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Methods For The Detection Of Microorganisms In The Environment

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METHODS FOR THE DETECTION OF MICROORGANISMS IN THE ENVIRONMENT

Developed by

The Commission of the European Communities

The US Department of Agriculture
Animal and Plant Health Inspection Service

The US Environmental Protection Agency
Office of Pesticides and Toxic Substances

as part of the EC/US Cooperation
in the Permanent Technical Working Group
on Biotechnology and the Environment

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METHODS FOR THE DETECTION OF MICROORGANISMS IN THE ENVIRONMENT

1. PREFACE 3

2. INTRODUCTION 4

3. OVERVIEW 6
   Introduction 6
   Direct Detection Techniques 6
   Detection by Culturing of the Microorganism 8
   Detection by Nucleic Acid Hybridization 10
     General Considerations 10
     Nucleic Acid Hybridization Techniques 13

4. USE OF STATISTICS IN THE DETECTION OF MICROORGANISMS 16

5. ANALYSIS OF TECHNIQUES 18
   Direct Detection Techniques 18
     Microscopy - detection of viable microorganisms 18
     Microscopy - detection of total viable and nonviable microorganisms. 20
     Microscopy - fluorescent antibody 22
     Summary of direct detection techniques 23

   Detection Techniques that Require Culturing of the Microorganism 24
     Selective media 24
     Differential media 30
     Most probable number technique 31
     Fluorescent antibody - colony ELISA blots 33
     Summary of cell culture techniques 35

   Techniques to Label and Detect Nucleic Acids 39
     Nucleic acid probe construction 39
     Hybridization techniques 47
     Probe visualization 67
     Use of nucleic acid hybridization in analyzing microbial species composition 68

6. REFERENCES 70

7. TABLES 94

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1. PREFACE

This document has been jointly developed by the Commission of the European Communities, the Office of Pesticides and Toxic Substances of the US Environmental Protection Agency, and the Animal and Plant Health Inspection Service of the US Department of Agriculture, as part of the EC/US Cooperation in the Permanent Technical Working Group on Biotechnology and the Environment (TWGBE). The TWGBE was established in the framework of the EC/US Bilateral Environmental Consultations by agreement in a plenary session of the EC/US Bilateral Environmental Consultations in Brussels in 1990. The TWGBE was established with the aim of promoting closer bilateral cooperation on technical issues associated with evaluating and validating data to assess the environmental risk of releases of genetically modified organisms.

The document presents an overview and summary of methods currently in use for detecting and monitoring microorganisms in the environment. It is intended to be used as a reference document for regulatory officials and scientists. Given the rapid developments in this area the guide may have to be updated periodically, however an attempt was made to provide information that was current at the time of publication.

2. INTRODUCTION

The choice of a detection technique(s) depends greatly on the the purpose of the intended use of the microorganism, whether there is a need for quantitative versus qualitative data, and whether the microorganism itself or its genetic material is to be detected. Thus, the purpose of a particular field test may include assessing the efficacy of a microbial product under commercial development, assessing safety to human health and the environment, or to gain information as part of basic research in microbial ecology. In addition, the monitoring techniques described in this document have been used for developing both qualitative and quantitative data, as appropriate for the purposes of the study. Finally, studies on microbial survival, dissemination, and beneficial or adverse impacts in the environment have relied classically on the detection of specific microorganisms, but more recently methods that allow the direct detection of nucleic acid have been developed. These include studies on the transfer of genetic material to other microorganisms in the environment usually involve some method detection of specific gene sequences. This document attempts to provide a source of information and references about the expanding variety of detection techniques and their uses.

The document is divided into two principal sections and a brief synopsis of statistical considerations in environmental microbiology. The principal sections are a general overview and an analysis of individual techniques. The Overview section is intended to provide readers with a general interest in the topic of microbial detection methods with a survey and discussion of the variety of techniques used in the study of microorganisms in the environment.

The Analysis of Techniques section provides a more detailed examination of individual detection methods. The discussion proceeds from an analysis of direct detection techniques to culture based identification and finally to identification of specific nucleic acid sequences.

The individual analyses are designed to provide information in five general categories: (1) specificity, (2) sensitivity, (3) ease of use and time necessary to execute the technique, (4) cost and (5) constraints on use in different environmental media. The five evaluation criteria may be defined as follows:

1. Specificity: The ability of the technique to distinguish between the target microbe and other microbes. Specificity could also include whether the technique distinguishes viable and non-viable organisms.

2. Sensitivity: The minimum detection limit, e.g., (1) the number of cells needed for detection or (2) mass units of nucleic acid target sequence required for detection.

3. Ease of use & time necessary: Self-explanatory, techniques are compared for their relative ease of use.

4. Cost: Self-explanatory

5. Effect of environmental media: The degree to which a given technique is able to detect a microorganism when used in different environmental media, e.g., soil, water, plant tissue, air, or sediment.

3. OVERVIEW

Introduction

The variety of techniques available to researchers in microbial ecology has expanded greatly in recent years. In addition to some of the classic techniques such as direct detection techniques and culture-based identification, the researcher now has molecular techniques based on the identification of specific nucleic acid sequences. The expansion in the number of techniques is due in part to an increased interest in the commercial use of microorganisms in the environment for applications such as biopesticides, symbiotic nitrogen fixation, bioremediation, animal vaccines, feed additives for livestock and other uses. A further impetus has come from research in both basic and applied microbial ecology. As a result, the relatively recent use of molecular techniques in the detection of microorganisms in the environment offers the potential for increased sensitivity. However, the more traditional methods such as dilution plating are still the most commonly used and provide the simplest (and in some cases still the most sensitive) means of enumerating a microorganism in the environment, provided it is in a culturable state.

These advances in methodology have increased our understanding of the manner in which microorganisms interact with their physical/chemical environment, and with other organisms, and the factors that affect their environmental fate and impacts (either beneficial or adverse). A concise summary of the variety of techniques available for use in environmental microbiology is given below.

Direct Detection Techniques

The section on direct detection techniques covers the following: microscopy, flow cytometry and antibody capture. Direct detection allows the enumeration of microorganisms without culturing the sample. These methods do not discriminate per se between viable and dead cells. However, direct viable counts (DVC) may be determined by incubating the sample with nalidixic acid prior to counting. Such techniques yield consistently higher counts than those obtained with classical methods (plate counts, most probable number, etc.) which can be hampered by the problem of viable but nonculturable microorganisms or inefficient recovery of the desired microorganism.

With microscopy, the sample is visualized by staining with fluorescent dyes such as 4'6-diamidino-2-phenylindole (DAPI) or acridine orange. Cells can then be detected using epifluorescence microscopy. Microscopic techniques can also be

combined with hybridization techniques using oligonucleotide probes tagged with dyes such as fluorescein isothiocyanate (FITC) and tetrazolium isothiocyanate (TITC) (Delong et al., 1989, Amann et al., 1990a). For example, 16S/23S oligonucleotide probes (15 to 25 bases in length for in situ use) can be used to differentiate organisms at the species level. Antibodies raised against strains of microorganisms may also be used to determine direct counts for a specific subpopulation. It is also possible to detect microorganisms in the environment by means of antibodies using the enzyme-linked immunosorbent assay (ELISA) (Morgan et al., 1989). To eliminate background signals from indigenous bacteria, the antibody/oligonucleotide probe must be specific for the target organism. Environmental samples may exhibit nonspecific binding of antibodies and oligonucleotide probes due to the presence of phenolic compounds and organic matter particularly in soils, sediments, and plant tissue. Such interference can be reduced by counterstaining with gelatin-rhodamine conjugants. For these reasons, detection by microscopy has been more frequently and successfully used for microorganisms in aquatic environments.

Oligonucleotide probes (Amann et al., 1990b) or antibodies (Saunders et al., 1990) can be used for enumeration of the microorganisms with flow cytometry/fluorescence-activated cell sorting which allows rapid identification, sorting, and counting of specific microorganisms. This technique has not been used extensively in environmental microbiology; however, it has promise for future use. Flow cytometry can be directly employed with aquatic samples. Use with soil samples would require extensive treatment to remove particulates that would interfere with accurate enumeration of microorganisms.

Techniques using antibody capture require raising a monoclonal antibody to the microorganism of interest. These antibodies can then be secured to a magnetized polystyrene bead and introduced into the environmental sample. Any organisms which express the appropriate antigen will then bind to the beads, and be retrieved with a magnet (Saunders et al., 1990). Problems associated with this method include nonspecific binding of the antibody and (potentially) low recovery of target organism, particularly in environments with organic matter such as soil. These methods should work well with aquatic samples, and find greater use in the future.

Detection by Culturing of the Microorganism

Detection techniques that require culturing microorganisms rely on the ability of the target organism to grow on or in a suitable growth medium. Not all microorganisms isolated from the environment will be culturable and thus detectable by these methods. In addition, the accuracy of these methods (especially dilution plate counts) may be affected by cell clumping and inaccuracies in preparing dilutions of the sample. Since these methods require the growth of target microorganisms, they are susceptible to microbial contaminants that interfere with the detection of the desired organism, especially in environments such as soil which contain relatively high numbers of indigenous microorganisms. Sensitivity can be improved by the use of biomass concentration techniques (Herron and Wellington, 1990). These methods have been used in conjunction with direct counting for the detection of nonculturable cells.

Methods for detecting specific phenotypes within a complex community of heterogenous bacteria, such as soil, are summarized in Tables 1 and 2. Such methods were first developed in sterile soil (Table 1) and many have been used for the detection of survival and gene transfer events in nonsterile soil (Table 2). These tables serve to illustrate the continued predominance of the dilution plate count technique for detection, monitoring, and enumeration of bacteria in natural environments. Many studies on the use of dilution plate count techniques have been published in the literature on gene transfer in bacteria, and Table 3 provides a summary of these references.

Culture techniques that use selective media rely on the ability of the selective agent to either kill or prevent the growth of competitive microorganisms, usually on solid growth media, thus providing relatively good sensitivity in detection. Commonly, the gene that encodes the trait(s) that enable the bacterium to resist the toxic agent is introduced through genetic engineering. Selective agents used in media, and discussed below, include antibiotics, heavy metals, and unique carbon sources.

(1) Resistance to Antibiotics

Markers conferring on antibiotic resistance can either be transferred into a host from another organism or obtained by selection. One concern is the transfer of antibiotic resistance determinants *in situ* to pathogenic microorganisms or the indigenous microbial population. Efforts have been made to avoid the use of resistance markers for clinically significant antibiotics.

(2) Heavy Metal Markers (Bale et al., 1987, Höfte et al., 1990 citation for Warwick meeting, November, 1990)

The use of heavy metal markers is basically similar to that of antibiotic resistance, although potential risks concerning transfer to pathogens are not as great. However, resistance to heavy metals and antibiotics can often reside on the same plasmid. Theoretically heavy metal resistances could be used to detect microorganisms in polluted environments provided that the natural resistance of the indigenous population does not result in high background levels.

(3) Rare Substrates

The ability to grow on rare substrates such as pollutants can be found in some microorganisms, and can be used both to study the degradative capacity of the host organism in the environment and to prevent the growth of indigenous microflora (counterselection) in selective media (Dwyer et al., 1988).

(4) Unique Environmental Conditions

Harsh environmental conditions, such as high temperature, extreme pH, or high osmotic pressure, can be used to select for microorganism that are adapted to these conditions. In addition the ability to survive environmental extremes can be selected in the laboratory.

Differential media exploit the unique metabolic capacities of individual species of microorganisms. Recently, genetic markers have been introduced into microorganisms in order to provide a differential phenotype. A marker(s) is chosen for its ability to confer on the host organism a capacity to stand out from the indigenous population on isolation plates. Some examples of markers used are the lacZY genes from Escherichia coli, lux cassette from Vibrio fischeri, and xylE from Pseudomonas putida. The lacZY marker from Escherichia coli confers the ability to metabolize of the chromogenic galactose derivative, X-gal. The lux cassette encodes the enzyme, luciferase. When isolation plates are sprayed with an aldehyde substrate, light is emitted from colonies carrying this marker (Shaw and Kado, 1986). The PROSAMO initiative (Planned Release of Selected and Manipulated Organisms, Rees et al., 1990) is using the luxAB genes for monitoring and detection in soil. The xylE gene codes for catechol 2,3-dioxygenase and is derived from the Pseudomonad TOL plasmid. Colonies carrying this marker produce yellow pigment on isolation plates sprayed with catechol (Morgan et al., 1989).

False positives can be reduced and sensitivity optimized by using multiple types of markers e.g., antibiotic resistance markers, metabolic markers such as XylE, and nonexpressed markers such as

nucleotide sequences which are targets for nucleic acid probe hybridization.

Detection by Nucleic Acid Hybridization

1) General Considerations

Classical identification procedures depend upon the detection of metabolic products, enzymatic activities, specific cell constituents or antigenic structures. Nucleic acid hybridization on the other hand is based on the detection of specific gene sequences. Hybridization is an important tool for distinguishing between closely related bacteria. Once the gene coding for the differentiating trait has been isolated, the gene can be detected by hybridization with specific DNA probes. With careful probe selection, virulent strains can often be distinguished from avirulent ones and genetically marked strains can be distinguished from nearly isogenic strains.

The sensitivity and specificity in a given hybridization reaction depend on probe construction and labeling, the target gene sequence, and detection methodology. These methods can be used to either qualitatively or quantitatively study microbial populations in the environment. However, the difficulty in extracting sufficiently pure nucleic acids from an environmental sample for use in hybidization studies is often a problem. Extraction of hybridizable DNA from the environment can be carried out by two general procedures, either by direct extraction of soil (Ogram et al., 1988; Steffan et al., 1988) or aquatic (Somerville et al., 1989) samples or by using an indirect method in which the bacteria are first separated from non-cellular material and then the nucleic acids are extracted (Holben et al., 1988).

Examples of direct DNA extraction are provided in Table 4. Direct extraction does not allow distinction between cellular and free DNA or between bacterial, fungal, or other classes of organisms. The indirect method may be also prone to inaccuracies since only approximately 30% of soil bacteria are recovered (Holben et al., 1988) and thus, the extract may not represent a truly statistical sample of the overall community, although the recovery efficiency of this technique has been improved.

With both methods, it is necessary to confirm complete lysis of the microbial population. The most important considerations governing nucleic acid yield and purity are lysis of bacterial cells, the removal of nucleases and hybridization inhibitors, and gently handling of DNA. Most of the rapid lysis and purification techniques are derived from Marmur (1961), a method originally developed for pure cultures of laboratory strains. The method may not always be applicable to microorganisms isolated from the environment.

Gram-negative bacteria are readily lysed by treatment with detergent (sodium dodecylsulfate or sarcosyl), high pH, cell wall lytic enzymes, or a combination of these reagents (Maniatis, 1982). Proteins and other cell components are removed by proteinase digestion and repeated phenol and chloroform extractions. A variety of commercial kits often containing chromatography columns are available for nucleic acid purification.

The procedures outlined for the extraction of nucleic acid from Gram-negative bacteria can be applied only for certain Gram-positive bacteria. In general, the lysis of Gram-positive is more difficult and may require treatment with cell wall lytic enzymes (lysozyme, lysostaphin, mutanolysin) often combined with proteinase treatment and detergent application (Klinger et al., 1988). Pretreatment of cells with organic solvents (acetone) facilitates subsequent lysis of some Gram-positive bacteria (Heath et al., 1986). DNA and RNA can often be released from Gram-positive cells by hot phenol treatment of cell suspensions. If sufficient lysis cannot be achieved with these techniques, physical methods such as sonnication (Sharrock and Rabinowitz, 1979) or shaking with glass beads in a cell mill (Stahl et al., 1988) may be required.

Additional problems can be encountered in the preparation of nucleic acid for colony hybridization. The DNA has to be released from cells grown or concentrated on solid supports (nylon or nitrocellulose membranes), and fixed in situ. Gram-negative cells usually can be lysed on filters by simple alkali treatment (0.5 M NaOH) of the cells (Grunstein and Hogness, 1975). Pretreatment of cells with 10% sodium dodecylsulfate at room temperature has been effective for the in situ lysis of some Gram-positive bacteria (Betzl et. al., 1990). An alternative technique is to incubate filter grown colonies with detergent in a microwave oven (Buluwela et al., 1989).

There are different approaches to the preparation of the second hybridization component, the nucleic acid probe. Probes can be derived from randomly cloned DNA fragments or DNA or RNA fragments of known sequence and function. For the detection of a microorganism in the environment, specific probes must be used to differentiate it from a background in which closely related strains are found. Probes which identify organisms on the species level, for example whole-cell DNA (Table 5) or ribosomal RNA (Table 6) probes, are generally not useful for the detection of a specific microorganism introduced to the environment. Examples of DNA probes from randomly cloned DNA fragments and from specific genes can be found in Tables 7 and 8, respectively.

Probes are frequently derived from the plasmid used to construct the target microorganism. Either the whole plasmid can be used

or a defined gene fragment can be isolated by restriction digestion and gel purification. A second strategy is to use chemically synthesized oligonucleotides. One advantage of oligonucleotide probes is that large quantities of consistent quality can be quickly prepared. A second is the high specificity they exhibit, since single nucleotide mismatches may be sufficient to prevent hybridization of a short oligonucleotide to a potential target (Wallace et al., 1981; Ikuta et al., 1987). Specificity is especially important samples derived from the natural environment. However, the sensitivity of oligonucleotide probes is lower than polynucleotides because they are shorter and thus have fewer labeling sites. A third approach is to use single-stranded RNA probes. These offer the advantage of high specific activity and no self-annealing because they are single stranded. Examples of these approaches with environmental samples can be found in Tables 10 and 11.

Before hybridization, the nucleic acid probe has to be labeled to enable detection of the resulting hybrid. There are two principal types of labeling in use: direct and indirect labeling. With direct labeling, a label is covalently bound to the probe. With indirect labeling, an reporter group is attached to the probe and is subsequently detected by a labeled binding protein. The label can either be measured directly (e.g. fluorescent label) or indirectly by the reaction product formed by an enzyme conjugated to the binding protein.

Labels can be radioactive or non-radioactive. Originally, radioactive isotopes were used as markers and are still preferred in research studies where high sensitivity and low non-specific background are required. Safety considerations, the lack of stability, and waste disposal problems associated with radioactive labels have spurred continuing efforts to develop and improve non-radioactive alternatives (see Table 9 for examples).

The sensitivity of non-radioactive labels is now approaching that of radioactive methods and in certain applications (e.g. in situ hybridizations) is already superior. Radioactive as well as fluorescent-labeled nucleotide analogues can be introduced into nucleic acid probes directly by using polymerases. Some methods for the incorporation of nonradioactive labels are nick translation (Rigby et al., 1977), specific or random primed synthesis of complementary strands (Feinberg et al., 1983), in vitro transcription for generating labeled RNA (Melton et al., 1984) and polymerase chain reaction (Saiki et al., 1988). These techniques are used to label polynucleotides.

These labeling techniques can be applied to either single stranded RNA or DNA. Single stranded probes can only hybridize to the target and cannot self-anneal which makes them more sensitive than double stranded probes. However, their preparation requires cloning of the fragment of interest proximal

to a phage promotor, which is time-consuming. On the other hand, double stranded probes also have disadvantages. These probes have to be denatured prior to labeling and competition between probe and target DNA for their respective complementary strands during hybridization decreases sensitivity.

Oligonucleotide probes are commonly end-labeled at: i) 5' terminal by using T4 kinase (Maxam and Gilbert, 1980), or ii) 3' terminal by using terminal transferase (Radcliff, 1981). Direct labeling of oligonucleotides can also be achieved by attaching primary amino-groups to the probe during the synthesis. These linkers can be labeled with activated fluorescent dyes or enzymes (Jablonski et al., 1986; Smith et al., 1985; Inoue et al., 1985).

A variety of reporter groups are available for use in conjunction with nonradioactive labelling (indirect labeling). Table 9 lists those that are available commercially. One of the most commonly used reporter groups is biotin. A second reporter group that has been gaining increased use is digoxigenin - a steroid from Digitalis purpurea. These reporter groups have been reviewed by Matthews and Kricka (1988). Indirect labels often allow signal amplification and may be more versatile than direct labels. However, some reporter groups such as biotin exhibit nonspecific binding to protein contaminants resulting in a decrease in specificity. Thus, these techniques are more difficult to apply to complex environmental samples.

2) Nucleic Acid Hybridization Techniques

Hybridization involves two major steps: 1) binding of the probe to the target (hybridization); and, 2) separation of specific probe-target hybrids from unbound probe (washing). The specificity can be controlled by the stringency of the conditions applied (temperature, salt concentration).

The most commonly used techniques involve a solid phase support matrix. The denatured target nucleic acids are immobilized on a solid support, such as nitrocellulose or nylon membranes. The most frequently used solid support, nitrocellulose, has the advantage of relatively low levels of nonspecific hybridization (Sambrook et al, 1989). Its two major limitations are: 1) it is extremely brittle when dry and 2) it binds small fragments (less than 200-300 nucleotides in length) poorly (Wahl et al., 1987). The major advantages of nylon are its structural stability (Wahl et al., 1987) and superior retention properties (Jagus, 1987). Once target DNA or RNA is applied, therefore, these filters can undergo multiple hybridizations with different probes. With nitrocellulose, the nucleic acid is gradually dissociated from the filter and thus the blot can only be reused a limited number of times.

The following three methods employing solid phase supports are distinguished by the way the target nucleic acid is applied to the solid support:

(1) Spot/Dot/Slot Blot (Kafatos, 1979)

DNA, isolated directly from environmental samples (Sayler and Barkley, 1987) or indirectly from pure culture isolates or enrichments from the environment (Ezaki et al., 1989), is applied to a defined membrane area with a slot/dot blot apparatus. Quantitation is achieved through a densitometer or liquid scintillation counting. Because cultivation is not required, culturable and non-culturable organisms may be enumerated in environmental samples.

(2) Colony and plaque hybridization

The cells are grown directly on hybridization membranes or cell material is transferred from a master plate onto the filters; in situ lysis and DNA binding is followed by hybridization. The method allows the rapid screening of multiple colonies that are culturable.

(3) Southern and Northern hybridization

Nucleic acid fragments, either DNA (Southern, 1977) or RNA (Northern) can be transferred to a membrane from a electrophoretic gel. In the original method, the gel is placed on a membrane in buffer and the fragments are deposited on the membrane by passive diffusion. Modifications to this method accelerate transfer by applying vacuum (Peferoen et al., 1982) or an electric current.

Since the original protocol was developed by Grunstein and Hogness (1975), colony hybridization has been the most widely used hybridization protocol for environmental studies (reviewed by Sayler & Layton, 1990), particularly those that require quantitation of specific microorganisms. Though more labor intensive than colony hybridizations, more detail can be obtained with Southern and Northern analyses. For instance, Southern blots can be used to analyze restriction fragment length polymorphisms (RFLPs). This method allows the identification of single strains; for example, parental strains and genetically modified strains can be differentiated in soil samples (Holben et al., 1988). Also the stability and fate of specific nucleotide sequences in microorganisms, i.e., rearrangements, deletions and gene transfer, can be detected.

The alternative to hybridization on a fixed matrix is to allow the reaction to proceed in solution and then to capture the probe/target duplex for quantitation. Because the target is not fixed and completely accessible to the probe, hybridization in solution has the advantage of a higher rate of hybridization than

membrane techniques. Disadvantages associated with solution hybridization include: 1) possible self-reassociation of target DNA; 2) difficulties in separation of probe-target hybrids from unbound probe; and, 3) added logistical difficulties with handling multiple samples simultaneously.

Solution reassociation hybridizations have recently been used to estimate the genetic diversity in soil ecosystems (Torsvik et al., 1990). Several companies offer nucleic acid probes for the rapid identification of specific pathogens or environmentally important microorganisms. These are mostly based on rRNA-targeted oligonucleotide probes. However, commercially available probes have not been widely employed for environmental studies.

There are several considerations associated with the choice of hybridization technique when used for environmental samples. The Analysis of Techniques section of this document covers a variety of hybridization techniques in detail. A summary of nucleic acid hybridization techniques used in environmental studies can be found in Tables 10 and 11. The majority of applications cited in the literature report the use of dot/slot blots and colony hybridizations. The polymerase chain reaction is relatively new and there are fewer studies in which it has been used. These studies are listed in Table 12. Some permutations of the these techniques such as multiplex amplification using the polymerase chain reaction have yet to be applied, but are included in the Analysis of Techniques to demonstrate possible applications.

4. USE OF STATISTICS IN THE DETECTION OF MICROORGANISMS

Much of the research on the fate of microorganisms in the environment has relied on quantitative enumeration of microbial populations. One consideration when using quantitative analyses of data derived from any method of detection is the ability to make inferences about the microbial population of interest, which requires statistical tests. The use of most common statistical tests demands certain conditions be met by both the sample and the sampled population.

One of the first requirements is that the sample be taken at random and "representative" of the true population. Being representative is a difficult state to define. It is always possible that the chosen parameter to measure is invalid or skewed due to inappropriate sampling. However, any sampling must be replicated to be able to derive mathematically valid conclusions about either the samples themselves or the true population. Once replicate measures are taken it is possible to examine characteristics such as the mean and variability of the sample population and infer about the true population. Generation of statistics requires replication of samples and measurements.

Once a mean and variance are generated for a sample population it becomes possible to infer about the sample population and the true population. One of the most frequently abused assumptions of the most common statistical tests is that the population is normally distributed about the measured parameter. This assumption implies that the measured mean and variance are independent and as one increases it has no effect on the other. Often microbial populations show a linkage between the size of the mean and its variance and approach a Poisson rather than a normal distribution.

Another assumption that is frequently found invalid is that the true population is randomly distributed in the sampled media, e.g., soil, water, or plant tissue. This implies that recovering any individual in any one sample does not affect the likelihood of finding an individual in subsequent samples. This distribution results in measured means being much greater than their associated variance. Populations sampled in soil or other nonhomogenous media are often not randomly distributed but rather clustered. Clustered populations are characterized by having variances greater than their means and more easily fit a negative binomial rather than a normal distribution.

Many of the techniques discussed in this document seem very sensitive when the detection limits are examined. It must be remembered that many have not been utilized extensively in a variety of environments. For example, the distribution of the target population in media such as animal tissues may be expected

to be more homogenous or easily localized than those found in terrestrial or aquatic environments. In these cases the loss of the target population through sample processing may influence the results more than the sensitivity of the final detection method. Thus, the variability of the measured parameter will be more affected by the extraction or culturing step than the final detection method.

In the development of a detection method for quantitative analysis of microbial populations in a new environment, it is critical to repeat the method enough times with a given matrix to be able to generate an accurate representation of both the background and the minimum level of detection. This may be accomplished by adding known amounts of the target microorganism to a negative and known positive environmental sample to verify the extraction efficiency, minimum detection limit, and linearity of response. It is helpful to remember that a minimum detection limit is usually defined as the measured background mean plus 2 or 3 standard deviation units of that mean.

Several of the techniques mentioned such as in situ and filter hybridization involve the recognition of homology between complementary strands of DNA. The specificity of this reaction can be greatly affected by the temperature and osmotic conditions under which it is run as well as the % GC content of the components. This stringency can also be affected by contaminants present in the sample. As mentioned above it is useful if the system is replicated for each new media tested to determine the amount of interference and its affect on the detection limit.

In addition to stringency considerations, techniques such as restriction endonuclease digestions and polymerase chain reaction require purified nucleic acid preparations for proper enzyme activity. The purification process decreases the yield of nucleic acid due to sample preparation and may increase the variability of detection. The statistics related to data generated from enzymatically amplified DNA targets has not been well described for their detection limit. It may be helpful to generate the same sort of spiked samples for determining a background and minimum level of detection alluded to above.

4. ANALYSIS OF TECHNIQUES

DIRECT DETECTION TECHNIQUES

(a) Microscopy - Detection of viable microorganisms

DESCRIPTION: Direct count procedures consist of techniques in which microorganisms are enumerated, often without need for culturing or plating, generally through microscopic techniques. For large microorganisms (e.g., protozoa, algae), counting chambers are used, and special techniques exist for fungal counts (modified agar film techniques). This second direct count procedure combines direct microscopic examination with a stain or other technique that indicates whether the microorganism is metabolically active. One technique that indicates the number of actively respiring microorganisms is the AODC method combined with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (or INT). A second technique for use with bacteria involves using a fluorescent stain in combination with nalidixic acid. Nalidixic acid (an antibiotic that inhibits cell division through inhibition of DNA gyrase) will cause actively growing cells supplied with a carbon source to elongate, while dormant or dead microorganisms will retain their normal size and shape (Kogure, et. al., 1978). Novobiocin is an another DNA gyrase inhibitor. Third, autoradiography can be combined with direct microscopic observation: bacteria are incubated with a radiolabeled growth substrate, and actively metabolizing microorganisms can be differentiated as those which cause exposure of a photographic film coated over the cells. Fourth, there are techniques for fungal enumeration which involve the use of an agar film combined with fluorescence microscopy using fluorescein diacetate which only stains metabolically active mycelia. Techniques specific to certain species of microorganisms have been developed, with an emphasis on detection of aquatic microorganisms. Recently, however, a technique has been developed for detection of rhizobia in soil, using yeast extract and nalidixic acid (Bottomly and Magard, 1990).

SPECIFICITY: The identification of the specific isolates is determined by the skill of the microscopist, the unique morphological traits of the isolate, and the ability of the dye to discriminate between metabolically active and nonactive cells.

SENSITIVITY: A high background fluorescence resulting from the release of esterases which react with the fluorescein diacetate dye may make it difficult to distinguish metabolically active mycelia. Exposure to nalidixic acid may not elongate some cells if the septation mechanism involved in cell division is not linked to DNA replication.

EASE OF USE & TIME: Techniques that use nalidixic acid to detect viable rhizobia from soil samples require approximately 90 minutes to recover bacteria and initiate incubation in a substrate-antibiotic combination. On average, the incubation time in nalidixic acid is up to 24 hours for studies with soil rhizobia, and, by contrast, 6 to 8 hours for aquatic studies.

COST: Similar to the costs of the direct viable count technique described below except additional reagents are needed that are able to discriminate between those microorganisms which are metabolically active and those that are dormant or dead.

EFFECTS OF ENVIRONMENTAL MEDIA: See discussion on direct viable count techniques.

(b) Microscopy - Detection of total viable and nonviable microorganisms

DESCRIPTION: Direct count procedures consist of techniques in which microorganisms are enumerated, often without need for culturing or plating, generally through microscopic techniques. For large microorganisms (e.g., protozoa, algae), counting chambers are used, and special techniques exist for fungal counts (modified agar film techniques). Stains such as acridine orange (AODC method), 4'6-diamidino-2-phenylindole (DAPI), and fluorescein isothiocyanate (FITC) are used to assist in enumeration (and are detected by epifluorescence microscopy). Often, direct count techniques are used in conjunction with immunological techniques, such as fluorescent antibody methods, to detect viable, and viable but nonculturable microorganisms (Colwell et al., 1985 and 1988). Other instrumentation, such as electron microscope and particle counters (Coulter counters), has been used in place of light microscopes.

SPECIFICITY: The identification of specific isolates is determined by the skill of the light microscopist and the unique morphological characteristics of the isolate (fluorescent antibody techniques are extremely useful for identification of specific strains). Stains such as acridine orange stain bacteria and other organisms, but the intensities of the stain do not correlate well with specific isolates (or live or dead microorganisms). DAPI is preferable to acridine orange for counting small bacterial cells.

SENSITIVITY: Counts by direct epifluorescent microscopy are typically two orders of magnitude higher than plate count techniques would indicate. The AODC technique has resulted in counts of Vibrio cholerae that are 6 orders of magnitude higher than plate count techniques indicate (Colwell, et al., 1985). However, the epifluorescent microscopy technique is not a very sensitive one since it requires a high concentration of cells before a single cell can be seen in a microscopic field. The microorganisms which are detected by direct counts (and not be plate counts) may be viable but nonculturable due to insufficiencies in media, incubation time, etc. Direct counts are often directly proportional to the microbial biomass found in a variety of environmental media (soil, freshwater, and marine habitats).

EASE OF USE & TIME: Microscopic examination can be tedious and time-consuming, and is therefore impractical for a large number of samples. Conversion of microscopic counts to biomass approximations can also be tedious.

COST: This relatively low cost method does require both commonly available equipment such as microscopes, fluorescent stains, and possibly counting chambers and specialized equipment such for fluorescent .

EFFECTS OF ENVIRONMENTAL MEDIA: Direct counts allow enumeration of microorganisms in a variety of habitats (marine, freshwater, and soil) without the bias associated with plate count techniques (see "Sensitivity"). However, underestimation of microbial numbers may occur if high amounts of background debris exist in the sample (such as with soil or plant tissue).

(c) Microscopy - fluorescent antibody

DESCRIPTION: Fluorescent antibody (FA) techniques have been used successfully in environmental microbiology for the detection and enumeration of microorganisms at the species or even strain level (Bohlool and Schmidt, 1980). The detection of microbial cells that have reacted with labeled antibody is usually carried out through direct microscopic observation of the environmental sample.

SENSITIVITY AND SPECIFICITY: The sensitivity of fluorescent antibody microscopy is generally not as good as standard plate count, most probable number, or molecular detection methods. This is a result of the qualitative nature of this method based on microscopic observation of samples. The microscopic field that is viewed is very small in area and only a limited quantity of the environmental sample can be visualized in each field. This necessitates that a minimum of between 10^4 to 10^6 cells per gram of soil or milliliter of diluent be present in order to detect one cell in a microscopic field. Aquatic samples offer the advantage of allowing concentration of the sample before microscopic analysis. Quantitative FA techniques have been developed for detecting rhizobia bacteria in soil which have minimum detection limits approaching 10^4 CFU/g soil (Schmidt, 1974). Nevertheless, the specificity of this detection technique can be quite satisfactory as individual microbial strains can be distinguished in environmental samples. The degree of cross-reactivity between antisera prepared against the microorganism of interest versus reactivity against other related strains must be assessed to ensure satisfactory specificity with this method. However, one problem with FA specificity is that no distinction between viable and nonviable cells is possible. Furthermore, if one wants to detect a specific introduced microorganism or nucleic acid sequences within that microorganism, fluorescent antibody techniques alone are usually not sufficient. The exception to this would be a microorganism modified with novel DNA sequences which code for a unique antigenic product which allows detection with fluorescent antibodies.

Although polyclonal antisera are easier to prepare for use in fluorescent antibody methods, they are less specific than monoclonal antibodies. The use of monoclonal FA provides a highly specific technique that has been successfully used for the direct detection of microorganisms in the environment.

DETECTION TECHNIQUES THAT REQUIRE CULTURING OF THE MICROORGANISM

(a) Selective media

(i) Resistance to antibiotics

DESCRIPTION: Resistance to antibiotics is the most frequently used detection method in environmental microbiology for reasons of overall convenience and due to their successful use for over a decade. The antibiotics are usually added as components of a solid medium for plate counting of resistant colonies. As microbial resistance to clinically useful antibiotics is a major health concern, efforts have been made to develop alternative detection methods, particularly for microorganisms introduced to the environment.

SENSITIVITY AND SPECIFICITY: The sensitivity of this technique in detecting microorganisms introduced to soil microcosms or terrestrial environments is often in the range of 10^2 to 10^4 CFU/g soil, although reports of detection limits as low as 1-20 CFU/g soil have been reported (Devanas and Stotzky, 1986). Studies on microbial fate on plant surfaces (see e.g., Armstrong et al., 1987) or in aquatic environments (see e.g., Scanferlato et al., 1989; Amy and Hiatt, 1989) often report a greater sensitivity - as low as 10 to 100 CFU/per unit area or volume. Differential media may be necessary to improve specificity and to distinguish colonies of the introduced microorganism on selective media containing antibiotics. With experience an investigator may recognize colony phenotypes. Specificity can be improved by employing resistances to more than one antibiotic, so that a spontaneous mutation to sensitivity to one antibiotic will not eliminate the detectability of the introduced microorganism. Sensitivity may also be enhanced in this way, although the possibility of reduced recovery of microorganisms carrying additional resistance markers on selective media must be checked. Moreover, certain spontaneous antibiotic resistance mutations may reduce survival capabilities in situ (Compeau et al., 1988). All detection techniques based on plating or culturing have an advantage of detecting only viable microorganisms as compared to direct detection, e.g., serological techniques. Sensitivity and selectivity of the technique (relative to detection of the microorganism) may be affected since environmental influences may alter phenotypic expression of resistance, and resistance genes may be transferred from the GEM to other microorganisms.

EASE OF USE: Fluorescent antibody microscopy does not require culturing on solid or in liquid media. Moreover, the physiological state of the introduced microorganism is not critical for adequate detection in the environmental sample unless linked to antigenic expression. Preparation of labeled antibodies and processing of environmental samples for microscopy do result in this technique being more resource intensive than plate count or MPN techniques. However, automation of certain steps in this procedure reduces time and expense. Moreover, the requirement for extensive verification of antibody specificity, through testing of a variety of negative control microorganisms, adds to the resource requirements.

COST: The primary cost involves the preparation of polyclonal, and especially monoclonal, antibodies reactive against the desired microorganism.

EFFECTS OF ENVIRONMENTAL MEDIA: The specificity of fluorescent antibody microscopy can be hampered by nonspecific binding of the antibody to various chemical and biological components of the environmental sample. The natural sample may contain microorganisms, clays, humic materials, or inorganic constituents that are reactive with the labeled antibody. This can be particularly problematic when this technique is used with soil samples.

(d) Summary of direct detection techniques

Direct detection techniques have, in theory, an unlimited detection limit depending on how much of the environmental sample the observer is prepared to scan with a microscope. However, in practice technical restraints on this detection limit are governed by the presence and persistence of soil particles (practical detection limit = 1-10 CFU/g). The problems associated with these methods are ones of contamination with environmental material that results in false positives for direct counts by microscopy.

EASE OF USE: Selection of spontaneous resistance to antibiotics is significantly easier to develop and use in environmental microbiology than the available immunological, most probable number, or nucleic acid hybridization procedures. Genetic modification of microorganisms through the addition of specific resistance markers is becoming more routine, for example the use of Tn5 marked microorganisms for environmental studies which imparts both resistance to kanamycin/neomycin and provides a convenient target DNA sequence for hybridization in the microorganism. However, certain antibiotics are also toxic to humans and must be handled with appropriate precautions.

COST: The cost of many commercially available antibiotics is relatively low, and their use generally less expensive than immunological, MPN, or nucleic acid hybridization detection methods.

EFFECTS OF ENVIRONMENTAL MEDIA: Unequal dispersal of soil, sediment, or plant-associated microorganisms (due to associations with soil or plant material) may lead to poor population estimates. Moreover, indigenous microorganisms expressing resistance to antibiotic may have the ability to outgrow the introduced microorganism on selective media. This is particularly true if the added microorganisms is better adapted to laboratory, rather than environmental, conditions.

(ii) Resistance to heavy metals

DESCRIPTION: Resistance to heavy metals has been used to detect microorganisms in environmental samples. Heavy metals such as mercury and cadmium are added to plating media to allow selection of appropriately marked resistant microorganisms. Resistance to these antimicrobials is frequently encoded by genes found on bacterial plasmids. It has been pointed out that the use of microorganisms resistant to heavy metals in environmental studies can contribute to the spread of resistance to clinically important antibiotics as both are plasmid-borne.

SENSITIVITY AND SPECIFICITY: The sensitivity of techniques employing heavy metal resistance as the selective parameter is often in the range of 10^3 to 10^4 CFU/g soil in terrestrial studies. Sensitivity can be improved up to 10 to 100 fold in aquatic environments or in planta due to the presence of fewer indigenous bacteria resistant to heavy metals. Thus, antimicrobial resistance may be less sensitive than the use of resistance to antibiotics. Nevertheless, Top et al. (1990) reported a minimum detection limit of approximately 10 CFU/g soil in nonsterile soil with microorganisms carrying resistance to the heavy metals cadmium, zinc, and cobalt.

As discussed above for antibiotics, differential media may be necessary to improve specificity and allow the discrimination of colonies of the introduced microorganism on selective media containing the heavy metal, although with experience an investigator may recognize colony phenotypes. Likewise, specificity can be improved by employing resistances to more than one heavy metal or antibiotic. The ability to monitor microorganisms based on plasmid-borne heavy metal resistance can be hampered by the loss of the plasmid DNA after introduction of the microorganism to the environment. All detection techniques based on plating or culturing have an advantage of detecting only viable microorganisms as compared to direct detection, e.g., serological techniques. Sensitivity and selectivity of the technique (relative to the microorganism) may be affected since resistance genes may be transferred from the GEM to other microorganisms (see Tables 1 and 2).

EASE OF USE: The toxicity of heavy metals requires that they be handled with care when used in selective media. Nevertheless, as with antibiotic resistance, selection of microorganisms resistant to heavy metals can be relatively easy and the use of this technique in environmental microbiology more convenient than the available immunological, most probable number, or nucleic acid hybridization procedures. Resistance to heavy metals is often located on transposons which can be inserted into the genome of a microorganism through genetic manipulation in order to provide a means of selection in environmental studies.

COST: As with antibiotics, the cost of many commercially available antimicrobial agents is relatively low, and their use generally less expensive than immunological, MPN, or nucleic acid hybridization detection methods.

EFFECTS OF ENVIRONMENTAL MEDIA: Unequal dispersal of soil, sediment, or plant-associated microorganisms (due to associations with soil or plant material) may lead to poor population estimates. Moreover, indigenous microorganisms expressing resistance to antibiotic may have the ability to outgrow the introduced microorganism on selective media. This is particularly true if the added microorganisms is better adapted to laboratory, rather than environmental, conditions.

(iii) Unusual carbon sources

DESCRIPTION: Media for the detection and differentiation of microorganisms based on their ability to utilize unusual carbon sources are an effective means for the detection, identification, and enumeration of microorganism in the environment. Certain groups of microorganisms, e.g., the pseudomonads, are able to utilize carbon sources toxic to most microorganisms, such as toluene or 3-chlorobenzoate, which facilitates their selection on appropriate media. Genetic manipulation can also be a useful, for example Walter *et al*. (1988) cloned the *xylE* gene from the TOL plasmid of *Pseudomonas aeruginosa* in other genera of bacteria to provide a means of selection from other soil and plant bacteria. In addition, expression of this gene results in a yellow colony which can be readily distinguished on selective media from indigenous bacteria.

SENSITIVITY AND SPECIFICITY: The sensitivity of this technique is a function of the environment in which it is used as the numbers of indigenous microorganisms capable of utilizing the selective carbon source is highly variable. The use of the *xylE* gene described above resulted in a sensitivity comparable to that seen with antibiotic markers in environmental studies. The sensitivity was improved to 10 CFU/g soil or ml of wastewater when the *xylE* gene was used in conjunction with the selective antibiotic nalidixic acid. The selective capabilities of lignin utilization have been exploited by Wang *et al*. (1989) through the cloning of genes for lignin degradation into high expression vectors in streptomycetes used in soil studies. All detection techniques based on plating or culturing have an advantage of detecting only viable microorganisms as compared to direct detection methods, e.g., serological techniques. If expression of catabolic genes is inducible, their expression may be inhibited during primary recovery.

EASE OF USE: This technique requires only plating on, or growth in, selective media. Thus, overall ease of use can be significantly greater than immunological or nucleic acid hybridization methods. If genetic manipulation of the microorganism of interest is required to introduce the catabolic genes for a particular carbon source, considerable resources may be spent obtaining satisfactory expression of this trait.

COST: As with antimicrobial agents discussed above, the cost of many commercially available microbial carbon sources is relatively low, and their use generally less expensive than immunological or nucleic acid hybridization detection methods.

EFFECTS OF ENVIRONMENTAL MEDIA: Unequal dispersal of soil, sediment, or plant-associated microorganisms (due to associations with soil or plant material) may lead to poor population estimates. The use of minimal media is often required in order to obtain proper selection based on a sole carbon source. However, recovery of the desired microorganism on minimal media can be poor due to the exposure of the microorganism to relatively harsh environmental conditions. Furthermore, genes for utilization of certain unusual carbon sources are often found on plasmids or transposons which may be lost through plasmid segregation under environmental conditions.

(b) Differential media

DESCRIPTION: Plate enumeration techniques employing differential media have been used extensively in environmental microbiology to distinguish introduced microorganisms from the indigenous microflora. Often used in combination with a selective agent, differential media can increase sensitivity by at least an order of magnitude since the microorganism being enumerated on solid media can be distinguished from others present in the sample. Examples include the detection of pigment-producing fluorescent pseudomonads (Drahos et al., 1988), P. aeruginosa (Zechman and Casida, 1982), or various enteric bacteria capable of metabolizing lactose. Recently, investigators have genetically modified bacteria to impart a differential colony phenotype by the addition of genes for beta-galactosidase to non-enteric microorganisms (Jain et al., 1988; Drahos et al., 1988), for luciferase to non-marine microorganisms (Gutterson, 1988; Meighan, 1988), and for xylose utilization (Walter et al., 1988).

SENSITIVITY AND SPECIFICITY: Differential media are employed in plate enumeration of microorganisms to improve the specificity of this detection method. Sensitivity of detection is only as good as the selective capability of the medium that is employed. Since ATP is required for expression of the luciferase genes, environmentally starved microorganisms bearing a luciferase cassette may not be detected.

EASE OF USE: A variety of differential media or medium components are available commercially. However, the majority of microorganisms have not been studied sufficiently to design differential media. Thus, investigators interested in using particular microorganisms in environmental studies have used genetic manipulation, as discussed above, to introduce the genetic capacity to differentiate the microorganism on selective media.

COST: Commercially prepared differential media or reagents for incorporation into differential media components are often relatively inexpensive and readily available.

EFFECTS OF ENVIRONMENTAL MEDIA: Environmental interference problems for differential media are similar to those seen with selective media.

(c) Most probable number technique

DESCRIPTION: The most probable number (MPN) method provides an estimate of population density without a count of single cells or colonies. It is based on a determination of the presence or absence of microorganisms in replicate dilutions of soil or other material. Based on probability theory, tables have been developed that allow estimation of the numbers of microbes in an original sample, based on the numbers of positive and negative replicates which received a certain quantity of inoculum.

MPN techniques have been developed for a number of different microorganisms including algae, protozoa, denitrifiers, nitrifiers, and rhizobia. Often, the MPN technique is compared to the plate count method (or the roll-tube method for obligate anaerobes) in which dilutions of samples are spread on the surface of, or mixed with, agar. Both the MPN and plate count methods are called viable count procedures. Viable count procedures suffer from the drawback that they require separation of microbes into individual reproductive units.

SPECIFICITY: This method is limited in that the specificity and sensitivity is dependent on the technique used to detect the microorganism in the medium into which it is inoculated. As with enrichment techniques for plate counts, various subsets of the microbial community may be enriched for through the use of selective growth media.

SENSITIVITY: This method is less precise than the plate count technique. Also, both viable count procedures can be affected by microbial inhibitions caused by other microbes present in the growth medium. Viable but nonculturable microorganisms may not grow under conditions of MPN incubations. Also see limitations under "environmental interference" below. The lower range of reported sensitivity is 10 cells per unit; however, statistical variability can be relatively large, for examples compared to plate count techniques, and may prevent detection of statistically significant differences.

EASE OF USE & TIME: This is a relatively easy procedure which can require little time and minimal equipment, relative to certain direct detection or hybridization techniques discussed in this section. However, it is often more time-consuming than many plate count procedures and can be cumbersome if statistical tables for the number of replicates chosen are not available.

COST: Inexpensive technique requiring only glassware, appropriate media, and possibly instrumentation for detection of isolate growth.

EFFECTS OF ENVIRONMENTAL MEDIA: The environmental matrix is diluted out in a 10-fold dilution series which generally stops at 10^{-9}. In soil, water, sediment, and plant tissue samples it may be difficult to obtain individual reproductive units. Also, inhibition of the growth of the desired microorganisms by antagonistic indigenous microorganisms may present problems.

(d) Fluorescent antibody - colony ELISA blots

DESCRIPTION: A variety of immunological methods are available that employ monoclonal or polyclonal antibodies that react with specific antigens on the surface of microorganisms. Serological techniques have been used extensively in studies on aquatic microorganisms such as the pathogen Vibrio cholerae, and somewhat less frequently for soil or plant associated microorganisms, with the exception of the root nodulating Rhizobium and Bradyrhizobium. One of the most commonly used methods in terrestrial and aquatic microbiology, is the fluorescent antibody technique. A technique under development, based on enzyme-linked immunosorbant assay methods, is the colony ELISA blots.

This fluorescent antibody technique combines the specificity of antigen-antibody reactions with plate counts techniques in the identification of individual colonies on a solid medium in colony enzyme-linked immunosorbent assay (ELISA) blots. In this relatively new technique, the microorganism of interest produces an antigenic cellular constituent, often an enzyme, which can be detected in individual colonies through colony blots. Thus, dilutions of the environmental sample containing the antigen-producing microorganism, are plated on selective media, replica-plated onto nitrocellulose filters, lysed, and detected through the use of a fluorogenic or chromogenic label.

SENSITIVITY AND SPECIFICITY: As with any FA technique, specificity can be very good, allowing the detection of individual microbial strains. An advantage of colony ELISA blots, when compared to fluorescent antibody microscopy techniques, is that there is less non-specific interaction of the antibody to components of the environmental sample as the assay is carried out on membrane filters with isolated colonies. However, colony ELISA blots do require culturing of the microorganism of interest, and the sensitivity of this technique for environmental samples is only as good as that of the selective media used to isolate and enumerate the microorganism. Although polyclonal antisera are easier to prepare for use in colony blots, they can be less specific than monoclonal antibodies. The use of labeled monoclonal antibodies provides a highly specific technique that has been used for the direct detection of microorganisms in the environment.

EASE OF USE: Preparation of labeled antibodies and processing of environmental samples for microscopy do result in this technique being more resource intensive than plate count or MPN techniques. Moreover, the requirement for extensive verification of antibody specificity, through testing of a variety of negative control microorganisms, adds to the resource requirements.

COST: The primary cost involves the preparation of polyclonal, and especially monoclonal antibodies, reactive against the desired microorganism. Additional costs and time is involved in verification of the specificity of the antibody preparation.

EFFECTS OF ENVIRONMENTAL MEDIA: The specificity of FA-ELISA technique can be hindered by nonspecific binding of the FA to compounds introduced during plating of the environmental sample on solid media for colony development. This is largely a problem on plates containing low sample dilutions and can result in transfer of the contaminants to the membrane filter during replica plating.

(e) Summary of cell culture techniques

All of the methods classified as cell culture techniques often provide relatively good levels of detection with approximately 10^2 CFU/g soil or plant tissue being the accepted figure for traditional plate counts. This figure can be improved in aquatic samples, often through the use of biomass concentration techniques. The primary practical limitation with cell culture techniques is one of contamination due to growth of indigenous microflora, either on solid media for plate count techniques or in liquid media for MPN techniques. However, for many environmental studies a detection limit of 10^2 CFU is sufficient to allow a determination that the microorganism's survival or dissemination is following expected patterns. In this case, there is no real need to decrease limits of detection. Microbial populations below this level could only again become significant if selection pressure resulted in an increase in numbers. The likelihood of such selection would depend on the microorganism.

As the use of selective and differential media remains the most common technique employed in the detection microorganisms, much experience has been gained regarding its advantages and disadvantages. Consideration of the strengths and weaknesses of the technique will ensure that the desired sensitivity and specificity are attained. The considerations discussed below are based on the growing literature in microbial ecology. More detailed discussions of these topics can be found in reviews by Grainger and Lynch (1984), Colwell et al. (1985), Sayler and Stacy (1986), Stotzky et al. (1990).

In general, the choice of any particular selective medium approach depends on not only the characteristics of the microorganism, and the environment that it is introduced to, but also the monitoring endpoints that are to be measured, e.g., survival, dissemination, or gene transfer. Each of these monitoring endpoints has unique practical problems that should be considered in the planning of the monitoring procedures. As an example, resistance to an antibiotic may provide a sensitive phenotypic marker for monitoring the survival in soil of a microorganism, but may not be of adequate sensitivity to detect transfer of the resistance gene to recipient cells. Thus, several factors come into play in the choice of an appropriate selective or differential medium.

(i) Recovery of oligotrophic versus copiotrophic microorganisms

Oligotrophic microorganisms are those that preferentially utilize nutrients at relatively low concentrations, while copiotrophs exhibit optimal growth at relatively high nutrient concentrations. When sampling an environment particular consideration should be given to the oligotrophic or copiotrophic nature of the microorganism to be detected. For example, for

isolation of oligotrophic microorganisms from soil may require a soil extract medium while those from marine environments may require a defined seawater medium. These media often result in optimal recovery of oligotrophs compared to a more nutrient rich medium such as those commonly used in the laboratory for cultivation of microorganisms.

(ii) Selection of specific resistance to antibiotics

Resistance to antibiotics can be used with most microorganisms as a selective technique for quantitative or qualitative monitoring of environmental fate. A number of practical considerations arise in the use of this technique.

A common consideration is the stability of the resistance genotype in the microorganism to be monitored. Often, chromosomally located resistance genes offer greater stability than those located on plasmids. Resistance to nalidixic acid and rifampicin may be preferable for this reason (Stotzky et al. 1990), as other plasmid associated resistance markers, such as erythromycin, are more readily lost in the absence of selection pressure.

Resistance to nalidixic acid has been reported to be advantageous for studies of gene transfer among microorganisms (Walter, 1988). The presence of nalidixic acid in the plating medium used for colony counts of transconjugants effectively prevented artificially high gene transfer rates due to conjugal transfer events on the agar medium, rather than in the soil microcosm.

Not all resistance phenotypes are without secondary effects. For example, Compeau et al. (1988) showed that resistance to rifampicin in certain pseudomonads conferred a selective disadvantage to the microorganism when introduced into sterile soil microcosms. Resistance to tetracycline can also alter the colony phenotype of certain microorganisms, presumably due to alterations in cell membrane functions.

(iii) Stability and expression of the selective or differential phenotype

As mentioned above, plasmid-borne selective markers may be lost during incubation in the environment as a result of plasmid segregation. This could result in artificially low quantitative estimates of microbial populations. The possibility of this occurrence can be checked in microcosm experiments in which population estimates are compared on nonselective media and media containing the antibiotic.

Expression of a differential phenotype on a solid medium may allow the accurate detection of certain microorganisms, however, this can be confounded by the presence of large numbers of

indigenous microbial colonies. Competition for limited nutrients can result in colonies of small size which do not express the differential phenotype, e.g., the characteristic green colony produced by fluorescent pseudomonads may not be visible with small colonies on crowded plates.

(iv) Resistance to antimicrobials: increased sensitivity versus reduced recovery

The use of more than one antibiotic or heavy metal in the selective medium will often markedly increase the sensitivity of this detection method; however, a reduction in efficiency of recovery of the microorganism from environmental samples may also occur. Reasons for reduced recovery may be attributed to various phenomena including the potential for the microorganism to produce viable but non-culturable cells, the effects of antimicrobials in the selective medium that alter cell function or physiology, or other stresses on the microbial cells when placed in environmental conditions.

Available solutions include reliance on a single antimicrobial with shorter incubation times that prevent the overgrowth of indigenous microorganisms. Also, an initial plating of the environmental sample on a nonselective solid medium can allow growth and recovery of the introduced microorganism, followed by replicate plating on the desired selective medium for enumeration. Similarly, for aquatic environments, an initial growth and recovery in nonselective liquid media may be necessary although this can result in a loss of the ability to accurately quantitate the microorganism.

(v) Viable but nonculturable or somnicells in bacteria

The viable but nonculturable (or somnicell) phenomenon has been reported to occur in various gram negative bacteria including pathogens, and aquatic or soil-inhabiting microorganisms (Rozak and Colwell, 1987; Bottomley et al., 1990). Viable but non-culturable microorganisms are metabolically active, but not culturable by standard methods. It is thought that this dormant stage is a survival mechanism for the microorganism allowing it to exist under less than optimal conditions. Relatively few bacteria have been studied for their ability to exhibit this property, so it is not known whether this is a general characteristic of bacteria that do not produce typical resistant structures such as endospores or conidia. However, in those microorganisms in which the production of somnicells has been demonstrated, it is possible that populations in the environment can be underestimated.

(vi) Use of selective media for colony hybridization

While colony hybridization techniques have been applied to the detection of microorganisms in the environment with increasing frequency in both microcosm and field studies, a number of practical considerations apply to the use of this technique.

The primary consideration is that the sensitivity of detection by colony hybridization may be the same or only slightly greater than that obtained by selective media. This results from the fact that the colonies used as the source of target DNA are either picked or replicated from selective media plates. Thus, the lowest dilution of the environmental sample which results in discrete colonies on a plate determines the sensitivity of this technique (Saylor and Layton, 1990; see also discussion on hybridization in this paper).

TECHNIQUES TO LABEL AND DETECT NUCLEIC ACIDS

(a) Nucleic acid probe construction

(i) Labels available for nucleic acid probes

DESCRIPTION: The detection of microorganisms in environmental samples by nucleic acid hybridization has most frequently been accomplished by the use of radiolabeled probes, although nonradioactive probes have been employed with increasing frequency. Radio labeling of nucleic acid probes is carried out by the incorporation of a radioactive nuclide, such as ^{32}P, in the nucleotides of the probe sequence. Colony, slot/dot blot, or Southern hybridization procedures have all been used in the detection of specific target sequences in microorganisms employed in microcosm or field studies. Several types of nonradioactive labelling procedures have been developed including nick translation to incorporate biotin-labelled nucleotides, nick translation to incorporate an antigenic sulfone group, or direct photolabelling with biotin.

SENSITIVITY AND SPECIFICITY: The specificity and sensitivity of radiolabelled nucleic acid probes have been reported to be significantly greater (Forster et al., 1985) or only slightly greater (Zeph et al., 1990) than nonradioactive probes in detecting target nucleotide sequences. It appears that optimal sensitivity can be obtained with nonradioactive probes with Southern hybridizations or dot/slot blots (1-10 pg for single copy gene sequences); however, background nonspecific signal development can at times cause problems with colony lift hybridizations. Nevertheless, Yang (1985) reported the detection of one bacterial colony containing target sequences per 10^5 to 10^6 colonies from the environmental community (i.e., food products) which rivals detection limits with radiolabeled catabolic plasmid probes (1 colony per 10^6 colonies from natural soil; see, e.g., Sayler et al., 1985).

EASE OF USE: Nonradioactive probes are the easiest to use because their longer storage life (up to 6 months) compared to the common radioactive labels (approximately 2 weeks) allows a significantly larger number of hybridizations to be carried out. In addition, radiolabeled probes require special handling procedures and protective clothing. Although certain nonradioactive probes require relatively extensive post-hybridization washing procedures, visualization of the hybridization signal can often be accomplished in less than one hour as opposed to overnight time periods typical with radiolabeled probes.

COST: The cost of purchasing radioactive nuclides is significantly more than that of reagents or kits available for nonradioactive labeling. In addition specials materials and equipment are necessary for handling radioactive compounds. As mentioned above, nonradioactive probes have a longer storage life which also reduces their cost.

EFFECTS OF ENVIRONMENTAL MEDIA: Non-specific hybridization can be a problem with nonradioactive probes, particularly biotin-labeled probes. In addition to non-specific hybridization due to impurities in the environmental sample, proteinaceous compounds from the lysed cells can react with the avidin-phosphatase moiety used to visualize the bound probe when hybridizations are performed on membrane filters. It is for this reason that radiolabeled probes continue to be the method of choice in microbial ecology for hybridization.

(ii) Probes from random or defined nucleotide fragments

DESCRIPTION: DNA probes are generally derived from two sources. DNA isolated from the microorganism of interest can be isolated from total genomic DNA or from plasmid DNA, subjected to restriction digestion, and either an uncharacterized or random fragment is selected or a specific or defined fragment is used in the construction of a labeled probe.

Randomly generated DNA fragments are screened for the intended specificity. Table 7 lists several cases. These probes can be specific for a species (Totten et al., 1983) or only for certain strains (Tannock, 1989). The function of the gene detected by the random DNA probe is not known. It may be part of an important ubiquitously distributed gene or of a DNA sequence without any essential function. Therefore nothing is known about its stability or genetic variability. However, lateral transfer of these genes has to be ruled out before these probes are considered reliable tools.

Defined DNA probes are obtained through the selection of a specific restriction digestion fragment from either plasmid or genomic DNA. The choice of which restriction fragment gives optimal sensitivity and specificity is based on knowledge of the genetic map of the microorganism and the use of restriction fragments from genes with relatively unique nucleotide sequences. Specific target DNA sequences can be cloned into plasmid vectors for production of large quantities of probe DNA. For example, probes to the 16 S ribosomal DNA have been used to classify organisms (Jain, et al., 1988). For those microorganisms that are not well characterized genetically, an empirical approach must be employed, e.g., by the preparation and analysis of a genomic DNA library in phage vectors.

Nick-translation is the most widely used method for incorporating labeled nucleotides into double-stranded DNA. This procedure is based on the ability of DNA polymerase I to add nucleotide residues to the 3'-hydroxyl terminus of a nick while removing nucleotides from the adjacent 5' - phosphoryl-terminus (Meinkoth and Wahl, 1987). The nicks are generated with DNAase I. A second technique is random priming. The basis of this technique is the ability of DNA polymerase I to copy single-stranded DNA templates primed with random hexamers prepared from calf thymus DNA. A second method to obtain single-stranded probe uses M13 phage. The insert is cloned into M13 phage. The plus strand of the phage is then used as a template to synthesize the probe using DNA polymerase I and labeled nucleotides.

SENSITIVITY: Specificity and sensitivity of DNA probes constructed from restriction fragments varies considerably depending on the size of the probe, the amount of sequence homology between the DNA probe and target sequence, the labeling method and stringency conditions that are chosen, among other factors. In general, restriction fragment probes constructed from nucleotide fragments of 200 bp or more can be highly sensitive in DNA hybridizations. The sensitivity is determined by the amount of label that can be incorporated into the nucleic acid (Atlas and Sayler, 1988). Oligonucleotide probes (up to 100 nucleotides; Wallace and Miyada, 1987) are more specific. More radioactivity and thus a higher specific activity can be incorporated into longer probes (nick translation produces fragments between 500-1500 nucleotides; Meinkoth and Wahl, 1987). Oligonucleotides are end-labeled to incorporate one labeled nucleotide per molecule. Using 5' (alpha ^{32}P) ATP for example, 10^8 cpm/microgram DNA can be incorporated. With random priming, probes that have specific activities of 10^9 cpm/microgram DNA can be obtained. By preparing probes from M13 phage, specific activities of 10^9 per microgram of DNA can be obtained. Probes are added to the hybridization filter in excess. Double-strand probes must first be denatured to permit single-strand probes to anneal to the target DNA. During hybridization, double-stranded probes can reanneal, thus decreasing the effective amount of probe available to bind to the immobilized target DNA. With single-strand probes, reannealing is not a problem.

By extracting bacteria from the soil and probing without pre-culturing, Holben et al. (1988) were able to detect 4.3 X 10^4 cells of Bradyrhizobium japonicum per gram of soil or 0.2 picograms of DNA per 1 microgram of total DNA using a single-stranded ^{32}P-DNA probe prepared from M13. Other factors that affect sensitivity are the sequence complexity, the abundance of target sequences (i.e., are they multiple copies?), and the type of label (i.e., is it radioactive or biotinylated?). Inaccuracies in microorganism counts can result if changes in copy number of the target sequence occur as a result of environmental influences.

SPECIFICITY: Specificity is increased only if the DNA (or specific gene sequences), rather than the whole plasmid, is used. For example, the insert DNA can be purified by restriction digestion and isolation of the fragment using gel electrophoresis. Nick translation or random priming can then be used to label the fragment. Alternatively, synthesis off of a M13 phage template primed proximal to the insert results in insert-specific probes since synthesis does not proceed across the entire plasmid. A probe that is limited to a fragment of the gene rather than the whole gene or a whole plasmid carrying the gene is more selective, since in the latter case considerable cross-hybridization and non-specific reactions can occur (Sayler

et al. 1985). Tables 10 and 11 list examples of the use of defined polynucleotide gene probes.

EASE OF USE & TIME: This is dependent on the labeling procedure and the type of label used. Some laboratories find the use of radioactivity problematic; however, radioactive nucleotides allow for the maximum sensitivity. Increase probe specificity can be costly in terms of ease and time. For instance single-strand probes require cloning into M13. The isolation of fragments from a plasmid is time-consuming and cumbersome but results in a more specific probe.

COST: The cost incurred is from the purchase of enzymes and labeled nucleotides.

EFFECTS OF ENVIRONMENTAL MEDIA: Not applicable

(iii) Oligonucleotide probes

DESCRIPTION: Synthetic oligonucleotides are short chemically-synthesized nucleic acids. The oligonucleotide corresponds to a defined nucleotide sequence. It can be up to 100 nucleotides in length but in practice is usually between 15 and 30. One advantage of this technique is that if only the amino acid sequence is known, a mixture of oligonucleotides can be synthesized based on the deduced nucleotide sequences (Wallace and Miyada, 1987). For hybridization, the oligonucleotides are most commonly end-labeled with T4 polynucleotide kinase. This enzyme adds gamma-^{32}P ATP to the free 5'-OH end of the oligonucleotide (Wallace and Miyada, 1987; Berent et al., 1985). A second method for labeling is primer extension using Klenow fragment (DNA polymerase I; Wallace and Miyada, 1987; Berent et al., 1985).

SENSITIVITY: End-labeled oligonucleotide are 30-100 fold less sensitive as hybridization probes than nick-translated DNA fragments (Berent et al., 1985). The extent of the decrease in sensitivity varies with the method (dot or Southern blots) and target nucleic acid being probed (RNA or DNA). One reason for this decrease in sensitivity is inherent in end-labeling. In end-labeling, only one labeled phosphate can be added per molecule of probe whereas in nick-translation, 40 to 80 labeled phosphates are added per molecule of probe with one of the four nucleotides labeled. The decreased sensitivity resulting from end-labelling can be alleviated to some extent with the use of primer extension via Klenow fragment which results in labelling of all four nucleotides. Extrapolating from data using a synthetic oligonucleotide to detect rat ribosomal DNA (Berent et al., 1985), an end-labeled oligonucleotide can theoretically detect 1 microbial genome out of 10^4. Inaccuracies in microorganism counts can result if changes in copy number of the target sequence occur as a result of environmental influences.

SPECIFICITY: The main advantage of using an oligonucleotide probe is specificity. Specificity is dependent on the length of the probe: the longer the probe is, the lower the probability that a cross-reactive sequence will appear with a match to the total contiguous sequence contained in the probe. However, this is balanced by the higher probability of matches between small contiguous sequences within the probe. These partial duplexes will be less stable than the more extensive match between the target sequence and the oligonucleotide. Thus, oligonucleotides can be designed so that hybridization and subsequent washing of the blot can be performed under very stringent conditions. Longer probes are more stable and can be hybridized under higher temperatures, i.e. have a higher T_d, the temperature at which one-half of the duplexes are dissociated (Wallace and Miyada, 1987). Therefore, a probe can be designed to alleviate any problems of cross-reactivity if organisms containing sequences

closely-related to the target sequences are expected to be isolated with the organism of interest. Another factor that can affect specificity is the GC content. The higher the GC content, the higher the T_d. However, oligonucleotides containing high GC contents exact other problems such as self-complementarity which interferes with 5' labeling and purification during synthesis of the nucleotide itself (Wallace and Miyada, 1987).

EASE OF USE & TIME: The oligonucleotides must be synthesized. This can be done commercially or within the laboratory. The synthesis is automated, but it is technical and time-consuming. For example, the probe must be labeled (however labeling is usually a simple procedure with few steps involved). The use of radioactivity as a label may be a problem for some labs.

COST: The primary cost is due to the synthesis of the oligonucleotide. The cost of synthesis is dependent on the length, i.e. the number of nucleotides that must be incorporated. Synthesis can be done commercially or in the laboratory. Synthesis requires an automated DNA synthesizer which is very expensive. Therefore unless the use of oligonucleotides as probes and primers are routine, it is more cost-effective to have the oligonucleotide synthesized commercially. Additional cost is incurred with buying labeled nucleotide and enzymes that carry out the labeling reaction.

EFFECTS OF ENVIRONMENTAL MEDIA: Not applicable

(iv) Transcription probes

DESCRIPTION: Single-stranded RNA can be prepared for use as probes. Transcripts are synthesized *in vitro* using a transcription vector. The nucleotide sequence is cloned into one of the multiple sites in the polylinker of this specialized vector. The polylinker is 3' to the promoter so that cloning into one of these sites orients the insert with respect to the promoter. The alignment with respect to the promoter allows the transcription of the DNA so that one of the two strands will be transcribed. The most commonly-used promoters are from bacteriophage SP6, T3, and T7. Transcription via a DNA-dependent RNA polymerase is performed *in vitro* in the presence of all four nucleotides, one of which is labeled (Ford and Olson, 1988). Some vectors are constructed so that the polylinker lies between two different promoters so that transcripts of either polarity can be obtained.

SENSITIVITY: Probes of relatively high specific activity can be prepared, greater than 2 X 10^8 cpm per microgram of RNA (Wahl, *et al.*, 1987). In addition, the use of asymmetric probes increases hybridization efficiency since the complementary strand is not present in the hybridization mixture. The ability to obtain large quantities of high specific activity probe and the ability to limit the annealing of the probe to only the target DNA increases the sensitivity of RNA probes relative to double-stranded DNA probes.

SPECIFICITY: Specificity is dependent on the extent of complementarity between the probe and the target DNA. Sensitivity and specificity are increased because the probe is single-stranded and does not reanneal to itself.

EASE OF USE & TIME: The main disadvantage of using RNA probes is related to the extra cloning steps required to construct the vector.

COST: The major cost incurred is from the purchase of the labeled nucleotide. A secondary cost is from the purchase of the RNA polymerase. Overall, the cost is equivalent to that incurred using the nick-translation procedure.

EFFECTS OF ENVIRONMENTAL MEDIA: Not applicable

(b) Hybridization techniques

These methods provide different formats which allow nucleic acid probes to anneal to target DNA.

(i) Colony Hybridization

DESCRIPTION: This hybridization technique allows the screening of individual colonies isolated from an environmental sample. It can be used for screening microorganisms when traditional culturing techniques are insufficient to identify the introduced organism, for example screening for a bacterium containing beta-lactamase as a marker gene in a field of "naturally-occurring" ampicillin resistant bacterium.

The technique involves first culturing the microorganisms in the laboratory on an appropriate medium. A solid medium is prepared that will support growth and allows for the selection and/or enrichment of the microorganism and a nitrocellulose filter is placed on top of it thereby allowing the filter to become impregnated with an aliquot of the microorganisms. The plate is incubated at the appropriate temperature to allow the bacteria to grow. The growth conditions, temperature and time, will be dependent on the bacteria of interest, for E. coli 8 to 10 hours at 37^0 C is recommended. It is probably necessary to maintain the selection or enrichment at this step even if the original culturing of the organism contained it.

This technique was developed to screen libraries in E. coli, a rapidly growing organism. Most organisms introduced into the environment will be relatively slow growing and if the nitrocellulose is not presterilized, contaminants may be introduced via handling the filters and may grow out if a long time span is required to grow the bacterium. After the appropriate incubation time, the filters are removed and replica plated onto other hybridization membranes. The master copy is then reincubated on fresh media to regenerate the colonies. This plate is the master plate to which the results from hybridization of the replicas can be referred (Vogeli and Kaytes, 1987).

Depending on the efficiency of transfer and the amount of replicas made from each master, it may be necessary to incubate the replicas on fresh media to reach a cell density high enough to obtain a signal sufficient for the detection of the nucleotide sequence of interest. Before hybridization the cells must be lysed. Again these techniques have been developed for E. coli and may have to be adjusted for the organisms of interest. van Elsas et al. (1989) found that by adding an extra lysozyme step to the standard procedure, they were able to improve the lysis efficiency of soil isolates of Pseudomonas fluorescens. The membranes are pretreated with SDS to remove debris that can

interfere with hybridization. The cells are then lysed in situ and the DNA is denatured in an alkali solution. The alkali solution also destroys the RNA which can interfere with hybridization. The cells are placed in a neutralizing solution. If nitrocellulose is used, the membranes are baked to fix the DNA. In order to remove remaining debris, the baking is done in an EDTA and SDS solution. If a nylon filter is used, baking is unnecessary.

The probe can be composed of either RNA or DNA; an isolated DNA fragment, the whole plasmid, or a synthetic oligonucleotide; and be radiolabeled or labeled with a nonradioactive marker. The conditions of the hybridization will depend on the probe used, the T_m of the duplex, the complexity of the nucleotide sequence, the match of the probe to the target sequence, and the cation concentration (Wahl and Berger, 1987). The stringency of the wash is dependent on the specificity of the probe for the target sequence; i.e. the extent of match to the target sequence versus the presence of cross-reacting sequences in any of the background microorganisms.

SPECIFICITY: The specificity of this technique is dependent on the ability of the probe to differentiate between the target sequence and cross-reacting sequences in background microorganisms. Because of carry-over of components of culture media on the hybridization membrane such as salts and impurities from cell lysis, i.e., hybridization is in situ and the DNA is not purified away from the cellular debris, there are artifactual spots that arise that can be misinterpreted as false positives if they coincide with colonies (Amy and Hiatt, 1989). It is therefore necessary to hybridize, at the minimum, duplicate copies to identify artifacts, i.e. a true signal will be show up on both copies.

One advantage of the technique is that it detects only live organisms and there is a confirmatory method of identification, i.e., biochemical or immunological. Thus, the target DNA can be positively associated with a given strain and/or species and it can be determined if conjugation or transduction into new strains or species had occurred. The technique is inexact because of the impurity of the target DNA and therefore is limited to screening. If a more exact procedure is needed, the potential positives can be subjected to a more involved analysis such as hybridization of Southern blotted DNA for verification.

SENSITIVITY: The sensitivity of the technique is limited because of the need to culture out the organism. Thus this step is limited by the ease in which the bacteria can be grown under laboratory conditions. Organisms that are hard to grow may be underrepresented in this type of study. Also, the technique is limited by the inability of most cultivation methods to isolate but a small percentage of the total microbial population at the

time of sampling (Jain et al., 1988; Steffen et al., 1989). For those organisms that can be grown in culture, the method has the potential to be 4 to 5 times more sensitive than identification using traditional culturing techniques (Jain et al., 1988). Also because it is sometimes difficult to reisolate cells on a selective/differential media, the cells can be isolated on an enriched medium and then identified.

It has been shown that detection of 1 cell in 10^6 colonies is possible with this technique (Jain et al., 1988). Thus, this technique has the potential to allow for a more sensitive quantitation than traditional plate counts. In binary cultures of Pseudomonas putida and E. coli, Saylor et al. (1985) were able to detect 1 colony of P. putida in 10^6 nonhomologous cells using a nick translated ^{32}P-DNA probe specific for the TOL catabolic plasmid. In a fresh water microcosm containing Alcaligenes A5 and Pseudomonas cepacia, Steffen et al. (1989b) found the limits of detection for the bacteria to be 10^2 cells/ml using either a whole plasmid (Alcaligenes) or a species -specific (P. cepacia) radiolabeled probe. The limit of detection for Tn5-marked Azospirilum lipoferum in soil was found to be 10^3 colonies/ gram of soil using a radiolabeled plasmid probe containing the npt II gene (Bentjen et al., 1989).

EASE OF USE & TIME: Of all the hybridization techniques, this methods can generally be the easiest, fastest, and least expensive (Sayler and Layton, 1990). The speed of this method is limited by the growth rate of the organism. Large numbers of colonies can be screened simultaneously, thus saving time. Another time saving factor is the small amount of sample preparation once the organism is grown, i.e. DNA does not have to be isolated and purified.

COST: The cost is the same as traditional culture methods plus the cost of probe preparation (see above discussions).

EFFECTS OF ENVIRONMENTAL MEDIA: The environmental limitation of this technique is the same as that encountered with traditional isolation techniques, i.e. the growth of the organism on solid media under laboratory conditions. The ease of lysis of the bacterium could also be a limiting factor with those organisms that are difficult to lyse because of the presence of a capsule, slime layer etc. Moreover, the ability of the bacterium to adhere to the membrane support because of an extracellular layer may be a factor.
Colony hybridization was designed to screen high density libraries contained in a pure culture (Sayler and Layton, 1990). Screening environmental samples represents a different application. One major problem encountered is colony density (Sayler and Layton, 1990). The probe must be able to select a specific colony in a dense heterogeneous background. A problem can be encountered with environmental samples such as soil in

which their is an abundance of microorganisms and the target organism is a small percentage of the total.

Conversely, in dilute aqueous environments the same problem arises because a concentration step must be included. Steffen (1989a) found that if the target organism is less than 0.1% of the total viable population than the plates are totally overgrown by non-target organisms. For hybridization analysis, the ideal number of cells per plate is between 30 and 300. Dilutions to obtain this range will exclude the target organism if the total population is three orders of magnitude higher than the target organism (Steffen *et al*. 1989b). Often the colonial morphology and size of environmentally isolated colonies limit the application of this technique (Sayler and Layton, 1990). In a study in which genetically-engineered *E. coli* was introduced into lake water and allowed to compete with the indigenous organisms, it was found that after a few days the indigenous organisms overwhelmed the introduced one that was barely visible on the plate. A combination of greater cell volumes and selective pressure can alleviate this problem (Amy and Hiatt, 1989; Steffen *et al*. 1989b). Some organisms or a percentage of the introduced organism may be non-culturable (Sayler and Layton, 1990). In some cases, high cell densities are needed for cross-feeding in the initial isolation thereby hindering the ability to achieve colony counts low enough for hybridization (Mancini, 1987).

(ii) In situ hybridization

DESCRIPTION: This technique takes advantage of the observation that fixing whole cells makes them permeable to oligonucleotide probes. Identification of single microbial cells can then be achieved with radiolabelled, rRNA targeted, group-specific oligonucleotide probes (Giovannoni et al., 1988) that specifically hybridize to rRNA in situ. Identification can then be achieved without the need for cell lysis or nucleic acid extraction. The method is comparable to staining with fluorescent dyes (e.g. DAPl, Acridine Orange) or the immunofluorescence approach, in that the visualization of the results require an epifluorescence microscope or a flow cytometer (Amann et al., 1990b). Flow cytometry has the potential for rapid identification, counting and sorting of specific microorganisms.

SENSITIVITY AND SPECIFICITY: De Long et al. (1989) could demonstrate that fluorescently labelled oligonucleotides offer not only all the advantages of a non-radioactive label but are even more sensitive than radioactive probes. In situ detection of intestinal microorganisms has been shown in the extremely complex cecum content as well as differentiation at the genus, species and subspecies level (Amann et al., 1990a). The method has not been extensively used for environmental samples so far, but has several promising aspects: (a) cultivation of the organism of interest is not required for probe design and detection, (b) probe specificity is not restricted to strains or species but also larger phylogenetic groups of organisms may be studied (e.g. all eubacteria, SRBs), (c) physiological activity is correlated to the rRNA content (De Long et al., 1989) and therefore to the amount of bound probe per cell, and (d) the probe is synthesized chemically, quickly and inexpensively. Problems in the use of fluorescent, rRNA-targeted oligonucleotide probes are: auto-fluorescent background in some environments and low sensitivity (10^4 cells per g of soil are required for detection).

EFFECTS OF ENVIRONMENTAL MEDIA: In some environmental matrices, background auto-fluorescence may interfere with identification.

(ii) Dot/slot blot

DESCRIPTION: This procedure involves the immobilization of nucleic acid onto a solid support matrix such as nitrocellulose or nylon. Like the colony lift technique, it is a screening procedure for the detection of the inserted DNA. It does not require isolated cells but rather the DNA can be extracted either directly from the environmental sample itself or from batch culture grown from the environmental sample. RNA can also be immobilized though it is more unstable than DNA and not likely to be isolated directly from the environment. For direct detection, the DNA can be isolated and enriched from the environmental sample using known techniques such as that published by Sayler and Barkley (1987). Alternatively, cells can be lysed and DNA partially purified from bacteria separated out of the environmental sample, i.e., without laboratory culturing (Holben et al., 1988).

The DNA must be made single stranded for it to adhere to the support matrix and thus the first step must be either a denaturing step or if a covalently-closed circular plasmid contains the target sequence, digestion to a linear form and then denaturation (Costanzi and Gillespie, 1987). If enrichment for the insert has been performed by culturing techniques and a few microliters of sample is sufficient to verify the presence of the microorganism, purified denatured DNA can be applied directly to the filter. Under most circumstances, a much larger quantity of sample must be applied and it is necessary to use a minifiltration device such as a minifold or slot blotter which allows the application of the sample under vacuum. With nitrocellulose, the blot is baked to fix the DNA before hybridization. It is unnecessary to bake nylon filters prior to hybridizing.

All types of probes can be used with this technique, either RNA or DNA, random or defined nucleic acid fragments, whole plasmids or synthetic oligonucleotides, and radiolabeled or labeled with a nonradioactive marker. The conditions of the hybridization will depend on the probe used, the T_m of the duplex, the complexity of the nucleotide sequence, the match of the probe to the target sequence, and the cation concentration (Wahl and Berger, 1987). The stringency of the wash is dependent on the specificity of the probe for the target sequence; i.e. the extent of match to the target sequence versus the presence of cross-reacting sequences in any of the background microorganism.

SPECIFICITY: The specificity of the technique is dependent on the probe used and the nature of the target DNA. If the DNA is isolated directly either out of the soil or from cells separated from the soil, it is not possible to determine whether any positive signals are from viable cells or from extraneous DNA. Thus, it is not possible under these conditions to associate a

positive signal with the presence of the introduced genetically engineered microorganism. It is likewise not possible to determine if the DNA of interest is still contained in the introduced organism or has been transferred via conjugation for example to another strain or species.

The amount of target DNA sequence is low in relation to the background, especially if the DNA is probed directly from the environmental sample and an enrichment step for the recombinant organism has not been performed. Thus, a large amount of sample must be loaded onto the hybridization membrane. This can result in non-specific hybridization and false positives. Also, since the sample is loaded in bulk, cross-reactive sequences from indigenous microorganisms cannot be differentiated from the sequences of interest. Washing the membrane under conditions of high stringency helps reduce background caused by cross-reactive sequences. The probe is extremely important in this regard. The sequences chosen should be unique to the target DNA and there should be a perfect match, if possible, between the probe and the target sequence.

The stability of the duplex is dependent on the extent of matching sequences between the hybridizing nucleic acid molecules. Thus the higher the complementarity between the molecules the higher the T_m, and the more stable the duplex at higher temperatures. Salt helps to stabilize duplexes thus reducing the salt concentration will destabilize imperfect matches. If the probe is perfectly matched to unique insert sequences, the wash can be performed under high stringency conditions, high temperature/ low salt, thus destabilizing any duplexes formed by hybridization of the probe with similar but non-identical sequences from non-target organisms.

SENSITIVITY: The sensitivity of the technique is dependent on the probe and also the way the DNA is isolated. One advantage of the technique is that many bacteria do not survive the transfer from the environment to laboratory media. By direct probing, higher numbers of bacteria can be sampled. By extracting bacteria from the soil and probing without pre-culturing, Holben *et al*. (1988) were able to detect 4.3 X 10^4 cells of *B. japonicum* per gram of soil or 0.2 picograms of DNA per 1 microgram of total DNA using a single-stranded ^{32}P-DNA probe. They reported that their technique can possibly detect as few as 10^3 cells per gram of soil. Steffen and Atlas (1988) reported the detection of 10^3 cells of *P. cepacia* per gram of sediment using a nick-translated species-specific ^{32}P-labeled isolated DNA fragment as a probe (repeat sequence, 15-20 copies/ cell, RS-1100-1 that is located on plasmids and chromosomally). To detect *E. coli* and *P. putida* containing a xylE marker in lake water, Morgan *et al*. (1989), used an 822 base-pair fragment labeled with [^{32}P]dCTP by random priming. The limit of detection using this fragment for dot blot

analysis was 10^4 CFU/ml. In the same study, the limit of detection in an ELISA analysis was 10^3 CFU/ml.

A major limiting factor is the amount of DNA that can be bonded onto the nitrocellulose before the membrane is saturated (Steffen and Atlas, 1988). Since the amount of DNA attributable to the introduced microorganism is low compared to background, it may not be possible to load enough material to exceed the limits of detection. In many instances, the low sensitivity will be a problem if there is a need to determine die-back, i.e., survival in low numbers (Morgan et al., 1989). For example if there is a need to determine survival in reservoirs or overwintering of plant pathogens.

EASE OF USE & TIME: This technique is relatively easy however it does involve processing of the environmental sample before hybridization. The ease of the technique is dependent on the complexity of the DNA extraction procedure and/or the cell separation procedure used. In those cases where the cells are first cultured, the DNA must still be isolated however extensive DNA purification such as CsCl gradients is usually not necessary. The other procedure that is factored into the complexity is probe preparation.

COST: It allows multiple samples to be tested at the same time, which makes this method cost-effective. The major cost factor is incurred with probe labeling.

EFFECTS OF ENVIRONMENTAL MEDIA: It is advisable to use an isolation or enrichment protocol for the DNA or cells in order to obtain enough DNA to be within the limits of detection and to prevent adding extraneous contaminants such as salts and acids that can interfere with hybridization. A major problem with isolating DNA from soil samples is purity (Sayler and Layton, 1990). Steffen et al. (1988) found that high levels of humic acid inhibited the detection of target DNA by dot blot analysis. However, low amounts of humic acid and other contaminants such as clay did not interfere with detection by dot blot analysis. False positives can be obtained by non-specific binding to soil particles (Frederickson et al., 1989). Filtration methods need to be applied to aqueous matrices. However even so, the sensitivity of the technique may not be sufficient to detect organisms in water where bacteria are maintained in low concentrations (Saylor and Layton, 1990).

If a sufficient concentration of DNA can be isolated, DNA from water samples is usually of high-purity and large-fragment size (Sayler and Layton, 1990). One environmental factor that all direct DNA detection methods circumvent is the problem with organisms that are either non-culturable or difficult to isolate (Sayler and Layton, 1990).

(iii) Most-Probable Number-Hybridization

DESCRIPTION: Frederickson et al. (1988) developed methodology to enumerate the number of colonies by dot blot analysis by combining it with the most-probable-number technique. Batch cultures are grown in either selective or non-selective media and diluted out to infinity in microtiter plate. The dilutions are then transferred by aid of a filtration manifold onto nitrocellulose or nylon filters, lysed in situ, and hybridized. This technique allows cell enumeration without colony counts.

SENSITIVITY & SPECIFICITY: Frederickson et al. (1989) found that the limit of detection of an introduced P. putida and biovars of R. leguminosarum containing Tn5 was 10 and 10^2, respectively, cells per gram of soil using selective medium and probing with a 32P-labeled plasmid probe. The specificity is dependent on the probe.

EASE AND COST: Comparable to dot blot analysis (see above discussion).

EFFECTS OF ENVIRONMENTAL MEDIA: The quantitation depends both on the ability to recover viable cells in culture and the interactions of DNA with environmental contaminants (Frederickson, et al., 1989). These workers found that over time enumeration of cells being recovered from soil by MPN-hybridization was lower than the counts determined by florescent antibody enumeration. Moreover, using P. putida marked with Tn5, they found that cell counts were inversely proportional to clay and organic matter content. Steffen et al. (1989b) found this technique to give highly variable results in their monitoring of P. cepacia introduced into a freshwater microcosm.

(iv) Solution Hybridisation

DESCRIPTION: This technique involves the hybridization in solution of DNA with a specific probe. It is a screening procedure. As in the dot blot procedure, DNA is extracted directly from the environmental sample or from batch cultures thus avoiding the need to obtain isolated colonies. The DNA is isolated from the sample directly (Steffen and Atlas, 1990; Jain et al., 1988) or after enrichment steps for bacterial cells (Holben et al., 1988); or from a culture(s) grown from environmental samples. The DNA is then mixed with the probe and allowed to hybridize under standard conditions required for the type of probe used and the DNA being targeted (eg. 12 h\$50^0$ C). At the end of the hybridization period, the probe must be removed. This can be done with a combination of RNase and S1 nuclease to remove single stranded nucleic acid (if the probe is RNA) or hydroxyapatite chromatography (separates single from double stranded nucleic acid). The duplex DNA is recovered by ethanol precipitation and any free nucleotides are removed with a Sephadex G-25 column. The detection of positive cells is dependent on how the probe was labeled eg. measuring the amount of radioactivity in a scintillation counter.

SPECIFICITY: As in other hybridization techniques, the specificity of the technique is mainly dependent on the probe used and the nature of the target DNA. This technique does not detect viable cells unless a preculturing step is included. Like, the dot blot it is not possible to determine whether any positive signals are from viable cells or from extraneous DNA if this preculturing step is not included. Thus, it is not possible under these conditions to associate a positive signal with the presence of a specific microorganism. It is likewise not possible to determine if DNA sequences of interest are still contained in the introduced organism or has been transferred via conjugation for example to another strain or species.

Non-specific binding of the probe to sequences with some percentage of matching base pairs as a result of omission of the high stringency wash steps used with membrane hybridization procedure may give false positives. The purpose of the high stringency washes is to remove any duplexes formed by cross-reaction of the probe to similar sequences in DNA from non-target organisms. This procedure does not correct for this cross-reactivity. The specificity may be helped somewhat because there will be no non-specific binding of the DNA to the membrane itself or to an overloaded spot of DNA on the membrane.

SENSITIVITY: Since the amount of DNA present in the introduced microorganism is low in relation to the background, a concentration of total must be used that is within the limits of detection. Therefore, more DNA can be used in this procedure than in procedures that utilize DNA binding to a matrix support system. Steffen and Atlas (1990) reported detection limits in the range of 10^2 to 10^3 cells of P. cepacia per gram of sediment using a T7-generated radiolabeled RNA probe. This is approximately 10-fold more sensitive than that reported for the dot blot procedure.

EASE OF USE & TIME: This technique is relatively easy however it does involve processing of the environmental sample before hybridization. The ease of the technique is dependent on the complexity of the DNA extraction procedure and/or the cell separation procedure used. In those cases where the cells are first grown-up in the laboratory, the DNA must still be isolated however extensive DNA purification such as CsCl gradients is usually not necessary.

The other procedure that must be factored into complexity is probe preparation. The types of probes that can be used is more limited. Either an RNA probe generated by transcription from a vector designed for in vitro transcription or a single-strand DNA probe generated from a M13 phage system must be used. The reason single-stranded probes must be used is that the step to remove the labeled probe so that it will not interfere with the detection of true hybrids depends on the separation of single-stranded nucleic acids from duplexes. Nick-translated DNA probes rehybridize during the hybridization step thus forming duplexes that can not be differentiated in solution from the target duplex by procedures such as nuclease digestion or hydroxyapatite chromatography. Thus, this procedure introduces the necessity for an extra cloning step to prepare the vector. Column chromatography steps to replace the washing of the hybridization membrane can be more cumbersome. Since the kinetics of hybridization are faster in solution, this procedure can be adapted to yield more rapid assays than those using a solid-support matrix (Sayler and Layton, 1989).

COST: In addition to the cost of probe preparation, expensive equipment must be used such as a liquid scintillation counter or fluorometer since the procedure does not involve visual detection of label on a solid-support matrix or an autoradiogram. Also column chromatography can add to the expense.

EFFECTS OF ENVIRONMENTAL MEDIA: Extraneous material such as acids, alkalis, and salts can interfere with hybridization therefore it is necessary to isolate and purify the DNA. The major limitation in this regard is the ability to purify the DNA

from a given environmental medium or the ability to transfer bacteria out of that medium and into a laboratory medium.

(v) Southern blot analysis

DESCRIPTION: This technique also uses hybridization with a specific probe to detect DNA fragments that have been immobilized on a matrix support such as nitrocellulose. This procedure involves digesting the DNA with a restriction enzyme to obtain fragments of various sizes which can be fractionated by gel electrophoresis. The DNA in the gel is than transferred to a nitrocellulose or nylon filter for hybridization with the appropriate probe. The electrophoresis is usually done in an agarose gel and the DNA is transferred to the hybridization membrane by passive diffusion by denaturing the DNA in situ with alkali and neutralizing. The hybridization membrane is placed on the treated gel and a circuit of transfer buffer is created to allow the flow of buffer from the gel through the filter. The DNA that is carried in the buffer is trapped on the filter. Alternatively for small fragments that must be fractionated by polyacrylamide gel electrophoresis, the transfer is achieved by applying current through the system. After the DNA is deposited on the membrane, it is denatured and in the case of nitrocellulose, fixed onto the filter by baking (Wahl et al. 1987; Jain et al., 1988).

The probe can be either RNA or DNA, an isolated DNA fragment, the whole plasmid, or a synthetic oligonucleotide; radiolabeled or nonradioactive. The conditions of the hybridization will depend on the probe used, the T_m of the duplex, the complexity of the nucleotide sequence, the match of the probe to the target sequence, and the cation concentration (Wahl and Berger, 1987). The stringency of the wash is dependent on the specificity of the probe to the target sequence; i.e. the extent of match to the target sequence versus the presence of cross-reacting sequences in any of the background microorganism.

SPECIFICITY: The specificity of the technique is mainly dependent on the probe used and the nature of the target DNA. The restriction digestion and fractionation adds to the specificity of the analysis. The length of the fragment containing the foreign DNA is predictive for the restriction enzyme used. If the cut is internal in the DNA molecule inserted into the host, the length of the fragment isolated from any strain is expected to be the same as in the original plasmid molecule. Thus, the original plasmid is digested and electrophoresed along side of the isolated DNA as a reference. Because this procedure is more complex than solution hybridization or dot blots it is usually used for diagnosis and verification rather than screening.

False positives can be eliminated because a fragment of a predicted size could not be detected with the probe. The one exception would be if the insert had undergone a deletion or insertion, the fragment length will have changed. However, false

positives can usually be distinguished by the intensity of the band after reacting with the probe. Often false positives are not the result of one piece of DNA reacting but rather since the dot blot detects batch DNA, many closely-related sequences reacting. Once these sequences are fractionated out, the signal from the probe is dispersed and therefore much less intense and often can no longer be detected. An insertion or deletion does not usually affect the intensity unless it is very large and falls within the sequence that is complementary to the probe. If the DNA is isolated directly either out of the soil or from cells separated from the soil, it is not possible to determine whether any positive signals are from viable cells or from extraneous DNA. Thus, it is not possible under these conditions to associate a positive fragment with the presence of the introduced genetically engineered microorganism.

It is likewise not possible to determine if the DNA sequence of interest is still contained in the introduced organism or has been transferred (via conjugation) for example to another strain or species. However, if the cells can be cultured in the laboratory, Southern blot analysis can be used in conjunction with cell isolation procedures to verify the presence of the microorganism or the transfer of the DNA to a new organism. In the absence of being able to culture out the organisms, the movement of the DNA of interest can be discerned. By using a restriction enzyme that cuts once within the nucleotide sequence of interest, restriction length polymorphisms can be detected and can be one indicator of gene transfer. Restriction length polymorphisms can also indicate if the specific sequences have moved from the chromosome to a resident plasmid or from an introduced plasmid into the chromosome. Polymorphisms can also distinguish between two identically engineered organisms that have been co-introduced into the environment.

Jansson et al. (1989) could distinguish between two Pseudomonas spp. inoculated into a soil microcosm by the mobility of a ClaI restriction fragment containing the engineered nptII gene. Changes in restriction fragment mobilities can indicate a deletion or insertion in the target fragment. Using a marker gene, Jansson et. al (1989) were able to demonstrate by Southern hybridization a deletion in their engineered DNA sequence had occurred in cells after inoculation into soil. They used a ^{32}P single-stranded DNA probe to marker gene (npt II) inserted into Pseudomonas sp. strain B8. In addition to the major target fragment, an anomalous band that had a faster mobility than predicted was detected in Southern blots.

SENSITIVITY: Sensitivity is dependent on the 1) the probe used and 2) the efficiency of transfer. The efficiency of transfer is dependent on the size of the fragment and the gel concentration. Frequently, an acid depurination step is added prior to denaturing the DNA in the gel. The purpose of this step is to hydrolyze the DNA and thus increase the efficiency of transfer (Maniatis, 1984). Conversely, smaller fragments bind the nitrocellulose inefficiently. To detect a single copy gene in mammalian DNA, a minimum of 10 micrograms of DNA must be applied to the well. The mammalian haploid genome is 3×10^9 base pairs. The bacterial genome is in the range of 4×10^6 base pairs. A comparable quantity of DNA represents approximately 1000 bacterial cells; therefore, theoretically 1 microorganism in 1000 cells can be detected. In practice Holben et al. (1988) found that the hybridization of Southern blots was 5-fold less sensitive than dot blots, 0.02 picograms of the B. japonicum insert sequence per microgram of total DNA can be detected in dot blots using a ^{32}P single -stranded DNA probe as compared to 0.1 picograms of the sequence in Southern analysis using the same probe. The reduction in sensitivity was attributed to the inefficiency of transfer from the gel to the blot.

EASE OF USE & TIME: It is more time consuming and technically involved because it includes a digestion step(s), electrophoresis, and transfer (usually overnight) in addition to probe preparation and hybridization.

COST: Southern analysis is more costly than dot blots due to the restriction enzymes. In addition, expense is incurred due to the enzymes and labeled nucleotides required for probe preparation.

EFFECTS OF ENVIRONMENTAL MEDIA: Purity of DNA is very important in this technique. Restriction enzymes are very sensitive to impurities. Contaminants can alter the sequence specificity or inhibit cutting. Enzymes are sensitive to glycerol, magnesium concentration, and salts for example. Steffen et al. (1988) found that humic acid and montmorillonite clay inhibited restriction enzyme digestion. These investigators also showed that trace contaminants can also inhibit digestion. DNA preparations isolated form soil and sediment that were highly purified as determined by the $A_{260/280}$ ratios inhibited the digestion of target DNA by some restriction enzymes. Methylation of the DNA can inhibit cutting.

(vi) Polymerase chain reaction

DESCRIPTION: Table 12 lists studies in which PCR techniques have been used with environmental samples. This technique is used to amplify a targeted segment of DNA. Amplification is accomplished by repeated cycles of DNA synthesis primed at opposite ends of each complementary strand of the DNA duplex. The DNA between the these ends is replicated. Primers are prepared that are complementary to a short sequence at the 3' ends of each strand.

```
5' ------------------------------------------ 3'   strand 1
                                   3' ---- 5'     primer 1

3' ------------------------------------------ 5'   strand 2
5' ---- 3'                                        primer 2
```

The duplex DNA is denatured and allowed to anneal to the single stranded primers. DNA synthesis proceeds off of each target template via the annealed primers. The nucleotides are added to the 3'-OH of the primers, i.e., 5' to 3' chain elongation. The newly synthesized strand is then removed form its complement by denaturation and a new cycle of annealing and synthesis begins, now with double the number of templates so that amplification is exponential.

These cycles can be repeated with one reaction mixture 25 or more times to amplify a targeted sequence 4 X 10^6 times (Lewin, 1990). One reaction mixture is prepared for the 25 or more cycles. Included in this reaction mixture are the nucleotides needed for polymerization, the DNA preparation, the buffer, the specific primers, and the DNA polymerase.

The DNA polymerase used in this procedure is isolated from Thermus aquaticus, a thermophilic bacterium and is stable at temperatures up to 95^0 C, the temperature at which denaturation of the two strands is performed (Gelfand, 1989). Its optimal activity is in the range of 75-80^0 C. Typically, the DNA is denatured at 90-95^0 C, annealing of the primers proceeds at 40-60^0 C, and polymerization is carried out at 72^0 C. Heating and cooling intervals during the 25 cycles is controlled by a computerized temperature block. The primer length is around 20 base pairs and the fragment of DNA to be amplified can be up to 2 kilobases though for the purposes of diagnosis one considerable shorter is adequate.

Recently, two other thermostable polymerases have been identified that could be used for polymerase chain reaction amplification of DNA. The two are the VENT™ DNA polymerase, isolated from the thermophile Thermococcus litoralis which inhabits thermal vents on the ocean floor (Neuner et al., 1990), and the Pfu DNA polymerase from the thermophilic archaebacterium Pyrococcus furiosis (Bergseid et al., 1991). These two enzymes, unlike Taq

DNA polymerase, possess both the 5' to 3' DNA polymerase and 3' to 5' exonuclease-dependent proofreading activities. The proofreading activity will excise mismatched 3' terminal nucleotides from a primer:template complex and correctly incorporate nucleotides complementary to the template strand, a feature which enhances the fidelity of these DNA polymerases (Eckert and Kunkel, 1991).

SPECIFICITY: Specificity is determined by the primers. Sequences unique to the insert should be chosen for primer synthesis. The requirement that two separate sequences for each strand be complementary contributes to stringency of the technique. Stringency can be added by using a high annealing temperature. The kinetics of binding should also favor the primer binding to the targeted sequence over a competing sequence with imperfect match to the primers. Initially, primers will bind to the imperfect as well as the perfect matched sequences and the extent of the interference from cross-reacting sequences will depend on their molar concentration as compared to the target sequence and the amount of matched base pairs with the primer. As cycling and amplification proceeds, the target sequence will increase in concentration and should effectively out-compete any cross-reacting sequences.

$MgCl_2$ is an important component of the buffer that can significantly affect specificity. The optimal $MgCl_2$ concentration varies with the sequences being amplified and the primer. The concentration of primer can also affect specificity. Excessive primer concentration can amplify non-target cross-reacting sequences. Excessive concentrations of the Tag polymerase can also increase the non-specific products of the reaction (Saiki, 1990). Once optimization is achieved the reaction should be able to amplify a DNA sequence of a predicted length. The products of PCR can be electrophoresed in a sizing gel to identify the fragment. For reference, purified plasmid containing the insert DNA can be amplified in parallel and electrophoresed. It is highly improbable that cross-reacting non-target sequences even if they interfered and competed with the reaction would yield an identical fragment.

The availability of a restriction map of the fragment can improve specificity, i.e., the restriction map should match the restriction map of the insert in the reference plasmid. Lastly, any of the fragments can be hybridized to a specific probe using a Southern or dot blot By adding this step the identity of a specific DNA fragment is demonstrated by the complementarity of the two primers and a probe that matches a sequence unique from the primers.

Chaudhry et al. (1989) used this approach to study survival of bacteria in sterile lake water and sewage. A 0.3 kb sequence from napier grass was inserted into E. coli via pBR322 and three

complementary oligonucleotides were made, two to serve as primers and a third to serve as a probe in dot blot analysis. They found that the organism was undetectable by 6 days using plate counts on a selective medium however could still be detected up to 14 days by PCR combined with dot blot analysis. Since this technique utilizes isolated DNA it does not necessarily identify whether the DNA was isolated from a viable organism nor does it identify the organism that contained the DNA. These studies could not determine if the increased survival was an artifact of DNA from cell lysis or was a result of the detection of viable cells by the more sensitive method of PCR. Culturing techniques can be used in conjunction with PCR (provided the number of cells is in the range of detection by culturing methodology), either in isolating the DNA or to identify the source of specific DNA sequences.

SENSITIVITY: This technique can amplify DNA 1×10^6 times. Using PCR to amplify and dot blots with a ^{32}P-nick translated DNA probe, Steffen and Atlas (1988) were able to detect 1 cell of P. cepacia in 1 gram of sediment in a background of 10^{11} diverse organisms. This is a 10^3-fold increase in detection over hybridization procedures on non-amplified DNA (Steffen and Atlas, 1988). By using PCR it is possible to use the number of amplification cycles to calculate back to the original amount of DNA present and therefore, the number of cells in the sample.

EASE OF USE & TIME: The amplification is extremely easy to perform because it involves a computerized incubation system. The more technical aspects of the reaction are selecting the primers and optimizing the reaction.

COST: The expense is incurred in buying the cycler and synthesizing the target-specific primers. The reaction components (except for the primers) are sold as a kit and are fairly costly.

EFFECTS OF ENVIRONMENTAL MEDIA: The DNA can be fairly impure. However, contaminants from environmental samples may inhibit the polymerase (Chaudhry et al., 1989). Any requirements for purity that subsequent manipulations require, such as restriction digests, are bypassed because the sample is amplified.

(vii) Multiplex amplification

DESCRIPTION: A modification of the polymerase chain reaction, multiplex amplification, allows the simultaneous amplification of multiple genetic loci. Within one reaction mix, multiple sets of primers are used to amplify unique sequences to identify corresponding genes within a mixed DNA population. The technique was developed by Chamberlain et al. (1988) to detect multiple deletion mutations within different sites of the Duchenne muscular dystrophy locus but it should be readily adaptable to environmental analysis. For instance, it can be used to follow the fate of organisms in a field test involving a multi-species release or the fate of multiple genes in a field test involving one organism with multiple insertions.

The basic parameters in the use of multiplex amplification are as described above for the polymerase chain reaction. An increase in reagents including the Taq polymerase must be included in order to accommodate the increase in target DNA. Dot blots, reverse dot blots, or gel electrophoresis can be used to detect the diagnostic fragment. If identification is based on size fractionation, the primers must amplify corresponding fragments of unique lengths that can be differentiated in gel electrophoresis.

SPECIFICITY & SENSITIVITY: Optimization of conditions may be necessary for each set of primers since not all primer-target duplexes in the mix are expected to have identical melting temperatures.

EASE OF USE & TIME: This adaptation of PCR simplifies the analysis of complex field releases involving multiple organisms or multiple gene insertions in one organism. Using standard hybridization reactions or standard PCR, multiple probes or primers have to be used and this involves a corresponding number or reaction mixtures, blots, sample preparations etc. This technique especially when used in conjunction with reverse dot blot analysis reduces the number of steps and the amount of time required for detection. Chamberlain et al. (1988) reported a time of 5 hours for the total analysis. With the reduction in time and complexity of the analysis, a corresponding reduction in cost is achieved.

EFFECTS OF ENVIRONMENTAL MEDIA: Contamination from extraneous DNA could be a problem. If the reaction was limited to 25 cycles, Chamberlain et al. found that 3 - 5% contamination could be tolerated.

(viii) Reverse dot blot

DESCRIPTION: Reverse dot blot analysis is a detection method for polymerase chain reaction products. A modified nucleoside triphosphate, e.g. biotinylated uracil, is added to the reaction mix. During chain elongation the modified nucleoside triphosphate is incorporated into the growing chain resulting in an amplified product that is labeled. Alternatively, a labeled oligonucleotide can be used as a primer. In this case the label is incorporated in the 5' end of the product rather than dispersed through the extended portion of the chain. For example, biotin or fluorescent dyes such as fluorescein or rhodamine can be used to either end-label the 5' end of the primer or be incorporated into the oligonucleotide during synthesis. In either case, the amplified fragment is labeled during the reaction.

The PCR product can then be "captured" with a nonlabeled probe that has been immobilized on a solid-support such as nitrocellulose. Immobilization is accomplished by the addition of a polythymidine tail to each oligonucleotide by terminal deoxyribonucleotidyltransferase. Exposure to UV light causes covalent coupling of the thymidine residues to the nylon membrane. Detection of the product on the nitrocellulose membrane is via the fluorescent dye that remains after the appropriate wash has been performed or in the case of biotin through an enzymatic reaction via a streptavidin-enzyme complex. For example, Saiki et al. (1989) used a streptavidin-horseradish peroxidase conjugate to detect PCR fragments hybridized to probe. A third variation on this technique is to label the PCR products directly using biotin during first strands synthesis. Streptavidin is then used to "capture" a fluorescent probe-target duplex.

SPECIFICITY & SENSITIVITY: Hybridizations that employ short oligonucleotide probes are highly specific (see discussion above on nucleic acid probe construction). Under stringent hybridization conditions, a single base pair mismatch will destabilize the complex and prevent the formation of a probe-target duplex. One major difficulty encountered with the use of multiple probes is that not all the probes may be sequence-specific under identical hybridization conditions leading to inconclusive results. The length, choice of sequence, and/or concentration of the probe applied to the filters can be adjusted to meet uniform hybridization requirements. Another consideration, is that the length of the poly dT tail and the amount of UV exposure can effect the hybridization efficiency.

The main advantage of this technique is flexibility. For multiple gene sequences that require different primers and probes, reverse dot blots can be used to detect sequences synthesized via multiplex amplification. Alternatively if a

family of genes are inserted into a microbe, they can be followed simultaneously by amplifying the polymorphic region of the genes using one set of primers specific to common flanking sequences. Probes that incorporate the variation can be used to screen the environmental sample. Likewise, polymorphisms in a common sequence can be used to detect an introduced organism in a dense background or to differentiate between organism that have been released into the environment simultaneously.

Using species specific probes to a common gene, unrelated microbes can be monitored concomitantly. This technique can also be adapted to detect predicted changes in an inserted sequence. It can be used in conjunction with culturing techniques for screening species into which horizontal transfer of an introduced sequence has occurred. For instance if during plate hybridization it is determined that the insert has been transferred to an unidentified species, positive colonies can be pooled and DNA isolated. PCR amplification of a ribosomal gene, for example, followed by screening using a panel of immobilized species-specific probes can be used to screen possible recipients of the genetic exchange.

The sensitivity of the amplification can be decreased in protocols using one set of primers because of competition. Sensitivity could also be reduced as a result of the adjustments in the hybridization conditions that may be needed to accommodate differences in the oligonucleotide probes. Sensitivity can also be decreased if label is incorporated via 5' end-labeling of the primer.

EASE OF USE & TIME: This technique consolidates a number of separate procedures. The labeling is done during the amplification thereby eliminating the need for a separate labeling step. For those screening protocols requiring multiple probes, the immobilization of probe rather than target DNA eliminates the need for multiple hybridization reactions and subsequent filters to be washed. The use of a non-radioactive probe also eliminates the complexity inherent in the precautions required with the use of radioactivity.

Once conditions are optimized, the amplification, hybridization, and color development can be accomplished in as little as 3 to 4 hours. Used in conjunction with multiplex amplification, it permits the concurrent analysis of multiple genetic loci thereby readily allowing the simultaneous detection of organisms or insertions.

(c) Probe visualization

DESCRIPTION: Liquid scintillation is used in procedures that involve solution hybridization. Hybridization of DNA attached to a solid matrix routinely involves the use of autoradiography or some form of fluorography. Autoradiography is the exposure of film by radioactive particles whereas fluorography is the exposure of the film by light particles generated from the interaction of radioactive particles with added fluors. The type of fluor used is dependent on the energy and range of the emissions from the isotope. Gels or blots obtained using ^{35}S radiolabelled probes are treated with a solution containing 2,5 diphenyloxasole (PPO) or a commercially prepared fluor. ^{32}P has a longer range and therefore, an external $CaWO_4$ intensifying screen can be placed next to the film (Bonner, 1987). Autoradiography is used to quantitate the amount of target DNA since intensity is linear up to absorbances of 1 unit.

SENSITIVITY AND SPECIFICITY: Fluorography is commonly used to enhance the sensitivity. For instance, the use of an intensifying screen (s) can increase the sensitivity of detection with a ^{32}P-labeled probe 5 to 20 fold. Pretreating the blot or gel with a fluor can increase the sensitivity of detection using a ^{35}S-labeled probe 5 to 15 fold. The most sensitive method is the use of a sandwich composed of a screen-film-blot (dried gel)-screen arrangement. The second screen acts by increasing the backscatter of the beta rays from the ^{32}P from the blot. Longer exposure increases the sensitivity and allows detection of weak positives. However, this is accompanied by a decrease in resolution (Bonner, 1987). If the sample is very hot, autoradiography is used. Some combination of autoradiography followed by reexposure using fluorography can be used to obtain the best combination of resolution and sensitivity. In addition, exposure time can be varied to optimize resolution and sensitivity.

(d) Use of nucleic acid hybridization in analyzing microbial species composition

DNA-DNA hybridization studies are an important tool for distinguishing between closely related bacteria and can be used for their identification. However, the classical DNA hybridization methods are too cumbersome to be used to identify a large number of strains. DNA must be purified from each of the strains tested. Furthermore, the various DNA hybridization techniques are relatively tedious and time-consuming (Schleifer and Stackebrandt, 1983). Recent studies have shown that the blotting of whole cells onto membrane filters, followed by lysis and hybridization with labelled chromosomal DNA from reference strains, is a quick method of identifying bacterial isolates (Roberts et al., 1984, 1987; Moromoti et al. 1988). Examples of whole cell DNA probes are given in Table 5. Ezaki et al. (1989) are using photobiotin-labelled whole-cell-probes to hybridize reference DNAs bound to microdilution plates. For identification of an unknown strain it is then sufficient to extract its DNA from 1 to 3 ml of overnight culture, to label it with photobiotin and to probe the immobilized reference DNA.

The advantage of the whole-cell DNA probe method is that it is simple, quick and that a large number of strains can be handled at the same time. Moreover, specifically designed probes are not necessary. Disadvantages are mainly the lack of specificity and the fact that only culturable organisms can be identified. Cross-hybridization of whole cell DNA probes with other related bacteria is rather common (Grimont et al., 1985; Hyypiä et al., 1985) and stringency has to be carefully controlled.

Another approach is the use of comparative sequence analysis of 16S and 23S ribosomal RNA (or any gene of interest) in the elucidation of phylogenetic relationships in prokaryotes (Woese, 1987). The rRNA molecules contain regions of highly conserved sequences interrupted by more variable sequences. Because of the differences in the degree of conservation within the molecule, rRNA can be used to design probes with specificity ranging from species to kingdom level. The use of rRNA as target nucleic acid has even more advantages. First, rRNAs are present in very high copy numbers (about 10^4 molecules per E. coli cell). Thus, a considerable increase of sensitivity (at least thousand times) can be achieved by targeting rRNA instead of DNA. Another advantage is the single strandedness of rRNA which avoids problems with target renaturation. In contrast to mRNA, rRNA is quite stable and rigid methods assuring high yields, can be used for target extraction without any damage to rRNA (chromosomal DNA would be considerably degraded). The use of probes with different specificities allows rapid classification of an unknown isolate (Regensburger et al., 1988). As first step, a universal probe is applied as a control for bound target. By repeated use

of probes with increasing specificity unknown isolates are characterized with a few steps.

These polynucleotide probes are sensitive and are not affected by minor nucleotide mismatches. The first defined DNA probes were derived from cloned DNA. Therefore, it was rather time consuming and difficult to prepare these probes in large quantities and at constant quality. Moreover, care must be taken to prepare probes free of vector sequences in order to avoid unspecific hybridizations. More recently, the use of polymerase chain reaction methodologies allows preparation of vector-free nucleic acid probe in a relatively short amount of time.

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TABLE 1. DETECTION OF GENE TRANSFER IN STERILE SOIL: RECIPIENT SELECTION AND DONOR COUNTERSELECTION.

REFERENCE.	EXPERIMENTAL CONDITIONS.	MODE OF GENETIC TRANSFER.	METHOD FOR SELECTION OF EXCIPIENTS AND COUNTER-SELECTION OF DONORS.
Weinberg & Stotzky, (1972)	Intraspecific crosses between E. coli auxotrophic recipient & prototrophic donor.	Conjugal transfer of chromosomal genes, genetic recombination.	Selection of prototrophs / Resistance of recipients to Sm.
Graham & Istock, (1978 & 1979)	Intraspecific crosses between B. Subtilis in autoclaved potting soil.	Transformation of linked chromosomal genes.	Selection of auxotrophs / Resistance of recipients to Sp & Lm.
Trevors & Oddie, (1986)	Intraspecific crosses between E. coli in amended and unamended autoclaved soil.	Conjugal R-plasmid transfer.	Selection of transconjugants by expression of plasmid encoded Tc^r / Resistance of recipients to Nx.
van Elsas et al., (1987)	Intergeneric crosses between B. cereus and B. subtilis. Gamma irradiated soil, 20-22% moisture (60% MHC).	Conjugal plasmid transfer (pFT30).	Selection of transconjugants by expression of plasmid encoded Tc^r / Resistance of recipients to Em.
Krasovsky & Stotzky, (1987)	Intraspecific crosses between prototrophic & auxotrophic E. coli in autoclaved amended & unamended soil. Moisture -33kPa.	Conjugal transfer of chromosomal DNA, and genetic recombination.	Selection of prototrophic recombinants on minimal agar / Resistance of recipients to Sm.

TABLE 1. (Continued)

REFERENCE.	EXPERIMENTAL CONDITIONS.	MODE OF GENETIC TRANSFER.	METHOD FOR SELECTION OF EXCIPIENTS AND COUNTER-SELECTION OF DONORS.
Rafii & Crawford, (1988)	Inter- & Intraspecific crosses between streptomycetes. Autoclaved soil, moisture 60% MHC.	Conjugal plasmid transfer & plasmid mobilization.	Selection of transconjugants by expression of plasmid encoded Ts^r or Nm^r, and production of melanin/ Resistance of recipients to various antibiotics.
Lorenz *et al.*, (1988)	Competent *B. subtilis* (auxotrophic, TrpC2) attached to sterile sand grains.	Transformation of naked DNA isolated from *B. subtilis* 168 prototroph.	Transformants selected on modified minimal salts medium (Aardema *et al.*, 1983)
Wellington *et al.*, (1988)	Inter- & Intraspecific crosses between strains of streptomycetes. Autoclaved soil, amended & unamended, moisture 40% MHC.	Conjugal plasmid transfer (pIJ673).	Selection of transconjugants by expression of plasmid encoded Ts^r and Nm^r/ Resistence of recipients to Sm.
Zeph *et al.*, (1988)	Coinoculation of phage P1 lysates and *E. coli* recipients, inoculation of lysogenic *E. coli* donor and non-lysogenic recipient. Autoclaved soil, moisture 21.1-24.4% (wt./wt.).	P1 phage infection, formation of lysogens.	Selection of lysogens by the expression of phage Cm^r & Hg^r genes / Counterselection of auxotrophic *E. Coli* lysogenic donor using MM.

TABLE 1. (Continued)

REFERENCE.	EXPERIMENTAL CONDITIONS.	MODE OF GENETIC TRANSFER.	METHOD FOR SELECTION OF EXCIPIENTS AND COUNTER-SELECTION OF DONORS.
Richaume *et al.*, (1989)	Intergeneric crosses between *E. coli* donor and *Rhizobium freudii*, autoclaved soil, various soil conditions.	Conjugal plasmid transfer (CpRK2073:Tn5)	Selection of transconjugants by expression of antibiotic resistance genes / Counter-selection of donor by the use of a selective carbon source and resistance of recipient to Nx.
Herron & Wellington, (1990)	Co-inoculation of KC301 phage and *S. lividans*, soil amended with 1% soluble starch, 1% chitin.	Actinophage infection, formation of lysogens.	Selection of lysogens by expression of phage Ts^r gene.
Top *et al.*, (1990)	Intergeneric crosses between *E. coli* donor and *A. eutrophus*, autoclaved soils, moisture 75% field capacity.	Conjugal plasmid transfer, and plasmid mobilization.	Selection of transconjugants by expression of plasmid borne heavy metal resistance to Co, Cd & Zn / Counterselection of donor due to non-expression of heavy metal plasmid markers in donor *E. coli*

Abbreviations: Cd; Cadmium. Cm; Chloramphenicol. Co; Colbalt. Em; Erythromycin. Hg; Mercury. Lm; Lincomycin. MM; Minimal medium. Nx; Nalidixic Acid. Nm; Neomycin. Sp; Spectinomycin. Sm; Streptomycin. Tc; Tetracycline. Ts; Thiostrepton. Zn; Zinc.

(Table reproduced from, Cresswell & Wellington, 1991)

TABLE 2. DETECTION OF GENE TRANSFER IN NON-STERILE SOIL: RECIPIENT SELECTION AND DONOR COUNTERSELECTION.

REFERENCE.	EXPERIMENTAL CONDITIONS.	MODE OF GENETIC TRANSFER.	METHOD FOR SELECTION OF EXCIPIENTS AND COUNTER-SELECTION OF DONORS.
Schilf & Klingmuller, (1983)	Crosses with E. coli donor to artificially raised numbers of indigenous bacteria ca. 10^9 CFU/g soil.	Conjugal R-plasmid transfer.	Selection of transconjugants by selection of plasmid encoded antibiotic resistance genes. No counterselection, no transfer detected.
van Elsas et al., (1987)	As in Table 1.	As in Table 1.	As in Table 1.
Krasovsky & Stotzky, (1987)	As in Table 1.	As in Table 1.	Additional requirement for selection of auxotrophic recombinants with Pc 8µg/ml.
Trevors & Starodub, (1987)	Intraspecific crosses between E. coli strains. Various soil conditions.	Conjugal R-plasmid transfer.	Selection of transconjugants by expression of plasmid encoded Tc^r / Resistance of recipients to Nx.
Zeph et al., (1988)	As in Table 1.	As in Table 1.	As in Table 1.
van Elsas et al., (1988)	Intraspecific crosses between Pseudomonads. Rhizosphere, bulk & amended bulk soil, moisture 20% (wt./wt.)	Conjugal R-plasmid transfer.	Selection of transconjugants using Kings B medium and expression of plasmid encoded Km^r and Tc^r / Resistance of recipient to Rp.

TABLE 2. (Continued)

REFERENCE.	EXPERIMENTAL CONDITIONS.	MODE OF GENETIC TRANSFER.	METHOD FOR SELECTION OF EXCIPIENTS AND COUNTER-SELECTION OF DONORS.
Germida & Khachatourians, (1988)	Co-inoculation of transducing P1 phage lysates and auxotrophic *E. coli* K12-GK401, soil amended.	Transduction by phage P1.	Selection of recipient *E. coli* using EMB medium and Sm^r, selection of transductant phenotypes using MM, Tc and leucine or threonine.
Wellington *et al.*, (1990)	As in Table 1.	As in Table 1.	Selection as in Table 1, use of a streptomycete selective medium 'RASS' to aid the counterselection of indigenous organisms.
Herron & Wellington, (1990)	As in Table 1. Soil unamended.	As in Table 1.	Selection of *S. lividans* recipient by resistance to Sm. Use of streptomycete selective medium 'RASS'. No lysogens detected (Ts^r Sm^r). Phage survived for 39 days.
Top *et al.*, (1990)	As in Table 1. Soil unamended & amended.	As in Table 1.	Selection of transconjugants on Tris azelate medium and expression of plasmid encoded heavy metal resistance genes to Zn^{2+} & Cd^{2+}. Counter-selection of indigenous bacteria by replica plating onto 2nd medium containing Co^{2+} and T Tc.

TABLE 2. (Continued)

REFERENCE.	EXPERIMENTAL CONDITIONS.	MODE OF GENETIC TRANSFER.	METHOD FOR SELECTION OF EXCIPIENTS AND COUNTER-SELECTION OF DONORS.
Henscke & Schmidt, (1990)	E. coli SM10 plasmid donor, study of transfer to indigenous microflora.	Conjugal plasmid mobilization.	Selection of transconjugants by the expression of Tb^r & growth in the presence of IPTG / Counterselection of donor facilitated by the poor survival; rapid decline after 25 days.
Smit & van Elsas, (1990)	P. fluorescens plasmid donor (R2F RP4::pat Rp^r), study of transfer to indigenous microflora.	Conjugal RP4 plasmid transfer.	Selection of transconjugants by expression of plasmid encoded Tc^r / Deselection of donor facilitated by the use of a donor specific virulent phage.

Abbreviations: Cd; Cadmium. Co; Cobalt. EMB; Eosin methylene blue medium. IPTG; Isopropyl-β-D-thiogalactoside. Km; Kanamycin. MM; Minimal medium. Nm; Nalidixic acid. Pc; Penicillin. RASS; Reduced arginine starch medium. Rp; Rifampicin. Sm; Streptomycin. Tc; Tetracycline. Tb; Tobramycin. Zn; Zinc.

(Table reproduced from, Cresswell & Wellington, 1991).

TABLE 3. Plasmid transfer rates in laboratory, microcosm, and *in situ* soil and aquatic systems. Rates given in terms of transconjugants per donor cell (D) where possible; other rates are given in terms of the transconjugants per recipient cell (R).

PLASMID	PLASMID TYPE	DONOR/RECIPIENT	CONDITIONS	RATE	REF
pBC16	conjugative	*B. subtilus*	lab: broth	10^{-2}–10^{-5}D	Koehler and Thorne (1987)
pBC16		*B. subtilus*/ *B. licheniformis*	lab: broth	10^{-3}D	
pBC16		*B. subtilus*/ *B. megaterium*	lab: broth	10^{-6}D	
RP4	conjugative	*E. coli*/ *Thiobacillus novellus*	lab: filter mating	10^{-5}D	Davidson and Summers (1983)
RP4		*Thiobacillus novellus*/ *E. coli*		1.0D	
Sa	conjugative	*E. coli*/ *E. coli*	lab: combined spread plate	10^{-3}D	Walter *et al*. (1987)
Sa			lab: broth	10^{-6}D	
R68.45	conjugative	*P. aeruginosa*/ *P. aeruginosa*	lab: broth	10^{-4}D	O'Morchoe *et al*. (1988)
R68.45			lab: sterile lake water	10^{-5}D	
R68.45			lab: sterile lake water plus filter	10^{-2}D	
R68.45			field: non-sterile lake water	10^{-8}D	

TABLE 3. (Continued)

PLASMID	PLASMID TYPE	DONOR/RECIPIENT	CONDITIONS	RATE	REF
PMQ1	conjugative	*P. aeruginosa*/ *P. aeruginosa*	lab: broth with sterile stone & filter	10^{-1}R	Bale *et al*. (1987)
PMQ1			lab: sterile river water,	10^{-2}R	
PMQ1			field: unencl. nonsterile river water, sterile stone & filter	10^{-4}R	
PMQ1			field: unencl. nonsterile water, nonsterile stone , & filter	10^{-7}R	
pBR325	mobilizable	*E. coli*/*E. coli*	lab: broth & mobilizer plasmid	10^{-5}D	McPherson and Gealt (1986)
pBR325			lab: raw,sterile wastewater & mobilizer plasmid	10^{-7}D	
pFT30	conjugative	*Bacillus cereus*/ *B. subtilis*	lab: filter	10^{-6}D	Van Elsas (1987)
pFT30			lab: sterile soil	10^{-7}D	

TABLE 3. (Continued)

PLASMID	PLASMID TYPE	DONOR/RECIPIENT	CONDITIONS	RATE	REF
pFT30			lab: nonsterile soil	0D	
pFT30			lab: nonsterile soil & bentonite	10^{-7}D	
R plasmids	conjugative	*Klebsiella*/ *Klebsiella*	lab: nonsterile radish rhizosphere	10^{-6}D	Talbot *et al*. (1980)
pRTr5a	conjugative	*Rhizobium trifolii*/ *R. leguminsarum*	lab: in situ	?	Hooykaas *et al*. (1981)

TABLE 4. DNA extraction from the environment

Reference	Environment	Detection	Notes
Abbot *et al.* (1988)	HIV/HTLV in Human T-cells	?	Cells isolated, DNA extracted, PCR amplified.
Attwood *et al.* (1988)	Rumen	50ng DNA ($2x10^7$ cfu) *(Bacteroides)*	Indirect lysis, hybridisation.
Bej *et al.* (1990)	Water	1-10 fg DNA or 1-5 cfu/100ml *E. coli*	Indirect lysis, PCR, hybridisation.
Deflaun *et al.* (1986)	Water	volume dependent	Indirect lysis Hoechst 33258.
Deflaun & Paul (1989)	Fresh water/ marine	167 fg Herpes DNA in *E. coli* plasmid	Indirect lysis, hybridisation.
Fredrickson *et al.* (1988)	soil	10-100 cfu/g *(Rhizobium/ E. coli)*	Enrichment, in direct lysis, hybridisation, MPN.
Fuhrman *et al.* (1988)	Water	25-50% of total bacterial	Indirect lysis, agarose gel electrophoresis, Hoechst 33258.
Hay *et al.* (1990)	Cerebrospinal fluid	65 cfu-/ml *(Treponema)*	Direct lysis, PCR, hybridisation.
Holben *et al.* (1988)	Soil	0.2 pg DNA/10^4 cfu/g *(Bradyrhizobium)*	Indirect lysis, hybridisation.
Knight *et al.* (1990)	Water	7-15 ng DNA/2.6 x 10^3 cfu/ml *(Salmonella)*	Indirect lysis, hybridisation.
Kuritza *et al.* (1986)	Faeces	? *(Bacteroides)*	Indirect lysis, hybridisation, (radioactive count).

TABLE 4. (Continued)

Reference	Environment	Detection	Notes
Kuritza & Salyers (1985)	Faeces	2% of population (Bacteroides)	Indirect lysis, hybridisation (radioactive count)
Nannipieri et al. (1986	Soil	?	Indirect lysis, diphenylamine assay.
Ogram et al. (1987)	Sediment	?	Direct lysis, hybridisation.
Paul & Carlson (1984)	Fresh/marine water	40-91% total	Indirect lysis, Hoechst 33258.
Paul & Myers (1982)	Water	95-100% total DNA	Indirect lysis, Hoechst 33258.
Preston et al. (1990)	Waste water	10^4 pfu (Poliovirus)	Indirect lysis, hybridisation.
Somerville et al. (1989)	Marine	1-3 cfu/ml	Indirect lysis, agarose gel elect.
Steffan & Atlas (1988)	Sediment	100 cfu/100g (Pseudo monas)	Indirect lysis, PCR, hybridisation.
Steffan et al. (1988)	Soil/sediment	?	Direct lysis/ Indirect lysis, agarose gel, electrophoresis, absorbance.
Torsvik & Goksøyr (1977)	Soil	?	Indirect lysis, DABA.
Torsvik (1980)	Soil	?	Indirect lysis, absorbance.
Torsvik et al. (1990)	Soil	?	Indirect lysis, T_m analysis.

Also see reviews by Hazen & Jiminez (1988); Holben & Tiedje (1988); Ogram & Sayler (1988); Lidstrom (1989); Salyers (1989); Trevors & Van Elsas (1989).

TABLE 5: Examples of whole cell DNA probes

Identification	Method	Label	Reference
Acinetobacter spp.	whole-cell dot blot	^{125}I	Tjernberg et al., 1989
Bacteroides spp.	whole-cell dot blot	^{32}P	Roberts et al., 1987
Bacteroides spp.	whole-cell dot blot	^{32}P	Morotomi et al., 1988
Campylobacter pylori	dot blot	^{32}P	Wetherall et al., 1988
Campylobacter spp.	dot blot	Acetyl-amino-fluorene	Chevrier et al.,
Chlamydia trachomatis	dot blot	^{32}P	Hyypiä et al., 1985
Enterobacter agglomerans	whole-cell dot blot	^{125}I	Tjernberg et al., 1989
Mobiluncus spp.	whole-cell dot blot	^{32}P	Roberts et al., 1984
Mycobacterium leprae	dot blot	^{32}P	Athwal et al., 1984

TABLE 6: Examples of DNA probes derived from 16 or 23S rRNA targets

Specificity	Type of Probe	Detection	Reference
Universal probe	Oligonucleotide (16S)	all organisms	Giovannoni et al., 1988
	Cloned fragment (23S)	all organisms	Regensburger et al., 1988
Kingdom	Oligonucleotide (16S)	eucaryotes	Giovannoni et al., 1988
	Oligonucleotide (16S)	eubacteria	Giovannoni et al., 1988
	Cloned fragment of 23S rRNA	eubacteria	Regensburger et al., 1988
	Oligonucleotide (16S)	eubacteria	Chen et al., 1989
	Oligonucleotide (16S)	archaebacteria	DeLong et al., 1989
Supragenus	Cloned fragment (23S)	*Micrococcus* - *Arthrobacter*	Regensburger et al., 1988
Genus	Cloned fragment (16S)	*Mycoplasma*	Göbel and Stanbridge, 1984
	Cloned fragment (23S)	*Pseudomonas*	Festl et al., 1986
	Oligonucleotide (16S)	*Proteus*	Haun and Göbel, 1987
	Oligonucleotide (not specified)	*Legionella*	Enns, 1988
Species	Cloned rRNA gene fragment	*Legionella pneumophila*	Grimont et al., 1985
	Oligonucleotide (16S)	*Mycoplasma pneumoniae*	Göbel et al., 1987
	Oligonucleotide (16S)	*Proteus* species	Haun and Göbel, 1987
	Oligonucleotide (16S)	Species associated with human periodontitis	Chuba et al., 1988
	Oligonucleotide (16S)	*Bacteroides succinogenes*, *Lachnospira multiparus*	Stahl et al., 1988
	Oligonucleotide (16S)	*Clostridium difficile*	Wilson et al., 1988
	Oligonucleotide (16 and 23S)	*Neisseria gonorrhoeae*	Rossau et al., 1989
	Oligonucleotide (not specified)	*Mycoplasma pneumoniae*	Enns, 1988
		Mycobacterium avium complex	Drake et al., 1987
	Oligonucleotide (16S)	*Vibrio anguillarum*	Rehnstam et al., 1988
Subspecies	Oligonucleotide (16S)	*Bacteroides succinogenes* strains	Stahl et al., 1988

TABLE 7: Examples of DNA probes derived from randomly cloned DNA fragments

Specificity	Origin	Identification	Reference
Genus	Genomic DNA	*Salmonella* spp.	Fitts et al., 1983
	plasmid	*Spiroplasma* spp.	Nur et al., 1986
Species	Genomic DNA	*Bacteroides thetaiotaomicron*	Salyers et al., 1983
	Genomic DNA	*Bacteroides vulgaris*	Kuritza and Salyers, 1986
	Genomic DNA	*Bacteroides fragilis*	Kuritza et al., 1986
	Oligonucleotide (not specified)	*Campylobacter jejuni*	Bryan et al., 1986
	Genomic DNA	*Chlamydia trachomatis*	Palva, 1985
	Genomic DNA	*Corynebacterium sepedonicum*	Verreault et al., 1988
	Genomic DNA	*Mycobacterium tuberculosis*	Eisenach et al., 1988
	Genomic DNA	*Mycobacterium leprae*	Clark-Curtiss and Docherty, 1989
	Genomic DNA	*Mycoplasma hyorhinis*	Taylor et al., 1985
	Genomic DNA	*Mycoplasma pneumoniae*	Hyman et al., 1987
	Genomic DNA	*Mycoplasma gallisepticum*	Santha et al., 1987
	Genomic DNA	*Mycoplasma*-like organism	Lee et al., 1988
	Cryptic plasmid	*Neisseria gonorrhoeae*	Totten et al., 1983
	Genomic DNA	*Rickettsia prowazekii*	Regnery et al., 1986
	Genomic DNA	*Streptococcus oralis*	Schmidhuber et al., 1988
	Genomic DNA	*Thiobacillus* spp.	Yates et al., 1986
Subspecies	Genomic DNA	Strain of *Bacteroides ruminicola*	Attwood et al., 1988
	Cryptic plasmids	Strains of *Lactobacillus delbrueckii* and *L. reuteri*	Tannock, 1989
	Genomic DNA	Strains of *Thiobacillus ferrooxidans*	Yates et al., 1986

TABLE 8: Examples of DNA probes from genes

Specificity	Gene coding for	Identification	Reference
Strains of different species	Delayed hypersensitivity factor	*Listeria* strains	Notermans et al., 1989
	β-Lactamases	*E. coli* and *Pseudomonas aeruginosa*	Cooksey et al., 1985
	β-Lactamases	various gram-negative eubacteria	Huovinen et al., 1988
	Different antibiotic resistance genes	various eubacteria	Halbert, 1988
	Elongation factor Tu	*Mycoplasma* spp.	Yogev et al., 1988
	Pili and outer membrane proteins	*Neisseria* spp.	Aho et al., 1987
	Hemolysin	*Vibrio parahaemolyticus*, *V. hollisae*	Nishibuchi et al., 1985; 1986
	Virulence-associated plasmid	*Yersinia* spp.	Gemski et al., 1987; Robins-Browne et al., 1989
Species	Surface protein	*Anaplasma marginale*	Eriks et al., 1989
	Hemolysin	*Listeria monocytogenes*	Datta et al., 1988
	Capsular antigen	*Salmonella typhi*	Rubin et al., 1985
	Deoxyribonuclease	*Staphylococcus aureus*	Liebl et al., 1987
	Autolysin	*Streptococcus pneumoniae*	Pozzi et al., 1989

TABLE 8 (continued)

Specificity	Gene coding for	Identification	Reference
Strains	Delta-endotoxin	*Bacillus thuringensis* strains	Prefontaine et al., 1987
	Gentamicin resistance	*E. coli* strains	Groot Obbink et al., 1985
	Heat-labile and heat-stable toxins	enterotoxigenic *E. coli* strains	Moseley et al., 1982; Sommerfelt et al., 1988a,b
	Invasion process	*E. coli* and *Shigella* spp.	Wood et al., 1986; Gomes et al., 1987
	Shiga-like toxi	enterohemorrhagic *E. coli* strains	Bohnert et al., Meyer et al., 1989
	Adhesin-factor	enteropathogenic *E. coli* strains	Nataro et al., 1985
	Capsular antigens	encapsulated *E. coli* strains	Roberts et al., 1988
	Toluene degradation	*Pseudomonas putida* strains	Sayler et al., 1985
	Exfoliative toxin	*Staphylococcus aureus* strains	Rifal et al., 1989
	Virulence (plasmid)	*Yersinia enterocolitica*	Jagow and Hill, 1986
	Mercury resistence	whole-community genome (water)	Barkay et al., 1989

TABLE 9: Non-radioactive reporter groups for indirect detection of hybrids

Reporter group	Detection	Reference
Biotin	Labelled avidin, Labelled antibody	Langer et al., 1981; Leary et al., 1983; Kumar et al., 1988
N-2-Acetylamino-fluorene	Labelled antibody	Tchen et al., 1984; Landegent et al., 1984
Sulfone	Labelled antibody	Verdlov et al., 1974; Syvänen et al., 1986
4-dinitrophenyl	Labelled antibody	Vincent et al., 1982; Keller et al.,1989
Digoxygenin	Labelled antibody	Heiles et al., 1988

Table 10. Applications of hybridization methods in environmental studies.

ORGANISM	TARGET DNA	DETECTION METHOD	SAMPLE MATRIX	PURPOSE
E. coli[1]	P1 Cm *cts*::Tn*501*	dot blot on DNA extracted from clonies isolated on selective media using biotinylated probe to *repA*	sterile and non-sterile soil	verify transduction
Streptomyces sp.[2]	conjugative plasmid, pIJ303	colony hybridization with selective media using a nick translated biotinylated probe made from the whole plasmid	sterile soil	detect mobilization of conjugative plasmid in *Streptomyces* sp. recepients
		Southern hybridization on DNA isloated from colonies		verify the presence of the plasmid and determine modifications in new host by RFLP.
Pseudomonas sp.[3] *P. cepacia*	chromosomal insertion of vector sequences from suicde vector, pRL425, with *npt*II	Southern analysis on total DNA using a ^{32}P-labeled single-stranded DNA probe to *npt*II	non-sterile soil	use restrction length polymorphisms to detect plasmid sequence deletion as a result of a double cross-over recombination event

Table 10. (Continued)

ORGANISM	TARGET DNA	DETECTION METHOD	SAMPLE MATRIX	PURPOSE
		Colony blot analysis with probe made to ^{32}P-labeled pRL425 vector		verify the absence of vector sequence in kan^r cells
P. cepacia[4] Pseudomonas sp.	nptII chromosomal insert	slot blots on total DNA using a ^{32}P-labeled single- stranded DNA probe	soil microcosm	follow the population dynamics, movement, and leaching of introduced organism
P. putida[5] R. legumisarium	Tn5 chromsomal insertion	Dot blots on DNA from isolated colonies using nick translated ^{32}P-labeled vector containing Tn5 as probe	sterile soil	determine if Tn5 insert is stable in soil
		Dot blots on cells grown in selective medium and diluted for MPN analysis	sterile and non-sterile soil	determine recovery of and follow growth of organisms over time

Table 10. (Continued)

ORGANISM	TARGET DNA	DETECTION METHOD	SAMPLE MATRIX	PURPOSE
P. fluorescens[6]	conjugative, plasmid RP4	colony hybridization with selective media using a nick translated ^{32}P-labeled probe made from whole plasmid	non-sterile soil	detect plasmid transfer to homologous recepient
E. coli[7]	pBR322 modified with *Drosphila* gene	colony hybridization with selective and non-selective media using a ^{32}P-labeled probe to *Drosophila* insert	sterile and non-sterile soil	determine stability of plasmid and foreign DNA
E. coli[8] *Rhizobium fredi*	pRK2073::Tn*5*	Southern analysis on individual colonies grown in selective media using a probe to Tn*5* made by nick translation of a heterlogous plasmid containing Tn*5*	sterile soil	transfer of conjugative plasmid from *E. coli* and *R. fredii*

Table 10. (Continued)

ORGANISM	TARGET DNA	DETECTION METHOD	SAMPLE MATRIX	PURPOSE
E .coli[9]	non-conjugative plasmid containing herpes simplex thymidine kinase (tk) gene	colony hybridization with selective media using a nick translated ^{32}P-labeled 850 bp tk fragment as a probe	laboratory waste treatment facility	demonstrate mobilization of non-conjugative plasmid in a tripatental mating with recepient cells
P. putida[10]	plasmid RK2 and biodegredative plasmid TOL encoding the xyl operon colony	hybridization with non-selective media using nick-tanslated ^{32}P-labeled probes to RK2 and xylR gene on TOL plasmid	groundwater aqifer material: uncontaminated and experimentally contaminated	demonstrate stability and maintenance of introduced biodegredative strain and plasmids
Alcaligenes A5[11]	biodegredation plasmid, pSS50 allows the degredation of 4-chlorobiphenyl	colony hybridization with selective or non-selective media and dot blots on extracted DNA using ^{32}P-labeled probes, to RS-1100-1, 1.3 KD repeat fragment (15-20 copies/cell)	non-contaminated and contaminated freshwater microcosm	monitor growth and persistance of organisms
P.cepacia	genes involved in 2-4-5-trichloro-phenoxyacetic acid degredation			

Table 10. (Continued)

ORGANISM	TARGET DNA	DETECTION METHOD	SAMPLE MATRIX	PURPOSE
		located chromosomally and on plasmids in *P. cepacia* or to pSS50 for *Alcaligenes*		
E. coli[12]	hybrid plasmid between pBR325 and pEML159 from *Alcaligienes* sp. encoding mercury resistance and 2,4-dichloro-phenoxyacetic acid degredation	colony hybridization with selective and non-selective media using a nick translated biotinylated probe to whole plasmid	filtered and unfiltered lake water	survival and monitoring study
P. putida[13]	non-conjugative IncQ broad-host range plasmid in which *xylE* gene has been inserted	Dot blots of DNA isolated from filtered cells lysed *in situ* on nitrocellulose membranes using a 0.6 kb *xylE* fragment randomly primed and labeled with ^{32}P-dCTP	filtered and unfiltered lake water	survival and monitoring study

Table 10. (Continued)

ORGANISM	TARGET DNA	DETECTION METHOD	SAMPLE MATRIX	PURPOSE
Azospirillum lipoferum[14]	Tn*5* chromosomal insert	Colony blots from MPN dilutions with selective media using an oligo-nucleotide (30 base pair) probe to *nptII* end-labeled with ^{32}P-ATP.	corn seeds coated with *A. lipoferum* germinated in microcosm	determine levels in rhizosphere, endorhizo-sphere, and xylem exudates; determine extent of leaching into soils
Clavibacter cynodontis[15]	chromosomal insertion using insertion vector containing *Bacillus thuringensis* delta-endotoxin gene and *Streptococcal tet* gene	Hybridization blots on colonies grown in selective and non-selective media, transferred to nitrocellulose and lysed *in situ*; probed with isolated delta-entotoxin and *tet* genes labeled by random priming with digoxygenin-dUTP	corn plants planted in the field for a small-scale release	study gene loss and segregation rates of inserts in corn; determine rate of survival in plant debris post-harvest; monitor dissemination into trap plants

[1]Zeph et al. 1988
[2]Rafii and Crawford. 1988
[3]Jansson et al. 1989
[4]Jansson et al. 1989
[5]Fredrickson et al. 1989
[6]van Elsas et al. 1989
[7]Devanas and Stotzky. 1986
[8]Richaume et al. 1989
[9]Mancini et al. 1987

Table 10. (Continued)

ORGANISM	TARGET DNA	DETECTION METHOD	SAMPLE MATRIX	PURPOSE

[10]Jain et al. 1987
[11]Steffen et al. 1989
[12]Amy and Hiatt. 1989
[13]Morgan et al. 1989
[14]Bentjen et al. 1989
[15]Turner et al. USDA submission 90-016-01

TABLE 11. Hybridization techniques for detection of microorganisms introduced into the environment.

ORGANISM	TARGET SEQUENCE	PROBE	DETECTION METHOD	SAMPLE MATRIX	SENSITIVITY (DETECTION LIMIT)
Pseudomonas putida[1]	TOL plasmid	radiolabeled TOL plasmid	colony hybridization	sediment	1 colony in 10^6
Azospirilum lipoferum[2]	*nptII*	radiolabeled heterologous plasmid containing *nptII* sequence	colony hybridization	soil	10^3 colonies/ gram of soil
Pseudomonas cepacia[3]	species-specific repeat 1.3KB sequence	radiolabeled fragment containing 1 KB of RS1100-I	dot blot	sediment	10^3 cells/ gram of sediment
Bradyrhizobium japonicum[4]	*nptII*	radiolabeled single-stranded DNA	dot blot	soil	4 X 10^4/gram of soil or 0.02 picograms target DNA/microgram total DNA
E. coli[5] *P. putida*	*xylE*	radiolabeled 822 base-pair fragment	dot blot	lake water	10^4 colonies/ml
P. cepacia[6]	RS1100-I	T7-generated radiolabeled transcription product of RS1100-I	solution hybridization	sediment	10^2-10^3 cells/gram of sediment

TABLE 11. (Continued)

ORGANISM	TARGET SEQUENCE	PROBE	DETECTION METHOD	SAMPLE MATRIX	SENSITIVITY (DETECTION LIMIT)
B. japonicum[7]	*nptII*	radiolabeled single-stranded DNA	Southern blot	soil	0.1 picogram target DNA/microgram total DNA
Rhizobium *leguminosarum*[8]	Tn*5*	radiolabeled nick translated plasmid DNA containing Tn*5* sequences	MPN-DNA hybridization	soil	10^2 cells/gram of soil
P. putida					10 cells/ gram of soil

[1]Saylor et al. 1985.
[2]Bentjen et al. 1989
[3]Steffen and Atlas. 1988
[4]Holben et al. 1988.
[5]Morgan et al. 1989
[6]Steffen and Atlas. 1990.
[7]Holben et al. 1988.
[8]Fredrcikson et al. 1988

TABLE 12. Detection of bacteria in environmental samples by total community DNA extraction and polymerase chain reaction (PCR) enhancement

Environment	Strain detected	Seq. ampl.	Copy no.	Remarks/ problems	Detection limit	References
Soil	*Pseudomonas fluorescens*	0.7 kb *pat*	1-3	Persistent impurities; 1:10 dilution needed	10^3-10^4	Van Elsas et al., 1991
Soil	*P. fluor.*	0.7 kb	1-3	Idem above	~10^3	Cresswell et al., 1991
Soil	*Frankia* sp.	*nifH* (*Frankia*)	1?	Non-target amplification; (booster) optimization	~10^3(cfu); ~200(IU)	Myrold et al., 1990
Alnus nodules	*Frankia* sp.	0.2 kb *nifH*	1?	Dilution (1:4/1:9) needed for PCR	?	Simonet et al., 1990
Infected wheat	*G. graminis*	0.29/0.19 kb fragm.	1?	Nested primers; 2 rounds of amplification	?	Schesser et al., 1991
Sediment	*P. cepacia*	1.0 kb	15-20	Primer selection important	~1/0.3pg	Steffan & Atlas, 1988
Aquatic	Coliform bacteria	*LacZ*/ *LamB*	1?			
Aquatic (lake, sew.)	*E. coli*	0.2 kb	multiple (plasm.)		<10^3	Chaudry et al., 1990
Aquatic	*Legionella pneumophila*	0.65 kb *mip* fr.	1		1-10	Bej et al., 1991 Mahbubani et al., 1990 Starnbach et al., 1989

* Detection of microorganisms in clinical samples or food are not listed here. *Seq. ampl.:* sequence amplified; *Copy no.:* number of copies of the target sequence per cell; *detection limit:* minimum number of target organisms or amount of target DNA per ml. or g. of sample still detectable after PCR; *fragm./fr.:* fragment; *kb:* kilobases; *G. graminis:* *Gaeumannomyces graminis*; *cfu:* colony-forming units; *IU:* infective units.

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