5 Identification of Pathogens by Nonculturing Molecular Techniques

Natalie N. Whitfield[1] · *Donna M. Wolk*[2]
[1]University of Arizona Medical Center, Tucson, AZ, USA
[2]Department of Pathology, University of Arizona BIO5 Institute, Tucson, AZ, USA

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Abstract
This chapter will focus on the use of non-culture-based methods to identify prokaryotic human pathogens. The methods discussed rely primarily on nucleic acid detection and analysis for pathogens commonly encountered in diagnostic and clinical research settings. Diagnostic capabilities in clinical microbiology have exponentially grown due to the impact of molecular methods and tools, like (but not limited to) polymerase chain reaction (PCR) and DNA sequencing, for the detection of human pathogens. Increasingly, technologies such as mass spectrometry and next-generation sequencing that have been incorporated into routine research laboratory use for sometime will be implemented for routine use in a clinical setting along with PCR-based assays. In addition, the pace and continued development of molecular methods for clinical applications of pathogen detection may ultimately transform diagnostic microbiology into a “culture less” diagnostic science, in the future.

Introduction

This chapter will focus on non-culture-based methods to identify prokaryotic human pathogens, commonly encountered in diagnostic and clinical research settings. Diagnostic capabilities in clinical microbiology have exponentially grown due to the impact of molecular tools to detect human pathogens. New technology will continue to rapidly influence the diagnostic sciences in the future.

The current technologies have already improved diagnostic performance and stand ready to be used as tools to improve patient care and disease management. The benefits of these technologies are their speed, increased sensitivity and specificity, greater versatility, and more accurate results that are not dependent on isolation of pathogens in culture. Their limitations can include higher costs, inability to detect emerging genetic sequences that may not be targeted by primer or probe regions, and the inability to access cultivated bacteria for susceptibility testing or genotyping.

We will begin by reviewing real-time polymerase chain reaction (PCR), arguably the molecular method with most impact to the clinical laboratory in the last two decades. The focus of this chapter will be on the applied use of this technology and the circumstances and challenges faced by clinical and diagnostic laboratories as we strive to impact health care and serve as

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prudent stewards of resources. We will finish with a review of emerging technology and DNA sequencing methods that may impact clinical microbiology over the next two decades.

Real-Time PCR

Real-time PCR has quickly integrated into diagnostic microbiology practice as a rapid method to identify or quantify many common pathogens (Espy et al. 2006; Bravo and Procop 2009; Wittwer and Kusukawa 2011; Wolk and Hayden 2011). Related methods continue to evolve and redefine its role in clinical diagnostics. With the development of simpler and even more rapid methods, multi-target chemistries, and smaller instruments that integrate extraction and amplification, require minimal manipulation, and impose less-stringent biosafety requirements (Espy et al. 2002, 2006), real-time PCR methods are steadily impacting the laboratory's ability to improve patient care. Real-time PCR methods for pathogen identification are becoming commonplace, and there is an emerging trend to explore the use of this technology for determining antimicrobial resistance of organisms by analyzing the genes that confer resistance opposed to merely discerning the phenotypic determinants (Espy et al. 2006; Marlowe and Wolk 2006; Wolk et al. 2009c, d; Marner et al. 2011). The development of bacterial panels for direct detection of pathogens and their resistance mechanisms from blood cultures is likely to impact antimicrobial stewardship practices (Tenover and Rasheed 2004).

Such benefits currently decrease the time to result, the hands-on time demand, and the requirements for qualified molecular technologists to perform the testing, and although the evidence-based laboratory intervention literature is currently sparse, there is hope that these improvements will increase the cost-benefit of the test. There are now several assays and instruments available, which are classified as "moderate complexity" by the Clinical Laboratory Improvement Act, making them amenable to testing by most laboratory staff, 24/7 testing, and use in small community hospital laboratories and large reference laboratories alike (Espy et al. 2006; Bravo and Procop 2009). With the advent of completely integrated systems, the ability to apply such a technology to direct point-of-care testing and bedside identification is promising.

Currently, two main types of diagnostic real-time PCR assays are used for the detection of pathogens: (1) multi-step real-time PCR assays that require off-platform specimen processing, extraction of nucleic acid from the specimen, PCR assay setup and amplification/detection and (2) integrated assays that incorporate the three steps into the subsequent amplification/detection step. Examples of the latter type, in the order of appearance to the US market, include Cepheid's GeneXpert® system, Becton Dickinson's BD Max™ system, BioFire FilmArray®, and Focus Diagnostic's Simplexa Assays on the Integrated Cycler. The benefits of the integrated real-time PCR assays are decreased hands-on time, no manipulation of the extracted nucleic acids, which decreases the risk of contaminating the work area, and in most cases decreased reaction time and overall turnaround time.

Since there are hundreds of real-time PCR examples, all cannot be reviewed here and we refer readers to recent extensive review of methods (Wittwer and Kusukawa 2011; Wolk and Hayden 2011). Likewise, it is not possible to review the entire breadth and depth of laboratory developed tests (LDTs). We will review examples of common or emerging technology with impact to the clinical microbiology laboratories.

Multi-step Real-Time PCR Assays: Requirements for Upstream Nucleic Acid Extraction

Although, some of the newly developed PCR instruments are becoming fully integrated assays, there remains a large demand for multi-step PCR assays, which require upstream nucleic acid (NA) extraction; therefore, assays of this type continue to be created for Unites States and international patient care purposes. A summary of some common extraction methods used in diagnostic laboratories is presented in *Table 5.1*.

Nucleic extraction from clinical samples is accomplished by either manual or automated methods (see *Table 5.1* for commonly available methods). Determining which method to use is a critical step in successful nucleic acid purification and subsequent amplification assays. The extraction methods that may work for one pathogen and specimen type may not necessarily be effective for other specimen types and pathogens. In addition, structural characteristics of microorganisms and specimen-inhibiting substances can affect nucleic acid extraction.

Automated extraction systems tend to be more common and practical for clinical laboratories and are thought to provide several benefits over manual extraction methods. Automated NA extraction systems yield more reproducible NA recovery, while reducing the risk for contamination of other samples and the work environment (Hill 2011). Since they perform the NA extraction steps robotically, automated extraction instruments ensure greater workload efficiency; once the instrument is set up it requires very minimal manipulation or attention (Wolk et al. 2001; Knepp et al. 2003). An example of an automated extraction instrument, the Qiagen QIAsymphony SP system, is shown in *Fig. 5.1* and is designed to couple with the Qiagen Rotor-Gene Q real-time PCR instrument, pictured in front. Integrated extraction systems, such as the QIAsymphony, typically provide prefilled reagent cartridges, bar coding of samples and reagents, and touch-screen operation. Many systems allow continuous loading of 1–96 samples per run and can perform different purification procedures within the same run of 96 samples. In clinical laboratories, technology is expected to offer inventory control and full process documentation.

Despite the advantages of automated systems, there are circumstances when manual methods may be preferred or used in combination with automated methods. For example, manual methods may offer higher DNA yield or purity, or may be useful when microbial cell walls or inhibitors in specimen matrices become problematic. Manual extraction kits are usually less

Table 5.1
Manual and automated nucleic acid extraction methods for real-time PCR assays

	Kit/Platform	Manufacturer	Technology	Throughput samples/time	Specimen type
Manual	Highly pure	Roche Applied Science	Glass fiber fleece immobilized in a plastic filter	24/h	Serum, whole blood, plasma, urine, stool, sterile body fluids, respiratory tract specimens, genital and dermal swabs
	QIAamp	Qiagen	Silica gel membrane in column	DNA-24/h RNA-24/1.5 h	Serum, whole blood, plasma, urine, stool, sterile body fluids, respiratory tract specimens, nasal and fecal swabs
	IsoQuick	Orca Research	Ethanol precipitation	DNA-24/h RNA-24/2 h	Whole blood, plasma, stool, respiratory tract specimens, sterile body fluids, dermal, fecal, and genital swabs
	NucliSENS® miniMAG®	bioMeriéux	Proprietary Boom® technology with magnetic silica	DNA 12/ batch RNA 12/batch	Most sample types (plasma, serum, whole blood, stool, respiratory samples, etc.) 50–1,000 μL
Automated	MagNA Pure LC	Roche Applied Science	Magnetic silica particles	32/90 min	Serum, whole blood, plasma, stool, sterile body fluids, respiratory tract specimens, dermal and genital swabs
	MagNA Pure Compact			8/30 min	
	Cobas Ampliprep			96/2 h	
	BioRobot EZ1	Qiagen	Magnetic silica particles	6/20 min	Dermal, genital, and nasal swabs
	QIAcube			24/1.5 h	
	QIAagility			96/1.5 h	
	QIAsymphony			96/2 h	
	Need to add bioMerieux Easy Mag as they are common in clinical labs				
	Also M2000 from Abbott				
	NucliSENS® EasyMAG®	bioMeriéux	Proprietary Boom® technology with magnetic silica	DNA 24/ batch RNA 24/batch	Most sample types (plasma, serum, whole blood, stool, respiratory samples, etc.) 50–1,000 μL
	ABI Prism 6100	Applied Biosciences	Silica fiber membrane	96 per 30 min	Serum, plasma, swabs, cell culture media, blood, semen
	ABI Prism 6700		Magnetic particles	96 per 74 min	
	MagMax Express				
	MagMax Express-96				
	Maxwell 16	Promega	Paramagnetic particles	16 per 30–45 min	Blood, serum, plasma, cells, fresh/frozen or FFPE tissue

expensive, but do require several manipulations, offsetting the savings in reagent costs. Manual methods are time-consuming and are generally less reproducible than automated methods.

Trending Real-Time PCR Assays

Simplexa Assays

Simplexa (Focus Diagnostics; Cypress, CA) PCR assays on the 4-channel 3M Integrated Cycler (➲ *Fig. 5.2*) allow the processing of multiple pathogen target genes simultaneously. The Simplexa PCR assays are available in two formats, depending on the target pathogen(s): chemistries that require nucleic acid extraction prior to sample loading and direct specimen and reagent loading. PCR amplification and detection of up to 96 samples is rapid with results available in about an hour. The assays and instruments are extremely versatile with software programs for DNA and RNA, qualitative and quantitative PCR reactions, and a small instrument footprint. Their growing menu of molecular chemistries allow detection of *Clostridium difficile*, *Bordetella pertussis/parapertussis*, *Chlamydophila pneumoniae*, Group A streptococcus, *Mycoplasma pneumonia*, and *Borrelia* spp. from a patient specimen without requiring preliminary growth of the pathogen on agar media (Emmadi et al. 2011; Lanotte et al. 2011; Touati et al. 2009). The Simplexa assays that

Fig. 5.1
QIAsymphony SP instrument, an integrated extraction system from Qiagen, Valencia, CA, is designed to work in conjunction with the Qiagen Rotor-Gene Q, real-time PCR Instrument shown in front

Fig. 5.2
The 3M Integrated Cycler, sold by Focus Diagnostics

are fully integrated and do not require the upstream nucleic acid extraction steps allow for direct specimen testing, decreased turnaround time, and more widespread use by smaller laboratories where engineered molecular laboratory space is typically not available.

IntelligentMDx Assays

IntelligentMDx (Cambridge, MA) uses a unique proprietary bioinformatics process to provide analysis and verification of the genetic sequences, thus producing assays with the potential to quickly adapt to genetic change. The Intelligent MDx bioinformatics process PriMD has been specifically developed to address complex and evolving infectious targets. The PriMD system and IMDx processes enable a better understanding of nucleic acid interactions and thus can enhance the development of tests of any molecular application. Accelerated production of assays occurs by preselection of metrics, algorithms, and rules necessary to build robust, reproducible molecular assays for detection of pathogens. Theirs is the first process to use a systems approach and multifactorial computational capability that solves complex problems in assay commercialization. The *in silico* design and analysis process avoids the long trial-and-error approach and requires less wet lab experimentation prior to assay production. Built into this process is the integration of inputs from expert end-users to preset the clinical indications and specifications for an assay. In theory, the IMDx assay should be robust and easily adapted to emerging genetic sequences as pathogens evolve. Time will tell if the IMDx system will enhance clinical practice; yet, the strategy to prepare for genetic change, as pathogens evolve and mutate, and the ability to pretest assay design and check the cross-reactivity *in silico* prior to wet laboratory testing appear to add value to the existing commercial processes for diagnostic assay development and upkeep. Real-time PCR assays are designed with similar reaction conditions so that they can be multiplexed, run in parallel, or run separately, allowing clinical laboratories to optimize throughput and workflow.

Currently, there are two IMDx assays commercially available in Europe. The IMDx *C. difficile* assay for the Abbott *m*2000 is a PCR assay for the qualitative detection of conserved regions within toxin A and toxin B genes of the *C. difficile* genome in human symptomatic patients. The assay is intended for use on the Abbott *m*2000 RealTime system—a high-throughput system ranging from 24 to 96 samples/batch. The assay detects toxigenic *C. difficile*, including NAP1/027 hypervirulent strain and toxin B-variant type strains. In addition, the IMDx VanR assay detects *vanA* and *vanB* vancomycin resistance genes directly from human peri-rectal or rectal swabs and stool samples from patients at risk for Vancomycin Resistant Enterococcus (VRE) colonization.

Integrated PCR Assays

GeneXpert Assays

The Cepheid GeneXpert (Cepheid, Sunnyvale, CA) is a completely integrated single-use cartridge real-time PCR instrument for detection of pathogens directly from specimens. Specimen processing and decontamination occurs along with DNA extraction, amplification, and detection of the genetic target all

Fig. 5.3
Cepheid GeneXpert systems range in throughput from 1 cartridge to 96 cartridges

in the same cartridge. The result is rapid, that is, within 2 h, for all assays and requires minimal hands-on time. The system is composed of the GeneXpert instrument, with throughput designed to range from 1 to 96 samples simultaneously (Fig. 5.3) and a computer containing the required assay software to interpret the results. The various cartridges all incorporate *Bacillus globigii* spores, which serve as internal controls for extraction and amplification making the test technically effortless with little manipulation by the operator. Currently, Cepheid offers assay cartridges for several important pathogens (listed with reference to first published method comparisons), such as *Staphylococcus aureus* (Parta et al. 2009), *methicillin-resistant S. aureus* (Rossney et al. 2008), *C. difficile* (Novak-Weekley et al. 2010), vancomycin-resistant *Entercoccus* (Bourdon et al. 2010), Group B streptococcus (Gavino and Wang 2007), and *Mycobacterium tuberculosis* (Moure et al. 2011a, b), the latter assay being associated with a recent eruption of publications (Evans 2011; Hillemann et al. 2011; Ioannidis et al. 2011; Laudat et al. 2011; Lawn 2011; Marlowe et al. 2011; Miller et al. 2011; Spencer et al. 2011; Zeka et al. 2011).

The GeneXpert instrument is a random access instrument with STAT testing capabilities. Each individual reaction module can detect up to six genetic targets in a single cycler. Another advantage for pathogen detection is its multiplex capabilities, detecting the pathogen as well as some antibiotic-resistance genes. Strain typing capabilities are also available, as in the case of presumptive identification of *C. difficile 027/NAP1/B1 strains* (Babady et al. 2010). Methicillin resistance of *S. aureus*, vancomycin resistance of enterococci, and rifampin resistance of *M. tuberculosis* are discriminated by the system (Rossney et al. 2008; Shore et al. 2008; Wolk et al. 2009b, c, d; Bourdon et al. 2010; Dekeyser et al. 2011; Gazin et al. 2011; Hanif et al. 2011; Hillemann et al. 2011; Ioannidis et al. 2011; Marlowe et al. 2011; Marner et al. 2011; Miller et al. 2011; Moure et al. 2011a, b; Scanvic et al. 2011; Spencer et al. 2011; Zabicka et al. 2011; Zeka et al. 2011), but as with any laboratory method, vigilance is required, because antibiotic resistance genes can mutate quickly and exceptions can occur (Brenwald et al. 2010; Ciardo et al. 2010; Blanc et al. 2011; Shore et al. 2011).

Fig. 5.4
Idaho Technology's FilmArray

BioFire Film Array System: "The FilmArray"

The FilmArray system (BioFire Inc, Salt Lake City, UT) is a novel and completely integrated real-time multiplex PCR instrument (Fig. 5.4) that incorporates succeeding single-plex PCR reactions (Endimiani et al. 2011; Poritz et al. 2011). Originally launched for the detection of respiratory viruses (Loeffelholz et al. 2011; Pierce et al. 2011; Poritz et al. 2011; Rand et al. 2011), it does have the capability to identify bacterial pathogens, but

Fig. 5.5
The Luminex 100/200 system (*left*) and the Luminex MAGPIX instrument (*right*) provide detection of targets downstream to multiplex PCR

those targets have not yet been cleared by the Food and Drug Administration (FDA) for use in clinical laboratories. All nucleic acids are extracted and purified directly from the unprocessed sample. Then, the FilmArray performs a nested multiplex PCR assay. During the nested PCR, the FilmArray performs a single, large volume, multiplexed reaction with primers specific to several targets. Following the multiplex PCR reaction an individual single-plex second-stage PCR reaction detects the products that are amplified during the first round of PCR. Endpoint melting curve analysis of the amplicons and the FilmArray software generate a target result from the data. Targets in development include a 23 target gastrointestinal panel, 8 target sexually transmitted infection panel, and a 27 target Biothreat Panel.

Multiplex PCR

Bead-Based Assays

While none of the bacterial multiplex assays are currently cleared by the FDA, their widespread use and FDA product lines for Clinical Virology make it likely that panels of bacteriology targets will become the next stage of development. Luminex xTAG Technology (Luminex Corp, Austin TX) assays, performed on Luminex 100/200 instruments (Fig. 5.5), and Qiagen Liquichip (Qiagen Inc, Valencia, CA) assays are the two most common instrument platforms.

The Luminex assays are composed of a multiplexed PCR reaction that amplifies the regions of interest in the target pathogen genes followed by treatment with exonucleases to remove excess nucleotides and primers. Subsequently, a primer extension step that is specific for the pathogen target being analyzed is included in the PCR reaction. The 5' end of the primers is attached to a universal tag sequence. The 5' universal tag sequence is hybridized to the complementary anti-tag sequence coupled to a particular xMAP bead set. After performing a wash step, detection is initiated. The hybridized beads are read by aspirating the assay samples, one after another, into the reader. The hybridized beads are carried by a microfluidic system in a stream of fluid through the measurement cuvette where they are individually irradiated by dual reporter lasers. A red laser identifies each bead (or pathogen) by its color-coding, while a green laser detects the hybridization signal associated with each bead (indicating the presence or absence of a particular pathogen). Instrumentation reads the color-coded beads that attach to specific nucleic acid sequences, and results are analyzed by specific data analysis software (Dunbar 2006; Dunbar and Jacobson 2007a, b).

Luminex xMAP technology is supported by the principles of flow cytometry, and the major advantage of the technology is centered on the bead color-coding, which enables up to 100 possible separate interactions that can be analyzed simultaneously (Dunbar and Jacobson 2007a, b). The entire procedure can be completed in approximately 5 h making it a quick method to identify multiple genetic targets. Other significant advantages over traditional methods include: the speed and accuracy, the versatility of one system to analyze several target types, and the standardization that is observed because of the reproducibility of high-volume production of xMAP microspheres within a single lot. The new Luminex instrument, the MAGPIX (Fig. 5.5), is a compact instrument launched to support bacterial panel testing.

Luminex xTAG panels are available outside the USA as xTAG® Gastrointestinal Pathogen Panel (xTAG GPP) to detect *Salmonella, Shigella, Campylobacter, Yersinia enterocolitica*, Enterotoxigenic *E. coli* (ETEC), *E. coli* O157, Shiga-like Toxin-producing *E. coli* (STEC), *Clostridium difficile* Toxin A/B, and *Vibrio cholera* in addition to viruses and parasites associated with gastrointestinal disease (Battaglia et al. 2011; Dunbar and Jacobson 2007a, b). The multiplex aspect of the xMAP panels enables the identification of infections due to multiple pathogens with rapid diagnostic results for diagnosis and patient management.

Qiagen QIAplex Panels are commercially available for the detection of gram-positive cocci in clusters (StaphPlex) and pathogens associated with community-acquired pneumonia (Resplex I: *Mycoplasma pneumonia, Chlamydophila pneumonia, Legionella pneumophila, Streptococcus pneumonia, Neisseria*

meningitidis, and *Haemophilus influenza*) (Tang et al. 2007; Benson et al. 2008; Brunstein et al. 2008). The Staphplex differentiates between 18 different targets and provides species-level identification of *Staphylococci*, as well as the identification of antimicrobial resistance determinants and genes encoding Panton-Valentine leukocidin (PVL) (Tang et al. 2007). The QIAplex assays are performed on the Qiagen LiquiChip 200 Workstation, a branded form of the Luminex 100/200 instrument.

Transcription-Mediated Amplification (TMA)

Transcription-mediated amplification (TMA) is the synthesis of a DNA strand complementary to a target nucleic acid, usually RNA (Kwoh et al. 1989). The newly synthesized cDNA becomes the template for the subsequent in vitro transcription reactions. An excess of RNA is created and is used as the substrate for another round of transcription. Much like PCR this process continues at an exponential rate and several million copies of the target can be created. The amplified target is then detected using various methods. Gen-Probe (Gen-Probe Incorporated, San Diego, CA) has launched the PANTHER, the smaller version of the TIGRIS, a fully automated random-access TMA instrument for high-volume testing. Like the TIGRIS, the PANTHER also utilizes Gen-Probe's target capture technology for sample processing, TMA amplification, and HPA detection and decontamination into one instrument (Graber et al. 1998). The PANTHER boasts the ability to test for four targets from one specimen using Gen-Probe's APTIMA assays for *N. gonorrhoeae*/*C. trachomatis*, human papilloma virus, and *Trichomonas vaginalis*, providing a multi-targeted STD testing option. Currently, the assays are limited to endpoint assays; however, the PANTHER boasts of assays currently in development with real-time TMA capabilities to compete with quantitative PCR applications (Templeton et al. 2001).

Loop-Mediated Isothermal Amplification of DNA (LAMP)

Loop-mediated isothermal amplification of DNA (LAMP) is a novel method that is quickly impacting molecular detection of pathogens (Notomi et al. 2000). This method rapidly amplifies DNA under isothermal conditions with high specificity and efficiency. The *illumigene* (Meridian Bioscience Inc, Cincinnati OH) DNA amplification assays use specific primers that continuously facilitate isothermal amplification producing the by-product, magnesium pyrophosphate, in the reaction. The accumulation of magnesium pyrophosphate results in a precipitated solution. The resulting turbidity from the precipitate can be detected by the instrument, which functions as both an incubator and turbidometer (Mori et al. 2001). When the pathogen, specifically the gene target of interest, is not present, precipitate does not form and the amplification reaction is negative. When the gene target is present, the resulting amplification produces a turbid solution and a positive result. The *illumigene C. difficile* assay detects the pathogenicity locus (PaLoc) of toxigenic *C. difficile*, by targeting a region of *tcdA*, and the illumigene Group A Streptococcus assay targets the pyrogenic exotoxin B (speB) gene, with results available in about an hour (Kato and Arakawa 2011; Lalande et al. 2011; Noren et al. 2011). LAMP assays have also been published for *Legionella* spp., Group B strep, *Vibrio* spp., *Salmonella, Shigella, Listeria monocytogenes, Staphylococcus, Mycobacterium, Neisseria*, and *Brucella*.

During the LAMP process, six different primers, specifically designed to recognize six or eight distinct regions on the target sequence, participate in amplification and detection of the target sequence, which can be completed in a single step (Nagamine et al. 2001; Tomita et al. 2008). A DNA Polymerase with strand displacement activity forms a dumbbell-like DNA structure, which is the starting structure for LAMP cycling. A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. A primer anneals to the 5' end of the single-stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary regions. Next, starting from the 3' end, DNA synthesis starts using the DNA itself as a template and releases the complementary strand. The released single strand then forms a dumbbell-like structure at both ends. The steps are repeated through self-primed DNA synthesis starting from the 3' end. An additional primer anneals to the 3' end and primes strand displacement DNA synthesis, releasing the primed DNA strand with a stem-loop structure. Accordingly, similar stem-loop structures as well as the dumb-bell structure are produced. Another primer anneals to the single-stranded region of the produced stem-loop, and DNA synthesis continues by displacing double-stranded DNA sequence. Various-sized structures made up of alternately inverted repeats of the target sequence on the same strand are created. This cycling results in the amplification of the target.

Direct Probe Technology

FISH

Fluorescence in situ hybridization (FISH) utilizes fluorescent dyes linked to nucleic acid probes that target conserved molecules found abundantly in microorganisms (Muresu et al. 1994; Hayden, Kolbert et al. 2001a, b). Detection and identification can be accomplished without cultivation, and this method could potentially be applied directly to almost any sample. Following a simple hybridization procedure, fluorescence of probe-target hybrid molecules is visualized with a fluorescence microscope. Identification of samples containing mixed organisms can be achieved, although individual probes for each pathogen are required. If commercial or published probe

sequences are not available, they can be obtained by designing probes specific to the target sequence of interest (i.e., rRNA or DNA). Optimal probes for FISH range between 15 and 25 nucleotides to provide high specificity and stringency during the hybridization process, resulting in greater discrimination between closely related organisms. The number of copies of the target molecule(s) present in the cell determines the detection limit of the target probe, with $\geq$ 1,000 ribosome molecules needed for successful hybridization. There are currently no DNA-in situ hybridization assays that are commercially available.

PNA-FISH

Peptide nucleic acid (PNA) probes are fluorescent probes attached to PNA molecules that have a non-charged polyamide (peptide) backbone as opposed to the negatively charged sugar-phosphate backbone of DNA and RNA. PNA probes have the same nucleotides (adenine-A, guanine-G, cytosine-C, and thymine-T) and follow the same base-pairing rules allowing them to bind normally to complementary sequences of nucleic acid. There are several advantages to the structure of PNA probes over nucleic acid probes: (1) PNA probes bind stronger in the hybridization reaction than the DNA probe; PNA chemistry hybridizes more readily to the target of interest due to very small dye-labeled probes, approximately 12–20 nucleotides in size. (2) The neutral charge of the PNA backbone reduces natural repulsion with negatively charged backbones (i.e., DNA), and therefore, they bind tightly. (3) Greater specificity as a result of more precise binding to the target and exquisite base-discrimination. (4) PNA probes provide greater sensitivity; they add very low background with better visualization of the fluorescent signal.

Advandx, Inc. (Woburn, MA) commercially markets FDA-cleared PNA-FISH kits for clinical diagnostic use and identification of *Staphylococcus aureus*, *Enterococcus* spp., *Mycobacterium* spp., *Streptococcus agalactiae*, *E. coli*, *Klebsiella pneumoniae*, *and Pseudomonas aeruginosa*, among other targets (Chapin and Musgnug 2003; Stender 2003; Forrest 2007; Forrest et al. 2008; Hensley et al. 2009; Morgan et al. 2010, 2011). These kits are relatively easy to use and are now widely used as a method for rapid identification with an approximate turnaround time of about 1.5–3.5 h. A current disadvantage to the use of PNA-FISH is the ability to rapidly infer resistance to antimicrobials, based on identification of the organisms and local antibiograms.

For the identification of fastidious, non-cultivatable, and slow-growing pathogens, PNA-FISH is a quick and easy method. This method relies solely on the availability of nucleic acid targets rather than phenotypic expression of markers that are usually targeted by more extensive and time-consuming immunological methods. More cost-effective than PCR techniques, PNA-FISH does not require extensive knowledge or experience in molecular techniques. The chemistry of FISH probes makes this technology just as sensitive but more specific than gram staining and direct fluorescence staining, and will conceivably propel PNA-FISH to a more routinely used diagnostic method.

Emerging Technologies

Mass Spectrometry

Promising new broad-based techniques have merit, based on their ability to identify difficult-to-culture organisms or newly emerging strains, as well as their capacity to track disease transmission. Both techniques revolve around the mass spectrometry technology: matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS) and polymerase chain reaction electrospray ionization mass spectrometry (PCR-ESI/MS). The use of mass spectrometry methods is challenged by the high costs of instruments, yet there is potential for these technologies to supplant the foundation of clinical microbiology, replacing most of biochemical testing as we know it. Overall, both methods show promise for both routine and, in some cases, epidemiological use in hospital settings. These two techniques, in a direct comparison, show no statistically significant differences in their performance, but PCR-ESI/MS is substantially more expensive and has greater capabilities.

Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) Spectrometry

Mass spectrometry methods leverage their high sensitivity with links to large microbial databases, which enable users to obtain species-specific spectra that can be used to reproducibly identify microorganisms. MALDI-TOF/MS requires only media to culture the organism and a small quantity of matrix (typically less than 1 μg/sample), the cost of which is negligible.

Analysis of whole cells was first proposed in 1975 to study the biomarker profile of various bacterial species following pyrolysis-mass spectrometry for low molecular weight products (Anhalt and Fenselau 1975). However, it was not until 1996 that the first MALDI-TOF/MS experiment was successful in identifying bacteria directly from whole colonies based on protein biomarkers (Claydon et al. 1996; Holland et al. 1996). A great number of developments have been made over the last decade on whole organism MALDI-TOF/MS. The protein biomarkers that are measured in mass spectrometry of microorganisms are highly expressed proteins responsible for housekeeping functions, such as ribosomal components, chaperones, and transcription/translation factors.

Relying on identification of protein profiles, MALDI-TOF/MS derives its information from profiles of highly conserved bacterial proteins. For MALDI-TOF, the protein profiles are generated from direct ionization of an intact colony or a bacterial protein extract, after manual extraction. Identification occurs after correlating a protein's spectral signature to a database of spectra collected from reference strains. In its current form, MALDI-TOF/MS requires subculture prior to identification. An advantage of MALDI-TOF/MS is that it does not require batching.

MALDI-TOF/MS is widely used, for its high accuracy, low consumable cost, and speed of analysis. A typical experiment

consists of outgrowth of bacteria, colony selection and placement on a target, addition of matrix, and analysis with MALDI-TOF/MS. Mass spectrometry identification is broadband, such that it can measure multiple analytes simultaneously, does not require prior knowledge about the organism, and is both fast and sensitive by not requiring a pre-fractionation step. It generally measures all *m/z* between 2 and 20 kDa. Several reviews are available on this topic for a more in-depth overview (Fenselau and Demirev 2001; Lay 2001; Demirev and Fenselau 2008).

Limitations of Mass Spectrometry–Based Technologies

The major barrier to implementing mass spectrometry–based technologies to the clinical laboratory is the cost associated with capital purchase of a dedicated mass spectrometer (Wolk and Dunne 2011). Thus, as is true for most newly emerging technologies, these methods may be limited to larger reference laboratories until the cost-benefit is fully evaluated or until smaller more affordable benchtop options are developed. Nevertheless, the potential for high analytical sensitivity, accuracy, and broad-based applicability of mass spectrometry–based platforms have the potential to change the practice of clinical microbiology in the future and offers the promise of off-setting some portion of the complexity and workload in clinical microbiology.

Fig. 5.6
The Bruker MicroFlex Mass Spectrometer

Bruker MALDI Biotyper

Commercial mass spectrometry systems exist that can integrate with traditional antimicrobial susceptibility systems. Among them, the MALDI Biotyper from Bruker Daltonics (*Fig. 5.6*) is among the most widely tested and has proven accuracy in the identification of bacteria (Maier and Kostrzewa 2007). The system is partnered with Becton Dickenson Phoenix System, which supplies antimicrobial susceptibility results that pair with bacterial identification. Evaluations of the system show high accuracy, even for rare or fastidious bacteria (Alanio et al. 2010; Saffert et al. 2011), which represent a challenge to clinical laboratories using phenotypic identification evaluation due to their limited biochemical reactivity. Identification of yeast has also been reported (Marklein et al. 2009). The method finds common use in Europe where clinical microbiologists are using it to rapidly identify microbial colonies isolated from culture.

A few reports show promise for the identification of microorganisms using MALDI-TOF/MS without subculture (Ferreira et al. 2010; Moussaoui et al. 2010; Prod'hom et al. 2010; Stevenson et al. 2010). Recent evaluations show promise, but the methods were limited by the need for a large number of cells—adaptations showed successful identification for only approximately 80 % of blood cultures. A promising new technology, the MALDI Sepsityper system (Bruker Daltonics), is under evaluation and aims to identify bacteria and yeast directly from positive blood culture bottles. If successful, the MALDI Sepsitype system has the potential to reduce the identification time for many different bacterial species.

It is important to note some of the current limitations associated with MALDI-TOF/MS. The need for outgrowth of organisms from potentially contaminated material is still required in order to obtain isolated colonies of organisms as the technique's ability to resolve mixtures is lacking. Additionally, a high number of bacterial cells are required for identification, such that a whole intact colony is typically used for analysis, limiting the ability to rapidly identify microorganisms directly from biological fluids where the bacterial count is expected to be relatively low. Research is currently being done in the research field to mitigate some of these requirements. Finally, until accurate determinations of resistance factors can be made, parallel culture-based recovery of positive blood cultures will most certainly be required for the foreseeable future for antimicrobial susceptibility testing.

Vitek MS-Biomerieux/Shimadzu AXIMA Microorganism Identification System

Another MALDI-TOF system, the bioMerieux Vitek MS (*Fig. 5.7*), shows similar capabilities to the Bruker system; no statistically significant difference was identified between the two platforms for general bacteria (Cherkaoui et al. 2010). While

Fig. 5.7
The bioMériuex, Vitek MS with disposable array

fundamentally equivalent, the Vitek MS differs in its instrumentation scoring algorithm and databases. The mass spectrometer, designed by Shimadzu, is partnered with bioMerieux systems for antimicrobial susceptibility testing. The system is supported with a bacterial database from Anagnsotic Biosystems.

PCR Mass Spectrometry: Abbott IBIS

Brief History of PCR-ESI/MS

Emerging new technology, like Polymerase Chain Reaction Electrospray Ionization Mass Spectrometry (PCR-ESI/MS), sold by Abbott Molecular (DesPlaines, IL) has the potential to identify nearly all known human pathogens directly from clinical specimens and the potential to identify genetic evidence of undiscovered pathogens (Taylor et al. 2001) or genetic changes to diagnostic targets. Therefore, if a novel microbial genetic sequence is uncovered, it is reported and accompanied by information that describes its relationship to closely related organisms. The Ibis technology was originally developed for biodefense and public health safety, the first use of PCR-ESI/MS was reported to be in 2005, and the technology was further advanced in 2008 (Hofstadler et al. 2005).

The T5000™ Biosensor System was the PCR-ESI/MS prototype instrument (Ecker et al. 2008). Originally created by Ibis Biosciences (Carlsbad, CA), the system couples equipment that desalts PCR products to a time-of-flight (TOF) analyzer, providing mass accuracy of sufficient resolution to discern the base compositions of the amplicons produced in multiplex PCR. The Ibis technology has advanced the prototype to the refined current version of the instrument, now called the PLEX-ID (➲ *Fig. 5.8*). Abbott Molecular currently supports reagents that are classified as "not-for-diagnostic-use" (NFDU), but the system is being evaluated in public health and military laboratories as well as several large clinical reference laboratories.

The PLEX-ID enables rapid identification (approximately 6–8 h, depending on nucleic acid type) and characterization of bacterial, viral, fungal, and other infectious organisms as well as analysis of human DNA, achieving a wide breadth of pathogen

Fig. 5.8
The Abbot PLEX-ID system for PCR/ESI-MS

identification. As the PLEX-ID is relatively new, the published literature summarized in this review describes research performed on its prototype the Ibis T5000 (Hofstadler et al. 2005; Ecker et al. 2008).

Principles of PCR-ESI/MS

1. Sample Preparation
   As with other molecular sample preparation methods, nucleic acids must be extracted, either directly from clinical specimens or from cultivated microbial isolates. A wide variety of DNA extraction methods, both manual and automated, were successfully used to effectively isolate nucleic acid from bacteria prior to the upstream PCR-ESI/MS assays (Hannis et al. 2008; Baldwin et al. 2009; Ecker et al. 2009; Hall et al. 2009; Wolk et al. 2009a; Crowder et al. 2010; Emonet et al. 2010; Endimiani et al. 2010; Eshoo et al. 2010; Whitehouse et al. 2010; Kaleta et al. 2011; Massire et al. 2011). Sample types include bacterial colonies and bacterial cultures, clinical samples (throat swabs, nasal swabs, nasopharyngeal swabs, nasal washes, sputum, and skin swabs), etc.
2. Multiplex PCR Prior to PCR-ESI/MS
   After sample preparation, nucleic acids are dispensed into wells of a microtiter plate for downstream multiplex amplification. Each well contains one or more pairs of broad-range or target-specific primers (depending on the assay type) and other PCR components, to support amplification

via multiplex PCR. The PCR-ESI/MS assays contain a variety of purposefully designed primer sets that interrogate common conserved and variable sequences among various classes of organisms. Small amplicons (80–150 bp) of various sizes are created, depending on the species of microbes present in the original sample. Amplification produces genetic products, unique within a group of organisms, microbial domain or microbial division.

Proper amplification relies on the genetic similarities in microbial genomes. For example, bacteria have highly conserved sequences in a number of chromosomal locations, including the universally conserved regions of ribosomal, other noncoding RNAs, and essential protein-encoding genes. These conserved sequences, intercalated with regions of sequence diversity, serve as priming sites for broad-range primers to amplify sequences of various sizes and compositions.

A typical PCR-ESI/MS assay uses a master-mix of reagents, optimized for use in an amplification reaction using multiple primers. Multiplex amplification generally occurs under conditions of low stringency to allow for nonspecific primer annealing, a parameter that supports the mismatch amplification required to identify unknown genomic mutations within the targeted regions. Amplification typically requires a two-step PCR protocol to enrich amplification.

3. Desalting and Electrospray Ionization-Time of Flight
After amplification, products undergo an extensive desalting process prior to injection into a mass spectrometer. Negative ion-mode electrospray ionization is used for analysis. Electrospray ionization moves charged amplicon into the mass spectrometer, via processes optimized to detect negatively charged oligonucleotide ions. By definition, mass spectrometry (MS) is a sensitive analytical technique that measures the *mass/charge* (*m/z*) ratio of charged particles, and can do so with high mass-accuracy. To optimize the process, the electrospray ionization separates double-stranded amplicon, and intact strands are pulsed into the flight tube of the mass spectrometer under high vacuum and are separated based on *m/z* where lower *m/z* ions travel faster and reach the detector earlier than ions with higher *m/z*.

The resulting mass spectrum, which consists of a distribution of peaks corresponding to different charge states, is then "deconvoluted," a process by which the spectra are mathematically simplified. The mass of the intact amplicon is calculated and signals from low molecular weight chemical noise are eliminated on the ESI-TOF instrument. Once signals are digitally processed, the results are listed as highly accurate molecular weights (masses) of the forward and reverse strand of each amplicon.

Once the amplicon masses are established, software algorithmically predicts their base composition. Calculations rely on the known masses of the four nucleic acids (adenine, guanine, cytosine, and thymine bases) present in the amplicon and the knowledge of DNA strand complementarity. A joint least-square algorithm correlates potential organism identifications across multiple genetic regions to reveal the unique identity of the microbe. Since several genetic regions are amplified, multiple genetic compositions are compared to a curated database to narrow the genetic possibilities to one unambiguous genetic identity. The regions amplified vary by organisms and are assay dependent.

4. Curated Amplicon Reference Database
Undoubtedly, a key element of the PLEX-ID system is the Ibis Biosciences-curated database of genomic information that associates base composition with the identity of thousands of organisms. Despite the enormous class diversity of organisms, an Ibis database containing carefully selected and curated genetic sequences allows the interrogation to produce relevant and accurate results for surveillance, epidemiology, forensic, and biological research. The database is regularly updated with the latest information for newly identified microorganisms, based on input from users and review of web-based genomic databases.

For sequence matching to occur, the software bases its deductions on several known assumptions: (1) The potential genetic targets are known and present in each multiplex primer set. (2) Base compositions of forward and reverse amplicon strands must be complementary; therefore, for a particular *m/z* ratio, there are a limited number of possibilities. (3) For a single, unique base composition, only a small number of possible base compositions must be consistent with each measured mass. Finally, the unique base composition of an amplicon is compared to the Ibis database of over 750,000 entries, which links the base composition for each primer pair to a small list of candidate microbes.

Workflow Overview

The PLEX-ID Rapid Bioidentification System provides complete information management for instrument control, tracking, mass spectral signal processing, and analysis from original samples to organism identification. After the extraction and amplification step, the PLEX-ID process is fully automated with regard to sample tracking, instrument control and robotics, data processing and analysis, and comparison to the curated database. The entire PLEX-ID process requires approximately 6 h per batch of specimens tested, including 4 h for amplification. Processing events, which are the time-limiting factors, such as PCR amplification and sample dispensing, are scheduled in parallel to maximize the throughput of the instrument system. Post-PCR on-instrument throughput is approximately 1.25 h, such that approximately 20 plates or more can be analyzed each day, and, depending on the assay, can process between 6 and 12 patient samples per plate, resulting in 120–240 samples per day maximum. During the PLEX-ID testing process, robotics and sample tracking register and verify plate barcodes to ensure accurate tracking of the physical transfer of samples during the spray and data acquisition phases. The system also automatically triggers spectral processing and data analysis of the raw data. Coupled together, the instrument enables a continuous, automated workflow from physical sample to resulting organism identification.

Although the PLEX-ID system is in its infancy, it is a technology that could shift the way microbiologists think about the diagnosis of infections. It appears accurate and flexible, and has potential for implementation in large reference laboratories or in public health surveillance.

DNA Sequencing

Sequence-based identification, strain typing, and comprehensive isolate fingerprinting can be utilized for the tracking and control of pathogenic organisms; therefore, molecular methods like sequencing have been a widely used tool for epidemiological fingerprinting of isolates important to public health. However, in 1995, the first two complete DNA sequences of the bacterial genomes *Haemophilus influenzae* and *Mycoplasma genitalium* were revealed by scientists at the institute for genomic research (TIGR), followed in 1996 by the first complete genome sequencing of an archaea, *Methanococcus jannaschii*. Since then automated sequencing using fluorescent chain-terminating nucleotides during elongation of a DNA template (referred to as Sanger sequencing) has made it possible to sequence the genomes of many pathogens and environmental microorganisms rapidly both in research and diagnostic settings. A major advantage of sequencing is that it allows for the comparison of genomes identifying differences between virulent and avirulent medically important pathogens. The comparison data generated at the genomic level of a pathogenic species can provide information about host or tissue specificity and antimicrobial resistance patterns.

Short Read Pyrosequencing

Pyrosequencing is a DNA sequencing method that detects released pyrophosphate as DNA polymerase acts on a target sequence. Pyrosequencing assays have been developed for several applications, including genotyping, single nucleotide polymorphism (SNP) detection, and microorganism identification. Pyrosequencing is real-time sequencing and thus can be used for not only microbial species identification but for the discrimination of point mutations that confer antimicrobial resistance. There are several major advantages of pyrosequencing: it is sensitive, rapid, simple, customizable, highly specific and also provides high throughput (Quiles-Melero et al. 2011; Jordan et al. 2005, 2009; Zhang et al. 2011).

The principle of the pyrosequencing reaction relies upon annealing of a target primer to a single stranded template. After binding, DNA polymerase acts to elongate the sequence, much like PCR. As the polymerase adds deoxynucleotides, in a predefined dispensation pattern, the primers extend and release inorganic pyrophosphate (PPi) as part of the synthesis. Inorganic pyrophosphate is converted into ATP by ATP-sulfurylase. The accumulating ATP is utilized by firefly luciferase in a reaction to produce light. The pattern of light signals, correlating to the added nucleotide, is a true representation of the sequence that is being analyzed. The amount of light generated by the reaction is directly proportional to the PP_i produced or the number of nucleotides that were incorporated into the newly generated sequence. Any nucleotides that are not incorporated into the new sequence are enzymatically degraded. The sequence generated by pyrosequencing is quantitative in nature. Select pyrosequencing methods utilize a solid-phase template preparation of streptavidin beads that interact with biotinylated 5' ends of single-stranded DNA as opposed to the enzyme-based template preparation.

The reaction is rapid, as it takes less than 2 h to result, and DNA extracted from multiple microbial species in a single specimen can be sequenced in the same assay batch. Still, disadvantages to the technology consist of the method being relatively hands-on, labor intensive, and open to potential contamination events. In addition, the sequencing reaction is only able to produce sequence for short targets, in some cases <100 nucleotides. Qiagen Pyromark (Qiagen Inc., Valencia, CA), a commercial system, is currently available in three system formats—Q24, Q96, and Q96MD.

Next-Generation Sequencing

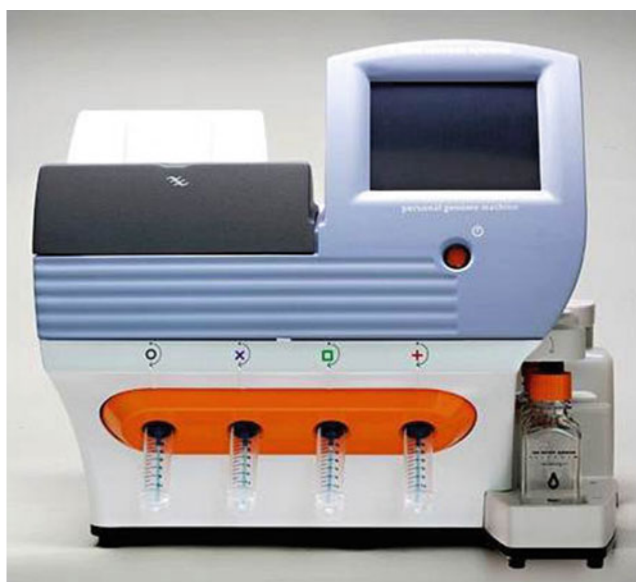
Traditionally, sequencing methods were based on slab gel electrophoresis that separated DNA fragments produced from in vitro Sanger sequencing reactions. These methods involved laborious molecular cloning and PCR techniques. Several instruments have recently been introduced that utilize molecular cloning techniques (minus any bacterial vectors), PCR amplification, and capillary electrophoresis to produce sequencing data. Next-generation sequencing takes amplified templates isolated by molecular cloning techniques within the instrumentation and discriminates the incorporation of nucleotides (adenosine, thymine, cytosine, or guanine) on two-dimensional arrays. Digital imaging monitors the nucleotides as they are incorporated on a cycle-by-cycle basis. Next-generation sequencing provide high throughput results, and these DNA sequencers can synthesize >100 megabases of DNA sequences per assay, drastically shortening the time required to sequence an entire bacterial genome. Though the principles are similar, the currently available systems use sequence synthesis chemistries that are distinct to each system.

The Illumina MiSeq (Illumina Inc., San Diego, CA) system uses fluorophore-labeled nucleotides with terminating 3' ends that are added to the end of a primer one at a time. The addition of the fluorophore-labeled nucleotides results in laser excitation of the fluorophore and detection by the imaging system; subsequently the 3' end terminators and fluorophore are cleaved, allowing for the addition of the next nucleotide. The nucleotides equally compete for incorporation resulting in superior strain specificity. MiSeq permits ~150 bp paired sequence reads and results can be achieved in less than 8 h. Any genome can be sequenced using this technology with the ability to uncover genetic variants and antimicrobial resistance mechanisms.

The ABI SOLiD (Applied Biosystems, Foster City, CA) utilizes a similar reaction chemistry of labeled nucleotides with blocked 3' termini that are enzymatically attached to the end of a primer; however, the central dinucleotide is flanked with universal and degenerated nucleotides. This provides added specificity to the sequencing chemistry. The nucleotides are fluorophore labeled and added in blocks of eight nucleotides or octamers that specify the sequence for two nucleotides. Once added and the fluorophore is detected, the entire octamer is removed. The subsequent ligation reactions use smaller and smaller primers until ultimately a 35 bp sequence is read. The ABI SOLiD sequencers can sequence multiple whole bacterial genomes in less than 2 weeks as opposed to months.

Roche 454 instruments (Roche Diagnostics Corp, Branford, CT) are high-throughput solid-phase pyrosequencers, with long sequence reads of approximately 400 bp with up to 99 % accuracy. For microbiology, the Roche GS Junior benchtop system can be used for whole genome sequencing. In these systems, beads are loaded onto a nanoscale well device with a single capture bead per well and all the required enzyme reagents. A camera detects chemilumenescent light signals as complementary nucleotides, which flow sequentially across the wells and are incorporated into the template strand attached to the beads. Applications for the 454 pyrosequencers include, but are not limited to, pathogen detection, direct amplicon sequencing, de novo microbial genome sequencing, and metagenomic characterization of environmental samples.

The Ion Torrent Personal Genome Machine (Life Technologies, Grand Island, NY) is centered on semiconductor technology as opposed to light chemistry to acquire genomic sequences (➋ *Fig. 5.9*). Chemical signals are translated into digital signals which are then detected by the proprietary ion sensor. The chemical signals are generated as nucleotides are added to the nucleic acid strand. When a complementary nucleotide is incorporated the release of hydrogen ions results in a pH change that is ultimately read as a voltage change. The chip is flooded sequentially with nucleotides and if the nucleotide is not a match, no change in voltage occurs. If two or more nucleotides are complementary to the strand, the voltage change is multiplied proportionally to the number of correctly added nucleotides. Recording of sequence synthesis by the ion sensor is analogous to a small pH-meter and is extremely rapid since laser scanning and cameras are not necessary for synthesis detection. Thus sequence acquisition occurs rather quickly, in about 2 hours time.



◘ Fig. 5.9
Ion Torrent sequencer (Life Technologies)

Summary

Although molecular and protein-based testing is becoming commonplace, and offers the prospect for major improvements in speed and accuracy of clinical microbiology methods, in the short term there remains a strong need to continue culture-based testing. Culture is still required for most antimicrobial susceptibility testing and will still be advantageous for identifying genotypes and mutated strains that may not be detected by molecular or protein-based techniques. While there are many advantages of rapid broad-range testing, the cost of implementation of molecular methods and mass spectrometry methods will keep traditional culture and phenotypic and biochemical characterization methods available in the clinical laboratories. Nevertheless, the trend toward multiplex and broad base testing is primed to continue and should greatly impact the practice of clinical microbiology in the future.

Specifically, today's molecular diagnostic platforms are providing unparalleled clinical value, especially for diagnosis of infectious diseases. Moreover, these molecular diagnostic technologies are smaller, less expensive, and more user-friendly than their predecessors, driving the adoption and utilization of new innovative tests. Through prudent and evidence-based use of new technology, we can improve both the care and the economics of patient care. Laboratory tests that streamline diagnostic laboratory processes can significantly impact our ability to advance clinical practice.

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