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.”
Molecular Biology

...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

\[ \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{OH} \quad \text{H} \]

\[ \text{O} \quad \text{HOCH}_2 \]

\[ \text{O} \quad \text{3'} \quad \text{OH} \quad \text{H} \]

\[ \text{O} \quad \text{5'} \]
PURINES

Adenine

Guanine

PYRIMIDINES

Thymine

Cytosine
PURINES

Adenine

Guanine

PYRIMIDINES

Thymine

Cytosine
HYDROGEN BONDING

Adenine

Guanine

Thymine

Cytosine
COMPONENTS

DNA

- Phosphate
- Pentose (deoxyribose)
- Base (thymine)

RNA

- Phosphate
- Pentose (ribose)
- Base (uracil)
NOMENCLATURE

ENERGY

[Diagram of nucleoside, nucleoside monophosphate, and nucleoside triphosphate with labels for base, sugar, nucleoside, and respective phosphate connections.]

[Diagram shows a nucleoside with a base bonded to a sugar, and three phosphate groups attached, forming a nucleoside triphosphate.]
DOUBLE STRANDED EVIDENCE

G + C content

Previous tetranucleotide hypothesis:

*DNA consists of equal quantities of 4 bases*

Chargaff ratios (1952)

<table>
<thead>
<tr>
<th>Organic Material</th>
<th>Percent adenine</th>
<th>Percent thymine</th>
<th>Percent guanine</th>
<th>Percent cytosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver</td>
<td>30.3</td>
<td>30.3</td>
<td>19.5</td>
<td>19.3</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>15.1</td>
<td>14.6</td>
<td>34.9</td>
<td>35.4</td>
</tr>
</tbody>
</table>
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
Molecular Diagnostics Classifications
Probe anneals to target of interest
Probe anneals to target of interest
NUCLEIC ACID HYBRIDIZATION

COGNATE HYBRIDIZATION

(62°C; following denaturation)

Nucleic acid probe

TGGCTAACGTT

Sequence-specific (single-stranded) DNA template

........ACCGATTGCAA........
Diagnostic Application: Hybridization
PROBE TECHNOLOGY

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

- Specificity or sensitivity depends upon size of probe (stringency)

- Shorter probes result in quicker completion

- 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 onto 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

- Streptavidin and alkaline phosphatase conjugate
- Biotin
- Substrate: NBT, BCIP (COLOR)
- Biotinylated Probe
- DNA/RNA on slide or membrane
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
Adenine

Cytosine

DNA polymerase

x 3
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
Okazaki fragment B synthesized as unreplicated loop elongates.

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
Amplify target of interest prior to detection
### Analytical Sensitivity

<table>
<thead>
<tr>
<th>Method</th>
<th>Approx copy no. detectable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide staining</td>
<td>$10^8$</td>
</tr>
<tr>
<td>Radiolabeled oligonucleotide probes</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Radiolabeled full-length probes</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Enzyme-coupled probes</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Chemiluminescent probes</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Compound or branched probes</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Nucleic acid amplification</td>
<td>$\leq 10$</td>
</tr>
</tbody>
</table>
Diagnostic Application: Polymerase Chain Reaction
“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

5' CATTCCGAA………………...TGTACATGC  3'

5' GTAAGGCTT….……...….….ACATGTACG  3'
REQUIREMENTS FOR PCR

- Known (unique) DNA sequence
- Oligonucleotide primers

**Forward primer**

5' CATTCCGAA 3'

**Reverse primer**

3' ACATGTACG 5'

*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
REQUIREMENTS FOR PCR

- Known (unique) DNA sequence
- Oligonucleotide primers
- DNA polymerase
- MgCl$_2$
- Deoxynucleotide triphosphates (dNTPs)
- Buffer
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
Isolated DNA
Denaturation

Cycle 1
Primer annealing

Cycle 1
Primer extension (polymerization)

Cycle 1

Klenow fragment

TARGET

3' 5' 0°

3'

ACATGTACG

5'

CATTCCGAA

3' 5' 1°

3'

TARGET

0° 3'

5'

Cycle 2

263
Primer annealing

Cycle 2
Primer extension (polymerization)

Klenow fragment

Cycle 2
Cycle 3
VISUALIZE PRODUCT

DNA of different sizes

agarose gel

- Power source
  - Cathode

+ Anode

-
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)