

Structure-guided molecular cloning for improving site-directed mutagenesis and stability in protein design

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Abstract

Protein stability is essential when optimizing the expression, purification, and formulation conditions for protein functional studies. Unstable proteins are often co-expressed with chaperones or fused to affinity tags to improve solubility and expression. These approaches are ineffective when constructs are prone to degradation or thermodynamic instability. In these cases, established protocols suggest removing protease sites or introducing mutations that improve thermodynamic stability. However, predicting the contextual effects of these changes requires a 3D structure and an approach to evaluate many sequence perturbations within the protein fold.

NovaDesign Benchmarks

NovaDesign uses the DEE/A* method to deterministically find the global minimum energy conformation (GMEC) for a protein design problem. Unlike stochastic approaches, NovaDesign guarantees it will find the GMEC for a given energy function and flexibility model. Our approach currently uses the Penultimate rotamer library to model side chain flexibility, the Backrub model to model backbone flexibility, and the DFIRE statistical potential to evaluate variants and conformations. NovaDesign can instantly model specific variants, while larger searchers for optimizing fold stability can take minutes to hours. We are also planning to introduce backbone-dependent rotamers and molecular mechanics force fields in the future.

We present a deterministic protein design algorithm that optimizes stability in a protein by identifying the sequence in a given search space associated with the Global Minimum Energy Conformation (GMEC) and pinpointing potential liabilities in the resulting molecule. When searching for the GMEC, variant and surrounding side chains are repacked and backbone atoms are perturbed in a chemically-plausible manner to accommodate the changes. We also report the accuracy of the algorithm using a benchmark of over 650 mutations with thermodynamically characterized changes in fold stability. In addition, we present a case study where we redesign the core of a protein to improve its fold stability and design cloning primers to synthesize the new molecule using an array of recombinant DNA technologies. The entire workflow can be completed within an hour using a Windows or macOS workstation, highlighting the approach's practicality for use by any life scientist.

Protein Design Tools to Improve Fold Stability

Nature commonly evolves proteins to provide sufficient fold stability to optimize its activity-not its energetic stability. Buried core residues can be altered to improve a protein's developability and stability, as is the case with Repressor protein cl. The variant p.(Val37Leu; Met41Leu; Val48IIe) (LLI) confers 0.5 kcal/mol more stability than wild type (Lim, et al., 1994, Proc. Natl. Acad. Sci. USA, 91:423-7). Using the wild type structure (PDB ID: 1LMB), NovaDesign reproduces this observation and suggest several other variant combinations that could provide even greater thermostability.

🔀 NovaDesign						_			
Substitutes									
Enter the substitut	ion variations you v	vant to model.							
Workflow	Structure: 1LMB (REFINED 1.8 ANGSTROM CRYSTAL STRUCTURE OF THE LAMB)						✓ Sho		
Substitutes	Chain	Residue	Position	Substitute	V	ariant			
Options	3	Ser	35	-Select-	•				
Vob Vol	3	Val	36	Nonpolar	- p	.(Val36Xaa)			
	3	Ala	37	-Select-	•				
	3	Asp	38	-Select-	-				
	3	Lys	39	-Select-	-				
	3	Met	40	Nonpolar	- p	.(Met40Xaa)			
	3	Gly	41	-Select-	•				
	3	Met	42	-Select-	•				
	3	Gly	43	-Select-	-				



The Fold-X Blind Test benchmark contains 664 substitution variants, each with an experimentally-determined change in free energy $(\Delta \Delta G)$ relative to wild type. NovaDesign calculated the change in fold stability ($\Delta \Delta E$) for the entire data set in less than 10 minutes on a single workstation with a Pearson's linear correlation coefficient (PCC) of 0.70, indicating a strong correlation between experimental and calculated values. Backrub backbone flexibility was used and side chains of neighboring residues were repacked.

Molecular Cloning Workflow

The SeqBuilder Pro primer workbench allows users to create PCR primer pairs at the exact desired location with respect to features and translational frames. The point-and-click "Introduce Mutation" tool allows a user to easily modify triplet codons within primers to introduce the desired mutations. SeqBuilder Pro provides multi-fragment PCR based cloning options so that the mutated gene of interest can be cloned into an appropriate expression vector. The automated virtual cloning process creates all the required PCR primers, a cloning history, and the clone sequences. After virtual cloning, users can visualize all the of the vector and insert feature annotations and apply ORF detection and translation to confirm that the gene of interest remains in-frame with the fusion protein.



		Report 4: Fold Stability - cl core	e \		🔻 🍸 📑
		Fold Stability - cl core			
		Molecule	Variant	ΔE (DFIRE-A)	Prediction
		3:1LMB	M40L, V36F, V47F	-3.275465141287924	stabilizing
		3:1LMB	M40F, V36F, V47F	-3.2447190589279558	stabilizing
		3:1LMB	M40I, V36F, V47F	-3.0360308661623208	stabilizing
KE GALLEN		3:1LMB	M40L, V36L, V47F	-3.0116922666325365	stabilizing
		3:1LMB	M40I, V36L, V47F	-2.867843956520801	stabilizing
		3:1LMB	M40F, V36L, V47F	-2.8542568442191794	stabilizing
		3:1LMB	M40L, V36I, V47F	-2.575720242077182	stabilizing
		3:1LMB	M40F, V36I, V47F	-2.569988044195412	stabilizing
		3:1LMB	M40I, V36I, V47F	-2.4801046836243756	stabilizing
		3:1LMB	M40L, V36F, V47L	-2.847947199858197	stabilizing
		3:1LMB	M40L, V36L, V47L	-2.7940506460562347	stabilizing
		3:1LMB	M40F, V36F, V47L	-2.726482958236346	stabilizing
Structure	🛱 🗐 🛛	3:1LMB	M40I, V36L, V47L	-2.653061599355823	stabilizing
		3:1LMB	M40I, V36F, V47L	-2.6113721881439176	stabilizing
DNA (5'-D(*AP*AP*TP*AP*CP*CP*AP*CP*TP*GP*GP*CP*GP*GP*TP*GP*A P*TP*AP*T)-3') (1LMB:1)		3:1LMB	M40F, V36L, V47L	-2.545897064380995	stabilizing
		3:1LMB	M40L, V36I, V47L	-2.2485912730428197	stabilizing
TATATCACCGCCAGTGGTAT		3:1LMB	M40I, V36I, V47L	-2.1558349780013373	stabilizing
Advance 40		3:1LMB	M40F, V36I, V47L	-2.127120862950136	stabilizing
Kuler 30 40		3:1LMB	M40L, V36F, V47I	-2.6216892420995634	stabilizing
PROTEIN (LAMBDA REPRESSOR) (1LMB:3)		3:1LMB	M40F, V36F, V47I	-2.5107110462626565	stabilizing
KSDSSP 2* Structure		3:1LMB	M40L, V36L, V47I	-2.4453015403461222	stabilizing
Sequence STKKK PLIQEQLEDARRLKATYEKKKNELGLSQESVADKMGMGQSGVGAL		3:1LMB	M40I, V36F, V47I	-2.4031666771259097	stabilizing
Ruler 10 20 30 40 50		3:1LMB	M40I, V36L, V47I	-2.3223649403863362	stabilizing
		3:1LMB	M40F, V36L, V47I	-2.2076340044558265	stabilizing
FNGINALNAYNAALLAKILKVSVEEFSPSIAREIYEMYEAVS		3:1LMB	M40L, V36I, V47I	-1.9581679838826176	stabilizing
Sequence		3:1LMB	M40I, V36I, V47I	-1.883464135581761	stabilizing
kuller 60 70 80 90 52		3:1LMB	M40F, V36I, V47I	-1.831941451816391	stabilizing
PROTEIN (LAMBDA REPRESSOR) (1LMB:4)					
Analysis RCG Sequence	🛱 🗐 🛛	😑 Report 🛛 🗙			(二)



First, positions Val 37, Met 41, and Val 48 are annotated to allow the residues to be replaced with any hydrophobic residue (AGILMPV). Next, pairwise energies are precalculated for all potential combinations, high-energy conformations are excluded, and the GMEC and alternate sequence permutations are identified in energetic order. NovaDesign identified the LLI sequence as the 21st lowest-energy sequence in its search, suggesting 20 other permutations that have even greater fold stability. All computations were completed in under a minute on a laptop computer, highlighting the accessibility of the software.

IDENTIFY VARIANTS

The cl gene is the first of three in the lambdap50 operon. There are three predicted mutations in the cl gene: Val37Leu; Met41Leu; and Val48lle.



CREATE & MUTATE PRIMERS

Mutated bases and amino acids are colored pink and bases are displayed in lower case in the primer list view. Both primers can be further adjusted to improve Tm differences and minimize primer-dimer formation. Primer statistics are updated instantly in header.

CREATE VIRTUAL CLONES

The mutated cl gene within the lambdap50 operon and the upstream (non-mutated) fragment of the cl gene have been cloned into an E.coli expression vector system (pBAM/Thio) using Gibson Assembly. The uninterrupted ORF (arrow) points to the atg start site of hpTrxA, a mutated version of the E. coli protein thioredoxin (trxA) used to create a metal-binding domain that allows purification of thioredoxin fusion on metal- chelating resins.





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