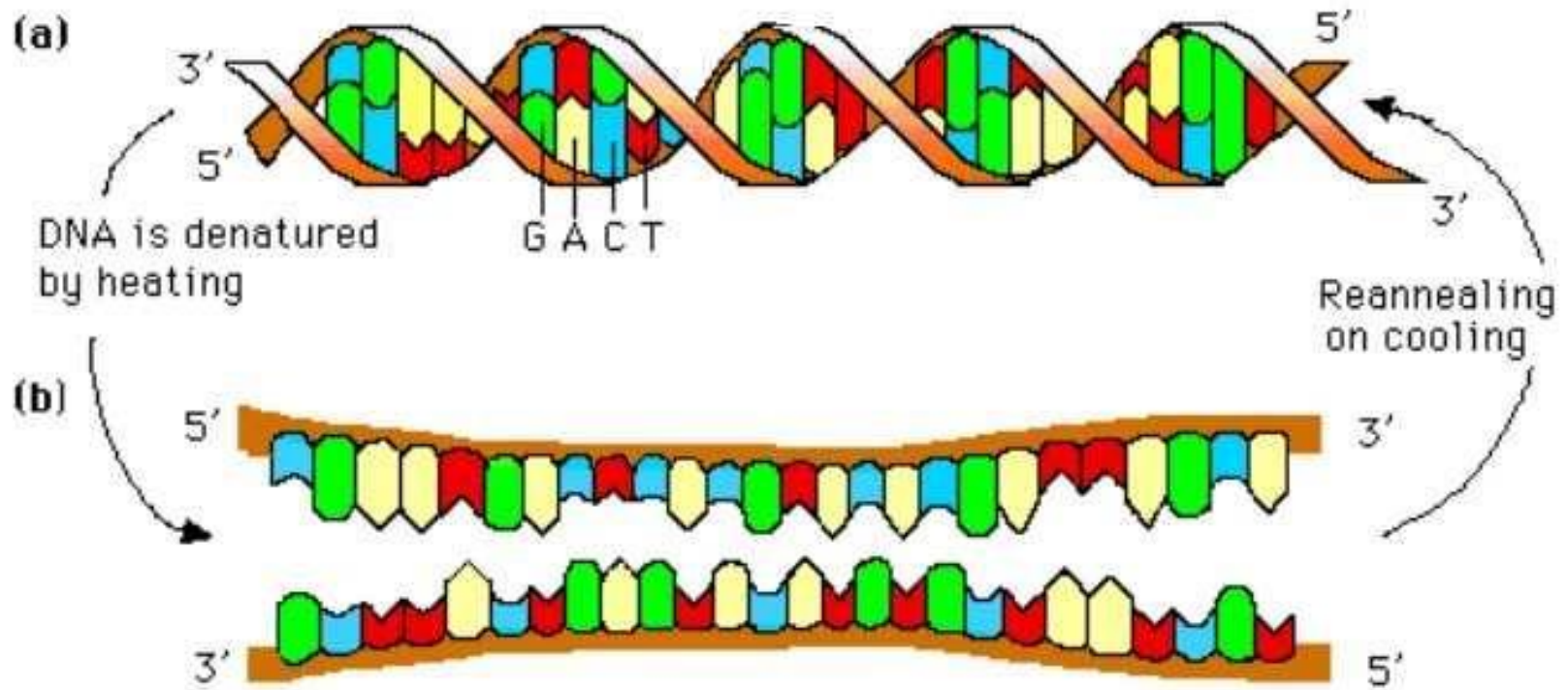
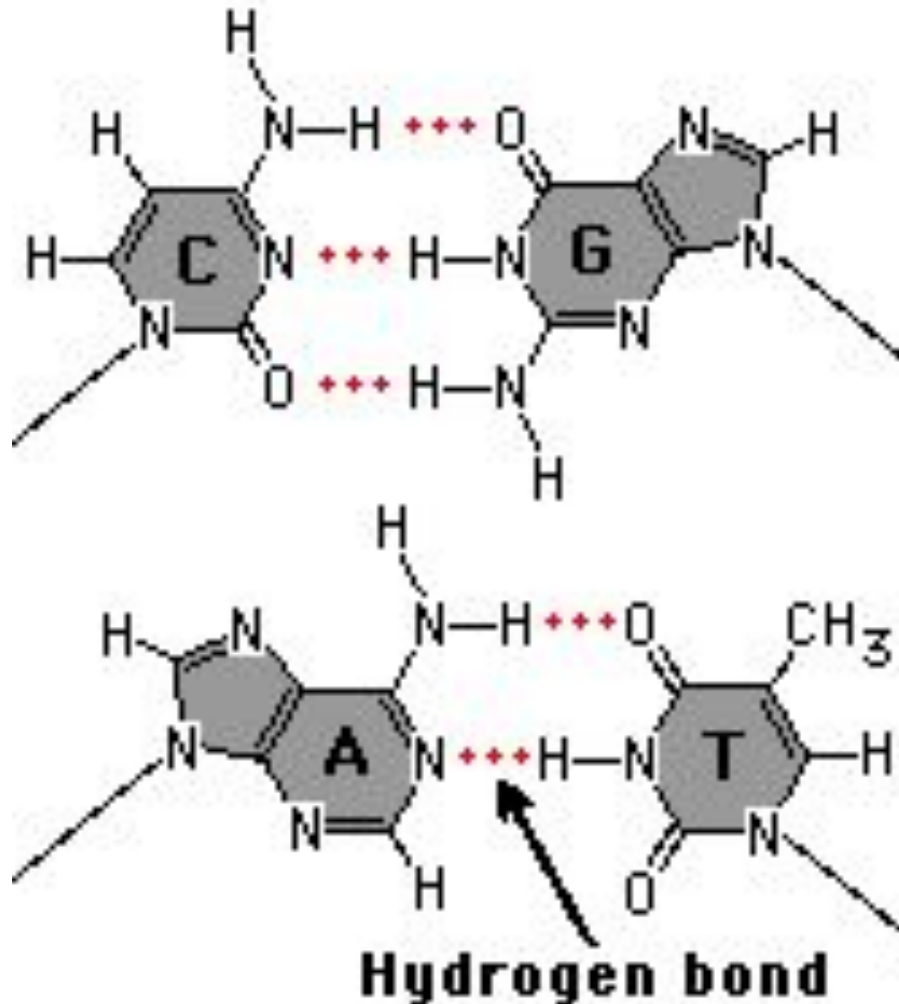


1. Hybridization (DNA-DNA or DNA-RNA)



HYBRIDIZATION? –

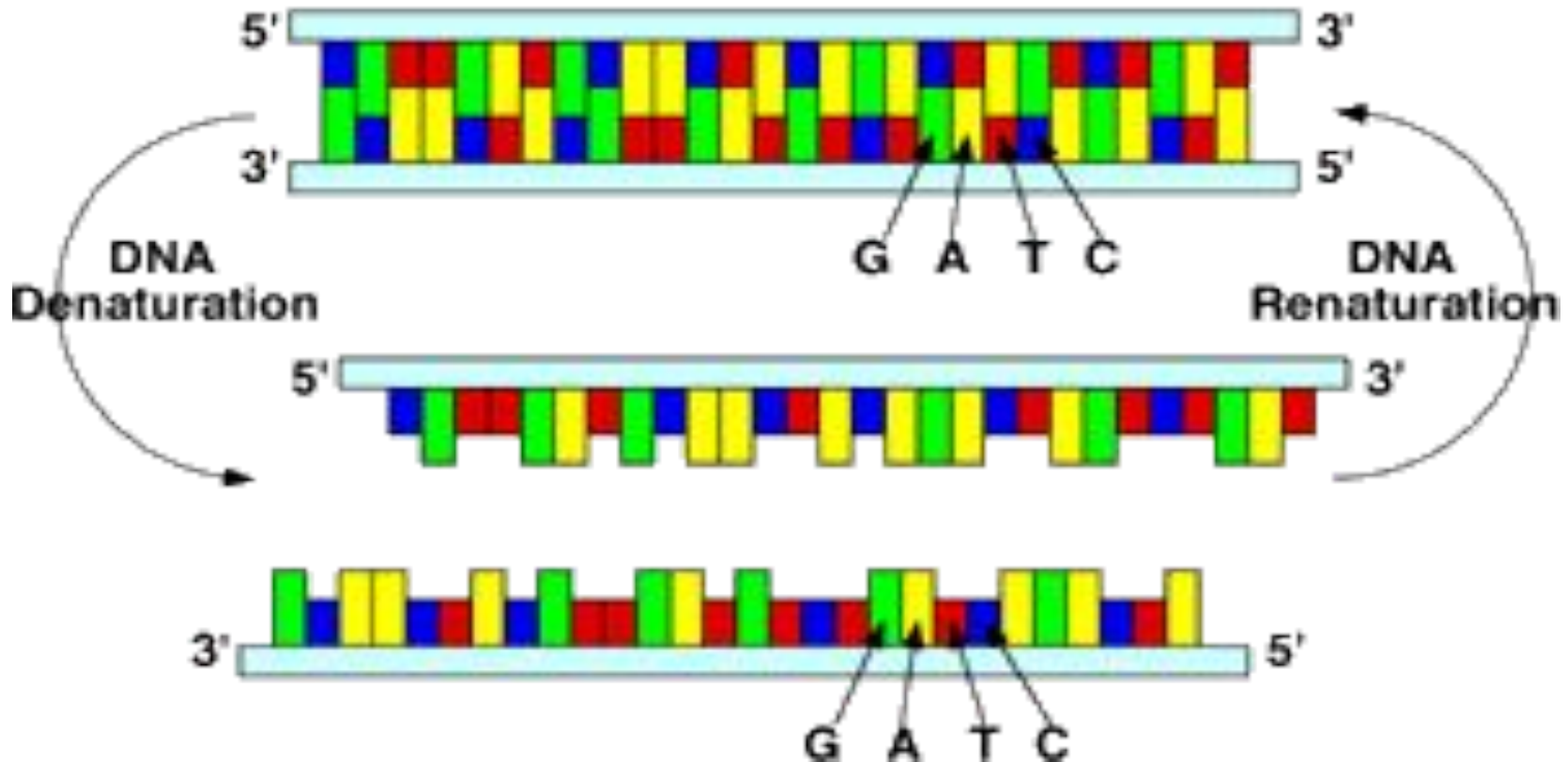
Yes, it is about this familiar picture



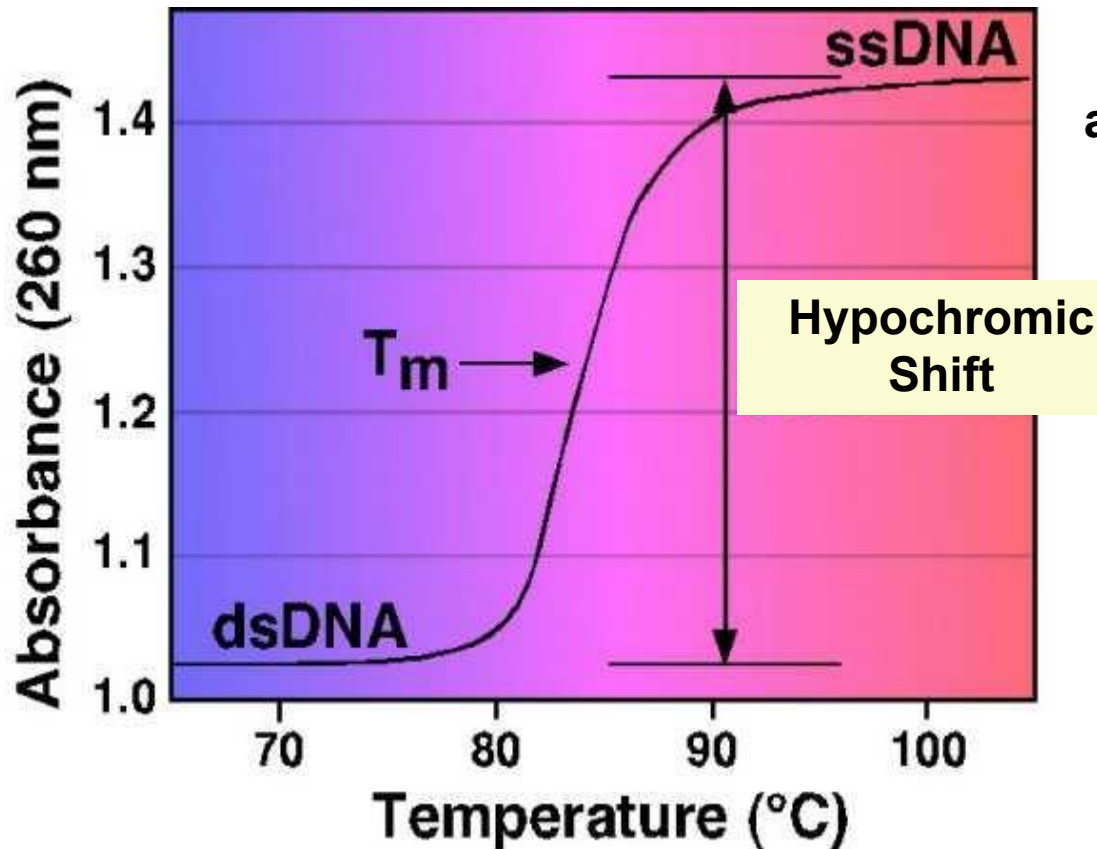
We can denature and renature DNA by heating/cooling

Denaturation/Renaturation

DNA



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

T_m (melting temp)

The temperature at which DNA is half unfolded

T_m is a measure of the stability of dsDNA under a given set of conditions

T_m of DNA is affected by:

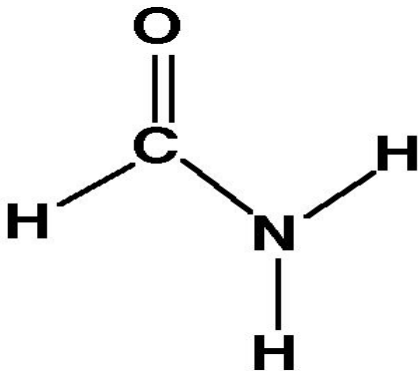
1. Base Composition :

higher the GC content, the higher the T_m.

2. Ionic Strength :

as the ionic strength increases, so does T_m.
Double helical DNA is stabilized by cations.

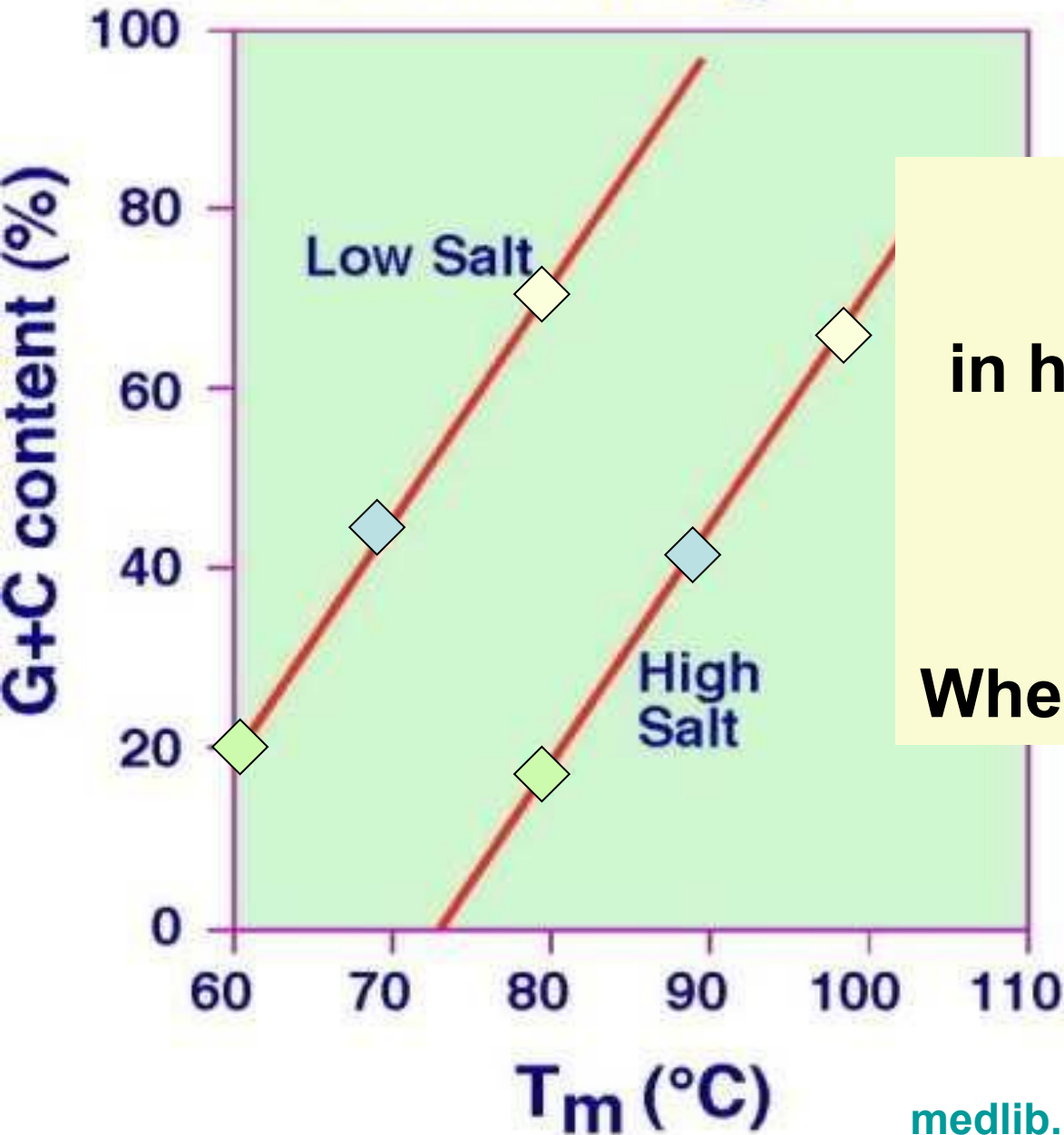
Divalent cations (Mg²⁺) are more effective
than monovalent cations (Na⁺ or K⁺).



3. Organic Solvents –

formamide for instance
lowers the T_m by weakening
the hydrophobic interactions.

T_m depends on G+C content and ionic strength

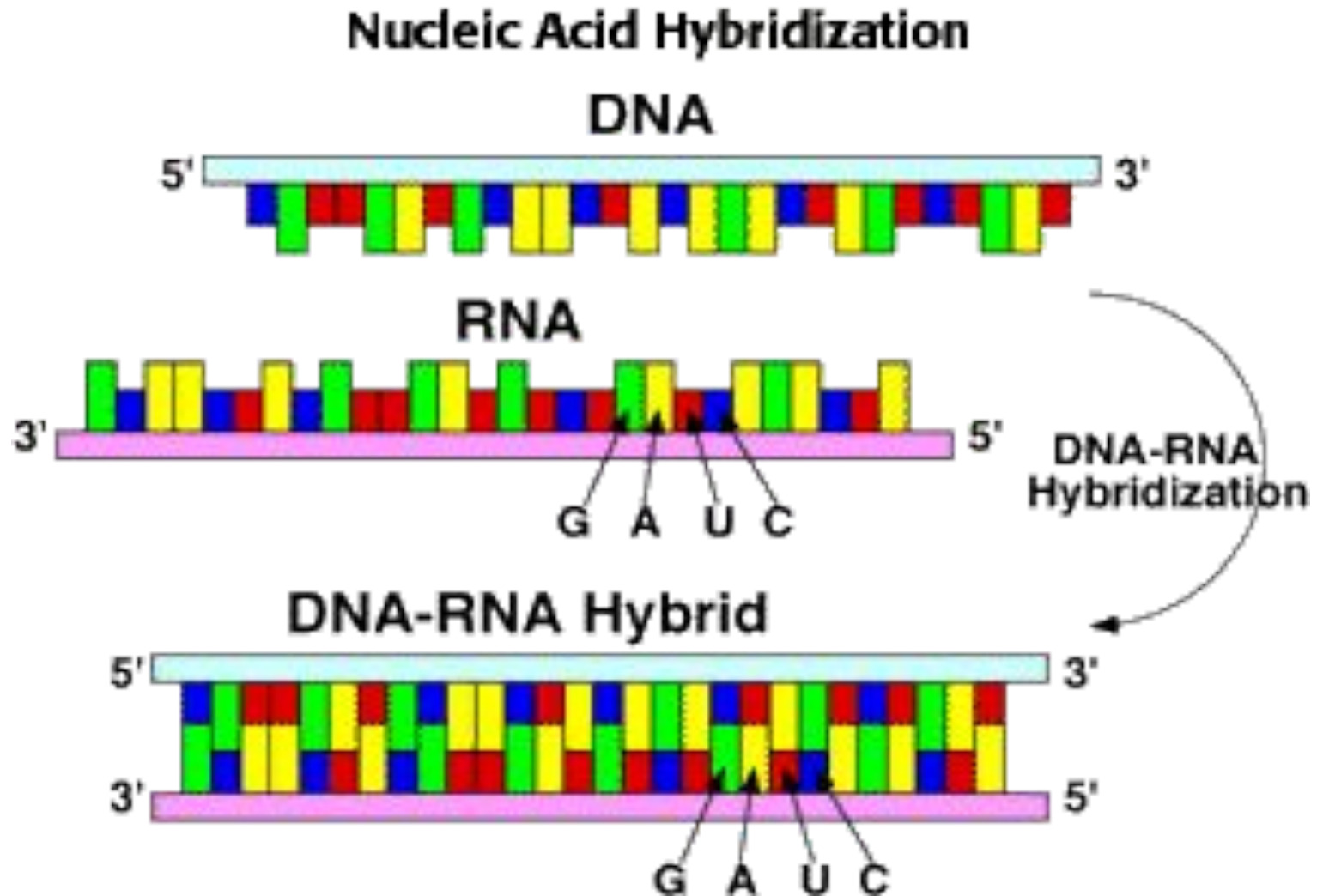


**DNA
more STABLE
in high-salt conditions.**

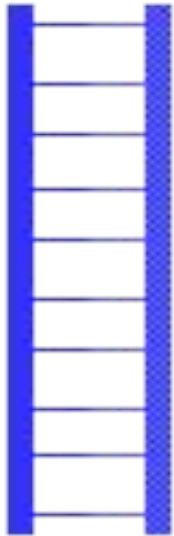
**DNA
more STABLE
When contains many GC**

RNA can bind DNA

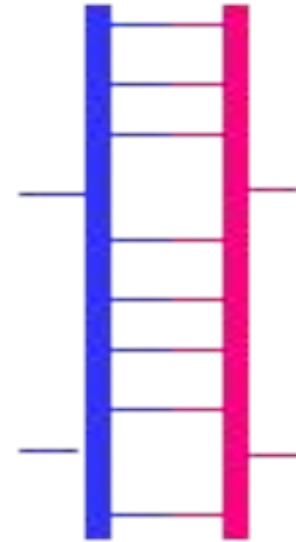
(U is equivalent of T in hybridization)



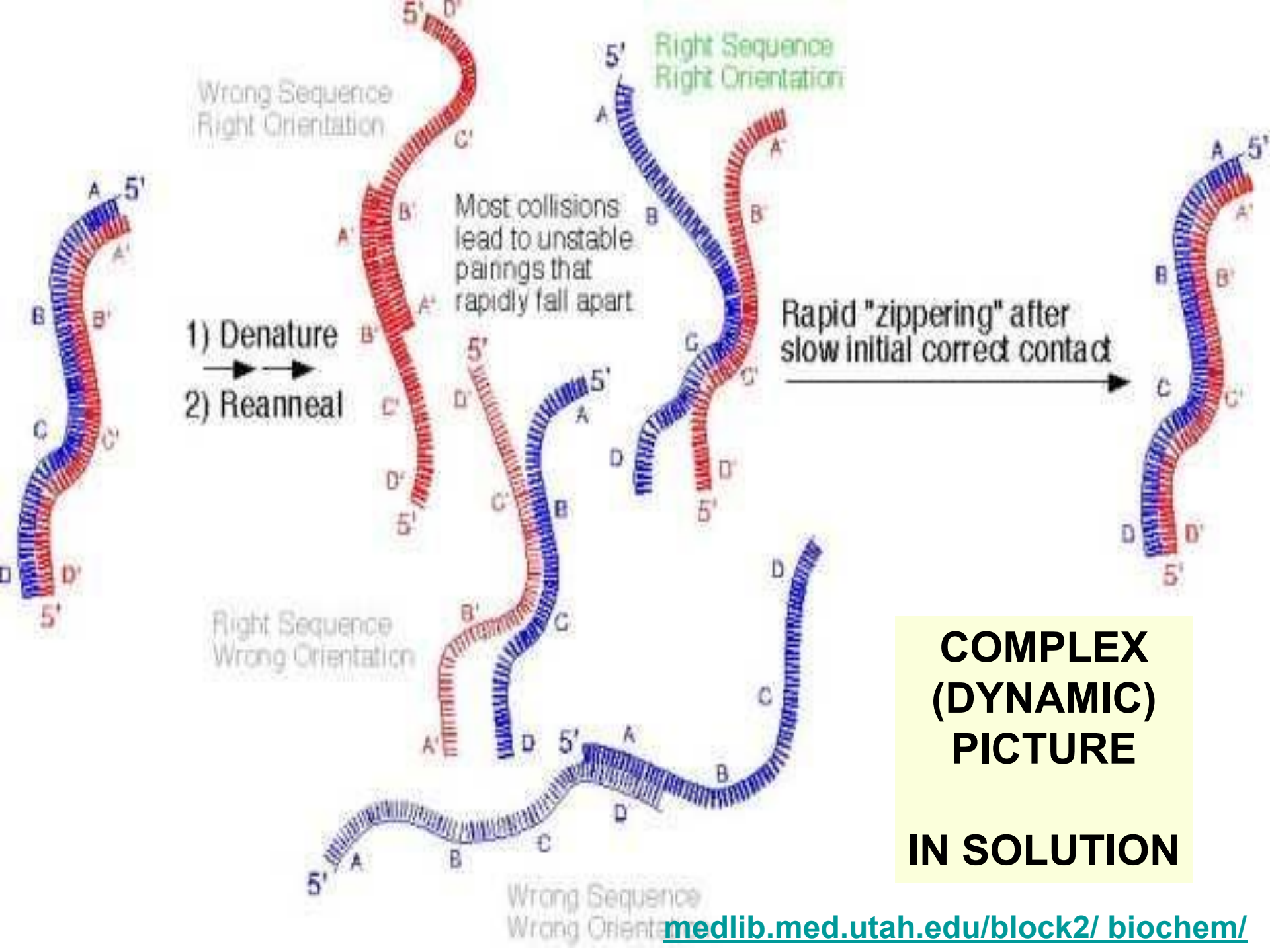
Hybridization could be less than perfect



Homoduplex DNA,
both strands from
the same species



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

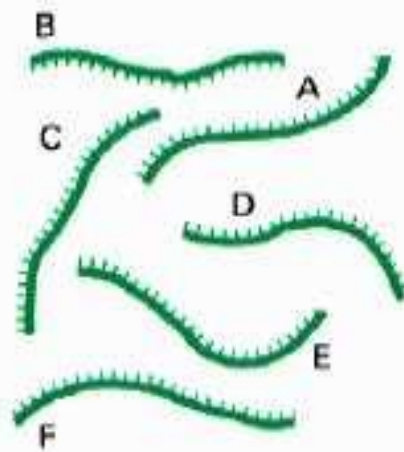


**COMPLEX
(DYNAMIC)
PICTURE
IN SOLUTION**

Single-stranded DNA probes for gene A

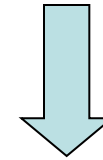


+



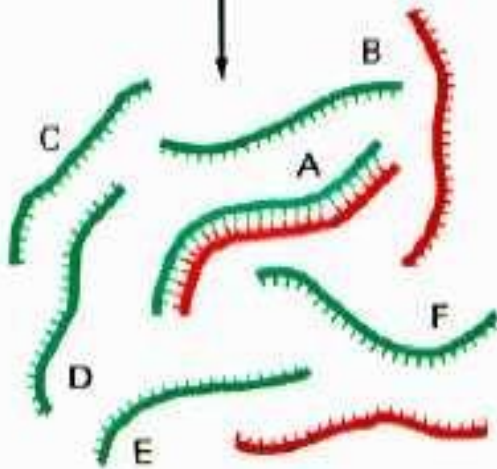
Mixture of single-stranded DNA molecules

42 C
is more stringent
condition
than 35 C



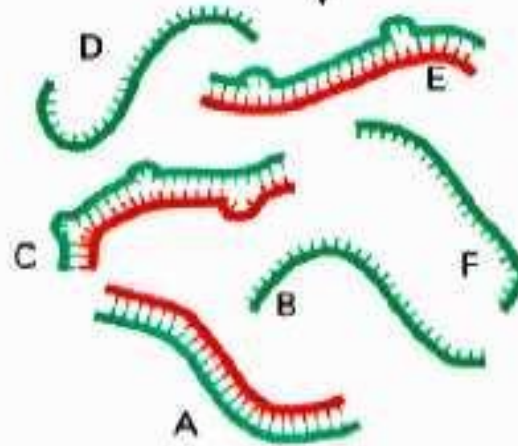
(hybridization
is more specific)

Hybridization in
50% formamide
at 42°C



Only A forms stable
double helix

Hybridization in
50% formamide
at 35°C



A, C, and E all form
stable double helices

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

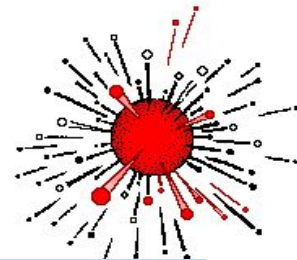
Specific probe self-anneals to target DNA

Only problem – DNA is invisible

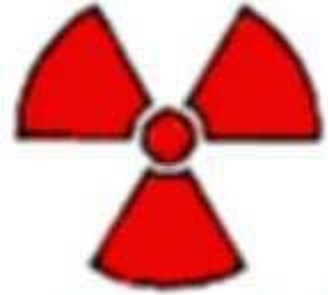
How to visualize DNA?



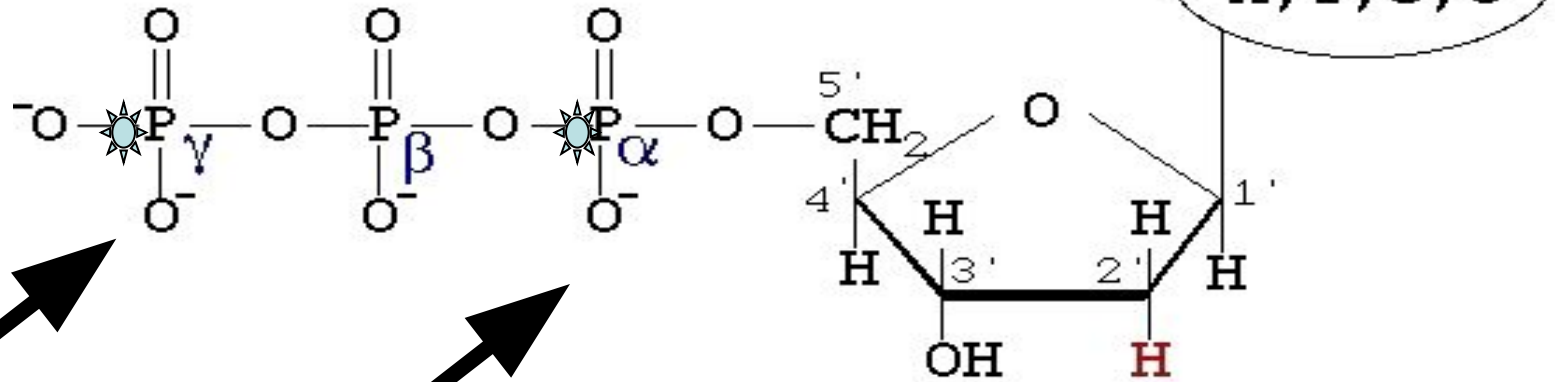
Radioactively



Fluorescently



Polymerase labeling of DNA

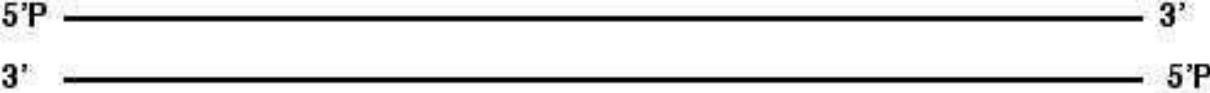


Gamma-33-ATP

Alpha-32-ATP

dNTP

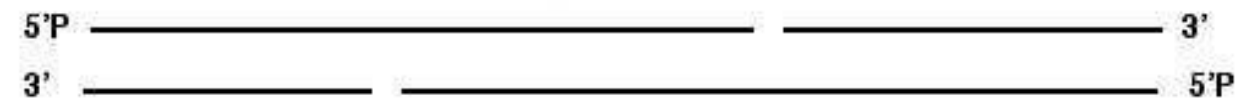




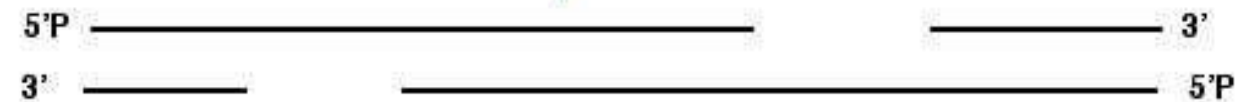
DNase I

Labeling by
NICK TRANSLATION

Will work without DNase,
as there are always nicks in DNA

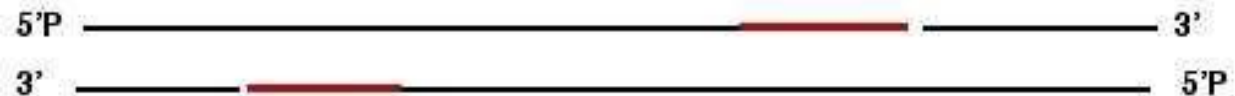


**Polymerase I
(exonuclease activity)**



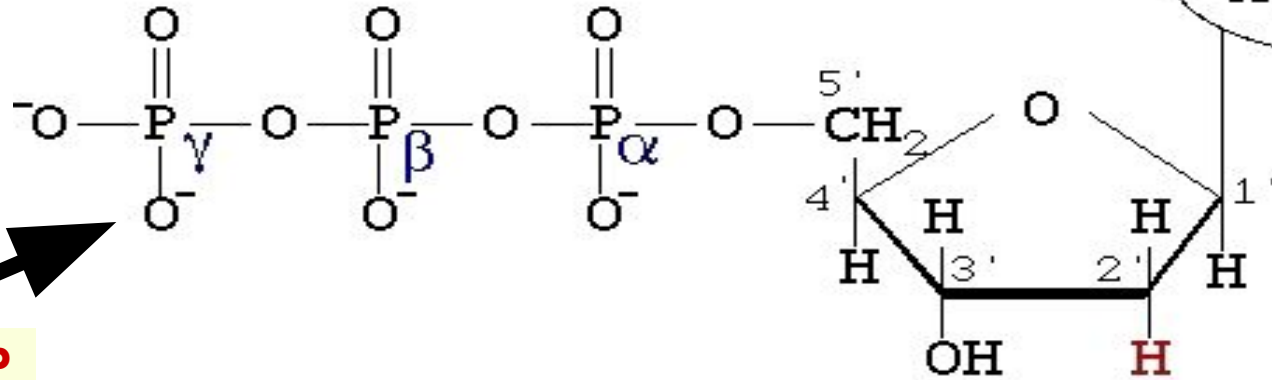
**Polymerase I
(polymerase activity)**

+ dTTP
+ dCTP
+ dGTP
+ dAT³²P

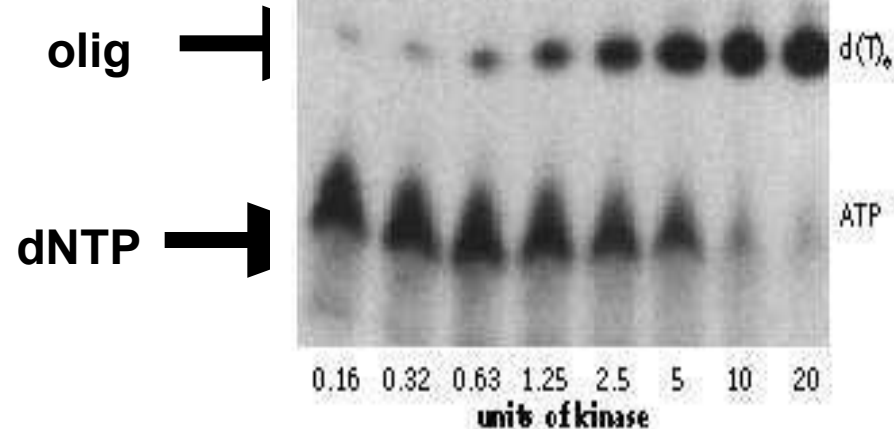
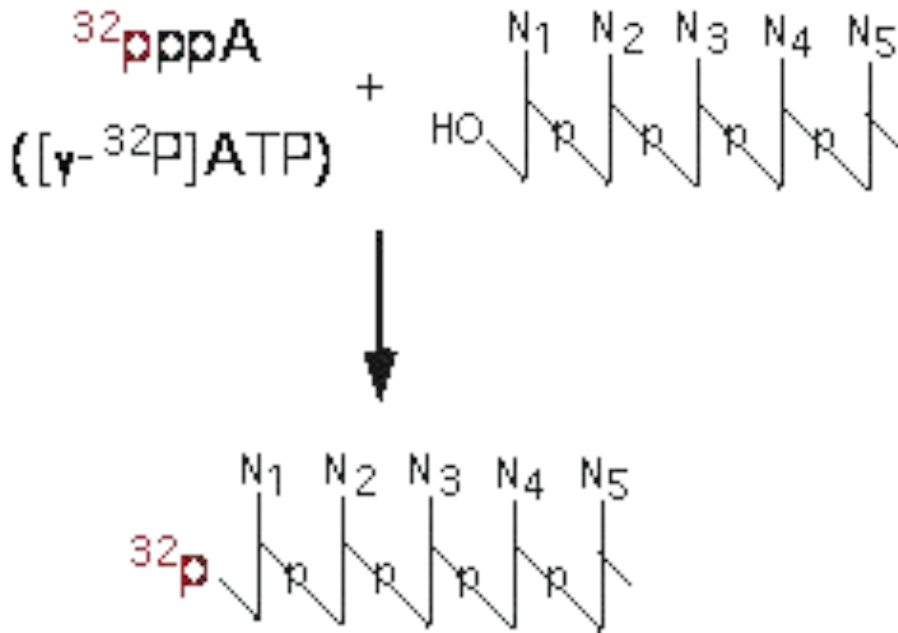


T4 polynucleotide kinase labeling

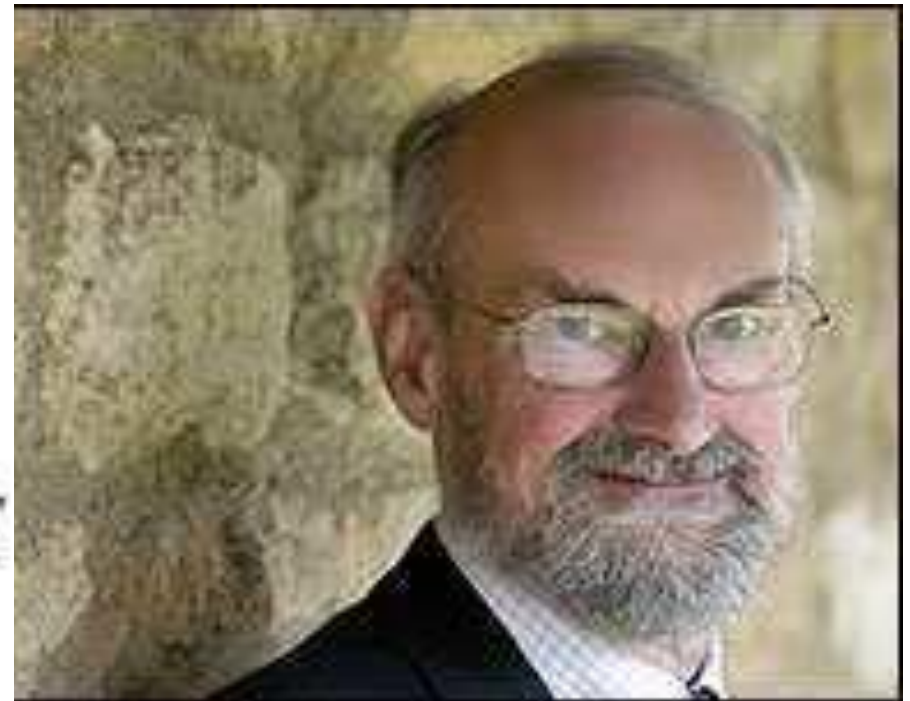
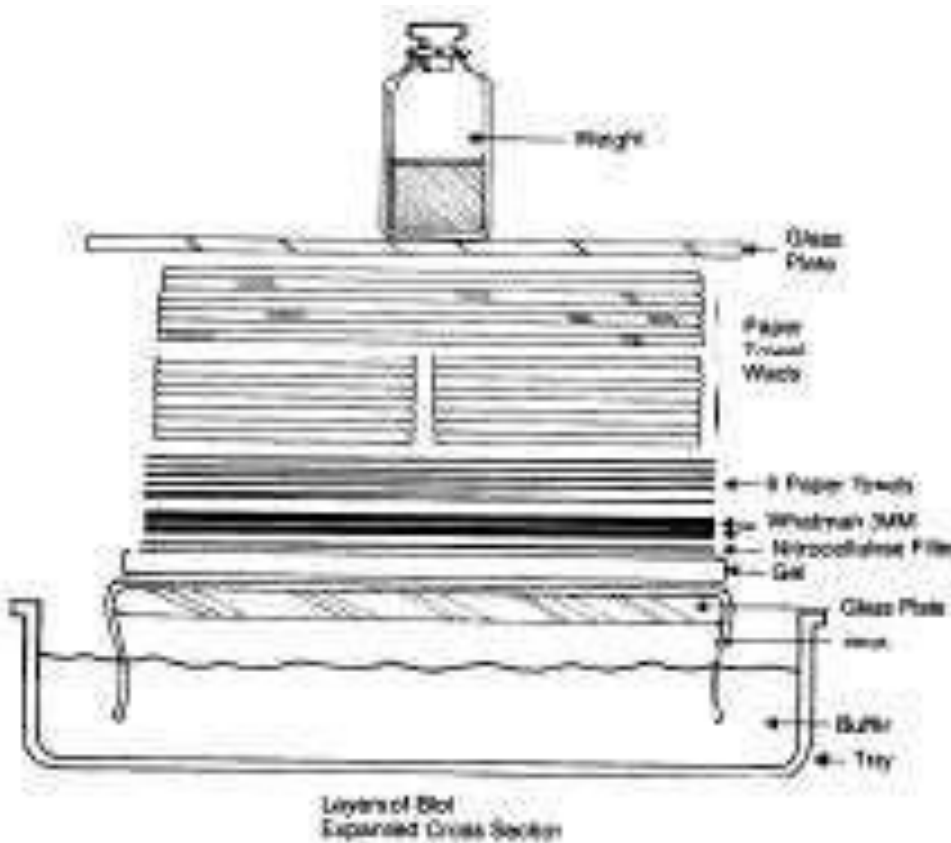
Used for oligonucleotide labeling

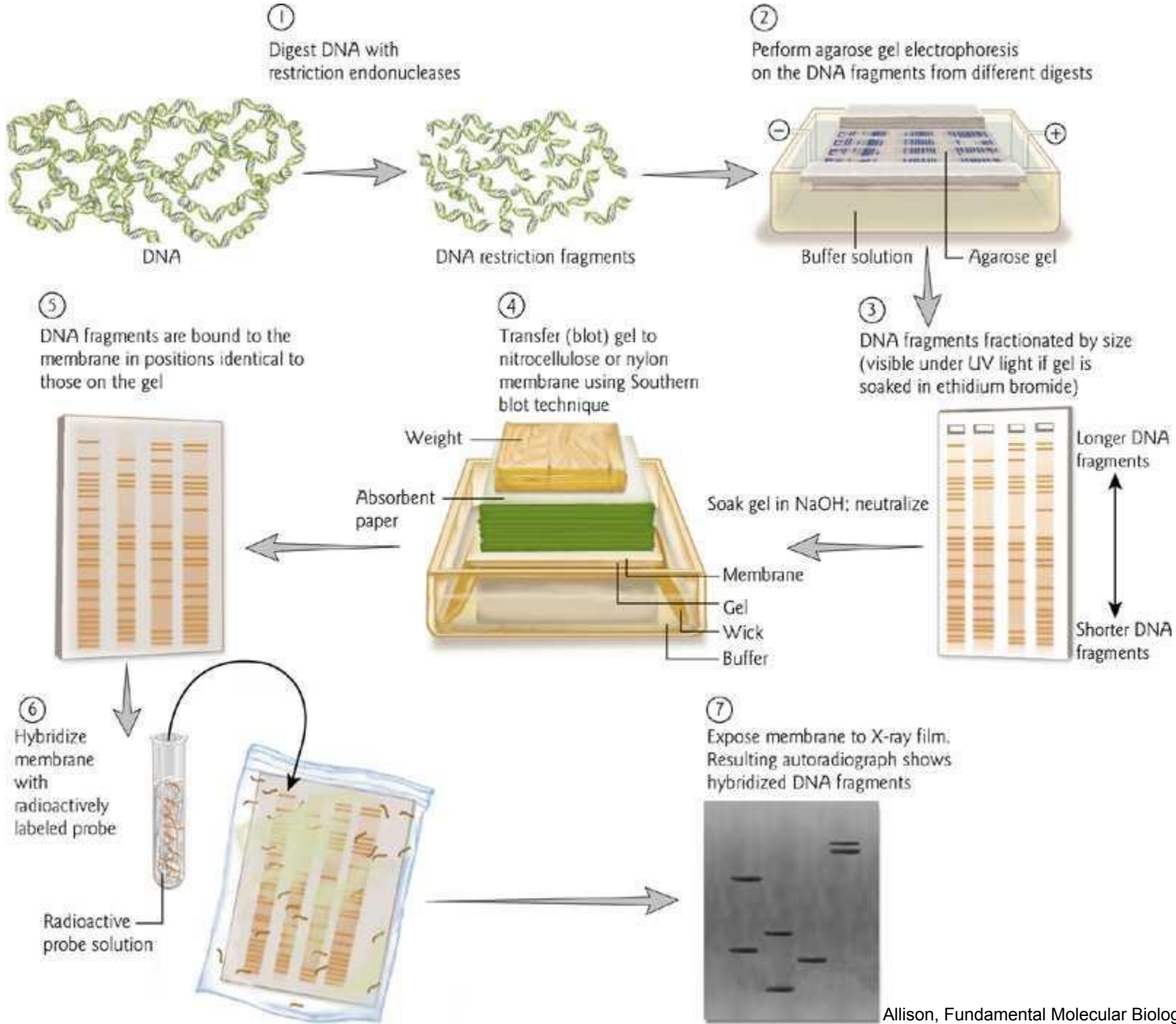


Gamma-33-ATP

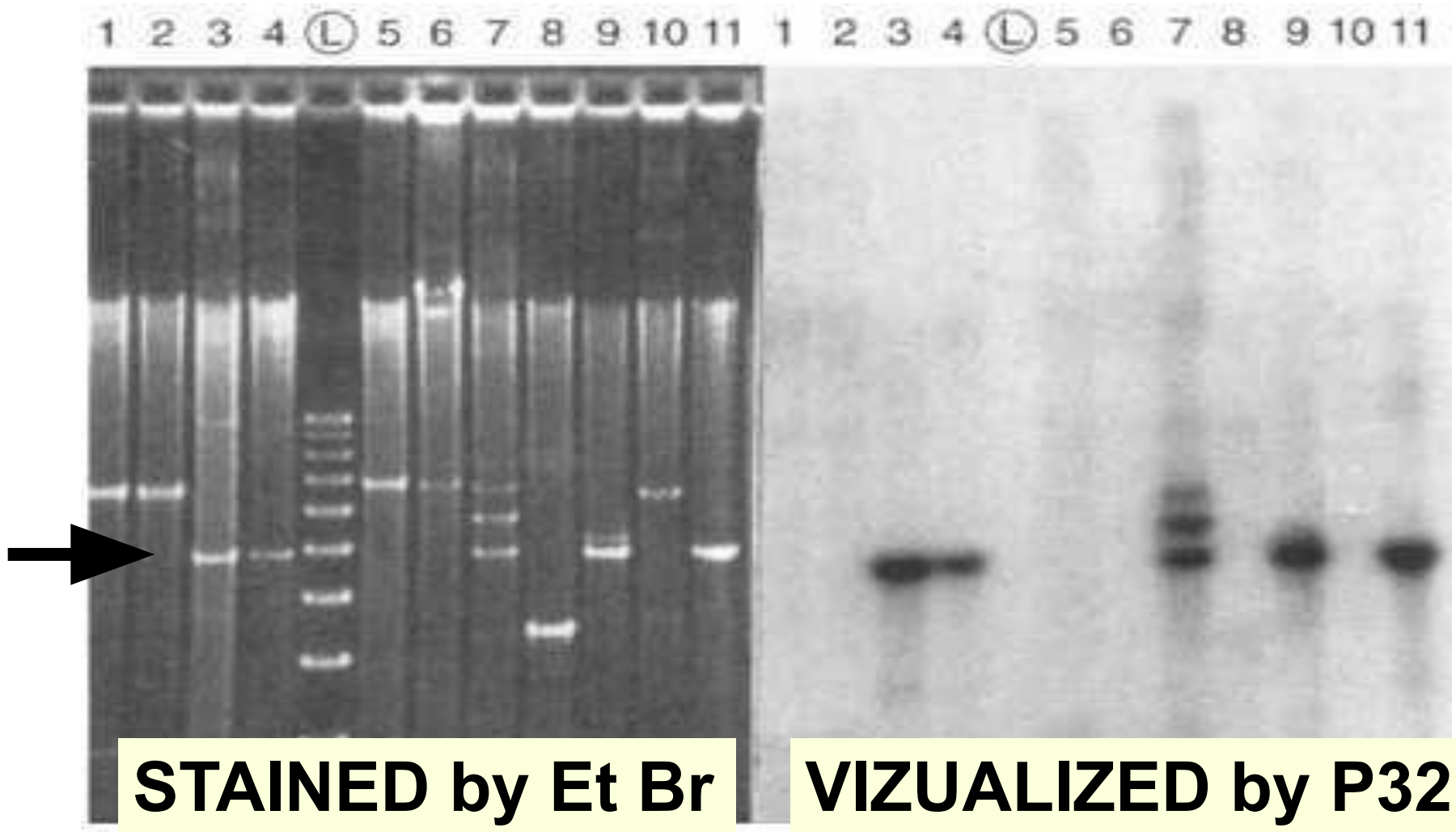


Professor Sir Ed Southern, Whitley Professor of Biochemistry at the University of Oxford

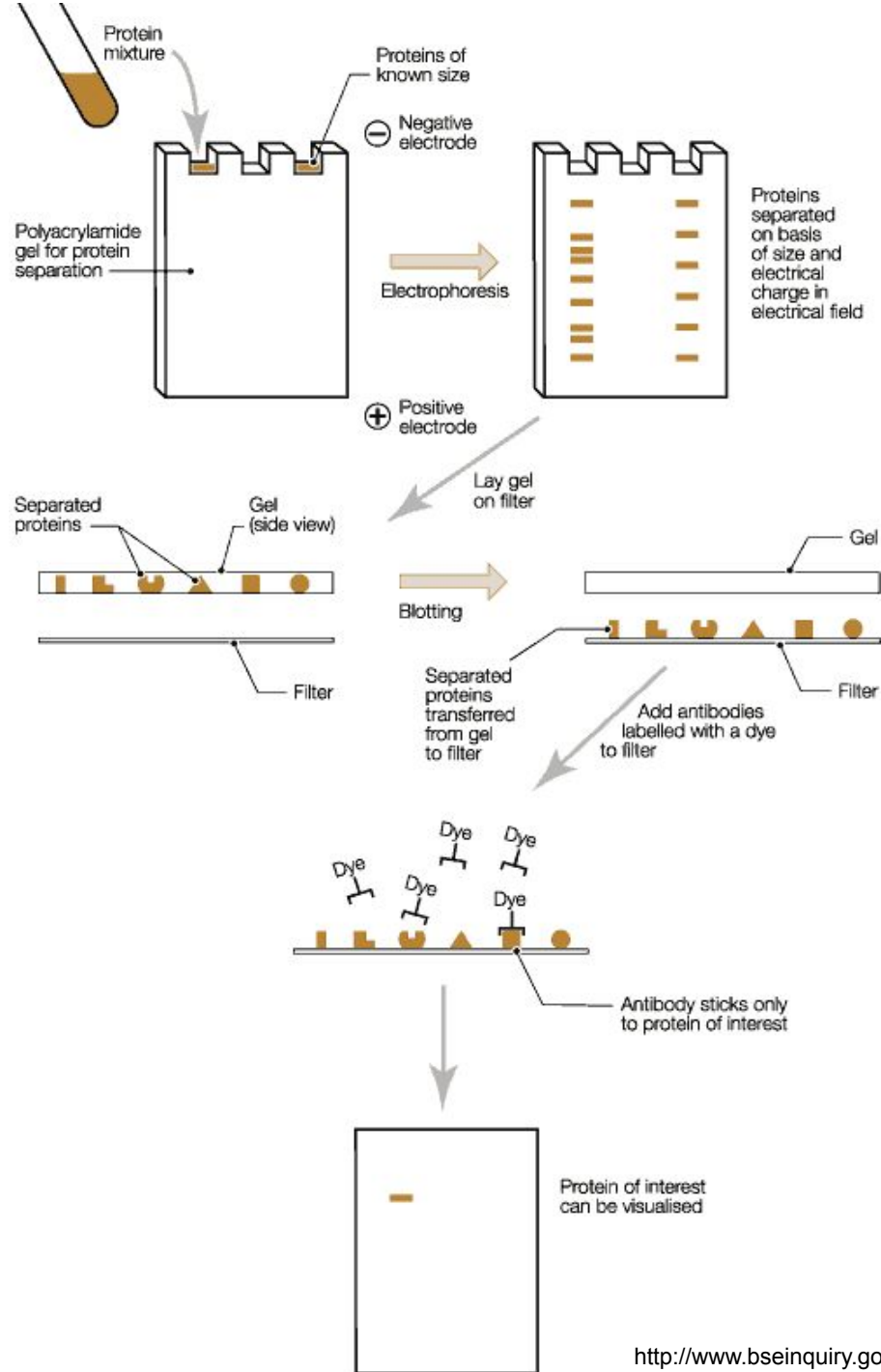




“Real” Southern blot (DNA-DNA blot)

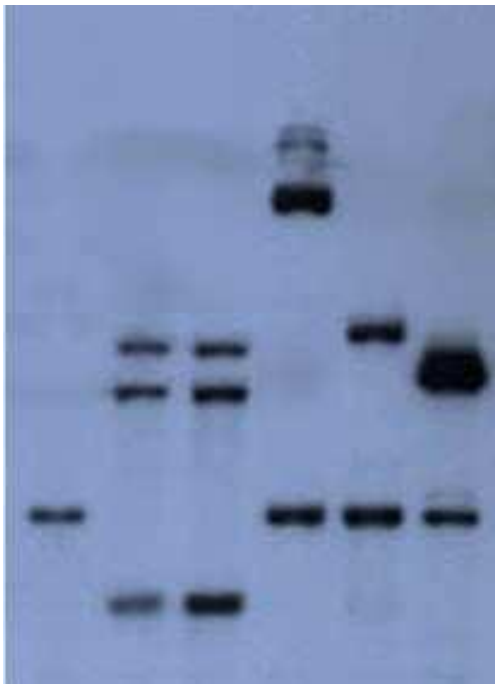


Western Blot



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

Southern



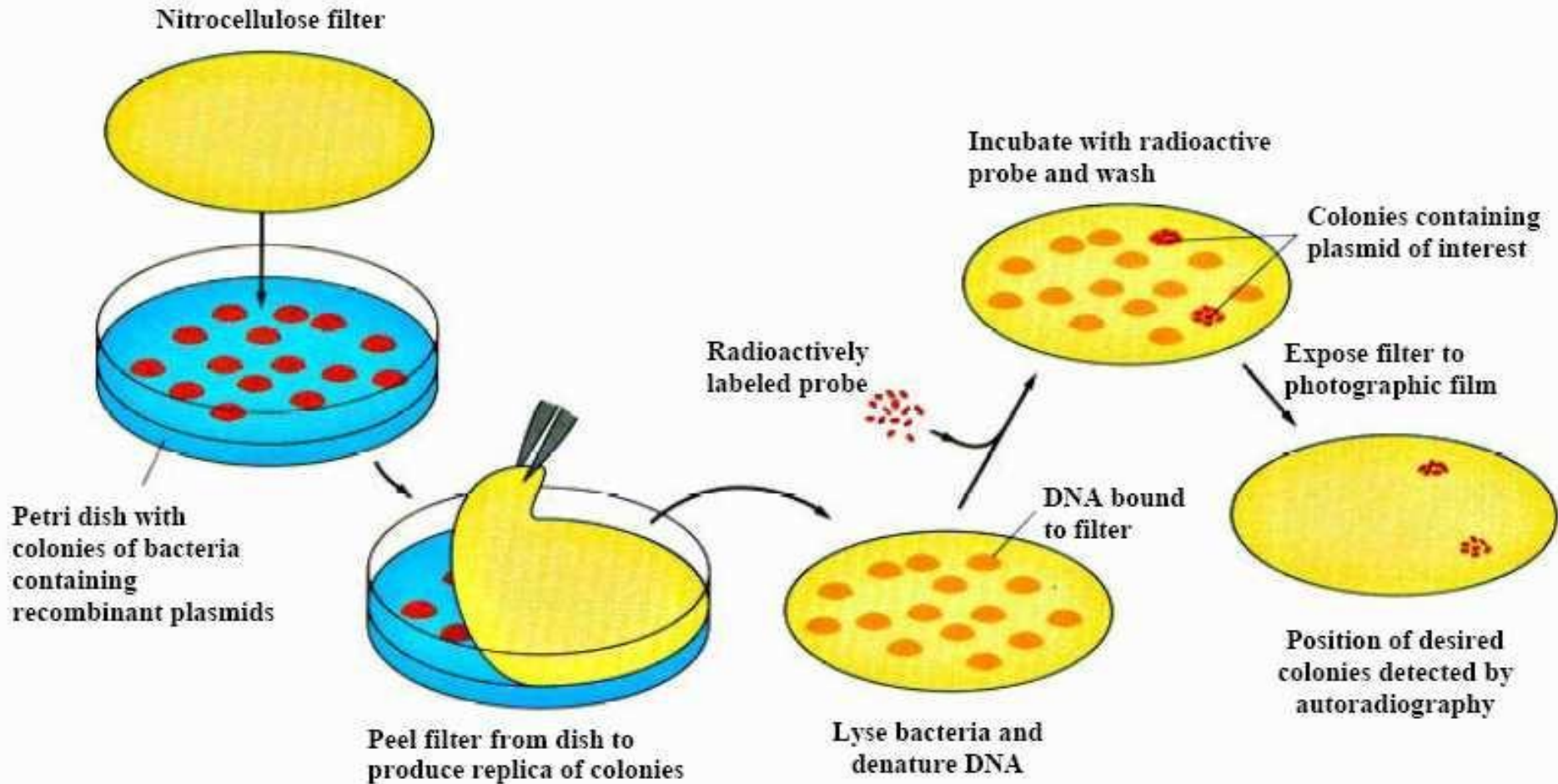
northern



western



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



Same for arrayed clones

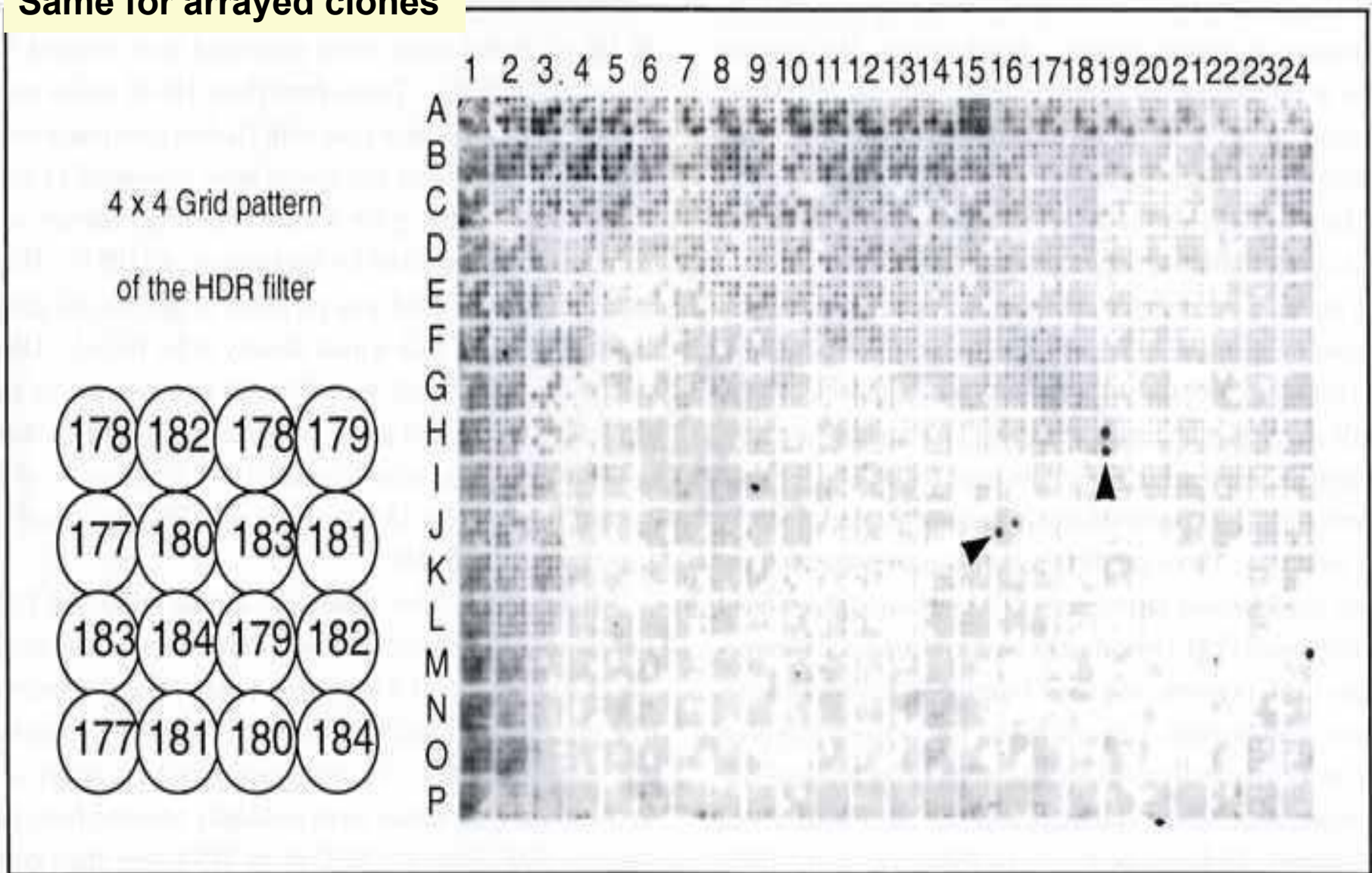
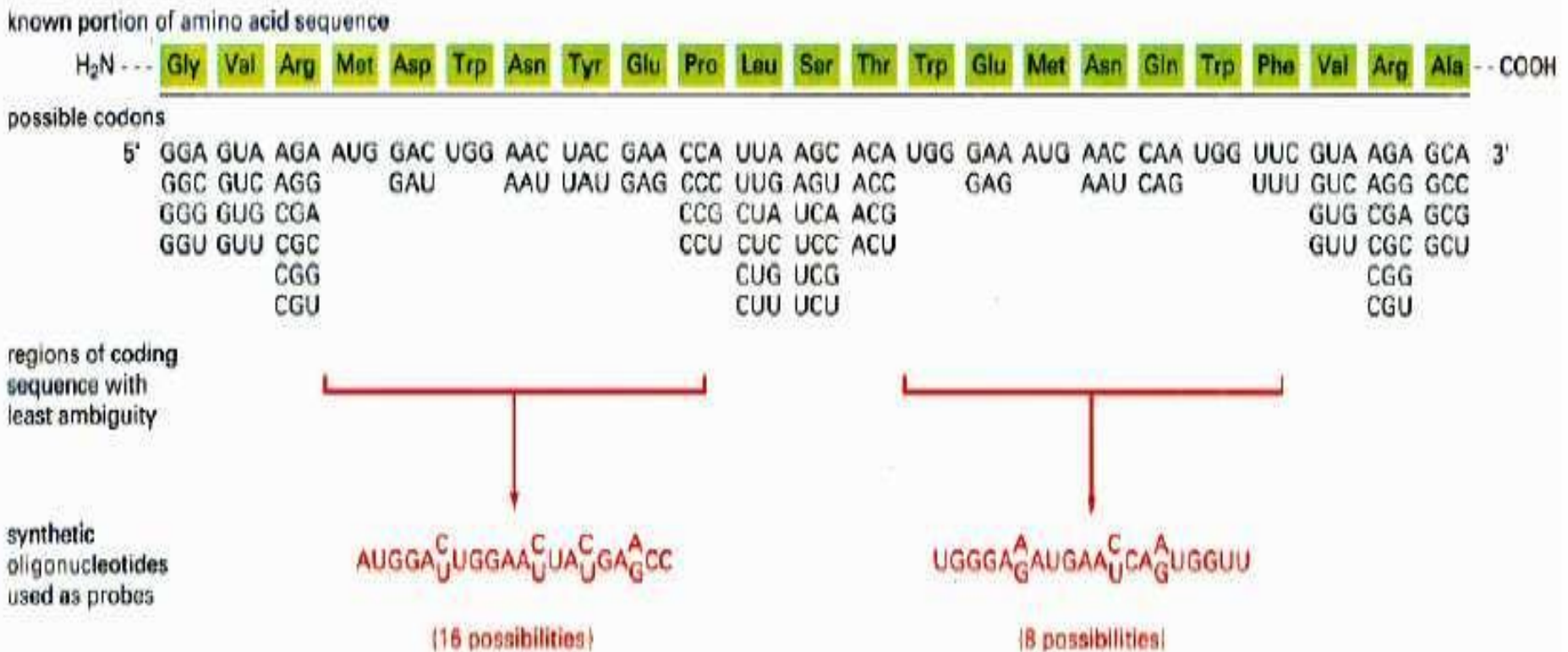


Fig. 2. Library screening by hybridization. Colony hybridization to a high density colony filter using the sequence of the single-copy gene *SLI* as a probe. 3072 BAC clones were gridded on a membrane with a 4 x 4 double offset pattern. Positive clones were detected as doublet signals.

Design of degenerated synthetic hybridization probes (for already known proteins only)



The **degeneracy** of a primer is the number of unique sequences it corresponds to (5 in one of the examples below).

TTGATTCTTAAGA.....CCAGTGTAGC
GGATTGTAGGCT.....ACTGAGGAGTTAT
CTGGACTGTTGCCA.....CGGGC GGAGTAC
GAGATTCTGCATC.....CTAGTGT C GGTAGC
AGACTCTTAAGA.....AGAGAGGAGCTA

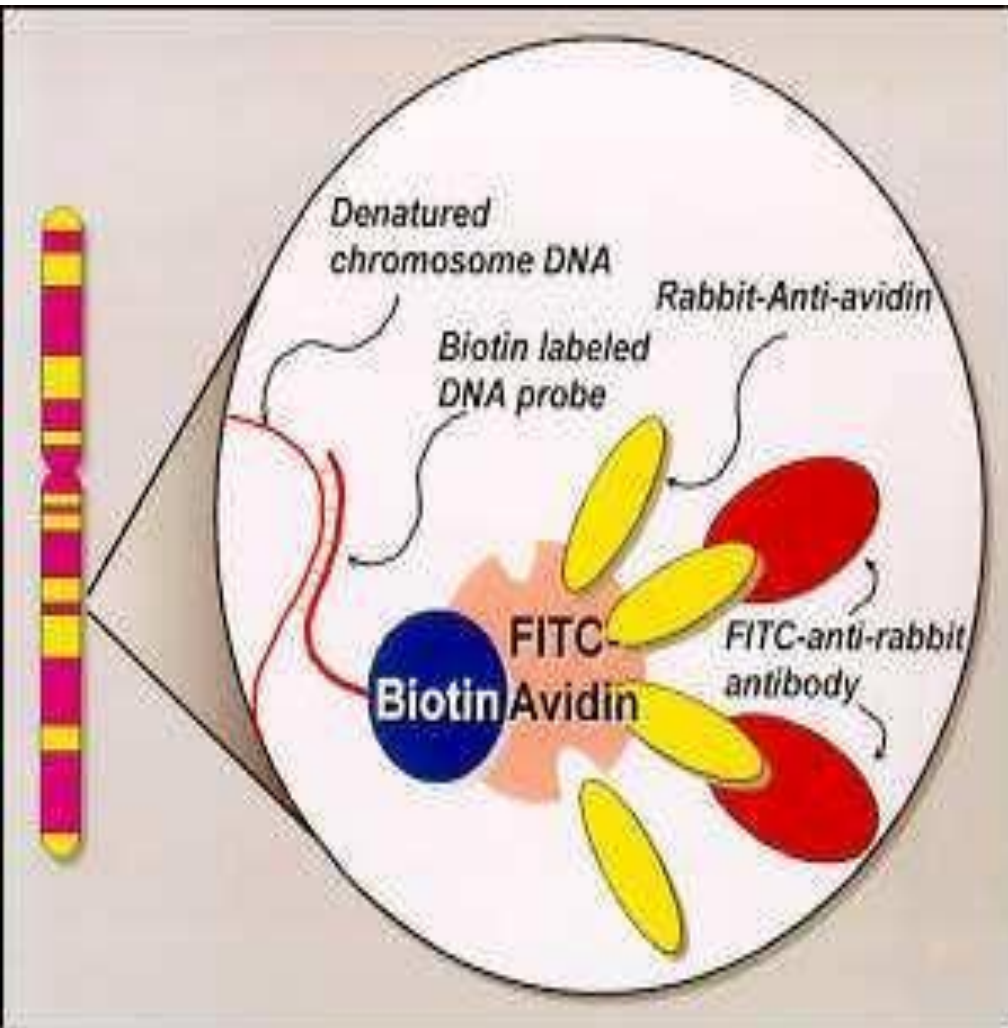
$\begin{matrix} & C & & C \\ & | & & | \\ G & A & T & G \\ & | & & | \\ & T & & T \end{matrix}$
5' primer

$\begin{matrix} & A & & G \\ & | & & | \\ G & C & G & T \\ & | & & | \\ & T & & A \end{matrix}$
3' primer

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.

How streptavidin/biotin binding is working?

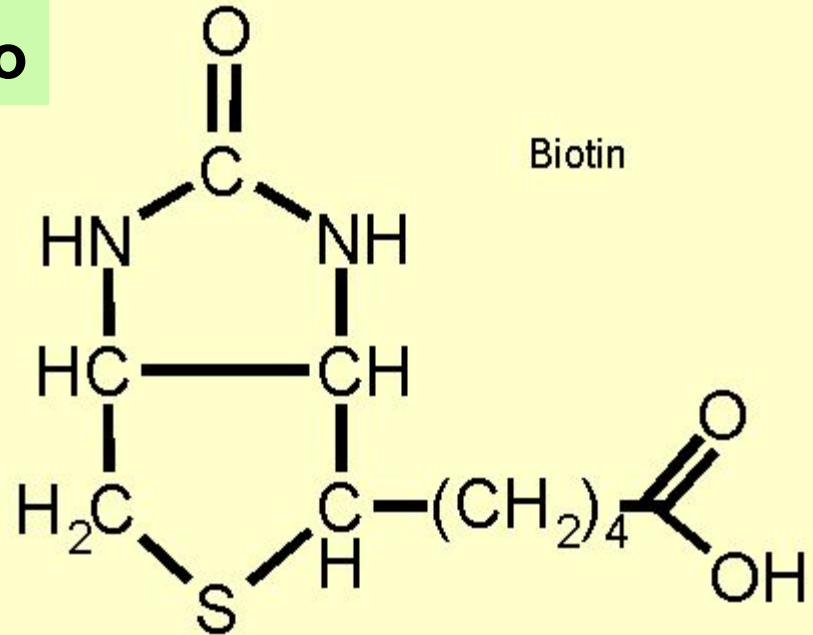
**Streptavidin is a protein.
1 mole of SA binds 4 moles Bio**

(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 ($K_{\text{assoc}} = 10^{14}$).
Complex is extremely stable.

***IN SITU* HYBRIDIZATION**

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

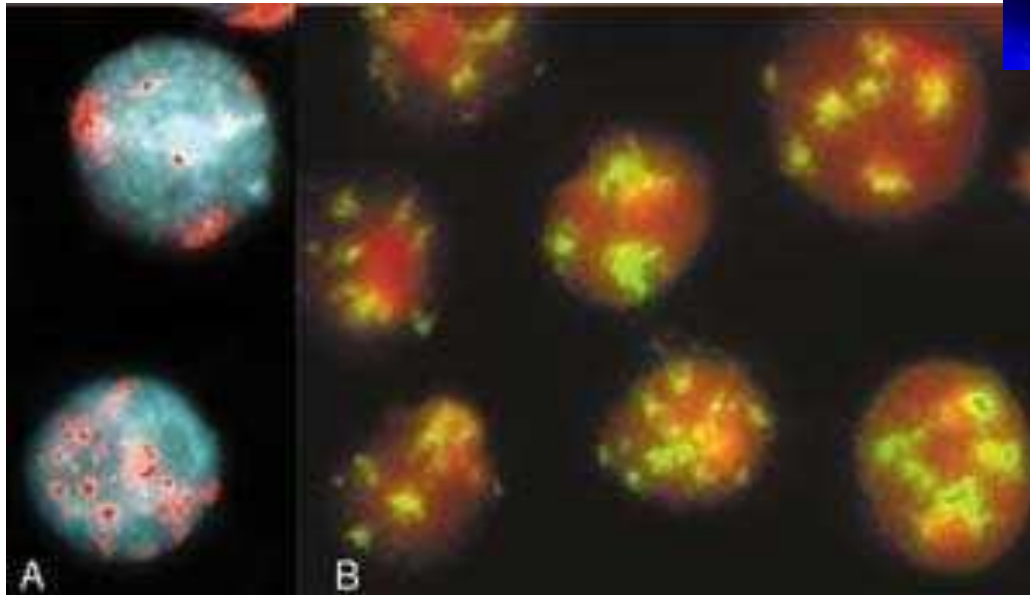
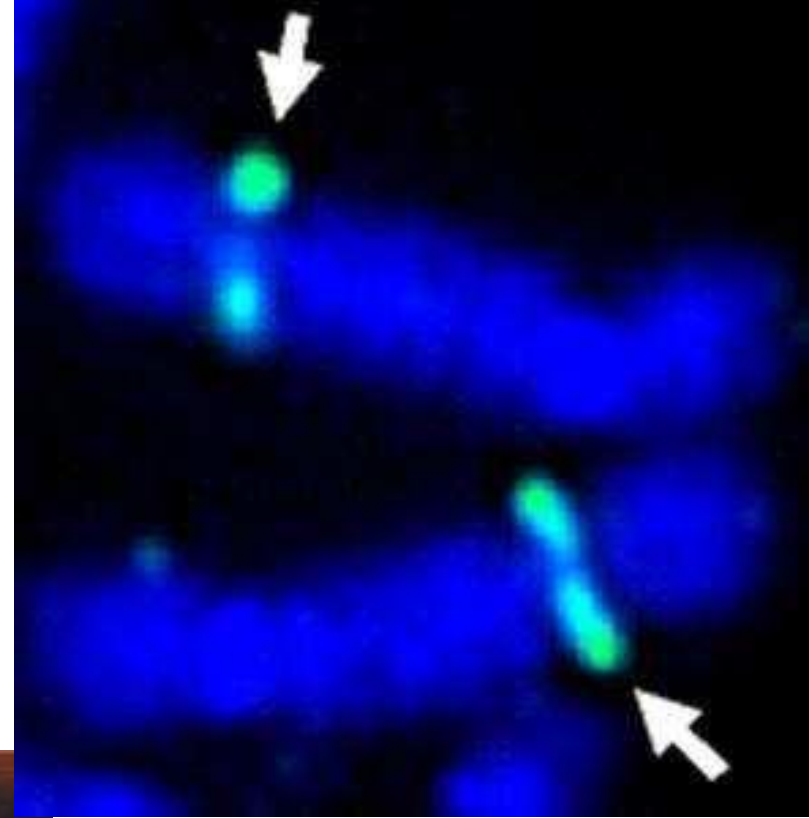


**Enkephalin gene expression
in the mouse brain**



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

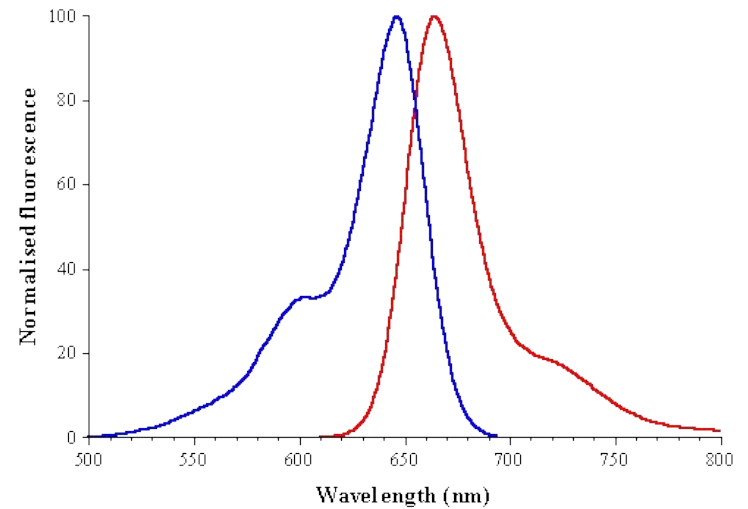
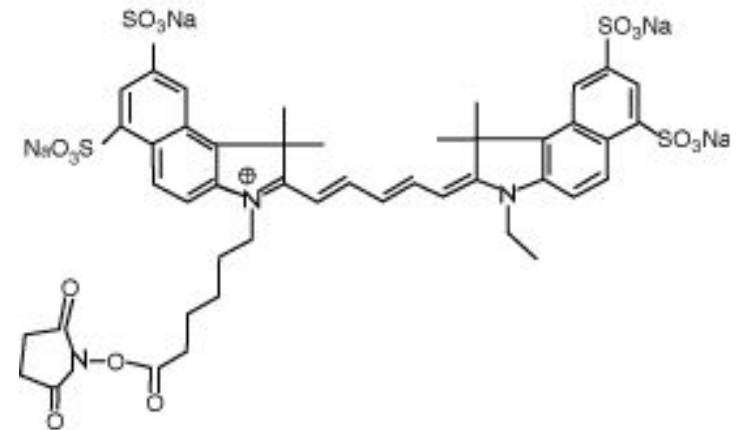
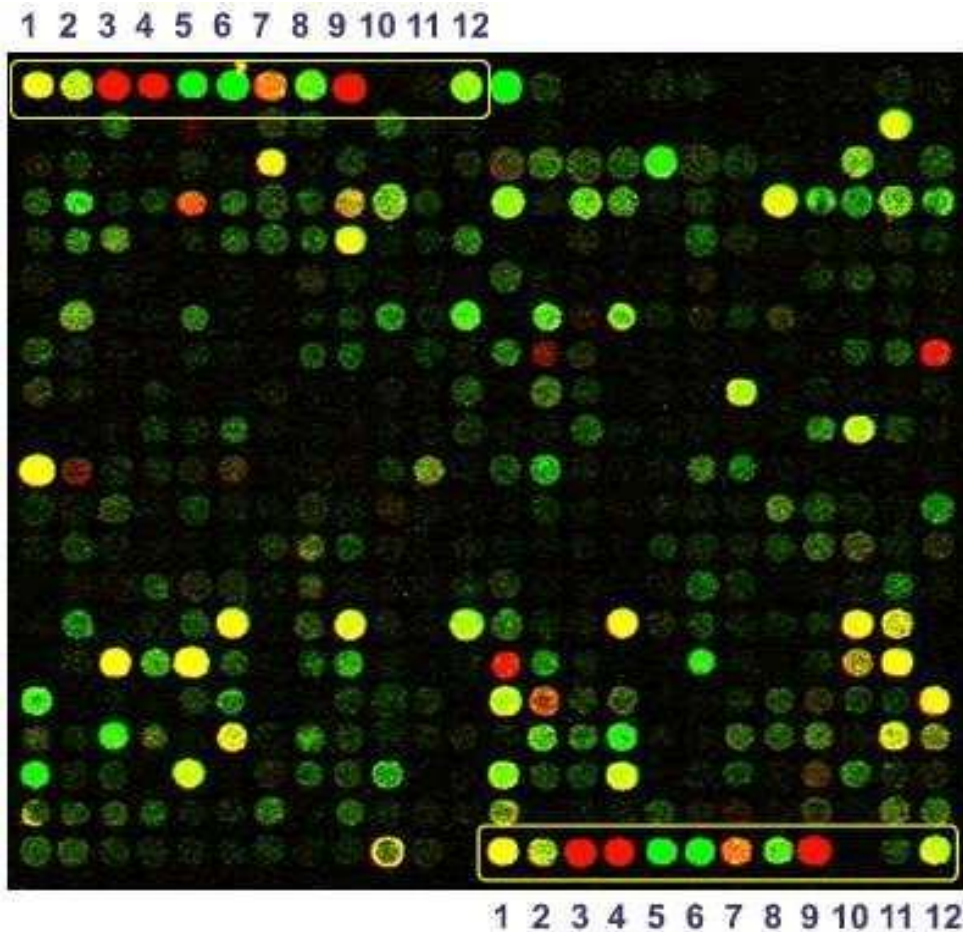
**Metaphase FISH analysis
using the BAC probe RP11-104M2
labeled with FITC (green)
hybridized to a normal metaphase cell
confirms the chromosomal localization
of the probe (gene) to 4q28.**



www.infobiogen.fr/.../

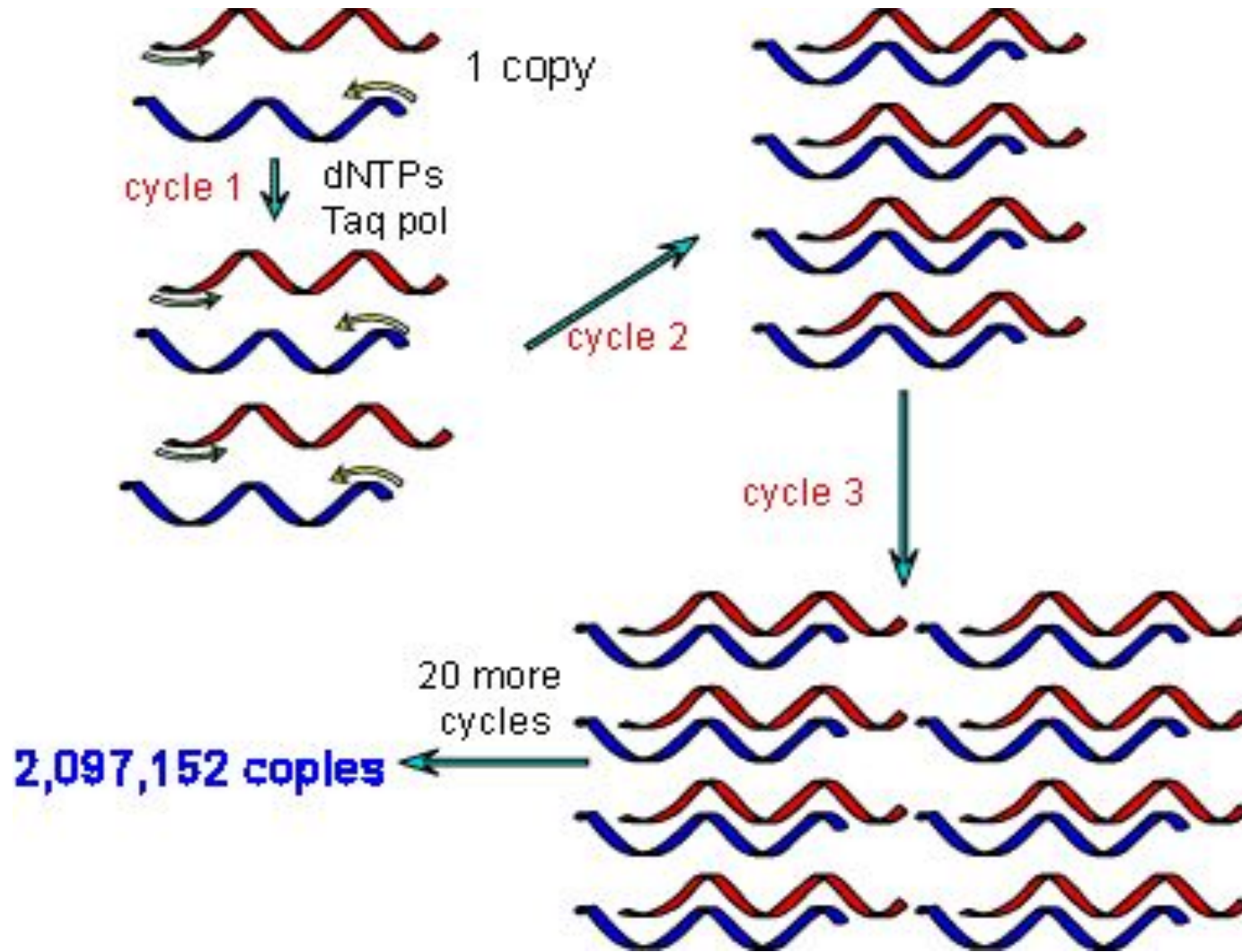
FISH labeling of the centromeric highly repeated DNA

Cy3/Cy5 direct labelling of DNA (for microarrays)

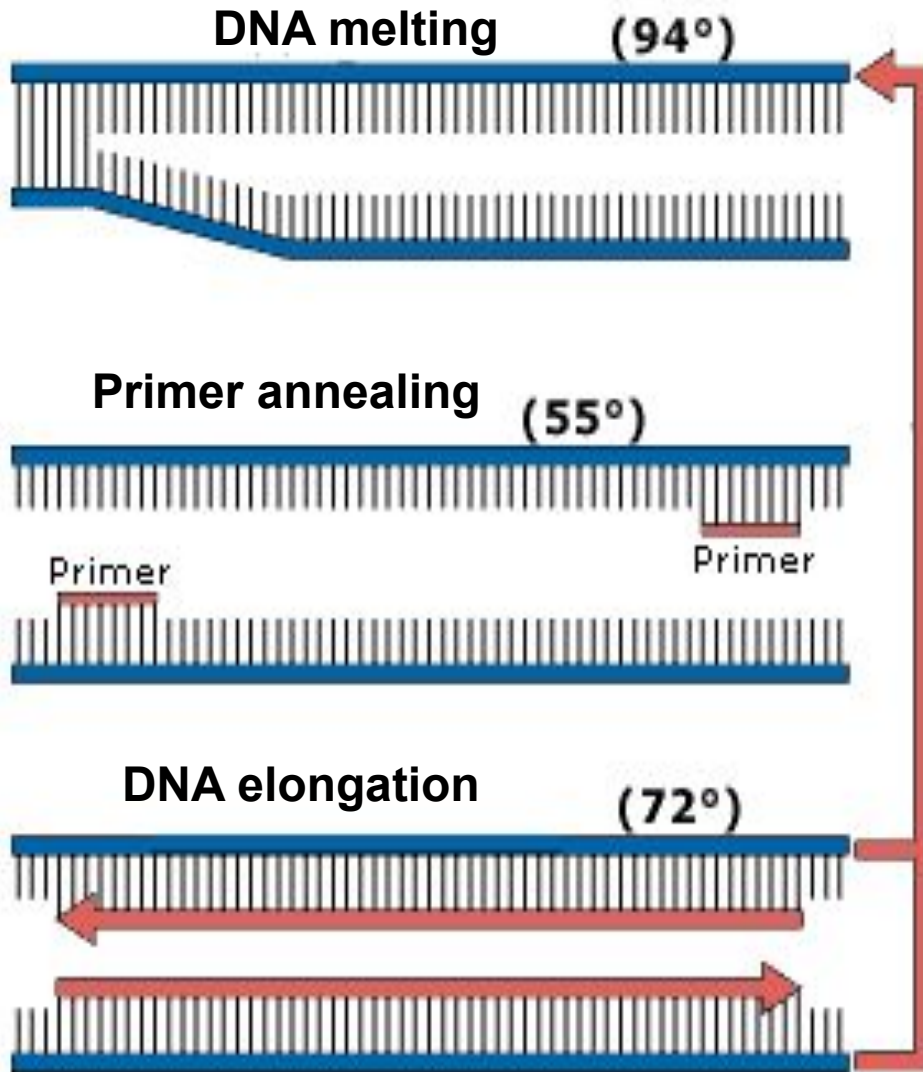


Cy5 -- RED

2. Polymerase chain reaction (PCR)



Polymerase Chain Reaction (PCR)



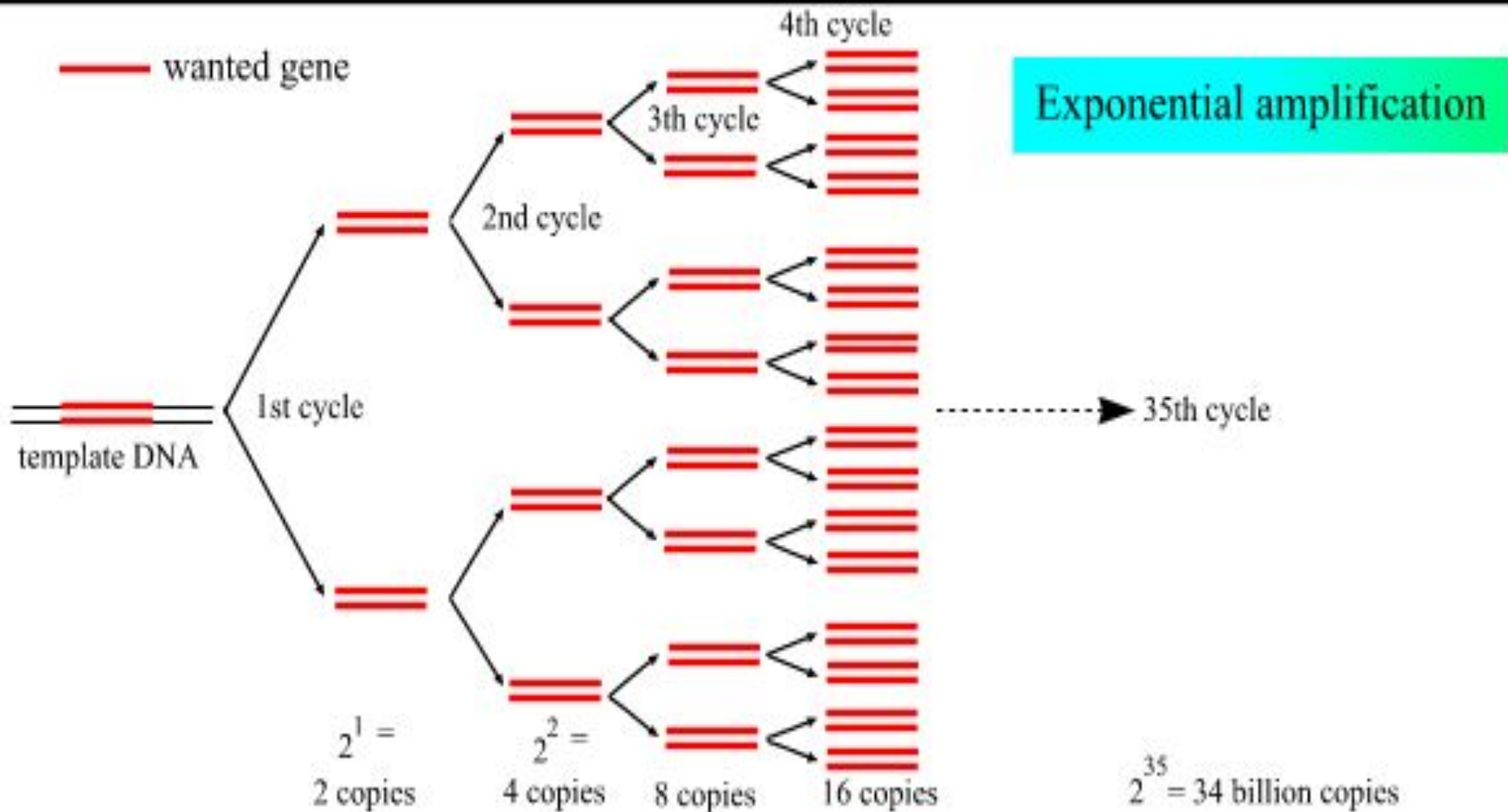
**Nobel Prize in Chemistry 1993,
at age 48**

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

PhD

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

Exponential nature of PCR amplification



PCR Reagent Components

- Template (DNA or RNA)
- Two primers
- dNTPs
- PCR buffer
 - KCl, Tris, MgCl₂
- Thermostable DNA Polymerase (*Taq*)



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 T_m for both primers

$$T_m = 69.3 + 0.41 * (\%GC) - 650/\text{length}$$

use OLIGONEW or PRIMER software

Avoid primer dimer formation

5' CCTGCAACGTGAACTTGGCAC3'
3' GCACGTTGCACTTGAACCGTGAAGTCT...5'

5' ...TTCATGTGTGCACTTGAACCAAGTTCAGG3'
3' CGTGAACTTGGTTCAGTCC5'



Primer-Dimer Formation

5' CCTGCAACGTGAACTTGGCAC3' →
← 3' CGTGAACTTGGTTCAGTCC5'

Marginally problematic primer

5' -GCTTTGTCCGATGAATCGT-3'

|||||
3' -TGCTAAGTAGCCTGTTTCG-5'

Use Software to avoid of such problems

General Primer Picking Conditions

Primer Size Min: Opt: Max:

Primer Tm Min: Opt: Max: Max Tm Difference:

Product Tm Min: Opt: Max:

Primer GC% Min: Opt: Max:

Max Self Complementarity: Max 3' Self Complementarity:

Max #N's: Max Poly-X:

Inside Target Penalty: Outside Target Penalty: Set Inside Target F

First Base Index: CG Clamp:

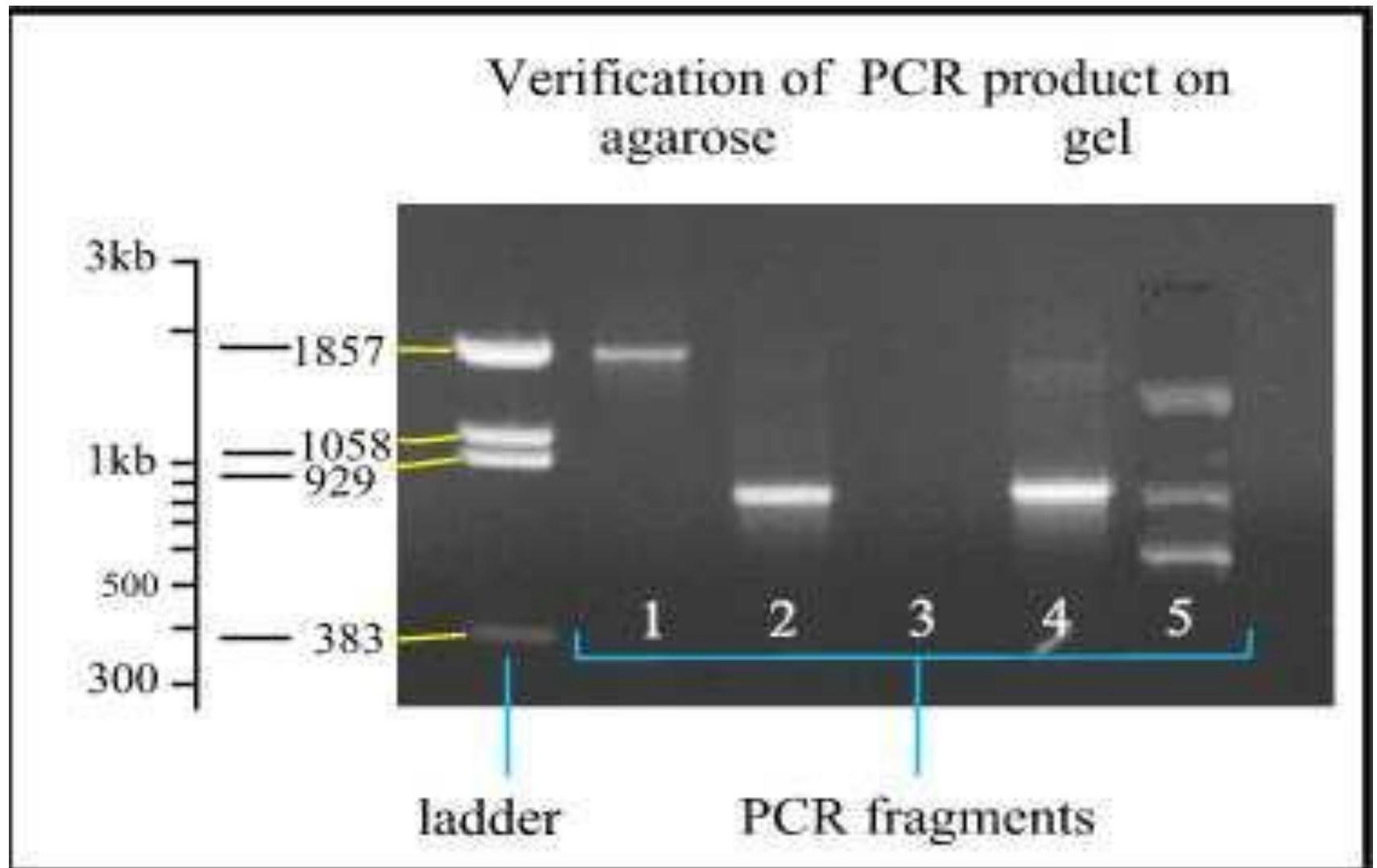
Salt Concentration: Annealing Oligo Concentration: (Not the concentra
template.)

Liberal Base Show Debugging Info

Pick Primers

Reset Form

Typical PCR gel (Every PCR should be gel-verified)



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

	<u>Primer Concentration*</u>	
	<u>Lower</u>	<u>Higher</u>
Product Specificity:	Increases	Decreases
Product Yield:	Decreases	Increases

* Compared to optimum

Input Copy Number, Number of Cycles, and Product Mass

Human Genomic 			Plasmid 6kb 		
Copies ^a	Cycles	Yield ^b	Copies ^a	Cycles	Yield ^b
100	33	160 pg	100	33	660 ag
20,000	25	32 ng	20,000	25	130 fg
600,000	20	960 µg	600,000	20	4 pg
			1.5x10 ¹¹	2	1 mg

^a Input copies

^b Product mass

If complete copies is amplified

Cofactor required for activation of Taq polymerase

- dNTPs bind free Mg²⁺ in a 1:1 molar ratio
 - Increases or decreases in dNTPs must be matched with equivalent changes in [Mg²⁺]
- Each PCR reaction has an optimum concentration of free [Mg²⁺]
- Different Enzymes require different [Mg²⁺]



Mg⁺⁺ Optimization

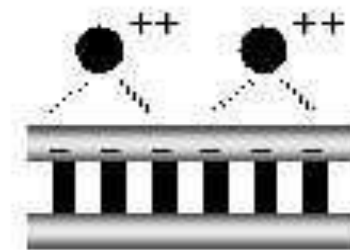
Suboptimal
Mg²⁺



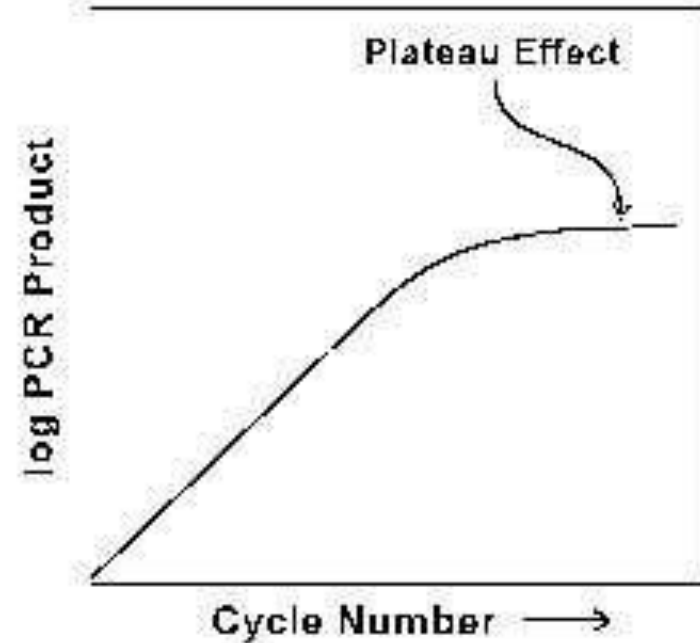
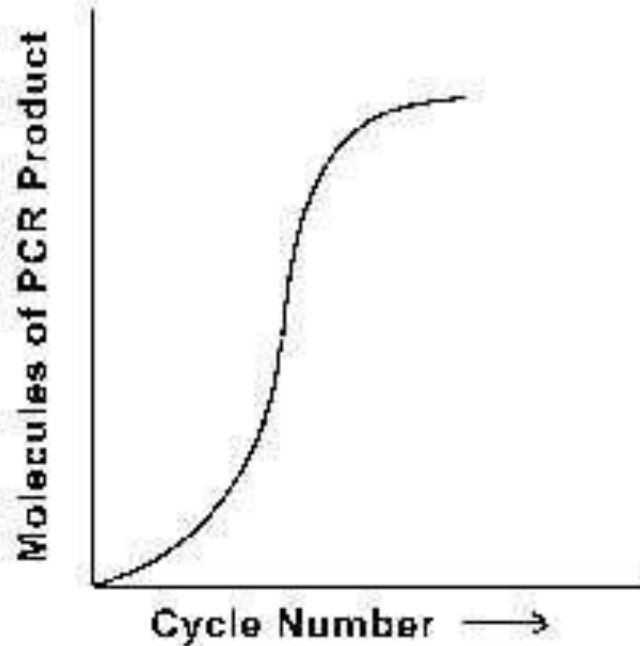
Optimal
Mg²⁺



Excess
Mg²⁺



Plateau effect in PCR reaction



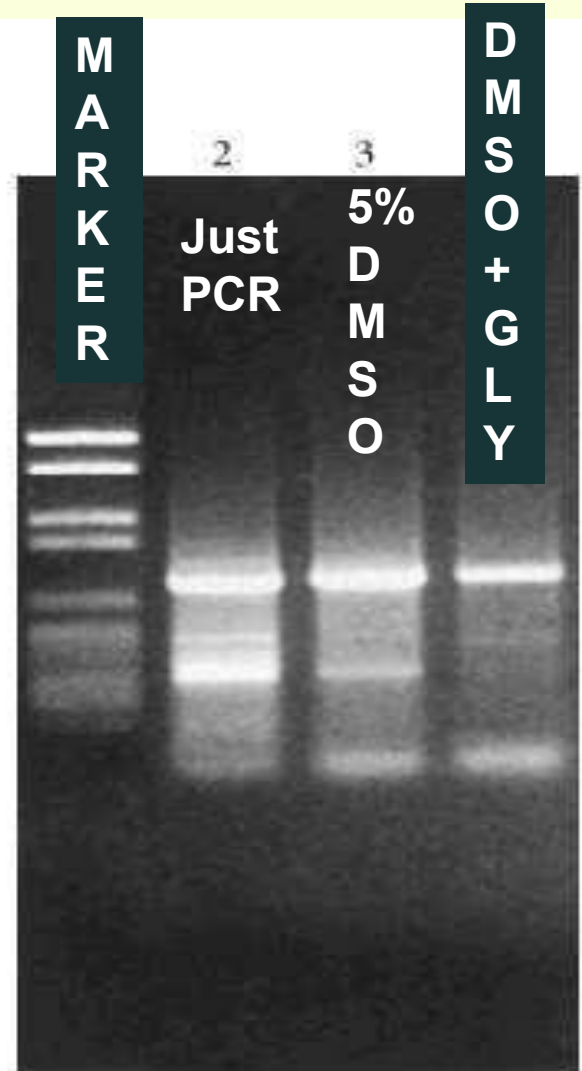
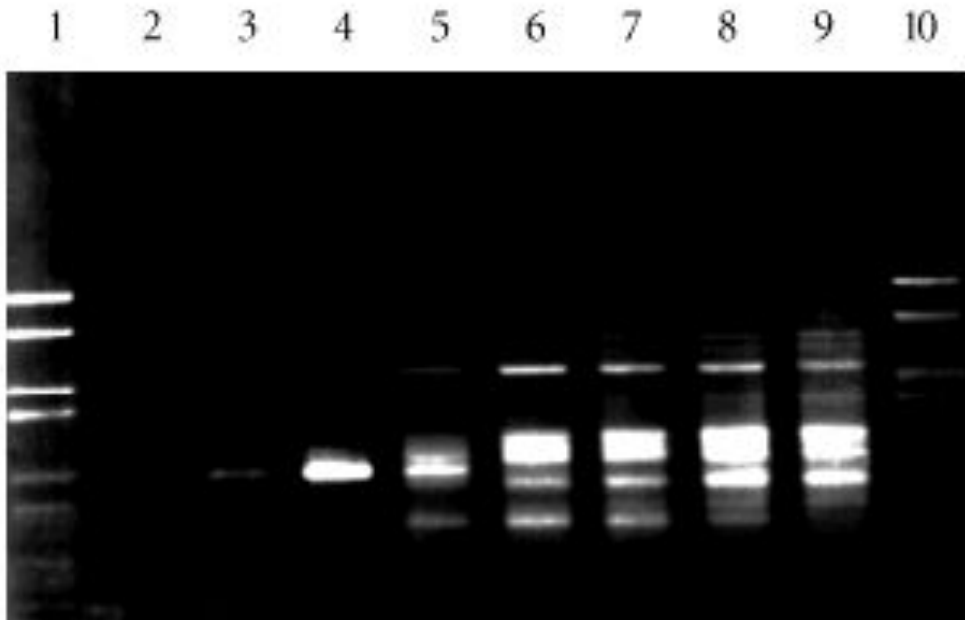
Plateau effect: The point at which product accumulation is no longer exponential (saturation), typically 10^8 to 10^{12} molecules of product.

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

Non-specific PCR and how to improve it

Decrease in Mg concentraton



PCR enzymes

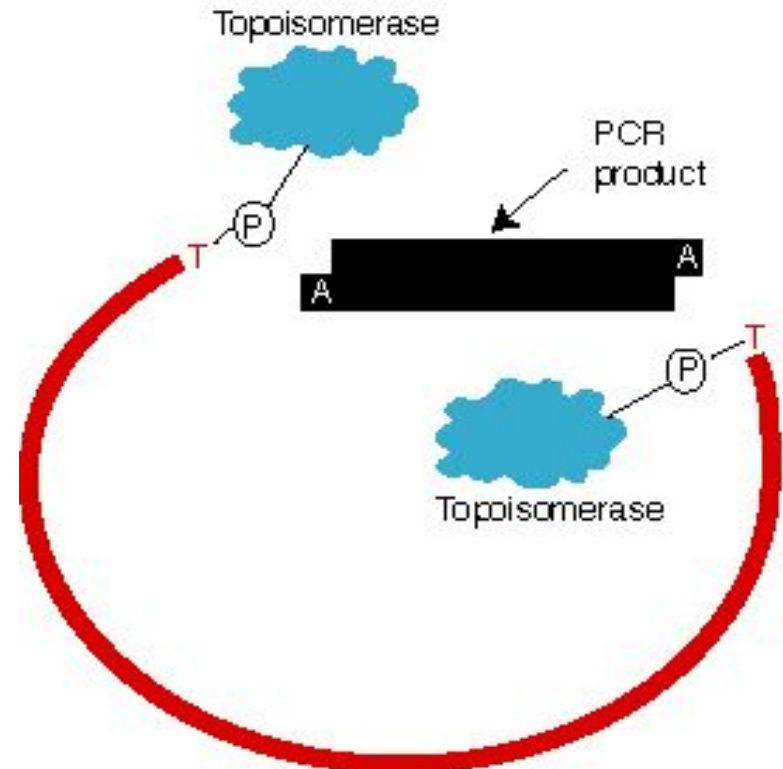
Taq DNA polymerase, the first enzyme used for PCR, is still the most popular.

- 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)

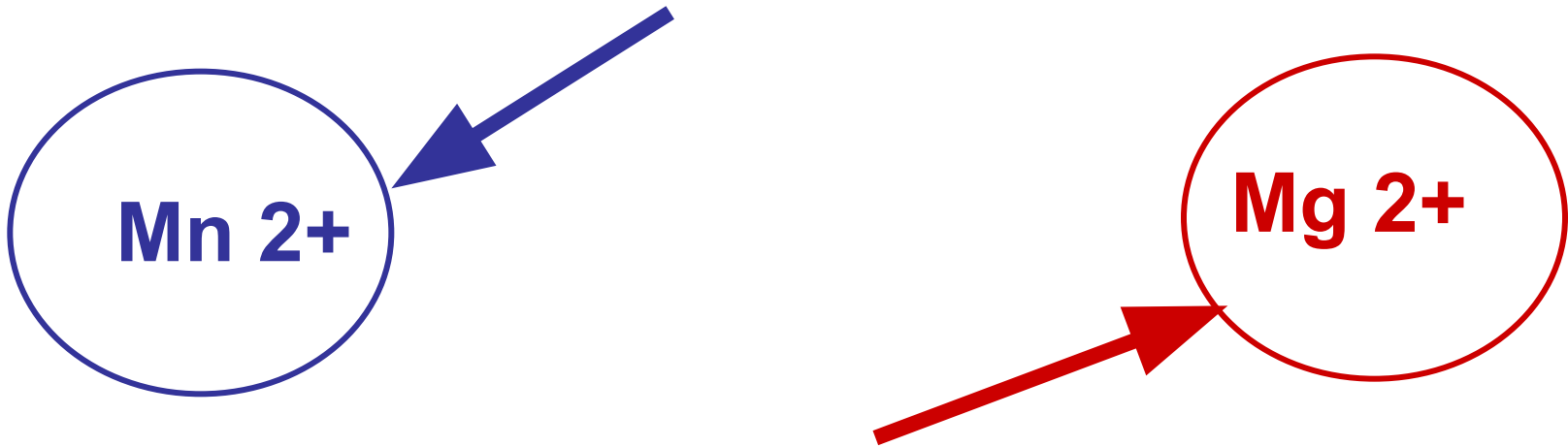
“Topo” cloning system (Invitrogen)



Tth polymerase

Thermus thermophilus strain HB8.

RNA-dependent DNA-polymerase activity in the presence of Mn^{2+} ions.



DNA-dependent DNA-polymerase activity in the presence of Mg^{2+} 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 thermostable: 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:

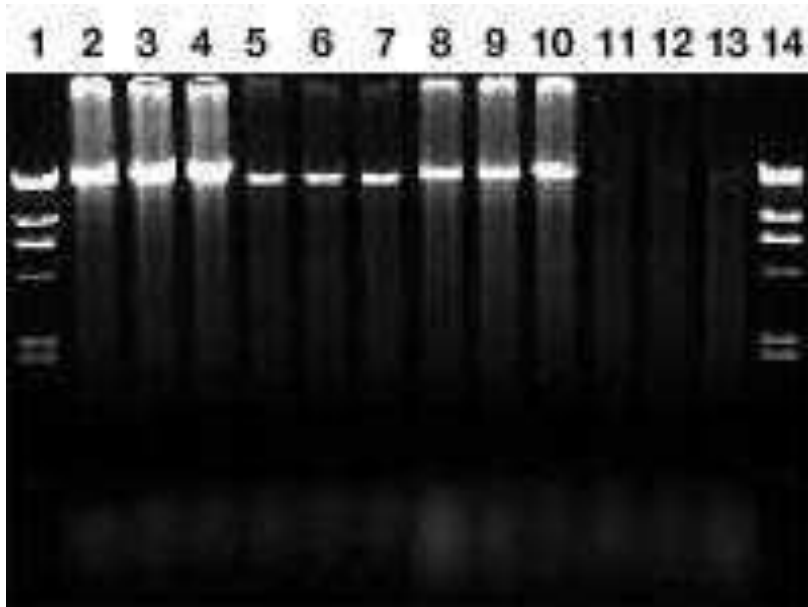
Deep Vent (Pyrococcus species GB-D) (New England Biolabs)
New England Biolabs claims fidelity is equal to or greater than that of Vent.

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

Long-Range PCR

Use of two polymerases:

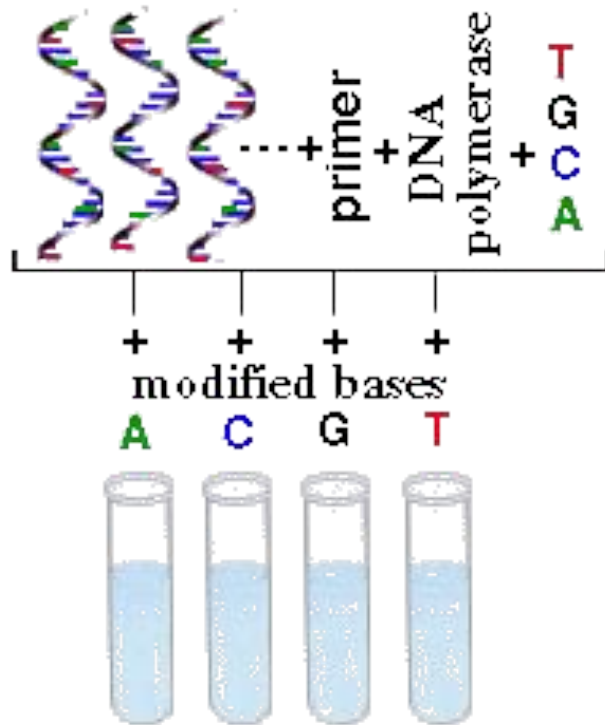
a non-proofreading polymerase **Taq** is the main polymerase in the reaction, a proofreading polymerase (3' to 5' exo) **Pwo** is present at a lower concentration.



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)

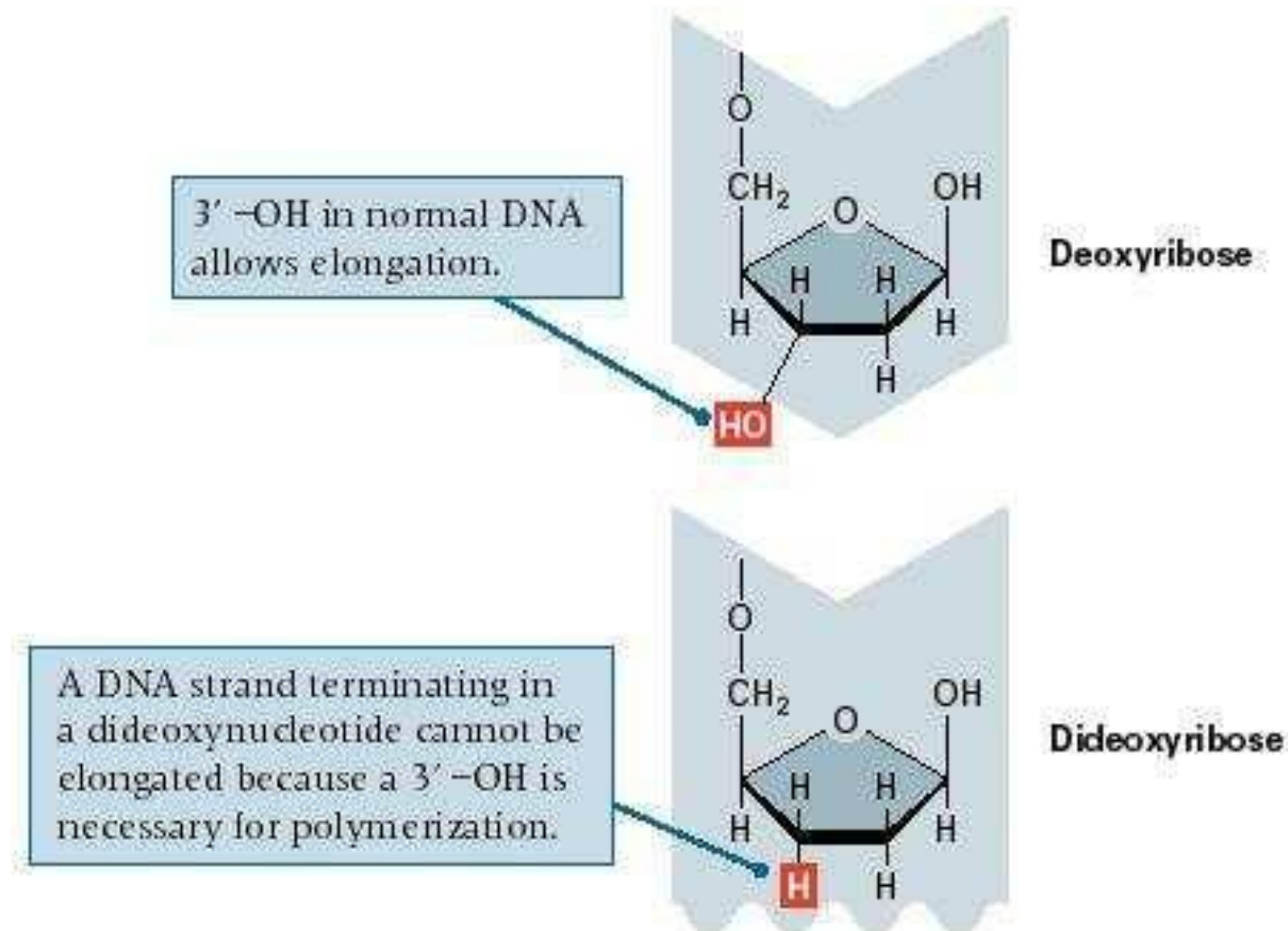


Sequencing of DNA
by the Sanger Method

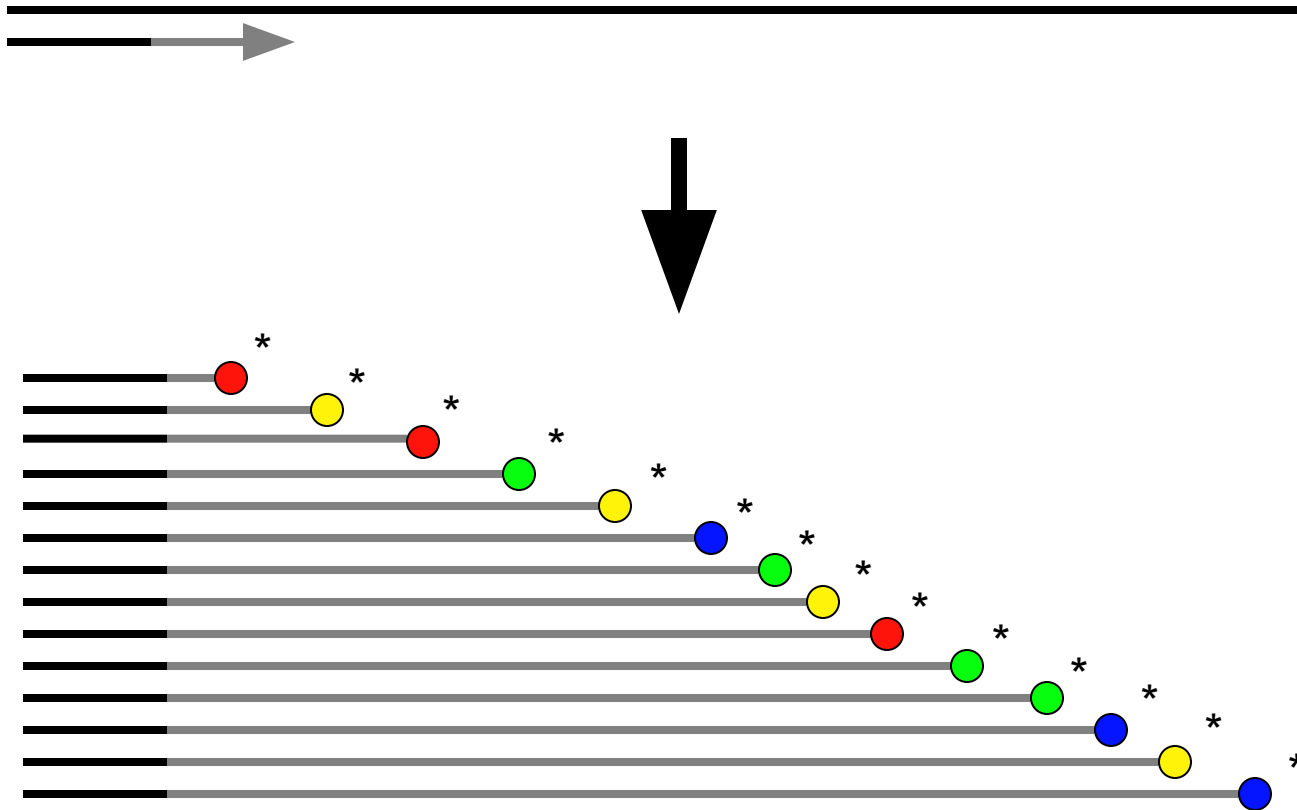


Frederick Sanger
(Nobel prize 1980 with Paul Berg and Walter Gilbert)

Dideoxynucleotide blocks chain elongation

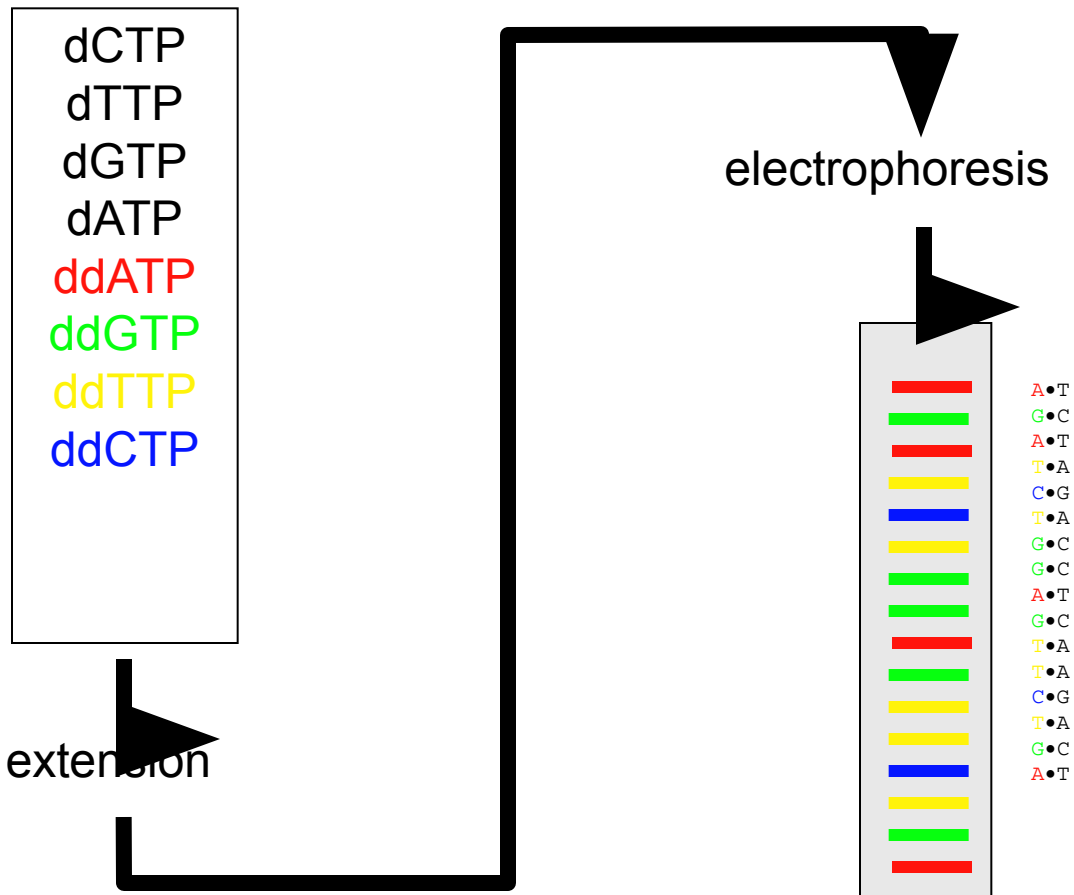


DNA sequencing: chemistry, with terminator dyes

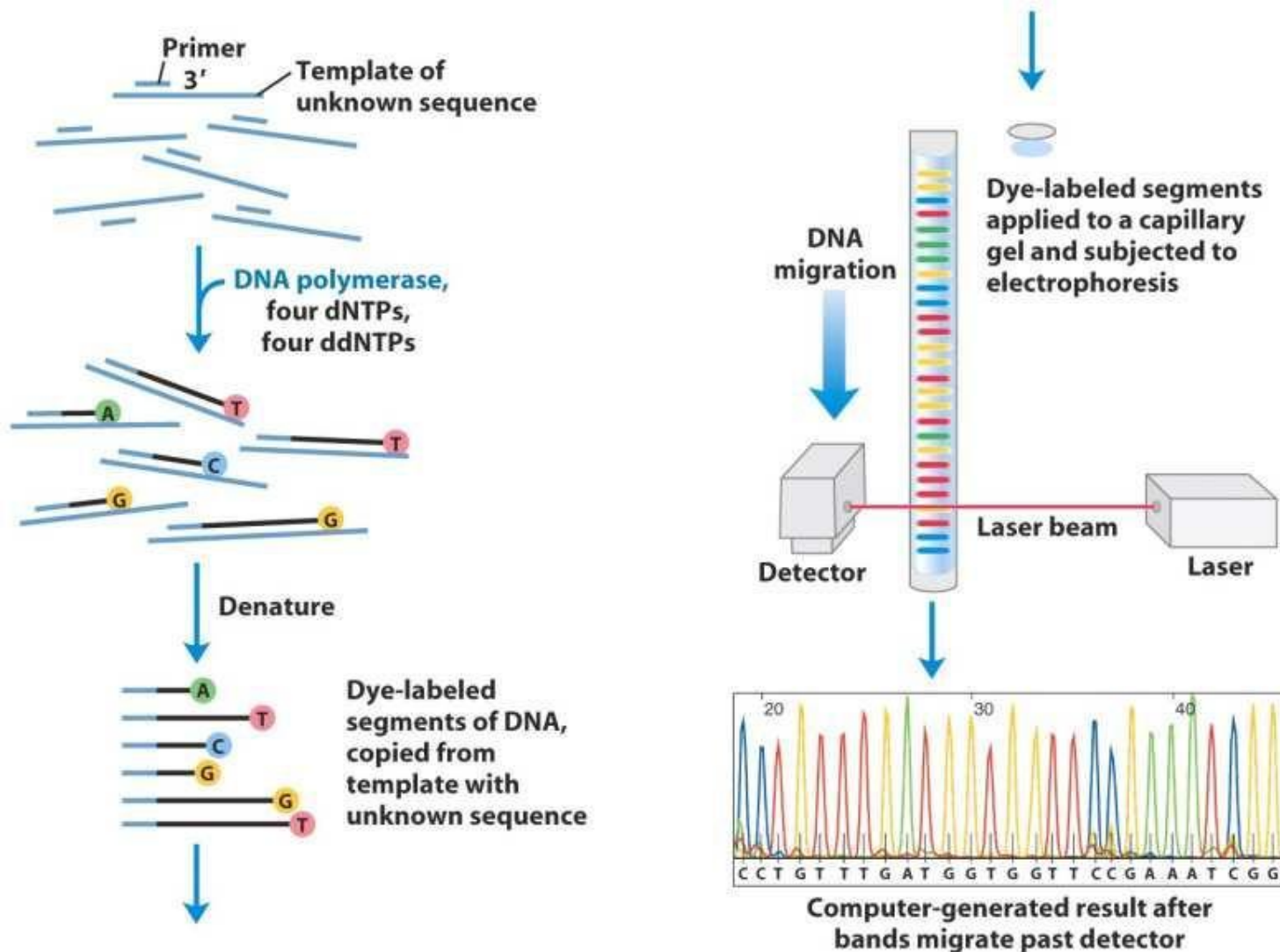


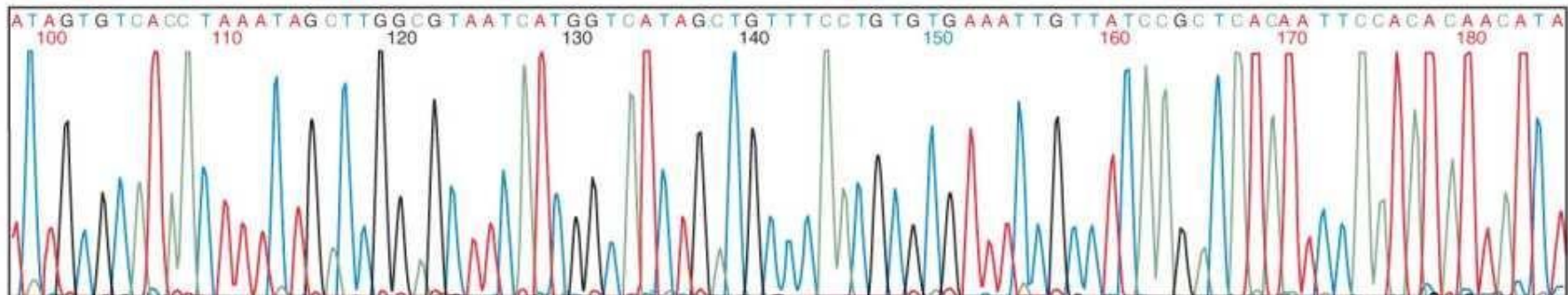
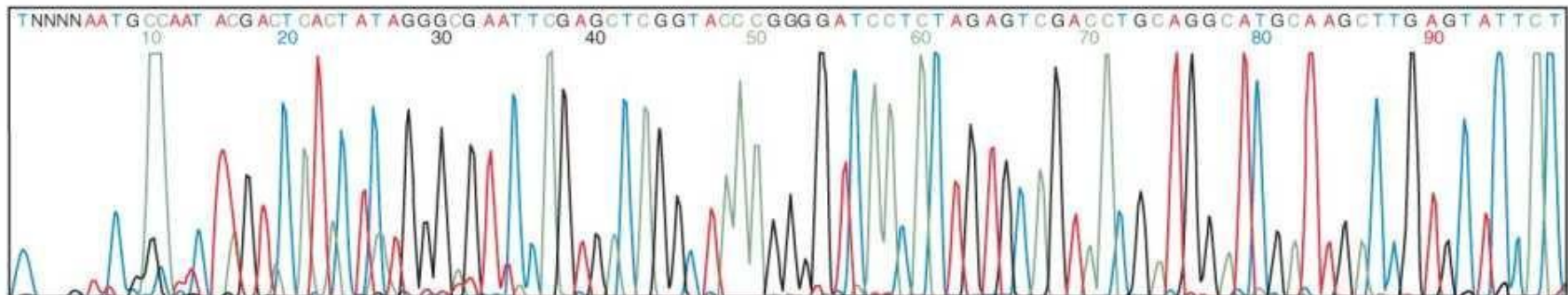
Semi-automated fluorescent DNA sequencing:

template + polymerase +



Cycle Sequencing - PCR



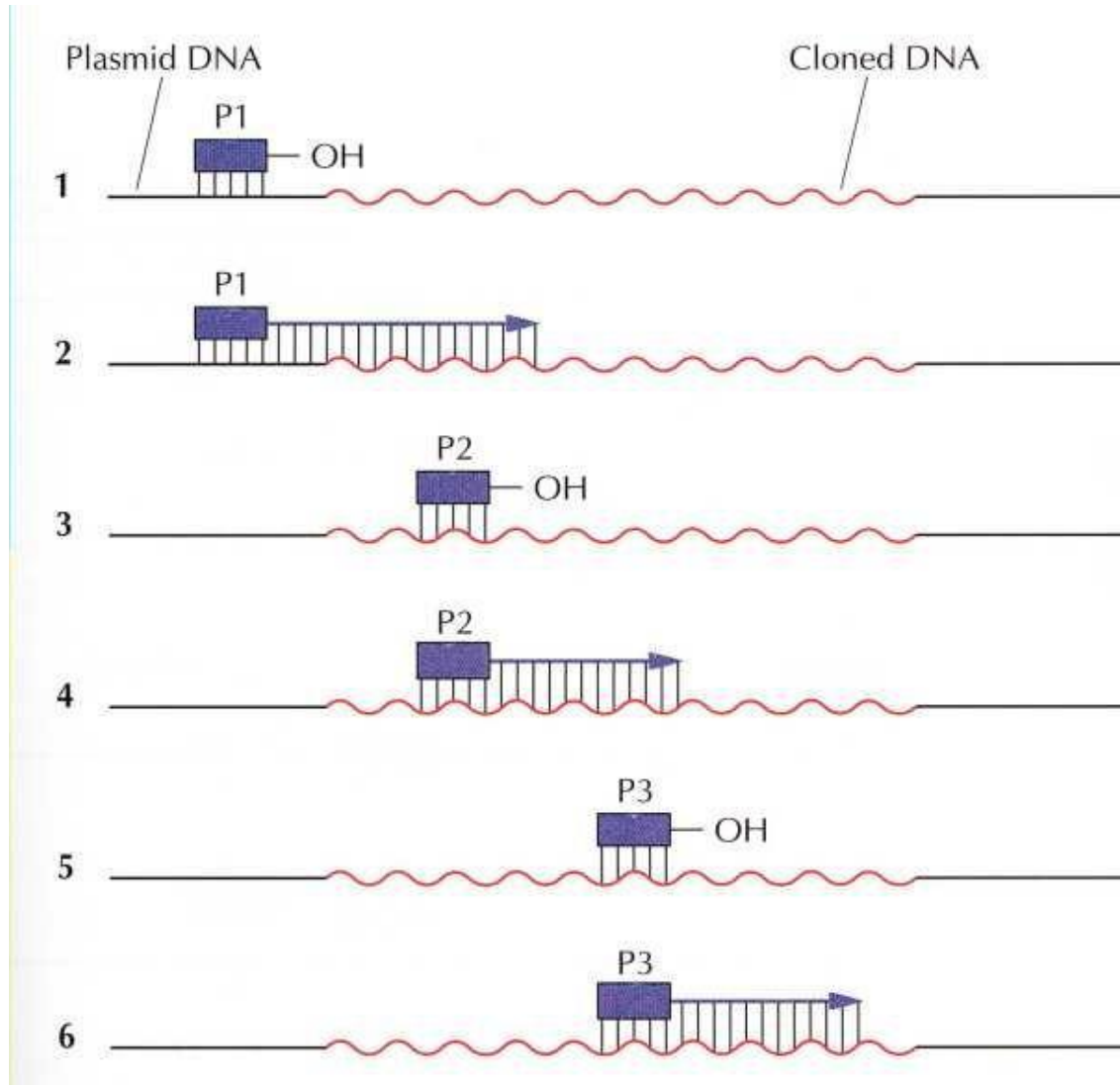


Applied Biosystems Inc., have designed an automated method that combines the PCR and actual sequencing

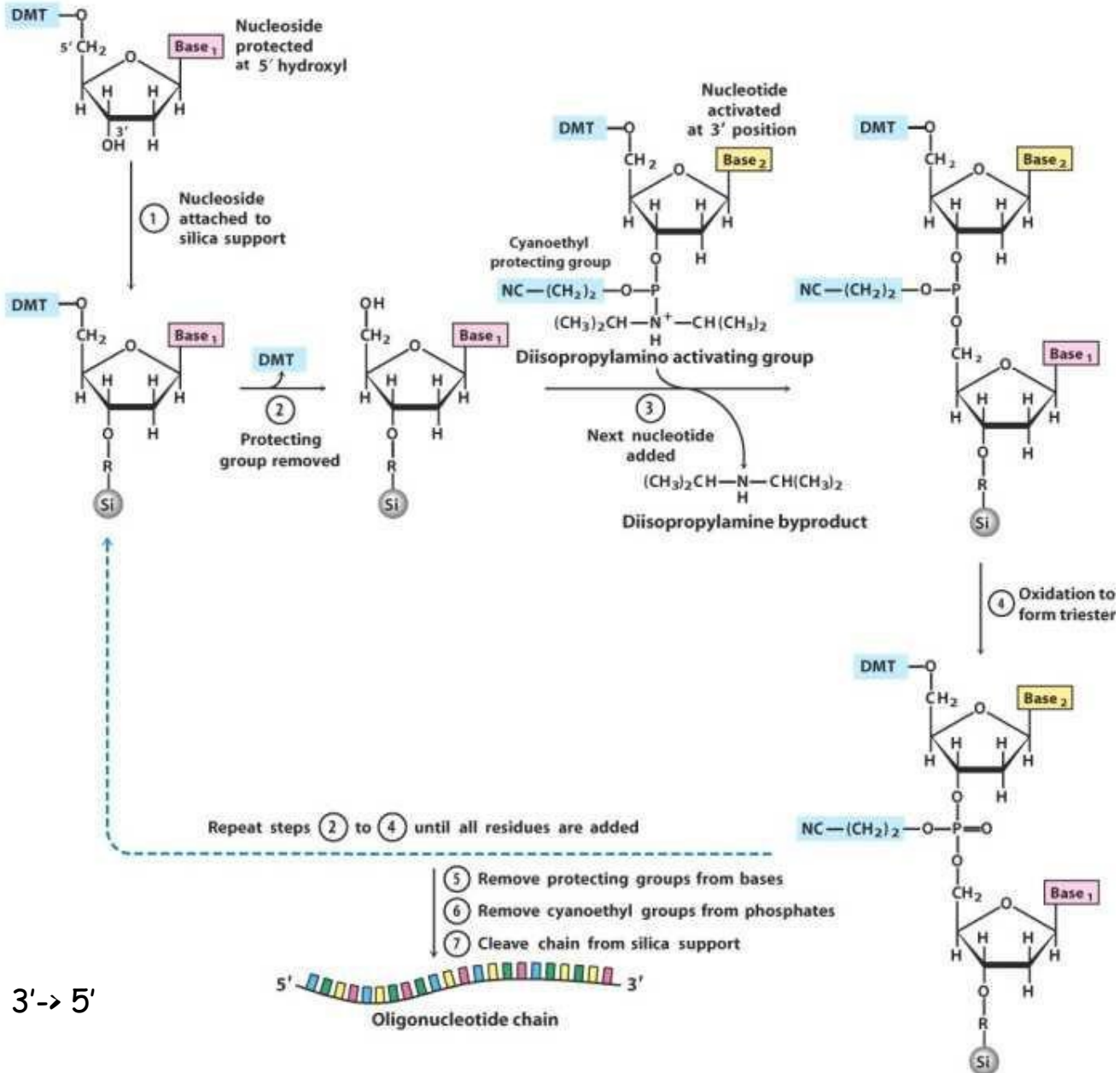


<<http://www.utmb.edu/proch/servo3.htm>>

DNA sequencing by primer walking



Chemical synthesis of DNA



Chain grows: 3' → 5'

General consideration about Gene Expression

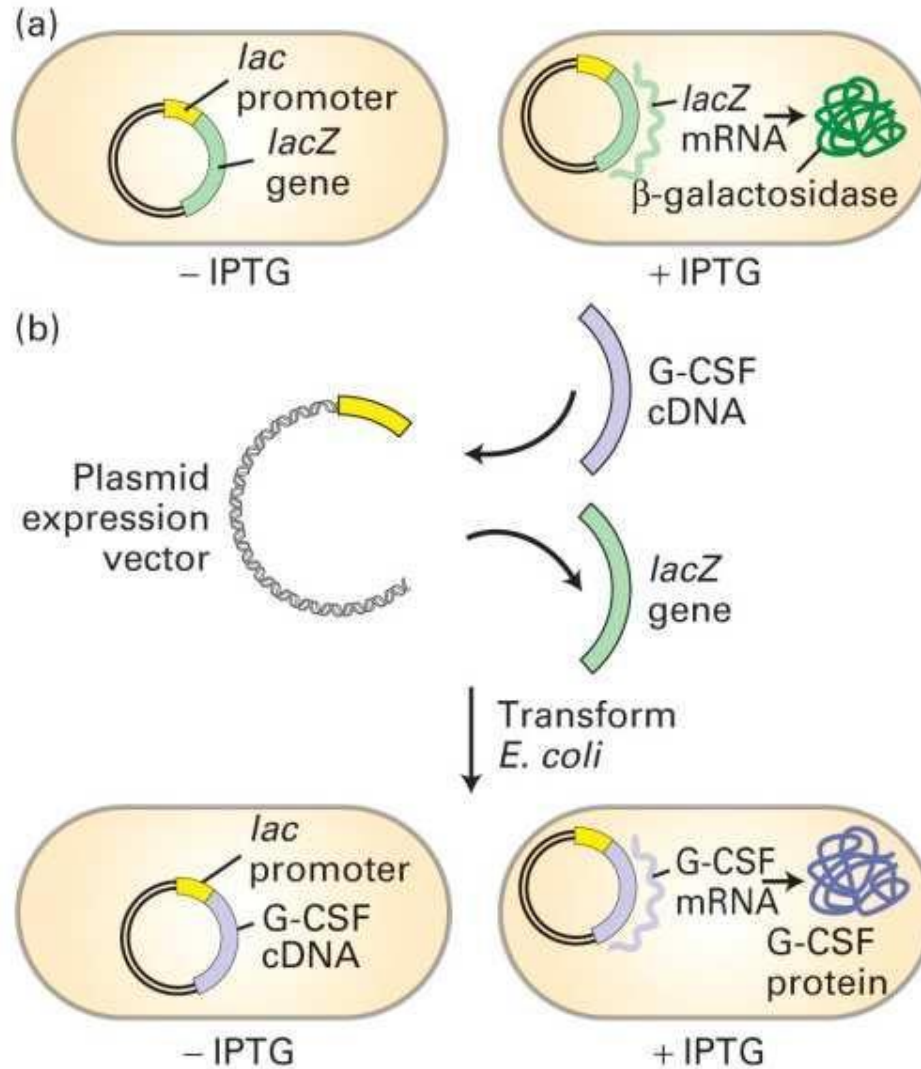
- Expression Host -> Expression System
- Promoter system -> expression vector
- Properties of product -> stability
- Production level

Comparison of expression systems

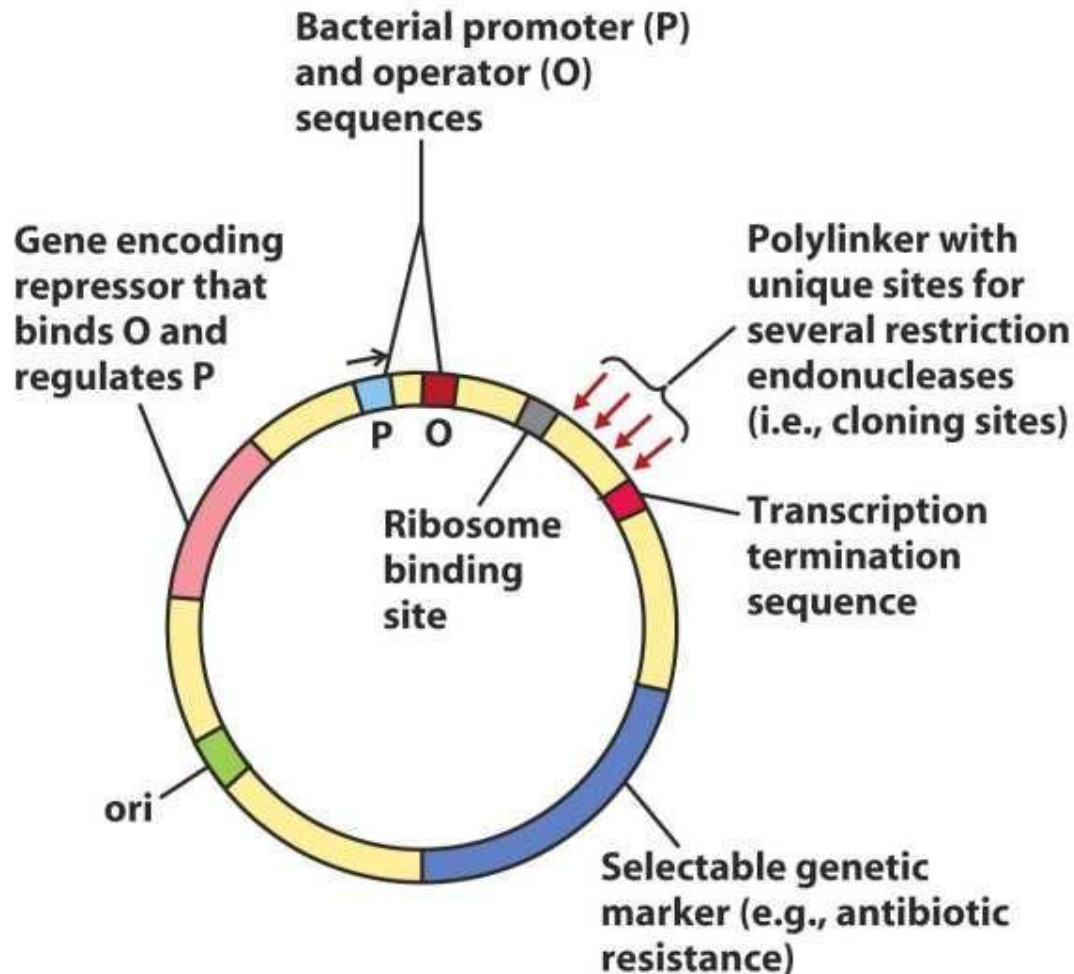
Table 1
Comparison of Expression Systems

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

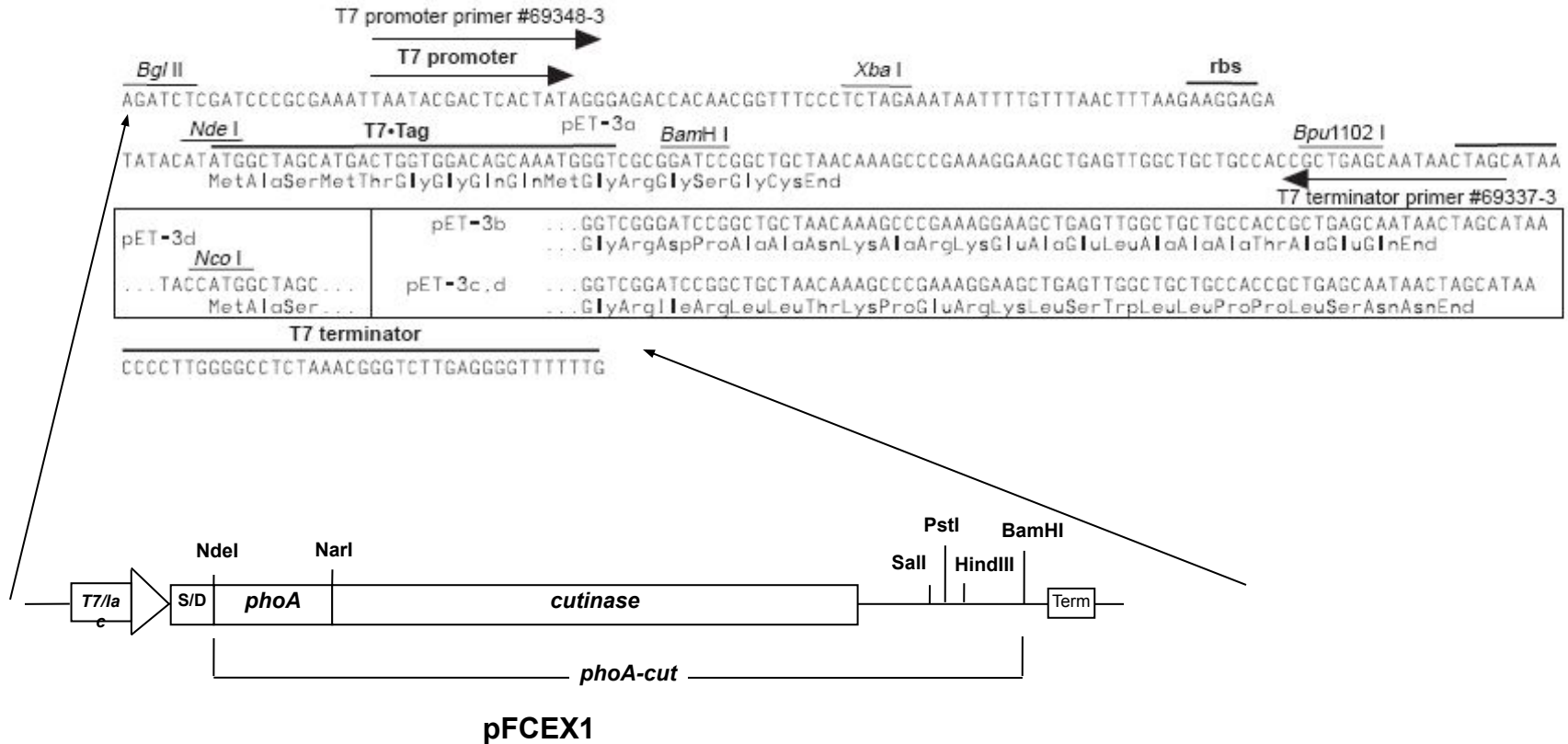
Use of *Lac* promoter (*pLac*) for expression of foreign protein



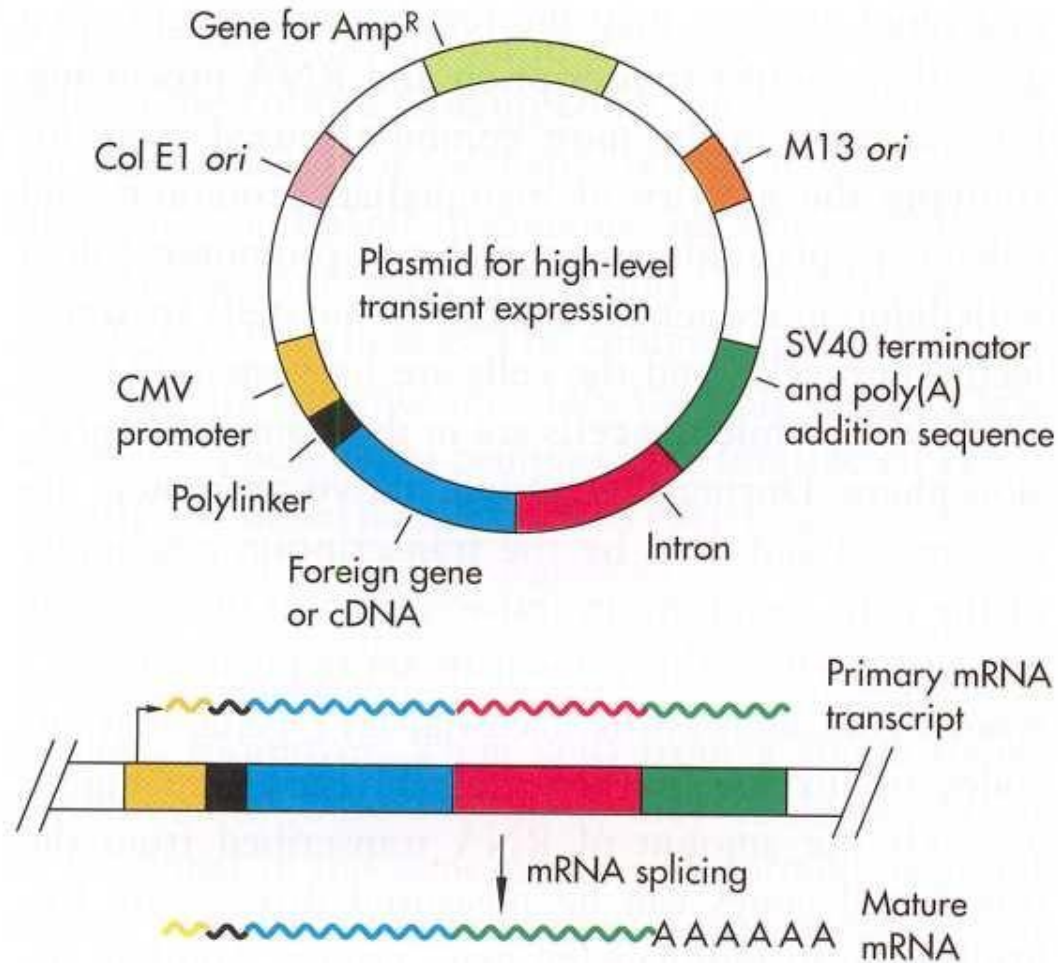
Prokaryotic Expression vector



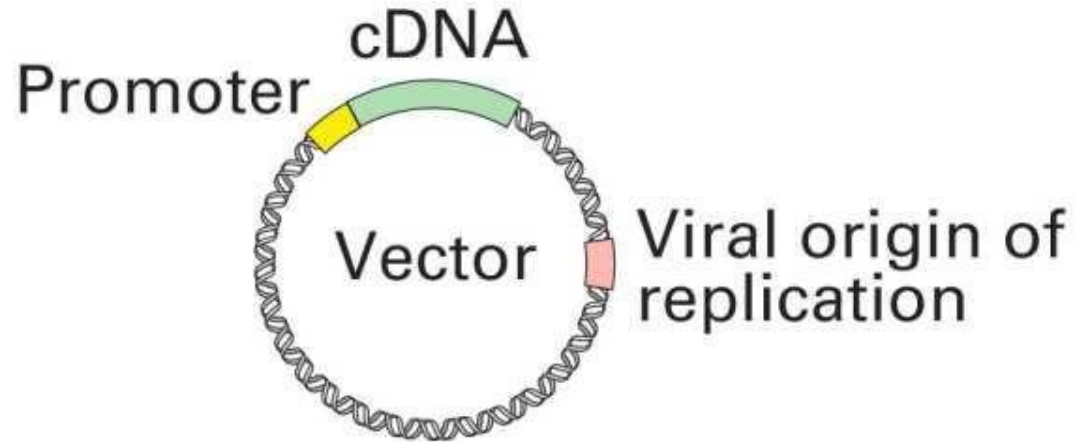
Prokaryotic Expression vector



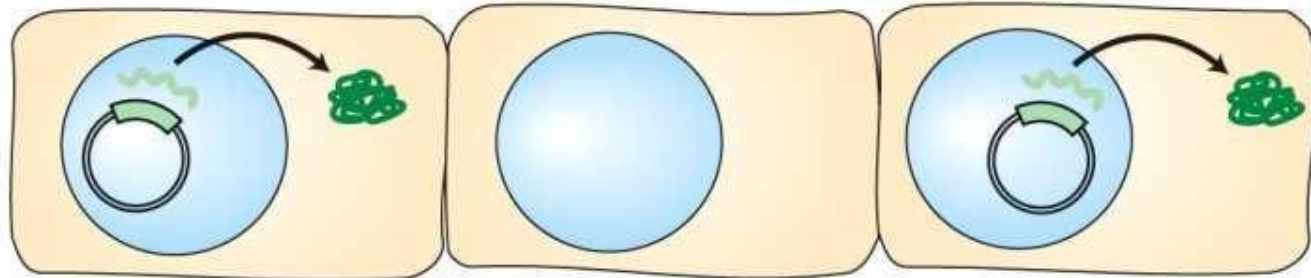
Eukaryotic Expression vector



(a) Transient transfection

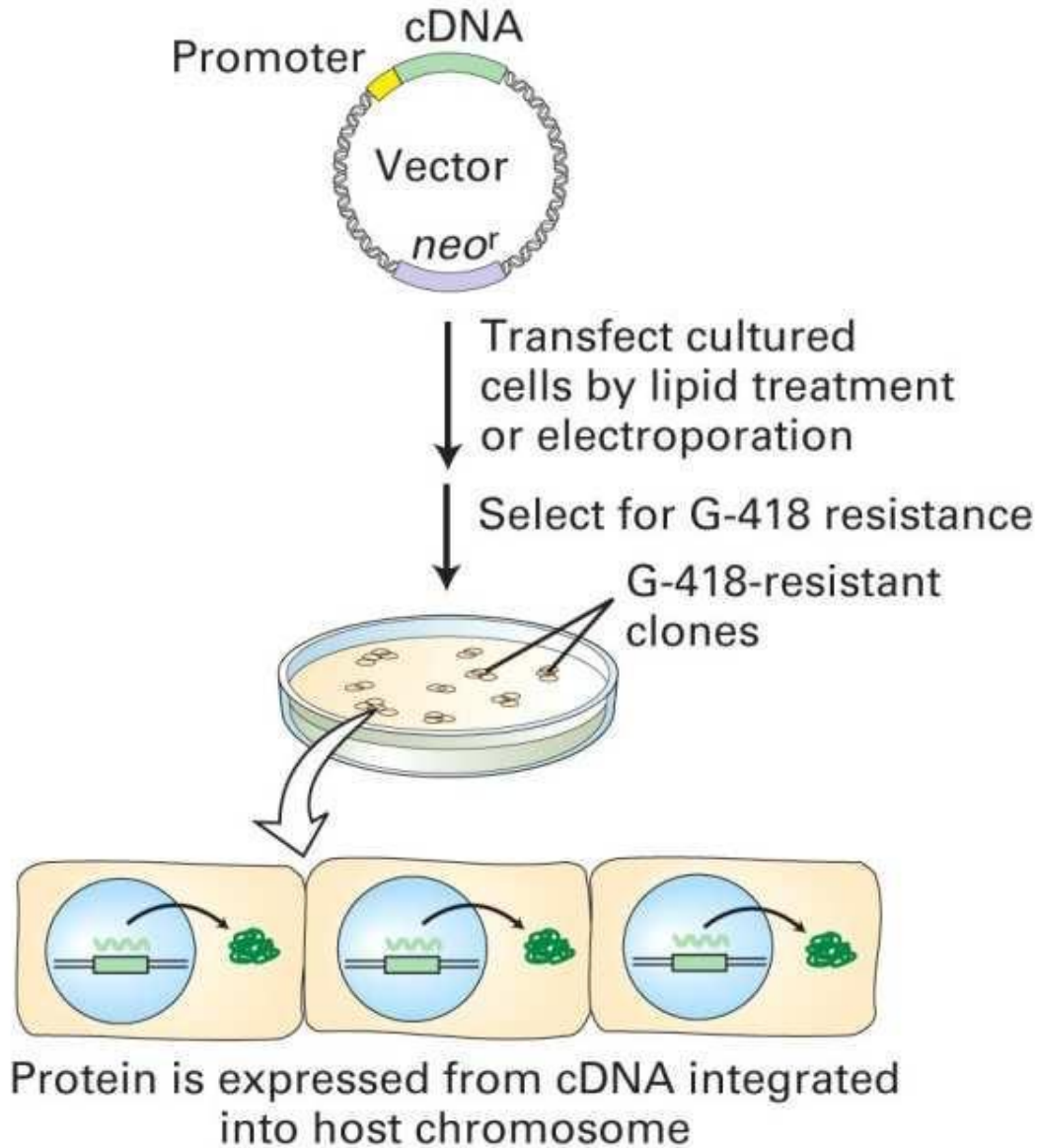


Transfect cultured cells by lipid treatment or electroporation

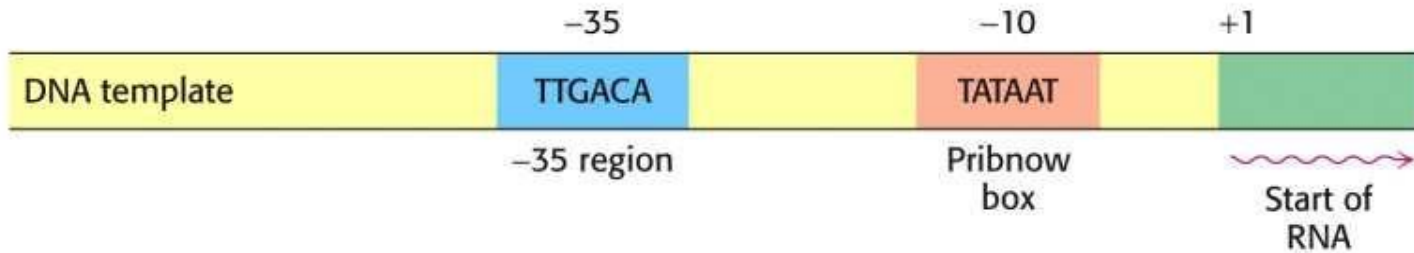


Protein is expressed from cDNA in plasmid DNA

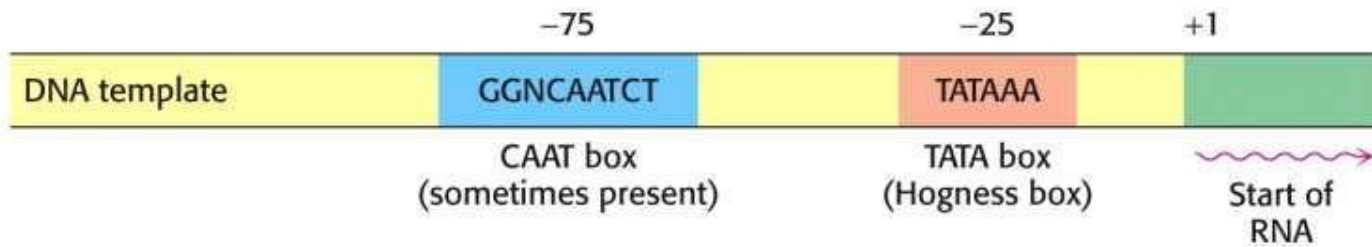
(b) Stable transfection (transformation)



Promoters

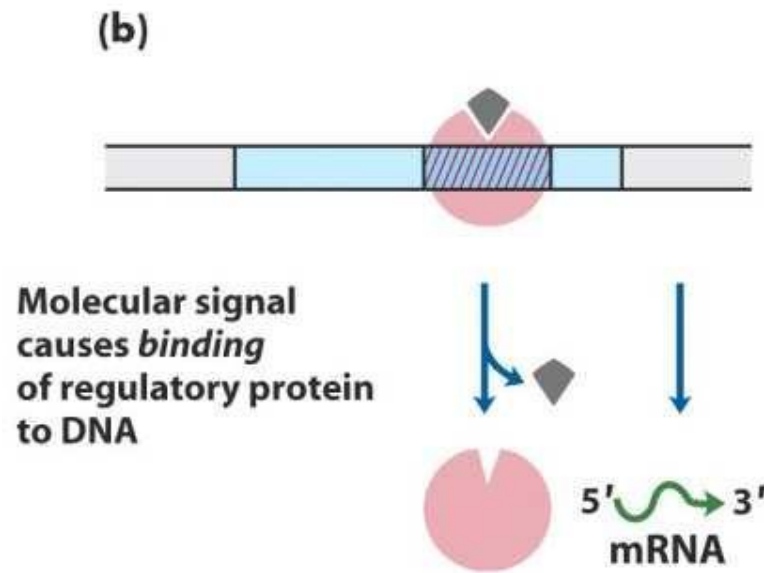
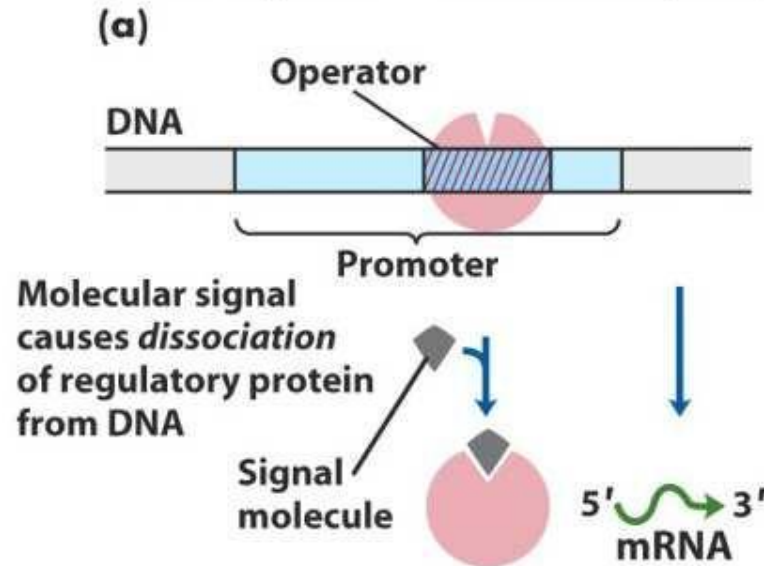


(A) Prokaryotic promoter site

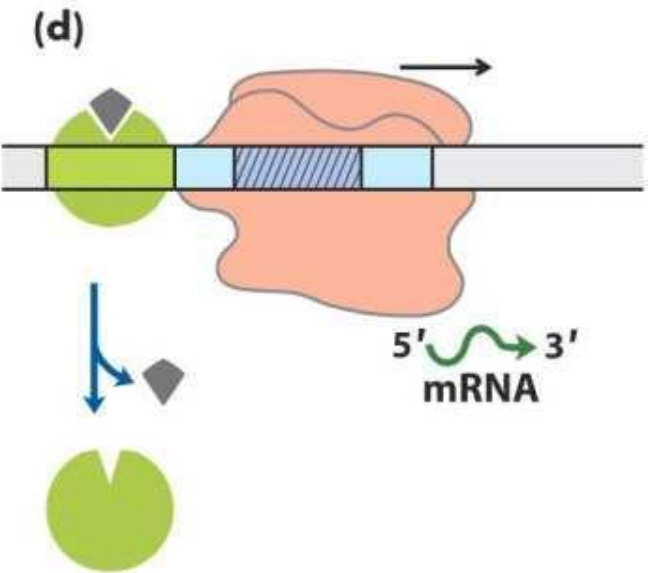
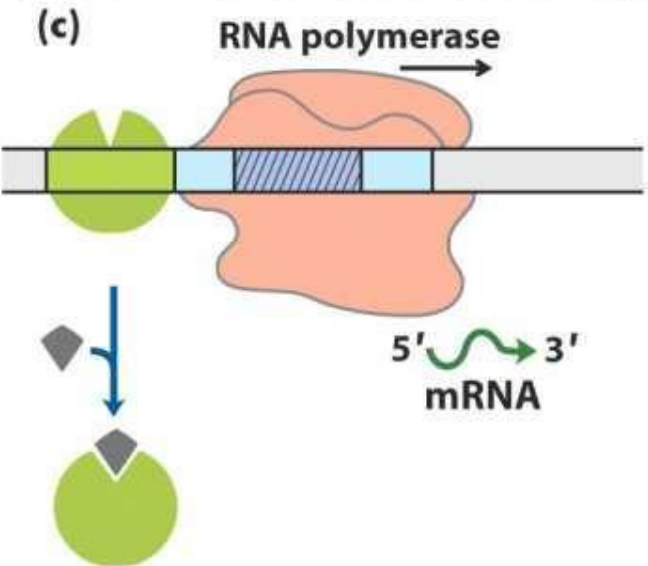


(B) Eukaryotic promoter site

Negative regulation
(bound repressor inhibits transcription)

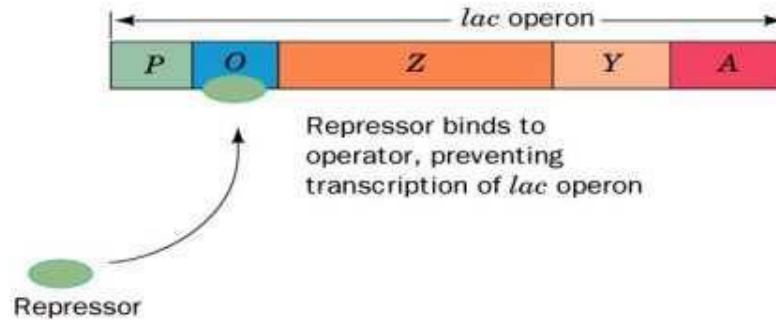


Positive regulation
(bound activator facilitates transcription)

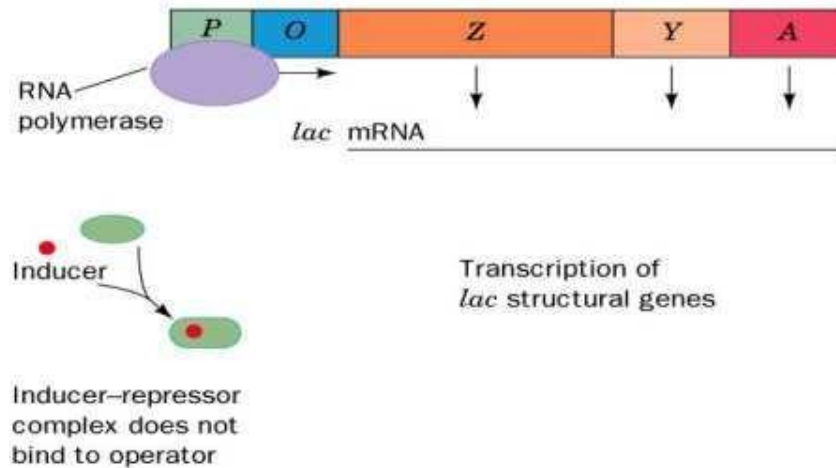


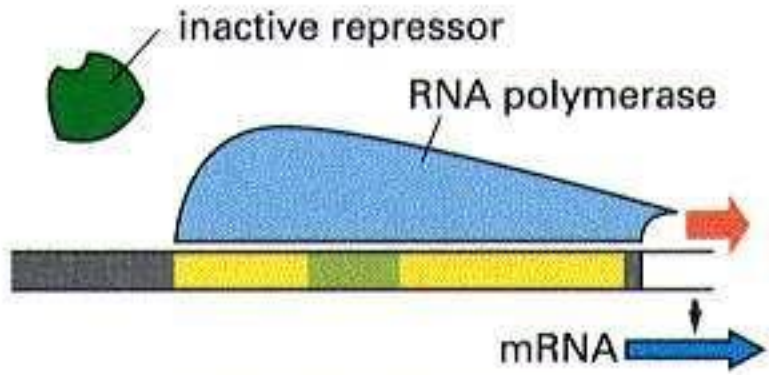
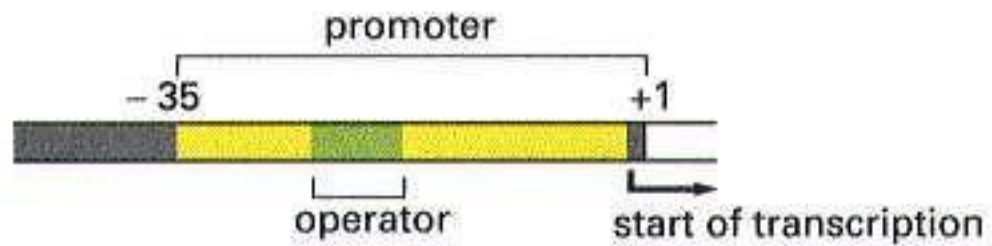
Control of transcription of the *lac* operon.

(a) Absence of inducer

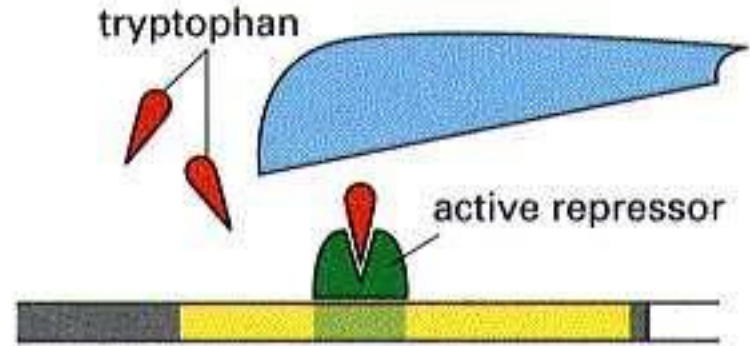


(b) Presence of inducer

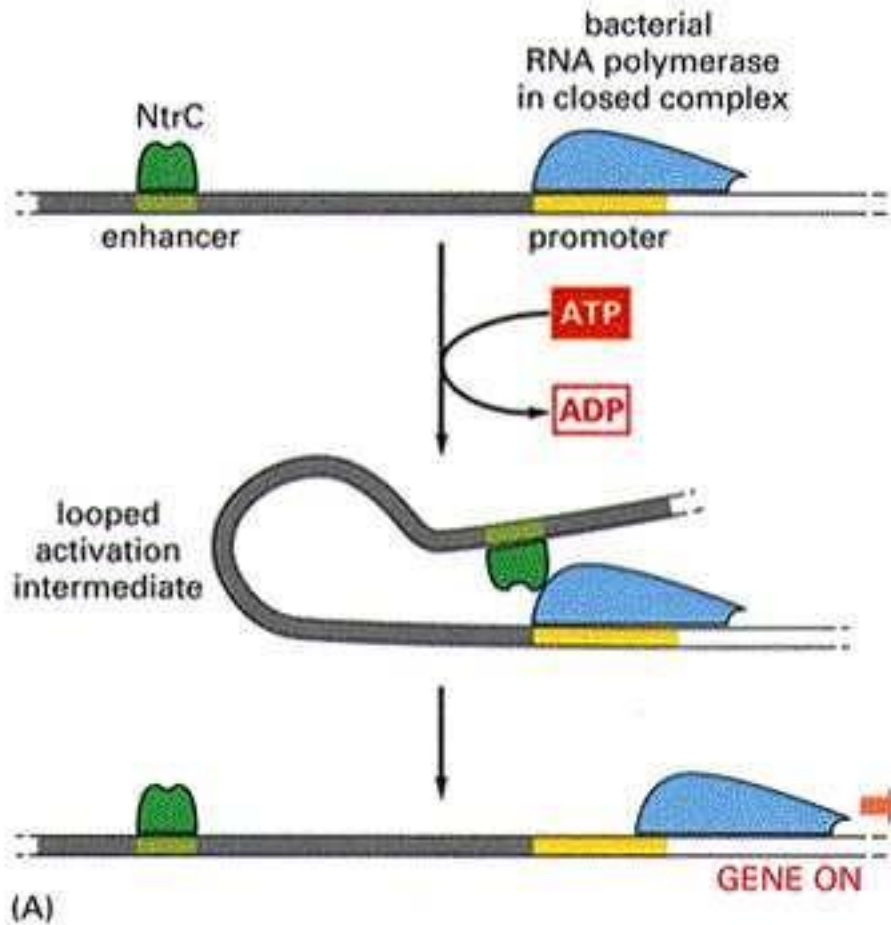


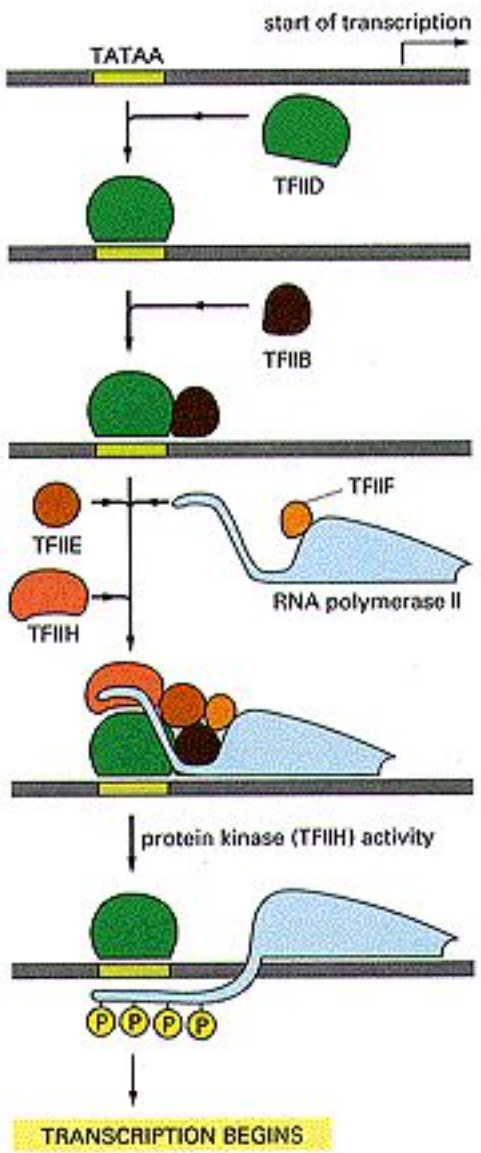


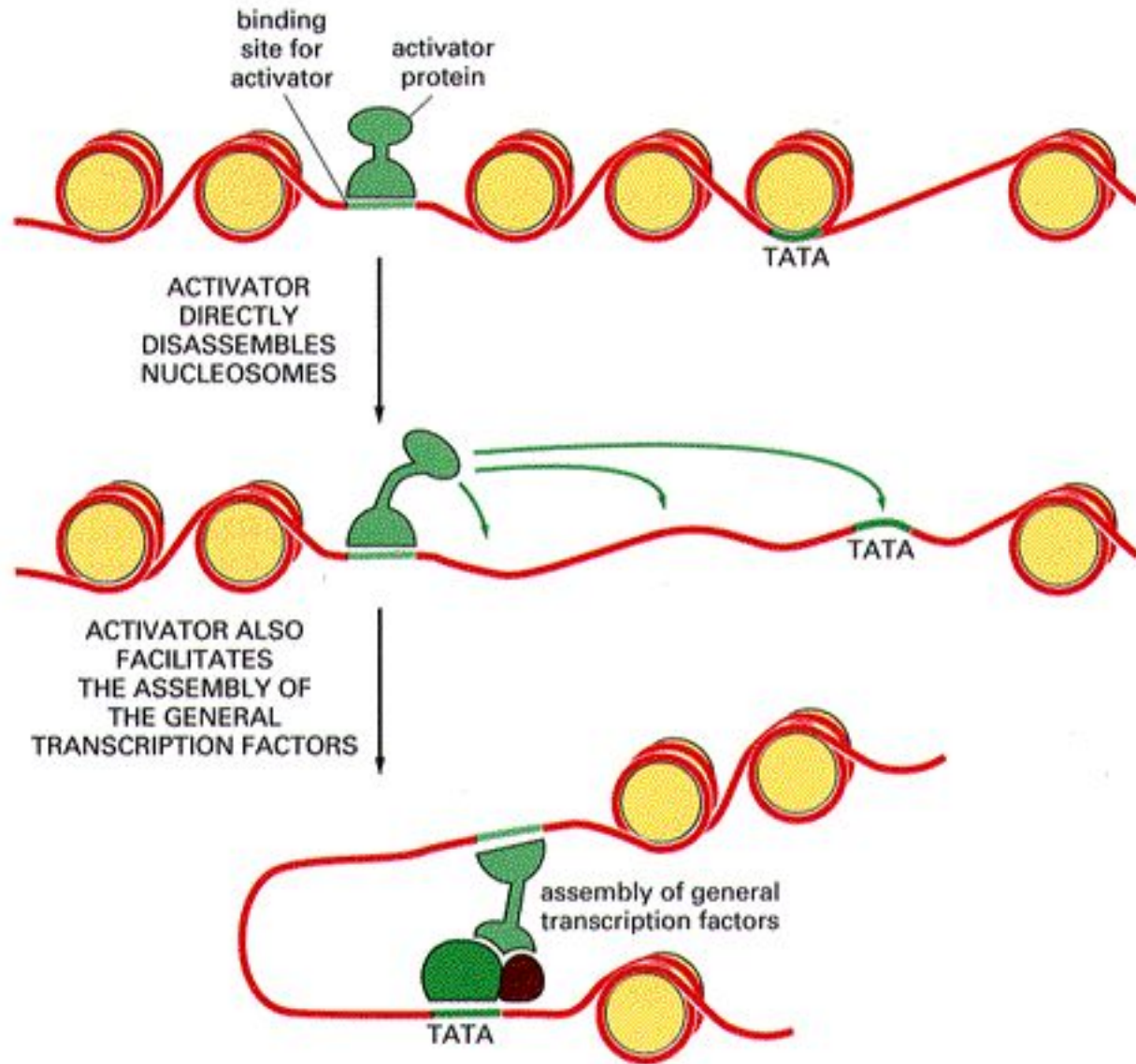
GENES ARE ON



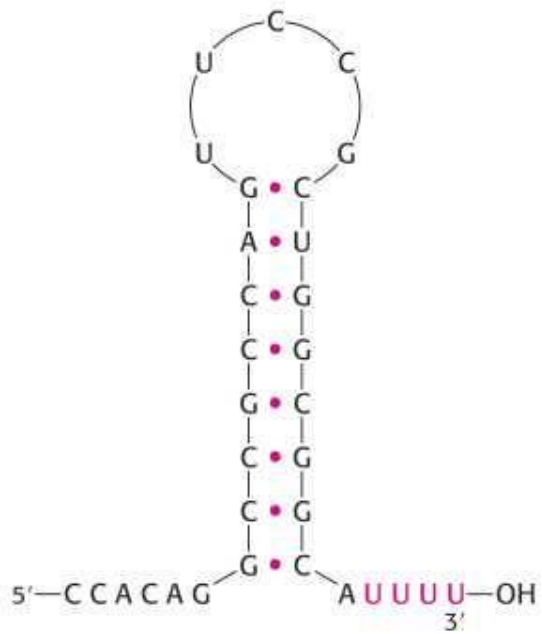
GENES ARE OFF



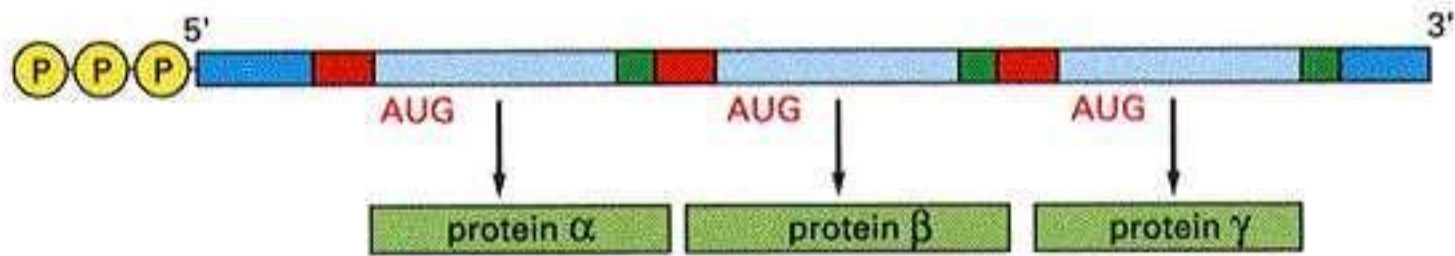




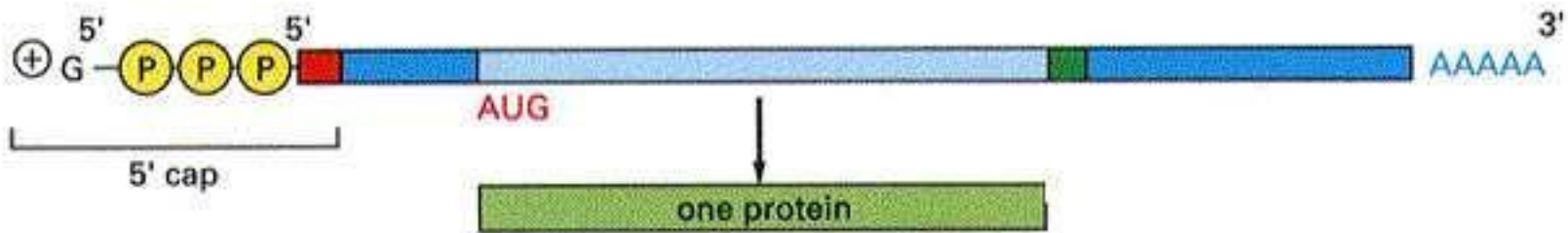
Terminators



procaryotic mRNA

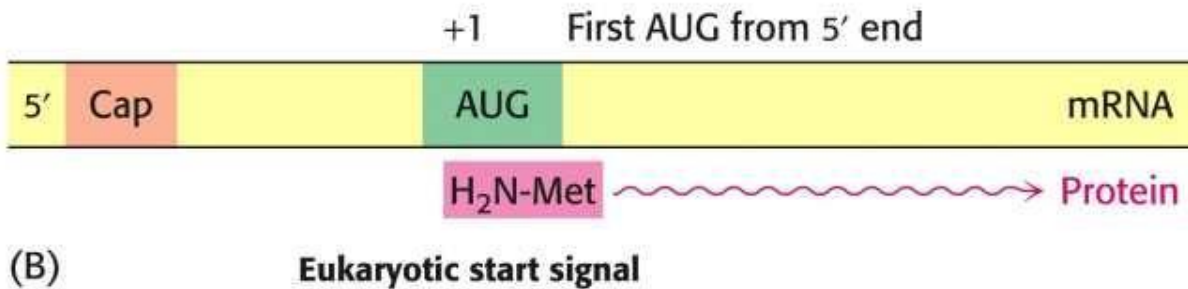
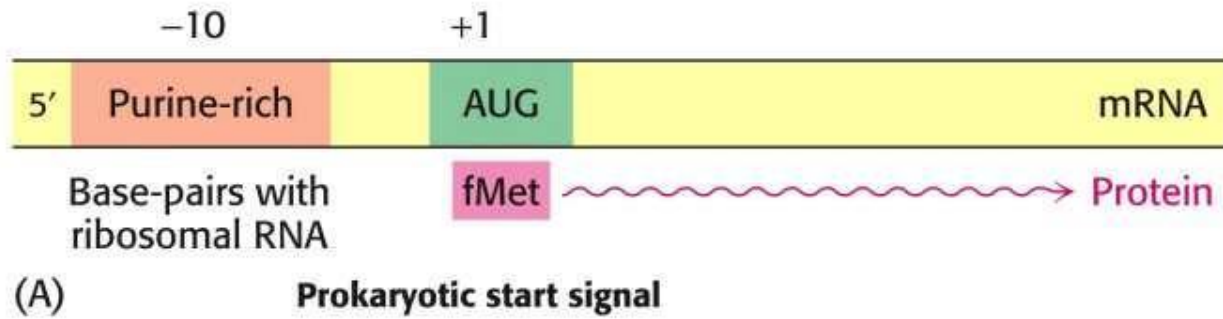


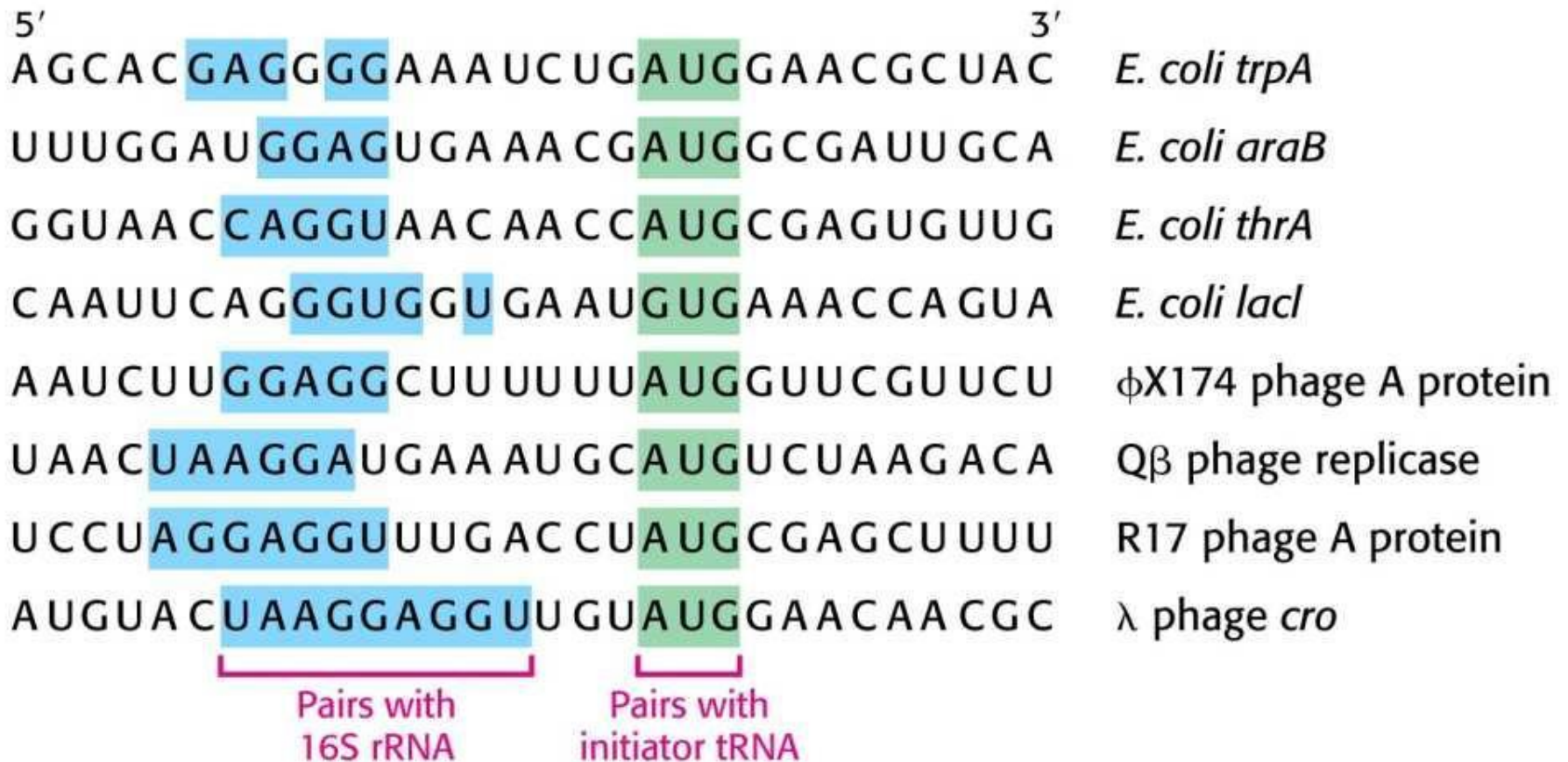
eucaryotic mRNA



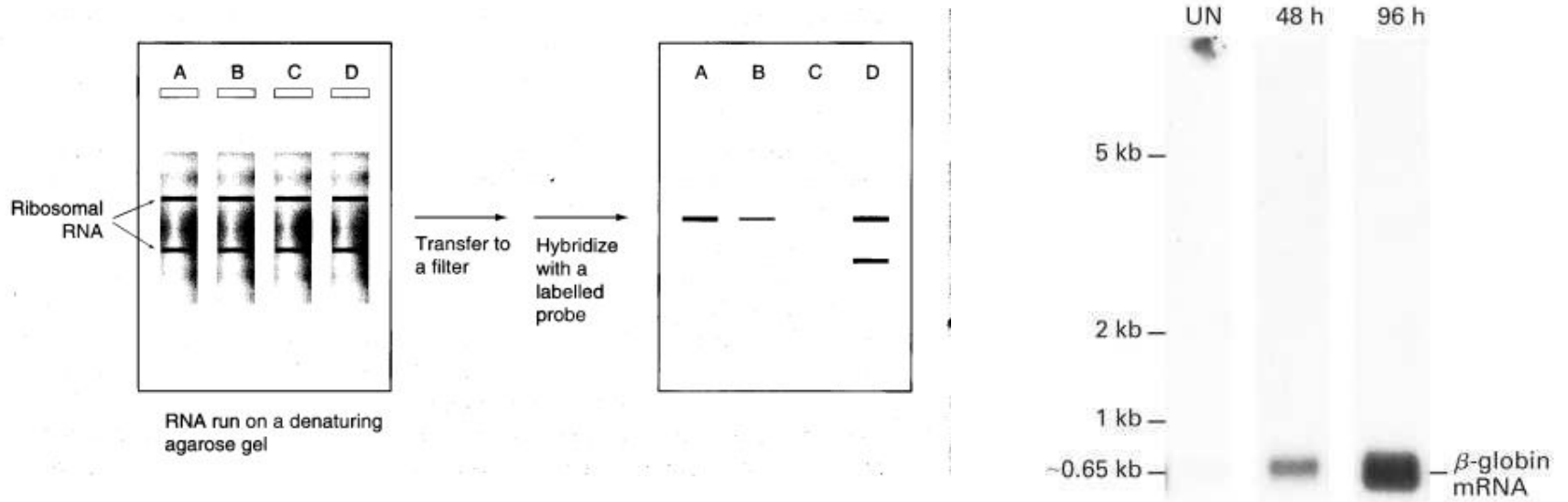
key:

-  ribosome-binding sites
-  coding sequences
-  noncoding sequences
-  stop codons





Northern Blot



-> to study transcription level

Expression studies by microarray technique

