

Probe Design, Production, and Applications

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1. Introduction

A probe is a nucleic acid molecule (single-stranded DNA or RNA) with a strong affinity with a specific target (DNA or RNA sequence). Probe and target base sequences must be complementary to each other, but depending on conditions, they do not necessarily have to be exactly complementary. The hybrid (probe–target combination) can be revealed when appropriate labeling and detection systems are used. Gene probes are used in various blotting and *in situ* techniques for the detection of nucleic acid sequences. In medicine, they can help in the identification of microorganisms and the diagnosis of infectious, inherited, and other diseases.

2. Probe Design

The probe design depends on whether a gene probe or an oligonucleotide probe is desired.

2.1. Gene Probes

Gene probes are generally longer than 500 bases and comprise all or most of a target gene. They can be generated in two ways. Cloned probes are normally used when a specific clone is available or when the DNA sequence is unknown and must be cloned first in order to be mapped and sequenced. It is usual to cut the gene with restriction enzymes and excise it from an agarose gel, although if the vector has no homology, this might not be necessary.

Polymerase chain reaction (PCR) is a powerful procedure for making gene probes because it is possible to amplify and label, at the same time, long stretches of DNA using chromosomal or plasmid DNA as template and labeled nucleotides included in the extension step (*see Subheadings 2.2. and 3.2.3.*). Having the whole sequence of a gene, which can easily be obtained from databases (GenBank, EMBL, DDBJ), primers can be designed to amplify the whole gene or gene fragments (see Chapter 28). A considerable amount of time can be saved when the gene of interest is PCR amplified, for there is no need for restriction enzyme digestion, electrophoresis, and elution of DNA fragments from vectors. However, if the PCR amplification gives nonspecific bands, it is recommended to gel purify the specific band that will be used as a probe.

Gene probes generally provide greater specificity than oligonucleotides because of their longer sequence and because more detectable groups per probe molecule can be incorporated into them than into oligonucleotide probes (**1**).

2.2. Oligonucleotide Probes

Oligonucleotide probes are generally targeted to specific sequences within genes. The most common oligonucleotide probes contain 18–30 bases, but current synthesizers allow efficient synthesis of probes containing at least 100 bases. An oligonucleotide probe can match perfectly

its target sequence and is sufficiently long to allow the use of hybridization conditions that will prevent the hybridization to other closely related sequences, making it possible to identify and detect DNA with slight differences in sequence within a highly conserved gene, for example.

The selection of oligonucleotide probe sequences can be done manually from a known gene sequence using the following guidelines (**1**):

- The probe length should be between 18 and 50 bases. Longer probes will result in longer hybridization times and low synthesis yields, shorter probes will lack specificity.
- The base composition should be 40–60% G-C. Nonspecific hybridization may increase for G-C ratios outside of this range.
- Be certain that no complementary regions within the probe are present. These may result in the formation of “hairpin” structures that will inhibit hybridisation to target.
- Avoid sequences containing long stretches (more than four) of a single base.
- Once a sequence meeting the above criteria has been identified, computerized sequence analysis is highly recommended. The probe sequence should be compared with the sequence region or genome from which it was derived, as well as to the reverse complement of the region. If homologies to nontarget regions greater than 70% or eight or more bases in a row are found, that probe sequence should not be used.

However, to determine the optimal hybridization conditions, the synthesized probe should be hybridized to specific and nonspecific target nucleic acids over a range of hybridization conditions.

These same guidelines are applicable to design forward and reverse primers for amplification of a particular gene of interest to make a gene probe. It is important to bear in mind that, in this case, it is essential that the 3' end of both forward and reverse primers have no homology with other stretches of the template DNA other than the region you want to amplify. There are numerous software packages available (LaserDNA™, GeneJockey II™, etc.) that can be used to design a primer for a particular sequence or even just to check if the pair of primers designed manually will perform as expected.

3. Labeling and Detection

3.1. Types of Label

3.1.1. Radioactive Labels

Nucleic acid probes can be labeled using radioactive isotopes (e.g., ^{32}P , ^{35}S , ^{125}I , ^3H). Detection is by autoradiography or Geiger–Muller counters. Radiolabeled probes used to be the most common type but are less popular today because of safety considerations as well as cost and disposal of radioactive waste products. However, radiolabeled probes are the most sensitive, as they provide the highest degree of resolution currently available in hybridization assays (**1,2**). High sensitivity means that low concentrations of a probe–target hybrid can be detected; for example, ^{32}P -labeled probes can detect single-copy genes in only 0.5 μg of DNA and Keller and Manak (**1**) list a few reasons:

- ^{32}P has the highest specific activity.
- ^{32}P emits β -particles of high energy.
- ^{32}P -Labeled nucleotides do not inhibit the activity of DNA-modifying enzymes, because the structure is essentially identical to that of the nonradioactive counterpart.

Although ^{32}P -labeled probes can detect minute quantities of immobilized target DNA (<1 pg), their disadvantages is the inability to be used for high-resolution imaging and their relatively short half-life (14.3 d); ^{32}P -labeled probes should be used within a week after preparation.

The lower energy of ^{35}S plus its longer half-life (87.4 d) make this radioisotope more useful than ^{32}P for the preparation of more stable, less specific probes. These ^{35}S -labeled probes, although less sensitive, provide higher resolution in autoradiography and are especially suitable for *in situ* hybridization procedures. Another advantage of ^{35}S over ^{32}P is that the ^{35}S -

labeled nucleotides present little external hazard to the user. The low-energy β -particles barely penetrate the upper dead layer of skin and are easily contained by laboratory tubes and vials.

Similarly, ^3H -labeled probes have traditionally been used for *in situ* hybridization because the low-energy β -particle emissions result in maximum resolution with low background. It has the longest half-life (12.3 yr).

The use of ^{125}I and ^{131}I has declined since the 1970's with the availability of ^{125}I -labeled nucleoside triphosphates of high specific activity. ^{125}I has lower energies of emission and a longer half-life (60 d) than ^{131}I , and are frequently used for *in situ* hybridization.

3.1.2. Nonradioactive Labels

Compared to radioactive labels, the use of nonradioactive labels have several advantages:

- Safety.
- Higher stability of probe.
- Efficiency of the labeling reaction.
- Detection *in situ*.
- Less time taken to detect the signal.

Concern over laboratory safety and the economic and environmental aspects of radioactive waste disposal have been key factors in their development and use. Some examples are as follows:

- Biotin: This label can be detected using avidin or streptavidin which have high affinities for biotin. Because the reporter enzyme is not conjugated directly to the probe but is linked to it through a bridge (e.g., streptavidin–biotin), this type of nonradioactive detection is known as an indirect system. Usually, biotinylated probes work very well, but because biotin (vitamin H) is a ubiquitous constituent of mammalian tissues and because biotinylated probes tend to stick to certain types of Nylon membrane, high levels of background can occur during hybridizations. These difficulties can be avoided by using nucleotide derivatives, including digoxigenin-11-UTP, -11-dUTP, and -11-ddUTP, and biotin-11-dUTP or biotin-14-dATP. After hybridization, these are detected by an antibody or avidin, respectively, followed by a color or chemiluminescent reaction catalysed by alkaline phosphatase or peroxidase linked to the antibody or avidin (1,2).
- Enzymes. The enzyme is attached to the probe and its presence usually detected by reaction with a substrate that changes color. Used in this way, the enzyme is sometimes referred to as a “reporter group.” Examples of enzymes used include alkaline phosphatase and horseradish peroxidase (HRP). In the presence of peroxide and peroxidase, chloronaphtol, a chromogenic substrate for HRP, forms a purple insoluble product. HRP also catalyzes the oxidation of luminol, a chemiluminogenic substrate for HRP (2,3).
- Chemiluminescence. In this method, chemiluminescent chemicals attached to the probe are detected by their light emission using a luminometer. Chemiluminescent probes (including the above enzyme labels) can be easily stripped from membranes, allowing the membranes to be reprobed many times without significant loss of resolution.
- Fluorescence chemicals attached to probe fluoresce under ultraviolet (UV) light. This type of label is especially useful for the direct examination of microbiological or cytological specimens under the microscope—a technique known as fluorescent *in situ* hybridization (FISH). Hugenholts et al. have some useful considerations on probe design for FISH (4).
- Antibodies. An antigenic group is coupled to the probe and its presence detected using specific antibodies. Also, monoclonal antibodies have been developed that will recognize DNA–RNA hybrids. The antibodies themselves have to be labeled, using an enzyme, for example.
- DIG system. It is the most comprehensive, convenient, and effective system for labeling and detection of DNA, RNA, and oligonucleotides. Digoxigenin (DIG), like biotin, can be chemically coupled to linkers, and nucleotides and DIG-substituted nucleotides can be incorporated into nucleic acid probes by any of the standard enzymatic methods. These probes generally yield significantly lower backgrounds than those labeled with biotin. An anti-digoxigenin an-

Table 1
Types of Label

Radioactive labels
³² P
³⁵ S
¹²⁵ I
¹³¹ I
³ H
Nonradioactive labels
Biotin
Chemiluminescent enzyme labels (acridinium ester, alkaline phosphatase, β-D-galactosidase, horseradish peroxidase [HRP], isoluminol, xanthine oxidase)
Fluorescence chemicals (fluorochromes)
Antibodies
Digoxigenin system

tibody–alkaline phosphatase conjugate is allowed to bind to the hybridized DIG-labeled probe. The signal is then detected with colorimetric or chemiluminescent alkaline phosphatase substrates. If a colorimetric substrate is used, the signal develops directly on the membrane. The signal is detected on an X-ray film (as with ³²P- or ³⁵S-labeled probes) when a chemiluminescent substrate is used. Roche Biochemicals has a series of kits for DIG labeling and detection, as well as comprehensive detailed guides (5,6) with protocols for single-copy gene detection of human genome on Southern blots, detection of unique mRNA species on Northern blots, colony and plaque screening, slot/dot blots, and *in situ* hybridization.

The one area in which nonradioactive probes have a clear advantage is *in situ* hybridization. When the probe is detected by fluorescence or color reaction, the signal is at the exact location of the annealed probe, whereas radioactive probes can only be visualized as silver grains in a photographic emulsion some distance away from the actual annealed probe (7).

3.2. Labeling Methods

The majority of radioactive labeling procedures rely upon enzymatic incorporation of a nucleotide labeled into the DNA, RNA, or oligonucleotide.

Table 1 summarizes the various types of label (2).

3.2.1. Nick Translation

Nick translation is one method of labeling DNA, which uses the enzymes pancreatic Dnase I and *Escherichia coli* DNA polymerase I. The nick translation reaction results from the process by which *E. coli* DNA polymerase I adds nucleotides to the 3'-OH created by the nicking activity of Dnase I, while the 5' to 3' exonuclease activity simultaneously removes nucleotides from the 5' side of the nick. If labeled precursor nucleotides are present in the reaction, the pre-existing nucleotides are replaced with labeled nucleotides. For radioactive labeling of DNA, the precursor nucleotide is an [α-³²P]dNTP. For nonradioactive labeling procedures, a digoxigenin or a biotin moiety attached to a dNTP analog is used (2).

3.2.2. Random-Primed Labeling (or Primer Extension)

Gene probes, cloned or PCR-amplified, and oligonucleotide probes can be random-primed labeled with radioactive isotopes and nonradioactive labels (e.g., DIG). Random-primed labeling of DNA fragments (double- or single-stranded DNA) was developed by Feinberg and Volgestein (8,9) as an alternative to nick translation to produce uniformly labeled probes.

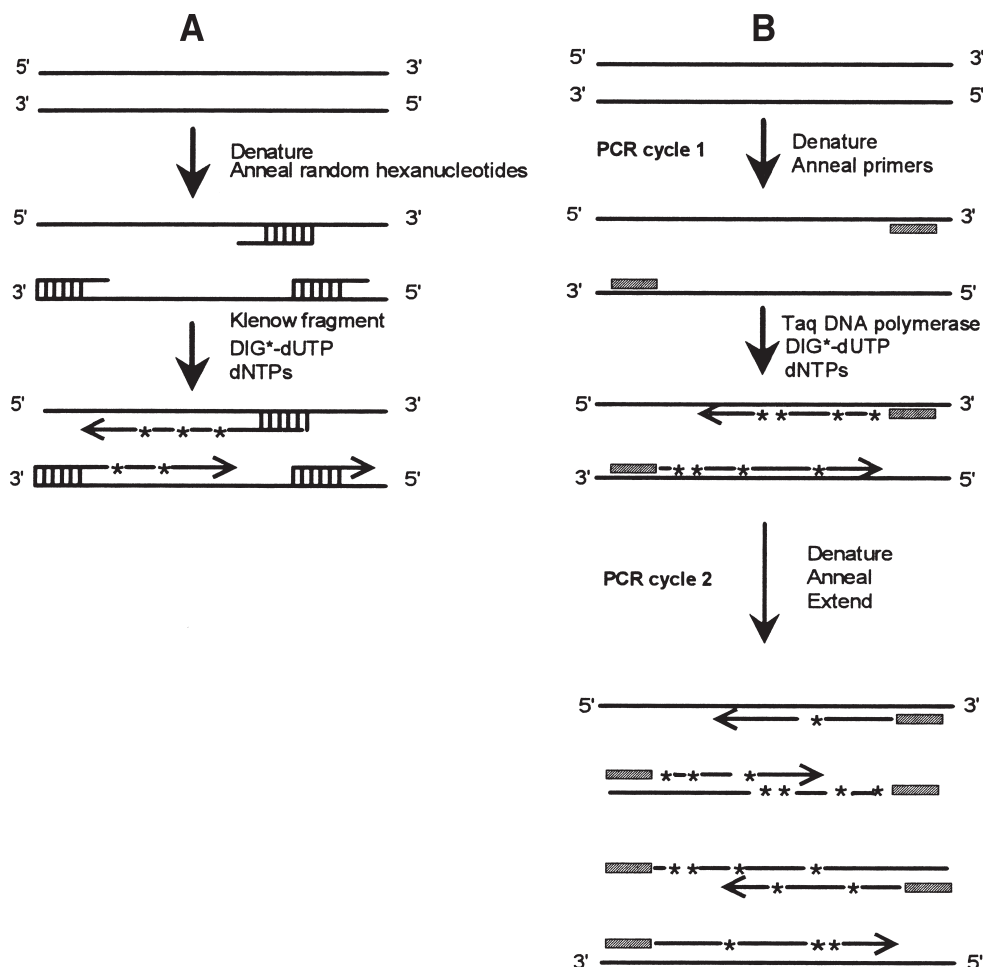


Fig. 1. Steps involved in the following: (A) random-primed DIG labeling: Double-stranded DNA is denatured and annealed with random oligonucleotide primers (6-mers); the oligonucleotides serve as primers for the 5' to 3' Klenow fragment of *E. coli* DNA polymerase I, which synthesizes labeled probes in the presence of DIG-dUTP. (B) PCR-DIG labelling: DIG-dUTP is incorporated during PCR cycles into the DNA strands amplified from the DNA target. The asterisk represents the digoxigenin molecule incorporated along the DNA strands.

Double-stranded DNA is denatured and annealed with random oligonucleotide primers (6-mers). The oligonucleotides serve as primers for the 5' to 3' polymerase (the Klenow fragment of *E. coli* DNA polymerase I), which synthesizes labeled probes in the presence of a labeled nucleotide precursor. **Figure 1A** shows the steps involved in random-primed DIG labeling as an example.

3.2.3. DIG-PCR Labeling

A very robust method for labeling a gene probe with DIG uses PCR. The probe is PCR-amplified using the appropriate set of primers and thermocycling parameters, however, the dNTP mixture has less dTTP because the labeled DIG-dUTP will also be added to the reaction. (Similarly, when this method is used with [α - 32 P]dCTP, the dNTP mixture will not have dCTP.) The advantage of PCR-DIG labeling, over random-primed DIG labeling, is the incorporation

of a higher number of DIG moieties along the amplified DNA strands during the PCR cycles. It is worth noting that the random incorporation of large molecules of DIG–dUTP along the DNA strands during the PCR cycles makes the amplified fragment run slower on an agarose gel. A control PCR reaction, without DIG–dUTP, should also be prepared at the same time to verify whether the size of the amplified fragment with incorporated DIG (labeled probe) corresponds to the desired gene fragment. **Figure 1B** shows the steps involved in PCR–DIG labeling, and refs. (10–12) describe successful examples of use of PCR–DIG labeling.

3.2.4. Photobiotin Labeling

Photobiotin labeling is a chemical reaction, not an enzymatic one. Biotin and DIG can be linked to a nitrophenyl azido group that is converted by irradiation with UV or strong visible light to a highly reactive nitrene that can form stable covalent linkages to DNA and RNA (2). The materials for photobiotin labeling are more stable than the enzymes needed in nick translation or oligonucleotide labeling and are less expensive, and it is a method of choice when large quantities of probe but not very high sensitivities (3,13).

3.2.5. End Labeling

End labeling of probes for hybridization is mainly used to label oligonucleotide probes (for a review, see **ref. 14**).

Roche Biochemicals (6) has developed three methods for labeling oligonucleotides with digoxigenin:

- The 3'-end labeling of an oligonucleotide 14–100 nucleotides in length with 1 residue of DIG-11-ddUTP per molecule
- The 3' tailing reaction, where terminal transferase adds a mixture of unlabeled nucleotides and DIG-11-dUTP, producing a tail containing multiple digoxigenin residues
- The 5' end labeling in a two-step synthesis with first an aminolinker residue on the 5' end of the oligonucleotide, and then after purification, a digoxigenin-*N*-hydroxy-succinimide ester is covalently linked to the free 5'-amino residue.

Oligonucleotides can also be labeled with radioisotopes by transferring the γ - ^{32}P from [γ - ^{32}P]ATP to the 5' end using the enzyme bacteriophage T4 polynucleotide kinase. If the reaction is carried out efficiently, the specific activity of such probes can be as high as the specific activity of [γ - ^{32}P]ATP itself (2).

Promega has a detailed guide (15) with protocols on radioactive and nonradioactive labeling of DNA. The choice of probe labeling method will depend on the following:

- Target format: Southern, Northern, slot/dot, or colony blot (*see Subheading 4.*)
- Type of probe: gene or oligonucleotide probe
- Sensitivity required for detection: single-copy gene or detection of PCR-amplified DNA fragments

For example, 3'- and 5'-end labeling of oligonucleotides give good results on slot and colony hybridization in contrast with poor sensitivity when using Southern blotting.

4. Target Format

4.1. Solid Support

A convenient format for the hybridization of DNA to gene probes or oligonucleotide probes is immobilization of the target nucleic acid (DNA or RNA) onto a solid support while the probe is free in solution. The solid support can be a nitrocellulose or Nylon membrane, Latex or magnetic beads, or microtiter plates. Nitrocellulose membranes are very commonly used and produce low background signals; however, they can only be used when colorimetric detection will be performed and no probe stripping and reprobing is planned. For these purposes, positively charged Nylon membranes are recommended, and they also ensure an optimal signal-to-noise ratio when the DIG system is used. Although nitrocellulose membranes are able to bind

large quantities of DNA, they become brittle and gradually release DNA during the hybridization step. Activated cellulose membranes, on the other hand, are more difficult to prepare, but they can be reused many times because the DNA is irreversibly bound (2).

After size fractionation of nucleic acids by electrophoresis, they are transferred to a filter membrane, which is then probed. The presence of target is confirmed by the detection of a probe on the filter membrane, for example, radiolabeled probe can be detected by autoradiography and the location of the target sequence in the bands in the original gel determined.

The different immobilization techniques include the following: Southern blots, when whole or digested chromosomal DNA is electrophoresed in an agarose gel, denatured, and blotted onto a membrane; Northern blots, when the same procedure is used for RNA; slot blots, when whole RNA or denatured DNA is loaded under vacuum into slots onto membranes (similar procedure for dot blots); colony blots, when colonies are treated with lysozyme on plates and further treatment with protease, and denaturation and neutralization solutions are applied and the procedure adjusted according the microorganism's peculiarities. The great advantage of colony blotting over slot blotting is that strains with a specific sequence can be rapidly detected from plates and the DNA preparation procedure can then be done only for the strains of interest. In a similar way, the slot blotting procedure has the advantage of quickly highlighting which DNA sample has the gene sequence of interest when a gene probe is hybridized to whole-DNA samples. The Southern blotting procedure, which involves the digest of DNA with restriction enzymes and gel electrophoresis, takes a longer preparation time than slot blotting but can provide information on the size and position of the gene as well as grouping the samples based on the similar patterns when different restriction enzymes are used to digest the samples. Schleicher and Schuell has a detailed manual on solid supports and DNA transfer (16).

4.2. In Solution

Both the probe and the target are in solution. Because both are free to move, the chances of reaction are maximized and, therefore, this format generally gives faster results than others.

4.3. In Situ

In this format, the probe solution is added to fixed tissues, sections, or smears, which are then usually examined under the microscope (see Chapter 29). The probe label (e.g., a fluorescent marker) produces a visible change in the specimen if the target sequence is present and hybridization has occurred. However, the sensitivity might be low if the amount of target nucleic acid present in the specimen is low. This can be used for the gene mapping of chromosomes and for the detection of microorganisms in specimens.

5. Hybridization Conditions

Many methods are available to hybridize probes in solution to DNA or RNA immobilized on nitrocellulose membranes (see Chapter 4). These methods can differ in the solvent and temperature used, the volume of solvent and the length of time of hybridisation, the method of agitation, when required, and the concentration of the labeled probe and its specificity, the stringency of the washes after the hybridization. However, basically, target nucleic acid immobilized on membranes by the Southern blot, Northern blot, slot or dot blot, or colony blot procedures are hybridized in the same way. The membranes are first prehybridized with hybridization buffer minus the probe. Nonspecific DNA binding sites on the membrane are saturated with carrier DNA and synthetic polymers. The prehybridization buffer is replaced with the hybridization buffer containing the probe and incubated to allow hybridization of the labeled probe to the target nucleic acid. The optimum hybridization temperature is experimentally determined, starting with temperatures 5°C below the melting temperature (T_m). The T_m is defined as the temperature corresponding to the midpoint in transition from helix to random coil and depends on length, nucleotide composition and ionic strength for long stretches of nucleic acids. G-C pairs are more stable than A-T pairs because G and C form three H bonds as

Table 2
Examples of the Applications of Nucleic Acid Probes in Medical Research

Application	Ref.
Detection of tumor suppressor genes in human bladder tumors	21
Identification of <i>Leishmania</i> parasites	22
Detection of malignant plasma cells of patients with multiple myeloma	23
Diagnosis of human papillomavirus	24
Visual gene diagnosis of HBV and HCV	25
Detection and identification of pathogenic <i>Vibrio parahaemolyticus</i>	26
Detection of <i>Vibrio cholerae</i>	27–29
Molecular analysis of tetracycline resistance in <i>Salmonella enterica</i>	30
Identification of fimbrial adhesins in necrotoxicogenic <i>E. coli</i>	31
Epidemiological analysis of <i>Campylobacter jejuni</i> infections	32
Molecular analysis of NSP4 gene from human rotavirus strains	33
Physical mapping of human parasite <i>Trypanosoma cruzi</i>	34
Detection and identification of African trypanosomes	35
Changes related to neurological diseases (Alzheimer's, Huntington's)	36
Detection and identification of pathogenic <i>Candida</i> spp.	37,38
Identification of <i>Mycobacterium</i> spp.	39
Detection of rifampin resistance in <i>Mycobacterium tuberculosis</i>	40
Identification of <i>Staphylococcus aureus</i> directly from blood cultures	41
Detection of rabies virus genome in brain tissues from mice	42

opposed to two between A and T. Therefore, double-stranded DNA rich in G and C has a higher T_m (more energy required to separate the strands) than A-T rich DNA. For oligonucleotide probes bound to immobilized DNA, the dissociation temperature, T_d , is concentration dependent. Stahl and Amman (17) discussed in detail the empirical formulas used to estimate T_m and T_d .

Following the hybridization, the unhybridized probe is removed by a series of washes. The stringency of the washes must be adjusted for the specific probe used. Low-stringency washing conditions (higher salt and lower temperature) increases sensitivity; however, these conditions can give nonspecific hybridization signals and high background. High-stringency washing conditions (lower salt and higher temperature, closer to the hybridization temperature) can reduce background and only the specific signal will remain. The hybridization signal and background can also be affected by probe length, purity, concentration, sequence, and target contamination (1).

In aqueous solution, RNA–RNA hybrids are more stable than RNA–DNA hybrids, which are, in turn, more stable than DNA–DNA ones. This results in a difference in T_m of approx 10°C between RNA–RNA and DNA–DNA hybrids. Consequently, more stringent conditions should be used with RNA probes (8).

In general, the hybridization rate increases with probe concentration. Also, within narrow limits, sensitivity increases with increasing probe concentration. The concentration limit is not determined by any inherent physical property of nucleic acid probes, but by the type of label and nonspecific binding properties of the immobilization medium involved.

6. Applications in Medical Research

At least three basic applications of nucleic acid probes in medical research can be mentioned: (1) detection of pathogenic microorganisms, (2) detection of changes to nucleic acid sequences, and (3) detection of tandem repeat sequences. **Table 2** presents only a few examples of recently published literature on applications of nucleic acid probes in medical research.

6.1. Detection of Pathogenic Microorganisms

The application of nucleic acid probes has particularly been evident in microbial ecology, where probes can be used to detect unculturable microorganisms and pathogens in the environment or simply provide rapid identification of species and group levels. Through the development of DNA–DNA and RNA–DNA hybridization procedures and recombinant DNA methodology, the isolation of species-specific gene sequences is readily achieved (18,19). Oligonucleotide hybridization probes complementing either small ribosomal subunits, large ribosomal subunits, or internal transcribed spacer regions have now been developed for a wide variety of microorganisms (20), such as *Actinomyces*, *Bacteriodes*, *Borrelia*, *Clostridium*, *Campylobacter*, *Candida*, *Haemophilus*, *Helicobacter*, *Lactococcus*, *Mycoplasma*, *Neisseria*, *Proteus*, *Rickettsia*, *Vibrio*, *Streptococcus*, *Plasmodium*, *Pneumocystis*, *Trichomonas*, *Desulfovibrio*, *Streptomyces*, including some uncultivated species such as marine proteobacteria and thermophilic cyanobacterium, and *Chlamydia* species, *Rickettsia* species, *Trypanosoma* species, *Treponema pallidum*, *Pneumocystis carinii*, and *Mycobacterium* species to mention only a few examples with medical relevance. Detection of a nucleic acid sequence unique to a particular microorganism would demonstrate its presence in a specimen and, perhaps, confirm an infectious disease.

6.2. Detection of Changes to Nucleic Acid Sequences

A change to the DNA sequence is a mutation, which could involve deletion, insertion, or substitution. Changes in certain gene sequences can cause inherited diseases such as cystic fibrosis, muscular dystrophies, phenylketonuria, apolipoprotein variants, and sickle cell anemia, and they can be diagnosed by probe detection. Nucleic acid probes have successfully been used to detect those mutations. With some inherited diseases, more than one type of mutation can cause the disease, in which case, a probe might have to be used under low stringency (to allow hybridization to a range of sequences) or several probes might be used to ensure hybridization to all target sequences.

6.3. Detection of Tandem Repeat Sequences

Tandem repeat sequences are usually 30–50 bp in length. Their size and distribution are distinctive for an individual. They can be detected using nucleic acid probes and PCR. They are the basis of so-called “DNA fingerprinting,” which can be used in forensic science to confirm the identity of a suspect from specimens (any body fluid, skin, and hair) left at the scene of a crime. This technique can also be used for paternity tests, sibling confirmation, and tissue typing.

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