridization. Conventionally, hybridization of polynucleotides where one type of polynucleotide is immobilized has been thought to

require a temperature above 50 degrees Celsius and an elongated reaction time (1 hr - 72 hrs) since the hybridization procedure is dependent on regular diffusion. In particular, long hybridization times have been used for long target polynucleotides because they diffuse slowly in solution.

Graham et al has shown that hybridization times can be decreased by concentrating the polynucleotides to the site of hybridization with the aid of magnetism, whereby paramagnetic particles are coupled to the probe and pulled to the support with the aid of an electric current [1]. Graham et al (figure 4a) uses a total hybridization time of 480 seconds, or 8 minutes. A disadvantage with the method described in Graham et al, beside the long hybridization time, is that a complicated type of array with integrated electric circuitry must be used for the generation of the electromagnetic field.

It would be advantageous if the hybridization time could be decreased. This would, for example, simplify the use of e DNA hybridization in diagnosis since patients and laboratory staff would not have to wait during the hybridization time.

Human Papilloma Virus is a virus that causes cervix cancer in humans. Current methods of diagnosis of cervix cancer are not sufficiently fast and easy to use, nor applicable outside of a laboratory and without expensive machinery. Thus in addition, there is a need for improved methods for diagnosis of HPV.

Summary

It is an object of the present invention to obviate at least some of the disadvantages in the prior art.

In a first aspect it is provided a method of hybridizing a first polynucleotide to a second polynucleotide wherein the second polynucleotide is immobilized to a surface of a solid support, comprising the steps of a) contacting a solution containing the first polynucleotide with the surface of the solid support to which the second polynucleotide is immobilized, b) concentrating the first polynucleotide to the surface of the solid support to which the second polynucleotide is immobilized and allowing the first

polynucleotide to hybridize to the second polynucleotide for a time period (hybridization time) of less than 300 seconds.

In a second aspect there is provided a method for diagnosis, in particular HPV diagnosis, comprising the inventive method for polynucleotide hybridization.

In a third aspect there is provided the use of the inventive method for diagnosis.

In a fourth aspect there is provided the use of the inventive method for quality control of a DNA microarray.

Further aspects and embodiments are defined in the appended claims, which are specifically incorporated herein by reference.

Definitions

Before the invention is disclosed and described in detail, it is to be understood that this invention is not limited to particular compounds, configurations, method steps, substrates, and materials disclosed herein as such compounds, configurations, method steps, substrates, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention is limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural referents unless the context clearly dictates otherwise.

If nothing else is defined, any terms and scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains.

The term "about" as used in connection with a numerical value throughout the description and the claims denotes an interval of accuracy, familiar and acceptable to a person skilled in the art. Said interval is ± 10 %.

Hybridization: binding of a polynucleotide to a complementary polynucleotide where one of the polynucleotides is immobilized on a solid support.

Hybridization time: The time from initiation of the concentration step until when steps are taken to detect to what extent hybridization has occurred. When a magnetic field is used to concentrate the first nucleotide bound to paramagnetic particles, the hybridization time starts when the magnetic field starts to attract the particles. One example of taking a step to detect to what extent hybridization has occurred is carrying out a washing step. Another example of such a step is manually reading off the array, for example, when washing is not necessary.

Particle size: average particle size.

Polynucleotide: a nucleic acid polymer comprising at least 5 base pairs.

Probe: the polynucleotide that, in a hybridization reaction, is immobilized to the surface of a solid support. It should be noted that this nomenclature has been chosen because it applies to microarrays. It is recognized that when discussing, for example, southern blots, the "probe" usually designates the non-immobilized polynucleotide.

Target: a polynucleotide in a hybridization reaction that is not immobilized to a solid support.

Particles may sometimes be referred to as "beads"

Brief description of the drawings

The invention is described in greater detail with reference to the drawings in which:

Figure 1a-e schematically shows one embodiment of the method according to the invention where paramagnetic particles are coupled to the target DNA and attracted to the surface of a DNA array with a magnetic mean. The drawing is not to scale.

Figure 2 a-d essentially shows the same as figure 1a-e, but with a larger scale.

Figure 3a-b schematically shows an embodiment of the method according to the invention where it is used together with Multiplex Competitive Hybridization. The drawing is not to scale.

Detailed description

The inventors have surprisingly found that the time necessary for hybridization reactions where a probe is immobilized on a solid support can be shortened considerably to the range of a few seconds, and that thus detection of hybridization can be made much earlier than with previous methods. This has the advantage that that the hybridization procedure can be carried out in a shorter time that with previous methods.

The invention provides methods for rapidly hybridizing polynucleotides such as DNA or RNA molecules to one another. The hybridization method can be used to save time in diagnostic procedures, quality control of microarrays, or research in general. The method can be used for various types of hybridization reactions such as DNA to DNA, RNA to RNA or DNA to RNA.

The invention is based on the surprising insight that polynucleotide hybridization occurs very rapidly once a local concentration of polynucleotide is achieved. The travel of the polynucleotide in solution is the rate-limiting step for hybridization to occur. Based on this insight a novel hybridization method is provided in which a much shortened hybridization time can be used.

Even comparatively long and slow-diffusing target polynucleotides, such as polynucleotides up to thousands of base pairs can be hybridized by using the disclosed method because the rate limiting step is overcome by achieving a high local concentration. Thus, polynucleotides of up to 5, 10, 20, 40, 100, 500, 1000, or 2000 base pairs, can be hybridized by using the invention. The local concentration is achieved by having one type of polynucleotide, the probe, immobilized to the surface of a solid support and concentrating the second type of polynucleotides, the target, to that surface. This considerably speeds up the hybridization reaction as a whole. The concentration step can be achieved in various manners, for example with suction, centrifugation or magnetic force.

If centrifugation is used, the second type of polynucleotide (the probe) can be immobilized to a membrane in a column that is suitable for use in a table-top mini-centrifuge. The first type of polynucleotide (the target) can then be applied to the top of the column and brought in contact with the second type of polynucleotide by running the centrifuge and driving the liquid containing the first type of polynucleotide through the membrane. It is then conceivable that the steps a) (contacting) and b) (concentrating) takes place at the same time.

The concentration step can be carried out with the aid of magnetic force. When a magnetic force is used,paramagnetic particles can be coupled to the first polynucleotide and the concentration step is carried out by providing at least one magnetic field that attracts the first polynucleotide towards the surface of the solid support to which the second polynucleotide has been immobilized. The paramagnetic particles can be superparamagnetic.

Alternatively, the particles coupled to the target DNA can be magnetic, in which case they can be attracted to the probe DNA by placing an amount of a paramagnetic material, such as, for example, iron, so that the particles with DNA are attracted to the surface of the solid support with the probe DNA. Under the influence of the magnetic field, the particles are attracted towards the surface of the solid support, and a high local concentration of target DNA is generated, which allows for the hybridization to

take place. Hybridization starts immediately and the rate limiting step for the completion of hybridization is the movement of the particles trough the fluid. The coupling of paramagnetic particles to macromolecules is well known in the art.

The solid support is preferably made of glass, plastic, paper, cellulose slilicon or non-magnetic metals such as gold, platinium or aluminium.

The solid support can be a flat object with roughly dimension of a standard microscope slide. However, the invention is off course not limited to arrays of that size.

Hybridization times may vary according to the specific combination of magnetic particles, and magnetic field strength. Thus, the hybridization time can be 300 seconds. More preferably, the hybridization time is 150, 90, 70, 50, 30, 20, 15, 10, 5, 1, 0.5, or, most preferably, 0.25 seconds.

When paramagnetic particles are used, they may conveniently be made of a super paramagnetic material, that is, a material that has high magnetic mass susceptibility. Superparamagnetic particles are well known in the art and available from manufacturers of lab supplies.

Superparamagnetic particles are preferably made of a polymer doped with gamma-Fe_2O_3 or Fe_3O_4. They can, for example, have a mass of $1.1 \pm 0.4 \times 10^{-15}$ kg and have a magnetic mass susceptibility of about 825×10^{-6} m^3/kg. Preferably, the magnetic mass susceptibility is more than 50×10^{-6} m^3/kg. More preferably, the magnetic mass susceptibility is more than 75×10^{-6} m^3/kg, more preferably more than 100×10^{-6} m^3/kg

Preferably the particles are of a size of between about 0.5 micrometers and about 3 micrometers. Even more preferably the particles have an average size of about 1 micron in diameter, for example Dynal myOne beads. The method is not limited to the use of particles of this size. Thus, the average size of the paramagnetic particles can be from about 0.5 micrometers to about 3 micrometers, in particular from about 0.75 to about 1.5 micrometers. When the particles are of this size, they are suitably attracted by magnet with a magnetic field of from 0.5 Tesla to 2.0 Tesla for the concentration step.

In particular the magnetic field can be from 1.0 Tesla to 1.5 Tesla. For these particles and this magnet less than 3 seconds is sufficient to concentrate the DNA-bead particles at the surface of the solid support when the maximum distance that the beads have to travel is about 5 mm. That is the case when the first nucleotide is contained in a liquid drop of less than 30 µl that is placed on the solid support. Thus the solution containing the first polynucleotide can be contacted with the surface of the solid support in a manner such that the maximum distance that the first nucleotide has to travel in order to become concentrated to the surface of the solid support is less than 5 mm. Preferably the solution containing the first polynucleotide is contacted with the surface of the solid support in the form of a drop with an extent of less than 5 mm in any direction. Under these conditions, the lower limit of the hybridization time is about 2 seconds.

Surprisingly, hybridization of the polynucleotides on one individual bead occurs instantly as soon as the bead becomes in contact with the surface of the solid support. Three seconds is the time that is necessary for the beads to travel though the liquid to the surface of the solid support under these conditions and thus the hybridization process is initiated immediately when the concentration is initiated. Therefore, the hybridization time is the time from initiation of the concentration step until when steps are taken to detect to what extent hybridization has occurred.

Under these conditions, the hybridization time can be 300 seconds or less. More than 300 seconds of hybridization time provides no additional benefits. On the contrary, times in excess of 300 seconds tend to increase the background signal due to unspecific binding. However, the background may vary due to the specific polynucleotides involved. Some polynucleotides give more background than others.

Meanwhile, it is convenient not to be restricted to short hybridization times because if short hybridization times makes exact timing necessary. Thus, it is an advantage to have a hybridization time that is short but still gives the laboratory staff freedom to have a longer incubation time if the situation so requires (if, for example, something unexpected happens, for example, if the phone rings).

More preferably, the hybridization time is less than 180 seconds, more preferably less

than 150 seconds, more preferably less than 90 seconds, more preferably less than 70 seconds, more preferably less than 60 seconds, more preferably less than 50 seconds, more preferably less than 40 seconds, more preferably less than 30 seconds, more preferably less than 20 seconds, more preferably less than 15 seconds, more preferably less than 10 seconds, more preferably less than 8 seconds, more preferably less than 5 seconds, more preferably less than 3 seconds and most preferably less than 2 seconds.

Thus the hybridization time can be from 2 seconds to 300 seconds, from 2 seconds to 180 seconds, from 2 seconds to 150 seconds, from 2 seconds to 90 seconds, from 2 seconds to 70 seconds, from 2 seconds to 60 seconds, from 2 seconds to 50 seconds, from 2 seconds to 40 seconds, from 2 seconds to 30 seconds, from 2 seconds to 20 seconds, from 2 seconds to 15 seconds, from 2 seconds to 10 seconds, from 2 seconds to 8 seconds.

Thus the hybridization time can be from 3 seconds to 300 seconds, from 3 seconds to 180 seconds, from 3 seconds to 150 seconds, from 3 seconds to 90 seconds, from 3 seconds to 70 seconds, from 3 seconds to 60 seconds, from 3 seconds to 50 seconds, from 3 seconds to 40 seconds, from 3 seconds to 30 seconds, from 3 seconds to 20 seconds, from 3 seconds to 15 seconds, from 3 seconds to 10 seconds, from 3 seconds to 8 seconds.

In particular the hybridization time can be less than 30 seconds in order to avoid background. Thus the hybridization time can be 28, 26, 24, 22, 20 or 18 seconds. Thus the hybridization time can be from 3 seconds to 28 seconds, from 3 seconds to 26 seconds, from 3 seconds to 24 seconds, from 3 seconds to 22 seconds, from 3 seconds to 20 seconds from 3 seconds to 18 seconds, from 3 seconds to 17 seconds, from 3 seconds to 16 second or from 3 seconds to 15 seconds.

Further, the hybridization time can be from 2 seconds to 28 seconds, from 2 seconds to 26 seconds, from 2 seconds to 24 seconds, from 2 seconds to 22 seconds, from 2 seconds to 20 seconds from 2 seconds to 18 seconds, from 2 seconds to 17 seconds, from 2 seconds to 16 seconds or from 2 seconds to 15 seconds.

Particles of 100 nm in diameter and below are too small and therefore move too slowly under these conditions (when the magnetic field is about from 0.5 Tesla to 2.0 Tesla). Basically this is due to the drag that the surrounding water applies to the particle in relation to its mass. A stronger magnetic field (for instance a magnetic field created by an electromagnet) is then advantageously used. Larger particles of 3 μm in diameter may be used, but introduce a narrow dynamic range in the detection step after hybridization, since fewer particles will fit on the surface.

When the particles are of a paramagnetic material, the magnetic field can be provided by a magnet. The magnet can be a permanent magnet, such as a neodymium magnet; or an electromagnet, as described in Graham et al. Preferably the magnet is a neodymium magnet. A neodymium magnet is a type of permanent magnet made from an alloy of neodymium, iron and boron in the form $Nd_2Fe_{14}B$. This material is currently the strongest type of permanent magnet.

A magnetic field of suitable strength can be achieved by placing a neodymium magnet with a magnetic field of 0.5-2.0 Tesla, a weight of approximately 20 grams and a diameter of about 2 cm about 1 cm below the solid support on which the polynucleotide are immobilized.

Advantageously, the magnet is not integrated in the solid support. This has the advantage that an "off-the-shelf" type of solid support can be used for immobilizing the polynucleotide, enabling the development of low-cost assays based on the method. Otherwise a more complicated DNA array which electric circuitry integrated into it must be used.

The procedure can be carried out at room temperature, which is approximately 18°C to 25°C.

When the hybridization time has come to an end the user can determine to what extent hybridization has occurred. Advantageously, the method comprises the step of determining to what extent hybridization has occurred.

Detection and quantification can be carried out by various methods known to a person

skilled in the art such as radioactivity, chemiluminescence, fluorescence, photography, laser scanning, or microscopy.

Detection may or may not be preceded by a washing step. This is dependent on the background which in turn is dependent on the sequences being hybridized. A washing step may comprise providing a second magnetic field of roughly the same strength as the first magnetic field that moves at least some of the first polynucleotide that has not hybridized away from the surface of the solid support to which the second polynucleotide has been immobilized. Washing can efficiently and conveniently be carried out by placing the same magnet that has been used for concentrating the polynucleotide above the surface of the solid support just touching the liquid phase for about 5 seconds to attract and lift away any non-hybridized DNA-particles

Figures 1a-1e are schematic figures of sequential steps in one embodiment of the method, where the paramagnetic particles are used for the concentration step. The different components are not drawn to scale.

Fig 1a shows double stranded DNA 1 comprising two single strands of target DNA 4 and 4', coupled to biotin 2. Biotin can be incorporated by, for example, using biotin-labelled primers during the amplification of the target DNA. Fig 1b shows how a magnetic bead 3 has been coupled to biotin 2 on the target DNA 1 via avidin (not shown). The coupling may be streptavidin-biotin interaction but other coupling chemistries may also be employed.

The double stranded DNA is then converted to single stranded DNA, shown in fig. 1c. This step is generally carried out by heating the double stranded DNA. The single stranded target DNA 4 together with biotin 2 and the magnetic bead 3 is a DNA-bead complex 8.

Fig 1d shows the hybridisation step. DNA probes 5 are immobilised on the surface of a solid support 6. The DNA probe is immobilised on a discrete zone 12 on the surface of the solid support 6. A multitude of different probes can be spotted in several discrete zones or spots on the solid support 6 to form a DNA array.

The solid support 6 can be made of glass, plastic, paper or other material, preferably a material that is non-magnetic and does not shield a magnetic field. The solid support can also have a microfluid architecture or be equipped with electronic circuitry, such as a DNA chip. Preferably, the solid support has a thickness of less than 2 cm.

For the concentration step, a permanent magnet 7 is placed directly underneath the bottom surface of the solid support 6 so that it is approximately 0.5 to 2 centimetres from the surface 6 of the solids support where the probes are immobilized. Preferably the magnet 7 is positioned directly below the discrete zone 12.

Figure 2a shows the target DNA-bead complexes 8 as a slurry in solution in a test tube 9. Figure 2b shows how the solution containing the target DNA-bead complexes 8 are placed on the surface of the solid support 6 where the DNA probes are immobilised. Typically, 10-100 microliters of fluid, or a volume large enough to fully cover the probe surface, is added to the array. Fig 2b also shows how a magnet 7 positioned directly below the discrete zone is used to attract the target DNA particles 8 to the surface 6 of the solid support where the DNA probes are immobilised, as shown in 2c. The beads travel trough the liquid phase towards the surface of the solid support in a direction perpendicular to the surface, but as can be seen in figure 2b compared to figure 2c, also in a direction parallel to the surface of the solid support. Thus 2c compared to 2b shows how the beads are concentrated on an area directly above the magnet. 2d shows the signal from a zone 12 where hybridization has occurred when liquid has been removed.

An advantage with this embodiment is that it is very robust and only simple and low cost components, such as a permanent magnet, are required to achieve the magnetic field. Also, no centrifugation and a minimum of pipetting is required. Detection is immediate as the washing step can be carried out very quickly.

An additional advantage when particles are coupled to the target polynucleotide is that the detection step detection can comprise visually observing the surface to which the second polynucleotide has been immobilized to see if the first polynucleotide has

hybridized. The detection may be carried out instantaneously since the particles become visible on the surface. This is possible because an aggregation of particles, even if their average size is on the scale of micrometers, is visible to the human eye [2]. Thus no washing step and no steps involving further reagents have to be used, which is an advantage. This can be carried out if the detection area is sufficiently large so that it is visible by the human eye.

It is also provided a method for diagnosis comprising the inventive method for hybridization. There is also provided a method for diagnosis of Human Papillomavirus (HPV).

A step comprising making a decision about diagnosis may be included in the method. Suitably, a DNA array is used where probes comprising sequences that are unique for various HPV strains are immobilized. Such a DNA array may suitable also includes positive and negative controls.

Alternatively MUCH (Multiplex Competitive Hybridization) may be used, for example as described in Example 2, below. MUCH is described in reference 3. In MUCH, the sequence to be detected, in this case a HPV sequence, is first allowed to hybridize in solution with a MUCH probe which is a "two headed" probe, of which one part is specific for a HPV subtype and one part is an artificial tag sequence that is designed to hybridize to a corresponding artificial sequence on an array.

Figure 3a-b shows, schematically, sequential steps of the combination of MUCH with hybridization according to the invention. As shown in fig 3a, the sequence of the MUCH probe 10 is composed of two parts: one target-specific part 14 that it complementary to the target DNA 4, in this case a HPV subtype; and a tag sequence 15 that is artificially generated. When the HPV-specific part 14 of the MUCH probe 10 has bound in solution to the target DNA 4 coupled to a paramagnetic bead 3 it is added to the solid support 6 where zones 12 and 12' on which different target probes 5 and 11 are immobilized. In this schematic example the probe 5 is complementary to the tag sequence of the MUCH probe whereas the probe 11 is not complementary. Fig 3b shows how the hybridization step is enhanced with a magnet 7 which is positioned

below the solid support 6 in order to focus the complex 13 comprising magnetic bead 3, target DNA 4 and MUCH probe 10 to the surface of the solid support 6 where it can hybridize to the target probe 5. The target DNA – MUCH probe complex 13 does not hybridize to the non-complementary probe 11.

It is also provided the use of the above mentioned method for diagnosis of a disease, such as the presence of Human Papilloma Virus in a sample.

In a second aspect the invention provides use of the method for polynucleotide hybridization for diagnostics or for quality control of a DNA array. When making use of microarray technology to carry out hybridization experiments on surfaces, it is of importance to retain a good quality of the features present on the detection surface. If a spot is missing after printing of the surface, the particular spot could generate a false negative signal during a genotyping experiment, which in the worst case could lead to an incorrect diagnosis of a test subject. Quality control of a microarray where a set of different probes are arranged on a solid support is suitably carried out by using a soluble target that has such a sequence that it can hybridize with every probe on the array. If the probes are very different a target sequences that can hybridize to the spacer sequence of the probes can be used because such spacer sequences usually does not vary between the different probes. If for example the spacer is a poly(T) sequence, a poly (A) target sequence can be used for the quality control of the DNA array. Example 1 is an example of how the method according to the invention can be used for this purpose. For the sake of clarity it should be pointed out that the poly(T) spacer is, however, not used for the quality control in Example 1.

It is to be understood that this invention is not limited to the particular embodiments shown here. The following examples are provided for illustrative purposes and are not intended to limit the scope of the invention since the scope of the present invention is limited only by the appended claims and equivalents thereof.

The invention will now be described in further detail with reference to the following non-limiting examples.

Example 1

Samples and amplification

Sample DNA was extracted from canine blood and a selected 169 base pair region of mitochondrial DNA was amplified as previously described [2] using one biotinylated and one non-biotinylated primer with the sequences (SEQ ID NO 1 and 2) shown in Table 1, to produce target DNA (SEQ ID NO 3).

Table 1. Primers, target DNA and probes for quality control experiment

Primers for making target DNA in Example 1
Forward Biotin (SEQ ID NO 1) GGTTTGCCCCATGCATATAAG
Reverse (SEQ ID NO 2) ATTACGAGCAAGGGTTGATGG

Target DNA sequence for Example 1 (5' to 3')
Biotin – (SEQ ID NO 3)
GGTTTGCCCCATGCATATAAGCATGTACATAATATTATATCCTTACATAGGACATATTAACT
CAATCTCATAATTCACTGATCTTTCAACAGTAATCGAATGCATATCACTTAGTCCAATAAGG
GCTTAATCACCATGCCTCGAGAAACCATCAACCCTTGCTCGTAAT

Surface bound probes for Example 1 (5' to 3')

Name	SEQ ID NO	Sequence
QC1	4	GAGTTAATATGTCCTATGTAAGG
QC2	5	GAGTTAATATGTCCTATGTAAGT
QC3	6	GAGTTAATATGTCCTATGTAAGA
QC4	7	GAGTTAATATGTCCTATGTAAGC
QC5	8	TTGAGTTAATATGTCCTATGTAAG
QC6	9	AGATTGAGTTAATATGTCCTATGT
QC7	10	AGATTGAGTTAATATGTCCTATGA
QC8	11	AGATTGAGTTAATATGTCCTATGC
QC9	12	AGATTGAGTTAATATGTCCTATGG
QC10	13	GAGATTGAGTTAATATGTCCTATG
QC11	14	TATGAGATTGAGTTAATATGTCCT
QC12	15	TATGAGATTGAGTTAATATGTCCA
QC13	16	TATGAGATTGAGTTAATATGTCCC
QC14	17	TATGAGATTGAGTTAATATGTCCG
QC15	18	TTATGAGATTGAGTTAATATGTCC
QC16	19	TGAATTATGAGATTGAGTTAATATG
QC17	20	TGAATTATGAGATTGAGTTAATATT
QC18	21	TGAATTATGAGATTGAGTTAATATA

QC19	22	TGAATTATGAGATTGAGTTAATATC
QC20	23	GTGAATTATGAGATTGAGTTAATAT
QC21	24	CAGTGAATTATGAGATTGAGTTAC
QC22	25	CAGTGAATTATGAGATTGAGTTAG
QC23	26	CAGTGAATTATGAGATTGAGTTAT

Microarray preparation

23 different sequences for surface bound probes (SEQ ID NO 4-26) that are complementary to different subsequences of the 169 pb (SEQ ID NO 3) region were randomly selected. The 23 probes with the sequences as described in Table 1 were synthesized by MWG-Biotech AG (Ebersberg, Germany). The probes were synthesized with a 5'-poly (T) spacer of 15 thymine residues and a 5'-terminus amino link with a C6 spacer. The probes were suspended at a concentration of 20 μM in 150 mM sodium phosphate, pH 8.5 and 0.06% sarkosyl solution (sarkosyl for improved spot uniformity) and were printed using a Q-array (Genetix, New Milton, Hampshire, UK) onto CodeLink™ Activated Slides (Surmodics, Eden Prairie, MN, USA). After printing, surface blocking was performed according to the manufacturer's instructions. The 23 probes were printed in 12 identical arrays on the slide. The 12 sub-arrays were separated during hybridization by a 16-pad mask (ChipClip™ Schleicher & Schuell BioScience, Keene, NH, USA).

Preparation of on bead-coupled single-stranded target DNA

A bead mixture consisting of 10 μl of 1 μm-diameter streptavidin-coated superparamagnetic beads solution and 90 μl 2x bind and wash solution (10mM Tris-HCl, 1mM EDTA, 2M NaCl, 1mM Beta-Mercaptoethanol, 0.1% Tween, pH 7.5) was prepared. 30 μl of PCR product (SEQ ID NO 3) were mixed with 30 μl of the bead mixture, followed by a 5-minute incubation. In this way approximately 1-3 pmol product was mixed with 3 μl superparamagnetic beads. Following binding of DNA the beads were washed with 1x TE buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA). Elution of the non-biotinylated strand was carried out using 0.1 M NaOH and incubating for 4 minutes, after which the supernatant was discarded.

Hybridization

After removal of the non-biotinylated strand the beads were washed twice in 40 ul 1x PBS (Phosphate buffered saline; 137mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), after which the beads were resolved in 30 ul 1x PBS. All of the prepared target DNA-bead solution was deposited onto the microarray surface. The beads were then attracted towards the surface by holding a circular 20 gram neodymium magnet with a diameter of 2 cm and a thickness of 1 cm directly underneath the array area where the array, at a distance of about 1 cm from the slide for 15 seconds to allow migration of the beads to the surface of the array to allow for hybridization of target DNA to the spotted polynucelotides on the surface. The magnet had a magnetic field of 1.27-1.29 Tesla and a pull of 8.4 kg. After hybridization, washing was carried out by moving the magnet from below the array to a position above the surface of the array where it was just touching the liquid phase for about 5 seconds to attract and remove any non-hybridized target DNA-beads.

Detection and results

The results were detected by visual inspection of the surface where aggregation of beads as determined by the human eye was considered as hybridization.

Target DNA hybridized to all sequences on the array (SEQ ID NO 4 to SEQ ID NO 26). No binding occurred outside the printed areas. Thus, 15 seconds of hybridization time was sufficient for hybridization to take place.

Example 2 HPV typing by using MUCH

This is an example of typing of Human Papillomavirus (HPV) by using the method of the invention in combination with MUCH (Multiplex Competitive Hybridization) [3].

Microarray preparation

16 different random DNA sequences each consisting of 25 bp were generated to serve as tag sequences (Table 2). Polynucleotides with these sequences were spotted on glass slides to function as surface bound capture probes. The capture probes were synthesized with a 5'-poly (T) spacer of 15 thymine residues and a 5'-terminus amino link with a C6 spacer. All probes where synthesized by MWG-Biotech AG (Ebersberg, Germany). The capture probes were suspended at a concentration of 20 µM in 150 mM sodium phosphate, pH 8.5 and 0.06% sarkosyl solution (sarkosyl for improved spot uniformity) and were spotted using a Q-array (Genetix, New Milton, Hampshire, UK) onto CodeLink™ Activated Slides (Surmodics, Eden Prairie, MN, USA). After printing, surface blocking was performed according to the manufacturer's instructions. The capture probes were printed in distinct zones where each zone contained one type of probe, thus corresponding to one HPV strain. There were two different probes for each HPV subtype, allowing for detection of each subtype using two independent MUCH target oligos.

Table 2. Surface bound capture probes

Name	SEQ ID NO	Artificial tag sequence
LT3	31	CCTCATGTCAACGAAGAACAGAACC
LT4	32	ATTGAAGCCTGCCGTCGGAGACTAA
LT6	33	TTATGGTGATCAGTCAACCACCAGG
LT7	35	GAGACACCTTATGTTCTATACATGC
LT9	35	GCCTTACATACATCTGTCGGTTGTA
LT10	36	CACAAGGAGGTCAGACCAGATTGAA
LT12	37	ACACATACGATTCTGCGAACTTCAA
LT13	38	TTACAGGATGTGCTCAACAGACGTT
LT15	39	CTGCACTGCTCATTAATATACTTCTGG
LT16	40	TTCACGCACTGACTGACAGACTGCTT
LT18	41	GCATCAGCTAACTCCTTCGTGTATT
LT19	42	GGCGTTATCACGGTAATGATTAACAGC
LT21	43	GCCTTATGCTCGAACTGACCATAAC
LT22	44	CGGATATCACCACGATCAATCATAGGTAA
LT24	45	TAGCTCTCCGCCTACAATGACGTCA
LT25	46	AGGAACGCCTTACGTTGATTATTGA

Samples and amplification

The L1 region of the HPVs 18 (SEQ ID NO 95) and 45 (SEQ ID NO 96) were PCR amplified using the general primer set GP5+/GP6+ with the sequences GP5+ TTTGTTACTGTGGTAGATACTAC (SEQ ID NO 29) and GP6+ GAAAAATAAACTGTAAATCATATTC (SEQ ID NO 30) where the GP6+primer was biotinylated. The amplification reactions were performed as previously described [4] The GP6+ primer was biotinylated to allow coupling to streptavidin-coated beads. The HPV plasmids were normalized to 100 ng/ml by using a ND-100 spectrophotometer (NanoDrop, Wilmington, DE).

Preparation of on bead coupled single-stranded DNA

A bead mixture consisting of 10 µl of 1 µm-diameter streptavidin-coated superparamagnetic beads solution and 90 µl 2x bind and wash solution (10mM Tris-HCl, 1mM EDTA, 2M NaCl, 1mM Beta-Mercaptoethanol, 0.1% Tween, pH 7.5) was prepared. 30 µl of PCR product was mixed with 30 µl of the bead mixture, followed by 5-minute incubation. In this way approximately 1-3 pmol product was mixed with 3 µl superparamagnetic beads. Following binding of DNA the beads were washed with 1x TE buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA). Elution of the non-biotinylated strand was carried out using 0.1 M NaOH and incubating for 4 minutes, after which the supernatant was discarded.

Multiplex competitive hybridization (MUCH)

After removal of the non-biotinylated strand the beads were washed twice in 40 µl 1x AB (Annealing buffer; 20 mM Tris-Acetate, 2 mM $MgAc_2$, pH 7.6), after which the beads were resolved in 30 µl 1x AB. The bead-bound single-stranded DNA was then divided into two reaction tubes (tube 1 and tube 2). Each reaction tube was subjected for a multiplex competitive hybridization (MUCH). In tube 1, MUCH probes HPV-6-1-LT3, HPV-11-1-LT6, HPV-16-1-LT9, HPV-18-1-LT12, HPV-31-1-LT15, HPV-33-1-

LT18, HPV-40-1-LT21 and HPV-45-1-LT24 was added while in tube 2, MUCH probes HPV-6-2-LT4, HPV-11-2-LT7, HPV-16-2-LT10, HPV-18-2-LT13, HPV-31-2-LT16, HPV-33-2-LT19, HPV-40-2-LT22 and HPV-45-2-LT25 was added (see also Table 3). The concentration of each MUCH probe was 0.1 µM. As listed in Table 3, each MUCH probe contains a 5´-signature tag sequence that is complementary to a specific capture probe on the array. In table 3 the HPV sequence as well as the artificial sequence is listed. The sequence of the MUCH probe is the combined sequence of the HPV sequence and the artificial sequence. These sequences as well as the sequence of the entire probe have been assigned a separate SEQ ID NO.

The MUCH probes were allowed to anneal to the bead-coupled template in solution by heating the solution to 78° C and allowed to cool down to room temperature. After removal of unbound MUCH probes the beads were washed twice in 40 µl 1x PBS (Phosphate buffered saline; 137mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), after which the beads were resolved in 30 µl 1x PBS. The content of the two reaction tubes were then mixed to allow for simultaneous detection of the two independent MUCH probe sets.

Hybridization

30 µl of prepared DNA-bead solution was deposited onto the microarray surface prepared with tags complementary to the tag sequences on the MUCH probes. The beads were then attracted towards the surface by holding a circular 19 gram neodymium magnet with a diameter of 2 cm and a thickness of 1 cm directly underneath the array for 15 seconds to allow migration of the beads to the surface of the array to allow for hybridization of target DNA to the spotted polynucelotides on the surface. After hybridization a washing step was performed by removing the magnet and placing it above the surface of the array, just touching the liquid phase, for 5 seconds to attract and lift away any non-hybridized DNA-beads.

Detection and results

The results were detected by visual inspection of the surface and documented by photography with a digital camera, and scanning using a microarray laser scanner. Aggregation of beads were clearly visible to the eye for four sequences of which two were representative of HPV 18 and two of HPV 45 (SEQ NO ID 37, 38, 45 and 46) and thus 15 seconds of total reaction time was sufficient for hybridization to take place. No signal was detected for other sequences. Thus, the hybridization step is highly specific.

Example 3

Example 3 was carried out essentially as example 1. The hybridization time was varied according to table 4. The strength of the hybridization signal and the background noise was estimated by visual inspection. Data is shown in Table 4.

Table 4

Time	Signal	Background noise
3s	Weak but detectable	None
8s	Strong	Low
10s	Strong	Low
15s	Strong	Low
30s	Strong but limited by noise	High

References

1. Graham D, Ferreira H, Felicianoa N, Freitas P, Clarke L, et al. (2005) Magnetic field-assisted DNA hybridisation and simultaneous detection using micron-sized spin-valve sensors and magnetic nanoparticles. Sensors and Actuators B: Chemical 107: 936-944.
2. Ståhl PL, Gantelius J, Natanaelsson C, Ahmadian A, Andersson-Svahn H, et al. (2007) Visual DNA -- identification of DNA sequence variations by bead trapping. Genomics 90: 741-745.
3. Käller M, Hultin E, Zheng B, Gharizadeh B, Wallin KL, et al. (2005) Tag-array based HPV genotyping by competitive hybridization and extension. Journal of Virological Methods 129: 102-112.
4. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ (1995) The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 76 (Pt 4): 1057-1062.

Table 3

Name	SEQ ID NO (entire probe)	5'-Tag sequence (artificial sequence complementary to the surface bound capture probes)	SEQ ID NO (tag)	HPV-type-specific sequence-3'	SEQ ID NO (HPV)
HPV-6-1-LT3	47	ggttctgttcttcgttgacatgagg	63	CGTAACTACATCTTCCACA	79
HPV-6-2-LT4	48	ttagtctccgacggcaggcttcaat	64	TGATTATAAAGAGTACATGC	80
HPV-11-1-LT6	49	cctggtggttgactgatcaccataa	65	TGTGTCTAAATCTGCTACA	81
HPV-11-2-LT7	50	gcatgtatagaacataaggtgtctc	66	AGATTATAAGGAATACATGC	82
HPV-16-1-LT9	51	tacaaccgacagatgtatgtaaggc	67	CATATCTACTTCAGAAACT	83
HPV-16-2-LT10	52	ttcaatctggtctgacctccttgtg	68	TACTAACTTTAAGGAGTACC	84
HPV-18-1-LT12	53	ttgaagttcgcagaatcgtatgtgt	69	TACACAGTCTCCTGTACCT	85
HPV-18-2-LT13	54	aacgtctgttgagcacatcctgtaa	70	TGCTACCAAATTTAAGCAGT	86
HPV-31-1-LT15	55	ccagaagtatattaatgagcagtgcag	71	AATTGCAAACAGTGATACT	87
HPV-31-2-LT16	56	aagcagtctgtcagtcagtgcgtgaa	72	TAGTAATTTTAAAGAGTATT	88
HPV-33-1-LT18	57	aatacacgaaggagttagctgatgc	73	AGTAACTAGTGACAGTACA	89
HPV-33-2-LT19	58	gctgttaatcattaccgtgataacgcc	74	GAATTTTAAAGAATATATAA	90
HPV-40-1-LT21	59	gttatggtcagttcgagcataaggc	75	CACACAGTCCCCCACACCA	91
HPV-40-2-LT22	60	ttacctatgattgatcgtggtgatatccg	76	TAACAGTAATTTCAAGGAAT	92
HPV-45-1-LT24	61	tgacgtcattgtaggcggagagcta	77	TACACAAAATCCTGTGCCA	93
HPV-45-2-LT25	62	tcaataatcaacgtaaggcgttcct	78	TCCTACTAAGTTTAAGCACT	94

Claims

1. A method of hybridizing a first polynucleotide to a second polynucleotide wherein the second polynucleotide is immobilized to a surface of a solid support, comprising the steps of
    a) contacting a solution containing the first polynucleotide with the surface of the solid support to which the second polynucleotide is immobilized,
    b) concentrating the first polynucleotide to the surface of the solid support to which the second polynucleotide is immobilized and allowing the first polynucleotide to hybridize to the second polynucleotide for a time period (hybridization time) of less than 300 seconds.

2. The method according to claim 1 where the concentration step b) is carried out with the aid of magnetic force.

3. The method according to claim 2 where paramagnetic particles are coupled to the first polynucleotide and step b) is carried out by providing at least one magnetic field that attracts the first polynucleotide towards the surface of the solid support to which the second polynucleotide has been immobilized.

4. The method according to claim 3 wherein the particles are paramagnetic and of an average size of from 0.5 micrometers to 3 micrometers and the particles are attracted by a magnet with a magnetic field of from 0.5 Tesla to 2.0 Tesla.

5. The method according to claim 4 wherein the particles are of an average size of from 0.75 to 1.5 micrometers, and the particles are particles are attracted by a magnet with a magnetic field of from 0.5 Tesla to 2.0 Tesla.

6. The method according to any one claims 1 to 5 wherein the hybridization time in step b) is less than 180 seconds.

7. The method according to any one claims 1 to 5 wherein the hybridization time in step b) is less than 60 seconds.

8. The method according to any one of claims 1 to 6 wherein the hybridization time in step b) is less than 30 seconds.

9. The method according to any one of claims 2 to 8 where the magnet that supplies the magnetic force is not integrated into the solid support.

10. The method according to any one of claims 2 to 8 where the magnet is a permanent magnet.

11. A method for diagnosis comprising the method according to any one of claims 1-10.

12. A method for diagnosis of Human Papillomavirus (HPV) according to claim 11.

13. A method according to any one of claims 11 or 12 further including the step of using multiplex competitive hybridization.

14. The use of a method according to any one of claims 1-11 for diagnosis of a disease.

15. The use of a method according to any of claims 1-10 for quality control of a DNA array.

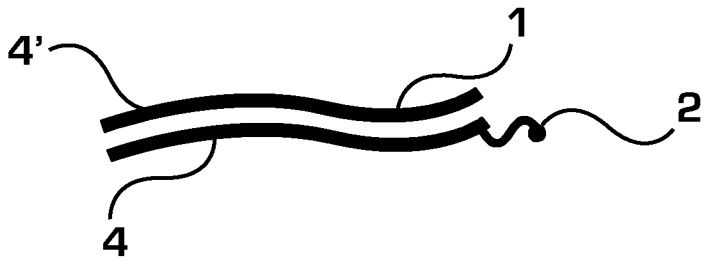


Fig. 1a

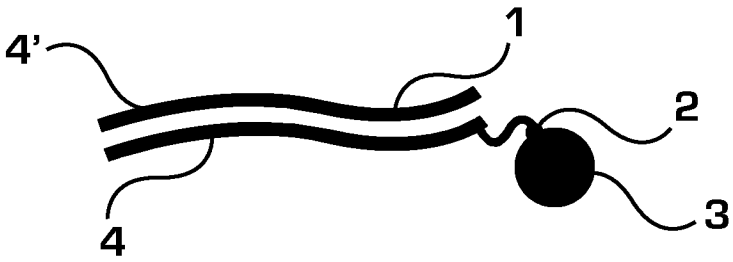


Fig. 1b

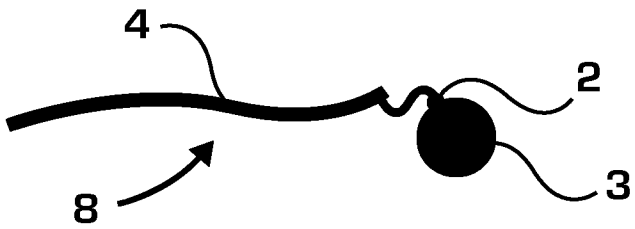


Fig. 1c

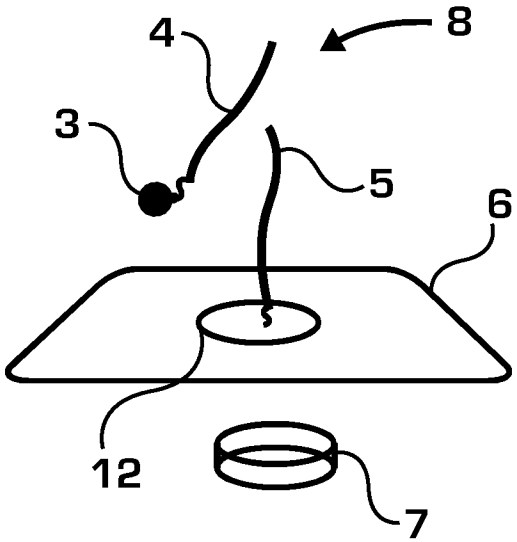


Fig. 1d

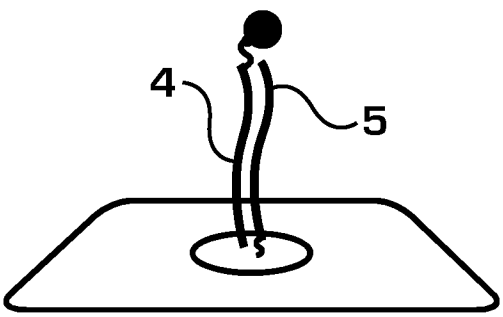


Fig. 1e

Fig. 2a

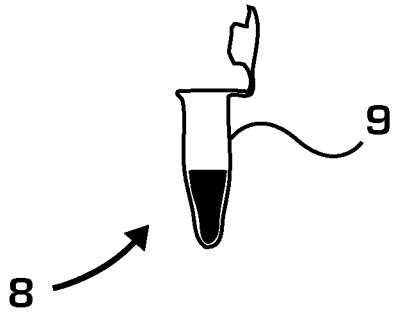


Fig. 2b

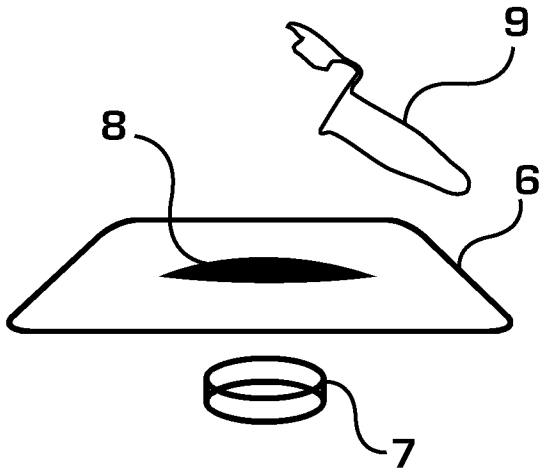


Fig. 2c

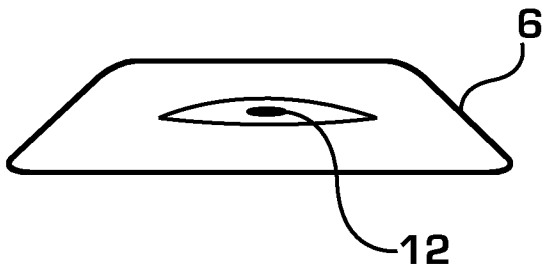
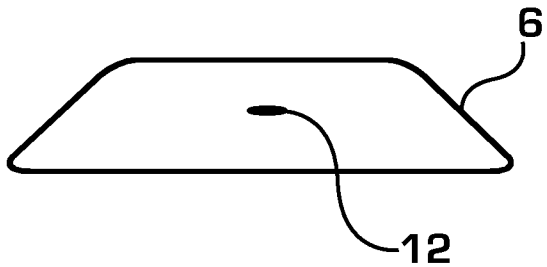


Fig. 2d



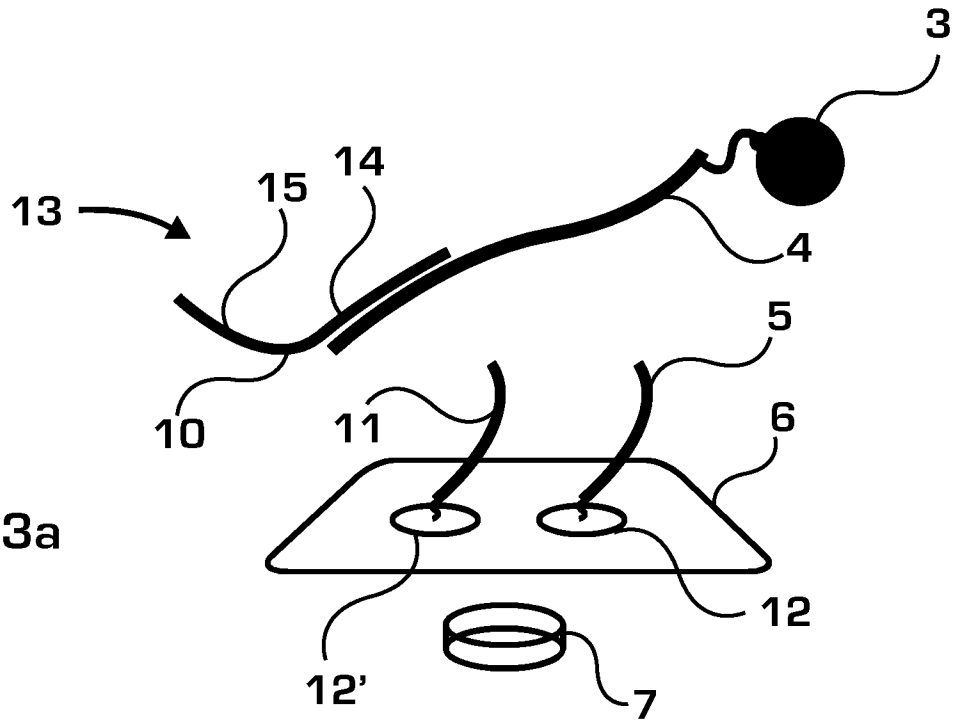


Fig. 3a

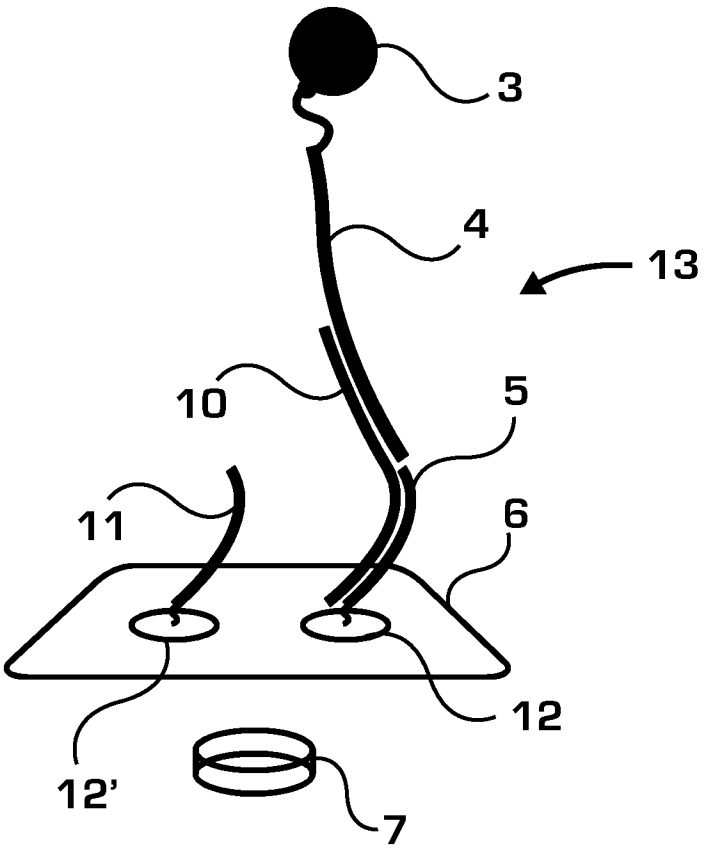


Fig. 3b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/069617

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/069617

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, WPI Data, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRAHAM DFERREIRA HFELICIANOA NFREITAS PCLARKE L ET AL.: "Magnetic field-assisted DNA hybridisation and simultaneous detection using micron- sized spin-valve sensors and magnetic nanoparticles", SENSORS AND ACTUATORS B: CHEMICAL, vol. 107, 20 May 2001 (2001-05-20), pages 936-944, XP25328713, cited in the application Whole document, in particular Fig. 4 -----	1-15
A	US 2002/164607 A1 (RAMIREZ-VICK JAIME E [US] ET AL) 7 November 2002 (2002-11-07) paragraphs [0012], [0020], [0023], [0025]; claims 1-16 ----- -/--	1-15

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

[X] See patent family annex.

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

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"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
18 May 2011	25/05/2011

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	Schmitt-Humbert, C

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007/021502 A1 (GE HEALTHCARE BIO SCIENCES [US]; PALANIAPPAN CHOCKALINGAM [US]) 22 February 2007 (2007-02-22) page 3, line 1 - line 12 claims 1-18	15
A	WO 2009/091148 A2 (JUNG WOON-WON [KR]; KIM HYUN-SOOK [KR]) 23 July 2009 (2009-07-23) page 2 - page 3; claims 7-15	11-14
A	US 2009/064800 A1 (FASCHING GERHARD [AT] ET AL) 12 March 2009 (2009-03-12) paragraphs [0009], [0013], [0014]	10
A	WO 2006/018811 A1 (INST DE ENGENHARIA DE SIST S E [PT]; UNIV COLLEGE CORK NAT UNIV IE [IE) 23 February 2006 (2006-02-23) paragraphs [0056], [0057], [0058], [0061]	11,14
A	US 2008/188374 A1 (CHEN CHUNG-HSUAN [TW] ET AL) 7 August 2008 (2008-08-07) figure 2	1-15

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/069617

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2002164607 A1	07-11-2002	NONE	
WO 2007021502 A1	22-02-2007	US 2009143234 A1	04-06-2009
WO 2009091148 A2	23-07-2009	NONE	
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		JP 2009072774 A	09-04-2009
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