DDT Vol. 7, No. 21 November 2002

Rapid molecular theranostics in infectious diseases

François J. Picard and Michel G. Bergeron

The increasing availability of rapid and sensitive nucleic acid testing assays for infectious diseases will revolutionize the practice of medicine by gradually reducing the need for standard culture-based microbiological methods that take at least two days. Molecular theranostics in infectious diseases is an emerging concept in which molecular biology tools are used to provide rapid and accurate diagnostic assays to enable better initial management of patients and more efficient use of antimicrobials. Essential conditions and the quality control required for the development and validation of such molecular theranostic assays are reviewed.

François J. Picard and Michel G. Bergeron*
Centre de Recherche en Infectiologie de l'Université Laval
Centre Hospitalier Universitaire de Québec
Pavillon CHUL
2705 Boul. Laurier
Québec
Canada G1V 4G2
*e-mail: michel.g.bergeron@crchul.ulaval.ca

Each year, infectious diseases are responsible for more than 17 million deaths worldwide, most of which are associated with bacterial infections. The use of antimicrobials to treat patients without bacterial infection, as well as the common use of broad-spectrum antimicrobials, are associated with an increasing rate of resistance which complicates treatment. This inappropriate use of antimicrobials is well illustrated in a study from Spain, which showed that the number of antimicrobial prescriptions was three times higher than the number of patients diagnosed with a bacterial infection [1]. Clearly, the healthcare costs associated with infectious diseases are enormous and growing.

Microbial identification and antimicrobial susceptibility testing methods currently used in clinical microbiology laboratories require at least two days because they rely on the growth and isolation of micro-organisms. This long, but necessary, delay has enormous consequences on antimicrobial usage [2]. It forces physicians to frequently treat patients empirically with broad-spectrum antimicrobials, which are often toxic and expensive [3]. The slowness of the microbiology laboratory contrasts with the time required (less than one hour) to get the results from other hospital laboratories or departments, such as biochemistry, hematology and radiology. Indeed, clinical microbiology procedures remain based on the use of a variety of growth-dependent biochemical tests developed by Pasteur and others during the 19th and 20th centuries. Consequently, physicians rarely consult the microbiology results unless the patient is not responding to the initial antimicrobial therapy, which is based on key information obtained during the first hour after patient admission, thereby excluding any microbiology results. Clearly, there is a need for rapid (less than one hour) and accurate diagnostic tests for use in clinical microbiology laboratories to enable optimal patient management and treatment [4,5]. Rapid detection and identification of microbial pathogens and their antimicrobial resistance profiles would have a tremendous impact on the practice of medicine by providing physicians with key microbiology results when needed. The consequent improvement in antimicrobial usage should lead to a better control of antimicrobial resistance and to a reduction of healthcare costs. It would also provide essential diagnostic tools for the rapid investigation and development of new and more specific antimicrobial agents or futuristic drugs, such as anti-toxins and biological response modifiers [6].

Molecular theranostics

Molecular theranostics for infectious diseases is an emerging concept in which molecular biology tools are used to provide rapid (less-than-one-hour), accurate and more informative diagnostic microbiology assays, thus enabling better therapeutic interventions (Fig. 1). It is anticipated that the optimal selection of appropriate antimicrobials by clinicians, and the ability to monitor the efficacy of prescribed therapies, will be gradually improved as an increasing number of rapid molecular diagnostic tools for the detection, identification and characterization of infectious agents become

 1359-6446/02/$ – see front matter PII: S1359-6446(02)02497-2

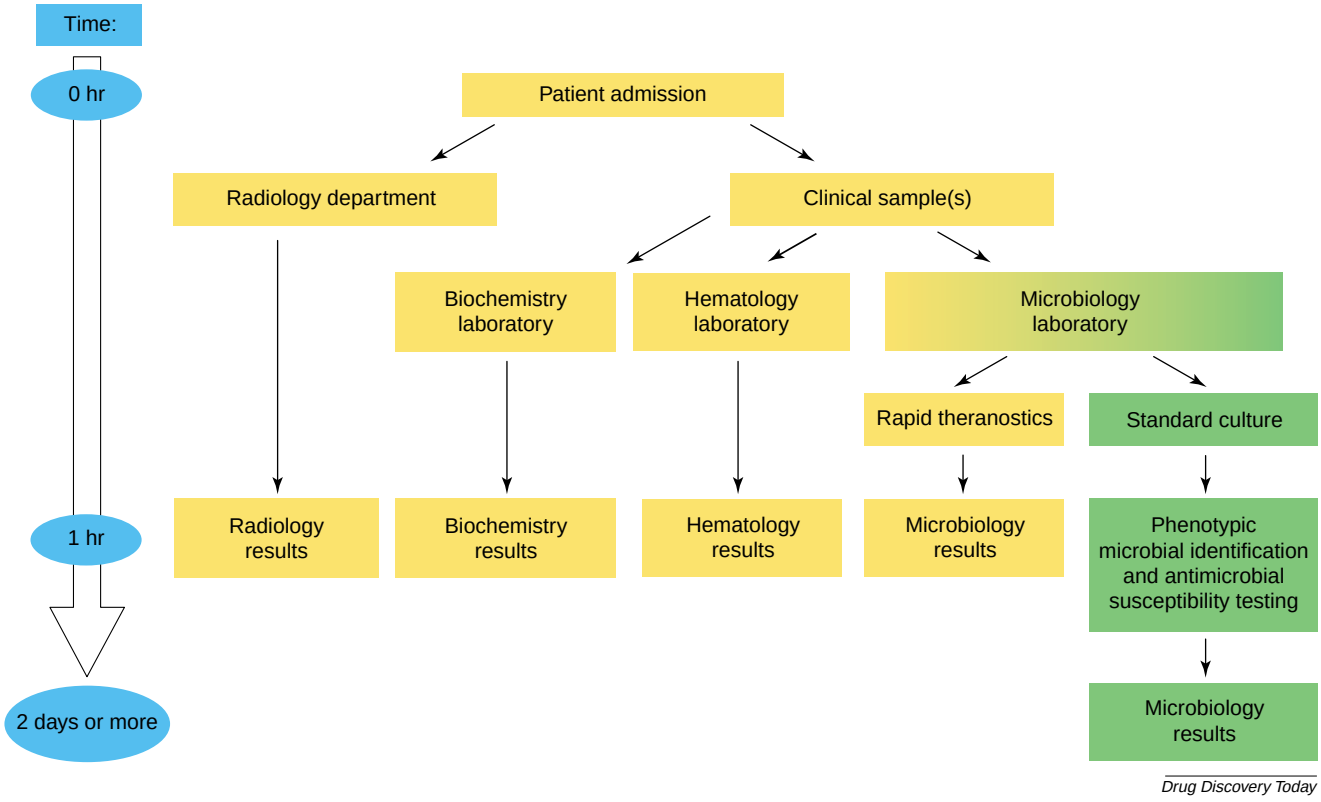

Figure 1. Rapid molecular theranostics in infectious diseases. Currently, microbiology results are usually not available for at least two days. The rapid theranostic approach will provide results within one hour, at the same speed as most other hospital laboratories or departments. This will revolutionize the practice of medicine by enabling appropriate management and immediate prescription of the optimal treatment, if necessary.

commercially available. Assays based on the detection of nucleic acids offer enormous potential for the rapid and accurate diagnosis of microbial infections including detection and identification of the causal infectious agent(s), as well as the detection and characterization of genes or mutations associated with antimicrobial resistance and virulence. Diagnostic tools based on amino-acid sequence detection and protein expression patterns should also have an important role in the future. However, currently available protein-based diagnostic assays are generally not sensitive enough for direct detection from clinical samples and are not as specific as diagnostic tests based on nucleic acid detection [7]. Furthermore, protein information available in public databases is limited compared with the enormous amount of available nucleic acid sequences. Hence, this review will focus primarily on nucleic acid-based theranostics.

Essential conditions for rapid molecular theranostic assays

The development of molecular theranostic assays for diagnostic purposes in microbiology relies on the following: (1) genomics or proteomics to provide the essential nucleotide or amino-acid sequence data required to design accurate assays; (2) the development of rapid, simple and efficient methods for microbial nucleic acid or protein extraction from a variety of clinical samples; (3) rapid, specific and ubiquitous (i.e. able to detect all or most targeted micro-organisms) multiplex target amplification and detection, or signal amplification technologies required to reach a level of analytical sensitivity appropriate for testing directly from clinical samples without previous growth enrichment; and (4) the careful monitoring of assay performance with quality control reactions.

Genomics and proteomics

In 1995, *Haemophilus influenzae* became the first bacterial pathogen for which the entire genome sequence was determined [8]. Subsequently, the amount of nucleotide and amino-acid sequences in public databases has increased at an accelerating pace because of technological advances in sequencing and bioinformatics. In June 2002, the complete sequence of 67 microbial genomes was publicly available and the sequencing of ~200 additional microbial genomes was in progress [The Institute for Genomic Research (TIGR);

http://www.tigr.org/tdb/mdb/mdbinprogress.html]. Until recently, microbiologists were sequencing only one genome per microbial species. However, the emerging gold standard is to generate multiple complete genome sequences from strains of a single species and compare them because intra-species variations could be of great biological importance [9]. The first comparison of genome sequences from the same species was performed with *Escherichia coli* strain K-12 and a representant of *E. coli* serogroup O157:H7, which is associated with deadly food- and water-borne disease outbreaks. This study revealed that the O157:H7 genome had approximately one million more base pairs than strain K-12 and almost 1400 new genes [10]. In a similar fashion, and as a consequence of such profound differences between strains of the same species, TIGR is now sequencing multiple strains of the same species, including 20 different *Bacillus anthracis* strains from around the world [9]. The combination of whole-genome shotgun sequencing approaches with powerful computational algorithms to facilitate sequence data assembly, gene prediction and functional annotation has created a revolution in our understanding of the biology of micro-organisms [11]. Bioinformatics, comparative genomics, proteomics and functional genomics are thus molecular biology tools essential for the progress of molecular theranostics. These tools generate the genetic and protein information required for the development of diagnostic assays.

The growth of nucleotide sequence databases over the past decade has enabled the development of a wide array of molecular diagnostic assays targeting infectious disease agents including viruses, bacteria, fungi and parasites [12–20]. Most of these assays are based on nucleic acid amplification using PCR. They target a variety of nucleotide sequences including sets from different genes that have evolved at different rates. PCR technology has the flexibility to adjust to the desired stringency of detection. For example, assays for the universal detection of bacteria or for bacterial detection at the genus or family level must target highly conserved nucleotide sequences that are associated with genes involved in essential functions of the cell and constantly challenged by a strong selective pressure during evolution. By contrast, less conserved sequences must be used for detection and identification at the species level or for epidemiological purposes, such as strain typing.

The most widely used genetic targets for diagnostic purposes in infectious diseases are ribosomal RNA (rRNA) genes [mainly those coding for the bacterial and archaeal 16S small subunit (SSU) rRNA] because these genes have been used extensively for microbial evolution studies and phylogenetic analysis over the past 20 years. This has created an intense need to generate the widest spectrum of nucleotide sequences for a given genetic target covering most known bacteria, fungi and parasites [5,21]. Indeed, the Ribosomal Database Project (http://rdp.cme.msu.edu/), which provides data, programs and services related to rRNA sequences, consisted of 16,277 bacterial and archaeal, 2055 eucaryotic and 1503 mitochondrial aligned SSU rRNA sequences as of 1 June 2000 [22]. More recently (June 2002), there were more than 75,000 entries of 16S rRNA bacterial and archaeal sequences available in public databases (http://www.ncbi.nlm.nih.gov/). rRNA genes are particularly flexible for diagnostic purposes because they are composed of (1) highly conserved regions suitable for the universal detection of bacteria, fungi or parasites and (2) variable regions appropriate for detection and identification at the genus or species level. Other conserved genetic targets, including *tuf* (encodes translation elongation factor), *groEL* (encodes HSP60 heat-shock protein), *recA* (encodes the recA homologous DNA recombination and repair protein) and 16S–23S intergenic spacer region of bacterial rRNA operons, have also been used for a variety of diagnostic purposes [23–29].

Nucleotide sequences for genes and polymorphisms associated with antimicrobial resistance and virulence factors, such as the production of toxin or capsule, are also rapidly accumulating in public databases. The genetic diversity of factors contributing to resistance in viruses and micro-organisms is particularly striking when currently available databases for antimicrobial resistance genes and polymorphisms are examined [30,31]. Indeed, there are variable and multiple mechanisms of resistance to many classes of antimicrobials. Antimicrobial resistance in bacteria is associated with more than a hundred different clinically relevant genes, as well as hundreds of mutations in key chromosomal or plasmid-borne genetic loci or regulatory elements. The multiplicity of resistance mechanisms is particularly high in Gram-negative bacteria, a situation that will complicate the development of molecular theranostic approaches to detect this diversity of resistance genotypes. However, the task of detecting resistance genotypes in Gram-positive bacteria should be easier because fewer genes and mutations are involved.

It is estimated that ~90% of bacterial resistance mechanisms are mediated by the acquisition of one or more genes, whose presence is sufficient for a resistance phenotype [30,32–34]. However, culture-based antimicrobial susceptibility testing has revealed rare situations in which resistance genes are present but not expressed, probably because of mutations in regulatory sequences [35–38]. Several studies have demonstrated that it is possible to select resistant cells from originally susceptible strains by *in vitro* selection in the presence of increasing gradients of an antimicrobial [35,36,38]. These findings suggest that a

susceptible strain harboring, but not expressing, an antimicrobial resistance gene should be regarded as potentially resistant to that antimicrobial. The remaining 10% of bacterial resistance mechanisms is associated with chromosomal or plasmidic mutations mainly mediating resistance to quinolones, β-lactams in *Streptococcus pneumoniae*, rifampin in *Mycobacterium* and extending the spectrum of substrates of β-lactamases [32–34]. By contrast, toxin and capsule production genes exhibit a much lower genetic diversity and the presence of these virulence factors is usually associated with the acquisition of gene(s) [39].

Methods for microbial nucleic acid extraction

Extraction of nucleic acids from a wide variety of clinical samples is a challenging and crucial step in molecular microbiology. It is compliated by the difficulty in lysing microbial cells with sturdy cell walls to release nucleic acids. An optimal sample preparation procedure for nucleic acid testing (NAT) from clinical specimens must: (1) efficiently release nucleic acids (DNA, RNA or both) from the target micro-organism(s), (2) protect the released nucleic acids from degradation by nucleases, (3) remove or neutralize clinical specimen substances that are frequently present in clinical specimens that inhibit reverse transcription, molecular amplification and/or detection of nucleic acids, and (4) take into account the analytical sensitivity of the molecular assay required for the specimen-type tested, based on clinical data.

Over the past decade or so, an impressive variety of nucleic acid extraction procedures and commercial reagents and kits have been used in molecular diagnostics [15–17,40]. Initially, laborious protocols based on phenol–chloroform extraction, proteinase K digestion and ethanol precipitation were used. However, the use of these protocols is not recommended for NAT in infectious diseases because they are time-consuming, labour-intensive and lead to an increased risk of false-positive results associated with carry-over contamination between samples, attributable to the large number of required manipulations [15,41]. Currently, most molecular diagnostic microbiology laboratories use commercially available prepackaged extraction kits or reagents for extraction of DNA and/or RNA (Table 1). These commercial systems for nucleic acid extraction and purification are either manual or automated and are often based on nucleic acid capture technologies using, for example, magnetic beads, silica matrix or resins [15,42,43]. The automated systems have the added advantages of enabling high-throughput extraction and often providing closed systems designed to reduce or eliminate carry-over contamination. However, there is no commercially available system, to date, that reliably lyses all types of microbes that might be found in a clinical sample and that is sufficiently rapid to be compatible with the development of less-than-one-hour molecular theranostic tools (Table 1). The comparison of a variety of classical and commercial nucleic acid extraction methods by various investigators has revealed variable performances associated with incomplete cell lysis and/or PCR inhibition [15,44–46]. Clearly, extensive validation of these methods is required to evaluate their potential to extract and purify nucleic acids from a variety of microbial cells and viruses. The real challenge is to extract rapidly (ideally in less than 15 min) and efficiently microbial nucleic acids from low amounts of hard-to-lyse target cells, such as Gram-positive bacteria, bacterial spores and yeasts, from within a variety of clinical samples. Cepheid (http://www.cepheid.com) has addressed this challenge elegantly by developing a single-use disposable cartridge for rapid (~10 min) sample preparation [47,48]. This cartridge is a closed microfluidic system containing all necessary reagents, components and chambers for microbial cell lysis and test sample preparation for NAT. It has been validated for DNA extraction from *Bacillus* spores using a system currently under development at Cepheid called GeneXpert™, enabling subsequent PCR amplification and fluorescence-based detection. Infectio Diagnostic (IDI; http://www.infectio.com) has also developed a rapid (10–15 min) sample preparation method that has been validated for the detection of both Gram-positive and Gram-negative bacteria with various types of clinical samples including vaginal and/or anal swabs, nasal swabs, feces and urine [49–51]. Recently, this nucleic acid extraction method has been shown to detect as few as two *Bacillus anthracis* spores per PCR reaction from clinical and environmental samples [52].

The efficiency of the sample nucleic acid preparation procedure is also highly dependent on the ability of the specimen collection and transport device to: (1) stabilize microbial cells and virus particles and more particularly their nucleic acids, and (2) not interfere significantly with the performance of NAT assays. Currently, most commercially available sample collection and transport devices are developed and validated exclusively for standard culture-based diagnostic microbiology. However, over the past few years several manufacturers have developed sample collection and transport systems that are compatible and validated with NAT assays, and in which nucleic acids are stable. Examples of such systems designed to improve the efficiency of molecular diagnostic test procedures include the DNA/RNA Protect™ system from Sierra Diagnostics (http://www.sierradiagnostics.com), the BD Vacutainer™ systems, CPT™ and PPT™, from Becton Dickinson (http://www.bd.com/vacutainer) and the swab systems for

Table 1. Examples of commercially available kits and automated systems for rapid nucleic acid extraction from micro-organisms in various clinical samples

Company	Commercial Kit[a]	Time (min)	Samples
Amersham Biosciences	GFX Genomic Blood DNA Purification Kit	30	Blood, buccal cells, yeast, bacteria
Q-BIOgene (BIO-101)	Fast DNA/RNA Kits	30	Plant and animal tissues, bacteria, yeast, fungi
bioMérieux	Nuclisens™ Isolation Kits	90	Serum, plasma, whole blood, genital secretions, tissues, cells, virus
	Nuclisens™ Extractor System[a]	45	Serum, plasma, whole blood, genital secretions, tissues, cells, virus
Bio-Rad	AquaPure DNA/RNA Isolation Kits	30	Cultured cells, plant and animal tissues, buffy coat, blood, bacteria
Dynal	Dynabeads® DNA DIRECT™ Universal	10	Cultured cells, clinical specimens, bacteria, fungi, tissues from various species
	Dynal MPC®-auto 96[a]	60–80	Blood
Epicentre	MasterPure™ Complete DNA and RNA Purification Kit	60	Blood, cell cultures, plant tissues, mammalian tissues, fluids, bacteria, yeast
MBI Fermentas	Genomic DNA Purification Kit	20–25	Blood, serum, cell culture, plant, bacteria, mammalian tissues
MoBio	UltraClean Microbial DNA/RNA Kit	30–35	Yeast, bacteria, fungi, fecal samples
QIAGEN	QIAamp DNA/RNA Mini Kits	20–60	Mammalian tissues and fluids, bacteria, parasites, virus
	QIAamp 96 Virus Biorobot Kit[a]	150	Cell-free body fluids
Roche	High Pure™ PCR Template Preparation Kit	35	Blood, buffy coat, cultured cells, yeast, bacteria
	MagNA Pure LC DNA Isolation Kits[a]	60	Blood, tissues, cultured cells, yeast, bacteria

[a]Automated high-throughput systems for nucleic acid extraction.
Amersham Biosciences (http://www.amershambiosciences.com), Q-BIOgene (http://www.qbiogene.com), bioMérieux (http://www.biomerieux-vitek.com), Bio-Rad (http://www.bio-rad.com), Dynal (http://www.dynalbiotech.com), MBI Fermentas (http://www.fermentas.com), QIAGEN (http://www.qiagen.com), Roche (http://www.roche.com).

NAT assays developed by Medical Packaging Corporation (http://www.medicalpackaging.com).

Towards real-time molecular theranostic tools

Over the past decade, several companies have developed various NAT assays for direct detection of viral pathogens and some fastidious bacteria from clinical samples (Table 2). However, most of these commercial tests require hours (time to results range from 2–30 h) and numerous (up to 75) technical steps and are thus incompatible with one-hour molecular theranostics in infectious diseases. Moreover, these assays focus on few bacterial pathogens (*Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Mycobacterium tuberculosis*) and on viruses, and consequently, do not address the crucial identification of bacterial pathogens responsible for the most treatable bacterial infections, where rapid and appropriate intervention is necessary for quick prevention measures (i.e. isolation of patients) or proper treatment. Assays based on strand displacement amplification (SDA) developed by Becton Dickinson are among the most rapid on the market (approximately 2.5 hours including sample preparation). For these assays, isothermal amplification by SDA at 60°C was coupled with real-time detection of amplicons using fluorescent probes [53,54]. Amplification by SDA coupled with real-time detection of amplified products by fluorescence polarization in 15–30 min has been reported [55,56]. However, this technology is not commercially available.

Regarding PCR, the recent availability of rapid cycling instruments thus enabling fluorescence measurements during each amplification cycle [e.g. the LightCycler™ from Roche (http://www.roche.com) and the SmartCycler® from Cepheid] coupled with recent advances in nucleic acid extraction and homogeneous nucleic acid amplification based on fluorescence detection has led to the development of less-than-one-hour PCR assays [43,57–62]. In these systems, real-time fluorescence monitoring can be performed by using fluorescent dyes such as SYBR-Green I, which binds non-specifically to double-stranded DNA generated during the PCR amplification. Alternatively, fluorescent probes (such as the popular TaqMan® system) that bind specifically to amplicon target sequences can also be used. Besides their speed, real-time PCR assays using rapid thermocycling technologies

Table 2. Main commercial molecular diagnostic tests for detection of infectious disease agents directly from clinical samples

Company	Method	Target (specimen type)
Abbott	LCR	*Chlamydia trachomatis* [endocervical, urethral (male), urine (male and female)][a]
		Neisseria gonorrhoeae [endocervical, urethral (male), urine (male and female)][a]
	PCR	HCV (blood)
		HIV-1 (blood)[a]
Bayer	bDNA	HBV (blood)
		HCV (blood)
		HIV (blood)
bioMérieux	NASBA	CMV (blood)[a]
		HIV-1 (blood)[a]
Becton Dickinson	SDA	*C. trachomatis*/*N. gonorrhoeae* (urine)[a]
		Mycobacterium tuberculosis (clinical respiratory samples)
Digene	Hybrid capture	*C. trachomatis* (urine)[a]
		CMV (peripheral white blood cells isolated from whole blood)[a]
		EBV (cerebrospinal fluid)
		HPV (cervical swab or brush)[a]
		N. gonorrhoeae (urine)[a]
		HBV (serum)
Gen-Probe	Hybridization	*C. trachomatis*/*N. gonorrhoeae* [endocervical, urethral (male)][a]
		Streptococcus pyogenes (throat swab)[a]
	TMA	*C. trachomatis* [endocervical, urethral (male), conjunctive][a]
		HIV/HCV (blood)[a]
		M. tuberculosis (clinical respiratory samples)[a]
Roche	PCR	*C. trachomatis*/*N. gonorrhoeae* [endocervical, urethral (male), urine (male and female)][a]
		CMV (blood)
		HBV (blood)
		HCV (blood)[a]
		HIV-1 (blood)[a]
		M. tuberculosis (clinical respiratory samples)[a]
Visible Genetics	PCR and DNA sequencing	HIV (blood)[a]

Abbreviations: bDNA, branched-DNA; CMV, cytomegalovirus; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HIV-1, type 1 human immunodeficiency virus; HPV, human papillomavirus; HSV, herpes simplex virus; LCR, ligase chain reaction; NASBA, nucleic acid sequence-based amplification; PCR, polymerase chain reaction; SDA, strand displacement amplification; TMA, transcription-mediated amplification.
[a]FDA-approved molecular diagnostic tests.
Abbott (http://www.abbott.com), Bayer (http://www.bayer.com), bioMérieux (http://www.biomerieux-vitek.com), Becton Dickinson (http://www.bd.com), Digene (http://www.digene.com), Gen-Probe (http://www.gen-probe.com), Roche (http://www.roche.com), Visible Genetics (http://www.visgen.com).

offer additional advantages including: (1) reduced risks of carry-over contamination by amplicons because amplification and detection occur within a closed system, and (2) the ability to quantify more accurately the starting amount of target nucleic acids because fluorescence is measured at the end of the primer annealing step of each amplification cycle. Presently, there is no real-time PCR assay for infectious agents approved by the US Food and Drug Administration (FDA).

A real-time, fast PCR assay for the detection of Group B streptococci from combined vaginal and anal swabs in parturient women at delivery to prevent meningitis in newborns, is likely to be commercialized by IDI in the near future. This assay, developed in our laboratory, can identify this pathogen directly from the swabs in less than 45 min [50], and has been shown to have good sensitivity and specificity (Table 3). Other examples of useful real-time molecular theranostic tools, which will greatly impact on clinical practice include: (1) assays for the rapid screening of methicillin-resistant *Staphylococcus aureus* (MRSA) from nasal swabs, and of vancomycin-resistant enterococci (VRE) from rectal swabs to enable better management of both colonized and infected patients; (2) broad-range assays for the universal detection of bacteria from blood samples; and (3) species-specific assays for the rapid detection of *Bacillus*

Table 3. Clinical evaluation of conventional and real-time PCR assays for the detection of GBS in combined vaginal and anal specimens from 112 pregnant women at delivery

PCR method	Sensitivity[a]	Specificity[b]	Positive predictive value[c]	Negative predictive value[d]
Conventional	97.0% (32/33)	100% (79/79)	100% (32/32)	98.8% (79/80)
Real-time	97.0% (32/33)	100% (79/79)	100% (32/32)	98.8% (79/80)

[a]Number of both culture-positive and PCR-positive/number of culture-positive.
[b]Number of both culture-negative and PCR-negative/number of culture-negative.
[c]Number of both culture-positive and PCR-positive/number of PCR-positive.
[d]Number of both culture-negative and PCR-negative/number of PCR-negative.
Source: Ref. [50]

anthracis from clinical and environmental samples. In July 2002, the first vancomycin-resistant *Staphylococcus aureus* (VRSA) was described. If this resistance disseminates like most other resistant bacteria have done in the past, a rapid test could again be extremely useful in the future. In the years to come, a myriad of other rapid DNA-based tests for immediate patient care in hospitals and medical clinics will probably be developed using these real-time technologies.

Oligonucleotide microchips for more complex and more informative NAT assays

Real-time NAT technologies are limited by the number of different wavelengths of fluorescent emission that can be simultaneously monitored (i.e. maximum of four wavelengths) [43,61]. The challenge of detecting and identifying the wide variety of genetic polymorphisms important for microbiological diagnostic purposes requires the development of much more informative assays. In this respect, the biochip technology is extremely promising. Oligonucleotide microchips have been used for amplicon detection after nucleic acid amplification for microbial identification at the species level and resistance genotyping [63–65]. However, complex multiplex PCR assays, which are often difficult to optimize, must be developed with these systems because of the wide variety of genes involved, particularly for antimicrobial resistance. The use of oligonucleotide analogs, such as locked nucleic acids (LNAs), peptide nucleic acids (PNAs) or various DNA ligands, is known to facilitate the development of multiplex PCR assays by reducing secondary structures and improving the binding properties of oligonucleotides [66,67]. However, emerging biochip technologies, such as nucleic acid amplification directly onto the oligonucleotide biochip or the use of arrays coupled with ultrasensitive detection methods not requiring previous amplification of the nucleic acid target, represent more promising strategies for the development of highly informative NAT assays for infectious diseases theranostics [68]. PCR amplification onto a microarray of gel-immobilized oligonucleotides, anchored SDA on microelectronic chip arrays, rolling circle amplification directly onto a solid support and array-based ultra-sensitive electrical detection of nucleic acids with gold nanoparticle probes are particularly promising [68–72].

Most microchip technologies require tagging of the analyte with fluorescent, radioactive or electroactive labels [73,74], which require additional transformation steps that are time-consuming and costly. Future technologies will incorporate more active electronics and optics to improve sensitivity, reduce costs and increase speed of analysis. An example of this is a recently developed methodology using a polymeric biosensor enabling optical (colorimetric or fluorometric) or electrical detection of nucleic acids [75]. This method is rapid, simple, sensitive (detection limit of 1.5×10^4 target nucleic acids per µl) and does not require any chemical reaction of the probes or the analytes because it is based on conformational modification of the polymer when mixed with single-stranded or double-stranded target nucleic acids. Such technologies should lead to solution and solid-state colorimetric or electrical nucleic acid sensors that can be incorporated into microfluidic systems for point-of-care diagnostics in the future.

Monitoring of assay performance using quality control reactions

The following controls must be included to verify the performance of a NAT assay:

Contamination control reactions These control reactions not containing any target nucleic acid must be performed with each run to exclude false-positive results associated with carry-over contamination by target nucleic acids. The contaminating nucleic acids can originate from one or more of the four following sources: (1) target amplification products from previous reactions, (2) microbial cultures of the target organism, (3) test samples positive for the target infectious agent(s), and (4) NAT reagents including lytic enzymes used for DNA extraction, *Taq* polymerase and water (which have all been shown to be contaminated by microbial genomic DNA [76–78]).

Carry-over contamination by positive test samples, microbial cultures or amplicons can be efficiently prevented by using good laboratory practices [76,79,80]. Degradation of uracil-containing DNA by the uracil-*N*-glycosylase is often

used in combination with good laboratory practice to further reduce the risks of carry-over contamination by amplicons [16]. By contrast, NAT reagents contaminated by target microbial nucleic acids must be submitted to a chemical or enzymatic inactivation treatment before testing to prevent their amplification and/or detection. This type of contamination particularly hampers the development of sensitive broad-range molecular diagnostic assays based on nucleic acid detection. Indeed, analysis of the conserved bacterial rRNA gene sequences contaminating different preparations of *Taq* DNA polymerase revealed that these nucleic acids originated from various bacteria [78,81]. DNA inactivation using UV light exposure in the presence of photoreactive compounds (e.g. psoralen or isopsoralen) represents the most widely used method to prevent amplification of microbial DNA contaminating PCR reagents [77,82]. Psoralens and isopsoralens form covalent monoadducts and crosslinks with nucleic acids upon activation with UV light.

Internal controls Internal controls (IC) must be performed. They are integrated into the NAT assay, and are designed to verify the efficiency of each amplification and/or detection reaction. The IC must always show a uniform positive signal for all test samples negative for the target microorganism(s) and/or associated resistance and virulence genes. An absent or low IC signal is commonly associated with total or partial inhibition of the NAT assay by the test sample. Technical failures at the reagent or instrument level can also prevent normal amplification of the IC. Thus, all NAT reactions showing (1) a negative signal for the target infectious agent(s) and (2) a lower than expected IC signal must be considered non-conclusive because assay performance is not optimal for these reactions.

External controls These are positive and negative controls that must be incorporated into each run to validate reagents integrity and monitor assay specificity. The positive control should be at or near the detection limit of the NAT assay to efficiently detect any technical failure that might reduce its performance. Ideally, the negative control should be nucleic acids similar to those targeted by the assay, such as DNA from a closely related microbial species or virulence factor.

Dried reagents

The need for standardized and quality controlled reagents for NAT renders the use of frozen ingredients impractical. The availability of ready-to-use freeze-dried NAT reagent mixes of proven quality would greatly facilitate implementation of these assays in microbiology laboratories including those with relatively limited facilities. Indeed, dried reagent formulations, which are already used for the manufacturing of some commercially available molecular diagnostic tests, will become increasingly popular because they ensure maximum performance and highest reproducibility of NAT assays and are stable for at least a year at 4°C or at ~20°C [83].

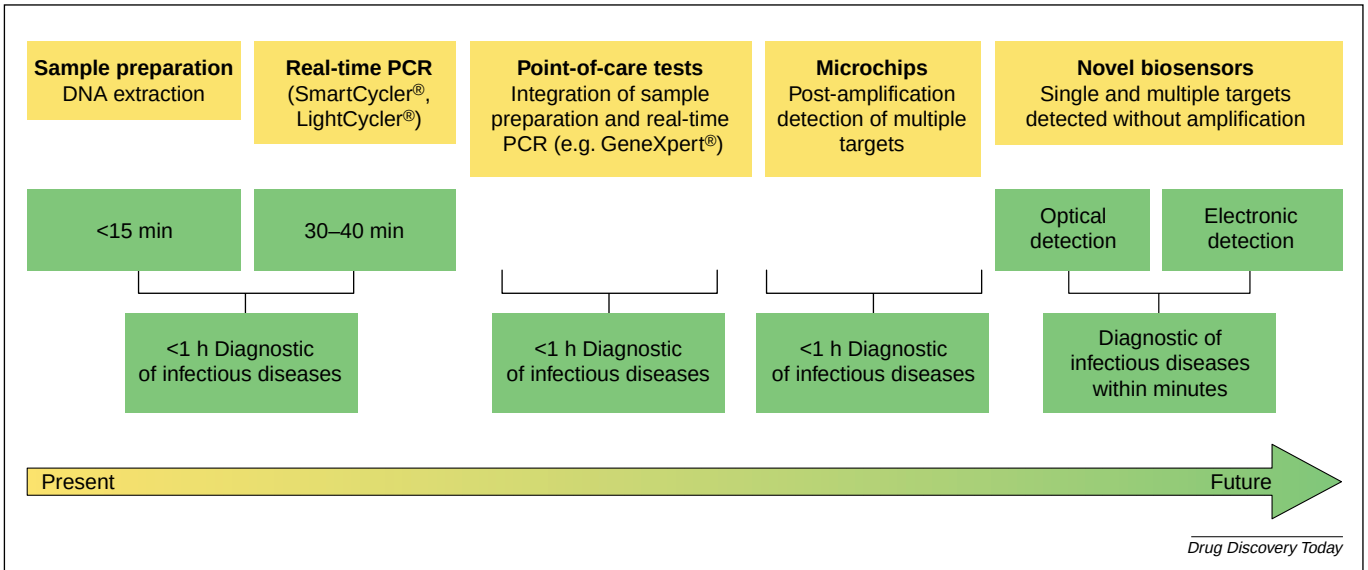

Figure 2. The future of DNA-based theranostic technologies. Currently available real-time PCR technologies, coupled with rapid sample preparation, enable less-than-one-hour diagnostic of infectious diseases. Within the next few years, microfluidic devices will integrate sample preparation and nucleic acid extraction with amplification and detection technologies. Subsequently, post-amplification detection on microchips, and eventually, ultra-sensitive detection technologies enabling direct detection from clinical samples without prior nucleic acid amplification should become available. These novel molecular diagnostics tools will bring faster and more comprehensive diagnostic information that will enable optimal management of patients.

Conclusions and perspectives

Stat molecular microbiology is here! The rapidly growing database of nucleotide sequences combined with recent technological advances in the fields of microbial nucleic acid extraction from biological samples, real-time NAT assays and microchips, form the basis for a wide array of rapid diagnostic tools for microbiology laboratories and point-of-care testing. Integration of these technologies into microfluidic systems will ensure a single-step assay not requiring technical skills. Simple dipstick diagnosis using polymeric detection without amplification also seems possible. Decision algorithms for diagnostic and therapeutic guidance will be needed to facilitate clinical practice. These novel diagnostic tools will also have an important role in the rapid detection and characterization of biothreat agents from various clinical and environmental samples to effectively protect military and civilian populations against biological weapons. Overall, these new technologies will offer multiple rapid diagnostic possibilities that will slowly replace classical phenotypic methods for identification of microbes and determination of their antimicrobial susceptibility pattern (Fig. 2).

The increasing availability of rapid (less-than-one-hour), sensitive, accurate and highly informative NAT assays for the detection of infectious agents and associated resistance and virulence genes will revolutionize the practice of medicine by gradually reducing the reliance on standard culture-based microbiological methods that require at least two days. Eventually, physicians will be able to simultaneously obtain microbiology, biochemistry, hematology and radiology results for their patients. This will enable better initial management of patients including the need for isolation, the choice of appropriate routine examinations and, if required, the selection of an optimal treatment with antimicrobials. Consequently, more rational and effective use of antimicrobials should lead to a better control of the emergence of microbial drug resistance. Thus, novel rapid molecular theranostic tools will provide clinicians with real-time, crucial, clinical information that should greatly improve the management of microbial infections and, ultimately, will save lives, improve the quality of life of infected patients and reduce healthcare costs.

Acknowledgements

The molecular diagnostic group of the Centre de Recherche en Infectiologie de l'Université Laval is supported by Infectio Diagnostic (IDI) Inc. and by grant PA-15586 from the Canadian Institutes of Health Research. We thank Jean-Luc Simard, Maurice Boissinot, Luc Bissonnette and Myrian Grondin for their important contribution in the creation and writing of this review.

References

1 Baquero, F. (1996) Antibiotic resistance in Spain: what can be done? Task force of the general direction for health planning of the spanish ministry of health. *Clin. Infect. Dis.* 23, 819–823
2 Bergeron, M.G. and Ouellette, M. (1998) Preventing antibiotic resistance through rapid genotypic identification of bacteria and of their antibiotic resistance genes in the clinical microbiology laboratory. *J. Clin. Microbiol.* 36, 2169–2172
3 McCaig, L.F. and Hughes, J.M. (1995) Trends in antimicrobial drug prescribing among office-based physicians in the United States. *J. Am. Med. Assoc.* 273, 214–219
4 Bergeron, M.G. and Ouellette, M. (1995) Diagnosing bacterial infectious diseases in one hour: An essential upcoming revolution. *Infection* 23, 69–72
5 Picard, F.J. and Bergeron, M.G. (1999) Rapid diagnosis of bacterial infections using technologies based on nucleic acid detection. *Can. J. Infect. Dis.* 10, 16C–24C
6 Bergeron, M.G. (2001) *Molecular Testing in Laboratory Medicine* (Bruns, D.E., ed.), pp. 217–218, AACC Press
7 Mahony, B.J. and Chernesky, A.M. (1999) *Manual of Clinical Microbiology* (Murray, P.R., ed.), pp. 202–214, ASM Press
8 Fleischmann, R.D. *et al.* (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 269, 496–512
9 Enserink, M. (2002) Microbial genomics: TIGR begins assault on the anthrax genome. *Science* 295, 1442–1443
10 Perna, N.T. *et al.* (2001) Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409, 529–533
11 Subramanian, G. *et al.* (2001) Microbial disease in humans: a genomic perspective. *Mol. Diagn.* 6, 243–252
12 Persing, D.H. (1996) *PCR Protocols for Emerging Infectious Diseases*, ASM Press
13 Persing, D.H. *et al.* (1993) *Diagnostic Molecular Microbiology – Principles and Applications*, ASM Press
14 Ehrlich, G.D. and Greenberg, S.J. (1994) *PCR-Based Diagnostics in Infectious Diseases*, Blackwell Scientific Publications
15 Jungkind, D. and Kessler, H.H. (2002) *Manual of Commercial Methods in Clinical Microbiology* (Truant, A.L., ed.), pp. 306–323, ASM Press
16 Tang, Y. and Persing, D.H. (1999) *Manual of Clinical Microbiology* (Murray, P.R., ed.), pp. 215–244, ASM Press
17 Tang, Y. *et al.* (1997) Molecular diagnostics of infectious diseases. *Clin. Chem.* 43, 2021–2038
18 Greisen, K. *et al.* (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *J. Clin. Microbiol.* 32, 335–351
19 Krzanowski, J.J. (1994) Broad-range polymerase chain reaction for detection and identification of bacteria. *JFMA* 81, 835–837
20 McCabe, K.M. *et al.* (1999) Bacterial species identification after DNA amplification with a universal primer pair. *Mol. Genet. Metab.* 66, 205–211
21 Woese, C.R. (1987) Bacterial evolution. *Microbiol. Rev.* 51, 221–271
22 Maidak, B.L. *et al.* (2001) The RDP-II (ribosomal database project). *Nucleic Acids Res.* 29, 173–174
23 Martineau, F. *et al.* (2001) Development of a PCR assay for identification of staphylococci at genus and species levels. *J. Clin. Microbiol.* 39, 2541–2547
24 Garcia-Martinez, J. *et al.* (2001) RISSC: A novel database for ribosomal 16S-23S RNA genes spacer regions. *Nucleic Acids Res.* 29, 178–180
25 Ke, D. *et al.* (1999) Development of a PCR assay for rapid detection of enterococci. *J. Clin. Microbiol.* 37, 3497–3503
26 Blackwood, K.S. *et al.* (2000) Evaluation of *recA* sequences for identification of *Mycobacterium* species. *J. Clin. Microbiol.* 38, 2846–2852
27 Kwok, A.Y. *et al.* (1999) Species identification and phylogenetic relationships based on partial HSP60 gene sequences within the genus *Staphylococcus*. *Int. J. Syst. Bacteriol.* 49, 1181–1192
28 Goh, S.H. *et al.* (1996) HSP60 gene sequences as universal targets for microbial species identification: Studies with coagulase-negative staphylococci. *J. Clin. Microbiol.* 34, 818–823
29 Kostman, J.R. *et al.* (1995) A universal approach to bacterial molecular epidemiology by polymerase chain reaction ribotyping. *J. Infect. Dis.* 171, 204–208
30 Fluit, A.C. *et al.* (2001) Molecular detection of antimicrobial resistance. *Clin. Microbiol. Rev.* 14, 836–871

31 Pfaller, M.A. (2001) Molecular approaches to diagnosing and managing infectious diseases: Practicality and costs. *Emerg. Infect. Dis.* 7, 312–318
32 Quintiliani, R. *et al.* (1999) *Manual of Clinical Microbiology* (Murray, P.R., ed.), pp. 1505–1525, ASM Press
33 Tenover, F.C. and Rasheed, J.K. (1999) *Manual of Clinical Microbiology* (Murray, P.R. *et al.*, eds.), pp. 1578–1592, ASM Press
34 Guillaume, A. and Philippon, A. (1994) *PCR-based Diagnostics in Infectious Disease* (Ehrlich, G.D. and Greenberg, S.J., eds.), pp. 665–687, Blackwell Scientific Publications
35 Martineau, F. *et al.* (2000) Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* 44, 231–238
36 Martineau, F. *et al.* (2000) Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. *J. Antimicrob. Chemother.* 46, 527–533
37 Niemeyer, D.M. *et al.* (1996) Role of *mecA* transcriptional regulation in the phenotypic expression of methicillin resistance in *Staphylococcus aureus*. *J. Bacteriol.* 178, 5464–5471
38 Kolbert, C.P. *et al.* (1998) Branched-DNA assay for detection of the *mecA* gene in oxacillin-resistant and oxacillin-sensitive staphylococci. *J. Clin. Microbiol.* 36, 2640–2644
39 Deisingh, A.K. and Thompson, M. (2002) Detection of infectious and toxigenic bacteria. *Analyst* 127, 567–581
40 Greenfield, L. and White, T.J. (1993) *Diagnostic Molecular Microbiology: Principles and Applications* (Persing, D.H. *et al.*, eds), pp. 122–137, ASM Press
41 Kwok, S. and Higuchi, R. (1989) Avoiding false positives with PCR. *Nature* 339, 237–238
42 Grisold, A.J. *et al.* (2002) Detection of methicillin-resistant *Staphylococcus aureus* and simultaneous confirmation by automated nucleic acid extraction and real-time PCR. *J. Clin. Microbiol.* 40, 2392–2397
43 Wolk, D.M. and Persing, D.H. (2002) *Manual of Commercial Methods in Clinical Microbiology* (Truant, A.L., ed.), pp. 429–450, ASM Press
44 Dion, M. *et al.* (1999) Comparison of nine commercial kits for rapid nucleic acid extraction from microbial cultures. In *99th General Meeting of the American Society for Microbiology*, Chicago, IL, USA, (abstract no. C-481)
45 Echavarria, M. *et al.* (1998) PCR method for detection of adenovirus in urine of healthy and human immunodeficiency virus-infected individuals. *J. Clin. Microbiol.* 36, 3323–3326
46 Muller, F.C. *et al.* (1998) Rapid detection of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. *J. Clin. Microbiol.* 36, 1625–1629
47 Belgrader, P. *et al.* (2000) A microfluidic cartridge to prepare spores for PCR analysis. *Biosens. Bioelectron.* 14, 849–852
48 Taylor, M.T. *et al.* (2001) Lysing bacterial spores by sonication through a flexible interface in a microfluidic system. *Anal. Chem.* 73, 492–496
49 Bélanger, S.D. *et al.* (2002) Rapid detection of Shiga toxin-producing bacteria in feces by multiplex PCR with molecular beacons on the Smart Cycler. *J. Clin. Microbiol.* 40, 1436–1440
50 Bergeron, M.G. *et al.* (2000) Rapid detection of group B streptococci in pregnant women at delivery. *N. Engl. J. Med.* 343, 175–179
51 Martineau, F. *et al.* (2000) Development of a rapid PCR assay specific for *Staphylococcus saprophyticus* and application to direct detection from urine samples. *J. Clin. Microbiol.* 38, 3280–3284
52 Leblanc, E. *et al.* (2002) Less than one hour detection of *Bacillus anthracis* spores and vegetative cells from clinical specimens by fluorescence-based PCR. In *102nd General Meeting of the American Society for Microbiology*, Salt Lake City, UT, USA, (abstract no. C-257)
53 Templeton, K. *et al.* (2001) The detection of *Chlamydia trachomatis* by DNA amplification methods in urine samples from men with urethritis. *Int. J. STD AIDS* 12, 793–796
54 Nadeau, J.G. *et al.* (1999) Real-time, sequence-specific detection of nucleic acids during strand displacement amplification. *Anal. Biochem.* 276, 177–187
55 Spears, P.A. *et al.* (1997) Simultaneous strand displacement amplification and fluorescence polarization detection of *Chlamydia trachomatis* DNA. *Anal. Biochem.* 247, 130–137
56 Walker, G.T. and Linn, C.P. (1996) Detection of *Mycobacterium tuberculosis* DNA with thermophilic strand displacement amplification and fluorescence polarization. *Clin. Chem.* 42, 1604–1608
57 Belgrader, P. *et al.* (1999) PCR detection of bacteria in seven minutes. *Science* 284, 449–450
58 Foy, C.A. and Parkes, H.C. (2001) Emerging homogeneous DNA-based technologies in the clinical laboratory. *Clin. Chem.* 47, 990–1000
59 Mackay, I.M. *et al.* (2002) Real-time PCR in virology. *Nucleic Acids Res.* 30, 1292–1305
60 Ke, D. *et al.* (2000) Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. *Clin. Chem.* 46, 324–331
61 Cockerill, F.R. and Smith, T.F. (2002) Rapid-cycle real-time PCR: a revolution for clinical microbiology. *ASM News* 68, 77–83
62 Nadkarni, M.A. *et al.* (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 148, 257–266
63 Troesch, A. *et al.* (1999) *Mycobacterium* species identification and rifampin resistance testing with high-density DNA probe arrays. *J. Clin. Microbiol.* 37, 49–55
64 Westin, L. *et al.* (2001) Antimicrobial resistance and bacterial identification utilizing a microelectronic chip array. *J. Clin. Microbiol.* 39, 1097–1104
65 Chizhikov, V. *et al.* (2002) Detection and genotyping of human group A rotaviruses by oligonucleotide microarray hybridization. *J. Clin. Microbiol.* 40, 2398–2407
66 Leumann, C.J. (2002) DNA analogues: From supramolecular principles to biological properties. *Bioorg. Med. Chem.* 10, 841–854
67 Stender, H. *et al.* (2002) PNA for rapid microbiology. *J. Microbiol. Methods* 48, 1–17
68 Schweitzer, B. and Kingsmore, S. (2001) Combining nucleic acid amplification and detection. *Curr. Opin. Biotechnol.* 12, 21–27
69 Tillib, S.V. *et al.* (2001) Integration of multiple PCR amplifications and DNA mutation analyses by using oligonucleotide microchip. *Anal. Biochem.* 292, 155–160
70 Westin, L. *et al.* (2000) Anchored multiplex amplification on a microelectronic chip array. *Nat. Biotechnol.* 18, 199–204
71 Park, S.J. *et al.* (2002) Array-based electrical detection of DNA with nanoparticle probes. *Science* 295, 1503–1506
72 Tillib, S.V. and Mirzabekov, A.D. (2001) Advances in the analysis of DNA sequence variations using oligonucleotide microchip technology. *Curr. Opin. Biotechnol.* 12, 53–58
73 Ramsay, G. (1998) DNA chips: state-of-the art. *Nat. Biotechnol.* 16, 40–44
74 Wang, J. (2000) From DNA biosensors to gene chips. *Nucleic Acids Res.* 28, 3011–3016
75 Ho, A-H. *et al.* (2002) Colorimetric and fluorometric detection of nuleic acids using cationic polythiophene derivatives. *Angew. Chem. Int. Edit* 41, 1548–1551
76 Millar, B.C. *et al.* (2002) Risk assessment models and contamination management: Implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. *J. Clin. Microbiol.* 40, 1575–1580
77 Corless, C.E. *et al.* (2000) Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. *J. Clin. Microbiol.* 38, 1747–1752
78 Hughes, M.S. *et al.* (1994) Identification and elimination of DNA sequences in *Taq* DNA polymerase. *J. Clin. Microbiol.* 32, 2007–2008
79 Furrows, S.J. and Ridgway, G.L. (2001) 'Good laboratory practice' in diagnostic laboratories using nucleic acid amplification methods. *Clin. Microbiol. Infect.* 7, 227–229
80 Burkardt, H.J. (2000) Standardization and quality control of PCR analyses. *Clin. Chem. Lab. Med.* 38, 87–91
81 Maiwald, M. *et al.* (1994) Characterization of contaminating DNA in *Taq* polymerase which occurs during amplification with a primer set for *Legionella* 5S ribosomal RNA. *Mol. Cell. Probes* 8, 11–14
82 Klausegger, A. *et al.* (1999) Gram type-specific broad-range PCR amplification for rapid detection of 62 pathogenic bacteria. *J. Clin. Microbiol.* 37, 464–466
83 Klatser, P.R. *et al.* (1998) Stabilized, freeze-dried PCR mix for detection of Mycobacteria. *J. Clin. Microbiol.* 36, 1798–1800