

# DNA Probes: Applications of the Principles of Nucleic Acid Hybridization

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**ABSTRACT:** Nucleic acid hybridization with a labeled probe is the only practical way to detect a complementary target sequence in a complex nucleic acid mixture. The first section of this article covers quantitative aspects of nucleic acid hybridization thermodynamics and kinetics. The probes considered are oligonucleotides or polynucleotides, DNA or RNA, single- or double-stranded, and natural or modified, either in the nucleotide bases or in the backbone. The hybridization products are duplexes or triplexes formed with targets in solution or on solid supports. Additional topics include hybridization acceleration and reactions involving branch migration. The second section deals with synthesis or biosynthesis and detection of labeled probes, with a discussion of their sensitivity and specificity limits. Direct labeling is illustrated with radioactive probes. The discussion of indirect labels begins with biotinylated probes as prototypes. Reporter groups considered include radioactive, fluorescent, and chemiluminescent nucleotides, as well as enzymes with colorimetric, fluorescent, and luminescent substrates.

**KEY WORDS:** Nucleic acid hybridization, thermodynamics, kinetics, single-stranded DNA branch migration, labeled probes.

## I. INTRODUCTION

Nucleic acid hybridization with a labeled probe is the only practical way to detect the presence of a complementary target sequence in a complex nucleic acid mixture. The goal of this article is to develop a unified approach to all types of nucleic acid hybridization.

Quantitative aspects, thermodynamics, and kinetics of hybridization are explained below in Section II. Three temperatures are distinguished and calculated for (1) melting of polynucleotides, (2) melting of oligonucleotides, and (3) dissociation of oligonucleotide probes from targets immobilized on solid supports. The hybrids considered include DNA duplexes and triplexes, RNA duplexes, and RNA-DNA hybrids, including in each case structures with nucleotides modified in the bases or in the backbones. Hybridization rates

are calculated for the same probes reacting with targets in solution and on solid supports. Additional topics include acceleration of hybridization using inert polymers, emulsions, or proteins as well as quantitative aspects of reactions involving single-stranded DNA branch migration, including D-loop formation, R-loop formation, displacement reactions, and branch capture reactions.

The synthesis or biosynthesis and the detection of labeled probes are considered below in Section III, with a discussion of their sensitivity and specificity limits. Direct labeling is illustrated with radioactive probes, including end labels and internal labels. Indirect labels are detected after they bind proteins or other molecules that act directly or indirectly as reporters. The discussion of indirect labels begins with biotinylated probes as prototypes. Reporter groups include radioactive, fluorescent, and chem-

luminescent nucleotides, as well as enzymes with colorimetric, fluorescent, and luminescent substrates.

## II. HYBRIDIZATION

The hybridization section is intended to be a thorough consideration of the fundamental aspects of the subject without reference to the choice of detection methods.

### A. Denaturation

There are three *different* temperatures that are often called melting temperatures. We will consider each of these temperatures separately. The associated physical phenomena are illustrated in Figures 1 to 3, respectively. The temperatures and their characteristics are<sup>1</sup>

- (1)  $T_m^\infty$ , the melting temperature of a polynucleotide duplex, including long DNA molecules
- (2)  $T_m$ , the melting temperature where at least one duplex strand is an oligonucleotide
- (3)  $T_d$ , the dissociation temperature for an oligonucleotide bound to a polynucleotide on a solid support, such as a blot

#### 1. Melting Temperatures of Polynucleotides

The melting temperature of a polynucleotide duplex,  $T_m^\infty$ , as illustrated in Figure 1a, is the temperature at which 50% of the base pairs in a duplex have been denatured, leading to molecules containing alternating duplex and denatured (loops or ends) regions. Because the reaction is intramolecular and equilibrium is achieved,  $T_m^\infty$  is independent of polynucleotide concentration and time. The only variables are the composition of the polynucleotide duplex and the composition of the solvent. The polynucleotide duplex may be DNA, RNA, or RNA-DNA hybrid and may vary in base composition, expressed as % G + C,

with possible substitution of unusual bases affecting the results.

In  $\text{Na}^+$  salt solutions with pH of  $7 \pm 1$ ,  $T_m^\infty$  may be expressed as a function of % G + C and  $[\text{Na}^+]$ , the salt concentration.<sup>2</sup> Upper and lower case Ts are used for °K and °C, respectively ( $T = t + 273.16$ ). The summary Equations 1a, 1b, and 1c include these variables with the salt concentration term modified to extend the practical range to 1 M  $\text{Na}^+$ , a concentration routinely employed to maximize hybridization rates on blots.<sup>3</sup> The equations also include terms for duplex length (D) and percent mismatching (P) to be discussed below.

#### DNA:

$$\begin{aligned} t_m^\infty = & 81.5 \\ & + 16.6 \cdot \log_{10} \left\{ \frac{[\text{Na}^+]}{1.0 + 0.7[\text{Na}^+]} \right\} \\ & + 0.41 \cdot (\% \text{ G} + \text{C}) \\ & - \frac{500}{D} - P \end{aligned} \quad (1a)$$

#### RNA:

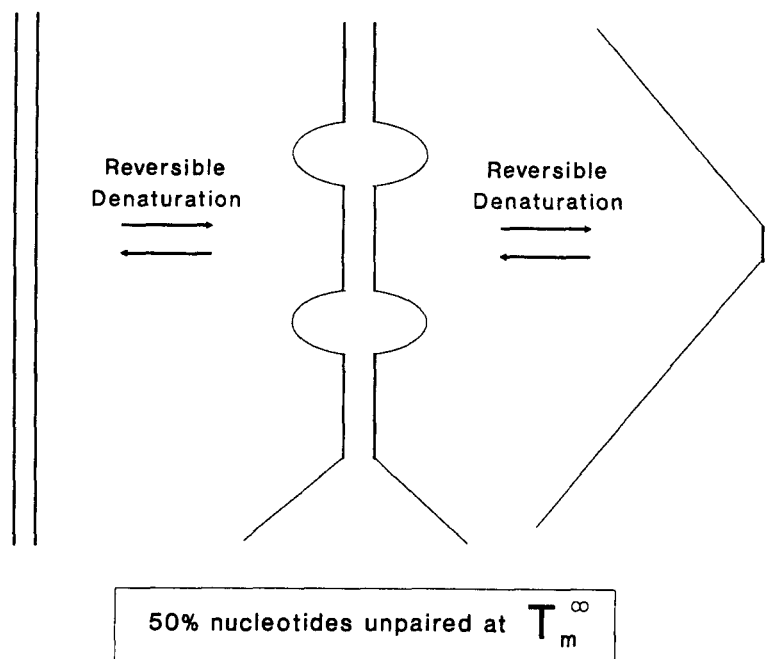
$$\begin{aligned} t_m^\infty = & 78 \\ & + 16.6 \cdot \log_{10} \left\{ \frac{[\text{Na}^+]}{1.0 + 0.7[\text{Na}^+]} \right\} \\ & + 0.7 \cdot (\% \text{ G} + \text{C}) \\ & - \frac{500}{D} - P \end{aligned} \quad (1b)$$

#### RNA-DNA Hybrids:

$$\begin{aligned} t_m^\infty = & 67 \\ & + 16.6 \cdot \log_{10} \left\{ \frac{[\text{Na}^+]}{1.0 + 0.7[\text{Na}^+]} \right\} \\ & + 0.8 \cdot (\% \text{ G} + \text{C}) \\ & - \frac{500}{D} - P \end{aligned} \quad (1c)$$

The dependence of  $T_m^\infty$  of native DNA on

## a. Polynucleotide Melting Temperature



## b. Kinetics of Hybridization

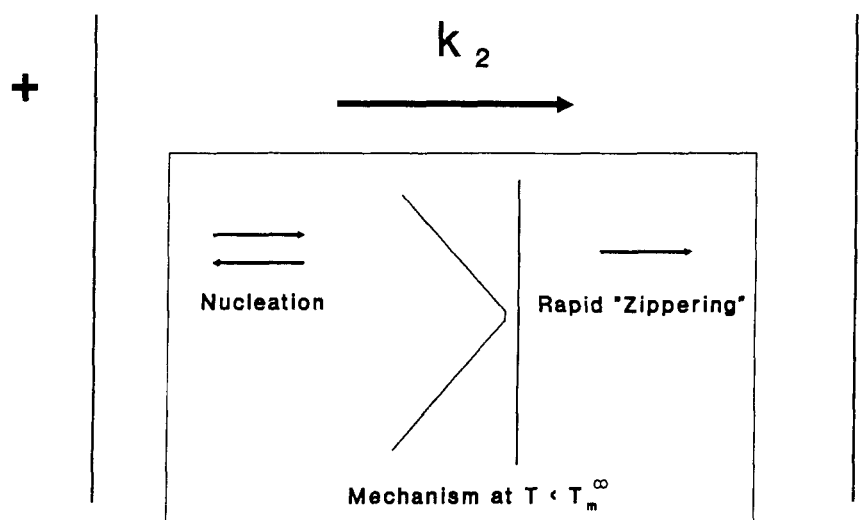


FIGURE 1. Thermodynamics and kinetics of polynucleotide hybridization.

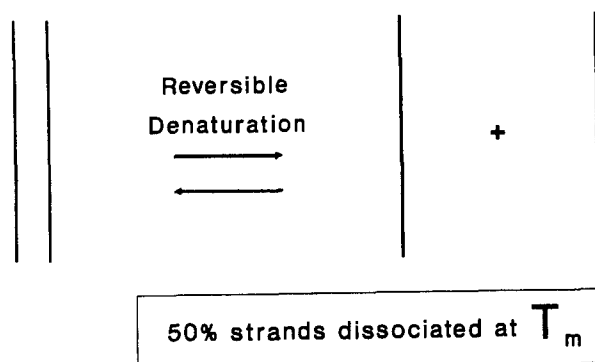
$[Na^+]$  and % G + C (Equation 1a) has been known for almost 30 years.<sup>2</sup> Polymerase chain reaction (PCR)<sup>4</sup> buffer containing 1.5 mM  $Mg^{++}$  and 0.05M  $K^+$  is equivalent to  $[Na^+] = 0.20$ .<sup>5</sup> Note that the DNA denaturation temperature in PCR

is really a strand separation temperature for a homogeneous DNA, as illustrated in Figure 3a, and must be higher (typically 92°C for 50% G + C) than  $T_m^{\infty}$ .

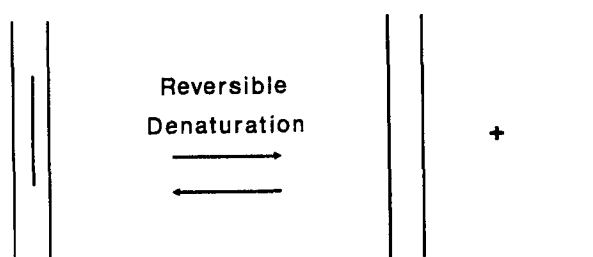
Base substitutions that raise  $T_m^{\infty}$  include

## a. Oligonucleotide Melting Temperatures

### i. Duplexes

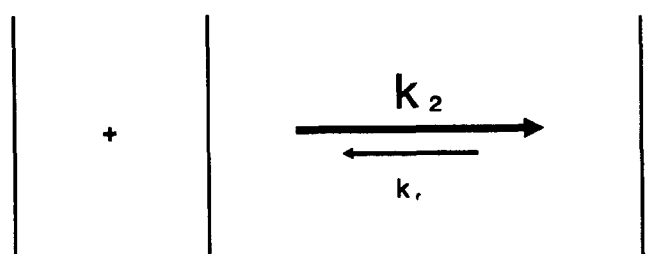


### ii. Triplexes



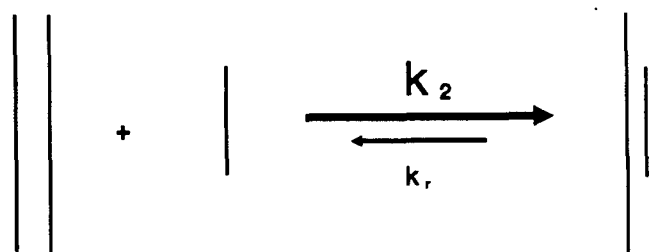
## b. Kinetics of Hybridization

### i. Duplexes



Reactions at  $T < T_m$

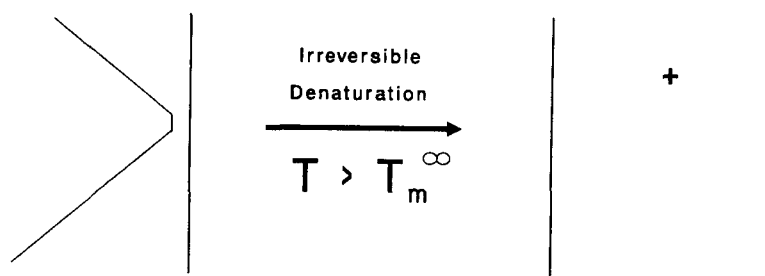
### ii. Triplexes



**FIGURE 2.** Thermodynamics and kinetics of oligonucleotide hybridization.

## Kinetics of Strand Separation; Dissociation Temperature

### a. Strand Separation (continuation of Figure 1a)



### b. Oligonucleotide Dissociation Temperature

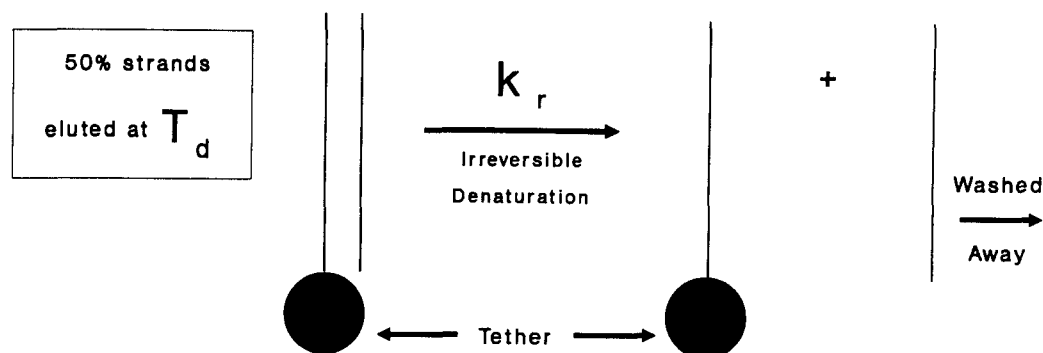


FIGURE 3. Irreversible dissociation of nucleic acid duplexes.

5-methyldeoxycytidine (MedC)<sup>6</sup> in place of deoxycytidine (dC), which results in an increase in the 0.41% G + C factor to 0.53, and 5-bromodeoxyuridine (BrdU)<sup>7</sup> in place of thymidine (dT), which increases the factor to 0.53 or 0.49 in 0.15 or 1.35M K<sup>+</sup>, respectively. On the other hand, decreased dependence of  $T_m^\infty$  on % G + C may be obtained, while retaining base pair specificity in PCR products, with 7-deaza-2'-deoxyguanosine<sup>8</sup> replacing guanosine.

Data for melting of more stable, double-stranded RNAs with complex sequences are less complete. However, Equation 1b may be used to predict the results compiled from many sources,<sup>9-11</sup> generally to within 1°C. The data for RNA-DNA hybrids with complex sequences are even less complete. Equation 1c is based on the measurement of  $t_m^\infty = 92^\circ\text{C}$  for a 42% G + C RNA-DNA hybrid<sup>12</sup> in 0.4M Na<sup>+</sup> and limited data on % G + C dependence of rRNA-DNA hybrids.<sup>13</sup> Homopolymer  $T_m^\infty$  data<sup>14,15</sup> are in general agreement, but were not used to derive the equations. Examples of calculations using these equa-

tions for polynucleotides with 42% G + C in 0.4M Na<sup>+</sup>, all of which agree with results in the literature, are as follows:

#### Example 1a DNA

$$\begin{aligned} t_m^\infty &= 81.5 + 16.6 \cdot \log_{10}(0.4/1.28) \\ &\quad + 0.41 \cdot 42 \\ &= 81.5 - 8.4 + 17.2 \\ &= 90^\circ\text{C} \end{aligned}$$

#### Example 1b RNA

$$\begin{aligned} t_m^\infty &= 78 + 16.6 \cdot \log_{10}(0.4/1.28) \\ &\quad + 0.7 \cdot 42 = 78 - 8.4 + 29.4 = 99^\circ\text{C} \end{aligned}$$

#### Example 1c RNA-DNA hybrid

$$t_m^\infty = 67 + 16.6 \cdot \log_{10}(0.4/1.28)$$

$$+ 0.8 \cdot 42 = 67 - 8.4 + 33.6 = 92^{\circ}\text{C}$$

Several other solvents are commonly employed for hybridization experiments. Formamide may be used with DNA,<sup>13,16</sup> RNA,<sup>11</sup> and RNA-DNA hybrids.<sup>13</sup>  $T_m^{\infty}$  is lowered by  $0.63^{\circ}\text{C}/\%$  formamide for DNA, significantly less for RNA-DNA hybrids, and even less for RNA. Thus, at high formamide concentrations, conditions spanning a wide range of % G + C exist that favor formation of RNA-DNA hybrids over DNA-DNA hybrids, thus facilitating formation of R-loops.<sup>17</sup> Guanidinium cations and thiocyanate anions may be employed to denature proteins while allowing rapid hybridization for DNA,<sup>18</sup> RNA,<sup>19</sup> and RNA-DNA<sup>19</sup> hybrids. Tetraalkylammonium salt solutions may be employed to decrease the % G + C dependence of DNA<sup>20</sup> and RNA<sup>9</sup> melting. In particular, for all long DNAs of any % G + C,  $T_m^{\infty} = 94^{\circ}$  and  $63^{\circ}\text{C}$  in  $3.2M$   $\text{Me}_4\text{NCl}$  and  $2.4M$   $\text{Et}_4\text{NCl}$ , respectively. For long RNAs, the concentration of  $\text{Et}_4\text{NCl}$  necessary to completely eliminate the larger dependence on % G + C is impractically high ( $3.5M$ ) because the  $T_m^{\infty}$  is below room temperature.

The  $500/D$  term in Equations 1a through 1c derives from a compilation<sup>21</sup> of measurements from several laboratories of the effect of duplex length ( $D$ ) on  $T_m^{\infty}$ . The lower  $T_m^{\infty}$  with decreasing  $D$  is due both to the equilibrium property of opening at the newly created ends and to the irreversible denaturation of lower % G + C duplexes within the population. Strand separation is irreversible because the rate of the reverse reaction, hybridization (renaturation, reassociation), approaches zero at  $T_m^{\infty}$ . Experimental verification of theoretical models<sup>22</sup> for the melting of DNA domains in duplexes where the  $500/D$  term becomes significant have been obtained by denaturing gradient<sup>23</sup> or temperature gradient<sup>24</sup> gel electrophoresis.

Studies of molecular evolution often take advantage of the effects of various mismatches in heteroduplexes on  $T_m^{\infty}$ , especially as measured in  $2.4M$   $\text{Et}_4\text{NCl}$ .<sup>21,25,26</sup> The consensus from many measurements, including mismatches and bulges<sup>27</sup>

and synthetic oligodeoxynucleotides with apurinic and abasic sites,<sup>28</sup> is that  $\Delta T_m = 1^{\circ}\text{C}$  for  $P = 1\%$  mismatch, although  $\Delta T_m/P$  measurements<sup>25,29</sup> have varied from  $0.6$  to  $1.5^{\circ}\text{C}$  per % mismatch.

## 2. Melting Temperatures of Oligonucleotides

### a. Duplexes

The melting temperature of an oligonucleotide bound to a complementary oligonucleotide or polynucleotide ( $T_m$ ) as illustrated in Figure 2a, is the temperature at which 50% of the duplexes have strand separated. Because in this case the reaction is intermolecular and equilibrium is achieved,  $T_m$  is dependent upon oligonucleotide concentration, but still independent of time.  $T_m$  is the temperature of importance for reaction of oligonucleotides with complementary sequences, whether in solution, as in the annealing step of PCR, as primers for various polymerases, and as substrates for ligases, or on solid support prior to washing. However,  $T_m$  is *not*  $T_d$ , the dissociation temperature for *retention* of probes on blots or other solid supports subsequent to one or more washing steps.  $T_d$  is discussed below in Section II.C.

In addition to concentration,  $T_m$  is dependent upon the oligonucleotide sequence and the composition of the solvent. The use of % G + C for a DNA, RNA, or RNA-DNA duplex containing an oligonucleotide is insufficient for prediction of  $T_m$ . A nearest neighbor model permits incorporation of sequence-related thermodynamic data, although the method is not perfect.<sup>30</sup> Several basis sets are available for RNA<sup>31</sup> and DNA.<sup>1,32</sup>

Defining  $\Delta G^{\circ}$  and a temperature-independent  $\Delta H^{\circ}$  to be the standard free energy and enthalpy for the sum of all nearest neighbor contributions (terms labeled  $_{nn}$ ) in the coil  $\rightarrow$  helix transition and letting  $C$  be the *total molar strand concentration* with equal concentrations of both of the complementary oligodeoxynucleotides,  $t_m$  may be calculated from Equation 2a.

$$t_m = \frac{T^\circ \cdot \Delta H^\circ}{(\Delta H^\circ - \Delta G^\circ + R \cdot T^\circ \ln[C/4])} + 16.6 \cdot \log_{10} \left\{ \frac{[Na^+]}{1.0 + 0.7 [Na^+]} \right\} - 269.3 \quad (2a)$$

where

$$\Delta H^\circ = \sum_{nn} (N_{nn} \cdot \Delta H_{nn}^\circ) + \Delta H_p^\circ + \Delta H_e^\circ \quad (2b)$$

$$\text{and} \quad \Delta G^\circ = \sum_{nn} (N_{nn} \cdot \Delta G_{nn}^\circ) + \Delta G_i^\circ + \Delta G_e^\circ \quad (2c)$$

A standard  $[Na^+] = 1$  is assumed, with  $-269.3 + \text{salt term} = -273.2$  for the  $^\circ K$  to  $^\circ C$  conversion.  $T^\circ$  is usually  $298.2^\circ K$ .  $R$  is  $1.99 \text{ cal/mol} \cdot ^\circ K$ . The term  $[C/4]$  in Equation 2a becomes  $[C/2]$  for self-complementary oligonucleotides and  $[C]$  for one excess oligonucleotide probe binding to a polynucleotide. In addition to the basis set of nearest neighbor thermodynamic values ( $\Delta H_{nn}^\circ$  and  $\Delta G_{nn}^\circ$ ) in Equations 2b and 2c,  $\Delta G^\circ$  includes a temperature-independent  $\Delta G_i^\circ$ , the initiation term, which is included in each basis set. Average nearest neighbor enthalpies and free energies are  $8.0$  and  $-1.6 \text{ kcal/mol}$ , respectively, with the initiation term being  $+2.2 \text{ kcal/mol}$ .<sup>1</sup>

If  $\Delta H_e^\circ$ , an average dangling end enthalpy, is estimated to be  $-8 \text{ kcal/mol}$  per end, the same as average  $\Delta H_{nn}^\circ$ , then  $\Delta G_e^\circ$  is about  $-1 \text{ kcal/mol}$  per end, which is less stabilizing than an additional average  $\Delta G_{nn}^\circ$ .<sup>33</sup> These terms are important for blotting or other hybridizations of *short* oligonucleotides to polynucleotides attached to solid supports. Extensive measurements of thermodynamic parameters exist for duplexes with single-strand breaks (nicks),<sup>34</sup> mismatched base pairs,<sup>35</sup> and bulge defects.<sup>36,37</sup> Internal mismatched base pairs and bulge defects result in the loss of two nearest neighbor contributions. This loss is partially compensated by a  $\Delta H_p^\circ$  term, which may again be estimated to be  $-8 \text{ kcal/mol}$ . The salt dependence of  $T_m$  and  $T_m^\infty$  is similar.<sup>1,38</sup> As for long DNA,  $T_m$  is independent of base composition in tetraalkylammonium salt so-

lutions for a limited set of oligodeoxynucleotides.<sup>39</sup> Examples using average  $\Delta H_{nn}^\circ$  and  $\Delta G_{nn}^\circ$  in Equations 2a through 2c are presented below for excess probe and  $1.0 M Na^+$ :

### Example 2b

$$\Delta H^\circ = \sum_{nn} (N_{nn} \cdot \Delta H_{nn}^\circ) + \Delta H_p^\circ + \Delta H_e^\circ$$

e.g., 14-mer

$$\begin{aligned} \Delta H^\circ &= \sum_{nn} (N_{nn} \cdot \Delta H_{nn}^\circ) + 0 + 0 \\ &= 13 \cdot (-8.0) = -104 \text{ kcal/mol} \end{aligned}$$

Add 2 dangling ends

$$\begin{aligned} \Delta H^\circ &= 13 \cdot (-8.0) + \Delta H_e^\circ + 0 \\ &= -104 + 2 \cdot (-8.0) \\ &= -120 \text{ kcal/mol} \end{aligned}$$

And 1 mismatch/loop

$$\begin{aligned} \Delta H^\circ &= 11 \cdot (-8.0) + 2 \cdot (-8.0) + \Delta H_p^\circ \\ &= -104 + 1 \cdot (-8.0) \\ &= -112 \text{ kcal/mol} \end{aligned}$$

### Example 2c

$$\Delta G^\circ = \sum_{nn} (N_{nn} \cdot \Delta G_{nn}^\circ) + \Delta G_i^\circ + \Delta G_e^\circ$$

e.g., 14-mer

$$\begin{aligned} \Delta G^\circ &= \sum_{nn} (N_{nn} \cdot \Delta G_{nn}^\circ) + 2.2 + 0 \\ &= 13 \cdot (-1.6) = -18.6 \text{ kcal/mol} \end{aligned}$$

Add 2 dangling ends

$$\begin{aligned} \Delta G^\circ &= 13 \cdot (-1.6) + 2.2 + \Delta G_e^\circ \\ &= -18.6 + 2 \cdot (-1.0) \\ &= -20.6 \text{ kcal/mol} \end{aligned}$$



And 1 mismatch

$$\begin{aligned}\Delta G^\circ &= 11 \cdot (-1.6) + 2.2 \\ &+ 2 \cdot (-1.0) \\ &= -17.4 \text{ kcal/mol}\end{aligned}$$

**Example 2a:**

All cases:

$$[\text{Na}^+] = 1.0M$$

$$[\text{C}] = 1.0 \cdot 10^{-6}$$

$$\begin{aligned}\text{or: } R \cdot T^\circ \ln[\text{C}] &= 1.99 \cdot \ln(1.0 \cdot 10^{-6}) \\ &= -8.2 \text{ kcal/mol}\end{aligned}$$

$$\begin{aligned}t_m &= \frac{T^\circ \cdot \Delta H^\circ}{(\Delta H^\circ - \Delta G^\circ + R \cdot T^\circ \ln[\text{C}])} \\ &= 273.2\end{aligned}$$

Sample calculation: 14-mer, no dangling ends or mismatches, 50% G + C:

$$t_m = \frac{298 \cdot (-104)}{\{-104 - (-18.6) - 8.2\}} \quad \begin{array}{l} \text{Infinite} \\ \text{(No C} \\ \text{term)} \end{array}$$

Length L	=	13	14	15	
$t_m$	=	55°C	58°C	61°C	99.5°C

			2 dang- ling ends	2 dang- ling ends and 1 mismatch
Structure	Oligo- nucleotides			
L = 14	$t_m =$	58°C	59.5°C	52°C
L = 20	$t_m =$	70°C	70°C	65°C

PCR primers are typically 20 nucleotides long and present at  $C = 1 \mu M$ . In PCR buffer, equivalent to 0.20M NaCl,  $t_m = 61^\circ\text{C}$ . The  $t_m$  may be increased by using longer primers or primers with nucleotide sequences with stronger nearest neighbor interactions. Depression of the melting temperature of PCR primers at mismatched sites is the key to specificity.<sup>40,41</sup> Extensions that tolerate low levels of mismatching in Alu PCR<sup>42</sup> may be

achieved by using reduced annealing and extension temperatures.

Ligation of adjacent hybridized oligonucleotides is the basis for a second signal amplification-based DNA diagnostic system involving successive ligation steps,<sup>43</sup> either alone or coupled to PCR.<sup>44</sup> The oligonucleotides are approximately the same length as used for PCR, the buffer has similar characteristics, but the concentration is 10 nM, giving an expected  $t_m = 55^\circ\text{C}$ . Specificity of hybridization may be increased by using 25% formamide for room temperature ligation or ligation at elevated temperatures using a thermostable ligase.<sup>45</sup>

Very short oligonucleotides, where dangling end effects become very important, are not usually employed by molecular biologists because of their limited specificity, with four<sup>8</sup> possible 8-mers implying a random occurrence very 65 kb or once per cosmid clone insert. However, complete sets of very short oligonucleotides can be synthesized and stored for subsequent use. A new sequencing method has been described with an initial low temperature extension of very short primers using T7 DNA polymerase,<sup>46</sup> eliminating the need for continuous synthesis of new primers dictated by newly obtained sequence during a sequencing project.

The thermodynamic basis sets may be expanded to include modified bases using a  $\Delta G^\circ$  correction term<sup>1</sup> or incorporating average or specific nearest neighbor thermodynamic values. Modified oligonucleotides may include base substitutions such as MedC<sup>5,47</sup> or 5-bromodeoxycytidine (BrdC)<sup>48</sup> for dC for increasing  $T_m$  and 2,6 diaminopurine for deoxyadenine increasing<sup>49</sup> or decreasing<sup>49,50</sup>  $T_m$ , depending on the sequence. Use of 5-fluorodeoxyuridine to pair with purines,<sup>51</sup> dG to pair with pyrimidines, and deoxyinosine to pair with dA or dC<sup>52</sup> may be employed when broader base-pairing specificity is desired. Modified backbones may include methylphosphonates,<sup>1,53</sup> phosphorothioates,<sup>54</sup> phosphorosenoates,<sup>55</sup> and  $\alpha$ -anomers.<sup>56</sup> These structures are nuclease-resistant and, thus, potential antisense agents.<sup>54,57-59</sup> Except for probes attached to intercalating agents,<sup>60</sup> most oligonucleotide derivatives, including the probes to be discussed in Section III, decrease  $T_m$ .

When the furocoumarin 4'-hydroxymethyl-



4,5',8-trimethylpsoralen is attached to a radio-labeled oligonucleotide, a duplex with a long complementary polynucleotide may be trapped by photocrosslinking and  $T_m$  may be determined.<sup>61,62</sup> The partial duplex product may be detected following separation by alkaline agarose gel electrophoresis<sup>63</sup> or other methods. This photocrosslinking method extends the temperature range where oligonucleotide binding may be investigated, thus permitting more complete measurements of the effect of polynucleotide secondary structure on oligonucleotide binding.<sup>61,62</sup> Methylphosphonate oligonucleotides may be synthesized with attached 4'-(aminoalkyl)-4,5',8-trimethylpsoralen,<sup>64</sup> for photocrosslinking, as well as with attached EDTA,<sup>65</sup> which, when activated by  $Fe^{++} + DTT$ , will cleave the target, acting as an artificial restriction enzyme.

### b. Triple Helices (Triplexes)

Many recent studies of the hybridization of oligonucleotides with native DNA at sites permitting triple helix (triplex) formation have been carried out to explore the potential of these oligonucleotides or their derivatives as artificial restriction endonucleases or antisense oligonucleotides. Triplexes have been formed *in vitro* at pH below 7 between polypurine-polypyrimidine stretches in DNA and polypyrimidine oligodeoxynucleotides with the sequence parallel to the purine-rich strand in the DNA. Systematic studies have been directed toward several variables:<sup>66</sup> pH, salt concentration, % G + C, and mismatches. The thermodynamic parameters for triplex formation have been determined.<sup>67</sup> It is interesting to note that the enthalpy per base pair associated with forming a triplex from an oligonucleotide and a duplex was only one third that for forming a duplex from oligonucleotides. The triplets contained Hoogsteen base pairs between the T in the oligonucleotide and the A in DNA, and between protonated dC ( $dC^+$ ) in the oligonucleotide and dG in the DNA. Substitution of either BrdU for thymidine or MedC for dC, or substitution of both, increased the stability of the triplexes at pH 7.0, or 7.4, respectively.<sup>68</sup> A construct containing a 3'-3' bridge between opposite sense oligonucleotide segments permitted

triplex formation at adjacent polypurine and polypyrimidine sequences on a duplex DNA strand.<sup>69</sup> More recently, a purine·purine·pyrimidine triple helix motif (T may substitute for A) has been described with antiparallel polypurine strands, increasing the range of potential targets.<sup>70</sup>

Triplex-forming oligonucleotides containing EDTA· $Fe^{++}$  have been activated to produce specific, although very incomplete, cleavage of the DNA.<sup>71</sup> To date, the greatest specificity demonstrated has been a single cleavage of a yeast chromosome into which a 20 nucleotide target had been engineered.<sup>72</sup>

Intercalating agents have been added to increase binding affinity by short triplexes. Triplex-forming oligo-dT oligonucleotides containing both 5'-EDTA· $Fe^{++}$  and 3'-acridine have been investigated.<sup>73</sup> Sequence specificity of binding was demonstrated by footprinting for an oligonucleotide with acridine attached to the 5'-end,<sup>74</sup> although the specificity test was quite limited compared with that demonstrated above for a 20-mer oligonucleotide and yeast DNA.<sup>72</sup>

In addition to EDTA· $Fe^{++}$ , other covalent additions used to mediate detection have included ellipticine,<sup>75</sup> which permits photocleavage, *p*-azidophenacyl,<sup>76</sup> which permits photocrosslinking, a N-bromoacetyl group,<sup>77</sup> which mediates alkylation and staphylococcal nuclease.<sup>78</sup>

## B. Hybridization

We are using the term hybridization to include all intermolecular duplex formation by complementary nucleic acid strands, including not only RNA-DNA hybridization, but also DNA renaturation or reassociation. Hybridization reactions are illustrated in Figures 1b and 2b.

### 1. Hybridization with Polynucleotides

The fundamental studies of hybridization, including the second-order kinetics<sup>79</sup> and the dependence on polynucleotide length<sup>80</sup> and information content or complexity,<sup>79-81</sup> were completed in 1968. The theoretical and practical aspects of hybridization have not changed appreciably since

the subject was reviewed in 1974.<sup>82,83</sup> A comprehensive book on hybridization was published in 1985.<sup>84</sup> Thus, what appears below will be a short summary of the subject, initially directed toward quantitative interpretation of solution hybridization reactions.

Because the individual polynucleotide strands often do not contain the same sequences, the concentration term used for hybridization of polynucleotides is molar nucleotide residue concentration ( $C_o$ ), not strand concentration ( $C$ ), as is used for both oligonucleotide denaturation or hybridization.

The rate constant, ( $k_2$ ), in  $M^{-1} s^{-1}$ , for hybridization reactions is given by

$$k_2 = \frac{k'_N \sqrt{L_s}}{N} \quad (3)$$

where  $L_s$  is the length of the shortest strand participating in duplex formation,  $N$  is the complexity or the total number of base pairs present in non-repeating sequences, and  $k'_N$  is the nucleation rate constant. The inverse dependence of  $k_2$  on  $N$  results from mass action, where at constant  $C_o$  increasing  $N$  means a lower concentration of any particular sequence.

Because the yield of base pairs for a given nucleation increases as  $L_s$ , the dependence of  $k_2$  on  $\sqrt{L_s}$  implies that fewer nucleation sites are available for reaction as the molecules get longer. Both experiments<sup>12,80,85</sup> and excluded volume theory<sup>80,85,86</sup> agree that circular permutation does not affect the yield of base pairs formed.

Hybridization reactions are second order if the concentrations of the complementary strands are equal and pseudo-first order when the concentration of one strand is in excess. In Equations 4a and 4c,  $f_{ss}$  is the fraction of potential duplex remaining single-stranded at time ( $t$ ).

Second order:

$$\frac{1}{f_{ss}} = k_2 \cdot \frac{C_o}{2} \cdot t + 1 \quad (4a)$$

for which the half time (seconds) is

$$t_{1/2} = \frac{2}{k_2 \cdot C_o} \quad (4b)$$

Pseudo-first order:

$$f_{ss} = e^{-k_2 \cdot C_o \cdot t} \quad (4c)$$

for which the half time (seconds) is:

$$t_{1/2} = \frac{\ln 2}{k_2 \cdot C_o} \quad (4d)$$

Equations 4a and 4c are used for plotting rate data. For the second-order reaction, a plot of  $1/f_{ss}$  vs.  $t$ , in seconds, gives a slope equal to  $k_2 \cdot C_o / 2$ . For the first-order reaction, a plot of  $\ln (1/f_{ss})$  vs.  $t$  gives a slope equal to  $k_2 \cdot C_o$ .

Many hybridization reactions are carried out with *excess target* DNA or RNA, using tracer concentrations of labeled probe compared with the concentration of complementary sequences present in the target. Equations 4a and 4b describe reactions between single- or double-stranded probes and double-stranded targets in solution. For all excess target reactions,  $C_o$  is the total (mostly target) nucleotide concentration.

#### Example 4b

Let  $G_o = 1.5 \cdot 10^{-4} M$  (the same as 50  $\mu g/ml$  and, for native DNA, absorbance 260 nm = 1). Let  $[Na^+] = 1$  such that  $k'_N = 3.5 \cdot 10^5 M^{-1} sec^{-1}$ . Digest the DNA with a restriction enzyme with a 4-base recognition sequence (frequency =  $1/4^4$ ). The weight average length (nucleotides) is twice the number average (strands), giving  $L_s = 512$ . For *Escherichia coli* DNA:  $N = 4.2 \cdot 10^6$ .

$$k_2 = \frac{k'_N \sqrt{L_s}}{N} = 3.5 \cdot 10^5 \cdot \sqrt{512} / N \\ = 1.9 M^{-1} sec^{-1}$$

$$\text{and } t^{1/2} = \frac{2}{k_2 \cdot C_o} = 2 / (1.9 \cdot 1.5 \cdot 10^{-4})$$

$$= 7000 \text{ seconds} = 1.95 \text{ hours}$$

Single-copy target DNA:

	pUC19	$\lambda$	<i>E. coli</i>	<i>S. cerevisiae</i>	Man
N:	2686	48,502	$4.2 \cdot 10^6$	$2.3 \cdot 10^7$	$3 \cdot 10^9$
$t_{1/2}$	4.5 s	81 s	1.9 h	10.6 h	58 d

Clearly, hybridization reactions of trace quantities of probe with mammalian DNA cannot be done at 50  $\mu\text{g/ml}$ . Increasing the concentration to several milligrams per milliliter and/or acceleration of the reaction (see Section II.B.4) are necessary to make the experiment practical.

Note that the probe length should not be less than the length of a double-stranded target, or the rate of target-target hybridization will exceed the rate of probe-target hybridization, and the kinetics will become much more complicated. Typically, the probe and target DNA strands are of equal average length. Sheared DNA, unlike DNA digested with a restricted endonuclease, is circularly permuted. If partially duplex molecules are captured by hydroxylapatite chromatography, instead of assaying for base pair formation, approximately 50% of the captured probe nucleotides will be single-stranded and the observed  $k_2$  (and hence  $k'_N$ ) will be doubled. If, after correcting for the measurement method, predicted and observed  $C_o t_{1/2}$  values differ, the sequences complementary to the probe are repeated in the target DNA with a copy number,  $n$ :

$$n = C_o t_{1/2} (\text{predicted}) / C_o t_{1/2} (\text{observed}) \quad (5)$$

Equations 4c and 4d describe reactions between single- or double-stranded probes and single-stranded targets in solution. The term  $f_s$  refers only to that fraction of a probe complementary to target sequences, which is at most half of a double-stranded probe. The definition of complexity for target DNA or RNA is the same for single-stranded or double-stranded nucleic acids.

#### Example 4d

Again use  $C_o = 1.5 \cdot 10^{-4} M$  and let  $k'_N = 2.8 \cdot 10^5$ . Probe = cloned cDNA with  $L = 1600$ . Target = total mRNA. Measure  $t_{1/2} = 66$  sec.

$$\begin{aligned} N &= C_o \cdot t_{1/2} \cdot k'_N \cdot \sqrt{L_s} / \ln(2) \\ &= 1.5 \cdot 10^{-4} \cdot 60 \cdot 2.8 \cdot 10^5 \cdot 40 / 0.69 \\ &= 1.8 \cdot 10^5 \end{aligned}$$

One copy of  $L = 1600$  mRNA in  $1.4 \cdot 10^5$  nucleotides or about 1% of total mRNA.

Far more hybridization reactions (e.g., blots) use *excess probe* DNA or RNA concentrations compared with concentrations of complementary sequences present in the target. Equations 4a and 4b describe reactions between double-stranded probes and single- or double-stranded targets in solution. Equations 4c and 4d describe reactions between single-stranded probes and single- or double-stranded targets in solution. Also, in contrast to target-excess hybridization,  $C_o$  is now the probe nucleotide concentration (e.g., plasmid plus insert) and  $N$  is the complexity of the probe (e.g., plasmid plus insert), which is again the same whether the probe is single-stranded or double-stranded. Excess probe reactions are usually fast. *Excess probe hybridization rates are the same for targets of plasmid, viral, bacterial, or mammalian DNA.* Note that the length of double-stranded probes must not be greater than that of the target, or the rate of probe-probe hybridization will exceed the probe-target hybridization rate, and the kinetics will become much more complicated.

The nucleation rate constant ( $k'_N$ ) is affected by temperature,<sup>80</sup> ionic strength,<sup>80</sup> and viscosity,<sup>87</sup> but is not significantly pH-dependent<sup>80</sup> for pH of  $7 \pm 2$ . It is not affected by % G + C<sup>83</sup> or substitution of other bases capable of duplex formation. The value of  $k'_N$  for RNA-DNA hybridization at 42% G + C is reduced about 20% (assumed in Example 4d), compared with DNA hybridization,<sup>12</sup> but may be reduced as much as 50% at higher % G + C when RNA secondary structure interferes more with nucleation.

The rate of hybridization reactions, and hence  $k'_N$ , goes to zero at  $T_m^\infty$ . The term criterion<sup>82</sup> is defined as the difference between  $T_m^\infty$  and the hybridization temperature. The  $k'_N$  rises steadily as the criterion is increased to 25°C. As the criterion exceeds 25°, intramolecular base pair formation occurs, leading to structures that are no longer capable of presenting all possible sequences as nucleation sites. Although the maximum rate occurs at a criterion of 25°, higher hybridization temperatures are often employed to

increase the stringency or fidelity of hybridization with excess target. Alternately, with excess probe, reactions may be carried out at  $T_m - 25^\circ\text{C}$ , leading to mismatched products, and these structures may be subsequently eliminated by melting (e.g., washing at low salt).

Because  $k'_N$  is a very strong function of ionic strength at salt concentrations below  $0.2M$ , hybridization reactions should not be carried out in low salt. In the range of interest ( $0.25 \leq [\text{Na}^+] \leq 4.0$ ), the dependence of  $k'_N$  on salt concentration is given by Equation 6.<sup>88</sup>

$$k'_N = \{4.35 \cdot \log_{10}[\text{Na}^+] + 3.5\} \cdot 10^5 \quad (6) \\ \text{for } 0.2 \leq [\text{Na}^+] \leq 4.0$$

Hybridization reactions may be carried out in denaturing solvents while maintaining high ionic strength. In these cases,  $k'_N$  is reduced if the denaturing solvent has a higher viscosity than  $k'_N$  in  $1.0M$  NaCl at  $70^\circ\text{C}$ . Examples include DNA hybridization in  $2.4M$  Et<sub>4</sub>NCl or other tetraalkylammonium salt solutions<sup>88</sup> and formamide,<sup>89</sup> where  $k'_N$  decreases 1.1% for each 1% addition of formamide. RNA hybridization may also be carried out in tetraalkylammonium salt solutions. In Et<sub>4</sub>NCl,  $k'_N$  for RNA hybridization is almost an order of magnitude lower than  $k'_N$  for RNA hybridization. The origin of this difference is unknown.

In most solution hybridization reactions, the rate of diffusion of the probe is sufficient to assure reaction homogeneity. However, in reactions with trace probe concentrations, stirring may be necessary to assure homogeneity during the latter part of the reaction.<sup>90</sup>

Mismatches of up to 10%, although easily measured as a depression in  $T_m^\infty$ , have essentially no effect on hybridization rates at the temperature of the maximum rate and may be ignored.<sup>29</sup> Thus, most of the modifications used for labeling probes described below in Section II, even if they significantly decrease  $T_m^\infty$  and  $T_m$ , have little or no effect on  $k_2$ . As mismatching is increased to 20 and to 30%, where  $T_m$  is nearing the normally optimum hybridization temperature,  $k'_N$  falls to half and then to zero.

Subtractive hybridization, based on duplex structure with<sup>91</sup> or without<sup>92</sup> an affinity label, has been used to deplete one complex nucleic acid

mixture, such as human DNA, of all sequences present in a second complex mixture, such as human DNA containing a deletion mutation. Subtractive hybridization with mammalian DNAs is facilitated by multiple copies of the desired product in the second mixture,<sup>92</sup> as well as by using one of the methods of acceleration of hybridization described below in Section II.B.4.<sup>92</sup> Recently, subtractive hybridization has been carried out without multiple copies or use of an acceleration method by using PCR to amplify the products following subtraction.<sup>93</sup>

Possible DNA hybridization reactions in agarose gels include reassociation of bacterial DNA,<sup>94</sup> permitting detection of restriction fragment length polymorphisms, and mammalian DNA,<sup>95-97</sup> permitting detection of amplified sequences. The method may be extended to permit differential cloning of restriction fragment length polymorphisms in single copy mammalian DNA<sup>98</sup> using another of the hybridization acceleration methods described below in Section II.B.4. As a first approximation, the hybridization rates in gels may be predicted by assuming the same parameters in the  $k_2$  calculation for hybridization in the appropriate solvent and the same  $C_o$  as was present in the well prior to electrophoresis.

## 2. Hybridization with Oligonucleotides

### a. Duplexes

Hybridization rates with oligonucleotides are also described by Equations 3 and 4a through d. The reactions are usually reported in terms of molar concentration of oligonucleotide ( $C$ ), and not the nucleotide concentration ( $C_o$ ).  $C_o$  is  $C$  multiplied by the length of the oligonucleotide.

The definition of complexity remains the same for oligonucleotides and polynucleotides. It is important to note that the increased complexity of mixed oligonucleotide probes used for screening cDNA libraries<sup>99</sup> increases the half-time for hybridization proportionally. The  $\sqrt{L}$  dependence of  $k_2$  has not been strictly verified for oligonucleotides, although estimates based on Equation 3 are remarkably accurate.<sup>15</sup> Equation 3 may underestimate  $k'_N$  for the shortest oligonucleotides where the availability of nucleation



sites may not decrease with increasing  $L_s$ . The  $k'_N$  for poly(rA) + poly(rU)<sup>14</sup> is  $8 \cdot 10^4 M^{-1} s^{-1}$  after correction from 0.4 to 1.0M Na<sup>+</sup>.

Compare the forward rate constant<sup>100</sup> for self-association of r(A<sub>7</sub>U<sub>7</sub>) at  $100 \cdot 10^4 M_{\text{strands}}^{-1} s^{-1}$ : dividing by the length of 14 to get units of nucleotides and correcting the four-fold increase in rate for self-complementary reactions,  $k_2 = \{100/(4 \cdot 14)\} \cdot 10^4 M^{-1} s^{-1}$ . Equation 3 with  $L_s = N = 14$  for r(A<sub>7</sub>U<sub>7</sub>) predicts:  $k'_N = k_2 \cdot \sqrt{L_s} = \{100 \cdot \sqrt{14}/(4 \cdot 14)\} \cdot 10^4 M^{-1} s^{-1} = 7 \cdot 10^4 M^{-1} s^{-1}$ .

In this case, the same result is found for both oligonucleotides and polynucleotides. On the other hand, a similar calculation using a forward rate constant<sup>38</sup> of  $4 \cdot 10^4 M^{-1} s^{-1}$  for d(GGAATTCC) gives  $k'_N = 35 \cdot 10^4 M^{-1} s^{-1}$  in 0.25M Na<sup>+</sup>, which is about fourfold higher than predicted by Equation 6.

With polynucleotides,  $k'_N$  is affected by temperature, ionic strength, and viscosity. Just as with polynucleotides, oligonucleotide RNA hybridization rates are less than DNA hybridization rates, which are nearly the same as RNA-DNA hybridization, depending on % G + C.<sup>101</sup>

The temperature dependence of the rate of oligonucleotide hybridization is simpler than the bell-shaped dependence of  $k_2$  on temperature. With polynucleotides near  $T_m^\infty$ ,  $k_2$  decreases with increasing temperature as base pair formation rates at the end of duplex regions approach base pair dissociation rates. Hybridization with oligonucleotides usually takes place far below  $T_m^\infty$ . In fact, there is little temperature dependence of  $k_2$  for oligonucleotide-oligonucleotide reactions.<sup>38,101</sup> Reaction rates between oligonucleotides and polynucleotides at temperatures above  $T_m$  but still well below  $T_m^\infty$ , determined using psoralen photocrosslinking, indicate a temperature dependence attributable to intramolecular pairing of the polynucleotide strand.<sup>61,62</sup> The effect of solvent viscosity on oligonucleotide hybridization rates has not been studied adequately.

The effect of ionic strength on  $T_m$  is the same for oligonucleotides and polynucleotides. For example, the slope of a plot of  $(\log k_2)$  vs.  $(-\log [Na^+])$  is 1.3 for DNA<sup>80</sup> in the interval  $0.1 \leq [Na^+] \leq 0.7$  and 1.4 for self-complementary d(GGAATTCC)<sup>38</sup> in the interval  $0.05 \leq [Na^+] \leq 0.3$ . Because the  $[Na^+]$  dependence of  $T_m$  decreases for both oligonucleotides and polynu-

cleotides at high  $[Na^+]$ , Equation 6 may be used for all hybridizations in the practical interval indicated.

One of the steps in PCR with thermostable polymerases is an annealing step.<sup>4</sup> PCR primers are typically present at  $C = 1 \mu M$  and are commonly 20 nucleotides long. Given that  $k'_N$  is  $5 \cdot 10^4 M^{-1} s^{-1}$  in PCR buffer (equivalent to 0.20M NaCl), using Equations 3 and 4d, we predict  $t_{1/2} = 3$ . By the time the annealing step temperature is reached, annealing is completed. At  $C = 0.01 \mu M$  used for oligonucleotide ligation assays, although  $t_{1/2}$  is increased to 300 due to lower  $C$  and may be further increased in formamide-containing solvents. The  $t_{1/2}$  is still much less than the ligation time and is not a variable in the assay.

### b. Triple Helices (Triplexes)

A recent study of the kinetics of triplex formation, employing a restriction endonuclease protection assay, has led to the surprising result that triplex formation is quite slow.<sup>102</sup> All of the conditions studied, with 0.07 or 0.6M NaCl, corresponded to high salt conditions because of the presence of 400  $\mu M$  spermine and 20 mM Mg<sup>++</sup>. The forward strand rate constant observed at 37°C was approximately  $1800 M^{-1} s^{-1}$  or, dividing by the length of 21,  $k_2 = 86 M^{-1} s^{-1}$ . Even after correcting  $k'_N$  to  $2 \cdot 10^5 M^{-1} s^{-1}$  at 37°,  $k_2$  from Equation 3 for duplex formation was 500 times greater than  $k_2$  observed for triplex formation.

## 3. Reactions on Solid Supports

### a. Blots

Most hybridization reactions on solid supports occur with target nucleic acids noncovalently or covalently linked to nitrocellulose or to a nylon-based (or other) membrane. If a probe is hybridized to a membrane replica of a library of plasmid-containing bacteria, or of bacteriophages, following alkaline lysis *in situ* and immobilization of the DNA on the membrane, the procedures are called colony or plaque hybridizations, respectively. A similar screening procedure may be used with yeast artificial chro-

mosome clones.<sup>103</sup> If the target DNA or RNA is applied directly to the membrane, the probe hybridization reaction is a dot blot, a slot blot, or simply a filter hybridization. Sandwich hybridizations may be carried out using two adjacent, nonoverlapping probes.<sup>104,105</sup>

If the target is DNA that has been separated by gel electrophoresis prior to transfer (blotting) to the solid support, the reaction is called Southern hybridization.<sup>3</sup> Southern hybridization may be carried out with large DNAs separated by pulsed field gel electrophoresis using several types of apparatuses.<sup>106-110</sup> Southern hybridization may also be carried out with small DNA fragments that are obtained from sequencing gels, allowing multiplex,<sup>111</sup> as well as direct<sup>112</sup> sequencing of complex DNAs. If the target is RNA that has been separated by gel electrophoresis, the reaction is called Northern hybridization.

The fundamental aspects of hybridization on solid supports have not changed appreciably since the topic was the subject of a review in 1984<sup>113</sup> and a chapter in a comprehensive book on hybridization published in 1985,<sup>84</sup> although additional practical details may be found in recently updated cloning manuals.<sup>114,115</sup> All of the results of variables affecting melting temperatures, detailed above for solution hybridization, are applicable for hybridization on solid supports. The effects of temperature, ionic strength, denaturing solvents, and solvent viscosity on  $k_N'$ , and the lack of effect of base composition or limited mismatching on  $k_N'$ , are the same in solution and on solid supports.

Equation 3 needs to be reconsidered. The effect of complexity on  $k_2$  is the same for hybridization in solution and on solid supports, but the effect of length is more complicated. When the probe is an oligonucleotide, Equation 3 is found to be valid. The same rates of hybridization are also found for 50-nucleotide probes simultaneously hybridizing with 150-nucleotide DNA in solution and tethered once to latex beads.<sup>116</sup> The newly described method for introduction of a residue during phosphoramidite synthesis, which may be used later with a variety of tethers,<sup>117</sup> should permit a more thorough study of surface effects on hybridization with bound molecules.

More is known about filter hybridization. In this case, the target nucleic acids are tethered to the solid support at many sites. Each short domain between tethers acts as an independent target. Thus, as the length of a polynucleotide RNA probe is increased, the rate of hybridization to immobilized DNA is unchanged following RNase digestion and washing of the hybrids on the membranes.<sup>118</sup> At  $C_o = 2.75 \mu M$ ,  $t_{1/2} = 4800$  s for  $\phi X174$  ( $N = 5386$ ) RNA-DNA hybridization in high salt, 50% formamide. According to Equation 4d,  $k_2 = 53$ . According to Equation 3,  $k_N' \cdot \sqrt{L_s} = 5380 \cdot 53 = 2.9 \cdot 10^5 M^{-1} s^{-1}$ , which is 2 to 4 times the value of  $k_N'$  for RNA-DNA solution hybridization previously reported in this solvent. Apparently the first approximation to calculation of predicted  $k_2$  values is to use Equation 3 with  $L_s$  set equal to 10, no matter what the actual length of the probe.

Equations 4a through 4d also need to be reconsidered. For those extremely rare occasions where the probe is at trace concentrations compared with the complementary target sequences on the membrane, Equations 4c and 4d are applicable for both single- and double-stranded probes reacting with both single- and double-stranded target DNA or RNA, because the target DNA or RNA cannot hybridize with itself. The complexity and concentration of the target still govern the reaction, although this situation may be complicated if the probe is so dilute and the target is so concentrated that hybridization depletes the probe in the vicinity of the membrane. Then the reaction becomes dependent upon diffusion of the probe to the target.

The more common situation is excess probe. If the probe is single-stranded, Equations 4c and 4d again hold, but the complexity and concentration of the *probe* govern the reaction. If the probe is double-stranded, the problem of competing reactions occurs. The probe should not be longer than the effective length of the target strands, or the rate of probe-probe hybridization will exceed the rate of probe-target hybridization, and the kinetics will become much more complicated. A length mismatch does not affect the initial rate of hybridization, but becomes important at later times. Because such a length mis-

match is the norm with polynucleotide probes, studies of the time course of filter hybridization are difficult to interpret.

#### b. *In situ* Hybridization

Hybridization rates have been obtained for reaction of  $^{125}\text{I}$ -labeled 5S rRNA (120 base pairs) with DNA on nitrocellulose filters and, *in situ*, with *Drosophila melanogaster* DNA in polytene chromosomes using  $0.4M$   $\text{Na}^+$  and 50% formamide.<sup>119</sup> The use of formamide with a RNA *in situ* hybridization probe has been especially useful because RNA-DNA hybrid formation may be favored over DNA reassociation.<sup>120</sup> The filter hybridization result was  $k'_N \sqrt{L_s} = 1.8 \cdot 10^5 M^{-1} s^{-1}$ , consistent with  $2.9 \cdot 10^5 M^{-1} s^{-1}$  in  $1.0M$   $\text{Na}^+$  reported above. The *in situ* hybridization rate was approximately fivefold lower. In neither case was the sample agitated to promote mixing. Because similar rates had previously been found for rRNA hybridization *in situ* to diploid cells compared with filters, the reduced rate for *in situ* hybridization to *Drosophila* polytene chromosomes was attributed to a diffusion limited reaction due to a high local concentration of target DNA.

More recently, fluorescence *in situ* hybridization<sup>121,122</sup> has nearly replaced *in situ* hybridization using radioactive probes. By using suppression hybridization<sup>123</sup> to inhibit probe hybridization to repeated sequences, a method easier than removal of probe repeated sequences by subtractive hybridization,<sup>124</sup> DNA from a cosmid clone has been used directly as a probe.

### 4. Acceleration of Hybridization

#### a. Inert Polymers

One of the methods for increasing hybridization rates is to exclude much of the volume of the solution with inert polymers so that the apparent concentration of hybridizing strands increases.<sup>87</sup> Dextran sulfate allows  $k_2$  to be increased 10- or even 100-fold.<sup>125,126</sup> This acceleration also works for hybridization on solid supports.

Polyethylene glycol will accelerate hybridization sufficiently in agarose gels to obtain hybridization of single-copy mammalian DNA.<sup>98</sup> Using such a solvent where the increased  $k_2 = 0.02$  for single copy mammalian DNA and the initial DNA concentration is  $1 \mu\text{g}/\mu\text{l}$  ( $0.003M$  nucleotides), Equation 4b predicts the observed<sup>98</sup> half-time of 9.25 h.

#### b. PERT

Single-stranded DNA strands preferentially occupy the interface between phenol and water, leading to substantial increases in hybridization rates in phenol-water emulsions. The phenol emulsion reassociation technique (PERT)<sup>18</sup> is especially useful for dilute DNA solutions because the hybridizing strands are concentrated on the emulsion surface. At these low DNA concentrations ( $<10 \mu\text{g}/\text{ml}$ ), the reaction order is  $\leq 2$ , and there is no effect of length on the apparent  $k_2$ ,<sup>18,127</sup> just as was seen for hybridization on solid supports.

At high DNA concentrations ( $>100 \mu\text{g}/\text{ml}$ ) where the surfaces become saturated, the order of the reaction decreases by one, with the rate of hybrid formation becoming almost independent of DNA concentration. The apparent  $k_2$  decreases with increasing DNA concentration.<sup>18,127</sup> Furthermore, the apparent  $k_2$  actually decreases with increasing length, apparently because the longer DNA molecules saturate the emulsion surface at lower nucleotide concentrations than the shorter DNAs.

Using the original method, shaking of the phenol-water emulsions shears single-stranded DNA. A method using both phenol and formamide apparently reduces this cleavage.<sup>128</sup>

#### c. Proteins

Hybridization has been catalyzed by inclusion of certain proteins in the hybridization mixture. DNA hybridization kinetics in a low salt,  $\text{Mg}^{++}$ -containing buffer at  $37^\circ\text{C}$  in the presence of bacteriophage T4 gene 32 protein followed the standard form of Equations 3, 4a and 4b with a  $k'_N$  comparable to that seen in  $1M$   $\text{Na}^+$  at  $70^\circ\text{C}$ .<sup>129</sup>



A similar result was observed with *Escherichia coli* single strand binding protein (SSB) at low pH or in the presence of polyamines.<sup>130</sup> Another highly studied protein found to increase hybridization rates by a different mechanism was RecA protein.<sup>131-135</sup> A similar activity was detected in yeast.<sup>136</sup> Helicases with or without RecA protein may use native DNA as one of the reactants.<sup>137,138</sup> In higher eukaryotes, RNA-binding proteins with possible structural homology to T4 gene 32 protein<sup>139</sup> or proteins having helicase activities<sup>140</sup> have been shown to accelerate hybridization. Ribonuclear protein A1 has been found to accelerate DNA or RNA-DNA hybridization<sup>141,142</sup> by a mechanism that increases the local concentration of complementary strands such as at  $k'_N$  is 300-fold greater than seen in 1M Na<sup>+</sup> at 70°C.<sup>141</sup>

## C. Dissociation

### 1. The Dissociation Temperature, $T_d$

When discussing the denaturation temperature for PCR, we noted that the important temperature is the strand separation temperature (see Figure 3a) and not the melting temperature of the duplex (see Figure 1a). With oligonucleotides, bimolecular structures with both helical and random-coil regions are rare, and the reaction may be assumed to be an all-or-nothing conversion between helix and coil, with equal concentrations of helical and coil nucleotides at  $T_m$ . Thus, the reverse reaction to the hybridization reaction is another strand separation reaction.

$T_d$  is the corresponding temperature of importance for screening plasmid or bacteriophage recombinant DNA libraries using oligodeoxynucleotide probes. When oligonucleotides are hybridized to complementary sequences attached to solid supports, and subsequently washed to eliminate unhybridized oligonucleotide, both  $T_m$  and the rate of rehybridization of released oligonucleotides drop as C drops, until  $T_m$  becomes irrelevant and the specificity of hybridization to complementary targets depends entirely on the kinetics of release.<sup>1</sup> This reaction is illustrated in Figure 3b.  $T_d$  may be defined as the temperature at which 50% of correctly matched hybrid is released in a specified length of time ( $t_{wash}$ ). Be-

cause  $T_d$  depends on time and not concentration, unlike  $t_{1/2}$  for hybridization and  $T_m$ ,  $T_d$  is the same whether using unique or mixed probes.<sup>99</sup> At  $T_m$ , the half-time for the forward (hybridization) and reverse (release) reactions is the same. For average composition oligonucleotides in 1.0 Na<sup>+</sup> at C = 1  $\mu$ M:

1.  $t_m = 58^\circ\text{C}$  and  $70^\circ\text{C}$  for L = 14 and 20, respectively.
2.  $t_{1/2}$  (at  $t_m$ ) = 0.53 and 0.44 s for L = 14 and 20, respectively.

As presented above,  $k_2$  is only weakly dependent upon temperature, while melting curves are narrow, indicating a large temperature coefficient for the reverse reaction. An appropriate estimate for the activation energy for hybridization would be 4 kcal/mol, approximately that which could be accounted for by a diffusion-controlled rate determining step depending on T/microscopic solvent viscosity. The activation energy of the reverse reaction ( $E_a$ ) would be 4.0 kcal/mol plus  $-\Delta H^\circ$ , the total enthalpy for the coil to helix transition. For example, for an average 14-mer with dangling end contribution,  $E_a = 4.0 + 13 \cdot 8.0 + 2 \cdot 8.0 = 124$  kcal/mol. The value for a 20-mer would be 172 kcal/mol. Given  $t_{wash}$ ,  $T_d$  may be calculated using Equation 7.

$$\ln \frac{t_{wash}}{t_{1/2}} = \frac{E_a}{R} \left\{ \frac{1}{T_d} - \frac{1}{T_m} \right\} \quad (7)$$

### Example 7

Wash the filters for 3 h (10,800 s). L = 20.

$$\begin{aligned} \text{Term} &= \ln \frac{t_{wash}}{t_{1/2}} \\ &= \ln(10800/0.44) = 10.1 \\ t_d &= T_m / \{1 + (R \cdot T_m \cdot \text{term} / E_a)\} \\ &= 273.2 \\ &= \{343.2 / (1 + 1.99 \\ &\quad \cdot 343.2 \cdot 10.1 / 172,000)\} \\ &= 273.2 \\ &= 57^\circ\text{C} \end{aligned}$$

Similarly, Equation 7 predicts  $t_d = 41^\circ\text{C}$  for  $L = 14$ . This theoretical calculation may underestimate experimental  $t_d$  if  $C$  of the oligonucleotide is so high during washing that rebinding occurs. When  $t_d$  is a kinetic measurement, increasing the washing time, but not the volume, will lead to decreased retention of radioactivity on a dot. If rebinding is important, increasing the volume at constant washing time will lead to decreased retention of radioactivity on a dot.

The theoretical result may be compared with the empirical calculation<sup>143</sup> of  $t_d$  in 1M NaCl: the sum of  $2^\circ\text{C}$  for each dA·dT base pair and  $4^\circ\text{C}$  for each dG·dC base pair, which predicts  $42^\circ\text{C}$  and  $60^\circ\text{C}$  for average 14-mer and 20-mer, respectively. Theoretical and empirical  $t_d$  values agree remarkably well. The theoretical calculation has greater generality than the empirical calculation because it takes into account not only the overall % G + C but the actual nearest neighbor pairs.

Tetramethylammonium salt solutions may be used to decrease the dependence of  $T_d$  on % G + C without significantly lowering  $T_m$ .<sup>144</sup>

Interestingly, the major effect of changing the ionic strength is on the hybridization rate constant.<sup>38</sup> For an extreme example,  $A_N\text{GCU}_N$  duplex dissociation rates are the same at any temperature in both 0.05M NaCl and 1.0M NaCl for both  $N = 2$  and  $N = 4$ .<sup>145</sup> Thus, the  $T_d$  values calculated in 1.0M  $\text{Na}^+$  may be good approximations to  $T_d$  values in lower salt washes as well.

## 2. Sequencing by Hybridization

Sequencing by oligonucleotide hybridization has been suggested and partially explored as a potential but unproved method for rapid acquisition of sequence data.<sup>146,147</sup> Although the details are changing rapidly, the method, as currently being developed, uses an array of all possible 8-mer oligonucleotides, simultaneous detection of sequence-specific hybridization of fluorescently labeled DNA from a clone, and error-tolerant computer analysis of the data to reassemble the sequence from the overlapping data. The array can be constructed by embedding beads covalently linked to one of the oligonucleotides in a gel, or by covalently linking the oligonucleotides to sites in a gel or to another solid support.

The hybrids are detected using a fluorescence microscope and video camera. Whether  $T_m$  or  $T_d$  governs the results depends on the conditions for hybridization and washing of the arrays. The problem of homopolymeric runs within sequences would need to be addressed by another method.

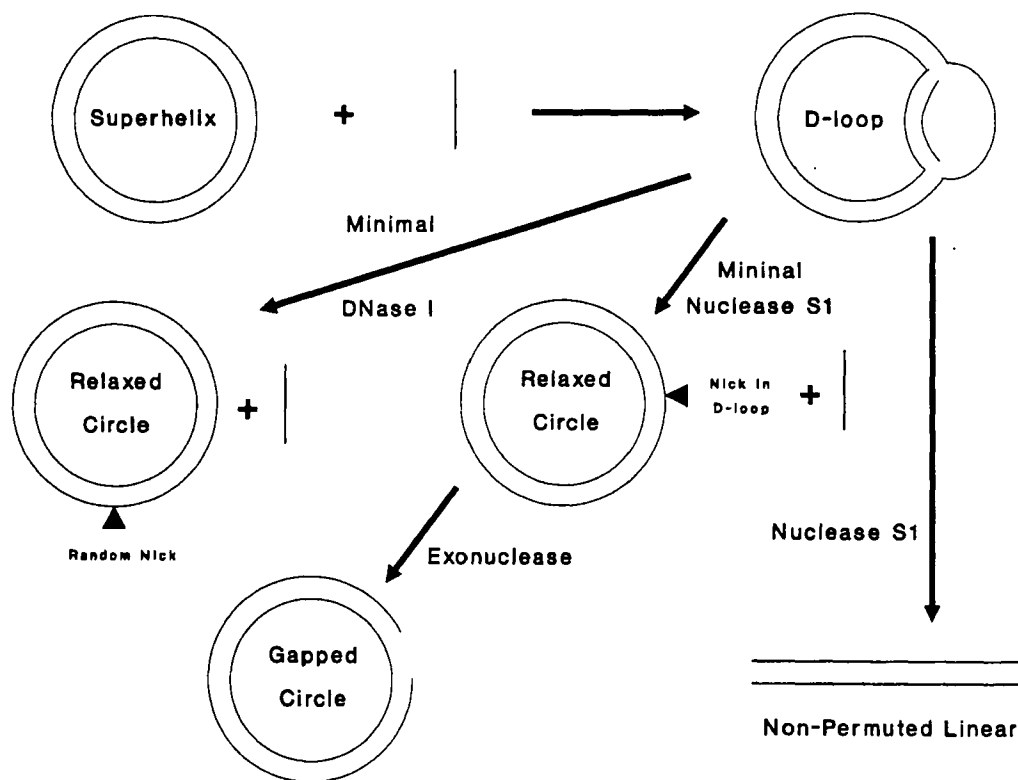
## D. Branch Migration

### 1. D-Loops and R-Loops

Figure 4a shows the formation of a D-loop, where single-stranded DNA, a *displacer*, is taken up by a superhelical DNA, a *recipient*, by an entropically driven process using the free energy stored in the negative supercoils.<sup>148</sup> The reaction may be initiated when a target region on the recipient duplex is transiently denatured, as at temperatures slightly below  $T_m^\infty$ . Base pair formation with the displacer DNA takes place at the expense of existing base pairs in the recipient DNA, a process known as single-strand branch migration. D-loop formation is thought to be one step in generalized recombination.<sup>149</sup> The analogous process where the single-stranded nucleic acid is RNA is called R-loop formation.<sup>17</sup> As illustrated in Figure 4c, R-loop formation differs from D-loop formation in that R-loops may be formed with linear duplex recipient DNA in solvents, including those with high concentrations of formamide, where  $T_m^\infty$  for RNA-DNA hybrids is greater than that for DNA duplexes. Again, the reaction may be initiated when a target region on the recipient duplex is transiently denatured, as at temperatures near  $T_m^\infty$ , but below the strand separation temperature. Similarly, as described below in Section II.D.3, substitution of MedC or BrdC in displacer DNA favors D-loop formation with linear duplex DNA.<sup>5,48,150</sup>

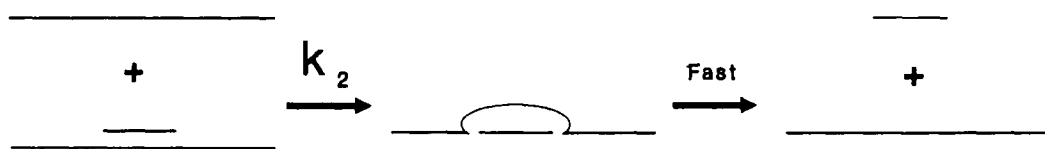
As illustrated in Figure 4a, D-loops in superhelical DNA may be the target for single strand-specific endonucleases, such as nuclease S1, which nick the displaced recipient DNA strand, relaxing the circular molecule. By a reverse branch migration process, the displacer strands are eliminated. Subsequent treatment with nuclease S1 leads to linear molecules with deleted base pairs.<sup>151</sup> Alternatively, subsequent treatment

## a. D-loop Formation and Disruption

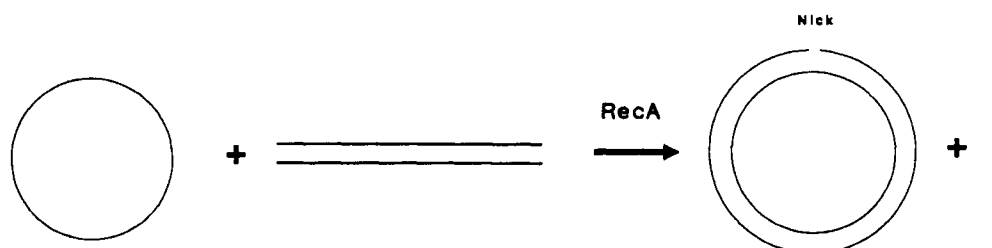


## b. DNA Displacement Reactions

### i. Hybridization



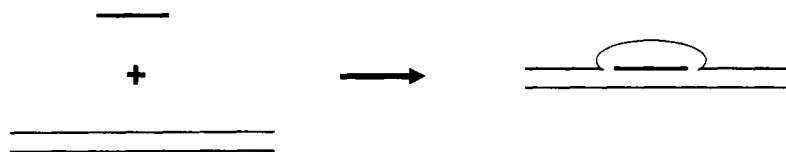
### ii. RecA Protein-Catalyzed Displacement



**FIGURE 4.** Nucleic acid displacement reactions.

### c. Displacement to Stronger Base Pairs

#### i. R-loops



#### ii. MedC- or BrdC-Modified Displacer Strands

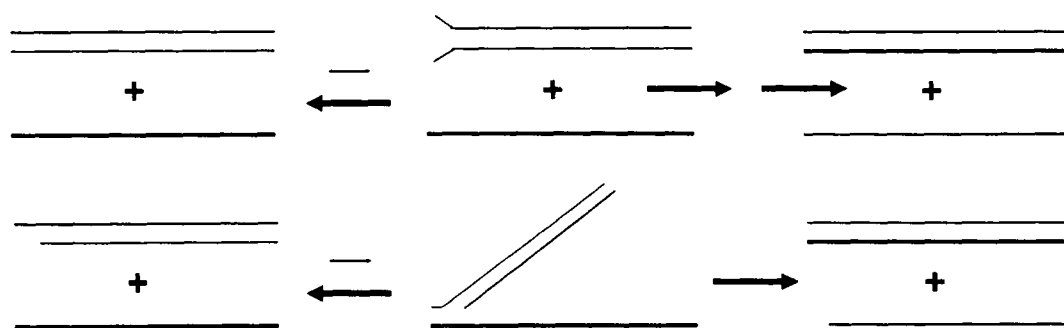


FIGURE 4C

with an exonuclease and a single-strand-specific reagent leads to a gapped circle with modified base pairs.<sup>152</sup> These reactions allow creation of site-specific deletion<sup>151</sup> or point mutations.<sup>152</sup> A more recent version of this experiment uses a displacer oligodeoxynucleotide covalently bonded to a nuclease.<sup>153</sup> Transient formation of D-loops is sufficient to lead to specific cleavage of the recipient DNA. Since the displacer and the nuclease are one molecule, this reagent is an artificial restriction enzyme.

Also as illustrated in Figure 4b, a transient D-loop may be formed when a long single strand reassociates with a long complementary single-stranded DNA containing a short, internal duplex region.<sup>154</sup> This type of reaction would be expected to occur frequently during the latter stages of hybridization with randomly cleaved DNAs. A transient D-loop also may be formed in a relaxed circular molecule by nicking a D-loop in a superhelical molecule with an endonuclease such as DNase I.<sup>155</sup> Without the contribution of superhelix free energy, and because of the greater entropy of linear molecules vs. loops, a D-loop

is unstable to dissociation. Transient D-loops have been used to study the rate of single strand branch migration. Branch migration rates are very fast unless the temperature is so low that the loop has substantial intramolecular base-pairing.<sup>155</sup> The branch migration part of the displacement reaction in the reassociation experiment is governed by the hybridization rate constant  $k_2$ .<sup>154</sup>

Displacement reactions have been studied for BrdC- or MedC-substituted oligodeoxynucleotide displacers reacting both with recipient oligonucleotide duplexes and recipient oligonucleotide partial duplexes with 4-nucleotide overhangs<sup>48,150</sup> analogous to the overhang resulting from cleavage with many restriction endonucleases, as illustrated in Figure 4c. With duplex recipients, the displacement rate increases with increasing temperature, consistent with a mechanism of displacement initiation at "frayed" or "breathing" ends. With the partial duplex recipients, the displacement rate decreases with increasing temperature, consistent with a mechanism involving nucleation at the 4-base overhang followed by branch migration. The rate of branch

migration is so fast, however, that the rate constant calculated using Equation 3, reduced by 4/L due to the limited nucleation site, correctly predicted the overall reaction rate.<sup>48,150</sup>

The rapidity of branch migration has led to the development of probes where the label is detected on the displaced strand of the recipient partial duplex.<sup>156-158</sup> Detection may depend upon separation of displaced probe and unreacted partial duplex<sup>156</sup> or upon the single-stranded nature of the displaced probe.<sup>157</sup> Under conditions where secondary structure of the displacement strand is significant, RecA protein may be used to catalyze displacement reactions.<sup>158</sup>

## 2. RecA Protein

RecA protein, which is central to generalized recombination in *Escherichia coli*,<sup>159</sup> may be used to promote D-loop formation with both superhelical and linear recipient duplexes, as illustrated in Figure 4b. RecA protein hydrolyzes ATP during displacement, allowing not only the otherwise thermodynamically unfavorable reaction with linear recipients<sup>160</sup> but also the formation of heteroduplexes with mismatched base pairs,<sup>161</sup> presumed intermediates in generalized recombination. The search for complementary sequences in recipient duplex DNA by RecA-coated single-stranded DNA takes place at the rate of the order of 6 kb pairs per min.<sup>162</sup>

## 3. Branch Capture Reactions (BCR)

Branch capture reactions (BCR) are illustrated in Figure 5. A complex DNA is digested with a restriction endonuclease. A specific displacer is synthesized for reaction at the end of one specific recipient duplex. The extremely rapid displacement reaction discussed above at a 4-base overhang of a recipient duplex is reversible when the recipient duplex is longer than the displacer. In one version of BCR, the displacer oligodeoxynucleotide contains two contiguous sequences, a sequence complementary to the recipient duplex strand containing the overhang and a sequence complementary to and hybridized with a linker oligodeoxynucleotide. The linker strand

may be covalently bonded to the recipient overhang using T4 or Taq DNA ligase, thus capturing the branched complexes composed of recipient and displacer-linker duplexes.<sup>5,48,150</sup>

The specificity of BCR was tested by comparing the reaction of a single displacer, capable of forming 20 base pairs following a 4-base *Pst*I overhang, with three relevant *Pst*I termini from a single plasmid.<sup>163</sup> The three *Pst*I ends had no ( $m = 1$ ), partial ( $m = 5$ ), and complete ( $m = 20$ ) nucleotide homology with the displacer following the *Pst*I sites. The reactions were carried out with unmodified displacers and with displacers containing no substitutions<sup>48</sup> and with BrdC<sup>48</sup> and MedC<sup>5</sup> for dC.

Let  $B_k$  be the branch migration equilibrium constant for branch migration step  $k$ .  $B_k = 1$  if displacer base  $k$  is dA, dG or dT, resulting in no nucleotide substitution.  $B_k = 1.8$  if displacer base  $k$  is MedC or BrdC. At temperatures where the recipient ends are not saturated, the relative rate of ligation at a recipient site compared with ligation at an unrelated site, the specificity of the reaction(s) is correctly predicted by Equation 8.

$$S = \sum_{j=1}^m \prod_{k=1}^j B_k/B_1 \quad (8)$$

### Example 8

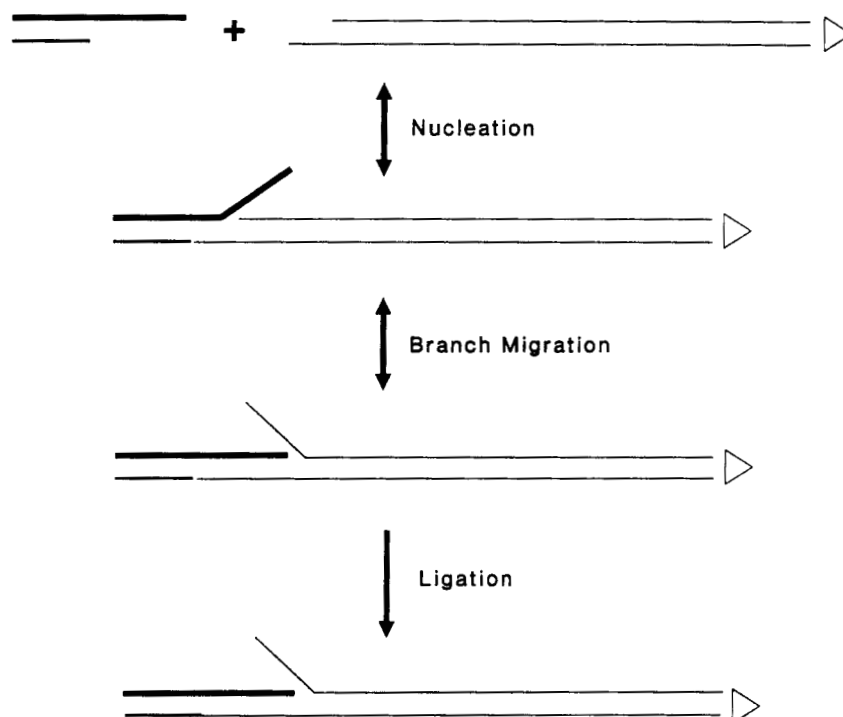
Displacer = NNNNNNNNNNSTBTCCA-  
ATGCCCCAGGAGCCCT.

CTGCAG is the *Pst*I site. Substituted bases are italicized.

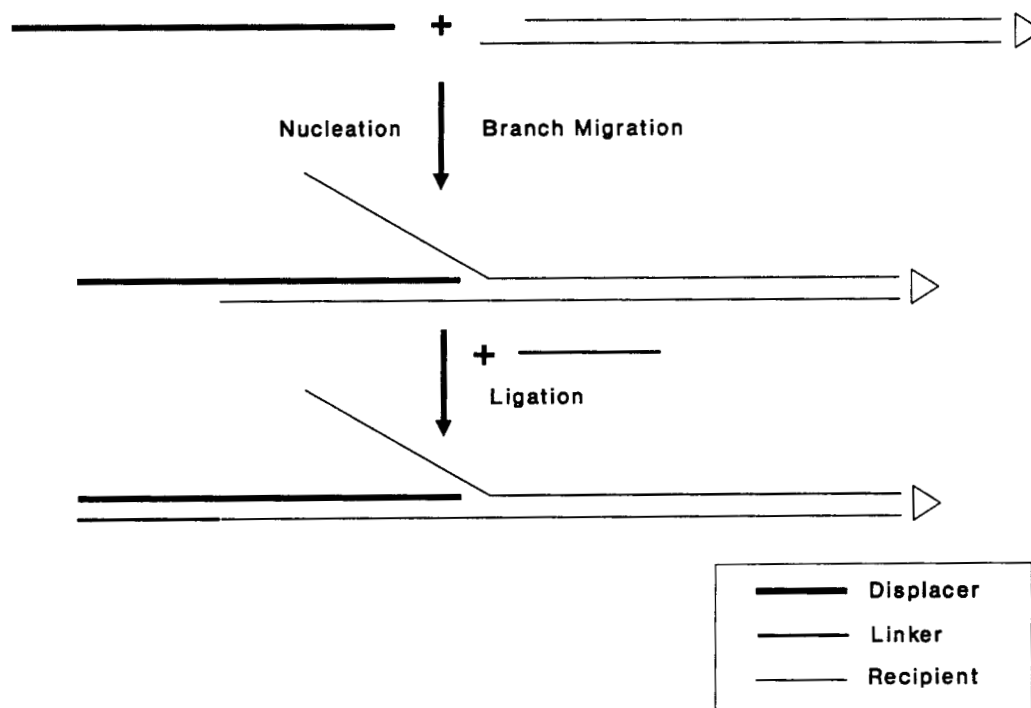
$$S = \{1.8 + 1.8^2 + 1.8^2 + 1.8^2 + 1.8^2 + 1.8^2 + 1.8^3 + 1.8^4 + 1.8^5 + 1.8^6 + 1.8^6 + 1.8^6 + 1.8^6 + 1.8^7 + 1.8^8 + 1.8^9 + 1.8^9\}/\{1.8\} = 458\text{-fold specificity}$$

Specificities of over 3000 were detected for displacers containing these substitutions, while the specificity with no substitutions was as predicted by Equation 8 with all  $B_k = 1$ . Similar reactions have been carried out at 4-base overhangs of different nucleotide composition.<sup>5,150</sup> BCR rates were always proportional to ligase concentration.

### a. Oligonucleotide Displacer-Linker Duplex



### b. Polynucleotide Displacer - Followed by Linker



**FIGURE 5.** Nucleic acid branch capture reactions.



A mismatch will lower  $B_k$  at any site to  $<10^{-2}$ . Results of the study of a construct with a single base mismatch demonstrate no contribution from sequences distal to the mismatch. The ability of a single mismatch to block branch migration means that BCR is much more stringent than hybridization, and even more sensitive to mismatched bases than  $T_m$  or  $T_m^\infty$ .

In another version of BCR, the MedC- or BrdC-substituted displacer oligodeoxynucleotide was so long that its reaction with the recipient duplex was effectively irreversible.<sup>5</sup> The displacers were prepared using asymmetric PCR in solvents containing ethylene glycol or glycerol to lower  $T_m^\infty$  while preserving or stabilizing the Taq polymerase. This reaction was comparable to R-loop formation. Linker attachment was carried out separately. The potential specificity of this system was even greater than for BCR with shorter oligonucleotides.

It is important to note that modifications of the nucleotides that lower  $T_m$  of the displacer-linker duplex are not detrimental as long as BCR is carried out below  $T_m$ . Practical applications of BCR include using a fluorescent linker, a biotinylated linker or a linker-displacer terminus with a different overhang sequence. BCR may thus be used for sequence-specific labeling of one DNA fragment in a population of DNA fragments for subsequent detection, affinity chromatography, or cloning, respectively.

### III. LABELING AND DETECTION

In the 1984 review on hybridization of nucleic acids immobilized on solid supports,<sup>113</sup> all of the probe labels discussed in detail were radioactive. The development of nonradioactive probes was in its infancy. A review 4 years later listed a dozen direct and a dozen indirect labels for nonradioactive detection.<sup>164</sup>

#### A. Radioactive Labels

Radioactive labels are direct labels, where the radioactivity incorporated into the probe molecules may be directly detected. Although non-radioactive labels may be preferable for reasons

of safety and convenience, sensitive detection of radioactive labels is still the "gold standard" of probe technology.<sup>165</sup> Phosphodiester linkages may contain  $^{32}\text{P}$  or  $^{35}\text{S}$ ; the nucleoside may contain  $^3\text{H}$  or  $^{14}\text{C}$ , and dU or dC may be modified to contain  $^{125}\text{I}$ . Details of the preparation and use of radioactive probes are available in recently updated cloning manuals.<sup>114,115</sup>

The choice of labeling method is dictated by the nature of the probe. Uniform labeling of double-stranded DNA probes may be accomplished by nick translation,<sup>166</sup> random priming,<sup>167,168</sup> and by PCR<sup>4</sup> with one or more radioactive deoxynucleotide triphosphates. Single-stranded DNA probes may be prepared by primer extension from a single-stranded DNA template (e.g., M13), by run-off polymerization, or asymmetric PCR using Taq polymerase.<sup>169</sup> Uniformly labeled RNA probes may be prepared by *in vitro* transcription, especially from bacteriophage promoters such as SP6<sup>170</sup> or T7.<sup>171</sup> The 3' end(s) of double-stranded DNA may be labeled by replacement synthesis using T4 DNA polymerase.<sup>114</sup> The 3' end(s) of oligodeoxynucleotides and double-stranded DNA also may be labeled using terminal transferase by addition of one<sup>172</sup> or more radioactive nucleotides. The 5' hydroxyl on an oligodeoxynucleotide, or on a DNA after treatment with alkaline phosphatase, may be phosphorylated using  $\gamma$ - $^{32}\text{P}$ -labeled ATP and T4 polynucleotide kinase.<sup>173</sup> The simultaneous application of probes labeled with different radioisotopes is unusual, but has recently been used to compare two-dimensional restriction enzyme fingerprint patterns of DNA from two strains of *Escherichia coli*.<sup>174</sup>

#### B. Biotin Labels

Biotin labels are the most commonly studied indirect labels, where the biotin incorporated directly into the probe molecules must be detected following binding of the biotin to a signal-generating system.

Oligonucleotides may be labeled at any position by reacting a biotinylating reagent, such as *N*-biotinyl-6-aminocaproic acid *N*-hydroxysuccinimide ester,<sup>175</sup> with an aliphatic amino group in the oligonucleotide, such as an allylamino-dU,<sup>175</sup> alkyne-containing alkylamino-dU,<sup>176</sup>



or alkylamino-dC<sup>177</sup> residue incorporated as a protected phosphoramidite. Protected amino-containing moieties (e.g., lysine) may be synthesized on a support, prior to addition of the first nucleotide, with subsequent phosphoramidite synthesis yielding a 3'-polyamide-oligonucleotide composite probe.<sup>178</sup> A 3'-ribonucleoside may be modified to incorporate an aliphatic amino group.<sup>179</sup> Aminoalkyl-phosphoramidites may be incorporated at the 5'-end.<sup>179,180</sup> These aliphatic amino-containing oligonucleotides may be biotinylated using the same reagents. The same aliphatic amino-containing intermediates may be used with different activated compounds for incorporation of fluorescent or other labels or for attachment of proteins.

As with radiolabeled probes, DNA may be uniformly labeled with biotin by incorporation of biotinylated nucleotides,<sup>181</sup> such as dU and dA linked to biotin by linker arms. Biotinylated deoxynucleoside triphosphates are good substrates for *Escherichia coli* DNA polymerase I<sup>181</sup> and Taq polymerases.<sup>182</sup> Nick translation, random priming, or PCR may be used to produce double-stranded probes, and runoff polymerization, asymmetric PCR, or primer extension may be used to produce single-stranded probes. Similarly, uniformly labeled RNA probes may be prepared by *in vitro* transcription because biotinylated nucleoside triphosphates are good substrates for *E. coli* and T7 RNA polymerases.<sup>181</sup> Preformed double-stranded DNA also may be uniformly labeled with biotin using reagents consisting of biotin linked to a photochemically activated DNA-binding moiety such as a psoralen<sup>183</sup> or isopsoralen.<sup>184</sup> The 3'-end(s) of double-stranded DNA may be labeled by filling in with *E. coli* DNA polymerase I (Klenow fragment)<sup>181,185</sup> or replacement synthesis using T4 DNA polymerase.<sup>181</sup> The 3' end(s) of oligodeoxynucleotides and double-stranded DNA also may be labeled using terminal transferase.<sup>186</sup> Although not demonstrated with biotin, the 3' end could be labeled by modified dideoxy chain terminators.<sup>187</sup>  $T_m$  or  $T_m^\infty$  of biotinylated probes is slightly reduced, but the labeling does not affect  $k_2$ .

Biotin detection usually involves secondary labeling systems with avidin or streptavidin, which binds very tightly to biotin, and a covalently

linked reporter group, such as an enzyme, fluorescent moiety, or luminescent moiety. Other detection systems use antibodies instead of avidin or streptavidin. Because in some probe formats the same reporter groups may be directly attached to a probe, reporter groups are considered separately below.

Because the binding of biotin by avidin or streptavidin is so tight, biotin-labeled probes may facilitate isolation of hybrid molecules by affinity chromatography.<sup>188</sup> RecA-coated biotinylated probes have been used for D-loop formation with recipients present in a cDNA library, permitting specific cDNA enrichment by affinity chromatography and subsequent cloning of an enriched cDNA library.<sup>189</sup> Using hybridization without strand displacement, a 350-fold enrichment has been achieved using a biotinylated RNA probe to hybridize with large recipient DNA molecules containing extended single-stranded termini resulting from exonuclease digestion. The hybrids were enriched by affinity chromatography, and the large DNA molecules were released by treatment with RNase.<sup>190</sup> In addition to chromatography on an avidin- or streptavidin-coated matrix, separations using streptavidin-coated magnetic beads have allowed direct cloning of a gene,<sup>191</sup> and even separation of chromosomes.<sup>192</sup>

Using one biotinylated PCR primer, one strand of a PCR product may be labeled and isolated for subsequent use as a probe or for sequencing.<sup>193</sup> Primer-extension DNA sequencing using blotting also may be carried out with biotinylated primers.<sup>194</sup> Chemical DNA sequencing may be carried out with biotin end-labeled restriction endonuclease fragments.<sup>195,196</sup> Potential detection methods following electrophoresis and blotting may be STBT<sup>194,195</sup> and chemiluminescence.<sup>196</sup>

### C. Other Indirect Labels

Modified bases potentially detectable by proteins other than antibodies<sup>164</sup> include the highly reactive mercuric-dU, an intermediate in the synthesis of biotin-dU,<sup>181</sup> which may be recognized by modified or unmodified glutathione or by sulfhydryl-containing chromatographic beads or resins. Glycosylated bases, as found in bacteri-

ophage T2 and T4 DNA, may be recognized by lectins, *Lac* operator DNA and poly(dT) or poly(dA) tracts may be recognized by *lac* repressor and poly(dA)- or poly(dT)-conjugated proteins, beads, or resins, respectively.

Antibody-based systems are more common. Specifically, hybridization-dependent detection may be achieved using anti-RNA-DNA hybrid antibody against biotinylated RNA-DNA hybrids captured on a solid support.<sup>197,198</sup> DNA-protein A conjugate probes, which nonspecifically bind antibody, also may be produced as probes.<sup>199</sup> More generally, the same methods used for incorporation of biotin into oligonucleotides and polynucleotides are used to produce probes with a wide variety of different modified nucleotides. By coupling the modified nucleotide to a protein carrier for immunization, antibodies are generated that recognize these modified nucleotides or haptens. In most formats, the reporter group is attached to a secondary anti-antibody, allowing the signal-generating system to be the same for a variety of probe modifications. Two such systems recognize probes labeled with DNP<sup>200</sup> and digoxigenin.<sup>201</sup> Using existing commercial systems based on DNP- or digoxigenin- or biotin-labeled probes, comparable results are achieved in fluorescent *in situ* hybridization.<sup>122</sup>

Phosphorothioate linkages, which may be incorporated into both oligonucleotides or DNA probes, are themselves chemically reactive and may be used for posthybridization fluorescent labeling of probe-target duplex products using monobromobimane.<sup>202,203</sup> All of these probes with base- or backbone-modified nucleotides have slightly reduced  $T_m$  or  $T_m^\infty$ , but again the label does not affect  $k_2$ .

#### D. Enzyme Labels and Colorimetric Reporter Groups

Enzymes that may be directly attached to probes include alkaline phosphatase<sup>204,205</sup> and horseradish peroxidase,<sup>204</sup> both with commercially available chromogenic substrates. Detection is reported at the level of one attomole.<sup>205</sup> Although detection requires blotting following electrophoresis, the sensitivity of chromogenic assays may be sufficient to substitute for radio-

active detection in DNA sequencing.<sup>194,195</sup> These same enzymes are commercially available as colorimetric reporter groups attached to avidin or streptavidin or attached to primary or secondary antibodies for the haptens described above. Horseradish peroxidase produces a color faster than alkaline phosphatase, but the sensitivity achievable with alkaline phosphate, and especially poly-alkaline phosphate,<sup>206</sup> is greater.

#### E. Fluorescent Labels and Fluorimetric Reporter Groups

NHS esters of fluorescein and rhodamine derivatives may be attached to aliphatic amine linker arms on oligonucleotides and polynucleotides, allowing these molecules to be used as direct probes. The ability to directly attach different fluorescent dyes to different probe molecules allows the simultaneous detection of hybridization and/or biosynthetic products involving more than one probe. Based on differential hybridization of mismatched and perfectly matched primers, a competitive PCR color complementation test is available to detect human mutations or polymorphisms.<sup>41</sup> Both automated fluorescent DNA sequencing without blotting<sup>187,207</sup> and restriction endonuclease fingerprinting without blotting<sup>208</sup> may be carried out using four different fluorophores per lane.

Many fluorescent dyes are commercially available as fluorimetric reporter groups attached to avidin or streptavidin or attached to primary or secondary antibodies for the haptens described above. Probes labeled with biotin, DNP, and digoxigenin may be simultaneously detected using secondary labeling with different fluorophores.<sup>122</sup> In an attempt to overcome background due to intrinsic fluorescence in a sample, delayed fluorescence may be employed with europium or terbium attached to avidin or other proteins through chelators.<sup>209,210</sup> The label also may be attached directly to chelator-containing nucleic acid.<sup>211</sup>

Homogeneous hybridization assays have been few and far between. One difficulty has been determining the difference between single-stranded and hybridized probes in solution. The first such assay was based on the difference in

fluorescence yield and polarization of a probe containing 1,*N*<sup>6</sup>-etheno-2'-deoxyadenosine residues.<sup>212</sup> The method was not very sensitive and, more importantly, would not operate in the more convenient format of excess probe, where the half-time for hybridization with complex systems can be reduced to practical times.

Potentially, fluorescence energy transfer may be a practical basis for a homogeneous hybridization assay. In one format, two probes binding to adjacent sequences on a target nucleic acid contained fluorescein and rhodamine, respectively.<sup>213</sup> When both probes are bound, fluorescein emission was quenched and rhodamine emission was enhanced. In another format, the donor molecule was intercalated into the hybrid duplex.<sup>213</sup> In a third format, one 5'-labeled fluorescent probe reacted with either target DNA or a complementary competitor probe with an attached 3'-quencher moiety.<sup>214</sup>

## F. Luminescent Labels and Luminimetric Reporter Groups

Direct attachment of chemiluminescent isoluminol<sup>177</sup> or acridinium<sup>215</sup> to probes has been accomplished using the standard chemistry for attachment of enzymes, biotin, or fluorescent labels. Chemiluminescence was produced by treatment with alkaline hydrogen peroxide. In both cases, the unenhanced yield was limited to one photon per reporter group and cannot produce the signal achieved by other methods.<sup>177</sup>

Horseradish peroxidase has been directly linked to probes for an assay using luminol, H<sub>2</sub>O<sub>2</sub>, and either *p*-iodophenol or hydroxycinnamic acid.<sup>216</sup> By using an enzyme, the sensitivity should be greater. Direct chemiluminescent substrates have been used for indirect detection of alkaline phosphatase,<sup>217-220</sup> with results superior to colorimetric substrates. Although indirect detection required blotting following electrophoresis, the sensitivity of the chemiluminescent assay was sufficiently increased to substitute for radioactivity in DNA sequencing.<sup>196</sup>

A homogeneous strand-displacement hybridization assay has been reported where the RNA displaced was degraded by ribonuclease and phosphorylated to the triphosphate. The hydro-

lysis of the substrate ATP by luciferase was detected by the resulting bioluminescence.<sup>157</sup>

In many applications, chemiluminescence or bioluminescence has derived from a coupled enzymatic assay for a reporter enzyme directly or indirectly attached to a probe. For example, a glucose-6-phosphate-dehydrogenase-containing reporter group may be detected in a coupled enzyme assay using FMN oxireductase and luciferase.<sup>221</sup> A more generally applicable system has been developed where NAD(P)H was produced by coupled systems utilizing alkaline phosphatase,  $\beta$ -galactosidase, or glucose-6-phosphate dehydrogenase. The bioluminescence resulted from the microperoxidase-catalyzed reaction of isoluminol and O<sub>2</sub><sup>-</sup>, and H<sub>2</sub>O<sub>2</sub> resulting from the reduction of O<sub>2</sub> in the presence of NAD(P)H and an electron mediator.<sup>222</sup> These coupled systems provided at least the same sensitivity as the best probe detection systems based on colorimetric or radioactive determinations. The choices for labeling probes and for detecting labels have expanded greatly over the past few years. Increasing competition between these systems should lead to increasing substitution of nonradioactive for radioactive methods in many laboratories over the next few years.

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