Methods in Molecular Biology 978

Springer Protocols

James C. Samuelson *Editor*

Enzyme Engineering

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY™

Series Editor
John M. Walker
School of Life Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For further volumes:
http://www.springer.com/series/7651

Enzyme Engineering

Methods and Protocols

Edited by

James C. Samuelson

New England Biolabs, Inc., Ipswich, MA, USA

Humana Press

Editor
James C. Samuelson
New England Biolabs, Inc.
Ipswich, MA, USA

ISSN 1064-3745 ISSN 1940-6029 (electronic)
ISBN 978-1-62703-292-6 ISBN 978-1-62703-293-3 (eBook)
DOI 10.1007/978-1-62703-293-3
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2013930234

Printed on acid-free paper

Humana Press is a brand of Springer
Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The following collection of chapters is intended to provide guidance to investigators wishing to create enzyme variants with desired properties. Whether the pursuit is commercially motivated or purely academic, engineering a novel biological catalyst is an enticing challenge. High-resolution protein structure analysis allows for rational alteration of enzyme function, yet many useful enzyme variants are the product of well-designed selection schemes or screening strategies. Accordingly, this volume contains examples where bugs are employed as workhorses in order to evolve enzyme function or to isolate enzyme variants with improved solubility or stability.

One step away from cell-based selection and screening is the use of in vitro compartmentalization to isolate enzyme variants and their respective nucleotide codes, as the most powerful in vitro methods for enzyme evolution link gene sequence to gene product function. In Chapter 6, Golynskiy et al. present a comprehensive review of the methods used for the in vitro evolution of protein enzymes. The general principles of ribosome display, mRNA display, and DNA display are outlined, and the advantages of each of these approaches are highlighted. For many years, in vitro translation systems have offered the opportunity to produce small quantities of protein containing unnatural amino acids. More recently this objective has been realized in genetically modified organisms so protein yield may no longer be a limiting factor. In Chapter 7, Singh-Blom et al. demonstrate that residue-specific incorporation of tryptophan analogs is possible in a Δ*trp*C derivative of BL21(DE3). Additionally, the same researchers present a step-by-step guide to prepare an S30 lysate from a tryptophan auxotroph so that cell-free synthesis of tryptophan-substituted protein may also be accomplished.

The potential for any directed evolution project is dependent upon the type of gene library and the degree of library diversity. Therefore, multiple chapters outline methods for gene mutagenesis, gene and operon assembly, and efficient do-it-yourself gene synthesis. Remarkably, today's researcher also has the option of purchasing mutant gene libraries now that gene synthesis costs have declined significantly. Once the task of gene library construction is completed and after promising enzyme variants are isolated, a further challenge is thorough protein characterization. When a novel enzyme variant is isolated, many concerns must be addressed: For example, what is the true enzyme specificity and is the turnover rate acceptable for the desired application? In Chapter 2, Demarse et al. showcase an underutilized, yet simple and effective technique for analyzing enzyme kinetics. Isothermal titration calorimetry (ITC) is a direct method for determining the basic parameters of an enzyme-catalyzed reaction (i.e. V_{max}, K_m and k_2). Since ITC is a non-destructive method, precious quantities of an enzyme variant are not consumed. But perhaps more importantly, ITC is suitable for many types of assays since substrate(s) do not require labeling and linkage to a secondary-detectable process is not necessary.

Today many engineering efforts are focused on creating protein-based therapeutics. For example, Chapter 3 presents two examples of a screen for evolving amino acid degradation enzymes. Such enzymes show promise in cancer therapy by limiting the nutrient supply for

tumor cells. Amino acid auxotrophic host cells were critical in this cell-based screen and a customized host strain is often the enabling element of a cell-based selection or screen. Therefore, this volume includes a simple method for generating site-specific mutations within bacterial chromosomes. In the last few years, Bryan Swingle (Chapter 9) and others have defined the requirements of oligonucleotide recombination and have found that the process is RecA-independent. In short, a bacterial cell is transformed with a single-stranded DNA oligonucleotide containing the desired mutation and the oligonucleotide anneals and becomes incorporated during replication of the host chromosome. This method is certainly a breakthrough and has already yielded impressive biocatalysts.

This volume also highlights the engineering of two different types of rare-cutting endonucleases that show great potential in gene therapy applications: The newest development is the emergence of TAL effector nucleases or TALENs. TALENS are derived from transcription factors (TAL effectors) and the DNA cleavage domain from the FokI restriction endonuclease. In Chapter 5, Li and Yang describe a simple method for creating designer TALENs (dTALENs) from modular motifs with defined DNA-binding specificities. A related chapter describes a method for characterizing the DNA-binding and cleavage properties of LAGLIDADG homing endonucleases. This family of endonuclease is being recruited for gene targeting due to a natural affinity for rare DNA target sites approximately 22 bp in length. Homing endonuclease specificity is especially difficult to characterize and Baxter et al. have developed a high-throughput method where homing endonucleases are expressed on the surface of yeast and specificity is evaluated against synthetic DNA target sequences using flow cytometry.

Finally, the vast amount of genome sequence data and protein structural data has allowed for the development of two new methods that incorporate rational design. First, the REAP method takes advantage of ancient sequences from a phylogenetic tree. Signatures of functional divergence are identified and used to design a library with a high density of viable protein variants. This focused library is then tested for desirable properties such as thermostability. In contrast, the DECAAF method takes advantage of existing protein structure data (PDB files) to search for "promiscuous" active sites that have the potential to catalyze a desired reaction. The DECAAF analysis arrives at a protein scaffold that serves as the basis for rational engineering of residues within the putative active site. Many natural enzymes are thought to have "moonlighting" domains based on the number of encoded proteins versus verified chemical transformations in simple organisms. The DECAAF method may also be useful in identifying these bona fide secondary active sites.

The contents of this book should be valuable for scientists with a budding interest in protein engineering as well as veterans looking for new approaches to apply in established discovery programs. The following chapters describe newly developed technologies in sufficient detail so that each method can be practiced in a standard molecular biology laboratory. Accordingly, I wish to thank each contributor for sharing his/her expertise with the research community. And finally, I thank my colleagues at New England Biolabs for their support and their commitment to the advancement of basic science.

Ipswich, MA, USA *James C. Samuelson*

Contents

Contributors

Giulia Agnello • *Institute for Cellular and Molecular Biology, University of Texas, Austin, TX, USA*

James C.A. Bardwell • *Howard Hughes Medical Institute, Chevy Chase, MD, USA; Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI, USA*

Sarah K. Baxter • *Department of Immunology, University of Washington, Seattle, WA, USA; Center for Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA, USA; Northwest Genome Engineering Consortium, Seattle, WA, USA*

Swapan K. Bhattacharjee • *Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India*

Jurate Bitinaite • *New England Biolabs, Inc., Ipswich, MA, USA*

Jason Cantor • *Department of Chemical Engineering, University of Texas, Austin, TX, USA*

Sandeep Chakraborty • *Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India*

Megan F. Cole • *School of Biology, Georgia Institute of Technology, Atlanta, GA, USA*

Virginia W. Cornish • *Department of Chemistry, Columbia University, New York, NY, USA*

Vanessa E. Cox • *School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA, USA*

T. Ashton Cropp • *Department of Chemistry, Virginia Commonwealth University, Richmond, VA, USA*

Abhaya M. Dandekar • *Plant Sciences Department, University of California, Davis, CA, USA*

Neil A. Demarse • *TA Instruments, Lindon, UT, USA*

Andrew D. Ellington • *Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX, USA*

Linda Foit • *Howard Hughes Medical Institute, Chevy Chase, MD, USA; Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI, USA*

Eric A. Gaucher • *School of Biology, Georgia Institute of Technology, Atlanta, GA, USA*

Eric R. Geertsma • *Department of Biochemistry, University of Zurich, Zurich, Switzerland*

George Georgiou • *Department of Chemical Engineering, Institute of Cellular and Molecular Biology, Section of Molecular Genetics and Microbiology, Department of Biomedical Engineering, University of Texas, Austin, TX, USA*

Misha V. Golynskiy • *Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, St. Paul, MN, USA; BioTechnology Institute, University of Minnesota, St. Paul, MN, USA*
Kelsey L. Gratton • *The Wallace H. Coulter, Department of Biomedical, Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA, USA*
Lee D. Hansen • *Department of Chemistry, Brigham Young University, Provo, UT, USA*
John C. Haugner III • *Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, St. Paul, MN, USA; BioTechnology Institute, University of Minnesota, St. Paul, MN, USA*
Hidehiko Hirakawa • *Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Tokyo, Japan*
Pei-Chung Hsieh • *New England Biolabs, Inc., Ipswich, MA, USA*
Randall A. Hughes • *Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX, USA*
Jordan Jarjour • *Northwest Genome Engineering Consortium, Seattle, WA, USA; Pregenen Inc., Seattle, WA, USA*
Marie C. Killian • *Department of Chemistry, Brigham Young University, Provo, UT, USA*
Abigail R. Lambert • *Center for Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA, USA; Northwest Genome Engineering Consortium, Seattle, WA, USA*
Ting Li • *Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA, USA*
Jia Liu • *Department of Chemistry, Virginia Commonwealth University, Richmond, VA, USA*
Jian-Wei Liu • *CSIRO Ecosystem Sciences, Canberra, Australia*
Renu Minda • *Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India*
Aleardo Morelli • *Department of Biochemistry, Molecular Biology and Biophysics & BioTechnology Institute, University of Minnesota, St. Paul, MN, USA*
Dana Morrone • *Department of Biochemistry, Molecular Biology and Biophysics & BioTechnology Institute, University of Minnesota, St. Paul, MN, USA*
Teruyuki Nagamune • *Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Tokyo, Japan*
David L. Ollis • *Research School of Chemistry, Australian National University, Canberra, Australia*
Nili Ostrov • *Department of Chemistry, Columbia University, New York, NY, USA*
Olga Paley • *Department of Chemical Engineering, University of Texas, Austin, TX, USA*
Colette F. Quinn • *TA Instruments, Lindon, UT, USA*
Basuthkar J. Rao • *Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India*
Lipika Salaye • *Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India*

Andrew M. Scharenberg • *Department of Immunology, University of Washington, Seattle, WA, USA; Center for Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA, USA; Northwest Genome Engineering Consortium, Seattle, WA, USA; Pregenen Inc., Seattle, WA, USA*

Burckhard Seelig • *Department of Biochemistry, Molecular Biology and Biophysics & BioTechnology Institute, University of Minnesota, St. Paul, MN, USA*

Amrita Singh-Blom • *Department of Molecular Genetics and Microbiology, The University of Texas at Austin, Austin, TX, USA*

Bradley J. Stevenson • *Research School of Chemistry, Australian National University, Canberra, ACT, Australia*

Everett Stone • *Department of Biomedical Engineering, University of Texas at Austin, Austin, TX, USA*

Bryan M. Swingle • *United States Department of Agriculture, Agricultural Research Service, Ithaca, NY, USA; Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, NY, USA*

Romualdas Vaisvila • *New England Biolabs, Inc., Ipswich, MA, USA*

Laura M. Wingler • *Department of Chemistry, Columbia University, New York, NY, USA*

Bing Yang • *Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA, USA*

Sylvia H.-C. Yip • *Research School of Chemistry, Australian National University, Canberra, Australia*

Tae Hyun Yoo • *Division of Applied Chemistry and Biological Engineering, Department of Molecular Science and Technology, Ajou University, Suwon, South Korea*

Chapter 1

A Tripartite Fusion System for the Selection of Protein Variants with Increased Stability In Vivo

Linda Foit and James C.A. Bardwell

Abstract

We describe here a genetic selection system that directly links protein stability to antibiotic resistance, allowing one to directly select for mutations that stabilize proteins in vivo. Our technique is based on a tripartite fusion in which the protein to be stabilized is inserted into the middle of the reporter protein β-lactamase via a flexible linker. The gene encoding the inserted protein is then mutagenized using error-prone PCR and the resulting plasmid library plated on media supplemented with increasing concentrations of β-lactam antibiotic. Mutations that stabilize the protein of interest can easily be identified on the basis of their increased antibiotic resistance compared to cells expressing the unmutated tripartite fusion.

Key words: Genetic selection, Protein stability, Protein evolution, Mutagenesis, Reporter protein, Tripartite fusion, Sandwich fusion

1. Introduction

Most soluble, globular proteins exhibit only marginal thermodynamic stabilities between approximately −5 and −10 kcal/mol (1, 2). Such low protein stability imposes significant challenges on the use of these polypeptides in many biotechnological, biomedical, and practical applications, where large amounts of stable and soluble protein are needed. The identification of stabilizing mutations, however, is difficult, since most random amino acid substitutions actually decrease stability (3–5). Computational methods that estimate the effect of mutations on protein stability are available but usually require detailed structural knowledge about the target protein, information that is often not available. Unfortunately, though computational methods are often good at predicting the destabilizing effect of mutations they are generally less accurate at predicting stabilizing mutations (6).

James C. Samuelson (ed.), *Enzyme Engineering: Methods and Protocols*, Methods in Molecular Biology, vol. 978,
DOI 10.1007/978-1-62703-293-3_1,

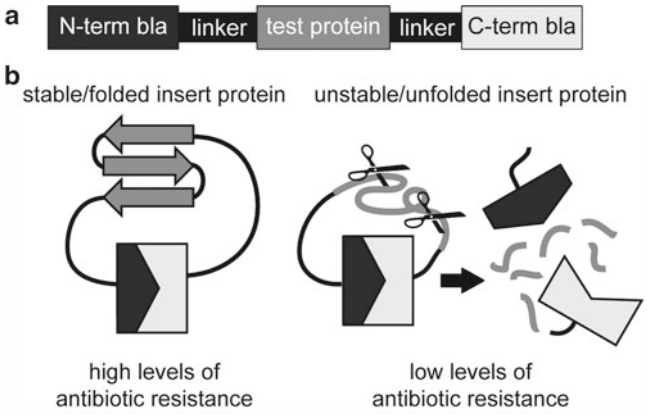

Fig. 1. Schematic representation of the tripartite fusion system. (**a**) The protein of interest is inserted into β-lactamase via a linker. N-term bla = N-terminal part β-lactamase, C-term bla = C-terminal part β-lactamase, linker = flexible glycine-serine linker. (**b**) If the test protein is folded properly (*left*), the two parts of β-lactamase can interact and provide β-lactamase activity. This results in high levels of antibiotic resistance. Unfolded proteins on the other hand (*right*) are subject to degradation by cellular proteases, leading to a large reduction in antibiotic resistance.

Recently, a number of approaches have been developed that utilize reporter proteins to monitor or increase the stability of proteins in the cellular environment (6–12). The genetic system we established (12) allows for monitoring and increasing protein stability and combines the following attractive features: (1) it directly links the in vivo stability of proteins to antibiotic resistance, a quantitative readout, (2) it is a *selection* rather than a *screen*, omitting laborious testing of individual protein mutants, (3) it does not require prior knowledge about structure or function of the insert protein, and (4) it can be used with a variety of different proteins.

Our approach is based on the reporter protein TEM1-β-lactamase. This enzyme is located in the periplasm of Gram-negative bacteria and confers resistance towards β-lactam antibiotics like ampicillin or penicillin (13). TEM1-β-lactamase is tolerant of insertions or deletions that occur in a solvent-exposed loop around residue 196 (14, 15). Based on this knowledge, we generated a tripartite fusion in which a test protein is inserted between residues 196 and 197 of the enzyme via flexible glycine-serine linkers (Fig. 1a) (12). Cleavage of the enzyme at this position results in two fragments that are catalytically inactive when expressed separately (16). However, when these fragments are fused to interacting partner protein as part of a protein complementation assay, activity will be restored (16).

The underlying principle for the use of this tripartite fusion as a readout for protein stability in our genetic selection system is the following: If the insert test protein is folded properly and is stable, the two fragments of β-lactamase can interact with each other, providing enzymatic activity. Cells expressing such a fusion construct

exhibit high levels of resistance towards β-lactam antibiotics (Fig. 1b, left). Unstable insert proteins on the other hand are subject to degradation by cellular proteases. Proteolysis of such unstable and unfolded insert protein leads to separation of the two β-lactamase fragments. The result is a substantial decrease in antibiotic resistance levels (Fig. 1b, right).

To select for protein variants with increased in vivo stability, the gene encoding the target protein is randomly mutated and the resulting plasmid library transformed into *Escherichia coli* cells. The cells are then spread on plates containing increasing concentrations of a β-lactam antibiotic. Colonies showing increased levels of antibiotic resistance compared to cells expressing a construct containing the wild-type protein are selected and the protein sequence is determined.

For the model protein immunity protein 7 (Im7), we found that the in vivo steady-state levels of tripartite fusions containing different Im7 variants correlated well with the resulting level of antibiotic resistance (12). Moreover, the vast majority of constructs selected for their increased antibiotic resistance encoded protein variants that were both thermodynamically and kinetically more stable in vitro when expressed in absence of the fusion partner.

Our tripartite fusion system is not just useful for identifying stabilized protein variants. It can also be utilized to select for mutant proteins with other improved properties that lead to increased steady-state levels of the protein in the periplasm. Examples are increased solubility (12), the elimination of disulfide bonds or kinetic traps that are problematic for protein folding in vivo (17), decreased proteolytic susceptibility, and possibly increased translocation efficiency or a combination of these factors.

In addition, alterations that improve the folding of proteins are in principle not limited to mutagenesis of the gene encoding the protein inserted into β-lactamase. The system could possibly also be used to select for increased activity and specificity of chaperones or other folding factors that are co-expressed with a tripartite fusion containing a target protein. For instance, we have shown that it is possible to randomly mutate the host chromosome to generate bacterial strains that enhance expression of target proteins. In doing so we have identified a novel molecular chaperone called Spy (18).

Our selection works in the bacterial periplasm, a very oxidizing environment (19). To avoid nonnative and unwanted disulfide bond formation within the protein of interest, we strongly recommend the use of insert proteins that (1) do not contain unpaired cysteines that are normally part of a disulfide and (2) If they possess disulfides that the number of disulfides present is small, zero is ideal, but up to a maximum of two disulfide bonds can be tolerated, especially if they occur between consecutive cysteines.

In this chapter, we will focus on the selection of protein variants with increased in vivo stability.

2. Materials

2.1. Biological and Chemical Materials

Prepare all stock solutions using double-distilled water or deionized and then distilled water (ddH_2O).

1. Plasmids.
   (a) pBR322, e.g., from New England Biolabs (NEB) (20, 21) (see Note 1).
   (b) pBAD33 (22) (see Note 2).
   (c) Plasmid or chromosomal DNA encoding the insert protein (for amplification with PCR).
2. Primer solutions, 10 or 100 μM, stored at –20°C.
   (a) Primer 1 (polyacrylamide gel electrophoresis purified): 5′-CCGCTCCCGGATCCTGAGCTCGAGCCACCACCACCAGAACCACCACCACCTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCC-3′.
   (b) Primer 2 (polyacrylamide gel electrophoresis purified): 5′-TTCCGGAAGCGGAGGAGGTGGTTCAGGCGGAGGTGGAAGCCTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCG-3′.
   (c) Primer 3: 5′-ATAGGTACCAGGAGGAATTCATGAGTATTCAACATTTCCGTGTCGC-3′.
   (d) Primer 4: 5′-GGTGGCAGTCTAGATTACCAATGCTTAATCAGTGAGGCACC-3′.
   (e) Primer 5: 5′-TATCGTGCGGCCGCTCATGTTTGACAGCTTATCATCG-3′.
   (f) Primer 6: 5′-AGCTAGTCTAGACCGCGGGAAGATCCTTTTTTGATAATCTC-3′.
   (g) Primer 7: 5′-GCTATACTAGTTCTTCCCCATCGGTGATGTCGGCG-3′.
   (h) Primer 8: 5′-ATCGATGCGGCCGCATGTATTTAGAAAAATAAACAAAAGAG-3′.
   (i) Primers 9 + 10: Forward and reverse primers containing appropriate restriction sites for cloning of the gene encoding the protein of interest into the tripartite fusion expression plasmid of choice (see Note 3).
   (j) Primers 11 + 12: Forward and reverse primers for the random mutagenesis of the gene encoding for the protein of interest (see Note 4).
3. Individual stock solutions of dNTPs (10 mM), stored at –20°C.

4. Highly competent *E. coli* cells, e.g., NEB10-beta electrocompetent *E. coli*, transformation efficiency: $2–4 \times 10^{10}$ colony forming units (cfu)/μg pUC19 (New England Biolabs, Ipswich, MA, USA).
5. SOC outgrowth medium: 2% Vegetable Peptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose.
6. Culture tubes with 5 ml Luria Broth (LB) medium.
7. LB medium: 1% Vegetable Peptone, 0.5% Yeast Extract, 1% NaCl.
8. LB plates containing 34 μg/ml chloramphenicol or 15 μg/ml of tetracycline.
9. Plates with medium of choice with appropriate concentrations of β-lactam antibiotic, e.g., ampicillin, penicillin V (see Note 5).
10. DNA polymerase with proofreading ability, 2 U/μl, e.g., Phusion® High Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA).
11. T4 Polynucleotide kinase, 10 U/μl, e.g., from New England Biolabs (Ipswich, MA, USA).
12. T4 DNA ligase, 400 cohesive end units/μl, e.g., from New England Biolabs (Ipswich, MA, USA).
13. GeneMorph II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA).
14. Pfu Turbo, 2.5 U/μl (Agilent Technologies, Santa Clara, CA, USA).
15. Restriction enzymes, e.g., from New England Biolabs (Ipswich, MA, USA) stored at –20°C.
    (a) *Dpn*I, 20 U/μl.
    (b) *Kpn*I, 10 U/μl.
    (c) *Not*I, 10 U/μl.
    (d) *Spe*I, 10 U/μl.
    (e) *Xba*I, 20 U/μl.
    (f) Restriction enzymes needed for cloning the gene encoding the insert protein into the tripartite fusion expression plasmid.
16. DNA gel extraction kit, e.g., QIAquick Gel extraction kit (Qiagen, Valencia, CA, USA).
17. PCR purification kit, e.g., QIAquick PCR purification kit (Qiagen, Valencia, CA, USA).
18. Plasmid DNA extraction kit, e.g., QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA).

19. 70% ethanol.
20. 100% ethanol.
21. Phosphate buffered saline (PBS): 1.35 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, adjusted to pH 7.4 with HCl.
22. L-(+)-arabinose.
23. Agarose gels: 1% electrophoresis grade agarose in TAE buffer: 40 mM Tris–acetate, 1 mM ethylene diamine tetraacetic acid (EDTA), 0.5 μg/ml ethidium bromide.
24. Pellet Paint® co-precipitant and supplied 3 M sodium acetate, pH 5.2 (Merck KGaA, Darmstadt, Germany).

2.2. Equipment and Consumables

1. Agarose gel running system.
2. Electroporator, e.g., Electroporator 2510 (Eppendorf, Hauppauge, NY, USA).
3. Incubator, capable of shaking.
4. Microcentrifuge, e.g., Centrifuge 5414 R (Eppendorf, Hauppauge, NY, USA).
5. Thermocycler, e.g., Veriti® Thermal Cycler (Life Technologies Corporation, Carlsbad, CA, USA).
6. Thermomixer, e.g., Thermomixer R (Eppendorf, Hauppauge, NY, USA).
7. UV-Spectrometer, e.g., Genesys 10vis (Thermo Scientific, Waltham, MA, USA).
8. 8- or 12-channel pipette, pipetting range 2–20 μl.
9. 8- or 12-channel pipette, pipetting range 20–200 μl.
10. Sterile electroporation cuvettes, 1 mm gap.
11. Sterile, thin-walled PCR tubes.
12. Sterile 17 × 100 mm round-bottom tubes.
13. Sterile 96-well plate (well volume >250 μl).

3. Methods

Perform all procedures at room temperature unless noted otherwise. When using DNA gel extraction, PCR purification, or plasmid DNA extraction kits, elute DNA from spin column with ddH_2O.

3.1. Construction of a Tripartite Fusion Expression Plasmid (see Note 6)

3.1.1. Construction of a Plasmid for the Expression of the Tripartite Fusion Under Its Native, Constitutive Promoter (pBR322-bla-link)

1. Mix 0.5 μM primer 1, 0.5 μM primer 2, 10 ng of *dam*-methylated pBR322 plasmid DNA, 200 μM of each dNTP, and 1 U Phusion® DNA polymerase in the supplied high fidelity (HF) buffer in a total volume of 50 μl in a thin-walled PCR tube. Keep mixture on ice (see Note 7).
2. Perform PCR in a thermocycler using the following program: 98°C for 5 min (initial denaturation); 25 cycles of: 98°C for 10 s (denaturation), 68°C for 30 s (annealing), 72°C for 3 min (elongation). Following these cycles perform a final elongation at 72°C for 10 min and then hold at 4°C (see Note 8).
3. Run sample of PCR product on an analytical agarose gel to assess yield of the full-length PCR product (see Note 9).
4. Remove the *dam*-methylated template DNA by adding 20 U *Dpn*I. Incubate at 37°C for 2 h. Heat inactivate the restriction enzyme by incubation at 80°C for 20 min.
5. Purify the linear PCR product using a PCR purification kit.
6. Phosphorylate the linear PCR product by combining 0.2–1 μg of the DNA and 10 U of T4 Polynucleotide Kinase in 1× T4 DNA ligase buffer in a total volume of 50 μl. Incubate at 37°C for 30 min. Heat inactivate the enzyme by incubation at 65°C for 20 min.
7. Add 400 cohesive end units T4 DNA ligase. Incubate at 16°C for 12 h. Heat inactivate the enzyme by incubation at 65°C for 10 min.
8. Transform 0.5 μl of ligation reaction into 25 μl of electrocompetent NEB10-beta cells in a chilled electroporation cuvette using the following conditions: 1.8 kV, 200 Omega, and 25 μF. Typical time constants are 4.8–5.1 ms. Immediately after electroporation, add 975 μl of pre-warmed SOC medium to the cuvette and transfer cells into a 17×100 mm round-bottom culture tube. Shake at 250 rpm at 37°C for 1 h (see Note 10).
9. Spread different cell dilutions on pre-warmed LB plates containing the appropriate antibiotic. Incubate plates overnight at 37°C.
10. Select a single colony and isolate plasmid DNA using a plasmid DNA extraction kit.
11. Verify accuracy of the nucleotide sequence by DNA sequencing. The resulting vector is called pBR322-bla-link (see Note 6 for availability of plasmid pBR322-bla-link).

3.1.2. Construction of a Plasmid for the Expression of the Tripartite Under an Arabinose-Inducible Promoter (pMB1-ara-bla-link)

1. For the amplification of the *bla-link* gene from pBR322-bla-link, mix 0.5 μM primer 3, 0.5 μM primer 4, 10 ng pBR322-bla-link plasmid DNA, 200 μM of each dNTP, and 1 U Phusion® DNA polymerase in the supplied high fidelity (HF) buffer in a total volume of 50 μl in a thin-walled PCR tube. Keep mixture on ice (see Note 8).

2. Perform PCR in a thermocycler using the following program: 98°C for 30 s (initial denaturation); 30 cycles of: 98°C for 10 s (denaturation), 69°C for 30 s (annealing), 72°C for 20 s (elongation). Following these cycles perform a final elongation at 72°C for 5 min and then hold at 4°C.
3. Run sample of PCR product on an analytical agarose gel to assess yield of the full-length PCR product (see Note 11).
4. Purify the PCR product using a PCR purification kit.
5. Digest 0.2–1 μg PCR product as well as 0.2–1 μg pBAD33 plasmid with restriction enzymes *Kpn*I and *Xba*I according to the manufacturer's instructions.
6. Heat inactivate the restriction enzymes at 65°C for 20 min. Purify the PCR product and the vector using a PCR purification kit.
7. Mix 50 ng of vector with a threefold molar excess of insert and 400 cohesive end units T4 DNA ligase in T4 DNA ligase buffer. Incubate at 16°C for 12 h. Heat inactivate the enzyme by incubation at 65°C for 10 min.
8. Perform transformation and verification of the vector sequence as described in Subheading 3.1.1, steps 8–11. The resulting vector is called pBAD33-bla-link.
9. Mix 200 μM of each dNTP, 1 U Phusion® DNA polymerase in the supplied high fidelity (HF) buffer in a total volume of 50 μl in a thin-walled PCR tube. Add either 0.5 μM primer 5, 0.5 μM primer 6, and 10 ng of pBR322 (PCR 1) or 0.5 μM primer 7, 0.5 μM primer 8, and 10 ng of pBAD33-bla-link (PCR 2). Keep mixtures on ice.
10. Perform the following PCR in a thermocycler using the following program: 98°C for 30 s (initial denaturation); 30 cycles of: 98°C for 10 s (denaturation), 67°C (PCR 1) or 65.3°C (PCR 2) for 30 s (annealing), 72°C for 1 min (elongation). Following these cycles perform a final elongation at 72°C for 5 min and then hold at 4°C.
11. Purify both full-length PCR products on a preparative agarose gel. Extract DNA from agarose gel using gel DNA gel extraction kit.
12. Digest PCR product 1 with *Not*I and *Xba*I according to the manufacturer's instructions (see Note 12).
13. Digest PCR product 2 with *Spe*I and *Not*I according to the manufacturer's instructions (see Note 12).
14. Heat inactivate the restriction enzymes according to the manufacturer's instructions. Purify the PCR products using PCR purification kit.

15. Mix 100 ng of PCR product 1 and 400 cohesive end units T4 DNA ligase with PCR product 2 in molar ratios of 1:3, 1:1, and 3:1 in supplemented T4 ligase buffer. Incubate at 16°C for 12 h. Heat inactivate the enzyme by incubation at 65°C for 10 min.
16. Perform transformation and verification of the vector sequence as described in Subheading 3.1.1, steps 8–11. The resulting vector is called pMB1-ara-bla-link (see Note 6 for availability of plasmid pMB1-ara-bla-link).

3.1.3. Insertion of the Target Protein Gene into a Tripartite Fusion Expression Plasmid

1. Perform a PCR to amplify the gene of interest with primers carrying appropriate restriction sites using a proofreading polymerase (see Note 3).
2. Clone the gene encoding the target protein into a tripartite fusion expression plasmid of choice, e.g., pMB1-ara-bla-link. Adapt steps 3–8, Subheading 3.1.2, followed by steps 8–11, Subheading 3.1.1. The resulting plasmid is called, e.g., pMB1-ara-bla-link-insert.

3.2. Construction of Expression Libraries

The construction of a library of tripartite fusion plasmids in which only the target gene (but not the *bla* gene or the vector backbone) is mutated is based on the MEGAWHOP technique (23). First, the target gene is amplified in an error-prone PCR, generating a pool of mutated target genes, also termed a megaprimer (23, 24) (see Note 13). This megaprimer is then used to substitute the wild-type version of the target gene in the tripartite fusion expression plasmid, involving a whole-plasmid PCR with a proofreading polymerase.

1. For the random mutagenesis of the gene encoding the target gene, combine 0.1–1,000 ng of target gene, 125 ng of each mutagenesis primer, 200 μM of each dNTP, and 2.5 U Mutazyme II DNA polymerase (from the GeneMorph® II Random Mutagenesis kit) in supplemented Mutazyme II reaction buffer in a total volume of 50 μl in a thin-walled PCR tube. Keep mixture on ice (see Note 4).
2. Perform PCR in a thermocycler using the following program: 95°C for 2 min (initial denaturation); 25–35 cycles of: 95°C for 30 s (denaturation), melting temperature of primers –5°C for 30 s (annealing), 72°C for 1 min for target DNA ≤1 kb or 1 min/kb for target DNA >1 kb (elongation). Following these cycles perform a final elongation at 72°C for 10 min and then hold at 4°C.
3. Purify pool of mutated PCR products on a preparative agarose gel. Extract amplicon band using a DNA gel extraction kit.
4. Combine 200–500 ng of the megaprimer, 50 ng of *dam*-methylated expression plasmid, 200 μM of each dNTP, and

5 U of high-fidelity polymerase Pfu Turbo® in the supplemented buffer in a total volume of 50 µl in a thin-walled PCR tube (see Note 14).

5. Perform PCR in a thermocycler using the following program: 95°C for 3 min (initial denaturation); 25–30 cycles of: 95°C for 2 min 30 s (denaturation), 55°C for 1 min (annealing), 72°C for 2 min/kb (elongation). Following these cycles perform a final elongation at 72°C for 20 min and then hold at 4°C (see Note 15).
6. Remove the *dam*-methylated template DNA by adding 20 U *Dpn*I. Incubate at 37°C for 2 h. Heat inactivate the restriction enzyme by incubation at 80°C for 20 min.
7. To concentrate the undigested PCR product and remove salts, add 2–4 µl Pellet Paint® Co-Precipitant and 0.1 volume of 3 M sodium acetate buffer to the PCR. Mix briefly.
8. Add one volume of isopropanol. Vortex briefly. Incubate at room temperature for 5 min.
9. Spin at 16,000×*g* for 20 min in a microcentrifuge (see Note 16).
10. Discard supernatant without disturbing the pellet. Add 500 µl of 70% ethanol. Spin at 16,000×*g* for 5 min in a microcentrifuge.
11. Discard supernatant without disturbing the pellet. Add 500 µl of 100% ethanol. Spin at 16,000×*g* for 5 min in a microcentrifuge.
12. Remove supernatant completely without disturbing pellet. Dry pellet in thermomixer at 55°C for 5–10 min, leaving the lid of the tube open.
13. Resuspend DNA pellet in 3 µl ddH_2O.
14. Transform 0.5–2 µl of the library into 25–100 µl of highly electrocompetent cells as described in Subheading 3.1.1, step 8 (see Note 17).
15. Spread different various cell dilutions (e.g., 1:1,000, 1:500, 1:100) on pre-warmed LB plates containing an antibacterial agent selecting for the chosen expression plasmid, not the antibiotic degraded by the tripartite fusion (any β-lactam antibiotic). Incubate plates at 37°C for 16 h.
16. Count the number of colonies on each plate and calculate the total library size (see Note 18).
17. Randomly select at least 20, better 40 single colonies, and isolate the plasmid DNA using a plasmid DNA extraction kit.
18. Sequence the target protein encoding sequence and determine the mutagenesis rate per kilobase (see Note 19).

3.3. Selection for Increased Levels of Resistance

1. Transform the library generated above into an *E. coli* strain of choice, using optimized conditions (see Note 18). As a control, transform the expression plasmid that was used as a template for the library construction (containing the wild-type version of the target protein gene).
2. Spread cells on pre-warmed LB plates spanning a range of different concentrations of β-lactam antibiotic (e.g., 0, 500, 1,000, … 3,000 μg/ml ampicillin). If expressing the tripartite fusion under an arabinose-inducible promoter, include arabinose in selection plates. Incubate plates at 37°C for 16–20 h (see Note 20).
3. Select single colonies from plates containing concentrations of the β-lactam antibiotic that did not permit growth of cell containing the control plasmid. Isolate plasmid DNA using a plasmid DNA extraction kit.
4. Sequence the target protein encoding sequence (see Note 21).
5. Optional: Repeat the mutagenesis, this time using a mixture of mutant plasmids (e.g., different plasmids encoding for various single point mutations in the protein of interest that have been selected for increased resistance) (see Note 22).

3.4. Determining the Level of Antibiotic Resistance Using Spot Titers

3.4.1. Spot Titer Experiment

1. Re-transform mutant DNA isolated in Subheading 3.3, step 3, into fresh competent cells using the same bacterial strain (e.g., commercially available NEB10-beta) that was originally used for the selection (see Note 23).
2. Inoculate 5 ml LB medium with a single colony expressing the mutated tripartite fusion. Cells expressing tripartite fusions containing the wild-type insert protein serve as a control (see Note 24).
3. Grow cells at 37°C until they reach A_{600nm} of about 0.5–0.7.
4. Harvest 1 ml culture by spinning at 16,000 × *g* for 5 min in a microcentrifuge. Discard supernatant. Keep cells on ice.
5. Adjust cells in 1× PBS to $A_{600nm} = 1$. Keep cells on ice.
6. Prepare tenfold dilutions of the cell suspension with 1× PBS (up to 10^{-6}) in a 96-well plate using a multichannel pipette. Keep cells on ice (see Note 25).
7. Using a multichannel pipette, spot 2 μl of each cell dilution onto pre-warmed LB plates containing increasing concentrations of β-lactam antibiotic. If expressing the tripartite fusion under an arabinose-inducible promoter, include arabinose in plates (see Note 20). Wait until spots are dry. Incubate at 37°C for 16–20 h. An example plate is shown in Fig. 3a (see Note 26).

3.4.2. Calculation of the Average Minimal Inhibitory Concentration

1. For each strain and each concentration of β-lactam antibiotic, score cell growth at each cell dilution with "growth" or "no growth" (Fig. 3a) (see Note 27).
2. Plot the maximal cell dilution allowing cell growth versus the antibiotic concentration for each strain (Fig. 3b).
3. For each strain and each cell dilution, assess the smallest concentration of β-lactam antibiotic that inhibits cell growth for this particular dilution, this is the minimal inhibitory concentration or MIC (see Table 1) (see Note 28).
4. For each cell dilution, normalize the MIC for each strain (e.g., expressing the tripartite fusion containing the mutant protein) to the MIC of the reference strain (e.g., expressing the tripartite fusion containing the wild-type (WT) protein) by calculating the value for MIC (mutant)/MIC (WT) (see Table 1) (see Note 29).
5. Average the MIC (mutant)/MIC (WT) ratios for at least three cell dilutions. Standard deviations should be calculated for independent experiments, not for different cell dilutions within one experiment (see Note 30).

Table 1
Example MIC values for three different strains

	MIC						
Cell dilution [10^{-x}]	**6**	**5**	**4**	**3**	**2**	**1**	**0**
Im7 WT	1,400	1,400	1,800	2,600	2,850	3,100	n.d.
Im7 L34A	700	900	1,000	1,400	1,900	3,100	n.d.
Im7 I54V	500	600	600	900	1,400	2,850	n.d.
	MIC_{mut}/MIC_{WT}						
Cell dilution [10^{-x}]	**6**	**5**	**4**	**3**	**2**	**1**	**0**
Im7 WT	1.00	1.00	1.00	1.00	1.00	1.00	n.d.
Im7 L34A	0.50	0.64	0.56	0.54	0.67	1.00	n.d.
Im7 I54V	0.36	0.43	0.33	0.35	0.49	0.92	n.d.

Upper part: Minimal inhibitory concentrations (MIC) for serial dilutions of NEB10β cells expressing tripartite fusions containing Im7 WT, Im7 L34A, or L54A, respectively. *Lower part*: MIC(mutant)/MIC(WT) ratios for serial dilutions of cells expressing tripartite fusions containing Im7 WT, Im7 L34A, or L54A, respectively

4. Notes

1. This plasmid is both ampicillin and tetracycline resistant.
2. This plasmid is chloramphenicol resistant.
3. The primers should be designed to have a similar melting temperature. Appropriate restriction sites (1) are present in the linker-encoding region of the bla-link gene (Fig. 2), (2) are unique within the chosen tripartite fusion expression plasmid, and (3) are not present in the target gene.
4. Mutagenesis primers should be designed to have a similar melting temperature (ranging from 55 to 72°C). The forward primer should cover the 5′ end of the target gene, the reverse primer the 3′ end of the target gene. Since the mutagenesis rate is decreased in the region covered by the primers, short primers are preferred. The amount of template DNA is determined by the amount of actual target DNA (length of the gene encoding for the protein of interest), not by the total amount of DNA added to the reaction in form of the tripartite fusion expression plasmid. The mutation frequency can be increased by lowering the amount of target DNA result and/or by increasing the number of cycles in the PCR program. Note that PCR yields might decrease at target DNA amounts below 0.1 ng. The mutation frequency can further be increased by using a pool of already mutated sequences as a template for the error-prone PCR.
5. Square plates are preferred over round plates, because they allow one to compare more strains on a single plate.
6. The tripartite fusion can be expressed from a variety of different plasmids. For smaller, nontoxic *E. coli* proteins (smaller than about 15 kDa), expression of the tripartite fusion can often be achieved simply by using the constitutive, native β-lactamase promoter (e.g., expression plasmid pBR322-bla-link, see Subheading 3.1.1). For heterologous, larger or more toxic *E. coli* proteins (larger than about 15 kDa), we found it

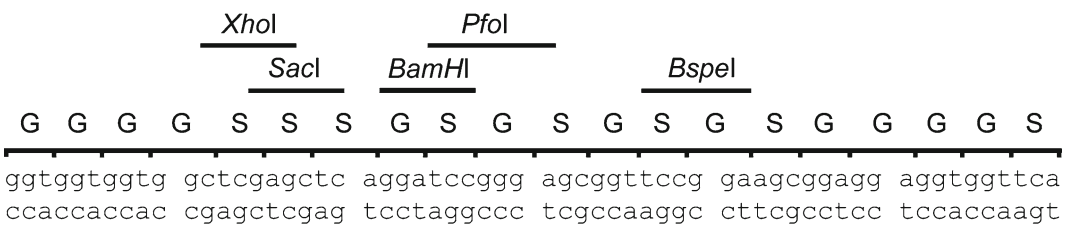

Fig. 2. Glycine-serine linker. Shown is the nucleotide sequence encoding for residues 6–25 of the glycine-serine linker. Restriction sites are indicated. The amino acid sequence of the entire linker is $(GGGGS)_2SSGSGSGSG(GGGGS)_2$.

beneficial to be able to fine-tune expression of the tripartite fusion and thus optimize the basal level of antibiotic resistance by using a regulatable promoter such as the arabinose promoter (e.g., expression plasmid pMB1-ara-bla-link, see Subheading 3.1.2). Choosing a pMB1 origin of replication (about 15–20 plasmid copies per cell, e.g., on pBR322) over lower copy number origins like pSC101 (about 5 copies per cell, e.g., on pBAD43) or p15A (10–12 copies per cells, on, e.g., pBAD33) will increase the basal level of antibiotic resistance conferred by the tripartite fusion and is preferred. Plasmids that already carry the intact β-lactamase (*bla*) gene absolutely need to be avoided since the wild-type *bla* gene will overwhelm any antibiotic resistance encoded by the tripartite fusion. Additionally, plasmids that even contain fragments of the *bla* gene should also be avoided to prevent unwanted recombination with the *bla* portions of the tripartite fusion encoding sequence. The standard length of the linker in the tripartite fusion length is 30 amino acids (aa), but can be adjusted to accommodate the insertion of larger test proteins into β-lactamase. We calculated that for a theoretical, perfectly spherical protein of 50 kDa with N- and C-termini at opposite sites, a 30 aa long linker should be more than long enough to allow interaction of the two β-lactamase fragments and therefore activity. For non-spherical proteins that have their termini far apart, the use of a longer linker, e.g., with 60 residues, is suggested. This however may lead to lower levels of antibiotic resistance.

Both plasmids, pBR322-bla-link and pMB1-ara-bla-link, can be requested from the authors. The description of their derivation is included simply to facilitate the reader to prepare similar constructs that are customized for their own purposes.

7. In this PCR, a linker-encoding sequence is inserted into the *bla* gene present on pBR322. The 5′ end of each primer encodes either for the first 17 residues (primer 1) or for the last 13 residues (primer 2) of the linker. The remaining nucleotide sequences of the two primers are complementary to the regions directly upstream (primer 1) or downstream (primer 2) of the insertion site for the linker-encoding sequence within the *bla* gene. The linker-encoding region contains restriction sites, allowing the insertion of a guest protein into approximately the middle of the linker (Fig. 2).
8. High fidelity (proofreading) DNA polymerases other than Phusion® can be used in this step. In this case, follow the instructions of the manufacturer.
9. If more than one PCR product is observed, gel-purification of the full-length PCR product after step 4 is recommended. In this case, omit step 5 and instead extract and purify the appropriately

sized DNA fragment from a preparative agarose gel using a DNA gel extraction kit.

10. Electrocompetent or chemically competent strains other than NEB10-beta can alternatively be used in this step as long as they are not tetracycline resistant.
11. If more than one PCR product is observed, gel-purification of the full-length PCR product after step 4 is recommended. In this case, omit step 4 and instead purify the appropriately sized DNA fragment from a preparative agarose gel using a DNA gel extraction kit.
12. The restriction enzymes *Xba*I and *Spe*I produce compatible ends.
13. In theory, the mutagenesis of the target gene can also be achieved by alternative mutagenesis techniques such as chemical mutagenesis, random insertion and deletion mutagenesis, random oligonucleotide mutagenesis, and so on. For reviews about mutagenesis methods see ref. 25–28.
14. Estimate megaprimer concentration by measuring absorption at 260 nm or by comparing the intensity of the corresponding band on an ethidium bromide stained agarose gel to the intensity of a band representing a DNA fragment of similar size and known concentration.
15. At this time, a PCR product may or may not be visible on an analytical agarose gel. Proceed either way with step 6.
16. At this point, a DNA pellet should have formed, easily visible due to co-precipitation of the dye. If no pellet can be observed, spin for additional 10 min at 16,000 × *g* in a microcentrifuge and/or add more isopropanol.
17. A variety of different strains can be used for the selection. To ensure large library sizes, however, highly competent cells should be used, e.g., commercially available NEB10-beta electrocompetent cells with a transformation efficiency of $2–4 \times 10^{10}$ cfu/μg pUC19.
18. Typical library sizes are 10^5 colonies and more for about 100 μl competent cells. If the yield of the synthesized plasmid is unsatisfactory, increase the amount of megaprimer used in the PCR and/or optimize the reaction by trying different ratios of megaprimer: plasmid. The total number of transformed cells can further be optimized by using different DNA amounts for the transformation, more cells and/or simply by performing multiple transformations.
19. It is crucial to determine the mutagenesis rate prior to any selection for increased levels of resistance to β-lactam antibiotics. The desired mutagenesis rate depends on the application. A rate that results in single point mutations can be useful for

initial experiments. Proceed with the selection described in Subheading 3.3 only when satisfied with the mutagenesis rate (see Note 4).

20. We found this genetic selection system to work with a variety of different β-lactam antibiotics (e.g., ampicillin, penicillin V), media (e.g., LB, minimal medium, MacConkey medium), *E. coli* strains (e.g., MG1655, NEB10-beta, BW25113), and incubation temperatures (30–42°C). Choose conditions suitable to address the scientific question of interest. We found that including an additional antibiotic (e.g., for maintenance of the expression plasmid) in the selection medium causes additional stress to the cells and is neither recommended nor necessary. When expressing the tripartite fusion under an arabinose-inducible promoter, it is further advisable to optimize the arabinose concentration used for induction prior any selection experiment. For this, perform spot titer experiments (see Subheading 3.4) using plates supplemented with different concentrations of arabinose (e.g., 0, 0.1, 0.2, 0.5, 0.75, 1, 1.5, and 2%) and one to three fixed concentrations of β-lactam antibiotic that prevent growth for some but not all cell dilutions. The arabinose concentration allowing the highest level of resistance (without causing any cell sickness on plates containing no β-lactam antibiotic) should be chosen for downstream experiments.
21. Although less common, mutations within the reporter protein can occur and could theoretically be selected for if the specific activity of β-lactamase was increased as a result of the mutation. To exclude this possibility, sequencing of the entire fusion protein gene and its promoter region, not only of the gene for the protein of interest, is recommended. We have not observed any mutations on the plasmid that should increase its copy number. However, if this is a concern, the rest of the plasmid can be sequenced as well.
22. This additional PCR step can result in recombination of different single point mutants in form of multiple mutations, as well as in the introduction of additional mutations. The consequence can be even higher levels of antibiotic resistance.
23. This step serves to exclude mutations in the host chromosome that could have occurred sporadically and caused an increased level of resistance.
24. If the overall level of antibiotic resistance is extremely low, a plasmid-free strain should be used as an additional control to monitor the basal level of antibiotic resistance this strain exhibits.
25. Depending on insert protein, strain, incubation temperature, and medium, cell dilutions of 10^{-5} or 10^{-6} might or might not

show cell growth. More important than absolute growth is relative growth compared to the control strain expressing a tripartite fusion containing the wild-type insert protein.

26. Include the control strain (strain expressing tripartite fusion containing the wild-type version of the target protein gene) on every plate.
27. For example, in Fig. 3a depicting a LB plate containing 0.9 mg/ml penicillin V, cells expressing a tripartite fusion containing the insert protein Im7 L34A grow at dilutions of 10^{0} to 10^{-4}, but not at dilutions of 10^{-5} or 10^{-6}. The maximal cell dilution allowing growth for this mutant at this concentration of β-lactam antibiotic is therefore 10^{-4}. The corresponding data point in Fig. 3b is marked with an asterisk symbol.
28. For example, in Fig. 3b, inspect the graph representing cells expressing a tripartite fusion containing the insert protein Im7 L34A. Cell dilutions of 10^{-3} show growth on plates containing 0–1.3 mg/ml penicillin V, but not on plates containing ≥1.4 mg/ml penicillin V. The MIC is the smallest concentration of β-lactam antibiotic tested that prevents cell growth (in this example, 1.4 mg/ml is the MIC for cell dilution of 10^{-3}). The smaller the concentration differences between plates, the more precise the calculated MIC will be. As an alternative to using the first concentration tested that prevents cell growth for a given dilution, extrapolation of the MIC value from the graphs in Fig. 3b is generally acceptable, too. For example, we often extrapolated the MIC to be, e.g., 0.1 mg/ml higher than the last concentration that showed cell growth for a particular dilution. In the case of cells expressing a tripartite fusion containing the insert protein Im7 L34A, for instance, cells show growth at dilutions of 10^{-1} up to 2.75 mg/ml. We estimated the MIC to be $2.75 + 0.1 = 2.85$ mg/ml (although the next tested concentration was 3 mg/ml). In our experience, extrapolation of MIC values does not significantly influence the ratio of MIC (mutant)/MIC (WT).
29. MIC (mutant)/MIC (WT) values smaller than one indicate decreased levels of antibiotic resistance compared to WT. MIC (mutant)/MIC (WT) values larger than one indicate increased levels of antibiotic resistance compared to WT.
30. Which cell dilutions show the highest phenotypic reproducibility depends on insert protein, strain, incubation temperature, and medium. We frequently average MIC (mutant)/MIC (WT) ratios for cell dilutions of 10^{-4} to 10^{-2}, 10^{-3} to 10^{-1}, or 10^{-4} to 10^{-1}. Phenotypes for cell dilutions of 10^{0}, 10^{-5}, and 10^{-6} are in general less reproducible and their MIC values should be excluded from the MIC average if possible.

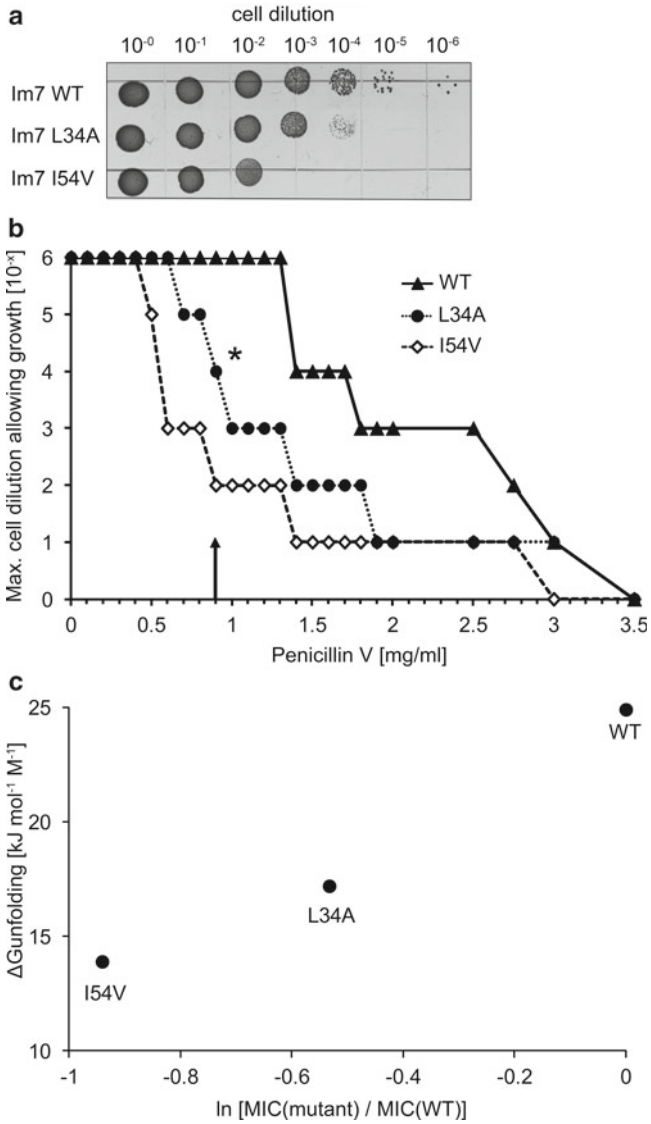

Fig. 3. Spot titer experiments and their analysis. (**a**) Mid-log phase cells of *E. coli* NEB10β expressing tripartite fusions with Im7 WT, Im7 L34A, or Im7 I54V, respectively, were adjusted to $A_{600nm} = 1$ with PBS. Serial cell dilutions of 10^0 to 10^{-6} were spotted on a LB plate containing 0.9 mg/ml penicillin V. (**b**) The maximal cell dilution allowing growth is plotted against the concentration of penicillin V used in LB plates. The *arrow* indicates the penicillin V concentration used in (**a**) (for *, see Note 27). (**c**) The free energy of unfolding ($\Delta G_{unfolding}$) is plotted against the natural logarithm of the average ratio MIC (Im7 mutant)/MIC (Im7 WT) (28) (see Note 31).

31. At constant temperature in vitro, the free energy of unfolding $\Delta G_{unfolding}$ is directly correlated to the equilibrium constant K of the unfolding reaction through the following equation: $\Delta G_{unfolding}(T) = -R \ln K$. K is defined as the number of molecules occupying the unfolded state divided by the number of

molecules occupying the folded state. In vivo, this ratio is reflected by the steady-state expression level of the tripartite fusion in the periplasm: the more insert proteins occupy the folded state, the higher is the level of the tripartite fusion. Since we found the steady-state expression level to be directly proportional to the level of antibiotic resistance, we plot the natural logarithm of MIC here, analogous to ln *K*.

References

1. Privalov PL, Khechinashvili NN (1974) A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. J Mol Biol 86:665–684
2. Giver L, Gershenson A, Freskgard PO, Arnold FH (1998) Directed evolution of a thermostable esterase. Proc Natl Acad Sci U S A 95:12809–12813
3. Taverna DM, Goldstein RA (2002) Why are proteins marginally stable? Proteins 46:105–109
4. Guo HH, Choe J, Loeb LA (2004) Protein tolerance to random amino acid change. Proc Natl Acad Sci U S A 101:9205–9210
5. Soskine M, Tawfik DS (2010) Mutational effects and the evolution of new protein functions. Nat Rev Genet 11:572–582
6. Ghaemmaghami S, Oas TG (2001) Quantitative protein stability measurement in vivo. Nat Struct Biol 8:879–882
7. Ignatova Z, Gierasch LM (2004) Monitoring protein stability and aggregation in vivo by real-time fluorescent labeling. Proc Natl Acad Sci U S A 101:523–528
8. Mayer S, Rudiger S, Ang HC, Joerger AC, Fersht AR (2007) Correlation of levels of folded recombinant p53 in Escherichia coli with thermodynamic stability in vitro. J Mol Biol 372:268–276
9. Barakat NH, Carmody LJ, Love JJ (2007) Exploiting elements of transcriptional machinery to enhance protein stability. J Mol Biol 366:103–116
10. Chautard H, Blas-Galindo E, Menguy T, Grand'Mourselle L, Cava F, Berenguer J, Delcourt M (2007) An activity-independent selection system of thermostable protein variants. Nat Methods 4:919–921
11. Waldo GS (2003) Improving protein folding efficiency by directed evolution using the GFP folding reporter. Methods Mol Biol 230:343–359
12. Foit L, Morgan GJ, Kern MJ, Steimer LR, von Hacht AA, Titchmarsh J, Warriner SL, Radford SE, Bardwell JC (2009) Optimizing protein stability in vivo. Mol Cell 36:861–871
13. Delaire M, Lenfant F, Labia R, Masson JM (1991) Site-directed mutagenesis on TEM-1 beta-lactamase: role of Glu166 in catalysis and substrate binding. Protein Eng 4:805–810
14. Hallet B, Sherratt DJ, Hayes F (1997) Pentapeptide scanning mutagenesis: random insertion of a variable five amino acid cassette in a target protein. Nucleic Acids Res 25:1866–1867
15. Zebala J, Barany F (1991) Mapping catalytically important regions of an enzyme using two-codon insertion mutagenesis: a case study correlating beta-lactamase mutants with the three-dimensional structure. Gene 100:51–57
16. Galarneau A, Primeau M, Trudeau L-E, Michnick SW (2002) [beta]-Lactamase protein fragment complementation assays as in vivo and in vitro sensors of protein-protein interactions. Nat Biotechnol 20:619–622
17. Foit L, Mueller-Schickert A, Mamathambika BS, Gleiter S, Klaska CL, Ren G, Bardwell JC (2011) Genetic selection for enhanced folding in vivo targets the Cys14-Cys38 disulfide bond in bovine pancreatic trypsin inhibitor. Antioxid Redox Signal 14:973–984
18. Quan S, Koldewey P, Tapley T, Kirsch N, Ruane KM, Pfizenmaier J, Shi R, Hofmann S, Foit L, Ren G, Jakob U, Xu Z, Cygler M, Bardwell JC (2011) Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. Nat Struct Mol Biol 18:262–269
19. Gleiter S, Bardwell JC (2008) Disulfide bond isomerization in prokaryotes. Biochim Biophys Acta 1783:530–534
20. Watson N (1988) A new revision of the sequence of plasmid pBR322. Gene 70: 399–403
21. Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JH, Falkow S (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113

22. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 177: 4121–4130
23. Miyazaki K (2003) Creating random mutagenesis libraries by megaprimer PCR of whole plasmid (MEGAWHOP). Methods Mol Biol 231:23–28
24. Miyazaki K (2011) MEGAWHOP cloning: a method of creating random mutagenesis libraries via megaprimer PCR of whole plasmids. Methods Enzymol 498:399–406
25. Labrou NE (2010) Random mutagenesis methods for in vitro directed enzyme evolution. Curr Protein Pept Sci 11:91–100
26. Rasila TS, Pajunen MI, Savilahti H (2009) Critical evaluation of random mutagenesis by error-prone polymerase chain reaction protocols, Escherichia coli mutator strain, and hydroxylamine treatment. Anal Biochem 388:71–80
27. Wong TS, Roccatano D, Zacharias M, Schwaneberg U (2006) A statistical analysis of random mutagenesis methods used for directed protein evolution. J Mol Biol 355: 858–871
28. Chusacultanachai S, Yuthavong Y (2004) Random mutagenesis strategies for construction of large and diverse clone libraries of mutated DNA fragments. Methods Mol Biol 270:319–334

Chapter 2

Determining Enzyme Kinetics via Isothermal Titration Calorimetry

Neil A. Demarse, Marie C. Killian, Lee D. Hansen, and Colette F. Quinn

Abstract

Isothermal titration calorimetry (ITC) has emerged as a powerful tool for determining the thermodynamic properties of chemical or physical equilibria such as protein–protein, ligand–receptor, and protein–DNA binding interactions. The utility of ITC for determining kinetic information, however, has not been fully recognized. Methods for collecting and analyzing data on enzyme kinetics are discussed here. The step-by-step process of converting the raw heat output rate into the kinetic parameters of the Michaelis–Menten equation is explicitly stated. The hydrolysis of sucrose by invertase is used to demonstrate the capability of the instrument and method.

Key words: Isothermal titration calorimetry, ITC, Enzyme kinetics, Michaelis–Menten kinetics

1. Introduction

Enzymes are biological macromolecules that catalyze the conversion of chemical precursor molecules (substrate) to essential chemical products. When linked in series, enzyme pathways perform numerous critical functions to maintain organismal life (i.e., cell growth, cell differentiation, breakdown of nutrients for energy, energy storage, etc.). When enzyme function is perturbed, serious disease can result. Thus, studying enzyme kinetics and determining the details of an enzyme's activity is a necessary prerequisite for developing novel therapeutics to treat and understand disease. Isothermal titration calorimetry (ITC) is a straightforward and direct method for determining the basic chemical details of an enzyme catalyzed reaction (i.e., V_{max}, K_m, and k_2). The advantages of ITC over other analytical methods are: (1) Substrate(s) do not require labeling or linkage to a secondary-detectable process.

James C. Samuelson (ed.), *Enzyme Engineering: Methods and Protocols*, Methods in Molecular Biology, vol. 978,
DOI 10.1007/978-1-62703-293-3_2,

(2) ITC is nondestructive to the enzyme. And (3) ITC is compatible with both physiological and synthetic substrates (1, 2). This study uses a standard experimental system (hydrolysis of sucrose by invertase) to illustrate measurement of the kinetics of a reaction by ITC (3). Because processing of enzyme kinetic data collected by ITC is over-simplified in the literature, details of the calculations to convert the heat generated into the kinetic parameters are given (2). Some of the instructions given here are specific to invertase catalyzed hydrolysis of sucrose, our test system, but these can easily be adapted for other enzymes.

2. Materials

2.1. Laboratory Supplies

1. Lint-free laboratory wipes (e.g., KimWipes®).
2. Analytical balance to weigh samples.
3. 12″ stainless steel tweezers.
4. 10 mL graduated cylinder or 10 mL volumetric flask.
5. 100 mL graduated cylinder or 100 mL volumetric flask.
6. 500 mL graduated cylinder or 500 mL volumetric flask.
7. 1 L graduated cylinder or 1 L volumetric flask.
8. 10 μL pipette.
9. 10 μL pipette tips.
10. Waste beaker.

2.2. Instrument Setup Components

1. Nano ITC Low Volume (part number 601000.901) (TA Instruments) (see Notes 1 and 2).
2. Degassing station (part number 6326) (TA Instruments).
3. 500 μL Hamilton filling syringe.
4. Computer running Windows XP or Windows 7.
5. Nano ITCRun data acquisition software (available at TAInstrument.com).
6. Data analysis software (e.g., MATHCAD).

2.3. Instrument Cleaning Components

1. Nano ITC cleaning tool (part number 601028.901) (TA Instruments).
2. Silicone rubber tubing (1/16 inside diameter).
3. 1 L side-arm vacuum flask with a #8 rubber stopper and tubing to connect to a vacuum source.
4. Cleaning solution: 100 mL 5% w/v SDS solution. Weigh 5 g of sodium dodecylsulfate and transfer to 100 mL graduated