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14. ABSTRACT The overall goal of the project was to evolve a highly thermostable enzyme (alcohol dehydrogenase D (AdhD) from <i>Pyrococcus furiosus</i>) to bind an explosive molecule, RDX. The enzyme naturally catalyzes the nicotinamide cofactor-dependent oxidation or reduction of alcohols, aldehydes, ketones and carbohydrates. Directed evolution techniques were used to convert the enzyme into a simple binder of the small molecule RDX. Novel binders have been identified and characterized with sub-millimolar dissociation constants. These could be further developed for use in robust and low cost biosensors.					
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a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER 212-854-7531

Report Title

Final Report: Engineering of an Extremely Thermostable Alpha/Beta Barrel Scaffold To Serve as a High Affinity Molecular Recognition Element For Use in Sensor Applications

ABSTRACT

The overall goal of the project was to evolve a highly thermostable enzyme (alcohol dehydrogenase D (AdhD) from *Pyrococcus furiosus*) to bind an explosive molecule, RDX. The enzyme naturally catalyzes the nicotinamide cofactor-dependent oxidation or reduction of alcohols, aldehydes, ketones and carbohydrates. Directed evolution techniques were used to convert the enzyme into a simple binder of the small molecule RDX. Novel binders have been identified and characterized with sub-millimolar dissociation constants. These could be further developed for use in robust and low cost biosensors.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Haghpanah, J. (presenter), Bulutoglu, B., and Banta, S. "Converting a Thermostable Enzyme into a Binder of Explosive Molecules" AIChE Annual Meeting, Salt Lake City, UT Nov. 2015

Number of Presentations: 1.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

<u>NAME</u>	<u>PERCENT_SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT_SUPPORTED</u>
Jennifer Haghpanah	1.00
FTE Equivalent:	1.00
Total Number:	1

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
Scott Banta	0.08	
FTE Equivalent:	0.08	
Total Number:	1	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Karina Montenegro	0.00	Chemical Engineering
Jennifer Xiong	0.00	Biomedical Engineering
FTE Equivalent:	0.00	
Total Number:	2	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 2.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 2.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 1.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

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The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:..... 0.00

Names of Personnel receiving masters degrees

<u>NAME</u>
Total Number:

Names of personnel receiving PHDs

<u>NAME</u>
Total Number:

Names of other research staff

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See Attachment

Technology Transfer

Engineering of an Extremely Thermostable Alpha/Beta Barrel Scaffold To Serve as a High Affinity Molecular Recognition Element For Use in Sensor Applications

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Statement of the Problem Studied

The overall goal of the project was to evolve a highly thermostable enzyme (alcohol dehydrogenase D (AdhD) from *Pyrococcus furiosus*) to bind an explosive molecule, RDX. The enzyme naturally catalyzes the nicotinamide cofactor-dependent oxidation or reduction of alcohols, aldehydes, ketones and carbohydrates. Directed evolution techniques were used to convert the enzyme into a simple binder of the small molecule RDX. Novel binders have been identified and characterized with sub-millimolar dissociation constants. These could be further developed for use in robust and low cost biosensors.

Summary of the most Important Results

The specific aims of the project were:

Specific Aim 1

To develop large randomized libraries of AdhD mutants as well as a selection method for identifying AdhD mutants with a high affinity to RDX crystals. Library diversity and efficient selection schemes will be generated. We will make libraries of AdhD mutants with the substrate and cofactor binding pockets randomized. Conditions where RDX-binding to AdhD mutants displayed on ribosomes can be separated using insoluble RDX crystals will be determined.

Specific Aim 2

Utilize directed evolution techniques to identify an AdhD mutant with both a high affinity and specificity to RDX crystals. Mutants from the randomized library will be identified and genetic diversity will be introduced for affinity maturation. Competition experiments with related compounds will be used to enhance selectivity of the selected mutants. AdhD mutants with high affinity to insoluble RDX crystals will be identified and characterized.

Specific Aim 3

Extensively characterize new RDX-binding reagents for future biosensing device applications. The dissociation constants for the mutants binding to soluble RDX will be determined via analytical methods. The selectivity and stability of the new binders will also be determined. Visualization software will be used to gain structural insights for high affinity binders with AdhD scaffolds.

We have developed three randomized libraries with mutations located in the native binding pocket of AdhD for use in ribosome display selections (Figure 1). There is always a trade-off in library development, as many sites can be chosen for randomization, but this can quickly lead to library sizes that are too large to fully explore. Therefore, we have chosen to take three different approaches with respect to AdhD libraries generated. The first library explores 5 randomized sites (library size of 20^5 possible combinations) within the cofactor binding pocket; the second explores 5 randomized amino acids (library size of 20^5 possible combinations) that were identified in a docking simulation between AdhD and RDX. The final library was created with all 10 sites from both libraries randomized, but the libraries were restricted to contain only serine or tyrosine substitutions (made with the TMT randomized DNA codon) and this should lead to a library size of 2^{10} possible combinations. A limited library was desired to enable a more of the library to be explored and serine and tyrosine were desired because tyrosine endows binding sites with a high affinity and specificity, while serine is flexible and can provide space for interactions.¹

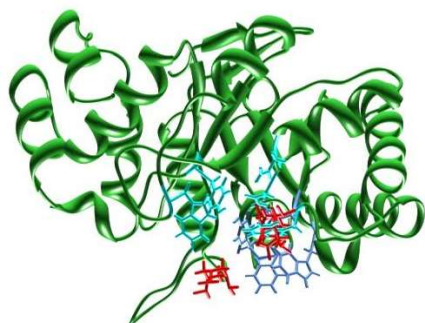


Figure 1: AdhD mutant library locations. The AdhD protein is shown in green with the 5 randomized sites near the cofactor binding pocket in blue, the 5 randomized sites away from the pocket in cyan and RDX molecules in red.

Previously, we explored using phage display to validate our selection strategy. A peptide with sequence K A S G P S G F W P S G G G C was identified with weak affinity ($K_d=6-12$ mM) as measured by isothermal titration calorimetry (ITC). Even though we acquired a weak binder, we were able to prove that we could select for an RDX binder using precipitated RDX for selections. We then moved on to develop that AdhD enzyme in the in vitro ribosome display format. We explored two selection protocol methods involving either multiple rounds of biopanning or extended off-rate selections. After multiple trials, we determined that biopanning was better than off-rate selection because the RNA was difficult to recover after adding RDX crystals to the plate for long periods of time.

We began our rounds of selection with the TMT library utilizing the method of biopanning and increasing the number of washes each round. After three rounds of selection, we noticed that randomized residues in the co-factor binding pocket were showing some sort of convergence, while the residues within the docking pocket were noisy and not converging (Table 1 and Figure 2). All sequences of interest were cloned into a pet20b (expression) vector with an N-terminal FLAG-tag (DYKDDDDK). We pre-screened all mutants for thermostability by ensuring solubility in lysate after heating to 80°C . The sequences with the randomized residues (Table 1), were soluble after heating to 80°C , and were further assessed for their affinity by both ELISA and ITC (Figure 2, Figure 3 and Table 2). A small amount of RDX was dissolved in aqueous

buffer (20 mM Tris and 100 mM NaCl at pH = 7.5) used for ribosome display and the solution was sonicated to homogenize the RDX crystals in solution.

sample	61	63	64	92	122	123	154	156	185	205
wt	A	F	Y	W	H	W	N	N	W	Y
A	Y	Y	Y	Y	S	Y	Y	S	Y	S
B	S	S	Y	Y	Y	S	Y	S	Y	Y
C	S	S	Y	Y	S	Y	G	M	I	Y
D	S	S	Y	W	H	T	C	C	K	S

Table 1: Shows amino acid residues that appeared in multiple sequences after three rounds of selection with the serine/tyrosine (TMT) library. The 5 randomized residues shown in dark blue are within the cofactor binding pocket and while 5 the randomized residues outside of the binding pocket are in cyan.

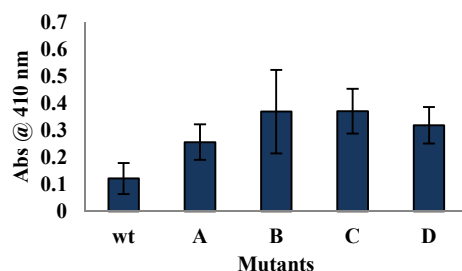


Figure 2: ELISA of the mutants from three rounds on selection with standard deviations representing the error of three trials.

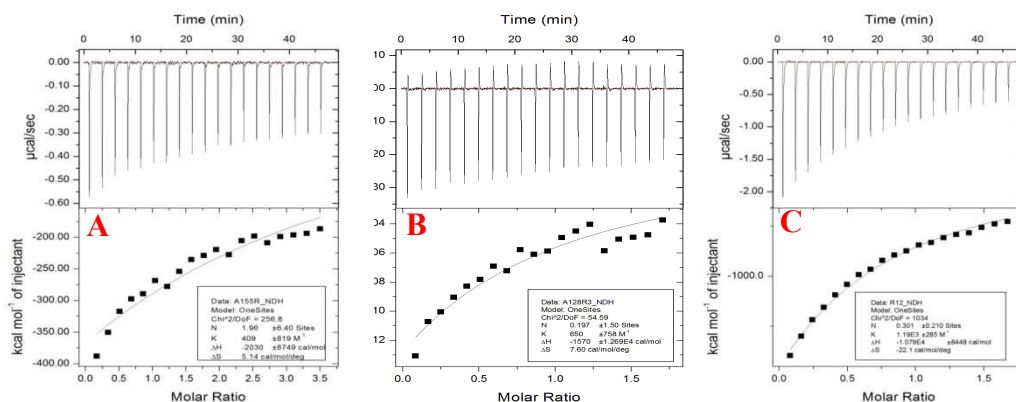


Figure 3: ITC result of three of the mutants obtained after three rounds of selection, with mutant D not showed because mutant D did not exhibit affinity for RDX via ITC experiments.

	A K_D (mM)	B K_D (mM)	C K_D (mM)
Average K_D	1.86 ± 0.56	1.18 ± 0.04	0.89 ± 0.36

Table 2: Average K_D 's determined from ITC with standard deviations from three trials.

After three rounds of selection with the serine/tyrosine (TMT) library, we wanted to confirm RDX was binding in the cofactor pocket only. The previous data showed a convergence in randomized residues within the cofactor binding pocket, so we engineered some of the mutants with the five residues outside the cofactor binding pocket mutated back to wild type. After mutations were made and sequences were confirmed, we assessed the binding affinities for RDX via ITC (Figure 4).

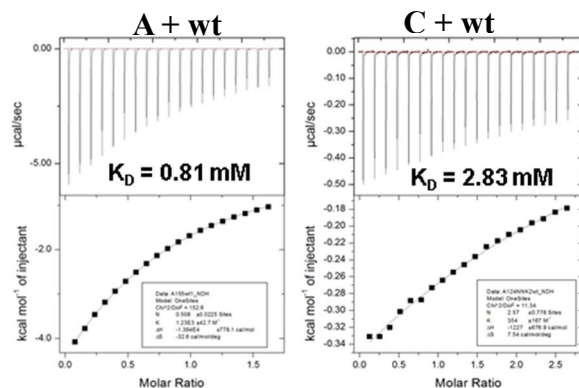
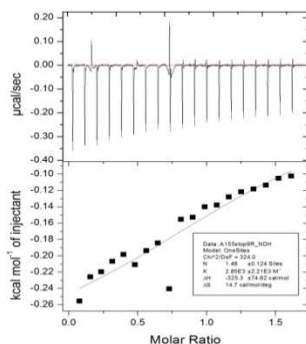


Figure 4: ITC of mutants A and C with five of the residues outside the co-factor binding pocket mutated to the wild type residues. Mutants were assessed for their affinity for RDX.

In a different project focused on engineering AdhD we had made the surprising discovery that AdhD could be split into two halves, and that the first half of the enzyme still folded into a stable conformation, but with a loss of activity. Since the mutations in the cofactor binding pocket are all in this first half, we explored whether mutations in truncated form of AdhD could also bind RDX. The results for truncated mutant A (Figure 5) show the same affinity for RDX as the full length enzyme.

Front Half of Mutant



$$K_D = 0.8 \text{ mM} \pm 0.435$$

Figure 5: ITC of front half of mutant A with RDX with standard deviation denoting three trials.

After we confirmed binding was taking place in the cofactor binding pocket, we went forward with ribosome display focusing on the library with the fully randomized sites within the cofactor binding pocket. After three rounds of selection, we found that our library was converging towards a single sequence (Table 3). We took the converged sequences from three rounds of

selection and compared their affinity for RDX with the previous via ELISA and ITC (Figure 6, Figure 7 and Table 4).

	61	63	64	92	122
wt	A	F	Y	W	H
E	P	T	T	R	H
F	C	M	C	Y	H

Table 3: The residues that showed up after three rounds of selection with RDX. The dark blue amino acids represent the randomized sites in the cofactor binding pocket.

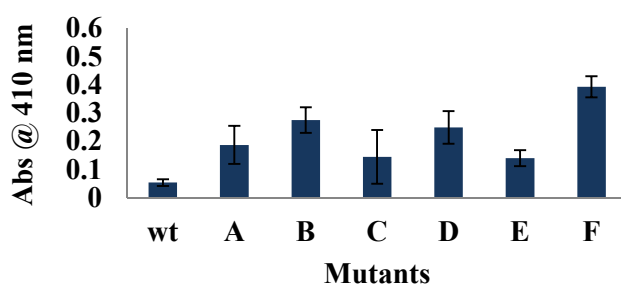


Figure 6: ELISA of mutants from the serine/tyrosine (TMT) library and the randomized cofactor library with standard deviations representing error from three trials.

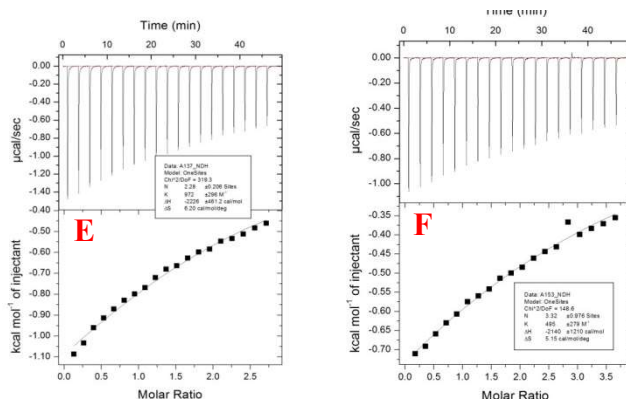


Figure 7: ITC of mutants from three rounds of selection with the cofactor library.

	E K_D (mM)	F K_D (mM)
Average K_D	1.45 ± 0.601	1.45 ± 0.80

Table 3: Average K_D 's determined from ITC with standard deviations from three trials of ITC.

Since the mutants obtained from the fully randomized cofactor binding pocket were not significantly better than what we obtained from the limited serine/tyrosine (TMT) library, we decided to proceed with a different approach. We began to perform error prone PCR on the best

mutants with a non-biased polymerase (Mutazyme), which introduces a spectrum of mutations throughout the AdhD gene. We are currently in the end stages of diversifying our library as we have gone through four rounds of selection with error prone PCR and have acquired some convergence of sequences. We also noticed an increase in the number of good reads in sequences from round 3 to round 4 with error prone PCR (Table 4). We are currently preparing this mutant G and H (see sequence below) for ITC experiments. Mutant G showed up 8 times while mutant H showed up once.

Library and Pan Round	# Samples	# Good Samples	# Bad Samples
TMT Pan Round 1	8	2	6
TMT Pan Round 2	8	6	2
TMT Pan Round 3	8	3	5
Co-Factor Library Pan Round 1	16	8	8
Co-Factor Library Pan Round 2	16	6	12
Co-Factor Library Pan Round 3	16	12	4
Round 1 Pan Error Prone PCR	8	4	4
Round 2 Pan Error Prone PCR	16	12	4
Round 3 Pan Error Prone PCR	16	6	10
Round 4 Pan Error Prone PCR	20	9	11

Table 4: Showing the overall samples with the amount sent for sequencing, good reads and bad reads.

While we have been diversifying the library, we have examined the secondary structures of the proteins via circular dichroism (Figure 8). We have also employed circular dichroism to investigate the stability of the proteins in the presence of the denaturant (guanidine hydrochloride) (Figure 9). All proteins had similar secondary structures when compared to the wild type protein (Figure 8). After performing denaturation experiments, we discovered that all of the mutants were as stable as the wild type, and some of the mutants may even have improved stability.

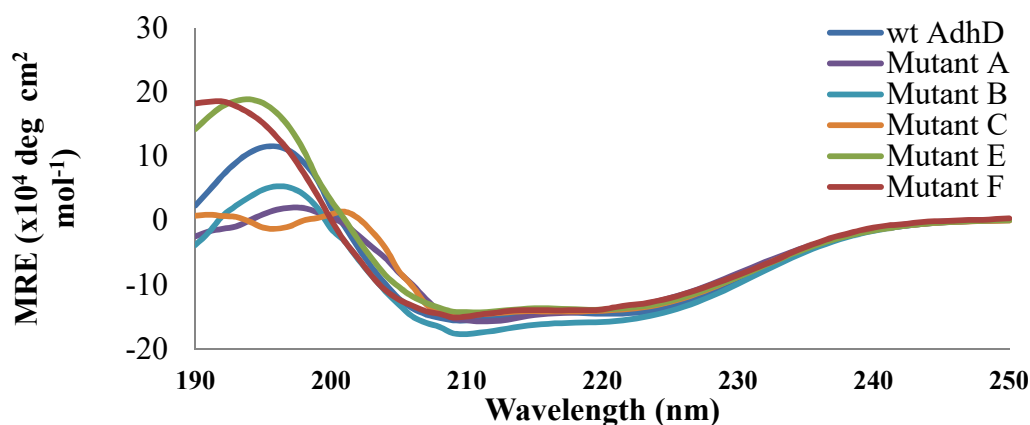


Figure 8: Circular dichroism of all AdhD mutants (10 uM) in 20 mM Tris-HCl with 100 mM NaCl at pH = 7.5.

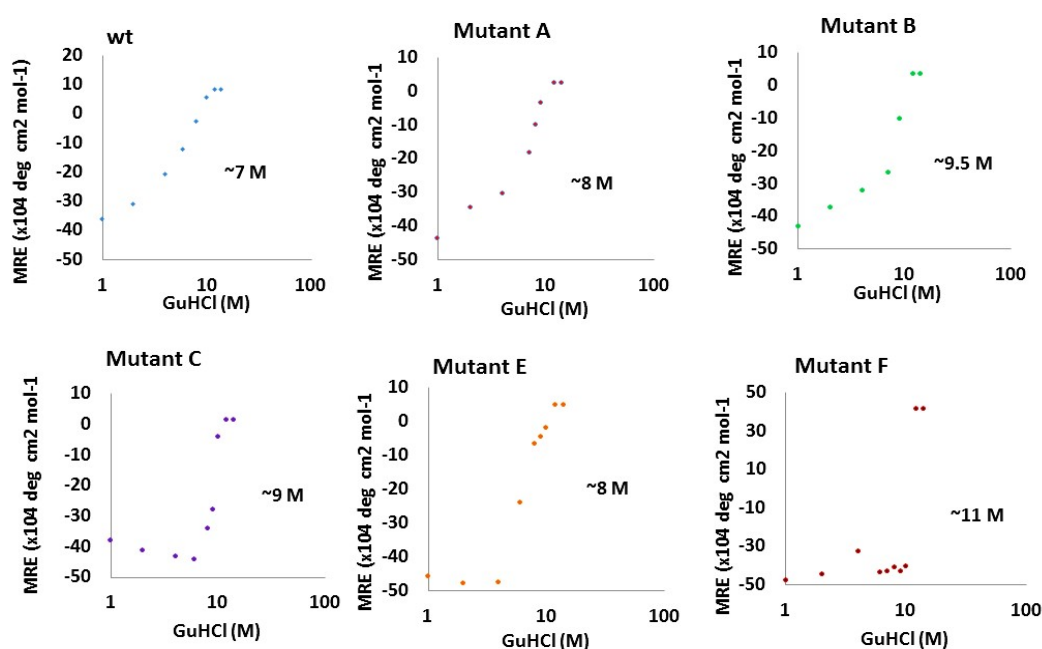


Figure 9: Stability studies of AdhD mutants in the presence of different amounts of Guanidine Hydrochloride. The concentration on the figure denotes the concentration of Guanidine Hydrochloride at which the enzyme denatures.

We are in the process of finalizing the characterization of the mutants from the error prone PCR experiments and then we will produce a manuscript for submission to a top-tier peer-reviewed journal.

References:

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10. Ng, D.T. & Sarkar, C.A. Model-guided ligation strategy for optimal assembly of DNA libraries. *Protein Engineering Design and Selection* **25**, 669-678 (2012).

Appendix: AdhD Mutant Sequences

Mutant A

MGDYKDDDDKAKRVNAFNDLKRIGDDKVTAIGMGTWGIGGRETPDYSRDKESIEAIRYGLELG
MNLIDTYEYYGAGHAEIVGEAIKEFEREDIFIVSKVYPHTFGYEEAKKAARASAKRLGTYIDL
LLSYPVDDFKKIEETLHALEDLVDEGVIRYIGVSYFSLELLQRSQEVMRKYEIVANQVKYSVKDR
YPETTGLLDYMKREGIALMASTPLEKGTXXVTSAWQRSVRSTVRRRHKL-

Mutant B

MGDYKDDDDKAKRVNAFNDLKRIGDDKVTAIGMGTWGIGGRETPDYSRDKESIEAIRYGLELG
MNLIDTSEYYGAGHAEIVGEAIKEFEREDIFIVSKVYPHTFGYEEAKKAARASAKRLGTYIDL
LYYPVDDFKKIEETLHALEDLVDEGVIRYIGVSSFSLELLQRSQEVMRKYEIVANQVKYSVKDRS
PETTGLLDYMKREGIALMASTPLEKGTXXVTSAWQRSVRSTVRRRHKL-

Mutant C

MGDYKDDDDKAKRVNAFNDLKRIGDDKVTAIGMGTWGIGGRETPDYSRDKESIEAIRYGLELG
MNLIDTYEYYGAGHAEIVGEAIKEFEREDIFIVSKVYPHTFGYEEAKKAARASAKRLGTYIDL
LLSYPVDDFKKIEETLHALEDLVDEGVIXYIGVSYFSLELLQRSQEVMRKYEIVANQVKYSVKDR
YPETTGLLDYMKREGIALMASTPLEKGTLARNECLAKIGKKYGKTAAQVALNYLIWEENVVAIP
KASNKEHLKENFGAMGWRLXEEDREMARRCVEDPNXSSXX

Mutant D

MGDYKDDDDKAKRVNAFNDLKRIGDDKVTAIGMGWTWGIGGRETPDYSRDKESIEAIRYGLELG
MNLIDTSESYGAGHAEIIVGEAIKEFEREDIFIVSKVWPTHFGYEEAKKAARASAKRLGTYIDL
YLHTPVDDFKKIEETLHALEDLVDEGVIXYIGVSCFCLELLQRSQEVMRKYEIVANQVKYSVKDRK
PETTGLLDYMKREGIALMASTPLEKGTLARNECLAKIGKKYGKTAAQVALNYLIWEENVVAIPK
ASNKEHLKENFGAMGWRLXEEDREMARRCVEDPNXSSXX

Mutant E

MAKRVNAFNDLKRIGDDKVTAIGMGWTWGIGGRETPDYSRDKESIEAIRYGLELGMNLIDTPETT
GAGHAEIIVGEAIKEFEREDIFIVSKVRSTHFGYEEAKKAARASAKRLGTYIDL
YLLPWPVDDFKKIEETLHALEDLVDEGVIRYIGVSNFNLELLQRSQEVMRKYEIVANQVKYSVKDRWPETTGLLD
YMKREGIALMAYTPLEEGTLARNECLAKIGEKYGKTAAQVALNYLIWEENVVAIPXASNKEHL
KENFGAMGWRLSEXIVRWRARCVEDPNSSSVDKLSXXFRWXXXLRSGSXXR-

Mutant F

MAKRVNAFNDLKRIGDDKVTAIGMGWTWGIGGRETPDYSRDKESIEAIRYGLELGMNLIDTCEMC
GAGHAEIIVGEAIKEFEREDIFIVSKVYPPTHFGYEEAKKAARASAKRLGTYIDL
YLLHWPVDDFKKIEETLHALEDLVDEGVIRYIGVSNFNLELLQRSQEVMRKYEIVANQVKYSVKDRWPETTGLLD
YMKREGIALMAYTPLEKGTLARNECLAKIGEKYGKTAAQVALNYLIWEENVVAIPKASNKEHL
KENFGAMGWRLSEEDREMARRCVEDRIXAPWXS-

Mutant G

MAKRVNAFNDLKRIGDDKVTAIGQGTWGIGGRETPDYSRDKESIEAIRYGLELGQNLIDTAEFYG
AGHAEIIVGEAIKEFEREDIFIVSKVMPPTHFGYEEAKKAARASAKRLGTYIDL
YLLHWPVDDFKKIEETLHALEDLVDEGVIRYIGVSNFNLELLQRSQEVQRKYEIVANQVKYSVKDRWPETTGLLDYQ
KREGIALQAYTPLEKGTLARNECLAKIGEKYGKTAAQVALNYLIWEENVVAIPKASNKEHLKEN
FGAXAGA-

Mutant H

MAKRVNAFNDLKRIGDDKVTAIGMGWTWGIGGRETPDYSRDKESIEAIRYGLELDMNLIDTCVMC
GAGHAEIIVGEAIKEFEREDIFIVSKVYPPTHFGYEEAKKAACASAKRLGTYIDL
YLLHWPVDDFKKIEETLHALEDLVDEGVIRYIGVSNFNLELLQRSQEVQRKYEIVANQVKYSVKDRWPETTGLLDY
MKREGIALMANTPLEKGTLARNECLAKIGEKYGKTAAQVALNYLNWEENVVAILKASNEEHQK
ENFGAMGWRLXEXIVRWRAGVLRIFELXXXLSLGLPVXRSKLRXGS