Chapter 34
Multiplex PCR in Molecular Differential Diagnosis of Microbial Infections: Methods, Utility, and Platforms

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Introduction: The Needs, Wants, and Challenges

We are entering the age of personalized medicine where treatments are designed to target specific causes, rather than a group of patients with similar symptoms. However, personalized medicine is impossible without a personalized diagnosis that considers all the possible causes of a person's disease. Traditional molecular diagnostic methods, such as PCR and qPCR, cannot provide the necessary information to practice personalized medicine, because they cannot be multiplexed, allowing the detection of only one or a few (no more than 3) targets at a time in one sample. Practicing personalized medicine, therefore, requires multiplex PCR (mPCR), which can evaluate many molecular targets at once, in one reaction, from one sample.

For most infectious diseases, the clinical presentations are often not specific enough to allow for a definitive diagnosis of the causative pathogen. Coughing and fever, for example, are symptoms that may be caused by many different bacterial or viral infections. Thus, for better treatment and disease control, a molecular differential diagnostic (MDD) assay that can pinpoint the offending pathogen associated with a clinical syndrome is needed. MDDs are essential tools for effective infectious disease surveillance, biodefense, and personalized medicine.

MDDs are *needed* for emerging infectious disease surveillance and control. When outbreaks such as SARS occur, public health officials and laboratory scientists often struggle for weeks, if not longer, to identify the offending pathogen. With MDDs available, scientists involved in an outbreak investigation can quickly rule out many pathogens associated with similar clinical symptoms and focus on new, emerging infections. An MDD test can also aid in the management of a public health crisis by helping healthcare personnel in triaging patients and determining

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which patients should be isolated, as well as identifying environmental sources of contamination within an intensive care unit (ICU) or patient hospital room.

MDDs are needed for homeland security and biodefense. With the current global political atmosphere, biodefense threats are a reality. A first-response technology could quickly identify a bioterrorism agent and control the spread of the pathogen. Without the availability of MDDs for rapid pathogen identification, the bioterrorism agent may go undetermined for days. Every hour wasted in determining the causative agent provides a greater opportunity for pathogen spread and panic to occur.

MDDs are needed for delivering true personalized medicine, which focuses on treating the patient, rather than the disease. It is genotype-based, rather than phenotype- or symptom-based medicine. An MDD test also makes it possible to practice *theranosis* (therapy guided by a diagnosis) by developing or reclassifying drugs that specifically target the molecular cause of the disease. If pharmacogenomics is the development of drugs based on individual genotypes, then theranosis is the administration of drugs based on individual (or infectious agent) genotypes.

It is clear that MDDs are *needed*, but in order to make the assays practical, we *want* them to have the following advanced features:

- *Multiplex capabilities*. The definition of multiplexing is "receiving multiple signals from the same source." For MDDs, multiplexing refers to the ability to conduct multiple genotyping tests at the same time and within the same sample. We *want* multiplexing because it requires only small amounts of precious patient sample; it allows the clinician to run fewer tests, while acquiring more relevant information; it reduces the amount of reagents, consumables, and time involved; and, most importantly, it can save lives. For infectious disease MDDs, we *want* a multiplex test that can identify all pathogens related to a clinical syndrome or detect all the genes and mutations responsible for the drug-resistance phenotype.
- *Specificity*. Even though multiple microorganisms are studied simultaneously, we want only the pathogens associated with the infection to be identified and with a high level of confidence.
- *Sensitivity*. We want an MDD to be able to identify a pathogen or drug resistance *directly* from a patient sample rather than from an enrichment culture. Using the patient sample directly eliminates the time required for bacterial or viral culture preparation and enzymatic testing. However, in order to bypass this propagation step, the assay must be sensitive enough to detect only a small amount of pathogen material in the patient sample.
- *Reliability*. For clinical application of MDDs, a consistent performance from assay to assay and from lot to lot is required.
- *Speed*. For an MDD to be practical for infectious disease diagnosis and treatment, it must be locally available and produce results within a few hours.
- *Automation*. An MDD should not require a PhD scientist: it should be user-friendly, and no special training should be required to perform the assay. The MDD system should also be easily integrated into standard molecular laboratory practice and should be automatable.

- *Closed system*. A challenge to widespread use of MDD is the risk of template or amplicon contamination of the work environment, which may lead to false positives. One advantage of qPCR is that the specific detection step is carried out inside the PCR tube, in real time, without the need to reopen the tube. To make MDD practical, it must be carried out in a closed system.
- *Affordability*. MDDs should be cost-effective.

The technology advances in this postgenomic era have made sequence information readily available for almost all known pathogenic microorganisms. Based on this information and armed with standard molecular tools, scientists have developed molecular assays, usually PCR-based, for almost every infectious pathogen. A simple internet keyword search for a pathogen name together with "PCR" will produce several pages referencing specific tests for that pathogen. From this exercise, it seems possible that the basic *needs* for molecular differential diagnosis can be met. However, to produce the MDD assay we really *want*, some unique technical challenges must be addressed.

The most difficult challenge is multiplexing. While PCR technology has been established for nearly 30 years, multiplex PCR is still very difficult to accomplish. There are many publications that claim multiplex PCR applications, but here we consider true multiplexing to be the detection of 5 or more targets in one reaction. The following is a list of common challenges associated with multiplexing.

- *Incompatible loci*. Each target in a multiplex PCR requires its own optimal conditions, and these may be incompatible between targets; therefore, increasing the number of multiplex targets makes finding satisfactory common conditions difficult and, in many instances, impossible.
- *Lack of specificity*. Multiple sets of high-concentration primers in a system often generate primer dimers or nonspecific background. Lack of specificity also adds operational burdens by requiring post-PCR cleanup and multiple posthybridization washes, which make automation very difficult.
- *Lack of sensitivity*. Crowded primers reduce amplification efficiency and waste resources by occupying enzymes and consuming substrates.
- *Uneven amplification*. Differences in amplification efficiency may lead to large discrepancies in amplicon yield. In a multiplex system, some loci may amplify very well, while others may amplify poorly or even fail to amplify. Uneven amplification makes it impossible to accurately perform end-point quantitative analysis.
- *Lot-to-lot variation*. Because large amounts of primers are consumed in each reaction, and manufacturers can generate only a limited number of assays per lot, quality control and quality assurance can be difficult. The resulting poor repeatability makes it very difficult to gain FDA approval and therefore restricts large-scale use of the technology.

In the following discussions of this chapter, we present several mPCR methods and describe their applications. We also introduce multiplex hardware platforms.

Novel Multiplex PCR Technologies

The difficulty of mPCR assays may be appreciated by an analogy to a three-legged race: each primer pair is like two people tied together at the ankle who must work together to reach the finish line (amplify their target). However, the goal of an mPCR race is not for one team to finish first, but for all teams (primer pairs) to reach the finish line at about the same time (obtain equal amplification of all targets).

This is why mPCR is so difficult. There are two major conflicts: for a particular amplification target, the forward and reverse primers need to be compatible and in sync, while for all the targets, the amplification efficiency also need to be in sync. The following table (Table 34.1) outlines four recently developed methods that have tried to resolve these conflicts to make mPCR work.

Target-Enriched Multiplex PCR or tem-PCR

The tem-PCR method was first reported in 2006 by Han and colleagues [1]. In 2010, the US patent (7,851,148) was awarded, and the technology has been commercialized by Qiagen (Products) and Diatherix (Services). Figure 34.1 illustrates the tem-PCR method.

For each target in the multiplex PCR reaction, nested gene-specific primers are designed and included in the reaction (*Fo* forward out, *Fi* forward in, *Ri* reverse in, and *Ro* reverse out). These primers are used at extremely low concentrations and are used only to enrich the targets during the first few cycles of PCR. Some of these gene-specific primers have tag sequences that can be recognized by a universal set of primers, called SuperPrimers™, which are included in the same reaction system together with the gene-specific nested primers. Only the SuperPrimers are included at a concentration necessary for exponential amplification, and only the reverse SuperPrimer is labeled. For detection, labeled PCR products are detected with a

Table 34.1 Current multiplex nucleic acid amplification techniques

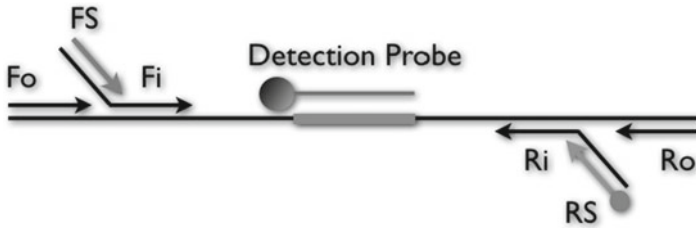| Method	Reference	Patent#	Company
Target enriched multiplex PCR (tem-PCR)	Han et al. [1]	7,851,148	Qiagen/Diatherix
Dual-priming oligonucleotide PCR (DPO)	Chun et al. [2]	WO/2008/143367	Seegene
Nested Patch PCR	Varley and Mitra [3]	20100129874 (pending)	Kailos Genetics
Amplicon rescued multiplex PCR (arm-PCR)	Wang et al. [4]	7,999,092	HudsonAlpha Institute for Biotechnology

Fig. 34.1 In the tem-PCR method (target-enriched multiplex PCR), nested gene-specific primers are designed to enrich the targets during the initial cycles. Later, a SuperPrimer™ pair is used to amplify all targets

complementary capture probe that is covalently coupled to a color-coded bead (Luminex platform) or printed array, or are sequenced.

tem-PCR addresses two of the most difficult problems inherent in multiplex PCR: (1) incompatibility of amplification conditions between different primer sets and (2) background amplification associated with high concentrations of primers.

First, in a standard multiplex PCR reaction, if there are six targets to be amplified, each may require a different optimal annealing temperature or buffer formula. When the number of multiplex targets increases, it forces all primer sets to work under a single amplification profile; therefore, multiplex PCR is nearly impossible under standard conditions. With tem-PCR, there are two sets of nested primers for each target in the enrichment stage. This design gives rise to four possible forward and reverse primer combinations for amplification. Each combination may have its own optimal amplification profile, but given four amplification opportunities, a common condition that satisfies all targets can be attained.

Second, standard multiplex PCR utilizes multiple sets of labeled primers at high concentrations, which can associate with one another to form dimers or create nonspecific background amplification. Reduced amplification efficiency can also occur when primers occupy active sites on the polymerase. In addition, unused labeled primers produce background signal and use up reagents during the detection part of the assay. Because of these issues, post-PCR cleanup (such as spin column purification) is often required to remove these labeled primers before they can be used as probes. Yet, high-concentration primers are only required in the last cycles of a PCR reaction. With tem-PCR, the amounts of gene-specific primers used is only enough to enrich the targets and incorporate the SuperPrimer tag into the PCR products. After enrichment and tag incorporation, amplification is carried out with only one pair of primers. Because only one pair of primers is labeled, the background is low; therefore, no post-PCR cleanup is required. The PCR reaction is also very specific and sensitive, and no posthybridization washes are necessary. This feature makes it feasible to fully automate the laboratory procedures and perform high-throughput clinical studies.

The tem-PCR method also allows semiquantitative analysis of coinfections. With traditional multiplex PCR, each primer set, or each locus, has its own amplification efficiency. Hence, at the end of amplification cycling, the signal ratio of
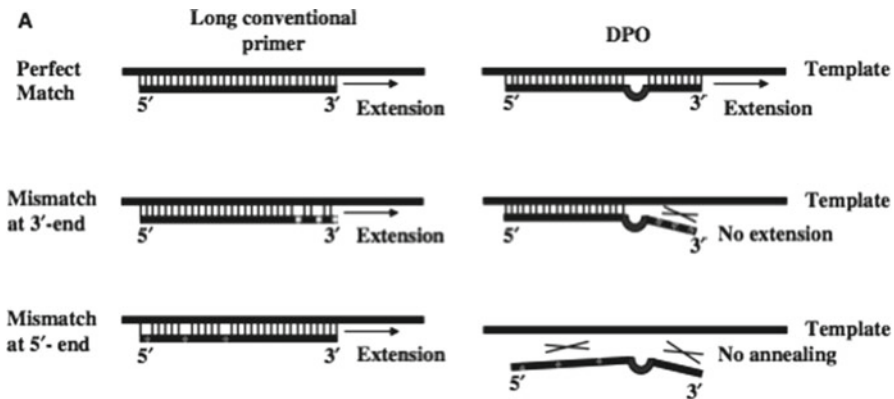
Fig. 34.2 Schematic diagram of long conventional primer-based and dual-primer oligonucleotide (DPO)-based PCR strategies

PCR products from different loci will not reflect the original ratio of the templates. With tem-PCR, the only primers used for exponential amplification are the pair of SuperPrimers. Consequently, all coamplified loci will have the same amplification efficiency. As a result, the end-point reading reflects the original copy number ratios between the coamplified targets.

tem-PCR is a flexible technology. Increased compatibility among multiple targets means that existing panels can be reorganized and remixed to build new panels. In addition, new amplification targets can be added without significantly reducing the sensitivity of the panel.

Continuing with the three-legged race analogy, the tem-PCR method does not rush all the paired runners to the finish line, but instead gives each time to run at their own pace at the beginning of the race. Once all the pairs (all the targets) have learned how to run in sync, the "SuperPrimer bus" will pick everyone up and carry them to the finish line together.

Dual-Priming Oligonucleotide (DPO) mPCR

The DPO mPCR method was first reported by Chun and colleagues [2]. The International patent (WO/2008/143367) describing the application of the method was awarded in 2008, and the technology has been commercialized by Seegene (Korea). Figure 34.2 illustrates the DPO method.

The DPO system has two separate primer segments, one of which is longer than the other, joined by a polydeoxyinosine linker. Deoxyinosine is known to have a relatively low melting temperature compared to the natural bases, due to weaker hydrogen bonding. Thus, the authors hypothesized that the poly(I) linker inserted between two stretches of natural bases would form a bubble-like structure and

separate a signal primer into two functional regions at a certain annealing temparature: a 5′-segment 18–25 nt in length and a 3′-segment 6–12 nt in length. This unequal distribution of nucleotides leads to a different annealing preference for each segment. The longer 5′-segment preferentially binds to the template DNA and initiates stable annealing, whereas the short 3′-segment selectively binds to its target site and blocks nonspecific annealing. Therefore, only target-specific extension will result from the successive priming of both the 5′ and 3′ segments of the DPO.

The DPO mPCR method successfully addresses the nonspecific priming issue. If we use the three-legged race analogy again here, the DPO method is successful by preselecting the athletes: only those fit to run are allowed in the race. There is no SuperPrimer bus to pick up teams in the middle of the race (no universal primers), so every pair has to run their own race from beginning to end.

Nested Patch PCR Method

The Nested Patch mPCR method was first reported by Varley and Mitra [3]. The patent (20100129874) is pending, and the technology is commercialized by Kailos Genetics (US). Figure 34.3 illustrates the Nested Patch method.

Like the DPO mPCR method, the Nested Patch PCR also intends to solve the problem of mispriming events that are typical of standard multiplex PCR. Like tem-PCR, the Nested Patch PCR method also requires four oligonucleotide hybridizations per locus, giving more specific amplification than standard multiplex PCR, which requires only two hybridizations per target locus.

Nested Patch PCR begins with a regular PCR reaction using primers with uracil substituted for thymine. PCR is first carried out for only a few cycles, which aims to define the ends of the targeted regions. The primers are then cleaved from the amplicons by the addition of an enzyme mix containing uracil DNA glycosylase. The ends of the target region are then internal to the PCR primer sequences. Next, Nested Patch oligonucleotides are annealed to the target amplicons and serve as a patch between the correct amplicons and universal primers. The universal primers are then ligated to the amplicons. The ligation reaction is highly specific, because thermostable ligases are used and will discriminate against mismatches at the junction. Signal is further enhanced by exonuclease digestion to remove mis-priming products or genomic DNA. The selected amplicons are protected from digestion by a 3′ modification of the universal primer. The selected amplicons are then amplified together by PCR with the universal primers. Even though there are many enzymatic steps involved in the protocol, it is an addition-only process; therefore, it is quite easy to perform and can be automated.

With Nested Patch PCR, the exponential phase of the amplification is carried out with the universal primers. Again, using the three-legged race analogy, all the paired runners are picked up by a universal primer bus after the initial enrichment of specific targets; in this case, the target enrichment is carried out with the aid of specially designed primers and a series of enzymes.
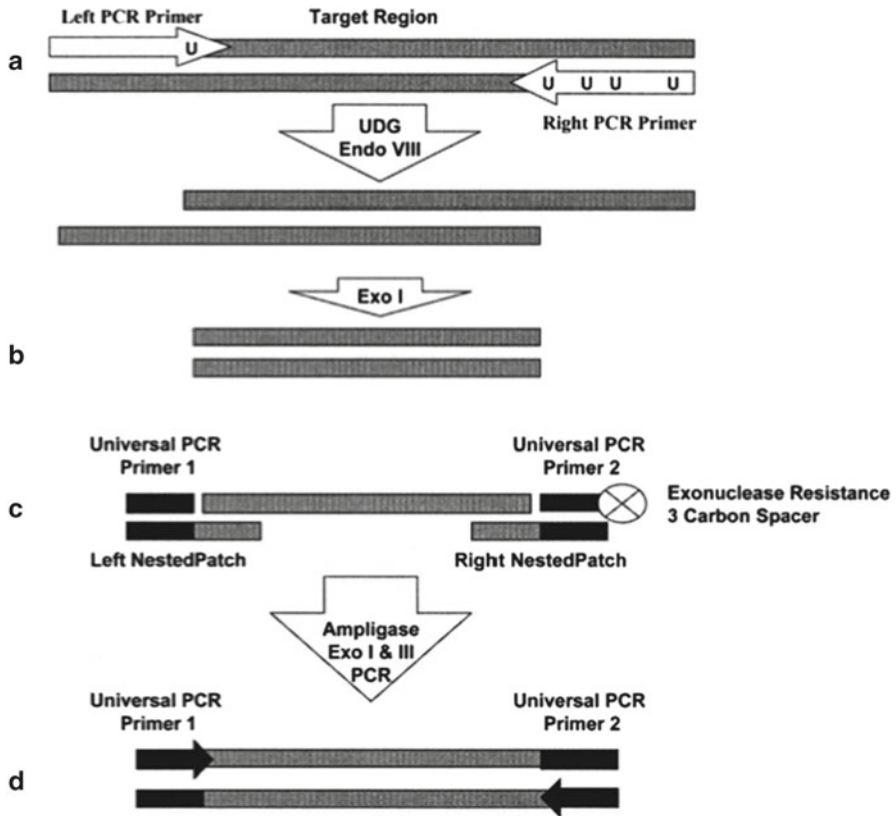
Fig. 34.3 Schematic diagram of Nested Patch PCR. (**a**) Using primers with uracil substituted for thymine, multiple primer pairs PCR amplify different targets from genomic DNA. The primers are then cleaved from the amplicons by the addition of heat-labile uracil DNA glycosylase, endonuclease VIII, and single-strand-specific exonuclease I. (**b**) The ends of the target regions are now internal to the PCR primers (nested). (**c**) Nested-patch oligonucleotides are annealed to the target amplicons and serve as a patch between the correct amplicons and universal primers. The universal primers are then ligated to the amplicons. The universal primer on the 3′ end of the amplicon is modified with a three-carbon spacer that protects the selected amplicon from the final exonuclease reaction that degrades nonspecific products. (**d**) The selected amplicons are then PCR amplified together using universal primers

Amplicon Rescued Multiplex PCR (arm-PCR)

The arm-PCR method was first reported in 2010 by Wang and colleagues [4]. The patent (7,999,092) describing the arm-PCR process was awarded in 2011, and the technology has been assigned to the HudsonAlpha Institute for Biotechnology (US) and licensed to iCubate and iRepertoire for commercialization in diagnostic and immune repertoire sequencing applications, respectively. Figure 34.4 illustrates the arm-PCR method.
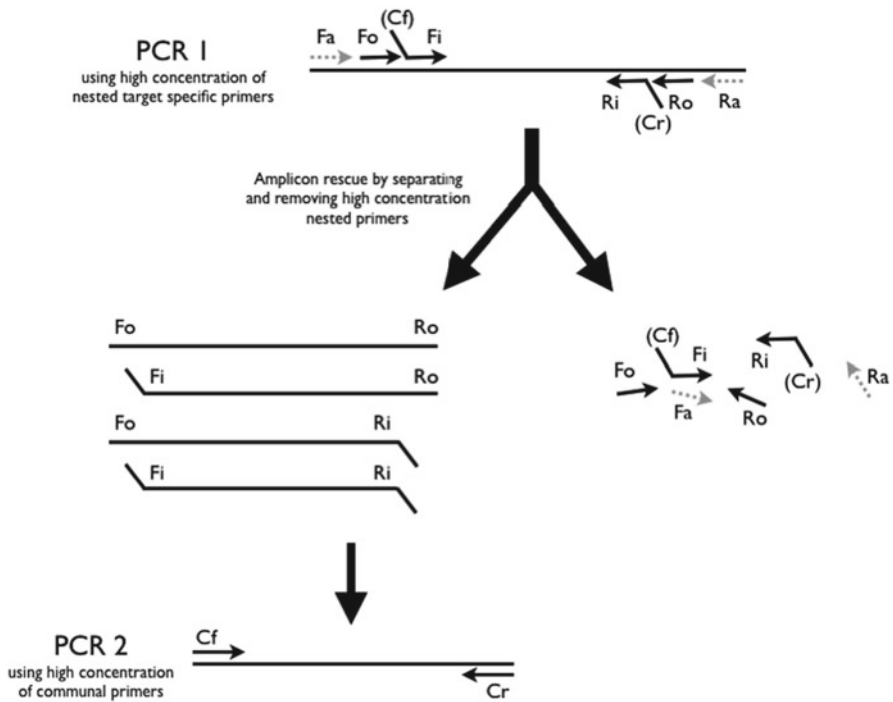
Fig. 34.4 Schematic diagram of amplicon-rescued multiplex PCR (arm-PCR). For each target in a mPCR reaction, a set of nested sequence-specific primers is designed (*Fo* forward-out, *Fi* forward-in, *Fa* additional forward primers, *Ro* reverse-out, *Ri* reverse-in, *Ra* additional reverse primers). A pair of common sequence tags is linked to all internal primers (Fi and Ri). Once these tags are incorporated into PCR products in the first few PCR cycles, an exponential phase of amplification can be carried out with a pair of communal primers, which can pair with the tag sequences. In the first round of amplification, only sequence-specific nested primers are used. The nested primers are then removed by exonuclease digestion and the first-round PCR products are used as templates for a second round of amplification by adding communal primers and a mixture of fresh enzyme and dNTP

Similar to tem-PCR, the arm-PCR method also uses nested gene-specific primers to solve the loci incompatibility problem. There are, however, two major differences between tem-PCR and arm-PCR: (1) with tem-PCR, the concentrations of gene-specific nested primers are very low, but with arm-PCR, the primer concentrations are high in order to increase amplification sensitivity and efficiency; (2) with tem-PCR, the universal SuperPrimers are included in the initial reaction together with the nested gene-specific primers, but with arm-PCR, the universal primers are not included in the initial reaction. The amplicons from the first round of PCR are rescued after removing the nested primers, and the universal, communal primers are then added for a second round of PCR.

If we refer to the three-legged race analogy again, like the tem-PCR and Nested Patched PCR methods, the arm-PCR method uses a SuperPrimer bus to carry all the

runners to the finish line once initial target enrichment is achieved. The first round of amplification is used to achieve specificity and introduce the universal tag into the PCR products. The second round of amplification is used to achieve sensitivity by efficient and semiquantitative amplification of all targets by using only one pair of primers.

We have described four mPCR methods, and there are many additional publications claiming multiplexing capabilities. When it is time to decide which method to use for infectious disease molecular diagnosis, however, we need to ask a few key questions: (1) Is the technology really a multiplex amplification method or it is only a multiplex detection method? (2) Is it easy to develop assays based on the method? (3) Is the method easy to use and can it be automated? (4) How is this multiplex amplification method incorporated with a downstream detection platform? (5) Finally, what about the assay specificity and sensitivity?

Vertical Integration to Provide a Complete Molecular Diagnostic Solution

MDD is a comprehensive process that includes three major steps: nucleic acid isolation, amplification, and detection (Fig. 34.5). There are many methods for completing each of the three steps in this process. Furthermore, a wide variety of instrument platforms are available to facilitate or automate each of these methods. To make MDD a routine clinical practice, these choices must be weighed against each other to obtain the best possible combination of methods and platforms to carry
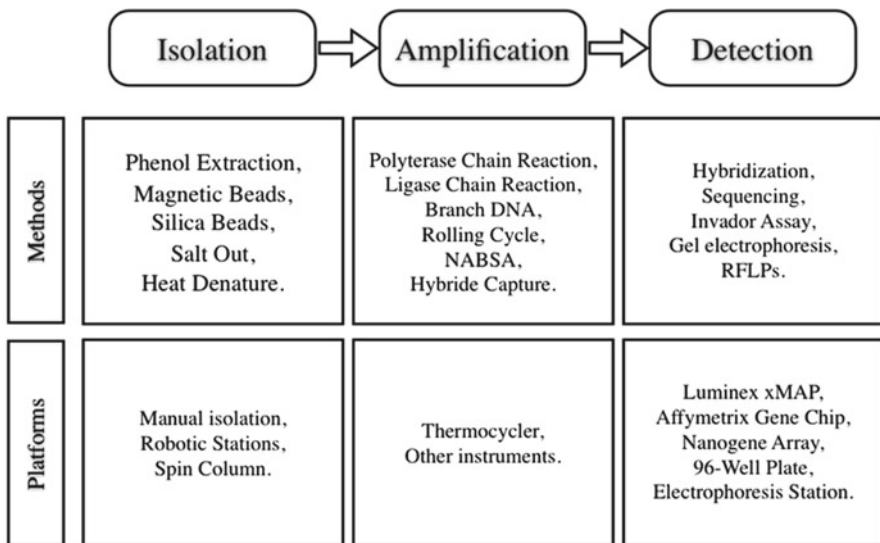

Fig. 34.5 System integration to achieve practical multiplex PCR solutions

Table 34.2 Comparison of information technology and biotechnology for multiplex PCR

	IT	Biotech
Hardware	PC or Apple	Luminex xMAP, Affymetrix chip, flow cytometry, next-gen sequencing, iCubate
Operation systems	Windows, Mac OS, Linux	PCR, qPCR, mPCR
Applications	Word, Excel, Powerpoint	HIV, bloodstream infection panels, HPV typing

Table 34.3 Comparison of different nucleic acid detection methods and platforms

Method	Platform	Throughput	Specificity	Sensitivity	Ease of use	Company	Price
Sequencing	HiSeq	+++++	+++++	++	++	Illumina	High
Sequencing	MiSeq	++++	+++++	++	++	Illumina	Medium
Sequencing	454	+++++	+++++	++	++	Roche	High
Sequencing	454 Jr	++++	+++++	++	++	Roche	Medium
Hybridization	Luminex	+++	+++	++++	+++	Luminex	Medium
Hybridization	eSensor	++	+++	+++	+++	GenMark Dx	Medium
Hybridization	Verigene	++	+++	+++	+++	Nanosphere	Medium
Hybridization	iCubate	++	+++	++++	++++	iCubate	Medium
Invader assay	Invader	++	+++	+++	++	Hologic	Medium
Electrophoresis	Gel box	+	+	+	+	Bio-Rad	Low

out the task. This process of vertical integration can produce multiple possibilities. Thus, if MDD is to be the next breakthrough in modern medicine, we must choose wisely which technology integration path to take.

The biotech industry is very much like the information technology industry (Table 34.2), where an application is developed by using a combination of hardware (the platform) and software (the basic methodology and reagent system). A research tools company may choose different combinations of methods and platforms, so that a particular amplification method can be followed by one of many different detection methods. For example, to build a molecular diagnostic system, PCR amplification may be paired with multiple detection methods, such as direct hybridization, gel analysis, or sequencing. One should also note that a particular method can be performed on multiple platforms. The most successful biotech companies and clinical laboratories are those that are able to develop such applications through technology integration and innovation.

Detection Methods and Platforms

We have described four novel mPCR technologies in this chapter. The next step after amplification is detection of the specific PCR products. Currently, there are two major detection methods: sequencing and hybridization. Table 34.3 lists several methods and representative platforms.

Before discussing detection platforms, it will be helpful to address a common misunderstanding regarding the use of next-generation sequencing in molecular diagnosis of infectious diseases. It is sometimes believed that, with the advance of high throughput sequencing technology and the accompanying rapid price drop, PCR will soon become an outdated technology and will no longer be needed. This is not true. Because sequencing is only a detection technology, it cannot increase the signal-to-noise ratio, which is critical in infectious disease diagnosis. For example, in bloodstream infections, peripheral blood samples from patients may have only 20 copies of the bacterial genome per milliliter of blood, but will have millions copies of the host genome. At that low signal-to-noise ratio, false positive rates will be extremely high and unacceptable for clinical applications. High-throughput sequencing will be useful in infectious disease diagnosis only if the signal-to-noise ratio can be increased significantly, and that requires mPCR.

mPCR products can be sequenced directly with relatively low cost thanks to technology advances in this field. To further reduce the cost, a molecular tag (barcode) system can be used during amplification; then, after amplification, hundreds of samples can be pooled together for one sequencing run, and software can be used to identify and differentiate the samples. The most promising sequencing platforms are Illumina MiSeq (www.ilumina.com) and Roche 454 Jr (www.roche.com), which both cost around $120,000 USD, while the reagents cost a few hundred dollars per run. If 10–20 samples are pooled in one run, the sequence analysis cost per sample will be under $50.

If abundant and specific DNA targets can be generated by an efficient amplification method, detection is more straightforward and rapid. The challenge then becomes providing an accurate measurement of the amplification products in a rapid, high-throughput, and low-cost format.

The simplest detection method is hybridization, which occurs without an enzymatic reaction. One strand of DNA binds to its complementary strand in solution via hydrogen bonding, and specificity is controlled by temperature and salt concentration. Typically, a detectable molecule (fluorescent dye or radioisotope) is attached to one strand of DNA, which can be recognized by a detection device. Because of its ease of use, hybridization is the method of choice for many detection platforms.

High-throughput DNA hybridization is performed with arrays. Currently, nucleic acids are arrayed on solid supports that are either glass slides or nylon membranes. Depending on the type of array, targets can be composed of oligonucleotides, PCR products, cDNA vectors, or purified inserts. The sequences on an array may represent entire genomes, including both known and unknown sequences, or they may be collections of sequences, such as apoptosis-related genes or cytokines. Many premade and custom arrays are available from commercial manufacturers, though many labs prepare their own arrays with the help of robotic arrayers. The methods of probe labeling, hybridization, and detection depend on the solid support to which the sequences are bound. Typically, fluorescent-labeled probes are used with glass arrays.

Luminex xMAP technology (www.luminexcorp.com) is also an array of sorts; however, unlike other arrays, the solid support for probe binding is provided by microspheres in suspension. Therefore, Luminex xMAP technology is also known

as a "liquid chip" or "suspension array." With xMAP technology, molecular reactions take place on the surface of color-coded beads (microspheres). For each pathogen, target-specific capture probes are covalently linked to a specific set of color-coded microspheres. Labeled PCR products are captured by the bead-bound capture probes in a hybridization suspension. A microfluidics system delivers the suspension hybridization reaction mixture to a dual-laser detection device. A red laser identifies each bead by its color-coding, while a green laser detects the hybridization signal associated with each bead. Software is used to collect the data and report the results in a matter of seconds.

This platform is specific, because only the probes that are captured by the beads are recognized by the green laser as signal. Any signal not associated with a specific set of color-coded beads is considered background. The platform is also very sensitive. Each bead has as many as 10^8 COOH groups on its surface for linking capture oligos. The green laser can detect the signal for as few as eight fluorescent-labeled probes that are captured by a bead. Another important feature of the xMAP platform is its repeatability. Because everything occurs in a homogeneous solution (from bead manufacture, color-code staining, and capture probe coupling to product hybridization and data collection), highly repeatable results are obtained with this platform. The xMAP method for collecting and reporting data also contributes to repeatability. Typically, there are 5,000 beads added per reaction for each color-coded bead set. Each bead set is specific for a particular disease marker, such as a mutation or a pathogen. The laser counts 100 microspheres from each bead set and reports the median fluorescent intensity (MFI). Thus, the data represents 100 microbead-associated data points, not just one data point produced by a standard array.

The eSensor XT-8 system developed and marketed by GenMark Dx (www.GenMarkdx.com) is also an array-based detection platform. Capture oligos are printed onto an electronic circuit board directly and eSensor detects electronic signals from the hybridized PCR products. The system is compact, very sensitive, and GenMark Dx has obtained FDA approval for several products for genetic mutation detection.

The Verigen® system developed by Nanosphere (www.nanosphere.us) is another very sensitive detection system, with a benchtop molecular diagnostic workstation that utilizes patented gold nanoparticle technology to detect nucleic acids. To use the Verigenii® system, target nucleic acid is simultaneously hybridized to capture oligonucleotides arrayed in replicate on a solid support (an array) and sequence-specific mediator oligonucleotides, with gold nanoparticle probes, that detect single-copy DNA regions in each target of interest. A washing step is carried out to remove unhybridized gold nanoparticle probes. Silver signal amplification is performed on the gold nanoparticle probes that are hybridized to captured DNA targets of interest. One more washing step is performed to remove unreacted signal amplification reagents. Qualitative analysis of results (reading the array) can then be performed on the Verigen® Reader.

The iCubate system (www.icubate.com) is a new molecular diagnostic platform that uses a disposable cassette, a processor, and a reader to carry out sample prep, mPCR, array hybridization, washing, and detection steps. The detection is carried

Table 34.4 Comparison of different integrated multiplex PCR solutions

Company	Amplification method	Detection platform	Multiplex capability	Sample-to-answer automation	Closed system
Luminex	PCR	Luminex xMAP	Yes	No	No
Qiagen/Diatherix	tem-PCR	Luminex xMAP	Yes	No	No
Seegene	DPO PCR	Gel electrophoresis	Yes	No	No
iCubate	arm-PCR	iCubate	Yes	Yes	Yes
Roche	Real-time PCR	Light Cycler™	Limited	No	Yes
Cepheid	Real-time PCR	GeneXpert™	Limited	Yes	Yes
Gentura Dx	Real-time PCR	IDbox™	Limited	Yes	Yes
Idaho Technology	Real-time PCR	FilmArray	Limited	Yes	Yes

out on a glass array, where capture probes are printed onto a 1-cm^2 piece of glass. mPCR products are captured by the array, and a gene-specific detection probe is then hybridized to the PCR products for detection.

Integrated Solutions

An integrated solution is one that incorporates different methodologies and instruments to allow sample-to-answer results. Table 34.4 lists some examples of companies providing integrated solutions for molecular diagnosis of infectious pathogens. These companies are compared in the following categories: amplification methods; detection platforms; multiplexing capability of more than five targets; fully integrating sample prep, amplification, and detection steps to allow a maximum hands-on time of <3 min; and a closed reaction system so that amplicon contamination can be eliminated.

The Luminex Corporation, for example, has incorporated PCR (also reverse transcription PCR) with their xMAP/xTag detection platform and is offering an FDA-approved respiratory viral panel (RVP) *FAST* that detects eight viruses and subtypes: influenza A, influenza A subtype H1, influenza A subtype H3, influenza B, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), rhinovirus, and adenovirus [5]. While Luminex is a highly efficient detection platform, if traditional PCR is used to amplify multiple targets, the poor amplification efficiency and low signal-to-noise ratio has forced users to perform post-PCR cleanup and posthybridization washes. These steps are difficult to automate and perform in an enclosed system, risking amplicon contamination that may lead to false-positive results or high background.

Qiagen (www.Qiagen.com) and Diatherix (www.Diatherix.com) are using tem-PCR and Luminex xMAP technologies to provide infectious disease detection products and services. The tem-PCR method was first reported for HPV typing,

where 25 types of HPV were amplified and detected with one assay [1]. Since then, this mPCR method has been used to develop many multiplexed assays.

In 2006, Brunstein and Thomas reported the use of a tem-PCR-based multiplex respiratory pathogen identification assay on clinical specimens [6]. The same group also conducted a large-scale clinical study. From 1,742 clinical samples, they found that rapid molecular multiplex assays significantly increased the detection rate when compared with rapid direct fluorescent-antibody (DFA) assays, with an overall detection rate of 68 %, compared to 35 %. More interestingly, they found that over 30 % of the patients had coinfections by detecting more than one pathogen in a sample [7]. Using the same tem-PCR technology, Li and coworkers evaluated a respiratory viral panel [8], Zou and coworkers reported the development of a human influenza typing panel that identified 15 human H5N1 infections in China [9], and Tang and coworkers reported an evaluation of the StaphPlex System with 360 GPCC (gram-positive cocci in clusters) specimens [10]. This system detected 18 molecular targets in a multiplex assay for Staph identification and drug resistance gene detection. The StaphPlex system demonstrated 100 % sensitivity and specificity ranging from 95.5 to 100 % when used for staphylococcal cassette chromosome *mec* typing and PVL detection [10]. Benson and coworkers at the CDC reported the development of a respiratory bacterial panel that detected six pathogens, including *S. pneumoniae*, *Neisseria meningitidis*, encapsulated or nonencapsulated *Haemophilus influenzae*, *L. pneumophila*, *Mycoplasma pneumoniae*, and *C. pneumoniae* [11], while Media Gegia and coworkers reported the evaluation of a tem-PCR based panel that detects 24 *Mycobacterium tuberculosis* gene mutations responsible for resistance to isoniazid, rifampin, streptomycin, and ethambutol [12].

Even though Qiagen/Diatherix has also used Luminex xMAP technology for detection, the incorporation of tem-PCR multiplex amplification technology has made their assay much more user friendly. The Luminex RVP product uses several enzymatic steps, but the Qiagen ResPlex uses only one enzymatic step and eliminates the washing steps after amplification and hybridization. However, the potential risk of amplicon contamination still exists, because the amplification and hybridization reactions are still set up in an open environment.

Seegene (www.Seegene.com) has also developed a mPCR method (DPO PCR) that has made them a powerhouse of multiplex assay development. However, their detection platform lags behind and still uses gel electrophoresis to separate PCR products. This low-cost solution, however, has made their products more acceptable in emerging markets. The lack of automation and potential amplicon contamination may limit the ability of their products to penetrate the western market.

iCubate (www.iCubate.com) has developed a novel mPCR method (arm-PCR) and also a fully automated system that can carry out magnetic bead-based DNA/RNA extraction, arm-PCR amplification, array hybridization, washing, and signal acquisition steps. At the core of the iCubate technology is a single-use cassette that comes preloaded with all the reagents necessary to perform extraction, amplification, and detection steps. The closed design of the cassette guarantees that the high-concentration amplicons contained inside have no chance of contaminating the lab. The iCubate iC-Processor allows for the automated processing of iCubate cassettes.

Computer-controlled robotics allow for automated sample prep, arm-PCR, hybridization, and washing procedures to be performed. Each processor can run from 1 to 4 cassettes in a random access fashion; if more throughput is needed, up to 12 units can be linked together to run up to 48 samples simultaneously. The iCubate iC-Reader allows for automated data collection from iCubate cassettes. A high-speed rotating platter, laser, and photomultiplier tube allow the acquisition of data from each cassette in just seconds. The iCubate iC-Report software performs automated data analysis and generates individual reports for each cassette. It also monitors and tracks cassette progress, as well as system performance.

iCubate is also an open platform. The company launched the iCubate 2.0 open platform (www.icubate2.com) recently, allowing researchers to develop their own assays with the aid of the free online software iC-Architect, which incorporates the patented arm-PCR technology and novel algorithm called PPI (Polymerase Preference Index, patent pending). The PPI can help improve primer design by identifying the priming sites that are preferred by thermostable polymerases.

Table 34.4 also lists several fully integrated platforms that are based on real-time PCR technology. Companies like Cepheid, Gentura Dx, and Idaho Technology have all developed sample-to-answer solutions that allow molecular assays to be performed in a contamination-free closed system. However, in these real-time PCR-based platforms, multiplexing to amplify more than five targets in one reaction is very difficult. Nevertheless, the ease of use of these platforms has revolutionized the molecular diagnostics industry and benefited millions of patients.

Delivering Value Through Reducing Cost and Saving Lives

The advances of genomic technology have changed the way we define diseases from a phenotypic, symptomatic description of clinical presentations to a genotypic, molecular classification of underlying causes. Molecular differential diagnosis has become the hallmark of 21st century medical practice.

Every infectious disease starts with an invasion by a microorganism's genetic material into the human body. The expression of pathogen genes inside human cells can interrupt normal cellular function and induce systemic responses or clinical syndromes. The goal for infectious disease MDD is to investigate all possible causes of a common clinical syndrome and identify the offending pathogen. To achieve this goal, we need a multiplex technology that uses one sample, one test, one technician, one machine, and a short period of time to obtain multiple answers.

MDD is necessary for controlling an outbreak, such as avian flu or SARS. Poorly controlled outbreaks that lead to public health crises are costly. During the SARS outbreak, for example, it is estimated that East Asian countries suffered a loss of almost 2 % GDP in the second quarter of 2003. A difficult cycle is often set into motion: a lack of rapid and accurate diagnostic tests combined with a lack of communication to the public and lack of scientific knowledge about the disease lead to panic and disruption of economic systems. With an early and accurate differential

diagnosis, infected patients can be identified, isolated, and treated. In addition, the general population can be informed and protected.

Following the recent swine flu pandemic, Koon and coworkers reported mPCR results on 10,624 clinical samples with respiratory symptoms [13]. Of those tested, about 71.5 % of the patients with respiratory symptoms were *not* sick from the pandemic strain of H1N1 [13]. Health-care practitioners therefore quickly identified and properly treated those with pandemic flu infection and those requiring regular care. Furthermore, these findings contradicted the conventional wisdom at the time, which was that anyone with flu-like symptoms probably had the H1N1 virus and should be treated accordingly.

The Koon study revealed a second critical point: among those with the H1N109 infection, 28 % were also infected with at least one other bacterial or viral pathogen [13]. These patients with coinfections require more medical care. A Center for Disease Control (CDC) study has shown that a large percentage of deaths associated with H1N109 were due to coinfection with pathogenic bacteria; thus, a multiplex diagnostic test could help physicians to better triage patients and better allocate treatment resources.

Antibiotic treatment depends even more on MDD. The genes and mutations that lead to resistance can be detected by studying bacterial DNA. Almost all genomes of human pathogens have been sequenced, and newly emerging resistant strain genomes are being sequenced as quickly as possible. This DNA sequence information is publicly available in the federally funded GenBank database, allowing scientists to develop specific assays to detect these genes and mutations. However, without mPCR, scientists usually study only one gene segment at a time. This is a problem because, for a particular bacterial strain, drug resistance capability may come from many different genes and mutations. Therefore, a multiplex PCR assay that detects multiple targets in one reaction, instead of just one molecular target like other PCR tests, allows scientists to immediately identify multiple pathogens and multiple segments of a specific viral or bacterial genome.

Healthcare-associated bloodstream infections (BSIs) are the tenth leading cause of death in the United States, with over 350,000 cases reported every year, resulting in an estimated 90,000 attributable deaths. Studies have estimated the cost of treating BSIs to be approximately $27,000 per patient for community-associated bloodstream infections and $58,000–101,000 per patient for healthcare-associated (hospital-acquired) bloodstream infections. Eiland and coworkers showed that for each BSI patient, when mPCR technology is used, drug costs can be reduced by $100 and ICU stay reduced by 3 days, which represents an additional savings of over $10,000 per patient after the cost of the test has been deducted [14]. Multiplex PCR technology allowed for the optimization antimicrobial therapy in 27 % of the patients and de-escalation therapy in 23 % of the patients evaluated [14].

mPCR-enabled MDDs are exciting methods that are bringing revolutionary changes to many aspects of medical practice, especially to infectious disease management. First, it changes the way a doctor treats a patient. Instead of waiting days for culture results, a doctor can now act immediately based on a comprehensive molecular diagnosis. Instead of guessing what may be the offending pathogen,

a doctor can identify the microorganism with confidence. Instead of ordering the blood cultures to gain knowledge for future empirical treatment, a doctor can prescribe the test to seek immediate solutions. Instead of offering antibiotics to put families or parents (and sometimes the doctor) at ease, a doctor can now provide accurate treatment to actually improve a patient's condition.

Second, MDDs will change the way hospitals operate. Hospitals can implement MDDs as an active surveillance measure to prevent hospital-acquired infections (HAIs). Many studies have shown that active surveillance, plus patient isolation, is one of the most effective methods to reduce HAIs [15]. Regularly scheduled surveys of critical environments (such as the ICU), instruments, and healthcare providers will raise the level of awareness and identify problems early. When an outbreak of HAI occurs, MDDs can quickly identify the source of an infection, helping healthcare providers determine which patients should be isolated to prevent the spread of the microorganisms. In an increasing number of states, hospitals are required to publish their rate of HAI, which is calculated based on discharge records. However, some patients may be misclassified as having an HAI because they were asymptomatic carriers before being admitted to the hospital. MDDs can help hospitals better identify, control, and report HAIs, thereby lessening their liability. As a result, MDDs can help reduce costs, shorten hospital stays, and improve the quality of care, while protecting profits.

Third, MDDs will lead to many changes in the healthcare industry. Healthcare spending in the United States has grown rapidly over the past few decades—from $27 billion in 1960 to $900 billion in 1993 to $1.8 trillion in 2004 [16]. Depending on how you measure it, the healthcare industry represents between 15 and 16 % of the gross domestic product. Traditionally, these financial activities occurred in three subcategories: providers (such as hospitals, nursing homes, and diagnostic laboratories), payers (such as insurance companies), and life sciences (such as biotechnology and pharmaceuticals). For example, the cost of developing a new drug can be as high as $800 million [17]. That cost is passed on from the life science sector to the payers and then to the providers. How could MDDs help in this situation? They can help by allowing the three healthcare sectors to work with each other instead of against each other.

In the life science sector, biotech companies with MDD technologies can work with pharmaceutical companies to develop pharmacogenomic or theranostic solutions. This kind of collaboration will improve treatment outcomes without significantly increasing development costs. Instead of developing blockbuster drugs that are one-size-fits-all, more effective treatment can be obtained by utilizing an MDD to tailor the treatment options to the patient's needs. MDDs will make drugs more effective by providing a genotype-based targeting system.

For payers in the healthcare industry, MDDs will change the risk calculation equations used by the insurance companies, such as health maintenance organizations (HMOs) and preferred provider organizations (PPOs). The healthcare payers make money by managing the "risk capital" associated with healthcare services. Reducing costs and risks will directly result in increased revenue. Hallin and coworkers studied the clinical impact of a PCR assay for identification of MRSA directly from blood cultures [18]. They found that, on average, results were available about 39 h earlier

than with the culture method, and about 25 % of the treatments were modified following diagnosis [18]. MDDs can provide faster, more accurate diagnosis that directly influences the clinical outcome and reduces the risks and costs associated with traditional diagnostic methods.

For healthcare providers, the benefit of MDDs is even more apparent. An MDD can help doctors make the right treatment decisions much sooner, thereby shortening the patients' hospital stay and improving the overall quality of care.

Fourth, MDDs will bring about societal changes. Society is threatened by emerging infectious diseases, including many drug-resistant super bugs. The global economy, with its traveling professionals, makes the spread of diseases much faster. Rising costs make quality healthcare more difficult to manage. Moreover, the cost of developing new antibiotics is too high and the process is too slow. We have been promised a better system, and have been awaiting the arrival of MDDs for a long time. Now that the technology has finally arrived, we must maximize its utility and benefit.

Finally, MDDs offer all of the benefits needed for patient care, at once. Using current bacterial or viral culture methods, patients and physicians often need to wait for days before a result is available. Conventional PCR-based molecular analyses are labor-intensive, expensive, and often inconclusive. Powerful mPCR methods can provide a faster answer, leading to a faster recovery. The ultimate value of MDDs is found in its ability to save lives.

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