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THE USE OF DNA PROBES TO DETECT AND IDENTIFY MICROORGANISMS

David E. Kohne

Chief Scientist
Gen-Probe Incorporated
San Diego, California

INTRODUCTION

The objective of this article is to provide the reader a basis for understanding what DNA probes are and how they can be used to detect microorganisms.

The general theme of this presentation is largely a result of my basic research interest over the last 15 years; which has been to describe the bacterial and viral flora of normal humans in order to assess the disease potential which humans carry with them. At the beginning of this study I searched for the best technical approach and decided to detect the microorganisms by detecting their nucleic acids using the process of nucleic acid hybridization. The advantages of this approach include the following points:

1. If the target organism is present in the sample, the nucleic acids of the target organism must be present, no matter what the state of genetic expression of the organism in that sample.

2. A nucleic acid sequence specific only for a particular microorganism can always be found.

3. Nucleic acid probes highly specific for narrow or broad groups of target organisms can be developed.

4. The nucleic acid hybridization approach is highly sensitive and has the potential of detecting a single organism.

At that time, however, DNA probes had been little used to detect organisms in clinical samples. In addition, sample preparation which required nucleic acid isolation and purification, and the hybridization assay were both lengthy, labor-intense processes. Many of the DNA probe methods used today still have these disadvantages.

Makeup of Nucleic Acids

DNA or RNA probes provide a powerful tool for the detection, identification and quantitation of microorganisms. The key property of DNA probes is that specific DNA probe molecules can recognize and

selectively interact with target nucleic acid sequences present only in the target organism. This process of recognition and interaction is called nucleic acid hybridization.

Let's briefly examine the molecular basis which makes it possible to use specific nucleic acid molecules as DNA probes.

Nucleic acids are polymers composed of four basic subunits (also called bases) which are covalently linked in a linear fashion. Two types of nucleic acid are present in nature. DNA, which is the genetic material or the genes, and RNA which is a direct gene product.

DNA in nature is almost always double stranded and is composed of 4 subunits or bases. These are Adenine, Guanine, Cytosine and Thymine; A, G, C, and T. The individual strands of the double-strand molecule are held together by specific interactions between the bases in each strand. A and T specifically interact together as do G and C. AT and GC are called complementary base pairs. In the double-strand molecule, each base in one strand is paired with its complementary base in the other strand. Each A is paired with T and each G is paired with C. The chemical glue which holds the double strands together is made up of the many complementary base pair interactions. The individual single-strand molecules which make up the double-strand molecule are called complementary single strands.

RNA is also composed of 4 subunits or bases, Adenine, Guanine, Cytosine and Uracil (rA, rG, rC, rU). Complementary RNA molecules can recognize each other and interact to form a double strand form which is held together by interactions between the complementary bases rArU and rGrC. RNA in nature is almost always single strand in form. Only one of the two complementary strands is present in the cell. RNA is almost always a direct gene product with DNA serving as a template for the enzymatic synthesis of RNA.

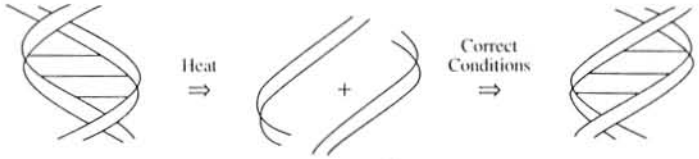
Complementarity can also exist between RNA and DNA molecules resulting in a double-strand molecule which is composed of one strand of RNA and one strand of DNA. Again, the molecules are held together by interactions between complementary base pairs. In this case, the pairs can be rAT, ArU, rGC, GrC.

For perspective, the number of bases present in the DNA of different organisms is presented in Table 1.

TABLE 1

NUMBER OF BASES IN THE DNA SEQUENCES OF AN ORGANISM VARIES

Mammal	7×10^9	bases, 23 chromosomes
Bacteria	10^7	bases, 1 chromosome
Large virus (HSV)	3×10^5	bases, 1 chromosome
Small virus (Polio)	8×10^3	bases, 1 chromosome
Smallest virus (viroid)	300	bases, 1 chromosome



- *E. coli* DNA contains 10^7 bases
- Each strand contains 5×10^6 bases
- Any complementary strand can hybridize to any other complementary strand

Figure 1. Schematic Representation of Hybridization

NUCLEIC ACID HYBRIDIZATION

The principle behind DNA probe technology is nucleic acid hybridization (1,2): Hybridization is the process of formation of stable double-strand nucleic acid molecules from complementary single-strand molecules. Nucleic acid hybridization is a rational chemical reaction and can be used in a predictable manner to detect and quantitate microorganisms.

Figure 1 presents a schematic illustration of hybridization using E. coli DNA as an example. E. coli DNA contains 10^7 bases. Since it is a double-strand molecule, each single strand is composed of 5×10^6 bases, and each base in one strand is paired with its complementary base in the other strand.

The double-strand nucleic acid molecule can be converted to two single-strand molecules by a variety of methods, heat being one. These complementary single strands wander around free in solution, and eventually a single-strand molecule will collide with its complementary molecule, chemical recognition (i.e., sequence recognition) will occur, and the two strands will interact to form a stable double-strand molecule. This process of collision, chemical recognition and interaction between two complementary strands is the process of nucleic acid hybridization. The basis for the selectivity of DNA probes lies in the ability of complementary sequences to specifically recognize each other and form a stable double-strand molecule.

IDEAL AND NON-IDEAL NUCLEIC ACID HYBRIDIZATION

Must two nucleic acid molecules be perfectly complementary in order to hybridize together? The answer is no. There are two different types of non-ideal hybridization. However, it will be useful to first describe ideal hybridization, again using E. coli DNA as an example.

E. coli DNA contains 10^7 bases and is a double-strand molecule. Each single-strand DNA molecule contains 5×10^6 bases. Each of the 5×10^6 bases in one strand is properly matched with its complementary base in the other strand resulting in: a) perfect end-to-end matching of the complementary DNA strands, and b) perfect base pair matching between the bases in each strand.

This double strand form can be converted to two complementary single-strand molecules by raising the temperature of the solution to a point where the double strand form is unstable. Under the proper conditions,

Figure 2a. Imperfect End to End Hybridization

Figure 2b.

these complementary single-strand molecules can chemically recognize each other and interact to form a stable double-strand molecule where the individual single strands are perfectly matched end to end, and all of the bases in one strand are paired with their proper complementary base in the other strand. This is ideal hybridization.

One type of non-ideal hybridization is imperfect end-to-end matching as illustrated in Figure 2. Two nucleic acid molecules which have complementary and non-complementary regions can hybridize together to form a partially double, partially single-strand hybrid molecule. A large number of permutations can occur under this format and many of these are illustrated in Figures 2A and 2B. Figure 2A illustrates various types of hybrids often formed when double-strand DNA is fragmented and hybridized. Figure 2B illustrates hybrids which are often formed by hybridizing small DNA probes to large target molecules. For example, a 30-base long DNA probe can hybridize with a 3000-base long RNA target molecule resulting in a hybrid in which the DNA probe is hybridized 100 percent, but only 1 percent of the RNA is in a hybrid state. At the other extreme, a probe mix consisting of one hundred different 30-base long DNA probes, each complementary to a different region of the 3000-base long molecule, can hybridize to the large molecule resulting in the entire target molecule being hybridized.

A second type of non-ideal hybridization results in imperfect base pair matching in the double strand region of a hybrid. This is illustrated in Figure 3. Two nucleic acid molecules can have perfect end-to-end matching but not have perfect sequence complementarity. Under the proper conditions, these partially complementary molecules can hybridize together to form a stable double-strand hybrid which is perfectly matched end to end but contains base pair mismatches. The imperfect base pair matching results in a double-strand molecule which is less stable (i.e., converts to a single strand form at a lower temperature) than if perfect base pair

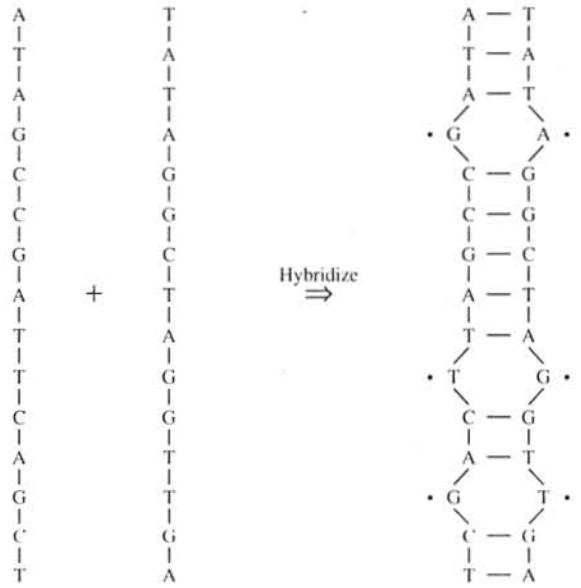


Figure 3. Imperfect Base Pair Matching

matching were present since the molecule with base pair mismatches has less "chemical glue" holding the two strands together. It is possible to get highly specific hybridization between partially complementary single strands resulting in double-strand molecules with 20-30% base pair mismatches in the double-strand region. Furthermore, it is possible for a hybridized molecule to have both imperfect end-to-end matching and imperfect base pair matching. This ability of partially complementary single-strand molecules to hybridize greatly enhances the power of DNA probes as a diagnostic tool. This will be illustrated below.

When put under the proper conditions, two nucleic acid molecules which are only partially complementary can hybridize together to form a stable double-strand molecule. What are these conditions? In order for complementary single-strand molecules to hybridize together, the resulting double-strand molecule must be stable under the conditions of hybridization. Therefore, the hybridization must be done at a temperature below the temperature at which the double-strand form is converted to single-strands.

How much below the dissociation temperature do you want to go? Hybridized molecules which have no mispaired bases have the highest dissociation temperature. If the hybridization is done at a temperature just below this dissociation temperature, then only hybrids with a high degree of base pair matching can form. However, if hybrids with a greater degree of mispaired bases are desired, then the temperature of hybridization is lowered. The lower the hybridization temperature, the greater the degree of base pair mismatches which can occur in the resulting hybrids. Thus by changing the hybridization temperature, one can select the degree of base pair matching which is required to form a stable hybrid.

This illustrates a very important property of DNA probes: the specificity of many DNA probes can be changed by simply changing the conditions of hybridization. The same DNA probe sequence can have broad or narrow specificity depending on the conditions of hybridization. When hybridized at higher temperature, it has narrow specificity and will detect only a limited number of related organism types. When hybridized at lower temperature, it has broader specificity and will detect a larger number of related organism types. In practical terms, this means that the same probe may be used at low temperature for screening purposes and at high temperature for a more precise identification.

THERMAL STABILITY OF HYBRIDIZED PROBE

The thermal stability (TS) of a Probe:Target hybrid is a measure of the temperature at which the double-strand hybrid is converted to single-strand molecules. TS is defined as the temperature at which 50 percent of the hybridized probe is converted to single-strand form. This can be measured by taking preformed hybrids and heating them to increasingly higher temperatures. After heating to each temperature, an assay is done to determine the fraction of probe which has been converted to the single-strand form.

Earlier, it was stated that DNA probes can have either broad or narrow specificity depending on the conditions of use. Whether a probe is useful to detect only a specific type of organism depends largely on the TS difference between Probe:Target (PT) hybrids and any Probe:Non-Target (PNT) hybrids which might be formed. What is wanted is a temperature of incubation where stable PT hybrids will form while no PNT hybrids will form because they are unstable. The (TS (PT) - TS (PNT)) difference should be as large as possible. The larger the difference, the easier it is to design the test. This is the basic principle behind the design of

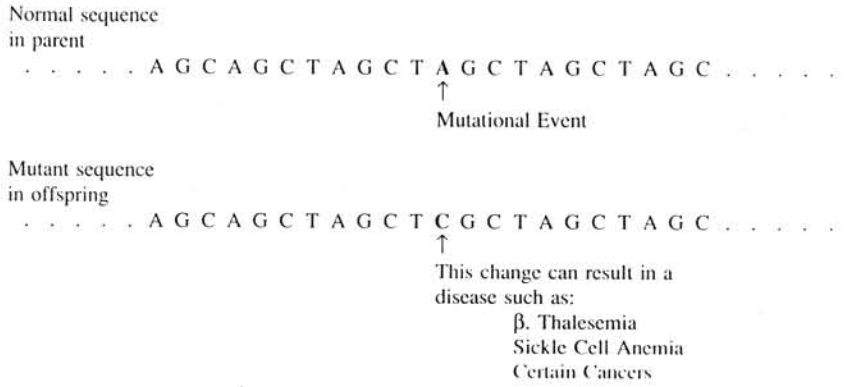


Figure 4a. Many Diseases Are Caused by a Single Base Change in the Sequence of the DNA of an Otherwise Normal Individual

all DNA probe diagnostic tests. Consequently, factors which affect the TS can affect the probe specificity, and these factors must be controlled when doing a DNA probe test.

To illustrate this principle, let us examine a DNA probe test designed to provide genetic information which can be used for human genetic counseling (3). Many diseases are caused by a single base change in the sequence of an otherwise normal individual. This can result in a genetic disease such as beta thalesemia or sickle cell anemia, and the onset of certain cancers can be triggered by such a single base mutation.

Figure 4A illustrates such a change. DNA probes can be used to detect a single specific base change which has occurred in the DNA. To accomplish this one first must know the sequence of the normal and mutant gene sequences. Then a short probe can be produced which is 100 percent complementary to the mutant sequence (Figure 4B) but has a single base pair mismatch with the normal or wild type DNA sequence (Figure 4C). The TS of the perfectly matched hybrid (40°C) is 10°C higher than that of the single mismatch hybrid (30°C) (Figure 4D). When hybridized with fetus DNA at 35°C only perfectly matched hybrids are stable and can form. Thus if this probe hybridizes at 35°C with fetus DNA then the fetus carries the mutant gene sequence.

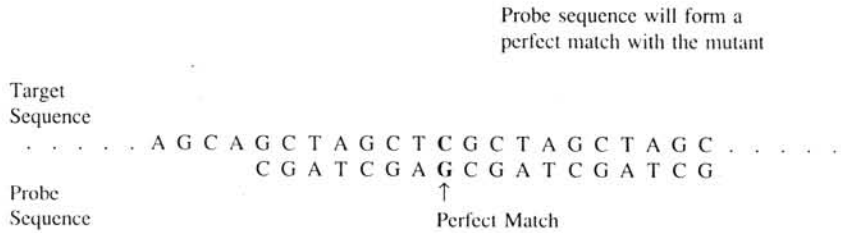


Figure 4b. DNA Probes Can Be Used to Detect a Specific Base Change Which Has Occurred in the DNA

Normal Sequence

. . . . . A G C A G C T A G C T A G C T A G C T A G C . . . . .
C G A T C G A G C G A T C G A T C G

Probe Sequence ↑ Mismatch

Figure 4c. Short Probe Sequence Will Form an Imperfect Match with the Normal Sequence

Hybrid	Number of Mispaired Bases	Thermal Stability
Probe:Mutant	0	40° C
Probe:Normal	1	30° C

1. When hybridized at 35° C only perfectly matched hybrids can form.
2. If this probe hybridizes at 35° C with DNA from a fetus, then the fetus carries the mutant gene.

Figure 4d. Thermal Stability of Probe and Probe Mutant Hybrids

SIMILARITY OF NUCLEIC ACID SEQUENCES AMONG DIFFERENT ORGANISM TYPES

The more closely related two organisms are, the more similar are their nucleic acid sequences (4,5). DNA sequences in members of the same species are virtually identical while DNA's from distantly related organisms have very little sequence similarity. The DNA's of man and chimp cross-hybridize to greater than 90% with 1-2% base pair mismatches while DNA's from man and mouse cross hybridize less than 5%. Among bacteria, the DNA's of Salmonella typh. and E. coli cross hybridize about 60% with a moderate number of base pair mismatches while DNA's from E. coli and Legionella bacteria cross hybridize very little.

Nucleic acid sequence specificity provides a powerful and general means for detecting and identifying organisms of a given type since: all organisms contain nucleic acids; a nucleic acid sequence specific for a given organism type can always be found; a specific probe can be developed for any specific sequence.

COMPONENTS OF DNA PROBE TEST

There are four basic components to a DNA probe test.

PROBE - a labeled single-strand sequence complementary to the target sequence to be detected.

SAMPLE - ranges from water to feces or sputum and may or may not contain the target organism.

HYBRIDIZATION METHOD - the conditions of solution composition and temperature under which hybridization occurs.

HYBRIDIZATION DETECTION METHOD - a method for detecting the presence of Probe: Target hybrids

The first two components will be discussed in some detail below.

GENERAL CHARACTERISTICS OF PROBES

An ideal probe is single-strand DNA or RNA which can hybridize only if the target is present in the sample. Such a probe contains only one of the complementary strands necessary for hybridization and when incubated alone, cannot form double-strand molecules because only one of the complements is present. The size of a probe can range 10 to over 10^4 bases in length. The length chosen depends on the specific requirements of the test.

Probe specificity lies in the probe sequence and the conditions under which it is used, as discussed earlier. The specificity requirements are that the probe must hybridize with the target nucleic acid but not hybridize with any non-target nucleic acids which may be present in a sample. Non-target nucleic acids which may be present in a human clinical sample are human and any contaminating microorganism nucleic acids. When properly designed and used a DNA probe has essentially absolute specificity and will hybridize only to target nucleic acid. In order to be useful as a diagnostic a probe need not have absolute specificity for the target organism. For example, the DNA probe used in Gen-Probe's diagnostic kit for Mycoplasma pneumonia will hybridize to Mycoplasma genitalium as well as the target. This does not affect the use of the probe detecting M. pneumonia in throat swab samples, however, since M. genitalium has never been found in the respiratory tract.

Probe must be marked either directly or indirectly in order to follow its fate. DNA probes can be marked with either radioactive (^{32}P, ^{35}S, ^{3}H, ^{14}C, ^{125}I) or non-radioactive (biotin-avidin systems, hapten-antibody systems, fluorescent, chemiluminescent) reporter molecules. In general, anything which can be used to mark antibodies can be used to mark probes.

Probes can be selected by determining the base sequence of target and non-target DNA or RNA, then comparing the sequences and selecting an appropriate sequence which is characteristic only of the target nucleic acids. Once the probe sequence is selected, the probe can be produced with a DNA synthesizer. Probes can also be selected on the basis of the hybridization characteristics desired. In this case, neither the probe sequence nor its function need be known.

The properly designed and used DNA probe can detect any type of genetic change. These include: single base changes as discussed earlier; multiple base changes; rearrangements of all kinds; additions and deletions; changes in genetic expression; and quantitative changes in genetic expression.

It should be emphasized that both the probe and the target sequences must be single stranded at the start of a DNA probe test in order for the test to work.

A PROBE TO POLIO I RNA: BASICS OF PROBE SELECTION

This example is derived from a research project the author was involved in some time ago. The object of the project was to determine whether normal and diseased human tissue contained RNA sequences similar to the sequence of Polio virus I RNA (6). A radioactive probe complementary to Polio RNA was produced by using Polio I RNA as a template for the enzyme reverse transcriptase to synthesize small pieces of radioactive DNA complementary (cDNA) to Polio I RNA. Each small piece of cDNA represented a small fraction of the Polio I RNA sequence, but the population of cDNA pieces represented the entire Polio RNA sequence.

TABLE 2

CHARACTERIZATION OF ^{3}H cDNA

10^{-5} microgram	Probe + Poly A	0.1%
10^{-5} microgram	Probe + 0	0.1%
10^{-5} microgram	Probe + 200 Micrograms Human RNA	0.1%
10^{-5} microgram	Probe + Mix of 200 Micrograms Human RNA and 0.1 Microgram Polio I RNA	99%

The hybridization characterization of this cDNA probe is shown in Table 2. The probe does not self hybridize and when incubated alone only 0.1% of the probe behaves as if hybridized. This 0.1% is not true hybridization but represents the non-specific background signal due to the assay. The negative control shows that probe incubated with human RNA also does not hybridize, even at a ratio of 10^{7} parts human RNA to 1 part probe. The same value would be obtained if the amount of RNA were 20 milligrams or 2 micrograms, and if RNA's or DNA's from widely different bacterial or mammalian sources were present in the hybridization mix. This gives an indication of the extremely high specificity of probes. The probe collides frequently with the heterologous nucleic acid but does not chemically recognize and specifically interact with them to form a stable double-strand hybrid. The positive control illustrates this also. The probe hybridizes almost completely with Polio I RNA even in the presence of a large excess of heterologous nucleic acid. The presence of the heterologous nucleic acid changes neither the kinetics nor extent of probe hybridization.

TABLE 3

SPECIFICITY OF POLIO I 3H cDNA
PROBE HYBRIDIZATION

		HYBRIDIZATION EXTENT OF PROBE
Polio I Probe	+ Polio I RNA	99%
Polio I Probe	+ Polio I RNA (5 Strains)	95-99%
Polio I Probe	+ Polio II RNA (8 Strains)	77-80%
Polio I Probe	+ Polio III RNA (8 Strains)	69-78%
Polio I Probe	+ Coxsackie RNA	5%
Polio I Probe	+ EMC RNA	3-5%
Polio I Probe	+ VSV RNA	0.1%
Polio I Probe	+ MS-2 RNA	0.1%

Probe for Polio I RNA	1'	2'	3'	4'	5'	6'	7'	8'	9'	10'
Polio I RNA	1	2	3	4	5	6	7	8	9	10
Polio II RNA	1	2	3	4	5	6	7	11	12	13
Polio III RNA	1	2	3	4	5	6	14	15	16	17
Coxsackie RNA	1	18	19	20	21	22	23	24	25	26
EMC RNA	1	27	28	29	30	31	32	33	34	35

Figure 5. Idealized Pattern of Sequence Relationships Among Related Viruses

This probe then is ready to use for viral detection. It does not hybridize with human cell nucleic acid but does hybridize to Polio I RNA. At this point, we must ask whether this probe hybridizes to any other virus nucleic acid besides Polio I RNA? The answer is yes it does, as can be seen in Table 3. This probe hybridizes to varying extent with all member viruses of the related group of viruses called "Enteroviruses" but does not hybridize to viruses which are not members of this group. Thus this cDNA probe, which represents all of the Polio I RNA sequence, can be used to detect a large number of different but related viruses. Such a probe can be used for screening samples for the presence of any member of the Enterovirus group. However, only in special circumstances can this cDNA probe be used to identify the type of Enterovirus present in a sample.

This raises another question. Can a probe which detects only Polio I RNA be produced?

The answer is yes. To illustrate this, it is necessary to examine an idealized pattern of nucleic acid sequence relationships among related viruses (Figure 5). It should be kept in mind that this pattern of sequence relationships is the same general pattern seen for nucleic acid sequence relationships among all related groups of organisms.

In this case, each virus RNA is segmented into 10 different subsequences. Each Enterovirus RNA is about 8000 bases long. A DNA probe for Polio I RNA contains 10 different subsequences, each of which is complementary to a different subsequence of the Polio RNA. By definition, this probe will hybridize to 100 percent with Polio I RNA. However, this probe cannot hybridize completely with any other Enterovirus RNA since the probe contains DNA subsequences (8, 9 and 10) which can only hybridize with Polio I RNA.

Probe complementary only to Polio I subsequences 8 or 9 or 10, or a mixture of 8, 9 and 10, will detect only Polio I RNA. This illustrates the basic pattern of nucleic acid sequence similarity seen for all related groups of organisms. Some sequences are held in common. Other sequences are not held in common, and these are the sequences which are characteristic of a specific group. Probes made to these sequences will detect only that group.

A probe specific only for each of the other Enteroviruses can also be produced by following this same principle. A variety of methods are available for selecting the proper subsequence to target.

If a probe were desired which would hybridize completely with all Enteroviruses, then subsequence 1 would be the chosen target. This would be a useful screening probe.

STRATEGY FOR DESIGNING A DNA PROBE APPROACH

The strategy for designing a DNA probe assay is very important and basically involves choosing the appropriate target and probe molecules with which to work. The conventional strategy involves using a DNA or RNA probe to detect a target DNA or gene sequence. Gen-Probe's strategy is to use DNA and RNA probes to target RNA sequences of the cell as a general class. Our initial efforts have focused primarily on using ribosomal RNA (rRNA) as the target molecule.

Ribosomes are present in all cellular life forms. There are always ribosomes present in a cell as they are an integral part of the protein synthetic machinery. Both procaryotic and eucaryotic cells contain large numbers of ribosomes and, therefore, rRNA molecules. rRNA is a direct gene product, and one strand of the rRNA gene is used as a template to produce rRNA which is then incorporated into a ribosome. A ribosome is composed of about 50% protein and 50% rRNA. In a typical bacterial cell such as E. coli there are about 10^4 ribosomes. The rRNA is in a single-strand form and RNA complementary to the rRNA is not present in the cell.

There are several dramatic advantage to this approach. One is a greatly enhanced (several thousandfold) sensitivity of detection. The other is that it allows the production of probes with specificities which were heretofore not attainable. These points are discussed in Tables 4 and 5.

There is a basic rationale for producing these probes targeted for rRNA, and it is based on the evolutionary history of the rRNA sequence. The total primary base sequence of rRNA in bacteria is about 4600 bases long. This sequence is generally considered to have been highly conserved during evolution (5). The rRNA sequence can be viewed as being composed of a population of short subsequences. During evolution, certain of these subsequences have been highly conserved and are present in all cellular

TABLE 4

THE ENHANCED SENSITIVITY OF THE GEN-PROBE RIBOSOMAL RNA DETECTION METHOD

- rRNA and DNA can be detected equally well by Nucleic Acid hybridization.
- An E. coli cell contains:

  10^4 ribosomes
  10^4 rRNA's
  2.5×10^{-14} grams rRNA

- That same E. coli cell contains:

  4 genes which code for rRNA
  10^{-17} grams of ribosomal DNA

Thus, by choosing rRNA as the target rather than DNA, the detection sensitivity is increased several thousandfold.

TABLE 5

SPECIFICITY OF DNA PROBES TO rRNA

1. The method allows probes with widely varying specificities to be produced. This allows:

   A. The detection of the presence of any life form (excluding viruses) in a sample, with the performance of a single lab assay.

   B. The specific detection of any member of a broadly related class (e.g., all bacteria) of organisms in a sample with just one lab assay, even in the presence of large numbers of unrelated organisms.

   C. A battery of different DNA probes to be used for the more precise identification (even down to the species level) of the organism present in the sample, even in the presence of large numbers of other organisms.

D. The ability to quantitate the number of organisms of a specific type even in the presence of other organisms.

organisms. Probes targeted to these sequences will detect any cellular life form. The remaining subsequences have diverged during evolution, and some of these subsequences have diverged faster than others.

The key to producing probes with the unique specificities described is to select the proper rRNA subsequences to serve as a target. Gen-Probe has developed a variety of methods for selecting only those rRNA subsequences which are unique to the group of bacteria of interest.

Figure 6 presents the structure of the small (16s) ribosomal subunit of E. coli. The heavy lines denote the sequences which have been highly conserved during evolution. These are interspersed among the changeable regions.

SAMPLE HANDLING

The basic requirement for sample handling is that the sample nucleic acid must be made available for hybridization with the probe. The conventional approach for sample handling involves immobilizing the target on an inert support. This requires at least a partial purification of the sample nucleic acid.

The Gen-Probe approach to sample handling is quite different and involves no immobilization step and no nucleic acid purification. The sample is mixed directly with a probe solution which contains the probe; lysing agents and solubilizer; nuclease inhibitors; and hybridization promoters. In certain cases, such as Mycobacteria, some sample handling steps may be necessary before mixing the probe solution and sample. Sample types which we have assayed using this approach are: lung and tissue homogenates; tissue culture medias; suspensions containing bacterial or mammalian cells; bacterial pellets; urine; feces; sputum; serum.

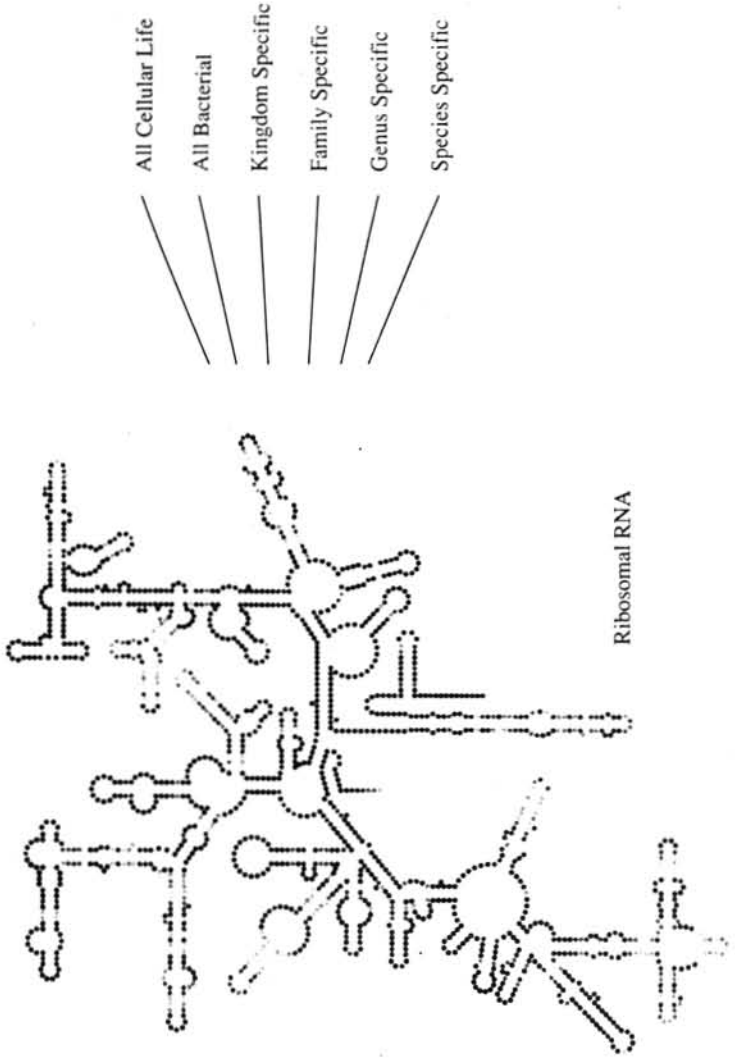


Figure 6. Specificity

TABLE 6

RNA:DNA HYBRIDIZATION IN HIGH CONCENTRATION OF CLINICAL SAMPLE

Clinical Sample	Concentration in Hybridization Mixture (Volume Percent)	% Hybridization of Probe
Sputum	56%	>90%
Feces	27% Solids	>90%
Serum (calf)	50% Serum	>90%
Legionella Infected Lung Homogenate	9% Lung Tissue	>90%

TABLE 7

RNA:DNA HYBRIDIZATION IN FECES

Sample		Probe	Percent Probe Hybridized
No feces, only bacteriophage MS-2 RNA	+	DNA complementary to bacteriophage MS-2 RNA	83%
Feces (final concentration of 27% solids) plus bacteriophage MS-2 RNA	+	DNA complementary to bacteriophage MS-2 RNA	83%
Feces (final concentration of 30% solids)	+	DNA complementary to bacteriophage MS-2 RNA	0.3%
No feces and no MS-2 RNA	+	DNA complementary to bacteriophage MS-2 RNA	0.3%
Feces (final concentration of 13% solids)	+	DNA complementary to E. coli rRNA	59%

DNA probes can be used in the presence of high concentrations of clinical samples. Table 6 summarizes data from such experiments. Table 7 presents details of RNA:DNA hybridization in high concentrations of feces. A probe which was partially damaged was used in these experiments in order to amplify any effect of the feces on the hybridization reaction. The same extent of hybridization was seen with or without feces present.

In order to rule out the possibility that the probe has bound to some feces component in a non-sequence dependent manner, probe was mixed with feces and no target RNA was added. None of the probe hybridized in this case. This demonstrated that the 83 percent signal was due to sequence-dependent hybridization of the probe to target RNA. The last result in Table 7 shows that rRNA endogenous to the feces can hybridize to a probe complementary to rRNA.

The sample handling step is the most difficult part of developing a DNA probe test. A variety of problems complicate this step.

Some samples, such as sputum, are highly viscous, and these samples must be liquified for easy handling. Hybridization inhibitors are present in many samples and can reduce the sensitivity of the test. Ribonuclease is in all samples and if the target is RNA, one must be able to keep it intact. Many organisms such as Mycobacteria, certain gram positives and yeast are difficult to break open to release the rRNA. Non-specific

TABLE 8

DNA PROBE TESTS FROM GEN-PROBE

	TEST	PROBE SPECIFICTY	MARKER
1.	Legionella Genus Culture Confirmation and Direct Specimen test	Genus	^{125}I
2.	Mycoplasma Direct	Species	^{125}I
3.	Mycobacterium Avium/Intracellullare Culture Confirmation	Species	^{125}I
4.	Mycobacterium tuberculosis Complex Culture Confirmation and Direct	Complex	^{125}I
5.	Mycobacteria Genus Culture Confirmation and Direct	Genus	^{125}I
6.	Chlamydia trachomatis Direct	Species	^{125}I, Chemi-luminescent
7.	Neisseria gonorrhoeae Culture Confirmation and Direct	Species	Chemilumi-nescent

binding (non-sequence dependent binding) of probe to a sample component can give false positive results.

Gen-Probe has been able to solve all of these problems and has incorporated the solutions into the test kits which are available.

DNA PROBE TESTS AVAILABLE

Gen-Probe has used its innovative DNA probe technology to produce simple, rapid and highly specific DNA probe tests for medically significant microorganisms. The first DNA probe test ever cleared by the FDA was a Gen-Probe test. Since that time, the FDA has cleared 15 DNA probe tests with 11 being Gen-Probe tests. Table 8 lists some of these tests. Certain tests employ isotopic labels while others utilize non-isotopic chemiluminescent markers. Culture confirmation tests are performed by assaying a suspension of cells made by putting a loop of a bacterial colony into water or saline. The direct tests are done directly on the clinical sample.

BASIC TEST FORMAT

The basic steps involved in these tests are: sample preparation, cell breakage, hybridization, separation of non-hybridized probe from hybridized, detection of hybridized marker group. Following is a skeleton description of a DNA probe test for Mycobacteria.

1. Sample Preparation

   a) Add 100 microliters of sample solution to tube containing glass beads.

2. Cell Breakage

   a) Place tube in ultrasonic cleaner for 15 minutes in order to break open bacteria and free rRNA.

3. Hybridization

   a) Add 1 ml probe solution to tube. Incubate 72°C for 1 hour.

   b) Hybridization times can vary from 15 minutes to 4 hours depending on the test.

4. Separation

   a) Add 4 ml of separation mix. Incubate at 72°C for 5 minutes.

   b) Centrifuge at 2000 RPM for 2 minutes. Decant supernatant.

   c) Add 4 ml wash solution, centrifuge at 2000 RPM for 2 minutes and decant supernatant.

   d) Separation mix contains a separation agent. Hybridized probe binds to the separation agent and non-hybridized probe does not and can be washed away. Separation agent in tests is either hydroxyapatite crystals which are centrifuged out of solution, or magnetic particles which are pulled out of solution by magnets.

TABLE 9

A LARGE NUMBER OF PEOPLE IN A HOSPITAL CAME DOWN WITH TB

1. The outbreak was traced to a patient admitted to the hospital with a hip abscess.
2. Biopsy was taken from abscess on hip and fixed in 10% formalin, processed with alcohols and xylene and embedded in paraffin.
3. Special stain showed tissue swarming with AFB.
4. Initial culture results showed only an avium-intracellulare complex bacteria.
5. Looked further and also isolated a TB bacteria.
6. Vast majority of organisms in culture were A-I organisms. Very few TB.

QUESTION: WAS THIS A MIXED INFECTION?

5. Detection

   a) Place tube in gamma counter and count 1 minute. Alternatively place tube in luminometer and measure light output.

AN EPIDEMIOLOGICAL PROBLEM

DNA probes can be very useful in certain epidemiological situations. Table 9 summarizes an epidemiological case where probes were used to resolve some questions concerning the epidemiology of the disease outbreak.

Portions of the formalin-fixed paraffin-embedded tissue were sent to the author. The samples were processed to remove the paraffin and formalin and put through a hybridization protocol similar to that described above for Mycobacteria. Aliquots of the processed tissue were hybridized with various Mycobacteria DNA probes as well as several control probes. Table 10 presents the results of this analysis.

Only two of the probes hybridized. Both the Mycobacteria Genus probe and the Tuberculosis complex probe hybridized well to the tissue preparation. Four other probes, including probes for M. avium and M. intracellulare, did not detectably hybridize. The lack of hybridization of these probes with the tissue preparation rules out any possibility of non-specific binding or non-sequence dependent binding of DNA probes to the tissue. This indicates that the signal seen with both the Genus and TB probes is true hybridization.

The process of nucleic acid hybridization can be used to quantitate the number of bacteria present as well as detect their presence. An estimate of the number of Mycobacteria per gram of tissue was obtained by hybridization kinetic analysis. The results indicated that the tissue contained about 3-4 x 10^9 TB bacteria per gram of tissue. The same type

TABLE 10

RESULTS OF TISSUE ANALYSIS

	HYBRIDIZATION WITH TISSUE	% HYBRIDIZATION OF PROBE WITH TISSUE
Mycobacteria Genus Specific Probe	+	66%
M. avium Specific Probe	-	<1%
M. intracellulare Specific Probe	-	<1%
Legionella Genus Specific Probe	-	<1%
Vesicular Stomatitis Virus Specific Probe	-	<1%
TB Complex Specific Probe	+	80%

Conclusion:

1. The tissue nucleic acids hybridized only with Mycobacteria genus specific probe and TB complex specific probe.
2. No detectable hybridization of M. avium or M. intracellulare probes was obtained.

of analysis indicated that there was at least 1000 times more TB bacteria in the tissue than M. avium-intracellulare complex bacteria.

From this data, it is clear that the hip abscess contained a preponderance of TB bacteria which then caused the TB epidemic.

This study was done in collaboration with Mary Hutton, George Kubica, Bob Good, Vella Silcox, Charles Woodley, Margaret Floyd, Alan Block, Bill Jones of the CDC and William Stead of the Arkansas State Public Health Service.

TROUBLESHOOTING

DNA probes, when properly designed and used, have essentially absolute specificity. Convincing microbiologists that this is true is a challenge for Gen-Probe as a young company. This process of education involves comparing results from probe tests to culture, the current gold standard for most labs. Our customer service department occasionally receives reports from customers regarding samples which test positive with the probe and are culture negative. We are developing a variety of strategies to resolve such discrepants. One of these is discussed below in the context of our direct test for Legionella bacteria.

We have examined a number of sputa which tested positive by the Legionella probe test and negative by culture. If the Legionella probe hybridizes significantly with a clinical sample, there are three possible interpretations.

1. The probe has bound in a sequence-dependent manner (true hybridization) to rRNA from a non-Legionella bacteria. In other words, the probe does not have the desired specificity.

2. The probe has bound in a sequence dependent manner to rRNA from a Legionella bacteria.

3. The probe has bound in a non-specific or non-sequence dependent manner to some component of the sample and subsequently behaves as if it is hybridized.

How do we distinguish among these possibilities?

There is no evidence that the Legionella genus probe will hybridize to non-Legionella bacteria when used under the conditions specified. The Legionella genus probe has been checked against rRNA's from a large number of different species and genera, including those found in the respiratory tract. The probe did not significantly hybridize with any of these rRNA's but did hybridize with all known Legionella species. Thus, the probe is specific only for Legionella species.

The physical chemical characteristics of DNA molecules make it possible to distinguish between sequence-specific binding (hybridization) and non-sequence specific binding to some sample component. All DNA probes are composed of the same 4 chemical sub-units, A, G, C and T. These bases are relatively simple chemical compounds and the base composition, as measured by G+C content, of the vast majority of DNA probes is similar. In general, therefore, the physical chemical characteristics of DNA probes are very similar. Consequently, if one probe will bind in a non-sequence dependent manner to a sample component, then any other probe will bind to that component in a non-sequence dependent manner. This suggests a simple method for determining whether non-sequence dependent binding has occurred in a sample which tests positive for Legionella genus probe and negative for culture. This is done by mixing the sample with a probe which has no target in the sample and then conducting the hybridization assay. If a "hybridization" signal

TABLE 11

MONOCLONAL ANTIBODY APPLICATIONS	AREAS OF OVERLAP	DNA PROBE APPLICATIONS
3 Immunoassay 3 Replacement	3 Infectious Diseases - Viruses - Bacteria	3 Genetic Diseases
	3 Cancer	3 Disease Susceptibility
	3 Food Testing 3 Plant Veterinary 3 Tissue Typing 3 Therapeutic	3 Drug Resistance

TABLE 12

	GEN-PROBE DNA PROBE TEST	MCA TEST
Ease of Detection	Good to Excellent	Generally Excellent
Rapidity	Excellent	Excellent
Shelf Life of Probe or Antibody	Excellent	Excellent
Ability to Quantitate Organisms	Good to excellent	Not Good
Capability of Detecting 1-10 Bacterial Cells	Feasible	Not Feasible
Capability of Developing Non-isotopic Tests	Excellent (on market)	Excellent (On Market)
Specificity	Essentially Absolute	Specificity is often a problem

is detected, then the probe has bound in a non- sequence dependent manner to a sample component. If no hybridization signal is seen, there is no non-sequence dependent binding of the probe occurring and Legionella target organisms are present in the sample. An example of such an analysis is discussed below.

We received a sputum sample which had tested positive with Legionella probe and negative with culture. After first confirming the probe-positive nature of the sample, a portion of the sample was hybridized with Mycobacteria genus probe. This probe did not significantly hybridize with the sample, thus ruling out non-sequence dependent binding of the Legionella probe and confirming the presence of Legionella in the sample.

TABLE 13

PROBE FORM	MCA FORM
1. Ideal probe is a single strand molecule which can only hybridize if target is present. 2. Probe can be either DNA or RNA.	1. MCA is a single molecule which can form Ag:Ab complex only if target antigen is present in the sample.

TABLE 14

PROBE SIZE	MCA SIZE
1. Length of probe can be as short as 10 bases (MW approx. 3.3×10^3) to greater than 10^4 bases (MW approx. 3.3×10^6).	1. IGG molecules have a MW approx. 1.5×10^5 equivalent to about 1500 amino acids.
2. The whole length of the probe is part of the combining site.	2. Size of combining site on antibody molecule is roughly 30 amino acids, a small fraction of the total.

DNA PROBE VERSUS MONOCLONAL ANTIBODY (MCA) TECHNOLOGY

These technologies overlap in certain areas and have unique roles in others (Table 11). DNA probe technology has only recently become commercialized while commercial antibody technology has been around for quite some time and such products are used widely in various areas. The main barrier to the widespread commercial use of DNA probes is the development of rapid, easy-to-use test formats. This is an area of intense investigation in the DNA probe industry and simple, easy-to-use formats will be forthcoming in the near future. Table 12 presents a summary of an overall comparison of the two technologies while Tables 13 thru 20 present detailed comparison of various aspects of probes and MCA's.

TABLE 15

SPECIFICITY OF PROBE	SPECIFICITY OF MCA
1. Depends upon:	1. Depends upon:
a) The base sequence of probe.	a) The primary, secondary and tertiary structure of the MCA.
b) The conditions under which the probe is used.	b) The primary, secondary and tertiary structure of the antigens.
2. Specificity requirements:	2. Specificty Requirements:
a) Probe must hybridize with target nucleic acid.	a) MCA must complex with target antigen.
b) Probe must not hybridize to other nucleic acids which may be present in the sample.	b) MCA must not complex with other antigens which may be present in the sample.
3. Specificity is essentially absolute.	3. MCA often complex with non-target antigens.

TABLE 16

PROBE PRODUCTION

PROBE TEST	MCA TEST
1. Probe sequence need not be known.	1. Ditto for MCA.
2. Sequence of target nucleic acid need not be known.	2. Ditto for MCA test.
3. Function of target sequence need not be known.	3. Ditto for MCA target.
4. Can select probe according to hybridization characteristic desired.	4. MCA selected to have desired specificty.
5. DNA probes can be developed very rapidly and cheaply. Gen-Probe has developed specific rationales for probe production which enable a probe to be identified and produced in 1-2 weeks.	5. MCA identification and production expensive and time-consuming. A short time for MCA development of an MCA is 6 months. The method used is basically a shotgun method with no rationale.

TABLE 17

RANGE OF REACTION CONDITIONS TOLERATED

PROBE TEST	MCA TEST
1. Nucleic acids are stable and will hybridize even in the presence of high concentrations of a wide variety of salts, organic solvent types and detergents, and over a wide range of temperatures.	1. The structure of the antigen and MCA are important to the association. Agents which greatly disturb the secondary and tertiary structure will make the antigens, the MCA, or both, incapable of associating. This greatly restricts the conditions used for the test.
2. Most DNA probe tests are done over a temperature range from room temperature to 72°C, and can be done over a range from -20°C to 100°C.	2. Most MCA tests are done over temperature range from room temperature to 37°C.

TABLE 18

"AVIDITY" OF PROBES

Probe + Target --> Probe:Target Hybrid	Antibody + Antigen<-----> Complex
Once Probe:Target Hybrid is formed it does not spontaneously dissociate.	Ag:Ab Complex spontaneously dissociates. Rate of dissociation depends on avidity of Ab.

TABLE 19

RATE OF COMPLEX
FORMATION AND HYBRIDIZATION

HYBRIDIZATION	ANTIGEN ANTIBODY COMPLEX FORMATION
1. Standard methods for doing hybridization are much slower than comparable antigen-antibody reaction rates.	1. Very fast. Close to theoretical for MCA-Hapten and within 10^2 - 10^3 of theoretical for MCA-large protein complexing. Much faster than standard hybridization methods.
2. The accelerated rate systems give rates comparable to the Ag:Ab rates.	

TABLE 20

ASSAY FOR PRESENCE
OF PROBE:TARGET HYBRIDS

HYBRIDIZATION	Ag + Ag COMPLEX FORMATION
1. Conventional methods are generally complex, lengthy and labor intense.	1. On the whole, the MCA tests are much easier and more rapid than conventional DNA probe tests.
2. Gen-Probe method is similar to many RIA type tests.	2. Generally comparable to the Gen-Probe test, although certain ones are faster and easier than the Gen-Probe tests.

CLINICAL SIGNIFICANCE OF DNA PROBES

DNA probes will allow the timely diagnosis of diseases caused by infectious agents. Early diagnosis and treatment will contribute greatly to both the patients' health and cost containment.

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