

# NUKLEOVÉ KYSELINY

1. H. Braconnot (30. léta 19. století)

- Strassburg – vinné kvasinky – izolace „**matière animale**“.

2. J.F. Meischer (1868-1869)

- experimenty z hnísem a mlíčím rýnského lososa – **NUKLEIN**

3. Altman (1899)

pokračoval v experimentech svého předchůdce ( živočišné tkáně – thymus a kvasinky) – nazývá izolovanou látku – **kyselina nukleová - DNA**

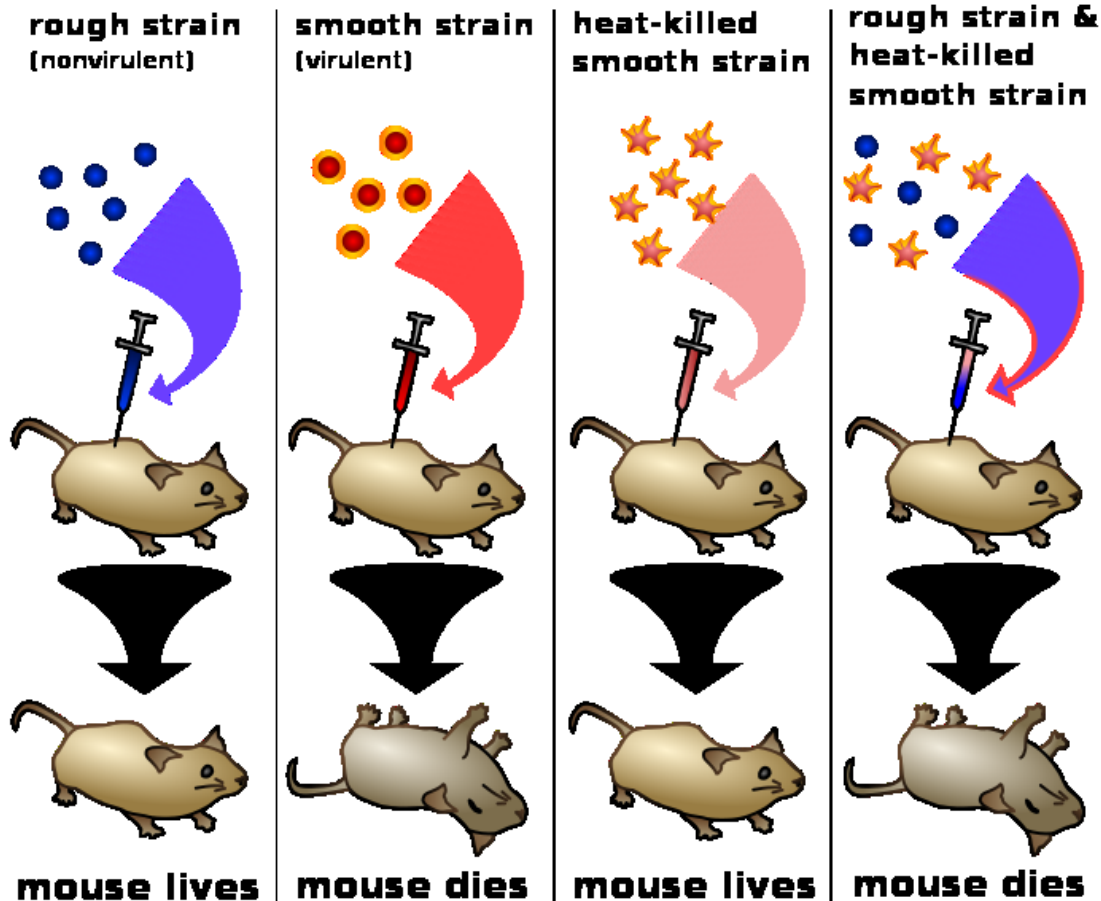
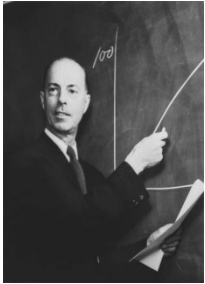
4. Leven (1909)

– **izoloval z NK kyselinu fosforečnou , cukr a báze – pojmenoval další typ NK – RNA**

5. Avery, MacLeod, McCarty (1943)

**DNA přenáší genetickou informaci – infekční princip**

# Avery-MacLeod-McCarty experiment – virulence DNA u *Pneumococcus*



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6. Watson, Crick, Wilkins, (1953) – **model sekundární struktury DNA – Nobelova cena 1962**

# NUKLEOVÉ KYSELINY

## Složení :

- Dusíkaté báze – purinové, pyrimidinové
- Sacharid – ribosa, deoxyribosa
- $\text{H}_3\text{PO}_4$



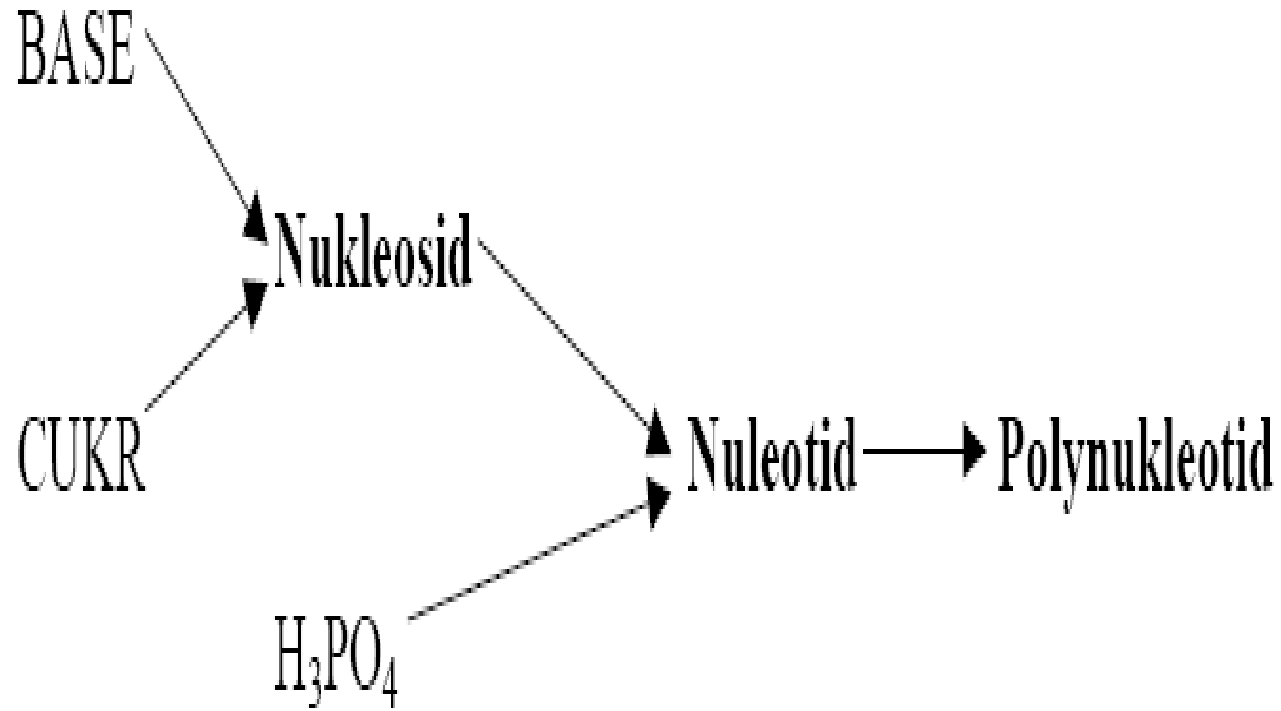
## Funkce :

DNA – nositel genetické informace

- Viry
- Prokaryonta – cytoplazma
- Eukaryonta – jádro, mitochondrie, chloroplasty

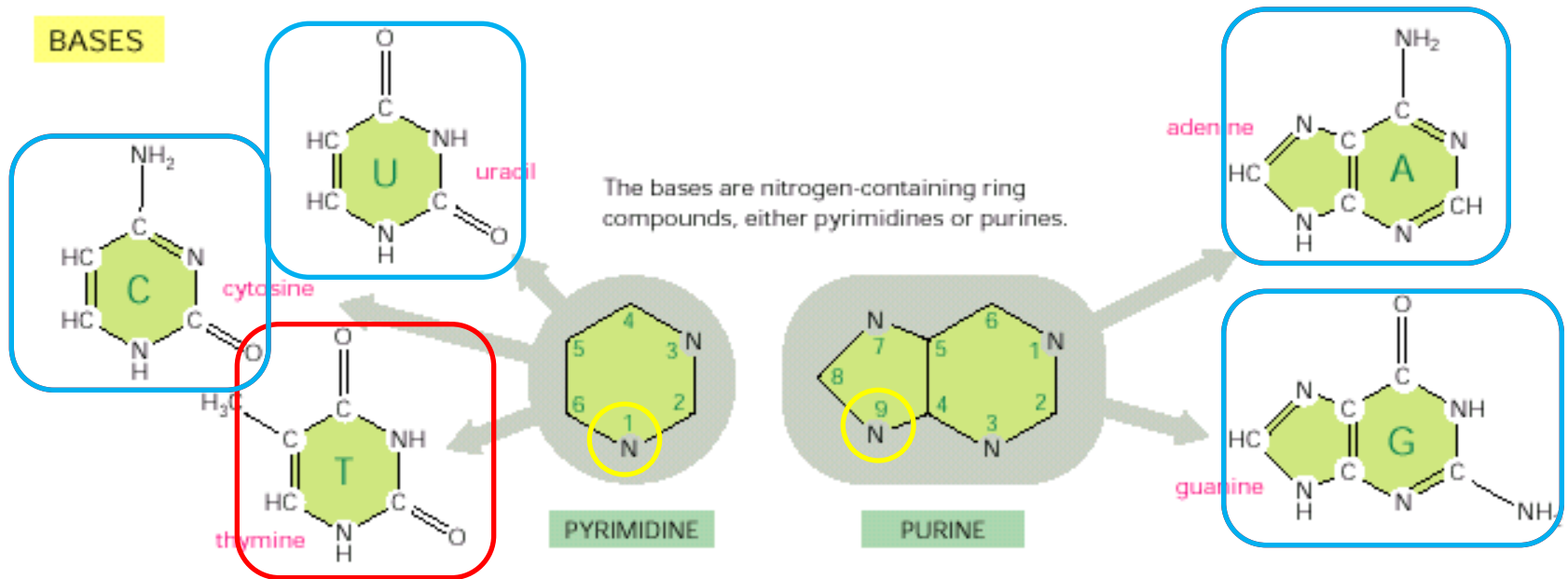
RNA – realizace genetické informace (u RNA virů i nositel genetické informace)

- Ribozymy - biokatalyzátory

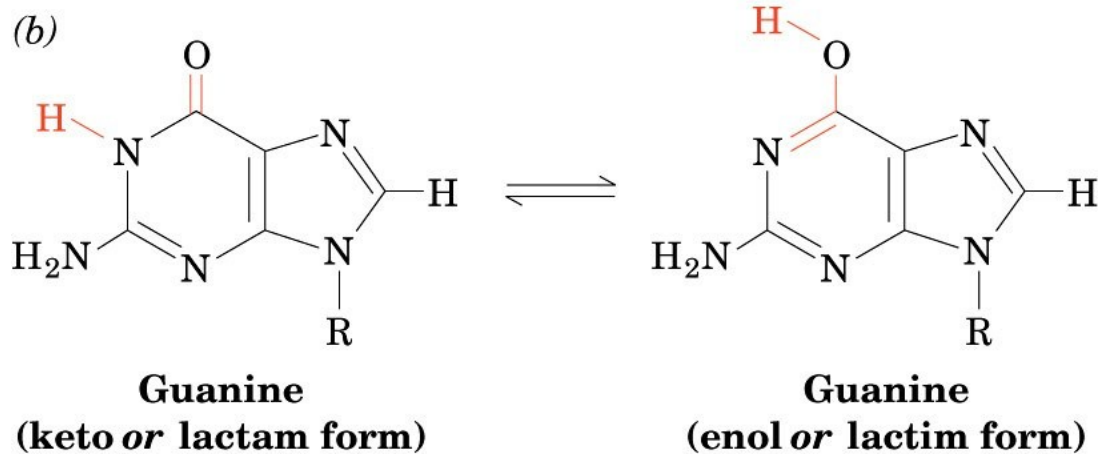
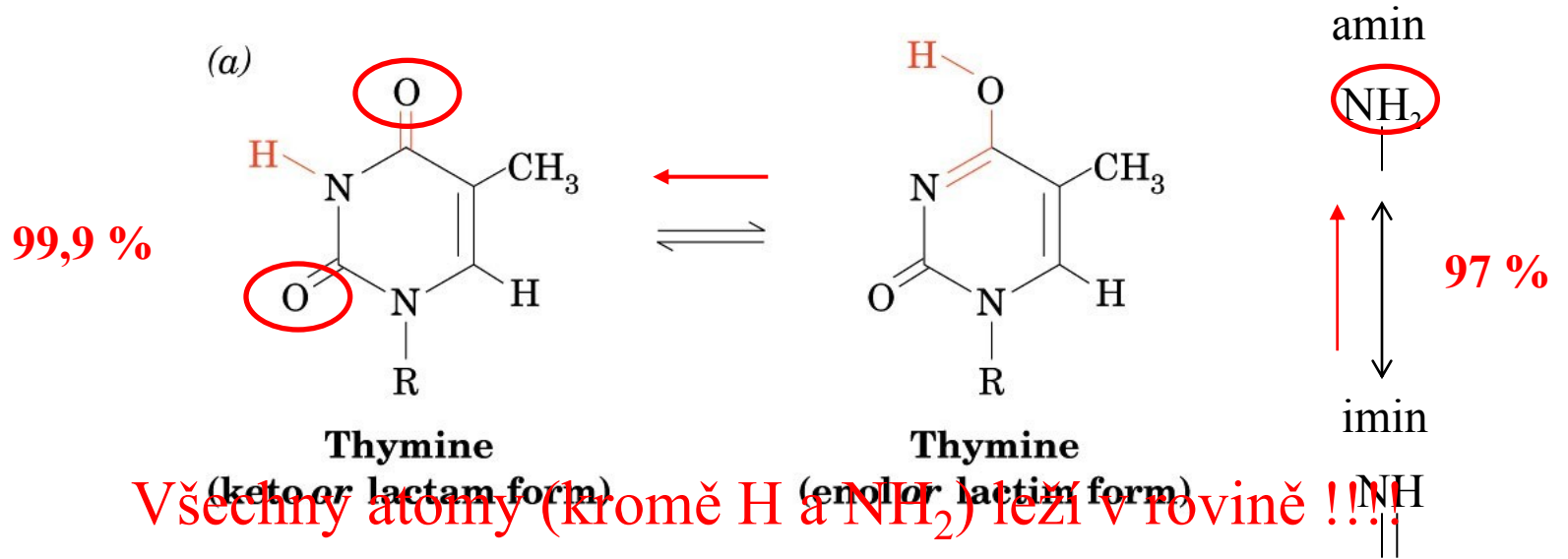


# Báze

## BNA



# Báze -tautomerie

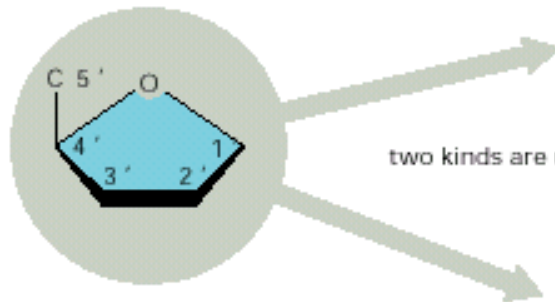


# Monosacharidy

## SUGARS

### PENTOSE

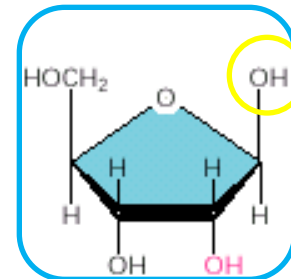
a five-carbon sugar



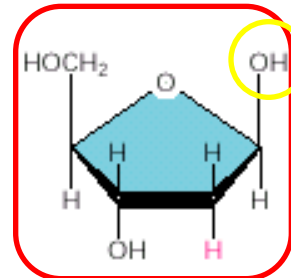
two kinds are used

Each numbered carbon on the sugar of a nucleotide is followed by a prime mark; therefore, one speaks of the "5-prime carbon," etc.

## RNA



$\beta$ -D-ribose  
used in ribonucleic acid

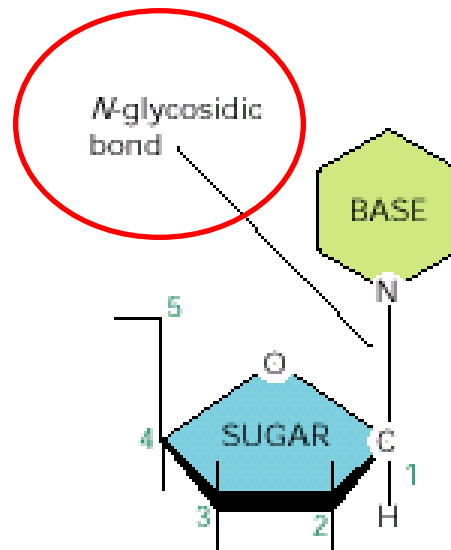


$\beta$ -D-2-deoxyribose  
used in deoxyribonucleic acid

## DNA

# Nukleosid

## BASIC SUGAR LINKAGE

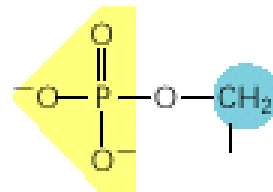


The base is linked to the same carbon (C1) used in sugar-sugar bonds.

# Kyselina fosforečná

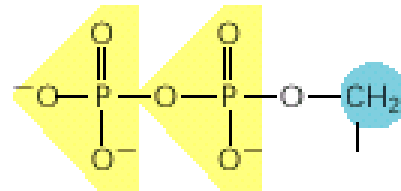
## PHOSPHATES

The phosphates are normally joined to the C5 hydroxyl of the ribose or deoxyribose sugar (designated 5'). Mono-, di-, and triphosphates are common.



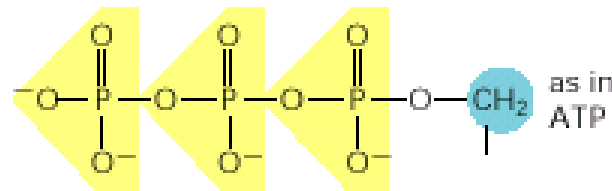
as in  
AMP

Ester



as in  
ADP

Anhydrid



as in  
ATP

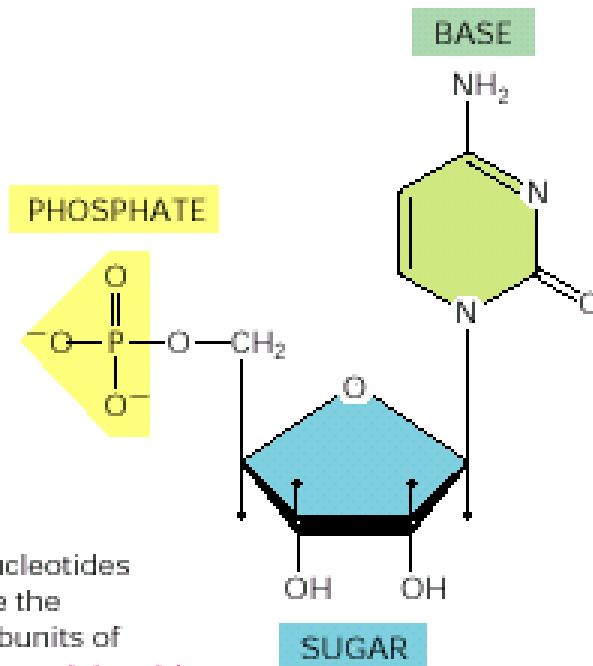
Anhydrid

The phosphate makes a nucleotide negatively charged.

# Nukleotid

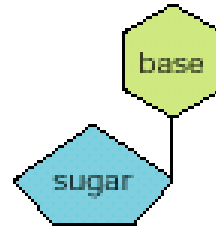
## NUCLEOTIDES

A nucleotide consists of a nitrogen-containing base, a five-carbon sugar, and one or more phosphate groups.

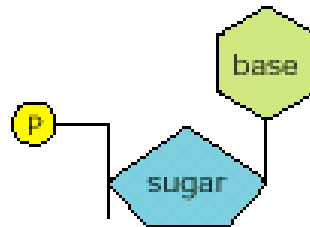




# Názvosloví



BASE + SUGAR = NUCLEOSIDE



BASE + SUGAR + PHOSPHATE = NUCLEOTIDE

BASE	NUCLEOSIDE	ABBR.
adenine	adenosine	A
guanine	guanosine	G
cytosine	cytidine	C
uracil	uridine	U
thymine	thymidine	T

Nucleotides are abbreviated by three capital letters. Some examples follow:

AMP = adenosine monophosphate

dAMP = deoxyadenosine monophosphate

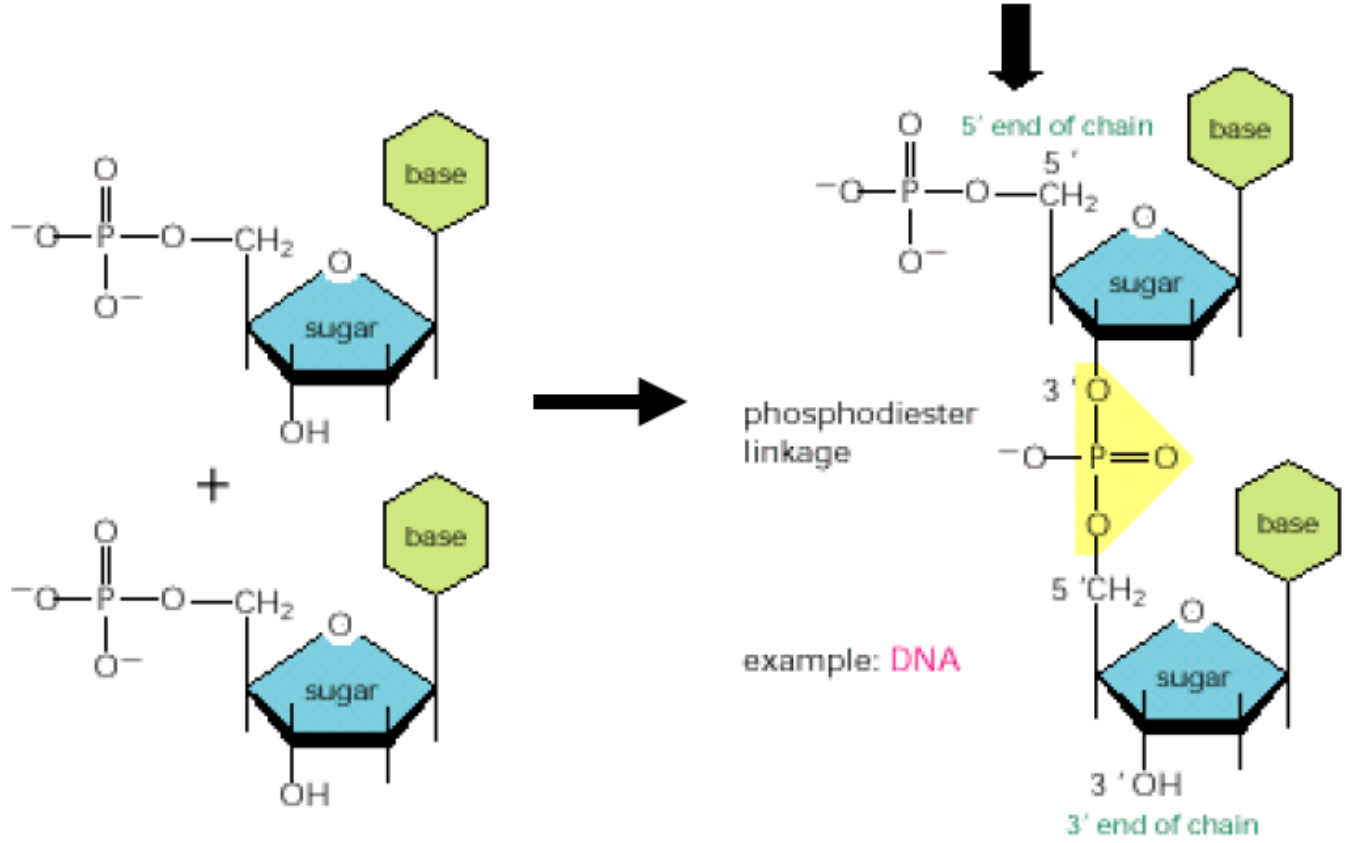
UDP = uridine diphosphate

ATP = adenosine triphosphate

# Funkce nukleotidů

- přenašeče energie (ATP, GTP)
- fosforylační činidla (ATP – kinasy)
- aktivátory meziproductů biosyntéz – UDP-glukosa, CTP-cholin
- součásti kofaktorů - NAD(P), FAD, PAPS,
- využití v terapii - antivirotika(AIDS, herpes) – AZT
- **stavební složky nukleových kyselin**

# Polynukleotid – nukleová kyselina



## Struktura a funkce DNA

A,T,G,C + deoxyribosa

Primární struktura – sekvence basí

Sekundární struktura – Watson, Crick (1953) – dvojšroubovice

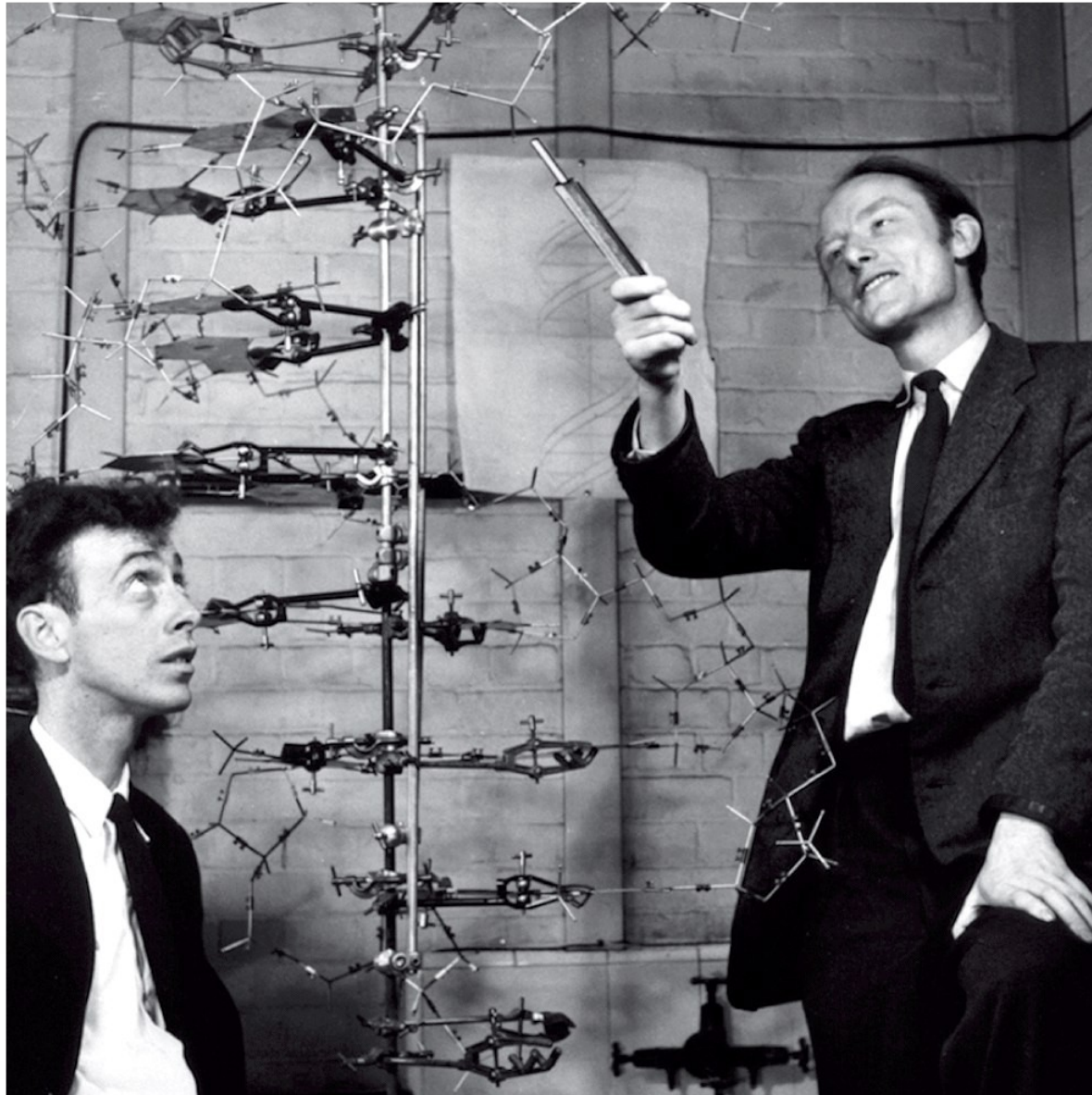
- Chragaffovy pravidla – poměr basí v DNA

$A+G=T+C$     $A=T$     $G=C$     $A+C=G+T$

- Donohue – báze v tautomerních ketoformách

- Franklinová – RTG difrakční analýza

Watson



Crick

Unnumbered figure pg 9 Concepts in Biochemistry, 3/e

# Chargaffovy pravidla

# Chargaffovy pravidla

## Zastoupení basí v DNA (molární %)

<u>Organismus</u>	<u>A</u>	<u>T</u>	<u>G</u>	<u>C</u>
Člověk	30.9	29.4	19.9	19.8
Kuře	28.8	29.2	20.5	21.5
Kobylka luční	29.3	29.3	20.5	20.7
Pšenice	27.3	27.1	22.7	22.8
Kvasinky	31.3	32.9	18.7	17.1
E. coli	24.7	23.6	26.0	25.7

nezávisí na tkáni

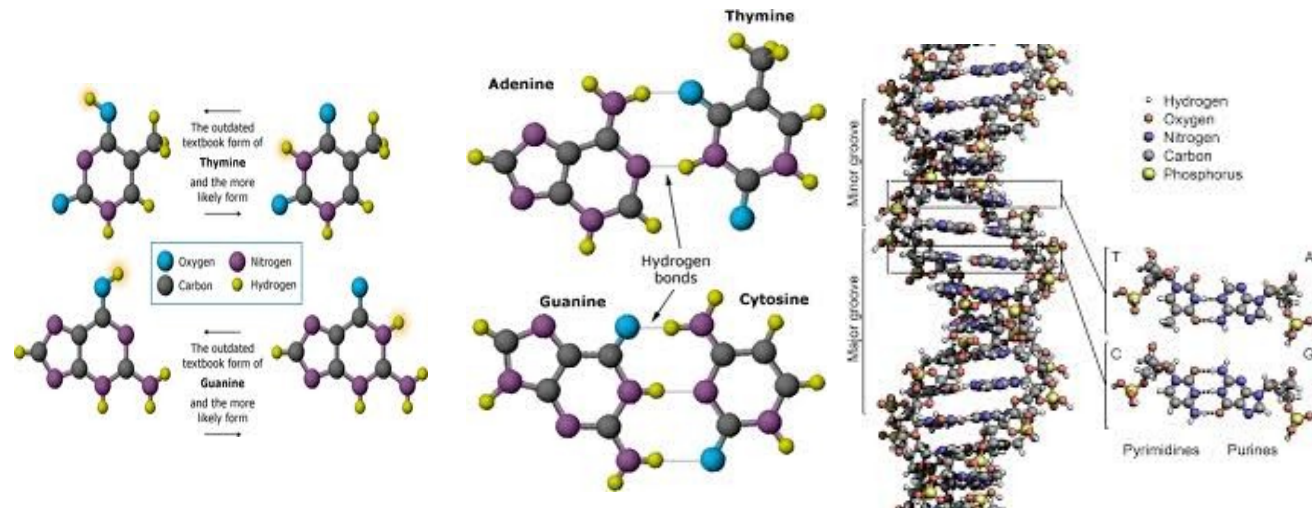
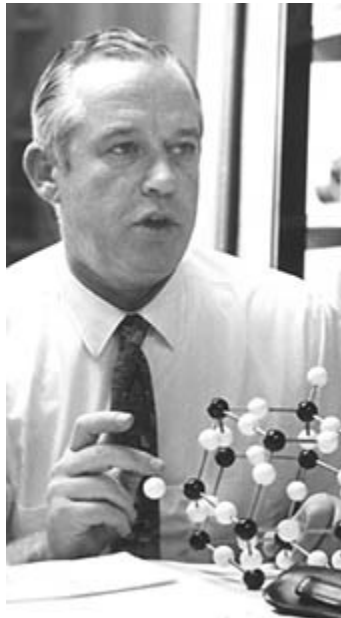
nezávisí na stáří

nezávisí na nutričních faktorech

nezávisí na životním prostředí

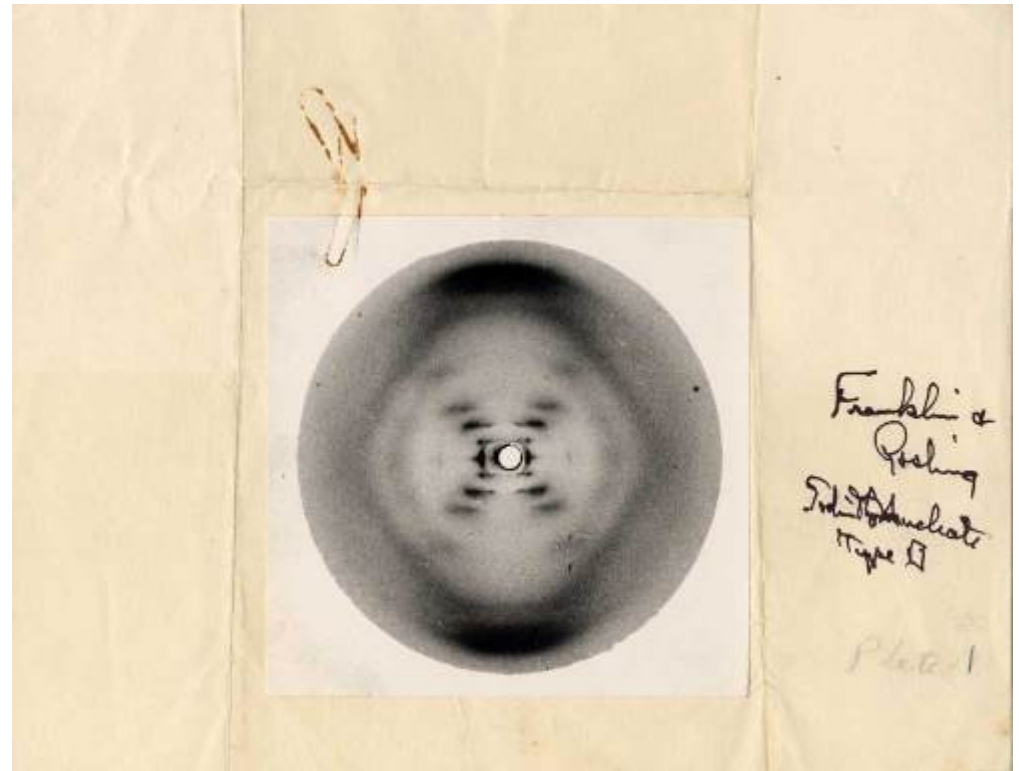
# Jerry Donohue

## tautomerní formy bází

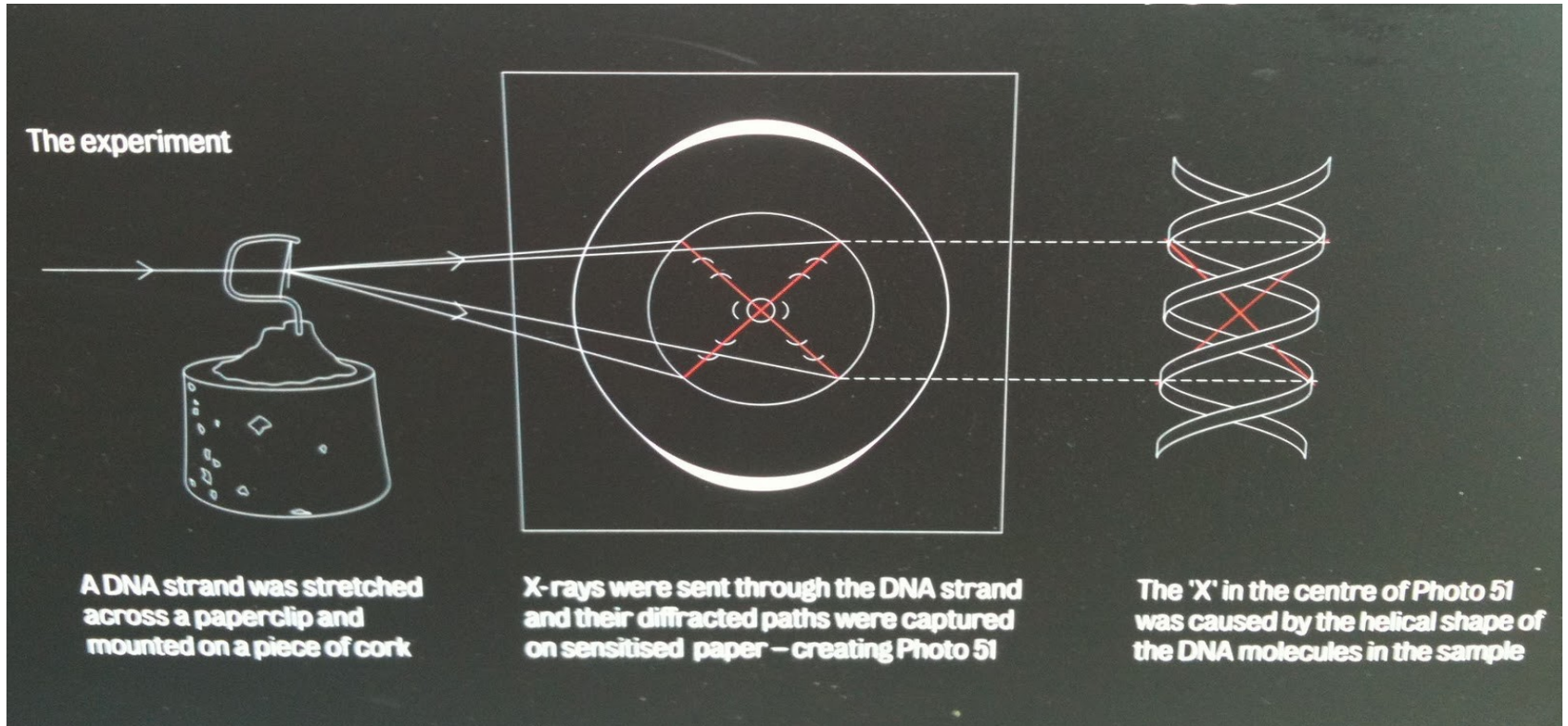




# Rosalind Franklin



## Obrázek 51



# Nobelova cena 1962



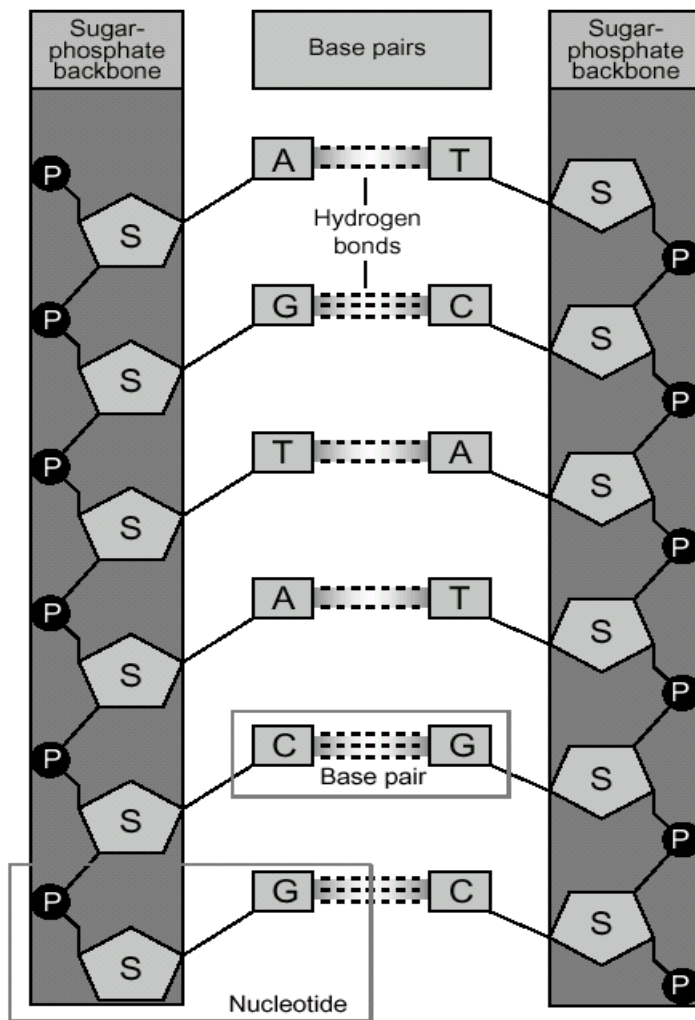
**Francis Harry  
Compton Crick  
(1916-2004)**



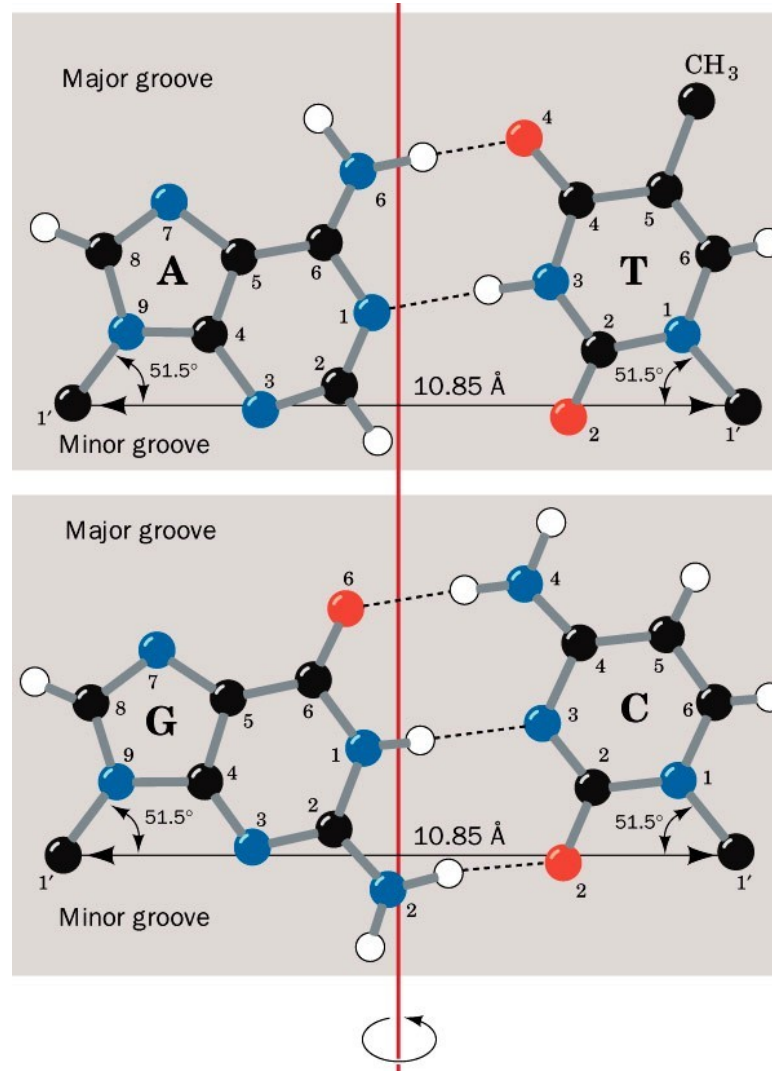
**James Dewey  
Watson  
(1928 - )**



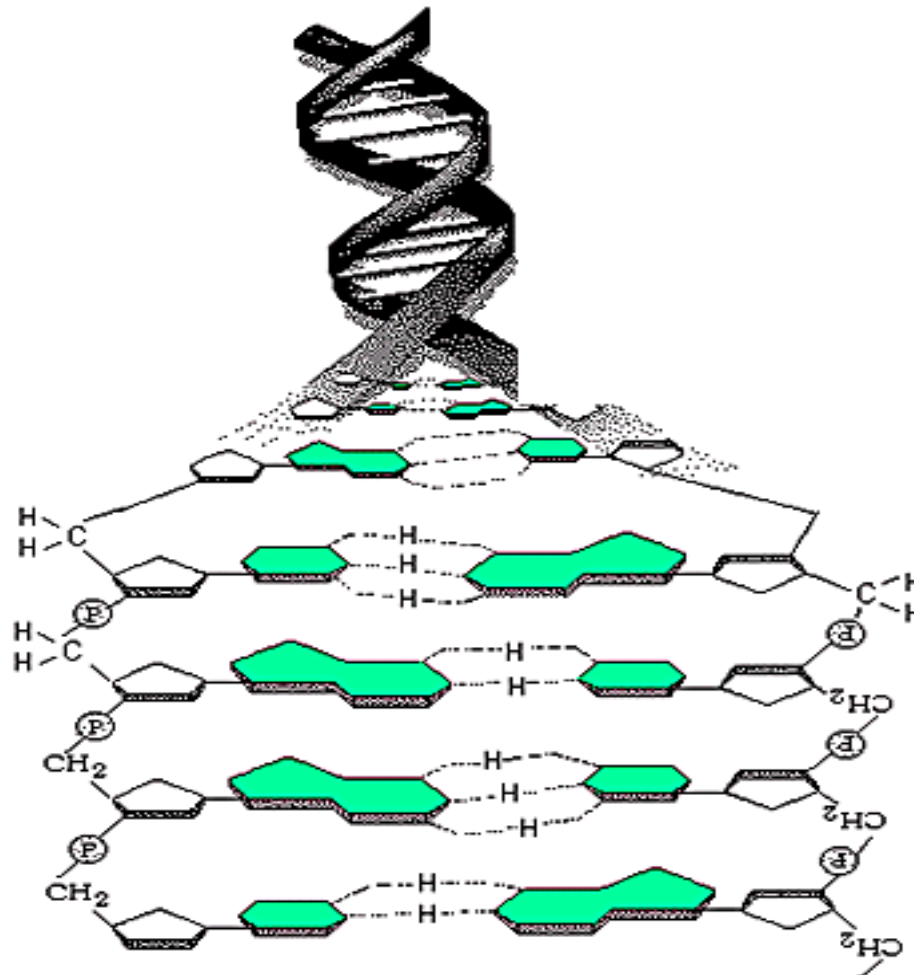
**Maurice Hugh  
Frederick Wilkins  
(1916-2004)**



# Párování basi – H můstky

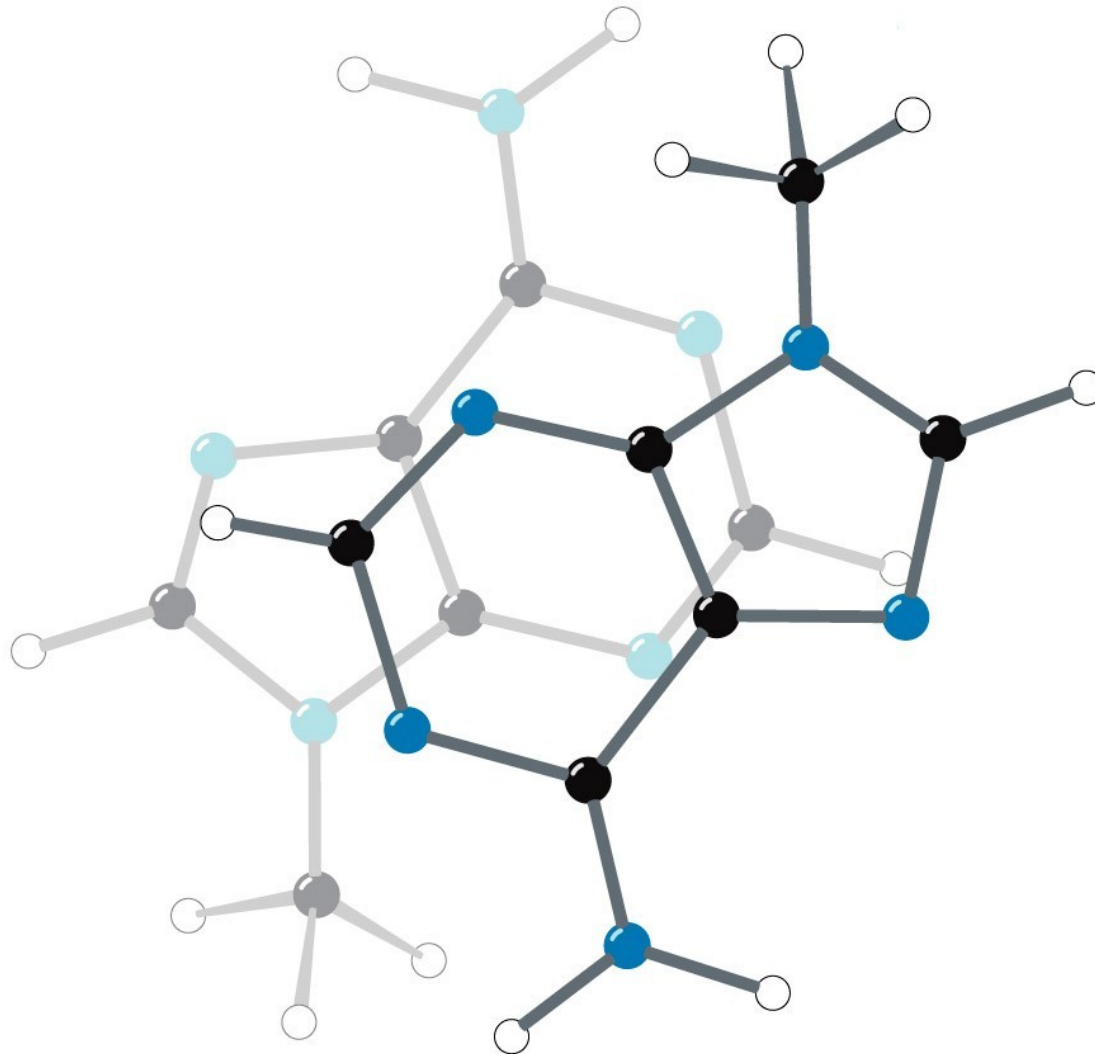


# Stabilizující vazby v DNA

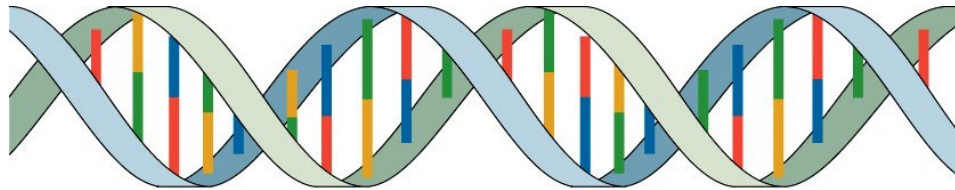




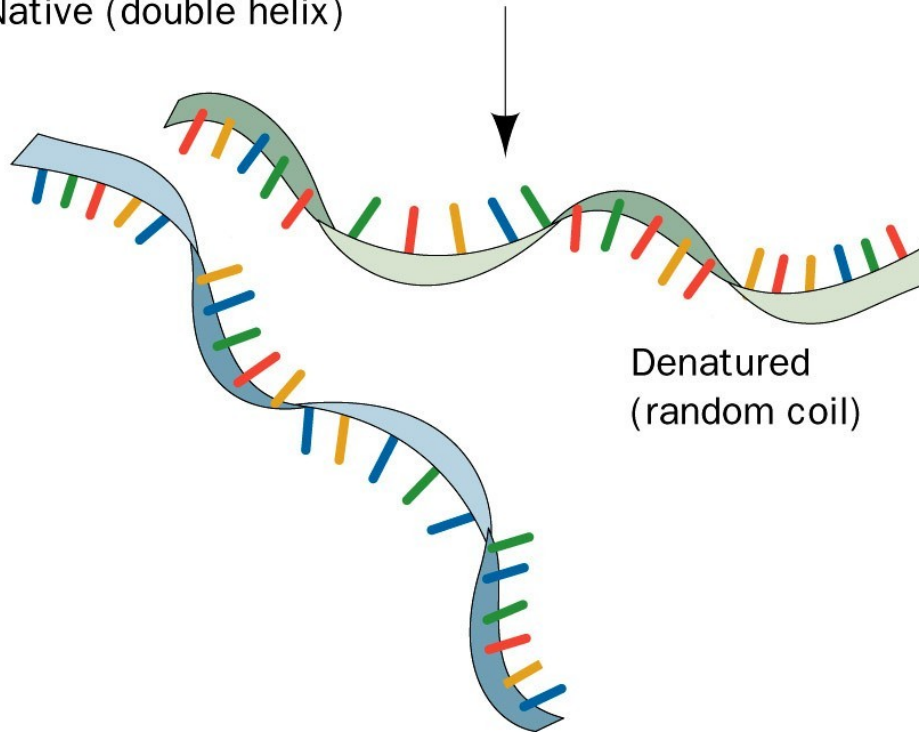
# Stohování bází



# Denaturace - renaturace



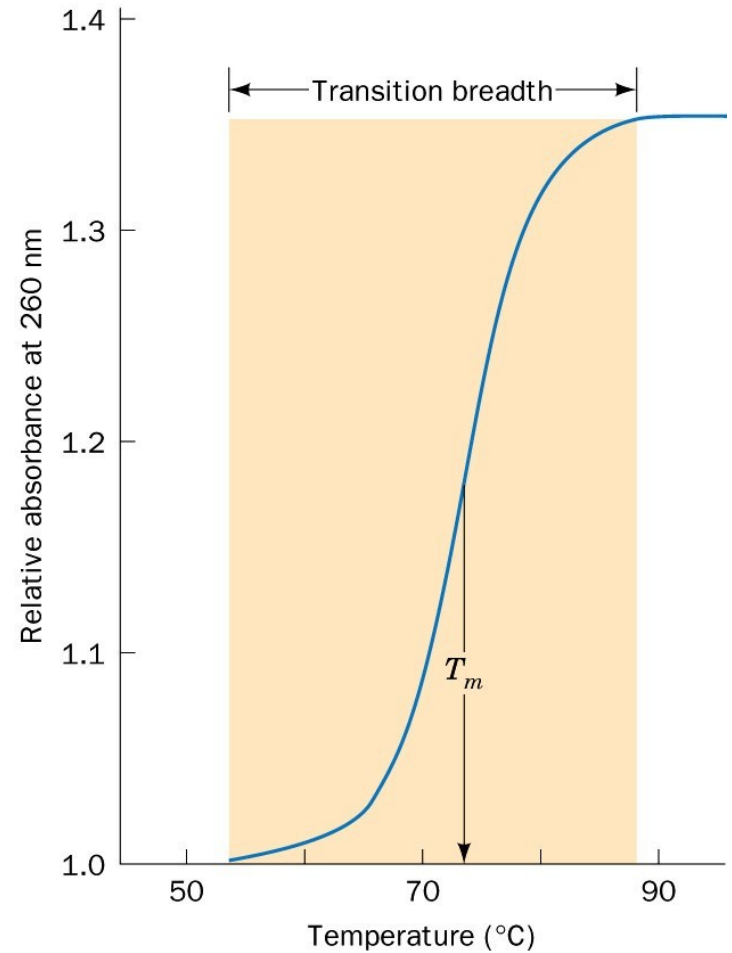
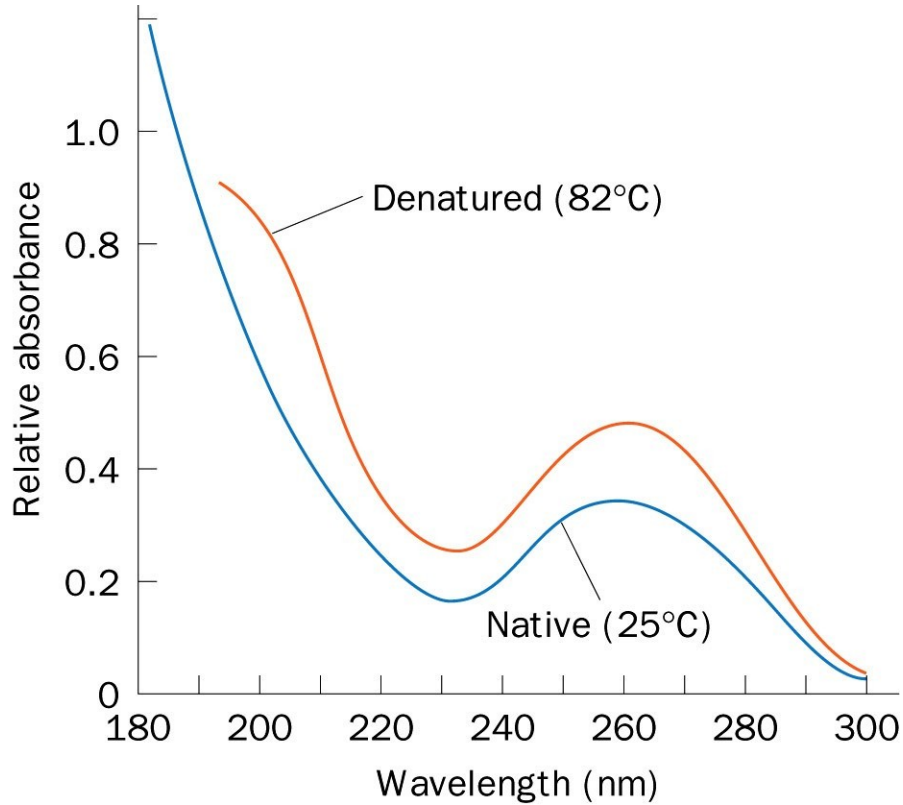
Native (double helix)



Denatured  
(random coil)



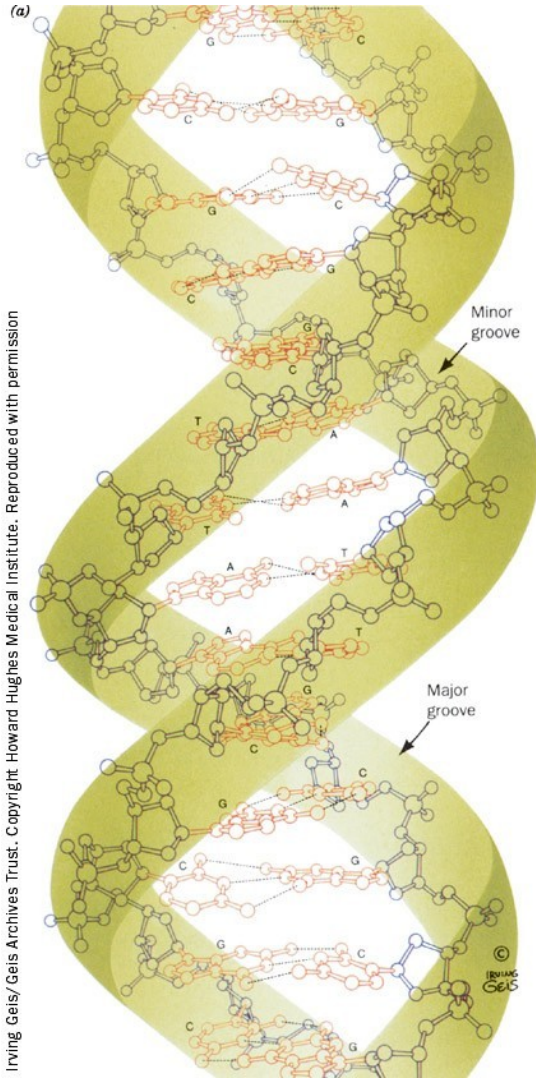
# Denaturace - renaturace



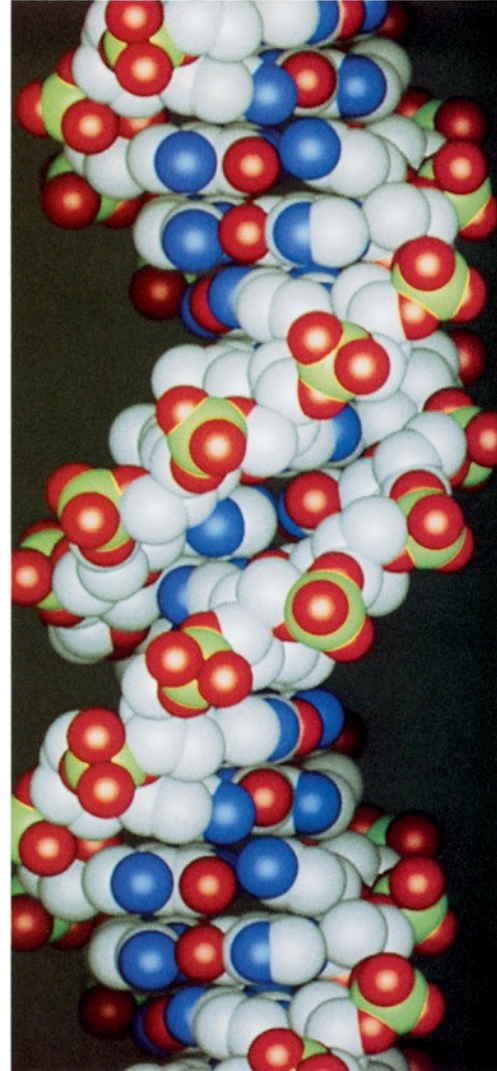
## Formy DNA

- B - DNA - nativní 92 % H<sub>2</sub>O, Na<sup>+</sup>  
pravotočivá - 10 párů bází na závit
- A - DNA - 75 % H<sub>2</sub>O, rovina bází 20°  
pravotočivá - 11 párů bází na závit
- Z - DNA - d(CGCGCG)  
levotočivá - 12 párů bází na závit

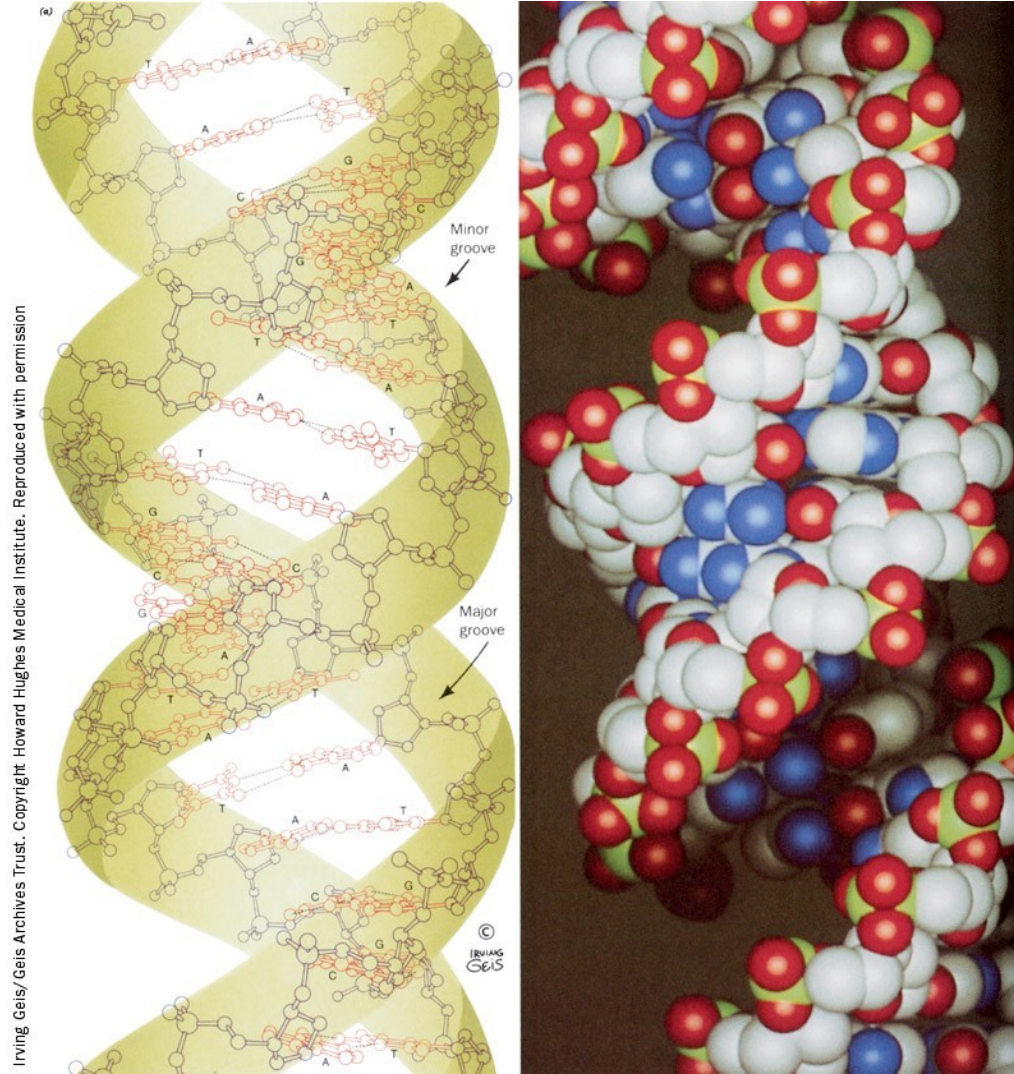
# B DNA



Irving Geis/Geis Archives Trust. Copyright Howard Hughes Medical Institute. Reproduced with permission

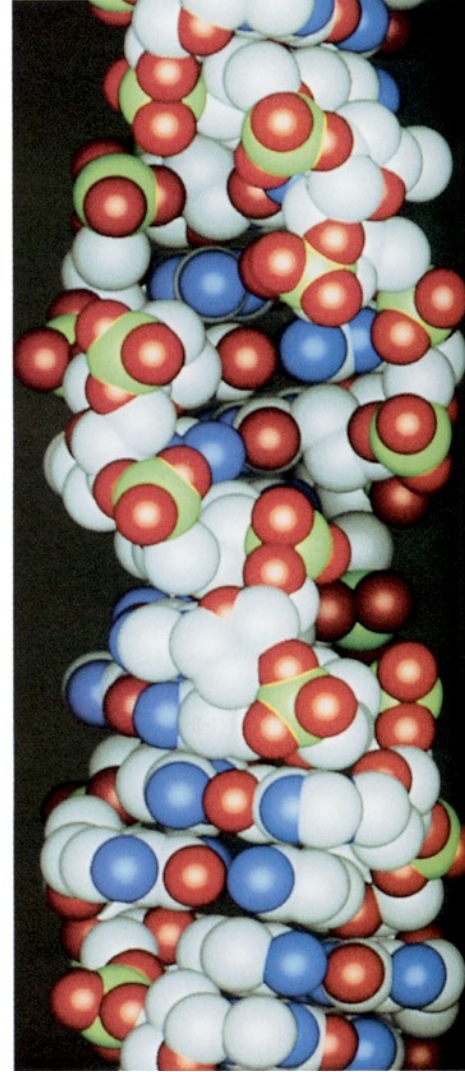
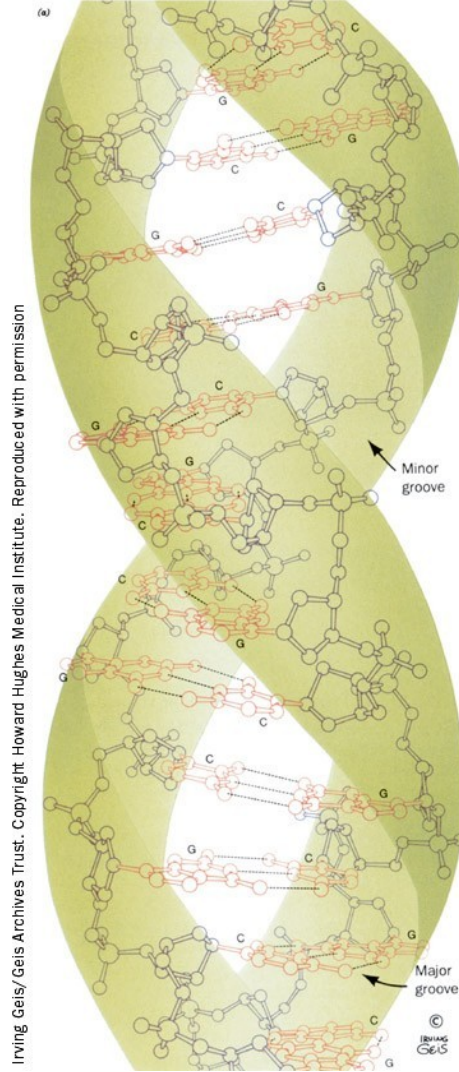


# A DNA



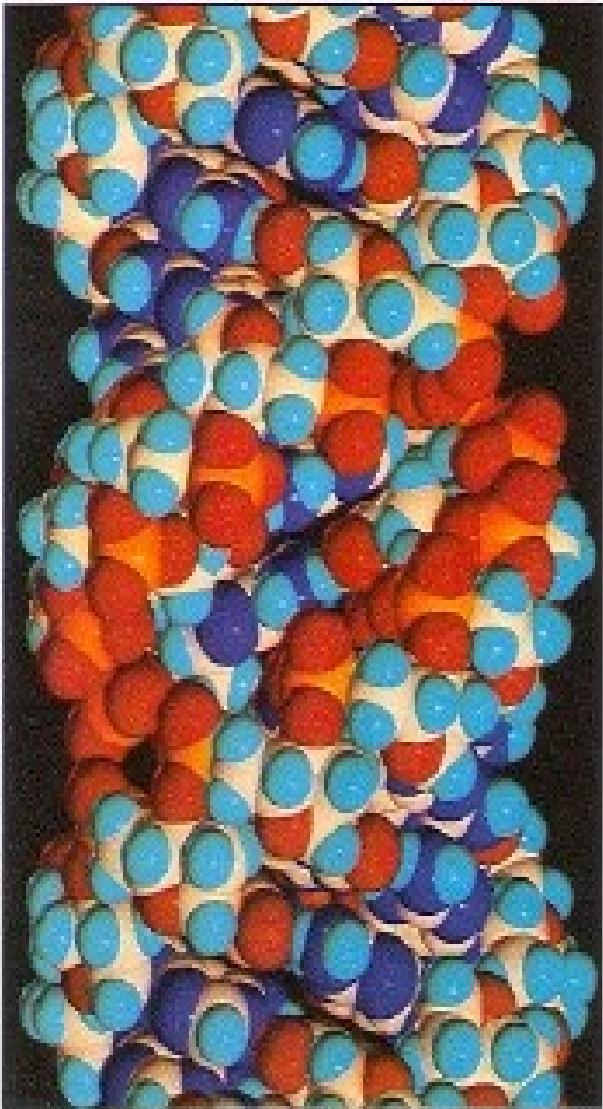


# Z DNA

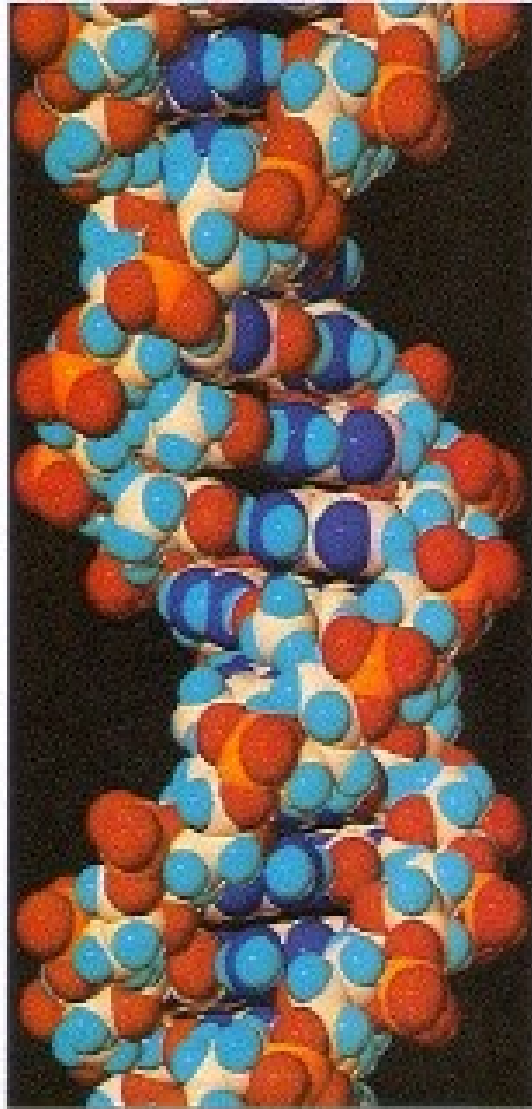


## Základní číselné charakteristiky tří nejznámějších helikálních forem DNA

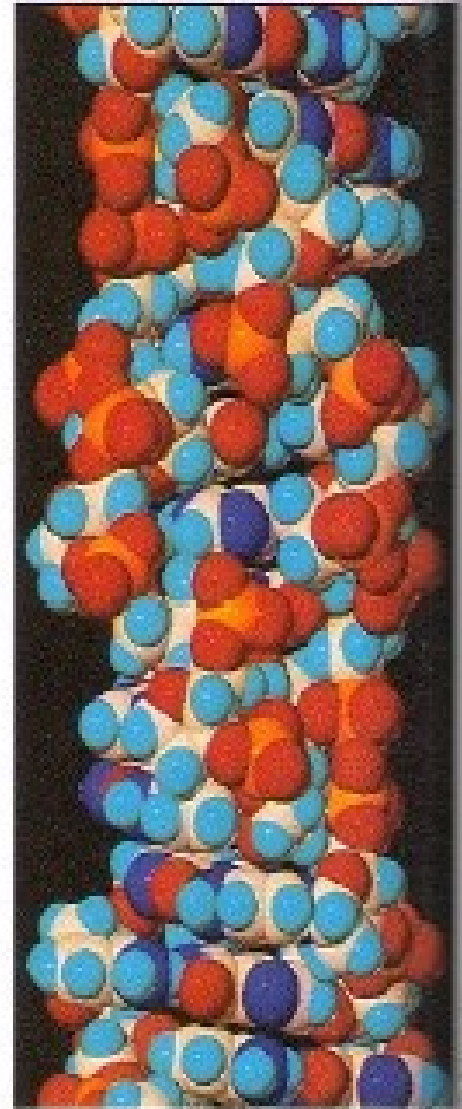
Atribut	A-DNA	B-DNA	Z-DNA
Točivá tendence šroubovice (chiralita) <sup>[1]</sup>	pravotočivá	pravotočivá	levotočivá
Opakování <sup>[2]</sup>	po každém páru	po každém páru	po každých dvou párech
Otočení po každém opakování <sup>[1]</sup>	32,7°	34,3°	
Průměrný počet párů na jedno otočení šroubovice <sup>[1]</sup>	11	10,5	12
Sklon páru k ose <sup>[2]</sup>	20°	6°	7°
Vzestup vůči ose na jeden pár <sup>[1]</sup>	2,55 Å (0,25 nm)	3,4 Å (0,34 nm)	3,7 Å (0,37 nm)
Průměr <sup>[1]</sup>	23 Å (2,3 nm)	20 Å (2,0 nm)	18 Å (1,8 nm)



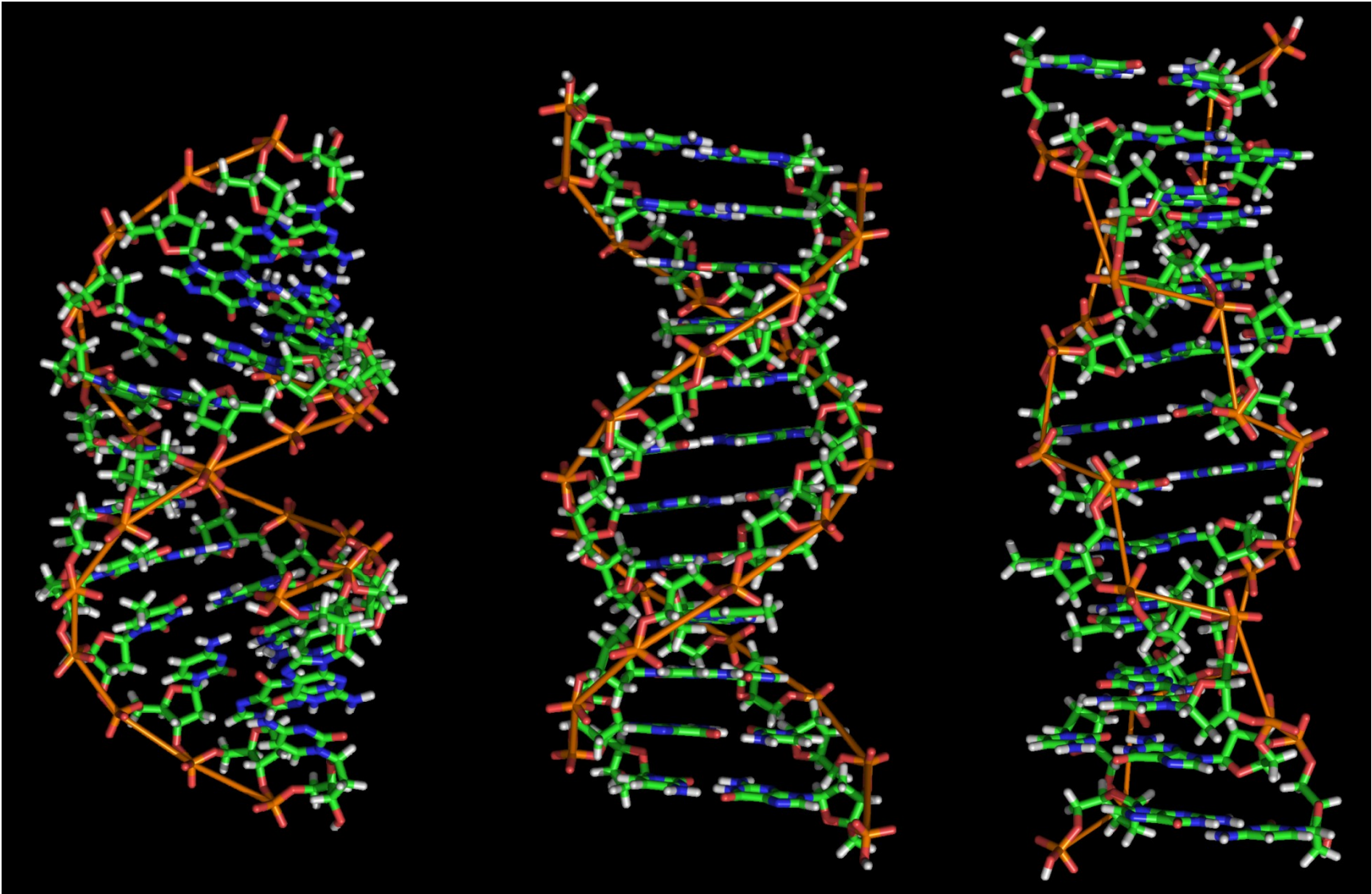
A-DNA



B-DNA

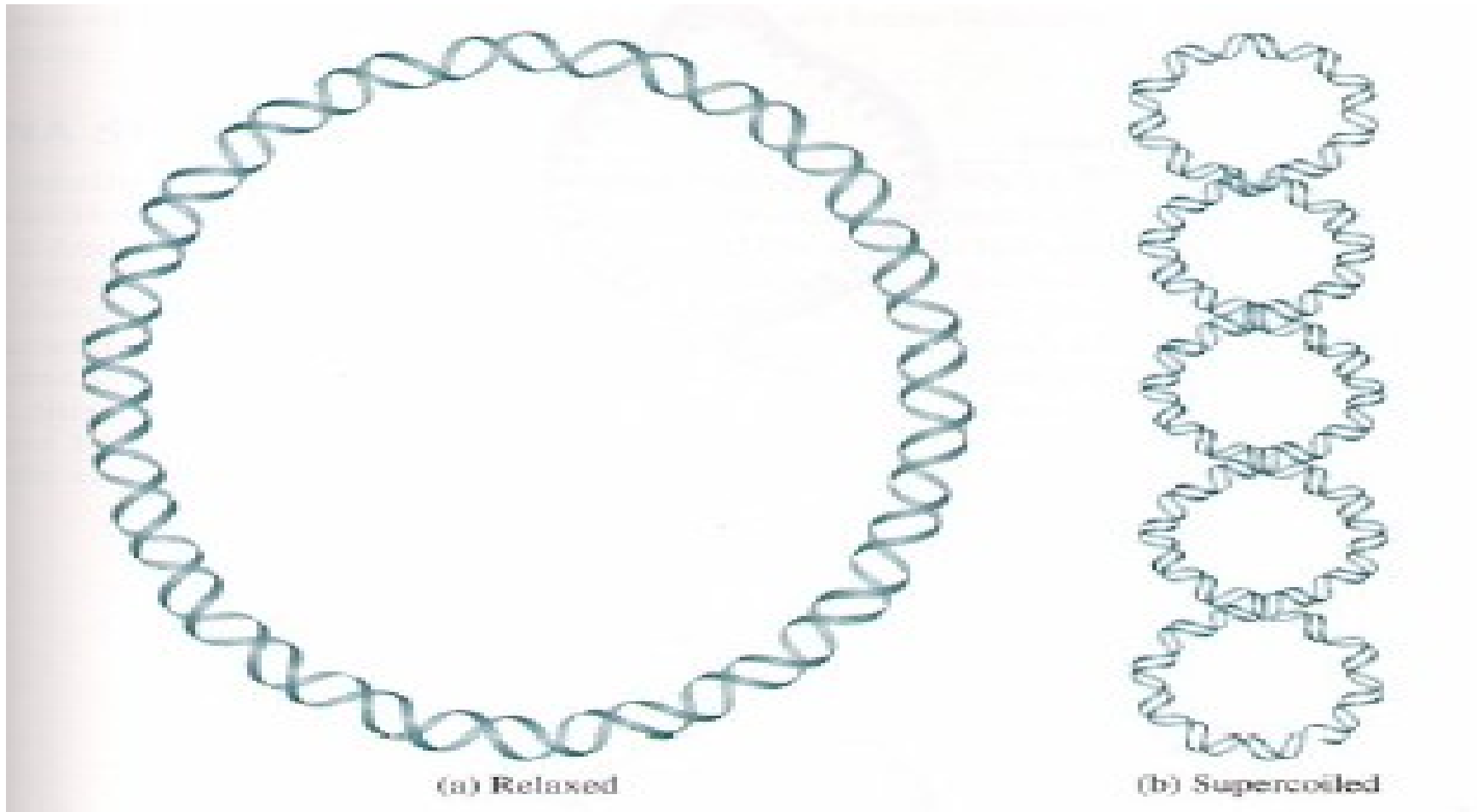


Z-DNA

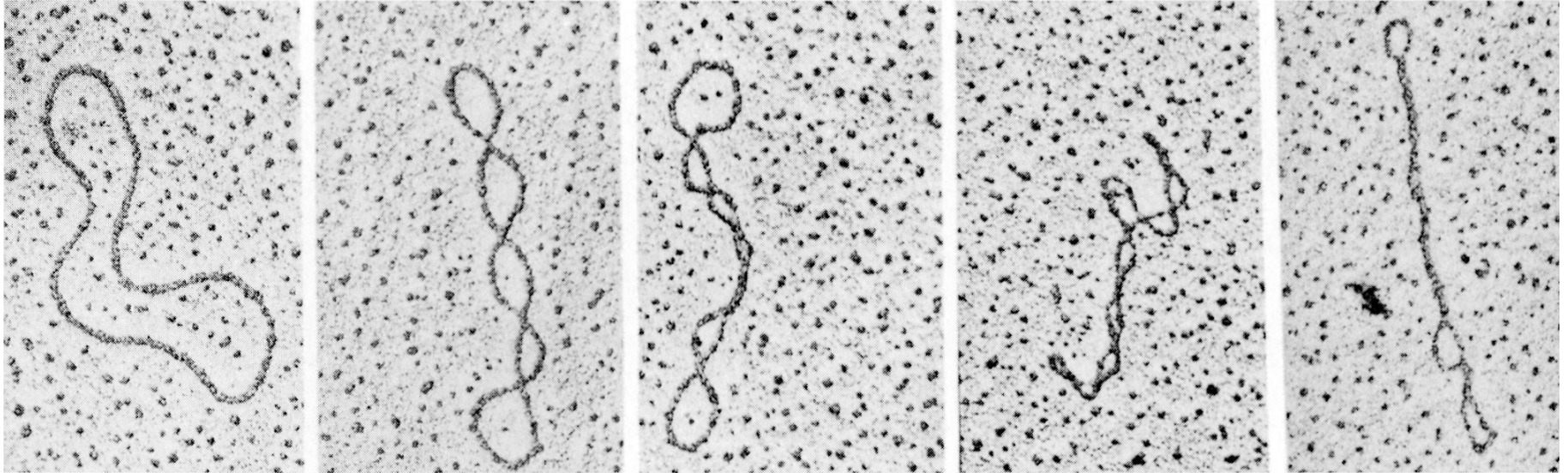




# Bakteriální DNA

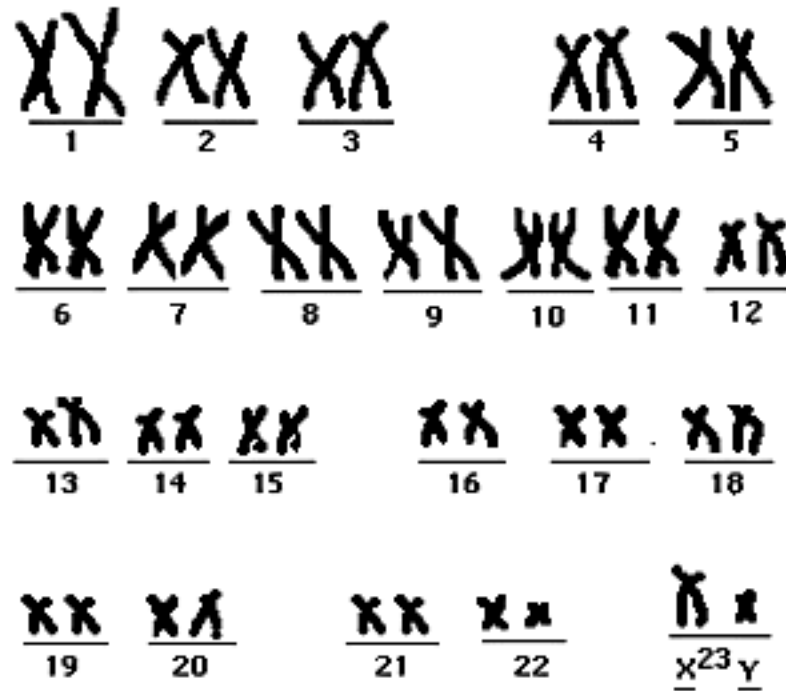


# Bakteriální DNA

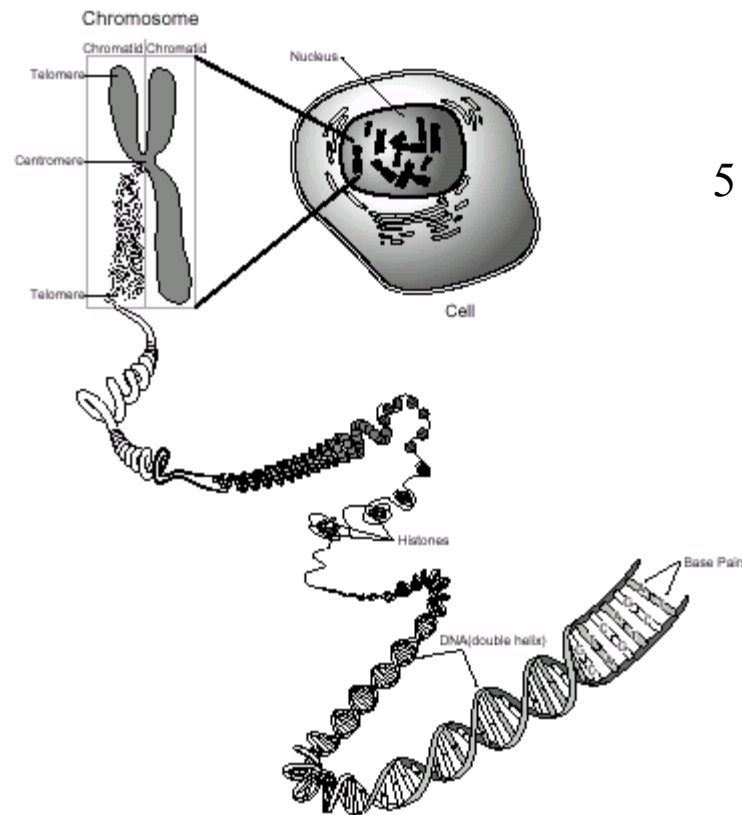


Electron micrographs by Laurien Polder. From Kornberg, A. and Baker, T.A., DNA Replication (2nd ed.), p. 36, W.H. Freeman (1992). Used with permission

# Lidské chromozomy

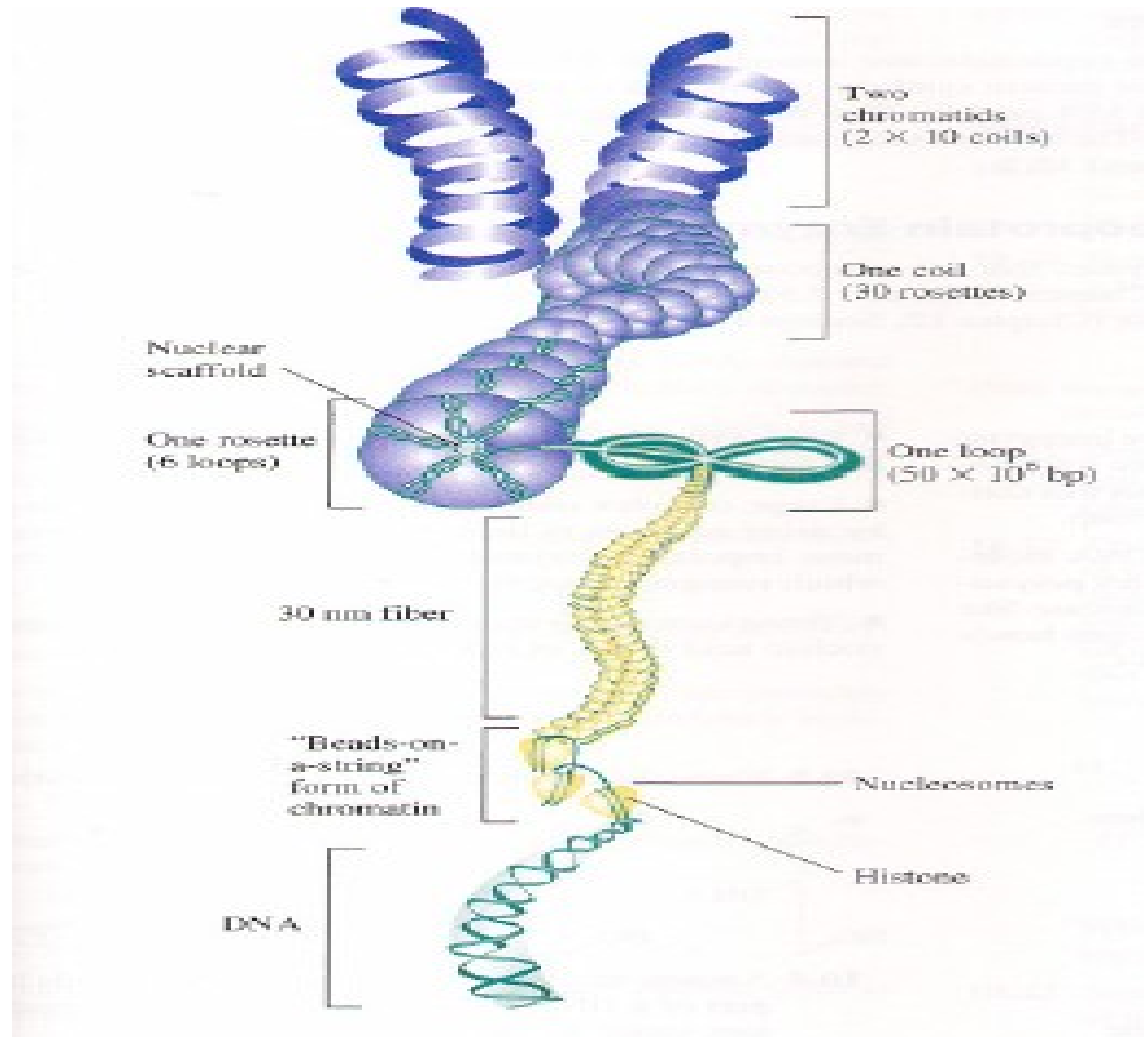


# Jádro – mitotický chromozom

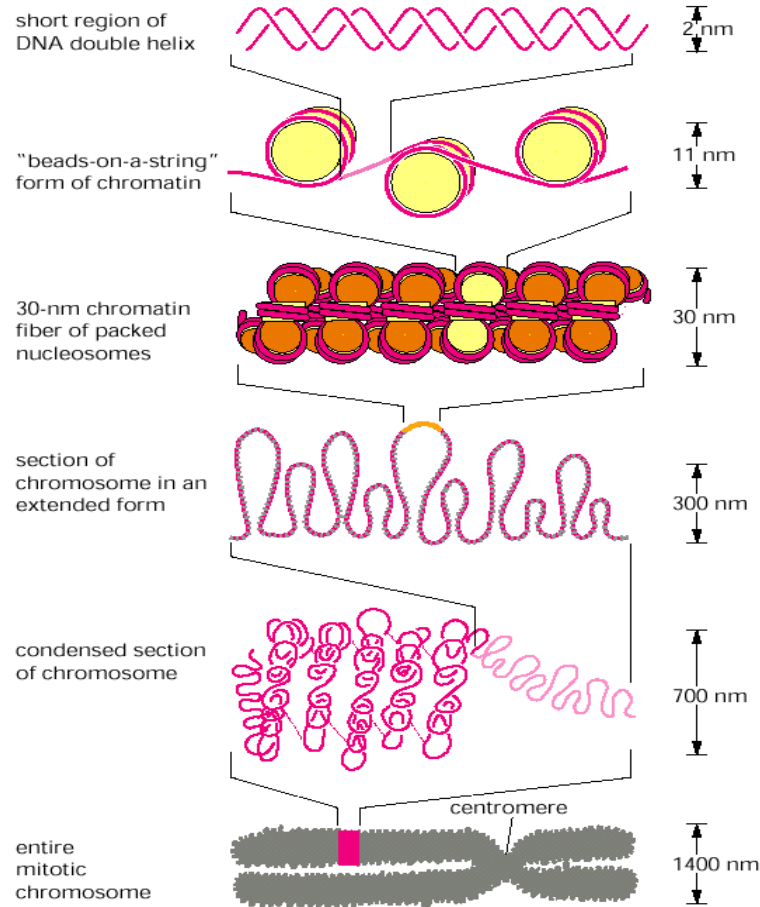


5 – 8  $\mu\text{m}$  – DNA 2 metry

# Chromozom



# Chromatin



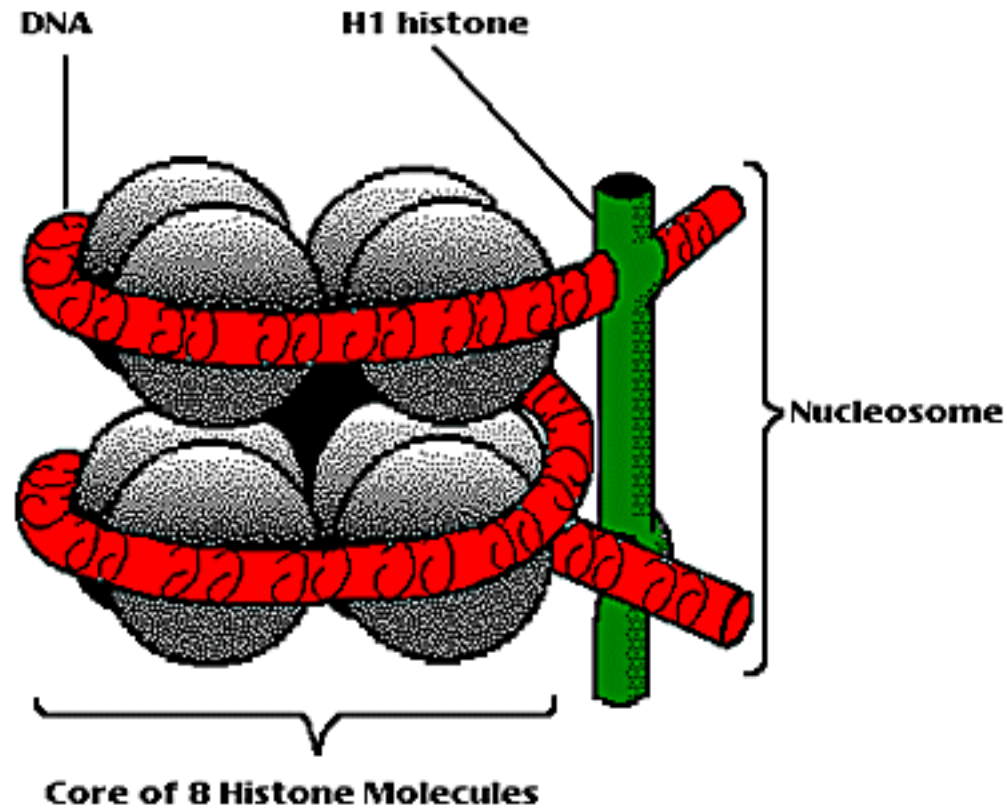
Nukleosom  
(korálky)

(cívka)

(smyčka)

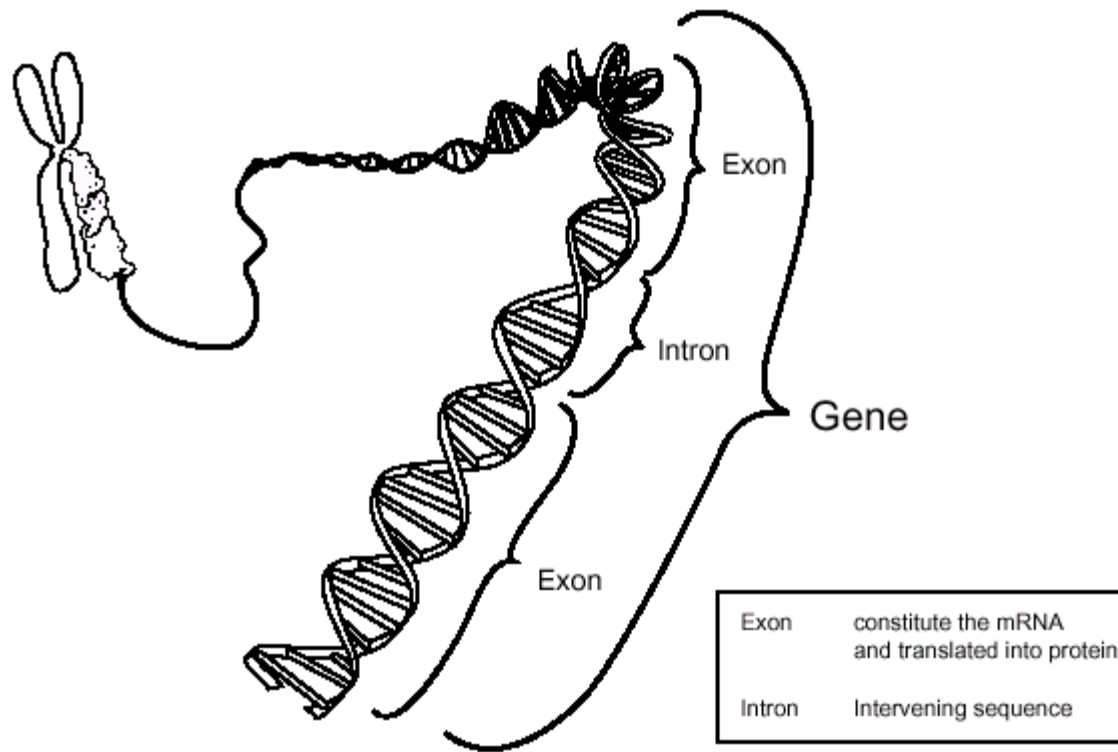
NET RESULT: EACH DNA MOLECULE HAS BEEN PACKAGED INTO A MITOTIC CHROMOSOME THAT IS 50,000x SHORTER THAN ITS EXTENDED LENGTH

# Nukleosom – DNA + 5 tříd histonů



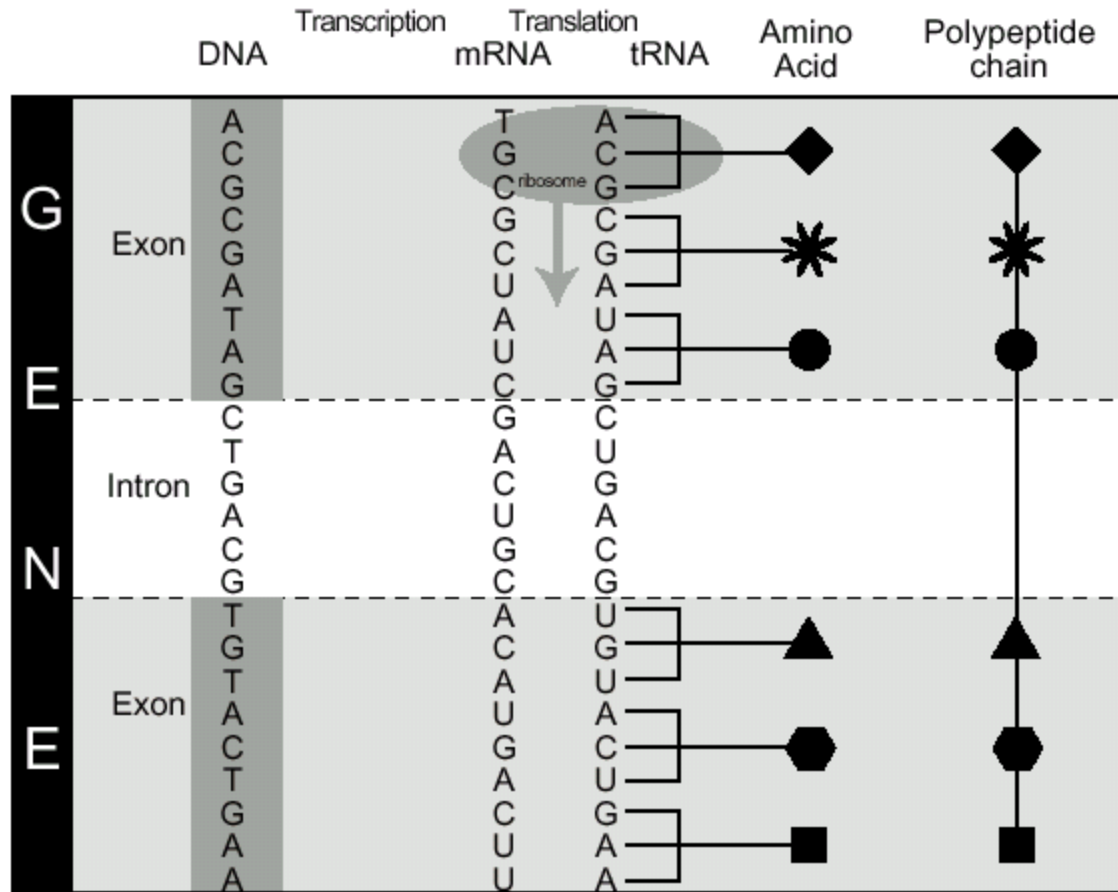
2 x H2A - 2 x H2B - 2 x H3 - 2 x H4

# Exon a intron





# Exon a intron



# Význam intronů

- v počátku evoluce genů urychlovaly vznik nových bílkovin pomocí rekombinace exonů
- možnost tzv. alternativního sestřihu, kdy může vznikat na základě vývojového stádia buňky hned několik různých typů mRNA - z jednoho genu tak může vznikat několik různých bílkovin

# Stanovení sekvence DNA

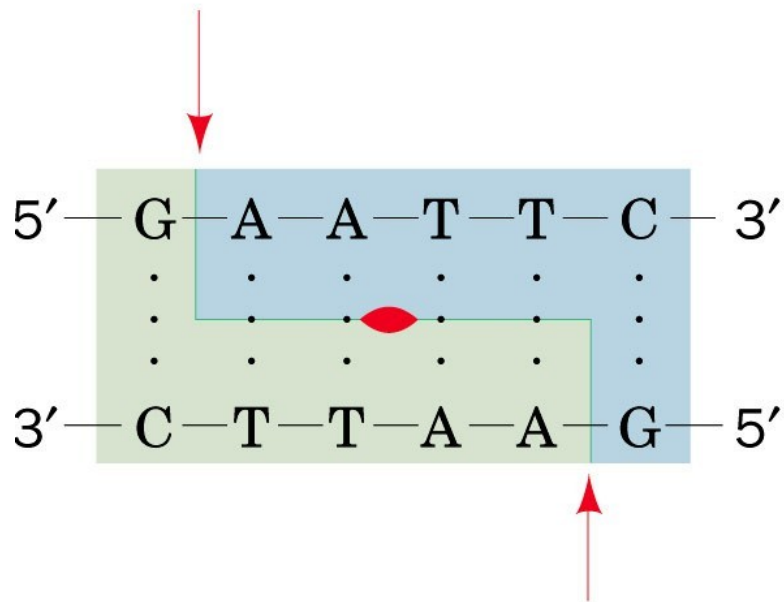
- Restrikční enzymy
- Chemické štěpení – Maxam Gilbertovo metoda
- Enzymová metoda
- Pyrosekvenování

# Restrikční enzymy

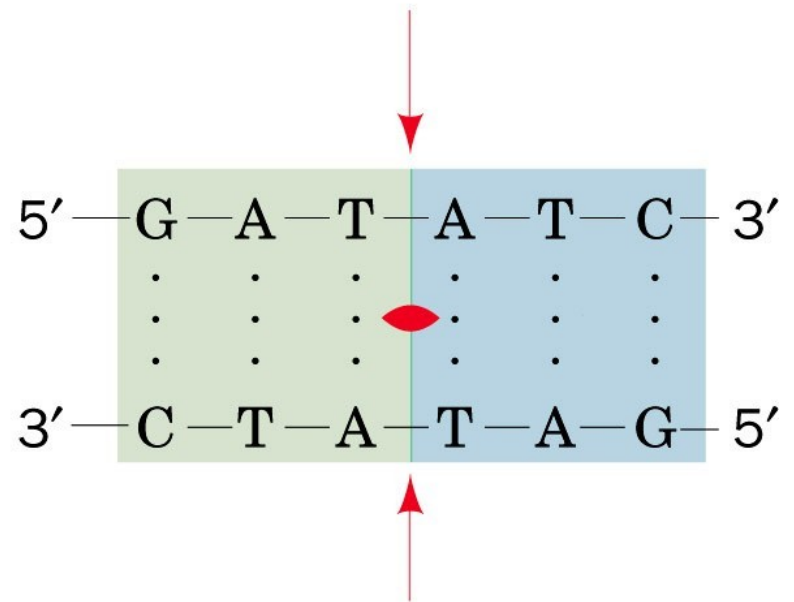
Enzyme	Recognition Sequence <sup>a</sup>	Microorganism
<i>AhaI</i>	AG↓C* <sup>a</sup> T	<i>Anthrobacter luteus</i>
<i>BamHI</i>	G↓GATC* <sup>a</sup> C	<i>Bacillus amyloliquefaciens</i> H
<i>BglI</i>	GCCNNNN↓NGCC	<i>Bacillus globigii</i>
<i>BglII</i>	A↓GATCT	<i>Bacillus globigii</i>
<i>EcoRI</i>	G↓AA* <sup>a</sup> TTC	<i>Escherichia coli</i> RY13
<i>EcoRII</i>	↓CC*( <sup>a</sup> )GG	<i>Escherichia coli</i> R245
<i>EcoRV</i>	GA* <sup>a</sup> T↓ATC	<i>Escherichia coli</i> J62 pLG74
<i>HaeII</i>	RGCGC↓Y	<i>Haemophilus aegyptius</i>
<i>HaeIII</i>	GG↓C* <sup>a</sup> C	<i>Haemophilus aegyptius</i>
<i>HindIII</i>	A* <sup>a</sup> ↓AGCTT	<i>Haemophilus influenzae</i> R <sub>1</sub>
<i>HpaII</i>	C↓C* <sup>a</sup> GG	<i>Haemophilus parainfluenzae</i>
<i>MspI</i>	C* <sup>a</sup> ↓CGG	<i>Moraxella</i> species
<i>PstI</i>	CTGCA* <sup>a</sup> ↓G	<i>Providencia stuartii</i> 164
<i>PvuII</i>	CAG↓C* <sup>a</sup> TG	<i>Proteus vulgaris</i>
<i>SalI</i>	G↓TOGAC	<i>Streptomyces albus</i> G
<i>TaqI</i>	T↓CGA* <sup>a</sup>	<i>Thermus aquaticus</i>
<i>XhoI</i>	C↓TCGAG	<i>Xanthomonas holcicola</i>

# Restrikční enzymy

(a) *EcoRI*



(b) *EcoRV*



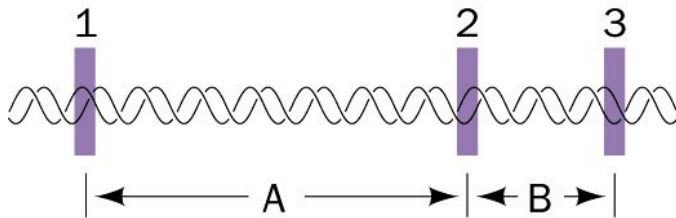
 Cleavage site

 Twofold symmetry axis

# Restrikční enzymy

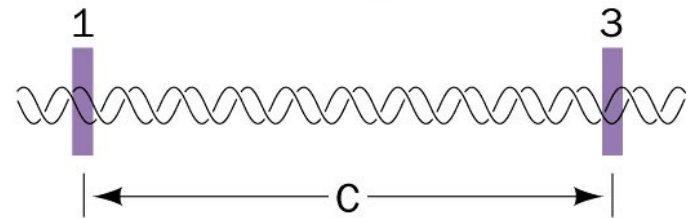
**Chromosome I**

DNA has  
3 target sites



**Chromosome II**

DNA has only  
2 of the target sites



Cleave with  
restriction enzyme  
and electrophoresis

Fragment C is  
the size of  
A + B combined

# Restrikční enzymy



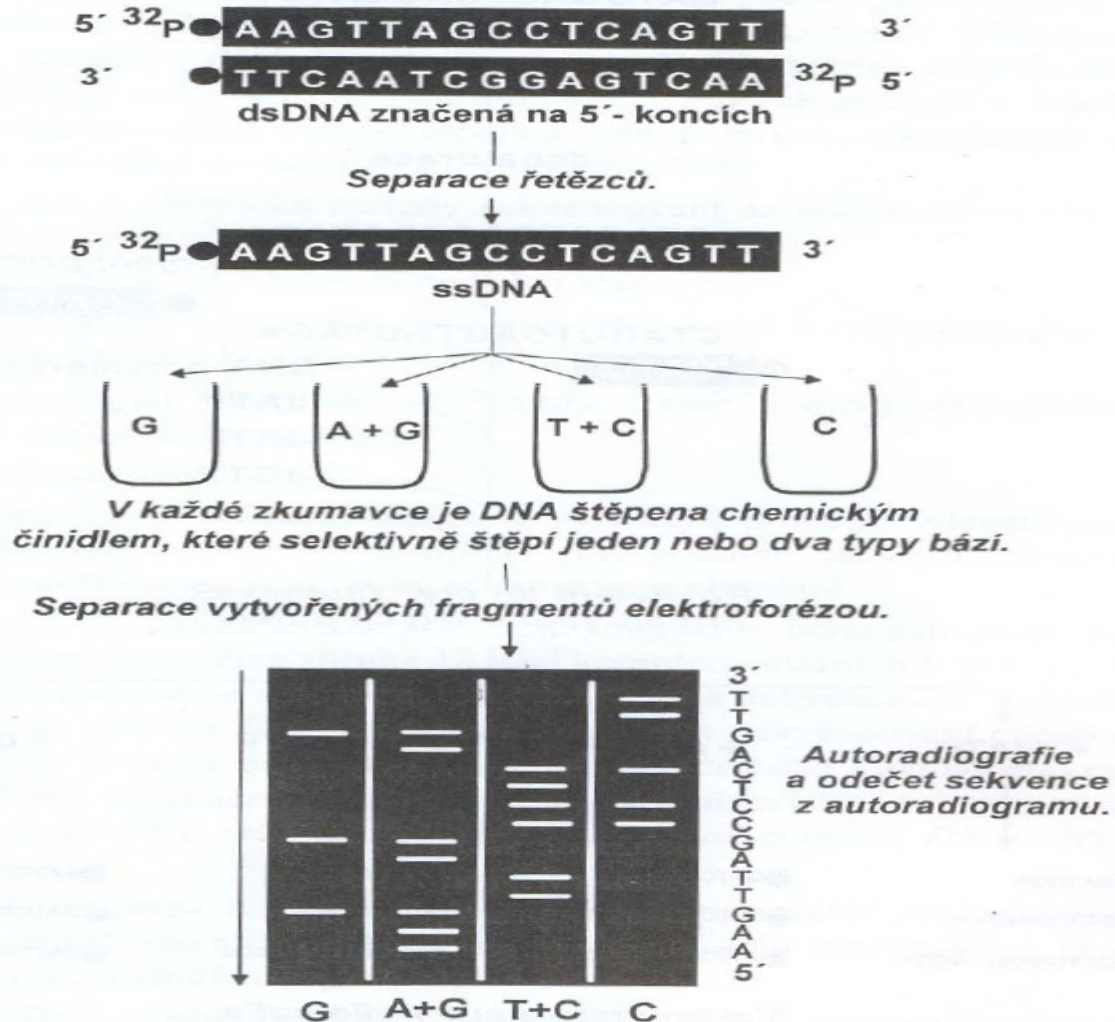
# Maxam Gilbertova metoda

## Maxam-Gilbertova metoda

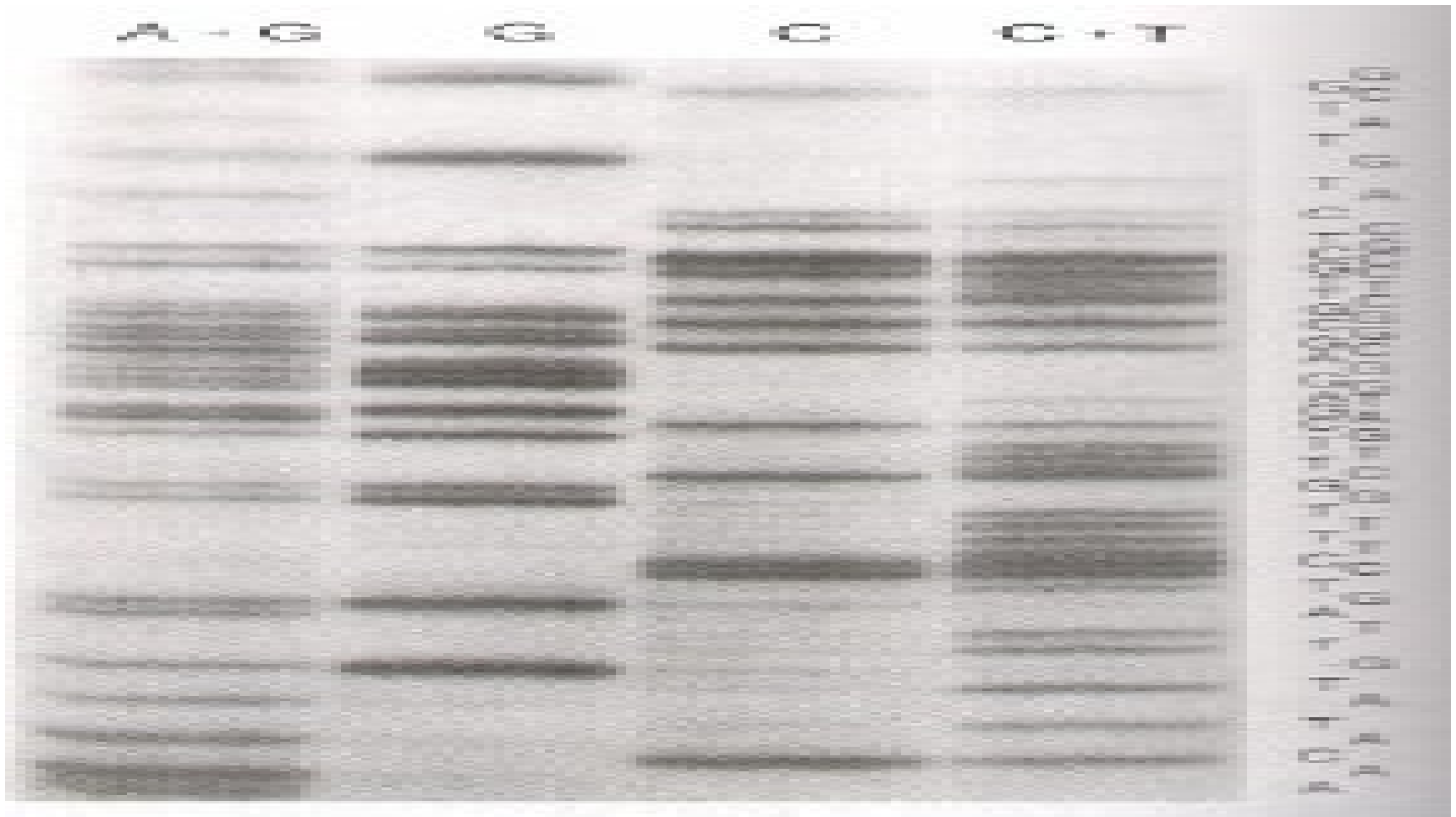
- značení 5' konce  $^{32}\text{P}$
- specifické chemické štěpení
- elektroforéza



# Maxam Gilbertova metoda

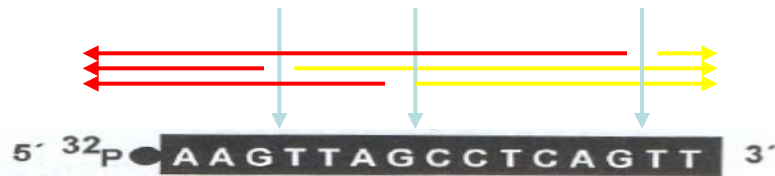


# Maxam Gilbertova metoda

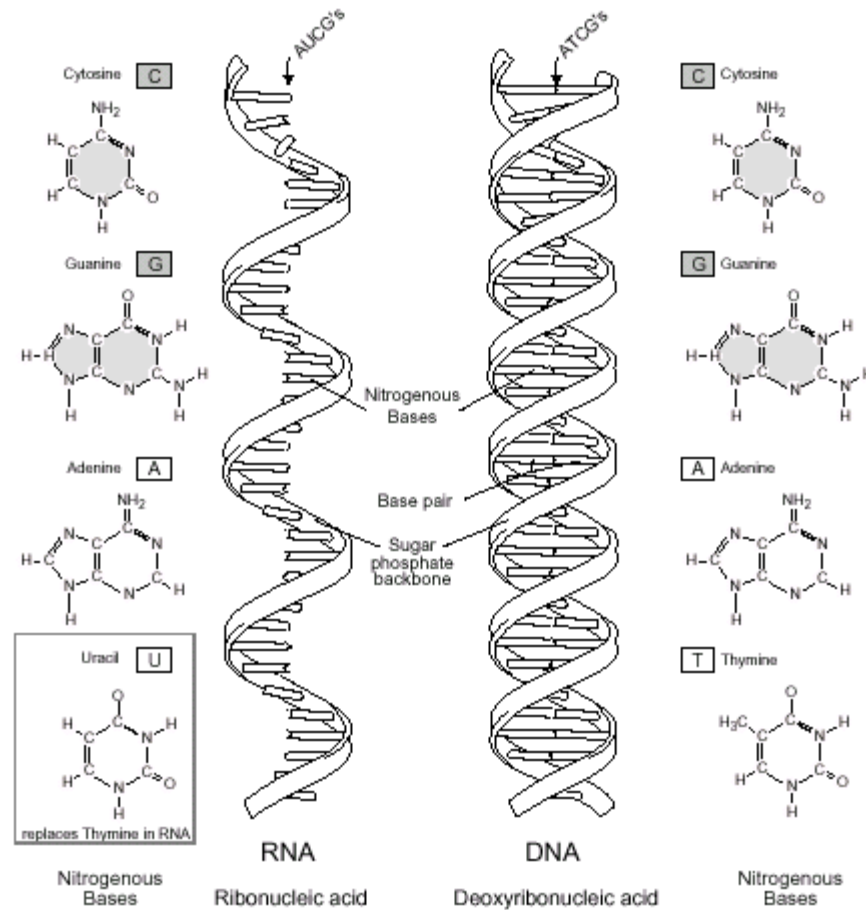


**kyselina a piperidin    dimetylsulfát a piperidin    kyselina, NaCl a piperidin    hydrazin a piperidin**

# Maxam Gilbertova metoda



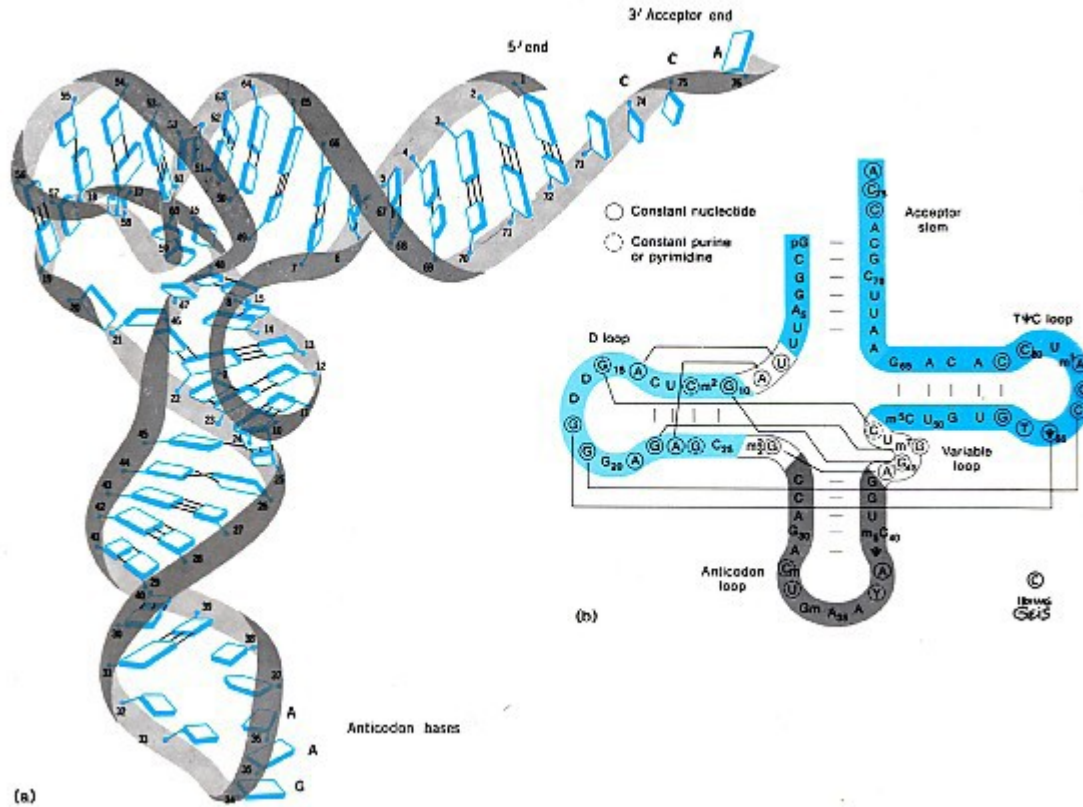
# RNA versus DNA



## Formy RNA

- mRNA – mediátorová, messenger,  
informační – 5-10 %
- rRNA – ribosomální – 80 %
- tRNA – transferová, přenosová – 10-15 %  
60 tRNA

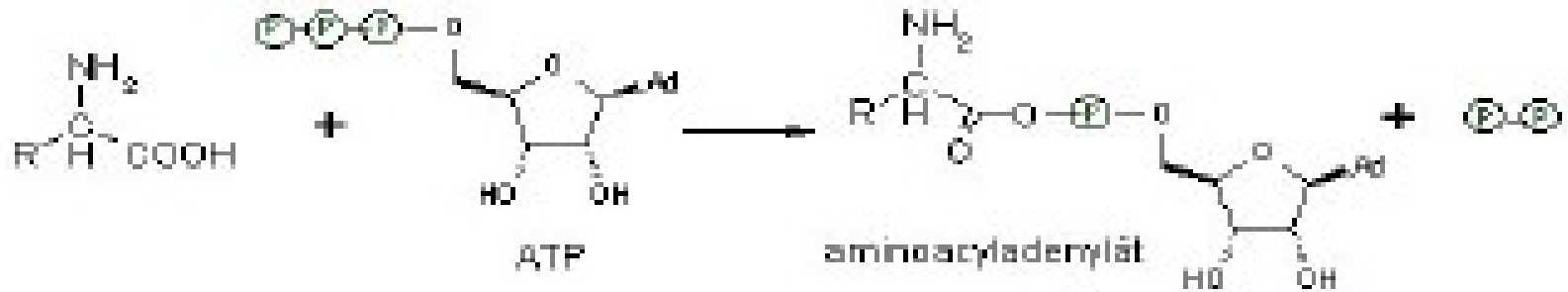
# tRNA



(a)

(b)

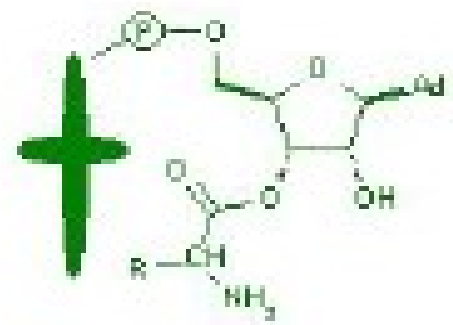
Anticodon bases



tRNA



aminoacyl-tRNA + AMP



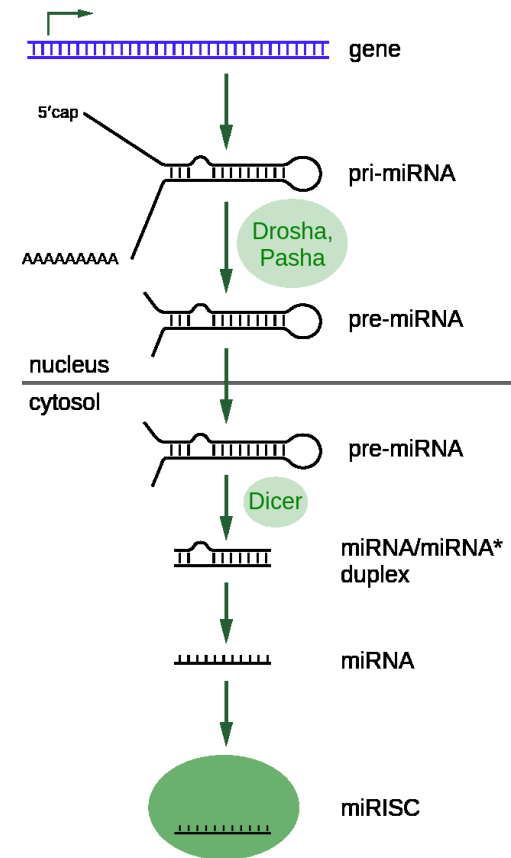
# Formy RNA

- mRNA – mediátorová, messenger,  
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60 tRNA

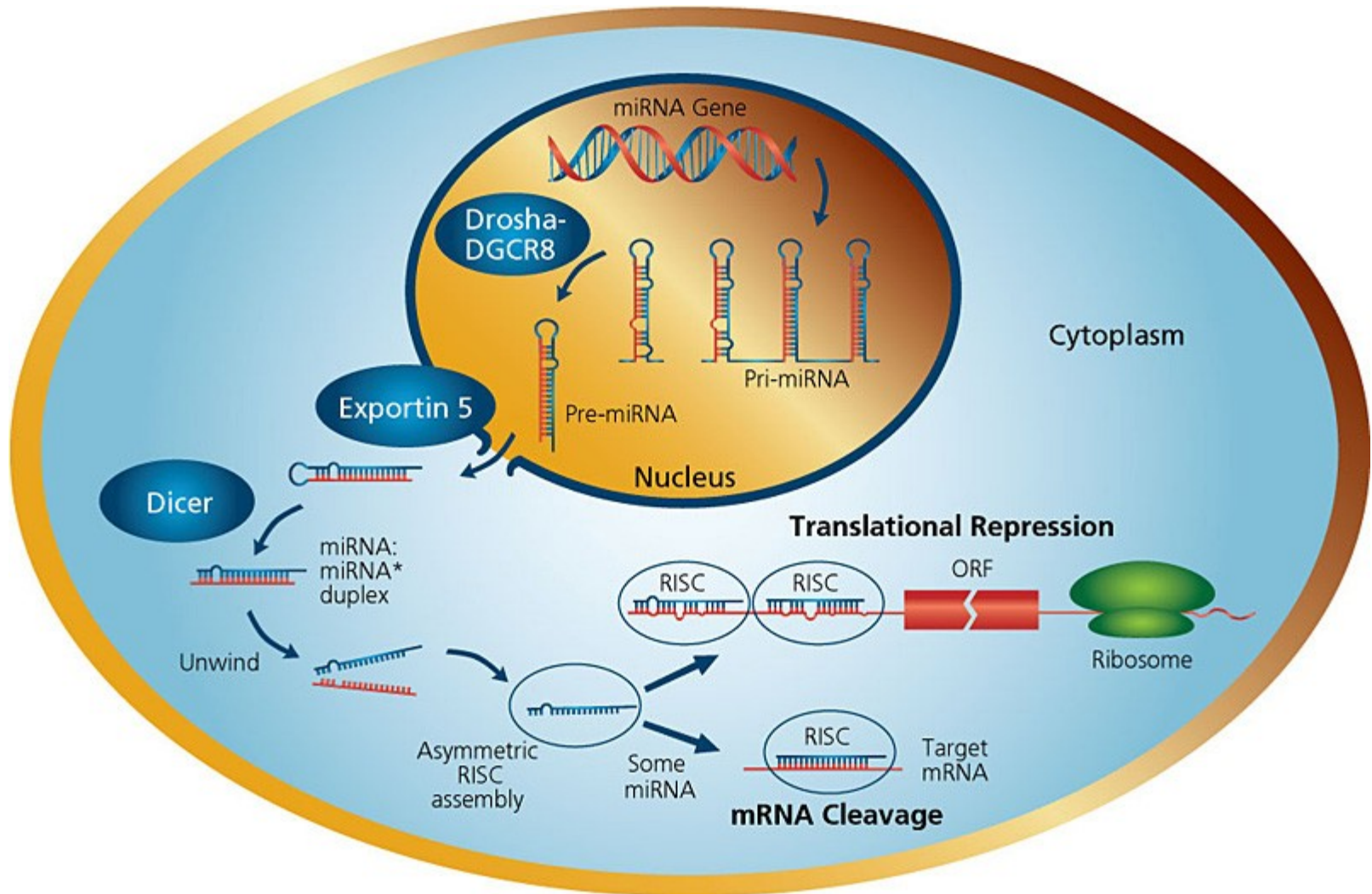
1993 Victor Ambrose

miRNA - microRNA



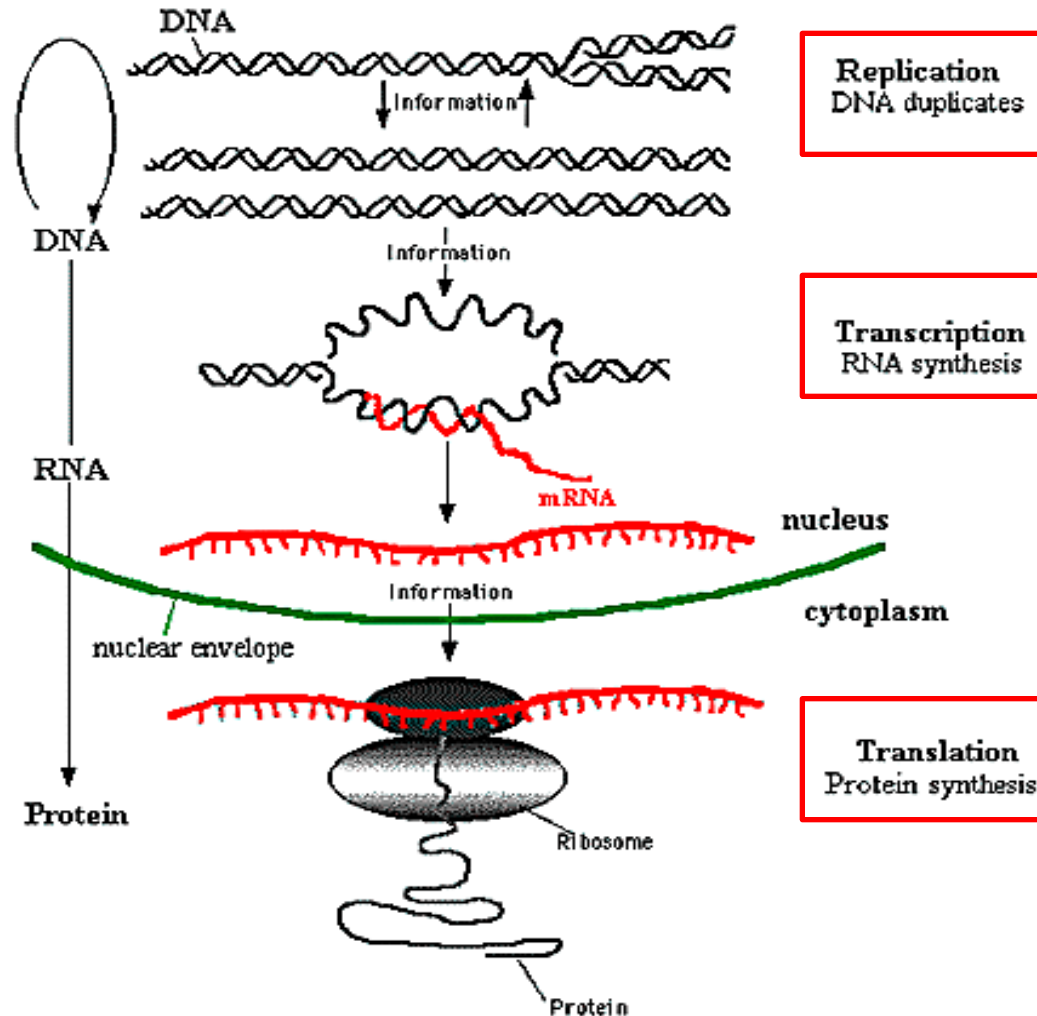


# Micro RNA

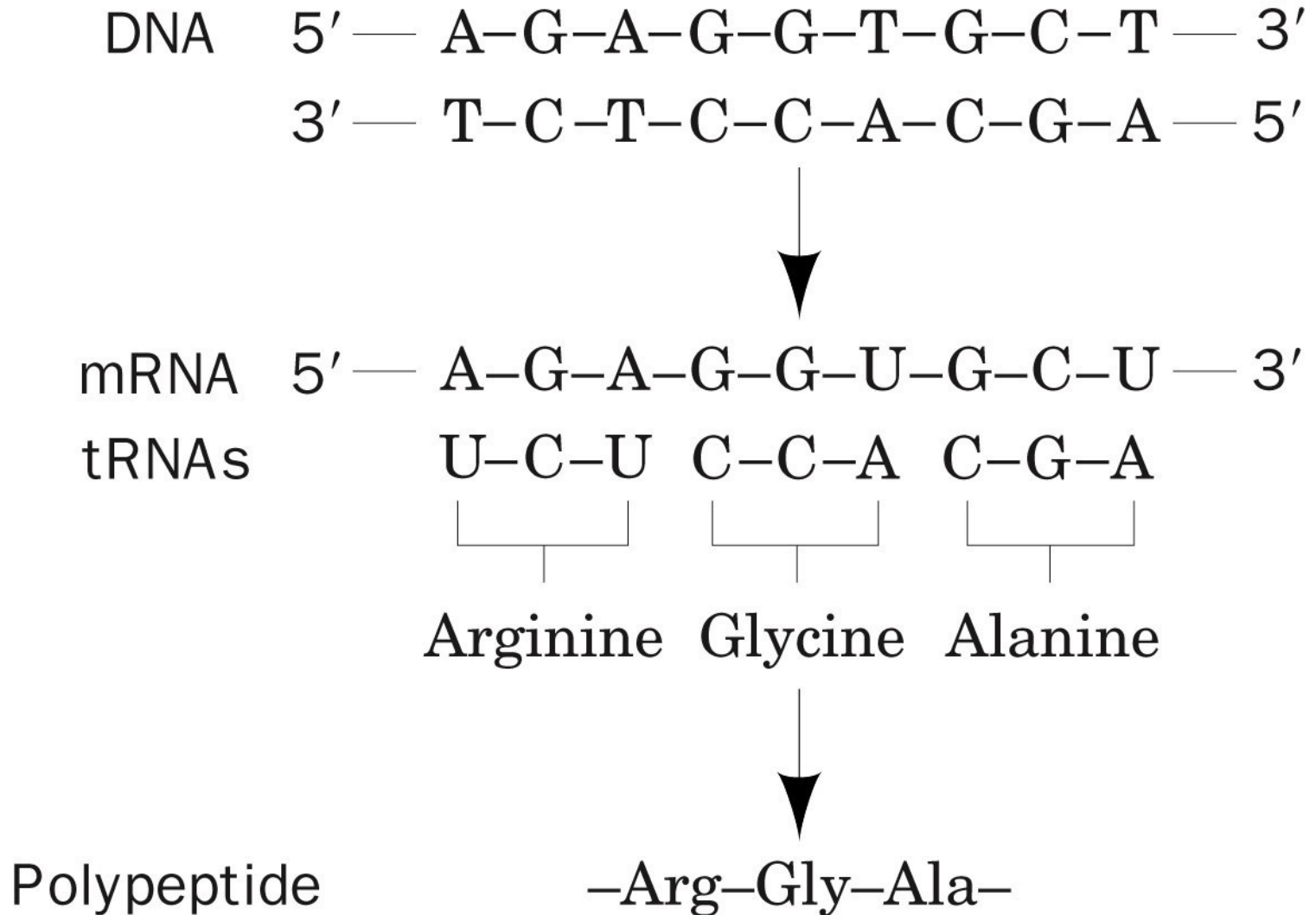


# Centrální dogma mol.biologie

**Crick 1958 - cesta od NK k proteinu**

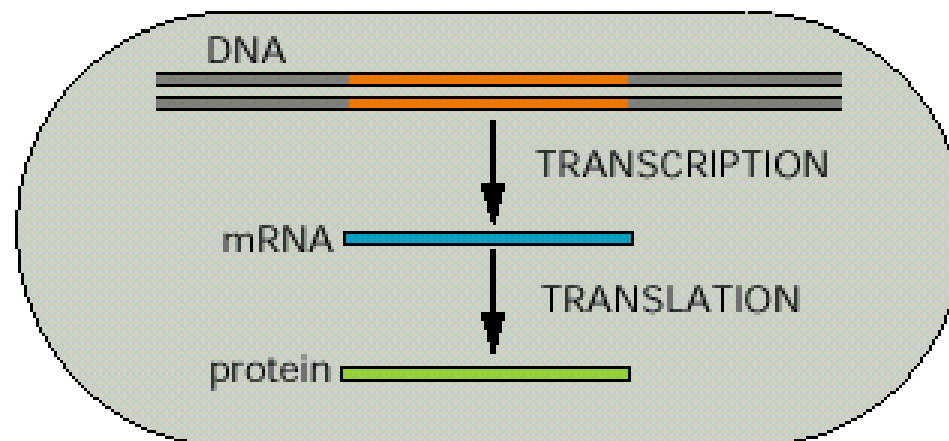


# Centrální dogma mol.biologie



# Prokaryota

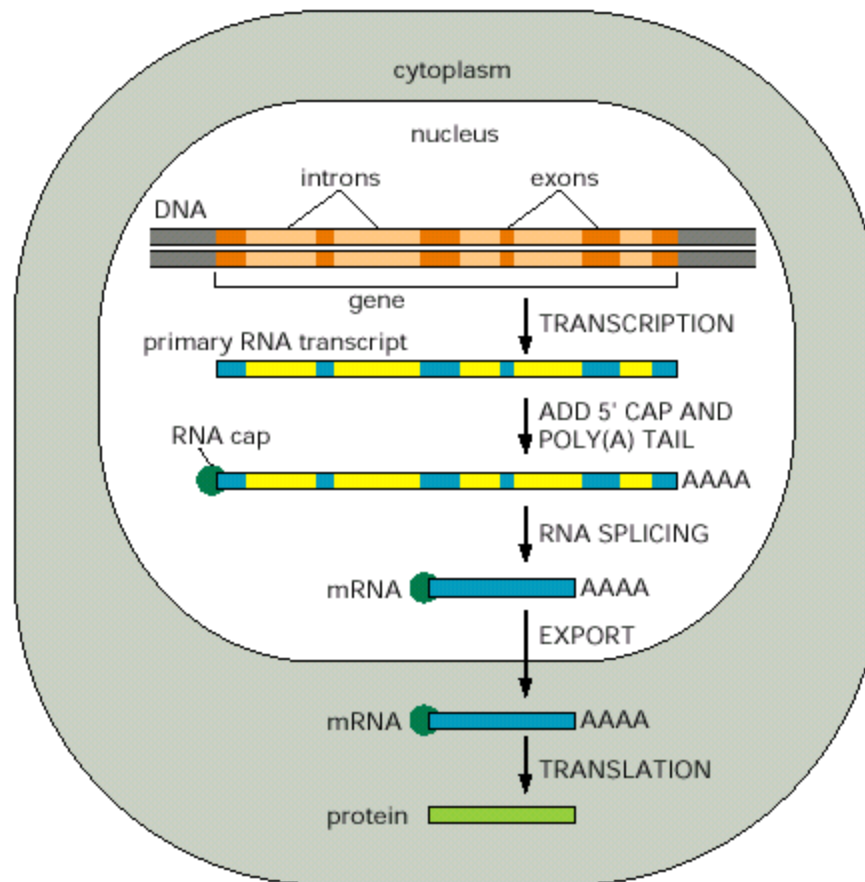
(B) PROCARYOTES



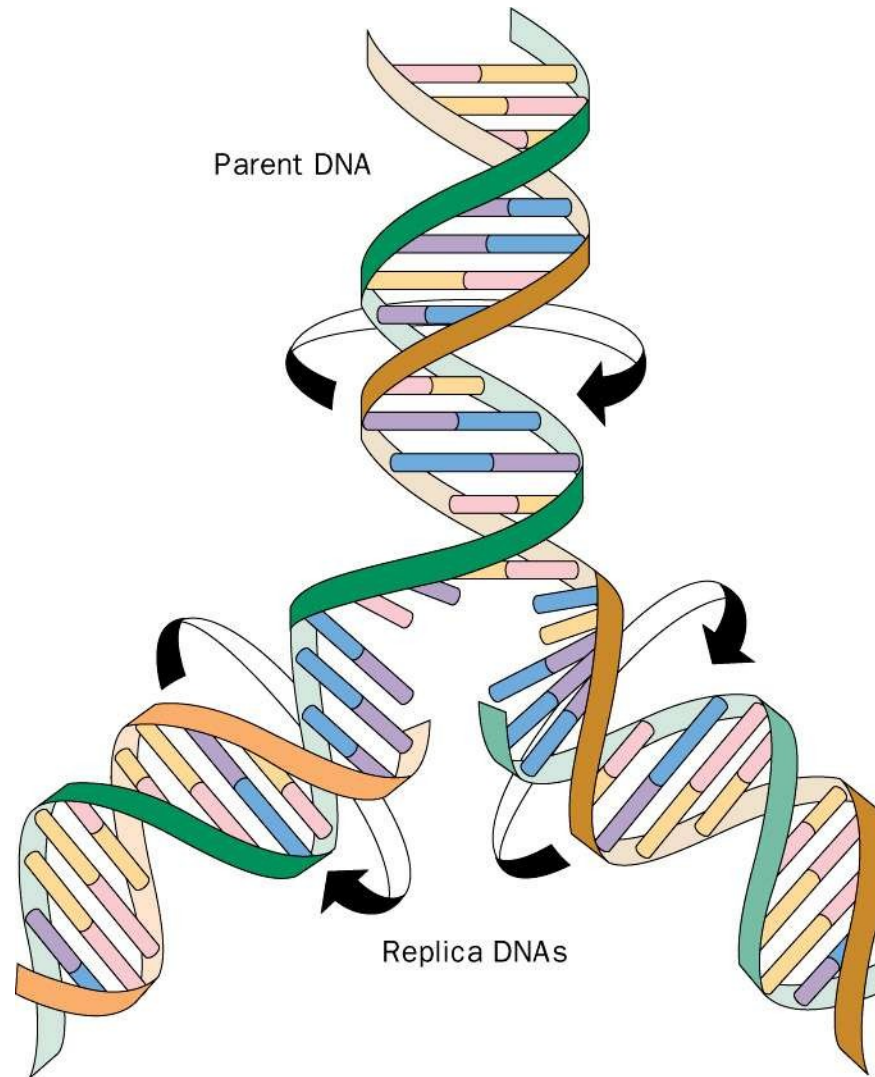
# Eukaryota

(A) EUCARYOTES

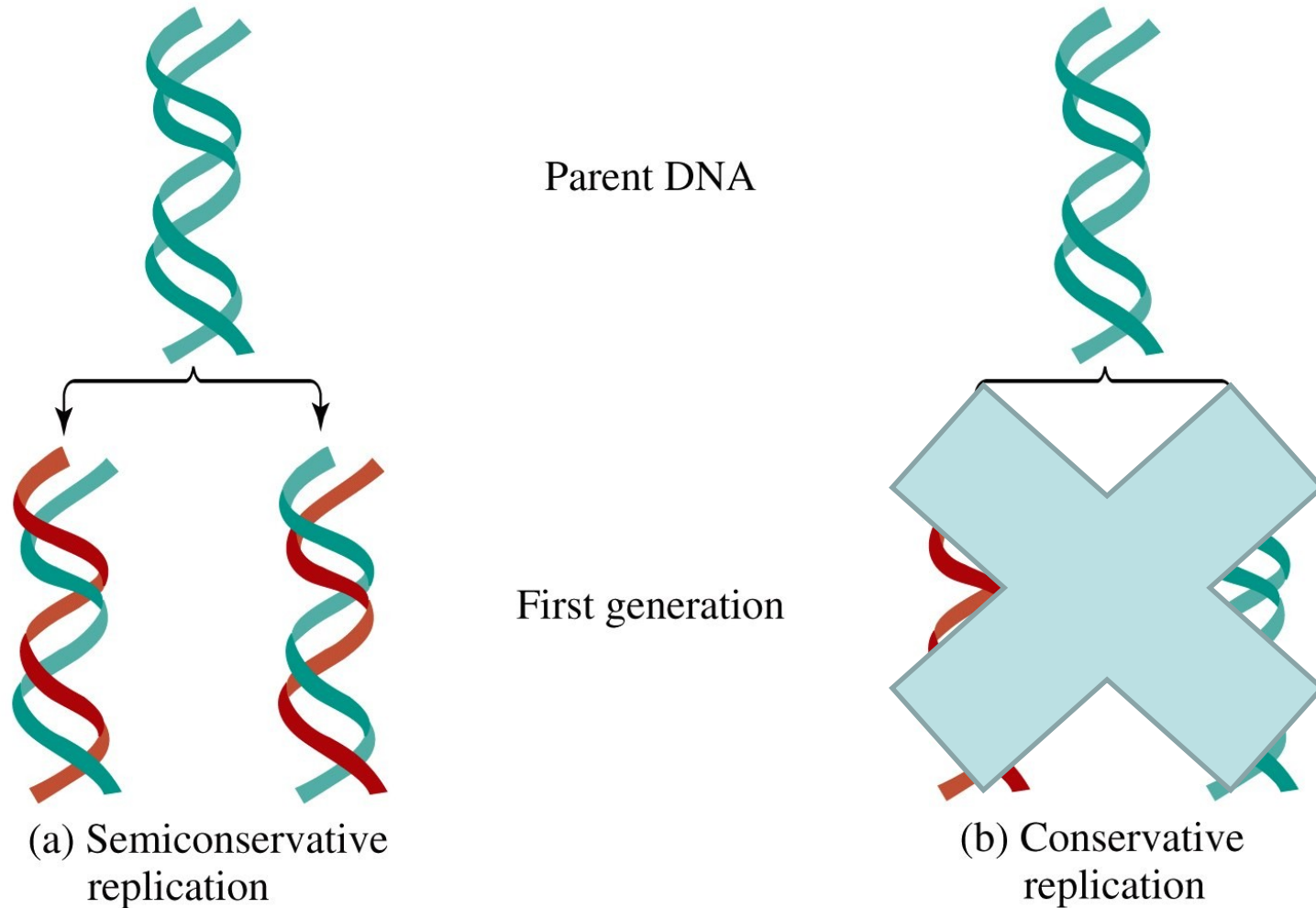
(B)



# Replikace – zdvojení - DNA → DNA



# Replikace DNA



# Meselsonův a Stahlův experiment

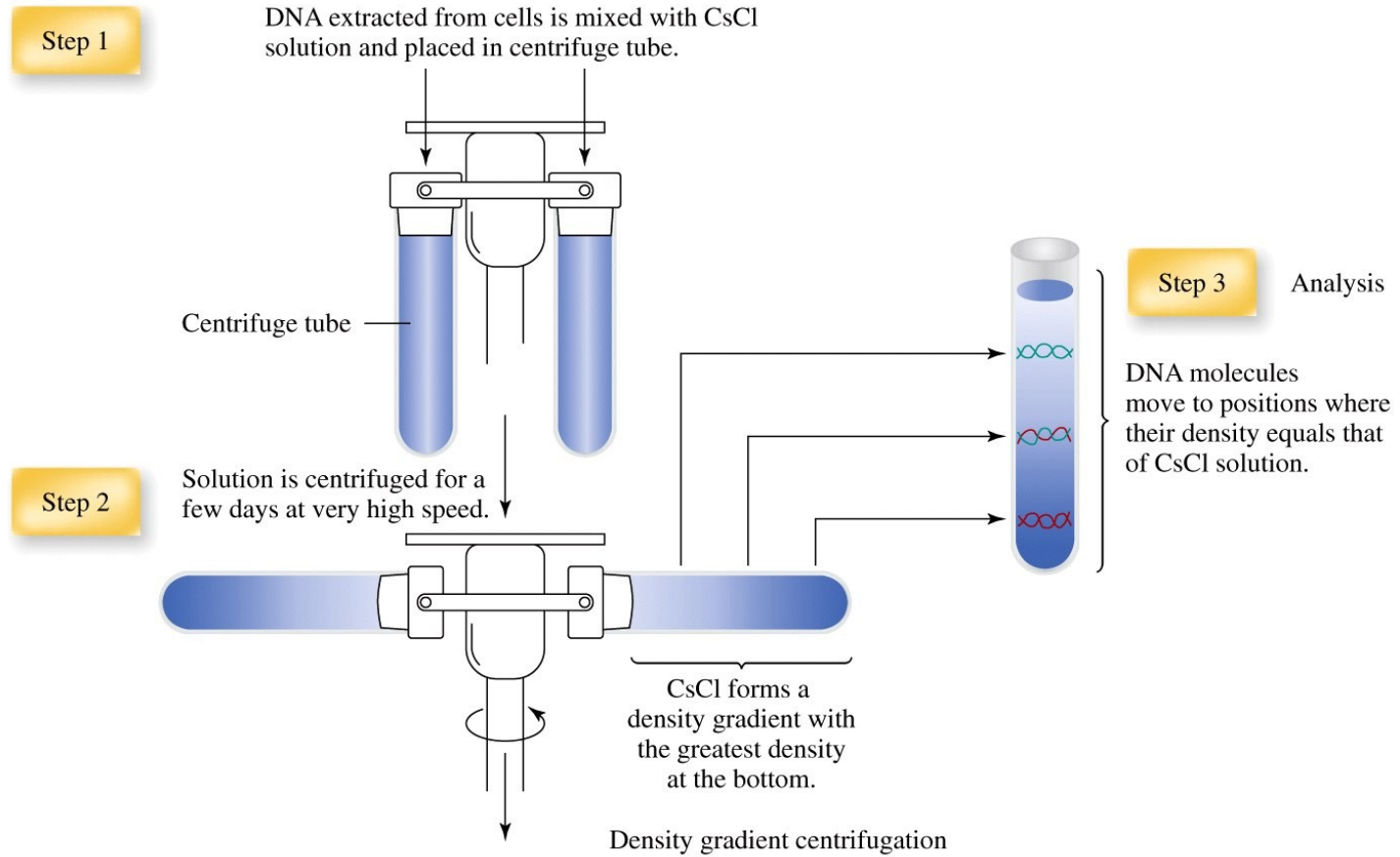


Figure 11-2a Concepts in Biochemistry, 3/e  
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# Ultracentrifugace

# Preparativní ultracentrifuga

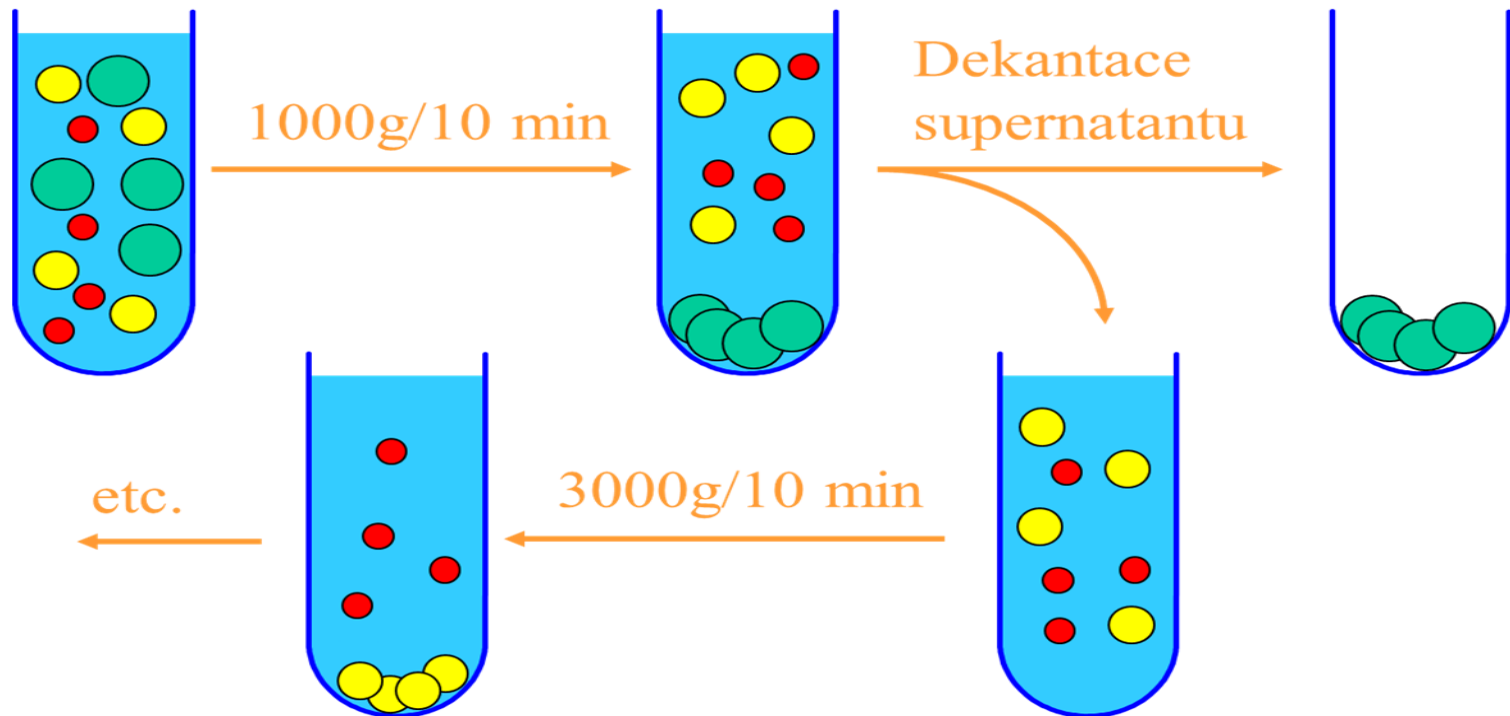


# Rotory

- Úhlový – diferenciální centrifugace
- Výkyvné – zonální centrifugace

# Diferenciální centrifugace

- opakovaná centrifugace se zvyšující se rychlostí otáček = gravitací



# Úhlový rotor

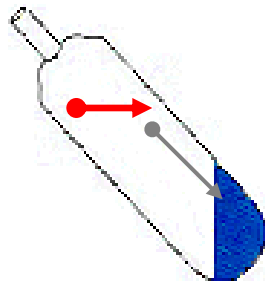
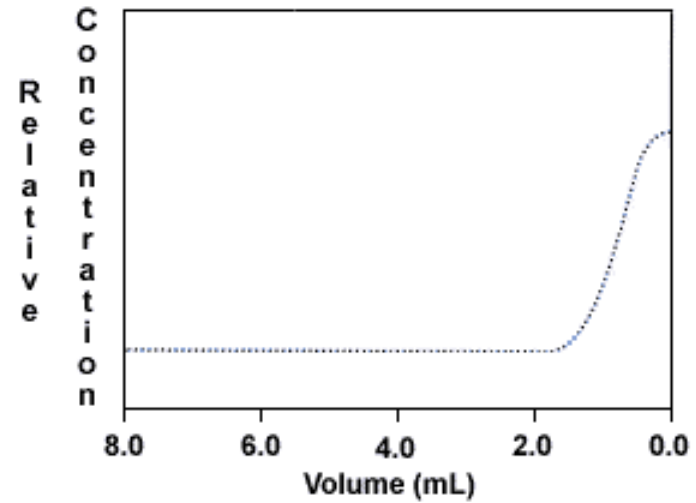
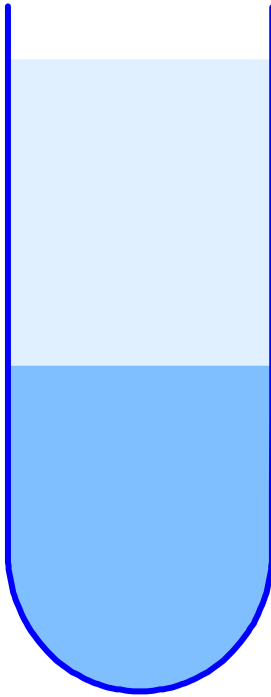


Figure 4



# Gradientová centrifugace

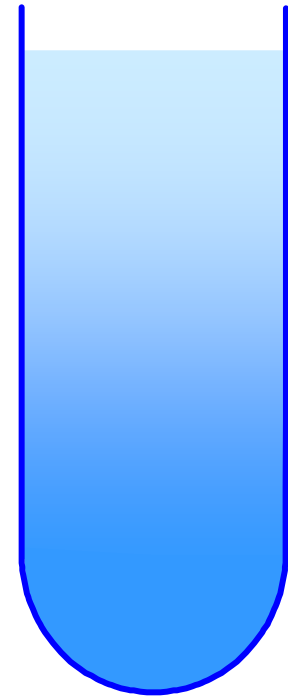
**Hustotní bariera**



**Diskontinuální**

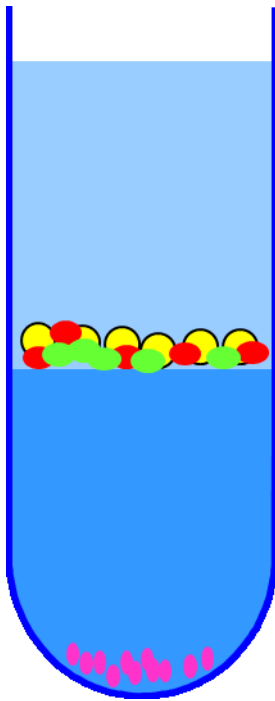


**Kontinuální**

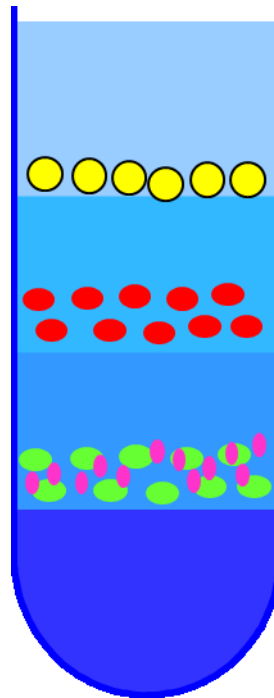


# Gradientová centrifugace

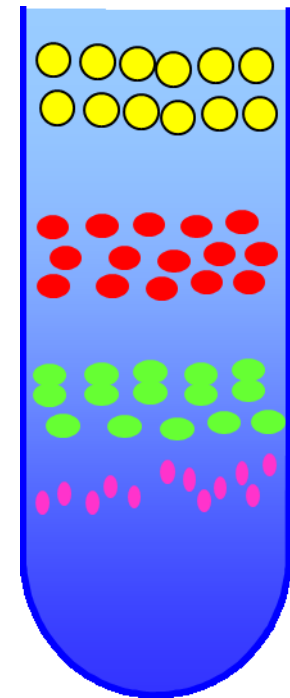
**Hustotní bariera**



**Diskontinuální**



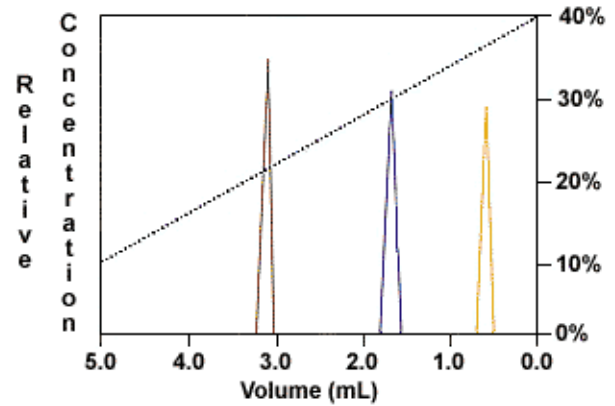
**Kontinuální**



# Výkyvný rotor



*Figure 2*





# Gradientová centrifugace média

- Sacharosa

- Glycerol

- Ficoll - dextran

- Percoll – SiO<sub>2</sub>

Hypertonické prostředí

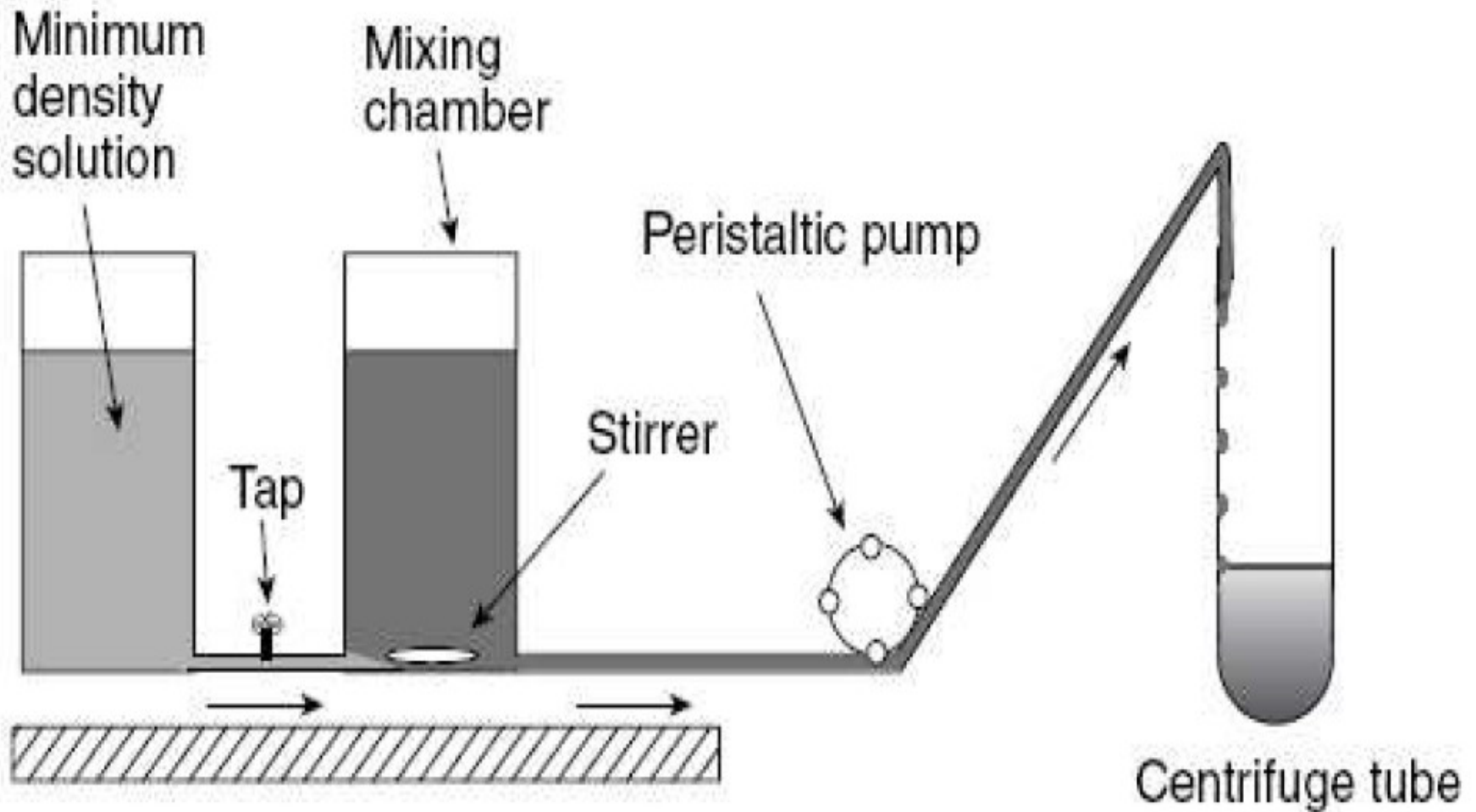
Nutno připravit gradient

- CsCl

- Cs<sub>2</sub>SO<sub>4</sub>

Gradient vzniká během centrifugace

# Gradientová centrifugace



# Gradientová centrifugace

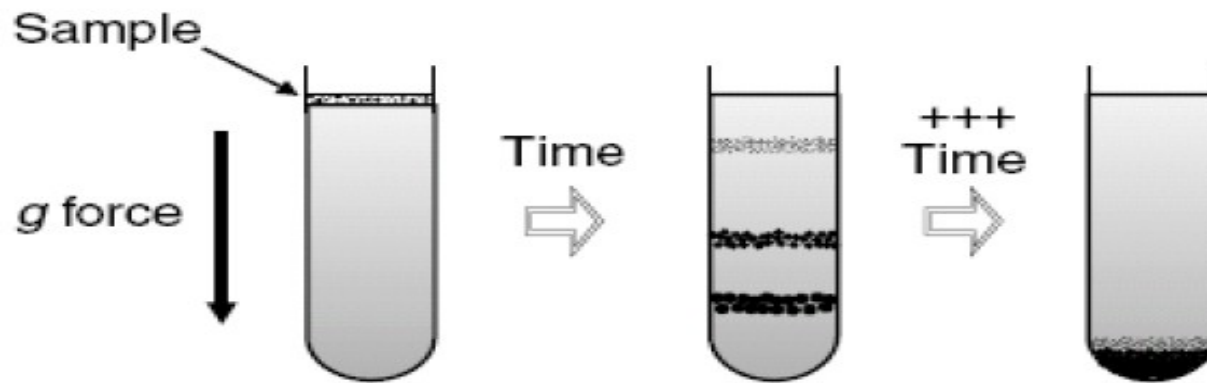
Metoda

Izopyknická

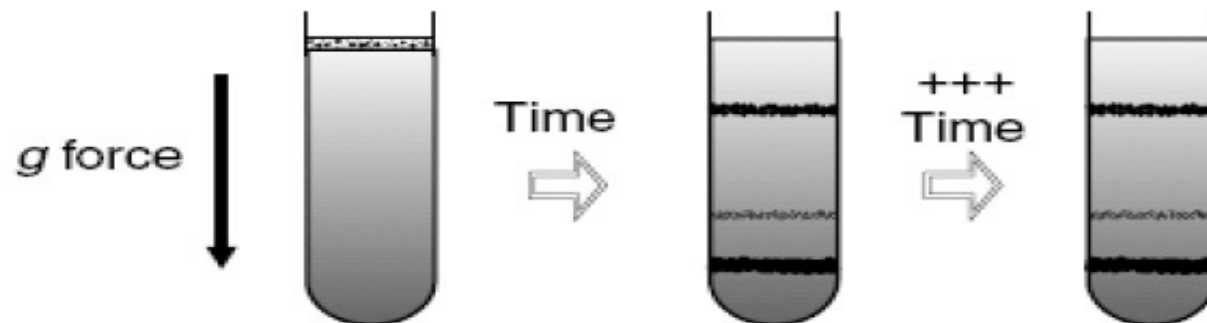
Nerovnovážná

# Gradientová centrifugace

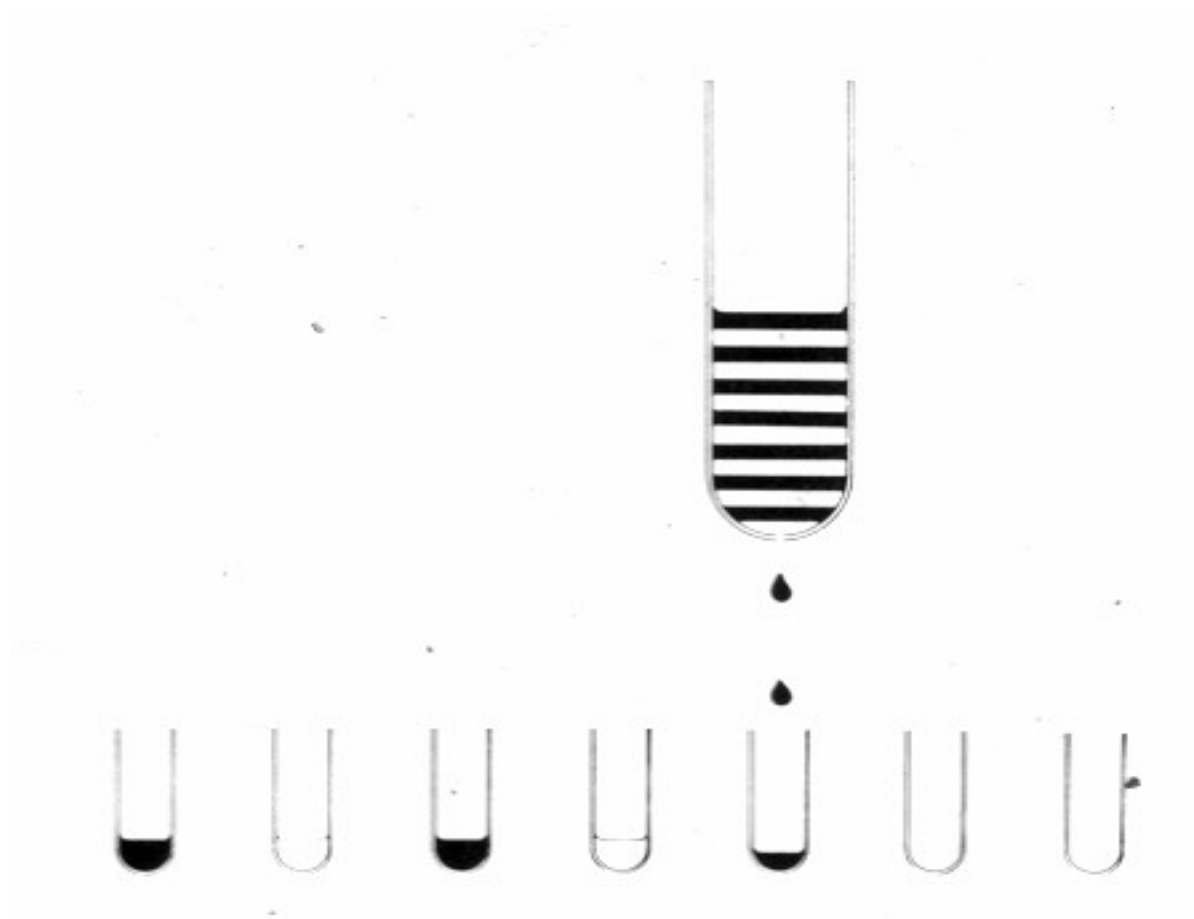
## Rate-zonal centrifugation



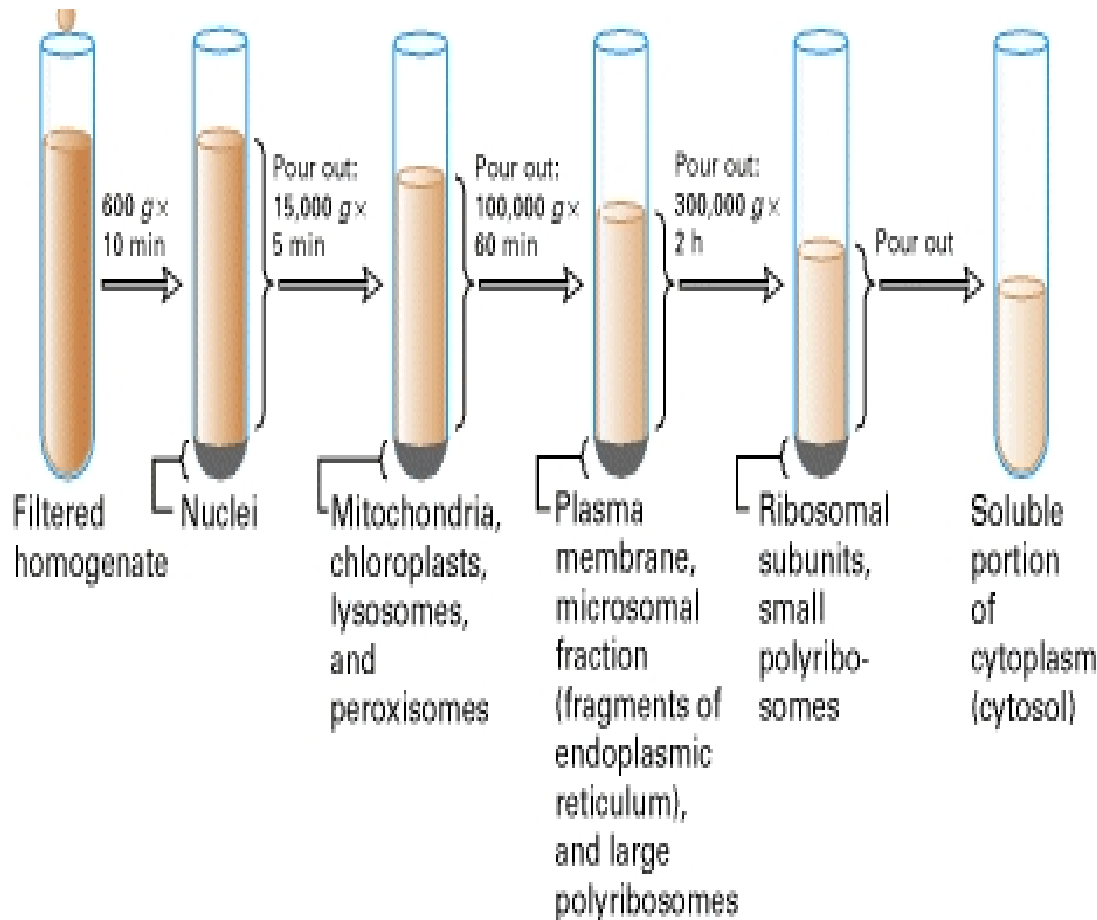
## Isopycnic centrifugation



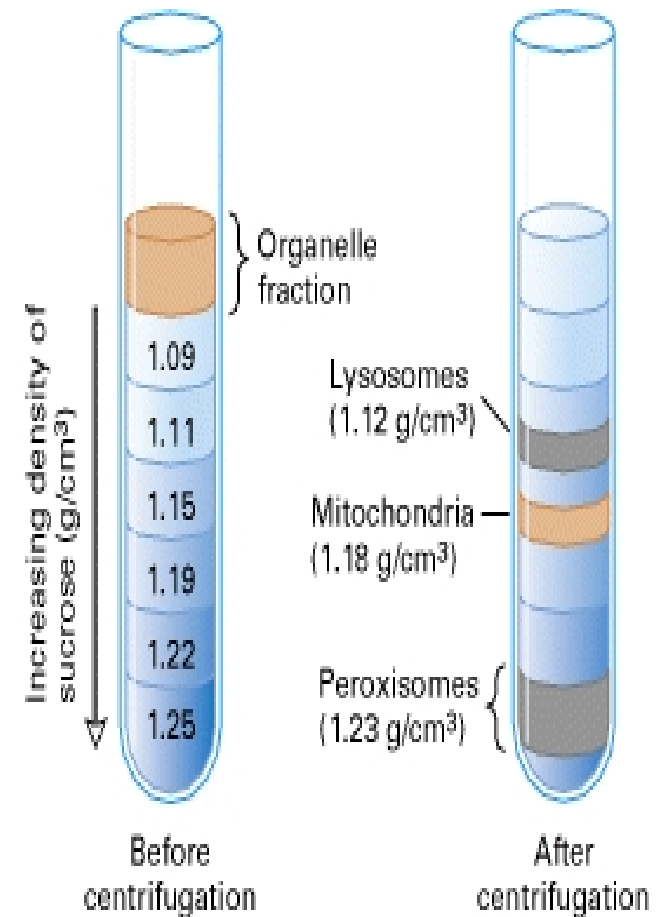
# Gradientová centrifugace



# Diferenciální versus gradientová centrifugace



4 x



1 x

# Meselsonův a Stahlův experiment

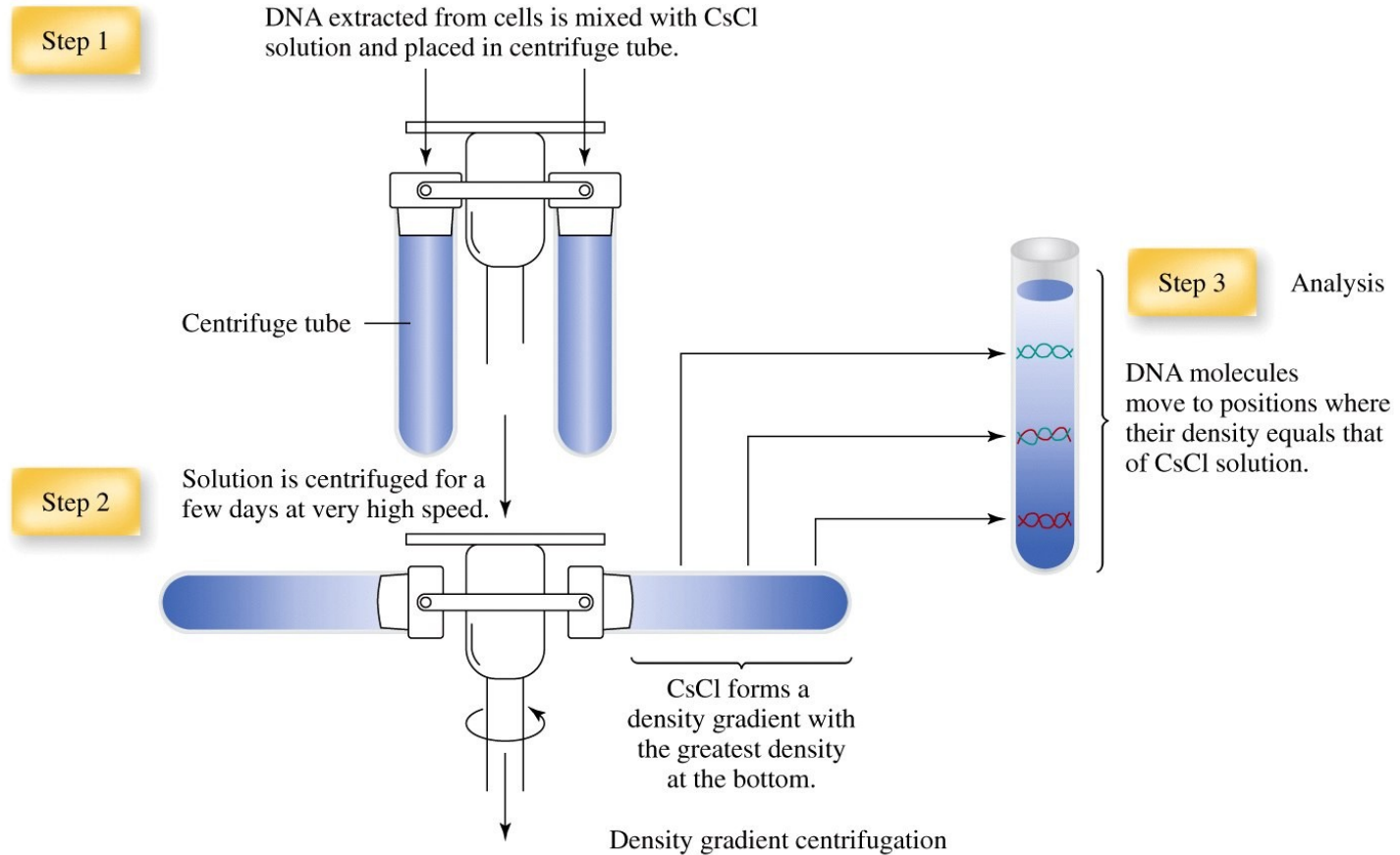


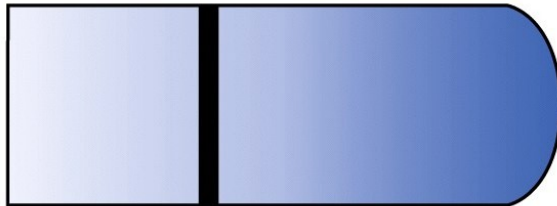
Figure 11-2a Concepts in Biochemistry, 3/e  
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# Meselsonův a Stahlův experiment

Direction of sedimentation



Heavy DNA



Light DNA



Parent  $^{15}\text{N}$ -DNA  
(both strands  
heavy)



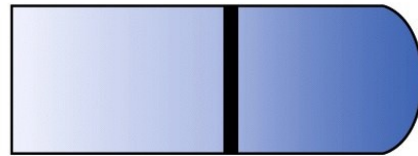
Normal  $^{14}\text{N}$ -DNA  
(with two light  
strands)

Preliminary experiment



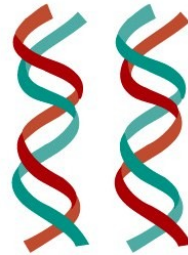
# Meselsonův a Stahlův experiment

## Experimental results



Hybrid DNA

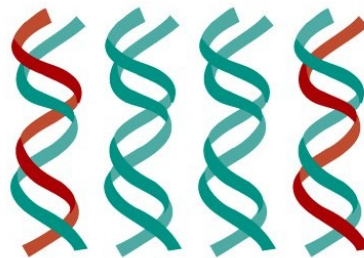
After one generation on  $^{14}\text{N-NH}_4\text{Cl}$



Light  
DNA

Hybrid  
DNA

After two generations on  $^{14}\text{N-NH}_4\text{Cl}$



## Conclusions

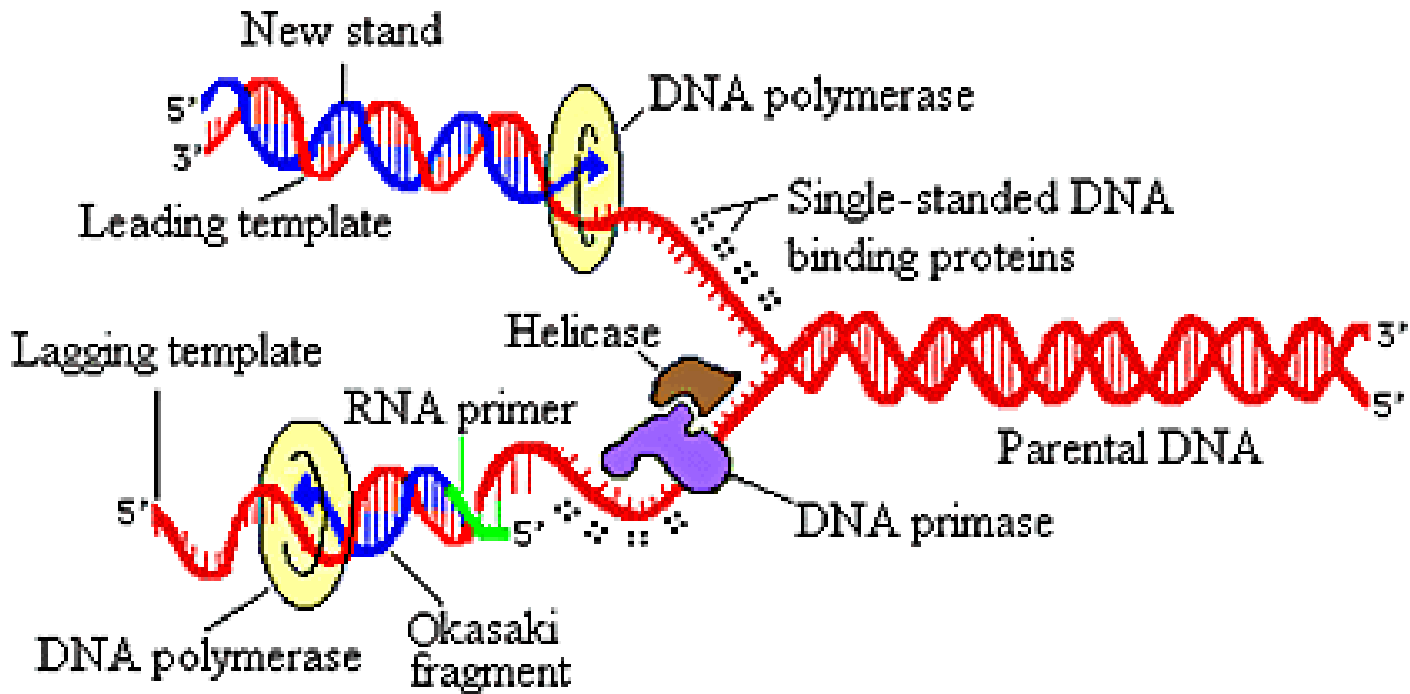
First generation:  
Both DNAs contain one light and one heavy strand.

Second generation:  
Two hybrid DNAs and two light DNAs are formed.

Actual experiment

# Replikace

**DNA + NTP + Mg<sup>2+</sup> + 7 enzymů**



# Účast enzymů na replikaci

**Table 11.2**

**Proteins necessary for DNA replication in *E. coli***

Protein	Function
Helicase	Begins unwinding of DNA double helix
DNA gyrase	Assists unwinding
SSB proteins	Stabilize single strands of DNA
Primase	Synthesis of RNA primer
DNA polymerase III	Elongation of chain by DNA synthesis
DNA polymerase I	Removal of RNA primer and filling in gap with DNA
DNA ligase	Closes last phosphoester gap to form phosphodiester bond

# Prokaryontní replikace

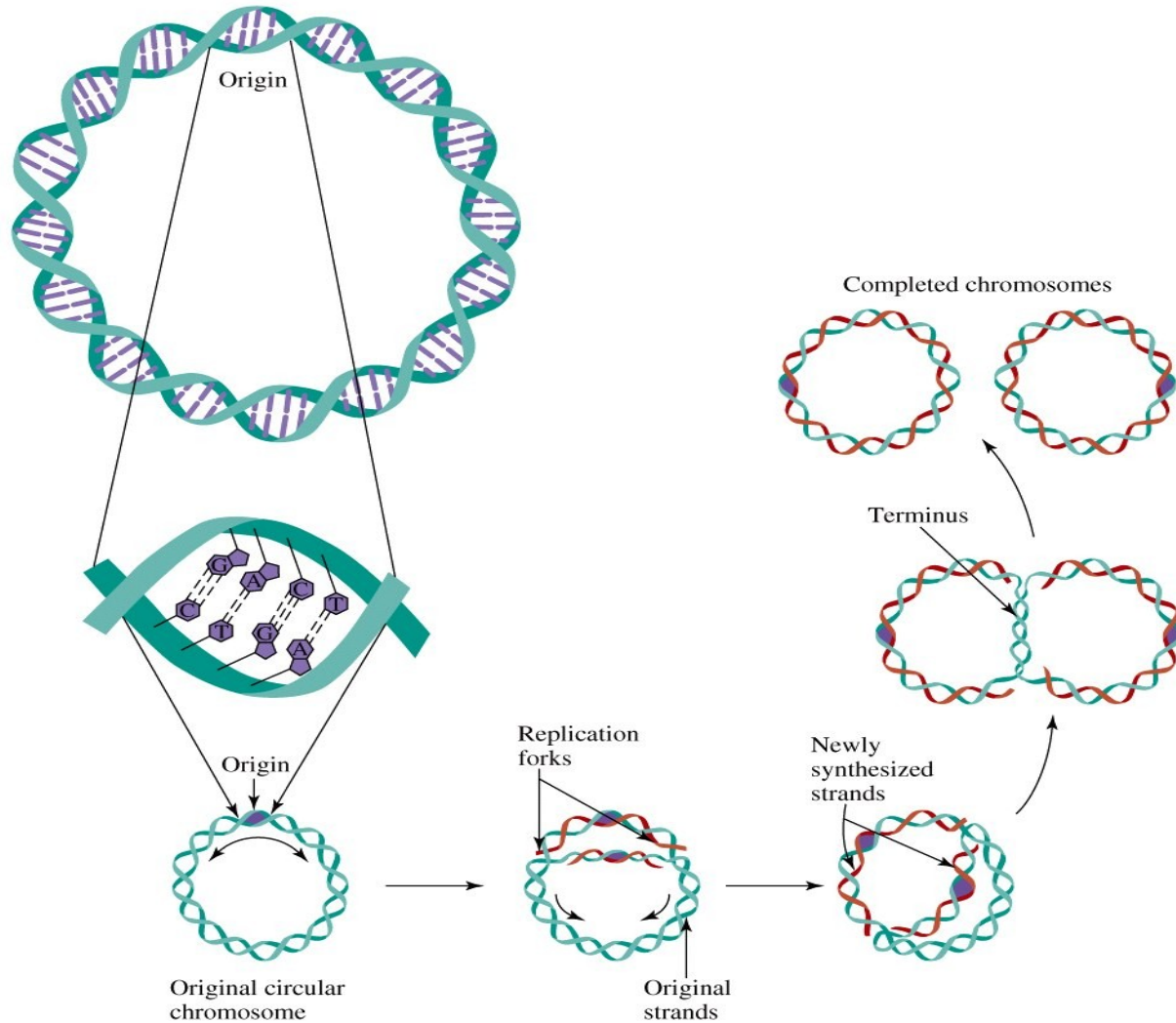
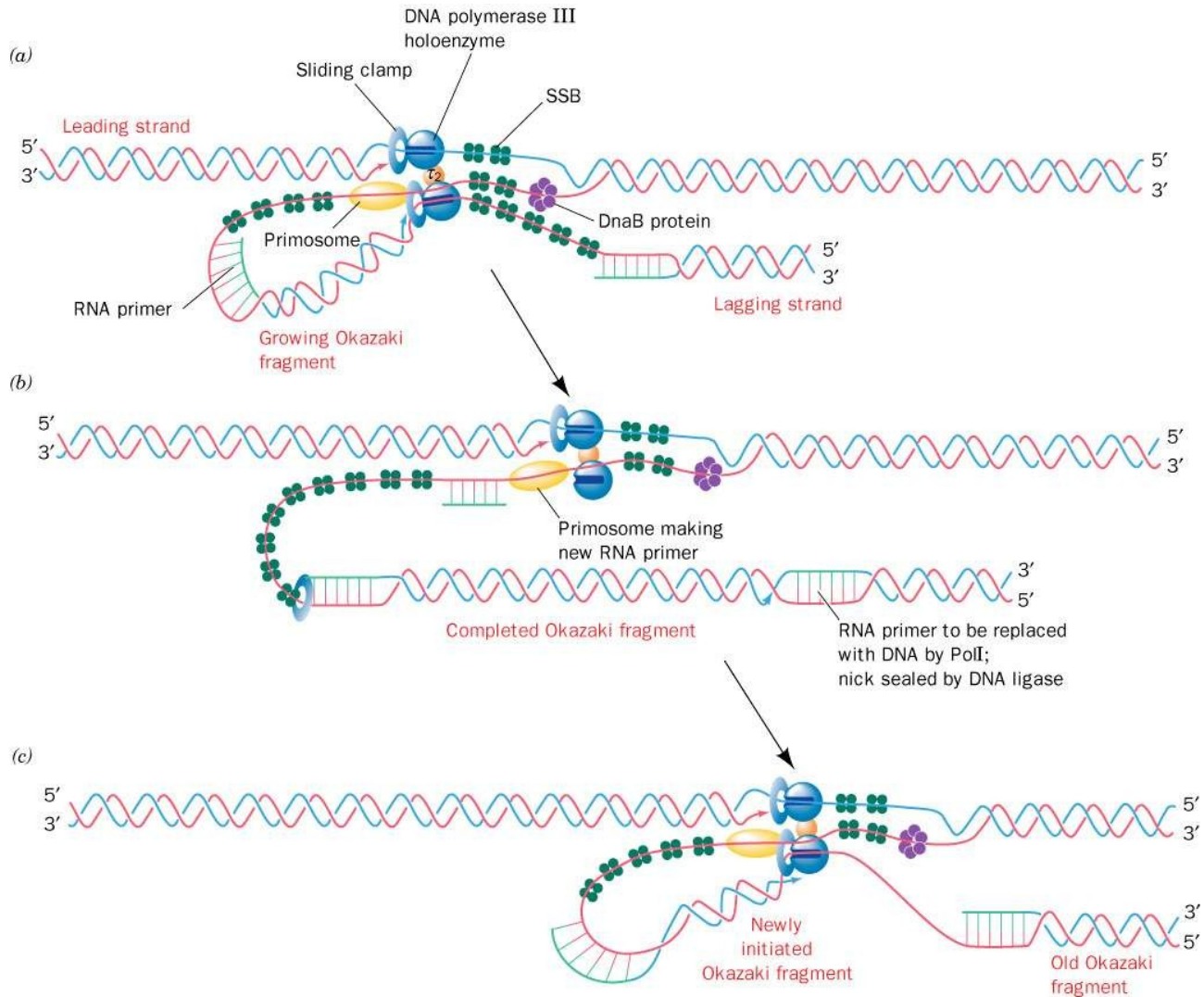


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# Prokaryontní replikace



# Replikace u E.coli

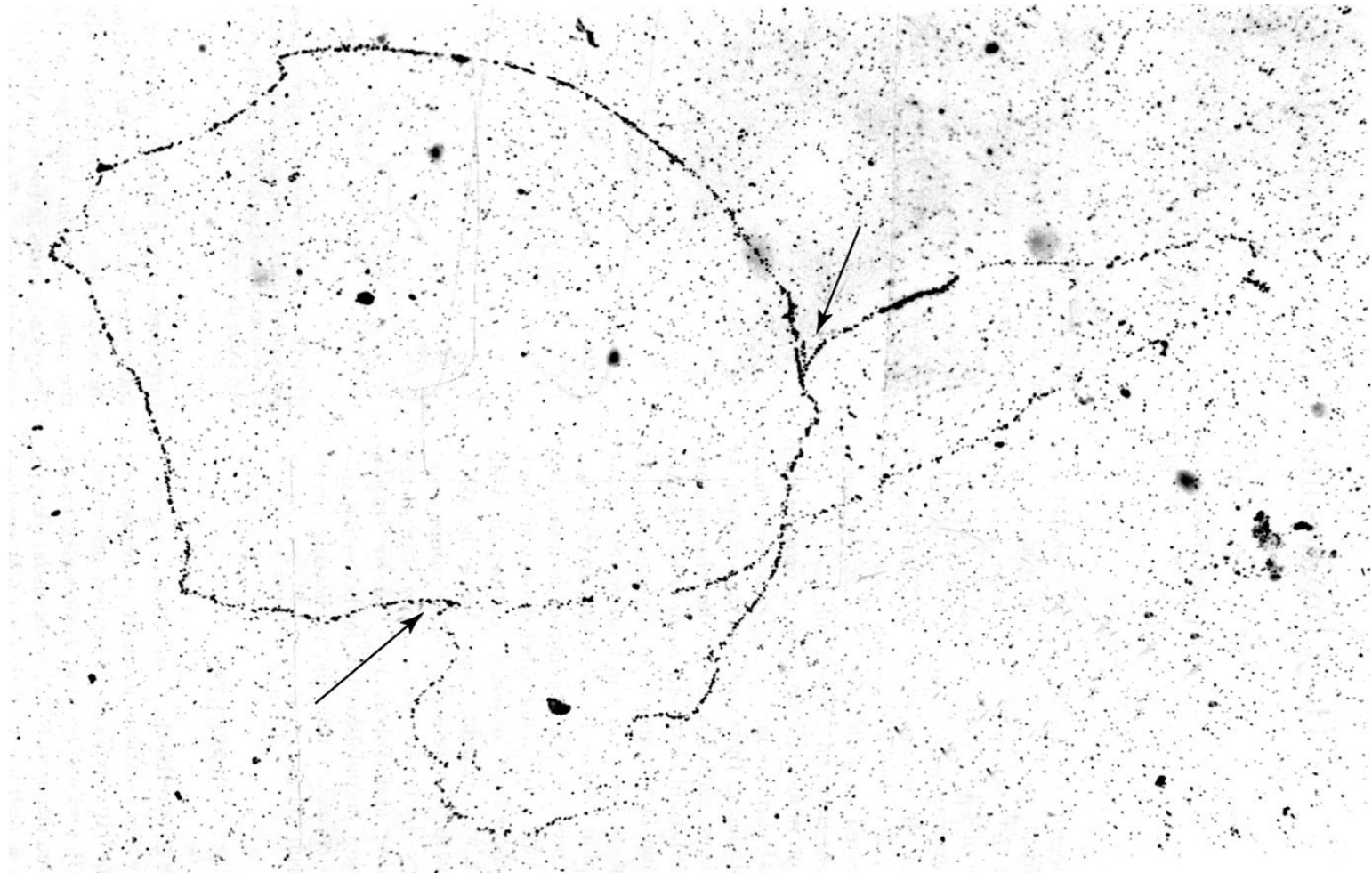


Figure 11-3 Concepts in Biochemistry, 3/e



# Eukaryontní replikaci

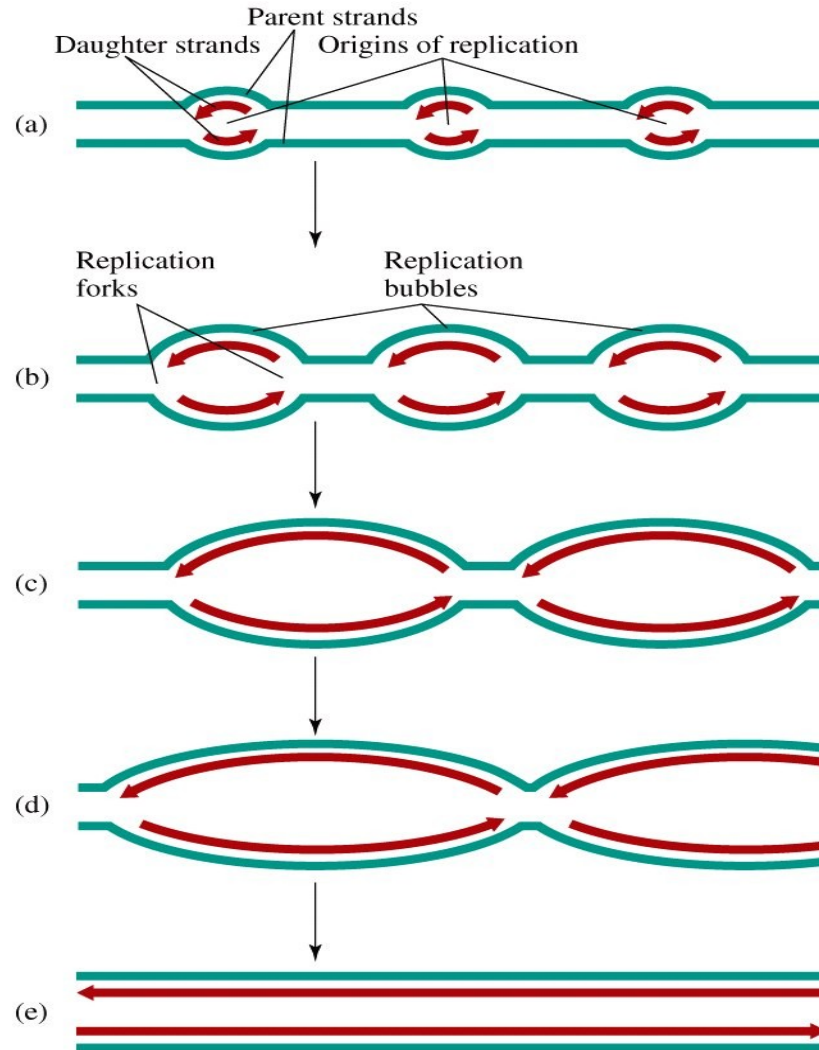
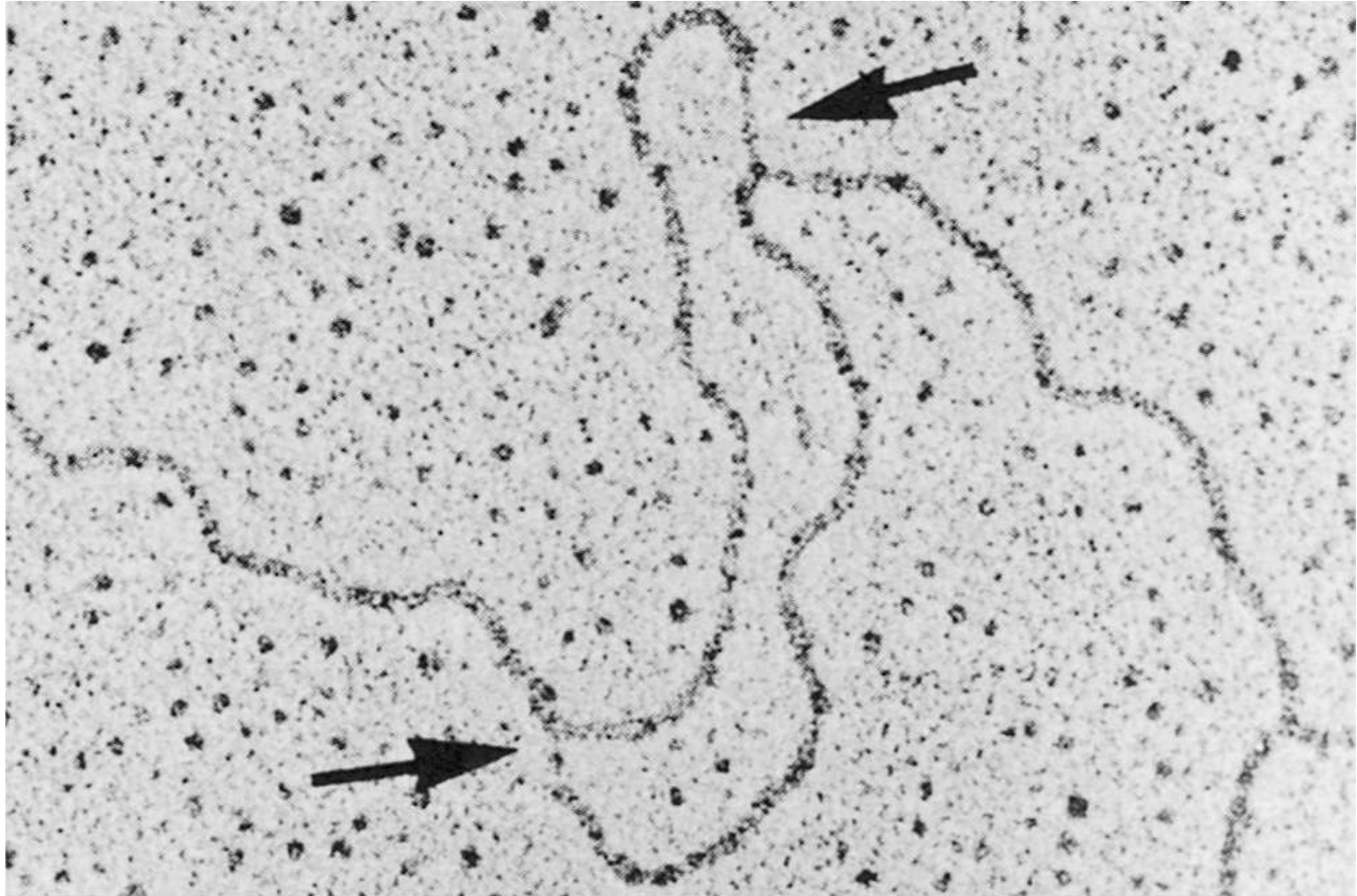


Figure 11-5 Concepts in Biochemistry, 3/e  
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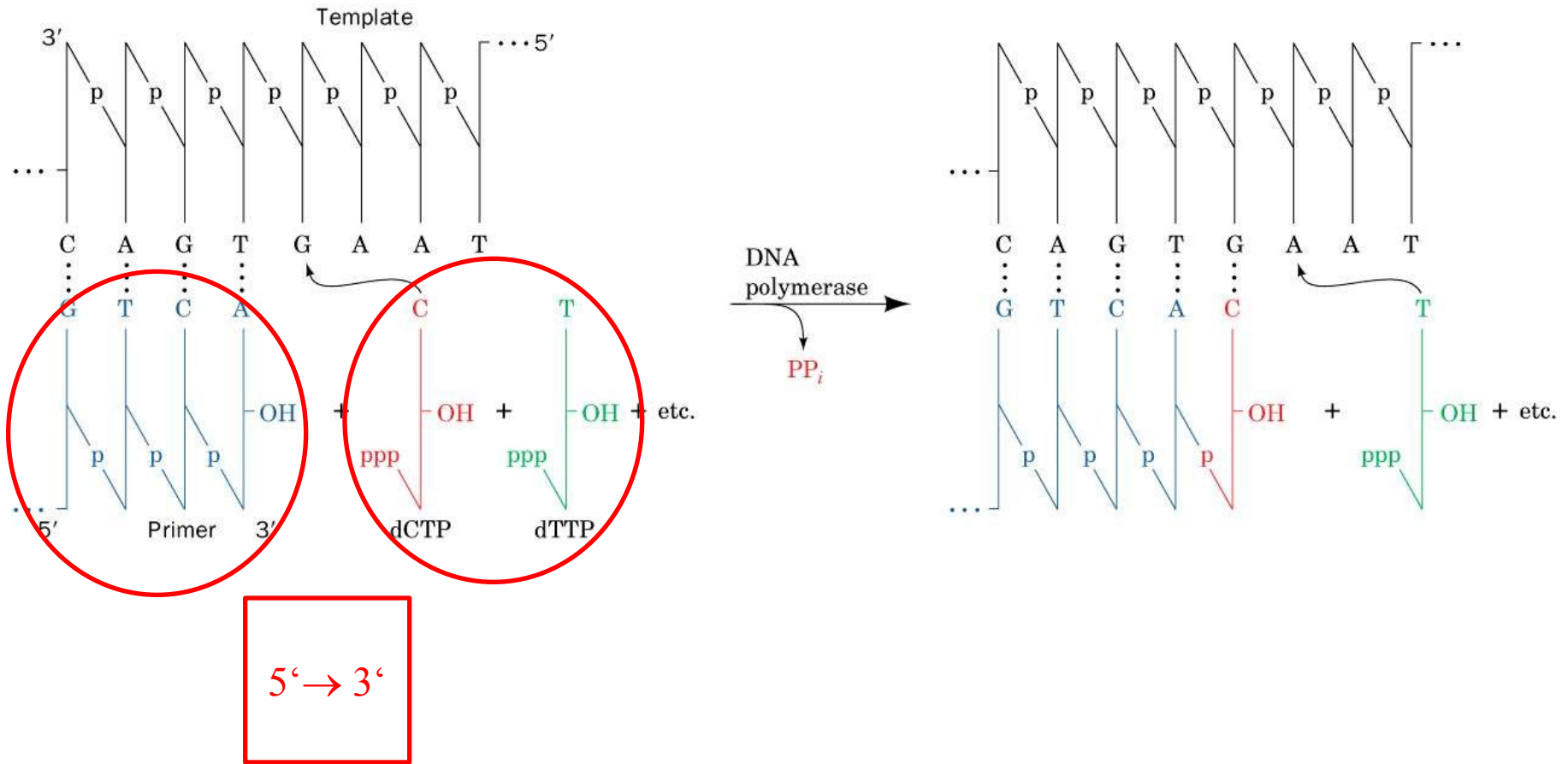
# Replikace u drosofil



From Kreigstein, H.J. and Hogness, D.S., *Proc. Natl. Acad. Sci.* **71**, 173 (1974)



# DNA polymeráza III



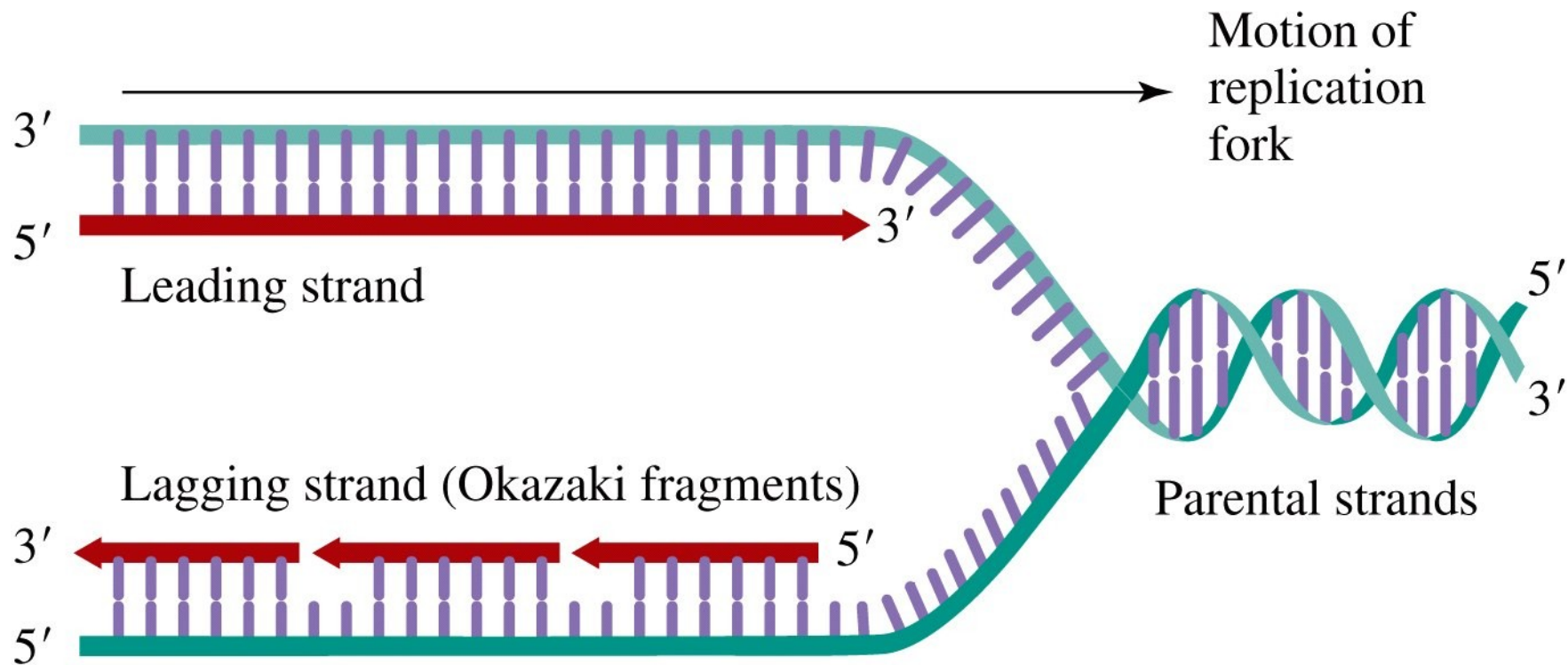
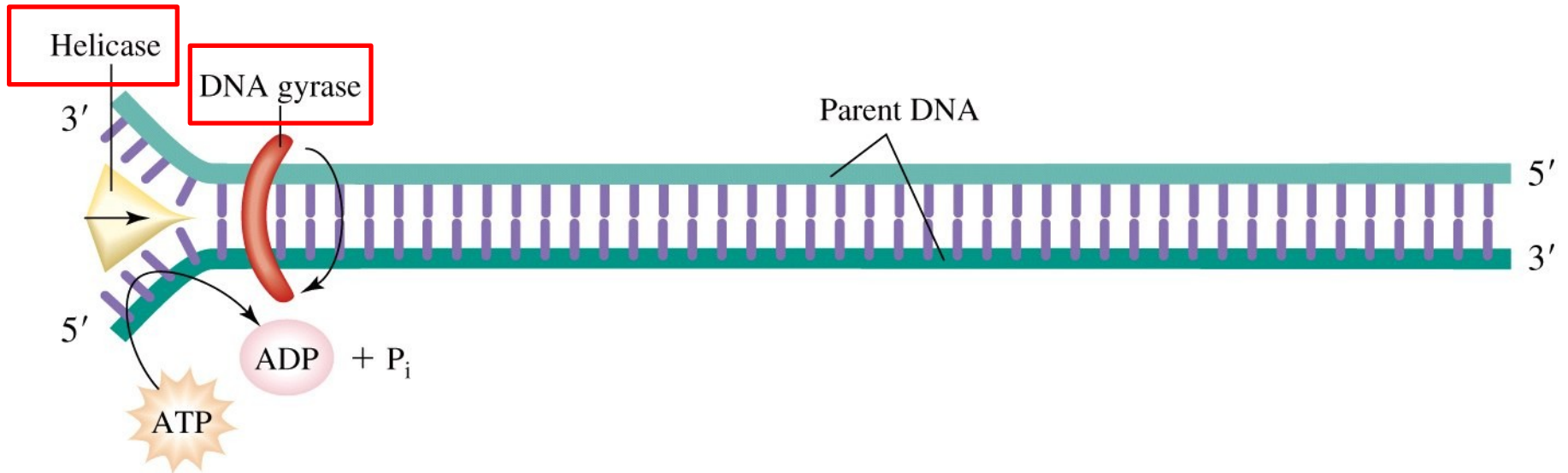
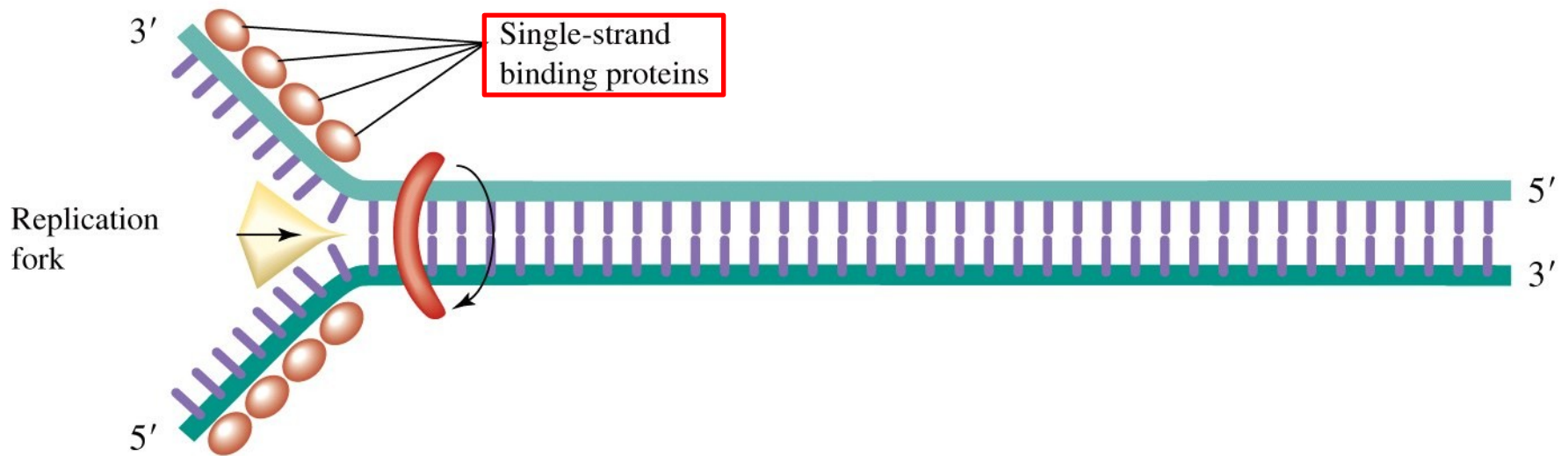


Figure 11-9 Concepts in Biochemistry, 3/e  
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**Figure 11-10a** Concepts in Biochemistry, 3/e  
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**Figure 11-10b** Concepts in Biochemistry, 3/e  
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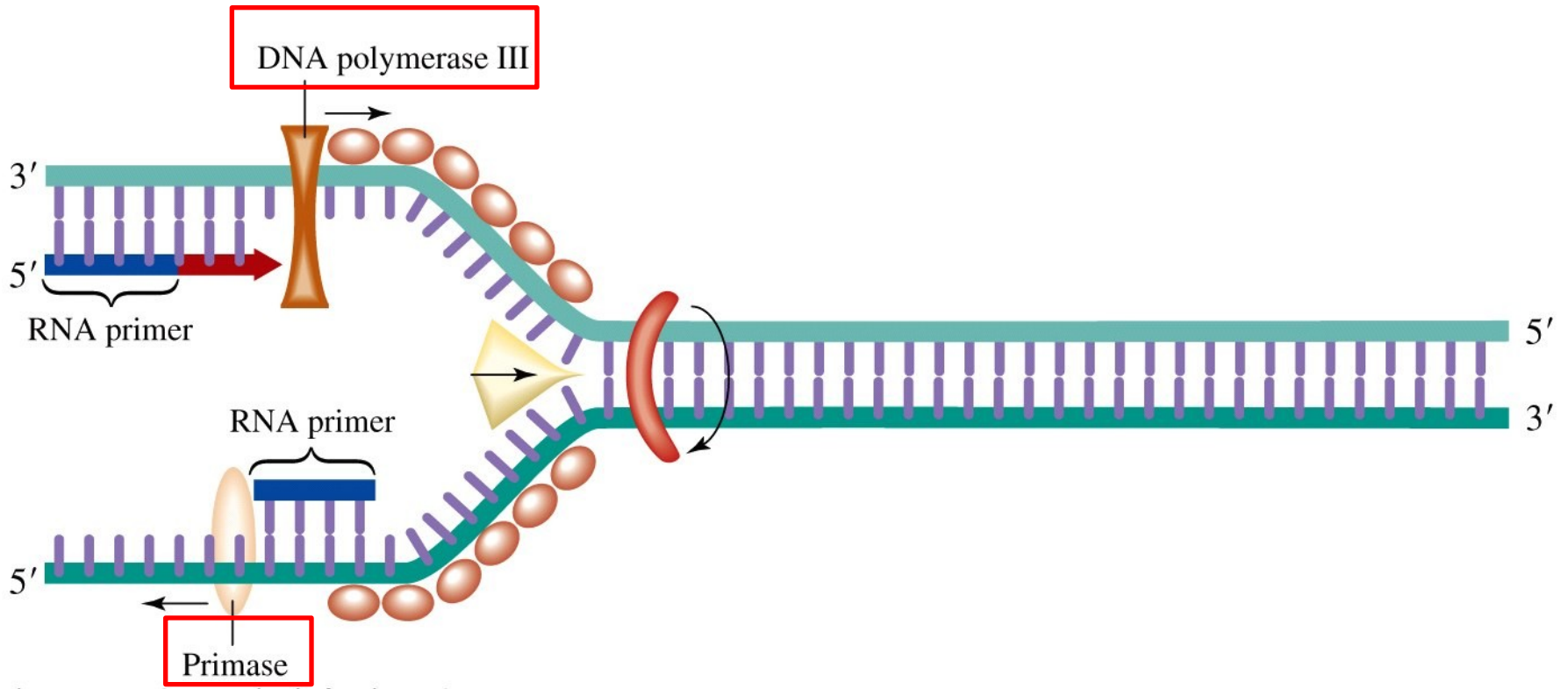


Figure 11-10c Concepts in Biochemistry, 3/e  
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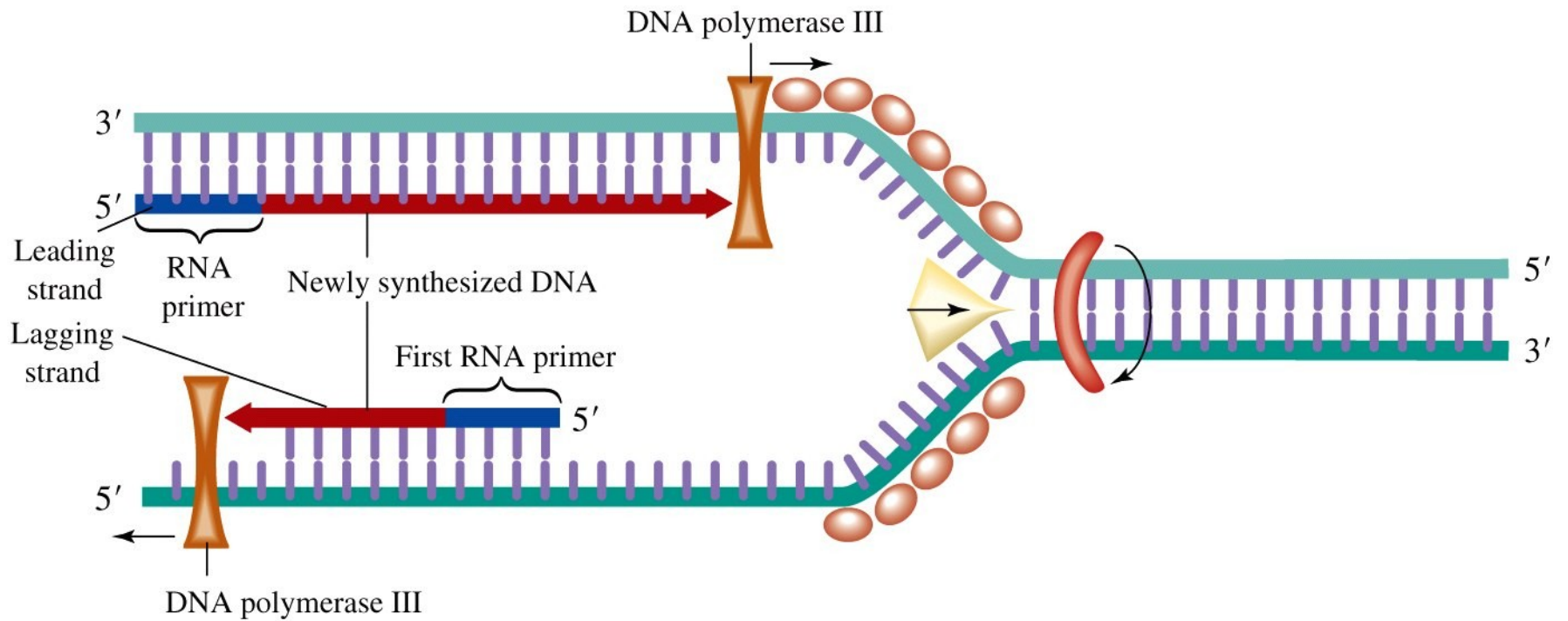


Figure 11-10d Concepts in Biochemistry, 3/e  
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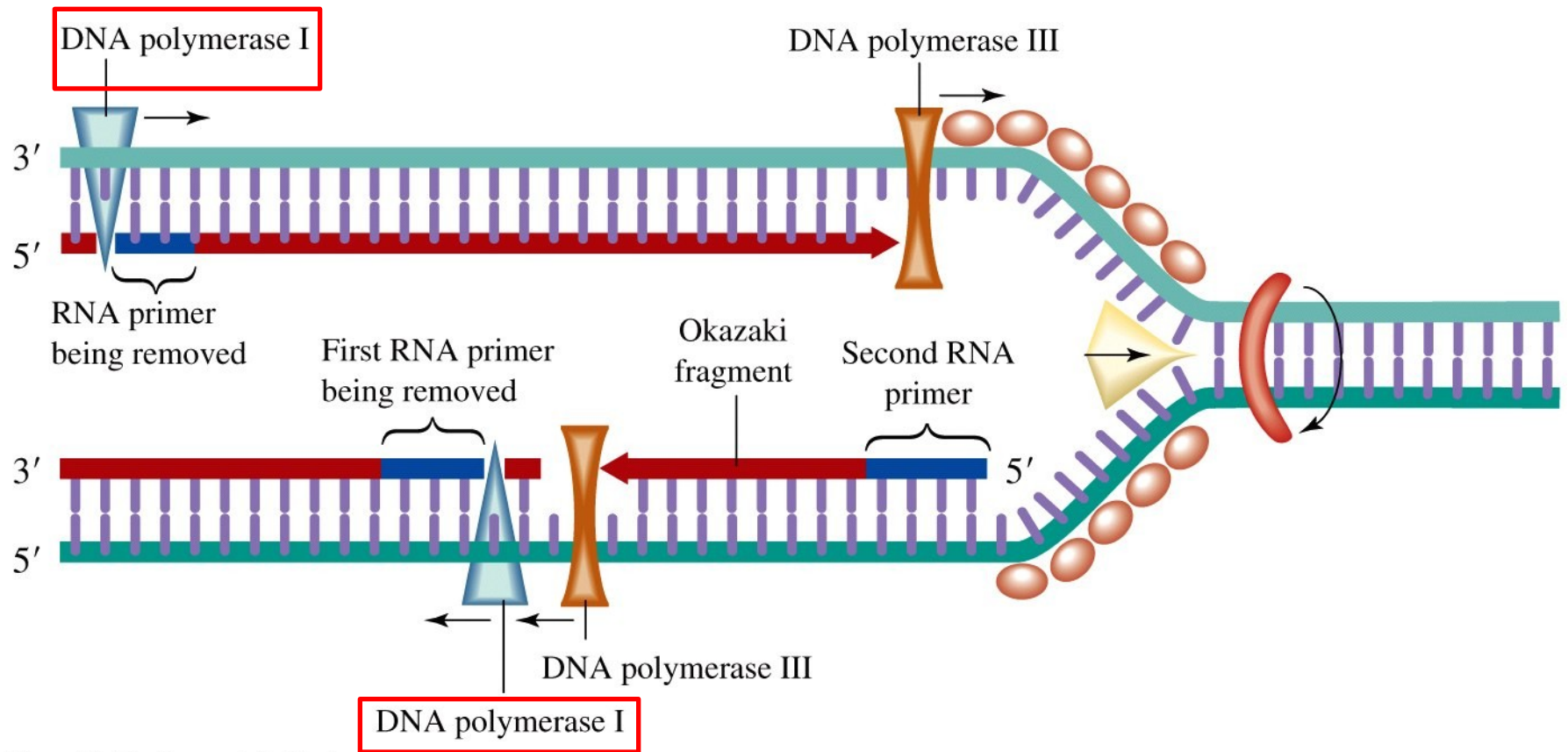


Figure 11-10e Concepts in Biochemistry, 3/e  
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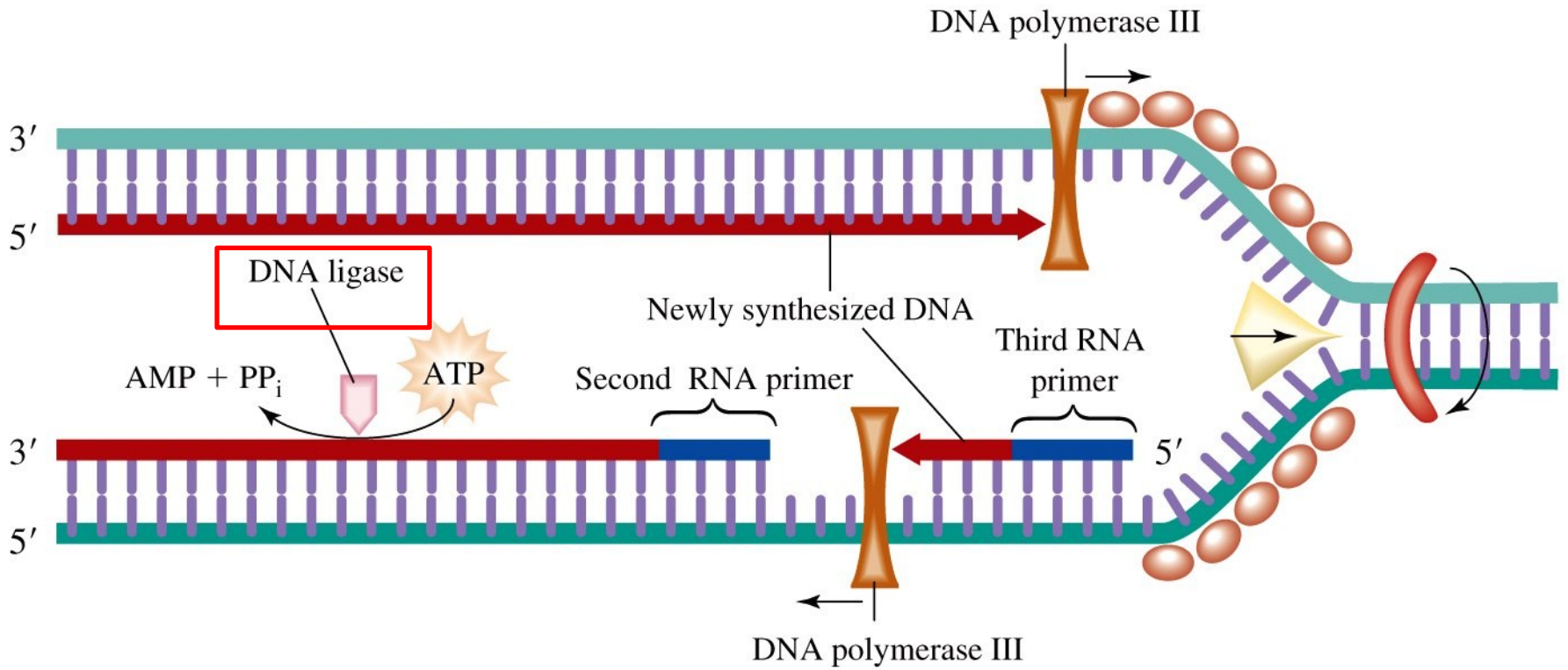
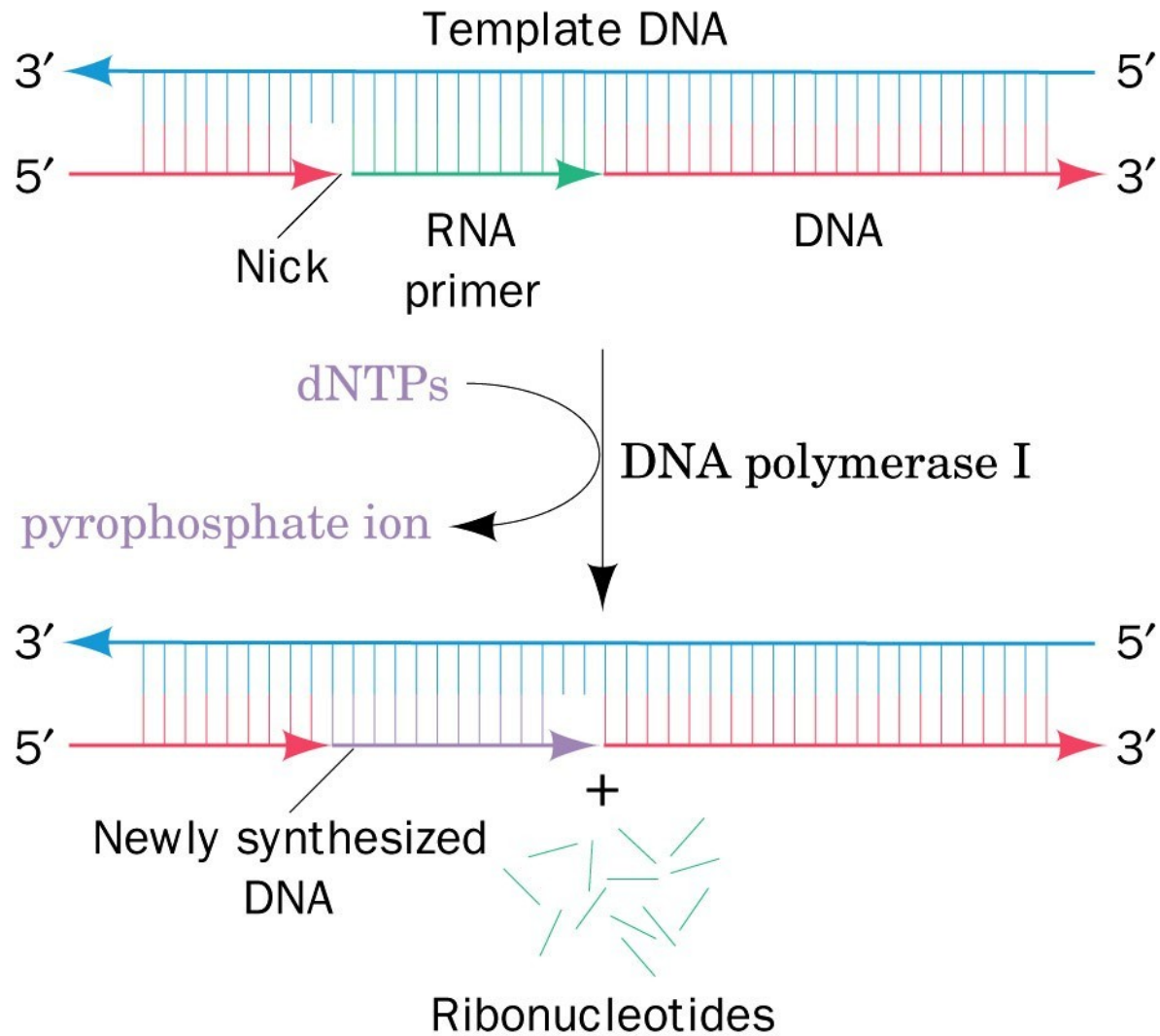
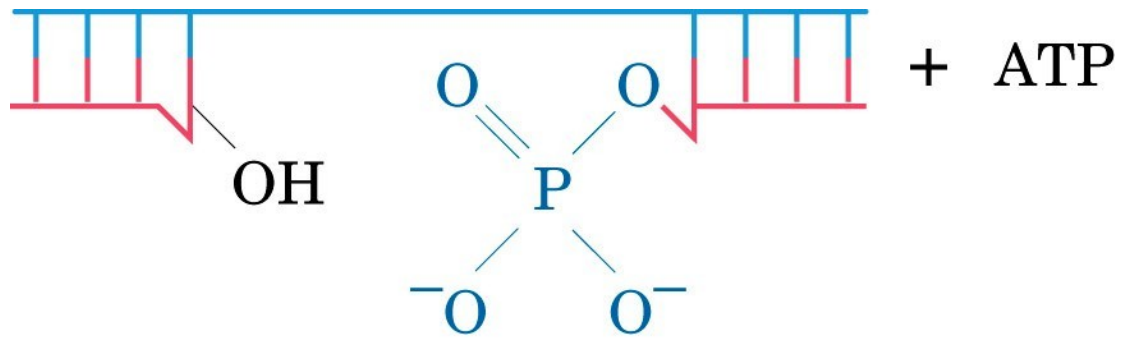


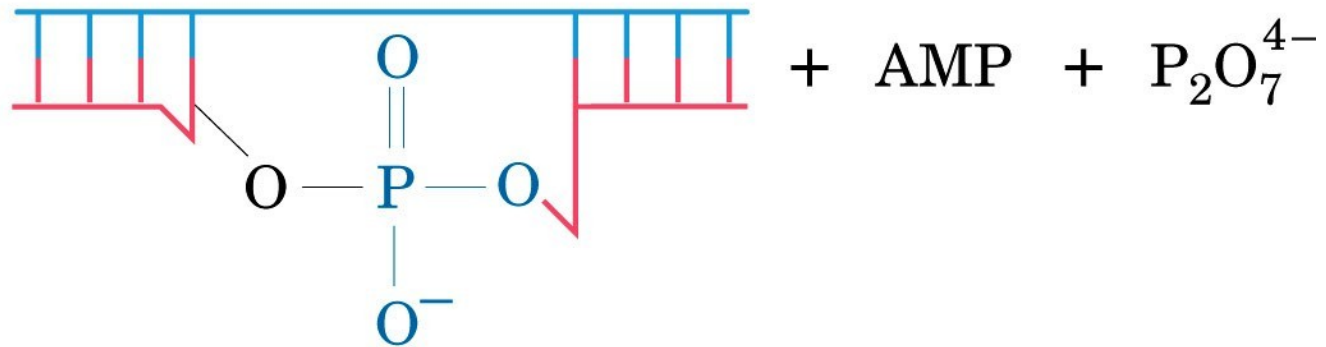
Figure 11-10f Concepts in Biochemistry, 3/e  
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DNA ligase



# Enzymová metoda

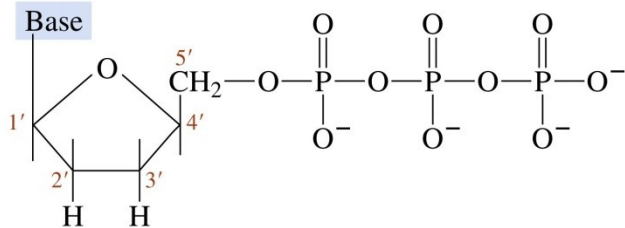


Figure 11-29 Concepts in Biochemistry, 3/e  
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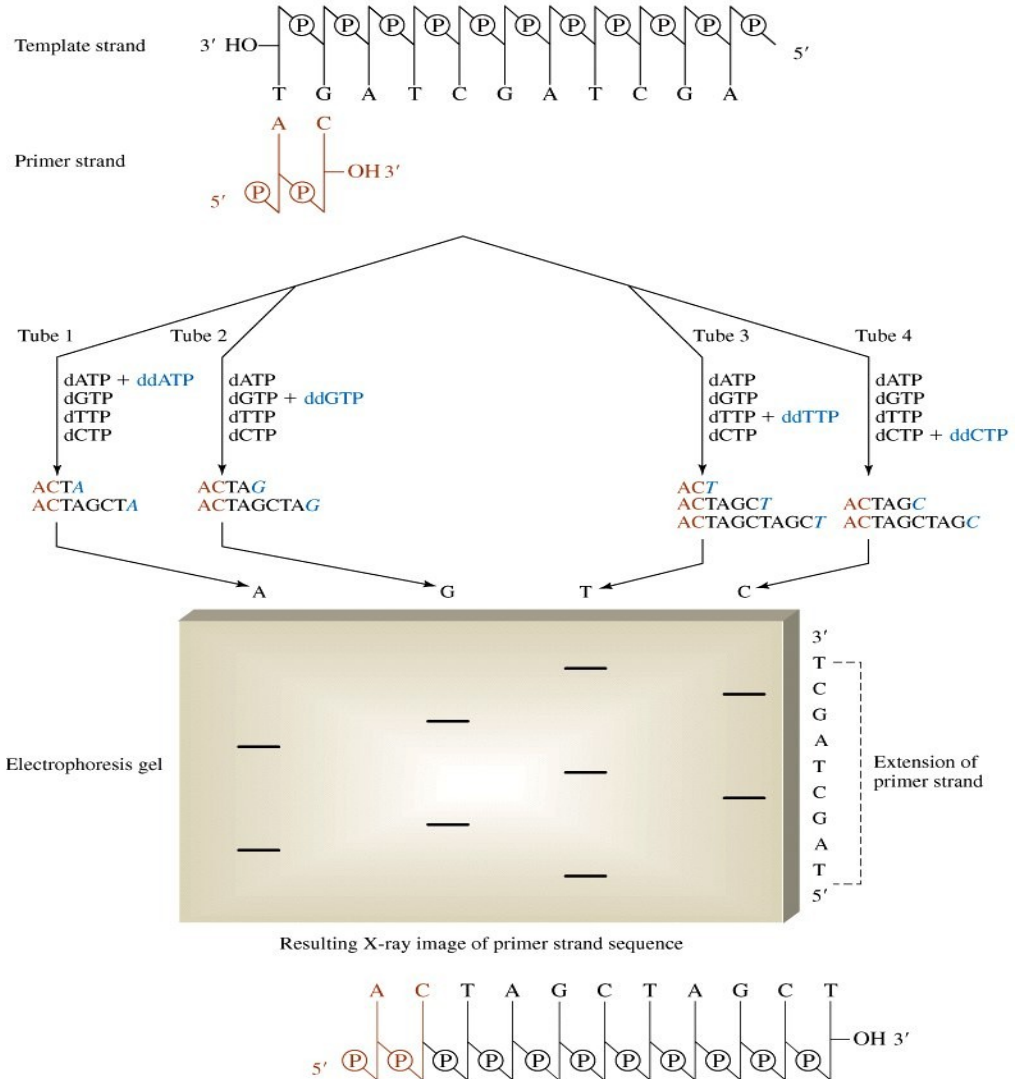
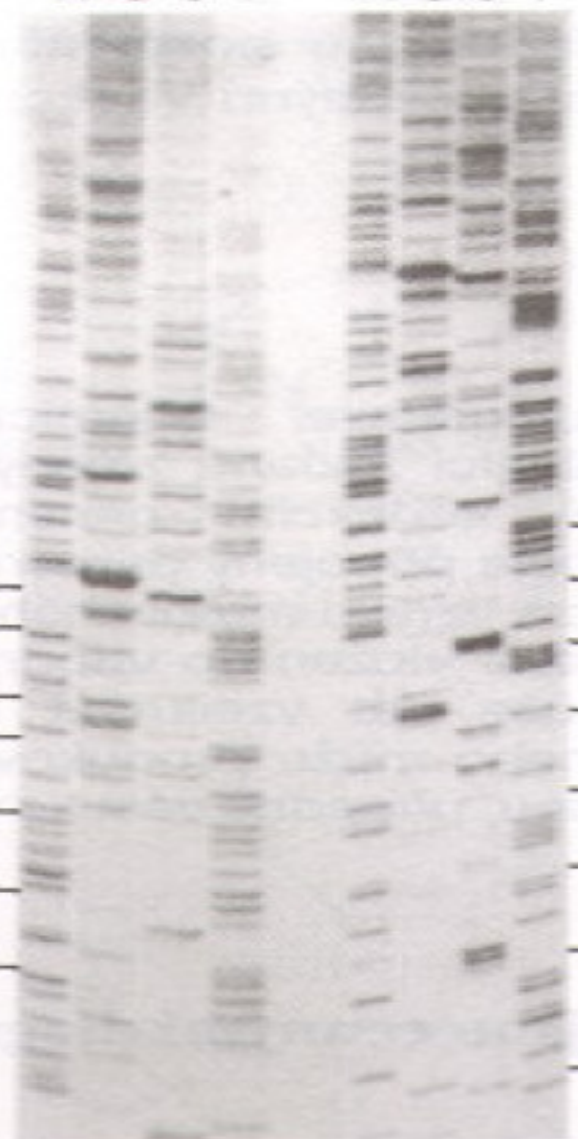


Figure 11-30 Concepts in Biochemistry, 3/e  
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A G C T

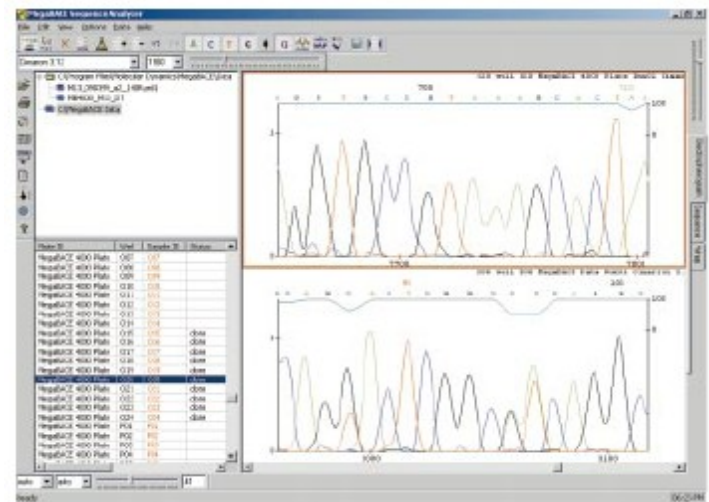
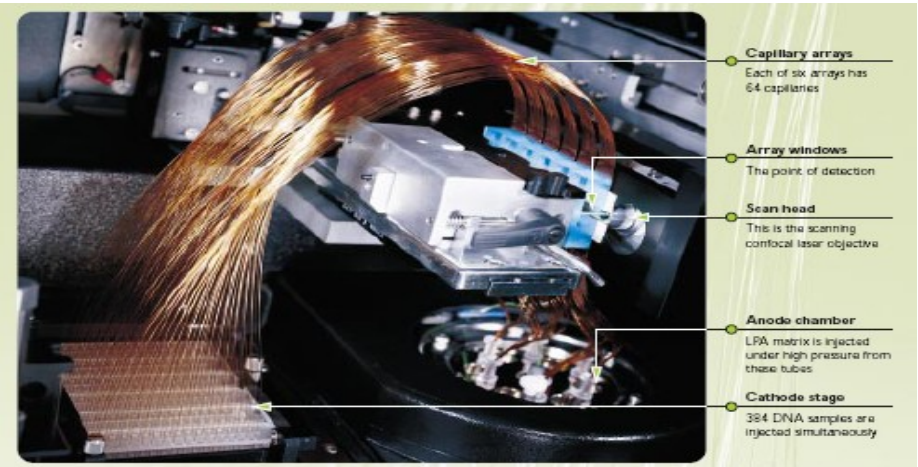
A G C T

140  
A G G T C C T T G G  
C T T T A T G T A T  
G T A A C A G A A G  
G C A T T T C G C G  
90 A T C T G A C T A T A  
T A A T A A T A T G  
T C C A A G T T T A

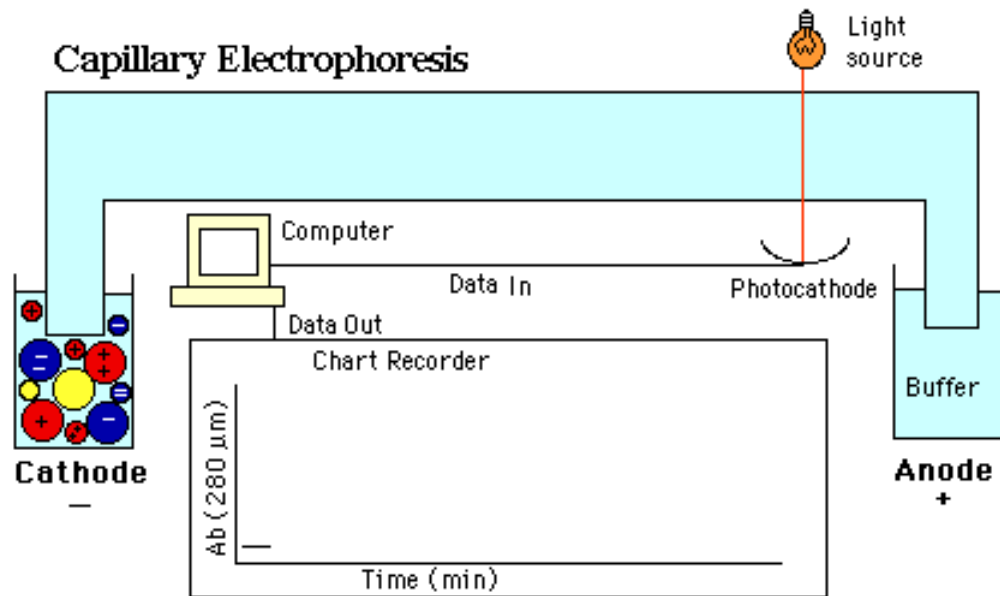


80  
A T T T G A A C C T  
C A G A T A G T A A  
T T C C C T G A A T  
C C T G G G A G T T  
A A C A T C C G A A  
T G A A C T T T T G  
T A C C C A T C A T  
T C G T A C T T G T

# 2003 - Projekt lidského genomu

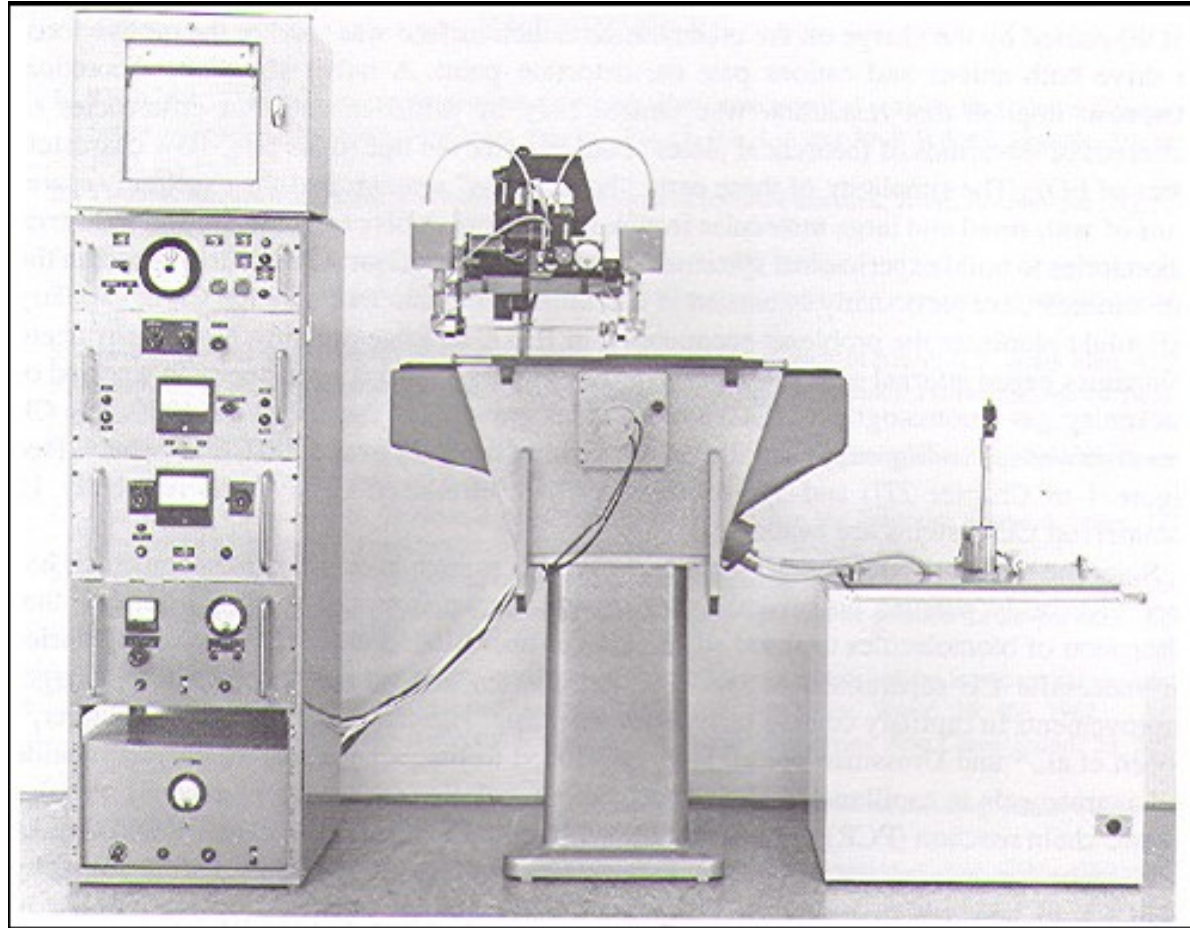


# Kapilární elektroforéza CZE





# Hjerten 1967



# 1981 - Jorgenson Lukacsová



CLIN. CHEM. 27/9, 1551-1553 (1981)

## Free-Zone Electrophoresis in Glass Capillaries

James W. Jorgenson and Kryn DeArman Lukacs

A simple theory of zone electrophoresis in open-tubular capillaries is developed. According to this theory, to achieve the highest resolution of zones, tubes with as small an inside diameter as possible should be used in combination with as high an applied voltage as feasible. To test this approach, we performed electrophoresis in glass capillaries with an internal diameter of  $75\ \mu\text{m}$  and a length of 100 cm. A special fluorescence detector was used to detect fluorescent zones while they migrated inside the capillary. With the application of 30 kV potentials to this system, rapid and efficient separations of amino acids, peptides, and urinary amines were demonstrated. In all cases fluorescent derivatives were necessary for detection. Preliminary results are encouraging, and with further development of sensitive detection devices, applications in clinical analyses may be feasible.

small diameter simultaneously facilitates heat transfer as well as stabilizes the medium. Regardless of the diameter, some radial temperature gradient will persist. However, this temperature gradient is undesirable only to the extent that a significant fraction of the solute molecules making up a zone spend longer than average times migrating within "cool" or "hot" regions of the tube radius. Here a more subtle effect of reduced tube diameters comes into play. If the diameter is small enough that solute molecules diffuse back and forth across the tube many times during their migration, then the probability that a significant fraction of molecules will spend excessive amounts of time in any one portion of the tube radius is greatly reduced. Thus the solute molecules have an excellent chance of traveling throughout all portions of the tube radius, and any variations in migration velocity will tend to average out.

To summarize, the possible advantages of performing zone electrophoresis in open tubes of small diameter are:

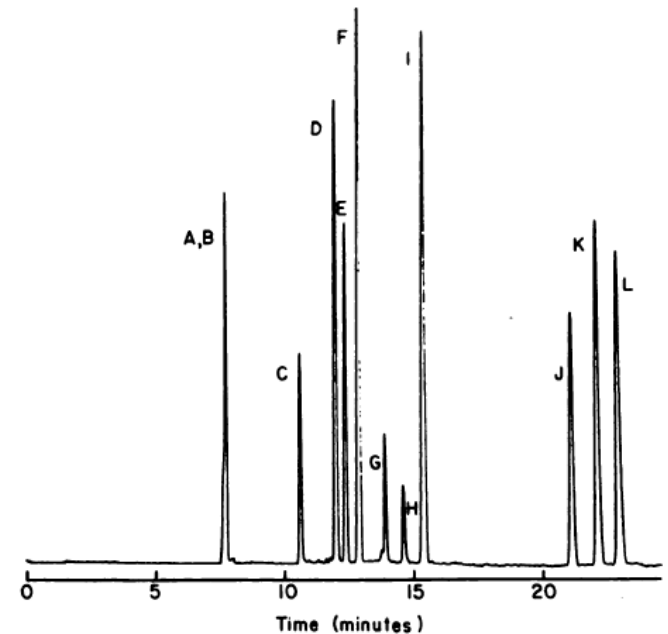
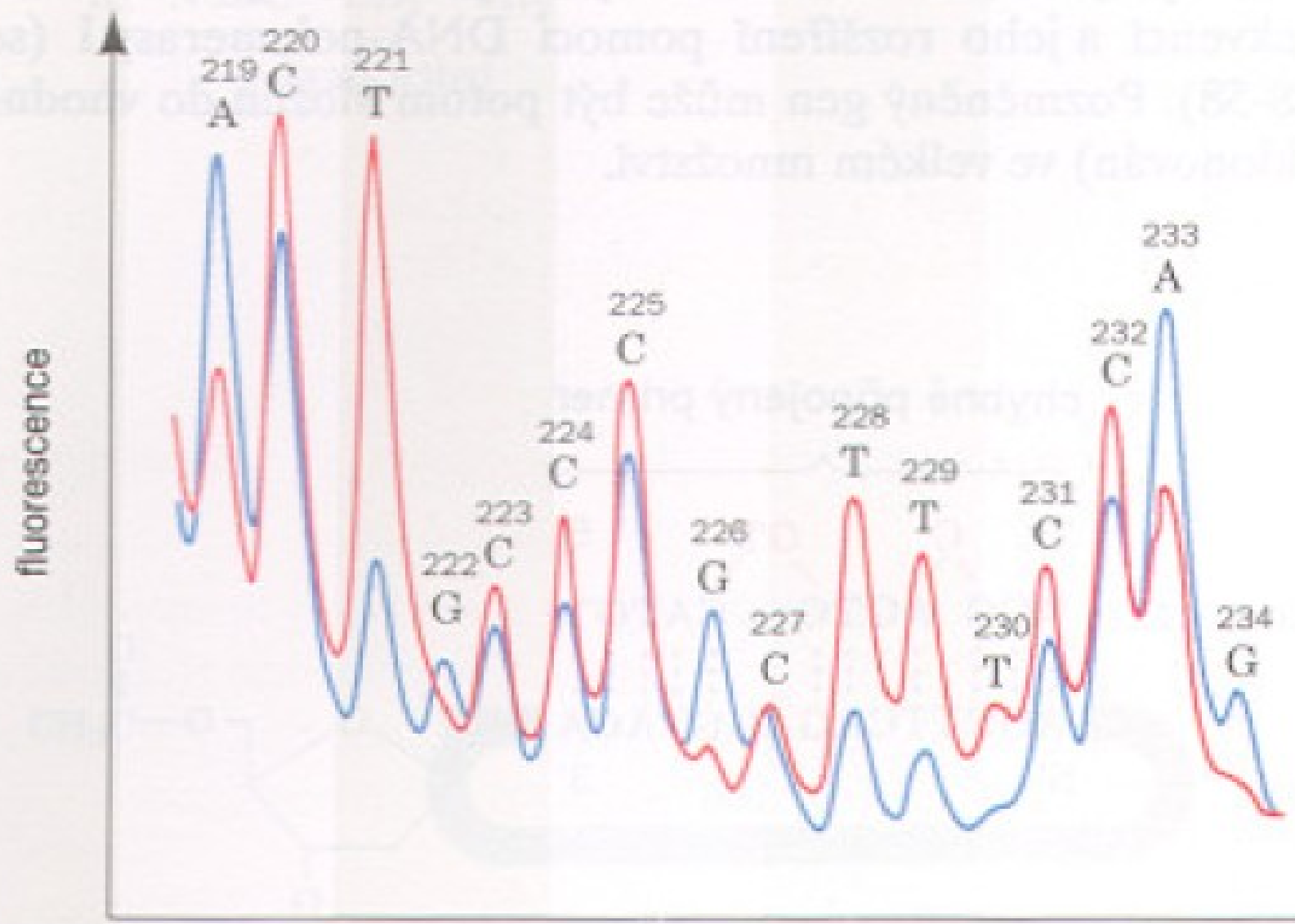


Fig. 1. Electropherogram of dansyl amino acids

# Beckman 1987











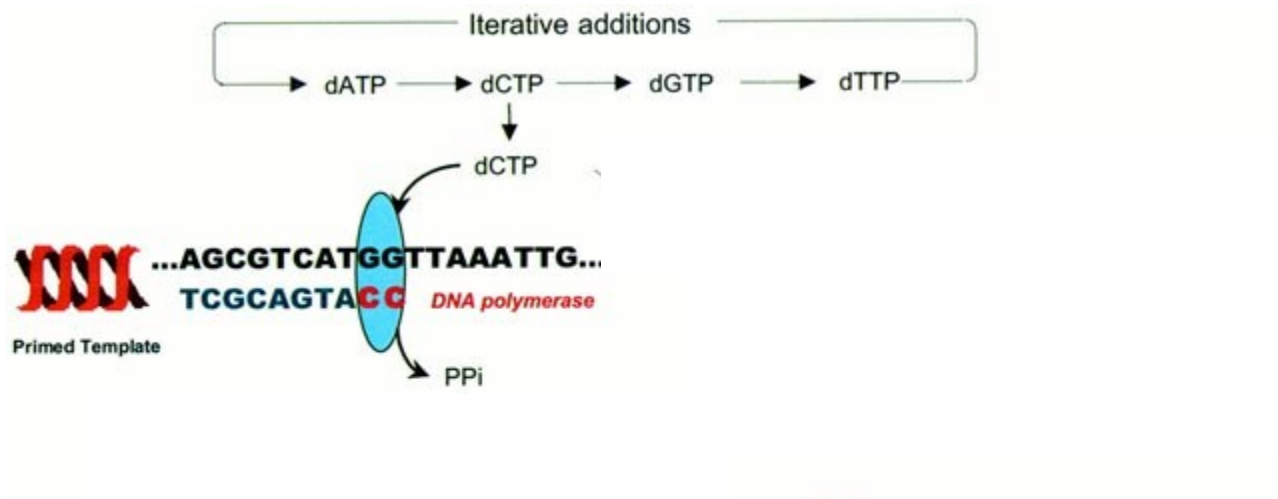
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# Pyrosekvenování

- První reakcí je DNA polymerace pomocí DNA polymerázy, kdy dochází k zařazení příslušného deoxynukleotid trifosfátu (dNTPs) za uvolnění pyrofosfátu.



# Pyrosekvenování



# Pyrosekvenování

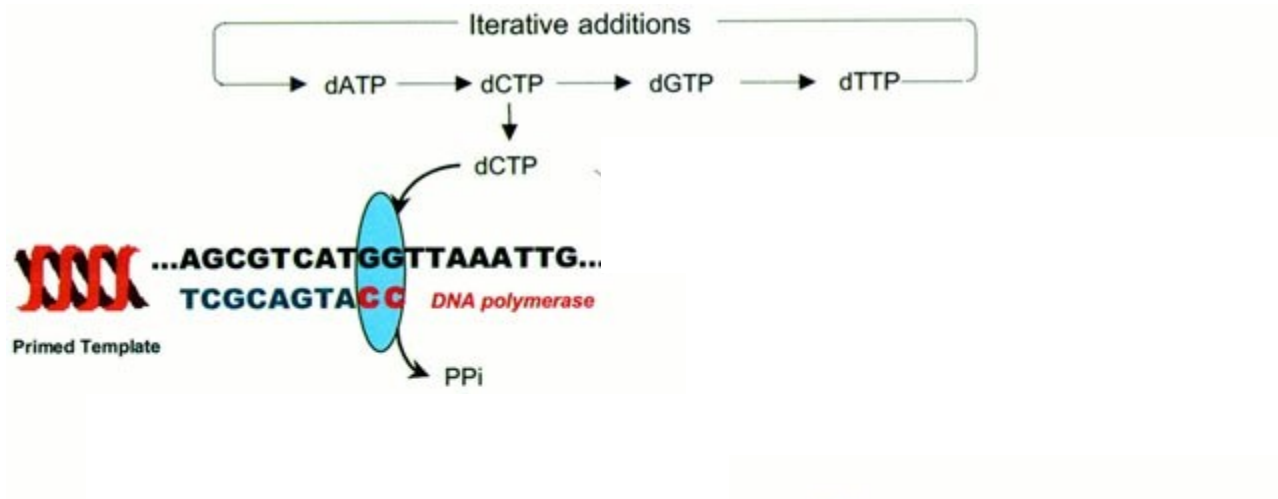
- První reakcí je DNA polymerace pomocí DNA polymerázy, kdy dochází k zařazení příslušného deoxynukleotid trifosfátu (dNTPs) za uvolnění pyrofosfátu.



- Vzniklý pyrofosfát je uvolněn z polymerázy a může sloužit jako substrát pro ATP sulfurylázu. Při této reakci dojde ke kvantitativnímu převedení pyrofosfátu na ATP.



# Pyrosekvenování



# Pyrosekvenování

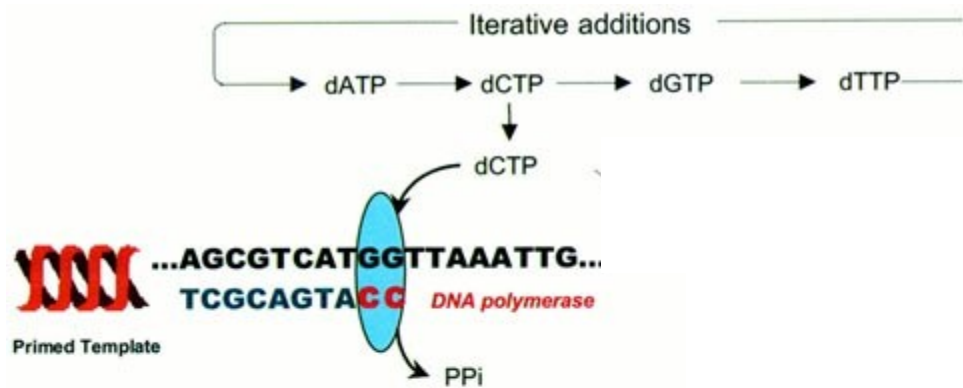
- Během třetí a čtvrté reakce je ATP převedeno na světelný signál pomocí enzymu luciferázy a následně je světelný signál detekován a vyhodnocen programem.

Luciferáza + D-luciferin + ATP → Luciferáza-luciferin-AMP + PPi

Luciferáza-luciferin-AMP + PPi + O<sub>2</sub> → Luciferáza + Oxyluciferin + AMP + CO<sub>2</sub> + světlo



# Pyrosekvenování

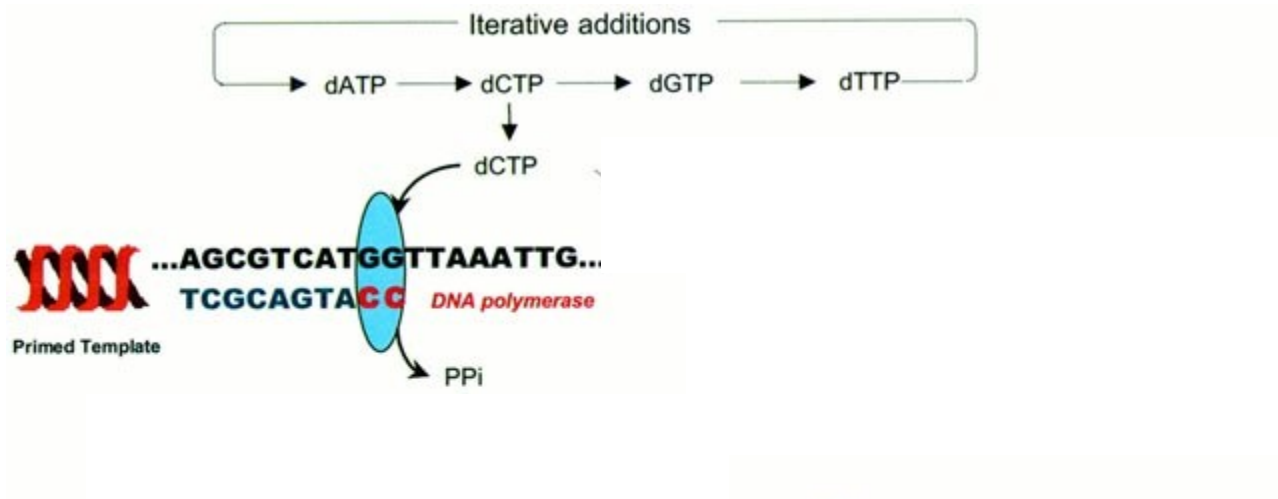


# Pyrosekvenování

- Poslední enzymatickou reakcí je reakce apyrázy, která odstraní nezainkorporované nukleotidy a ATP, aby následně mohlo dojít k zopakování celého výše popsaného procesu a mohlo být analyzováno zařazení dalšího nukleotidu. Tato degradace je nezbytná, aby bylo zajištěna synchronizace mezi syntézou a detekcí světelného signálu.



# Pyrosekvenování



# 454 pyrosekvenování

Technologie využívá paralelní sekvenace: více než

-2 milion sekvencí zároveň.

-Lze získat až 1GB (gigabázi) informace během jedné analýzy (cca4.5 h).

Využití:

-sekvenace genomů (náhodně naštěpená genomová DNA je sekvenována a sestavena)

- studium metagenomů (tj. souhrn všech genů, přítomných v daném prostředí, používá se DNA extrahovaná ze vzorku půdy, vody, sedimentu, mikroflóry střeva ad.)

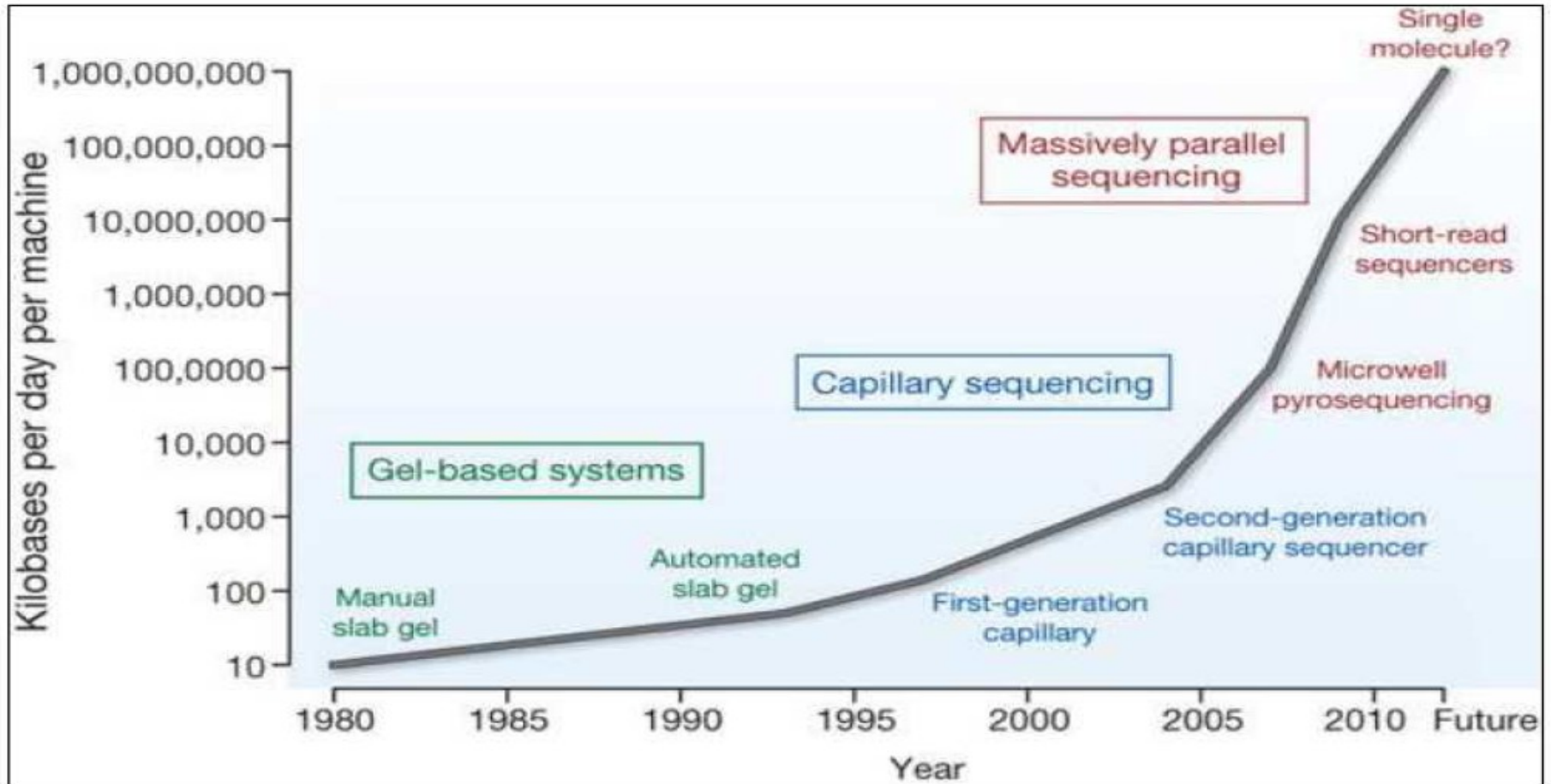
- tzv. ampliconové sekvenování. Vlastní sekvenaci předchází PCR zacílená na 16S nebo 18S geny prokaryot a eukaryot

- analýza typu „shotgun“ – veškerá DNA / RNA, získaná ze vzorku



Zařízení je velmi nákladné (cca 17 mil. Kč), analýzy jsou ale dostupné komerčně, takže většina laboratoří v současnosti využívá služeb externích sekvenačních středisek.

# Sekvenování



**Table 13.3**  
**Some Sequenced Genomes**

Organism	Genome size (kb)	Number of Chromosomes
<i>Mycoplasma genitalium</i> (human parasite)	580	1
<i>Borrelia burgdorferi</i> (agent of Lyme disease)	1444	1
<i>Haemophilus influenzae</i> (human pathogenic bacterium)	1830	1
<i>Mycobacterium tuberculosis</i> (cause of tuberculosis)	4412	1
<i>Escherichia coli</i> (bacterium)	4639	1
<i>Saccharomyces cerevisiae</i> (yeast)	11,700	16
<i>Drosophila melanogaster</i> (fruit fly)	137,000	4
<i>Oryza sativa</i> (rice)	430,000	12
<i>Homo sapiens</i> (human)	3,200,000	23

# PCR Mullis NC1993

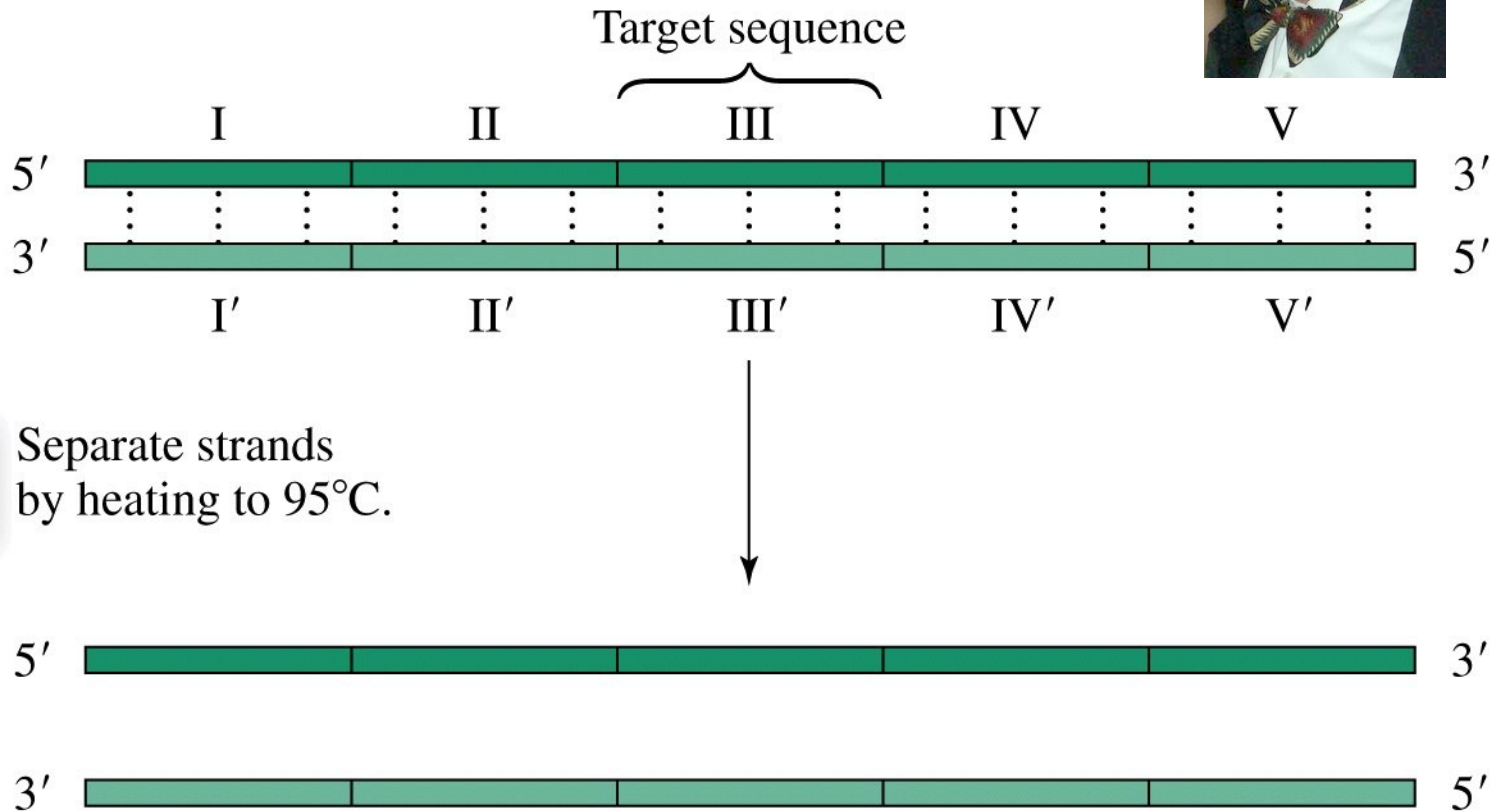
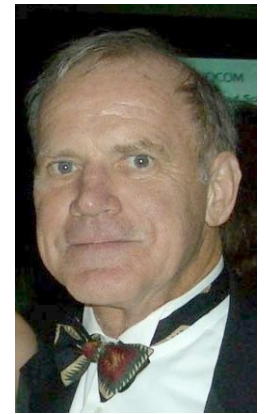


Figure 13-11 part 1 Concepts in Biochemistry, 3/e  
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Step 2

Hybridize primers by cooling to 50°C.

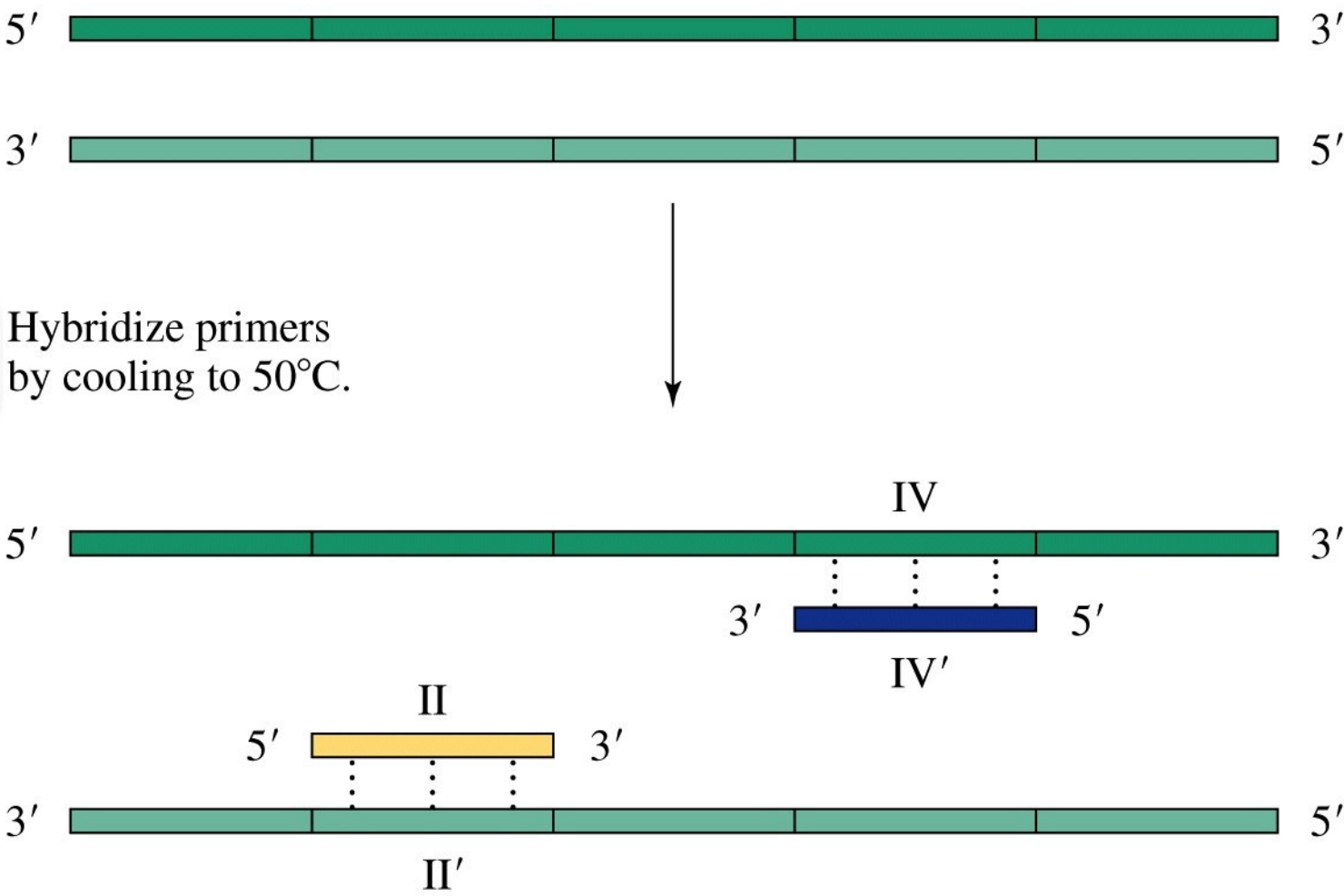
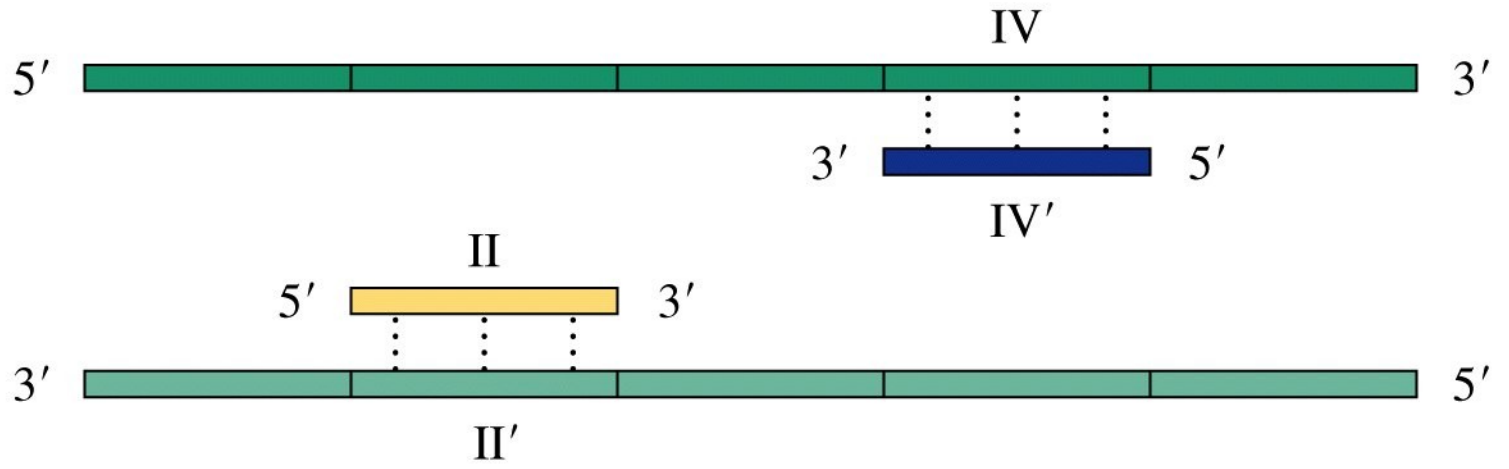


Figure 13-11 part 2 Concepts in Biochemistry, 3/e  
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Step 3

DNA is synthesized by extending the primers at 72°C.

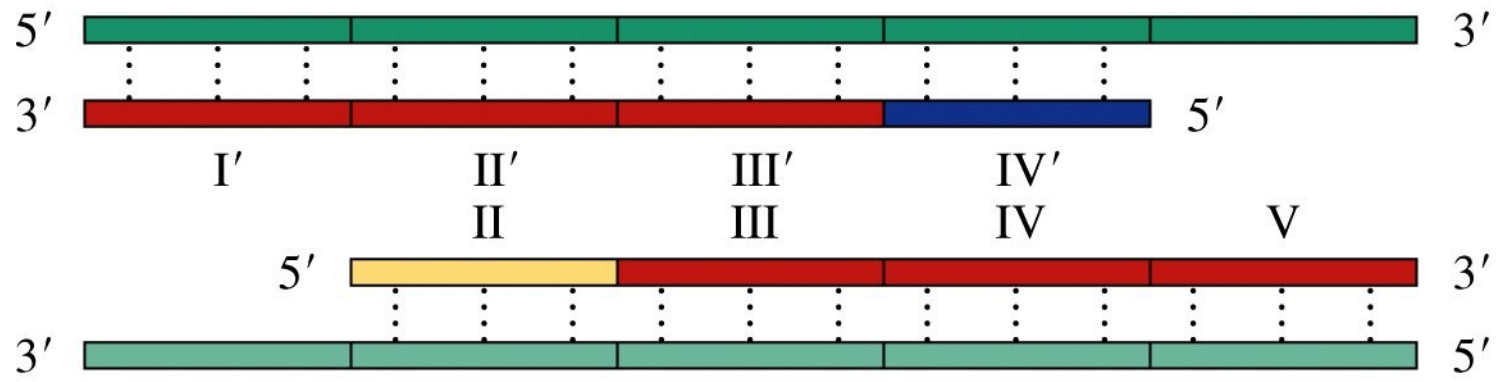
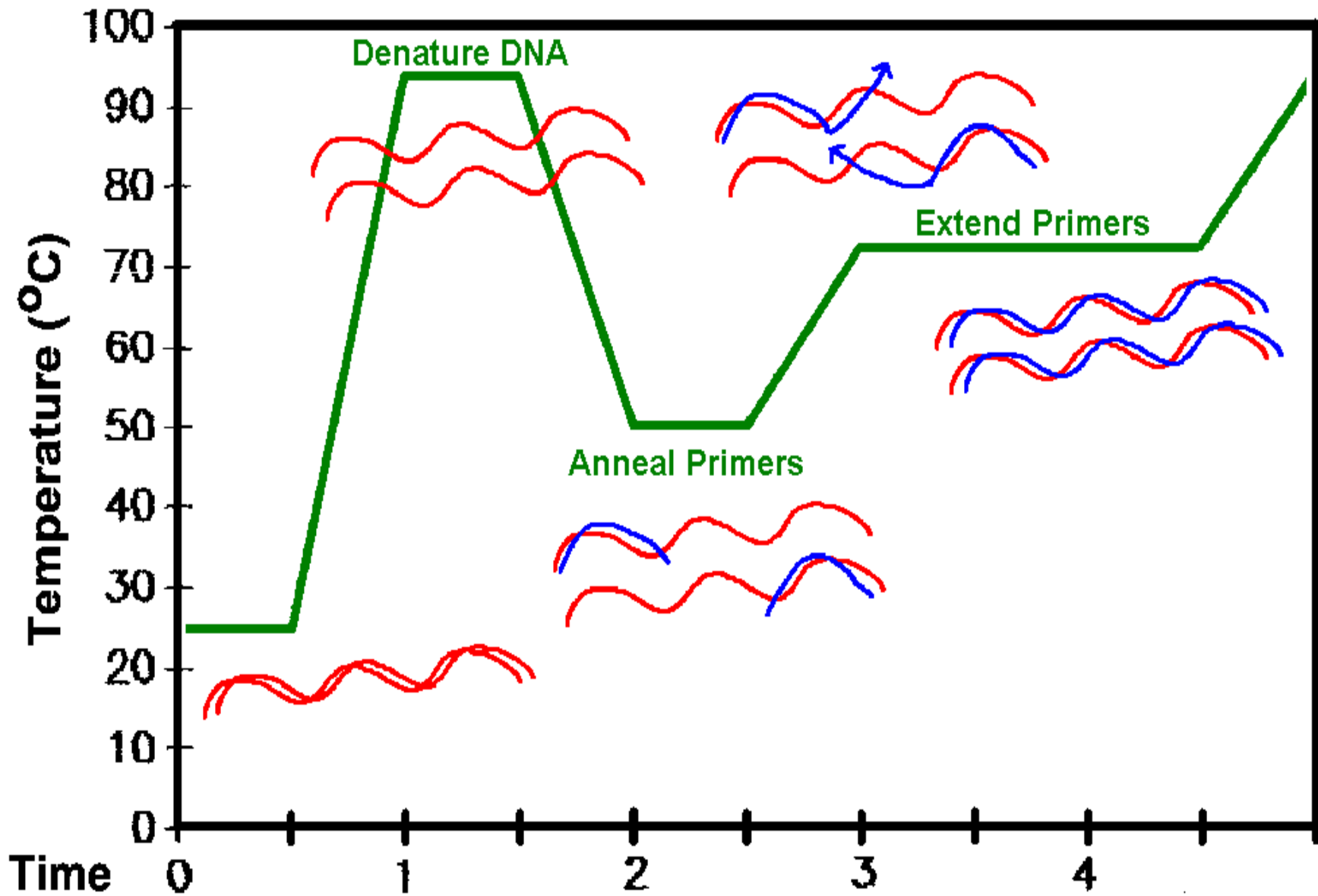
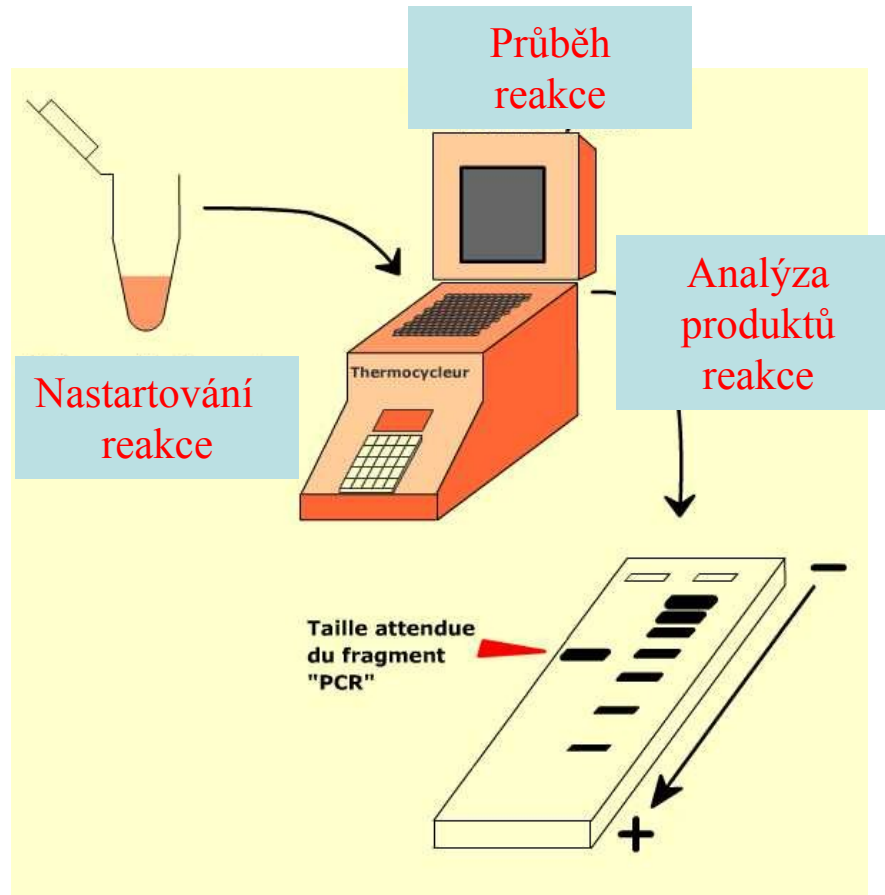


Figure 13-11 part 3 Concepts in Biochemistry, 3/e  
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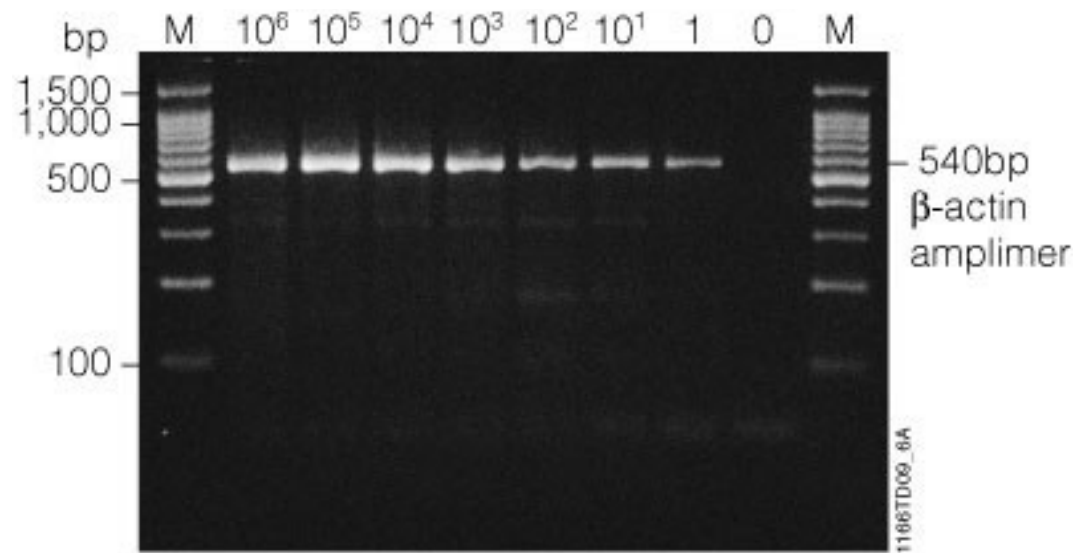
<http://www.dnalc.org/resources/animations/pcr.html>





# Thermocycler





# Real time PCR

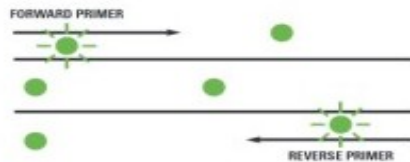
1. **Navázání:** SYBR® Green I se váže během každého cyklu na dvouvláknovou DNA.



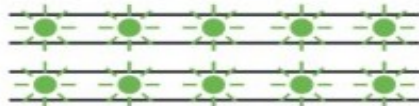
2. **Denaturace:** Ve fázi denaturace DNA je SYBR® Green I uvolněn z vazby na DNA a celková fluorescence dramaticky klesá.



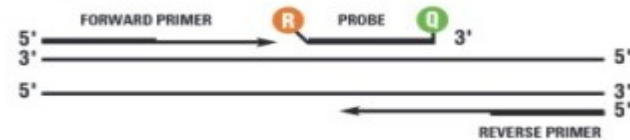
3. **Polymerizace:** Během annealingu primerů a elongace řetězce se Sybr Green opět začíná navazovat na vznikající dvouvláknovou DNA - fluorescence stoupá.



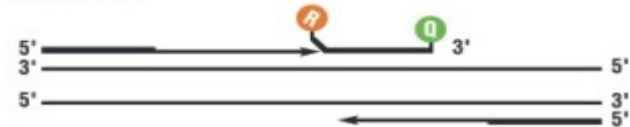
4. **Ukončení polymerizace:** Emitovaná fluorescence dosahuje maxima.



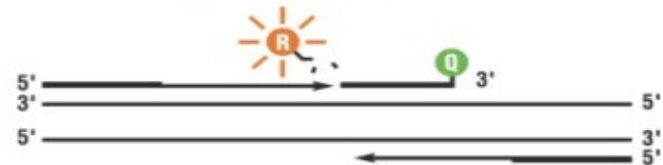
1. **Polymerizace:** Fluorescenční substrát (reporter - R) a jeho zhášec (Q) jsou navázány na 5' a 3' konce TaqMan sondy.



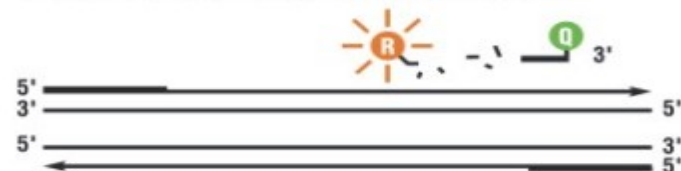
2. **Elongace řetězce:** Dokud je sonda intaktní záření emitované substrátem R je pohlcováno blízkým "zhášecem".



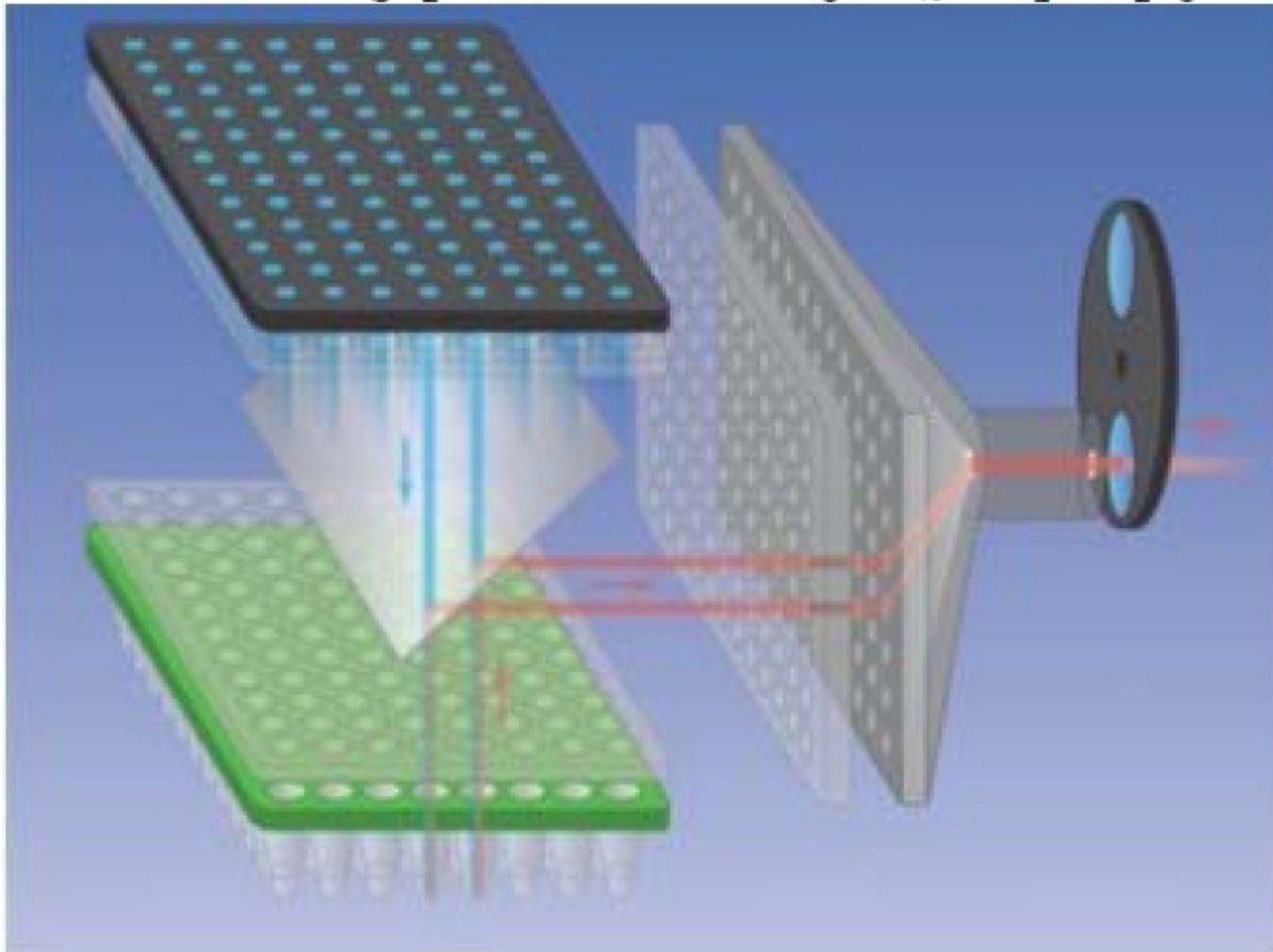
3. **Odštěpení:** když Tag polymeráza dorazí k začátku sondy, postupně ji odchlipuje až odštěpí fluorofor. Ten se tímto oddálí od zhášče a emitované světlo přestane být pohlcováno - detekovaná fluorescence stoupá.



4. **Polymerizace ukončena:** reporterová barva oddělená od zhášče emituje charakteristickou fluorescenci.



# Real time PCR

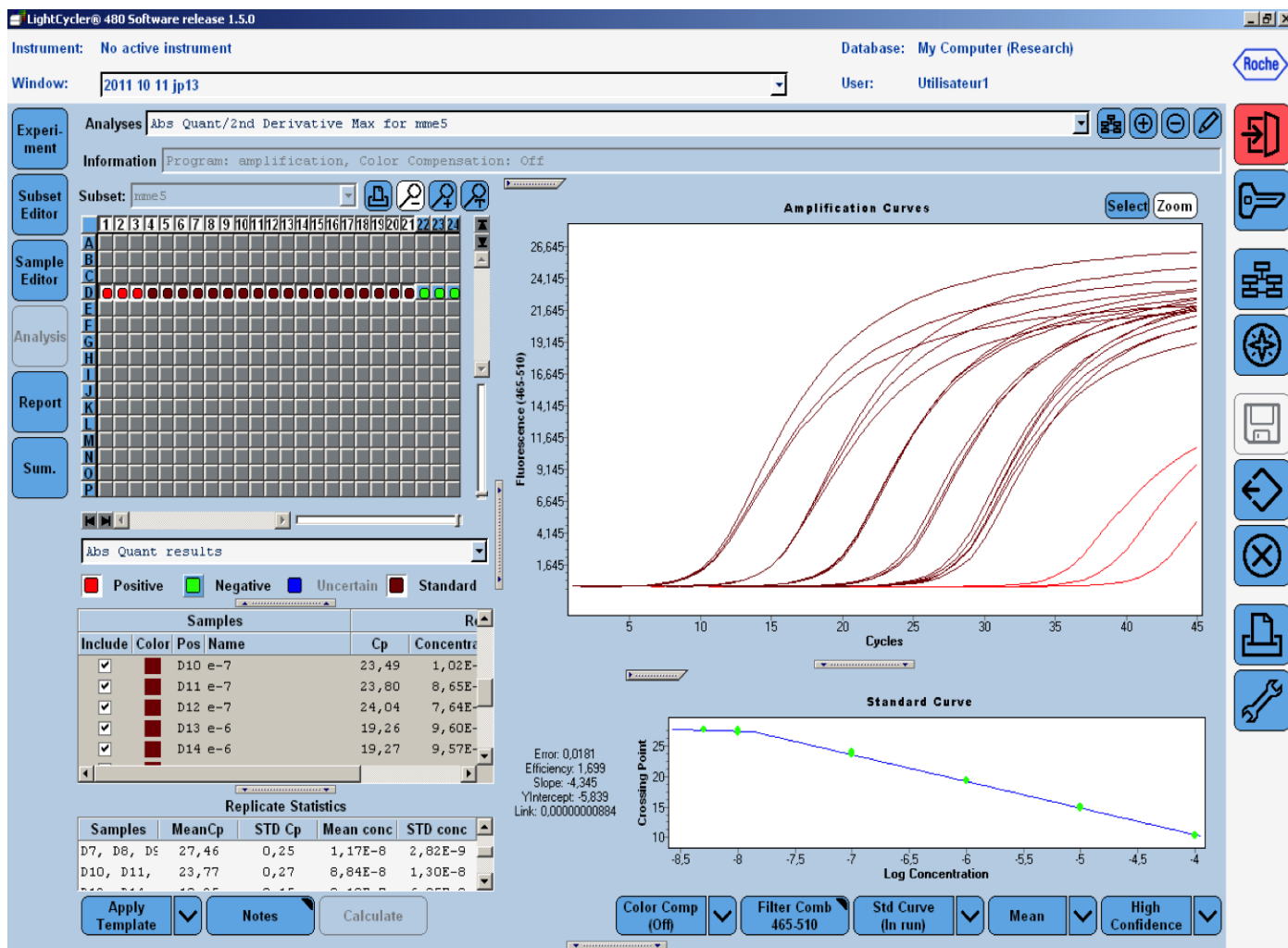


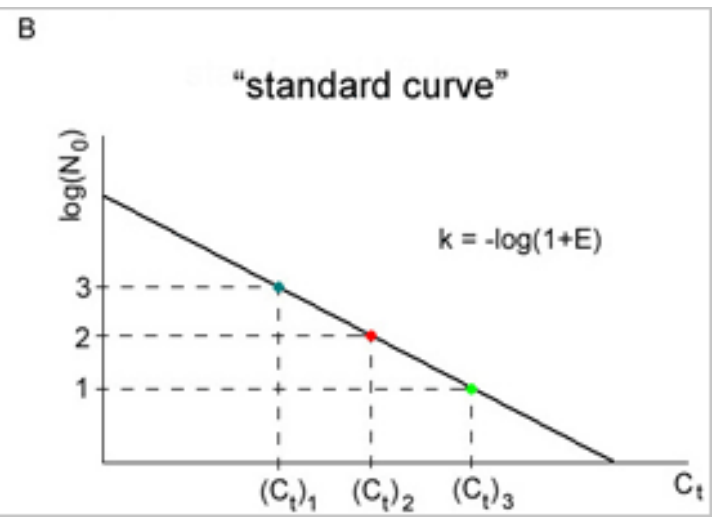
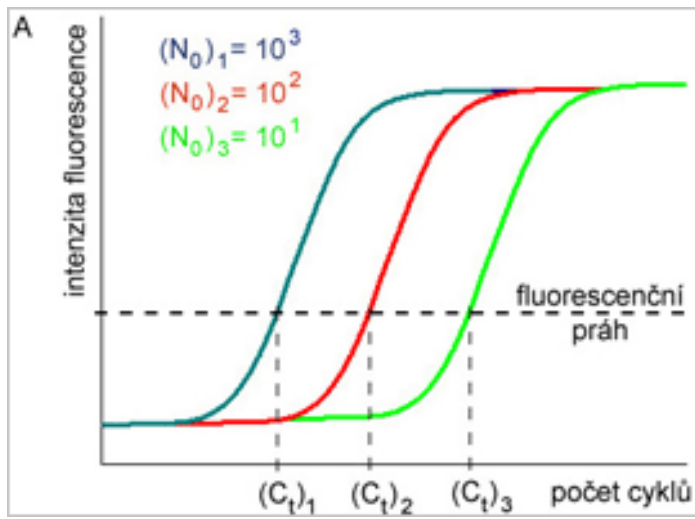


# Real time PCR

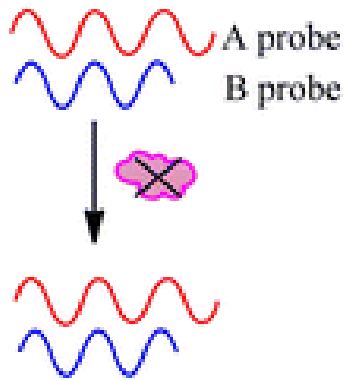


# Real time PCR

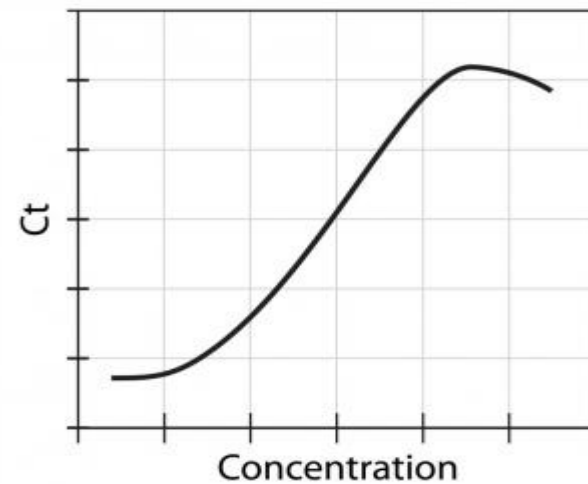
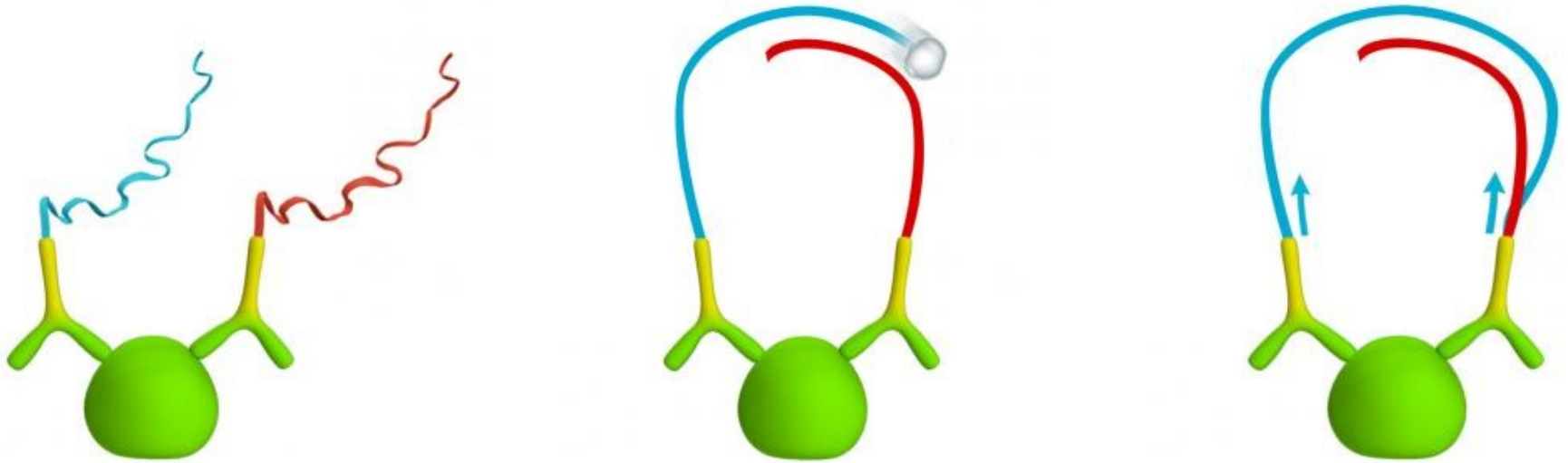




# Real time PCR proteinů

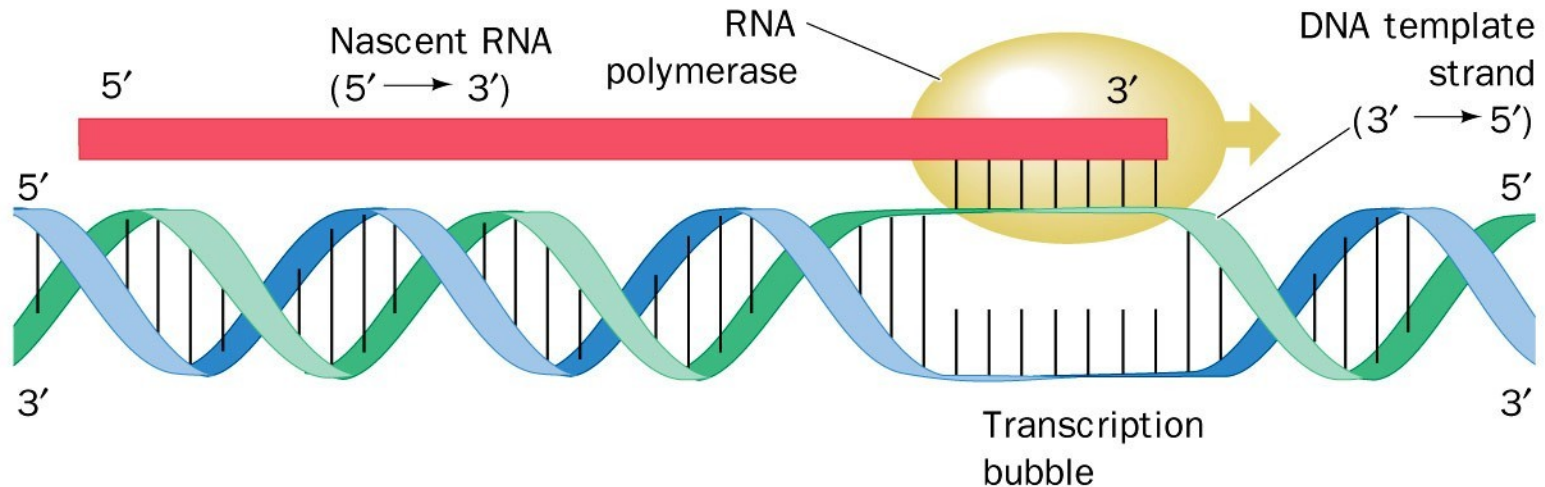


# Real time PCR proteinu

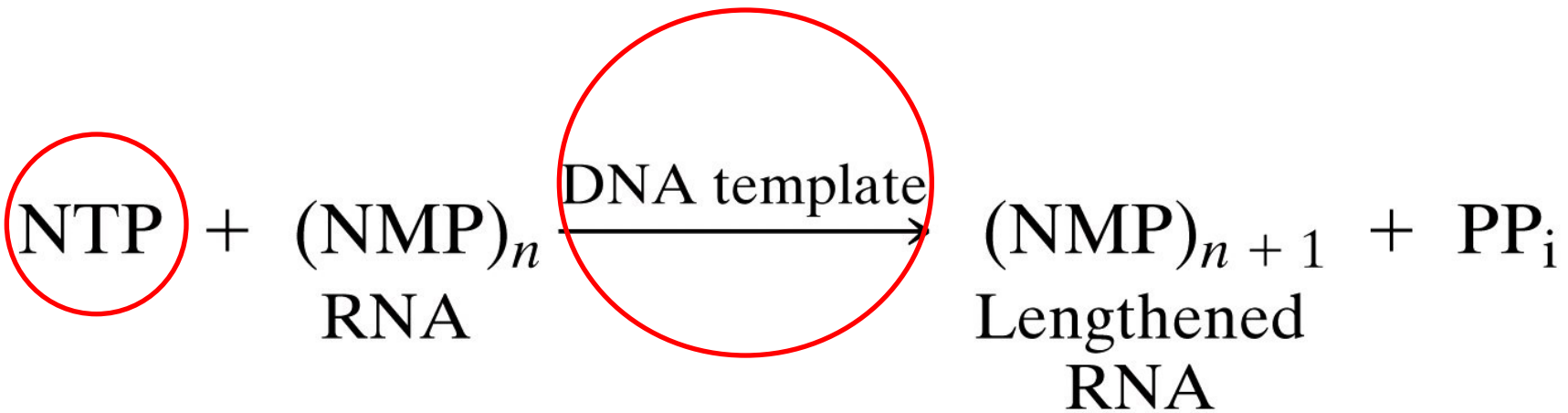


# Transkripce – přepis - DNA → RNA

**DNA + NTP + 1 enzym**



# RNA polymeráza



Unnumbered figure pg337 Concepts in Biochemistry, 3/e  
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5' → 3'



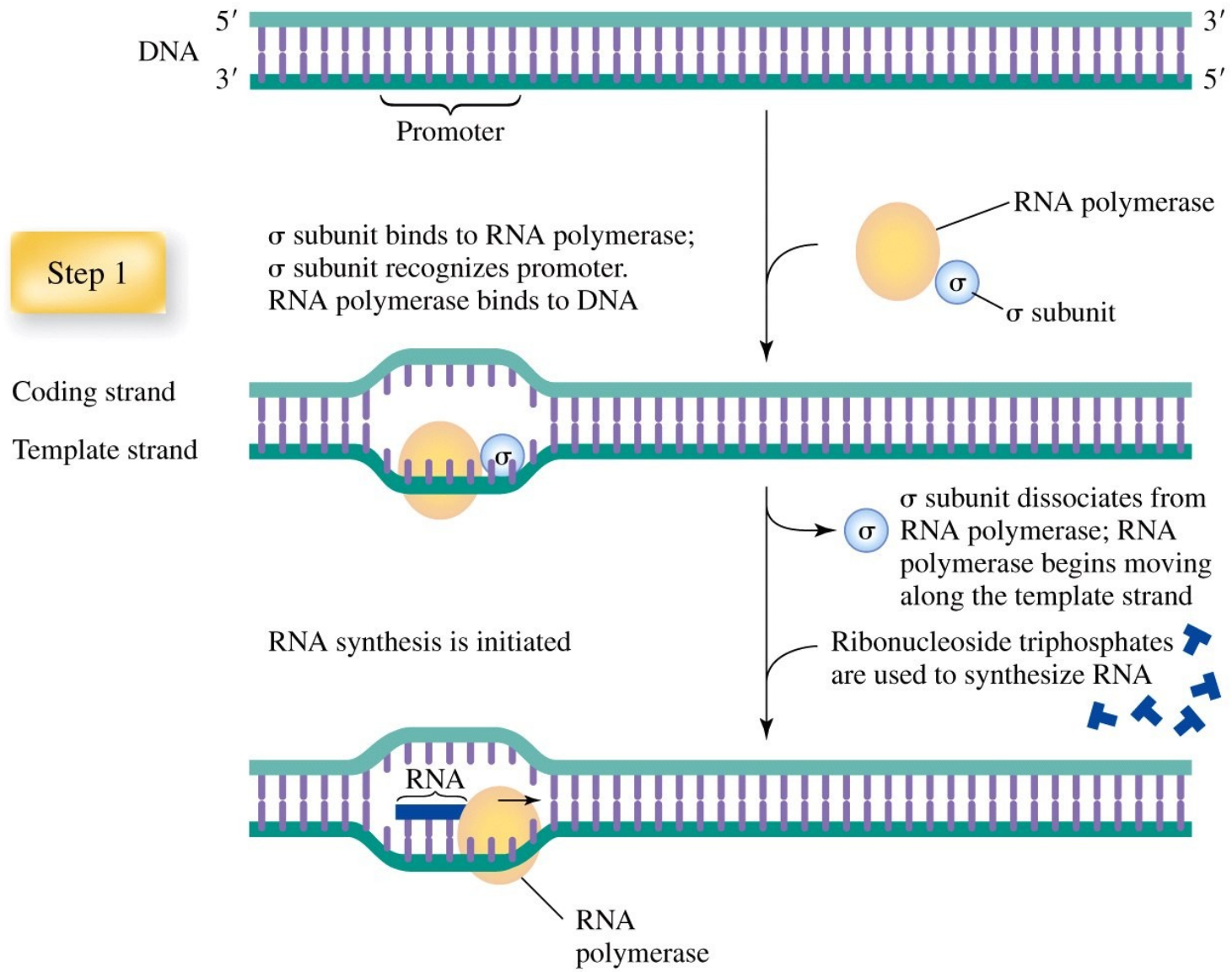


Figure 11-22 part 1 Concepts in Biochemistry, 3/e  
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Step 2

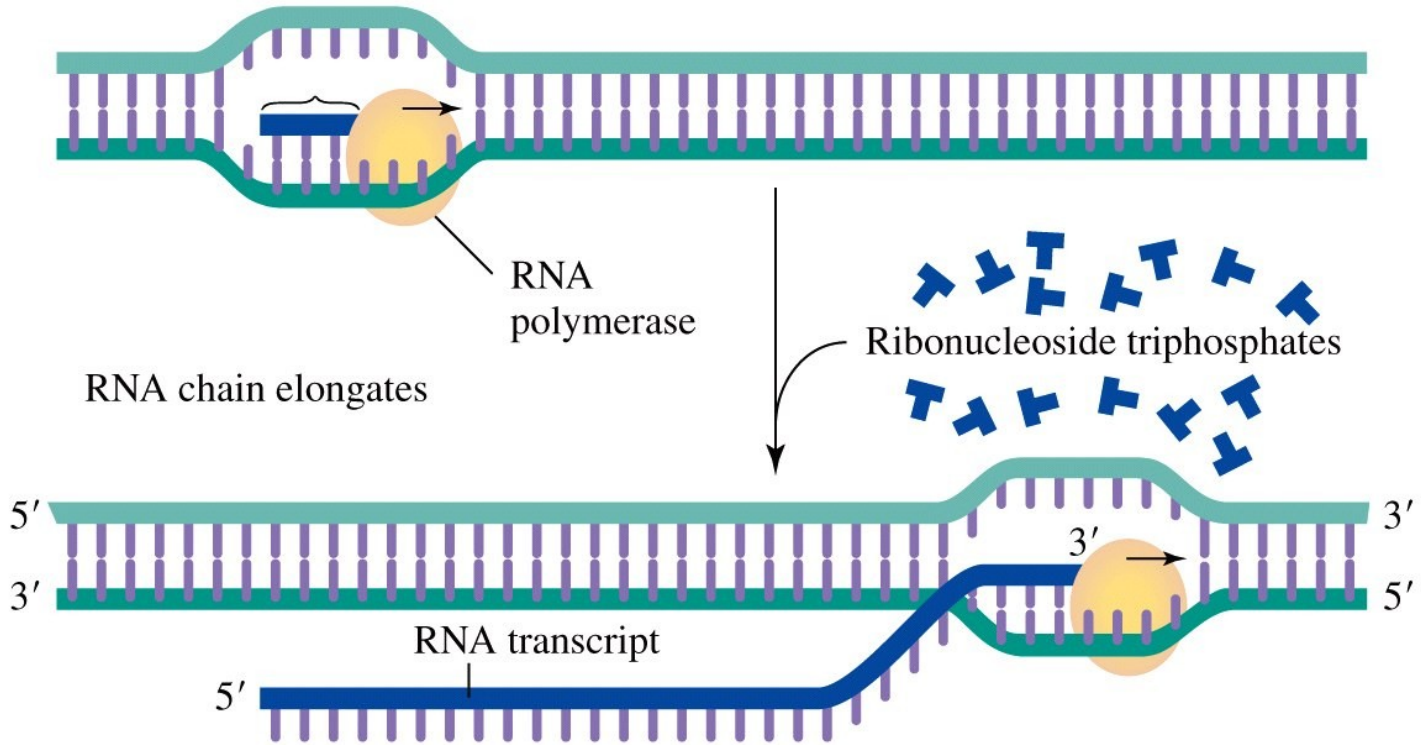


Figure 11-22 part 2 Concepts in Biochemistry, 3/e  
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Step 3

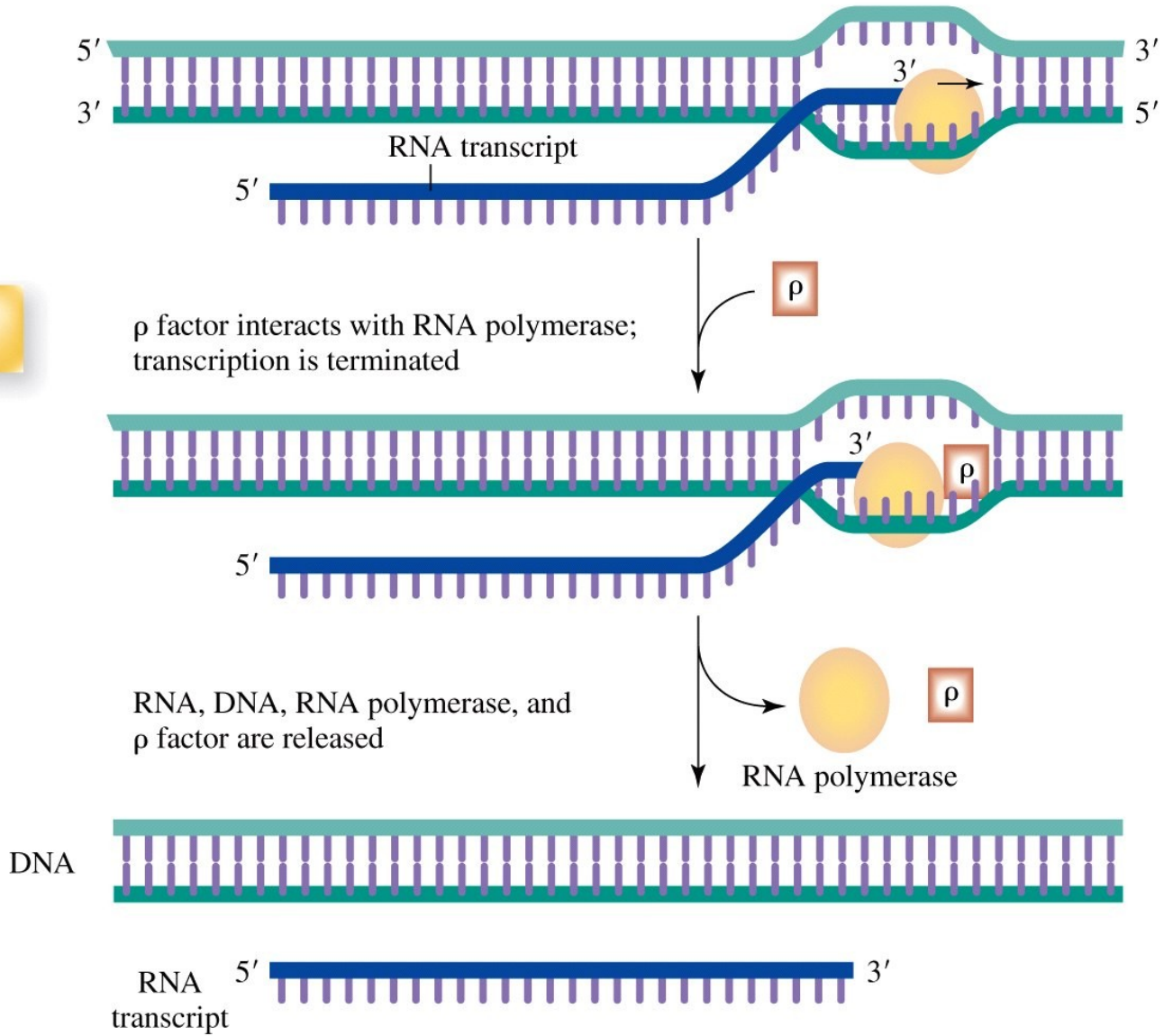
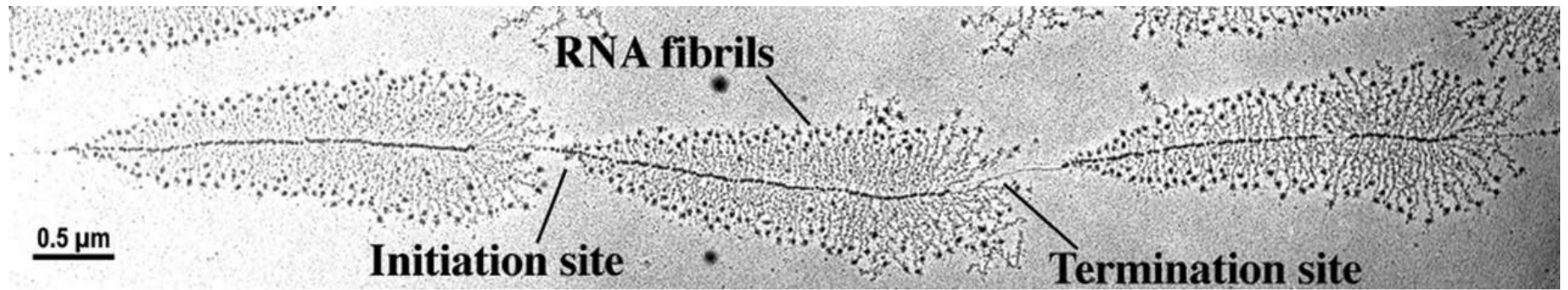
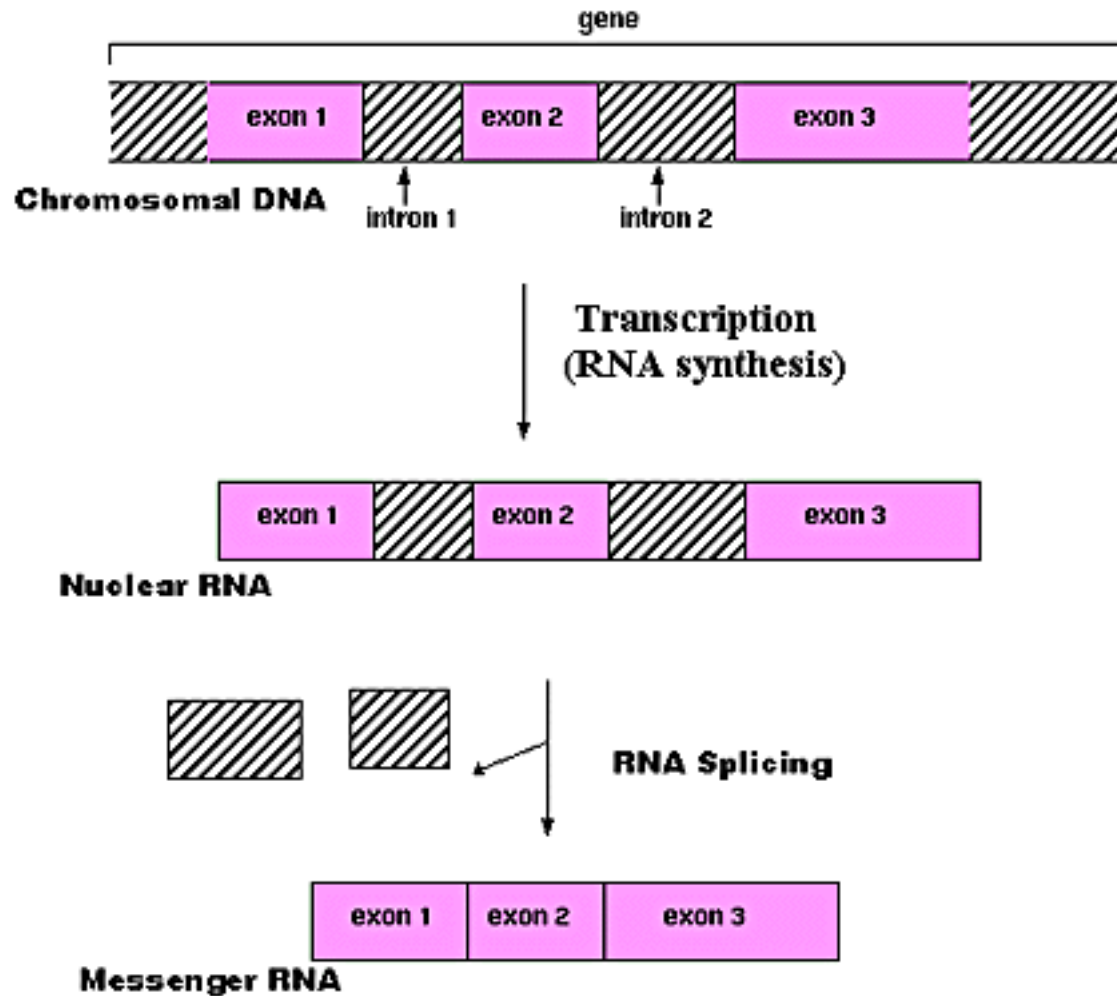


Figure 11-22 part 3 Concepts in Biochemistry, 3/e  
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Courtesy of Ulrich Scheer, University of Würzburg, Germany

# Syntéza eukaryontní RNA



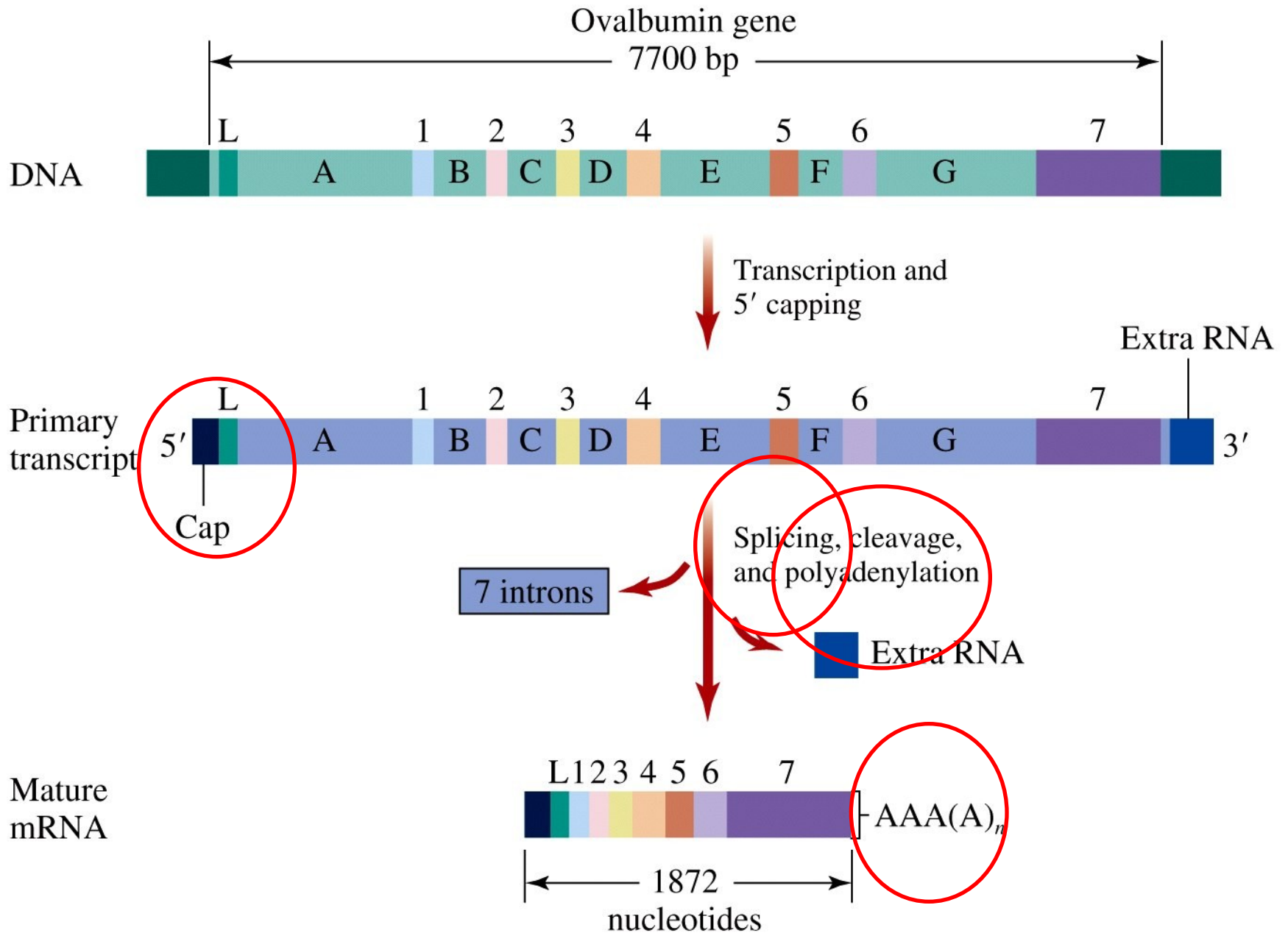
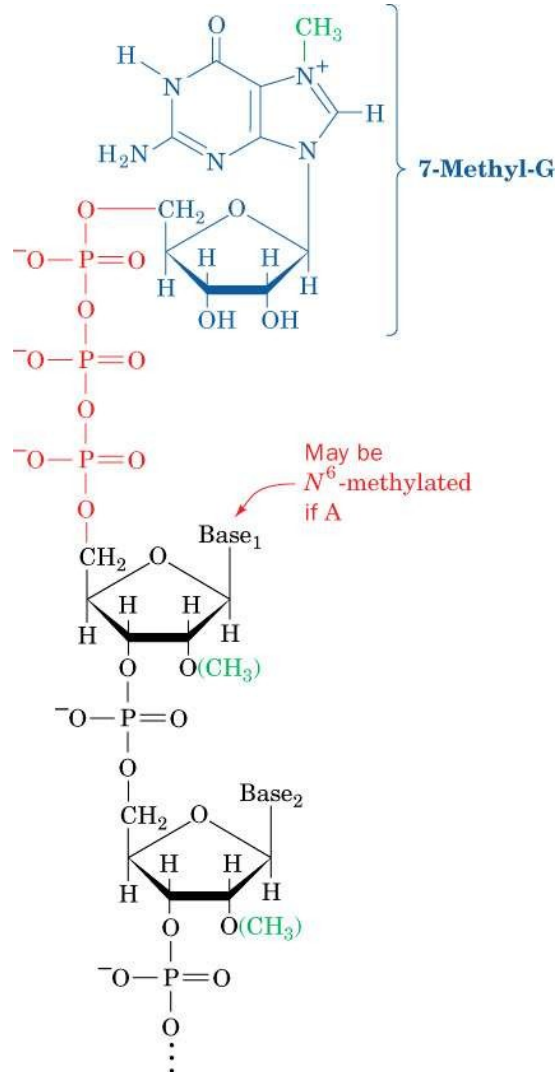


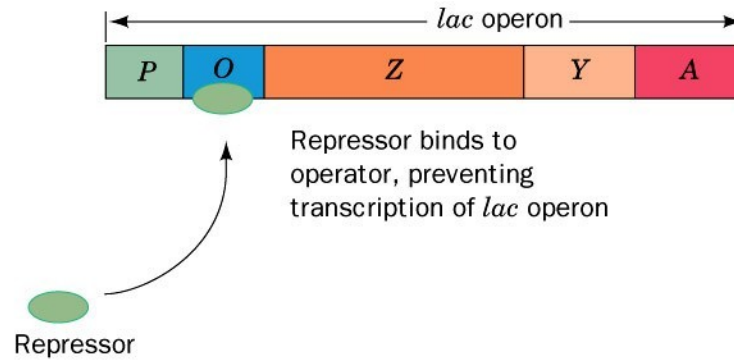
Figure 11-28 Concepts in Biochemistry, 3/e  
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# Cap

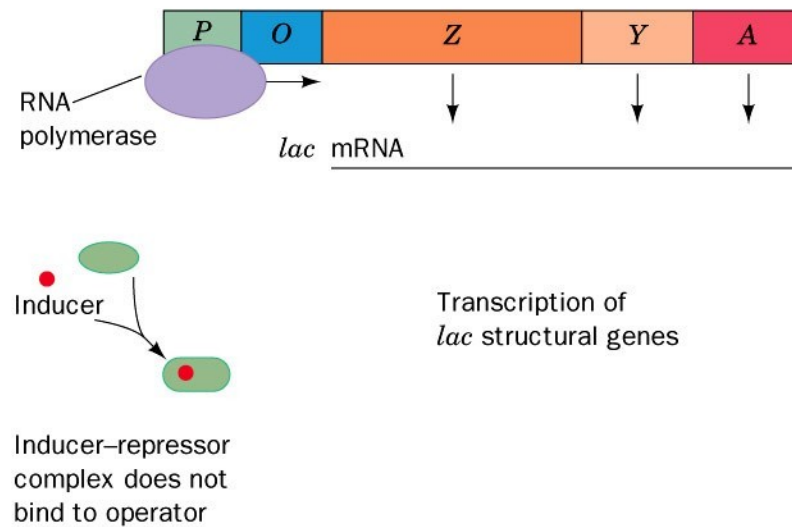


# Regulate transcribe

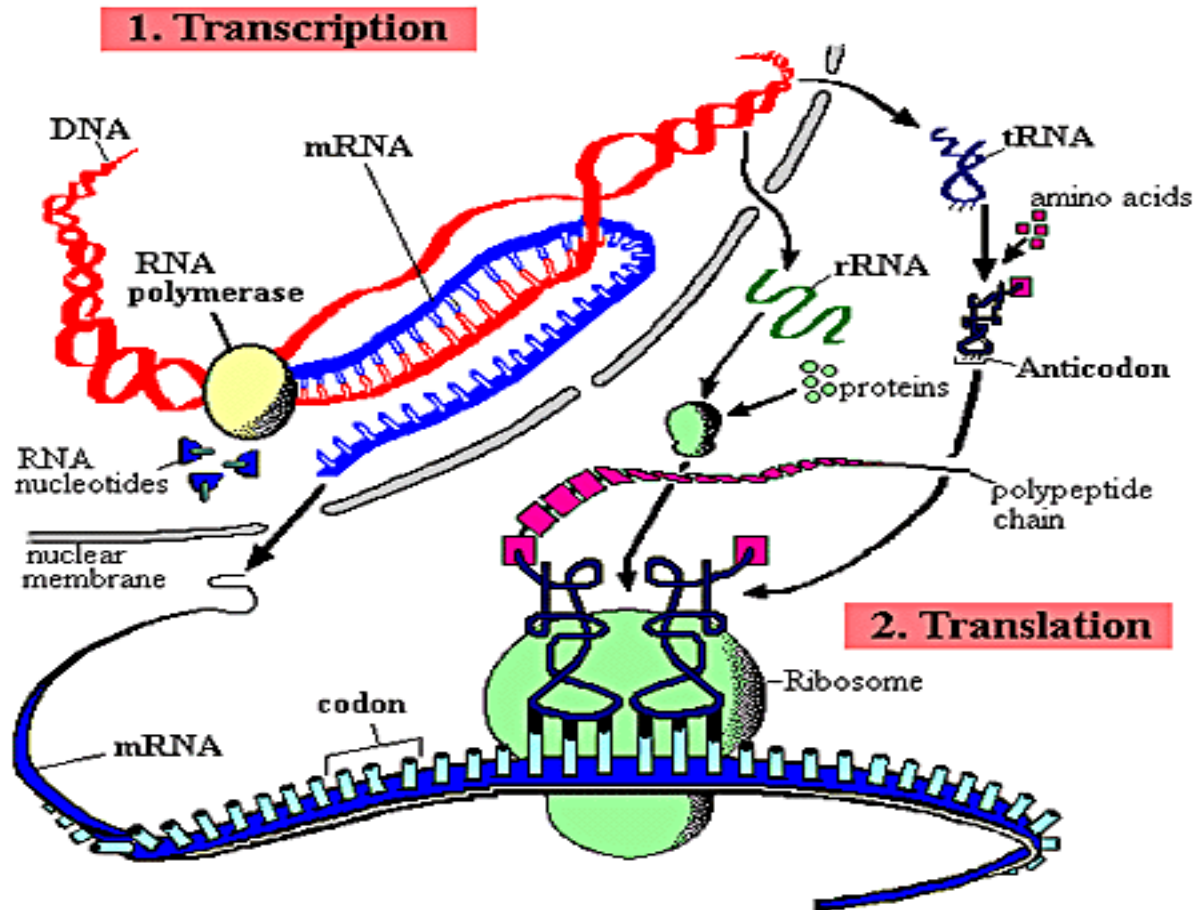
(a) Absence of inducer



(b) Presence of inducer



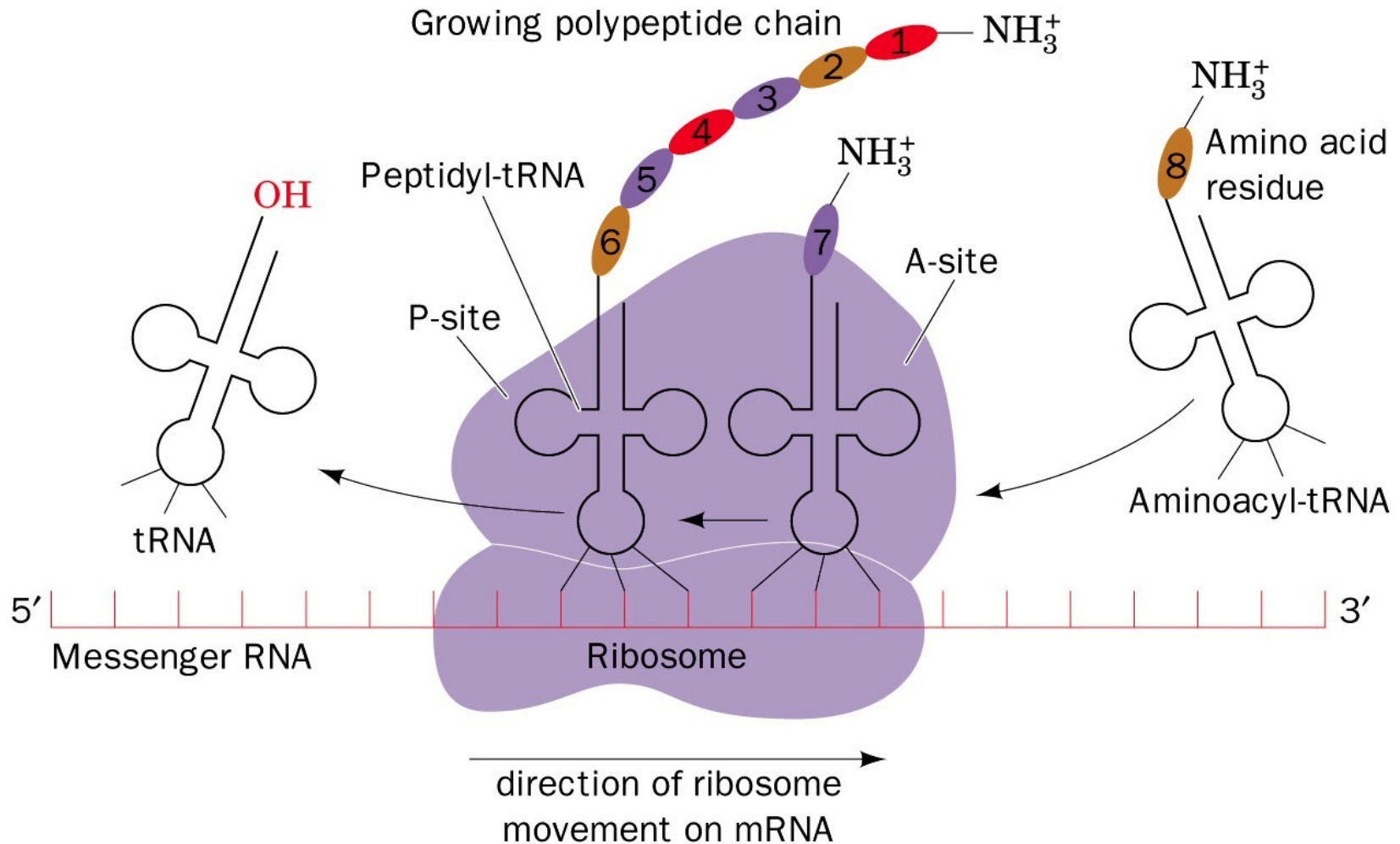
# Syntéza bílkovin





# Translaci – překladi - RNA → protein

**mRNA + ribozomy + (tRNA-AMK) + IFn + RFn**



# Ribozomy

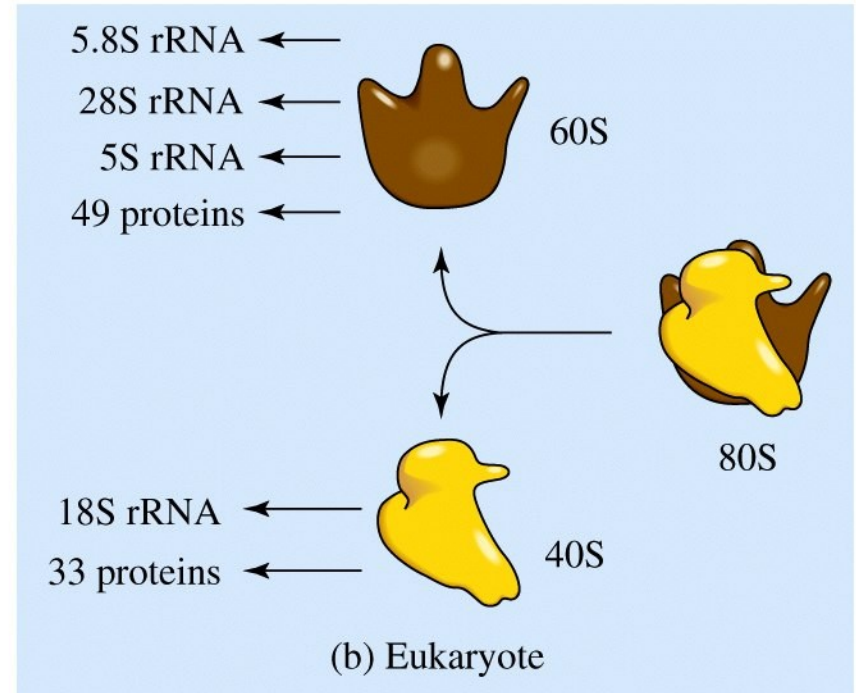
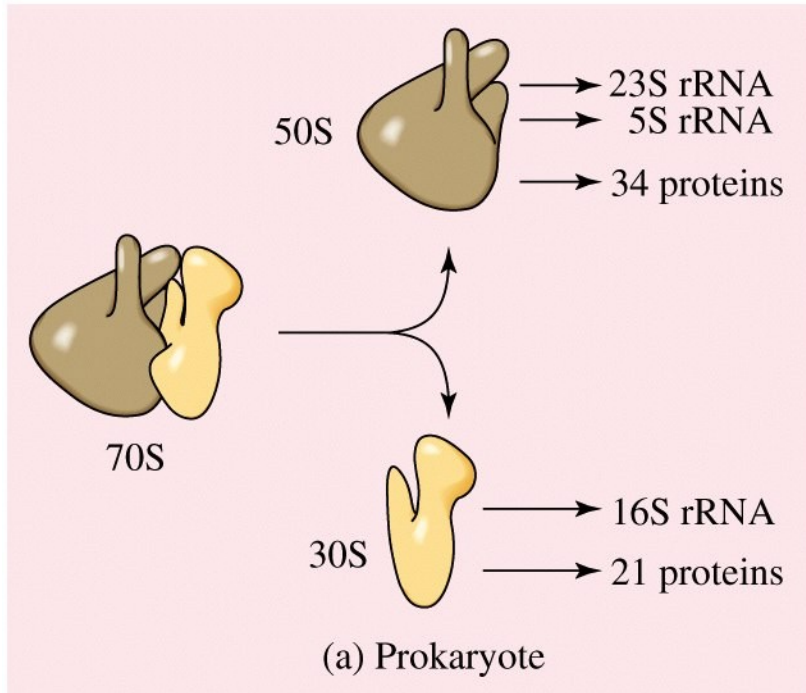
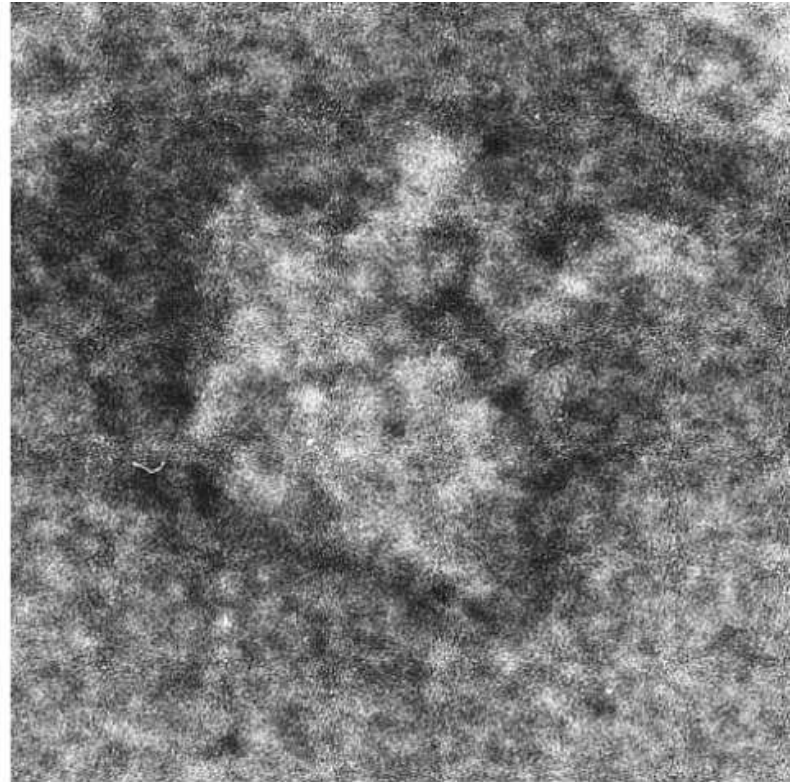
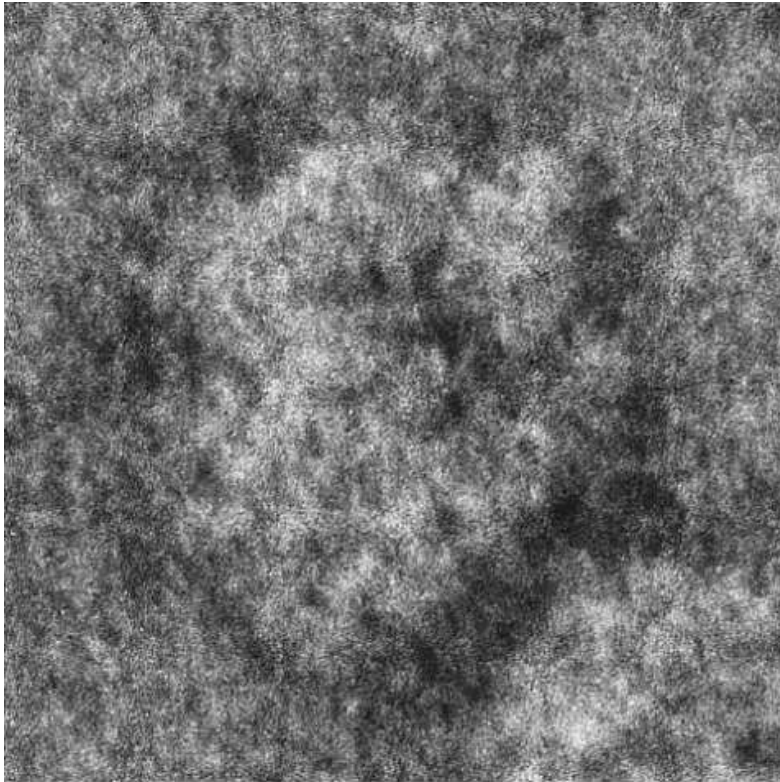


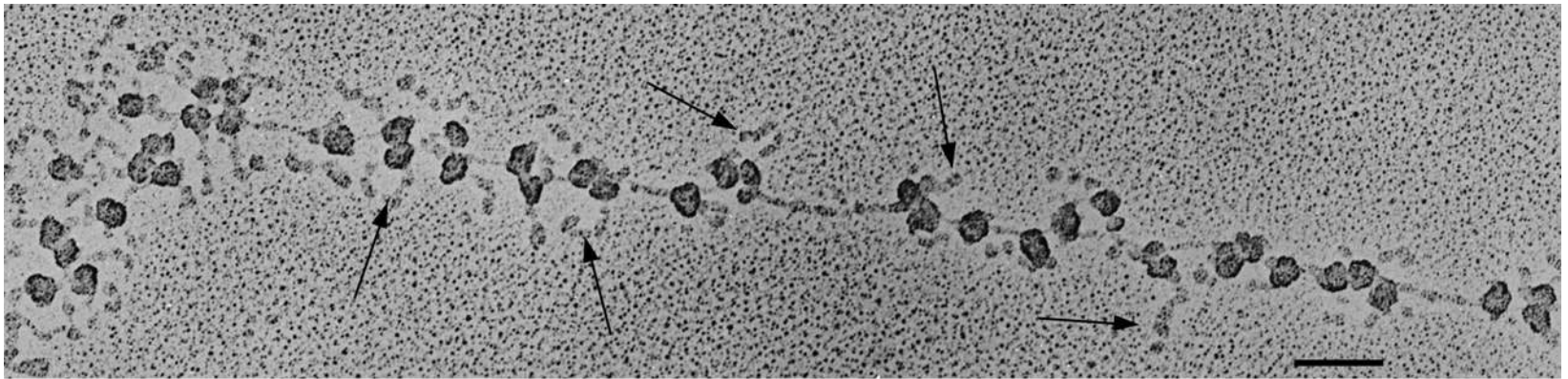
Figure 12-1 Concepts in Biochemistry, 3/e  
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# Ribozomy E.coli



Courtesy of James Lake, UCLA

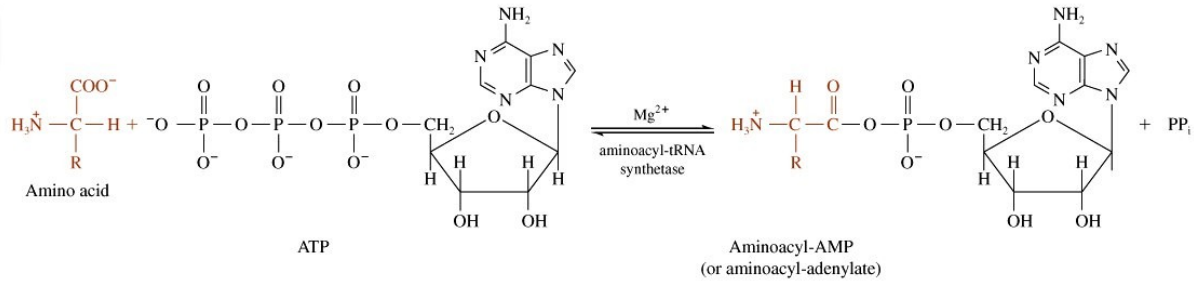
# Polyribozomy *Bombyx mori*



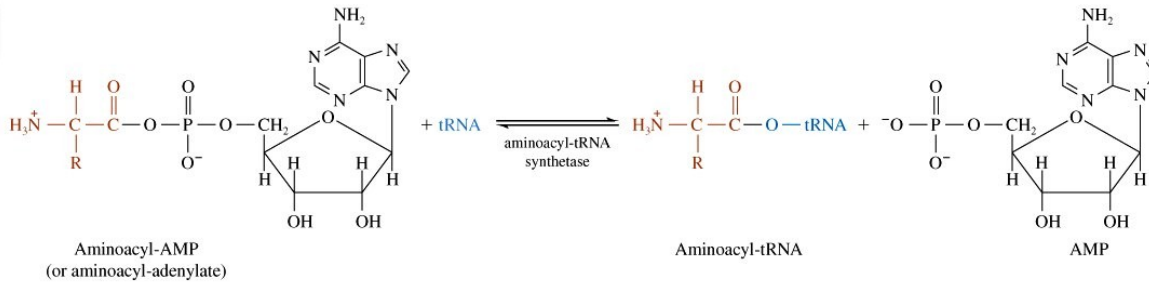
Courtesy of Oscar L. Miller, Jr. and Steven L. McKnight, University of Virginia

# Aktivace AMK

Step 1



Step 2



Overall

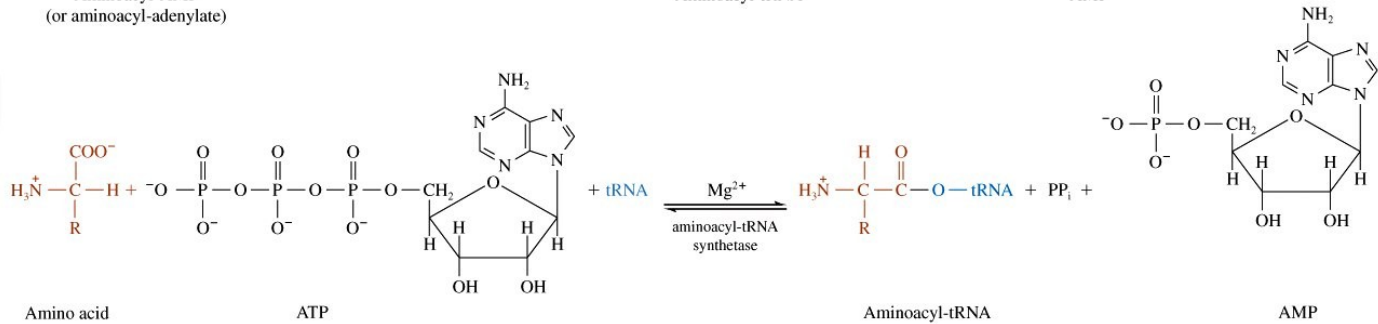
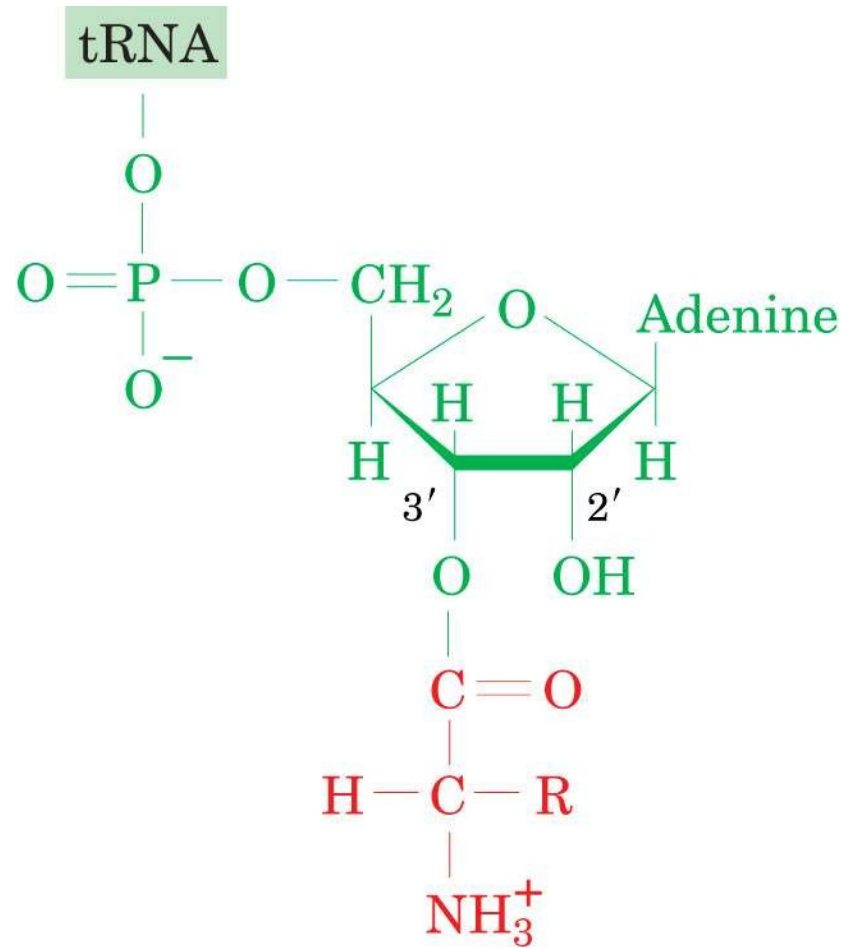
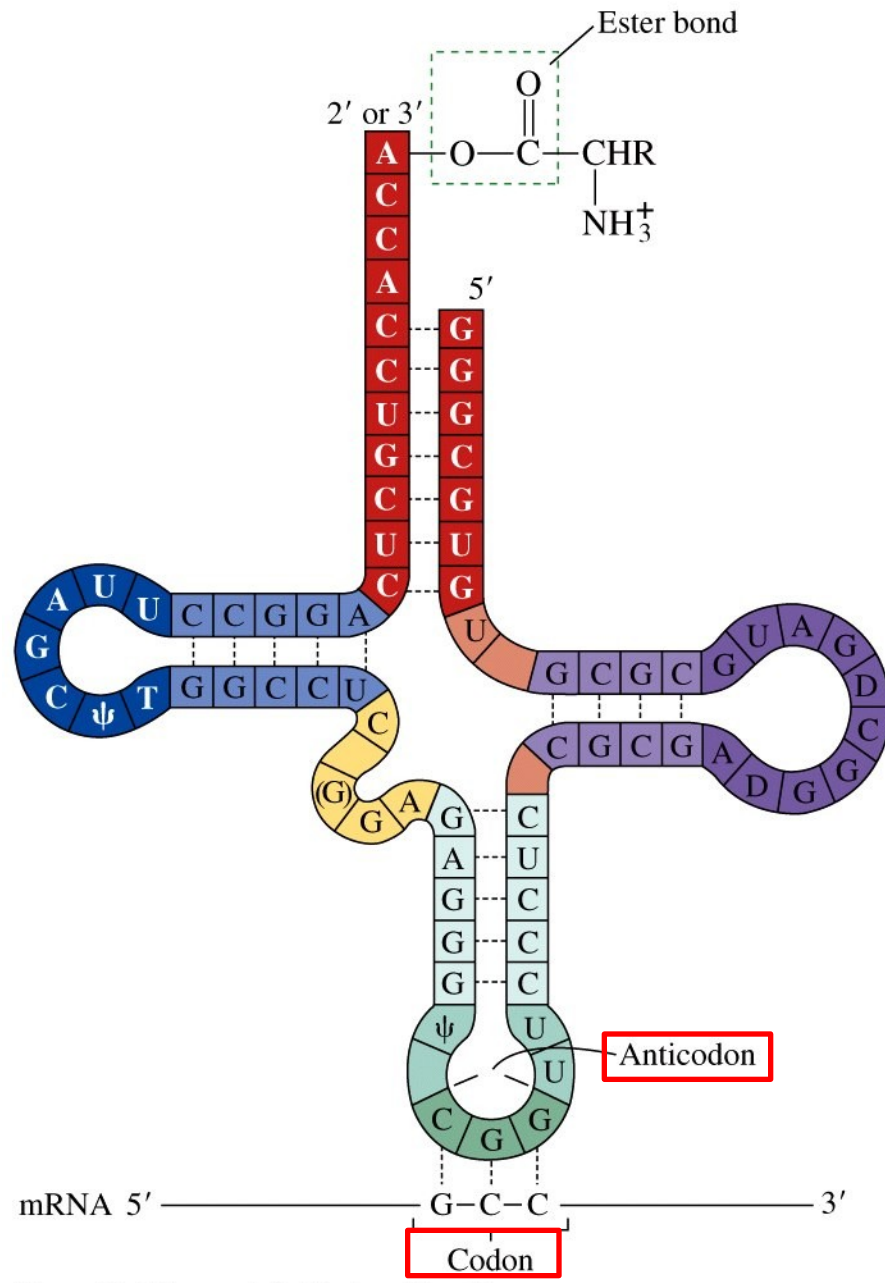


Figure 12-4 Concepts in Biochemistry, 3/e  
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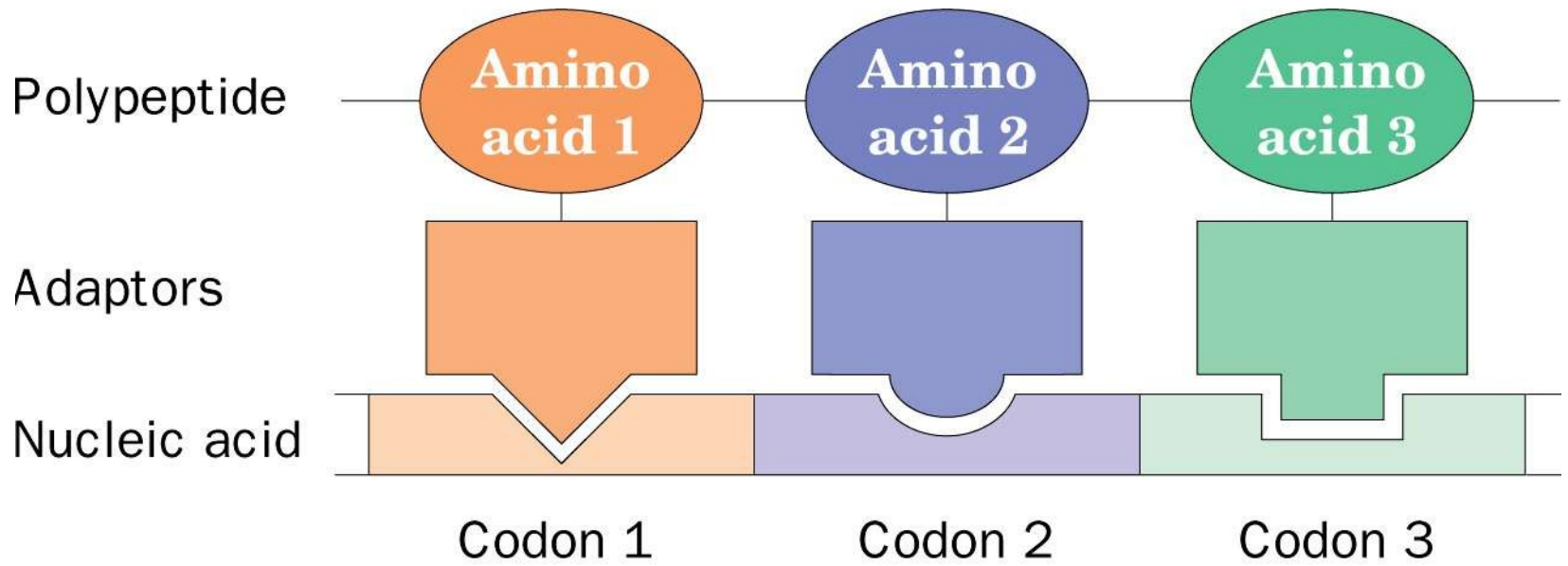


**Aminoacyl-tRNA**



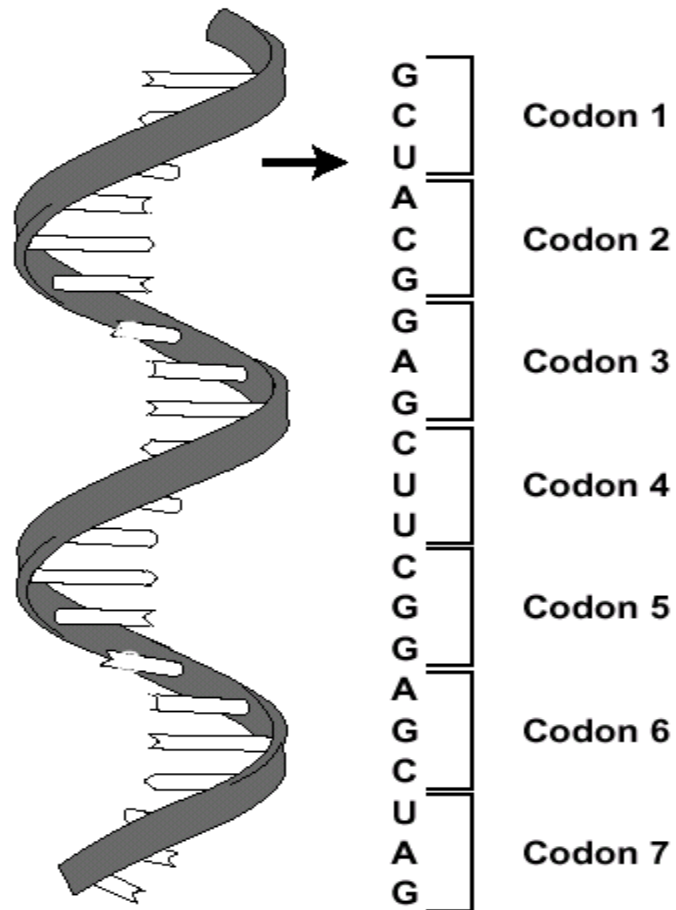
**Figure 12-3 Concepts in Biochemistry, 3/e**  
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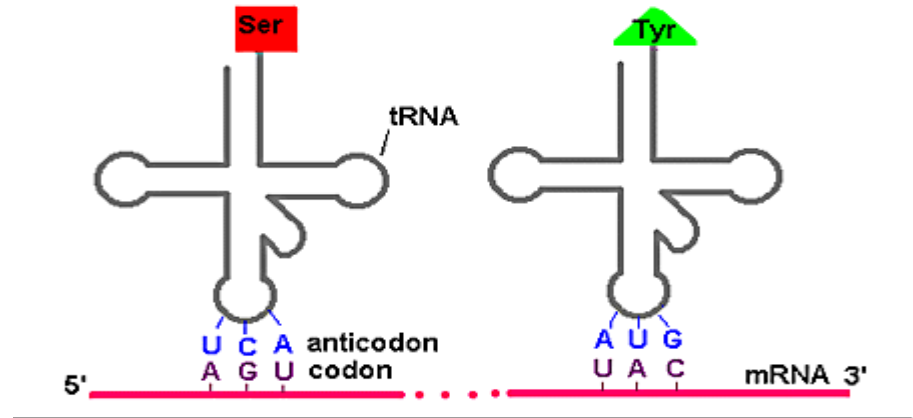


# Kodón

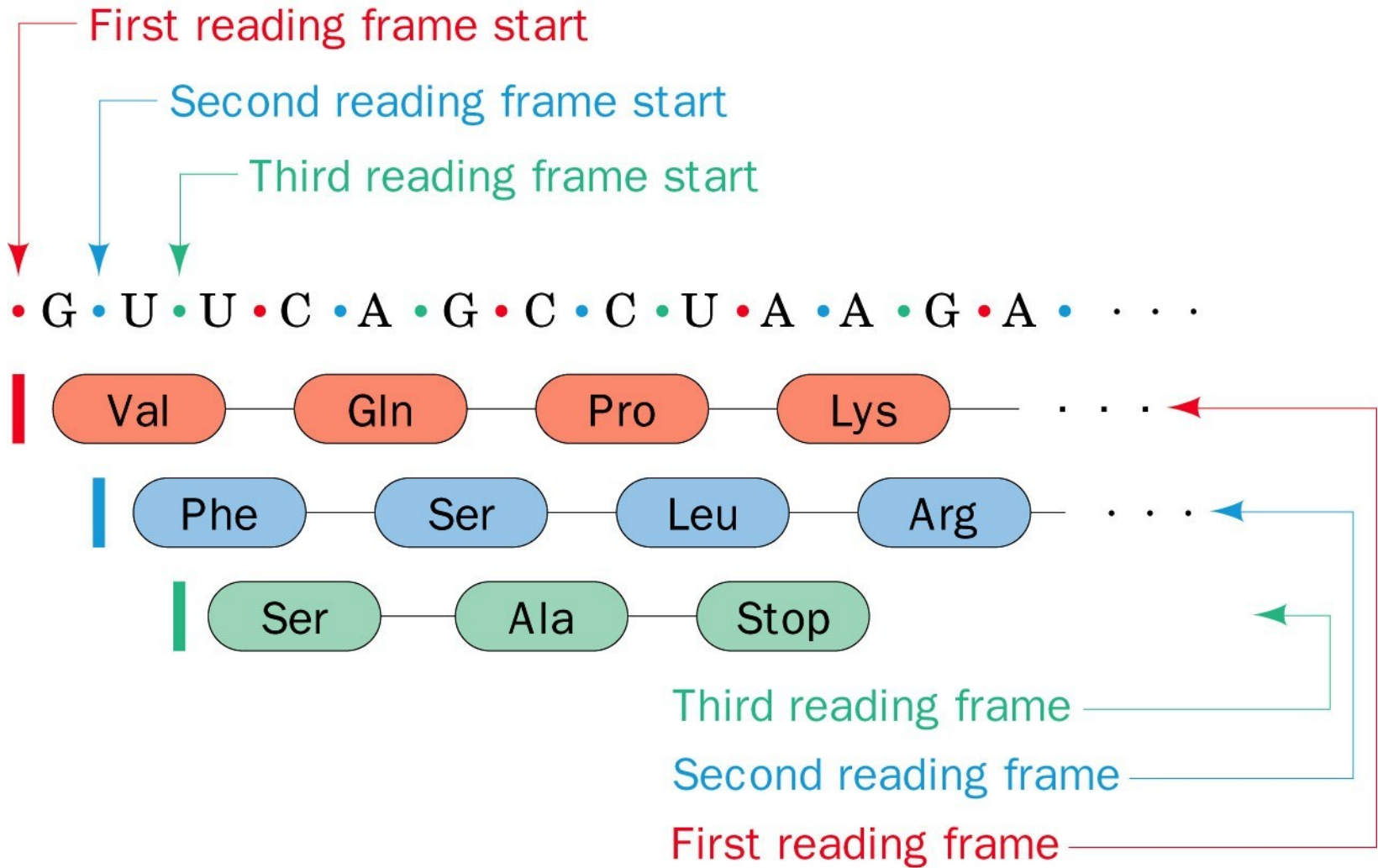


# Genetický kod

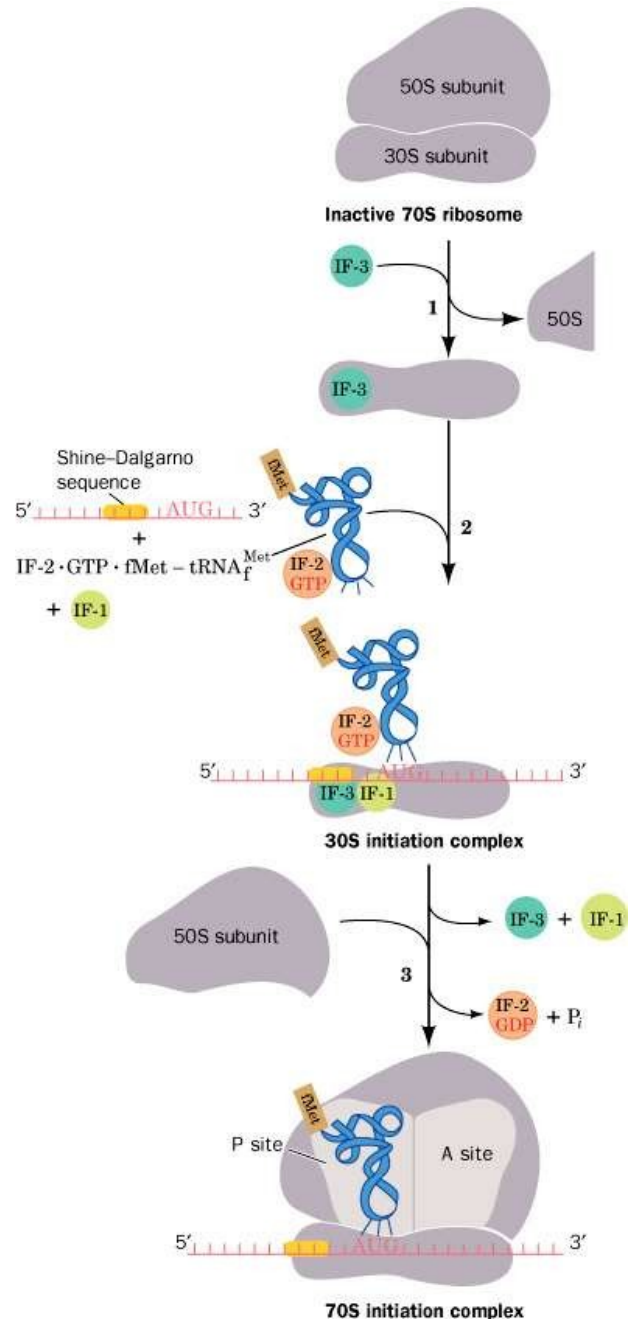
- Tripletový  $4^3 = 64$
- Degenerovaný
- Nepřekrývající se
- Universální

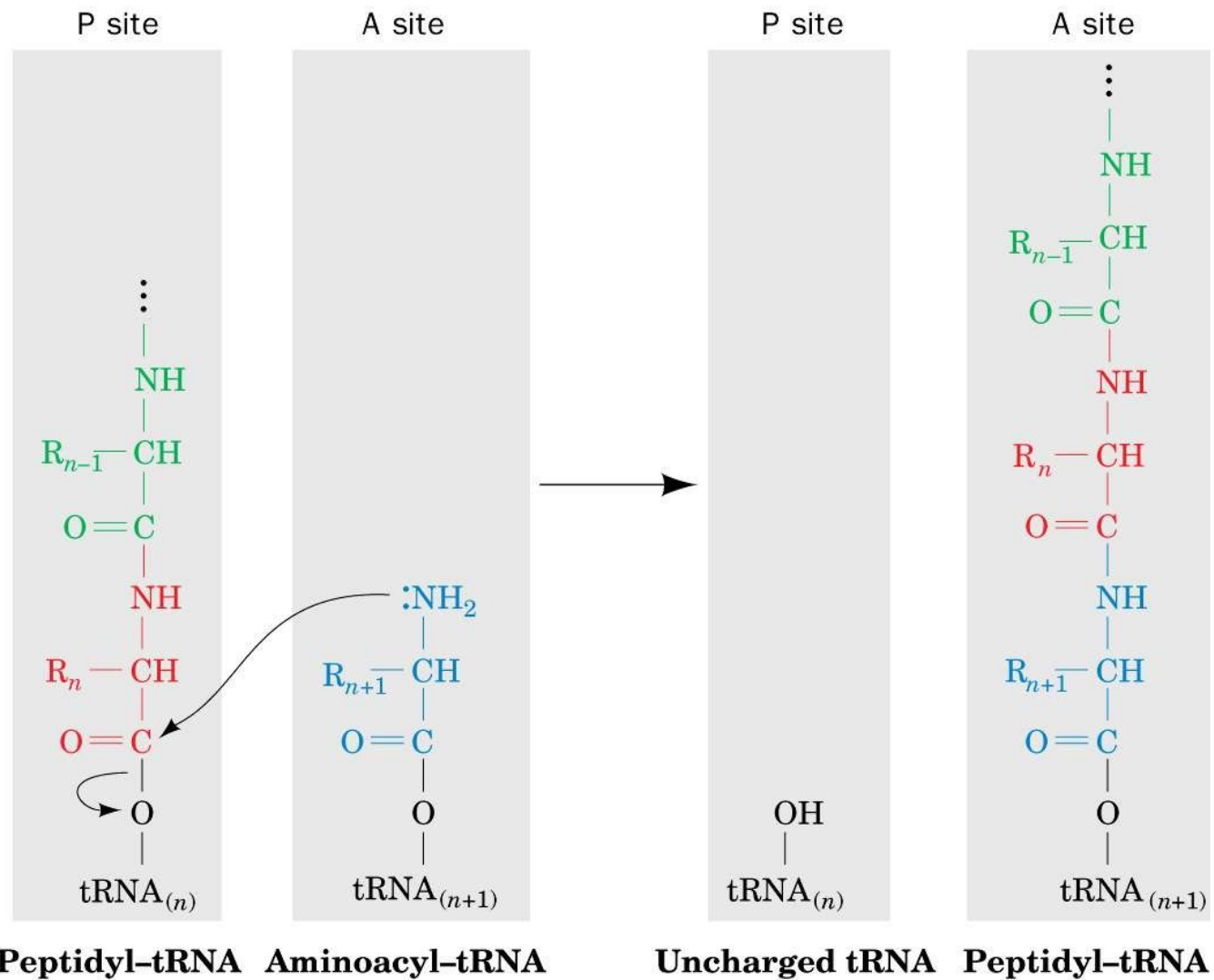


		2nd base in codon				
		U	C	A	G	
1st base in codon	U	Phe Phe Leu Leu	Ser Ser Ser Ser	Tyr Tyr <b>STOP</b> <b>STOP</b>	Cys Cys <b>STOP</b> Trp	U C A G
	C	Leu Leu Leu Leu	Pro Pro Pro Pro	His His Gln Gln	Arg Arg Arg Arg	U C A G
	A	Ile Ile Ile Met	Thr Thr Thr Thr	Asn Asn Lys Lys	Ser Ser Arg Arg	U C A G
	G	Val Val Val Val	Ala Ala Ala Ala	Asp Asp Glu Glu	Gly Gly Gly Gly	U C A G
						3rd base in codon

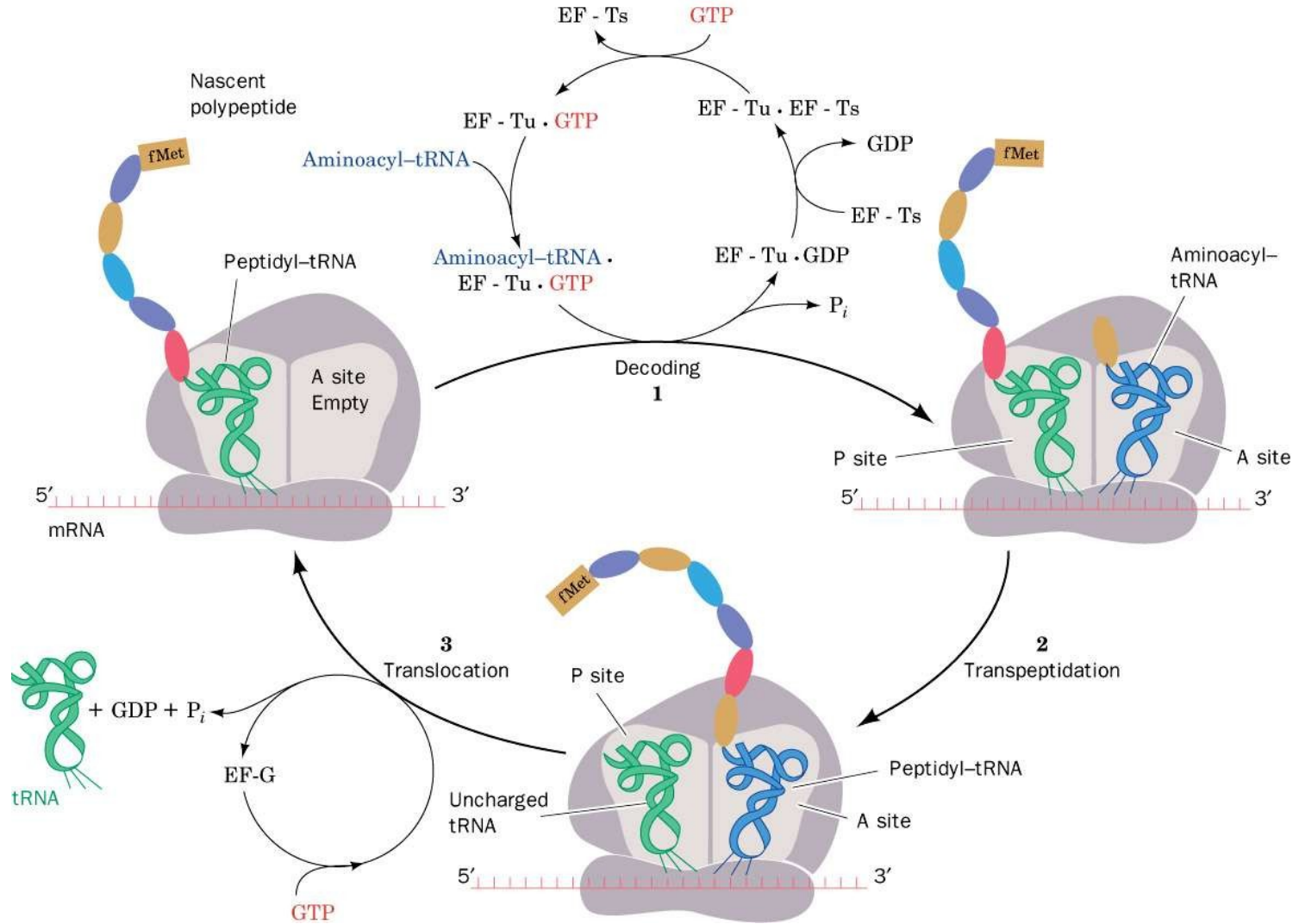


# Inicializace

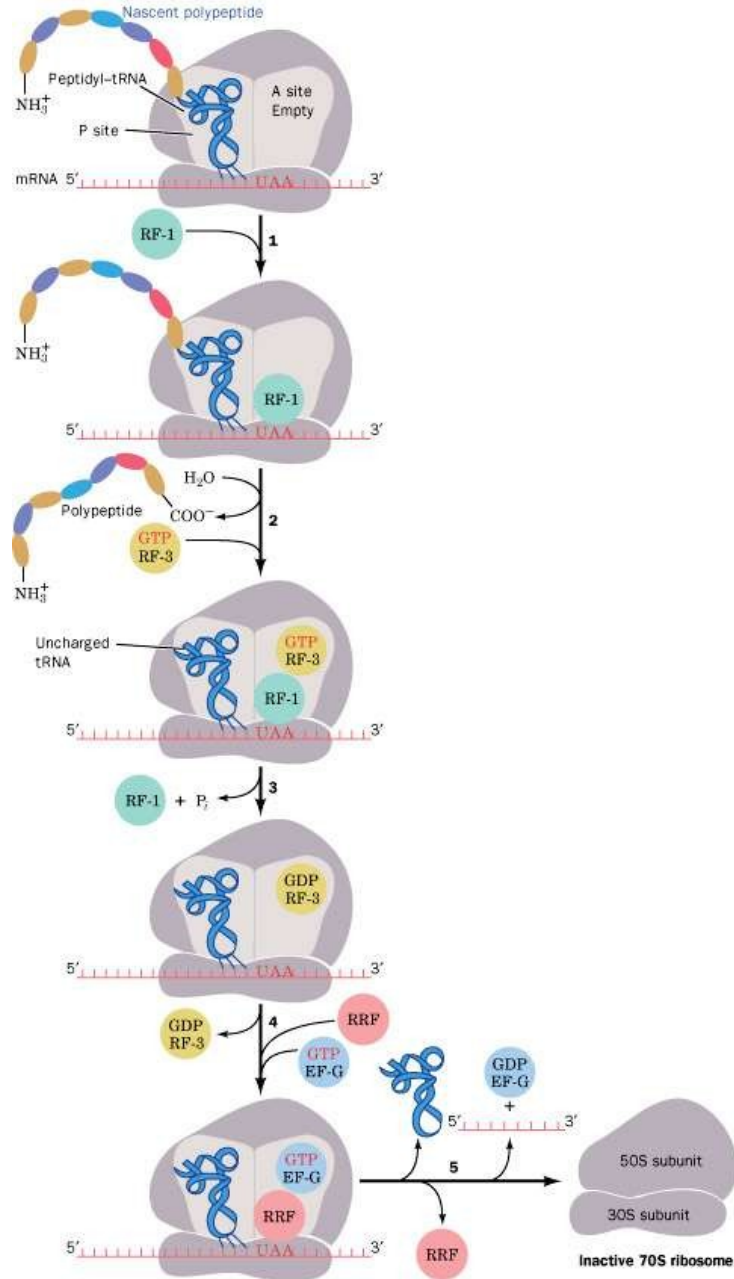


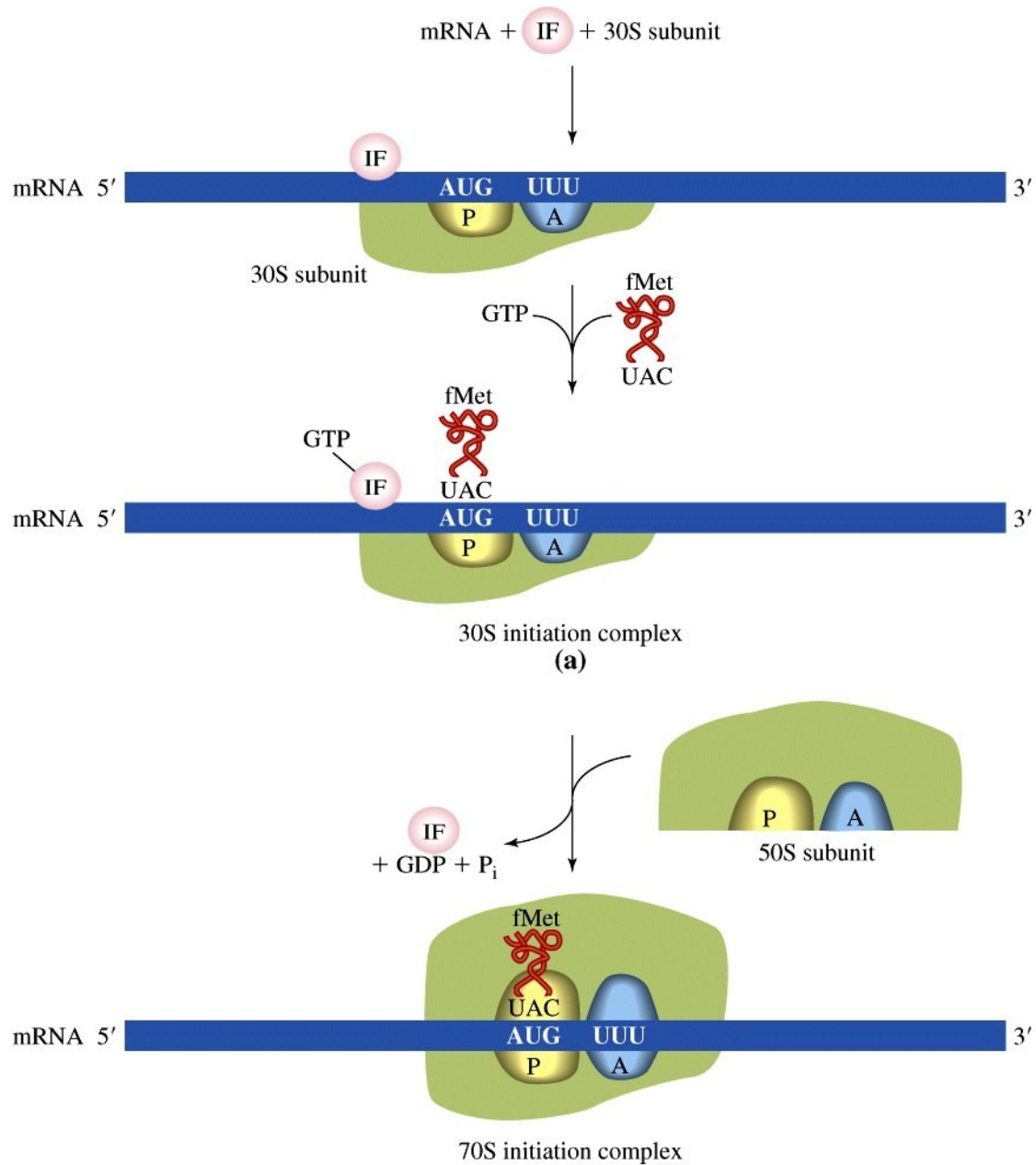


# Elongate



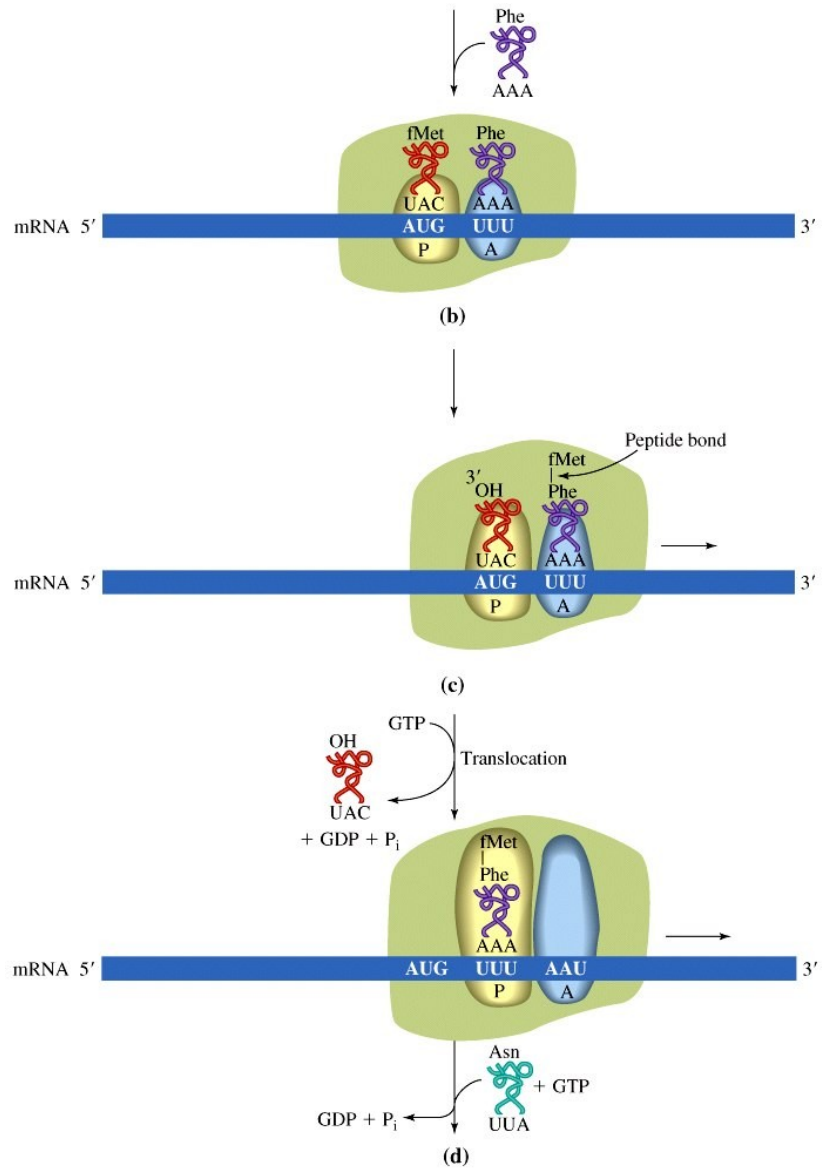
# Termination



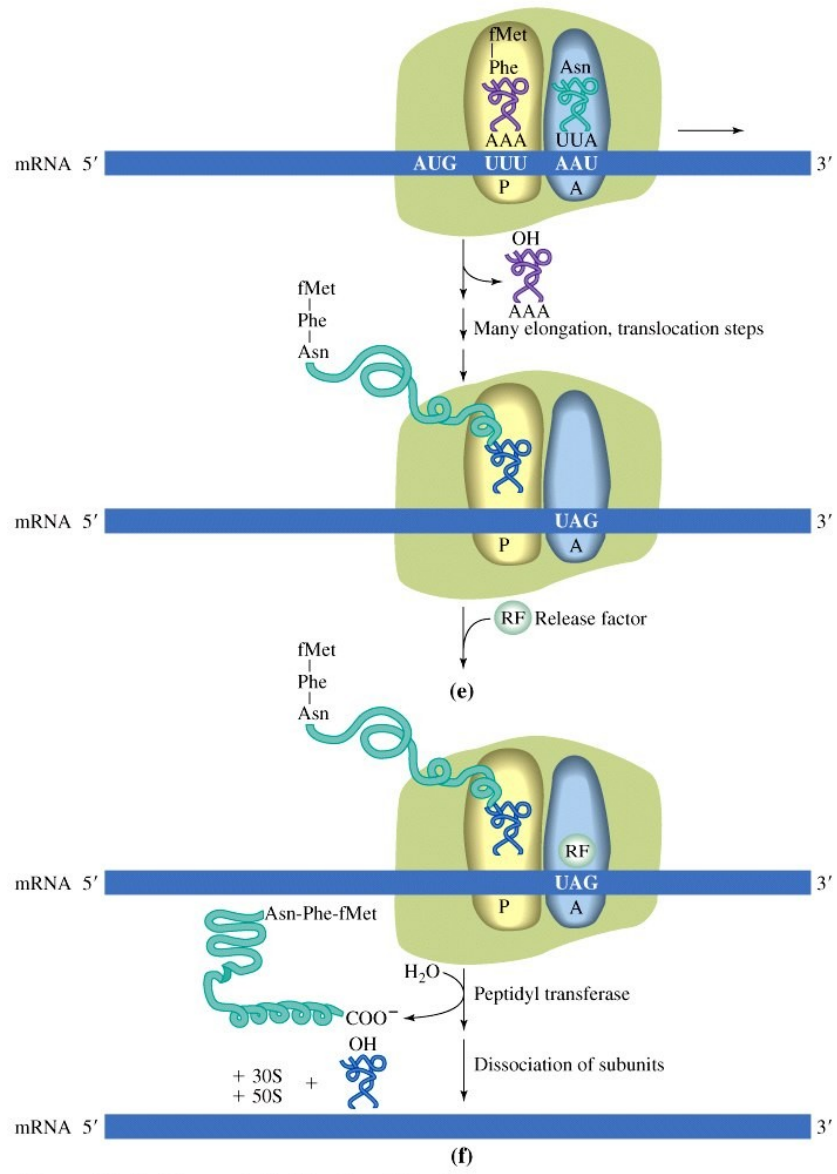


**Figure 12-5a Concepts in Biochemistry, 3/e**  
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**Figure 12-5b Concepts in Biochemistry, 3/e**  
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**Figure 12-5c Concepts in Biochemistry, 3/e**  
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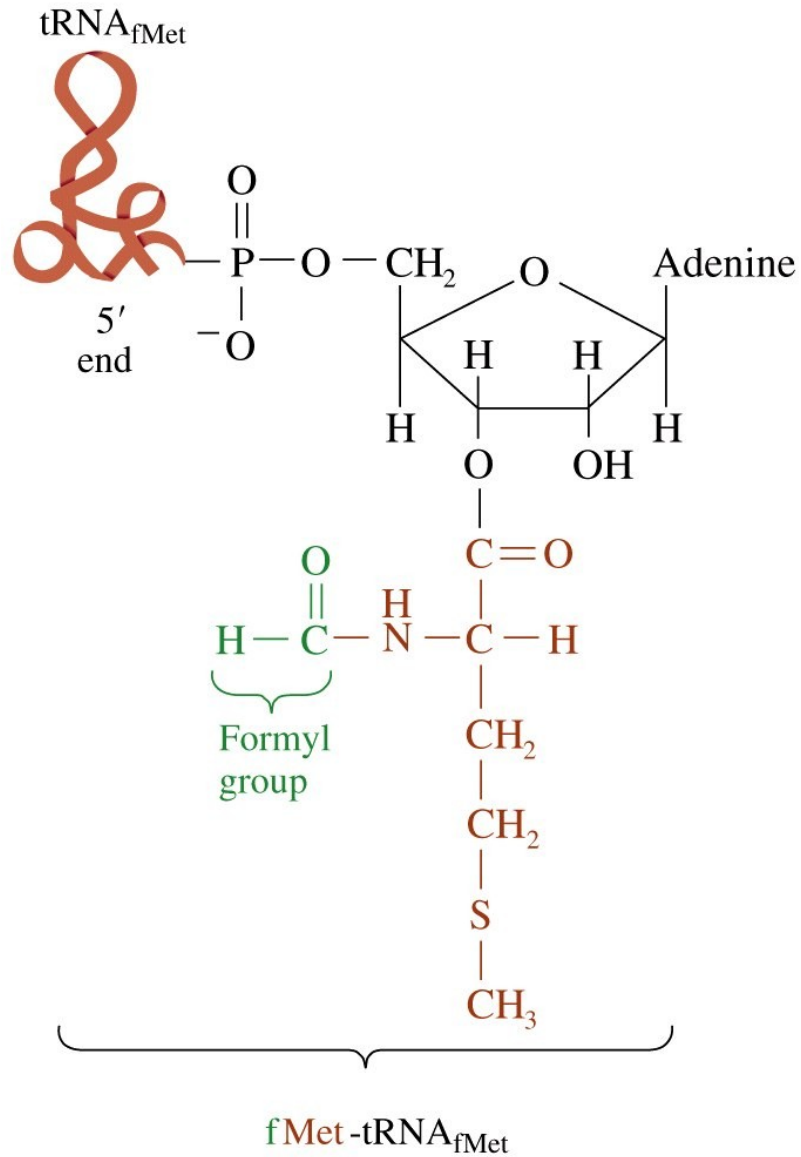


Figure 12-6 Concepts in Biochemistry, 3/e  
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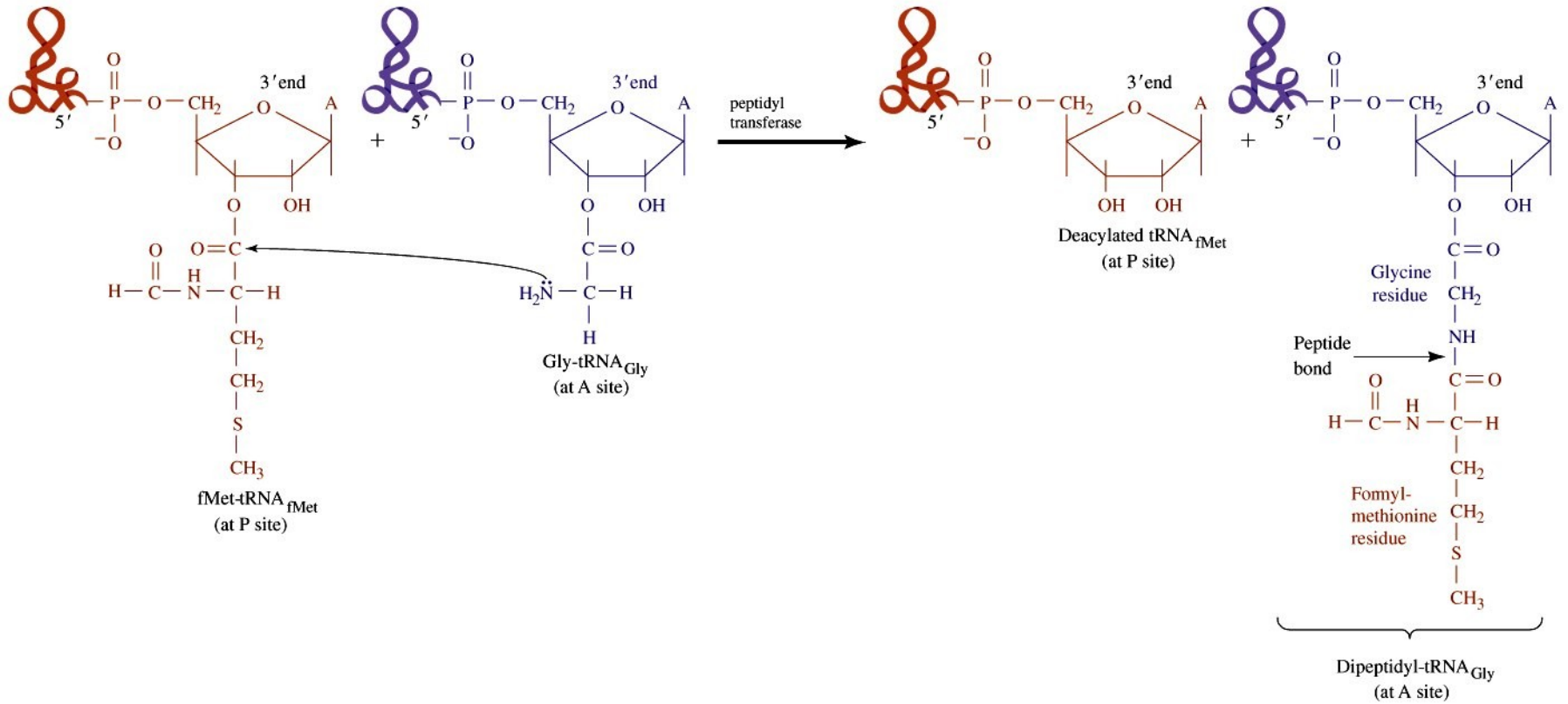
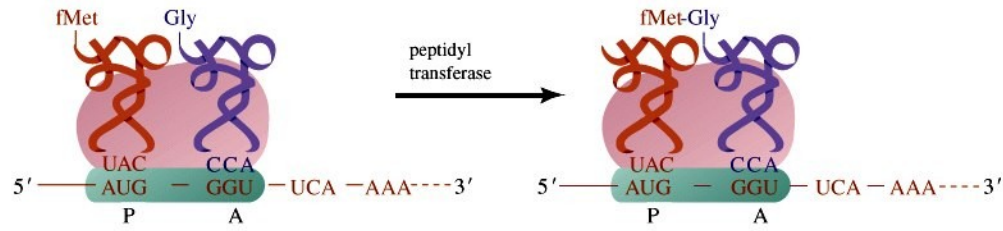
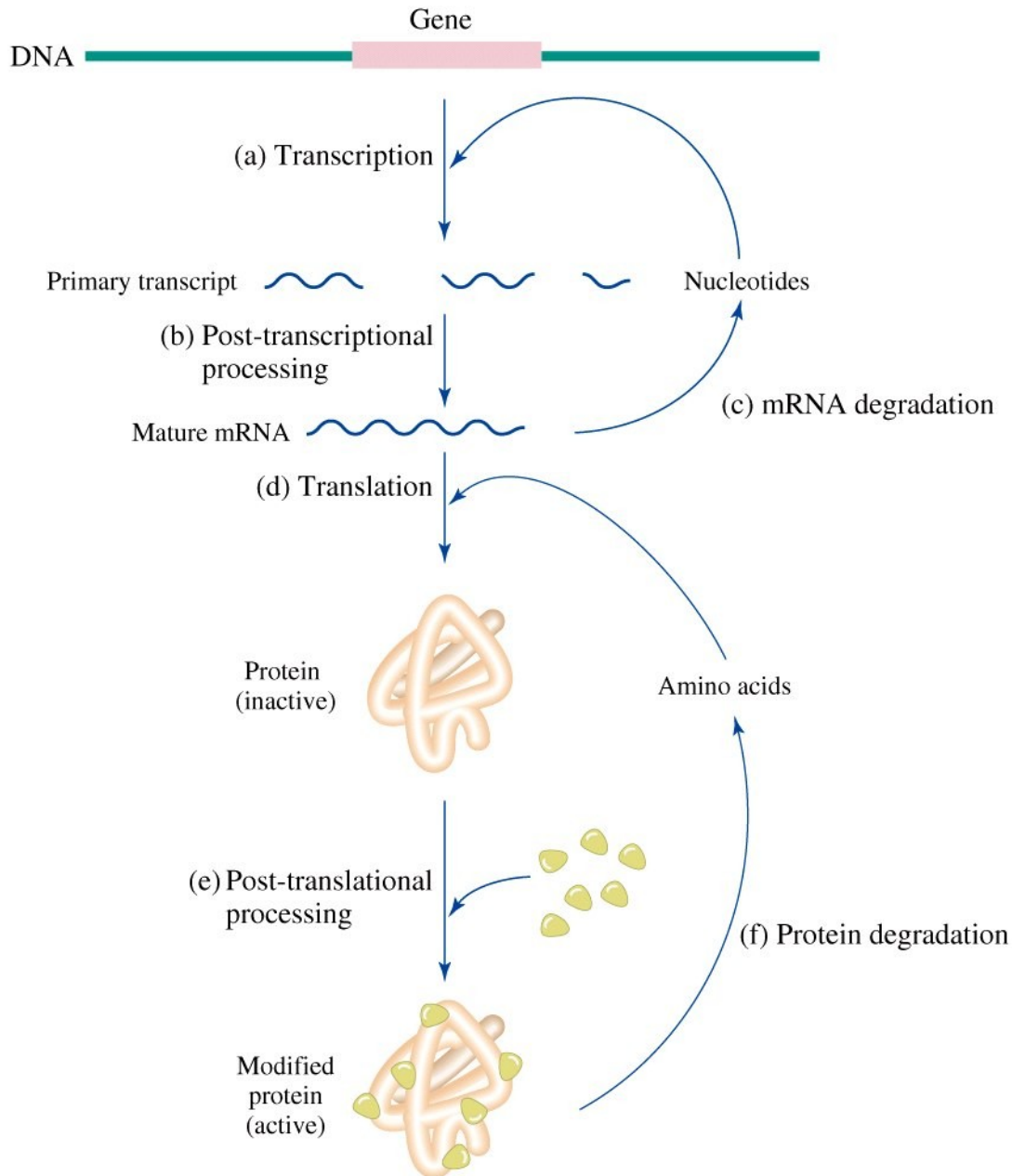


Figure 12-7 Concepts in Biochemistry, 3/e  
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**Figure 12-16 Concepts in Biochemistry, 3/e**  
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# Posttranslační modifikace

- fosforylace/defosforylace – enzymy kinázy a fosfatázy připojují či odpojují fosfátovou ( $\text{PO}_4^{3-}$ ) skupinu k proteinu na jeho serinové / threoninové zbytky nebo tyrosinové zbytky. Fosforylace/defosforylace často působí jako přepínač mezi aktivní a neaktivní formou proteinu.
- glykosylace – napojování sacharidů na protein. Sacharidové zbytky jsou nejčastěji připojovány na serin/ threonin – v případě tzv. O-glykoproteinů, nebo asparagin v případě N-glykoproteinů. Navázání sacharidů může stabilizovat konformaci proteinů; sacharidové složky mnoha proteinů se účastní rozpoznávacích interakcí (protein-sacharidové a nově objevené sacharid-sacharidové interakce)

# Posttranslační modifikace

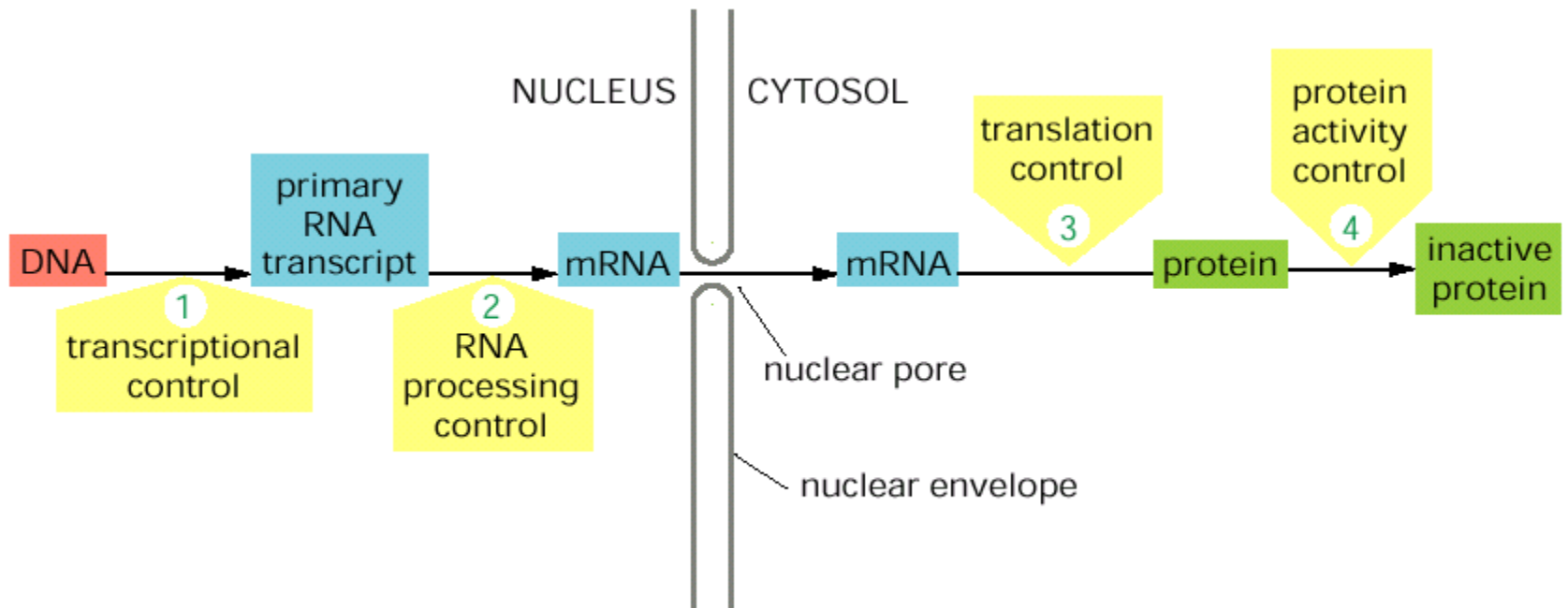
- ubikvitinace – připojení malého proteinu ubiquitinu k upravovanému proteinu přes aminokyselinu lysin ( její volný  $-NH_2$  konec). Připojování ubiquitinu na proteiny slouží jako molekulární hodiny, které určují stáří proteinu. Proteiny s mnoha navázanými ubiquitiny jsou degradovány v cytoplasmě pomocí proteazomu. Kromě této funkce, specifické navázání několika molekul ubiquitinu slouží k regulaci funkce některých proteinů
- sumoylace - připojení proteinu SUMO1, regulace funkce proteinů.
- proteolýza – odštěpení části molekuly proteinu – vede často k aktivaci nebo deaktivaci funkce proteinu.

# Posttranslační modifikace

- acetylace – acetylace koncové  $\text{-NH}_2$  lysinu snižuje jeho bazicitu a zeslabuje tak iontové interakce
- hydroxylace – hydroxylace prolinu nebo lysinu v kolagenu, slouží ke stabilizování specifické konformace molekuly kolagenu (trojitá šroubovice).
- disulfidické můstky - oxidace dvou -SH skupiny cysteinu na -S-S-
- vazba prostetických skupin – např. FAD, FMN, hem, nutné pro funkci některých enzymů



# Kontrola eksprese genu



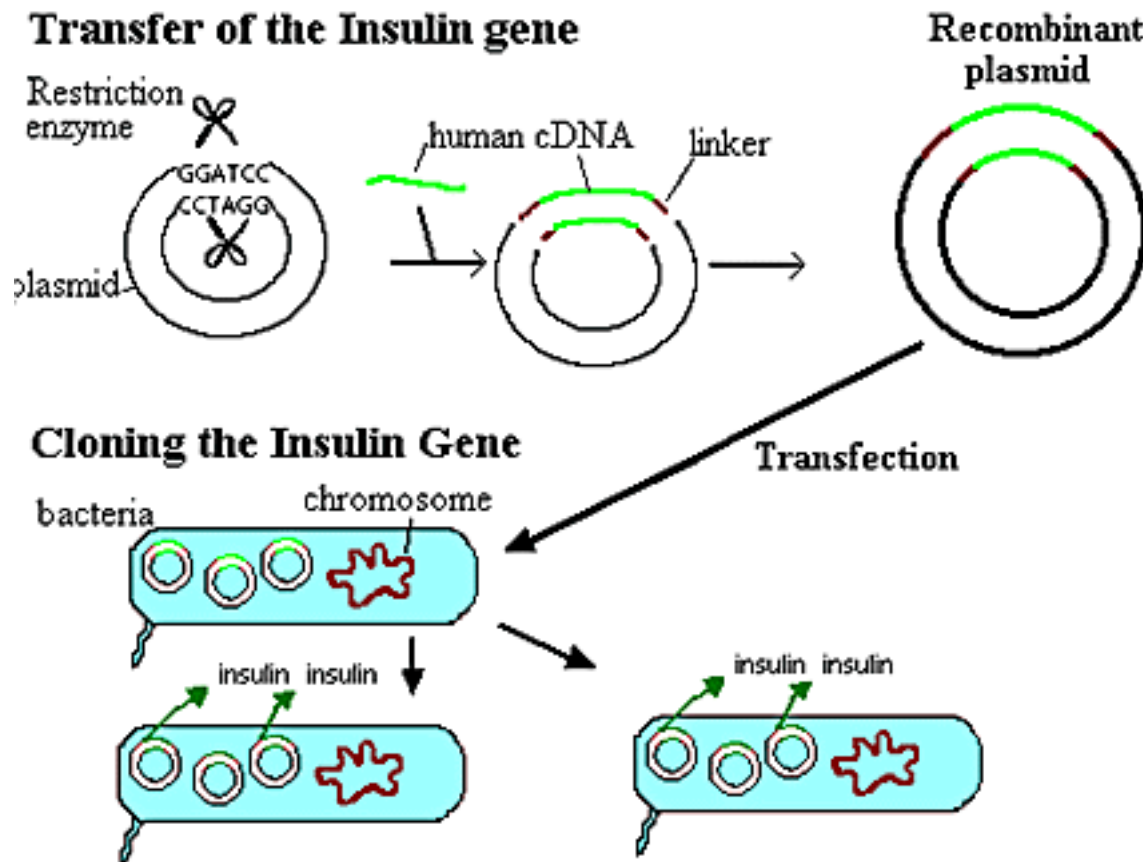
**Table 12.4****Antibiotic inhibitors of protein synthesis**

Antibiotic	Mode of Action
Puromycin	Causes early termination by mimicking the action of an aminoacyl-tRNA; acts on prokaryotes and eukaryotes
Streptomycin	Causes misreading of mRNA and inhibits initiation; acts on prokaryotes
Tetracycline	Binds to the A site of ribosomes and blocks entry of aminoacyl-tRNAs; acts on prokaryotes
Erythromycin	Binds to ribosome and inhibits translocation; acts on prokaryotes
Chloramphenicol	Binds to 50S subunit and inhibits peptidyl transferase; acts on prokaryotes
Cycloheximide	Inhibits translocation of eukaryotic peptidyl-tRNA
Linezolid	Blocks formation of 70S initiation complex in prokaryotes

**Table 12-4 Concepts in Biochemistry, 3/e**

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# Genetické inženýrství



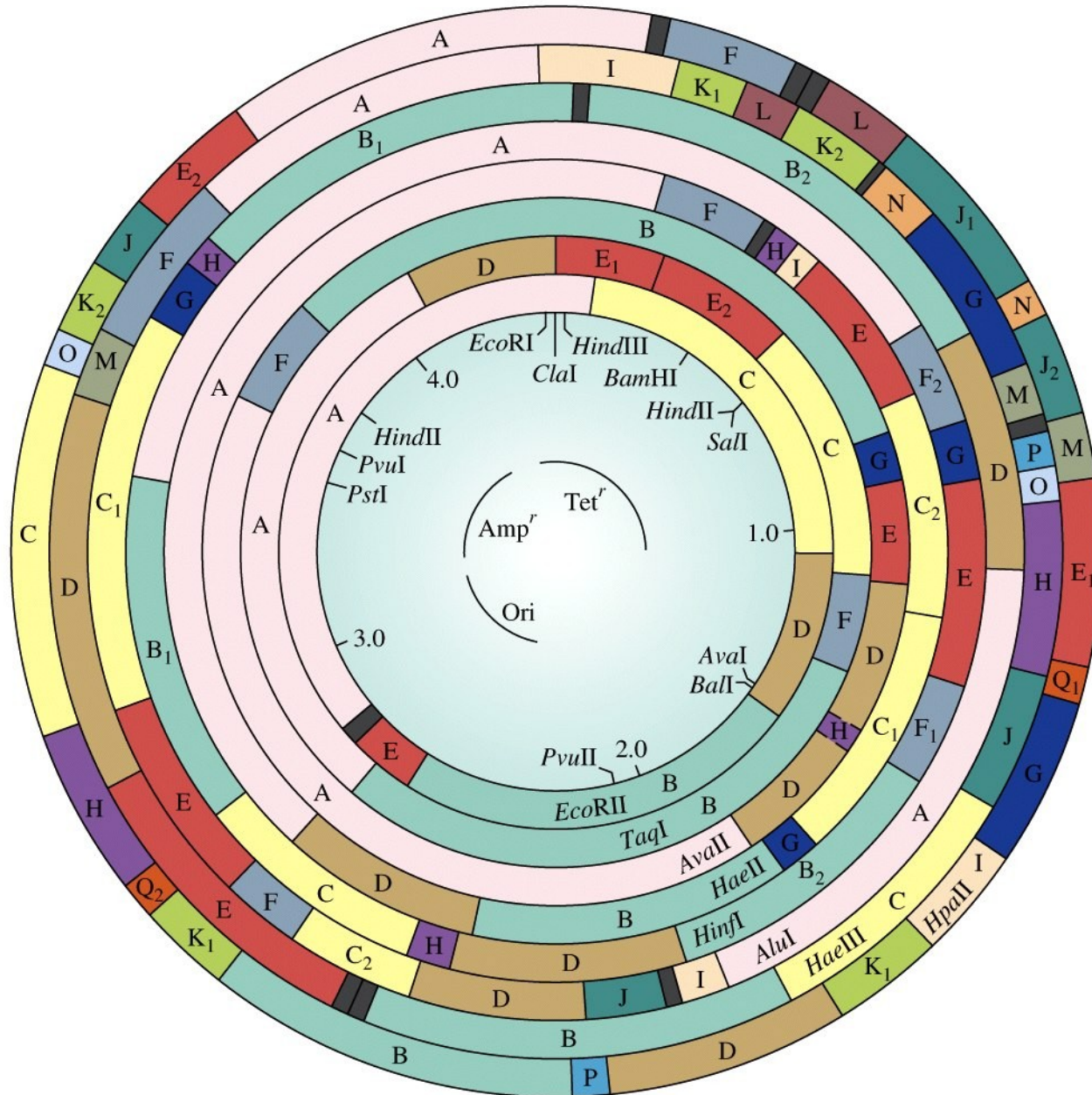


Figure 13-8 Concepts in Biochemistry, 3/e  
 © 2006 John Wiley & Sons

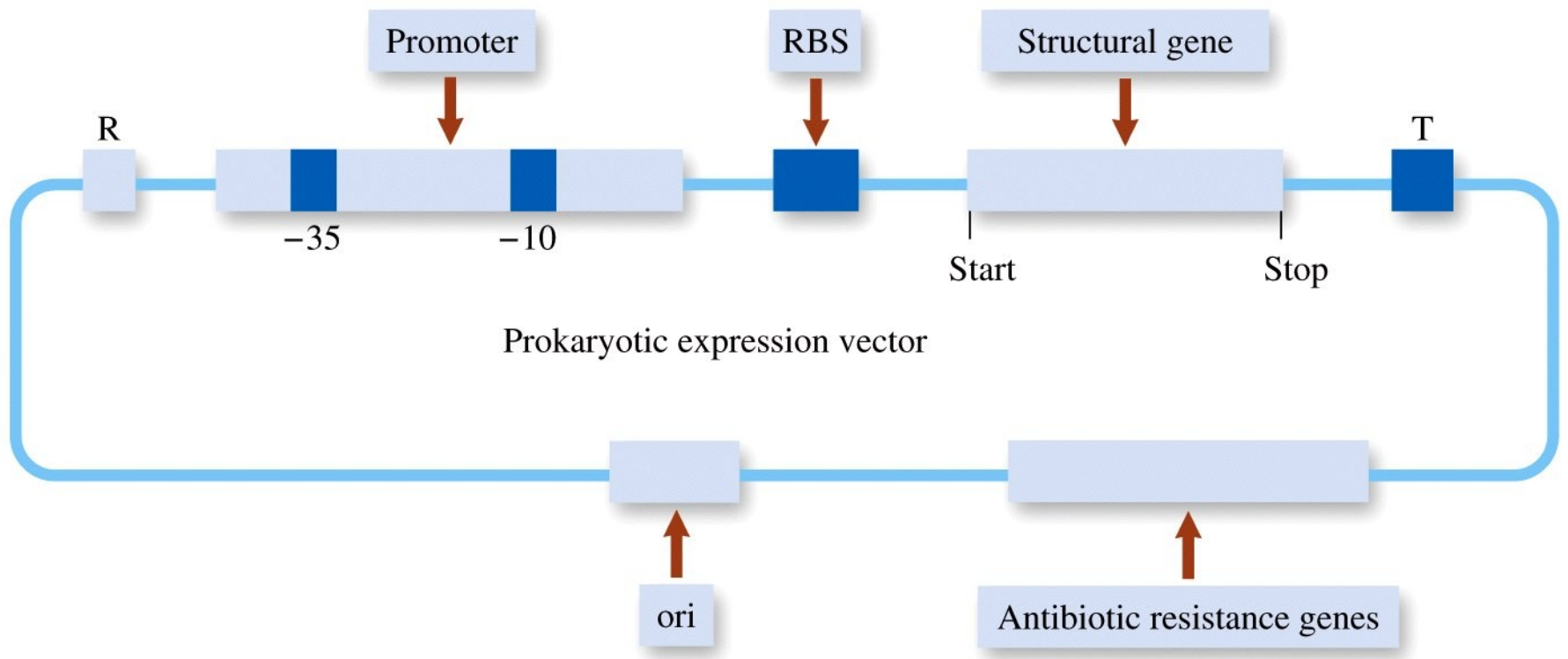
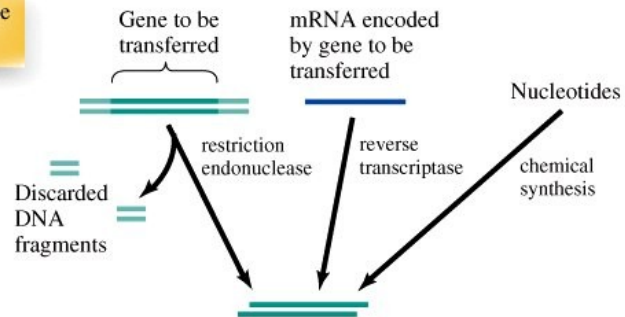
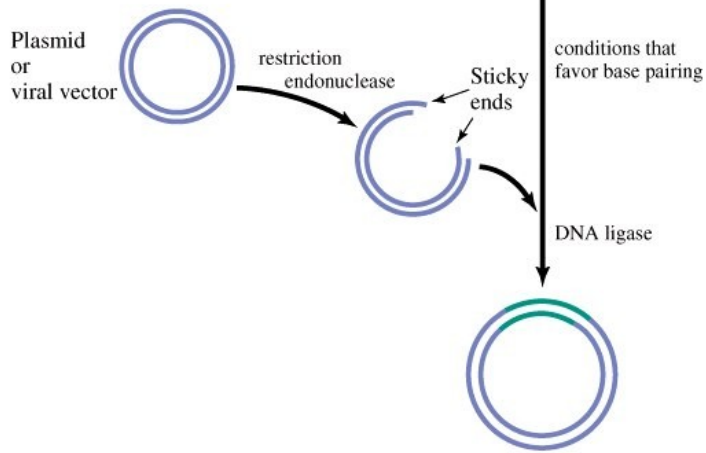


Figure 13-3 Concepts in Biochemistry, 3/e  
© 2006 John Wiley & Sons

Step 1. Obtain gene to be transferred.



Steps 2 and 3. Attach gene to vector



Steps 4 and 5. Introduce recombinant DNA into host cell.

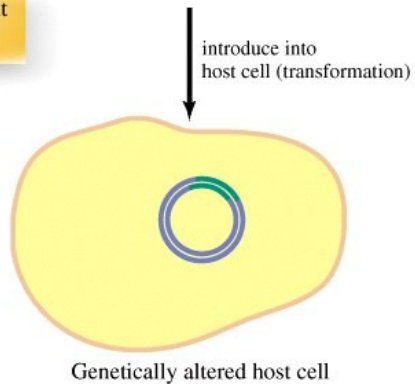


Figure 13-1 Concepts in Biochemistry, 3/e  
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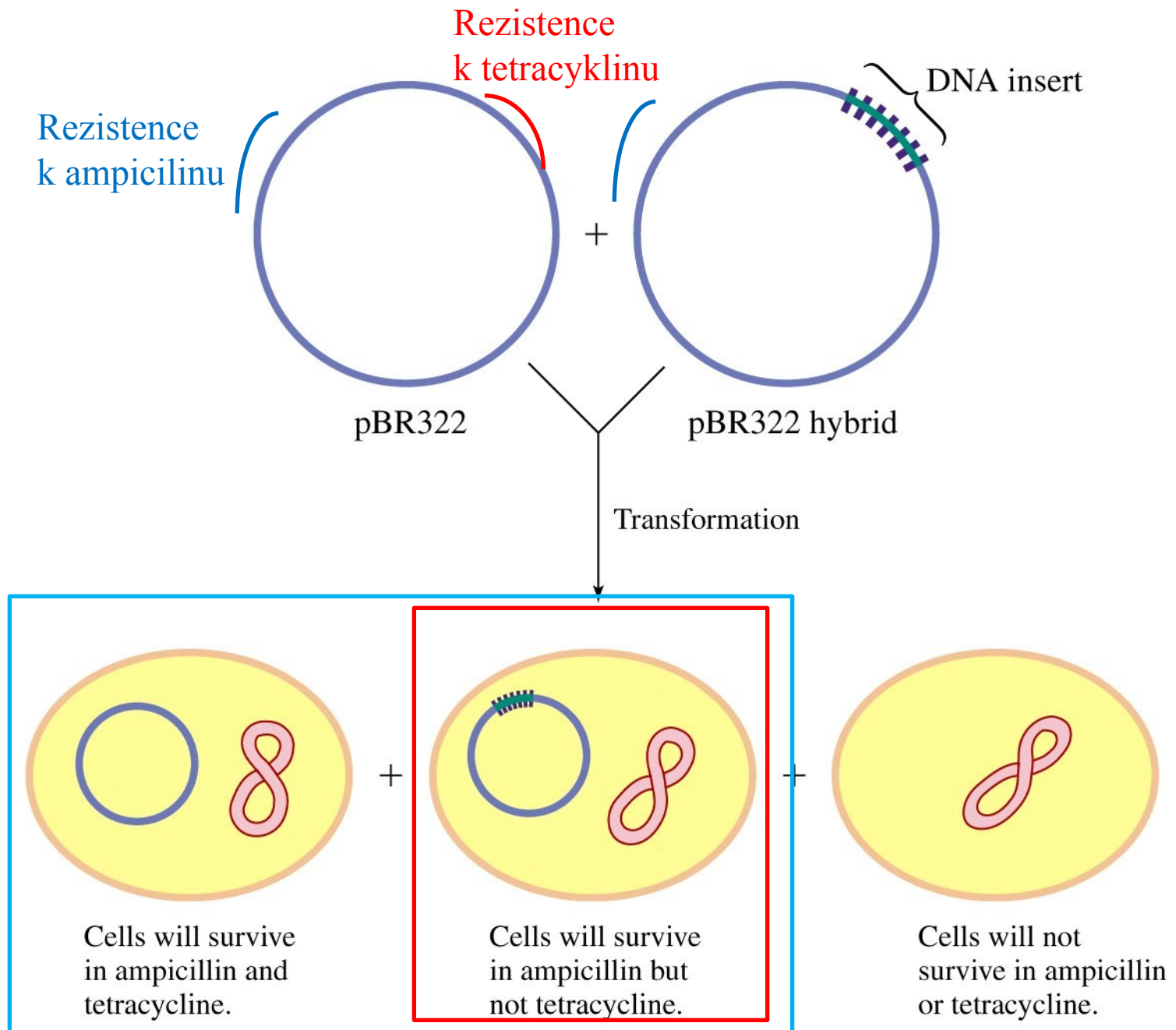
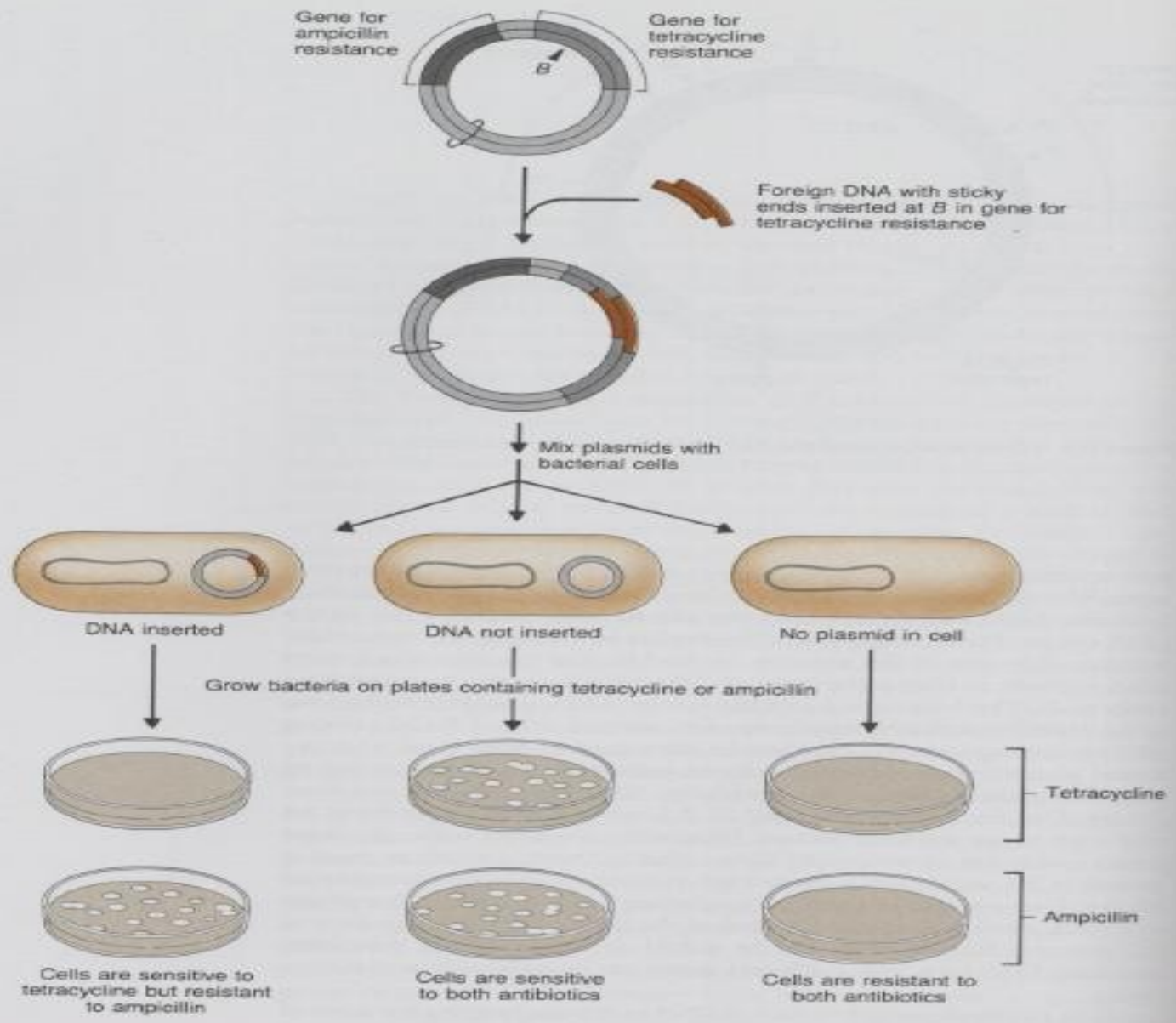
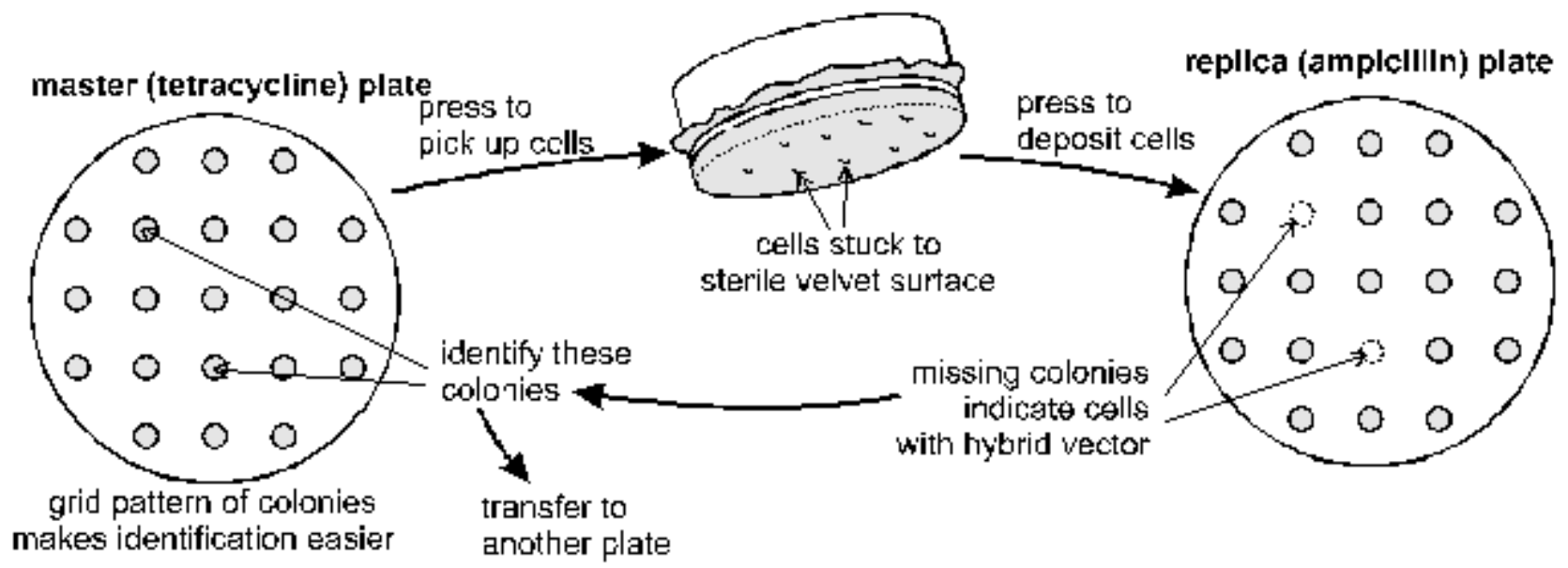


Figure 13-6 part 3 Concepts in Biochemistry, 3/e  
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**Figure 5.2** A plasmid vector with two genes for antibiotic resistance facilitates isolation of recombinants.



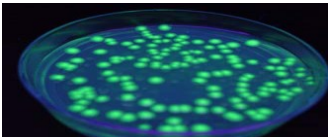


# Inklusní tělíska



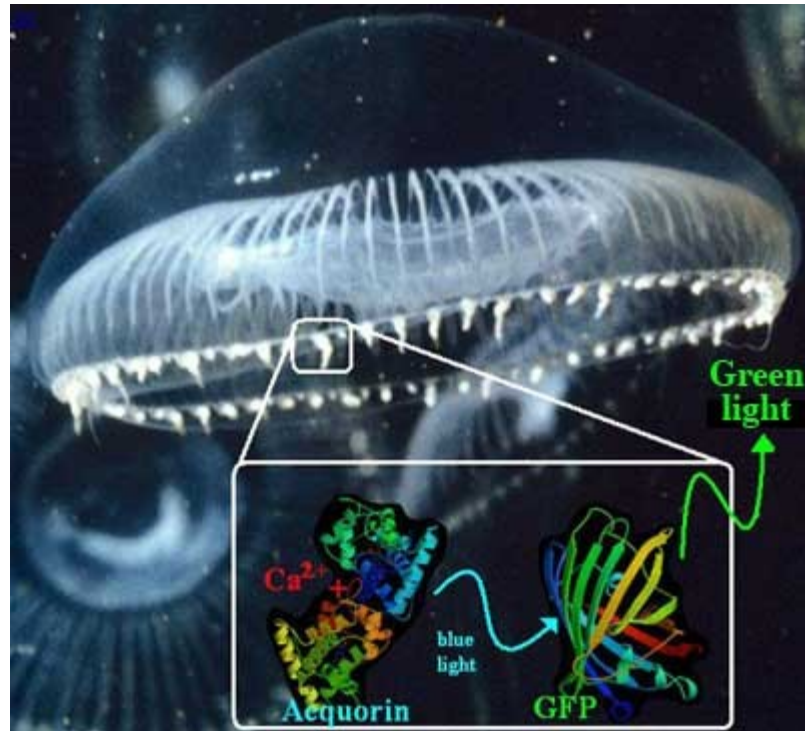
Courtesy of Teruhiko Beppu, Nihon University, Japan

# Využití AC pro purifikaci rekombinantních proteinů

fúzní kotva	imobilizovaný ligand	podmínky vazby	podmínky eluce
Glutathion S-transferasa <b>GST</b>	redukovaný glutathion	Neutrální pH, nedenaturující prostředí, glutathion musí být redukovaný a GST musí být aktivní	volný redukovaný glutathion
Histidinová kotva <b>His-tag</b>	Chelatovaný nikl nebo kobalt	Neutrální pH bez redukčních a oxidačních látek	>200 mM Imidazol, nízké pH, silné chelatační činidlo
Maltose Binding Protein <b>MBP</b>	Amylosa	Neutrální pH, nedenaturující prostředí; přídavek NaCl k snížení nespecifické sorbce	maltosa
Protein A	IgG	Neutrální pH, nedenaturující prostředí	změna pH, iontové síly
Green Fluorescent Protein <b>GFP</b>	Anti-GFP antibody 	Neutrální pH, nedenaturující prostředí	nízké pH, iontová síla

# Green Fluorescent Protein

*Aequorea victoria*



# Green Fluorescent Protein

## NC 2008

*Osamu Shimomura*

*Martin Chalfie*

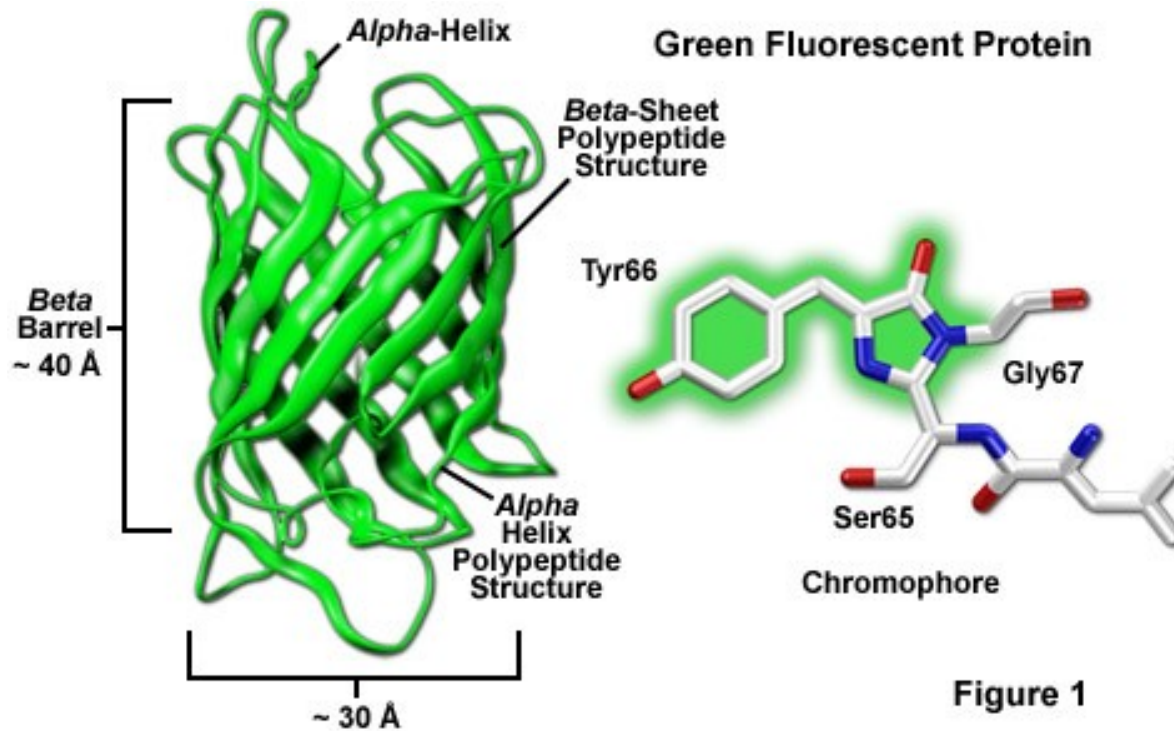
*Roger Tsien*



# Green Fluorescent Protein

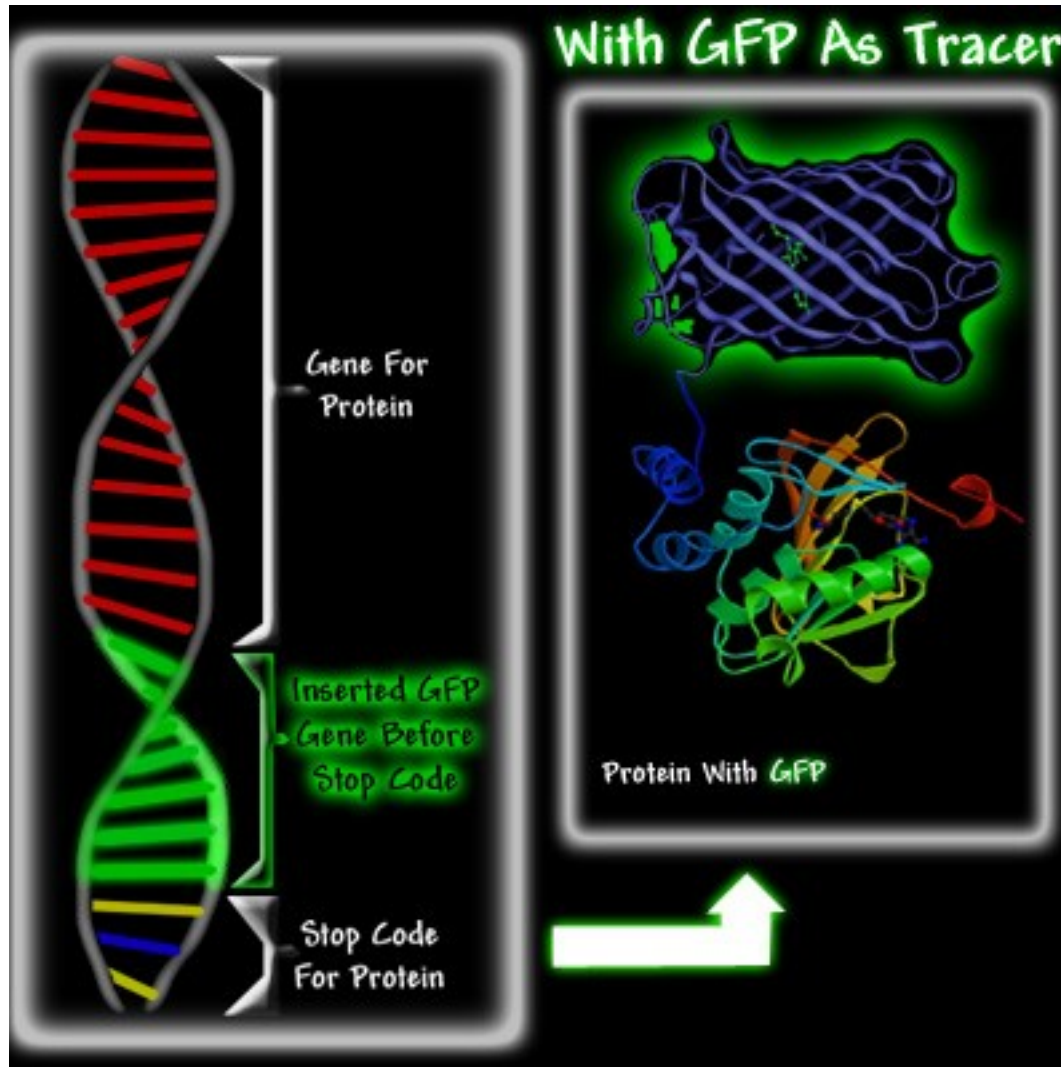
## GFP AMK sekvence – 238 AMK

MSKGEELFTGVVPLVELDGDVNGQKFSVSGEGEGDATYGKLTNFICT  
TGKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTI  
FYKDDGNYKTRAEVKFEEDTLVNRIELKGIDFKEDGNILGHKMEYNYNS  
HNVIYIMGDKPKNGIKVNFKIRHNIKDGSVQLADHYQQNTPIGDGPVLLP  
DNHYLSTQSALS KDPNEKRDHMILLEFVTAARITHGMDELYK





# Green Fluorescent Protein



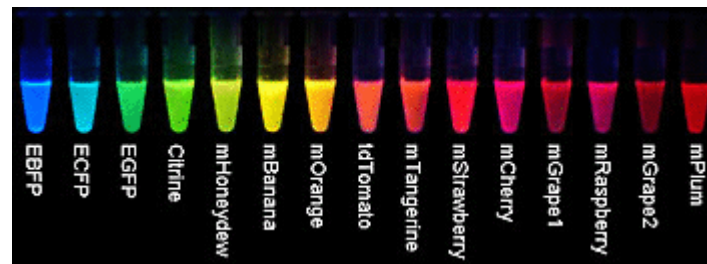
Reportérový gen

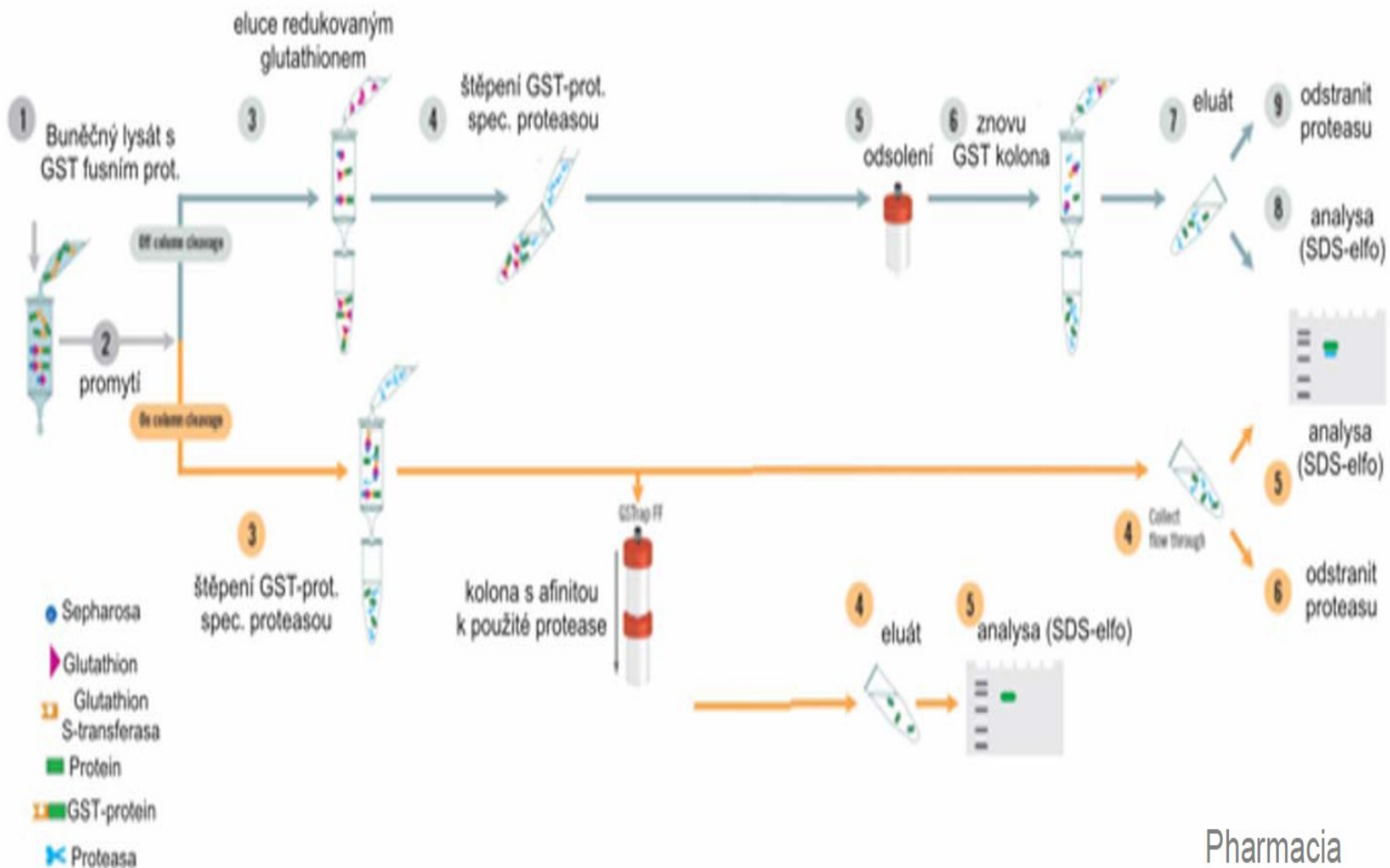
# Green Fluorescent Protein





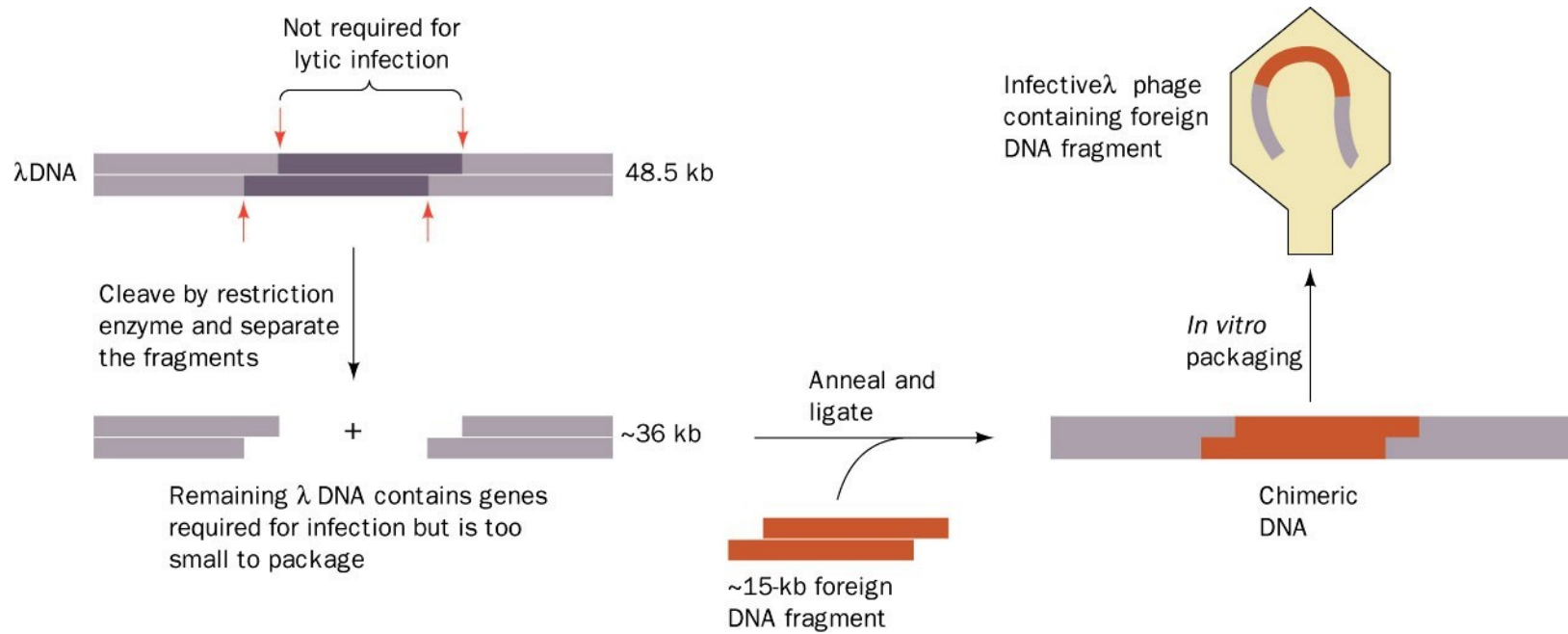
# Green Fluorescent Protein





**Table 13.1****Recombinant proteins and their use**

Protein	Use
Human insulin	Treatment of diabetes
Human somatotropin (growth hormone)	Treatment of dwarfism
Bovine somatotropin (BST)	Enhances milk production in dairy cattle
Porcine somatotropin	Enhances growth in pigs
Pulmozyme (DNase)	Treatment of cystic fibrosis
Tissue plasminogen activator (TPA)	Treatment of heart attack, stroke victims; dissolves blood clots
Erythropoietin	Stimulates erythrocyte production in anemia
Interferons	Antiviral agent; treatment of cancers
Atrial natriuretic factor	Reduces high blood pressure
Leptin	Treatment of obesity
Hepatitis B vaccine	Treatment of hepatitis
Herceptin	Monoclonal antibody to treat metastatic breast cancer
Superoxide dismutase	Destroys reactive oxygen species; treatment of arthritis

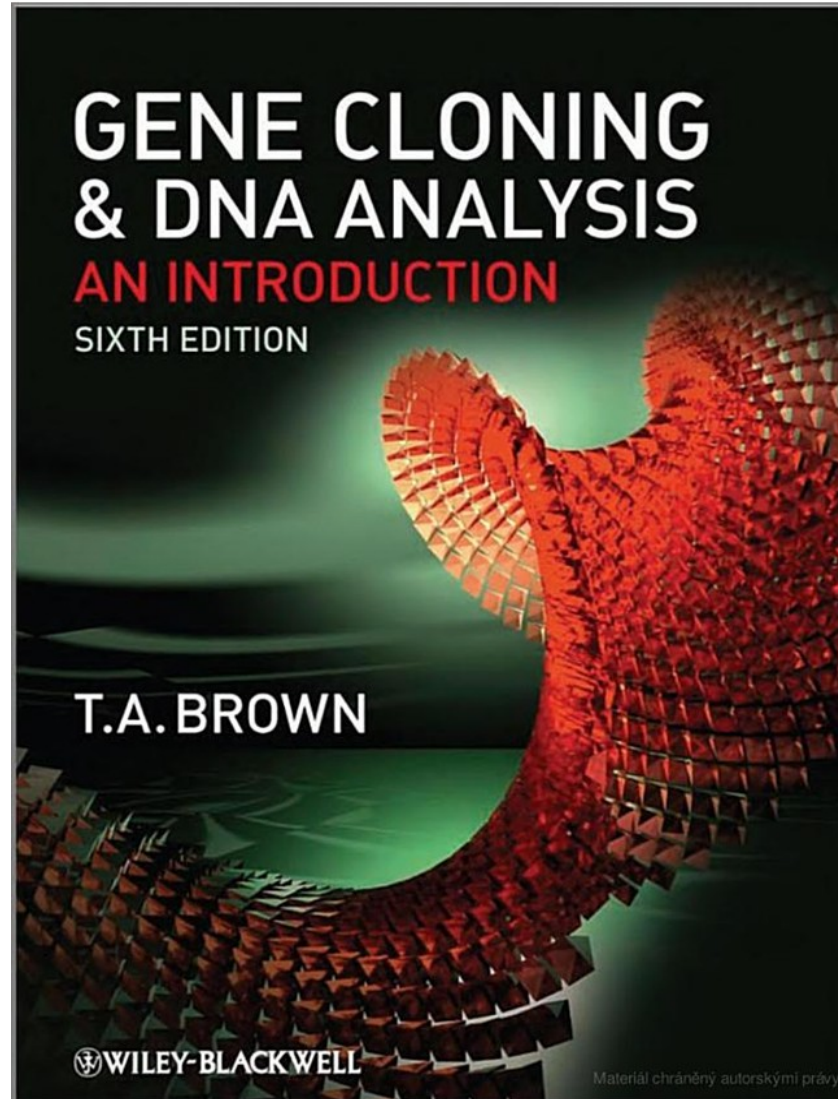


**Table 13.2****Human gene therapy projects currently in or preparing for clinical trials**

Disease	Defective Protein, Gene, or Inserted DNA
Lesch-Nyhan syndrome	Hypoxanthine–guanine phosphoribosyl transferase (Section 19.5)
Amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease)	Superoxide dismutase
Adrenoleukodystrophy (ALD)	Very long chain fatty acid synthetase transporting protein
Severe combined immunodeficiency (SCID)	Adenosine deaminase
$\beta$ -Thalassemia	$\beta$ -Globin, a polypeptide of hemoglobin
Familial hypercholesterolemia	Liver receptor for low density lipoprotein (LDL) (Section 18.5)
Hemophilia	Blood-clotting factors
Duchenne's muscular dystrophy	Dystrophin
AIDS	The gene to produce a ribozyme that cleaves HIV RNA
Inherited emphysema	$\alpha_1$ -Antitrypsin
Cystic fibrosis	A product that unclogs lung mucus is inhaled in a nasal spray
Cancer	E1A and p53 tumor suppressor genes

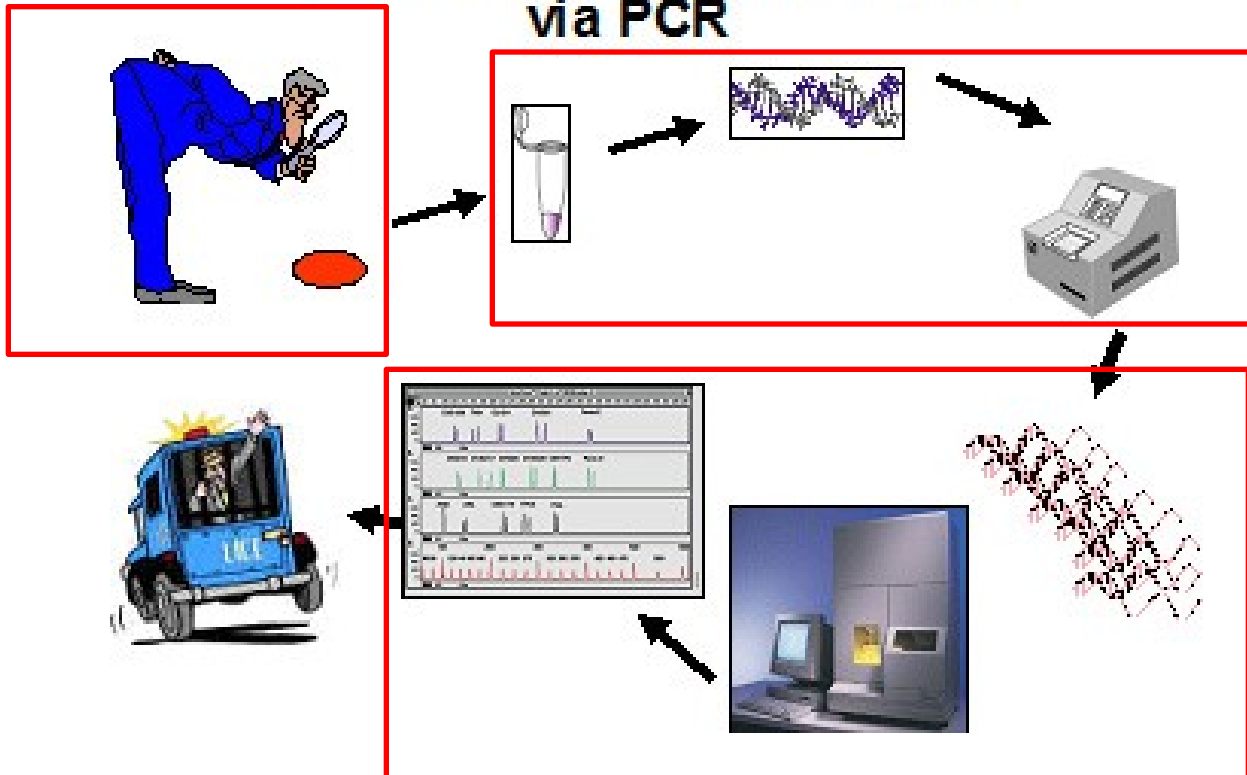


# Literatura



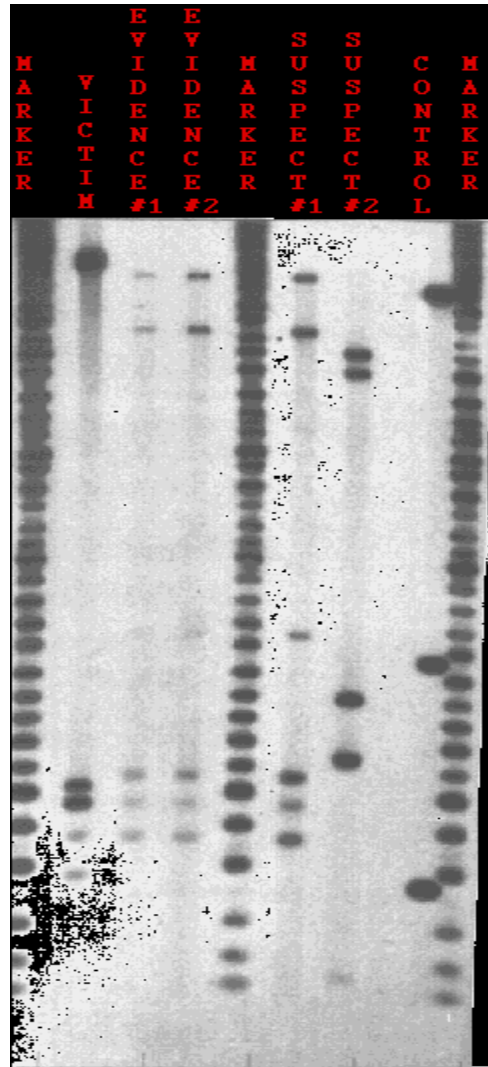
# Genetická daktyloskopie

## The Process of DNA Typing via PCR



# Použití restričních enzymů - RFLP

## Restriction Fragment Length Polymorphism

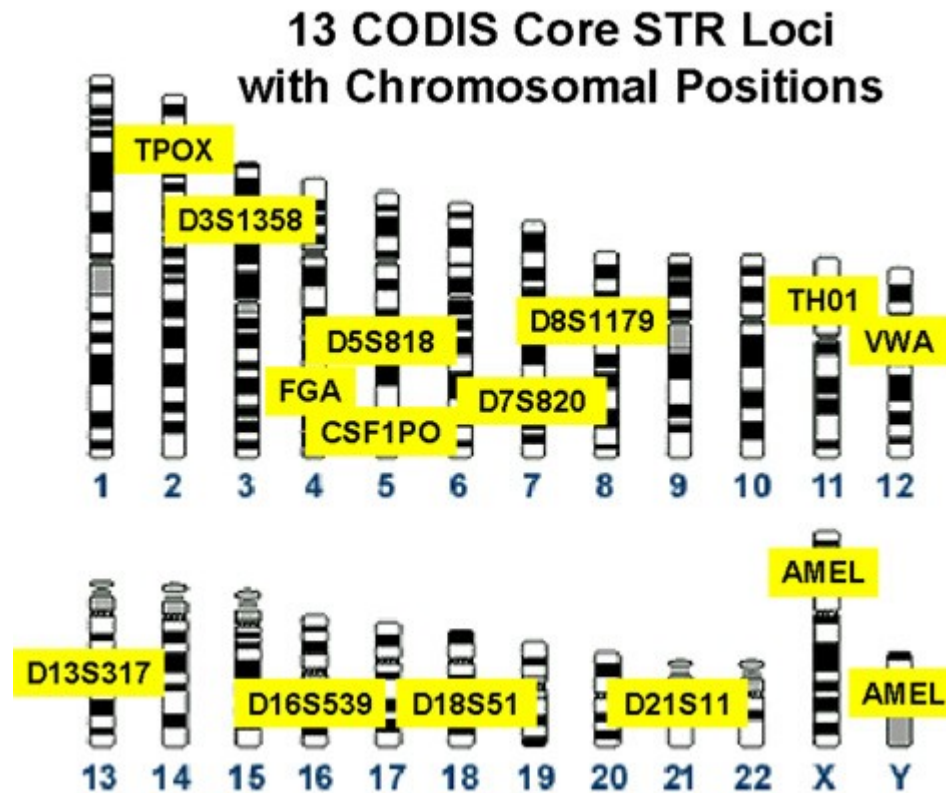




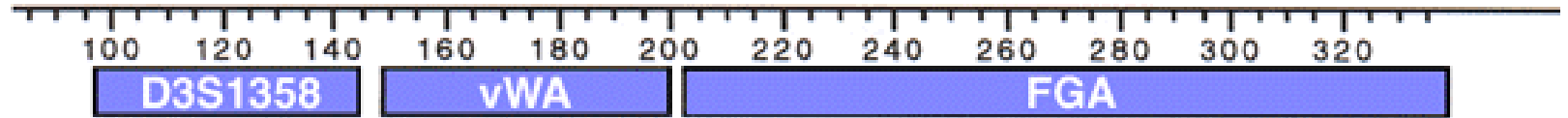
# Short tandem repeats

Repeat monomer	$P$ [%]	$REI$ [%]	$\alpha_B$	$\alpha_R$
A/T	59	1.75	1.0	0.2
C/G	41	0.12	1.1	0.4
AG/CT	27	0.27	2.6	0.24
AC/GT	20	0.66	2.6	
AT/TA	25	0.78	2.6	
CG/GC	10	0.02	2.6	
AAC/GTT	9	0.22	3.5	0.55
AAG/CTT	12	0.053	3.5	
AGG/CCT	11	0.077	3.5	

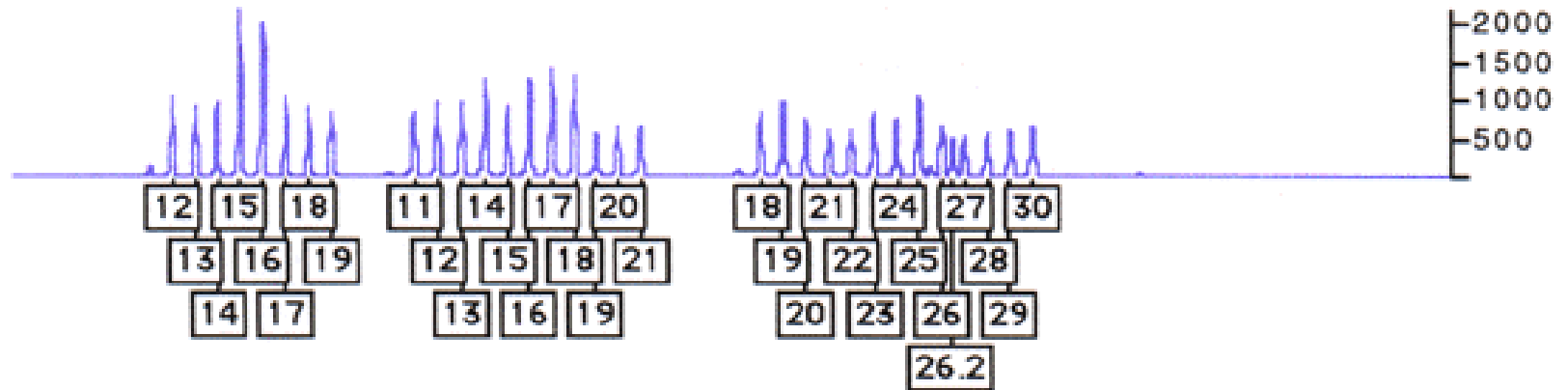
# Short tandem repeats



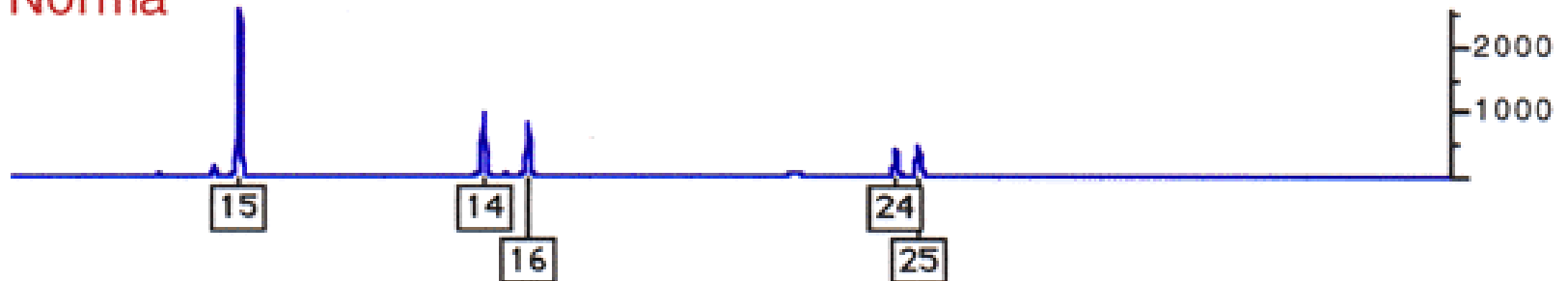
# Short tandem repeats



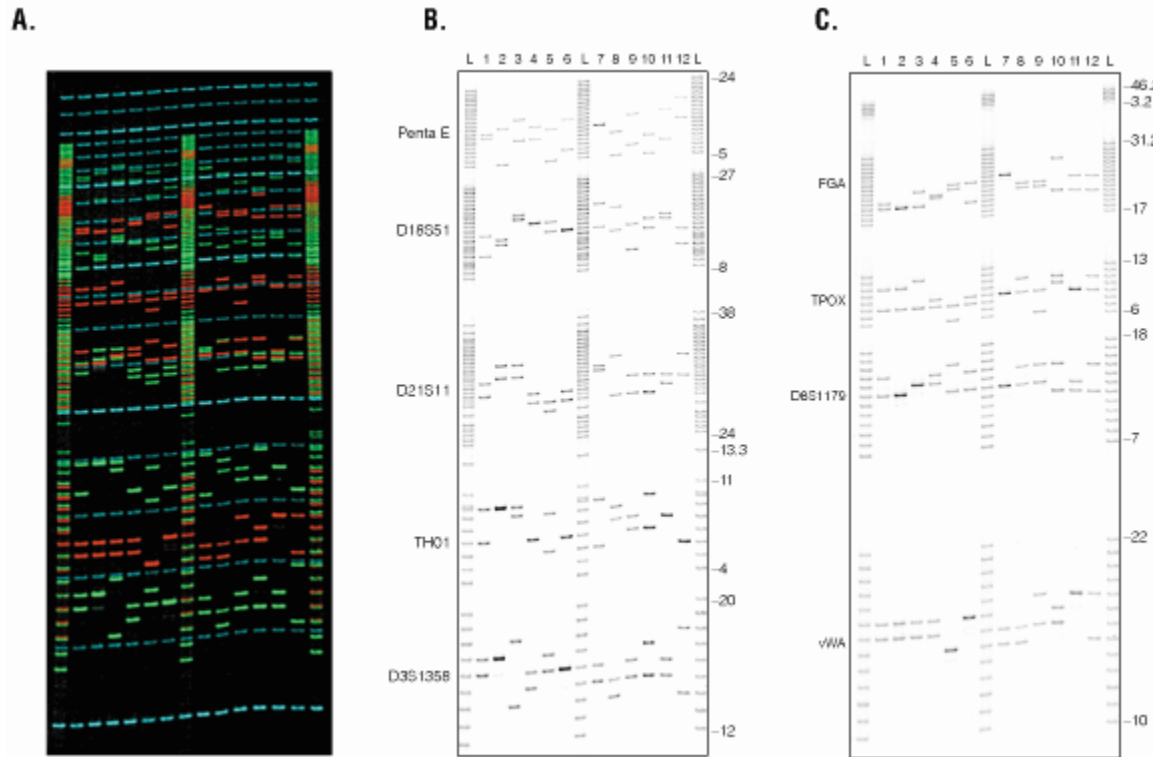
• A5\*PROFI...US LADDER 4 Blue PROFILER PLUS LADDER



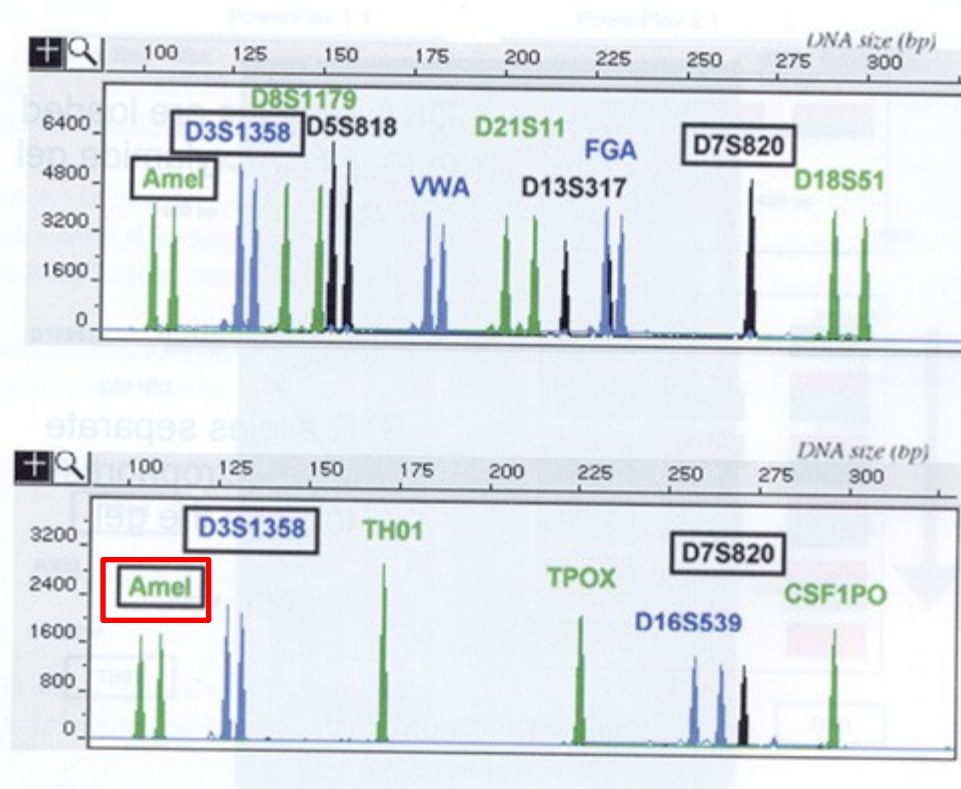
Norma



# Short tandem repeats



# Short tandem repeats



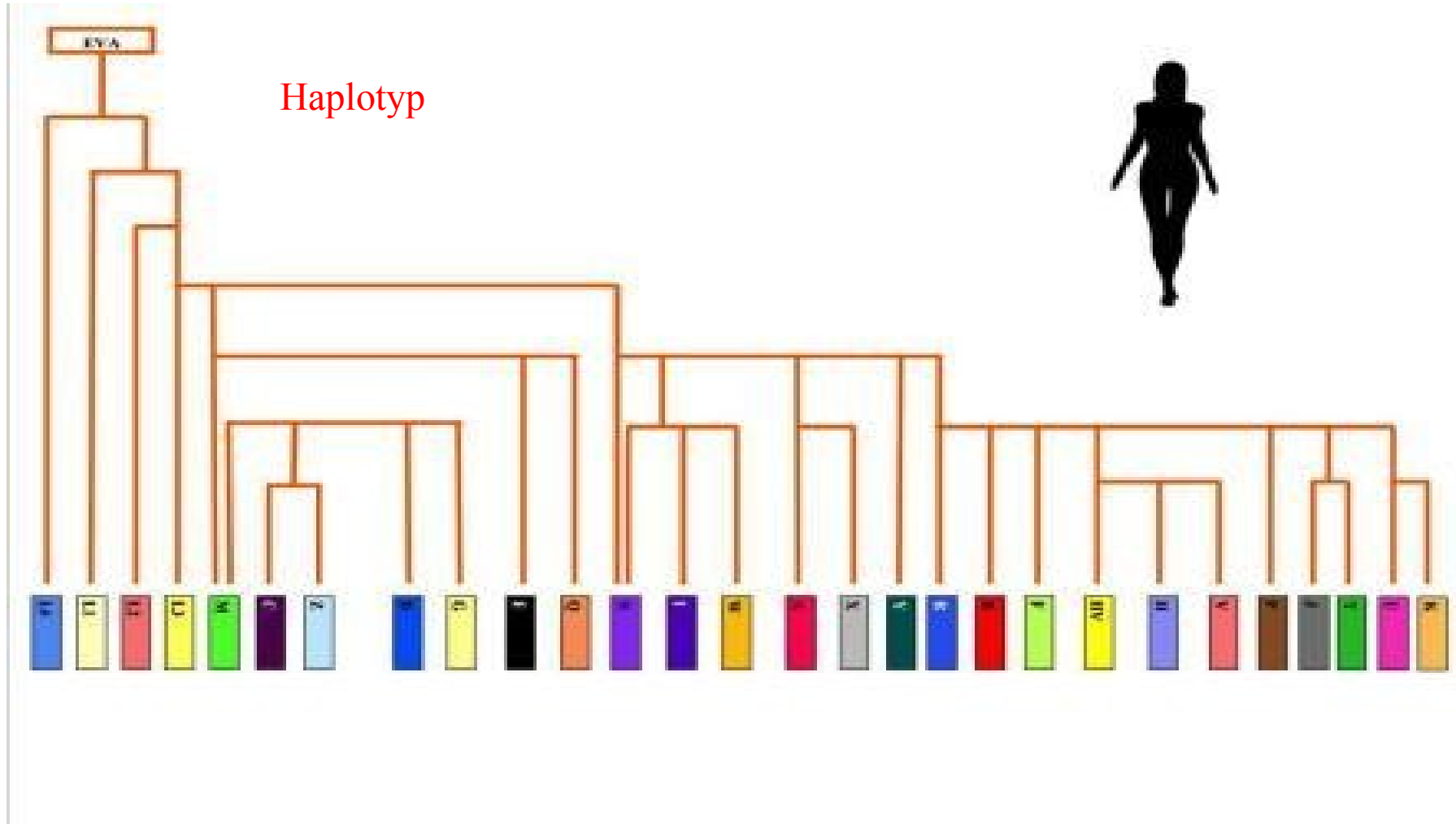
X - 106 Y - 112

# Testy paternity

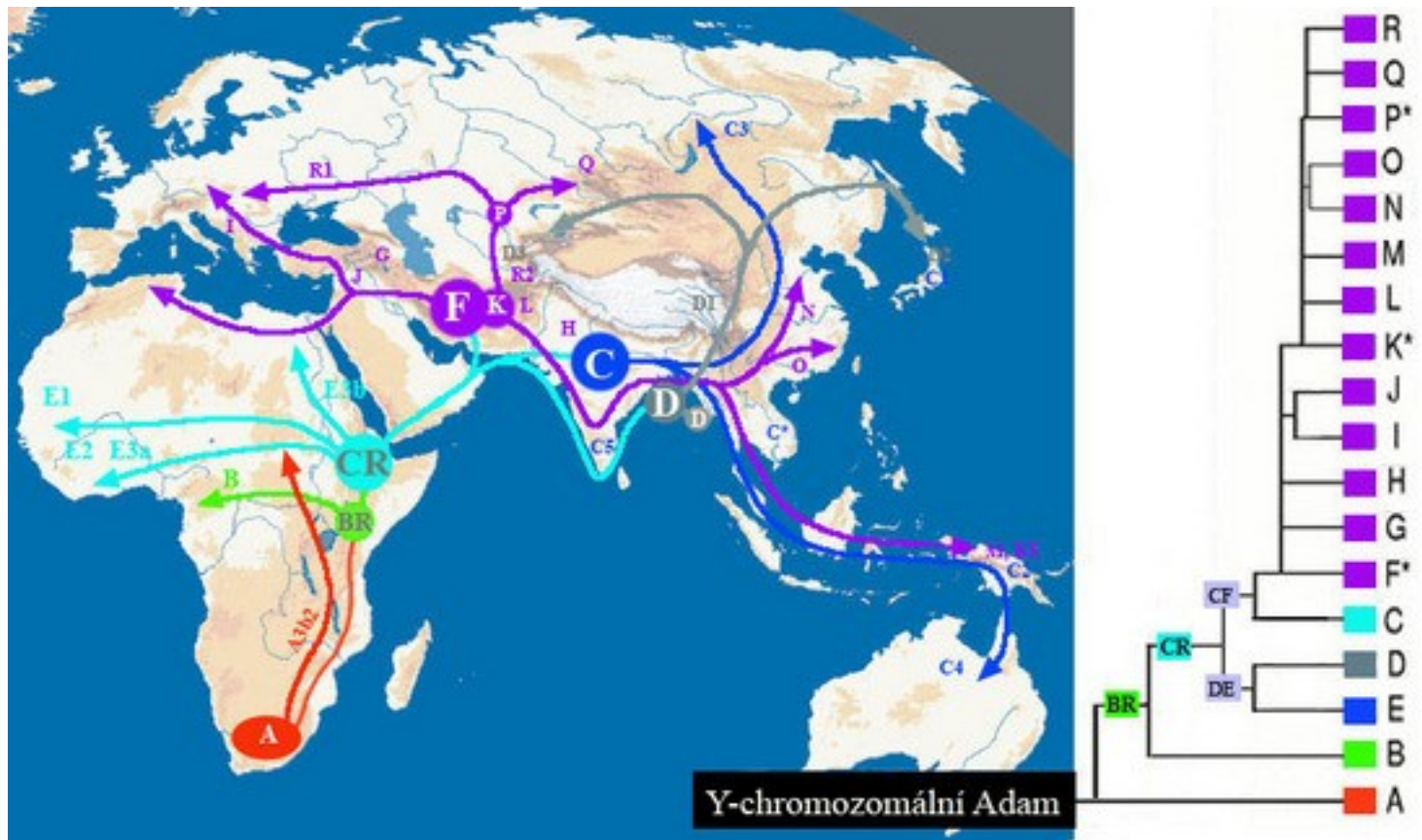
## Zjednodušené testy

- STR na Y chromosomu – mužských potomků srovnání s otcem
- Mitochondriální DNA – dědí se po matce – matroklinní dědičnost

# Mitochondriální Eva před 200 000 let v Africe

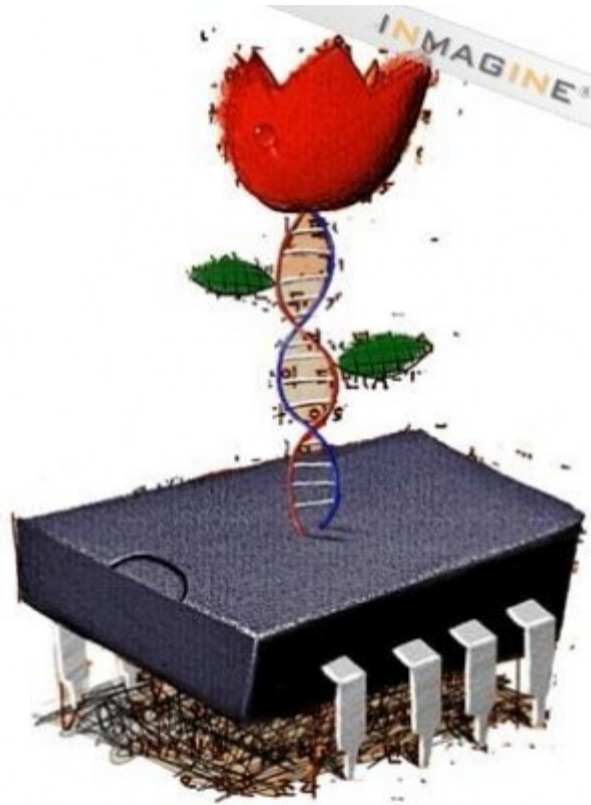


# Y-chromozomální Adam před 110 000 let v Africe

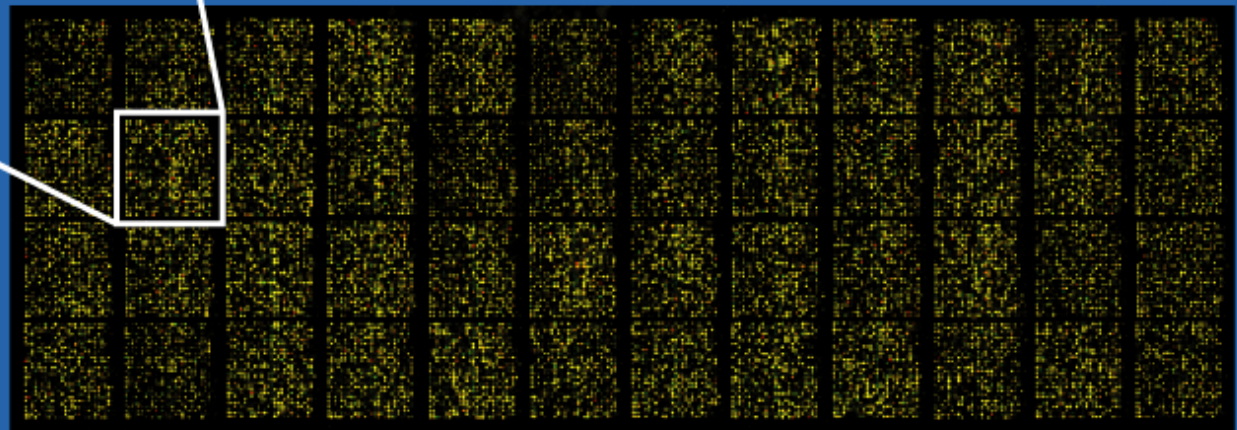
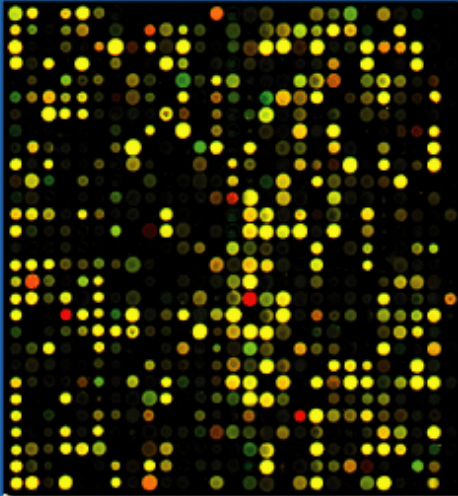




# DNA chipy



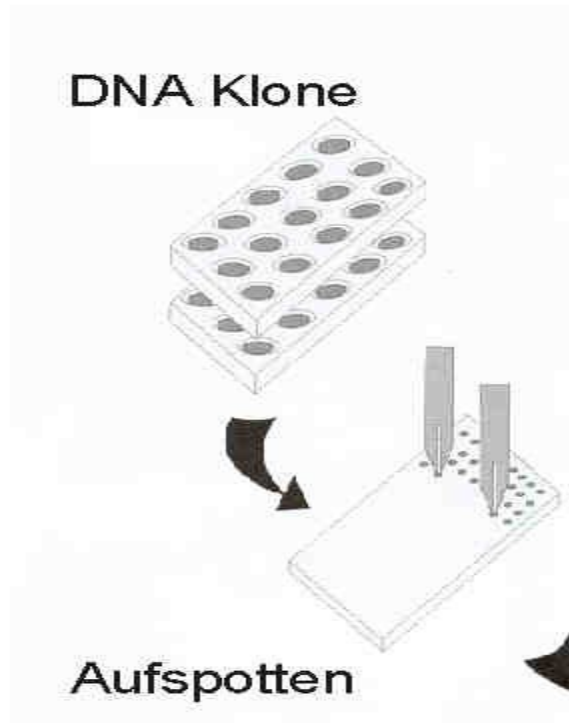
# DNA chipy



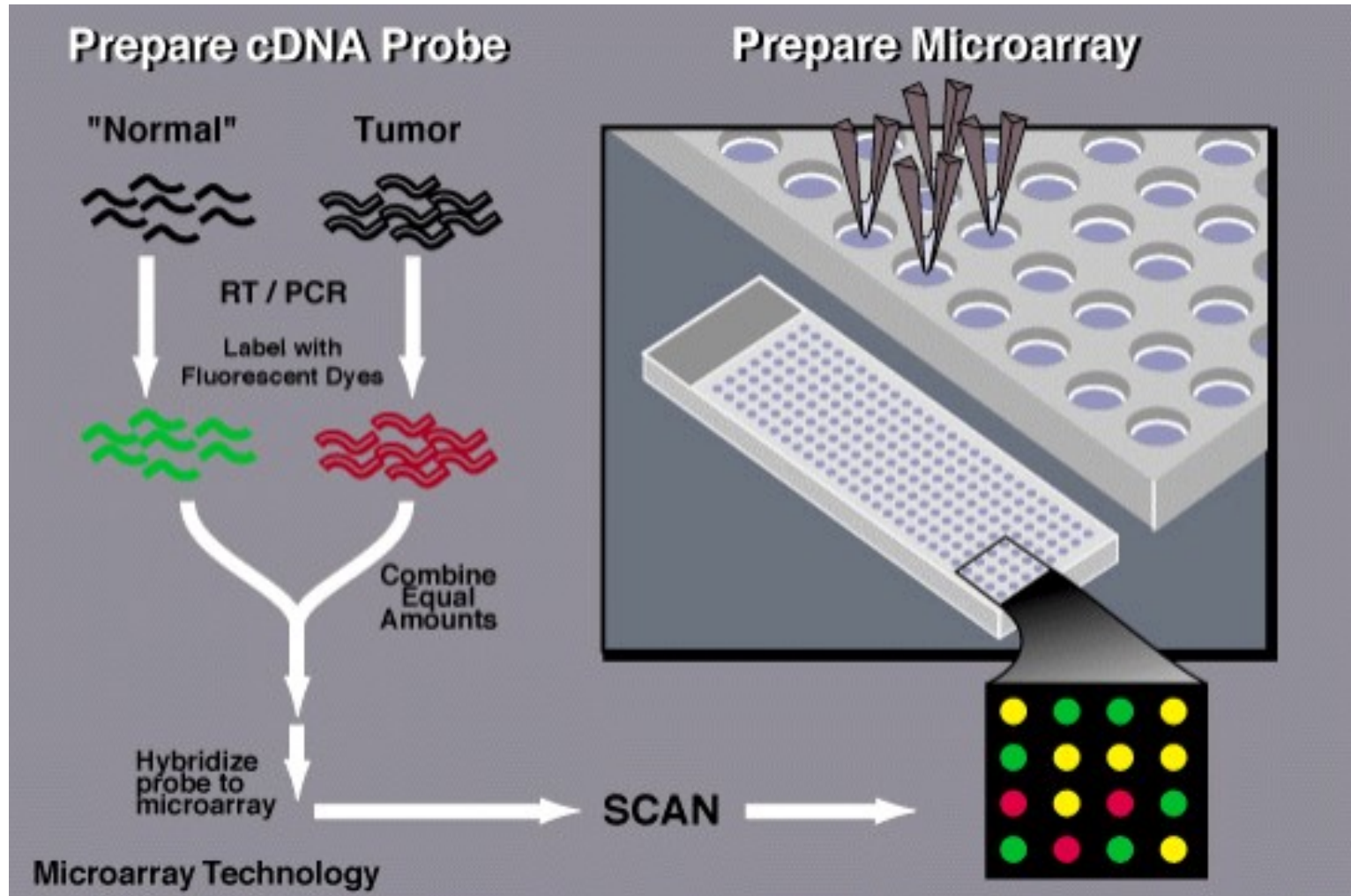
# DNA chipy

Fotolitografie

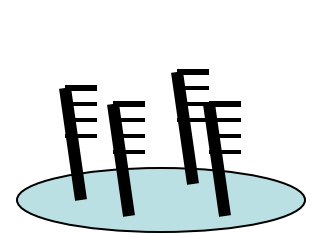
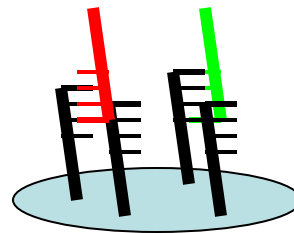
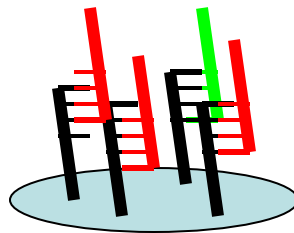
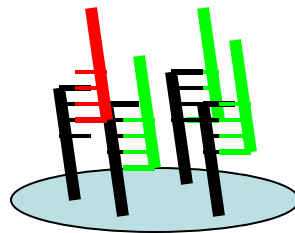
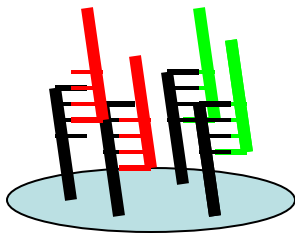
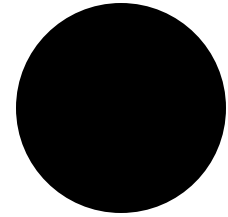
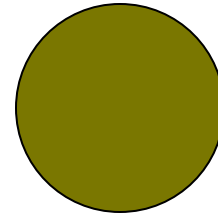
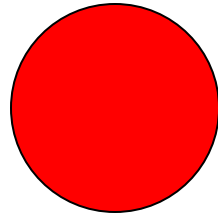
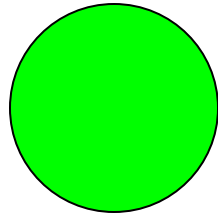
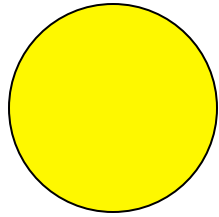
Kapkování



# DNA chipy



# DNA chipy - barva skvrn

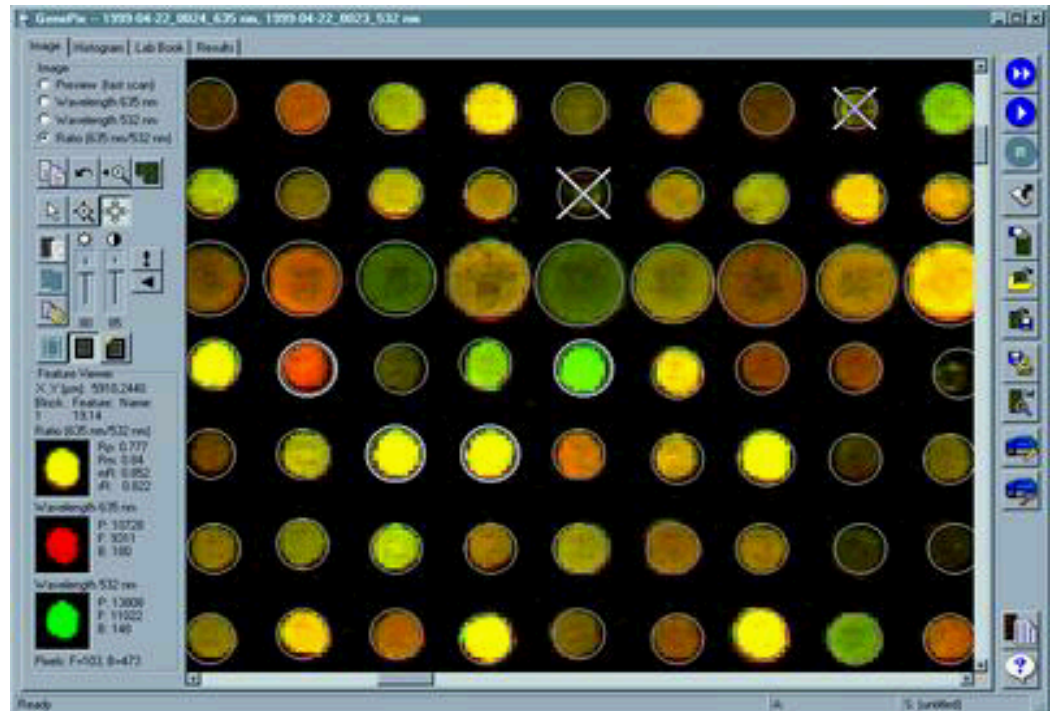
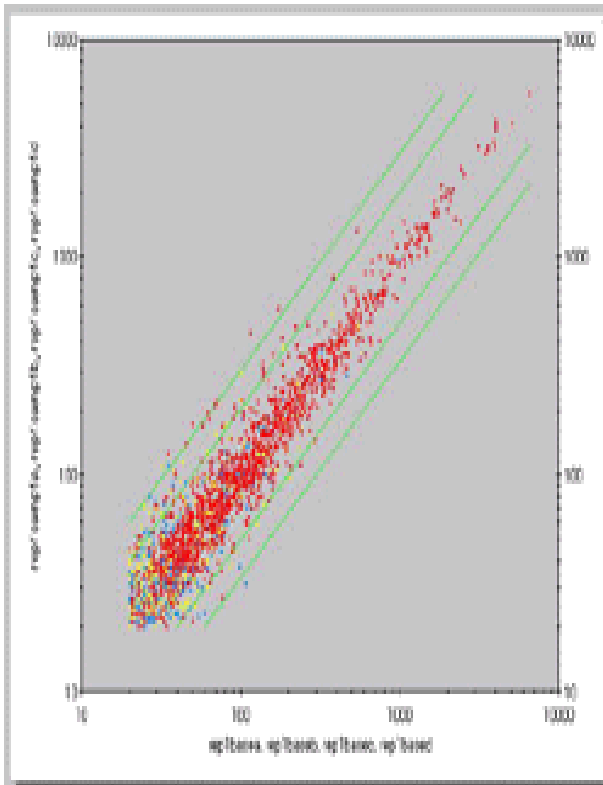


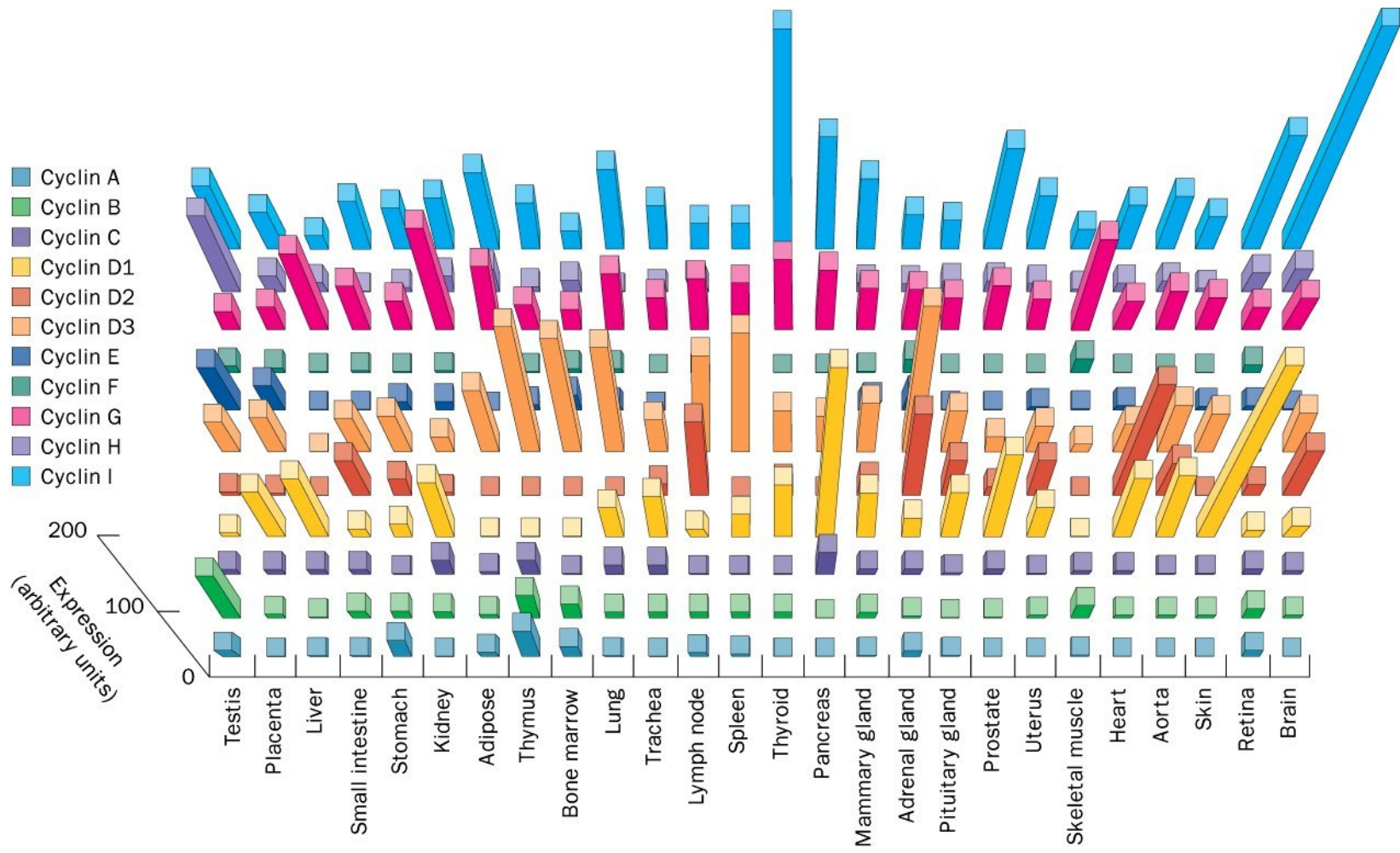
# DNA chipy - vybavení





# DNA chip software





Expression  
(arbitrary units)

200

100

0

Testis

Placenta

Liver

Small intestine

Stomach

Kidney

Adipose

Thymus

Bone marrow

Lung

Trachea

Lymph node

Spleen

Thyroid

Pancreas

Mammary gland

Adrenal gland

Pituitary gland

Prostate

Uterus

Skeletal muscle

Heart

Aorta

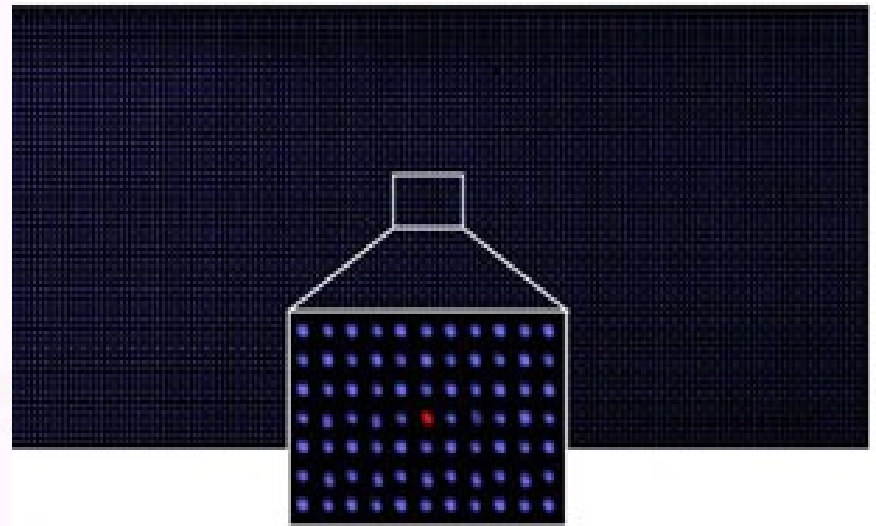
Skin

Retina

Brain

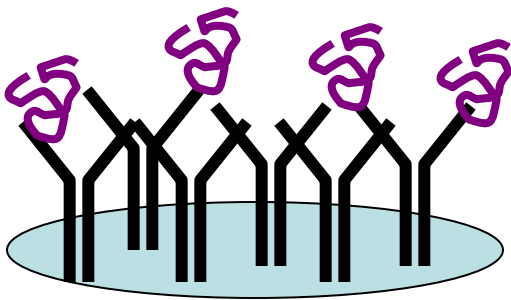


# Proteinové chipy

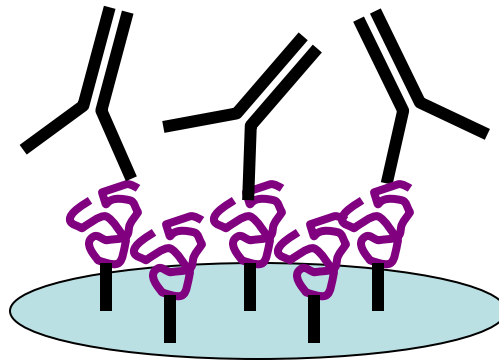


# Proteinové chipy – typy interakcí

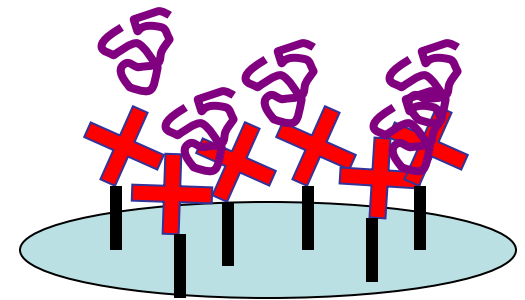
Protilátka



Antigen

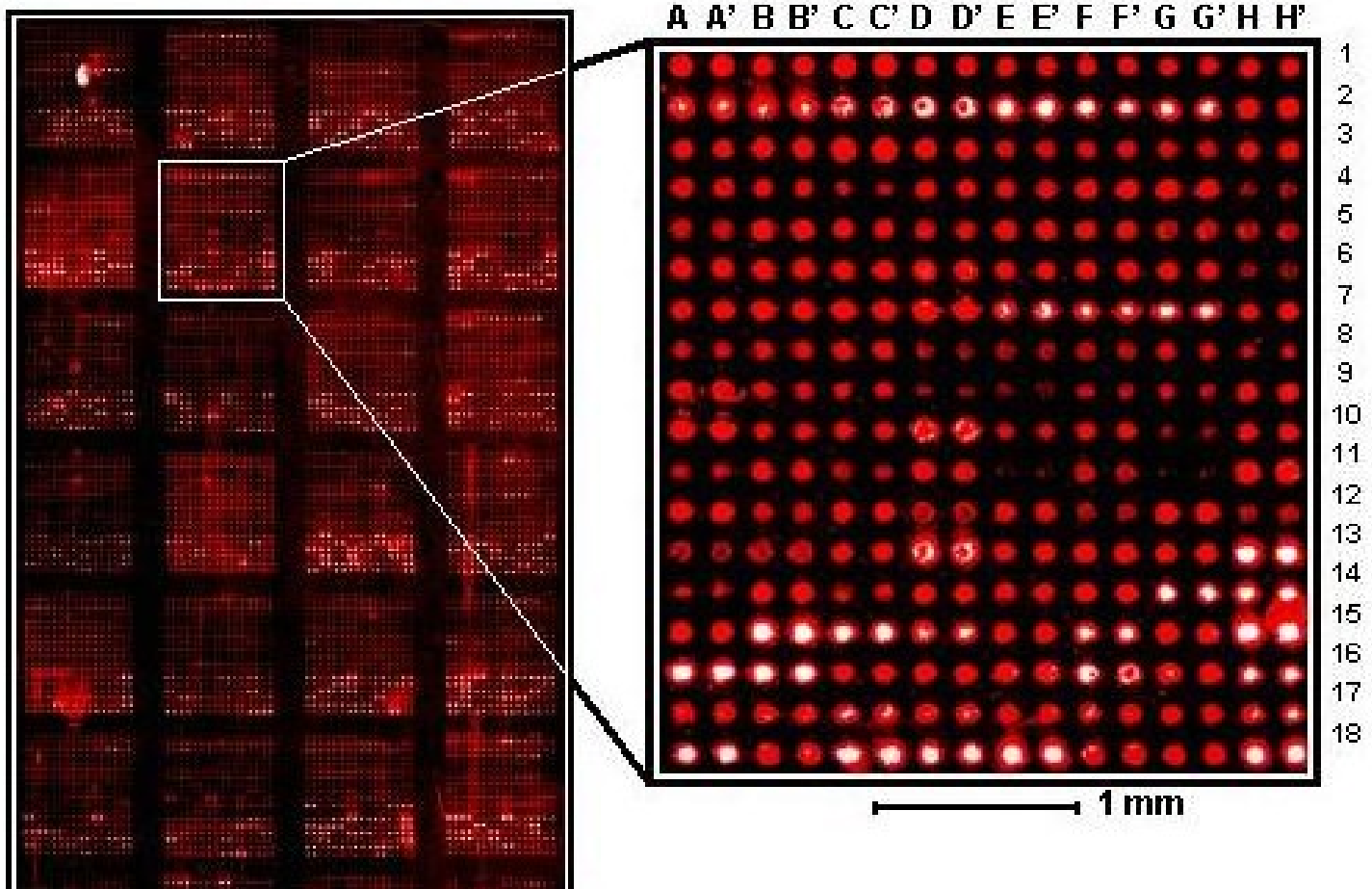


Ligand



Detekce: SELDI MS, fluorescence, SPR, electrochemická, radioaktivita,

# Anti-GST Probe



# Blotting

Side View:

Before Transfer:

(+) electrode



(-) electrode

bands in gel

nitrocellulose sheet



Direction of  
Transfer  
(electric field)

After Transfer:

(+) electrode

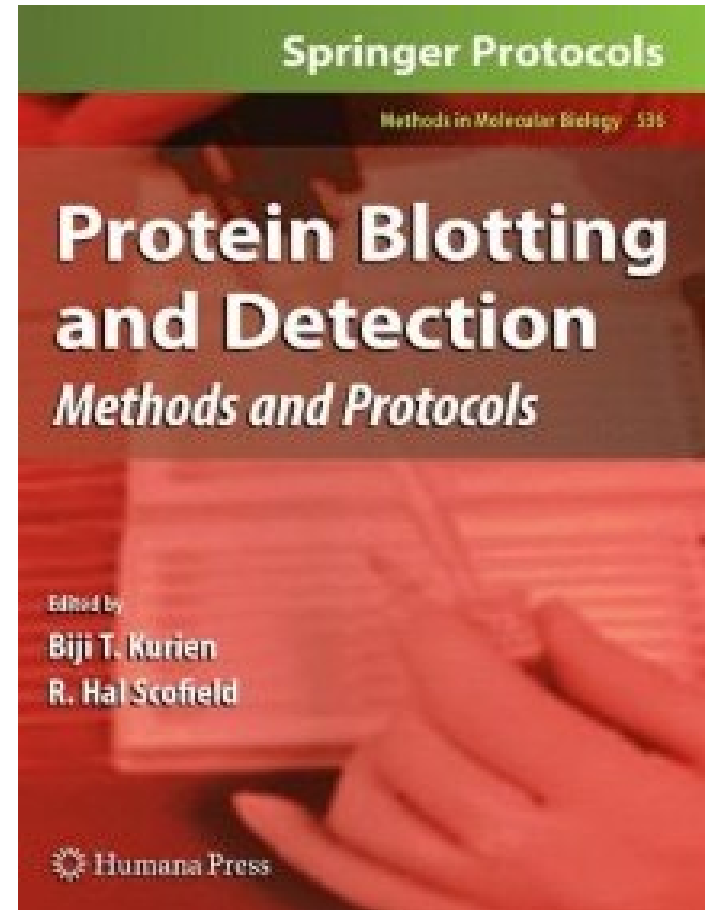
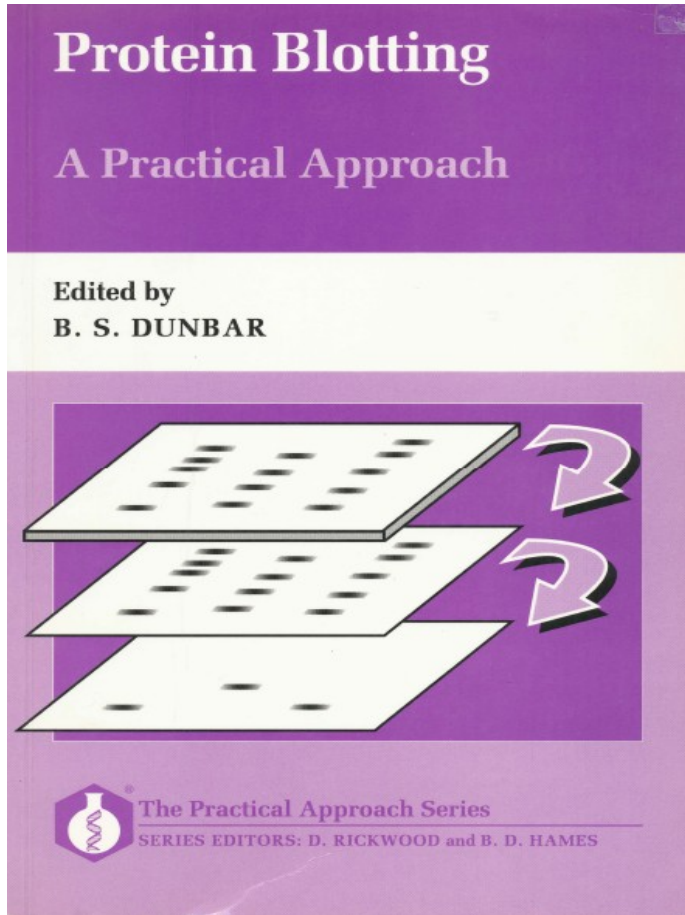


(-) electrode

bands on  
nitrocellulose

Note: All the layers are pressed tightly together.

# Blotting



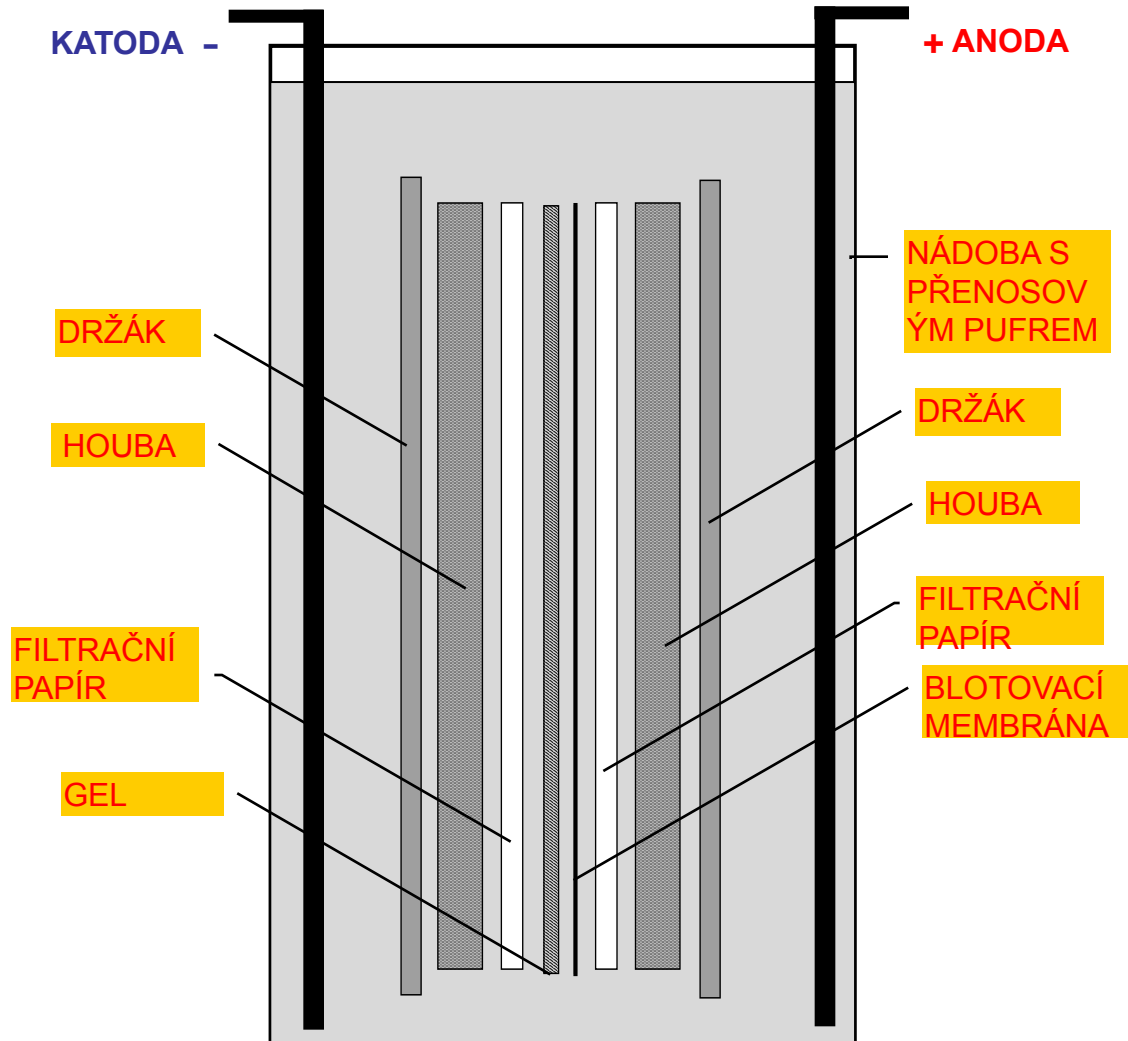
# Blotting

- Southern – DNA
- Northern – RNA
- Western - bílkoviny

# Výhody blottingu

- Dostupnost biomakromolekul
- Zakoncentrování biomakromolekul
- Redukce doby a množství potřebných chemikálií
- Imobilizace biomakromolekul – možnost uchovávání
- Možnost vícenásobné detekce
- Mechanická stabilita

# Tankový elektroblotting





# Tankový elektroblotting

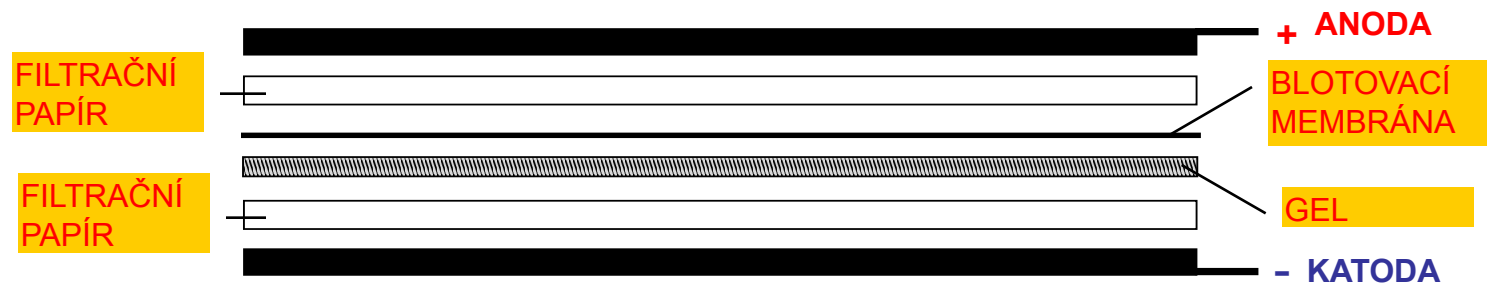


# Tankový elektroblotting

## Mini Protean Trans Blot Cell



# „Semi dry“ blotting



# „Semi dry“ blotting



# Membrány

- nitrocelulosa - nejběžnější
- polyviniliden difluorid – vysoká vazebná kapacita
- diazobenzoyloxymethyl – chemická aktivace
- ionexové membrány - preparativní
- aktivovaná skleněná vlákna – pro přímou sekvenaci

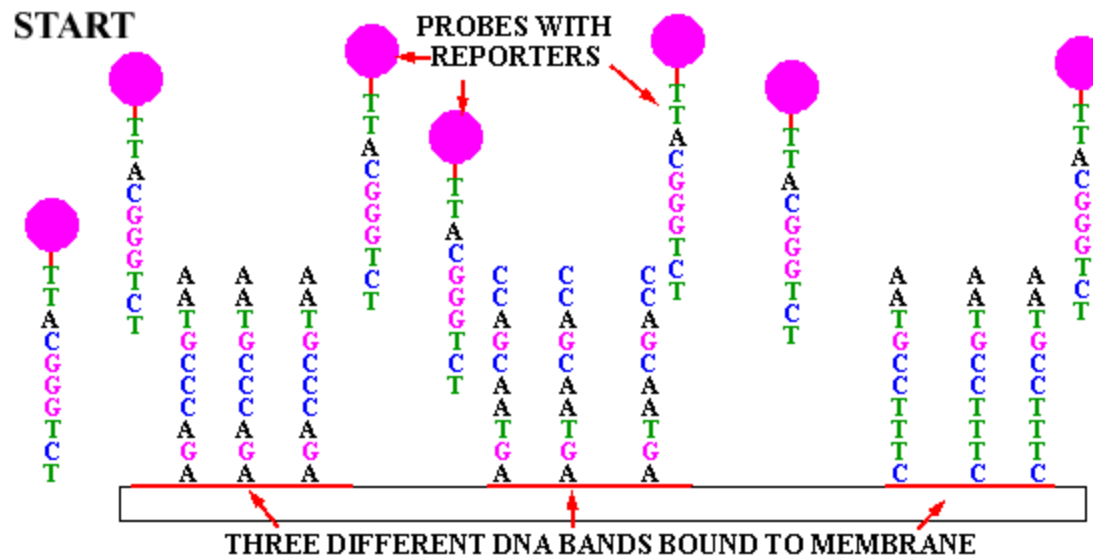
# Detekce

DNA

HYBRIDIZACE

- radioaktivní proba – vysoká senzitivita, Southern blot
- neradioaktivní proba – biotin – streptavidin, dioxigenin

# Detekce DNA



# Detekce

## Proteiny

REAKCE SE SUBSTRÁTEM    nativní enzym, nedifundující substrát

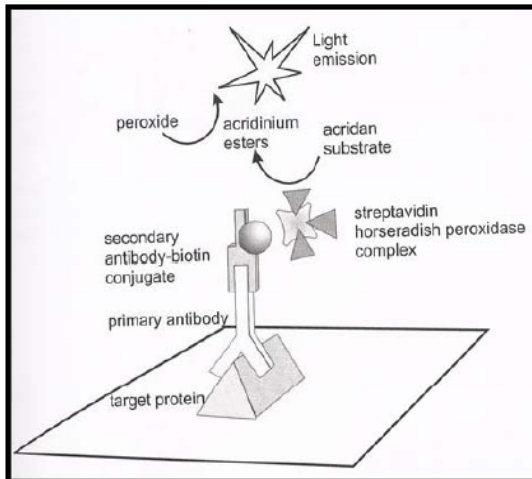
“IMUNOBLOTTING“

$^{125}\text{I}$ -protein A

enzymem značená sekundární protilátka – konjugace s peroxidasou (tetrazoliová sůl), **alkalickou** fosfatasou

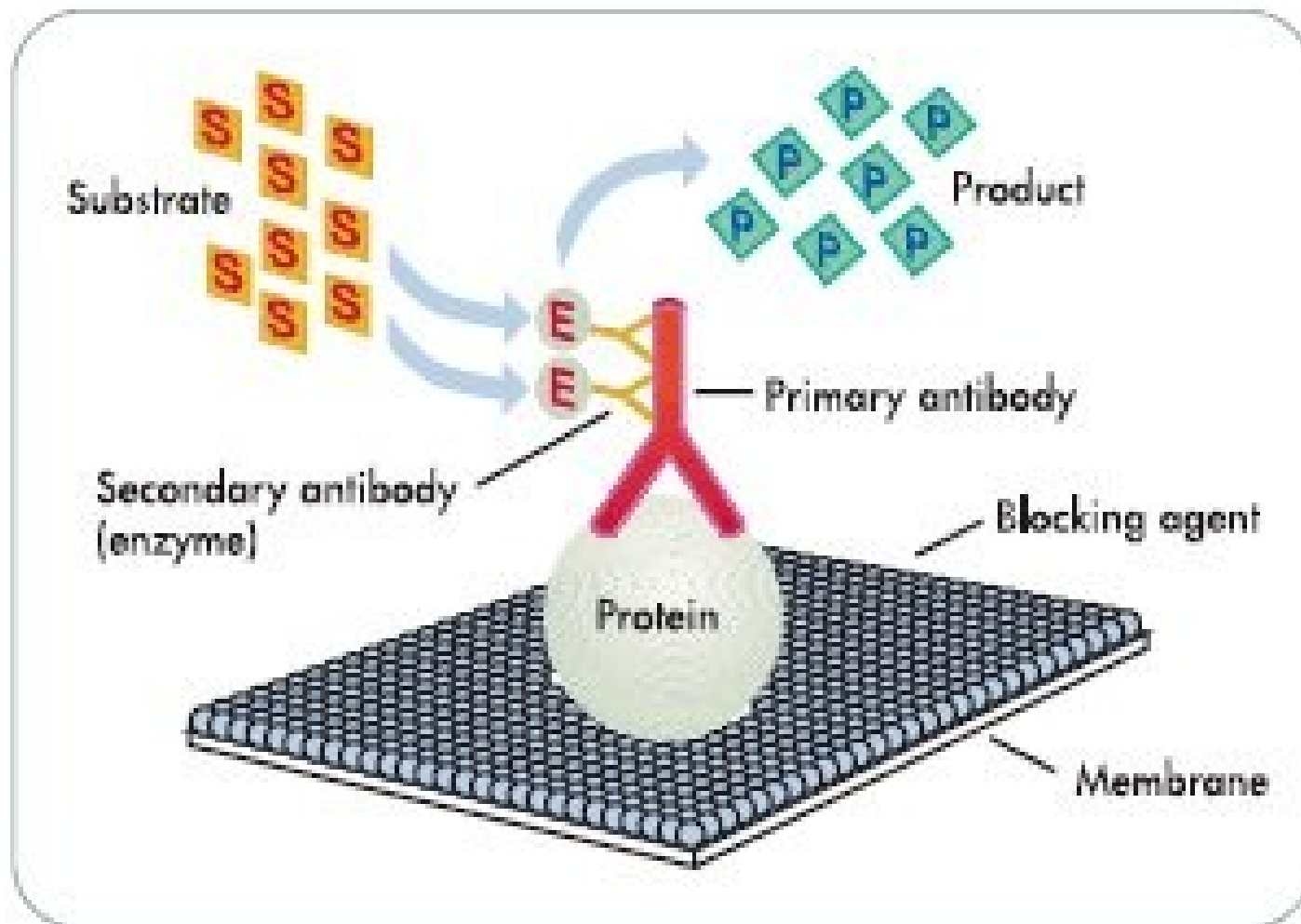
zlatem značená sekundární protilátka (100 pg)

chemiluminiscence – nejcitlivější





# Detekce proteinů



# Izolace nukleových kyselin

# Cíl izolace

- Odstranění proteinů
- DNA vs RNA
- izolace specifického typu NK

# Typy NK

- genomická (chromosomální)
- organelová (mitochondrie, chloroplasty)
- plasmidy (extra-chromosomální)
- virová (ds nebo ss)
- komplementární (mRNA)

# Nejpoužívanější metody

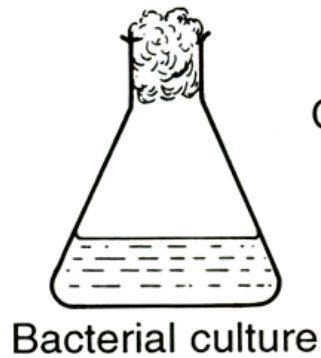
- na základě rozdílné rozpustnosti – extrakce, srážení
- na základě vlastností - chromatografie – polarita-adsorpční, náboj-ionexová
- sedimentace - gradientová ultracentrifugace

# Postup

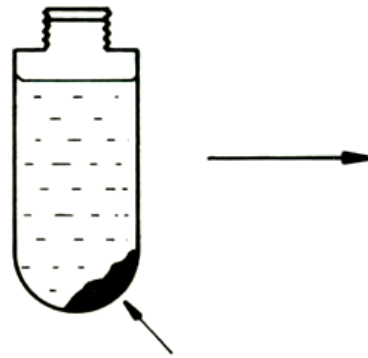
1. Rozbití buněk a membrán pro uvolnění NK
2. Inaktivace DNA- nebo RNA-degradujících enzymů (DNasy, RNasy).
3. Separace NK od dalších komponent uvolněných z buňky.
  - Extrakce/Precipitace
  - Chromatografie
  - Ultracentrifugace

Extrakce/Precipitace

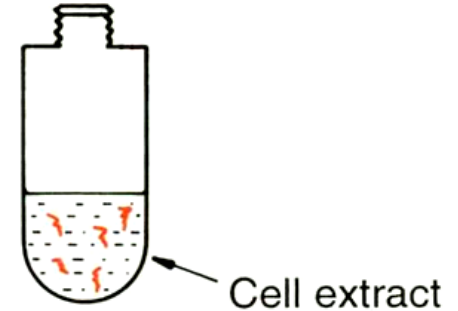
**1 A culture of bacteria is grown and then harvested**



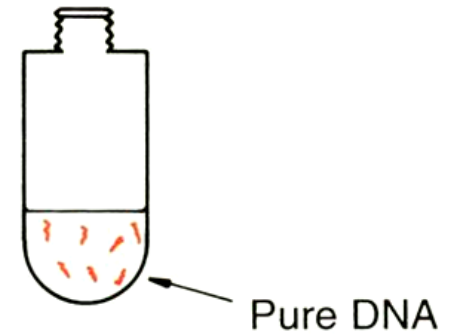
Centrifugation



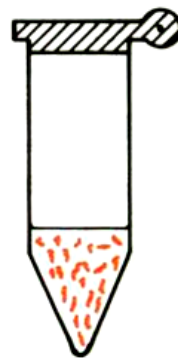
**2 The cells are removed and broken to give a cell extract**



Organic extraction



**4 The DNA is concentrated**



**3 The DNA is purified from the cell extract**



# Izolace genomické DNA

## Typická procedura

*1. Sklizení buněk*

*2. Lyse buněk*

- 0.5% SDS + proteinase K  
(55° několik hodin)

*3. Fenolová extrakce*

- Jemné třepání několik hodin  
(pH 8)

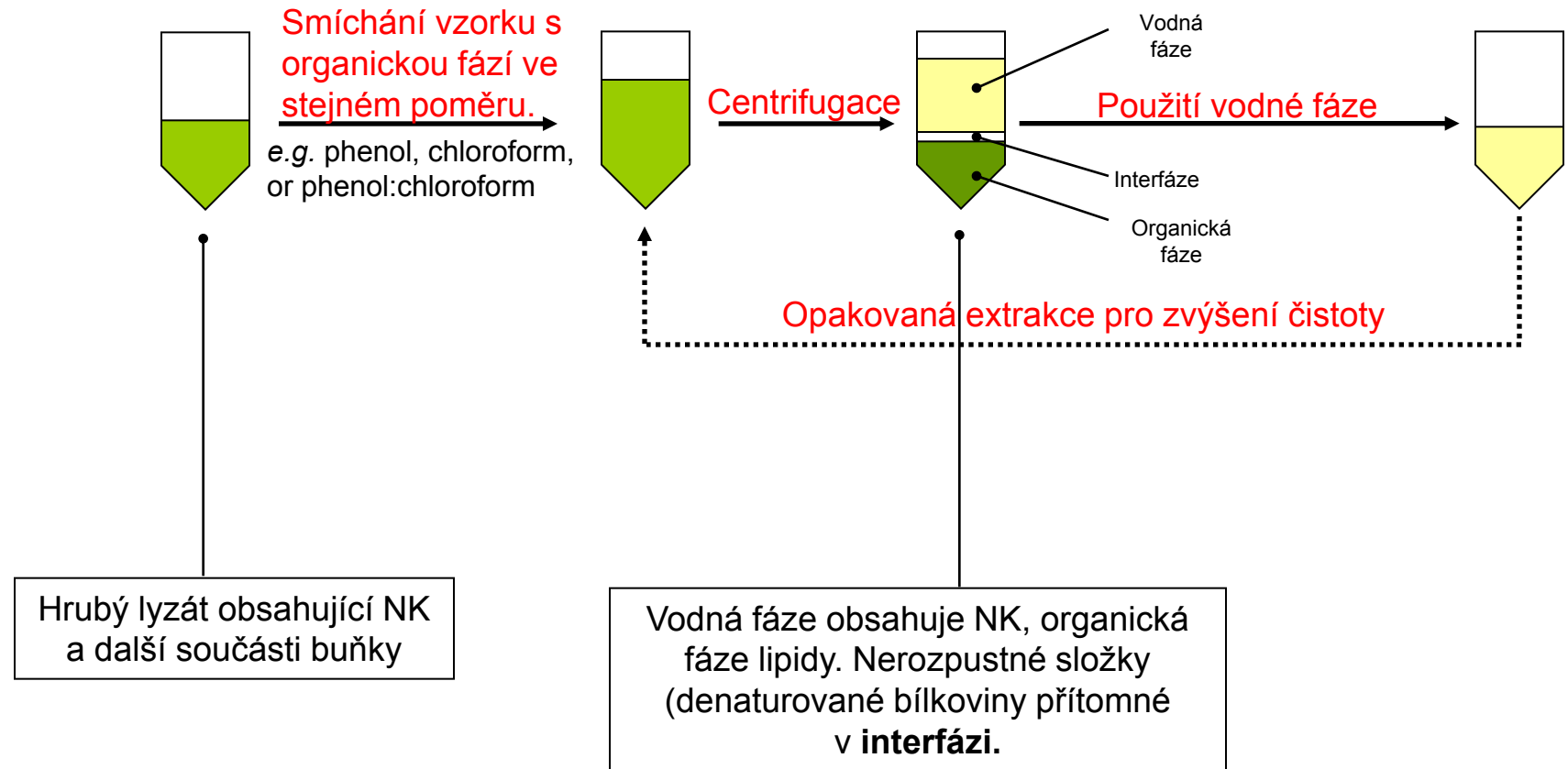
*4. Ethanolová precipitace*

*5. Působení RNAsy a proteinasy K*

*6. Opakování kroku 3 a 4.*

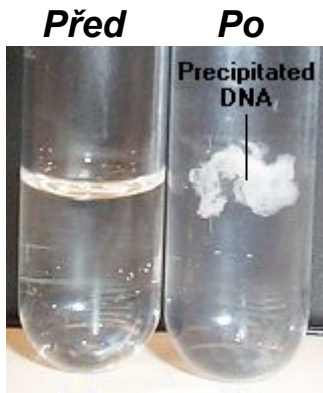
# Extrakce/Precipitace

## Krok 3: Organická extrakce



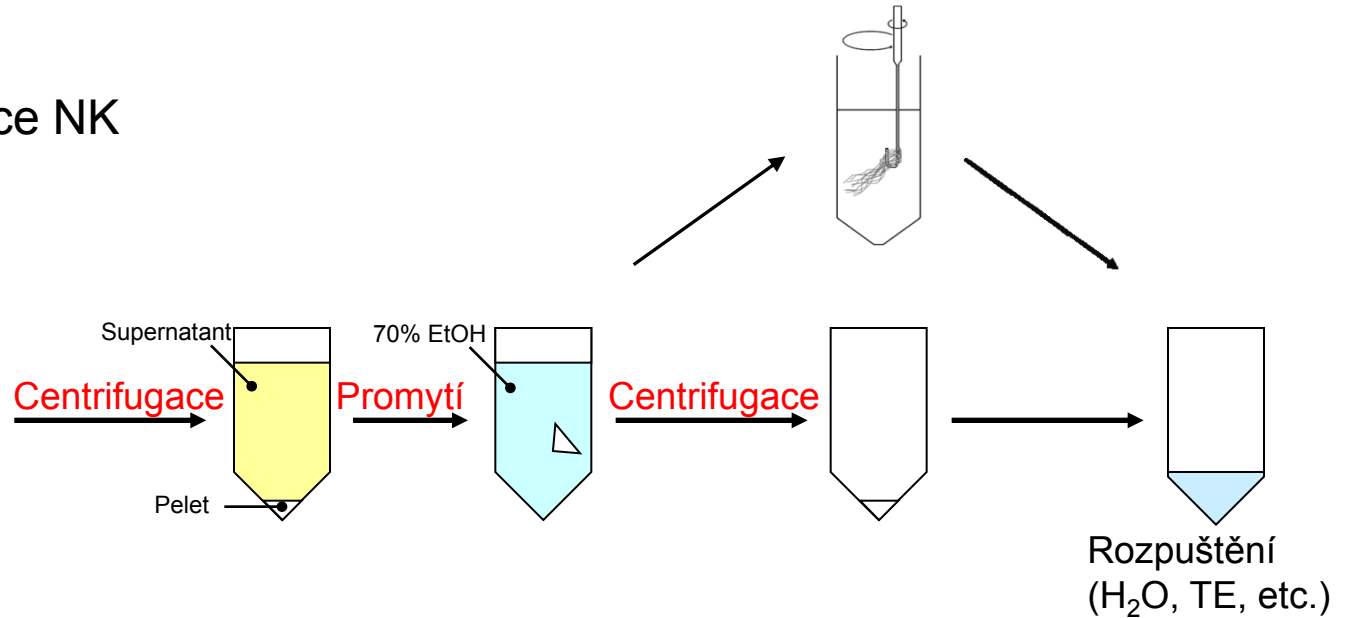
# Extrakce/Precipitace

## Krok 4: Precipitace NK



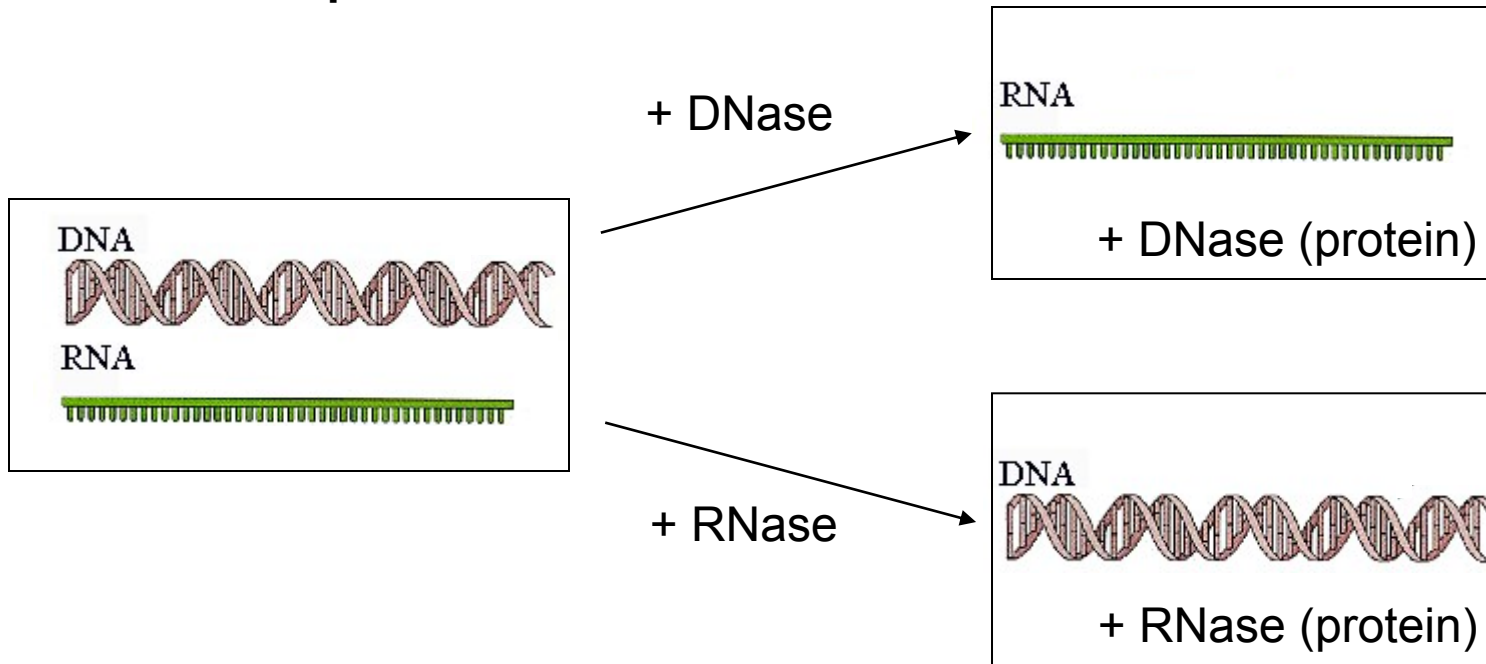
Přidání EtOH a soli

- 2-2,5 objem EtOH
- -20° C
- Vysoká I
- pH 5-5.5



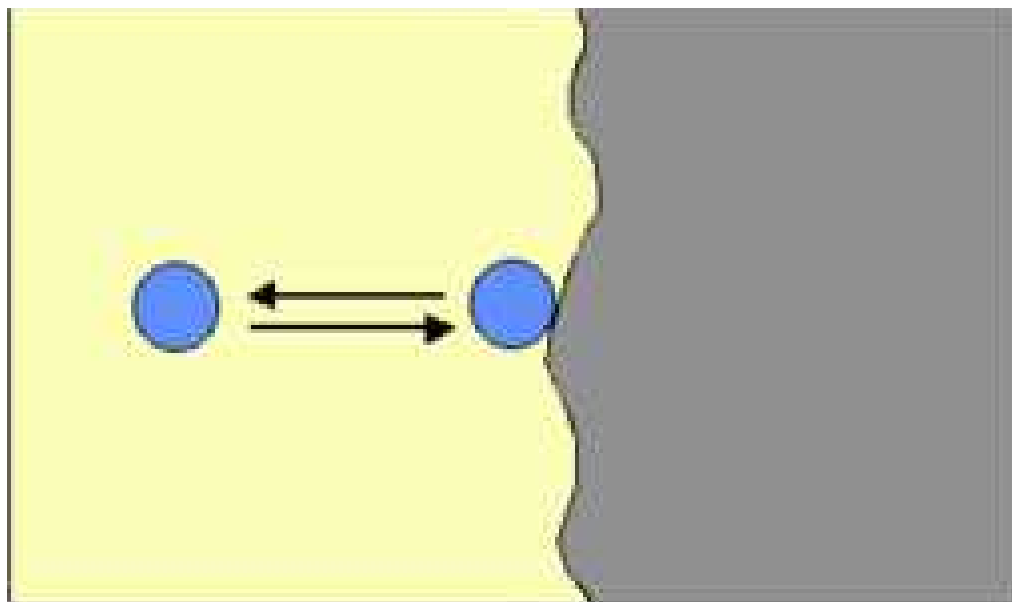
## Detail kroku 5

### Použití nukleas pro odstranění nechtěné DNA nebo RNA



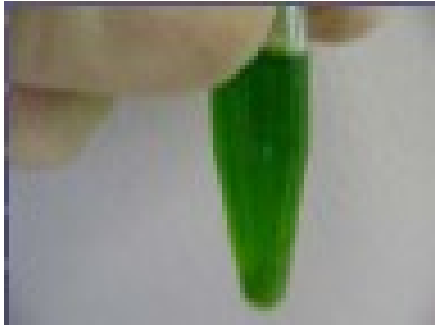
# Chromatografie

# Adsorpční chromatografie



# Adsorpční chromatografie

## Krok 1: Příprava lyzátu

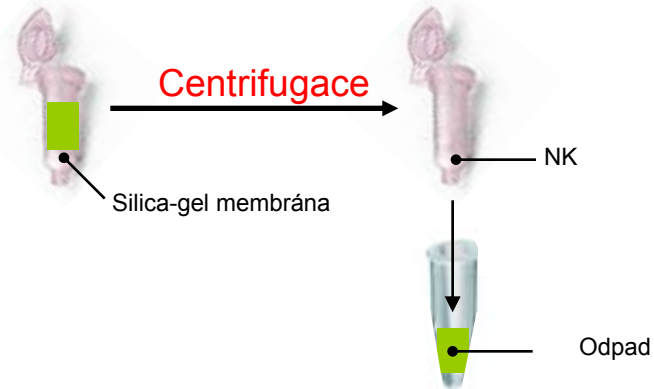


Extrakční pufr pro vazbu DNA a RNA na silikagel:

- nízké pH
- vysoká iontová síla
- chaotropní soli

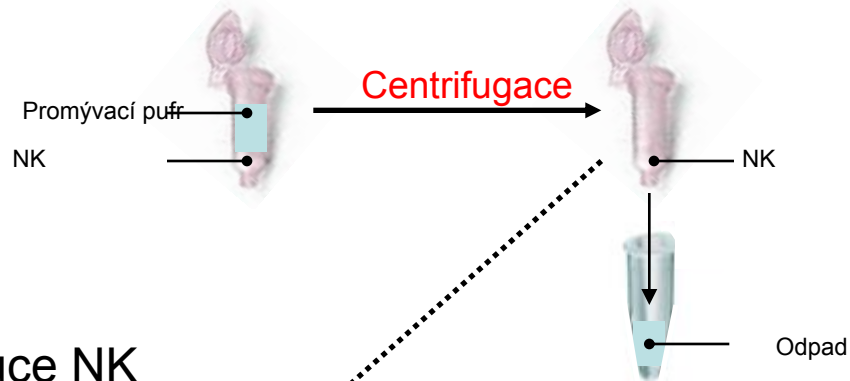
Aplikace na  
kolonku

## Krok 2: Adsorpce na silikagel

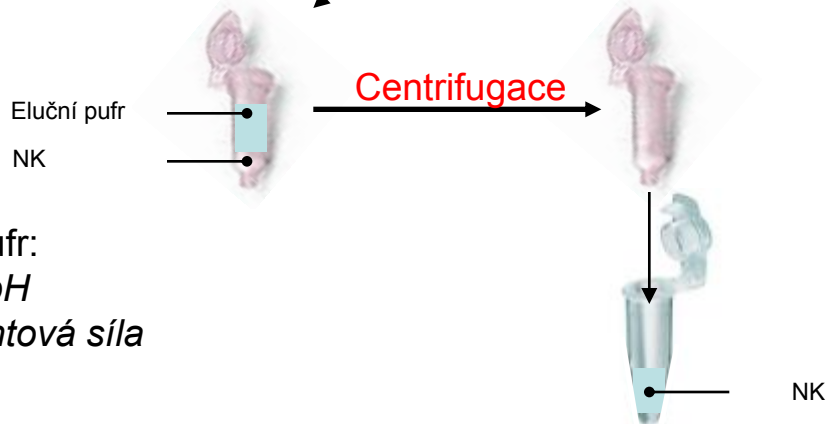


# Adsorpční chromatografie

Krok 3: Vymytí kontaminant



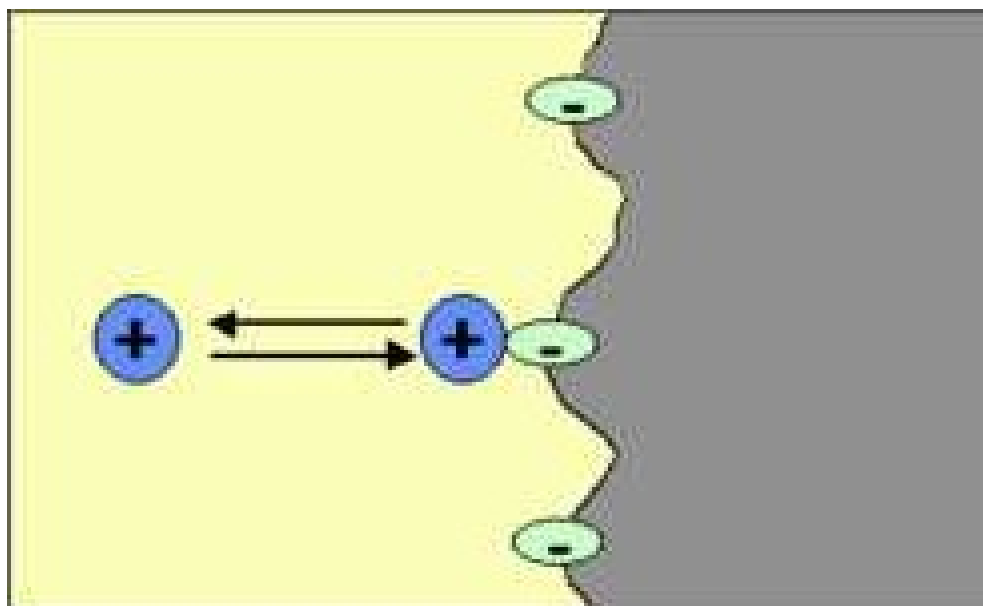
Krok 4: Eluce NK



Eluční pufr:  
*Vysoké pH*  
*Nízká iontová síla*

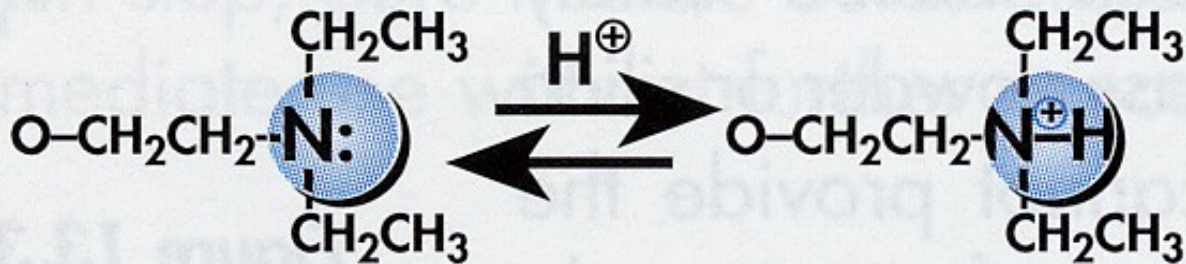


# Ionexová chromatografie



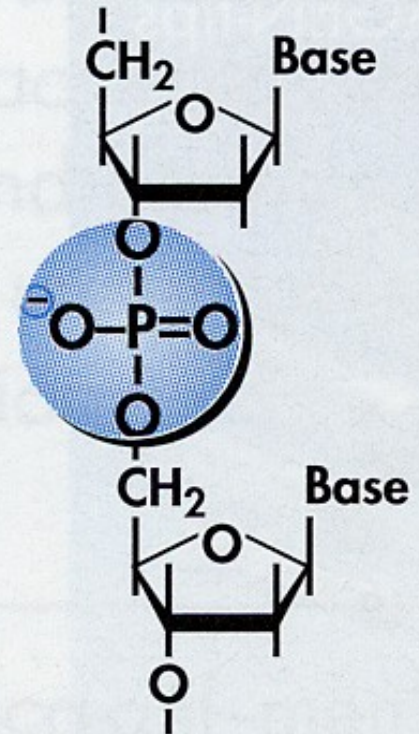
# Ionexová chromatografie

Vazba při nízkém pH nízké I



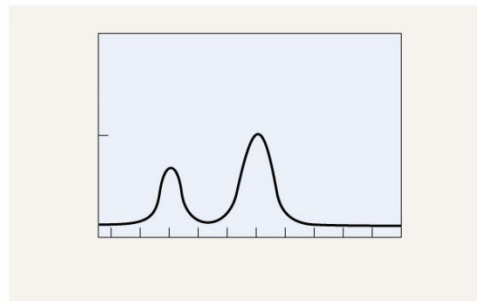
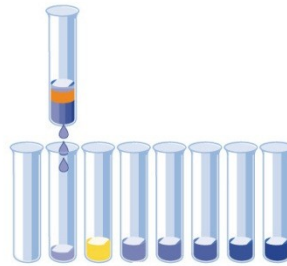
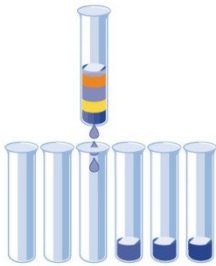
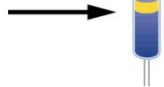
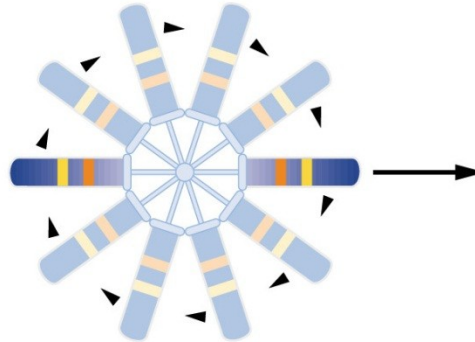
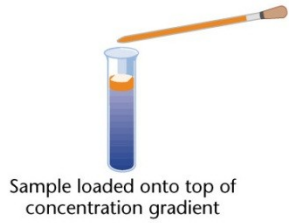
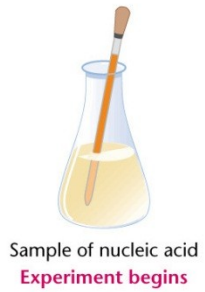
Eluce zvýšením pH nebo vysokou I

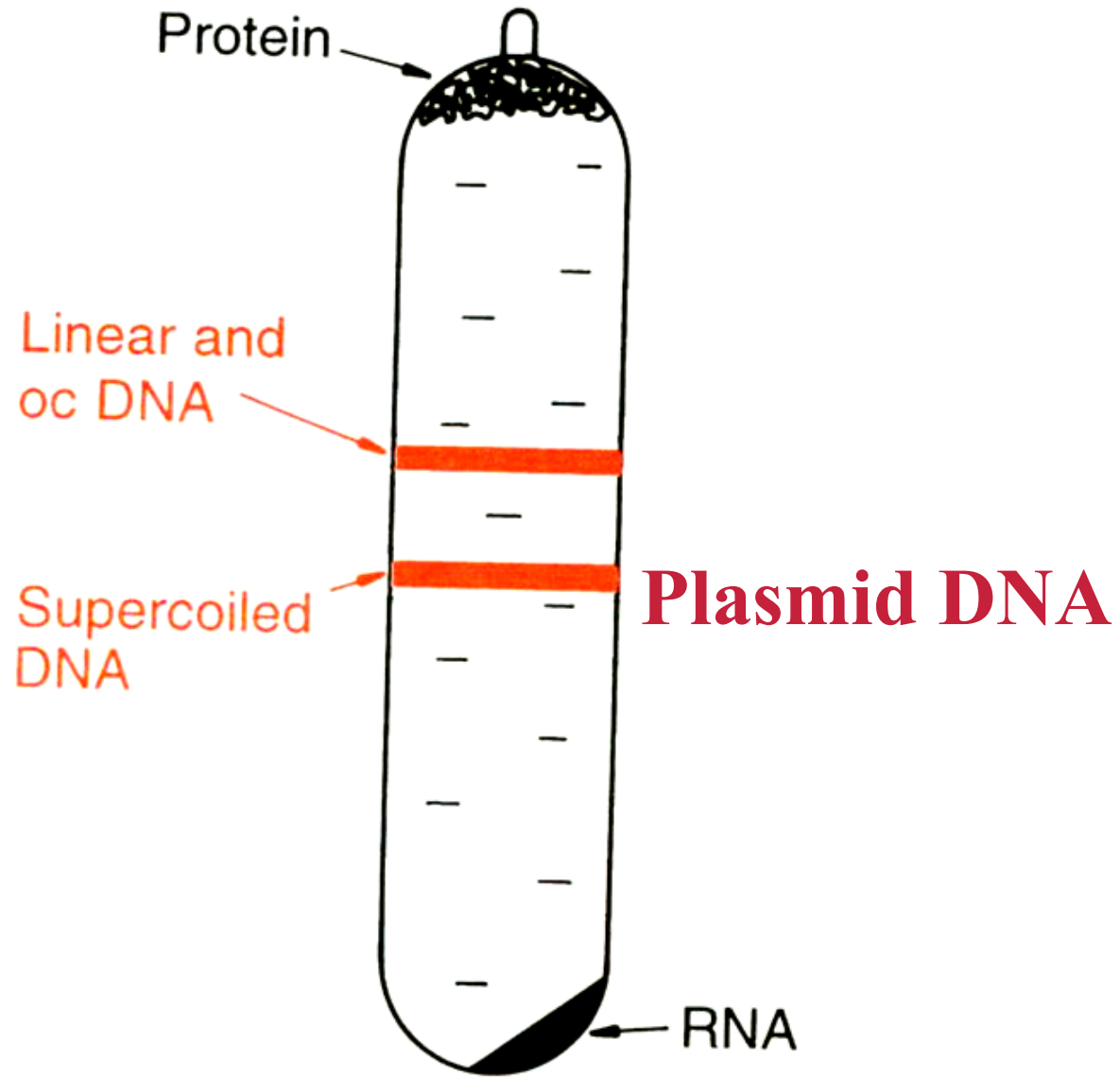
DEAE (diethylaminoethanol)



Chemical structure  
of DNA

# Separation of Nucleic Acids by CeCl Gradient Centrifugation

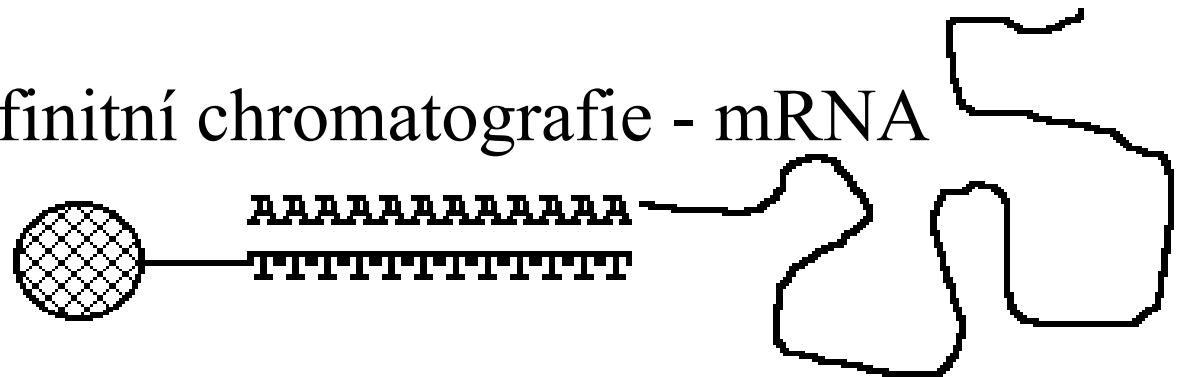




(a) An EtBr-CsCl density gradient

# Izolace RNA - speciální přístupy

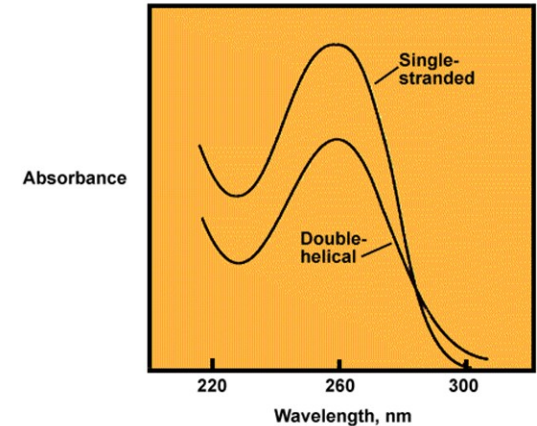
- nutno použít inhibitory RNAsy
- extrakce guanidium chloridem
- fenolová extrakce při  $\text{pH} < 4$  ( $\text{pH} 8$  pro DNA)
- působení RNase-free Dnase
- selektivní precipitace rRNA, mRNA s LiCl
- oligo-dT afinitní chromatografie - mRNA



# Kontrola čistoty a kvantifikace NK

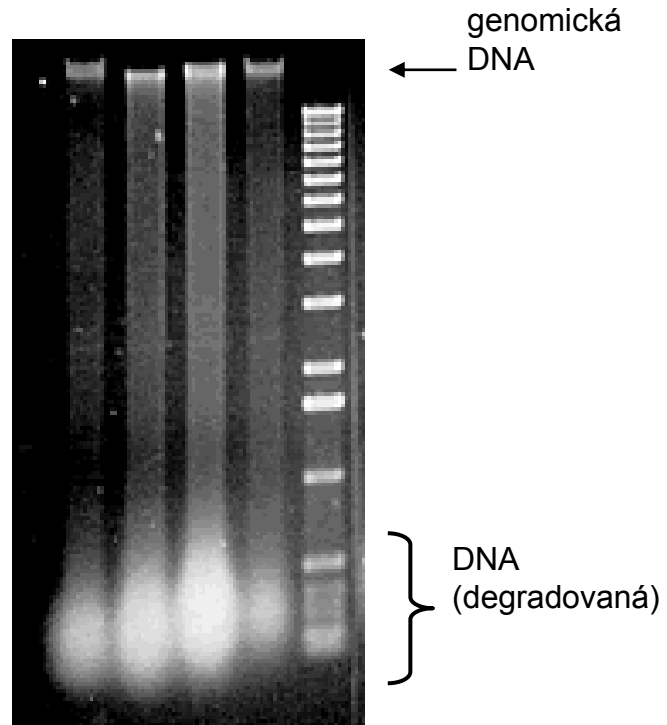
# Kontrola NK

- spektrofotometricky
  - kvalita
  - kvantita
- gelová elektroforéza
  - kvalita



DNA	$A_{260}$	$1.0 \approx 50 \mu\text{g/ml}_{\text{ds}} \approx 33 \mu\text{g/ml}_{\text{ss}}$
	$A_{260}/A_{280}$	1.6 - 1.8
RNA	$A_{260}$	$1.0 \approx 40 \mu\text{g/ml}$
	$A_{260}/A_{280}$	$\sim 2.0$

# Kontrola degradace: DNA





# Kontrola degradace: RNA

