Biotechnological Diagnostics for the Detection of Microbial Contamination of Food

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Food microbiology plays a critical role in providing consumers with a safe food supply. Foodborne illnesses are estimated to affect as many as 81 million people per year in the United States and to cost the American economy 40 billion dollars per year (Miller, 1990). The challenges of detecting pathogens in food matrices are substantial. A single organism in a 25 gram sample of food has the potential of growing to levels that can cause human illness. Recovery of these organisms in the laboratory is complicated by the fact that they may have suffered sublethal injury from heat, cold, drying or preservatives used in food processing. Since food is rarely a sterile medium, competition from other microorganisms can complicate isolation of pathogens as well. Because of the requirements on food quality control laboratories to provide accurate results for safe product release, assays for food pathogens must be rapid and should involve minimal training. Classical microbiology relies on the growth of pathogens in broths and on agars for presumptive identification. Other techniques are then applied to isolated colonies to determine the exact identity of suspect organisms. Although such techniques are the "Gold Standard" of food microbiology, they suffer from a number of limitations. Because food often contains nonpathogenic microorganisms that are closely related to important pathogens, appearance and biochemical reactions of these nonpathogens can mimic those of their more dangerous relatives. Highly trained personnel are thus needed to make these critical distinctions. Classical procedures are also very time consuming. It can take five days to several weeks to determine if food is free of certain pathogens. (Doyle *etal.*, 1988)

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Analytical procedures derived from biotechnology research have had a substantial impact on human health care in the past ten years. Assays based on monoclonal antibodies are commercially available for a wide range of drugs and hormones. Application of such assays to food microbiology has been slower to evolve for a number of reasons.

As outlined above, the problems of analysis of pathogens in food is quite complicated. An additional complication is the level of sensitivity required. Direct detection of a single organism in 25 grams of food is beyond the capabilities of even the best present assays and is likely to remain so for a number of reasons. All current biotechnology assays, therefore, require the cultural enrichment of pathogens to certain levels before they are detected. Detectable concentrations for both antibody based assays and DNA probe assays is about a million organisms per milliliter of enrichment broth. Most current procedures take two or more days to achieve this level, but efforts are underway to abbreviate this period without sacrificing assay sensitivity. This sensitivity level of a million organisms per milliliter is not a trivial task.

Table 1 Analyte Concentration

Target	Concentration (moles/L)
Glucose	10^{-3}
Theophylline	10^{-5}
hTSH	10^{-11}
1,000,000 E. coli/ml (rRNA)	10^{-12}
100 Hepatitis A/ml	10^{-}"

Table 1 shows a list of clinically relevant analytes typically determined by immunoassay and the target levels provided in a broth containing a million organisms per milliliter. Assays for human thyroid stimulating hormone (hTSH) are the most sensitive assays done in the clinical immunoassay laboratory today. Antibody assays targeting antigens with copy numbers of 10,000 per cell and DNA probe assays that target ribosomal RNA at the same copy number per cell must therefore be an order of magnitude more sensitive that these hTSH assays. Assays targeting viral agents where no ribosomal RNA is available must be even more sensitive.

Biotechnology assays face several design hurdles that must be overcome. Increasing target levels to detectable levels has already been discussed in the context of cultural enrichments. For probe assays, there is also a tantalizing opportunity for *in vitro* amplification. Because nucleic acids have evolved to be copied in order to transmit their information to the next generation or to the next process in a cell, very efficient enzyme systems exist in nature that can very rapidly produce millions of exact duplicate copies of an original nucleic acid sequence. These enzyme systems have been harnessed to provide nucleic acid amplification schemes to enhance the sensitivity of probe assays. The best known of these is a procedure known as the polymerase chain reaction (PCR) (Saiki *et al.*, 1985). It can amplify a target sequence a million fold in several hours. More recent discoveries have lead to a system based on an enzyme called Q-beta replicase that can amplify probe signals by a billion fold in as little as 15 minutes (Lomelli *et al.*, 1989).

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Application of the amplification technologies to food microbiology will, however, be limited by several problems that are related to the very sensitivity that makes them so attractive. These techniques are so sensitive that it is likely that they will detect dead microorganisms in food that are of little significance. The other issue that will need to be dealt with is the potential for cross contamination (Kwok and Higuchi, 1989). These detection systems are so sensitive that even the slightest cross contamination of a negative sample with materials from a positive sample can lead to a false positive result. It is most likely that nucleic acid amplification will be used to shorten enrichment times rather than to replace cultural enrichments altogether. The cross contamination issue will probably be minimized with automation.

Sample preparation is the next hurdle in the design of biotechnology assays. Bacterial antigens must be released from cell walls or internal structures so that they can bind to the detecting antibodies. Heating aliquots of the terminal enrichment culture in a boiling water bath is a common approach (D'Aoust and Sewell, 1988). Nucleic acids targets can be freed from the intracellular matrix of bacterial cells by treatment with strong base or by enzymatic processing, thus eliminating the need for a boiling water bath.

All of these processes also kill pathogens, thus providing a level of biosafety for the operator. Inherent in all high-sensitivity biotechnology as-

says are a separation step in which label bound to some solid phase as a result of the presence of target is separated from unbound label. Solid phase supports are usually made of plastic and facilitate the wash steps that enhance signal-to-noise ratios. Most immunoassays use a microtiter plate as a solid phase. Ninety-six wells that can hold approximately 0.3 milliliters are arranged in an 8 by 12 array. The wells are coated with a capture antibody by the manufacturer. The assay is run by adding the sample to a well and then adding the antibody-enzyme conjugate. If the desired antigen is present, it will be bound by the antibody on the well and the antibody-enzyme conjugate in solution will in turn bind to the antigen. This results in an antibody-antigen-antibody "sandwich" that forms only when antigen is present. Unbound antibody-enzyme conjugate is washed away and enzyme is detected as described below. A schematic representation of this format appears as Figure 1.

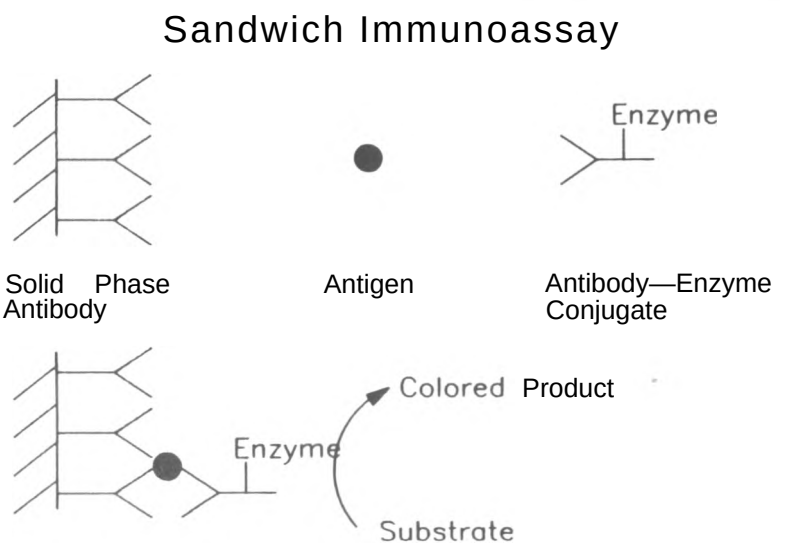

Figure 1 Antigen is released from cultured organisms by boiling. Solid phase antibody and solution phase enzyme labeled antibody react with antigen, if present. After a wash step, the amount of enzyme present is proportional to the amount of antigen present initially. Enzyme is detected by reaction with substrate and chromogen to produce color.

The DNA probe assays that are produced by GENE-TRAK Systems are based on another type of "sandwich". Target nucleic acid is allowed to react with two different probes in a test tube. These synthetic probes are exact matches for areas of the target that are fairly close together. One probe (reporter probe) is labeled with fluorescein and the other (capture probe) has a homopolymer tail of polydeoxyadenylic acid (dA). The target and these two probes form a probe-target complex. A plastic dipstick coated with the matching homopolymer polydeoxythymidylic acid (dT) is placed in the test tube. Any probe or probe-target complex that contains a poly dA tail is captured on the dipstick. A subsequent washing step removes any unbound material including fluorescein-labeled reporter probe not bound in a probe-target complex. The dipstick is now incubated with an antibody-enzyme conjugate which binds to any fluorescein residues present. Since the fluorescein-labeled reporter probe can only be present at this point as part of a probe-target complex, the amount of antibody-enzyme conjugate bound is proportional to the amount of target initially present. A second wash step removes unbound conjugate. Exposure of the dipstick bound enzyme to an appropriate substrate chromogen mixture produces a blue color in direct proportion to the amount of enzyme present on the dipstick. Removal of the dipstick and addition of dilute sulfuric acid stops the enzymatic reaction and intensifies the color, completing the assay. The results are read in a differential photometer at 450 nm. The entire reaction scheme appears as Figure 2.

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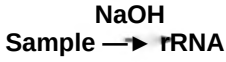
To ensure that the assay has been carried out correctly, two controls are run with each assay, a positive control and a negative control. Both controls must meet certain criteria for the assay to be considered valid. Samples that read 0.1 O.D. units above the negative control are considered presumptively positive for the organism in question.

DNA probe assays target the most fundamental level of information in a cell (Parsons, 1988). The practical significance of this fact is that they produce a better quality result in a shorter time. Table 2 illustrates the time savings possible with the use of our current generation *Salmonella* assay. Additional improvements in time frame to result are being actively researched. In addition, the quality of the result is also significantly better. One of the more dramatic examples of how much better these results can be was recently provided by some results of our quality control depart-

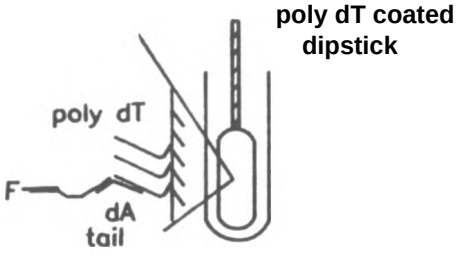
DNA Probe Assay

1. **Sample Lysis**

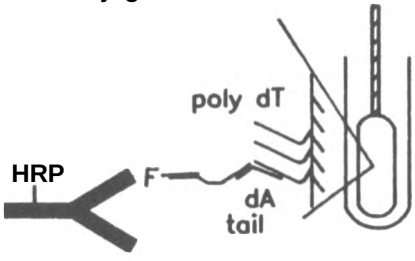


2. **Solution hybridization**

3. **Capture**



4. **Wash**
5. **Addition of HRP—conjugate**



6. **Wash**
7. **Add chromogen/substrate**
8. **Incubate and add stop reagent**
9. **Read color at 450 nm**

Figure 2 After organism lysis to expose the intracellular nucleic acid, the target nucleic acid is reacted with two probes to form a probe-target complex. The dA tail on the capture probe allows capture of this complex on a dT coated dipstick. Detector probe in the complex is detected with an antibody-enzyme conjugate. After a wash step, enzyme present is proportional to the amount of target nucleic acid initially present. Enzyme is detected by reaction with substrate and chromogen to produce color.

ment. We participate in a Check Sample program provided by the American Association of Cereal Chemists (AACC) (Sail *et al.*, 1988). Every eight weeks, this organization provides the subscribers of this Check Sample program with two unknown samples of flour or flour-based bakery mixtures. These samples may contain *Salmonella, Staphylococcus aureus, Escherichia coli* and / or other organisms of interest to food microbiologists. Alternatively, they may also be free of such organisms. Each subscriber laboratory tests these samples to the best of their ability and returns their results to the AACC under their own individual code number. The AACC then tabulates the results and reports the results by code number to assure anonymity. Only AACC and the respondent know their own code number. One can, however, compare one's own results against the results obtained

Table 2 Salmonella Microbiology

Steps	Conventional	Probe
Primary enrichment	18-24 hours	18-24 hours
Selective enrichment	6 hours	6 hours
Final enrichment	18 hours	18 hours
Plating	18-24 hours	—
Biochemical ID	5-24 hours	—
Serology	4 hours	—
Assay	2.5 hours	
Total Time	72 hours 95+ hours for a positive)	48-52 hours

by all of the other respondents. Since our colorimetric Salmonella assay was available in internal pilot lot form, we have been running these Check Samples in our Salmonella test. Our results represent perfect agreement with the stated AACC results for the entire duration of the present study with one exception. Despite exhaustive efforts, we were unable to find any viable Salmonella in the samples received in January of 1989. Twenty-two percent of the other respondents also reported an apparent false negative. We believe that there may have been a sampling problem with that particular Check Sample series or that there may have been some die off of the inoculated organisms.

What is striking about this study is the occasional spikes of high rates of false negatives experienced by other participants in this program. False negative rates have run as high as 38 percent for one recent sample. Al-

though we do not know the identity of any of the other participants in this study, their participation in such a check sample program speaks eloquently to their commitment to very high quality food microbiology. We believe that these results speak to the limitations of conventional food microbiology which is still used by a vast majority of the industry at this point. An assay such as ours removes the subjectivity and extensive work with various media to avoid missing biochemically atypical strains. These results are reported in greater detail at the Institute of Food Technologists (IFT) meeting in Anaheim, CA (McKenzie *et al.*, 1990).

The future of food microbiology has been brightened by the emergence of biotechnology diagnostic assays. Time to results have been shortened and the quality and universality of results has been improved by the current generation of tests. Yet there remain additional challenges. Tests for additional pathogens and spoilage organisms are in development and will make their appearance on the market in the next few years. Enrichment periods must become shorter to give even faster turnaround times. Minimal enrichments of 4-12 hours will probably always be necessary to avoid detection of dead organisms.

In vitro amplification methods will extend the range of present techniques so that viral agents such as Hepatitis A and Norwalk agent would be detectable in amounts sufficient to cause human disease. Agents causing diseases such as scrapie and bovine spongiform encephalopathy have the potential for causing serious economic loss (Holt and Philips, 1988). Amplification technology will provide the tools necessary for rapid, reliable detection of these agents as well.

Automation of these tests will also become important in the next decade. As more biotechnology pathogen tests become available, the volume of testing will mandate the implementation of cost effective automation to streamline and standardize testing in food microbiology laboratories.

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