LOSCHMIDT LABORATORIES

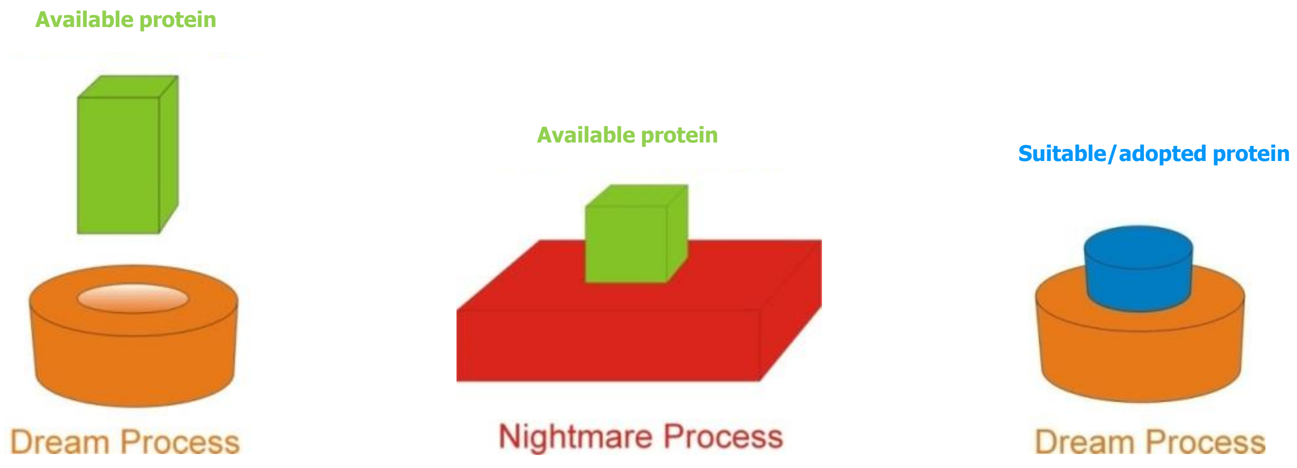
Protein Engineering

Outline

- Limitations of proteins in biotechnology processes
- Definition and aim of protein engineering
- Targeted properties of proteins
- Basic approaches in protein engineering
  - **DIRECTED EVOLUTION**
  - **RATIONAL DESIGN**
  - **SEMI-RATIONAL DESIGN**
- Examples

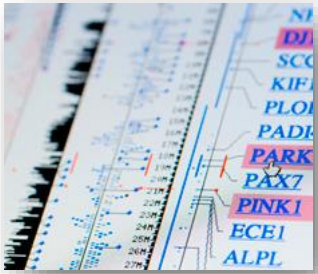
Proteins in biotechnology

- availability of optimal protein for specific process
- **traditional biotechnology** - adapt process
- **modern biotechnology** - adapt protein



Proteins in biotechnology

- **classical screening**
  - screening culture collections
  - polluted and extreme environment
- **environmental gene libraries**
  - metagenomic DNA
- **data-base mining**
  - gene databases
  - genome sequencing projects
  - numerous uncharacterised enzymes/proteins



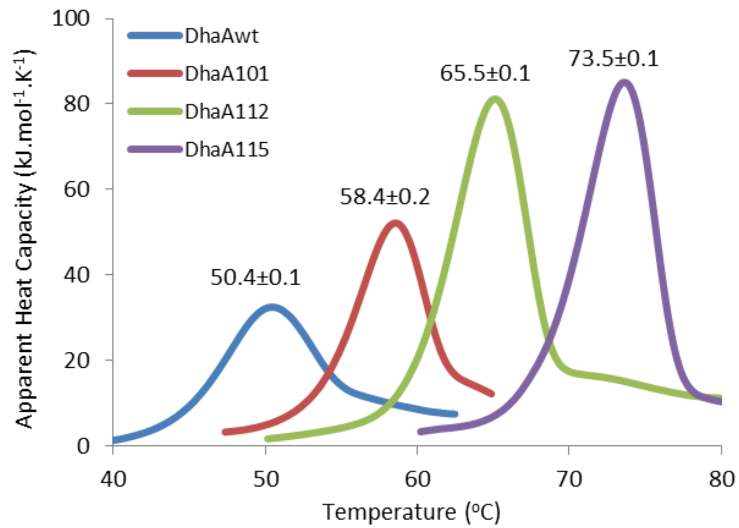
Proteins in biotechnology

- the process of **constructing novel protein** molecules by design first principles or altering existing structure
- use of genetic manipulations to alter the coding sequence of a gene and thus **modify the properties of the protein**

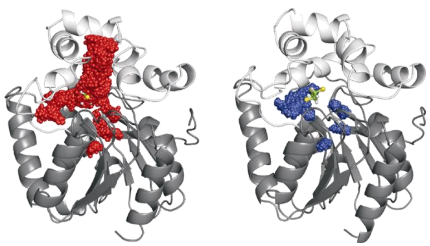
- AIMS AND APPLICATIONS
  - **technological** - optimisation of the protein to be suitable in particular technology purpose
  - **scientific** - desire to understand what elements of proteins contribute to folding, stability and function

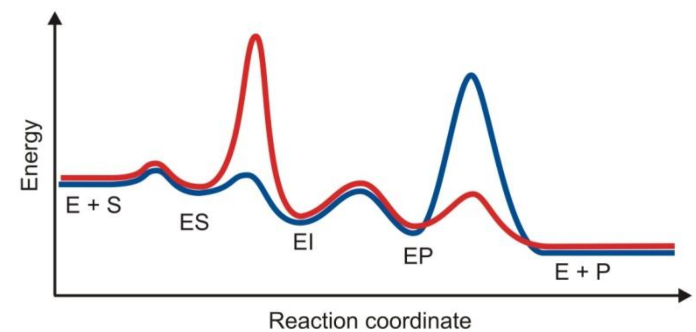
Targeted properties of proteins

- **structural** properties of proteins
  - stability (temperature, solvents)
  - tolerance to pH, salt
  - resistance to oxidative stress

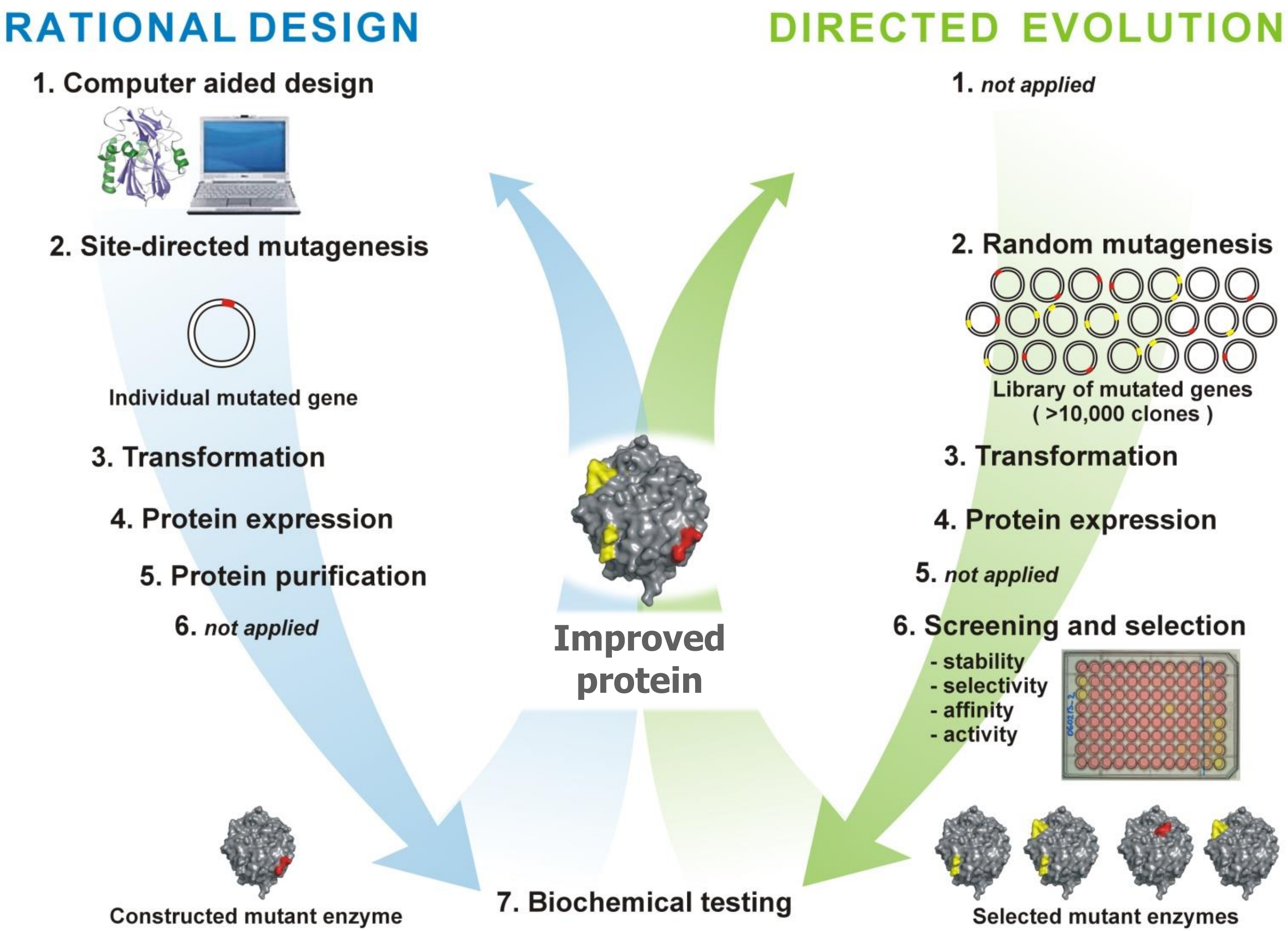


- **functional** properties of proteins
  - reaction type
  - substrate specificity and selectivity
  - kinetic properties (e.g., K_m, k_{cat}, K_i)
  - cofactor selectivity
  - protein-protein or protein-DNA interactions





Strategies in protein engineering



Directed evolution

- directed evolution techniques emerged during mid-1990s
- **inspired by natural evolution**
- this form of "evolution" does not match what Darwin had envisioned
  - requires **outside intelligence**, not blind chance
  - does not create brand new species, macroevolution,
    but only improvements of molecules, **molecular evolution**
  - does not take millions of years, but **happens rapidly**

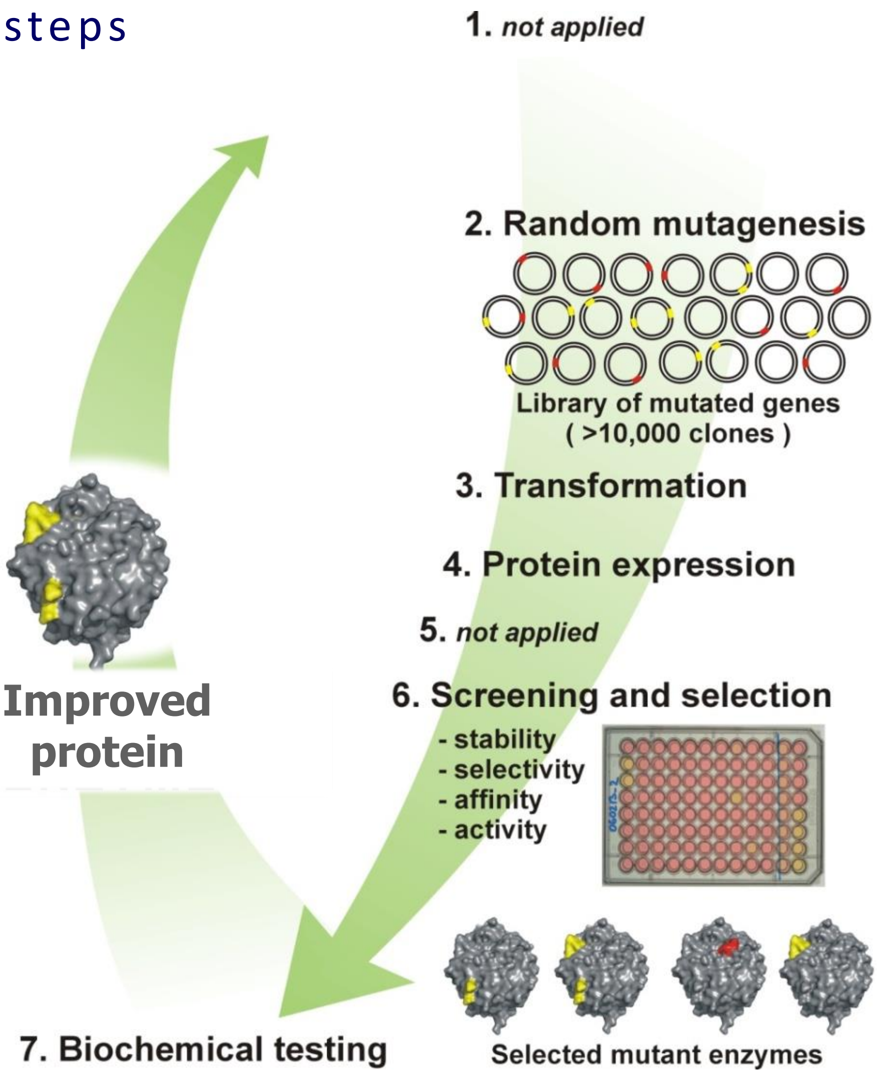
Directed evolution

- **evolution in test tube** comprises two steps
  - **random mutagenesis**

    mutant library building
  - **screening and selection**

    identification of desired biocatalyst

- **prerequisites** for directed evolution
  - gene encoding protein of interest
  - method to create mutant library
  - suitable expression system
  - screening or selection system

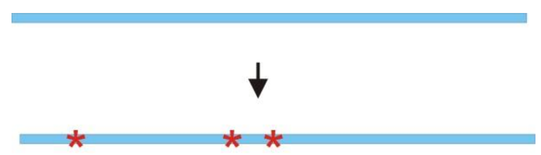


Methods to create mutant libraries

- technology to **generate large diversity**

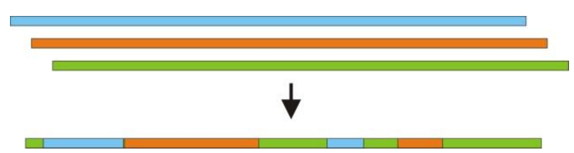
  - **NON-RECOMBINING**

    one parent gene -> variants with point mutations



  - **RECOMBINING**

    several parental homologous genes -> chimeras



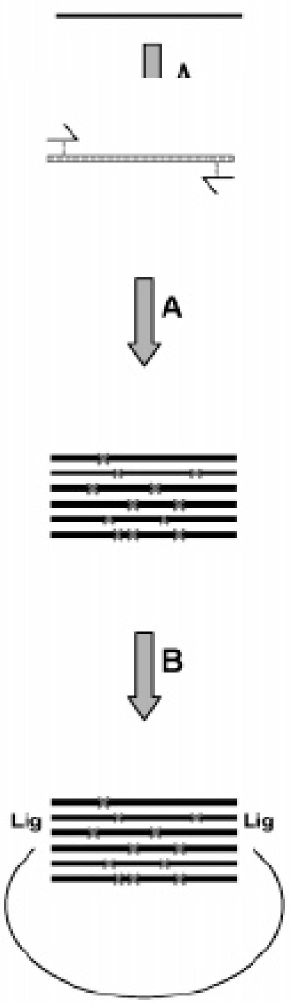
Non-recombining mutagenesis

- **UV irradiation** or **chemical mutagens** (traditional)
- **mutator strains** - lacks DNA repair mechanism

  mutations during replication (e.g., *Epicurian coli* XL1-Red)
- **error-prone polymerase chain reaction** (ep-PCR)
  - gene amplified in imperfect copying process

    (e.g., unbalanced deoxyribonucleotides concentrations,

    high Mg^{2+} concentration, Mn^{2+}, low annealing temperatures)
  - 1 to 20 mutation per 1000 base pairs
- **saturation mutagenesis**
  - randomization of single or multiple codons
- **other methods**
  - gene site saturation mutagenesis
  - cassette mutagenesis (region mutagenesis)

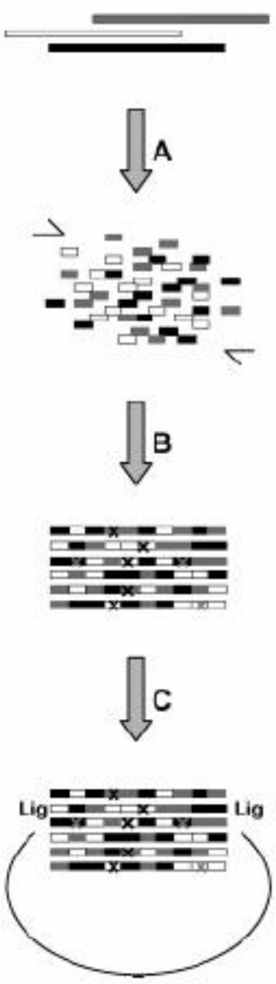


Recombining mutagenesis

- also refered to as **„sexual mutagenesis"**
- **DNA shuffling**
  - fragmentation step
  - random reassembly of segments
- **StEP** - staggered extension process
  - simpler then shuffling
  - random reannealing combined with limited primer extension
- **other methods**

  shuffling of genes with lower homology down to 70%

  (e.g., RACHITT, ITCHY, SCRATCHY)

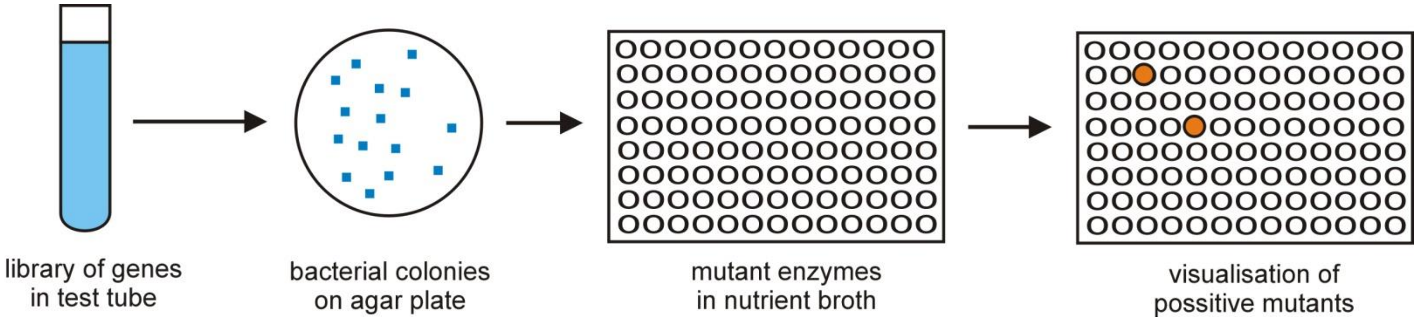


Screening and selection

- most **critical step** of direct evolution
- isolation of positive mutants hiding in library
  - **HIGH THROUGHPUT SCREENING**

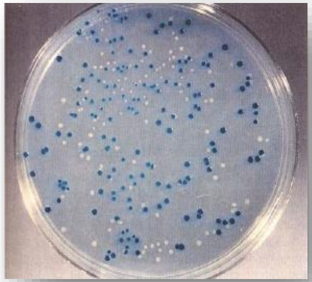
    individual assays of variants one by one
  - **DIRECT SELECTION**

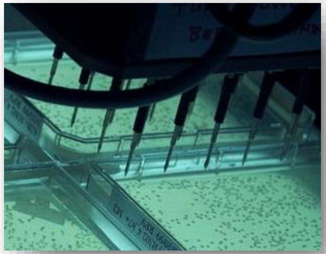
    display techniques (link between genotype and phenotype)

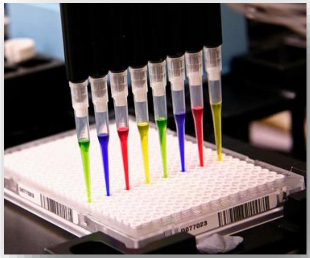


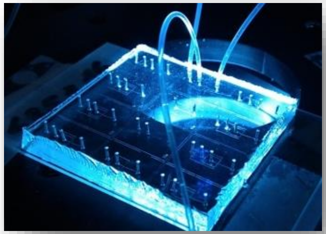
(Utra)High throughput screening

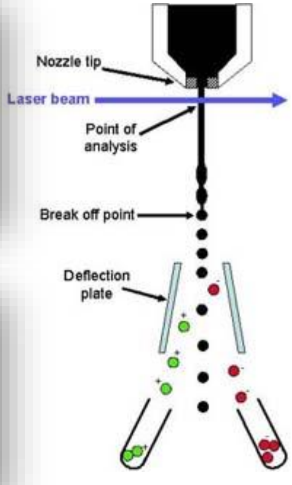
- common methods not applicable
- **agar plate (pre)screening**
- **microtiter plates screening**
  - 96-, 384- or 1536-well formate
  - robot assistance (colony picker, liquid handler)
  - 10^4 libraries
  - volume 10 – 100 uL
- **microfluidic systems**
  - water in oil emulsions (up to 10 kHz)
  - FACS sorting (10^8 events/hour)
  - 10^9 libraries
  - volume 1 – 10 pL

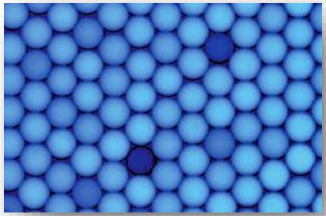






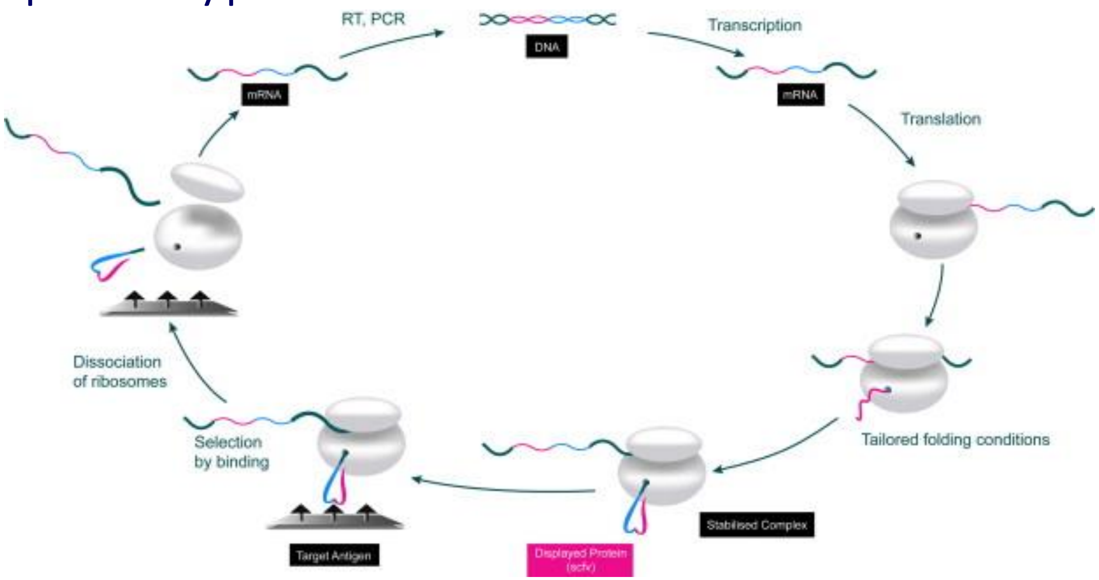


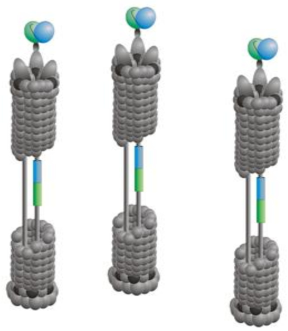




Direct selection

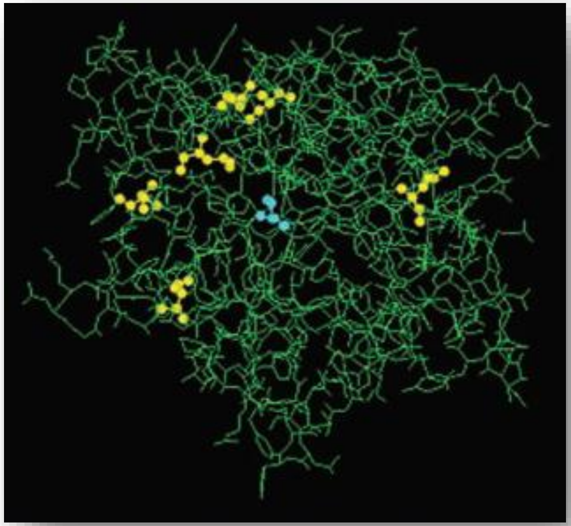
- not generally applicable (mutant libraries >10^6 variants)
- link between genotype and phenotype
- **display technologies**
  - ribosome display
  - phage display
- **life-or-death assay**
  - auxotrophic strain
  - toxicity based selection

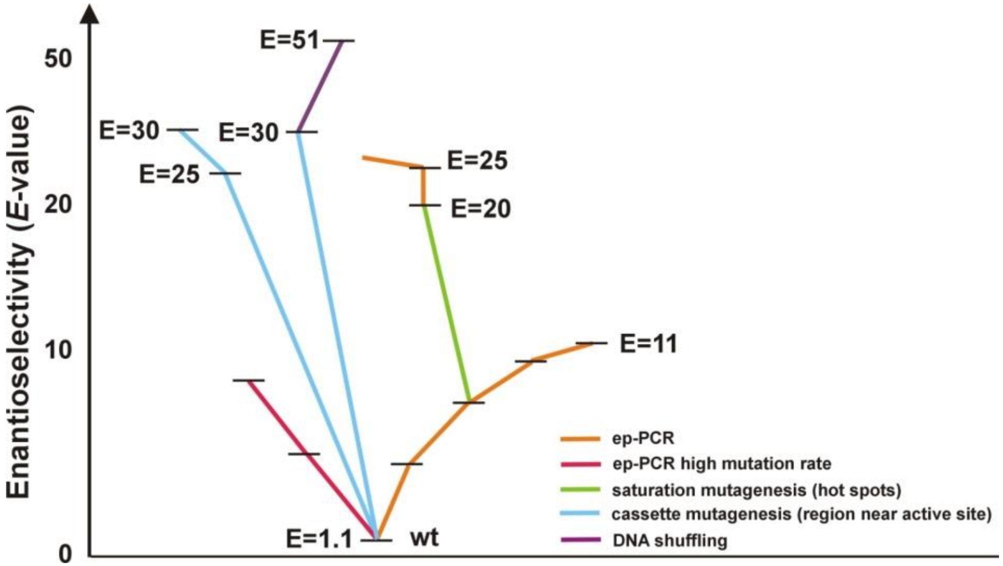




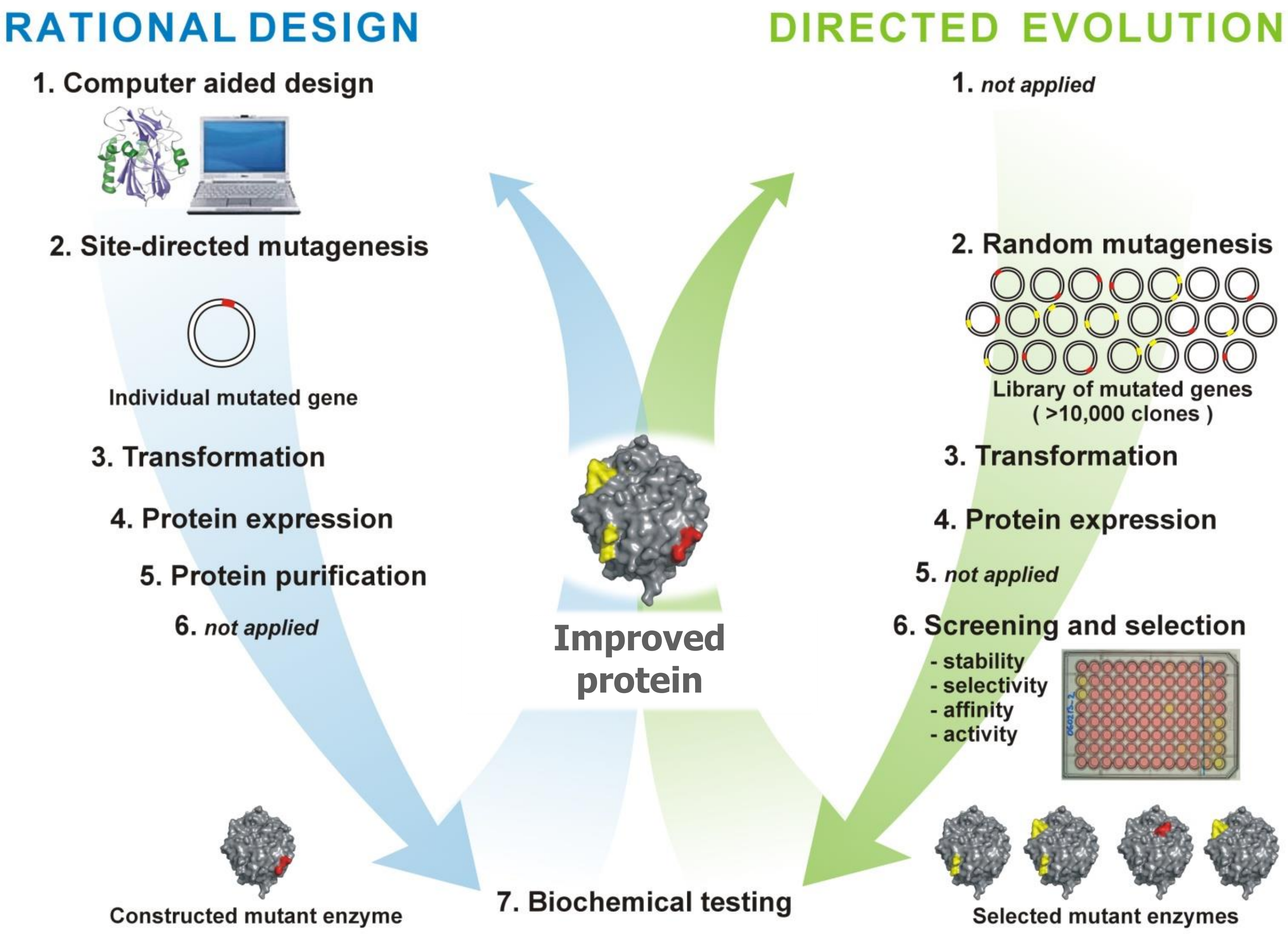
Example of Directed evolution

- directed evolution of **enantioselectivity**
  - lipase from *P. aeruginosa* (E-value improved from 1.1 into 51)
  - **spectrophotometric screening** of (*R*)- and (*S*)-nitrophenyl esters
  - **40 000 variants** screened
  - the best mutant contains six amino acid substitutions





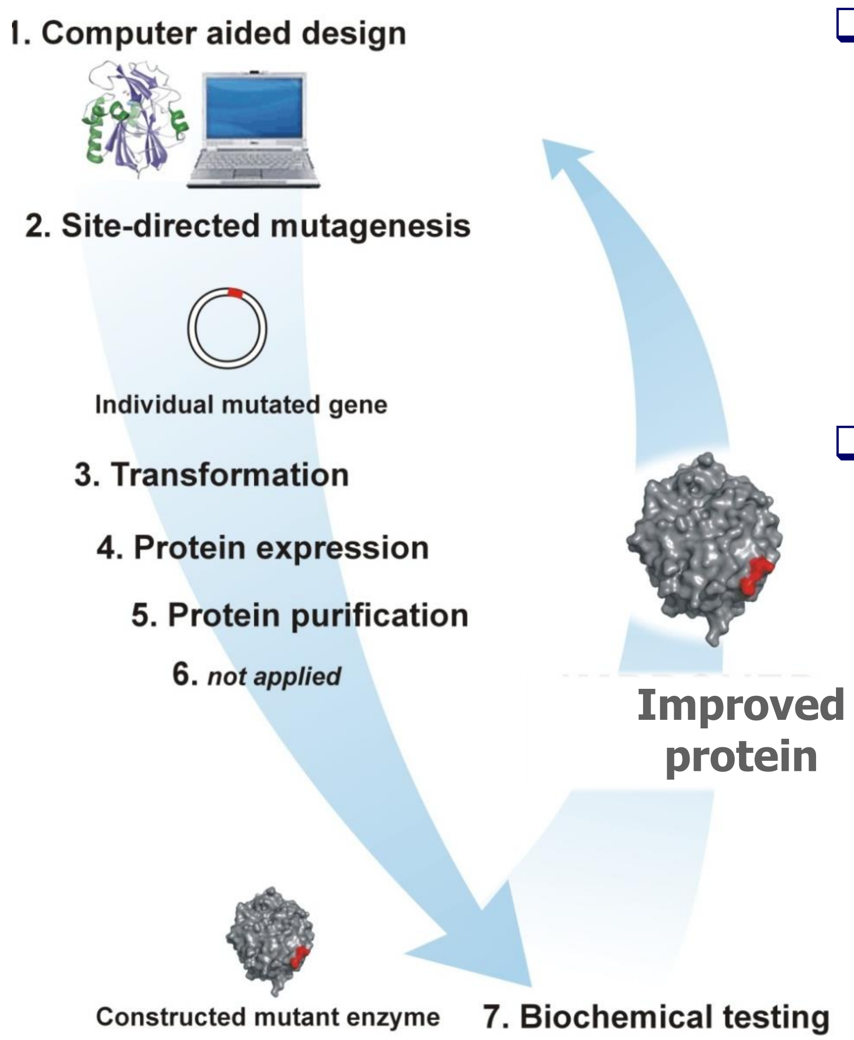
Strategies in protein engineering



Rational design

- emerged around 1980s as the original protein engineering approach
- **knowledge based** - combining theory and experiment
- protein engineering cycle:
  „structure-theory-design-mutation-purification-analysis“
- **difficulty in prediction** of mutation effects on protein property
- **de novo design**

Principal of rational design

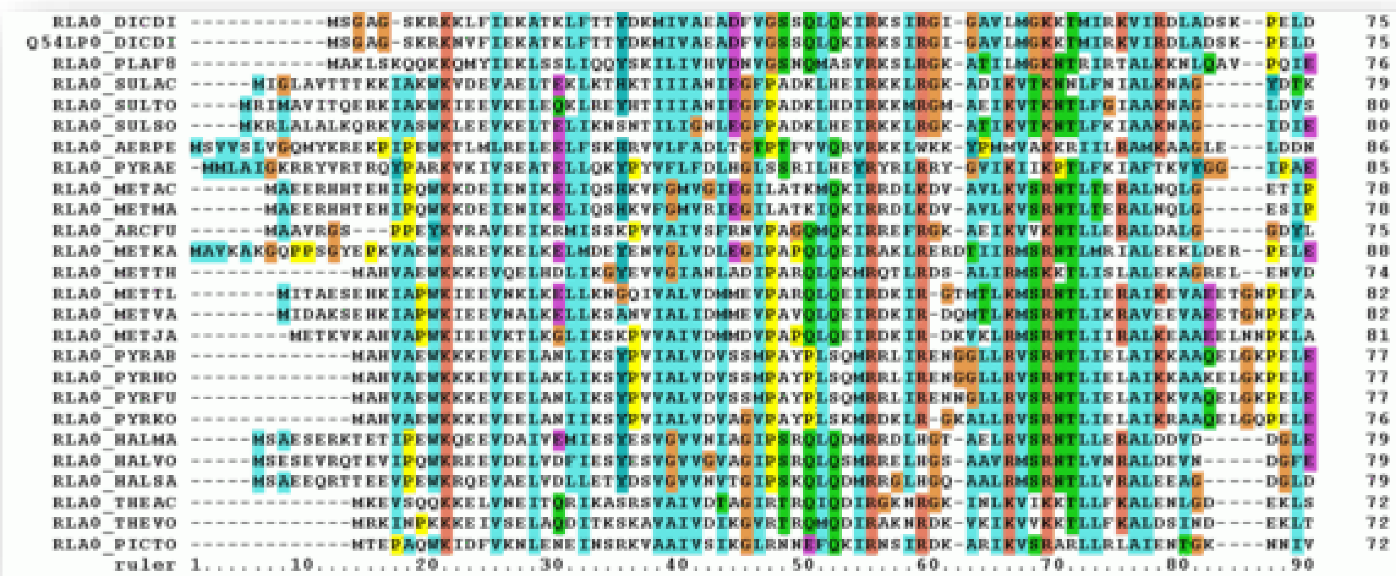


- rational design comprises:
  - **design** - understanding of protein functionality
  - **experiment** - construction and testing of mutants
- **prerequisites** for rational design:
  - gene encoding protein of interest
  - 3D structure (e.g., X-ray, NMR)
  - structure-function relationship
  - computational methods and capacity
  - (multi)side directed mutagenesis techniques
  - efficient expression system
  - biochemical tests

Design

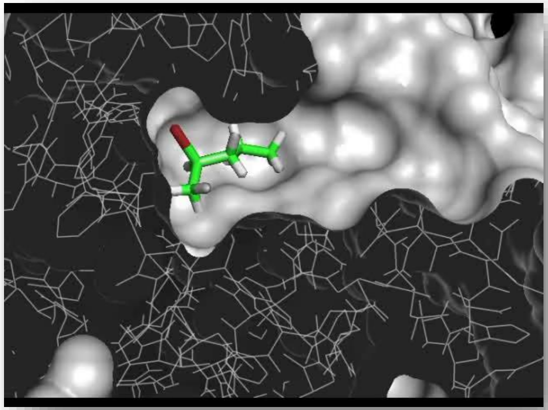
❑ HOMOLOGY APPROACH

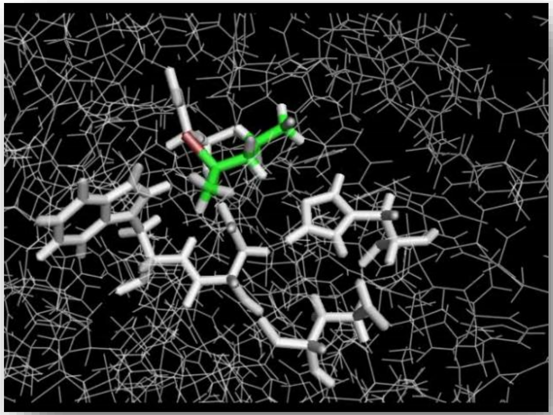
- homologous wild-type sequences are collected and compared
- identifying amino acid residues responsible for differences
- **reconstruction** - transfer differences from one enzyme to another
- **new design** - combination of possitive mutation from all parental proteins in one construct, new protein better than all parental

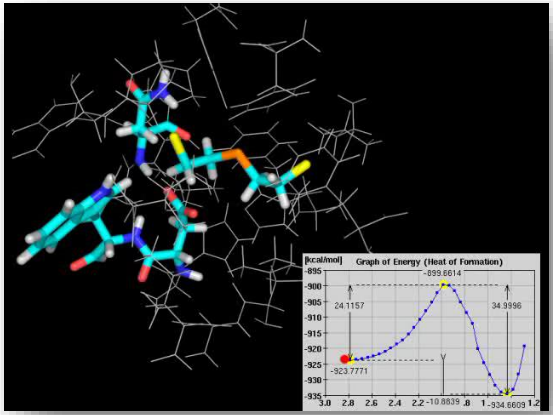


Design

- **STRUCTURE-BASED APPROACH**
  - **prediction** of enzyme function from structure alone is challenging
  - **protein structure** (X-ray crystallography, NMR, homology models)
  - **molecular modelling**
    - molecular docking
    - molecular dynamics
    - quantum mechanics/molecular mechanics (QM/MM)

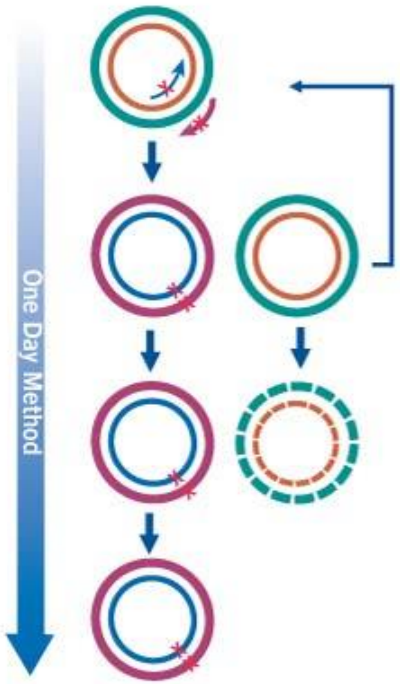






Construction

- site-directed mutagenesis
  - introducing point mutations
- multi site-directed mutagenesis
- gene synthesis
  - commercial service
  - codone optimisation



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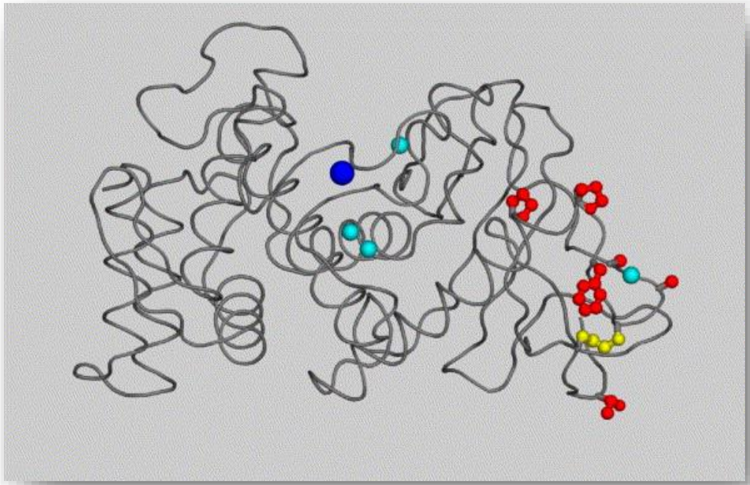
Example of rational design

- rational design of protein **stability**
  - stability to high temperature, extreme pH, proteases etc.
  - **stabilizing mutations** increase strength of weak interactions
    - **salt bridges and H-bonds**
      Eijsink et al., Biochem. J. 285: 625-628, 1992
    - **S-S bonds**
      Matsumura et al., Nature 342: 291-293, 1989
    - **addition of prolines**
      Watanabe et al., Eur. J. Biochem. 226: 277-283, 1994
    - **less glycines**
      Margarit et al., Protein Eng. 5: 543-550, 1992
    - **oligomerisation**
      Dalhus et al., J. Mol. Biol. 318: 707-721, 2002

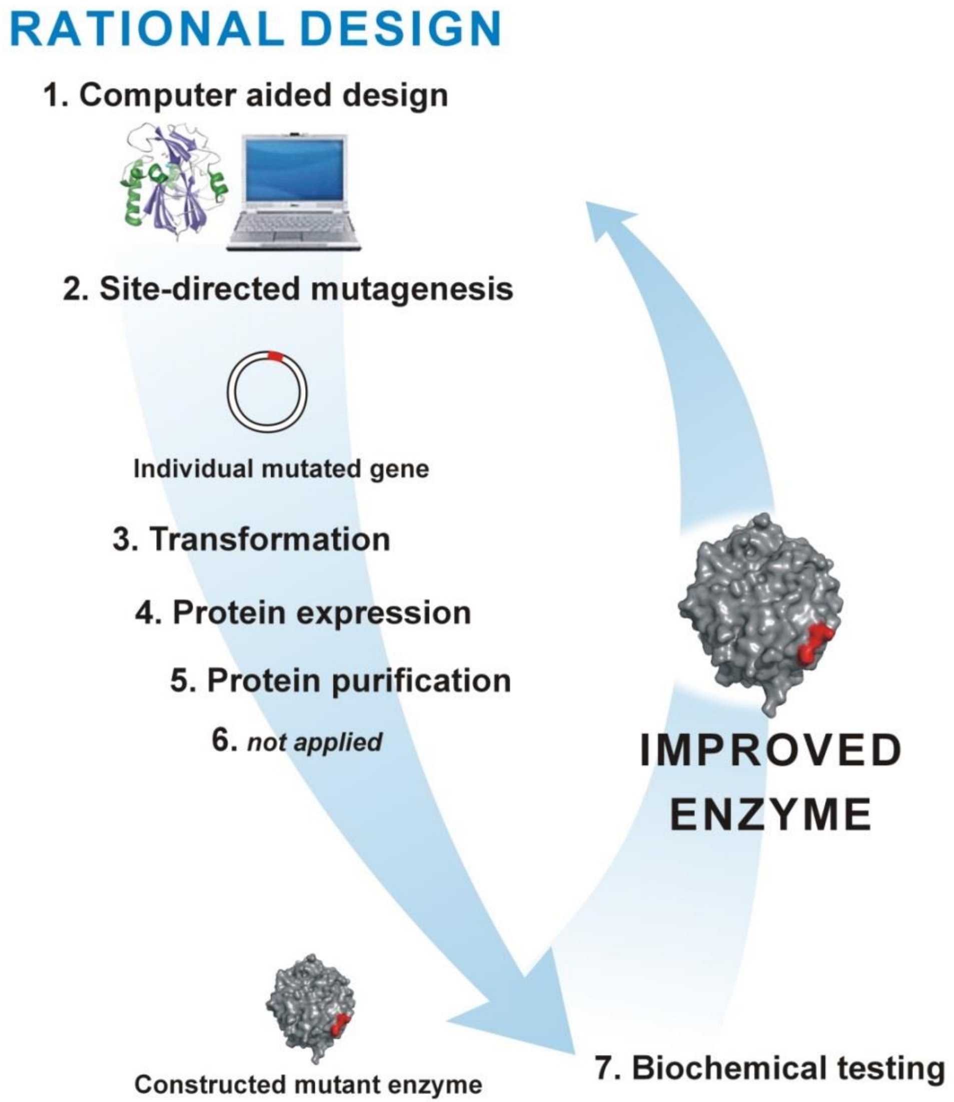
Example of rational design

- **engineering protein to resist boiling**
  - **reduced rotational freedom**
    Ser65Pro, Ala96Pro
  - **introduction of disulfide bridge**
    Gly8Cys + Asn60Cys
  - **improved internal hydrogen bond**
    Ala4Thr
  - **filling cavity**
    Tyr63Phe

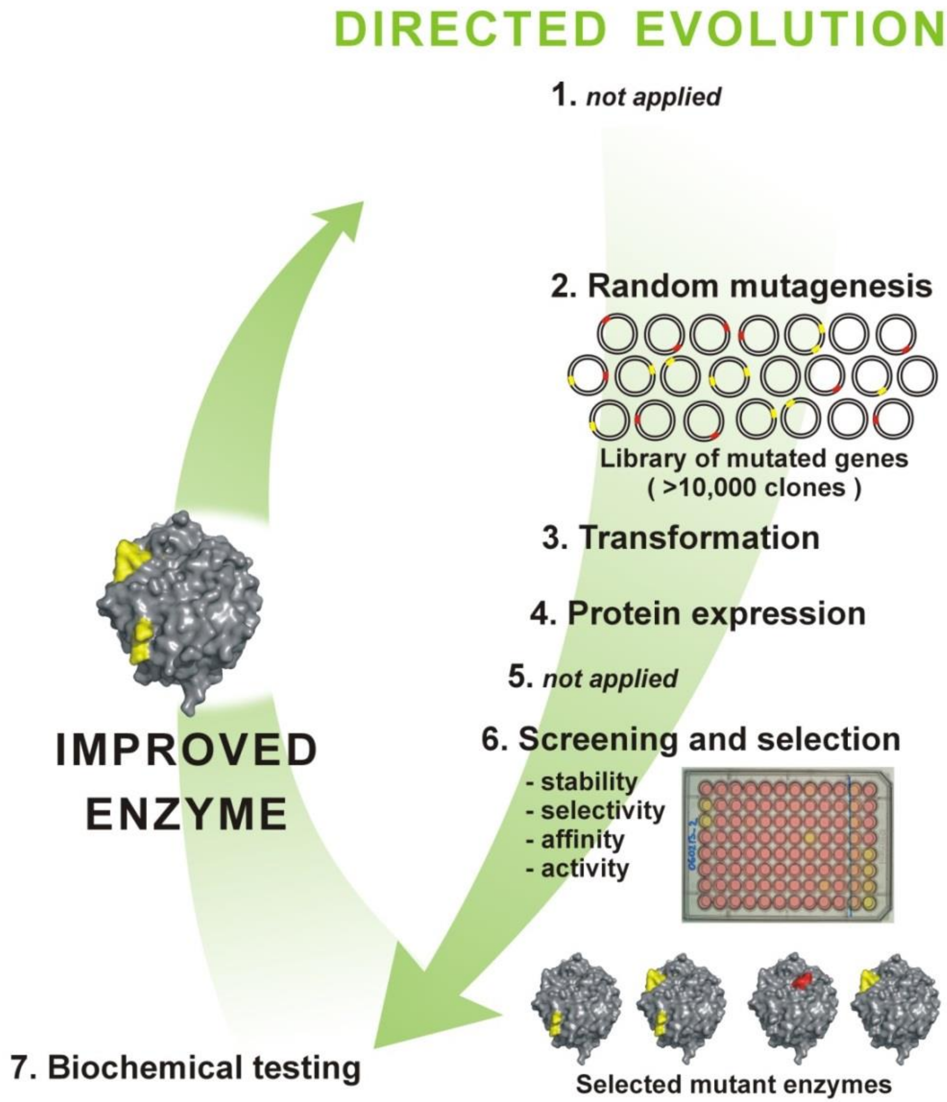


Half-lifes (min.)	80°C	100°C
wild type	17.5	>0.5
8-fold mutant	stable	170

Strategies in protein engineering



Strategies in protein engineering



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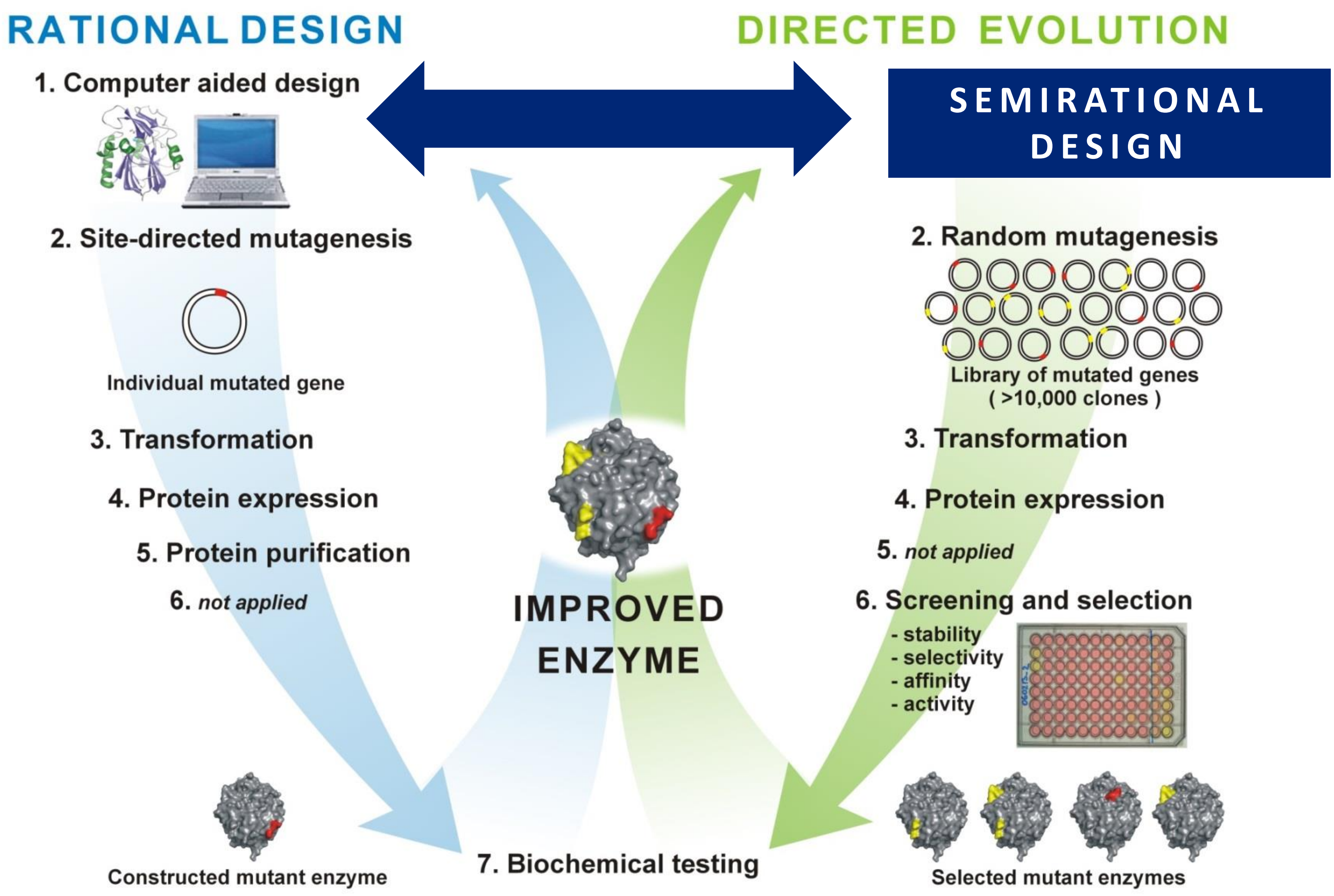
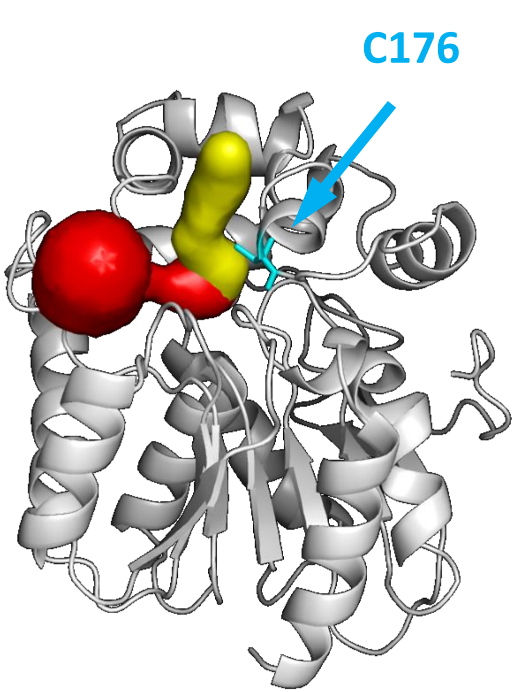
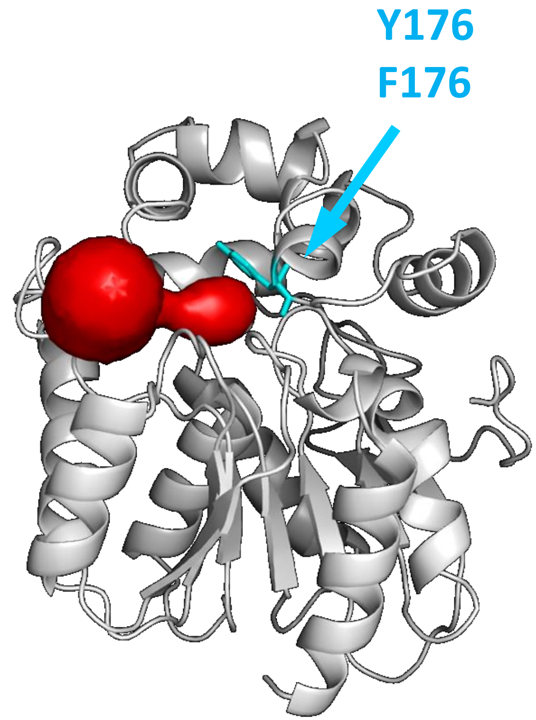


Example of rational design

- conversion of 1,2,3-trichloropropane
  by DhaA from *Rhodococcus erythropolis* Y2
- **DIRECTED EVOLUTION -** importance of access pathways



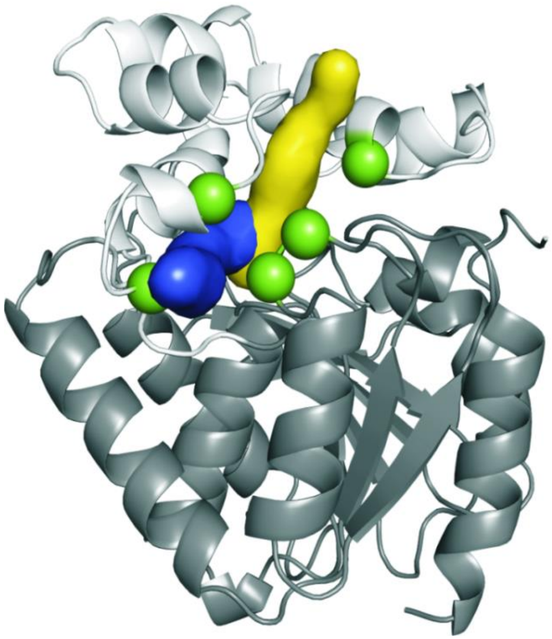


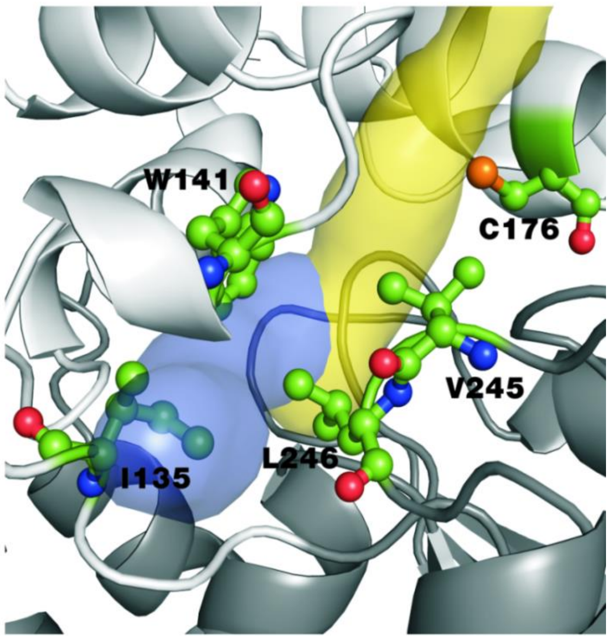
Bosma, T., et al. 2002: AEM 68: 3582-87

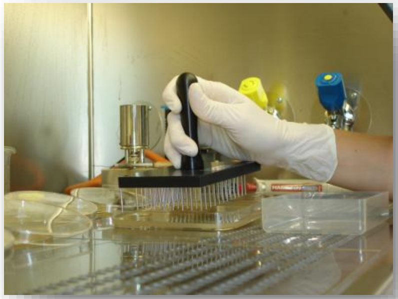
Gray, K.A., et al. 2003: Adv. Appl. Microbiol. 52: 1-27

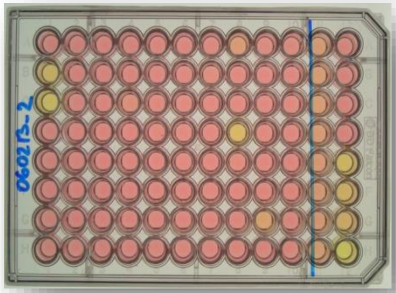
Example of rational design

- conversion of 1,2,3-trichloropropane by DhaA from *Rhodococcus erythropolis* Y2
- **DIRECTED EVOLUTION -** importance of access pathways
- **SEMI-RATIONAL DESIGN** - hot spots in access tunels
- library of **5,300 clones** screened



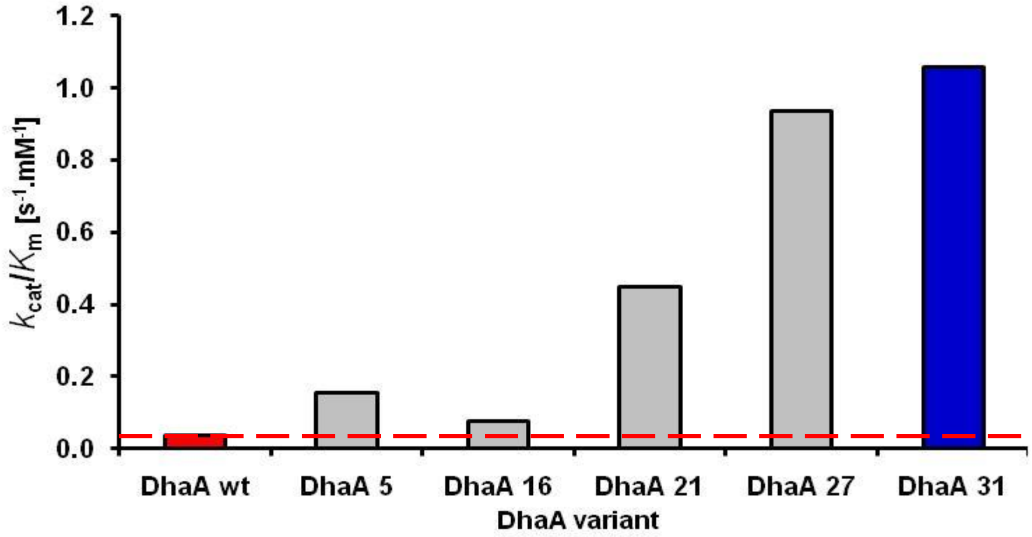


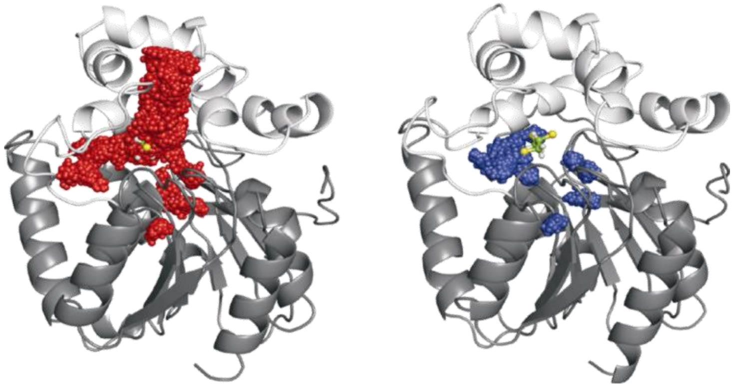


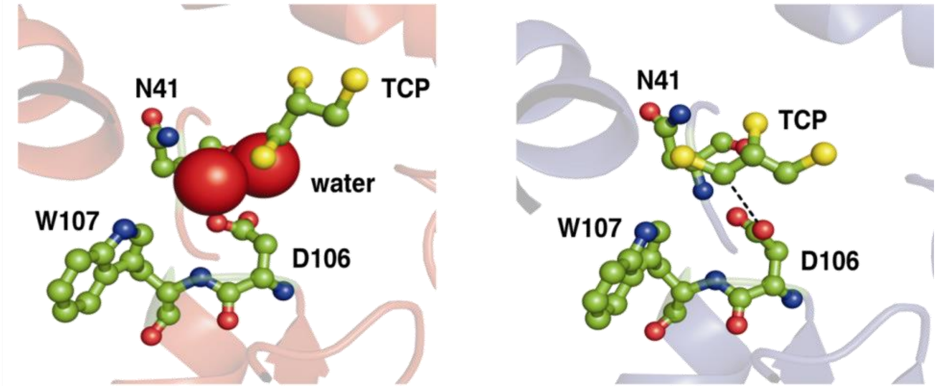


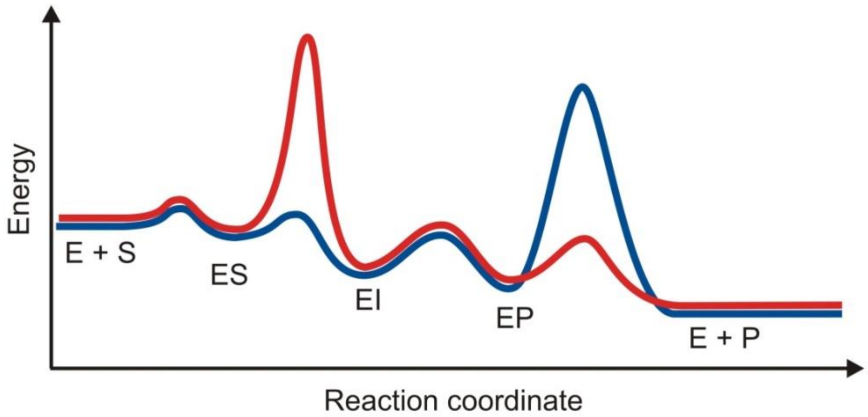
Pavlova, M., Klvana, M., Prokop, Z., et al. 2009: Nature Chem. Biol. 5: 727-733

Example of rational design



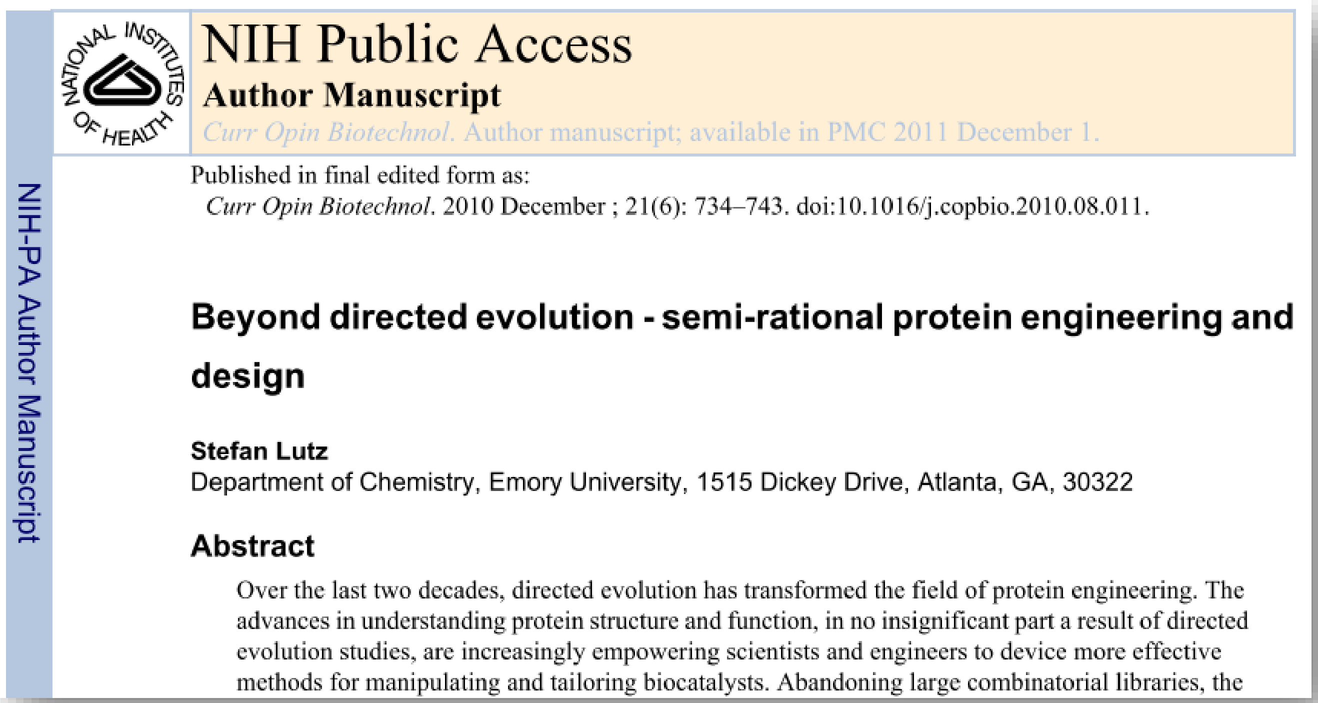






Reading

- Lutz, S. 2010: **Beyond directed evolution - semi-rational protein engineering and design**. *Curr Opin Biotechnol*. 21(6): 734–743
- *Computational enzyme redesign* and *Computational de novo enzyme design* (**page 5-7**)



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Beyond directed evolution - semi-rational protein engineering and design

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Abstract

Over the last two decades, directed evolution has transformed the field of protein engineering. The advances in understanding protein structure and function, in no insignificant part a result of directed evolution studies, are increasingly empowering scientists and engineers to device more effective methods for manipulating and tailoring biocatalysts. Abandoning large combinatorial libraries, the