Predavanje GIO 3

KAKO MIJENJATI GENE?

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Naučeno iz prirode:

- Stanice s mutator genima = mutator sojevi (1% u bakterijskoj populaciji)
- Indukcija SOS odgovora
- Indukcija rearanžmana u bakterijskom genomu uz Tn

Sve gore navedeno događa se u GASP mutantima = adaptivna evolucija!

- Indukcija točkastih mutacija kemijskim ili fizičkim mutagenim sredstvima

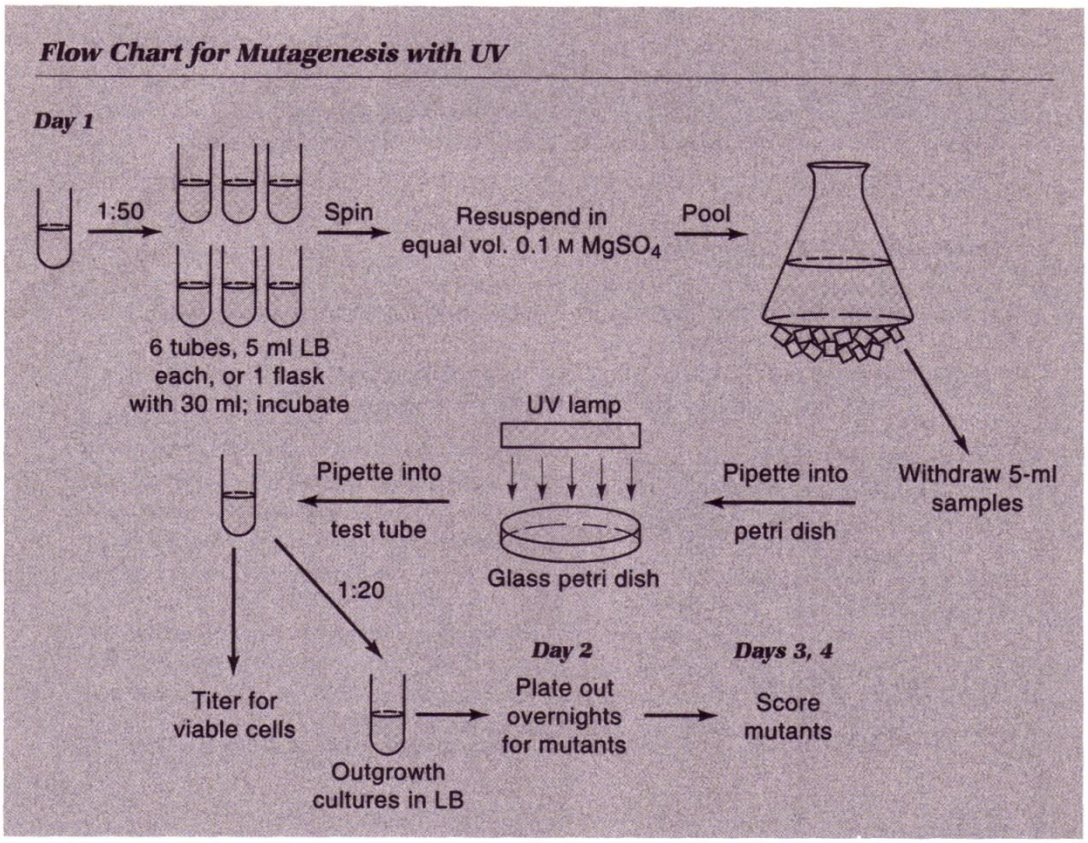
Klasične metode:

Kemijska i / ili fizička mutagena sredstava

- nekada jedina metoda i vrlo uspješna
- nedostaci: mutacije u željenom genu rijetke, mutacije slučajne na raznim mjestima, nedefinirane promjene (delecije, supstitucije, insercije)
- Kao u prirodi: dobro ili loše???

Mutageneza uz UV:

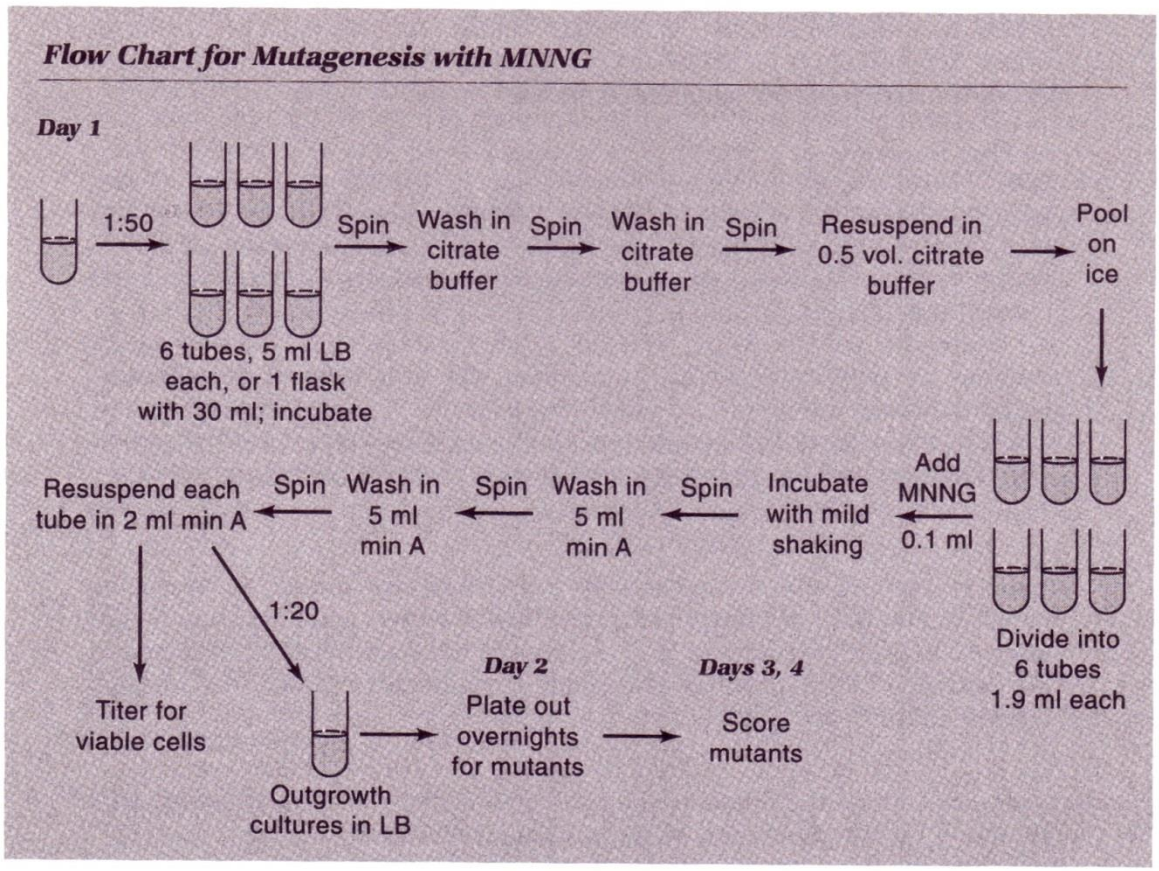
PROCEDURE



Slika 1. Primjena mutagenih svojstava UV svjetla

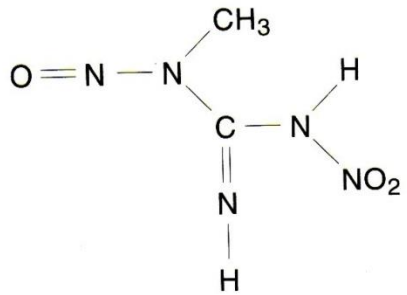
Mutageneza pomoću MNNG

PROCEDURE



Slika 2. korištenje alkilirajućeg agensa

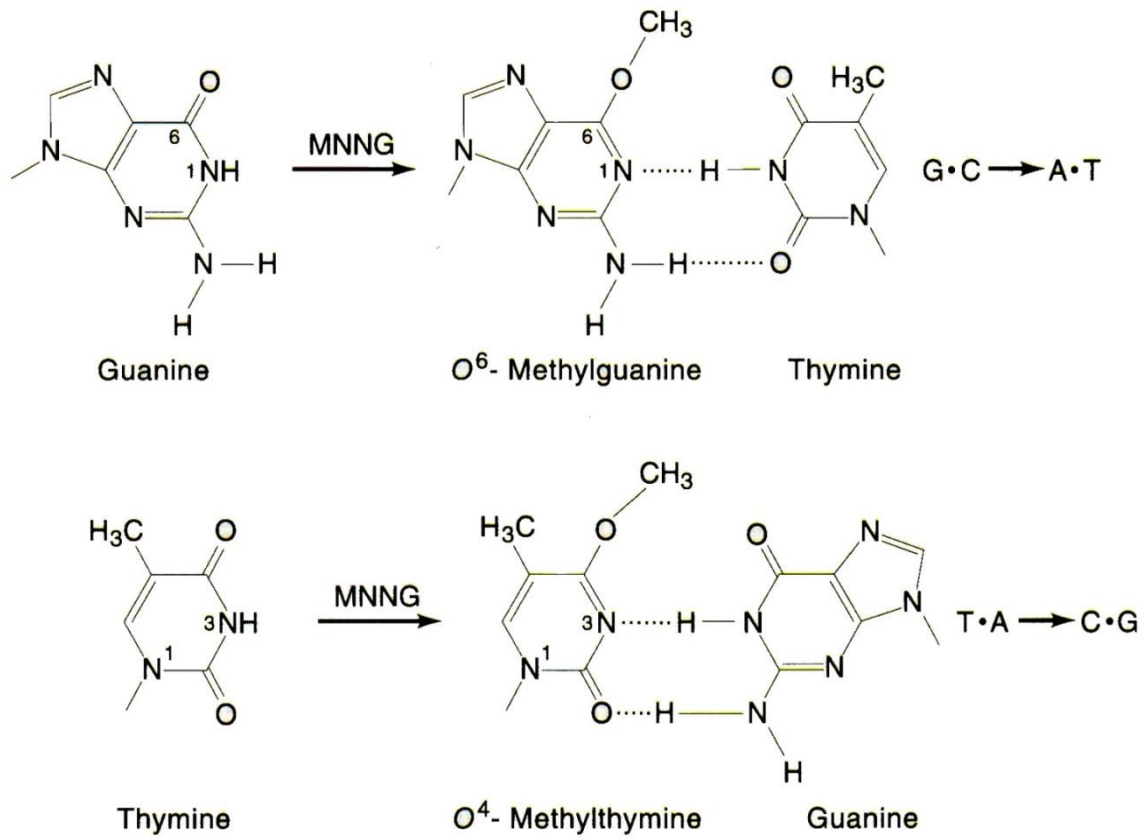
Kemiski mutagen MNNG



MNNG

Structure of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)

Slika 3. Kemijska struktura MNNG



Mutagenic Consequences of Alkylating Agents Alkylation, in this case methylation, of the O^6 position of guanine and the O^4 position of thymine results in direct mispairing with thymine or guanine, respectively.

Slika 4. Posljedice djelovanja MNNG

Kemijski mutageni i mutatori:

Tablica 1. Najčešće korišteni kemijski mutageni

Table 4.2 Mutagens Commonly Used in *E. coli*

Mutagen	Specificity	Mechanism	Additional advantages	Disadvantages
MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine)	Principally G:C→A:T transitions	Generates O^6-methylguanine	Very powerful mutagen	Dangerous to handle; frequent secondary mutations
EMS (ethylmethane sulfonate)	Principally G:C→A:T transitions	Generates O^6-ethylguanine	Powerful mutagen	Dangerous to handle; some secondary mutations
UV (ultraviolet) irradiation	All base substitutions, although favors G:C→A:T transitions; frequent hot spots; also induces frameshifts, deletions, and rearrangements	Generates photoproducts that require SOS bypass		High amount of killing required (relative to EMS) for mutagenesis; not a powerful mutagen; certain strains too sensitive
BPDE (benzo[a]pyrene diolepoxide)	Principally G:C→T:A transversions; frameshifts	Generates adducts that require SOS bypass; may stimulate depurination		Extremely dangerous to handle and difficult to obtain
2AP (2-aminopurine)	A:T→G:C and G:C→A:T transitions	Acts as a base analog	Safe and easy to use; works well on *recA* strains	Relatively weak mutagen
ICR 191	Frameshifts, mainly additions and deletions at monotonous runs of G (or C)	Probably stabilizes looped out bases by stacking between them	Causes only frameshifts, which are usually nonleaky	Some strains too sensitive
5AZ (5-azacytidine)	G:C→C:G transversions			Weak mutagen
NH_2OH (hydroxylamine)	G:C→A:T transitions when used in vitro	Reacts with cytosine to generate N^4-hydroxycytosine	Useful for treatment of phage or plasmid DNA in vitro; can be powerful mutagen under these conditions	Causes only one type of base change; more laborious to use than many mutagens

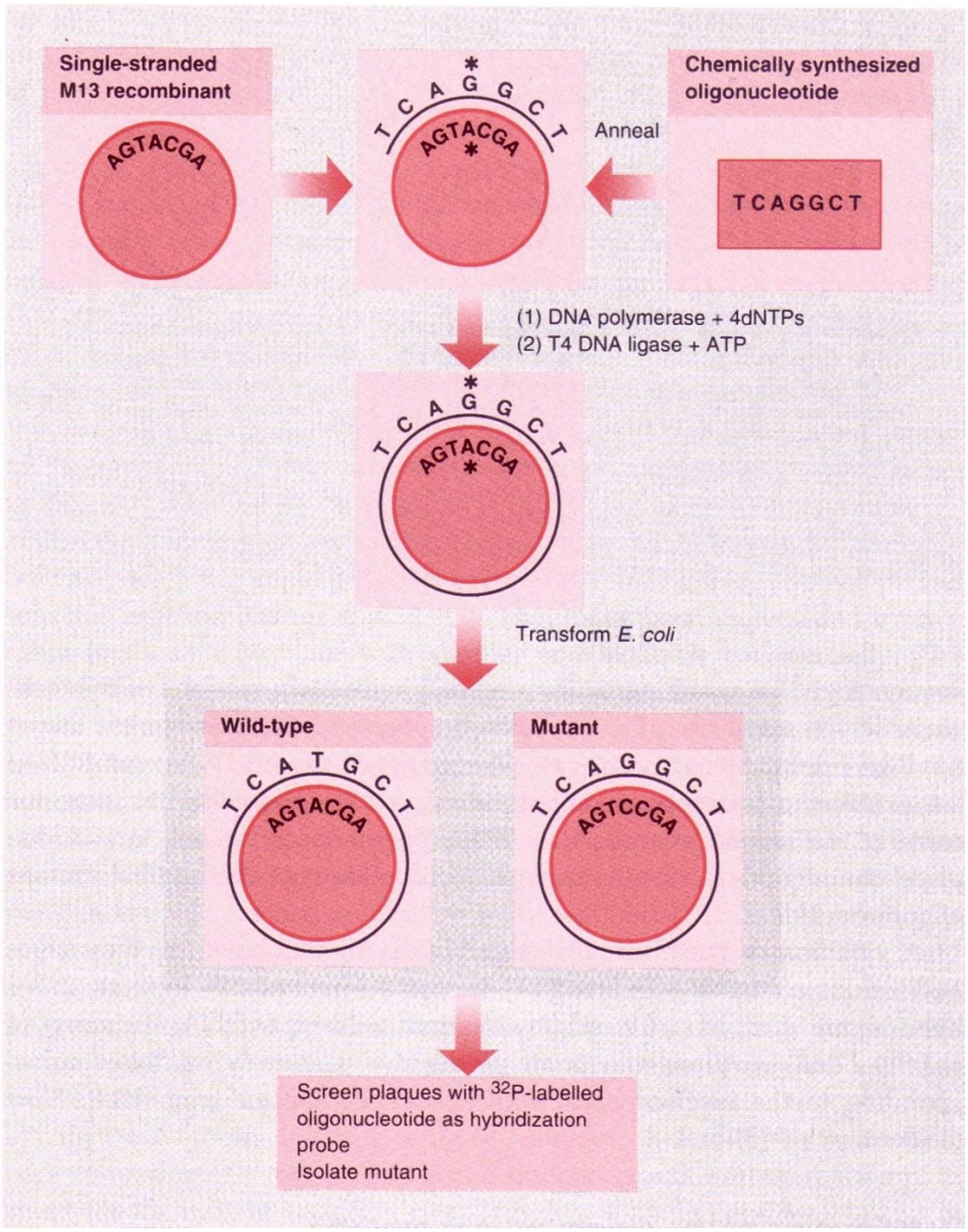
Tablica 2. Kemijski mutageni i mutatore geni

Nitrous acid	Principally transitions, deletions			High amount of killing required for good mutagenesis
Sodium bisulfite	G:C→A:T transitions		Can be used in vitro	Weak mutagen
NQO (4-nitroquinoline-1-oxide)	G:C→A:T transitions, and to a lesser extent G:C→T:A transversions; some frameshifts	Makes adducts that require SOS bypass		Extremely dangerous to handle
Mutator genes				
Nonspecific				
mutD	All base substitutions, frameshifts	Lacks editing function for DNA replication	No treatment required; convenient for phage and plasmids	Genetic construction required for chromosomal mutations; must move mutator out after use or move phage or plasmid
Specific				
mutT	A:T→C:G transversions	Lack different repair systems (see Table 4.3)	No treatment required	Not as strong as *mutD*; requires strain construction
mutY, mutM	G:C→T:A transversions			
mutH, mutL, mutS, uvrD (mutU)	A:T→G:C and G:C→A:T transitions; frameshifts			
mutY mutM (double)	G:C→T:A transversions	Inability to repair 8-oxodG lesions and mispairs	Very powerful (as strong as *mutD*)	Requires strain construction
Transposable elements	Insertions; can be used for deletions and other rearrangements		Generate nonleaky mutations; mutations are often associated with antibiotic resistance markers to facilitate mapping and cloning	Will not result in missense changes; some inserts are lethal; requires some genetic expertise
Spontaneous (no mutagen)	All base substitutions, frameshifts, deletions, insertions		Wide spectrum of mutations; ease of application; no secondary mutations	Low levels of mutants; many siblings in each culture

See Table 4.3 for references.

1. Mutageneza kazetom (eng. "cassette mutagenesis")

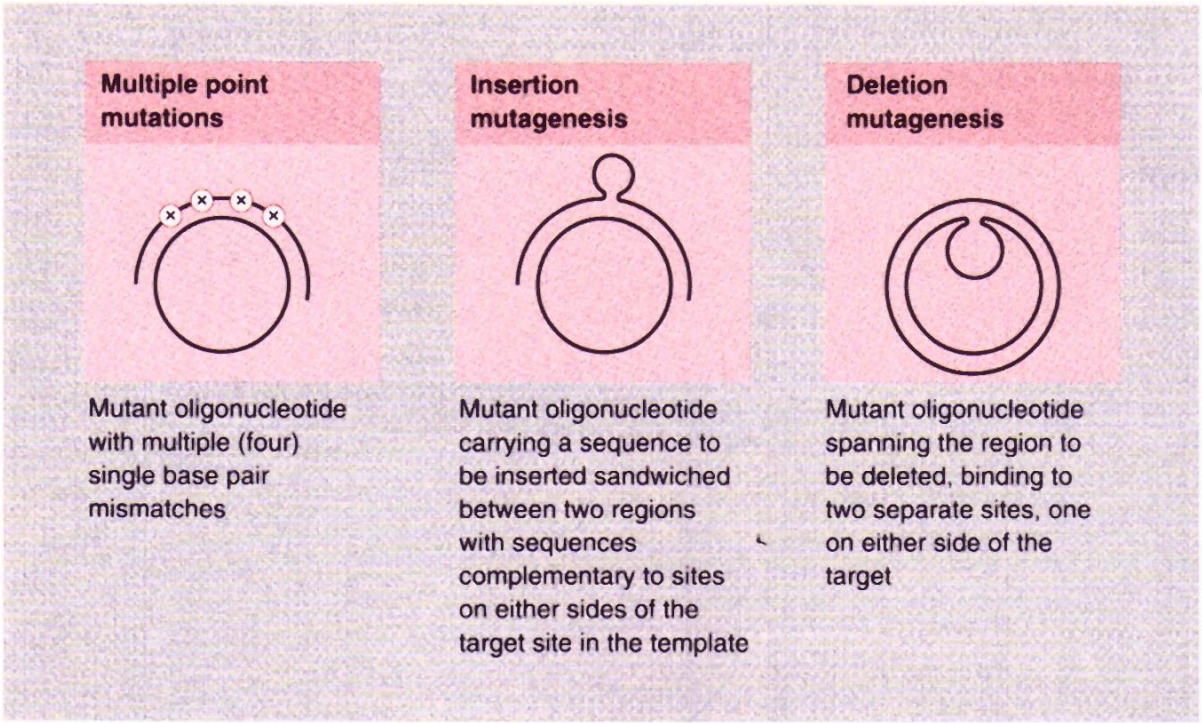
- sintetizirani DNA fragment sadrži željenu mutiranu sekvenciju koja se umetne umjesto postojećeg gena divljeg tipa (efikasnost mutageneze oko 100%):
- Nedostatak: potrebna jedinstvena restriktivna mjesta za uključivanje kazete; ograničeni broj sintetiziranih oligonukleotida što se može minimalizirati drugim metodama.



Slika 5. Mutageneza kazetom oligonukletida

B) Dodatak početnice (eng. "primer extension")

- povezivanje oligonukleotida (7-20 nukleotida), *in vitro* sintetiziranio, koji prozročuju nesparivanje (mismatch) na željenom mjestu te uz Klenow fragment DNA polimeraza sintetizira dlDNA;
- Početnica jlDNA: kloniranjem gena na bazi M13 vektora ili dlDNA vektor koji se prevede u jlDNA;
- Minimiziranje unošenja grešaka tijekom sinteze dlDNA korištenjem visoko preciznim DNA polimerazama iz T4 i T7.



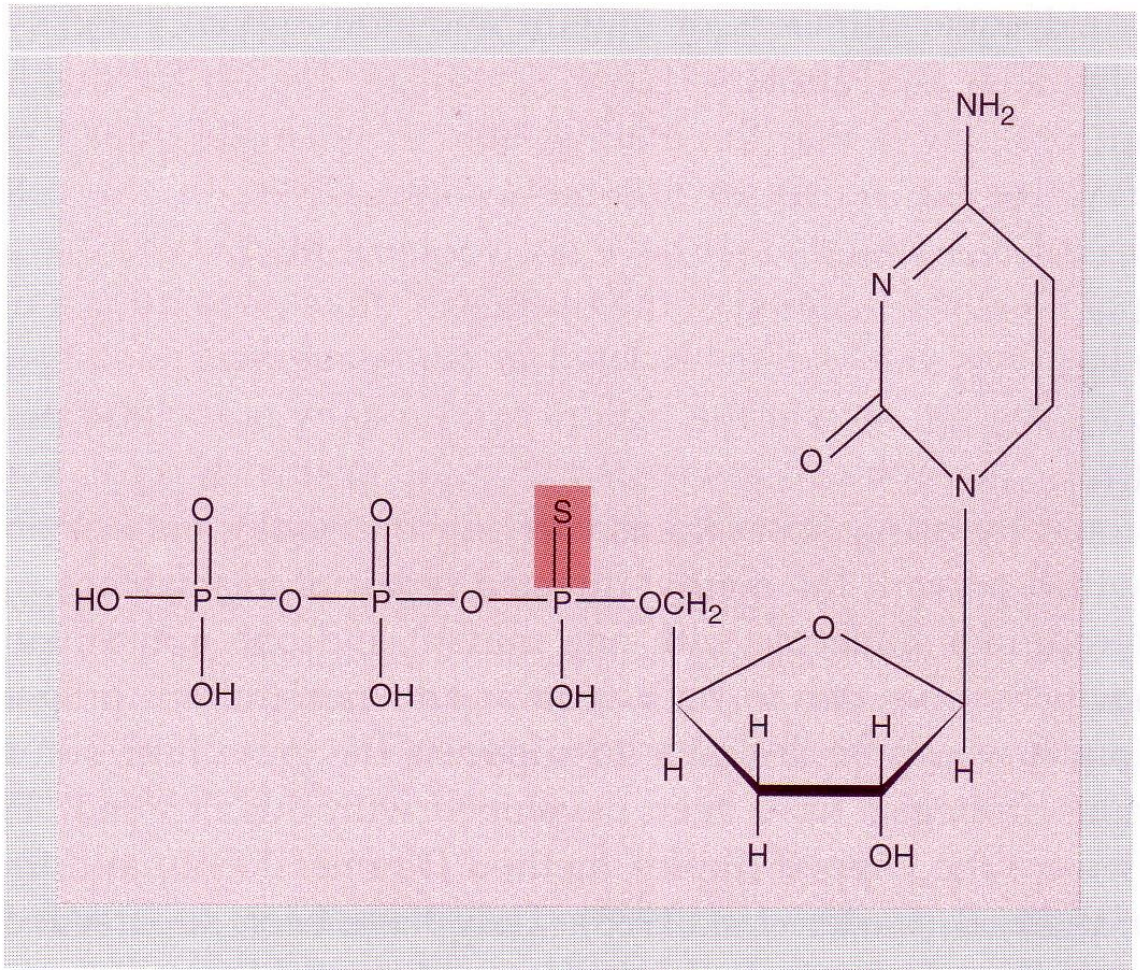
Slika 6. Mutageneza početnicom uz korištenje mutanata mismatch popravaka (*mutS*, *H*, *L*)

Nedostaci metode:

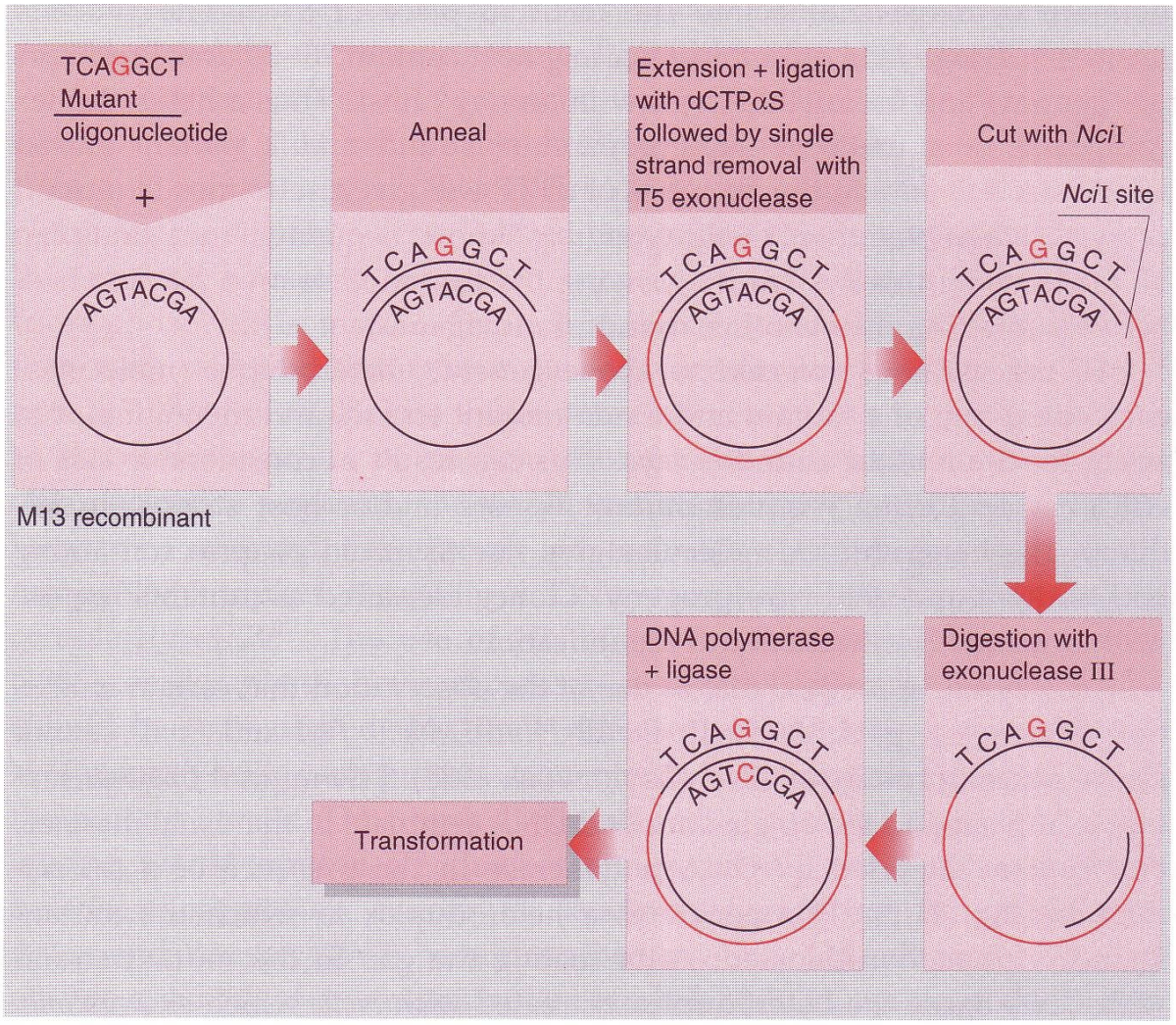
- Heterologne sintetizirane dlDNA molekule bit će kontaminirane preostalim nekopiranim jlDNA i djelomično dlDNA molekulama. Njihovo prisustvo značajno smanjuje udio mutiranih molekula te se moraju ukloniti centrifugiranjem u gradijentu saharaoze ili pomoću agarozne gel elektroforeze. Trošak vremena.

C) Dodatak početnice:

- Određeni restriktivnim enzimi ne režu prisutne sumporene nukleotide (alfa S) u sintetiziranom lancu (*Ava* I, *Ava* II, *Ban* II, *Hind* II, *Nci* I, *Pst* I, *Pvu* I)



Slika 7. Struktura sumpornog nukleotida dCTPαS (eng. “thionucleotide”)



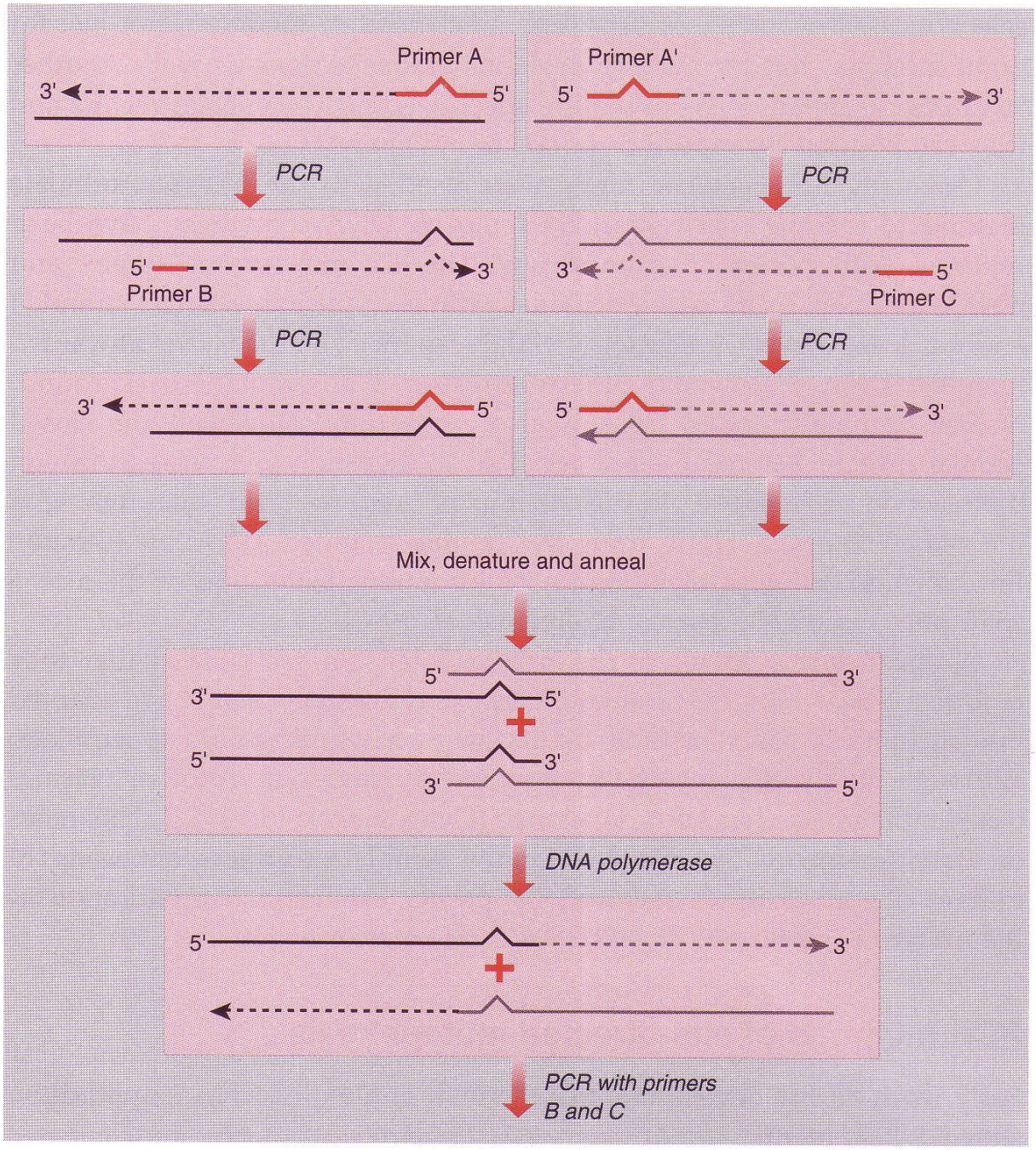
Slika 8. *In vitro* selekcija mutiranog lanca DNA, ne zahtjeva specijalnog domaćina ili vektor, može se više puta ponoviti.

3. Metode s PCR:

- Jedna nesparena baza u početnici bit će inkorporirana tijekom amplifikacije;
- Efikasnost 100 % ;
- Smeta slaba preciznost Taq polimeraze, postoje termostabilne DNA polimeraze s poboljšanom preciznošću.

Mutageneza PCR:

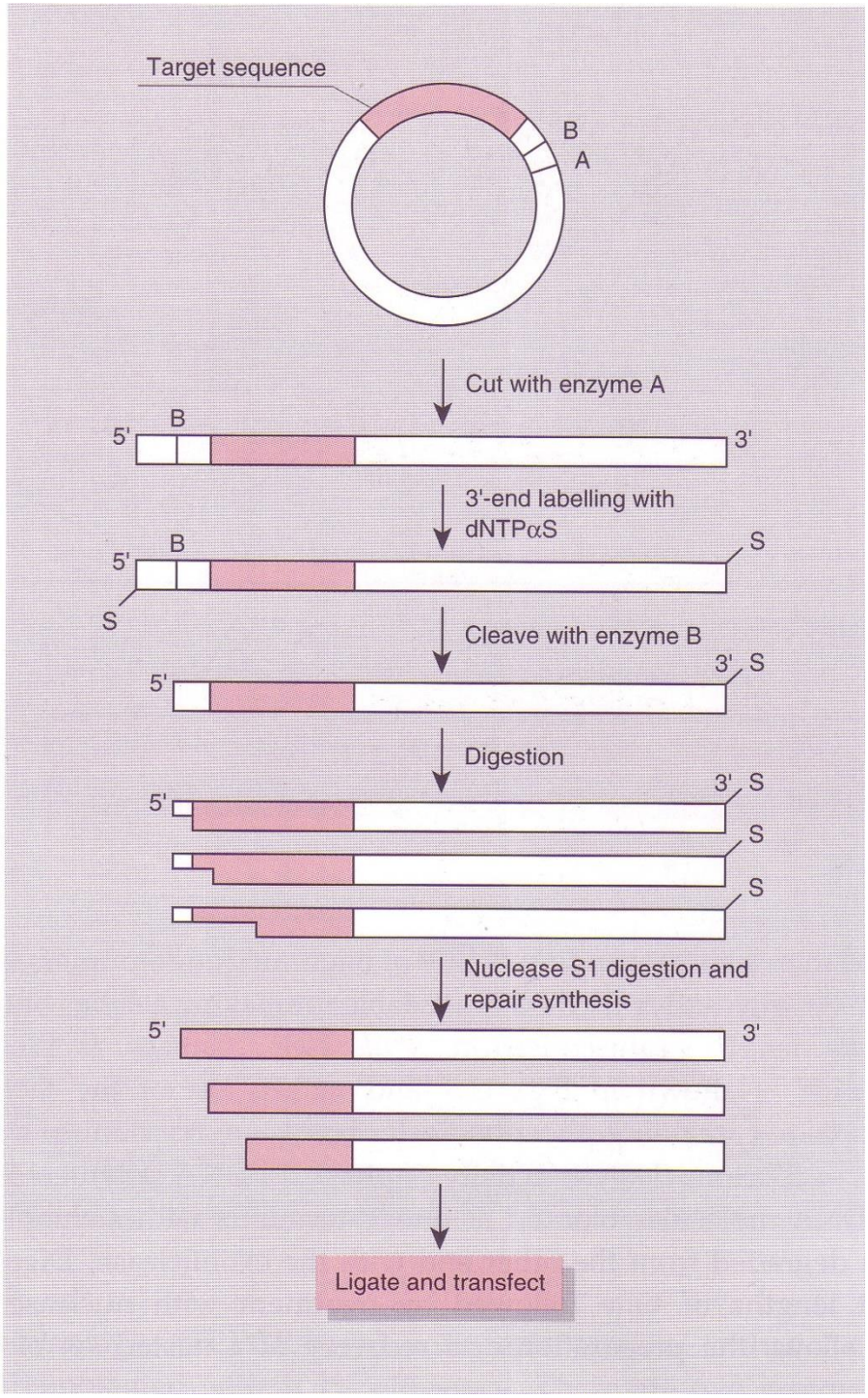
- početnice A i A'su komplementarne
-smještanje mutacije u sredinu DNA molekule



Slika 9. sk+hematski prikaz mutageneze PCR

4. Jednosmjerna delecija

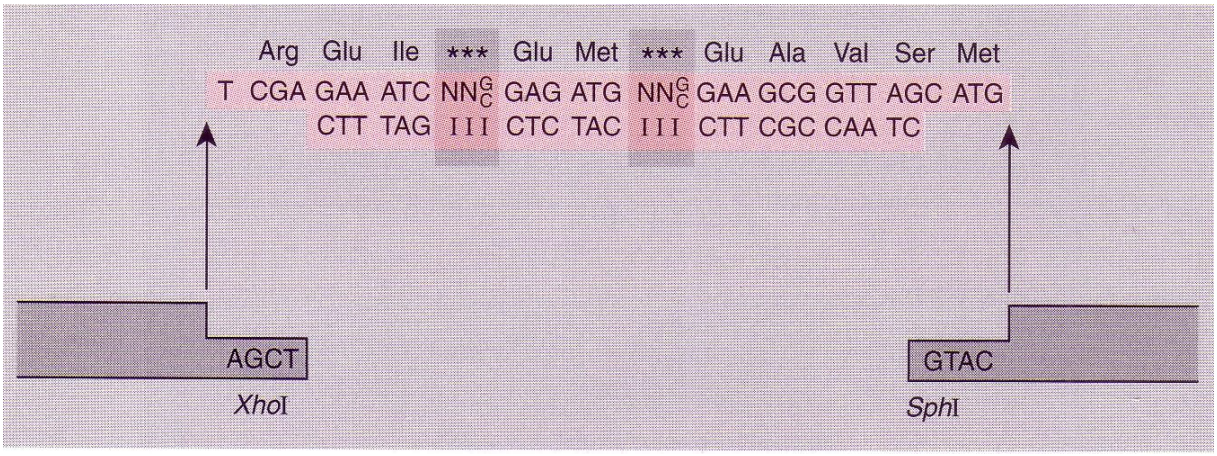
- potrebna obitelj oligonukleotidnih početnica,
- dobivaju se serije novih oligonukleotida različite duljine,
- linearna dlDNA označena na 3' kraju tiofosfatom,
- fosfodiesterska veza alfa-tiofosfata je rezistentna na hidrolizu 3'→5' egzonukleazne aktivnosti T4 DNA polimeraze



Slika 10. shematski prikaz mutageneze pomoću jednosmjerne delecije

5. Nasumična mutageneza:

- Sintezom prvog lanca četiri NTP, a drugi lanac na željena mjesta uklopljen inozin koji se sparuje sa sve četiri baze te se povežu – knjižnica mutanata;
- Nedostatak: ograničena duljina oligonukleotida.



Slika 11. shematski prikaz postupka nasumične mutageneze

6. Supresija amber mutacija:

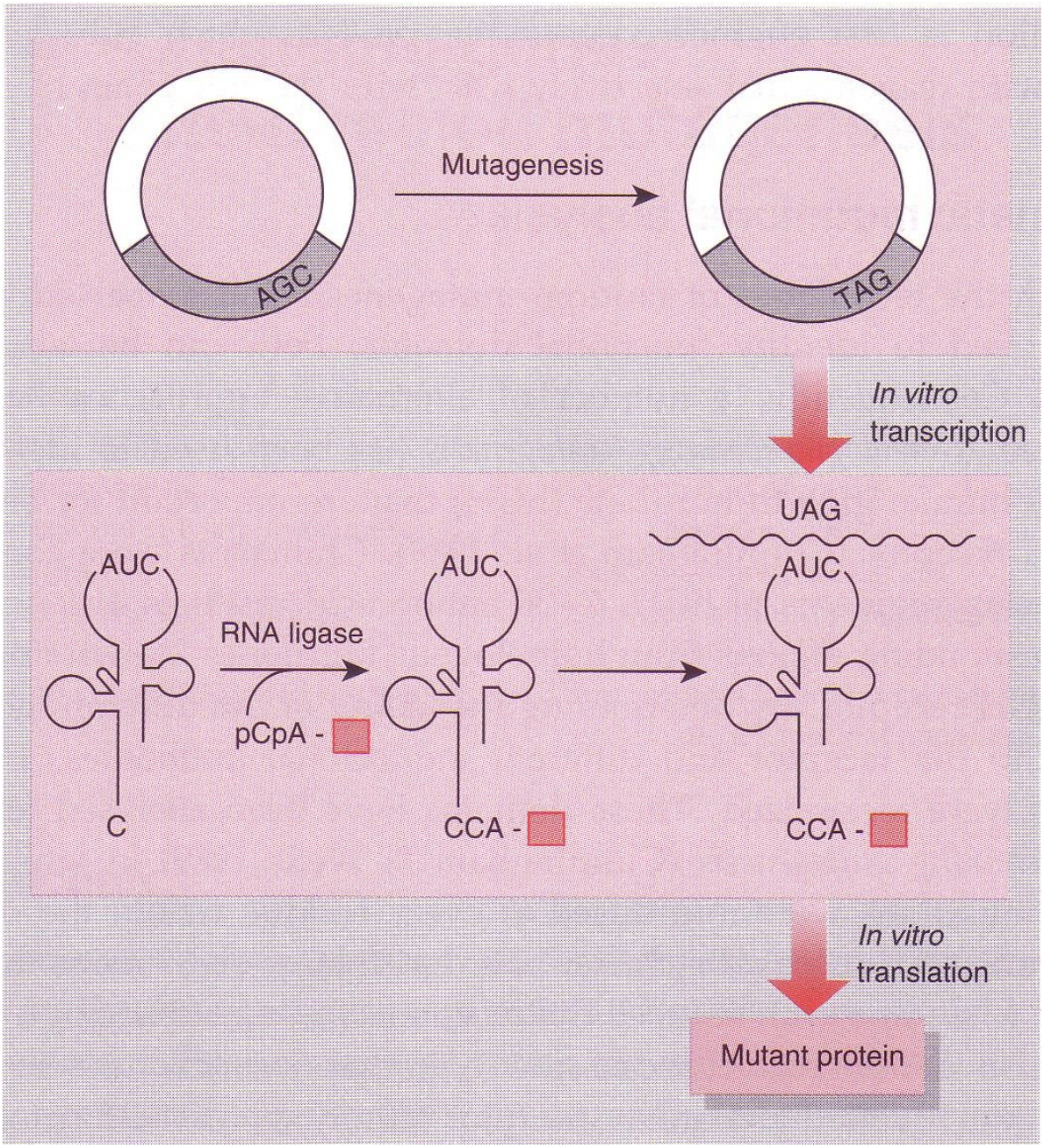
- stop kodon AUG,
- supresija s tRNA
- svaki soj umetne drugu aminikiselinu
- amber mutacija željeni gen

In vitro supresija amber mutacije upotrebom kemijski modificiranem tRNA

Stop kodoni:

u RNA: UAG ("amber") te UAA ("ochre") i UGA ("opal")

u DNA: TAG ("amber") te TAA ("ochre") i TGA ("opal" or "umber")



Slika 12. Shematski prikaz i*n vitro* supresije amber mutacijom upotrebom kemijski modificiranem tRNA

Tablica 3. prirodnih nonsens supresor mutacija

Nonsense Suppressors Employed to Generate Altered Proteins

Suppressor	Codons recognized	Amino acid inserted	Efficiency (%)	References
A. Natural				
Su1 (*supD*)	UAG	Serine	6–54	a, b
Su2 (*supE*)	UAG	Glutamine	0.8–20	a, b
Su2-89 (*supE*)	UAG	Glutamine	32–60	c, h
Su3 (*supF*)	UAG	Tyrosine	11–100	a, b
Su5 (*supG*)	UAA, UAG	Lysine	0.2–2 6–30*	a, b, h h
Su6 (*supP*)	UAG	Leucine	30–100	a, e
Su9	UGA	Tryptophan	0.1–30	a, f
B. Synthetic				
$tRNA^{Phe}_{CUA}$	UAG	Phenylalanine	48–100	g
$tRNA^{GluA}_{CUA}$	UAG	85% Glutamic acid 15% Glutamine	8–100	h, i
$tRNA^{Cys}_{CUA}$	UAG	Cysteine	17–51	g
$tRNA^{HisA}_{CUA}$	UAG	Histidine	16–100	h, i
$tRNA^{ProH}_{CUA}$	UAG	Proline	9–60	h, i
$tRNA^{Lys}_{CUA}$	UAG	Lysine	9–29	h, i
$tRNA^{Ala}_{CUA}$	UAG	Alanine	8–83	h, i
$tRNA^{Gly1}_{CUA}$	UAG	Glycine	39–67	h, i
FTORI 26	UAG	Arginine	4–28 4–47*	j h

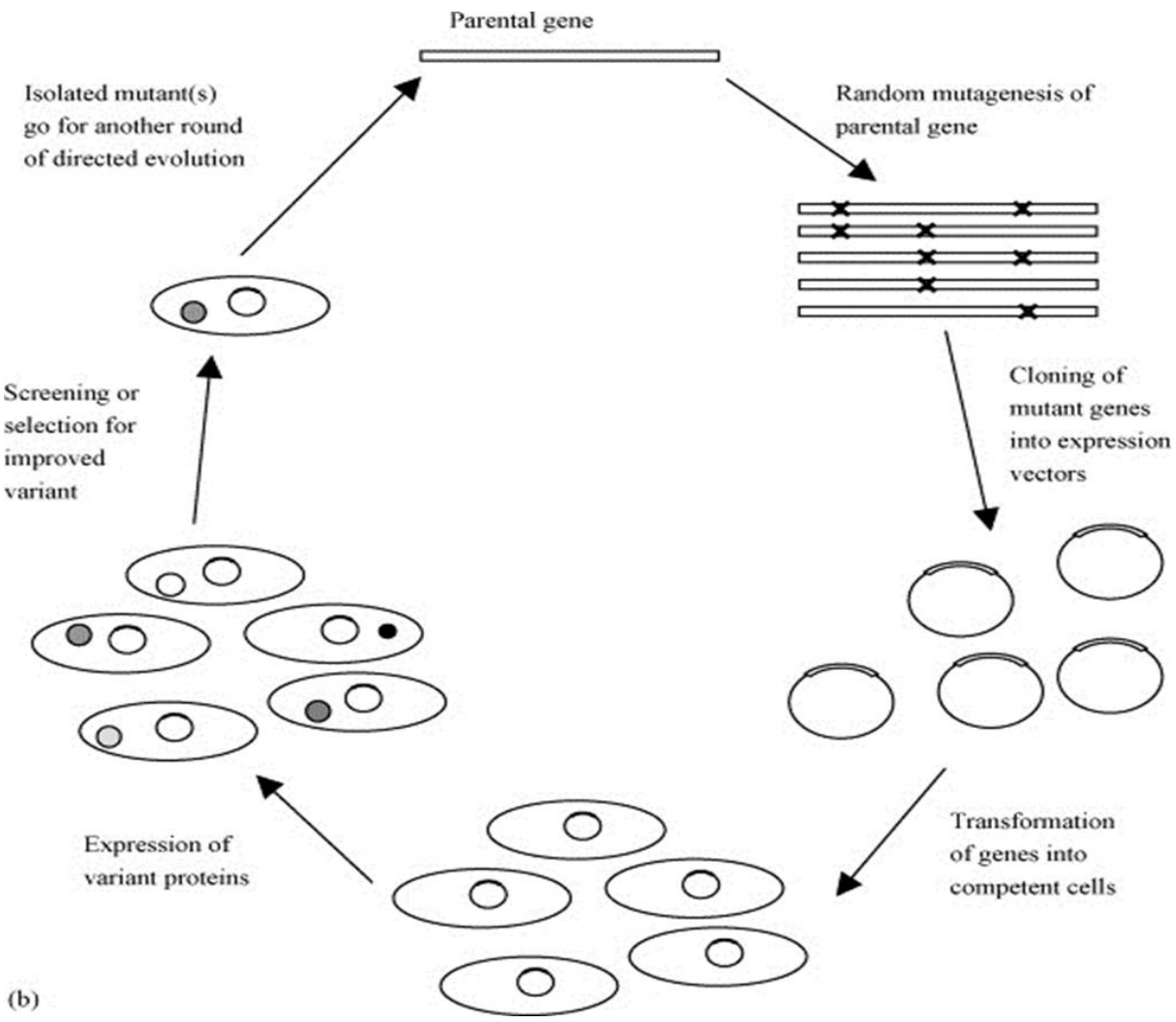
Supresija amber mutacija i korištenje:

- 163 različitih amber mutanata za T4 lizozim

- nalazi se u ekspresijskom vektoru koji se umetne u na pr. 13 supresorskih sojeva što generira preko 2000 različitih oblika proučavanog enzima
- nedostatak: ne može biti zastupljeno svih 20 aminikiselina.

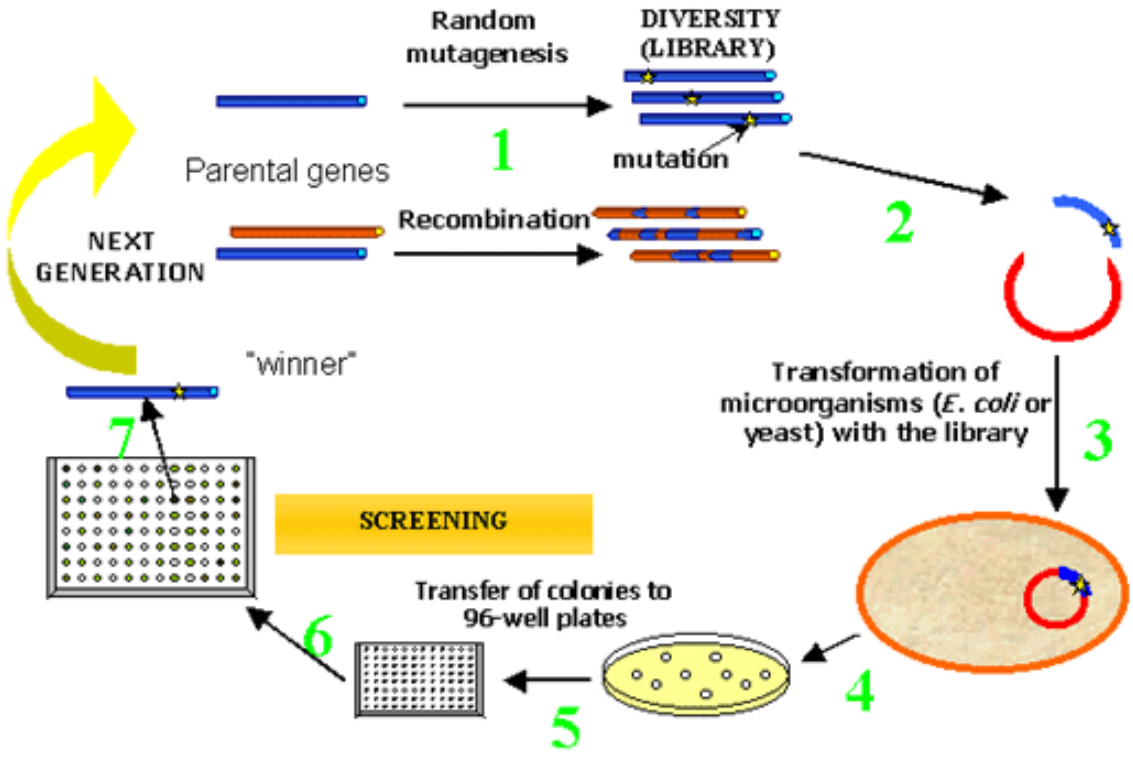
USMJERENA EVOLUCIJA (eng. "directed evolution"):

- Ne zahtjeva znanje proteinske strukture
- Imitiranje prirodnog procesa evolucije s određenim ciljem
- Stvaranje različitih knjižnica genskih varijanti pomoću NASUMIČNE mutageneze , što uključuje greške nastale PCR-om, tehnike rekombinacije gena ili *in vivo* miješanje DNA (eng. shuffling)
- Slijedi selekcija i analiza da bi se ustanovili enzimi sa željenim karakteristikama

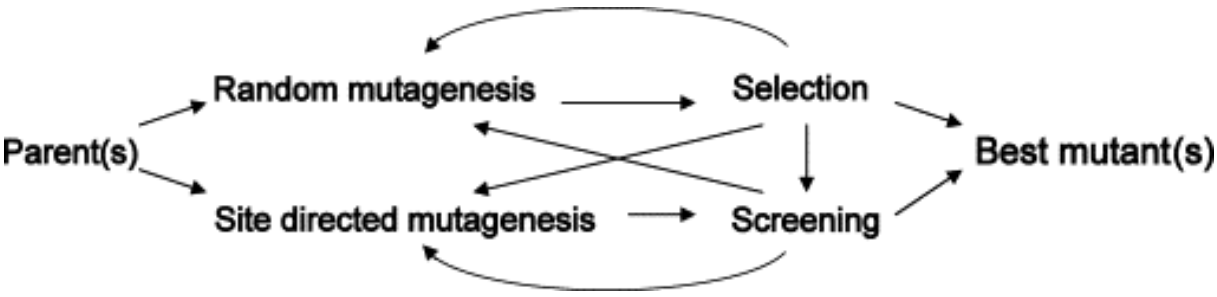


Slika 13. shematski prikaz postupka usmjerene evolucije

Kombinacija različitih postupaka mutageneze za dobivanje najuspješnijih mutanata.



Slika 14. Shematski prikaz kombiniranih postupaka mutageneze i pronnalaženje najboljeg konstrukta



Slika 15. Shematski prikaz kombiniranih postupaka mutageneze