

Protocol

Amber Suppressor tRNA-Based Mutagenesis for Positional Semi-Saturated Mutagenesis with Natural Amino Acid Substitutions: An Approach for Mapping Positional Contributions to Protein Function

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Abstract

There is a growing interest in methods that illuminate the contributions of individual positions to a protein's function by site-saturating mutagenesis. However, a commonly used approach for assessing variant libraries with deep mutational scanning relies on readouts of biological fitness, which is influenced by many protein properties (ligand binding, catalysis, allosteric effector binding, allosteric coupling between effector and substrate, etc.). Biochemical assays are required to distinguish among these factors. To facilitate the generation and biochemical evaluation of the functions of large numbers of substituted positions, we co-express updated plasmids coding a series of amber suppressor tRNA in a high-throughput workflow; these plasmids are available at Addgene. As an example, our goal is to evaluate whether allosteric mechanisms are conserved among homologs. Because homologs often have <50% identity, and up to 30% of a protein's positions can contribute to allosteric function, we reason that the set of "allosteric" positions likely differs among homologs. Our high-throughput workflow includes the following steps: Step (1) an amber suppressor tRNA-based mutagenesis protocol; Step (2) a robotic system for protein expression/purification and functional assays; and Step (3) a method for aggregating results from multiple substitutions at each position into a composite score.

Keywords: high-throughput mutagenesis; quantitative comparison; allosteric regulation; allostery; database



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1. Introduction

Understanding how each amino acid in a protein contributes to an overall function of that protein has long been an underlying goal of structure/function studies of proteins and enzymes [1–3]. However, a challenge quickly emerges when one outlines the many "functions" a residue can contribute to within a protein: stability, folding, ligand binding, catalysis, allosteric regulation, etc. [4–7]. Thus, attempts to investigate how each amino acid contributes to function require isolated quantitation of those individual attributes, often

through biochemical characterization (e.g., [8–10]). This characterization is expanded by substituting a residue position with a range of amino acids, a long-standing methodology for probing the role of a position [7,11–17]. As such, the need to generate and biochemically characterize a large number of protein variants presents a challenge to the field [18]. One of the main expenses of present approaches is building plasmid libraries to generate the protein variants. Here, we present an updated, plasmid-based co-expression of amber suppressor tRNAs that can be used to generate the needed numbers of substitutions in a protein. We also describe how this method can fit into pre-existing robotic protein purification/biochemical characterization pipelines as well as the needed framework for aggregating data outcomes. Our research focuses on the study of allosteric proteins. Therefore, herein we use allosteric regulation as the example function to be characterized by this method. It should not be overlooked that this amber suppressor tRNA-based mutational approach has broad application to the studies of many, if not most, protein functions.

For the uninitiated, an introduction to the field of allosteric regulation can be overwhelming [19]. A long list of experimental, theoretical, opinion, and speculative publications test and discuss the possible molecular mechanisms that underlie a protein's allosteric response (e.g., [20–34]). Despite their differences, the field ubiquitously agrees that more data are needed to illuminate the molecular mechanisms that underlie allosteric regulation [15,35,36]. That need includes both more observations per technique and more observations from different types of techniques. This is particularly true for our current question of interest: whether allosteric mechanisms are conserved among homologs of a protein family (e.g., [37]). That is, given one common protein architecture, are the same positions involved in the allosteric regulation of each homolog, or does each homolog use a unique set of positions to derive linked functions between two functional sites? Mechanistic conservation is often assumed but seldom evaluated. In fact, mechanistic conservation is an implicit assumption to any sequence alignment or structural comparison among homologs that is used to hypothesize which residues contribute to allosteric regulation (example: pyruvate kinase [38]).

Given our goal of evaluating the conservation of allosteric mechanism(s), one might expect our approach to center on comparing available structures of homologs. However, crystallized proteins do not capture all aspects of protein function, such as allostery [39]. In fact, many of the conditions used to crystalize the protein can alter allosteric regulation, including buffer type [40], pH [41,42], divalent cation type [40,43], monovalent cation type [40], anion type [40], temperature [44], and solvent (e.g., deuterated water) [45]. Thus, structures derived from X-ray crystallography may not capture allosterically relevant differences in all systems [32,46,47].

Instead, we have focused our studies on using functional data to evaluate conservation of allosteric mechanisms. Functional approaches typically rely on introducing perturbations, most often substitutions at individual protein positions and then assessing effects on allostery. For these studies, one must next consider which amino acid to substitute. For simplicity, one approach is to use alanine scans (e.g., [48,49]). However, at some positions, an alanine substitution could function similarly to the native residue, causing us to miss key positions [49,50]. Thus, to evaluate a position's contribution to allosteric regulation, it is advantageous to use a range of substitutions at each position [10,51–60]. This allows one to discriminate between instances for which (1) removal of the native residue causes a loss-of-function versus (2) addition of the substituted residue causes a gain-of-function. Restated, the use of a range of substitutions allows a discrimination between the case in which the natural amino acid is required from the case in which the substituted amino acid is prohibited [49,50]. Finally, a range of substitutions allows a study to focus on the overall

contribution of the position, rather than on the specific substitution (see positional types below) [51,52].

A focus on protein positions also parallels the type of information that is derived from the multiple sequence alignments used to determine homolog sequence identity. Indeed, in any protein family, homologs often have less than 50% identity. However, we previously determined that up to 30% of the positions in a protein can contribute to allosteric regulation [49]. Therefore, positions that contribute to allosteric mechanisms are not likely conserved among all homologs, which in turn implies that the study of positional contributions to allosteric regulation may be as valuable as attempting to understand how individual side chain chemistries contribute to that function [46].

There is a close connection between focusing on the functional side of a structure/function question and the use of perturbations. However, perturbation at any one position in a protein may only partially modify a given function. In fact, in our studies of allosteric regulation, we have demonstrated multiple times that this function is not an all-or-none phenomenon for which all influencing substitutions result in similar outcomes. Instead a set of substitutions (scattered throughout the protein [49] or a series made at one position [55]) can result in a range of allosteric regulation outcomes. Thus, as exemplified by our past studies of allosteric regulation, perturbation studies most often require quantitation of the change in allosteric function. Therefore, similar to many studies of proteins, the best method for quantifying those protein functions is made by using purified proteins and in vitro assays. In contrast, the use of deep mutational scanning that depends on one biological readout (i.e., fitness) will be avoided because that approach fails to quantify individual enzyme/protein functions (catalysis, substrate binding, allosteric effector binding, allosteric coupling between effector, and substrate, etc.), each of which can contribute to a single biological readout [61].

Based on these considerations, the amber suppressor tRNA mutagenesis approach described here facilitates an ultra-high-throughput generation of substituted proteins that facilitates the characterization of how individual positions in a protein contribute to a given function of that protein. Although not often highlighted, technological advancements can be forgotten in history. One example is the development of an amber suppressor tRNA-based mutagenesis (referenced as amber-based mutagenesis hereafter) approach for an ultra-throughput generation of singly substituted proteins. This approach was developed in the 1990s but has seldom been utilized [62–66]. Perhaps at the time of original development, few systems could accommodate an evaluation of thousands of individually substituted proteins. In contrast, the high-throughput generation of substituted proteins is complementary to the developing robotic high-throughput protein expression/purification and characterization methods. This amber-based mutagenesis approach relies on expressing a gene with a single amber stop codon in a series of *E. coli* strains. Each strain contains a unique suppressor tRNA. This series can be used to achieve semi-saturated substitution for single positions in a protein, a goal of our high-throughput method. While saturated mutagenesis is defined by substituting a single position to each of the 20 natural amino acids, semi-saturated mutagenesis relies on substituting a single position with at least 10 of the natural amino acids. In our previous works [67], we have demonstrated that the semi-saturated method is sufficient to determine a position's contribution to allostery.

The original design to use amber mutagenesis relied on the then available suppressor tRNAs that were characterized to insert specific amino acids at the introduced amber stop codon [62–66]. Originally, 13 amber suppressor tRNAs were identified, resulting in 13 substituted proteins (i.e., semi-saturated). However, one of the original suppressor tRNAs could insert either glutamic acid or glutamine, which results in a mixed population

unsuited for many protein structure/function studies. In addition, the uses of the original suppressor tRNAs used for His, Lys, and Pro substitutions have been questioned [68].

Our initial use of the amber-based mutagenesis has confirmed that the suppressor tRNA for Lys insertion failed to function, but the original suppressor tRNAs for His and Pro insertion inserted the respective amino acids. Fortunately, multiple suppressor tRNAs have been reported to result in Lys being substituted into the expressed protein and are available to replace the potentially problematic original [66]. In our experience, the Lys constructs suppress at different efficiencies (Lys2 > Lys1 > Lys4 > Lys3). Suppression efficiency can be dependent on adjacent sequences, so we have included all four Lys suppressors [69]. Therefore, it is our recommendation to first assess the Lys2 plasmid for its ability to insert lysine. If poor suppression is obtained, then we suggest empirically determining the best suppressing construct. Lastly, since the time of the original invention, new suppressor tRNAs have been reported. In particular, the orthogonal suppressor tRNA/amino acyl-tRNA synthetase pairs developed to insert non-natural amino acids provide options to expand the original substitution range [70].

The application of the amber-based mutagenesis is straightforward. A plasmid containing the gene for a protein, modified with a single codon mutated to an amber stop codon, is co-expressed in *E. coli* with a second plasmid that codes for the expression of one of the amber suppressor tRNAs. That same design is repeated with each of the amber suppressor tRNAs. The result is a series of substituted proteins, for which the single residue substitution is located at the targeted residue position. The benefit of using this system is the greatly reduced cost of producing a single modified gene vs. using a different gene variant for each substitution at the targeted residue position. The follow-up challenge is the need for a system to aggregate outcomes for a range of substitutions at any one position and repeat that aggregating process for many positions. Therefore, after briefly outlining our design for our own efforts to quantify allosteric properties, we reference our method for aggregating outcomes to modified function due to a series of substitutions at one position.

2. Experimental Design

2.1. Data Introduction

The field of allosteric regulation might best be considered as two subfields [19]. Since our goal is to report a proposed workflow that can evaluate allosteric regulation of each substituted protein, we first introduce these two subfields and describe how each derives a different perspective about what “allosteric mechanisms” can be. Most concepts related to allosteric mechanisms can be grouped as being derived from either (i) a two-ligand allosteric energy cycle (i.e., linked-binding-function) view of allostery or (ii) a “two-state” (e.g., two-conformation) concept of allostery; the latter includes both the classical MWC and Koshland models [71,72]. Both allostery subfields can be traced to the conceptualizations by Dr. Jefferies Wyman: Dr. Wyman had a mathematic-centric career exploring the uses of linked-functions, including a focus on the linkage between two distinct binding events on the same protein (e.g., homotropic cooperative responses in hemoglobin) [73]. As far as his contribution to the classic two-state field, Dr. Wyman also collaborated with Drs. Monod and Changeux in a theoretical exploration of whether a linked equilibrium between a binding event and conformational change could be used to model binding curve shapes that were consistent with data observed for homotropic cooperative responses [72].

The two subfields of allostery are distinguished by which details are focused on for their fundamental definitions of allosteric regulation. For the first subfield, two-ligand allosteric energy cycles require (1) two ligand-binding sites and (2) use regulation by the “other” binding site to define an allosteric mechanism [19,74–76]. This definition describes allosteric coupling as a Gibbs free energy term that intrinsically incorporates both enthalpy

and entropy (including protein dynamic) components and makes no restrictions on the number of potential conformations that can be assessed in the ensemble of each relevant liganded enzyme complex [77–79]. In simple binding terms, allosteric regulation is defined by how one ligand binds differently to the protein when a second ligand is absent vs. already bound to the protein [74].

For the second subfield, and despite using the term “allosteric” (which is defined as “other binding site”) [80], a later collaboration among Drs. Monod, Changeux, and Wyman led to the original “two-state” model [72]; a similar approach was adopted by Koshland [71,72]. This approach originally focused on the roles of two protein conformations and was later expanded to consider two-ensembles of conformations accessible by the protein structure [81–83]. The original articulation of two-state models focused on a restrictive assumption of two conformations. As such, two-state models often use the word “allosteric” to imply the “other” conformation. These classical two-state models have led this subfield to focus both on the percentage of the protein that is in each of the two accessible “states” in the absence of ligand vs. the presence of ligand and, primarily for the computational predictions, the sequential order of event and rates of those events that contribute to the transitions between those two states.

Importantly, the classic two-state models do not allow both a substrate and an allosteric inhibitor to simultaneously bind to the same protein conformation. In contrast, simultaneous binding of the substrate and effector (either an activator or inhibitor) to the same protein molecule is central to the two-ligand allosteric energy cycles. As such, an important conceptual difference of these two approaches is that the “allosteric coupling constant” (defined below) used to quantify the allosteric function in two-ligand allosteric energy cycle analyses is not compatible with the two-state model of allosteric inhibition. Thus, the two fields are largely mutually exclusive [19]. Notably, the two subfields can be bridged by expanding two-state models to thermodynamic cubes. While this does bring both the allosteric mechanism defined in the original two-state model and that defined by two-ligand allosteric energy cycles into the same model, it creates a new, complicated challenge of interpreting which mechanism is the source of the observed allosteric regulation [84].

These differences in conceptual definitions influence both experimental designs and data interpretations. In this study, we first consider that many of the protein model systems used in our laboratories are enzymes, so we choose to use enzyme terminology and focus on allosteric regulation that alters substrate affinity. As such, our definition of allosteric regulation starts with a simple statement: allosteric regulation is defined as how the enzyme (E) binds the substrate (A) differently when the effector (X) is absent vs. present (i.e., bound to the enzyme) (Figure 1). This definition implicitly assumes that X binds to a site on E that is distinct from the binding site for A. This definition is the same as that used by the two-ligand allosteric energy cycle subfield [50,74,79], is consistent with the earliest use of the word “allosteric” as the other binding site [80], and is agnostic to the number of conformations (or ensembles) that can be adopted by the protein structure. This definition for allosteric regulation results in a two-ligand allosteric energy cycle (i.e., a linked thermodynamic) scheme:

For allosteric regulation that alters substrate affinity (i.e., a K-type allosteric response), the allosteric coupling constant (Q_{ax}) is the ratio of the binding of one ligand to the protein in the absence vs. the presence of the second ligand (Equation (1)) using the K values defined from the allosteric energy cycle (Figure 1). As such, Q_{ax} is an equilibrium constant related to ΔG_{ax} by Equation (2). ΔG_{ax} has both ΔH_{ax} and ΔS_{ax} contributions that can be quantified by determining Q_{ax} (Equation (3)) over a range of temperatures.

$$Q_{ax} = \frac{K_{ia}}{K_{ia/x}} = \frac{K_{ix}}{K_{ix/a}} \quad (1)$$

$$\Delta G_{ax} = -RT \ln Q_{ax} \quad (2)$$

$$\Delta G_{ax} = \Delta H_{ax} - T \Delta S_{ax} \quad (3)$$

Therefore, the approach incorporated into the current workflow is to quantify these allosteric equilibrium constants by using purified proteins and by determining the substrate affinity (K_{app}) from initial velocity studies for one ligand across a range of concentrations for the allosteric effector (Figure 2). When the resulting substrate affinities are plotted over the effector concentration range on a log y-axis, the allosteric coupling is the difference between the horizontal plateau at low effector concentrations and the horizontal plateau at high, saturating concentrations (Figure 2). If $Q_{ax} = 1$, there is no free energy coupling between the two ligand-binding events (i.e., no change in substrate affinity over the concentration range of effector); if $Q_{ax} > 1$, there is positive coupling or an activating effect (i.e., Figure 2C, response to Fru-1,6-BP); and if $Q_{ax} < 1$ there is negative coupling or an inhibiting effect (i.e., Figure 2C, response to Ala).

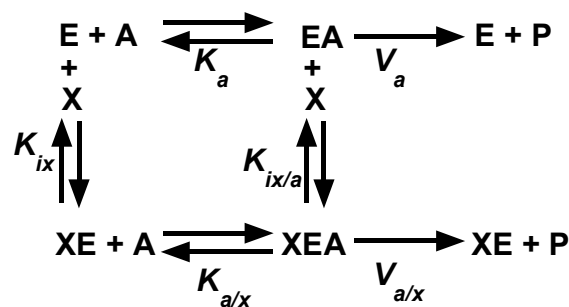


Figure 1. Allosteric coupling is defined by a two-ligand allosteric energy cycle scheme. The model enzymes used in our studies are K-type allosteric systems, which are described by the cycle above. “E” is the enzyme that binds the substrate “A.” “X” is an allosteric effector. When data are collected from initial ligand-binding experiments, K_{ia} and $K_{ia/x}$ are equilibrium binding constants for A in the absence and presence of the effector. To distinguish kinetically derived affinity values (as represented here), these parameters are replaced with K_a and $K_{a/x}$. For both binding and kinetic-derived data, K_{ix} and $K_{ix/a}$ are equilibrium binding constants for the effector to the enzyme in the absence and presence of substrate, respectively. Data collected with initial velocity measurements also includes turnover of both the EA and XEA complexes. The catalytic steps are represented by V_a and $V_{a/x}$. For simplicity, the allosteric systems that we will initially focus on do NOT have a V-type response (i.e., V_a and V_{ax} are equivalent).

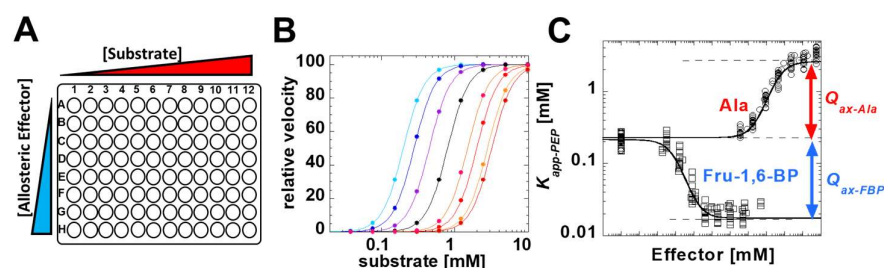


Figure 2. Allosteric assays and data analyses for a single purified protein. (A) Initial enzymatic rates at varied substrate and allosteric effector concentrations are collected in 96 deep-well plates. (B) For each row of effector concentration, initial velocity measurements across a concentration of substrates are used to generate a response curve from which the $K_{app-substrate}$ is determined. $K_{app-substrate}$ is the concentration that results in $\frac{1}{2} V_{max}$ activity (data shown are synthetic, colors correspond to different concentrations of allosteric effector). (C) Plots of $K_{app-substrate}$ versus effector concentration yield Q_{ax} ; the example used here consists of data for human liver pyruvate kinase (LPYK) [49] and panel (C) is modified from one published in [46]. In this example, the substrate is phosphoenolpyruvate (PEP), the inhibitor is alanine, and the activator is fructose-1,6-bisphosphate (Fru-1,6-BP). On the log y-axis, Q_{ax} is visualized as the difference between the two plateaus at high and low effector concentration.

Note that the example data in Figure 2 provide information for a single purified protein. For comparing the contributions of many substituted proteins in many homologs, this strategy must be repeated thousands of times. Hence our need for a higher-throughput workflow that massively scales up this data collection.

2.2. High-Throughput Mutagenesis via Amber Suppressor tRNAs

High-throughput studies that involve site-saturating mutagenesis (i.e., all 19 substitutions at each position) are daunting. Fortunately, none of our reasons for including multiple substitutions per position (see above) dictate that we need all 19 substitutions. Indeed, we have empirically determined that 10 randomly chosen substitutions are sufficient for positional classification (defined below) [51,53,55,85]. Nonetheless, our pipeline has the option to make 15 substitutions (minus one if the native residue is one of those 15) at any position.

Even so, this design has considerable financial ramifications. If we consider just three homologs in one protein family and sample 15% of each protein's positions (assuming 500 positions), our design requires 3375 mutant proteins. If we evaluate four protein families, we need to create 13,500 mutant proteins. The cheapest estimate for creating point mutations that we have identified (2026 pricing) is \$30/mutant. At this rate, the cost of obtaining mutations for our designed project would total \$400,000, which would dominate the budget of many NIH grant applications at this time.

To reduce this cost, our proposed workflow updates a classic, amber-based, high-throughput mutagenesis strategy. In the late 1980s and early 1990s, the Jeffery Miller laboratory used suppressor tRNAs to readthrough an introduced amber stop codon and thereby introduce a variant protein with a substitution at the targeted position (Figure 3). The advantage of this system is that, once an amber codon is introduced, the single mutation is grown in a series of *E. coli* strains, each of which contains a unique suppressor tRNA. The Miller laboratory designed expression plasmids and four suppressor tRNAs to be used in two plasmid co-expression designs [62–66]. They then relied on *E. coli* strains that carried natural suppressors for additional tRNAs to result in a total of 12 substitutions. A 13th tRNA was used that resulted in 85% Glu and 15% Gln.

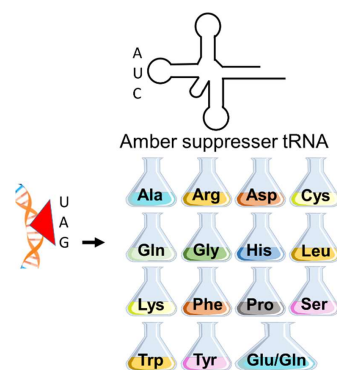


Figure 3. One mutation is made at the target codon, replacing the wild type with the amber stop codon. By expressing that mutated gene in a series of *E. coli*, each containing one of the 15 available suppressor tRNAs, we obtain up to 15 substitutions for a single mutated codon. Note that one suppressor tRNA previously used by the Miller laboratory results in Glu insertion in 85% of the produced proteins and Gln insertion in 15% of the produced proteins. Therefore, supplementing the Miller set of suppressor tRNAs with the orthogonal Asp system allows for an improved option for an acidic substitution to be included.

As a word of caution, the literature on suppressor tRNAs that have generated the tools for this amber-based mutagenesis strategy used nomenclature that can be misleading when used for our application. tRNAs are often named for the original amino acid type

associated with that tRNA. That name often does not imply which amino acid will be inserted once sequence modifications in the anticodon have been introduced. Furthermore, in orthogonal systems, a tRNA from a foreign species may insert a different amino acid when used in our *E. coli* expression system. It follows that the tRNAs that are useful for our amber-based mutagenesis design are those that recognize the amber stop codon and have been fully characterized for which amino acid is inserted into newly synthesized proteins, specifically, when expressed in *E. coli*. High selectivity for the amino acids inserted by each tRNA used in our design has been previously reported [86]. The sequences (including the promoter, the suppressor tRNA gene, and the terminator sequences used for suppressor tRNA expression) used in our design are included in Table 1.

3. Materials and Equipment

In the original, amber-based mutation strategy, the Miller laboratory co-expressed a few synthetic tRNAs using a tRNA gene on one plasmid and the target amber-mutated gene on a second plasmid. However, several natural suppressor tRNAs were used by expressing the target amber-mutated gene on a plasmid within the original *E. coli* strain found to possess that natural suppressor tRNA [62–66]. As the natural suppressors required cloning and the original Miller plasmids were no longer available, we synthesized both the Miller and natural suppressors into the same plasmid system. The plasmids that we generated used a single base modification when cloning the lipoprotein promoter that controls tRNA transcription, but otherwise reproduced the sequences for the tRNAs from Miller's previous design without any changes (see sequences in Table 1). This standardized the protocol across all amino acid types by cloning all suppressor tRNAs for use in two-plasmid co-expression designs described further below. These regenerated plasmids are available at Addgene.

During the cloning process, a few suppressor tRNA sequences, including His and Tyr, were difficult to generate. For these difficult tRNAs constructs, three major roadblocks hindered their construction: PCR template bleed-through, extremely low transformant counts and plasmid sequence alterations. Starting with the last roadblock, plasmid sequence alterations, the vast majority of sequenced plasmids have alterations in critical elements. Observed changes in critical elements included (i) deletion of the lipoprotein promoter, (ii) deletion of all or part of the tRNA cassette, and (iii) frameshift within the tRNA cassette. All of these changes would result in the same outcome—no functional suppressor tRNA present within the bacterium. We next sequenced the PCR products before transformation and discovered that the cloned tRNA was correctly generated. Therefore, the observed alterations after transformation were due to changes made within the *E. coli* cells. This suggests that some of the constructs were toxic. Furthermore, the other two common roadblocks (template bleed-through and low transformant counts), also are suggestive of plasmid toxicity. Cloning steps were carried out in the traditional cloning *E. coli* strains, which may indicate the suppression activity of specific tRNA constructs may be detrimental in bacterial strains that contain native amber codons.

Our proposed workflow will additionally use orthogonal suppressor tRNA/tRNA synthetases from other organisms (Table 1) that were developed to expand the use of non-natural amino acids in protein engineering [70]. We are not including non-natural amino acids in our design, but instead only using the orthogonal system that has been fully characterized by the non-natural amino acid field. Expression plasmids for those orthogonal systems include both the tRNA gene and the species-matched amino acyl tRNA synthetase gene cloned onto a single plasmid. Specifically, the orthogonal systems for suppressor tRNAs that insert Trp and Asp expand on the previous list of suppressor tRNAs used by Dr. Miller (Table 1).

Table 1. tRNA descriptions.

Suppressor tRNAs derived from <i>E. coli</i> that function with <i>E. coli</i> tRNA synthases				
Amino acid inserted	Suppressor tRNA (Gene)	Source	tRNA sequence (EcoRI-tRNA-PstI) ^{a,b,c}	Ref
Ala	<i>tRNA^{Ala2}_{CUA}</i>	synthetic	<u>gaattc</u> ggggctatagctcagctgggagagcgcttgc CTA aagcaagaggtcagcggttcgatcccgcttagctccaccactgcag	[64,66]
Arg	FTOIRΔ26	synthetic	<u>gaattc</u> gccggatagctcagctggatagagcggggatt CTA aatccccgtgtccttgggtcgaatccgagtcgggcaccactgcag	[64,66,87]
Cys	<i>tRNA^{Cys}_{CUA}</i>	synthetic	<u>gaattc</u> ggcgcgtaacaagcggttatgtagcggatt CTA aatccgtctagtcgggttcgactccggaacgcgcctccactgcag	[64–66]
Gln	Su2-89	supE	<u>gaattc</u> tgggggtatcgccaagcggtaaggcaccggatt CTA aatccggcattccgaggttcgaatcctcgtacccagccactgcag	[64,66,88]
Glu/Gln	<i>tRNA^{GluA}_{CUA}</i>	synthetic	<u>gaattc</u> gtcccttcgtctagaggccaggacaccgacct CTA aaggcggtaacaggggttcgaatccctaggggacgctgcag	[64,66]
Gly	<i>tRNA^{Gly1}_{CUA}</i>	synthetic	<u>gaattc</u> cgggcgtagttcaatggtagaacgagagctt CTA aagctctatacaggggttcgattcccttcgccgctccactgcag	[64,66]
His	<i>tRNA^{HisA}_{CUA}</i>	synthetic	<u>gaattc</u> ggtggctatagctcagttggttagagccctggatt CTA aatccagttgctgtgggttcgaatcccattagccacccactgcag	[64,66]
Leu	Su6	supP	<u>gaattc</u> gccgaagtggcgaaatcggtagacgcagttgatt CTA aatcaacgtagaaatcgtgccggttcgagtcggccttcggcaccactgcag	[64,66,89,90]
Phe	<i>tRNA^{Phe}_{CUA}</i>	synthetic	<u>gaattc</u> gccggatagctcagtcggtagagcaggggatt CTA aatccccgtgtccttgggttcgattccgagtcgggcactgcag	[64–66]
Pro	<i>tRNA^{ProH}_{CUA}</i>	synthetic	<u>gaattc</u> ggtgattggcgagcctggtagcgcaggggatt CTA aatccccgggtcggaggttcgaatcctctatcacgcaccactgcag	[64,66]
Ser	Su1	supD	<u>gaattc</u> ggagagatgccggagcggctgaacggaccggtct CTA aaacgggagtaggggcaactctaccgggggttcaaatccccctctctccgccactgcag	[64,66,91]
Tyr	Su3	supF	<u>gaattc</u> ggtggggttcccgagcggccaaagggagcagact CTA aatctgccgtatcgacttcgaaggttcgaatccttccccaccaccactgcag	[64,66,92]
Orthogonal tRNA systems that function in <i>E. coli</i>				
Amino acid inserted	Plasmid Name	Description	Ref	
Trp	pRST11B-AS3_4	Orthogonal yeast tRNA/amino acyl-tRNA synthetase pair	A gift from Andrew Ellington (Addgene plasmid #102789)	
Asp	pTAK-DRSE ^{188K} -YD _{CUA}	Orthogonal yeast tRNA/amino acyl-tRNA synthetase pair	[70]	
Suppressor tRNAs derived from <i>E. coli</i> that function with <i>E. coli</i> tRNA synthases and are reported to insert Lys				
Amino acid inserted	Suppressor tRNA (gene)	Source	tRNA Sequence (EcoRI-tRNA-PstI) ^{a,b,c}	Ref
Lys1	<i>tRNA^{Lys}_{CUA}</i>	synthetic	<u>gaattc</u> gggtcgtagctcagttggttagagcagttgact CTA aatcaattggtcgcaggttcgaatcctgcacgaccaccactgcag	[64,66]
Lys2	<i>tRNA^{mMet}_{CUA}</i> -Lys	synthetic	<u>gaattc</u> ggctacgtagctcagttggttagagcacatcact CTA aatgatggggtcacaggttcgaatcccgctgtagccaccactgcag	[66]
Lys3	<i>tRNA^{Ile2}_{CUA}</i> -Lys	synthetic	<u>gaattc</u> ggccccttagctcagttggttagagcaagcgact CTA aatcgcttggtcgtggttcgaatccagcagggggccaccactgcag	[66]
Lys4	<i>tRNA^{ASPM}_{CUA}</i> -Lys	synthetic	<u>gaattc</u> ggagcggtagttcagtcggttagaataccagact CTA aatctgggggtcgcgggttcgagtccttcgcttcgcgaactgcag	[66]

^a Following the Miller design, a 94 bp synthetic lipoprotein promoter was used for the expression of suppressor tRNAs in combination with a 31 bp synthetic *rrmC* terminator [63]. We inserted a point mutation in a second *NarI* site to prevent fragmentation of the promoter region during genetic manipulations. See the bolded capital-letter position. That promoter sequence (*NarI*-promotor-EcoRI) is GGCGCCGCTTCTTTGAGCGAACGATCAAAAAATAAGTGGGGCCCCATCAAAAAATATTCTCAACATAAAAACTTTGTGTAAT ACTGTAAACGCTGAATTC. Restriction sites are underlined; ^b Terminator sequence (PstI-terminator-HindIII) TGCAGATCCTTAGCGAAAGCTAAGGATTTTTTTAAGCTT; restriction sites are underlined; ^c the “CTA” anticodon recognition site in uppercase and bold. The plasmids that we generated with these tRNA expression clones are available at Addgene.

As a high-level introduction, pyruvate kinase (PYK) was used as a model protein example. In a plasmid encoding this protein, we changed the codon for V177 (GTG) into an amber codon (UAG) using standard mutagenesis practices: For the mutagenic primers, 10 to 15 base pairs before and after the target locus were used. PCR conditions depend on the individual protein/gene of interest, polymerase, and primers; in general, the mutagenic primers are annealed 5 °C below the calculated melting temperature and the sample is amplified for 1 min per kilobase of whole plasmid. After amplification, any non-mutated DNA (i.e., original template) is removed using DpnI digestion. Confirmation of mutagenesis should be performed by single-pass Sanger sequencing of the protein (once from each end of the gene), and it is advisable to perform whole-plasmid sequencing to provide information about the whole plasmid for approximately the same cost as single-pass sequencing. Expression of the PYK protein followed previously reported protocols (5), including inoculation of 750 mLs of LB medium in a 2.0 L flask using 250 µL of an overnight culture, induction with 5 mM lactose added at the time of inoculation, and growth at 37 °C and 225 rpm for 24 h before harvesting of cells.

Collectively, these natural suppressor tRNAs, engineered tRNAs, and orthogonal systems can be used to introduce 15 amino acids at a single amber codon mutation in *E. coli*. Thus, this high-throughput design for obtaining substituted proteins cuts the cost of mutagenesis by up to 19-fold (compared to saturating mutagenesis) and the resulting 15 substitutions per position exceeds the minimum 10 substitutions needed for positional characterization. The amino acids that can be used as substitutions are listed in Figure 3 and Table 1 [62,64–66,70,86–96].

4. Detailed Procedure

4.1. Modifying the *E. coli* Genome in Preparation for Protein Expression

In the non-natural amino acid field that routinely uses amber-based mutagenesis, a historic problem has been reduced protein yields. There are two likely reasons for that reduced yield. The first is the limitation of the non-natural amino acid crossing into the cell and then remaining in the cell without further modification due to cell metabolism. Fortunately, this problem does not affect our plan to incorporate natural amino acids. The second possible contribution to low protein yields is the competitive binding to the mutating-amber codon between a suppressor tRNA and the cellular machinery that causes translational termination at native amber stop codons (i.e., release factor 1). This latter possibility has been addressed in the non-natural amino acid field via engineering an *E. coli* strain that has all amber stop codons replaced with other stop codons and that has the related release factor 1 deleted [97]. The *E. coli* strain that we have used, C321.ΔA.opt, has been further optimized for increased growth rates [98].

Finally, in our study of allosteric systems, we consider that many allosteric proteins are multimeric. Therefore, when expressing a variant protein in an *E. coli* expression system, one concern is that the engineered variant could form hybrid multimers with endogenous encoded *E. coli* homologs. Thus, it is ideal to express target proteins in an *E. coli* for which the homolog gene has been knocked out. A number of advancements facilitate rapid generation of gene deletions in *E. coli*. In our workflow, we delete chromosomal *E. coli* genes from the C321.ΔA.opt strain before expressing targeted proteins from a plasmid system. The general outline for deleting genes follows that previously used to delete pyruvate kinase and phosphofructokinase genes out of the *E. coli* chromosome [41,99,100]. Notably, the C321.ΔA.opt is provided with notes that it should not be grown at temperatures above 34 °C. The gene deletion protocol includes “curing” a selection marker out of the genome using a temperature (37 °C) inducible expression of flp recombinase. By exploring the use

of 34 °C and 36 °C conditions, we found that the curing process in C321.ΔA.opt carrying a knockout cassette was indeed possible.

4.2. Co-Expression Considerations

The amber-based mutagenesis co-expresses a tRNA (and when needed for an orthogonal system, an acyl tRNA synthetase) using one plasmid with a second plasmid expressing the target protein of interest that contains an amber stop codon mutation. Co-transformation of *E. coli* with two plasmids requires that the two plasmids use different origins of replication (“ori”) and two different antibiotic resistance selection markers. Several commercial plasmids have been optimized for co-transformation and co-expression and may be useful to applications of co-expression of genes and suppressor tRNAs. The suppressor tRNA plasmid set that we have created, including the lipoprotein promoter and the synthetic rrnc terminator sequence (see Table 1 for sequences), have been cloned into pUC57 (ColE1 ori and AmpR antibiotic resistance) using the NarI and HindIII restriction sites. The lipoprotein promoter was selected to express the suppressor tRNAs because it is a strong, constitutive *E. coli* promoter, requiring no external additive for induction. Constructs with this promoter will be compatible with any induction system for the target protein. The kinetics of expression and amber suppression of a targeted protein of interest will depend only on its own induction. Thus, existing expression protocols (as dictated by targeted protein expression system) will not require any modifications for use with our amber suppression technique. The example data for amber-based mutation of human liver pyruvate kinase (LPYK) included the l-pyk cDNA gene in the pCDFDuet plasmid. This second plasmid includes the CloDF13 ori and Streptomycin antibiotic resistance, facilitating the co-expression with pUC57-based plasmids. As expected, control experiments, using *E. coli* transformed with only the amber-mutated expression plasmid did not express full-length protein. In contrast, most examples co-expressing one of the originally designed tRNA and the gene of interest showed strong expression based on SDS-PAGE.

In addition to these considerations, it is conceivable that the cell machinery responsible for initiating plasmid replication (i.e., interacting and functioning with an ori) could involve proteins that use amber stop codons. Therefore, readthrough translation during the synthesis of those ori-related proteins in the presence of a suppressor tRNA could result in lethality. This is one potential problem when co-transformation of both plasmids is attempted in *E. coli* strains that have not been modified as in the C321.ΔA.opt strain. This provides another rationale for using the C321.ΔA.opt strain in this design. C321.ΔA.opt is a highly preferred strain to use for the co-transformation and expression protocol. However, one disadvantage of both C321.ΔA.opt and its parent strain C321.ΔA is a slower growth phenotype [101] and lower growth temperature 30 °C [97,98,102]. In our hands, this lower temperature limit reduced the transformation efficiency and protein expression from these strains. In recent years, improved strains have been developed: C321.ΔA.exp (Addgene 49018), which can be grown at 37 °C, and C321.ΔA.exp.T7, which contains the machinery from BL21(DE3) cells to express proteins from T7 promoters [103].

4.3. High-Throughput Protein Purification and Characterization

Although we have no doubt that the amber-based mutagenesis will be useful to a range of studies, each with their own form of data collection, we express our substituted proteins in *E. coli*, purify them using cleavable purification tags, and collect enzymatic and/or binding data using our robust and validated robotic protein production pipeline (Figure 4) [104–107]. Our robotic system overcomes the technical challenge associated with generating the very large data sets that are needed for the positional mapping approaches (see Figure 4). Although our system can work with a range of vectors, affinity

tags, fusion proteins, helper plasmids, chaperones, temperature, shaker speed, affinity resin, and lysis buffer variables [108], one key variable that must be optimized for each target protein is the volume of culture necessary to purify enough of each protein to complete the study. The ability of our system to accommodate 384, 96 and 24 deep-well plates growth/expression/purification pipelines provide the needed flexibility for this optimization. Using this system, up to 2304 or 576 proteins (depending on the use of 96 or 24 deep-well plates for expression) can be purified and enzymatically analyzed per week [104,105].

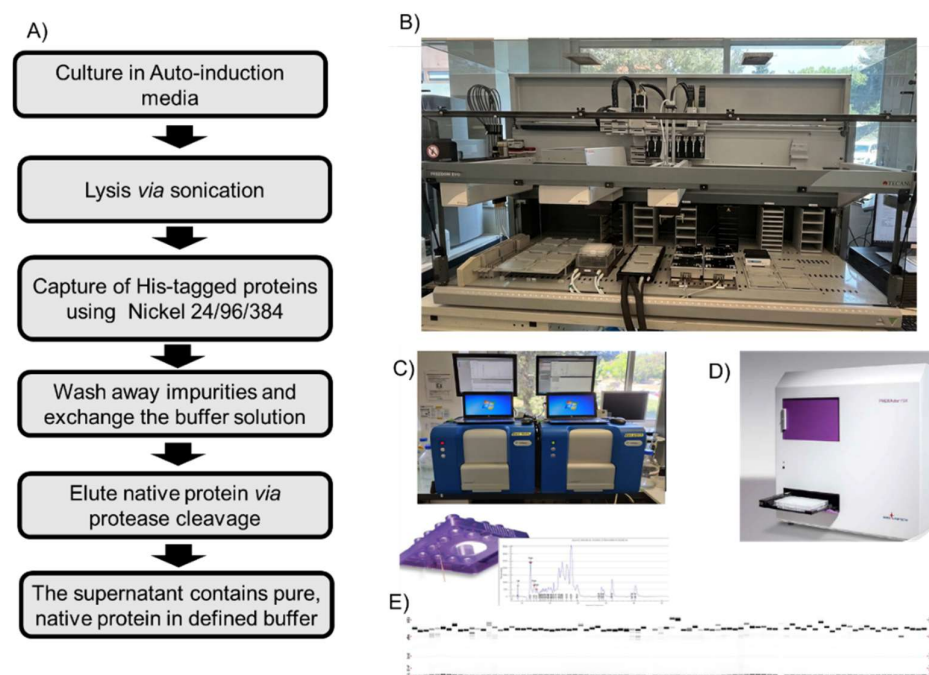


Figure 4. High-throughput automated protein purification and characterization. Protein purification follows an optimized plate-based workflow that produces highly pure native target proteins (i.e., no fusion domain) in 96 deep-well plates. (A) Representative steps in the protein purification protocol. (B) Purification is performed using a Tecan Evo liquid handling robot equipped with a 96-tip head, a span-8 $\times$ 1 mL pipet, and vacuum manifold. (C) Protein purity, molecular weight, and concentrations were determined on our two LabChip GXII (Perkin Elmer, Waltham, MA, USA). (D) Activity data were collected on our Pherastar (BMG LabTech, Baden-Wuerttemberg, Germany) plate reader equipped with a plate hotel. (E) Elution of the 96 purifications of a library.

In addition to optimization of the culture volume, many other variables can be explored to improve the yield of suppressed protein. These other variables include many of the items listed as compatible with our system (affinity tags, fusion proteins, helper plasmids, chaperones, temperature, shaker speed) as well as others (growth time, cell density (OD) at induction, and harvest OD). As the optimal conditions will depend on the target protein, optimization requires an iterative approach that is guided by relevant literature. Some general advice for increasing overall protein yield includes: (i) expression during early log-phase growth so that the cells are primed for protein production, (ii) harvesting at the end of log-phase growth before cells have depleted the nutrients in the media, and (iii) keeping the culture volume at 20% to 50% of the max container volume to ensure oxygen diffusion.

In addition, it may be useful to purify using C-terminal purification tags when possible. Although extensive efforts have been made throughout the literature to characterize the amino acid type inserted at an amber codon due to individual tRNAs, careful studies of the percentage of proteins that incorporate those amino acids compared to proteins that

are truncated are not always as fully characterized for all tRNAs. By purifying with a C-terminus purification tag, an intrinsic control is included so that only full-length (i.e., not truncated) proteins are further characterized.

5. Expected Results

Positional Classifications: A Method for Aggregating Outcome Scores from a Range of Substitutions at Each Position

An important aspect of studies that generate many substitutions at each position of a protein is the need to aggregate outcomes from individual substituted proteins to a limited number of “scores” for the position [51]. In the study of allosteric systems, it is primarily the allosteric coupling constant (Q_{ax} ; Equation (1)) that reflects the function of interest, but composite scores for positional contributions to substrate affinity and effector binding are also valuable. In our goal of determining whether allosteric mechanisms are conserved, these composite scores are extremely useful because the composite scores for the Q_{ax} parameter for various homologs can be mapped to their respective structures, thus allowing a visual comparison of how individual positions contribute to allosteric function among homologs. Independent of our own focus on allosteric regulation, composite scoring for any quantified protein function is based on recognizing three types of positions (Figure 5):

- A **neutral** position is one for which all substitutions retain wild-type-like function [53].
- A **toggle** position is one at which a few substitutions, with similar side-chain chemistry, may support wild-type function, but all other substitutions abolish function [16,109]. Toggle outcomes are associated with traditional textbook expectations, which were largely derived from substitutions at conserved positions.
- A **rheostat** position is one at which each type of substitution results in a unique change in the function being evaluated [51]. When a set of substitutions is compared at an individual rheostat position, their plot shows a continuum of functional responses. (Figure 5; notably, the function that is “better” than that of the wild type is frequently observed at these positions [10,51,52,54].)

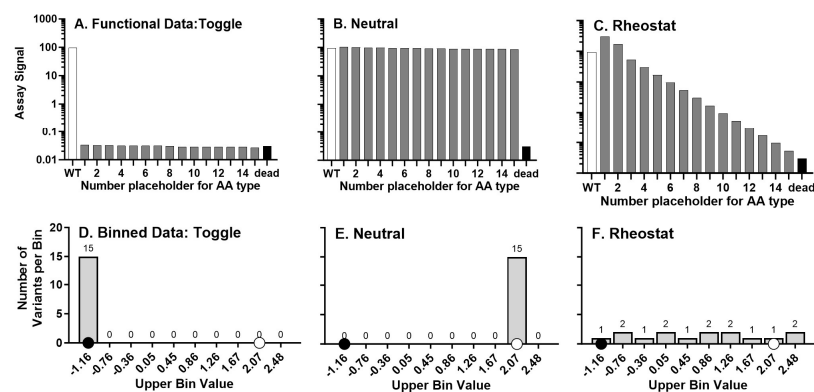


Figure 5. Synthetic data demonstrate how outcomes from multiple substitutions (A–C) made at one position for either (A,D) a toggle, (B,E) a neutral or (C,F) a rheostat position are represented in histogram bins (D–F). The occupancies of bins are used to derive the aggregate toggle, neutral, and rheostat scores for any one residue position evaluated. This image, for which the authors maintain copyright, was adapted and reproduced from [10].

To aggregate scores from all of a position’s substitutions and determine which of the three classifications best describes the role of that position, we have developed a histogram/binning-based analysis (Figure 5). Details of the histogram analysis have previously been described [51]. In brief, the toggle score reflects the percent of substitutions

that abolish function. A neutral score reflects how many substitutions exhibit wild-type-like function. The rheostat score reflects what fraction of bins are populated by any substitution. All three scores have associated cutoff thresholds to discriminate “high” vs. “low” scores [10,51,53]. As an example, positions with a high rheostat score but low toggle and low neutral scores are considered to be rheostat positions. Example data from LPYK and the accompanying toggle, neutral, and rheostat scores are in Figure 6.

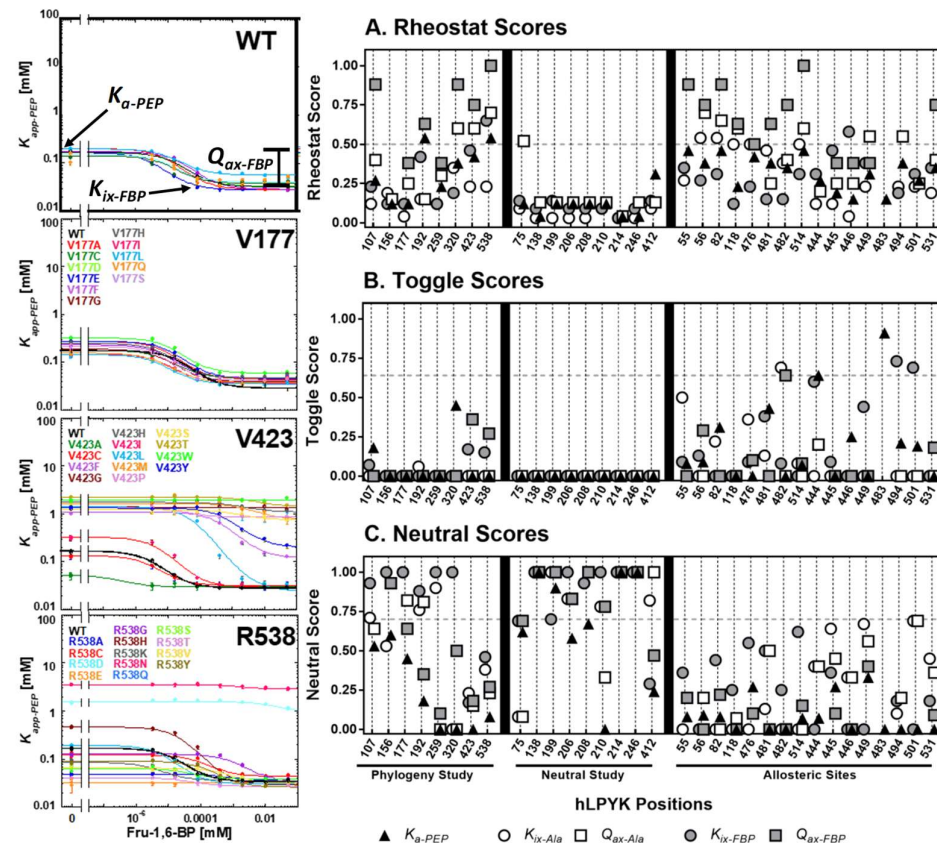


Figure 6. (Left) Examples of primary data for semi-saturated mutagenesis at individual positions in human LPYK, which are compared to repeatedly measured wild-type LPYK data to determine error (top panel). In subsequent panels, each line color represents a different substitution at the indicated position. For any one function (e.g., Q_{ax-Ala}), data for a range of substitutions at any one position, such as the examples shown here, can be evaluated using three composite scores. (Right) (A) The rheostat composite scores for five different functions (K_{ia-PEP} , K_{ix-FBP} , Q_{ax-FBP} , K_{ix-Ala} , and Q_{ax-Ala} ; defined in Figure 1 and Equation (1)) and using outcomes from semi-saturated mutagenesis at 33 different positions in LPYK. The horizontal dashed line is an established cutoff value; scores above this line indicate rheostat positions [10,49,51,53]. (B) The toggle composite scores for the same 33 positions. Values above the horizontal dashed line indicate toggle positions. (C) The neutral composite scores for the same 33 positions. Values above the horizontal dashed line indicate neutral contributions to any one function. Although a position can make different classifications of contributions to different functions (e.g., toggle to K_{ia-PEP} , but rheostat to Q_{ax-FBP}), the position must be neutral for all functions for the position to be classified as a neutral position. Determining the rheostat/toggle/neutral contributions to allosteric coupling and mapping those positional classifications on the structures of two homologs is one potential mechanism to evaluate conservation of allosteric mechanisms. Data for panels (A–C) were taken from [10,49,51,53,55,110–114]. A modified version of this figure was first published in [19].

6. Conclusions

There are growing lists of questions that benefit from knowledge gained by saturated or semi-saturated mutagenesis. Although many deep mutational scanning data sets have

been reported, the data produced by those designs can be challenging to interpret [61]. Therefore, the growing list of questions in the protein field stands in need of improved, cost-efficient, high-throughput mutagenesis designs that can easily be paired with high-throughput protein purification and characterization technologies. One example that highlights the need for high-throughput semi-saturated mutagenesis is our effort to develop methods capable of distinguishing the contribution patterns of individual positions to allosteric functions and then using the patterns to classify each position as toggle, neutral, or rheostat positions.

Many will likely find it surprising that much of the knowledge needed to facilitate sufficient throughput has been available for some time. Using the available information, we herein outline the combination of advancements from several fields that can generate biochemical characterization data for tens of thousands of substituted proteins. Thus, this system can generate extensive datasets that are needed to answer many protein structure/function questions, including our own question about whether allosteric mechanisms are conserved in homologs.

In addition to being useful for reaching designed goals, the proposed workflow will generate a database of substitution outcomes that is needed to train and validate computational analyses and predictive strategies.

Plasmids deposited in Addgene: pAla57 (211556), pArg57 (211557), pCys57 (211558), and pGln57 (211559), pGlu57 (211560), pGly57 (211561), pHis57 (211562), pLeu57 (211563), pLys57-1 (211564), pLys57-2 (211565), pLys57-3 (211566), pLys57-4 (211567), pPhe57 (211568), pPro57 (211569), and pSer57 (211570).

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Conflicts of Interest: Author Pierce T. O’Neil was employed by company Ceva Animal Health. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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