

Whole plasmid mutagenic PCR for directed protein evolution

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Abstract

Protein function can be engineered through iterated cycles of random mutagenesis and screening (directed evolution). Optimization of protein expression is essential for the development of sensitive and precise high throughput assays. Here we optimize the performance of a plasmid-borne *Escherichia coli lacZ* gene in two rounds of directed evolution. First, its promoter was “randomized” by whole plasmid polymerase chain reaction (PCR) and intra-molecular self-ligation. A genetically stable constitutive expression vector was isolated in an *in vivo* genetic selection. Second, the entire plasmid was randomly mutated in a slightly mutagenic long polymerase chain reaction. The PCR products were digested with a restriction enzyme, self-ligated by T4 DNA ligase and transformed into *E. coli*. The resulting library of beta-galactosidase (β -gal) mutants consisted mostly (~80%) of hypomorphs, suggesting that the mutation rate was appropriate for directed evolution applications. We isolated and characterized 14 variants with increased activity in reactions with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). The purified protein derived from one clone exhibited a 100-fold improvement in k_{cat} over its parent in reactions with *para*-nitrophenyl-beta-D-galactopyranoside (pNP-gal). This latter result clearly demonstrates the utility of whole plasmid mutagenic PCR for directed protein evolution.

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1. Introduction

Protein function can be optimized through directed evolution [12,15,29,31,34,37]. In its simplest form, random mutations are introduced into protein-coding genes by mutagenic polymerase chain reaction (PCR). The resulting library is cloned into an expression vector, and expressed in a microbial population. Clones with improvements in the desired properties are identified in high throughput screens. Protein function is further optimized through iterative cycles of random mutagenesis and screening. The outcome of a directed evolution experiment is primarily dependent upon the sensitivity, precision, dynamic range and throughput of the screen, and secondarily upon the quality of the library.

The optimization of heterologous gene expression levels is essential for directed evolution, because each parameter of the high throughput screen is affected by this variable. High

expression of the gene usually corresponds to high sensitivity, and is therefore essential for the directed evolution of properties that are weakly exhibited by the wild-type protein. Constitutive expression systems are potentially more efficient and convenient than inducible systems, at least for proteins that are stable and non-toxic. High levels of transcription, however, particularly of genes on plasmid expression vectors, can lead to genetic instability [2,41]. In the context of a high throughput screen, instability means phenotypic variation among clones (colonies or liquid micro-cultures) within an isogenic population. Excessive gene expression can also narrow the dynamic range of the high throughput assay, precluding further improvement beyond the first few rounds of evolution. This problem is particularly common for *in vivo* selection systems.

The optimization of gene expression is generally accomplished through extensive trial and error of multiple expression vectors, strains and growth/induction conditions. The sub-cloning steps generally cannot be done in parallel because different expression vectors utilize

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different multiple cloning sites. Here we demonstrate the utility of whole plasmid PCR for the optimization of gene expression and protein function. We employ the *Escherichia coli* beta-galactosidase (β -gal) as a model enzyme because its structure has been solved [19,21], and it is amenable to high throughput screening [43]. An expression vector constructed by conventional means proved genetically unstable. We directed the evolution of this “ancestral” vector into a stable and efficient constitutive *lacZ* expression vector in two rounds of mutagenesis and screening.

2. Materials and methods

2.1. Materials

The pBAD myc his *lacZ* vector was purchased from Invitrogen (Carlsbad, CA). 6his-*lacZ*-pET28 (induction control E) was from Novagen (Madison, WI), and *E. coli* strain DH5 Δ *lac* (DE3) was previously described [24]. *E. coli* strain W2244 (*lacZ39*, *rpsL110*), which contains a deletion of \sim 1/3 of its chromosomal *lacZ* gene, was from the *E. coli* Genetic Stock Center (New Haven, CT). The GeneAmp XL PCR kit was from Perkin-Elmer (Boston, MA). Molecular biology enzymes, including restriction enzymes, T4 DNA ligase and T4 DNA polymerase, were from New England Biolabs (Beverly, MA). Oligonucleotides were from IDT (Coralville, IA). All other chemicals including, M9 salts, ampicillin, lactulose, *para*-nitrophenyl-beta-D-galactopyranoside (pNP-gal) were from Sigma–Aldrich (St. Louis, MO).

2.2. Ancestral *lacZ* expression vector

The *lacZ* variant from pBAD myc his *lacZ* was subcloned downstream of the *lac* promoter in two steps. First, the *lacZ* gene was subcloned into pET28 (Novagen) using restriction enzymes *Nco* I and *Hind* III via standard cloning techniques [33]. Second, the wild-type *lac* promoter was from pBS KS+ (Stratagene) was PCR amplified and subcloned in place of the T7 promoter in pET20 using *Afl* III and *Xba* I (Novagen). Thus, the resulting “ancestral” P_{lac}-*lacZ*-pET20 plasmid

has a wild-type *lac* promoter upstream of the variant *lacZ* gene (Table 1).

2.3. Mutagenesis of *lac* promoter

We “randomized” the -10 region of the *lac* promoter by PCR amplification of the entire P_{lac}-pET20 plasmid using Vent polymerase and primers complementary to the *lac* promoter itself as previously described [26]. The nucleotides encoding the conserved -10 region of the promoter were degenerate (approximately equimolar mixtures of G, A, T and C). The PCR product was purified, phosphorylated with T4 Polynucleotide Kinase and blunt-end self-ligated (as described below). *E. coli* were transformed and propagated, and the resulting plasmid-borne library was purified. The *lacZ* gene was subcloned downstream of the randomized *lac* promoter using restriction enzymes *Xba* I and *Hind* III.

2.4. Whole plasmid mutagenic PCR and intra-molecular ligation

The long PCR reactions included: 50 ng of the ancestral *lacZ* expression vector, 500 nM primers (5'-GGGAAGCTT-GCGGCCGCACTCGAGCAC-3' and 5'-CCCGGGATCCT-TAAAGCTTTTTTTGACACCAGACCAACTGGTAATG-3'; these primers were designed to anneal to the 3' end of *lacZ* and to the region directly downstream, “back-to-back” in opposite directions), 200 nM of each dNTP, 1 \times ABI XL buffer II, and 0.8, 1.2 or 1.5 mM magnesium acetate. The 50 μ L reactions were overlaid with light mineral oil and heated to 80 $^{\circ}$ C in a thermal cycler for a “hot start”. 0.5 μ L of the Tth/Vent mixture (1 unit) was added, and the temperatures were raised to 94 $^{\circ}$ C for 1 min, followed by 25 cycles of 94 $^{\circ}$ C \times 15 s, 68 $^{\circ}$ C \times 6 min (1 min/kb). The reaction was further incubated at 72 $^{\circ}$ C for 10 min, and stored at 4 $^{\circ}$ C.

The mineral oil was removed, and the PCR reaction was incubated with 0.5% sodium dodecylsulfate and 50 μ g/mL proteinase K at 65 $^{\circ}$ C for 15 min to eliminate the thermostable polymerases. The PCR product was purified using the Promega Wizard PCR prep kit (Madison, WI) as directed by the manufacturer, with the final elution in 50 μ L water. The DNA was digested with restriction enzymes *Hind*

Table 1
Sequences of peptides fused to *lacZ* variants

	N-terminus	Internal <i>lacZ</i> seq	C-terminus
Wild-type	TMITDSLAVVL...	Wild-type	... <u>CQK</u> stop
<i>lacZ</i> -pET28 (Novagen)	MGSSHHHHHHSSGLVPRGSHMASMTGG-QQMGRDPVVL...	Wild-type	... <u>CQK</u> stop
pBAD myc his <i>lacZ</i> (Invitrogen)	MDPLVTAASVLEFGLFETMIDPVVL...	K774N	... <u>CQK</u> AAASRILQISITLAAARDL-QLVPYGNKSLGPEQKLISEEDLNS-AVDHHHHHH stop
“ancestral”/clone 1.1 (this study)	MDPLVTAASVLEFGLFETMIDPVVL...	K774N	... <u>CQK</u> AAASRILQISITLAAARDLQLPV-YGNKSLAAALEHHHHHH stop

III and *Dpn* I (to eliminate methylated parental template). The sticky-ended PCR product was gel purified using a Qiaquick spin column as directed by the manufacturer (Qiagen, Valencia, CA). The concentration of eluted DNA was estimated via agarose gel electrophoresis.

For intra-molecular self-ligation of sticky-ended DNA, we recommend low concentrations of DNA and moderate concentrations of T4 DNA ligase. Four femtomoles of purified PCR product was reacted with one Weiss unit T4 DNA ligase in a 20 μ L reaction containing 50 μ M each dNTP, 1 mM ATP, 50 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM DTT, at 16 °C for 1 h. For blunt-ended recircularization, such as the one used to “randomize” the *lac* promoter, we employ an additional 5 units of T4 DNA polymerase, 200 nM each dNTP (to insure blunt-ended DNA), 6 total Weiss units of T4 DNA ligase and 25 μ M bovine serum albumin, with a 1 h incubation at 23 °C. The T4 DNA ligase was heat-killed at 65 °C for 10 min, the DNA was butanol precipitated [40], and electroporated into freshly prepared *E. coli* Inv α F' cells as described by Dower et al. [10].

2.5. Characterization

Clones 1.1 and 2.38 were sequenced by the Applied Biosystems Big Dye protocol, using the following primers (5'–3'):

rev -270	TAACCGTATTACCGCCTTTGAGTGAGC
rev -80	ATGCTTCCGGCTCGTATGTTGTGTGG
<i>lacZ</i> 377	AATCCGACGGGTGTTACTCGCTCAC
<i>lacZ</i> 770	GAGTTGCGTGACTACCTACGGGTAAC
<i>lacZ</i> 1154	CTGAACGGCAAGCCGTTGCTGATTC
<i>lacZ</i> 1536	CGCGTGGATGAAGACCAGCCCTTC
<i>lacZ</i> 1920	CGGGCAAACCATCGAAGTGACCAGC
<i>lacZ</i> 2269	CATCGAGCTGGGTAATAAGCGTTGGC
<i>lacZ</i> 2679	TGCCAGCTGGCGCAGGTAGCAGAG

The hexahistidine-tagged β -gal proteins were purified to homogeneity by nickel chelate chromatography by a procedure similar to that developed for beta-glucuronidase [27,32]. The proteins were quantified by the Bradford protein assay (Bio-Rad, Hercules, CA), and reacted with varying concentrations of pNP-gal; the steady-state kinetic parameters were determined by fitting the data to the Michaelis–Menten equation.

3. Results

Our goal is to streamline the development of high throughput assays, and the process of directed evolution in general. Here we demonstrate the utility of whole circle PCR by optimizing the performance of the *E. coli lacZ* model system. The wild-type *lac* operon was optimized by natural selection, but directed evolution can further enhance the performance of enzymes above the levels required for the

survival of the organism [14]. The *lac* promoter and a commercially available *lacZ* variant (from pBAD myc his *lacZ*, described below) were cloned in tandem into a pBR322-based plasmid. Expression from this multi-copy vector, hereafter called the “ancestral” vector, in *E. coli* strain W2244 (*lacZ*⁻, *lacY*⁺) proved genetically unstable (data not shown). Apparently, cells that inherit fewer copies of plasmid have a selective growth advantage over those burdened with high copy numbers [20].

The level of *lacZ* transcription within W2244 *E. coli* cells, and the function of the β -gal protein, were optimized in two rounds of random mutagenesis and screening (Fig. 1). We first “randomized” the conserved –10 region of the *lac* promoter by site-saturation mutagenesis [26]. The *lacZ* variant from the commercially available pBAD myc his *lacZ* vector (Invitrogen) was subcloned downstream of the library of randomized *lac* promoters. This *lacZ* variant was chosen because it contained convenient restriction sites compatible with our expression vector; the restriction site at the 3' end (*Hind* III) enabled fusion to a C-terminal six histidine tag encoded in the vector. The non-native sequences at the ends of the *lacZ* gene encode 21 and 46 amino acid extensions of the N- and C-termini, respectively (Table 1); we were initially unconcerned because β -gal has been shown to accommodate fusions at either end [6].

E. coli W2244 (*lacZ*⁻, *lacY*⁺) cells were transformed with the library of promoter variants and spread onto M9

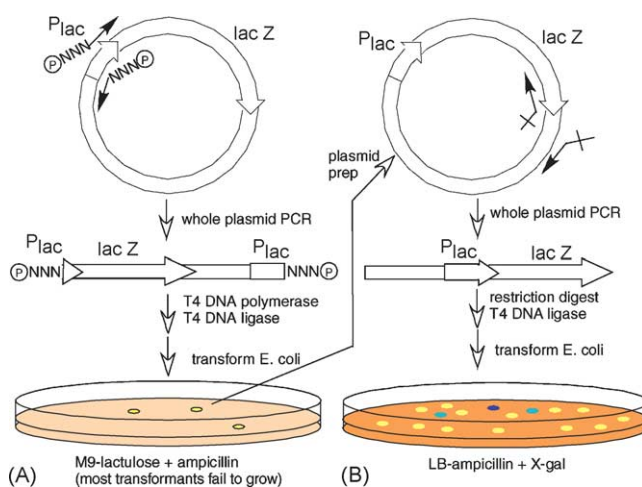


Fig. 1. Two variations of whole plasmid PCR. (A) “Back-to-back” mutagenic primers complementary to the *lac* promoter were used to PCR amplify the entire ancestral expression vector. The ends of the resulting linear PCR products were degenerate in sequence; intra-molecular blunt-end ligation produced a library of *lac* promoters “randomized” at the –10 region. A constitutive and genetically stable *lacZ* expression vector (plasmid 1.1) was identified by its ability to complement an auxotrophic (*lacZ*⁻) *E. coli* strain. (B) A different pair of “back-to-back” primers complementary to the 3' end of *lacZ* was used to PCR amplify plasmid 1.1. The resulting PCR products contained random point mutations, and the ends contained a unique restriction site. Sticky ends were created with a restriction enzyme, and the plasmids were self-ligated. The auxotrophic *E. coli* strain was transformed with the new library, and hypermorphic *lacZ* alleles were identified on X-gal plates.

minimal media agar plates containing ampicillin and lactulose as a sole carbon source. A colorimetric high throughput assay (such as the ones described below) could have been employed at this step, since the total number of sequence variants should be $4^6 = 4096$. Clones that formed colonies under these conditions constitutively expressed β -gal at moderate levels. One clone that consistently formed colonies under selective conditions after several rounds of serial passage in LB-ampicillin (LB-amp) medium was designated clone 1.1 (round one, clone one). The plasmid from clone 1.1 was purified and utilized as a template for the next round of evolution. The DNA sequence of the lac promoter of clone 1.1 was identical to that of the wild-type, except that the -10 region had changed from TATAAG (ancestor) to AAAAGG.

A variation of mutagenic PCR [3,4,25] was utilized to introduce random mutations throughout the ancestral *lacZ* expression vector. A slightly mutagenic long PCR protocol using the thermostable Tth and Vent polymerases [5] was employed to amplify the entire expression plasmid. We chose this polymerase mixture because the reported mutation rate, 1/700 nucleotides [39], is appropriate for the 3 kb *lacZ* gene [28]. The virtue of “whole plasmid mutagenic PCR” is that it obviates the subcloning of the mutant library back into the expression vector. In short, “back-to-back” (non-overlapping) oligonucleotides with *Hind* III sites at the 5' ends were used to amplify the ancestral expression vector. The PCR product was separated from the template by *Dpn* I restriction digestion, which fragmented the methylated template DNA, and gel purification. The ends of the PCR product were cut with restriction enzyme *Hind* III and self-ligated by T4 DNA ligase. The re-circularized plasmids were electroporated into *E. coli* producing 2,000,000 transformants ($6 \times 10^6 \mu\text{g}^{-1}$). We sequenced a total of 8500 bases from 11 randomly selected clones and identified 8 mutations; this suggests that the mutation rate in our hands was similar to the published value.

We spread $\sim 70,000$ *E. coli* transformants on LB-amp agar plates and propagated them for 16 h at 37 °C. The colonies were adsorbed to nitrocellulose filters and transferred (colony side up) to a second LB-amp agar plate containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Most ($\sim 80\%$) of the colonies exhibited less β -gal activity than control colonies carrying the ancestral *lacZ* expression vector. The distribution of mutations among alleles should follow the Poisson distribution [3]; 20–30% of all possible single mutations are silent [29], so it is likely that most of the *lacZ* alleles contained multiple mutations. The remainder ($\sim 20\%$) of the colonies turned dark blue at approximately the same rate as the ancestral control colonies (~ 30 min). Blue halos formed around some ($\sim 0.1\%$) of the colonies. We picked and re-streaked 38 of these on fresh LB-amp plates. All retained their hypermorphic phenotypes in the filter-lift assay, but the most hypermorphic colonies tended to produce heterogeneous mixtures of large and small colonies.

The improvements in fitness were measured in a microplate assay, and in one instance (described below) by kinetic characterization of purified protein. The 13 clones that exhibited the most activity and stability in the X-gal filter-lift screen, the parental clone (1.1), and a *lacZ*⁻ (empty vector) control, were propagated to saturation in parallel in liquid LB-ampicillin. Each culture was reacted separately (in triplicate) with 0.5 mM *para*-nitrophenyl-beta-D-galactopyranoside. The selected clones exhibited 8–36-fold more activity than the parental clone 1.1 (Fig. 2). The clones that formed the most pronounced halos when reacted with X-gal exhibited the most activity in reactions with pNP-gal (data not shown).

Clone 2.38 (round 2, clone 38) exhibited the most consistency between replicate cultures (Fig. 2, error bars), so it was more thoroughly studied than the others. The plasmid rescued W2244 cells in M9 minimal medium containing lactulose as a sole carbon source, but the transformants grew at the same rate as the parental clone (1.1, data not shown). This result simply shows that the dynamic range of the in vivo selection is less than those of the X-gal and pNP-gal screens. We sequenced the ancestral and clone 2.38 *lacZ* genes and identified three mutations, each encoding an amino acid change. K774N was found in both clone 2.38 and the ancestor (derived from the commercially available pBAD myc his *lacZ*). Two clone 2.38-specific mutations, V5L and E17N were identified, both occurred in the synthetic 25 amino acid N-terminus (Table 1). The entire lac promoter of clone 2.38 was identical to that of clone 1.1, suggesting that the two mutations in the N-terminus account for the improvement in activity.

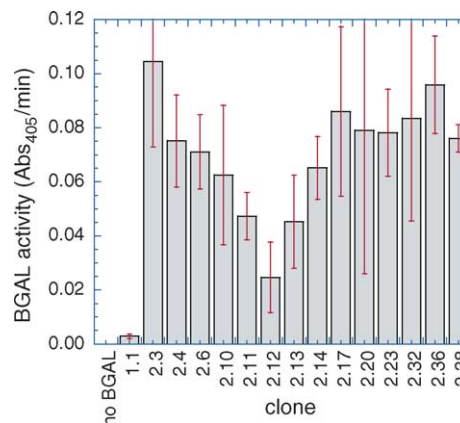


Fig. 2. Beta-galactosidase (β -gal) activities of selected clones. *E. coli* W2244 (*lacZ*⁻, *lacY*⁺) expressing sequence variants of β -gal were propagated in triplicate to saturation in liquid LB-ampicillin. The cultures (2 μL of each mutant culture or 20 μL of the clone 1.1 and β -gal-deficient controls) were reacted with 0.5 mM *para*-nitrophenyl-beta-D-galactopyranoside (pNP-gal) in 300 μL 50 mM Tris buffer, pH 7.6, 10 mM MgCl_2 for 90 min at 25 °C. The formation of the *para*-nitrophenol product was followed at 405 nm, and the rates of change (corrected for volume) are presented. The error bars (standard error) reflect the genetic instability of each expression vector.

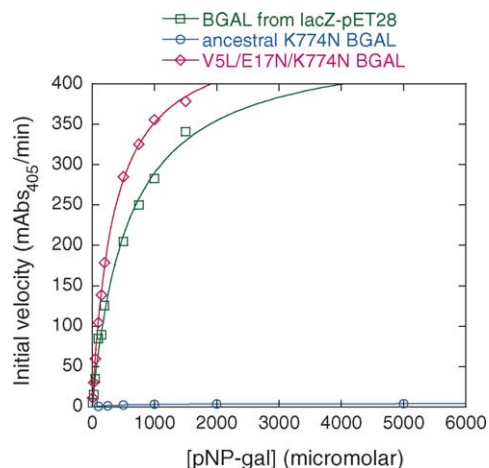


Fig. 3. Michaelis–Menten plots β -gal variants. One nanomolar purified 6his- β -gal (from *lacZ*-pET28a+), K774 β -gal-6his (clone 1.1, originally derived from pBAD myc his *lacZ*) and the clone 2.38 (V5L/E17N/K774) β -gal-6his were separately reacted with varying concentrations of pNP-gal in 50 mM sodium phosphate, pH 7.6, 10 mM MgCl₂. The formation of *para*-nitrophenol was monitored in a spectrophotometer (405 nm). The initial velocities are shown here; the lines represent fits to the Michaelis–Menten equation.

The “ancestral”/clone 1.1 (K774N) and clone 2.38 (V5L/E17N/K774N) β -gal proteins were purified in order to determine whether the two N-terminal mutations affected the properties of the enzyme. The k_{cat} of clone 2.38 in reactions with pNP-gal (33 s⁻¹) was \sim 100-fold higher than the comparable ancestral value (0.31 s⁻¹). A commercially available 6his- β -gal variant (Novagen, induction control E) exhibited catalytic activity similar to that of clone 2.38 (Fig. 3); the k_{cat} values for the reactions of both clone 2.38 and 6his- β -gal are close to (\sim 30%) the reported value for the wild-type *lacZ* enzyme [23]. Together these results suggest that the synthetic 25 amino acids fused to the N-terminus of the ancestral β -gal are disabling, and that mutation of one or two particular residues restores activity.

4. Discussion

4.1. Adaptive mechanism

We superimposed the selected mutations upon the published co-crystal of β -gal bound to the pNP-gal substrate [21]. The K774N mutation, which was present in the ancestor, clone 2.38 and presumably all of the other selected clones, is remote from the active-site (>45 Å, not shown); it is unclear whether this mutation is beneficial or neutral in effect. The two unique mutations in the N-terminus of clone 2.38 are also distant from the active-site, suggesting that improvement in k_{cat} is due to some change in overall conformation. The N-terminus of the wild-type β -gal is part of the “activating” subunit interface [19]. We speculate that

the synthetic N-terminus of the β -gal derived from pBAD myc his *lacZ* partially interferes with subunit association; the V5L and/or E17N mutations somehow alleviate this interference (Fig. 4).

4.2. Whole plasmid PCR

We have found in this and other experiments in our laboratories, that whole plasmid mutagenic PCR and self-ligation is less labor intensive than the more conventional procedure that we described previously [25]. The traditional approach is to generate molecular diversity in mutagenic PCRs [3,4] and to utilize T4 DNA ligase to clone the amplification products with ends that are blunt [18] or “sticky” [22,33]. Cloning efficiency is primarily determined by the uniformity (or raggedness) of the ends of the DNA, and secondarily by the concentration of DNA, the concentration of enzyme, polyethylene glycol, reaction temperature and time. The rigorous removal of the thermostable polymerase prior to restriction digestion [42] is helpful, but some restriction endonucleases do not reliably produce ligatable ends in our hands, particularly when the restriction sites are close to the ends of the DNA.

The intra-molecular self-ligation of whole plasmid PCR products is inherently more efficient and easier to control than inter-molecular ligation reactions [7]. PCR amplification of entire plasmids using primers containing mismatches at particular loci is commonly used for site-directed mutagenesis [8,9,16,17], and site-saturation mutagenesis [11,30,38]. At least one kit (ExSite, Stratagene, La Jolla, CA) is available for these purposes, but we are unaware of previous studies that exploited mutations that were not encoded in the primers. We utilize a mixture of Tth and Vent DNA polymerases [5] instead of Pfu to randomly mutate and recombine genes for directed evolution. In our hands, the Tth/Vent polymerase mixture amplifies product more reliably and is more mutagenic [39] than Pfu DNA pol alone.

The methods described here could be applied to optimize the performance of almost any expression vector or protein. Whole plasmid mutagenic PCR can introduce beneficial mutations into any part of the plasmid. We do not consider this feature disadvantageous, as the possible occurrence of such mutations did not preclude the improvement of beta-galactosidase in this study. The whole plasmid mutagenic PCR protocol offers a versatile and rapid way to generate libraries. If higher mutation rates are desired, the fidelity of Tth DNA polymerase could likely be further decreased by the addition of up to 0.5 mM manganese chloride to the PCR reaction [1]. We [13] and others [35] have also found that long PCR amplification of mixed alleles using the Tth and Vent polymerases leads to the random recombination of point mutations. This template switching process offers the advantages of DNA shuffling [36] and StEP [44], albeit at a lower rate.

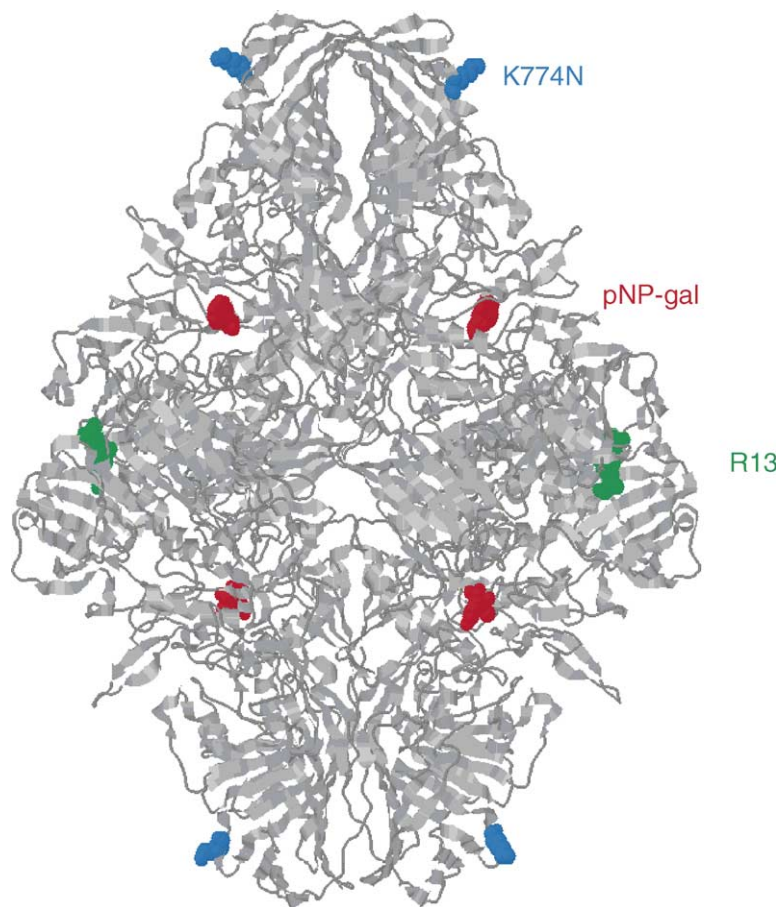


Fig. 4. Mapping the mutations in β -gal variant 2.38. The structure of the β -gal tetramer is shown in gray [21]. The pNP-gal substrate (red) illustrates the active-site. The K774N mutation, which is present in the commercially available pBAD myc his *lacZ* (and our “ancestral” vector), is colored blue. The N-terminus (R13, green) represents the start of the “activating” subunit interface. The beneficial mutations in variant 2.38 occur in a synthetic peptide upstream of R13 (Table 1).

Acknowledgements

LR sequenced the DNA, purified the proteins and measured their enzyme activities in vitro. Both LR and IM were supported by NSF BIO/MCB (#0109668); the FAME Center is supported by NSF/DBI (#0320786).

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