

# Directed Mutagenesis and Protein Engineering

# Mutagenesis

Mutagenesis -> change in DNA sequence

-> Point mutations or large modifications

Point mutations (directed mutagenesis):

- Substitution: change of one nucleotide (i.e. A-> C)
- Insertion: gaining one additional nucleotide
- Deletion: loss of one nucleotide

# Consequences of point mutations within a coding sequence (gene) for the protein

(a) Point mutations and small deletions

## Wild-type sequences

Amino acid	N-Phe	Arg	Trp	Ile	Ala	Asn-C
mRNA	5'-UUU	CGA	UGG	AUA	GCC	AAU-3'
DNA	3'-AAA	GCT	ACC	TAT	CGG	TTA-5'
	5'-TTT	CGA	TGG	ATA	GCC	AAT-3'

## Missense

	3'-AAT	GCT	ACC	TAT	CGG	TTA-5'
	5'-TTA	CGA	TGG	ATA	GCC	AAT-3'
N-	Leu	Arg	Trp	Ile	Ala	Asn-C

-> Change of amino acid

## Nonsense

	3'-AAA	GCT	ATC	TAT	CGG	TTA-5'
	5'-TTT	CGA	TAG	ATA	GCC	AAT-3'
N-Phe	Arg	Stop				

-> truncation of protein

## Frameshift by addition

	3'-AAA	GCT	ACC	ATA	TCG	GTT A-5'
	5'-TTT	CGA	TGG	TAT	AGC	CAAT-3'
N-Phe	Arg	Trp	Tyr	Ser	Gln	

-> change of c-terminal part of protein

## Frameshift by deletion

		GCTA				
		CGAT				
	3'-AAA	↓ CCT	ATC	GGT	TA-5'	
	5'-TTT	GGA	TAG	CCA	AT-3'	
N-Phe		Gly	Stop			

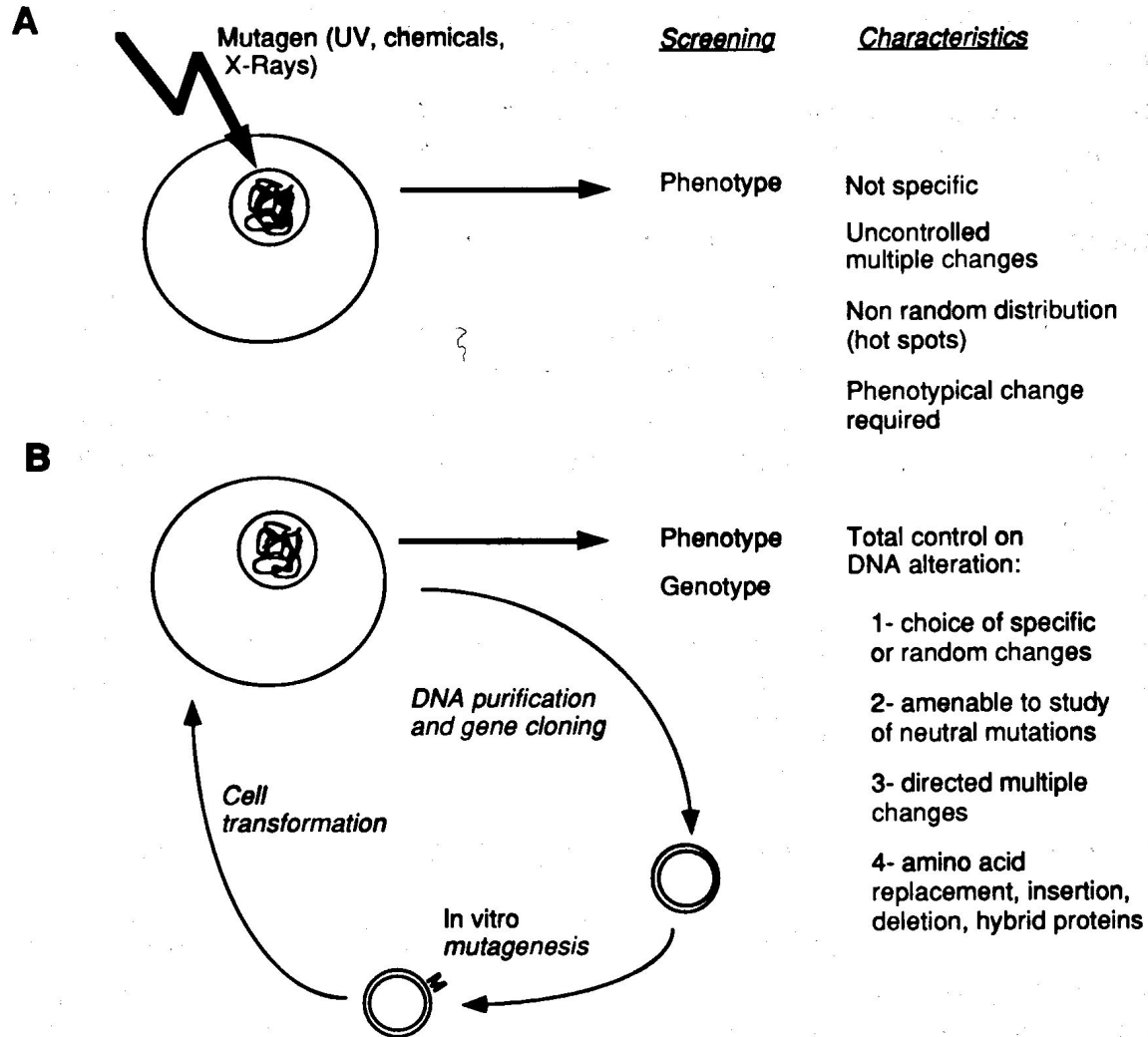
-> change of c-terminal part of protein

Silent mutations:

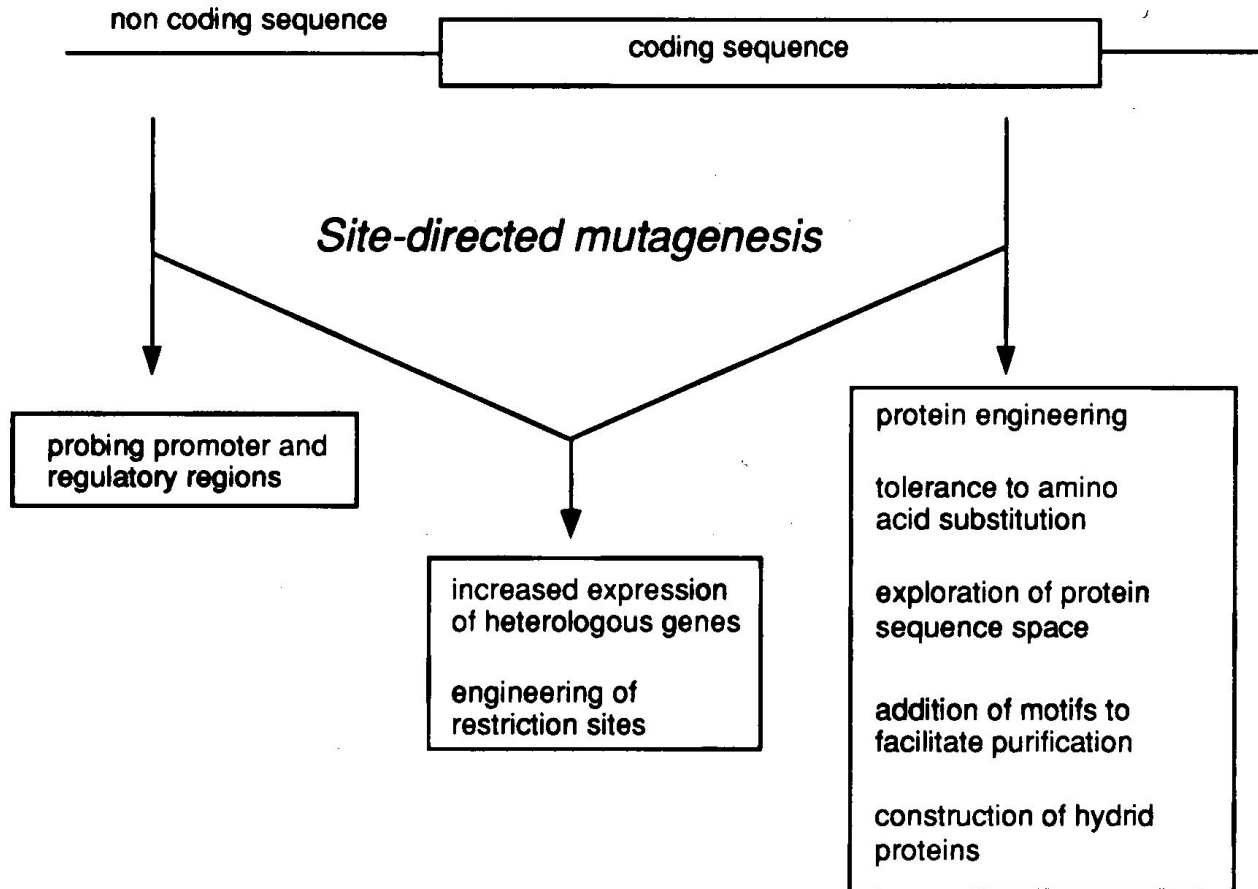
-> change in nucleotide sequence with **no consequences** for protein sequence

# Mutagenesis

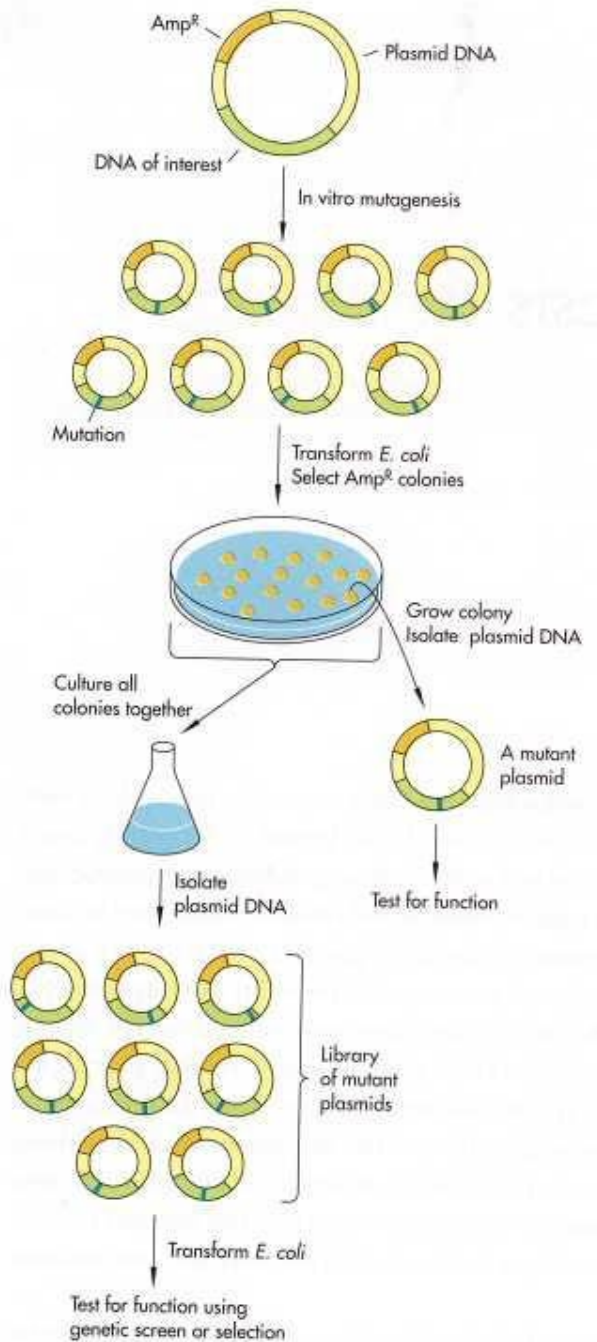
## Comparison of cellular and invitro mutagenesis



# Applications of directed mutagenesis



# General strategy for directed mutagenesis



## Requirements:

- DNA of interest (gene or promoter) must be cloned
- Expression system must be available -> for testing phenotypic change

# Approaches for directed mutagenesis

## -> site-directed mutagenesis

-> point mutations in particular known area

result -> library of wild-type and mutated DNA (site-specific)  
not really a library -> just 2 species

## -> random mutagenesis

-> point mutations in all areas within DNA of interest

result -> library of wild-type and mutated DNA (random)  
a real library -> many variants -> **screening !!!**

if methods efficient -> mostly mutated DNA

# Protein Engineering

-> Mutagenesis used for modifying proteins  
Replacements on protein level -> mutations on DNA level

Assumption : Natural sequence can be modified to  
improve a certain function of protein

This implies:

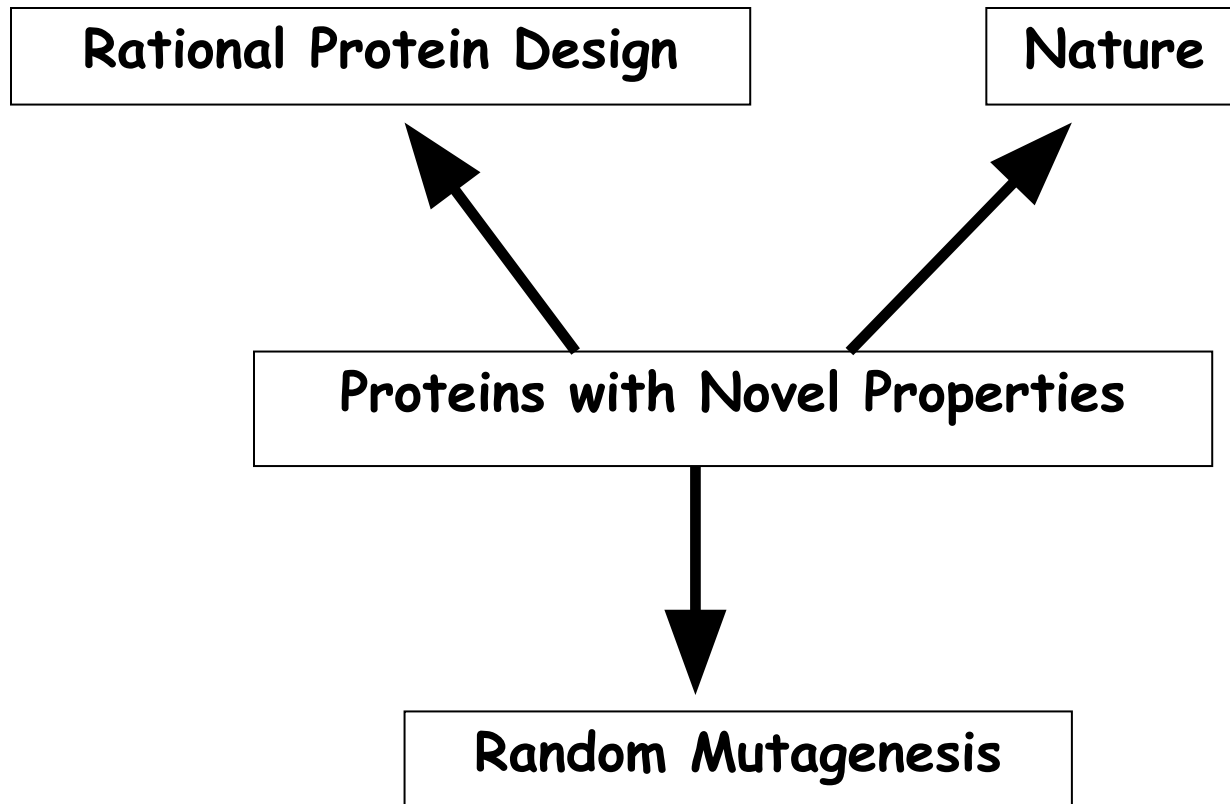
- Protein is NOT at an optimum for that function
- Sequence changes without disruption of the structure
- (otherwise it would not fold)
- New sequence is not TOO different from the native sequence (otherwise loss in function of protein)

**consequence -> introduce point mutations**



# Protein Engineering

Obtain a protein with improved or new properties



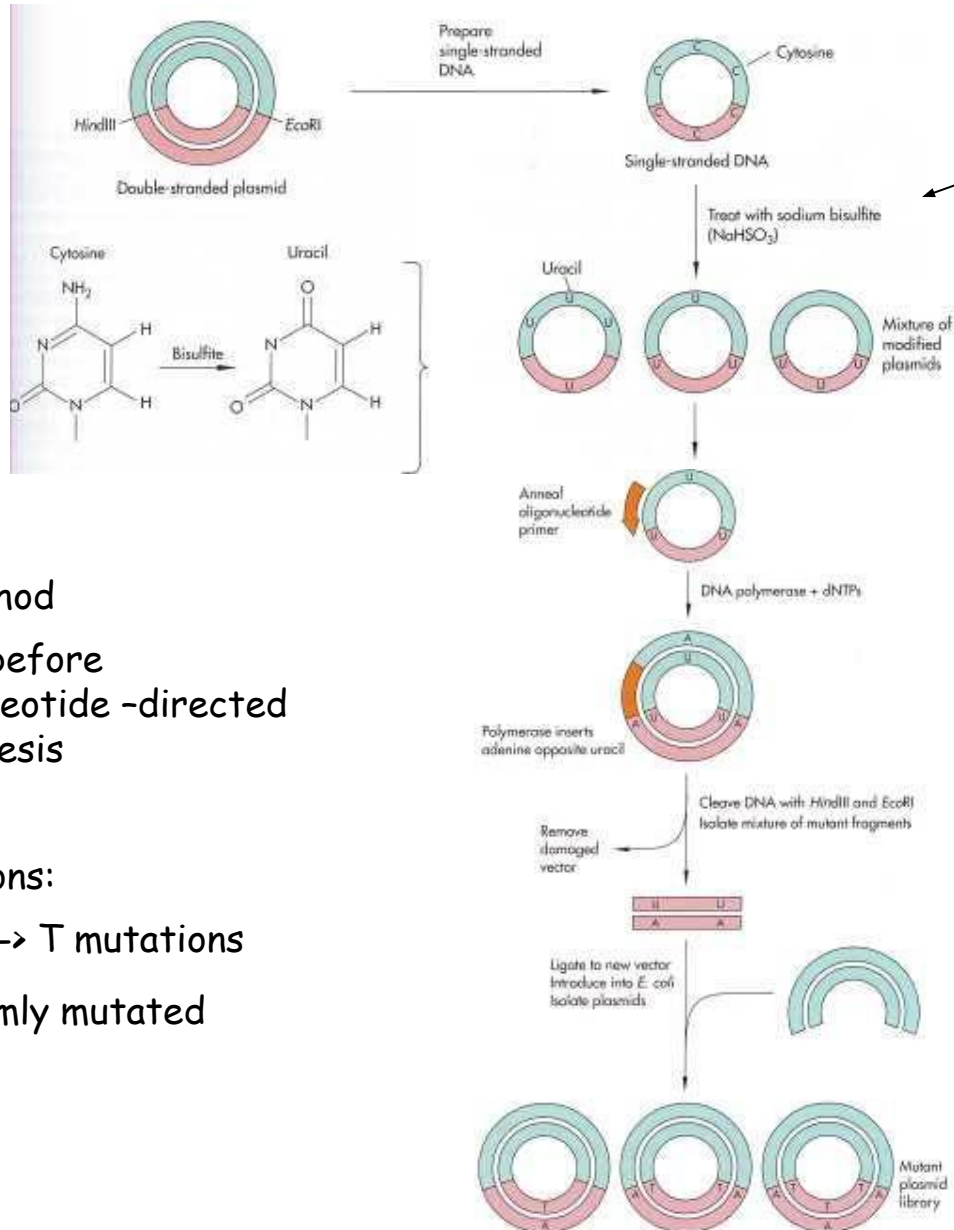
# Rational Protein Design

⇒ Site -directed mutagenesis !!!

Requirements:

- > Knowledge of sequence and preferable Structure (active site,....)
- > Understanding of mechanism (knowledge about structure - function relationship)
- > Identification of cofactors.....

# Site-directed mutagenesis methods



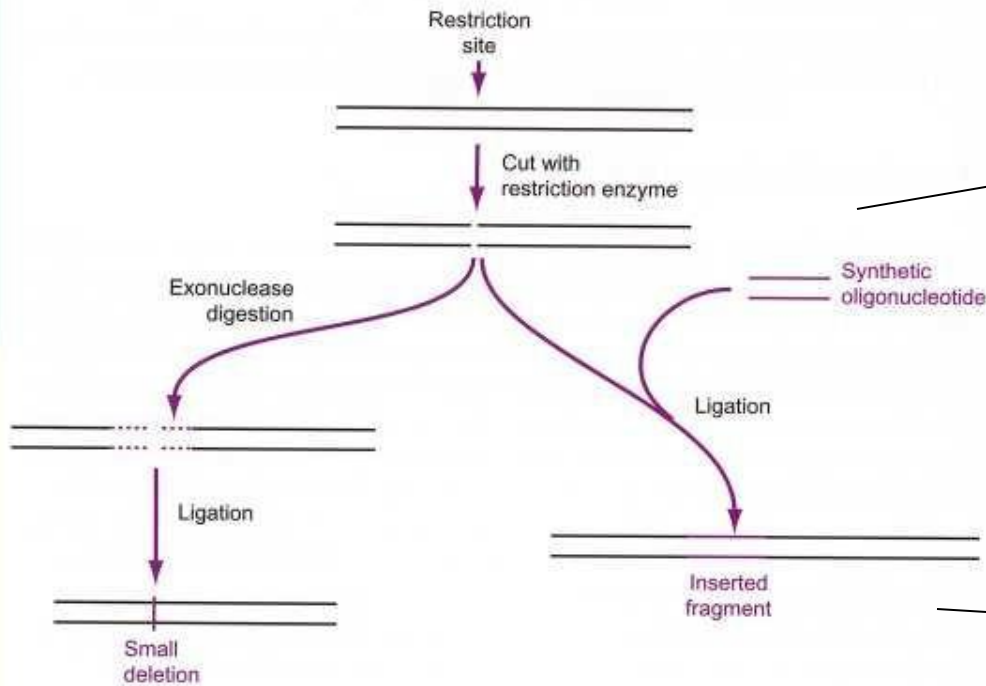
Old method

-> used before  
oligonucleotide -directed  
mutagenesis

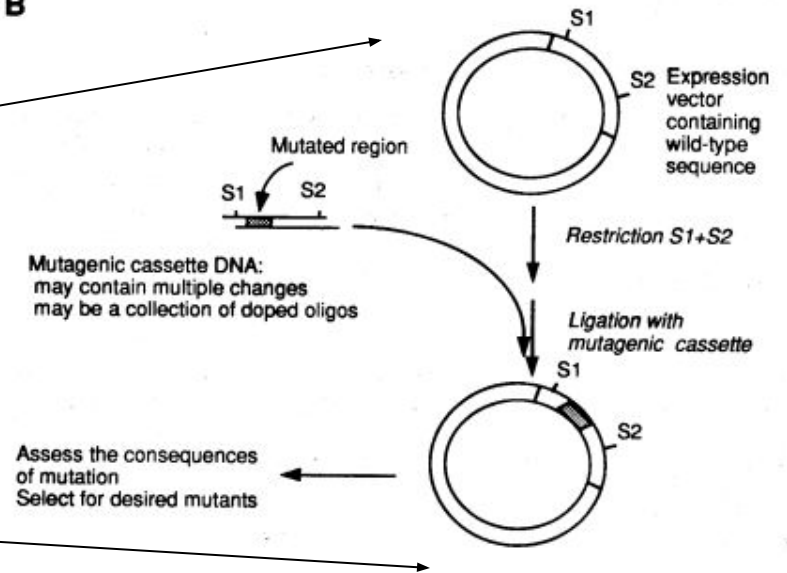
Limitations:

- > just C-> T mutations
- > randomly mutated

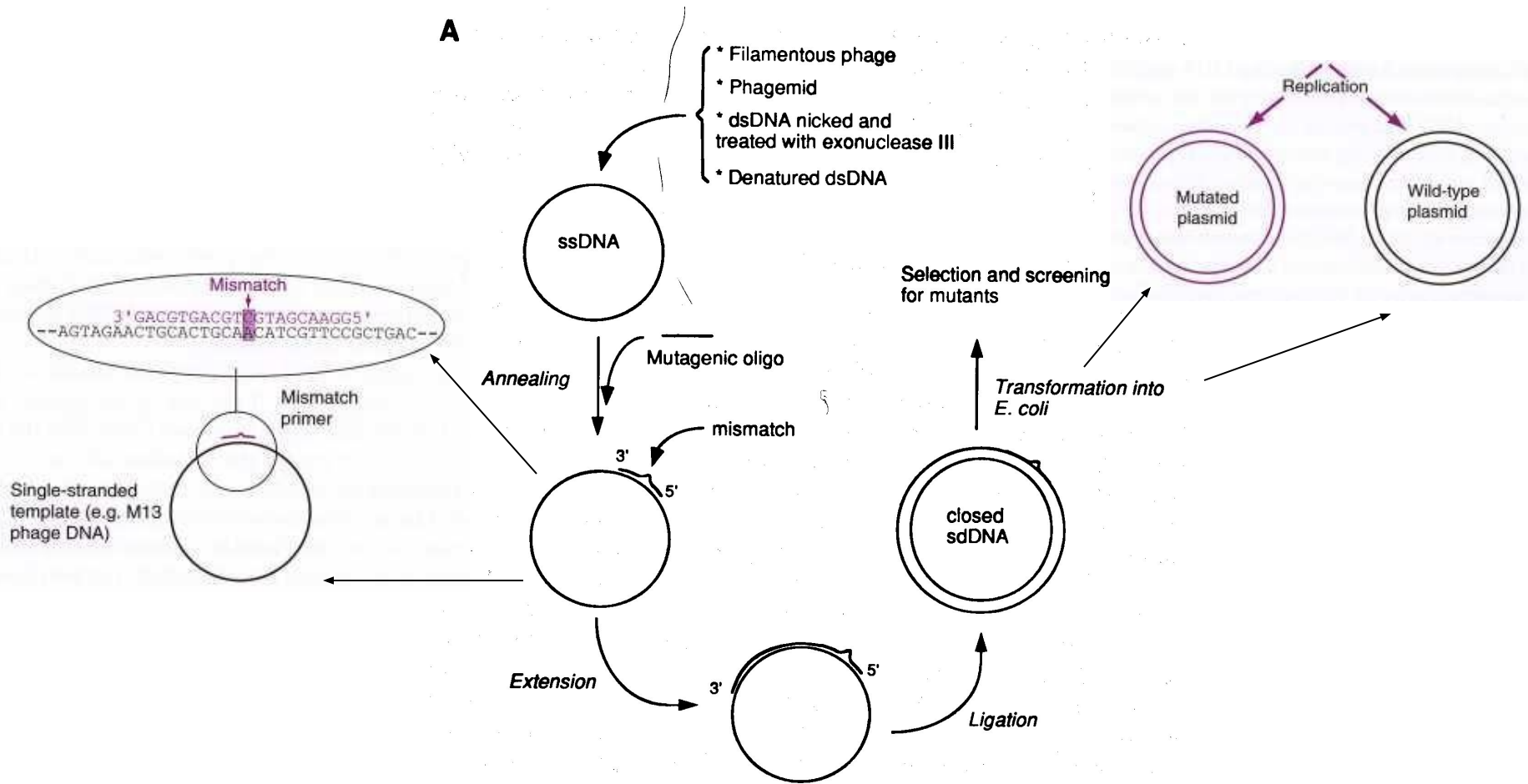
# Site-directed mutagenesis methods



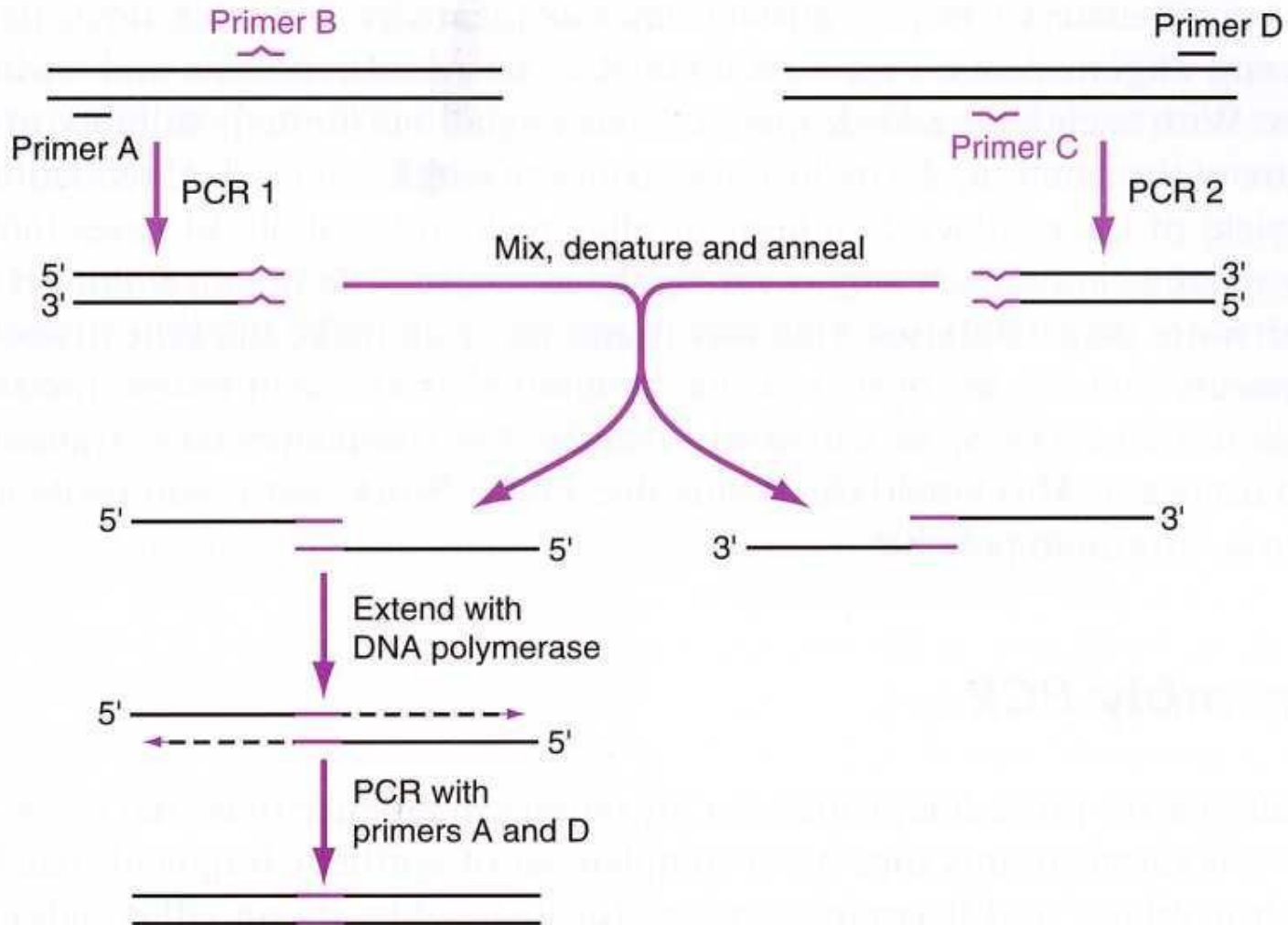
**B**



# Site-directed mutagenesis methods - Oligonucleotide - directed method



# Site-directed mutagenesis methods - PCR based



# Directed Evolution - Random mutagenesis

-> based on the process of natural evolution

- **NO structural information required**
- **NO understanding of the mechanism required**

## General Procedure:

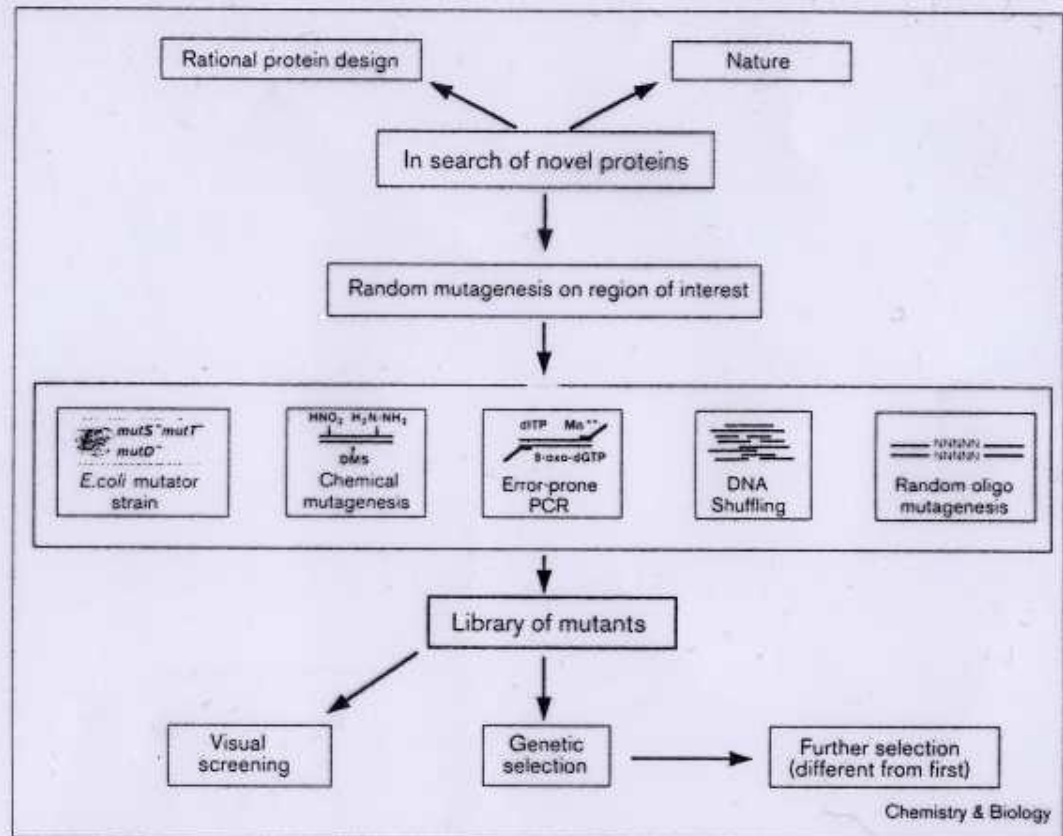
Generation of genetic diversity

⇒ Random mutagenesis

Identification of successful variants

⇒ Screening and selection

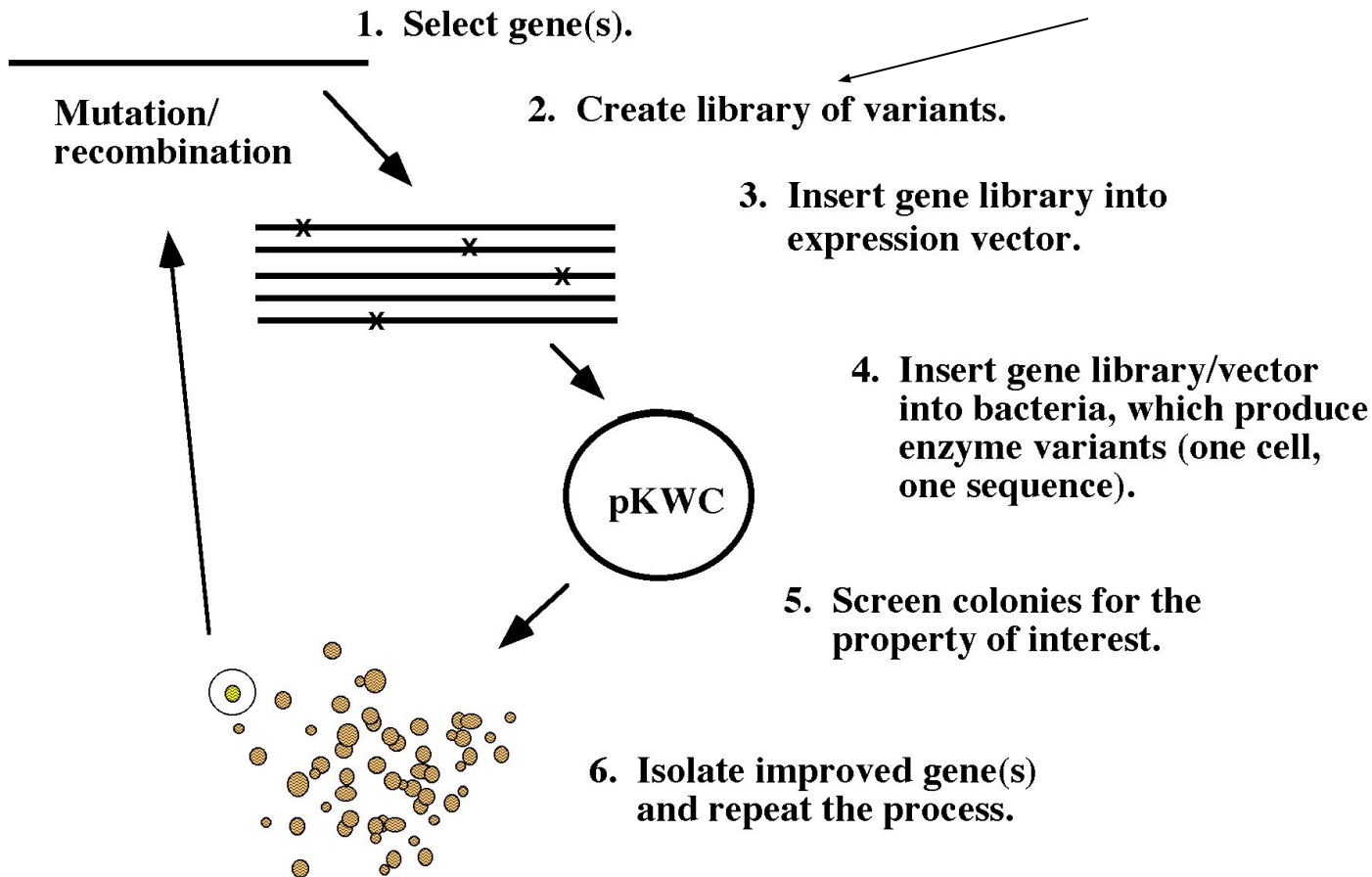
The creation of random libraries by applied molecular evolution. A gene target can be randomized by one of several methods, followed by ligation of the library into a vector backbone and transformation into an appropriate strain of *Escherichia coli* for selection/screening.





# General Directed Evolution Procedure

Random mutagenesis methods



# Directed Evolution Library

Even a large library  $\rightarrow$  ( $10^8$  independent clones)  
will not exhaustively encode all possible single point mutations.

Requirements would be:

$20^N$  independent clones  $\rightarrow$  to have all possible variations in a library  
(+ silent mutations)

N..... number of amino acids in the protein

For a small protein:  $\rightarrow$  Hen egg-white Lysozyme (129 aa; 14.6 kDa)  
 $\rightarrow$  library with  $20^{129}$  ( $7 \times 10^{168}$ ) independent clones

Consequence  $\rightarrow$  not all modifications possible

$\rightarrow$  modifications just along an evolutionary path !!!!

# Limitation of Directed Evolution

1. Evolutionary path must exist - > to be successful

2. Screening method must be available

-> You get (exactly) what you ask for!!!

-> need to be done in -> High throughput !!!

# Typical Directed Evolution Experiment

Successful experiments involve generally  
less than 6 steps (cycles)!!!

Why?

1. Sequences with improved properties are rather close to the parental sequence -> along a evolutionary path
2. Capacity of our present methods to generate novel functional sequences is rather limited -> requires huge libraries

⇒ Point Mutations !!!

# Evolutionary Methods

- **Non-recombinative methods:**
  - > Oligonucleotide Directed Mutagenesis (saturation mutagenesis)
  - > Chemical Mutagenesis, Bacterial Mutator Strains
  - > Error-prone PCR
  
- **Recombinative methods** -> Mimic nature's recombination strategy  
Used for: Elimination of neutral and deleterious mutations
  - > DNA shuffling
  - > In vivo Recombination (Yeast)
  - > Random priming recombination, Staggered extension process (StEP)
  - > ITCHY

# Evolutionary Methods

## Type of mutation - Fitness of mutants

Type of mutations:

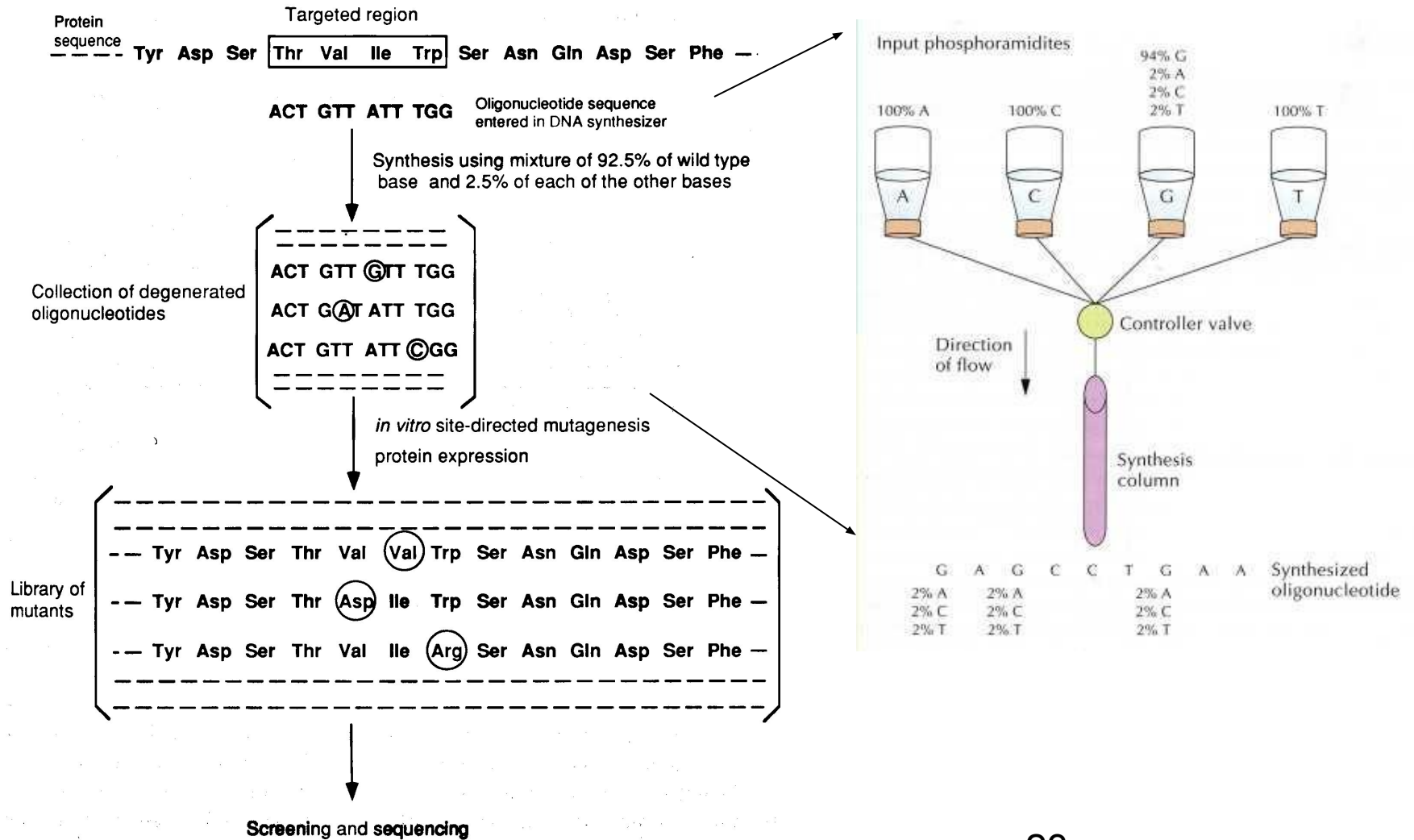
- ⇒ Beneficial mutations (good)
- ⇒ Neutral mutations
- ⇒ Deleterious mutations (bad)

⇒ Beneficial mutations are diluted with neutral and deleterious ones

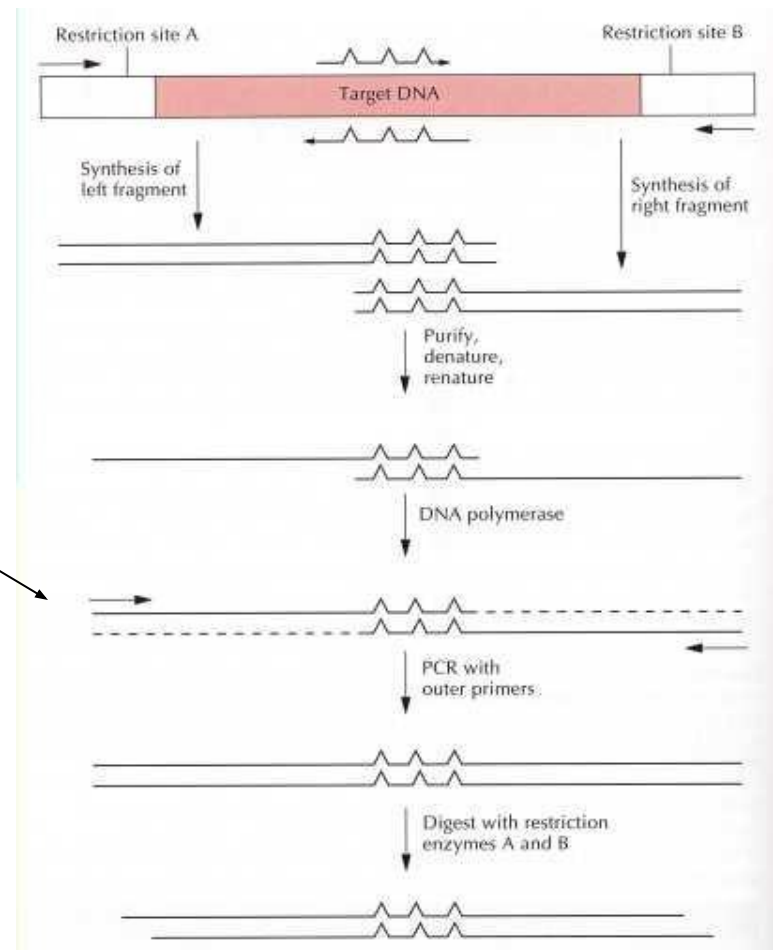
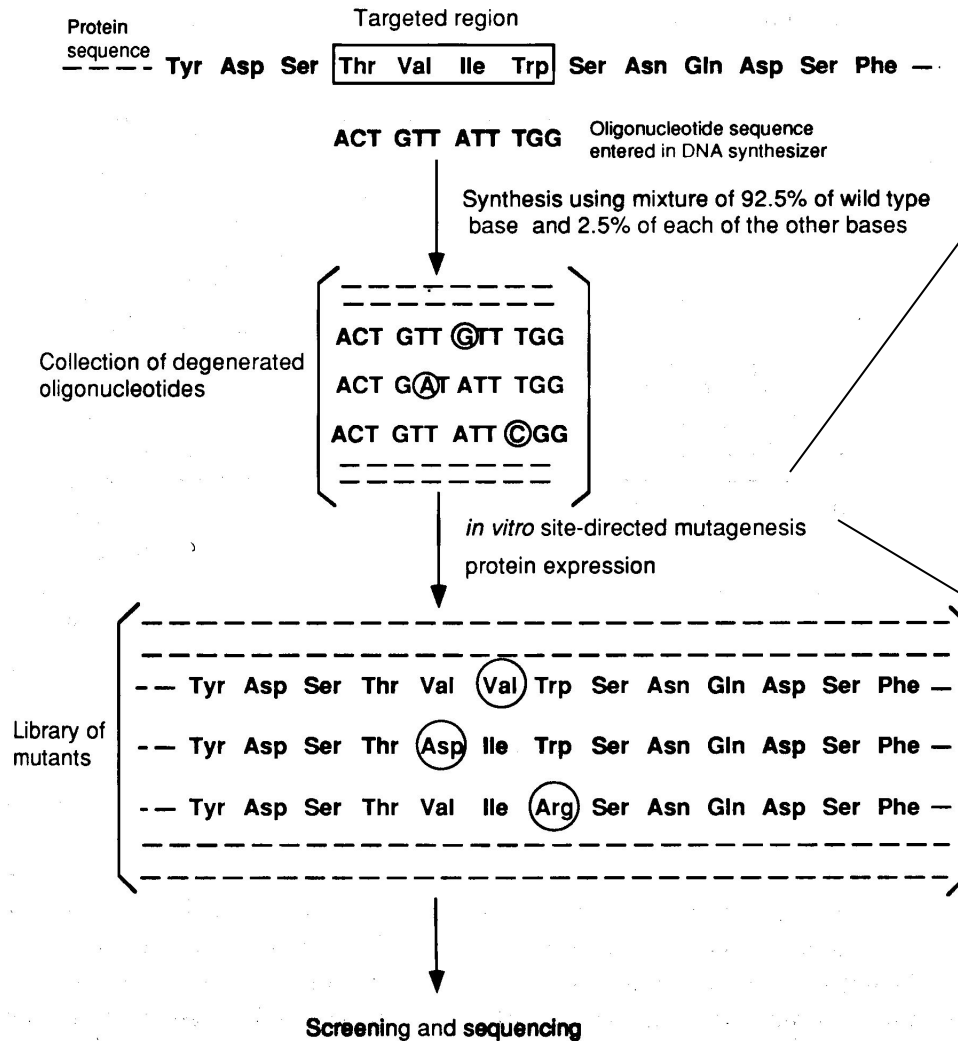
**!!! Keep the number of mutations low per cycle**

**-> improve fitness of mutants!!!**

# Random Mutagenesis (PCR based) with degenerated primers (saturation mutagenesis)

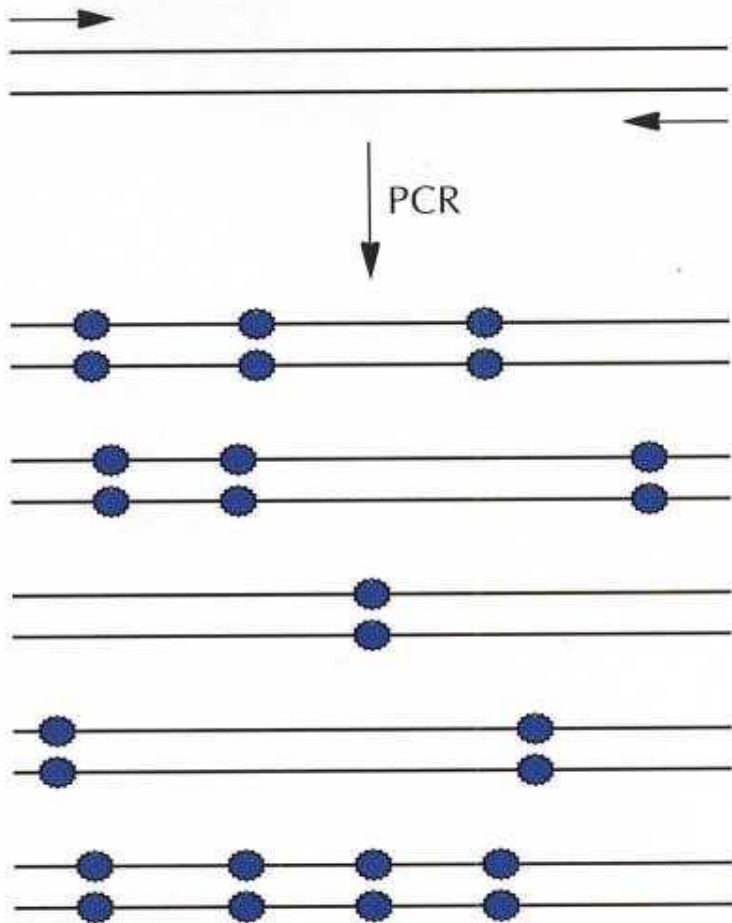


# Random Mutagenesis (PCR based) with degenerated primers (saturation mutagenesis)





# Random Mutagenesis (PCR based) Error -prone PCR

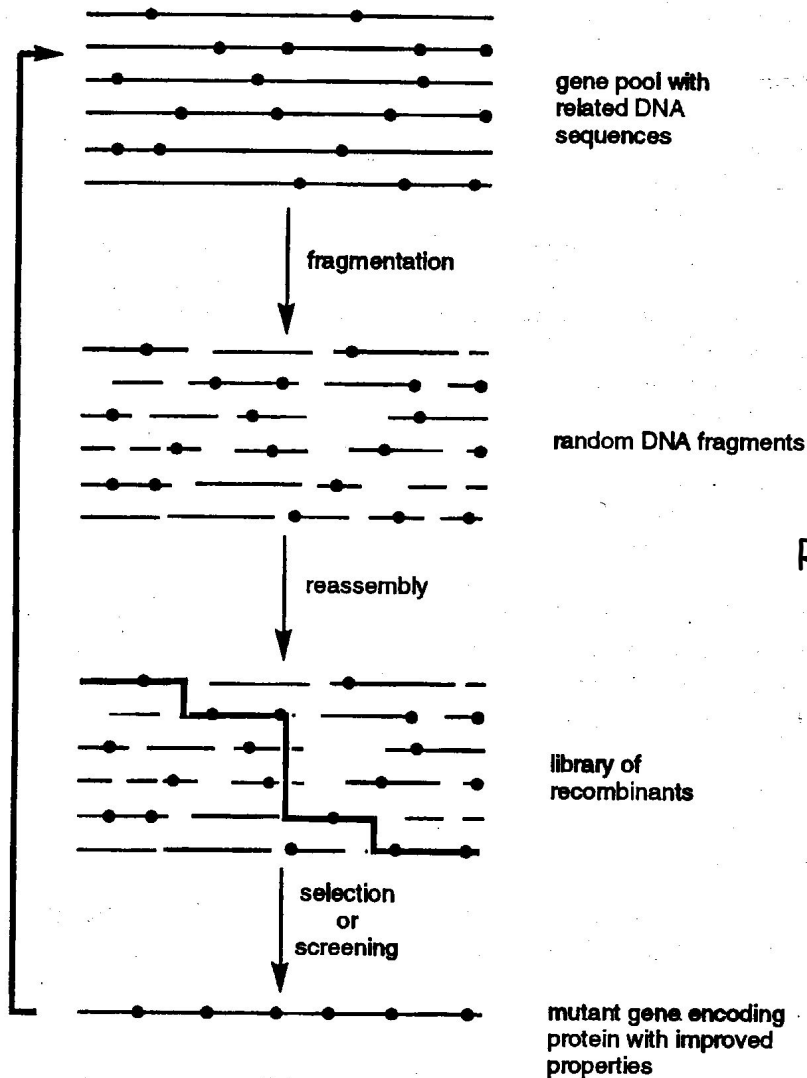


-> PCR with low fidelity !!!

Achieved by:

- Increased  $Mg^{2+}$  concentration
- Addition of  $Mn^{2+}$
- Not equal concentration of the four dNTPs
- Use of dITP
- Increasing amount of Taq polymerase (Polymerase with NO proof reading function)

# Random Mutagenesis (PCR based) DNA Shuffling

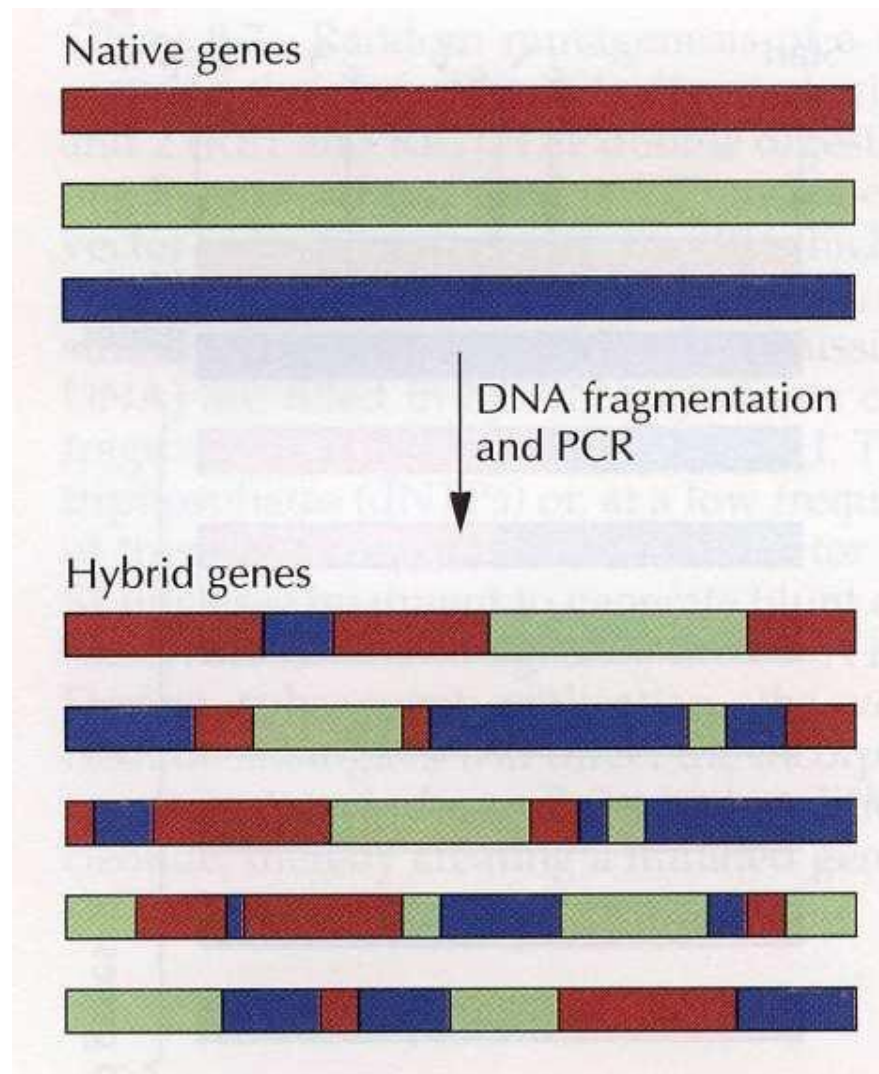


DNase I treatment (Fragmentation,  
10-50 bp,  $Mn^{2+}$ )

Reassembly (PCR without primers,  
Extension and Recombination)

PCR amplification

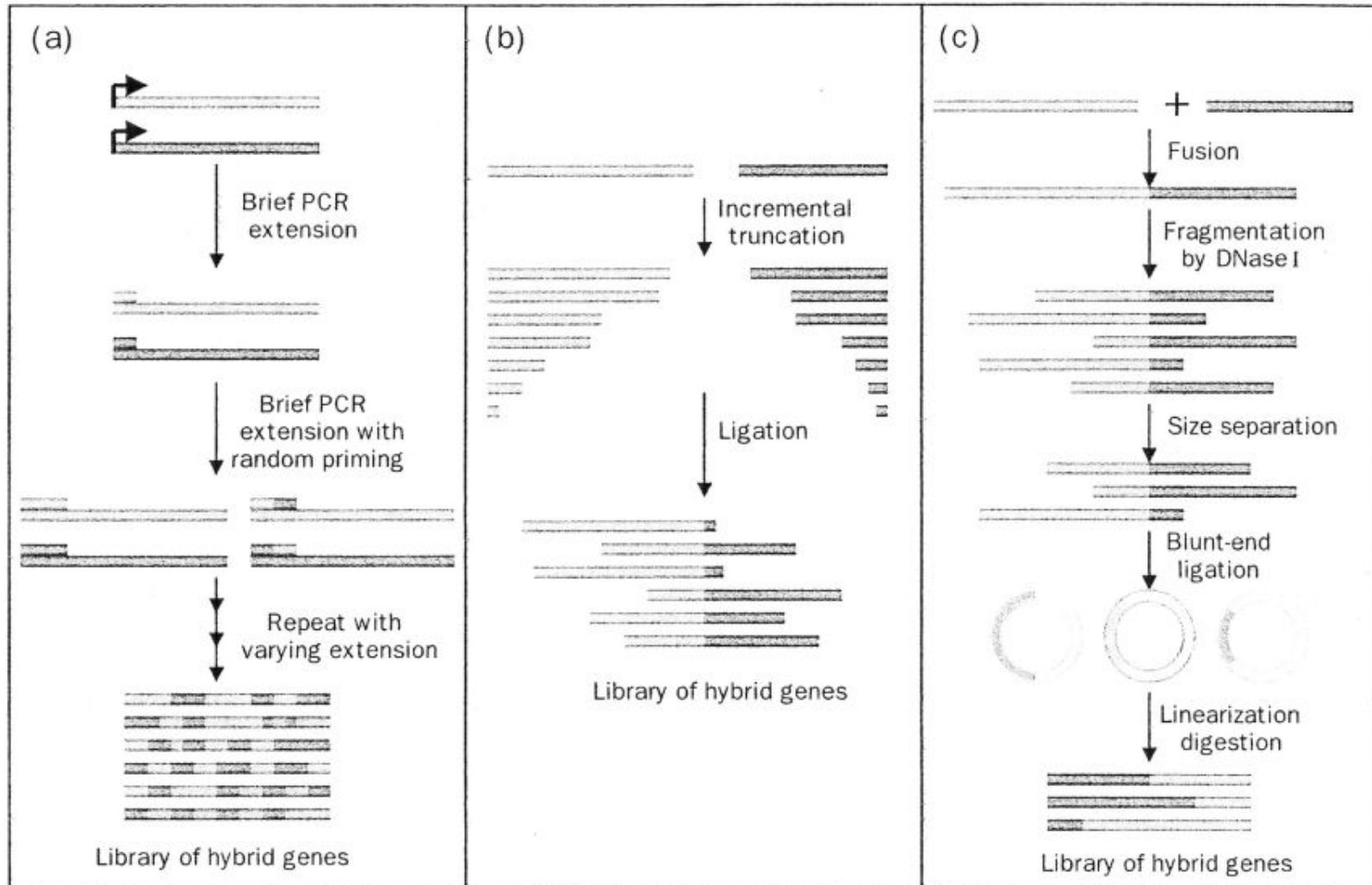
# Random Mutagenesis (PCR based) Family Shuffling



Genes coming from the same  
gene family -> highly  
homologous

-> Family shuffling

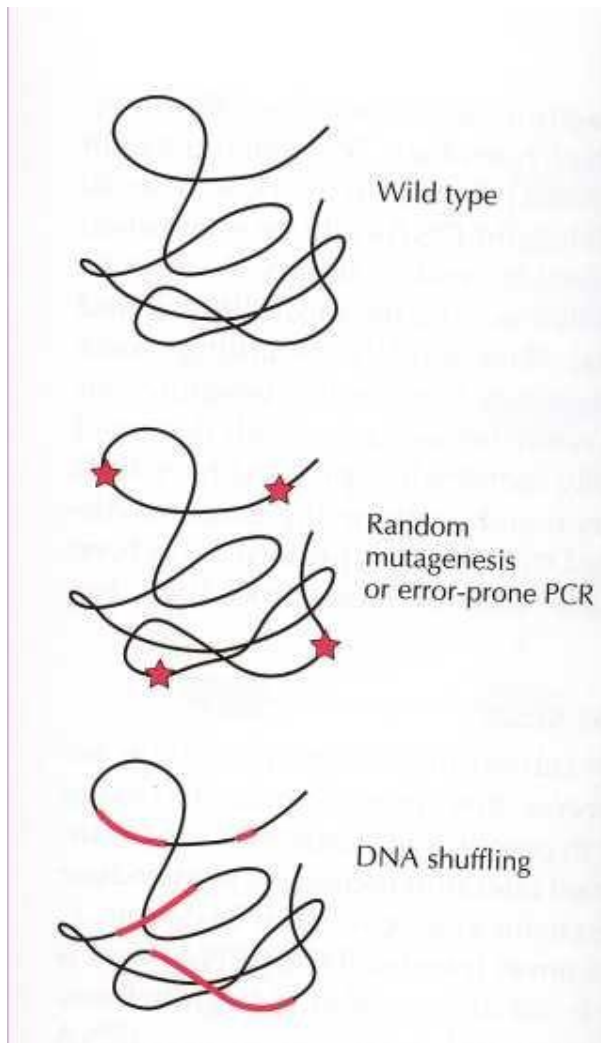
# Random Mutagenesis (PCR based)



**Figure 11** Procedures for the (a) StEP, (b) the ITCHY, and (c) the SHIPREC methods.

# Directed Evolution

Difference between non-recombinative and recombinative methods



Non-recombinative methods

recombinative methods ->  
hybrids (chimeric proteins)

# Protein Engineering

What can be engineered in Proteins ?

## -> Folding (+Structure):

### 1. Thermodynamic Stability

(Equilibrium between: Native  $\leftrightarrow$  Unfolded state)

### 2. Thermal and Environmental Stability (Temperature, pH, Solvent, Detergents, Salt .....

# Protein Engineering

What can be engineered in Proteins ?

- > **Function:**

1. **Binding** (Interaction of a protein with its surroundings)

How many points are required to bind a molecule with high affinity?

2. **Catalysis** (a different form of binding - binding the transition state of a chemical reaction)

Increased binding to the transition state  $\Rightarrow$  increased catalytic rates !!!

Requires: Knowledge of the Catalytic Mechanism !!!

-> engineer  $K_{cat}$  and  $K_m$

# Protein Engineering

## Factors which contribute to stability:

1. Hydrophobicity (hydrophobic core)
2. Electrostatic Interactions:
  - > Salt Bridges
  - > Hydrogen Bonds
  - > Dipole Interactions
3. Disulfide Bridges
4. Metal Binding (Metal chelating site)
5. Reduction of the unfolded state entropy with  
X → Pro mutations



# Protein Engineering

## Design of Thermal and Environmental stability:

1. Stabilization of  $\alpha$ -Helix Macrodipoles
2. Engineer Structural Motives (like Helix N-Caps)
3. Introduction of salt bridges
4. Introduction of residues with higher intrinsic properties for their conformational state (e.g. Ala replacement within a  $\alpha$ -Helix)
5. Introduction of disulfide bridges
6. Reduction of the unfolded state entropy with  
X  $\rightarrow$  Pro mutations

# Protein Engineering - Applications

## Engineering Stability of Enzymes - T4 lysozyme

-> S-S bonds introduction

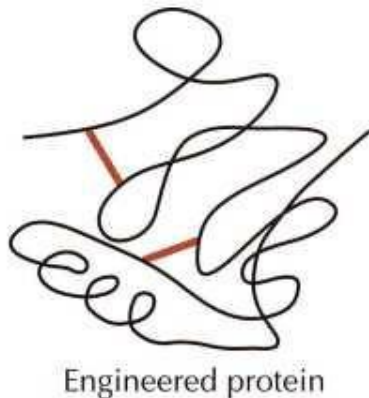


Table 8.2 Properties of T4 lysozyme and six engineered variants

Enzyme	Amino acid at position:							No. of -S-S-	% Activity	$T_m$ (°C)
	3	9	21	54	97	142	164			
wt	Ile	Ile	Thr	Cys	Cys	Thr	Leu	0	100	41.9
pwt	Ile	Ile	Thr	Thr	Ala	Thr	Leu	0	100	41.9
A	Cys	Ile	Thr	Thr	Cys	Thr	Leu	1	96	46.7
B	Ile	Cys	Thr	Thr	Ala	Thr	Cys	1	106	48.3
C	Ile	Ile	Cys	Thr	Ala	Cys	Leu	1	0	52.9
D	Cys	Cys	Thr	Thr	Cys	Thr	Cys	2	95	57.6
E	Ile	Cys	Cys	Thr	Ala	Cys	Cys	2	0	58.9
F	Cys	Cys	Cys	Thr	Cys	Cys	Cys	3	0	65.5

Adapted from Matsumura et al., *Nature* 342:291-293, 1989.

wt, wild-type T4 lysozyme; pwt, pseudo-wild-type enzyme; A through F, six engineered cysteine variants; -S-S-, disulfide bonds;  $T_m$ , "melting" temperature (a measure of thermostability).

# Protein Engineering - Applications

Engineering Stability of Enzymes - triosephosphate isomerase from yeast

-> replace Asn (deaminated at high temperature)

*Table 8.3* Stability at 100°C of the yeast enzyme triosephosphate isomerase and its engineered derivatives

Enzyme	Amino acid at position:		Half-life (min)
	14	78	
Wild type	Asn	Asn	13
Variant A	Asn	Thr	17
Variant B	Asn	Ile	16
Variant C	Thr	Ile	25
Variant D	Asp	Asn	11

Adapted from Ahern et al., *Proc. Natl. Acad. Sci. USA* 84:675–679, 1987.

Enzyme stability is expressed as the half-life, or rate of enzyme inactivation, at 100°C. A longer half-life indicates a more stable enzyme.

# Protein Engineering - Applications

Engineering Activity of Enzymes - tyrosyl-tRNA synthetase from *B. stearothermophilus*

-> replace Thr 51 (improve affinity for ATP) -> Design

*Table 8.4* Aminoacylation activity of native (Thr-51) and modified (Ala-51 and Pro-51) tyrosyl-tRNA synthetases

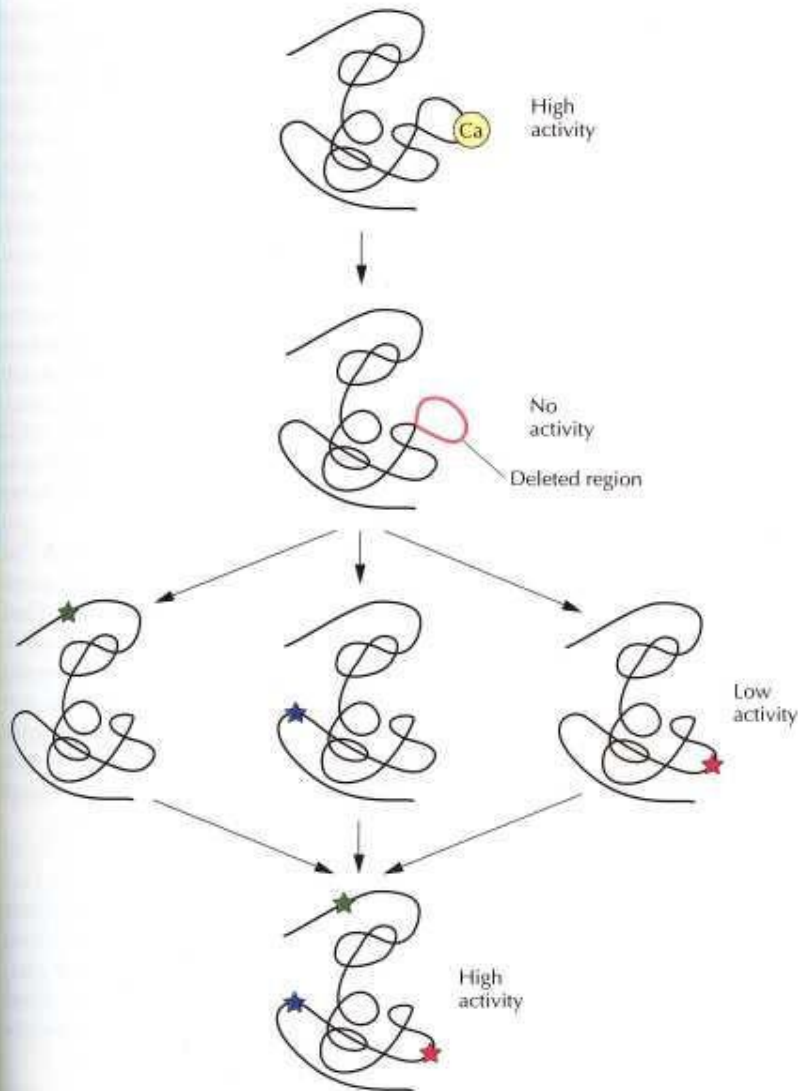
Enzyme	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ ( $\text{s}^{-1} \text{M}^{-1}$ )
Thr-51	4.7	2.5	1,860
Ala-51	4.0	1.2	3,200
Pro-51	1.8	0.019	95,800

Adapted from Wilkinson et al., *Nature* 307:187–188, 1984.

The units for  $K_m$ , the binding constant of the enzyme for ATP, are millimolar units (mM); the units for  $k_{\text{cat}}$ , the catalytic rate constant, are reciprocal seconds ( $\text{s}^{-1}$ ); and the units for  $k_{\text{cat}}/K_m$ , the catalytic efficiency, are  $\text{s}^{-1} \text{M}^{-1}$ .

# Protein Engineering - Applications

## Engineering Ca-independency of subtilisin



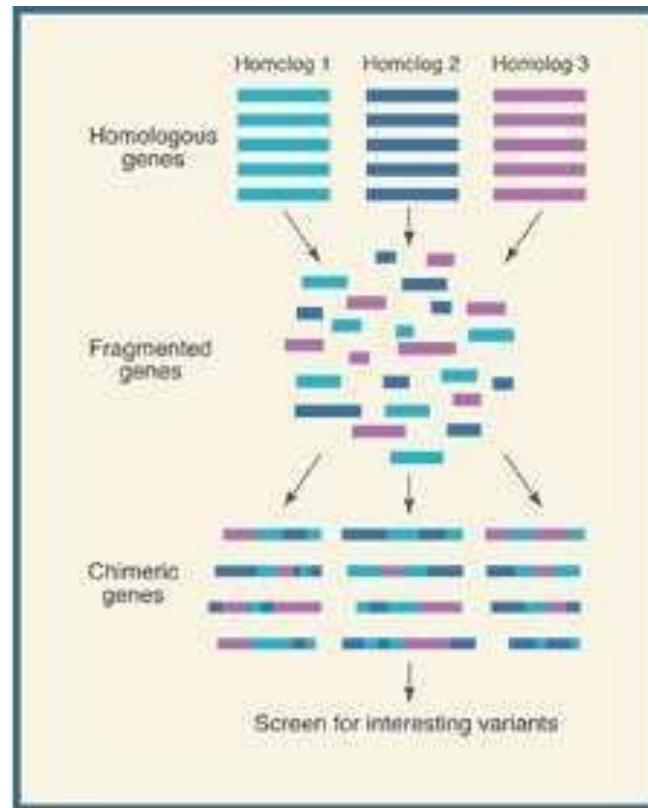
Saturation mutagenesis → 7 out of 10 regions were found to give increase of stability

Mutant:

10x more stable than native enzyme in absence of Ca

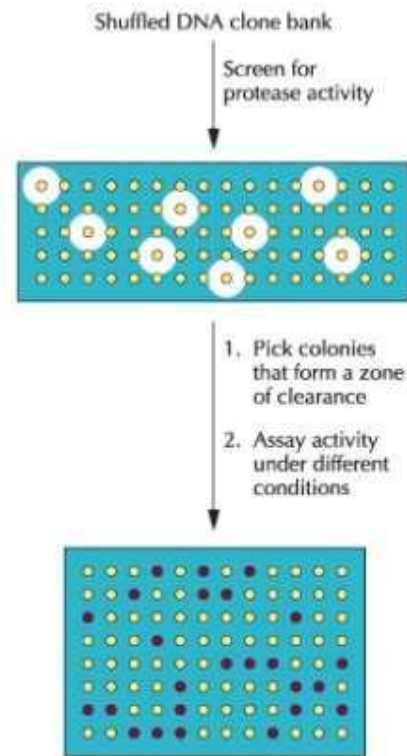
50% more stable than native in presence of Ca

# DNA shuffling



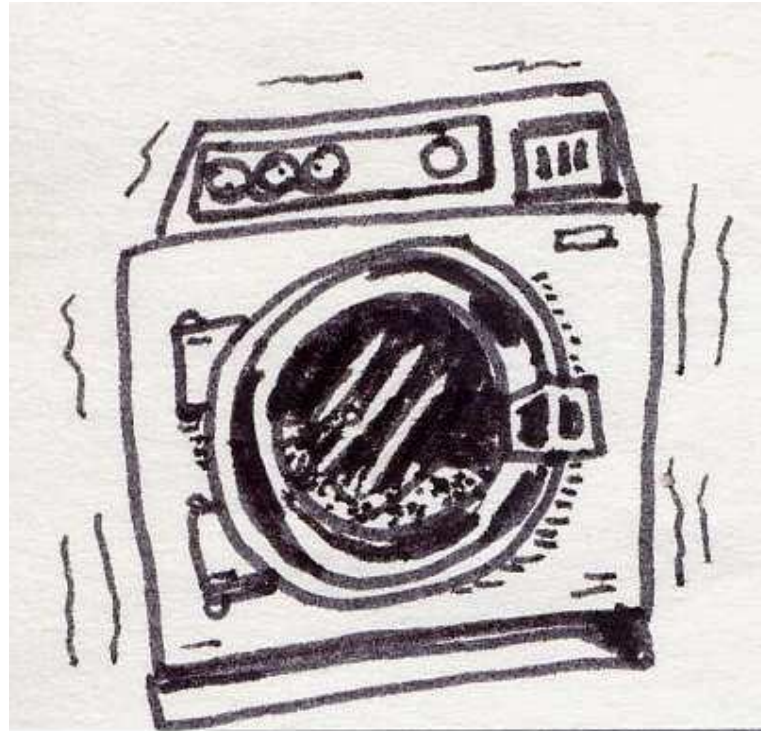
- JCohen. News note: How DNA shuffling works. *Sci* 293:237 (2001)
- Maxygen, PCR without synthetic primers
- Using family of related genes, digest into fragments
- Heat and renature randomly
- Use as PCR primers

# Altering multiple properties: rapid high-throughput screening



- ex., subtilisin
- Use 26 different subtilisin genes
- Shuffle DNA*, construct library of 654 clones, and Tf B. subtilis
- Assay in microtiter plates: originals plus clones
- Activity at 23C; thermostability; solvent stability; pH dependence
- Of 654 clones, 77 versions performed as well as or better than parents at 23C
- Sequencing showed chimeras; one has 8 crossovers with 15 AAc substitutions

# Laundry, detergent and mushrooms



- Peroxidase, ink cap mushroom; dye transfer inhibitor
- Wash conditions: bleach-containing detergents, pH 10.5, 50C, high peroxide concentration (inactivates peroxidase)
- Random mutagenesis or error-prone PCR, followed by DNA shuffling
- One construct had 114x increase in thermal stability, 2.8x increase in oxidative stability



# Mushroom peroxidase

**Table 1. Improved peroxidase stability**

Sense mutations (mutant number)	Source	Yeast broth screen data		Purified mutants index (mutant/wt)			
		Initial POXU/ml	% residual activity	Thermostability (% remaining)	H <sub>2</sub> O <sub>2</sub> stability	pH 7 specific activity	pH 10.5 specific activity
CiP	—	7	0	0.5	1.00	1.00	1.00
V53A, V319A	R1	34	1	1	2.93	1.13	0.58
M166F	SD	2	10	0	2.41	0.94	0.58
M166L	SD	4	9	nd	nd	nd	nd
M242I	SD	nd	nd	3	1.21	0.92	0.79
Y272F	SD	nd	nd	9	1.29	1.05	0.77
T34A, S198T, E239G	R1	5	22	29	1.04	0.69	0.70
I195V, E239V	R1	6	22	nd	nd	nd	nd
E239G	R1	11	38	73	1.13	0.96	0.78
E239K	SD	4	38	67	1.17	0.67	0.85
M166F E239K	SD	nd	nd	70	2.84	0.81	0.60
E239K M242I Y272F (Ø72)	SD	34	59	78	2.27	0.72	0.61
M166F E239K M242I Y272F	SD	15	63	62	4.65	0.74	0.36
E239G M242I Y272F	SD	19	44	77	5.07	nd	nd
I49T E239G M242I Y272F	R2	13	66	nd	nd	nd	nd
V53A E239G M242I Y272F	R2	16	80	nd	nd	nd	nd
T121A E239G M242I Y272F	R2	20	69	55	2.77	nd	nd
I49T V53A E239G M242I Y272F (Ø72)	SH	32	94	nd	nd	nd	nd
I49T V53A T121A M166F E239G M242I Y272F	SH	47	92	95	50	1.06	0.08
I49S V53A T121A M166F E239G M242I Y272F	SH	40	100	87	100	1.15	0.05

Table 1. Characterization of mutants. Assays were performed on yeast broth samples during mutant screening or on mutants purified from *A. oryzae* culture broths. Initial and residual activities were determined on yeast culture supernatants using ABTS as the colorimetric substrate at pH 7. Residual activities were determined after a 20 min incubation at 50°C in pH 10.5 buffer containing 0.2 mM H<sub>2</sub>O<sub>2</sub>. Thermostability of purified mutants is expressed as percent activity remaining after treatment for 5 min. at 40°C, pH 10.5. Hydrogen peroxide stability was determined by measuring enzyme half-life at 40°C in pH 10.5 buffer, 0.2 mM H<sub>2</sub>O<sub>2</sub>, and is expressed relative to wild-type CiP. pH 7 and 10 enzyme activities were determined at 30°C using a model dye as substrate as described in the Experimental Protocol. Source refers to the method used to generate the mutant; SD, site-directed mutagenesis; R1, first round of random mutagenesis; R2, second round of random mutagenesis; and SH, in vivo shuffling. "nd" indicates assay values were not determined.

- ex., Coprinus cinereus heme peroxidase (ink cap mushroom); 343 AAC, heme prosthetic group
- Multiple rounds of directed evolution to generate mutant for dye transfer inhibitor in laundry detergent
- Native form or WT is rapidly inactivated under laundry conditions at pH 10.5,
- 50C and high peroxide concentrations (5-10mM)
- Combined mutants from site-directed and random mutagenesis led to mutant with
- 110x thermal stability, 2.8x oxidative stability
- Additional in vivo shuffling of pt mutations -> 174x thermal stability and 100x oxidative stability
- Cherry...Pedersen. 99. Nat Biotech “Directed evolution of a fungal peroxidase”

# Molecular analysis of hybrid peroxidase

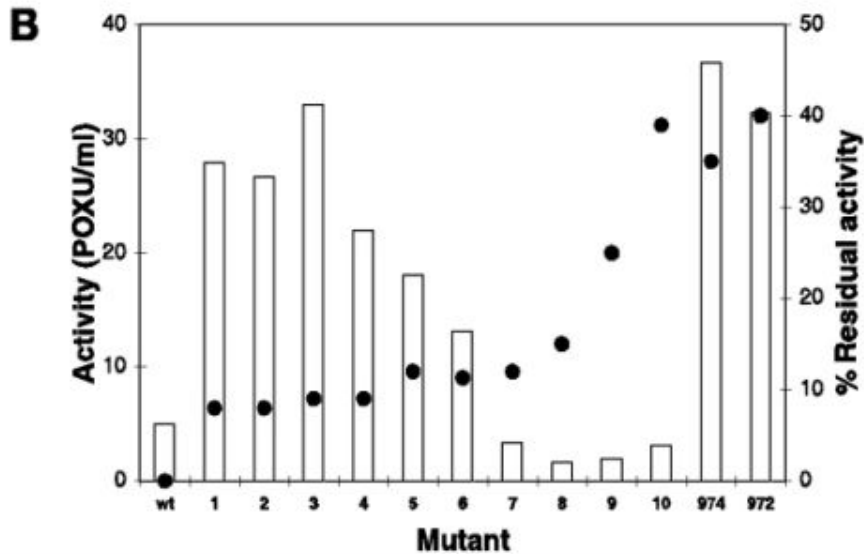
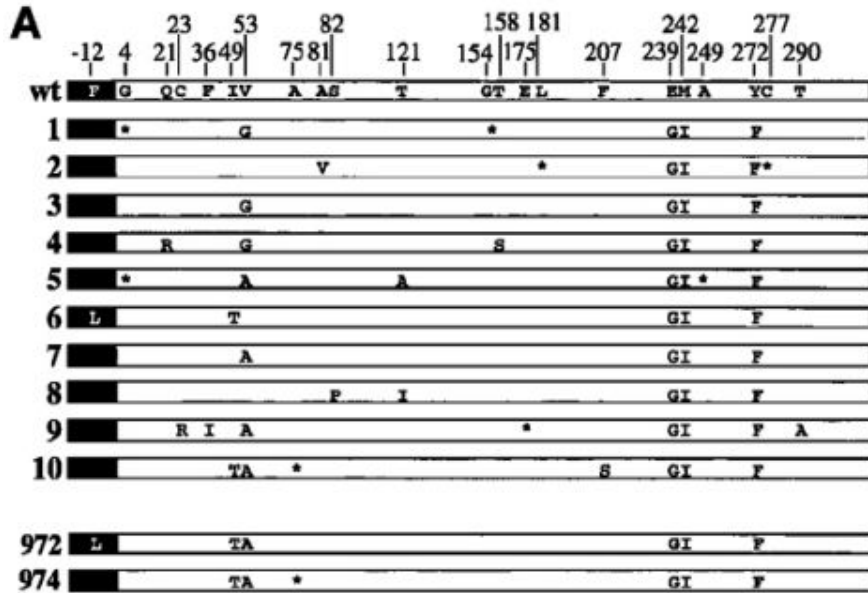
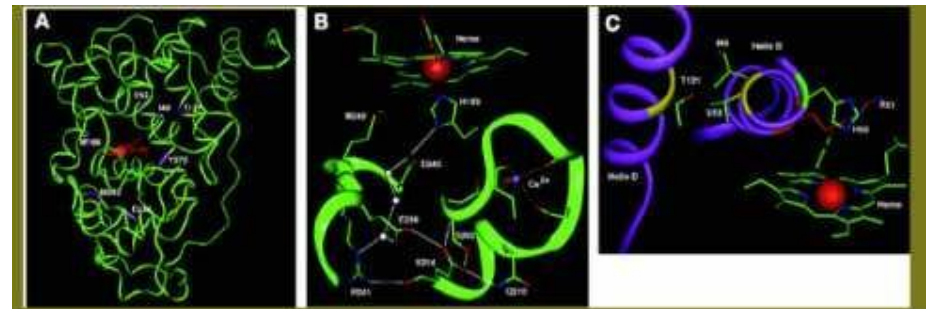


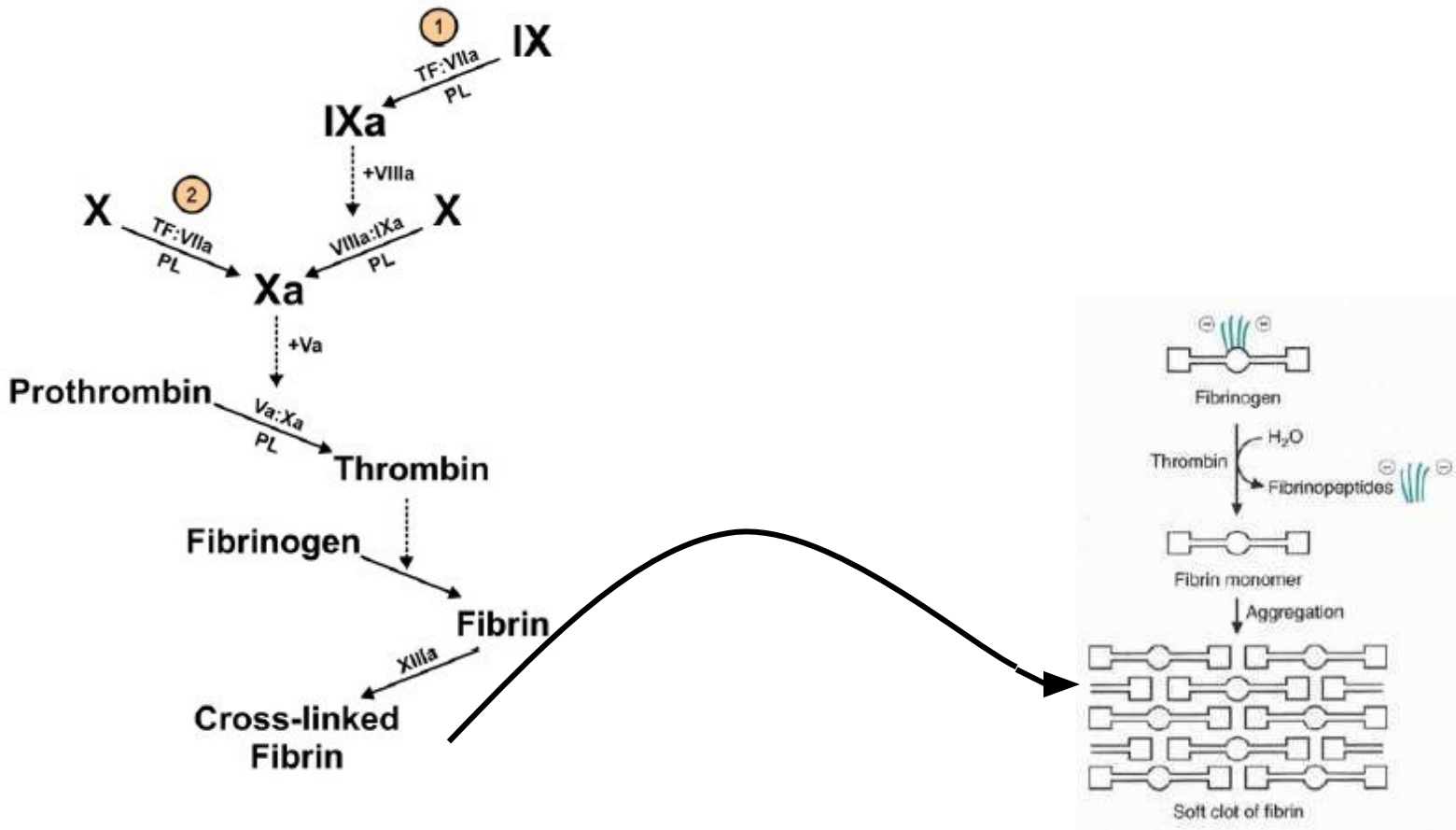
Figure 2. Results of in vivo shuffling of improved mutants.



**Figure 4. Structural mapping of amino acid substitutions.**

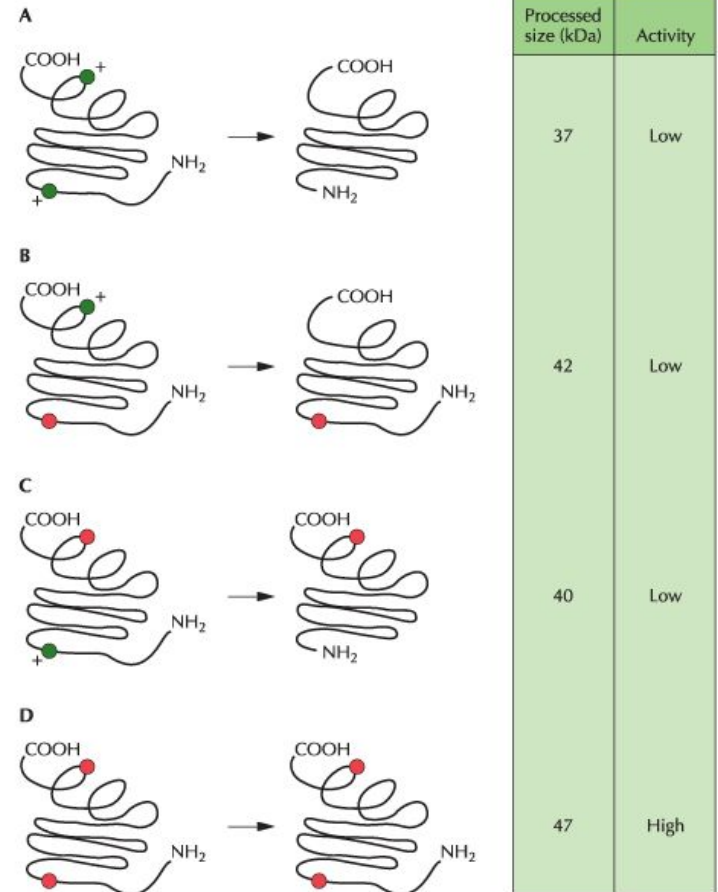
(A) Overview of the seven amino acids identified to be important in thermal and oxidative stability. (B) Partial view of the proximal side of CIP heme showing the water (white) network connecting E239 and H183 and stabilizing interactions between E214 and the calcium (Ca) binding loop. (C) Helices B and D showing interaction between 149, V53, and T121 and catalytic residues H55 and R51.

# Decreasing protein sensitivity



- Streptococcus streptokinase, 47 kDa protein that dissolves blood clots
  - Complexes with plasminogen to convert to plasmin, which degrades fibrin in clots
  - Plasmin also degrades streptokinase [feedback loop]
- In practice, need to administer streptokinase as a 30-90 min infusion [heart attacks]
- A long-lived streptokinase may be administered as a single injection

# Decreasing protein sensitivity



- Streptococcus streptokinase, plasmin sensitivity domain
- Attacks at Lys59 and Lys382, near each end of protein
- Resultant 328 AAc peptide has ~16% activity
- Mutate Lys to Gln
- Gln has similar size/shape to Lys also no charge
- Single mutations similar to double to native in binding and activating plasminogen;
- In plasmin presence, half-lives increased with double as 21x more resistant to cleavage
- TBD...(2003) longer life wanted

# Protein Engineering - Applications

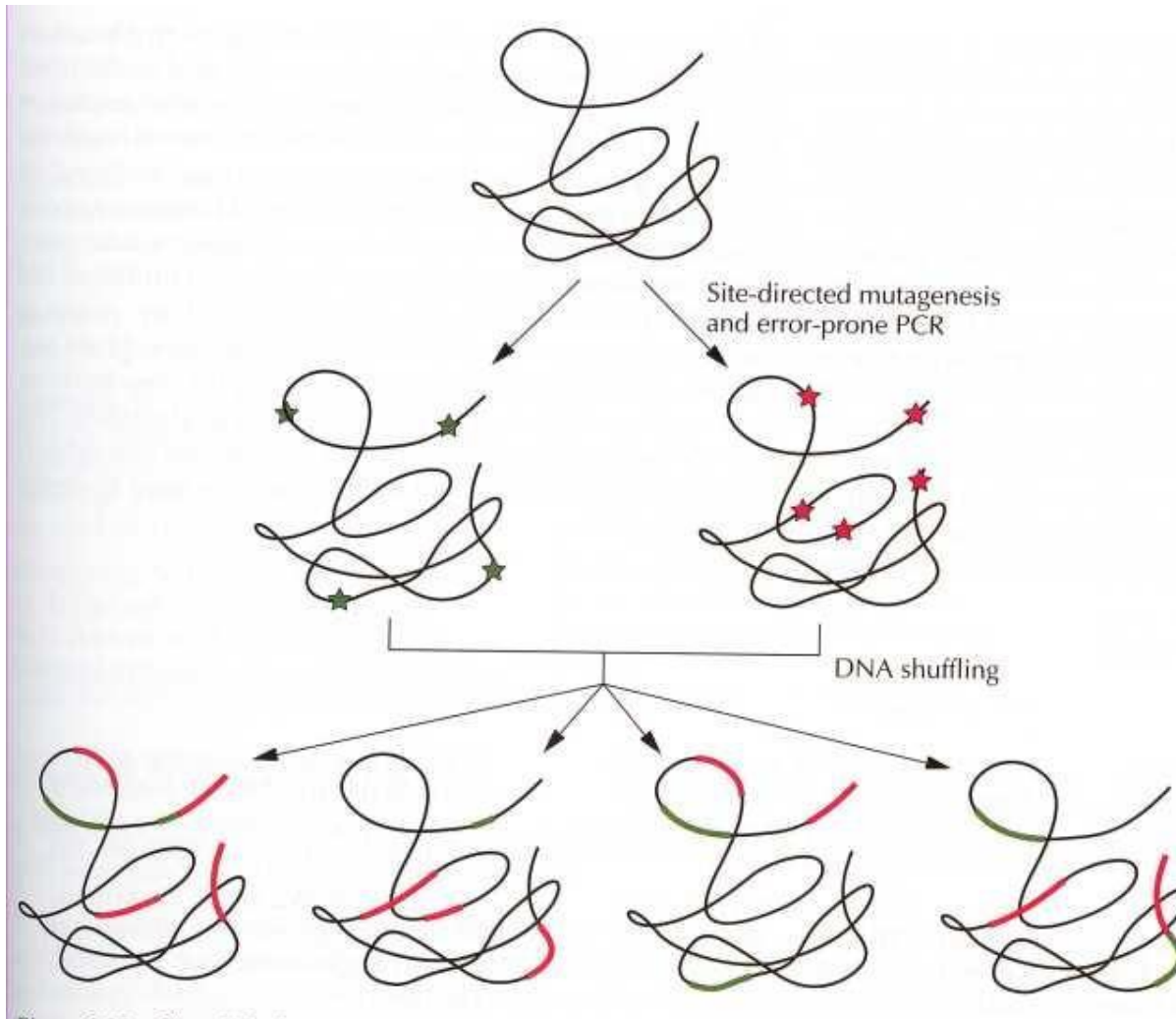
Site-directed mutagenesis -> used to alter a single property

Problem : changing one property -> disrupts another characteristics

Directed Evolution (Molecular breeding) -> alteration of multiple properties

# Protein Engineering - Applications

## Directed Evolution



# Protein Engineering - Applications

## Directed Evolution

**Table 1.** Examples of the diversity of properties that have been improved by evolutionary engineering

Protein	Altered function	Reference
Barley $\alpha$ -amylase	Thermostability: ten-fold increase of half-life at 90°C	JOYET et al. 1992
Subtilisin	Alkaline stability: doubling the autolytic half-time at pH 12	CUNNINGHAM and WELLS (1987)
Subtilisin	Tolerates loss of stabilizing divalent cations	STRAUSBERG et al. (1995)
Subtilisin E	Active in 60% DMF	YOU and ARNOLD (1996)
<i>Streptomyces griseus</i> protease B	Broadened substrate specificity	SIDHU and BORGFORD (1996)
Green fluorescent protein	40-fold brighter fluorescing bacterial colonies	CRAMERI et al. (1996)
Immunoglobulin constant domain	Preferential formation of heterodimers	ATWELL et al. (1997)
Immunoglobulin variable domain	Tolerates loss of structural disulfide bridge	MARTINEAU et al. (1998)

# Protein Engineering - Applications

## Directed Evolution

**Table 2.** Representative experiments using successive cycles of variation and selection

Protein	Property	Number of cycles for success	Number of nucleotide changes required	Number of amino acid changes required	Reference
$\beta$ -Lactamase	Increased activity	3	4	4	STEMMER (1994b)
GFP	Improved folding and expression	3	3	3	CRAMERI et al. (1996)
Subtilisin E	Stability in aqueous DMF	2	3	3	YOU and ARNOLD (1996)
Arsenite membrane pump	Increased activity	3	3	3	CRAMERI et al. (1997)
FLP-recombinase	Thermostability	8	3-4	3-4	BUCHHOLZ et al. (1998)

In almost all cases a single nucleotide change leading to a single amino acid change was sufficient per cycle, the number of silent mutations was approximately the same. No amino acid change was reported that would have required more than one nucleotide change. Thus current protocols appear to sample sequence space in a biased fashion, in single mutation steps.



# Protein Engineering - Applications

## Directed Evolution

**Table 1. Summary of key directed-enzyme-evolution experiments utilizing sequential generations of random mutagenesis and recombination with selection or screening.**

Enzyme	Altered property	Mutagenesis method	Screened or selected	Refs
Kanamycin nucleotidyltransferase	Thermostability	Mutator strain	Selected	4
Subtilisin E	Activity in organic solvents	Error-prone PCR	Screened	5,6
$\beta$ -Lactamase	Total activity and substrate specificity	DNA shuffling	Selected	7
Subtilisin BPN'	Stability	Cassette	Screened	8
Paranitrobenzyl esterase	Substrate specificity and activity in organic solvents	Error-prone PCR and recombination	Screened	9
Thymidine kinase	Substrate specificity	Cassette	Both	10
$\beta$ -Galactosidase	Substrate specificity	DNA shuffling	Screened	11
Arsenate detoxification pathway	Arsenic resistance	DNA shuffling	Screened	12
Paranitrobenzyl esterase	Substrate specificity and activity in organic solvents	Error-prone PCR and DNA shuffling	Screened	13

# Protein Engineering - Directed Evolution

Table 1

**Genes and operons evolved by DNA shuffling.**

System	Improvement	Size	Cycles	Mutations	Comments	Reference
TEM-1 $\beta$ -lactamase	Enzyme activity 32,000-fold	1 kb	3 + 2 <sup>†</sup>	6 aa	Selection MIC 0.02 $\mu$ g/ml to 640 $\mu$ g/ml In comparison, three cycles of mutagenic PCR and selection=16-fold	[12]
$\beta$ -lactamase family	Enzyme activity 270-540-fold	1 kb	1	Chimerics	Selection 500-fold jump in fitness in one round of shuffling and screening 50,000 colonies	[16**]
$\beta$ -galactosidase	Fucosidase activity 66-fold Substrate specificity 1,000-fold	4 kb	7	6 aa 13 bp	Screen 10,000 colonies per round, best 20-40 chosen per cycle 66-fold fucosidase activity: 2-3-fold increase expression plus 20-fold increase activity 11/13 base mutations in coding sequence	[30**]
Green fluorescence protein	Protein folding 45-fold ( <i>E. coli</i> and mammalian cells)	0.8 kb	3	3 aa 6 bp	Screen 10,000 colonies per round, best 20-40 chosen per cycle Protein folding improvement	[31]
Antibody (scFv)	Avidity >400-fold	0.8 kb	8 + 2 <sup>†</sup>	34 aa	Phage panning Naive human antibody library Stop codon modified to suppressible stop codon	[32]
Antibody (scFv)	Expression level 100-fold	0.8 kb		5	Phage panning Murine hybridoma library	[32]
Arsenate operon	Arsenate resistance 40-fold	2.3 kb	3	3 aa 13 bp	Selection Three genes; 5.5 kb plasmid shuffled 10,000 colonies per cycle All three mutations in <i>arsB</i> efflux pump, <i>arsC</i> was predicted to be rate limiting Arsenate reduction activity up 5-10-fold Growth in 0.5 M arsenate Plasmid integrated in round 3	[19*]
Atrazine degradation	Atrazine degradation 80-fold	5.6 kb	4	8 aa <sup>‡</sup>	Screen Two genes: 8.5 kb plasmid shuffled Atrazine chlorohydrolase activity increased Wild-type inactive versus terbutylazine, evolved enz active Screen: insoluble atrazine to soluble product gives zone of clearing	
Alkyl transferase	DNA repair 10-fold	0.5 kb	6	7 aa	Selection Suicide enzyme: limited potential for improvement	FC Christians, G Dawes, WPC Stemmer, unpublished data
Benzyl esterase	Antibiotic deprotection 150-fold	1.5 kb	2	8 aa	Screen Four rounds of PCR mutagenesis and screening for improved variants prior to shuffling	[20]
tRNA synthetase	Charging of engineered tRNA 180-fold	2.0 kb	7	nd	Selection	[22]

<sup>†</sup>Additional backcross cycles. <sup>‡</sup>Rare mutation: 3 NA insertions in 5 codons restored frame, inserted 1 aa with 5 other aa, changed *atz* gene lost 2 kb in final mutant, no loss of function. aa, amino acid changes; bp, base pair changes; nd, not determined.

# Protein Engineering - Applications

*Table 8.1* Some industrial enzymes and their commercial uses

Enzyme	Industrial use(s)
$\alpha$ -Amylase	Beer making, alcohol production
Aminoacylase	Preparation of L-amino acids
Bromelain	Meat tenderizer, juice clarification
Catalase	Antioxidant in prepared foods
Cellulase	Alcohol and glucose production
Ficin	Meat tenderizer, juice clarification
Glucoamylase	Beer making, alcohol production
Glucose isomerase	Manufacture of high-fructose syrups
Glucose oxidase	Antioxidant in prepared foods
Invertase	Sucrose inversion
Lactase	Whey utilization, lactose hydrolysis
Lipase	Cheese making, preparation of flavorings
Papain	Meat tenderizer, juice clarification
Pectinase	Clarifying fruit juices, alcohol production
Protease	Detergent, alcohol production
Rennet	Cheese making

**Table 3** Some useful web sites for protein engineering and PCR applications

Web address	Information
<a href="http://www.expasy.ch">www.expasy.ch</a>	Range of proteomic tools, from translation to homology modeling
<a href="http://biotech.embl-heidelberg.de:8400/">http://biotech.embl-heidelberg.de:8400/</a>	Checking protein models for problems
<a href="http://www.bmm.icnet.uk/~3dpssm/">www.bmm.icnet.uk/~3dpssm/</a>	Recognition of protein folds
<a href="http://www.firstmarket.com">www.firstmarket.com</a>	Restriction map generator
<a href="http://www.chemie.uni-marburg.de/becker/welcome.html">http://www.chemie.uni-marburg.de/becker/welcome.html</a>	Provides useful links to useful software for PCR and primer design
<a href="http://www.ebi.ac.uk/software/software.html">http://www.ebi.ac.uk/software/software.html</a>	Archives of software for molecular biologists
<a href="http://endeavor.med.nyu.edu/download/molbio/">http://endeavor.med.nyu.edu/download/molbio/</a>	
<a href="http://bioinformatics.weizmann.ac.il/mb/software.html">http://bioinformatics.weizmann.ac.il/mb/software.html</a>	
<a href="http://alces.med.umn.edu/VGC.html">http://alces.med.umn.edu/VGC.html</a>	Useful for oligonucleotide T <sub>m</sub> and protein coding regions
<a href="http://bimas.dcrn.nih.gov/sw.html">http://bimas.dcrn.nih.gov/sw.html</a>	Useful links to software sites and molecular biology protocols
<a href="http://www.yk.rim.or.jp/~aisoai/soft.html">http://www.yk.rim.or.jp/~aisoai/soft.html</a> and <a href="http://iubio.bio.indiana.edu/soft/molbio/Listings.html">http://iubio.bio.indiana.edu/soft/molbio/Listings.html</a>	Useful collection of www links for information and services to molecular biologists
<a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a>	Links to GenBank <sup>®</sup> and sequence databases