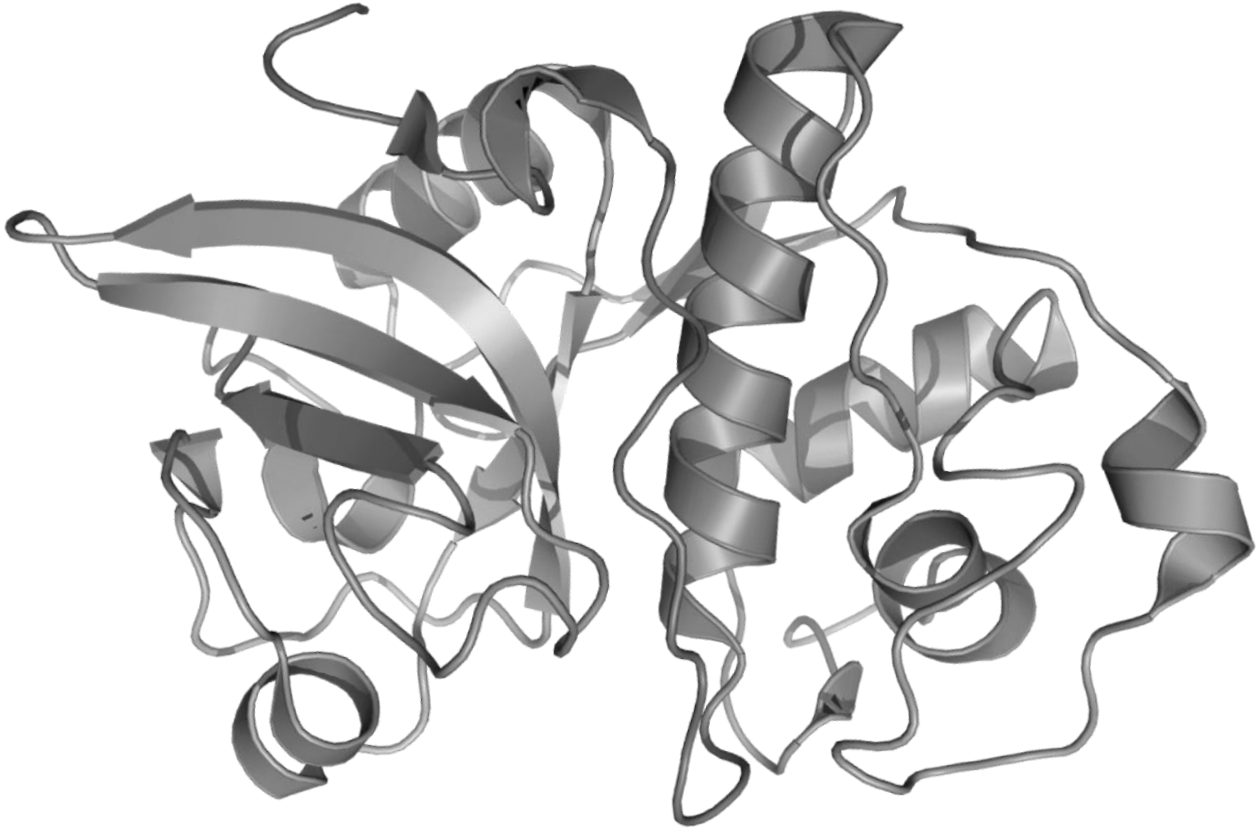
How to win the Cup

PRAXISforum, Frankfurt 08/11/2016

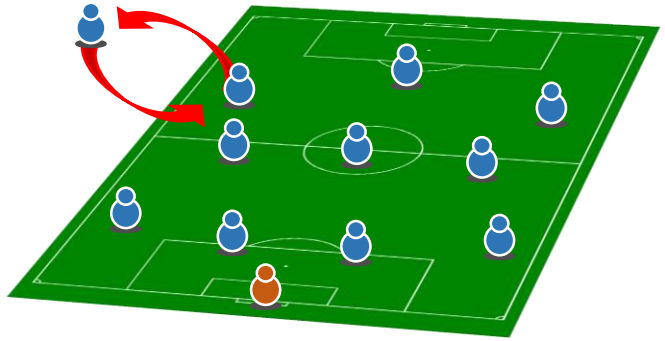
bebam BIOTECH
Quality Enzyme Solutions

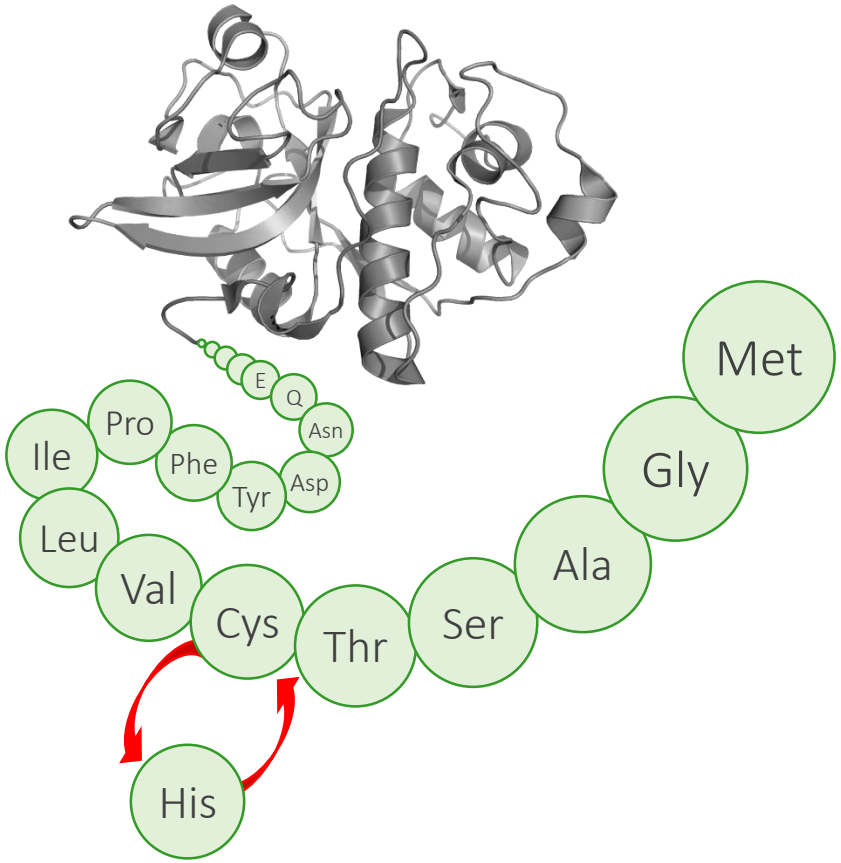
Football – 11 players per team

Protein – 100-500 amino acids per enzyme



Rational Mutagenesis





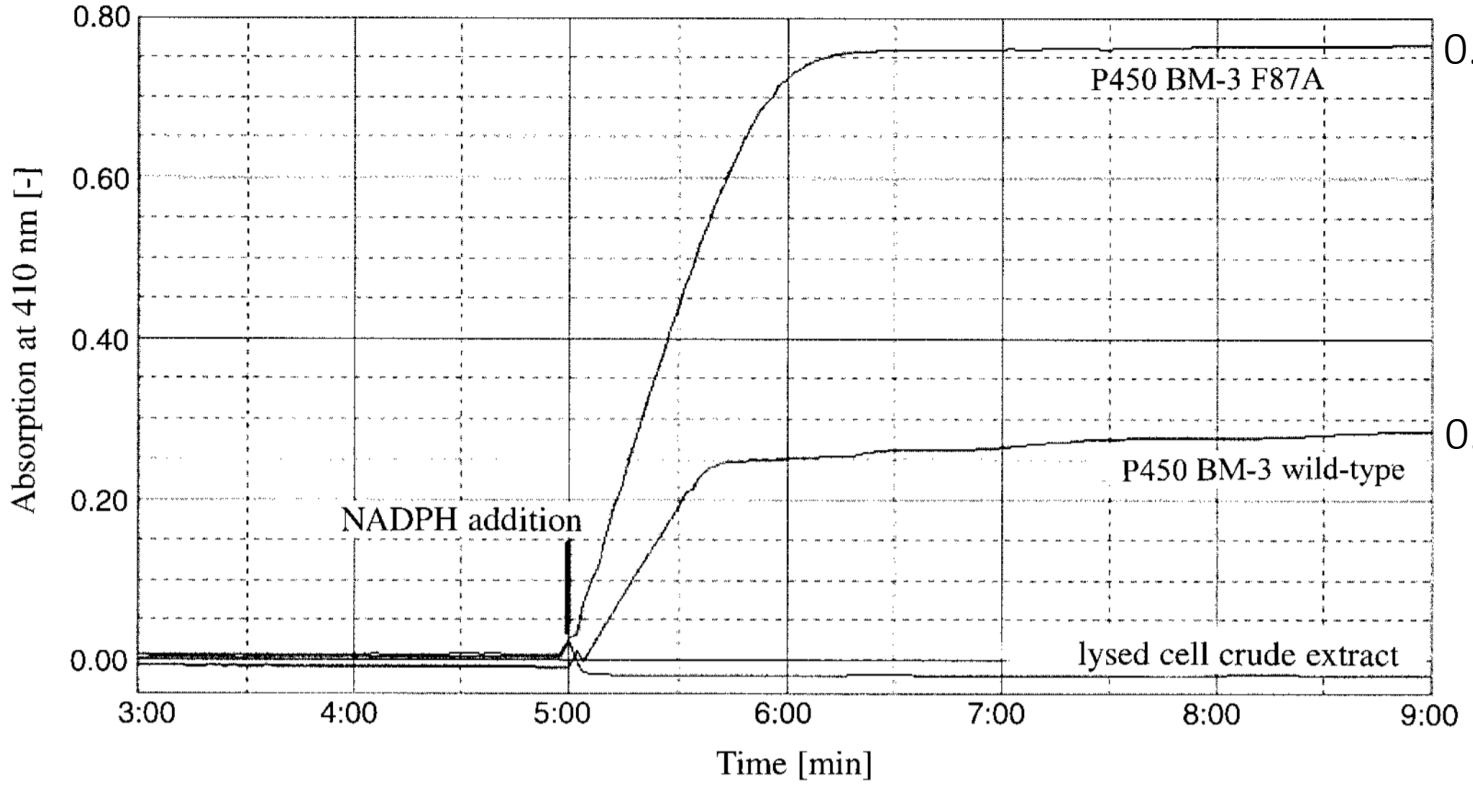
- **Site Directed Mutagenesis** (SDM)
  – single exchange of amino acids

- **Site Saturation Mutagenesis** (SSM)
  – substitute amino acid with all other 19 amino acids

Theoretical diversity: 20*x
(for x corresponding to number of mutated positions)

Example: SSM at 4 positions: 20 * 4 = 80 variants to screen
Screening: HPLC, GC, TLC, MTP

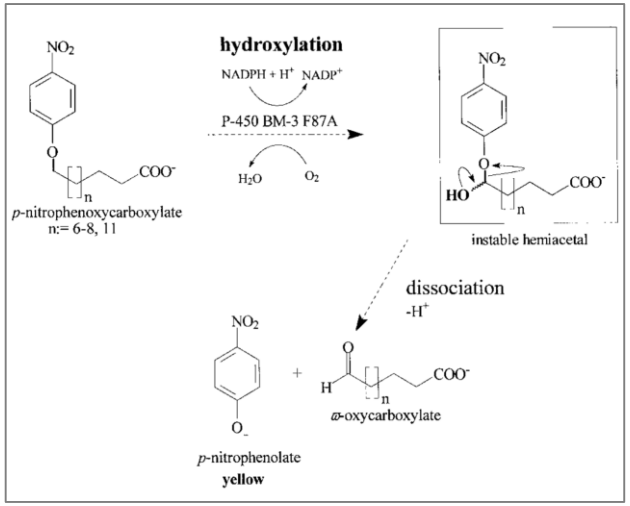
Rational Mutagenesis – Examples for SDM/SSM



0.77

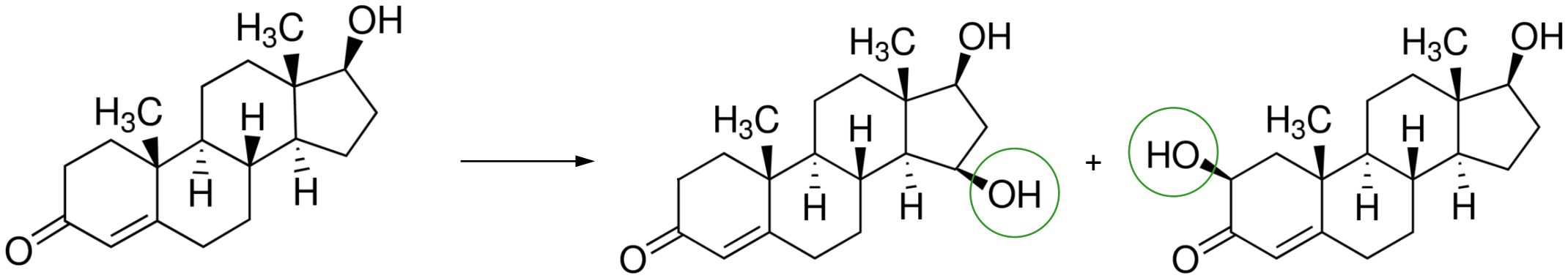
+0.49
(factor 2.75)

0.28



Schwaneberg *et al.* (1999) Anal. Biochem. 269:359-366

Rational Mutagenesis – Examples for SDM/SSM



Testosterone | *15β-Hydroxytestosterone* | *2β-Hydroxytestosterone*

Mutant	Conversion	Concentration *15β-Hydroxytestosterone*	Concentration *2β-Hydroxytestosterone*
P450 BM-3 F87A	21 %	52 %	45 %
P450 BM-3 F87A + A330W	79 %	97 %	3 %
P450 BM-3 F87A + R47Y/T49F/V78I/A82M	91 %	3 %	94 %

Kille *et al.* (2011) Nature Chemistry 3:738-743

Rational Mutagenesis - Summary

Rational Mutagenesis is suitable to generate improved enzyme variants!

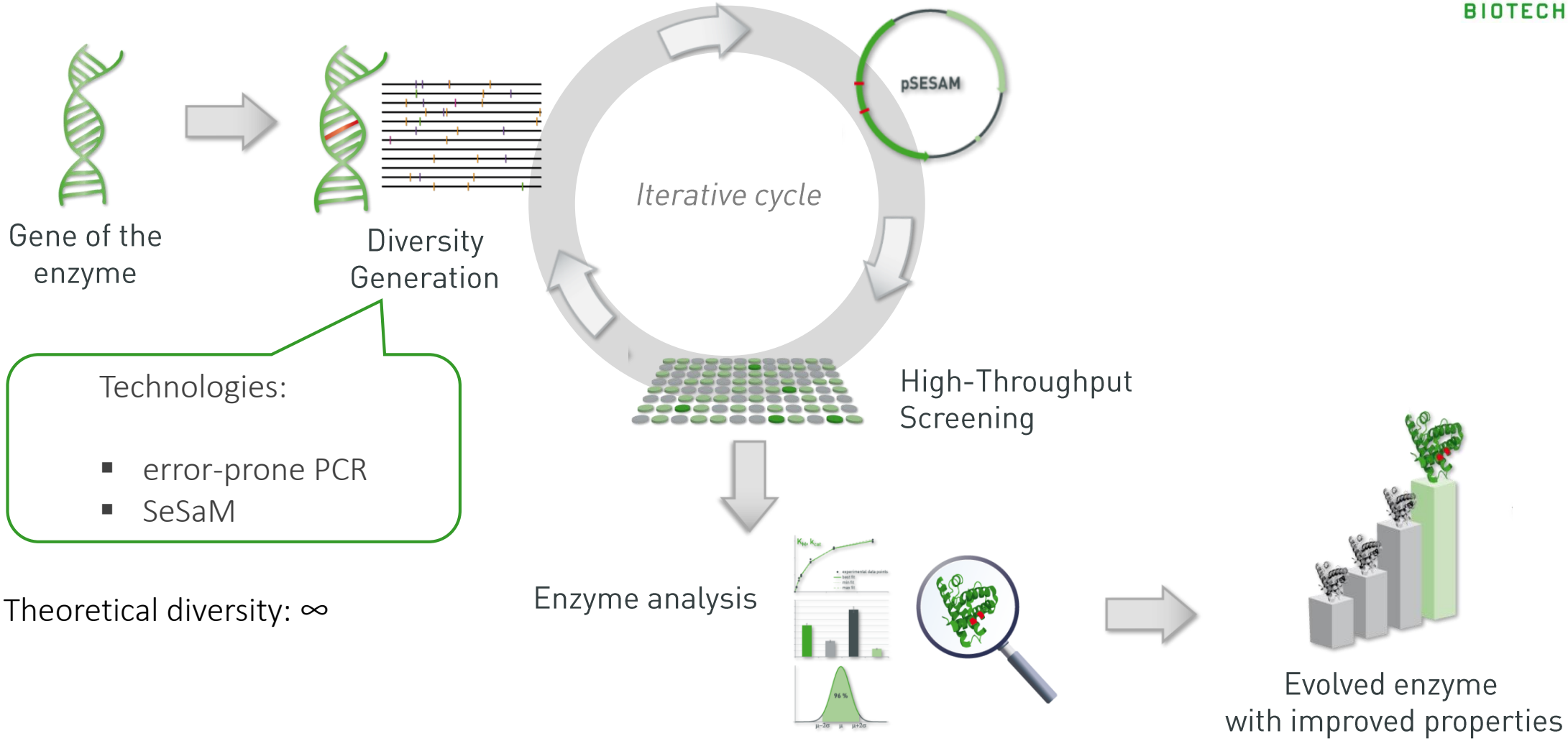
Learnings:

- ✓ Focused mutation allows selective improvement of enzyme properties
- ✓ Multiple substitutions often have ‘additive‘ effects

Required: Knowledge of enzyme‘s structure or location of beneficial amino acids

What if the enzyme‘s structure or relevant amino acid positions are unknown?

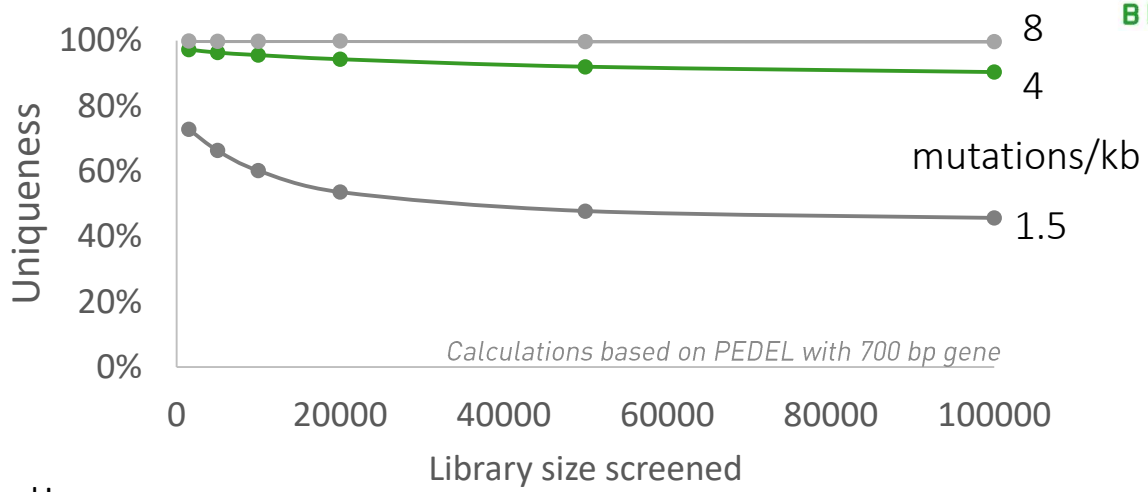
Random Mutagenesis



Random Mutagenesis - Statistics

Diversity is theoretically endless…

…but **Uniqueness** is a good measure



Caution: Keep fraction of active population in mind!

	Sequenced mutations	No. of sequenced *bsla* genes (kb sequenced)	Mutation frequency per kb[a]	No. of amino acid substitutions per BSLA protein	Fraction of wild-type protein sequence	Active fraction of the population
epPCR-low	1,000	611 (318)	3.1	1.1	33	55
epPCR-high	1,000	164 (85)	11.7	4.1	2	15
SeSaM-Tv P/P	1,000	373 (194)	5.1	1.6	13	52

What is the recommended size of the library to still find improvements?

Zhao *et al.* (2014) Biotechn. Bioeng. 111(12):2380-2389

Random Mutagenesis - Example

Comprehensive *bsla* study for [BMIM]-based Ionic liquids
by Prof. Jaeger (HHU Düsseldorf) and Prof. Schwaneberg (RWTH Aachen)

Bacillus subtilis Lipase A: 181 AA
⇒ All possible 3620 single mutants generated (1 aa mutated per *bsla* variant)

Findings:

- 66-95 % of beneficial amino acid substitutions were chemically distinct to 'original'
  ⇒ Use transversion enriched random mutagenesis technologies for IL resistance

- 50 -70 % of positions contribute towards IL resistance
  ⇒ Explains why libraries of 1000-2000 variants yield improved variants despite infinite sequence space

- epPCR-based methods identified only 20 % of beneficial positions
  ⇒ Use advanced random mutagenesis technologies (e.g. SeSaM) to increase to 36 %[1] = doubled success chance

Frauenkorn *et al.* (2015) ChemBioChem 16:937-945 | [1]Manuscript in preparation

Random Mutagenesis - Summary

Random Mutagenesis is a useful tool to identify beneficial amino acids and improve enzyme properties.

Learnings:

- ✓ Libraries of 1000-2000 variants are sufficient as **>50% positions affect properties**
- ✓ Mutational loads >3 mutations/kb are recommended to achieve high library **uniqueness**
- ✓ Beware of high fraction of **lethal variants** at high mutational loads (>10 mutations/kb) and **oversampling**
- ✓ Statistical tools may support library design, but lack experimental factors (WT preferences, sample loss, …)
- ✓ Use advanced, **un-biased random mutagenesis technologies** to increase success chances

Frauenkorn *et al.* (2015) ChemBioChem 16:937-945

How to win the Cup?

For protein engineering:

⇒ Find the best **TEAM** of amino acids

Source: twitter.com

...by Directed Evolution 2.0

Directed evolution 2.0: improving and deciphering enzyme properties

Feng Cheng,†[a] Leilei Zhu†[a] and Ulrich Schwaneberg*[ab]

Directed evolution has matured to a routinely applied algorithm to tailor enzyme properties to meet the demands in various applications. In order to free directed enzyme evolution from methodological restraints and to efficiently explore its potential, many different strategies have been used in directed

Developed in 2015 by Prof. Schwaneberg (RWTH Aachen):

Strategy: **KnowVolution**
"Knowledge-gaining
Directed Evolution"

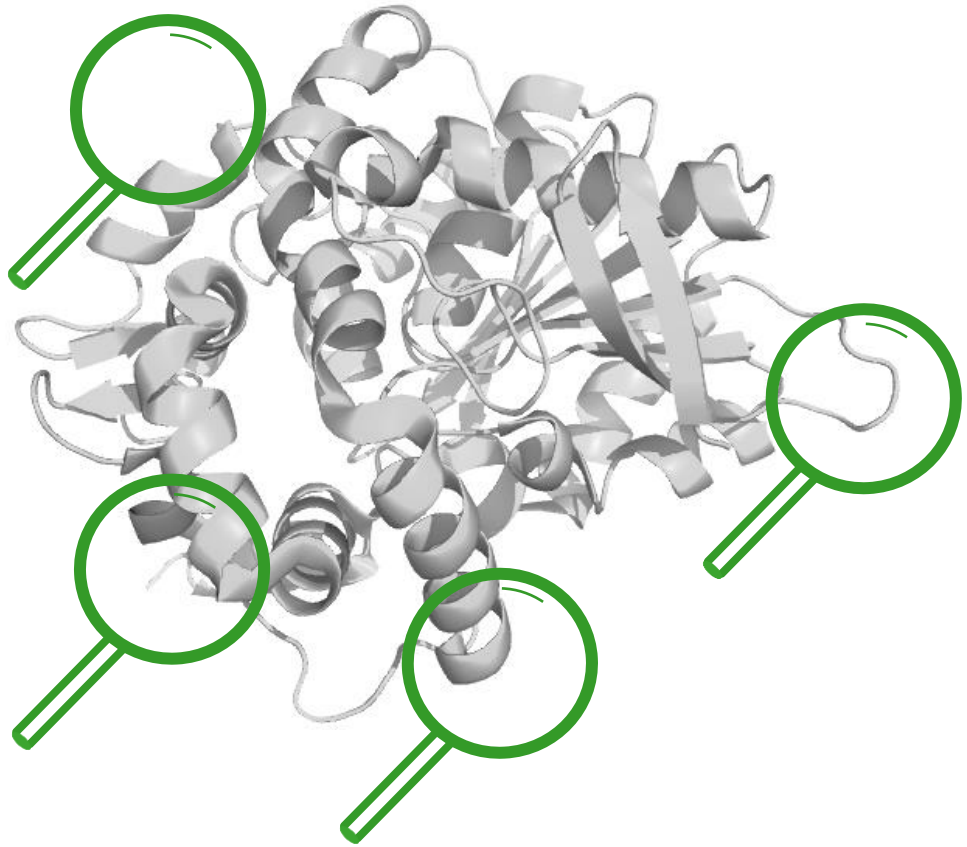
Cheng, Zhu and **Schwaneberg** (2015) Chem. Commun. 51:9760-9772

KnowVolution strategy comprises 4 Phases

Phase 1 - Identification

Goal:
Identify potential beneficial amino acid positions

Methods:
- Computational Modelling and conservation analysis
- Random Mutagenesis
- (Literature)

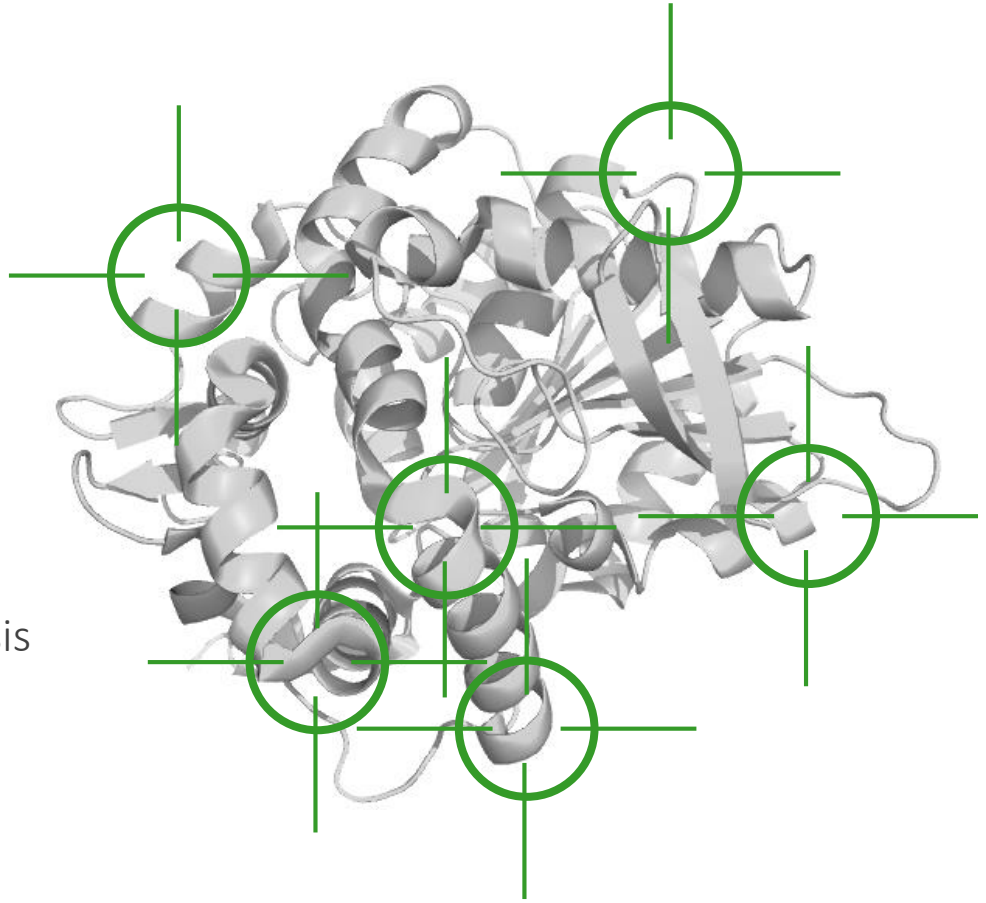


KnowVolution strategy comprises 4 Phases

Phase 2 - Determination

Goal:
Determine beneficial amino acid positions
(>50 % of positions are irrelevant)

Methods:
- Rational Mutagenesis by Site Saturation Mutagenesis
- Each position individually



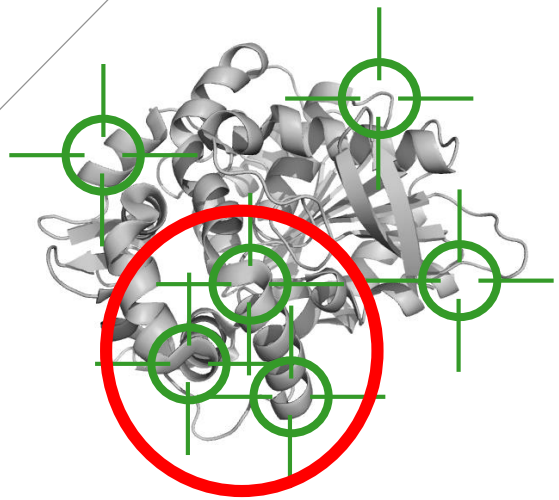
KnowVolution strategy comprises 4 Phases

Phase 3 – Analysis and Clustering

Goal:
Analyse preferred amino acid substitutions +
Locate interacting amino acids (TEAM compositions)

Methods:

- Computational modelling and simulations to rank and cluster amino acids



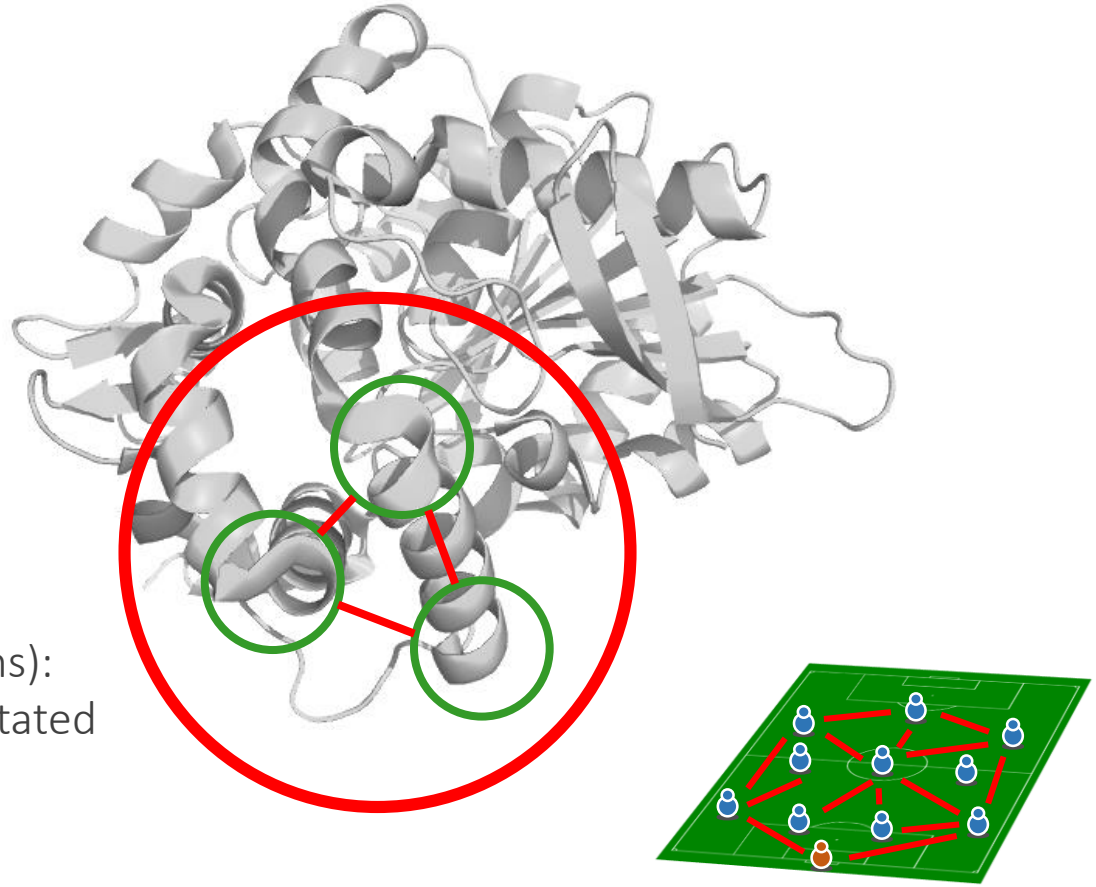
KnowVolution strategy comprises 4 Phases

Phase 4 – Recombination

Goal:
Find most suitable amino acid TEAM

Method:

- **Multi-Site** Saturation Mutagenesis (using smart Codons): OmniChange, up to 5 aa positions simultaneously mutated
- Gene synthesis
  (beware of up to 50 % reduced viability)



Dennig *et al.* (2011) PLoS One 6(10):e26222

KnowVolution: Success stories

1) **Phytase** from *Yersinia mollaretii*[2]
used as feed additive, has to withstand high temperatures (>80 °C) and acidic pH (<3, animal stomach)

KnowVolution result: 4 mutations improve pH stability at 2.8 by factor 2, Tm + 2 °C, 41 U/mg increased activity

2) **Protease Subtilisin E** from *Bacillus subtilis*[2]
has to withstand chaotropic salts like guanidinium chloride to efficiently degrade proteins in blood samples

KnowVolution result: 4 mutations (2 cooperative) increase chaotolerance by factor 192 (from <2 min to 385 min)

3) **Cellulase CelA2**
used for cellulose and hemicellulose degradation in ILs and deep-eutectic solvents

KnowVolution result: 2 cooperative mutations increase specific activity by **factor 41** compared to WT[3]

[2]Cheng *et al.* (2015) Chem. Commun. 51:9760-9772 | [3]Manuscript in preparation

KnowVolution strategy: Summary

Innovative concept to select the most suitable amino acid TEAMs based on their synergy

Key advantages over traditional Directed Evolution approaches:

- ✓ Lesser screening needs
- ✓ Higher protein property improvements due to cooperative effects
- ✓ Increased success chance
- ✓ Faster and more cost effective way to an improved enzyme

KnowVolution at SeSaM-Biotech

R&D services employing KnowVolution last 6-11 months:

START: Delivery of gene sequence to SeSaM-Biotech

- Codon-optimization and gene synthesis
- Cloning, Transformation and Expression
- Verification of correct fold / enzymatic activity

MILESTONE

- Screening assay development
- Screening assay adaption and validation

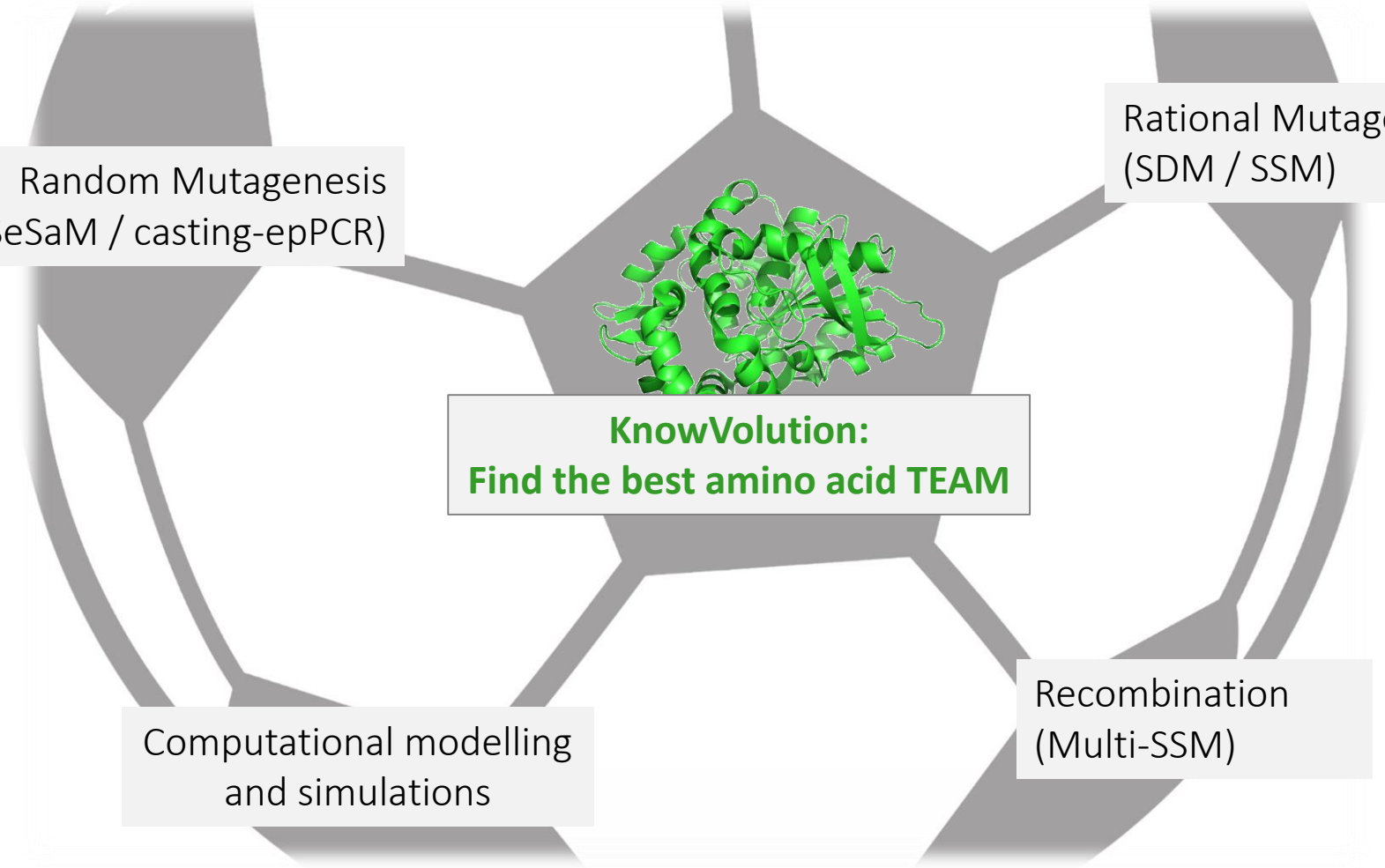
- Homology model generation and computational simulations

MILESTONE

- KnowVolution Phase 1-4

FINISH: Delivery of optimized protein/gene sequence including IP ownership

Take home


Random Mutagenesis
(epPCR / SeSaM / casting-epPCR)
Rational Mutagenesis
(SDM / SSM)
KnowVolution:
Find the best amino acid TEAM
Computational modelling
and simulations
Recombination
(Multi-SSM)

Thank you!

Mr. David Schönauer
Managing Director
schoenauer@sesam-biotech.com
www.sesam-biotech.com

sesam BIOTECH
Quality Enzyme Solutions