CHAPTER 4

# Making PCR a Normal Routine of the Food Microbiology Lab

Susan Sanchez, Ph.D.
*Athens Diagnostic Laboratory, and the Department of Infectious Diseases, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602*



## INTRODUCTION

In food microbiology, polymerase chain reaction (PCR) should not be considered a substitute for conventional microbiology techniques. The rationale of employing PCR technology, as well as any other molecular diagnostic technique, should be founded on the following key consideration: (1) simplicity, (2) throughput, (3) cost, (4) speed, and (5) appropriateness (37). Conventional bench microbiology is often considered to be less technically demanding than polymerase chain reaction, however, in reality, the techniques of PCR are easier to master and usually requires less time to achieve competence than conventional microbiology. Experience in our laboratory shows that personnel of varied technical and educational backgrounds, and absolutely no training in microbiology, can master polymerase chain reaction in no more than a week. By

contrast, competence in conventional microbiology techniques and their interpretation requires significant training and experience. Polymerase chain reaction can be mastered in less than a week with very little or no previous training in microbiology, whereas conventional microbiological techniques will require several weeks of training and a background in microbiology. A technique, as simple as PCR, can be applied in more laboratories and it is more amenable to field applications, which could allow for data collection right at the food-processing plant. The food microbiologists' constant aim is to make our food supply safer; this is only achieved with large-scale screening of foodstuffs. Polymerase chain reaction is a test process that allows for high throughput and is amenable to automation. Over the past few years, molecular reagents have become more affordable, and easier to obtain, with longer shelve life (37). Naturally, the cost and accessibility varies greatly with location. Except for primers and probes, most PCR reagents can be used and shared among different PCR tests, as well as for other molecular-based techniques used in food microbiology (e.g., strain typing) (37). The decision to use PCR needs to be made by the laboratory based on clients' requirements for quick turnaround, reliability, and confidence in the laboratory for correctly reporting results. Real-time PCR allows not only the detection of suspect pathogen in food but its enumeration as well (36, 37, 38). These molecular tools are essential to a contemporary food laboratory. Although the cost to equip a laboratory is high, PCR complements and enhances the traditional microbiological methods; by increasing speed, sensitivity, and, specificity for detecting pathogens in foods. Polymerase chain reaction can be performed rapidly in the field, and limits the number of cultures and isolations to the few samples identified as positive by PCR (28). This reduces labor, conserves resources, and holds down costs.

## SETTING UP YOUR LABORATORY FOR PCR

The PCR is a very sensitive, exceptionally powerful, and relatively simple method that can unlock the door to many a genetic mystery after a few rounds of cycling temperatures. However, PCR can return false positives or negatives if care is not taken in the proper setup, standardization, and implementation of quality controls. Careful planning needs to be given to standardizing a routine protocol(s) for processing and testing of samples by PCR. This includes: the physical setup of the PCR laboratory; quality control; storage and selection of reagents; deciding which PCR detection platform to use; and finally, a critical component—well-trained personnel. With good laboratory practices, the diagnostic laboratory avoids the possible pitfalls with PCR that lead to erroneous reporting of laboratory results.

**Physical Setup of PCR.** Avoiding sources of PCR contamination is paramount when dedicating laboratory space to PCR. Ideally there should be 4 to 5 distinctly separate rooms or areas in the laboratory for: (1) sample preparation, which includes sample enrichment, clean reagent preparation, and DNA extraction; (2) PCR setup; (3) thermocycler; and (4) detection of PCR products or amplicons by

agarose gel electrophoresis, or enzyme-linked immunosorbent assay (ELISA). Realistically, having this much room may not be possible in existing food microbiology laboratory, although it should be kept in mind if future renovations are planned, or a new building is being designed. In most instances, this physical separation can be accomplished by the development of one-way traffic flow (Fig. 1) within a laboratory, assigning particular areas to each step in the process. This will hopefully prevent cross-contamination from sample to sample and PCR to PCR. The assignment of areas and/or rooms needs to be intuitive and easy to follow so that it does not influence the speed and normal work flow of the laboratory. Ideally, personnel will start the day in the clean area and move to those areas where there is higher risk of aerosols that could be transported to clean areas and become sources of PCR contamination. In those laboratories that process a very large number of samples, dedicated personnel to each area is a good idea with rotation of these people to a different area every week to avoid boredom and maintain full competence in all aspects of the PCR protocol. Laboratory coats or scrubs should be worn always, but these garments need to remain in each area except when cleaned. DNA present on a bench top can easily contaminate a sleeve. If a technologist later uses the same laboratory coat, then the jacket could serve as the source of PCR carryover contamination when the individual sets up the next PCR reaction. Gloves should be used at all times and should never be worn in the different PCR areas. *The sample preparation area is one of the most important areas in the laboratory and it should be divided in three individual areas for: (1) food sample preparation, (2) clean reagent preparation for DNA extraction, where no sample or template should ever be present; and (3) processing and extraction of DNA from foods or enrichments*. This final step should be conducted in a biological safety cabinet to avoid sample cross-contamination. The technician can inadvertently contaminate sample(s), from which PCR template is prepared by

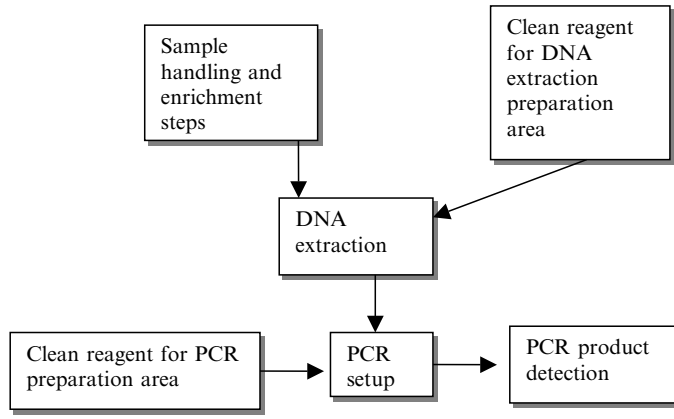


**Figure 4.1.** Chart delineating the workflow in the PCR laboratory. Some of the PCR work areas need to be physically separated in order to minimize the potential for PCR carryover contamination, or sample cross-contamination. DNA template or PCR product should never enter the clean reagent preparation area.

touching a small droplet from an enrichment broth, positive for the microbe in question, and subsequently can transfer this to another tube containing a different sample, by touching the second sample with contaminated gloved hand. This cross-contamination is insidious as it will not be detected by our PCR controls and, even if contamination is suspected, retesting of the contaminated sample template will only yield the same spurious false-positive results. The sample will have to be reextracted, adding time and cost to the process. *The PCR can be exquisitively sensitive, even detecting a few cells that may cross-contaminate a "negative" sample*. To avoid this scenario, care should be taken in cleaning the outside of the sample tubes with a disinfectant to kill bacteria and DNAse, or 10% bleach solution to destroy any contaminating DNA. It is also recommended that the cabinet and the pipettes are wiped with bleach solution to eliminate sample cross-contamination, and subsequently wiped clean with ethanol or water to avoid corrosion by the bleach, before the next DNA extractions (13). Contamination by aerosols can also be minimized if supernatants are aspirated and not decanted. When large volumes of sample need to be handled, disposable individually wrapped sterile pipettes should be used. DNA extraction from already enriched samples is one of the PCR steps that is amiable to automation, which reduces the risk for intersample cross-contamination and sample exchange (30, 49). *The PCR setup* and *thermoycler area* can be physically separated but they can also be placed together as long as there is a biosafety cabinet in this area, or a PCR box for the PCR setup. "PCR clean hoods" are relatively inexpensive, sit on a table or bench and they are easy to clean. Their relatively small size allows for their placement in most laboratories. PCR clean hoods also have germicidal ultraviolet (UV) lights, which aid in maintaining sample purity by denaturing DNA contaminants (13). The use of UV light to decontaminate the PCR setup area, where reagents are openly handled can minimize the risk of carryover contamination (40).

The *detection area* itself, where tubes or capillaries are opened and loaded into agaraose gels or ELISA microplates, can serve as a source of PCR contamination. PCR can amplify DNA from very small amounts of template to large quantities of amplicon, which once aerosolized, aspirated, or spilled, can easily contaminate surfaces. The glass capillaries used with the hot-air thermocyclers can break within their carrier during transport to and from thermocycler and detection area, or required glasscutter to free it from its block. Breakage can result in contamination of this block with amplicon. Therefore, pipettes as well as racks and carriers, used to transport samples from PCR setup to thermocycler to detection area and back, can provide an opportunity for contamination of the next scheduled PCR run. This issue is largely avoided with thin-walled microcentrifuge tubes and other specialized microtubes for real-time PCR. These tubes are not likely to break and contaminate the holders, the only piece of equipment that will go back in the PCR setup area for future use. Barrier tips can prevent inadvertent suction of fluids into the barrel of pippettors. Also a separate set of pipettors also eliminates pipettors as vehicles for introducing PCR carryover contamination during setup. Real-time PCR avoids carryover contamination due to the "real-time" detection of the amplicons as

they are produced, and thus eliminates the necessity from ever having to open the tube once the PCR reaction has been set up to run.

With each additional PCR run, the risk of false-positives increases for that test. Therefore, minimizing the risk using the best work flow and work practices possible is paramount. The size and the number of working areas that can be used simultaneously at one given time will have to be anticipated based on the number of samples that the laboratory plans to process daily, and how many personnel will be working in each area at the same time.

**Personnel.** The PCR is simpler than traditional microbiology, not only regarding the procedure itself, but also because mastering this new technology takes less time than required to learn conventional microbiology. It can be mastered in less than a week with very little or no previous training in microbiology, whereas conventional techniques will require several weeks of training and a background in microbiology. Competent conventional microbiologists can be easily trained to perform PCR and will quickly discover the advantages of this methodology. It can provide rapid same or next day results and, as an initial screen, it can streamline culture and isolations to the few presumptively positive samples (Fig. 2). If we only check PCR-positive samples, we decrease our workload. For the laboratory, this reduces labor, resources, and finally cost. Labor costs rise exponentially as the number of microorganisms to be ruled out increases when comparing PCR to bacterial culture methods (47). Multiplex PCR and microarrays discussed in Chapter 1, reduces these pathogen screens to a single test (12, 26, 41, 46). Unfortunately, several of the commercial tests discussed later in this chapter are detection tests for single foodborne pathogen, and single multipathogen-detection systems are in their infancy. While detection and final confirmation of foodborne pathogen may take up to 6 days with standard microbiology protocols, PCR can provide clientele with preliminary results while the microbiology lab continues to work up the submission. Laboratory supervisory personnel need to be knowledgeable in interpreting PCR results, recognizing and correcting problems as they develop, and in explaining the significance of PCR results to the clientele. Chapter 2 discusses interpretation of PCR results. Personnel with enough molecular training needs monitor result output and keep track of the number of positive samples, to request the retesting of positive samples suspected of being cross-contaminated. The personnel should also keep abreast of the literature regarding PCR to discover improvements and problems with current tests and identify new primers, PCR assays, or methodology available for foodborne pathogens confronting the food industry.

## REAL-TIME VS. STANDARD FORMAT PCR

The PCR amplicons are detected either in "real time" as they are synthesized, or following PCR run on an agarose gel, or in an ELISA microplate. The suitability of either real-time or standard format PCR will differ according to the laboratory's needs, resources, and expertise.

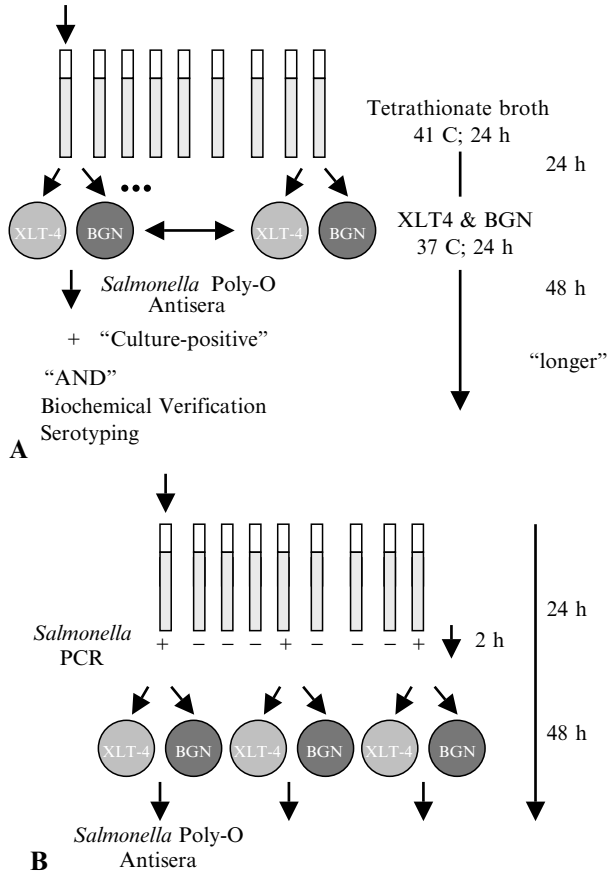


**Figure 4.2.** PCR as tool to identify samples or enrichments, which need to be processed further. (A) Steps and time required to identify sample(s) contaminated with PCR. (B) Incorporation of PCR into screen of samples, or culture enrichments to identify samples requiring additional culture/platings to identify the foodborne pathogen, in this example *Salmonella enterica*.

Amplicon detection can be done without agarose gels. In PCR-ELISA, the amplicon hybridizes with a specific, internal oligo probe tagged with biotin, which is subsequently bound and captured by streptavidin-coated ELISA microplate. As the amplicon was labeled with digoxygenin-tagged nucleotides during PCR, the bound amplicon-oligoprobe complex is detected colorometricly with antidigoxygenin antibody conjugated either to the enzymes alkaline phosphatase or horseradish peroxidases. Color change is subsequently recorded using an ELISA plate reader. With real-time PCR, a fluorimeter is built into the thermocycler to monitor fluorescence as the machine cycles through its denaturing, annealing, and extension step. As the double stranded amplicon is

produced, a fluorescent dye, called SYBR Green, or specific probes labeled with a fluorescent dye bind to the PCR product and the detector measures the resulting fluorescence. The advantageous of either PCR-ELISA or real-time PCR over agarose gels in the detection of amplicons is their amendability to 96-well-microplate formats and automation (31, 43, 46). However, the need for agarose gels for detecting amplicons is not eliminated with either PCR detection format, as it still has a use in validation; the occasional trouble shooting, as problems arise (see Chapter 2); and implementation of real-time PCR or PCR-ELISA into the laboratory's routine.

**Equipment.** When talking about the laboratory adopting PCR, the first and in most cases the only piece of equipment that comes to mind to most readers is the thermocycler. Although, by far it is the most expensive individual piece of equipment, we should not lose sight of other expenses accrued in setting up the laboratory to perform PCR. Several sets of pipettes are needed for DNA extraction and PCR setup, a minimum of four sets. If the laboratory does not own enough biosafety cabinets, PCR clean hoods can be used. Electrophoresis equipment is required for amplicon detection in gels, the microwave oven needed for melting agarose, and enough –20˚C freezer space to keep all the necessary PCR reagents separated from the sample template to be tested. For PCR-ELISA, it will be necessary to also possess an ELISA reader, although this is an essential component already in place for any laboratory that does serology (46). Finally, the laboratory will need to record PCR results. For standard PCR format, where agarose gels are used to detect amplicons, the laboratory will need UV transilluminator and a camera to capture the gel image.

There are a number of factors that will influence which type of thermocycler is purchased. The overall cost of the machine itself might be a major deciding factor in a laboratory's ability to take advantage of PCR technology at all. A simple, hot-air, or conventional heating block thermocycler cost a few thousand dollars. However, the price can vary depending on user's needs and requirements regarding sample throughput (48 vs. 96), sample format (tube vs. 96-well microplate), and PCR reaction volume. The laboratory also needs to consider the cost of the PCR assay with regards to the individual reagents, disposables and ancillary components (e.g., wax beads or mineral oil) in selecting a thermocycler. For example, thermocyclers with heated lids allow for smaller PCR reaction volumes and eliminates the need for mineral oil in the PCR reaction. The speed of a 30-cycler program, 10 vs. 90 min, may be another factor in the purchase of a thermocycler. The laboratory may want to consider the benefits of a gradient thermocycler, which allows users to perform, simultaneously, several PCR tests that require different PCR cycle parameters. These gradient thermocylers also have the advantage of identifying optimal-annealing temperature for a single or multiple PCR primer sets in a single run, due to the machine's ability to assign separate programs to each well. The price for real-time PCR technology jumps to between $30,000 and $140,000, depending on the components of the unit such as 96-well format and automation module. In selecting a thermocycler, the laboratory also needs to determine if the PCR will

be an in-house validated assay or a commercial assay, as the latter will require specific PCR equipment. The following questions need to be addressed before purchasing a PCR thermocycler: Will there be many samples submitted on a regular basis or only periodically that require this type of technology? Will the samples only be a part of the usual submitted workload? Will the samples require detection of only one gene of interest, or are there various genes to identify, which would require different programs for the thermocycler? The PCR format chosen by the laboratory is a big deciding factor to determine the needs for equipment as we have seen before, because initial cost of equipment and applications of the equipment vary.

All instruments, from pipettes to freezers, must be calibrated and certified according to the respective institutional standard operating procedures, which in most cases are dictated by the institutions accreditation agency. For most institutions it is at least once a year. Most large equipment manufacturers offer yearly contracts for calibration of their equipment.

**Reagents and Disposables.** Reagents for the PCR consist of those used for the reaction that takes place in the thermocycler, and those used for the detection of amplicons. Purchasing of reagents should be done from a reputable company and molecular grade should be requested for any reagent that is going to be used for PCR. Primers and probes as well as *Taq* DNA polymerase can be contaminated with extraneous DNA. Top quality components should only be used and when handling these reagents, gloves should be worn as to prevent the introduction of metal ions, nucleases, or other contaminants to the PCR reaction (48). Water is an important component. Always use it as aliquoted, single use volumes that have been filtered (0.22 μm), and autoclaved. If money is not a problem, then ideally DNA-free and DNAse-free water should be purchased for PCR. We must not forget that the PCR reaction requires a DNA template, therefore; the required reagents for extraction or cell lysis need to be taken into account. Consumption and cost of reagents is related to the machine and the DNA extraction method that is used and the number of samples to be analyzed. Depending on the machine, volumes for each reaction can vary from 10 to 100 μl. The average volume used in PCR reactions is 25–50 μl. The smaller the PCR reaction volume, the cheaper the cost per PCR test is. Costs for *Taq* DNA polymerase, dNTPs, and other components of the PCR can turn PCR into an expensive venture for a laboratory very quickly. Lastly, one must consider the cost of labor for PCR setup as well as for sample preparation. It has been reported that the overall costs of the reagents and materials involved in identifying specific bacterial agents by PCR were 2 to 5 times higher than the costs involved with bacterial culture identification (17). As molecular reagents are continuously getting better in quality and longer in shelf life making them overall less expensive, this may not hold true. A good example of this is the reduction in cost of oligonucleotide synthesis.

In cost analysis, one must consider whether certain alternative traditional microbiological methods are feasible or practical with the laboratory's current resources and manpower. For example, *Salmonella* serotyping requires the lab-

oratory to keep an extensive battery of antisera to identify the thousands of different *Salmonella* serovars. Considering the limited shelf-life of the necessary serotyping reagents, the laboratory's sample volume, and time it takes to identify the *Salmonella* serovar, a PCR-based approach might be a more time- and cost-effective approach (19, 21). Likewise, several of foodborne pathogens described in Chapters 6 and 7 are either recalcitrant to current culture, or isolation methods and PCR is more cost effective compared to the alternative isolation procedures. Where PCR trumps culture-based detection methods is that it can provide rapid preliminary results for making important decisions (15, 39, 41). For the laboratory, the decision as to which samples to work up further, and for the clientele, which lots to hold and which ones to ship.

The thermocycler and its throughput capacity will dictate the type of container used to hold PCR reaction: glass capillary tubes vs. thin-walled plastic; PCR microfuge tubes vs. 96-well microplate. Pipette tips must have filter barriers to avoid contamination of the inside of the pipette.

All newly prepared and purchased PCR reagents require quality control before use. This is necessary for any food microbiology laboratory to become successfully proficient at PCR.

**Quality Control and Quality Assurance.** Food microbiology laboratories are used to working under standard operating procedures (SOPs), as their laboratories are accredited to perform food microbiology. Each SOP not only contains the information on precisely how to conduct the procedure, but will also have information regarding when and how much quality control (QC) needs to be performed. A QC program usually requires all products and reagents (from DNA extraction to PCR) of each lot received to be tested to make sure they meet the same standard as previous lots so they can be utilized with confidence in the procedures. A good practice is to aliquot all reagents in single-use format after QC testing. Sometimes the testing of several aliquots is a good idea, especially if the laboratory does large volume of PCR routinely. Preparation of an aliquoted ready-to-use PCR master mix, that has been QC tested, is a good practice because this minimizes technician error resulting from miscalculation, or from forgetting to add a key component in the master mix. Commercially available kits should come with all reagents previously tested and this information if not in the packet insert should be available upon request. SOPs must include information regarding the required positive and negative amplification controls, as these will determine the stringency and accuracy of our PCR tests. The basic PCR controls are: DNA extraction controls, sample purposely spiked with the organism of interest, and another spiked with a different, unrelated organism; and PCR controls, pure, known amount of DNA from the organism of interest, and negative, no DNA, control. A QC for PCR can be made even more robust if an internal amplification control is included to check for the presence of PCR inhibitors in our samples (22, 23, 34). For more information about PCR inhibitors (see Chapters 3 and 4). Good SOPs and a good QC program will help minimize mistakes due to bad reagents and human error. Every laboratory should run a quality control program that is applicable and relevant

for this methodology, and which is capable of detecting deficiencies at any level. This process can be made simpler most times by consulting with other labs performing similar PCR assays and purchasing reagents from suggested reputable vendors, although this will not eliminate the need for QC testing. SOPs are good training guides for new staff. Good record keeping is essential to any laboratory, where a bound laboratory notebook is kept with detailed and dated descriptions of protocols, reagents (including lot numbers, purchase, and expiration dates), controls, and results (32). Furthermore, a good quality assurance (QA) program at the institution ensures that there is compliance with SOPs for the PCR assays and consistency is achieved. Additional measures to consider are addressed in Chapter 2, and for a more thorough review of PCR laboratory setup, see *Methods in Molecular Medicine, Vol. 16: Clinical Applications of PCR* (29).

**Where to Locate Vendors.** For setting up your laboratory to do diagnostic PCR, you will need equipment and reagents to routinely perform PCR. Reagent-wise, you will need the enzyme, buffers, nucleotides, and barrier tips for dispensing reagents into thin-walled PCR tubes that contain the PCR reaction. In addition to this, you will need a source for custom synthesis of your oligonucleotides and probes needed for PCR. You will need to purchase agaraose gel electrophoresis and photo documentation equipment (film or digital-based), for analysis and documentation of conventional PCR results, as well as source for agarose, electrophoresis buffers, loading dye, and molecular weight (MW) standards. We have listed in Table 1, several sources for reagents and equipment listed above. *This list of companies is not an endorsement of the companies or their products;* rather, the table provides the reader an idea of what will be needed to implement PCR into food microbiology laboratory.

## NONCOMMERCIAL TESTS FOR FOODBORNE PATHOGENS

Your laboratory after much consultation has decided that you need PCR as an additional method to evaluate your food samples. Now you need to make a decision which PCR format you want, provided you have a good physical infrastructure that will allow you to have the required physically separated areas in your laboratory, and you also have capable and trained personnel and a good QC program. Your laboratory has also decided that the use of a commercial test is not applicable, but instead the laboratory is going to use currently published primers, or even design for a much better PCR primer set. Published data by one laboratory can sometimes be difficult to reproduce due to the nature of the reagents, the variation in equipment, and the personnel training. Validation based on consensus criteria, detection limit, diagnostic accuracy (the degree of correspondence between the response obtained by the PCR method and the response obtained by the reference method on identical culture samples [AC = (PA + NA)/total number of samples; where PA = positive agreement; NA = negative agreement], diagnostic sensitivity, diagnostic specificity, and robusteness, is a must for a successful microbiology laboratory (24, 25, 33). Your

**Table 4.1.** Molecular biology vendors of PCR

| *Vendor* | *Web Address* | *Product(s)* |
|---|---|---|
| BIO-RAD | www.bio-rad.com | Thermocyclers, Electrophoresis apparatus, buffers, and MW standards, Photo documentation system |
| Dupont Qualicon | www.qualicon.com | PCR diagnostic tests (e.g., Bax *Salmonella*) |
| EPICENTRE | www.epicentre.com | PCR reagents, DNA cloning reagents |
| Fisher Scientific Co. | www.fishersci.com | PCR tubes, barrier tips, etc. Electrophoresis apparatus, Photo documentation, Agarose, Electro-phoresis buffer, Micropipettors, –20˚C Freezer |
| Idaho Technology Inc. | www.idahotech.com | Thermocyclers, design and synthesis of primers and probes |
| Invitrogen | www.invitrogen.com | PCR reagents, PCR cloning vectors |
| MO BIO Laboratories Inc. | www.mobio.com | Nucleic acid extraction kits |
| Molecular Probes, Inc. | www.probes.com | Fluorescent dyes |
| Promega | www.promega.com | PCR reagents, PCR cloning vectors, MW standards, gel loading dye |
| Roche Applied Science | www.roche-applied-science.com | PCR reagents, Thermocycler |
| SeqWright | www.seqwright.com | DNA sequencing |
| Sigma-Genosys | www.sigma-genosys.com | Custom oligonucleotide synthesis |
| USA/Scientific Inc. | www.usascientific.com | PCR clean hood |

laboratory will need to implement and then validate the PCR tests, benchmarking performance by demonstrating that the new method can generate results that are equal, or better, than those obtained by the current gold standard for detection. For a commercial PCR test that has already been validated, implementation is the only step required by your laboratory (34). One last point, when deciding which PCR format your laboratory is going to select, keep in mind the compatibility of the PCR tests chosen with the laboratory's current instrumentation and training. Molecular diagnostic tests that require new equipment, more laboratory space, and more training may not be the best choice. Therefore, a concerted effort at the initial planning stages should be made to foresee future demands.

**Validation.** There is no perfect PCR test and interlaboratory variation in performance of a PCR does occur. However, before diagnostic labs accept a PCR,

there is a requirement for multilaboratory confirmation of the tests, specificity, sensitivity, and reproducibility. (See Chapter 2, discussion of validation.) Spiked samples as well regular samples should be used in the validation process as to mimic as much as possible everyday samples and situations. The extent your lab plays in the validation process of a PCR will be directly related to the target organism, food matrix, and previous work describing validation of PCR in peer-reviewed publications.

Currently, there is not a single harmonized validation protocol available. In 1999, the European Union (EU), through an initiative titled "the FOOD-PCR Project" (http://www.PCR.dk), set out to validate and standardize PCR for the detection of pathogenic bacteria in food using nonproprietary primers. The intended outcomes of this project were production of guidelines and kits for proficiency testing of different brands and types of thermocyclers, method for DNA extraction and purification, production of reference DNA material, and an online database containing validated PCR protocols. These protocols and results are available from their website. A further attempt has been made by the EU through the ISO/TC34 committee in collaboration with CEN/TC275, through the proposal EN ISO/FIDS 16140 "Microbiology of food and animal feeding stuffs—Part 42: Protocol for the validation of alternative methods" (1). Validation through this method seems more suited for commercially developed tests as the process is costly for nonproprietary, "home brew" PCR (34). Several commercial diagnostic tests have been validated in a very extensive manner and have been accredited by organizations on standards both at the international and national level (14, 20, 44). Current commercially available tests for the detection of foodborne pathogens will be reviewed later in this chapter.

**Standardization.** Standardization of PCR tests and extraction protocols at the national and international level will allow for accurate interlaboratory comparison. This can be achieved either with commercial tests, or with validated published primers. Standardization allows for fast implementation of tests, warranted accuracy, and detection limits, as well as known strength to allow for some variation in the tests procedure without giving misleading results. Standardization will also guarantee continued research and improvement of the PCR assay and protocols. Thus, the PCR test will fulfill its promise of being simple, high-yield, fast, appropriate, and even cheaper than the traditional culture (24, 34).

## AVAILABLE COMMERCIAL PCR TESTS FOR FOODBORNE PATHOGENS

There is a wealth of ready-to-use PCR-based tests for the most common foodborne pathogens: *Salmonella* spp., *Listeria* sp. and *E. coli* O157:H7. The availability of commercial PCR tests for other foodborne pathogens is sparse and laboratory "home-brewed" PCR tests may be a better, if not the only, option. Chapters 5–7 describe several published PCR tests for bacterial, viral, and

protozoal-foodborne pathogens. Several real-time and standard format PCR are commercially available. These two formats need to be studied carefully by each individual laboratory introducing PCR into their routine, chiefly to determine whether the equipment cost in comparison with potentially improved diagnostic ability is a worthwhile endeavor to pursue. One or more national and international agencies have validated commercial PCR tests described in this section (14, 20, 44). Their sensitivities and specificities are well known (14, 18, 20, 35, 41, 44, 45), and information is readily available form the products websites (www.andiatec.com; www.bio-rad.com; www.qualicon.com/bax.html; and www.roche-applied-science.com). Once a decision has been made on a PCR-detection format, then the choice of brands should be determined by the individual laboratories based on following consideration: (1) simplicity, (2) throughput, (3) cost, (4) speed, and (5) appropriateness (37). Table 2 lists the currently available PCR-based diagnostic tests and identifies which type of format they are based. The commercial PCR tests described in the next section is not an endorsement of any one product, but rather presents the reader with the commercial kits available for PCR detection of pathogens in foods.

**Real-Time PCR.** *BAX Dupont-Qualicon* This PCR test is based on the use of pathogen-specific primers combined with a dye that allows for detection of amplicon formation during each cycle. A selective enrichment step appropriate for each food is required before the DNA extraction step. Testing is carried

**Table 4.2.** Commercial validated and approved test for the detection of bacterial pathogens in food

| *Test Format* | *Brand Name* | *Certification Agency* | *Organisms Detected* |
|---|---|---|---|
| Real-Time PCR | BAX Dupont-Qualicon | AOAC-RI | *Salmonella, E. coli* O157:H7, |
| | | USDA-FSIS | *Listeria monocytogenes* |
| | | AFNOR | |
| | | NorVal | |
| | Food-proof Roche | AOAC-RI | *Salmonella, E. coli* O157:H7, *Listeria* |
| | IQ-Check BioRad | AFNOR | *Salmonella* |
| PCR-ELISA | Probelia BioRad | AFNOR | *Salmonella, E. coli* O157:H7, *Listeria monocytogenes, Campylobacter jejuni* and *Campylobacter coli.* |
| | AnDiaTec | | *Salmonella* |
| | | | *Listeria monocytogenes* |

*Notes*: AOAC-RI: Association of Official Analytical Chemists-Registration International; AFNOR: Association Française de Noramlisation; NorVal: Nordic Validation Organ; USDA-FSIS: United States Department of Agriculture-Animal and Food Safety and Inspection Service.

out in a 96-well type matrix and up to 94 samples can be tested at once with one positive and one negative control. The product amplification is detected real time by including the fluorescent SYBR Green I. This fluorogenic reporter dye is not specific for the desired target molecule, therefore, post-PCR melting curve analysis is required in the protocol, and spurious, nonspecific amplicons are easy to identify. The BAX system has been developed for the detection of *Salmonella* spp., *Listeria monocytogenes*, *E. coli* O157:H7, and *Campylobacter* spp (12, 16, 20) (Table 2).

The BAX *Salmonella* spp has been accepted as an official method by several accreditation bodies; the Association of Official Analytical Chemists (AOAC) has accepted it as an official method (#2003.09) for use in raw beef, raw chicken, raw frozen fish, cheese, frankfurters, and orange juice (5). The AOAC-RI Performance Tested Method license #100201 applies to food: tested on milk, black pepper, chilled ready meal, chipped ham, chocolate, cooked chicken, cooked fish, custard, dry pet food, elbow macaroni, frozen peas, hot dogs, non-fat dry milk, orange juice, peanut butter, pizza dough, seafood-prawns, alfalfa sprouts, ground beef, and liquid egg (3). The USDA-FSIS (MLG 4C.00) has adopted the BAX *Salmonella* spp. for use in ready-to-eat meat, poultry, and pasteurized eggs (6). The AFNOR (certificate QUA-18/3-11/02) applies to all human and animal food (2). The NordVal (certificate 2003-2-5408-00023) applies to all foods and animal feed (7).

The BAX *Listeria monocytogenes* has been approved by the AOAC. It has been accepted as an official method (#2003.12 AOAC-RI Performance Tested Method license #070202) for use in a wide variety of foods including raw meats, fresh produce/vegetables, processed meats, seafood, dairy cultured/noncultured, egg and egg products, and fruit juices (4). The USDA-FSIS (MLG 8A.00) has adopted the BAX *Listeria monocytogenes* for use in red meat, poultry, egg, and environmental samples (11). The BAX *E. coli* O157:H7 has been approved by the AOAC, and has accepted as an official method (#2004.8 AOAC-RI Performance Tested Method license #010402) for use in apple cider, orange juice, and ground beef (9). The USDA-FSIS is currently in the process of validating this technology.

*Food-proof Roche* This PCR test is based on real-time detection of either *Salmonella spp*. or *Listeria monocytogenes* DNA in raw materials and food samples through the use of a combination of primers and sequence-specific taqman probes with hot start methodology. An internal control is added to each sample prior to extraction, in order to assess the presence of PCR inhibitors. Additionally, this commercial test contains uracil-DNA glycosidase to avoid PCR carryover contamination. The *Salmonella spp*. test method is certified by the AOAC-RI with license #12030 (8), as a performance-tested method for detecting *Salmonella* in food products. Some raw materials are highly inhibitory for the PCR reaction and the use of a proprietary sample preparation kit (High Pure Food-proof kit; Roche; Indianapolis, IN) seems to ensure DNA of high quality for PCR. The *Listeria monocytogenes* test method has also been certified by the AOAC-RI with license #12030 (10) as a performance-tested method for the detection *Listeria monocytogenes* in food products when used in

combination with ShortPrep foodproof II Kit. These foodstuffs include peanut butter, dried whole eggs, dry whole milk, dry pet food, milk chocolate, melon cubes, white cabbage, pizza, vanilla ice cream, paprika emulsion dye, spaghetti, sausage, gravlax, "harzer" cheese, raw ground chicken, raw ground pork, bean sprouts, parsley flakes, ham, and Pollack fillet.

*IQ-Check BioRad* This commercial test (BioRad; Hercules, CA) uses primers and a molecular beacon probe tagged with a fluorescent label specific for the target organism. Amplified products are detected real time by detection of the fluorescence. This system also contains an internal control, present in the amplification mix that assesses the presence of PCR inhibitors. The internal control is detected real time using another florescent beacon labeled with a different fluoroprobe (27, 45). The BioRad's IQ-Check *Salmonella* detection kit has been approved by AFNOR as a valid method for the detection of *Salmonella* in all human and animal food products, and environmental samples.

**PCR-ELISA.** *Probelia BioRad* This PCR test is based on the enzymatic detection of a PCR product that combines DNA: DNA hybridization with its capture and in a microtitier plate with an internal oligoprobe. For the detection of *Salmonella*, this PCR-ELISA can detect 3 CFU/25g sample with 99.6% specificity, following an 18 h of preenrichment step (14, 18). It includes an internal control to evaluate PCR inhibitors in samples, which are monitored in a parallel well. Results depend on the optical density obtained on the detection microplate relative to the internal control well. *Salmonella* and *Listeria* applications have been approved AFNOR for all foodstuffs.

*AnDiaTec Salmonella sp. PCR-ELISA* This commercial kit (AnDiaTec GmbH & Co.; Kornwestheim, Germany) comes in two modules. Module one includes all reagents needed for DNA extraction, amplification mixture in a ready-to-use format, and negative and positive controls. The second module consists of a microtiter plate, probes, the peroxidase conjugate, and all the buffers required for DNA: DNA hybridization and enzymatic detection of the amplified PCR products. For *Salmonella* spp. detection, this test has a demonstrated 98% agreement with bacterial culture when it is conducted according to the ISO 6579 standards. Only samples that had high levels of inhibitors, such as bitter chocolate and herbs required a different extraction method than the one included in the tests kit (35). There is also a kit for the detection of *Listeria monocytogenes*.

## REFERENCES

1. Anonymous. 2002. Microbiology of food and animal feeding stuffs—Protocol for the validation of alternative methods (EN ISO/FIDS 16140). European Committee for Standardization, AFNOR, Paris, France.
2. Anonymous. 2002b. AFNOR Certificate QUA-18/3-11/02: Dupont Qualicon BAX® system PCR assay for screening *Salmonella*. www.afnor.fr/portail.asp.
3. Anonymous. 2002c. BAX® system with automated detection PCR assay for screening for *Salmonella*. www.aoac.org/testkits/testedmethods.html.

4. Anonymous. 2002d. BAX® system with automated detection PCR assay for screening for *L. monocytogenes*. www.aoac.org/testkits/testedmethods.html.
5. Anonymous. 2003a. Evaluation of the BAX® system for the detection of *Salmonella* in selected foods (DuPont Qualicon, Inc.; Willmington, DE). www.aoac.org/vmeth/newsmtd.htm#faoma.
6. Anonymous. 2003b. FSIS procedure for the use of the BAX® system PCR assay for screening *Salmonella* in raw meat, carcass sponge samples, whole bird rinses, ready-to-eat meat and poultry products and pasteurized egg products. www.fsis.usda.gov/ophs/microlab/mlg4c01.pdf.
7. Anonymous. 2003c. NordVal Certificate 2003-20-5408-00023: Dupont Qualicon BAX® system PCR assay for screening *Salmonella*. www.nmkl.org/NordVal/NordVal.htm.
8. Anonymous. 2003d. Roche Diagnostics LightCycler® foodproof *Salmonella* detection kit for *Salmonella spp*. in combination with ShortPrep foodproof I Kit. www.aoac.org/testkits/testedmethods.html
9. Anonymous. 2004a. BAX® system PCR assay for screening *E. coli* O157:H7 kit. www.aoac.org/testkits/testedmethods.html.
10. Anonymous. 2004b. Roche/BIOTECON diagnostics LightCycler® *Listeria monocytogenes* detection systems in combination with the *Listeria* ShortPrep foodproof® II kit. www.aoac.org/testkits/testedmethods.html.
11. Anonymous. 2005. FSIS procedure for the use of *Listeria monocytogenes* BAX® screening test. www.fsis.usda.gov/Ophs/Microlab/Mlg_8A_01.pdf.
12. Bhagwat, A.A. 2003. Simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR. Int. J. Food Microbiol. **84**:217–224.
13. Borst, A., A.T. Box, and A.C. Fluit. 2004. False-positive results and contamination in nucleic-acid amplification assays: Suggestions for a "prevent and destroy" strategy. Eur. J. Clin. Microbiol. Infect. Dis. **23**:289–299.
14. Coquard, D., A. Exinger, and J.M. Jeltsch. 1999. Routine detection of *Salmonella* species in water: Comparative evaluation of the ISO and PROBELIA polymerase chain reaction methods. J. AOAC Int. **82**:871–876.
15. Cui, S., C.M. Schroeder, D.Y. Zhang, and J. Meng. 2003. Rapid sample preparation method for PCR-based detection of *Escherichia coli* O157:H7 in ground beef. J. Appl. Microbiol. **95**:129–134.
16. Englen, M.D. and P.J. Fedorka-Cray. 2002. Evaluation of commercial diagnostic PCR for the identification of *Campylobacter jejuni* and *Campylobacter coli*. Lett. Appl. Microbiol. **55**:353–356.
17. Erlandsson, A., A. Backman, E. Tornqvist, and P. Olsen. 1997. PCR assay or culture for diagnosis of *Bordetella pertussis* in the routine diagnostic laboratory? J. Infect. **35**:221–224.
18. Fach, P., F. Dilasser, J. Grout, and J. Tache. 1999. Evaluation of a polymerase chain reaction-based test for detecting *Salmonella spp*. in food samples: Probleia *Salmonella spp*. J. Food Prot. **62**:1387–1393.
19. Herrera-Leon, S., J.R. McQuiston, M.A. Usera, P.I. Fields, J. Garaizar, and M.A. Echeita. 2004. Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella spp*. J. Clin. Microbiol. **42**:2581–2586.
20. Hochberg, A.M., A. Roering, V. Gangar, M. Curiale, and W.M. Barbour. 2001. Sensitivity and specificity of the BAX for screening/*Listeria monocytogenes* assay: Internal validation and independent laboratory study. J. AOAC Int. **84**:1087–1097.

21. Hong, Y., T. Liu, C. Hofacre, M. Maier, S. Ayers, D.G. White, L. Wang, and J. J. Maurer. 2003. A restriction fragment length polymorphism based polymerase chain reaction as an alternative to serotyping for identifying *Salmonella* serotypes. Avian Dis. **47**:387–395.
22. Hoorfar, J., N. Cook, B. Malorny, M. Wagner, M.D. De, A. Abdulmawjood, and P. Fach. 2003. Making internal amplification control mandatory for diagnostic PCR. J. Clin. Microbiol. **41**:5835.
23. Hoorfar, J., N. Cook, B. Malorny, M. Wagner, M.D. De, A. Abdulmawjood, and P. Fach. 2004. Diagnostic PCR: Making internal amplification control mandatory. Lett. Appl. Microbiol. **38**:79–80.
24. Hoorfar, J. and N. Cook. 2003. Critical aspects of standardization of PCR. *In* K. Sachese and J. Frey (eds.), PCR Detection of Microbial Pathogens: Methods in Molecular Microbiology, Vol. 216, pp. 51–64.
25. Josefsen, M.H., S.T. Lambertz, S. Jensen, and J. Hoorfar. 2003. Food-PCR. Validation and standardization of diagnostic PCR for detection of Yersinia enterocolitica and other foodborne pathogens. Adv. Exp. Med. Biol. **529**:443–449.
26. Keramas, G., D.D. Bang, M. Lund, M. Madsen, H. Bunkenborg, P. Telleman, and C.B. Christensen. 2004. Use of culture, PCR analysis, and DNA microarrays for detection of *Campylobacter jejuni* and *Campylobacter coli* from chicken feces. J. Clin. Microbiol. **42**:3985–3991.
27. Liming, S.H. and A.A. Bhagwat. 2004. Application of a molecular beacon-real-time PCR technology to detect *Salmonella* species contaminating fruits and vegetables. Int. J. Food Microbiol. **95**:177–187.
28. Liu, T., K. Liljebjelke, E. Bartlett, C.L. Hofacre, S. Sanchez, and J.J. Maurer. 2002. Application of nested PCR to detection of *Salmonella* in poultry environments. J. Food Prot. **65**:1227–1232.
29. Lo, Y.M. 1998. Methods in Molecular Medicine: Clinical Applications of PCR, Vol. 16. Humana Press Inc., Totowa, NJ.
30. Loeffler, J., K. Schmidt, H. Hebart, U. Schumacher, and H. Einsele. 2002. Automated extraction of genomic DNA from medically important yeast species and filamentous fungi by using the MagNA pure LC system. J. Clin. Microbiol. **40**:2240–2243.
31. Luk, J.M., U. Kongmuang, R.S. Tsang, and A.A. Lindberg. 1997. An enzyme-linked immunosorbent assay to detect PCR products of the *rfbS* gene from serogroup D salmonella: A rapid screening prototype. J. Clin. Microbiol. **35**:714–718.
32. Macrina, F.L. 1995. Scientific Integrity: An Introductory Text with Cases. ASM Press, Washington, DC.
33. Malorny, B., E. Paccassoni, P. Fach, C. Bunge, A. Martin, and R. Helmuth. 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Appl. Environ. Microbiol. **70**:7046–7052.
34. Malorny, B., P.T. Tassios, P. Radstrom, N. Cook, M. Wagner, and J. Hoorfar. 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food.
35. Metzger-Boddien, C., A. Bostel, and J. Kehle. 2004. AnDiaTec *Salmonella sp*. PCR-ELISA for analysis of food samples. J. Food Prot. **67**:1585–1590.
36. Nogva, H.K., K. Rudi, K. Naterstad, A. Holck, and D. Lillehaug. 2000. Application of 5'-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. Appl. Enivorn. Microbiol. **66**:4266–4271.
37. Riley, L. W. 2004. Molecular epidemiology of infectious diseases: principles and practices. ASM Press, Washington, DC.

38. Rudi, K., H.K. Hoidal, T. Katla, B.K. Johansen, J. Nordal, and K.S. Jakobsen. 2004. Direct real-time PCR quantification of *Campylobacter jejuni* in chicken fecal and cecal samples by integrated cell concentration and DNA purification. Appl. Environ. Microbiol. **70**:790–797.
39. Sails, A.D., A.J. Fox, F.J. Bolton, D.R. Wareing, and D.L. Greenway. 2003. A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture. Appl. Environ. Microbiol. **69**:1383–1390.
40. Sarkar, G. and S.S. Sommer. 1993. Removal of DNA contamination in polymerase chain reaction reagents by ultraviolet irradiation. Methods Enzymol. **218**:381–388.
41. Sergeev, N., M. Distler, S. Courtney, S.F. Al-Khaldi, D. Volokhov, V. Chizhikov, and A. Rasooly. 2004. Multipathogen oligonucleotide microarray for environmental and biodefense applications. Biosens. Bioelectron. **20**:684–698.
42. Shearer, A.E., C.M. Strapp, and R.D. Joerger. 2001. Evaluation of a polymerase chain reaction-based system for detection of *Salmonella enteritidis*, *Escherichia coli* O157:H7, *Listeria spp.,* and *Listeria monocytogenes* on fresh fruits and vegetables. J. Food Prot. **64**:788–795.
43. Shi, P.Y., E.B. Kauffman, P. Ren, A. Felton, J.H. Tai, A.P. Dupuis, II, S.A. Jones, K.A. Ngo, D.C. Nicholas, J. Maffei, G.D. Ebel, K.A. Bernard, and L.D. Kramer. 2001. High-throughput detection of West Nile virus RNA. J. Clin. Microbiol. **39**:1264–1271.
44. Silbernagel, K., R. Jechorek, C. Carver, W.M. Barbour, and P. Mrozinski. 2003. Evaluation of the BAX system for detection of *Salmonella* in selected foods: Collaborative study. J. AOAC Int. **86**:1149–1159.
45. Uyttendaele, M., K. Vanwildemeersch, and J. Debevere. 2003. Evaluation of real-time PCR vs. automated ELISA and a conventional culture method using a semi-solid medium for detection of *Salmonella*. Lett. Appl. Microbiol. **37**:386–391.
46. Wellinghausen, N., B. Wirths, A. Essig, and L. Wassill. 2004. Evaluation of the Hyplex Bloodscreen multiplex PCR-enzyme-linked immunosorbent assay system for direct identification of gram-positive cocci and gram-negative bacilli from positive blood cultures. J. Clin. Microbiol. **42**:3147–3152.
47. Wiedbrauk, D.L. and R.L. Hodinka. 1998. Applications of the polymerase chain reaction. *In* S. Specter, M. Bendinelli, and H. Friedman (eds.), Rapid Detection of Infectious Agents. Plenum Press, New York.
48. Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. **63**:3741–3751.
49. Wolk, D.M., S.K. Schneider, N.L. Wengenack, L.M. Sloan, and J.E. Rosenblatt. 2002. Real-time PCR method for detection of *Encephalitozoon intestinalis* from stool specimens. J. Clin. Microbiol. **40**:3922–39288.