Method and/or apparatus of oligonucleotide design and/or nucleic acid detection

Field of the invention

The present invention relates to the field of oligonucleotide design and/or nucleic acid detection. The method, apparatus and/or product according to the invention may be used for the detection of pathogens, for example for the detection of viruses.

Background of the invention

The accurate and rapid detection of viral and bacterial pathogens in human patients and populations is of critical medical and epidemiologic importance. Historically, diagnostic techniques have relied on cell culture passaging and various immunological assays or staining procedures. Accurate and sensitive detection of infectious disease agents is still difficult today, despite a long history of progress in this area. Traditional methods of culture and antibody-based detection still play a central role in microbiological laboratories despite the problems of the delay between disease presentation and diagnosis, and the limited number of organisms that can be detected by these approaches. Faster diagnosis of infections would reduce morbidity and mortality, for example, through the earlier implementation of appropriate antimicrobial treatment. During the past few decades, various methods have been proposed to achieve this; with those based on nucleic acid detection, including PCR and microarray-based techniques, seeming the most promising. In particular, PCR-based assays have been implemented, allowing for more rapid diagnosis of suspected pathogens with higher degree of sensitivity of detection. In clinical practice, however, the etiologic agent often remains unidentified, eluding detection in myriad ways. For example, some viruses are not amenable to culturing. At other times, a patient's sample may be of too poor quality or of insufficient titre for

pathogen detection by conventional techniques. Moreover, both PCR- and antibody-based approaches may fail to recognize suspected pathogens simply due to natural genetic diversification resulting in alterations of PCR primer binding sites and antigenic drift.

DNA and oligonucleotide microarrays with the potential to detect multiple pathogens in parallel have been described (Wang et al. 2002; Urisman et al. 2005). However, unresolved technical questions prevent their routine use in the clinical setting. For example, how does one select the most informative probes for comprising a pathogen "signature" in light of amplification and cross-hybridization artifacts? What levels of fluorescent signal and signature probe involvement constitute a detected pathogen? What is the accuracy and sensitivity of an optimized detection algorithm? (Striebel et al. 2003; Bodrossy and Sessitsch, 2004; Vora et al. 2004).

Accordingly, there is a need in this field of technology for alternative and improved methods of detection of nucleic acids. In particular, there is a need for alternative and/or improved diagnostic methods for the detection of pathogens.

Summary of the invention

The present invention addresses the problems above, and in particular provides a method, apparatus and/or product of oligonucleotide design. In particular, there is provided a method, apparatus and/or product of oligonucleotide probe and/or primer design. There is also provided a method, apparatus and/or product of nucleic acid detection.

According to a first aspect, the present invention provides a method of designing at least one oligonucleotide for nucleic acid detection comprising the following steps in any order:

(I) identifying and/or selecting at least one region of at least one target nucleic acid to be amplified, the region(s) having an efficiency of amplification (AE) higher than the average AE; and

(II) designing at least one oligonucleotide capable of hybridizing to the selected region(s).

The at least one oligonucleotide may be at least one probe and/or primer. In particular, in step (I) a score of AE is determined for every position i on the length of the target nucleic acid(s) or of at least one region thereof and subsequently, an average AE score is obtained. Those regions showing an AE score higher than the average may be selected as the region(s) of the target nucleic acid to be amplified. In particular, the AE of the selected region(s) may be calculated as the Amplification Efficiency Score (AES), which is the probability that a forward primer r_i can bind to a position i and a reverse primer r_j can bind at a position j of the target nucleic acid, and $|i-j|$ is the region of the target nucleic acid desired to be amplified. In particular, the region $|i-j|$ may be $\leq$ 10000 bp, more in particular $\leq$ 5000 bp, or $\leq$ 1000 bp, for example $\leq$ 500 bp. In particular, the forward and reverse primers may be random primers.

According to another aspect, the step (I) comprises determining the effect of geometrical amplification bias for every position of a target nucleic acid, and selecting at least one region(s) to be amplified as the region(s) having an efficiency of amplification (AE) higher than the average AE. For example, the geometrical amplification bias is the PCR bias.

The step (II) of designing at least one oligonucleotide capable of hybridizing to the region(s) selected in step (I) may be carried out according to any

oligonucleotide designing technique known in the art. In particular, the oligonucleotide(s) capable of hybridizing to the selected region(s) may be selected and designed according to at least one of the following criteria:

(a) the selected oligonucleotide(s) has a CG-content from 40% to 60%;

(b) the oligonucleotide(s) is selected by having the highest free energy computed based on Nearest-Neighbor model;

(c) given oligoncleotide s_a and oligonucleotide s_b substrings of target nucleic acids v_a and v_b, s_a is selected based on the hamming distance between s_a and any length-*m* substring s_b and/or on the longest common substring of s_a and oligonucleotide s_b;

(d) for any oligonucleotide s_a of length-*m* specific for the target nucleic acid v_a, the oligonucleotide s_a is selected if it does not have any hits with any region of a nucleic acid different from the target nucleic acid, and if the oligonucleotide s_a length-m has hits with the nucleic acid different from the target nucleic acid, the oligonucleotide s_a length-*m* with the smallest maximum alignment length and/or with the least number of hits is selected; and

(e) a oligonucleotide p_i at position i of a target nucleic acid is selected if p_i is predicted to hybridize to the position i of the amplified target nucleic acid.

In particular, the oligonucleotide may be a probe and/or primer.

Accordingly, two or more of the criteria indicated above may be used for designing the oligonucleotide(s). For example, the oligonucleotide(s) may be designed by applying all criteria (a) to (e). Other criteria not explicitly mentioned herein but which are within the knowledge of a skilled person in the art may also be used.

In particular, under the criterion (e), a oligonucleotide p_i at position i of a target nucleic acid v_a is selected if $P(p_i \mid v_a) > \lambda$, wherein λ is 0.5 and $P(p_i \mid v_a)$ is the probability that p_i hybridizes to the position i of the target nucleic acid v_a. More in particular, λ is 0.8.

In particular, $P(p_i \mid v_a) \approx P(X \leq x_i) = \frac{c_i}{k}$, wherein X is the random variable representing the amplification efficiency score (*AES*) values of all oligonucleotides of v_a, k is the number of oligonucleotides in v_a, and c_i is the number of oligonucleotides whose *AES* values are $\leq x_i$.

According to another aspect of the invention, the method of designing the oligonucleotide(s) as described above further comprises a step of preparing the selected and designed oligonucleotide(s). The oligonucleotide, which may be at least one probe and/or primer, may be prepared according to any standard method known in the art. For example, by chemical synthesis or photolithography.

According to another aspect, the present invention provides a method of detecting at least one target nucleic acid comprising the steps of:

(i) providing at least one biological sample;

(ii) amplifying nucleic acid(s) comprised in the biological sample;

(iii) providing at least one oligonucleotide capable of hybridising to at least one target nucleic acid, if present in the biological sample, wherein the oligonucleotide(s) is designed and/or prepared by using a method according to any aspect of the invention described herein; and

(iv) contacting the oligonucleotide(s) with the amplified nucleic acids and/or detecting the oligonucleotide(s) hybridised to the target nucleic acid(s).

In particular, the oligonucleotide is a probe.

The amplification step (ii) may be carried out in the presence of random primers. For example, the amplification step (ii) may be carried out in the presence of at least one random forward primer, at least one random reverse primer and/or more than two random primers. Any amplification method known in the art may be used. For example, the amplification method is a RT-PCR.

In particular, a forward random primer binding to position *i* and a reverse random primer binding to position j of a target nucleic acid v_a are selected among primers having an amplification efficiency score (AES_I) for every position *i* of a target nucleic acid v_a of :

$$AES_i = \sum_{j=i-Z}^{i} \left\{ P^f(j) \times \sum_{k=\max(i+1,\, j+500)}^{j+Z} P^r(k) \right\},$$

wherein $\sum_{k=\max(i+1,\, j+500)}^{j+Z} P^r(k)$ = $P^r(i+1) + P^r(i+2) + \ldots\ldots P^r(j+Z)$,

$P^f(i)$ and $P^r(i)$ are the probability that a random primer r_i can bind to position *i* of v_a as forward primer and reverse primer, respectively, and Z ≤ 10000 bp is the region of v_a desired to be amplified. More in particular, Z may be ≤ 5000 bp, ≤ 1000 bp, or ≤ 500 bp.

The amplification step may comprise forward and reverse primers, and each of the forward and reverse primers may comprise, in a 5'-3' orientation, a fixed primer header and a variable primer tail, and wherein at least the variable tail hybridizes to a portion of the target nucleic acid v_a. In particular, the amplification step may comprise forward and/or reverse random primers having the nucleotide sequence of SEQ ID NO:1 or a variant or derivative thereof.

The biological sample may be any sample taken from a mammal, for example from a human being. The biological sample may be tissue, sera, nasal pharyngeal washes, saliva, any other body fluid, blood, urine, stool, and the like. The biological sample may be treated to free the nucleic acid comprised in the biological sample before carrying out the amplification step. The target nucleic acid may be any nucleic acid which is intended to be detected. The target nucleic acid to be detected may be at least a nucleic acid exogenous to the nucleic acid of the biological sample. Accordingly, if the biological sample is from a human, the exogenous target nucleic acid to be detected (if present in the biological sample) is a nucleic acid which is not from human origin. According to an aspect of the invention, the target nucleic acid to be detected is at least a pathogen genome or fragment thereof. The pathogen nucleic acid may be at least a nucleic acid from a virus, a parasite, or bacterium, or a fragment thereof.

Accordingly, the invention provides a method of detection of at least a target nucleic acid, if present, in a biological sample. The method may be a diagnostic method for the detection of the presence of a pathogen in the biological sample. For example, if the biological sample is obtained from a human being, the target nucleic acid, if present in the biological sample, is not from human.

The oligonucleotide(s) designed and/or prepared according to any method of the present invention may be used in solution or may be placed on an insoluble support. For example, the oligonucleotide probe(s) may be applied, spotted or printed on an insoluble support according to any technique known in the art. The support may be a microarray, a biochip, a membrane/synthetic surface, solid support or a gel.

The probes are then contacted with the nucleic acid(s) of the biological sample, and, if present, the target nucleic acid(s) and the probe(s) hybridize, and the presence of the target nucleic acid is detected. In particular, in the detection

step (iv), the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

More in particular, in the detection step (iv), the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, and the method further comprises the step of computing the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of the probes used in the detection method having high signal intensities, the density distribution of the signal intensities of probes v_a being more positively skewed than that of probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

For example, in the detection step (iv), at least one target nucleic acid in a biological sample is detected if the density distribution of its probe signal intensities is not normal, i.e. more positively skewed, given by Anderson-Darling test value ≤ 0.05 and/or a value of t-test ≤ 0.1 and/or a value of Weighted Kullback-Leibler divergence of ≥ 1.0, preferably ≥ 5.0., In particular, the t-test value is ≤ 0.05.

More in particular, the method of the detection step (iv), further comprises evaluating the probe signal intensity of probe(s) in each pathogen specific signature probe set (SPS) for the target nucleic acid(s) v_a by calculating the distribution of Weighted Kullback-Leibler (WKL) divergence scores:

$$WKL(P_a \mid \overline{P_a}) = \sum_{j=0}^{k-1} \frac{Q_a(j)\log(\frac{Q_a(j)}{Q_{\bar{a}}(j)})}{\sqrt{Q_{\bar{a}}(j)\left[1-Q_{\bar{a}}(j)\right]}}$$

where $Q_a(j)$ is the cumulative distribution function of the signal intensities of the probes in P_a found in bin b_j ; $Q_{\bar{a}}(j)$ is the cumulative distribution function of the signal intensities of the probes in $\overline{P_a}$ found in bin b_j. $Q_{\bar{a}}(j)$ is the cumulative distribution function of the signal intensities of the probes in $\overline{P_a}$ found in bin b_j. P_a is the set of probes of a virus v_a and $\overline{P_a} = P - P_a$.

For example, each signature probe set (SPS) which represents the absence of target nucleic acid(s) v_a has a normally distributed signal intensity (assessed by Anderson-Darling test value ≤ 0.05) and/or a Weighted Kullback-Leibler (WKL) divergence score of WKL<5. Each signature probe set (SPS) which represents the presence of at least one target nucleic acid v_a has a positively skewed signal intensity distribution and/or a Weighted Kullback-Leibler (WKL) divergence score of WKL>5.

The method may further comprise performing Anderson-Darling test on the distribution of WKL score(s), wherein a result of P>0.05 thereby indicates the absence of target nucleic acid(s) v_a, or wherein a result of P<0.05 thereby indicates the presence of target nucleic acid(s) v_a. Additionally, a further Anderson-Darling test may be performed thereby indicating the presence of further co-infecting target nucleic acid(s). According to another aspect, the present invention provides a method of determining the presence of a target nucleic acid v_a comprising detecting the hybridization of at least one oligonucleotide probe (the probe being selected and designed according to any known method in the art and not necessary limited to the methods according to the present invention) to at least one target nucleic acid v_a and wherein the mean of the signal intensities of the probe(s) which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, thereby indicating the presence of v_a. In particular, the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, and the method

further comprises the step of computing the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of the probes used in the detection method having high signal intensities, the density distribution of the signal intensities of probes v_a being more positively skewed than that of probes $\notin v_a$, thereby indicating the presence of v_a. More in particular, the presence of a target nucleic acid in a biological sample is given by a value of t-test $\leq$ 0.1 and/or Anderson-Darling test value $\leq$ 0.05 and/or a value of Weighted Kullback-Leibler divergence of $\geq$ 1.0, preferably $\geq$ 5.0. For example, the t-test value may be $\leq$ 0.05.

According to another aspect, the present invention provides a method of detecting at least one target nucleic acid, comprising the steps of:

(i) providing at least one biological sample;

(ii) amplifying at least one nucleic acid(s) comprised in the biological sample;

(iii) providing at least one oligonucleotide capable of hybridizing to at least one target nucleic acid, if present in the biological sample; and

(iv) contacting the oligonucleotide(s) with the amplified nucleic acids and detecting the oligonucleotide(s) hybridized to the target nucleic acid(s), wherein the mean of the signal intensities of the oligonucleotide(s) which hybridize to v_a is statistically higher than the mean of the oligonucleotide(s) $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

In particular, the oligonucleotide is an oligonucleotide probe.

In step (iv), the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, and the method further comprises the step of computing the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of the probes used in the detection method having high signal intensities, the density distribution of

the signal intensities of probes v_a being more positively skewed than that of probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample. In particular, in step (iv) the presence of at least one target nucleic acid in a biological sample is given by a value of t-test ≤ 0.1 and/or Anderson-Darling test value ≤ 0.05 and/or a value of Weighted Kullback-Leibler divergence of ≥ 1.0, preferably ≥ 5.0. The t-test value may be ≤ 0.05. The nucleic acid to be detected is nucleic acid exogenous to the nucleic acid of the biological sample. The target nucleic acid to be detected may be at least one pathogen genome or fragment thereof. The pathogen nucleic acid may be at least one nucleic acid from a virus, a parasite, or bacterium, or a fragment thereof. In particular, when the sample is obtained from a human being, the target nucleic acid, if present in the biological sample, is not from the human genome. The probes may be placed on an insoluble support. The support may be a microarray, a biochip, or a membrane/synthetic surface.

The present invention provides an apparatus of the invention, comprising an apparatus for performing the methods according to the invention. In particular, the apparatus may be for designing oligonucleotide(s) for nucleic acid detection and/or amplification, the apparatus being configured to identify and/or select at least one region(s) of at least one target nucleic acid to be amplified, the region(s) having an efficiency of amplification (AE) higher than the average AE; and design at least one oligonucleotide(s) capable of hybridizing to the identified and/or selected region(s). More in particular, the apparatus may be configured to detect at least one target nucleic acid comprising any one of the steps of: providing at least one biological sample; amplifying nucleic acid(s) comprised in the biological sample; providing at least one oligonucleotide capable of hybridizing to at least one target nucleic acid, if present in the biological sample, wherein the oligonucleotide(s) is designed and/or prepared according to the apparatus being configured according to the invention; and

contacting the oligonucleotide(s) with the amplified nucleic acids and/or detecting the oligonucleotide(s) hybridized to the target nucleic acid(s).

The present invention also provides at least one computer program product configured for performing the method according to the invention. There is also provided at least one electronic storage medium storing the configuration of the apparatus according to the invention. According to one aspect, the invention provides a removable electronic storage medium comprising a software configured to perform the method(s) according to the invention. In particular, the removable electronic storage medium may comprise a software configured to determine the WKL divergence score and/or Anderson-Darling test for designing at least one oligonucleotide probe and/or primer, and/or detecting at least one target nucleic acid. More in particular, the removable electronic storage comprising a software configuration may comprise the WKL, Anderson-Darling test, the designing of probe(s) and/or the detecting of target nucleic acid(s) as defined according to the invention. Accordingly, there is also provided a software configured as described above.

Brief description of the figures

Figure 1 shows a RT-PCR binding process of a pair of random primers on a virus sequence (SEQ ID NOS: 1to 9). The labels for Figure 1 are as follows:

A: Reverse transcription (RT). Primer binds to template.

B: Tagged RT products are generated (in detail with hypothetical viral sequence template, and hypothetical specific random primer).

C: Second strand synthesis is completed incorporating tags.

D: Amplification of tagged RT product using PCR Primer GTTTCCCAGTCACGATA (SEQ ID NO:8).

Figure 2 shows an Amplification Efficiency Scoring (AES) Map for the RSV B genome.

Figure 3 shows oligonucleotide probe signal intensities for 1 experiment for RSV B.

Figures 4(A, B). Figure 4A shows the density distribution of signal intensities of a virus that is the sample tested. An arrow indicates the positive skewness of the distribution. This indicates that although there is noise, there is significant amount of real signals as well. Figure 4B shows the density distribution of signal intensities of a virus not in the sample. It is noise dominant.

Figure 5 shows an analysis framework of pathogen detection chip data.

Figure 6. Oligonucleotide probe design schema. This illustrates the tiling probes created across the genome of NC_001781 Human respiratory syncytial virus (RSV). The numbers represent the start and end positions of each probe. 1948 probes were synthesized to cover the entire 15225 bp RSV genome. This process was repeated for the remaining 34 viral genomes.

Figure 7(A,B,C) Key to labels of microarray bars:

Virus family	Virus genus/species
Orthomyxoviridae	Sars Sin2500 OC41 229E
Coronaviridae	Flu A Flu B
Picornaviridae	Entero D Entero C Echo 1 Entero B

	Entero A Rhino 89 Rhino B Hep A Foot & mouth C
Bunyaviridae	Hantaan Sin Nombre
Flaviviridae	West Nile Jap enceph Dengue 3 Dengue 1 Dengue 2 Dengue 4 Yellow fever
Paramyxoviridae	Paraflu 1 Paraflu 3 Nipah Paraflu 2 Newcastle RSV (B1) Metapneumovirus
Others	HPV type 10 HIV 1 Hep B Rubella LCMV-S LCMV-L PMMV Human controls

RNA isolated from SARS Sin850-infected cell line **(A)** or Dengue I-infected cell line **(B)** was hybridized onto the pathogen microarray following SARS-specific or Dengue I-specific RT-PCR respectively. SARS cross-hybridized (shown in black colour) to other coronaviridae genomes, particularly to the highly conserved middle portion of the genome (Ruan et al. 2003). Dengue I cross-hybridized to probes derived from flaviviridae and other genomes based on their sequence similarity. By examining the Hamming Distance (HD) and Maximum Contiguous Match (MCM) scores, we established thresholds to predict whether

cross-hybridization would occur and utilized this information to generate in silico hybridization signatures. **(C)** RNA isolated from a clinical patient diagnosed with RSV was amplified using random RT-PCR and hybridized onto the pathogen microarray.

Figure 8 Relationship between probe Hamming Distance (HD), probe Maximum Contiguous Match (MCM) and probe Signal Intensity. Average probe signal intensity decreases as HD increases and MCM decreases. This correlates with a reduction in the percentage of detectable probes (signal intensity > mean + 2 SD). At the optimal cross-hybridization thresholds HD≤4 or MCM≥18 (shaded), >98% of probes can be detected. At HD=5 or MCM=17, the detection rate falls to 85%.

Figure 9(A, B) RNA isolated from a RSV-infected patient was hybridized onto a pathogen detection array. **(A)** Distribution of probe signal intensities all 53,555 probes show a normal distribution (grey solid line). Non-RSV probes, when examined on a genome-specific level, e.g. parainfluenza-1 (grey dotted line), also show a normal distribution. Signal intensity of RSV-specific probes have a positive skew, with higher signal intensities in the tail of the distribution (black solid line). **(B)** Distribution frequency of WKL scores for the 35 SPS with majority ranging between -5 and 3. However the WKL score for the RSV genome is 17, so the distribution is not normal ($P<0.05$ by Anderson Darling test). Excluding the outlier genome results in a normal distribution. From this computation, we conclude that RSV is present in the hybridized sample.

Figure 10 AES is indicative of probe amplification efficiency. Higher proportion of probes with high AES are detectable above signal intensity thresholds over 5 experiments.

Figure 11 Schema showing the processes necessary for pathogen detection using microarray.

Figure 12 Hybridization signal intensity correlates to Amplification Efficiency Score (AES), $P=2.2 \times 10^{-16}$. A RSV patient sample was hybridized onto a microarray, and signal intensities of each probe were plotted together with the computed AES. The signal threshold for high-confidence detection on a typical array is indicated by the green line.

Figure 13 Using AES-optimized primer tags for random RT-PCR increases the AES by 10-30-fold. The optimized primers were predicted to have the same performance across all 35 genomes represented on the microarray. Most patient samples were amplified using the AES-optimized primer A2.

SEQ ID NO:	Primer	Nucleotide sequence
10	A1	GTTTCCCAGTCACGATA
11	A2	GATGAGGGAAGATGGGG
12	A3	CTCATGCACGACCCAAA
13	A4	AGATCCATTCCACCCCA

Figure 14(A,B) Key to labels of microarray bars:

Virus family	Virus genus/species
Orthomyxoviridae	Sars Sin2500 OC41 229E
Coronaviridae	Flu A Flu B
Picornaviridae	Entero D Entero C Echo 1

	Entero B Entero A Rhino 89 Rhino B Hep A Foot & mouth C
Bunyaviridae	Hantaan Sin Nombre
Flaviviridae	West Nile Jap enceph Dengue 3 Dengue 1 Dengue 2 Dengue 4 Yellow fever
Paramyxoviridae	Paraflu 1 Paraflu 3 Nipah Paraflu 2 Newcastle RSV (B1) Metapneumovirus
Others	HPV type 10 HIV 1 Hep B Rubella LCMV-S LCMV-L PMMV Human controls

Choice of primer tag in random RT-PCR has significant effect on PCR efficiency. Heatmap showing probes hybridizing to a clinical hMPV sample following RT-PCR using **(A)** original primer described by Bohlander, et. al. 1992, or **(B)** primer designed following PCR modeling to ensure that it will efficiently amplify all genomes (high AES) represented on the microarray.

Figure 15 Diagnostic PCR results for RSV Patient #412 confirm that patient does not have a coronavirus infection. **(A)** PCR using Pancoronavirus primers. Lane 1: OC43 coronavirus positive control, Lane 2: 229E coronavirus positive control, Lane 3: RSV patient #412, Lane 4: PCR primers and reagents only negative control. 1 kb ladder. **(B)** PCR using OC43 specific primers. Lane 1: OC43 coronavirus positive control, Lane 2: RSV patient #412, Lane 3: purified RSV from ATCC, Lane 4: PCR negative control. 50bp ladder. **(C)** PCR using 229E specific primers. Lane 1: 229E coronavirus positive control, Lane 2: RSV patient #412, Lane 3: PCR negative control. 1 kb ladder.

Detailed description of the invention

Bibliographic references mentioned in the present specification are, for convenience, listed in the form of a list of references and added to the end of the examples. The whole content of such bibliographic references is herein incorporated by reference.

The present invention addresses the problems of the prior art, and in particular provides at least one method, apparatus and/or product of oligonucleotide design. In particular, thre is provided a method, apparatus and/or product of probe and/or primer design. There is also provided a method, apparatus and/or product of nucleic acid(s) detection.

While the concept of using oligonucleotide hybridization microarrays as a tool for determining the presence of pathogens has been proposed, significant hurdles remain, thus preventing the use of these microarrays routinely (Striebel, H.M., 2003). These hurdles include probe design and data analysis (Striebel, H.M., 2003; Bodrossy, L. & Sessitsch, A., 2004; Vora, G.J., et al., 2004). The present inventors observed in a pilot microarray that despite meticulous probe selection, the best *in silico* designed probes do not necessarily hybridize well to

patient samples. The inventors realized that to generate probes which would hybridize consistently well to patient material, it was necessary to develop a new and/or improved method of probe design so as to determine the optimal design predictors. In particular, as described in the Example section, the present inventors created a microarray comprising overlapping 40-mer probes, tiled across 35 viral genomes. However, the invention is not limited to this particular application, probe length and type of target nucleic acid.

According to a particular aspect of the invention, the present inventors describe how a support, in particular a microarray platform, is optimized so as to become a viable tool in target nucleic acid detection, in particular, in pathogen detection. The inventors also identified probe design predictors, including melting temperature, GC-content of the probe, secondary structure, hamming distance, similarity to human genome, effect of PCR primer tag in random PCR amplification efficiency, and/or the effect of sequence polymorphism. These results were considered and/or incorporated into the development of a method and criteria for probe and/or primer design. According to a more particular aspect, the inventors developed a data analysis algorithm which may accurately predict the presence of a target nucleic acid, which may or may not be a pathogen. For example the pathogen may be, but not limited to, a virus, bacteria and/or parasite(s). The algorithm may be used even if probes are not ideally designed. This detection algorithm, coupled with a probe design methodology, significantly improves the confidence level of the prediction (see Tables 6 and 7).

According to a particular aspect, the method of the invention may not require a prediction of the likely pathogen, but may be capable of detecting most known human viruses, bacteria and/or parasite(s), as well as some novel species, in an unbiased manner. Genome or a fragment thereof is defined as all the genetic material in the chromosomes of an organism. DNA derived from the

genetic material in the chromosomes of a particular organism is genomic DNA. A genomic library is a collection of clones made from a set of randomly generated overlapping DNA fragments representing the entire genome of an organism. The rationale behind this detection platform according to the invention is that each species of virus, bacteria and/or parasite(s) contains unique molecular signatures within the primary sequence of their genomes. Identification of these distinguishing regions allows for rational oligonucleotide probe design for the specific detection of individual species, and in some cases, individual strains. The concomitant design and/or preparation of oligonucleotide (oligo) probes that represent the most highly conserved regions among family and genus members, will enable the detection and partial characterization of some novel pathogens. Furthermore, the inclusion of all such probes in a single support may allow the detection of multiple viruses, bacteria and/or parasite(s) that simultaneously co-infect a clinical sample. The support may be an insoluble support, in particular a solid support. For example, a microarray or a biochip assay.

According to a particular aspect, the invention may be used as a diagnostic tool, depending on the way in which oligonucleotide probes are designed, and/or how the data generated by the microarray is interpreted and analyzed.

Determination of efficiency of amplification

According to a first aspect, the present invention provides a method of designing oligonucleotide probe(s) for nucleic acid detection comprising the following steps in any order:

(i) identifying and/or selecting at least one region of at least one target nucleic acid to be amplified, the region(s) having an efficiency of amplification (AE) higher than the average AE; and

(ii) designing at least one oligonucleotide probe capable of hybridizing to the identified and/or selected region(s).

In particular, in step (i) a score of AE is determined for every position i on the length of the target nucleic acid or of a region thereof and an average AE is obtained. Those regions showing an AE higher than the average are selected as the region(s) of the target nucleic acid to be amplified. In particular, the AE of the selected region(s) may be calculated as the Amplification Efficiency Score (AES), which is the probability that a forward primer r_i can bind to a position i and a reverse primer r_j can bind at a position j of the target nucleic acid, and $|i - j|$ is the region of the target nucleic acid desired to be amplified. In particular, the region $|i - j|$ may be $\leq$ 10000 bp, more in particular $\leq$ 5000 bp, or $\leq$ 1000 bp, for example $\leq$ 500 bp. In particular, the forward and/or reverse primers may be random primers.

According to another aspect, the step (i) of identifying and/or selecting region(s) of a target nucleic acid to be amplified comprises determining the effect of geometrical amplification bias for every position of a target nucleic acid, and selecting the region(s) to be amplified as the region(s) having an efficiency of amplification (AE) higher than the average AE. The geometrical amplification bias may be defined as the capability of some regions of a nucleic acid to be amplified more efficiently than other regions. For example, the geometrical amplification bias is the PCR bias.

Modeling of amplification efficiency

Since it is not known what target nucleic acid (for example a pathogen) exists within the patient sample, random primers may be used during the amplification step and/or the reverse-transcription (RT) process to ensure unbiased reverse-transcription of all RNA present into DNA. Any random amplification method known in the art may be used for the purposes of the present invention. In the present description, the random amplification method may be RT-PCR.

However, it will be clear to a skilled person that the method of the present invention is not limited to RT-PCR. In particular, the RT-PCR approach may be susceptible to signal inaccuracies caused by primer-dimer bindings and poor amplification efficiencies in the RT-PCR process (Bustin, S.A., et al, 2004). To overcome this hurdle, the inventors have modeled the RT-PCR process by using random primers.

According to a particular aspect of the invention, the amplification step comprises forward and reverse primers, and each of the forward and reverse primers comprises, in a 5'-3' orientation, a fixed primer header and a variable primer tail, and wherein at least the variable tail hybridizes to a portion of the target nucleic acid v_a. The size of the fixed primer header and that of the variable primer tail may be of any size, in mer, suitable for the purposes of the method according to the present invention. The fixed header may be 10-30 mer, in particular, 15-25 mer, for example 17 mer. The variable tail may be 1-20 mer, in particular, 5-15 mer, for example 9 mer. An example of these forward and reverse primers is shown in Figure 1. More in particular, the amplification step may comprise forward and/or reverse random primers having the nucleotide sequence 5'-GTTTCCCAGTCACGATANNNNNNNNN-3', (SEQ ID NO:1), wherein N is any one of A, T, C, and G or a derivative thereof.

According to a particular embodiment, also exemplified in Figure 1, the present inventors have modeled the random RT-PCR process as follows. Let v_a be the actual virus in the sample. The random primer used in the RT-PCR process was preferably a 26-mer primer having a fixed 17-mer header and a variable 9-mer tail of the form (5'-GTTTCCCAGTCACGATANNNNNNNN-3')(SEQ ID NO:1 and, in particular, SEQ ID NOS:2-7). However, it is clear to a skilled person the that primer according to the invention is not limited to the sequence(s) of SEQ ID NOS:1-7 and Figure 1. In fact, nucleotide size of the primer, and in particular of the header and variable tail may be varied and chosen within the ranges

discussed above. To obtain a RT-PCR product in a region between positions *i* and *j* of v_a, the inventors required (1) a forward primer binding to position *i*, (2) $|i - j| \leq 10000$, and (3) a reverse primer binding to position *j*. In particular, $|i - j|$, which is the region of the target nucleic acid desired to be amplified, may be $\leq$ 5000 bp, more in particular $\leq$ 1000, for example $\leq$ 500 bp. The quality of the RT-PCR product depends on how well the forward primer and/or the reverse primer bind to v_a. Some random primers can bind to v_a better than others. The identification of such primers and where they bind to v_a gives an indication of how likely a particular region of v_a will be amplified. Using this approach, there is provided an amplification efficiency model which computes an Amplification Efficiency Score (*AES*) for every position of v_a.

For a particular position *i* of a target nucleic acid v_a, $P^f(i)$ and $P^r(i)$ are the probabilities that a random primer r_i can bind to position *i* of v_a as forward primer and reverse primer respectively. For simplicity, it is assumed that a random primer can only bind to v_a if the last 9 nucleotides of the random primer is a substring of the reverse complement of v_a (forward primer) or a substring of v_a (reverse primer). This is shown in Figure 1. Based on well-established primer design criteria (Wu, D.Y., et al., 1991), the $P^f(i)$ was estimated to be low if r_i forms a significant primer-dimer or has extreme melting temperature. On the other hand, if r_i does not form any significant primer-dimer and has optimal melting temperature, then $P^f(i)$ will be high. Note that if the header of the random primer is similar to v_a, it may also aid in the binding and thus result in a higher $P^f(i)$. Similarly, the $P^r(i)$ was computed.

The binding of the random primer r_i at position *i* of v_a as a forward primer affects the quality of the RT-PCR product for at least 10000 nucleotides upstream of position *i*. Similarly, the binding of the random primer r_i at position *i* of v_a as a reverse primer affects the quality of the RT-PCR product for at least 10000

nucleotides downstream of position *i*. Thus, an amplification efficiency score, AES_i, for every position *i* of v_a can be computed by considering the combined effect of all forward and reverse primer-pairs that amplifies it:

$$AES_i = \sum_{j=i-Z}^{i} \left\{ P^f(j) \times \sum_{k=\max(i+1, j+500)}^{j+Z} P^r(k) \right\}$$

wherein $\sum_{k=\max(i+1, j+500)}^{j+Z} P^r(k)$ = $P^r(i+1) + P^r(i+2) + \ldots\ldots P^r(j+Z)$

$P^f(i)$ and $P^r(i)$ is the probability that a random primer r_i can bind to position *i* of v_a as forward primer and reverse primer, respectively, and Z ≤ 10000 bp is the region of v_a desired to be amplified.

Accordingly, Z may be ≤ 10000 bp, ≤ 5000 bp, ≤ 1000 bp or ≤ 500 bp.

To verify if the variation in signal intensities displayed by different regions of a virus has direct correlation with their corresponding amplification efficiency scores, several microarray experiments (in the particular case, a total of five microarray experiments) were performed on a common pathogen affecting human, the human respiratory syncytial virus B (RSV B).

Modeling of RT-PCR for amplification efficiency

According to the method of the invention, which is an improvement of the method of (Sung et al. 2003, CSB) the primer used for the reverse transcription comprises a fixed oligonucleotide tag (header) and a random oligonucleotide tail. In theory, the random oligonucleotide tail should bind indiscriminately to all

nucleic acids in the patient sample, initiating first strand synthesis. After the second strand synthesis, all reversed-transcribed sequences will have the fixed oligonucleotide tag (header) at both ends. These sequences are amplified by PCR, using the fixed oligonucleotide tag (header) as the primer to generate PCR products of at least 10000 bp in length. In particular, the majority of the amplified PCR products are between 500-1000 bp in length. According to the particular embodiment, the 26-mer primer used for reverse transcription (RT) comprises a fixed 17-mer tag with a 9-mer random tail: 5'-GTTTCCCAGTCACGATANNNNNNNNN-3' (SEQ ID NO:1).

In our model, v_a represents the pathogen in the clinical sample. To generate at least one PCR product, for example of 500-1000 bp, in any region of the genome, defined by positions i and j of v_a requires a forward primer binding to position I and a reverse primer binding to position j in the anti-sense direction such that $500 = |i - j| = 10000$, and in particular, such that $500 = |i - j| = 1000$. The binding affinity of a primer is determined by at least two factors: (1) primer dimer formation, and (2) hybridization affinity of the primer to the virus v_a. Genomic regions which can be successfully amplified by virtue of having ideal primer binding locations within 10000 nucleotides, in particular within 1000 or 500 nucleotide, can be predicted for by calculating an Amplification Efficiency Score (*AES*) for every position of v_a: Figure 1.

Amplification Efficiency Score (AES)

For each position i of v_a, let $P^f(i)$ and $P^r(i)$ be the probability that a random primer r_i can bind to position i of v_a as forward primer and reverse primer respectively. For simplicity, we assumed that a random primer can only bind to v_a if the nucleotide of the random tail of the primer (for example, the last 9 nucleotides of the random primer as shown in Figure 1) is a substring of the

reverse complement of v_a (forward primer) or a substring of v_a (reverse primer; Figure 1). Based on well-established primer design criteria (Wu and Ugozzoli, 1991), we estimated $P^f(i)$ to be low if r_l formed a significant primer-dimer or had extreme melting temperature. On the other hand, if r_l did not form any significant primer-dimer and had optimal melting temperature, then $P^f(i)$ will be high. If the fixed oligonucelotide tag (header) of the random primer (for example, the fixed 17-mer tag shown in Figure 1) is similar to v_a, it may also aid in the binding and thus result in a higher $P^f(i)$. Similarly, we computed $P^r(i)$.

The binding of the random primer r_l at position i of v_a as a forward primer affects the quality of the RT-PCR product for the nucleotides upstream of position i (for example, for the 500 to 1000 nucleotides upstream of position i). Similarly, the binding of the random primer r_l at position i of v_a as a reverse primer affects the quality and coverage of the RT-PCR product for the nucleotides downstream of position i (for example, for the 500 to 1000 nucleotides downstream of position i). Consider a position x of v_a. All effective primer pairs that reside at positions i and j respectively contribute to the quality of the RT-PCR product at x. Note that $i = x = j$ and $i - j = 10000$. For example, $500 = i - j = 1000$ since our RT-PCR product when 500 to 1000 basepairs long. Thus, an Amplification Efficiency Score, AES_x, for every position x of v_a can be computed by considering the combined effect of all primer pairs that amplifies it:

$$AES_x = \sum_{j=x-1000}^{x} \left\{ P^f(j) \times \sum_{k=\max(x+1,\, j+500)}^{j+1000} P^r(k) \right\}$$

AES threshold predictive of successful RT-PCR

The threshold for amplification efficiency scores for probe selection for a virus v_a is determined by the cumulative distribution function of the *AES* values v_a. Let X be the random variable representing the *AES* values of all probes of v_a. Let k be the number of probes in v_a. Then, we denote the probability that the *AES* value is less than or equal to x be $P(X \leq x) = c/k$ where c is the number of probes which have *AES* values less than or equal to x. For a probe p_i at position i of v_a, let x_i be its corresponding *AES* value. Since the signal intensity of a probe is highly correlated to its *AES* value, we estimated $P(p_i \mid v_a)$, the probability that p_i has high signal intensity in the presence of v_a, to be $P(X \leq x_i)$. Thus,

$$P(p_i \mid v_a) \approx P(X \leq x_i)$$

$$= \frac{c_i}{k}$$

where c_i is the number of probes whose *AES* values are less than or equal to x_i.

For probe selection, probe p_i is selected if $P(p_i \mid v_a) > \lambda$. In our experiments, we set $\lambda = 0.8$. At this threshold (top 20% AES), we observed that more than 50% of expected probes would hybridize reproducibly to different clinical samples. While using probes with higher AES (eg. top 10% AES) would improve reproducibility, this would reduce the number of unique probes remaining for some genomes to <10 at the species level, consequently eroding the ability of the array to specifically identify pathogen. Thus top 20% AES was used.

Empirical Determination of Cross-Hybridization Thresholds on a Pathogen Detection Microarray:

Probe Design

The step (ii) of designing oligonucleotide probe(s) capable of hybridizing to the selected region(s) may be selected to any one of the probe designing techniques known in the art. The following description relates to probe design, however, it will be clear to a skilled person to apply the same principle also for designing primer(s), in particular, for designing primer(s) for RT-PCR.

For example, given a set of target nucleic acids (for example, viral genomes) $V = \{v_1, v_2, \ldots, v_n\}$, for every v_i e V, a set of length-m probes (that is a substring of v_i) which satisfies the following conditions may be designed taking into consideration, for example, at least one of the following:

(a) established probe design criteria of homogeneity, sensitivity and specificity (Sung, W.K. et al, 2003, CSB);

(b) no significant sequence similarity to human genome; and

(c) efficiently amplified using AE score,for example by RT-PCR, as herein described.

Noisy signals caused by cross-hybridization artifacts present a major obstacle to the interpretation of microarray data, particularly for the identification of rare pathogen sequences present in a complex mixture of nucleic acids. For example, in clinical specimens, contaminating nucleic acid sequences such as those derived from the host tissue, will cross-hybridize with pathogen-specific microarray probes above some threshold of sequence complementarity. This can result in false-positive signals leading to erroneous conclusions. Similarly, the pathogen sequence, in addition to binding its specific probes, may cross-hybridize with other non-target probes (i.e., designed to detect other pathogens). This latter phenomenon, though seemingly problematic, could

provide useful information for pathogen identification to the extent that such cross-hybridization may be accurately predicted. With various metrics to assess annealing potential and sequence specificity, microarray probes have traditionally been designed to ensure maximal specific hybridization (to a known target) with minimal cross-hybridization (to non-specific sequences). However, in practice we have found that many probes, though designed using optimal in silico parameters, do not perform according to expectations for reasons that are unclear.

To systematically investigate the dynamics of array-based pathogen detection, we created an oligonucleotide array using Nimblegen array synthesis technology (Nuwaysir et al. 2002). The array was designed to detect up to 35 RNA viruses using 40-mer probes tiled at an average 8-base resolution across the full length of each genome (53,555 probes; Figure 6, Table 1).

Table 1. List of Genomes represented on the pathogen detection microarray. (column 1) Number of probes for each genome synthesized on the microarray. (column 2) Number of probes for each genome remaining following application of probe design filters. (column 3) Number of probes for each genome which are unique to the genome and do not cross-hybridize with human.

Ge-nome	Original No. of Probes (1)	Filtered No. of Probes (2)	Unique Probes (3)	NCBI GI number	Ref type	Accession no.	Description
1	1948	537	271	9629198	RefSeq	NC_001781.1	Human respiratory syncytial virus, complete genome
2	1995	550	295	19718363	RefSeq	NC_003461.1	Human parainfluenza virus 1 strain Washington/1964, complete genome
3	2002	762	474	19525721	RefSeq	NC_003443.1	Human parainfluenza virus 2,

							complete genome
4	1979	701	345	10937870	RefSeq	NC_001796.2	Human parainfluenza virus 3, complete genome
5	3805	588	444	30468042	Genbank	AY283794.1	SARS coronavirus Sin2500, complete genome
6	3937	604	356	38018022	RefSeq	NC_005147.1	Human coronavirus OC43, complete genome
7	3495	182	112	12175745	RefSeq	NC_002645.1	Human coronavirus 229E, complete genome
8	1705	292	177	46852132	RefSeq	NC_004148.2	Human metapneumovirus, complete genome
9	296	118	101	8486138	RefSeq	NC_002023.1	Influenza A virus RNA segment 1, complete sequence
10	282	69	42	8486136	RefSeq	NC_002022.1	Influenza A virus RNA segment 3, complete sequence
10	296	81	54	8486134	RefSeq	NC_002021.1	Influenza A virus RNA segment 2, complete sequence
10	110	69	57	8486131	RefSeq	NC_002020.1	Influenza A virus RNA segment 8, complete sequence
10	196	71	62	8486129	RefSeq	NC_002019.1	Influenza A virus RNA segment 5, complete sequence
10	177	75	59	8486127	RefSeq	NC_002018.1	Influenza A virus RNA segment 6, complete sequence
10	225	70	51	8486125	RefSeq	NC_002017.1	Influenza A virus RNA segment 4, complete sequence
10	300	105	48	8486164	RefSeq	NC_002204.1	Influenza B virus RNA-1, complete sequence

10	293	113	74	8486148	RefSeq	NC_002205.1	Influenza B virus RNA-2, complete sequence
10	279	94	59	8486150	RefSeq	NC_002206.1	Influenza B virus RNA-3, complete sequence
10	237	70	53	8486152	RefSeq	NC_002207.1	Influenza B virus RNA-4, complete sequence
10	232	90	82	8486154	RefSeq	NC_002208.1	Influenza B virus RNA-5, complete sequence
10	195	64	32	8486156	RefSeq	NC_002209.1	Influenza B virus RNA-6, complete sequence
10	150	47	37	8486159	RefSeq	NC_002210.1	Influenza B virus RNA-7, complete sequence
10	136	59	50	8486161	RefSeq	NC_002211.1	Influenza B virus RNA-8, complete sequence
11	1401	85	54	11528013	RefSeq	NC_001563.2	West Nile virus, complete genome
12	1389	145	123	9627244	RefSeq	NC_002031.1	Yellow fever virus, complete genome
13	2335	235	171	13559808	RefSeq	NC_002728.1	Nipah virus, complete genome
14	1943	244	211	11545722	RefSeq	NC_002617.1	Newcastle disease virus, complete genome
15	1174	208	128	9629357	RefSeq	NC_001802.1	Human immunodeficiency virus 1, complete genome
16	409	134	106	21326584	RefSeq	NC_003977.1	Hepatitis B virus, complete genome
17	1011	169	135	9627257	RefSeq	NC_001576.1	Human papillomavirus type 10, complete genome
18	1036	325	299	10445391	RefSeq	NC_002554.1	Foot-and-mouth disease virus C,

							complete genome
19	1246	211	209	9790308	RefSeq	NC_001545.1	Rubella virus, complete genome
20	955	309	172	9626732	RefSeq	NC_001489.1	Hepatitis A virus, complete genome
21	834	103	29	38371716	RefSeq	NC_005222.1	Hantaan virus, complete genome
22	837	188	98	38371727	RefSeq	NC_005217.1	Sin Nombre virus, complete genome
23	430	100	86	23334588	RefSeq	NC_004294.1	Lymphocytic choriomeningitis virus segment S, complete sequence
23	853	455	286	23334585	RefSeq	NC_004291.1	Lymphocytic choriomeningitis virus segment L, complete sequence
24	1404	204	122	9626460	RefSeq	NC_001437.1	Japanese encephalitis virus, genome
25	1370	284	91	51850386	DNA Database of Japan	AB189128.1	Dengue virus type 3 genomic RNA, complete genome, strain: 98902890 DF DV-3
26	1361	130	57	12659201	Genbank	AF326573.1	Dengue virus type 4 strain 814669, complete genome
27	1370	142	21	19744844	Genbank	AF489932.1	Dengue Virus Type 2 strain BR64022, complete genome
28	1370	152	52	323660	Genbank	M87512.1	DENT1SEQ Dengue virus type 1 complete genome
29	944	175	87	9626436	RefSeq	NC_001430.1	Human enterovirus D, complete genome
30	945	183	122	9626433	RefSeq	NC_001428.1	Human enterovirus C, complete genome
31	946	196	148	9627719	RefSeq	NC_001612.1	Human enterovirus A, complete

							genome
32	945	364	154	21363125	RefSeq	NC_003986.1	Human echovirus 1, complete genome
33	944	94	12	9626677	RefSeq	NC_001472.1	Human enterovirus B, complete genome
34	913	283	190	9627730	RefSeq	NC_001617.1	Human rhinovirus 89, complete genome
35	920	426	291	9626735	RefSeq	NC_001490.1	Human rhinovirus B, complete genome

Together with 7 replicates for each viral probe, and control sequences for array synthesis and hybridization (as described below), the array contained a total of 390,482 probes.

Homogeneity, sensitivity and specificity

Homogeneity requires the selection of probes which have similar melting temperatures. It was found that probes with low CG-content did not produce reliable hybridization signal intensities, and that probes with high CG-content had a propensity to produce high signal intensities through non-specific binding. Thus, it could be established that the CG-content of probes selected should be from 40% to 60%.

Accordingly, the present invention provides a method of designing oligonucleotide probe(s) for nucleic acid detection, comprising selecting the probes having a CG-content from 40% to 60%.

The term "hybridization" refers to the process in which the oligo probes bind non-covalently to the target nucleic acid, or portion thereof, to form a stable double-stranded. Triple-stranded hybridization is also theoretically possible.

Hybridization probes are oligonucleotides capable of binding in a base-specific manner to a complementary strand of target nucleic acid. Hybridizing specifically refers to the binding, duplexing, or hybridizing of a molecule substantially to or only to a particular nucleotide sequence or sequences under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) of DNA or RNA. Hybridizations, e.g., allele-specific probe hybridizations, are generally performed under stringent conditions. For example, conditions where the salt concentration is no more than about 1 Molar (M) and a temperature of at least 25°C., e.g., 750 mM NaCl, 50 mM NaPhosphate, 5 mM EDTA, pH 7.4 (5 times SSPE) and a temperature of from about 25°C to about 30°C. Hybridization is usually performed under stringent conditions, for example, at a salt concentration of no more than 1 M and a temperature of at least 25°C. For stringent conditions, see also for example, Sambrook and Russel, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2001) which is hereby incorporated by reference in its entirety for all purposes above.

Sensitivity requires that probes that cannot form significant secondary structures be selected in order to detect low-abundance mRNAs. Thus, probes with the highest free energy computed based on Nearest-Neighbor model are selected (SantaLucia, J., Jr., et al., 1996).

Accordingly, the present invention provides a method of designing at least one oligonucleotide probe for nucleic acid(s) detection, wherein the probe(s) are selected by having the highest free energy computed based on Nearest-Neighbor model.

Specificity requires the selection of probes that are most unique to a viral genome. This is to minimize cross-hybridization of the probes with other non-target nucleic acids (for example, viral genomes). Given probe s_a and probe s_b

substrings of target nucleic acids v_a and v_b, s_a is selected based on the hamming distance between s_a and any length-m substring s_b from the target nucleic acid v_b and/or on the longest common substring of s_a and probe s_b. In particular, let s_a and s_b be length-m substrings from viral genome v_a and v_b respectively, where ($v_a \neq v_b$).

The length of the probe(s) to be designed may be of any length useful for the purposes of the present invention. The probes may be less than 100 mer, for example 20 to 80 mer, 25 to 60 mer, for example 40 mer. The hamming distance and/or longest common substring may also vary.

According to Kane's criteria (Kane, M.D., et al., 2000), s_a is specific to v_a if:

(a) the hamming distance between s_a and any length-m substring s_b from viral genome v_b, is more than $0.25m$;

(b) the longest common substring of s_a and s_b is less than 15.

The cutoff value(s) for the hamming distance may be chosen according to the stringency desired. It will be evident to any skilled person how to select the hamming distance cutoff according to the particular stringency desired. According to a particular example of the herein described probe design, the inventors used hamming distance cutoffs of >10 with respect to other target nucleic acids for specific probes, and < 10, preferably < 5 for conserved probes. With a specific probe, it indicates a probe which only hybridizes to a specific target nucleic acid, while with a conserved probe it indicates a probe which may hybridize to any member of the family of the target nucleic acid.

Accordingly, the present invention also provides a method of designing oligonucleotide probe(s) for nucleic acid detection, wherein given probe s_a and probe s_b substrings of target nucleic acids v_a and v_b comprised in the biological sample, s_a is selected if the hamming distance between s_a and any length-m

substring s_b from the target nucleic acid v_b is more than 0.25m, and the longest common substring of s_a and probe s_b is less than 15.

To study array hybridization dynamics without the complexity of cross-hybridization from human RNA, SARS coronavirus and Dengue serotype 1 viral RNA were purified from the media of infected cell lines, reverse-transcribed, and PCR-amplified using virus-specific primers (Wong, et. al., 2004). Each genome cDNA was amplified in its entirety (as confirmed by sequencing), labeled with Cy3 and hybridized separately on microarrays. The SARS sample hybridized well to the SARS tiling probes, with all 3,805 SARS-specific probes displaying fluorescent (Cy3) signal well above the detection threshold (determined by probe signal intensities >2 standard deviations above the mean array signal intensity; Figure 7A) Cross-hybridization with other pathogen probe sets was minimal, observed only for other members of Coronaviridae and a few species of Picornaviridae and Paramyxoviridae, consistent with the observation that SARS shares little sequence homology with other known viruses (Ksiazek et al. 2003). The hybridization pattern of Dengue 1, on the other hand, was more complex (Figure 7B). First, we observed that hybridization to the Dengue 1 probe set was partially incomplete (i.e., regions absent of signal) due to sequence polymorphisms. The Dengue 1 sample hybridized on the array was cultured from a Hawaiian isolate in 1944 (ATCC Catalog #VR-1254), whereas the array probe set is based on the sequence of strain S275/90, isolated in Singapore in 1990 (Fu et al. 1992). The Dengue 1 probes that failed to hybridize with the cDNA target each contained at least 3 mismatches (within a 15-base stretch) with the target sequence. Second, we observed that cross-hybridization occurred to some degree with almost all viral probe sets present on the array, particularly with probes of other Flaviviridae members, consistent with the fact that the 4 Dengue serotypes share 60-70% homology. To understand the relationship between hybridization signal output and annealing specificity, we first compared all probe sequences to each viral genome using 2 measures of

similarity: probe hamming distance (HD) and maximum contiguous match (MCM). HD measures the overall similarity distance of two sequences, with low scores for similar sequences (Hamming, 1950). MCM measures the number of consecutive bases which are exact matches, with high scores for similar sequences (Kane et al. 2000).

We calculated the HD and MCM scores for every probe relative to the Hawaiian Dengue 1 isolate and observed that these scores are inversely and directly correlated respectively to probe signal intensity. All probes on the array with high similarity to the Hawaiian Dengue I genome, i.e. HD = 2 (n=942) or MCM = 27 (n=627), hybridized with median signal intensity 3 logs above background. Although 98% of probes were detectable at the low HD range from 0-4, or high MCM range from 18-40, median probe signal intensity decreased at every increment of sequence distance. Median signal intensity dropped off sharply to background levels at HD=7 and MCM=15, with 43% and 46% detectable probes respectively. The majority of probes (>96%, n>51,000) had HD scores between 8-21 and/or MCM scores between 0-15, of which 1.23% and 1.57% were detectable respectively.

The ideal cross-hybridization similarity threshold would be one where all probes identifying a specific pathogen would always have detectable signal intensity above background noise, even in the presence of polymorphisms in the pathogen sequence. At the optimal similarity thresholds HD = 4 and MCM = 18, >98% of probes could be detected with median signal intensity 2 logs above background, whereas adjusting the threshold down 1 step to HD=5 and MCM = 17 would result in only ~85% probe detection and median signal intensity ~1.2 logs above background (Figure 8)

Using these optimal HD and MCM thresholds to predict for cross-hybridization, we binned all probes into groups most likely to detect a given pathogen. We

refer to these groups as specific signature probe sets (SPSs), and we defined SPSs for each of the 35 pathogen genomes represented on the array (Table 2).

Table 2 Each pathogen signature probe set (SPS) comprise its probes with AES in the top 20th percentile [column (1)]. Probes that do not have GC between 40-60% [column (2)] or high similarity to human genome [column (3)] were removed. Probes derived from other pathogens which will cross-hybridize to the pathogen based on HD and MCM [column (4)] were added to the SPS [column (5)].

	Pathogen	Family	Total tiling probes	AES (1)	GC content filter (2)	Human Genome filter (3)	No. of filtered probes left	No. of predicted cross-hybridizing probes (HD≤4 and MCM≥18) (4)	No. of probes in SPS (5)
1	LCMV	Arenaviridae	1283	574	1	18	555	0	555
2	Hantaan	Bunyaviridae	834	131	6	22	103	2	105
3	Sin Nombre	Bunyaviridae	837	225	8	29	188	3	191
4	229E	Coronaviridae	3495	196	2	12	182	2	184
5	OC43	Coronaviridae	3937	663	16	43	604	3	607
6	SARS	Coronaviridae	3805	672	6	78	588	3	591
7	Dengue serotype 1	Flaviviridae	1370	201	2	47	152	50	202
8	Dengue serotype 2	Flaviviridae	1370	178	0	36	142	71	213
9	Dengue serotype 3	Flaviviridae	1370	336	1	51	284	69	353
10	Dengue serotype 4	Flaviviridae	1361	172	1	41	130	44	174
11	Japanese encephalitis	Flaviviridae	1404	274	6	64	204	40	244
12	West Nile	Flaviviridae	1401	111	4	22	85	22	107
13	Yellow Fever	Flaviviridae	1389	151	0	6	145	10	155
14	Hepatitis B	Hepadnaviridae	409	146	2	10	134	0	134
15	Influenza A	Orthomyxoviridae	1582	601	2	46	553	0	553
16	Influenza B	Orthomyxoviridae	1822	718	7	69	642	2	644
17	Human papillomavirus type 10	Papillomaviridae	1011	177	1	7	169	0	169
18	hMPV	Paramyxoviridae	1705	375	23	60	292	8	300
19	Newcastle disease	Paramyxoviridae	1943	252	0	8	244	0	244
20	Nipah	Paramyxoviridae	2335	274	22	17	235	0	235
21	Parainfluenza 1	Paramyxoviridae	1995	625	13	62	550	3	553

22	Parainfluenza 2	Paramyxoviridae	2002	838	31	45	762	0	762
23	Parainfluenza 3	Paramyxoviridae	1979	834	29	104	701	9	710
24	RSV B	Paramyxoviridae	1948	655	52	66	537	4	541
25	Echovirus 1	Picornaviridae	945	439	3	72	364	59	423
26	Enterovirus A	Picornaviridae	946	205	0	9	196	21	217
27	Enterovirus B	Picornaviridae	944	109	0	15	94	47	141
28	Enterovirus C	Picornaviridae	945	202	0	19	183	31	214
29	Enterovirus D	Picornaviridae	944	191	0	16	175	15	190
30	Foot and mouth disease	Picornaviridae	1036	356	26	5	325	0	325
31	Hepatitis A	Picornaviridae	955	355	9	37	309	0	309
32	Rhinovirus A (type 89)	Picornaviridae	913	333	2	48	283	13	296
33	Rhinovirus B	Picornaviridae	920	464	3	35	426	11	437
34	HIV 1	Retroviridae	1174	229	4	17	208	0	208
35	Rubella	Togaviridae	1246	748	534	3	211	0	211
		Total	**53555**				**10955**		**11497**

Each pathogen's SPS comprised tiling probes derived from its genome sequence (HD=0, MCM=40), as well as cross-hybridizing probes derived from other pathogens (HD = 4, MCM = 18).

We next considered other non-specific hybridization phenomena that could affect performance of our SPS probes. For example, we observed a general relationship between probe signal and %GC content. Consistent with previous observations, we found that probes <40% GC resulted in diminished signal intensities, while probes >60% GC content showed higher signal intensities (Wong et al. 2004; Maskos and Southern, 1993). Thus, we utilized %GC content as an additional selection filter, whereby probes with <40% GC and >60% GC were excluded from our SPSs, despite optimal HD and MCM values.

Sequence similarity to human genome

In case the target nucleic acid to be detected is extracted from humans (for example, human samples containing viral genomes), probes with high homology to the human genome should also be avoided. Accordingly, for any probe s_a of length-*m* specific for the target nucleic acid v_a, the probe s_a is

selected if it does not have any hits with any region of a nucleic acid different from the target nucleic acid, and if the probe s_a length-m has hits with the nucleic acid different from the target nucleic acid, the probe s_a length-*m* with the smallest maximum alignment length and/or with the least number of hits is selected. In particular, for any length-*m* probe s_a, hits of s_a with the human genome are found with the BLAST algorithm (Altschul, S.F., et al., 1997). A BLAST word size of (W = 15) and an expectation value of 100 was used to find all hits. s_a is selected if it does not have any hits with the human genome, that is, it is specific to v_a. However, if all length-*m* substrings of v_a have hits with the human genome, those with the smallest maximum alignment length and with the least number of hits was selected.

Furthermore, as cross-hybridization with human sequences could also confound results, we compared all probes to the human genome assembly (build 17) (International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. Nature 409(6822), 860-921 (2001).) by BLAST using a word size of 15 (Altschul et al. 1997). Probes with expectation value of 100 were further filtered from the SPSs (see Table 2 above).

Accordingly, the present invention provides a method of designing oligonucleotide probe(s) for nucleic acid detection, wherein for any probe s_a of length-*m* specific for the target nucleic acid v_a, the probe s_a is selected if it does not have any hits with any region of a nucleic acid different from the target nucleic acid, and if the probe s_a length-m has hits with the nucleic acid different from the target nucleic acid, the probe s_a length-*m* with the smallest maximum alignment length and/or with the least number of hits is selected.

Further, the design of the oligonucleotide probe(s) may be also carried out by AES according to the invention. In particular, the invention provides a method of selecting and/or designing probes wherein a probe p_i at position i of a target

nucleic acid is selected if p_i is predicted to hybridize to the position i of the amplified target nucleic acid.

In particular, the oligonucleotide probe(s) capable of hybridizing to the selected region(s) may be selected and/or designed according to at least one of the following criteria:

(a) the selected probe(s) has a CG-content from 40% to 60%;

(b) the probe(s) is selected by having the highest free energy computed based on Nearest-Neighbor model;

(c) given probe s_a and probe s_b substrings of target nucleic acids v_a and v_b, s_a is selected based on the hamming distance between s_a and any length-*m* substring s_b from the target nucleic acid v_b and/or on the longest common substring of s_a and probe s_b;

(d) for any probe s_a of length-*m* specific for the target nucleic acid v_a, the probe s_a is selected if it does not have any hits with any region of a nucleic acid different from the target nucleic acid, and if the probe s_a length-m has hits with the nucleic acid different from the target nucleic acid, the probe s_a length-*m* with the smallest maximum alignment length and/or with the least number of hits is selected; and/or

(e) a probe p_i at position i of a target nucleic acid is selected if p_i is predicted to hybridize to the position i of the amplified target nucleic acid.

According to a particular aspect of the invention, two or more of the criteria indicated above may be used for designing the oligonucleotide probe(s). For example, the probe(s) may be designed by applying all criteria (a) to (e). Other criteria, not explicitly mentioned herein but which are evident to a skilled person in the art may also be used.

In particular, under the criterion (e), a probe p_i at position i of a target nucleic acid v_a is selected if $P(p_i \mid v_a) > \lambda$, wherein λ is 0.5 and $P(p_i \mid v_a)$ is the probability that p_i has to hybridize to the position i of the target nucleic acid v_a. More in particular, λ is 0.8.

According to another aspect, the invention provides a method as above described wherein $P(p_i \mid v_a) \approx P(X \leq x_i) = \frac{c_i}{k}$, wherein X is the random variable representing the amplification efficiency score (*AES*) values of all probes of v_a, k is the number of probes in v_a, and c_i is the number of probes whose *AES* values are $\leq x_i$.

According to another aspect, the AES can also be used to design random primer tags to facilitate random amplification of sample by random PCR (for use in applications such as detection of pathogens, detection of gene expression, constructing clonal DNA libraries, and other applications a skilled person would employ random PCR).

Synthesis of oligonucleotide probes on a support

According to another aspect of the invention, the method of selecting and/or designing at least one oligonucleotide probe(s) as described above further comprises a step of preparing the selected and/or designed probe(s). Designing a probe comprises understanding its sequence and/or designing it by any suitable means, for example by using a software. The step of preparing the probe comprises the physical preparation of it. The probe may be prepared according to any standard method known in the art. For example, the probes may be chemically synthesized or prepared by cloning. For example, as described in Sambrook and Russel, 2001.

There is also provided a support, for example a microarray or biochip, prepared according to any embodiment according to the present invention.

The probe(s) designed and prepared according to any method of the present invention may used in solution or may be placed on an insoluble support. For example, may be applied, spotted or printed on an insoluble support according to any technique known in the art. The support may be a solid support or a gel. The support with the probes applied on it, may be a microarray or a biochip.

More in particular, the present invention provides an oligo microarray hybridization-based approach for the rapid detection and identification of pathogens, for example viral and/or bacterial pathogens, from PCR-amplified cDNA prepared from primary tissue samples. In particular, from random PCR-amplified cDNA(s).

In the following description, the preparation of probes is made with particular reference to a microarray. However, the support, as well as the probes, may be prepared according to any description across the whole content of the present application. In particular, an "array" is an intentionally created collection of molecules which may be prepared either synthetically or biosynthetically. The molecules in the array may be identical or different from each other. The array may assume a variety of formats, e.g., libraries of soluble molecules; libraries of compounds tethered to resin beads, silica chips, or other solid supports. Array Plate or a Plate is a body having a plurality of arrays in which each array is separated from the other arrays by a physical barrier resistant to the passage of liquids and forming an area or space, referred to as a well.

Sample preparation and hybridization onto the Microarray

The biological sample may be any sample taken from a mammal, for example from a human being. The biological sample may be blood, a body fluid, saliva,

urine, stool, and the like. The biological sample may be treated to free the nucleic acid comprised in the biological sample before carrying out the amplification step. The target nucleic acid may be any nucleic acid which is intended to be detected. The target nucleic acid to be detected may be at least a nucleic acid exogenous to the nucleic acid of the biological sample. Accordingly, if the biological sample is from a human, the exogenous target nucleic acid to be detected (if present in the biological sample) is a nucleic acid which is not from human origin. According to an aspect of the invention, the target nucleic acid to be detected is at least a pathogen genome or fragment thereof. The pathogen nucleic acid may be at least a nucleic acid from a virus, a parasite, or bacterium, or a fragment thereof.

According to an aspect of the present invention, there is provided a method of target nucleic acid detection analysis. The target nucleic acid(s) from a biological sample desired to be detected may be any target nucleic acid, RNA and/or DNA. For example, mRNA and/or cDNA. More in particular, the target nucleic acid to be detected may be a pathogen or non-pathogen. For example, it may be the genome or a fragment thereof of at least one virus, at least one bacterium and/or at least one parasite. The probes selected and/or prepared may be placed, applied and/or fixed on a support according to any standard technology known to a skilled person in the art. The support may be an insoluble support, for example a solid support. In particular, a microarray and/or a biochip.

According to a particular example, RNA and DNA was extracted from patient samples e.g. tissues, sera, nasal pharyngeal washes, stool using established protocols and commercial kits. For example, Qiagen Kit for nucleic acid extraction may be used. Alternatively, Phenol/Chloroform may also be used for the extraction of DNA and/or RNA. Any technique known in the art, for example as described in Sambrook and Russel, 2001 may be used. RNA was reverse-

transcribed to cDNA using tagged random primers, based on a protocol described by Bohlander et. al., 1992 and Wang et. al., 2003. The cDNA was then amplified by random PCR. Fragmentation, labeling and hybridization of sample to the microarray were carried out as described by Wong et. al., 2004.

Microarray synthesis

According to a particular experiment described in the Examples section, the present inventors selected several viral genomes representing the most common causes of viral disease in Singapore. Using the complete genome sequences downloaded from Genbank, 40-mer probes which tiled across the entire genomes and overlapping at five-base resolution were generated. Seven replicates of each virus probe were synthesized directly onto the microarray using Nimblegen technology (Nuwaysir, E.F., et al., 2002). The probes were randomly distributed on the microarray to minimize the effects of hybridization artifacts. To control for non-specific hybridization of sample to probes, 10,000 oligonucleotide probes were designed and synthesized onto the microarray. These 10,000 oligonucleotides did not have any sequence similarity to the human genome, or to the pathogen genomes. They were random probes with 40-60% CG-content. These probes measured the background signal intensity. As a positive control, 400 oligonucleotide probes to human genes which have known or inferred functions in immune response were synthesized on the array. A plant virus, PMMV, was included as a negative control, for a total of approximately 380,000 probes. In the following description, the invention will be described in more particularity with reference to a pathogen detection chip analysis (also referred to as PDC). However, the analysis (method) is not limited to this particular embodiment, but encompasses the several aspects of the invention as described across the whole content of the present application.

Method of detecting target nucleic acid(s)

According to another aspect, the present invention provides a method of detecting at least one target nucleic acid comprising the step of:

(i) providing a biological sample;

(ii) amplifying nucleic acid(s) comprised in the biological sample;

(iii) providing at least one oligonucleotide probe capable of hybridizing to at least one target nucleic acid, if present in the biological sample, wherein the probe(s) is prepared by using a method according to any aspect of the invention herein described;

(iv) contacting the probe(s) with the amplified nucleic acids and detecting the probe(s) hybridized to at least one target nucleic acid.

The amplification step (ii) may be carried out in the presence of random, partially random (that is, comprising a fixed portion and a random portion) or specific primers. In particular, the amplification step (II) may be carried out in presence of at least one random primer. More in particular, in the presence of at least one random forward primer and/or at least one random reverse primer. For example, the amplification step (ii) may be carried out in the presence of more than two random primers. Any amplification method known in the art may be used. For example, the amplification method is a RT-PCR.

In particular, the present inventors developed a method of detecting the probe(s) hybridized to the to the target nucleic acid based on the amplification efficiency score (AES). This may herein also be referred to as the algorithm according to the present invention. In particular, a forward random primer binding to position *i* and a reverse random primer binding to position j of a target nucleic acid v_a are selected among primers having an amplification efficiency

score (AES_i) for every position i of a target nucleic acid v_a of:

$$AES_i = \sum_{j=i-Z}^{i} \left\{ P^f(j) \times \sum_{k=\max(i+1, j+500)}^{j+Z} P^r(k) \right\},$$

wherein $\sum_{k=\max(i+1, j+500)}^{j+Z} P^r(k)$ = $P^r(i+1) + P^r(i+2) + \ldots\ldots P^r(j+Z)$

$P^f(i)$ and $P^r(i)$ are the probabilities that a random primer r_i can bind to position i of v_a as forward primer and reverse primer, respectively, and $Z \leq$ 10000 bp is the region of v_a desired to be amplified. More in particular, Z may be $\leq$ 5000 bp, $\leq$ 1000 bp, or $\leq$ 500 bp.

The amplification step may comprise forward and reverse primers, and each of the forward and reverse primers may comprise, in a 5'-3' orientation, a fixed primer header and a variable primer tail, and wherein at least the variable tail hybridizes to a portion of the target nucleic acid v_a. In particular, the amplification step may comprise forward and/or reverse random primers having the nucleotide sequence of any of SEQ ID NOS:1-7, or a variant, or derivative thereof.

The biological sample may be any sample taken from a mammal, for example from a human being. The biological sample may be tissue, sera, nasal pharyngeal washes, saliva, any other body fluid, blood, urine, stool, and the like. The biological sample may be treated to free the nucleic acid comprised in the biological sample before carrying out the amplification step. The target nucleic acid may be any nucleic acid which is intended to be detected. The target nucleic acid to be detected may be at least a nucleic acid exogenous to the nucleic acid of the biological sample. Accordingly, if the biological sample is from a human, the exogenous target nucleic acid to be detected (if present in the biological sample) is a nucleic acid which is not from human origin.

According to an aspect of the invention, the target nucleic acid to be detected is at least a pathogen genome or fragment thereof. The pathogen nucleic acid may be at least a nucleic acid from a virus, a parasite, or bacterium, or a fragment thereof.

Accordingly, the invention provides a method of detection of at least a target nucleic acid, if present, in a biological sample. The method may be a diagnostic method for the detection of the presence of a pathogen into the biological sample. For example, if the biological sample is obtained from a human being, the target nucleic acid, if present in the biological sample, is not from human.

The probe(s) designed and/or prepared according to any method of the present invention may used in solution or may be placed on an insoluble support. For example, may be applied, spotted or printed on an insoluble support according to any technique known in the art. The support with the probes applied on it may be a solid support or a gel. In particular, it may be a microarray or a biochip.

The probes are then contacted with the nucleic acid of the biological sample, and if present the target nucleic acid(s) and the probe(s) hybridize, and the presence of the target nucleic acid is detected. In particular, in the detection step (iv), the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

More in particular, in the detection step (iv), the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, and the method further comprises the step of computing the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of the probes used in the detection method having high signal

intensities, the density distribution of the signal intensities of probes v_a being more positively skewed than that of probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

For example, in the detection step (iv), the presence of a target nucleic acid in a biological sample is given by a value of t-test $\leq$ 0.1 and/or Anderson-Darling test value $\leq$ 0.05 and/or a value of Weighted Kullback-Leibler divergence of $\geq$ 1.0, preferably $\geq$ 5.0. In particular, the t-test value is $\leq$ 0.05.

According to another aspect, the present invention provides a method of determining the presence of a target nucleic acid v_a comprising detecting the hybridization of a probe to a target nucleic acid v_a and wherein the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, thereby indicating the presence of v_a. In particular, the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, and the method further comprises the step of computing the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of the probes used in the detection method having high signal intensities, the density distribution of the signal intensities of probes v_a being more positively skewed than that of probes $\notin v_a$, thereby indicating the presence of v_a. More in particular, the presence of a target nucleic acid in a biological sample is given by a value of t-test $\leq$ 0.1 and/or Anderson-Darling test value $\leq$ 0.05 and/or a value of Weighted Kullback-Leibler divergence of $\geq$ 1.0, preferably, $\geq$ 5.0. For example, the t-test value may be $\leq$ 0.05.

According to another aspect, the present invention provides a method of detecting at least one target nucleic acid, comprising the steps of:

(i) providing at least one biological sample;

(ii) amplifying nucleic acid(s) comprised in the biological sample;

(iii) providing at least one oligonucleotide probe capable of hybridizing to at least one target nucleic acid, if present in the biological sample;

(iv) contacting the probe(s) with the amplified nucleic acids and detecting the probe(s) hybridized to target nucleic acid(s), wherein the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

In step (iv), the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, and the method further comprises the step of computing the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of the probes used in the detection method having high signal intensities, the density distribution of the signal intensities of probes v_a being more positively skewed than that of probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample. In particular, in step (iv) the presence of a target nucleic acid in a biological sample is given by a value of t-test $\leq$ 0.1 and/or Anderson-Darling test value $\leq$ 0.05 and/or a value of Weighted Kullback-Leibler divergence of $\geq$ 1.0, preferably $\geq$ 5.0. The t-test value may be $\leq$ 0.05. The nucleic acid to be detected is nucleic acid exogenous to the nucleic acid of the biological sample. The target nucleic acid to be detected may be at least a pathogen genome or fragment thereof. The pathogen nucleic acid may be at least a nucleic acid from a virus, a parasite, or bacterium, or a fragment thereof. In particular, when the sample is obtained from a human being, the target nucleic acid, if present in the biological sample, is not from the human genome. The probes may be placed on an insoluble support. The support may be a microarray or a biochip.

Test using the template sequence of RSV B

To verify if the variation in signal intensities displayed by different regions of a virus has direct correlation with their corresponding amplification efficiency

scores, a total of five microarray experiments were performed on a common pathogen affecting human, the human respiratory syncytial virus B (RSV B).

Next, the probe design criteria, as described above, were applied on the template sequence of RSV B obtained from NCBI (NC_001781). This resulted in 1948 probes spotted onto each microarray. The amplification efficiency map for RSV B was also computed prior to the actual experiments and shown in Figure 2. This figure shows the peaks having the AES higher than the average AES and indicating the regions of the RSV B with higher probability of amplification.

Using 5 samples containing the human respiratory syncytial virus B (RSV B), independent microarray experiments were conducted. The resultant signal intensities for one such experiment is shown in Figure 3.

For each experiment, the signal intensities of the 1948 probes were ranked in decreasing order and were correlated with their corresponding *AES* value. The *p*-value was found to be $< 2.2e^{-16}$ on the average. This indicates that the correlation between the signal intensity of probe at position *i* of RSV B with AES_i is not at all random. Further investigations revealed that about 300 probes, which consistently produced high signal intensities in all five experiments, have amplification efficiency scores in the 90^{th} percentile level.

Having shown that the described amplification efficiency model works well on the RSV B genome, it was desired to show that the model according to the invention may be extended to other viral genomes as well. Another microarray experiment was performed on the human metapneumonia virus (HMPV). This time, there were 1705 probes on the microarray. Again, the amplification efficiency map for HMPV was computed. In this experiment, the correlation test

between signal intensities and amplification efficiency scores gave a *p*-value of $1.335e^{-9}$.

Accordingly, the amplification efficiency model according to the invention is able to predict the relative strength of signals produced by different regions of a viral genome in the described experiment set-up. Probes from regions with low amplification efficiency scores have a high tendency to produce no or low signal intensities. This would result in a false negative on the microarray. Such probes will complicate the analysis of the microarray data and this is made even more complicated since a probe with a low signal intensity may be due to its target genome not being present or simply that it was not amplified. As such, probes in regions with reasonably high amplification efficiency scores should be selected to minimize inaccuracies caused by the RT-PCR process using random primers.

The threshold for amplification efficiency scores for probe selection for a virus v_a is determined by the cumulative distribution function of the *AES* values v_a. Let X be the random variable representing the *AES* values of all probes of v_a. Let k be the number of probes in v_a. Then, we denote the probability that the *AES* value is less than or equal to x be $P(X \leq x) = c/k$, where c is the number of probes which have *AES* values less than or equal to x. For a probe p_i at position i of v_a, let x_i be its corresponding *AES* value. Since the signal intensity of a probe is highly correlated to its *AES* value, we estimate $P(p_i \mid v_a)$, the probability that p_i has high signal intensity in the presence of v_a, to be $P(X \leq x_i)$. Thus,

$$P(p_i \mid v_a) \approx P(X \leq x_i)$$

$$= \frac{c_i}{k}$$

where c_i is the number of probes whose *AES* values are less than or equal to x_i.

For probe selection, probe p_i is selected if $P(p_i \mid v_a) > \lambda$. In the present experiments, λ was set as λ = 0.8.

Accordingly, the present invention also provides a method of probe design and/or of target nucleic acid detection wherein a probe p_i at position i of a target nucleic acid v_a is selected if $P(p_i \mid v_a) > \lambda$, wherein λ is 0.75 and $P(p_i \mid v_a)$ is the probability that p_i has a high signal intensity in the presence of v_a. More in particular, $P(p_i \mid v_a) \approx P(X \leq x_i) = \frac{c_i}{k}$, wherein X is the random variable representing the amplification efficiency score (*AES*) values of all probes of v_a, k is the number of probes in v_a, and c_i is the number of probes whose *AES* values are less than or equal to x_i.

Target nucleic acid detection analysis

In the following description, the invention will be described in more particularity with reference to a pathogen detection chip analysis (also referred to as PDC). However, the analysis (method) is not limited to this particular embodiment, but encompasses the several aspects of the invention as described across the whole content of the present application. Therefore, in particular, given a PDC with a set of length-m probes $P = \{p_1, p_2, \ldots, p_l\}$, which is designed for a set of viral genomes $V = \{v_1, v_2, \ldots, v_n\}$, the pathogen detection chip analysis problem is to detect the virus present in the sample based on the chip data. The chip data here refers to the collective information provided by the probe signals on the PDC. Thus, the chip data $D = \{d_1, d_2, \ldots, d_x\}$ is the set of corresponding signals of the probe set P on the PDC.

Given a sample, it is not known what pathogens are present in the sample, how many different pathogens there are, if present at all. However, if a virus v_a is

indeed in the sample, then the signal intensities of the probes of v_a should differ significantly from the signal intensities of probes from other viruses. Specifically, a higher proportion of probes of v_a should have high signal intensities compared to other viruses. Hence, it would be expected that the mean of the signal intensities of the probes in v_a should be statistically higher than that of probes $\notin v_a$.

Accordingly, the invention provides a method wherein the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, which may indicate the presence of v_a in the biological sample.

However, having a statistically higher mean may still be insufficient to conclude that v_a is in the sample. Preferably, an additional step may be required. We need to compute the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of probes on the PDC having high signal intensities. This is based on the observation that the distribution of the signal intensities of probes $\in v_a$ is more positively skewed than that of probes $\notin v_a$ (see the arrow in Figure 4 A. For comparison see Figure 4B).

Based the above observations, the chip data D for the presence of viruses was analyzed as follows. For every virus $v_a \in V$, we used a one-tail t-test (Goulden, C.H., 1956) to determine if the mean of the signal intensities of the probes $\in v_a$ was statistically higher than that of the signal intensities of the probes $\notin v_a$. Thus, the t-statistic was computed:

$$t_i = \frac{\mu_a - \mu_{a'}}{\sqrt{\frac{s_a^2}{n_a} + \frac{s_{a'}^2}{n_{a'}}}}$$

where μ_a, σ_a^2 and n_a is the mean, variance, and size of the signal intensities of the probes $\in$ v_a respectively and $\mu_{a'}$, $s_{a'}^2$ and $n_{a'}$ is the mean, variance, and size of the signal intensities of the probes $\notin$ v_a respectively.

To test the significance of the difference, the level of significance was set to 0.05. This means that the hypothesis that the mean of the signal intensities of the probes $\in$ v_a is higher than that of the signal intensities of the probes $\notin v_a$ would only be accepted if the p-value of $t_a < 0.05$. In this case, v_a is likely to be present in the sample.

The t-test alone, which allows the inventors to know if the distribution of the signal intensities of a virus is different from that of other viruses, may not be sufficient to determine if a particular virus is in the sample. It is also essential to know how similar or different the two distributions are. A ruler that can be used to measure the similarity between a true distribution and a model distribution is the Kullback-Leiber divergence (Kullback and Leiber, 1951) (also known as the relative entropy). In this application, the probability distribution of the signal intensities of the probes in v_a is the true distribution while the probability distribution of the signal intensities of all the probes in P is the model distribution. Let P_a be the set of probes in v_a. The Kullback-Leibler (KL) divergence of the probability distribution of the signal intensities of P_a and P is:

$$KL(P_a \| P) = \sum_{\mu \le x \le \max(D)} f_a(x) \log\left(\frac{f_a(x)}{f(x)}\right)$$

where μ is the mean signal intensity of the probes in P; $f_a(x)$ is the fraction of probes in P_a with signal intensity x; and $f(x)$ is the fraction of probes in P with signal intensity x. It follows that if $KL(P_a \| P) = 0$ then the probability distribution of P_a is exactly the same as that of P. Otherwise they are different.

Since a virus that is present in the sample would have signal intensities higher than that of the population, this implies that v_a has a chance of being present in the sample if $KL(P_a \| P) > 0$. Thus, the larger the value of $KL(P_a \| P)$, the more different are the two probability distributions and the more likely that v_a is indeed present in the sample.

It is important to note that the Kullback-Leibler divergence is the collective difference over all x of two probability distributions. Thus, while the Kullback-Leibler divergence is good at finding shifts in a probability distribution, it is not always so good at finding spreads, which affect the tails of the probability distribution more. As described in Figure 4(A,B), the tails of the probability distribution provides the most information about whether a virus is present in the sample. Hence, the Kullback-Leibler divergence statistic must be improved to reflect more accurately such an observation.

To increase its sensitivity out on the tails, we introduced a stabilized or weighted statistic to the Kullback-Leibler divergence, the Anderson-Darling statistic (Stephens, M. A. (1974). EDF Statistics for Goodness of Fit and Some Comparisons, Journal of the American Statistical Association, Vol. 69, pp. 730-737). Thus the Weighted Kullback-Leibler divergence (*WKL*) is:

$$WKL(P_a \| P) = \sum_{\mu \le x \le \max(D)} \frac{f_a(x) \log \frac{f_a(x)}{f(x)}}{\sqrt{Q(x)[1-Q(x)]}}$$

where $Q(x)$ is the cumulative distribution function of the signal intensities of the probes in P.

Empirical tests show that in samples where there are no viruses, viruses that pass the t-test with significance level 0.05 have $WKL < 5.0$. In samples where there is indeed a virus present, the actual viruses not only pass the t-test with significance level 0.05 but are also the only viruses to have $WKL \geq 5.0$. Thus

we set the Weighted Kullback-Leiber divergence threshold for a virus to be present in the sample to be 5.0.This analysis framework is shown in Figure 5.

Apparatus and/or product performing the method according to the invention

It is well-known to a skilled person in the art how to configure software which can perform the algorithms and/or methods provided in the present invention. Accordingly, the present invention also provides a software and/or a computer program product configured to person the algorithms and/or methods according to any embodiment of the present invention There is also provided at least one electronic storage medium. The electronic storage medium may be a computer hard-drive, a CD-ROM, a flash memory device (e.g. USB thumbdrive), a floppy disk, or any other electronic storage medium in the art. The software may be run on personal computers, mainframes, and any computing processing unit, and the particular configurations are known to a person skilled in the art.

It will be appreciated that the present invention has been described by way of example only and that various modifications in design may be made without departure from the spirit and scope of the invention.

Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russel, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2001).

Microarray synthesis

We selected 35 viral genomes representing the most common causes of viral disease in Singapore (see Table 1 above).

Complete genome sequences were downloaded from NCBI Taxonomy Database (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) to generate 40-mer probe sequences tiled across the entire genomes and overlapping at an average 8-base resolution. 7 replicates of each virus probe was synthesized directly onto the microarray using Nimblegen proprietary technology (Nuwaysir et al. 2002). The probes were randomly distributed on the microarray to minimize the effects of hybridization artifacts. To control for non-specific hybridization of sample to probes and measure background signal, 10,000 oligonucleotide probes were designed and synthesized onto the microarray. They are random probes with 40-60% GC-content with no sequence similarity to the human genome, or to the pathogen genomes. As a positive control, 400 oligonucleotide probes to human genes which have known or inferred functions in immune response were synthesized on the array. A plant virus, PMMV, was included as negative control, for a total of 390,482 probes.

Sample preparation, microarray hybridization and staining

Dengue cell line (ATCC #VR-1254) was cultured as per ATCC recommendations and Sin850 SARS cell line was cultured as described (Vega et al. 2004). Clinical specimens (nasopharyngeal washes) were obtained from an Indonesian pediatric population and stored at -80 °C in RNAzol (Leedo Medical Laboratories, Inc., Friendswood, TX). All were suspected pneumonia patients aged between 7 to 38 mths demonstrating specific clinical signs of respiratory illnesses. RNA was extracted with RNAzol according to manufacturer's instructions (Smalling et al. 2002; Tang et al. 1999). Extracted

RNA was resuspended in RNA storage solution (Ambion, USA) and stored at -80°C until needed. RNA was reverse transcribed to cDNA using tagged random primers, based on a protocol described by Bohlander et al and Wang et al (Wang et al. 2002; Bohlander et al. 1992). The cDNA was then amplified by random PCR, fragmented, end-labeled with biotin labeling, hybridized onto the microarray and stained as previously described (Wong et al. 2002). In our initial experiments, we found that probe GC content could create artifacts in signal intensity measurements, with increasing signal directly proportional to probe GC content. Adding 0.82 M TMAC to Nimblegen's proprietary TMAC hybridization buffer eliminated this artifact.

Real-time Diagnostic RT-PCR for RSV and hMPV

A 20µl reaction mixture containing 2µl of the purified patient RNA, 5U of MuLV reverse transcriptase, 8U of recombinant RNase inhibitor, 10µl of 2X universal PCR Master Mix with no UNG (all from Applied Biosystems) 0.9 µM primer and 0.2 µM probe. The real-time RT-PCRs were carried out in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). RT was performed at 48°C for 30 min followed by 10 min at 95°C for activation of DNA polymerase. Amplification of RT products achieved by 40 cycles of 15 s at 95°C and 1 min at 60°C. Negative controls and serial dilutions of a plasmid clone (positive control) were included in every PCR assay. During amplification, fluorescence emissions were monitored at every thermal cycle. The threshold (CT) represents the cycle at which significant fluorescence is first detected. CT value was converted to copy number using a control plasmid of known concentration. For RSV, 2.61 x 10^9 copies had a CT value of 11.897 while for hMPV, 7.51 x 10^9 copies had a CT value of 10.51.

1-step Diagnostic RT-PCR for coronavirus and rhinovirus

Frozen live cultures of human coronavirus OC43, 229E and rhinovirus 16 were purchased from ATCC (Cat #VR-1558, VR-740, VR-283) for use as positive

controls. RNA was extracted from these cultures using RNA Mini Kit (Qiagen, Germany) in accordance with manufacturer's instructions. The samples were amplified as previously described using the following diagnostic primer pairs: pancoronavirus (Cor-FW, Cor-RV), OC43 (OC43-FW, OC43-RV), 229E (229E-FW, 229E-RV), rhinovirus (Amplimer 1, Amplimer 2) (Moës et al. 2005; Deffernez et al. 2004).

Analysis of Pathogen Microarray Data

Our Pathogen Microarray contains a set of 40-mer probes $P = \{p_1, p_2, \ldots, p_s\}$, binned into distinct probe hybridization signatures for 35 viral genomes $V = \{v_1, v_2, \ldots, v_{35}\}$. Upon hybridization of pathogen nucleic acids, a set of probe signal intensity data $D = \{d_1, d_2, \ldots, d_s\}$ corresponding to probe set P is generated.

1-tail T-test

If virus v_a is present, then probes comprising its hybridization signature (probes $\in v_a$) should have statistically higher signal intensities than probes $\notin v_a$ determined by the t-statistic (1-tail T-test):

$$t_i = \frac{\mu_a - \mu_{a'}}{\sqrt{\frac{s_a^2}{n_a} + \frac{s_{a'}^2}{n_{a'}}}}$$

where μ_a, σ_a^2 and n_a are the mean, variance, and size of the signal intensities of the probes $\in v_a$ respectively and $\mu_{a'}$, $s_{a'}^2$ and $n_{a'}$ are the mean, variance, and size of the signal intensities of the probes $\notin v_a$ respectively.

The level of significance was set to 0.05. This means that we would only accept the hypothesis that the mean of the signal intensities of the probes $\in v_a$ is higher than that of the signal intensities of the probes $\notin v_a$ if the *p*-value of t_a < 0.05. In this case, v_a is likely to be present in the sample. However, the T-test method of detection results in many false positive calls.

PDA v. 1

PDA v.1 comprises a series of statistical tests, beginning with a Weighted Kullback-Leibler test and Z-score transformation (WKL score) followed by Anderson-Darling test for normality.

Consider the virus v_a. Let P_a be the set of probes of a virus v_a and $\overline{P_a} = P - P_a$. Let $[r_{low}, r_{high}]$ be the signal intensity range. We partitioned it into *c* bins $[r_{low} + j(\frac{r_{high} - r_{low}}{c}), r_{low} + (j+1)(\frac{r_{high} - r_{low}}{c})]$ for j=0, 1, ..., c-1. The unmodified Kullback-Leibler divergence may be computed by

$$KL(P_a \mid \overline{P_a}) = \sum_{j=0}^{c-1} f_a(j) \log(\frac{f_a(j)}{f_{\overline{a}}(j)})$$

where n_a^j and $n_{\overline{a}}^j$ are the number of probes in P_a and probes in $\overline{P_a}$ contained in the bin b_j respectively. $f_a(j) = \frac{n_a^j}{\sum_{h=0}^{c-1} n_a^h}$ is the fraction of probes in P_a found in bin b_j; and $f_{\overline{a}}(j) = \frac{n_{\overline{a}}^j}{\sum_{h=0}^{c-1} n_{\overline{a}}^h}$ is the fraction of probes in $\overline{P_a}$ found in bin b_j.

To compare the signal difference of the tail of the probability distribution, we set $r_{low} = \overline{\mu_a}$, the mean signal intensity of the probes in $\overline{P_a}$, and r_{high} = maximum signal intensity. We set the default number of bins, c = 20.

To further stabilize and/or increase the sensitivity of the Kullback-Leibler divergence on the tail of the probability distribution, two modifications were made. First, we introduced the Anderson-Darling type weight function to the Kullback-Leibler divergence. This gave more weight to the tails than the middle of the distribution. Next, we applied the statistic over the two corresponding cumulative distribution functions instead of their probability density functions. We call our improved Kullback-Leibler divergence the Weighted Kullback-Leibler divergence (*WKL score*):

$$WKL(P_a \mid \overline{P_a}) = \sum_{j=0}^{k-1} \frac{Q_a(j)\log(\frac{Q_a(j)}{Q_{\bar{a}}(j)})}{\sqrt{Q_{\bar{a}}(j)[1-Q_{\bar{a}}(j)]}}$$

where $Q_a(j)$ is the cumulative distribution function of the signal intensities of the probes in P_a found in bin b_j; $Q_{\bar{a}}(j)$ is the cumulative distribution function of the signal intensities of the probes in $\overline{P_a}$ found in bin b_j.

Thus for each hybridized sample, we computed the *WKL* score of every virus $v_a \in V$. Next, we claimed that the distribution of WKL scores of all viruses $v_a \in V$ was approximately normal if there was no virus present in a sample. We empirically verified if our claim was correct by a bootstrapping process: Let n be the number of viruses in V. For each virus $v_k \in V$ where $k = 1, \ldots, n$, we choose $|v_k|$ probe signal intensities from a real dataset D randomly with replacement to form a "perturbed" signal intensity distribution of v_k. Such distribution can mimic the situation where virus v_k is not present in the sample D. Thereafter, n *WKL* scores are generated for the set of n viruses. Next, we checked if the n WKL scores follow a normal distribution by the Anderson-Darling test for normality at 95% confidence interval. The bootstrap was repeated 100,000 times. The distribution was found to be normal in more than 99% of the time. (NB: since there are 35 viral genomes represented on our microarray, n =35)

Based on the above discussion, we can test if a sample contains virus(es) by making the following null and alternative hypothesis:

H_0: The distribution of *WKL* scores is normal, i.e. viruses are not present in the sample.

H_1: The distribution of *WKL* scores is not normal, i.e. at least 1 virus is present in the sample.

Definition The Anderson-Darling test is defined as:

H_0: The data follow a specified distribution.

H_a: The data do not follow the specified distribution

Test Statistic: The Anderson-Darling test statistic is defined as

$$A^2 = -N - S$$

where

$$S = \sum_{i=1}^{N} \frac{(2i-1)}{N} \left[\ln F(Y_i) + \ln\left(1 - F(Y_{N+1-i})\right)\right]$$

F is the cumulative distribution function of the specified distribution. Note that the Y_i are the *ordered* data.

Significance Level: α

Critical Region: The critical values for the Anderson-Darling test are dependent on the specific distribution that is being tested. Tabulated values and formulas have been published (Stephens, 1974, 1976,

1977, 1979) for a few specific distributions (normal, lognormal, exponential, Weibull, logistic, extreme value type 1). The test is a one-sided test and the hypothesis that the distribution is of a specific form is rejected if the test statistic, A, is greater than the critical value.

We proceed to apply the Anderson-Darling test for normality on the distribution of *WKL* scores to reject H_0 with 95% confidence interval. If the distribution of *WKL* scores is not normal, then we exclude the virus with the outlying *WKL* score and apply the Anderson-Darling test again. This process is repeated (to identify the presence of co-infecting pathogens) until H_0 is accepted.

We denote the distribution of *WKL* score when H_0 is accepted as the background *WKL* distribution. The viruses excluded are thus very likely to be present in the sample since their *WKL* score does not follow the background *WKL* distribution.

In our experiments, we observed that P, the probability that a non-normal distribution occurring by random chance with a given WKL score, in samples which contain a virus is very low i.e. $P < 1.0 \times 10^{-6}$(obtained via Z-score transformation of WKL score). Box 1 shows the pseudo-code for our virus-detection algorithm.

Box 1: Virus detection algorithm

Given a pathogen microarray data *D* with virus set *V* and probe set *P*,
Let $V_{present} = F$

Let D_{WKL} be the set of $WKL(P_v \| P_{\bar{v}})$ for all $v \in V$;

1. Determine normality of D_{WKL} with Anderson Darling test for normality. If D_{WKL} is a normal distribution with significance level 0.05, return $V_{present}$. Else, go to step 2.
2. Find the virus v_a with the highest $WKL(P_a \| P_{a'})$ from D_{WKL}.
   Let $V_{present} = V_{present} \cup \{ v_a \}$; $D_{WKL} = D_{WKL} - \{ WKL(P_a \| P_{a'}) \}$; Go to step 1.
3. Remove detected SPS and verify that WKL distribution is normal.
4. If distribution is not normal, go back to step 2 to find co-infecting pathogen.

Predicting Genome-wide Amplification Bias

Random primer amplification, rather than primer-specific amplification is preferred for identifying unknown pathogens in clinical specimens. However, in initial experiments using random priming amplification to identify known pathogens, we frequently observed incomplete hybridizations spanning genomic regions not explained by sequence polymorphisms (Figure 7C) Genome secondary structure, probe secondary structure and probe GC content also failed to explain these low signal intensity probes. Thus, we hypothesized that incomplete hybridization might owe to PCR bias stemming from differential abilities of the random primers to bind to the viral genome at the reverse transcription (RT) step. The random primer used in our experiments was a 26-mer comprised of a random nonamer (3') tagged with a fixed 17-mer sequence (5'-GTTTCCCAGTCACGATA)(SEQ ID NO:1)(see also Figure 1), where the purpose of the fixed 5' tag was to facilitate PCR of the RT product, generating PCR fragments of less than 10000bp, in particular 500-1000 bp PCR fragments (Pang et al. 2005; Wang et al. 2002; Wang et al. 2003). To study this phenomenon, we designed an algorithm (AES) to model the RT-PCR process using experimental data. Successful RT-PCR is dependent on the ability of

primers to bind to template. Intra-primer secondary structure formation, such as dimer and hairpin formation between to template. Intra-primer secondary structure formation, such as dimer and hairpin formation between the tag and nonamer, and probe melting temperature are known to influence binding efficiency(Nguyen and Southern, 2000; Ratushna et al. 2005).

Assuming that a nonamer in the random primer mix complements the sequence of the viral genome perfectly, the algorithm determines the probability that a 500-1000 bp product can be generated from each possible starting position in the genome. Thus, for every nucleotide in a sliding window of 1000 bases, the probability that it will be successfully amplified is reflected in its Amplification Efficiency Score (AES; See Amplification Efficiency Score above). To validate the algorithm, we ranked the hybridization signal intensities for all 1,948 SPS probes for the RSV genome and compared them to their AES values. Across the RSV genome, we observed that AES correlates remarkably well to hybridization signal intensities (Fisher's Exact Probability Test $P=2.2x10^{-16}$) demonstrating the strong correlation between AES and probe detection (Figure 12). Another comparison using 1,705 SPS probes for metapneumovirus showed a similar result, $P=1.3x10^{-9}$. The importance of AES in predicting SPS probe detection in clinical samples is demonstrated in Figure 10. Notably, we observed that higher values of AES correlated with greater proportions of detectable probes, particularly in the top 20% of AES values. Therefore, while HD, MCM, %GC and sequence uniqueness are valuable parameters of probe performance, they do not take into account PCR bias, and thus are insufficient predictors of probe performance when considered in the absence AES. Using top 20th percentile AES as the first filter in the selection of pathogen SPS significantly improved pathogen prediction as evidenced by higher WKL scores and elimination of false-positive calls (Table 3).

Table 3: Detecting pathogens using only mean probe signal intensities (T-test) results in high number of false-positive calls. Optimized hybridization signatures

and removal of probes which cross-hybridize to human genome (filtered) reduces false-positive calls but is not sufficient for detection accuracy. PDA v.1 is able to make an accurate diagnosis using the entire unfiltered probe set. A virus is "detected" if WKL score > 5. Using optimized hybridization signatures (filtered) increases the WKL score, corresponding to increased confidence of the diagnosis. Virus CT value: the real-time PCR cycle when virus was detected (see above).

		Detection using PDA v.1			Virus CT Value	Virus copy no.
Chip #	Pathogen	Max WKL score (no filters)	Max WKL score (filtered)	No. of viruses Detected		
32272	Pure SARS	5.007	5.803	1	--	--
34959	Pure Dengue	14.351	20.373	1	--	--
35259	RSV patient 324	18.288	20.611	1	21.4366	9.8×10^7
35179	hMPV patient 122	1.747	8.439	1	25.5388	50384
35253	RSV patient 841	12.056	12.069	1	20.8619	14×10^7
36042	RSV patient 412	16.466	17.531	1	23.5804	2.5×10^7
36053	RSV patient 483	12.089	12.168	1	24.8340	1.2×10^7
35915	non-pneumonia patient (negative control)	3.916	4.284	0	0	0

Data for all patient samples hybridized on the array are shown in Table 4 below.

Table 4. Complete list of clinical patients hybridized onto pathogen microarrays.

Array	Patient ID	WKL	P-value	PDA v.1 diagnosis	Clinical diagnosis*	Initial PCR diagnosis	PCR CT value	Virus copy no.	RT-PCR Primer
35179	122	8.439216	1.34×10^{-71}	hMPV	LRTI	hMPV	24.8	5.0×10^4	A1
35887	122	18.312077	2.98×10^{-22}	hMPV	LRTI	hMPV	24.8	5.0×10^4	A2
71180	133	17.359597	2.42×10^{-37}	hMPV	LRTI	hMPV	25.1159	4.0×10^4	A2
66691	165	8.56786	1.84×10^{-4}	hMPV	pneumonia	hMPV	27.9	3.9×10^3	A2
70935	254	21.348515	8.70×10^{-30}	hMPV	LRTI	hMPV	21.9518	5.4×10^5	A2

63781	283	16.680752	3.97×10^{-12}	hMPV	pneumonia	unknown			A2
73067	769	24.006323	1.34×10^{-51}	hMPV	LRTI	hMPV	25.6715	2.5×10^{4}	A2
66690	853			none detected	pneumonia	hMPV	36	0.5	A2
68359	892	12.534284	5.66×10^{-5}	Rhinovirus genus	pneumonia	hMPV	33.8	27	A2
35915	111			none detected	Negative ctrl	None			A1
70927	818			none detected	Negative ctrl	None			A2
66701	312			none detected	pneumonia	RSV A	33.7	44	A2
71006	321			none detected	pneumonia	RSV A	31.1	340	A2
66702	368			none detected	pneumonia	unknown			A2
71025	414	25.406289	3.80×10^{-24}	RSV B	pneumonia	RSV A	22.3	3.9×10^{5}	A2
71027	478			none detected	pneumonia	RSV A	34.8	18	A2
73068	832	59.275233	1.91×10^{-102}	RSV genus	LRTI	RSV A	23.7681	1.2×10^{5}	A2
71028	913	25.897084	3.23×10^{-30}	RSV B	pneumonia	RSV A	19.1	4.7×10^{6}	A2
66703	924	12.673149	9.71×10^{-6}	RSV genus	pneumonia	RSV A	31.5	250	A2
35259	324	20.61147	3.55×10^{-94}	RSV B	LRTI	RSV B	21.4366	3.0×10^{6}	A1
35662	355	17.999418	2.97×10^{-40}	RSV B	LRTI	RSV B	20.2642	6.7×10^{6}	A1
66695	374			none detected	pneumonia	RSV B	34.1	500	A2
70933	378	13.81578	7.77×10^{-17}	RSV B	LRTI	RSV B	23.9204	5.4×10^{5}	A2
36042	412	17.531234	4.58×10^{-55}	RSV B	LRTI	RSV B	23.5804	6.9×10^{5}	A1
35890	412	17.214556	1.05×10^{-43}	RSV B	LRTI	RSV B	23.5804	6.9×10^{5}	A2+A3
36053	483	12.168025	1.47×10^{-12}	RSV B	LRTI	RSV B	24.834	2.9×10^{5}	A1
70997	554	76.5474183 54.013223	1.83×10^{-119} 2.45×10^{-61}	Rhinovirus genus; Enterovirid ae family	pneumonia	RSV B	35.1	240	A2
35253	841	12.069138	4.86×10^{-26}	RSV B	pneumonia	RSV B	20.8619	4.4×10^{6}	A1
73070	841	22.108575.708560	6.80×10^{-50} 5.66×10^{-6}	RSV B, hMPV coinfection	pneumonia	RSV B/ hMPV	20.861935.4	4.4×10^{6} 8	A2

68360	841	21.369516 9.647188	2.09 x 10^{-25} 1.23 x 10^{-8}	RSV B, hMPV coinfection	pneumonia	RSV B/ hMPV	20.8619 35.4	4.4 x 10^{6} 8	A2
66696	185			none detected	pneumonia	unknown			A2
66697	261			none detected	pneumonia	unknown			A2
66698	331			none detected	pneumonia	unknown			A2
71189	393			none detected	pneumonia	unknown			A2
66699	461			none detected	pneumonia	unknown			A2
66700	573	41.39705 1 27.44489 3	3.97 x 10^{-23} 1.34 x 10^{-11}	Rhinovirus genus; Enterovirid ae family	pneumonia	unknown			A2
71182	639			none detected	pneumonia	unknown			A2
71007	699			none detected	pneumonia	unknown			A2
71188	859			none detected	pneumonia	unknown			A2

*LRTI: lower respiratory tract infection

The importance of AES suggested that amplification efficiency and subsequent probe detection could be improved by using optimized RT-PCR primer tags. Thus, we calculated AES scores using randomly generated 17-mer tag sequences, and selected the top 3 most divergent primers which resulted in the greatest overall increase in AES scores (Figure 13). Using the AES optimized primers, we amplified metapneumovirus and RSV from clinical samples with improved PCR efficiency and detection sensitivity (Figure 14, Table 5)

Table 5: Comparison of E-Predict and PDA v.1 algorithms on patient samples #412 and #122. Array 35179 was amplified using the original PCR primer described in Results. Arrays 36731 and 35887 were amplified using primer A2, and Array 35890 was amplified using both primers A2 and A3. PDA v.1 returned only the correct pathogen in all cases. The authors of E-Predict use $P<0.01$ as significance cutoff on their platform (Urisman et al. 2005). A lower cutoff appears to be necessary if this algorithm is used to analyze our array data. The

new primers designed by PCR modeling result in better prediction scores using either algorithms (arrays 35179 vs 35887). Having a second primer during the PCR process offered incremental improvement in WKL scores and P-values (arrays 36731 vs 35890).

Array	Patient	PCR amplifi-cation primers	E-Predict algorithm			GISPathogen algorithm	
			Genome	Similarity_Score	P-value	Genome	WKL
36042	412 (RSV)	Original primer A1	RSV	0.35128	0	RSV	21.526316
			OC43 coronavirus	0.350264	6.84E-20		
			229E coronavirus	0.323503	1.77E-10		
			Hepatitis B	0.134825	3.03E-04		
			SARS coronavirus	0.338911	0.00299		
			Hepatitis A	0.229589	0.00847		
36731	412 (RSV)	A2	RSV	0.335389	0	RSV	21.836754
			OC43 coronavirus	0.348043	2.29E-13		
			229E coronavirus	0.322055	2.00E-09		
			Hepatitis B	0.135222	1.02E-06		
			Rubella	0.164332	0.00919		
35890	412 (RSV)	A2 + A3	RSV	0.334602	0	RSV	22.093258
			OC43 coronavirus	0.348969	3.63E-23		
			229E coronavirus	0.322805	3.20E-14		
			Hepatitis B	0.13436	6.74E-04		
			SARS coronavirus	0.338609	0.03060		
35179	122 (hMPV)	Original primer A1	hMPV	0.260110695	5.01E-28	hMPV	9.763149
			Rubella	0.164784981	1.20E-17		
			Foot-and-mouth C	0.206747816	4.66E-11		
			Jap encephalitis	0.201347222	1.65E-04		
			Hepatitis B	0.133407622	1.98E-04		
			Yellow Fever	0.200500564	0.00567		
			Echovirus 1	0.222002025	0.01740		
			Newcastle	0.234481686	0.01820		
35887	122 (hMPV)	A2	hMPV	0.299655	0	hMPV	39.677149
			Rubella	0.169626	3.40E-19		
			Hepatitis B	0.137703	5.84E-12		
			OC43 coronavirus	0.347685	5.06E-10		

			229E coronavirus	0.321702	1.72E-06		
			SARS coronavirus	0.340504	1.76E-06		
			Foot-and-mouth C	0.2075	1.31E-04		
			Newcastle	0.23453	0.04310		

PDA v.1 – an algorithm for detecting pathogens

Clinical specimens are often sub-optimal for genomic amplification: they may have low viral titres, have sequence polymorphisms from the reference strain on the array, or have co-infecting pathogens. Microarrays also have an inherent noise from non-specific hybridization and other artifacts. Thus, interpreting microarray data is not a simple matter of matching probe signal intensity profiles to the SPS, or using simple statistical methods (e.g. T-test, ANOVA,and the like). To address this issue, we established a robust statistical software, PDA v.1, which analyzes the distribution of probe signal intensities relative to the *in silico* predicted SPS to identify pathogens present in a hybridized sample (See above).

Based on our observations that while the signal intensities for all probes on the array would fall in a normal distribution, a large proportion of probes comprising a pathogen SPS which is present in the sample would have very strong signal intensities resulting in a distribution skewed to the right; we deduced that we could detect the presence of pathogens by analyzing the distribution of probe signal intensities (Figure 9A). Examining the tails of the signal intensity distributions for each SPS would also enable us to identify the presence of co-infecting pathogens in the sample.

Thus, PDA v.1 comprises 2 parts: (1) Weighted Kullback-Leibler Divergence (WKL; our enhanced Kullback-Leibler test) to evaluate the probe signal intensity of probes in each pathogen SPS, and (2) an Anderson-Darling test to determine if the distribution of WKL scores for each SPS is normal.

The original Kullback-Leibler cannot reliably determine differences in the tails of a probability distribution, and is highly dependent on the number of probes/genome and the size of each signal intensity bin (Kullback and Leibler, 1951). We overcame these deficits by incorporating the Anderson-Darling statistic to give more weight to the tails of each distribution, and by using a cumulative distribution function instead of the original probability distribution (Anderson and Darling, 1952). We call our enhanced KL divergence the Weighted Kullback-Leibler divergence (WKL):

$$WKL(P_a \mid \overline{P_a}) = \sum_{j=0}^{k-1} \frac{Q_a(j)\log(\frac{Q_a(j)}{Q_{\bar{a}}(j)})}{\sqrt{Q_{\bar{a}}(j)\left[1-Q_{\bar{a}}(j)\right]}}$$

where $Q_a(j)$ is the cumulative distribution function of the signal intensities of the probes in P_a found in bin b_j; $Q_{\bar{a}}(j)$ is the cumulative distribution function of the signal intensities of the probes in $\overline{P_a}$ found in bin b_j. SPS representing absent pathogens should have normal signal intensity distributions and thus relatively low WKL scores, whereas those representing present pathogens should have high, statistically significant outlying WKL scores (Figure 9B). In the second part of PDA v.1, the distribution of WKL scores is subjected to an Anderson-Darling test for normality. If $P<0.05$, the WKL distribution is considered not normal, implying that the pathogens with outlying WKL score is present. Upon identification of a pathogen, a separate Anderson-Darling test is performed in the absence of its WKL score to test for the presence of co-infecting pathogens. In this manner, the procedure is iteratively applied until only normal distributions remain (i.e., $P>0.05$; see Table 3 and Table 4 above). PDA v.1 is extremely fast, capable of making a diagnosis from a hybridized microarray in about 10 secs.

Pathogen Diagnosis on 33 Clinical Patient Samples

We evaluated our platform by hybridizing 33 clinical specimens onto our pathogen microarray platform, according to the workflow illustrated in Figure 11. Of these, 27 specimens had been previously diagnosed as RSV A, RSV B or metapneumovirus. Our platform accurately detected pathogens from 21/27 samples. The 6 samples where no virus was detected (false-negative) were at the detection limit by real-time PCR (<10 viral copies/reaction), and such low viral loads were unlikely to be the etiologic agent responsible for the patient's severe disease. 2 of these were correctly diagnosed by microarray to be infected with rhinovirus. In a screen of another 6 patients with severe respiratory disease caused by unknown pathogen, the microarray identified the etiologic agent (rhinovirus) in 1 of the samples (Table 4 above). These results were validated by real-time PCR. As expected, we did not detect any pathogens when we hybridized samples extracted from pneumonia patients with non-viral etiology.

Data analysis

Microarrays were scanned at 5μm resolution using the Axon 4000b scanner and Genepix 4 software (Axon Instruments). Signal intensities were extracted using Nimblescan 2.1 software (NimbleGen Systems). Using an automated script, we calculated the median signal intensity (to eliminate hybridization artifacts) and standard deviation from the 7 replicates of each probe. The probe signal intensities were sorted by genome and arranged in sequence order, then reformatted into CDT format for graphical viewing of signal intensities in Java Treeview (http://jtreeview.sourceforge.net). In parallel, the probe median signal intensities were analysed using PDA v.1 to determine which pathogen is present, and associated confidence level of prediction. The present inventors carried out experiments to demonstrate the effects of probe design on

experimental results and then to show the robustness of the analysis algorithm according to the present invention.

Effects of probe design on experimental results

A PDC containing 53555 40-mer probes from 35 viruses affecting human was used for 4 independent microarray experiments. These 53555 probes were chosen based on a 5-bps tiling of each virus and were not subjected to any of our probe design criteria. Thus, we would expect errors arising due to CG-content, cross-hybridization and inefficient amplification to be significantly more than that of a PDC with well-designed probes. We tested our analysis algorithm in such an adverse setting for 4 experiments.

In this example, a human sample with an unknown pathogen was amplified by the RT-PCR process using random probes and then hybridized onto the PDC. We subjected the probes for each of the 35 viruses on our PDC to the one-tailed t-test with significance level 0.05 and computed the Weighted Kullback-Leibler (WKL) divergence of their signal intensities to the signal intensities of all the probes on the chip to determine which virus was in the sample for each experiment. Confirmation of the accuracy of the analysis by our program was done by wet-lab PCR to identify the actual virus in the sample. We present the results of our analysis for the 4 experiments of Table 6 and their corresponding PCR verifications in Table 6.

Table 6: Analysis results done on a PDC with no probe design criteria applied. The virus determined by our analysis algorithm to be the actual virus in the sample tested for each experiment is highlighted in light gray colour.

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
Sample Name	35259_324		35179_122		35253_841		35915_111	
ID	53555		53555		53555		53555	
Viruses (Accession No.)	t-test p-value	WKL	t-test p-value	WKL	t-test p-value	WKL	t-test p-value	WKL
NC_001781.1	0	16.391	1	NA	0	10.85635	1	NA
NC_003461.1	1	NA	1	NA	1	NA	1	NA
NC_003443.1	0.999324	NA	0.873017	NA	0.99802	NA	0.999961	NA
NC_001796.2	1	NA	1	NA	1	NA	1	NA
AY283794.1	0	0.5435	0.108141	NA	0	0.775959	0	0.435427
NC_005147.1	0	1.2896	1	NA	0	1.399591	0	1.762912
NC_002645.1	0	1.2943	0.999847	NA	0	1.655888	0	2.079334
NC_004148.2	1	NA	0.002733	5.762907	1	NA	1	NA
NC_002023.1 NC_002022.1 NC_002021.1 NC_002020.1 NC_002019.1 NC_002018.1 NC_002017.1	1	NA	0.579561	NA	1	NA	1	NA
NC_002204.1 NC_002205.1 NC_002206.1 NC_002207.1 NC_002208.1 NC_002209.1 NC_002210.1 NC_002211.1	1	NA	1	NA	1	NA	1	NA
NC_001563.2	1	NA	0.000001	0.537826	1	NA	0.995013	NA
NC_002031.1	1	NA	0.000005	0.758758	0.998873	NA	0.363947	NA
NC_002728.1	1	NA	0.999062	NA	1	NA	1	NA
NC_002617.1	0.999994	NA	0	0.571844	1	NA	0.769098	NA
NC_001802.1	1	NA	0.999966	NA	1	NA	1	NA
NC_003977.1	0	2.7424	0	2.189827	0	3.978747	0	1.490665
NC_001576.1	0.371224	NA	0.004643	0.94841	0.009599	1.257041	0	3.961532
NC_002554.1	0.000062	0.7146	0	1.527292	0.299334	NA	0.000002	0.166239
NC_001545.1	0	1.4545	0	2.438558	0	0.869782	0	0.989592
NC_001489.1	0	1.7088	0.319125	NA	0	2.593065	0	1.510399
NC_005222.1	0.999757	NA	0.646314	NA	0.773912	NA	0.807875	NA
NC_005217.1	0.60477	NA	0.999903	NA	0.354358	NA	0.000871	0.626818
NC_004294.1	0	1.8411	0.000523	0.902399	0	2.43215	0.000007	0.537531
NC_004291.1	0.662386	NA	0.954137	NA	0.255422	NA	0.099148	NA
NC_001437.1	1	NA	0	0.593093	1	NA	1	NA
AB189128.1	1	NA	0.906213	NA	1	NA	1	NA
AF326573.1	1	NA	0.038503	0.539783	1	NA	1	NA
AF489932.1	1	NA	0.899797	NA	1	NA	1	NA
M87512.1	1	NA	0.759668	NA	1	NA	1	NA
NC_001430.1	1	NA	0.912496	NA	1	NA	0.999912	NA
NC_001428.1	0.999988	NA	0.284792	NA	0.999346	NA	0.957164	NA

NC_001612.1	0.970379	NA	0.000001	0.557865	0.998878	NA	0.061226	NA
NC_003986.1	1	NA	0.000012	0.604474	1	NA	0.997945	NA
NC_001472.1	0.999999	NA	0.0046	0.455194	0.999579	NA	0.143404	NA
NC_001617.1	0.721465	NA	0.98373	NA	0.178733	NA	0.414209	NA
NC_001490.1	0.999808	NA	0.995029	NA	0.997369	NA	0.859025	NA
Deduction Virus	NC_001781.1 (RSV)		NC_004148.2 (HMPV)		NC_001781.1 (RSV)		None	
Confirmation Virus (PCR)	NC_001781.1 (RSV)		NC_004148.2 (HMPV)		NC_001781.1 (RSV)		None	

The present results show that the analysis algorithm accurately deduces the actual virus in the sample tested in the first 3 experiments (results shown in Table 6 above). Furthermore, we were able to deduce that the sample has no viruses in the last experiment. Note that if we had just used the t-test with level of significance 0.05, then the number of viruses detected to be present for each sample is shown in Table 7 below.

Table 7: False positive detection of viruses using t-test alone

Sample Name	35259_324	35179_122	35253_841	35915_111
Viruses Detected Using T-test	9	14	9	10
False Positives	8	13	8	10
Max KL divergence (> 5.0)	16.391	5.76	10.85	-
Viruses Detected Using T-test followed by KL divergence	1	1	1	0

By using the Weighted Kullback-Leibler divergence of the viruses that pass the t-test, we were able to remove all false positive viruses and identify the actual virus. Thus, our analysis algorithm can robustly determine the virus under a high level of noise.

Next, we investigated the effects of using a PDC with probe design criteria applied on our analysis results. Firstly, the amplification efficiency map for each

of the 35 viruses was computed. Then, the exact 53555 probes on the original PDC were subjected to probe design criteria. Probes which had extreme levels of CG-content, high similarity to human and non-target viruses, and low amplification efficiency scores were removed from the chip. A total of 10955 probes were retained for the second set of experiments. Using the samples used in the first set of experiments, we repeated the 4 experiments in Table 8 below with the new chip. The experimental results are presented in Table 8.

Table 8: Analysis results done on a PDC with probe design criteria applied. The virus determined by our analysis algorithm to be the actual virus in the sample tested for each experiment is highlighted in light gray colour.

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
Sample Name	35259_324		35179_122		35253_841		35915_111	
ID	10955		10955		10955		10955	
Viruses (Accession No.)	t-test p-value	WKL	t-test p-value	WKL	t-test p-value	WKL	t-test p-value	WKL
NC_001781.1	0	18.54859	1	NA	0	11.17914	1	NA
NC_003461.1	1	NA	1	NA	1	NA	1	NA
NC_003443.1	0.548718	NA	0.53727	NA	0.002783	0.837121	0.020436	0.603552
NC_001796.2	1	NA	0.999907	NA	1	NA	1	NA
AY283794.1	0	1.347801	0.024116	0.858364	0	1.523272	0	1.128637
NC_005147.1	0	1.604381	0.999697	NA	0	2.150019	0	2.893555
NC_002645.1	0	2.802742	0.999895	NA	0	4.612482	0	3.635771
NC_004148.2	1	NA	0.000003	9.324785	1	NA	1	NA
NC_002023.1 NC_002022.1 NC_002021.1 NC_002020.1 NC_002019.1 NC_002018.1 NC_002017.1	1	NA	0.124517	NA	1	NA	0.999163	NA
NC_002204.1 NC_002205.1 NC_002206.1 NC_002207.1 NC_002208.1 NC_002209.1 NC_002210.1 NC_002211.1	1	NA	0.998724	NA	1	NA	1	NA
NC_001563.2	0.986443	NA	0.428418	NA	0.76002	NA	0.112011	NA
NC_002031.1	0.998103	NA	0.003435	2.52162	0.278672	NA	0.409527	NA
NC_002728.1	0.999375	NA	0.30951	NA	0.969492	NA	0.297244	NA

NC_002617.1	0.63418	NA	0.003578	0.965856	0.247148	NA	0.025188	0.861163
NC_001802.1	1	NA	0.998118	NA	1	NA	1	NA
NC_003977.1	0	3.062956	0.000028	3.027442	0	4.574591	0	3.277708
NC_001576.1	0.579342	NA	0.101093	NA	0.155219	NA	0.026417	3.280335
NC_002554.1	0.6722	NA	0	2.289379	0.80654	NA	0.106683	NA
NC_001545.1	0	2.225817	0	2.794877	0.000019	1.674329	0	1.97064
NC_001489.1	0.099427	NA	0.999985	NA	0.000366	1.829543	0.000006	3.023235
NC_005222.1	0.999735	NA	0.294141	NA	0.974031	NA	0.356952	NA
NC_005217.1	0.916186	NA	0.994358	NA	0.600759	NA	0.032616	2.105628
NC_004294.1	0.867625	NA	0.235197	NA	0.100961	NA	0.052759	NA
NC_004291.1	0.992032	NA	0.964128	NA	0.714211	NA	0.206422	NA
NC_001437.1	1	NA	0.001058	1.563913	1	NA	0.857228	NA
AB189128.1	1	NA	0.732737	NA	0.999997	NA	0.98859	NA
AF326573.1	1	NA	0.435629	NA	0.999986	NA	0.905393	NA
AF489932.1	1	NA	0.322655	NA	0.999996	NA	0.996837	NA
M87512.1	0.999617	NA	0.057346	NA	0.999758	NA	0.937937	NA
NC_001430.1	1	NA	0.865038	NA	1	NA	0.882339	NA
NC_001428.1	1	NA	0.522986	NA	0.999351	NA	0.749412	NA
NC_001612.1	0.991708	NA	0.751091	NA	0.990929	NA	0.257635	NA
NC_003986.1	0.999997	NA	0.02014	0.93616	0.937996	NA	0.708985	NA
NC_001472.1	0.99959	NA	0.977242	NA	0.957869	NA	0.692936	NA
NC_001617.1	0.435562	NA	0.474076	NA	0.028549	1.699567	0.079676	NA
NC_001490.1	1	NA	0.90881	NA	0.996231	NA	0.518662	NA
Deduction Virus	NC_001781.1 (RSV)		NC_004148.2 (HMPV)		NC_001781.1 (RSV)		None	
Confirmation Virus (PCR)	NC_001781.1 (RSV)		NC_004148.2 (HMPV)		NC_001781.1 (RSV)		None	

In the following set of experiments, the analysis algorithm correctly detected the actual virus in the 3 samples and also the negative sample. After designing good probes for our chip, the Weighted Kullback-Leibler divergence of the actual viruses in Experiment 1, 2 and 3 was greater than that of the corresponding experiments without probe design. This means that the signal intensities from the actual virus were relatively higher than the background noise in the PDC. This showed that our probe design criteria had removed some bad probes from the PDC, which resulted in a more accurate analysis.

Again, we present results of the 4 experiments shown in Table 9 below, if we had just used the t-test with a level of significance 0.05. This time, the number of viruses detected to be present for each sample is shown in Table 9:

Table 9: False positive detection of viruses using t-test alone in a PDC with probe design.

Sample Name	35259_324	35179_122	35253_841	35915_111
Viruses Detected Using T-test	6	9	9	10
False Positives	5	8	8	10
Max KL divergence (> 5.0)	18.54859	9.324785	11.17914	-
Viruses Detected Using T-test followed by KL divergence	1	1	1	0

From Table 9, it can be seen that probe design has reduced the number of false positive viruses detected by the t-test for samples 35259_324 and 35179_122. A more important observation is that the Weighted Kullback-Leiber divergence for the actual virus has increased for all 4 samples. This means that the signals of the actual virus are more differentiated than the background signals when probe design criteria are applied on the PDC.

In conclusion, we showed that using the one-tailed t-test with significance level 0.05, followed by computing the Weighted Kullback-Leibler divergence for the signal intensities of each virus, we were able to accurately analyze the data on the PDC and determine with high probability the actual pathogen in the sample. Although the analysis algorithm works well even under a high level of noise, we showed that the accuracy of the analysis is improved by using the above-described probe design criteria to select a good set of probes for the PDC.

Alternative methods for probe design and pathogen detection

Very few algorithms are available for predicting cross-hybridization on microarrays and only 1 algorithm, E-predict, has been reported and validated for detecting pathogens on microarrays (Urisman et al. 2005; Li et al. 2005). E-predict matches hybridization signatures with predicted signatures derived from

the theoretical free energy of hybridization for each microarray probe. However, using E-predict to analyze our microarrays resulted in a number of false positive calls (see Table 5 above). For example, E-Predict detected coronavirus in RSV patient 412 (Figure 15). Diagnostic PCR using pancoronavirus primers as well as specific diagnostic primers for OC43 and 229E coronavirus confirmed the absence of coronavirus from patient 412 (see Table 4 above). We hypothesized that false positive calls using E-Predict resulted from coronavirus probes which cross-hybridized with human or RSV genomes. Indeed, 85% of the 50 coronavirus probes with highest signal intensity were predicted to cross-hybridize with human genome and 65% had HD<17 relative to RSV, which is just above our HD threshold of 12 for familial cross-hybridization. Furthermore, E-Predict was optimized to work on a microarray which contained probes that are highly conserved among viral genomes regions instead of tiling arrays where cross-hybridization to human genome would be a key consideration. Thus it is likely that these 2 factors – different microarray design strategy and cross-hybridization to human genome, contributed to the poor performance of E-predict on our platform. From our experience with E-predict, it would not be fair for us to compare PDA v1 with the other algorithms as they were designed for different probe lengths and optimized for other applications and platforms.

Conclusion

By empirically determining cross-hybridization thresholds, we created in silico pathogen signature probe sets comprising only probes which would hybridize well to specific viruses present in clinical samples. The AES algorithm allowed us to design universal primer tags to efficiently amplify entire viral genomes. Together with the PDA v.1 detection algorithm, we can confidently identify any of the pathogens represented on the microarray from clinical samples. This approach eliminates the requirement for empirical validation of each pathogen hybridization signature and allows for future microarrays containing probes for

>10000 pathogens to become powerful diagnostic platforms for pathogen identification.

We have optimized the design and analysis for pathogen detection microarrays, facilitating their use in a hospital setting. We discovered that primer tags routinely used in random PCR are biased, resulting in non-uniform amplification of pathogen genomes. This bias can be avoided by designing primers using our AES algorithm. Our in silico signature probe sets allow us to predict accurately which probes would hybridize to any pathogen represented on the array. Together with the PDA v.1 detection algorithm, this approach eliminates the requirement for empirical validation of each pathogen hybridization signature and allows for future microarrays containing probes for >10000 pathogens to become powerful diagnostic platforms for pathogen identification.

Here, we report the results of a systematic investigation of the complex relationships between viral amplification efficiency, hybridization signal output, target-probe annealing specificity, and reproducibility of pathogen detection using a custom designed microarray platform. Our findings form the basis of a novel methodology for the in silico prediction of optimal pathogen signature probe sets (SPS), shed light on the factors governing viral amplification efficiency (prior to microarray hybridization) and demonstrate the important connection between a viral amplification efficiency score (AES) and optimal probe selection. Finally, we describe a new statistics-based pathogen detection algorithm (PDA), that can rapidly and reproducibly identify pathogens in clinical specimens across a range of viral titers.

We have demonstrated the feasibility of using viral genome sequence obtained from publicly available databases, to detect viruses in clinical samples with a high degree of certainty if at least 4000 virus copies are present (see Table 3 above). Its sensitivity approaches that of antigen detection methods, making it a

clinically relevant detection tool (Liu et al. 2005; Marra et al. 2003). The ability to predict in silico pathogen hybridization signatures accurately presents a significant advance over current microarray methods, which require empirical validation by first hybridizing the array with pure pathogen samples. Besides specific identification of pathogens represented on the array, PDA v.1 allows identification of the pathogen class, family or genus for those genomes which are not specifically represented on the array (by relaxing thresholds for HD and MCM). This information is often sufficient for treatment decisions in the clinic. With an AES-optimized tag, we were able to identify virus from clinical samples which could not be detected earlier when amplified using a non-AES-optimized tag. Thus selection of tags by AES increased PCR efficiency and sensitivity of detection. The algorithm according to the invention may be applied to other tagged-based PCR applications, such as generation of DNA libraries and enrichment of RNA for resequencing.

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Claims

1. A method of designing at least one oligonucleotide for nucleic acid detection comprising the following steps in any order:

   (I) identifying and/or selecting at least one region of at least one target nucleic acid to be amplified, the region(s) having an efficiency of amplification (AE) higher than the average AE; and

   (II) designing at least one oligonucleotide capable of hybridizing to the identified and/or selected region(s).

2. The method according to claim 1, wherein the AE of the selected region(s) is calculated as the Amplification Efficiency Score (AES), which is the probability that a forward primer r_i can bind to a position i and a reverse primer r_j can bind at a position j of the target nucleic acid, and $|i - j|$ is the region of the target nucleic acid desired to be amplified.

3. The method according to any one of the preceding claims, wherein step(I) comprises determining the effect of geometrical amplification bias for every position of a target nucleic acid, and selecting at least one region to be amplified as the region(s) having an efficiency of amplification (AE) higher than the average AE.

4. The method according to claim 3, wherein the geometrical amplification bias is the PCR bias.

5. The method according to any one of the preceding claims, wherein the at least one oligonucleotide capable of hybridizing to the selected region(s) is selected and designed according to at least one of the following criteria:

   (a) the selected oligonucleotide(s) has a CG-content from 40% to 60%;

   (b) the oligonucleotide(s) is selected by having the highest free energy computed based on Nearest-Neighbor model;

(c) given oligonucleotide s_a and oligonucleotide s_b substrings of target nucleic acids v_a and v_b, s_a is selected based on the hamming distance between s_a and any length-*m* substring s_b from the target nucleic acid v_b and/or on the longest common substring of s_a and oligonucleotide s_b;

(d) for any oligonucleotide s_a of length-*m* specific for the target nucleic acid v_a, the oligonucleotide s_a is selected if it does not have any hits with any region of a nucleic acid different from the target nucleic acid, and if the oligonucleotide s_a length-m has hits with the nucleic acid different from the target nucleic acid, the oligonucleotide s_a length-*m* with the smallest maximum alignment length and/or with the least number of hits is selected; and

(e) an oligonucleotide p_i at position i of a target nucleic acid is selected if p_i is predicted to hybridize to the position *i* of the amplified target nucleic acid.

6. The method according to claim 5, wherein under the criterion (e), an oligonucleotide p_i at position *I* of a target nucleic acid v_a is selected if $P(p_i \mid v_a) > \lambda$, wherein λ is 0.5 or 0.8 and $P(p_i \mid v_a)$ is the probability that p_i hybridizes to the position *i* of the target nucleic acid v_a.

7. The method according to any one of the preceding claims, wherein $P(p_i \mid v_a) \approx P(X \leq x_i) = \frac{c_i}{k}$, wherein *X* is the random variable representing the amplification efficiency score (*AES*) values of all oligonucleotide(s) of v_a, *k* is the number of oligonucleotide(s) in v_a, and c_i is the number of oligonucleotide(s) whose *AES* values are $\leq x_i$.

8. A method of detecting at least one target nucleic acid comprising the steps of:

(i) providing at least one biological sample;

(ii) amplifying nucleic acid(s) comprised in the biological sample;

(iii) providing at least one oligonucleotide capable of hybridizing to at least one target nucleic acid, if present in the biological sample, wherein the oligonucleotide(s) is designed and/or prepared according to the method of any one of claims 1 to 7; and

(iv) contacting the oligonucleotide(s) with the amplified nucleic acids and/or detecting the oligonucleotide(s) hybridized to the target nucleic acid(s).

9. The method according to any one of the preceding claims, wherein the amplification step is carried out in the presence of at least one random forward primer and/or at least one reverse random primer.

10. The method according to any one of the preceding claims, wherein the amplification step is a RT-PCR.

11. The method according to any one of claims 2 to 10, wherein the forward random primer binding to position *i* and the reverse random primer binding to position j of a target nucleic acid v_a are selected among primers having an amplification efficiency score (AES_i) for every position *i* of a target nucleic acid v_a of :

$$AES_i = \sum_{j=i-Z}^{i} \left\{ P^f(j) \times \sum_{k=\max(i+1,\, j+500)}^{j+Z} P^r(k) \right\}$$

wherein $\sum_{k=\max(i+1,\, j+500)}^{j+Z} P^r(k)$ = $P^r(i+1) + P^r(i+2) + \ldots\ldots P^r(j+Z)$;

$P^f(i)$ and $P^r(i)$ are the probabilities that a random primer r_i binds to position i of v_a as forward primer and reverse primer respectively, and Z ≤ 10000 bp is the region of v_a desired to be amplified.

12. The method according to any one of the preceding claims, wherein the amplification step comprises forward and reverse primers, and each of the forward and reverse primers comprises, in a 5'-3' orientation, a fixed primer header and a variable primer tail, and wherein at least the variable tail hybridizes to a portion of the target nucleic acid va and/or the amplification step comprises forward and/or reverse random primers having the nucleotide sequence of SEQ ID NO:1 or a variant or derivative thereof.

13. The method according to any one of claims 8 to 12, wherein in the detection step (iv), the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

14. The method according to any one of claims 8 to 12, wherein in the detection step (iv), the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, and the method further comprises the step of computing the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of the probes used in the detection method having high signal intensities, the density distribution of the signal intensities of probes v_a being more positively skewed than that of probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

15. The method according to any one of claims 8 to 12, wherein in the detection step (iv), the presence of at least one target nucleic acid in a biological sample is given by a value of t-test ≤ 0.1 and/or Anderson-Darling test

value ≤ 0.05 and/or a value of Weighted Kullback-Leibler divergence of = 1.0.

16. The method according to claim 15, wherein the t-test value is ≤ 0.05 and/or the value of Weighted Kullback-Leibler divergence is = 5.0.

17. The method according to any one of claims 8 to 12, wherein the detection step (iv) comprises evaluating the signal intensity of probe(s) in each signature probe set (SPS) for the target nucleic acid(s) v_a by calculating the distribution of Weighted Kullback-Leibler (WKL) divergence scores:

$$WKL(P_a \mid \overline{P_a}) = \sum_{j=0}^{k-1} \frac{Q_a(j)\log(\frac{Q_a(j)}{Q_{\bar{a}}(j)})}{\sqrt{Q_{\bar{a}}(j)\left[1-Q_{\bar{a}}(j)\right]}}$$

where $Q_a(j)$ is the cumulative distribution function of the signal intensities of the probes in P_a found in bin b_j; $Q_{\bar{a}}(j)$ is the cumulative distribution function of the signal intensities of the probes in $\overline{P_a}$ found in bin b_j. P_a is the set of probes of a virus v_a and $\overline{P_a}$ = P - P_a.

18. The method according to claim 17, wherein each signature probe set (SPS) which represents the absence of target nucleic acid(s) v_a has a normally distributed signal intensity and/or a Weighted Kullback-Leibler (WKL) divergence score of WKL<5 or alternatively, each signature probe set (SPS) which represents the presence of at least one target nucleic acid v_a has a positively skewed signal intensity distribution and/or a Weighted Kullback-Leibler (WKL) divergence score of WKL>5.

19. The method according to any claim 17 or 18, further comprising performing Anderson-Darling test on the distribution of WKL scores, wherein a result of $P<0.05$ thereby indicates the presence of target nucleic acid(s) v_a .

20. A method of determining the presence of at least one target nucleic acid v_a comprising detecting the hybridization of at least one oligonucleotide probe designed and/or prepared according to the method of any one of claims 1 to 7, to at least one target nucleic acid v_a and wherein the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, thereby indicating the presence of v_a.

21. The method according to claim 20, wherein the mean of the signal intensities of the probe(s) which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, and the method further comprises the step of computing the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of the probes used in the detection method having high signal intensities, the density distribution of the signal intensities of probes v_a being more positively skewed than that of probes $\notin v_a$, thereby indicating the presence of v_a.

22. The method according to claim 20 or 21, wherein the presence of at least one target nucleic acid in at least one biological sample is given by a value of t-test ≤ 0.1 and/or Anderson-Darling test value ≤ 0.05 and/or a value of Weighted Kullback-Leibler divergence of = 1.0.

23. The method according to claim 22, wherein the t-test value is ≤ 0.05 and/or the value of Weighted Kullback-Leibler divergence is = 5.0.

24. An apparatus for designing at least one oligonucleotide for nucleic acid detection, the apparatus being configured to

(I) identify and/or select at least one region(s) of at least one target nucleic acid to be amplified, the region(s) having an efficiency of amplification (AE) higher than the average AE; and

(II) design at least one oligonucleotide capable of hybridizing to the identified and/or selected region(s).

25. The apparatus according to claim 24, wherein the AE of the selected region(s) is calculated as the Amplification Efficiency Score (AES), which is the probability that a forward primer r_i can bind to a position i and a reverse primer r_j can bind at a position j of the target nucleic acid, and $|i - j|$ is the region of the target nucleic acid desired to be amplified.

26. The apparatus according to claim 24 or 25, wherein step(I) comprises determining the effect of geometrical amplification bias for every position of a target nucleic acid, and selecting at least one region to be amplified as the region(s) having an efficiency of amplification (AE) higher than the average AE.

27. The apparatus according to claim 26, wherein the geometrical amplification bias is the PCR bias.

28. The apparatus according to any one of claims 24 to 27, wherein the oligonucleotide(s) capable of hybridizing to the selected region(s) is selected and/or designed according to at least one of the following criteria:

(a) the selected oligonucleotide(s) has a CG-content from 40% to 60%;

(b) the oligonucleotide(s) is selected by having the highest free energy computed based on Nearest-Neighbor model;

(c) given oligonucleotide s_a and oligonucleotide s_b substrings of target nucleic acids v_a and v_b, s_a is selected based on the hamming

distance between s_a and any length-*m* substring s_b from the target nucleic acid v_b and/or on the longest common substring of s_a and oligonucleotide s_b;

(d) for any oligonucleotide s_a of length-*m* specific for the target nucleic acid v_a, the oligonucleotide s_a is selected if it does not have any hits with any region of a nucleic acid different from the target nucleic acid, and if the oligonucleotide s_a length-m has hits with the nucleic acid different from the target nucleic acid, the oligonucleotide s_a length-*m* with the smallest maximum alignment length and/or with the least number of hits is selected; and

(e) at least one oligonucleotide p_i at position i of a target nucleic acid is selected if p_i is predicted to hybridize to the position i of the amplified target nucleic acid.

29. The apparatus according to claim 28, wherein under the criterion (e), an oligonucleotide p_i at position *I* of a target nucleic acid v_a is selected if $P(p_i \mid v_a) > \lambda$, wherein λ is 0.5 or 0.8 and $P(p_i \mid v_a)$ is the probability that p_i hybridizes to the position *i* of the target nucleic acid v_a.

30. The apparatus according to any one of claims 24 to 29, wherein $P(p_i \mid v_a) \approx P(X \leq x_i) = \frac{c_i}{k}$, wherein *X* is the random variable representing the amplification efficiency score (*AES)* values of all oligonucleotides of v_a, *k* is the number of oligonucleotides in v_a, and c_i is the number of oligonucleotides whose *AES* values are $\leq x_i$.

31. An apparatus configured to detect at least one target nucleic acid comprising any one of the steps of:

(i) providing at least one biological sample;

(ii) amplifying nucleic acid(s) comprised in the biological sample;

(iii) providing at least one oligonucleotide capable of hybridizing to at least one target nucleic acid, if present in the biological sample, wherein the oligonucleotide(s) is designed and/or prepared according to the apparatus being configured according to any one of claims 53 to 63; and

(iv) contacting the oligonucleotide(s) with the amplified nucleic acids and/or detecting the oligonucleotide(s) hybridized to the target nucleic acid(s).

32. The apparatus according to any one of claims 24 to 31, wherein the amplification step is carried out in the presence of at least one random forward primer and/or at least one reverse random primer.

33. The apparatus according to any one of claims 24 to 32, wherein the amplification step is a RT-PCR.

34. The apparatus according to any one of claims 24 to 33, wherein the forward random primer binding to position *i* and the reverse random primer binding to position j of a target nucleic acid v_a are selected among primers having an amplification efficiency score (AES_i) for every position *i* of a target nucleic acid v_a of :

$$AES_i = \sum_{j=i-Z}^{i} \left\{ P^f(j) \times \sum_{k=\max(i+1,\, j+500)}^{j+Z} P^r(k) \right\}$$

wherein $\sum_{k=\max(i+1,\, j+500)}^{j+Z} P^r(k)$ = $P^r(i+1) + P^r(i+2) + \ldots\ldots P^r(j+Z)$;

$P^f(i)$ and $P^r(i)$ are the probabilities that a random primer r_i binds to position *i* of v_a as forward primer and reverse primer respectively, and $Z \leq 10000$ bp is the region of v_a desired to be amplified.

35. The apparatus according to any one of claims 24 to 34, wherein the amplification step comprises forward and reverse primers, and each of the forward and reverse primers comprises, in a 5'-3' orientation, a fixed primer header and a variable primer tail, and wherein at least the variable tail hybridizes to a portion of the target nucleic acid v_a and/or the amplification step comprises forward and/or reverse random primers having the nucleotide sequence of SEQ ID NO:1 or a variant or derivative thereof.

36. The apparatus according to any one of claims 31 to 35, wherein in the detection step (iv), the mean of the signal intensities of the probe(s) which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

37. The apparatus according to any one of claims 31 to 35, wherein in the detection step (iv), the mean of the signal intensities of the probes which hybridize to v_a is statistically higher than the mean of the probes $\notin v_a$, and the apparatus being configured further comprises the step of computing the relative difference of the proportion of probes $\notin v_a$ having high signal intensities to the proportion of the probes used in the detection method having high signal intensities, the density distribution of the signal intensities of probes v_a being more positively skewed than that of probes $\notin v_a$, thereby indicating the presence of v_a in the biological sample.

38. The apparatus according to any one of claims 31 to 35, wherein in the detection step (iv), the presence of a target nucleic acid in a biological sample is given by a value of t-test $\leq$ 0.1 and/or Anderson-Darling test value $\leq$ 0.05 and/or a value of Weighted Kullback-Leibler divergence of = 1.0.

39. The apparatus according to claim 38, wherein the t-test value is ≤ 0.05 and/or the value of Weighted Kullback-Leibler divergence is = 5.0.

40. The apparatus according to any one of claims 31 to 35, wherein the detection step (iv) comprises evaluating the signal intensity of probe(s) in each signature probe set (SPS) for the target nucleic acid(s) by calculating the distribution of Weighted Kullback-Leibler (WKL) divergence scores:

$$WKL(P_a \mid \overline{P_a}) = \sum_{j=0}^{k-1} \frac{Q_a(j)\log(\frac{Q_a(j)}{Q_{\bar{a}}(j)})}{\sqrt{Q_{\bar{a}}(j)\left[1-Q_{\bar{a}}(j)\right]}}$$

where $Q_a(j)$ is the cumulative distribution function of the signal intensities of the probes in P_a found in bin b_j; $Q_{\bar{a}}(j)$ is the cumulative distribution function of the signal intensities of the probes in $\overline{P_a}$ found in bin b_j, and where P_a is the set of probes of a virus v_a and $\overline{P_a} = P - P_a$.

41. The apparatus according to claim 40, wherein each signature probe set (SPS) which represents the presence of at least one target nucleic acid has a positively skewed signal intensity distribution and/or a Weighted Kullback-Leibler (WKL) divergence score of WKL>5.

42. The apparatus according to any one of claims 40 to 41, further comprising performing Anderson-Darling test on the distribution of WKL score(s), wherein a result of P>0.05 thereby indicates the absence of target nucleic acid(s) or alternatively, a result of P<0.05 thereby indicates the presence of target nucleic acid(s).

43. The apparatus according to claim 42, wherein a further Anderson-Darling test is performed thereby indicating the presence of further co-infecting target nucleic acid(s).

44. A computer program product configured to perform the method according to any one of claims 1 to 23 and/or a computer program product comprising a software configured to determine the WKL divergence score and/or Anderson-Darling test for designing at least one oligonucleotide and/or detecting at least one target nucleic acid, wherein the WKL, Anderson-Darling test, the designing of oligonucleotide probe(s), the designing of oligonucleotide primer(s), and/or the detecting of target nucleic acid(s) are defined according to any one of claims 1 to 23.

45. A removable electronic storage medium comprising a software configured to perform the method according to any one of claims 1 to 23 and/or a software configured to determine the WKL divergence score and/or Anderson-Darling test for designing at least one oligonucleotide probe, the designing of oligonucleotide primer(s), and/or detecting at least one target nucleic acid, wherein the WKL, Anderson-Darling test, the designing of oligonucleotide probe(s), the designing of oligonucleotide primer(s), and/or the detecting of target nucleic acid(s) are defined according to any one of claims 1 to 23.