CHAPTER 3

Sample Preparation for PCR

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INTRODUCTION

Adding PCR-detection to a laboratory's repertoire of tools can improve sample turn-around time and accuracy. Yet, PCR is not a universal solution for pathogen detection problems. For pathogens that are rapidly growing and contaminate foods in high numbers, culture onto selective and differential media may actually be more rapid and cost-effective than PCR. However, PCR can greatly improve turn-around time in instances of slow-growing pathogens and can improve detection of pathogens present at low concentrations. Nevertheless, like any other protocol, correct preparation of the samples is key to PCR's success. Figure 3.1 shows a sample processing strategy for PCR. Specific steps in the processing strategy will vary depending on pathogen and foodstuff. Prior research may have shown whether pathogen amplification steps, such as enrichment culture, are needed prior to PCR, so check the published literature for relevant protocols.

HOW DO YOU GET STARTED?

Sample preparation serves several functions for PCR detection (14, 19). It initially decreases sample volume and concentrates the PCR template into a workable volume. The first challenge in choosing a good sample preparation protocol is to know whether the pathogen contaminates food at high levels or whether it

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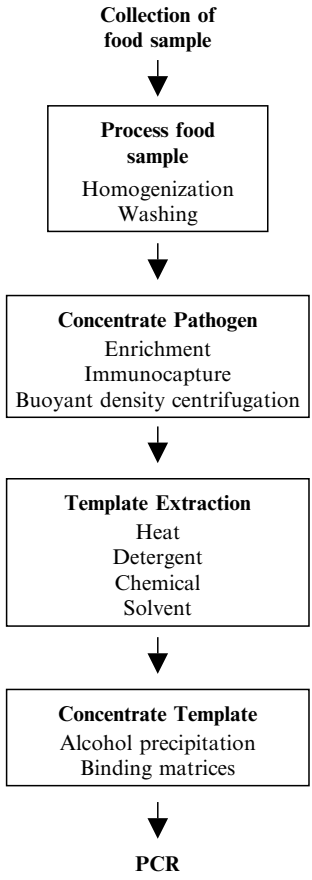

Figure 3.1. Sample preparation for PCR detection of foodborne pathogens.

will be necessary to amplify the bacteria with an enrichment culture. For example, very few *Listeria* cells may be present on a slice of deli meat but these few bacteria may be enough to cause serious illness in a pregnant woman. It may be impossible to directly collect a few bacterial cells and detect them using PCR. An enrichment culture can amplify the bacterial cells and the PCR can detect the bacteria in the enrichment broth more rapidly than they can be identified using standard bacteriological methods. In this instance PCR can aid in the rapid detection of *Listeria* and the sample preparation protocol will include performing the enrichment culture, collection of bacteria from the enrichment broth, extraction of DNA from the bacterial cells and then performing the PCR test.

Once the pathogen is collected, PCR template must be prepared from its DNA (or RNA). The first step in preparing template from a pathogen requires lysis (rupture) of the cells (or viruses) to release the nucleic acids (DNA and RNA). Specific organisms may require specific protocols for efficient template extraction. For example, there are a few basic approaches to extracting nucleic acids from bacteria but their effectiveness depends on several features of the bacterial cell wall. Gram-negative bacteria lack a thick cell wall, thus heat or detergent can lyse the cells. Many of the published protocols for *E. coli, Salmonella,* and *Campylobacter* use this approach for lysis of the cells (see Table 3.1 for applications). Gram-positive bacteria have a thick cell wall that must be removed or disrupted in order to lyse the cells. Lysozyme (plus lysostaphin for *Staphylococcus*) digestion is commonly used, prior to detergent treatment, for nucleic acid extraction from gram-positive and gram-negative bacteria. A third method of bacterial cell lysis involves a high salt/chemical lysis with guanidium salts. This method is most commonly used for gram-positive bacteria but will work for gram-negative cells as well. Solvent extraction of nucleic acids, with organic solvents such as ether, can be used for bacteria, viruses, and protozoa. Commercial PCR detection kits will incorporate one or some derivation of these methods, but the methods have to be optimized for the specific organism.

Table 3.1. Sample preparation of foods for PCR

Food Category (Challenges)	*Sample*	*Method to Concentrate Pathogen*	*DNA (RNA) Extraction method*	*Pathogens (Reference)*
Dairy				
PCR inhibitors (fat, protein, calcium, chelators), dead cells, low numbers of pathogen cells, other bacteria	Skim milk, pasteurized milk, dry milk, hard and soft cheese, reconstituted whey powder	Differential centrifugation or none	Solvent-based nucleic acid extraction or guanidinium isothiocyanate extraction	*E. coli O157* (16) *Listeria* (12) *Staphylococcus, Yersinia* (20) *Campylobacter* (24)
	Raw milk	Centrifugation	Boiled cells with Chelex-100 removal of inhibitors; *Tth* polymerase improved sensitivity	*Staphylococcus* (10)
	Raw milk	Enrichment and centrifugation	Commercial kits	*Salmonella* (5)
	Soft cheese	None	Detergent lysis with NaI extraction of DNA	*Listeria* (15)
Meat and poultry rinses				
PCR inhibitors (fat, protein, collagen, blood), small numbers of bacteria,	Chicken carcass rinses, red meat	Enrichment and centrifugation	Commercial kits	*Listeria Salmonella E. coli* (4, 5)
	Homogenates of chicken skin, whole chicken leg, chicken sausages, turkey leg meat, ground beef, mince meat, beef, pork	Buoyant density centrifugation	Guanidinium isothiocyanate and detergent extraction	*Campylobacter* (24)
	Raw whole chicken rinses	Buoyant density centrifugation and enrichment culture	Boiled cells	*Campylobacter* (27)
	Chicken and turkey muscle, skin, internal organs; raw carcasses	Enrichment	Multiple methods firmed including boiled cells, alkaline lysis, and commercial kits	*Salmonella* (6)

Continued

Table 3.1. Sample preparation of foods for PCR—*cont'd*

Food Category (Challenges)	*Sample*	*Method to Concentrate Pathogen*	*DNA (RNA) Extraction method*	*Pathogens (Reference)*
Meat and poultry rinses	Ground beef	Buoyant density centrifugation, immunomagnetic separation, enrichment	Boiled cells or Chelex-extraction	*E. coli* (25)
	Ham	Immunomagnetic separation	Lysozyme and detergent extraction	*Listeria* (8)
	Minced Pork meat, raw whole pork leg	Enrichment and buoyant density centrifugation,	Commercial extraction buffer and heat	*Yersinia* (13)
	Ground pork	Enrichment	Chelex resin-based commercial kit	*Yersinia* (26)
	Sausage and meat rolls (Korean ethnic foods)	Homogenization of food then filtration and centrifugation	Commercially available kits but increased Mg++ levels in samples	*Clostridium* (11)
	Deli meats: ham, turkey, roast beef	None	Commercial extraction solution	*Norwalk-like virus Hepatitis A virus* (22)
Seafood PCR inhibitors (phenolics, cresols, aldehydes, proteins, fats), low numbers of bacteria	Smoked salmon	Homogenization of food	Detergent extraction and Tween 20 facilitator; PCR inhibitors removed by solvent extraction or column purification	*Listeria* (23)
	Fish cakes, fish pudding, peeled frozen shrimp, salted herring, marinated and sliced coalfish in oil	Enrichment	Detergent and boiling for extraction	*Listeria* (1)

	Shellfish: muscles and oysters	Homogenization of food then high-speed centrifugation	Guanidinium thiocyanate and silica purification	*Norwalk-like virus, Adenovirus Enterovirus, Hepatitis A virus* (7)
	Raw Oysters	Homogenization then buoyant density centrifugation	Commercial kit	*Norovirus* (17)
Produce PCR inhibitors (chelators), few bacteria	Whole raspberries	Column filtration and centrifugation	Commercial kit (FTA filter)	Protozoa (18)
	Lettuce	Homogenization, centrifugation, and precipitation with polyethylene glycol	Commercial kits	*Hepatitis A virus Norwalk virus* (21)

The next step in sample processing is to concentrate the template and reduce the concentration of PCR inhibitors. The specific approach will vary depending on whether DNA or RNA is desired as template and the chemical composition of the PCR inhibitors present in the sample. Phenol/chloroform extraction steps can reduce protein and lipid inhibitors. Other chemical inhibitors can be diluted by washing bound (silica beads or column matrices) or precipitated (ethanol or propanol) nucleic acids. An effective protocol for removing inhibitors must be developed for each specific food. Then the specific PCR can be performed for the pathogens of interest.

WHAT CONDITIONS AFFECT THE SUCCESS OF THE PCR?

The purpose of PCR is to detect the organism's specific nucleic acids in the sample so that time-consuming biochemical and immunological assays are not needed. PCR causes the synthesis of DNA using an enzymatic reaction that cycles over and over due to the temperature cycling of the thermal cycler. Enzymes, including the polymerases that are used in PCR, must have specific chemical conditions in order to do work effectively. One of the major concepts of PCR is that the polymerase can exponentially increase the amount of DNA in the sample because of the temperature cycling (9). However, if the conditions are not optimal, the polymerase may not be able to synthesize enough DNA for the reaction to be detected as positive; these are called "false-negative" reactions. There are many situations where the PCR reaction can be suboptimal and

produce false negative results. Incorrect primers, buffer composition, cation (Mg++) concentration, nucleotide concentration (dNTPs), the wrong annealing temperature, extension cycles that are too brief, and incorrect template can cause the reaction to be falsely positive or negative. Always include two negative controls: a different organism's DNA and a control with no DNA template. These will help you determine the specificity of your PCR and whether you have sample contamination. In addition, always include a positive control with DNA template that you know will amplify in the PCR. These controls can help identify the problem when the PCR is inhibited. For example, different polymerases need different cations in the buffer in order to synthesize DNA. Taq polymerase uses magnesium (Mg++) therefore too little Mg++ in the master mix will result in a negative PCR reaction. The nucleotide concentration is important as well; they will chelate the Mg++ if you use too much of the dNTP mix. However, too much Mg++ will also result in a false negative reaction because there is a narrow window of effectiveness for the PCR to work. Every PCR reaction must be tested to determine the optimal concentration of Mg++ for the specific primers, buffer, and cycling temperatures. A similar situation also exists for the template concentration, too much or too little will result in a false negative reaction. You should optimize the PCR, by running different concentrations of cation and template to find the concentrations that will produce the amplicon of the correct size (or melting temperature if you are using real-time PCR). If you are setting up a new PCR that you found in published literature, do not just assume that the published conditions will work for you. If you get into the habit of optimizing your PCR reaction conditions, you will seldom have a problem with your routine PCRs that you cannot quickly solve.

WHAT ARE PCR INHIBITORS?

The PCR reaction can be inhibited when substances bind (chelate) or degrade a component in the reaction and prevent it from participating in the synthesis of DNA (9, 28). These substances are called "PCR inhibitors" and include chelators of cations and substances that bind or degrade the polymerase or the DNA template. When pathogens are grown to high levels in culture, PCR template can often be made directly by chemical or enzymatic lysis of the organism's cells. For example, DNA can easily be extracted from gram-negative bacteria by boiling the cells in water and using the boiled lysate as template. One important caveat, most enrichment broths and selective agars contain substances that inhibit PCR so it will be important to wash the cells collected from an enrichment or agar plate. You can do this with bacteria by pelleting the cells using centrifugation, removing the liquid and resuspending the cells in saline or water for the DNA extraction.

The real challenge is to isolate the pathogen and/or its DNA directly from a food matrix. The great variety of foodstuffs complicates any quest to produce a single sample preparation protocol that will work for every application. Unique PCR inhibitors are found in just about any food type including meat, milk,

cheese, produce, and spices (28). Many of these have not been identified but some are known substances. For example, milk contains high levels of cations (Ca++), proteases, nucleases, fatty acids, and DNA. In addition, heme, bile salts, fatty acids, antibody, and collagen are PCR inhibitors that may be present in meat or liver. The inhibitors have variable effects on the PCR reaction but in general, they will make it more difficult to detect low numbers of bacterial cells or viruses. A good sample preparation protocol will focus on collecting the pathogen, removing the inhibitors present in the foodstuff (or culture medium) and concentrating the template for PCR. In addition, use of a polymerase that is less susceptible to the effects of inhibitory substances is a possible solution to some PCR problems. For example a number of the newer polymerases, such as *Tfl* and *rTth*, are more reliable than *Taq* polymerase when using PCR template made from meat or cheeses (2). Moreover, the activity of the polymerases, in the presence of inhibitors, can be improved with the use of some facilitators such as bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), Tween 20 and betaine (2, 3, 19, 28). If you are trying to adapt a published PCR to a different food type, you may have to consider adding a facilitator or using a different polymerase to enhance sensitivity of the reaction.

POTENTIAL SOLUTIONS TO THE CHALLENGES OF USING PCR TO DETECT PATHOGENS IN FOODS

Foods differ greatly in their composition. The presence of fats, proteins, enzymes, chemical additives, fiber, and bacteria as well as ranges of pH influence your ability to isolate the organism, its nucleic acids, and amplify its nucleic acids using PCR. In addition, nonpathogenic organisms present in fermented foods, the contaminating soil and manure organisms present on produce, and fecal contamination of meat will produce competing DNA that may reduce the sensitivity of the PCR reaction. Unless you are experienced in developing PCR reactions, you may not want to solve all of these problems yourself. Use protocols developed and validated by reputable labs. Note specific steps in the protocol. Are enrichment steps such as immunomagnetic capture or enrichment culture needed to collect the organism from the sample? How are the organisms collected from the sample or the enrichment? How is the DNA extracted from the organism? What are the specific conditions of the PCR reaction? How will you detect the PCR amplicon? Do you have a positive control organism (or template) for the reaction? Once you have dissected out these important components from the publication or protocol, you can determine, which components can be modified for your specific needs.

Table 3.1 illustrates some of the challenges and solutions for PCR detection of pathogens that contaminate foods. If the challenges are acknowledged, then possible solutions become feasible. If the pathogen contaminates the food in low numbers, then the pathogen must be amplified in some way. The important thing to know is whether the PCR can detect very low numbers of bacteria. Theoretically PCR can detect 1 pathogen in the reaction. Yet realistically,

because of PCR inhibitors and other factors, you will usually need a substantial amount of template, from a few hundred to thousands of pathogen cells or viruses, in the PCR reaction in order to reliably detect the presence of the pathogen. In addition, if you consider that some food samples may only contain a few hundred pathogen cells per gram of food, the need for an enrichment step becomes apparent. Figure 3.2 shows how common pathogen amplification methods work to concentrate the pathogen in a volume that can be used for the next steps in the sample preparation . Enrichment culture is commonly used to amplify the bacterial numbers although immunological capture can theoretically be used for bacteria, viruses, and protozoa. Nevertheless, immunocapture requires the availability of an antibody that is specific for the pathogen. An immunocapture system works by binding one end of the antibody to a handling apparatus (such as magnetic beads in immunomagnetic separation) then exposing the other end of the antibody to the contaminated food. If the pathogen is present in the food, many of the cells or virus particles will be bound to the antibody. This means that the immunocapture system can concentrate the pathogen onto the magnetic beads, which can then be used for enrichment or directly processed in a DNA extraction for PCR. Similarly, pathogens can be isolated from liquid samples by using centrifugation protocols that either float the bacteria or virus particles out of the sample (buoyant density centrifugation) or pellet the cells in the tube. Filtration of liquid samples may be effective for

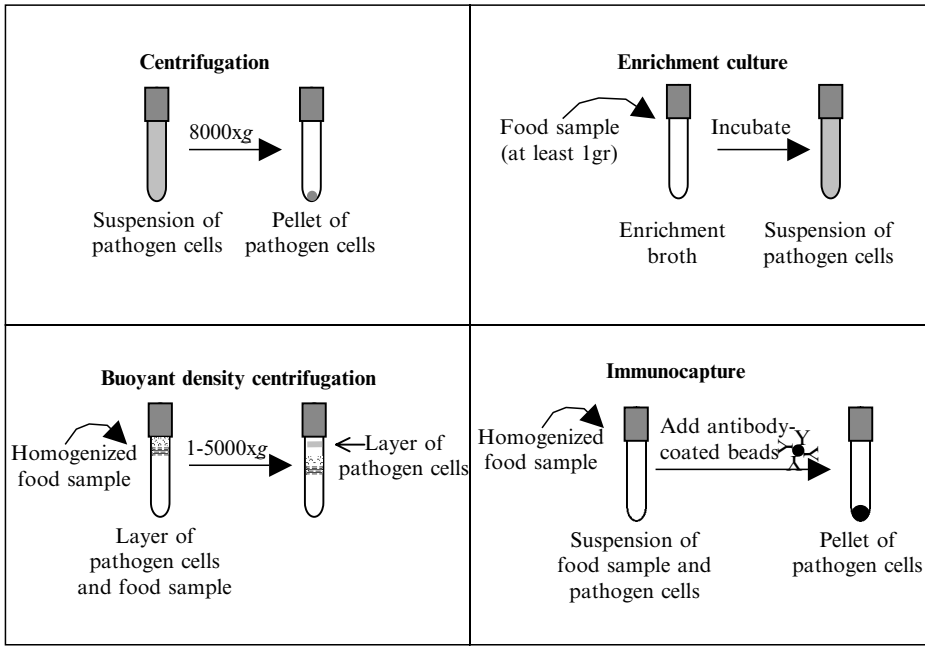

Figure 3.2. Approaches to concentrate or amplify pathogens.

concentrating pathogens that pass through a 0.45 micron filter (such as *Campylobacter* and virus particles). For some pathogens where the infectious dose is very low, more than one concentration step may be needed in order to amplify the pathogen to a detectable level. Many studies have addressed these detection issues in developing PCRs for specific applications (Table 3.1).

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