M2/L1: Enzyme sources, screening and selection

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Enzyme sources

Enzymes can be produced from any living organism, either by extracting them from their cells or by recovering them from cell exudates.

Industrial Enzymes produced from

1. Plant
2. Animal
3. Microorganisms

In 1960, about 70% of the enzymes extracted from plant tissues or exudates and animal organs.

Enzyme sources

Nowadays, Animal derived and Plant derived enzymes roughly represent about 10% and 5% respectively of the total enzyme market.

Some example of most relevant animal and Plant enzymes,widely used in the industries

- Catalase (EC 1.11.1.6) from liver (Yildiz et al. 2004),
- lipase (EC 3.1.1.3)
- Chymotrypsin (EC 3.4.21.1),
- Trypsin (EC 3.4.21.4) from pancreas (Underkofleret al. 1958)
- Rennin from calf stomach
- Papain (a cysteine protease obtained from the latex of papaya for meat tenderization, beer clarification, yeast extract production, stain removal, and, in highly purified form, in several cosmetic and medical applications)
- Bromelain (a complex of cysteine proteases extracted from pineapple stems with different applications mainly the medical area as a wound healing, anti-inflammatory, digestive-aid and appetite suppressant agent.

Enzyme sources

Limitations

Plant Sources:

- Restricted Cultivation
- Concentrations of Enzymes in plant tissues is generally low

Animal Sources:

- Enzymes produced as by products in meat industries
- Low Concentration

20 years later the situation had reversed and most industrial enzymes were produced from microbial sources.

Why microorganisms preferred for enzyme production over Plant and Aminal sources

- metabolically vigorous & versatile
- easy to propagate on a large scale by submerged or solid-state fermentation,
- simple to manipulate both environmentally and genetically
- Simple nutritional requirements
- Supply not conditioned by seasonal fluctuations
- profound technological implications,
- more reliable, simpler and cheaper enzyme production
- Satisfy the demands of the market

Advent of rDNA technology

- Most enzymes are produced by mesophilic organisms
- Research, various tests and screening performed on enzymes from Extremophiles in search of potential Industrial enzymes to perform under extreme conditions
- Genes of Thermophilic and psychophysics enzymes cloned into suitable mesophilic or other convenient microbial host as such extremophiles are difficult to grow under laboratory conditions
- In 1994 it was estimated that about 50% of the industrial enzymes (on a mass basis) were produced from genetically engineered organisms
- The advances in recombinant DNA technology and protein engineering increases the production of specialty enzymes for the pharmaceutical and fine-chemicals industries

An example that illustrates this evolution

- Chymosin (EC 3.4.23.4: very specific aspartic acid protease) best choice for Cheese making

- High yields of curdling
- Proper flavor development in aged cheese

- Traditional source: calf rennet, an extract from the inner mucosa of the fourth stomach (abomasus) of suckling calves obtained as a by-product of veal production.

- Crisis: Shortage of rennet as source of chymosin for cheese making became critical

- 1st Approach: Partial substitution of chymosin for Pepsin despite the sacrifice in quality of the cheeses so produced

In the early 1960s, most successful microbial chymosin proteases obtained from *Mucor miehei* and *Mucor pusillus* (Hiramatsu et al. 1989).

Drawbacks:

- not as acceptable as calf chymosin because low Curd yields are lower
- not so pleasant flavor is developed upon maturation.
- microbial rennets are more thermostable and its inactivation at high temperature impairs the texture of the product so that the enzyme requires being chemically modified (van den Berg et al. 1987).

Advanced genetic engineering Approach:

Initial host for (pro)chymosin : *Escherichia coli*;

Disadvantage:

- the enzyme produced in the form of insoluble inclusion bodies
- Required post-transcriptional processing
- Complex process of denaturation and renaturation

Better hosts for chymosin expression:
Fungi*: Trichoderma, Neurospora*, *Aspergilli*

Aspergillus oryzae have been claimed as the best hosts & has been in the market for over a decade

Drawback: the enzyme was poorly excreted in an active form

Kluyveromyces lactis soon considered as the best option (van den Berg et al. 1990; Morris and Anderson 1991)

- Impressive secretory capacity,
- excellent fermentation characteristics at large scale,
- Food grade status
- availability of both episomal and integrative expression vectors
- synthesizing and excreting fully active prochymosin

The recombinant chymosin from *K. lactis* has been in the market for more than 20 years now with great commercial success and it is probably the most widely used enzyme in cheese-making.

Protein engineering has also been applied to improve chymosin performance by conferring the enzyme an increased specificity and better pH profile (Mantafounis and Pitts 1990).

Enzyme Screening

- Screening and selection are early processes in the research and development cycle of a product.

- Enzyme screening and selection strategies are based on knowledge of the application and the physical and chemical conditions under which the enzyme must operate.

- In the past this involved exclusively the screening of living microorganisms (classical microbial screening)

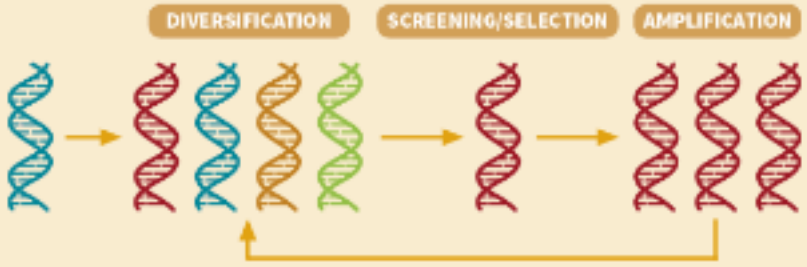
Enzyme Screening

- Access to a large gene pool is another prerequisite for a successful screening program.

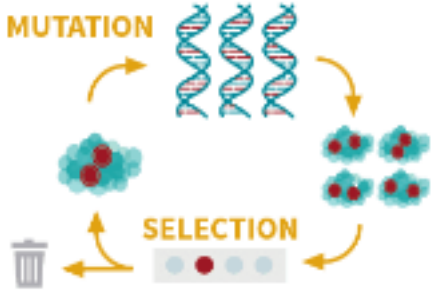
- With application of combining enzyme screening with modern techniques such as protein engineering and directed evolution, screening can be performed without the need to culture the organisms involved.

2018 NOBEL PRIZE IN CHEMISTRY

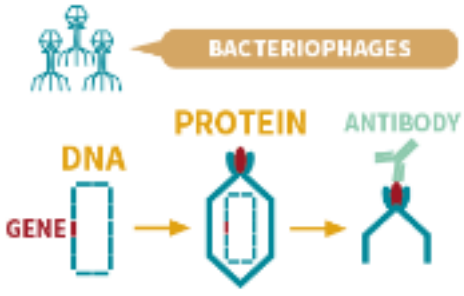
The Nobel Prize in Chemistry 2018 was awarded to Frances H Arnold, George P Smith and Sir Gregory P Winter for their use of directed evolution to produce new enzymes and antibodies.



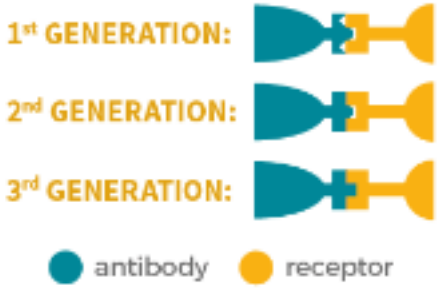
Directed evolution allows chemists to speed up the evolution process in the laboratory. Using it, enzymes can be tailored to catalyse reactions better, or catalyse new ones. Proteins can be made more selective for targets, leading to useful medicines.



Arnold pioneered directed evolution of enzymes. She created random changes in an enzyme's DNA, then selected the variant that was most effective in a certain role.



Smith used bacteriophages (viruses that infect bacteria). He realised that if a gene was added to phage DNA, the protein it produces could be identified on the phage surface.

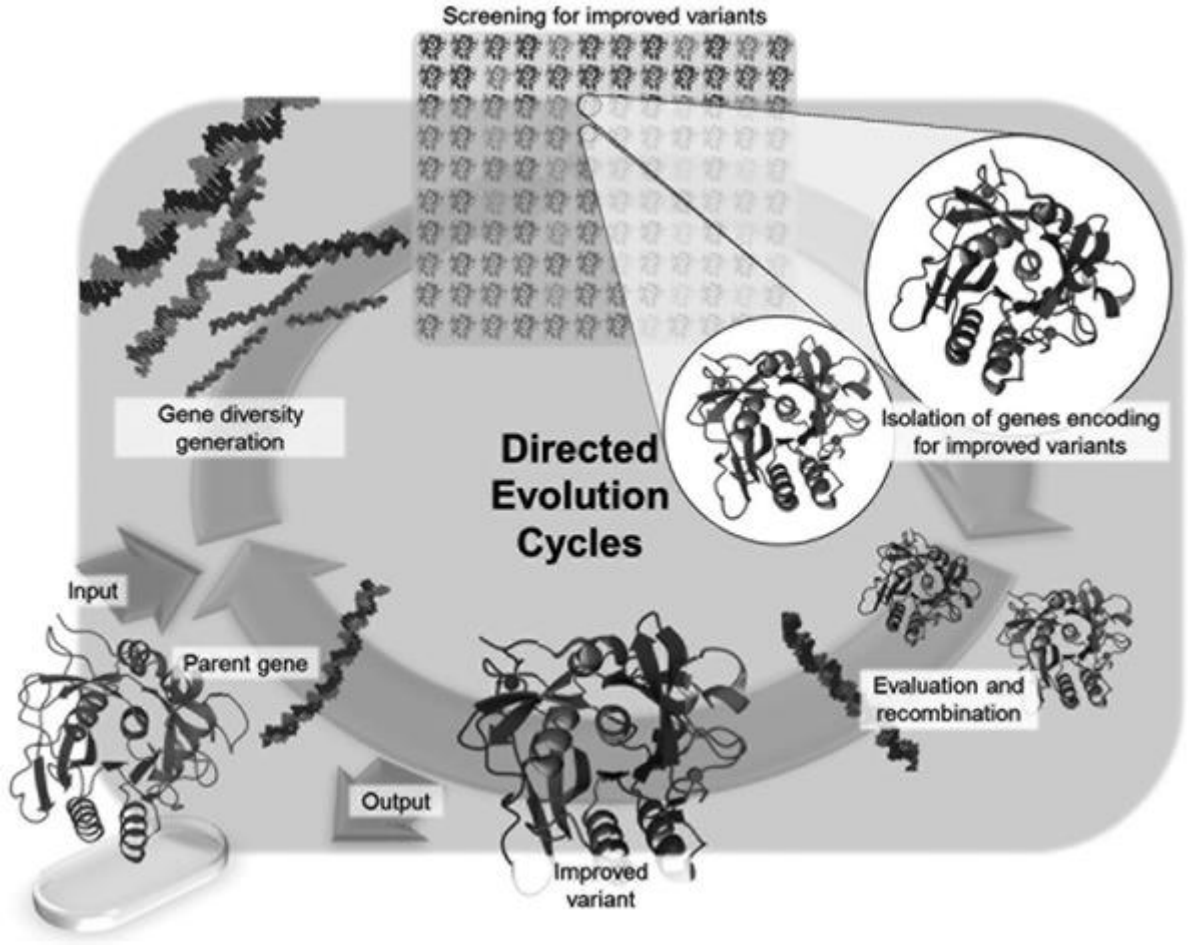


Winter genetically tweaked phages to produce antibodies on their surface. Through directed evolution, he made antibodies with stronger attachments to their targets.

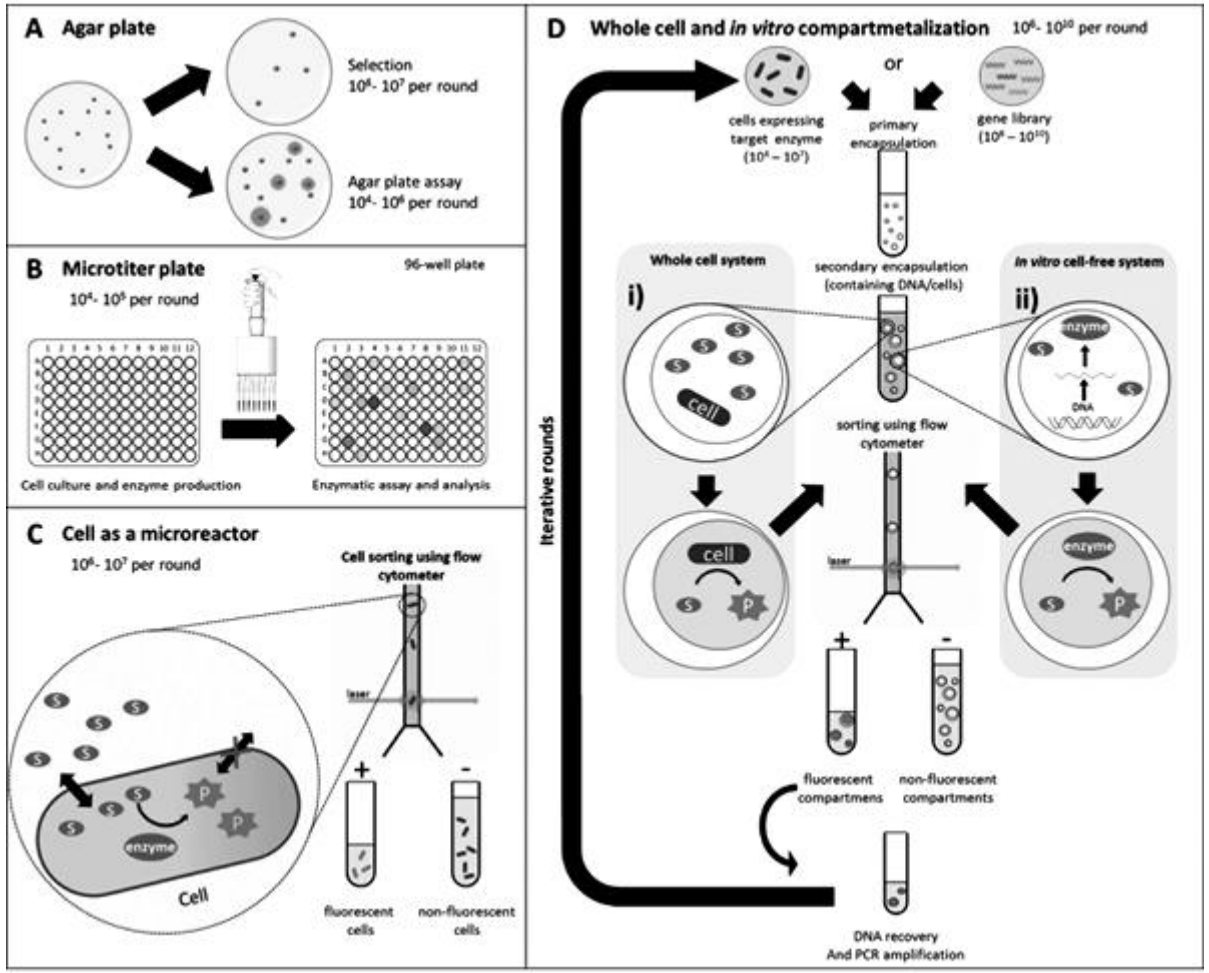
WHY DOES THIS RESEARCH MATTER?

Custom enzymes produced via directed evolution are now used in the production of biofuels and medicines, while evolved antibodies can be used against autoimmune diseases and metastatic cancer.

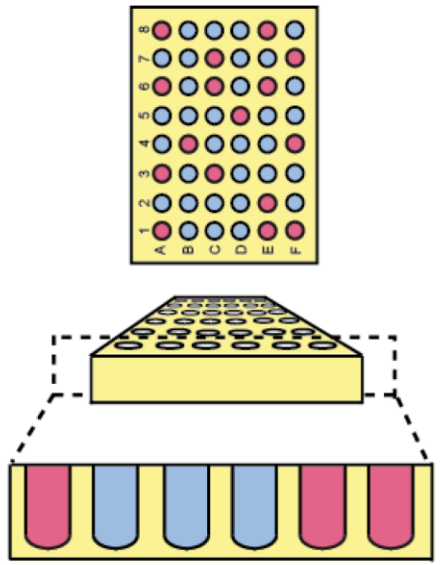
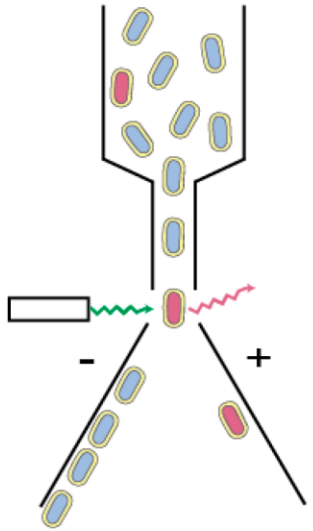
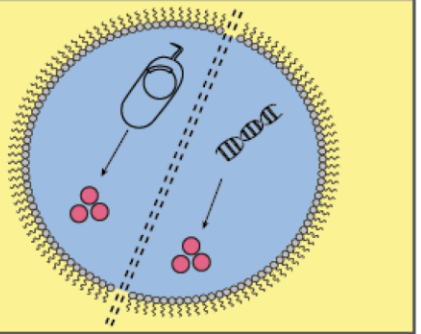
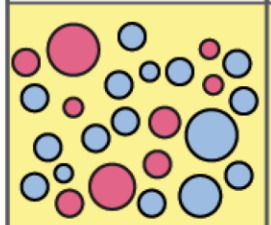
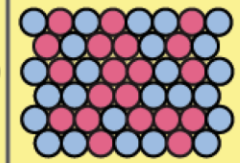
Enzyme engineering!



Various options for screening



Enzyme screening

	Microtiter plate	FACS	Emulsion
			 IVC  µIVC 
Throughput	High ($<10^5$ per day)	Ultrahigh ($>10^5$ per day)	Ultrahigh ($>10^5$ per day)
Genotype/phenotype link	Well boundary	Cell membrane	Droplet boundary
Assay type*	Versatile	Cell only	Versatile
Single-cell compatible	No	Yes	Yes
Cost per reaction	High	Low	Low

Lab on a Chip

PAPER

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High-throughput screening for industrial enzyme production hosts by droplet microfluidics†

Cite this: *Lab Chip*, 2014, **14**, 806

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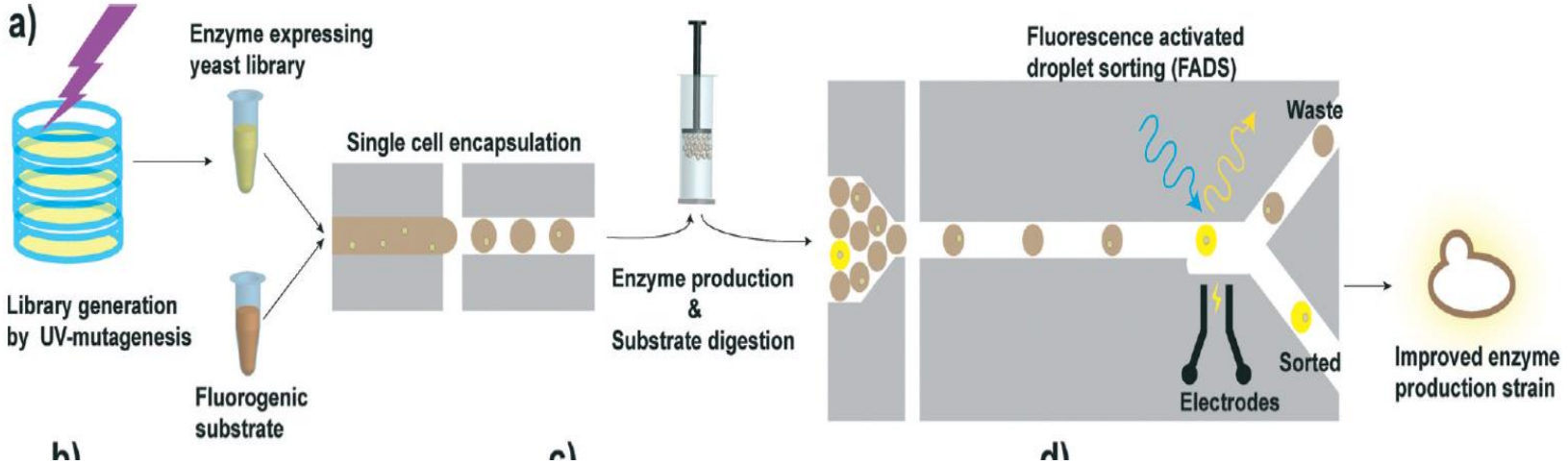
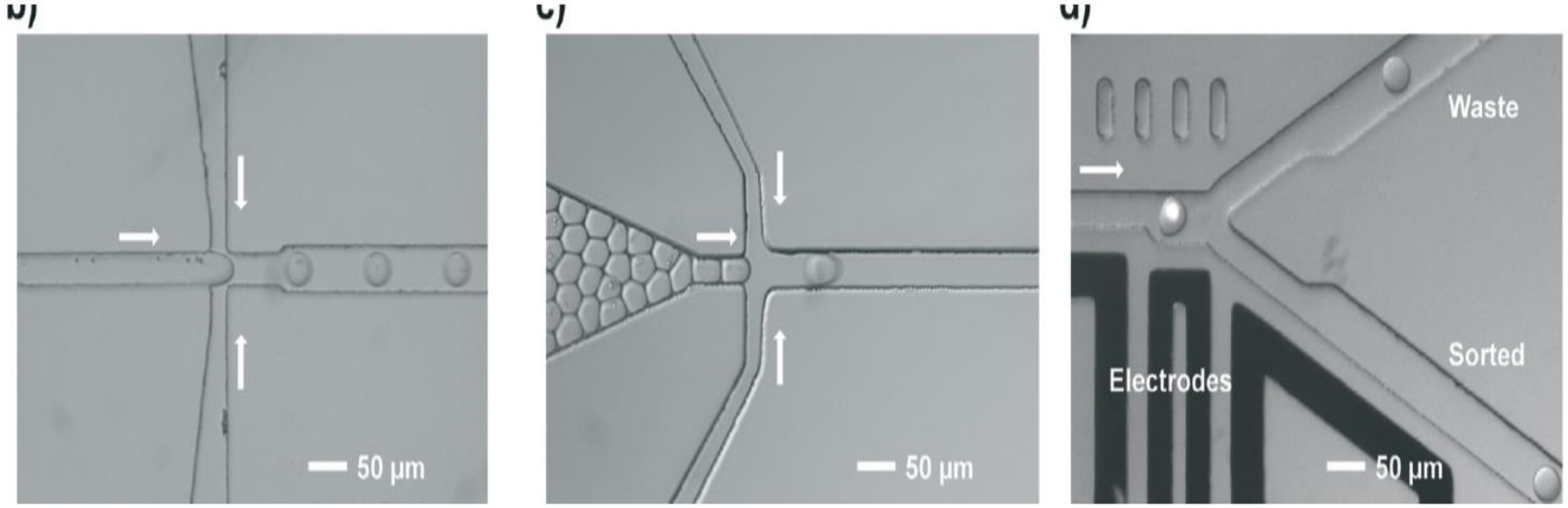
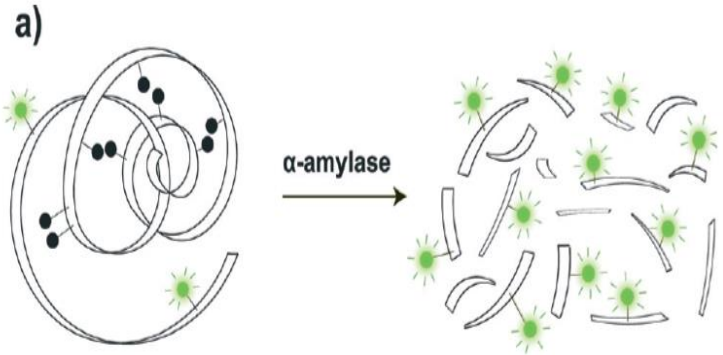
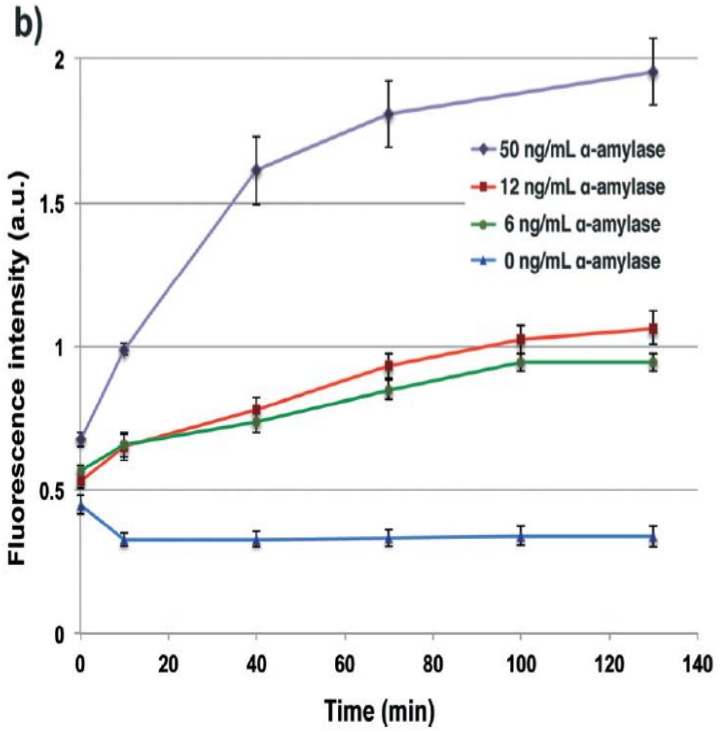
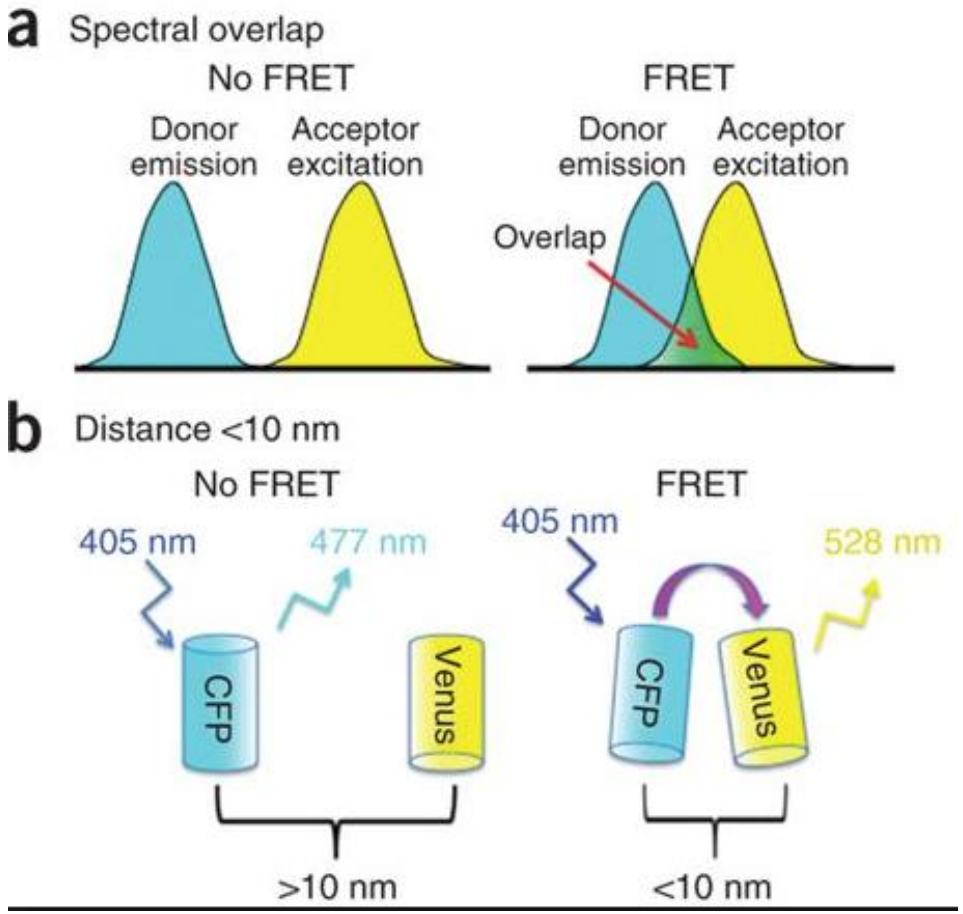
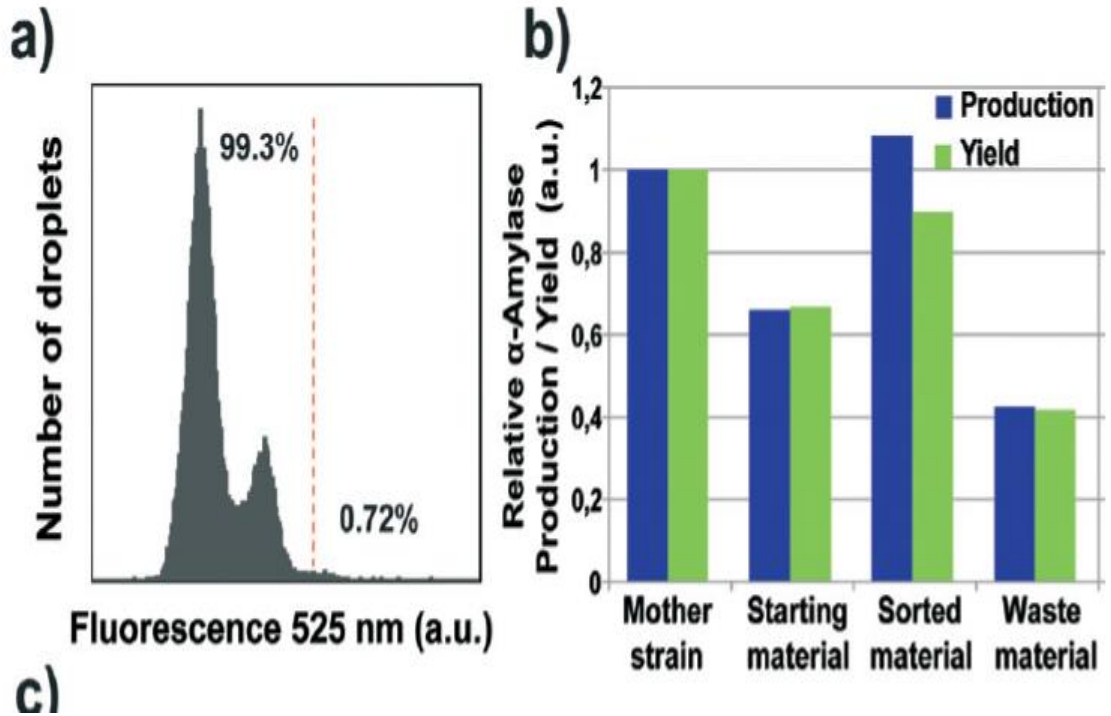

a)
Enzyme expressing
yeast library
Library generation
by UV-mutagenesis
Fluorogenic
substrate
Single cell encapsulation
Enzyme production
&
Substrate digestion
Fluorescence activated
droplet sorting (FADS)
Waste
Sorted
Electrodes
Improved enzyme
production strain

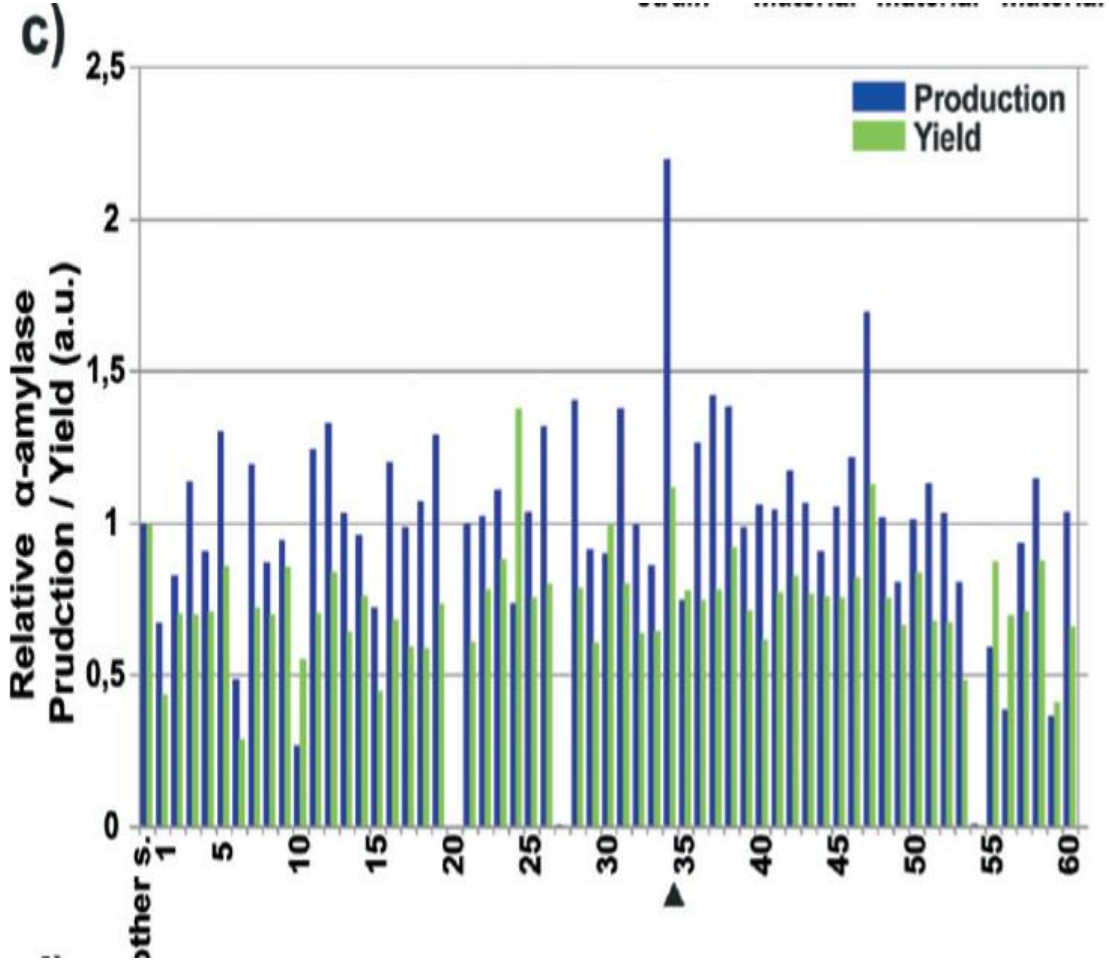

b)
50 µm
c)
50 µm
d)
Waste
Sorted
Electrodes
50 µm

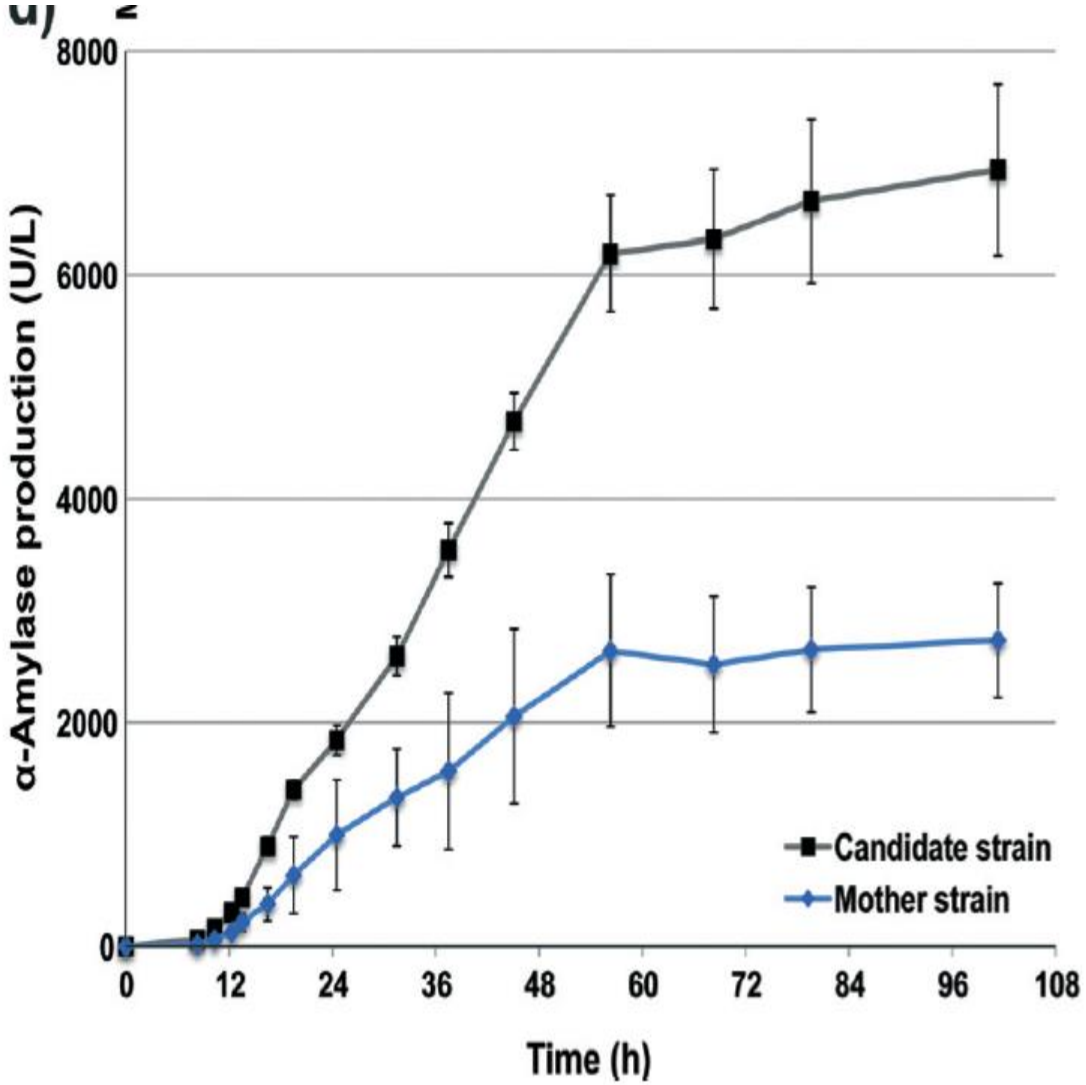

a)
α-amylase


b)
Fluorescence intensity (a.u.)
Time (min)
50 ng/mL α-amylase
12 ng/mL α-amylase
6 ng/mL α-amylase
0 ng/mL α-amylase
0
0.5
1
1.5
2
0
20
40
60
80
100
120
140


a Spectral overlap
No FRET
FRET
Donor emission
Acceptor excitation
Donor emission
Acceptor excitation
Overlap
b Distance <10 nm
No FRET
FRET
405 nm
477 nm
405 nm
528 nm
CFP
Venus
CFP
Venus
>10 nm
<10 nm


a)
Number of droplets
99.3%
0.72%
Fluorescence 525 nm (a.u.)
b)
Relative α-Amylase Production / Yield (a.u.)
1,2
1
0,8
0,6
0,4
0,2
0
Production
Yield
Mother strain
Starting material
Sorted material
Waste material
c)


c)
Production
Yield
Relative α-amylase
Prudction / Yield (a.u.)
2,5
2
1,5
1
0,5
0
other s.
1
5
10
15
20
25
30
35
40
45
50
55
60


8000
6000
4000
2000
0
α-Amylase production (U/L)
0
12
24
36
48
60
72
84
96
108
Time (h)
Candidate strain
Mother strain

Thank you

Summary of HTSOS Methods

methods	screening/library size	applications
high throughput screening methods		
microtiter plates	$<10^4$/day	enzyme reactions leading to change in color, fluorescence, pH, cell growth, etc.
Digital Imaging	limited by transformation efficiency	restricted to colorimetric activity assays
FACS	up to 3×10^4/s	enzyme reactions leading to change in fluorescence
Cell surface Display	limited by transformation efficiency	screening for bond-forming enzymes
IVTC	limited by the throughput of the detection method (e.g., FACS) but not transformation efficiency	screening enzymatic activities combined with FACS
Reporter based selection	limited by transformation efficiency	selecting enzymes that produce transcription regulation molecules

Summary of HTSOS Methods

methods	screening/library size	applications
high throughput selection methods		
Plasmid Display	limited by transformation efficiency	selecting protein binders
Phage Display	limited by transformation efficiency	extremely efficient in affinity-based selections
SNAP Display	limited to 10^9/(mL of emulsion)	selecting protein binders
Ribosome Display	not limited by transformation efficiency	selecting protein binders, high affinity antibodies, and catalytic enzymes
Growth complementation	limited by transformation efficiency	selecting enzyme properties that can be coupled to host fitness
Reporter based selection	limited by transformation efficiency	selecting enzymes that produce transcription regulation molecules

Enzyme Screening

Different screening methods currently used in industry to obtain enzymes with desired properties

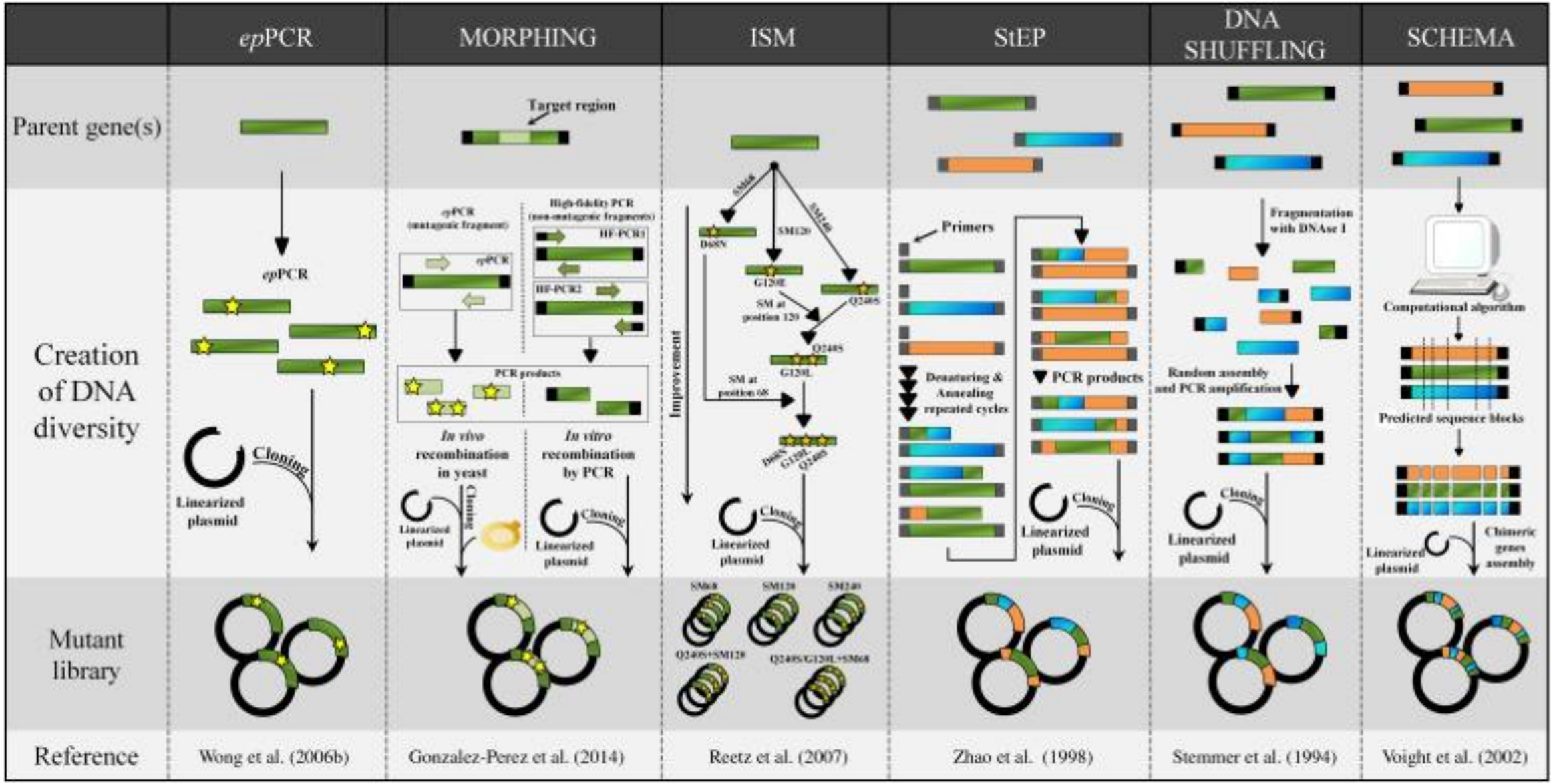
- Natural Isolate Screening
- Molecular Screening
- Environmental Gene Screening
- Genomic Screening
- Proteomic Screening
- Protein Engineering

Enzyme Screening

- The first 5 screening methods rely on the natural evolution of genes in the microbial species over million of years.
- All in all these screening methods work on the principle of isolating homologous enzymes or their cDNA using a custom made probe of conserved sequence of either amino acid or its cDNA.
- For example, if a suitable enzyme has been identified in a particular species then a search can be made for homologous enzymes in related species.
- Drawback: all these methods rely on natural evolution and recorded genomic or proteomic database of microorganisms which is estimated to be less than 1% of all the microorganisms that exists in nature.

Enzyme Screening

- Protein engineering via directed evolution is proven to be a promising tool to engineer enzymes with a wide variety of properties such as substrate specificity, organic solvent resistance, thermostability, and optimum working pH.
- It allow any amino acid in the amino acid sequence of a protein to be replaced by one of the other 19 naturally occurring amino acids as well as nonnatural amino acids in order to change their properties
- A successful directed evolution experiment depends on two aspects:
  - Genetic diversity or Library generation
  - High throughput screening or selection (HTSOS) methods

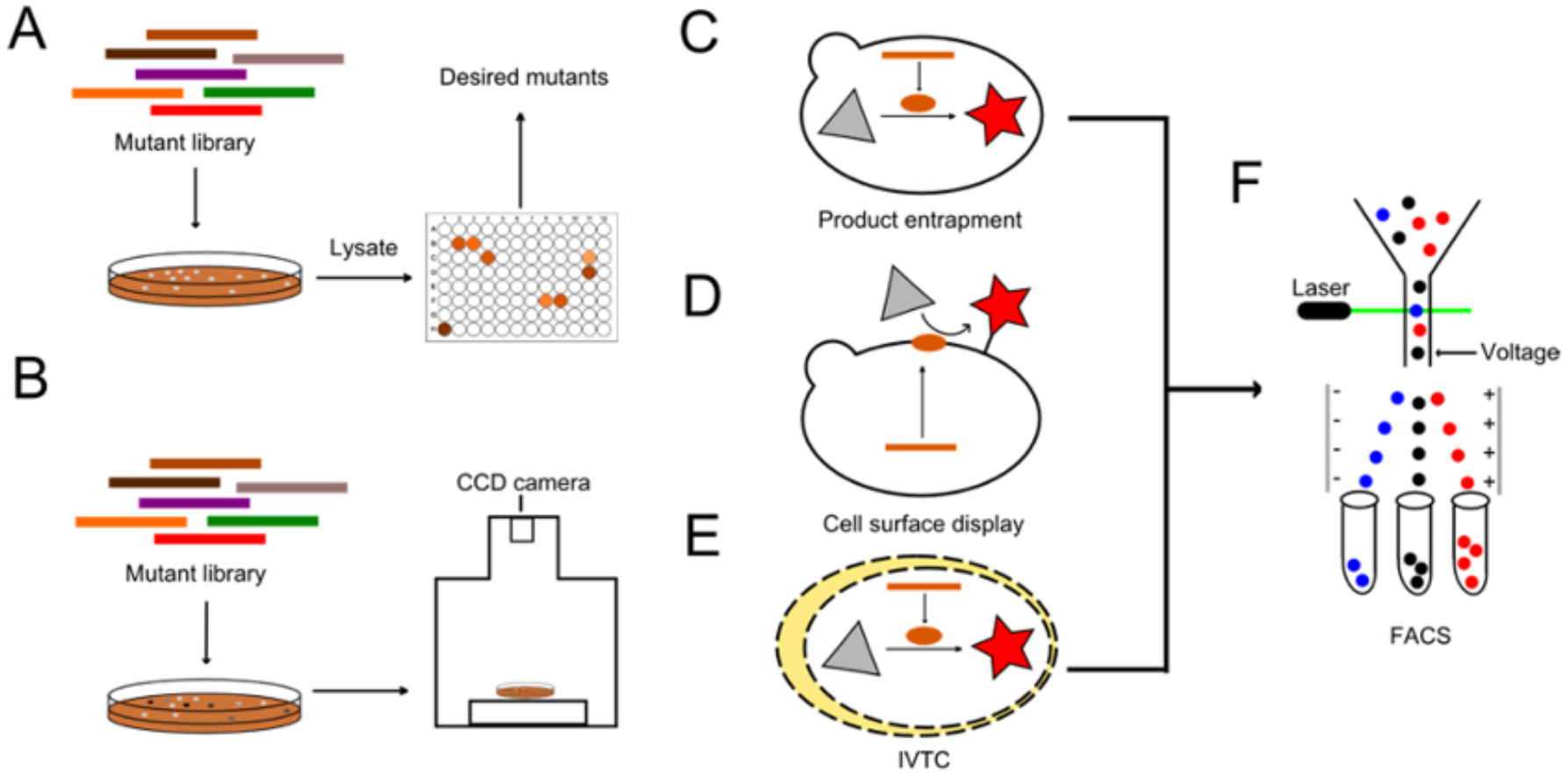


Various methods including error prone PCR, random mutagenesis, gene recombination, and semi rational mutagenesis have been developed to introduce sufficient genetic diversity or Library.

Enzyme Screening

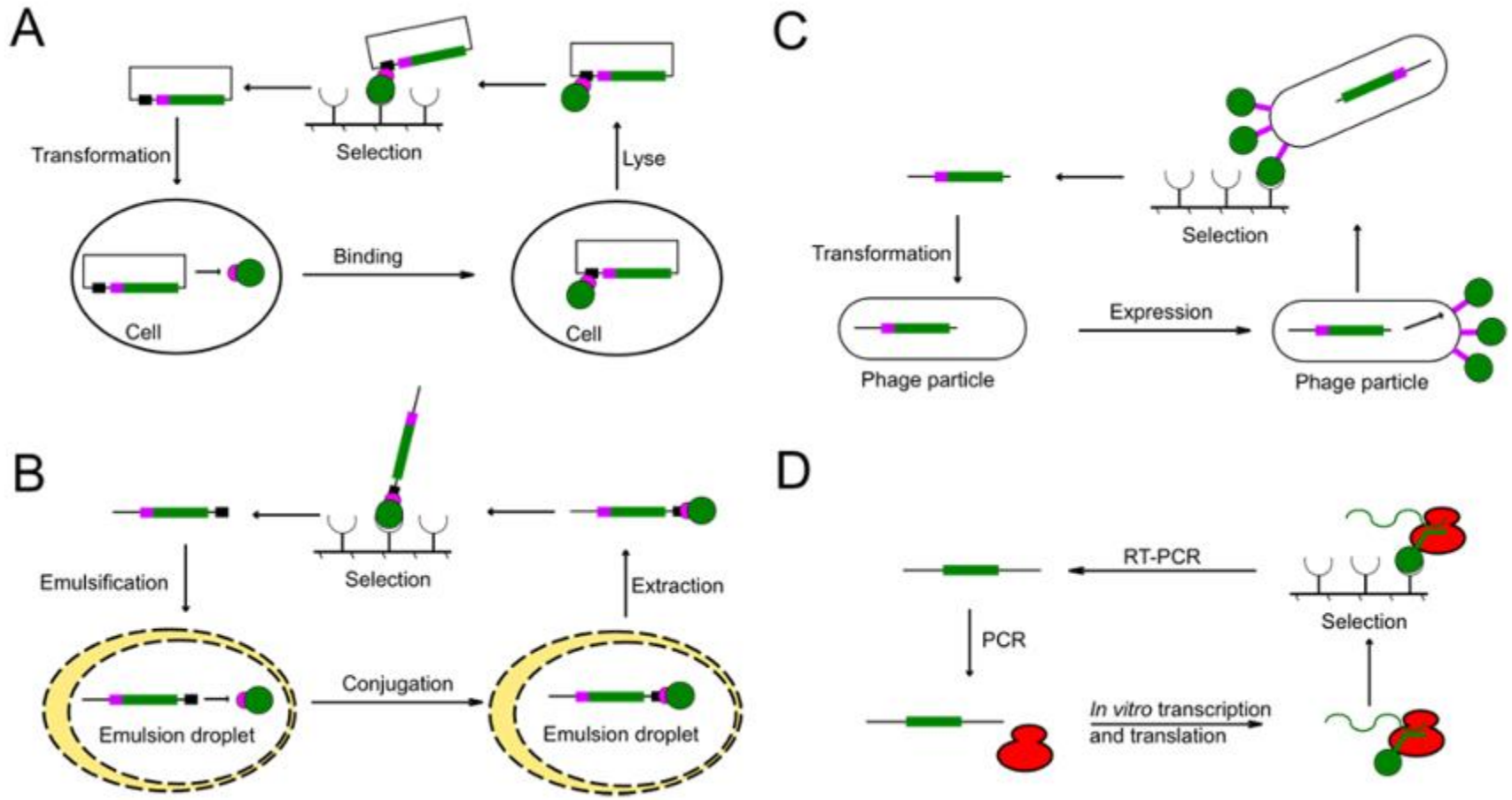
- Screening and selection are the two main methods of library analysis
- Screening refers to evaluation of every protein for the desired property, while selection automatically eliminates nonfunctional variants
- High throughput screening and selection methods enable the rapid identification of desirable traits from multifarious candidates

Enzyme Screening



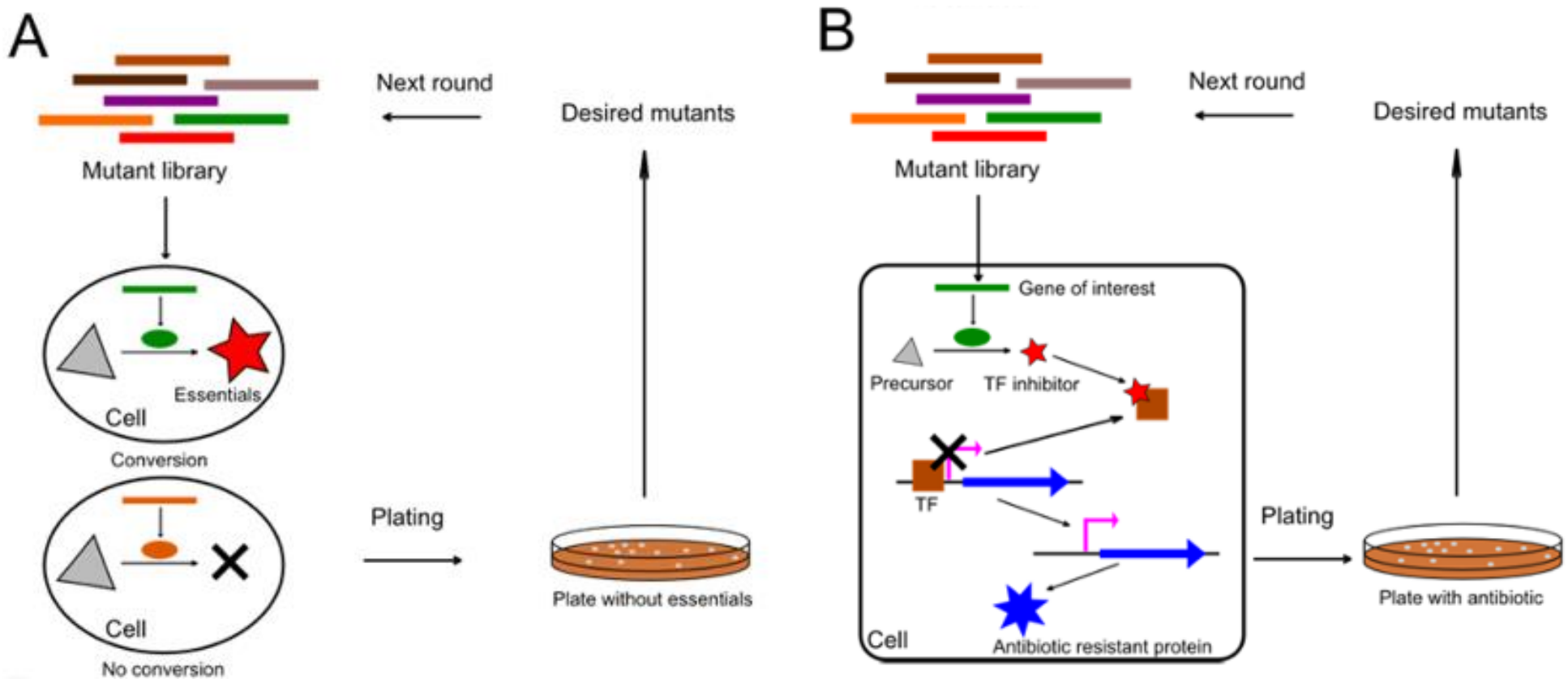
Schematic overview of high throughput screening methods. (A) Microtiter plates (B) Digital imaging. (C) Product entrapment(D) Cell surface display(E) In vitro compartmentalization (IVTC) (F) FACS

Enzyme Screening



Schematic overview of four display techniques for high throughput selection. (A) Plasmid display (B) SNAP display (C) Phage display (D) Ribosome display

Enzyme Screening



Schematic overview of growth complementation and reporter-based selection. (A) Growth complementation (B) Reporter-based selection.