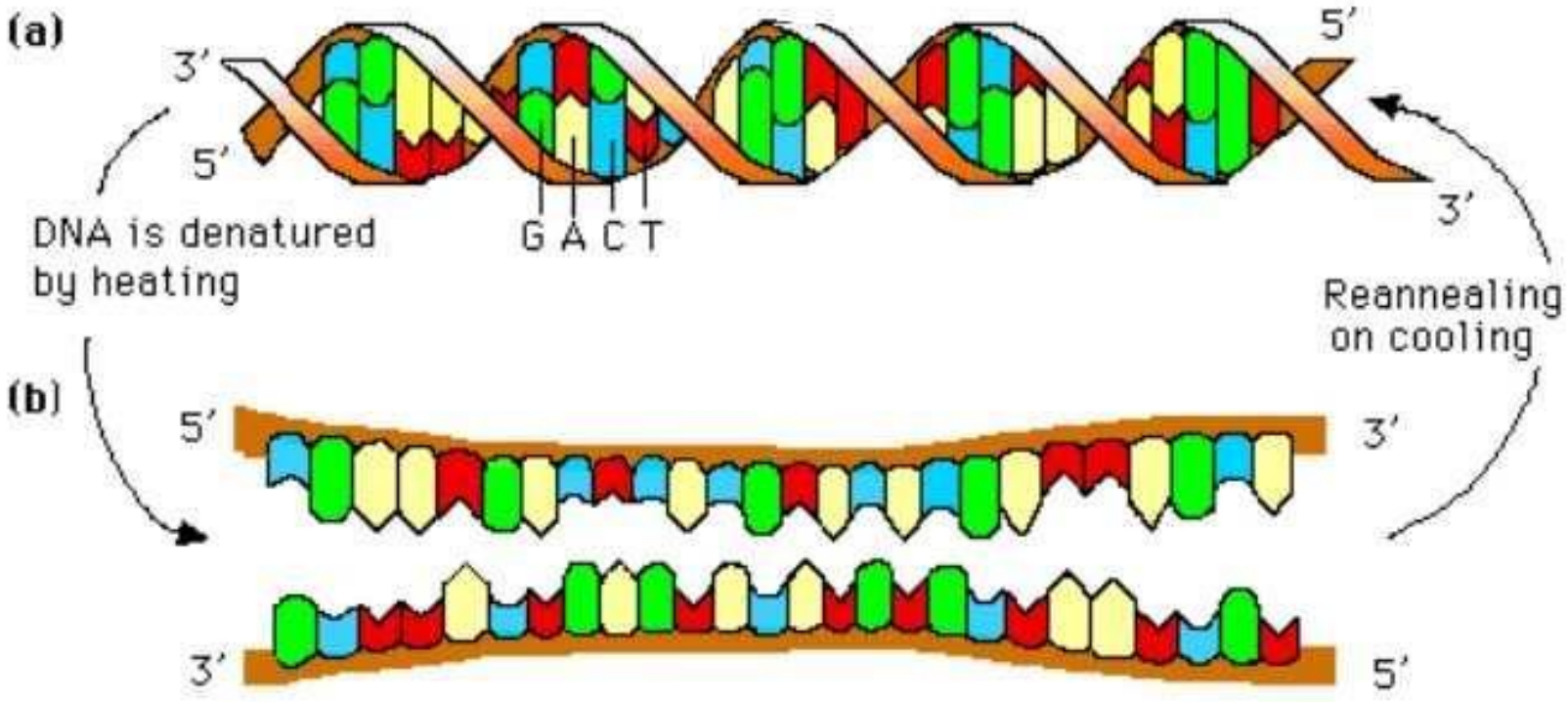
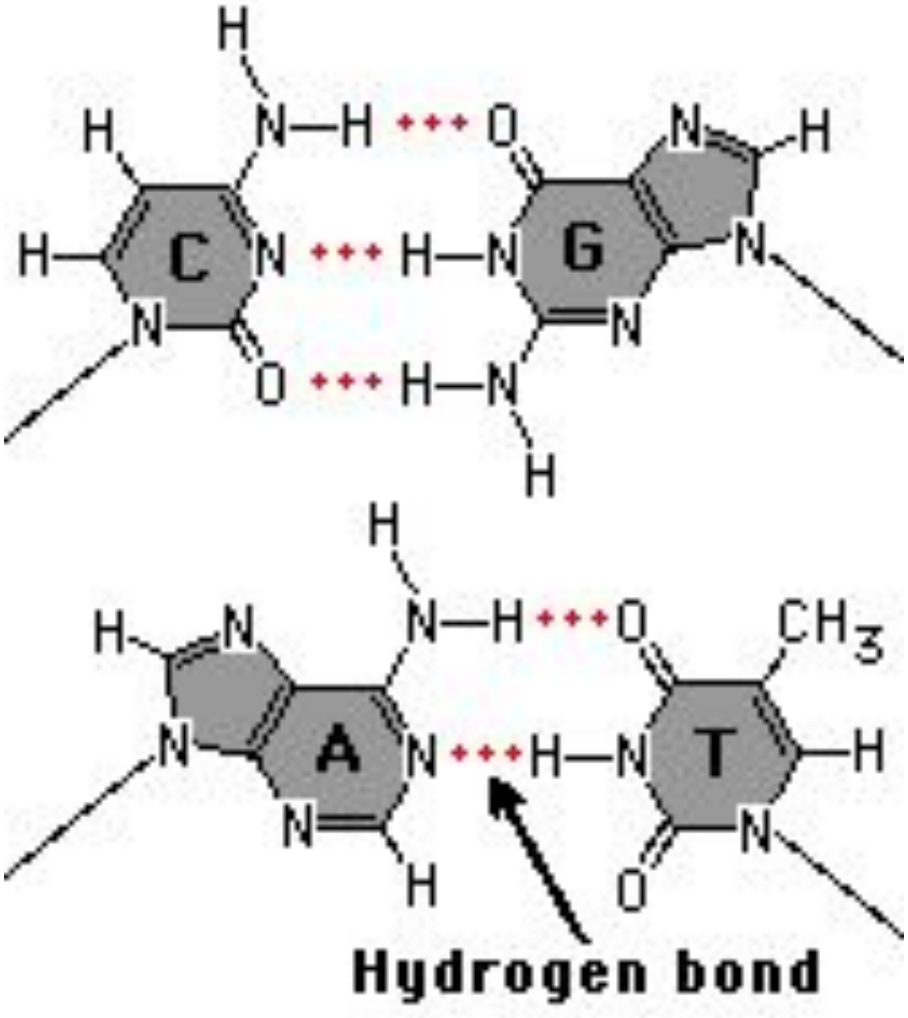
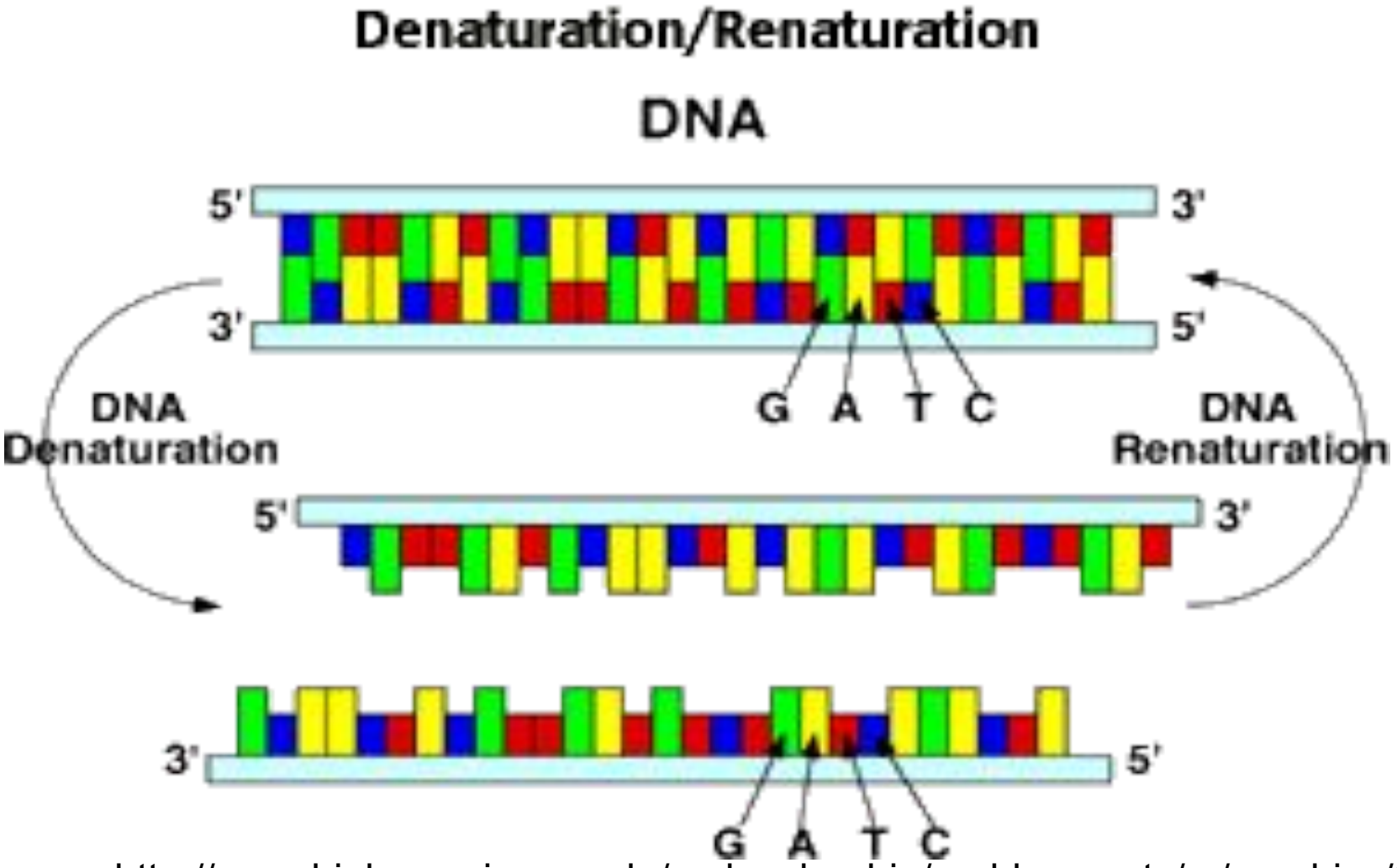
1. Hybridization (DNA-DNA or DNA-RNA)



HYBRIDIZATION? – Yes, it is about this familiar picture

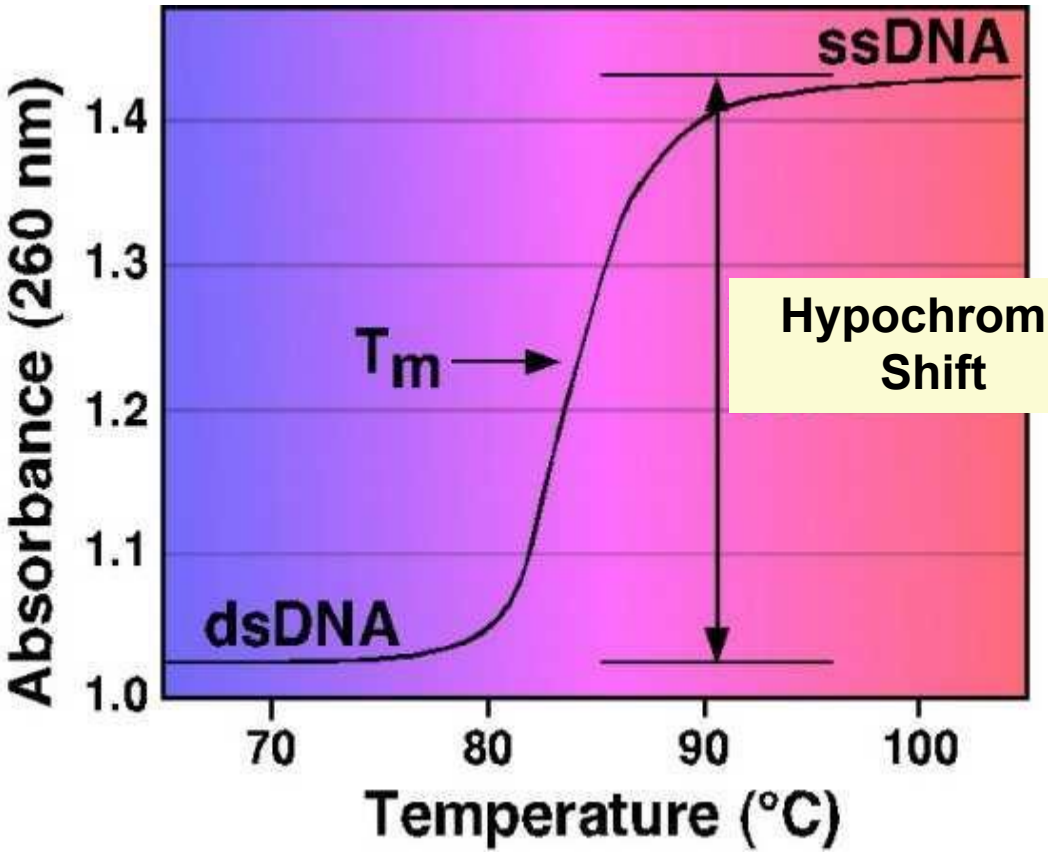


We can denaturate and renaturate DNA by heating/cooling



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

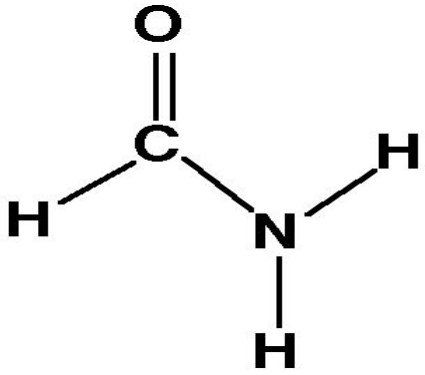
medlib.med.utah.edu/block2/ biochem/

Tm of DNA is affected by:

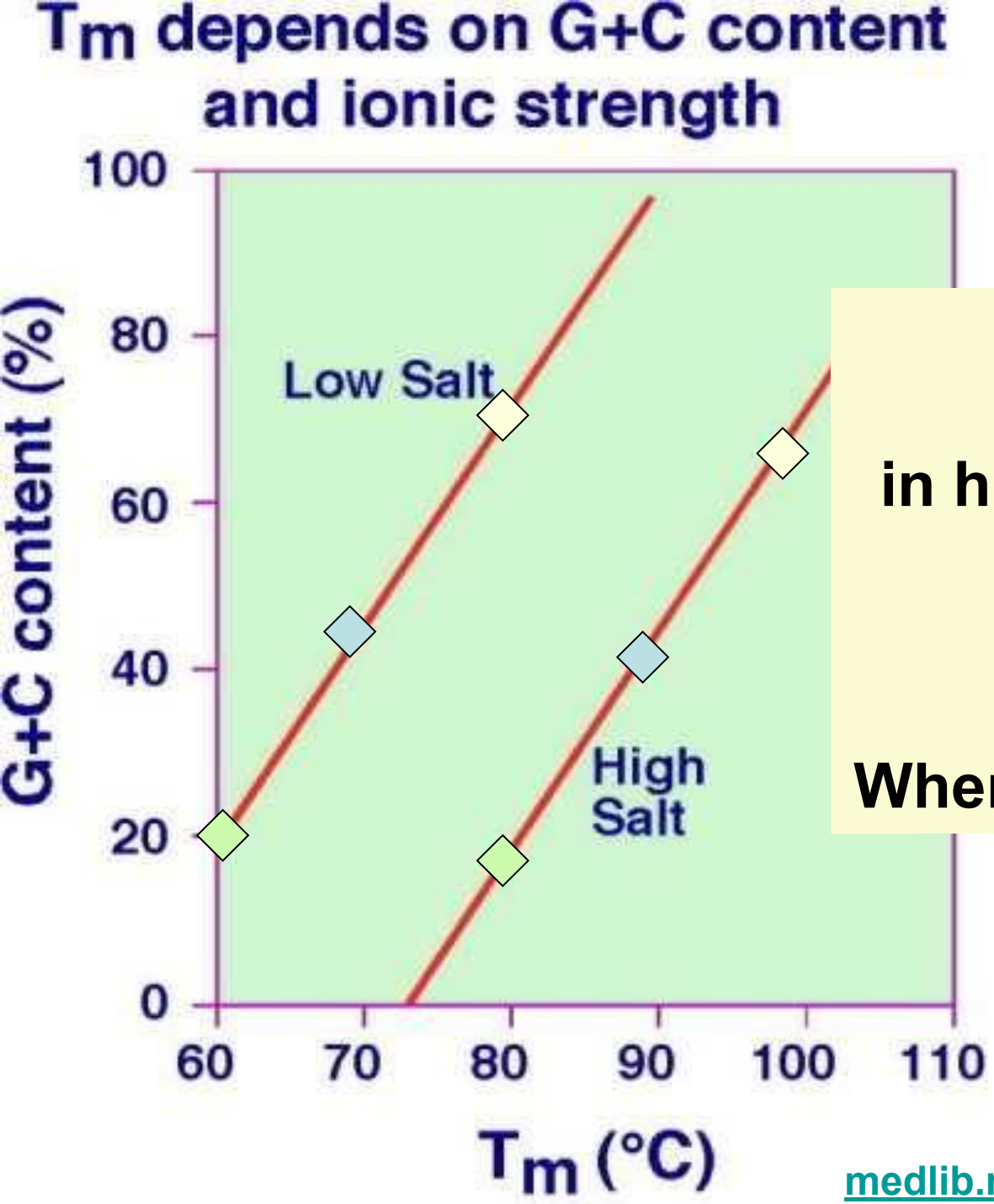
1. Base Composition :
higher the GC content, the higher the Tm.

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.

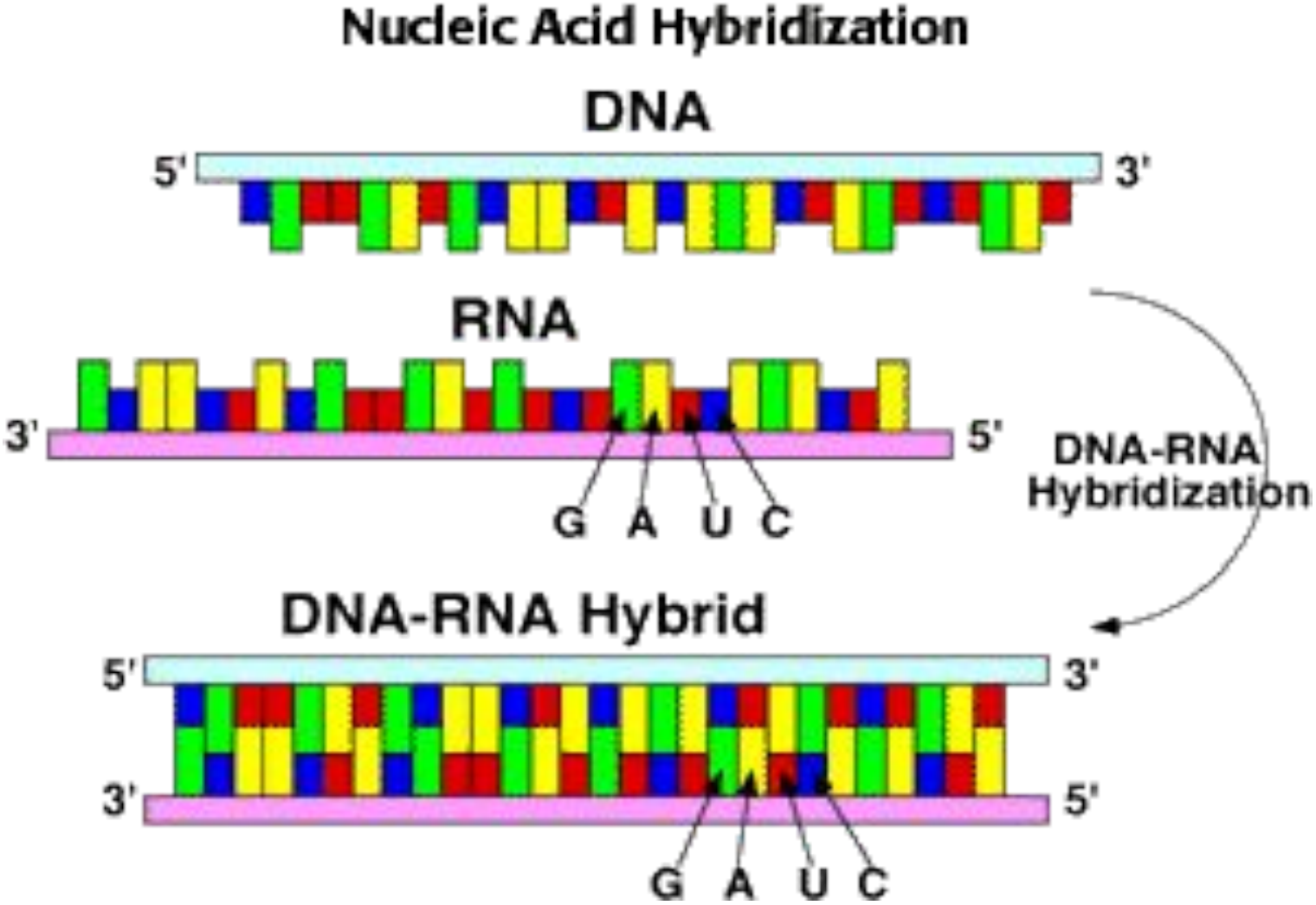


DNA
more STABLE
in high-salt conditions.

DNA
more STABLE
When contains many GC

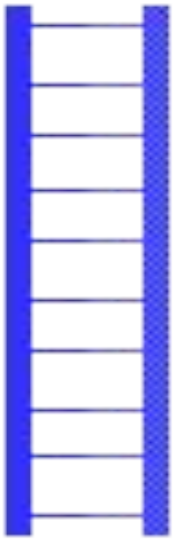
medlib.med.utah.edu/block2/ biochem/

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

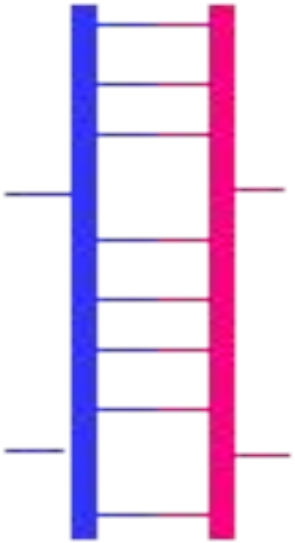


http://www.biology.arizona.edu/molecular_bio/problem_sets/m/graphics/05ta.gif

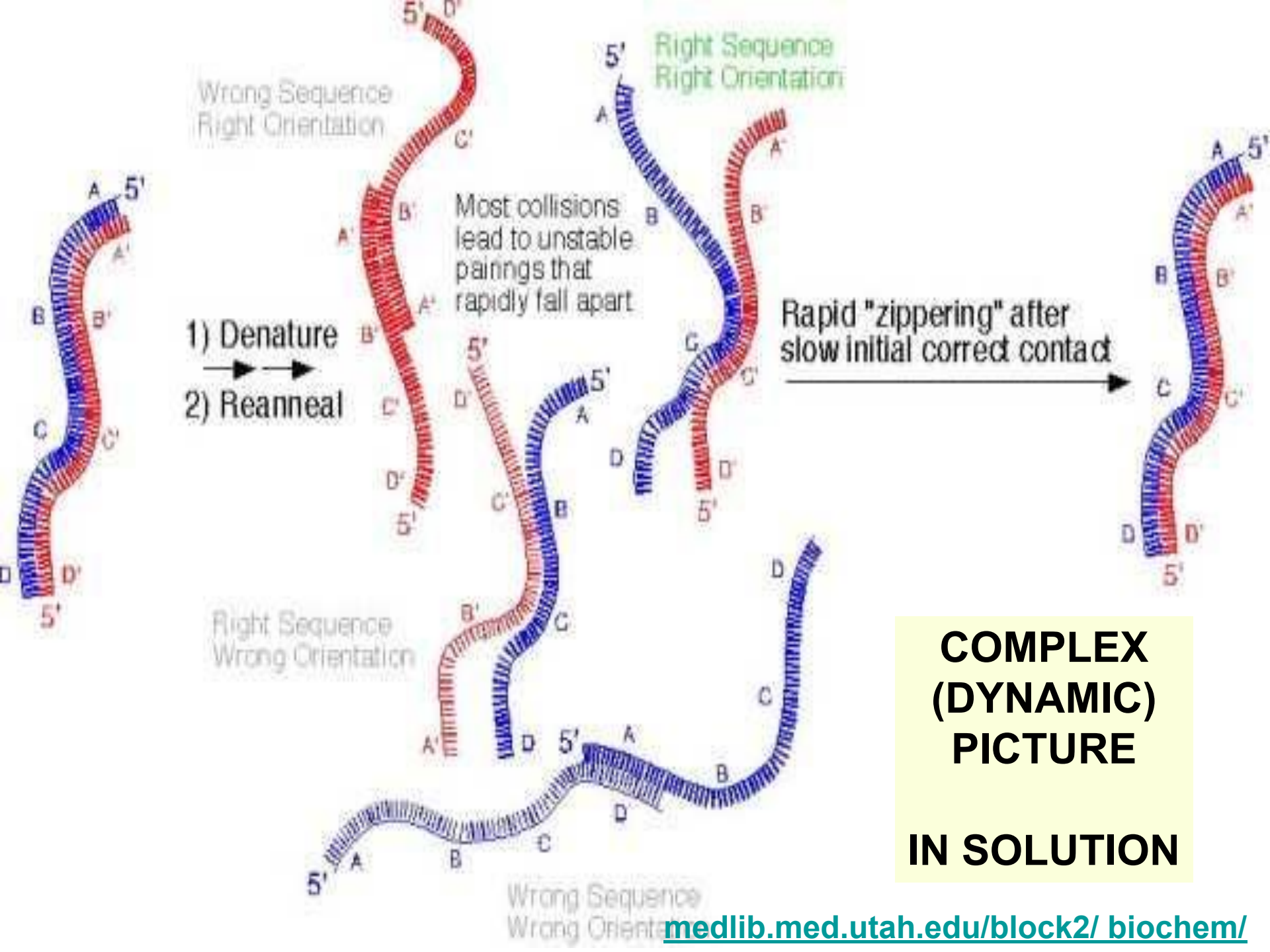
Hybridization could be less than perfect



Homoduplex DNA, both strands from the same species



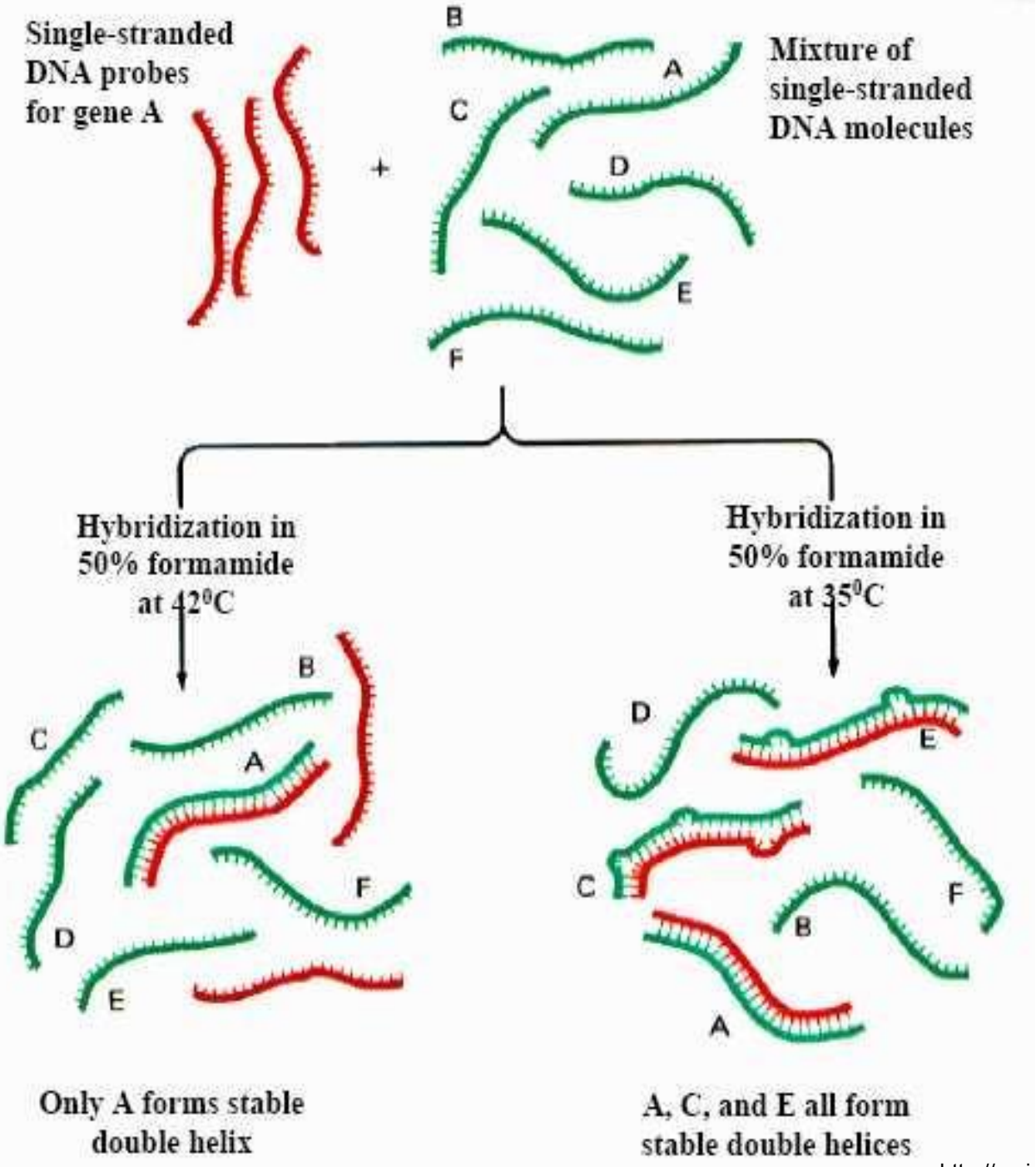
Heteroduplex DNA, strands from different species, with some bases unpaired



COMPLEX (DYNAMIC) PICTURE

IN SOLUTION

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



42 C
is more stringent
condition
that 35 C

(hybridization
is more specific)

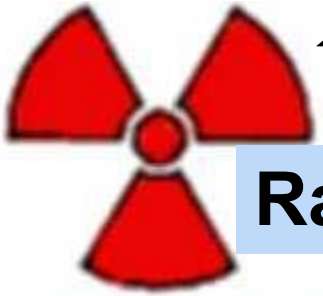
http://amiga1.med.miami.edu/Medical/PDF-Files/Lecture10.pd

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

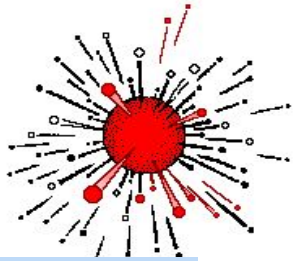
Specific probe self-anneals to target DNA

Only problem – DNA is invisible

How to visualize DNA?

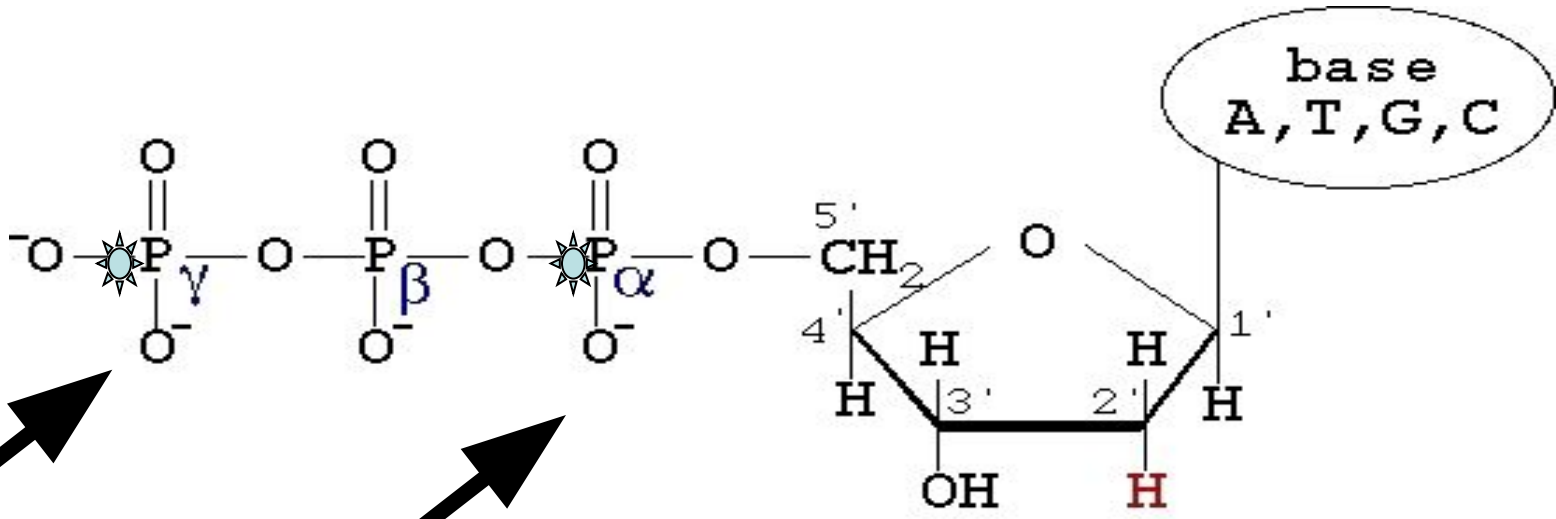


Radioactively



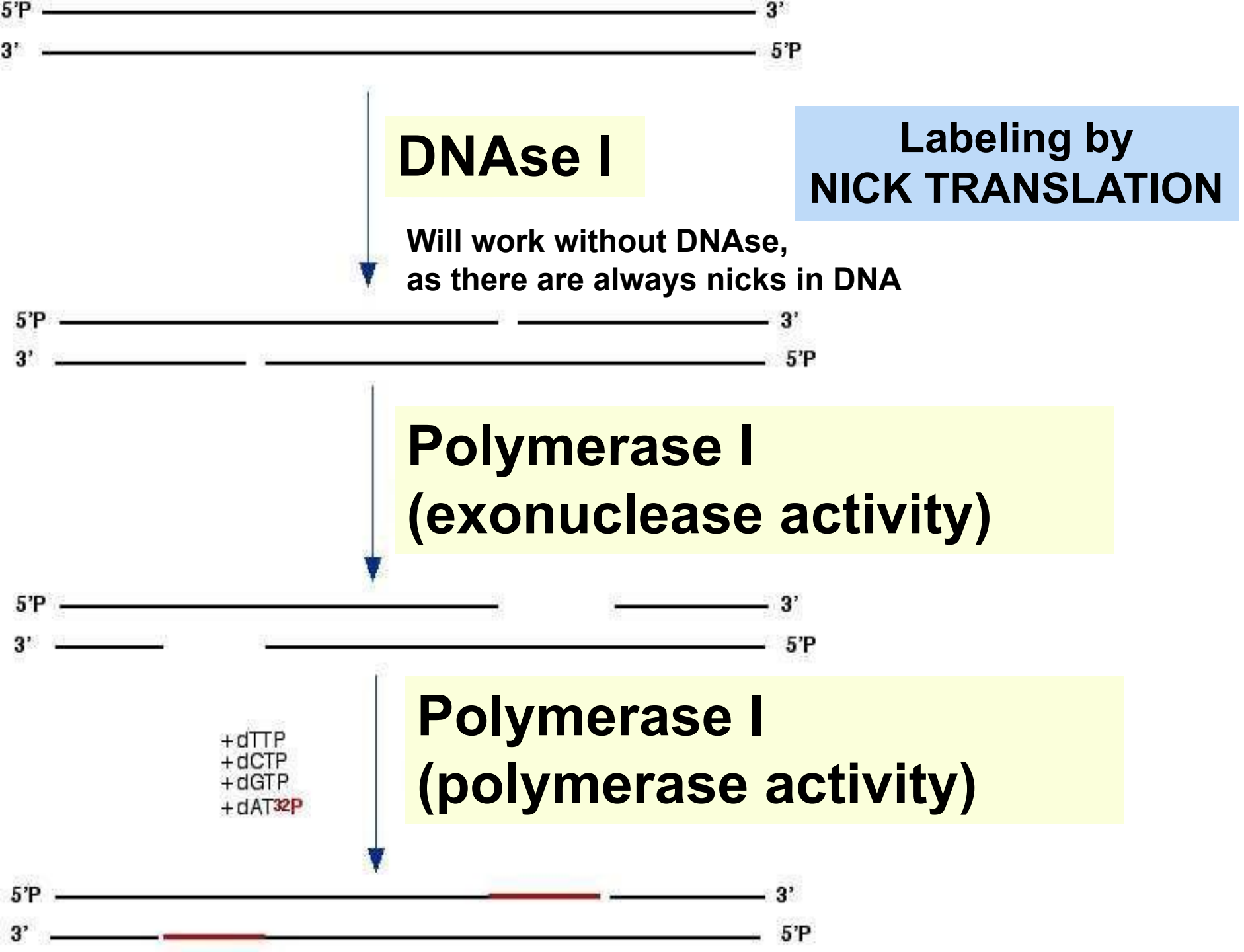
Fluorescently

Polymerase labeling of DNA



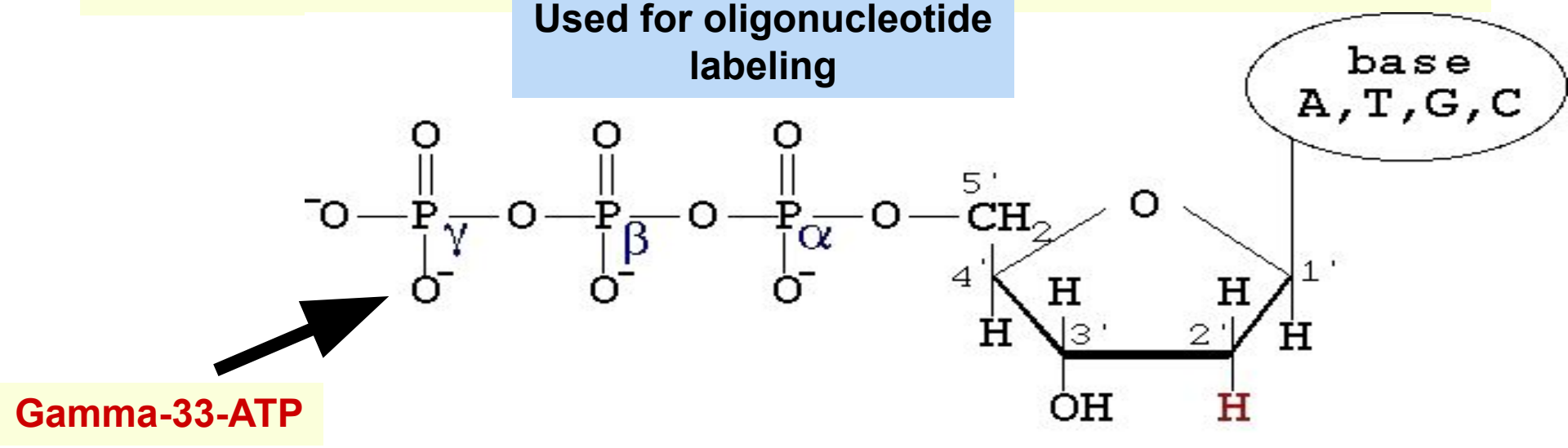
Gamma-33-ATP

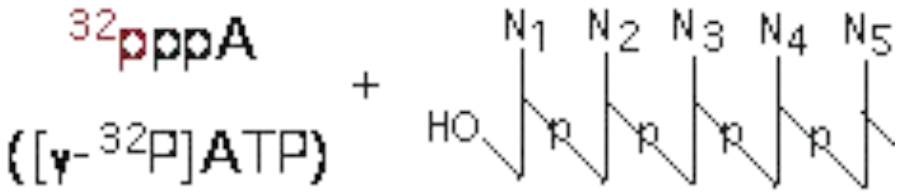
Alpha-32-ATP

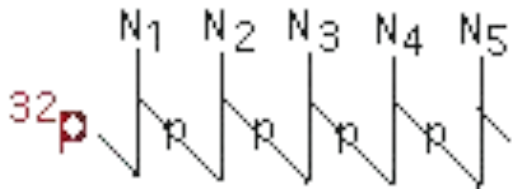

5'P
3'
3'
5'P
DNAse I
Labeling by
NICK TRANSLATION
Will work without DNAse,
as there are always nicks in DNA
5'P
3'
3'
5'P
Polymerase I
(exonuclease activity)
5'P
3'
3'
5'P
+dTTP
+dCTP
+dGTP
+dAT32P
Polymerase I
(polymerase activity)
5'P
3'
3'
5'P

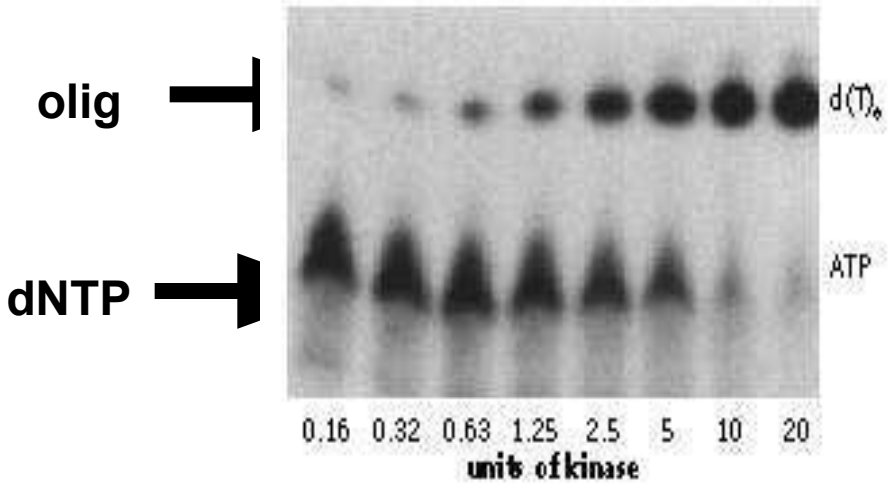
T4 polynucleotide kinase labeling

Used for oligonucleotide labeling

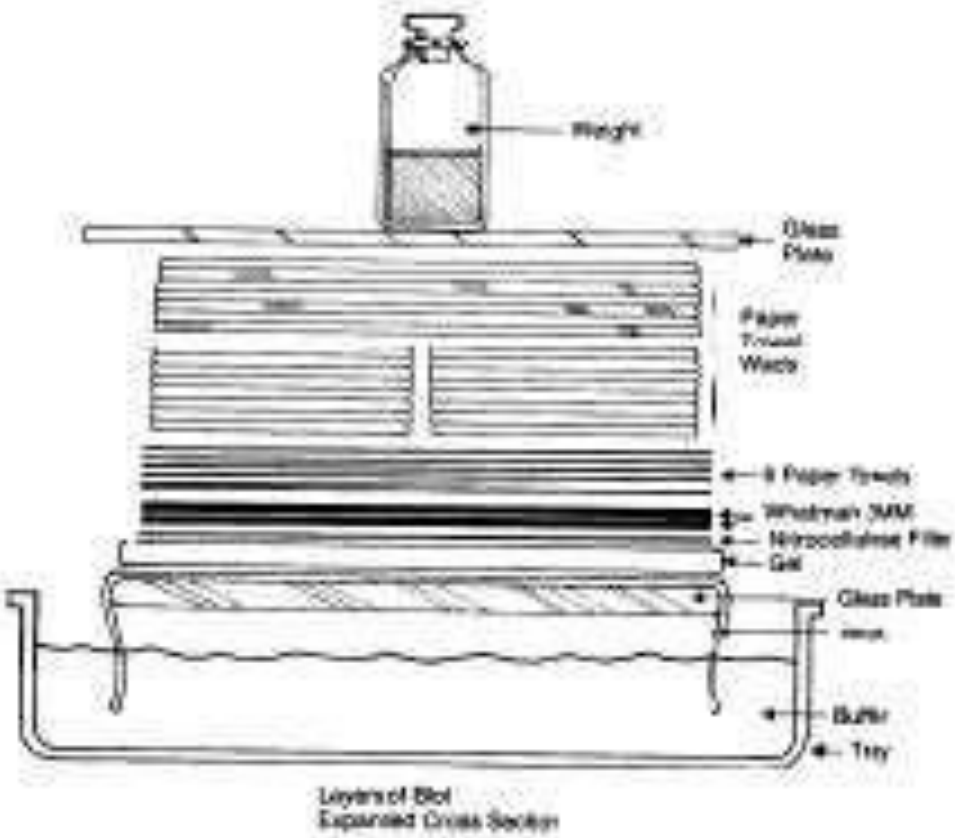




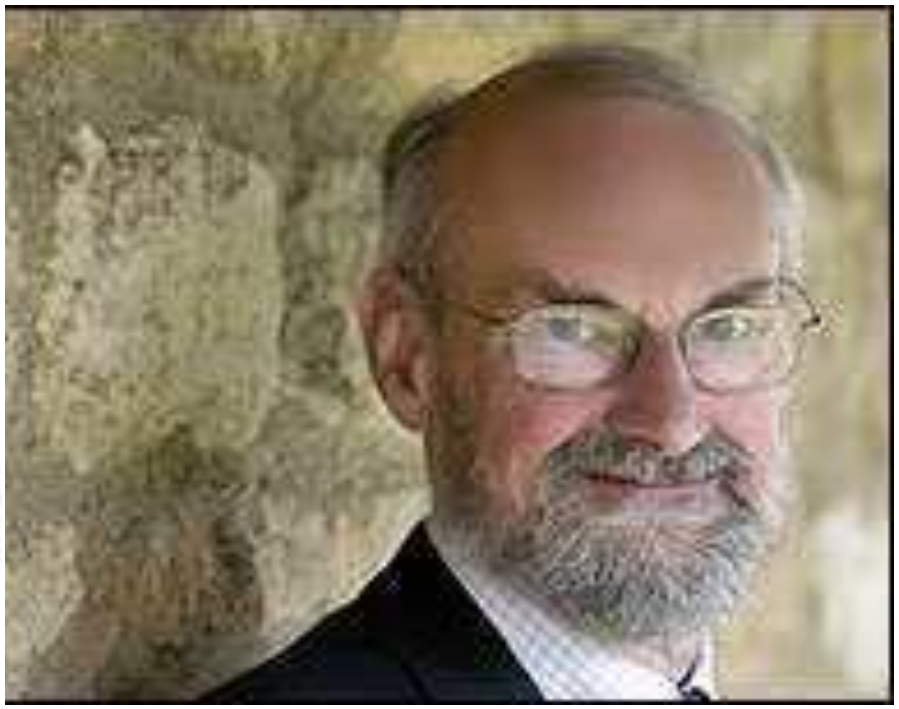


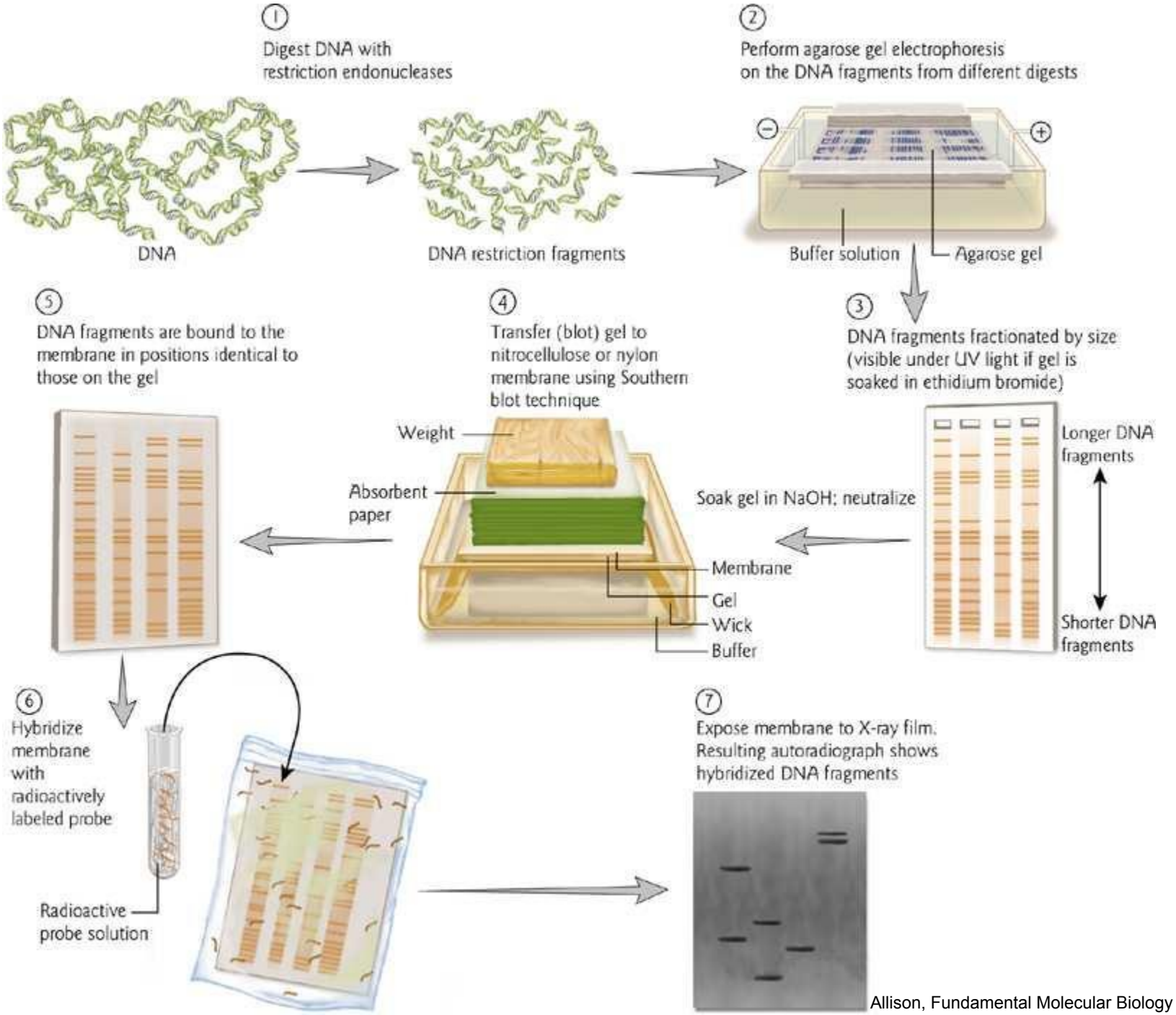


Professor Sir Ed Southern, Whitley Professor of Biochemistry at the University of Oxford



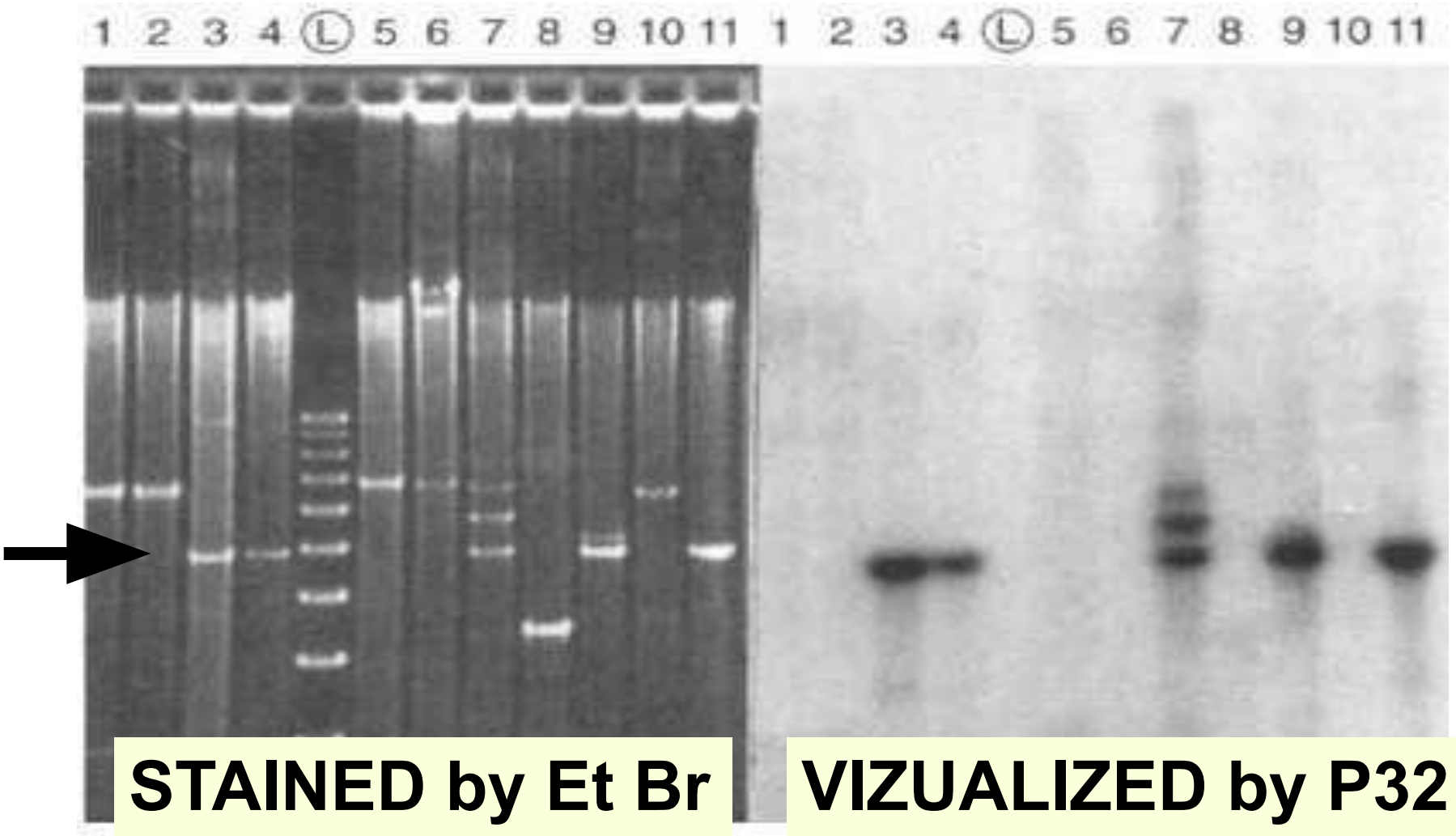
Layers of Blot
Expanded Cross Section





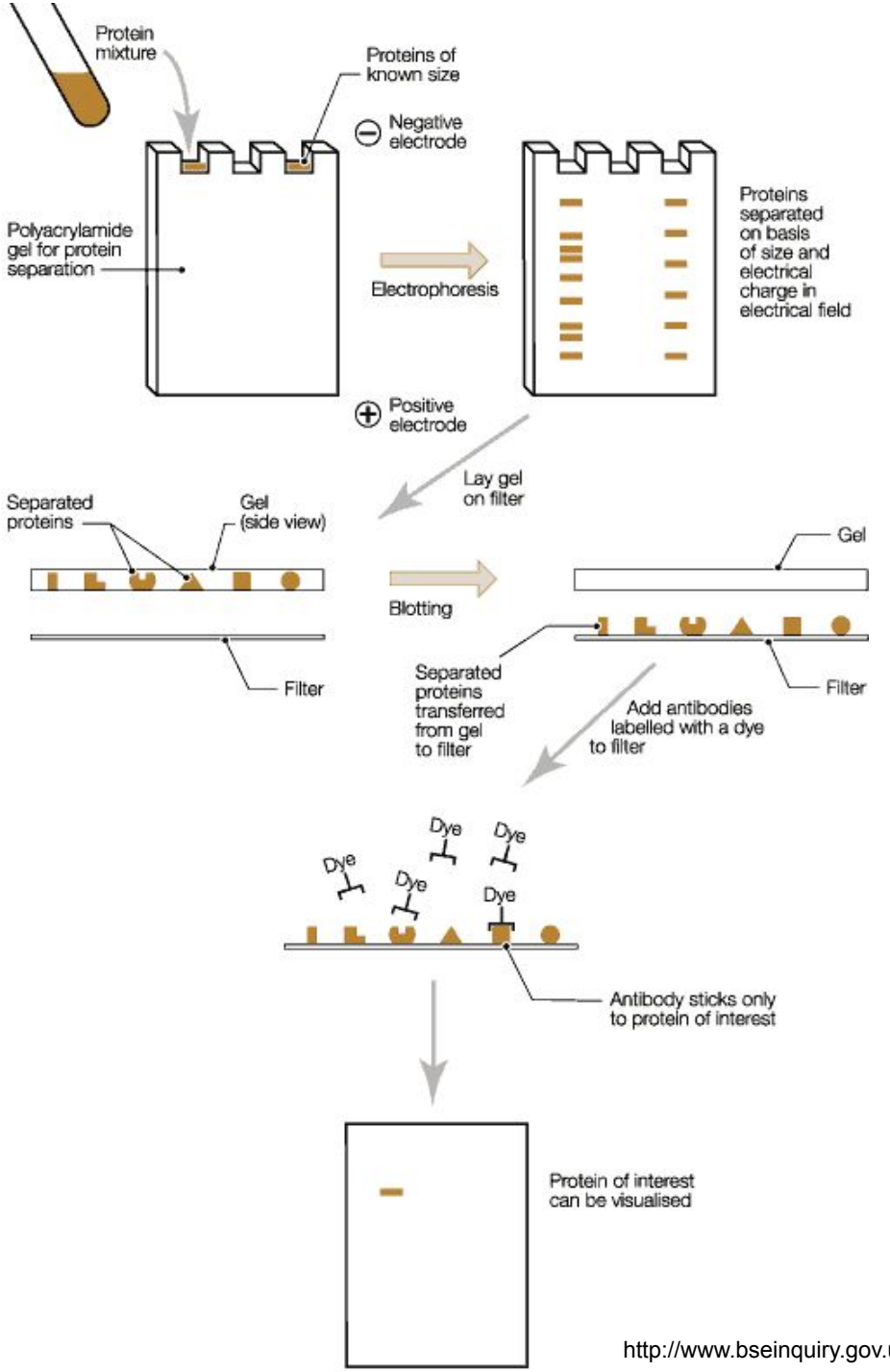
Allison, Fundamental Molecular Biology

“Real” Southern blot (DNA-DNA blot)



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

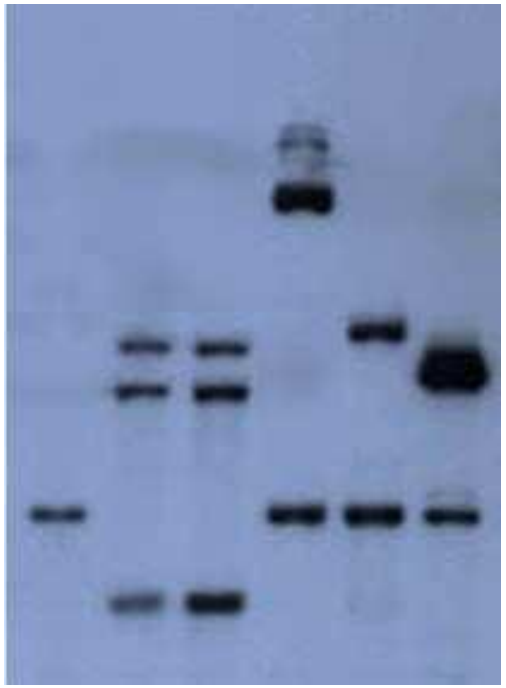
Western Blot



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

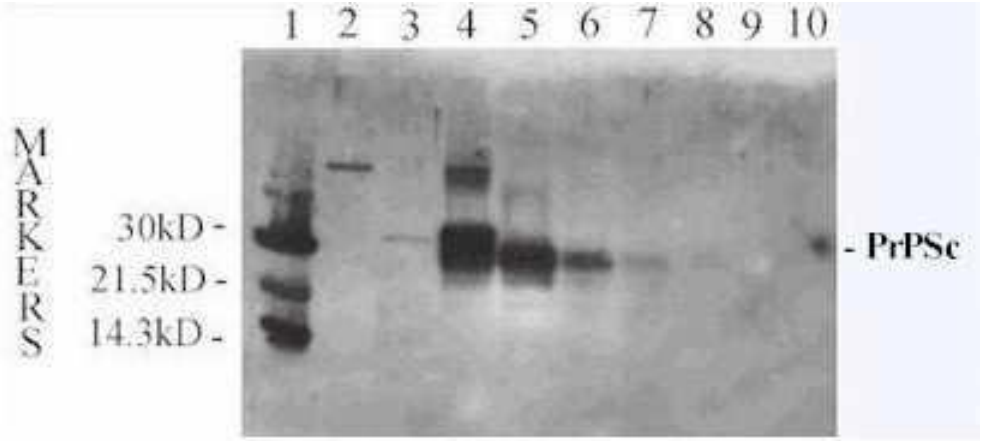
What different types of information can be provided by each different blot type?

Southern

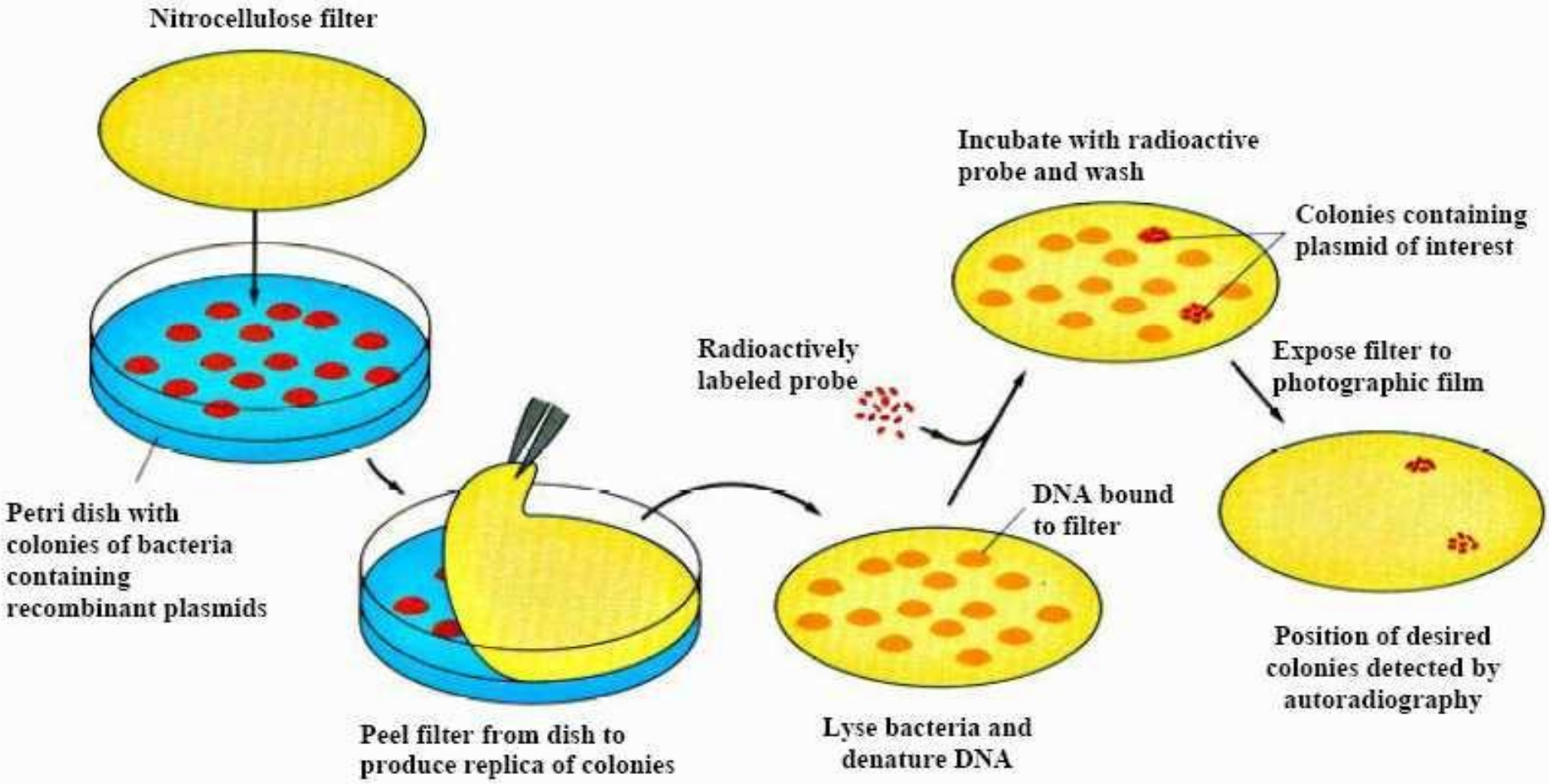


northern

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

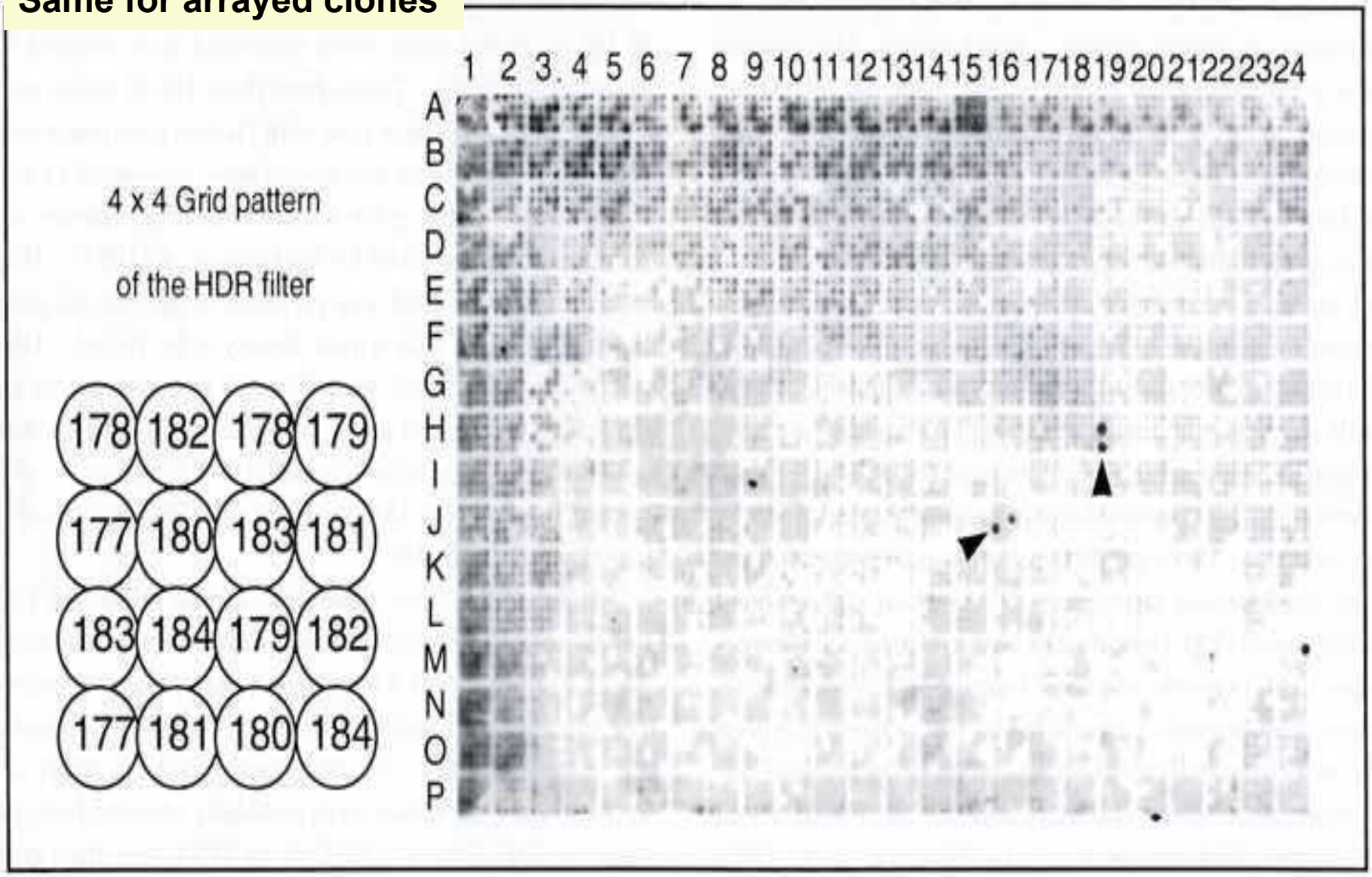
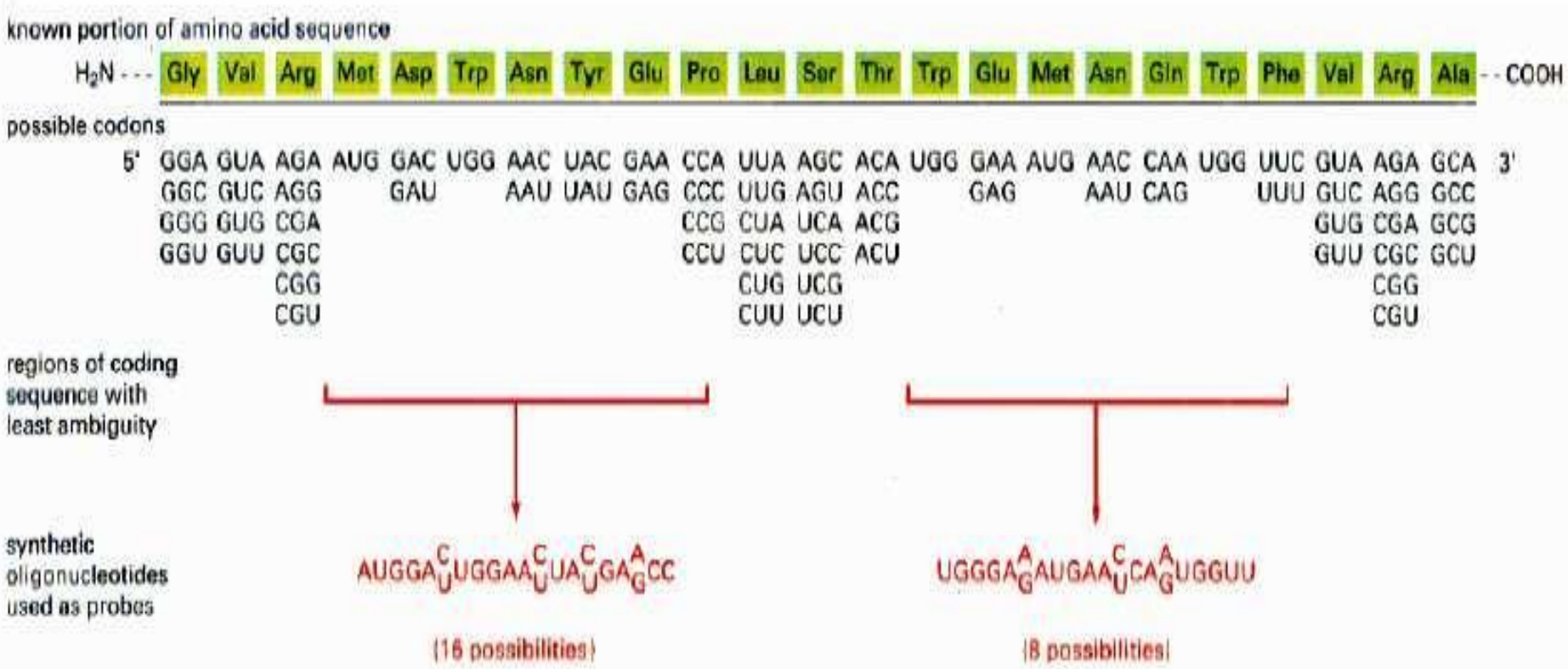


Fig. 2. Library screening by hybridization. Colony hybridization to a high density colony filter using the sequence of the single-copy gene *SL1* as a probe. 3072 BAC clones were gridded on a membrane with a 4 × 4 double offset pattern. Positive clones were detected as doublet signals.

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

Design of degenerated synthetic hybridization probes

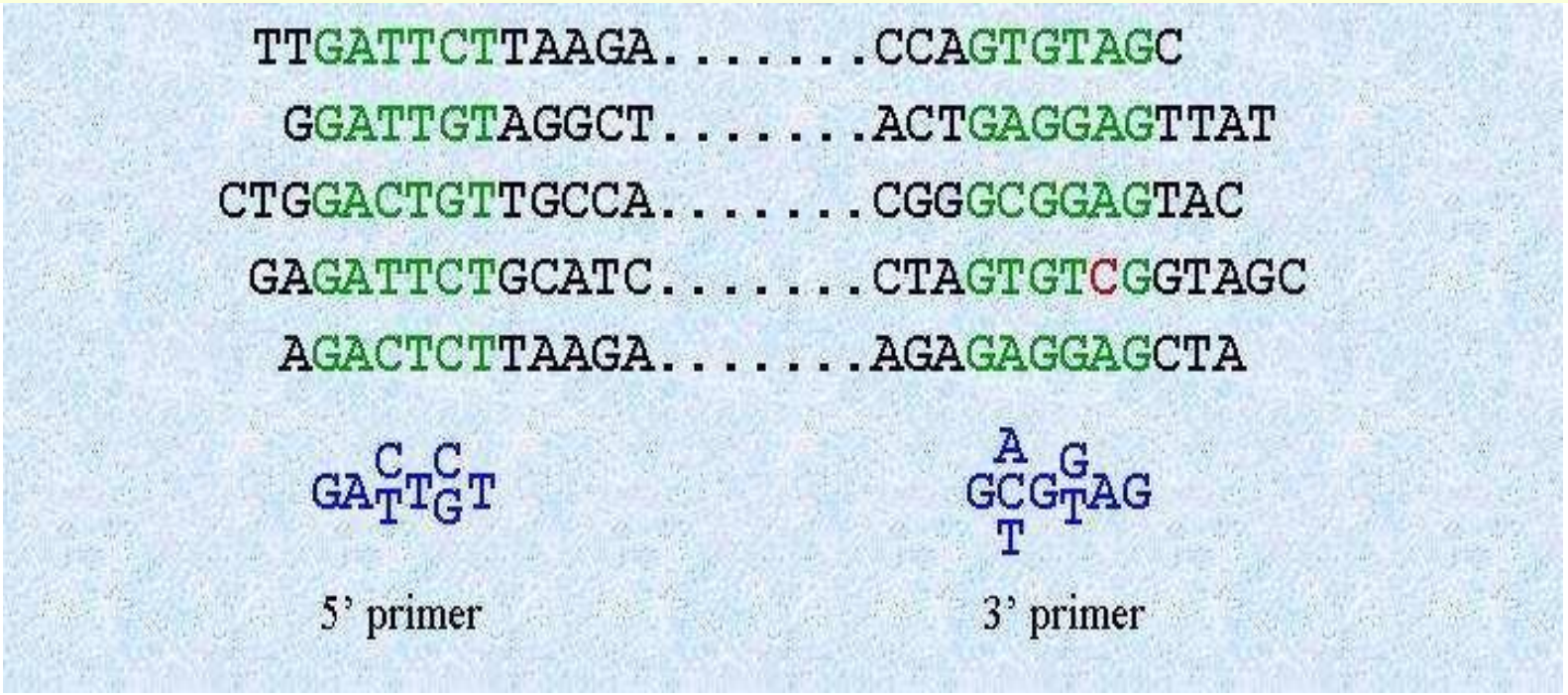
(for already known proteins only)



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

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

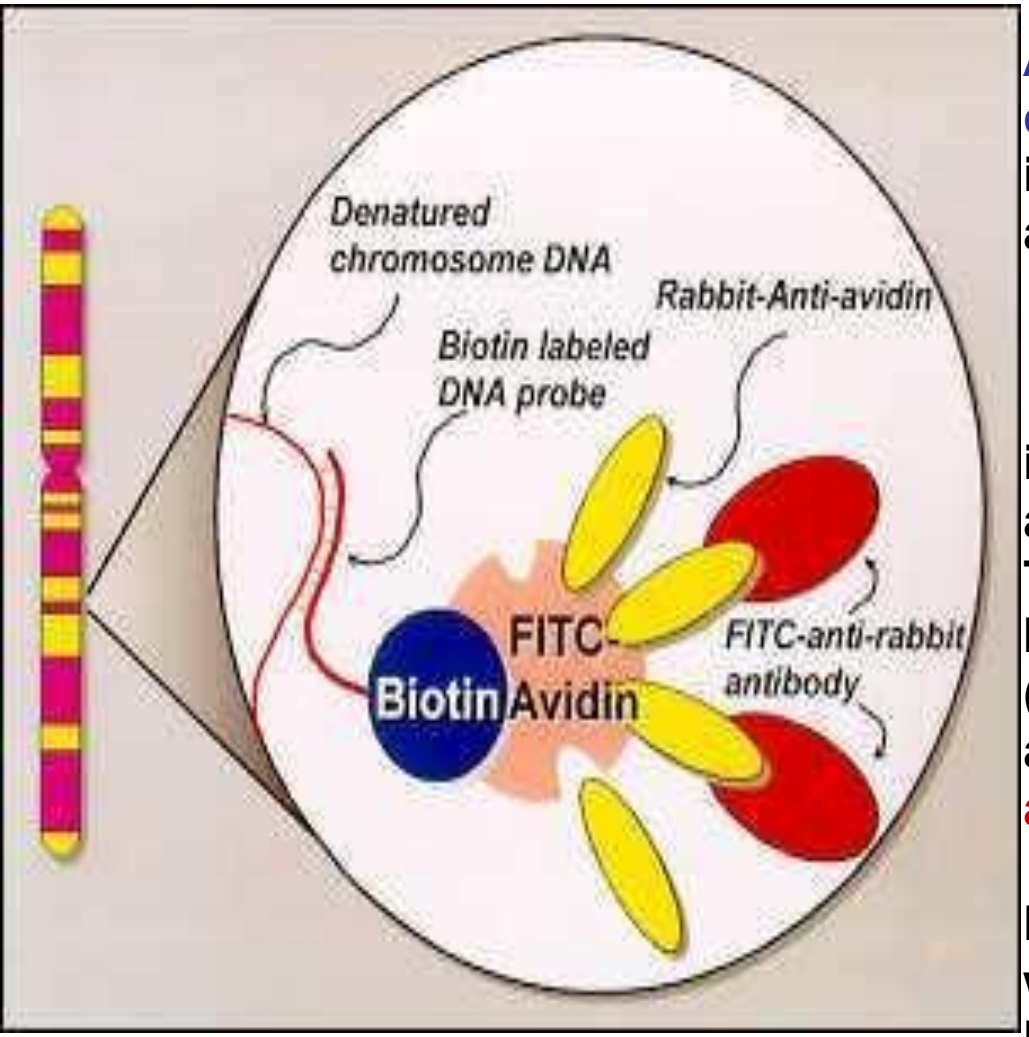
(5 in one of the examples below).



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

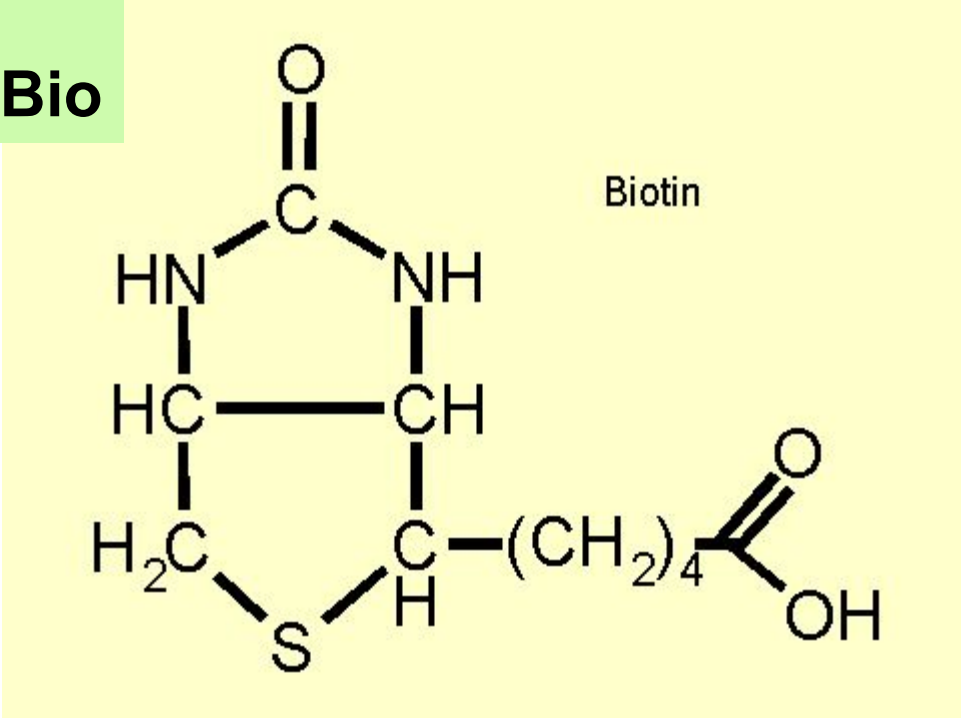
(67 kD protein

from Streptococcus avidinii)

BIOTIN is a vitamin B (small thing)

Biotin could be added to nucleotide and incorporated into the probe

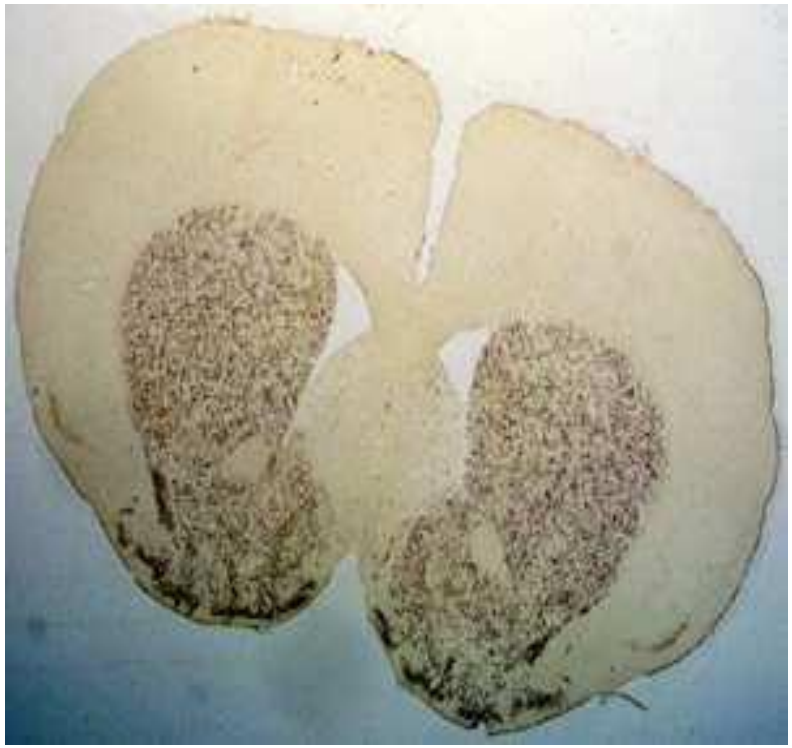
Avidin could be conjugated with fluorophore



Largest free energies of association of yet observed for noncovalent binding of a protein and small ligand in aqueous solution (K_assoc = 1014). Complex is extremely stable.**

IN SITU HYBRIDIZATION

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

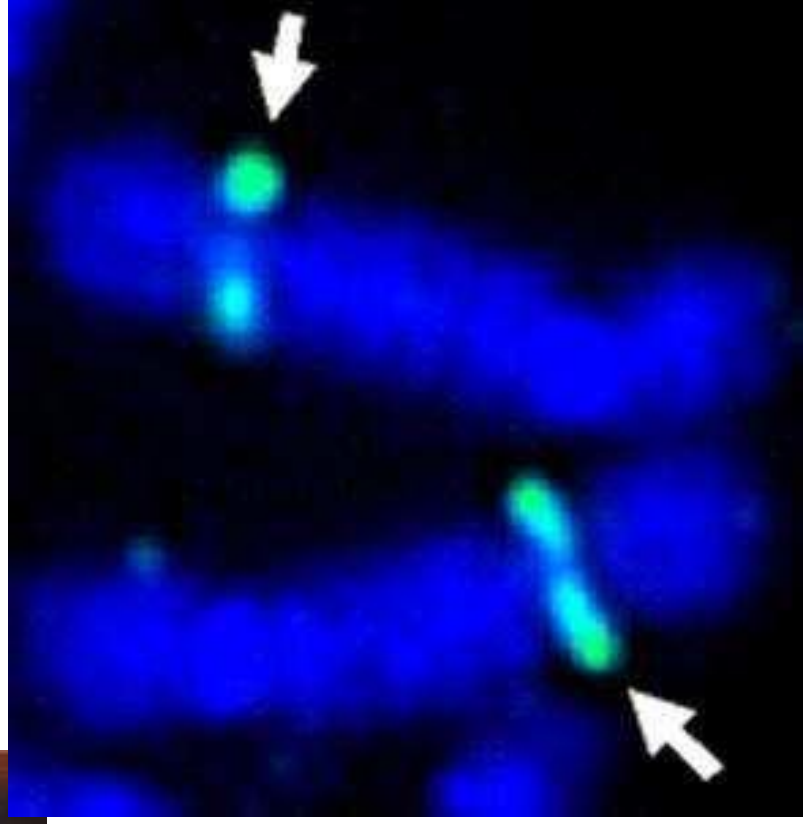


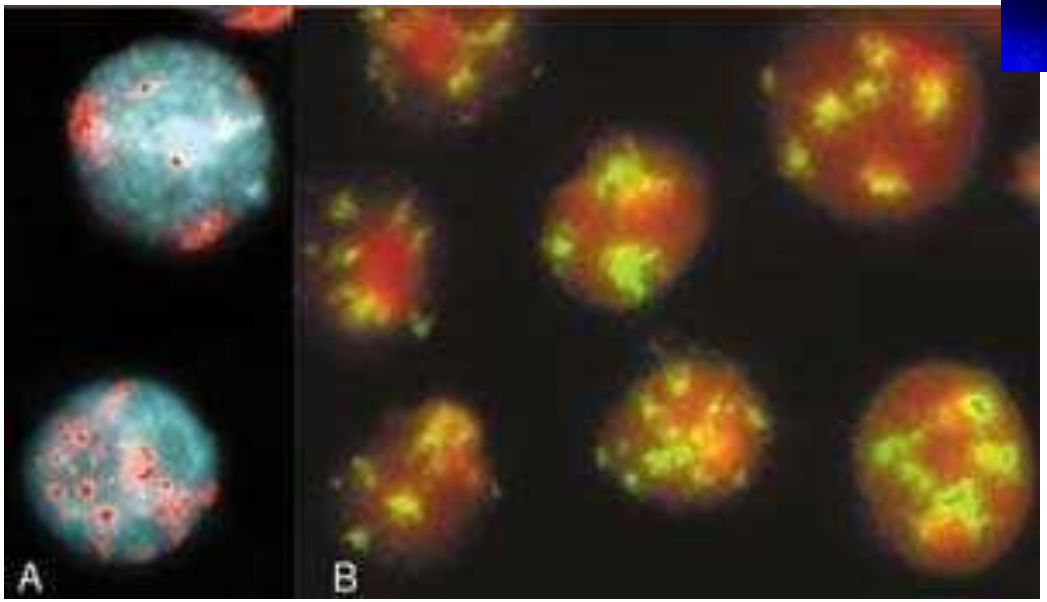
Encephalin gene expression in the mouse brain

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.

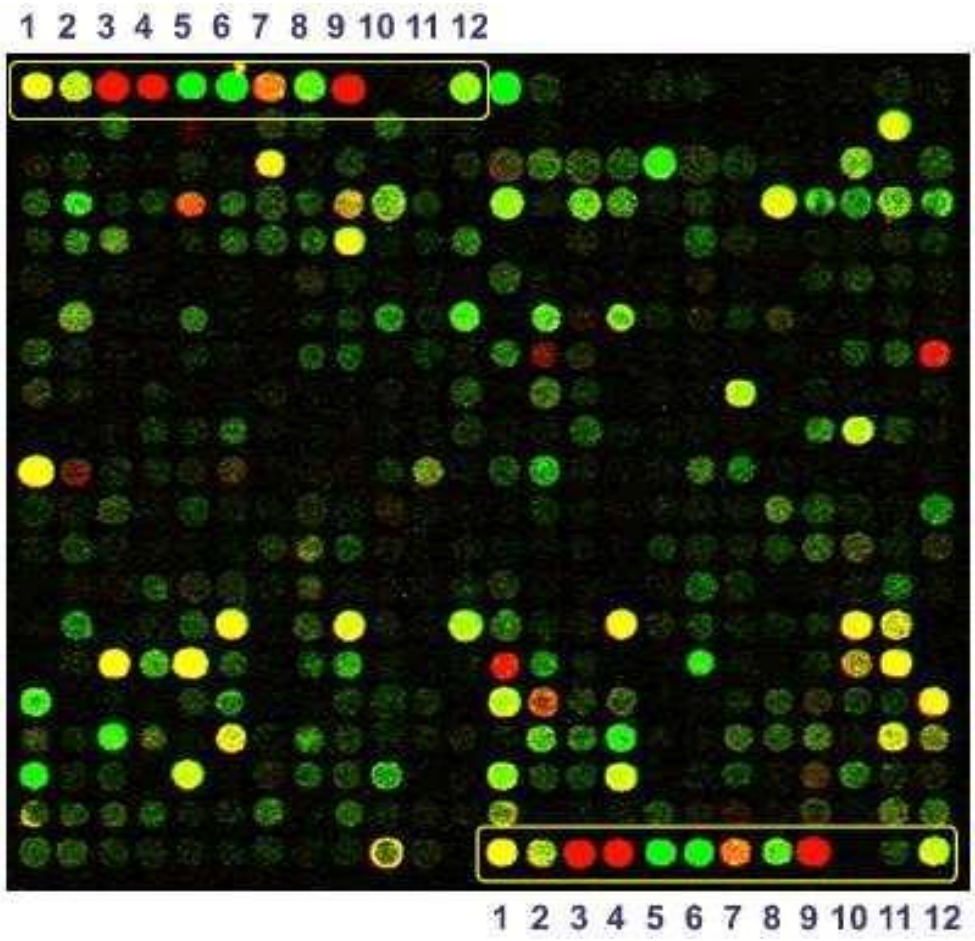


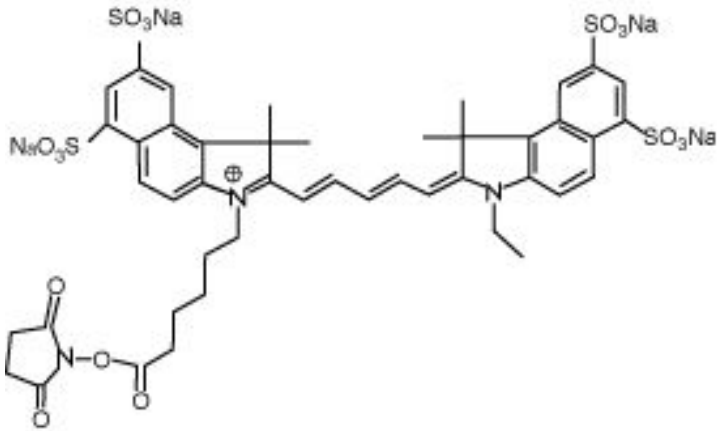


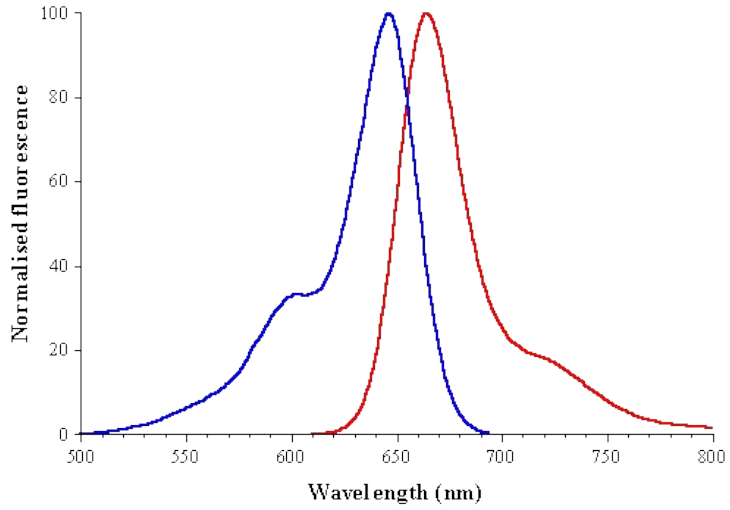
www.infobiogen.fr/.../

FISH labeling of the centromeric highly repeated DNA

Cy3/Cy5 direct labelling of DNA (for microarrays)

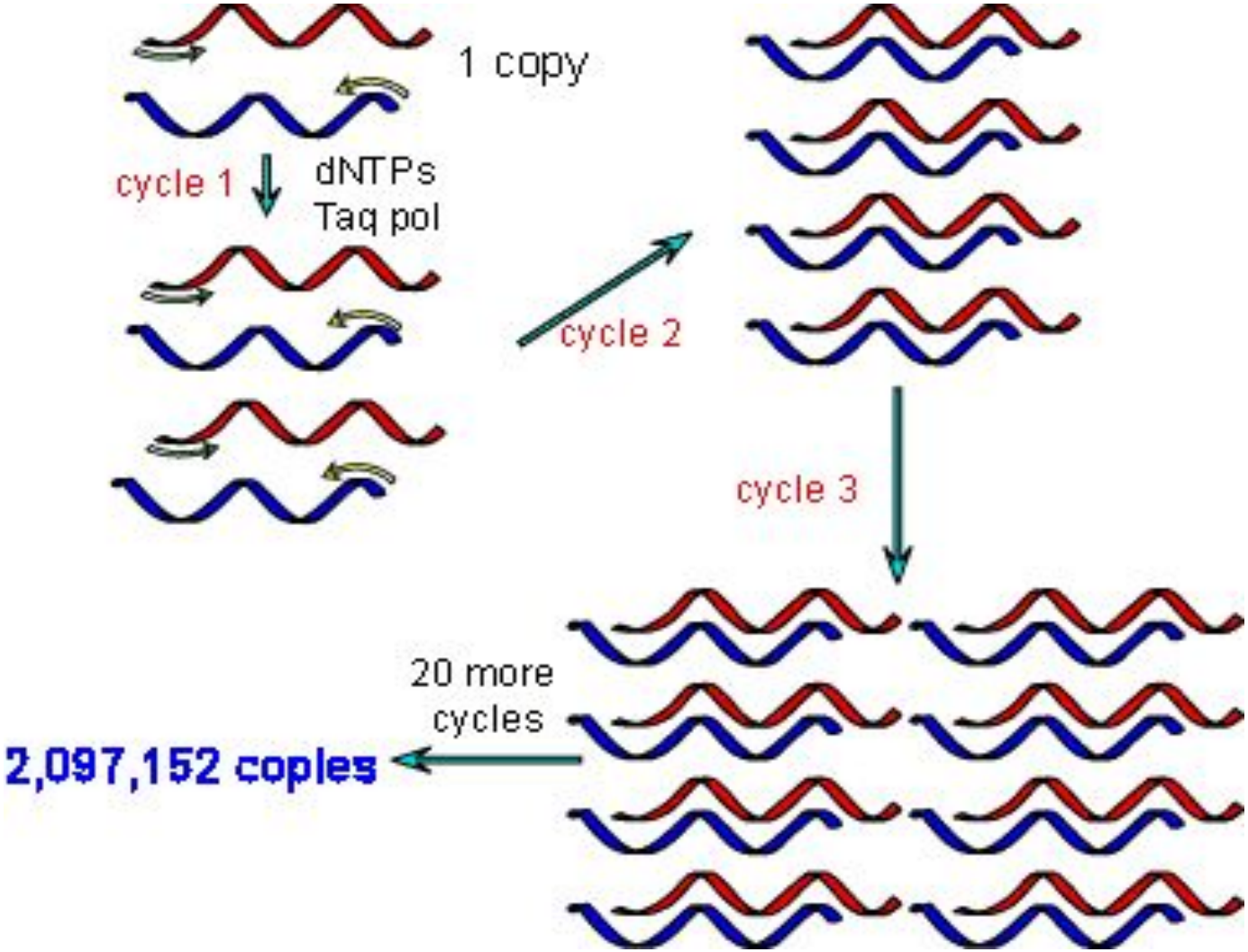






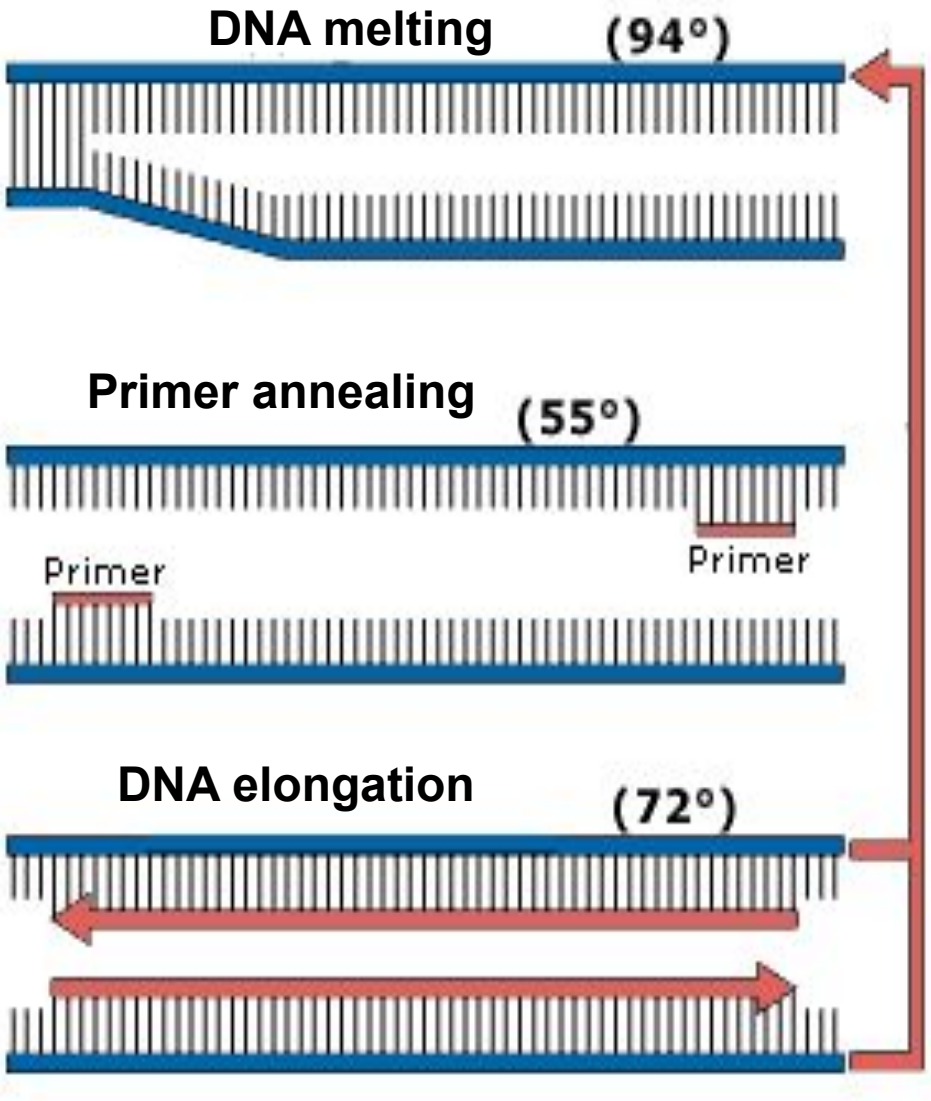
Cy5 -- RED

2. Polymerase chain reaction (PCR)



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

Polymerase Chain Reaction (PCR)



Biochemistry from U.C. Berkeley

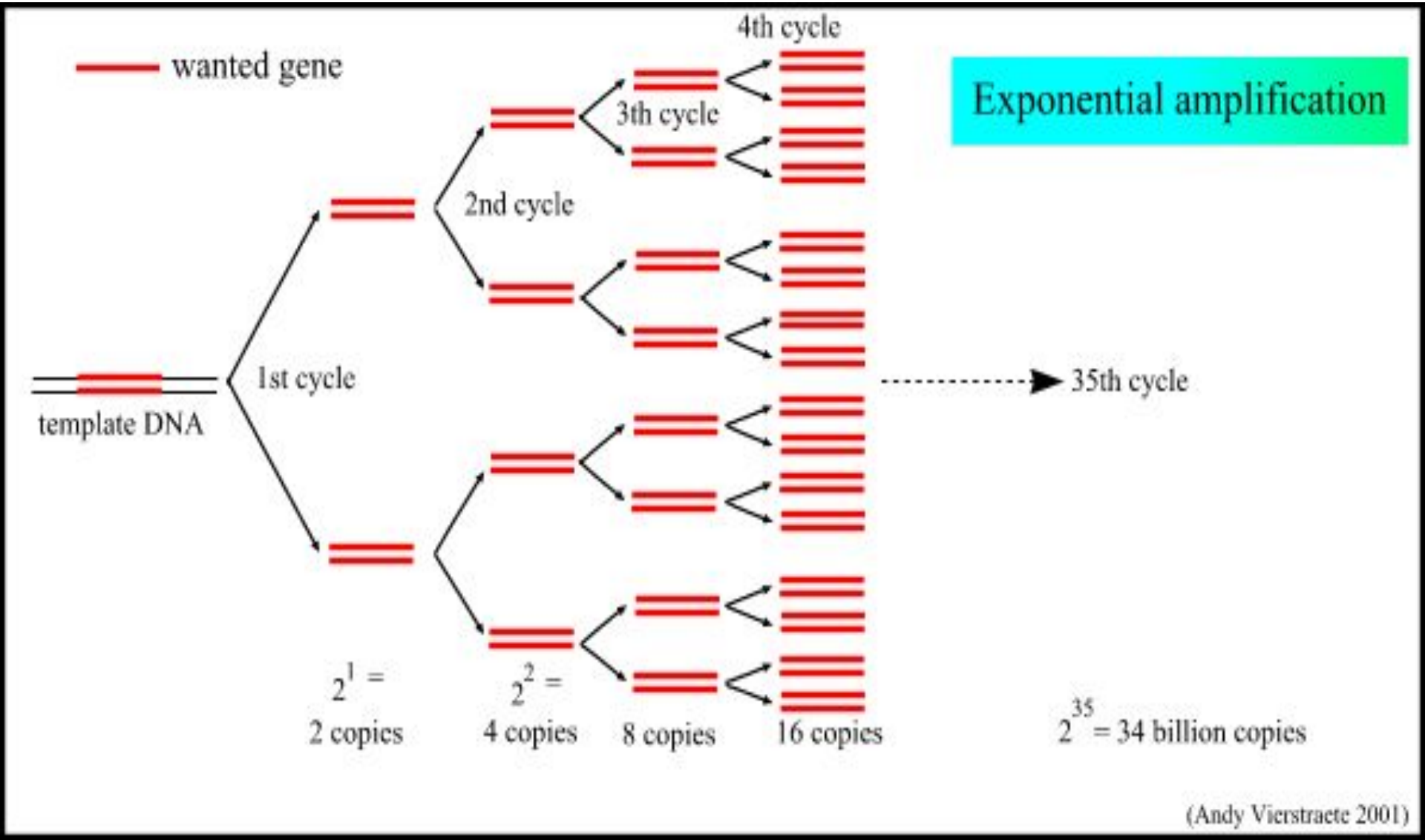
Nobel Prize in Chemistry 1993, at age 48

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

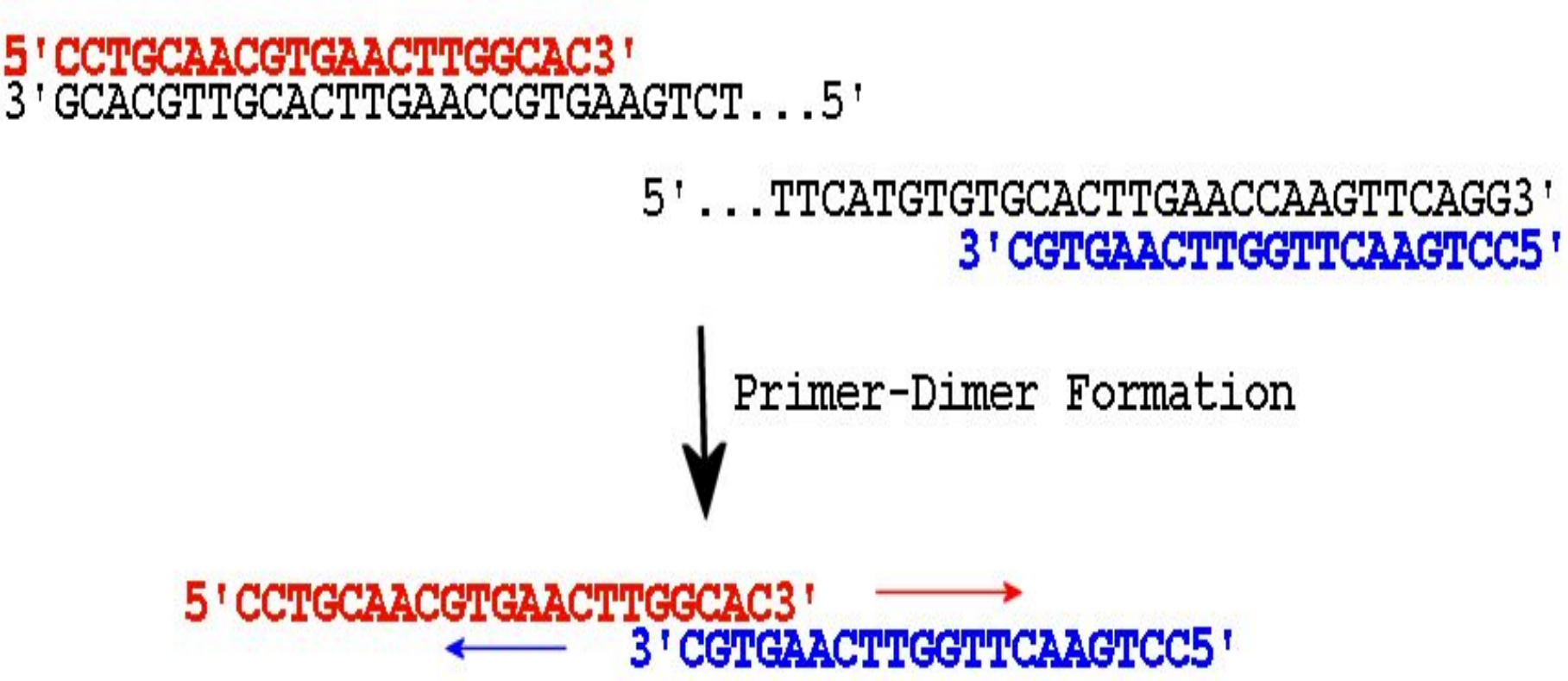
Basic Primer Design

- 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

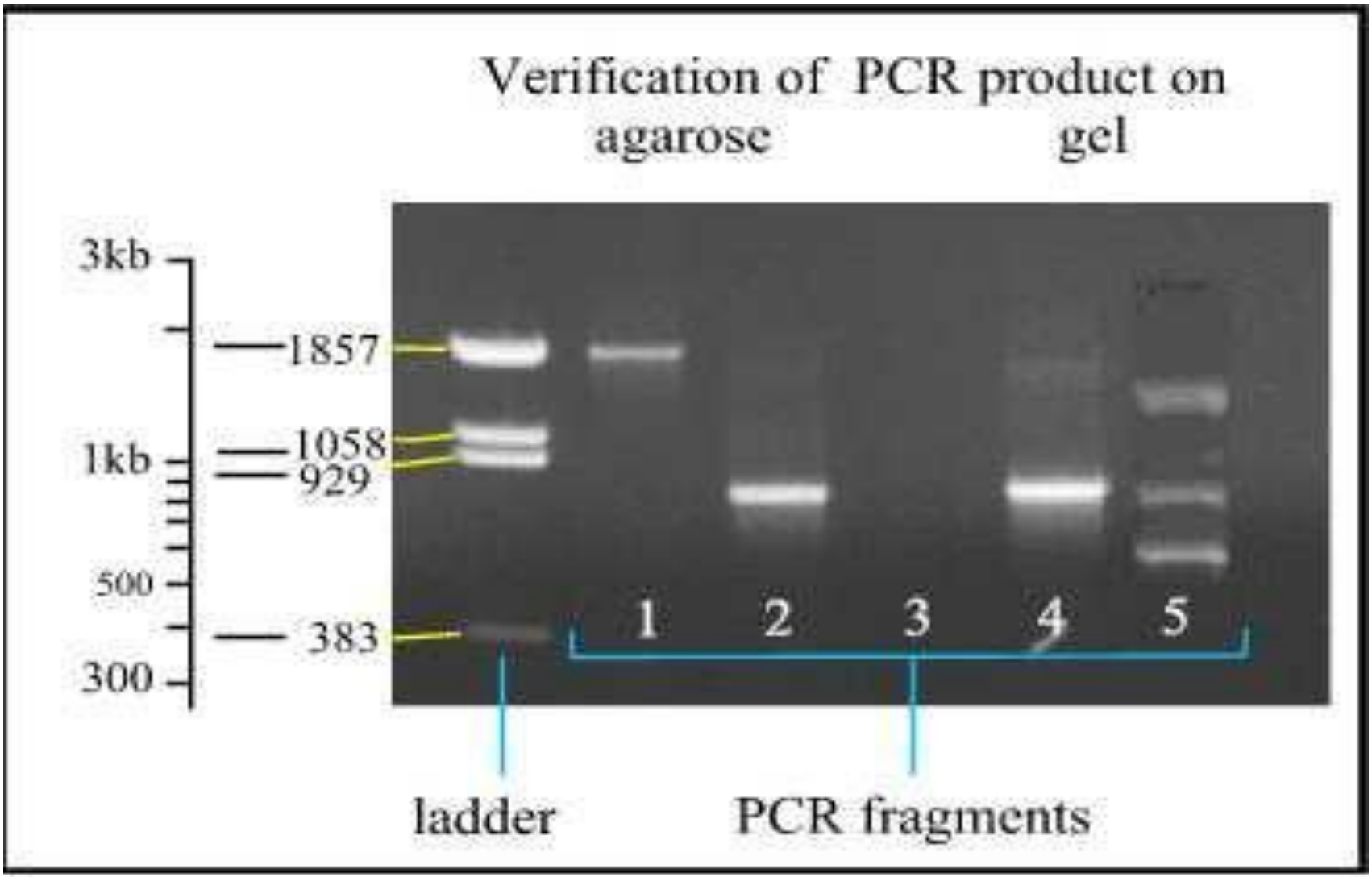
General Primer Picking Conditions

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

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

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
1.5×10^{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 $[Mg^{2+}]$

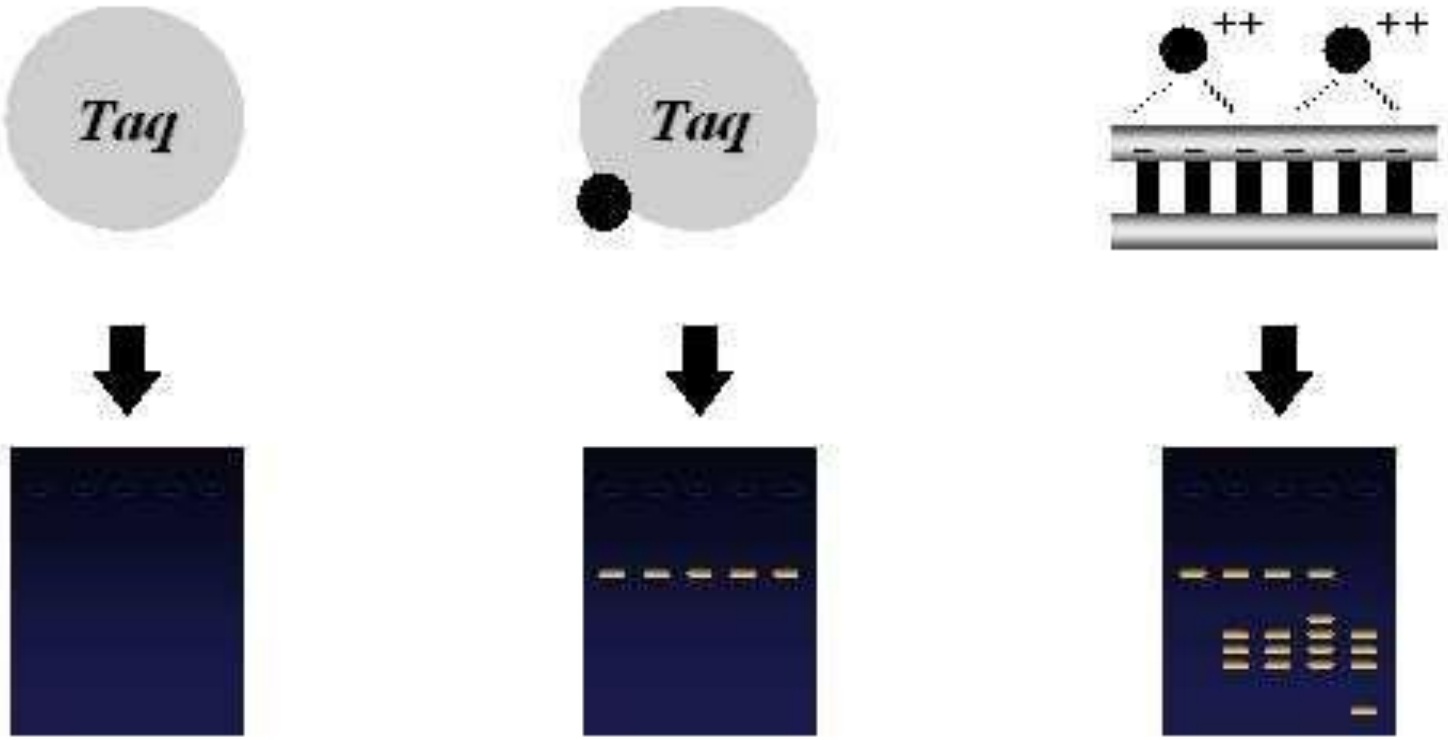
www.biotechlab.nwu.edu/ pe/Sld022.htm

Mg^{++} Optimization

Suboptimal Mg^{2+}

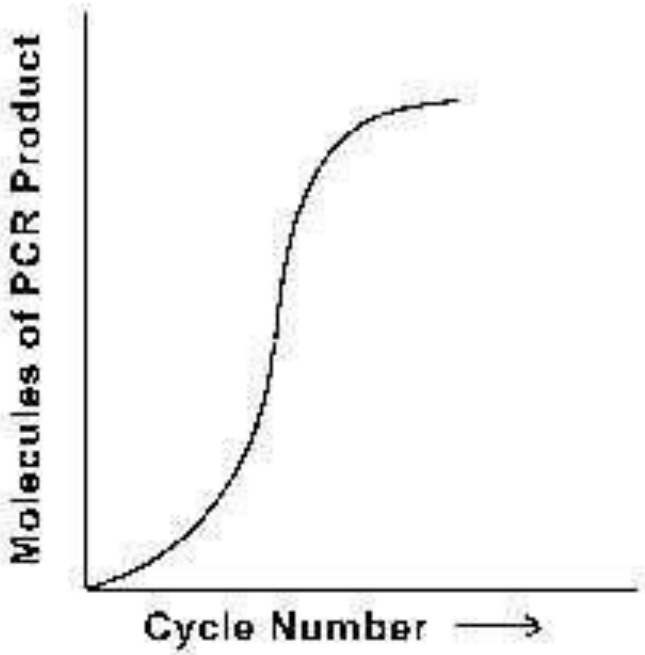
Optimal Mg^{2+}

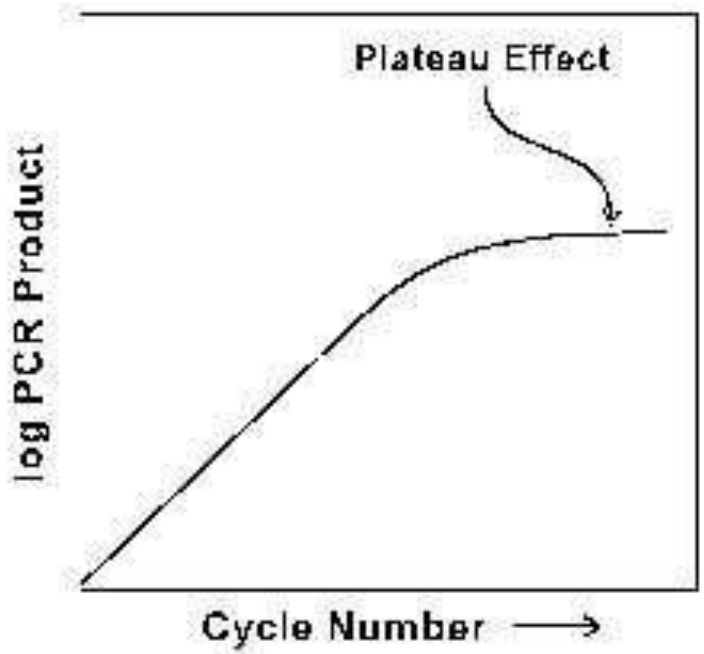
Excess Mg^{2+}



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.

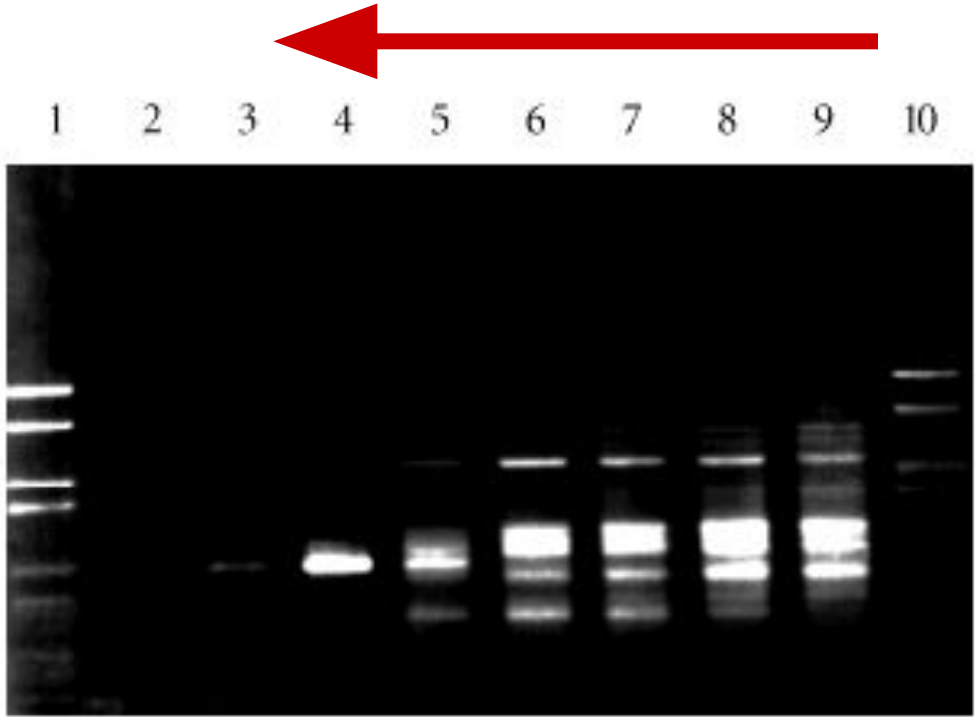
Plateau effect in PCR reaction

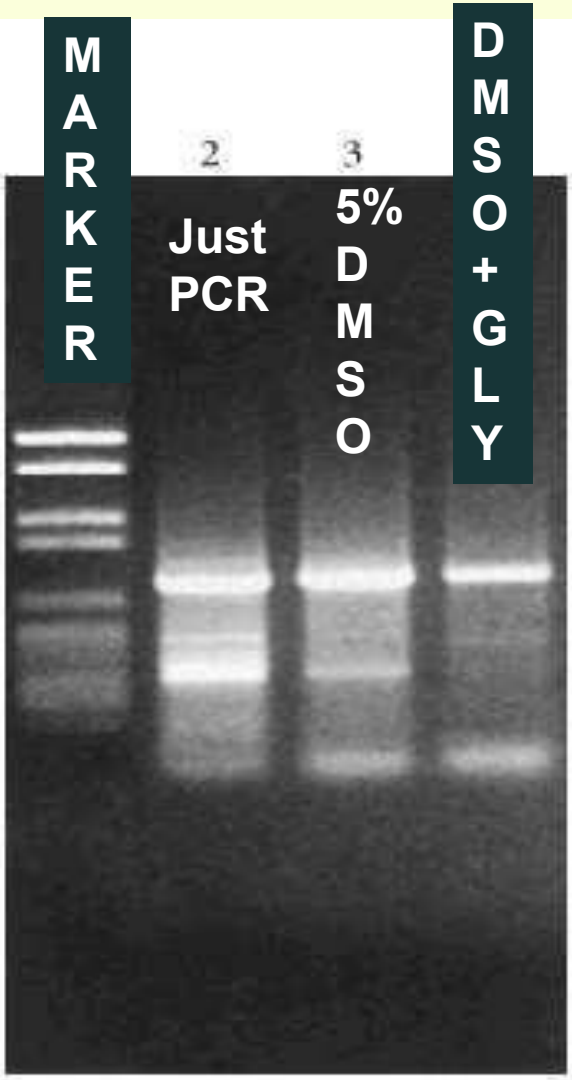
- The point in a PCR where running more cycles does not result in a net gain of specific PCR
- Amplicons not in plateau - including nonspecific fragments - may continue to grow exponentially
- Hence, running PCRs into plateau may result in high background or smearing

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

Non-specific PCR and how to improve it

Decrease in Mg concentraton





PCR enzymes

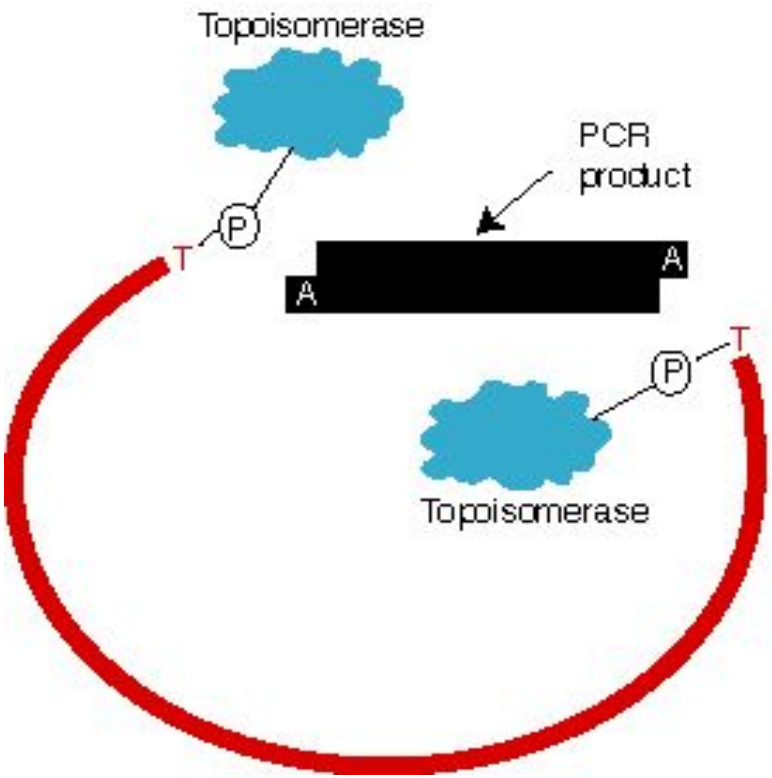
Taq DNA polymerase, the first enzyme used for PCR, is still the most popular.

-- 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)

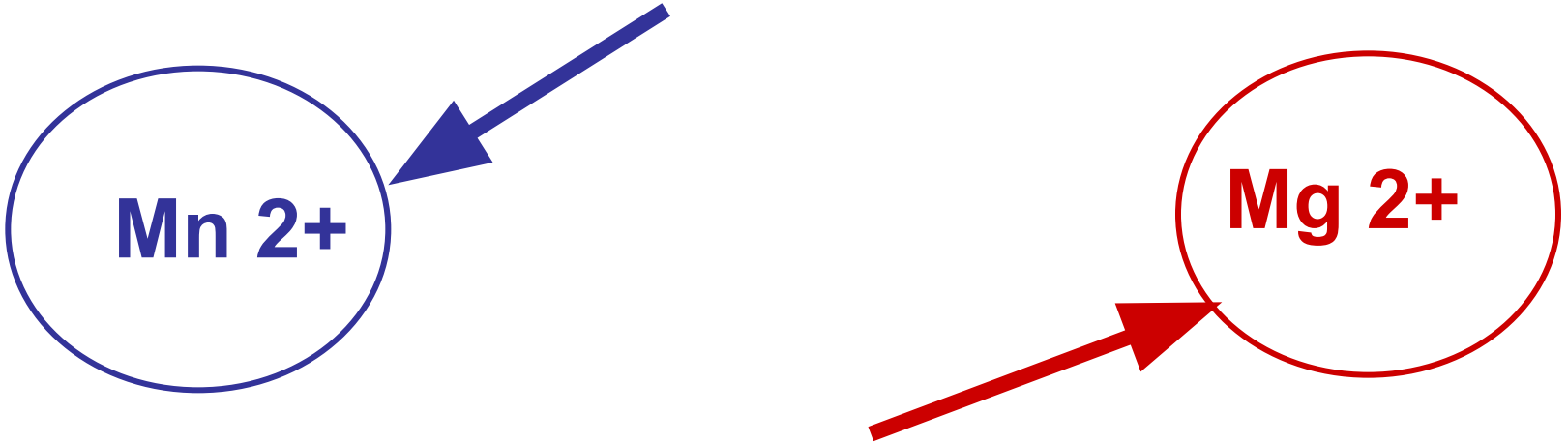
"Topo" cloning system (Invitrogen)



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:

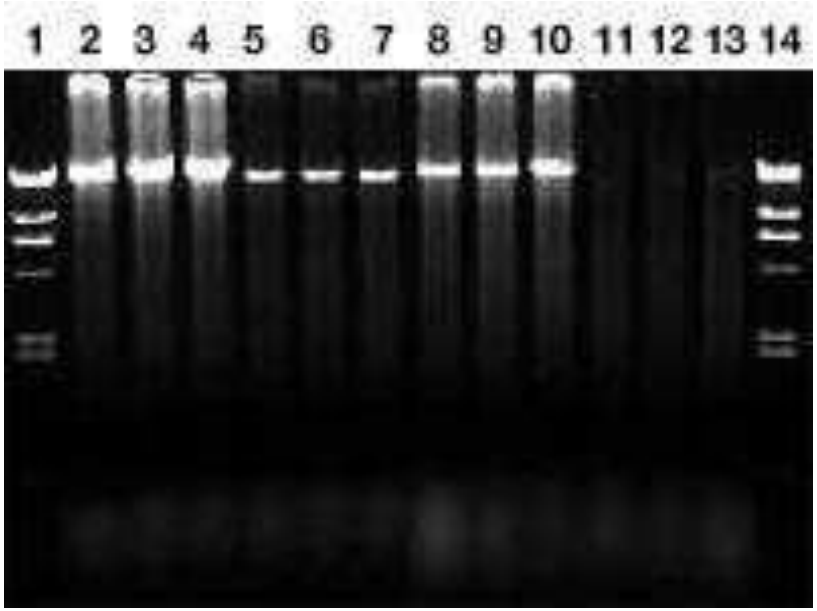
Deep Vent (Pyrococcus species GB-D) (New England Biolabs)
New England Biolabs claims fidelity is equal to or greater than that of Vent.

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

Long-Range PCR

Use of two polymerases:

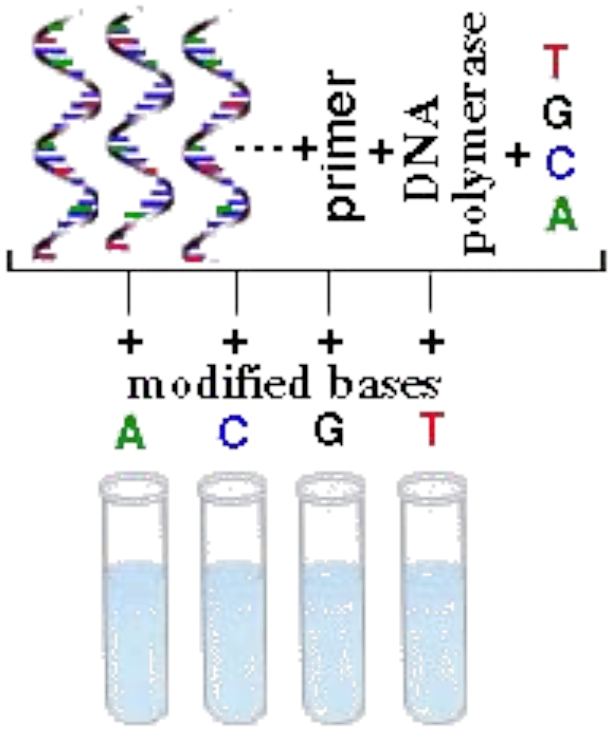
a non-proofreading polymerase Taq
is the main polymerase in the reaction,
a proofreading polymerase (3' to 5' exo) Pwo
is present at a lower concentration.



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)

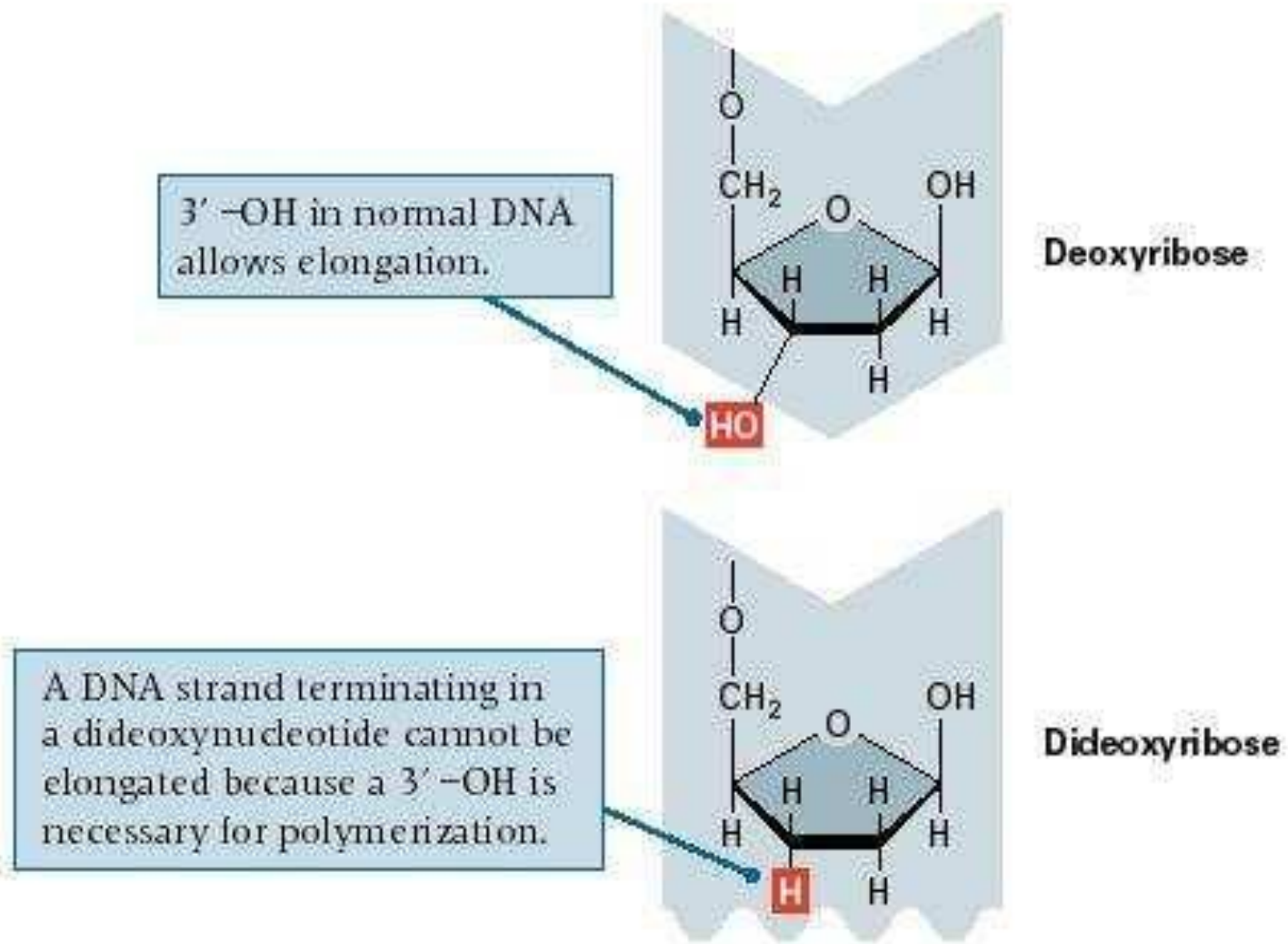


Sequencing of DNA by the Sanger Method

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

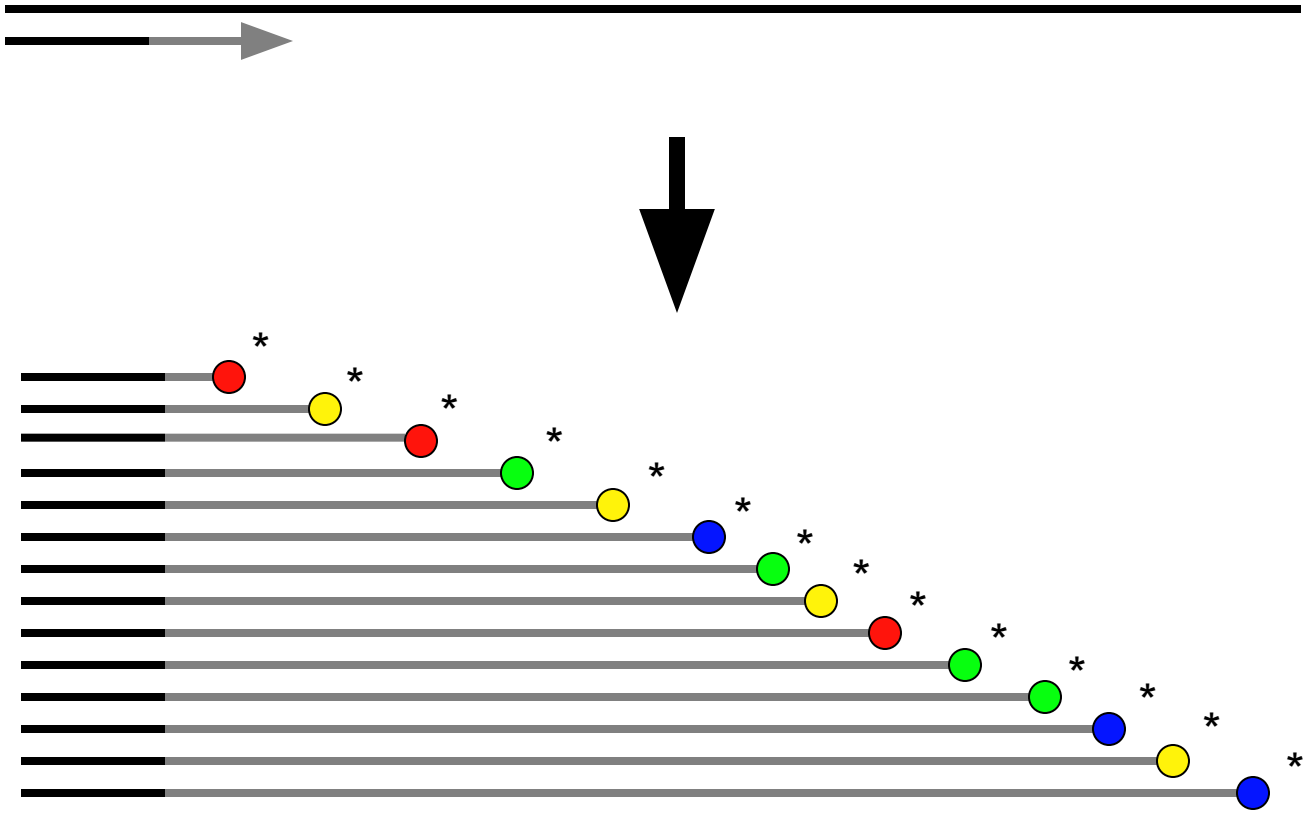
http://www.kids-dna.com/dnatube.gif

Dideoxynucleotide blocks chain elongation

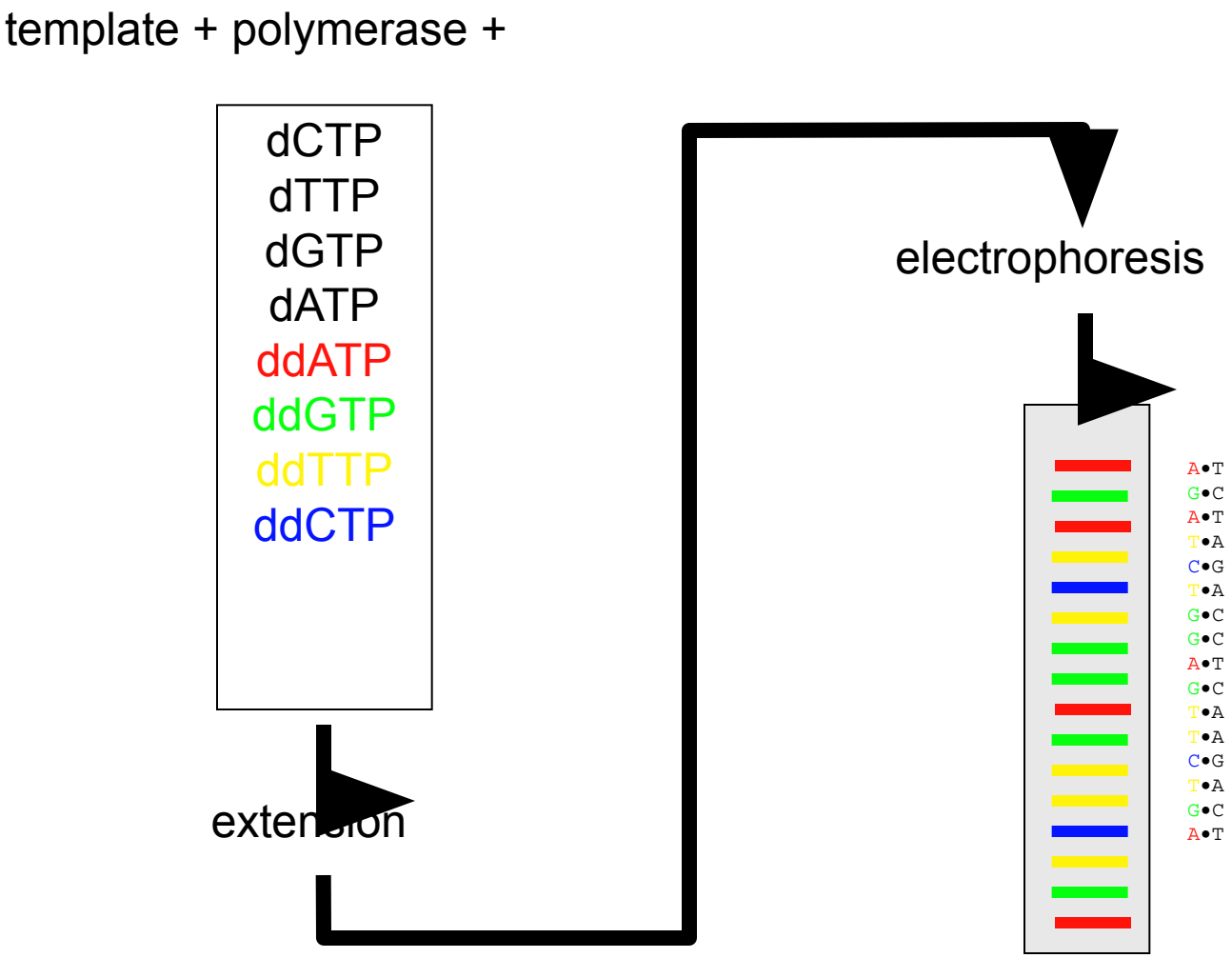


http://www.cbs.dtu.dk/staff/dave/roanoke/fig5_38.jpg

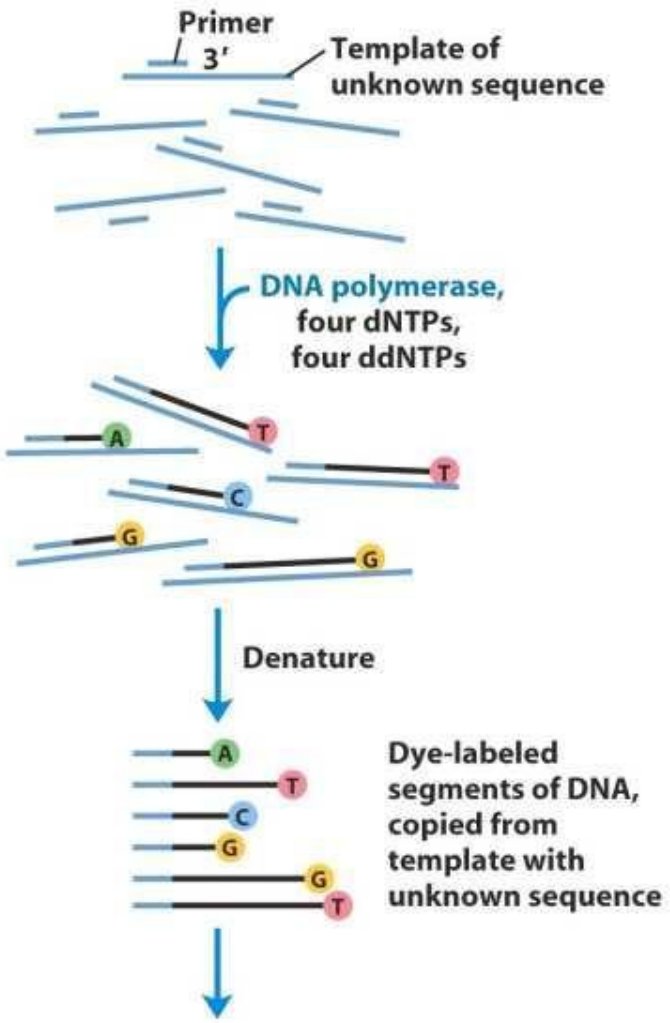
DNA sequencing: chemistry, with terminator dyes

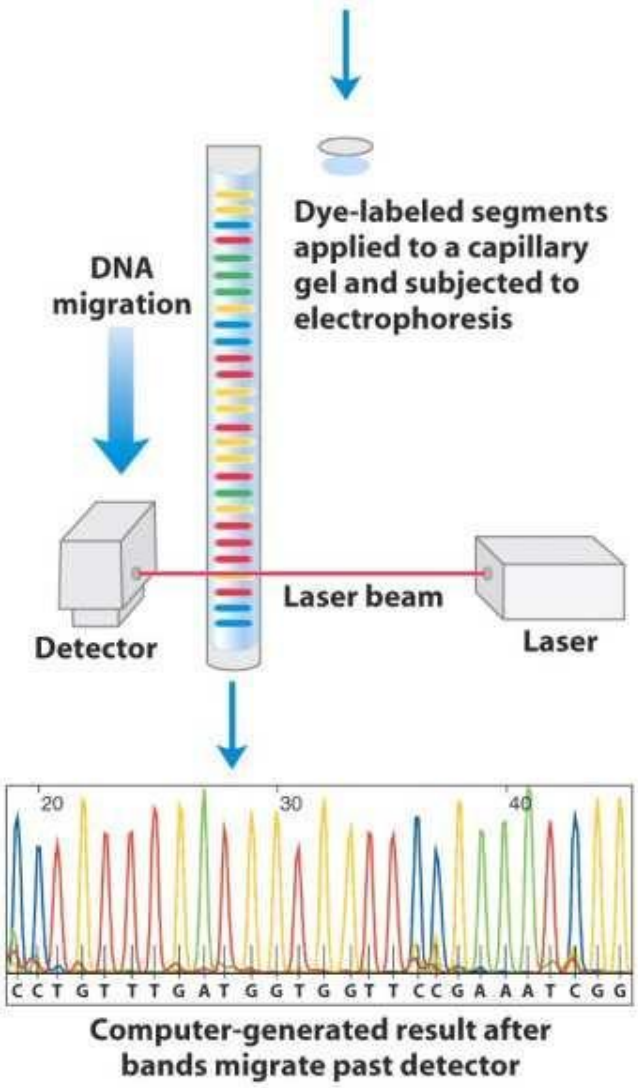


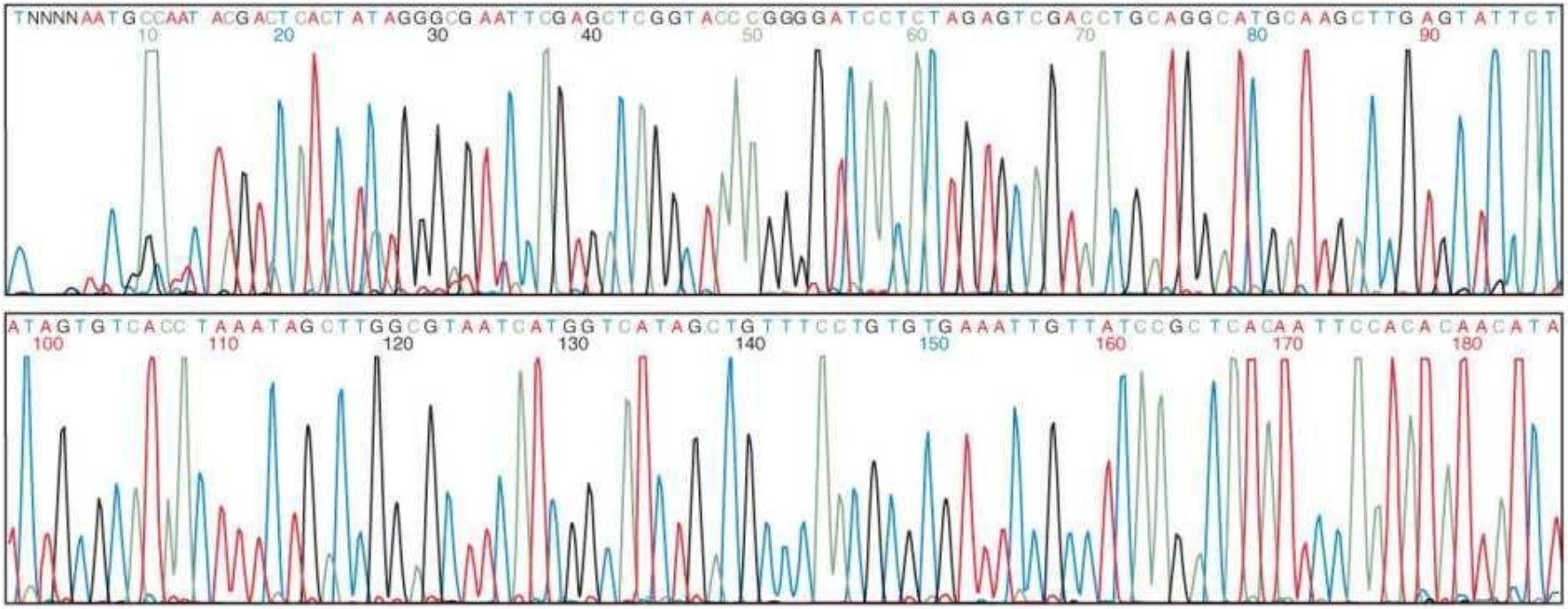
Semi-automated fluorescent DNA sequencing:



Cycle Sequencing - PCR



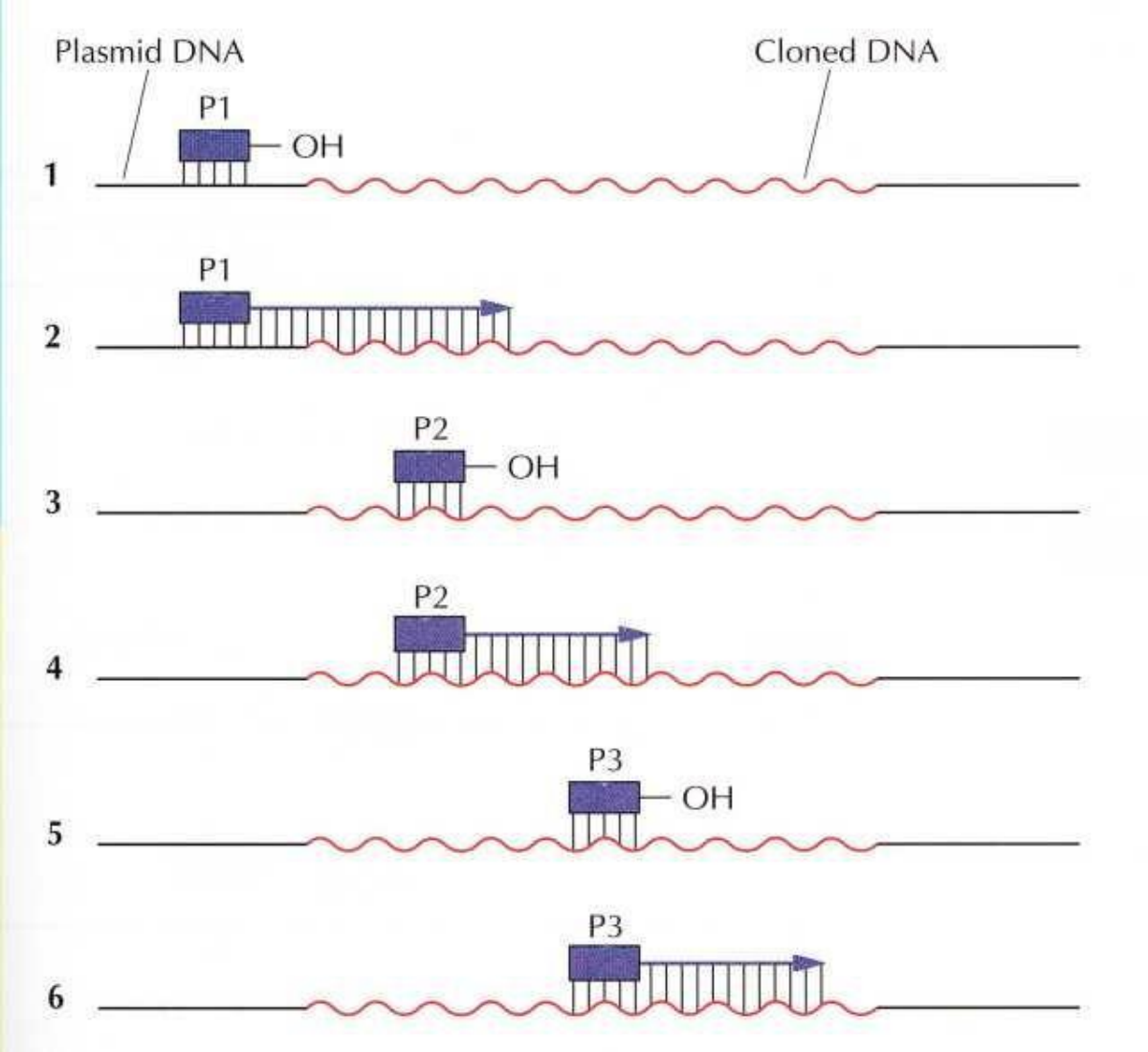



TNNNNAATGCCAAT ACGACTCACTATAGGGCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGAGTATTCT
10 20 30 40 50 60 70 80 90
ATAGTGTCACC TAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATA
100 110 120 130 140 150 160 170 180

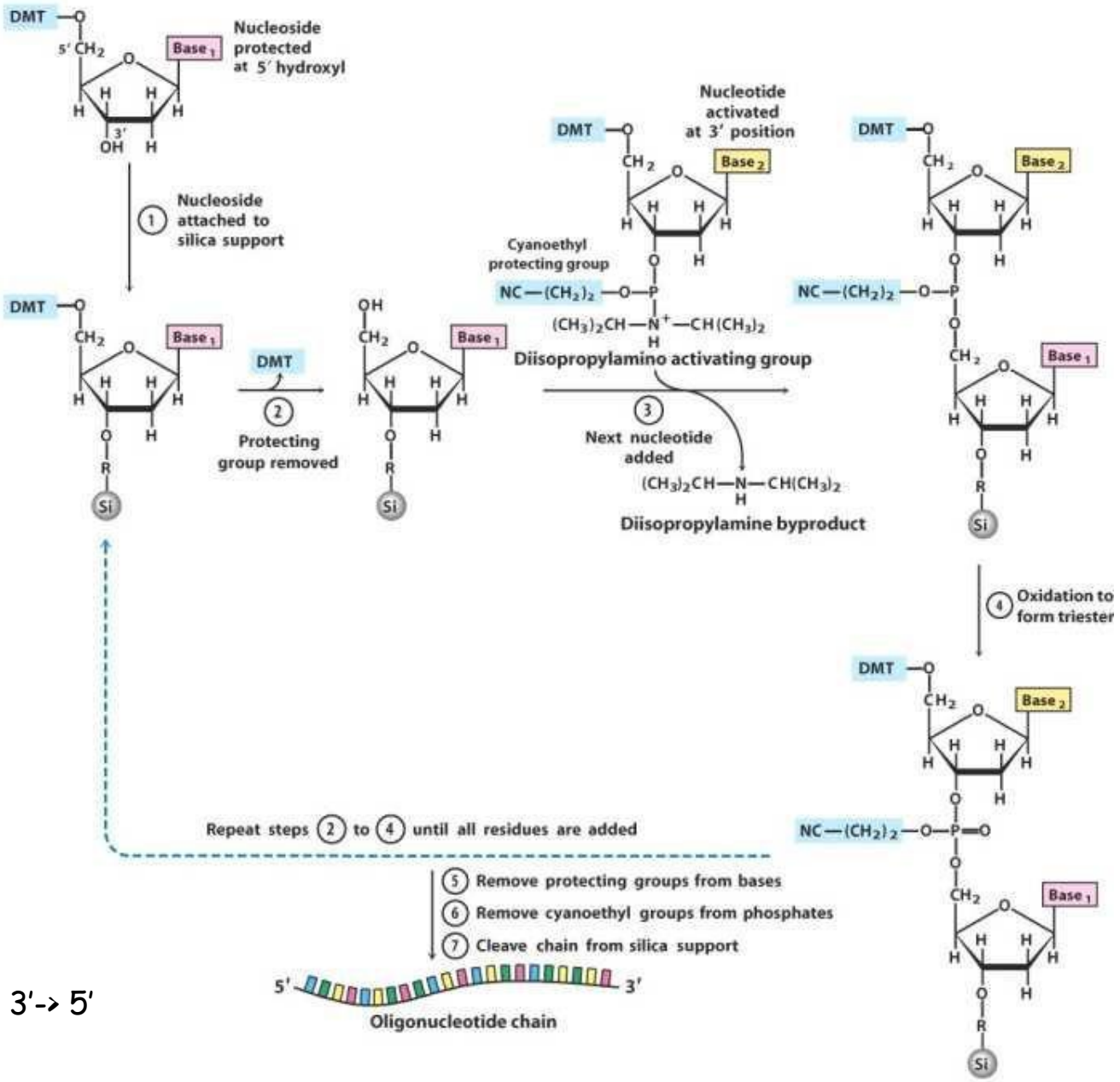
Applied Biosystems Inc., have designed an automated method that combines the PCR and actual sequencing

<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

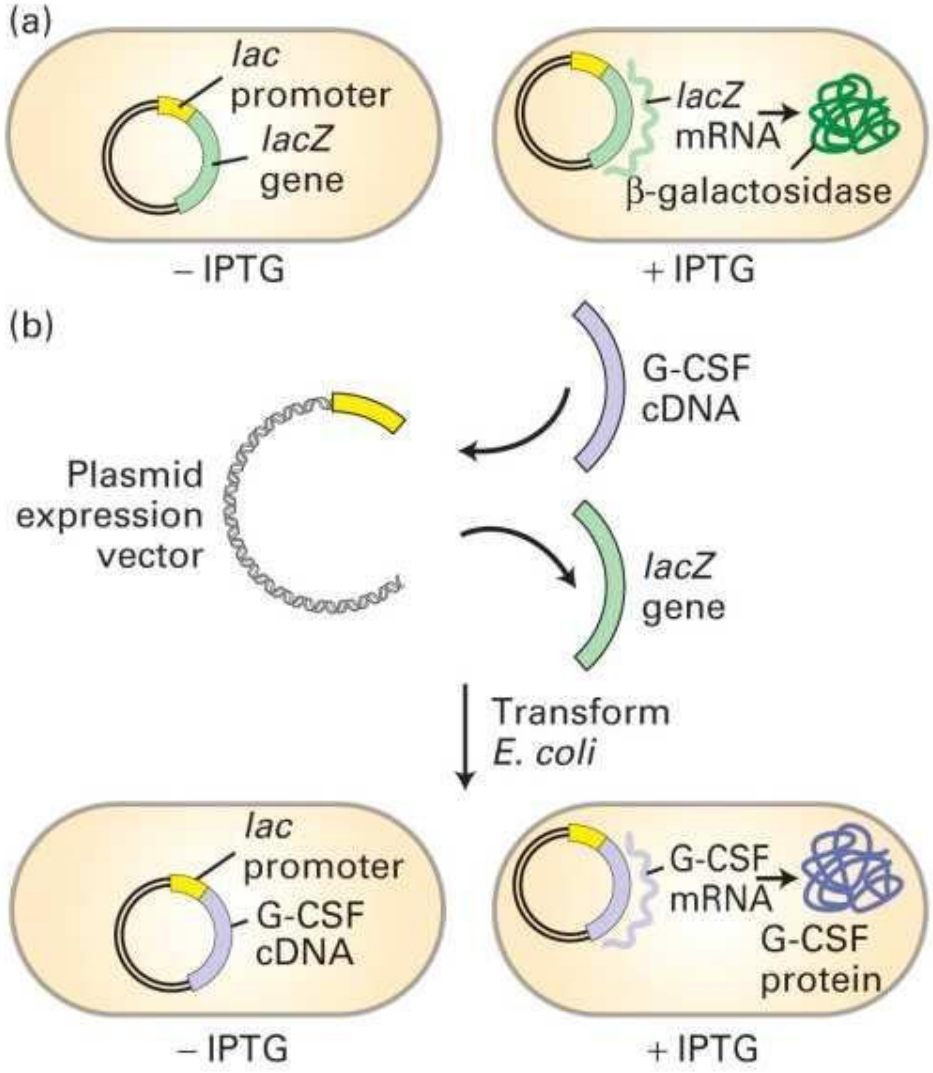
- Expression Host -> Expression System
- Promoter system -> expression vector
- Properties of product -> stability
- Production level

Comparison of expression systems

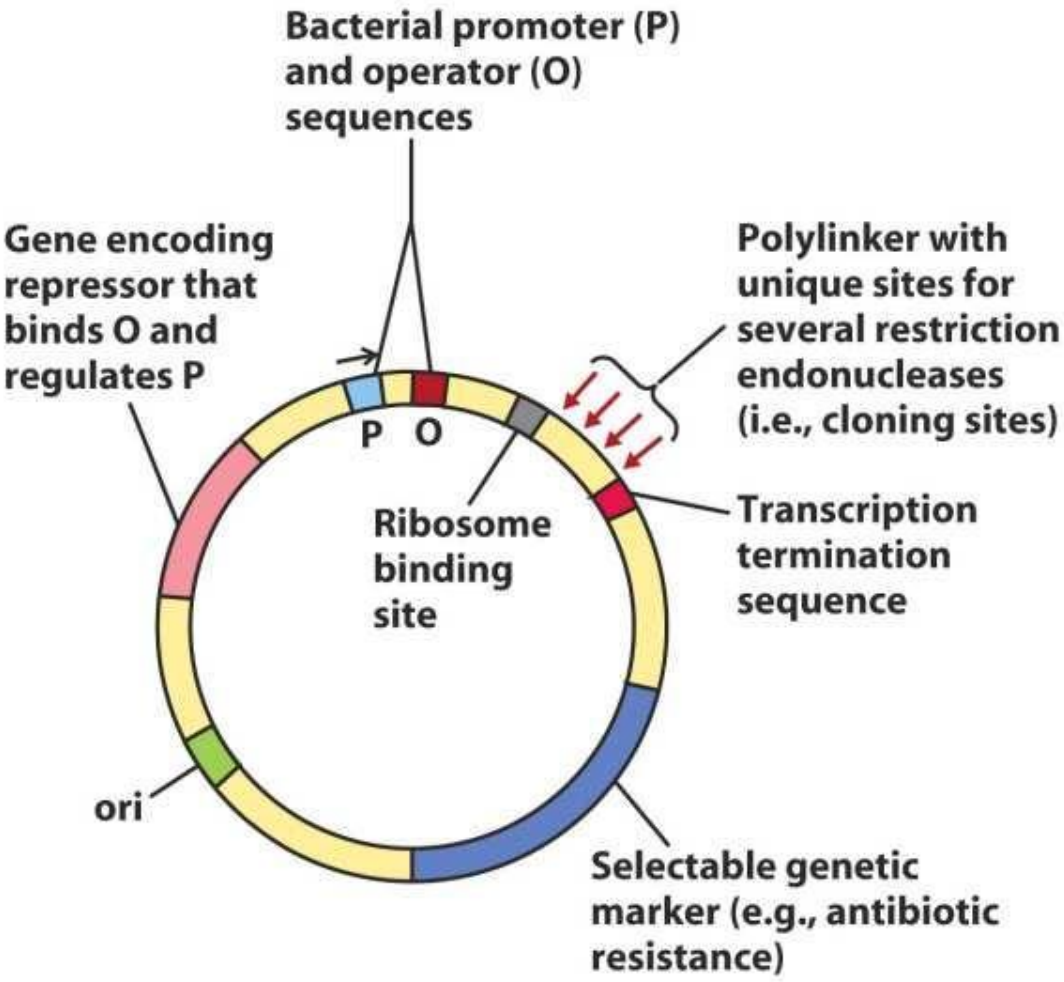
Table 1
Comparison of Expression Systems

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

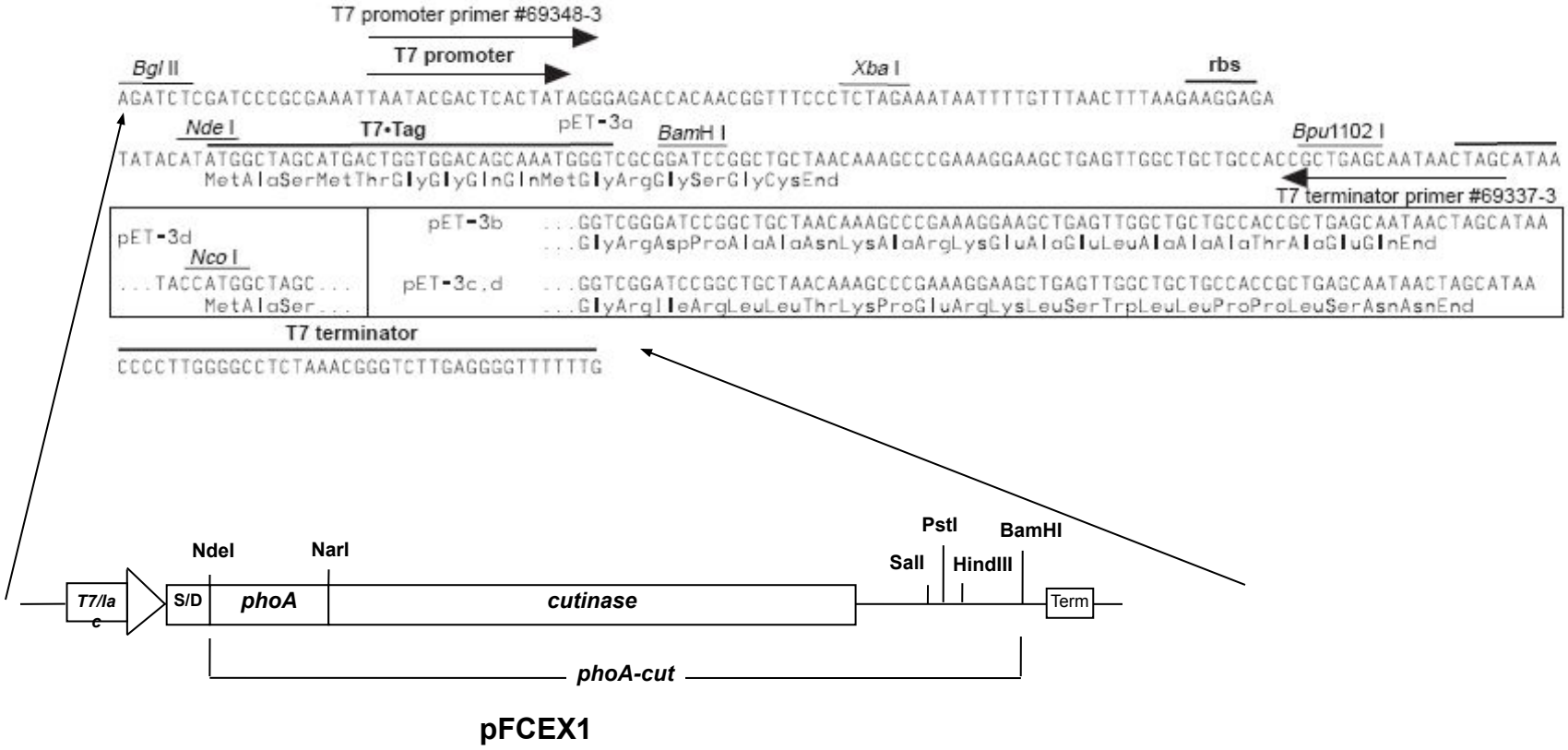
Use of *Lac* promoter (*pLac*) for expression of foreign protein



Prokaryotic Expression vector

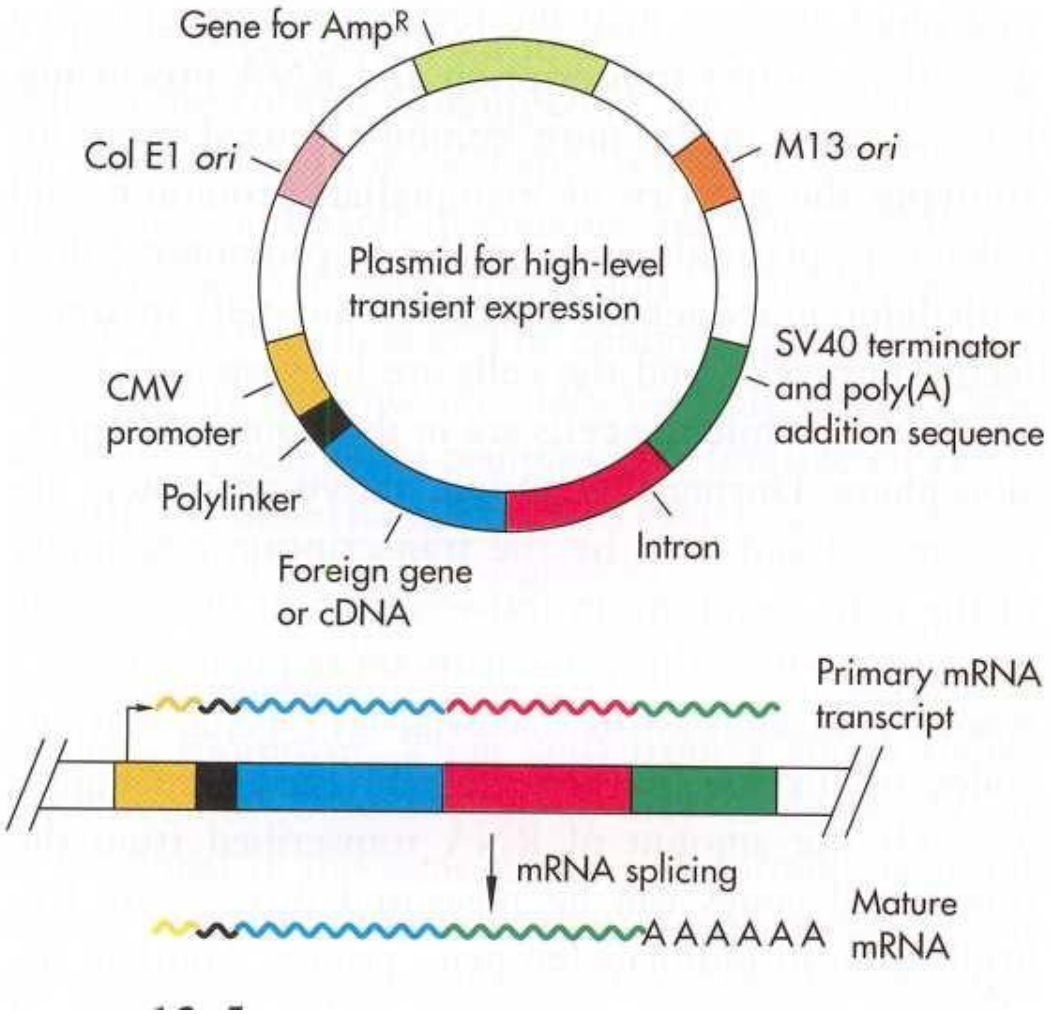


Prokaryotic Expression vector

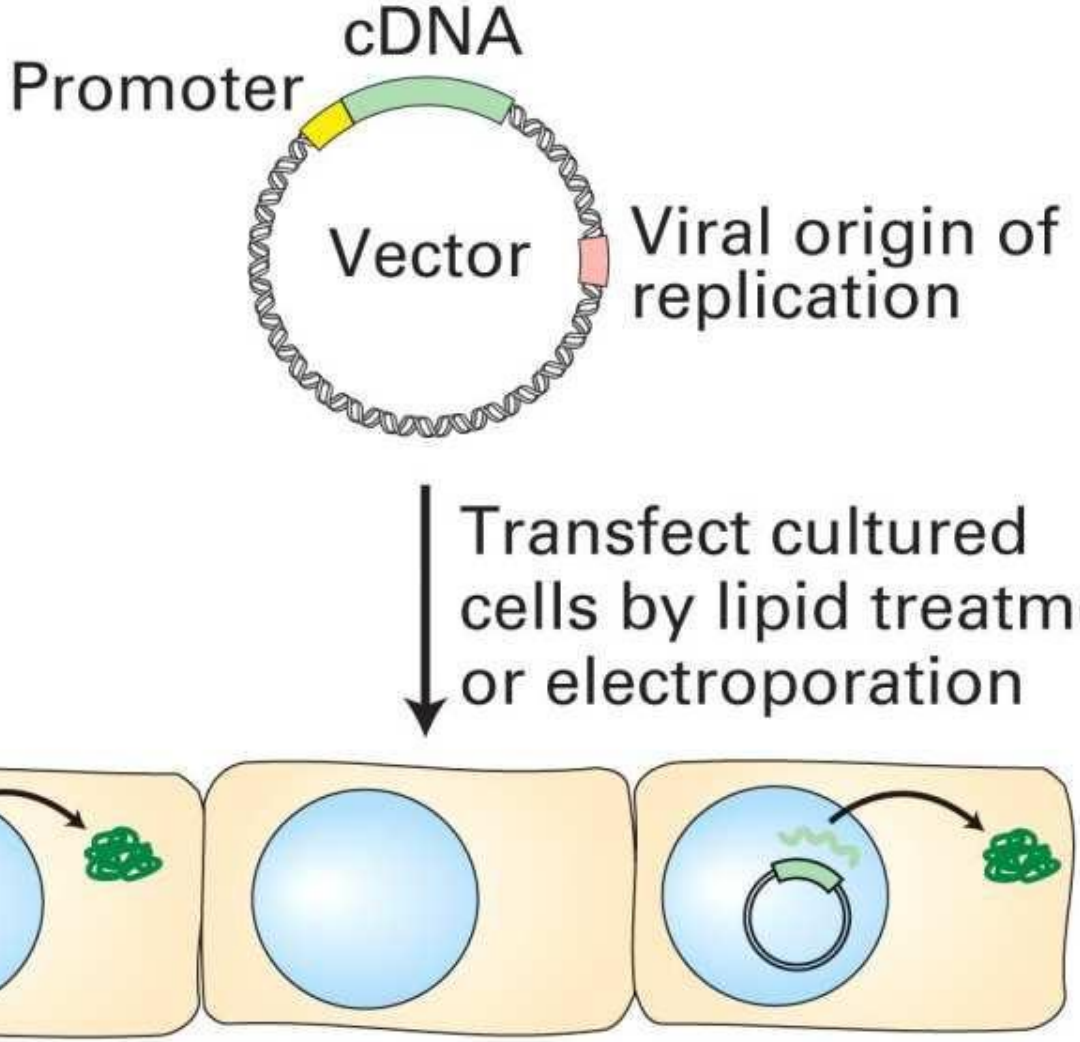


pFCEX1

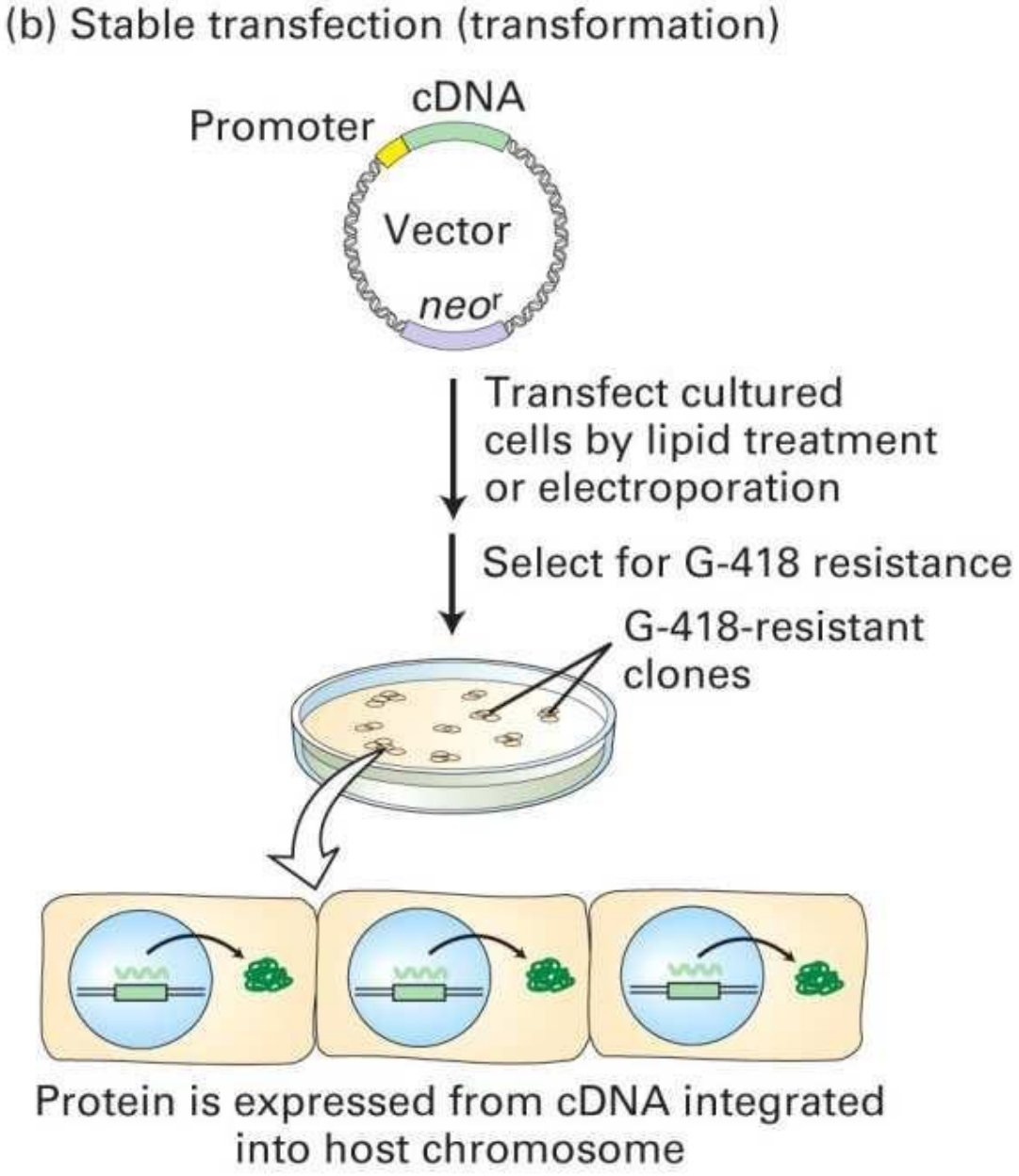
Eukaryotic Expression vector



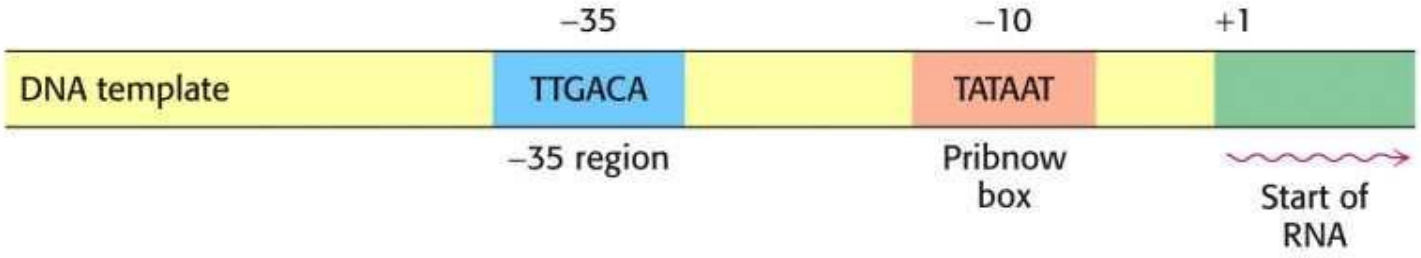
(a) Transient transfection



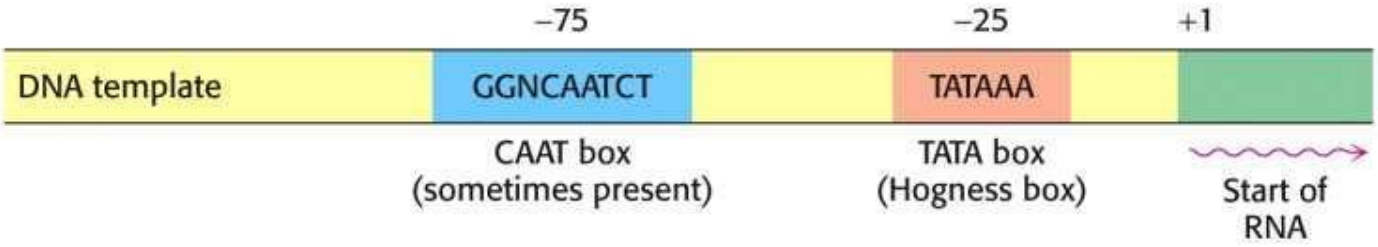
Protein is expressed from cDNA in plasmid DNA


(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

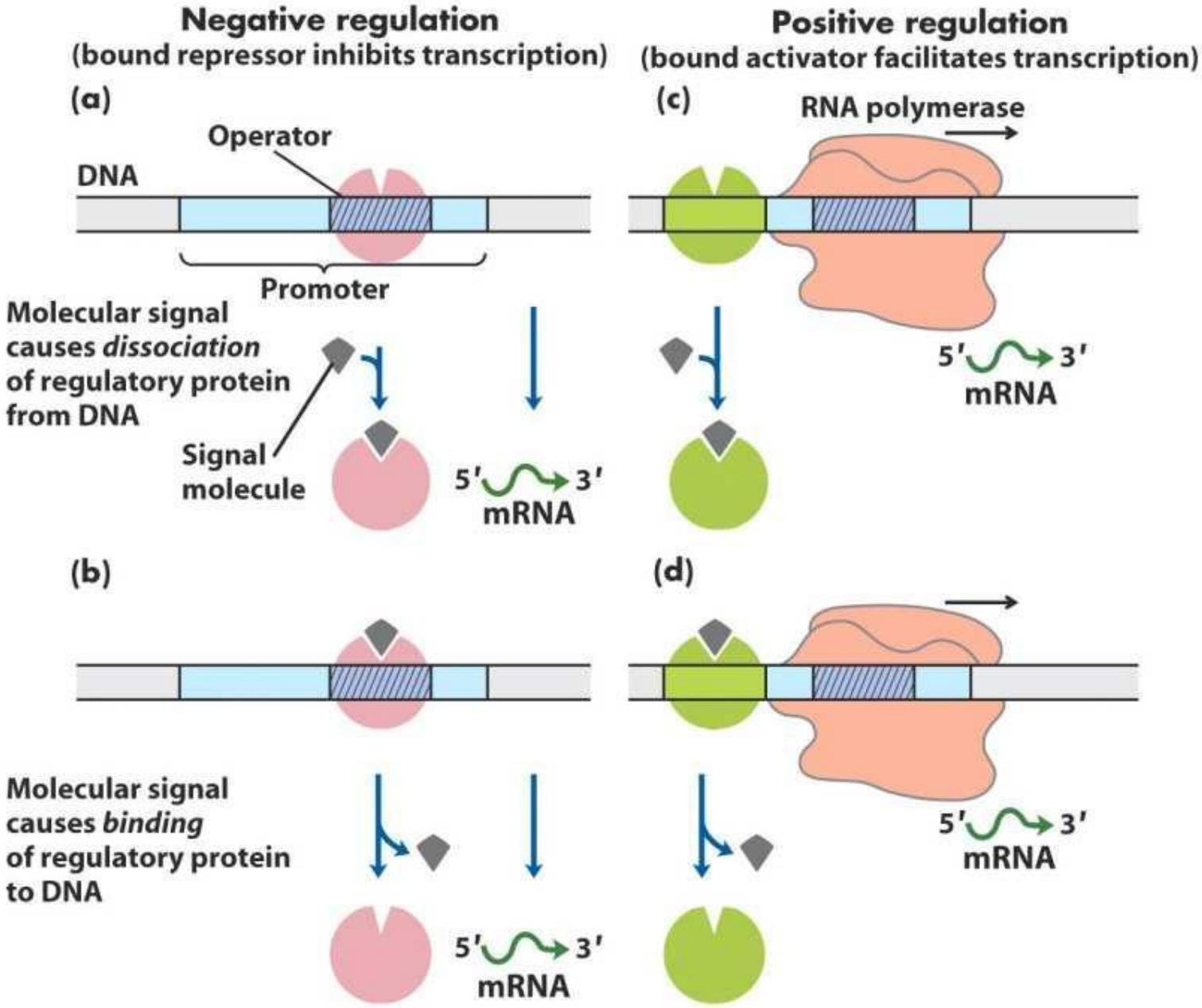
Promoters



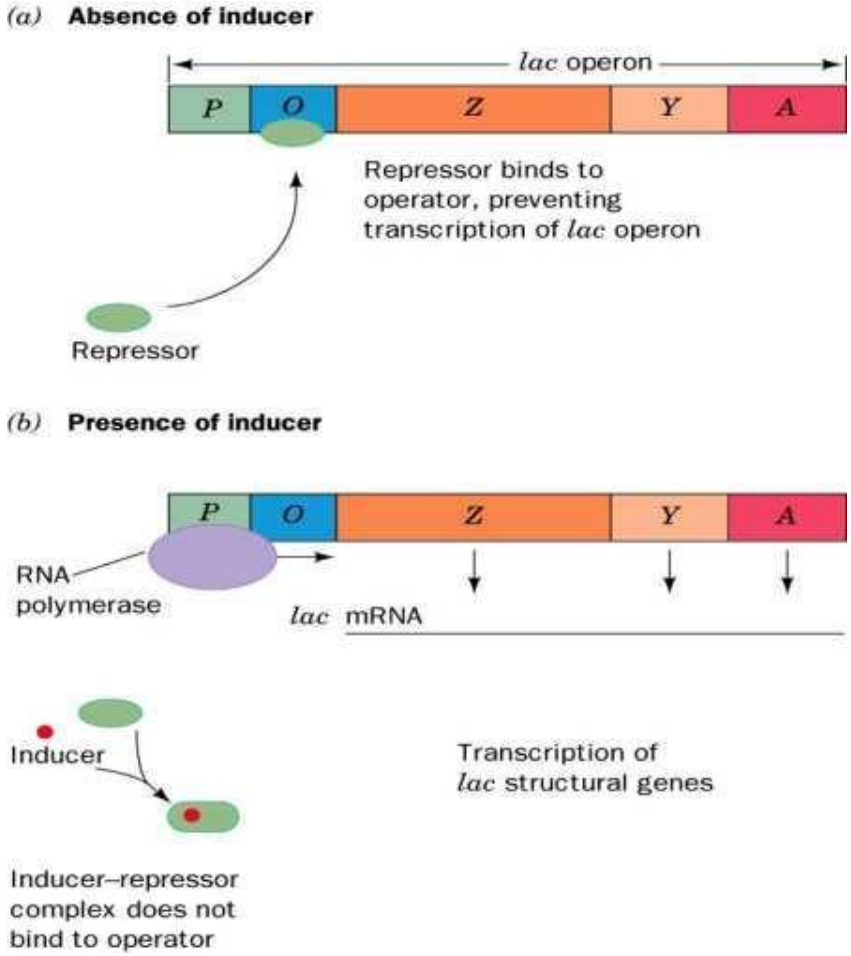
(A) **Prokaryotic promoter site**

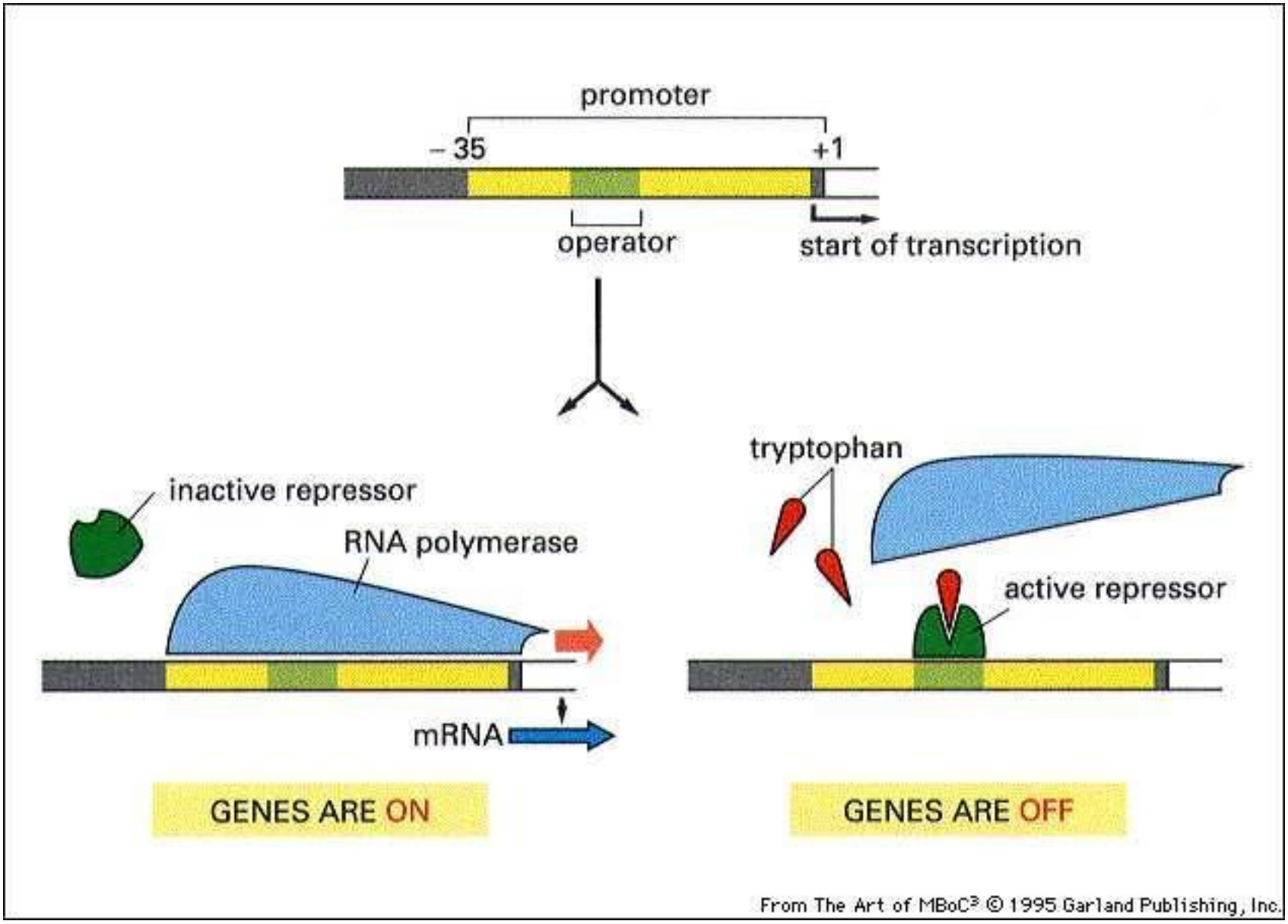


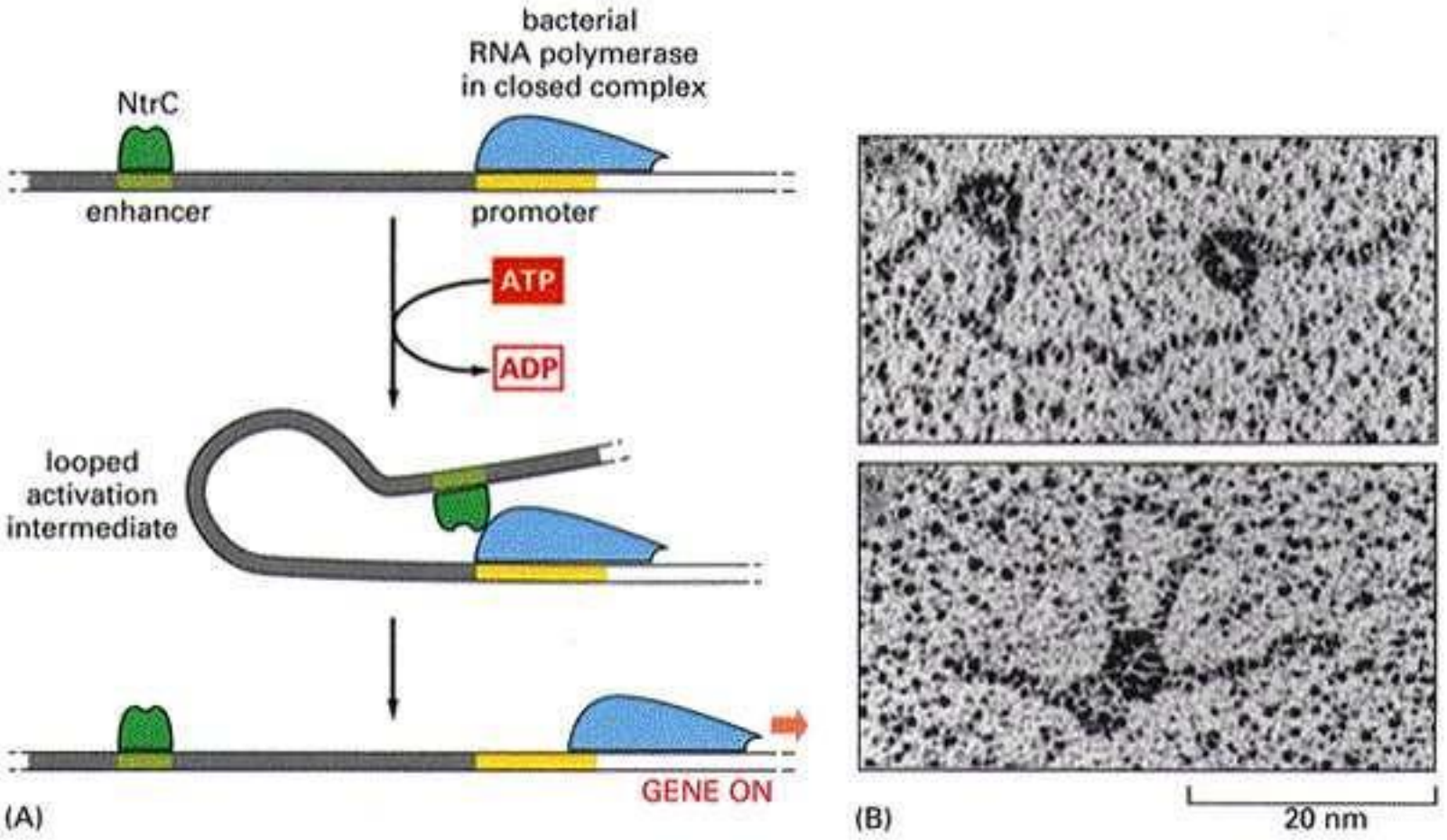
(B) **Eukaryotic promoter site**

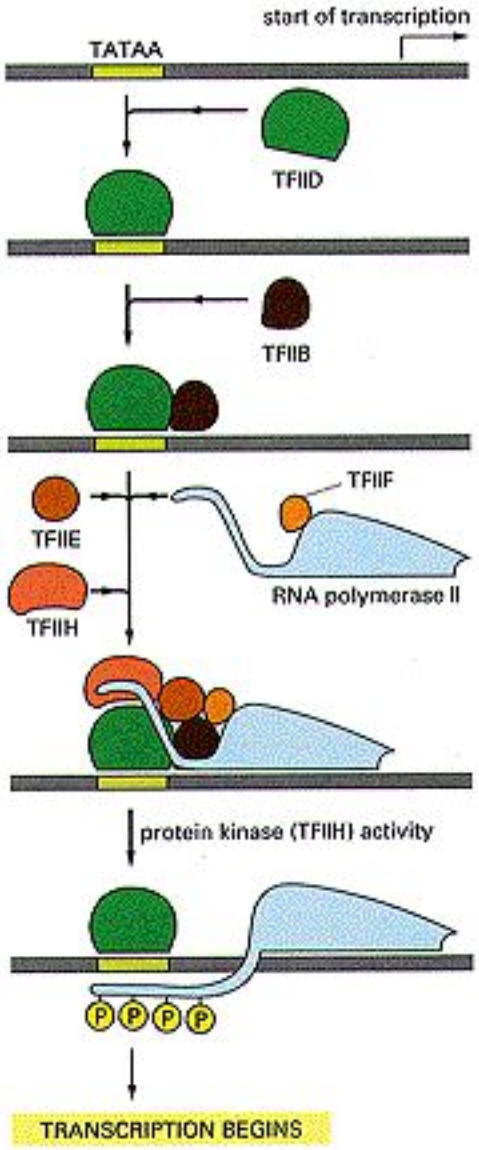

Negative regulation
(bound repressor inhibits transcription)
Positive regulation
(bound activator facilitates transcription)
(a)
Operator
DNA
Promoter
Molecular signal causes dissociation of regulatory protein from DNA
Signal molecule
5′
3′
mRNA
(c)
RNA polymerase
5′
3′
mRNA
(b)
Molecular signal causes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

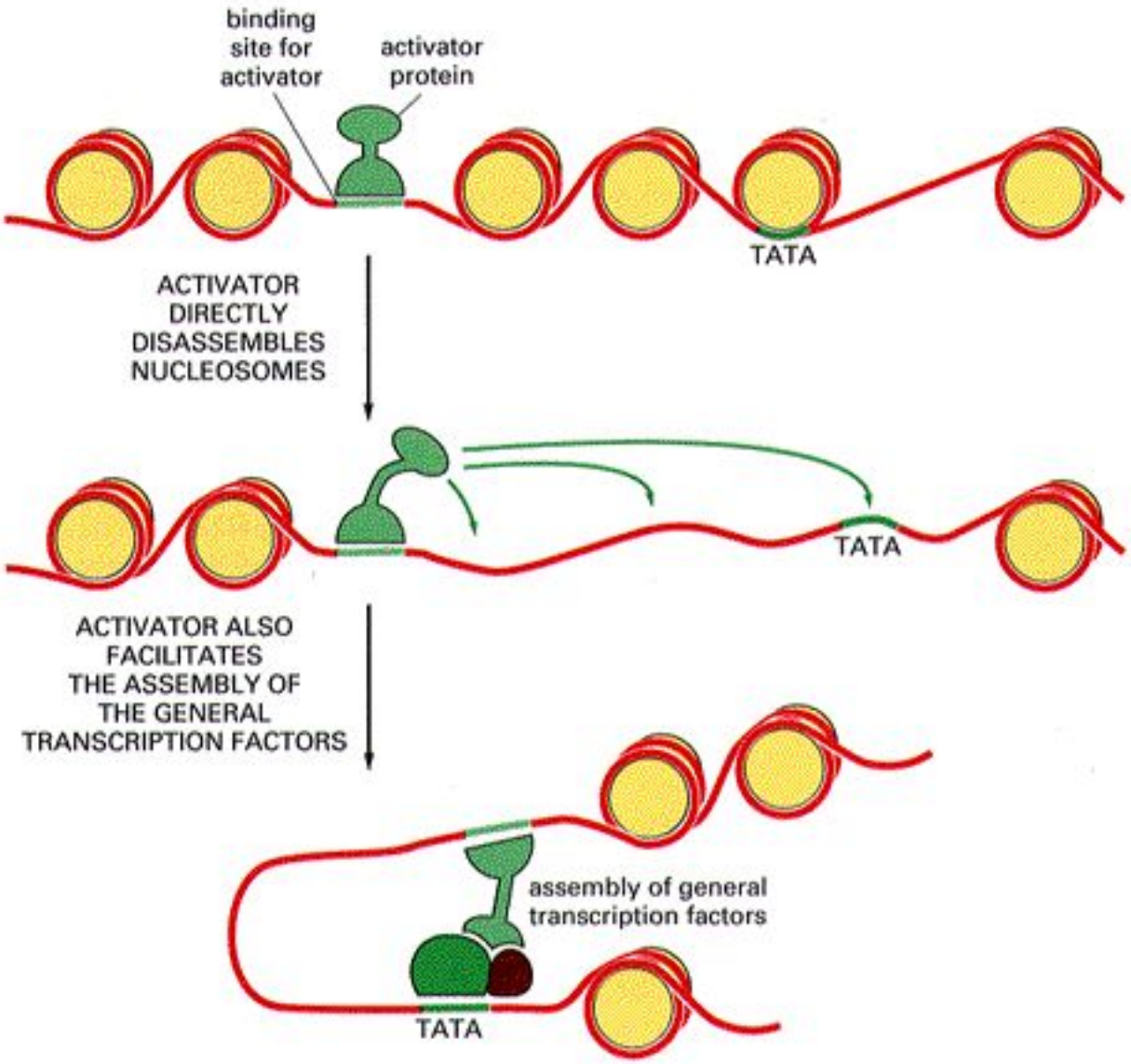
Control of transcription of the *lac* operon.



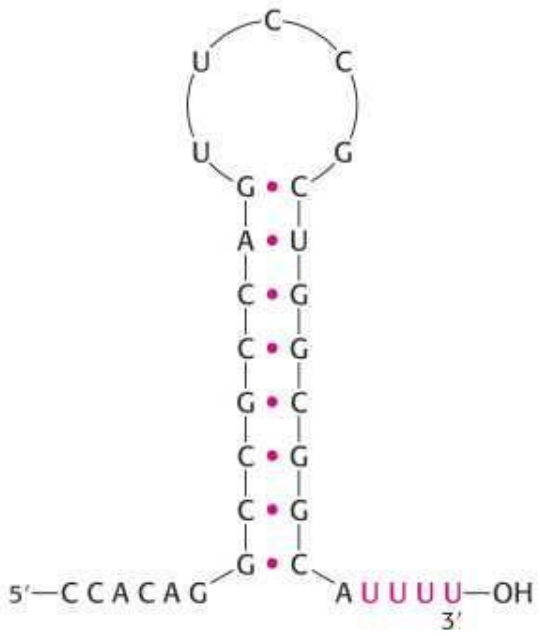


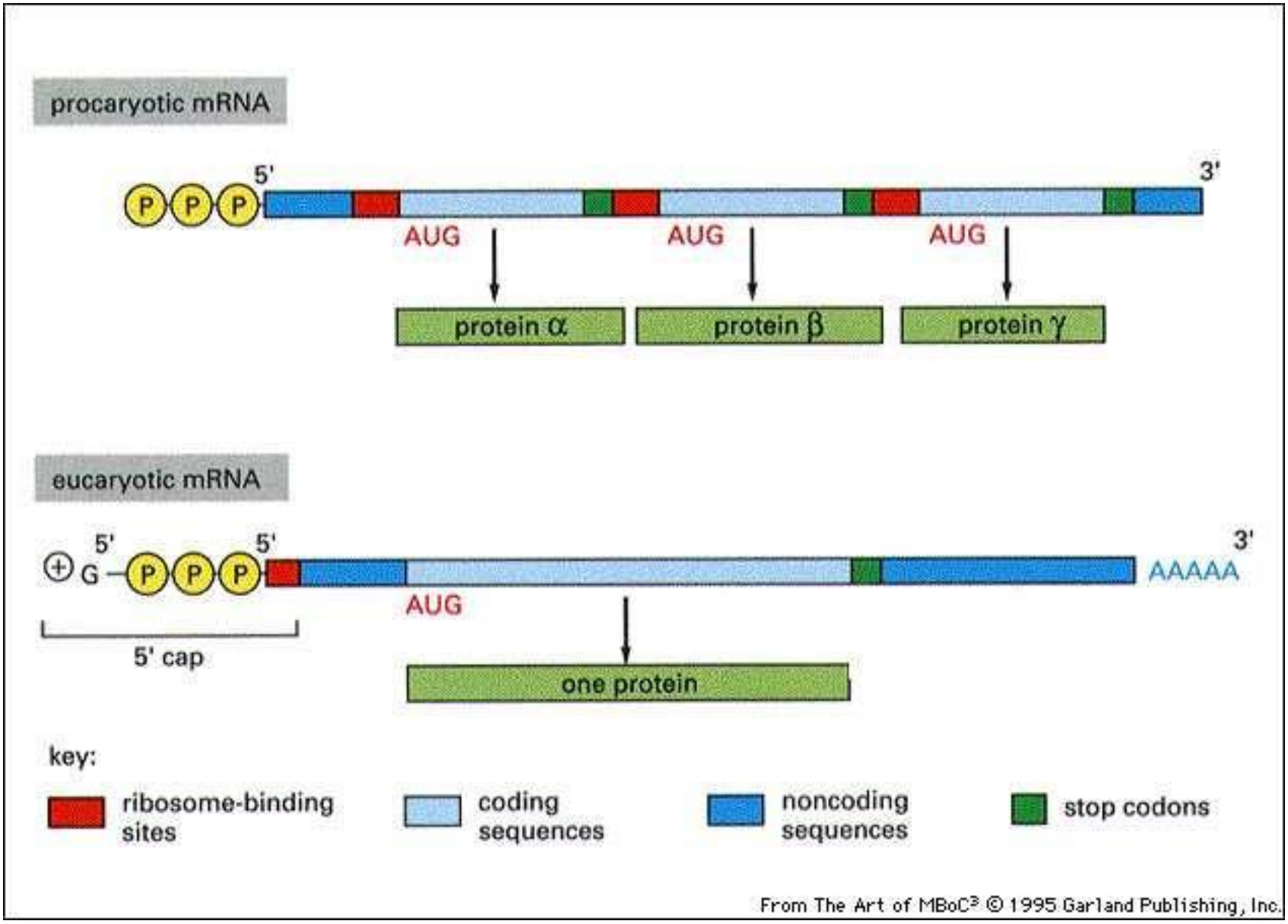


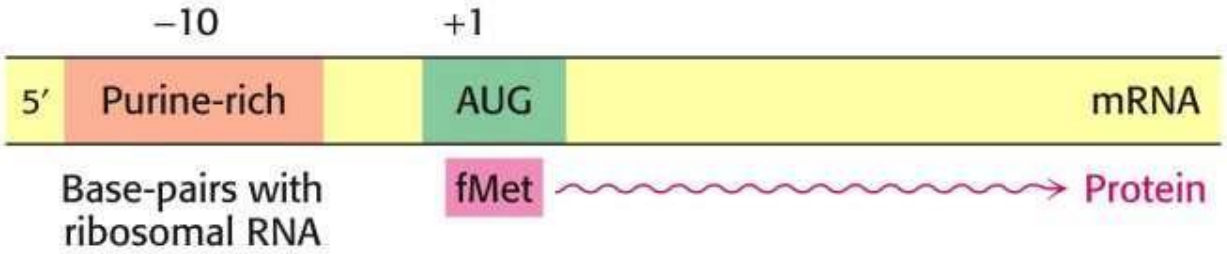




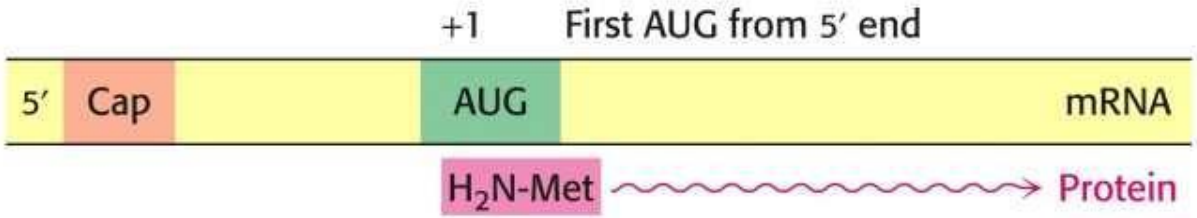
Terminators



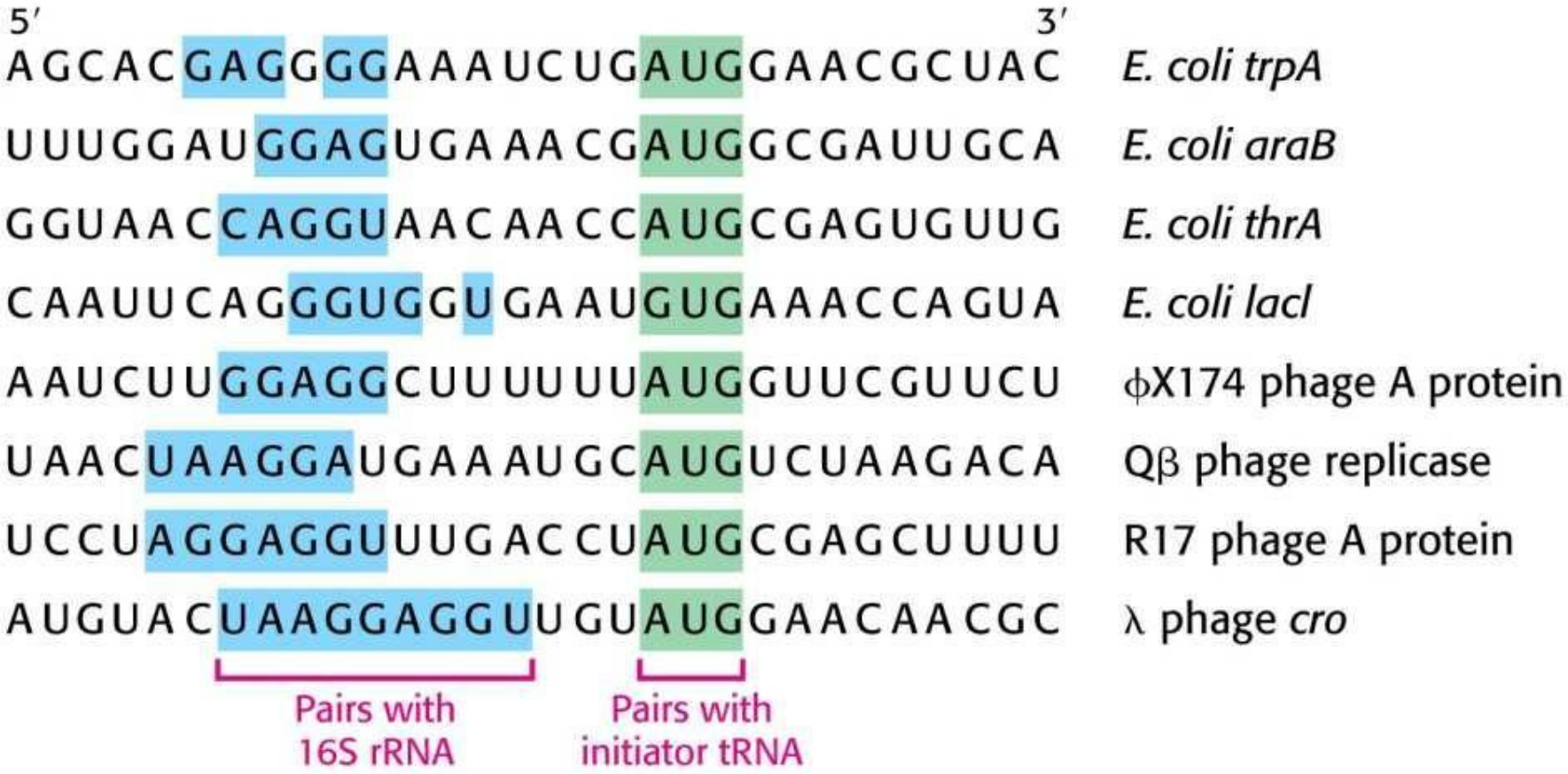




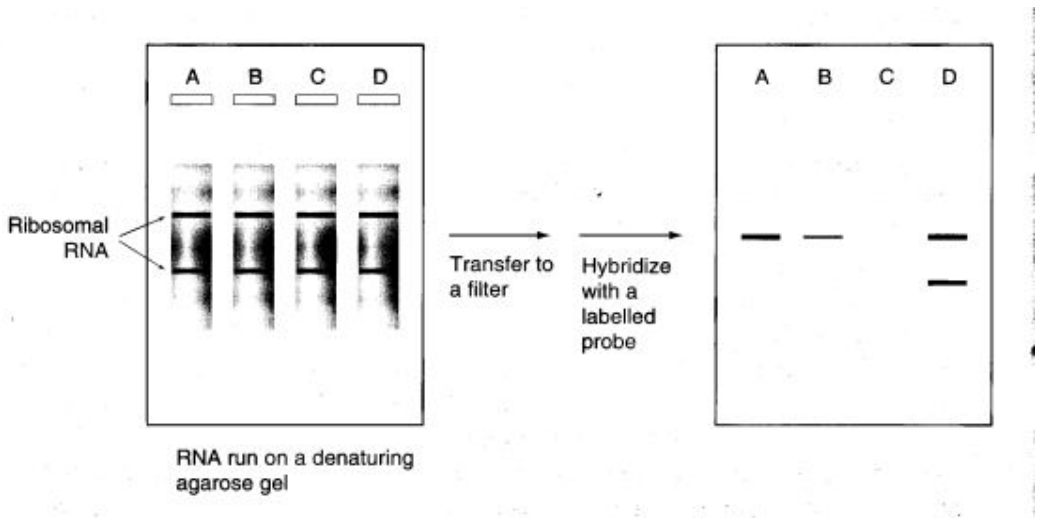
(A) **Prokaryotic start signal**

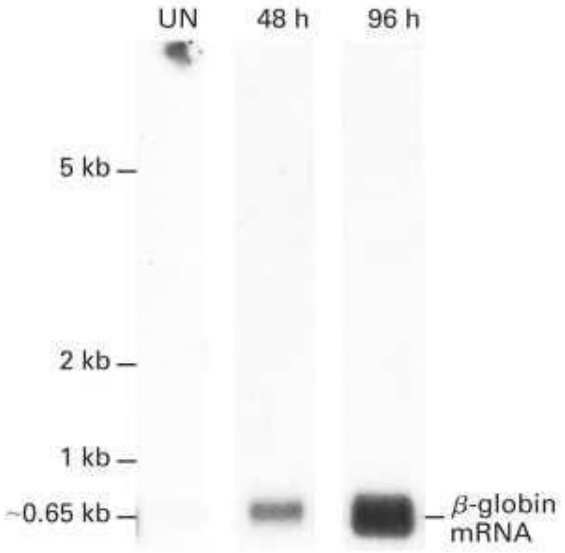


(B) **Eukaryotic start signal**


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