Molecular Biology 101 for Laboratory Professionals: Part One

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The presenter states no conflict of interest and has no financial relationship to disclose relevant to the content of this presentation.

OUTLINE

I. Cell biology vignette

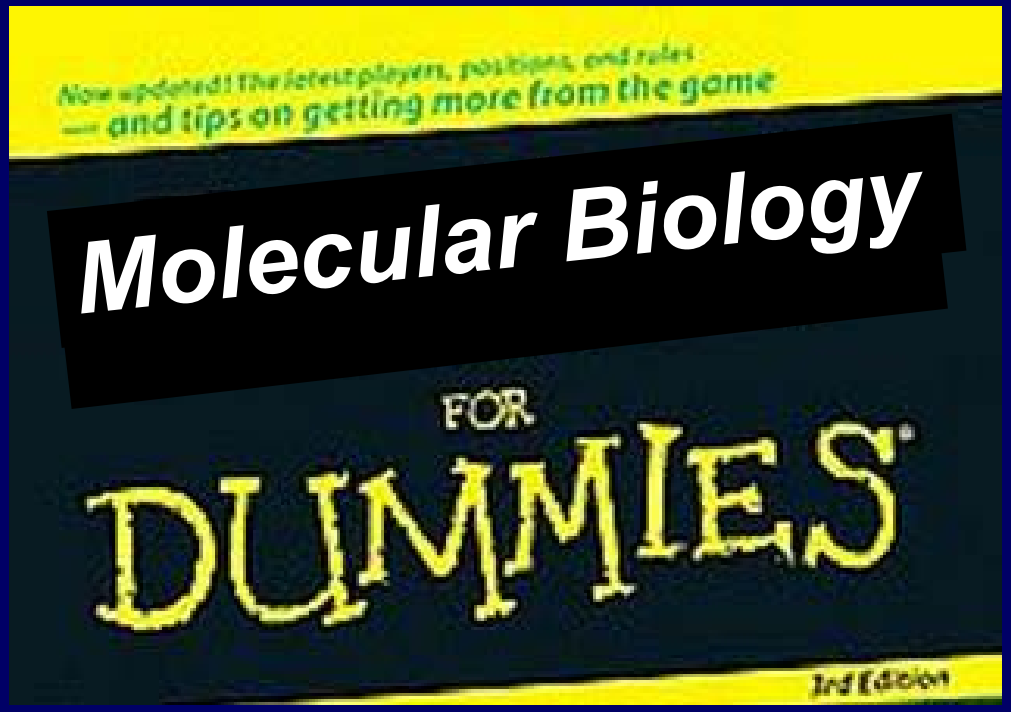
II. Molecular diagnostic application

III. Life-creating, life-changing events

   A. DNA structure

   B. DNA replication

"D#*%it, Jim,
I'm not a physician."

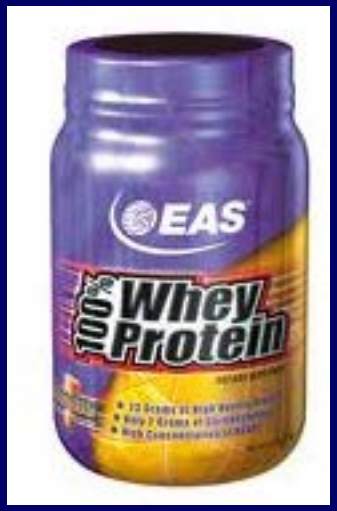


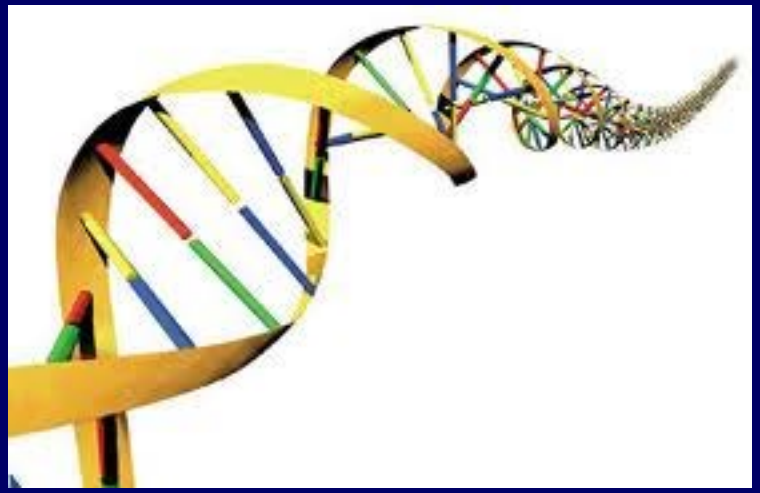
…including myself

Some Perspective

MACROMOLECULES

- Proteins
- Lipids
- Carbohydrates
- Nucleic acids

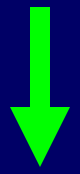




CENTRAL DOGMA

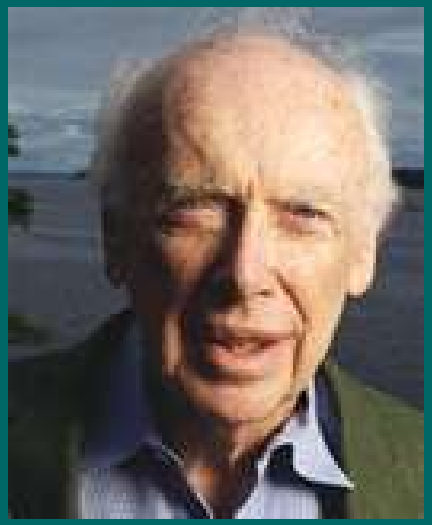
DNA

RNA

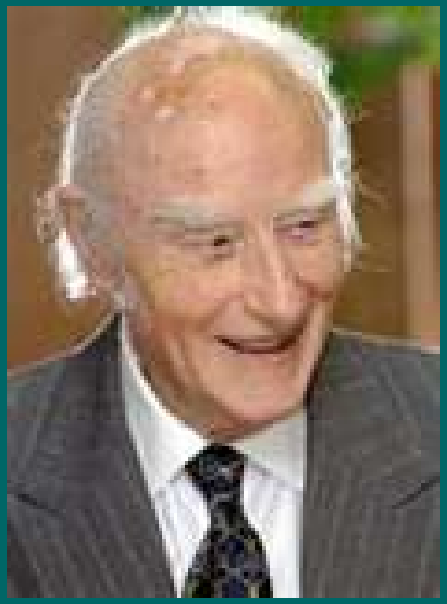


protein

DNA Structure



Watson & Crick



Nature **171:** 737-738; 1953

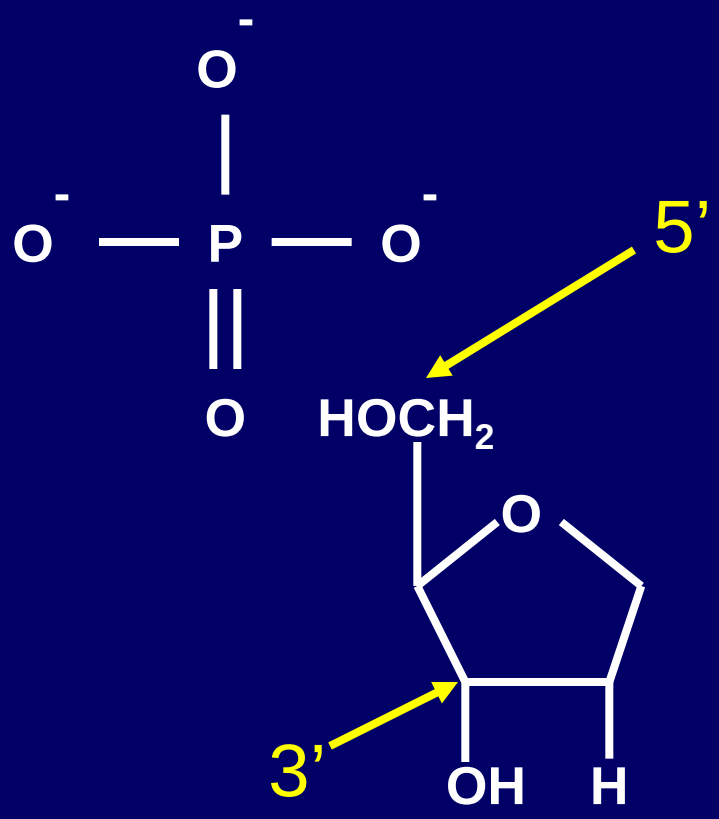
COMPONENTS

DNA

Phosphate

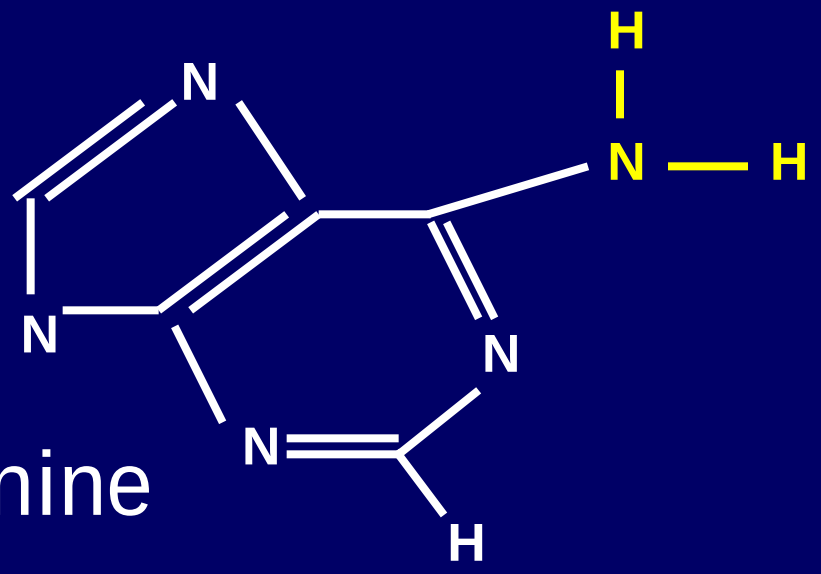
Pentose
(deoxyribose)

Base



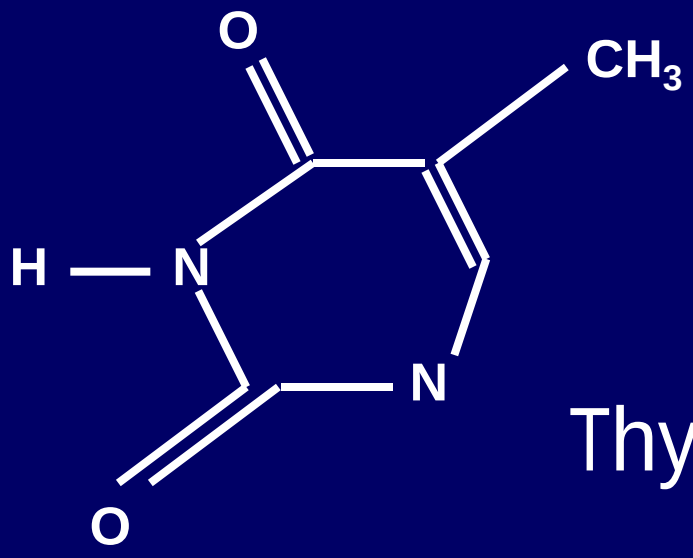
PURINES

PYRIMIDINES

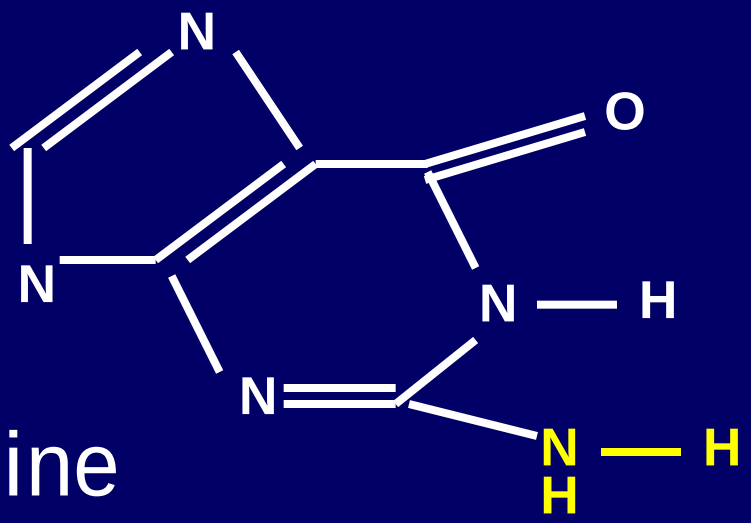


Adenine

AMINO



Thymine

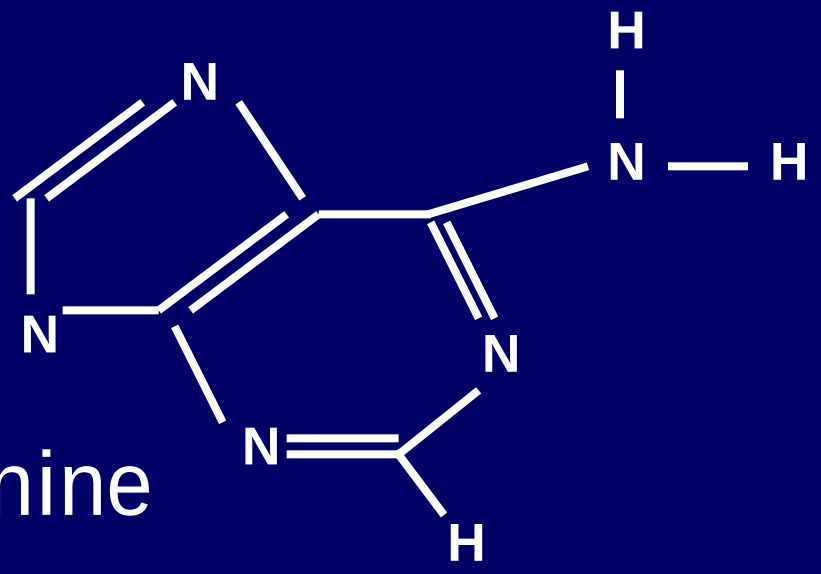


Guanine

Cytosine

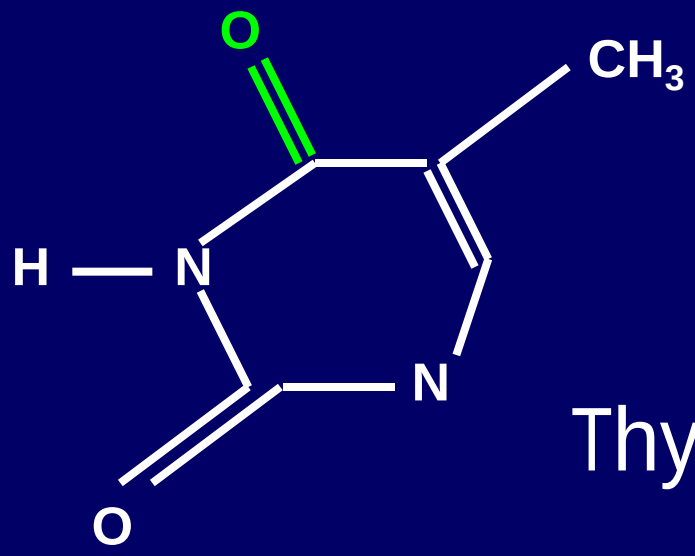
PURINES

PYRIMIDINES

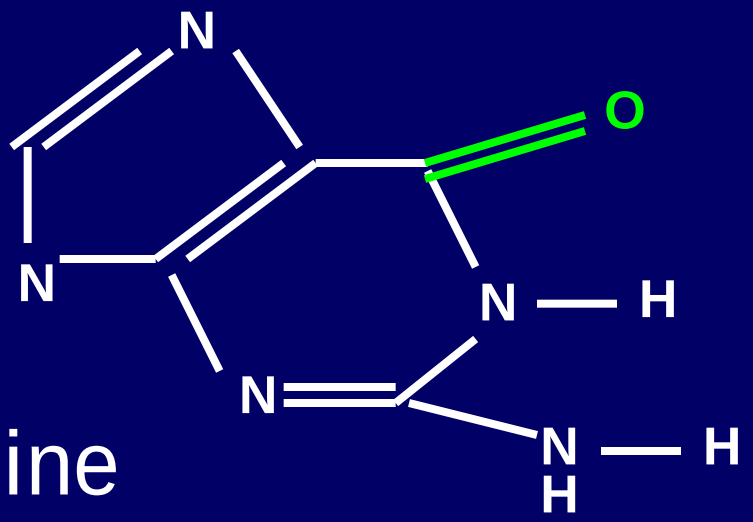


Adenine

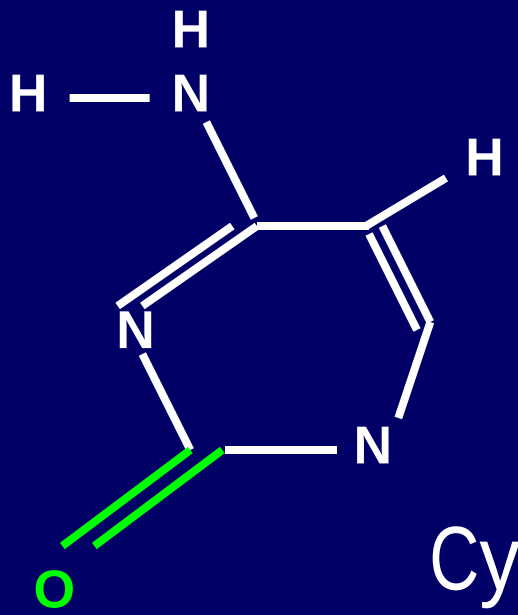
KETO



Thymine

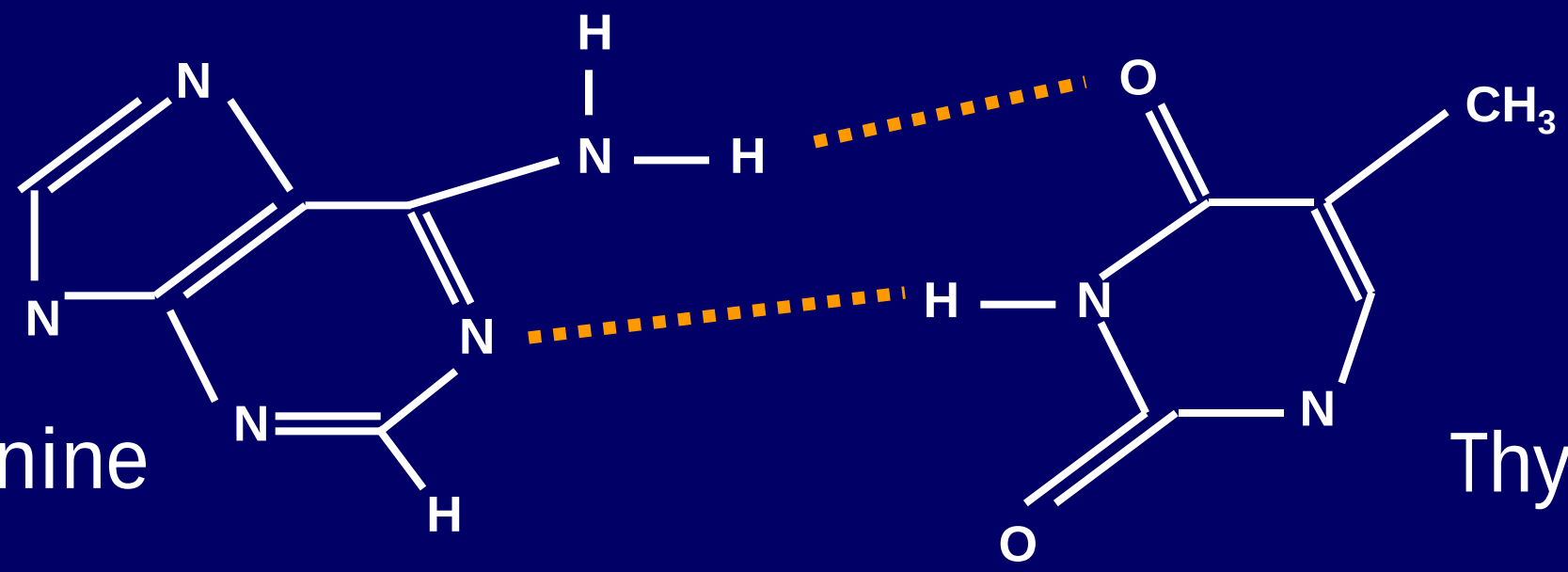


Guanine



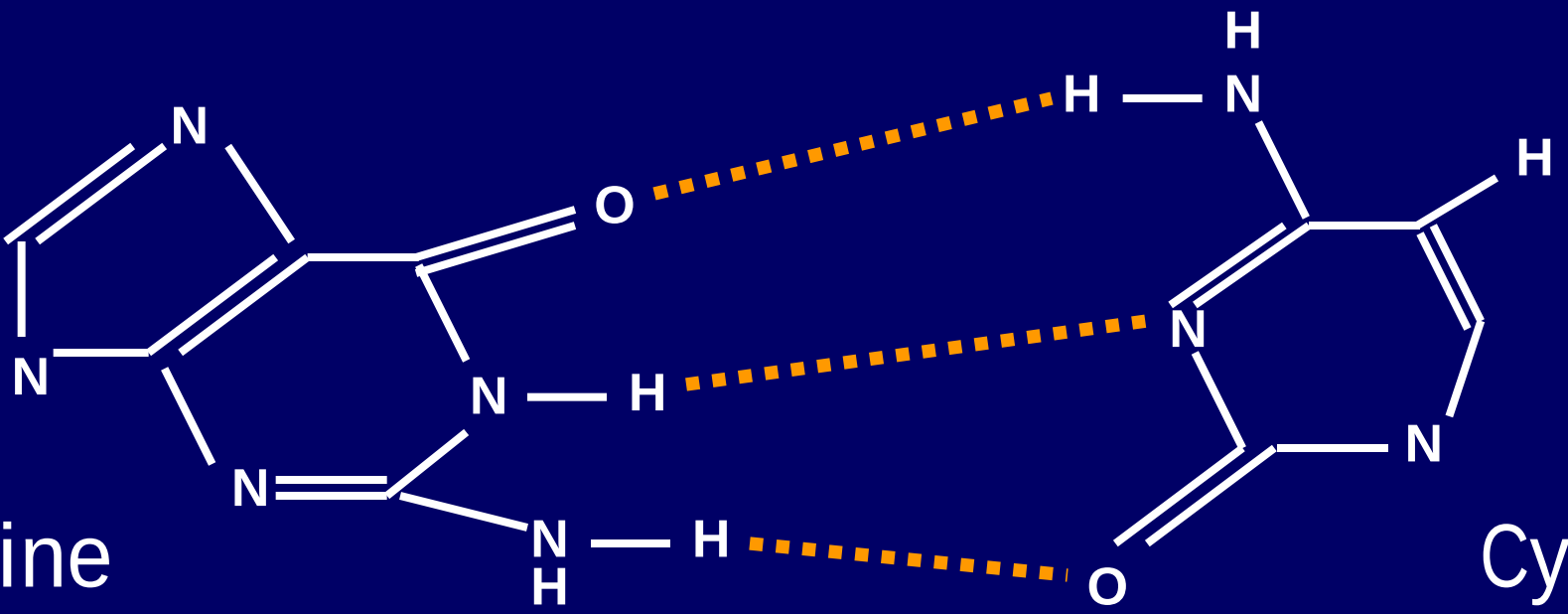
Cytosine

HYDROGEN BONDING



Adenine

Thymine



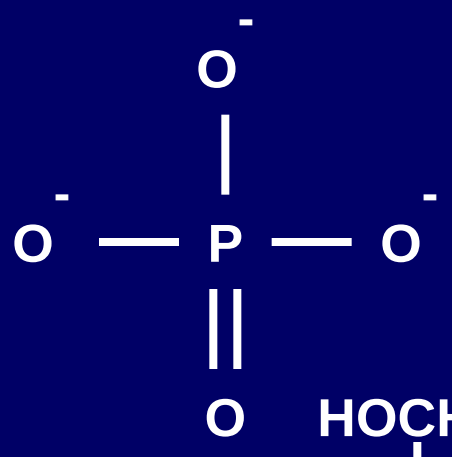
Guanine

Cytosine

COMPONENTS

DNA

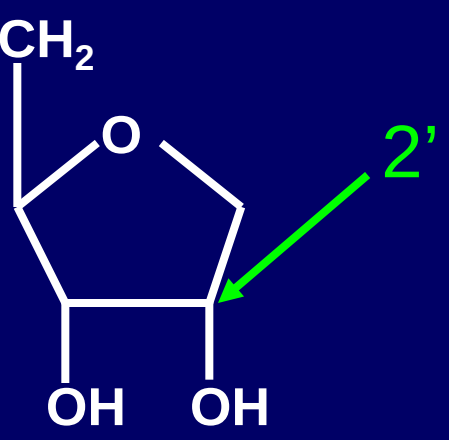
Phosphate



RNA

Phosphate

Pentose
(deoxyribose)

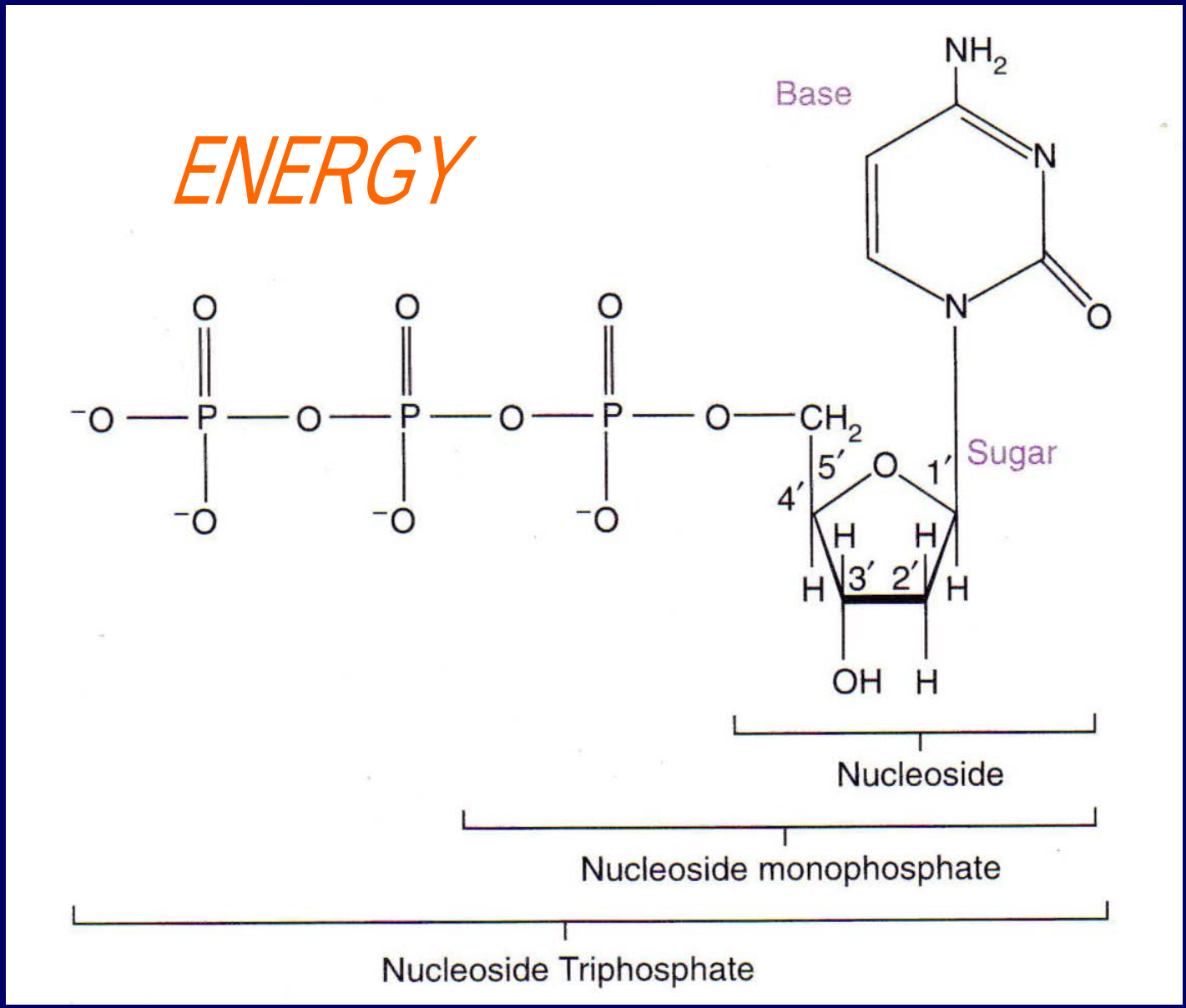


Pentose
(ribose)

Base
(thymine)

Base
(uracil)

NOMENCLATURE



DOUBLE STRANDED EVIDENCE

- G + C content

  Previous tetranucleotide hypothesis:

  DNA consists of equal quantities of 4 bases

  Chargaff ratios (1952)

Organic Material	Percent adenine	Percent thymine	Percent guanine	Percent cytosine
Human liver	30.3	30.3	19.5	19.3
Mycobacterium tuberculosis	15.1	14.6	34.9	35.4

DOUBLE STRANDED EVIDENCE

- G + C content

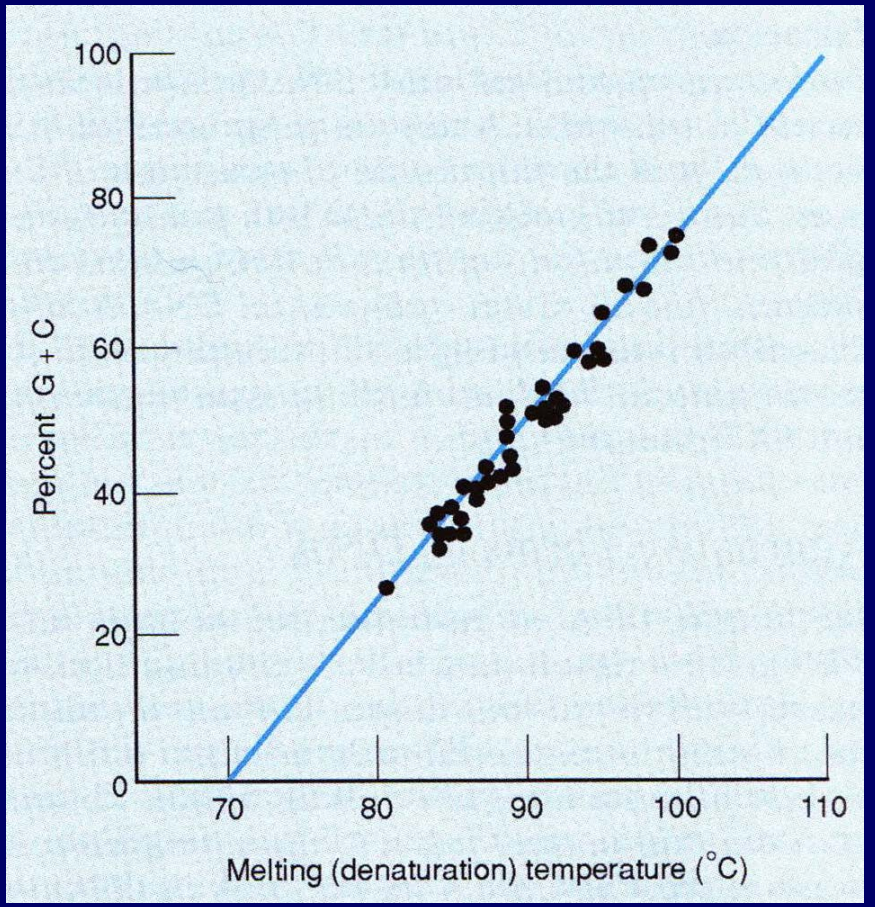
- Polarity

  Hydrogen bonding can only occur if chains go in opposite directions

  5’ end is phosphate; 3’ end is hydroxyl

DOUBLE STRANDED EVIDENCE

- G + C content
- Polarity
- Hydrogen bonding

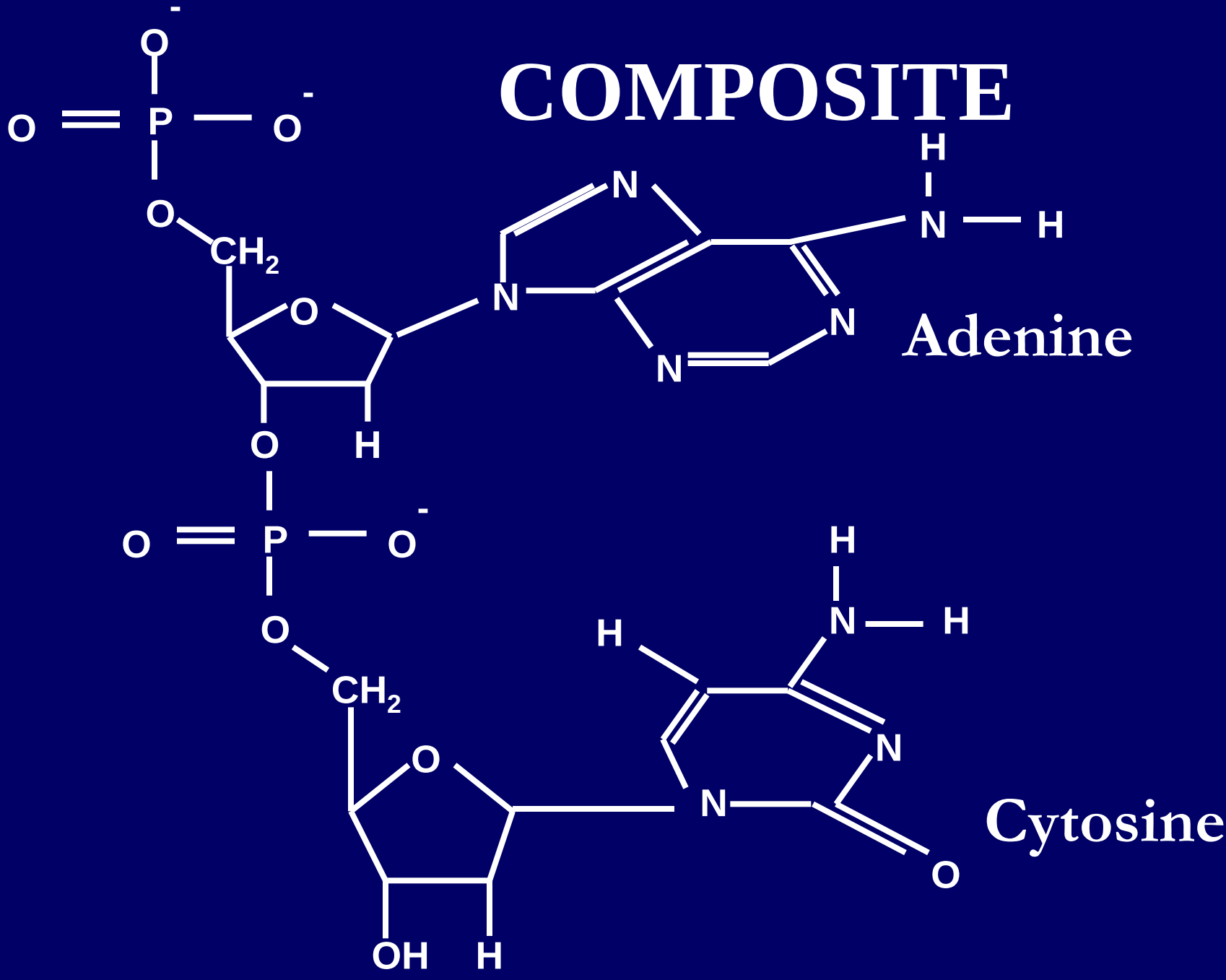


Intrinsically weak
Susceptible to heat (denaturation)

WHY PHOSPHATE??

- Easily form linking bonds
  - Ester bonds stable, yet can be hydrolyzed
  - Removal of nucleotide (repair) without denaturation
- Phosphate remains negatively charged
  - ↓ chance of spontaneous nucleophilic attack
  - Nucleotides & DNA stay inside membranes

COMPOSITE


O
P
O
O
CH2
O
N
N
N
N
H
N
H
Adenine
O
H
O
P
O
O
CH2
O
H
N
H
H
N
N
O
OH
H
Cytosine

Molecular Diagnostics Classifications

NUCLEIC ACID HYBRIDIZATION

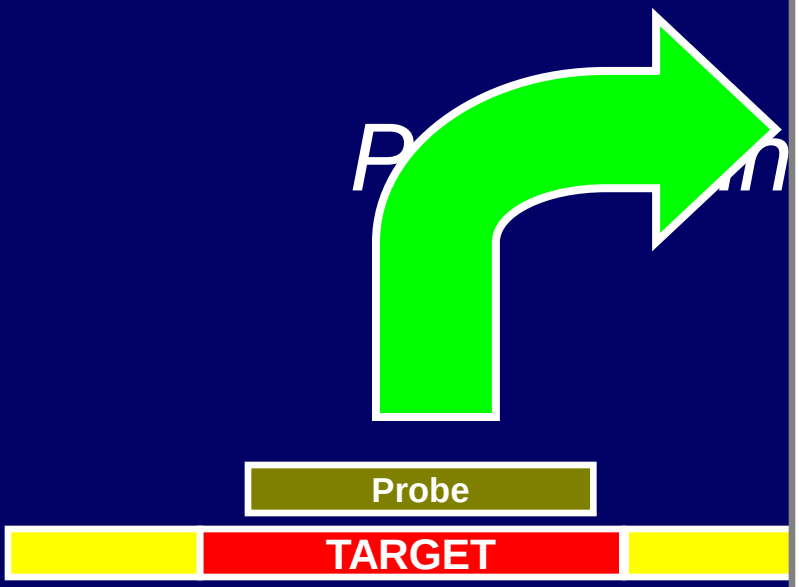
Probe anneals to target of interest

NUCLEIC ACID HYBRIDIZATION

Probe anneals to target of interest

DETECT

NUCLEIC ACID HYBRIDIZATION



COGNATE HYBRIDIZATION

(62° C; following denaturation)

Nucleic acid probe

TGGCTAACGTT

…….ACCGATTGCAA…….

Sequence-specific
(single-stranded) DNA template

Diagnostic Application: Hybridization

PROBE TECHNOLOGY

- Specificity/sensitivity dependent upon size of probe (stringency)
- Shorter probes → quicker completion

PROBE TECHNOLOGY

- Sp… …vity o… of… …cy)
- Sl… …quic…

- More effective on colonial growth than on primary clinical specimens

1. LIQUID PHASE HYBRIDIZATION

- Target and single-stranded (ss) DNA free to "interact" in aqueous mixture (fast reaction)
- Digestion of non-hybridized ssDNA
- Recovery of remaining dsDNA hybrids

  Tricholoroacetic acid precipitation
  Hydroxyapatite column
  Hybridization protection assay

2. SOLID PHASE HYBRIDIZATION

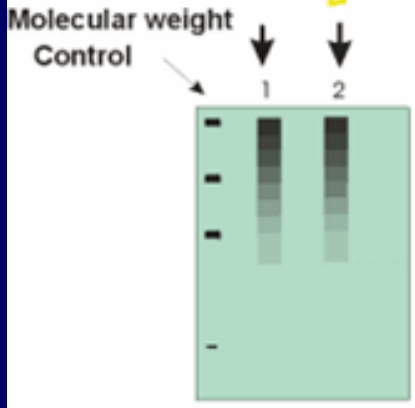
- Nucleic acid embedded on nitrocellulose membrane hybridized with nucleic acid probe in solution
- Unbound probe washed away
- Bound probe detected by fluorescence, radioactivity, luminescence, enzyme

SOUTHERN HYBRIDIZATION

- Facilitates size determination of DNA fragments
- Purified DNA digested with restriction endonuclease; electrophoresis
- Transfer to membrane for hybridization
- Inherited diseases; prenatal diagnosis

1. DNA is prepared

2. The DNA is cut with restriction enzymes and then loaded unto the gel

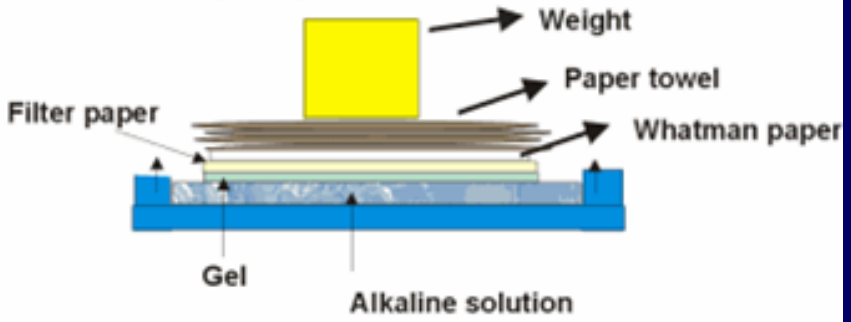


3. Gel Electrophoresis:

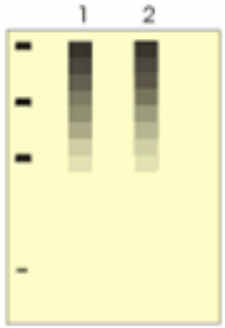
DNA looks like a smear after running through gel electrophoresis

4. Blotting:

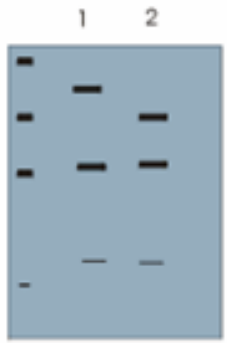
The gel is transferred to a nylon by capillary action



5. Nylon with the transferred DNA



6. The filter is hybridized with labeled DNA



7. Autoradiography

The DNA of interest is visible

NORTHERN HYBRIDIZATION

- Facilitates size determination of RNA fragments
- Electrophoretic separation of purified RNA
- Transfer to membrane for hybridization
- Not routinely utilized in diagnostic setting

3. *In situ* HYBRIDIZATION

- Advantages

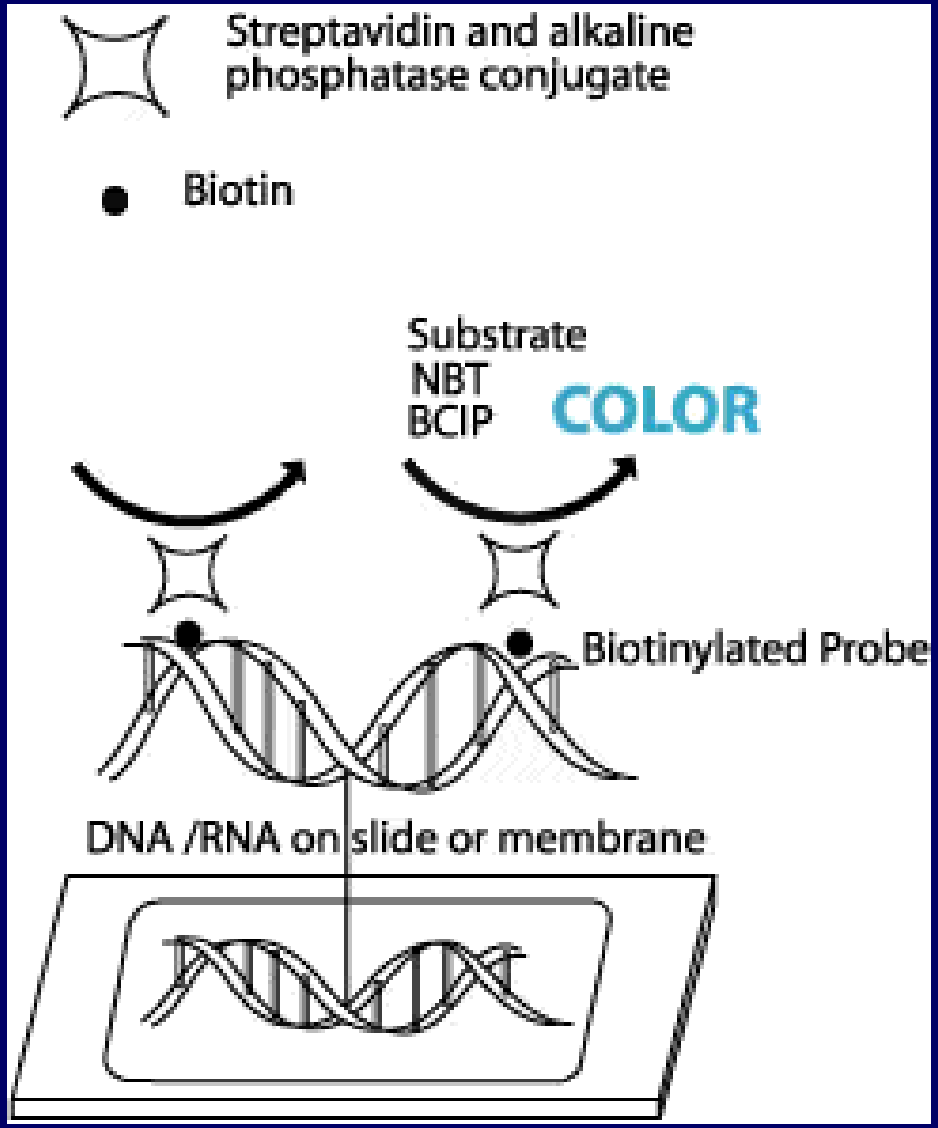
  Hybridizes target of interest

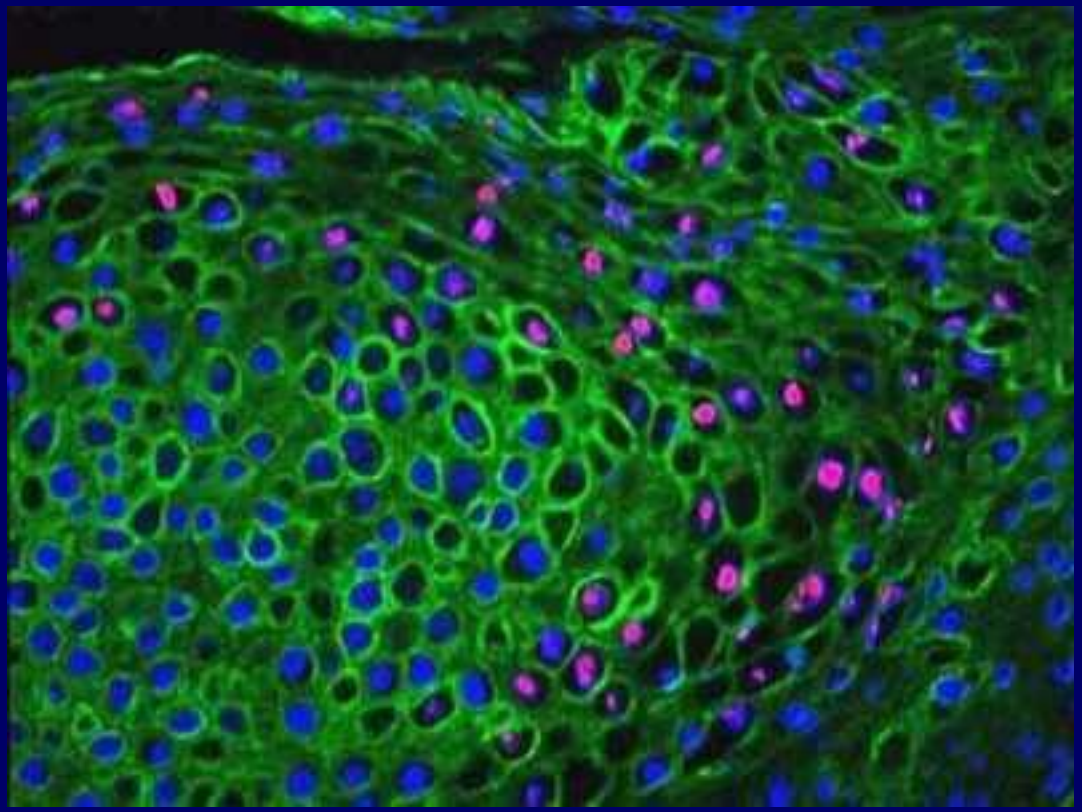
  At same time, can provide data on tissue morphology or host cell response

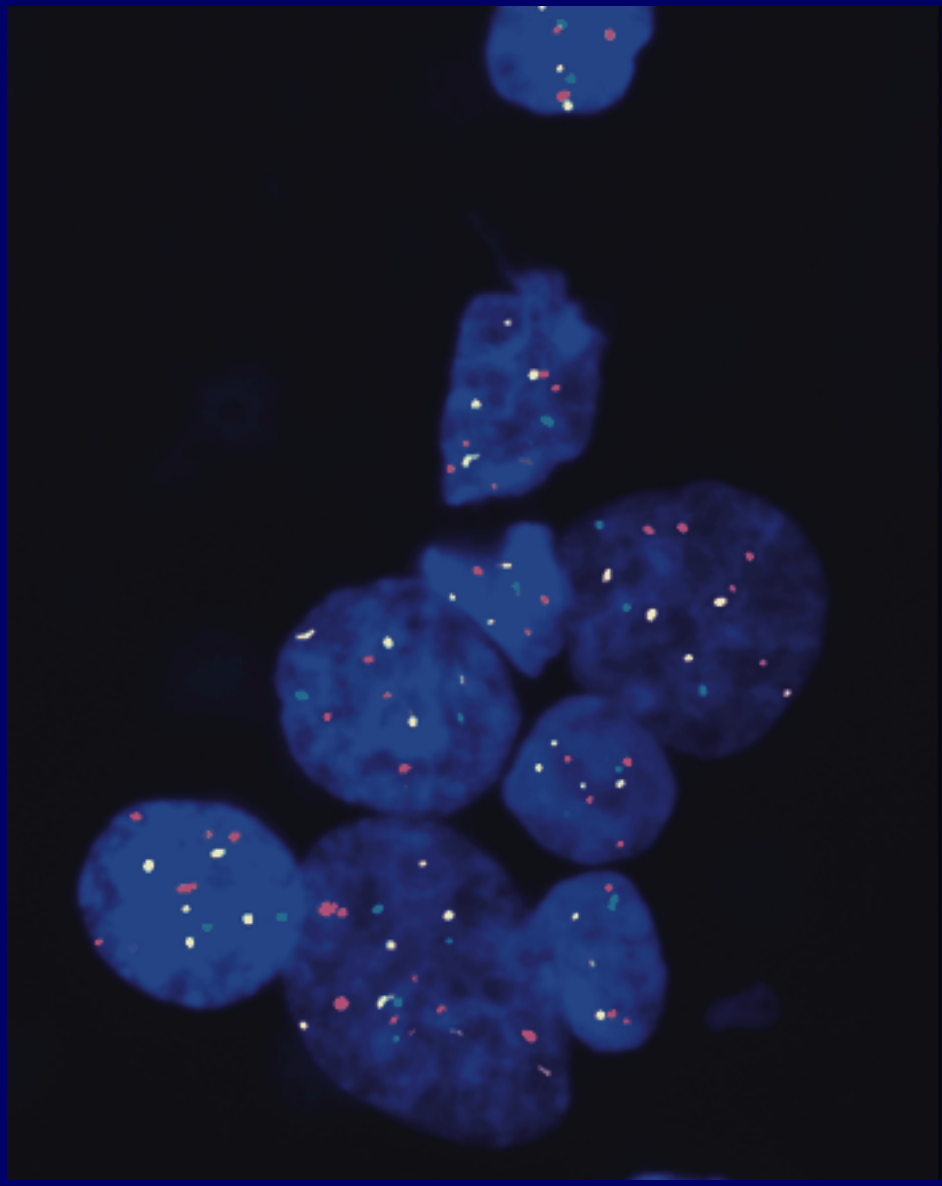
- Specimen preparation

  Whole cells or tissue embedded to slides
  Permeabilize cells while preserving structure
  Denature nucleic acid

In situ HYBRIDIZATION







DNA Replication

ORIGIN OF REPLICATION

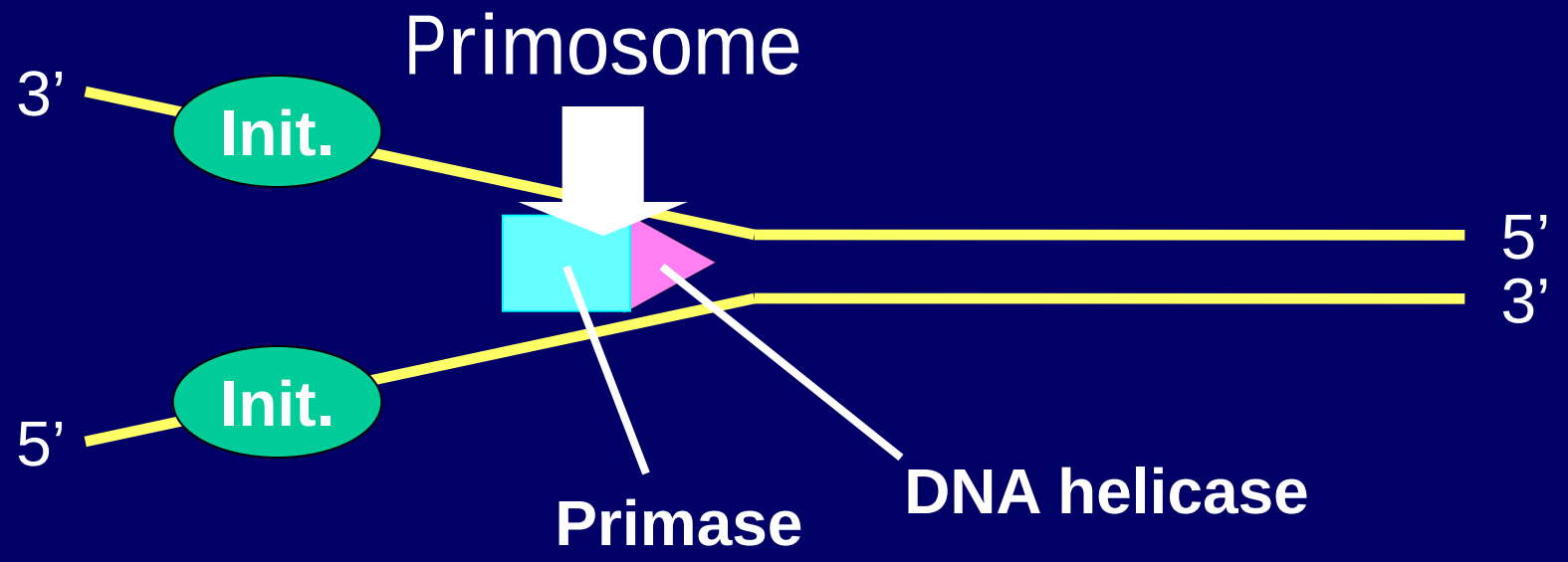
- 245-base pair sequence in *Escherichia coli*
- Initiator proteins

  Bind *oriC* to open double helix
  Assist in attachment of primosome

PRIMOSOME

- Complex of two proteins

  Primase (generates RNA primers)
  DNA helicase (unwinds DNA)



REPLICATION ENZYMES

- DNA polymerase I

  Fills in small DNA segments during replication and repair process

- DNA polymerase II

  Alternate repair polymerase if DNA polymerase I is damaged by mutation

- DNA polymerase III

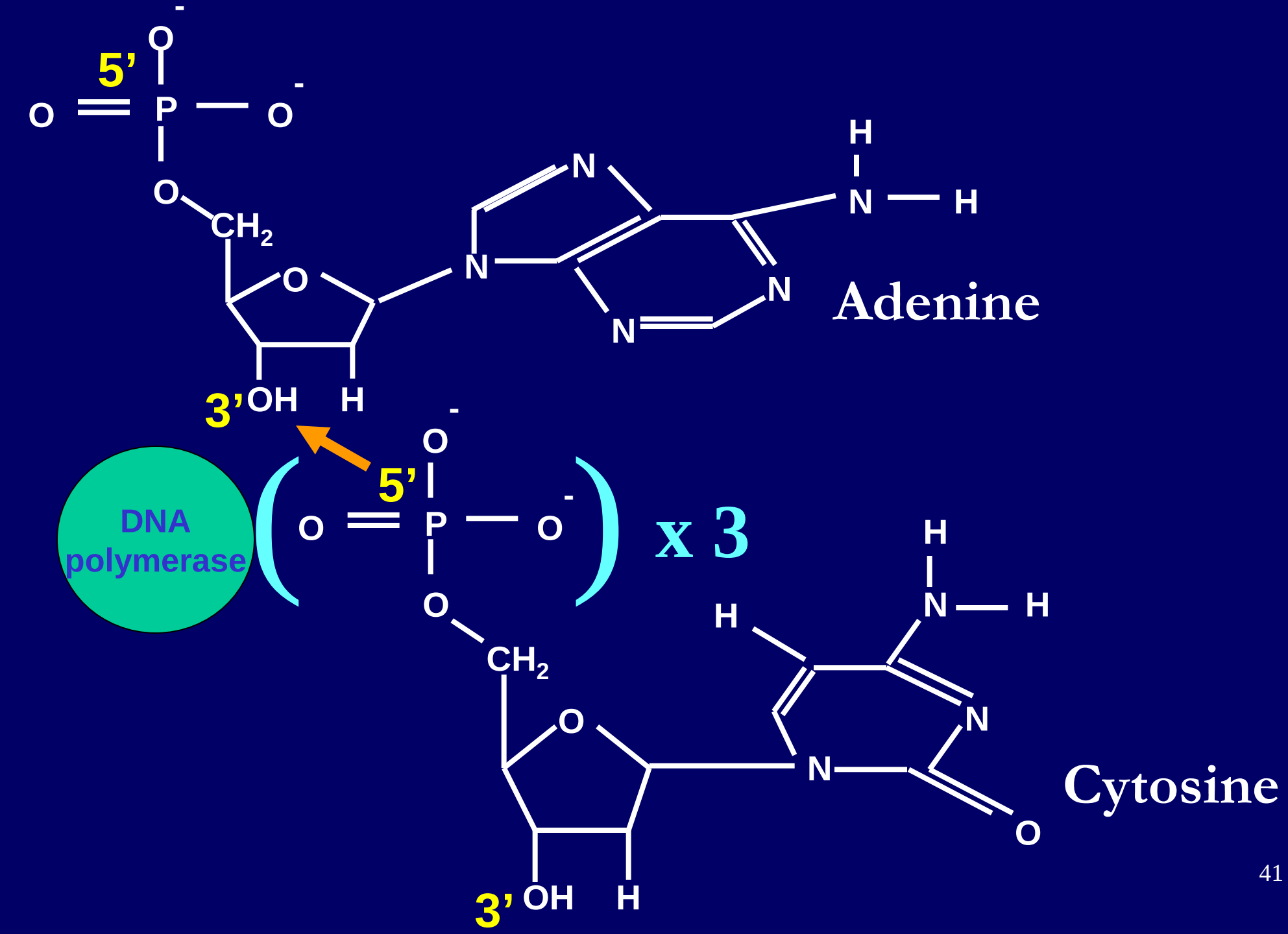
  Primary active polymerase during normal DNA replication

POLYMERASE SPECIFICITY

- Catalyze ester bond ONLY between first 5’ phosphate of new nucleotide and 3’ hydroxyl of previous nucleotide

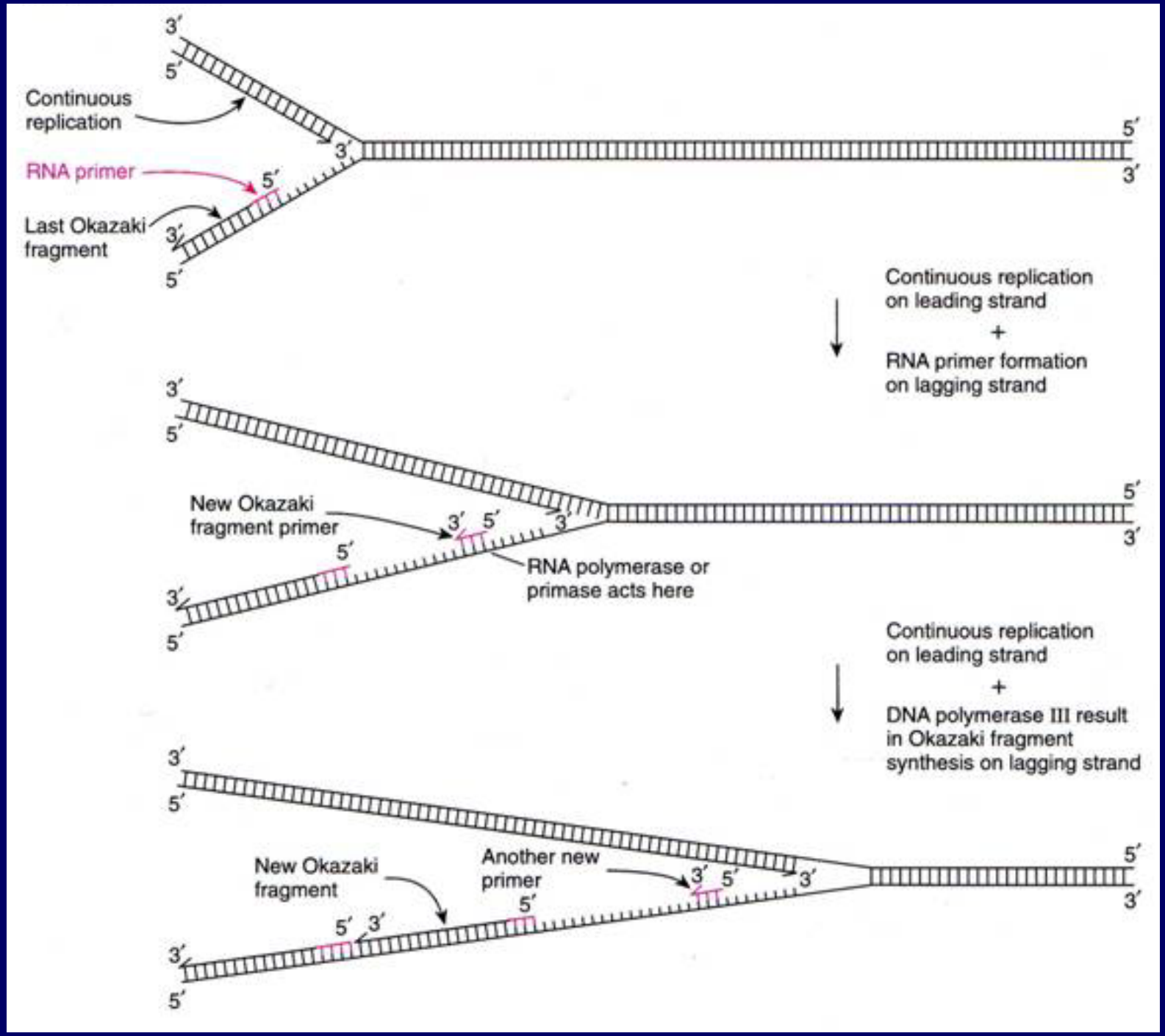
5’ to 3’ direction

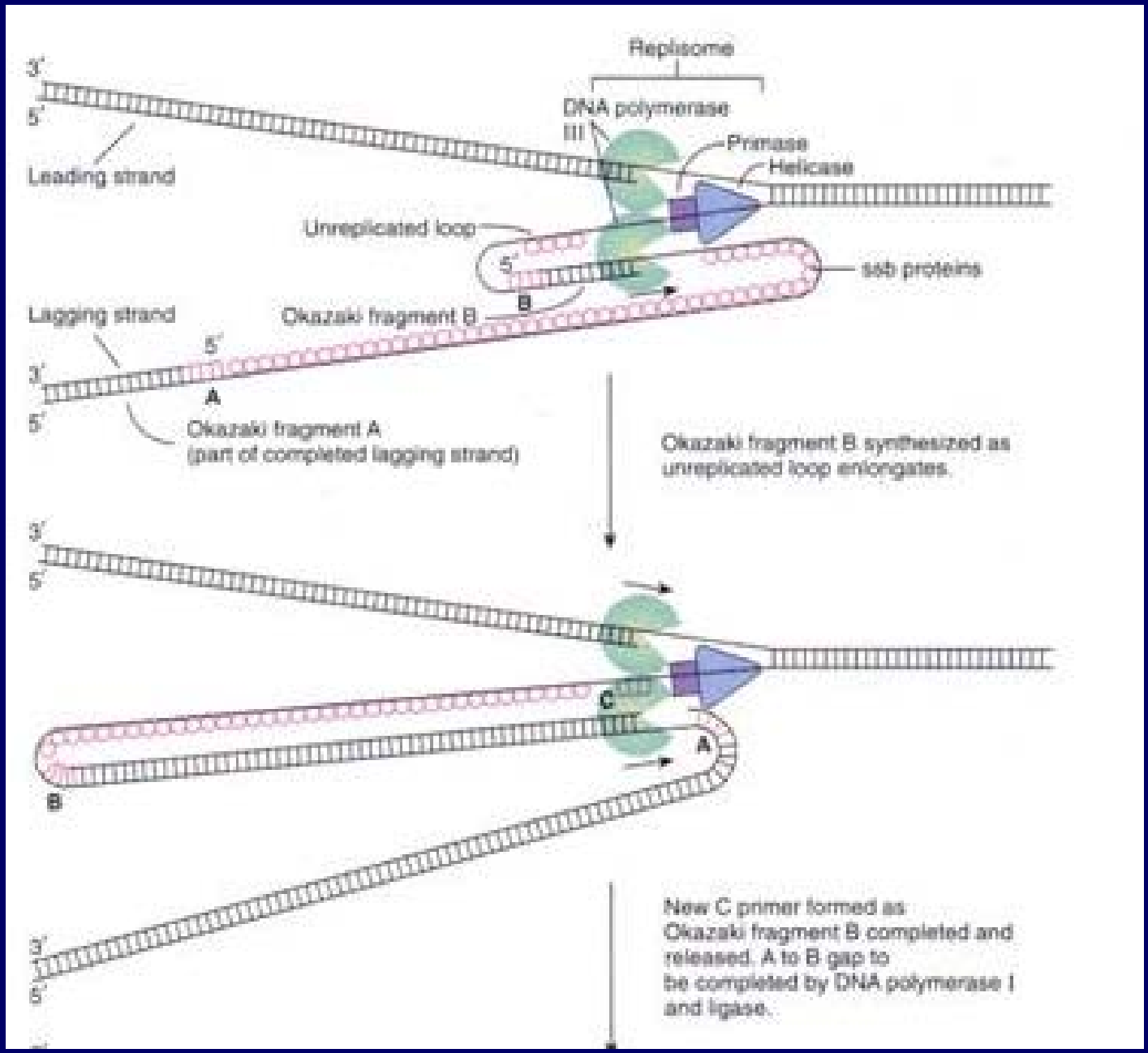
- Polymerase ONLY allows addition of phosphate to pre-existing hydroxyl


5’
3’OH
H
Adenine
DNA
polymerase
x 3
5’
3’ OH
H
Cytosine

WHY 5’ → 3’ ???

- Specificity of DNA polymerase
- Triphosphate energy source
- *In toto*, this creates necessity of both lagging strand (Okazaki fragments), leading strand templates for DNA replication


Continuous replication
RNA primer
Last Okazaki fragment
Continuous replication on leading strand
+
RNA primer formation on lagging strand
New Okazaki fragment primer
RNA polymerase or primase acts here
Continuous replication on leading strand
+
DNA polymerase III result in Okazaki fragment synthesis on lagging strand
New Okazaki fragment
Another new primer


Replisome
3′
5′
DNA polymerase III
Primase
Helicase
Leading strand
Unreplicated loop
5′
ssb proteins
B
Lagging strand
Okazaki fragment B
5′
3′
A
5′
Okazaki fragment A
(part of completed lagging strand)
Okazaki fragment B synthesized as unreplicated loop enlongates.
3′
5′
C
A
B
3′
5′
New C primer formed as Okazaki fragment B completed and released. A to B gap to be completed by DNA polymerase I and ligase.

Molecular Diagnostics Classifications

NUCLEIC ACID AMPLIFICATION TESTING (NAAT)

Amplify target of interest prior to detection

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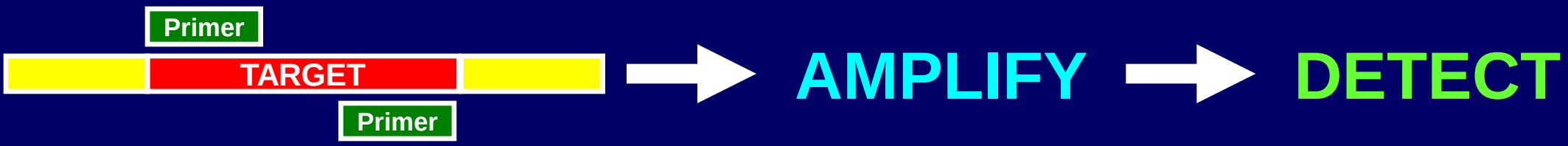
Amplify target of interest prior to detection



ANALYTICAL SENSITIVITY

Method	Approx copy no. detectable
Ethidium bromide staining	10^8
Radiolabeled oligonucleotide probes	10^6
Radiolabeled full-length probes	10^4
Enzyme-coupled probes	10^4
Chemiluminescent probes	10^4
Compound or branched probes	10^4
Nucleic acid amplification	≤ 10

Diagnostic Application: Polymerase Chain Reaction

Kary Mullis, Ph.D.

RESEARCH ARTICLE

Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia

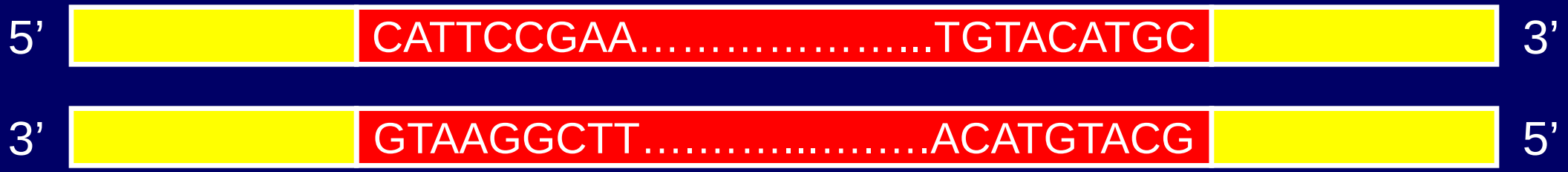
Randall K. Saiki, Stephen Scharf, Fred Faloona, Kary B. Mullis
Glenn T. Horn, Henry A. Erlich, Norman Arnheim

"The ability of the PCR procedure to amplify a target DNA segment in genomic DNA raises the possibility that its use may extend beyond that of prenatal diagnosis to other areas of molecular biology."

Science **230:** 1350-1354; 1985

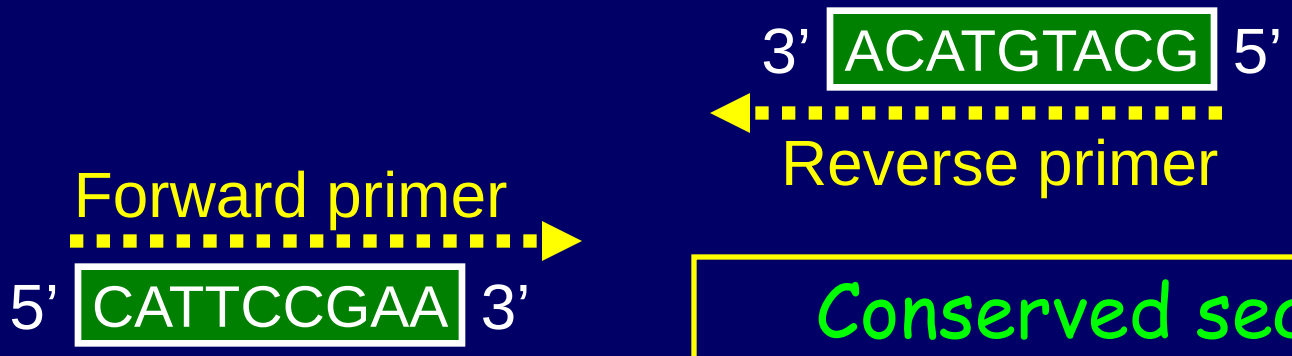
REQUIREMENTS FOR PCR

- Known (unique) DNA sequence



REQUIREMENTS FOR PCR

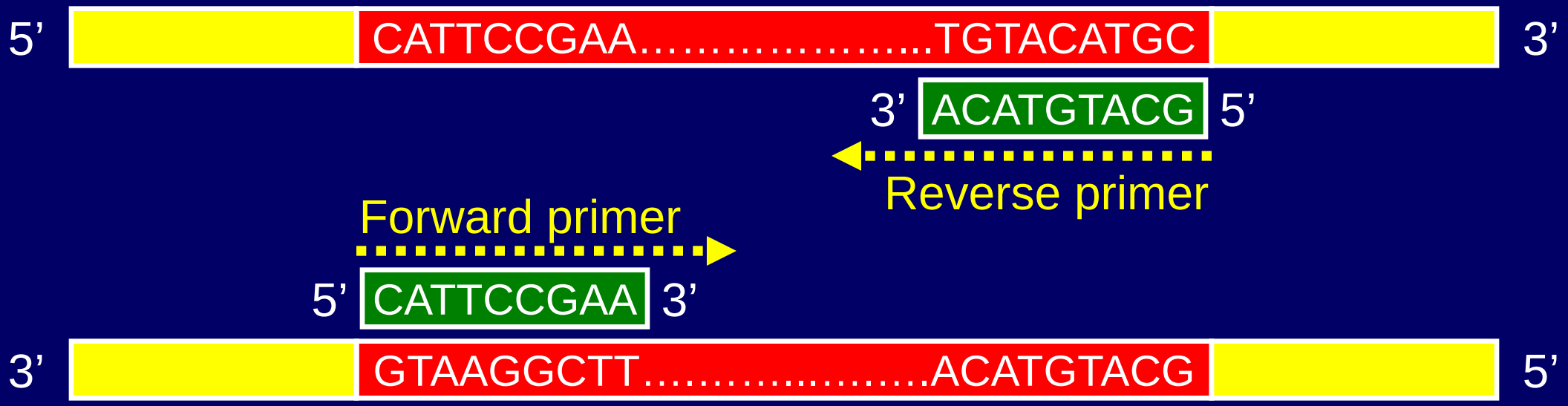
- Known (unique) DNA sequence
- Oligonucleotide primers



Conserved sequence
18-28 base pairs optimum
Avoid complementarity

REQUIREMENTS FOR PCR

- Known (unique) DNA sequence
- Oligonucleotide primers



REQUIREMENTS FOR PCR

- Known (unique) DNA sequence
- Oligonucleotide primers
- DNA polymerase

  Isolated from *Escherichia coli* in 1958
  Klenow fragment


ROSE
14

REQUIREMENTS FOR PCR

- Known (unique) DNA sequence

- Oligonucleotide primers
- DNA polymerase
- $MgCl_2$
- Deoxynucleotide triphosphates (dNTPs)
- Buffer

Master Mix

REQUIREMENTS FOR PCR

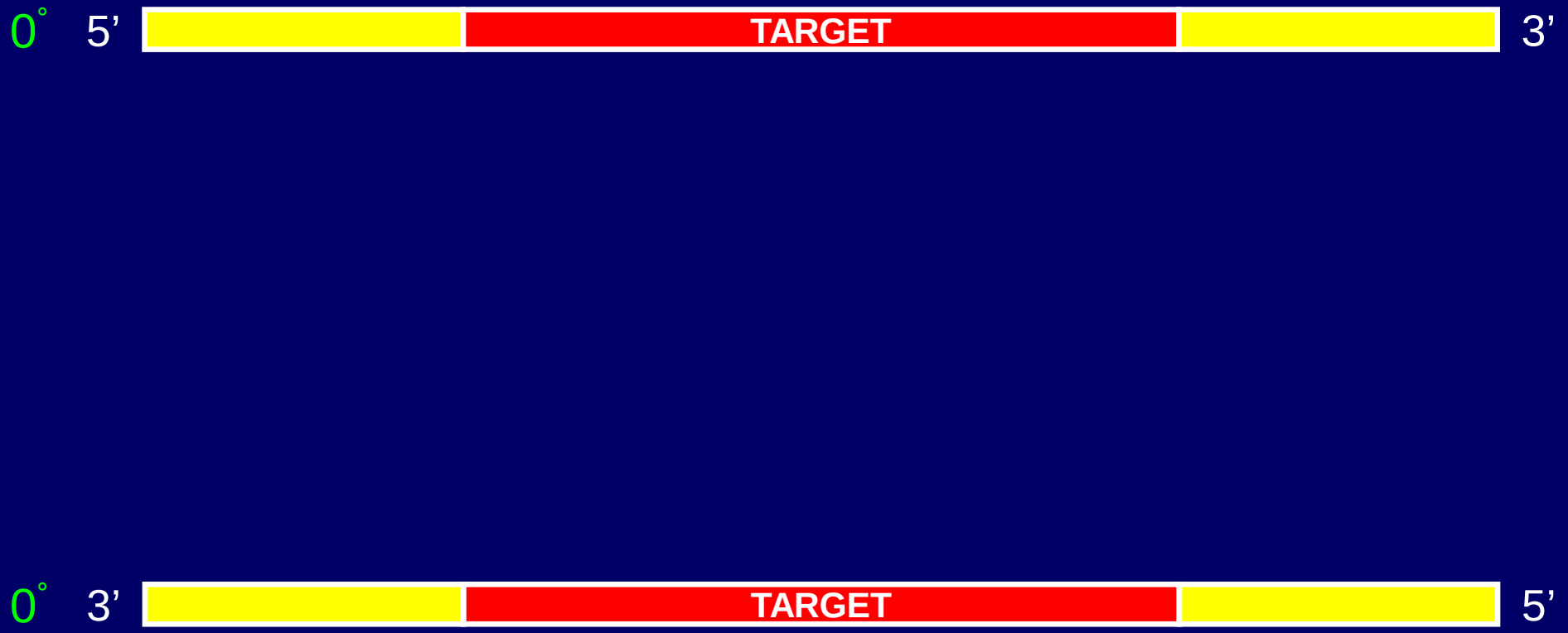
- Known (unique) DNA sequence
- Oligonucleotide primers
- DNA polymerase
- $MgCl_2$
- Deoxynucleotide triphosphates (dNTPs)
- Buffer
- Temperature modulation

PROTOCOL (Mullis *et al.*)

- Denature
  (95˚ C, 5 min)
- Anneal/hybridize
  (30˚ C, 2 min)
- Klenow extension
  (30˚ C, 2 min)
- Repeat 19 times;
  add Klenow each time

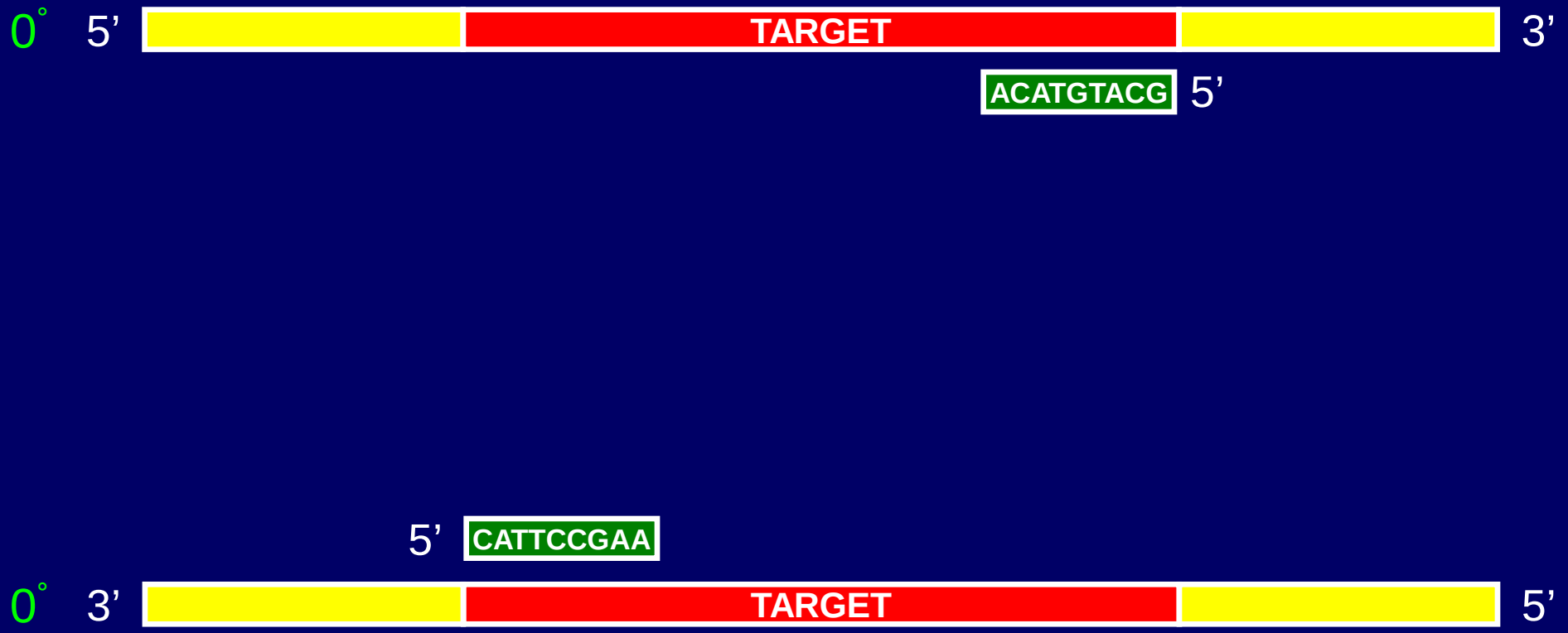
1

Isolated DNA



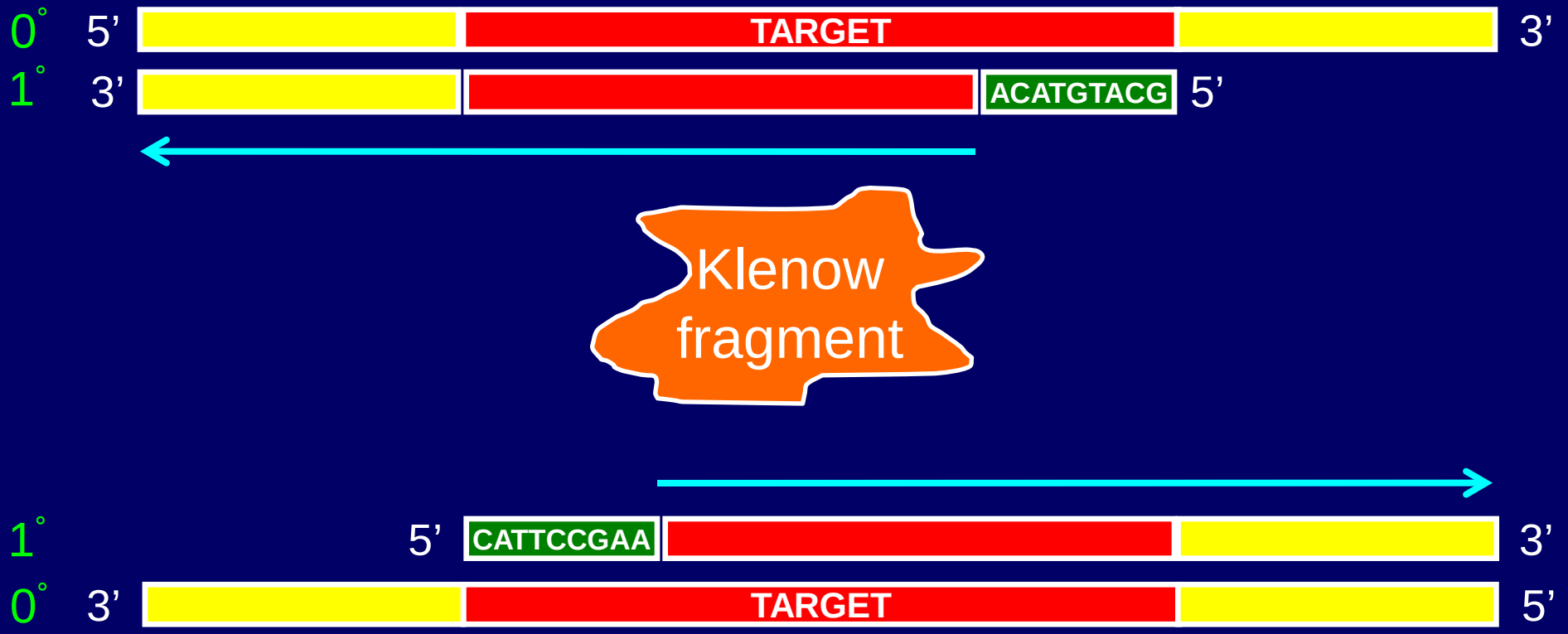
Denaturation

Cycle 1



Primer annealing

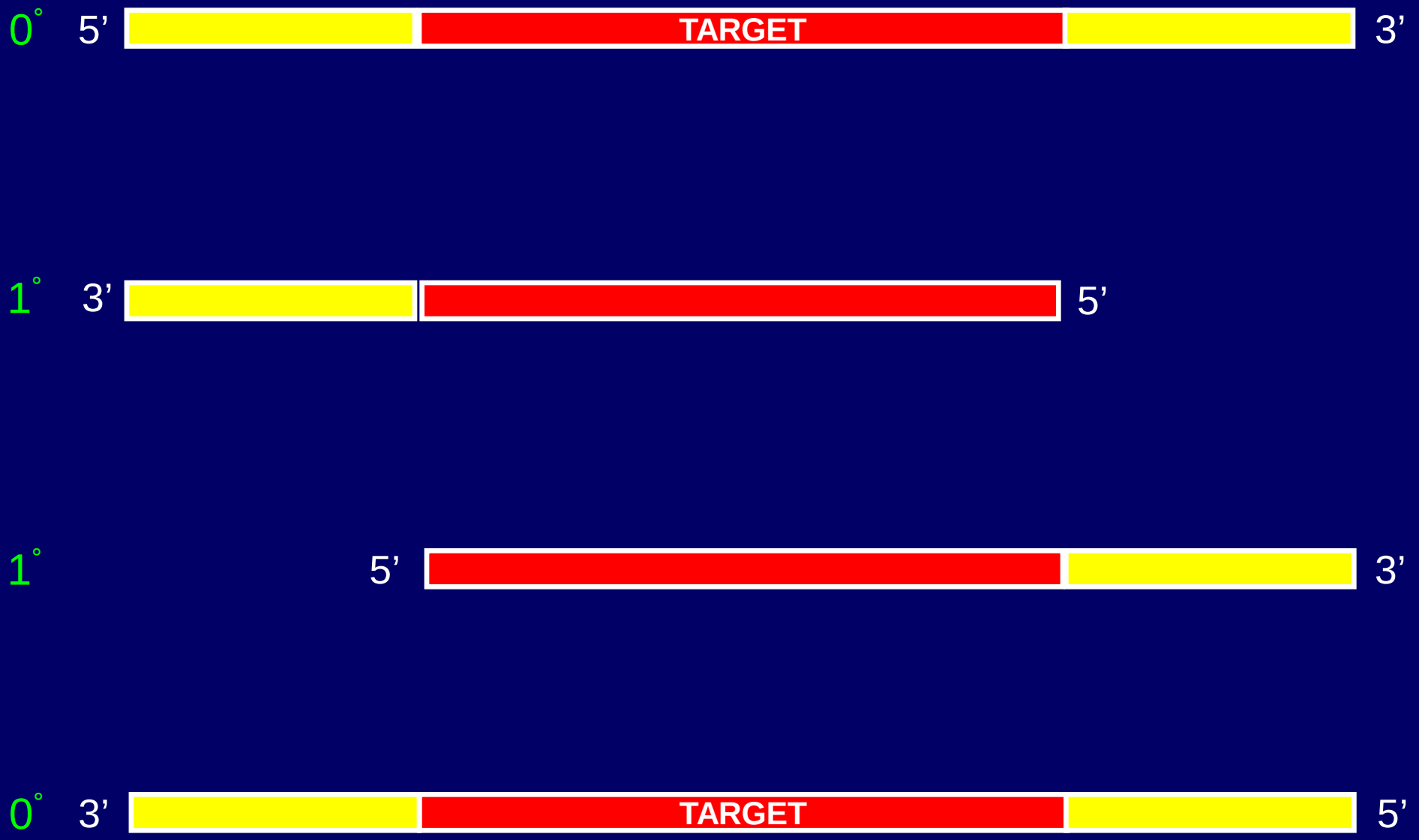
Cycle 1



2

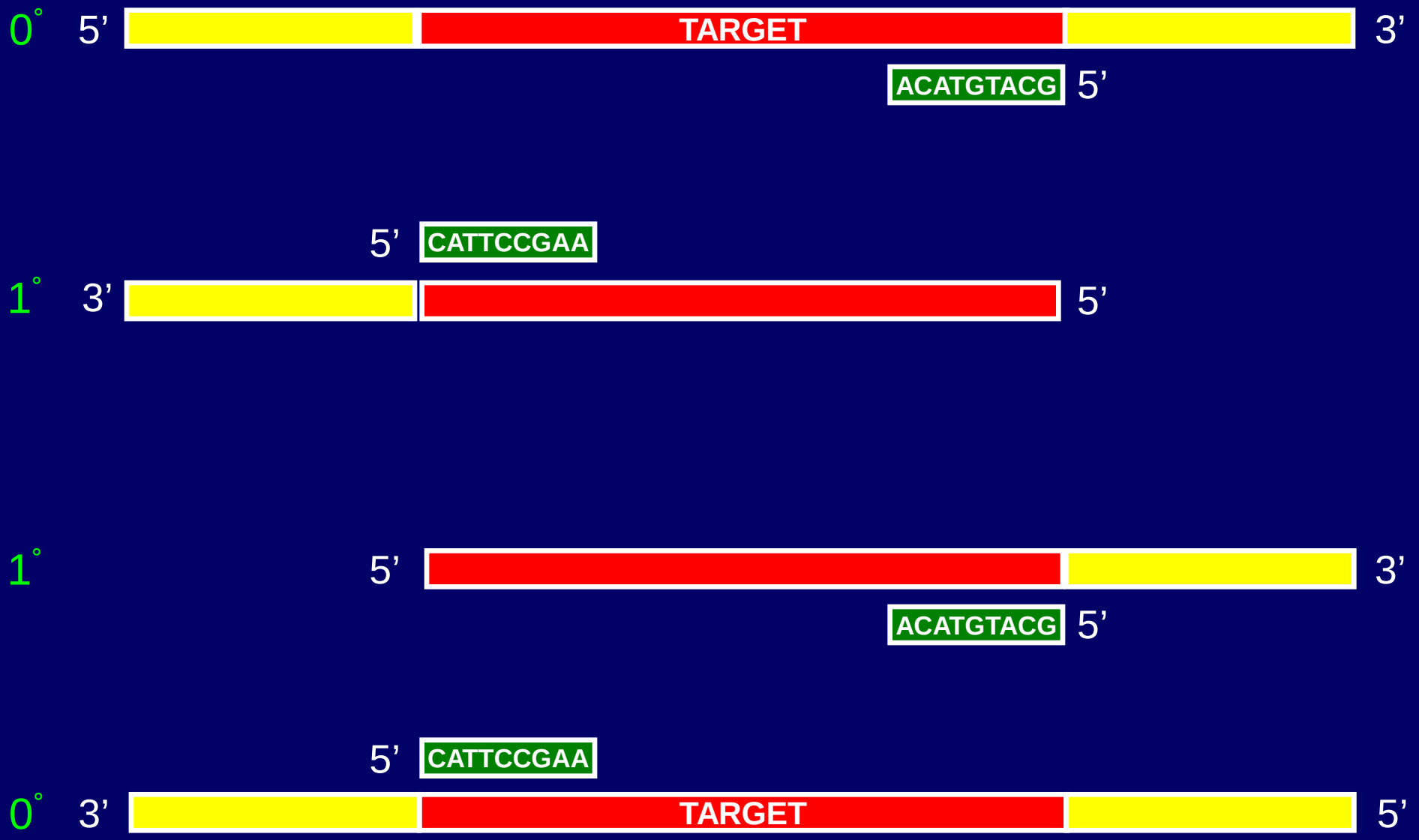
Primer extension (polymerization)

Cycle 1



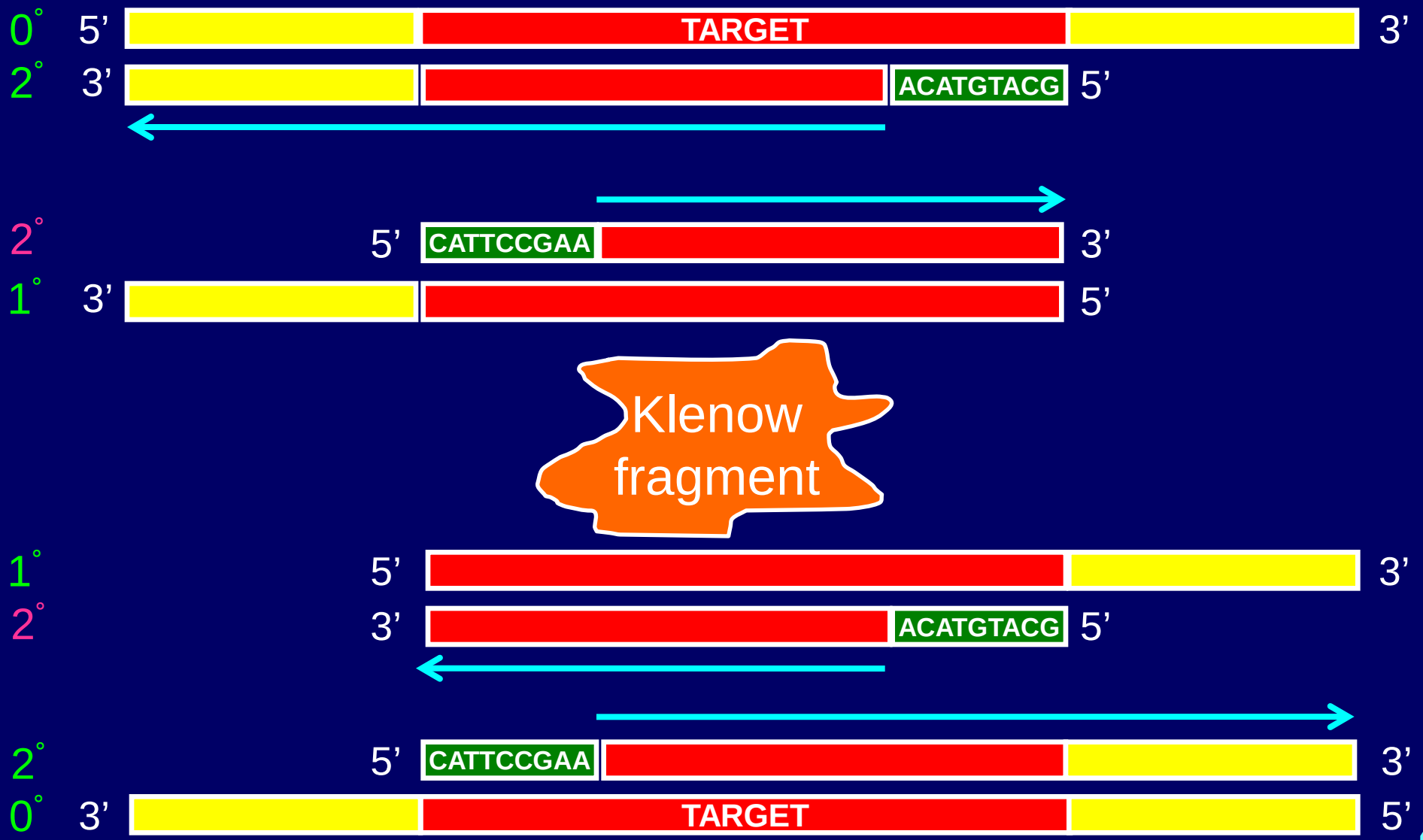
Denaturation

Cycle 2



Primer annealing

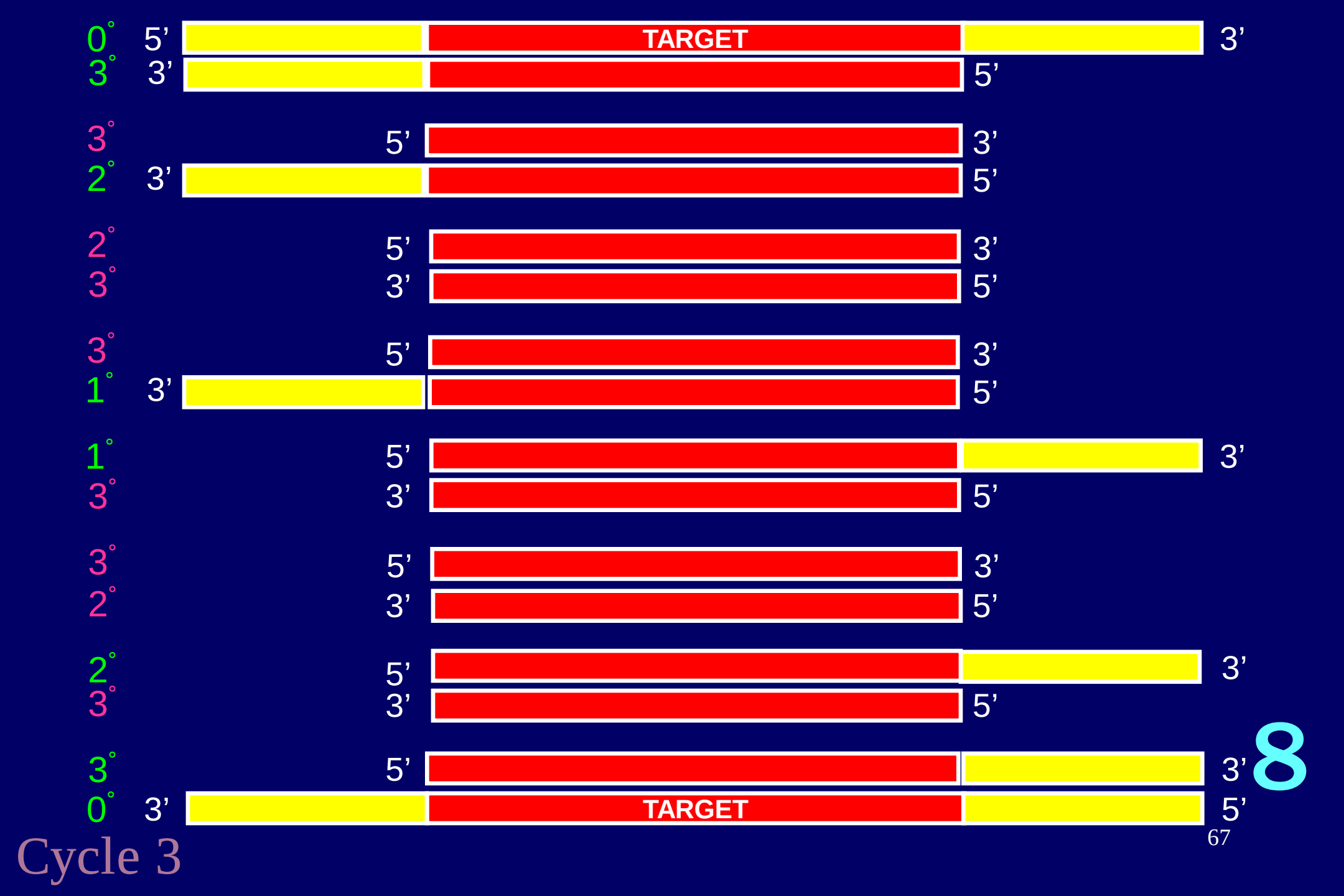
Cycle 2



4

Primer extension (polymerization)

Cycle 2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Cycle 3


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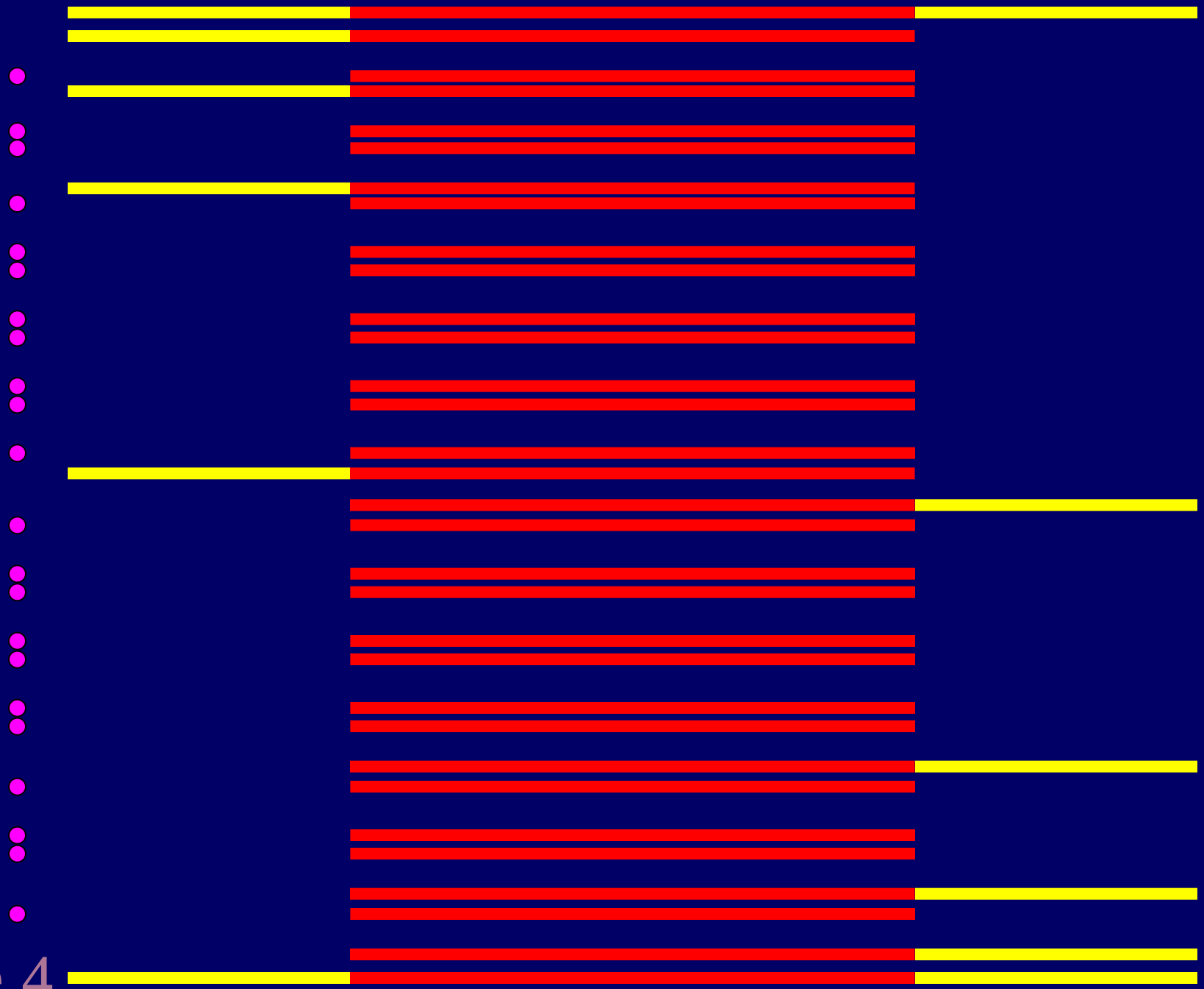
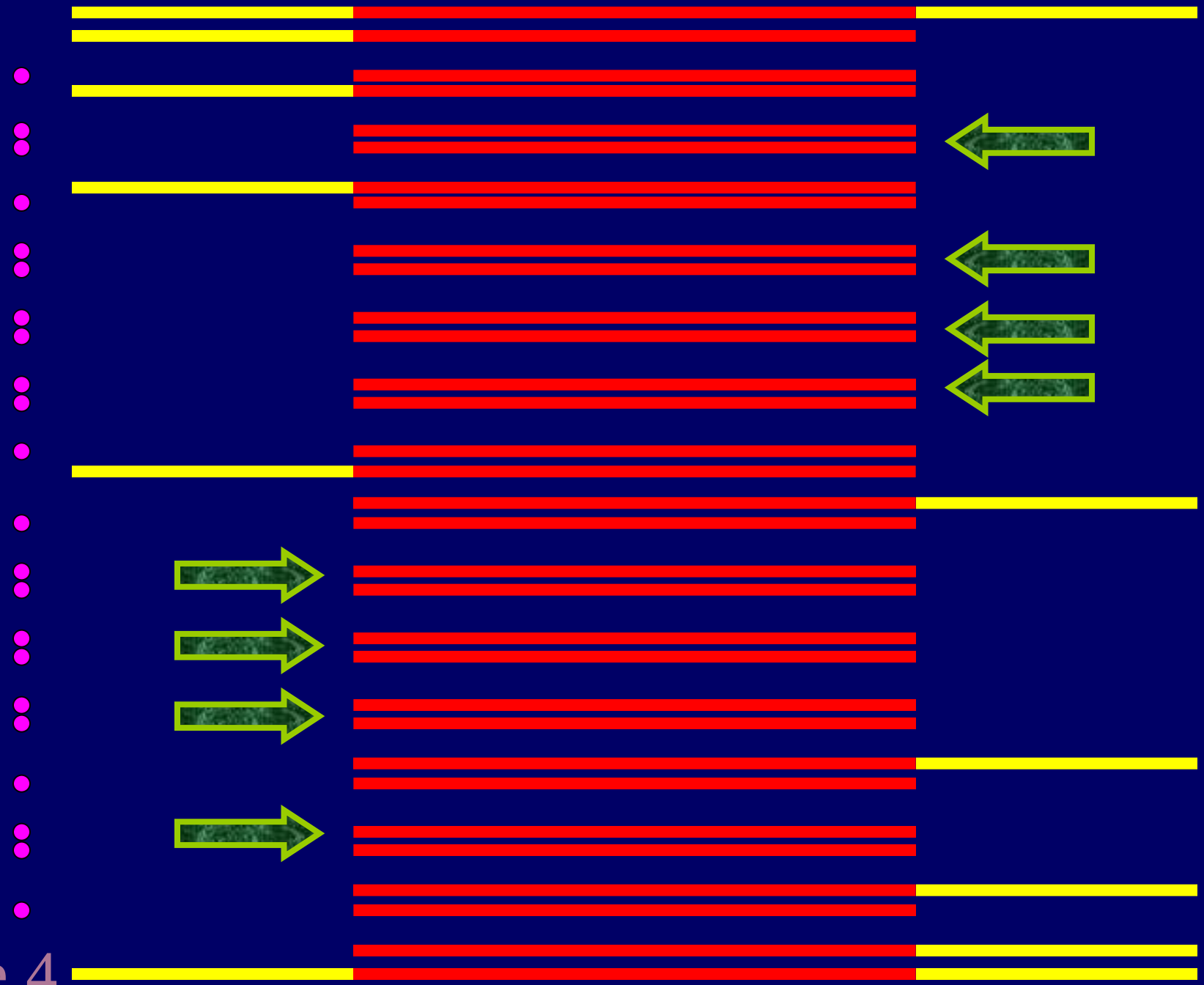
Cycle 3



16

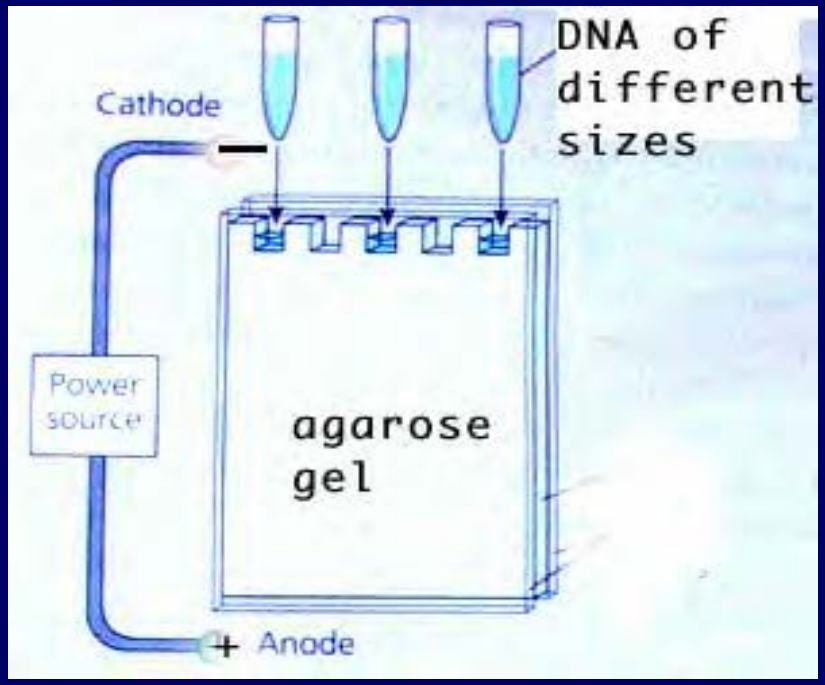
Cycle 4

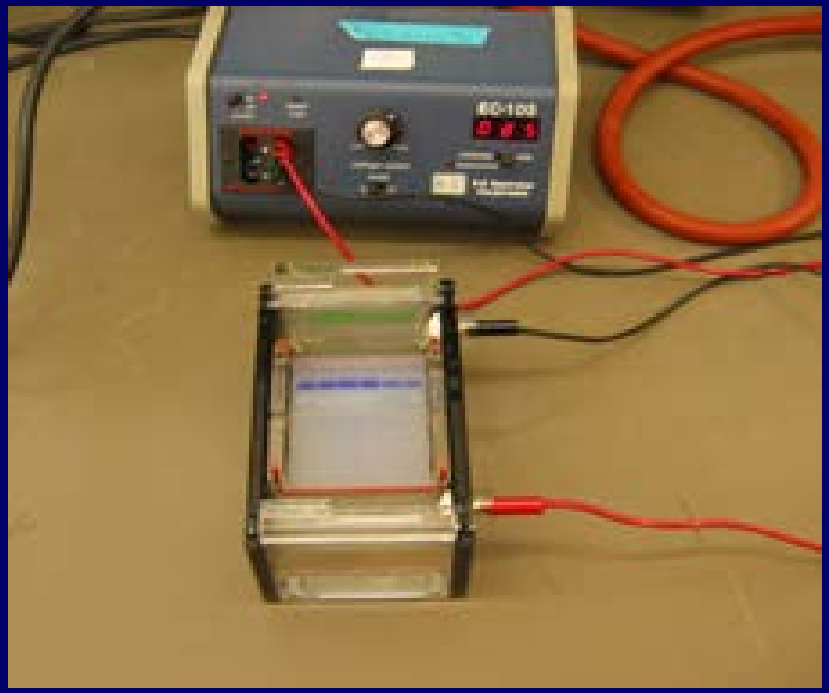


16

Cycle 4

VISUALIZE PRODUCT





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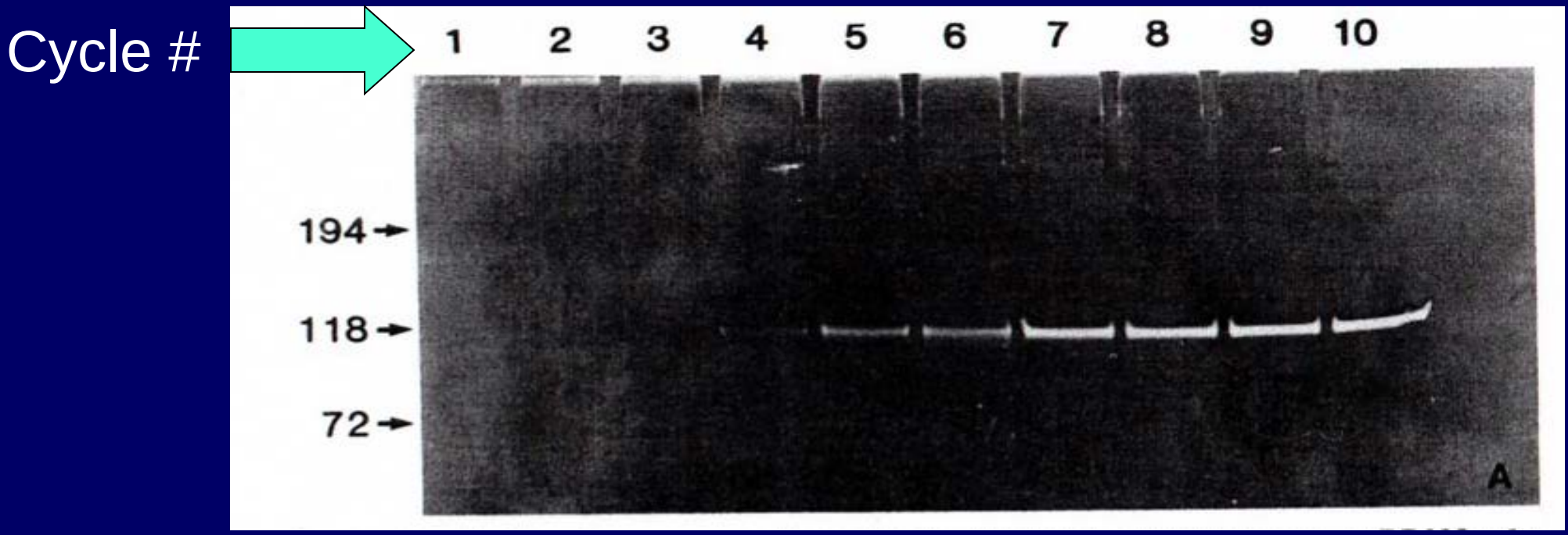
INCREASED YIELD PER CYCLE



Method Enzymol. **155:** 335-350; 1987

Theoretical yield: 2^N fold amplification

N = cycle number

REVOLUTIONARY FINDING

- Taq polymerase isolated from extreme thermophile *Thermus aquaticus*
- Thermostability eliminates necessity to replenish enzyme with each new cycle

Science **239:** 487-491; 1988

“OPTIMIZED” PCR PROTOCOL

- Denature (95˚ C)
- Anneal/hybridize (62˚ C)
- Extension (72˚ C)
- ~40 cycles

THE END

- DNA structure (hybridization)

  HER2-*neu*
  Dimorphic fungi
  Cystic fibrosis screening
  Human papillomavirus
  Factor V Leiden
  Prothrombin mutation
  Parental screening
  et cetera

- DNA replication (PCR)