Topic Introduction

Preparation of Labeled DNA, RNA, and Oligonucleotide Probes

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Labeled nucleic acids and oligonucleotides are typically generated by enzymatic methods such as end-labeling, random priming, nick translation, in vitro transcription, and variations of the polymerase chain reaction (PCR). Some of these methods place the label in specific locations within the nucleic acid (e.g., at the 5' or 3' terminus); others generate molecules that are labeled internally at multiple sites. Some methods yield labeled single-stranded products, whereas others generate double-stranded nucleic acids. Finally, some generate probes of defined length, whereas others yield a heterogeneous population of labeled molecules. Options available for generating and detecting labeled nucleic acids, as well as advice on designing oligonucleotides for use as probes, is included here.

INTRODUCTION

Labeled nucleic acids and oligonucleotides are used in molecular cloning either as reagents or as probes.

- Labeled fragments of cloned DNA and oligonucleotides of defined size are used as reagents in a variety of methods to analyze RNA, including digestion with S1 nuclease, RNase protection, and primer extension. In these cases, the labeled reagent is converted to products of different sizes that can be detected by a variety of methods, the most common being gel electrophoresis followed by autoradiography or phosphorimaging. Labeled DNA and RNA can also be used as size markers in gel electrophoresis.
- Labeled DNA, RNA, LNA, and oligonucleotide probes are used in hybridization-based techniques to locate and bind DNAs and RNAs of complementary sequence. These techniques include Southern and northern analyses and in situ hybridization.

Success in all these applications depends on the facile introduction of label(s) into the nucleic acid or oligonucleotide. Tables 1 and 2 summarize the options available for labeling nucleic acids and guide investigators to a method of labeling that suits the task at hand.

RADIOACTIVE VERSUS NONRADIOACTIVE LABELING OF NUCLEIC ACIDS

In conventional in vitro radiolabeling, radioactive isotopes—usually ^{32}P —are woven into the natural fabric of the probe in place of their nonradioactive homologs. In 5'-terminal-labeling reactions of DNAs and oligonucleotides, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is used to provide the ^{32}P moiety, which is transferred to the 5'-most deoxyribonucleotide triphosphate (dNTP) using bacterial T4 polynucleotide kinase. This phosphorylation reaction results in the incorporation of one atom of ^{32}P per nucleic acid molecule.

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TABLE 1. Methods used to label nucleic acids in vitro

Starting material	Method	Enzyme used	Type of probe generated	Position of label	Size of probe	Application	Specific activity if radiolabeled	Can be nonradio-labeled?
DNA	Random priming ^a	Klenow	dsDNA	Internal	~400–600 nt	5' nuclease mapping	5 × 10 ⁸ to 5 × 10 ⁹ dpm/μg	Yes
	Nick translation ^b	DNase I + DNA Pol I	dsDNA	Internal	~400 nt	Primer extension RNase protection Size markers Southern and northern blotting	5 × 10 ⁸ to 5 × 10 ⁹ dpm/μg	Yes
	PCR ^c	Thermostable DNA polymerase (e.g., <i>Taq</i>)	ss or dsDNA	Internal	Depends on the positions of primers; generates probes of defined length	•	~1 × 10 ⁹ to 2 × 10 ⁹ dpm/μg	Yes
	Primer extension	T4 polynucleotide kinase+Klenow	ssDNA	Internal	Generates probes of defined length following restriction enzyme digestion	•	~1 × 10 ⁹ dpm/μg	No
	In vitro transcription ^d	DNA-dependent RNA polymerase (e.g., T3, T7, or SP6)	ssRNA	Internal	Generates probes of defined length equivalent to template	•	~1 × 10 ⁹ dpm/μg	Yes
	Addition of nucleotides to recessed 3' termini ^e	Klenow	dsDNA	3' terminus	Generates probes of defined length approximately equivalent to template	•	1 × 10 ⁸ to 2 × 10 ⁸ dpm/μg	Yes
RNA	Phosphorylation of 5' termini ^{f,g}	T4 polynucleotide kinase	dsDNA	5' terminus	Generates probes of defined length equivalent to template	•	1 × 10 ⁸ to 2 × 10 ⁸ dpm/μg	No
	Random priming ^{h,i}	Reverse transcriptase, Klenow	ssDNA or DNA–RNA hybrids	Internal	~400–600 nt	•	~1 × 10 ⁹ dpm/μg	No

(continued)

TABLE 1. Continued

Starting material	Method	Enzyme used	Type of probe generated	Position of label	Size of probe	Application	Specific activity if radiolabeled	Can be nonradio-labeled?
Oligonucleotide or LNA	Phosphorylation of 5' termini ^g	T4 polynucleotide kinase	ssDNA	5' terminus	Generates probes of defined length equivalent to oligo	5' nuclease mapping	1×10^8 to 4×10^8 dpm/ μ g	No
	Addition of nucleotides to 3' termini ⁱ	Terminal deoxynucleotidyl transferase	ssDNA	3' terminus	Generates probes of defined length approximately equivalent to template	Primer extension Size markers Southern and northern blotting In situ hybridization Differential screening	3×10^{10} dpm/ μ g	Yes
	Primer extension ^k	Klenow	dsDNA, whose strands must then be separated	Internal	Generates probes of defined length approximately equivalent to template		2×10^{10} dpm/ μ g	No

^aSee Protocol: Random Priming: Labeling of Purified DNA Fragments by Extension of Random Oligonucleotides (Green and Sambrook 2019a).^bSee Protocol: Labeling of DNA Probes by Nick Translation (Green and Sambrook 2020a).^cSee Protocol: Labeling of DNA Probes by Polymerase Chain Reaction (Green and Sambrook 2021a).^dSee Protocol: Synthesis of Single-Stranded RNA Probes by In Vitro Transcription (Green and Sambrook 2020b).^eSee Protocol: Labeling 3' Termini of Double-Stranded DNA Using the Klenow Fragment of *E. coli* DNA Polymerase I (Green and Sambrook 2020c).^fSee Protocol: Dephosphorylation of DNA Fragments with Alkaline Phosphatase (Green and Sambrook 2020d).^gSee Protocol: Labeling the 5' Termini of DNA with Bacteriophage T4 Polynucleotide Kinase (Green and Sambrook 2021b).^hSee Protocol: Synthesis of cDNA Probes from mRNA Using Random Oligonucleotide Primers (Green and Sambrook 2019b).ⁱSee Protocol: Radiolabeling of Subtracted cDNA Probes by Random Oligonucleotide Extension (Green and Sambrook 2021c).^jSee Protocol: Labeling the 3' Termini of Oligonucleotides Using Terminal Deoxynucleotidyl Transferase (Green and Sambrook 2021d).^kSee Protocol: Labeling of Synthetic Oligonucleotides Using the Klenow Fragment of *E. coli* DNA Polymerase I (Green and Sambrook 2021e).

TABLE 2. End-labeling of DNA

Template	Desired position of label	Preferred methods
ssDNA or RNA	5' Terminus	<p>Kinase reaction: A hydroxyl group is first created by removing the unlabeled phosphate residue from the 5' end of the nucleic acid with an alkaline phosphatase such as CIP or SAP (Protocol: Dephosphorylation of DNA Fragments with Alkaline Phosphatase [Green and Sambrook 2020d]). Bacteriophage T4 polynucleotide kinase is then used to transfer the label from the γ position of ATP to the newly created 5'-hydroxyl group (Wu et al. 1976) (Protocol: Labeling the 5' Termini of DNA with Bacteriophage T4 Polynucleotide Kinase [Green and Sambrook 2021b]). These reactions can be performed sequentially in one tube but require inhibition of phosphatase with inorganic phosphate (Chaconas and van de Sande 1980; Cobianni and Wilson 1987). An alternative procedure has been described in which digestion with a restriction endonuclease and 5'-end labeling with polynucleotide kinase can be performed in one step without the benefit of alkaline phosphatase (Ommen et al. 1990). The mechanism of this reaction is obscure.</p> <p>Exchange reaction: The unlabeled phosphate residue is transferred from the 5' terminus of the nucleic acid to ADP and is then replaced by labeled phosphate from the γ position of ATP. Both of these reactions are catalyzed by bacteriophage T4 polynucleotide kinase and proceed simultaneously in the same test tube in the presence of excess ADP and limiting amounts of γ-labeled ATP (Berkner and Folk 1977). When labeling ssDNAs that are >300 nucleotides in length, the overall efficiency of the exchange reaction is greatly improved by including a macromolecular crowding agent such as PEG (4%–10%) in the reaction mixture (Harrison and Zimmerman 1986a,b). Even so, the specific activity of the 5'-labeled DNA is always less than can be achieved with the kinase reaction.</p> <p>Note: Bacteriophage T4 polynucleotide kinase carries a 3'-phosphatase activity that degrades ATP. Several commercial suppliers prepare the enzyme from cells infected with a strain of bacteriophage T4 (amN81 pseT1) that carries a mutated version of the gene encoding polynucleotide kinase. This mutant form of the enzyme lacks 3'-phosphatase activity.</p>
ssDNA	3' Terminus	<p>Template-independent polymerization of [α-^{32}P]NTP to the 3' terminus of ssDNA is catalyzed by calf thymus terminal deoxy-DNA nucleotidyl transferase and requires Co^{2+} as a cofactor (Protocol: Labeling the 3' Termini of Oligonucleotides Using Terminal Deoxynucleotidyl Transferase [Green and Sambrook 2021d]; Deng and Wu 1983). The radiolabeled DNA is then exposed to alkali and treated with alkaline phosphatase to generate a uniformly labeled DNA fragment of defined length (Roychoudhury et al. 1976, 1979; Wu et al. 1976). Alternatively, incorporation at the 3' terminus can be limited to just one nucleotide by using [α-^{32}P]ddATP (Yousaf et al. 1984) or [α-^{32}P]cordycepin triphosphate (Tu and Cohen 1980) as a substrate for terminal transferase. Because neither of these molecules carries a 3'-hydroxyl group, no additional molecules can be incorporated. Under most circumstances, [α-^{32}P]ddATP is the preferred substrate for labeling 3' termini because it is incorporated more efficiently than [α-^{32}P]cordycepin triphosphate. However, DNA molecules carrying cordycepin residues at their 3' termini have one advantage: They are resistant to digestion with contaminating exonucleases that may remove 3'-terminal ribonucleotide labels from ssDNA.</p> <p>Terminal transferase can also be used to add biotinylated or fluoresceinated deoxynucleotides or dideoxynucleotides to the 3' termini of ssDNA fragments (see Protocol: Labeling the 3' Termini of Oligonucleotides Using Terminal Deoxynucleotidyl Transferase [Green and Sambrook 2021d], Alternative Protocol: Synthesizing Nonradiolabeled Probes Using TdT; e.g., see Vincent et al. 1982; Schneider et al. 1994).</p>
dsDNA with protruding 5' terminus	5' Termini	Labeling is performed with polynucleotide kinase as described above for labeling of the 5' termini of ssDNA (Protocol: Labeling the 5' Termini of DNA with Bacteriophage T4 Polynucleotide Kinase [Green and Sambrook 2021b]).
dsDNA with blunt-ended or recessed 5' termini	5' Termini	Labeling is performed with polynucleotide kinase as described above for labeling of the 5' termini of ssDNA (Protocol: Labeling the 5' Termini of DNA with Bacteriophage T4 Polynucleotide Kinase [Green and Sambrook 2021b]). However, dsDNA molecules with blunt ends or recessed 5' termini are labeled less efficiently by polynucleotide kinase than molecules with protruding 5' termini. Even a partial reaction at recessed 5' termini requires large amounts of enzyme and [γ - 32 P]ATP or the addition of a crowding agent such as PEG (Lillehaug and Kleppe 1975; Harrison and Zimmerman 1986a).
dsDNA with blunt-ended or recessed 3' termini	3' Termini	<p>The Klenow fragment, which retains the template-dependent deoxynucleotide polymerizing activity and the 3' $\rightarrow$ 5' exonuclease of the holo-enzyme but lacks its powerful 5' $\rightarrow$ 3' exonuclease activity, is used to fill recessed 3' termini of dsDNA (Protocol: Labeling 3' Termini of Double-Stranded DNA Using the Klenow Fragment of <i>E. coli</i> DNA Polymerase I [Green and Sambrook 2020c]). Usually, only one of the four dNTPs present in the reaction mixture is radiolabeled. The presence of the three unlabeled dNTPs serves two purposes: It blocks exonucleolytic removal of nucleotides from the 3' terminus of the template, and it allows the radiolabeled dNTP to be the second, third, or fourth nucleotide added to the recessed 3' terminus. The presence of only one radiolabeled dNTP in</p>

(continued)

TABLE 2. Continued

Template	Desired position of label	Preferred methods
		<p>the reaction mixture sometimes permits dsDNA that has been cleaved with two different restriction endonucleases to be labeled selectively at only one end. For example, a DNA that has been cleaved with</p> <div><div>G</div><div>G</div><div>BamHI CCTAG and EcoRI CTTAA</div></div> <p>can be selectively labeled at the BamHI terminus by using $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$ as the sole source of radioactivity in the fill-in reaction. Alternatively, DNA can be cleaved with one restriction enzyme, radiolabeled, and then cleaved with a second enzyme to generate two DNA fragments, each of which carries radiolabel at only one end.</p> <p>Specialized vectors have been constructed that facilitate the asymmetric labeling of DNA fragments. For example, pSP64CS and pSP65CS contain sites that are cleaved by the restriction enzyme Tth111, which recognizes the redundant sequence</p> <div><div>↓</div><div>5'GACNNNGTC3'</div><div>3'CTGNNNCAG5'</div><div>↑</div></div> <p>and generates a terminus with a single protruding 5' nucleotide. The DNA of interest is cloned between two Tth111 sites that yield DNAs with different protruding nucleotides at their termini. After cleavage, the DNA can be selectively labeled at one or both ends by the Klenow fragment and the appropriate $[\alpha\text{-}^{32}\text{P}]\text{dNTP(s)}$ (Volkaert et al. 1984; Eckert 1987).</p> <p>Blunt-ended DNA fragments can be labeled by template-independent addition of a single nucleotide (usually $[\alpha\text{-}^{32}\text{P}]\text{dATP}$) catalyzed by <i>Taq</i> polymerase.</p> <p>Bacteriophage T4 DNA polymerase carries a 5' → 3' polymerase and 3' → 5' exonuclease activity that is more active on ssDNA than dsDNA. The exonuclease of bacteriophage T4 DNA polymerase is ~200 times more potent than the equivalent exonuclease of the Klenow fragment. T4 DNA polymerase is used to end-label DNA molecules with protruding 3' termini. This reaction works in two stages. First the powerful 3' → 5' exonuclease removes the protruding tails from the DNA and creates recessed 3' termini. Then, in the presence of high concentrations of radiolabeled precursor(s), exonucleolytic degradation is balanced by incorporation of dNTPs at the 3' termini. This reaction, which consists of cycles of removal and replacement of the 3'-terminal nucleotides from recessed or blunt-ended DNA, is sometimes called an exchange or replacement reaction (O'Farrell et al. 1980).</p> <p>Alternatively, protruding 3' termini can be labeled by template-independent addition of nucleotides catalyzed by calf thymus terminal deoxynucleotidyl transferase, poly(A) polymerase (see 3'-terminal labeling of ssDNA) or certain thermostable DNA polymerases, (e.g., <i>Taq</i>).</p> <p>Bacteriophage T4 RNA ligase catalyzes the joining of a terminating radioactive bisphosphate nucleotide (5' - ^{32}PpNp) to a 3'-hydroxyl terminus of RNA (Uhlenbeck and Gumpert 1982). The reaction extends the length of the RNA molecule by one nucleotide and generates a phosphorylated 3' terminus with a $[\gamma\text{-}^{32}\text{P}]\text{phosphate}$ in the last internucleotide linkage (England et al. 1977; Kikuchi et al. 1978; Tyc and Steitz 1989). The terminal 3'-phosphate group acts as a chain terminator by preventing formation of further phosphodiester bonds. This method of labeling has been chiefly used with small RNAs. However, perhaps because of steric effects caused by secondary structure, the amount of label incorporated seriously misrepresents the relative abundance of the RNAs used as substrates.</p> <p>Poly(A) polymerase catalyzes the template-independent addition of A residues to the 3' terminus of RNA. When $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ is used as a substrate, the phosphodiester bonds in the poly(A) tract contain ^{32}P atoms (Lingner and Keller 1993). Substitution of cordycepin triphosphate (3'-dATP) for ATP results in the addition of a single 3'-dA residue to the end of the RNA, which is a useful method for radiolabeling RNA at the 3' end. Yeast poly(A) polymerase preferentially labels longer RNA molecules, whereas short RNA molecules are labeled more efficiently by RNA ligase.</p> <p>Terminal transferase has been used to add a biotin-labeled dideoxynucleotide to the 3' terminus of RNA (Schneider et al. 1994). However, the efficiency of this reaction has not been thoroughly investigated.</p>
dsDNA with protruding 3' termini	3' termini	
RNA	3' termini	

In internal labeling methods, radioactive derivatives of [α - ^{32}P]dNTP are substituted for their non-radioactive counterparts in reactions that are typically performed in the presence of one ^{32}P -dNTP and three unlabeled dNTPs, resulting in the incorporation of multiple ^{32}P atoms per nucleic acid molecule. Radioactive nucleotides can be incorporated internally using one of several enzymatic methods, such as random priming or primer extension (using the Klenow fragment of *Escherichia coli* DNA polymerase I [Pol I]; see Introduction: *E. coli* DNA Polymerase I and the Klenow Fragment [Green and Sambrook 2020e]), nick translation (using a combination of DNase I and DNA Pol I), PCR (using a thermostable DNA polymerase such as *Taq*), and in vitro transcription (using a bacteriophage DNA-dependent RNA polymerase; see Introduction: **In Vitro Transcription Systems** [Green and Sambrook 2020f]). Finally, DNAs can be labeled at their 3' end by the addition of one or more radiolabeled [α - ^{32}P]dNTPs to recessed 3' termini using the Klenow fragment of *E. coli* DNA polymerase, whereas oligonucleotides are labeled at their 3' termini using terminal deoxynucleotidyl transferase (TdT), which catalyzes non-template-directed nucleotide incorporation onto the 3'-hydroxyl end of single-stranded DNA (ssDNA).

In contrast to radiolabeling methods, nonradioactive labeling methods involve the incorporation of a chemical group or compound that is not normally found in nucleic acids. The nonradioactive label can be conjugated to the probe via a photochemical or synthetic reaction (for reviews, see Kricka 1992; Mansfield et al. 1995) or, more commonly, a labeled nucleotide can be incorporated into the probe using the same enzymatic reactions (e.g., random priming, nick translation, in vitro transcription) used to incorporate a radioactive label. However, as described in more detail below, unlike radiolabeling, nonradioactive labeling of the 5' terminus cannot be done enzymatically. After hybridization to the target nucleic acid, the modifying groups on the probe are detected by an appropriate indicator system.

Choosing a Label: Radioactive or Nonradioactive?

The main advantage of radioactive probes is their high sensitivity; the chief disadvantage is the use of hazardous radioactive isotopes. The exposure of personnel to radiation from the energetic β particles released by decaying ^{32}P may be significant. Not surprisingly, the amount of paperwork and equipment required to order and monitor the use and disposal of radiolabeled materials has increased significantly over the years to the point where it has become a real burden. In addition, the physical and political difficulties in the storage and disposal of low-level radioactive waste have become significant and continue to grow. Finally, there are also practical inconveniences; for example, experiments must be scheduled around the delivery of radiolabeled compounds.

The advantages claimed for nonradioactive methods include faster results, more stable probes, and lower cost. However, choosing a nonradioactive detection method is not a trivial task: A plethora of nonradioactive detection kits are available on the commercial market and more are appearing all the time. Claims on the sensitivity and signal-to-noise ratios made on their behalf should be viewed with caution. Many of these claims are based on experiments performed under idealized conditions that cannot be immediately or easily reproduced by inexperienced investigators in the hurly-burly of a working laboratory. Frequently, the signal-to-noise ratio is higher than advertised and the neophyte will likely struggle to match the sensitivity of more traditional methods of radiolabeling. Many of the kits are expensive, and nonradioactive detection methods only become cost-effective after they have been optimized for the particular task at hand.

Perhaps the chief factor to consider when choosing between a radioactive or nonradioactive label is the type of experiment being performed. For some techniques, the use of radioactivity is still the predominant labeling method. For example, differential display, subtractive hybridization, and RNA analyses using S1 nuclease, RNase protection, or primer extension almost exclusively rely on radioactively labeled probes. In these methods, the radiolabeled products are first separated by gel electrophoresis and then detected by autoradiography or phosphorimaging. It should be noted, however, that nonradioactive modifications of these methods have been described (see, e.g., Turnbow and Garner 1993; Chan et al. 1996).

For Southern and northern hybridization-based techniques, researchers have a choice between using radioactive and nonradioactive labels. ^{32}P -labeled probes offer high sensitivity—they can detect minute quantities of immobilized target DNA (<1 pg)—but also suffer from a short half-life (14.3 d) and the inability to be used for high-resolution imaging. Nonradioactive probes offer several advantages: Not only are they less hazardous and less costly than radiochemical techniques, but their half-life and shelf life are longer, and detection of hybrids is much faster than for radioactive probes.

Nowadays, in situ hybridization techniques rely exclusively on the use of nonradiolabeled probes. Traditionally, in situ hybridization for the detection of messenger RNA (mRNA) in tissues was performed on sectioned material using probes that were labeled with ^3H or ^{35}S , which required the use of a photographic film emulsion covering the sections for the detection of the signal. In addition to the inherent problems associated with radioactive probes, such as safety and limited shelf life, the scatter associated with radioactive decay greatly limited the spatial resolution of the technique. The development of highly sensitive nonradioactively labeled probes has allowed in situ hybridization to be performed directly in whole embryos, tissues, and, in the case of fluorescence in situ hybridization (FISH), in single cells with incredibly high resolution.

The protocols cited in this introduction provide detailed methods for generating radiolabeled probes. Where applicable, the materials and steps for synthesizing nonradioactive probes are integrated into the protocol.

TYPES OF NONRADIOACTIVE DETECTION SYSTEMS

Indirect Detection Systems

The first nonradioactive methods of detection developed in the 1980s were based on the labeling of the nucleic acid probe with dinitrophenol (Keller et al. 1988), bromodeoxyuridine (Traincard et al. 1983), or biotin (Langer et al. 1981; Chu and Orgel 1985; Reisfeld et al. 1987). It was this latter compound that provided the most durable and sensitive of these prototypic systems and remains one of the most commonly used nonradioactive labels today (see Introduction: **Biotin** [Green and Sambrook 2021f]). After hybridization, the biotinylated probe is detected via interaction with streptavidin that has been conjugated to a reporter enzyme—usually alkaline phosphatase. The membrane is then exposed to an enzyme capable of hydrolyzing a colorigenic, fluorogenic, or chemiluminescent substrate. The sensitivity of this system derives from the rapid enzymatic conversion of the substrate to a product that is colored or emits visible or UV light at a specific wavelength. The distribution and intensity of the product correspond to the spatial distribution and concentration of target sequences on the membrane.

Because the reporter enzyme is not conjugated directly to the probe but is linked to it through a bridge (in this case, streptavidin-biotin), this type of nonradioactive detection is known as an indirect system. Many of the kits marketed today by commercial companies rely on indirect detection systems that are elaborations of the original biotin:streptavidin:reporter enzyme method. Most of these kits use biotinylated nucleotides (e.g., biotin-11-dUTP, biotin-12-dUTP, or biotin-16-dUTP) (UTP, uridine triphosphate) as labeling agents. The biotin moiety is conjugated to the nucleotide via a spacer that is 11–16 carbon atoms long and that reduces the possibility of steric hindrance during synthesis and detection of biotinylated nucleic acid probes. Under most circumstances, biotinylated probes work very well and are free from problems. However, because biotin (vitamin H) is a ubiquitous constituent of mammalian tissues and because biotinylated probes tend to stick doggedly to certain types of nylon membranes, high levels of background can occur during in situ, northern, and Southern hybridization. These difficulties can be avoided by labeling probes with a compound that (1) has a low affinity for membranes of all types and (2) does not occur naturally in eukaryotic animals. The cardenolide digoxigenin (DIG) (Pataki et al. 1953), which is synthesized exclusively by *Digitalis* plants (Hegnauer 1971), fulfills both of these criteria (see Fig. 1 and Introduction: **Digoxigenin** [Green and Sambrook 2021g]).

FIGURE 1. *Digitalis purpurea*. (Redrawn from Culbreth 1927.)

DIG, like biotin, can be chemically coupled to linkers and nucleotides and, as described below, DIG-substituted nucleotides can be incorporated into nucleic acid probes by standard enzymatic methods. These probes generally yield significantly lower backgrounds than those labeled with biotin. DIG-labeled hybrids are detected with an anti-DIG Fab (fragment antigen-binding) fragment conjugated to a reporter enzyme (usually, alkaline phosphatase; less frequently, horseradish peroxidase (HRP); and even less frequently, β -galactosidase, xanthine oxidase, or glucose oxidase) (Höltke et al. 1990, 1995; Seibl et al. 1990; Kessler 1991).

A third class of nonradioactive probes, fluorescein-labeled probes (fluorescein-12-UTP or -dUTP), are detected by anti fluorescein antibodies conjugated to alkaline phosphatase. Labeling of nucleic acids with fluorescein, biotin, or DIG is equally easy, and the sensitivity of indirect systems based on fluorescein is at least equal to those based on biotin and DIG. All three labeling systems are capable of detecting subpicogram quantities of target nucleic acids on Southern and northern blots. Nevertheless, for reasons that are unclear, indirect systems based on fluorescein are not as commonly used as those based on biotin and DIG.

Methods to Label Probes with Biotin, Digoxigenin, or Fluorescein

Enzymatic Methods

Biotinylated, digoxigeninated, or fluoresceinated nucleotides can be used to internally label DNA by any of the standard enzymatic techniques such as random priming, nick translation, and PCR (see Table 1). Labeled RNA can be synthesized in vitro in transcription reactions catalyzed by SP6, T3, or T7 RNA polymerases. Labeling of the 3' terminus can be performed by a 3'-end-filling reaction for DNA probes or via TdT for oligonucleotide probes. It is important to note that, unlike radiolabeling, nonradioactive labeling of the 5' terminus cannot be done enzymatically. Instead, nonradioactive 5' labeling of oligonucleotides is performed directly during oligonucleotide synthesis by chemical addition of a biotin- or DIG-succinimide ester. Most oligonucleotide synthesis companies provide a service for direct labeling of oligonucleotides with biotin or DIG.

The label in all of these reactions is provided as modified uridine ribotriphosphates or deoxyribotriphosphates that are used in place of UTP or deoxythymidine triphosphate (dTTP) in the labeling reaction. Commonly used modified nucleotides include biotin-11-dUTP, biotin-16-dUTP, DIG-11-dUTP, and fluorescein-12-dUTP. In this nomenclature, the number preceding "dUTP" refers to the

number of carbon atoms in the backbone of the linker between dUTP and the label; a linker length of “11” is optimal for the majority of applications.

Biotin-, DIG-, or fluorescein-conjugated nucleotides typically are purchased from a commercial source, either individually or in combination with a labeling kit that provides all reagents required for probe synthesis. Such kits are marketed by many commercial suppliers (e.g., Ambion, Enzo Life Sciences, PerkinElmer, Roche Applied Science, Thermo Scientific). It is worth noting that it is also possible to synthesize conjugated nucleotides “in house”; for example, labeled nucleotides can be synthesized by chemically coupling allylamine-dUTP to succinimidyl-ester derivatives of biotin or DIG (Henegariu et al. 2000).

In contrast to radiolabeling with [^{32}P]NTPs or [^{32}P]dNTPs, where the efficiency of incorporation can be monitored and the specific activity of the final probe easily calculated, there is no simple or rapid method to monitor the progress of the nonisotopic labeling reactions or to calculate their efficiency. In the case of biotin- and DIG-labeled probes, the manufacturers recommend that no more than 30%–35% of the thymidine residues in the probe be replaced by labeled uridine residues (see Gebeyehu et al. 1987; Lanzillo 1990). Higher levels of replacement cause a reduction in the sensitivity with which target sequences can be detected, presumably because of the steric hindrance between closely spaced adducts during the hybridization step. However, because of a lack of an effective monitoring system, synthesis of nonradioactive probes remains an ad hoc affair. It is therefore best to perform a pilot reaction in which samples are withdrawn at intervals during the course of the labeling reaction. The labeled products are tested in dot or slot blots against dilutions of the target DNA. Large-scale labeling reactions can then be designed and performed with some confidence that the labeled product will have the required sensitivity.

Photolabeling

Biotin and DIG can also be attached to nucleic acids in photochemical reactions. In this method, the label is 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 (Forster et al. 1985; Habili et al. 1987). Photochemical labeling is far less efficient than enzymatic labeling: At best, only one base in 150 becomes modified, and the resulting probe is not potent enough to detect single-copy sequences in Southern analyses of mammalian DNA. However, newer versions of biotinylated probes combine biotin with psoralen, a nucleic acid intercalating moiety (e.g., BrightStar Psoralen-Biotin labeled probes from Applied Biosystems). The psoralen-biotin moiety intercalates within the RNA and is then covalently bound by irradiation with UV light; such probes are reported to be two to four times more sensitive than enzymatically labeled biotinylated nucleotides.

Detection of Nonradioactively Labeled Probes after Hybridization

Southern and Northern Blotting

Two steps are required to detect probes labeled with biotin, fluorescein, or DIG residues after hybridization to Southern and northern blots. The membrane is first exposed to a synthetic conjugate consisting of the reporter enzyme and a ligand that binds tightly and with high specificity to the labeling moiety. In the case of biotin, most of the detection procedures exploit the high-affinity reaction between biotin and enzyme-conjugated streptavidin. Alternative procedures rely on specific monoclonal or polyclonal anti-biotin antibodies. Fluoresceinated or digoxigeninated probes are routinely detected by interaction with specific enzyme-conjugated antibodies. The reporter enzyme is then assayed with colorimetric, fluorogenic, or chemiluminescent substrates.

In colorimetric assays (e.g., see Leary et al. 1983; Urdea et al. 1988; Rihn et al. 1995), a combination of two dyes is used to detect alkaline phosphatase that has been captured by labeled hybrids. The enzyme catalyzes the removal of the phosphate group from BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Horwitz et al. 1966), generating a product that oxidizes and dimerizes to dibromodichloro indigo. The reducing equivalents produced during the dimerization reaction reduce NBT

(nitroblue tetrazolium) (McGadey 1970) to an insoluble purple dye, diformazan, that becomes visible at sites where the labeled probe has hybridized to its target (Leary et al. 1983; Chan et al. 1985). The results can be analyzed visually and recorded on conventional photographic film. Colorimetric detection works well with nitrocellulose and PVDF membranes, but not as well with nylon and charged nylon. Unfortunately, even when working at their best, colorimetric methods are two or more orders of magnitude less sensitive than other nonradioactive detection methods, for example, chemiluminescence (Bronstein et al. 1989a; Beck and Köster 1990; Kerkhof 1992; Kricka 1992). Detection of single-copy sequences in mammalian genomes is therefore barely within reach of colorimetric assays (Düring 1993). A final disadvantage is that the colored precipitates are difficult to remove from membranes, making reprobing difficult or impossible. In some cases, the procedures used to strip the precipitates (100% formamide at 50°C–60°C) either dissolve the membrane or greatly increase its fragility.

Fluorescent assays for alkaline phosphatase make use of HNPP (2-hydroxy-3-naphthoic acid 2'-phenylanilide phosphate) (Kagiyama et al. 1992). After dephosphorylation, HNPP generates a fluorescent precipitate on membranes that can be excited by irradiation at 290 nm from a transilluminator. Light emitted at 509 nm can be captured by charged couple device (CCD) cameras or on Polaroid film using the same photographic setup (filters, etc.) as that used for conventional ethidium-bromide-stained gels. Fluorescent assays using HNPP are more sensitive than colorimetric methods and are capable of detecting single-copy sequences in Southern blots of mammalian genomic DNA when the membrane is incubated with substrate for 2–10 h (Diamandis et al. 1993; Hölte et al. 1995). The fluorescent precipitate can be easily removed by washing in ethanol and the filters used for several more rounds of hybridization before the signal-to-noise ratio increases to unacceptable levels.

Chemiluminescence is the fastest and most sensitive assay to detect DNA labeled with biotin, fluorescein, or DIG, via conjugates containing HRP or alkaline phosphatase (Beck et al. 1989; Bronstein et al. 1989b,c; Schaap et al. 1989). An HRP-luminol detection system (Thorpe and Kricka 1986) is marketed by several companies (e.g., BioRad, Life Technologies, Sigma-Aldrich). HRP catalyzes the oxidation of luminol in the presence of hydrogen peroxide, generating a highly reactive endoperoxide that emits light at 425 nm during its decomposition to its ground state. A wide range of compounds, including benzothiazoles, phenols, naphthols, and aromatic amines, can significantly enhance the amount of light generated during the reaction (Thorpe and Kricka 1986; Pollard-Knight et al. 1990; Kricka and Ji 1995). This signal amplification, which can be as much as 1000-fold (Whitehead et al. 1983; Hodgson and Jones 1989), increases the sensitivity of the HRP-luminol system to the point where it can detect single-copy genes in Southern blots of mammalian DNA. Light is produced at a rapid rate for the first few minutes of the reaction but thereafter declines and is no longer detectable after 1–2 h. Under ideal conditions, ~ 1 pg (5×10^{-17} M) of target DNA can be detected on Southern blots after 1 h of exposure on gray-tinted, blue-light-sensitive X-ray film or a cooled CCD camera (Beck and Köster 1990; Durrant et al. 1990; Kessler 1992). The probes can be stripped from filters by heating them to 80°C (Dubitsky et al. 1992).

Many commercial suppliers (e.g., Applied Biosystems, Life Technologies, Roche Applied Science, Sigma-Aldrich) sell alkaline phosphatase detection systems that use dioxetane-based chemiluminescent substrates such as AMPPD and Lumigen-PPD and their more recent derivatives CSPD and CDP-Star. One of the most sensitive chemiluminescent substrates available (Edwards et al. 1994; Hölte et al. 1994), CDP-Star differs from AMPPD in that it carries halogen substituents at the five positions in its adamantyl and phenyl rings. This suppresses the tendency of the 1,2-dioxetane to aggregate and reduces background caused by thermal degradation (Bronstein et al. 1991). Cleavage of the phosphate ester of CDP-Star by alkaline phosphatase drastically reduces the thermal stability of the dioxetane ring, which consequently decomposes with emission of light at 465 nm. Nylon membranes (either amphoteric or positively charged) strongly enhance the signal (Tizard et al. 1990) by providing hydrophobic domains into which the dephosphorylated intermediate produced in the chemiluminescent reaction becomes sequestered. This stabilizes the intermediate and reduces its nonluminescent decomposition. In turn, the intensity of the light emitted from the excited dioxetane anion begins as a glow that increases in intensity for several minutes and then persists for several hours (Martin and

Bronstein 1994). The hydrophobic interactions between the nylon and the anion also cause a “blue shift” of ~ 10 nm in the emitted light, that is, from 477 to 466 nm (Beck and Köster 1990; Bronstein 1990).

In most experimental situations, the extended kinetics of chemiluminescence on nylon filters are advantageous because they allow time to capture images at several exposures. However, the slow kinetics may be disadvantageous when alkaline phosphatase–triggered chemiluminescence is used to detect extremely low concentrations of DNA, RNA, or protein, for example, when the target band on a filter is expected to contain only 10^{-18} mol. In such cases, a halogen-substituted derivative of AMPPD, for example, CSPD, may be a better choice. The addition of a chlorine atom to the five position of the adamantyl group restricts its interactions with nylon filters. With this compound, the time to reach maximum light emission is markedly reduced so that very small quantities of target molecules can be detected rapidly (e.g., see Martin et al. 1991).

Under ideal conditions, as little as 1 zmol (10^{-21} M) of alkaline phosphatase or 1×10^{-19} mol of DNA can be detected (Schaap et al. 1989; Beck and Köster 1990; Bronstein 1990; Kricka 1992). Such sensitivity means that a single-copy gene in a Southern blot of mammalian genomic DNA can be detected after the nylon membrane has been exposed for ~ 5 min to gray-tinted, blue-light-sensitive X-ray film or a cooled CCD camera (Höltke et al. 1995). The probes can be stripped by using 50% formamide at 65°C (Dubitsky et al. 1992) and the nylon membranes can then be used for several additional rounds of hybridization.

In Situ Hybridization

Detection methods for in situ hybridization are similar to those used in Southern and northern hybridization and generally rely on colorimetric assays following detection of biotin or DIG using streptavidin or a specific antibody. Following hybridization, samples are incubated with alkaline phosphatase color reagents NBT and BCIP, followed by visual detection using a bright field microscope.

For FISH, detection of the biotin or DIG moiety is performed using a fluorochrome-conjugated detection reagent, such as streptavidin, or an antibiotin or anti-DIG antibody conjugated to fluorescein isothiocyanate (FITC), rhodamine, or Texas Red. The probe is then detected by immunofluorescence microscopy.

Direct Detection Systems

In this type of detection, nucleic acid probes are labeled directly with fluorescent dyes such as fluorescein, Texas Red, rhodamine, Cy3, or Cy5 (Agrawal et al. 1986); lanthanide chelates (europium) (Templeton et al. 1991; Dahlén et al. 1994); acridinium esters (Nelson et al. 1992); or enzymes, for example, alkaline phosphatase (Jablonski et al. 1986) or HRP (Renz and Kurz 1984). The presence of such covalently attached adducts is assayed by any one of a number of techniques, including colorimetry, chemiluminescence, bioluminescence, time-resolved fluorometry, or energy transfer/fluorescence quenching. Of course, direct detection methods can be converted to indirect methods if an enzyme-antibody conjugate specific for the adduct is available.

Because it forms the basis of automated DNA sequencing methods (Smith et al. 1986; Ansorge et al. 1987; Prober et al. 1987), direct labeling of nucleic acids with fluorescent compounds has revolutionized molecular biology. A single fluorescent adduct per DNA molecule is sufficient for detection by automated DNA sequencers when the DNA is in the gel. Furthermore, the ability of ABI-type automated sequencers to discriminate accurately among different fluors signifies that all four sequencing reactions can be analyzed in a single lane of a gel. The stunning success of direct labeling in automated DNA sequencing has, however, not spread to other areas of molecular cloning, where direct nonradioactive methods of detection are rarely used. This is because of the following.

- Traditional methods used for the direct conjugation of enzymes such as alkaline phosphatase require the extensive use of column chromatography, polyacrylamide gel electrophoresis, and/or high-performance liquid chromatography (HPLC) to obtain a pure product (Jablonski et al. 1986;

Urdea et al. 1988; Farmar and Castaneda 1991). Commercial kits are available but are expensive and typically yield an impure product. Newer methods of conjugation (e.g., see Reyes and Cockrell 1993) are simpler but slightly less efficient.

Whatever method is used, a separate conjugation step is required for each different probe. Once attached, large enzymes such as alkaline phosphatase may reduce the rate of annealing of probes and may be labile under the conditions conventionally used for annealing of nucleic acids. Hybridization and washing must therefore be performed at low temperature (usually $<50^{\circ}\text{C}$) in aqueous solvents. Under such nonstringent conditions, high backgrounds are an inevitable problem. Because of the presence of bulky, charged adducts, probes directly labeled with enzymes may bind nonspecifically but tightly to certain types of membranes. For example, the background signal from oligonucleotide probes directly conjugated to alkaline phosphatase is considerably greater on charged nylon than on neutral nylon membranes (Benzinger et al. 1995).

- The detection of single-copy sequences in Southern hybridization of mammalian DNA lies close to the limit of sensitivity of many direct methods unless specialized equipment is available (Urdea et al. 1988). Detection of fluorescent adducts, for example, requires irradiating with light of one wavelength and acquiring data at another. The signal must then be enhanced electronically and stored digitally.

Because of these problems, it is not surprising that direct detection techniques have fallen from favor and that indirect detection has become the dominant nonradioactive method to locate target nucleic acids in Southern and northern hybridization. However, despite these problems, probes directly labeled with alkaline phosphatase are now produced commercially for forensic and pathological analyses. The sensitivity of these probes can approach that of isotopically labeled probes (Dimo-Simonin et al. 1992; Klevan et al. 1993; Benzinger et al. 1995). The use of such standardized nonisotopically labeled probes is expected to increase greatly in laboratories involved, for example, in constructing large numbers of DNA profiles or in diagnosing infectious diseases.

One area in which direct detection techniques have become more popular is in the use of FISH, which allows probe-target hybrids to be visualized under a fluorescence microscope immediately after the hybridization reaction. Direct fluorescent labeling can be performed in one of two ways: fluorochromes can be terminally added to the 5' or 3' end of the nucleic acid (Bauman et al. 1980) or fluorochrome-conjugated dNTPs can be used in enzyme labeling reactions (Renz and Kurz 1984). Fluorescently labeled probes offer several advantages over biotin- or DIG-labeled probes including increased specificity (because shorter probes can be used) and reduced background signal. However, they also tend to generate a lower signal intensity and, as noted above, are costly to synthesize, which has somewhat hampered their popularity as reagents in FISH.

DESIGNING OLIGONUCLEOTIDES FOR USE AS PROBES

Success with oligonucleotide probes begins with the design of an oligonucleotide (1) that hybridizes specifically to its target and (2) whose physical properties (e.g., length and GC content) do not impose unnecessary constraints on the experimental protocol. In a few cases, the investigator has little latitude in the design of the oligonucleotide. For example, when oligonucleotides are used as allele-specific probes for the presence or absence of defined mutations, the salient properties of the oligonucleotides are defined to a very large extent by the particular sequences in which the mutation is embedded. In most other circumstances, however, the chance of success can be increased by careful adjustment of parameters defined by the investigator, such as the length, sequence, and base composition of the oligonucleotide. The rules outlined below were developed to optimize the design of oligonucleotide hybridization probes.

Melting Temperature and Hybridization Temperature

An ideally designed oligonucleotide probe should form a perfect duplex only with its target sequence. These duplexes should be sufficiently stable to withstand the posthybridization washing steps used to remove probes nonspecifically bound to nontarget sequences. For oligonucleotides of 200 nt or less, the reciprocal of the melting temperature of a perfect hybrid (T_m^{-1} , measured in degrees Kelvin) is approximately proportional to n^{-1} , where n is the number of bases in the oligonucleotide (see Gait 1984).

Several equations are available to calculate the melting temperature of hybrids formed between an oligonucleotide primer and its complementary target sequence. Because none of these is perfect, the choice among them is largely a matter of personal preference (for additional information on calculating melting temperatures, see More on Melting Temperatures below). The melting temperatures of each member of a primer:target duplex should obviously be calculated using the same equation.

An empirical and convenient equation known as the “Wallace rule” (Suggs et al. 1981b; Thein and Wallace 1986) can be used to calculate the T_m of perfect duplexes, 15–20 nt in length, in solvents of high ionic strength (e.g., 1 M NaCl or 6× SSC):

$$T_m(\text{in } ^\circ\text{C}) = 2(A + T) + 4(G + C),$$

where $(A + T)$ is the sum of the A and T residues in the oligonucleotide and $(G + C)$ is the sum of G and C residues in the oligonucleotide.

The equation derived originally by Bolton and McCarthy (1962) and later modified by Baldino et al. (1989) predicts reasonably well the T_m of oligonucleotides, <100 nt long, in cation concentrations of 0.5 M or less and with a $(G + C)$ content between 30% and 70%. It also compensates for the presence of base-pair mismatches between the oligonucleotide and the target sequence:

$$T_m(\text{in } ^\circ\text{C}) = 81.5^\circ\text{C} + 16.6 (\log_{10}[\text{Na}^+]) + 0.41(\%[G + C]) - 675/n - 1.0m,$$

where n is the number of bases in the oligonucleotide and m is the percentage of base-pair mismatches. This equation can also be used to calculate the melting temperature of an amplified product whose sequence and size are both known. When PCR amplification is performed under standard conditions, the calculated T_m of the amplified product should not exceed $\sim 85^\circ\text{C}$, thus ensuring complete separation of its strands during the denaturation step. Note that the denaturation temperature in PCR is more accurately defined as the temperature of irreversible strand separation of a homogeneous population of molecules. The temperature of irreversible strand separation is several degrees higher than the T_m (typically, 92°C for DNA whose content of $G + C$ is 50%) (Wetmur 1991).

Neither of the above equations takes into account the effect of base sequence (as opposed to base composition) on the T_m of oligonucleotides. A more accurate estimate of the T_m can be obtained by incorporating nearest-neighbor thermodynamic data into the equations (Breslauer et al. 1986; Freier et al. 1986; Kierzek et al. 1986; Rychlik et al. 1990; Wetmur 1991; Rychlik 1994). A relatively straightforward equation, described by Wetmur (1991), is

$$T_m(\text{in } ^\circ\text{C}) = (T^0 \Delta H^0) / (\Delta H^0 - \Delta G^0 + RT^0 \ln[c]) + 16.6 \log_{10}([\text{Na}^+] / \{1.0 + 0.7[\text{Na}^+]\}) - 269.3,$$

where

$$\begin{aligned} \Delta H^0 &= \sum nn(N_{nn} \Delta H_{nn}^0) + \Delta H_p^0 + \Delta H_e^0, \\ \Delta G^0 &= \sum nn(N_{nn} \Delta G_{nn}^0) + \Delta G_i^0 + \Delta G_e^0, \end{aligned}$$

N_{nn} is the number of nearest neighbors (e.g., 13 for a 14-mer), $R = 1.99$ cal/mol (in degrees Kelvin), $T^0 = 298.2^\circ\text{K}$, c is the total molar strand concentration, and $[\text{Na}^+] \leq 1$ M.

Some of the thermodynamic terms can be approximated as follows: average nearest-neighbor enthalpies,

$$\Delta H_{nn}^0 = -8.0 \text{ kcal/mol};$$

average nearest-neighbor free energies,

$$\Delta G_{nn}^0 = -1.6 \text{ kcal/mol};$$

initiation term,

$$\Delta G_i^0 = +2.2 \text{ kcal/mol};$$

average dangling end enthalpy,

$$\Delta H_e^0 = -8.0 \text{ kcal/mol/end};$$

average dangling end free energy,

$$\Delta G_e^0 = -1 \text{ kcal/mol/end};$$

and average mismatch/loop enthalpy,

$$\Delta H_p^0 = -8.0 \text{ kcal/mol/mismatch}.$$

Note that for each mismatch or loop, N_{nn} is reduced by 2.

When the oligonucleotide is used as a probe, hybridization is usually performed at $\sim 5^\circ\text{C}$ – 12°C below the calculated T_m , and stringent posthybridization washing is performed at $\sim 5^\circ\text{C}$ below the T_m . Working so close to the calculated T_m has two consequences: (1) It reduces the number of mismatched hybrids and (2) less desirably, it reduces the rate at which perfect hybrids form.

For all practical purposes, hybrid formation between complementary DNA molecules >200 nt in length is an irreversible reaction when annealing is performed at $\sim 10^\circ\text{C}$ below the calculated T_m . The chance that such a long stretch of perfect double helix will unwind under the conditions conventionally used for hybridization (68°C and $\sim 1 \text{ M } [\text{Na}^+]$) is small. However, hybrids (even perfect hybrids) formed between short oligonucleotides and their target sequences at 5°C – 10°C below the T_m are far easier to unwind; thus, hybridization reactions of this type can be considered reversible. This behavior has important practical consequences. The concentration of oligonucleotide in the hybridization reaction should be high (0.1 – 1.0 pmol/mL) so that the annealing reaction rapidly reaches equilibrium within 3–8 h. Posthybridization washing, however, should be brief (1–2 min) and should be initially performed under conditions of low stringency and then under conditions of stringency that are at least equal to those used for hybridization (Miyada and Wallace 1987).

Length of Oligonucleotide Probes

The longer an oligonucleotide, the higher its specificity for the target sequence. The following equations can be used to calculate the probability that a specific string of nucleotides will occur within a sequence space (Nei and Li 1979):

$$K = \frac{[g/2]^{G+C} \times [(1-g)/2]^{A+T}}{N},$$

where K is the expected frequency of occurrence within the sequence space, g is the relative G + C content of the sequence space, and G , C , A , and T are the number of specific nucleotides in the oligonucleotide. For a double-stranded genome of size N (in nucleotides), the expected number (n) of sites complementary to the oligonucleotide is $n = 2NK$.

These equations predict that an oligonucleotide of 14–15 nt would be represented only once in a mammalian genome where N is $\sim 3.0 \times 10^9$. In the case of a 16-mer, there is only one chance in 10 that a typical mammalian complementary DNA (cDNA) library (complexity of $\sim 10^7$ nt) will fortuitously contain a sequence that exactly matches that of the oligonucleotide. However, these estimates are based on the assumption that the distribution of nucleotides in mammalian genomes is random. Unfortunately, this is not the case, due to bias in codon usage (Lathe 1985) and because a significant fraction of the genome is composed of repetitive DNA sequences and gene families. Because of the presence of these elements, no more than 85% of the mammalian genome can be targeted precisely, even by probes and primers that are 20 nt in length (Bains 1994).

To minimize problems of nonspecific hybridization, it is advisable to use oligonucleotides longer than the statistically indicated minimum. Any clones that then hybridize to the probe are likely to be derived from the gene of interest. Bear in mind that when a cDNA library is screened with an oligonucleotide probe, no relationship exists between the observed number of positive clones and their frequency predicted by statistics. For example, if by chance the oligonucleotide should match an expressed sequence that is abundantly represented in mRNA, the number of clones that hybridize to the probe will be much larger than theory predicts. Before synthesizing an oligonucleotide probe or primer, it is advisable to scan DNA databases to ensure that the proposed sequence occurs only in the desired gene and not in vectors, undesired genes, or repetitive elements (e.g., see Mitsuhashi et al. 1994).

The effect of hybridization between imperfectly matched sequences cannot be easily quantified because different types of mismatching (mispairing between single bases, loopouts on either strand, or multiple mismatches, whether closely or distantly spaced) have different effects on the stability of double-stranded DNA (dsDNA). For example, a single mismatch in the center of a short oligonucleotide (of ~ 16 nt in length) will destabilize the hybrid by $\sim 7^\circ\text{C}$ (Wetmur 1991) and, consequently, may attenuate the hybridization signal to a very considerable degree. On the other hand, a mismatch at the 3' end of a probe will have little effect on hybridization signal (Ikuta et al. 1987) but greatly diminish the ability of an oligonucleotide to prime DNA synthesis.

Locked Nucleic Acids

Locked nucleic acids (LNAs) are a class of nucleic acid analogs in which the ribose ring is “locked” into a particular conformation by virtue of a methylene bridge connecting the 2'-oxygen atom and the 4'-carbon atom (for review, see Vester and Wengel 2004). This restricted conformation promotes optimal Watson–Crick binding. When incorporated into a DNA oligonucleotide, LNAs increase the stability of the resulting duplex, thereby increasing the hybridization properties (i.e., melting temperature) of the oligonucleotide. Incorporation of an LNA into an oligonucleotide increases the T_m of the oligonucleotide by 2°C – 8°C per LNA. The high binding affinity of LNA-enhanced oligonucleotides allows for the use of shorter probes.

First synthesized by Jesper Wengel and colleagues in 1998 (Koshkin et al. 1998), LNA-enhanced oligonucleotides are now used in hybridization-based assays when extra specificity and sensitivity are required. They have become particularly prevalent in probes used in *in situ* hybridization assays (Thomsen et al. 2005; Kubota et al. 2006). The only disadvantage of LNAs is their high cost relative to DNA oligonucleotides; for this reason, DNA oligonucleotides are still widely preferred for most applications.

LNAs are commercially available from many sources (e.g., Exiqon, Integrated DNA Technologies, Bio-Synthesis Inc.). The synthesis and incorporation of LNA bases are achieved using standard DNA synthesis chemistry, and LNA-enhanced oligonucleotides can be purified and analyzed using the same standard methods as those used for DNA. Due to the high affinity and thermal stability of

the LNA:DNA duplex, it is not advisable to have >15 LNA bases in an oligonucleotide, because it induces strong self-hybridization; for hybridization-based assays, it is generally recommended to substitute four to six bases with LNAs. Placement of the LNA analogs within the oligonucleotide is important. To assist the researcher, several software programs are available for LNA design. One of the most popular programs is OligoDesign (Tolstrup et al. 2003), which is freely accessible at <http://www.exiqon.com/oligo-tools>. OligoDesign software features recognition and filtering of the target sequence by genome-wide BLAST analysis to minimize cross-hybridization with nontarget sequences. Furthermore, it includes algorithms for prediction of melting temperature, self-annealing, and secondary structure for LNA-substituted oligonucleotides, as well as secondary structure prediction of the target nucleotide sequence.

Like DNA oligonucleotides, LNA oligonucleotide probes are labeled enzymatically at either the 5' or 3' end: 5'-end-labeling reactions are performed using T4 polynucleotide kinase in the presence of [γ - 32 P]ATP, whereas 3'-end-labeling is performed using terminal transferase and a radioactive, biotin, DIG, or fluorescent nucleotide.

Degenerate Pools of Oligonucleotides

In molecular cloning, the situation frequently arises in which a short sequence of amino acids has been obtained by sequencing a purified protein. Because of the degeneracy of the genetic code, many different oligonucleotides can potentially code for a given tract of amino acids. For example, 64 possible 18-mers can code for the sequence Asn Phe Tyr Ala Trp Lys. However, only one of these oligonucleotides will exactly match the coding sequence used in the gene of interest. Because there is no way to know a priori which of these oligonucleotides has its true counterpart in the gene, pools of oligonucleotides containing all potential coding combinations are synthesized and used as probes. Depending on the length of the amino acid sequence and the amount of degeneracy at each position, pools can contain up to several hundred oligonucleotides. If hybridization conditions can be found under which only perfectly matched sequences form stable duplexes, cloned copies of the gene of interest can be readily isolated from genomic or cDNA libraries (e.g., see Goeddel et al. 1980; Agarwal et al. 1981; Sood et al. 1981; Suggs et al. 1981b; Wallace et al. 1981; Toole et al. 1984; Jacobs et al. 1985; Lin et al. 1985).

Pools of degenerate oligonucleotides are generally used to select a series of candidate clones that can then be analyzed further to identify the true target. To ensure that the target clone is represented in the initial group of candidates, screening conditions are usually chosen that allow detection of perfect hybrids formed between the most A/T-rich oligonucleotide in the pool and its potential target. Other hybrids, both perfect and imperfect, that are stable under such conditions will identify additional candidate clones.

It is easy to calculate accurately the T_m of a perfectly matched hybrid formed between a single oligonucleotide and its target sequence. However, when using pools of oligonucleotides whose members have greatly different contents of G + C, it is not feasible to estimate a consensus T_m . Because it is impossible to know which member of the pool will match the target sequence perfectly, conditions must be used that allow the oligonucleotide with the lowest content of G + C to hybridize efficiently. Usually, conditions are chosen to be 2°C below the calculated T_m of the most A/T-rich member of the pool (Suggs et al. 1981a). However, the use of such “lowest common denominator” conditions can generate false positives: Mismatched hybrids formed by oligonucleotides of higher G + C may be more stable than a perfectly matched hybrid formed by the correct oligonucleotide. In most cases, this problem is not serious, because the number of positive clones obtained by screening cDNA libraries with pools of nucleotides is quite manageable. It is therefore easy to distinguish false positives from true positives by another test (e.g., DNA sequencing or hybridization with a second pool of oligonucleotides corresponding to another segment of amino acid sequence).

Quarternary Alkylammonium Salts

When the number of apparent positives is unacceptably high, it is worthwhile using hybridization solvents that contain quarternary alkylammonium salts such as tetraethylammonium chloride

(TEACl) or tetramethylammonium chloride (TMACl) (Melchior and von Hippel 1973; Jacobs et al. 1985, 1988; Wood et al. 1985; Gitschier et al. 1986; DiLella and Woo 1987; see also Protocol: **Hybridization of Oligonucleotide Probes in Aqueous Solutions: Washing in Buffers Containing Quaternary Ammonium Salts** [Green and Sambrook 2022]). By binding to A/T-rich polymers (Shapiro et al. 1969), quaternary alkylammonium salts reduce the preferential melting of A:T versus G:C base pairs (Melchior and von Hippel 1973; Riccelli and Benight 1993). In TEACl or TMACl, the T_m of an oligonucleotide–DNA hybrid becomes less dependent on its base composition and more dependent on its length. By choosing a hybridization temperature appropriate for the lengths of the oligonucleotides in a pool, the effects of potential mismatches can be minimized.

It is important to obtain an accurate estimate of the T_m in TMACl or TEACl before using pools of oligonucleotides to screen cDNA or genomic libraries. Jacobs et al. (1988) measured the T_i (irreversible melting temperature) of the hybrid formed between the probe and its target as a function of chain length for a number of oligonucleotides of different (G + C) content in solvents containing either sodium ions or tetraalkylammonium ions. Hybrids involving oligonucleotides 16 and 19 nt in length melt over a smaller range of temperatures in solvents containing TMACl than in solvents containing sodium salts. For example, 16-mers melt over a 3°C temperature range in solvents containing TMACl, compared with 17°C in SSC.

The optimal temperature for hybridization is usually chosen to be 5°C below the T_i for the given chain length. The recommended temperature for 17-mers in 3 M TMACl is 48°C–50°C; for 19-mers, 55°C–57°C; and for 20-mers, 58°C–66°C. Two points are worth emphasizing. First, the T_i of hybrids is uniformly 15°C–20°C higher in solvents containing TMACl than in solvents containing TEACl. The higher T_i in solvents containing TMACl allows hybridization to be performed at temperatures that suppress nonspecific adsorption of the probe to solid supports (such as nylon membranes). Second, hybridization solvents containing TMACl do not have significant advantages over those containing sodium ions until the length of the oligonucleotide exceeds 16 nt.

Guessmers

A guessmer is a long synthetic oligonucleotide (usually 30–75 nt in length) whose sequence corresponds to an amino acid sequence of 15–25 residues. Because of the degeneracy of the genetic code, many thousands of possible nucleotide sequences could code for such a sizable tract of amino acids. Unless the designer is incredibly lucky and chooses the correct codon in every single position, the guessmer will not form a perfect hybrid with the target gene. However, because most amino acids are specified by codons that differ only in the third position, at least 2 of the 3 nt of each codon are likely to be perfectly matched. In addition, the number of mismatches in the third position can be minimized by selecting codons that are used preferentially by a particular organism, organelle, or cell type. In this way, it is possible to make guessmers of sufficient length so that the detrimental effects of mismatches are outweighed by the stability of extensive tracts of perfectly matched bases. For further details, see Box 1.

No rules guarantee selection of the correct codon at a position of ambiguity; neither is it possible to predict how many correct choices must be made to achieve success. On the basis of mathematical calculations (Lathe 1985), a probe would be expected to have at least 76% identity with the authentic gene even if all codon choices were made on a random basis. If substitutions are chosen on the statistical basis of known codon usage in the species of interest, the expected homology increases to 82%; it rises still further (to 86%) if regions lacking leucine, arginine, and serine are chosen (each of these three amino acids is specified by six codons). Clearly, as the degree of homology increases, the guessmer becomes a more specific probe for the gene of interest and forms hybrids that are stable under a wider range of hybridization conditions. If the sequences of selected genes, successfully isolated by probing with guessmers, are compared with their corresponding guessmer sequences, the (respective) homologies range from a low of 71% to a high of 97%. Unless the choice of codons is extremely unfortunate, guessmers can be designed whose homology with their target genes lies near the upper end of this range. However, in addition to overall homology, the presence of a perfectly

BOX 1. DESIGNING A GUESSMER

- Eliminate codon choices that generate the sequence CpG between codons. This dinucleotide is significantly underrepresented in mammalian DNA (Bird 1980), and CpG (C-phosphate-G) occurs at the junction between codons at about one-half of the expected frequency (Lathe 1985).
- Choose the codon that most commonly codes for a particular amino acid in the species under study. Comprehensive lists of relative codon frequencies in genes have been published by Wada et al. (1992) or may be found at <http://www.kazusa.or.jp/codon>.
- Remember that specific gene families occasionally display a marked bias for or against certain codons. For example, histone genes show a marked preference for codons enriched in A and T residues, whereas proteins expressed abundantly in yeast are biased toward a set of highly preferred codons (Bennetzen and Hall 1982) and, for example, hardly ever use the codons UCG and UAU for serine and isoleucine (Ogden et al. 1984).
- Different mammalian tissues display different patterns of codon usage (e.g., see Newgard et al. 1986). It would therefore be worthwhile to determine the codon usage in any genes that have already been cloned and are known to be expressed in the same tissue as the gene of interest.
- At positions where the choice is between C and T and there is no strong codon preference, choose T. This rule is still somewhat controversial. There is good evidence indicating that rG:rU base pairs are more stable than rA:rC base pairs (Uhlenbeck et al. 1971). However, the extension to dG:dT base pairs (Wu 1972) has been both challenged (Smith 1983) and upheld (Martin et al. 1985).
- Examine the sequence of the proposed nucleotide for regions of internal complementarity that might reduce hybridization efficiency. Wherever possible, avoid sequences that could form stable duplexes under the conditions used for hybridization.
- Ensure that the 5'-terminal position of the oligonucleotide is not occupied by a cytosine residue. For reasons that are unknown, the efficiency of radiolabeling of oligonucleotides depends on the sequence of the oligonucleotide (van Houten et al. 1998). Oligonucleotides with a cytosine residue at their 5' termini are labeled fourfold less efficiently than oligonucleotides beginning with A or T and sixfold less efficiently than oligonucleotides beginning with G.

matching sequence of contiguous nucleotides within the guessmer may also be important for success. Almost all guessmers that have been used successfully contain regions that exactly match sequences in their target genes. Although it is not possible to guarantee that a guessmer will contain an exactly matching sequence, the chances increase dramatically as the degree of overall homology rises.

It is sometimes possible to synthesize a small pool containing two to eight guessmers that includes all possible codon choices at certain amino acid positions. This kind of limited substitution is most useful when an amino acid, whose codon is highly degenerate, separates two tracts containing only a few potential mismatches. In this way, it may be possible to generate a continuous sequence within the guessmer that is a perfect match for the target gene. However, when using a mixture of guessmers as probes, the strength of the hybridization signal generated by the "correct" probe is usually reduced. For guessmers labeled by phosphorylation or by filling of recessed ends, an eightfold reduction in signal strength typically does not compromise the identification of clones of interest.

Perhaps the most critical step in the use of guessmers is the choice of conditions for hybridization. The temperature should be high enough to suppress hybridization of the probe to incorrect sequences but must not be so high as to prevent hybridization to the correct sequence, even though it may be mismatched. Before using an oligonucleotide to screen a library, perform a series of trial experiments in which a series of northern or genomic Southern hybridizations are performed under different degrees of stringency (Anderson and Kingston 1983; Wood et al. 1985). Lathe (1985) presents a set of theoretical curves relating the temperature of the washing solution to the length and homology of the probe. Using these curves as a guide, determine the optimal conditions for detection of sequences complementary to the probe by hybridizing the oligonucleotide to a series of nitrocellulose or nylon membranes at different temperatures. The membranes are washed extensively in 6× SSC at

room temperature and then briefly (5–10 min in 6× SSC) at the temperature used for hybridization. This method, in which both hybridization and washing are performed under the same conditions of temperature and ionic strength, appears to be more discriminating than the more commonly used procedure of hybridizing under conditions of lower stringency and washing under conditions of higher stringency. If trial experiments are not possible, estimate the melting temperature (T_m) as follows.

1. Calculate the minimum G + C content of the oligonucleotide, assuming that A or T is present at all positions of ambiguity.
2. Use the following formula to calculate the T_m of a dsDNA with the calculated G + C content:

$$T_m = 81.5^\circ\text{C} + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%[\text{G} + \text{C}]) - (500/n),$$

where n is the number of nucleotides. The term $500/n$ is derived from a compilation (Hall et al. 1980) of measurements, from several laboratories, of the effect of duplex length on melting temperature. This formula only works for Na^+ concentrations of 1.0 M or less.

3. Calculate the maximum amount of possible mismatches assuming that all choices of degenerate codons are incorrect. Subtract 1°C from the calculated T_m for each 1% of mismatch. The resulting number should be the T_m of a maximally mismatched hybrid formed between the probe and its target DNA sequence.

Almost certainly, the actual T_m will be higher than that predicted by this worst-case calculation. If the bases used at positions of ambiguity were chosen at random, one out of four should be correct, and approximately half of these would be expected to be G or C. The observed T_m should therefore be significantly higher than that estimated. However, to minimize the risk of missing the clone of interest, it is best to hybridize and wash at 5°C–10°C below the T_m estimated as described above. If, under these conditions, the probe hybridizes indiscriminately, repeat the hybridization at a higher temperature or wash under conditions of higher stringency.

Guessmers reached their height of popularity in the mid 1980s, a time when the fledgling recombinant DNA companies were desperately trying to clone genes of commercial value. cDNA and genomic clones of many useful genes were obtained during this period. In recent years, however, guessmers have been eclipsed by probes generated in PCRs using sets of redundant primers. This is chiefly because PCR-based methods require far shorter tracts of unambiguous amino acid sequence. Nevertheless, when an amino acid sequence of sufficient length is available, guessmers remain the probes of choice. They have a strong track record of success and are free of the artifacts that PCR can visit upon even the best of investigators.

Universal Bases

Universal bases have reduced hydrogen-bonding specificity and can therefore “pair” with natural bases at positions of ambiguity without disrupting the DNA duplex. For several years, the “universal” base of choice has been the purine nucleoside inosine, whose neutral base, hypoxanthine, forms stable base pairs with cytosine, thymine, and adenine. Because of the absence of a 2-amino group, base pairing between inosine and cytosine involves two hydrogen bonds (as in A:T base pairs) instead of the three that occur in C:G base pairs (Corfield et al. 1987; Xuan and Weber 1992). In most other respects, however, inosine behaves like guanosine.

- Inosine occurs naturally in the first (wobble) position of some transfer RNAs (tRNAs), where it pairs with adenosine in addition to cytidine and uridine, the nucleosides that normally pair with guanosine in that position (Crick 1966).
- Inosine is able to occupy the middle position of the anticodon, where it again pairs with adenosine (Davis et al. 1973). However, in inosine:adenosine pairs in B-DNA duplexes, inosine adopts a *trans* (anti) orientation with respect to the furanose moiety, whereas adenosine is in the *syn* configu-

ration (Corfield et al. 1987). This is similar to the arrangement that is formed when guanosine mispairs with adenosine (Brown et al. 1986).

- Poly(rI) and poly(dI) form helices with poly(rC) and poly(dC) that are stable enough to serve as templates used by various RNA and DNA polymerases. The enzymes can incorporate cytosine into the polymerization products (for review, see Felsenfeld and Miles 1967; Hall et al. 1985).

Pools of synthetic oligonucleotides and guessmers containing inosine have been used extensively as hybridization probes to screen cDNA or genomic libraries for genes encoding proteins whose amino acid sequence is only partly known. However, although inosine is able to form hydrogen bonds with the three other nucleotides, the resulting base pairs are less stable than the equivalent guanosine-containing base pairs (Martin et al. 1985). The melting temperatures of duplexes containing inosine vary widely depending on the base to which the analog is paired and on the surrounding sequence. In the worst case, the melting temperature of synthetic oligonucleotide duplexes containing inosine opposite any base may be depressed by 15°C, which corresponds to a difference in base-pair stability of 2–3 kcal/mol⁻¹ (Martin et al. 1985; Kawase et al. 1986). Although this situation is less than ideal, it has surprisingly few practical consequences: Inosine-containing oligonucleotides have been used successfully to clone many genes from genomic and cDNA libraries of high complexity (e.g., see Jaye et al. 1983; Ohtsuka et al. 1985; Takahashi et al. 1985; Bray et al. 1986; Nagata et al. 1986).

Table 3 contains a list of codons that are recommended when designing oligonucleotides that are to be used to screen primate or mammalian cDNA libraries and that contain inosine at positions of ambiguity. The recommendations take into account the natural usage of codons in human genes and the fact that the sequence CpG is underrepresented in human DNA.

Hybridization of Oligonucleotides that Contain Inosine at Positions of Degeneracy

Although the conditions for hybridization of probes that contain the neutral base inosine have not been extensively explored, it is possible to make a conservative estimate of the T_m as follows.

- Subtract the number of inosine residues from the total number of nucleotides in the probe to give a value S .
- Calculate the G + C content of S .
- Estimate the T_m of a perfect hybrid involving S using either the Wallace rule, the Baldino algorithm, or the Wetmur equation.
- Use conditions for hybridization that are 15°C–20°C below the estimated T_m .

The T_m of hybrids involving oligonucleotides that contain neutral bases can also be estimated empirically. Oligonucleotides containing inosine can be used with washing buffers containing TMACl or TEACl (e.g., see Andersson et al. 1989).

TABLE 3. Inosine-containing codons recommended for use in oligonucleotide probes

Amino acid	Codon	Amino acid	Codon
A (Ala)	GCI	M (Met)	ATG
C (Cys)	TGC ^a	N (Asn)	AAC ^a
D (Asp)	GAT	P (Pro)	CCI
E (Glu)	GAI	Q (Gln)	CAI
F (Phe)	TTC ^a	R (Arg)	CGI ^c
G (Gly)	GGI	S (Ser)	TCC ^{b,c}
H (His)	CAC ^a	T (Thr)	ACI
I (Ile)	ATI	V (Val)	GTI
K (Lys)	AAI	W (Trp)	TGG
L (Leu)	CTI ^c	Y (Tyr)	TAC ^a

^aIf the first nucleotide of the succeeding codon is G, use T in the third position.

^bIf the first nucleotide of the succeeding codon is G, use I in the third position.

^cTry to avoid amino acids with six codons if at all possible.

MORE ON MELTING TEMPERATURES

Heating a solution of dsDNA unravels the duplexes by disrupting the hydrogen bonds that pin together complementary base pairs. Individual hydrogen bonds are relatively weak (~ 5 kcal/mol/bond) and are thus easily disrupted by heat. However, the greater the number of hydrogen bonds holding any two complementary strands of DNA together, the higher the temperature necessary to cause all of those bonds to break, separating the nucleic acid into two strands. This transition from double-stranded (helix) to single-stranded (coil) conformations as a function of temperature can be monitored as an increase in absorbance and is marked by a sharp increase in the extinction coefficient at the temperature at which the conformational transition takes place. The temperature corresponding to the midpoint of the absorbance rise is called the melting temperature (T_m). In structural terms, the T_m is the temperature at which 50% of the base pairs in a duplex have been denatured. This relationship between nucleic acid structure and thermal stability has been experimentally exploited for four decades.

Marmur and Doty (1959, 1962) used this relationship to establish that for a wide range of DNA samples, the T_m is a linear function of the sample's base composition. The slope of the line is 0.41°C per 1% increase in the (G + C) content. For thermal denaturation profiles of DNA samples (with a G + C content between 30% and 75%) run in 0.15 M NaCl and 15 mM sodium citrate, the melting temperature is

$$T_m = 69.3^\circ\text{C} + 0.41(\%[\text{G} + \text{C}]). \quad (1)$$

This equation makes it possible to estimate accurately the base composition of a DNA sample simply by measuring the T_m of the sample. To determine the T_m of DNA samples run under other salt conditions, use the more general equation

$$T_m = 81.5^\circ\text{C} + 16.6 \log_{10}[M^+] + 0.41(\%[\text{G} + \text{C}]), \quad (2)$$

where $[M^+]$ is the monovalent cation concentration for $M^+ \leq 0.5$ M.

Molecular Hybridization

The molecular hybridization techniques developed in the 1960s continue to be some of the most useful tools of the molecular biologist. Their simplicity and sensitivity have helped to drive a revolution in our understanding of gene structure, gene expression, and genome organization. These techniques have also been adapted as diagnostic tools in clinical laboratories and pharmaceutical companies.

Establishing optimal conditions for hybridization of a nucleic acid probe and its target sequence requires knowledge of the T_m of the target:probe duplex. Although under some circumstances, the T_m can be experimentally determined, it is more common to calculate the T_m . A variety of equations have been derived that can be used to estimate the T_m of a target:probe duplex under a range of conditions.

At the T_m , when the probe and target are both polynucleotides, the molecules contain stretches of native duplex separated by denatured regions. The transition then from helix to coil is intramolecular and is thus independent of polynucleotide concentration (Wetmur 1991). The T_m is a function of base composition, solvent composition, duplex length, and extent of base-pair mismatches (Hall et al. 1980; Wahl et al. 1987; Baldino et al. 1989). For DNA duplexes, Equation 2 has been modified by Wetmur (1991) to permit hybridization in salt up to 1 M NaCl:

$$T_m = 81.5 + 16.6 \log_{10}([\text{Na}^+]/\{1.0 + 0.7[\text{Na}^+]\}) + 0.41(\%[\text{G} + \text{C}]) - 500/n - P - F, \quad (3)$$

where n is the length of the duplex, P is the temperature correction for the percent mismatch of base pairs (which is typically 1°C per 1% mismatch), and F is 0.63°C per 1% formamide. For RNA

duplexes,

$$T_m = 78 + 16.6 \log_{10}([Na^+]/\{1.0 + 0.7[Na^+]\}) + 0.7\%(G + C) - 500/n - P - F, \quad (4)$$

where F is 0.35°C per 1% formamide. For hybrids of RNA and DNA,

$$T_m = 67 + 16.6 \log_{10}([Na^+]/\{1.0 + 0.7[Na^+]\}) + 0.8\%(G + C) - 500/n - P - F, \quad (5)$$

where F is 0.5°C per 1% formamide. Variations on Equations 4 and 5 have been derived by Bodkin and Knudson (1985) and Casey and Davidson (1977), respectively.

When the probe is an oligonucleotide, at the T_m , half of the duplexes have separated. The transition from helix to coil is intermolecular, and thus the T_m is dependent on the oligonucleotide concentration (Wetmur 1991), the oligonucleotide sequence, and the composition of the solvent. Although various permutations of Equations 3–5 are frequently used, the use of $\%(G + C)$ is not sufficient to predict the T_m . A more accurate estimate of T_m can be obtained from a nearest-neighbor model that permits the incorporation of sequence-related thermodynamic data (Breslauer et al. 1986; Freier et al. 1986; Kierzek et al. 1986; Rychlik et al. 1990; Wetmur 1991; Rychlik 1994). One equation derived from such a model is

$$T_m(\text{in degrees Celsius}) = (T^0\Delta H^0)/(\Delta H^0 - \Delta G^0 + RT^0\ln[c]) + 16.6 \log_{10}([Na^+]/\{1.0 + 0.7[Na^+]\}) - 269.3, \quad (6)$$

where

$$\Delta H^0 = \sum nn(\Delta H_{nn}^0) + \Delta H_p^0 + \Delta H_e^0,$$

$$\Delta G^0 = \sum nn(N_{nn}\Delta G_{nn}^0) + \Delta G_i^0 + \Delta G_e^0,$$

N_{nn} is the number of nearest neighbors (e.g., 13 for a 14-mer), $R = 1.99 \text{ cal/mol}$ (in degrees Kelvin), $T^0 = 298.2^\circ\text{K}$, C is the total molar strand concentration, and $[Na^+] \leq 1 \text{ M}$.

As described above in Melting Temperature and Hybridization Temperature, a number of the thermodynamic terms can be approximated: average nearest-neighbor enthalpies,

$$\Delta H_{nn}^0 = -8.0 \text{ kcal/mol};$$

average nearest-neighbor free energies,

$$\Delta G_{nn}^0 = -1.6 \text{ kcal/mol};$$

initiation term,

$$\Delta G_i^0 = +2.2 \text{ kcal/mol};$$

average dangling end enthalpy,

$$\Delta H_e^0 = -8.0 \text{ kcal/mol/end};$$

average dangling end free energy,

$$\Delta G_e^0 = -1 \text{ kcal/mol/end};$$

and average mismatch/loop enthalpy,

$$\Delta H_p^0 = -8.0 \text{ kcal/mol/mismatch.}$$

Note that for each mismatch or loop, N_{nn} is reduced by 2. At the opposite end of the complexity spectrum is the equation

$$T_m = 2(A + T) + 4(G + C), \quad (7)$$

where $(A + T)$ is the number of adenines and thymines in the oligonucleotide and $(G + C)$ is the number of guanines and cytosines in the oligonucleotide, which works for short oligonucleotides that perfectly match their target sequence (Suggs et al. 1981b). More precisely, this equation predicts the dissociation temperature (T_d) of an oligonucleotide hybridized to a target sequence that is bound to a solid support. For a detailed discussion of T_d , see Wetmur (1991).

The differences in calculated T_m can be significant. For example, the predicted T_m for hybridization, in 0.5 M NaCl, of a 14-bp oligonucleotide probe that perfectly matches a sequence (with a % $[G + C] = 50$) in the target DNA will be 59°C using Equation 3, 55°C using Equation 6, and 42°C using Equation 7.

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