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.

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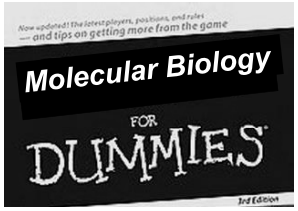
OUTLINE

I. Cell biology vignette
II. Molecular diagnostic application
III. Life-creating, life-changing events
    A. DNA structure
    B. DNA replication

2

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

3



…including myself

4

Some Perspective

5

MACROMOLECULES

- Proteins
- Lipids
- Carbohydrates
- Nucleic acids

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CENTRAL DOGMA

DNA

↓

RNA

↓

protein

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DNA Structure

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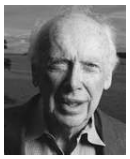

Watson & Crick

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

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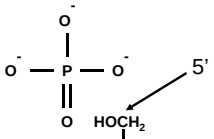
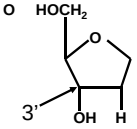
COMPONENTS

DNA

Phosphate



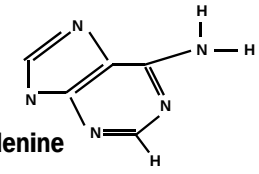
Pentose
(deoxyribose)



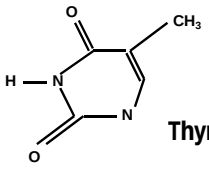
Base

10

PURINES PYRIMIDINES

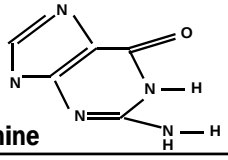


Adenine

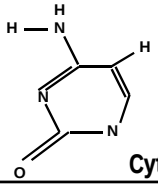


Thymine

AMINO



Guanine



Cytosine

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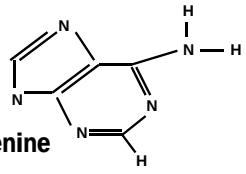
PURINES PYRIMIDINES



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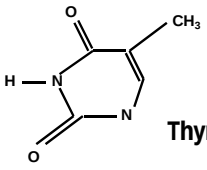
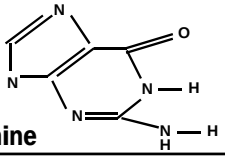
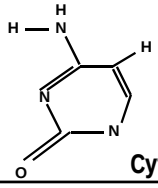


Thymine

KETO



Guanine



Cytosine

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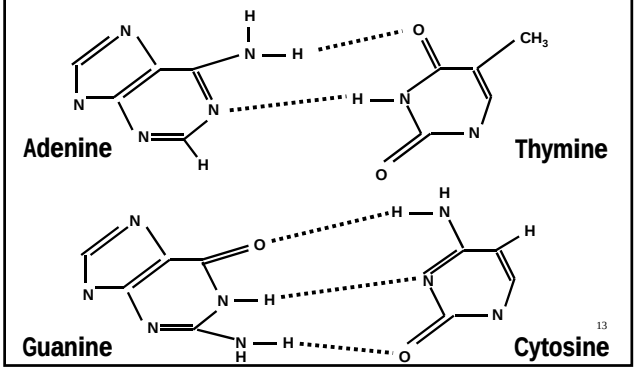
HYDROGEN BONDING



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COMPONENTS

DNA

Phosphate

Pentose (deoxyribose)

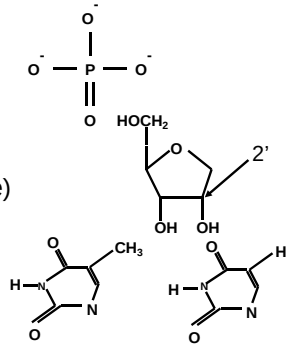
Base (thymine)

RNA

Phosphate

Pentose (ribose)

Base (uracil)



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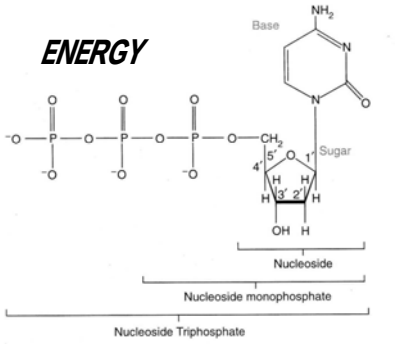
NOMENCLATURE

ENERGY



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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

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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

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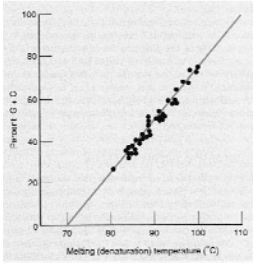
DOUBLE STRANDED EVIDENCE

- G + C content
- Polarity
- Hydrogen bonding



  Intrinsically weak
  Susceptible to heat (denaturation)

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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

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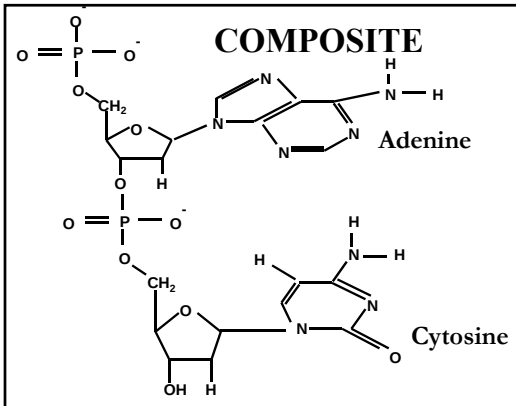
COMPOSITE



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Molecular Diagnostics Classifications

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NUCLEIC ACID HYBRIDIZATION

Probe anneals to target of interest

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NUCLEIC ACID HYBRIDIZATION

Probe anneals to target of interest

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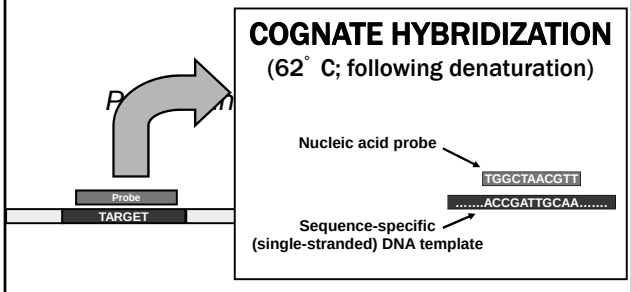
NUCLEIC ACID HYBRIDIZATION



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Diagnostic Application: Hybridization

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PROBE TECHNOLOGY

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

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PROBE TECHNOLOGY

- S ity
  of cy)
- S qui

- More effective on colonial growth than on primary clinical specimens

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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

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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

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SOUTHERN HYBRIDIZATION

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

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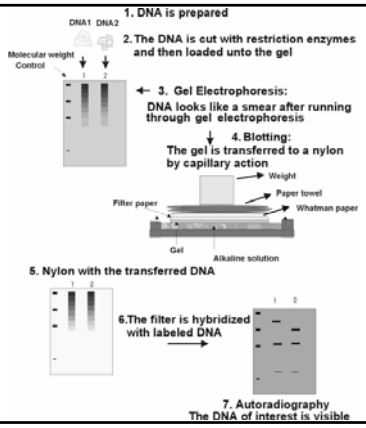


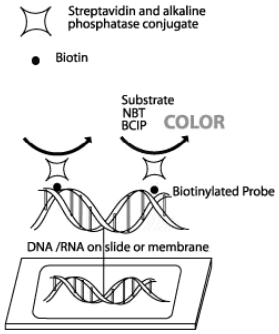
NORTHERN HYBRIDIZATION

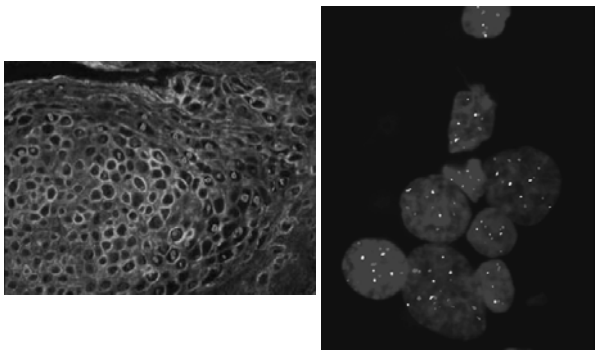
- Facilitates size determination of RNA fragments
- Electrophoretic separation of purified RNA
- Transfer to membrane for hybridization
- Not routinely utilized in diagnostic setting

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



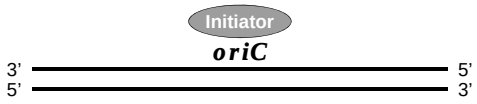


DNA Replication

ORIGIN OF REPLICATION

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

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



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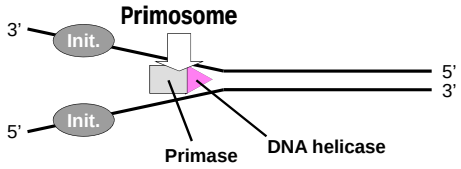
PRIMOSOME

- Complex of two proteins

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



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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

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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

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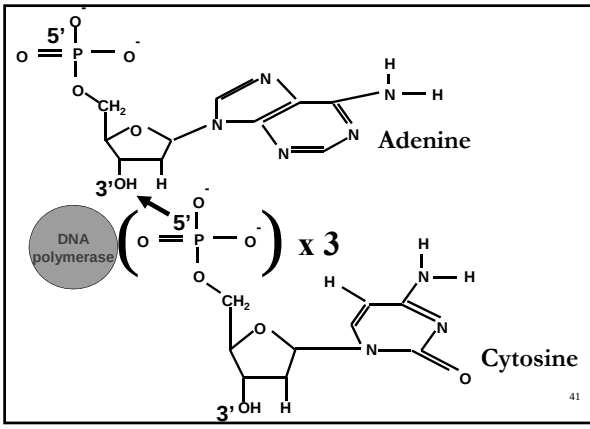

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WHY 5' → 3' ???

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

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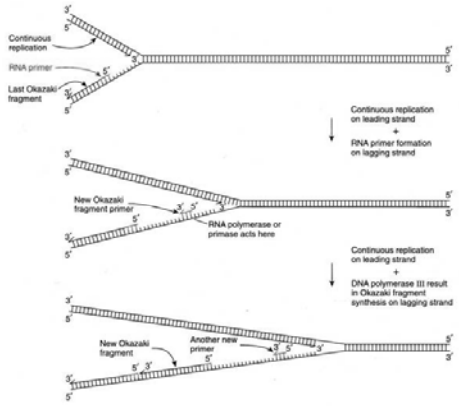

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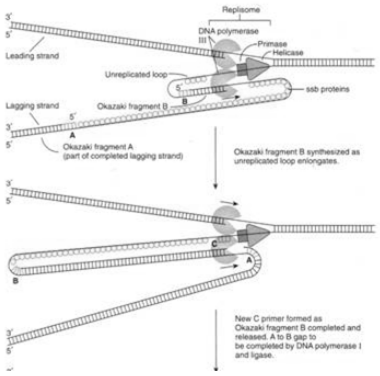

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Molecular Diagnostics Classifications

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NUCLEIC ACID AMPLIFICATION TESTING (NAAT)

Amplify target of interest prior to detection

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NUCLEIC ACID AMPLIFICATION TESTING (NAAT)

Amplify target of interest prior to detection

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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

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Diagnostic Application: Polymerase Chain Reaction

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Kary Mullis, Ph.D.

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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

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REQUIREMENTS FOR PCR

- Known (unique) DNA sequence

5' CATTCCGAA……..TGTACATGC 3'
3' GTAAGGCTT……..ACATGTACG 5'

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REQUIREMENTS FOR PCR

- Known (unique) DNA sequence
- Oligonucleotide primers

3' ACATGTACG 5'
Reverse primer

Forward primer
5' CATTCCGAA 3'

Conserved sequence
18-28 base pairs optimum
Avoid complementarity

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REQUIREMENTS FOR PCR

- Known (unique) DNA sequence
- Oligonucleotide primers

5' CATTCCGAA……..TGTACATGC 3'
3' ACATGTACG 5'
Reverse primer
Forward primer
5' CATTCCGAA 3'
3' GTAAGGCTT……..ACATGTACG 5'

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REQUIREMENTS FOR PCR

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

  Isolated from *Escherichia coli* in 1958
  Klenow fragment

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REQUIREMENTS FOR PCR

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

Master Mix

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REQUIREMENTS FOR PCR

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

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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

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1

Isolated DNA

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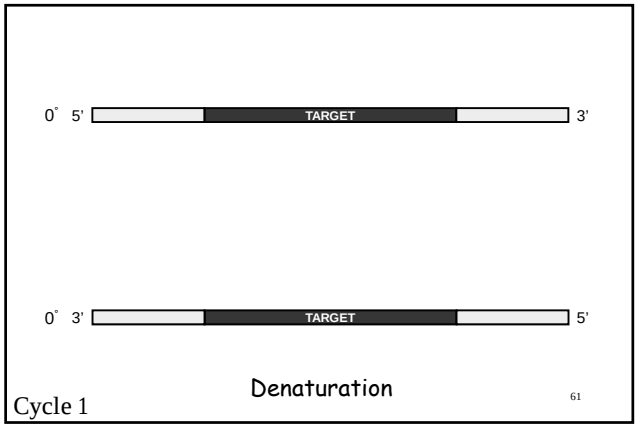
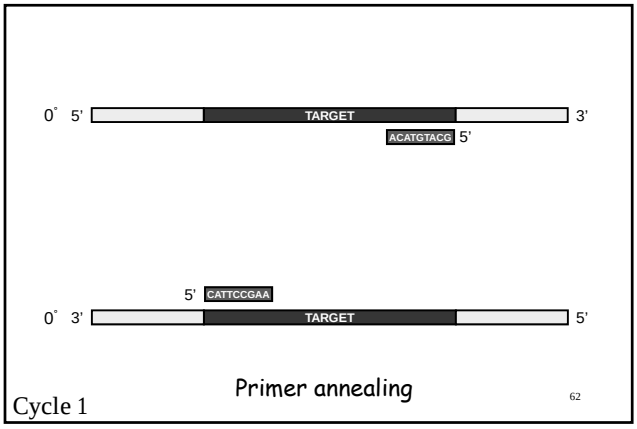


Denaturation

Cycle 1

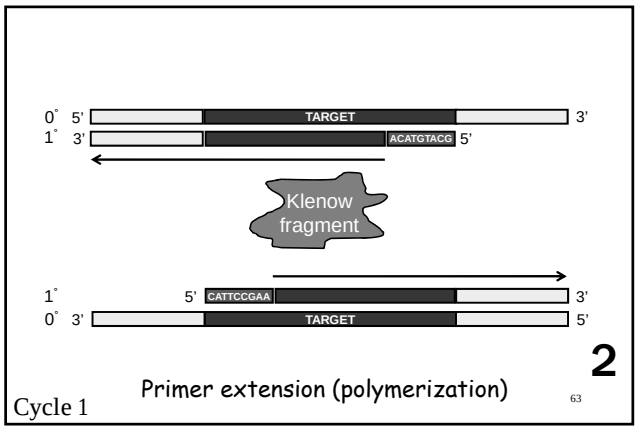
61



Primer annealing

Cycle 1

62



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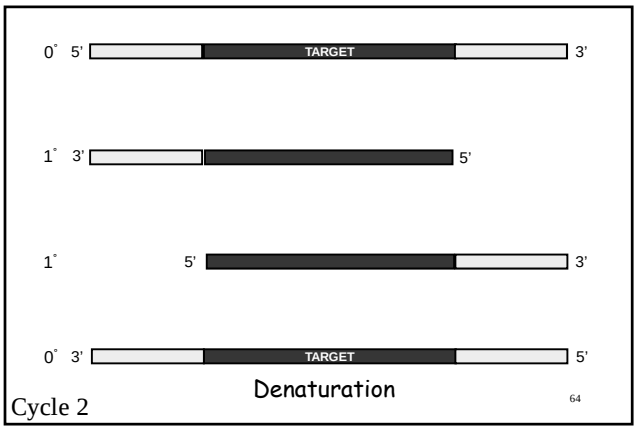
Primer extension (polymerization)

Cycle 1

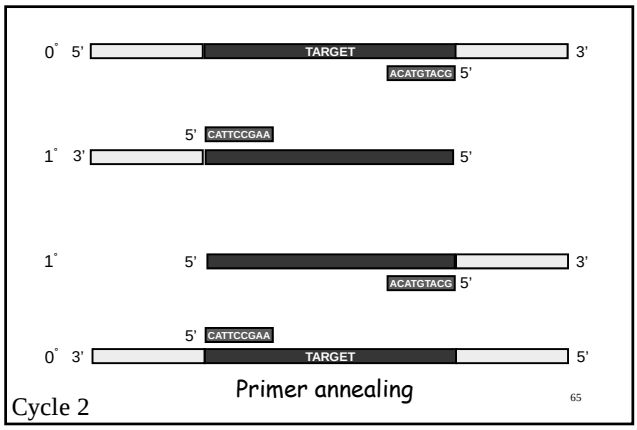
63



Denaturation

Cycle 2

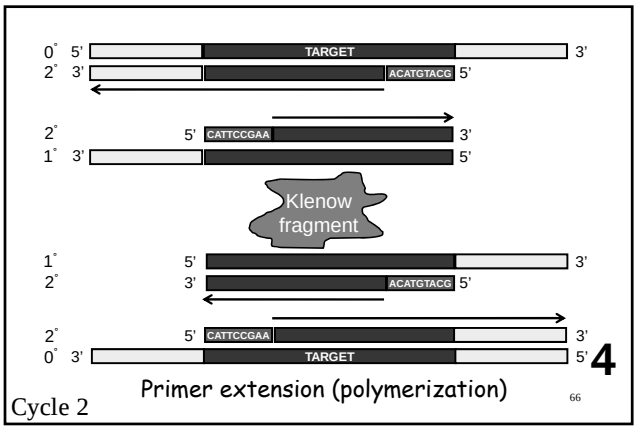
64



Primer annealing

Cycle 2

65



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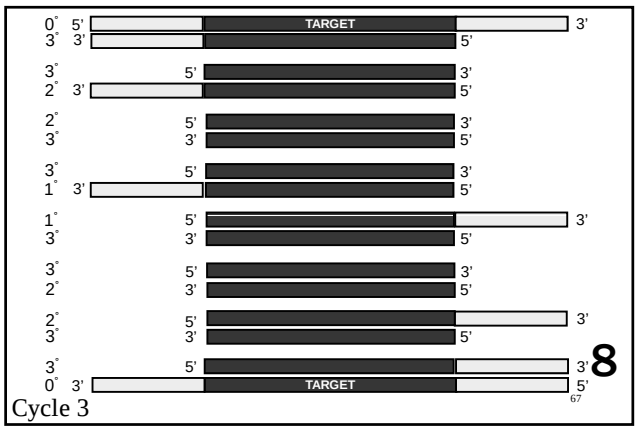
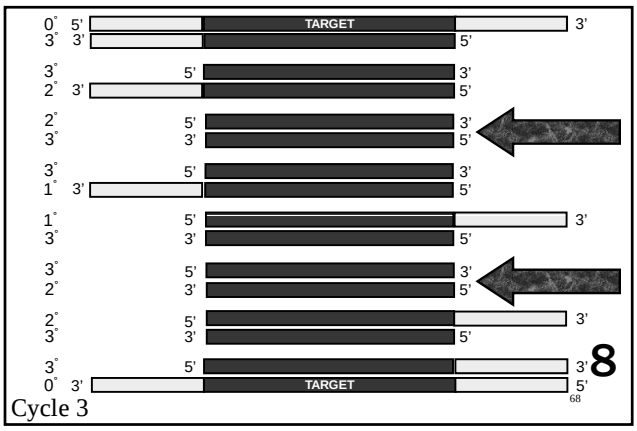
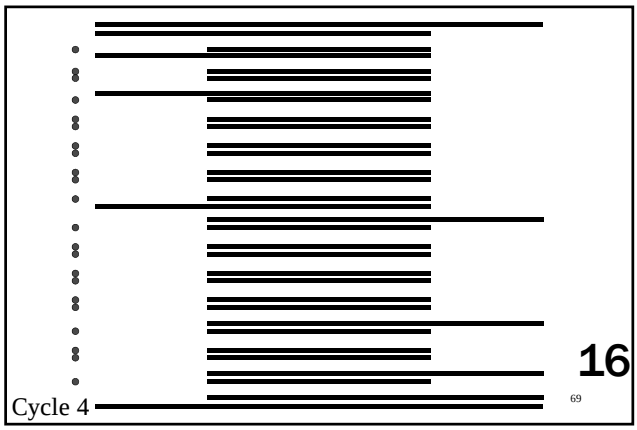
Primer extension (polymerization)

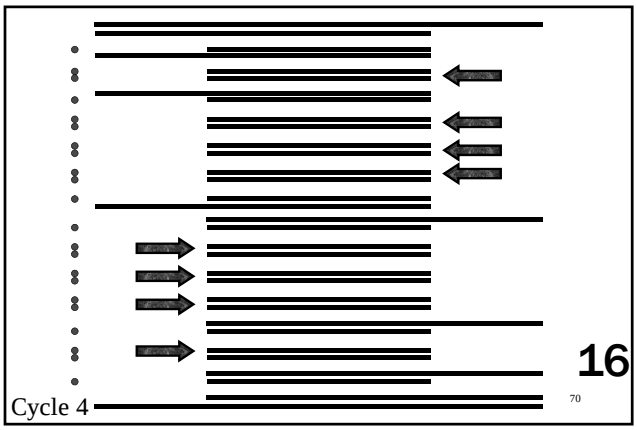
Cycle 2

66

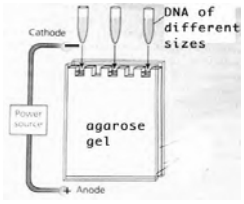


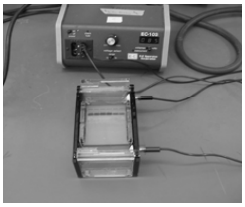






VISUALIZE PRODUCT





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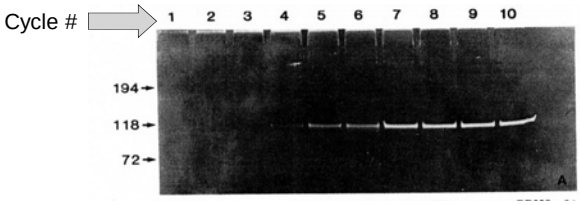
INCREASED YIELD PER CYCLE



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

Theoretical yield: 2^N fold amplification

N = cycle number

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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

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"OPTIMIZED" PCR PROTOCOL

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

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THE END

- DNA structure (hybridization)

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

- DNA replication (PCR)

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