Title	Recent advances in the development of nucleic acid diagnostics
Author(s)	O'Connor, Louise; Glynn, Barry
Publication Date	2010
Publication Information	O'Connor L, Glynn B. (2010) 'Recent advances in the development of nucleic acid diagnostics' Expert Rev Med Devices. 2010 Jul 7(4):529-39
Link to publisher's version	http://www.expert-reviews.com/doi/abs/10.1586/erd.10.22
Item record	http://hdl.handle.net/10379/2263

EXPERT REVIEWS

Recent advances in the development of nucleic acid diagnostics

Louise O'Connor[†1] and Barry Glynn[1]

[1]*National University of Ireland, Galway, Ireland*
[†]*Author for correspondence: louise.oconnor@nuigalway.ie*

Since the early 1970s, the use of nucleic acid sequences for specific diagnostic applications has followed a somewhat linear pattern of development. Early methods for restriction enzyme digestion as well as reverse transcription were followed in the late 1970s by Southern, northern and dot blotting, as well as DNA sequencing. In 1985, the description of PCR and the routine laboratory manipulation of sufficient quantities of DNA for diagnostics, resulted in the exponential growth of molecular biology. Subsequently, alternative DNA and RNA amplification protocols followed. The last 10 years have seen the second explosion in molecular biology with the development of real-time quantitative PCR and oligonucleotide microarrays. This advancement continues with the development of methods for 'direct' nucleic acid target detection from samples without *in vitro* amplification, and enhanced transduction elements for improved sensitivity of nucleic acid detection. In this article, we will describe the current state of the art in nucleic acid diagnostics, the use of nucleic acid-based diagnostics in clinical practice and the emerging technologies in the field. Finally, we will describe future trends and expected advances in the field.

Nucleic acid diagnostics

Within each species of microorganism there exist unique nucleic acid signature sequences that can be exploited to determine the presence of that specific microorganism. Nucleic acid-based diagnostics (NADs) refers to the use of these specific sequences of nucleic acid (either DNA or RNA) to detect the presence of a pathogenic microorganism in a clinical sample. In addition to identification of the particular microorganism, clinically relevant information related to that particulate strain can also be elucidated, including its resistance to various antibiotics and its virulence related to other strains. NAD techniques have several advantages over alternative technologies, such as microbiological culture and immunological-based methods. These include faster turnaround time and improved sensitivity and specificity for the detection of organisms of interest relative to traditional methods. Currently, NAD systems are available in a variety of formats ranging from simple nucleic acid probe hybridization systems, to tests incorporating amplification of a specific genomic target utilizing one of many available *in vitro* amplification technologies. At their core, all NADs require a nucleic acid target sequence, unique to the microorganism of interest. Ideally, this candidate NAD target should be present in the cell at relatively high copy number, while being sufficiently heterologous at the sequence level to allow for differentiation of the pathogen at both genus and species levels.

The basis of most NAD tests is DNA. DNA is a very stable molecule, which makes it particularly suitable as a molecular target for NAD tests as it can be isolated relatively simply from a variety of complex biological samples [1–3].

To date, NAD tests have utilized a wide variety of genomic targets, including multicopy genes, such as those coding for ribosomal RNA (rRNA), toxin encoding genes or virulence factors and genes involved in cellular metabolism. For example, our research group at the National University of Ireland (Galway, Ireland) has successfully employed the 16S/23S rRNA intergenic spacer region in bacteria as a molecular target for the development of a variety of NAD tests for a variety of microorganisms [1–4].

10.1586/ERD.10.22   ISSN 1743-4440   **1**

In addition to using DNA as a target in NADs, RNA is a useful alternative nucleic acid target. Compared with DNA, RNA is an especially labile molecule that is degraded quickly and easily, particularly once the organism is killed. This property makes handling RNA much more difficult than DNA but means RNA has the potential advantage of enabling viable organisms to be distinguished from nonviable organisms.

In terms of using RNA for diagnostics, rRNA remains the 'gold standard' target for pathogen identification and has been exploited in a range of NADs for identification of food pathogens [5]. Gen-Probe Inc. (CA, USA) have patented the use of rRNA as a target technology and have used it in a range of tests [101].

At the National University of Ireland, we have also investigated the possibility of using functional, high copy number RNAs other than rRNA for the rapid, sensitive and specific detection of microbial pathogens. For example, the bacterial *ssrA* gene and its encoded transcript, transfer-messenger RNA (tmRNA), present in all bacterial phyla, has many of the desired properties of a NAD target [6]. tmRNA is present in all bacterial species at relatively high transcript numbers [7], for example *Escherichia coli* has been shown previously to have approximately 5×10^2 tmRNA molecules per cell. This high copy number of tmRNA is advantageous in developing NADs [8,9] and makes tmRNA a useful target for species-specific bacterial detection [6,10,11].

Detection formats

Formats for the detection of nucleic acid signature sequences of interest can be divided into two categories. These are direct detection methods, which utilize a probe to hybridize directly to the target sequence of interest, and those employing an *in vitro* amplification technology to increase the amount of the target sequence followed by detection of the amplified target.

Direct detection systems are available in multiple formats; for example, direct nucleic acid probe hybridization tests are applied widely in food testing laboratories for confirmation of the identity of organisms following culture-based isolation of the food-borne pathogen of concern [12]. Such test systems require that the samples are culture-enriched before analysis. This is then followed by a simple lysis procedure to release target nucleic acid material before probe analysis is performed. FISH is another example of a direct detection method, which was first reported by DeLong *et al.* in 1989, and involves the hybridization of the fluorescent probe to intact nucleic acid [13]. While the technique is most commonly used in the environmental field [14], it also has clinical applications [15]. An advantage of such direct detection methods is that there is no requirement for sophisticated equipment; they are simple to perform and have detection limits of 10^4–10^5 bacterial cells [16].

The second format involves the detection of nucleic acid following an *in vitro* amplification technique. Common amplification techniques employed to increase the target nucleic acid prior to detection include PCR, nucleic acid sequence-based amplification (NASBA) and strand displacement amplification (SDA).

Initially, these amplification based technologies relied on what was called 'end point' analysis. This involved, in the case of PCR for example, allowing the amplification reaction to proceed to completion before taking an aliquot of the amplification reaction and running it through an agarose gel. Arguably, the most significant advance in molecular diagnostics since the initial description of PCR for target amplification has been the development of real-time detection formats. Real-time PCR monitors the accumulation of the amplification product in a reaction while it is taking place compared with end point detection of the amplification product in conventional PCR. These technologies provide sensitive, quantitative detection of PCR products in a fast turnaround time in a closed tube format, thereby significantly reducing the risk of contamination [17,18]. For clinical diagnostic applications, virtually all conventional amplification techniques have been superseded by their 'real-time' equivalents. These techniques have the advantage of not only being faster and more sensitive, but also introducing the concept of quantitative nucleic acid detection, as the rate of increase of amplification product is found to be proportional to the amount of target material present in the starting sample.

The popularity of these *in vitro* amplification-based diagnostic techniques mostly relates to simplicity of use, robustness and sensitivity.

The following section describes the most common amplification techniques used.

PCR

The first report of specific DNA amplification using the PCR was in 1985. The basic method was developed by Kary Mullis [19]. Since this time, the field of application has expanded enormously to include clinical, veterinary, food and environmental areas. As mentioned earlier, the basic PCR technique has, in recent years, been combined with fluorescently labeled probe hybridization in the same reaction, allowing real-time monitoring of the target amplification. This development, known as real-time PCR, has been responsible for the increase in popularity of molecular assays in diagnostic laboratories. Most real-time methods are based on the principle of fluorescence resonance energy transfer. Fluorescence resonance energy transfer occurs when two fluorescently labeled molecules are in close proximity to each other and the energy from an excited donor molecule is transferred to an acceptor molecule.

Real-time PCR platforms utilize different detection formats. Fluorescent technologies employed are either nonspecific, using dyes such as SYBR Green I or SYBR Gold, which intercalate into the PCR product during the reaction, or specific, using probes to detect specific sequence amplification in the PCR. A number of different fluorescent probe chemistries have been employed in real-time PCR assays including TaqMan® (5′exonuclease) probes, HybProbes, molecular beacons and Scorpion probes. While the mechanism of fluorescent signal generation is different for each of the probe chemistries, the fluorescent signal generated by the probes or minor groove binding dyes is directly proportional to the amount of PCR product generated [20,21]. Real-time PCR is quantitative with a broader dynamic range than conventional PCR. Informative reviews of the mechanisms of real-time PCR are available, including Bustin and Mueller [22] and Dorak [102].

The most commonly used chemistries utilized in real-time platforms are the hydrolysis or TaqMan probe [23] and HybProbes. Taqman probes consists simply of an oligonucleotide probe that has a fluorescent label attached to the 5′ end and a quencher at the 3′ end. Once bound to the target during the PCR reaction, the probe is cleaved by DNA polymerase, which separates the fluorescent label from the quencher. When the quencher and the label are no longer in close proximity, the signal is emitted and detected in the real-time machine. **Figure 1** outlines the mechanism of action of TaqMan probes. The fluorescence is directly proportional to the amount of PCR product produced. The hybridization probe or HybProbe format was developed specifically for use on the LightCycler® instrument (Roche, Basel, Switzerland). In this detection format, two separate probes are designed to hybridize next to each other on the target DNA. HybProbes are most commonly used for melting curve analysis, which is based on the probes dissociating from the target DNA at a certain temperature. Numerous protocols are described in the literature for the use of hybridization probes [24]. Another chemistry less often utilized but none the less extremely specific is molecular beacons. These are probes that form a stem–loop structure when in solution and emit a signal only when bound to their specific target [25]. An example of a commercial assay using molecular beacons is the methicillin-resistant *Staphylococcus aureus* (MRSA) assay from Cepheid (CA, USA), which is performed on the Smartcycler® instrument. Real-time PCR is most often used for detection and quantification of microorganisms but another use of real-time PCR is the ability to quantify and genotype genetic variations, such as single nucleotide polymorphisms (SNPs), using a set of specific probes for each possible SNP nucleotide within the same assay. An example of a commercially available test for SNP identification would be the Factor V Leiden test from Roche.

Advantages of real-time PCR include that it is a rapid, accurate and highly sensitive technique and can be utilized for both qualitative and quantitative analysis, and for genotyping **(Table 1)**. These advantages have led to an explosion in the development of real-time PCR assays for diagnostic purposes driven by the need for faster and more accurate diagnostic tests.

Alternative in vitro *amplification technologies*

A wide variety of alternative *in vitro* amplification-based technologies have been described including ligase chain reaction, branched DNA amplification, NASBA, SDA, transcription-mediated amplification, loop mediated amplification, rolling circle amplification and TwistAmp™ **(Table 1)**.

Many of these amplification technologies have been applied to detect pathogens in clinical microbiology. For instance SDA allows for 10^{10}-fold amplification of a DNA target sequence in
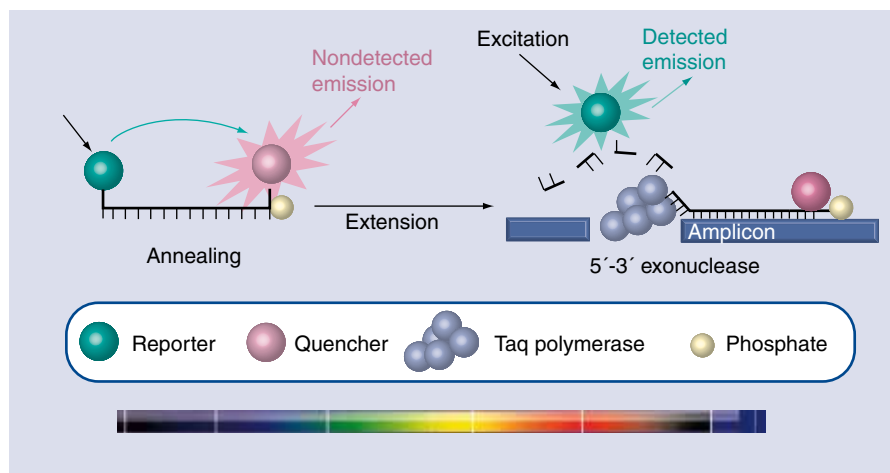

Figure 1. Schematic of TaqMan® probe technology.
Courtesy of TibMolBiol Berlin.

as little as 15 min [26]. It was the first nucleic acid amplification technology to be coupled with real-time homogeneous fluorescence-based detection for routine application in the clinical laboratory. The isothermal nature of the reaction process offers distinct advantages with regard to the cost and simplicity of instrumentation, while a universal detection format permits the use of the same fluorescent detector probes across multiple analytes [27]. SDA, for example, has been applied to the identification of pathogenic *E. coli* in water samples [28]. Transcription-mediated amplification, a proprietary technology of Gen-Probe Inc., is another isothermal RNA-based amplification system that combines the use of two enzymes to amplify either a DNA or RNA target. NASBA is yet another popular and commonly used amplification technique. It is an isothermal amplification technology that uses three enzymes to amplify RNA or DNA targets and it can be used to detect viable cells [29]. As NASBA can be employed as an RNA-based amplification technology, it is particularly suited for applications where the assessment of the viability status of a pathogen is a requirement [30–33]. In fact, NASBA was initially designed to only amplify from RNA templates.

Most of these platforms enable pathogen detection based on *in vitro* amplification of a specific DNA and RNA target; however, some technologies, for example branched DNA, enable detection and identification through probe-mediated signal amplification. Other proprietary *in vitro* amplification technologies include ligase chain reaction (Abbott Diagnostics, IL, USA) and Hybrid Capture (Digene, MD, USA). A common feature of many of these alternative amplification methods is that they are isothermal; that is, they only require one temperature to work instead of the thermal cycling required for PCR. The major advantage of isothermal technologies is that they lend themselves easily to automation since no heating and cooling elements need to be included in the diagnostics instrument. This may be of particular interest in the area of microdevices and point-of-care technologies, which will be referred to later in this article.

Table 1. Summary of *in vitro* amplification technologies.

Amplification technology	Features	Company	Ref.
PCR	DNA/RNA, efficient, specific	Roche Cepheid	[105] [103]
Nucleic acid sequence-based amplification	RNA/DNA, isothermal, efficien	bioMérieux	[106]
Strand displacement amplification	DNA, isothermal	Becton Dickinson	[107]
Ligase chain reaction	DNA, specific	Abbott	[104]
Branched DNA amplification	DNA/RNA, isothermal	Chiron	[108]
Loop-mediated amplification	DNA, isothermal	Eiken Chemical Company	[109]
Rolling circle amplification technology	DNA/RNA, isothermal	Molecular Staging Inc.	[110]
Transcription-mediated amplification	DNA/RNA, isothermal, rapid	GenProbe	[101]
TwistAmp™	DNA, rapid, isothermal	TwistDx	[111]

Microarrays

Microarrays for microbial identification have been used in environmental and clinical settings, among others. For use in diagnostics, the principle advantage of array-based detection formats is the very high probe density that can be incorporated into a single assay. Clinical microarrays (applied in medical, veterinary, food and biodefence scenarios) are required to rapidly and reliably detect the presence of specific species and strain types of only a few potentially interesting microbes. The ability to immobilize large numbers of oligonucleotide probes onto an array allows for simultaneous multiorganism detection in a single experiment [34].

Clinically relevant species identified on microarrays have included *Chlamydia* and *Chlamydophila spp.* [35]. The authors reported the possibility of direct detection of pathogens from clinical tissue, although their then reported detection limit was in the order of 6×10^6 target molecules. However, this is still far superior to sensitivities reported for gel-electrophoresis-based detection (1×10^{11}). Bacterial rRNA genes have many of the properties associated with an ideal diagnostics target. It is no surprise, therefore, to find in the literature many instances of the microarray-based detection of this nucleic acid diagnostic target. The advantage of this target on a microarray is the possibility of differentiating between very large numbers of species potentially present in a single assay. The high intracellular levels of rRNA in bacteria also make it an interesting target for microarray detection without *in vitro* amplification [36,37].

One serious difficulty associated with the successful penetration of microarray detection systems into the market place is the extremely high cost of the associated infrastructure (hybridization stations, detector and spotters). This is potentially offset by the possibility of highly parallel detection and the massive number of potential oligonucleotides deployed. However, only one sample is measured at a time.

As microarray technology matures, these planar arrays are being supplemented by further evolutions, including microbead and suspension microarray formats. Suspension microarrays are a modification of the original planar microarrays with the differentiating probes being immobilized onto the surface of polystyrene microbeads containing internal fluorescent dyes [38,39].

A microarray-based assay incorporating signal amplification and suspension microarray technologies has been reported for the identification and subtyping of *Listeria monocytogenes* from genomic DNA [39]. Microbead-type arrays have been developed for identification of *Salmonella spp.* Detection sensitivities of 1×10^3 CFU/ml were reported [38]. A multiplex PCR for *E. coli* O157:H7 and *Salmonella* with a suspension microarray detection system had a similar sensitivity [40].

Use of NADs in clinical practice & disease management

While NADs have been in existence for many years, it has taken some time for this to translate into the clinical diagnostics setting. However, the emergence from research to clinical practice is now complete and the area of nucleic acid testing is one of the fastest growing segments of the diagnostics market [41]. While the majority of NADs are used for the detection of infectious microorganisms, the technology can also be utilized in the fields of inherited diseases and disorders, tissue typing, oncology and cardiology. As infectious disease testing is by far the most popular area for use of NADs we will focus our attention on this application. Within the field of infectious disease testing, key areas where NADs are employed include microbial identification, detection of pathogen antibiotic susceptibility [42] and monitoring and surveillance of routes of infection [43]. The use of NADs in these areas has provided rapid sensitive tools for pathogen detection and identification, and for establishing antibiotic susceptibility, ultimately leading to improved treatment options.

First-generation real-time PCR assays concentrated solely on single-parameter tests [44,45]. As the technology developed, additional assays were developed with particular focus on the introduction of genetic markers for antimicrobial resistances, for example, MRSA real-time assays (Cepheid Xpert® MRSA, BD GeneOhm MRSA).

While many hospital laboratories have in-house validated nucleic acid methods, the major diagnostics companies also provide NADs for a variety of infectious diseases. For example, Cepheid currently markets *in vitro* diagnostic tests for use in the areas of healthcare acquired infections, critical infectious disease and women's health [103]. Their test menu includes real-time PCR assays for MRSA, MRSA and *S. aureus*, enteroviruses and others. In the future, the company expects to add immunocompromised, oncology and genetic disease tests to their portfolio. In addition, they also provide analyte-specific reagents comprising easy-to-use primer and probe sets for organisms such as *Bordatella pertussis*,

parapertussis, herpes simplex virus and norovirus. These are designed for rapid results on real-time PCR instruments such as the Cepheid SmartCycler System.

Another major diagnostics company, Roche Molecular Diagnostics, provides virology (HIV, hepatitis C virus), microbiology (MRSA/*Mycobacterium tuberculosis*), genomics and oncology (factor V leiden, prothrombin) products. One of the company's more high profile products is the LightCycler® SeptiFast Test MGRADE. The test allows rapid detection, in one step, of bacterial and fungal DNA, directly from a 1.5 ml whole blood sample, without prior incubation or culture steps in less than 6 h. The test menu includes gram-negative organisms, such as *E. coli*, *Klebsiella pnuemoniae/oxytoca*, *Serratia marcescens*, *Enterobacter cloacase/aerogenes*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter buamannii*, *Stenotrophomonas maltophilia*, and gram-positive organisms such as *S. aureus*, *CnNS*, *Streptococcus pneumoniae*, *Streptococcus spp.*, *Enterococcus faecium* and *Enterococcus faecalis*. Additionally, fungal pathogens *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida glabrata* and *Aspergillus fumigatus* can also be detected. This test provides rapid species identification of pathogens causing blood stream infections, thus allowing for earlier treatment and reduction of the overuse of broad-spectrum antibiotics.

Another company providing NADs is Abbott Diagnostics (IL, USA) [104]. The company's tests include real-time PCR tests for *M. tuberculosis*, Coronvirus and Epstein–Barr virus.

The NADs such as those described earlier are revolutionizing the way in which clinical laboratories are carrying out their diagnostic testing. These tests are accurate, rapid and sensitive. Their use is changing disease management, allowing early disease detection, enabling tailored treatment regimes and predicting patient response to specific therapies. The current challenge for all involved in the field of NAD development is to offer simple, automated systems that will integrate easily into the routine clinical laboratory providing information within hours instead of days or weeks, thus improving the outcome for patients.

Development in the field will be driven further by the changes in healthcare reform and the need for more streamlined processes within the sector, specifically the need for advanced tests that are user friendly and, most importantly, cost effective. New systems are now providing the user with a large menu of tests that can use a variety of sample types, such as blood, urine, saliva and cerebrospinal fluid. In addition, it is possible to achieve faster turnaround time from sample collection to result with NADs compared with standard techniques. The overall result is faster sample to result time with improved patient care.

New developments in NADs

Many new and exciting technologies are currently under development for specific detection of nucleic acid sequences, these include biosensors, nanopores and next-generation sequencing technologies. A description of some of these technologies and their potential use in NADs will be described in the following sections.

Biosensors
The first published demonstration of an enzyme/electrode combination for biological sensing was reported in 1967 [46]. Since this time, there has been much advancement in the biosensor field. Biosensors are defined as analytical devices incorporating a biological material (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies and nucleic acids), a biologically derived material or a biomimic intimately associated with, or integrated within, a physiochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical.

Biosensor technologies developed for the specific detection of microorganisms have included those that utilize metabolism-, antibody- and DNA-based systems. While immunodiagnostic biosensors are currently faster and more robust [47] than nucleic-acid based methods, the latter are more specific and sensitive, especially when combined with an *in vitro* amplification step. Indeed, novel biosensors have been described that utilize advanced visualization and signal amplification technologies, allowing the possibility of monitoring single molecular interactions in real-time [48]. The next generation of biosensors will have application in all sectors of the molecular diagnostics market. It is envisaged that for applications in microbial diagnostics, the ideal device would be a self-contained, automated system capable of organism detection directly from different sample types without pre-enrichment, and also be capable of differentiating live from dead cells [49].

Assay formats
Two basic formats of diagnostic biosensors are generally available; direct and indirect assays [50]. In the case of direct assays, the recognition element (oligonucleotide probe) is attached to the transducer, and attachment or capture of the target element is detected directly. This format of detection is the basis of many devices that detect changes in mass, refractive index or impedance. Indirect biosensor formats require the addition of at least one additional recognition element to the assay before the target molecule can be detected. These forms of assays are well characterized for immunoassays and form the basis of the well established sandwich assay format. Sandwich assays have also been described in nucleic acid diagnostics [51].

Indirect assays may ultimately prove to be most useful for dealing with complex clinical sample types where nonspecific absorption to biosensor surfaces is seen as a significant problem. The extra layer of specificity introduced by an additional recognition element (labeled oligonucleotide probes) goes some way towards reducing these spurious detections.

Biosensor systems for NADs
Optical-based systems
Optical-based detection systems are the most commonly used form of biosensor. These can be divided into systems that use labels, typically fluorescence, and those that are unlabeled. The most common form of unlabeled optical detection is based upon surface plasmon resonance (SPR).

Surface plasmon resonance-based biosensors have been developed by a number of companies (including Biacore International and Texas Instruments, TX, USA) to monitor biomolecular interactions on a surface in real-time [52]. An advantage of SPR-based systems is that no labeling of the target molecule is required. The Biacore system uses SPR that occurs in thin metal (gold) films under total internal reflection. The gold transducer surface is modified with dextran onto which the oligonucleotide probe is attached, typically by avidin to biotin linkages. Hybridization takes place within a continuous flow system and oligonucleotide hybridizations are monitored in real-time. Typical analysis takes approximately 5 min. Probe surfaces are very easily regenerated, allowing many repeat hybridizations to be performed on the same surface. However, the applications of this technology for pathogen identification have yet to be fully realized [53,54]. To date, the potential of SPR-based biosensors has been demonstrated by the detection of genetically modified soybeans and maize following PCR amplification of transgenic and wild-type sequences [55].

Sensitivity of these systems remains poor, requiring approximately 1 µM target concentration which would correspond to 1.5×10^{13} target molecules in typical reaction volumes. However, combination of such a system with *in vitro* amplification should not be ruled out [56,57]. Much progress will be made in the coming years in the integration of high throughput parallel plasmonic detectors with integrated lab-on-a-chip systems [58].

Piezoelectric biosensors

The piezoelectric principle describes the property of some materials, typically crystals, to generate an electrical potential in response to a mechanical force. The addition of mass to the sensor surface will cause a measurable change in the resonance frequency of the crystal proportional to the added mass. Put simply, these sensors are weighing the addition of nucleic acid targets.

Piezoelectric biosensors consist of specific oligonucleotides immobilized on the surface of an appropriate crystal (typically quartz). The most common type of piezoelectric biosensor is the quartz crystal microbalance. This sensor is placed in a solution containing potential target nucleic acids, which bind to their complementary oligonucleotides. Therefore, the mass at the surface of the crystal increases and the resonance frequency of the quartz oscillation will decrease proportionally. Piezoelectrical biosensors may be used for direct, label-free detection of specific nucleic acid targets. These systems combine real-time readout of hybridization measurements with relative simplicity of use. These sensor surfaces can be difficult to regenerate following hybridization. However, economy of scale may serve to make these detectors essentially disposable. Other difficulties remain to be overcome, including lack of specificity and sensitivity, as well as considerable interference at the sensor surface with buffer components and media. Quartz crystal microbalances have been described for the sequence-specific capture of oligonucleotides corresponding to pathogenic microalgae species *Alexandrium minutum* (Dinophyceae) with a detection limit of 20 µg/ml [59]. This is still outside the useful sensitivity range required, but, if such a system could be integrated with an *in vitro* amplification or

a high copy number RNA target then it may have genuine application in the area of environmental monitoring; an interesting solution to the issue of sensitivity may involve using nanoparticles as 'mass-enhancers' [60].

Electrochemical biosensors

These devices work by detecting current or potential changes caused by binding reactions occurring on or near the sensor surface. Different devices are classed depending on their observed parameter. Devices that measure current are amperometric, those that measure electrical potential are potentiometric, while impedance based devices are impedometric. In principle, nucleic acid can be incorporated into each of these types of device.

Electrochemical devices are an attractive technology in that they are label-free, are not affected by the turbidity or other optical properties of the sample matrix and also offer the potential for signal amplification. Some progress has been made in demonstrating the selectivity of these devices for nucleic acid-based detection of microorganisms [61]. As with other label-free systems, sensitivity remains poor and these devices remain susceptible to nonspecific interference. Practical demonstrations of these sensors have used environmental samples. The waterborne pathogen *Cryptosporidium* was detected by an oligonucleotide immobilized onto a carbon-paste transducer [62,63] that employed a sensitive chronopotentiometric transduction mode for hybridization monitoring. Detection of nanograms per milliliter of target nucleic acid was achieved following a 30-min hybridization. Electrochemical methods are directly adaptable to direct electronic readout perhaps coupled with signal amplification. The amplification of an electrochemical detection signal by use of nanoparticles has shown particular promise [64]. Liposome signal amplification has been incorporated into an electrochemical microfluidic biosensor for nucleic acid target detection by using an integrated potentiostat [65]. Sensitivity was in the range of 0.01 µM while functionality was demonstrated using Dengue virus RNA.

Application of nucleic acid biosensors for rapid pathogen detection

While the biosensor technologies described previously show potential for use in the detection of nucleic acids, and many of these are still in development stages, it is important to understand the context in which such biosensors could offer advantages over the current technologies in use. For example, the area of biothreat monitoring is one in which biosensor technology could offer significant advantages. Biothreat monitoring is an area that has received particular attention in recent times. Technologies currently in use such as the Razor (Idaho Technologies, UT, USA), the Bio-Seeq Plus (Smiths Detection, MD, USA) and the GeneXpert system (Cepheid), represent significant advances in that they allow detection within a 30-min timeframe. However, future devices will be required to operate within the 'detect to protect' window, which requires results within 5 min. There has, therefore, been a recent surge in developing technologies that bridge the gap between the 30-min 'detect to treat' and the 5-min 'detect-to-protect' time criteria. When considering the detection

of biological threat agents, it is necessary to consider that these may exist as bacteria (both vegetative and as spores), viruses and toxins. In a real sense, nucleic acid-based biosensor detection of biothreat agents is an extension of the technologies employed for environmental monitoring with the same basic considerations. Further considerations specific to 'field-deployable' assays include low energy, user friendliness and sufficient robustness to work in a variety of challenging environments, and not necessarily a laboratory. Military applications of nucleic acid biosensors are associated with the rapid ultrasensitive detection of selected biowarfare agents. Detection devices are required to be portable, robust, rapid and user friendly. To date, direct detection biosensors have been described that take advantage of changes at a solid/liquid interface that measure pH, oxygen consumption, ion concentration, potential difference, current or optical properties. Of these, only optical biosensors have so far shown genuine utility as nucleic acid-based biosensors. Undoubtedly, this is a field where advances in nucleic acid biosensors could have significant impact.

Nanopores

In reference to their use as a biosensor, nanopores relate to a specific class of electrochemical sensor in which an electrically insulating material is permeated by one or more pores with diameters ranging from 10 to 150 nM. The insulating layer may be an organic or synthetic material. For example, nanopores have been introduced into lipid bilayers using self-assembling protein tetramers [66]. Other, highly innovative, work has exploited the fact that cell membranes are naturally permeated by stable nanoscale ion channels. A feature of some of these naturally occurring structures is that they have an extremely narrow diameter (such that single-stranded nucleic acid molecules can only pass through one nucleotide at a time) [67,68]. Finally, so called 'solid-state' nanopores containing membranes can be fabricated in synthetic materials such as silicon nitride. The fabrication methodologies owe much to the technologies used in microprocessor fabrication and include such techniques as ion-beam sculpting [69] or electron beams [70].

The nanopore-containing membrane is placed in an electrolyte solution in an electrical cell and a voltage is applied across the membrane. Detection is based upon monitoring of the ionic current through the pores. The sensing potential of these devices depends on the interruption of this ion flow due to 'blocking' of the opening by different biological molecules. For nucleic acid-based nanopore detection, specific capture oligonucleotides are immobilized on the surface of the nanopores and used to detect complementary single stranded sequences as they pass through the opening. Electric current is used to electrophoretically move the charged nucleic acid molecules through the pores. The observation that different strands of DNA or RNA can have a different effect on the ionic current has also led directly to the field of naonpore sequencing [71].

Next-generation sequencing

Possibly one of the most interesting developments at the interface between detection/diagnostics technologies and next-generation sensor devices is in the area of rapid gene sequencing. The ability to rapidly and reliably obtain the nucleotide sequence of pathogenic microorganisms present in a sample would radically change nucleic acid-based diagnostics. Massively parallel sequencers, chain extension on array and nanopore sequencing are just some of the technologies that are being evaluated [71–73]. The focus of these technology developments has been primarily in the area of whole genome sequencing [74] and expression profiling [75], and would represent somewhat of an overkill related to diagnostics. However, it is inevitable that these tools will find application in clinical microorganism diagnostics in the near future at least. Some potential areas include array-based sequencing of a number of genes present in all, or at least a large subset of all, bacteria.

Direct RNA sequencing is a recently described technology that can be used for the microarray-based sequencing of all transcribed RNAs present in a sample without the requirement to convert RNA to cDNA or perform any *in vitro* amplification of target material [76]. The authors used poly-T-containing oligonucleotides to capture femtomolar quantities of mRNAs from *Saccharomyces cerevisiae.* Sequencing reactions were performed using 'virtual terminator' nucleotides, which are fluorescent nucleotide analogues that contain a fluorescent dye and chemically cleavable groups that allow stepwise sequencing. Following addition of each base in the sequencing reaction, an image of the array is taken. The fluorescent group is then cleaved for the nucleotide analogue and the next base in the sequence is added. Another array image is taken and, in this way, the sequence of each RNA molecule captured at different positions on the array is determined.

Expert commentary & five-year view

The driver for innovation in the field of nucleic acid testing is the requirement for simple, cost-effective, easy to use systems for routine use. Tests developed previously are now being re-formatted for integration onto novel platforms. The trend is for systems where multiple sample types can be used, with little sample preparation, fast turnaround time and a large test menu. In addition, these instruments must be of low cost and have a small footprint. The trend in device manufacture related to biosensor development is towards miniaturization. Micro- and nano-scale fabrication methods developed for the electronics industry are being applied to biosensors. In some cases, their application is leading to a change in the way these devices are designed and manufactured. Development of rapid detection systems for pathogens is progressing towards integrated devices utilizing solid state technology that do not require introduction of reagents or the separation of unbound probe molecules. Electrochemical and optical detection methods are attractive in this context as they couple a signal generated by hybridization with instrument readout. These assay formats do not require the use of labeled reporter molecules, such as enzymes or flurophores. Further advantages of microscale devices include cheaper unit cost owing to mass production, and much reduced assay cost owing to smaller amounts of reagent used. Microfluidic devices also have improved mixing rates resulting in shorter analysis times. Technologies such as biosensors promise to shorten the time to result but must match the performance of pre-existing technologies. In order to gain a significant market share, biosensor

systems for microbial diagnostics have to be capable of sensitivity and specificity comparable with the current state of the art *in vitro* amplification assays. Only when this improvement in sensitivity is achieved will the secondary advantages of biosensor systems (speed, sensitivity, unit cost) come to the fore. Multianalyte detection on the same device is now an absolute requirement. Advances in detection systems have led to several systems that do not require *in vitro* amplification. Nanosphere Inc. (Northbrook, IL, USA) has succeeded in developing two automated systems that combine advanced optical detection with microfluidic sample handling. The Verigene Autolab™ and the Verigene Mobile™ use DNA probes conjugated to gold nanoparticles for detection in combination with the company's proprietary Biobarcode technology. This technology has been demonstrated for detection of DNA and RNA targets at sensitivities comparable with those associated with PCR. The Autolab system can also be used for identification of amplified DNA samples, while multiplexing is achieved by using a mixture of different oligonucleotide–nanoparticle conjugates in the same assay. The Verigene Mobile is capable of detection of nonamplified product in a total assay time of less than 1 h.

The incorporation of nucleic acid diagnostic biosensors has benefited from close integration with the physical sciences. The area of micro- and nano-biosensors involves the application of nanotechnology, microfluidics, bioinformatics and molecular biology. System engineers will play a key role in future biosensor development as advances in filtration, flow-injection, microfabrication and detection are combined. In order to be suitable for routine applications, biosensor devices must have the capability to distinguish target sequences in a multiorganism background, often in the presence of complex sample matrix. Devices should be rapid, sensitive and adaptable (allow interchangeable probe cartridges), in a user-friendly and inexpensive configuration.

Future technologies will move nucleic acid diagnostics away from centralized laboratory facilities and into locations such as emergency rooms, physician's offices and even into the home. Point of care (generally referring to hospitals and clinics) and point of patient (referring to at-home or physician's offices) testing will be the way of the future, with systems providing integrated information transfer from one location to another, allowing remote diagnosis by clinicians. To enable provision of such diagnostics requires the evolution of novel, miniature, easy to use portable devices that provide clinically relevant results. The application of these principles has already been applied to the measurement of disease states, such as diabetes and hypertension. The long-term view is that this type of technology development will eventually translate into the area of nucleic acid testing. Further reviews on point of care testing and the associated challenges are available [77,78].

Financial & competing interests disclosure

Key issues

- Challenges to be overcome before development of future diagnostics systems include determining appropriate sample type and volume, developing efficient nucleic acid extraction systems and integrating novel detection systems.
- Integration of devices that combine the sensitivity of *in vitro* amplification with the advantages related to portability and size possible with sensor systems.
- Improved integration of diagnostics assays on next generation.
- Instrumentation in order to develop true point-of-care diagnostics.
- Improved sensitivity for biosensors to potentially allow for diagnostics systems that do not require an enzymatic *in vitro* amplification step.
- Miniaturization similar to the developments that have characterized the electronics industry.

References

1   Grennan B, O'Sullivan NA, Fallon R *et al.* PCR-ELISAs for the detection of *Campylobacter jejuni* and *Campylobacter coli* in poultry samples. *Biotechniques* 30(3), 602–606, 608–610 (2001).

2   Maher M, Finnegan C, Collins E, Ward B, Carroll C, Cormican M. Evaluation of culture methods and a DNA probe-based PCR assay for detection of Campylobacter species in clinical specimens of feces. *J. Clin. Microbiol.* 41(7), 2980–2986 (2003).

3   O'Connor L, Joy J, Kane M, Smith T, Maher M. Rapid polymerase chain reaction/DNA probe membrane-based assay for the detection of Listeria and *Listeria monocytogenes* in food. *J. Food Prot.* 63(3), 337–342 (2000).

4   O'Connor L. Detection of *Listeria monocytogenes* using a PCR/DNA probe assay. *Methods Mol. Biol.* 216, 185–192 (2003).

5   Pfaller MA. Molecular approaches to diagnosing and managing infectious diseases: practicality and costs. *Emerg. Infect. Dis.* 7(2), 312–318 (2001).

6   Schonhuber W, Le Bourhis G, Tremblay J, Amann R, Kulakauskas S. Utilization of tmRNA sequences for bacterial identification. *BMC Microbiol.* 1, 20 (2001).

7   Williams KP. The tmRNA Website: invasion by an intron. *Nucleic Acids Res.* 30(1), 179–182 (2002).

8   Glynn B, Lacey K, Reilly J, Barry T, Smith T, Maher M. Quantification of bacterial tmRNA using *in vitro* transcribed RNA standards and two-step qRT-PCR. *Res. J. Biol. Sci.* 2(5), 564–570 (2007).

9   Lee SY, Bailey SC, Apirion D. Small stable RNAs from *Escherichia coli*: evidence for the existence of new molecules and for a new ribonucleoprotein particle containing 6S RNA. *J. Bacteriol.* 133(2), 1015–1023 (1978).

10   O' Grady J, Sedano-Balbas S, Maher M, Smith T, Barry T. Rapid real-time PCR

detection of Listeria monocytogenes in enriched food samples based on the ssrA gene, a novel diagnostic target. *Food Microbiol.* 25(1), 75–84 (2008).

11 Wernecke M, Mullen C, Sharma V *et al.* Evaluation of a novel real-time PCR test based on the *ssrA* gene for the identification of group B streptococci in vaginal swabs. *BMC Infect. Dis.* 9, 148 (2009).

12 Fung DY. Predictions for rapid methods and automation in food microbiology. *J. AOAC Int.* 85(4), 1000–1002 (2002).

13 DeLong EF, Wickham GS, Pace NR. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science* 243(4896), 1360–1363 (1989).

14 Amann RI, Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* 172(2), 762–770 (1990).

15 Kempf VA, Trebesius K, Autenrieth IB. Fluorescent *in situ* hybridization allows rapid identification of microorganisms in blood cultures. *J. Clin. Microbiol.* 38(2), 830–838 (2000).

16 Todd EC, Szabo RA, MacKenzie JM *et al.* Application of a DNA hybridization-hydrophobic-grid membrane filter method for detection and isolation of verotoxigenic *Escherichia coli. Appl. Environ. Microbiol.* 65(11), 4775–4780 (1999).

17 Csordas AT, Barak JD, Delwiche MJ. Comparison of primers for the detection of *Salmonella enterica* serovars using real-time PCR. *Lett. Appl. Microbiol.* 39(2), 187–193 (2004).

18 Raoult D, Fournier PE, Drancourt M. What does the future hold for clinical microbiology? *Nat. Rev. Microbiol.* 2(2), 151–159 (2004).

19 Mullis KB, Faloona FA. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155, 335–350 (1987).

20 Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* 29(1), 23–39 (2002).

21 McKillip JL, Drake M. Real-time nucleic acid-based detection methods for pathogenic bacteria in food. *J. Food Prot.* 67(4), 823–832 (2004).

22 Bustin SA, Mueller R. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin. Sci. (Lond.)* 109(4), 365–379 (2005).

23 Fitzmaurice J, Glennon M, Duffy G, Sheridan JJ, Carroll C, Maher M.

Application of real-time PCR and RT-PCR assays for the detection and quantitation of VT 1 and VT 2 toxin genes in *E. coli* O157:H7. *Mol. Cell. Probes* 18(2), 123–132 (2004).

24 O'Connor L, Lahiff S, Casey F, Glennon M, Cormican M, Maher M. Quantification of *ALS1* gene expression in *Candida albicans* biofilms by RT-PCR using hybridisation probes on the LightCycler. *Mol. Cell. Probes* 19(3), 153–162 (2005).

25 Templeton KE, Scheltinga SA, van der Zee A *et al.* Evaluation of real-time PCR for detection of and discrimination between *Bordetella pertussis, Bordetella parapertussis,* and *Bordetella holmesii* for clinical diagnosis. *J. Clin. Microbiol.* 41(9), 4121–4126 (2003).

26 Walker GT, Little MC, Nadeau JG, Shank DD. Isothermal *in vitro* amplification of DNA by a restriction enzyme/DNA polymerase system. *Proc. Natl Acad. Sci. USA* 89(1), 392–396 (1992).

27 Chen Q, Bian Z, Chen M *et al.* Real-time monitoring of the strand displacement amplification (SDA) of human cytomegalovirus by a new SDA-piezoelectric DNA sensor system. *Biosens. Bioelectron.* 24(12), 3412–3418 (2009).

28 Ge B, Larkin C, Ahn S *et al.* Identification of *Escherichia coli* O157:H7 and other enterohemorrhagic serotypes by EHEC-hlyA targeting, strand displacement amplification, and fluorescence polarization. *Mol. Cell. Probes* 16(2), 85–92 (2002).

29 Manji R, Lotlikar M, Zhang F, Ginocchio CC. Clinical evaluation of NucliSENS magnetic extraction and NucliSENS analytical specific reagents for the real-time detection of respiratory syncytial virus (RSV) in paediatric respiratory specimens. *J. Clin. Pathol.* 62(11), 998–1002 (2009).

30 Uyttendaele M, Bastiaansen A, Debevere J. Evaluation of the NASBA nucleic acid amplification system for assessment of the viability of *Campylobacter jejuni. Int. J. Food Microbiol.* 37(1), 13–20 (1997).

31 Simpkins SA, Chan AB, Hays J, Popping B, Cook N. An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica. Lett. Appl. Microbiol.* 30(1), 75–79 (2000).

32 Cook N. The use of NASBA for the detection of microbial pathogens in food and environmental samples. *J. Microbiol. Methods* 53(2), 165–174 (2003).

33 Rodriguez-Lazaro D, Lloyd J, Herrewegh A

et al. A molecular beacon-based real-time NASBA assay for detection of *Mycobacterium avium* subsp. paratuberculosis in water and milk. *FEMS Microbiol. Lett.* 237(1), 119–126 (2004).

34 Wilson WJ, Strout CL, DeSantis TZ, Stilwell JL, Carrano AV, Andersen GL. Sequence-specific identification of 18 pathogenic microorganisms using microarray technology. *Mol. Cell. Probes* 16(2), 119–127 (2002).

35 Sachse K, Hotzel H, Slickers P, Ellinger T, Ehricht R. DNA microarray-based detection and identification of *Chlamydia* and *Chlamydophila spp. Mol. Cell. Probes* 19(1), 41–50 (2005).

36 Small J, Call DR, Brockman FJ, Straub TM, Chandler DP. Direct detection of 16S rRNA in soil extracts by using oligonucleotide microarrays. *Appl. Environ. Microbiol.* 67(10), 4708–4716 (2001).

37 Anthony RM, Schuitema AR, Oskam L, Klatser PR. Direct detection of *Staphylococcus aureus* mRNA using a flow through microarray. *J. Microbiol. Methods* 60(1), 47–54 (2005).

38 Ahn S, Walt DR. Detection of *Salmonella spp.* Using microsphere-based, fiber-optic DNA microarrays. *Anal. Chem.* 77(15), 5041–5047 (2005).

39 Borucki MK, Reynolds J, Call DR, Ward TJ, Page B, Kadushin J. Suspension microarray with dendrimer signal amplification allows direct and high-throughput subtyping of *Listeria monocytogenes* from genomic DNA. *J. Clin. Microbiol.* 43(7), 3255–3259 (2005).

40 Straub TM, Dockendorff BP, Quinonez-Diaz MD *et al.* Automated methods for multiplexed pathogen detection. *J. Microbiol. Methods* 62(3), 303–316 (2005).

41 Carlson B. Sky could be the limit for DNA testing. *GEN* 29(4) (2009).

42 Rolain JM, Mallet MN, Fournier PE, Raoult D. Real-time PCR for universal antibiotic susceptibility testing. *J. Antimicrob. Chemother.* 54(2), 538–541 (2004).

43 Vainio K, Nordbo SA, Njolstad G *et al.* Detection and characterization of group A rotaviruses in children hospitalized with acute gastroenteritis in Norway, 2006–2008. *J. Med. Virol.* 81(10), 1839–1844 (2009).

44 Ballard AL, Fry NK, Chan L *et al.* Detection of Legionella pneumophila using a real-time PCR hybridization assay. *J. Clin. Microbiol.* 38(11), 4215–4218

(2000).

45   Drosten C, Panning M, Kramme S. Detection of *Mycobacterium tuberculosis* by real-time PCR using pan-mycobacterial primers and a pair of fluorescence resonance energy transfer probes specific for the *M. tuberculosis* complex. *Clin. Chem.* 49(10), 1659–1661 (2003).

46   Updike SJ, Hicks GP. The enzyme electrode. *Nature* 214(5092), 986–988 (1967).

47   Iqbal SS, Mayo MW, Bruno JG, Bronk BV, Batt CA, Chambers JP. A review of molecular recognition technologies for detection of biological threat agents. *Biosens. Bioelectron.* 15(11–12), 549–578 (2000).

48   Yao G, Fang X, Yokota H, Yanagida T, Tan W. Monitoring molecular beacon DNA probe hybridization at the single-molecule level. *Chemistry* 9(22), 5686–5692 (2003).

49   Ivnitski D, Abdel-Hamid I, Atanasov P, Wilkins E, Stricker S. Application of electrochemical biosensors for detection of food pathogenic bacteria. *Electroanalysis* 12(5), 317–325 (2000).

50   Baeumner AJ. Biosensors for environmental pollutants and food contaminants. *Anal. Bioanal. Chem.* 377(3), 434–445 (2003).

51   Baeumner AJ, Jones C, Wong CY, Price A. A generic sandwich-type biosensor with nanomolar detection limits. *Anal. Bioanal. Chem.* 378(6), 1587–1593 (2004).

52   Rand AG, Ye JM, Brown CW, Letcher SV. Optical biosensors for food pathogen detection. *Food Technol.* 56(3), 32–39 (2002).

53   Mannelli I, Minunni M, Tombelli S, Wang R, Michela Spiriti M, Mascini M. Direct immobilisation of DNA probes for the development of affinity biosensors. *Bioelectrochemistry* 66(1–2), 129–138 (2005).

54   Wang R, Tombelli S, Minunni M, Spiriti MM, Mascini M. Immobilisation of DNA probes for the development of SPR-based sensing. *Biosens. Bioelectron.* 20(5), 967–974 (2004).

55   Feriotto G, Borgatti M, Mischiati C, Bianchi N, Gambari R. Biosensor technology and surface plasmon resonance for real-time detection of genetically modified Roundup Ready soybean gene sequences. *J. Agric. Food Chem.* 50(5), 955–962 (2002).

56   Nelson BP, Grimsrud TE, Liles MR, Goodman RM, Corn RM. Surface plasmon resonance imaging measurements of DNA and RNA hybridization adsorption onto DNA microarrays. *Anal. Chem.* 73(1), 1–7 (2001).

57   Spadavecchia J, Manera MG, Quaranta F, Siciliano P, Rella R. Surface plamon resonance imaging of DNA based biosensors for potential applications in food analysis. *Biosens. Bioelectron.* 21(6), 894–900 (2005).

58   Hoa XD, Kirk AG, Tabrizian M. Towards integrated and sensitive surface plasmon resonance biosensors: a review of recent progress. *Biosens. Bioelectron.* 23(2), 151–160 (2007).

59   Lazerges M, Perrot H, Antoine E, Defontaine A, Compere C. Oligonucleotide quartz crystal microbalance sensor for the microalgae *Alexandrium minutum* (Dinophyceae). *Biosens. Bioelectron.* 21(7), 1355–1358 (2006).

60   Mao X, Yang L, Su XL, Li Y. A nanoparticle amplification based quartz crystal microbalance DNA sensor for detection of *Escherichia coli* O157:H7. *Biosens. Bioelectron.* 21(7), 1178–1185 (2006).

61   Lazcka O, Del Campo FJ, Munoz FX. Pathogen detection: a perspective of traditional methods and biosensors. *Biosens. Bioelectron.* 22(7), 1205–1217 (2007).

62   Wang R, Zhang L, Feng Y *et al.* Molecular characterization of a new genotype of *Cryptosporidium* from American minks (Mustela vison) in China. *Vet. Parasitol.* 154(1–2), 162–166 (2008).

63   Wang J, Fernandes JR, Kubota LT. Polishable and renewable DNA hybridization biosensors. *Anal. Chem.* 70(17), 3699–3702 (1998).

64   Peng H, Soeller C, Cannell MB, Bowmaker GA, Cooney RP, Travas-Sejdic J. Electrochemical detection of DNA hybridization amplified by nanoparticles. *Biosens. Bioelectron.* 21(9), 1727–1736 (2006).

65   Kwakye S, Goral VN, Baeumner AJ. Electrochemical microfluidic biosensor for nucleic acid detection with integrated minipotentiostat. *Biosens. Bioelectron.* 21(12), 2217–2223 (2006).

66   Terrettaz S, Follonier S, Makohliso S, Vogel H. A synthetic membrane protein in tethered lipid bilayers for immunosensing in whole blood. *J. Struct. Biol.* 168(1), 177–182 (2009).

67   Braha O, Walker B, Cheley S *et al.* Designed protein pores as components for biosensors. *Chem. Biol.* 4(7), 497–505

(1997).

68   Kang XF, Gu LQ, Cheley S, Bayley H. Single protein pores containing molecular adapters at high temperatures. *Angew. Chem. Int. Ed. Engl.* 44(10), 1495–1499 (2005).

69   Li J, Stein D, McMullan C, Branton D, Aziz MJ, Golovchenko JA. Ion-beam sculpting at nanometre length scales. *Nature* 412(6843), 166–169 (2001).

70   Storm AJ, Chen JH, Ling XS, Zandbergen HW, Dekker C. Fabrication of solid-state nanopores with single-nanometre precision. *Nat. Mater.* 2(8), 537–540 (2003).

71   Branton D, Deamer DW, Marziali A *et al.* The potential and challenges of nanopore sequencing. *Nat. Biotechnol.* 26(10), 1146–1153 (2008).

72   Kurg A, Tonisson N, Georgiou I, Shumaker J, Tollett J, Metspalu A. Arrayed primer extension: solid-phase four-color DNA resequencing and mutation detection technology. *Genet. Test* 4(1), 1–7 (2000).

73   Dahl F, Stenberg J, Fredriksson S *et al.* Multigene amplification and massively parallel sequencing for cancer mutation discovery. *Proc. Natl Acad. Sci. USA* 104(22), 9387–9392 (2007).

74   Wheeler DA, Srinivasan M, Egholm M *et al.* The complete genome of an individual by massively parallel DNA sequencing. *Nature* 452(7189), 872–876 (2008).

75   Wang WC, Lin FM, Chang WC, Lin KY, Huang HD, Lin NS. miRExpress: analyzing high-throughput sequencing data for profiling microRNA expression. *BMC Bioinformatics* 10, 328 (2009).

76   Ozsolak F, Platt AR, Jones DR *et al.* Direct RNA sequencing. *Nature* 461(7265), 814–818 (2009).

77   Holland CA, Kiechle FL. Point-of-care molecular diagnostic systems – past, present and future. *Curr. Opin. Microbiol.* 8(5), 504–509 (2005).

78   Kiechle FL, Holland CA. Point-of-care testing and molecular diagnostics: miniaturization required. *Clin. Lab. Med.* 29(3), 555–560 (2009).

Websites

101   Gen Probe, Inc.
www.gen-probe.com

102   Review of the mechanisms of real-time PCR
http://dorakmt.tripod.com/genetics/realtime.html

103 Cephid
www.cepheid.com

104 Abbott Diagnostics Ltd
www.abbottmolecular.com

105 Roche Diagnostics Corporation
www.roche-applied-science.com

106 bioMérieux Clinical Diagnostics
www.biomerieux.com

107 Becton Dickinson Co.
www.bd.com

108 Novartis Diagnostics
www.chiron.com

109 Eiken Chemical Co.
www.loopamp.eiken.co.jp

110 Molecular Staging Inc.
www.molecularstaging.com

111 TwistDx Ltd
www.twistdx.co.uk