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(54) **Title:** MEANS AND METHODS FOR QUANTIFYING NUCLEIC ACIDS

(57) **Abstract:** The present invention relates to a method for comparing the amounts of a first and a second polynucleotide com-
prised in a sample, said method comprising the steps of a) amplifying said first and said second test polynucleotide in a test sample
and a first and a second control polynucleotide in a reference sample, wherein said first and said second control polynucleotide is
present in the reference sample in amounts sufficient to allow for generation of maximal amounts of amplification products of the
said control polynucleotides, b) determining the amounts of the first and the second test amplification product of said first and said
second polynucleotide and the first and the second control amplification product of said first and said second control polynucleotide,
c) calculating (i) the ratio of the amounts of the first test amplification product and the first control amplification product and (ii) the
ratio of the second test amplification product and the second control amplification product; and, d) comparing the ratios calculated in
c) (i) and (ii), whereby the amount of the first test polynucleotide and the amount second test polynucleotide comprised in the test
sample are compared. Furthermore, the present invention relates to a device for comparing the amounts of a first and a second poly-
nucleotide comprised in a sample by the method of the present invention.



Means and methods for quantifying nucleic acids

5 The present invention relates to a method for comparing the amounts of a first and a second polynucleotide comprised in a sample, said method comprising the steps of a) amplifying said first and said second test polynucleotide in a test sample and a first and a second control polynucleotide in a reference sample, wherein said first and said second control polynucleotide is present in the reference sample in amounts sufficient to allow for generation of maximal
10 amounts of amplification products of the said control polynucleotides, b) determining the amounts of the first and the second test amplification product of said first and said second polynucleotide and the first and the second control amplification product of said first and said second control polynucleotide, c) calculating (i) the ratio of the amounts of the first test amplification product and the first control amplification product and (ii) the ratio of the second
15 test amplification product and the second control amplification product; and, d) comparing the ratios calculated in c) (i) and (ii), whereby the amount of the first test polynucleotide and the amount second test polynucleotide comprised in the test sample are compared. Furthermore, the present invention relates to a device for comparing the amounts of a first and a second polynucleotide comprised in a sample by the method of the present invention.

20 Nucleic acid detection methods are widely utilized in research and development, drug discovery and diagnostic applications. Detection methods of nucleic acids for identification of targets, e.g., those aiming to detect whether a subject is infected with bacteria or viruses, already has been described (WO/2009/027403). However, such methods, in principle, could also be used for
25 identification of chromosomal aberrations such as deletions or duplications which are often related with severe phenotypes (Krajewski et al. (2000). Neurological dysfunction and axonal degeneration in Charcot-Marie-Tooth disease type 1A. *Brain* 123, 7: 1516–27; Jacobsen 1973. An (11;21) translocation in four generations with chromosome 11 abnormalities in the offspring. A clinical, cytogenetical, and gene marker study. *Hum Hered.*;23(6):568-85).

30 Further, somatic aberrations in multiple genes, pathways and chromosomal regions have been associated with certain cancers, suggesting that abnormal genomic content and consequent changes in gene expression patterns underlie the aberrant biological behavior of these cancers. Overexpression or increased activity of many oncogenes, including MYC, KRAS, EGFR, Cyclin
35 D1 and BCL2, has been implicated in the pathogenesis of certain cancers as well as abnormal expression or impaired function of tumor suppressor genes including, p53, p16, Rb, FHIT, RASSF1A, SEMA3B and PTEN (Dehan et al. Chromosomal aberrations and gene expression profiles in non-small cell lung cancer *Lung Cancer* (2007) 56, 175-184).

Further, the differential expression of several genes have been associated with diseases. For example, regulation of expression of calreticulin has been associated with several diseases, including neurodegenerative problems, cancers, autoimmune diseases and wound healing (Qiu et al Transcriptional control of the calreticulin gene in health and disease. *Int J Biochem Cell Biol.* 2009 41(3):531-8). An efficient, reliable and precise determination of the amount of multiple polynucleotides within a sample would be of value in the diagnosis of these disease and disorders.

The identification of specific genotypes or differential expression of genes is currently almost exclusively based on the detection of molecular and biochemical markers, such as DNA and RNA. Hybridization techniques for example have been developed using a single-stranded polynucleotide probe that is complementary to a specific target polynucleotide to selectively identify the presence of a particular target polynucleotide via hybridization. Furthermore, several PCR-based methods were developed within the last years. The majority of these PCR systems use consensus or general primers that bind to highly conserved regions of the target region. The amplified PCR products may then be subjected to further analysis (e.g. sequencing, restriction fragment length polymorphism (RFLP) analysis or hybridization) in order to identify a specific target. The identification of one or more polynucleotides related to diseases or polynucleotides which are differentially expressed due to certain circumstances can be detected by PCR techniques, such as multiplex PCR. Such methods have been developed to amplify multiple nucleic acids within a sample allowing a simultaneous detection of several targets in a single reaction.

However, the interpretation of results of such multiplex PCR techniques is very complex and demanding. Ideally, all primer pairs in a multiplex PCR should enable similar amplification efficiencies for their respective target. In contrast, a preferential amplification of one target sequence over another is a known phenomenon in multiple PCRs (Mutter and Boynton. 1995. PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. *Nucleic Acids Res.* 23:1411-1418; Polz and Cavanaugh. 1998. Bias in template-to-product ratios in multitemplate PCR. *Appl. Environ. Microbiol.* 64:3724-3730). It has been shown that amplification biases are strongly dependent on the choice of primers and dependent to a lesser extent on the templates (Suzuki and Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *App. Environ. Microbiol.* 62:625-630). In addition, quantification of multiple templates in a test samples can only be accomplished using single-, double-, triple- or quadruplex real-time PCR techniques. Thus, when using higher multiplexing degrees, quantification of multiple templates in the test sample is not possible by standard endpoint detection systems. So far, only qualitative analyses can be performed in techniques multiplexing more than five or six amplification reactions.

Moreover, the amplified polynucleotides can be determined by methods involving hybridization to poly- or oligonucleotides that are complementary to individual genotypic sequences in the amplified polynucleotides. The hybridization and the detection of the occurrence of a hybridization event may be carried out by any method under any conditions deemed appropriate, e.g., by Southern blot assays, dot blot assays, or by membrane-based reverse line blot (Melchers et al., Prevalence of genital HPV infections in a regularly screened population in The Netherlands in relation to cervical cytology. 1988. J Med Virol 25:11-6; van den Brule et al., GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. 2002. J Clin Microbiol 40:779-87; Melchers et al., Optimization of human papillomavirus genotype detection in cervical scrapes by a modified filter in situ hybridization test. 1989. J Clin Microbiol 27:106-10). A particularly preferred method for the detection of amplified polynucleotides is the hybridisation to specific probes coupled to fluorescence-labelled polystyrene beads (Luminex suspension array technology) under suitable, preferably, stringent conditions. Moreover, the amplification products may be identified by use of DNA-Chips which contain specific oligonucleotides linked to a suitable carrier.

Ideally, all hybridization oligonucleotides probes in a multiplex hybridization should enable similar annealing efficiencies for their respective target. However, it is well recognized that the hybridization efficiency and thus, the hybridization signal, may vary substantially due to biochemical differences between distinct oligonucleotides probes. Thus, there is a need for an efficient, reliable and precise determination of the amount of multiple polynucleotides within a sample, in particular by avoiding the aforementioned hybridization problems.

Thus, the technical problem underlying the present invention may be seen as the provision of means and methods for efficiently, reliably and precisely detecting multiple polynucleotides in a sample. The technical problem is solved by the embodiments characterized in the claims in herein below.

Accordingly, the current invention relates to a method for comparing the amounts of a first and a second polynucleotide comprised in a sample, said method comprising the steps of:

- a) amplifying said first and said second test polynucleotide in a test sample and a first and a second control polynucleotide in a reference sample, wherein said first and said second control polynucleotide is present in the reference sample in amounts sufficient to allow for generation of maximal amounts of amplification products of the said control polynucleotides;
- b) determining the amounts of the first and the second test amplification product of said first and said second polynucleotide and the first and the second control amplification product of said first and said second control polynucleotide;

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- c) calculating (i) the ratio of the amounts of the first test amplification product and the first control amplification product and (ii) the ratio of the second test amplification product and the second control amplification product; and
- d) comparing the ratios calculated in c) (i) and (ii), whereby the amount of the first test polynucleotide and the amount second test polynucleotide comprised in the test sample are compared.

The method of the present invention, preferably, is an *in vitro* method. Moreover, it may comprise steps in addition to those explicitly mentioned above. For example, further steps may relate to sample pre-treatments or evaluation of the results obtained by the method. The method of the present invention, preferably, is used for comparing the amounts of a first and a second polynucleotide comprised in a sample. However, it is to be understood that the amount of more than two polynucleotides can be compared by the method of the invention as well, preferably 3, 4, 5, 6, 7, 8, 9, 10, 20, 30 or more polynucleotides. Thus, the amount of a further polynucleotide, i.e. a third, fourth, fifth etc. polynucleotide, can be determined by the method of the present invention and compared to the amount of the first and/or second polynucleotide. To this end, preferably, the amount of the said further polynucleotide can be determined as specified for the first and/or second polynucleotide above. The method may be carried out manually or assisted by automation. Preferably, steps a) and / or b) and / or c) and / or d) may in total or in part be assisted by automation, e.g., by a suitable robotic and sensory equipment for quantification or determination in step a) and / or b), or a computer-implemented calculation or comparison step in steps c) and / or d).

The term "polynucleotide" as used herein means a double or single stranded DNA including cDNA and genomic DNA or RNA. The term encompasses single- as well as double- stranded polynucleotides. Moreover, comprised are also chemically modified polynucleotides including naturally occurring modified polynucleotides such as glycosylated or methylated polynucleotides or artificial modified ones such as biotinylated polynucleotides.

The term "test sample" refers to a biological sample. Such a biological sample, preferably, can be a sample of a body fluid, a sample of separated cells or tissue culture cells, a sample from a tissue or an organ, a sample of wash/rinse fluid obtained from an outer or inner body surface or an environmental sample. It will be understood that the sample shall comprise at least a first and a second polynucleotide the amounts of which shall be compared. Preferably, the said first and second polynucleotides are comprised by a genome or transcriptome of an organism or part thereof which is comprised in the sample. Samples can be obtained by well known techniques from an organism and include, preferably, scrapes or biopsies from the urogenital tract, perianal regions, anal canal, the oral cavity, the upper aerodigestive tract and the epidermis. The organisms from which the aforementioned samples are, preferably, obtained encompass animals,

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preferably, mammals such as rats, mice, sheep, dogs, cats, horses, and most preferably, humans. Such samples can be obtained by use of brushes, (cotton) swabs, spatula, rinse/wash fluids, punch biopsy devices, puncture of cavities with needles or surgical instrumentation. Preferably, the sample contains mucosal cells, more preferably the sample comprises mucosal cells from the cervix. However, samples of blood, plasma, serum, urine, saliva, lacrimal fluid, stool are also encompassed by the method of the present invention. Tissue or organ samples may be obtained from any tissue or organ by, e.g., biopsy or other surgical procedures. Separated cells may be obtained from the body fluids or the tissues or organs by separating techniques such as filtration, centrifugation or cell sorting. Environmental samples as referred to herein may, preferably, comprise microorganisms such as fungi or bacteria.

It is to be understood that the sample may be further processed in order to carry out the method of the present invention. Particularly, the polynucleotides might be extracted and/or purified from the obtained sample by methods and means known in the art or as described in the accompanying Examples, below. Thus, the term sample also relates to partially or entirely purified and/or extracted polynucleotides from any sample as mentioned above.

The term "reference sample" refers to a sample comprising a first and second control polynucleotide in an amount which allows for the generation of a maximum number of amplification products by a given amplification reaction used for amplifying the first and second control and/or test polynucleotides. Thus, the reference sample reflects an optimal amplification reaction for the first and second polynucleotide since the amount of template to be amplified by the reaction is chosen as to allow for the generation of the maximum number of amplification products. The amount of a first and a second polynucleotide which allows for the said generation of a maximum number of amplification products, i.e. the optimal template amount, can be, preferably, experimentally determined for given amplification parameters used in an amplification reaction. To this end, a calibration curve with different amounts of amplification products is set up wherein the said amounts of the amplification products are derived by the said amplification reaction starting from different template amounts. Based from the calibration curve, the optimal template amount can be derived. Such an optimal template amount for the first and second polynucleotide can be included into the reference sample in an appropriate form, e.g., as linear DNA or as plasmid DNA. In an analogous manner, the optimal template concentration for a further control polynucleotide can be determined.

The term "amplifying" as used herein relates to nucleic acid PCR-based amplification techniques well known in the art. It will be understood that the amplification parameters used for the first polynucleotide in the test sample and the first control polynucleotide in the reference sample are, preferably, identical. Preferably, the amplification is carried out using a portion of the test sample to be investigated and a portion of the control sample, i.e. aliquots of the respective

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samples. Moreover, the amplification parameters used for the second polynucleotide in the test sample and the second control polynucleotide in the reference sample are, preferably, identical. Amplification parameters as referred to herein encompass oligonucleotides used as primers, nucleotide concentration, nucleic acid polymerases used for the amplification, buffer composition, number of amplification cycles, and temperatures during the cycles.

The PCR-based amplification techniques allow for specific amplification of a first and/or second polynucleotide by utilizing oligonucleotide primers which hybridize to the said polynucleotides under the specific conditions of the envisaged amplification reaction. Preferably, such an oligonucleotide primer comprises or essentially consists of a nucleic acid sequence being either identical or similar to a sequence of the target to be amplified or being reverse complementary thereto. Similar as used in this context means that the sequence is at least 80%, at least 90%, at least 95% or at least 99% identical to the nucleic acid sequence of the target or its reverse complement over the entire length of the oligonucleotide. Preferably, an oligonucleotide primer as meant herein has between 15 and 30 nucleotides in length, more preferably between 18 and 28 nucleotides in length, and most preferably between 23 to 25 nucleotides in length. Preferably, the oligonucleotide primer is a single-stranded oligonucleotide. Preferably, the oligonucleotide primers used for the method of the present invention may be labeled or may contain other modifications which allow for detection and/or analysis of an amplification product and/or the binding to a carrier. Labeling can be done by various techniques well known in the art and depending of the label to be used. Particularly, the oligonucleotide primers may be biotinylated in order to enable the binding of the amplification products to a streptavidin surface or fluorescent conjugate. Moreover, labels to be used in the context of the present invention may be, but are not limited to, fluorescent labels comprising, inter alia, fluorochromes such as R-phycoerythrin, Cy3, Cy5, fluorescein, rhodamin, Alexa, or Texas Red. However, the label may also be an enzyme or an antibody. It is envisaged that an enzyme to be used as a label will generate a detectable signal by reacting with a substrate. Suitable enzymes, substrates and techniques are well known in the art. An antibody to be used as label may specifically recognize a target molecule which can be detected directly (e.g., a target molecule which is itself fluorescent) or indirectly (e.g., a target molecule which generates a detectable signal, such as an enzyme). The oligonucleotide primers of the present invention may also contain 5' restriction sites, locked nucleic acid molecules (LNA) or be part of a peptide nucleotide acid molecule (PNA). Such PNA can be, in principle, detected via the peptide part by, e. g., antibodies. Quantitative PCR methods with the above mentioned labeled oligonucleotides can be used for relative quantification; if a calibration curve is incorporated in such an assay, the relative quantification can be used to obtain an absolute quantification. Other methods known for amplification are, e.g. nucleic acid sequence-based amplification (NASBA).

The term “determining the amount” as used herein refers to determining the number of generated amplification products of the first and second test as well as control polynucleotides. The amount can be determined quantitatively or semi-quantitatively, i.e. as an absolute amount or a relative amount. In order to determine the amount, at least one characteristic feature of each
5 amplification product will be determined. Characteristic features in accordance with the present invention are features which characterize the physical and/or chemical properties including biochemical properties of an amplification product. Such properties include, e.g., molecular weight, viscosity, density, electrical charge, spin, optical activity, colour, fluorescence, chemo-
10 luminescence, elementary composition, chemical structure, capability to react with other compounds, and the like. Values for said properties may serve as characteristic features and can be determined by techniques well known in the art. Moreover, the characteristic feature may be any feature which is derived from the values of the physical and/or chemical properties of an amplification product by standard operations, e.g., mathematical calculations such as multiplication, division or logarithmic calculus. Most preferably, the at least one characteristic
15 feature allows the determination and/or chemical identification of the amplification product and its amount. Accordingly, the characteristic value, preferably, also comprises information relating to the abundance of the amplification product from which the characteristic value is derived.

The term „calculating“ as used in accordance with the method of the present invention relates to
20 mathematical operations which allow for determining the ratio of the test amplification product and the control amplification product. Preferably, the amount of a test amplification product is divided by the amount of a corresponding control amplification product. Accordingly, the performance of the amplification reaction for amplifying a test polynucleotide is normalized with respect to the optimal performance for the said amplification reaction, i.e. the amplification
25 reaction carried out on the reference sample.

The term “comparing” as used herein relates to assessing whether the ratios to be compared are identical or differ from each other and to what extent they differ from each other. Based on the said comparison of the ratios, it can be furthermore assessed whether the amount of a first test
30 polynucleotide and a second test polynucleotide comprised in the test sample are identical or differ from each other and to what extent they differ. Preferably, comparing as used herein also encompasses assessing whether an observed difference between two ratios is statistically significant, or not. To this end, known statistical techniques can be applied such as Student’s t-test and the like. The comparison referred to in step d) of the method of the present invention
35 may be carried out manually or computer-assisted. The computer program may further evaluate the result of the comparison, i.e. automatically provide the desired assessment in a suitable output format. Based on the comparison of the amounts of at least two polynucleotides in a sample the polynucleotide is analyzed which is most likely to be biologically relevant.

Advantageously, the method of the present invention allows for a robust and reliable determination and comparison of the amounts of two or more polynucleotides comprised in a test sample. In particular, the method is useful for determining and comparing the amounts of two or more polynucleotides comprised in a genome of an organism or part thereof comprised in the sample. Therefore, the method can be applied, for instance, in order identify the strength of expression or the abundance of a polynucleotide or to make a ranking for different polynucleotides with respect to their strength of expression/presence or their abundance in a genome. Polynucleotides showing a particular strong expression or high abundance in a sample shall, most likely, be of biological relevance, e.g., they may be associated with diseases or disorders. Thus, the method of the present invention is particularly advantageous since it allows for rapid, efficient, reliable and precise detection of the amounts of multiple polynucleotides in a sample. Moreover, this method is technically simple and, preferably, is amenable to automation in a high-throughput format. In particular, the method can be applied in biomarker development, clinical assessment, routine diagnostics, environmental analyses and monitoring and the like.

In a preferred embodiment of the method of the present invention, a ratio calculated in c) i) which is increased compared to the ratio calculated in c) ii) is indicative for an amount of the first test polynucleotide which is increased compared to the amount of the second test polynucleotide, a ratio calculated in c) i) which is decreased compared to the ratio calculated in c) ii) is indicative for an amount of the first test polynucleotide which is decreased compared to the amount of the second test polynucleotide, and a ratio calculated in c) i) which is identical to the ratio calculated in c) ii) is indicative for an amount of the first test polynucleotide which is identical compared to the amount of the second test polynucleotide.

In another preferred embodiment of the method of the present invention, said first and said second control polynucleotide is each present in an amount of at least 10^4 copies in the reference sample. It has been found in the studies underlying this invention that an amount of at least 10^4 copies is, preferably, required for a PCR-based amplification reaction to give a maximum number of amplification products.

In a preferred embodiment of the present invention said first test polynucleotide and said first control polynucleotide is a polynucleotide of a first HPV and said second test polynucleotide and said second control polynucleotide is a polynucleotide of a second HPV. The term "HPV" as used herein relates to the human papillomavirus. Infection of the cervix with human papillomavirus (HPV) is the predominant cause for cervical cancer, particularly with high-risk HPV genotypes. Human papillomaviruses form a large group of viruses and are small, non-enveloped DNA viruses that infect almost exclusively skin and mucosal cells. To date, the genomes of > 120 various genotypes of human papillomaviruses have been characterized (de Villiers, E. M., C. Fauquet, T. R. Broker, H. U. Bernard, and H. zur Hausen. 2004. Classification

of papillomaviruses. Virology 324:17-27), and a much larger number of human papillomaviruses is thought to exist (Hazard K, Andersson K, Dillner J, Forslund O. Human papillomavirus subtypes are not uncommon. Virology. 2007 May 25;362(1):6-9).

5 In a preferred embodiment of the method of the present invention, the amplification products are determined by immobilized oligonucleotide probes. Preferably, an immobilized oligonucleotide probe is capable of specifically hybridizing to an amplification product (i.e. polynucleotide) to be detected in accordance with the method of the present invention. The oligonucleotide shall, preferably, comprise a sequence of sufficient length and complementarity for specific binding.
10 Moreover, upon specific binding, the amount of amplification products shall be detectable, e.g., in an ELISA format or by determining fluorescence or radioactivity of the bound amplification products. Based on the determined amount and the predefined information on the immobilized oligonucleotide probe, the amount of a certain polynucleotide species can be determined. The oligonucleotide probe is, preferably, immobilized to a matrix achieved by covalent direct or
15 indirect binding of the oligonucleotide probe to the said matrix. Materials for said matrix are well known in the art and include, inter alia, commercially available polysaccharide matrices comprising sepharose, sephadex, agarose, sephacell, micro-cellulose, and alginate-beads, polypeptide matrices, polystyrene beads, latex beads, magnetic beads, colloid metal particles, glass, plastic and/or silicon chips and surfaces, nitrocellulose strips, membranes, sheets,
20 duracytes, wells and walls of reaction trays, plastic tubes.

In a more preferred embodiment of the method of the present invention, said oligonucleotide probes are immobilized on a microbead. The overall size of a small particle or bead, preferably, may be in the micrometer or nanometer range. Preferably, said beads and particles may be
25 stained with a specific dye, more preferably with a specific fluorescent dye. Preferably, by staining various carriers with various dyes, the carriers can be distinguished from each other. By using a carrier with a specific dye for a specific probe oligonucleotide (thus, a oligonucleotide targets the amplified polynucleotides), said carrier is distinguishable from other carriers comprising different dyes.

30 In yet another preferred embodiment of the method of the present invention, said microbead is a Luminex™ bead. Such microbeads are commercially available (Luminex Corp., Austin, Texas, USA). Thus, for determining the amounts of the first and second polynucleotide, the first and second polynucleotide type-specific oligonucleotides are coupled to fluorescence-labelled
35 polystyrene beads (Luminex suspension array technology) which are hybridized with amplification products as specified herein above under suitable, preferably, stringent conditions. It is, however, also contemplated by the current invention that the oligonucleotides are linked to a suitable carrier in a spatially separated way, e.g. in the form of microarrays, Reverse-Line blots (RLB), dot blots or similar technologies.

In a preferred embodiment of the method of the present invention the polynucleotides are amplified by PCR or NASBA, the terms "PCR" and "NASBA" already have been specified above.

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In a preferred embodiment of the method of the present invention, the ratios calculated in step c) i) and ii) are normalized. It will be understood that such normalization, preferably, made with respect to the total nucleic acids present in the sample or to a certain nucleic acid species which is presumably always present in similar amounts. Thus, preferably, the reference amplification product is a product obtained from a polynucleotide known to have a constant abundancy in each cell, i.e. a polynucleotide comprised in most, preferably all, cells of a sample in approximately the same amount. More preferably, the reference amplification product is amplified from a chromosomal or mitochondrial gene or from the mRNA of a housekeeping gene. Endogenous polynucleotide sequences are, preferably, polynucleotide sequences of genes selected from the group consisting of [beta]-globin, GAPDH, Actin and Ubiquitin C. In another preferred embodiment of the method of the present invention, the reference amplification product is amplified from an artificial exogenous calibrator polynucleotide sequences. Preferably, these exogenous polynucleotide sequences contain the same oligonucleotide primer binding sites but different oligonucleotide probe regions. Normalization and thus quantification is preferably achieved by adding a predefined amount of calibrator DNA or RNA to the amplification mixture. Said calibrator RNA, preferably, shall be *in vitro*-transcribed RNA that can be amplified by the same oligonucleotides that are capable of specifically amplifying the transcripts to be analyzed. Said calibrator DNA, preferably, shall be cloned DNA that can be amplified by the same oligonucleotides that are capable of specifically amplifying the polynucleotides to be analyzed. However, said calibrators shall comprise a specific target region for a probe oligonucleotide (i.e. a target region not comprised by the polynucleotide to be analyzed). Said specific target region shall allow for differentiating between the amplification product of the polynucleotide to be analyzed and the amplification product of the calibrator.

The principle of the normalization is the competitive co-amplification of the calibrator and the polynucleotide to be analyzed with the same oligonucleotide pair. It is to be understood that calibrator amounts, preferably, need to be titrated for each polynucleotide to be analyzed in the context of the present invention. For quantification expression levels can be compared to a standard curve or to suitable reference material. This can be done by the skilled person without further ado.

30

The present invention relates to a device for comparing the amounts of a first and a second test polynucleotide comprised in a sample, said device comprising:

- 5 a) an analysis unit which comprises a detector for determining the amount of the first and the second test amplification products and the first and the second control amplification products; and
- b) an evaluation unit comprising a data processor having implemented an algorithm for calculating (i) the ratio of the amounts of the first test amplification product and the first control amplification product and (ii) the ratio of the second test amplification product
10 and the second control amplification product.

The term "device" as used herein relates to a system comprising the aforementioned units operatively linked to each other as to allow the comparison of the amounts of a first and a second test polynucleotide comprised in a sample according to the methods of the invention. The term
15 "analysis unit" as used herein refers to an agent which is capable of specifically quantifying the amount of the first and the second test amplification products present in a sample. Preferably, the first and the second test amplification products are amplified by one of the methods described herein before quantification occurs. Preferred detection methods are detection of luminescence, fluorescence, or absorbance. The determined amount of polynucleotides can be transmitted to the
20 evaluation unit. Said evaluation unit comprises a data processing element, such as a computer, with an implemented algorithm for carrying out a comparison between the determined amount of the first and the second test amplification products. It is to be understood that the data obtained will need interpretation by the clinician. However, also envisaged are expert system devices wherein the output comprises processed diagnostic raw data the interpretation of which does not
25 require a specialized clinician.

In a preferred embodiment of the device of the present invention, said data processor further having implemented an algorithm for comparing the calculated ratios with each other. Preferably, according to such an algorithm, a ratio calculated in c) i) which is increased
30 compared to the ratio calculated in c) ii) is indicative for an amount of the first test polynucleotide which is increased compared to the amount of the second test polynucleotide, a ratio calculated in c) i) which is decreased compared to the ratio calculated in c) ii) is indicative for an amount of the first test polynucleotide which is decreased compared to the amount of the second test polynucleotide, and a ratio calculated in c) i) which is identical to the ratio calculated
35 in c) ii) is indicative for an amount of the first test polynucleotide which is identical compared to the amount of the second test polynucleotide.

In a preferred embodiment of the device of the present invention, the device further comprises an amplification unit for the amplification of the polynucleotides. An amplification unit as used

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herein refers preferably to a thermo-cycler or similar device which is capable of carrying out nucleic acid amplification reactions in an automated fashion.

5 All references cited in this specification are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this specification.

10 FIGURES

Figure 1 shows HPV16 quantification by net MFI [A] or HPV16 to beta-globin ratios [B] in HPV16 plasmid dilution series in 100 ng human placenta DNA per PCR.

15 **Figure 2** shows Lactobacillus quantification by net MFI [A] or Lactobacillus to calibrator ratios [B] in Lactobacillus dilution series in 100 ng human placenta DNA per PCR.

The invention will now be illustrated by the following Examples which shall, whatsoever, not be
20 construed as limiting the scope.

EXAMPLES

25 **Example 1: Quantification of HPV subtypes by BSGP5+/6+-PCR/MPG**

The detection of 51 HPV types and three subtypes, including Hr-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a/b and pHr-HPV types 26, 53, 67, 70, 73, 82, and beta-globin by the BSGP5+/6+-PCR/MPG assay was performed as described with some modifications in
30 WO2009/027403. HPV amplification was carried out using the Multiplex PCR Kit (Qiagen, Hilden, Germany). 0.2 to 0.5 μ M each of the BSGP5+ and 5'-biotinylated BSGP6+ primers, and 0.15 μ M each of the beta-globin primers MS3 and 5'-biotinylated MS10 were added to the PCR mixture. The primers have a sequence as disclosed in WO2009/027403. The cutoff value (5 net Median Fluorescence Intensities (MFI)) to define HPV DNA positivity was applied as described
35 previously in WO2009/027403. For the colony PCR, Escherichia coli DH5 α , transformed by high-copy-number plasmids containing the viral genome, replaced the template DNA. Depending on the amount of transferred bacteria, colony PCRs are estimated to contain $>10^6$ HPV copies per PCR. Quantification of HPV signals was accomplished by computing for each positive reaction the relative HPV MFI signal (%) by dividing the measured HPV MFI value

with the maximum value detected of this HPV type using colony PCR products. Finally, the relative MFI (%) was divided by the measured beta-globin MFI value to form a non-descriptive viral load value (BS viral load) (%HPVMFI / beta-globin MFI).

BSGP5+/6+-PCR/MPG allowed the semi-quantitative analysis of HPV containing plasmid dilution series between 10 and 10,000 copies per PCR (Schmitt et al., 2008). Due to amplification competition of beta-globin with HPV, it was further observed that specimens with high viral loads showed reduced amplification of beta-globin (unpublished data). In order to strengthen this effect, beta-globin primer concentrations were titrated during the amplification of 10-fold dilution series of HPV16 containing plasmid DNA (Fig. 1). While HPV16 net MFI values reached the plateau phase with 1,000 to 10,000 copies per PCR, beta-globin signals declined with increased HPV copy number input (Fig. 1). The HPV16/beta-globin ratios, however, were able to quantify over 7 logs when beta-globin primer concentrations between 0.1 and 0.2 μ M were used.

Example 2: Quantification by genital microorganisms using calibrators

Amplification and quantification of *Lactobacillus crispaticus*, using dilution series of a plasmid containing cloned *Lactobacillus crispaticus* sequences, was carried out using the Multiplex PCR Kit (Qiagen, Hilden, Germany). One primer of each pair was biotinylated at the 5' terminus for labelling the target strand of the amplified product. The 50 μ l reactions comprised 1x QIAGEN Multiplex PCR Master Mix (containing 3 mM $MgCl_2$, dNTP mix, 0.5 x Q-solution and HotStarTaq DNA polymerase), 0.2 μ M each primer, 100,000 copies of the respective calibrators (plasmid containing cloned *Lactobacillus crispaticus* sequences with exchanged probe binding regions), and 1-2 μ L of template DNA. Using a Mastercycler (Eppendorf, Germany) PCR products were amplified by incubation at 95°C for 15 minutes to activate the enzyme, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 61°C for 90 seconds, and extension at 72°C for 60 seconds. Final extension was performed at 72°C for 10 minutes and reactions were stored at 4°C.

Following multiplex PCR amplification, 10 μ l of each reaction mixture were transferred to 96-well plates containing 33 μ l of tetramethylammonium chloride (TMAC) hybridization solution (0.15 M TMAC, 75 mM Tris-HCl, 6 mM EDTA, 1.5 g/liter Sarkosyl, pH 8.0), 7.0 μ l of 1 x TE, and a mixture of 2,000 probe-coupled beads of each set. The mixture was heated to 95°C for 10 min in a laboratory oven, immediately placed on ice for 1 min, and then transferred to a thermomixer. Hybridization was performed at 41°C for 30 min under agitation. The samples were transferred to a 96-well wash plate (Millipore, Bedford, MA), pre-equilibrated with washing buffer (phosphate-buffered saline, 0.02% Tween). Subsequently, the beads were washed

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once with 100 µl of washing buffer on a vacuum wash station (Millipore). On a horizontal shaker at room temperature, beads were resuspended for 20 min in 50 µl of streptavidin-R-phycoerythrin (Strep-PE; Molecular Probes, Eugene, OR) diluted 1:1,600 in 2.0 M TMAC, 75 mM Tris-HCl, 6 mM EDTA, 1.5 g/liter Sarkosyl, pH 8.0. Beads were then washed three times
 5 with 100 µl washing buffer and finally resuspended in 100 µl washing buffer for 5 min on a shaker. Beads were analyzed for internal bead color and R-phycoerythrin reporter fluorescence on a Luminex 100 analyzer. The median reporter fluorescence intensity (MFI) of at least 100 beads was computed for each bead set in the sample.

10 Quantification of signals was accomplished by dividing the measured *L. crispaticus* MFI values by the corresponding calibrator MFI value to form a non-descriptive quantitative value. While *Lactobacillus* net MFI values reached the plateau phase with 1,000 to 10,000 copies per PCR, calibrator signals declined with increased *Lactobacillus* copy number input (Fig. 2). The *Lactobacillus* / calibrator ratios, however, was able to quantify over 6 logs when calibrator
 15 concentrations of 100,000 copies per PCR were used.

Example 3: Risk of cytological abnormality associated with multiple high viral load

20 DNA aliquots of 1,000 consecutive samples, collected during routine gynaecological health checks from women in Flanders (Belgium), and 100 smears each from patients with ASC-US, LSIL and HSIL were analysed by the BSGP5+/6+-PCR-MPG assay. Multiple infections were most prevalent in LSIL (75.9% of HPV-positive smears), followed by HSIL (65.5%), ASC-US (64.6) and NIL/M (36.8%) (Table 1). In a single ASC-US sample, 17 concurrent HPV genotypes
 25 could be detected. Restricting the analysis to high viral load infections, there was a reduction in the number of multiple infections, however, the order remained the same with LSIL (62.6%), followed by HSIL (51.9%), ASC-US (40.7) and NIL/M (19.3%). Multiple high viral load infections with up to 7 (HSIL), 6 (ASC-US) and 12 concurrent HPV types (LSIL) could be detected. In contrast, in NIL/M only triple high viral load infections were observed. Age adjusted
 30 OR showed a significant positive association of multiple infections with the presence of LSIL and HSIL compared to NIL/M, which was stronger for multiple high viral load infections than for multiple DNA positivity alone (Table 1). Patients with multiple high viral loads showed a 5- to 6-fold increased risk of having cervical lesions than patients with single high viral loads.

Table 1: Age adjusted Odd Ratios for disease categories associated with multiple infection compared to single infections or multiple high viral loads compared to single high viral loads.

Assay	Disease category	Multiple infection ¹	OR (95% CI)	
			Positivity	High load
BSGP5+/6+-PCR/MPG	HSIL vs NIL/M	single infection	1.0 (reference)	1.0 (reference)
		14	1.78 (1.08 - 2.95)	5.81 (1.90 - 17.8)
		20	2.63 (1.62 - 4.25)	5.28 (2.16 - 12.9)
		54	3.00 (1.89 - 4.76)	4.37 (2.35 - 8.13)
	LSIL vs NIL/M	single infection	1.0 (reference)	1.0 (reference)
		14	2.35 (1.41 - 3.93)	6.58 (2.14 - 20.2)
		20	2.60 (1.59 - 4.26)	5.82 (2.38 - 14.2)
		54	4.74 (2.87 - 7.83)	6.39 (3.43 - 11.9)
	HSIL vs LSIL	single infection	1.0 (reference)	1.0 (reference)
		14	0.77 (0.44 - 1.35)	0.88 (0.48 - 1.60)
		20	1.03 (0.59 - 1.79)	0.91 (0.52 - 1.62)
		54	0.66 (0.36 - 1.21)	0.70 (0.40 - 1.24)
	CIN3 vs NIL/M	single infection	1.0 (reference)	1.0 (reference)
		14	1.57 (0.78 - 3.16)	4.22 (1.23 - 14.5)
		20	2.16 (1.09 - 4.26)	4.15 (1.50 - 11.5)
		54	2.86 (1.45 - 5.65)	4.12 (1.88 - 9.02)
	CIN2+ vs NIL/M	single infection	1.0 (reference)	1.0 (reference)
		14	1.71 (0.94 - 3.11)	4.77 (1.49 - 15.2)
		20	2.58 (1.44 - 4.62)	5.16 (2.01 - 13.2)
		54	3.20 (1.79 - 5.71)	4.82 (2.38 - 9.76)
	CIN2 vs NIL/M	single infection	1.0 (reference)	1.0 (reference)
		14	2.17 (0.88 - 5.40)	7.03 (1.78 - 27.7)
		20	4.07 (1.57 - 10.6)	9.10 (2.78 - 29.8)
		54	4.17 (1.57 - 11.1)	6.58 (2.39 - 18.1)
	CIN1 vs NIL/M	single infection	1.0 (reference)	1.0 (reference)
		14	2.16 (0.92 - 5.09)	5.64 (1.37 - 23.2)
		20	2.56 (1.09 - 6.00)	9.38 (2.87 - 30.6)
		54	4.14 (1.67 - 10.3)	7.48 (2.83 - 19.8)
	CIN2+ vs CIN1	single infection	1.0 (reference)	1.0 (reference)
		14	0.79 (0.30 - 2.07)	1.05 (0.38 - 2.89)
		20	1.01 (0.37 - 2.73)	0.67 (0.26 - 1.75)
		54	0.71 (0.24 - 2.12)	0.61 (0.22 - 1.70)
	CIN3+ vs CIN1	single infection	1.0 (reference)	1.0 (reference)
		14	0.71 (0.25 - 1.98)	0.85 (0.28 - 2.56)
		20	0.82 (0.28 - 2.37)	0.50 (0.17 - 1.42)
		54	0.64 (0.20 - 2.04)	0.52 (0.17 - 1.57)

5 ¹ number of HPV types analysed: 14: multiple infections with the 14 HPV types included in GP5+/6+-PCR/EIA; 20: additional types 26, 53, 67, 70, 73 and 82 included according to IARC Monograph V. 100B.

Claims

1. A method for comparing the amounts of a first and a second polynucleotide comprised
5 in a sample, said method comprising the steps of:
 - a) amplifying said first and said second test polynucleotide in a test sample and a first
and a second control polynucleotide in a reference sample, wherein said first and
said second control polynucleotide is present in the reference sample in amounts
sufficient to allow for generation of maximal amounts of amplification products of
10 the said control polynucleotides;
 - b) determining the amounts of the first and the second test amplification product of
said first and said second polynucleotide and the first and the second control
amplification product of said first and said second control polynucleotide;
 - c) calculating (i) the ratio of the amounts of the first test amplification product and the
15 first control amplification product and (ii) the ratio of the second test amplification
product and the second control amplification product; and
 - d) comparing the ratios calculated in c) (i) and (ii), whereby the amount of the first test
polynucleotide and the amount second test polynucleotide comprised in the test
sample are compared.
- 20 2. The method of claim 1, wherein a ratio calculated in c) i) which is increased compared
to the ratio calculated in c) ii) is indicative for an amount of the first test
polynucleotide which is increased compared to the amount of the second test
polynucleotide, a ratio calculated in c) i) which is decreased compared to the ratio
25 calculated in c) ii) is indicative for an amount of the first test polynucleotide which is
decreased compared to the amount of the second test polynucleotide, and a ratio
calculated in c) i) which is identical to the ratio calculated in c) ii) is indicative for an
amount of the first test polynucleotide which is identical compared to the amount of
the second test polynucleotide.
- 30 3. The method of claim 1 or 2, wherein said first and said second control polynucleotide
is each present in an amount of at least 10^4 copies in the reference sample.
4. The method of claim 3, wherein said first test polynucleotide and said first control
35 polynucleotide is a polynucleotide of a first HPV and said second test polynucleotide
and said second control polynucleotide is a polynucleotide of a second HPV.

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5. The method of claim 1, wherein the amplification products are determined by immobilized oligonucleotide probes.
6. The method of claim 5, wherein said oligonucleotide probes are immobilized on a microbead.
7. The method of claim 6, wherein said microbead is a Luminex™ bead.
8. The method of any one of claims 1 to 7, wherein the polynucleotides are amplified by PCR or NASBA.
9. The method of any one of claims 1 to 8, wherein the ratios calculated in step c) i) and ii) are normalized.
10. The method of claim 9, wherein said normalization is made with respect to a third normalization polynucleotide suspected to be present in the test sample, preferably a house keeping gene, the total amount of nucleic acids present in the test sample or a calibrator polynucleotide.
11. A device for comparing the amounts of a first and a second test polynucleotide comprised in a sample according to the method of claim 1, said device comprising:
 - a) an analysis unit which comprises a detector for determining the amount of the first and the second test amplification products and the first and the second control amplification products; and
 - b) an evaluation unit comprising a data processor having implemented an algorithm for calculating (i) the ratio of the amounts of the first test amplification product and the first control amplification product and (ii) the ratio of the second test amplification product and the second control amplification product.
12. The device of claim 11, wherein said data processor further having implemented an algorithm for comparing the calculated ratios with each other.
13. The device of claim 11 or 12, further comprising an amplification unit for the amplification of the polynucleotides.

35

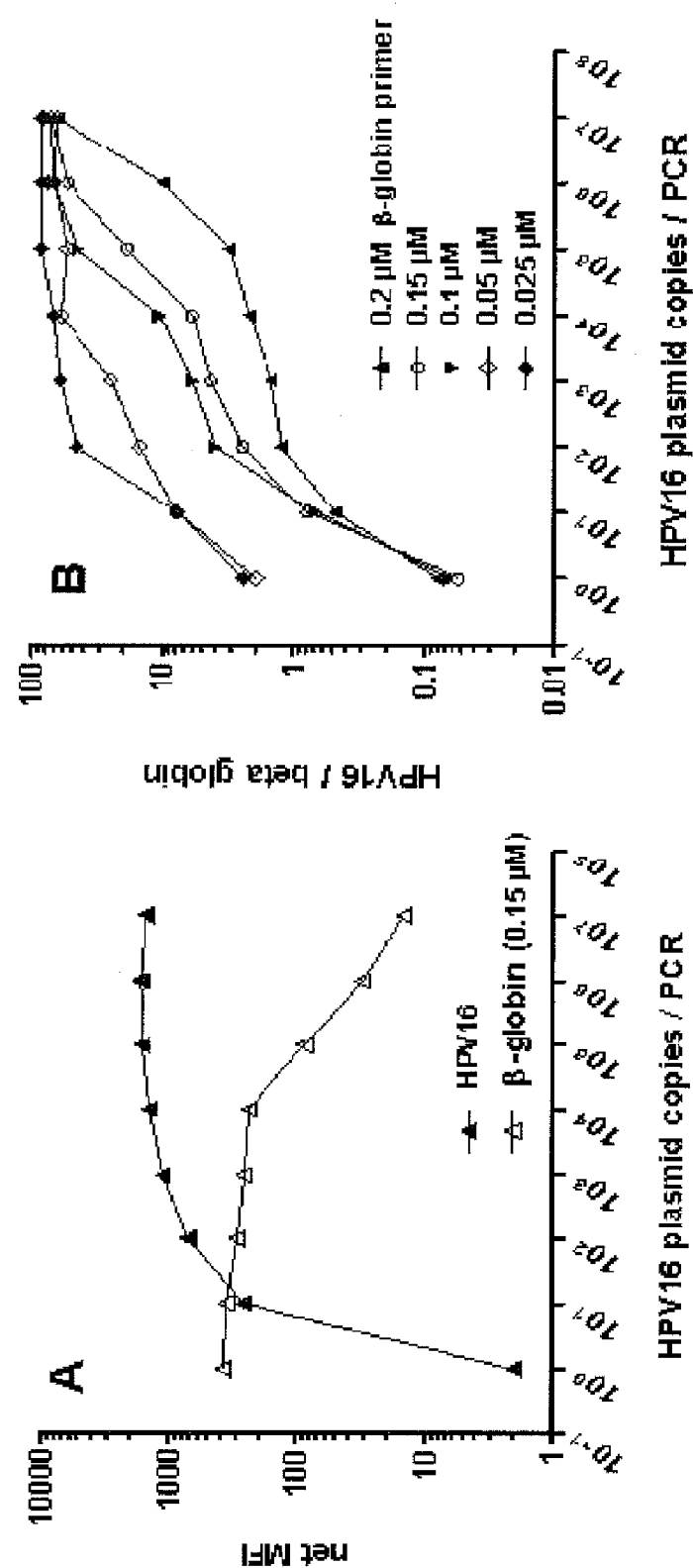


Fig. 1

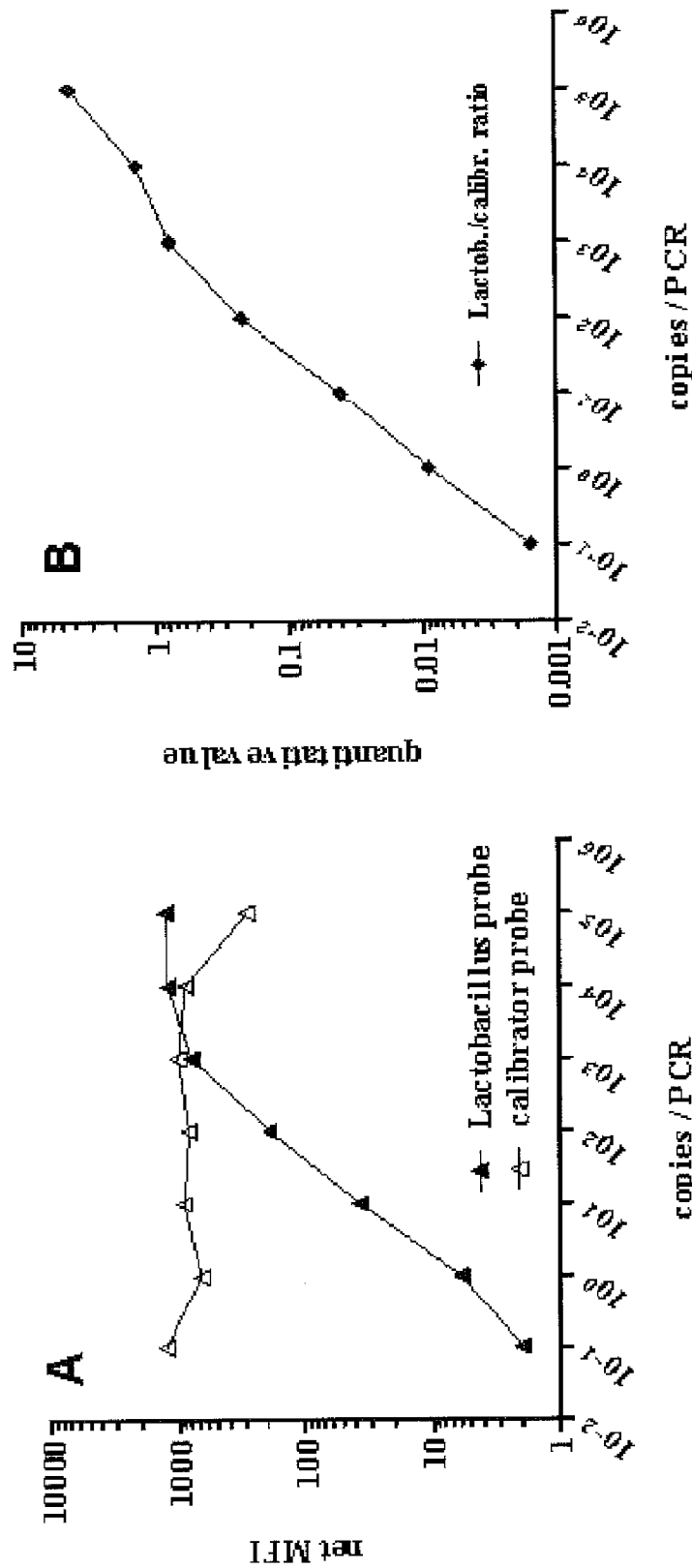


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/056178

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 C12Q1/70
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 practicable, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/035697 A1 (US GOV HEALTH & HUMAN SERV [US]; HUIZING MARJAN [US]; KLOOTWIJK ENRIKO) 29 March 2007 (2007-03-29)	1-3,8-13
Y	claims 1,11,38,39 example 3	4-7
X	----- US 2010/009372 A1 (TANABE TETSUYA [JP]) 14 January 2010 (2010-01-14)	1-3,8-13
Y	claims 1-4 paragraph [0071]	4-7
Y	----- EP 2 184 368 A1 (DKFZ KREBSFORSCHUNGSZENTRUM [DE]) 12 May 2010 (2010-05-12)	4-7
A	claims 1-4,8,13,15-16 paragraphs [0072], [0195], [0205] -----	1-3,8-13



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/056178

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007035697	A1	29-03-2007	NONE

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