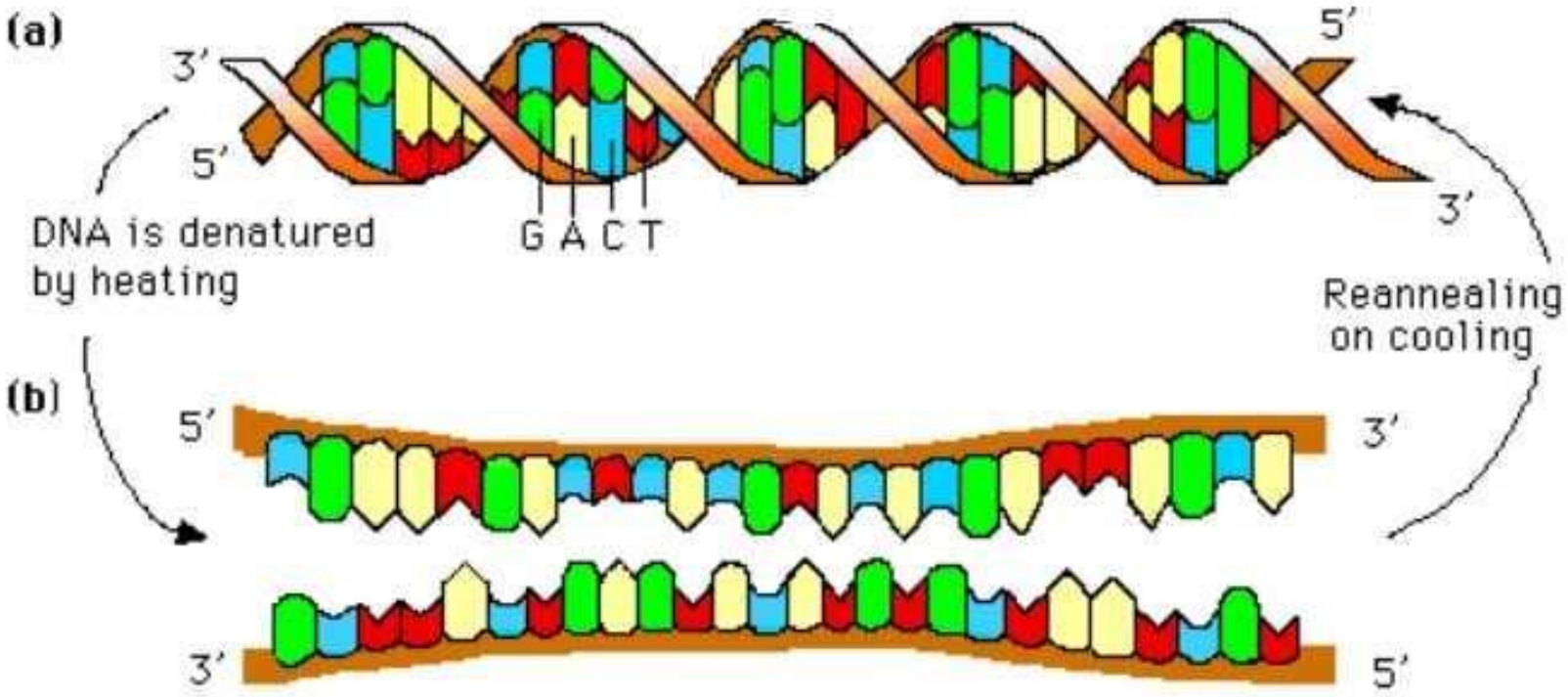
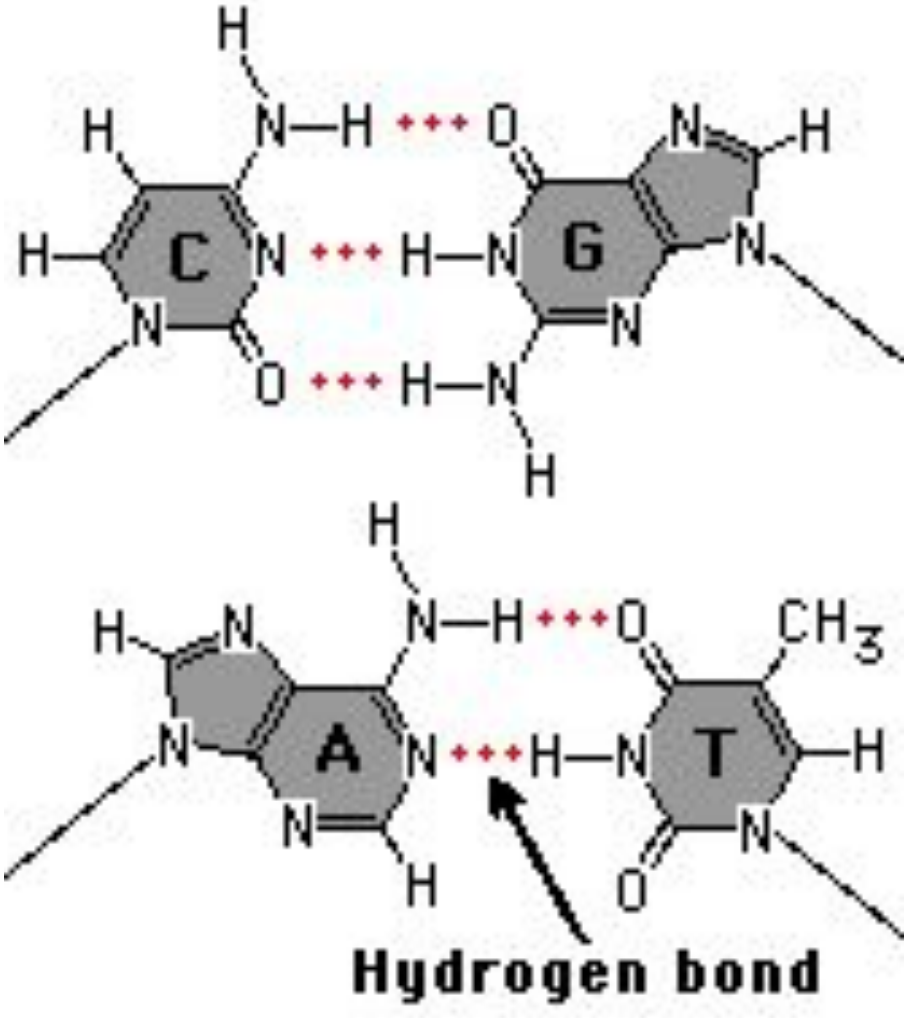
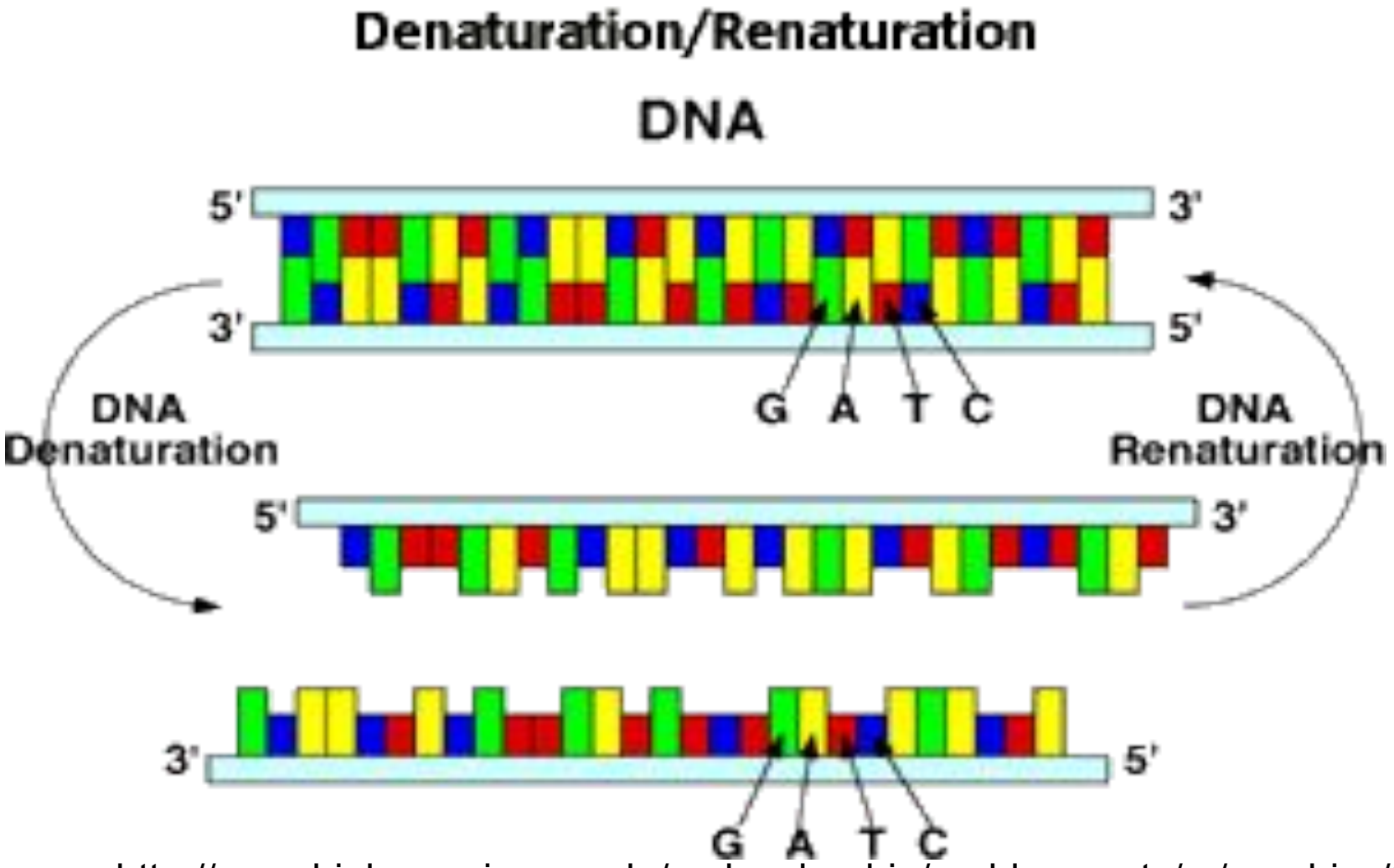
# 1.Hybridization (DNA-DNA or DNA-RNA)



# HYBRIDIZATION? – Yes, it is about this familiar picture

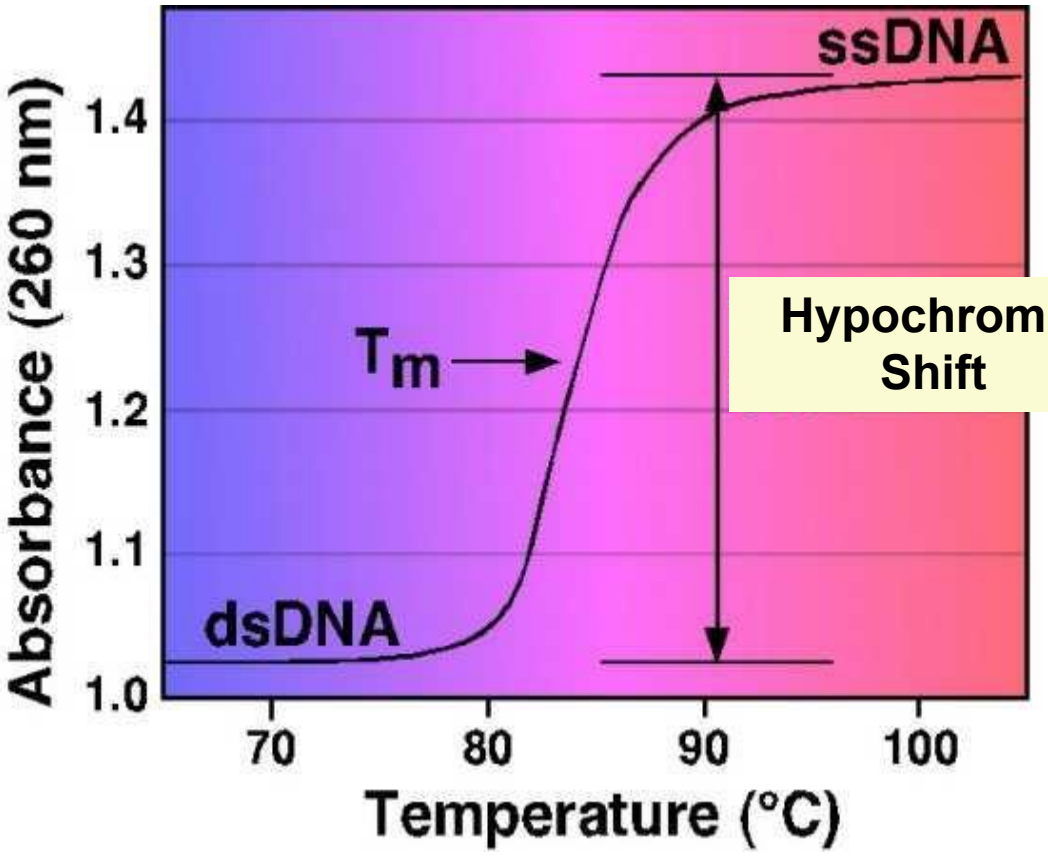


# We can denaturate and renaturate DNA by heating/cooling




http://www.biology.arizona.edu/molecular_bio/problem_sets/m/graphics/05ta.gif

As DNA is heated, it reaches a temperature where the strands separate (DNA melts).




**ssDNA**

**H-bonds between basepairs are broken and the strand unwind.**

**Melting curve.**

**Unstacked bases (random orientation) absorb more light than neatly stacked (oriented) base-pairs**

**Tm (melting temp)**

**The temperature at which DNA is half unfolded**

**Tm is a measure of the stability of dsDNA under a given set of conditions**

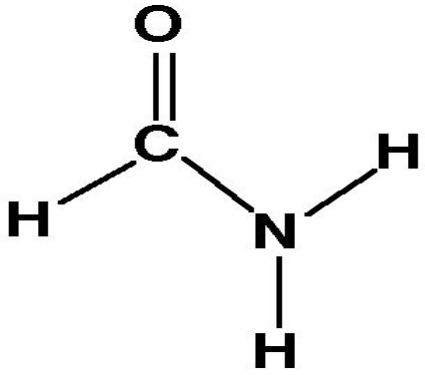
medlib.med.utah.edu/block2/ biochem/

# Tm of DNA is affected by:

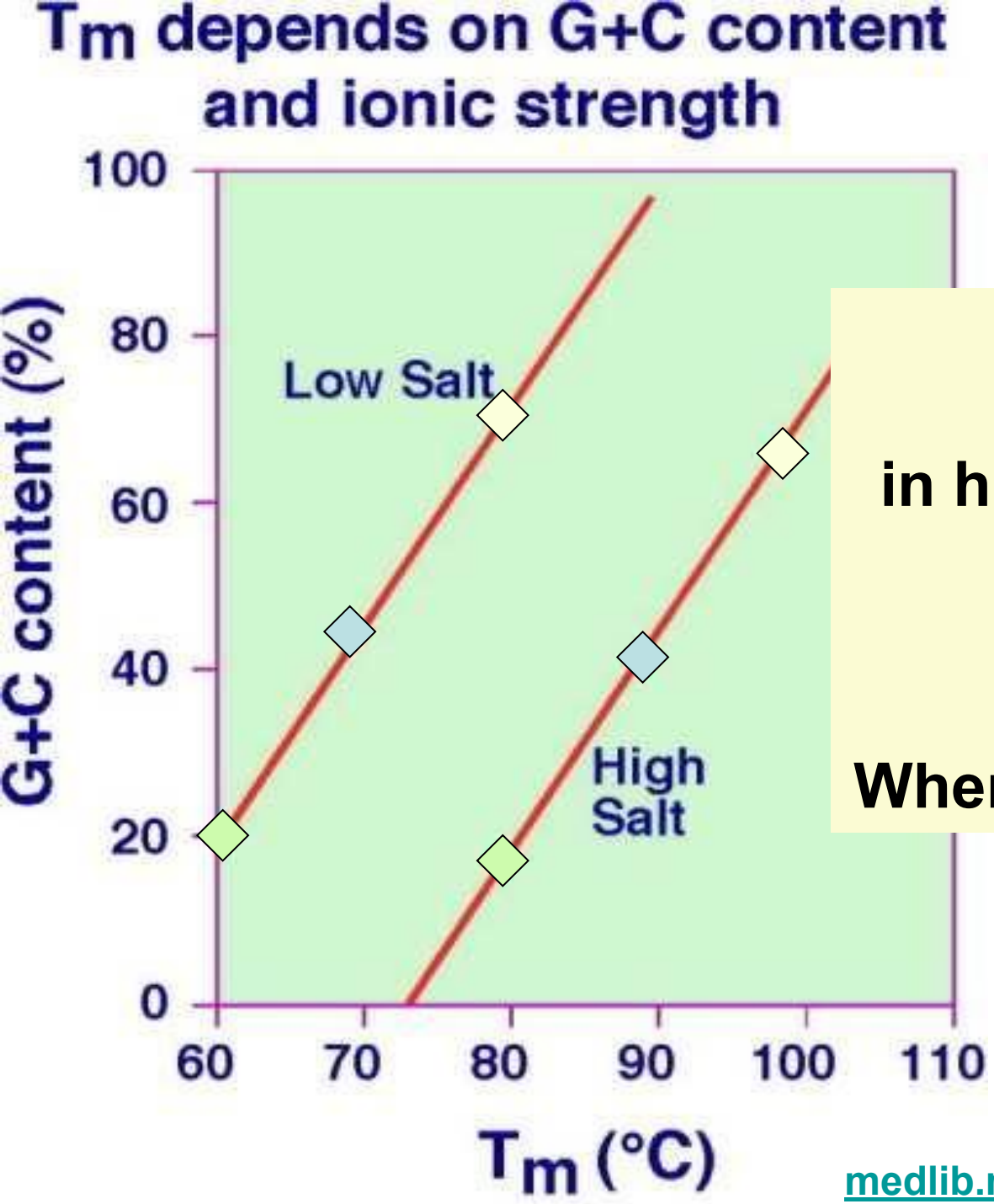
**1. Base Composition** :
higher the GC content, the higher the Tm.

**2. Ionic Strength** :
as the ionic strength increases, so does Tm.
Double helical DNA is stabilized by cations.

**Divalent cations** (Mg2+) **are more effective** than monovalent cations (NA+ or K+).




**3. Organic Solvents –**
formamide for instance
lowers the Tm by weakening
the hydrophobic interactions.

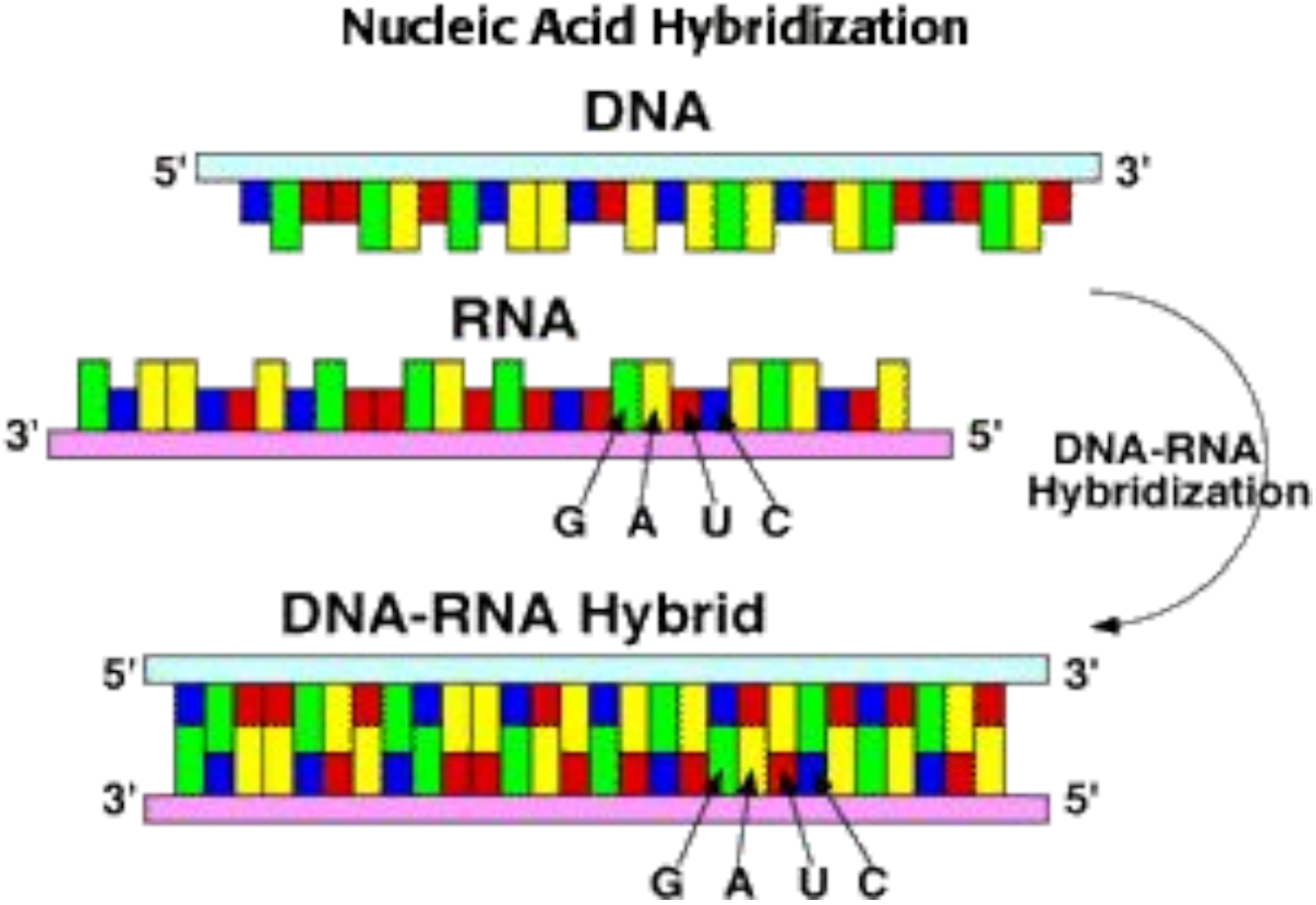



**DNA**
**more STABLE**
**in high-salt conditions.**

**DNA**
**more STABLE**
**When contains many GC**

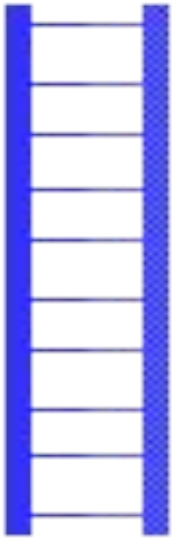
medlib.med.utah.edu/block2/ biochem/

# RNA can bind DNA
# (U is equivalent of T in hybridization)

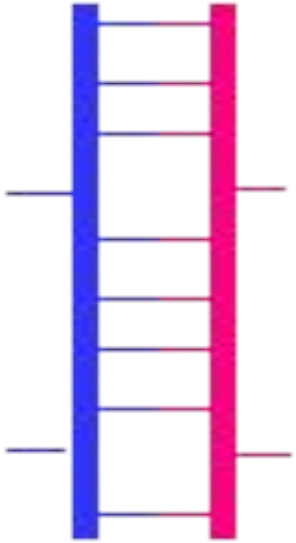



http://www.biology.arizona.edu/molecular_bio/problem_sets/m/graphics/05ta.gif

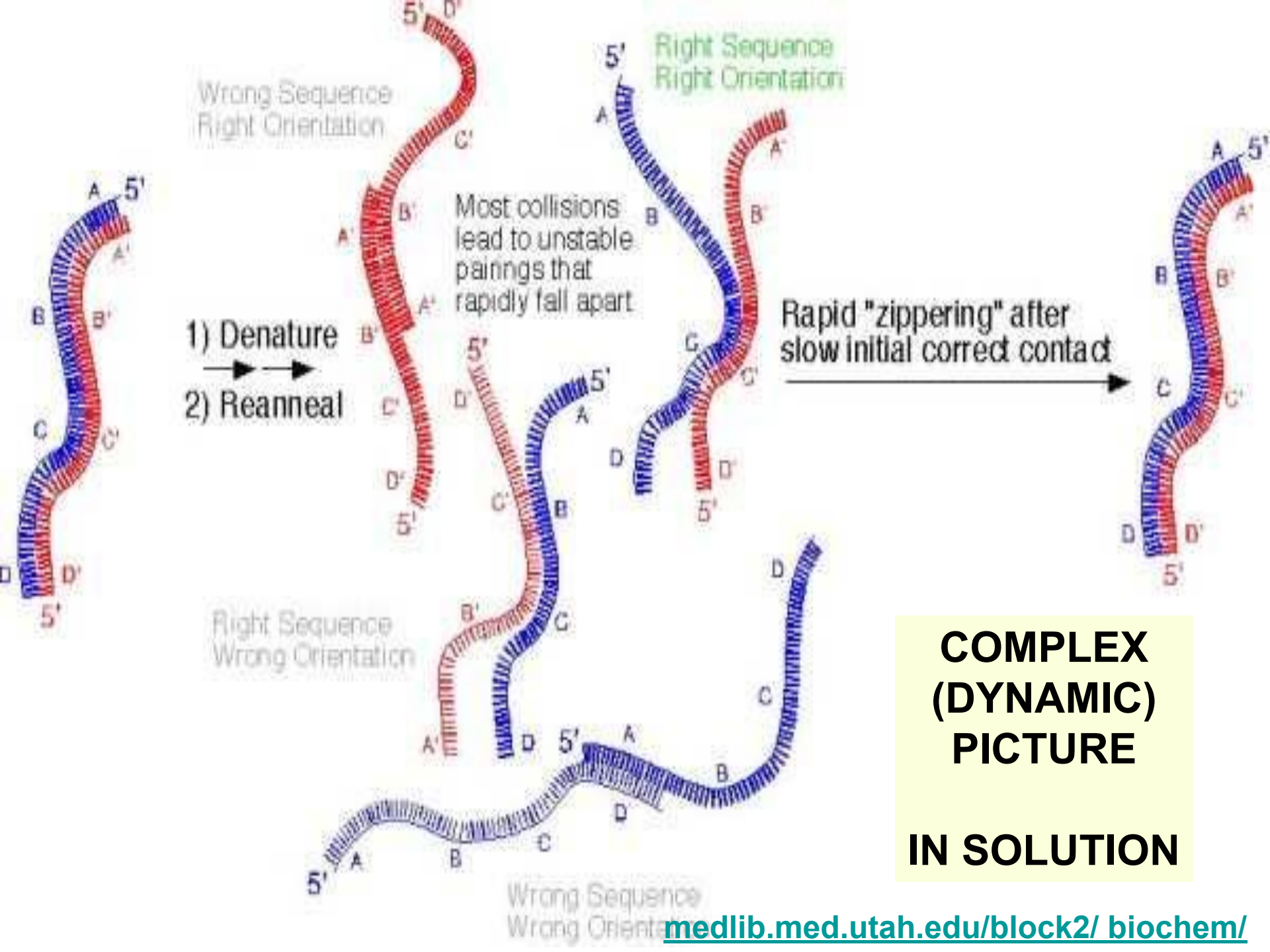
# Hybridization could be less than perfect



Homoduplex DNA, both strands from the same species



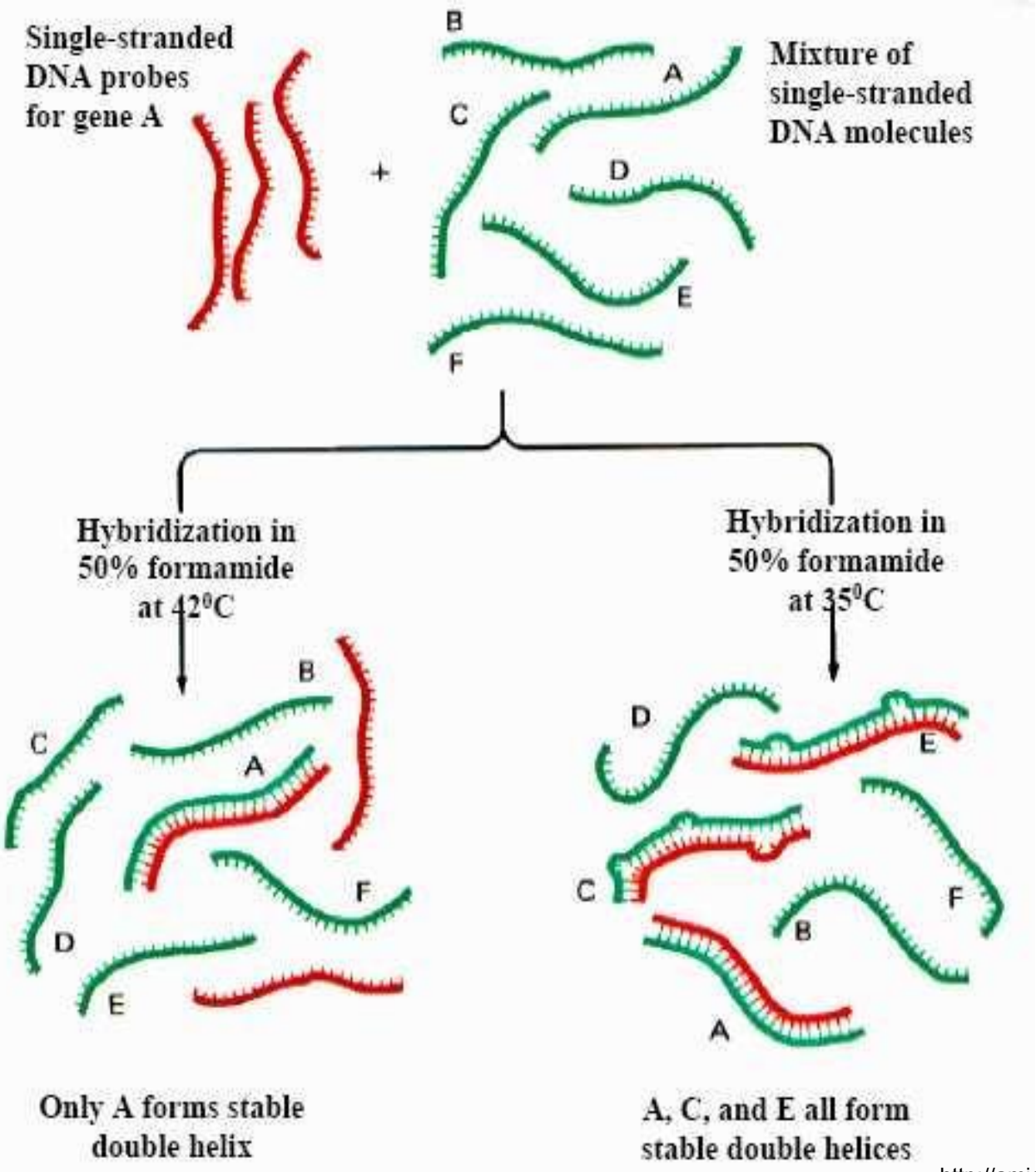
Heteroduplex DNA, strands from different species, with some bases unpaired




**COMPLEX (DYNAMIC) PICTURE**

**IN SOLUTION**

medlib.med.utah.edu/block2/ biochem/




**42 C**
**is more stringent**
**condition**
**that 35 C**

**(hybridization**
**is more specific)**

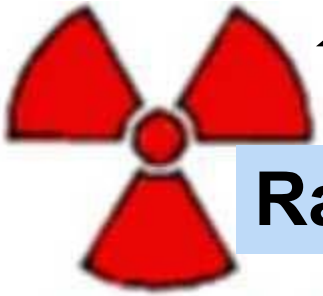
http://amiga1.med.miami.edu/Medical/PDF-Files/Lecture10.pd

# So, hybridization is a most obvious phenomenon to use for specific DNA detection

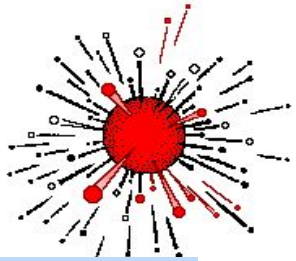
**Specific probe self-anneals to target DNA**

**Only problem – DNA is invisible**

## How to visualize DNA?

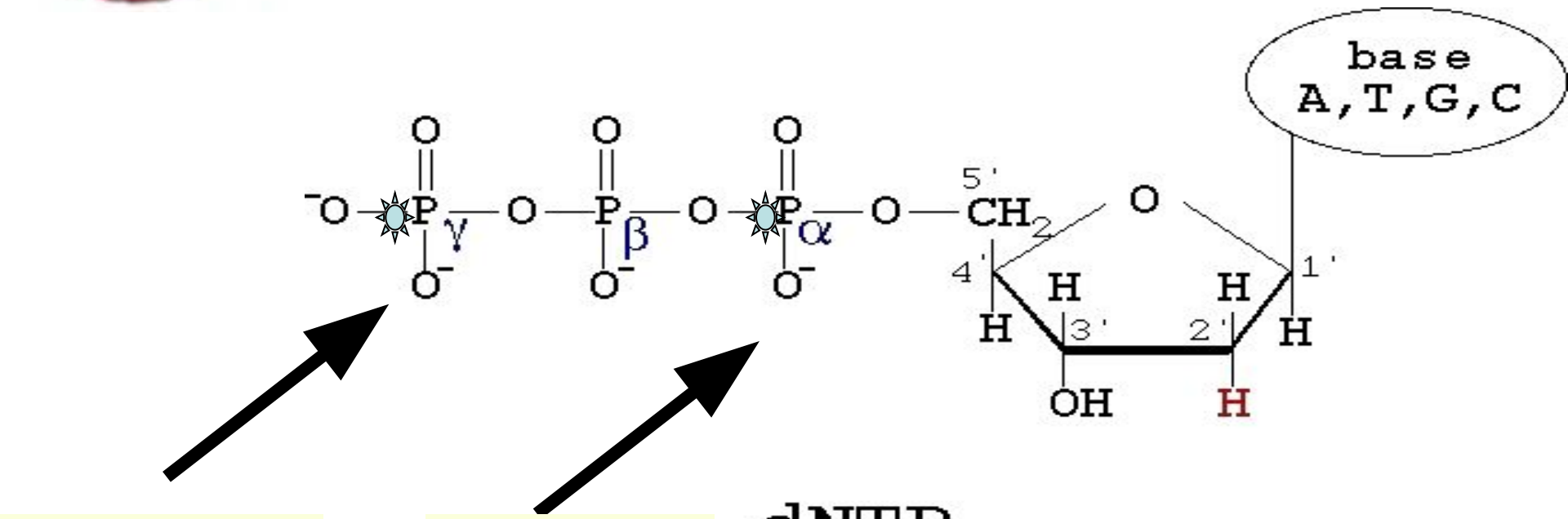


**Radioactively**



**Fluorescently**

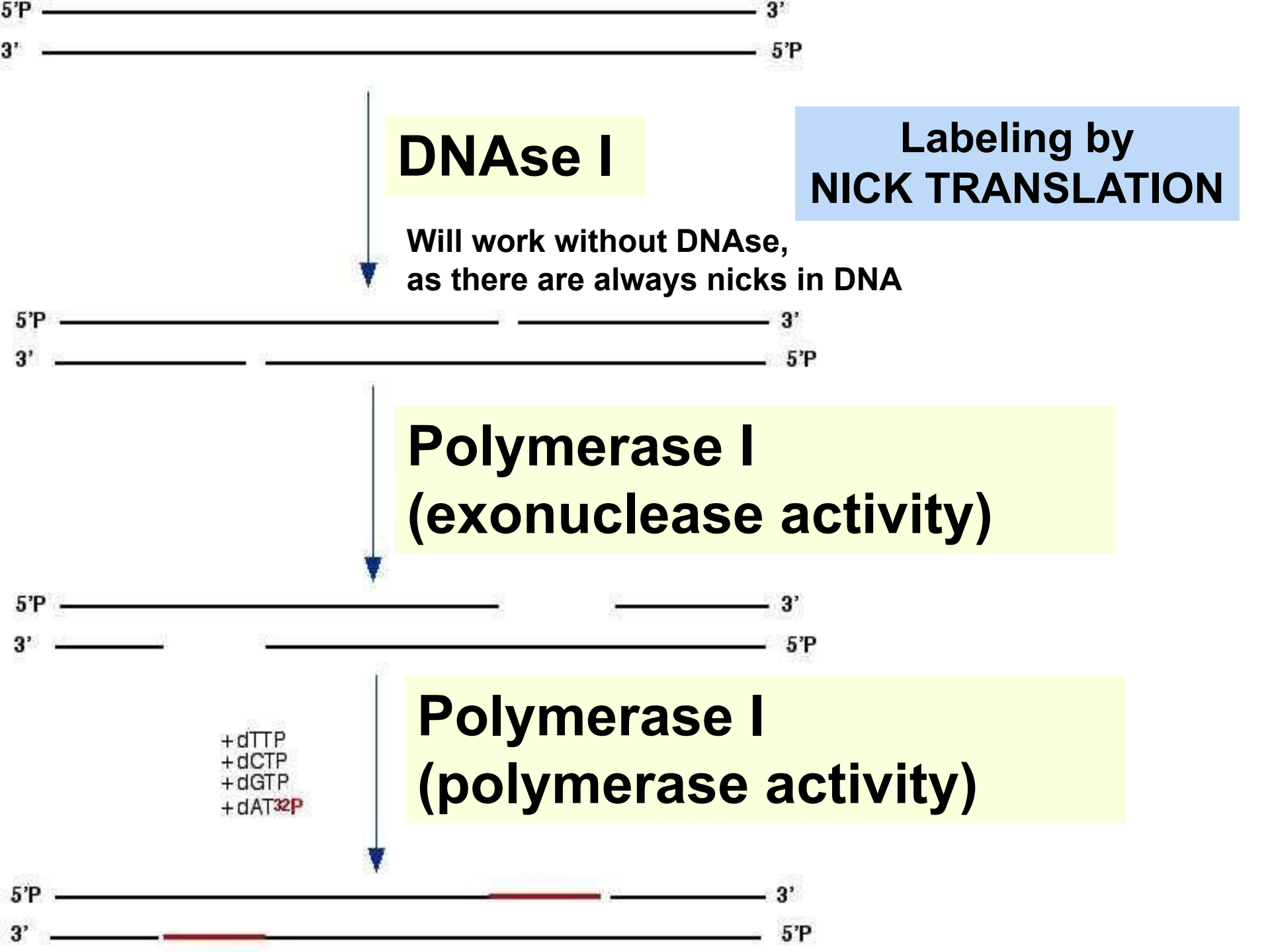
# Polymerase labeling of DNA




**Gamma-33-ATP**

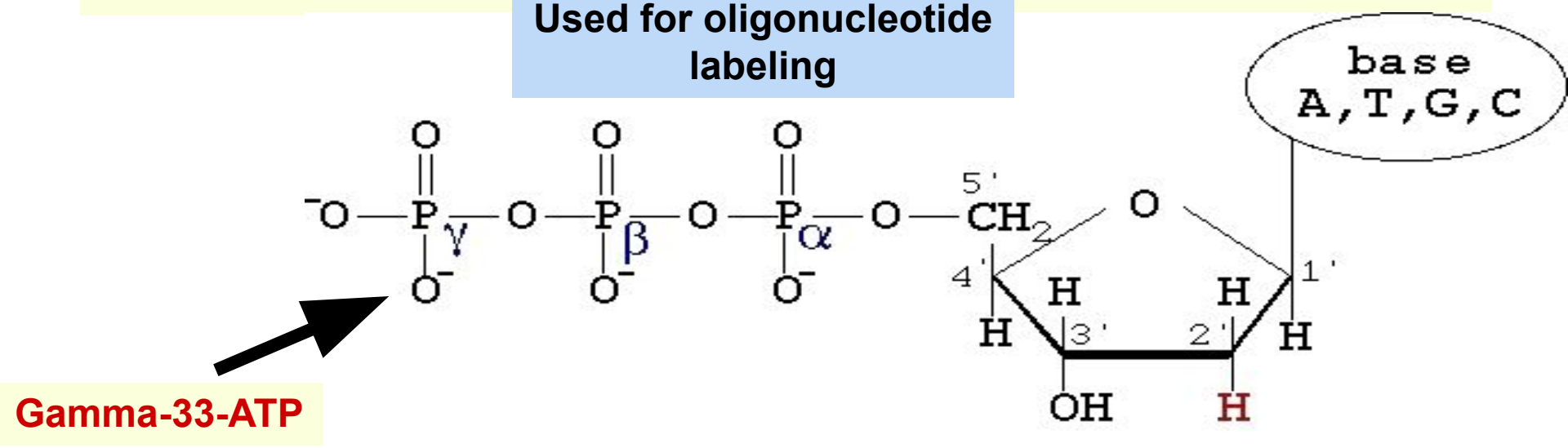
**Alpha-32-ATP**

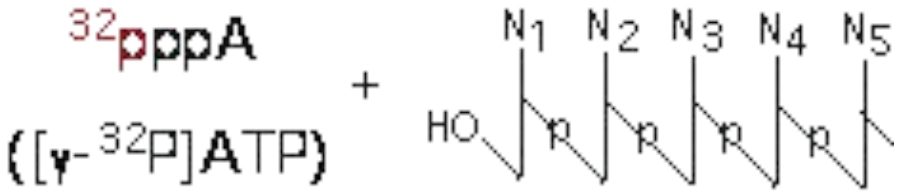

# Labeling by NICK TRANSLATION

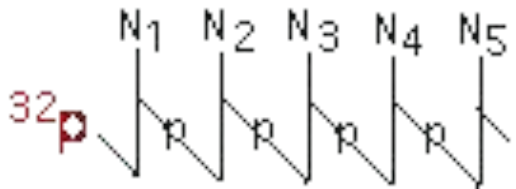


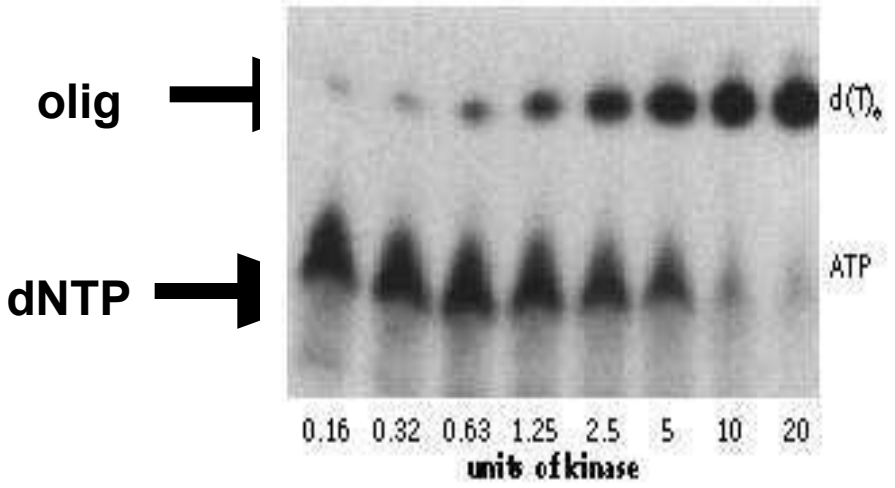
# T4 polynucleotide kinase labeling

**Used for oligonucleotide labeling**

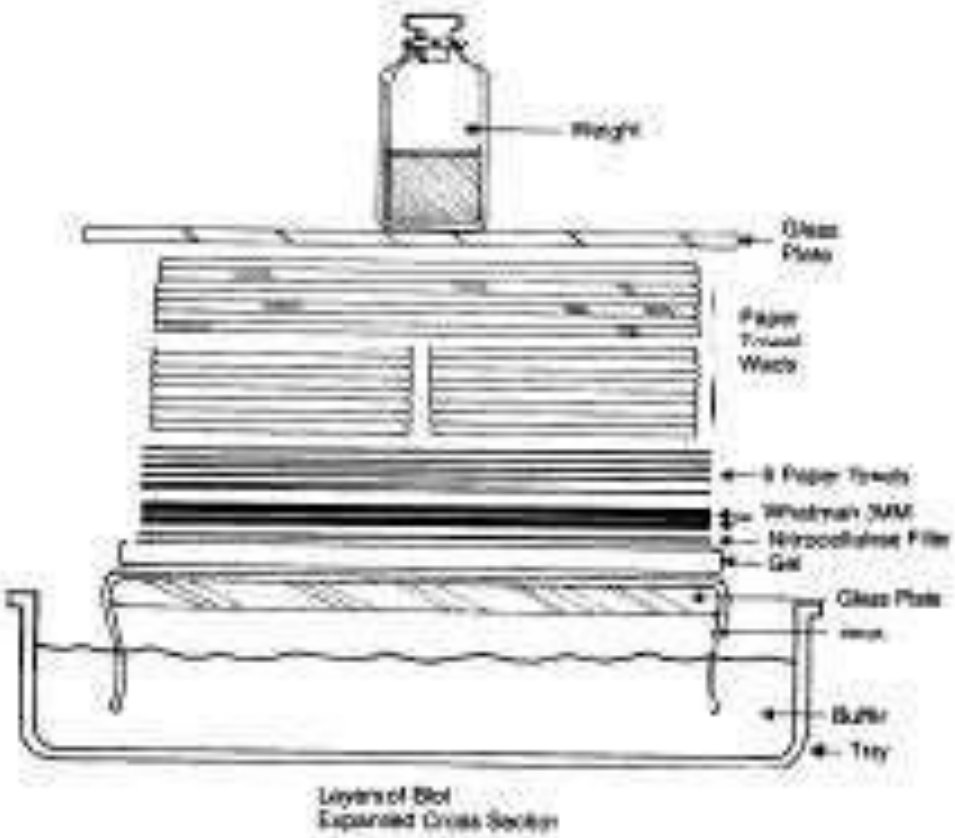


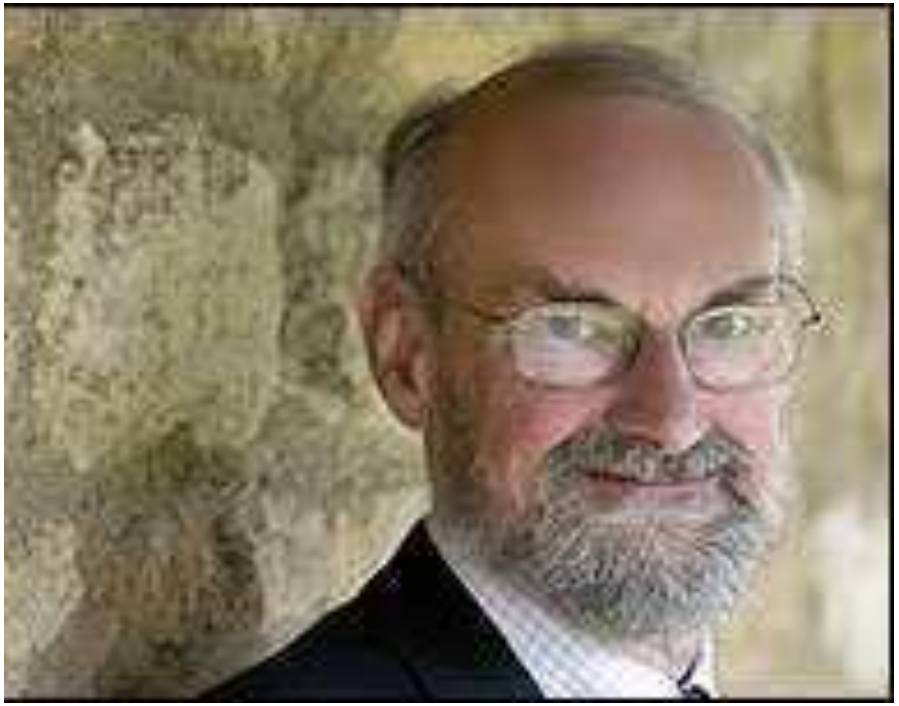



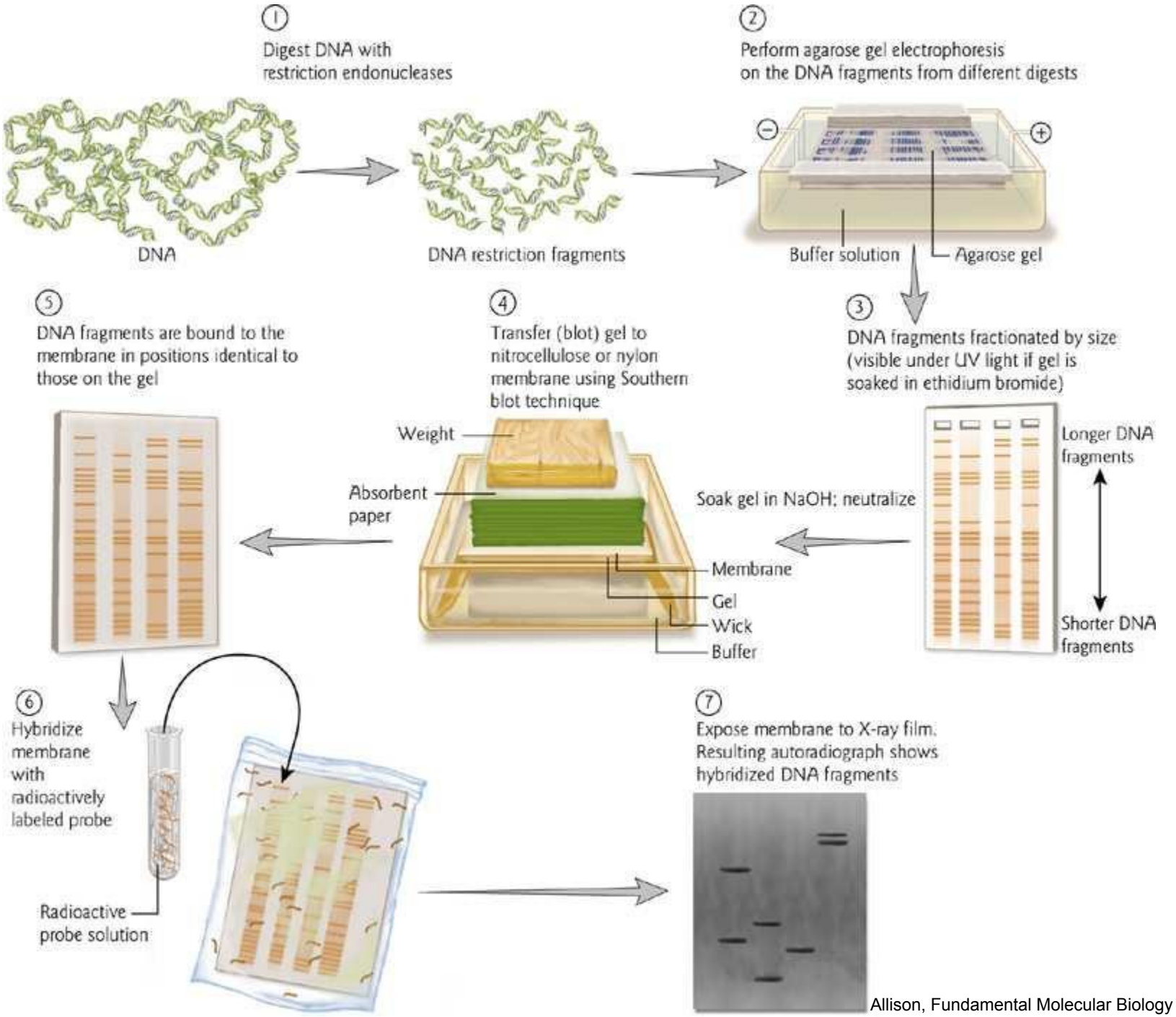



# Professor Sir Ed Southern, Whitley Professor of Biochemistry at the University of Oxford

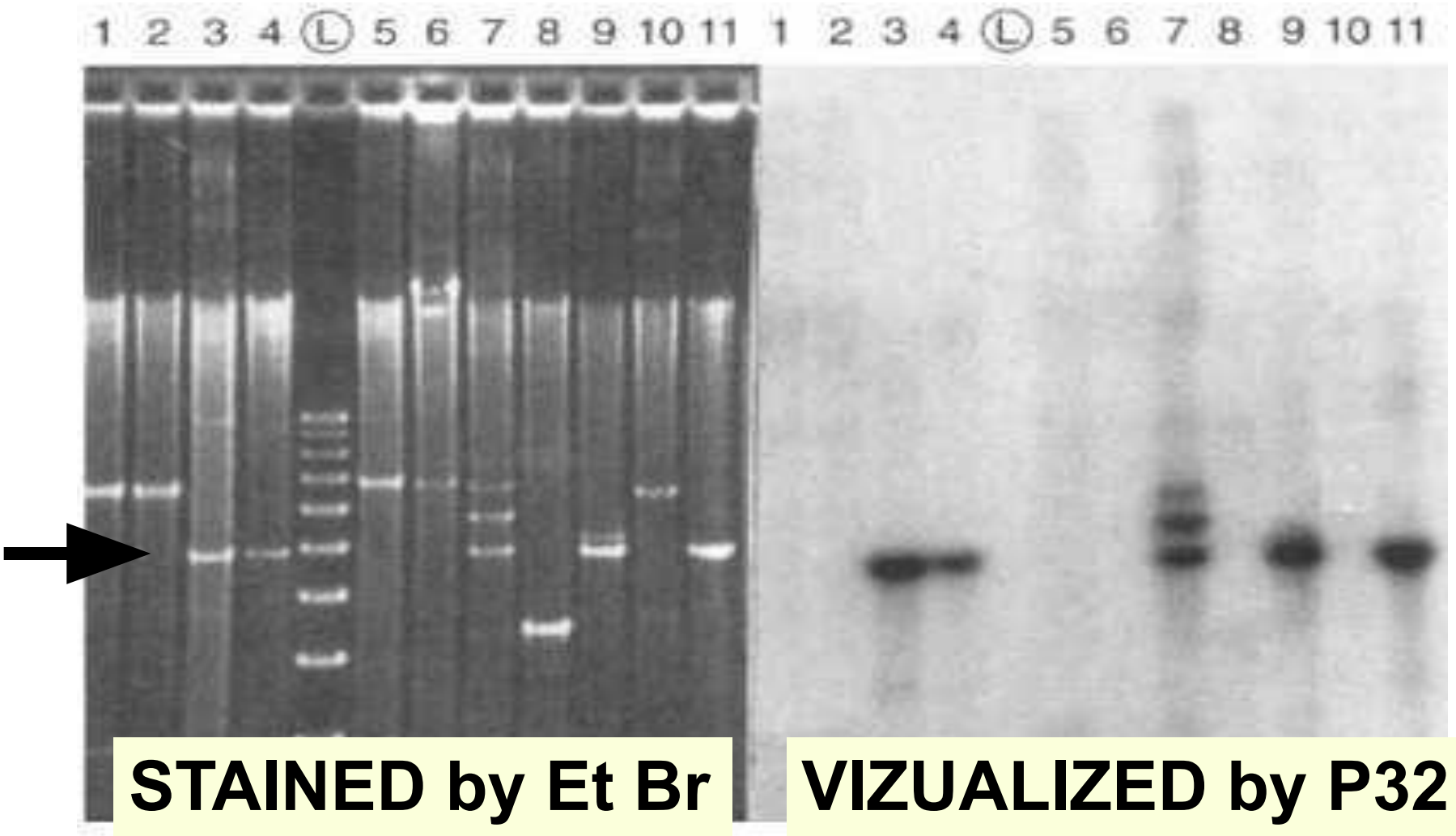




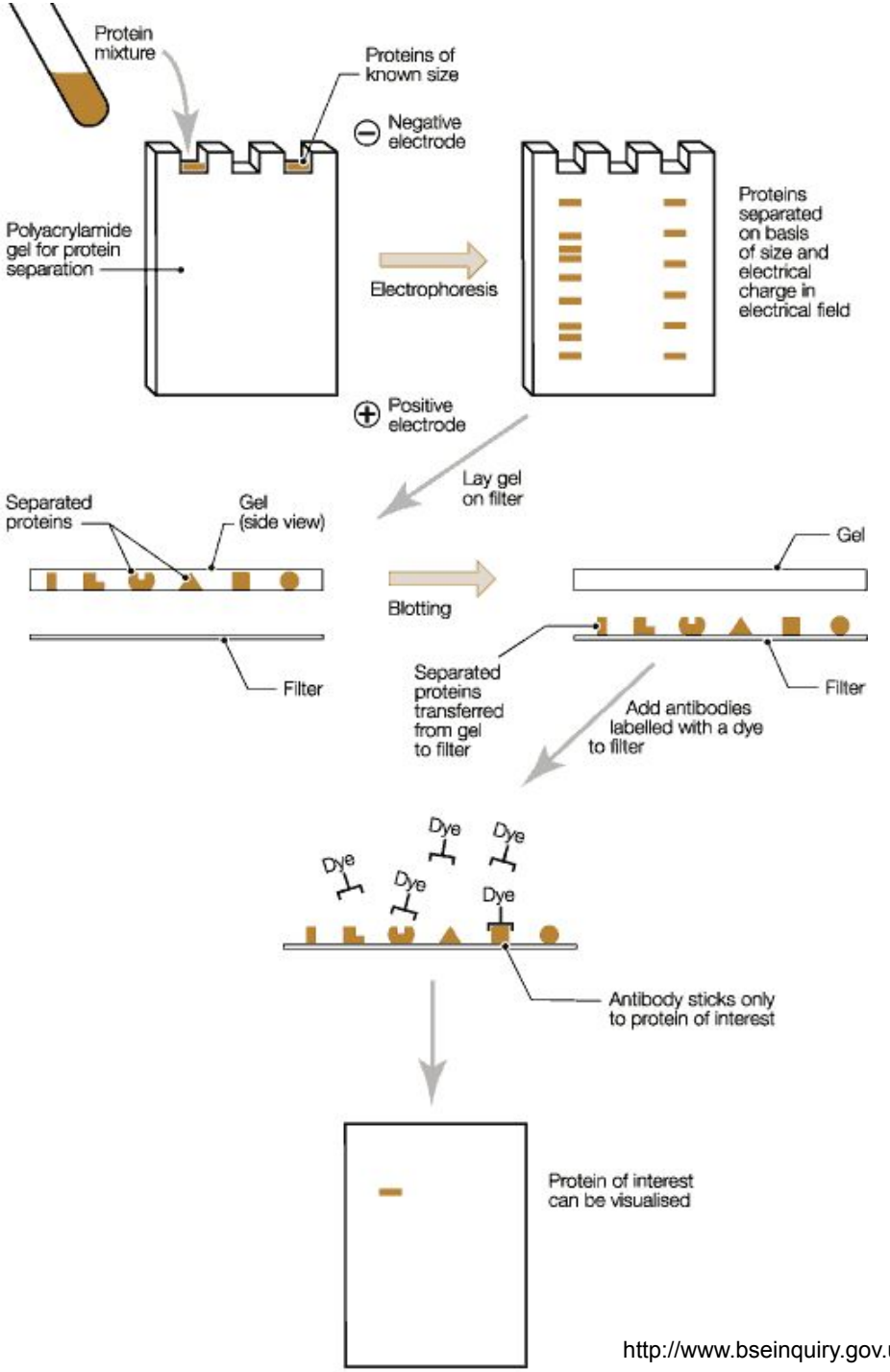

Allison, Fundamental Molecular Biology

# “Real” Southern blot (DNA-DNA blot)




www.mun.ca/biology/scarr/ 3250_Southern_Blot_analysis.htm

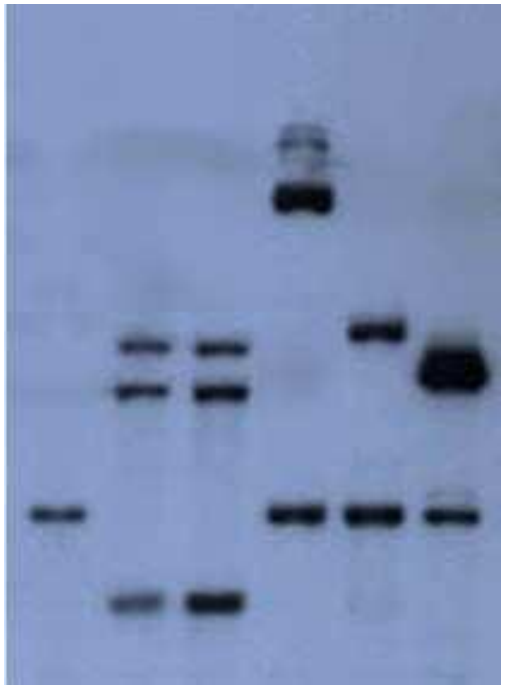
# Western Blot




http://www.bseinquiry.gov.uk/report/volume2/fig1_8.htm

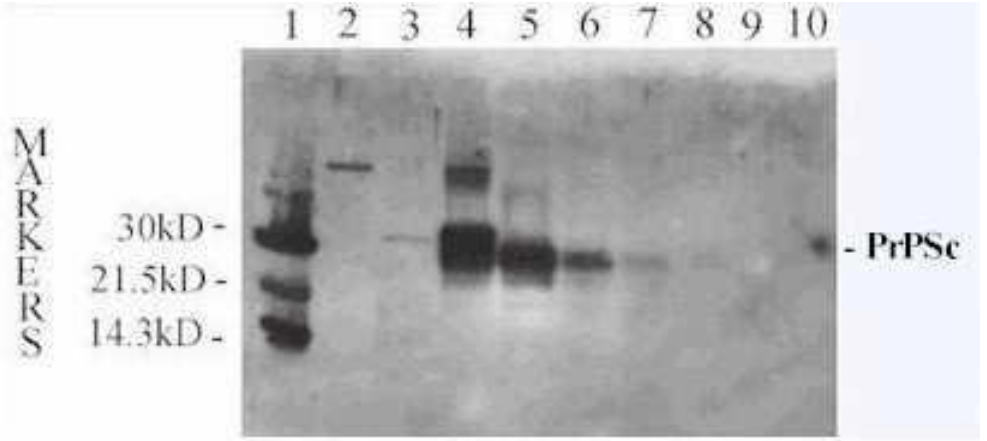
What different types of information can be provided by each different blot type?

Southern

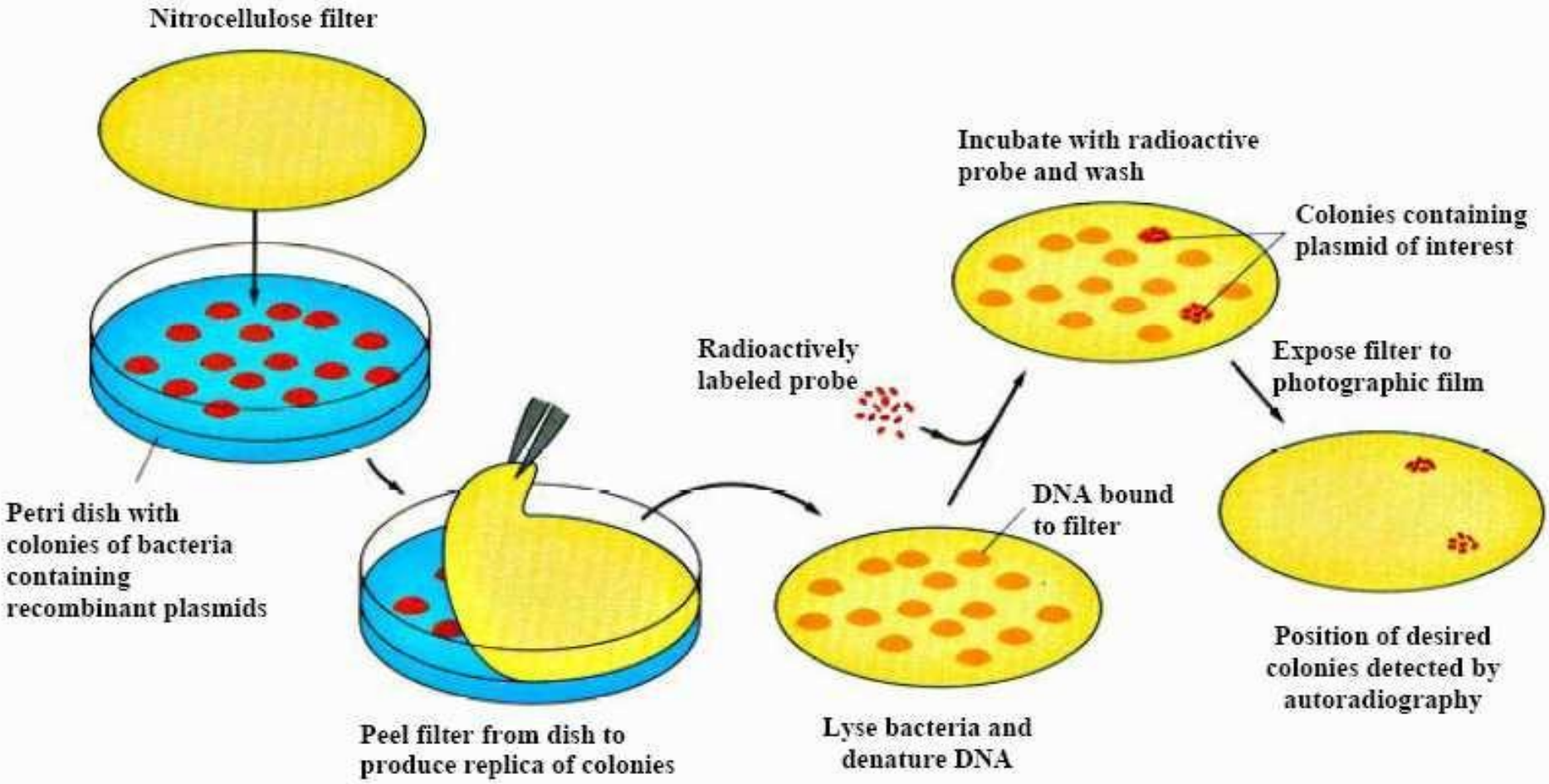


northern


western



# Colony hybridization assay for the identification of bacterial colonies carrying a particular DNA clone




http://amiga1.med.miami.edu/Medical/PDF-Files/Lecture10.pdf

**Same for arrayed clones**

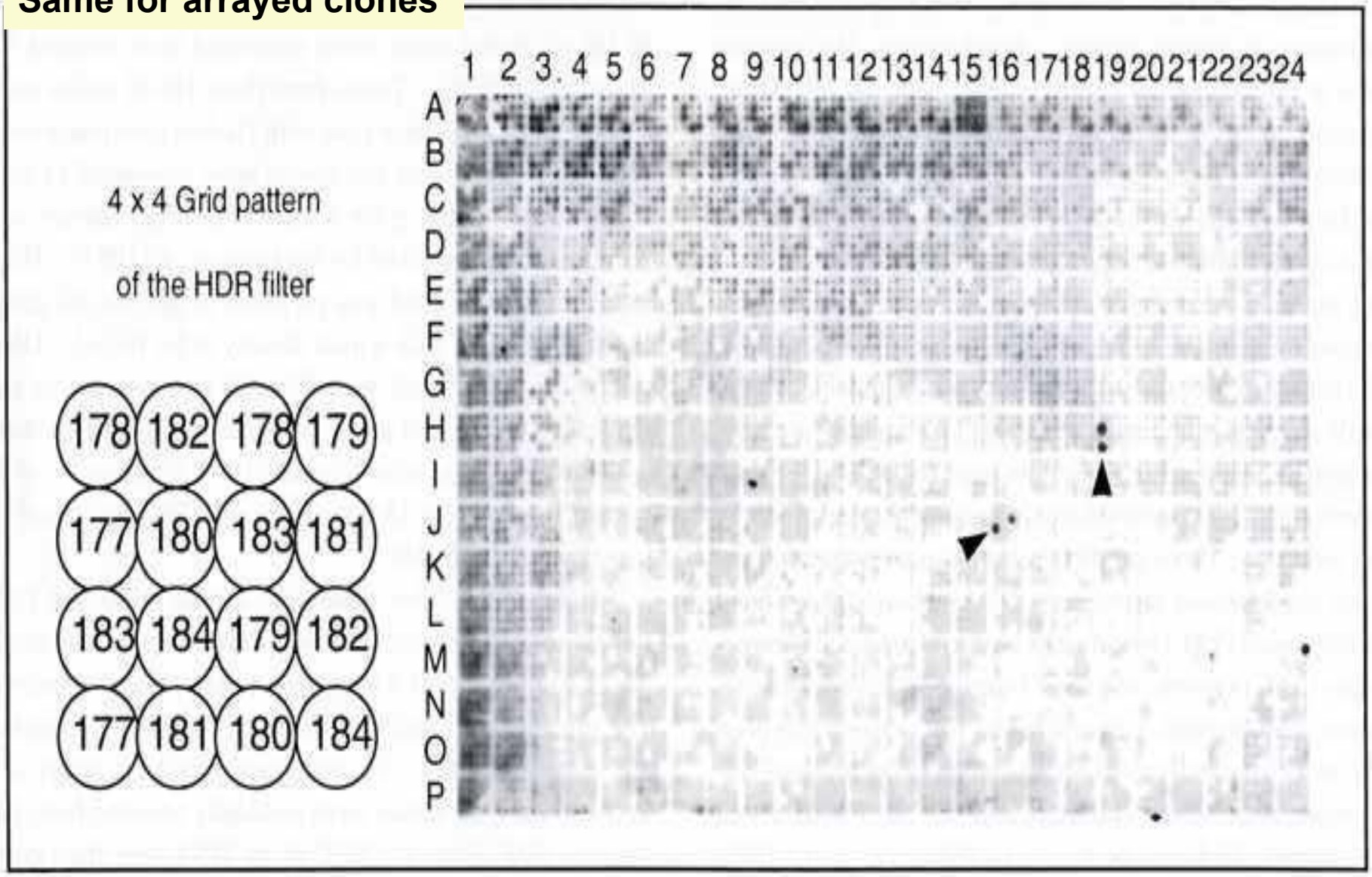
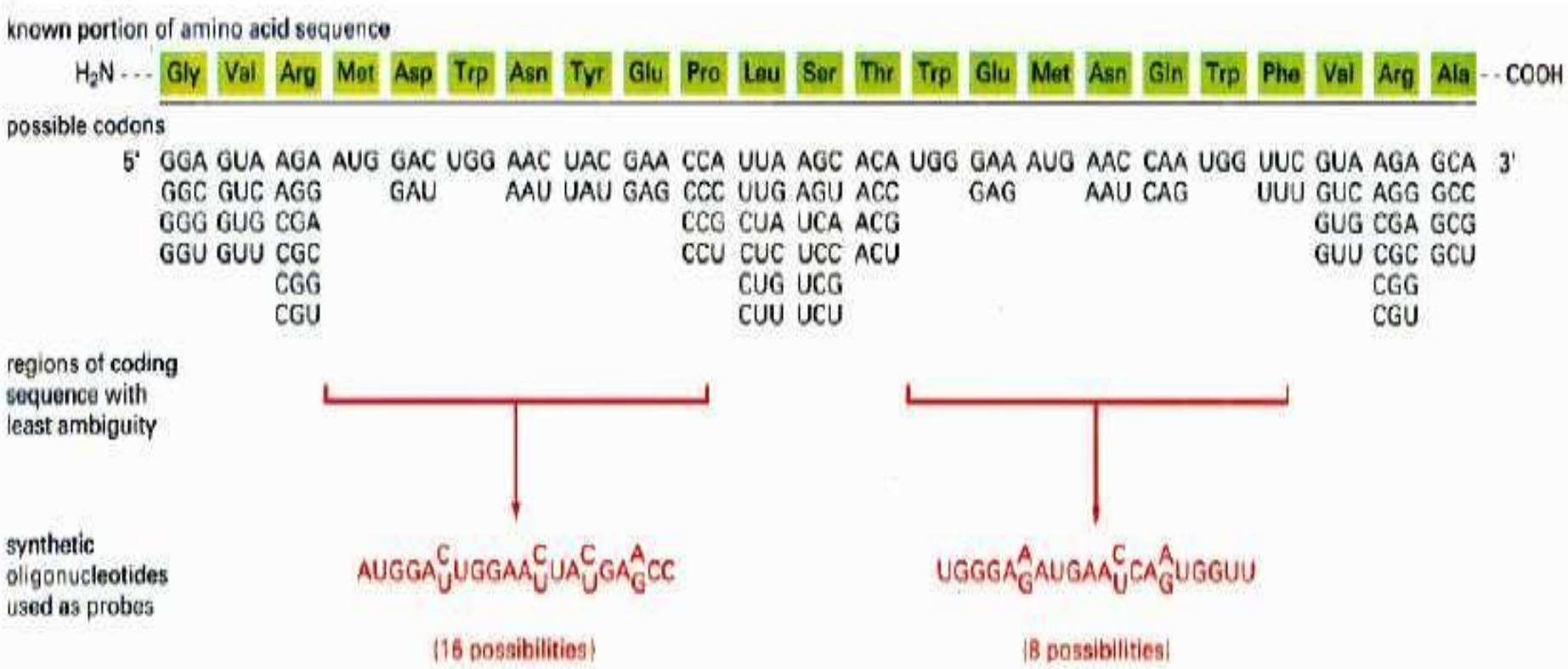



Fig. 2. Library screening by hybridization. Colony hybridization to a high density colony filter using the sequence of the single-copy gene *SL1* as a probe. 3072 BAC clones were gridded on a membrane with a 4 × 4 double offset pattern. Positive clones were detected as doublet signals.

http://biol1.bio.nagoya-u.ac.jp:8000/MatsudaM01fig2.jpg

# Design of degenerated synthetic hybridization probes

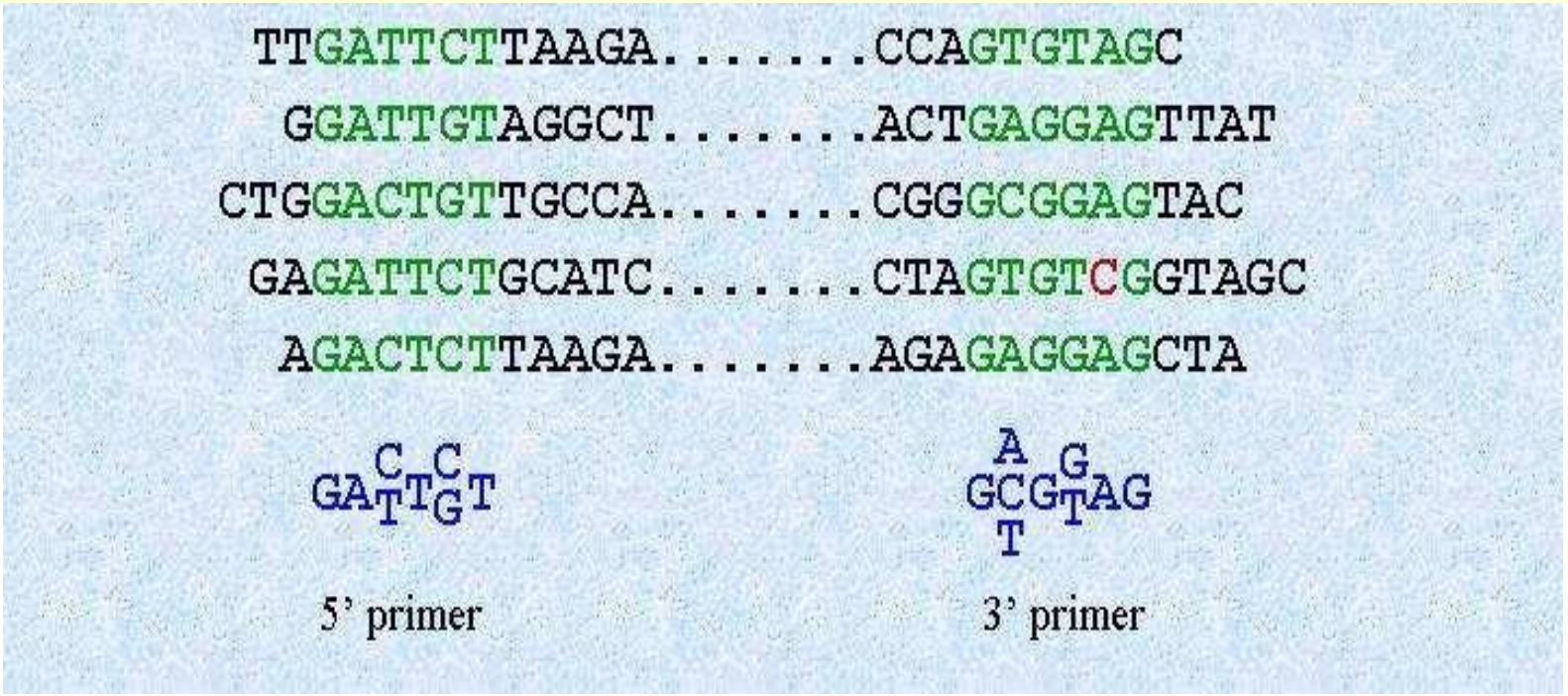
## (for already known proteins only)




http://amiga1.med.miami.edu/Medical/PDF-Files/Lecture10.pdf

The **degeneracy** of a primer is the number of unique sequences it corresponds to

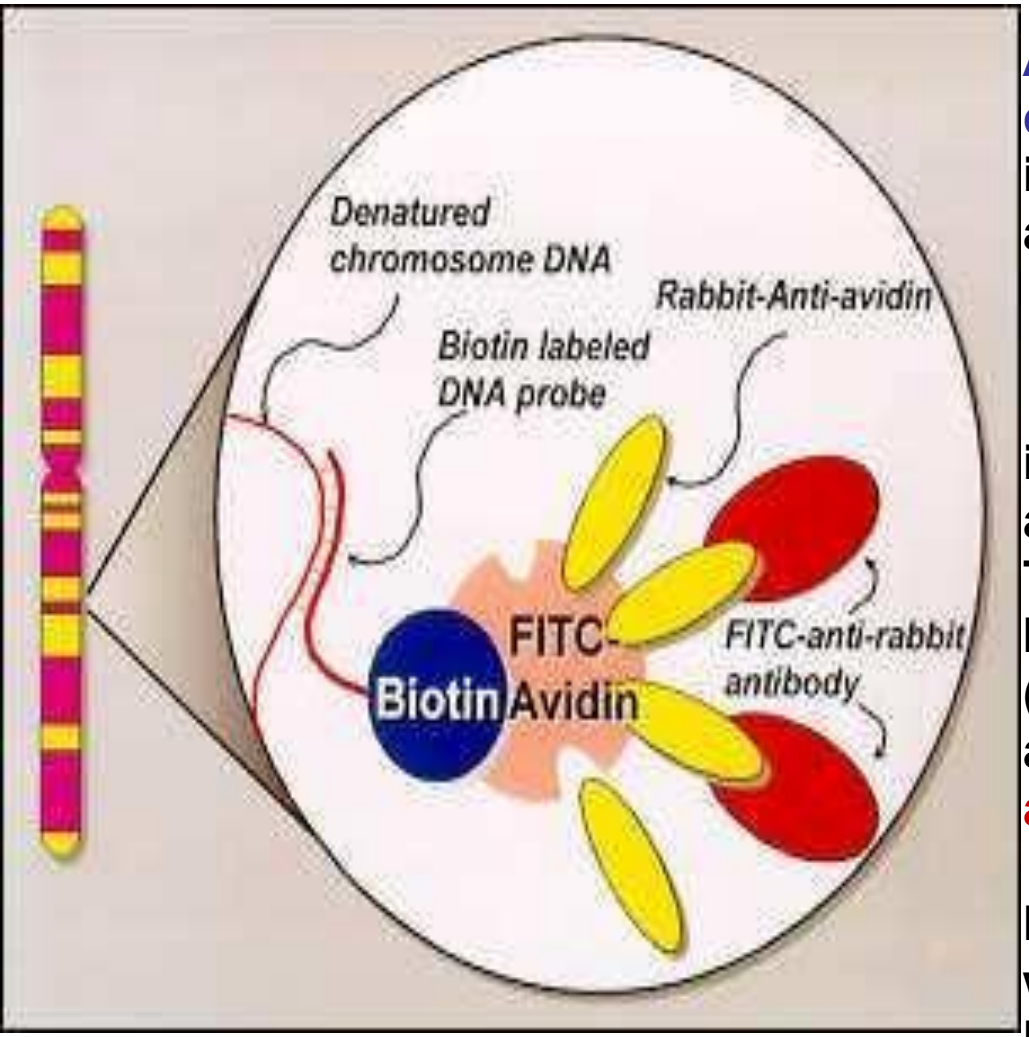
(5 in one of the examples below).




**can be used when some of the related genomic sequences are unknown, or known only in a related species.**

**Up to $10^{10}$ degeneracy is tolerated**

# Fluorescent probe hybridization




**A DNA probe,**
**covalently bound to biotin,**
**is hybridized to**
**a denatured chromosome preparation.**

**An avidin-bound fluorescent label (FITC)**
**is layered on top of the cells,**
**and the avidin-FITC binds the biotin.**
**The signal is amplified further**
**by layering rabbit anti-avidin antibody**
**(which binds the avidin-FITC),**
**and then layering FITC-labeled**
**anti-rabbit antibody on top.**

**Fluorescence will be detected only**
**where the DNA probe**
**has hybridized to the chromosome.**

http://www.childsdoc.org/spring2000/missinggenes.asp

# How streptavidin/biotin binding is working?

**Streptavidin is a protein.**
**1 mole of SA binds 4 moles Bio**

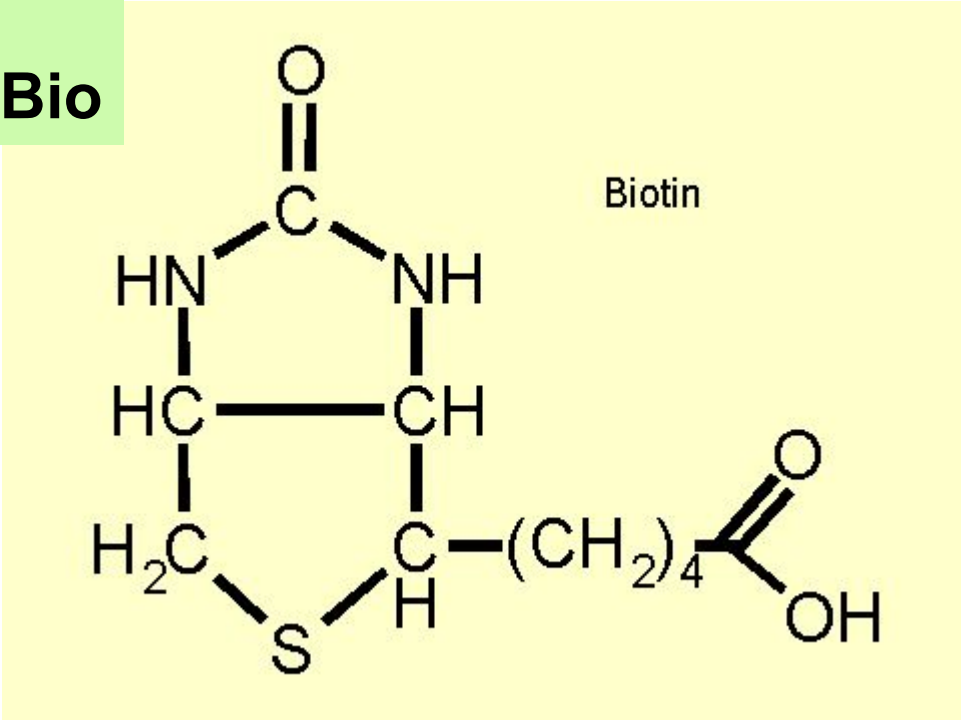
**(67 kD protein**

**from Streptococcus avidinii)**

**BIOTIN is a vitamin B (small thing)**

**Biotin could be added to nucleotide and incorporated into the probe**

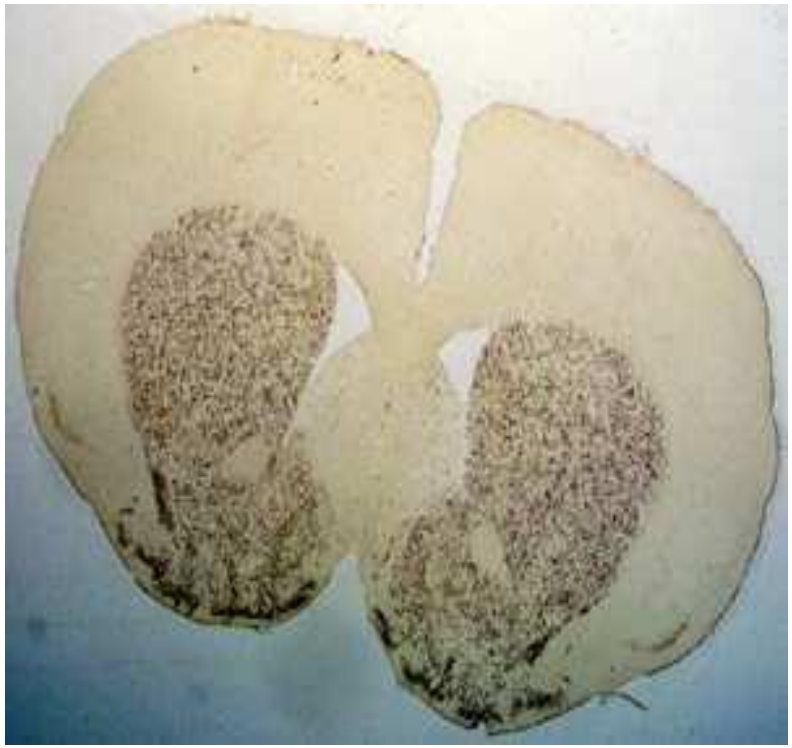
**Avidin could be conjugated with fluorophore**




**Largest free energies of association of yet observed for noncovalent binding of a protein and small ligand in aqueous solution (K_assoc = 10**14). Complex is extremely stable.**

# *IN SITU* HYBRIDIZATION

is an imaging method to visualize mRNA expression in tissues and cells.

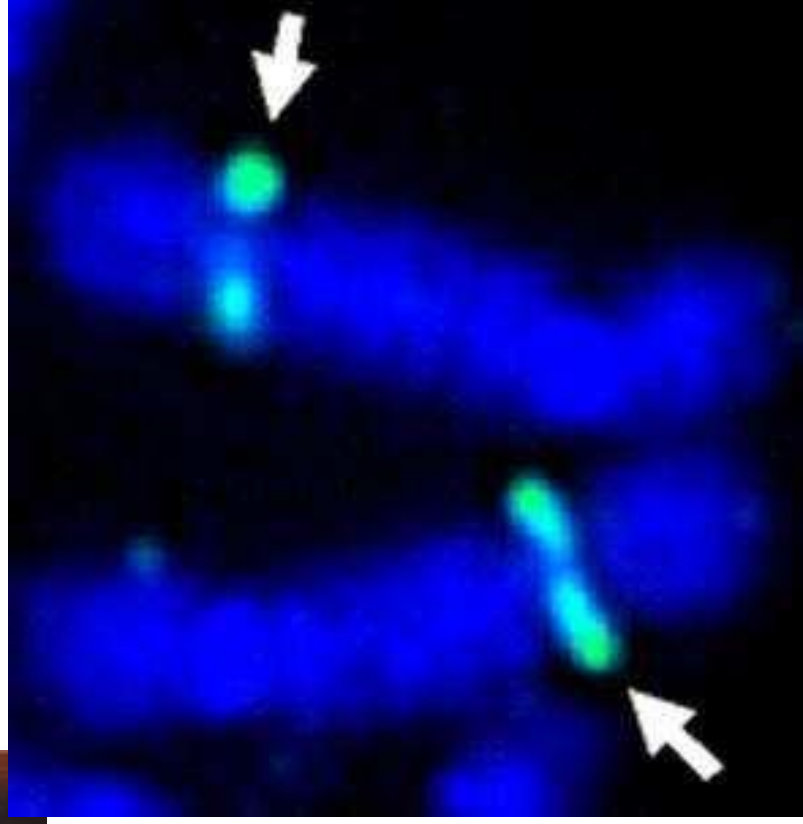


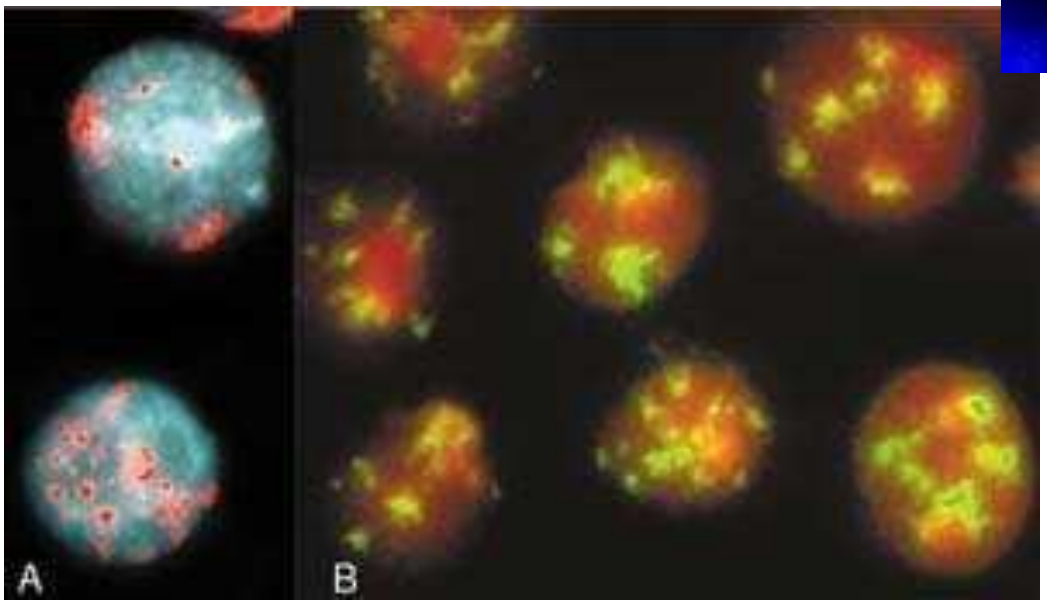
**Encephalin gene expression in the mouse brain**


**The HuC transcript is expressed specifically in the nervous system of this E18 mouse**

www.omrf.org/OMRF/ Core/InSitu.asp

**Metaphase FISH analysis using the BAC probe RP11-104M2 labeled with FITC (green) hybridized to a normal metaphase cell confirms the chromosomal localization of the probe (gene) to 4q28.**

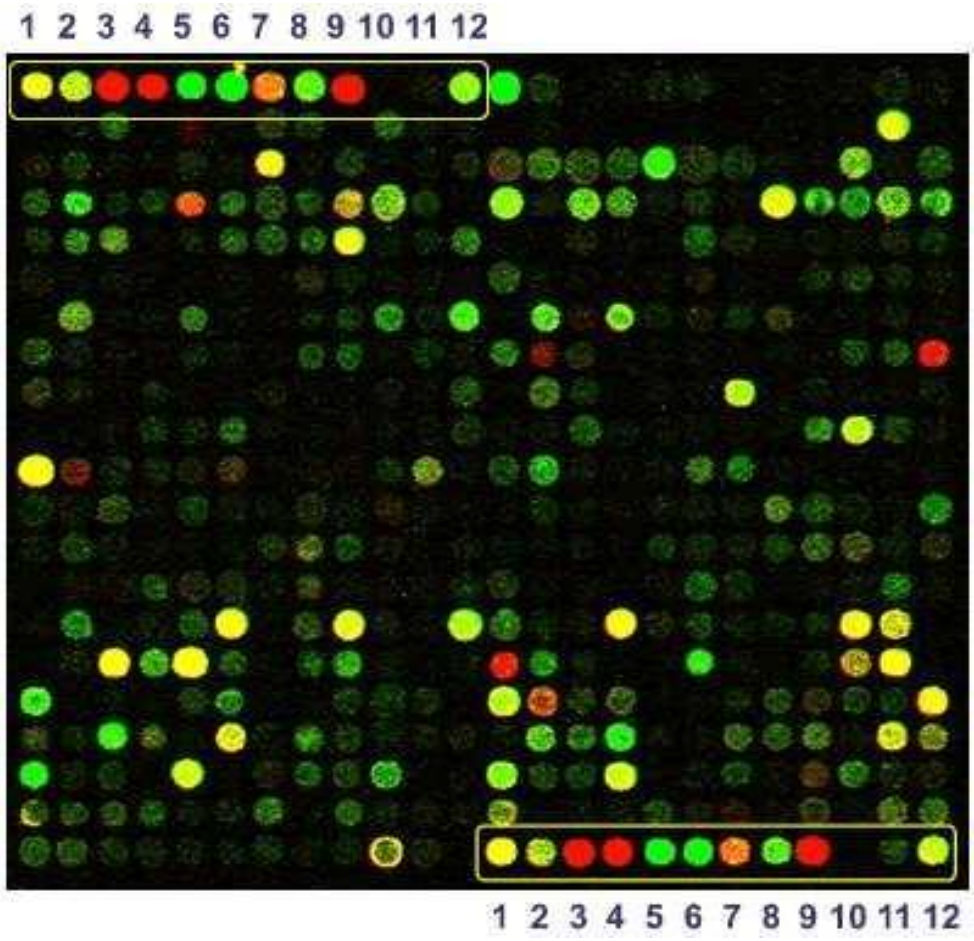


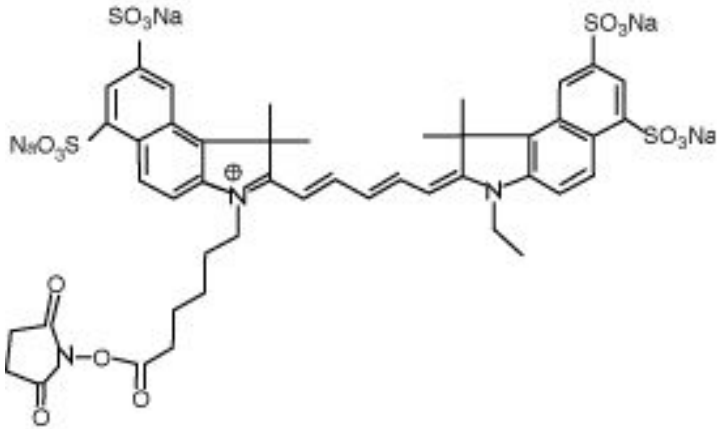


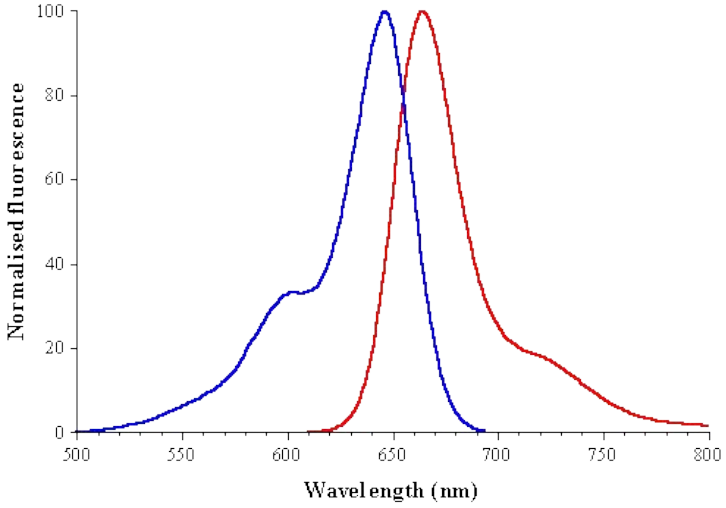

www.infobiogen.fr/.../

**FISH labeling of the centromeric highly repeated DNA**

# Cy3/Cy5 direct labelling of DNA (for microarrays)

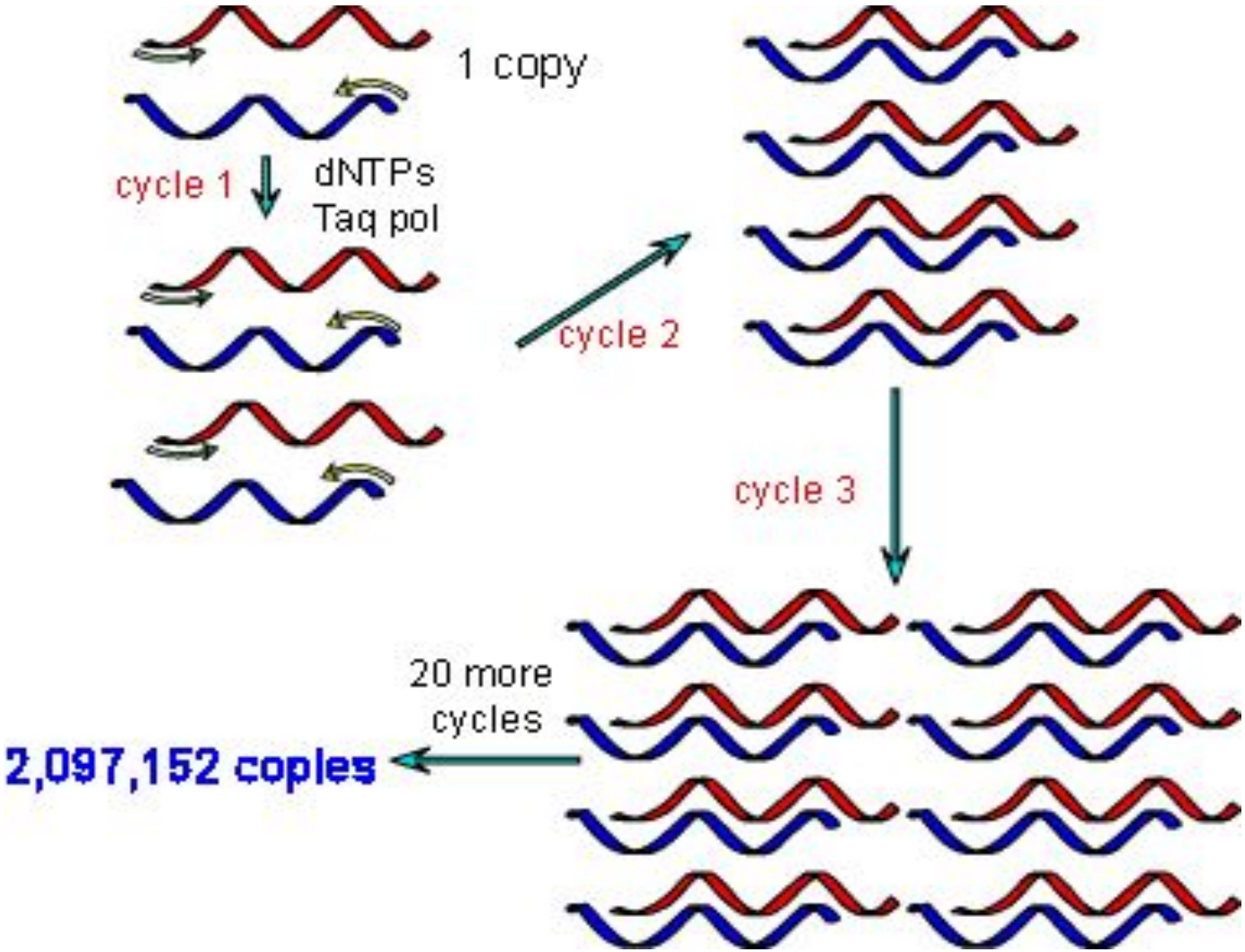


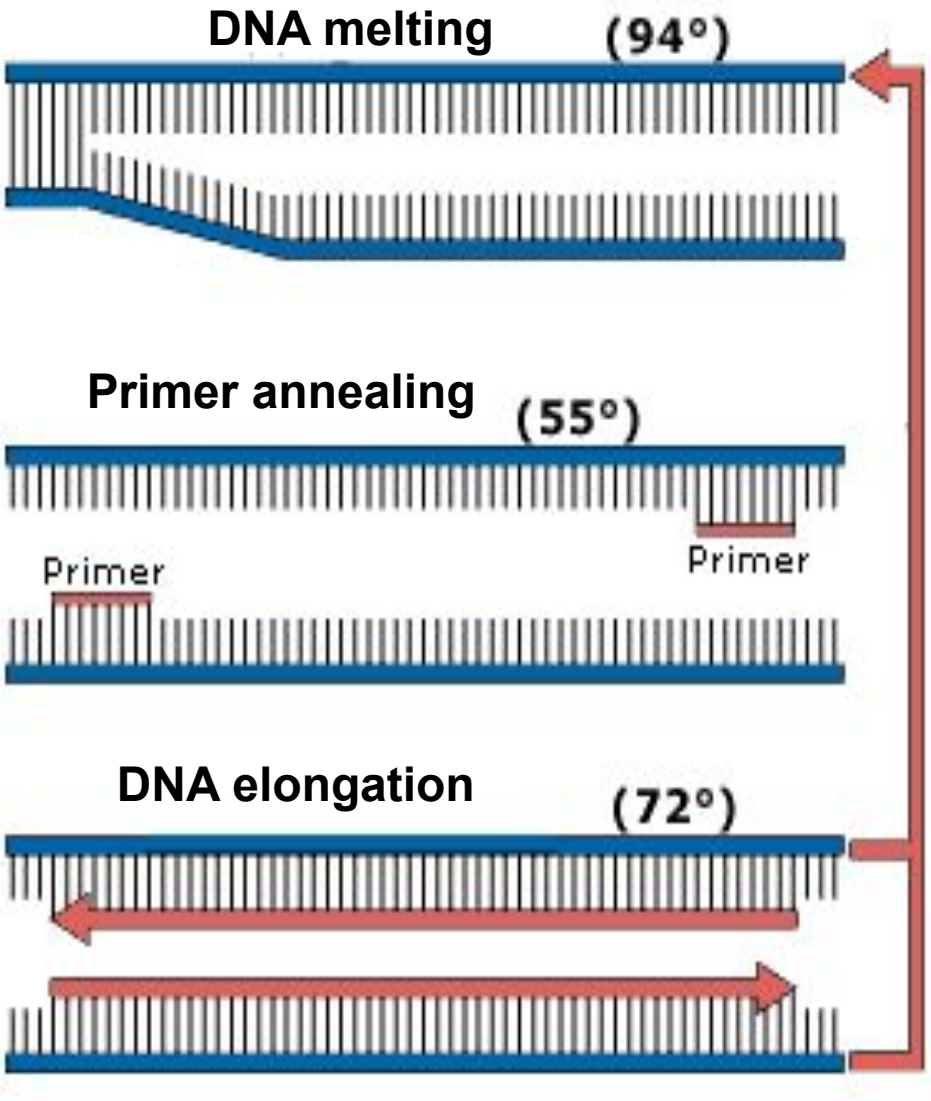

**Cy5 -- RED**

# 2. Polymerase chain reaction (PCR)




http://www.bioteach.ubc.ca/MolecularBiology/PolymeraseChainReaction/PCR.gif

# Polymerase Chain Reaction (PCR)




Biochemistry from U.C. Berkeley

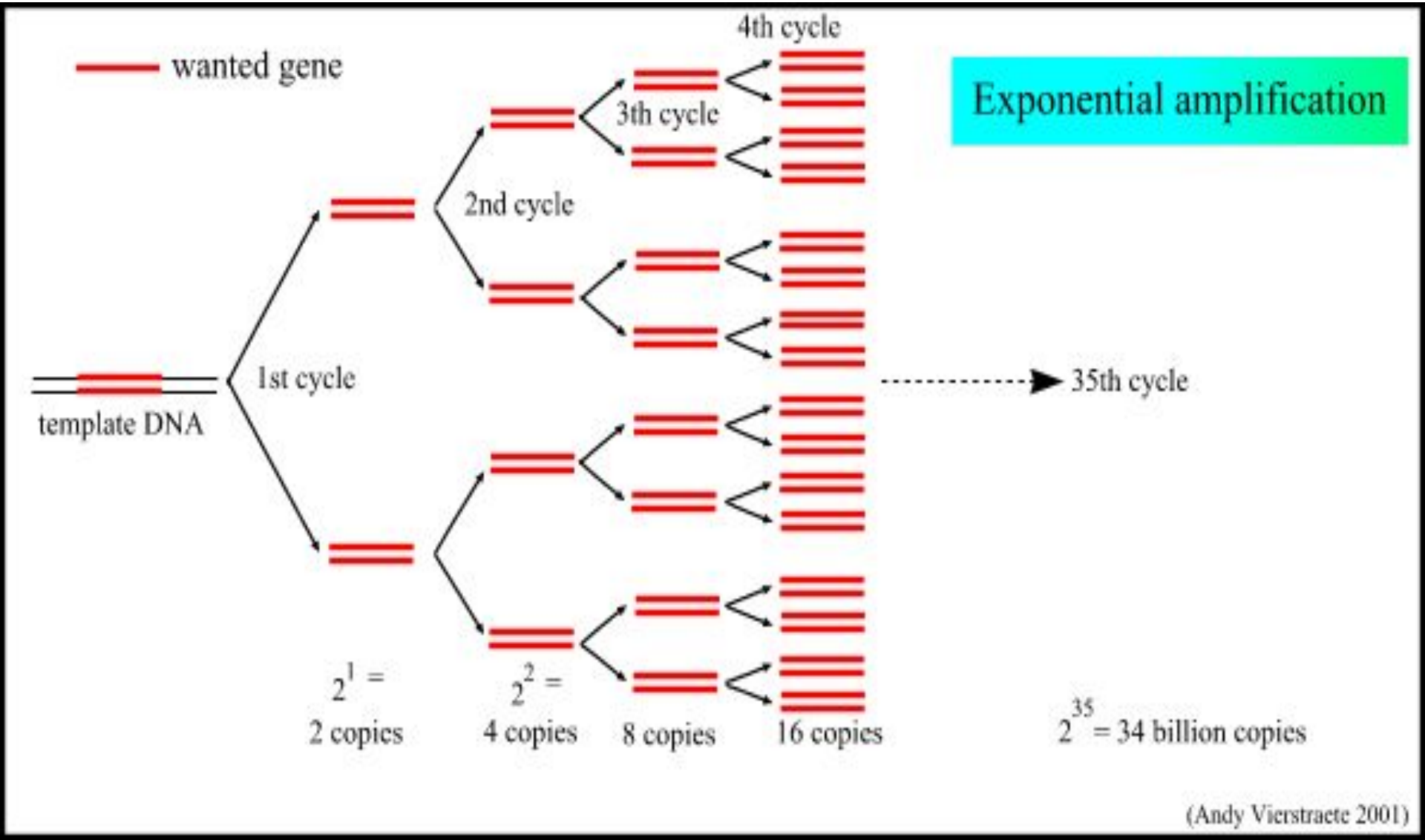
**Nobel Prize in Chemistry 1993, at age 48**

**Kary Mullis**
**(invented PCR in 1983)**

***PhD***
***"The Cosmological Significance of Time Reversal,"***

**ww2.mcgill.ca/biology/undergra/ c200a/f07-16.gi**

# Exponential nature of PCR amplification



# PCR Reagent Components

- Template (DNA or RNA)
- Two primers
- dNTPs
- PCR buffer
  - KCl, Tris, $MgCl_2$
- Thermostable DNA Polymerase (*Taq* )



www.biotechlab.nwu.edu/ pe/Sld022.htm

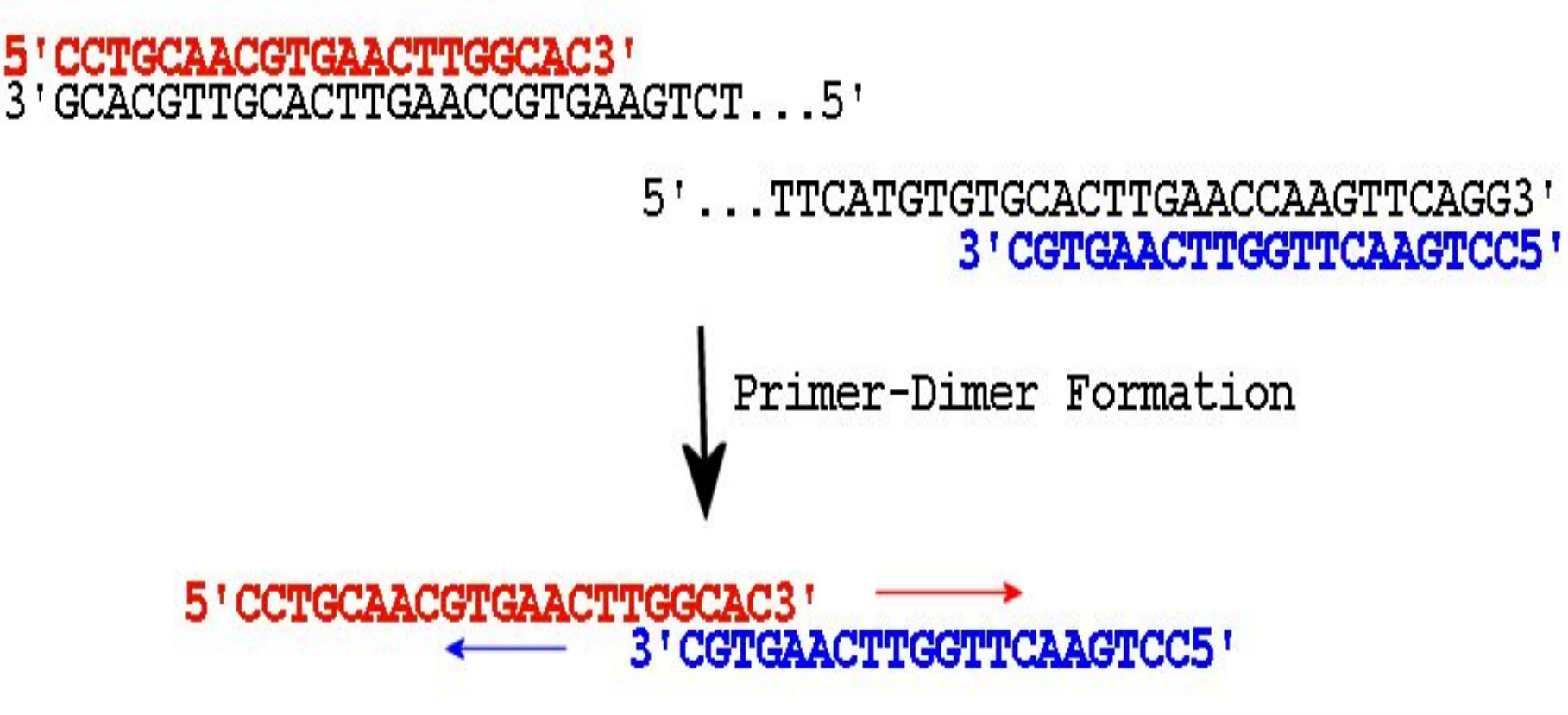
# Basic Primer Design

- Usually 15 - 30 nucleotides in length
- Confirm uniqueness of primers with computer programs
- Optimize base pairing at 3' end is critical
- Random base distribution and average G+C content
  - Avoid long A+T and G+C-rich regions if possible
- Minimize internal secondary structure
- **Try for equal Tm for both primers**

$$Tm = 69.3 + 0.41 * (\%GC) - 650/length$$

**use OLIGONEW or PRIMER software**

# Avoid primer dimer formation




**Marginally problematic primer**

```
5'-GCTTTGTCCGATGAATCGT-3'
          ||||   ||||
      3'-TGCTAAGTAGCCTGTTTCG-5'
```

# Use Software to avoid of such problems

**General Primer Picking Conditions**

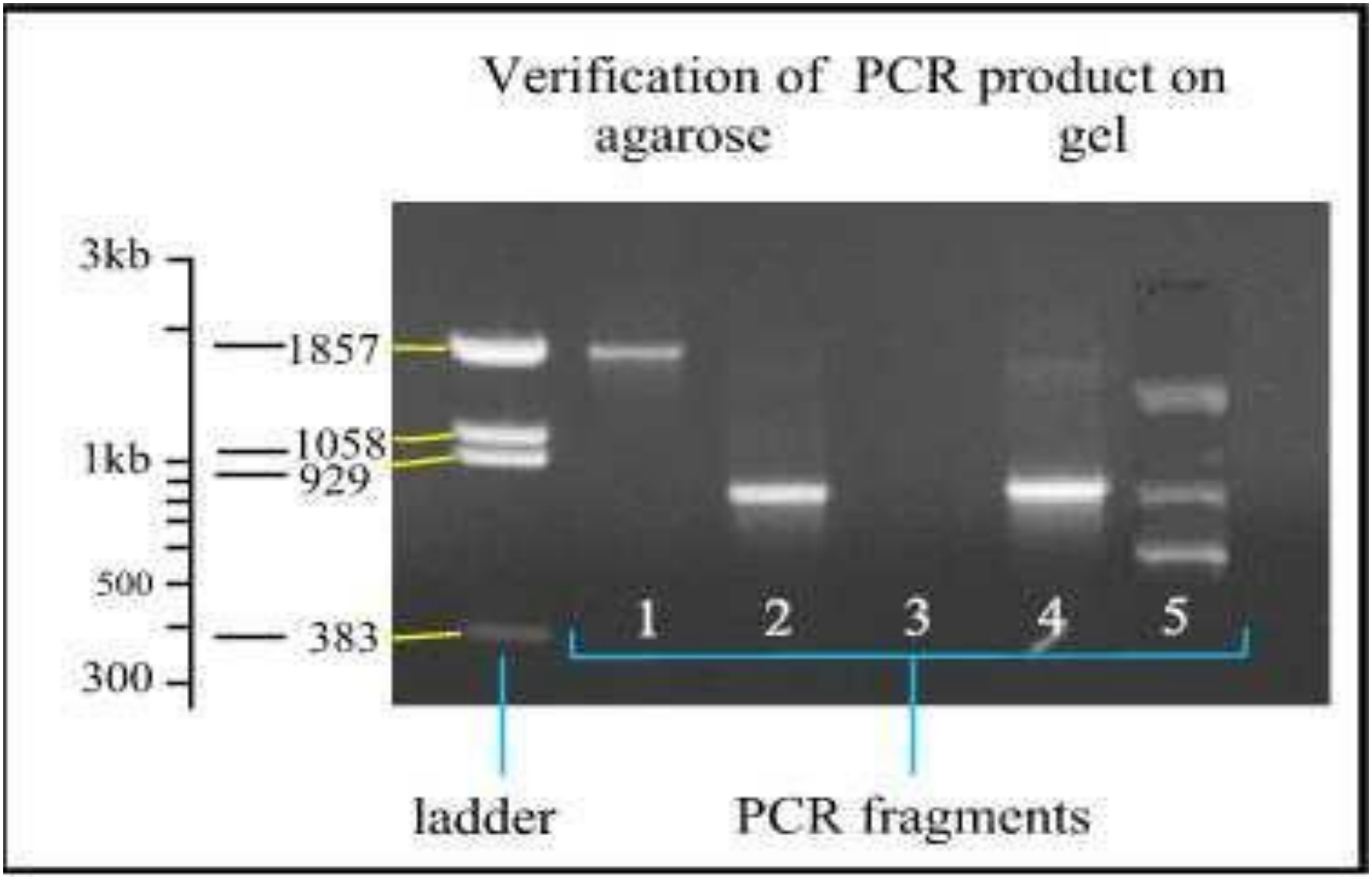
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Primer Size | Min: 18 | Opt: 20 | Max: 27 | | | | |
| Primer Tm | Min: 57.0 | Opt: 60.0 | Max: 63.0 | Max Tm Difference: | 100.0 | | |
| Product Tm | Min: | Opt: | Max: | | | | |
| Primer GC% | Min: 20.0 | Opt: | Max: 80.0 | | | | |
| Max Self Complementarity: | 8.00 | Max 3' Self Complementarity: | 3.00 | | | | |
| Max #N's: | 0 | Max Poly-X: | 5 | | | | |
| Inside Target Penalty: | | Outside Target Penalty: | 0 | Set Inside Target F | | | |
| First Base Index: | 1 | CG Clamp: | 0 | | | | |
| Salt Concentration: | 50.0 | Annealing Oligo Concentration: | 50.0 | (Not the concentra | | | |

emplate.)

☑ Liberal Base ☐ Show Debuging Info

Pick Primers | Reset Form

# Typical PCR gel
# (Every PCR should by gel-verifyed)



# Fidelity of PCR is often an issue

## Conditions Affecting Fidelity

| | Increase Fidelity | Decrease Fidelity |
|---|---|---|
| [dNTP] | 40 - 50 **mkM** all equimolar | 1 - 2mM with different molarities |
| [$Mg^{++}$] | 1:1 with dNTP | 6 - 8mM $Mg^{2+}$ + 0.5mM $Mn^{2+}$ |
| $Temp_{anneal}$ | Increase Temp | Decrease Temp |
| [Enzyme] | Decrease [Enz] | Increase [Enz] |
| Extension time | Decrease Time | Increase Time |
| # cycles | Decrease Cycles | Increase Cycles |

# Proof-reading activity enzymes are required for

## High Fidelity PCR

- Requires enzymes with 3'-5' nucleolytic properties
- Reaction conditions are
  - Designed to "slow down" the reaction
  - Limit reagents to favor correct base pair matches

**High Yield and High Fidelity are mutually exclusive**

# PCR Primer Concentrations

Normal Primer Range: 0.1 - 1.0 mM

| | Primer Concentration* | |
|---|---|---|
| | Lower | Higher |
| Product Specificity: | Increases | Decreases |
| Product Yield: | Decreases | Increases |

* Compared to optimum



www.biotechlab.nwu.edu/ pe/Sld022.htm

# Input Copy Number, Number of Cycles, and Product Mass

| Human Genomic | | |
|---|---|---|
| Copies[a] | Cycles | Yield[b] |
| 100 | 33 | 160 pg |
| 20,000 | 25 | 32 ng |
| 600,000 | 20 | 960 µg |

| Plasmid 6kb | | |
|---|---|---|
| Copies[a] | Cycles | Yield[b] |
| 100 | 33 | 660 ag |
| 20,000 | 25 | 130 fg |
| 600,000 | 20 | 4 pg |
| $1.5 \times 10^{11}$ | 2 | 1mg |

[a] Input copies

[b] Product mass

**If complete copies is amplified**



www.biotechlab.nwu.edu/ pe/Sld022.htm


# $Mg^{2+}$ in PCR

Cofactor required for activation of Taq polymerase

- dNTPs bind free $Mg^{2+}$ in a 1:1 molar ratio
  - Increases or decreases in dNTPs must be matched with equivalent changes in $[Mg^{2+}]$
- Each PCR reaction has an optimum concentration of free $[Mg^{2+}]$
- Different Enzymes require different $[Mg^{2+}]$



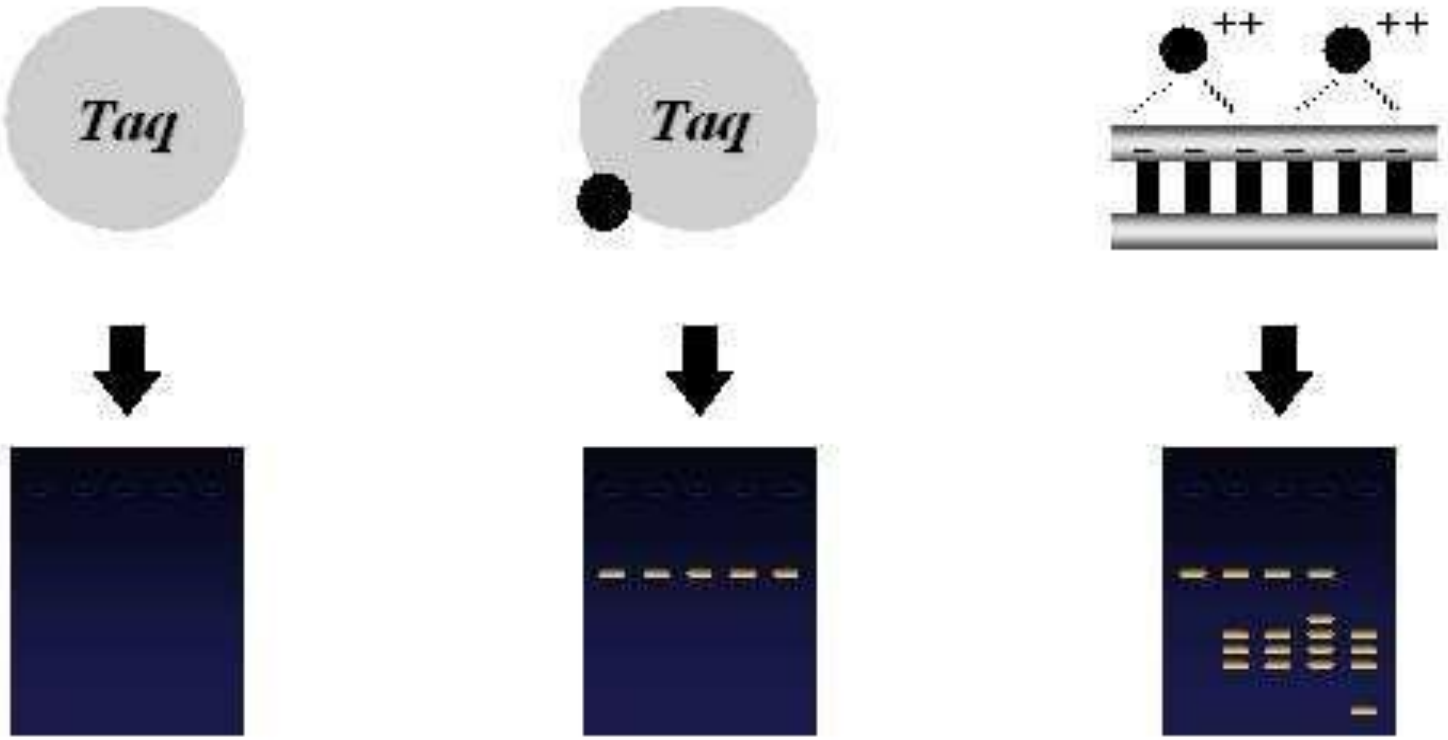
www.biotechlab.nwu.edu/ pe/Sld022.htm


# $Mg^{++}$ Optimization

Suboptimal $Mg^{2+}$

Optimal $Mg^{2+}$

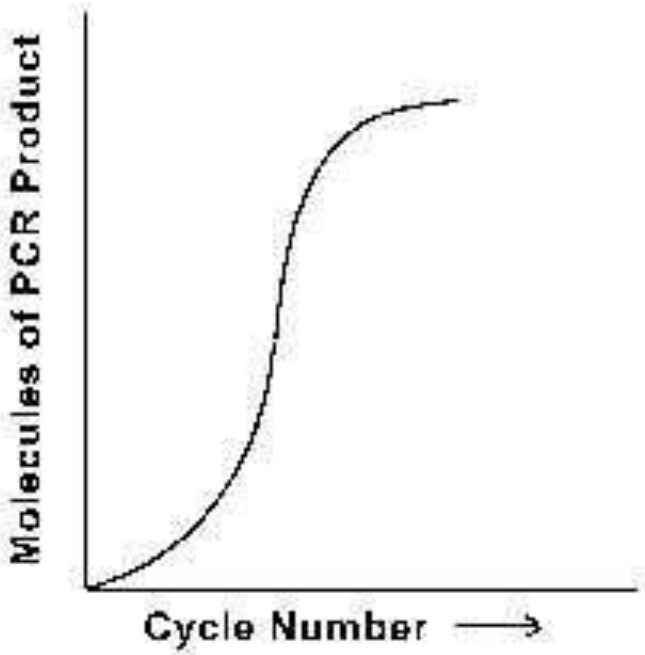
Excess $Mg^{2+}$

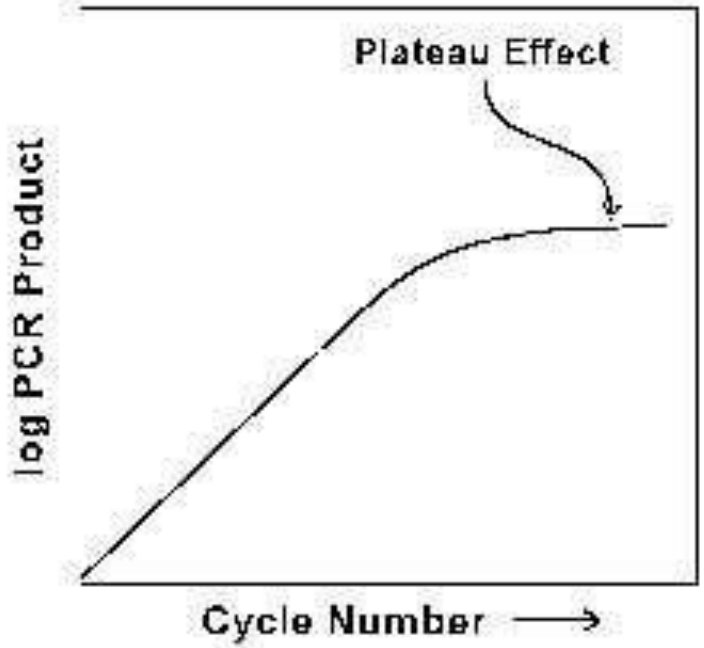





www.biotechlab.nwu.edu/ pe/Sld022.htm

# Plateau effect in PCR reaction




Plateau effect: The point at which product accumulation is no longer exponential (saturation), typically $10^8$ to $10^{12}$ molecules of product.

# Plateau effect in PCR reaction

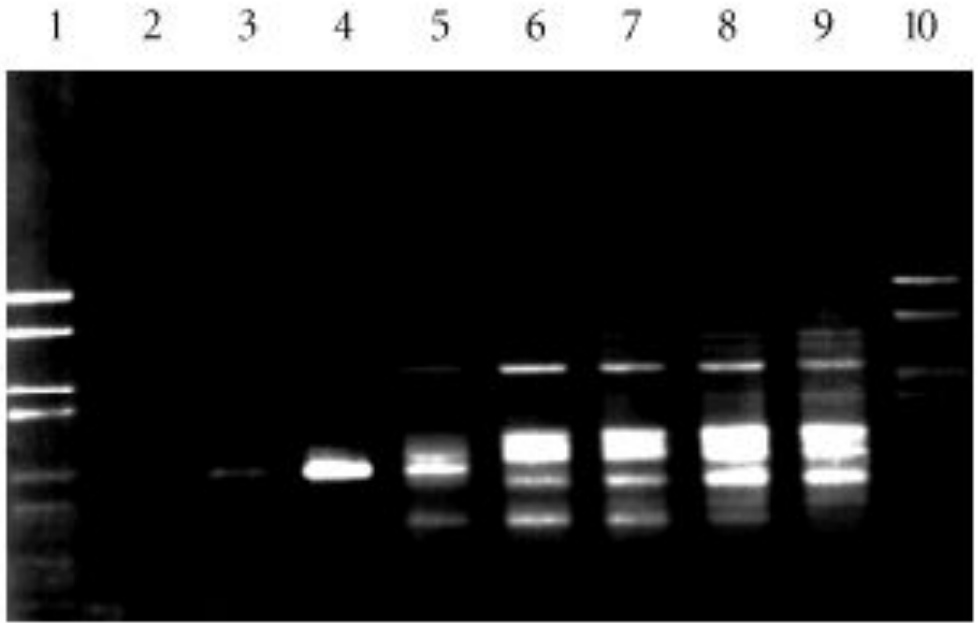
- The point in a PCR where running more cycles does not result in a net gain of specific PCR
- Amplicons not in plateau - including nonspecific fragments - may continue to grow exponentially
- Hence, running PCRs into plateau may result in high background or smearing

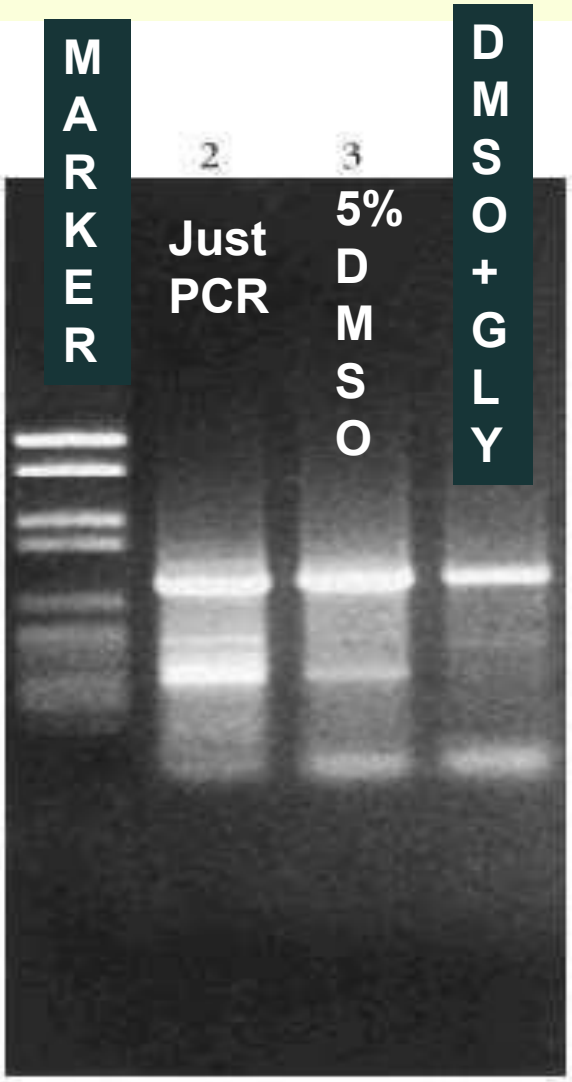


www.biotechlab.nwu.edu/ pe/Sld022.htm

# Non-specific PCR and how to improve it

**Decrease in Mg concentraton**



# PCR enzymes

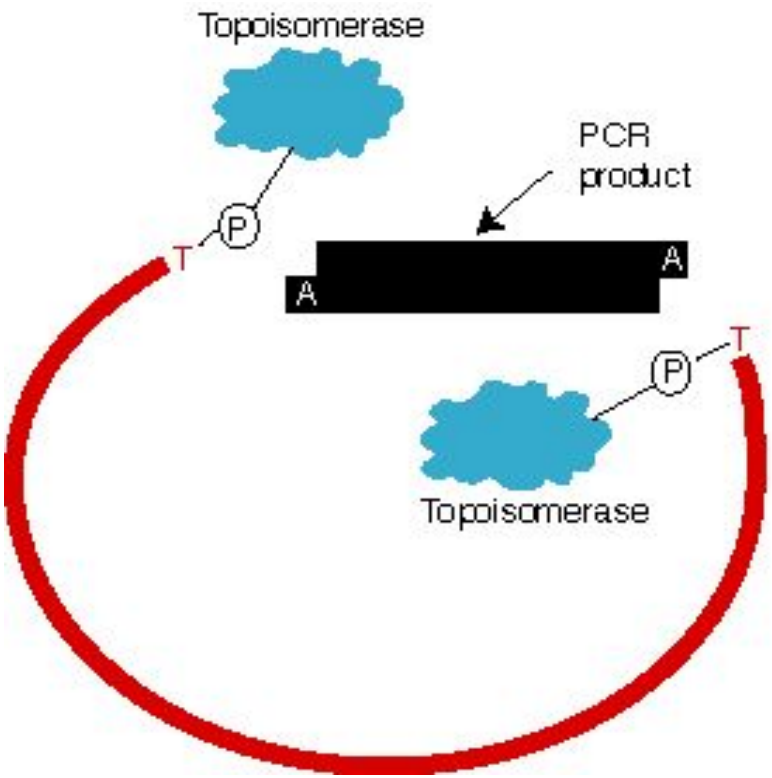
*Taq* DNA polymerase, the first enzyme used for PCR, is still the most popular.

**-- high processivity**
**-- is the least expensive choice**

**Half-life at 95C is 1.6 hours**

**-- generates PCR products with single A overhangs on the 3´-ends**
**(Suitable for TOPO-cloning)**

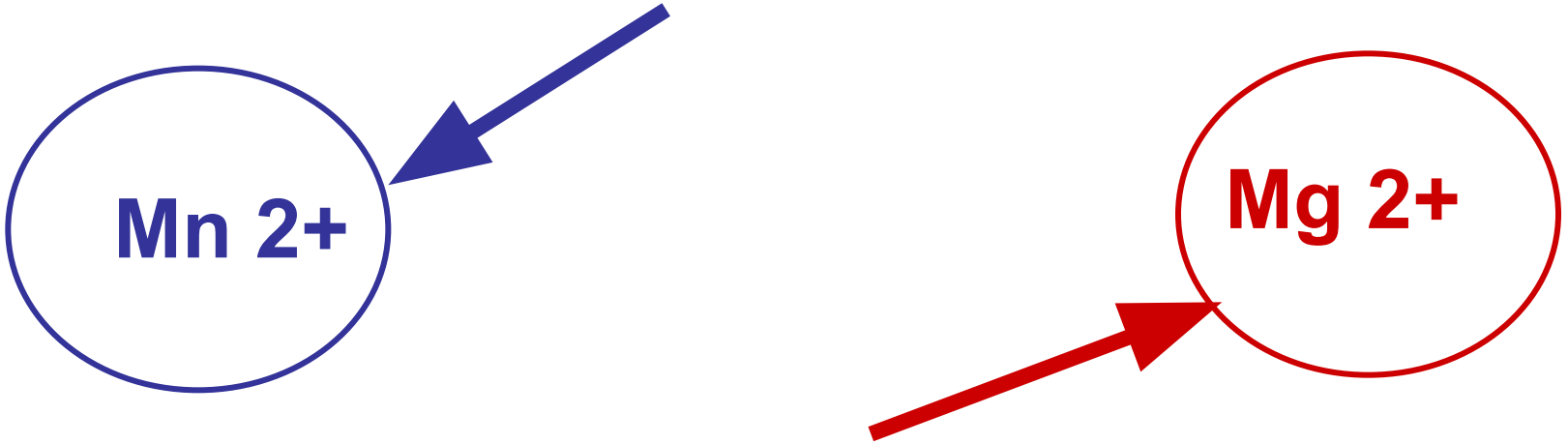
**"Topo" cloning system (Invitrogen)**



# Tth polymerase

***Thermus thermophilus* strain HB8.**

**RNA-dependent DNA-polymerase activity in the presence of Mn2+ ions.**




**DNA-dependent DNA-polymerase activity in the presence of Mg2+ ions.**

**The fragment should be ideally smaller 1 kb.**

# Pfu polimerase

Proofreading or high fidelity DNA polymerases

(from *Pyrococcus furiosus*).

**approx.1 / 2, 000,000 nucleotides before making an error.**

**In comparison Taq DNA polymerase**
**makes an error in approx. every 1/ 10,000 nucleotides.**

**can tolerate temperatures exceeding 95°C,**
**enabling it to PCR amplify GC-rich targets.**

**more expensive**

# Pol Vent (From *Thermococcus litoralis)*

**also known as Tli polymerase**

**Very termostable: Half-life at 95 C is approximately 7 hours**

**3'->5' exonuclease activity presents**

**Vent error rate is intermediate between Taq and Pfu.**

**2-5 x 10-5 errors/bp**

**Other polymerases:**

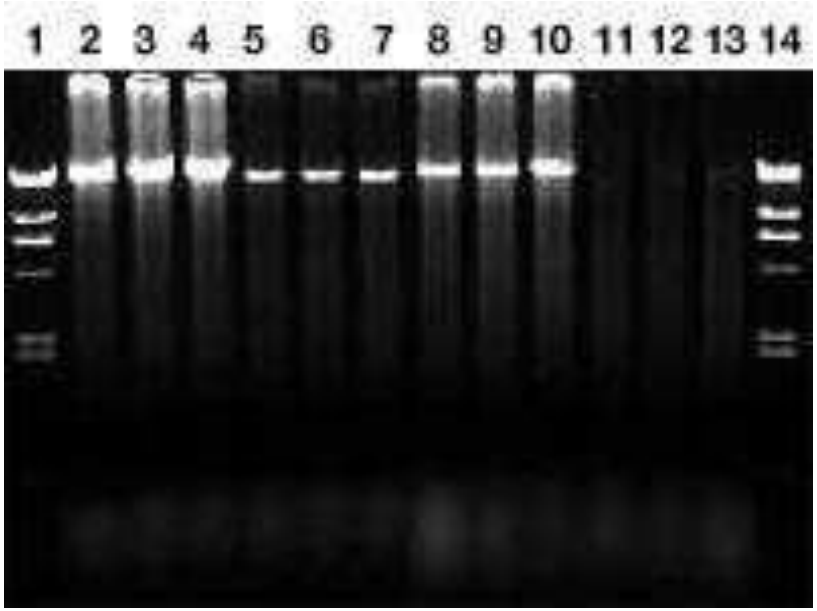
**Deep Vent (Pyrococcus species GB-D) (New England Biolabs)**
**New England Biolabs claims fidelity is equal to or greater than that of Vent.**

**Replinase (Thermus flavis) □1.03 x 10-4 errors/base**

# Long-Range PCR

## Use of two polymerases:

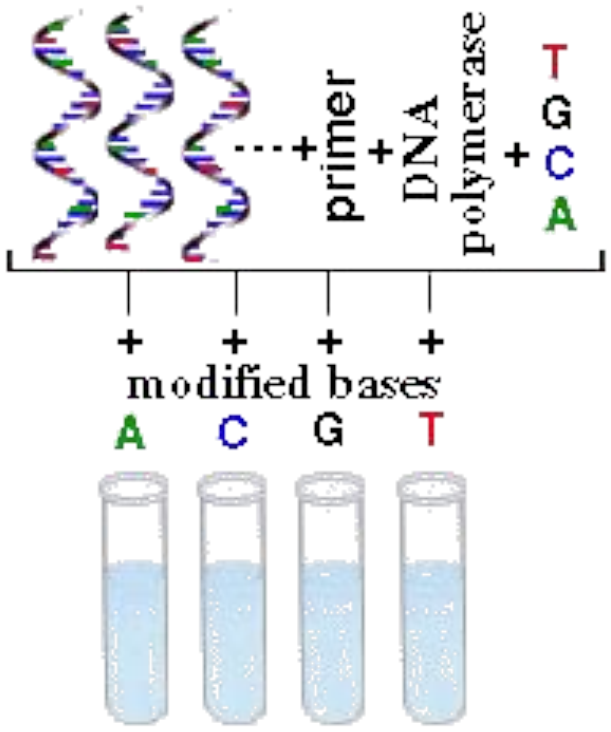
**a non-proofreading polymerase Taq**
**is the main polymerase in the reaction,**
**a proofreading polymerase (3' to 5' exo) Pwo**
**is present at a lower concentration.**




**22-24 kb PCR products are achieved on Qiagen and Eppendorf PCR mixes**

***Taq+ Pwo***
***(Pyrococcus woesei)* ;**
**Pwo is very stable,**
**2 hrs at 100 C**

# 3. SEQUENCING: (Sanger method)

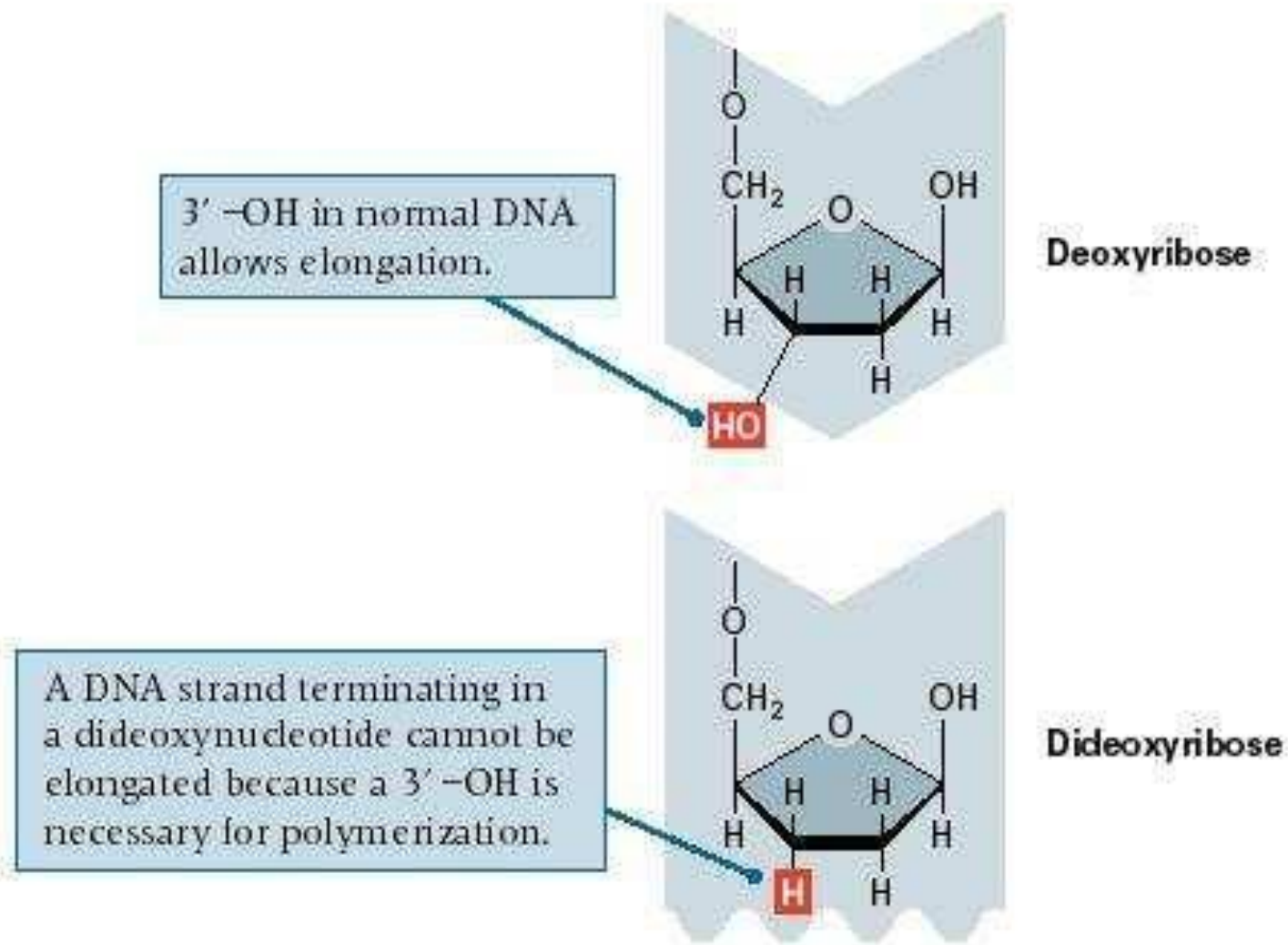



Sequencing of DNA by the Sanger Method

**Frederick Sanger**
**(Nobel prize 1980 with Paul Berg and Walter Gilbert)**

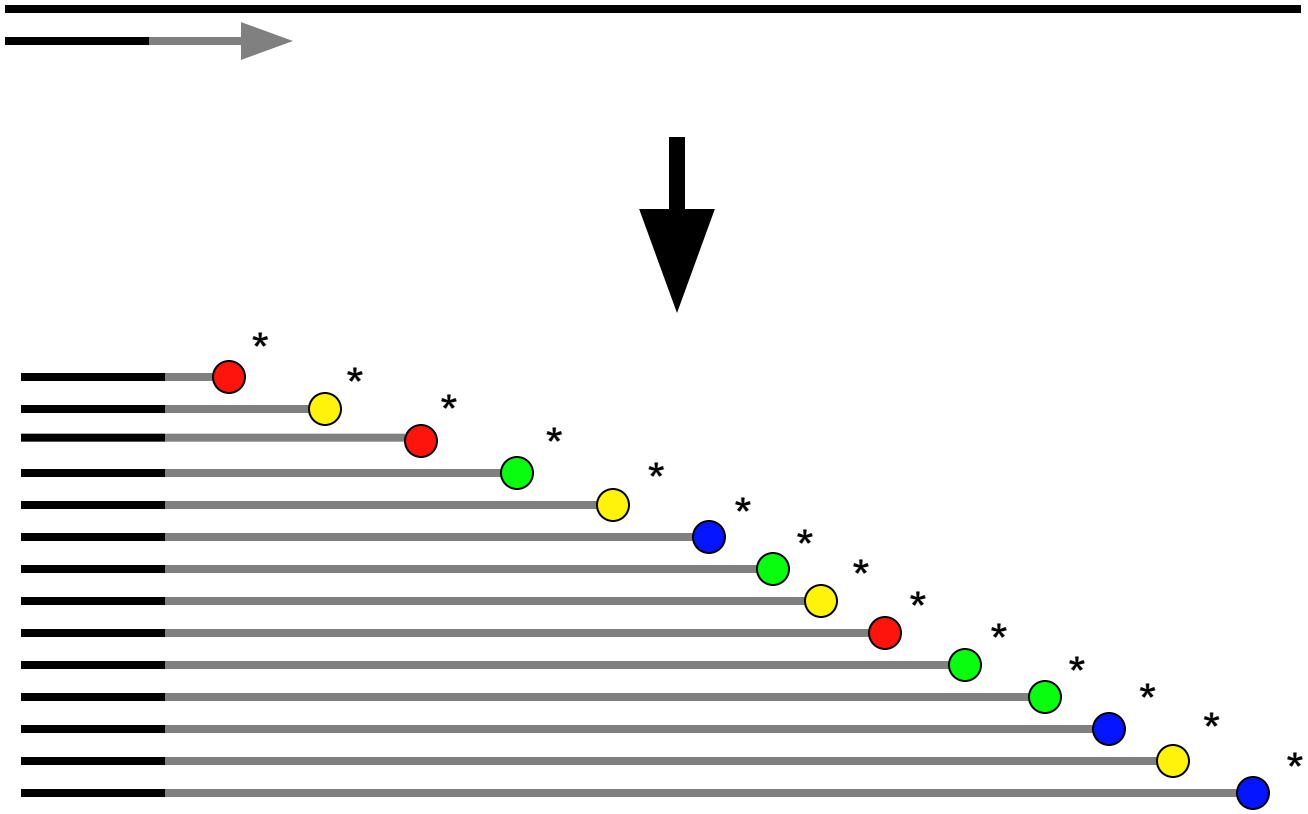
**http://www.kids-dna.com/dnatube.gif**

# Dideoxynucleotide blocks chain elongation

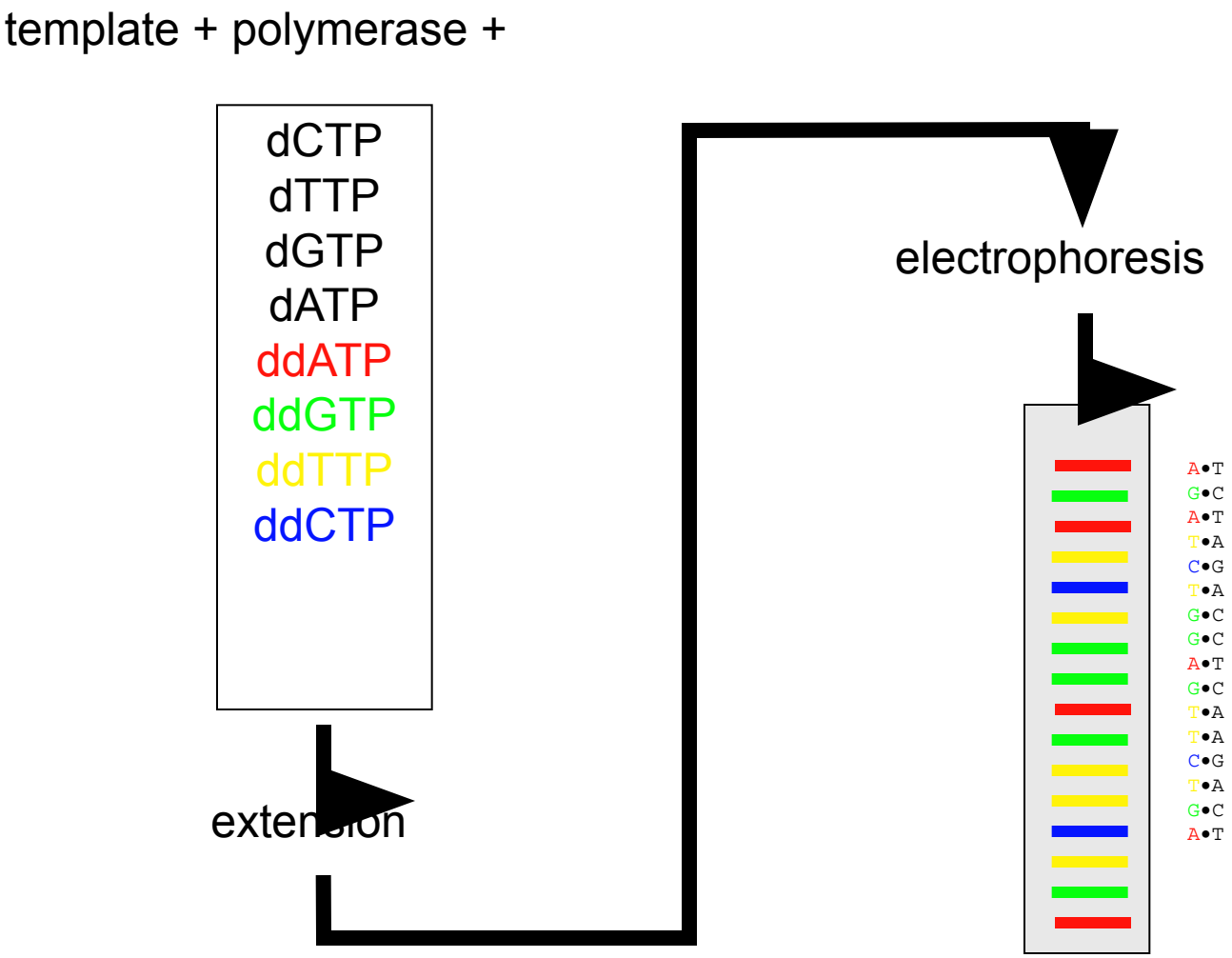



http://www.cbs.dtu.dk/staff/dave/roanoke/fig5_38.jpg

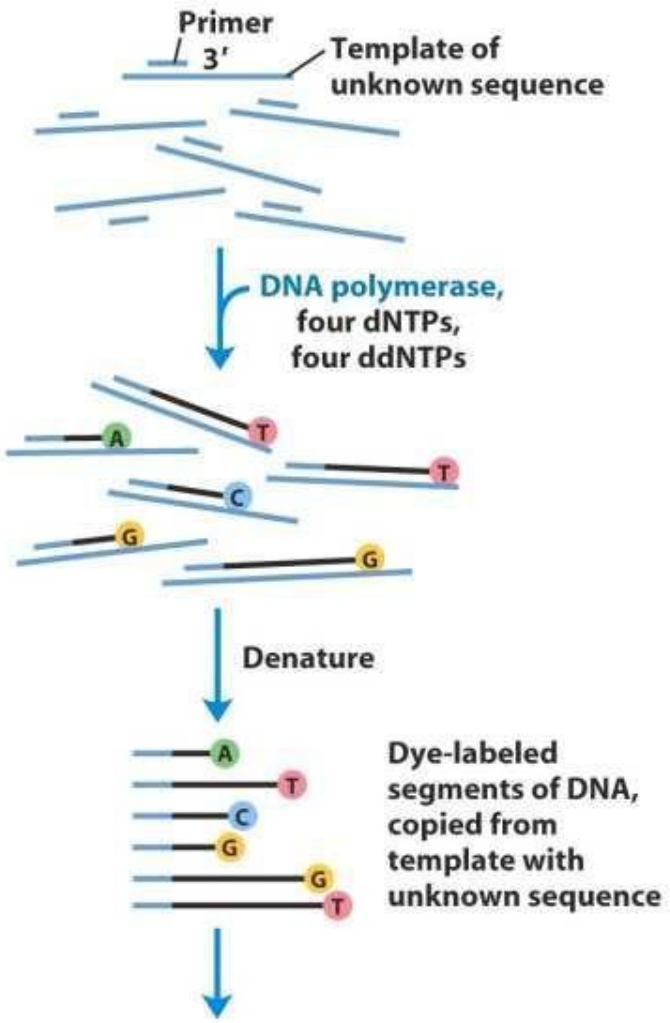
# DNA sequencing:  chemistry, with terminator dyes

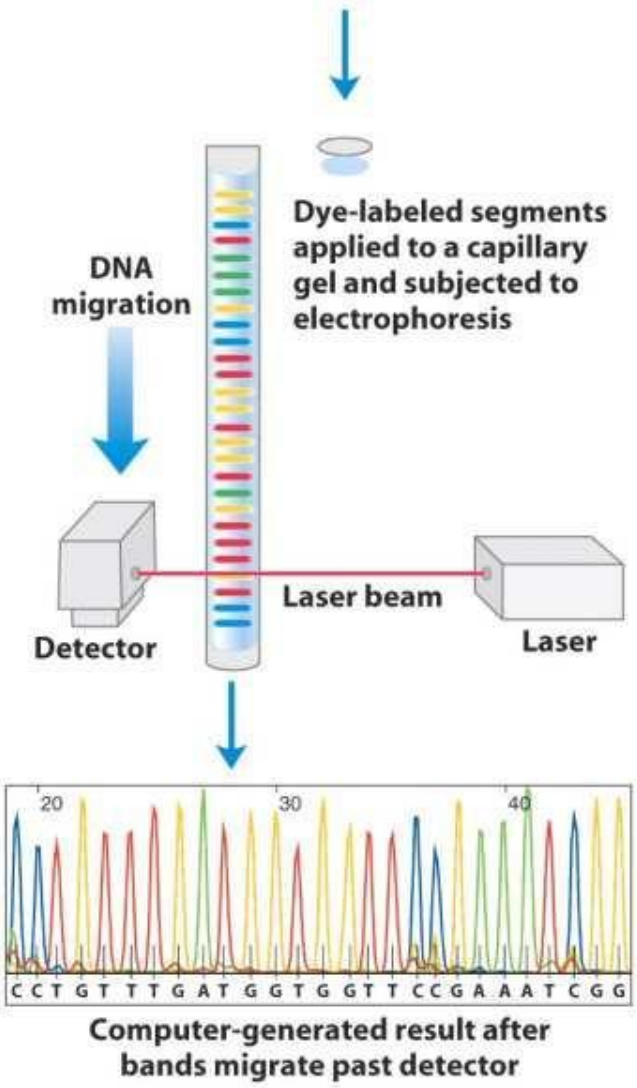


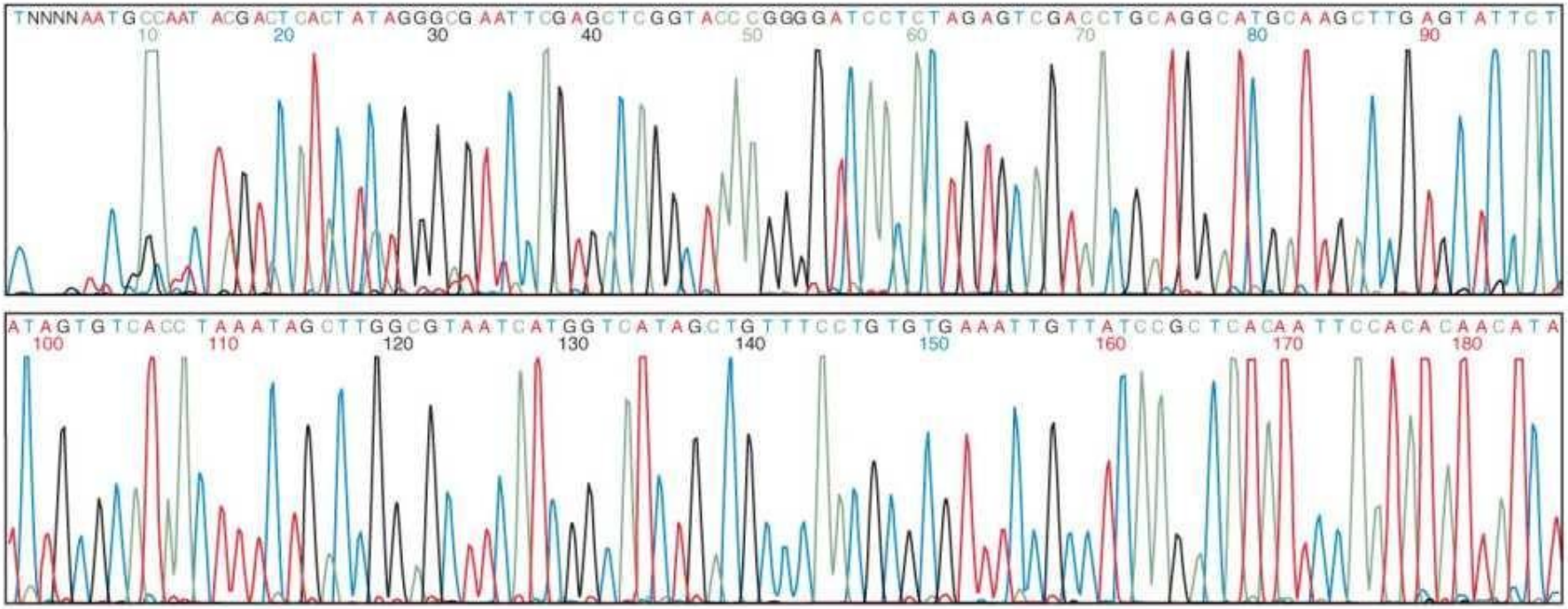
# Semi-automated fluorescent DNA sequencing:



# Cycle Sequencing - PCR



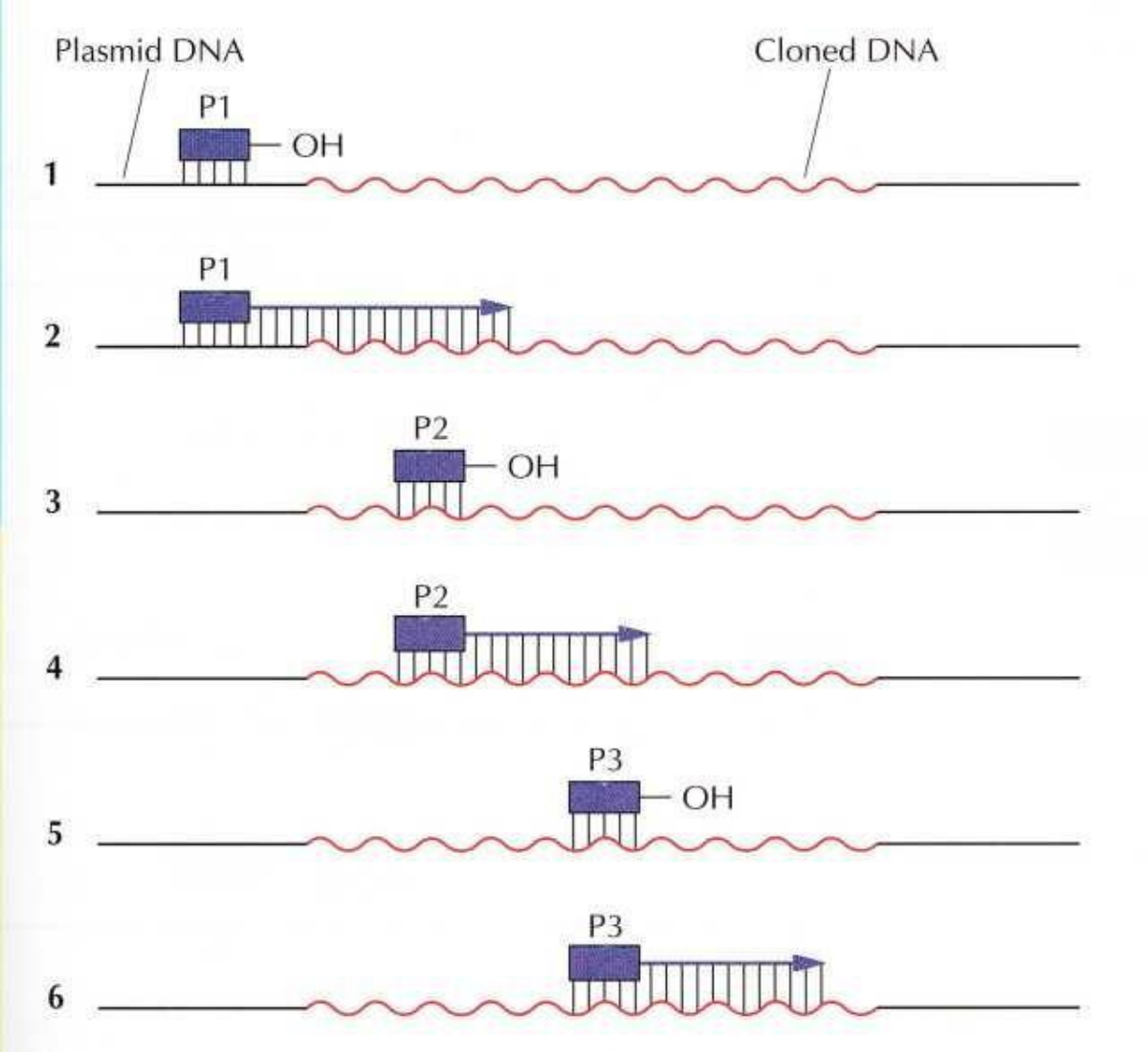




TNNNNAATGCCAAT ACGACTCACTATAGGGCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGAGTATTCT
10 20 30 40 50 60 70 80 90
ATAGTGTCACC TAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATA
100 110 120 130 140 150 160 170 180

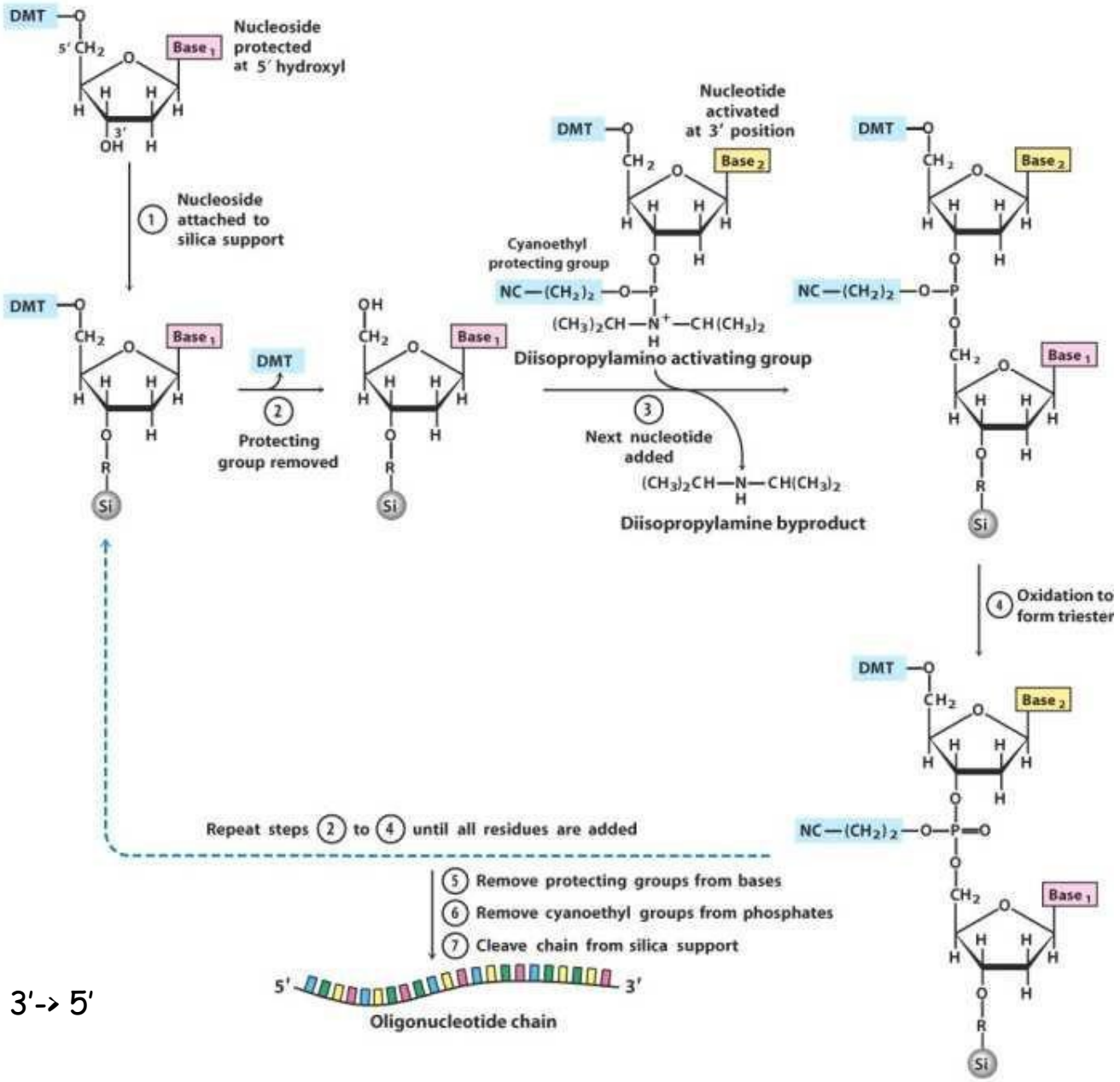
# Applied Biosystems Inc., have designed an automated method that combines the PCR and actual sequencing

<http://www.utmb.edu/proch/servo3.htm>

# DNA sequencing by primer walking



# Chemical synthesis of DNA




Chain grows: 3′-> 5′

# General consideration about Gene Expression

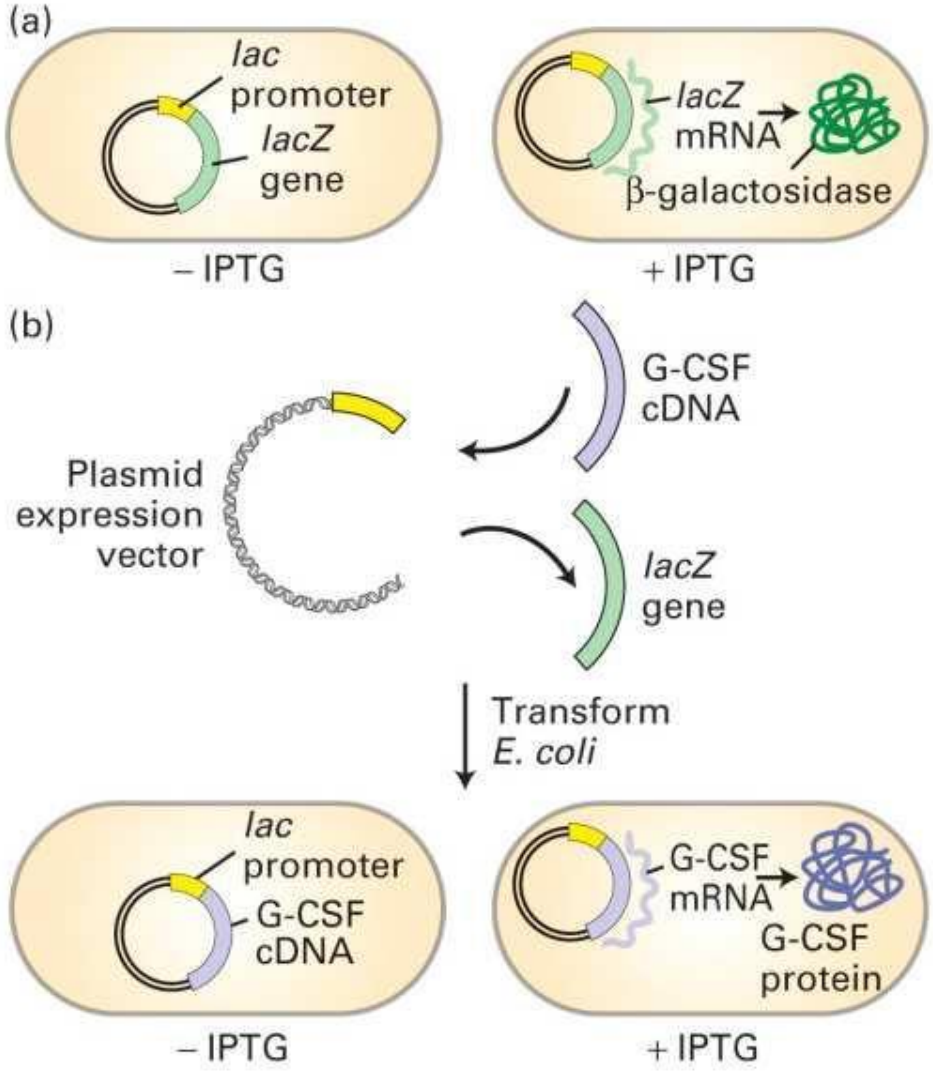
- Expression Host -> Expression System
- Promoter system -> expression vector
- Properties of product -> stability
- Production level

# Comparison of expression systems

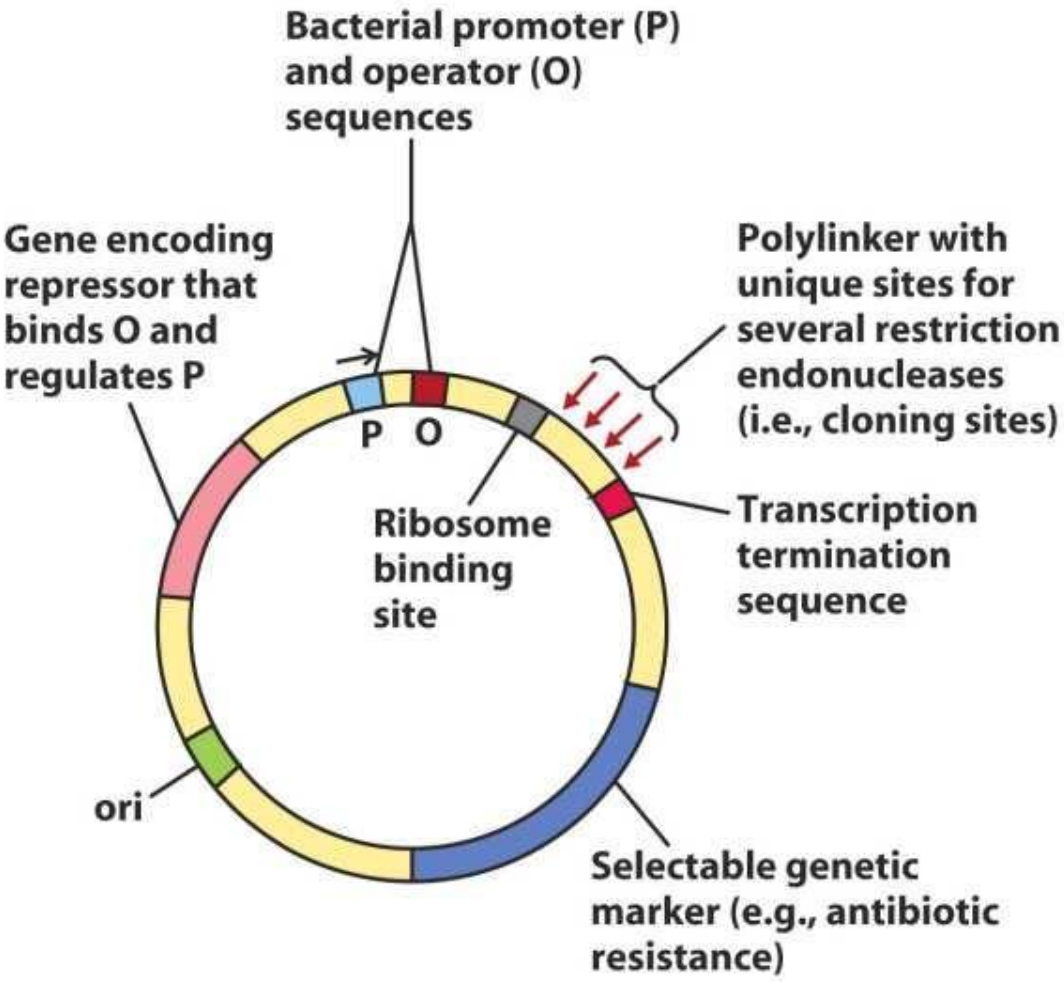
**Table 1**
Comparison of Expression Systems

| Desired characteristics | Expression system | | | |
|---|---|---|---|---|
| | Bacteria | Yeast | Insect | Mammalian cell culture |
| Cell growth | Rapid | Rapid | Slow | Slow |
| Complexity of growth medium | Minimum | Minimum | Complex | Complex |
| Cost of growth medium | Low | Low | High | High |
| Expression level | High | Low to high | Low to high | Low to moderate |
| Extracellular expression | Secretion to periplasm | Secretion to medium | Secretion to medium | Secretion to medium |
| Posttranslational modifications | | | | |
| Protein folding | Refolding usually required | Refolding may be required | Proper folding | Proper folding |
| N-linked glycosylation | None | High mannose | Simple, no sialic acid | Complex |
| O-linked glycosylation | No | Yes | Yes | Yes |
| Phosphorylation | No | Yes | Yes | Yes |
| Acetylation | No | Yes | Yes | Yes |
| Acylation | No | Yes | Yes | Yes |
| γ-Carboxylation | No | No | No | Yes |

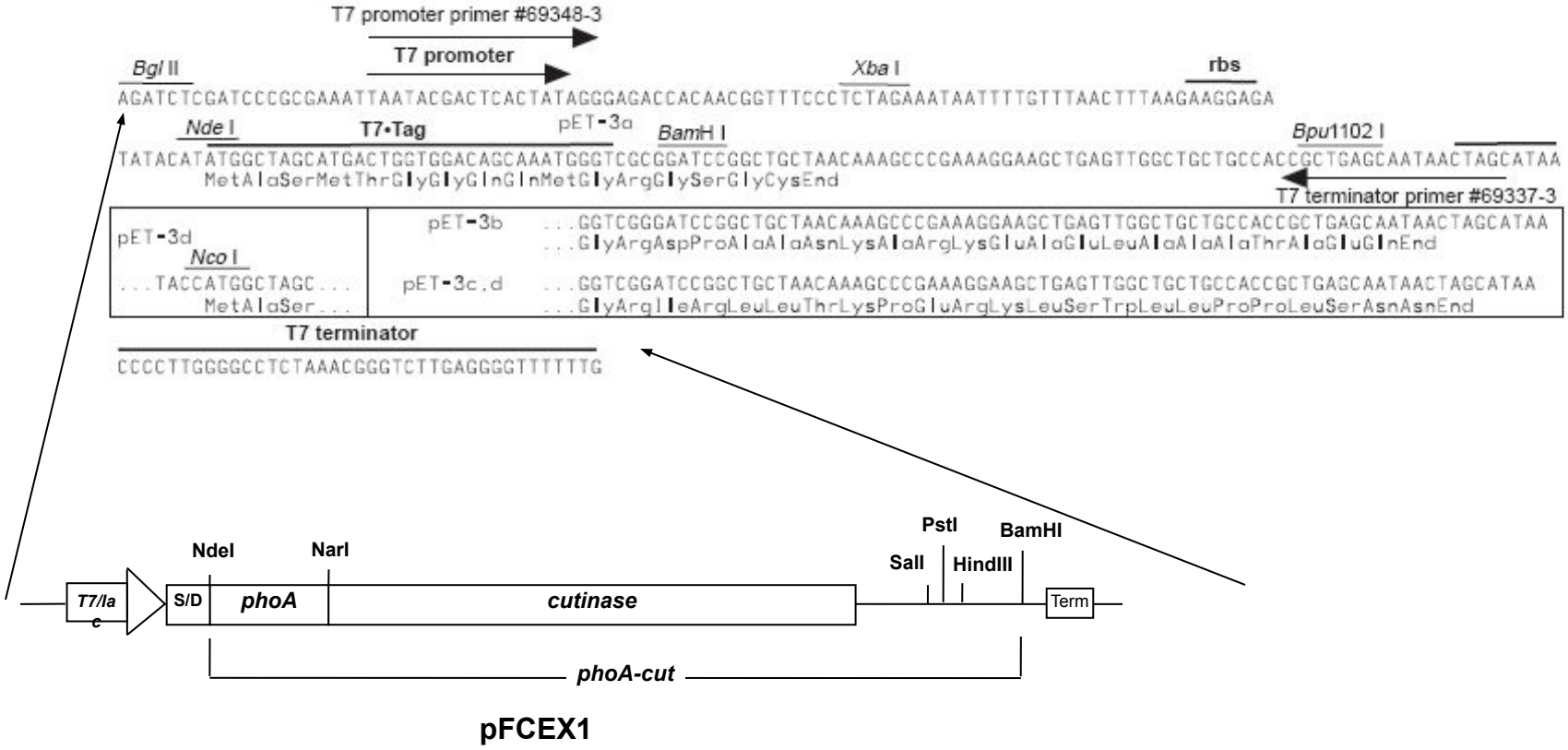
# Use of *Lac* promoter (*pLac*) for expression of foreign protein



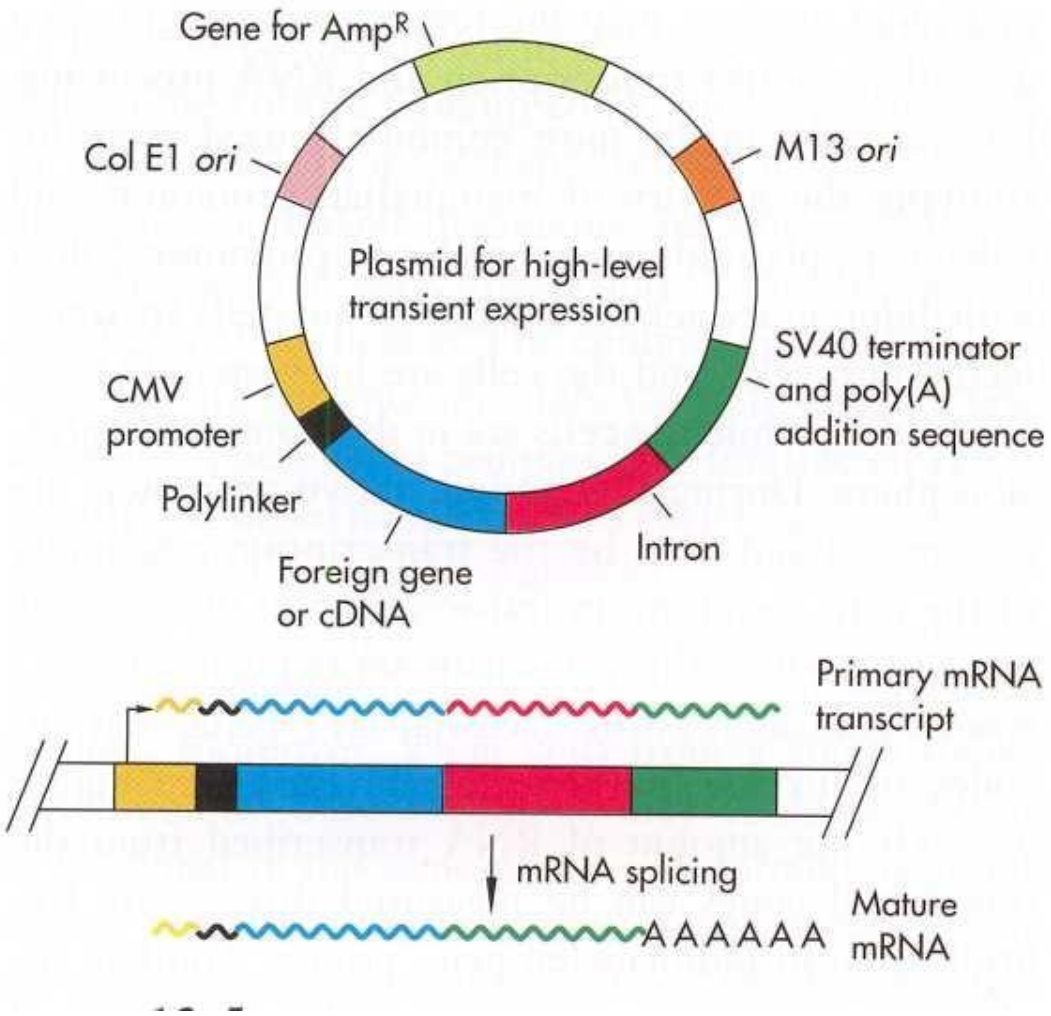
# Prokaryotic Expression vector



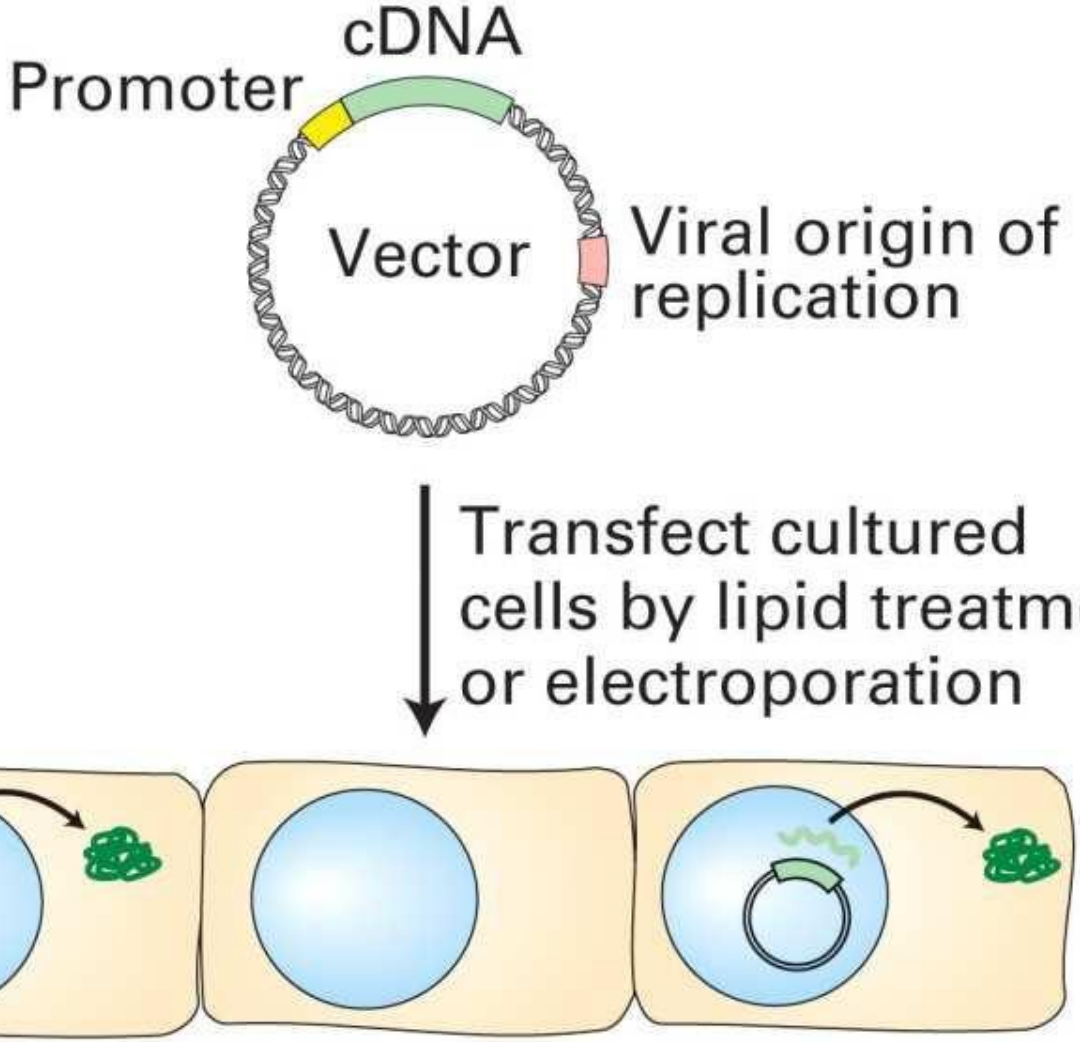
# Prokaryotic Expression vector



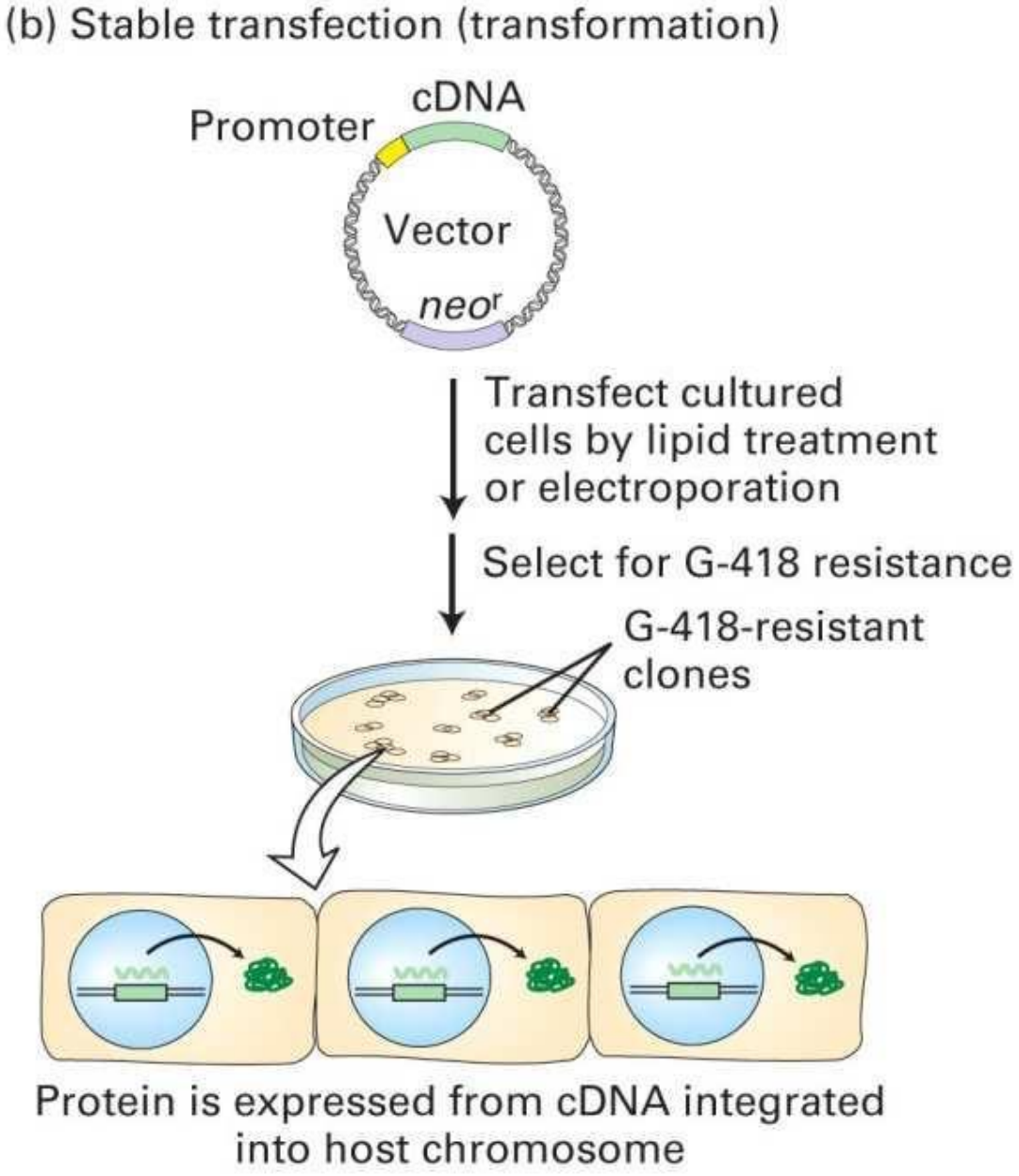
# Eukaryotic Expression vector



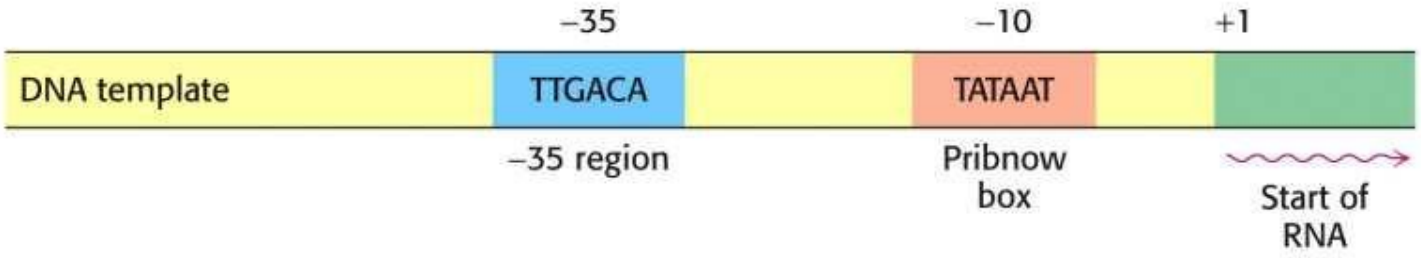
(a) Transient transfection



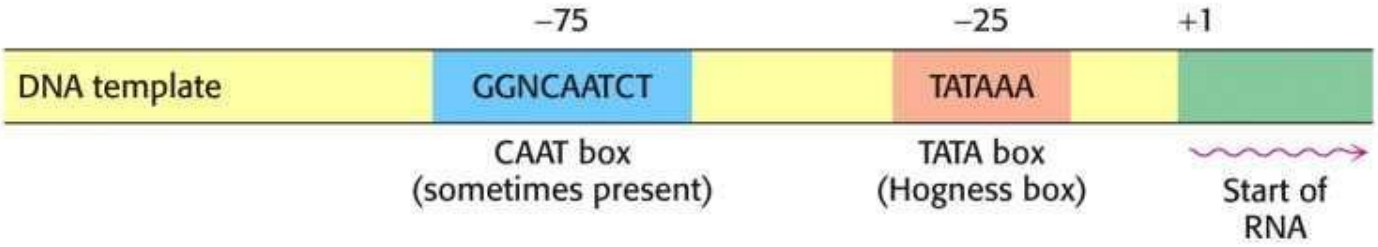

Protein is expressed from cDNA in plasmid DNA


(b) Stable transfection (transformation)
Promoter
cDNA
Vector
neor
Transfect cultured cells by lipid treatment or electroporation
Select for G-418 resistance
G-418-resistant clones
Protein is expressed from cDNA integrated into host chromosome

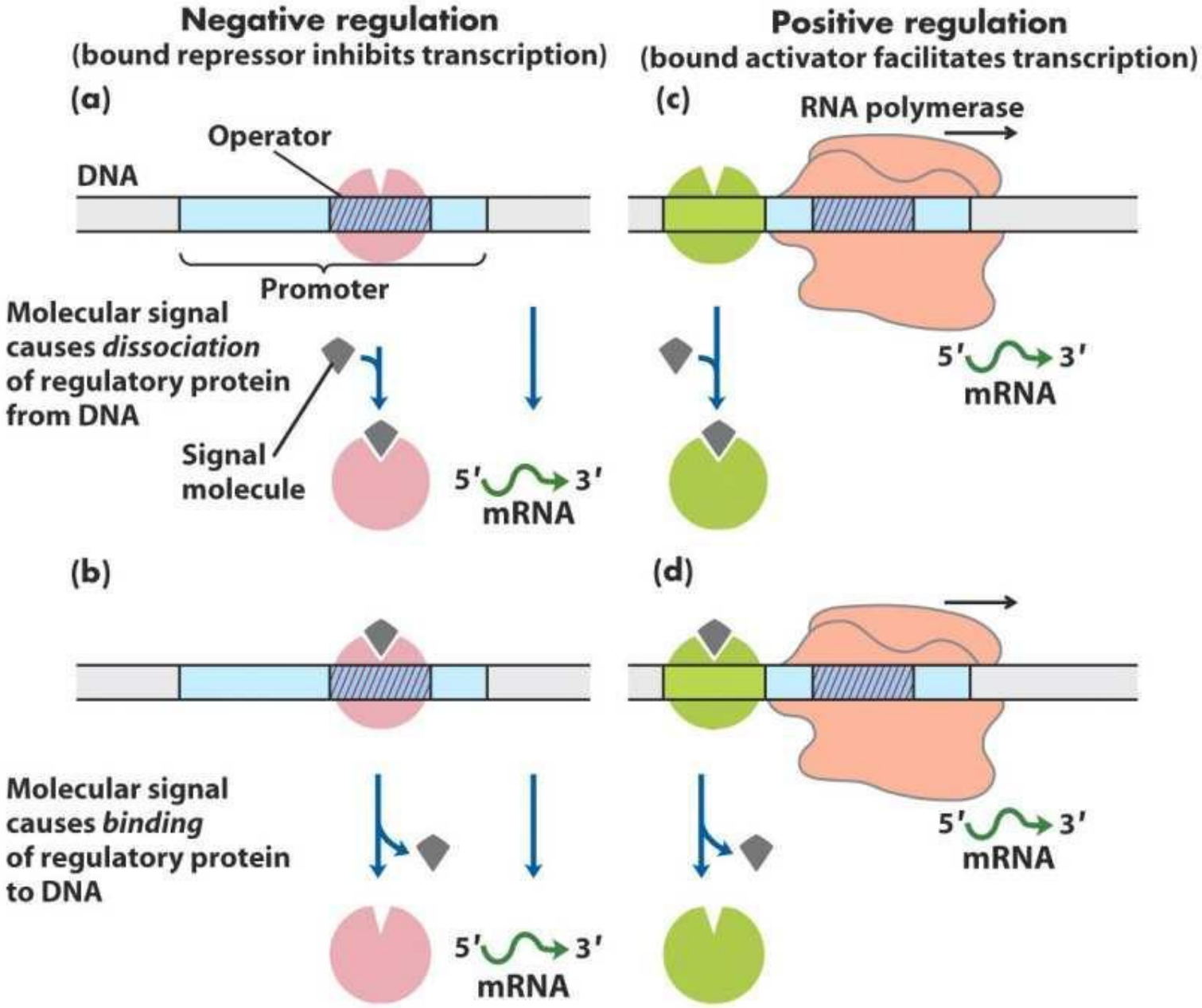
# Promoters



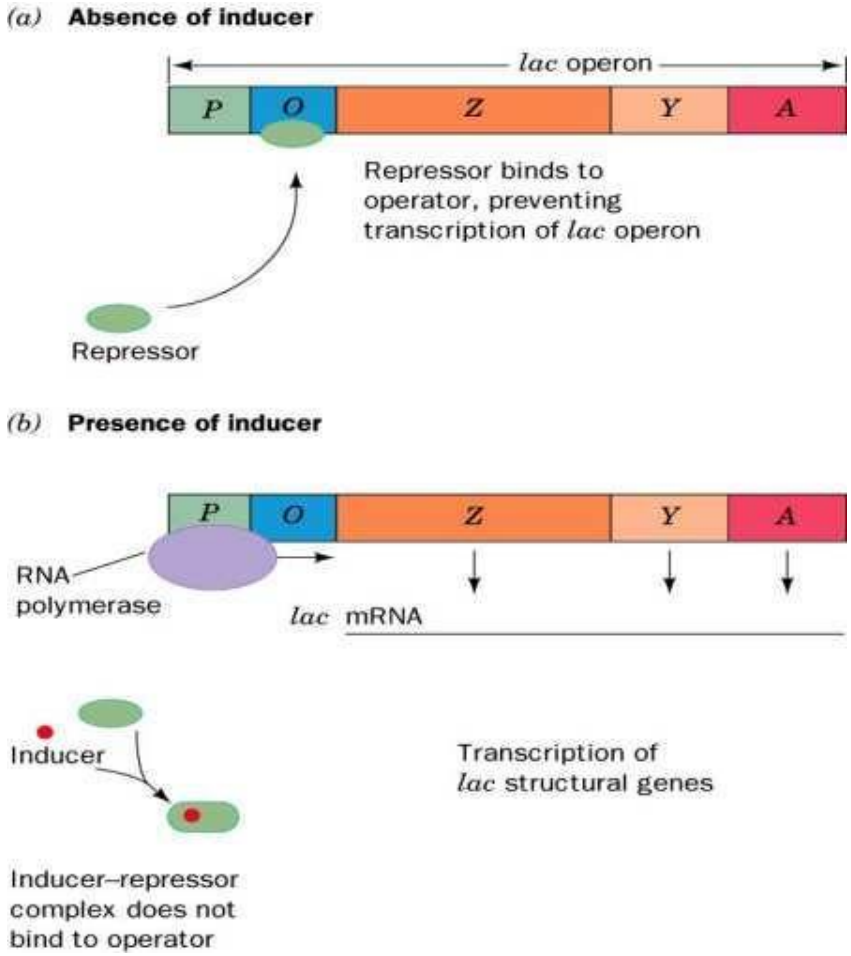

(A) **Prokaryotic promoter site**

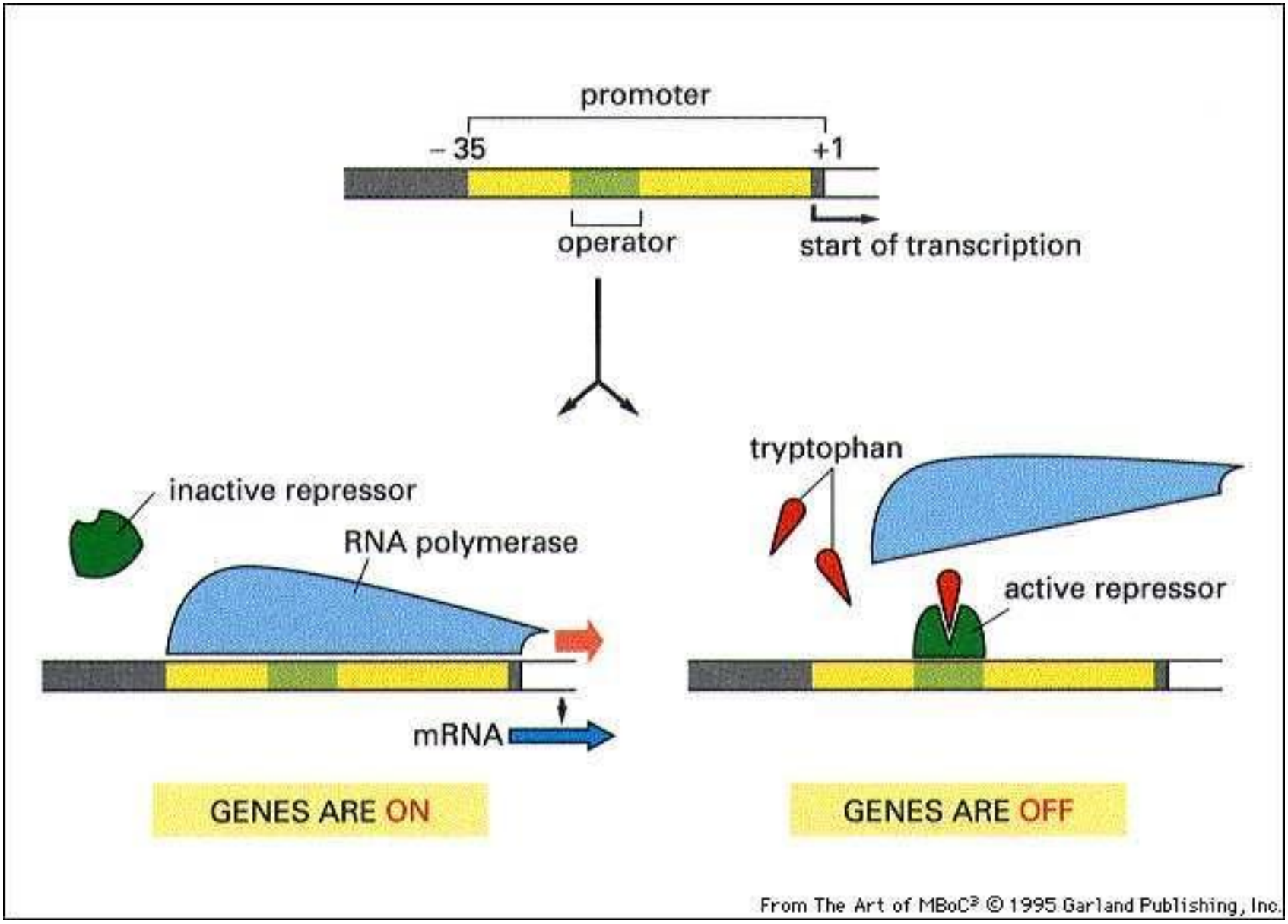


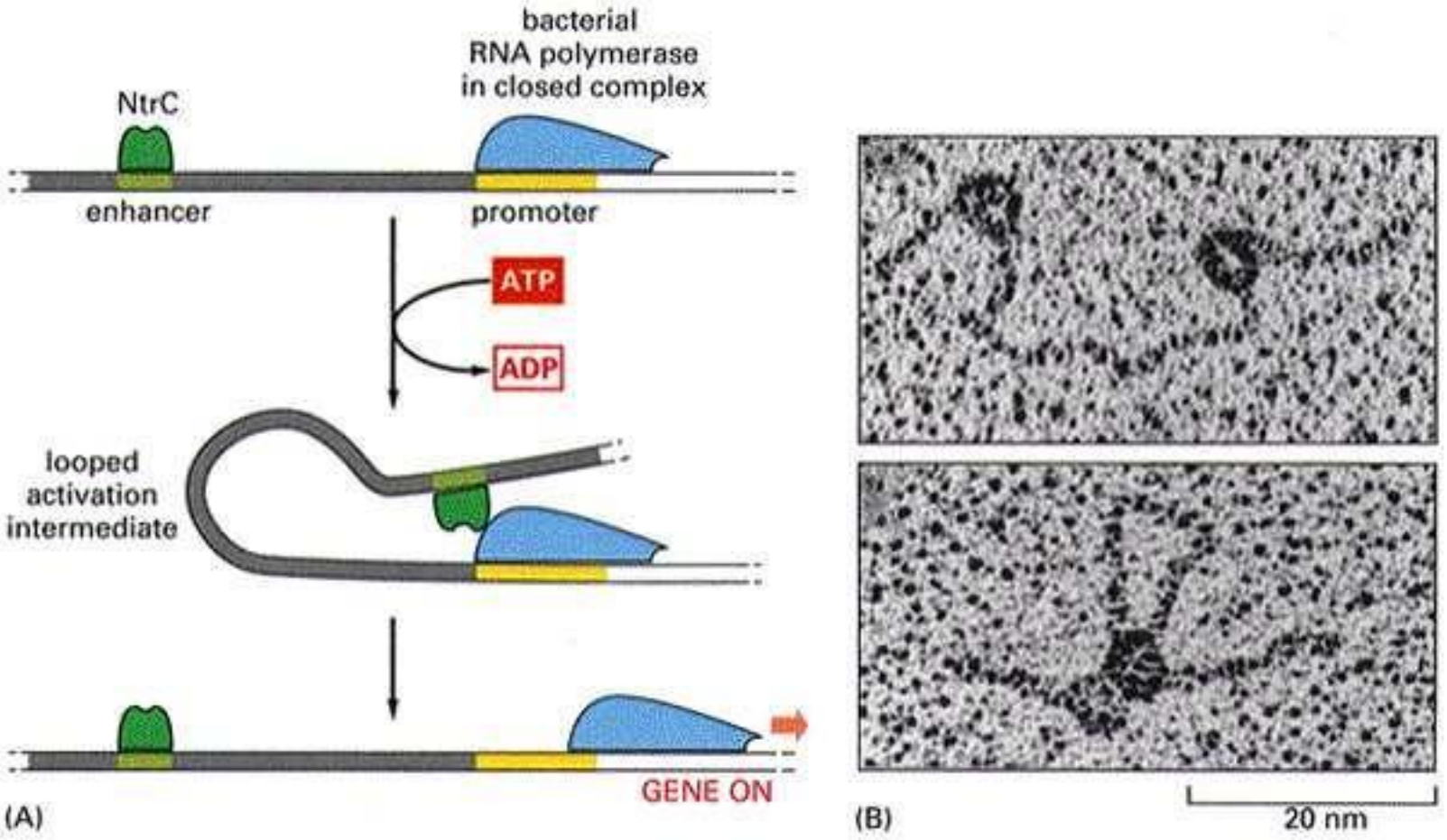

(B) **Eukaryotic promoter site**

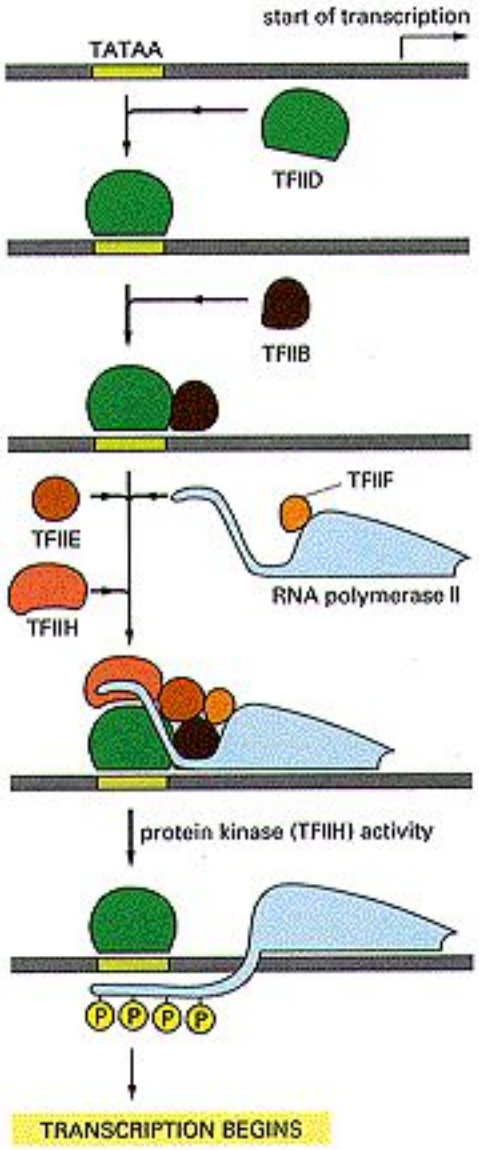

Negative regulation
(bound repressor inhibits transcription)
Positive regulation
(bound activator facilitates transcription)
(a)
Operator
DNA
Promoter
Molecular signal causes dissociation of regulatory protein from DNA
Signal molecule
5′
3′
mRNA
(c)
RNA polymerase
5′
3′
mRNA
(b)
Molecular signal causes binding of regulatory protein to DNA
5′
3′
mRNA
(d)
5′
3′
mRNA

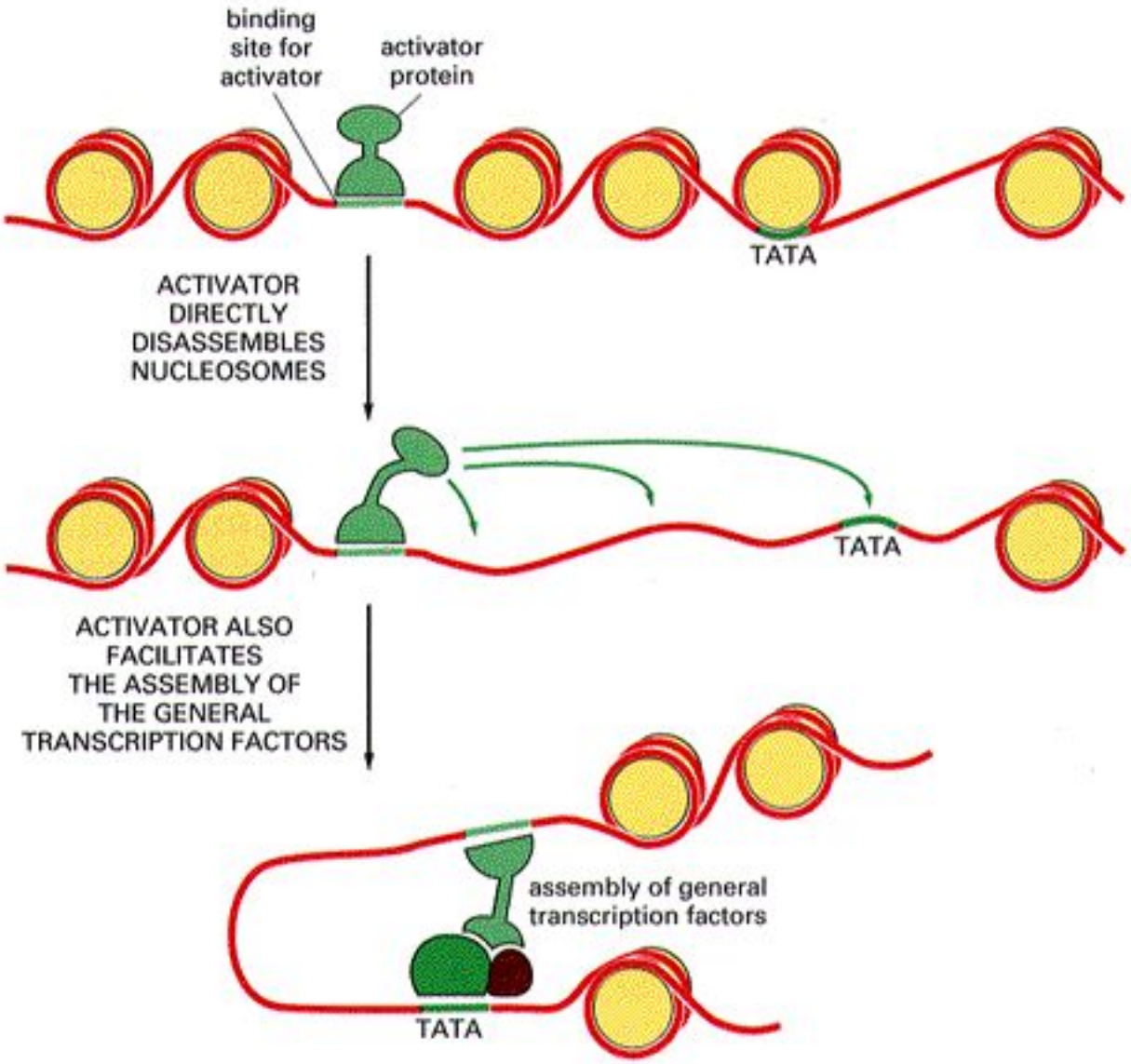
# Control of transcription of the *lac* operon.



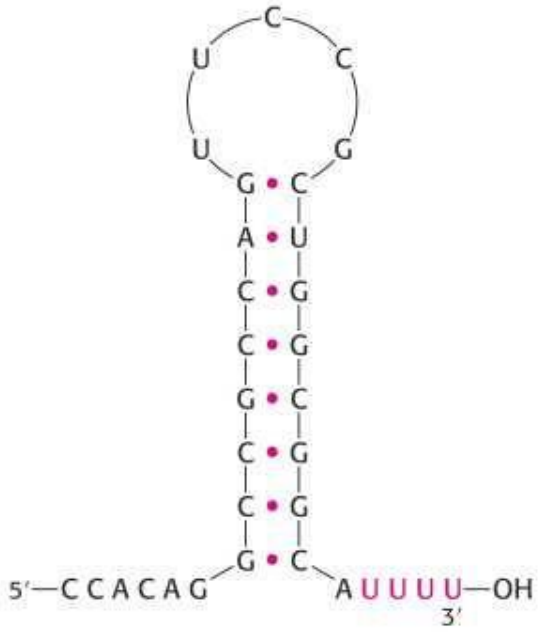


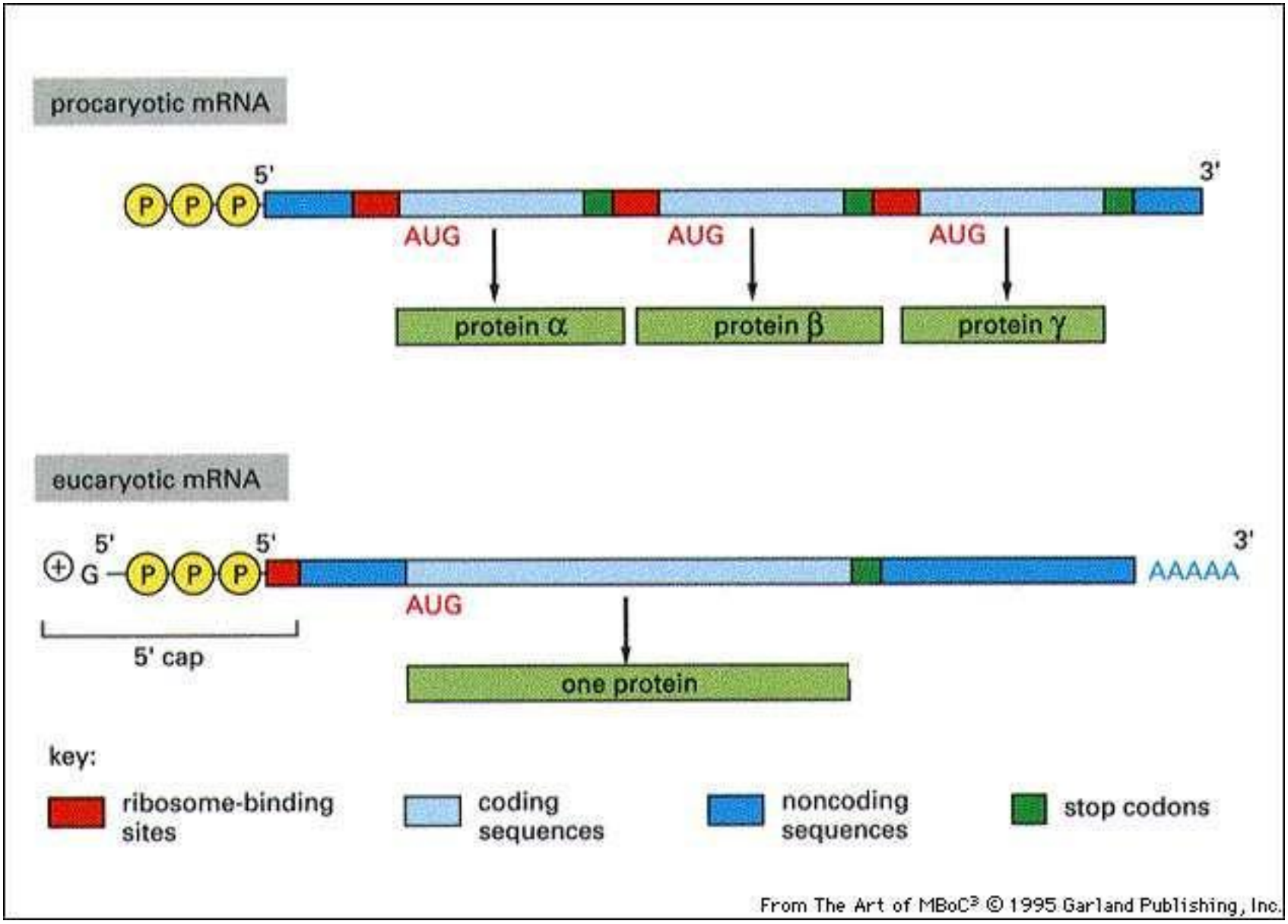


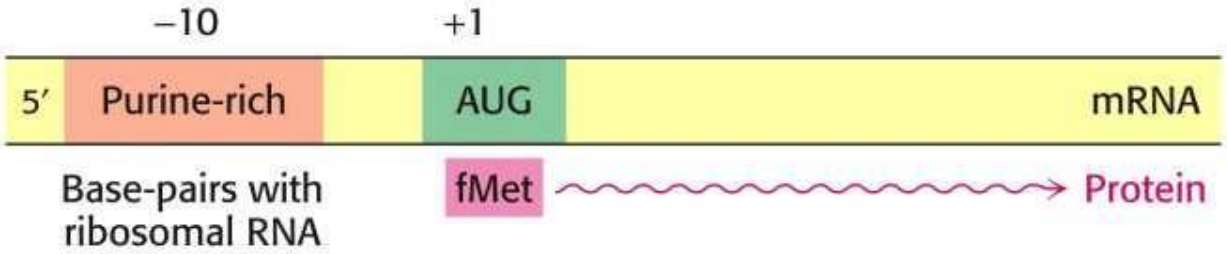




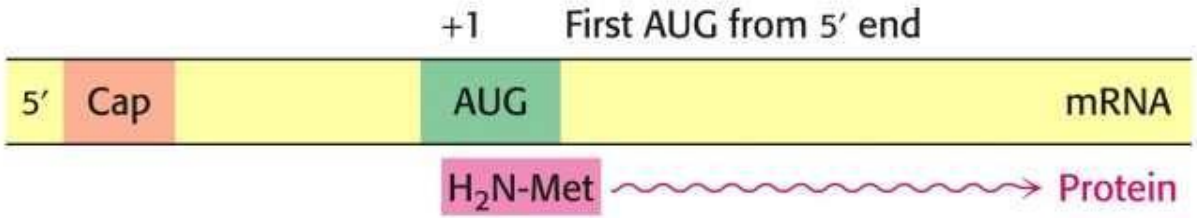
# Terminators



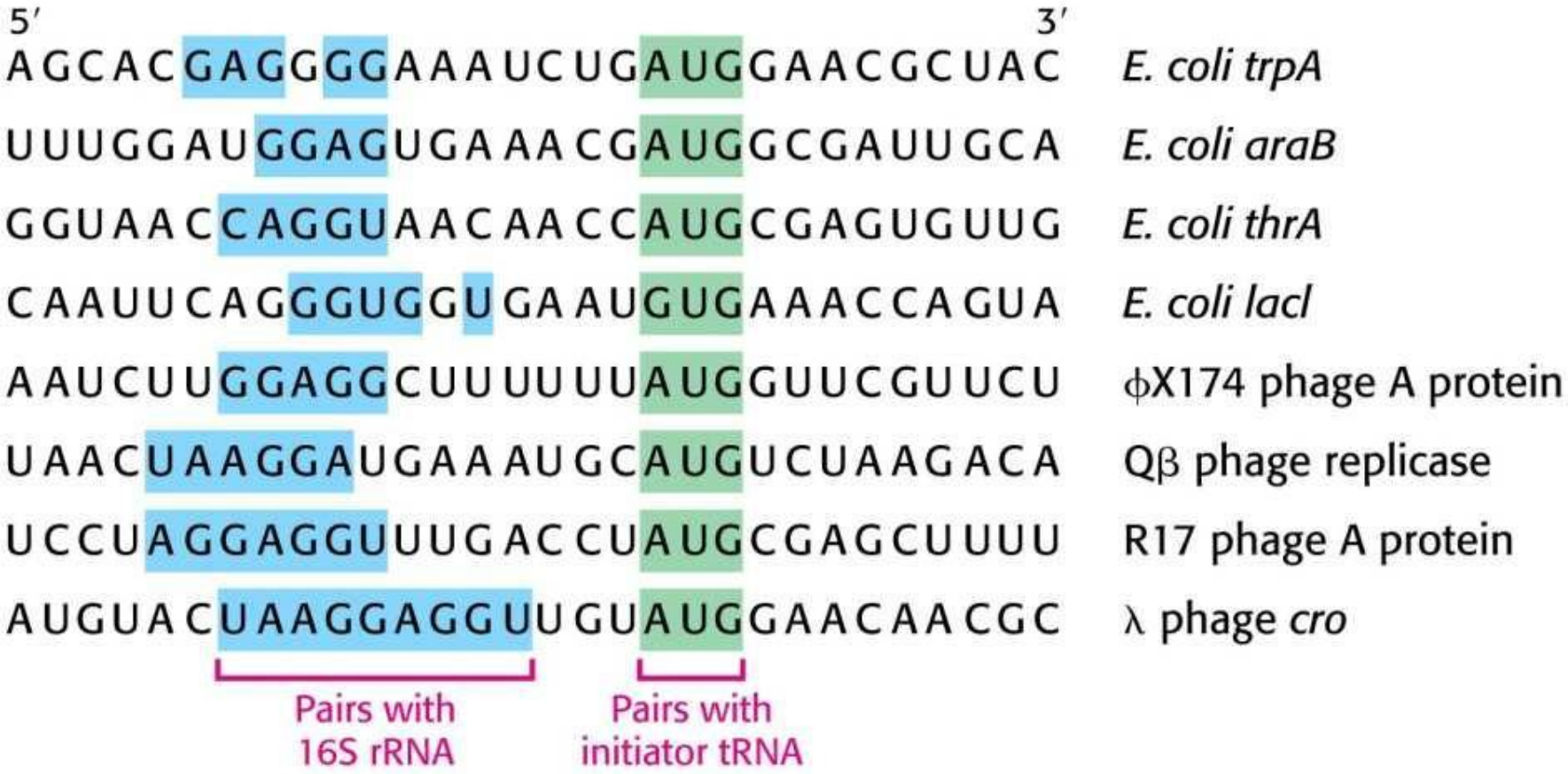




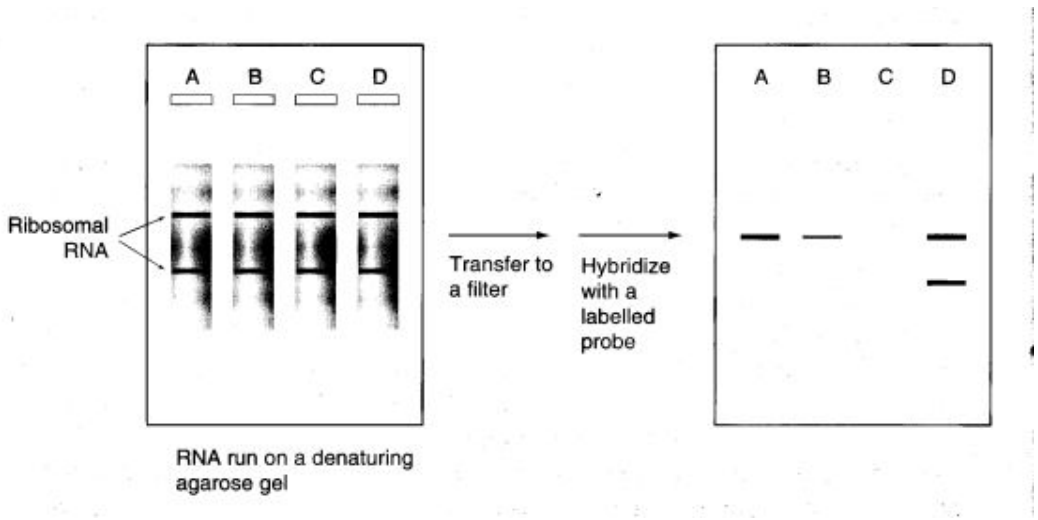

(A) **Prokaryotic start signal**

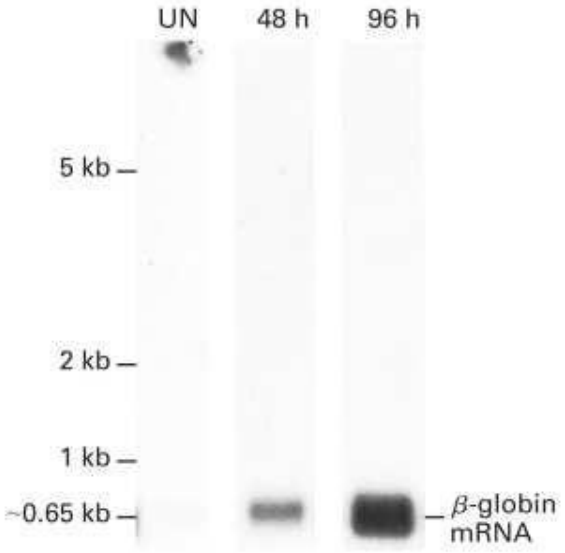



(B) **Eukaryotic start signal**


5′
3′
AGCACGAGGGGAAAUCUGAUGGAACGCUAC
E. coli trpA
UUUGGAUGGAGUGAAACGAUGGCGAUUGCA
E. coli araB
GGUAACCAGGUAACAACCAUGCGAGUGUUG
E. coli thrA
CAAUUCAGGGUGGUGAAUGUGAAACCAGUA
E. coli lacI
AAUCUUGGAGGCUUUUUUAUGGUUCGUUCU
ϕX174 phage A protein
UAACUAAGGAUGAAAUGCAUGUCUAAGACA
Qβ phage replicase
UCCUAGGAGGUUUGACCUAUGCGAGCUUUU
R17 phage A protein
AUGUACUAAGGAGGUUGUAUGGAACAACGC
λ phage cro
Pairs with 16S rRNA
Pairs with initiator tRNA

# Northern Blot




-> to study transcription level

# Expression studies by microarray technique