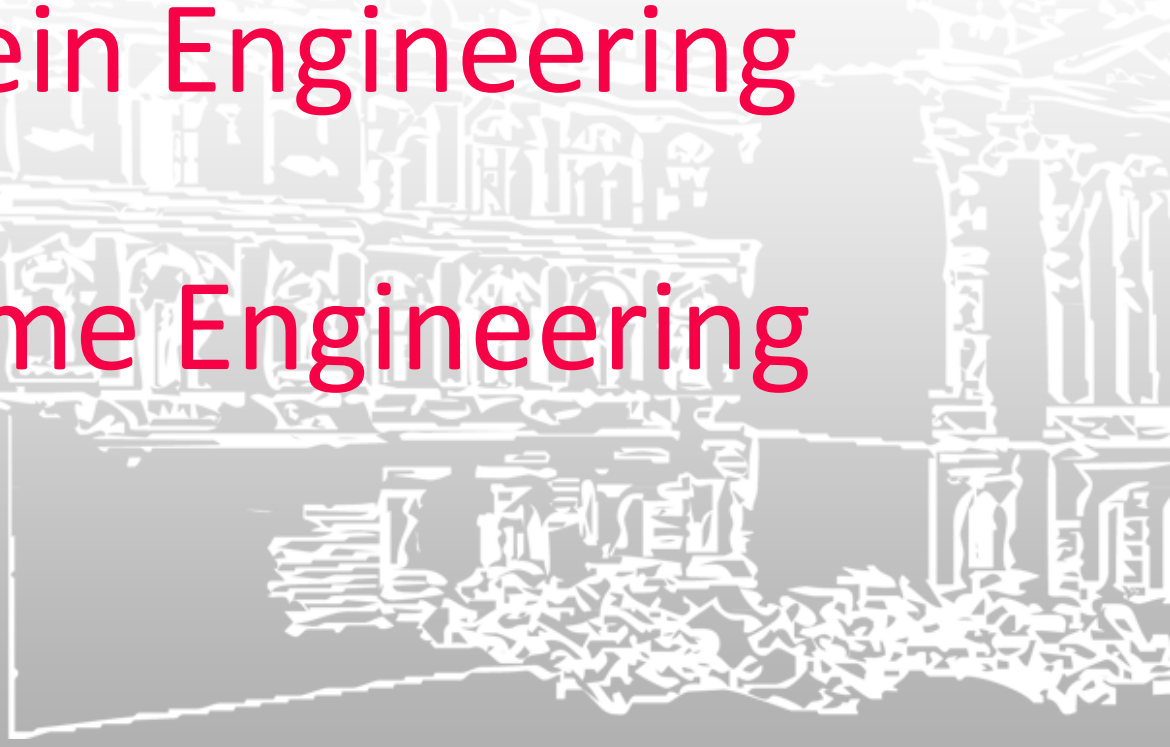


Protein Engineering

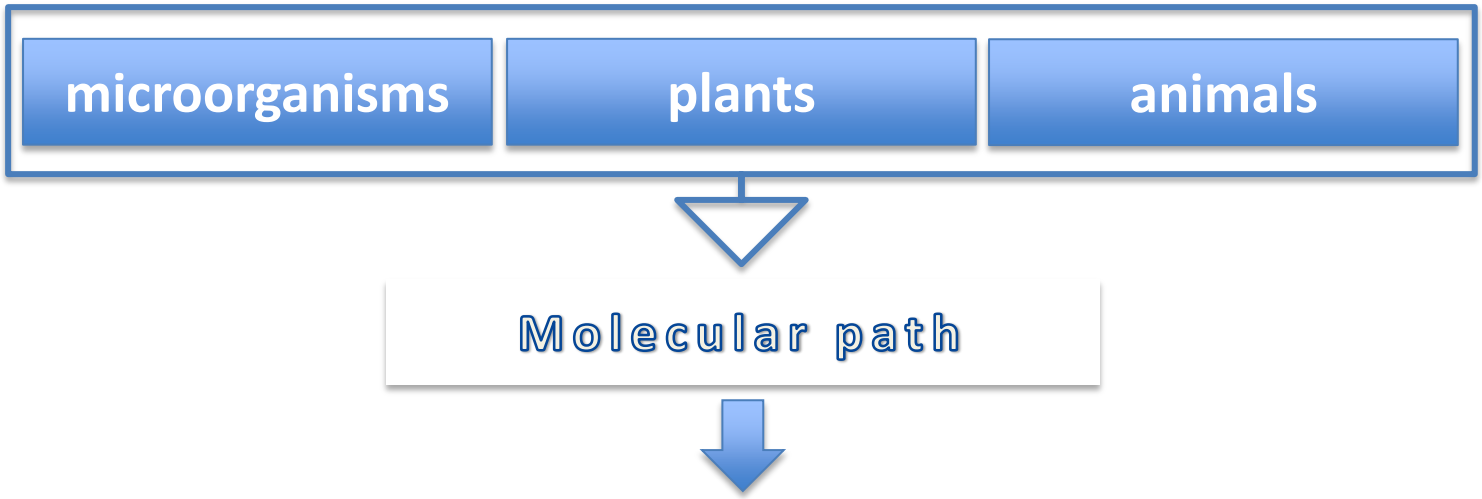
Enzyme Engineering

Molecular Biotechnology - Biocatalysis

- **Access to a broad diversity of biocatalysts**
  - -- natural diversity → „GENOMICS“
  - -- artificial diversity → „SYNBIO“
- **Economic production of enzymes**
  - -- recombinant enzymes
- **Efficient biocatalysts for any application**

  (fast and efficient methods for the development of enzymes)
  - -- enzyme engineering → “DIRECTED EVOLUTION“
    → “RATIONAL DESIGN“
- **Novel biocatalysts**
  - -- Nanobiotechnology
  - -- novel catalytic structures

Recruitment of enzymes from natural biodiversity



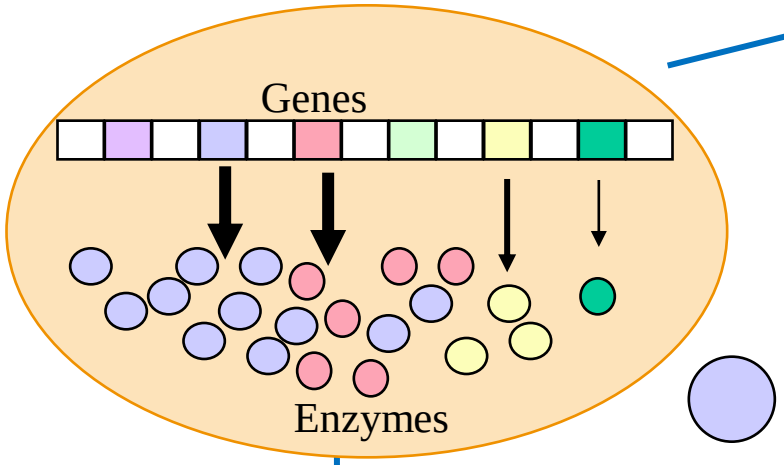
Isolation and cloning of gene(tic) material

Identification of clones with specific enzyme activity

Gene expression – gene technological production of enzymes

Recruitment of enzymes from natural biodiversity

Isolated Organism



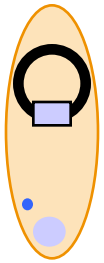
Screening

Natural diversity

Organism producing an interesting enzyme was identified

Isolation, purification and characterization of the enzyme

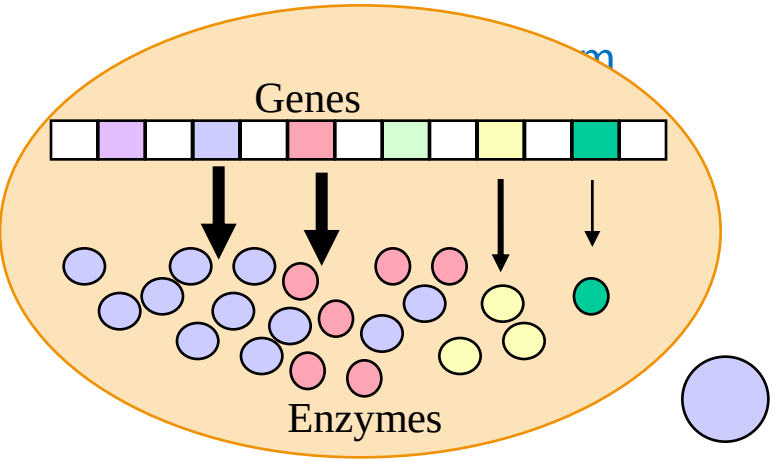
Cloning and expression of the specific gene



Recombinant microorganism

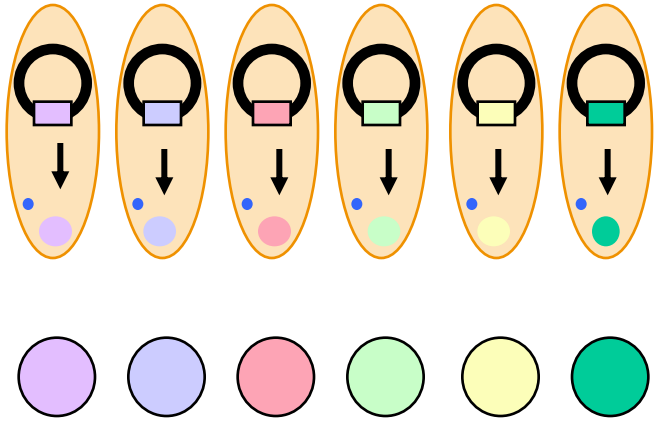
Recombinant protein

Recruitment of enzymes from natural biodiversity



Genomic Library

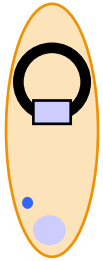
Gene Libraries



Screening

Natural diversity

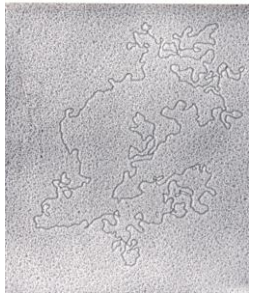
Screening



Recombinant microorganism

Recombinant protein

Recruitment of enzymes from natural biodiversity



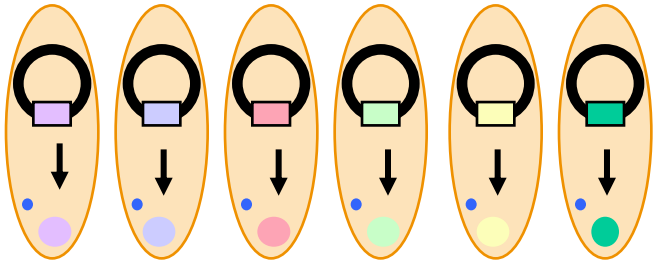
Cloning of DNA fragments

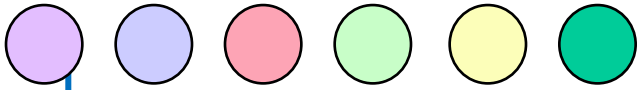
Gene libraries

Total DNA isolation

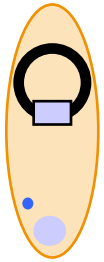
Non-cultivatable diversity

Metagenome Library





Screening



Recombinant microorganism

Recombinant protein

Recruitment of enzymes from natural biodiversity

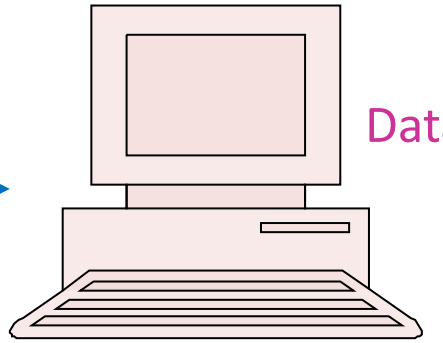
(Meta) Genome sequencing

David Parker/Science Photo Library/Photo Researchers, Inc.

Much of the speed with which recent advances in genetics research have been made results from the use of high-throughput DNA sequencers coupled with computerized sequence acquisition, like these devices at the Sanger Centre near Cambridge, England. This technology has made it possible to determine the complete DNA sequence of the human genome.

Natural diversity

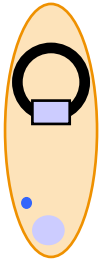
Sequence Database



Data Mining

Protein Sequence

Gene Design
Gene Synthesis



Recombinant microorganism

Recombinant protein

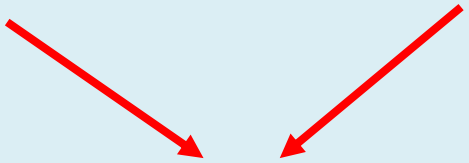
Designed Evolution

Concept of „Process Designed Enzymes“

Establish set of key enzymes – e.g. esterases
key structures / functionalities
genes - expression

Develop efficient routes to enzyme engineering
tuning enzymes towards specific process needs

Directed Evolution – Rational Design



Designed Evolution

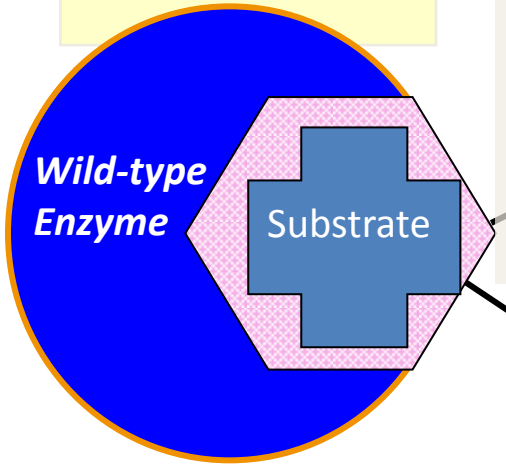
Enzyme-Engineering → basic routes

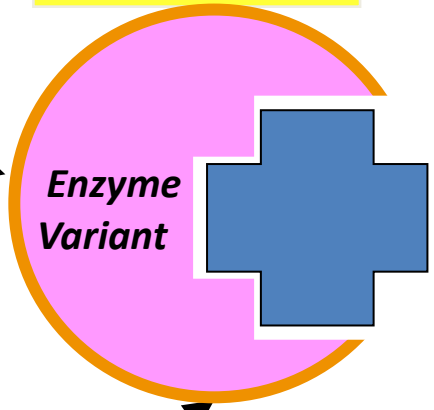
low activity
insufficient stability
unfavoured specificity
bad selectivity

Molecular Evolution
random mutagenesis
gene shuffling

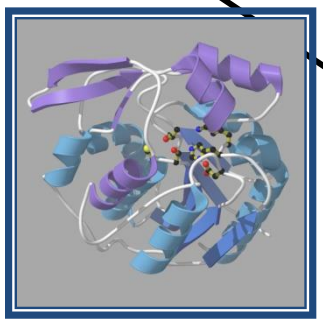
Variant library
Screening - Selection

high activity
excellent stability
proper specificity
good selectivity





Functional and molecular Characterization



defined specific variant
targeted construction

Rational Engineering
Structure-Function Relationships
Modelling, Design

Prerequisites for Protein Engineering by Rational Design

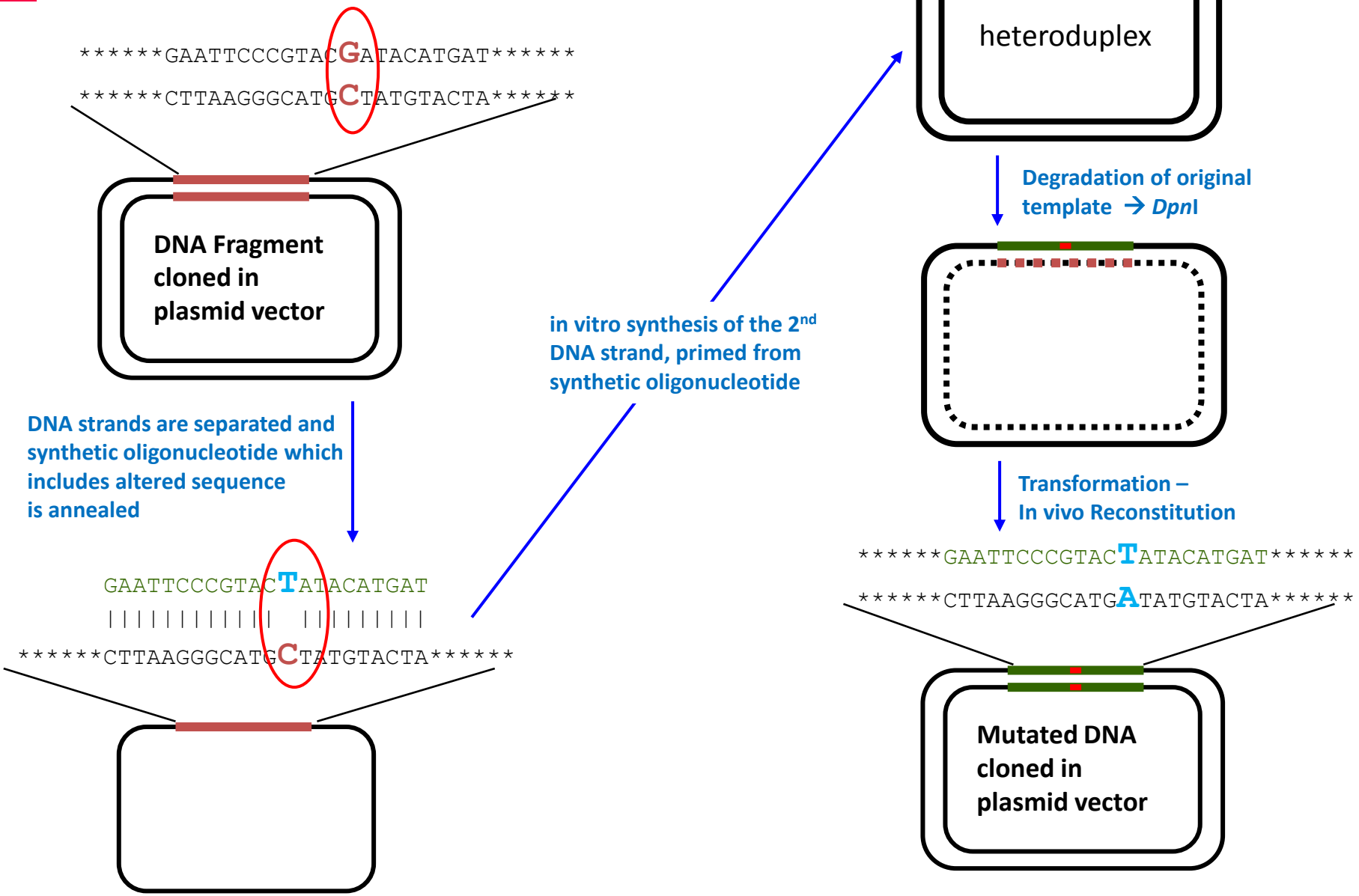
Availability of structure information

- **X-ray crystallography**
  - „frozen structure information“
  - need for crystallization
- **NMR structure analysis**
  - restricted to small proteins
  - information on protein dynamics possible
- **Modelling of structures based on aa sequences and homologies**
- **Modelling of substrate-Protein interactions - docking**

Availability of information on structure-function relations

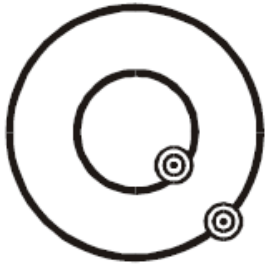
- **Information on molecular mechanisms of biological functions**
  - e.g. reaction mechanism, protein-small molecule interaction (e.g.
- **Information on aa residues involved in biological function**
  - e.g. active site residues…
  - e.g. sites for cofactor binding …

Site Specific Mutagenesis



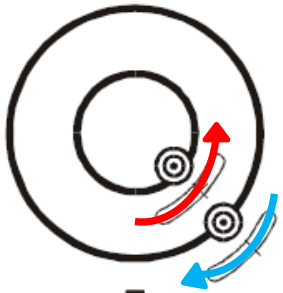
QuickChange™ Mutagenesis System

Step 1
Plasmid preparation

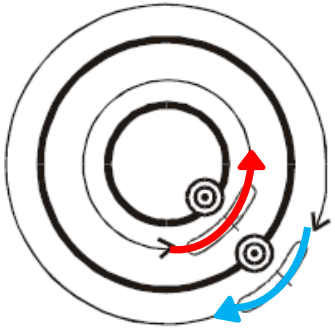


Gene in plasmid with target site for mutation

Step 2
Temperature Cycling

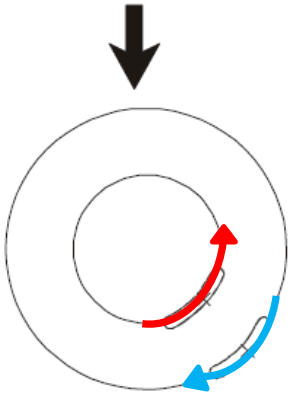


Plasmid denaturation
Annealing of oligonucleotid primers containing the desired mutation



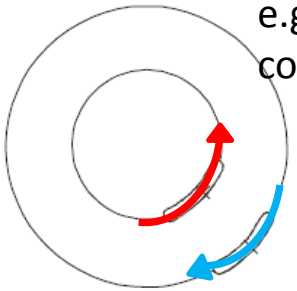
Extension and incorporation of mutagenic primers by *PfuTurbo* DNA Polymerase resulting in nicked circular strands

Step 3
Digestion



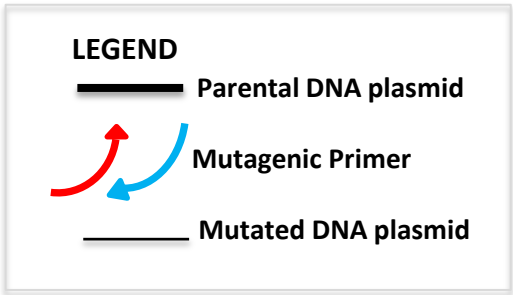
DpnI digestion of the non-mutated, parental DNA template

Step 4
Transformation



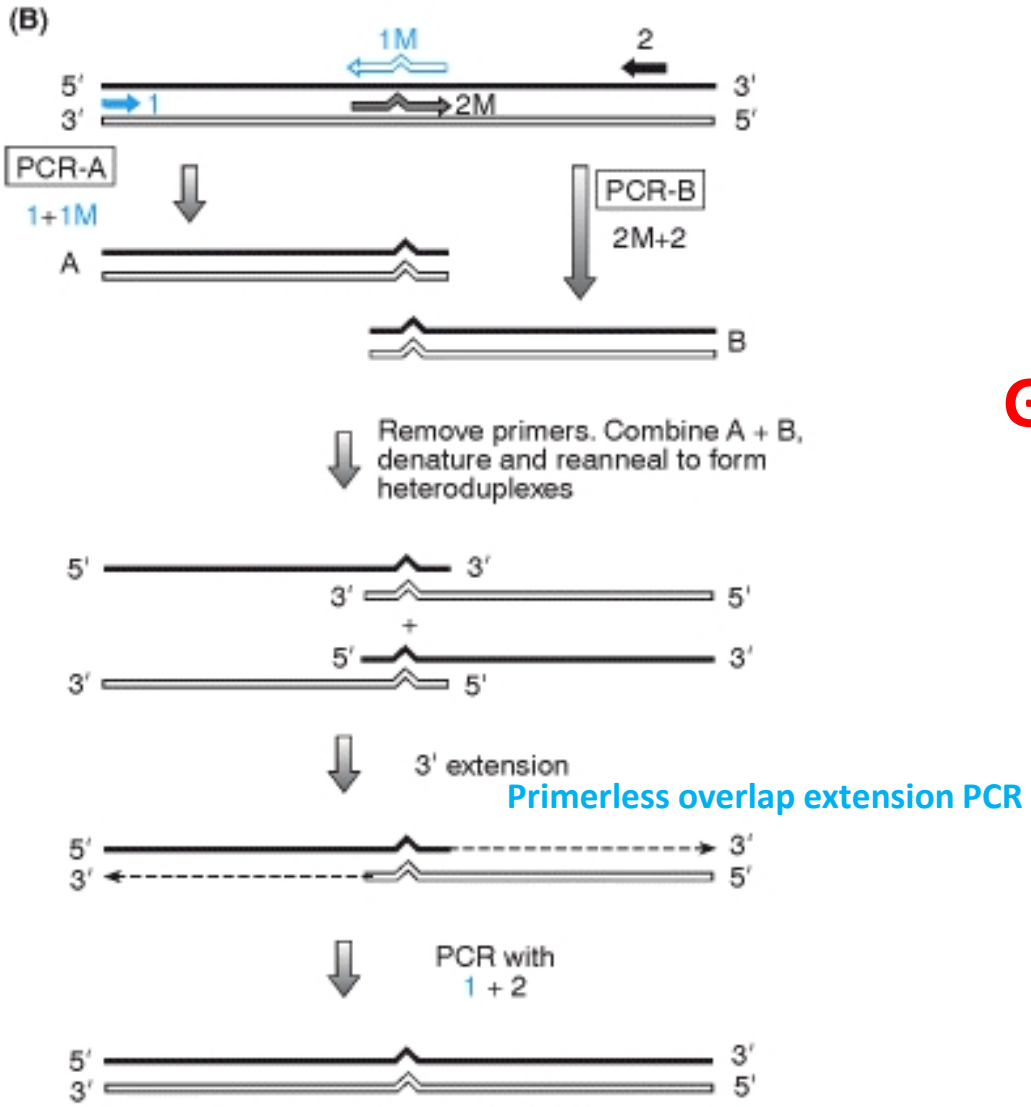
Transformation of the circular, nicked dsDNA into e.g. *E.coli* XL1-Blue competent cells

***E.coli* cells repair the nicks in the mutated plasmid**



Taken from: Stratagene QuikChange Manual

PCR- mediated, site directed Mutagenesis



Gene synthesis

Primerless overlap extension PCR

Taken from: Human Molecular Genetics. 2nd edition. Strachan T, Read AP. New York: Wiley-Liss; 1999.

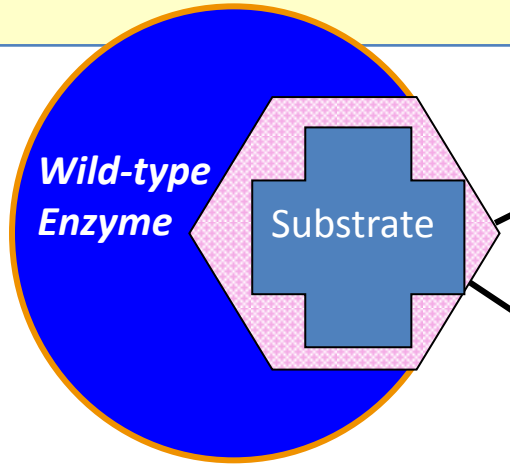
21.4.16

Enzyme-Engineering → basic routes

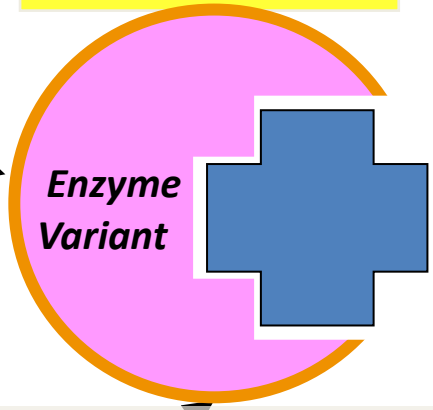
low activity
insufficient stability
unfavoured specificity
bad selectivity

Molecular Evolution
random mutagenesis
gene shuffling

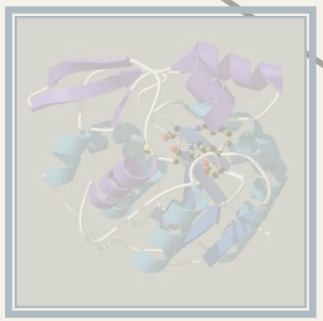
high activity
excellent stability
proper specificity
good selectivity



Variant library
Screening - Selection



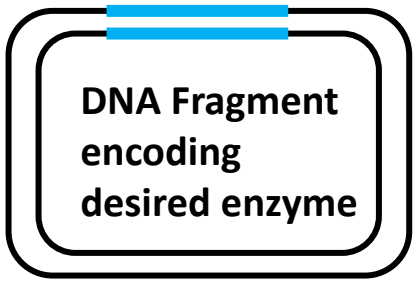
Functional and molecular Characterization



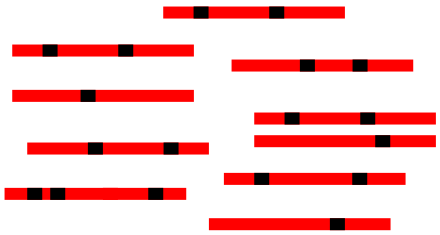
defined specific variant
targeted construction

Rational Engineering
Structure-Function Relationships
Modelling, Design

Directed Evolution - outline

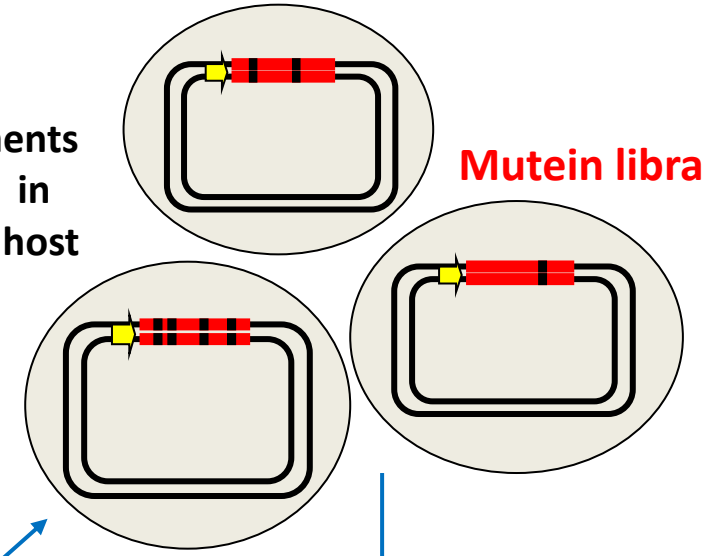


DNA fragments
derived by synthesis
PCR synthetic gene



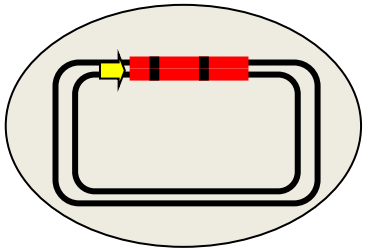
Generation of artificial diversity
Random Mutagenesis
Gene Shuffling

DNA Fragments
Introduced in
expression host



Mutein library

Screening
Selection

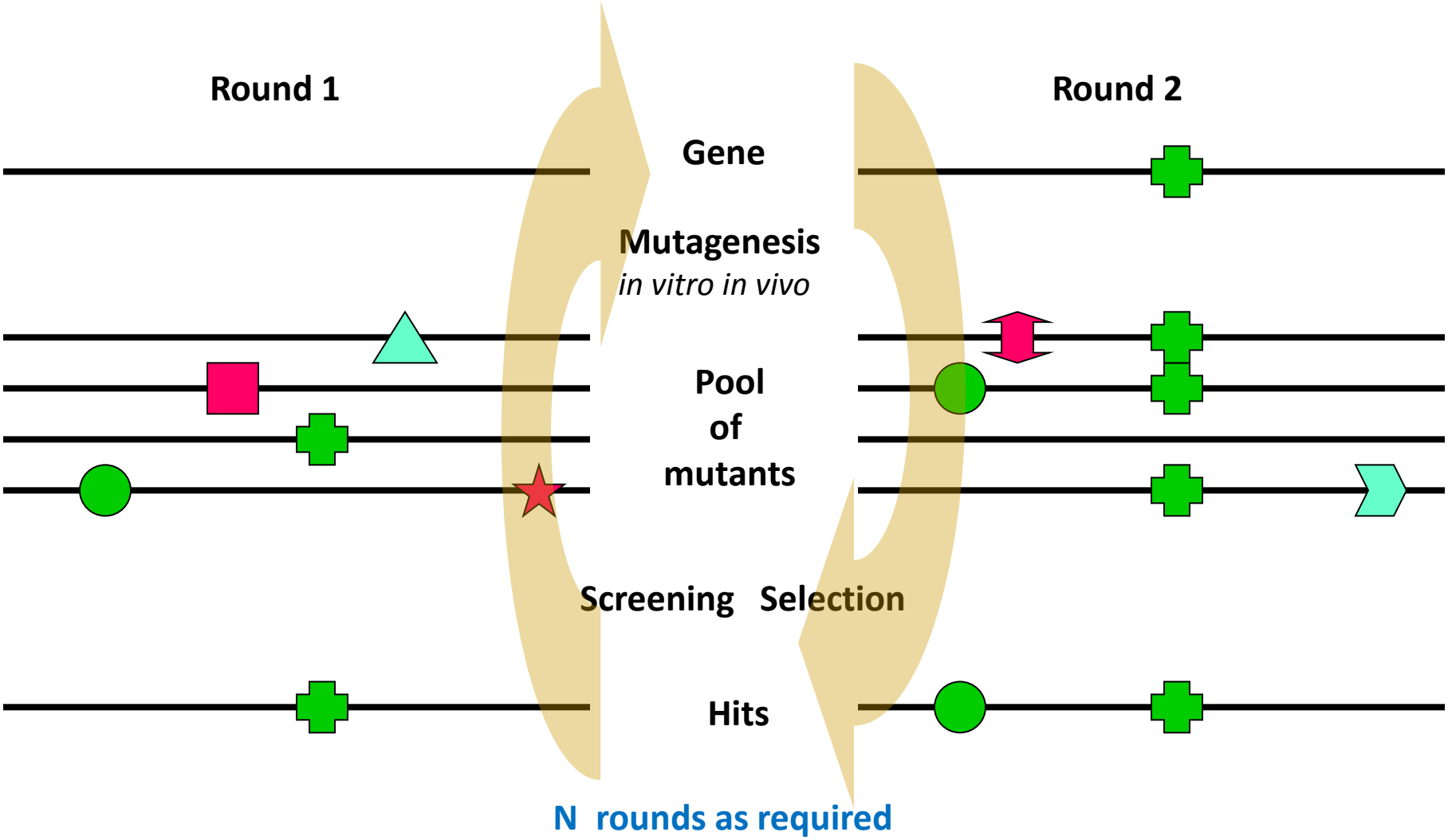


Hits

Cultivation
Functional *in vivo* expression
of enzyme variants

Molecular Evolution of Enzymes

Random mutagenesis



Generation of Mutant Libraries

Random mutagenesis of entire coding region

- error prone PCR, SeSaM
- *in vivo* mutation systems (mutator strains, transposons)
- deletion and insertion strategies (scanning mutagenesis)

Random mutagenesis of selected parts of coding region

- cassette mutagenesis with degenerate oligonucleotides
- megaprimer PCR

Site Saturation Mutagenesis

- All possible amino acids at specific position(s)

Mutagenesis by Error prone PCR

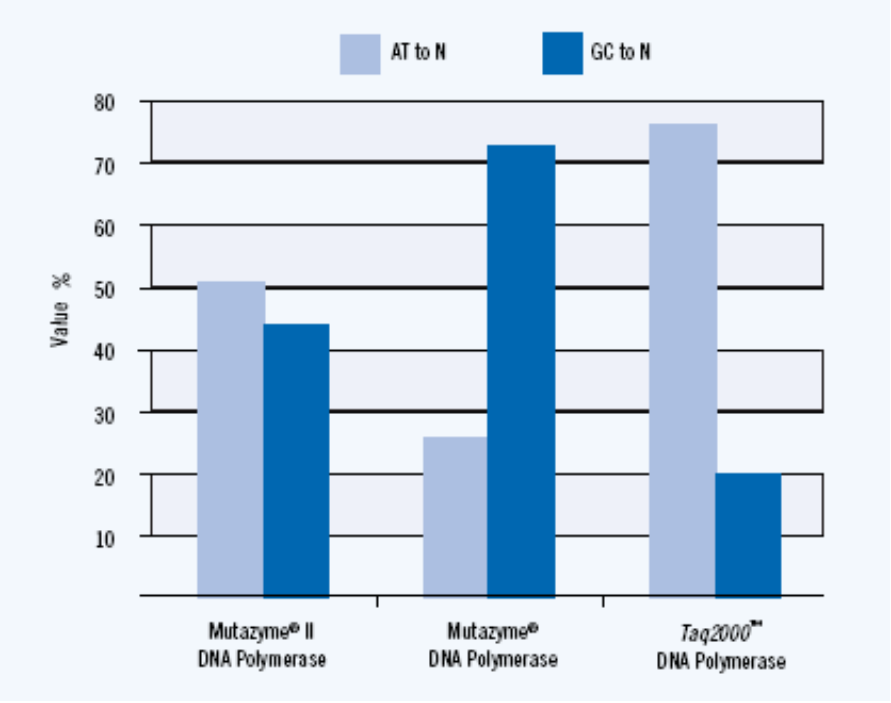
Error Prone PCR – mutagenic conditions

↑

$[Mg^{++}] \leftarrow\rightarrow [Mn^{++}]$

[dNTP]

Taq Polymerase → Mutazyme



Taq DNA Polymerase

Bias for mutating A and T under error-prone conditions,

Mutazyme® DNA polymerase:

Bias for mutating G and C

Mutazyme II DNA polymerase:

Blend of Mutazyme DNA polymerase and a novel *Taq* DNA polymerase mutant exhibiting a higher error rate

http://www.genomics.agilent.com/article.jsp?pageId=376&_requestid=1212515

Error Prone PCR - conditions

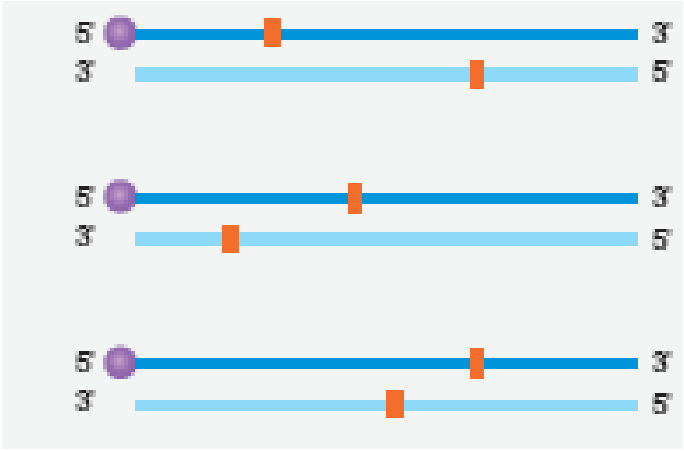
S A B C D F

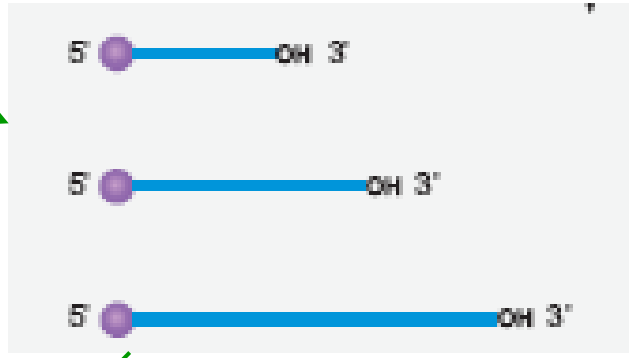
Condition	$[MgCl_2]$	$[MnCl_2]$	[dNTPs]
Standard	1,5mM		0,1mM each
A	7mM		1mM each
B	7mM	0,2mM	1mM each
C	7mM	0,5mM	1mM each
D	7mM	1,0mM	1mM each
F	7mM	0,5mM	1,0mM dCTP + dTTP each 0.2 mM dATP + dGTP each

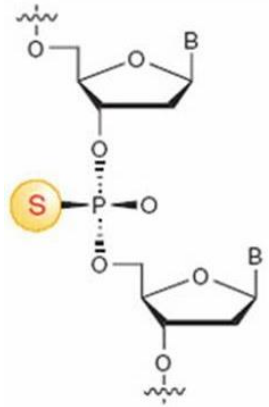
Conditon	sequenced basepairs	A -> C T -> G	A -> G T -> C	G -> A C -> T	G -> T C -> A	A -> T T -> A	G -> C C -> G	Insertion Deletion	Mutation rate
Standard	2388							-	0,00%
A	2388	1	1			2		-	0,17%
B	2388		7	2				-	0,34%
C	2388		10	2	1	4	1	-	0,76%
D	2388		10	1	1	4	3	-	0,85%
F	2388		2	2				-	0,17%

Mutagenesis by Sequence Saturation Mutagenesis

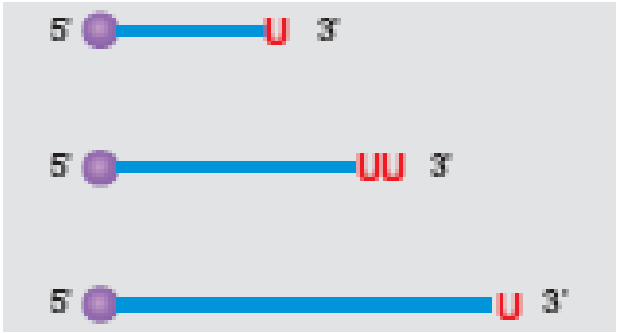
PCR amplification with biotinylated primer in presence of thiophosphate-dNTP







Cleavage at thiophosphate-dNTP positions with iodine under alkaline conditions

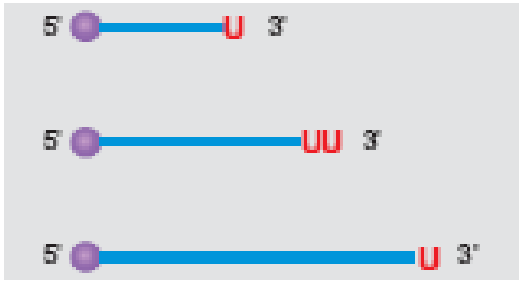


Addition of dUTP using terminal transferase, thereby at one position several nucleotides can be incorporated giving the chance to produce any triplett

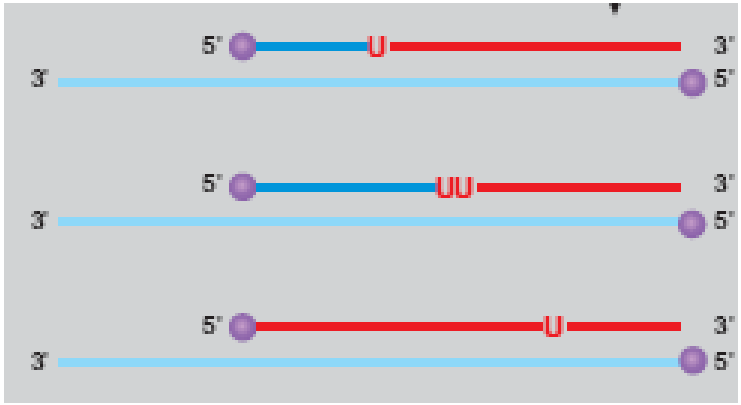
SeSaM

Schenk et al. (2006) Biospektrum 3, 277

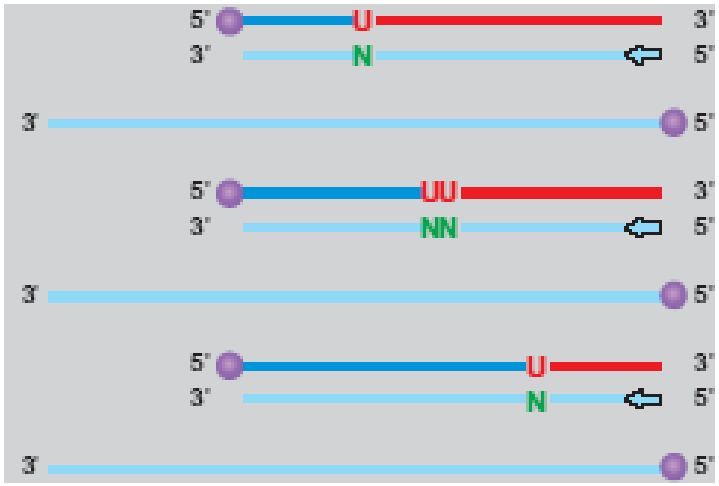
Mutagenesis by Sequence Saturation Mutagenesis



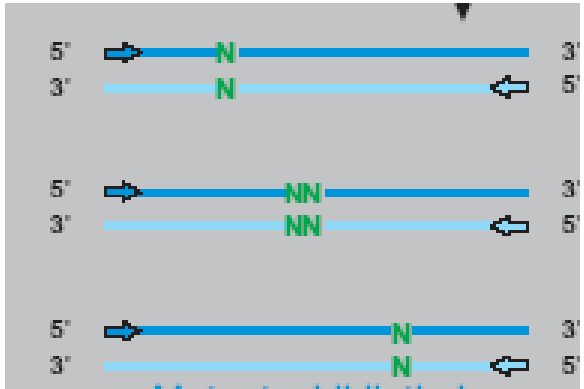
Annealing of wild type strand to biotinylated template and elongation



2nd strand synthesis with dNTPs



PCR amplification



Schenk et al. (2006) Biospektrum 3, 277

SeSaM

Experimental Library Limits

Length N , M aa => M^N aa sequences

	N	M^N	Mass of Library
	3	10^4	
	5	10^6	
	10	10^{13}	Milligrams
	20	10^{26}	Tons
	50	10^{65}	Mass of Earth
	100	10^{130}	
Typical Protein Size	200	10^{260}	

2.20.2000

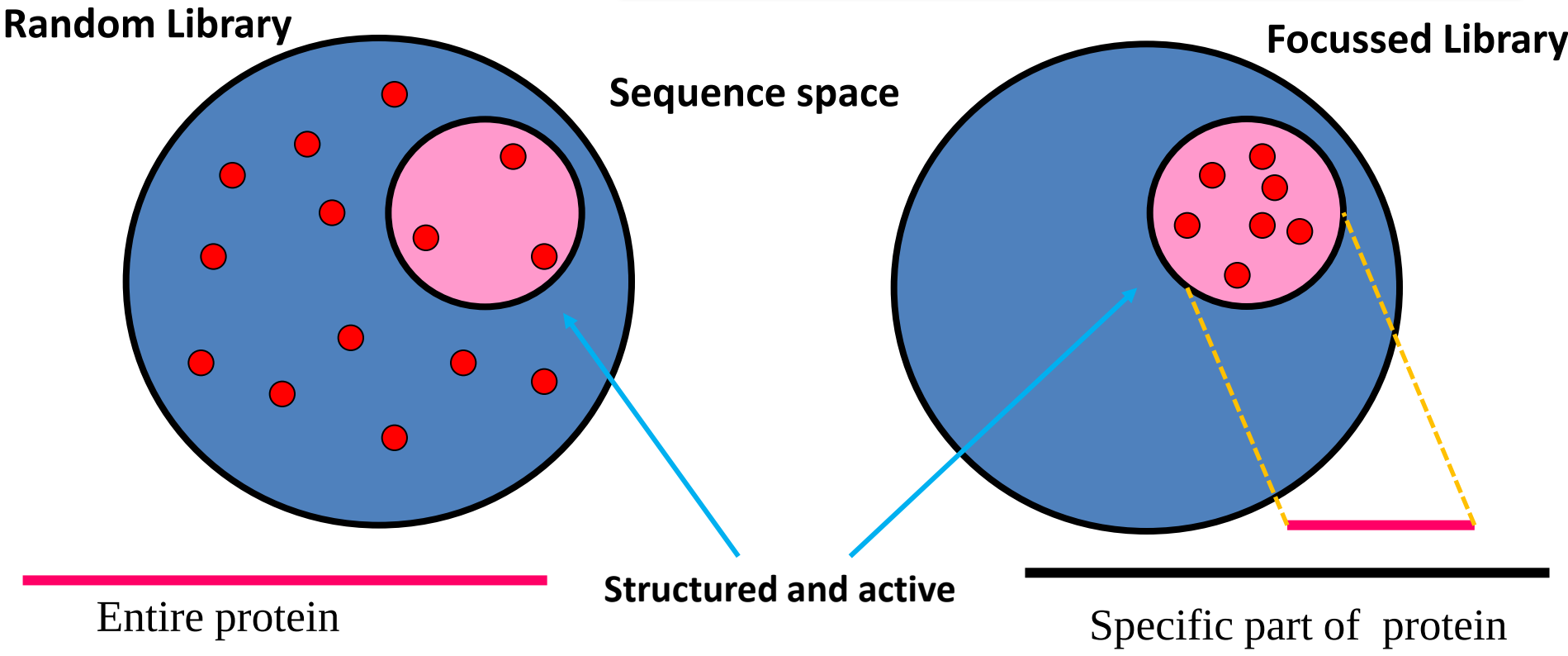
XENCOR

Library management

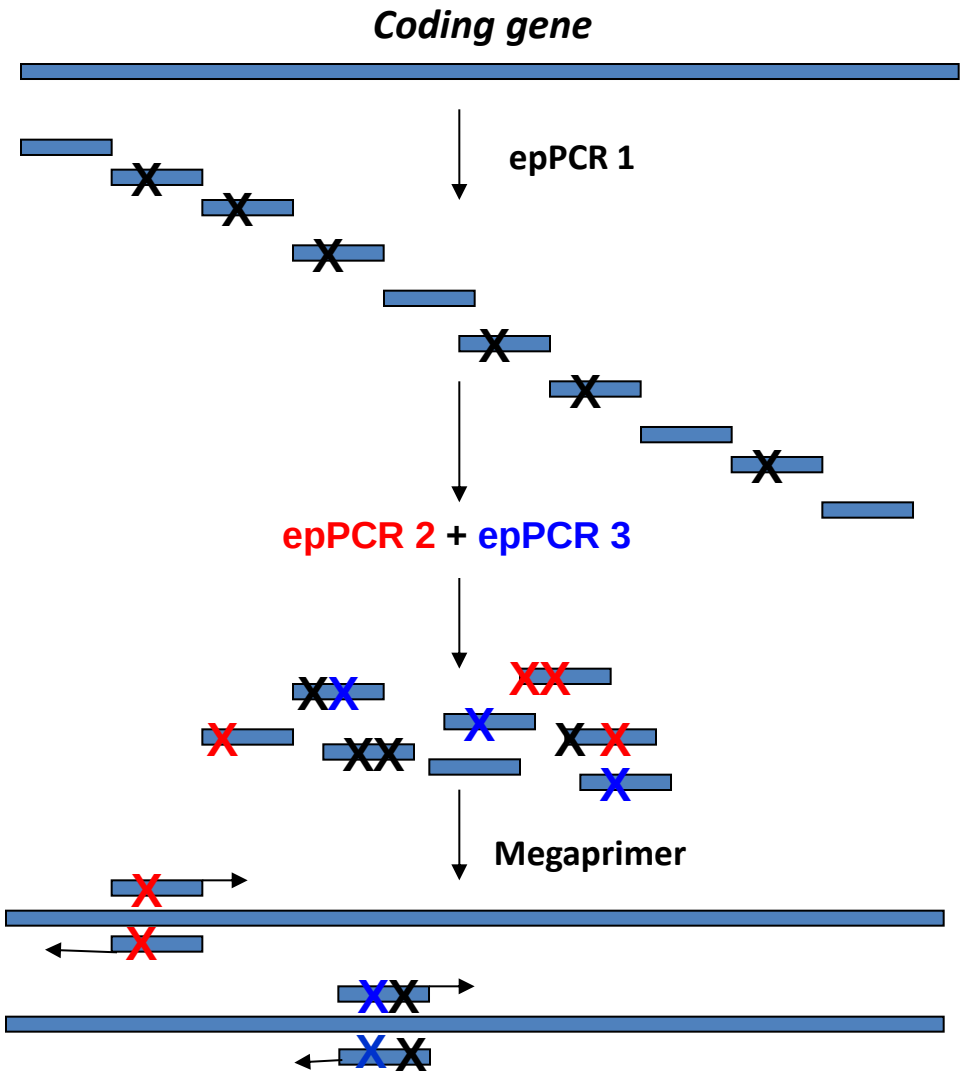
Numbers of possible protein variants (Kuchner and Arnold, 1997)

$$V = (19)^M \times \frac{N!}{(N-M)! \times M!}$$

Number of aa changed simultaneously (M)	Sequence length (N)	
	5	477
	Number of possible variants	
1	95	9063
2	3610	40982886
3	68590	1.239×10^{11}



Library management
Partial fragment mutagenesis



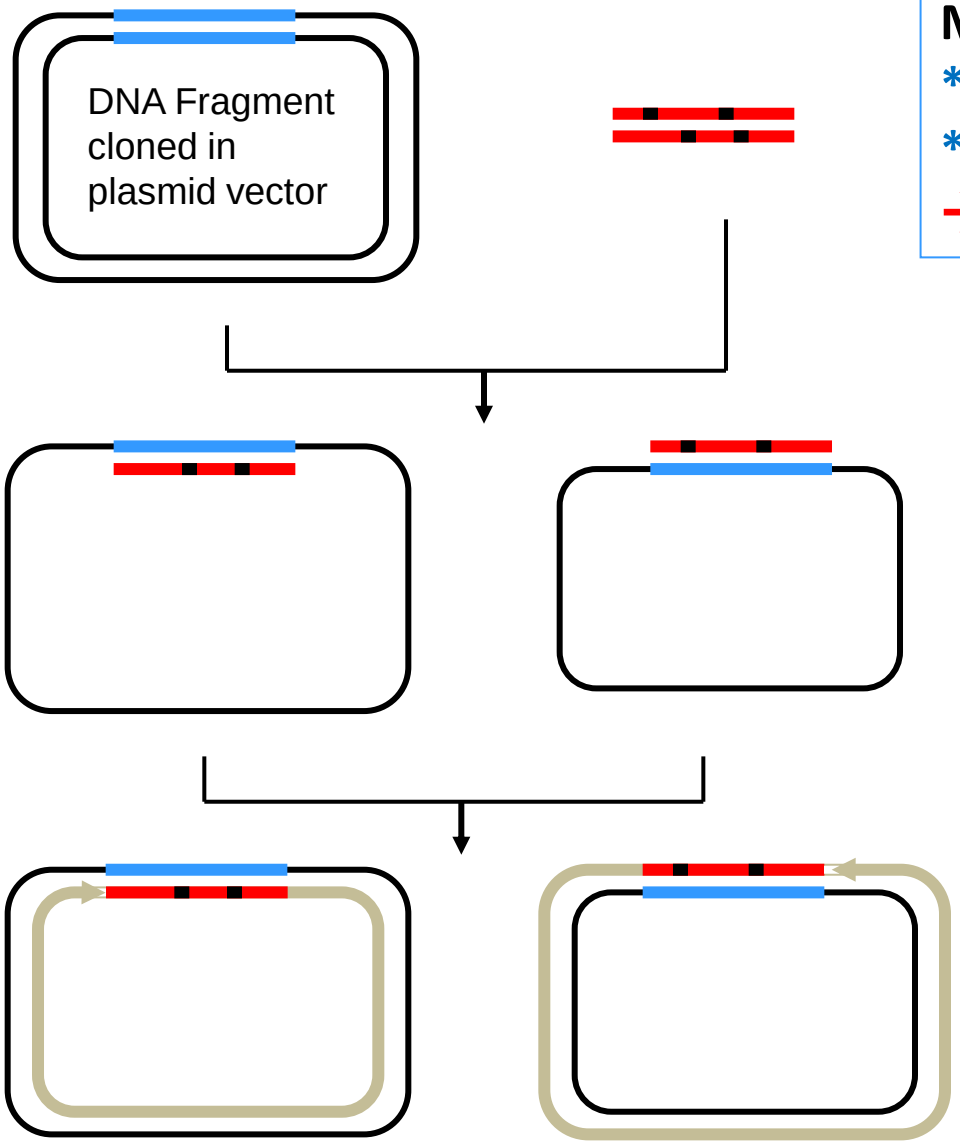
⇨Mutagenesis of subfragments

⇨DNA from epPCR 1 used as template for epPCR 2, etc…

⇨ Mutagenized fragments are introduced in expression vector by megaprimer PCR

⇨ 1- 3 mutations per ~100bp (fragment)

Random Mutant Libraries by Megaprimer PCR



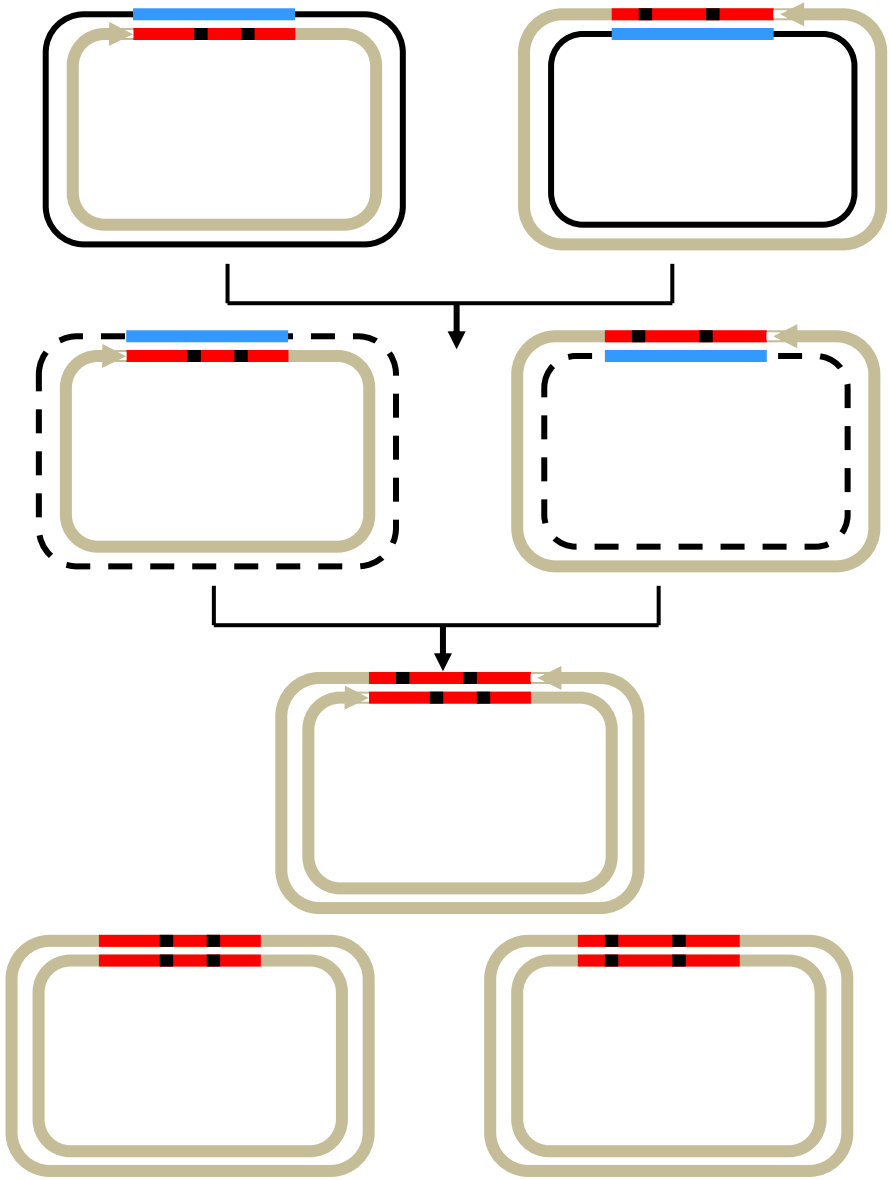
Mutagenize DNA fragment
* PCR
* Degenerate gene synthesis
→ Megaprimer

Denaturing
Megaprimer annealing

Extension with
DNA polymerase
(PCR)

K. Miyazaki (2003)
Methods Mol Biol, 231,23

Random Mutant Libraries by Megaprimer PCR



***DpnI* digestion**
(metylated template DNA)

denaturation
annealing

transformation
segregation

K. Miyazaki (2003)
Methods Mol Biol, 231,23

Generation of Diversity by Recombination

In vitro methods

Random fragmentation

- DNase I digestion;
  Stemmer (1998) *Nature* **370,** 389

Random priming synthesis

Shao *et al.* (1998) *Nucleic Acids Res.* **26,** 681

Staggered Extension Process (StEP)

Zhao *et al.* (1998) Nature Biotechnol. **16,** 258

RACHITT (**R**andom **C**himeragenesis on **T**ransient **T**emplates)

Coco *et al.* (2001) *Nat. Biotechnol.***19,** 354

In vivo gene recombination

Site-specific recombination

Cre-lox; Lambda

Homologous recombination

E.coli, Lambda phage, *Saccharomyces cerevisiae*

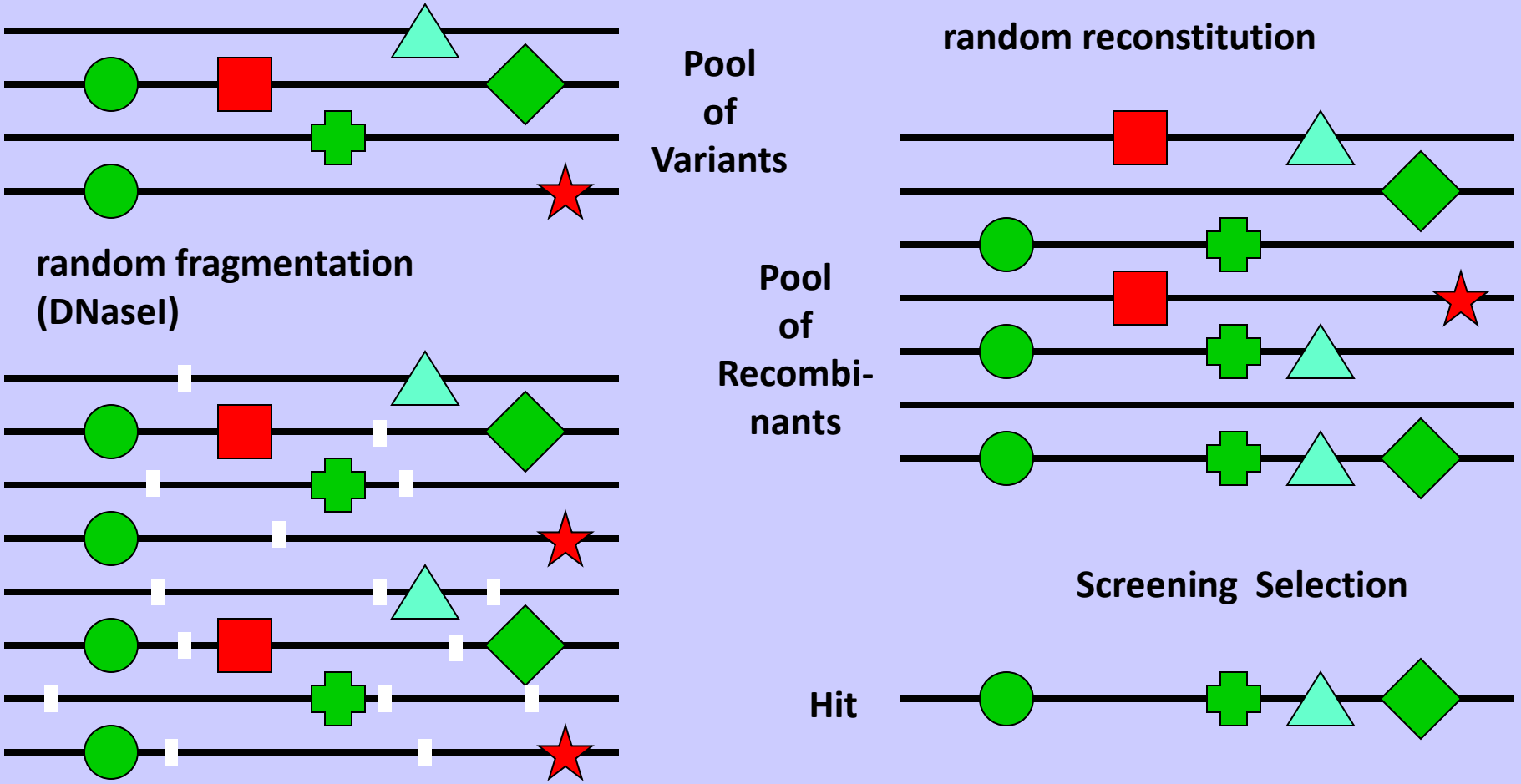
Recombination of non-homologous sequences

ITCHY, SCRATCHY, SHIPREC

Molecular Evolution of Enzymes

In vitro Recombination of Sequences

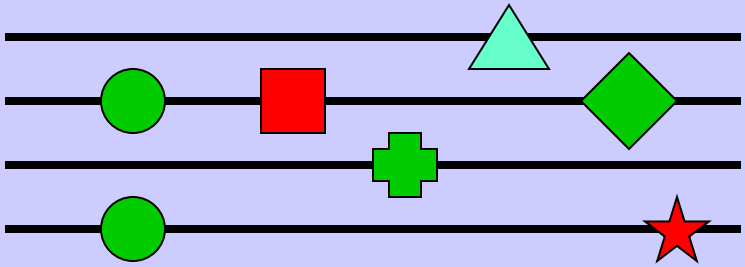
"Gene Shuffling" "Family Shuffling"



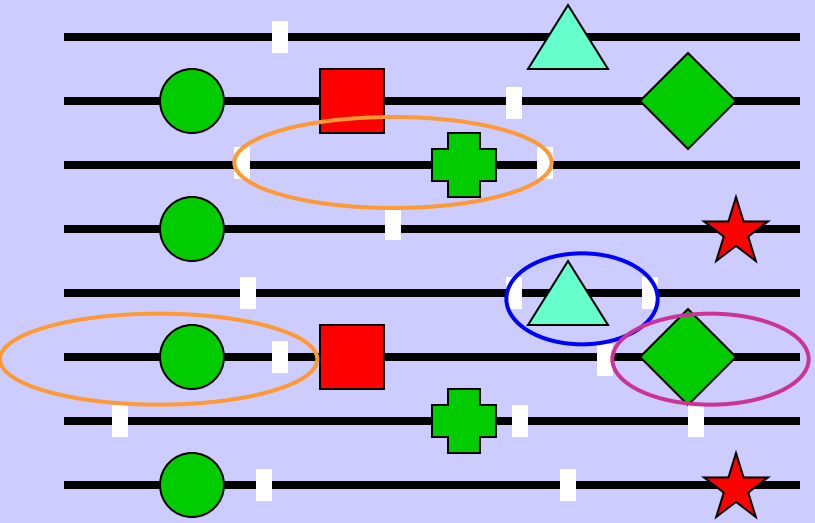
Molecular Evolution of Enzymes

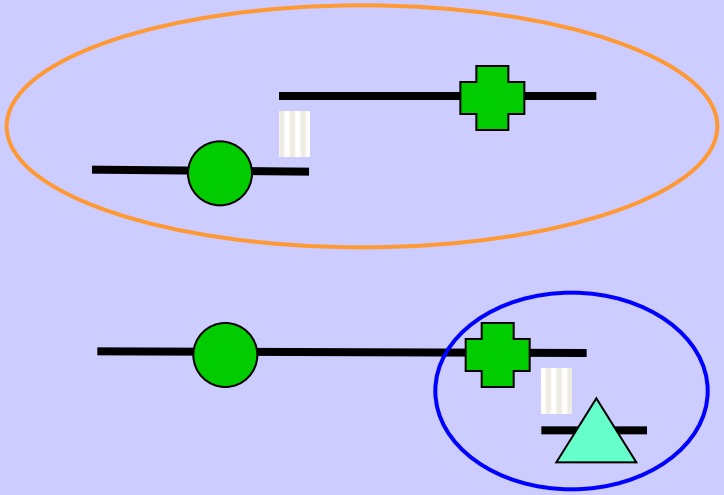
In vitro Recombination of Sequences

"Gene Shuffling" "Family Shuffling"

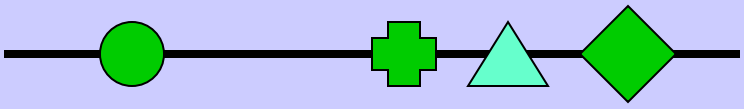


random fragmentation
(DNaseI)





Hit

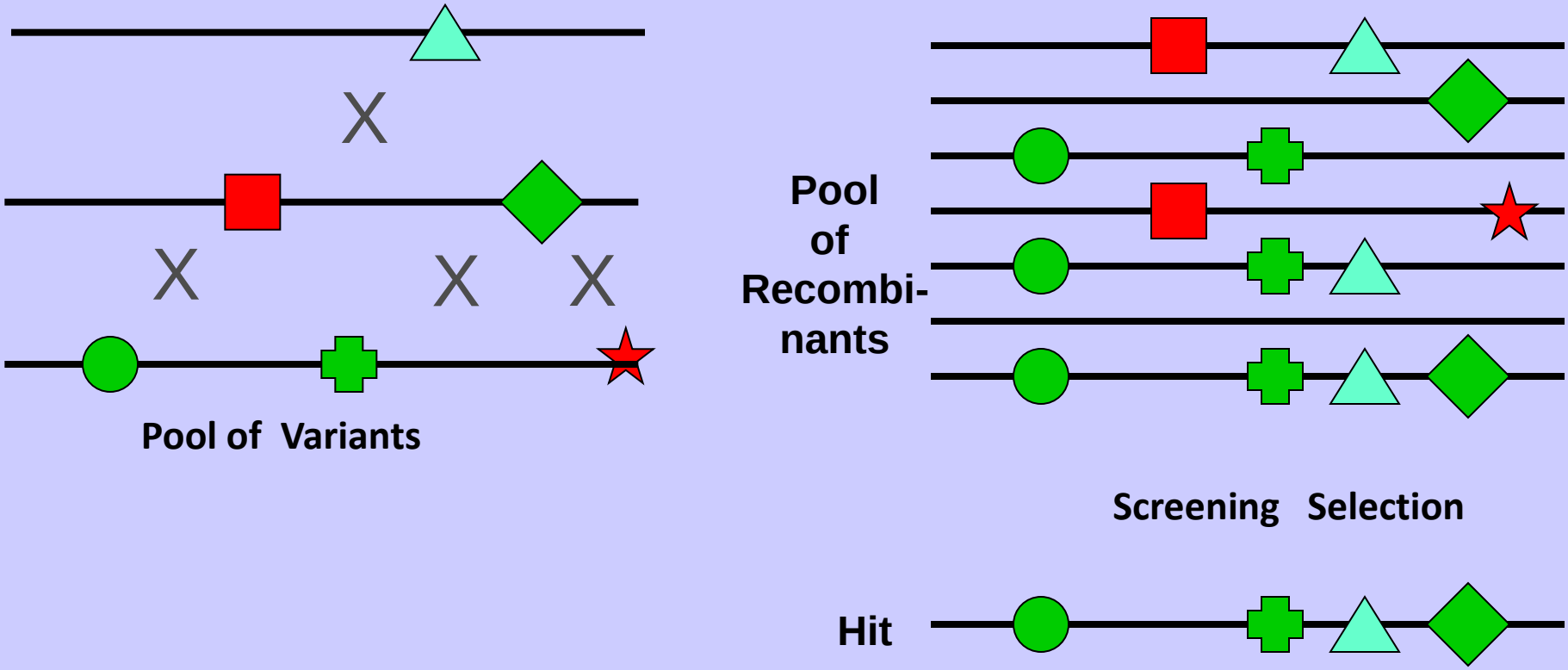


Molecular Evolution of Enzymes

In vivo Recombination of Sequences

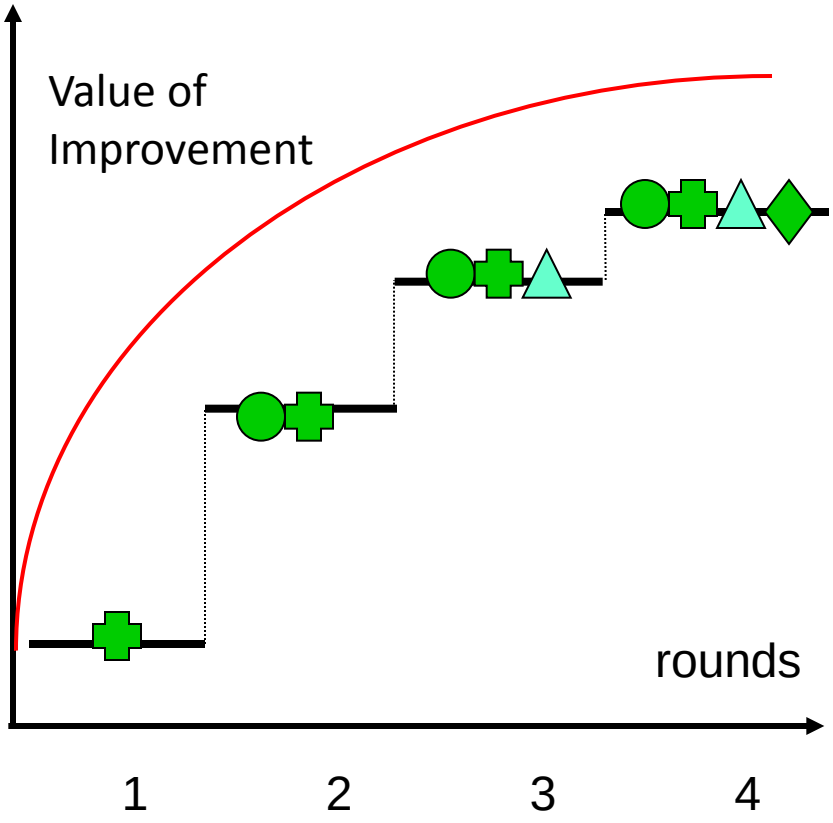
"Gene Shuffling" "**Family Shuffling**"

random recombination

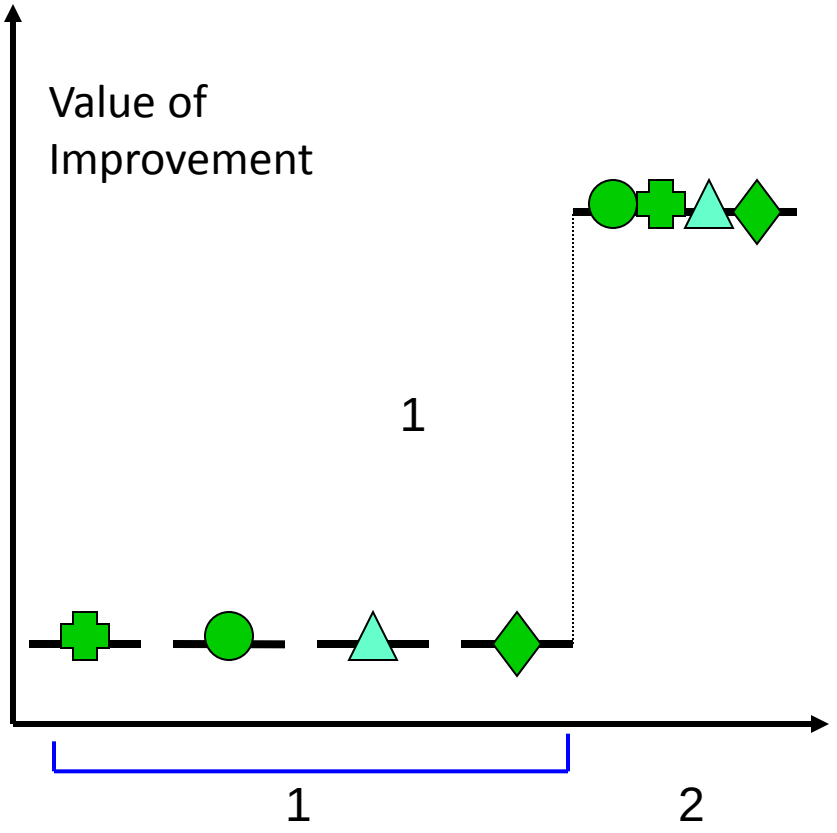


Molecular Evolution of Enzymes

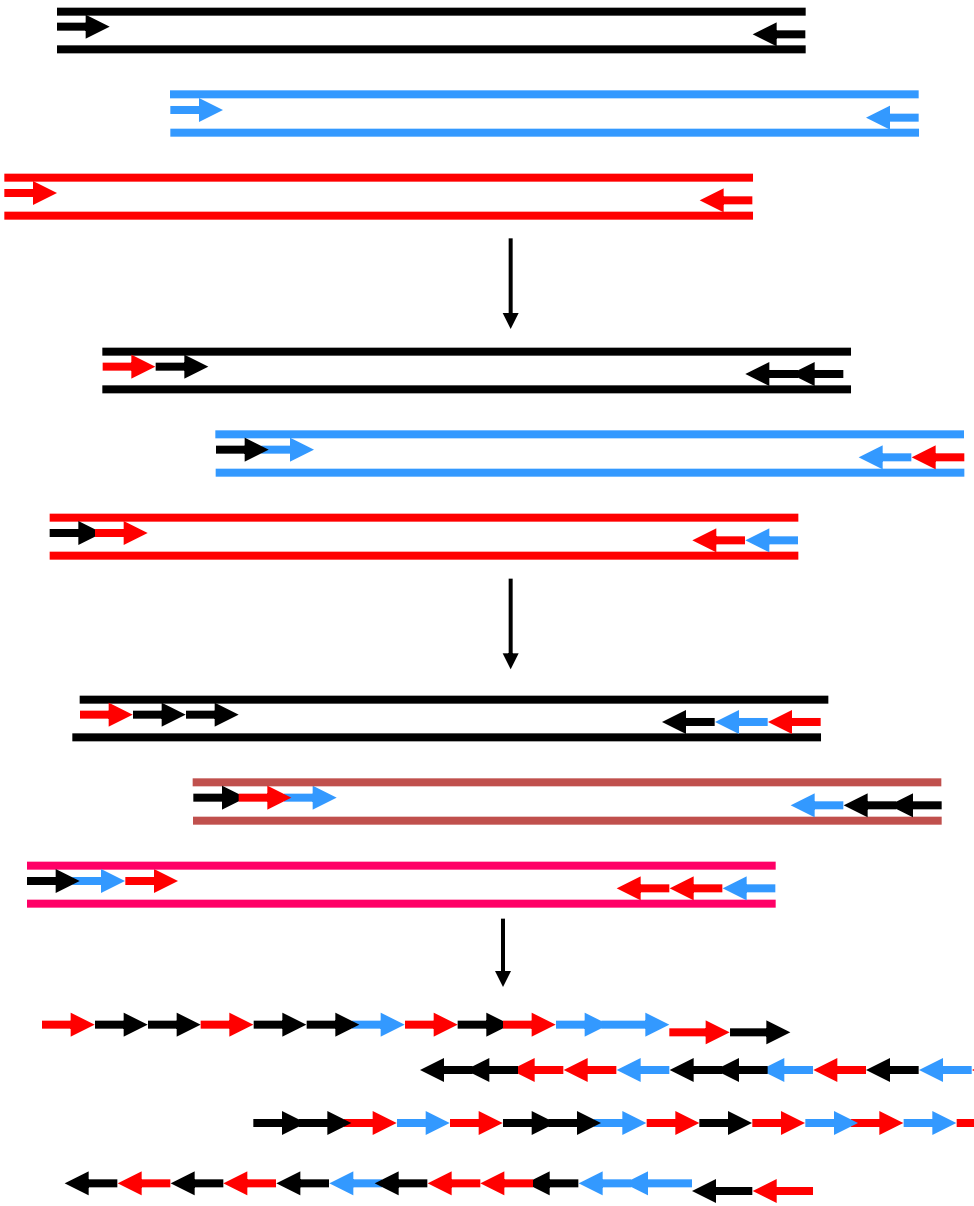
Rounds of Random Mutagenesis



Recombination of Sequences



Random Recombination Libraries by StEP



round 1
- denaturation
- primer annealing
- extension

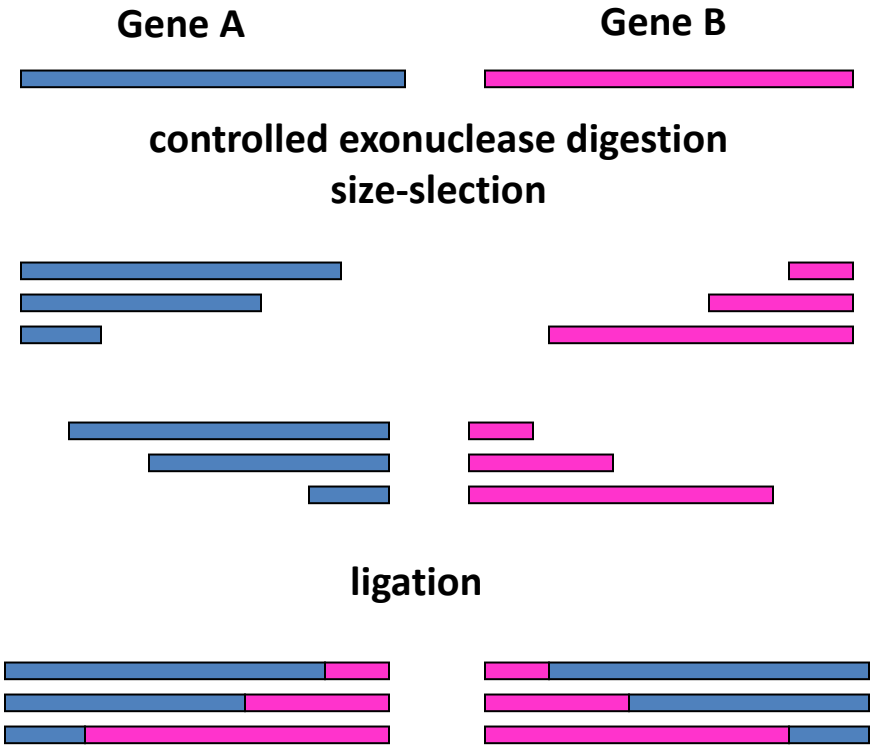
round n
- denaturation
- primer annealing
- extension

Library of mosaic genes

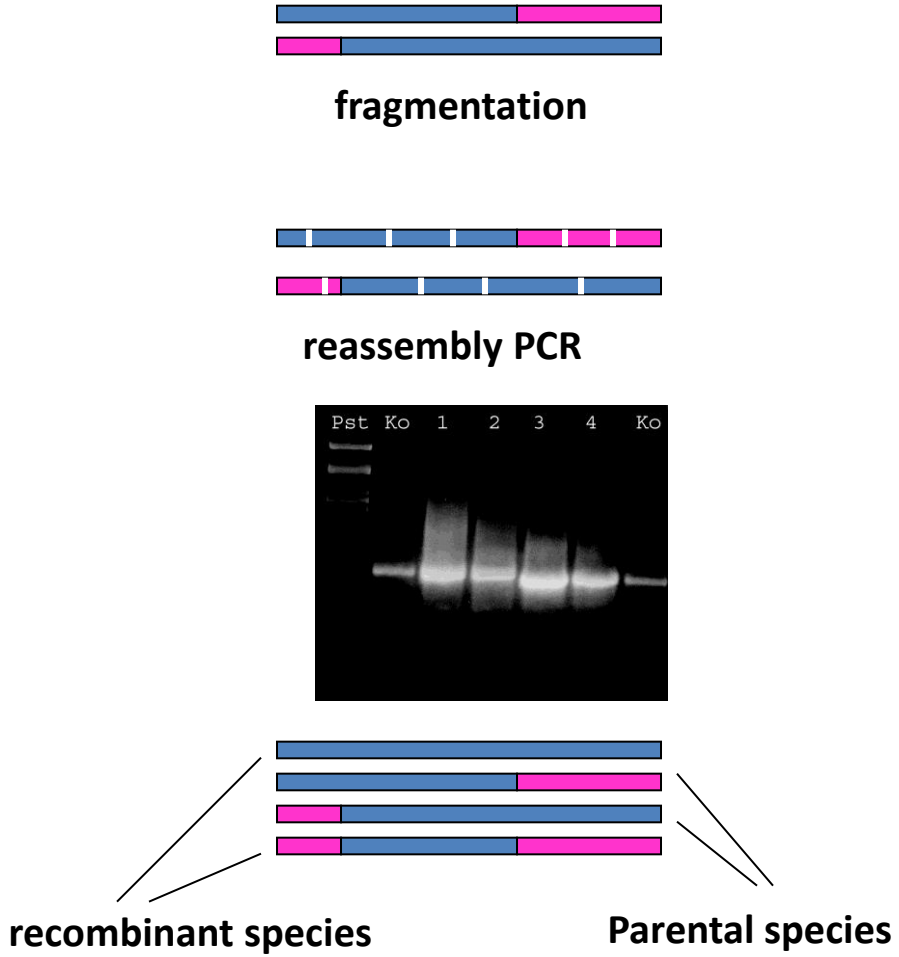
Aguinaldo & Arnold (2003)
*Methods Mol Biol, **231**,105*

Recombination of non-homologous sequences

Step 1:
random truncation - fusions



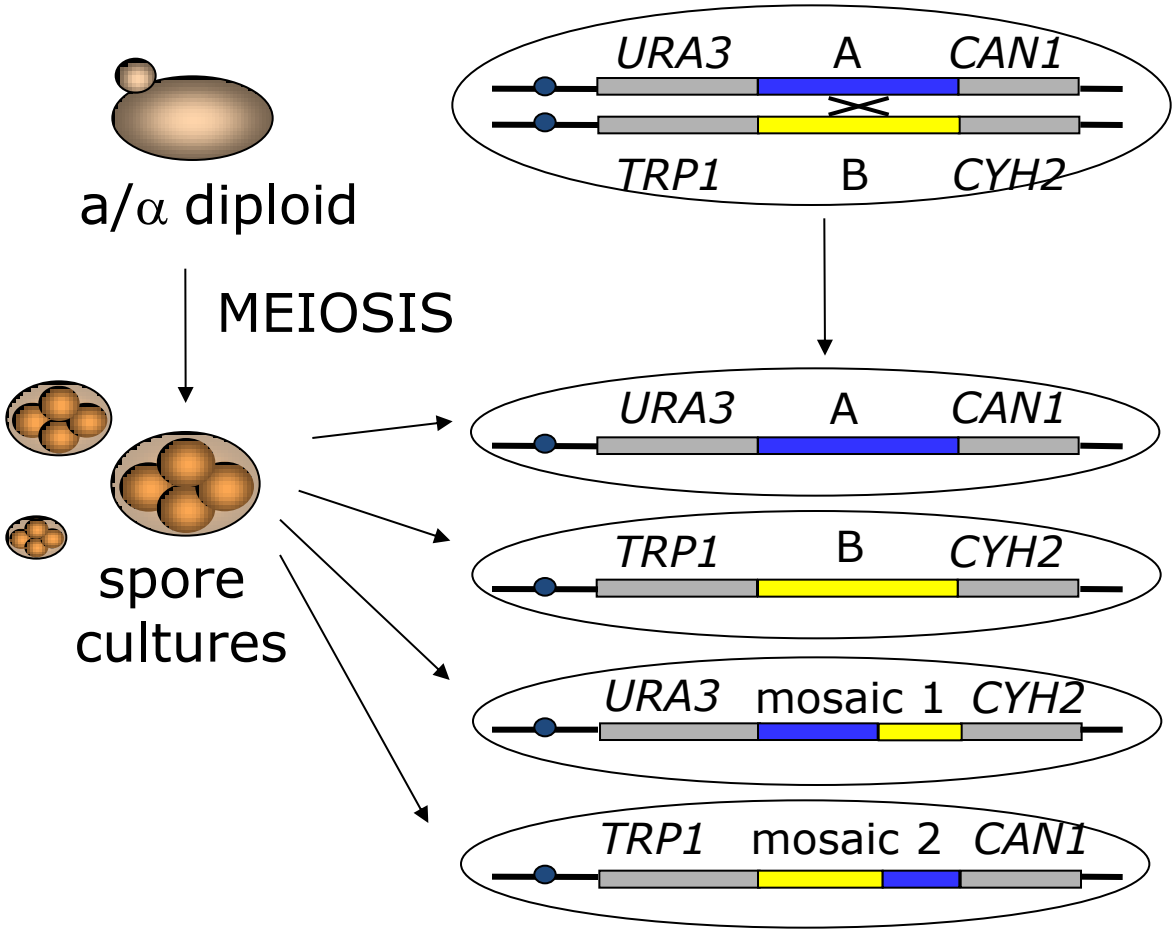
Step 2:
Shuffling of fused variants



Pool of random point fusions

B.Brunner, 2001

Overview of the yeast shuffling strategy



Meiosis :

high levels of genome-wide recombination

Selection for recombinants by marker exchange
Uneven numbers of recombination detectable

MSH2 **:**
key player in homeologous recombination, mismatch repair and mutagenesis

Screening - Selection

First law of directed evolution:

“You get what you screen for“

Frances Arnold

Screening - Selection

Screening → Individual Analysis of clones

Essentials:

- **high throughput - HTP**
- **simple and robust**
- **good discriminatory capacity**
- **accessible to robot handling**
- **application of process-near conditions (e.g. organic solvents)**
- **allows work with desired substrate → no surrogates**

Selection → Growth advantage

Prerequisite:

Bio-compatibility

Screening – Selection: Problems to consider

- **Uniform growth of individual clones**
  - Substrate supply
  - Mass transfer (oxygen supply, CO_2 emmission
  - heat transfer (e.g. position on plate/shaker)
  - inoculation
  - cross contamination

- **Homogeneous expression levels**
  - Host system
  - Vector copy number
  - Induction conditions
  - Functional expression (e.g. folding, post-translational modifications

- **Equal access/release of reactants to/from enzyme**
  - membrane/cell wall transfer
  - cell disruption

Screening Systems → hosts

Hosts for Enzyme Screening

Bacterial Hosts

E.coli strains	→	**"Golden Standard"**
Bacillus strains	→	secretory enzymes
Streptomyces sp.	→	expression background

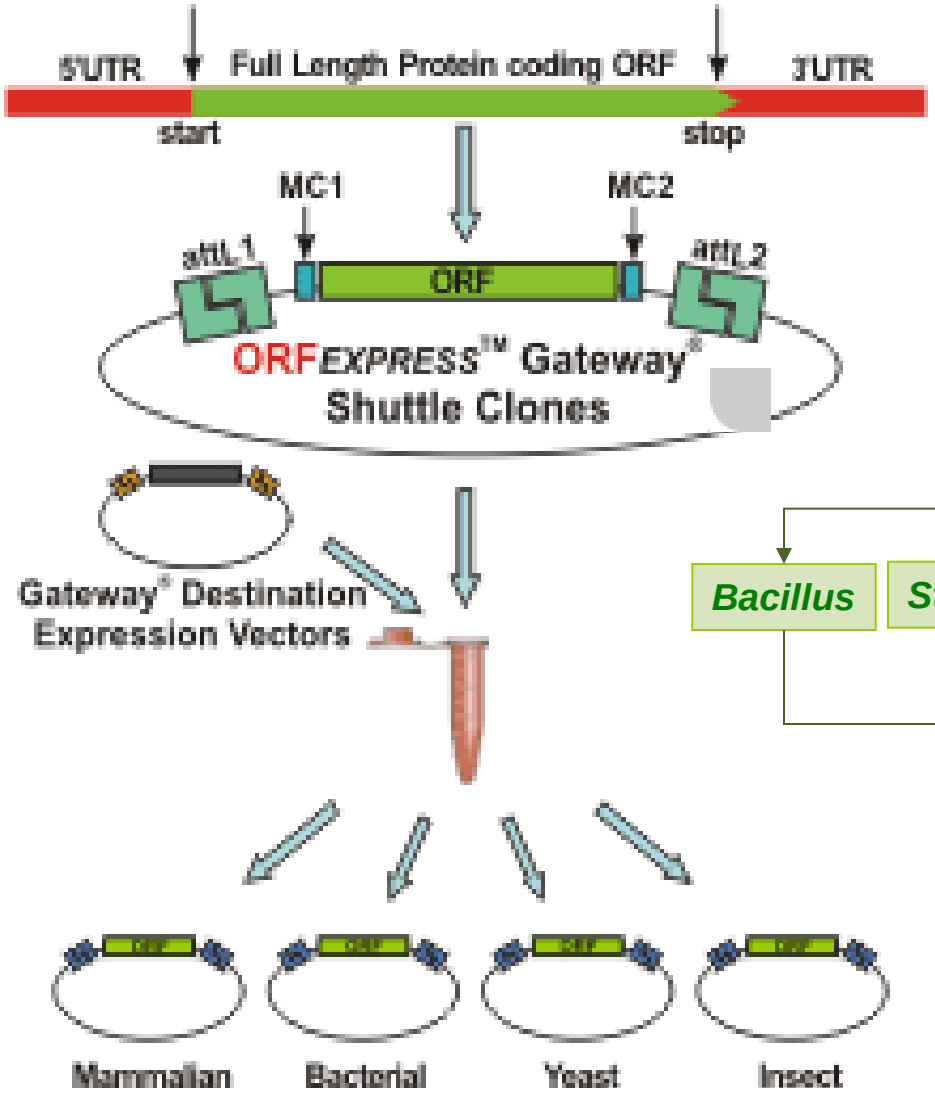
Fungal Hosts

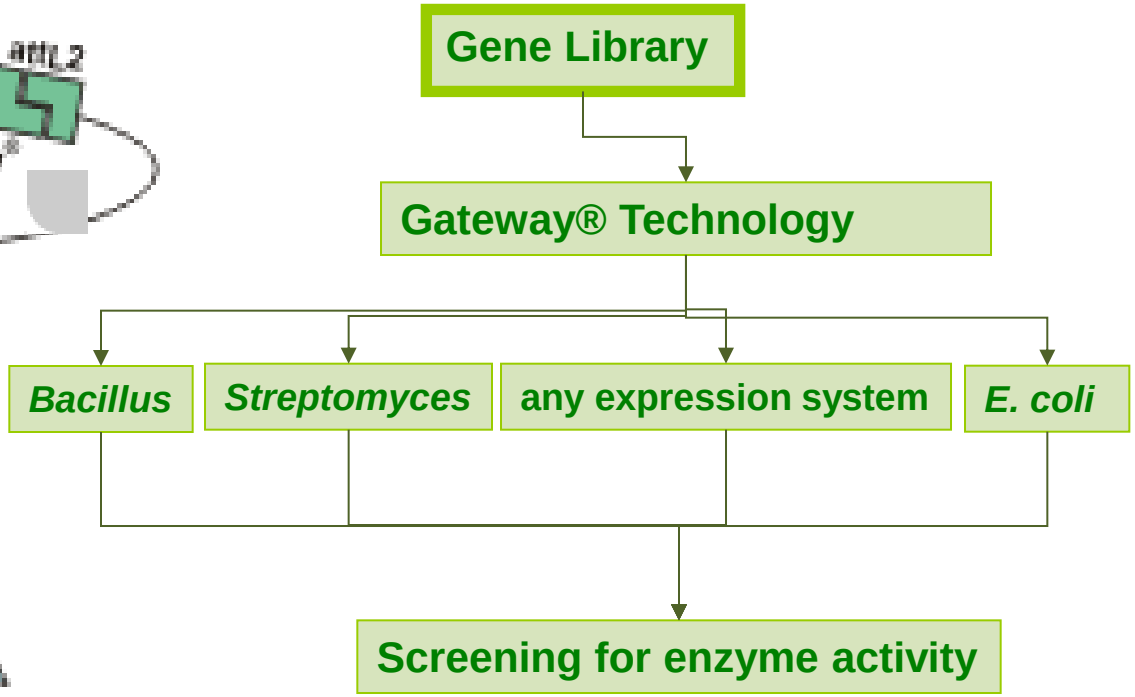
Pichia pastoris	→	enzymes of eukaryotic origin

New Library Concepts

Gateway Technology
in vivo Transfer Systems
Genome integration

Screening Systems → hosts



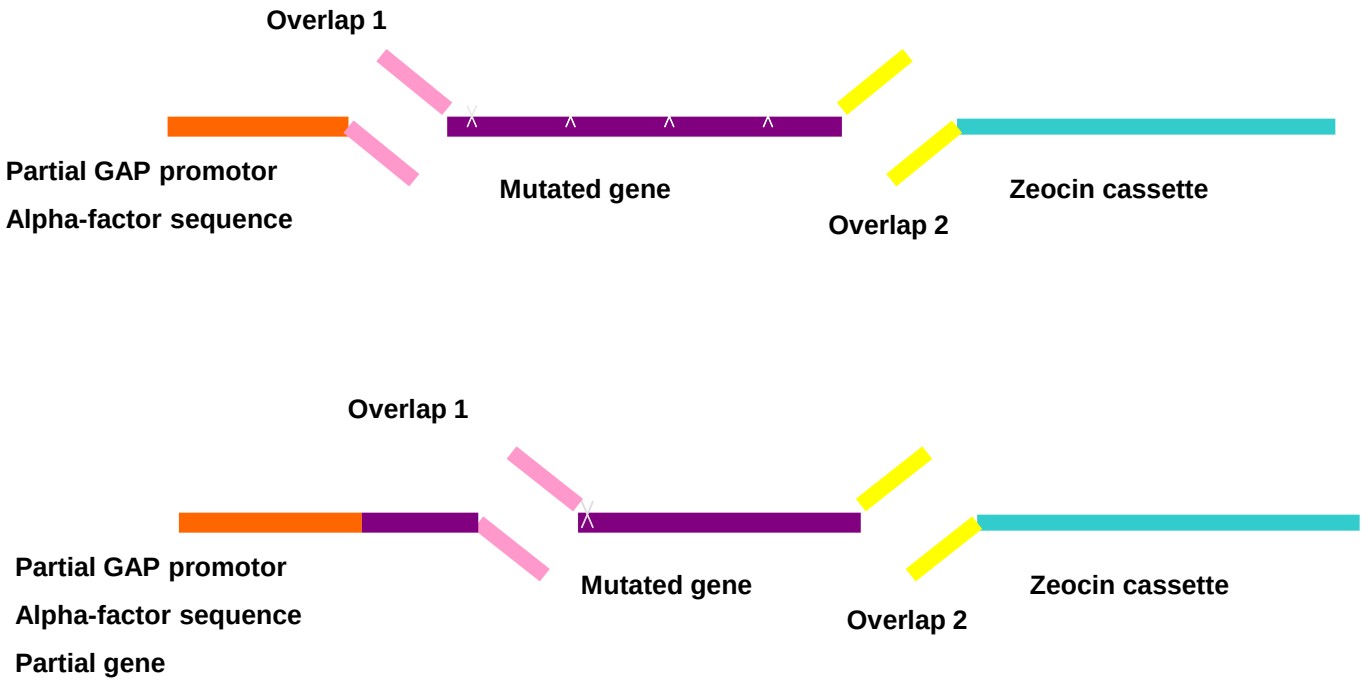


Gateway® Technology .

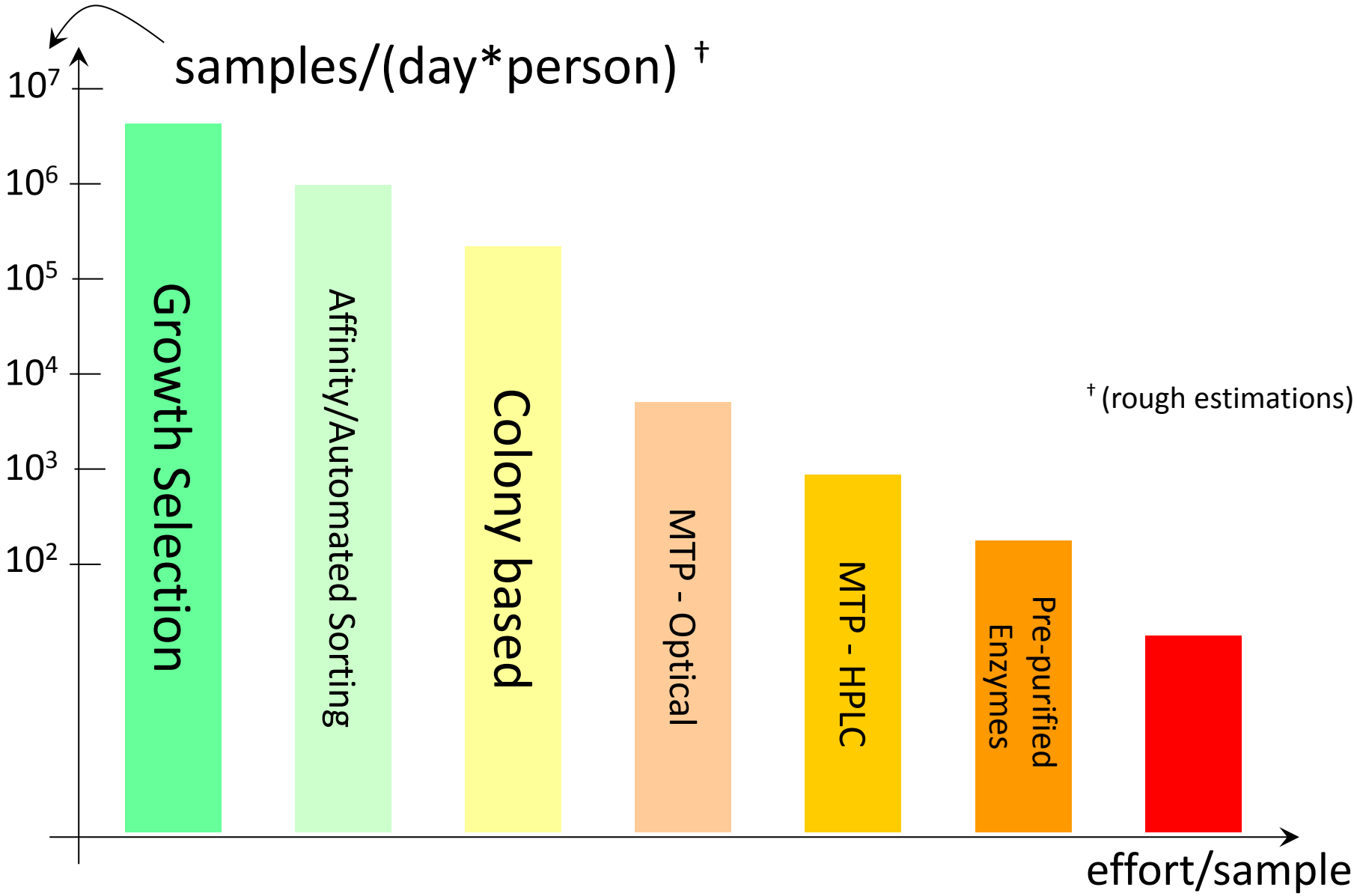
New Strategy for Library Generation

Random & Site Directed mutagenesis

Directed Evolution in *Pichia pastoris*

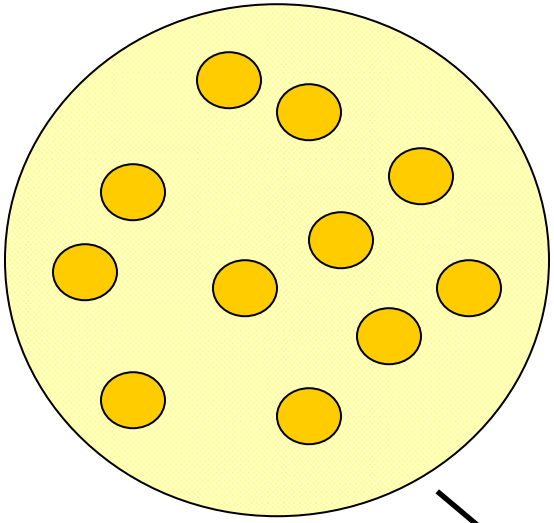


High Throughput Screening -Detection of Enzymatic Activity

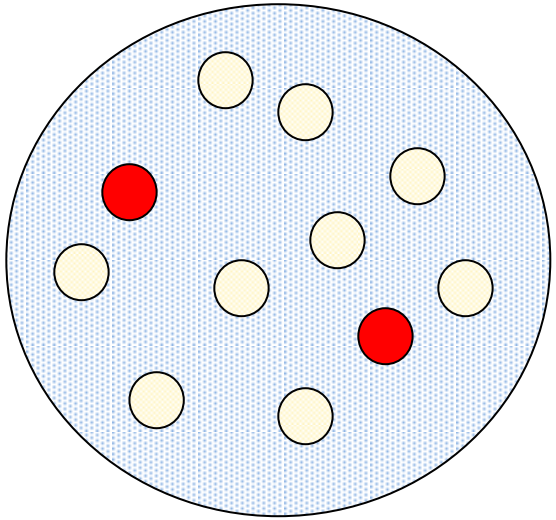


Screening by filter assays

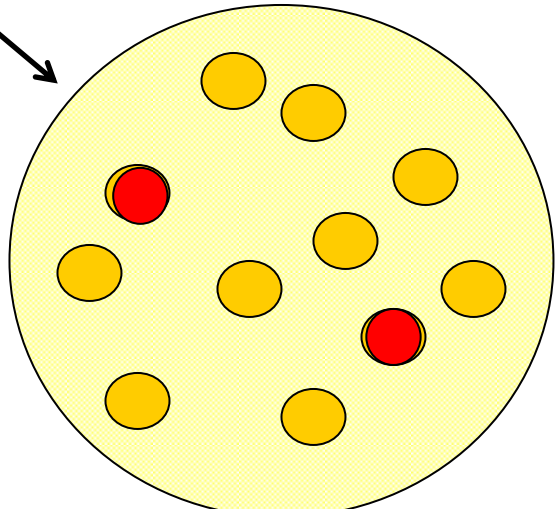
Colonies on agar plates



Transfer to filter



Substrate – Reaction Detection on filter



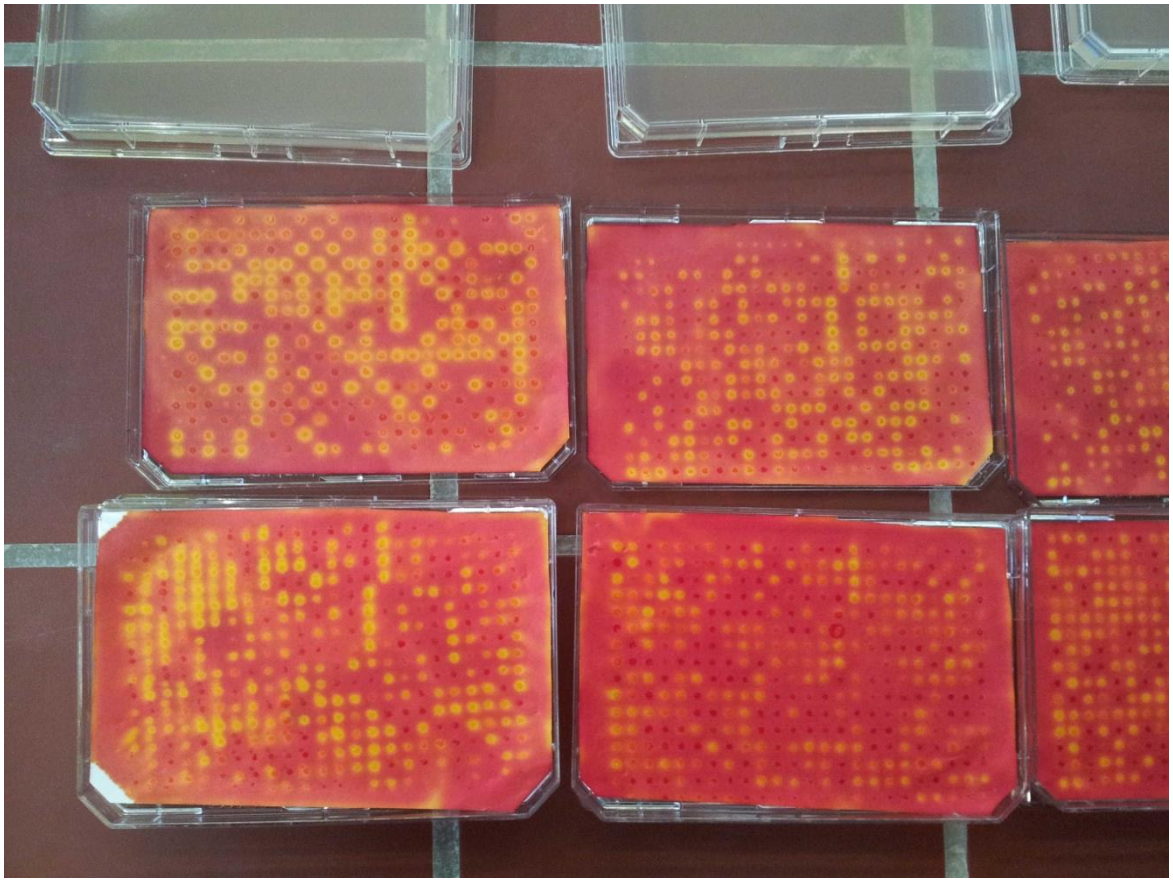
Direct substrate - reaction and detection on agar plate

Advantages:

- **No interference with media**
- **Use of solvent possible**
- **Cell lysis possible**
- **Pre-treatment (e.g. T, pH)**

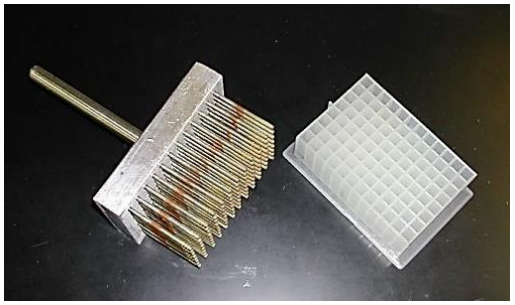
Screening by filter assays

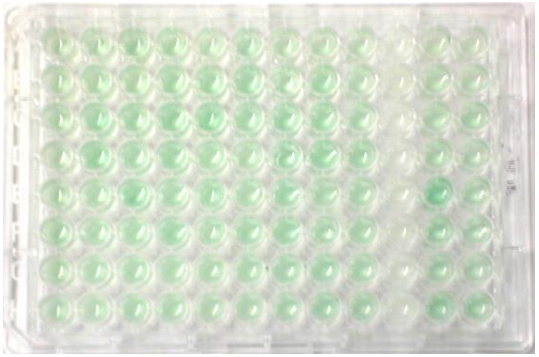
Screening based on detection of pH shift
Example: Esterases



Screening - Selection

Cultivation in Liquid Culture





Deepwell plates

Shake flasks

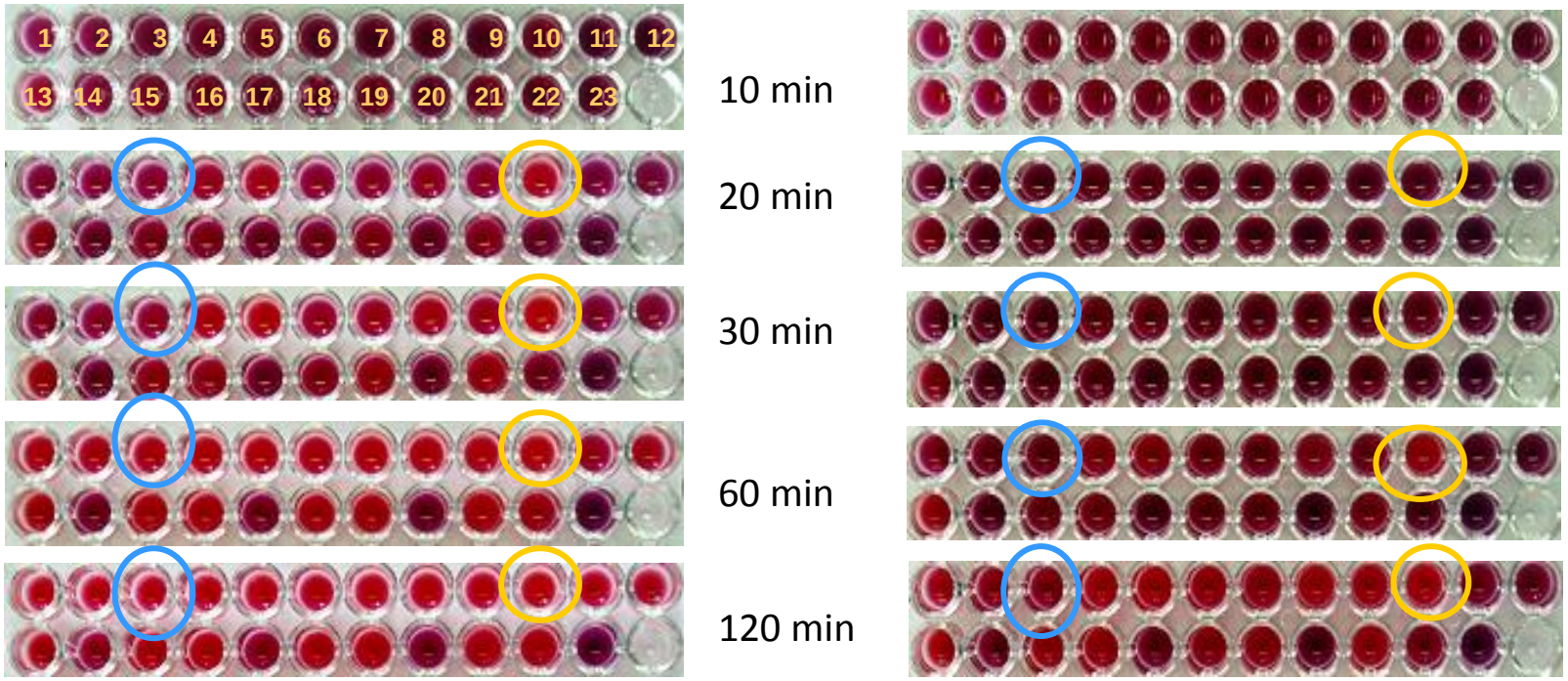
Lab fermenters

Detection

Microplate assays – Photometric Fluorometric
HTP chromatography (e.g. HPLC)
HTP MS methods
HTP NMR methods

Screening systems

Microplate Assays



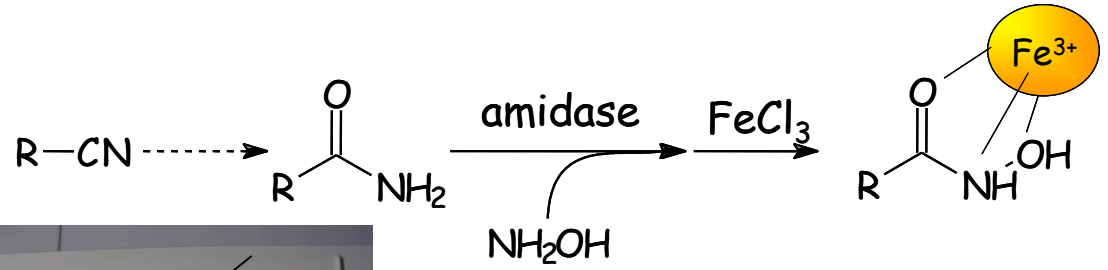
22: wt enzyme
23: blank lysate

New Nitrile Hydratases

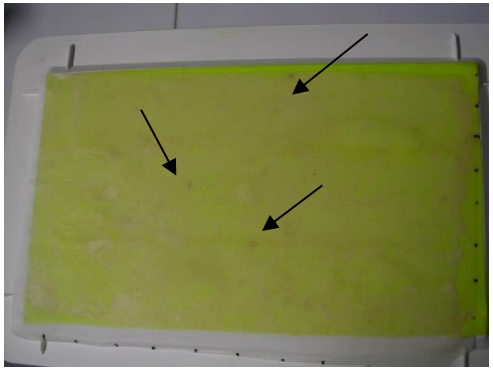
2.1

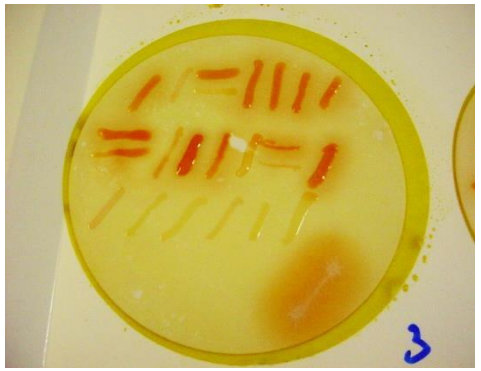
Screening assays

In vitro screening assay

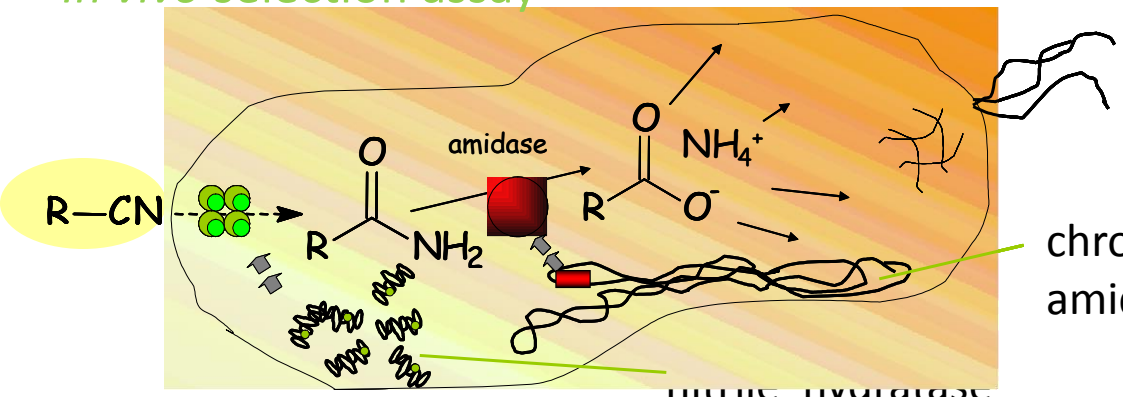


hydroxamic acid





In vivo selection assay

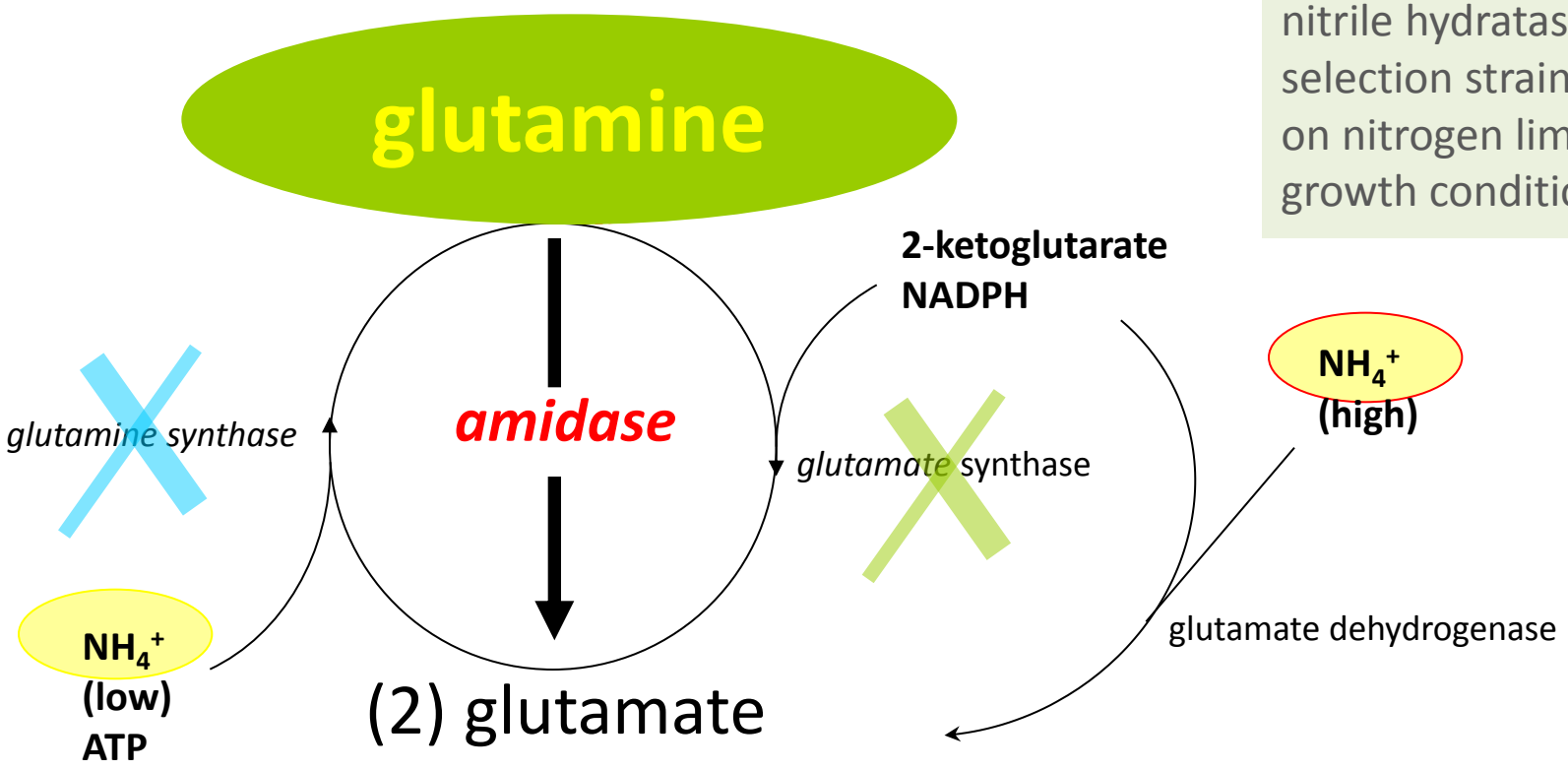


chromosome with amidase integration

nitrile hydratase expression plasmids

Selection Systems

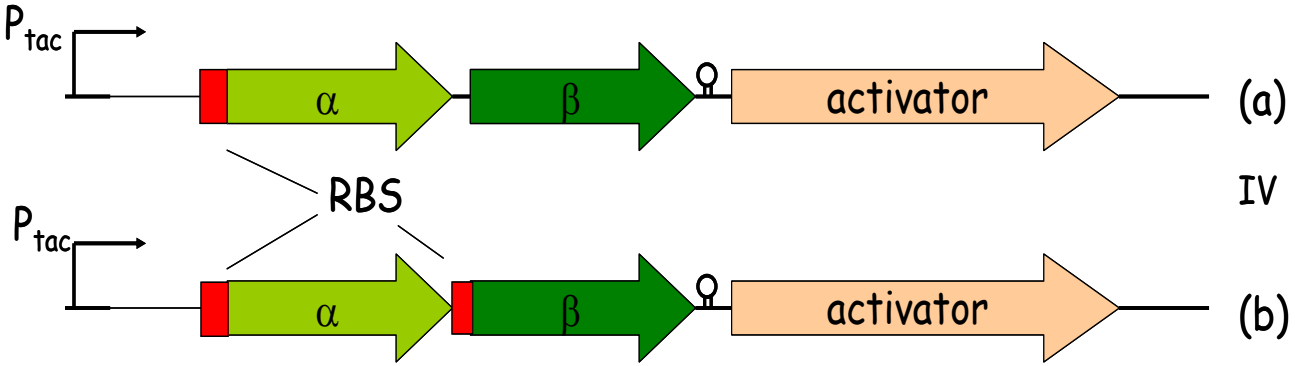
Development of a nitrile hydratase selection strain based on nitrogen limited growth conditions

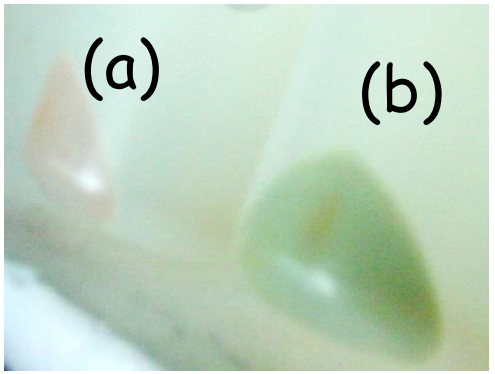


nitrile —*nitrile hydratase*→ amide —*amidase*→ acid + NH_4^+

New Nitrile Hydratases

2.1

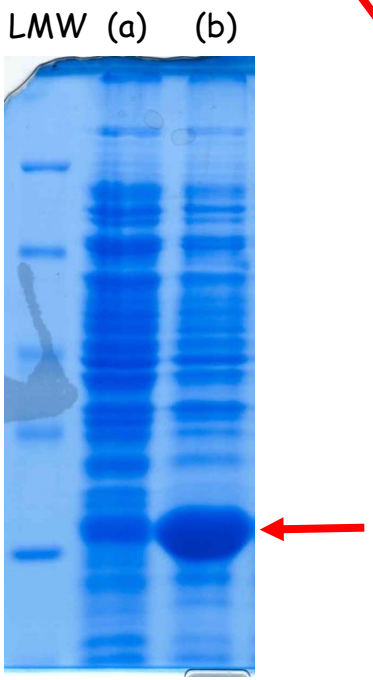




Improvement of the nitrile hydratase expression level by re-design of the expression cassette

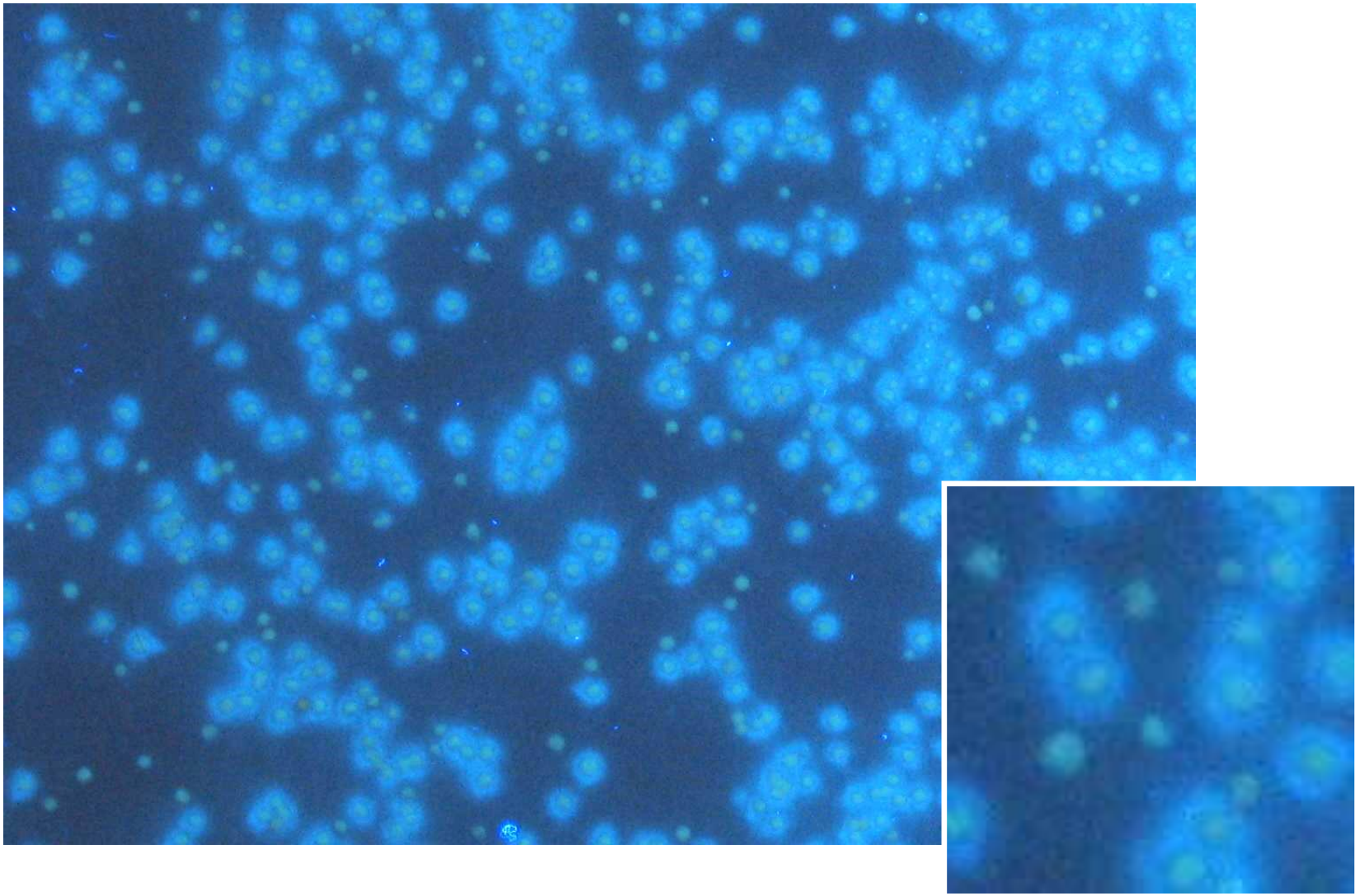
Introduction of a second copy of the optimized ribosome binding site upstream of the beta subunit

→ **dramatic increase** in soluble (and active) nitrile hydratase formation.
→ green appearance of the pellet of cells producing high amounts of the iron containing enzyme.



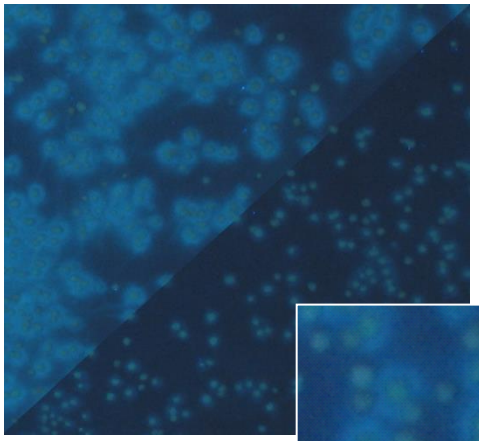
→ Industrial Application

NADH Fluorescence Coupled Assay

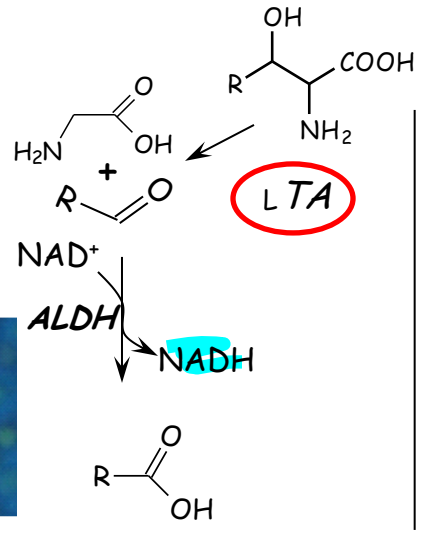


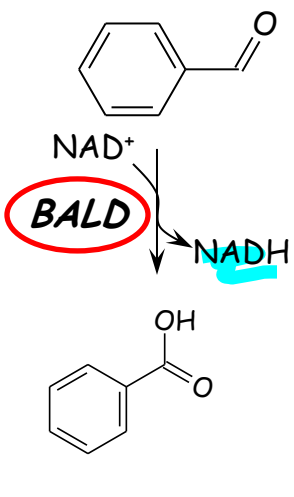
Mixture of *E. coli* colonies with and w/o threonine aldolase activity

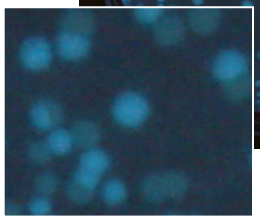
NADH – A Versatile Reporter

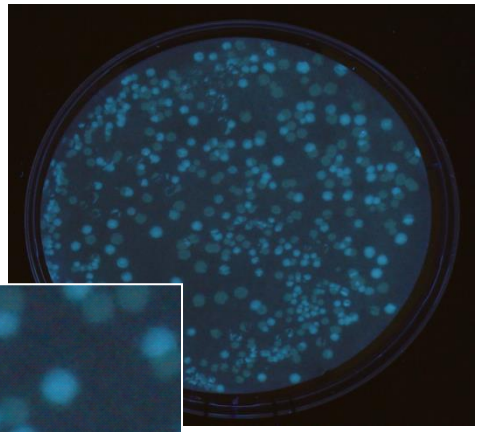


L-threonine aldolase



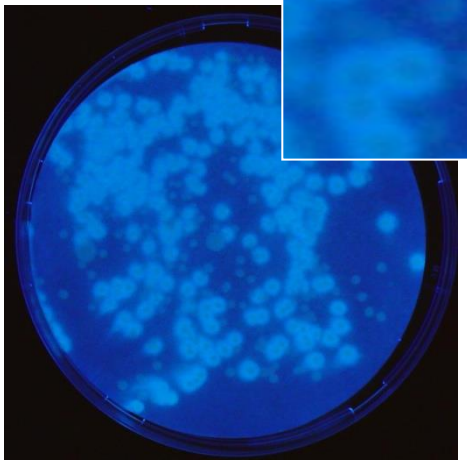


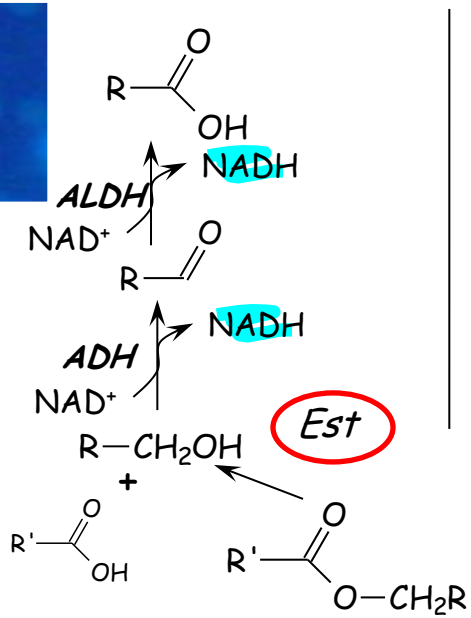




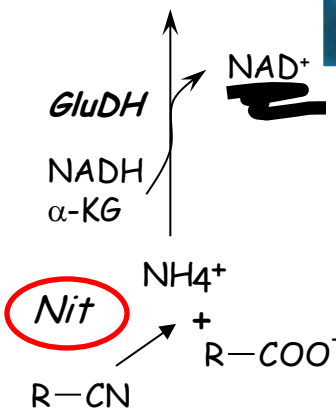
(benzaldehyde) dehydrogenase

esterase

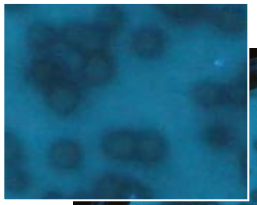




glutamate

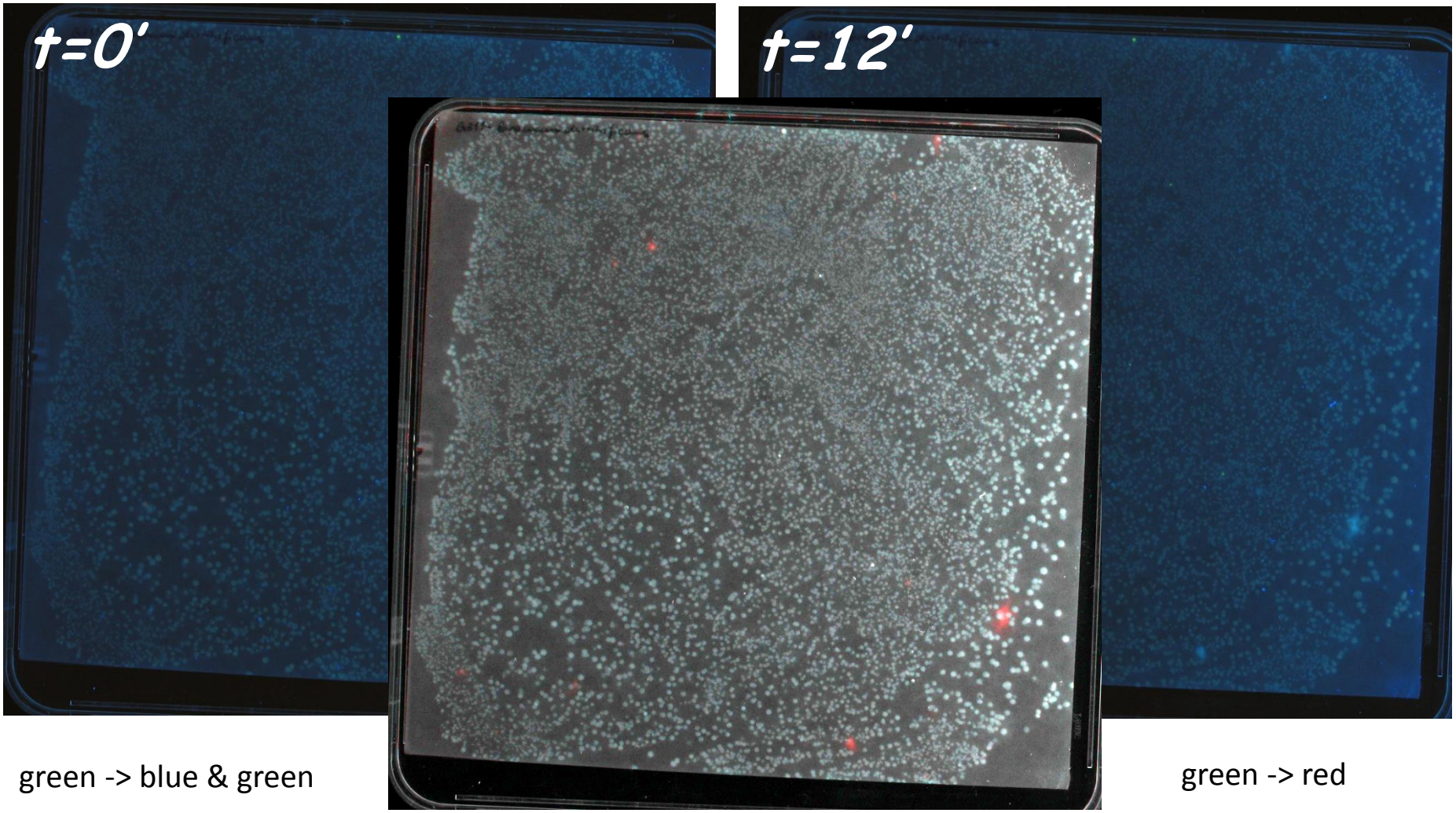


nitrilase



Reisinger *et al. J. Mol. Cat. B,* **2006**, 39, 149-155

Data evaluation

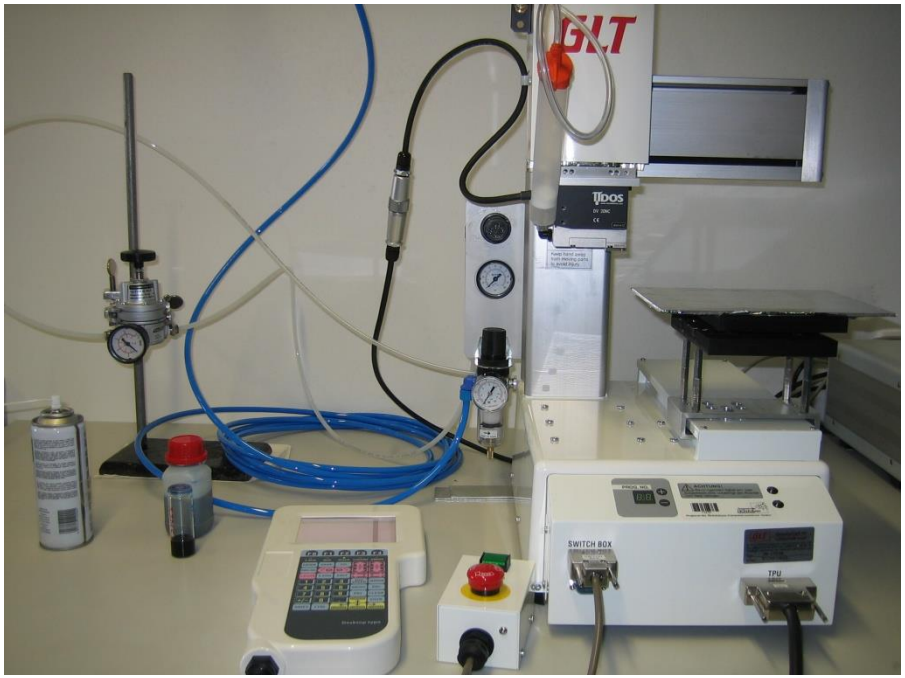


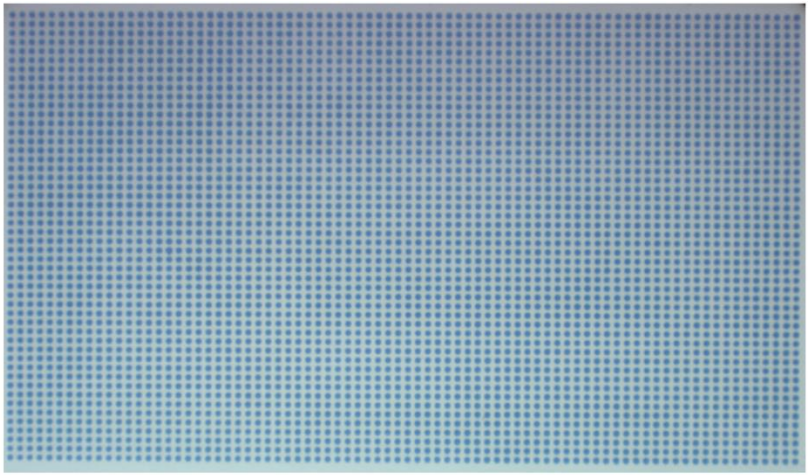
green -> blue & green

green -> red

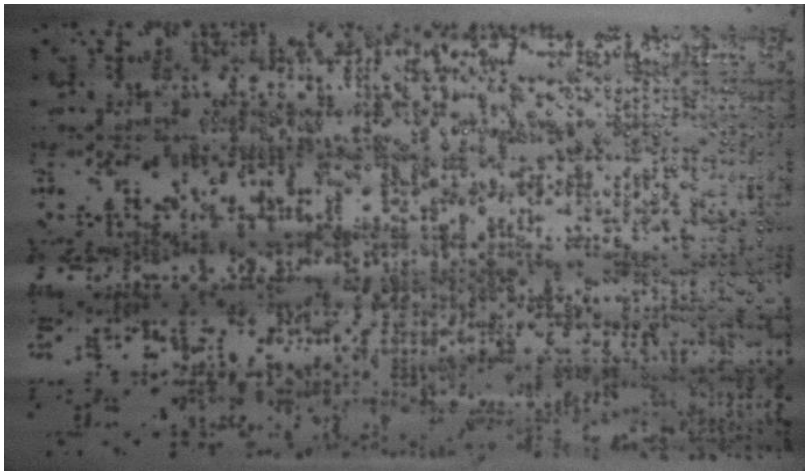
*1.Rasband, W.S., **ImageJ**, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006.*

Micro-colony Arrays





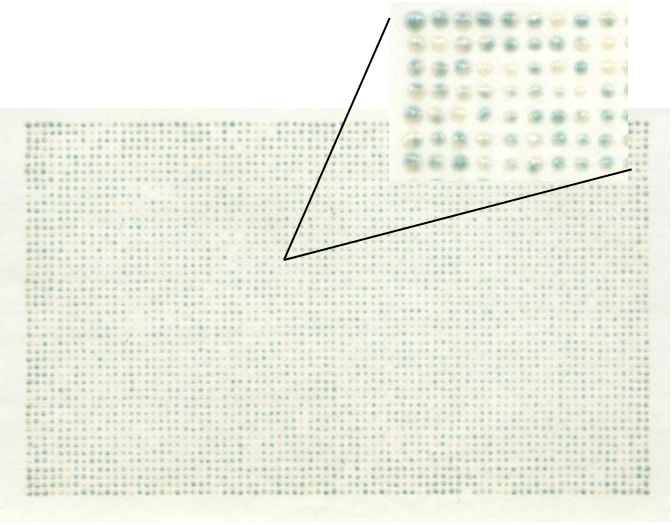
Ordered Arrays



Micro-colony Array Screening Platform

High throughput detection of enzyme activity

Generation of a high density ordered colony array



6000 colonies on filter (microplate formate)

Direct spotting from library pool – no colony picking

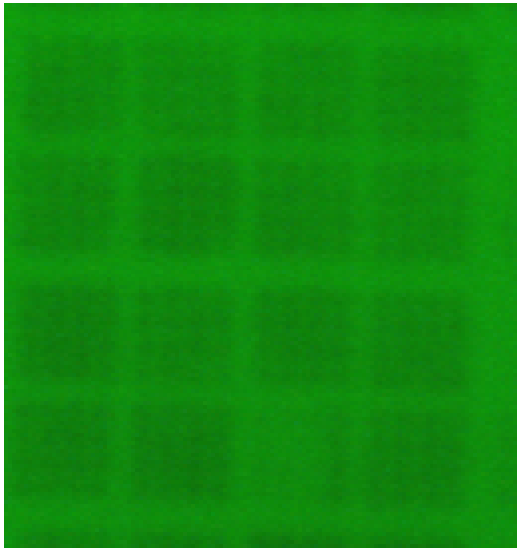
Replicas of filters

Pre-treatment of arrays possible (e.g. solvents, T , pH, etc.)

Enzyme reaction

Detection system (e.g. chemosensor)

Simultaneously monitoring CCD camera



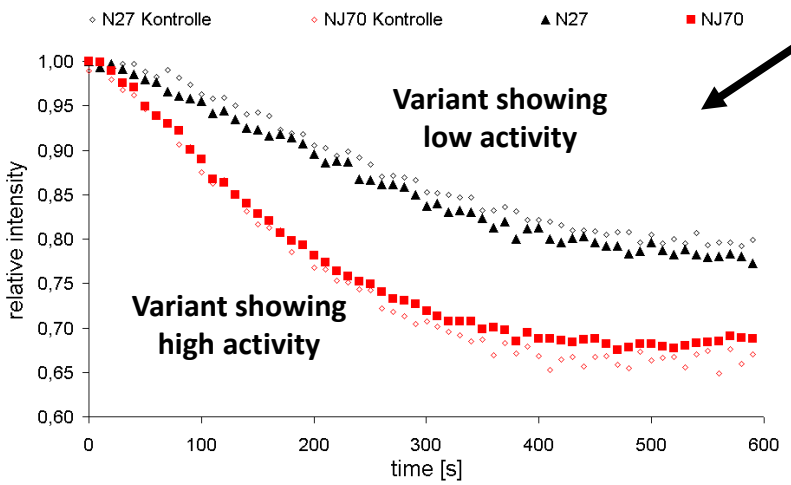
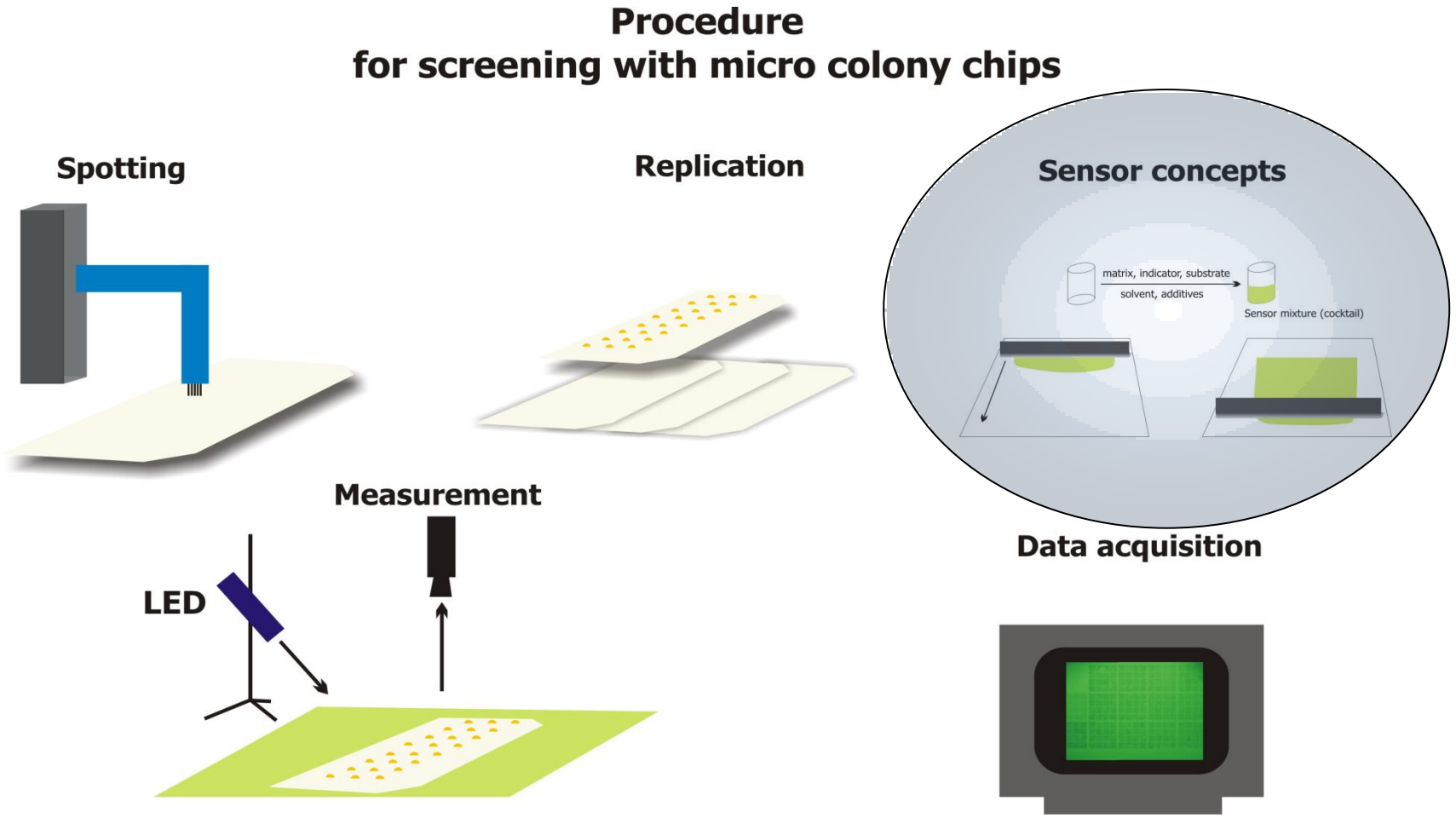
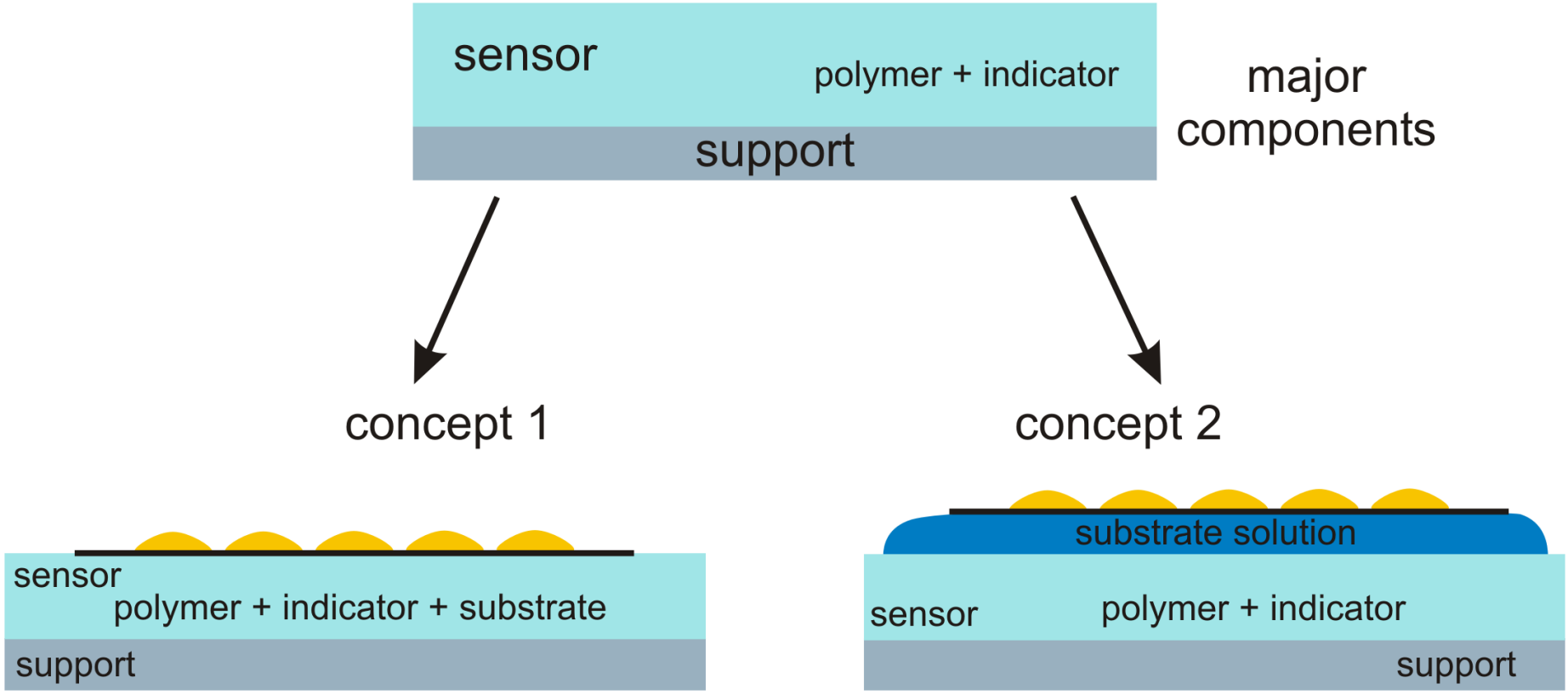


Image analysis & automated hit detection

Micro-colony Array Screening Platform

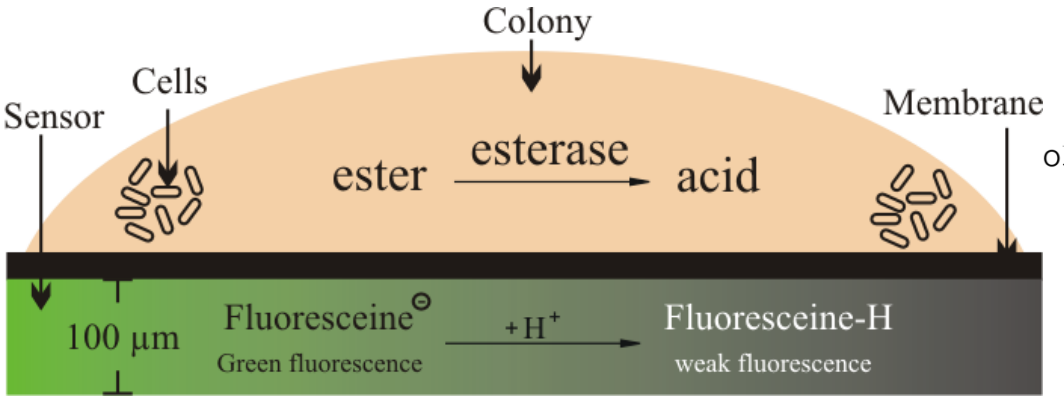


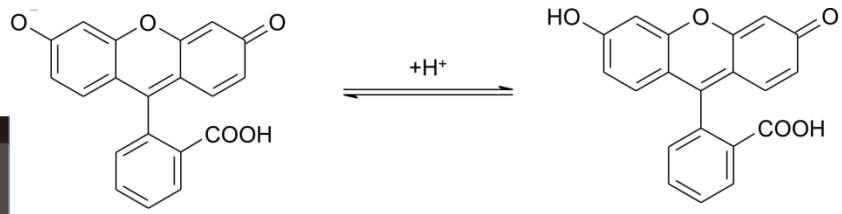
Sensor design

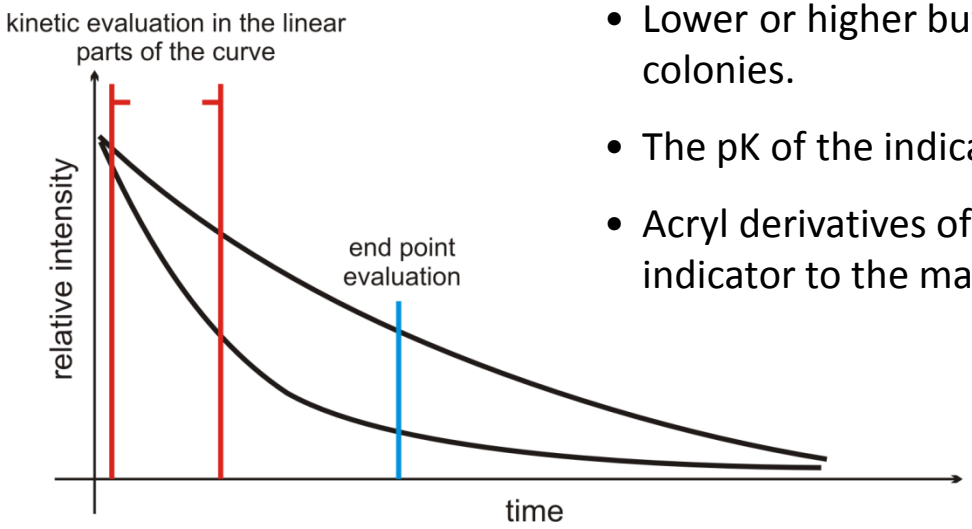


Sensor design: pH-Sensor

Esterase screening





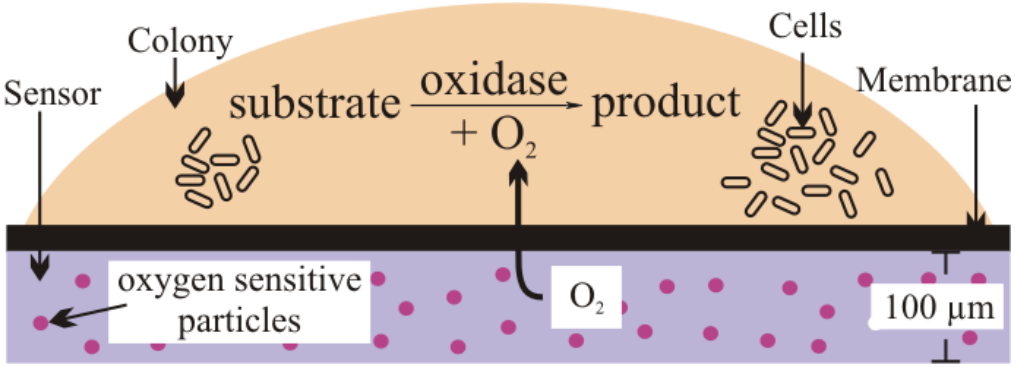


Features:

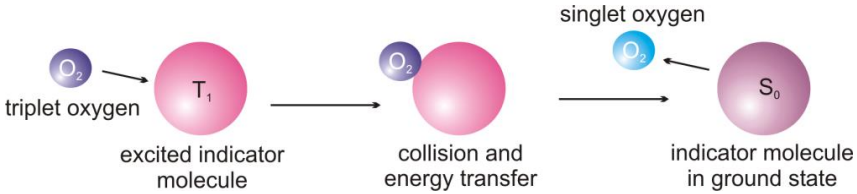
- Lower or higher buffer capacity allows adjustment to activity of the colonies.
- The pK of the indicator defines the sensitive pH window.
- Acryl derivatives of fluoresceine allow covalent linkage of the indicator to the matrix.

This system is usable for every enzymatic reaction which releases or consumes protons.

Sensor design: pO_2-Sensor

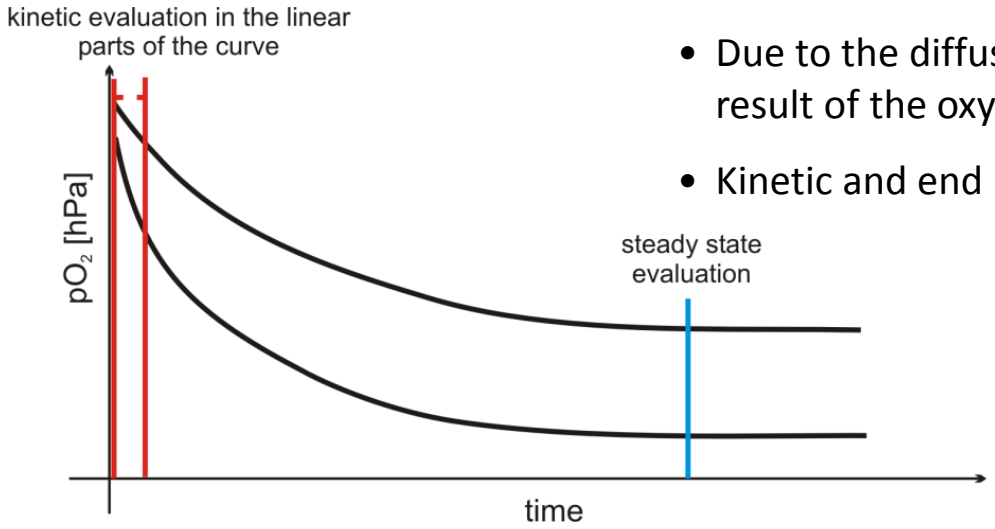


Oxidase screening



Features:

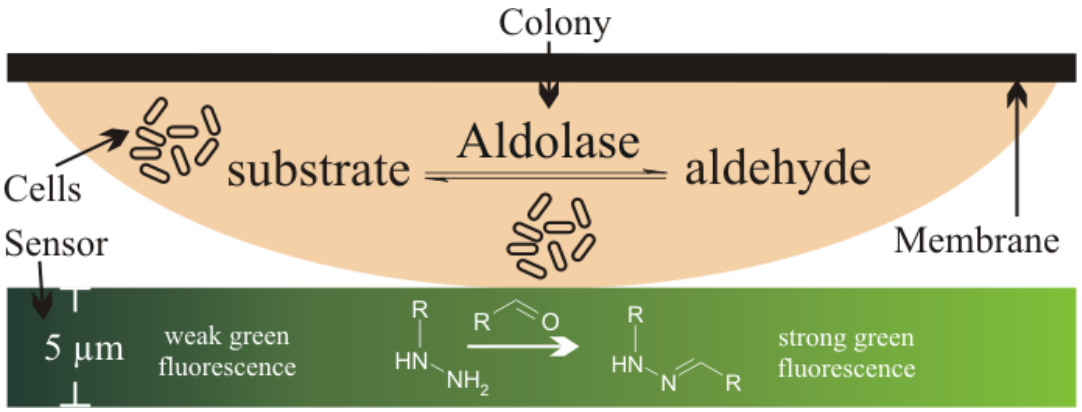
- Material of the particles allows adjustment to activity of the colonies.
- Due to the diffusion of oxygen in the sensor a steady state is the result of the oxygen consuming reaction.
- Kinetic and end point measurements are possible.

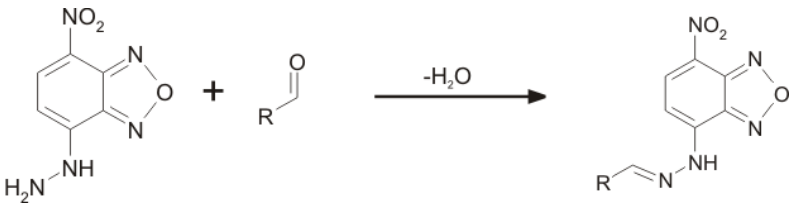


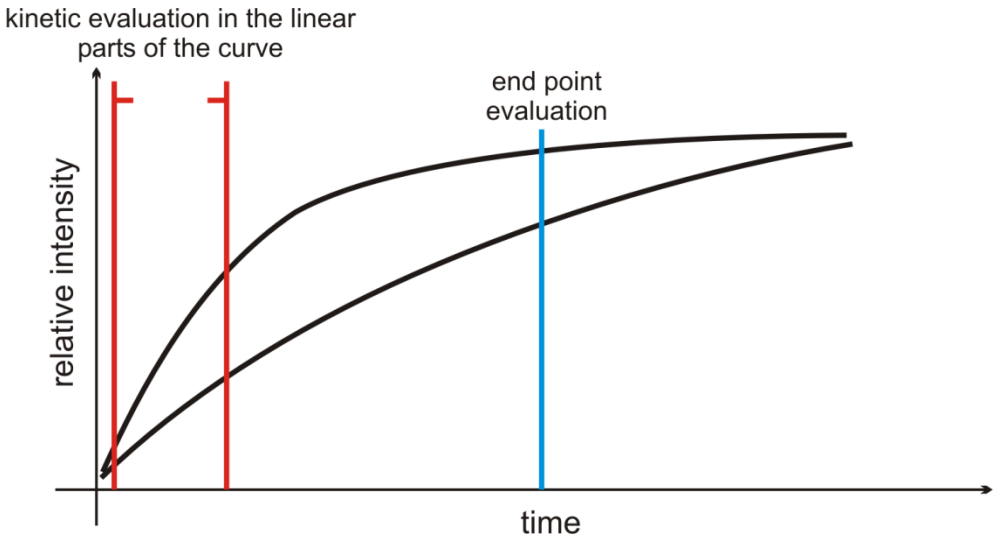
This system is usable for every enzymatic reaction which consumes oxygen.

Sensor design: Aldehyde sensor

Aldolase screening







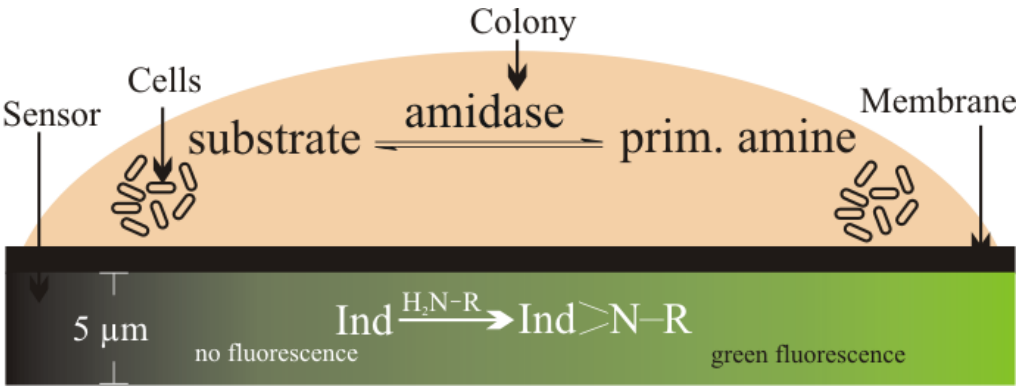
Features:

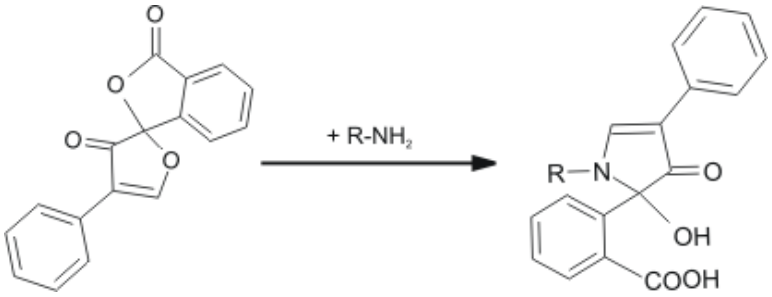
- Different matrices for different aldehydes.
- Screening in non-aqueous environment is possible.
- Incorporation in MTP should be possible.
- Other indicators have to be examined.

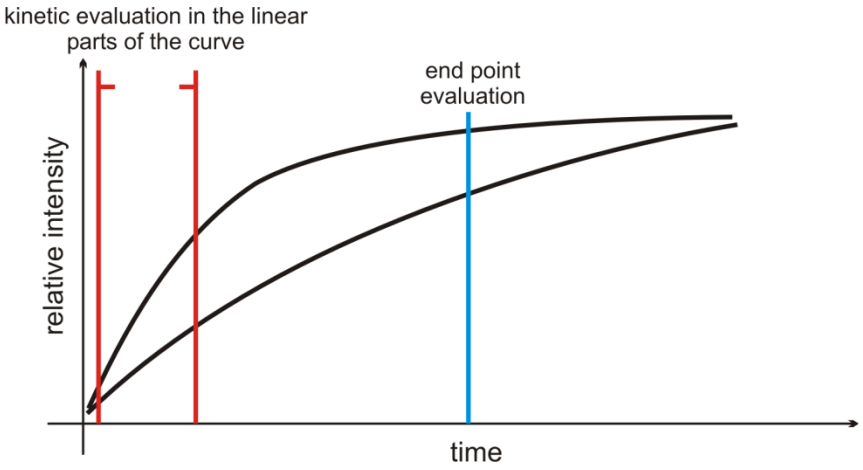
This system is usable for every enzymatic reaction which releases aldehydes.

Sensor design: prim. Amine sensor

Amidase screening







Features:

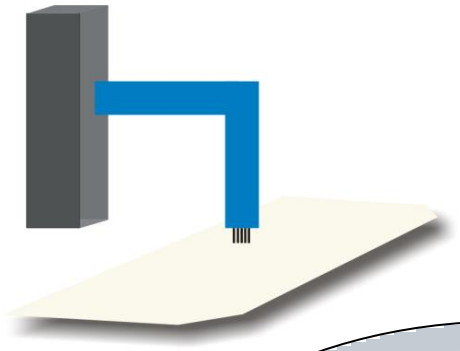
- matrix determines the screenable activity range.
- Sensor is easily adaptable for other amine indicators.
- Kinetic and end point measurements are possible.
- Incorporation in MTP is possible.
- Protease reactions are visualisable.

This system is usable for every enzymatic reaction which releases prim. amines.

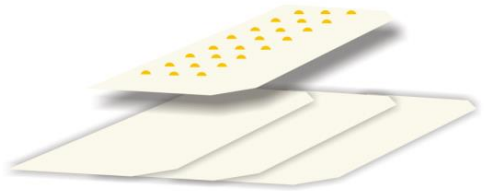
Micro-colony Array Screening Platform

Procedure for screening with micro colony chips

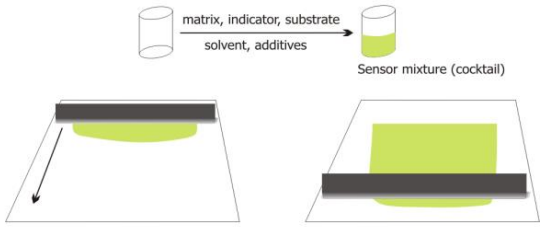
Spotting



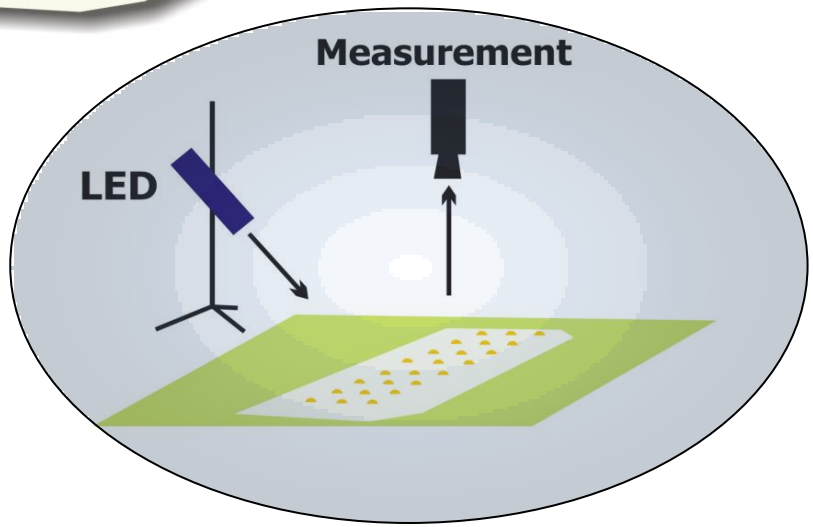
Replication



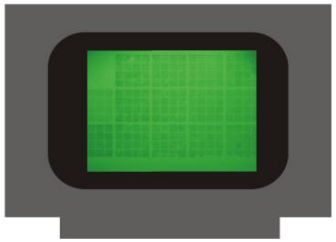
Sensor concepts



Measurement

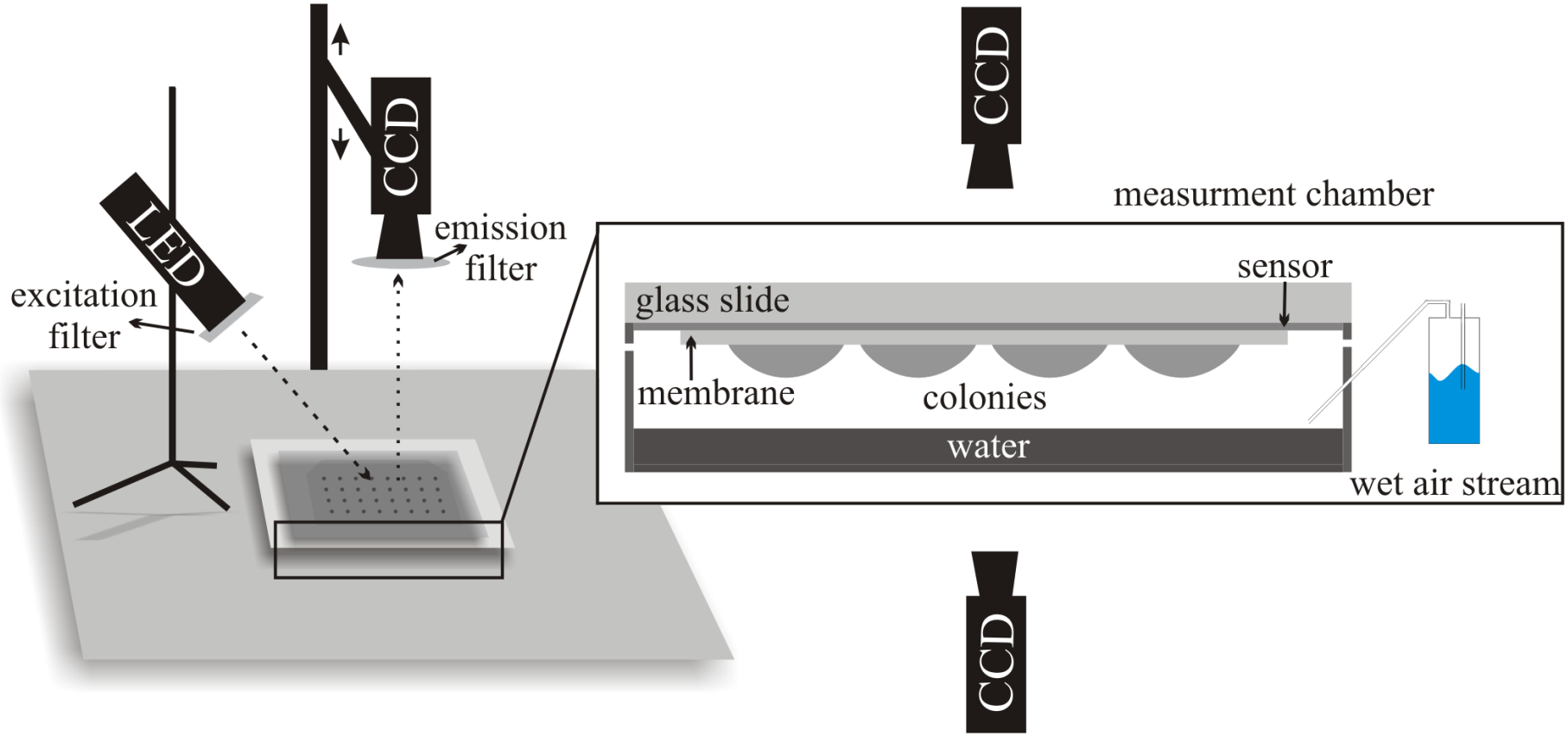


Data acquisition



Measurement – instrumentation

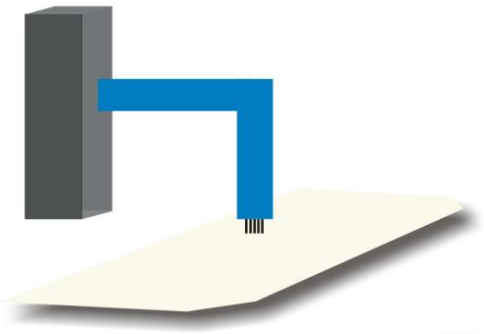
Overview



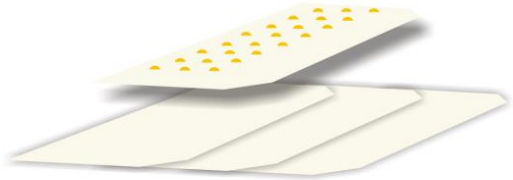
Micro-colony Array Screening Platform

Procedure
for screening with micro colony chips

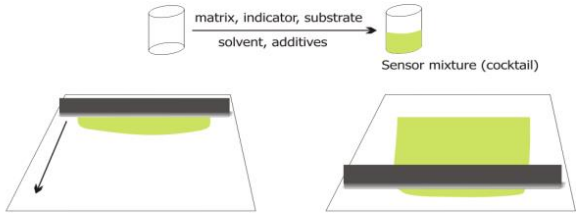
Spotting



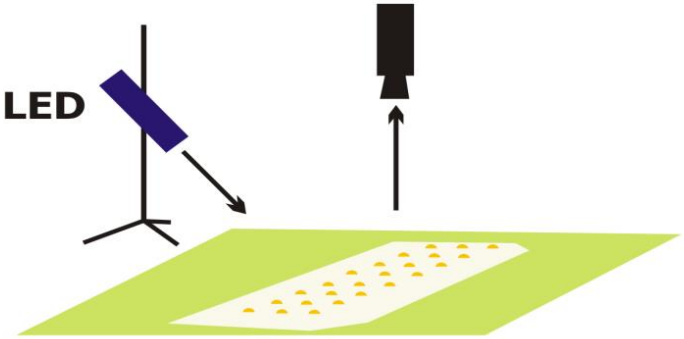
Replication



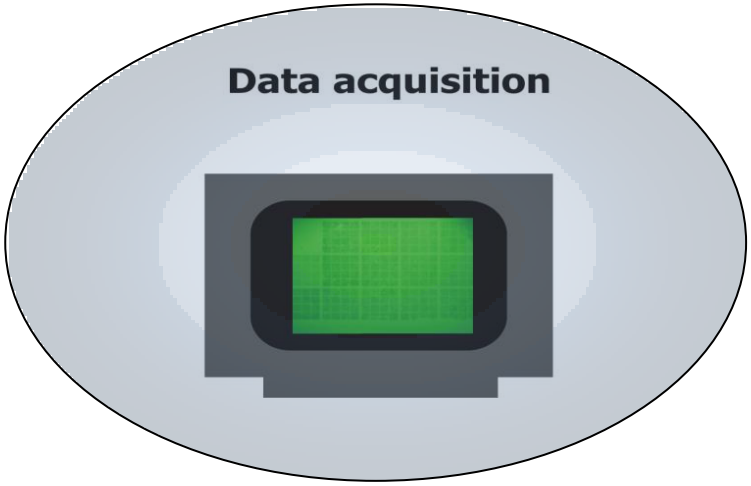
Sensor concepts



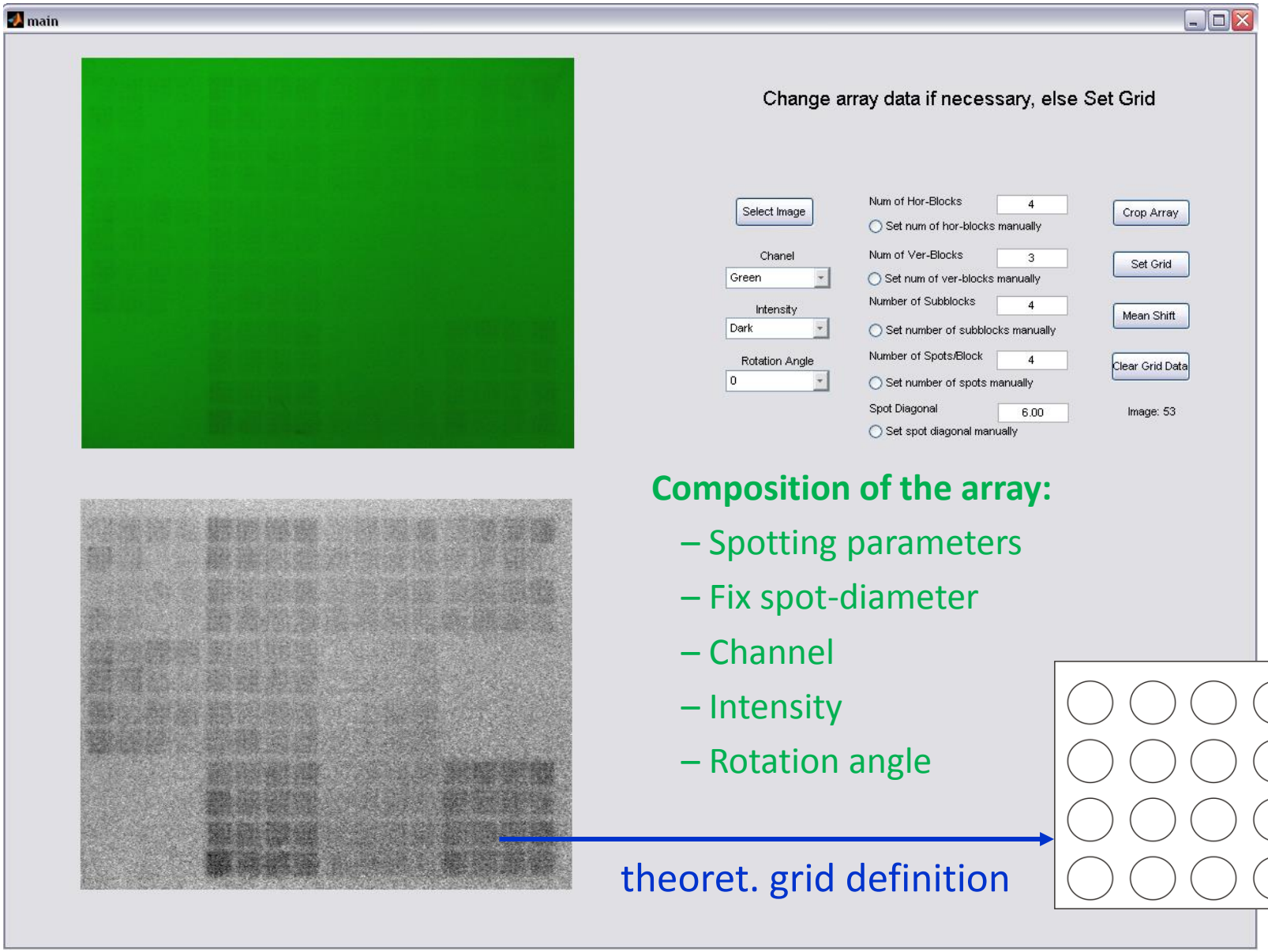
Measurement



Data acquisition



Measurement – Data Evaluation manually



Composition of the array:

- Spotting parameters
- Fix spot-diameter
- Channel
- Intensity
- Rotation angle

theoret. grid definition

Measurement – Data Evaluation automatically

… **normalise image for comparison**

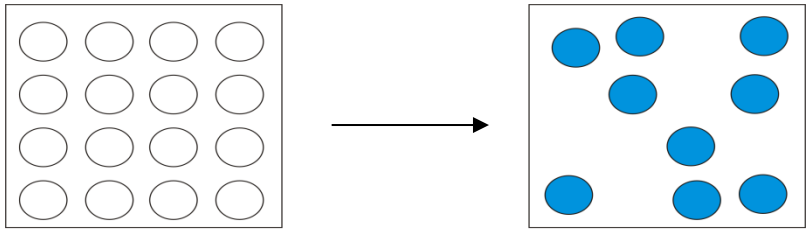
… **find the position of the spots**

- Corresponding block
- Situation inside the block

… **find the centre of the colonies**

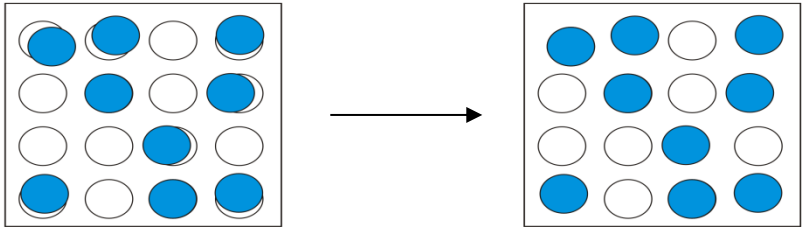
- Spot-content from centre and diameter

theoret. grid definition



real grid

Finding the centre of the colonies



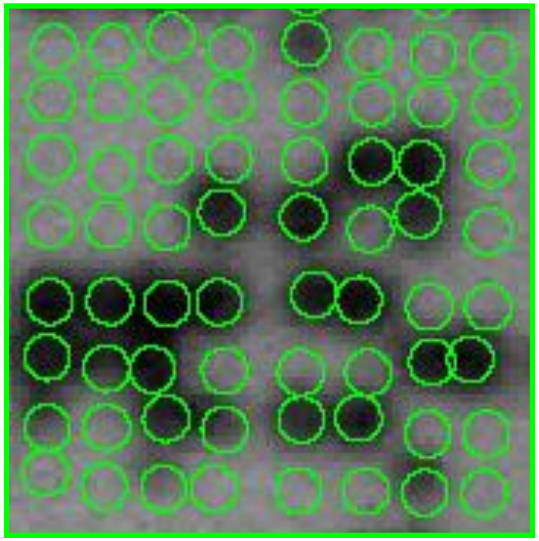
at found positions, calculation can be done

Measurement – Data Evaluation

Results

Spot positions are …

- clearly identified as long as there is a signal.
- approximated when there is no signal.
- missed if the real centre is too far from the first grid approximation.



Colony chips with less than ~ 30% visible spots:

- alignment of the theoret. grid with the real grid not possible.
- guide spots are not replicable.
- an image of the grid is needed – second camera for colony mage