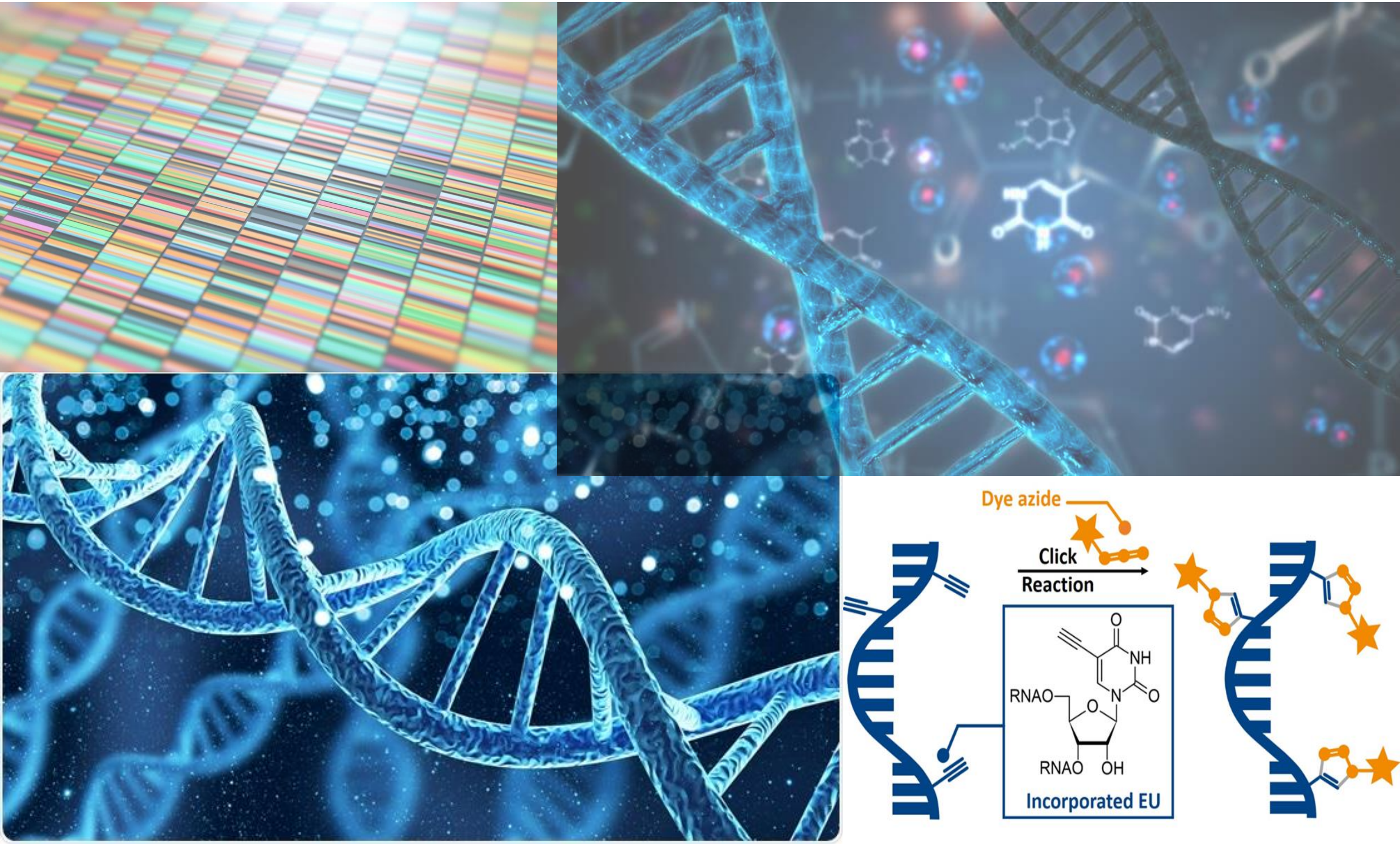
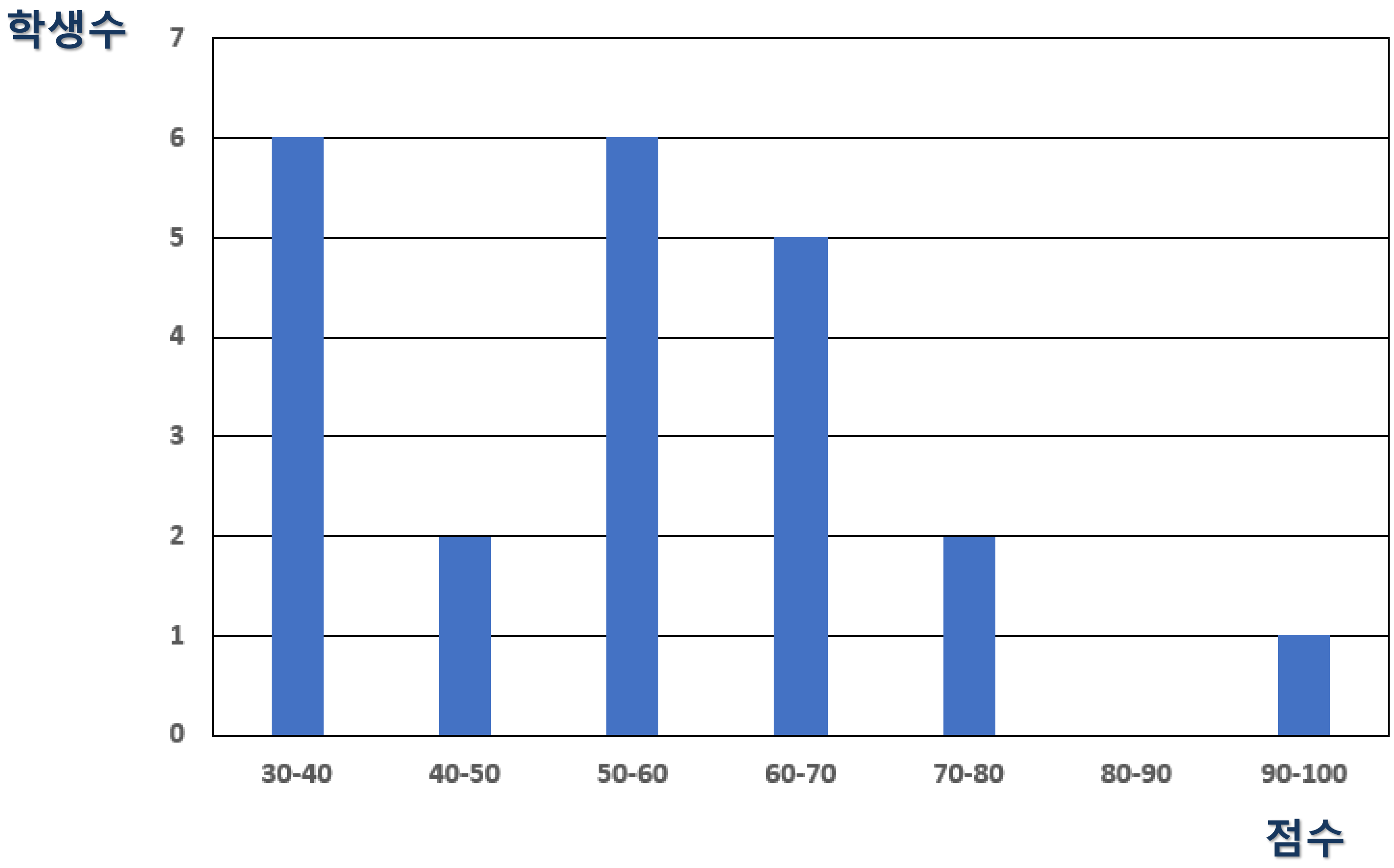
CHAPTER 7

Nucleic Acid Modification



중간고사 성적 분포



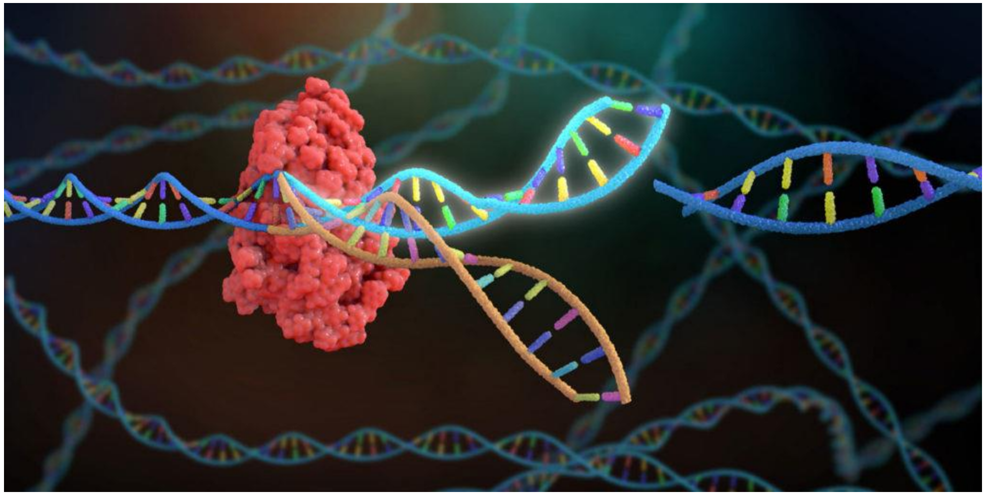
평균 54점
최고점 90점

Nucleic Acids

- The nucleic acid polymers DNA and RNA form the most basic units of information storage within cells
- The conversion of DNA′s unique information code into RNA and proteins is the fundamental step in controlling all cellular processes
- Despite the complexity of the genetic code, the base-pairing process that causes one oligonucleotide to bind to its complementary sequence is rather simple to predict and decipher
- Nucleic acids are the only type of complex biological molecule wherein their binding properties can be fully anticipated and incorporated into synthetic oligonucleotide probes

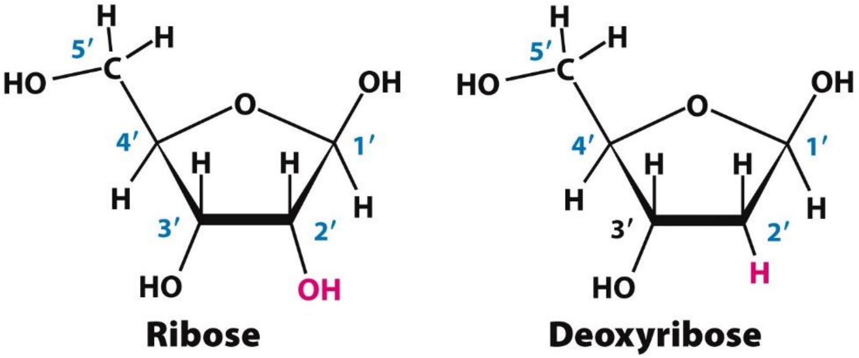
◆ Targeting and Probing Nucleic Acids

- Targeting specific sequences of DNA or RNA with labeled probes that are able to bind to specific genetic regions allows detection, localization, or quantification of discrete oligonucleotide sequences
- A short DNA segment can be synthetically designed and used to target and hybridize to a complementary DNA strand within much larger chromosomal material or extracted genomic DNA
- This targeting capability is made possible by the predictable nature of nucleic acid interactions

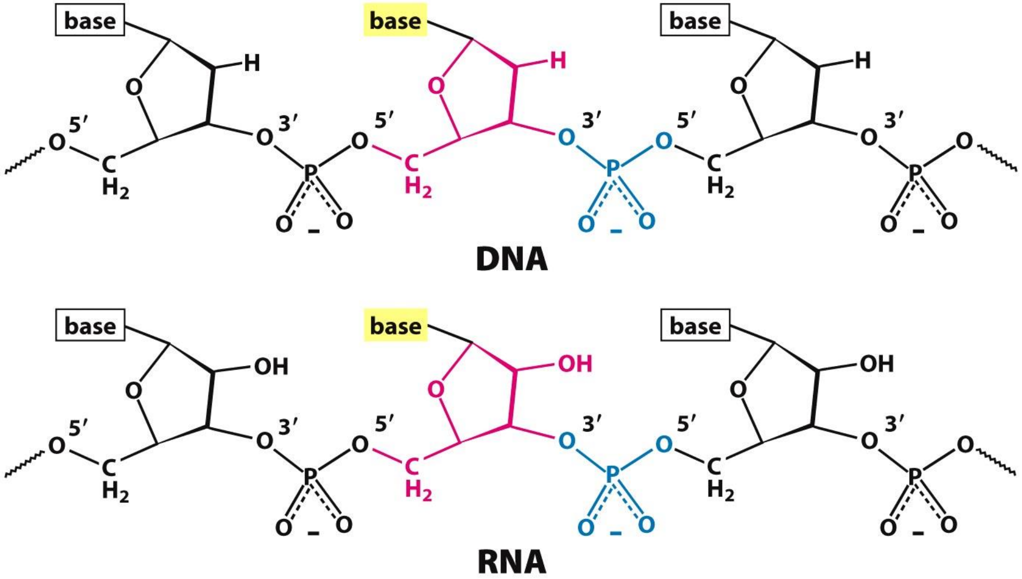


◆ Components of Nucleic Acids

- Sugar components



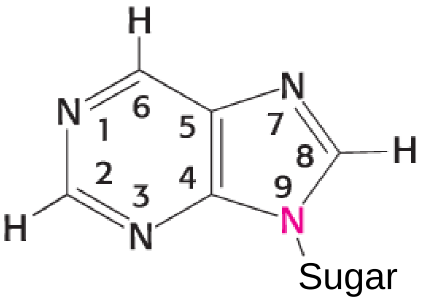
- Backbones



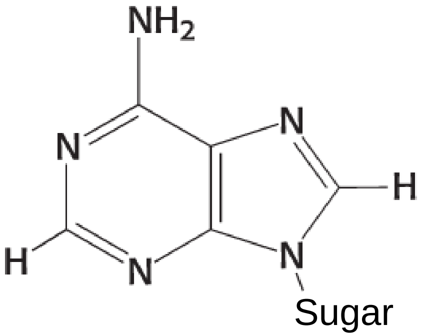
◆ Components of Nucleic Acids

- Nucleobases
  - Base-ribose linkage at N^9 and N^1

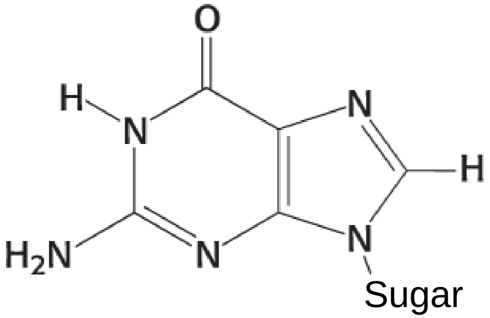
PURINES



Purine

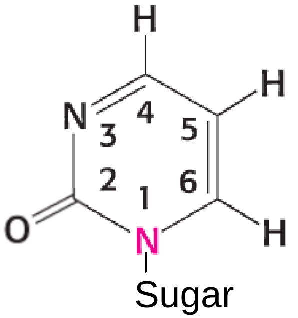


Adenine

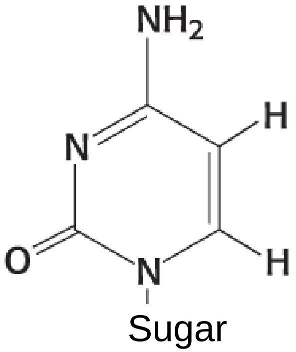


Guanine

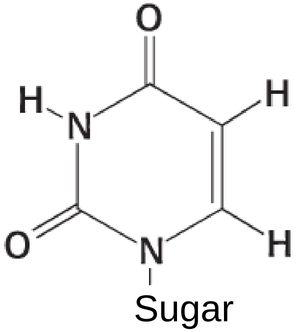
PYRIMIDINES



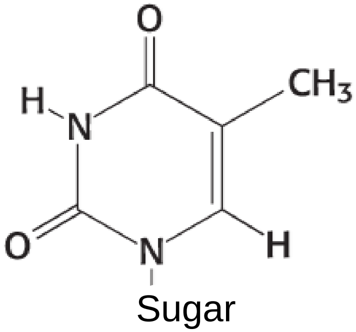
Pyrimidinone



Cytosine



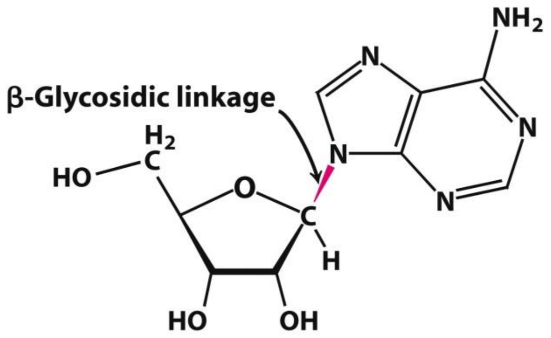
Uracil



Thymine

◆ Nucleic Acid nomenclatures

- A nucleoside contains only a base group and an attached sugar
- A nucleotide consists of a base and a sugar plus a phosphate group.



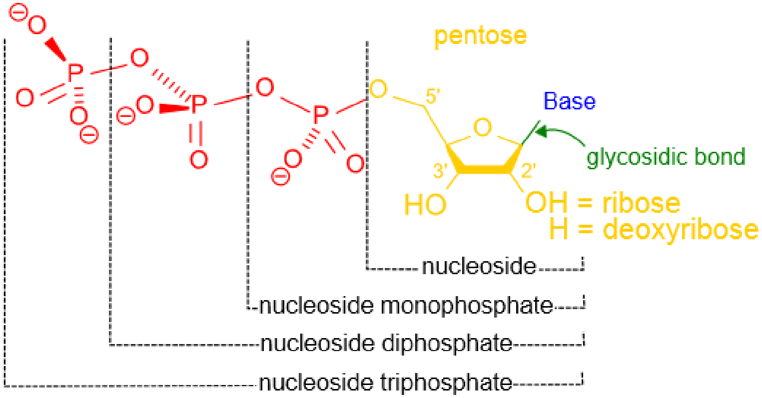


Table Nucleic acid nomenclatures

Base Name	Nucleoside Name[a] (Base + Sugar)	Nucleotide Name[b] (Base + Sugar+Phosphate)
Adenine	Adenosine	Adenosine monophosphate (AMP)
Guanine	Guanosine	Guanosine monophosphate (GMP)
Cytosine	Cytidine	Cytidine monophosphate (CMP)
Thymine	Thymidine	Thymidine monophosphate (TMP)
Uracil	Uridine	Uridine monophosphate (UMP)

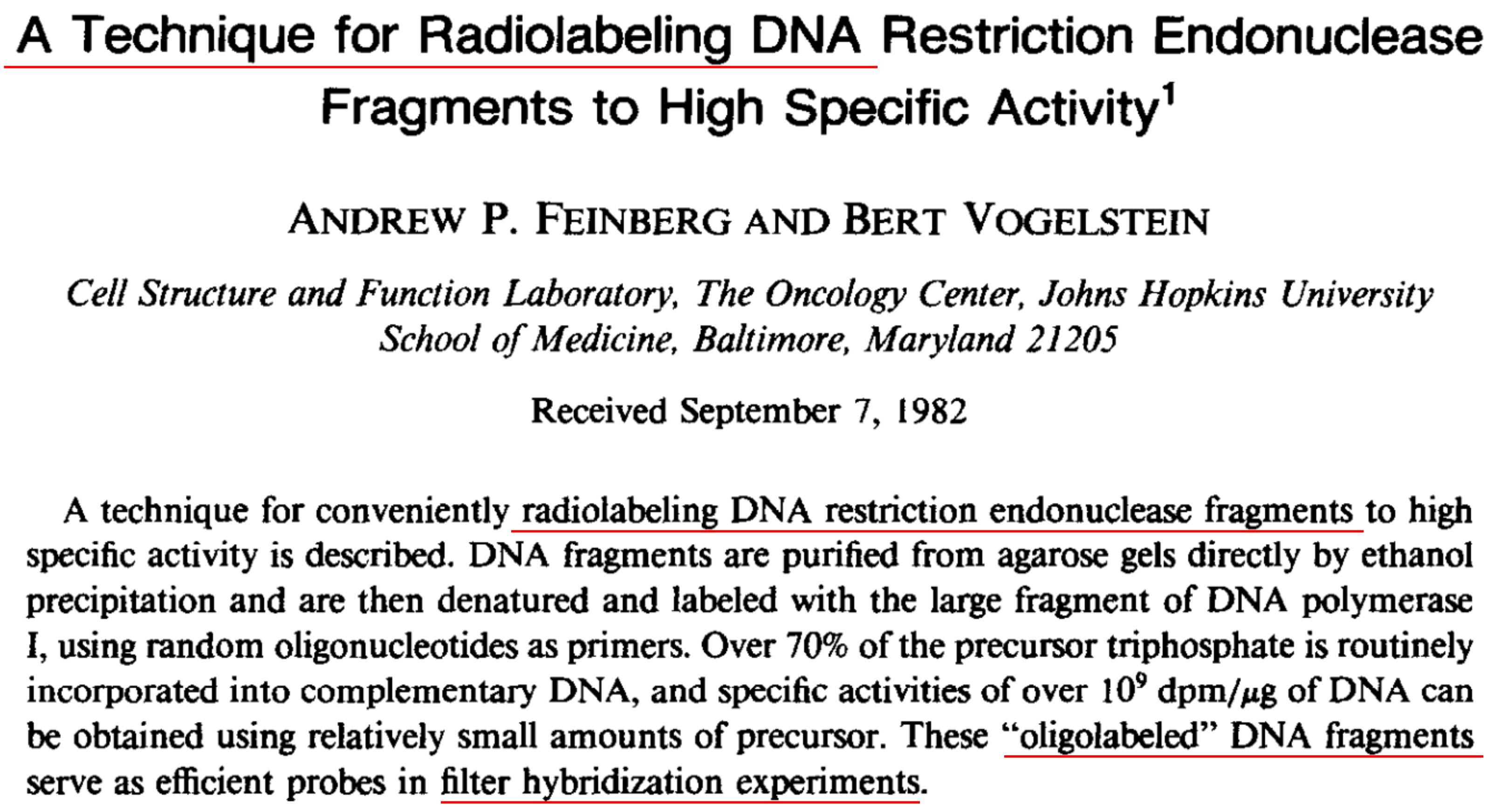
◆ Nucleic acid modification

- The methods developed to crosslink or label proteins do not always apply to nucleic acids
    - The major reactive sites on proteins involve primary amines, sulfhydryls, carboxylates, or phenolates which are relatively easy to derivatize
    - RNA and DNA contains functionalities that are relatively unreactive with many of the common bioconjugate reagents
- Unique chemistry to DNA and RNA has been developed and can be used with the same ease as with proteins
    - Understanding the reactivity of the functional groups in NAs is crucial for the development of methods for NA modification

◆ Enzymes used for NA modification

- Enzymes used for NA modification include DNA polymerase I, terminal deoxynucleotide transferase (TdT), or T4 RNA ligase 1
- The polymerase is most often used to add modified nucleotide to the end of a DNA molecule or to various sites within the middle of a sequence
- The terminal transferase enzyme can add modified nucleotides to the 3′ end of a chain without a template
- The T4 RNA ligase 1 enzyme similarly can add a single modified 5′ phosphoryl-terminated nucleotide to the 3′ hydroxyl terminated end of an RNA strand

◆ Random-primed labeling



A Technique for Radiolabeling DNA Restriction Endonuclease Fragments to High Specific Activity[1]

ANDREW P. FEINBERG AND BERT VOGELSTEIN

Cell Structure and Function Laboratory, The Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

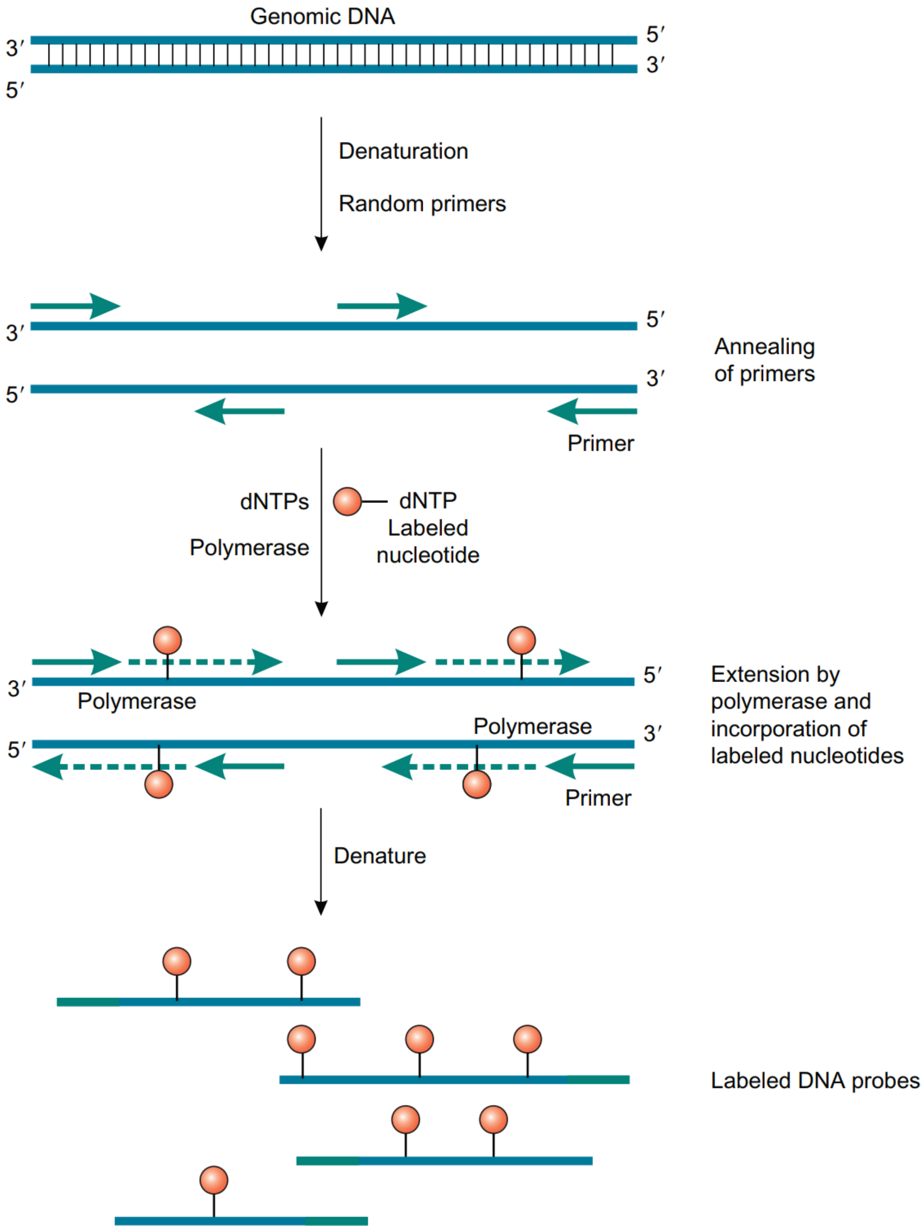
Received September 7, 1982

A technique for conveniently radiolabeling DNA restriction endonuclease fragments to high specific activity is described. DNA fragments are purified from agarose gels directly by ethanol precipitation and are then denatured and labeled with the large fragment of DNA polymerase I, using random oligonucleotides as primers. Over 70% of the precursor triphosphate is routinely incorporated into complementary DNA, and specific activities of over 10^9 dpm/μg of DNA can be obtained using relatively small amounts of precursor. These "oligolabeled" DNA fragments serve as efficient probes in filter hybridization experiments.

Anal. Biochem. **1983**, *132*, 6.

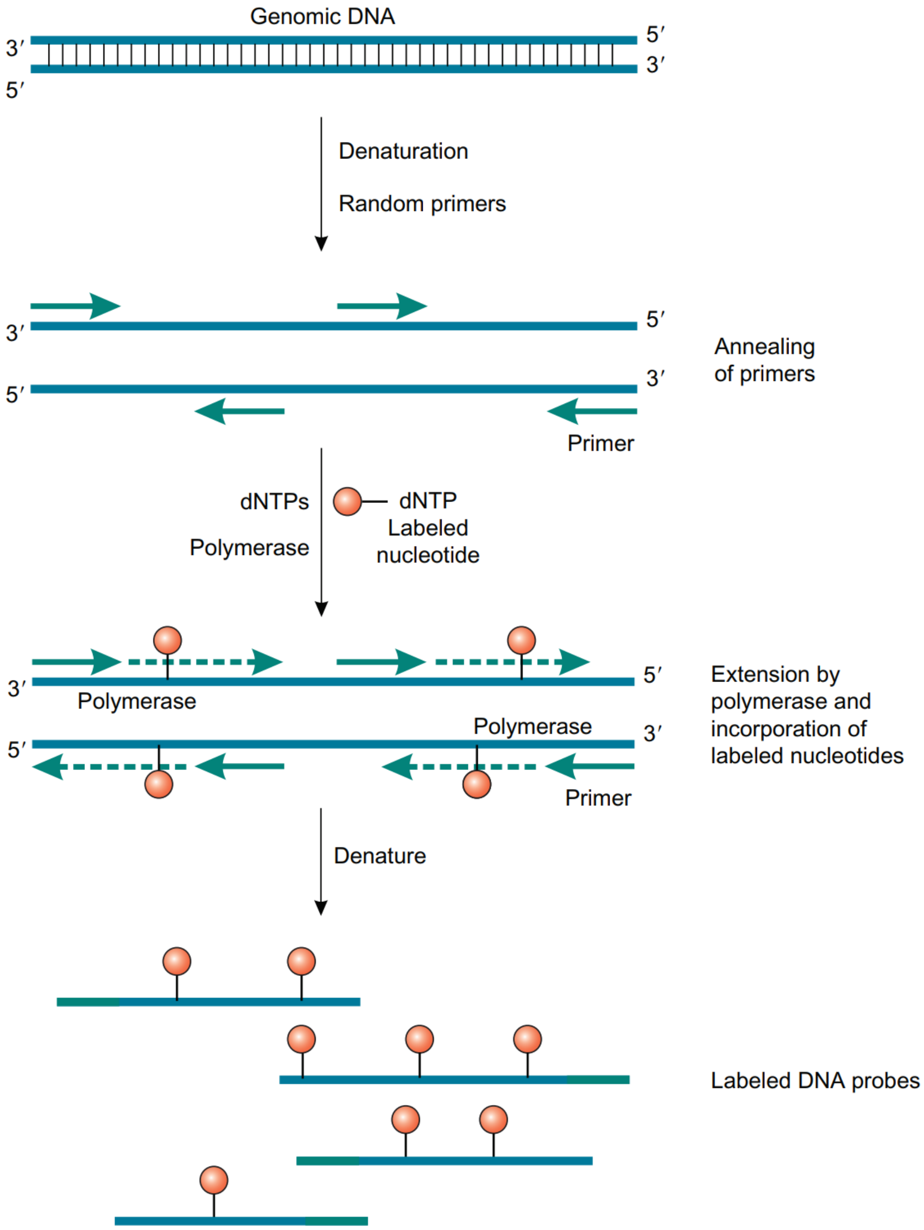
◆ Random-primed labeling

- Modified and unmodified dNTPs are added to a DNA template
- A random mixture of short deoxynucleotides is used to serve as 3′-OH primers
- The form of polymerase typically used is the Klenow fragment which lacks the 5′–3′ exonuclease activity
- The reaction creates a large selection of random complementary strands



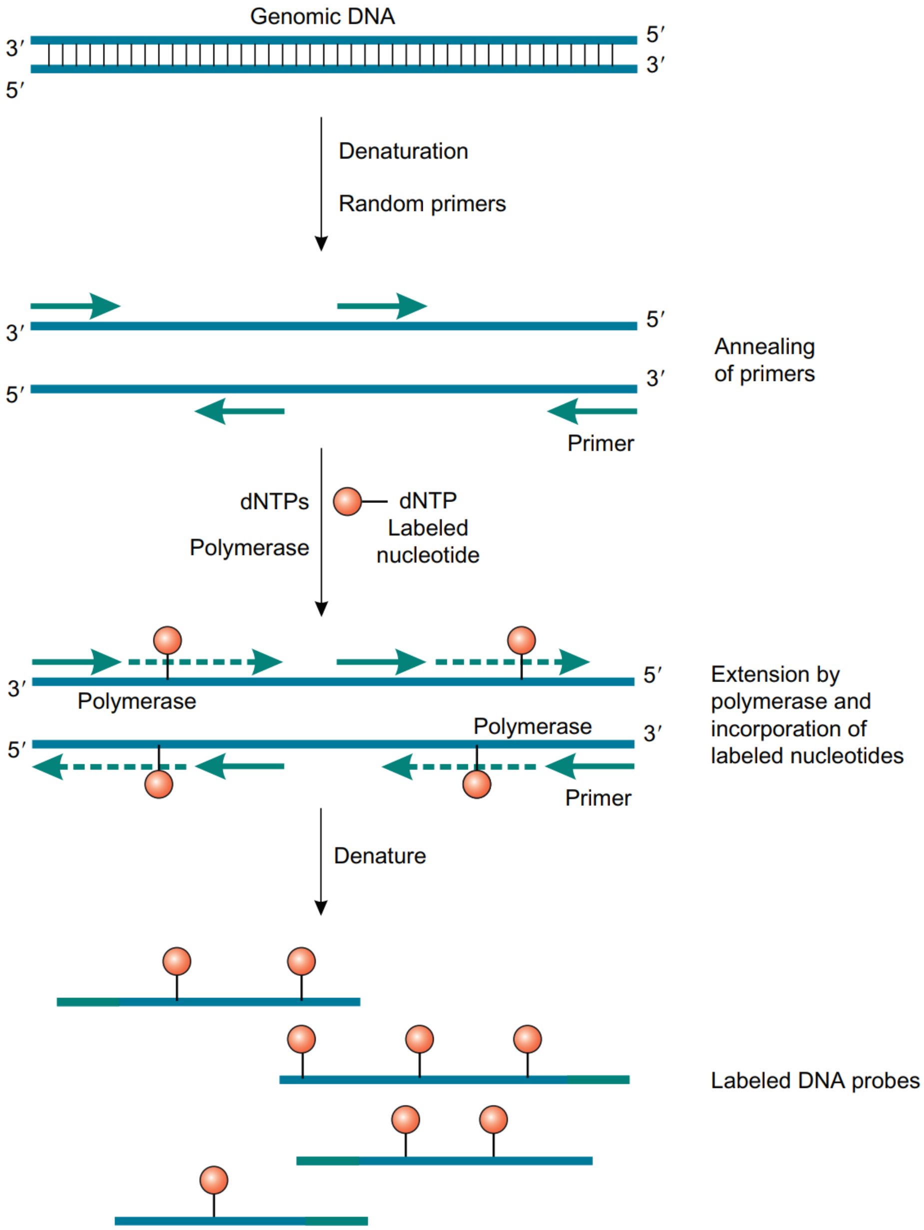
◆ Random-primed labeling

- Labeling components should be compatible with the enzyme
- One of the most proficient methods of adding modified nucleotides in multiple positions
- ^{32}P radio-labeled nucleotides: high density labeling w/o any functional disturbance
- A simple way of tagging probes prepared from a restriction digest template with randomly incorporated, labeled nucleotides

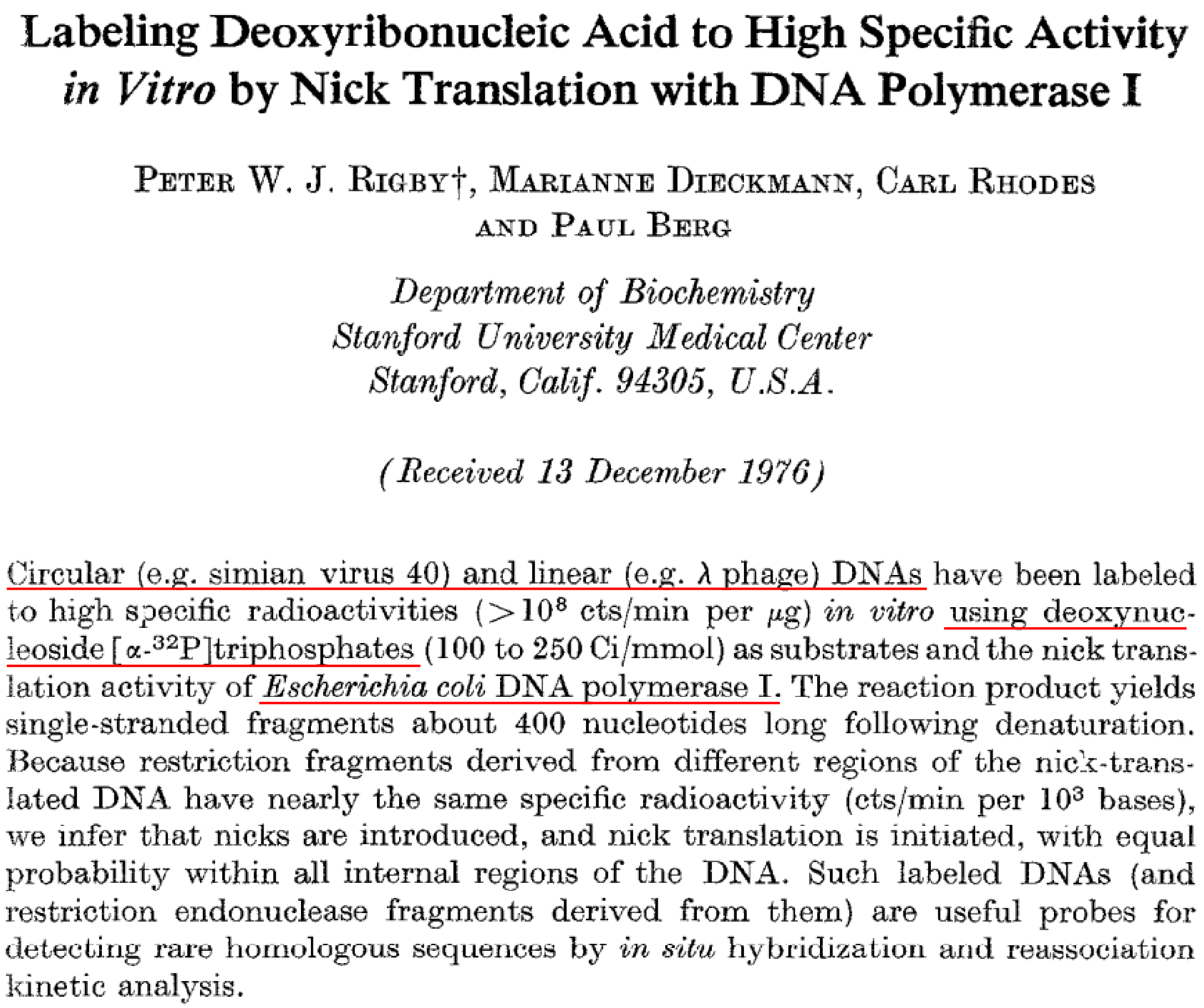


◆ Random-primed labeling

- The modified dNTPs can include detection molecules such as fluorescent dyes or affinity molecules such as a biotin
- The amount of labeling that is done on the complementary oligo needs to be controlled to not affect hybridization
- The ratio of modified to unmodified dNTPs ultimately determines the degree of labeling



◆ Nick translation labeling



Labeling Deoxyribonucleic Acid to High Specific Activity *in Vitro* by Nick Translation with DNA Polymerase I

PETER W. J. RIGBY†, MARIANNE DIECKMANN, CARL RHODES
AND PAUL BERG

Department of Biochemistry
Stanford University Medical Center
Stanford, Calif. 94305, U.S.A.

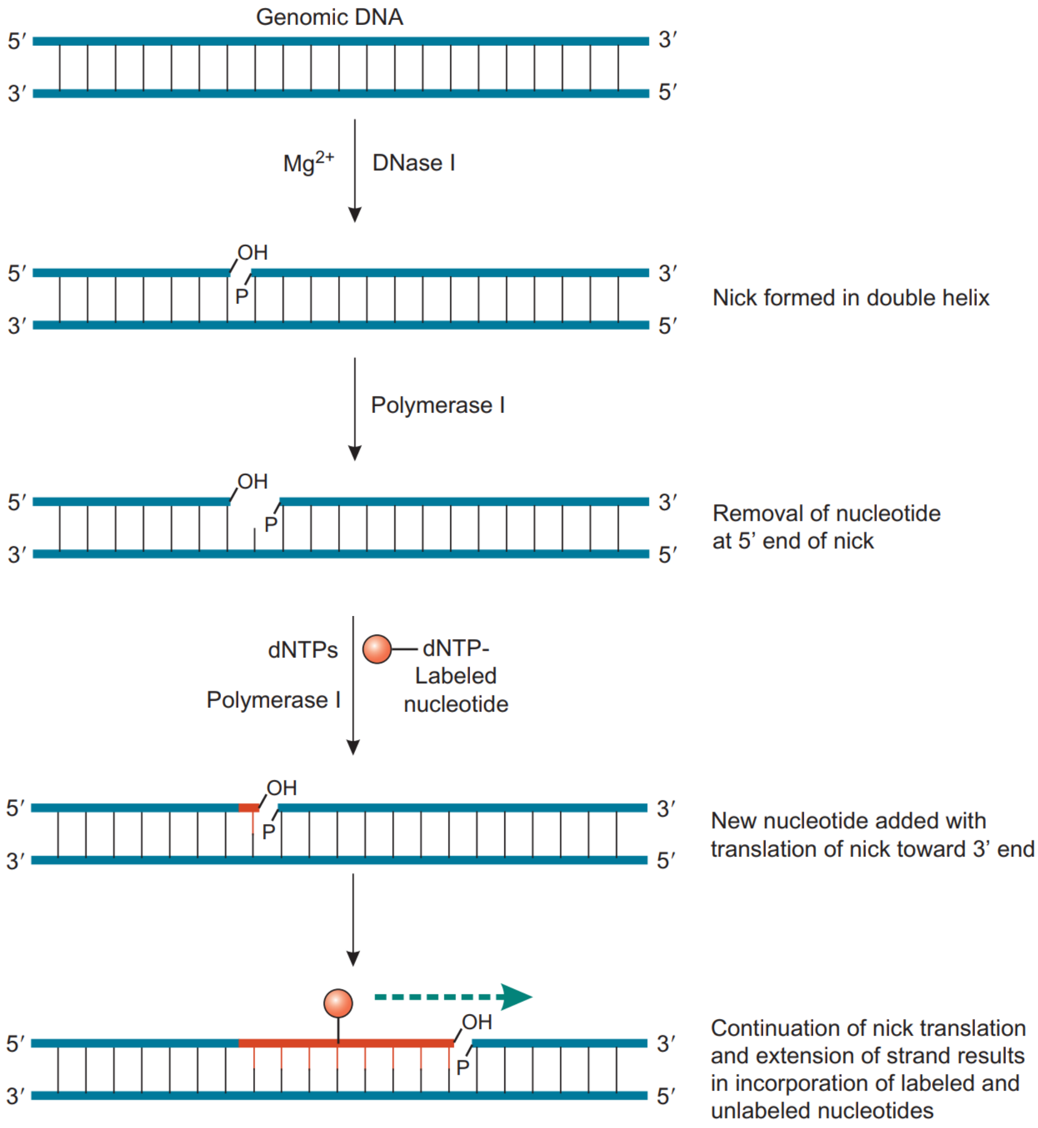
(Received 13 December 1976)

Circular (e.g. simian virus 40) and linear (e.g. λ phage) DNAs have been labeled to high specific radioactivities ($>10^8$ cts/min per μg) *in vitro* using deoxynucleoside $[\alpha\text{-}^{32}P]$triphosphates (100 to 250 Ci/mmol) as substrates and the nick translation activity of *Escherichia coli* DNA polymerase I. The reaction product yields single-stranded fragments about 400 nucleotides long following denaturation. Because restriction fragments derived from different regions of the nick-translated DNA have nearly the same specific radioactivity (cts/min per 10^3 bases), we infer that nicks are introduced, and nick translation is initiated, with equal probability within all internal regions of the DNA. Such labeled DNAs (and restriction endonuclease fragments derived from them) are useful probes for detecting rare homologous sequences by *in situ* hybridization and reassociation kinetic analysis.

J. Mol. Biol. **1977**, *113*, 237

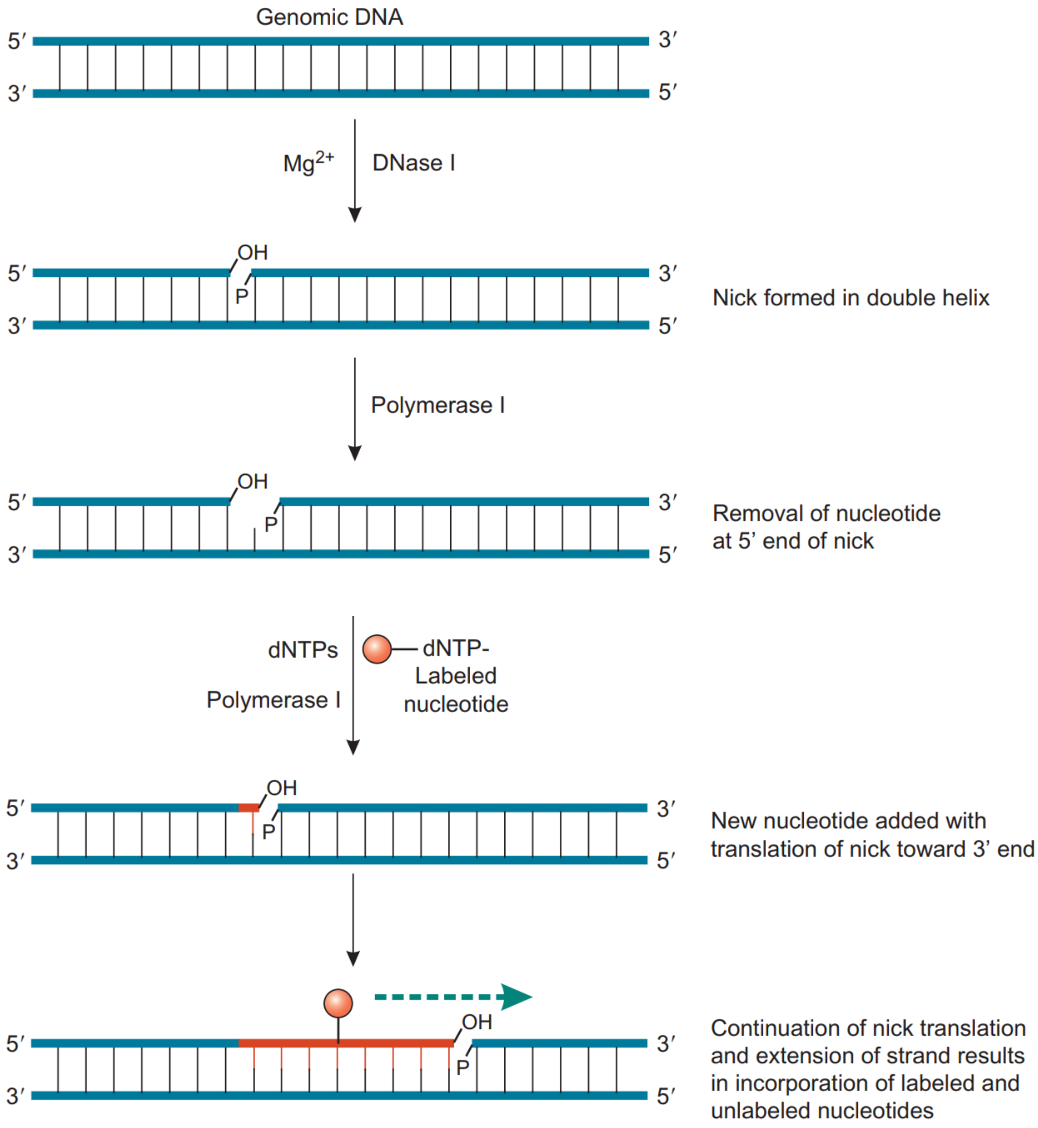
◆ Nick translation labeling

- Nick translation is a DNA-tagging technique using DNA polymerase I
- DNase I is used to generate nicks in intact DNA double-stranded molecules
- DNA polymerase removes the base at the 5′ end of the nick and replaces it with a new nucleotide by attachment to the open 3′ OH group



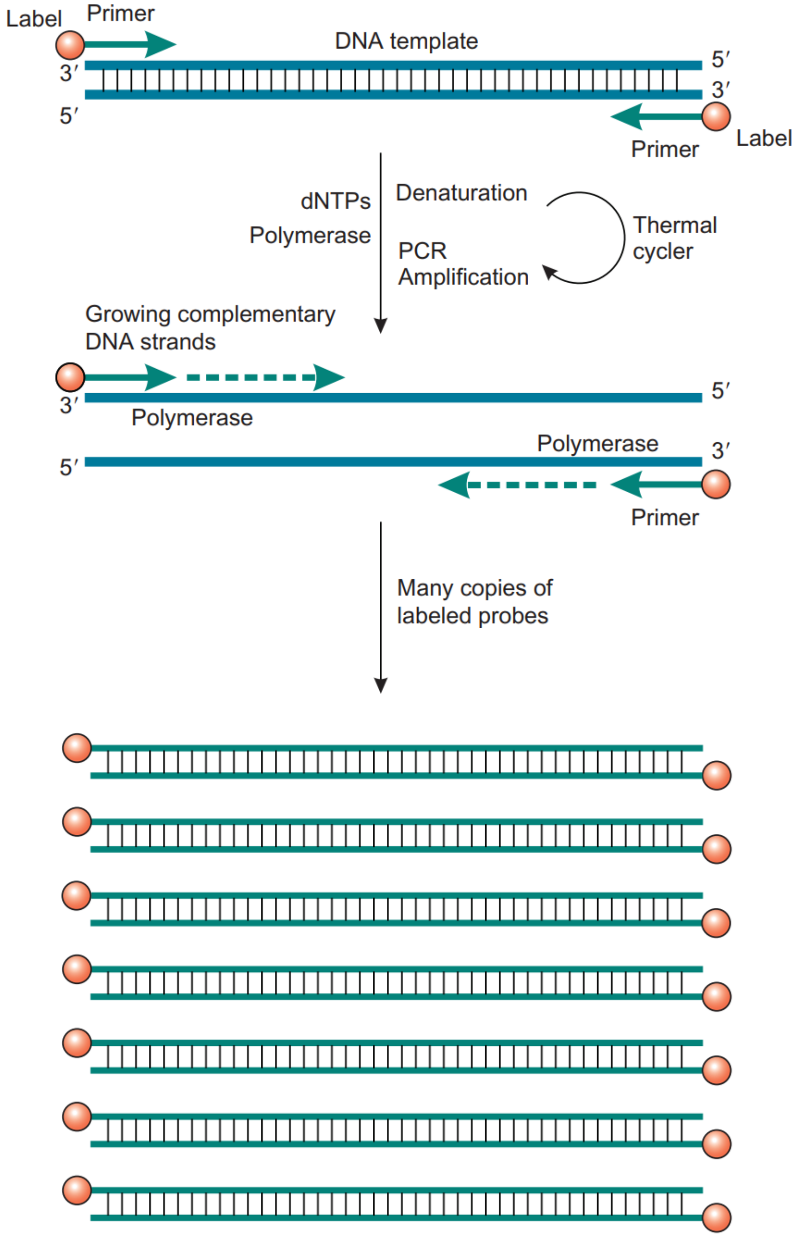
◆ Nick translation labeling

- The labeled and unlabeled nucleotides are incorporated into the growing sequence
- This nick could be sealed by DNA ligase, or its 3' hydroxyl group could serve as the template for further DNA polymerase I activity
- Nick translation can be used to incorporate affinity tags such as biotin and fluorescent labels into an oligo probe

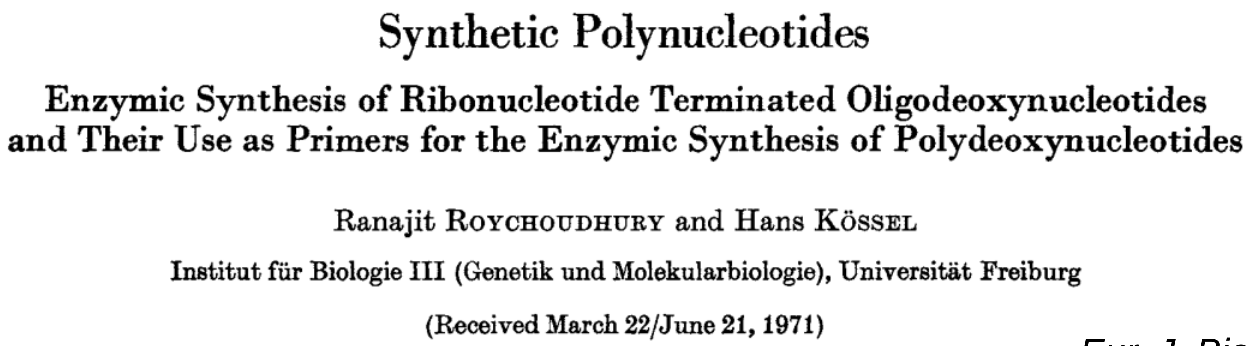


PCR labeling

- Enzymatic labeling of DNA by use of PCR techniques allows to add a label and amplify the labeled polymer
- PCR products are synthesized from primers and contain their sequence and modification
- This method can be used to produce cDNA probes and to detect specific sequences from viruses by RT-PCR



◆ Terminal transferase labeling



Synthetic Polynucleotides

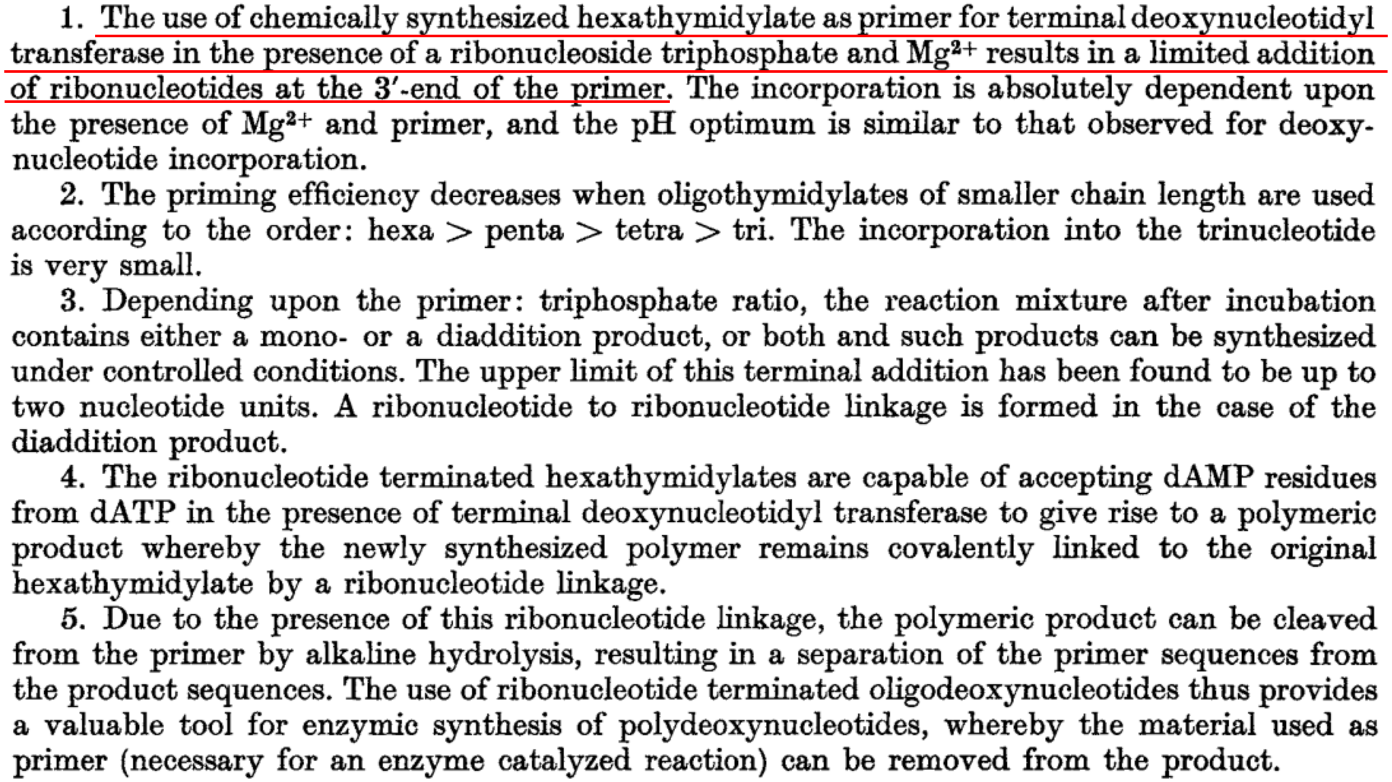
Enzymic Synthesis of Ribonucleotide Terminated Oligodeoxynucleotides and Their Use as Primers for the Enzymic Synthesis of Polydeoxynucleotides

Ranajit Roychoudhury and Hans Kössel

Institut für Biologie III (Genetik und Molekularbiologie), Universität Freiburg

(Received March 22/June 21, 1971)

Eur. J. Biochem. **1971**, *22*, 310.



1. The use of chemically synthesized hexathymidylate as primer for terminal deoxynucleotidyl transferase in the presence of a ribonucleoside triphosphate and Mg^{2+} results in a limited addition of ribonucleotides at the 3′-end of the primer. The incorporation is absolutely dependent upon the presence of Mg^{2+} and primer, and the pH optimum is similar to that observed for deoxynucleotide incorporation.

2. The priming efficiency decreases when oligothymidylates of smaller chain length are used according to the order: hexa > penta > tetra > tri. The incorporation into the trinucleotide is very small.

3. Depending upon the primer: triphosphate ratio, the reaction mixture after incubation contains either a mono- or a diaddition product, or both and such products can be synthesized under controlled conditions. The upper limit of this terminal addition has been found to be up to two nucleotide units. A ribonucleotide to ribonucleotide linkage is formed in the case of the diaddition product.

4. The ribonucleotide terminated hexathymidylates are capable of accepting dAMP residues from dATP in the presence of terminal deoxynucleotidyl transferase to give rise to a polymeric product whereby the newly synthesized polymer remains covalently linked to the original hexathymidylate by a ribonucleotide linkage.

5. Due to the presence of this ribonucleotide linkage, the polymeric product can be cleaved from the primer by alkaline hydrolysis, resulting in a separation of the primer sequences from the product sequences. The use of ribonucleotide terminated oligodeoxynucleotides thus provides a valuable tool for enzymic synthesis of polydeoxynucleotides, whereby the material used as primer (necessary for an enzyme catalyzed reaction) can be removed from the product.

◆ Terminal transferase labeling

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 1366

www.rsc.org/obc

PAPER

Tail-labelling of DNA probes using modified deoxynucleotide triphosphates and terminal deoxynucleotidyl tranferase. Application in electrochemical DNA hybridization and protein-DNA binding assays

Petra Horáková,[a] Hana Macíčková-Cahová,[b] Hana Pivoňková,[a] Jan Špaček,[a] Luděk Havran,[a] Michal Hocek*[b] and Miroslav Fojta*[a]

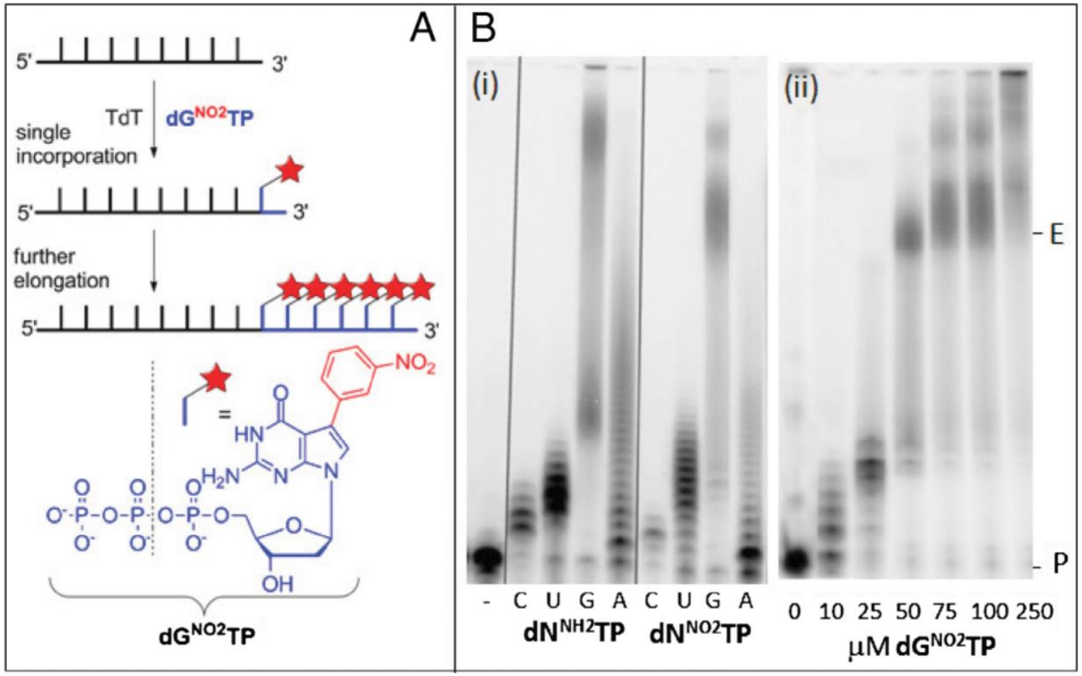
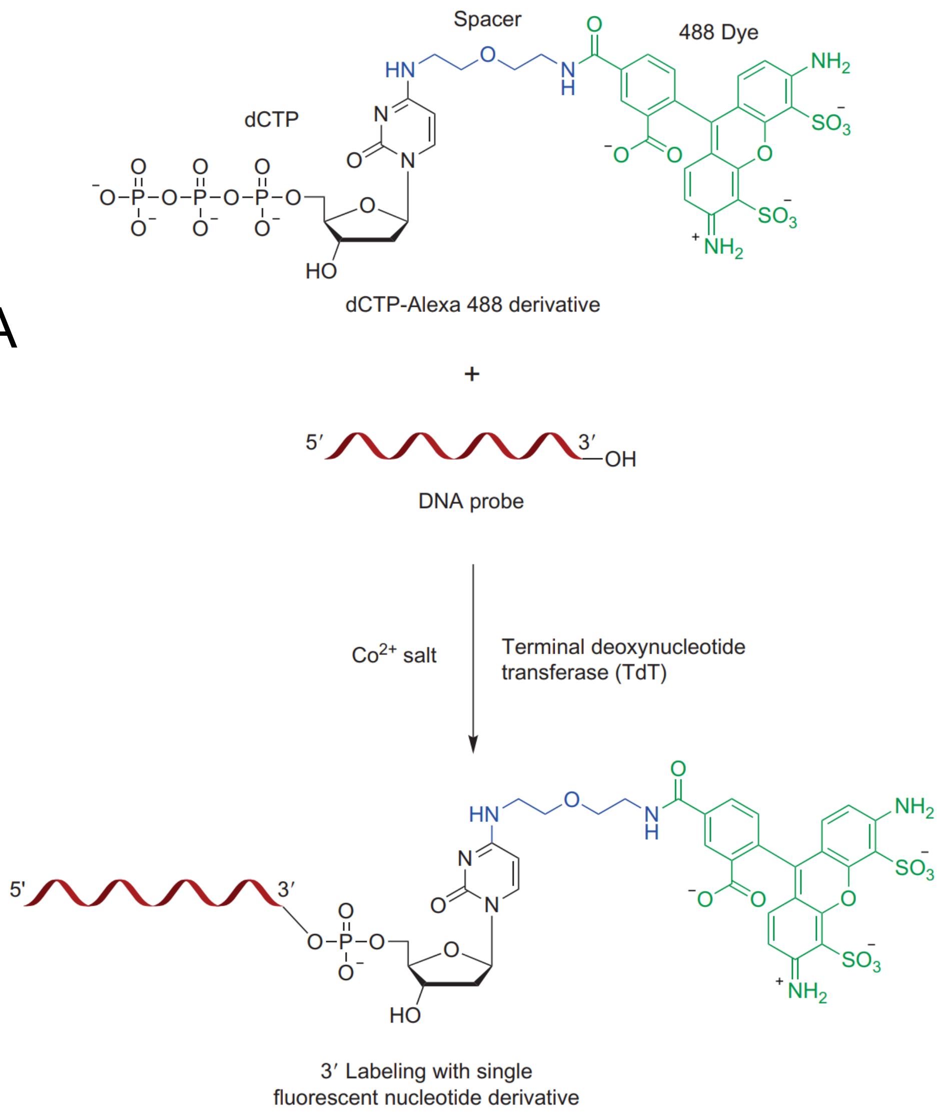


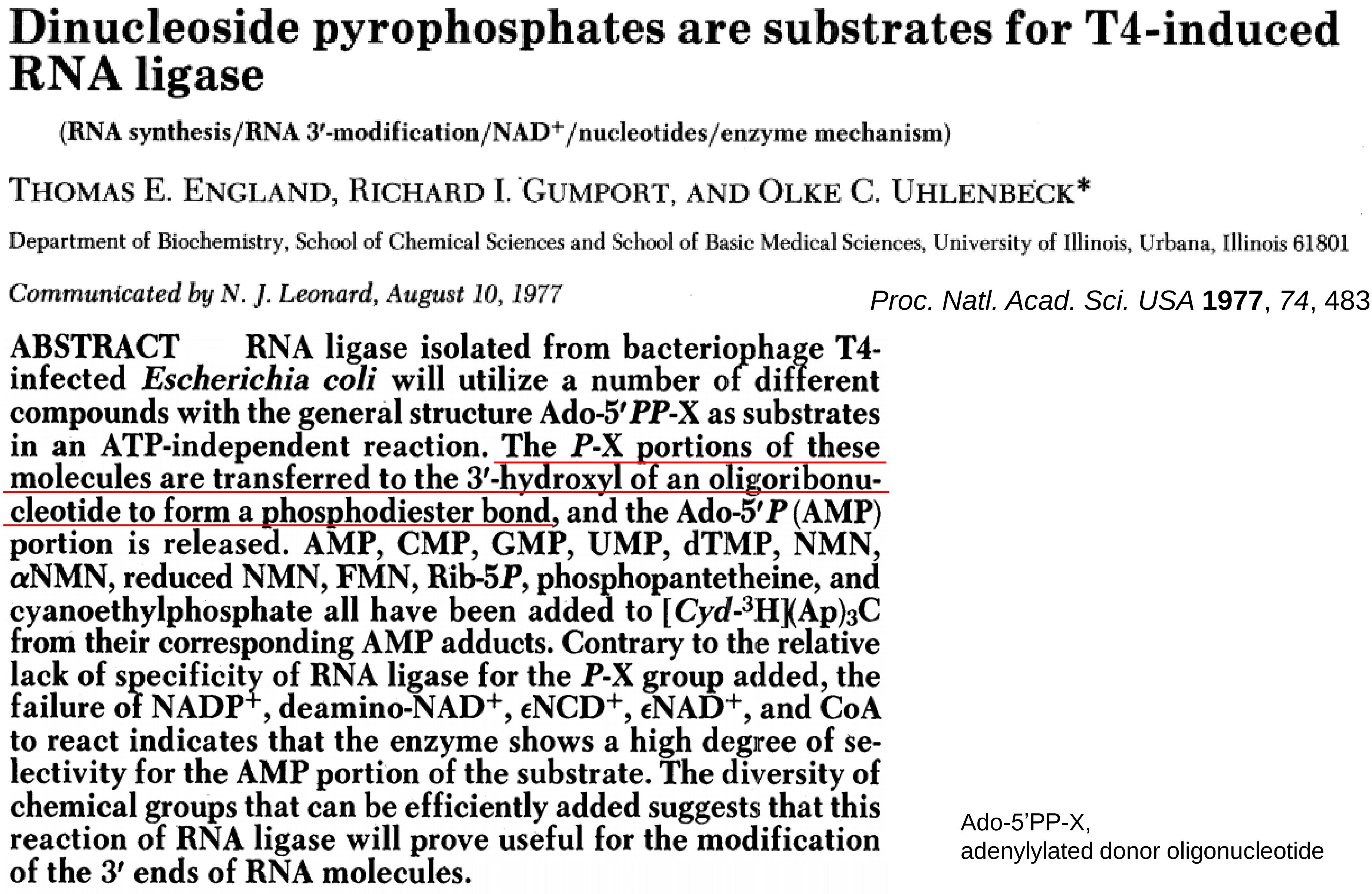
Fig. 1 (A) Scheme of end- or tail-labelling of an ON probe with an electroactive marker G^{NO2} using $dG^{NO2}TP$ and TdT. (B) Denaturing PAGE of products of TdT-catalyzed extension of ^{32}P-labelled prim-15 ON: (i), reactions with $dN^{NH2}TP$ and $dN^{NO2}TP$ (200 μM); (ii), reactions with $dG^{NO2}TP$ at concentrations given in the figure; P, primer; E, long extended products.

◆ Terminal transferase labeling

- Terminal transferase (TdT) is a template independent polymerase that catalyzes the addition of deoxynucleotides to the 3' hydroxyl terminus of DNA molecules
- Protruding, recessed or blunt-ended double or single-stranded DNA molecules serve as a substrate for TdT
- The technique is especially convenient for adding a single tag to the 3′ end of an oligo probe



◆ T4 RNA Ligase 1 Labeling



Dinucleoside pyrophosphates are substrates for T4-induced RNA ligase

(RNA synthesis/RNA 3′-modification/NAD$^+$/nucleotides/enzyme mechanism)

THOMAS E. ENGLAND, RICHARD I. GUMPORT, AND OLKE C. UHLENBECK*

Department of Biochemistry, School of Chemical Sciences and School of Basic Medical Sciences, University of Illinois, Urbana, Illinois 61801

Communicated by N. J. Leonard, August 10, 1977

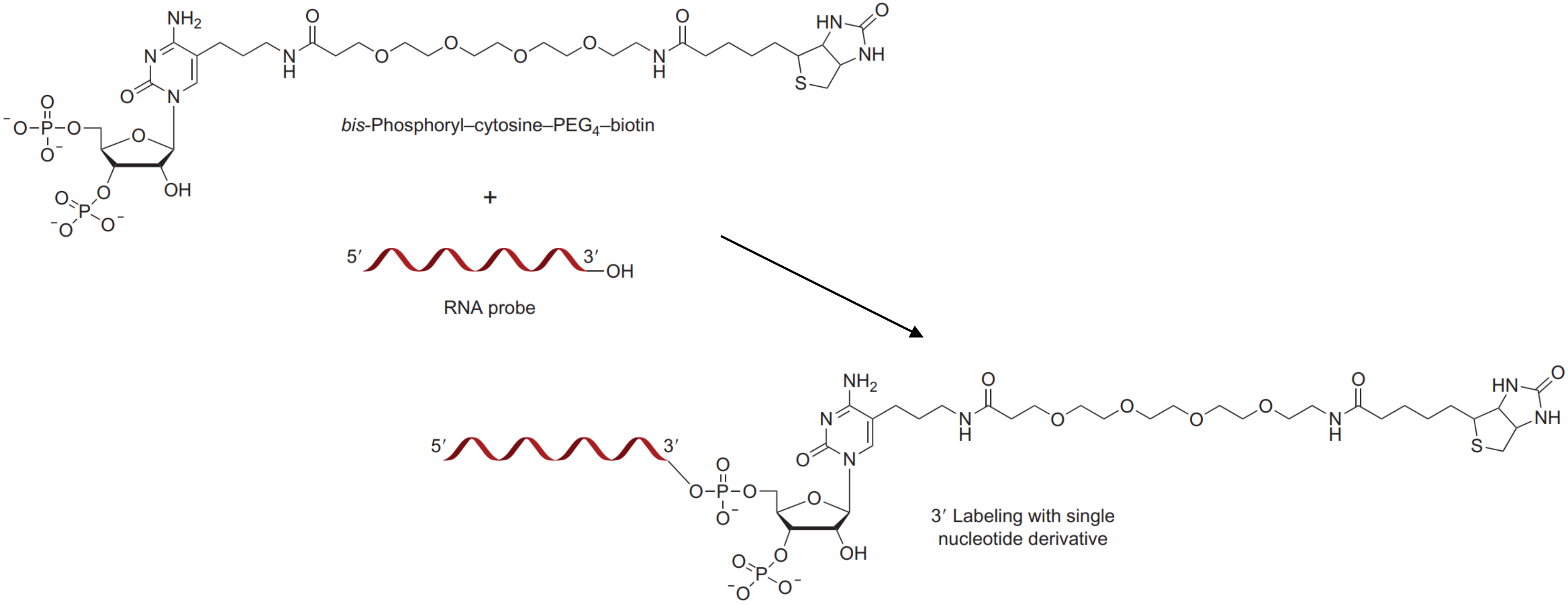
Proc. Natl. Acad. Sci. USA **1977**, *74*, 4839.

ABSTRACT RNA ligase isolated from bacteriophage T4-infected *Escherichia coli* will utilize a number of different compounds with the general structure Ado-5′*PP*-X as substrates in an ATP-independent reaction. The *P*-X portions of these molecules are transferred to the 3′-hydroxyl of an oligoribonucleotide to form a phosphodiester bond, and the Ado-5′*P* (AMP) portion is released. AMP, CMP, GMP, UMP, dTMP, NMN, αNMN, reduced NMN, FMN, Rib-5*P*, phosphopantetheine, and cyanoethylphosphate all have been added to [*Cyd*-^{3}H](Ap)$_3$C from their corresponding AMP adducts. Contrary to the relative lack of specificity of RNA ligase for the *P*-X group added, the failure of NADP$^+$, deamino-NAD$^+$, ϵNCD$^+$, ϵNAD$^+$, and CoA to react indicates that the enzyme shows a high degree of selectivity for the AMP portion of the substrate. The diversity of chemical groups that can be efficiently added suggests that this reaction of RNA ligase will prove useful for the modification of the 3′ ends of RNA molecules.

Ado-5’PP-X,
adenylylated donor oligonucleotide

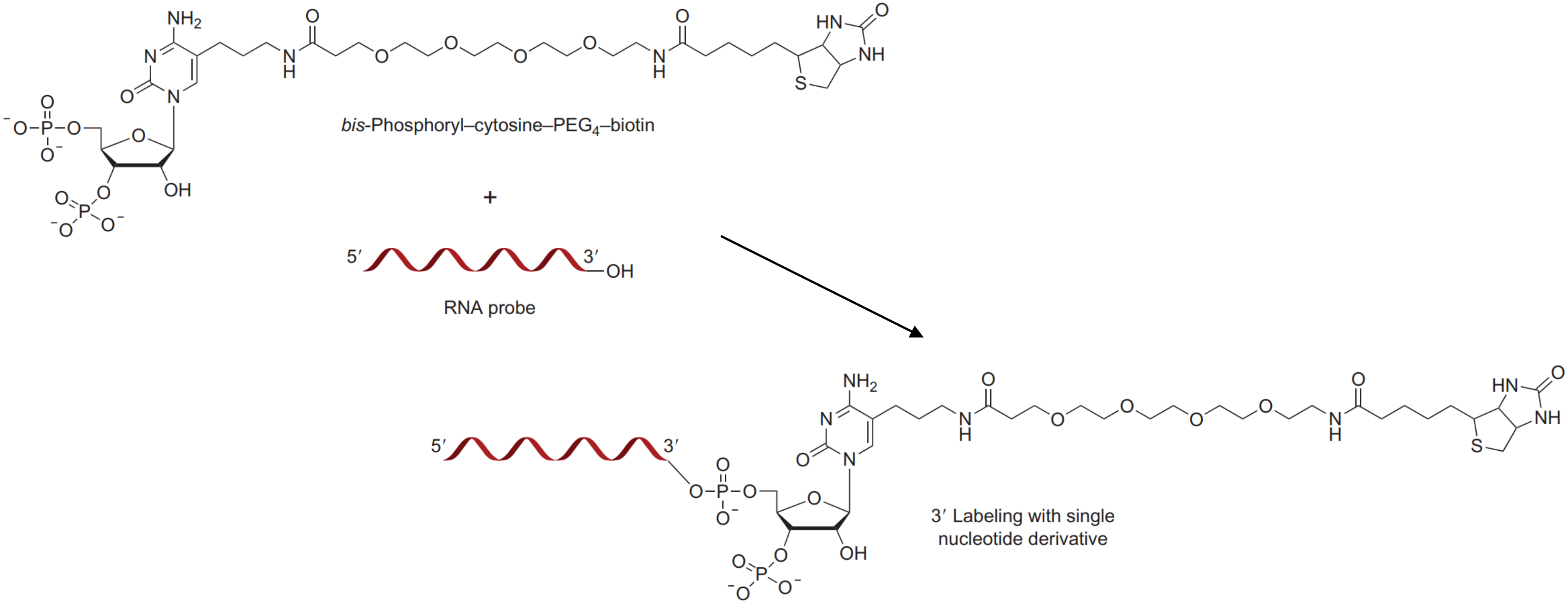
◆ T4 RNA Ligase 1 Labeling

- T4 RNA Ligase 1 is used for labeling of the 3′ hydroxyl end of RNA
- Biotinylated or fluorescently labeled *bis*-phosphoryl–cytosine derivatives can be introduced



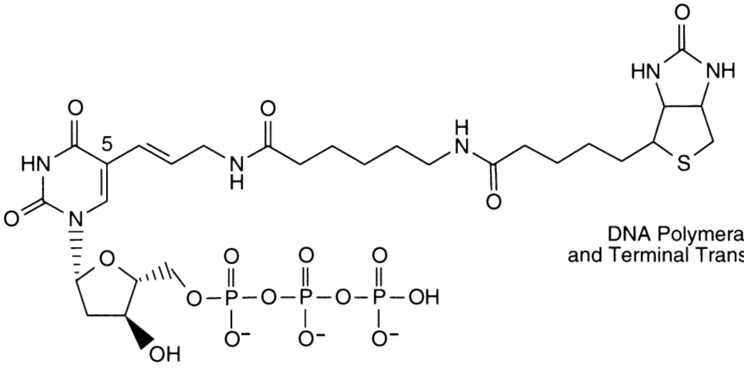
◆ T4 RNA Ligase 1 Labeling

- The enzymatic techniques for end labeling of DNA and RNA have an advantage over the PCR methods, because they add only a single label to each oligo probe at a position that will not affect hybridization efficiency with DNA or RNA targets



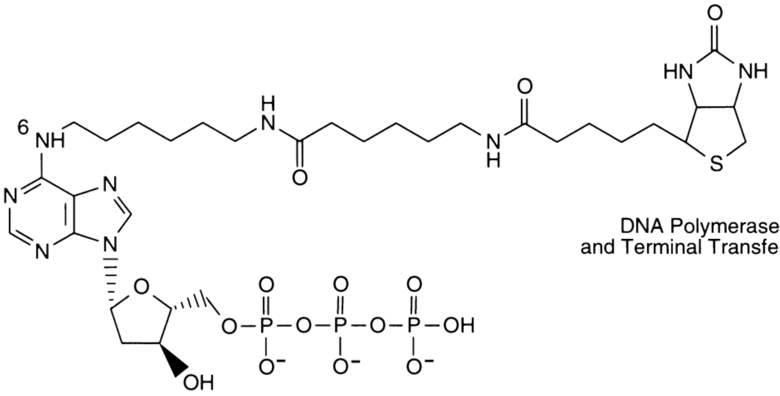
◆ Common nucleoside triphosphate derivatives

- Three common nucleoside triphosphate derivatives that can be incorporated into oligonucleotides by enzymatic means



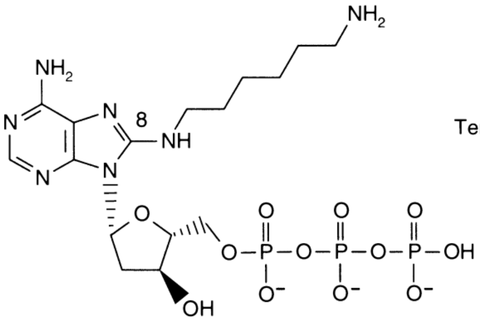
DNA Polymerase
and Terminal Transferase

Biotin–11–dUTP
Derivatized at the C-5 Position



DNA Polymerase
and Terminal Transferase

Biotin–14–dATP
Derivatized at the N-6 Position



Terminal Transferase

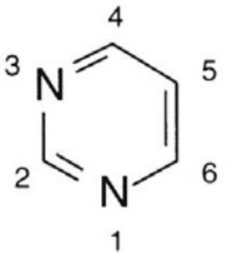
8–Aminohexyl–dATP
Derivatized at the C-8 Position

General strategies

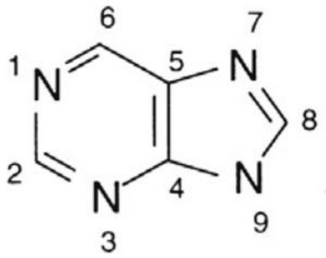
- If enzymatic modification is to be performed, the initial label still must be incorporated into an individual nucleoside triphosphate, which is then polymerized into an existing oligonucleotide strand
- Many useful modified nucleoside triphosphates are now available from commercial sources, often eliminating the need for custom derivatization of individual nucleotides
- Chemical modification may also be used to directly label an oligonucleotide
  - Initial installation of a reactive group (an amine) in oligonucleotide, and then coupling with a probe-containing compound

◆ Polynucleotide structure and functionality

- Knowledge of polynucleotide structure and functionality is crucial for both chemical and enzymatic modification of nucleic acids
  - Note that enzymatic modification also requires modified nucleotides as a substrate
- The pyrimidine and purine ring structures common to nucleic acids



Pyrimidine;
1,3-Diazine



Purine;
7*H*-Imidazo[4,5-*d*]pyrimidine

◆ Polynucleotide structure and functionality

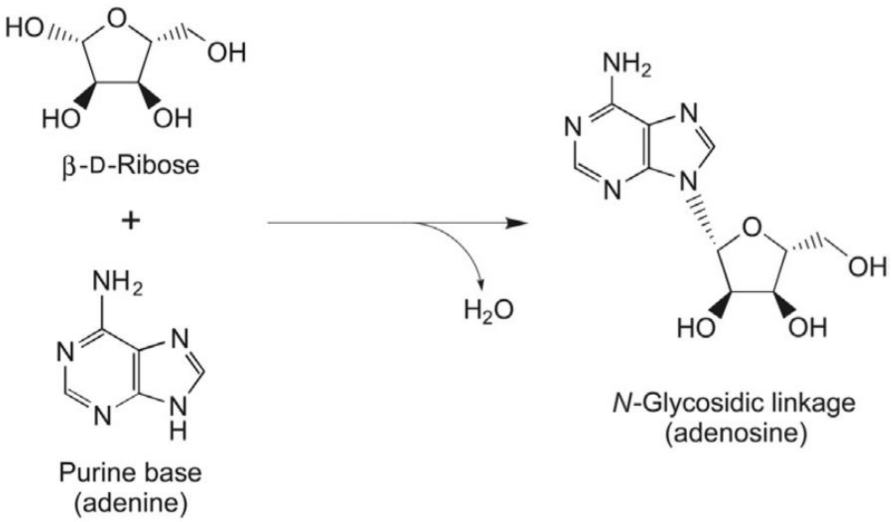


Figure 1. The formation of an N-glycosidic bond links the base unit of nucleic acids to the associated ribose derivative

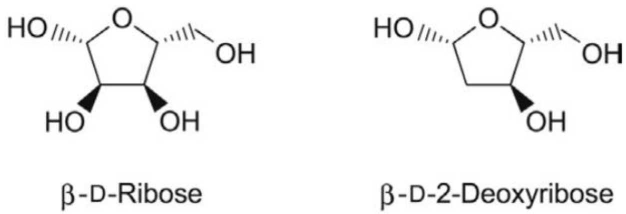


Figure 2. The two forms of sugar residues commonly found in nucleic acids. β-D-Ribose is the sugar constituent of RNA, while β-D-2-deoxyribose is a component of DNA

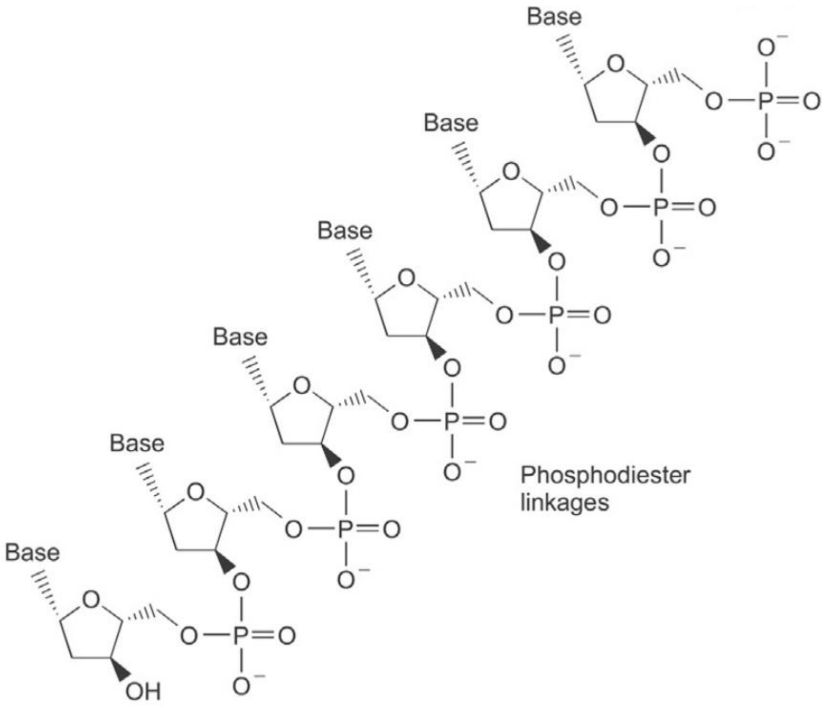


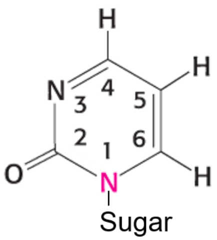
Figure 3. Polynucleotides are formed through phosphodiester bonds linking the associated sugar groups together. In DNA, the 3′-hydroxyl of one deoxyribose unit is bound to the 5′-hydroxyl of the next, creating direction in the polymer backbone

◆ Nucleotide functional groups

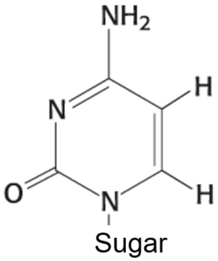
- Chemical attachment of a detectable component to an oligonucleotide forms the basis for constructing a sensitive hybridization reagent
    - The methods used for protein modification do not always apply to nucleic acids
    - The reactive functional groups found in proteins are not found in nucleic acids
    - Most functional groups in NAs are relatively unreactive with many of the common bioconjugate reagents discussed so far
- However, there are particular sites that can be modified on the bases, sugars, or phosphate groups of NAs to produce derivatives able to couple with a second molecule

◆ Pyrimidine (Pyrimidinone) residues

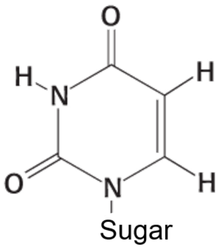
- The pyrimidine base units cytosine, thymine, and uracil contain six-membered nitrogenous ring structures with various points of unsaturation - *Note their structural differences*



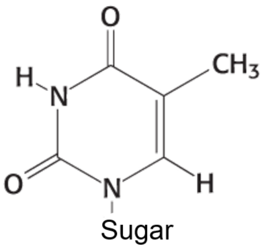
Pyrimidinone



Cytosine

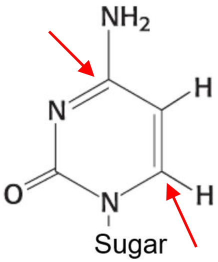


Uracil

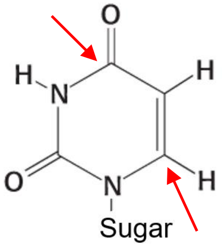


Thymine

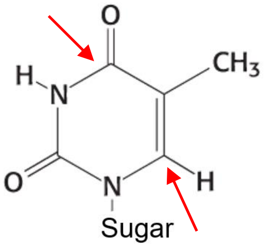
- Pyrimidine bases are subject to nucleophilic displacement reactions primarily at the C-4 and C-6 positions



Cytosine



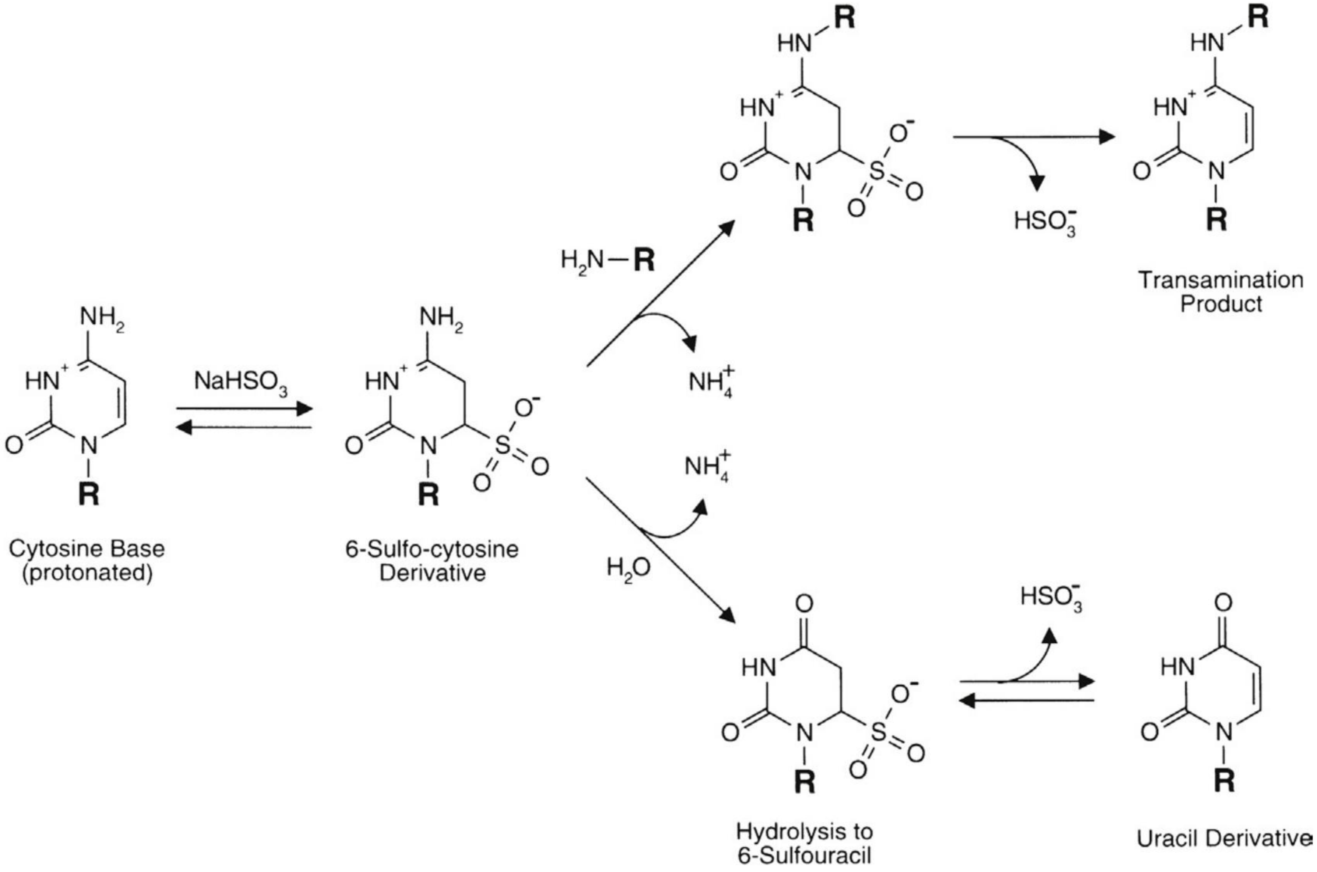
Uracil



Thymine

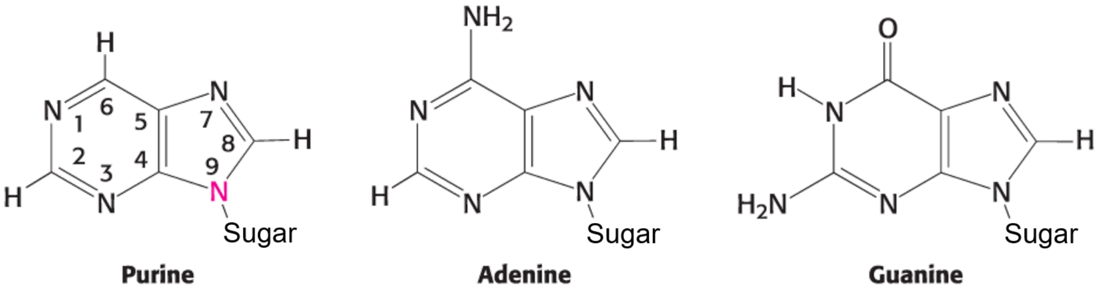
◆ Pyrimidine (Pyrimidinone) residues

- Reaction of bisulfite with cytosine bases is an important route of derivatization. It can lead to uracil formation or, in the presence of an amine- (or hydrazide)-containing compound, transamination can occur, resulting in covalent modification

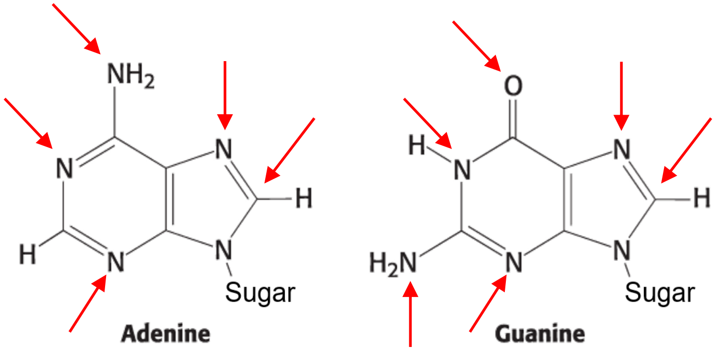


◆ Purine residues

- The structures of the common purine bases of RNA and DNA. The associated sugar groups are bound in N-glycosidic linkages to the N-9 position - *Note their structural differences*

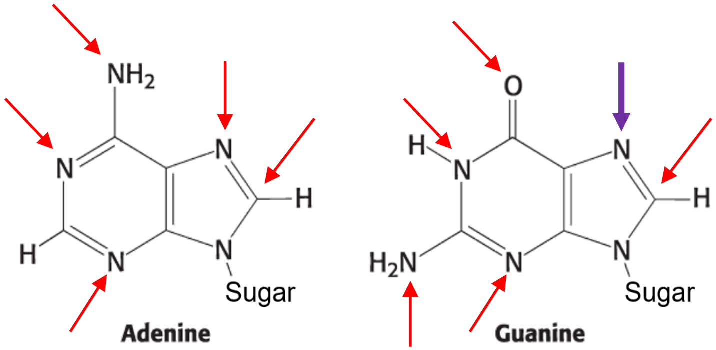


- Electrophilic attack can occur at a number of sites on both purine bases



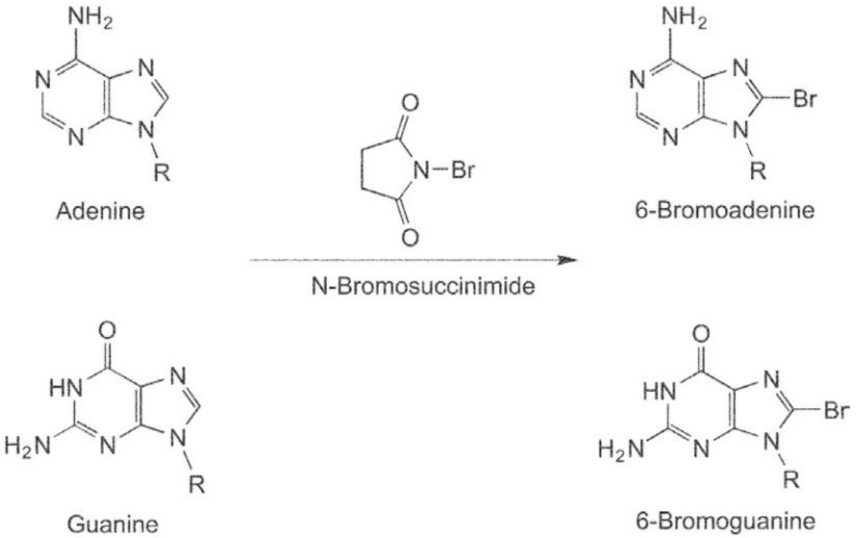
◆ Purine residues

- With purines, reaction with electrophilic species is the most important route to derivatization
- On both bases, it is the heteroatoms that make up the majority of sites. Alkylation reactions can occur at N-1, N-3, and N-7 in adenine or N-3 and N-7 in guanine
- However, the greatest location of electron density (nucleophilicity) occurs at *N-7 on the imidazole ring of guanine*, followed by N-1 of adenine
- The order of reactivity of nucleosides toward alkylation by esters of strong acids is guanine > adenine > cytidine >> uridine (nearly unreactive).



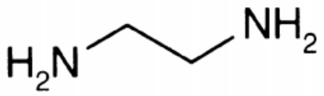
Purine residues

- One of the most important reactions of purines is the bromination of guanine or adenine at the C-8 position
- This site is the most common point of modification for bioconjugate techniques using purine bases
- Either an aqueous solution of bromine or the compound N-bromosuccinimide can be used for this reaction
- The brominated derivatives can then be used to couple amine-containing compounds to the pyrimidine ring structure by nucleophilic substitution

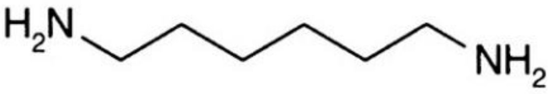


◆ Diamine or bis-hydrazide modification of DNA

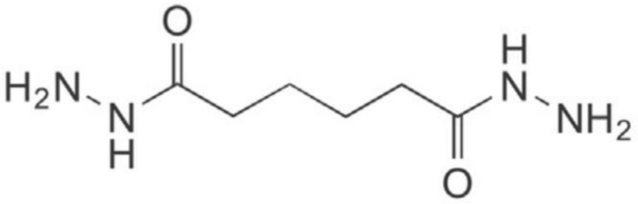
- One of the most useful chemical modifications that can be performed on nucleic acids or oligonucleotides is to add an amine-terminal spacer arm using a diamine compound
  - The resultant amine derivative can be targeted by numerous amine-reactive crosslinkers or modification reagents to create a detectable conjugate



Ethylene Diamine



1,6–Diaminohexane



Adipic acid dihydrazide

Diamine or bis-hydrazide modification of DNA

- Single-stranded DNA molecules can react with sodium bisulfite, adding a sulfonate group across the 5,6-double bond of cytidine bases and creating 6-sulfocytosine derivatives
  - The reaction also catalyzes the deamination of cytosine to uracil by loss of the 4-amino group

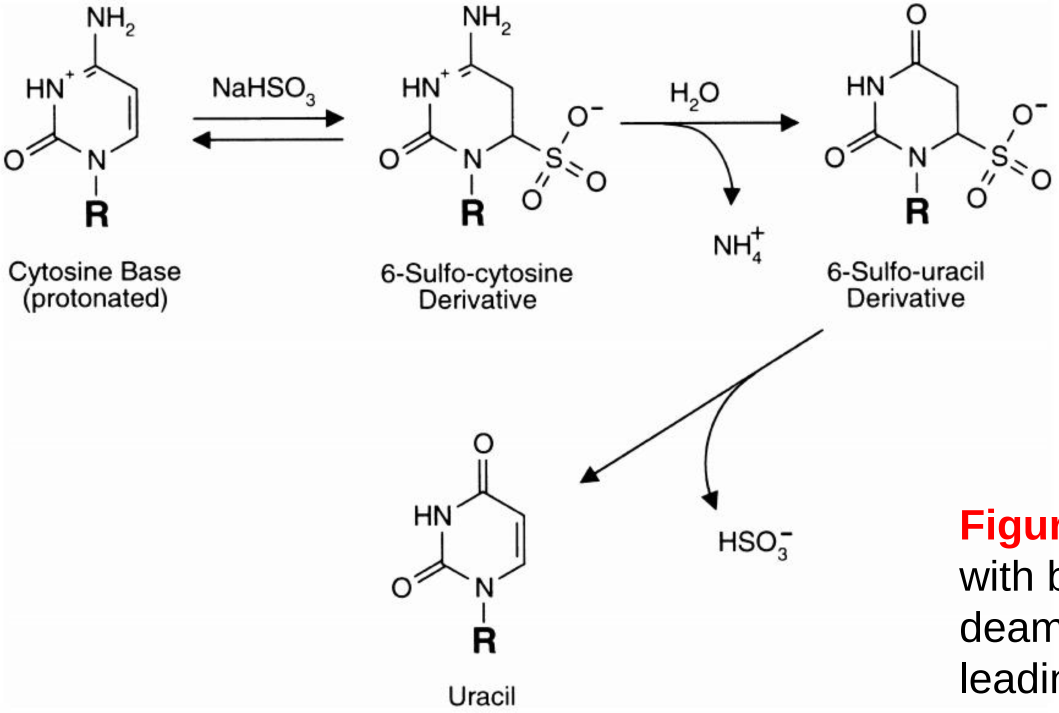
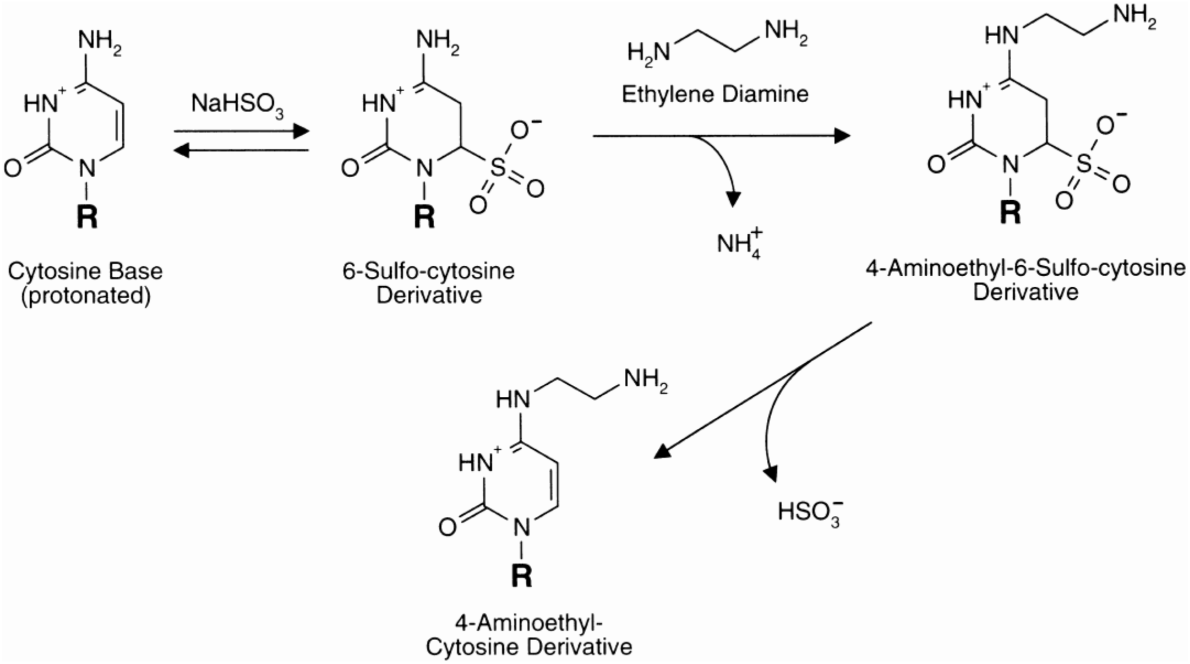


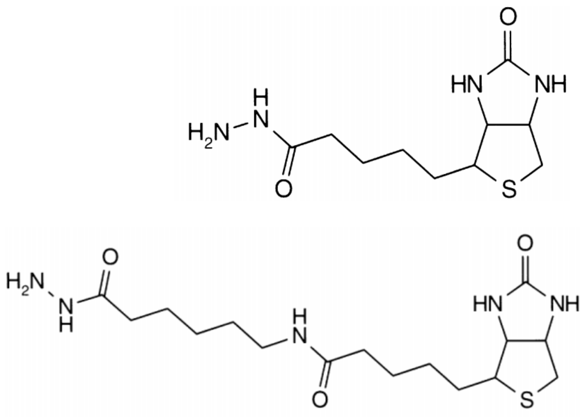
Figure. Treatment of cytosine bases with bisulfite results in a multistep deamination reaction, ultimately leading to uracil formation.

Diamine or bis-hydrazide modification of DNA

- The bisulfite reaction also can cause transamination to occur at the N-4 position of cytosine
  - In the presence of an amine-containing molecule, sodium bisulfite will cause the exchange of the N-4 amine for another amine-containing compound, effectively forming a new covalent linkage with release of ammonium ion



This method can also be used to label oligonucleotide probes



◆ Conjugation via bromine activation

- The nucleotide bases of DNA and RNA can be activated with bromine to produce reactive intermediates capable of coupling to nucleophiles
  - Bromination occurs at the C-8 position of guanine residues and the C-5 of cytosine, yielding reactive derivatives which can be used to couple diamine spacer molecules by nucleophilic substitution

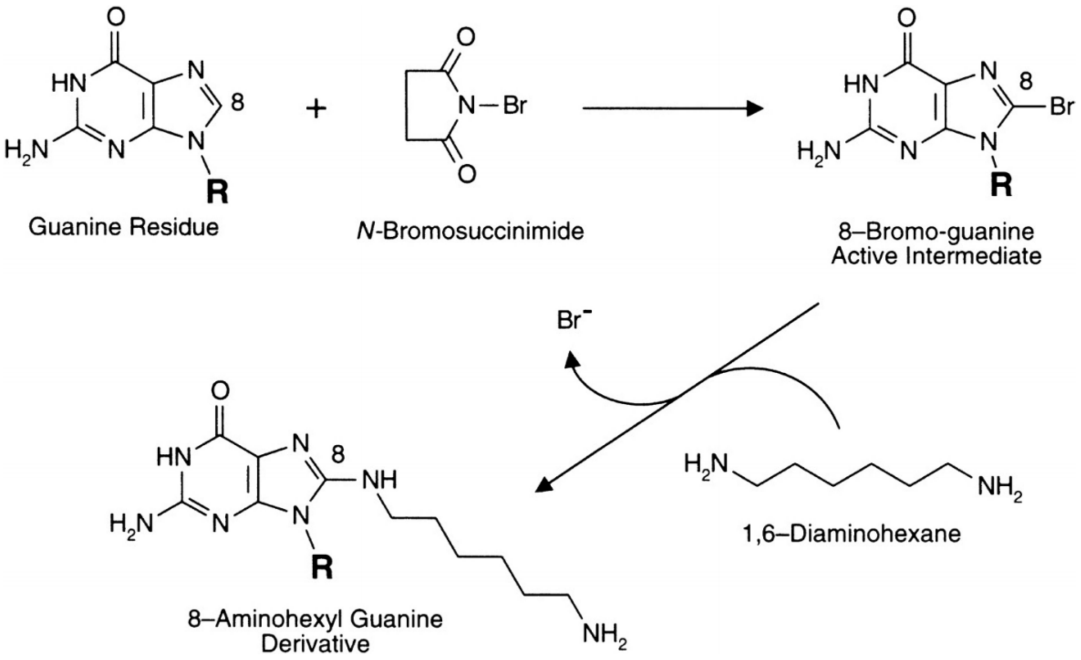
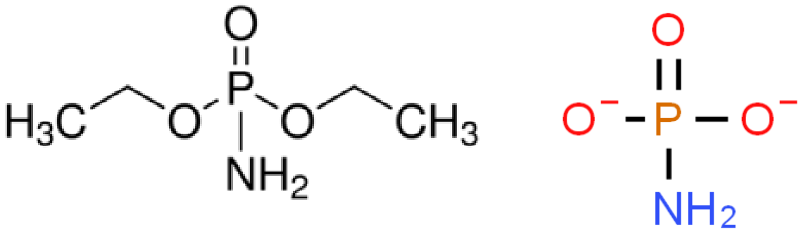


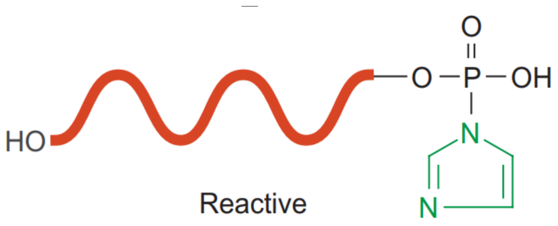
Figure. Reaction of guanine bases with N-bromosuccinimide causes bromination at the C-8 position of the ring. Amine nucleophiles can be coupled to this active derivative by nucleophilic displacement. Reaction of diamine compounds results in amine terminal spacers that can be further modified to contain detectable components.

Conjugation via phosphoramidate formation

- The water soluble carbodiimide EDC reacts with phosphates to form an active complex able to couple with amine-containing compounds
- Diamine spacer molecules or amine-containing probes then may react with this active species to form a stable phosphoramidate bond
- Specific labeling of DNA probes only at the 5′ end is possible using these techniques
- A reactive phosphorimidazolide will rapidly couple to amine-containing molecules to form a phosphoramidate linkage



phosphoramidates



Reactive phosphorimidazolide

◆ Conjugation via phosphoramidate formation

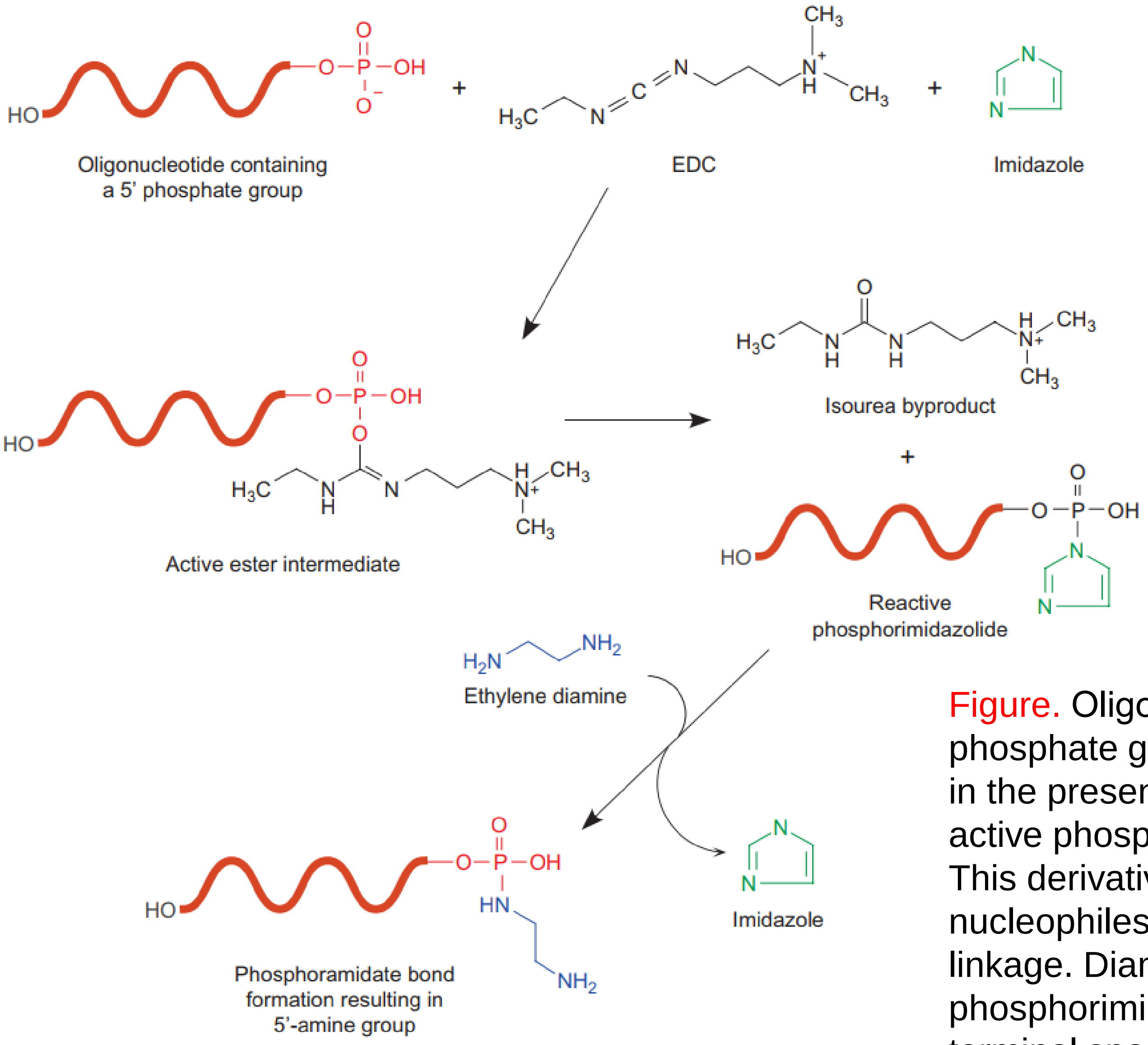
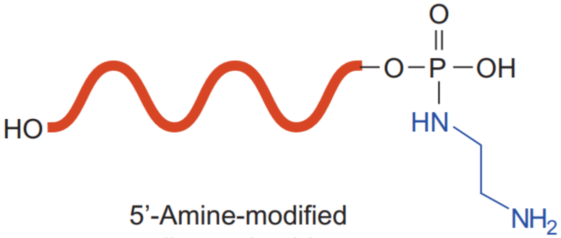
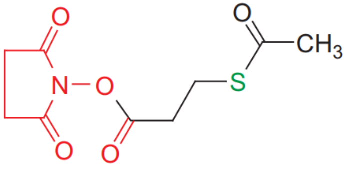


Figure. Oligonucleotides containing a 5′-phosphate group can be reacted with EDC in the presence of imidazole to form an active phosphorimidazolide intermediate. This derivative is highly reactive with amine nucleophiles, forming a phosphoramidate linkage. Diamines reacted with the phosphorimidazolide result in amine terminal spacers that can be modified with detectable components.

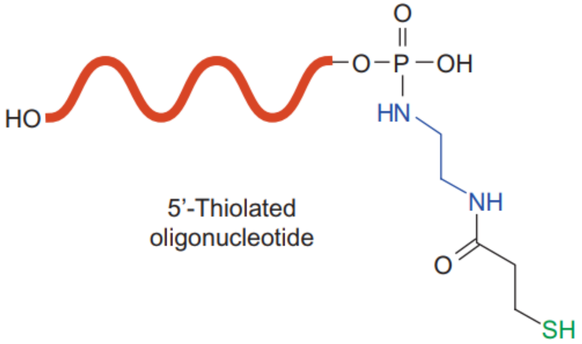
◆ Other modifications



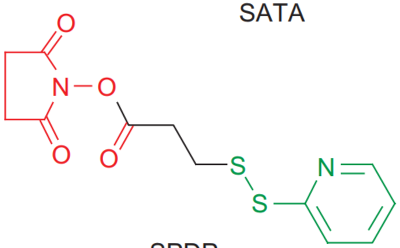
5’-Amine-modified oligonucleotide



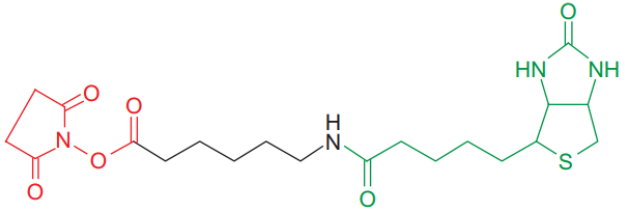
SATA



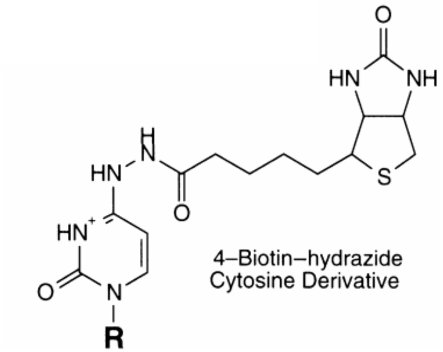
5’-Thiolated oligonucleotide



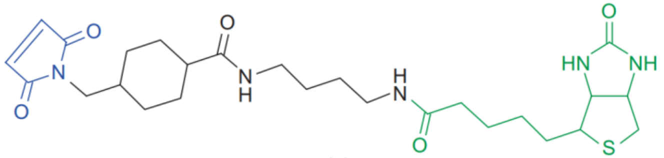
SPDP



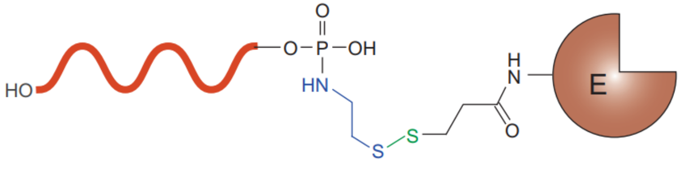
NHS–LC-biotin



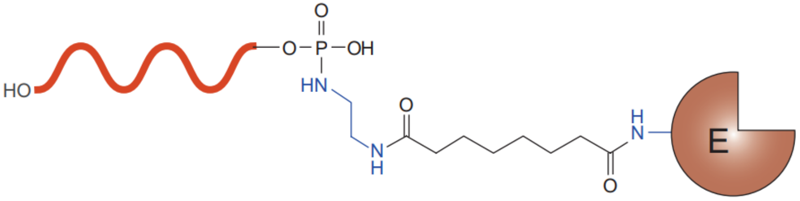
4–Biotin–hydrazide Cytosine Derivative



Biotin–BMCC



Conjugate formation via disulfide linkage



Conjugate formation via amide linkage