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FTA Filter Applications: A PCR Format to Alleviate Technical Barriers in the Detection of Food-borne Pathogens in Complex Matrices

BY PALMER A. ORLANDI
and KEITH A. LAMPEL

The safety of our food supply is a major public health concern for consumers, government regulatory agencies, and the food industry. Earlier generations may recall when fresh produce was largely domestic and seasonal. Today we live in a global marketplace, where fresh fruits and vegetables may be on the vine overseas one day and on our grocer's shelf the next. Although this has provided more year-round variety of foods for the consumer, a lack of uniformity in established agricultural standards and practices among international trading partners (e.g., sanitary issues and inspections) may ultimately lead to deleterious health effects. This is evident in the number of food-borne illness outbreaks and associated deaths. Negative economic consequences also result through lost wages and productivity, and health care costs.¹ The impact can be far greater in developing nations. Therefore, refining domestic and international food safety policies is at the forefront of many government agencies' efforts toward protecting the public health. Reducing the number of such incidences has become a priority for government regulatory agencies and the food industry. Similarly, public

awareness of microbial pathogens associated with food-borne illness has risen with such programs as FDA's Food Safety Initiative (instituted in the mid-1990s) and continues with national food safety programs instituted by the U.S. Department of Agriculture, FDA, the Centers for Disease Control and Prevention (CDC), and the U.S. Environmental Protection Agency.²

The new millennium has brought with it a new public health issue involving foods. Since the events of 2001, the specter of bioterrorism and the many forms in which it may manifest has been an impetus for federal, state, and local agencies to establish preventive measures to ensure the safety of the U.S. food and water supply. At the federal level, agencies within the Department of Health and Human Services (principally FDA, CDC, and NIH) and the U.S. Department of Agriculture have established a coordinated series of programs and research efforts designed to enhance food security and to provide for a rapid response to potential bioterrorist threats.³ Therefore, food safety issues, new and old, have created a need for a new generation of rapid and sensitive detection techniques to meet the challenges facing both the food industry and government regulatory agencies.

Food-borne Pathogen Detection

Just as government regulations and policies have had to adapt to ever-

changing times, so too has there been a need for adaptation, refinement, and development of detection methods. A large portion of applied food safety research during the last several years has been directed toward incorporating a multitude of novel molecular techniques designed to rapidly detect minute levels of food adulterations. These span the spectrum of chemical, bioengineered, and microbial events; the latter includes emerging pathogens that until recently were not commonly associated with human illness. Such efforts are intended not only for diagnostic purposes but also for epidemiological investigations. They are also in response to an increasing public demand to become more proactive, with a focus on preventing and reducing pathogenic microbes in foods.

Food analysis for bacterial pathogens has relied heavily on conventional, culture-based methods to enrich microbial growth to a level that can be confidently detected. Bacteriological media, serological reagents, and biochemical assays continue to be the standard means of isolating and identifying contaminating microorganisms. In some instances however, the microbe may be present but non-culturable in artificial media, or may take an inordinate amount of time to culture. In addition, many other food-borne pathogens, such as viruses and protozoan parasites, may not be capable of enrichment in culture at all. Current methods to detect these

Palmer A. Orlandi, Ph.D. (porlandi@cfsan.fda.gov) is a biochemical parasitologist and Keith A. Lampel, Ph.D. is a microbiologist are in the Division of Virulence Assessment, Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, MD.

pathogens can take days to weeks to confirm their presence and identity. Meanwhile, the source of contamination remains unidentified and the etiological agent continues to disseminate through a susceptible population.

More technologically advanced, molecular-based assays are a viable alternative to traditional methods; they provide results more rapidly and with greater sensitivity. Some applications advocate a universal approach that does not require the selective enrichment of the suspected agent. The current generation of detection methods for food-borne pathogens, therefore, has begun to incorporate many of these techniques. Protein-based assays, such as ELISAs (enzyme-linked immunosorbent assays), target selected gene products or specific cellular microbial prod-

ucts such as toxins or cell surface antigens. Alternatively, DNA methods test for the presence of a specific sequence of nucleic acids. One such method, the polymerase chain reaction (PCR), has undergone a significant transition from its initial description more than 15 years ago. End-point PCR, mutation analysis PCR, and real-time PCR protocols have become powerful analytical tools. As such, our laboratories and others have incorporated these techniques in applications involving food and environmental analyses, and clinical diagnostics. Other technologies (e.g., biosensing, nanotechnology, and microarray analysis) may offer alternative means of detection but the level of sensitivity, timeliness, and cost effectiveness critical for routine use has not been achieved.

In general terms, PCR is an amplification system that selectively increases the number of copies of a specific region of DNA or RNA to levels that can be readily detected. The latter process requires a reverse transcription step prior to DNA amplification. Although PCR is based on simple concepts, the success of each reaction depends on many factors. The specificity and sensitivity of each reaction are governed by oligonucleotide primer design, sensitivity of post-processing analysis, the incorporation of conventional versus real-time thermal cycling platforms, and the quantity and quality of the nucleic acid template. The latter two are the primary focus of this technical review.

PCR-based assays are becoming more rapid and sensitive as newer,

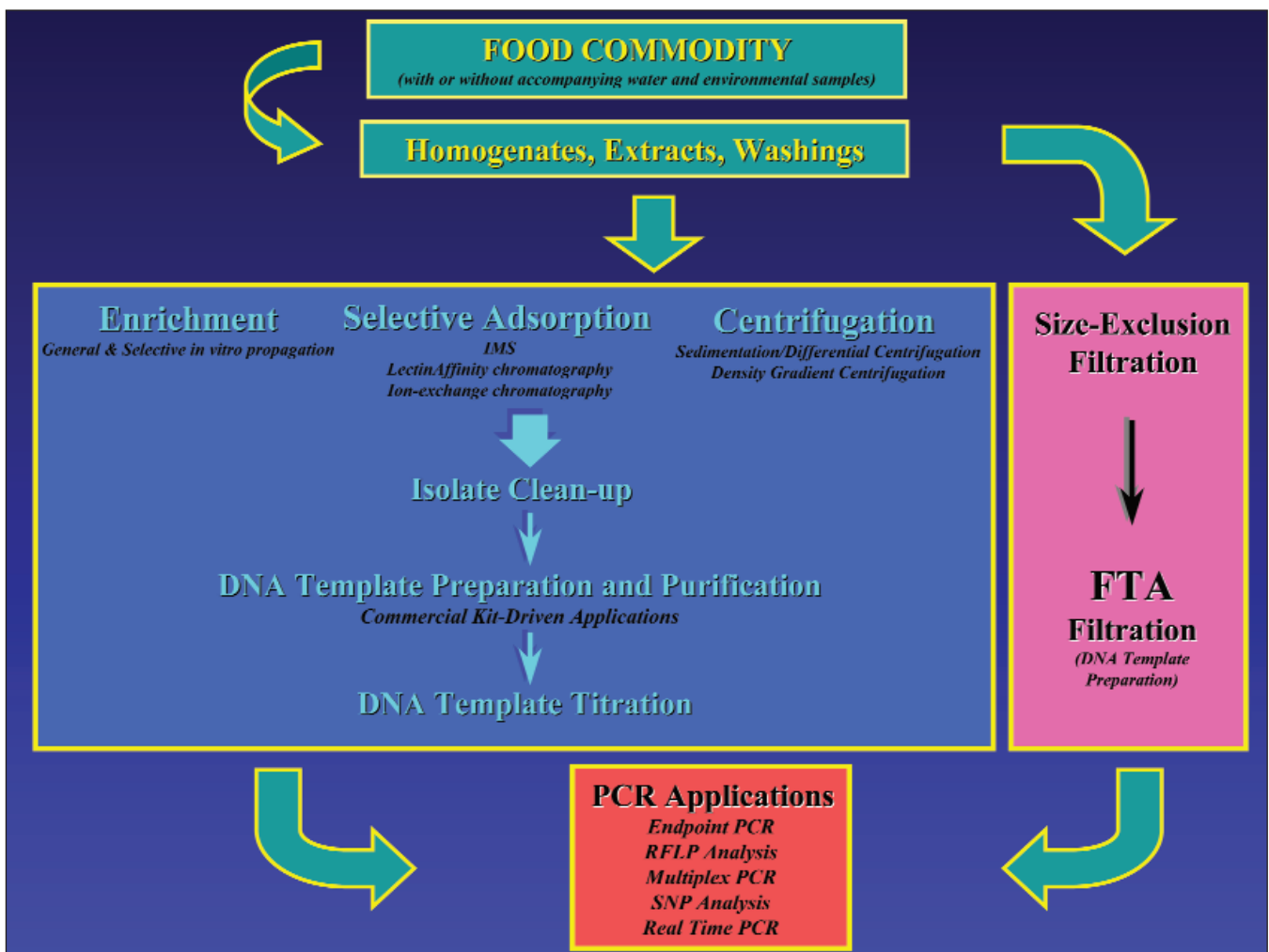


Figure 1. Flow diagram of sample handling alternatives in the analysis of fresh and processed foods for the presence of microbial pathogens by PCR-based applications.

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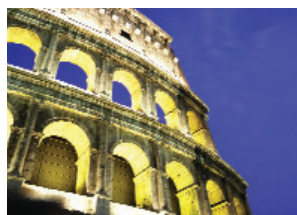
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sophisticated instrument technologies are introduced. However, even with continual improvements in the designs of PCR components, the quantity and quality of nucleic acid templates remain major barriers to reliable, reproducible, and robust PCR-assays. As such, these factors tend to dictate more than any other how sensitive a method will be. Therefore, the development of PCR-based protocols for the detection and identification of foodborne pathogens must address several unique concerns that directly influence the integrity of the nucleic acid template during its preparation. These include the heterogeneous nature of foods, the myriad potential inhibitory substances and nucleases, and the innumerable matrices that may need to be analyzed.

Low pathogen numbers in large sample sizes are an additional variable. Analytical methods to detect foodborne pathogens must have very low detection thresholds because the potential for human illness is directly related to infectious dose numbers. The importance of assessing the sensitivity of any assay is apparent when one considers that the infectious dose, that number of organisms that are required to cause disease or damage to the host, can be as low as 10 cells in a standard adult-size food portion.

As with any method, there are caveats to the overall utility of PCR amplification from complex matrices such as foods. The potential for false negative results can neither be ignored nor underestimated. False negative results occur when a microbial pathogen is present but a PCR-based assay fails to detect its presence. Underlying reasons can include the quantity of DNA template isolated, the integrity of the template, and the quality or purity of the template. Although there is very little control over the yield of template from any one particular sample, template integrity and quality can vary significantly among various food matrices and among preparative techniques. A variety of compounds can act as inhibitors in PCR assays. These can range from soil components to agricultural extracts and organics to other substances derived from food

processes.⁴ Specific compounds include polyphenolics, polysaccharides, and lipids. Unless eliminated during template isolation, they will significantly alter the outcome of the reaction. Therefore, the combination of low pathogen numbers, a large sample size, and a heterogeneous sample matrix has the potential to limit the success of any detection system, particularly those based on PCR methodologies. PCR-based assays that can routinely detect a single cell from a pure isolate would fail to amplify target DNA if the sample from which the template was derived was contaminated with reaction inhibitors.

Extraction-free, Filter-based Template Preparation

In a regulatory setting or for epidemiological purposes, the sensitivity of a particular method must be paramount. It cannot be overstated that the complex and heterogeneous nature of food matrices presents considerable difficulty for the detection of low numbers of pathogenic organisms. For molecular-based methods, the template is frequently DNA isolated from specific target organisms. The literature is replete with such protocols and commercially available kits are designed to provide a desired amount of template per starting material, free of inhibitors that can be amplified by PCR. Many existing commercial protocols profess to be efficient at removing such inhibitors. Though it is not within the scope of this article to review these products, even when given their claims of success, kit-driven assays can add significant extra expense and can be labor intensive when processing a large number of samples. The level of sensitivity in such cases is largely dependent on the quality and quantity of isolated nucleic acid templates. Thus, for food analyses, sample-processing protocols must include enrichment and/or concentration steps as a means to increase the likelihood that a particular pathogen can be detected. In addition to traditional culture-based enrichment, concentration techniques range in nature from general to highly specif-

ic, from differential and density gradient centrifugation to selective adsorption techniques that require specific reagents such as immunological or other affinity matrices (Fig. 1).

Although the general advantages and disadvantages of PCR-based assays are well documented and accepted, the potential for significant levels of PCR inhibitors is a constant problem, particularly in food analysis. To overcome the effects of such inhibitors and concurrently amplify the target of interest, enrichment in broth cultures for 4–18 hours is usually recommended. However, it has become increasingly clear that this option is no longer the norm, but rather the exception. The need for rapid analysis may preclude the use of traditional microbiological techniques for pathogen enrichment. In addition, many microorganisms are not amenable to broth culture; some are poor competitors with normal microbial flora of foods, some display *in vitro* growth patterns so delayed that this is not a viable option, and still others are newly recognized as human pathogens and are so ill-defined that an *in vitro* culture method is not yet available.

Alternative methods for isolating and concentrating microbial pathogens are also available and can be generally categorized into centrifugation, selective adsorption, and filtration techniques (Fig. 1). These have been applied with varying success not only to food analysis, but also to environmental surveillance and clinical diagnostics.^{5–8} But, the specific concentration and clean-up of microorganisms either by immuno-magnetic bead separation or lectin-affinity adsorption has drawbacks. Both require a general knowledge of the cell surface characteristics of the pathogen and require specific reagents. Most currently used concentration methods, however, can incur substantial and variable sample recovery. Hence, a universal method for preparing PCR templates has broad appeal. Such a method should be characterized by its ability to provide target DNA or RNA templates in sufficient quantity and largely free of PCR inhibitors; be independent of sample matrices; be applicable to a broad spec-

trum of pathogens; be sensitive, and not depend on enrichment protocols for detection. As with any useful protocol, its appeal should also be based on ease of sample handling, the ability to process multiple samples, no requirement for specialized equipment, and its applicability in a variety of physical settings.

Filter-based applications have long been a fixture in research and diagnostic protocols. In the past they have been employed for sample collection, transportation, pathogen concentration and isolation, and long-term specimen storage.^{8–14} Several commercially available filter formats are marketed specifically for processing whole blood samples for PCR amplification and nucleic acid storage. Of these, IsoCode® paper (Schleicher & Schuell, Keene, NH) and FTA® Gene Guard cards (Whatman Biosciences, Newton, MA) are well suited for molecular diagnostic purposes. The FTA Gene Guard format in particular has shown remarkable versatility as a means to isolate, transport, and store samples for nucleic acid processing. FTA filters were originally designed and marketed as a solid medium for the long-term storage of blood and other clinical samples. Impregnated with chelators and denaturants, FTA filters lyse most cells on contact and trap nucleic acids in the fibrous matrix of the filter.^{15,16} Potential PCR inhibitors (e.g., contaminating proteins, chemicals, and other debris) are subsequently removed with a quick series of washes. Through a series of investigations, we have shown that this filter format can be extended to other arenas with equally beneficial results.^{17,18} As an analytical and diagnostic tool, it has broad applicability in the molecular analysis of such complex matrices as foods, environmental samples, and clinical isolates. In practical terms, it has the potential to provide clean nucleic acid templates in a simple, timely manner that is amenable to processing one, several, or many samples from most matrices. As stated previously, food-borne illnesses affect an estimated 76 million people each year in the United States alone with causative agents encompassing a wide range of viruses, bacteria, and parasitic

protozoa. Each class of pathogen tested thus far can be captured on FTA filters. What distinguishes FTA filters from other conventional formats is the ease with which targeted organisms can be simultaneously concentrated and lysed onto the filter, thus eliminating the need for lengthy enrichment procedures. Processed FTA filters that contain sequestered DNA can then be used directly in PCR assays (Fig. 2).

The full potential of FTA filters as a practical means of preparing PCR-quality DNA templates from large samplings of foods (or environmental or clinical isolates) rests in sample processing prior to the time it comes into contact with the filter. Strategies for gross sample clean up are necessary to ensure maximum contact between microbial pathogens and the FTA filter. This can be accomplished in a relatively universal manner, with slight modifications depending on the commodity to be analyzed. Simple size exclusion filtration in the presence of a non-selective adsorption medium can accommodate the clean up of most crude samples for their filtration through FTA (Fig. 2) while other samples may require more extensive processing depending on the nature of the sample.

FTA Filters and Protozoan Parasites

Cyclospora cayetanensis, *Cryptosporidium* species such as *C. hominis* and *C. parvum*, and the Microsporidia are three classes of newly emerging parasitic pathogens that cause gastrointestinal illness in humans. *Cyclospora* infections through the consumption of fresh produce have resulted in several thousand confirmed illnesses.^{19–22} It is suspected that there are many more unreported cases of infection than are documented. Unpasteurized apple cider has been cited as a source for *C. parvum* infections and contaminated water sources have been suspected in illnesses from all three parasites.^{23,24} Detecting and identifying such important food- and water-borne protozoan parasites however, has collectively been extremely difficult. Previous analytical methods relied on traditional microscopic tech-

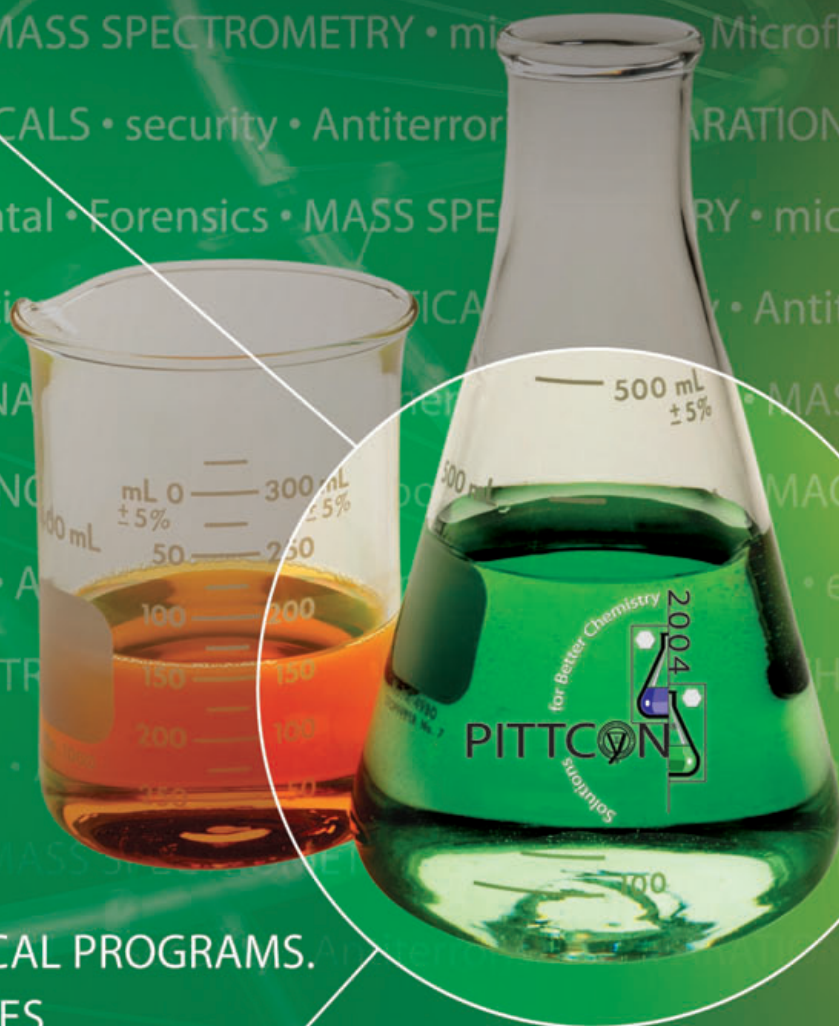
niques. This was adequate for detecting large numbers of parasites in clinical samples but unsuitable for screening foods. Multiple molecular detection methods for these environmentally hardened microorganisms can be found throughout the literature, though the challenge still remains in detecting small numbers in complex and heterogeneous matrices. Enrichment protocols using animal or tissue culture-based models have either not been developed or cannot be performed in a timely manner.

FTA filter incorporation into PCR detection methodologies for food-borne pathogens was first demonstrated by the detection of *C. cayetanensis*, *Cryptosporidium parvum*, and several Microsporidia species.¹⁷ Either as pure isolates or in matrices such as fresh produce, water, urine, fecal material, and sputum, the use of FTA-bound DNA as template in PCR assays had a demonstrable sensitivity range of 10–30 organisms. We have since refined the methodology to handle larger sample sizes and to include a variety of fresh and processed foods deemed susceptible to contamination with these pathogens. These include juices, cider, milk, fresh vegetables, and fruit.²⁵ The practical utility of this FTA-PCR format was illustrated in two outbreaks of cyclosporiasis attributed to the consumption of contaminated produce. In both instances, FTA filter usage was central to the identification of *C. cayetanensis* as the causative agent.^{20,26}

Bacteria Detection with FTA

Gram-negative and gram-positive bacteria were applied to FTA filters and tested in PCR-based assays. An initial concern was whether the level of sensitivity of using DNA impregnated into FTA filters from lysed cells would be as sensitive as adding identical number of cells directly into the reaction. Under the same conditions, the level of sensitivity was comparable.¹⁸ Without any time-consuming enrichment period to increase target cell number, the number of cells from pure cultures detected by PCR ranged from 30–50. In artificially seeded experiments where known amounts of

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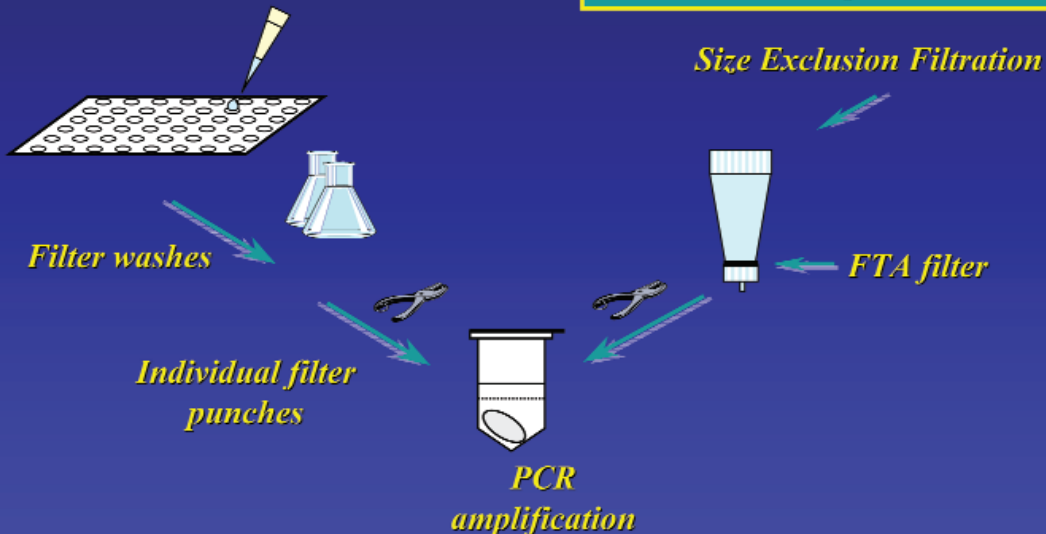
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(A)

Practical Use of FTA Filters: Detection of Human Pathogens in Foods and Water by PCR

*Direct application
of small volume samples
onto FTA filters*

*Washings, extractions, etc.
Direct clean-up, concentration, &
application of sample onto
FTA filters*



bacterial cells are added to foods, FTA filters provided the same level of detection as pure cultures. It was quite apparent from these results that the brief washing conditions were quite effective at removing PCR inhibitors. As stated before, this attribute is critical for analyzing foods. Chemicals from many different types of food matrices can easily be removed with this format. Although the same results have been achieved with clinical specimens, the number of these is somewhat dwarfed by the number of food matrices that must be considered. Therefore, processing food samples either directly or from enrichment cultures through FTA filters is an efficient alternative.

FTA and Novel PCR Applications

PCR template preparations using FTA filters are also being combined

(B)

Assembly of the FTA Filter Funnel Unit

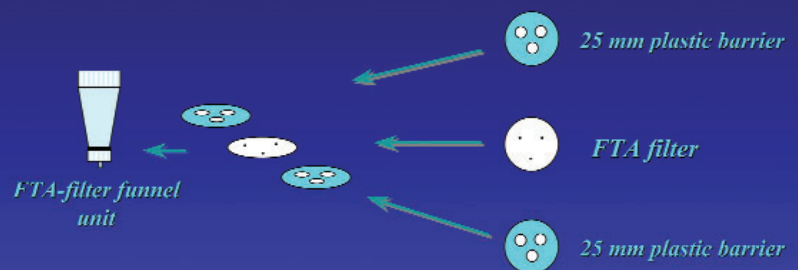


Figure 2. (A) Practical applications of the FTA filter format for the preparation of PCR-suitable nucleic acid templates. Samples can be applied either directly in small volumes ($\leq 15 \mu\text{l}$) onto FTA filters or in larger volumes (90–100 ml) when FTA filters are used as an insert in analytical filter funnels in combination with other sample clean up and concentration techniques. (B) The assembly of such an analytical filter unit containing an FTA filter can reasonably be accomplished as shown in the lower panel. Two 25-mm diameter plastic septa containing 3- to 6-mm diameter holes are used as a barrier to direct filtration onto selected areas of the FTA filter insert. These areas will be punched out once sample has been passed through the filter unit and subsequently washed. Such a format allows for PCR amplification of an FTA-applied sample in triplicate. The assembly of impermeable septa and FTA filter is then inserted into the bottom of a disposable filter funnel unit.

with other detection stratagems including those for bacterial spores and viruses (K.A. Lampel, personal communication). In such instances where reliable and timely enrichment protocols exist, the use of FTA template preparations has increased the level of sensitivity by a factor of ten; in some cases, 1–5 cells or spores can be detected. In light of heightened bioterrorism concerns, the ability to rapidly detect and identify microbial agents is critical in responding to any biological threat. PCR template preparation from any of these microbes can be achieved by FTA filters.

In addition to conventional PCR applications, pathogen detection using FTA filters has also been incorporated into real-time PCR formats. In contrast to the inclusion of DNA-laden FTA filters directly into reaction tubes in conventional amplification protocols, real-time PCR formats with FTA template preparations can be accomplished by the simple elution of FTA-bound DNA. A brief elution step at 95°C is sufficient to release DNA into solution. An aliquot of the eluted DNA can then be added to the reaction mixture. The utility of an adaptable format such as this, in conjunction with real-time applications, will allow multiple analyses for multiple pathogens with timeliness and sensitivity. While the research efforts reviewed here have centered on the practicality of FTA-filter PCR in the analysis of food-borne pathogens, clinical applications are equally promising. The evaluation of disease frequencies, outbreak epidemiology, and rapid phylogenetic analysis may be enhanced.

As technology in the pathogen detection arena advances, one can envision portable, field-hardened instruments for real-time PCR. When used in conjunction with FTA applications and protocols, on-site analysis of foods, such as fresh produce (on the farm or at the docks and food-processing sites), may be more feasible and timely. The potential applications include evaluating imported foods for contamination with human microbial pathogens, surveys of selected foods (e.g., produce), Hazard Analysis and Critical Control Point programs, epidemiological stud-

ies, and preventing the spread of disease by identifying and removing the source. These envisioned real-time protocols can be equally applied to bioterrorism threats.

Although the basis for such applications has now been established by incorporating FTA filter technology into detection methodologies, much remains to be done for putting into concert the application of template preparation with downstream portable and deployable instrumentation.

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