UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

Merck Sharp & Dohme LLC,
Petitioner,

v.

Halozyme Inc.,
Patent Owner.

Case No. PGR2025-00017
U.S. Patent No. 12,110,520

Declaration of Michael Hecht, Ph.D.

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B. C.V of Michael Hecht, Ph.D.

I. Introductory Matters

A. Background and Qualifications

1. My educational background, career history, and other relevant qualifications are summarized below. I attach to this Declaration my curriculum vitae, which provides a full and accurate description of my educational background, professional experience, and qualifications.

2. I received my Ph.D. in Biology from Massachusetts Institute of Technology in 1984. My Ph.D. thesis was on the topic of "The Effect of Amino Acid Replacement on the Structure and Stability of the N-terminal Domain of λ-Repressor." I received a Bachelor of Arts in Chemistry from Cornell University in 1977. For my B.A., I wrote a Thesis regarding Studies of the α-helical Propensities of Amino Acids in Synthetic Copolymers. I completed my Post-Doctoral work at Duke University from 1986 to 1989 in the Department of Biochemistry, where I conducted research on the Design of Novel Proteins.

3. I currently serve as Professor of Chemistry at Princeton University. I have been a professor at Princeton since 1990. I served as Associate Chair of the Chemistry Department from 2004-2007. From 2011-2008 I was Director of Undergraduate Studies for the Department of Chemistry. I was Master (Head) of Forbes College at Princeton from 2010-2018.

4. I am currently teaching courses in General Chemistry and Principles of Macromolecular Structure: Protein Folding, Structure and Design.

5. I have over 4 decades of experience in the field of protein structure and design. My research interests include de novo protein design, synthetic biology, and protein folding and design. In my laboratory, we explore protein design and synthetic biology to explore novel proteins. Since 1978, I have authored over 90 peer-reviewed publications, most of which are directed to these topics.

6. In 2024, one half of the Nobel Prize in chemistry was awarded to David Baker for "computational protein design," and the other half went to Demis Hassabis and John Jumper for their work in "protein structure prediction," specifically their AI model called AlphaFold2. The Nobel committee's write-up of other notable contributions in the field of protein design included a description of my earlier work. See https://www.nobelprize.org/uploads/2024/10/advanced-chemistryprize2024.pdf ("Four-helix bundles thus became common targets for protein design in the early years of this field, and the concept of a 'binary code' with hydrophobic and hydrophilic amino acid residues was further elaborated by Hecht and coworkers. These researchers conducted a large library of synthetic genes coding for the same pattern of polar and nonpolar residues and showed that most of the designed protein sequences folded into compact α-helical structures.")

(citing my 1993 paper entitled "Protein design by binary pattering of polar and nonpolar amino acids" in Science, 262, 1680-1685).

7. Since 2003, I have been on the Editorial Advisory Board of Protein Science and Protein Engineering, Design & Selection. In 2014, I organized a National Science Foundation Workshop on the Future of Protein Engineering & Design.

8. I was a National Science Foundation Graduate Fellow from 1979-1983, and a Life Sciences Research Foundation Burroughs-Wellcome Post-doctoral Fellow from 1986-1989. I won the Protein Society's Kaiser Award in 2003.

9. Since 1991, I have supervised 14 post-doctoral researchers, 32 graduate students in chemistry and molecular biology, and the senior thesis of 75 undergraduate students in chemistry and molecular biology.

B. Compensation

10. I am being compensated for my time at the rate of $700 per hour for my work in connection with this matter. I am being reimbursed for reasonable and customary expenses associated with my work in this investigation. This compensation is not dependent in any way on the contents of this Declaration, the substance of any further opinions or testimony that I may provide, or the ultimate outcome of this matter.

C. Person of Ordinary Skill in the Art

11. I understand that my analysis and opinions are to be provided using the perspective of a person of ordinary skill in the art in the timeframe before December 29, 2011. I will refer to this as the "2011 timeframe" in this declaration.

12. The scientific field of the patent concerns proteins, and more particularly, protein structures and modification of them. I am very familiar with this field, and the individuals who work within it, including in the 2011 timeframe.

13. I have been informed by counsel that a person of ordinary skill in the art is a hypothetical person who is presumed to have the typical skills and knowledge of someone working in the field of the invention. Based on my review of the patent and my experience, I believe a person of ordinary skill in the art (who I may refer to as "a skilled artisan") would have had an undergraduate degree, a Ph.D., and post-doctoral experience in scientific fields relevant to study of protein structure and function (*e.g.*, chemistry, biochemistry, biology, biophysics). From training and experience, the person would have been familiar with factors influencing protein structure, folding and activity, production of modified proteins using recombinant DNA techniques, and use of biological assays to characterize protein function, as well with techniques and tools used to analyze protein structure (*i.e.*, sequence searching and alignments, protein modeling software, etc.).

14. In the 2011 timeframe, I had at least the qualifications I outline above for a person of ordinary skill in the art. The opinions I provide in this declaration are provided from the perspective of a person of ordinary skill in the art in the 2011 timeframe as I have described it above.

D. Terminology Used in this Declaration; Patent Documents

15. I will use the following abbreviations and terminology in this declaration:

(a) "PH20" refers to the human PH20 protein. In its full-length form (including its 35 amino acid signal sequence) it has 509 amino acids. Its amino acid sequence was first published in 1993.[1] Its sequence also is reported as:

- UniprotKB Accession ID P38567;
- SEQ ID NO:1 in U.S. Patent No. 7,767,429; and
- SEQ ID NO:6 in U.S. Patent No. 12,110,520.

(b) When I refer to the "mature" protein sequence of PH20, I am referring to the form of the protein without the signal sequence. So, for example, the mature form of PH20 having amino acids from positions

[1] EX1029 (Gmachl), 546, Fig. 1.

36 to 509 of Uniprot ID: P38567, would have numbering that starts at position 1 and ends at position 474.

(c) I will use "$PH20_{1\text{-}n}$" to refer to a human wild-type PH20 polypeptide sequence that starts at position 1 and terminates at position "n" of the PH20 sequence. If I do not indicate otherwise, the sequence that is being referred to is the mature form of the protein without the signal sequence. For example, $PH20_{1\text{-}447}$ means the polypeptide starting at position 1 and ending at position 447 of the mature human wild-type PH20 sequence (which would be 36 to 482 if numbering includes the signal sequence).

(d) "AxxxB" refers to an amino acid substitution at position xxx, where the wild-type residue is A and the residue after the substitution is B.

16. I was asked to assess two patent documents. One is an issued U.S. Patent, which has the number 12,110,520, which I refer to as the '520 Patent (EX1001). The other is a U.S. patent application having the number 13/694,731, which I will refer to as the '731 Application (EX1026).

17. I understand that the disclosure of a patent consists of a narrative section called the specification, often includes drawings, and may contain sequence listings, which are nucleotide or amino acid sequences. I understand that each

sequence in the patent will be assigned a number for easy reference (*e.g.*, SEQ ID NO: 3). I understand that patents end with claims that define the invention.

18. I reviewed the contents of the ’520 Patent, as well as the ’731 application. I also reviewed a redline comparison of the specifications of the ’520 Patent and the ’731 application (EX1045). Based on that review, the two specifications appear to be substantively the same. Because the two patent documents have the same disclosures, I will refer to the two of them together as the “common disclosure” in this declaration. For convenience, citations will be the disclosure in the ’520 Patent.

E. Materials Considered

19. My opinions are based on my years of education, research, and experience, as well as my investigation and study of relevant materials. I reviewed a number of publications in the course of my assessment, including those listed in the Exhibit List. I also relied on my extensive familiarity with the scientific literature in this field.

20. I also reviewed a declaration by Dr. Sheldon Park provided to me by counsel (EX1004). Based on my review, Dr. Park’s declaration provides an accurate description of how a person of ordinary skill in the art in the 2011 timeframe would have approached the task of identifying single amino acid substitutions in non-essential regions of PH20 that would have been expected to be

tolerated by the protein, and is the type of analysis I would rely upon from others working in my lab in evaluating mutated proteins. Dr. Park's declaration also provides an accurate description of the techniques that were being used in the 2011 timeframe to find structurally similar proteins, analyze them using sequence alignment tools, identify conserved and evolutionarily varying positions in the related proteins, and use protein structure models to assess the tolerance of the protein to individual amino acid substitutions.

21. I reviewed Dr. Park's discussion of the tools he used in his analysis, such as BLAST, Clustel Omega, SWISS-MODEL and PyMol. See EX1004 (Park Dec.), ¶¶ 20-24, 154-179. I am familiar with these tools. I also agree with Dr. Park that while the tools in their modern incarnation have different forms and additional capabilities relative to the versions of the tools that existed in 2011, the analyses Dr. Park performed using them relied on features that would have been present in the versions that existed in the 2011 timeframe. For example, sequence alignments performed by the Clustal Omega tool rely on algorithms that were largely developed in the 1990s and produce outputs that are typically evaluated manually by the user to confirm the accuracy of the alignments.

22. Like Dr. Park, I also would have expected that structural models produced by the SWISS-MODEL tool today for PH20 would be very similar the models for PH20 that would have been produced by that tool in the 2011

timeframe. EX1004 (Park Dec.), ¶¶ 161-174. As Dr. Park points out, the template that SWISS-MODEL used to produce the PH20 models was the HYAL1 structure published by Chao et al. in 2007 (EX1006) (structure ID: 2PE4). I also agree with Dr. Park's observation that even if there were subtle differences in the positioning of certain side chains in the PH20 model relative to a model generated in the 2011 timeframe, those differences would not have affected the overall assessment that a scientist would have made, which are based on a visual assessment of individual substitutions within the PH20 structure.

F. Legal Principles

23. I am not a lawyer and am not offering opinions on the law. However, I have been provided a general explanation of some of the legal requirements for obtaining a patent.

24. I have been informed that a patent's disclosure consists of the information in the specification, along with any drawings and sequence listings that accompanied the patent application. When I use the term "patent disclosure," I am using it with this understanding.

25. I have been informed that a patent claiming a set (or "genus") of chemical compounds (*e.g.*, polypeptides) must be supported by the patent disclosure, and that there are two distinct disclosure requirements, as summarized in the following paragraphs.

26. I have been informed that one of the disclosure requirements is that the patent disclosure provide a sufficient written description of the claimed set of polypeptides ("written description" requirement). I have been informed that this can be achieved by a patent disclosure that describes either (i) "a representative number of species falling within the scope of the genus" or (ii) "structural features common to the members of the genus," with either being such that "one of skill in the art can 'visualize or recognize' the members of the genus." I have been informed that an adequate written description of a genus of polypeptides "requires a precise definition, such as by structure, formula, chemical name, physical properties, or other properties, of species falling within the genus sufficient to distinguish the genus from other materials." I also have been informed that a description that is "merely drawing a fence around the outer limits of a purported genus is not an adequate substitute for describing a variety of materials constituting the genus and showing that one has invented a genus and not just a species."

27. I have been informed a second disclosure requirement is that the patent disclosure provide a description that enables a skilled artisan to practice the claimed invention without engaging in undue experimentation ("enablement" requirement").

28. I have been informed that if a patent claims an entire class of compounds (*e.g.*, polypeptides), the patent's specification must enable a person

skilled in the art to make and use the entire class, which means the specification must enable the full scope of the invention as defined by its claims. I have also been informed that a research plan that requires a skilled artisan to perform trial and error procedures in order to discover which members of a large class of polypeptides have a desired functional property is not sufficient to satisfy the enablement requirement.

29. I have also been informed that factors such as the breadth of the claims, unpredictability in the field, the degree of guidance in the prior art and in the patent disclosure, and the level of skill of the skilled artisan are factors, among others, that are considered in assessing the question of enablement.

30. I have been informed that a separate patentability requirement is that an invention must not have been obvious to a person of ordinary skill in the art in view of what was known in the prior art before the filling date of the patent. I also have been informed that if a patent claim encompasses a compound that would have been obvious in light of the prior art, that claim is unpatentable.

31. I have been informed that for a claimed compound to be found obvious, a person of ordinary skill in the art must have found a reason in the prior art to make that compound, and must have had a reasonable expectation of success in achieving the claimed invention. I have been informed this does not require the skilled artisan to have absolute certainty about achieving a desired result, and that

an invention can be found obvious if a result is expected but still requires some experimentation to confirm.

32. I have been informed that if a particular compound exhibits unexpected properties, that evidence can support a finding that the compound is not obvious. For a claim defining a large class of compounds, the members of the class must share the unexpected property to support a finding that the class of compounds is not obvious. A claim defining a large class of compounds cannot benefit from evidence showing only one compound exhibits a particular unexpected property. Instead, the evidence must demonstrate that the unexpected property is shared by the entire class of claimed compounds.

II. Scientific Principles

33. The common disclosure discusses modified forms of a human hyaluronidase enzyme called PH20, which is one of five related hyaluronidase enzymes found to occur in humans.

34. The focus of the patent claims, as I explain below, are modified PH20 polypeptides that have incorporated at least one amino acid substitution at position 324, and optionally may incorporate 20 to 41 additional substitutions at any other position in the wild-type sequence of 37 different PH20 polypeptides of varying length, depending on the parameters of each claim.

35. The scientific fields relevant to the subject matter of the patent are protein structure and engineering. Two textbooks that provide a useful orientation to protein structure principles are: (i) Brandon & Tooze (EX1014) (chapters 1-6 and 17) and (ii) Alberts (EX1039) (chapter 3).[2]

A. Protein Structure Basics

36. Proteins are formed by sequences of amino acids, but the feature of each protein that confers its unique biological function(s) is its overall three-dimensional structure.[3] That overall structure results from folding of the amino acid sequence of the protein (its "primary structure"). The sequences initially fold into regions of characteristic "secondary structures" (*e.g.*, alpha-helices, beta-strands, loop regions). Sets of secondary structures then are positioned and arranged to form structural motifs, and those motifs and other sequences pack together to form compact globular units called domains, of which there may be one or several in the protein. That higher order structure is the "tertiary structure" of the protein. Multiple polypeptides can also form complexes, referred to as the "quaternary structure" of the protein. PH20 consists of a single polypeptide chain.

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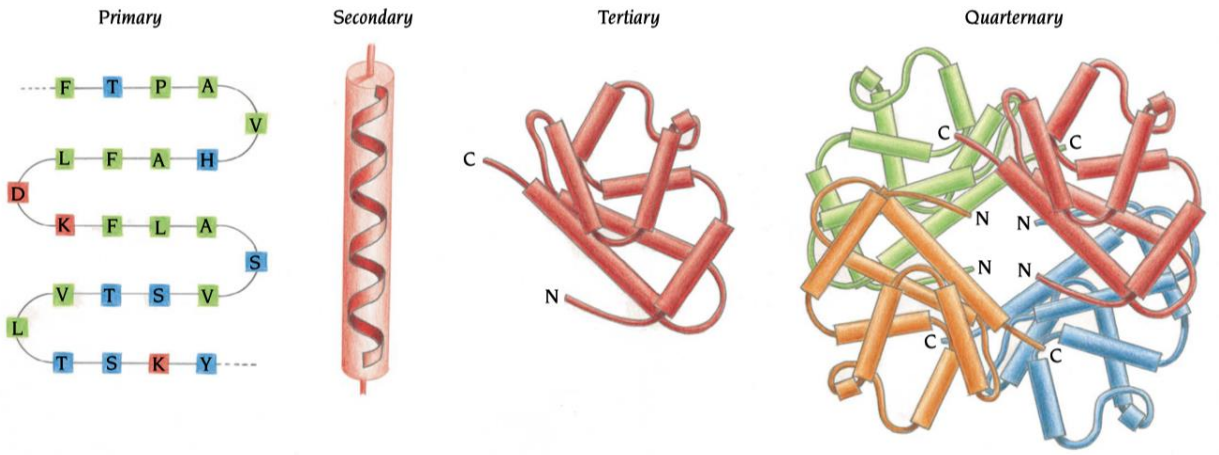
[2] Brandon & Tooze (EX1014) ("Brandon") is a textbook which I have used in the graduate course that I teach for many years.

[3] EX1014 (Brandon), 3-11.



37. Amino acids are generally classified based their chemical attributes, which are dictated by the side chain of the amino acid. Amino acids can be classified in different ways. For example, at a high-level, amino acids can be classified as being polar or non-polar, with subsets of polar residues being charged (positively or negative) or uncharged.[4] Amino acids also have varying sizes, which can influence their ability to fit into defined protein structures.

AMINO ACID			SIDE CHAIN
Aspartic acid	Asp	D	negative
Glutamic acid	Glu	E	negative
Arginine	Arg	R	positive
Lysine	Lys	K	positive
Histidine	His	H	positive
Asparagine	Asn	N	uncharged polar
Glutamine	Gln	Q	uncharged polar
Serine	Ser	S	uncharged polar
Threonine	Thr	T	uncharged polar
Tyrosine	Tyr	Y	uncharged polar

POLAR AMINO ACIDS

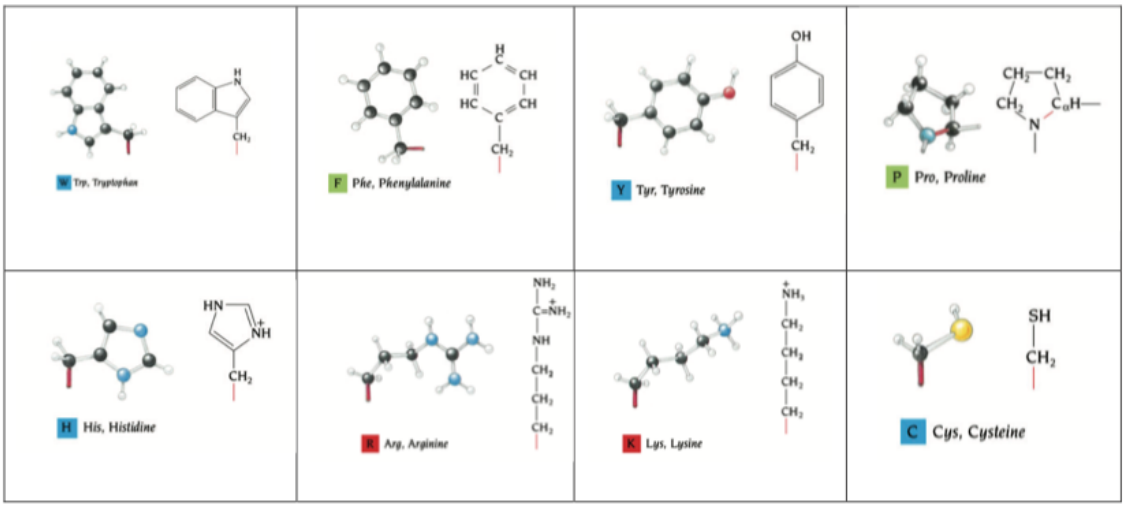
AMINO ACID			SIDE CHAIN
Alanine	Ala	A	nonpolar
Glycine	Gly	G	nonpolar
Valine	Val	V	nonpolar
Leucine	Leu	L	nonpolar
Isoleucine	Ile	I	nonpolar
Proline	Pro	P	nonpolar
Phenylalanine	Phe	F	nonpolar
Methionine	Met	M	nonpolar
Tryptophan	Trp	W	nonpolar
Cysteine	Cys	C	nonpolar

NONPOLAR AMINO ACIDS

Figure 3–2 The 20 amino acids found in proteins. Each amino acid has a three-letter and a one-letter abbreviation. There are equal numbers of polar and nonpolar side chains; however, some side chains listed here as polar are large enough to have some non-polar properties (for example, Tyr, Thr, Arg, Lys). For atomic structures, see Panel 3–1 (pp. 128–129).

[4] EX1039 (Alberts), 127.

38. Some amino acids have diverse chemical characteristics. For example, lysine (K) and arginine (R) have terminal amine groups (which are hydrophilic) and long aliphatic chains (which have a hydrophobic character).[5] Other amino acids incorporate ring structures (*e.g.*, tryptophan (W), phenylalanine (F), tyrosine (Y), histidine (H), proline (P)), which are rigid and may be aromatic. Cysteine (C) has the unique ability to form covalent disulfide bonds with other cysteines, which confers significant structural stability to areas of protein structure. The diversity of characteristics of amino acid side chains contributes to the incredible diversity of structures that proteins have.



39. Folded proteins generally are arranged to have a hydrophobic interior and a hydrophilic surface. The backbone or primary chain has a polar character,

[5] EX1014 (Brandon), 6-7.

which is hydrophilic and not energetically favored within a hydrophobic environment.[6]

40. Secondary structures formed out of characteristic patterns of amino acids, however, offset this intrinsically polar character of the backbone chain within the hydrophobic interior of proteins. Secondary structures form energetically favored structures within the backbone via hydrogen bonding between backbone NH and C'=O groups. The secondary structures then pack together to form motifs and higher order structures, both of which further stabilize the interior of the protein structure.

41. There are two general classes of secondary structures: (i) the alpha-helix (α-helix), and (ii) the beta-sheet (β-sheet) (illustrated below).[7]

[6] EX1014 (Brandon), 14.

[7] EX1039 (Alberts), 134-135.

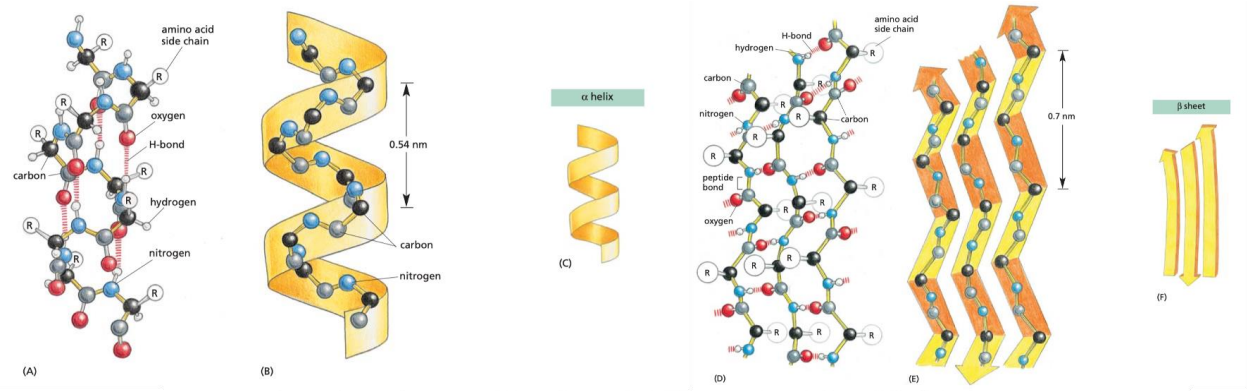


Figure 3–7 The regular conformation of the polypeptide backbone in the α helix and the β sheet. <GTAG> <TGCT> (A, B, and C) The α helix. The N–H of every peptide bond is hydrogen-bonded to the C=O of a neighboring peptide bond located four peptide bonds away in the same chain. Note that all of the N–H groups point up in this diagram and that all of the C=O groups point down (toward the C-terminus); this gives a polarity to the helix, with the C-terminus having a partial negative and the N-terminus a partial positive charge. (D, E, and F) The β sheet. In this example, adjacent peptide chains run in opposite (antiparallel) directions. Hydrogen-bonding between peptide bonds in different strands holds the individual polypeptide chains (strands) together in a β sheet, and the amino acid side chains in each strand alternately project above and below the plane of the sheet. (A) and (D) show all the atoms in the polypeptide backbone, but the amino acid side chains are truncated and denoted by R. In contrast, (B) and (E) show the backbone atoms only, while (C) and (F) display the shorthand symbols that are used to represent the α helix and the β sheet in ribbon drawings of proteins (see Panel 3–2B).

42. There are numerous variants of each class of secondary structure, each associated with characteristic patterns, spacing and types of amino acids in the polypeptide sequence. The structures are, to varying degrees, sensitive to the positioning and patterns of residues, as well as to the types of amino acids that may be in those positions.[8] For example, certain amino acids tend to favor being within a helical structure (*e.g.*, leucine), while others disfavor such structures if incorporated into a sequence that would ordinarily form an α-helix or β-sheet.[9]

43. The side chains of amino acids participating in a α-helix or β-sheet will either be directed to the interior of the secondary structure or directed to the

[8] See generally, EX1014 (Brandon), 16-20; EX1039 (Alberts), 131-135.

[9] EX1014 (Brandon), 353-4.

exterior of it. The interior is hydrophobic, while the exterior will generally be solvent accessible. However, when the secondary structures are packed together, the exterior facing side chains may interact with side chains of amino acids in other secondary structures (which may be a hydrophobic environment), and the effects of those interactions may be stabilizing or destabilizing.

44. Structural motifs form from combinations and arrangements of secondary structures, and those structural motifs, in turn, pack together to form the higher order structure that characterizes a protein domain. There are numerous influences on how these structural motifs are formed and stabilized.

45. For example, sequences that form secondary structures are often separated by stretches of amino acids of varying lengths. The nature and length of those intervening sequences influence how secondary structures on either end of the intervening sequence can interact with each other or with other secondary structures in the protein. To illustrate this, consider the figures below, which shows schematically how different lengths of intervening sequences between β-sheet secondary structures influence the packing of pairs of beta sheets and then the structural motifs created by those pairs into a higher order structure.[10]

[10] EX1014 (Brandon), 77-78.

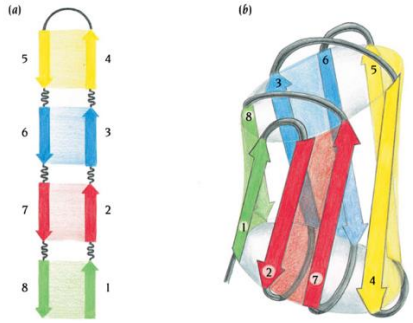


Figure 5.17 A simple illustration of the way eight β strands are arranged in a jelly roll motif. (a) The eight β strands are drawn as arrows along two edges of a strip of paper. The strands are arranged such that strand 1 is opposite strand 8, etc. The β strands are separated by loop regions. (b) The strip of paper in (a) is wrapped around a barrel in the same way as the string in Figure 5.16, such that the β strands follow the surface of the barrel and the loop regions (gray) provide the connections at both ends of the barrel. The β strands are now arranged in a jelly roll motif.

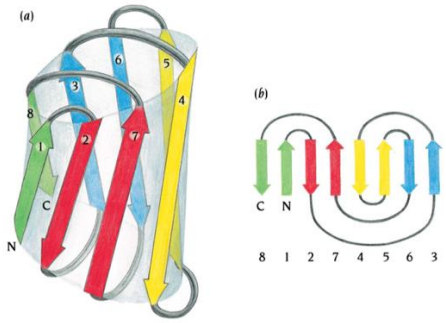


Figure 5.18 Topological diagrams of the jelly roll structure. The same color scheme is used as in Figure 5.17.

46. The specific amino acids in specific positions of an amino acid sequence also play a critical role in inducing the folding of the protein into its destined structure, as well as in maintaining that structure.[11]

B. Protein Engineering

1. Rational Design vs. Directed Evolution

47. Protein engineering aims to create novel proteins not found in nature with desired properties. At a conceptual level, there are two approaches for

[11] EX1039 (Alberts), 125-130.

creating engineered proteins, referred to as "rational design" and "directed evolution."[12]

48. "Rational design" involves an in-depth study of the structural features of a protein and their contribution to the stability and function(s) of the protein. The insights derived from that study are used with knowledge of protein structure principles to devise a modified sequence that will alter the protein's structural features in an intended manner to yield a protein that meets the goal of the engineering project (*e.g.*, greater stability, higher activity).[13]

49. Various computational tools are used in rational protein design. For example, an important part of the analysis of a protein is one focused on evolutionary-related, homologous proteins. Creating an alignment of homologous proteins helps to identify conserved residues and positions where variation in the residue occurs (and thus is tolerated in the proteins).[14] The other important tool is the structural model of the protein, which can be generated using automated procedures, such as the SWISS-MODEL server. The protein engineer used these

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[12] EX1059 (Leisola), 1225-1227; EX1018 (Chica), 378-379; EX1017 (Green), 223.

[13] EX1018 (Chica), 378.

[14] EX1017 (Green), 224-228.

models to visualize parts of the protein structure, and consider spatial interactions between the different residues.[15]

50. There were limits to using rational design techniques in the 2011-timeframe.[16] For example, modifying a known protein to incorporate a single amino acid change in a sequence responsible for a known secondary structure that was not essential to the protein's functions was fairly straightforward. In that setting, one determines the interactions of the new amino acid with other residues in the static environment of the position being changed. In contrast, devising a sequence incorporating 10 substitutions into a region where the set of ten can influence each other or several discrete structures in the protein was an exponentially more challenging exercise. There, the environment of each change is not static and will likely be influenced by the other changes being made. The

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15 EX1017 (Green), 227-230.

16 EX1018 (Chica), 378 (rational design "requires an in-depth knowledge of the structural features of the enzyme active site and their contribution to function. The complexity of the structure/function relationship in enzymes has proven to be the factor limiting the general application of rational design."); EX1059 (Leisola), 1225-1226.

complexity escalates rapidly with the number of changes being made, with a corresponding increase in the magnitude of work that must be performed.

51. Directed evolution arose out of the limits of rational design.[17] It has the same goal of producing a protein with a modified sequence that gives it desired properties. However, it aims to bypass the complexity and difficulty of devising that modified sequence *a priori*. In "directed evolution," one generates large libraries of mutant proteins with randomly distributed mutations using genetic methods, then screens those libraries to find mutants having the desired properties. The mutants with those properties are then isolated and characterized. Those mutant proteins with enhanced properties are then chosen and the modified amino acid sequence of the mutant protein is recorded.

52. The challenge with directed evolution is scale.[18] One has to identify the successful mutant out of an immense number of possibilities, which presents

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[17] EX1059 (Leisola), 1225-1226 ("However, because the difficulty is likewise indisputable, any approach that might succeed sooner is worth exploring. That realization has motivated work at the other end of the spectrum, where the emphasis is on finding what works rather than predicting what works.").

[18] EX1018 (Chica), 378 ("Because large numbers of mutants must generally be screened to obtain a significant, desired effect on enzyme activity, the main

different kinds of challenges. Most significantly, it requires sophisticated methodologies for generating mutations likely to yield beneficial changes, highly efficient, rapid and accurate screening methods that identify mutants which have desired properties (and which avoid those that do not), and efficient recovery and characterization techniques. It also depends on the science—the desired function must be physically possible in the protein, and it must be possible for a protein to incorporate mutations that deliver that function. A 2009 paper summarizes factors first articulated by Dr. Francis Arnold at Caltech that provide a good description of these requirements for a successful directed evolution campaign:

1. The desired function must be physically possible.

2. The function must also be biologically or evolutionarily feasible. In practice, this means that there exists a mutational

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limitation of directed evolution is the necessity of developing a high-throughput screening methodology that allows identification of the desired property under relevant conditions. Not all enzyme activities are readily amenable to developing a high-throughput screening method, nor are all screening methodologies easy to implement at the required scale."); EX1059 (Leisola), 1227 ("You must have a rapid screen or selection that reflects the desired function.").

> pathway to get from here to there through ever-improving variants.
>
> 3. You must be able to make libraries of mutants complex enough to contain rare beneficial mutations.
>
> 4. You must have a rapid screen or selection that reflects the desired function.[19]

53. A concise summary of the two alternative approaches was provided in a 2009 paper by Leisola and Turunen:

> At one end is an approach commonly referred to as a rational design, which aims to understand the principles of protein structure and function well enough to apply them in designing new properties or even novel proteins using de novo design. The value of this approach in purely scientific terms is indisputable. However, because the difficulty is likewise indisputable, any approach that might succeed sooner is worth exploring. That realization has motivated work at the other end of the spectrum, where the emphasis is on finding what works rather than predicting what works.[20]

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19 EX1059 (Leisola), 1226-1227.

20 EX1059 (Leisola), 1226, Figure 1.

2. Challenges with Predicting the Effects of Multiple Mutations in Proteins

54. Introducing a single mutation into a protein's sequence can be highly impactful if the substituted residue plays a critical role in the function of the protein (*e.g.*, a residue involved in catalysis mediated by the protein) or is a conserved amino acid necessary to an essential structure or function of the protein. By contrast, single amino acid substitutions at positions that are fully solvent exposed (*i.e.*, interacts primarily with water molecules rather than other residues of the protein) or at a non-conserved position that varies extensively within a set of homologous, naturally occurring variants of the protein are often tolerated. Of course, each change needs to be assessed on a case-by-case basis that considers each amino acid's unique characteristics and the environment within the protein where the change is being made.

55. Introducing multiple mutations into the amino acid sequence of a protein is a different story. Because so many secondary structures are dependent on particular patterns of amino acids with the correct spacing and character of amino acids at different positions, changing many amino acids simultaneously risks disrupting the pattern necessary to induce formation of the original secondary

structure. Eliminating or altering a secondary structure can prevent the protein from folding and be highly destabilizing to the overall protein structure.[21]

56. Introducing multiple substitutions into a protein's amino acid sequence will introduce many new steric and/or chemical interactions between amino acids that can influence the protein's overall structure. There are a variety of such interactions possible depending on each pair of amino acids being considered (illustrated in figure below).[22] Any single substitution may impact numerous interactions, and potentially with competing effects (*e.g.*, some beneficial and some adverse to the protein's stability). Examples of non-covalent interactions that occur between residues within a protein structure are listed below:

(a) Hydrogen bonds: These occur between a hydrogen atom and an electronegative atom like oxygen or nitrogen and are energetically favorable.

(b) Ionic interactions: Also known as salt bridges, these occur between positively and negatively charged side chains and are energetically

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[21] EX1046 (Beasley), 2034. *Also* EX1047 (Xiong), 6349, 6352 ("the choice between α-helical and β-sheet secondary structure is controlled by the sequence periodicity of polar and nonpolar amino acids.")

[22] EX1039 (Alberts), 126-127, 130.

favorable. Where the side chains have the same charge, interactions between those side chains are energetically unfavorable.

(c) Van der Waals forces: These are weak interactions that occur between all atoms when they are in close proximity and are energetically favorable.

(d) Hydrophobic interactions: These occur between nonpolar side chains, driving them to the interior of the protein to avoid water. Where both residues are hydrophobic, the interactions are energetically favorable. If one residue is hydrophobic and the other is polar, the interaction is energetically unfavorable.

(e) Pi-pi interactions: These occur between aromatic side chains, such as those of phenylalanine, tyrosine, and tryptophan, and are energetically favorable.

(f) Cation-pi interactions: These occur between a positively charged side chain and an aromatic side chain and are energetically favorable.

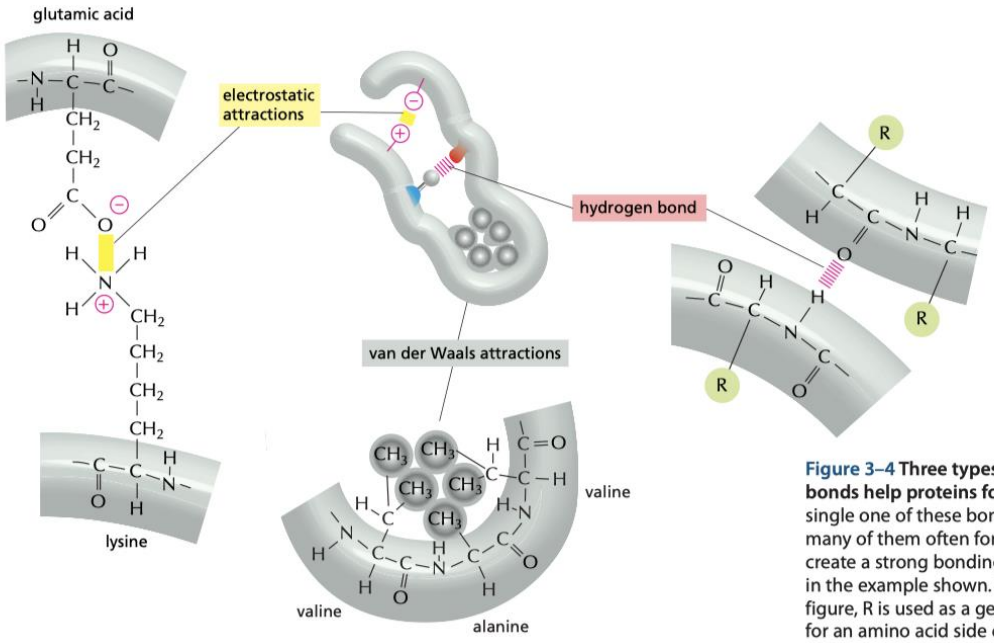

Figure 3–4 Three types of noncovalent bonds help proteins fold. Although a single one of these bonds is quite weak, many of them often form together to create a strong bonding arrangement, as in the example shown. As in the previous figure, R is used as a general designation for an amino acid side chain.

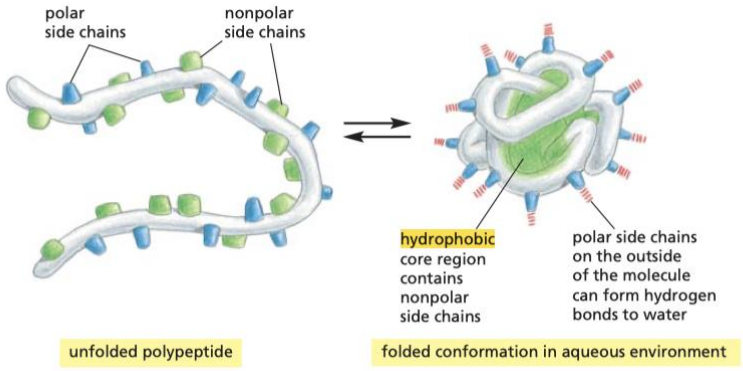

Figure 3–5 How a protein folds into a compact conformation. The polar amino acid side chains tend to gather on the outside of the protein, where they can interact with water; the nonpolar amino acid side chains are buried on the inside to form a tightly packed hydrophobic core of atoms that are hidden from water. In this schematic drawing, the protein contains only about 30 amino acids.

57. In any region of a protein structure, there are myriad interactions that occur between residues in the protein that define the structure of the protein in that region. The collective effect of those interactions dictates the stability and thus maintenance of that region of the protein structure.

58. Introducing one substitution in a region of a protein can create a new set of interactions with neighboring amino acids in that position that are favorable

to the stability of the protein. Introducing nine more substitutions in that region along with the first one, where some of the other nine causes unfavorable interactions, may collectively destabilize that region of the protein structure, despite the positive contribution of the first substitution. There are approximately 6×10^{12} different scenarios of 10 substitutions in just this one example (*i.e.*, 10 positions, with 19 different alternative amino acids, or 19^{10}).

59. The folding of secondary structures and higher order structures is often sensitive to the correct positioning of parts of the polypeptide chain during the folding process. Multiple substitutions made in a sequence may alter that timing and the presentation of those portions of the polypeptide chain that are necessary to form secondary structures or position them to form higher order structures. Introducing multiple amino acid changes simultaneously in this example could prevent the folding of sequences into secondary structures and structural motifs and can destabilize those structures if they do form.

60. Making multiple changes to an amino acid sequence can also cause formation of different types of secondary structures within the protein, which are highly disruptive to the original structure of the protein. For example, a group at Yale demonstrated that changing 28 residues caused a domain of a protein having a fold made up of four-stranded β sheet and one α-helix structures to adopt a different fold made up of all α-helical structures. In this example, the investigators

had the starting structure and the ending structure and engaged in experiments to convert one into the other.[23]

61. An enzyme, like PH20, with one amino acid substitution that exhibits increased enzymatic activity does not prove that the same protein with 9 more changes in addition to the first will also do so. The other 9 substitutions may individually or collective disrupt the structure of the enzyme in a way that causes it to exhibit decreased activity or to be rendered inactive. That effect may occur independently of the effect of the first substitution, or the other 9 changes may collectively disturb the environment of the first substitution in a way that negates the first change's effect. The effects caused by one substitution in a protein like PH20 thus cannot predict the effects on a modified form of that protein that incorporates 5, 10, 15 (or more) substitutions. A skilled artisan would not view the first, single amino acid substituted PH20 to as be representative of all modified PH20 proteins having that one substitution, along with 5, 10, 15, or more additional substitutions.

[23] EX1014 (Brandon), 368-370.

C. The Stability of a Protein Is Measured by Assessing the Free Energy Difference Between Its Folded and Unfolded States

62. Many proteins, particularly globular ones like PH20, are inherently unstable. Slight changes in pH or in temperature can convert a properly folded and active protein into an unfolded and inactive form (figure below).[24]

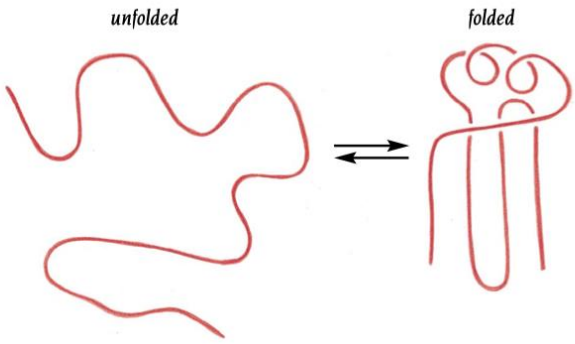

Figure 6.1 A polypeptide chain is extended and flexible in the unfolded, denatured state whereas it is globular and compact in the folded, native state.

63. The stability of a protein corresponds to the energy required to induce a transition from an unfolded state (an ensemble of disordered chains) to the folded state.[25] For many proteins, the total energy difference between the two states (the "free energy" difference) is small (*e.g.*, 5-15 kcal/mol), which is not much greater than the energy contribution of a single hydrogen bond.[26]

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[24] EX1014 (Brandon), 90.

[25] EX1014 (Brandon), 90; EX1039 (Alberts), 126.

[26] EX1014 (Brandon), 90.

64. There are two major contributors to the energy difference between the folded and unfolded states of a protein: enthalpy and entropy. Entropy derives from the second law of thermodynamics, which holds that energy is required to create order. Enthalpy is the energetic contribution that provides order and is the net result of a myriad of non-covalent interactions that occur between the residues within the folded form of the protein.

65. There are numerous types of non-covalent interactions that can occur between residues in a protein structure, and each can contribute to or detract from the net stability of the protein. For example, non-covalent interactions occur between atoms within the side chains of amino acids that are near each other within the protein structure. The interactions can be energetically favorable or unfavorable, and each can vary in magnitude of its energetic contribution. These interactions also are not static, as the residues within proteins are in continuous motion, and that motion varies in response to the environment of the protein (*e.g.*, changes in pH, temperature, salts, etc.).[27]

66. The "free energy" difference of a protein can be determined using a variety of experimental techniques. Generally, these techniques expose the protein to varying concentrations of a denaturing agent (urea, guanidine HCL) or varying

[27] EX1039 (Alberts), 126-131; EX1014 (Brandon), 13-14.

temperatures, and then detect the transition point between the folded and unfolded states of the protein using a scientific instrument (illustrated below).[28] For example differential scanning calorimetry (DSC) measures the heat capacity of the protein as it is heating, which allows one to determine the melting temperature and the enthalpy change associated with unfolding. Other instruments that can be used to measure the folding-to-unfolded transition include circular dichroism, fluorescence or nuclear magnetic resonance (NMR) spectrophotometers.

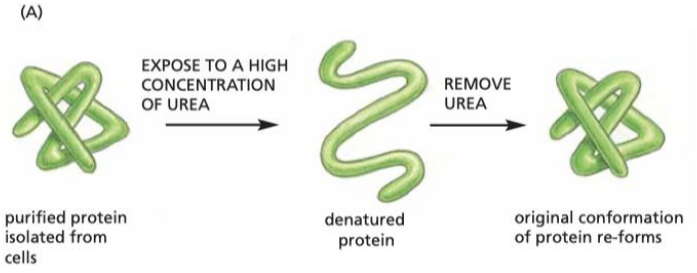

Figure 3–6 The refolding of a denatured protein. (A) This type of experiment, first performed more than 40 years ago, demonstrates that a protein's conformation is determined solely by its amino acid sequence. (B) The structure of urea. Urea is very soluble in water and unfolds proteins at high concentrations, where there is about one urea molecule for every six water molecules.

D. Activity Is Not Synonymous with Stability

67. Portraying an increase or decrease in biological activity of a mutated form of a protein as being indicative of a change in the stability of a specific aspect of a protein relative to the unmodified form of the protein is unwarranted without some direct basis for connecting the change in activity to the change in stability. To determine if a mutation affects the stability of a protein, it is necessary to use an

[28] EX1039 (Alberts), 130-131.

appropriate experimental technique to assess the stability of protein structure, which is one that compares the energy required for state transitions for the wild-type and mutant proteins. Without some evidence that directly connects a change in activity to a change in the stability of the protein, it is not appropriate to simply equate a change in biological activity with a change in stability.

68. The common disclosure makes this mistake. It provides two tables measuring the effects on hyaluronidase activity of 409 mutants at two temperatures (4°C and 37°C), and in the presence of a preservative (a "phenophile" called m-cresol).[29] One table (Table 11) reports measured hyaluronidase activities of the mutants while the other table (Table 12) divides the measured hyaluronidase activities for each mutant at two different conditions (*i.e.*, activity of a mutant at 37°C vs. 4°C, or activity of a mutant at 37°C with m-cresol vs. without m-cresol at 4°C or 37°C).

69. No direct thermodynamic assessment is provided for 408 of the 409 mutants that would indicate that the measured activity under the tested conditions is due to increased stability, or is due to other factors, such as more efficient catalysis within an equivalently stable protein. The tables show that most of the

[29] EX1001 ('520 Patent), 257:6-258:56, Tables 11, 12; *also* Appendix A-6 (Table 12 w/ colors).

tested mutants showed a significant reduction in activity in the presence of m-cresol, with the vast majority showing less than 20% activity. The common disclosure also does not characterize the effects of any particular mutation on the structural features of PH20, such as, for example, the catalytic site, substrate binding site, or other portions of the protein known to be important to hyaluronidase activity (discussed further below). There is also no discussion of whether particular mutations affected the secondary structure or structural motifs within PH20 proteins, and whether and why such structural changes would impact stability or hyaluronidase activity of the protein.

70. A second problem with the data presented in Tables 11 and 12 is that the positive control showed significant variability in the assays being used. I note that the patent as printed does not make clear what the last set of values in Tables 11/12 is referring to, but the originally filed '731 patent application does. They are values for the positive control (EX1026) (comparison below for Table 11):[30]

[30] The positive control was also used in additional testing of the F204P mutant. EX1001 ('520 Patent), 277:7-17 ("The positive control (WT PH20-OHO) showed a reduction in activity of 75% and 83% on the day of the assay (as assayed from two different OHO transfections). This demonstrated that the

TABLE 11: Absolute Hyaluronidase Activity

Mutant	No incubation (4 °C)		37 °C no cresol (37 °C)		37 °C with m-cresol (37 °C plus m-cresol)	
positive control (OHO)	2.919	2.173	2.773	2.105	0.145	0.178
	3.984	4.463	4.215	4.823	0.189	0.253
	3	2.725	3	3.325	0.1	0.125
	2.501	2.883	2.370	3.158	0.452	0.522
	7.629	2.989	10.835	3.914	0.485	0.219
	5.783	5.356	2.609	3.643	0.542	0.402
	5.279	5.422	2.815	4.026	0.618	0.401
	4.775	4.385	2.845	3.327	0.718	0.540
	3.617	4.264	3.322	3.427	0.633	0.479
	5.881	4.511	5.518	4.359	0.743	0.848
	6.754	4.932	3.902	4.120	0.665	0.724
	3.911	3.494	3.911	5.179	0.726	0.841
	5.406	7.559	4.018	4.620	0.735	0.429
	4.015	3.887	3.9400	3.4080	0.3340	0.3410
	2.604	2.339	2.4430	2.3910	0.2350	0.2330
	3.736	3.473	3.6210	3.0560	0.3100	0.2770
	3.759	3.509	3.6330	3.0490	0.3600	0.3030

TABLE 11-continued

Absolute Hyaluronidase Activity

Mutant	No incubation (4° C.)		37° C. no cresol (37° C.)		37° C. with m-cresol (37° C. plus m-cresol)	
positive control (OHO)	2.919	2.173	2.773	2.105	0.145	0.178
	3.984	4.463	4.215	4.823	0.189	0.253
	3	2.725	3	3.325	0.1	0.125
	2.501	2.883	2.370	3.158	0.452	0.522
	7.629	2.989	10.835	3.914	0.485	0.219
	5.783	5.356	2.609	3.643	0.542	0.402
	5.279	5.422	2.815	4.026	0.618	0.401
	4.775	4.385	2.845	3.327	0.718	0.540
	3.617	4.264	3.322	3.427	0.633	0.479
	5.881	4.511	5.518	4.359	0.743	0.848
	6.754	4.932	3.902	4.120	0.665	0.724
	3.911	3.494	3.911	5.179	0.726	0.841
	5.406	7.559	4.018	4.620	0.735	0.429
	4.015	3.887	3.9400	3.4080	0.3340	0.3410
	2.604	2.339	2.4430	2.3910	0.2350	0.2330
	3.736	3.473	3.6210	3.0560	0.3100	0.2770
	3.759	3.509	3.6330	3.0490	0.3600	0.3030

71. The data from Table 11 was used to recreate the % activity values in Table 12 for the positive controls. The colors indicate ranges of activity. The activity ranges, averages and mean values for the control unmodified $PH20_{1-447}$ are summarized below. Also, the values are plotted in Appendix A-8.

F204P phenophile was able to retain 60% to 90% or greater of its activity above the residual activity of the wildtype PH20 control enzyme.")

Positive Control ("PC") (OHO)	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol / 37°C	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol / 37°C	% Activity at 37°C+mcr/4 °C
PC1	94.998	5.230	4.970	96.871	8.456	8.190
PC2	105.798	4.480	4.740	108.066	5.246	5.670
PC3	100.000	3.330	3.330	82.778	3.759	4.590
PC4	94.762	19.070	18.070	109.539	16.529	18.110
PC5	142.024	4.480	6.360	130.947	5.595	7.330
PC6	45.115	20.770	9.370	68.017	11.035	7.510
PC7	53.324	21.950	11.710	74.253	9.960	7.400
PC8	59.581	25.240	15.040	75.872	16.231	12.310
PC9	91.844	19.050	17.500	80.371	13.977	11.230
PC10	93.828	13.470	12.630	96.630	19.454	18.800
PC11	57.773	17.040	9.850	83.536	17.573	14.680
PC12	100.000	18.560	18.560	148.226	16.239	24.070
PC13	74.325	18.290	13.600	61.119	9.286	5.680
PC14	98.132	8.480	8.320	87.677	10.006	8.770
PC15	93.817	9.620	9.020	102.223	9.745	9.960
PC16	96.922	8.560	8.300	87.993	9.064	7.980
PC17	96.648	9.910	9.580	86.891	9.938	8.630

KEY
Coloration of Percent (%) Activity Values
n/a
>120
between 100 and 120
between 80 and 100
between 40 and 80
between 20 and 40
between 10 and 20
between 0 and < 10

	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/ 4°C
High	142.02	25.24	18.56	148.23	19.45	24.07
Low	45.12	3.33	3.33	61.12	3.76	4.59
Range	96.91	21.91	15.23	87.11	15.70	19.48
Average	88.17	13.38	10.64	93.00	11.30	10.64
Mean	94.76	13.47	9.58	87.68	9.96	8.63

72. As the tables and plots (Appendix A-8) show, the positive control exhibited a significant amount of variation in its measured activity, which raises serious doubts about how probative or instructive the values for individual tested mutants are that fall within the range of variability observed for the control. There are also no statistical measurements of the data that is reported. A skilled artisan

would not view a measured value that is in the range of values of the control to a reliable indicator of a difference.

73. The common disclosure also suggests that 37°C is a denaturing temperature for PH20.[31] That, however, is the normal human body temperature in which PH20 exists naturally. It is unsurprising that single-replacement $PH20_{1-447}$ polypeptides showed higher activity at 37°C than at 4°C, given that PH20 exists at that temperature in humans. Also, only 5 out of 17 of the duplicate #1 runs for the positive control, and 4 out of 17 of the duplicate #2 runs showed significant decreased activity for the wild-type $PH20_{1-447}$ polypeptide at 37°C vs 4°C. Given the variability of the data for the control, portraying 37°C as a denaturing condition is not warranted. Instead, most of the relative activity values at 37°C vs. 4°C cannot be meaningfully differentiated from the control.

74. The common disclosure identified just two mutants (F204P and V58R mutants) out of 409 that were tested that retained more than 70% of their activity in the presence of m-cresol preservative in both of the tested duplicates. In Example 9 (Table 15), the data shows the F204P variant remained active over a longer period (4 weeks to 6 days) than the wild-type in the presence of m-cresol. In Example 11 (Table 22), both mutants retained more activity over time with m-

[31] EX1001 (’520 Patent), 171:11-20.

cresol than the unmodified control. The data as a whole is not indicative of a broader trend of stability in the 409 PH20 mutants.

75. The one direct assessment of stability is in Example 13 and it tested only one mutant: F204P $PH20_{1-447}$. This example reported that F204P $PH20_{1-447}$ had a melting point (Tm) that was 9°C higher than unmodified $PH20_{1-447}$. If the data were generated by experimentally valid procedures, that would suggest that the F204P $PH20_{1-447}$ is more stable than unmodified $PH20_{1-447}$. It is not possible to determine if it was, because experimental details were omitted. The higher stability also does not tell the skilled artisan ***why*** it is more stable, or whether it will retain this increased stability if other substitutions are made to the F204P $PH20_{1-447}$. Other mutations may and usually do independently influence stability, which can be near the structure containing F204P or somewhere else in the protein that, in the aggregate, offsets the reported stabilizing effect of F204P.

76. Overall, the data for testing the 409 mutants reported in Tables 11 and 12 does not provide any meaningful guidance to a skilled artisan about the types of mutations would improve the stability of PH20 polypeptides generally, or for the $PH20_{1-447}$ form specifically. It also does not provide insights for the skilled artisan regarding the impact of multiple substitutions on the PH20 structure in addition to one of the tested changes because there are no mutants being evaluated with more than a single amino acid change. In fact, the data on single-substitutions is

inconsistent between the two duplicate experiments for many individual mutants, and few show changes outside the range seen for the positive control. The increased activity (where it is observed) cannot be ascribed solely to improved stability and does not meaningfully guide a skilled artisan in how to create a more stable multiply-modified PH20 polypeptide, or a multiply-modified PH20 polypeptide that will exhibit increased resistance to or stability under any denaturing conditions.

E. Hyaluronidases and PH20

77. PH20 is a member of a family of five human hyaluronidase enzymes and is similar to hyaluronidase enzymes found in a wide range of organisms (*e.g.*, bacterial, fungal, insect, mammalian).[32] PH20 selectively catalyzes the hydrolysis of β1,4 glycosidic bonds in hyaluronan (also called "HA" or hyaluronic acid) (below). It does not act on β1,3 glycosidic bonds in HA.

[32] EX1008 (Stern), 819.

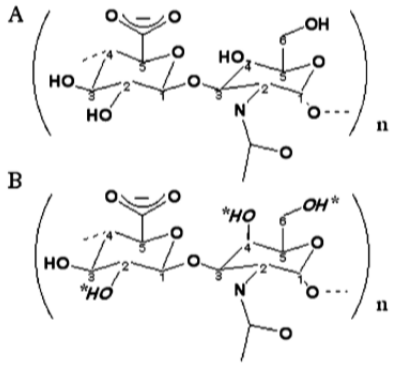

Figure 1. Chemical structures of (A) hyaluronan and (B) unsulfated chondroitin and chondroitin sulfates. The substrate structures differ only in the anomericity at the C4 position of the *N*-acetyl-D-glycosamine, *N*-acetyl-D-glucosamine in hyaluronan, and *N*-acetyl-D-galactosamine in chondroitin. Potential sulfation sites within the chondroitin molecule are indicated by italicized hydroxyl groups and asterisks. Both glycans are substrates for the human hyaluronidase enzymes. For Hyal-1 and -2, HA is the predominant substrate. However, binding and degradation of Ch/ChS also occurs, albeit at a slower rate, as observed experimentally in vitro. Hyal-4 appears to be a chondroitinase, with high specificity for Ch and ChS.

1. Characterization of Hyaluronidases

78. An early study (Arming (EX1011)) reported a number of conserved residues between human PH20 and bee venom hyaluronidase ("bvH"). Arming also reported that mutating five different residues individually resulted in a significant loss of activity ("This indicated that three of the mutants, [Glnl13]PH-20, [Gln249]PH-20, and [Thr252]PH-20 were devoid of enzymatic activity, while the two other mutants, [Asnlll]PH-20 and [Gly176]PH-20, had residual activities in the range of one to a few percent of wild-type PH-20 hyaluronidase.").[33]

[33] EX1011 (Arming), 813.

79. Arming also identified a number of conserved cysteine residues in the PH20 protein, which are identified in Figure 1 of Arming.[34] These conserved cysteines are also highlighted in a later alignment of the human hyaluronidases reported in Chao.[35] From Arming and Chao it was known that these cysteine residues are critical residues because they form disulfide bonds necessary to main the structure of PH20 and other hyaluronidases.

80. The first experimentally determined structure of a hyaluronidase was of bvH, both alone and in complex with HA (published in 2007). Markovic-Housley identified the catalytic site and residues involved in catalytic activity using this structure.[36] It also provided a sequence alignment comparing PH20, human HYAL1, human HYAL2 and bvH which identified conserved residues between the proteins.[37]

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[34] EX1011 (Arming), 811.

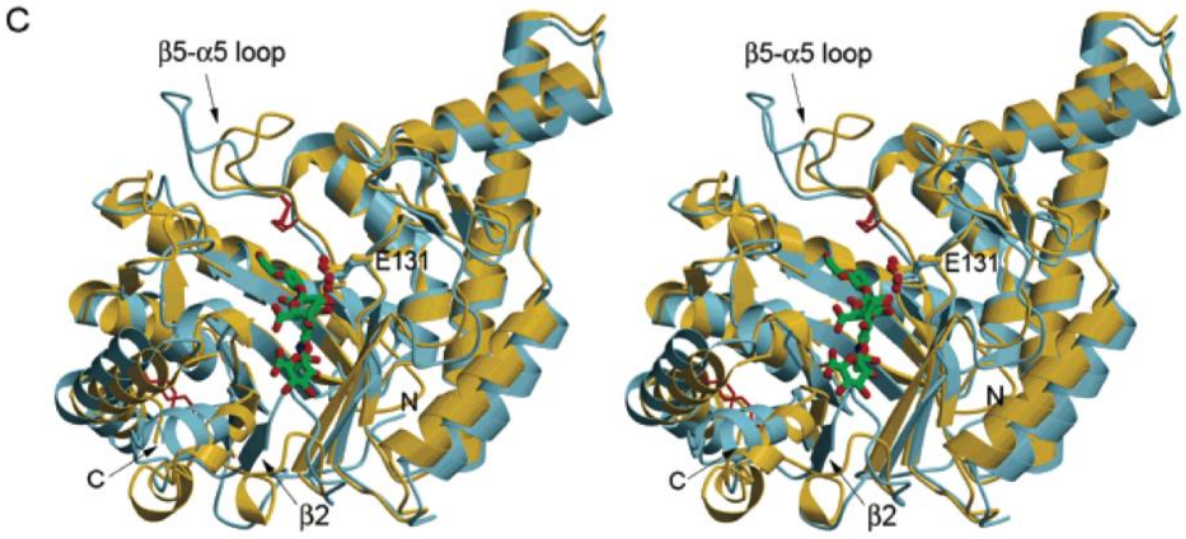
[35] EX1006 (Chao), 6916.

[36] EX1033 (Markovic-Housley), 1028-1031 (PDB identifiers: 1FCU, 1FCQ).

[37] EX1033 (Markovic-Housley), 1026.

2. Chao Reports Structure of Human HYAL1 and the Hyal-EGF Domain

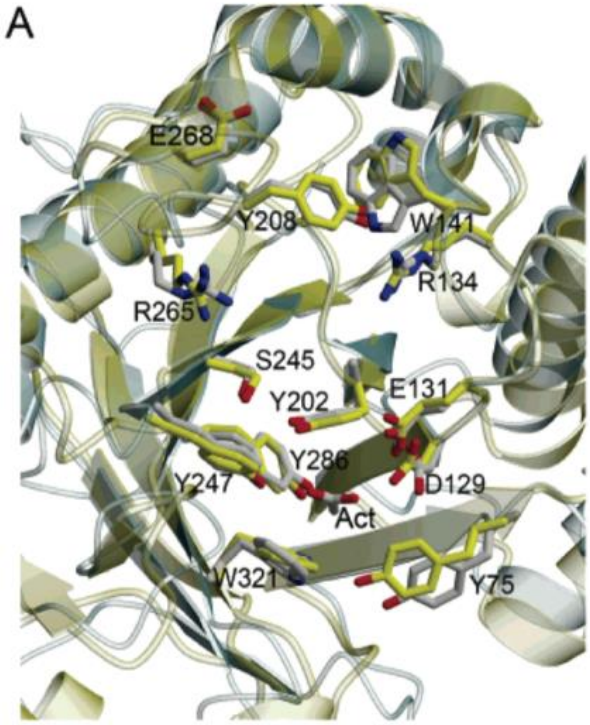
81. A structure for human HYAL1 was published by Chao and Herzberg in 2007 ("Chao") (EX1006). The Chao paper compared the human HYAL1 structure with the bvH structure that had previously been reported and observed that the two proteins had extensive overall structural similarity (Figure 2C, Chao).[38]



82. Chao also compared the catalytic sites of the two proteins, and found that they also exhibited extensive structural similarity (Figure 4A, Chao)[39]:

[38] EX1006 (Chao), 6915.

[39] EX1006 (Chao), 6917.



83. Chao provided an annotated alignment of the five human hyaluronidase enzymes which identified conserved residues among the set of five related proteins, identified cysteine residues involved in disulfide bonds, and included annotations identifying secondary structures with the proteins (*i.e.*, α-helices indicated by coiled illustration, and β-sheets by an arrow).[40]

[40] EX1006 (Chao), 6916.

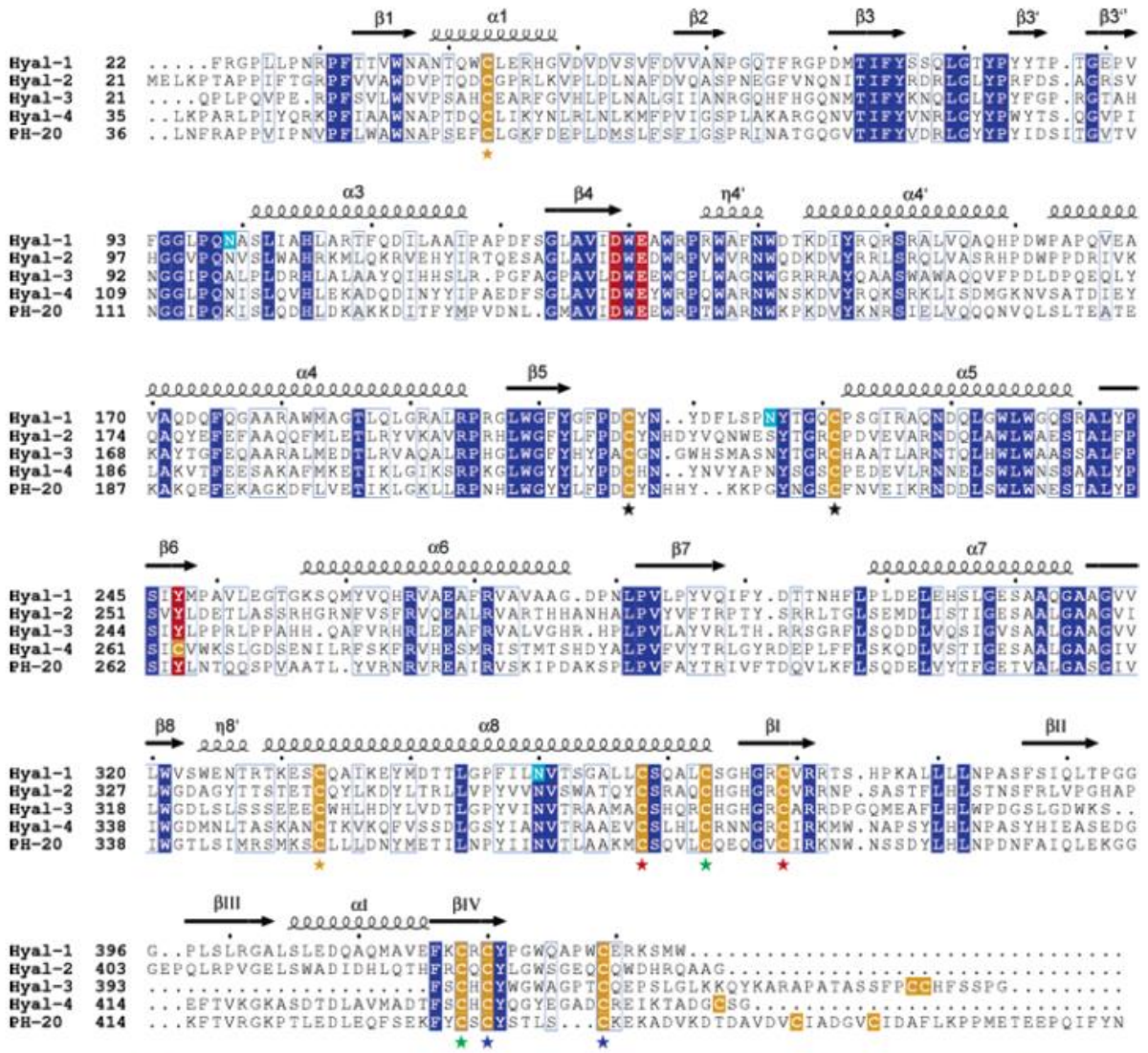


FIGURE 3: Structure-based sequence alignment of human hyaluronidases. Invariant residues are shown in blue except for three key catalytic residues that are colored red. Cysteine residues are colored yellow. The hHyal-1 N-glycosylated asparagines residues are colored turquoise. Residues exhibiting conservative replacements are blocked in blue. Pairs of cysteine residues that form disulfide bonds are indicated by stars with matching colors. Secondary structure units are labeled as in Figure 2B.

84. A notable finding in Chao was its identification of the "Hyal-EGF" domain in the C-terminal region of human hyaluronidases. Chao observed that the C-terminal region of mammalian hyaluronidases does not exhibit significant homology but does contain a cysteine-rich pattern of residues recognized by sequence analysis tools as an epidermal growth factor (EGF)-like motif (below).

Chao identified the pattern of sequences that generates this Hyal-EGF structural motif (*i.e.*, "x4Cx0-48Cx3-12Cx1-70Cx1-6Cx2Gax0-21Gx2C, where "a" denotes a hydrophobic residue, "x" denotes any residue, and the gaps between cysteine residues vary in length as indicated by the subscripts.").[41] This is a good illustration of how a pattern of amino acids shared within a family of related proteins can induce formation of a similar structural motif in those family members.

Figure 4B

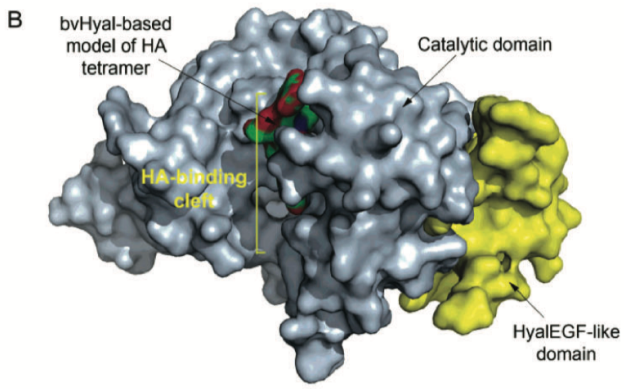
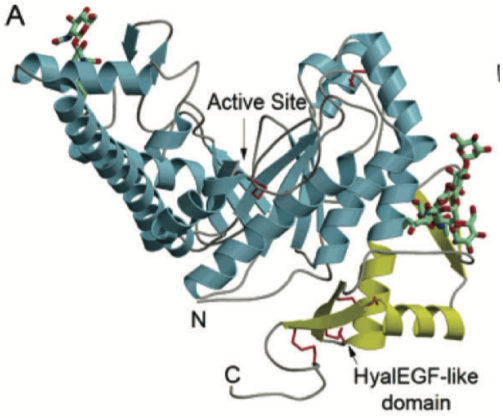


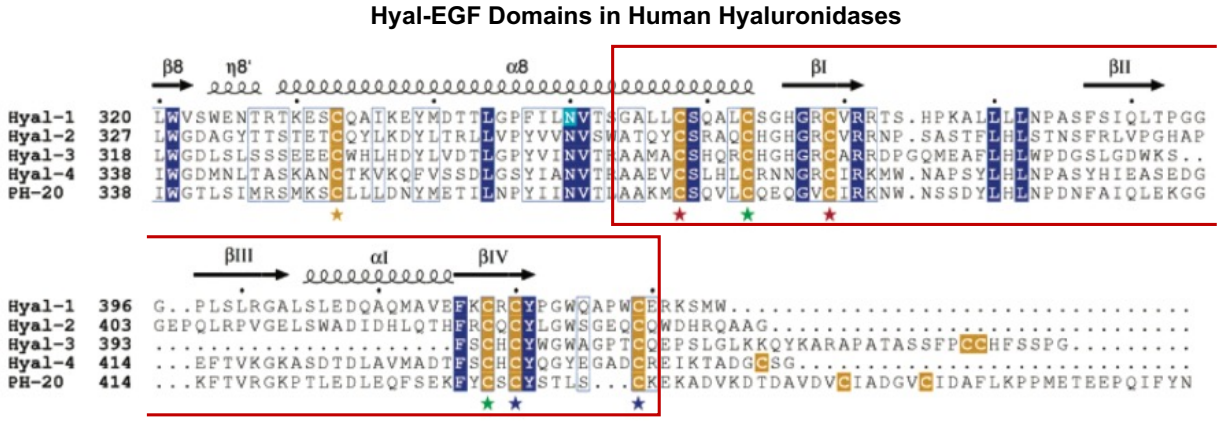
Figure 2A



85. Below is an excerpt from the Chao alignment annotated to show the location of the sequences that constitute the Hyal-EGF domain (*i.e.*, positions 337-409 in PH20), which Dr. Park identified. The constituent cysteines of the pattern are at positions 341, 346, 352, 400, 402, and 408, while the constituent glycines are

[41] EX1006 (Chao), 6912.

at positions 350, 377, 378, and 384.[42] Appendix A-9 also shows the substitutions that were classified as "active" or "inactive" mutants in Tables 3 and 5 of the common disclosure at positions between 337-409, plus 410-412.



86. The Chao paper provided new, highly relevant information for evaluating structural features of PH20, particularly by someone interested in modifying the structure of PH20. For example, its sequence alignment identified secondary structures and sites of conserved amino acids in both PH20 and HYAL1. Its structure of HYAL1 provided important insights, such as the existence of the Hyal-EGF domain, and provided a template to use in more accurate modeling of PH20. I did not find any discussion of Chao in the common disclosure of the patents, even though Chao was published many years before those patents were first filed in late 2011.

[42] EX1004 (Park Dec.), ¶ 98.

87. A 2009 paper by Zhang et al. (EX1010) reported that deleting the portion of the HYAL1 sequence containing its Hyal-EGF domain substantially eliminated HYAL1's hyaluronidase activity (*i.e.*, activity of ~6%).[43] It also tested the effects of mutating residues in proximity to the HA substrate within the active site. It identified those ligand-interacting residues by overlaying the HYAL1 structure on the bvH structure in complex with HA ligand, and measured distances to different residues indicative of interactions. This comparison was possible because of the highly conserved structure of the active site of hyaluronidases. Zhang also showed that single substitution mutants at each of these identified positions in HYAL1 rendered the enzyme inactive or significantly reduced its hyaluronidase activity (summarized in Table 1) (below).[44]

[43] EX1010 (Zhang), 9437-9439.

[44] EX1010 (Zhang), 9435-9438, Table 1.

TABLE 1
Summary of Hyal1 wild-type (WT) and mutant kinetic constants

Enzyme	K_m	V_{max}	% WT activity at 50 μM HA
	μM	μmol/min/mg	
Hyal1 wild-type	38.1 ± 4.8	12.5 ± 0.7	100.0 ± 1.8
Catalytic mutants			
E131Q	NA[a]	NA	0.08 ± 0.01
Y247F	NA	NA	0.04 ± 0.01
D129N	181 ± 19[b]	1.9 ± 0.1[b]	5.10 ± 0.09
Substrate binding mutants			
Y202F	367 ± 37[b]	6.7 ± 0.5[b]	11.1 ± 0.0
S245A	110 ± 19	10.7 ± 1.1	41.1 ± 0.4
Putative structural mutants			
R265L	NS[c]	NS	4.18 ± 0.09
N216A	103 ± 14	10.0 ± 0.8	44.6 ± 1.3
N350A	NS	NS	0.12 ± 0.01
N350tr	NS	NS	2.49 ± 0.17[d]
L356tr	NS	NS	6.29 ± 1.50[d]

[a] NA indicates no measurable activity at any HA concentration.
[b] Indicates extrapolated value from saturable curve fit.
[c] NS, not saturable, indicates data do not fit a saturation curve.
[d] Values measured at 125 μM HA.

88. Thus, before 2011, a number of residues within the region of the catalytic site in PH20 or HYAL1 had been experimentally shown to be necessary or important to the catalytic activity of hyaluronidases. For example, Arming (EX1011) identified positions D111, E113, R176, E249, and R252. Zhang (EX1010) identified positions in HYAL1 corresponding to D111, D113, D184, S227, Y229, R246, W304, and N333 in PH20, as well as the Hyal-EGF domain from 337-409 in PH20 identified by Chao.

3. Modifications at the C-Terminus of PH20 Were Poorly Understood in the 2011 Timeframe

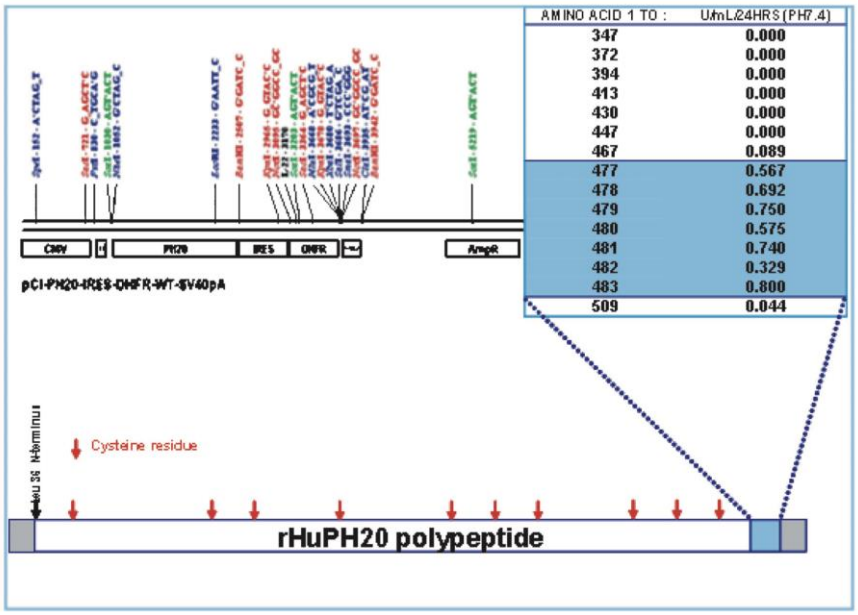
89. A patent filed by Halozyme in 2004 and issued in 2010 (the ’429 Patent) reported that a soluble, neutral active form of PH20 could be produced by

truncating the PH20 sequence just before the start of the glycosylphosphatidyl inositol (GPI) anchor sequence in the protein (position N483).[45]

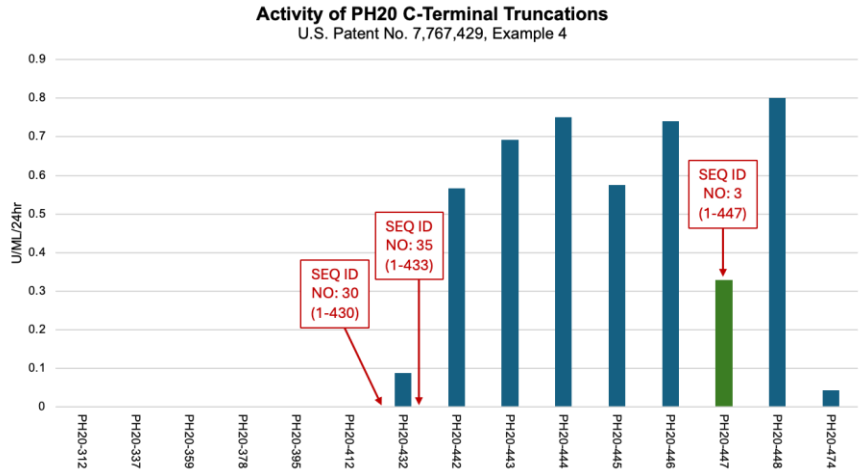
90. Data in the ’429 Patent and a 2007 paper by Frost (EX1013) also showed that truncations of varying length at the C-terminus of PH20 caused significant variations in hyaluronidase activity (below).[46] For example, both observed that when the PH20 protein was terminated at position 443 (mature protein), it only had 10% of the mutants that terminate at positions 443 or 448. Also, Frost reported that PH20 had to terminate between 442 and 448 to recover “soluble hyaluronidase activity” and the ’429 Patent reported that only a narrow range of truncation mutants (*i.e.*, those terminating between 438 and 448) “defined the minimally active domain” of PH20. Figure 2 from Frost is shown below.

[45] EX1005 (’429 Patent), 86:18-26 (“…the GPI anchor cleavage site was located around amino acid position N 483 in the full-length GPI-anchored protein.”); 3:51-62.

[46] EX1005 (’429 Patent), 87:52-88:24; EX1013 (Frost), 430-432, Fig. 2.



91. The data reported in the ’429 Patent and Frost show that C-terminal truncations of varying length had an unusual effect on activity (below). If the data is to be believed, the activity of two mutants ($PH20_{1-446}$ and $PH20_{1-448}$) with one more and one fewer residue was more than twice the activity of the PH_{1-447} truncation mutant. The terminal residues of these mutants are phenylalanine (F446), tyrosine (Y447), and asparagine (N448). This is highly unusual behavior for three highly similar mutants, but no explanation is provided.



Position (w/signal)	Mutant (mature #)	U/ML/24hr	Normalized to PH20(1-447)
347	PH20-312	0	0.00
372	PH20-337	0	0.00
394	PH20-359	0	0.00
413	PH20-378	0	0.00
430	PH20-395	0	0.00
447	PH20-412	0	0.00
467	**PH20-432**	**0.089**	**27.05**
477	PH20-442	0.567	172.34
478	PH20-443	0.692	210.33
479	PH20-444	0.75	227.96
480	PH20-445	0.575	174.77
481	PH20-446	0.74	224.92
482	**PH20-447**	**0.329**	**100.00**
483	PH20-448	0.8	243.16
509	PH20-474	0.044	13.37

92. The ’429 Patent and Frost report that certain of the PH20 truncation mutants had no hyaluronidase activity. Several of these terminate within the region of PH20 that was later identified by Chao as containing the Hyal-EGF domain (which is at positions 337-409 in the mature PH20 sequence), and one ($PH20_{1-412}$) terminates three residues downstream from the end of the Hyal-EGF domain (indicated in table below). The truncation mutants within the Hyal-EGF domain terminate at positions 312, 337, 359, 378, and 395. The Zhang paper reported that a truncation just upstream of the start of the Hyal-EGF domain in HYAL1 reduced its activity to ~6%.

AMINO ACID 1 TO:	U/ML/24 HRS PH 7.4
347	0.000
372	0.000
394	0.000
413	0.000
430	0.000
447	0.000
467	0.089
477	0.567
478	0.692

Residues within Hyal-EGF Domain

$PH20_{1-412}$ (+3 residues downstream from Hyal-EGF domain)

93. The common disclosure references these experimental results and reports that PH20 must contain residues that extend at least to position 429 in the mature protein (position 464 w/signal sequence) to have hyaluronidase activity:

> A mature PH20 polypeptide lacking the signal sequence and containing a contiguous sequence of amino acids having a C-terminal amino acid residue corresponding to amino acid residue 464 [429] of SEQ ID NO:6 (*e.g.*, amino acid residues corresponding to positions 36-464 [1-429] of the amino acid sequence set forth in SEQ ID NO:6) is the minimal sequence required for hyaluronidase activity [citing '429 Patent].[47]

94. The common disclosure also refers to the truncation mutants of PH20 discussed in the '429 Patent and Frost paper but does not discuss the unusual

[47] EX1001 ('520 Patent), 70:2-11.

variations in the activity of those C-terminal truncations.[48] Neither the scientific literature existing by 2011 nor the common disclosure provides an explanation why these PH20 truncation mutations that differ by one residue (*i.e.*, $PH20_{1-446}$ vs. $PH20_{1-447}$ vs. $PH20_{1-448}$) exhibit variability in their activity.

95. Before 2011, a skilled artisan would have connected the dots between the data reported in the '429 Patent and the Chao report of the existence of the Hyal-EGF domain. He or she would have reasoned that the loss in activity of truncation mutants ending below position 430 was likely due to disruption of the Hyal-EGF domain structure. That would have been reinforced by the experimental results reported in Zhang in 2009 that showed that deletion of the entire Hyal-EGF domain substantially eliminated hyaluronidase activity in HYAL1.

96. The data in the patent (particularly Tables 3 and 5 for "active mutants" and "inactive mutants," respectively) shows that making single amino acid substitutions in the region from 337-409 resulted in both active mutants and

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[48] EX1001 ('520 Patent), 74:13-19 ("Soluble PH20 enzymes also include truncated forms of non-human or human membrane-associated PH20 hyaluronidases that lack one or more amino acid residues of a … (GPI) anchor attachment signal sequence and that retain hyaluronidase activity (see e.g., U.S. Pat. No. 7,767,429…).").

inactive mutants for most of the range of positions. See Appendix A-9. In the aggregate, there appear to be somewhat more single mutations that result in inactive mutants than those that result in active mutants, but that is a qualitative assessment. Unsurprisingly, mutations that changed the cysteine residues (which are also highly conserved) were uniformly inactive. The varying effects of changing residues in the Hyal-EGF region of PH20 show that a skilled artisan's belief that changes in this region would be unpredictable were warranted and would be more so if multiple changes were made concurrently.

97. Overall, there was uncertainty in the scientific literature about the activity of truncation mutants in the C-terminal region of PH20 (*i.e.*, between positions 337 and 448). Truncations into the C-terminal region of the wild-type PH20 corresponding to the Hyal-EGF domain rendered the protein inactive, and those at its terminus (446-448) caused unpredictable changes in activity. Because there are no examples in the common disclosure testing the effects of introducing one or more substitutions into a PH20 polypeptide truncated to a position below position 447 (*e.g.*, 433, 430 or within the Hyal-EGF region at 337 to 409), there is no basis for predicting what might happen if one made such mutants, especially if that truncated form of the wild-type sequence was inactive. Also, the examples of single substitutions in $PH20_{1-447}$ are not analogous to substitutions in PH20 proteins truncated below 433, given the latter’s reduced or ablated activity.

III. The Common Disclosure Defines Two Mutually Exclusive Types of Modified PH20 Polypeptides

A. Two Types of Modified PH20 Polypeptides Are Differentiated Based on Possession or Absence of Hyaluronidase Activity

1. The Common Disclosure Draws a Clear Line Between Two Alternative Types of Modified PH20 Polypeptides

98. The common disclosure describes two, mutually exclusive types of modified PH20 polypeptides. First, it says "active mutants" are those having at least 40% hyaluronidase activity of the unmodified form of PH20.[49] Then, it says that "inactive mutants" are mutants that do not have significant hyaluronidase activity, which it indicates is 20% or less of the activity of the unmodified PH20.[50] This is a fairly straightforward delineation of two categories of proteins that either have or do not have an activity (here hyaluronidase activity).

99. Each type of mutant is addressed in a different section. The "active mutants" section runs from column 79, line 28 to column 115, line 40 and includes a compilation of the "active mutants" that were produced in Table 3, all of which have a single amino acid replacement in the $PH20_{1-447}$ wild-type sequence. The patent also lists single-replacement $PH20_{1-447}$ "active mutants" in Table 9 with reports of their relative activity to unmodified $PH20_{1-447}$.

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[49] EX1001 ('520 Patent), 79:28-44.

[50] EX1001 ('520 Patent), 115:41-47.

100. The common disclosure consistently uses the 40% activity threshold to classify a mutant as an "active mutant." For example, it classified individual modified PH20 polypeptides in Table 3 ("active mutants") "so long as the resulting modified PH20 polypeptide exhibits at least 40% of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid replacement."[51] It similarly explains in connection with Table 9 that "[a]ctive mutants were selected whereby at least one duplicate sample exhibited greater than 40% of wildtype activity when normalized to SEAP activity."[52]

101. "Inactive mutants" are discussed from column 115, line 41 to column 123, line 20 in the common disclosure. Examples of "inactive mutants" are compiled in Tables 5 and 10. The specification explains that "inactive mutants" are mutants with 20% or less of the activity of unmodified PH20, explaining:

> Provided herein are modified PH20 polypeptides that contain one or more amino acid replacements in a PH20 polypeptide and that are inactive, whereby the polypeptides do not exhibit hyaluronidase activity or exhibit low or diminished hyaluronidase activity. The modified PH20 polypeptides provided herein that are inactive generally exhibit less than 20%,

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51 EX1001 ('520 Patent), 79:28-82:11.

52 EX1001 ('520 Patent), 228:7-9.

> such as less than 10%, of the hyaluronidase activity of a wildtype or reference PH20 polypeptide…[53]

102. Example 4 reports that "inactive mutants" were experimentally confirmed to be inactive. These so-called "dead mutants" are compiled in Table 10.[54]

> The other mutants that exhibited less than 20% hyaluronidase activity of wildtype PH20, in at least one of the duplicates, were rescreened to confirm that the dead mutants are inactive. To confirm the inactive mutants, the hyaluronidase activity assay described in Example 3 was modified to incorporate an overnight 37° C. substrate-sample incubation step prior to measurement of enzymatic activity. The modified assay is intended to detect PH20 activities below 0.2 U/mL.

2. The Experimental Results Classify Single Replacement Mutants of $PH20_{1-447}$ as Active or Inactive Mutants

103. The common disclosure provides a compilation of all the mutants that apparently were produced by the inventors in Table 8. There are 6,753 entries in this table. These are all mutants generated by substituting one amino acid from

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53 EX1001 ('520 Patent), 115:41-48.

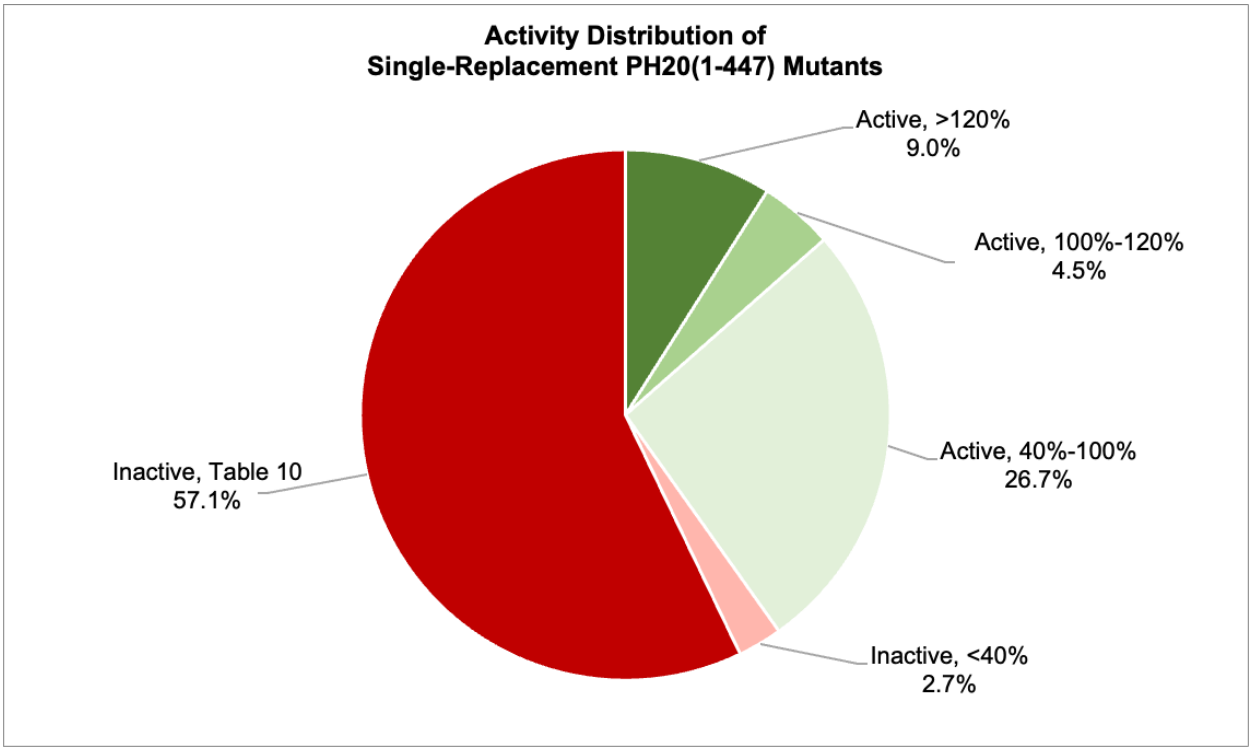
54 EX1001 ('520 Patent), 259:1-11, 259:29-32, Table 10 runs from column 251, line 34 to the end of 256.

$PH20_{1-447}$. There are 2,537 entries in Table 9. Table 10 contains a compilation of tested "inactive mutants" with 3,380 entries in it. The common specification thus provides test results for 5,917 mutants but does not report test results for the 836 other mutants that were apparently made.

104. The numbers of "active mutants" listed in Table 3 does not match the number of "active mutants" tested and shown to have at least 40% activity in Table 9 (*i.e.*, 2,516 vs. 2,376). Table 3 also does not match the total number of entries in Table 9 (*i.e.*, 2,516 vs. 2,536). Similarly, the number of "inactive mutants" listed in Table 5 does not match the number of tested inactive mutants (< 20% activity) listed in Table 10 (*i.e.*, 3,368 vs. 3,380). There is no explanation for these discrepancies in the common disclosure.

105. The table and graph below show that most of the single-replacement $PH20_{1-447}$ mutants that were tested exhibited less activity than the unmodified $PH20_{1-447}$ (*i.e.*, 57.1% were inactive, and 29.4% others had activity <100%).

Activity vs. Unmodified PH20	Number	% of Tested (5916)
Active Mutants (Table 9)		
>120%	532	9.0%
100%-120%	267	4.5%
40%-100%	1577	26.7%
Inactive Mutants (Table 9)		
<40%	160	2.7%
Inactive Mutants (Tables 5 and 10)		
Table 5 “inactive mutants”	3,368	56.9%
Table 10 ‘inactive mutants”	3,380	57.1%



106. The relative activities reported are difficult to assess because the specification does not provide measured values for the activity of the unmodified $PH20_{1-447}$ used as the reference for percentages reported in Table 9. There are also

no indications about how many replicates of each test were performed, and there is no statistical analysis of the data provided. That is a concern because the values of the positive control reported in Table 11 and 12 varied so extensively from run to run. If the control (the unmodified $PH20_{1-447}$) used as the reference assay for these relative activity measurements exhibits a variation of +/- 25% or more in its replicates, it would be difficult to meaningfully differentiate mutants exhibiting 125% vs 75% from the unmodified PH20.

107. Overall, between the explanations provided in the common specification and the approach it uses to classify mutants based on measured activity levels, a skilled artisan reading the common disclosure would have understood it to be describing two, mutually exclusive types of modified PH20 polypeptides: (i) active mutants are those with significant levels of hyaluronidase activity (*i.e.*, above 40% of the activity of unmodified PH20), and (ii) inactive mutants, which do not exhibit significant hyaluronidase activity (*i.e.*, less than 20% of the activity of the unmodified PH20).

B. Proposed Uses for Active Mutant Modified PH20 Polypeptides Are Different from Those for Inactive Mutants

108. The common disclosure identifies different uses for "active" and "inactive" mutants. For example, columns 174-188 are devoted to therapeutic uses of modified PH20 polypeptides that have the ability to degrade hyaluronan (HA),

which requires the protein to have meaningful hyaluronidase activity, which is why those uses are associated with "active mutant" modified PH20 polypeptides that have at least 40% of the activity of the unmodified PH20 (*e.g.*, $PH20_{1-447}$).

109. By contrast, one paragraph suggests using "inactive enzymes" in contraceptive vaccines:

> Modified PH20 polypeptides provided herein can be used as vaccines in contraceptive applications. … Immunization with PH20 has been shown to be an effective contraceptive in male guinea pigs (Primakoff et al. (1988) Nature 335:543-546, Tung et al. (1997) Biol. Reprod. 56:1133-1141). It also has been shown to be an effective contraceptive in female guinea pigs due to the generation of anti-PH20 antibodies that prevent sperm and egg binding. In examples herein, the modified PH20 polypeptides can be inactive enzymes, such as any described in Sections C.2. …[55]

110. I am aware of publications subsequent to the two publications that the patent portrays as demonstrating that inactive PH20 polypeptides can be used as the immunogen in a contraceptive vaccine (apparently in humans). These subsequent publications reported negative results in experiments attempting to

[55] EX1001 ('520 Patent), 188:8-27.

induce contraceptive by immunizing mammals (rats, mice) with PH20.[56] For example, Hardy reported from its experiments that "recombinant mPH20 is not a useful antigen for inclusion in immunocontraceptive vaccines that target mice."

111. I also reviewed publications reporting on the human testing of Hylenex® (wild-type $PH20_{1-447}$). One (a 2015 paper) reported that some subjects given Hylenex produced detectable antibodies (2.5-18%).[57] It also addressed possible concerns that such antibodies target cells involved in reproduction and affect fertility. The authors, which included scientists from Halozyme, reported that the results from the clinical trials alleviated that concern:

> These observations serve to alleviate potential concerns raised by the apparent binding (although not neutralization) of a treatment-induced antibody to an endogenous protein involved in aspects of reproduction and are further supported by published reports in

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[56] EX1019 (Hardy), 325; EX1020 (Pomering), 181 ("immunization [of rabbits] with reproductive antigens expressed only in the reproductive tract using routes which induce predominantly plasma IgG are unlikely to result in reduced fertility…"); EX1021 (Baba), 30310 ("PH-20 is not essential for fertilization, at least in the mouse, suggesting that the other hyaluronidase(s) may play an important role in sperm penetration…").

[57] EX1024 (Rosengren), 1146, 1147 (Table II reporting antibody production),

> which several attempts were made to immunize males with PH20 as an immunocontraceptive approach in animal models. These studies involved rabbits (45,46), mice (47), and guinea pigs (48), and only the latter experienced infertility following PH20 immunization with a crude testicular extract that resulted in autoimmune orchitis (49). Furthermore, sperm from mice lacking PH20 were able to fertilize eggs, albeit in a somewhat delayed manner (50).[58]

112. The reports on the lack of contraceptive effects of PH20 proteins in mice and rabbits were published before December 2011, while the reports on clinical testing of Hylenex were published in 2015 and 2018. They all suggest that PH20 does not appear to induce formation of antibodies that affect fertility in many rodents or in humans. The brief suggestion in the common disclosure about possibly using inactive mutant forms of PH20 as the immunogen of a contraceptive vaccine does not seem credible given these other experimental results.

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[58] EX1061 (Rosengren-2015), 1154; *also* EX1024 (Rosengren), 87 ("Although some antisperm antibodies are associated with decreased fertility [], no evidence of negative effects on fertility could be determined in rHuPH20-reactive antibody-positive subjects of either sex.")

113. Additionally, I note that the common disclosure does not identify any mutated PH20 proteins that were shown to be effective in contraceptive vaccines. It also does not provide guidance regarding how to identify candidate inactive PH20 mutants that may be useful as contraceptive vaccines (such as by identifying common structural or functional characteristics that would be shared by such inactive mutants). A skilled artisan could not predict from the common disclosures' limited discussion of contraceptive vaccines which, if any, mutated PH20 polypeptides would confer contraceptive effect in humans. And more generally, a skilled artisan would have believed inactive forms of an enzyme, like PH20, have no utility at all.

IV. The Claims Capture an Immense Number of Distinct PH20 Polypeptides

A. The Claims Define Large Sets of Modified PH20 Polypeptides Using Sequence Identity Parameters

114. I reviewed the claims of the '520 Patent. Claims 1-2, 6-15, 22, and 25-26 (below) define slightly different sets of modified PH20 polypeptides.

What is claimed:

1. A modified PH20 polypeptide, comprising one or more amino acid modifications in an unmodified PH20 polypeptide, wherein:

the unmodified PH20 polypeptide consists of the amino acid sequence selected from the group consisting of SEQ ID NO: 3, 7 and 32-66;

amino acid modifications are selected from the group consisting of amino acid replacements(s), deletion(s), and/or insertion(s);

the modified PH20 polypeptide comprises an amino acid replacement at a position corresponding to residue 324, with reference to amino acid positions set forth in SEQ ID NO: 3;

the replacement at the position corresponding to residue 324 is selected from the group consisting of A, D, H, M, N, R and S;

corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide having the amino acid sequence of SEQ ID NO: 3; and

the modified PH20 polypeptide has at least 91% sequence identity to a polypeptide having the amino acid sequence selected from the group consisting of SEQ ID NO: 3, 7 and 32-66.

2. The modified PH20 polypeptide of claim **1**, wherein the modified PH20 polypeptide has at least 95% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NO: 3, 7, and 32-66.

6. The modified PH20 polypeptide of claim **1**, wherein the replacement at the position corresponding to residue 324 is D.

7. The modified PH20 polypeptide of claim **1**, wherein the replacement at the position corresponding to residue 324 is N or R.

8. The modified PH20 polypeptide of claim **1**, wherein the unmodified PH20 polypeptide consists of the amino acid sequence selected from the group consisting of SEQ ID NO: 3 and 32-66.

9. The modified PH20 polypeptide of claim **7**, wherein the unmodified PH20 polypeptide consists of the amino acid sequence selected from the group consisting of SEQ ID NO: 3 and 32-66.

10. The modified PH20 polypeptide of claim **6**, wherein the unmodified PH20 polypeptide consists of the amino acid sequence selected from among the group consisting of SEQ ID NO: 3 and 32-66.

11. The modified PH20 polypeptide of claim **1**, wherein the unmodified PH20 polypeptide consists of the amino acid sequence of SEQ ID NO:35.

12. The modified PH20 polypeptide of claim **1**, wherein the unmodified PH20 polypeptide consists of the amino acid sequence of SEQ ID NO:32.

13. The modified PH20 polypeptide of claim **6**, wherein the unmodified PH20 polypeptide consists of the amino acid sequence of SEQ ID NO:35.

14. The modified PH20 polypeptide of claim **6**, wherein the unmodified PH20 polypeptide consists of the amino acid sequence of SEQ ID NO:32.

15. The modified PH20 polypeptide of claim **1**, comprising a sequence of amino acids that exhibits at least 91% sequence identity to the sequence of amino acids selected from the group consisting of SEQ ID NO: 3, and 32-66 and that contains an amino acid replacement D at the residue corresponding to residue 324 with reference to SEQ ID NO: 3.

22. The modified PH20 polypeptide of claim **1**, further comprising a heterologous signal sequence, wherein the unmodified PH20 polypeptide consists of the amino acid sequence selected from the group consisting of SEQ ID NO: 3 and 32-66.

25. The modified PH20 polypeptide of claim **6**, wherein:

the unmodified PH20 polypeptide consists of the amino acid sequence of SEQ ID NO:32; and

the amino acid sequence of the modified PH20 polypeptide has at least 95% sequence identity to the amino acid sequence of SEQ ID NO:32.

26. The modified PH20 polypeptide of claim **6**, wherein:

the unmodified PH20 polypeptide consists of the amino acid sequence of SEQ ID NO:35; and

the amino acid sequence of the modified PH20 polypeptide has at least 95% sequence identity to the amino acid sequence of SEQ ID NO:35.

115. The claims address two aspects of the modified PH20 polypeptides in each set defined by the claims.

- First, they require the wild-type glutamic acid (E) at position 324 to be replaced with one of seven amino acids: alanine (A), aspartic acid (D), histidine (H), methionine (M), asparagine (N), arginine (R), or serine (S). Claim 6 requires the amino acid at position 324 to be aspartic acid (D). Claim 7 requires the amino acid at position 324 to be asparagine (N) or arginine (R).
- Second, they permit (but do not require) the modified PH20 polypeptide to contain a certain number of additional changes besides the replacement at position 324.

116. The number of additional changes that each claim permits besides the replacement at position 324 varies but is defined via a percentage sequence identity calculation. This involves counting up the total number of changes in the modified PH20 relative to the unmodified (wild-type) sequence, and then dividing that number by the total number of amino acids in the unmodified PH20 sequence being referenced.

117. For example, claim 1 requires the modified PH20 polypeptide to have at least 91% sequence identity to one of 37 unmodified PH20 sequences (SEQ ID

NOs: 3, 7, or 32-66) having between 430 and 474 amino acids.[59] This means the maximum number of changes each PH20 polypeptide can have is equal to 9% of the number of amino acids in the unmodified PH20 (*i.e.*, 9% of 430 = 38, 9% of 465 = 41, 9% of 447 = 40, 9% of 474 = 42). Claim 2 tightens the sequence identity requirement to 95% as compared to the same unmodified PH20 sequences, which somewhat reduces the maximum number of changes each PH20 polypeptide can have (*i.e.*, 5% of 430 = 21, 5% of 465 = 23, 5% of 447 = 22, 5% of 474 = 23). Claims 8, 9, 10, 15, and 22 remove SEQ ID NO: 7 (length 474) from the reference unmodified sequences that may be used for the sequence identity comparison. Because SEQ ID NO: 7 is the longest of the reference unmodified sequences, the maximum number of amino acid changes permitted by these claims is slightly smaller (41 vs. 42) (*i.e.*, 9% of 430 = 38, 9% of 465 = 41, 9% of 447 = 40). In each of these scenarios, one change is accounted for by the required replacement at position 324.

118. Claims 11, 12, 13, 14, 25, and 26 specify the reference unmodified sequence to be used as either SEQ ID NO: 32 (claims 12, 14, 25) or SEQ ID NO: 35 (claims 11, 13, 26). SEQ ID NO: 32 has a length of 430 amino acids, and so

[59] SEQ ID NO:32 has 433 residues, SEQ ID NO:66 has 465 residues, and SEQ ID NO:3 has 447 residues.

permits either 21 changes (95% sequence identity) or 38 changes (91% sequence identity). SEQ ID NO: 35 has a length of 433 amino acids, and so permits either 21 changes (95% sequence identity) or 38 changes (91% sequence identity). Again, in each of these scenarios, one change is accounted for by the required replacement at position 324.

119. The claim language does not require the additional changes (besides the position 324 change) to be restricted to any region of the polypeptide. In addition, the common disclosure explains that changes can be additions, deletions or replacements, and for replacements, can be at any position in the sequence and to any other amino acid (*i.e.*, 19 alternatives).[60]

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[60] EX1001 ('520 Patent), 60:61-61:1 ("Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they can be clustered in one or more locations of varying length up to the maximum allowable, e.g., 10/100 amino acid difference (approximately 90% identity). Differences can also be due to deletions or truncations of amino acid residues."), 47:43-47 ("As used herein, modification is in reference to modification of a sequence of amino acids of a polypeptide or a sequence of nucleotides in a nucleic acid molecule and includes deletions, insertions, and replacements of amino acids and nucleotides, respectively."),

120. The sequence identity language causes the claims to encompass an immense number of distinct PH20 polypeptides. This is the consequence of the claims allowing up to 21-42 changes, with each additional change (except at position 324) being to 1 of 19 other amino acids. But the up to 21-42 changes also can be at any of between 430 and 465 (or, in the case of the broadest claims, 474) different positions depending on which unmodified PH20 sequence is used.

121. The Alberts textbook illustrated the scale of amino acid sequences captured by the language used in the patent claims:

> Since each of the 20 amino acids is chemically distinct and each can, in principle, occur at any position in a protein chain, there are 20 x 20 x 20 x 20 = 160,000 different possible polypeptide chains four amino acids long, or 20^n different possible polypeptide chains n amino acids long. For a typical protein length of about 300 amino acids, a cell could theoretically make more than 10^{390} (20^{300}) different polypeptide chains. This is such

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47:56-58 ("The modification can be an amino acid replacement (substitution), insertion (addition) or deletion of one or more amino acid residues."); 42:2-8 ("[T]he plurality of modified enzymes are such that the amino acid at each modified position is replaced by up to 1-19 other amino acids other than the original amino acid at the position…").

> an enormous number that to produce just one molecule of each kind would require many more atoms than exist in the universe.[61]

122. I have reviewed the analysis performed by Dr. Park of the number of distinct PH20 polypeptides that meet the parameters in claims 1-2, 6-15, 22, and 25-26.[62] Similar to the illustration in Alberts (above), the calculation permits 19 choices at up to 41 positions in the protein, and accounts for the fact that the 41 positional changes can be at any of between 430 and 474 positions in the reference protein. It also accounts for the fact that one change must be at position 324, and must be to one or one of seven alternatives. Consistent with my expectations, his calculations yielded immense numbers of distinct modified PH20 polypeptides based on the different sets of parameters used in claims 1-2, 6-15, 22, and 25-26 (reproduced below).

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61 EX1039 (Alberts), 136-137.

62 EX1004 (Park Dec.), ¶¶ 180-184, Appendix F-1, F-2.

PH20 length	*Sequence Identity %*	*# Changes*	*Pos. 324 Choices*	*Add'l Changes*	*# of Distinct Polypeptides*
430	91	38	7	37	5.36×10^{101}
430	91	38	1	37	7.66×10^{100}
430	95	21	7	20	3.08×10^{60}
430	95	21	1	20	4.40×10^{59}
430	91	38	2	37	1.53×10^{101}
433	91	38	7	37	7.02×10^{101}
433	91	38	1	37	1.00×10^{101}
433	95	21	1	20	5.08×10^{59}
447	91	40	7	39	9.77×10^{106}
447	91	40	1	39	1.40×10^{106}
447	95	22	7	21	2.63×10^{63}
447	91	40	2	39	2.79×10^{106}
465	91	41	7	40	9.88×10^{109}
465	91	41	1	40	1.41×10^{109}
465	91	41	2	40	2.83×10^{109}
474	91	42	7	41	1.41×10^{109}
474	91	42	1	41	6.32×10^{111}
474	95	23	7	22	3.63×10^{66}
474	91	42	2	41	1.26×10^{112}

123. One way to put this scale into perspective is to consider the aggregate weight of one set of these mutants from the '520 Patent claims, where one assumes one molecule of each mutant is in the set. The weight in grams of 1 molecule of an unmodified $PH20_{1-447}$ is ~8.94 x 10^{-20} grams.[63] For simplicity, assume that all the modified PH20 polypeptides have the same weight. With that assumption, the aggregate weight of the smallest set containing one molecule of each of the PH20 mutants would be 4.40 x 10^{59} x 8.94 x 10^{-20} = 3.93 x 10^{40} kg. The weight of Earth is "only" ~5.97 x 10^{24} kg.[64]

124. I also published on a similar topic years ago. As my colleague, James Beasley, and I explained:

> [F]or a relatively short sequence of 100 residues composed of the 20 naturally occurring amino acid, there are 20^{100} possibilities.

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[63] The Expasy website (https://web.expasy.org/compute_pi/) calculated the molecular weight of a polypeptide having residues 1-447 of SEQ ID NO:3 of the '520 Patent as 53870.95 Daltons. The weight of one molecule of that polypeptide is determined by multiplying 53870.95 D by 1.66063906660 x 10^{-24} g/D, or approximately 8.94 x 10^{-20} grams.

[64] *See* "Earth Fact Sheet" available from NASA web site (*i.e.*, https://nssdc.gsfc.nasa.gov/planetary/factsheet/earthfact.html).

> This number is so large ($20^{100} > 10^{130}$) that if one synthesized a single molecule of each sequence and put the entire collection into a box, the resulting box would be larger than Avogadro's number of universes.[65]

125. The calculations by Dr. Park show a minimum of 4.40×10^{59} potential sequences encompassed by the narrowest of the claims, which is certainly smaller than Avogadro's number of universes. Yet even this relatively "smaller" number is still astronomical in size.

B. The Claims Would Be Understood to Concern Active Mutant PH20 Modified Polypeptides

126. As I explained in ¶¶ 98-102, the common disclosure portrays modified PH20 polypeptides as being either "active mutants" or "inactive mutants." The former are mutants that exhibit meaningful hyaluronidase activity (>40% of the unmodified PH20). The latter are enzymatically inactive proteins (less than 20% activity of the unmodified PH20).

127. The claims require each set of modified polypeptides to contain a substitution of the glutamic acid at position 324 to a different amino acid. The claims allow for varying numbers of alternative amino acid choices at position 324: (i) claims 6 and 15 each permit one alternative, aspartic acid (E324D), (ii)

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65 EX1046 (Beasley), 2031.

claim 7 permits two alternatives, either asparagine (E324N) or arginine (E324R), and (iii) claim 1 permits seven alternatives, alanine (E324A), aspartic acid (E324D), histidine (E324H), methionine (E324M), asparagine (E324N), arginine (E324R), or serine (E324S). The common disclosure identifies the activity of each of these substitutions when the mutation is incorporated as the only change in the $PH20_{1-447}$ sequence.[66] As the table below shows, the common disclosure classified all seven substitutions as "active mutants," but only three of these single-mutation $PH20_{1-447}$ enzymes (E324D, E324N, and E324R) exhibited increased hyaluronidase activity relative to the unmodified form of $PH20_{1-447}$.

TABLE 9-continued

ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
E324A		0.59
E324D		1.15
E324H		0.79
E324M		0.50
E324N	623	1.01
E324R	624	2.28
E324S		0.62

[66] EX1001 ('520 Patent), column 231 (Table 9).

128. The common disclosure also says that "modified PH20 polypeptides contain an amino acid replacement at one or more amino acid positions identified as being associated with increased hyaluronidase activity."[67] The common disclosure then explains that a modified PH20 polypeptide with a mutation associated with increased hyaluronidase activity "can contain other modifications … so long as the resulting modified PH20 polypeptide exhibits increased hyaluronidase activity compared to the PH20 not containing" those modifications.[68]

129. The common disclosure reiterates several times this point about preserving hyaluronidase activity in a PH20 polypeptide that has incorporated a first change that causes it to be an "active mutant" when making additional changes to the protein (citations to EX1001 ('520 Patent), emphases added):

> As used herein, "modified PH20 polypeptide" or "variant PH20 polypeptide" refers to a PH20 polypeptide that contains at least one amino acid modification, such as at least one amino acid replacement as described herein, in its sequence of amino acids compared to a reference unmodified PH20 polypeptide. A modified PH20 polypeptide can have up to 150 amino acid

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67 EX1001 ('520 Patent), 97:34-37.

68 EX1001 ('520 Patent), 97:34-46.

replacements, ***so long as the resulting modified PH20 polypeptide exhibits hyaluronidase activity***. [48:38-46]

The modifications described herein can be in any PH20 polypeptide, including, including precursor, mature, or C-terminal truncated forms, ***so long as the modified form exhibits hyaluronidase activity***. [76:7-10]

The C-terminal truncation can be a truncation or deletion of [8-50] or more amino acids at the C-terminus, ***so long as the resulting C-terminally truncated polypeptide exhibits hyaluronidase activity*** and is secreted from cells (e.g., into the media) when expressed. [77:2-9]

In particular examples, the amino acid replacement(s) can be at the corresponding position in a PH20 polypeptide as set forth in any of SEQ ID NOs: 2, 3, 6-66, 68-72, 856-861, 869 or 870 or a variant thereof having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto, ***so long as the resulting modified PH20 polypeptide exhibits at least 40% of the hyaluronidase activity*** of the corresponding PH20 polypeptide not containing the amino acid replacement. … In one example, any one or more of the replacements are in SEQ ID NO:3, ***so long as the resulting modified PH20 polypeptide exhibits at least 40% of the hyaluronidase activity*** of the PH20 polypeptide set forth in SEQ ID NO:3. [81:3-82:11]

130. The common disclosure does not show any examples of a modified PH20 polypeptide (regardless of its length) that (i) incorporates one amino acid substitution that causes it to have hyaluronidase activity or increased hyaluronidase activity, and then (ii) introduces additional changes that render that multiply-modified PH20 protein an "inactive mutant." I can identify no scientific or practical reason it would because a skilled artisan would not pursue such a path. It makes no sense for a skilled artisan to go to the effort of making a modified PH20 with hyaluronidase activity (or greater activity than the unmutated protein), and then intentionally introduce additional mutations to make that modified protein inactive. If the goal were to make inactive mutants, the skilled artisan would have started by selecting one of the thousands of examples of PH20 polypeptides reported as having one amino acid replacement that produced inactive mutants.

131. The inventors appear to have stopped after making only single-replacement mutants. For example, there are no mutants with a first mutation that led to its classification as an "active mutant" and that then acquired a second mutation. More importantly, there are no "double" or "triple" (or more) mutants that combined sets of single mutations classified as causing both "active" mutants" and "inactive" mutants or were within particular regions of the PH20 sequence. Consequently, there is no meaningful guidance in the common disclosure on what

happens when different kinds of mutations are combined, or different mutations in a region of the PH20 are combined.

132. There is also a dependent claim that affirmatively requires the modified PH20 polypeptide to have increased hyaluronidase activity. Specifically, claim 4 requires that the modified PH20 polypeptide exhibit increased hyaluronidase activity compared to the unmodified PH20 polypeptide that does not contain the amino acid replacement at position 324.

133. I have been informed and understand that a dependent claim inherits all of the requirements of its parent claim, which for claim 4 is claim 1. I understand that also means that all of the modified PH20 polypeptides within the scope of claim 4 are also included in the scope of the set defined by claim 1.

134. I therefore believe a skilled artisan would have understood the claims to necessarily cover modified PH20 polypeptides that are active mutants and would not view them as including inactive mutants.

135. Even if the claims are interpreted to encompass inactive mutants, they would still include an immense number of "active mutant" modified PH20 polypeptides. As I discuss further below, it is my opinion that the common disclosure does not describe or enable this immense number of "active" mutants.

C. All of the Claims Encompass a Single-Replacement $PH20_{1-447}$ Mutant Where E at Position 324 is Changed to D, N, or R

136. I also was asked to consider whether three particular modified PH20 polypeptides, each with a single amino acid substitution (*i.e.*, E324D, E324N, or E324R in $PH20_{1-447}$), meet the sequence identity percentage parameters in the claims. Each does.

- Each mutant meets the requirements of claims 1, 2, 8, 9, and 10. That is because each of these claims encompasses a modified PH20 polypeptide that has only one amino acid difference from SEQ ID NO:3, which can be to any amino acid at any position in that sequence. The E324D, E324N, and E324R $PH20_{1-447}$ mutants meet that requirement: they each have only one difference from SEQ ID NO: 3 and are 99.7% identical (*i.e.*, 446 /447 = 99.7%).
- Each mutant also meets the requirements of claims 11 and 12, each of which depend from claim 1. Claim 11 requires a comparison of the mutant 447-residue sequence (*e.g.*, E324D, E324N, or E324R in $PH20_{1-447}$) to the 433-residue unmodified PH20 (SEQ ID NO:35). That translates into a total of up to 15 changes (447-433) (*i.e.*, 1 change for E324D, E324N, or E324R plus 14 more changes due to deletions), which make the E324D, E324N, or E324R $PH20_{1-447}$

proteins 3.5% different, or 96.5% identical, to SEQ ID NO:35. Claim 12 requires a comparison of the three mutants to SEQ ID NO:32, which has 430 residues. That translates into 17 + 1 or 18 total differences, which is 4.1% different and 95.9% identical to the unmodified 430 residue PH20. Each mutant thus has more than 91% identity to each of SEQ ID NO: 32 and SEQ ID NO: 35.

- Claim 6 depends from claim 1 and adds the requirement for a E324D substitution in a modified PH20 polypeptide. Claims 13, 14, 25 and 26 then add the requirement that the reference unmodified PH20 sequence be either SEQ ID NO: 35 or SEQ ID NO: 32. The E324D $PH20_{1-447}$ mutant meets the E to D substitution requirement, and, because it only has one change, it meets the at least 91% or 95% sequence identity requirement relative to SEQ ID NO: 35 and 32 (as I explained above).
- Claim 7 depends from claim 1 and requires a substitution at position 324 from E to either N or R. The E324N or E324R $PH20_{1-447}$ mutants both meet that additional requirement.

137. All of the other claims have structural limitations similar to those described above, and therefore would capture either the E324D, E324N, or E324R

$PH20_{1-447}$ mutants (or all three). I address these particular PH20 mutants in § VI, below.

V. Observations on the Common Disclosure

A. The Common Disclosure Does Not Identify the Modified PH20 Polypeptides with Multiple Amino Acid Substitutions Encompassed by the Sequence Identity Parameters in the Claims

1. The Data from Testing Single Replacement $PH20_{1-447}$ Mutants Does Not Identify a Correlation Between PH20 Polypeptides with 2-42 Substitutions and PH20 Proteins Having > 40% Hyaluronidase Activity

138. The common disclosure provides a report on a random mutagenesis experiment that generated a large number of single substitutions within the human $PH20_{1-447}$ sequence. The disclosure utilizes what would be considered a directed evolution approach to making and testing single-mutated proteins. The data showed that ~40% of the mutations were tolerated, resulting in $PH20_{1-447}$ mutants retaining at least 40% of the hyaluronidase activity of the unmodified parent, while ~57% were not tolerated, with no or <20% hyaluronidase activity. See ¶¶ 103 to 107. A significant number of the mutants (~12%) made were not characterized, and around 2.7% of the mutants had activity between 20% and 40%.

139. Random mutagenesis experiments like the one reported in the common disclosure provide empirical results. Typically, they are followed by additional analyses and experimentation to understand why the results were

observed, and to determine what changes influenced discrete structures within the protein. This work, if it was performed, is not documented or discussed in the common disclosure beyond experiments concerning two specific mutants (F204P and V58R). See ¶¶ 74-75. There are no observations from the experimental results on any specific secondary structures or structural motifs within the PH20 protein that were influenced (positively or negatively) by individual mutations. There also is no guidance regarding additional mutations that could be made to further enhance or alter the characteristics of these mutants.

140. The common disclosure does not provide any information that a skilled artisan could use to predict the effect of incorporating into a PH20 protein the myriad different sets of between 2 and 42 substitutions drawn from the thousands of individual mutations in $PH20_{1-447}$ listed in the common disclosure. It does not, for example, suggest that incorporating one of the specific single substitutions that caused that $PH20_{1-447}$ mutant to exhibit increased activity will cause a similar increase in the activity of any other PH20 polypeptide that contains additional substitutions, regardless of their number, location, or identity. That also would not be scientifically plausible. In other words, the functional and other characteristics of a PH20 protein that incorporates a first single amino acid substitution cannot be extrapolated to modified PH20 polypeptides that incorporate the myriad sets of combinations of multiple substitutions in addition to the first.

141. As I discussed above (§ II.B.2), including additional substitutions after a first may adversely impact the structure modified by the first substitution, may affect the region of the protein having that first change, or may introduce impactful changes in an unrelated part of the protein. Because the common disclosure has no examples of any PH20 with multiple substitutions and does not characterize the effects of the mutations on the PH20 protein structure, a skilled artisan could not realistically predict whether an effect observed in an active single-modified PH20 polypeptide would be observed the trillions and trillions of modified PH20 polypeptides that incorporate that first mutation plus the myriad other sets of 2 to 41 additional substitutions.

142. The positions of any particular set of substitutions and the identity of the amino acids being inserted at those positions can dramatically influence the structure of the PH20 protein. For example, multiple substitutions could be made at locations within a sequence in PH20 responsible for forming an α-helix that will disrupt the pattern necessary to form that α-helix, or even convert it into a different secondary structure, like a β-sheet. See ¶¶ 55 to 60, above. The effects of such a dramatic change in one or more of the secondary structures of PH20 could not have been predicted in 2011 based on the guidance in the common disclosure. And because the claims encompass making up to 21 to 42 substitutions at any positions in PH20 (and, except at position 324, changing wild type residues to any of the

other amino acids at these positions), there are many, many billions (and more) of possible scenarios where the changes may materially affect the folding and maintenance of the secondary structure(s) within the PH20 polypeptide.

143. The results of the random mutagenesis study are simply compiled in in the common disclosure. Those results by themselves do not identify any defined correlation between PH20 polypeptides having sets of 2 to 42 amino acid substitutions and PH20 polypeptides that retain 40% or more of the hyaluronidase activity of the unmodified form of the PH20 polypeptide. As the reported data confirms, the individual substitutions did not yield PH20 mutants with consistently observed effects; rather, the effects observed were random and unexplained among the tested mutants. The examples of single-replacement $PH20_{1-447}$ mutants are not representative of the incredible diversity of possible modified PH20 polypeptides having different sets of 1 to 41 *additional* substitutions that are within the scope of the claims.

2. The Common Disclosure Does Not Identify Any Specific PH20 Polypeptides Having 2-42 Substitutions that Retain >40% Hyaluronidase Activity

144. With one exception (addressed in § V.A.3), there are no examples of any modified PH20 polypeptide with between 2 and 22 amino acid substitutions described in the common disclosure. It also does not identify any specific sets of 2 to 42 single amino acid substitutions that will, in combination, confer improved

stability or activity on PH20 proteins, even with respect to the $PH20_{1-447}$ polypeptide.

145. The common disclosure lists ranges of sequence identity percentages relative to a set of PH20 sequences (*i.e.*, SEQ ID NO:3, SEQ ID NO: 7, or any of SEQ ID NO: 32 to 66). It also includes lists of total numbers of amino acid substitutions, which are the mathematical consequence of applying those percentage-based sequence identity parameters to PH20 polypeptides of different lengths.[69] Stating these parameters governing possible sets of PH20 polypeptides does not identify any specific PH20 polypeptides, much less identify those that retain >40% activity (or increased activity). That is because this general language does not restrict the positions into which substitutions can be made or which amino acid(s) can be incorporated into those different position(s). The sequence identity percentages and limits on total changes are simply identifying the boundaries of an immense group of different modified PH20 polypeptides, not modified PH20 polypeptides that will all share common structural or functional characteristics.

[69] EX1001 ('520 Patent), 9:19-27.

3. The Common Disclosure Says to Avoid Changing Certain Residues Involved in Glycosylation

146. The only examples of a PH20 polypeptides with more than one substitution that are discussed in the common disclosure are combinations of substitutions that the disclosures say to ***not*** include in modified PH20 polypeptides:[70]

> [W]here the modified PH20 polypeptide contains only two amino acid replacements, the amino acid replacements are ***not*** P13A/L464W, N47A/N131A, N47A/N219A, N131A/N219A or N333A/N358A. In a further example, where the modified PH20 polypeptide contains only three amino acid replacements, the amino acid replacements are ***not*** N47A/N131A/N219A.

147. Several of these positions are asparagine residues that were known to be glycosylation sites in PH20 and other hyaluronidases (N47A, N131A, N219A, N333A, N358A).[71] No other explanation is provided why these combinations should be excluded from modified PH20 polypeptides.

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[70] EX1001 (’520 Patent), 77:47-59 (emphases added).

[71] EX1001 (’520 Patent), 49:30-35; EX1007 (WO297), 36:11-26.

4. The Common Disclosure Says to Not Include Substitutions that Rendered $PH20_{1-447}$ Inactive in Modified PH20 Polypeptides that Are Active Mutants

148. The common disclosure says that substitutions that rendered $PH20_{1-447}$ an inactive mutant as a single amino acid replacement should be avoided in modified PH20 polypeptides intended to have hyaluronidase activity. It also makes these statements without regard to how many additional substitutions or other changes might be incorporated into the mutant. As it states:

> To retain hyaluronidase activity, modifications ***typically are not made*** at those positions that are less tolerant to change or required for hyaluronidase activity. For example, generally modifications are not made at a position corresponding to position … *[96 positions]* … with reference to amino acid positions set forth in SEQ ID NO:3. Also, in examples where modifications are made at any of *[… 313 positions …]* with reference to amino acid positions set forth in SEQ ID NO:3, the modification(s) is/are not the corresponding amino acid replacement(s) set forth in Table 5 or 10 herein, which are amino acid replacements that result in an inactive polypeptide.[72]

[72] EX1001 (’520 Patent), 80:15-57 (emphasis added).

149. A number of the single substitutions that the common disclosure reports as rendering $PH20_{1-447}$ inactive were known to be conserved residues in hyaluronidase proteins. As it observes:

> …confirm the requirement of PH20 amino acid residues corresponding to positions 25, 111, 113, 176, 189, 203, 246, 249, 252, 316, 341, 346, 352, 400, 402, 408, 423 and 429 of the sequence of amino acids set forth in a mature PH20 lacking the signal sequence such as set forth in SEQ ID NO: 3 or 7 for hyaluronidase activity, since mutagenesis of these residues results in an enzyme that is not active (e.g., it is not expressed or is inactive when expressed, see e.g., Tables 5 and 10). The exception is that amino acid replacement corresponding to R176K and C316D resulted in mutants that generated some residual hyaluronidase activity.[73]

150. A skilled artisan would not have been surprised that single amino acid replacements at highly conserved positions in the PH20 sequence would have an adverse effect on the protein's activity. The common disclosure, however, does

[73] EX1001 ('520 Patent), 70:49-59. The common disclosure does not refer to or discuss the findings reported before 2011 in Chao and Zhang about residues involved in the catalytic site of hyaluronidases. EX1006 (Chao), 6914-6916; EX1010 (Zhang), 9435-38.

not provide any explanation for why substitutions at positions outside of these highly conserved residues were rendered inactive.

151. The absence of any explanation why single amino acid substitutions of non-conserved residues rendered the $PH20_{1-447}$ inactive limits the insights one can draw from the patent's disclosure. For example, there is no discussion of the effect on any secondary or other structure within the PH20 protein of the substitution. The insights one can draw from this set of data is even more limited relative to PH20 proteins that incorporate multiple substitutions. It is what the common disclosure says, which is to not include any of the substitutions in Tables 5 and 10 that rendered $PH20_{1-447}$ inactive as part of a set of substitutions in a multiply modified PH20 polypeptides that is intended to have activity, regardless of the length of the PH20 polypeptide or the number of additional amino acid replacements.

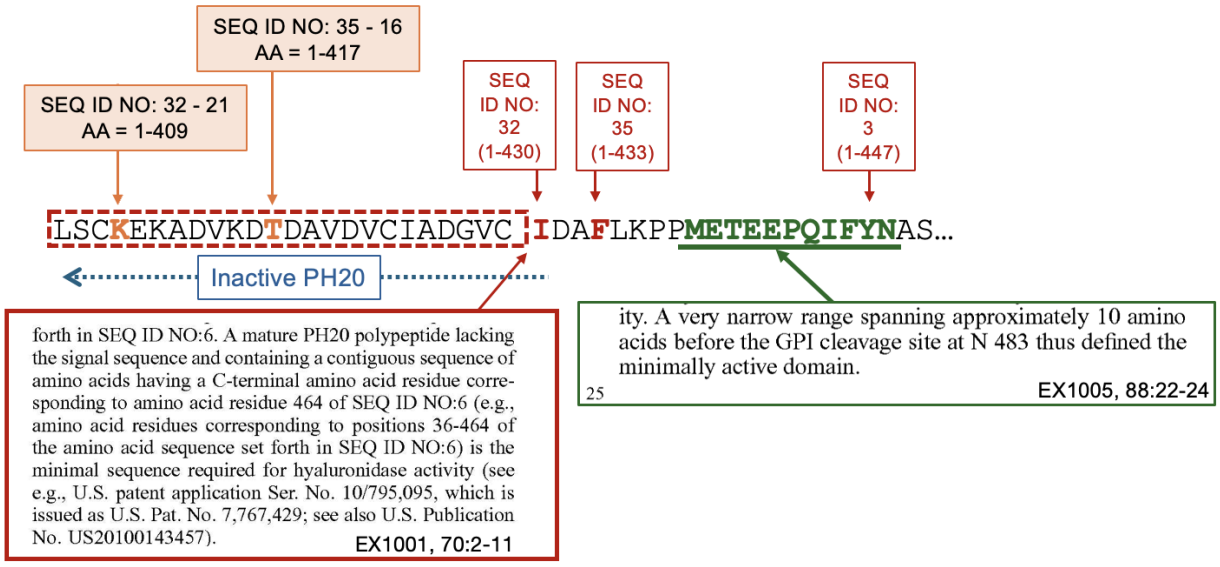
5. The Prior Art and the Common Disclosure Indicate that C-Terminal Truncations Can Render PH20 Polypeptides Inactive

152. Another type of change the common disclosure says to avoid is a truncation at the C-terminus that results in a PH20 sequence with fewer than 429 residues, as such a protein would be inactive:

> A mature PH20 polypeptide … containing a contiguous sequence of amino acids having a C-terminal amino acid residue

> corresponding to amino acid residue 464 of SEQ ID NO:6 [position 429] … ***is the minimal sequence required for hyaluronidase activity***.[74]

153. As I explained earlier (§ II.E.3), by 2011, there was substantial uncertainty surrounding truncations and other modification at the C-terminal region of PH20. The illustration below compiles this existing knowledge regarding truncations in C-terminal region of the PH20 and HYAL1 proteins.



154. A skilled artisan in 2011 would have approached making changes in the C-terminal region of PH20 with caution, as truncations resulting in PH20 polypeptides that terminate before position 430 yielded inactive proteins, and changes terminating at positions between 430 and 442 were not characterized,

[74] EX1001 (’520 Patent), 70:2-11 (emphasis added).

other than at position 432 (which exhibited ~27% of the activity of $PH20_{1-447}$).[75] Yet, as I discuss below, the claims purport to encompass mutated proteins truncated below all of these positions.

B. The Sequence Identity Parameters in the Claims Encompass Enzymatically Active Multiply-Modified PH20 Polypeptides the Common Specification Does Not Identify, Says to Not Make and For Which It Provides No Meaningful Guidance

155. The roughly 2,500 single-replacement $PH20_{1-447}$ polypeptides with hyaluronidase activity disclosed in the patent are an infinitesimally small fraction of the number of modified PH20 polypeptides encompassed by the sequence identity parameters used in the claims (*e.g.*, $2500 / 4.40 \times 10^{59}$ for the smallest group). In most settings, that fraction would be considered zero.

156. The remaining portion of the set of modified PH20 polypeptides captured by the sequence identity claim language is immense and will encompass PH20 polypeptides that incorporate 5, 10, 15, or more (for some claims upwards of 42) substitutions into the protein's structure. Many of these mutants will have multiple changes in the same region of the protein structure, which can cause energetically disfavored interactions not present in the native PH20 protein, disrupt patterns necessary for secondary structure formation and stability, induce changes

[75] EX1005 ('429 Patent), 87:52-88:24.

in positioning of secondary structures and structural motifs, impede folding of structurally significant regions of the protein, and any of a large number of other consequences that will disrupt the protein's structure.

157. The common disclosure does not even explain the effects that single amino acid substitutions had on the PH20 protein's native structure, let alone predict the effect additional amino acid substitutions proximate to the previously made single-substitutions would have on protein structure and activity. The common disclosure also does not identify any common structural features shared by the "active mutant" forms of the single-substitution $PH20_{1-447}$ polypeptides, or common structural features that should be shared by "active mutant" forms of multiple-modified PH20 polypeptides generally.

158. The effects of these myriad sets of combinations of multiple substitutions within PH20 could not have been predicted by a skilled artisan in the 2011 timeframe using the tools that were available then. For example, while the PH20 protein structure models Dr. Park used provided reliable insights when modeling the change of a single residue at a position where the model was, they cannot provide reliable insights when the modeled sequence incorporates many (*e.g.*, more than ~5) substitutions not found in a naturally occurring protein. That is because (i) if the modeled sequence incorporates multiple changes, it no longer has validity as a naturally occurring sequence, and (ii) the changes significantly

diminish the reliability of other positions of the model used to assess the change because they are no longer based on the structural positioning of residues within the template structure used to generate the model. Thus, a skilled artisan would have had to discover which combinations of substitutions to the PH20 protein would result in mutants that do exhibit hyaluronidase activity by making and testing all of them, an impossibly large undertaking.

159. Because of the complicated interactions multiple amino acid substitutions may have within a protein, the activity of multi-substituted polypeptides having sets of 5, 10, 15, or more changes could not generally be predicted from data regarding the activity of mutants containing only a single amino acid substitution. Put differently, the single-replacement $PH20_{1-447}$ polypeptides reported in the common disclosure are not representative of all the types of mutated $PH20_{1-447}$ polypeptides that have a particular substitution at position 324 and sets of between 1 and 41 *additional* substitutions at any of hundreds of positions within the PH20 protein.

160. Another problem caused by the use in the claims of sequence identity language to define the sets of proteins is that it captures many multiply-modified PH20 polypeptides with changes that common disclosure says are deleterious or eliminate hyaluronidase activity in PH20 enzymes.

161. First, the sequence identity language, read literally, would capture multiply-modified PH20 polypeptides that include substitutions listed in Tables 5 and 10. These are the substitutions that, when made as a single substitution in $PH20_{1-447}$, rendered the $PH20_{1-447}$ protein inactive. Also, there are no examples of any multiply-modified PH20 polypeptides in the specification, much less ones that combine substitutions that rendered the protein inactive from Tables 5 and 10 with other substitutions that together cause $PH20_{1-447}$ to have hyaluronidase activity (or increased levels of that activity).

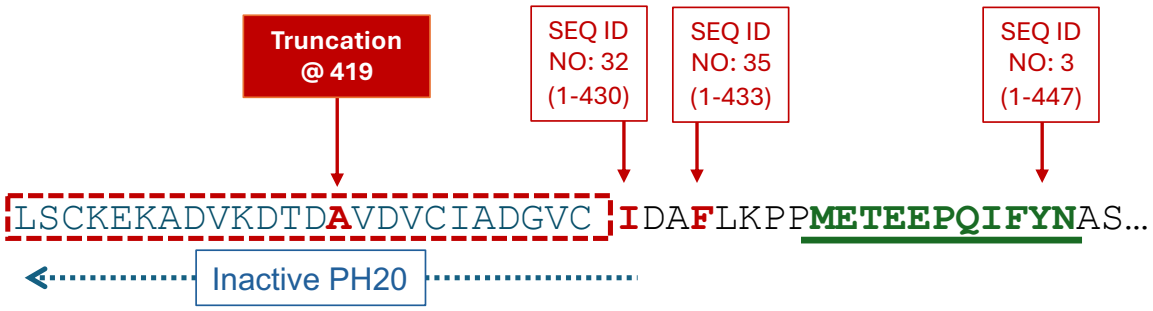
162. In fact, the common disclosure instructs skilled artisans to not include substitutions from Table 5/10 in any PH20 polypeptides with hyaluronidase activity, including those with multiple substitutions. See § V.A.4. To make all the multiply-modified PH20 polypeptides covered by the claims' sequence identity parameters, the skilled artisan would have to disregard the guidance in the common disclosure indicating that substitutions listed in Tables 5/10 should not be included in PH20 polypeptides intended to have hyaluronidase activity.

163. Second, the sequence identity language in the claims captures the six modified PH20 polypeptides with two or three specific combinations of substitutions that the common disclosure says to not make. See ¶¶ 146 to 147.

164. Third, the sequence identity language causes the claims to capture multiply-mutated PH20 polypeptides with C-terminal truncations that truncate the

sequence below position 429 and thus (according to the common disclosure) render the PH20 proteins inactive.[76] This happens when you apply the 91%/95% sequence identity language criteria to shorter reference PH20 sequences (*i.e.*, SEQ ID NO: 32 with 430 residues, and SEQ ID NO: 35 with 433 residues), as is permitted by the claims.

165. To illustrate this, consider a hypothetical modified PH20 polypeptide truncated to position 419 of PH20 (below).



166. Truncating PH20 to position 419 would require 11 changes relative to SEQ ID NO:32 (430 residues), and 14 changes relative to SEQ ID NO: 35 (433 residues). Applying the 95% identity requirement in claims 2 and 25 to SEQ ID NO:32 permits 21 total changes, of which 11 would be for the truncation and one for E324D, allowing 9 additional substitutions at any of 419 positions to any of 19 other amino acids. Similar claims that allow 91% sequence identity to SEQ ID NO: 32 (for example, claims 1, 6-10, 12, 14, 15, 22) would also capture this

[76] EX1001 ('520 Patent), 70:2-11.

truncated PH20 with an E324D substitution (in the case of claim 6) or either E324N or E324R substitutions (in the case of claim 7). For claim 25, the truncation to 419 consumes 14 of the 17 allowed changes at 95% sequence identity, and the position 324 substitution takes one more, leaving 2 additional substitutions across 419 positions to any of 19 amino acids. Similar claims that allow 91% sequence identity to SEQ ID NO: 35 (for example, claims 1, 6-11, 13, 15, and 22) would also capture this truncated PH20 with an E324D substitution (in the case of claim 6) or either E324N or E324R substitutions (in the case of claim 7).

167. The common disclosure describes no multiply-modified "active mutant" PH20 polypeptides, including those having fewer than 447 residues and provides no guidance about making enzymatically active mutants based on PH20 sequences ending before position 447 and containing 2 or more substitutions.

168. The common specification also did not report an experiment showing that introduction of an E324D, E324N, or E324R mutation into an inactive $PH20_{1-419}$ polypeptide would restore its hyaluronidase activity. There is no basis from the common specification or the scientific literature existing in late 2011 that would suggest that such a change would restore activity. The skilled artisan also could not predict whether any combinations of up to 9 or up to 2 additional (or more) substitutions could be made anywhere in the $PH20_{1-419}$ sequence or comparably

truncated PH20 polypeptide that would restore hyaluronidase activity to an inactive E324D, E324N, or E324R containing $PH20_{1-419}$ mutant.

169. In other words, the common disclosure not only does not help the skilled artisan identify which of the trillions of possible PH20 polypeptides of varying length with 2 to 42 substitutions have hyaluronidase activity; to practice the full scope of the claims it requires the skilled artisan to ignore what little guidance is in the specification about single-substitutions and truncations that render PH20 polypeptides inactive.

C. A Skilled Artisan Would Have to Engage in an Impossible Scale of Experimentation to Make and Identify All the Multiply-Modified PH20 Proteins with Hyaluronidase Activity Within the Sequence Identity Parameters of the Claims

170. Making and identifying all of the multiply-modified PH20 polypeptides that are within the immense set of polypeptides (between 10^{59} and 10^{112} distinct mutants) defined by the claims' sequence identity parameters would require not only an undue amount of experimentation, it likely is impossible.

171. The common disclosure contains a mixture of information that had been reported in the pre-2011 scientific literature. It also provided a description of the production, characterization and results from testing a library of single-replacement $PH20_{1-447}$ mutants, and as well as additional characterization and testing of two mutants (F204P and V58R). None of this information provides any

meaningful guidance to a skilled artisan that can be used to identify which of the myriad possible combinations of 5, 10, 15, or more substitutions can be incorporated into a PH20 protein to yield an enzymatically active protein.

172. The remainder of the common disclosure is largely hypothetical. This includes its descriptions about possible PH20 polypeptides that include one of the tested single substitutions, but then might have numerous additional, unidentified changes. Unlike its description of single-replacement $PH20_{1-447}$ mutants, the common disclosure identifies no examples of PH20 polypeptides with multiple amino acid substitutions at different positions (*i.e.*, specific amino acids being inserted into two or more different positions of the same PH20 polypeptide) that rendered active proteins. This appears to be the case because no such multiply-modified PH20 polypeptides appear to have actually been made or tested. That seems consistent with the common disclosure's description of a theoretical way of making such multiply-substituted PH20 polypeptides. That description is essentially a research plan that someone might use to discover multiply-modified PH20 polypeptides that retain hyaluronidase activity (through extensive time and effort), and not a description that demonstrates the inventors had already identified and were in possession of any such multiply-modified, active proteins.

1. The Common Disclosure Provides Only a Research Plan for Discovering Multiply-Modified, Enzymatically Active PH20 Polypeptides

173. The common disclosure's description of how to make modified PH20 polypeptides, including those with more than one amino acid substitution, are found in a section of the patent titled "Methods for Identifying Modified Hyaluronan-Degrading Enzymes with Altered Properties or Activities."[77] This section is describing a directed evolution experiment. A majority of this section is simply describing at a very general level the well-known techniques for using mutagenesis techniques to produce and screen libraries of mutated proteins.[78] The methodology being described in this section is best described as a research plan, as it generally outlines the types of steps one might take to carry out a mutagenesis and screening research program.

174. The part of this research plan that might be pursued to create multiply-modified PH20 polypeptides involves an iterative process of mutagenesis, screening and selection steps. An excerpt is provided below.

> The method can be performed a plurality of times, whereby the steps of the method are repeated 1, 2, 3, 4 or 5 times. The method provided herein also is iterative. In one example, after

[77] EX1001 ('520 Patent), 44:1-3.

[78] EX1001 ('520 Patent), 127-20-65, 128:7-129:49, 130:9-134:52.

> the method is performed, any identified modified hyaluronan-degrading enzyme can be modified or further modified to increase or optimize the activity.[79]

175. The mutagenesis methodology being described involves performing site-directed mutagenesis in which "single amino acid residues" are replaced at "target positions one-by-one, such that each individual mutant generated is the single product of each single mutagenesis reaction."[80] It says these techniques also can be configured to introduce any of 10 to 19 alternative amino acids in these single mutations.[81]

176. The screening clearly is geared to finding modified PH20 polypeptides that have hyaluronidase activity. For example, the common disclosure suggests that mutants can be screened to find those that retain hyaluronidase activity "…whereby the activity of the enzyme is indicative of the stability of the enzyme as a measure of its resistance to denaturation.[82] It also

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79 EX1001 ('520 Patent), 127:66-128:4.

80 EX1001 ('520 Patent), 129:57-62.

81 EX1001 ('520 Patent), 129:50-130:7.

82 EX1001 ('520 Patent), 133:1-5. *Also* EX1001 ('520 Patent) 133:16-21 ("In examples of the methods herein, the activity of the modified hyaluronan

suggests using cutoffs for relative activity, and that a mutant may be assumed to exhibit stability "if any detectable activity is measured or assessed upon exposure or incubation with a denaturation condition or denaturing agent."[83]

177. The common disclosure provides two general plans for producing modified PH20 polypeptides that may contain multiple substitutions. Each proposes using successive rounds of mutagenesis and screening to introduce single amino acid changes "one-by-one." In one, mutants are to be selected if they are "exhibiting stability, such as increased stability [that] can be modified or further modified to increase or optimize the stability." Then, a "secondary library can be created by introducing additional modifications in a first identified modified hyaluronan-degrading enzyme" and then testing that secondary library "using the assays and methods described herein."[84]

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degrading enzyme is assessed upon exposure to a first denaturation condition and also assessed upon exposure to a second condition that is a control or non-denaturation condition, and the resulting hyaluronidase activities [are] compared.").

83 EX1001 ('520 Patent), 133:40-54.

84 EX1001 ('520 Patent), 134:54-67.

178. The second approach proposes starting with mutants that "are identified as not exhibiting stability such as increased stability (e.g., such that they are not active or do not have increased activity under [] a denaturation condition)" and "can be further modified and retested for stability under a denaturation condition."[85] The targeting of these further mutations is described as follows:

> The further modifications can be targeted near particular regions (e.g., particular amino acid residues) associated with activity and/or stability of the molecule. For example, residues that are associated with activity and/or stability of the molecule generally are critical residues that are involved in the structural folding or other activities of the molecule. Hence, such residues are required for activity, generally under any condition. Critical residues can be identified because, when mutated, a normal activity of the protein is ablated or reduced. For example, critical residues can be identified that, when mutated in a hyaluronan-degrading enzyme, exhibit reduced or ablated hyaluronidase activity under a normal or control assay condition.[86]

179. This largely conceptual, trial-and-error research plan assumes that "ablated or reduced" enzymatic activity identifies critical residues "associated with

[85] EX1001 ('520 Patent), 135:1-6.

[86] EX1001 ('520 Patent), 135:1-19.

activity and/or stability of the molecule."[87] This assumption is not warranted. The loss of activity, as I explained previously, is not a direct measure of stability. It may be caused by mutations the influence the rate of catalysis without altering the overall stability of the protein (*e.g.*, influencing presentation or catalysis of the substrate). Conversely, changes that do affect the stability of the protein may not materially impair the activity of the catalytic active site.

180. The common disclosure's theoretical research plan is also largely meaningless when it is applied to the data in the common disclosure. The plan proposes to introduce modifications "near particular regions (e.g., particular amino acid residues) associated with activity and/or stability of the molecule." The data reported in Tables 5 and 10 show that at least one substitution at each of 405 different positions between positions 1 and 444 of the sequence rendered the 447 amino acid PH20 polypeptide inactive.[88] In other words, under the logic of the common disclosure's research plan, the skilled artisan should "target" mutations to positions "near" 90% of the protein sequence. Thus, the research plan provides little to no guidance on how to design and/or choose mutations in this protein.

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87 EX1001 ('520 Patent), 135:1-19.

88 EX1001 ('520 Patent), Tables 5, 10.

181. As I explained above (§ II.B.1, ¶¶ 50-52), the major challenge in using directed evolution techniques in protein engineering is scale. It is critical that each phase of the process is designed to navigate the challenge of making and screening such a huge scale of mutants.

182. For example, mutagenesis techniques must be focused on regions of the PH20 that are likely to productive mutations. The common disclosure does not provide any real guidance on this part of the process. It basically leaves the entire protein open as a target for mutagenesis.

183. When the mutagenesis methodology is unfocused as it is here, the importance of rapid, efficient and accurate screening and selection assays is paramount. The assays have to identify a characteristic of the mutated PH20 proteins that will narrow the massive collection of proteins in a productive manner. The assays described in the common disclosure do not do that. They instead say the mutants "can be tested using the assays and methods described herein." EX1001 ('520 Patent), 135:24-26. The only example in the patent showing a screening assay being applied to a library of mutants is in Examples 3 and 4.[89] This assay is based on a simple hyaluronidase activity screen, which selects active

[89] EX1001 ('520 Patent), 225:11-227:28 (Example 3), 227:30-228:35 (Example 4).

mutants if they show at least 40% of the hyaluronidase activity of a control. As I pointed out previously, the control values seen with this type of SEAP assay had a very wide range of activity. The assay being described is not one that would remedy the problems of the unfocused mutagenesis methodology of this procedure.

184. A scientist following this iterative mutagenesis and screening research plan cannot know in advance of conducting multiple rounds of experiments, whether modified PH20 polypeptides will be produced that have sets of 5, 10, 15, or more substitutions and retain sufficient activity that will be selected for the next round of the process. More directly, until a modified polypeptide with multiple substitutions is identified and characterized, the structural or functional properties of that mutant protein are not known (beyond possession of some threshold of relative activity used to select it). In other words, a skilled art cannot know ***which*** combinations of amino acids in ***which*** positions will be in the sequence of a modified PH20 polypeptide that is enzymatically active until that mutant is actually made, tested, isolated and characterized.

185. Likewise, until multiply-mutated PH20 polypeptides are actually made, isolated, characterized and tested, the skilled artisan could not know which of them would yield modified PH20 polypeptides with particular functional profiles, such as exhibiting more than 100% of the activity of the unmodified PH20 polypeptides or exhibiting increased stability or resistance in denaturing conditions

relative to unmodified forms of the protein. There is no guidance in the patents that helps the skilled artisan predict which particular combinations of 2 to 42 amino acid substitutions will yield modified PH20 polypeptides with these types of functional profiles (let alone why any particular combination of changes would yield increased activity and/or stability).

2. Discovering All the Active Mutant Multiply-Modified PH20 Polypeptides Within the Scope of the Claims Is Impossible

186. The directed evolution methodology used in the common disclosure to create mutant PH20 polypeptides, as I have discussed, was not new in the 2011 timeframe. The technique, however, is the quintessential "make and test" trial and error technique. By definition, the scientist carrying out a directed evolution protocol does not know which of the potentially trillions of possible mutants might incorporate a substitution that causes the protein to exhibit an improved characteristic, whether that is measured as increased stability, activity or something else.

187. As I explain in the preceding section, the common disclosure suggests that one can use an iterative, single mutation mutagenesis methodology to make modified PH20 polypeptides that would contain more than one substitution. In that process, a first set of mutants with one substitution are created, screened and those with some level of activity are selected. Then, the process is repeated, but here a

second single substation is introduced into each of the singly-substituted mutants that were selected in the first round, that set of "doubly-substituted" mutants is screened, and the doubly-substitute mutants above the cutoff activity threshold are selected.

188. To produce a set of multiply-modified PH20 polypeptides matching the limits of substitutions defined by the sequence parameters in the claims (*i.e.*, up to between 21 and 42 substitutions) pursuant to the methodology in the common disclosure, its process would be repeated 20-41 times (assuming one starts with a E324D, E324N, or E324R mutant in the first round). This methodology conceptually would narrow the number of n-substituted mutants in the (n)-library being screened in each step, like an inverted funnel. At the end of this iterative process, there may be one, none or some unidentified number of mutants having 21 to 41 single substitutions that was active. But because each step (n) of the process will have eliminated some (unknowable) number of (n)-substituted PH20 polypeptides during its activity screening step, the (n+1) round of the process will introduce single substitutions in a (presumably) smaller set of (n+1)-substituted PH20 polypeptides. In other words, after performing between 20 and 41 rounds of this iterative mutate/screen/select process, one would not have all the multiply-substituted PH20 polypeptides with activity, they would have only those that survived the screening steps in each round of the sequence.

189. Given the massive number of possible distinct PH20 polypeptides having between 2 and 42 substitutions that the sequence identity parameters of the claims capture, one would have to repeat this iterative process innumerable times to identify all of the enzymatically active multiply-mutated PH20 polypeptides in the scope of the claims. As I noted earlier, the aggregate mass of the collection of individual modified PH20 polypeptides covered by the claims (all of which have one of the three specified position 324 mutations of the narrowest claims) that would have to be produced and screened is greater than the mass of the earth. This task is simply impossible!

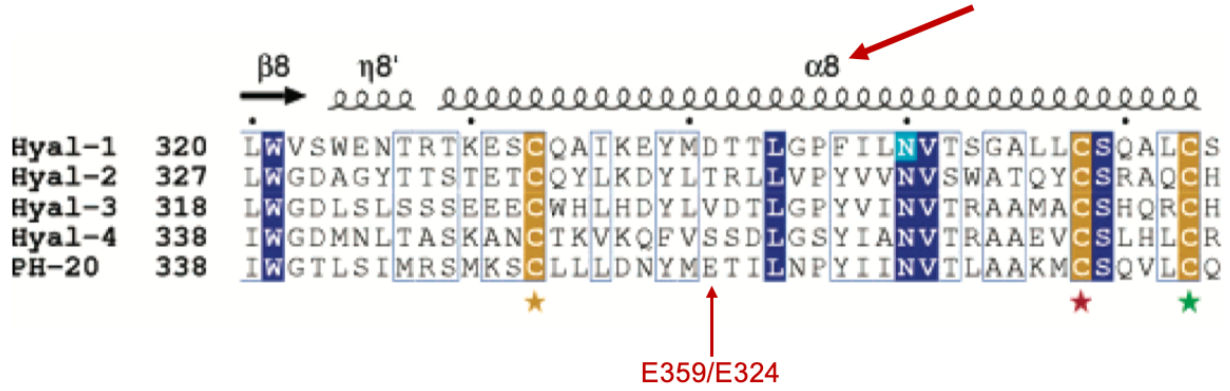
190. The common disclosure does not provide guidance that a skilled artisan could have used to identify which of the trillions of possible combinations of 2 to 42 substitutions in PH20 would retain 40% of the activity of the unmodified parent PH20 polypeptide. It also does not provide any guidance that would enable a skilled artisan to identify the subset of that massive (and unknowable) set of enzymatically active PH20 polypeptides having the myriad possible combinations of between 2 and 42 substitutions that result in the PH20 polypeptide having greater than 100% of the activity of the parent unmodified PH20. A skilled artisan, for example, could not, as a practical matter, use rational design techniques to assess all these possible variant sequences of PH20. There is not enough time in a person's lifetime to do that. Also, protein structural models available in the 2011

timeframe would not be able to reliably assess sequences that incorporated more than a few variant residues in one region of the protein.

191. Also, a skilled artisan would not have assumed that a singly-substituted PH20 polypeptide that exhibited increased activity would retain that activity if 1 to 41 *additional* substitutions were incorporated into any arbitrary position of its sequence. One can readily imagine scenarios where additional substitutions are made in portions of a sequence responsible for particular secondary structures of the protein, and which would disrupt those structures. See § II.B.2, above. Changes could also be made in the region of the first substitution that would have disruptive effects on secondary structure.

192. For example, position 324 is located near the middle of the "α8" α-helix structure in PH20 based on the information reported in Chao's alignment (below). As I have explained in my publications in the scientific literature, α-helices typically require a characteristic pattern of polar and non-polar residues to form and maintain the helical structure.[90] Introducing random amino acids could disrupt that pattern, which could have a range of effects in this region of the helical structure.

[90] EX1046 (Beasley), 2031-2032; EX1047 (Xiong), 6349, 6351.



193. The absence of any experimental characterization of any examples of multiply-substituted PH20 polypeptides here is fatal to being able to predict the effects of making 1 to 41 additional substitutions beyond the E324D, E324N, or E324R substitutions. The skilled person would have to perform innumerable rounds of mutagenesis and screening, a "make-and-test" type of experimental protocol, to discover all possible multiply-substituted, enzymatically active mutants in the scope of the claims.

VI. The E324D, E324N, and E324R PH20$_{1-447}$ Mutants Would Have Been Obvious

194. As I have explained above, the effects of making numerous substitutions to a naturally occurring protein, particularly when they are proximate to each other or are in related or proximate structures in the protein, would have been unpredictable in 2011. By contrast, it was possible, by studying a particular protein structure, to assess whether a single amino acid substitution within a

defined structure would be likely to be tolerated or not. This is the essence of the rational design methodology used in protein engineering.

A. The '429 Patent Suggests Making Single-Amino Acid Substitutions in Non-Essential Regions of $PH20_{1-447}$

1. The '429 Patent Describes $PH20_{1-447}$, Its Production and Its Uses

195. I reviewed the '429 Patent, which is owned by the same company that owns the '520 Patent (Halozyme) and which produces the Hylenex® human PH20 biological product that was approved in 2005. I understand that Hylenex® consists of the human PH20 protein having residues 1-447 of the mature form of PH20 (*i.e.*, without the signal sequence).[91] Because the $PH20_{1-447}$ form of PH20 was already an approved therapeutic product, it would be a focus of interest by persons of ordinary skill in the art in 2011 investigating therapeutic uses of human hyaluronidase enzymes.

196. The '429 Patent describes production of soluble, neutral active PH20 human hyaluronidase proteins, which it refers to using the abbreviation "sHASEGPs."[92] It explains these proteins can be made soluble by truncating the PH20 sequence before the start of the GPI anchor sequence of the protein, which it

[91] EX1049 (Hylenex sequence), 1.

[92] EX1005 ('429 Patent), 3:51-56.

identifies as being at position 483 in the full-length sequence (including the signal sequence).[93] One of these soluble, neutral, truncated proteins is $PH20_{1-447}$, which consists of residues 36 to 482 of the full-length PH20 sequence.[94]

197. The ’429 patent explains PH20 enzymes must be glycosylated to exhibit their catalytic activity.[95] As it states:

> N-linked glycosylation of the sHASEGP’s are critical for their catalytic activity and stability. While altering the type of glycan modifying a glycoprotein can have dramatic affects on a protein's antigenicity, structural folding, solubility, and stability, most enzymes are not thought to require glycosylation for optimal enzyme activity. sHASEGPs are thus unique in this regard, that removal of N-linked glycosylation can result in near complete inactivation of the Hyaluronidase activity.

198. A skilled artisan would have taken away from this observation that PH20 polypeptides should be produced in mammalian host cells to ensure they are glycosylated and retain enzymatic activity. The ’429 Patent also describes conventional methods of producing enzymatically active $PH20_{1-447}$ in CHO cells

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93 EX1005 (’429 Patent), 3:58-3, 86:7-88:24.

94 EX1005 (’429 Patent), 87:52-88:10.

95 EX1005 (’429 Patent), 7:9-20.

transfected with a bicistronic vector containing a DNA sequence encoding the 1-182 sequence of PH20.[96]

199. The '429 Patent explains that human PH20 enzymes (including $PH20_{1-447}$) are useful in various human therapeutic applications and provides a lengthy list of those therapeutic uses at columns 54 to 83. These various therapeutic uses of PH20 enzymes are based on the ability of the PH20 enzyme to selectively degrade hyaluronic acid/hyaluronan, a class of negatively charged polysaccharides.[97] Some of the therapeutic uses of enzymatically active PH20 polypeptides that the '429 Patent describes address a hyaluronan-associated disease or condition, and involve administering the PH20 polypeptide to the subject, and include the following:

- reversing the inhibition caused by chondroitin sulfate proteoglycans in glial scars and promoting axon regeneration following injury;[98]

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[96] EX1005 ('429 Patent), 89:53-61 (describing HZ24 vector containing "DNA encoding 1-482 of human PH20 hyaluronidase"); 90:19-91:67 (production of CHO cell expressing HZ24); 92:1-40 (expression and recovery of $PH20_{1-447}$).

[97] EX1005 ('429 Patent), 2:11-16, 26:11-14, 70:38-41.

[98] EX1005 ('429 Patent), 71:48-72:6.

- treatment of herniated disks;[99]
- removal of the cumulus cells surrounding an egg in connection with in vitro fertilization procedures;[100]
- treating various ophthalmic disorders (*e.g.*, injuries or increased eye pressure following cataract surgery);[101]
- during organ transplantation, treating interstitial edemas and degrading accumulated glycosaminoglycans;[102]
- treating pathologic accumulations of glycosaminoglycans in the brain or in cardiovascular disease;[103] and
- treating cysts or infiltrations of the skin by glycosaminoglycans. [104]

200. The ’429 Patent also portrays PH20 enzymes (including $PH20_{1-447}$) and pharmaceutical compositions that contain them as being useful in the treatment

[99] EX1005 (’429 Patent), 72:15-31.

[100] EX1005 (’429 Patent), 72:62-73:3.

[101] EX1005 (’429 Patent), 77:54-78:33, 81:21-82:29.

[102] EX1005 (’429 Patent), 78:60-79:22.

[103] EX1005 (’429 Patent), 79:23-80:43.

[104] EX1005 (’429 Patent), 80:7-52.

of cancer.[105] One example is to use PH20 to increase the sensitivity of cancers to chemotherapeutic agents or treating cancers with decreased to undetectable hyaluronidase activity.[106] Another example is using soluble, enzymatically active forms of hyaluronidase in combination with "anti-cancer agents" (*e.g.*, chemotherapeutic agents or other cancer treatments, such as antibodies, peptides, or gene therapies).[107] It also indicates that hyaluronidase enzymes can be used as a "delivery or 'spreading' agent in combination with a second active compound, such as a drug or prodrug, to facilitate delivery of or to enhance the activity of the second active ingredient," and particularly for subcutaneous administration and

[105] EX1005 ('429 Patent), 73:4-74:58.

[106] EX1005 ('429 Patent), 73:21-58.

[107] EX1005 ('429 Patent), 73:4-16.

delivery of therapeutically active proteins.[108] It points out this is useful when subcutaneously injecting these therapeutic agents.[109]

201. The ’429 Patent also describes pharmaceutical compositions that contain a PH20 protein, either by itself or in combination with another therapeutic agent.[110] One benefit the ’429 Patent identifies for combining a PH20 polypeptide with another therapeutic agent in a pharmaceutical composition is to facilitate “delivery or ‘spreading’” of the therapeutic agent.[111] As it explains, the inclusion

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[108] EX1005 (’429 Patent), 56:34-57:21 (contemplating co-formulation of PH20 enzymes with “various chemotherapeutics”). Other examples include chemotherapeutic agents, anti-inflammatory agents, analgesic agents, etc. EX1005 (’429 Patent), 60:38-61:4.

[109] EX1005 (’429 Patent), 63:41-44 (contemplating “[p]arenteral administration of the sHASEGP or a soluble human hyaluronidase domain thereof, generally characterized by injection, either subcutaneously, intramuscularly or intravenously”); EX1005 (’429 Patent), 76:18-77:37.

[110] EX1005 (’429 Patent), 54:40-65 (“The sHASEGP polypeptide and a second agent can be packaged as separate compositions for administration together or sequentially or intermittently” or “as a single composition”).

[111] EX1005 (’429 Patent), 56:66-57:21.

of the PH20 in the pharmaceutical composition with the other therapeutic agent is done "to increase diffusion of other injected molecules less than 200 nm in diameter," and that this enables the combined pharmaceutical compositions to be injected subcutaneously into patients.[112]

202. The '429 Patent portrays the therapeutic uses and pharmaceutical compositions I discuss in ¶¶ 199-201 as being shared by the class of hyaluronidase enzymes that the patent refers to as "sHAGEGPs," which include both the wild-type, C-terminally truncated (soluble) forms of PH20 and variants having a single amino acid substitution in a non-essential region of that protein.[113]

203. I note that conventional procedures relating to production of the wild-type $PH20_{1-447}$ protein that are described in the '429 Patent could be applied to produce forms of $PH20_{1-447}$ that incorporate a single amino acid substitution (*e.g.*, the E324D, E324N, or E324R substitutions I discuss below) with little effort.[114] It involves using the conventional techniques of creating a modified nucleotide sequence encoding the $PH20_{1-447}$ sequence with the single amino acid change,

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[112] EX1005 ('429 Patent), 1:16-27, 8:1-10, 8:60-9:4, 76:18-38.

[113] See ¶ 206, below; EX1005 ('429 Patent), 39:54-40:1-20, 39:8-16, 10:6-13; *also* 16:4-13.

[114] See EX1005 ('429 Patent), 39:54-40:21.

inserting it into the vector described in the common disclosure, and then using the vector to transfect a CHO cell, again as is described in the common disclosure.[115] The '429 Patent also indicates that different heterologous signal sequences can be used when expressing PH20 sequences, and was generally a known technique.[116] The modified $PH20_{1-447}$ proteins would be C-terminally truncated and therefore would be expected to be soluble.

204. The '429 Patent reports that expressing $PH20_{1-447}$ mutants in a CHO cell yields a glycosylated form of the protein that is enzymatically active.[117] The '429 Patent explains that PH20 must be glycosylated to exhibit enzymatic activity, and that its techniques of expressing the wild-type $PH20_{1-447}$ protein in CHO cells yielded enzymatically active forms of that protein. There is no reason to believe the E324D, E324N, or E324R $PH20_{1-447}$ mutants would not be equivalently glycosylated.

205. The '429 Patent describes modifying $PH20_{1-447}$ mutant proteins to "prolong its half life by way of masking the protein with polyethylene glycol" and that "[c]hemical modifications of a sHASEGP with polymers such as polyethylene

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115 EX1005 ('429 Patent), 89:51-90:16 (Example 6), 90:19-91:67 (Example 7).

116 EX1005 ('429 Patent), 6:50-55, 14:1-55, 15:19-54, 37:21-36.

117 EX1005 ('429 Patent), 89:43-91:67 (Example 7).

glycol and dextran are provided."[118] It also explains that sHASEGPs can be conjugated with "one or more targeting agents," joined with other proteins to yield "fusion proteins," or conjugated to a drug or prodrug.[119] Targeting agents include multimerization domains, such as Fc domains. $PH20_{1-447}$ mutant proteins can also comprise a "chimeric protein."[120] I would expect that single-substitution $PH20_{1-447}$ mutants including the E324D, E324N, and E324R single substitution mutants, could be similarly modified and/or conjugated, following the guidance in the examples in the '429 Patent.

2. The '429 Patent Says to Make Single Amino Acid Substitutions in Non-Essential Regions of PH20

206. The '429 Patent describes a class of soluble neutral active PH20 hyaluronidases that it calls "sHASEGP" proteins, including variants with "protein

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[118] EX1005 ('429 Patent), 3:64-4:1, 4:45-53; *also* EX1005 ('429 Patent), 26:20-28:4 (describing conjugation of modified PH20 proteins to polymers, including PEG).

[119] EX1005 ('429 Patent), 18:33-52, 54:20-37.

[120] EX1005 ('429 Patent), 51:11-41.

level" modifications, particularly amino acid substitutions that "do not substantially alter biological activity of" the proteins.[121] It also indicates that:

> Suitable substitutions, including, although not necessarily, conservative substitutions of amino acids, are known to those of skill in this art and can be made without eliminating the biological activity, such as the catalytic activity, of the resulting molecule.[122]

207. The '429 Patent then explains that a skilled artisan would "recognize that, in general, single amino acid substitutions in non-essential regions of polypeptides" (like PH20) "do not substantially alter biological activity" (*i.e.*,

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[121] EX1005 ('429 Patent), 39:6-16, 9:46-52, 10:6-13.

[122] EX1005 ('429 Patent), 10:9-13; *also* 40:1-20, 39:6-16, 10:6-13, *also* 16:4-13 ("For purposes herein, amino acid substitutions can be made in any of sHASEGPs and Hyaluronidase domains thereof provided that the resulting protein exhibits Hyaluronidase activity. Amino acid substitutions contemplated include conservative substitutions, such as those set forth in Table 1, which do not eliminate proteolytic activity.").

hyaluronidase activity).[123] In other words, the '429 Patent is explaining the prevailing beliefs among skilled artisans that making a single amino acid substitution within a non-essential region of PH20 would be tolerated by the enzymatically active forms of PH20 being described in the '429 Patent. Those would be the forms that contain the "minimally active domain" (*i.e.*, terminating at residues 438 to 448). A skilled person would have generally agreed with this explanation in the 2011-timeframe, particularly with respect to the $PH20_{1-447}$ protein that was shown to be enzymatically active. That person thus would expect that the $PH20_{1-447}$ protein incorporating a single amino acid substitution in a non-essential region would retain much of its activity, depending on the position of the substitution and the amino acid being substituted into that position. A skilled artisan also would have expected that the $PH20_{1-447}$ protein incorporating a single amino acid substitution in a non-essential region would generally have the same therapeutic uses and utilities as described with respect to the $PH20_{1-447}$ protein (discussed above).[124]

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[123] EX1005 ('429 Patent), 16:4-21; *also* 9:47-52 ("Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity…")

[124] EX1005 ('429 Patent), 56:66-57:21, 73:4-74:29.

208. A skilled artisan also would not have read the ’429 Patent as suggesting that the only amino acid substitutions that should be considered for non-essential positions in PH20 are the “conservative” substitutions are those listed in Table 1. The ’429 Patent describes those as only being examples and explains that “other substitutions are also permissible and can be determined empirically or in accord with known conservative substitutions.”[125] In any event, I note that aspartic acid (D) is identified as being a conservative amino acid substitution for glutamic acid (E) in Table 1 of the ’429 Patent. A skilled artisan following this guidance in Table 1 of the ’429 Patent would have viewed the E324D substitution in $PH20_{1\text{-}447}$ to be an example of what the ’429 Patent is describing to be a conservative substitution that would maintain the enzymatic activity of the $PH20_{1\text{-}}$

[125] EX1005 (’429 Patent), 16:24-36.

$_{447}$ protein (below).

TABLE 1 Original residue Conservative substitution Ala (A) Gly; Ser, Abu Arg (R) Lys, orn Asn (N) Gln; His Cys (C) Ser Gin (Q) Asn Glu (E) ASP Gly (G) Ala; Pro His (H) Asn; Gin Ile (I) Leu; Val; Met; Nle; Nva Leu (L); Val; Met; Nle; Nv Lys (K) Arg; Gin; Glu Met (M) Leu; Tyr; Ile; NLe Val Ornitine Lys; Arg Phe (F) Met; Leu; Tyr Ser (S) Thr Thr (T) Ser Trp (W) Tyr Tyr (Y) Trp; Phe Val (V) ILE; Leu; Met; Nle; Nv Other substitutions are also permissible and can be determined empirically or in accord with known conservative substitutions.

B. The Skilled Artisan Would Consider Chao for Structural Insights into Making Modified PH20 Polypeptides

209. In 2011, a skilled artisan interested in producing modified PH20 polypeptides as the ’429 Patent proposes would have certainly reviewed the Chao paper (EX1006) in conjunction with the ’429 Patent.

210. A skilled artisan would have viewed the Chao paper as being highly relevant to process of implementing single amino acid substitutions in non-essential regions of $PH20_{1-447}$ because it contains the type of information they would use to carry out a rational design project, particularly an experimentally-determined structure for the human HYAL1 hyaluronidase protein. It also

provided extensive discussion on structural similarities among human and non-human hyaluronidase proteins.[126] See § II.E.2.

211. Chao also provided an annotated sequence alignment of the five human hyaluronidase enzymes. In that alignment, Chao identified: (i) 76 invariant conserved positions (blue), (ii) 3 residues involved in catalysis (red), (iii) 10 conserved cysteine residues that form disulfide bonds (gold) and (iv) 3 conserved asparagine residues that are glycosylated (turquoise).[127] This type of information, again, was the type of information used routinely to perform rational design protein engineering in the 2011 timeframe.

C. The Skilled Artisan Would Have Identified Non-Essential Regions in PH20 and Suitable Amino Acid Substitutions Using a Multiple Sequence Alignment of Homologous Hyaluronidase Proteins

212. As I explained in § VI.A.2, the ’429 Patent would have encouraged a skilled artisan to make modified PH20 proteins having single amino acid substitutions in non-essential regions. The skilled artisan would have understood these non-essential regions to be the regions between the conserved residues within PH20, which are residues that are generally considered essential to the structure and functions of proteins like hyaluronidase enzymes. The skilled artisan also

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126 EX1006 (Chao), 6915.

127 EX1006 (Chao), 6916.

would assess the conserved residues using sequences of homologous hyaluronidase proteins that were available in 2011.

213. To identify conserved residues, and thereby simultaneously identify the non-essential regions of PH20, the skilled artisan would have produced and analyzed a multi-sequence alignment based on protein sequences having significant sequence homology to PH20. Protein scientists routinely used sequence alignments, and particularly multiple sequence alignments, to identify conserved residues within families of related proteins.[128] For example, both the '429 Patent and Chao discuss using sequence alignments in their analyses.[129]

214. A multiple-sequence alignment also identifies the different amino acids that occur in non-essential positions in a protein and allows one to calculate the frequency with which each amino acid occurs at each position in the set of proteins being aligned. It is important to remember that these different amino acids are found in actual, naturally occurring proteins that have evolved over

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[128] EX1014 (Brandon & Tooze), 351.

[129] EX1005 ('429 Patent), 12:46-49 ("By sequence identity, the numbers of conserved amino acids is determined by standard alignment algorithms programs, and are used with default gap penalties established by each supplier."); EX1006 (Chao), 6913, 6915-16, Figure 3.

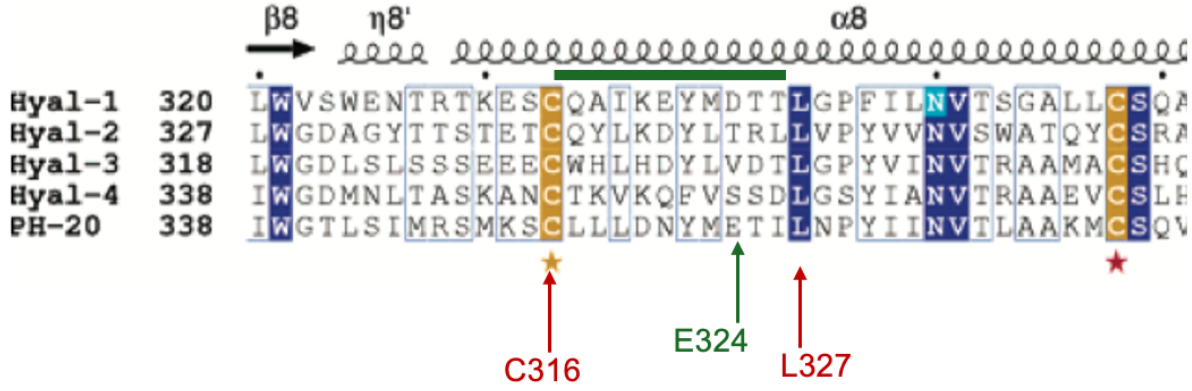
millions of years. The presence of these amino acids at the non-essential positions in hyaluronidase enzymes from different species demonstrates that proteins containing them are stable enough to survive evolutionary pressure that would have been eliminated from the genomes of organisms if they were not stable or were inactive.

215. I reviewed Dr. Park's report and its analyses (EX1004). Dr. Park performed an analysis that I believe a skilled artisan would have performed in 2011. His methodology included (i) using a multiple-sequence alignment to identify non-essential regions of PH20 (including position 324), (ii) identifying the amino acids that occur at those non-essential regions in the proteins in the set used for the alignment, and (iii) assessing whether amino acid substitutions appearing in nature at position 324 would be tolerated by PH20.

216. I believe Dr. Park's techniques and analysis—which follow a rational design approach to analyzing mutated proteins—were used by scientists I worked with in the 2011 timeframe in similar projects. I am familiar with these types of analyses. I also would have obtained these types of analyses and compilations of data from a similarly qualified colleague in 2011 when engaged in such a project. I found Dr. Park's analyses to be objective, thorough, useful, and reliable.

D. Both Chao and a Multi-Sequence Alignment of Proteins Homologous to PH20 Would Have Identified Position 324 Being in a Non-Essential Region and Suggested Aspartic Acid (D), Asparagine (N), and Arginine (R) as a Single Substitution at Position 324

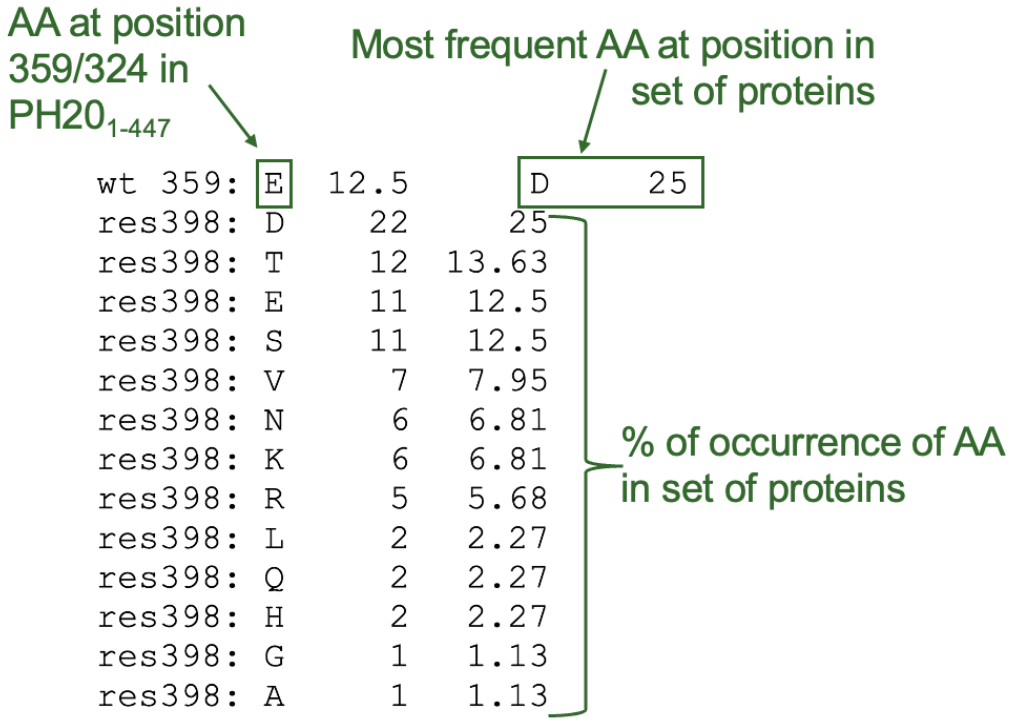
217. Position 324 is within a non-essential region of the PH20 sequence, based on my review of Dr. Park's analysis[130] and the sequence alignment in Chao. The nearest conserved residues to position 324 are C316 and L327. Chao identifies the region between C316 and L327 as containing 10 non-conserved residues, which includes glutamic acid at position 324. This region and position 324 are annotated in the excerpt from Figure 3 of Chao below.



218. Dr. Park's report identifies the frequencies of amino acids that occur in sequences homologous to PH20 as of December 2011. As shown below, at positions corresponding to 324 in PH20, the most prevalent amino acid is aspartic

[130] EX1004 (Park Dec.), ¶¶ 31-32, 106-107 and appendices thereto.

acid (D), which appears in approximately 25% of the proteins homologous to PH20. Threonine is the second-most prevalent amino acid at this position (13.6%, 12 homologous proteins), while the wildtype amino acid (*i.e.*, glutamic acid) is the third-most prevalent amino acid (approximately 12.5%, 11 homologous proteins). Asparagine (N) and arginine (R) appeared in 7% (6 proteins) and 6% (5 proteins) of the set of homologous proteins, respectively.



Pos (w/s)	Pos (wo/s)	WT	Alt	Frequency (%)
359	324	E	-	12.50
359	324	-	D	25.00
359	324	-	T	13.63
359	324	-	S	12.50
359	324	-	V	7.95
359	324	-	N	6.81
359	324	-	K	6.81
359	324	-	R	5.68
359	324	-	L	2.27
359	324	-	Q	2.27
359	324	-	H	2.27
359	324	-	G	1.13
359	324	-	A	1.13

219. Chao identifies the glutamic acid at position 324 of the wild-type PH20 sequence as being located near the middle of the α-helix secondary structure designated α8.[131] Dr. Park's MSA shows that that a large number (12) of other amino acids occur in homologous proteins at positions corresponding to position 324 in PH20. It also shows that these alternative amino acids have diverse properties (*e.g.*, polar vs. non-polar, small vs. large side chains, charged or uncharged residues, etc.).

[131] EX1006 (Chao), Figure 3.

220. A skilled artisan, in 2011, would have readily identified position 324 as being in one of the non-essential regions of $PH20_{1-447}$ contemplated by the '429 Patent. A skilled artisan also would have considered aspartic acid, asparagine, and arginine to be obvious choices for substitutions for glutamic acid at position 324 under the rationale of the '429 Patent in 2011. There are several reasons for this conclusion.

(a) Dr. Park's multiple sequence alignment of hyaluronidase proteins homologous to PH20 available in December of 2011 identifies aspartic acid, asparagine, and arginine as being amino acids that are tolerated at positions corresponding to position 324 in PH20 in many homologous hyaluronidase proteins.[132]

(b) Aspartic acid was the most prevalent amino acid observed to occur in the 88 published homologous sequences. Aspartic acid also occurs in a position corresponding to 324 in one other human hyaluronidase enzyme (Hyal1) as Chao illustrates.[133]

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[132] EX1006 (Chao), 6916, Figure 3; EX1004 (Park Dec.), ¶¶ 106, 111; Appendix D.

[133] EX1006 (Chao), 6916, Figure 3.

(c) The environment at position 324 is solvent exposed, which favors hydrophilic residues like aspartic acid, asparagine, and arginine.[134]

(d) Table 1 of the '429 Patent identifies aspartic acid as a conservative substitution for glutamic acid, which would have provided another reason to substitute aspartic acid for glutamic acid at position 324.[135]

221. The skilled artisan also would have reasonably expected, consistent with the '429 Patent's guidance and their knowledge of protein structure, that substituting aspartic acid (D), asparagine (N) or arginine (R) for glutamic acid (E)at position 324 of $PH20_{1-447}$ would not substantially alter the biological activity of $PH20_{1-447}$ protein. In other words, the skilled artisan would have expected the E324D, E324N, and E324R $PH20_{1-447}$ mutants to retain most of the hyaluronidase activity of the unmodified $PH20_{1-447}$ protein. As I noted above, position 324 in PH20 is solvent accessible and many different amino acids are tolerated at it in naturally occurring homologous hyaluronidase proteins. In addition, there a large number of homologous hyaluronidase proteins that have aspartic acid, asparagine, and arginine at positions corresponding to 324 in PH20: aspartic acid occurs in that position in 22 such proteins and 1 other human hyaluronidase enzyme while

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134 EX1004 (Park Dec.), ¶ 110.

135 EX1005 ('429 Patent), 16:7-36.

asparagine (N) and arginine (R) appear at that position in 6 and 5 homologous proteins, respectively. The probability that aspartic acid, asparagine, or arginine would not be tolerated in the PH20 structure around position 324 is very low, given the high degree of homology that a single-substitution PH20 mutant containing any of those three amino acids would have with the enzymatically active wild-type PH20 protein and the homologous proteins that do include them at positions corresponding to position 324. A skilled artisan thus would have expected that the E324D, E324N, and E324R substitutions in $PH20_{1-447}$ to be tolerated, and the resulting mutant would exhibit comparable activity to the unmodified $PH20_{1-447}$ enzyme, as the ’429 Patent suggests.

222. A skilled artisan would have expected a $PH20_{1-447}$ protein containing any of these three single substitutions at position 324 (*i.e.*, the E324D, E324N, or E324R mutants) to retain the general properties of the wildtype $PH20_{1-447}$ protein. Given this, a skilled artisan would have expected the E324D, E324N, or E324R single substituted $PH20_{1-447}$ proteins to have the same therapeutic uses that were identified in the ’429 Patent for the wildtype $PH20_{1-447}$ protein.[136] In addition, the person would have expected the E324D, E324N, and E324R $PH20_{1-447}$ proteins to

[136] EX1005 (’429 Patent), 56:66-57:21, 73:4-74:29.

be soluble, which results from truncating the PH20 protein sequence before the start of the GPI anchor sequence in it at around position 448.[137]

E. Inspection of the E324D, E324N, and E324R Substitutions in a PH20 Structural Model Confirms that Each Substitution Would be Tolerated in $PH20_{1-447}$

223. I believe the availability of an experimentally-determined structure of human HYAL1 would have prompted a skilled artisan in 2011 to produce a PH20 structural model and use it to assess single amino acid substitutions in $PH20_{1-447}$. This precise point is made in the Brandon & Tooze textbook, which captured prevalent thinking in the field in the 2011 timeframe. As it explains:

> If significant amino acid sequence identity is found with a protein of known crystal structure, a three-dimensional model of the novel protein can be constructed, using computer modeling, on the basis of the sequence alignment and the known three-dimensional structure. This model can then serve as an excellent basis for identifying amino acid residues involved in the active site or in antigenic epitopes, and the model can be used for protein engineering, drug design, or immunological studies.[138]

[137] EX1005 (’429 Patent), 3:58-3, 86:7-88:24.

[138] EX1014 (Brandon), 348.

224. Dr. Park explains in his report that he produced a PH20 structural model using Chao's HYAL1 structure as a template with the SWISS-MODEL tool.[139] Dr. Park's use of a HYAL1 structure to develop a model of PH20 in SWISS-MODEL is justified given Chao's observation that the HYAL1 sequence shares a high degree of sequence identity with PH20.

225. To assess the tolerability of a single amino acid change using a protein structural model, a skilled artisan would use the model to visualize the environment of the change and determine the nature of interactions the new amino acid in a position would have with its neighboring residues (both in the modeled sequence and in the experimental protein structure). Relevant types of interactions include: (1) whether the substitution will change the hydrophobicity or hydrophilicity of the environment; (2) whether the new residue has the potential to change secondary structure at the position; (3) whether the new residue would result in steric clashes within the protein environment; (4) whether the new residue would have tertiary interactions with neighboring residues; and (5) whether the substitution is made at a position that is solvent exposed or buried within the protein structure.[140]

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[139] EX1004 (Park Dec.), ¶¶ 161-163.

[140] EX1004 (Park Dec.), ¶¶ 45-52.

226. Dr. Park also indicates that he did this type of analysis.[141] He assessed the interactions between the wild-type residue (E) and the substituted amino acids aspartic acid (D), asparagine (N) and arginine (R) with neighboring residues around position 324 in PH20. He also documented the interactions that he observed based on his analysis of the E324D, E324N, and E324R substitutions, and provided his assessment of how those factors collectively would have influenced the tolerability of aspartic acid, asparagine, or arginine at position 324 of PH20.

227. Dr. Park's analysis is similar to assessments of substituted residues that were described in peer-reviewed publications from scientists working in this field in the 2011 timeframe. For example, Dr. Moult's lab at UMBI published work with single nucleotide polymorphisms that created single amino acid mutations in human proteins. They explain that they used a very similar methodology of building protein model, visually assessing the interactions between a substituted amino acid and its neighboring residues at a defined position in the model, and ranking the substitution based on an assessment of the interactions.[142]

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141 EX1004 (Park Dec.), ¶¶ 53-83, 104, 113, 122, 130.

142 EX1031 (Yue), 460, 462-463.

228. I note that there were limits to using protein structural models in 2011 to assess the effects of modifications to a modeled protein structure. Dr. Park explained some of those limitations in his report.[143] One was that the model could provide reliable insights only for those portions of the model that were based on the experimentally determined structure, and which was within quality metrics for the model as a whole and for local regions of the model. For the PH20 model based on the HYAL1 structure, this limited use of the model beyond position 403 of PH20, as there is no corresponding sequence (and thus no structure) in HYAL1 for that part of PH20. Dr. Park also limited the N-terminal assessments to position 7 and higher. A second limitation is that the PH20 model built with SWISS-MODEL could not reliably assesses multiple amino acid changes, and would have very low reliability when assessing modified sequences containing 10-20 or more concurrent changes. This is because each additional amino acid change from the originally modeled structure will significantly degrade the ability of the model to predict the modified structure, as the model is no longer based on the existing, known structure or a naturally occurring sequence. Multiple amino acid changes in proximity to each other further reduce the reliability of the model, as interactions

[143] EX1004 (Park Dec.), ¶¶ 164-165, 172-174.

between changes caused by each amino acid change become more and more complex.

229. I reviewed Dr. Park's assessment of the single amino acid substitutions E324D, E324N, and E324R in $PH20_{1-447}$ and agree with his conclusion that, based on modeling techniques available in 2011, the E324D and E324N substitutions would each be expected to be a neutral change, while the E324R substitution would be expected to be a beneficial change.[144] As such, a skilled artisan would have expected the E324D, E324N, and E324R substitutions in $PH20_{1-447}$ to each be tolerated, with each yielding a mutant that maintains comparable hyaluronidase activity to the wild-type $PH20_{1-447}$ protein.

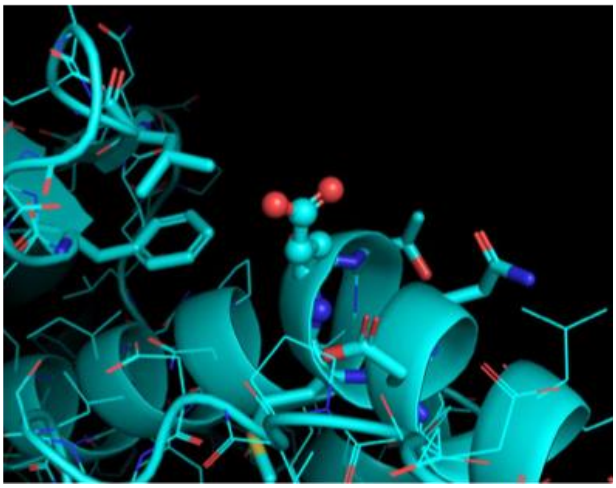
230. Dr. Park's model shows that the side chain of the glutamic acid at position 324 is solvent exposed (below).[145] The hydrophilic side chains of aspartic acid, asparagine, and arginine, if they were substituted at position 324 in PH20, would be positioned equivalently to the hydrophilic side chain of glutamic acid at position 324 in a solvent environment.[146] As such, one would expect the structure

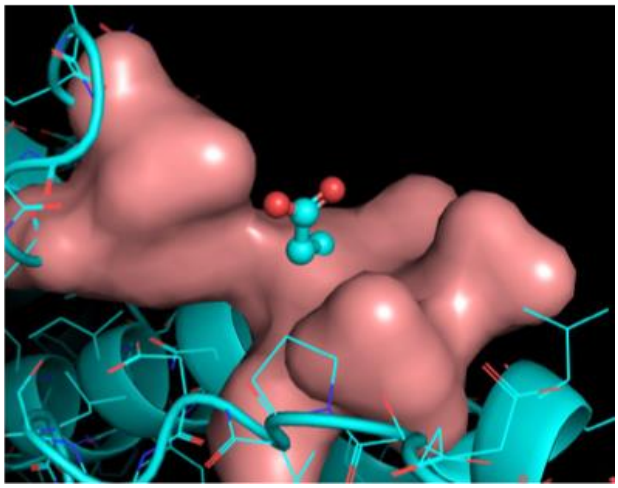
[144] EX1004 (Park Dec.), ¶¶ 121, 129, 137.

[145] EX1004 (Park Dec.), ¶ 110.

[146] EX1004 (Park Dec.), ¶¶ 116, 124, 132.

around position 324 to accommodate replacing the glutamic acid (a hydrophilic residue) at position 324 with aspartic acid, asparagine, or arginine.

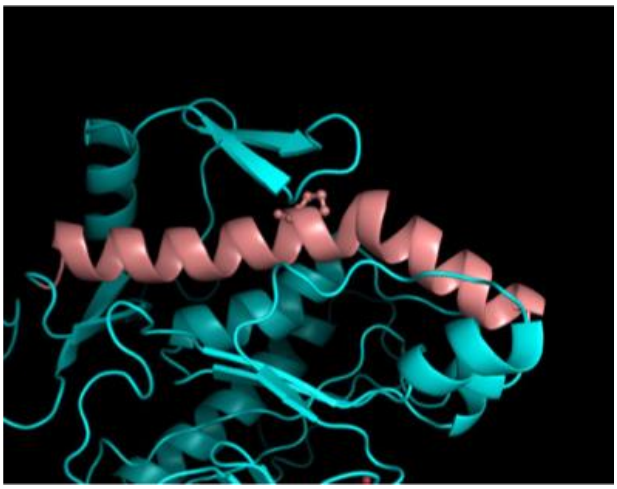




231. Dr. Park's modeling shows that position 324 is near a proline residue at position 329 within the α8 helix of PH20.[147] When a proline residue occurs within an α-helix sequence, it typically disrupts the alpha-helix structure. The proline at position 329 in PH20 disrupts the α8 helix structure including around position 324. As the model shows, P329 introduces a "kink" or bend into the α8 structure.[148]

[147] EX1004 (Park Dec.), ¶ 109.

[148] EX1004 (Park Dec.), ¶ 109.

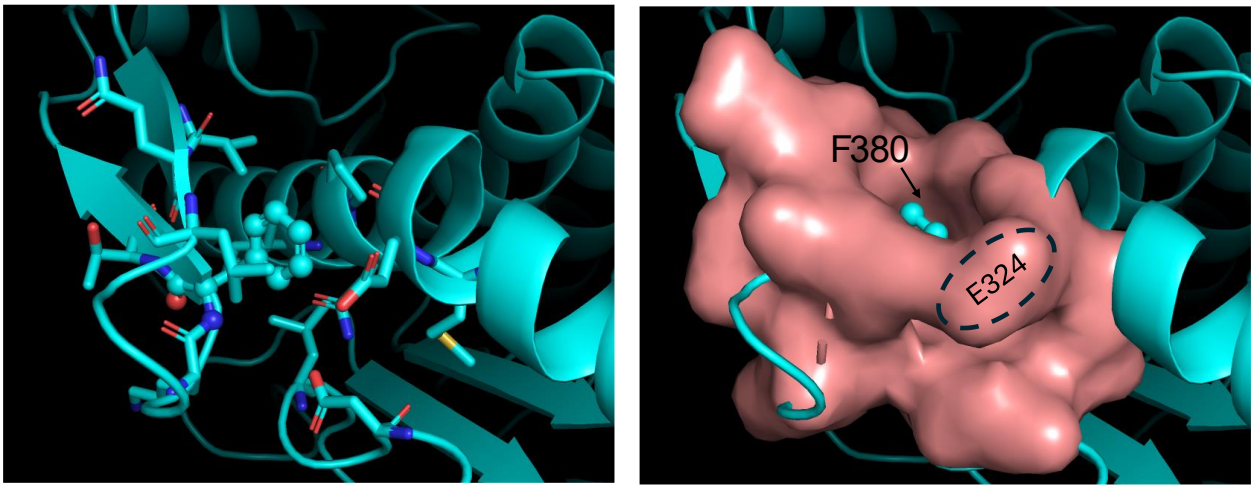


232. The disruption of the alpha-helix structure within the α8 helix in PH20 caused by the proline at position 329 makes the position 324 residue more solvent exposed, which is favorable to hydrophilic residues like arginine, aspartic acid, and arginine. It also lessens the importance of the amino acid at position 324 having a high helix propensity, as that residue will not need to support the helix structure to the same degree it might need to if there were no disruption of the alpha-helix structure.

233. Dr. Park's model shows that the side chain of the wild-type glutamic acid plays a role in restricting solvent access to the phenylalanine (F) residue at position 380 (below).[149] This appears to be due to the residue at position 324 sterically obstructing access by solvent to the F380 residue. That is consistent with the fact that many different amino acids with varying sizes and chemical

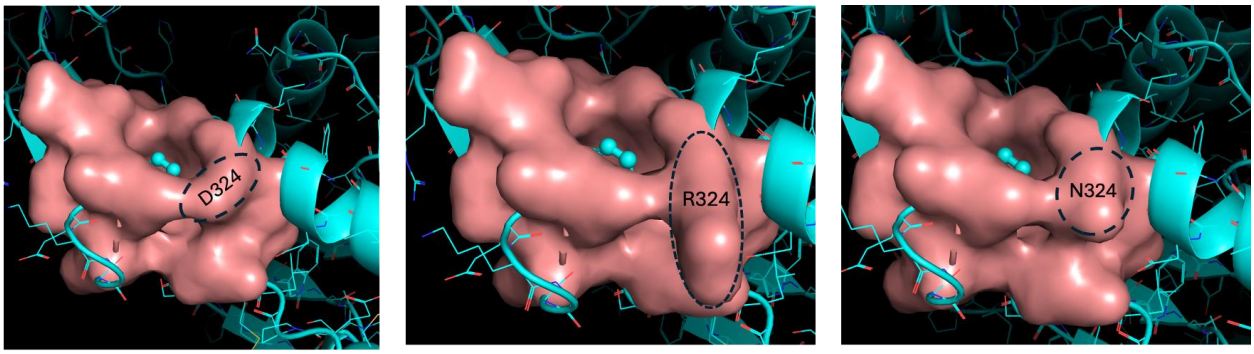
[149] EX1004 (Park Dec.), ¶ 111.

characteristics are found at positions corresponding to position 324 in PH20, as it is more likely that these residues limit solvent access to F380 by their size or positioning, rather than due to the charge or polarity of their side chains.



234. Dr. Park's models show that the side chains of aspartic acid, asparagine, and arginine would play a similar role in sterically obstructing solvent access to the F380 residue if any of those amino acids were substituted at position 324 in PH20. This is illustrated by the images below, which model the structures at position 324 with the D324, R324, and N324 single substitutions in PH20.[150]

[150] EX1004 (Park Dec.), ¶¶ 117, 125, 134.



235. Dr. Park also analyzed the QMEAN score associated with the PH20 model, including when assessing changes at position 324.[151] The QMEAN scores Dr. Park observed for the entire protein and for the changes at position 324 indicate that the model was of acceptable quality.

236. Based on the assessment above, it is my opinion that one of skill in the art would reasonably expect that the E324D, E324N, and E324R substitutions in PH20 would each be tolerated, yielding a protein that exhibits at least comparable hyaluronidase activity as unmodified $PH20_{1-447}$.

237. Finally, I note that Dr. Park's analysis of the substitution to position 324 appears to have been done with no pre-existing knowledge or review of the '520 Patent,[152] yet his conclusions regarding the tolerability of the substitutions

[151] EX1004 (Park Dec.), ¶¶ 120, 128, 136, 163-165.

[152] EX1004 (Park Dec.), Exhibit List.

E324D, E324N, and E324R are consistent with the ’520 Patent’s report that the E324D, E324N, and E324R substitutions resulted in an “active mutant.”[153]

[153] EX1001 (’520 Patent), Table 9 (col. 231).

I, Michael Hecht, do hereby declare and state, that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, under Section 1001 of Title 18 of the United States Code.

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Executed on: January 16, 2025

Exhibit List

The list of exhibits that I relied upon in forming my opinions is below.

EXHIBIT #	REFERENCE NAME
1001	U.S. Patent No. 12,110,520
1004	Declaration of Dr. Sheldon Park
1005	U.S. Patent No. 7,767,429
1006	Chao et al., "Structure of Human Huyaluronidase-1, a Hyaluronan Hydrolyzing Enzyme Involved in Tumor Growth and Angiogenesis," Biochemistry, 46:6911-6920 (2007)
1008	Stern et al., "Hyaluronidases: Their Genomics, Structures, and Mechanisms of Action," Chem. Rev. 106:818-839 (2006)
1010	Zhang et al., "Hyaluronidase Activity of Human Hyal1 Requires Active Site Acidic and Tyrosine Residues," J. Biol. Chem., 284(14):9433-9442 (2009)
1011	Arming et al., "In vitro mutagenesis of PH-20 hyaluronidase from human sperm," Eur. J. Biochem., 247:810-814 (1997)
1013	Frost, "Recombinant human hyaluronidase (rHuPH20): an enabling platform for subcutaneous drug and fluid administration," Expert Opinion on Drug Delivery, 4(4):427-440 (2007)
1014	Brandon & Tooze, "Introduction to Protein Structure," Second Ed., Chapters 1-6, 11-12, 17-18 (1999)
1017	Green, "Computer Graphics, Homology Modeling, and Bioinformatics," Protein Eng'g & Design, Ch. 10, 223-237 (2010)
1018	Chica et al., "Semi-rational approaches to engineering enzyme activity: combining the benefits of directed evolution and rational design," Curr. Opin. Biotechnol., (4):378-384 (2005)
1019	Hardy et al., "Assessment of contraceptive vaccines based on recombinant mouse sperm protein PH20," Reprod., 127:325-334 (2004)

EXHIBIT #	REFERENCE NAME
1020	Pomering et al., "Restricted Entry of IgG into Male and Female Rabbit Reproductive Ducts Following Immunization with Recombinant Rabbit PH-20," Am. J. Reprod. Immunol., (3):174-82 (2002)
1021	Baba et al., "Mouse Sperm Lacking Cell Surface Hyaluronidase PH-20 Can Pass through the Layer of Cumulus Cells and Fertilize the Egg," J. Biol. Chem., 277(33):30310-4 (2002)
1029	Gmachl et al., "The human sperm protein PH-20 has hyaluronidase activity," FEBS Letters, 3:545-548 (1993)
1031	Yue et al., "Loss of Protein Structure Stability as a Major Causative Factor in Monogenic Disease," J. Mol. Biol., 353:459-473 (2005)
1033	Marković-Housley et al., "Crystal Structure of Hyaluronidase, a Major Allergen of Bee Venom," Structure, 8:1025-1035 (2000)
1039	Alberts, "Molecular Biology of the Cell," Fifth Edition, Chapter 3 (2007)
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APPENDIX A

Data, Tables, and Analysis from U.S. Patent 12,110,520

Source(s) – Tables 3, 5, 8-12

APPENDIX A-1

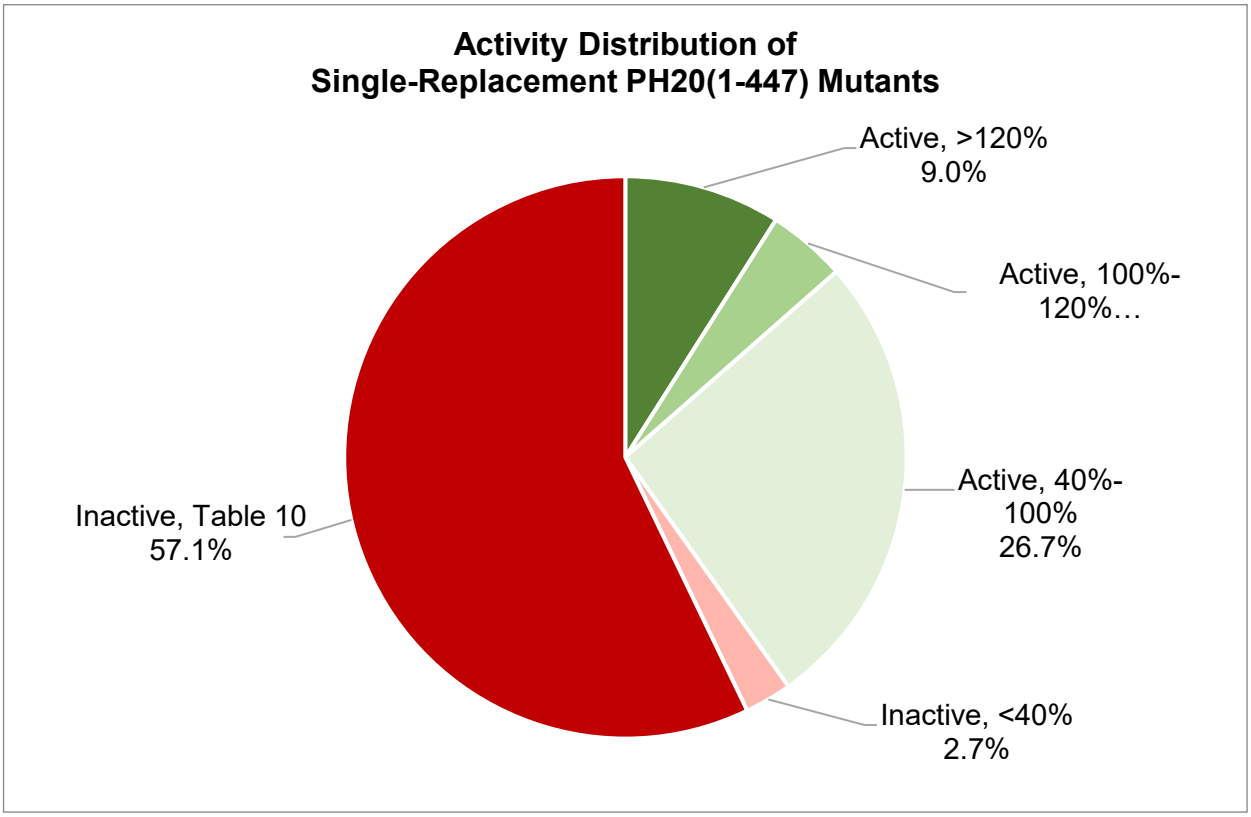
Activity Distribution – Total Mutants

Source(s) – Tables 3, 5, 8-10

Total Entries	
Table 3 (Active Mutants)	2516
Table 5 (Inactive Mutants)	3368
Table 8 (mutants made)	6,753
Table 9 (40% or greater)	2376
Table (<40%)	160
Table 9 (total)	2536
Table 10 (Inactive Mutants)	3380

Totals from Table 9 and 10		
% Activity	Number	
Active, >120%	532	8.99%
Active, 100%-120%	267	4.51%
Active, 40%-100%	1577	26.66%
Inactive, <40%	160	2.70%
Inactive, Table 10	3380	57.13%

Total Tested	5916



APPENDIX A-2

Composite List of Active Mutants

Source(s) – Tables 3, 9

Position	Wild-type AA	Substituted AA																	
1	L	A	C	E	F	G	H	K	N	P	Q	R	S	T	V	W		
2	N	A	C	G	I	L	P	Q	S	T	V							
3	F	E	H	L	Y													
4	R	A	I	S	T	V												
5	A	H																
6	P	A	H	K	L	N	Q	R										
7	P	M																
8	V	I	L	M	P													
9	I	K	L	Q	R	S	V											
10	P	D	E	G	H	N	Q	R	S	W								
11	N	D	G	H	K	S												
12	V	A	E	I	K	L	N	R	S	T								
13	P	H	S	T	Y													
14	F	D	I	M	V													
15	L	A	M	V														
16																		
17																		
18																		
19																		
20	A	S																
21																		
22	S	H	M	T	Y													
23	E	D																
24	F	A	E	G	H	I	K	L	M	N	R	T	V	Y				
25																		
26	L	A	E	G	H	I	K		M	P	Q	R	S	T	V	W	Y	
27	G	A	D	E	F	H	I	K	L	P	Q	R	S	T	W			
28	K	A	D	E	F	I	L	M	N	P	R	S	T	V	W			
29	F	A	E	G	H	I	K	L	M	P	R	S	T	V	W			
30	D	A	F	G	H	K	L	M	P	Q	R	S	T	V	W			
31	E	A	C	G	H	I	K	L	P	R	S	T	V	W	Y			
32	P	A	C	F	G	H	K	L	M	N	Q	R	S	T	V	W	Y	
33	L	G	M	P	Q	R	S	T	W									
34	D	A	E	H	K	Q	R	W										
35	M	F	H	L	Q	T	V	Y										
36	S	A	D	G	H	K	L	N	R	T								

Position	Wild-type AA	Substituted AA																
37	L	F	I	K	M	P	R	W	V									
38	F	Y																
39	S	A	L	N	Q	R	T	Y										
40	F	L	W															
41	I	A	C	D	E	G	H	N	T	V	W							
42	G	A																
43	S	T	N															
44	P	E																
45	R	I	K															
46	I	A	C	E	F	H	L	M	N	R	S	T	V	Y				
47	N	A	D	F	G	H	K	M	Q	R	S	T	W	Y				
48	A	F	G	H	I	K	M	N	Q	R	S	V	Y					
49	T	I	K	R	S	V												
50	G	A	C	D	E	H	L	M	Q	R	S	V	Y					
51	Q	A	N	R	S													
52	G	N	P	Q	R	S	T											
53																		
54	T	A	F	N	Q	S	V											
55																		
56																		
57																		
58	V	C	G	H	I	K	L	N	P	Q	R	S	W	Y				
59	D	Q	N															
60	R	K																
61	L	F	I	M	V													
62																		
63	Y	A	H	I	K	L	M	N	R	S	T	V	W					
64																		
65	P	R																
66	Y	H	R															
67	I	F	L	R	V	Y												
68	D	E	G	H	K	L	P	Q	R	S	T							
69	S	A	C	E	F	G	I	L	M	P	R	T	W	Y				
70	I	A	C	F	G	H	K	L	M	Q	R	S	T	W				
71	T	A	D	G	H	L	M	N	Q	R	S							
72	G	A	D	E	H	K	L	M	Q	R	S	T	Y					

Position	Wild-type AA	Substituted AA																
73	V	A	C	D	G	H	K	L	M	Q	R	S	T	W				
74	T	A	C	E	F	G	H	K	L	M	N	P	R	S	V	W		
75	V	A	C	F	H	L	M	N	Q	R	S	T	Y					
76																		
77	G	H	K															
78																		
79	I	L	T	V														
80																		
81	Q	P																
82	K	A	E	G	H	I	L	M	N	Q	R	S	T	V	Y			
83	I	F	G	H	K	L	N	Q	R	S	T	V						
84	S	D	E	F	G	H	I	L	M	N	P	Q	R	T	W	Y		
85	L	V																
86	Q	A	D	E	F	G	H	I	K	L	M	N	P	R	S	T	V	W
87	D	A	C	E	G	H	I	L	M	P	Q	R	S	T	V	Y		
88																		
89	L	C	K	M	P	R	W											
90	D	A	E	G	H	I	K	L	N	Q	R	S	T	W				
91	K	A	Q	R														
92	A	C	H	L	M	T	V											
93	K	D	E	F	G	H	I	L	M	N	P	Q	R	S	T	V		
94	K	A	C	D	E	F	H	L	M	N	Q	R	S	T				
95																		
96	I	D	L	V														
97	T	A	C	D	E	F	G	I	L	N	P	Q	R	S	W	Y		
98	F	A	C	D	E	H	I	L	M	Q	R	S	V	W				
99	Y	A	R	S														
100																		
101																		
102	V	A	C	E	G	H	K	L	M	N	Q	R	S	T	W			
103	D	N																
104	N	A	C	G	I	K	M	R	S	T								
105	L	A	C	G	H	I	P	Q	R	S	T	W	V					
106	G	V																
107	M	F	I	L														
108	A	G																

Position	Wild-type AA	Substituted AA																
109																		
110	I	V																
111																		
112																		
113																		
114	E	A	G	H	M	S												
115																		
116																		
117	P	D																
118	T	H	K	L	M	N	Q	V										
119	W	F	P	Q	Y													
120	A	D	F	G	H	I	L	N	P	R	S	T	V	W	Y			
121																		
122	N	M																
123																		
124	K	H	L	R														
125	P	A	H	R	S													
126																		
127	D	A	E	G	H	L	M	N	Q	R	S	T	V	W				
128	V	A	C	G	I	K	L	Q	R	S	W							
129																		
130	K	I	R															
131	N	C	E	F	G	H	I	L	M	Q	R	S	T	V	Y			
132	R	A	C	E	F	H	I	K	L	N	Q	S	T	V	Y			
133	S	I																
134	I	L	T	V														
135	E	A	C	D	F	G	H	K	L	N	Q	R	S	W	Y			
136	L	A	C	D	F	H	I	M	N	Q	R	S	T	W				
137	V	A	C	I	T	H	L	M	N	R	S	W	Y					
138	Q																	
139	Q	A	C	D	E	F	G	H	K	L	M	R	S	T	V			
140	Q	A	C	D	F	G	H	I	K	L	M	R	V	W	Y			
141	N	A	D	E	F	G	H	L	M	Q	R	S	T	V	W	Y		
142	V	C	D	E	G	H	I	K	L	M	N	P	Q	R	S	T		
143	Q	C	E	G	I	K	L	N	V									
144	L	R	T	W														

Position	Wild-type AA	Substituted AA																	
145	S	A	C	D	E	G	H	L	M	N	P	R							
146	L	A	C	E	G	H	I	K	N	P	Q	R	S	T	V	Y			
147	T	A	C	D	F	G	I	L	M	P	Q	R	S	V	W	Y			
148	E	C	F	G	H	I	K	L	Q	R	S	T	V	W	Y				
149	A	C	G	K	L	M	Q	R	S	T	V								
150	T	A	C	D	E	F	G	I	L	N	P	R	S	W	Y				
151	E	A	C	G	H	K	L	M	N	Q	R	S	T	V	W	Y			
152	K	A	C	F	I	M	R	T	V	W	Y								
153	A	I	L	S															
154	K	I	R	T	V														
155	Q	A	C	D	F	G	H	K	L	M	R	S	T	V	W				
156	E	A	C	D	G	I	L	M	Q	R	S	T	V	W					
157	F	W																	
158	E	A	F	G	H	L	Q	S											
159	K	A	D	E	G	H	L	M	N	Q	R	S	V						
160	A	C	F	G	H	I	K	L	M	N	Q	R	S	W	V	Y			
161	G	A	C	D	E	R	S	V											
162	K	A	D	E	F	G	H	L	M	P	Q	R	S	V	W	Y			
163	D	A	E	F	G	K	L	Q	R	S	T	V	W						
164	F	L	M	V	W														
165	L	A	C	D	F	N	R	S	V	W	Y								
166	V	A	C	E	F	G	H	L	N	Q	R	T	W	Y					
167	E	A	D	G	H	K	M	N	P	R	S	T	Y						
168	T	H																	
169	I	L	R	V															
170	K	A	Q	N	R	V													
171	L	I	V																
172	G	A	C																
173	K	Q	N	R															
174	L	A	G	H	K	M	N	Q	R	S	T	V	W	Y					
175	L	E	H	T	V	Y													
176	R	K	L																
177	P	V																	
178	N	G	K	M	R														
179	H	A	C	E	G	I	K	L	M	N	P	R	S	T	V				
180	L	F	G	I	K	M													

Position	Wild-type AA	Substituted AA																
181	W	K	M	Q														
182	G	L																
183	Y	E	L															
184	Y	W																
185																		
186	F	Y																
187																		
188																		
189																		
190																		
191																		
192	H	S	T															
193	H	F	G	Q	R	S	Y											
194																		
195	K	A	G	H	I	L	N	Q	R	S	T	W	V					
196	K	E	G	L	N	R	S	T	W	Y								
197	P	A	D	E	F	G	H	K	L	M	Q	R	S	T	W			
198	G	A	D	E	H	L	N	Q	R	S	T	W	Y					
199																		
200	N	D	T															
201																		
202	S	M																
203																		
204	F	P	W															
205	N	A	D	E	F	G	K	L	M	P	R	S	T	V	W	Y		
206	V	H	I	K	L	M	Q	R	S	T								
207																		
208	I	A	C	K	L	M	Q	R	S	T	V							
209	K	A	E	D	G	L	N	R	S	T								
210																		
211	N	L	W															
212	D	N	S	T														
213	D	A	E	G	H	K	L	M	N	Q	R	V	W	Y				
214	L	Q																
215	S	A	D	E	G	H	K	L	M	Q	R	T	V	W	Y			
216	W	Y																

Position	Wild-type AA	Substituted AA																
217	L	M																
218	W	F	M	V														
219	N	A	C	D	E	H	I	K	L	M	R	S	T	W				
220	E	A	D	H	I	L	M	S	T	V								
221	S	A	C	I	M	Q	T	V										
222	T	D	F	G	I	K	L	N	R	S	V							
223																		
224	L	I																
225																		
226	P	W																
227																		
228																		
229																		
230	L	I																
231	N	T																
232	T	S	F															
233	Q	A	F	G	K	L	R	Y										
234	Q	L	M															
235	S	A	E	G	H	K	T											
236	P	A	G	H	K	R	S											
237	V	A	C	E	F	H	L	N	Q	R	S	T	W					
238	A	D	E	H	K	Q	R	S	T									
239	A	N																
240	T	K	A	M	P	Q	R	S	V									
241																		
242	Y	F																
243																		
244																		
245	N	H																
246																		
247	V	I	L	M														
248	R	A	H	W	Y													
249	E	V																
250																		
251	I	L	M	Y														
252																		

Position	Wild-type AA	Substituted AA																
253	V	I																
254																		
255	K	A	G	N	Q	R	S											
256	I	A	H	L	V													
257	P	A	C	G	I	K	L	M	N	Q	R	T	V					
258	D	G	H	N	R	S												
259	A																	
260	K	A	D	E	H	L	M	Q	R	S	G	Y						
261	S	A	F	K	M	N	Q	R	T	V	W							
262																		
263	L	A	H	K	M	R	T	V										
264	P	A	H															
265	V	I																
266	F	Y																
267	A	M	T															
268																		
269	T	A	C	D	S													
270	R	M	N	S	T													
271	I	F	G	L	M	S	V											
272	V	D	E	M	R	S	T											
273	F	H	T	Y														
274	T	A	F	S														
275																		
276	Q	C	D	E	G	H	I	L	M	R	S	Y						
277	V	A	C	D	E	G	H	K	M	N	Q	R	S	T	Y			
278	L	A	E	F	G	H	I	K	N	R	S	T	V	Y				
279	K	A	H	Q	R	T												
280	F	G	Q															
281																		
282	S	D	G	M	Q													
283	Q	E	P	R	S	T												
284	D	A	E	G	H	L	M	N	Q	S	T	Y						
285	E	A	F	G	H	M	N	Q	Y									
286	L	R	S	W														
287	V	I	N	T														
288	Y	L	W															

Position	Wild-type AA	Substituted AA																
289	T	K	S															
290	F	I	M															
291	G	C	Q	R	S	V												
292	E	A	C	F	G	H	K	N	P	R	V	W						
293	T	A	C	D	F	G	K	L	M	P	Q	S	V	Y				
294	V	M																
295																		
296																		
297	G	A																
298	A	G	I															
299																		
300	G	R																
301	I	A	V															
302	V	I	W															
303	I	D	V															
304	W	G	I															
305	G	D	E	N														
306	T	D	E	S														
307	L	G	K	N	Q	S	T	V	W	Y								
308	S	D	G	H	K	N	P	R	T									
309	I	D	E	G	H	K	L	M	N	Q	R	S	T	V	W			
310	M	A	F	G	Q	R	S	T	V	Y								
311	R	G	H	K	Q	S	T											
312	S	G	K	L	N	T												
313	M	A	E	G	H	K	L	P	R	S	T	V	Y					
314	K	A	D	H	I	N	Q	R	S	T	Y							
315	S	A	E	G	H	K	L	M	R	T	Y							
316	C	D																
317	L	A	D	H	I	K	M	N	Q	R	S	T	W					
318	L	D	F	G	H	I	K	L	M	R	T	Y						
319																		
320	D	E	G	H	I	K	L	M	N	R	S	W	V	Y				
321	N	A	D	H	K	R	S	T	Y									
322																		
323	M	F	I	L														
324	E	A	D	H	M	N	R	S										

Position	Wild-type AA	Substituted AA																
325	T	A	D	E	G	H	K	M	N	Q	S	V	W					
326	I	C	K	L	S	V	Y											
327	L	M																
328	N	A	C	G	H	I	K	L	Q	R	S	T	V	W	Y			
329																		
330																		
331	I	E	C	V														
332																		
333																		
334	V	P	T															
335	T	S																
336																		
337																		
338	A	Q																
339	K	M																
340																		
341																		
342	S	A																
343	Q	T	V															
344																		
345																		
346																		
347	Q	A	E	G	L	M	R	S										
348	E	D	G	S														
349	Q	A	E	K	M	N	R	T										
350																		
351	V	A	C	I	Q	S												
352																		
353	I	T	V															
354																		
355																		
356	N	A	D	H	S													
357	W	A	C	K	S	T												
358	N	C	G	L	T													
359	S	D	E	H	K	M	T	V										
360	S	T																

Position	Wild-type AA	Substituted AA																
361	D	H																
362																		
363																		
364																		
365																		
366																		
367	P	A	C	G	K	R	S											
368	D	A	E	G	H	K	L	M	R	S	T	V						
369	N	H	R	S														
370																		
371	A	E	F	G	H	I	K	L	M	R	S	V						
372																		
373	Q	A	E	F	K	L	M	R	S	V								
374	L	A	H	I	M	N	P	R	S	T	V	W	Y					
375	E	A	G	I	K	L	M	N	R	S	T							
376	K	A	D	E	L	M	Q	R	S	T	V	Y						
377	G	D	E	H	K	P	R	S	T									
378	G	K	N	R														
379	K	G	H	R	S	T												
380	F	I	L	P	T	V	W	Y										
381	T	E	H	K	N	Q	R	S	V									
382																		
383	R	A	E	H	I	K	L	M	N	S	T	V						
384																		
385	K	A	G	H	Q	R	S	T	V									
386																		
387	T	S																
388	L	F	H	I	M	R	T	V	W	Y								
389	E	A	G	H	K	L	M	P	Q	R	S	T	Y					
390																		
391	L	C																
392	E	A	G	K	L	M	R	Q	S	T	V	W	Y	F				
393	Q	A	D	F	H	K	L	M	N	R	S	T						
394	F	L	W															
395	S	A	G	H	K	R	T	W										
396	E	A	D	H	L	Q	R	S	T									

Position	Wild-type AA	Substituted AA																
397	K	R																
398	F	L																
399	Y	A	C	E	K	M	N	Q	R	S	T	V	W					
400																		
401	S	A	E	G	Q	N												
402																		
403	Y	F																
404	S	A	P	T														
405	T	A	F	G	K	M	P	Q	R	S	W	Y						
406	L	A	C	E	F	G	I	N	Q	S	T	V	Y					
407	S	A	D	E	F	G	H	L	M	N	P	Q	R	V	W			
408																		
409	K	A	D	E	G	H	I	P	Q	R	S	T	V					
410	E	D	K	M	N	P	Q	R	S	T	V	Y						
411	K	A	H	N	P	R	S	T	V									
412	A	D	G	H	I	L	N	Q	P	R	S	V	W	Y				
413	D	A	E	H	K	N	Q	R	S	T								
414	V	I	K	L	M													
415	K	G	S	W	V	Y												
416	D	F	G	H	I	K	L	N	Q	R	T	V	Y					
417	T	I																
418	D	A	E	F	G	I	L	M	N	P	Q	R	S	V	Y			
419	A	E	F	G	H	I	K	L	N	R	S	W	Y					
420	V	I	P															
421	D	A	E	G	H	I	K	L	M	N	Q	R	S	T	Y			
422	V	I	T															
423																		
424																		
425	A	G	I	K	M	N	R	S	Y									
426	D	E	G	K	N	P	Q	S	Y									
427	G	H	I	K	Q	S	T											
428	V	L	M	P	T													
429																		
430																		
431	D	A	E	G	H	I	K	L	N	Q	R	S	V	W	Y			
432	A	E	G	H	N	S	V											

Position	Wild-type AA	Substituted AA																
433	F	A	C	D	E	G	H	I	K	L	P	R	S	T	V	W		
434	L	F	G	I	M	V												
435	K	A	C	E	G	H	R	S	T	V	Y							
436	P	C	D	E	G	H	I	K	L	M	Q	R	S	T	W	Y		
437	P	A	D	G	H	I	K	L	M	Q	R	S	Y					
438	M	A	C	D	E	G	L	N	P	Q	R	S	T	V	W			
439	E	A	C	F	G	H	K	L	P	Q	S	T	V	W				
440	T	A	D	E	G	H	I	L	M	P	R	S	V	Y				
441	E	A	D	F	G	H	K	L	N	Q	S	T	V	Y				
442	E	C	G	H	K	L	P	Q	R	T	V	W	Y					
443	P	A	E	F	G	H	L	M	N	Q	R	S	T	W				
444	Q	D	E	F	G	H	I	K	M	N	R	V	W	Y				
445	I	A	G	H	L	M	N	P	Q	R	S	T	V	W	Y			
446	F	A	C	D	E	G	H	I	K	L	M	Q	R	T	V	W		
447	Y	D	E	F	G	I	L	M	N	O	Q	R	T	V	W			

Key:
- Yellow Highlight = AA in T9 activity data, but not T3 active mutant table
- Blue Highlight = AA in T3 active mutant table, but not T9 activity data
- Grey Fill-in = no mutants tested at respective $PH20_{1\text{-}447}$ position

APPENDIX A-3

Composite List of Inactive Mutants

Source(s) – Tables 5, 10

Position	Wild-type AA	Substituted AA																
1																		
2	N	H	K	W	Y													
3	F	A	G	K	P	T	V											
4	R	D	E	F	G	L	P	W	Y									
5	A	D	G	I	L	M	N	P	Q	R	T	V	W	Y				
6	P	E	F	T	V	Y												
7	P	C	D	F	G	H	I	K	L	Q	R	S	T	W	Y			
8	V	D	E	G	H	N	R	S	W									
9	I	C	D	E	G	N	P											
10	P	F	I	L	M	Y												
11	N	A	C	F	I	L	P	T	W	Y								
12	V	G	H	W														
13	P	E	G	I	L	M	V											
14	F	A	E	G	H	K	N	P	Q	W								
15	L	E	F	G	K	N	P	Q	R	S	Y							
16	W	A	C	D	E	F	G	H	K	M	P	R	S	T	Y			
17	A	D	E	G	H	I	L	N	P	Q	R	S	T	V	W	Y		
18	W	C	D	F	G	H	I	L	M	P	Q	S	T	V	Y			
19	N	A	C	F	G	H	I	L	M	P	Q	R	S	V	W	Y		
20	A	D	E	F	H	K	L	N	P	R	T	V	Y					
21	P	A	C	D	E	G	H	I	L	M	R	S	T	V	W			
22	S	C	E	G	K	P												
23	E	A	F	L	M	N	P	R	S	T	V							
24																		
25	C	D	E	F	G	H	I	K	L	N	P	R	S	T	V	Y		
26																		
27	G	C																
28																		
29																		
30																		
31																		
32																		
33	L	C	D	H	N	V	Y											
34	D	I	L	N	S	T	V											
35	M	A	D	G	P	R	S											

Position	Wild-type AA	Substituted AA																
36	S	C	F	W	V	Y												
37	L	C	E	G	N	S												
38	F	E	G	K	L	N	Q	R	T	W								
39	S	C	D	F	W													
40	F	A	D	E	G	K	N	R	S	T	V							
41	I	Q																
42	G	D	E	H	I	K	L	M	P	Q	R	S	T	V				
43	S	A	E	F	G	I	K	L	Q	R	V							
44	P	A	C	F	G	H	I	L	N	Q	R	S	T	W	Y			
45	R	A	D	F	G	P	W											
46	I	P	W															
47	N	V																
48	A	P																
49	T	C	D	G	H	P												
50	G	V																
51	Q	C	F	I	M	P	T	W	Y									
52	G	C	E	F	W	Y												
53	V	A	C	D	E	G	H	L	N	P	Q	R	S	T	W	Y		
54	T	D	E	G	P	R	Y											
55	I	A	D	G	H	N	P	Q	R	T	V	Y						
56	F	A	C	E	G	H	I	K	L	P	R	S	T	V	W			
57	Y	A	D	F	G	I	L	M	P	Q	R	V	W					
58	V	A																
59	D	A	E	I	L	M	P	R	T	V	W	Y						
60	R	A	D	F	G	H	I	L	N	P	Q	S	T	V	Y			
61	L	A	E	F	G	H	N	P	Q	R	T	W	Y					
62	G	A	C	D	F	I	K	L	M	P	Q	R	S	T	V	Y		
63	Y	C	G	P														
64	Y	A	C	D	E	F	G	H	I	K	L	P	Q	R	S	T	V	W
65	P	A	C	D	G	H	I	K	N	R	S	T	V	W	Y			
66	Y	A	C	D	E	G	I	K	L	N	P	S	T	V				
67	I	D	E	G	P	R	T	W										
68	D	A	C	G	I	L	P	V	Y									
69	S	N	T															
70	I	Q																

Position	Wild-type AA	Substituted AA																
71	T	P																
72	G	C	F	H	I	P	V	W										
73	V	P																
74																		
75	V	D	G	P														
76	N	A	C	F	G	I	K	L	P	Q	R	S	T	V	W			
77	G	D	E	L	P	Q	R	T	V									
78	G	A	D	I	M	P	T	Y										
79	I	A	D	F	G	H	K	N	P	S	W	Y						
80	P	A	D	E	F	G	I	K	L	M	N	R	S	T	V	Y		
81	Q	A	C	E	G	H	L	N	P	S	V	W	Y					
82	K	Y	E	K	W													
83	I	E	K															
84	S	Y																
85	L	A	C	D	E	F	G	H	N	Q	S	T						
86	Q	C	P															
87	D	P																
88	H	A	C	E	F	G	I	K	L	M	P	R	S	T	V	Y		
89	L	A	D	E	G	Q	S	T	W	Y								
90	D	C	G															
91	K	D	E	F	G	H	I	L	N	T								
92	A	E	F	H	K	P	Q	R	W	Y								
93																		
94	K	G	P															
95	D	A	C	E	F	G	H	K	L	M	P	Q	S	V	W	Y		
96	I	A	C	G	S	V	H	P	R	T	W							
97																		
98	F	P																
99	Y	C	E	G	I	N	P	V	W									
100	M	C	E	F	G	N	P	R	S	T	W	Y						
101	P	A	C	F	H	I	K	L	M	N	Q	R	S	T				
102	V	P																
103	D	A	E	F	G	H	I	L	Q	R	T	V	W	Y				
104	N	F	P	W														
105	L	C	M	N														

Position	Wild-type AA	Substituted AA																
106	G	A	C	D	F	H	L	M	N	P	S	W	Y					
107	M	A	C	H	K	P	Q	S	V	W								
108	A	D	E	F	K	L	M	P	Q	T	V	Y						
109	V	C	D	E	L	M	R	T	W									
110	I	F	K	L	M	P	W											
111	D	H	I	Q														
112	W	C	E	G	H	L	N	P	S									
113	E	R	V															
114	E	I	L	P	T	V												
115	W	A	C	D	F	G	H	I	K	L	M	R	S	V	Y			
116	R	A	C	D	E	G	H	I	L	N	P	Q	S	V	W			
117	P	D	G	I	K	N	Q	R	S	V	W							
118	T	C	D	E	G	P	R	W	Y									
119	W	A	K	I	L	N	P	R										
120																		
121	R	A	C	E	F	G	H	K	L	M	P	W	Y					
122	N	A	C	E	F	I	K	Q	R	S	T	V						
123	W	A	C	D	E	H	L	M	P	Q	R	S	T	V	Y			
124	K	C	D	E	F	N												
125	P	C	D	G	L	N	W											
126	K	F	H	I	L	N	P	Y										
127	D	K																
128	V	E	P															
129	Y	A	C	D	E	G	H	L	P	Q	S	T	V	W				
130	K	C	D	G	H	L	N	S	T	W	Y							
131	N	P																
132	R	P																
133	S	D	E	F	G	H	L	M	N	P	R	T	V	W				
134	I	A	C	D	F	G	H	K	P	Q	R	S	W					
135	E	P																
136	L	P																
137	V	F	G	H	N	P	R	W	Y									
138	Q	V																
139	Q	P																
140																		

Position	Wild-type AA	Substituted AA																
141																		
142																		
143	Q	C	H	P	R	S	T											
144	L	A	E	F	I	K	P	Q	S	V	Y							
145	S	T	W															
146																		
147																		
148																		
149	A	E	P															
150	T	V																
151																		
152	L	L																
153	A	E	F	M	P	R	T	V										
154	K	D	E	G	P	S	W	Y										
155	Q	P	Y															
156	E	P																
157	F	A	C	D	E	G	H	I	K	L	M	P	Q	R	S	T	V	
158	E	D	K	P	R	Y												
159	K	W	Y															
160																		
161	G	W																
162																		
163	D	C	P															
164	F	A	C	D	E	G	H	N	P	Q	R							
165	L	C	H	P	T													
166	V	D																
167	E	V																
168	T	A	C	D	E	F	G	K	L	P	R	S	V	W	Y			
169	I	A	D	F	G	H	K	N	P	Q	S	T	Y					
170	K	C	D	E	G	M	P	W	Y									
171	L	C	D	H	M	N	R	S	W	Y								
172	G	D	E	I	L	P	Q	T	V	W	Y							
173	K	D	E	G	H	I	L	M	P	S	V	W	Y					
174	L	P																
175	L	C	D	G	K	P	R	S										

Position	Wild-type AA	Substituted AA																
176	R	A	C	E	F	G	H	I	P	Q	S	T	V	W				
177	P	A	C	D	F	G	H	L	M	Q	R	S	T	V	W			
178	N	E	I	L	V	W	Y											
179																		
180	L	A	C	E	P	R	S											
181	W	A	C	D	E	F	H	I	K	L	R	S	V					
182	G	A	C	D	E	H	N	P	Q	R	S	T	V	Y				
183	Y	C	D	E	G	I	K	N	P	Q	R	S	V					
184	Y	A	C	D	E	F	G	H	K	L	M	P	R	S	V			
185	L	A	D	E	F	G	I	K	P	R	S	T	V	W	Y			
186	F	A	D	G	H	I	K	L	N	P	Q	R	S	V	W			
187	P	A	F	G	H	I	L	M	N	Q	R	S	T	V	W	Y		
188	D	A	C	F	G	H	L	M	N	P	Q	R	S	T	V	W		
189	C	A	E	G	H	K	L	M	N	P	R	S	T	V	W	Y		
190	Y	C	E	F	G	H	K	L	N	Q	R	S	T	V	W			
191	N	A	E	F	G	K	L	M	P	Q	R	S	T	V	W	Y		
192	H	C	F	G	K	L	M	N	P	Q	R	V	W	Y				
193	H	A	D	K	L	M	P	V										
194	Y	A	C	I	L	P	S	T	V									
195	K	S																
196																		
197	P	C																
198	G	V	W															
199	Y	E	G	H	I	K	L	P	R	S	W							
200	N	A	F	G	H	K	L	M	P	Q	R	S	W	Y				
201	G	A	F	L	M	N	P	R	S	T	V	W						
202	S	A	E	F	G	H	K	N	P	Q	R	V	W	Y				
203	C	A	D	E	G	H	L	M	N	Q	R	S	T	V				
204	F	A	C	E	G	H	I	K	Q	R	S	T						
205																		
206	V	C	D	F	G	P	Y											
207	E	A	F	G	M	P	Q	R	S	T	V	W						
208	I	D	G	P	W													
209	K	C	P															
210	R	A	C	D	E	G	K	M	N	P	S	T	V	W	Y			

Position	Wild-type AA	Substituted AA																
211	N	C	F	G	H	I	K	M	P	R	S	T	V	W				
212	D	A	G	H	I	K	L	M	P	V	W							
213	D	P	S															
214	L	A	C	D	E	G	H	K	N	P	R	S	T	Y				
215	S	C	P															
216	W	D	E	G	H	I	K	L	M	N	P	Q	R	T	V			
217	L	A	C	G	H	P	Q	S	T	V	W							
218	W	A	I	K	L	P	S	V										
219	N	P																
220	E	G	K	N	P	R	W											
221	S	D	E	H	K	P	R											
222	T	P	Y															
223	A	C	D	E	G	H	K	L	P	Q	R	S	T	V	W	Y		
224	L	A	D	E	F	G	M	P	Q	R	S	T	W	Y				
225	Y	A	D	E	G	H	K	P	Q	R	T	V	W					
226	P	A	C	D	E	F	G	L	N	Q	R	S	T	V	W	Y		
227	S	A	F	G	H	I	K	L	M	P	Q	R	T	V	W	Y		
228	I	A	E	F	G	H	L	M	N	P	R	S	T	W				
229	Y	E	F	G	K	L	P	Q	T	V	W							
230	L	A	E	G	H	K	M	N	P	R	S	T	V	W	Y			
231	N	A	C	D	F	G	H	I	K	L	P	Q	R	S	V			
232	T	C	G	H	K	L	N	P	Q	V	Y							
233	Q	D	I	P	S	T												
234	Q	A	D	E	G	H	N	P	S	T	V	W						
235	S	F	L	M	R	W	Y											
236	P	C	I	L	N	Q	T	Y										
237																		
238	A	F	G	L	P	V	W	Y										
239	A	C	F	G	H	I	L	P	R	S	T	V	W	Y				
240	T	E	F	G	N	W	Y											
241	L	A	C	D	E	G	I	P	R	S	T	V	W					
242	Y	A	C	D	G	I	L	M	P	S	T	V	W					
243	V	C	D	F	G	H	L	M	P	Q	R	S	W	Y				
244	R	A	D	G	I	V	Y											
245	N	A	C	F	L	P	Q	R	S	T	V							

Position	Wild-type AA	Substituted AA																	
246	R	A	C	D	E	G	H	I	K	L	M	P	S	T	V	W			
247	V	A	C	F	H	N	P	Q	R	S	T	W	Y						
248	R	C	D	E	G	I	M	P	T										
249	E	A	G	H	I	K	M	Q	S	Y									
250	A	C	F	G	H	K	L	M	N	P	Q	R	S	T	V	W			
251	I	D	F	G	H	K	P	S	T	W									
252	R	A	D	E	F	G	H	I	K	L	N	P	S	T	Y				
253	V	A	D	E	G	H	L	M	N	Q	R	S	W						
254	S	C	D	E	G	I	K	L	P	Q	R	T	V	W	Y				
255	K	C	D	L	P	V	W												
256	I	C	D	E	G	P													
257	P	D																	
258	D	L	P	V	W														
259																			
260	K	C	P																
261	S	P																	
262	P	A	D	E	F	G	H	I	K	Q	R	S	T	V	W	Y			
263	L	E	F	P	Q	W													
264	P	D	E	F	G	L	M	R	T	V	W	Y							
265	V	A	D	F	G	H	K	L	M	N	Q	R	S						
266	F	A	C	G	H	M	P	Q	R	S	T	V	W						
267	A	D	G	H	I	K	N	R	S	W									
268	Y	A	C	F	G	H	K	L	N	P	Q	S	T	V	W				
269	T	E	K	L	M	N	P	Q	R										
270	R	A	C	E	F	G	H	I	P	Y									
271	I	A	D	E	H	K	T	W											
272	V	A	H	L	N	P	W												
273	F	A	C	D	G	I	L	P	Q	S	V	W							
274	T	C	E	G	H	N	Q	W	Y										
275	D	A	F	G	I	K	L	M	Q	T	V	W							
276	Q	F	P	W															
277																			
278	L	M	P																
279	K	A	C	F	G	L	W	Y											
280	F	D	I	L	M	N	R	S	T	V	W								

Position	Wild-type AA	Substituted AA																
281	L	A	D	G	H	I	K	N	P	Q	R	S	V	W				
282	S	F	L	V	W	Y												
283	Q	A	C	D	F	W												
284	D	C	I	P														
285	E	K	P	R	T	V												
286	L	A	C	D	F	H	K	M	P	T	Y							
287	V	A	C	D	E	G	K	L	N	P	Q	R	S					
288	Y	D	E	F	G	H	I	K	P	R	T							
289	T	A	C	E	G	H	L	P	Q	R	S	Y						
290	F	D	Q	Y														
291	G	A	C	D	E	F	M	N	T	W	Y							
292	E	I	L	T														
293	T	E	N															
294	V	A	E	G	H	K	L	N	P	Q	R	S	T	W				
295	A	C	G	H	I	L	N	P	T	V	Y							
296	L	C	F	G	I	K	M	Q	R	S	T	V	W	Y				
297	G	C	E	H	L	N	P	Q	R	S	T	Y						
298	A	C	E	L	M	N	P	Q	S	T	W	Y						
299	S	A	C	D	F	G	H	L	M	P	Q	T						
300	G	A	C	D	E	F	L	M	N	P	Q	S	T	V	W			
301	I	E	G	H	K	M	N	P	Q	R	S	W	Y					
302	V	C	D	E	F	G	H	L	M	P	R	S	T	Y				
303	I	A	C	D	E	F	G	K	L	M	R	W	Y					
304	W	A	C	D	G	I	M	N	P	Q	S	T	V	Y				
305	G	L	P	Q	R	S	T	V	Y									
306	T	A	C	H	I	L	V	W	Y									
307	L	C	I	P														
308	S	C	F	L	M	V	W	Y										
309																		
310	M	C	E	F	K	L												
311	R	C	E	F	I	L	P	V	W									
312	S	C	E	M	V	W												
313	M	C																
314	K	C	L	W														
315	S	C	I	V														

Position	Wild-type AA	Substituted AA																
316	C	E	G	I	K	L	M	P	R	S	T	V	W	Y				
317	L	G	P															
318	L	C	P	W														
319	L	C	E	F	G	H	I	K	M	P	Q	R	S	V	W	Y		
320	D	C	P	V														
321	N	E	M	P														
322	Y	C	D	E	G	I	L	N	P	R	S	T	V	W				
323	M	A	C	E	G	H	K	N	R	S	T	V						
324	E	C	F	P	V	W	Y											
325	T	C	R	E	G	H	N	W										
326	I	E	G	H	N	W												
327	L	A	E	F	G	H	N	Q	R	S	T	V	W	Y				
328																		
329	P	C	F	G	H	I	K	L	N	Q	R	S	T	V	W	Y		
330	Y	A	C	D	E	G	I	L	M	N	P	R	S	V	W			
331	I	A	C	D	E	F	H	K	Q	R	S	T	W	Y				
332	I	A	C	D	E	F	G	H	K	L	N	P	R	S	T	Y		
333	N	G	H	I	K	P	R	S	T	W	Y							
334	V	A	C	D	E	G	M	N	R	S								
335	T	F	G	H	I	K	L	P	V	W	Y							
336	L	A	E	F	G	K	N	P	R	S	T	V	W	Y				
337	A	C	F	G	I	K	L	M	R	T	W							
338	A	C	D	E	F	G	H	I	K	L	P	R	T	V				
339	K	D	E	F	G	H	L	N	P	S	T	V	W	Y				
340	M	A	C	D	E	F	G	H	K	P	R	S	T	V	W			
341	C	A	E	G	H	K	L	M	N	Q	R	S	T	V	Y			
342	S	D	E	F	H	K	L	M	P	Q	R	T	Y					
343	Q	C	D	F	I	P	W											
344	V	F	G	H	L	M	N	P	Q	R	S	T	W	Y				
345	L	A	C	E	H	K	N	Q	R	T	V	Y						
346	C	A	D	F	G	I	K	L	M	P	R	S	T	V	W			
347	Q	C	F	I	P	T	V	W										
348	E	C	H	I	L	P	Q	R	T	V	W	Y						
349	Q	D	F	G	P	V	W	Y										
350	G	A	D	E	F	H	K	L	M	N	P	R	S	T	V	Y		

Position	Wild-type AA	Substituted AA																
351	V	C	D	E	F	H	N	R	W	Y								
352	C	A	D	E	F	G	K	M	P	Q	R	S	T	V	W	Y		
353	I	C	F	G	H	K	L	M	Q	R	S	W						
354	R	C	D	E	G	H	I	K	L	M	P	Q	S	V	W	Y		
355	K	D	F	G	H	L	M	N	P	Q	R	S	T	V	W	Y		
356	N	C	G	K	L	P	R	T	V	W								
357	W	D	E	F	G	L	M	Q	R									
358	N	E	H	I	K	P	Q	R	W									
359	S	A	F	G	L	P	W											
360	S	A	C	E	F	G	I	K	L	M	P	Q	R	V				
361	D	A	C	E	G	M	N	P	Q	R	S	V	W					
362	Y	A	C	E	G	H	K	L	M	N	P	R	S	T	V	W		
363	L	A	C	D	E	F	G	H	I	P	Q	R	S	T	V	W		
364	H	A	C	D	E	F	G	K	L	M	P	R	S	T	V	Y		
365	L	A	C	D	E	G	M	N	P	Q	R	S	T	W	Y			
366	N	A	C	E	F	G	K	M	P	Q	R	T	W					
367	P	E	F	I	L	M	Q	V										
368	D	C	P	W														
369	N	C	E	F	I	K	L	P	Q	V	W							
370	F	A	D	E	G	H	K	L	N	P	Q	R	S	V	Y			
371	A	P	W															
372	I	A	D	E	F	G	H	K	L	N	P	R	S	T	V	W		
373	Q	C	P	W														
374	L	D	E															
375	E	C	F	P	V	Y												
376	K	I	P	W														
377	G	C	I	L	V													
378	G	D	E	F	I	L	M	Q	T	W	Y							
379	K	A	C	E	F	I	L	M	W									
380	F	C	D	E	G	Q	R	S										
381	T	G	L	P	W	Y												
382	V	E	G	H	K	L	M	N	P	Q	R	S	T	W	Y			
383	R	G	P															
384	G	C	F	M	Q	S	T											
385	K	C	L	M	P	W	Y											

Position	Wild-type AA	Substituted AA																	
386	P	A	C	F	G	H	I	L	M	N	Q	R	S	T	V	Y			
387	T	C	E	F	G	H	I	L	M	N	V	W	Y						
388	L	C	G	P	Q	S													
389	E	F	V																
390	D	A	C	E	F	G	H	L	N	P	R	S	T	V	W	Y			
391	L	A	D	G	H	K	N	P	Q	R	S	T	V	W	Y				
392	E	C	P																
393	Q	C	P																
394	F	A	D	E	G	I	K	N	P	Q	R	S	T	V					
395	S	C	L	M	P														
396	E	C	F	G	I	P	Y												
397	K	A	C	E	F	G	I	L	M	P	Q	T	V						
398	F	A	C	E	G	H	I	L	N	P	R	S	T	V	W	Y			
399	Y	D	P																
400	C	A	D	E	F	G	I	L	M	P	Q	R	S	T	V	Y			
401	S	C	F	H	K	R	W	Y											
402	C	A	D	E	F	L	M	P	Q	R	S	T	V	W	Y				
403	Y	A	C	E	G	H	K	L	M	N	P	Q	R	T					
404	S	C	D	F	G	H	L	M	N	R	V	W	Y						
405	T	C	I	V															
406	L	P	R																
407																			
408	C	A	E	F	G	I	K	L	P	R	S	T	V	W	Y				
409																			
410	E	W																	
411	K	D	E	F	G														
412	A	E	H																
413	D	H	I	K	L	P													
414	V	A	D	E	G	H	K	R	S	T									
415	K	C	D	E	P														
416	D	C	S																
417	T	A	D	E	F	G	H	K	M	P	Q	R							
418																			
419	A	D	P																
420	V	A	D	F	G	H	K	L	N	R	S	T	W	Y					

Position	Wild-type AA	Substituted AA																
421																		
422	V	C	D	G	H	L	M	N	Q	R	S	Y						
423	C	A	D	E	F	G	H	L	M	P	Q	R	S	T	V	W		
424	I	A	C	E	G	H	N	Q	R	S	W	Y						
425	A	E	L	P	W	Y												
426	D	C	F	M	R													
427	G	A	C	F	L	P	V	W	Y									
428	V	A	C	D	E	G	H	N	R	S	Y							
429	C	A	D	K	L	N	P	S	T	V	W	Y						
430	I	A	D	E	L	M	N	S	T	V								
431	D	P																
432	A	C	F	I	K	L	M	P	Y									
433																		
434	L	H	K	P	Q	R	W											
435																		
436																		
437	P	T																
438	M	Y																
439	E	N	R															
440	T	Q																
441	E	R																
442	E	M	N	S														
443	P	D																
444																		
445																		
446																		
447																		

Key:
- Yellow Highlight = AA in T10 inactive mutant table, but not T3 inactive mutant table
- Blue Highlight = AA in T3 inactive mutant table, but not T10 inactive mutant table
- Grey Fill-in = no mutants tested at respective $PH20_{1-447}$ position

APPENDIX A-4

List of Active Mutant Activity Data (Original)

Source(s) – Table 9

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
L001A	74	0.95	V008M	81	0.47	S022T	94	0.48
L001C		0.89	V008P		0.33	S022Y		0.45
L001E	75	0.55	I009K		0.69	E023D		0.97
L001F		0.41	I009L		1.08	F024A		0.69
L001G	76	0.62	I009R		0.53	F024E	95	3.99
L001H	73	1.90	I009S		0.98	F024G		0.75
L001K	77	1.39	I009V		0.84	F024H	96	2.07
L001N		0.87	I009Q	82	0.40	F024I		0.70
L001P		0.92	P010D		0.62	F024K		0.96
L001Q	78	3.27	P010E		0.66	F024L		0.62
L001R	79	0.72	P010G	83	0.55	F024M		0.85
L001S		0.74	P010H	84	0.43	F024N		0.60
L001T		0.99	P010N		0.55	F024R	97	1.22
L001V		1.00	P010Q		0.89	F024T		1.18
L001W		0.88	P010R		0.73	F024V		1.15
N002A		0.61	P010S		0.55	F024Y		0.90
N002C		0.40	P010W		0.59	L026A	98	1.30
N002G		0.44	N011D		0.54	L026E	99	3.22
N002L		0.46	N011G		0.45	L026G		0.81
N002P		0.54	N011H		0.69	L026H		0.97
N002Q		0.84	N011K		0.58	L026I		0.51
N002S		0.78	N011S	85	0.39	L026K	100	1.88
N002T		1.05	V012A		0.56	L026M	101	1.43
N002V		0.65	V012E	86	1.86	L026P		0.55
N002I		0.37	V012I	87	0.68	L026Q	102	1.44
F003E		0.42	V012K	88	0.65	L026R	103	1.43
F003H		0.68	V012L		0.44	L026S		0.78
F003L		0.59	V012N		0.46	L026T		0.87
F003Y		0.50	V012R		0.50	L026V		0.52
R004A		0.73	V012S		0.75	L026W		0.53
R004I		0.54	V012T	89	1.50	L026Y		0.52
R004S		0.60	P013H		0.46	G027A		0.79
R004T		0.66	P013S		0.68	G027D	104	1.22
R004V		1.09	P013T		0.90	G027E		1.18
A005H		0.44	P013Y		0.51	G027F		0.61
P006A	80	0.78	F014D		0.64	G027H		1.11
P006H		0.58	F014I		0.42	G027I		0.41
P006K		0.80	F014M		0.47	G027K	105	2.71
P006L		0.76	F014V	90	0.46	G027L		0.76
P006N		0.40	L015A		0.65	G027P		0.46
P006Q		0.89	L015M	92	0.45	G027Q		1.12
P006R		0.56	L015V	91	2.20	G027R	106	1.88
P007M		0.57	A020S	93	0.50	G027S		0.94
V008I		1.17	S022H		0.57	G027T		0.61
V008L		0.53	S022M		0.49	G027W		0.76

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
K028A		0.78	E031H	128	2.74	M035T		0.83
K028D		0.62	E031I	129	3.89	M035Y		0.78
K028E		0.54	E031K	130	3.13	M035Q		0.37
K028F		0.75	E031L	131	2.62	M035V	146	0.37
K028I		0.55	E031P	132	1.51	M035Q	145	0.37
K028L		0.51	E031R	133	2.27	S036A		0.45
K028M		0.67	E031S	134	1.70	S036D		0.32
K028N		0.58	E031T	135	3.96	S036G		0.64
K028P		0.40	E031V	136	4.57	S036H	147	0.54
K028R	107	0.71	E031W	137	1.26	S036K		0.83
K028S		0.46	E031Y		1.13	S036L		0.71
K028T		0.68	P032A		0.92	S036R		1.09
K028V		0.76	P032C	138	0.40	S036T		0.51
K028W		0.51	P032F	139	2.71	S036N	148	0.38
F029A		0.90	P032G	140	1.60	L037F	149	3.33
F029E	108	4.03	P032H	141	2.08	L037I		0.62
F029G		1.05	P032K		1.04	L037K		0.43
F029H		0.82	P032L		0.82	L037M	150	1.46
F029I	109	1.53	P032M		0.67	L037P		0.63
F029K	110	1.34	P032N		0.70	L037R		0.51
F029L	111	2.36	P032Q		1.11	L037V		0.57
F029M	112	2.08	P032R		1.17	L037W		0.36
F029P	113	3.79	P032S		1.01	F038Y	151	1.29
F029R	114	1.24	P032T		0.77	S039A	152	1.06
F029S	115	2.21	P032V		0.81	S039L	153	0.80
F029T	116	0.85	P032W		0.54	S039N	154	2.32
F029V	117	1.65	P032Y		1.01	S039Q		1.10
F029W		0.48	L033G	143	0.57	S039R		0.56
D030A		1.12	L033M		0.69	S039T	155	1.57
D030F		0.84	L033P		0.87	S039Y		0.56
D030G	118	2.02	L033Q		0.45	F040L	156	0.92
D030H	119	1.69	L033R		0.61	F040W		1.11
D030K	120	2.63	L033S		0.48	I041A		0.67
D030L	121	1.32	L033T		0.45	I041C		0.53
D030M	122	1.85	L033W	142	1.58	I041D		0.78
D030P		1.19	D034A		0.38	I041E		0.51
D030Q		0.84	D034E		0.58	I041G		0.76
D030R	123	1.82	D034H		0.41	I041H		0.77
D030S	124	1.62	D034K		0.54	I041N		0.40
D030T		0.57	D034Q		0.59	I041T	157	1.47
D030V		0.46	D034R		1.17	I041V		0.73
D030W		0.62	D034W	144	0.46	I041W		0.66
E031A	125	2.05	M035F		0.87	G042A		0.64
E031C	126	2.95	M035H		0.60	S043T		0.43
E031G	127	1.27	M035L		0.52	S043N		0.34

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
P044E		0.59	G050A		0.93	L061M		0.73
R045K		0.53	G050C		0.41	L061V		0.59
I046A		1.04	G050D	169	1.37	L061F		0.30
I046C		0.37	G050E		0.78	Y063A		0.63
I046E		0.43	G050H		0.74	Y063H		1.07
I046F		0.73	G050L		0.43	Y063I		1.03
I046H		0.82	G050M	171	0.47	Y063K	187	1.36
I046L	158	1.08	G050Q		0.86	Y063L	188	1.33
I046M		1.00	G050R		0.86	Y063M	189	1.32
I046N		0.66	G050S	170	1.24	Y063N		0.96
I046R	159	2.29	G050V		0.30	Y063R	190	1.40
I046S		0.64	G050Y		0.58	Y063S		1.00
I046T		0.55	Q051N		0.60	Y063T		1.07
I046V		1.01	Q051S		0.46	Y063V		0.43
I046Y		0.76	Q051A		0.34	Y063W	191	1.53
N047A		0.48	Q051R		0.36	P065R		0.57
N047D	160	0.82	G052N	172	0.89	Y066H		0.47
N047F	161	1.32	G052P		0.43	Y066R		0.51
N047G		0.82	G052Q	173	3.71	I067F		1.00
N047H		1.16	G052R	174	0.53	I067L		0.45
N047K		0.67	G052S	175	1.32	I067R		0.24
N047M		0.77	G052T	176	0.49	I067V	192	1.80
N047Q		0.69	T054A		0.43	I067Y		0.55
N047R		0.84	T054F		0.56	D068E		0.72
N047S		0.85	T054N		0.48	D068H	193	2.06
N047T	162	1.49	T054Q		0.91	D068K		1.08
N047W	163	0.63	T054S		0.70	D068L		0.43
N047Y		0.45	T054V		0.66	D068P	194	0.50
A048F	164	2.51	V058C	177	0.55	D068Q	195	1.67
A048G		0.83	V058G		0.54	D068R		0.70
A048H	165	1.99	V058H	183	1.09	D068S		0.81
A048I		0.64	V058I		0.57	D068T		0.75
A048K	166	1.28	V058K	178	4.08	D068G		0.37
A048M		0.76	V058L	179	1.54	S069A	196	22.06
A048N	167	4.25	V058N	184	0.49	S069C	197	1.97
A048Q		1.05	V058P	180	0.90	S069E	198	1.48
A048R		0.66	V058Q	181	4.54	S069F	199	8.75
A048S		1.06	V058R	182	1.92	S069G	200	6.06
A048V		0.60	V058S		0.83	S069I	201	3.12
A048Y		0.81	V058W		0.65	S069L	202	3.44
T049I		0.42	V058Y	185	1.07	S069M	203	2.67
T049K		0.85	D059Q		0.40	S069P	204	8.14
T049R	168	1.41	D059N	186	1.27	S069R	205	14.06
T049S		0.92	R060K		0.69	S069T	206	0.58
T049V		0.45	L061I		0.42	S069W	207	2.18

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
S069Y	208	2.71	V073R	235	0.72	K082Q		0.76
I070A	209	27.00	V073S		0.86	K082R		0.85
I070C	210	2.57	V073T	236	1.34	K082S		0.62
I070F	211	5.69	V073W	237	1.91	K082T		0.56
I070G	212	6.22	T074A	238	2.28	K082Y		0.32
I070H	213	9.09	T074C	239	2.18	K082V		0.57
I070K	214	14.64	T074E	240	1.38	I083F		0.57
I070L	215	3.05	T074F	241	1.43	I083G	264	1.05
I070N	216	6.19	T074G	242	2.75	I083L		0.93
I070P	217	3.03	T074H	243	1.40	I083N		0.82
I070R	218	13.95	T074K	244	1.29	I083Q	262	1.07
I070S	219	3.63	T074L	245	1.43	I083R		0.45
I070T	220	5.43	T074M	246	0.52	I083S	263	0.79
I070V	221	6.34	T074N	247	2.12	I083T		0.95
I070Y	222	1.26	T074P	248	2.45	I083V	261	0.99
T071A		0.86	T074R	249	2.22	I083H		0.40
T071D		0.50	T074S	250	1.80	I083K		0.30
T071G	223	1.41	T074V	251	2.27	S084D		0.98
T071H		0.93	T074W	252	2.13	S084E	265	0.52
T071L		1.09	V075A		0.71	S084F	266	0.72
T071M		0.89	V075C		0.46	S084G	267	8.68
T071N	224	1.21	V075F	253	2.00	S084H		0.96
T071Q		0.68	V075H		0.62	S084I		0.90
T071R	225	2.17	V075L	254	5.22	S084L		0.92
T071S	226	1.54	V075M	255	1.16	S084M		0.77
G072A		0.45	V075N		0.81	S084N	268	0.89
G072D		0.60	V075Q		1.51	S084P		0.57
G072E		0.69	V075R	256	3.02	S084Q		0.86
G072H		0.46	V075S		0.76	S084R	269	1.89
G072K	227	1.39	V075T	257	4.34	S084T		0.82
G072L		0.43	V075Y		0.63	S084W		0.86
G072M	228	3.11	G077H		0.32	S084Y		0.30
G072Q	229	2.33	G077K		0.32	L085V		0.42
G072R		0.65	I079L	258	1.44	Q086A	270	2.70
G072S		0.51	I079T		0.79	Q086D		0.88
G072Y		0.35	I079V		1.01	Q086E		1.18
V073A	230	1.38	Q081P		0.60	Q086F		0.54
V073C		0.84	K082A		0.94	Q086G		1.02
V073D		0.94	K082E		0.50	Q086H	271	1.70
V073G		1.17	K082G		0.64	Q086I		0.65
V073H	231	1.54	K082H		0.44	Q086K	272	0.97
V073K	232	1.42	K082I		1.01	Q086L		0.92
V073L	233	1.59	K082L	259	0.87	Q086M		1.06
V073M		0.68	K082M		0.58	Q086N	273	1.28
V073Q	234	0.96	K082N	260	0.96	Q086P		0.42

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
Q086R		0.93	A092M		0.86	T097R		0.95
Q086S	274	0.85	A092T		0.70	T097S	310	1.21
Q086T	275	0.58	A092V		1.09	T097W		0.53
Q086V		0.97	K093D		0.71	T097Y		0.74
Q086W	276	1.21	K093E		0.83	F098A		0.60
D087A		1.00	K093F		0.50	F098C		0.58
D087C	277	1.77	K093G		0.97	F098D		0.47
D087E		0.86	K093H		0.61	F098E		0.44
D087G	278	1.00	K093I	295	3.25	F098H		1.06
D087H		0.72	K093L	296	1.53	F098I		0.52
D087I		0.53	K093M		0.70	F098L		0.58
D087L	279	0.55	K093N		0.71	F098M		0.87
D087M	280	0.58	K093Q	297	0.84	F098Q		0.65
D087P		0.31	K093R	298	1.52	F098R		0.72
D087Q		1.05	K093S	299	1.25	F098S		0.56
D087R	281	1.28	K093T	300	3.93	F098V		0.46
D087S	282	0.99	K093V		0.24	F098W		0.81
D087T	283	1.70	K093P		0.38	Y099A		0.33
D087V	284	0.66	K094A		0.64	Y099R		0.53
D087Y	285	2.72	K094D	301	0.93	Y099S		0.43
L089C	286	1.46	K094E		0.79	V102A		0.83
L089R		0.34	K094F		0.59	V102C		0.69
L089K		0.45	K094H		0.72	V102E		0.90
L089M		0.63	K094L		0.52	V102G		0.67
L089W		0.26	K094M		0.66	V102H		0.88
L089P		0.38	K094N		0.99	V102K		1.03
D090A	287	1.48	K094Q	302	1.22	V102L		0.71
D090E	288	1.15	K094R	303	3.94	V102M		0.77
D090G		0.41	K094S		0.94	V102N		1.02
D090H	289	1.24	K094T		1.14	V102Q		1.03
D090I		1.10	K094C		0.33	V102R		0.94
D090K	290	1.36	I096D		0.69	V102S	311	1.41
D090L		1.15	I096L		0.46	V102T	312	1.26
D090N	291	1.18	I096V		0.68	V102W		0.76
D090Q		1.11	T097A	304	1.25	D103N		0.39
D090R	292	1.49	T097C	305	0.53	N104A		0.69
D090S		1.15	T097D	306	1.31	N104C		0.41
D090T		1.02	T097E	307	1.19	N104G		0.48
D090W		0.81	T097F		0.75	N104K		0.88
K091A		0.89	T097G	308	4.84	N104M		0.61
K091Q		0.43	T097I		0.85	N104R	313	1.25
K091R		0.67	T097L	309	1.22	N104S		1.03
A092C	293	1.97	T097N		1.10	N104T		0.71
A092H		0.22	T097P		0.62	N104I		0.35
A092L	294	1.29	T097Q		1.17	L105A		0.54

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
L105G		0.51	A120V	321	1.53	N131R	334	2.81
L105I		0.94	A120W		0.59	N131S		0.76
L105P		0.84	A120Y	322	1.95	N131T		1.02
L105Q		0.90	N122M		0.56	N131V	335	2.08
L105R		0.65	K124L		0.34	N131Y		0.85
L105S		0.61	K124R		0.62	R132A		0.68
L105T		0.51	K124H		0.35	R132C		0.58
L105W		0.34	P125H		0.43	R132E		0.70
L105V		0.99	P125R		0.63	R132F		0.60
L105C		0.33	P125S		0.54	R132H		0.66
L105H		0.36	P125A		0.36	R132I		0.56
G106V		0.43	D127A		0.89	R132K		1.05
M107F		0.91	D127E	323	1.31	R132L	337	0.76
M107I		0.67	D127G		0.97	R132N	336	1.28
M107L	314	1.32	D127H	324	2.33	R132Q		0.69
A108G		0.47	D127L		0.84	R132S		0.79
I110V		0.51	D127M		0.40	R132T		0.61
E114A	315	1.44	D127N	325	1.69	R132V		0.73
E114G		0.73	D127Q	326	1.21	R132Y		0.78
E114H		0.75	D127R	327	0.51	S133I		0.54
E114M		0.44	D127S		0.77	I134L		1.04
E114S		0.69	D127T		1.11	I134T		0.60
P117D		0.56	D127V		0.56	I134V		1.08
T118H		0.47	D127W		0.44	E135A		0.99
T118K		0.53	V128A		0.53	E135C		0.77
T118L		1.09	V128C		0.68	E135D	338	2.68
T118M		0.53	V128G		0.49	E135F		0.73
T118N		0.67	V128I	328	1.25	E135G	339	2.79
T118Q	316	3.37	V128K		1.16	E135H		0.79
T118V		0.79	V128L		0.95	E135K		1.15
W119F		0.53	V128Q		0.55	E135L		0.82
W119P		0.36	V128R		0.74	E135N		0.56
W119Y		1.08	V128S		0.53	E135Q		1.59
W119Q		0.72	V128W		0.50	E135R	340	2.08
A120D		0.76	K130I		0.50	E135S		1.13
A120F	318	2.62	K130R	329	1.42	E135W		0.63
A120G		1.03	N131C		0.60	E135Y		0.50
A120H	317	1.11	N131E		0.44	L136A		0.73
A120I	319	1.33	N131F		0.63	L136C		0.56
A120L		1.25	N131G	330	2.47	L136D		0.47
A120N		0.81	N131H		0.80	L136F		0.96
A120P		0.42	N131I	331	1.40	L136H		1.00
A120R		0.82	N131L		0.82	L136I		0.65
A120S	320	1.21	N131M	332	0.99	L136M		1.05
A120T		0.62	N131Q	333	1.24	L136N		0.48

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
L136Q		0.61	Q140M		0.80	S145A		0.58
L136R		0.74	Q140R		0.85	S145C		0.44
L136S		0.80	Q140V		0.61	S145D		0.48
L136T		0.72	Q140W		0.59	S145E		0.56
L136W		1.11	Q140Y		0.41	S145G		0.94
V137A		0.48	N141A		1.12	S145H		0.56
V137I		1.01	N141D		1.09	S145L		0.44
V137T		0.51	N141E		0.67	S145M		0.56
V137C		0.37	N141F		0.81	S145N		0.58
V137S		0.36	N141G		1.15	S145P		1.04
V137L		0.21	N141H	344	2.03	S145R		0.97
Q138A		0.69	N141L		0.61	L146A		0.52
Q138C		0.65	N141M		0.48	L146C		0.42
Q138H		0.71	N141Q		1.16	L146E		0.50
Q138I		0.54	N141R	345	1.40	L146G		0.62
Q138L	341	0.59	N141S	346	0.72	L146H		0.78
Q138M		0.68	N141T		0.45	L146I		0.82
Q138N		0.61	N141V		0.50	L146K		0.84
Q138R		0.53	N141W	347	0.83	L146N		0.57
Q138S		0.48	N141Y	348	1.55	L146P	362	0.93
Q138W		0.41	V142C		0.61	L146Q		0.84
Q138Y		0.60	V142D	349	0.71	L146R	363	1.47
Q139A		0.92	V142E		0.87	L146S		0.71
Q139C		0.44	V142G	350	0.98	L146T		0.74
Q139D		0.48	V142H		1.11	L146V		0.84
Q139E		0.94	V142I		0.81	L146Y		0.80
Q139F		0.53	V142K	351	1.40	T147A	364	1.20
Q139G		0.65	V142L		0.75	T147C		0.47
Q139H		0.56	V142M		0.76	T147D		0.71
Q139K		0.73	V142N	352	0.98	T147F	365	1.24
Q139L		0.70	V142P	353	0.88	T147G		1.05
Q139M		0.95	V142Q	354	1.04	T147I		0.85
Q139R		0.79	V142R	355	1.53	T147L	366	1.30
Q139S		0.81	V142S	356	0.93	T147M		0.79
Q139T	342	1.31	V142T	357	1.19	T147P		1.09
Q139V		0.77	Q143E		0.77	T147Q		1.29
Q140A		0.96	Q143G	358	0.62	T147R	367	2.11
Q140C		0.50	Q143K	359	1.30	T147S	368	1.27
Q140D		0.59	Q143L		0.56	T147V	369	2.04
Q140F		0.66	Q143N		0.73	T147W		0.97
Q140G		0.73	Q143V		0.57	T147Y		1.04
Q140H		0.84	Q143C		0.28	E148C		0.66
Q140I		0.75	L144T	361	1.02	E148F		0.42
Q140K	343	0.93	L144W		0.79	E148G		1.05
Q140L		0.51	L144R	360	0.26	E148H	370	1.24

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
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Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
E148I		0.73	E151T	390	1.21	E156V		0.45
E148K	371	1.63	E151V	391	1.38	E156W		0.49
E148L		0.85	E151W	392	1.31	E156C		0.35
E148Q	372	1.44	E151Y	393	1.31	F157W		0.61
E148R		0.97	K152A		0.51	E158A		0.56
E148S		1.15	K152C		0.52	E158F		0.51
E148T		0.82	K152F		0.61	E158H		0.54
E148V		0.99	K152I		0.65	E158L		0.44
E148W		0.43	K152M		0.75	E158Q	402	1.25
E148Y		0.95	K152R	394	1.85	E158S	403	0.95
A149C		1.15	K152T	395	1.20	E158G		0.37
A149G		0.52	K152V		0.82	K159A		0.64
A149K		0.51	K152Y		0.67	K159D		0.52
A149L		0.88	K152W	396	0.37	K159E		0.49
A149M		0.88	A153I		0.93	K159H		0.74
A149Q		1.15	A153L		0.51	K159L		0.62
A149R		1.02	A153S		0.34	K159M		0.66
A149S		1.08	K154R		0.86	K159N		0.73
A149T	373	1.24	K154T		0.83	K159Q		0.92
A149V	374	1.34	K154V		0.46	K159R		0.88
T150A	375	1.21	K154I		0.38	K159S		0.67
T150C		0.70	Q155A		0.91	K159V		0.41
T150D	376	1.24	Q155C		0.60	K159G		0.38
T150E		1.05	Q155D	397	1.49	A160C		0.61
T150F		0.71	Q155F		0.70	A160F		0.79
T150G	377	2.19	Q155G	398	1.61	A160G		0.75
T150I		0.52	Q155H		1.03	A160H		0.47
T150L		0.70	Q155K	399	1.57	A160I		0.43
T150N	378	0.91	Q155L		0.86	A160K		0.91
T150P		0.88	Q155M		0.97	A160L		0.67
T150R		0.90	Q155R	400	1.27	A160M		0.77
T150S	379	0.92	Q155S		0.77	A160N		0.56
T150W	380	1.25	Q155T		0.76	A160Q		0.65
T150Y	381	1.36	Q155V		0.73	A160R		0.89
E151A	382	1.27	Q155W		0.91	A160S	404	1.35
E151C		1.00	E156A		0.79	A160V		0.73
E151G		1.06	E156D	401	1.95	A160Y		1.07
E151H	383	1.34	E156G		0.49	A160W		0.39
E151K	384	2.05	E156I		0.51	G161A		0.99
E151L	385	1.03	E156L		0.43	G161C		0.44
E151M	386	1.26	E156M		0.87	G161D		0.86
E151N		0.95	E156Q		0.84	G161E		0.49
E151Q	387	2.01	E156R		0.43	G161R		0.48
E151R	388	1.61	E156S		0.62	G161S		0.77
E151S	389	1.28	E156T		0.69	G161V		0.42

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
G161V		0.42	V166G		1.11	L174S		0.85
K162A		0.50	V166H	422	1.74	L174T	438	1.12
K162D		0.77	V166L	423	4.38	L174V		0.62
K162E	405	0.51	V166Q	424	3.61	L174W		0.78
K162G		0.56	V166R	425	5.56	L174Y		1.06
K162H		0.62	V166T	426	4.26	L174H		0.38
K162L		0.54	V166W	427	1.26	L175E		0.43
K162M		1.04	V166Y	428	2.08	L175H		0.57
K162P		0.64	V166N		0.47	L175T	439	1.43
K162Q		0.58	E167A		0.84	L175V		0.94
K162R		0.52	E167D	429	0.69	L175Y		0.66
K162S		0.47	E167G		0.60	R176K		0.67
K162V		0.52	E167H		0.89	R176L		0.40
K162W		1.01	E167K		0.91	P177V		0.36
K162Y		0.72	E167M		0.87	N178G		0.85
D163A	406	1.52	E167N		0.83	N178K	440	0.85
D163E	407	1.63	E167P		0.58	N178M		0.88
D163G		1.15	E167R		1.02	N178R	441	1.10
D163K	408	1.90	E167S		1.17	H179A		1.06
D163L		1.18	E167T		0.59	H179C		0.94
D163Q	409	1.40	E167Y		0.55	H179E		0.62
D163R	410	1.80	E167F		0.31	H179G		0.86
D163S	411	1.34	T168H		0.46	H179I		0.90
D163T		1.13	I169L	430	2.08	H179K	442	1.39
D163V		0.76	I169R		0.54	H179L		0.73
D163W		0.38	I169V		0.74	H179M		0.63
D163F		0.39	K170N		0.72	H179N		0.96
F164L		1.13	K170R	431	2.58	H179P		0.44
F164M	412	1.66	K170V		0.58	H179R		0.96
F164V	413	1.23	K170A		0.40	H179S		0.51
F164W		0.72	K170Q		0.40	H179T		0.43
L165A		0.48	L171I		0.73	H179V		0.42
L165D	414	5.79	L171V		0.64	L180F		0.59
L165F	415	1.23	G172A	432	1.20	L180G		0.62
L165N	416	2.19	G172C		1.03	L180K		0.44
L165R		0.59	K173N		0.44	L180M		0.64
L165S	417	1.31	K173R	433	0.82	L180I		0.38
L165V	418	1.22	K173Q		0.32	W181M		0.88
L165W		1.14	L174A		1.20	W181Q		0.88
L165Y		0.66	L174G	434	0.40	W181K		0.29
L165C		0.27	L174K	435	2.39	G182L		0.90
V166A	419	2.85	L174M		0.79	Y183L		0.70
V166C		1.16	L174N	436	1.36	Y183E		0.32
V166E	420	1.28	L174Q		0.99	Y184W		0.39
V166F	421	1.67	L174R	437	1.50	F186Y		0.59

KEY
Coloration of Percent (%) Activity Values
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120% - 200%
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Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
H192S		0.49	G198E		0.49	I208S		0.62
H192T		0.50	G198H		0.84	I208T		1.01
H193G		0.68	G198L		0.48	I208V		1.07
H193Q	443	0.82	G198N		0.80	K209A		0.53
H193S		0.42	G198Q		0.55	K209E		0.46
H193Y		0.58	G198R		0.58	K209G		0.44
H193R		0.33	G198S		0.76	K209N		0.50
H193F		0.38	G198T		0.41	K209R	458	0.68
K195A		0.51	G198Y		0.81	K209S		0.50
K195G		0.45	G198W		0.29	K209T		0.50
K195H		0.45	N200D		0.46	K209F		0.40
K195I		0.50	N200T		0.37	K209L		0.38
K195L		0.45	S202M		0.40	N211L		0.41
K195N	445	0.74	F204P	449	0.63	N211W		0.51
K195Q		0.71	F204W		0.39	D212N	459	1.52
K195R		0.85	N205A	450	1.30	D212S	460	0.93
K195S		0.42	N205D		0.85	D212T		0.76
K195T	444	0.58	N205E	451	1.94	D213A	461	0.85
K195W		0.49	N205F		0.52	D213E		0.79
K195V		0.36	N205G		0.79	D213G		0.81
K196E	446	0.43	N205K		0.76	D213H		0.75
K196G		0.41	N205M		0.58	D213K		0.82
K196L		0.65	N205P		0.75	D213L		0.56
K196R	447	0.58	N205R		0.54	D213M	462	1.56
K196S		0.68	N205S		0.80	D213N	463	1.53
K196T		1.18	N205T	453	0.85	D213Q		1.04
K196W		0.55	N205V		0.49	D213R		0.92
K196N		0.39	N205W		0.41	D213V		0.47
K196Y		0.39	N205L	452	0.39	D213W		0.49
P197A		0.81	N205Y		0.40	D213Y		0.49
P197D		0.58	V206H		0.50	L214Q		0.57
P197E		0.52	V206K	455	1.75	S215A		0.74
P197F		0.48	V206L	456	1.57	S215D		0.62
P197G		0.75	V206M		0.43	S215E		0.74
P197H		0.62	V206R	457	1.30	S215G		0.88
P197K		0.99	V206S		0.72	S215H	464	0.91
P197L		0.56	V206T		0.59	S215K		0.99
P197M		1.03	V206Q		0.33	S215L		0.60
P197Q		0.69	I208A		0.62	S215M	465	1.77
P197R		0.58	I208C		0.48	S215Q		0.79
P197S		0.70	I208K		0.91	S215R		0.71
P197T		0.41	I208L		0.84	S215T		0.80
P197W		0.39	I208M		0.88	S215V		0.69
G198A		0.80	I208Q		0.77	S215W		0.52
G198D	448	1.99	I208R		1.14	W216Y		0.48

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
L217M		0.51	L230I		0.87	A239N		0.32
W218F		0.57	N231T		1.10	T240K		1.13
W218M		0.38	T232F	476	0.73	T240A	491	0.48
W218V		0.28	T232S		0.76	T240M		0.48
N219A	466	1.29	Q233A		0.71	T240P		0.56
N219C		0.43	Q233F		0.53	T240Q	492	0.75
N219D		0.75	Q233G	477	0.46	T240R		0.91
N219E		0.95	Q233K	478	1.69	T240S		0.74
N219H		0.97	Q233L		0.69	T240V		0.77
N219I	467	0.60	Q233R	479	1.50	Y242F		1.08
N219K	468	1.45	Q233Y		0.50	N245H		0.50
N219L		0.72	Q234M	480	1.65	V247I	493	2.01
N219M		1.02	Q234L		0.40	V247L		0.83
N219R		1.10	S235A	481	0.47	V247M		0.52
N219S	469	2.48	S235E		1.00	R248H		0.40
N219T		0.82	S235G		0.95	R248A	494	0.43
N219W		0.48	S235H		0.44	R248W		0.52
E220A		0.75	S235K		0.53	R248Y		0.67
E220H	470	1.40	S235T		0.66	I251Y		0.37
E220I	471	1.34	P236A		1.07	I251L		0.58
E220L	472	1.45	P236G		1.09	I251M		0.43
E220S		0.62	P236H		0.46	V253I		0.76
E220T		0.91	P236K		0.71	K255A		0.40
E220V	473	1.35	P236R	482	3.09	K255N		0.52
E220D		0.39	P236S		0.91	K255Q		0.91
E220M		0.36	V237A		0.90	K255R		0.71
S221I		0.35	V237E	484	1.93	K255S		0.43
S221A		0.72	V237F		0.41	K255G		0.39
S221C		0.59	V237H	485	0.75	I256A		0.42
S221M		0.46	V237L		1.12	I256H		0.51
S221Q	474	1.37	V237N		0.67	I256L		0.64
S221T		0.94	V237Q	486	1.46	I256V		0.51
S221V		1.04	V237R		0.71	P257C		0.36
T222D		0.43	V237S		1.03	P257A		0.82
T222F		0.43	V237T	487	1.01	P257G	496	0.51
T222G	475	0.49	V237W		0.52	P257I		1.07
T222K		0.75	V237C	483	0.35	P257K		0.92
T222L		0.64	A238D		0.75	P257L		0.69
T222N		0.80	A238E	488	0.59	P257M		0.90
T222R		0.75	A238H	489	0.60	P257N		0.69
T222I		0.40	A238K		0.60	P257Q		0.61
T222S		0.63	A238Q		1.02	P257R	498	1.38
T222V		0.79	A238R		0.49	P257T	497	2.04
L224I		0.61	A238S	490	2.62	P257V		0.88
P226W		0.51	A238T		0.44	D258H		0.84

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
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Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
D258N	499	1.44	L263H		0.36	Q276G		0.36
D258R		0.45	P264A		0.43	V277A	524	0.65
D258S	500	1.44	P264H		0.60	V277C		0.41
D258G		0.39	V265I		0.58	V277D		0.79
A259E		0.85	F266Y		0.58	V277E	525	1.02
A259G		0.68	A267M		0.45	V277G		1.18
A259I		0.46	A267T	509	1.34	V277H	526	1.09
A259K		0.76	T269A	510	1.63	V277K	527	1.51
A259L		0.53	T269C		0.75	V277M	528	0.94
A259N		0.49	T269D		0.76	V277N	529	1.15
A259P	501	1.54	T269S		1.01	V277Q	530	0.82
A259Q		0.70	R270M		0.46	V277R	531	1.63
A259R		0.72	R270N		0.52	V277S	532	0.83
A259S		0.63	R270S		0.69	V277T	533	1.94
A259T		0.51	R270T		0.40	V277Y		0.66
A259V		0.41	I271F		0.72	L278A		1.13
A259W		0.55	I271G		1.29	L278E	534	1.03
A259Y		0.51	I271L	511	10.62	L278F	535	1.26
K260A		0.66	I271M	512	3.24	L278G	536	1.33
K260D		0.41	I271S		0.42	L278H	537	4.50
K260E		0.58	I271V		1.05	L278I		0.93
K260H		0.87	V272E		0.39	L278K	538	1.75
K260L		0.60	V272D	513	1.36	L278N	539	1.74
K260M	502	0.85	V272R		0.74	L278R	540	5.87
K260Q		0.58	V272S		0.96	L278S	541	1.67
K260R		0.83	V272T	514	1.61	L278T	542	1.66
K260S		0.66	V272M		0.31	L278V		0.44
K260G		0.37	F273H	515	1.41	L278Y	543	1.51
K260Y	503	1.73	F273T		0.48	K279A		0.27
S261A	504	0.74	F273Y	516	0.90	K279H	544	0.44
S261F		0.73	T274A		0.51	K279Q		0.84
S261K	505	2.54	T274F	517	1.28	K279R		1.10
S261M		0.56	T274S		0.62	K279T		0.86
S261N	506	1.98	D275V		0.40	F280G		0.47
S261Q		0.76	D275L		0.24	F280Q		0.43
S261R		1.19	Q276C		0.88	S282D		0.41
S261T		0.66	Q276D	518	1.69	S282G		0.54
S261V		0.48	Q276E		1.05	S282M	545	2.64
S261W		0.44	Q276H	519	1.20	S282Q		0.41
L263A		0.76	Q276I		0.51	Q283E		0.63
L263K	507	2.73	Q276L		0.48	Q283P		1.18
L263M		0.89	Q276M	520	1.14	Q283R		0.59
L263R	508	1.63	Q276R	521	1.30	Q283S	546	1.73
L263T		0.49	Q276S	522	1.63	Q283T		0.65
L263V		0.75	Q276Y	523	1.94	D284A		0.58

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
D284E		1.21	E292W		0.83	S308H		1.15
D284G		0.60	T293A	558	1.90	S308K	573	1.33
D284H		0.51	T293C	559	1.67	S308N	574	2.33
D284L		0.50	T293D	560	1.46	S308P		0.65
D284M		0.56	T293F	561	1.94	S308R	575	1.34
D284N		0.40	T293G		1.00	S308T		0.72
D284Q		0.95	T293K	562	1.35	I309D		0.72
D284S		0.99	T293L		1.00	I309E	576	1.99
D284T		0.39	T293M	563	2.29	I309G	577	1.44
D284Y		0.37	T293P	564	1.64	I309H	578	1.30
E285A		0.34	T293Q	565	1.83	I309K		0.98
E285F		0.47	T293S		0.89	I309L	579	1.72
E285G		0.52	T293V	566	2.15	I309M	580	1.47
E285H	547	1.30	T293Y	567	1.49	I309N	581	3.11
E285M		0.43	V294M		0.41	I309Q	582	1.64
E285N		0.40	K297R		0.34	I309R	583	2.27
E285Q		0.59	G297A		0.57	I309S	584	1.16
E285Y		0.99	A298G	568	0.43	I309T	585	2.09
L286S		0.46	A298I		0.41	I309V	586	0.60
L286R		0.53	G300R		0.42	I309W		0.88
L286W		0.38	I301A		0.88	M310F		0.30
V287I		0.51	I301V		0.88	M310Y		0.38
V287T	548	0.50	V302W		0.46	M310A	587	1.50
V287N		0.35	V302I		0.45	M310G	588	2.73
Y288L		0.79	I303V		0.47	M310Q	589	0.59
Y288W		0.49	I303D		0.34	M310R		0.50
T289K		0.75	W304G		1.13	M310S	590	1.61
T289S	549	0.48	W304I		1.17	M310V		0.70
F290I		0.41	G305N		0.36	R311G		0.53
F290M		1.03	G305D		1.00	R311G		0.54
G291C		0.27	G305E	569	1.62	R311H		0.48
G291Q		0.80	T306D		0.76	R311K		0.72
G291R		0.45	T306E		0.52	R311Q		0.43
G291S	550	0.41	T306S		1.02	R311S		0.84
G291V	551	1.63	L307K		0.43	R311T		0.52
E292A		0.66	L307N		0.76	S312K		0.38
E292C	552	0.71	L307Q		0.61	S312G		0.49
E292F	553	0.90	L307S		0.86	S312N		1.26
E292G		0.41	L307T		1.08	S312T		0.75
E292H	554	1.26	L307V		0.48	S312L		0.38
E292K	555	1.27	L307W		0.64	M313A	591	1.34
E292N		0.99	L307Y		0.60	M313E		0.63
E292P		1.05	L307G	570	0.32	M313G	592	0.56
E292R	556	0.42	S308D	571	0.92	M313H	593	1.23
E292V	557	1.28	S308G	572	1.73	M313K	594	2.85

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Coloration of Percent (%) Activity Values
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Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
M313L		1.05	L318K	616	1.36	T325N	632	4.64
M313P	595	1.11	L318M	613	1.68	T325Q	633	5.08
M313R	596	2.30	L318N		0.52	T325S	634	3.19
M313S		0.88	L318Q		0.71	T325V	635	1.24
M313T	597	0.67	L318R	617	1.34	T325W		0.62
M313V		0.99	L318S		0.71	I326C		0.39
M313Y	598	1.12	L318T		0.63	I326S		0.95
K314A		0.82	D320L		0.37	I326K		0.95
K314D		0.53	D320E		0.78	I326L	636	1.50
K314H		1.10	D320G		0.83	I326V	637	6.29
K314I		0.54	D320H	618	1.75	I326Y		0.77
K314N		0.57	D320I		1.00	L327M		0.52
K314Q		0.62	D320K	619	6.42	N328A		0.67
K314R		0.95	D320M		0.79	N328C	638	1.25
K314S	599	0.61	D320N		0.52	N328G	639	0.56
K314T		0.61	D320R	620	3.19	N328H		0.88
K314Y	600	0.45	D320S		1.19	N328I	642	1.85
S315A	601	0.85	D320W		0.40	N328K	640	2.12
S315E		0.41	D320V		0.35	N328L	641	2.01
S315G		0.72	D320Y		0.86	N328Q		1.13
S315H	602	2.04	N321A		1.01	N328R		0.68
S315K		0.62	N321D		1.25	N328S	643	2.22
S315L		0.42	N321H		0.92	N328T		0.59
S315M		0.63	N321K		1.29	N328V		1.16
S315R		1.04	N321R	621	1.23	N328Y	644	1.66
S315T		0.97	N321S	622	1.26	N328W		0.33
S315Y	603	0.50	N321T		0.64	I331C		0.27
C316D		0.41	N321Y		0.40	I331E		0.34
L317A	604	1.27	M323F		0.64	I331V		0.94
L317D		0.61	M323I		0.55	V334T		0.39
L317H		1.05	M323L		0.55	V334P		0.46
L317I	605	1.76	E324A		0.59	T335S	645	0.47
L317K	606	5.11	E324D		1.15	A338Q		0.63
L317M		1.20	E324H		0.79	K339M		0.61
L317N	607	0.73	E324M		0.50	S342A		0.68
L317Q	608	1.67	E324N	623	1.01	Q343T		0.49
L317R	609	2.41	E324R	624	2.28	Q343V		0.51
L317S	610	1.03	E324S		0.62	Q347L		0.39
L317T	611	0.93	T325A	625	1.87	Q347A	646	0.78
L317W	612	0.84	T325D	626	1.78	Q347E		0.78
L318D	614	0.46	T325E	627	4.03	Q347G	647	2.68
L318F		0.51	T325G	628	4.21	Q347M		0.61
L318G		0.49	T325H	629	3.45	Q347R		0.55
L318H	615	0.45	T325K	630	4.37	Q347S	648	2.38
L318I		0.70	T325M	631	2.11	E348D		0.67

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
E348G		0.55	D368E	663	1.28	E375I		0.36
E348S		0.44	D368G		0.49	E375A	680	0.42
Q349A		0.47	D368H		0.96	E375G	681	0.90
Q349E		0.83	D368K	664	1.31	E375K	682	1.49
Q349K		0.93	D368L	665	0.64	E375L		0.46
Q349M	649	0.70	D368M	666	0.78	E375M		0.54
Q349N		0.44	D368R	667	1.31	E375N		0.81
Q349R	650	0.73	D368S		0.93	E375R	683	0.43
Q349T		0.49	D368T	668	0.80	E375S		0.77
V351Q		0.34	D368V		0.41	E375T		1.17
V351C		0.35	N369H	669	1.33	K376L		0.37
V351A		1.14	N369R	670	0.55	K376A		0.95
V351S	651	0.92	N369S		0.54	K376D	684	0.78
V351I		0.36	A371G		0.38	K376E	685	0.88
I353T		0.42	A371E		1.05	K376M		0.46
I353V	652	1.61	A371F	671	0.52	K376Q	686	0.69
N356A		0.41	A371H	672	1.20	K376R	687	0.67
N356D		0.79	A371I		0.50	K376S		0.80
N356H	653	0.82	A371K	673	1.76	K376T	688	0.53
N356S	654	0.46	A371L	674	0.57	K376V	689	0.58
W357K		0.36	A371M		0.57	K376Y	690	0.42
W357A		0.80	A371R	675	1.51	G377D	691	1.35
W357C		0.67	A371S	676	1.45	G377E	692	0.59
W357S		0.41	A371V		0.94	G377H	693	1.49
W357T		0.62	Q373A		0.65	G377K	694	1.50
N358C		0.66	Q373E		0.81	G377P	695	2.30
N358G		0.41	Q373F		0.62	G377R	696	1.28
N358T		0.58	Q373K		0.73	G377S	697	1.80
N358L		0.38	Q373L		0.84	G377T	698	3.83
S359D		0.45	Q373M	677	1.43	G378K		1.22
S359E	655	1.05	Q373R		0.68	G378N		0.64
S359H	656	0.44	Q373S		0.87	G378R		1.03
S359K		0.66	Q373V		1.05	K379G		0.52
S359M		0.63	L374W		0.34	K379H		0.57
S359T	657	2.11	L374A		0.60	K379R		0.74
S359V		0.65	L374H	678	1.42	K379S		0.46
S360T		0.50	L374I		0.80	K379T		0.40
D361H		0.37	L374M		1.11	F380V		0.39
P367A	658	0.55	L374N		0.43	F380T		0.39
P367C		0.83	L374P	679	0.43	F380I		0.56
P367G	659	0.47	L374R		0.83	F380L		0.67
P367K	660	0.57	L374S		0.58	F380P		0.47
P367R		0.46	L374T		0.47	F380W	699	2.15
P367S	661	0.52	L374V		0.56	F380Y	700	1.50
D368A	662	1.34	L374Y		0.66	T381H		0.48

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Coloration of Percent (%) Activity Values
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Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
T381K		1.06	E389R		0.94	E396L		0.39
T381N		0.51	E389S	714	1.08	F398L		0.35
T381Q		0.84	E389T		0.70	Y399A		1.01
T381R		0.87	E389Y		0.77	Y399C		0.46
T381S	701	0.87	L391C		0.90	Y399E		1.49
T381V		0.89	E392W		0.31	Y399K	730	1.94
T381E		0.35	E392A	715	0.58	Y399M	731	2.70
R383A		0.51	E392F	716	0.54	Y399N		0.52
R383E		0.51	E392G		1.00	Y399Q		1.18
R383H		0.71	E392K		0.66	Y399R		1.20
R383I	702	0.71	E392L		0.80	Y399S		1.01
R383K	703	1.30	E392M	717	1.54	Y399T	732	2.40
R383L	704	1.31	E392Q	718	1.01	Y399V	733	1.44
R383M		0.61	E392R	719	0.66	Y399W	734	1.92
R383N		0.77	E392S		0.52	S401Q		0.39
R383S	705	0.87	E392T		0.72	S401A	735	0.82
R383T		0.98	E392V	720	1.27	S401E	736	0.46
R383V		1.05	E392Y		0.92	S401N		0.42
K385A	706	1.12	Q393A		1.26	S401G		0.38
K385G		0.62	Q393D		0.45	Y403F		0.62
K385H		0.50	Q393F	721	1.23	S404T		0.37
K385N		0.41	Q393H		1.05	S404A	737	0.63
K385Q	707	0.73	Q393K		0.80	S404P		0.64
K385R		0.94	Q393L		0.91	T405F		0.36
K385S		1.05	Q393M	722	0.80	T405A		0.56
K385T		0.46	Q393N		0.72	T405G	738	2.32
K385V	708	0.43	Q393R		0.74	T405K		0.74
T387S		0.93	Q393S		1.15	T405M		0.48
L388F		0.92	Q393T		0.41	T405P		0.64
L388H		0.47	F394L		0.56	T405Q		0.75
L388I		0.98	F394W		0.41	T405R		0.60
L388M		0.79	S395W		0.40	T405S		0.94
L388R		0.60	S395T		0.39	T405W		0.73
L388T		0.51	S395A	723	1.10	T405Y		0.44
L388V		0.78	S395G		0.77	L406A		0.70
L388W		0.77	S395H	724	0.56	L406C		0.98
L388Y		1.18	S395K		0.96	L406E		0.73
E389A	709	1.14	S395R	725	1.98	L406F	739	1.42
E389G	710	0.91	E396A	726	0.52	L406G		1.00
E389H		1.17	E396D		0.64	L406I		0.61
E389K	712	1.91	E396H	727	0.47	L406N	740	0.76
E389L	711	0.65	E396Q	728	0.73	L406Q		0.93
E389M		0.60	E396R		0.61	L406S		0.47
E389P		0.75	E396S	729	0.61	L406T		0.83
E389Q	713	0.69	E396T		0.89	L406V		0.87

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
L406Y		0.74	K411V		0.99	D418G		0.45
S407L		0.40	A412H		0.39	D418A		0.92
S407A	741	1.16	A412Q	751	0.35	D418E	755	1.31
S407D	742	1.52	A412Y		0.66	D418F		0.81
S407E	743	1.38	A412D		0.74	D418G		0.45
S407F	744	1.42	A412G		0.80	D418I		0.99
S407G		0.75	A412I		0.81	D418L	756	1.28
S407H	745	1.34	A412L		0.65	D418M		1.09
S407M		0.74	A412N		0.86	D418N		0.91
S407N		0.72	A412P		0.77	D418P	757	2.11
S407P	747	0.94	A412R	752	0.66	D418Q		1.05
S407Q	746	1.71	A412S		0.86	D418R	758	1.18
S407R		1.04	A412V	753	0.53	D418S		0.78
S407V		0.56	A412W		0.54	D418V	759	1.43
S407W		0.41	D413H		0.31	D418Y		0.97
K409A	748	2.18	A413Q		0.38	A419E		0.45
K409D		0.65	D413E		0.52	A419F	760	2.17
K409E		0.62	D413K		0.42	A419G		0.42
K409G		0.50	D413N		0.94	A419H	761	1.21
K409H		0.64	D413R		0.50	A419I	762	1.64
K409I		0.51	D413T		0.41	A419K	763	1.88
K409P		0.48	D413A		0.38	A419L		0.56
K409Q	749	3.33	D413S		0.39	A419N		0.53
K409R		0.84	V414K		0.30	A419R	764	1.81
K409S		0.72	V414L		0.36	A419S	765	2.65
K409T		0.63	V414I		1.12	A419W		0.69
K409V		0.48	V414M		0.53	A419Y	766	1.44
E410D		0.47	K415V		0.39	V420I		1.04
E410K		0.70	K415G		0.40	V420P		0.48
E410M		0.42	K415S		0.42	V421I		0.39
E410N		0.67	K415W		0.42	D421A	767	1.28
E410P		0.73	K415Y		0.39	D421E		0.81
E410Q		0.85	D416F		0.41	D421G		0.62
E410R		0.61	D416G		0.67	D421H	768	1.98
E410S		0.81	D416H		0.57	D421K	769	2.42
E410T	750	1.54	D416I		0.63	D421L		0.73
E410V		0.65	D416K		0.76	D421M		0.94
E410Y		0.62	D416L	754	0.75	D421N	770	1.89
K411H		0.33	D416N		0.73	D421Q	771	1.54
K411A		0.48	D416Q		0.83	D421R	772	2.21
K411N		1.02	D416R		0.46	D421S	773	2.12
K411P		0.42	D416T		0.85	D421T		0.80
K411R		0.97	D416V		0.59	D421Y		0.66
K411S		1.21	D416Y		0.40	V422I		0.42
K411T		0.63	T417I		1.22	V422T		0.49

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
A425G	774	1.20	A432V		0.56	P436Y		0.49
A425I		0.44	F433A	790	0.97	P437A		0.56
A425K	775	1.75	F433C		0.69	P437D		0.62
A425M		0.70	F433D		0.95	P437G		0.50
A425N		0.46	F433E		0.82	P437H		1.11
A425R		0.49	F433G		0.54	P437I	800	2.46
A425S		0.47	F433H	791	0.83	P437K		0.83
A425Y		0.39	F433I	792	1.06	P437L		0.51
D426K		0.26	F433K	793	1.36	P437M	801	2.55
D426S		0.36	F433L	794	1.87	P437Q		0.96
D426E		0.62	F433P		0.95	P437R		0.85
D426G		0.85	F433R	795	1.63	P437S		0.57
D426N		0.61	F433S		0.86	P437Y		0.42
D426P		1.03	F433T	796	1.86	M438A	802	0.75
D426Q		0.42	F433V	797	1.63	M438C		0.63
D426Y		0.43	F433W	798	1.28	M438D	803	0.87
G427H		0.35	L434F		0.41	M438E	804	0.72
G427I		0.54	L434G		0.47	M438G		0.83
G427K		0.52	L434I		0.89	M438L	805	0.86
G427S		0.42	L434M		0.60	M438N	806	1.08
G427T	777	0.35	L434V		0.46	M438P		0.81
G427Q	776	0.39	K435A		1.08	M438Q		0.85
V428L	778	1.25	K435C		0.53	M438R		0.99
V428M		0.42	K435E		0.78	M438S		0.83
V428P		0.82	K435G		0.64	M438T	807	3.99
V428T		0.62	K435H		1.05	M438V		0.85
D431A	779	2.42	K435R		1.01	M438W		0.57
D431E	781	1.27	K435S		1.03	E439A	808	1.20
D431G	780	0.55	K435T		0.73	E439C	809	0.58
D431H	782	3.13	K435V		0.44	E439F		1.00
D431I		1.05	K435Y		0.50	E439G		1.22
D431K	783	1.83	P436C		0.39	E439H		0.74
D431L	784	0.62	P436D		1.19	E439K	810	1.20
D431N	785	1.30	P436E		0.74	E439L		0.88
D431Q	786	2.16	P436G		1.19	E439P	811	1.16
D431R	787	2.20	P436H		0.72	E439Q	812	1.32
D431S	788	1.91	P436I		0.84	E439S		1.02
D431V	789	1.52	P436K	799	2.05	E439T	813	1.15
D431W		0.56	P436L		0.63	E439V	814	1.57
D431Y		0.85	P436M		0.61	E439W		0.62
A432E		0.60	P436Q		0.86	T440A		1.22
A432G		0.52	P436R		1.00	T440D	815	1.03
A432H		0.34	P436S		0.92	T440E		1.00
A432N		0.51	P436T		0.59	T440F		0.85
A432S		0.61	P436W		0.43	T440G		0.86

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
T440H	816	3.00	P443T		0.87	Y447F		1.41
T440I		1.04	P443W		0.64	Y447G	849	0.92
T440L		0.97	Q444M		0.37	Y447I	850	1.36
T440M	817	1.08	Q444D		0.97	Y447L		1.09
T440P	818	0.88	Q444E	832	1.19	Y447M		0.90
T440R	819	1.77	Q444F		0.66	Y447N	851	1.58
T440S	820	1.17	Q444G		0.93	Y447P	852	1.46
T440V		1.02	Q444H	833	0.97	Y447Q	853	2.37
T440Y		1.11	Q444I		0.58	Y447R		1.12
E441A	821	1.47	Q444K		1.03	Y447T	854	1.90
E441D		0.67	Q444N		1.01	Y447V	855	1.38
E441F	822	3.91	Q444R		0.85	Y447W		1.07
E441G		0.87	Q444V	834	1.12	R0451		0.45
E441H		0.65	Q444W		0.64	Q1431		0.44
E441K		0.80	Q444Y		0.67	1445W	843	0.69
E441L		0.82	I445A		0.97	V2061	454	0.94
E441N		0.82	I445G		0.98			
E441Q		0.81	I445H	835	1.35			
E441S		0.79	I445L		1.06			
E441T		0.66	I445M	836	1.57			
E441V		0.54	I445N	837	1.24			
E441Y		0.51	I445P	838	1.67			
E442L		0.40	I445Q	839	1.26			
E442W		0.38	I445R		1.08			
E442C	823	1.38	I445S	840	1.21			
E442G	824	0.51	I445T	841	1.38			
E442H		0.76	I445V	842	1.25			
E442K		0.73	I445Y		0.53			
E442P		0.91	F446A	844	1.58			
E442Q		0.74	F446C		0.75			
E442R	825	3.94	F446D		1.18			
E442T		0.61	F446E		1.10			
E442V		0.65	F446G		1.12			
E442Y		0.60	F446H		1.28			
P443A	826	1.63	F446I		1.06			
P443E	827	1.07	F446K		0.94			
P443F	828	0.70	F446L		0.93			
P443G	829	1.12	F446M	845	1.31			
P443H		1.08	F446Q		0.72			
P443L		1.19	F446R		0.89			
P443M	830	1.99	F446T		0.89			
P443N	831	1.25	F446V		0.91			
P443Q		0.96	F446W	846	1.40			
P443R		1.04	Y447D	847	3.25			
P443S		0.99	Y447E	848	1.36			

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity

APPENDIX A-5

List of Active Mutant Activity Data – Sorted by High Activity to Low Activity

Source(s) – Table 9

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
I070A	209	2700%	G377T	698	383%	S282M	545	264%
S069A	196	2206%	F029P	113	379%	D030K	120	263%
I070K	214	1464%	G052Q	173	371%	E031L	131	262%
S069R	205	1406%	I070S	219	363%	A120F	318	262%
I070R	218	1395%	V166Q	424	361%	A238S	490	262%
I271L	511	1062%	T325H	629	345%	K170R	431	258%
I070H	213	909%	S069L	202	344%	I070C	210	257%
S069F	199	875%	T118Q	316	337%	P437M	801	255%
S084G	267	868%	L037F	149	333%	S261K	505	254%
S069P	204	814%	K409Q	749	333%	A048F	164	251%
D320K	619	642%	L001Q	78	327%	N219S	469	248%
I070V	221	634%	K093I	295	325%	N131G	330	247%
I326V	637	629%	Y447D	847	325%	P437I	800	246%
I070G	212	622%	I271M	512	324%	T074P	248	245%
I070N	216	619%	L026E	99	322%	D421K	769	242%
S069G	200	606%	D320R	620	319%	D431A	779	242%
L278R	540	587%	T325S	634	319%	L317R	609	241%
L165D	414	579%	E031K	130	313%	Y399T	732	240%
I070F	211	569%	D431H	782	313%	L174K	435	239%
V166R	425	556%	S069I	201	312%	Q347S	648	238%
I070T	220	543%	G072M	228	311%	Y447Q	853	237%
V075L	254	522%	I309N	581	311%	F029L	111	236%
L317K	606	511%	P236R	482	309%	G072Q	229	233%
T325Q	633	508%	I070L	215	305%	D127H	324	233%
T097G	308	484%	I070P	217	303%	S308N	574	233%
T325N	632	464%	V075R	256	302%	S039N	154	232%
E031V	136	457%	T440H	816	300%	T405G	738	232%
V058Q	181	454%	E031C	126	295%	M313R	596	230%
L278H	537	450%	V166A	419	285%	G377P	695	230%
V166L	423	438%	M313K	594	285%	I046R	159	229%
T325K	630	437%	N131R	334	281%	T293M	563	229%
V075T	257	434%	E135G	339	279%	T074A	238	228%
V166T	426	426%	T074G	242	275%	E324R	624	228%
A048N	167	425%	E031H	128	274%	E031R	133	227%
T325G	628	421%	L263K	507	273%	T074V	251	227%
V058K	178	408%	M310G	588	273%	I309R	583	227%
F029E	108	403%	D087Y	285	272%	T074R	249	222%
T325E	627	403%	G027K	105	271%	N328S	643	222%
F024E	95	399%	P032F	139	271%	F029S	115	221%
M438T	807	399%	S069Y	208	271%	D421R	772	221%
E031T	135	396%	Q086A	270	270%	L015V	91	220%
K094R	303	394%	Y399M	731	270%	D431R	787	220%
E442R	825	394%	E135D	338	268%	T150G	377	219%
K093T	300	393%	Q347G	647	268%	L165N	416	219%
E441F	822	391%	S069M	203	267%	S069W	207	218%
E031I	129	389%	A419S	765	265%	T074C	239	218%

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
K409A	748	218%	T293F	561	194%	E031S	134	170%
T071R	225	217%	Y399K	730	194%	Q086H	271	170%
A419F	760	217%	V237E	484	193%	D087T	283	170%
D431Q	786	216%	V058R	182	192%	D030H	119	169%
T293V	566	215%	Y399W	734	192%	D127N	325	169%
F380W	699	215%	V073W	237	191%	Q233K	478	169%
T074W	252	213%	E389K	712	191%	Q276D	518	169%
T074N	247	212%	D431S	788	191%	L318M	613	168%
N328K	640	212%	L001H	73	190%	D068Q	195	167%
D421S	773	212%	D163K	408	190%	V166F	421	167%
T147R	367	211%	T293A	558	190%	L278S	541	167%
T325M	631	211%	Y447T	854	190%	T293C	559	167%
S359T	657	211%	S084R	269	189%	L317Q	608	167%
D418P	757	211%	D421N	770	189%	I445P	838	167%
I309T	585	209%	L026K	100	188%	F164M	412	166%
F029M	112	208%	G027R	106	188%	L278T	542	166%
P032H	141	208%	A419K	763	188%	N328Y	644	166%
N131V	335	208%	T325A	625	187%	F029V	117	165%
E135R	340	208%	F433L	794	187%	Q234M	480	165%
V166Y	428	208%	V012E	86	186%	T293P	564	164%
I169L	430	208%	F433T	796	186%	I309Q	582	164%
F024H	96	207%	D030M	122	185%	A419I	762	164%
D068H	193	206%	K152R	394	185%	E148K	371	163%
E031A	125	205%	N328I	642	185%	D163E	407	163%
E151K	384	205%	T293Q	565	183%	L263R	508	163%
P436K	799	205%	D431K	783	183%	T269A	510	163%
T147V	369	204%	D030R	123	182%	Q276S	522	163%
P257T	497	204%	A419R	764	181%	V277R	531	163%
S315H	602	204%	I067V	192	180%	G291V	551	163%
N141H	344	203%	T074S	250	180%	F433R	795	163%
D030G	118	202%	D163R	410	180%	F433V	797	163%
E151Q	387	201%	G377S	697	180%	P443A	826	163%
V247I	493	201%	T325D	626	178%	D030S	124	162%
N328L	641	201%	D087C	277	177%	G305E	569	162%
V075F	253	200%	S215M	465	177%	E151R	388	161%
A048H	165	199%	T440R	819	177%	Q155G	398	161%
G198D	448	199%	L317I	605	176%	V272T	514	161%
I309E	576	199%	A371K	673	176%	M310S	590	161%
P443M	830	199%	V206K	455	175%	I353V	652	161%
S261N	506	198%	L278K	538	175%	P032G	140	160%
S395R	725	198%	D320H	618	175%	V073L	233	159%
D421H	768	198%	A425K	775	175%	E135Q		159%
S069C	197	197%	V166H	422	174%	L033W	142	158%
A092C	293	197%	L278N	539	174%	F446A	844	158%
A120Y	322	195%	K260Y	503	173%	Y447N	851	158%
E156D	401	195%	Q283S	546	173%	S039T	155	157%
N205E	451	194%	S308G	572	173%	Q155K	399	157%
Q276Y	523	194%	I309L	579	172%	V206L	456	157%
V277T	533	194%	S407Q	746	171%	E439V	814	157%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
I445M	836	157%	T293D	560	146%	Y447V	855	138%
D213M	462	156%	Y447P	852	146%	G050D	169	137%
N141Y	348	155%	N219K	468	145%	S221Q	474	137%
V058L	179	154%	E220L	472	145%	Y063K	187	136%
T071S	226	154%	A371S	676	145%	D090K	290	136%
V073H	231	154%	L026Q	102	144%	T150Y	381	136%
A259P	501	154%	I079L	258	144%	L174N	436	136%
E392M	717	154%	E114A	315	144%	V272D	513	136%
E410T	750	154%	E148Q	372	144%	L318K	616	136%
D421Q	771	154%	D258N	499	144%	F433K	793	136%
F029I	109	153%	D258S	500	144%	Y447E	848	136%
Y063W	191	153%	I309G	577	144%	Y447I	850	136%
K093L	296	153%	Y399V	733	144%	A160S	404	135%
A120V	321	153%	A419Y	766	144%	E220V	473	135%
V142R	355	153%	L026M	101	143%	T293K	562	135%
D213N	463	153%	L026R	103	143%	G377D	691	135%
K093R	298	152%	T074F	241	143%	I445H	835	135%
D163A	406	152%	T074L	245	143%	F029K	110	134%
D212N	459	152%	L175T	439	143%	V073T	236	134%
S407D	742	152%	Q373M	677	143%	A149V	374	134%
D431V	789	152%	D418V	759	143%	E151H	383	134%
E031P	132	151%	V073K	232	142%	D163S	411	134%
V075Q		151%	K130R	329	142%	E220I	471	134%
V277K	527	151%	L374H	678	142%	A267T	509	134%
L278Y	543	151%	L406F	739	142%	S308R	575	134%
A371R	675	151%	S407F	744	142%	M313A	591	134%
V012T	89	150%	T049R	168	141%	L318R	617	134%
L174R	437	150%	T071G	223	141%	D368A	662	134%
Q233R	479	150%	V102S	311	141%	S407H	745	134%
M310A	587	150%	F273H	515	141%	Y063L	188	133%
I326L	636	150%	Y447F		141%	A120I	319	133%
G377K	694	150%	Y063R	190	140%	L278G	536	133%
F380Y	700	150%	T074H	243	140%	S308K	573	133%
N047T	162	149%	N131I	331	140%	N369H	669	133%
D090R	292	149%	N141R	345	140%	D030L	121	132%
Q155D	397	149%	V142K	351	140%	N047F	161	132%
T293Y	567	149%	D163Q	409	140%	G052S	175	132%
E375K	682	149%	E220H	470	140%	Y063M	189	132%
G377H	693	149%	F446W	846	140%	M107L	314	132%
Y399E		149%	L001K	77	139%	E439Q	812	132%
S069E	198	148%	G072K	227	139%	T097D	306	131%
D090A	287	148%	H179K	442	139%	D127E	323	131%
I041T	157	147%	V073A	230	138%	Q139T	342	131%
L146R	363	147%	T074E	240	138%	E151W	392	131%
I309M	580	147%	E151V	391	138%	E151Y	393	131%
E441A	821	147%	P257R	498	138%	L165S	417	131%
L037M	150	146%	S407E	743	138%	D368K	664	131%
L089C	286	146%	E442C	823	138%	D368R	667	131%
V237Q	486	146%	I445T	841	138%	R383L	704	131%

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Coloration of Percent (%) Activity Values
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< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
D418E	755	131%	S312N		126%	A419H	761	121%
F446M	845	131%	N321S	622	126%	I445S	840	121%
L026A	98	130%	Q393A		126%	T147A	364	120%
Q143K	359	130%	I445Q	839	126%	K152T	395	120%
T147L	366	130%	K093S	299	125%	G172A	432	120%
N205A	450	130%	T097A	304	125%	L174A		120%
V206R	457	130%	N104R	313	125%	Q276H	519	120%
Q276R	521	130%	A120L		125%	L317M		120%
E285H	547	130%	V128I	328	125%	A371H	672	120%
I309H	578	130%	T150W	380	125%	Y399R		120%
R383K	703	130%	E158Q	402	125%	A425G	774	120%
D431N	785	130%	N321D		125%	E439A	808	120%
F038Y	151	129%	N328C	638	125%	E439K	810	120%
T074K	244	129%	V428L	778	125%	D030P		119%
A092L	294	129%	P443N	831	125%	T097E	307	119%
T147Q		129%	I445V	842	125%	V142T	357	119%
N219A	466	129%	F029R	114	124%	S261R		119%
I271G		129%	G050S	170	124%	D320S		119%
N321K		129%	D090H	289	124%	P436D		119%
A048K	166	128%	N131Q	333	124%	P436G		119%
Q086N	273	128%	T147F	365	124%	P443L		119%
D087R	281	128%	E148H	370	124%	Q444E	832	119%
R132N	336	128%	A149T	373	124%	F024T		118%
E151S	389	128%	T150D	376	124%	G027E		118%
V166E	420	128%	T325V	635	124%	Q086E		118%
T274F	517	128%	I445N	837	124%	D090N	291	118%
E292V	557	128%	F164V	413	123%	D163L		118%
D368E	663	128%	L165F	415	123%	K196T		118%
G377R	696	128%	M313H	593	123%	V277G		118%
D418L	756	128%	N321R	621	123%	Q283P		118%
D421A	767	128%	Q393F	721	123%	L388Y		118%
F433W	798	128%	F024R	97	122%	Y399Q		118%
F446H		128%	G027D	104	122%	D418R	758	118%
E031G	127	127%	K094Q	302	122%	F446D		118%
D059N	186	127%	T097L	309	122%	V008I		117%
T147S	368	127%	L165V	418	122%	P032R		117%
E151A	382	127%	G378K		122%	D034R		117%
Q155R	400	127%	T417I		122%	V073G		117%
E292K	555	127%	E439G		122%	T097Q		117%
L317A	604	127%	T440A		122%	E167S		117%
E392V	720	127%	T071N	224	121%	W304I		117%
D431E	781	127%	Q086W	276	121%	E375T		117%
E031W	137	126%	T097S	310	121%	E389H		117%
I070Y	222	126%	A120S	320	121%	T440S	820	117%
V102T	312	126%	D127Q	326	121%	N047H		116%
E151M	386	126%	T150A	375	121%	V075M	255	116%
V166W	427	126%	E151T	390	121%	V128K		116%
L278F	535	126%	D284E		121%	N141Q		116%
E292H	554	126%	K411S		121%	V166C		116%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
I309S	584	116%	A120H	317	111%	A160Y		107%
N328V		116%	D127T		111%	I208V		107%
S407A	741	116%	L136W		111%	P236A		107%
E439P	811	116%	V142H		111%	P257I		107%
F024V		115%	V166G		111%	P443E	827	107%
D090E	288	115%	M313P	595	111%	Y447W		107%
D090L		115%	L374M		111%	S039A	152	106%
D090S		115%	P437H		111%	A048S		106%
E135K		115%	T440Y		111%	Q086M		106%
N141G		115%	S039Q		110%	F098H		106%
E148S		115%	D090I		110%	E151G		106%
A149C		115%	T097N		110%	L174Y		106%
A149Q		115%	N178R	441	110%	H179A		106%
D163G		115%	N219R		110%	T381K		106%
V277N	529	115%	N231T		110%	F433I	792	106%
S308H		115%	K279R		110%	I445L		106%
E324D		115%	K314H		110%	F446I		106%
Q393S		115%	S395A	723	110%	N002T		105%
E439T	813	115%	F446E		110%	F029G		105%
K094T		114%	R004V		109%	A048Q		105%
L165W		114%	S036R		109%	I083G	264	105%
I208R		114%	V058H	183	109%	D087Q		105%
Q276M	520	114%	T071L		109%	R132K		105%
V351A		114%	A092V		109%	L136M		105%
E389A	709	114%	T118L		109%	T147G		105%
E031Y		113%	N141D		109%	E148G		105%
E135S		113%	T147P		109%	T150E		105%
D163T		113%	P236G		109%	I271V		105%
F164L		113%	V277H	526	109%	Q276E		105%
T240K		113%	D418M		109%	E292P		105%
L278A		113%	Y447L		109%	M313L		105%
W304G		113%	I009L		108%	L317H		105%
N328Q		113%	I046L	158	108%	S359E	655	105%
G027Q		112%	D068K		108%	A371E		105%
D030A		112%	W119Y		108%	Q373V		105%
N141A		112%	I134V		108%	R383V		105%
L174T	438	112%	A149S		108%	K385S		105%
V237L		112%	Y242F		108%	Q393H		105%
M313Y	598	112%	L307T		108%	D418Q		105%
K385A	706	112%	E389S	714	108%	D431I		105%
V414I		112%	K435A		108%	K435H		105%
P443G	829	112%	M438N	806	108%	P032K		104%
Q444V	834	112%	T440M	817	108%	I046A		104%
F446G		112%	P443H		108%	I134L		104%
Y447R		112%	I445R		108%	V142Q	354	104%
G027H		111%	V058Y	185	107%	S145P		104%
P032Q		111%	Y063H		107%	T147Y		104%
F040W		111%	Y063T		107%	K162M		104%
D090Q		111%	I083Q	262	107%	D213Q		104%

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
S221V		104%	E324N	623	101%	R383T		98%
S315R		104%	E392Q	718	101%	L388I		98%
S407R		104%	Y399A		101%	L406C		98%
V420I		104%	Y399S		101%	I445G		98%
T440I		104%	K435R		101%	E023D		97%
P443R		104%	Q444N		101%	L026H		97%
Y063I		103%	L001V		100%	Q086K	272	97%
V102K		103%	I046M		100%	Q086V		97%
V102Q		103%	Y063S		100%	K093G		97%
N104S		103%	I067F		100%	D127G		97%
A120G		103%	D087A		100%	S145R		97%
E151L	385	103%	D087G	278	100%	T147W		97%
Q155H		103%	L136H		100%	E148R		97%
G172C		103%	E151C		100%	Q155M		97%
P197M		103%	S235E		100%	N219H		97%
V237S		103%	T293G		100%	S315T		97%
L278E	534	103%	T293L		100%	K411R		97%
F290M		103%	G305D		100%	D418Y		97%
L317S	610	103%	D320I		100%	F433A	790	97%
G378R		103%	E392G		100%	T440L		97%
D426P		103%	L406G		100%	Q444D		97%
K435S		103%	P436R		100%	Q444H	833	97%
T440D	815	103%	E439F		100%	I445A		97%
Q444K		103%	T440E		100%	F024K		96%
Q086G		102%	L001T		99%	Y063N		96%
D090T		102%	I083V	261	99%	V073Q	234	96%
V102N		102%	D087S	282	99%	K082N	260	96%
N131T		102%	K094N		99%	S084H		96%
L144T	361	102%	L105V		99%	L136F		96%
A149R		102%	N131M	332	99%	Q140A		96%
E167R		102%	E135A		99%	H179N		96%
N219M		102%	E148V		99%	H179R		96%
A238Q		102%	G161A		99%	V272S		96%
V277E	525	102%	L174Q		99%	D368H		96%
T306S		102%	P197K		99%	S395K		96%
K411N		102%	S215K		99%	P437Q		96%
E439S		102%	D284S		99%	P443Q		96%
T440V		102%	E285Y		99%	L001A	74	95%
P032S		101%	E292N		99%	I083T		95%
P032Y		101%	M313V		99%	T097R		95%
I046V		101%	K411V		99%	V128L		95%
I079V		101%	D418I		99%	Q139M		95%
K082I		101%	M438R		99%	E148Y		95%
V137I		101%	P443S		99%	E151N		95%
K162W		101%	I009S		98%	E158S	403	95%
I208T		101%	S084D		98%	N219E		95%
V237T	487	101%	V142G	350	98%	S235G		95%
T269S		101%	V142N	352	98%	D284Q		95%
N321A		101%	I309K		98%	K314R		95%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
I326S		95%	S084L		92%	Y447M		90%
I326K		95%	Q086L		92%	L001C		89%
K376A		95%	Q139A		92%	P006Q		89%
F433D		95%	T150S	379	92%	P010Q		89%
F433P		95%	K159Q		92%	G052N	172	89%
G027S		94%	D213R		92%	T071M		89%
V073D		94%	P257K		92%	S084N	268	89%
K082A		94%	S308D	571	92%	K091A		89%
K094S		94%	N321H		92%	D127A		89%
V102R		94%	V351S	651	92%	A160R		89%
L105I		94%	L388F		92%	E167H		89%
Q139E		94%	E392Y		92%	L263M		89%
S145G		94%	D418A		92%	T293S		89%
L175V		94%	P436S		92%	T381V		89%
H179C		94%	Y447G	849	92%	E396T		89%
S221T		94%	T054Q		91%	L434I		89%
V277M	528	94%	M107F		91%	F446R		89%
I331V		94%	T150N	378	91%	F446T		89%
A371V		94%	Q155A		91%	L001W		88%
K385R		94%	Q155W		91%	Q086D		88%
E389R		94%	A160K		91%	V102H		88%
T405S		94%	E167K		91%	N104K		88%
S407P	747	94%	I208K		91%	V142P	353	88%
D413N		94%	S215H	464	91%	A149L		88%
D421M		94%	E220T		91%	A149M		88%
F446K		94%	P236S		91%	T150P		88%
V2061	454	94%	T240R		91%	K159R		88%
G050A		93%	K255Q		91%	N178M		88%
T071H		93%	E389G	710	91%	W181M		88%
I083L		93%	Q393L		91%	W181Q		88%
Q086R		93%	D418N		91%	I208M		88%
K094D	301	93%	E442P		91%	S215G		88%
Q140K	343	93%	F446V		91%	P257V		88%
V142S	356	93%	P013T		90%	Q276C		88%
L146P	362	93%	F024Y		90%	I301A		88%
A153I		93%	F029A		90%	I301V		88%
D212S	460	93%	V058P	180	90%	I309W		88%
L278I		93%	S084I		90%	M313S		88%
L317T	611	93%	V102E		90%	N328H		88%
Q349K		93%	L105Q		90%	K376E	685	88%
D368S		93%	T150R		90%	E439L		88%
T387S		93%	H179I		90%	T440P	818	88%
L406Q		93%	G182L		90%	L001N		87%
Q444G		93%	V237A		90%	L026T		87%
F446L		93%	P257M		90%	L033P		87%
L001P		92%	F273Y	516	90%	M035F		87%
P032A		92%	E292F	553	90%	K082L	259	87%
F040L	156	92%	E375G	681	90%	F098M		87%
T049S		92%	L391C		90%	V142E		87%

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
E156M		87%	N205T	453	85%	K260R		83%
E167M		87%	D213A	461	85%	V277S	532	83%
L230I		87%	A259E		85%	E292W		83%
K260H		87%	K260M	502	85%	D320G		83%
Q373S		87%	S315A	601	85%	Q349E		83%
T381R		87%	E410Q		85%	P367C		83%
T381S	701	87%	D416T		85%	L374R		83%
R383S	705	87%	D426G		85%	L406T		83%
L406V		87%	D431Y		85%	D416Q		83%
M438D	803	87%	P437R		85%	F433H	791	83%
E441G		87%	M438Q		85%	P437K		83%
P443T		87%	M438V		85%	M438G		83%
G050Q		86%	T440F		85%	M438S		83%
G050R		86%	Q444R		85%	F029H		82%
T071A		86%	N002Q		84%	P032L		82%
V073S		86%	I009V		84%	I046H		82%
S084Q		86%	D030F		84%	N047D	160	82%
S084W		86%	D030Q		84%	N047G		82%
D087E		86%	N047R		84%	I083N		82%
A092M		86%	V073C		84%	S084T		82%
K154R		86%	K093Q	297	84%	A120R		82%
Q155L		86%	L105P		84%	N131L		82%
G161D		86%	D127L		84%	E135L		82%
H179G		86%	Q140H		84%	L146I		82%
K279T		86%	L146K		84%	E148T		82%
L307S		86%	L146Q		84%	K152V		82%
D320Y		86%	L146V		84%	K173R	433	82%
A412N		86%	E156Q		84%	H193Q	443	82%
A412S		86%	E167A		84%	D213K		82%
F433S		86%	G198H		84%	N219T		82%
P436Q		86%	I208L		84%	P257A		82%
M438L	805	86%	D258H		84%	V277Q	530	82%
T440G		86%	K279Q		84%	K314A		82%
F024M		85%	R311S		84%	N356H	653	82%
F029T	116	85%	L317W	612	84%	S401A	735	82%
N047S		85%	Q373L		84%	V428P		82%
T049K		85%	T381Q		84%	F433E		82%
K082R		85%	K409R		84%	E441L		82%
Q086S	274	85%	P436I		84%	E441N		82%
T097I		85%	M035T		83%	L026G		81%
N131Y		85%	S036K		83%	P032V		81%
Q140R		85%	A048G		83%	A048Y		81%
T147I		85%	V058S		83%	D068S		81%
E148L		85%	K093E		83%	V075N		81%
L174S		85%	V102A		83%	D090W		81%
N178G		85%	N141W	347	83%	F098W		81%
N178K	440	85%	K154T		83%	A120N		81%
K195R		85%	E167N		83%	Q139S		81%
N205D		85%	V247L		83%	N141F		81%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
V142I		81%	S215Q		79%	P006L		76%
P197A		81%	T222V		79%	G027L		76%
G198Y		81%	V277D		79%	G027W		76%
D213G		81%	Y288L		79%	K028V		76%
Q373E		81%	D320M		79%	I041G		76%
E375N		81%	E324H		79%	I046Y		76%
E410S		81%	N356D		79%	A048M		76%
A412I		81%	L388M		79%	V075S		76%
D418F		81%	E441S		79%	K082Q		76%
D421E		81%	N002S		78%	V102W		76%
M438P		81%	P006A	80	78%	A120D		76%
E441Q		81%	L026S		78%	N131S		76%
P006K		80%	K028A		78%	R132L	337	76%
S039L	153	80%	M035Y		78%	V142M		76%
N131H		80%	I041D		78%	Q155T		76%
L136S		80%	G050E		78%	D163V		76%
Q140M		80%	R132Y		78%	G198S		76%
L146Y		80%	L146H		78%	N205K		76%
G198A		80%	L174W		78%	D212T		76%
G198N		80%	D320E		78%	T232S		76%
N205S		80%	Q347A	646	78%	V253I		76%
S215T		80%	Q347E		78%	A259K		76%
T222N		80%	D368M	666	78%	S261Q		76%
G291Q		80%	K376D	684	78%	L263A		76%
W357A		80%	L388V		78%	T269D		76%
D368T	668	80%	D418S		78%	T306D		76%
L374I		80%	K435E		78%	L307N		76%
K376S		80%	P032T		77%	L406N	740	76%
E392L		80%	I041H		77%	D416K		76%
Q393K		80%	N047M		77%	E442H		76%
Q393M	722	80%	S084M		77%	V012S		75%
A412G		80%	V102M		77%	F024G		75%
D421T		80%	D127S		77%	K028F		75%
E441K		80%	E135C		77%	D068T		75%
G027A		79%	Q139V		77%	T097F		75%
I079T		79%	Q143E		77%	E114H		75%
I083S	263	79%	Q155S		77%	Q140I		75%
K094E		79%	A160M		77%	V142L		75%
T118V		79%	G161S		77%	K152M		75%
R132S		79%	K162D		77%	A160G		75%
E135H		79%	I208Q		77%	P197G		75%
Q139R		79%	T240V		77%	N205P		75%
L144W		79%	I326Y		77%	D213H		75%
T147M		79%	E375S		77%	N219D		75%
E156A		79%	R383N		77%	E220A		75%
A160F		79%	L388W		77%	T222K		75%
L174M		79%	E389Y		77%	T222R		75%
N205G		79%	S395G		77%	V237H	485	75%
D213E		79%	A412P		77%	A238D		75%

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
T240Q	492	75%	A160V		73%	K093D		71%
L263V		75%	L171I		73%	K093N		71%
T269C		75%	H179L		73%	V102L		71%
T289K		75%	T232F	476	73%	N104T		71%
S312T		75%	S261F		73%	Q138H		71%
E389P		75%	L317N	607	73%	V142D	349	71%
T405Q		75%	Q349R	650	73%	L146S		71%
S407G		75%	Q373K		73%	T147D		71%
D416L	754	75%	K385Q	707	73%	T150F		71%
M438A	802	75%	E396Q	728	73%	K195Q		71%
F446C		75%	T405W		73%	S215R		71%
L001S		74%	L406E		73%	Q233A		71%
G050H		74%	E410P		73%	P236K		71%
T097Y		74%	D416N		73%	V237R		71%
V128R		74%	D421L		73%	K255R		71%
L136R		74%	K435T		73%	E292C	552	71%
L146T		74%	E442K		73%	L318Q		71%
K159H		74%	L001R	79	72%	L318S		71%
I169V		74%	D068E		72%	R383H		71%
K195N	445	74%	V073R	235	72%	R383I	702	71%
S215A		74%	S084F	266	72%	F024I		70%
S215E		74%	D087H		72%	P032N		70%
T240S		74%	K094H		72%	T054S		70%
S261A	504	74%	F098R		72%	D068R		70%
V272R		74%	W119Q		72%	A092T		70%
K379R		74%	L136T		72%	K093M		70%
Q393R		74%	N141S	346	72%	R132E		70%
T405K		74%	K162Y		72%	Q139L		70%
L406Y		74%	F164W		72%	T150C		70%
S407M		74%	K170N		72%	T150L		70%
A412D		74%	V206S		72%	Q155F		70%
P436E		74%	N219L		72%	Y183L		70%
E439H		74%	S221A		72%	P197S		70%
E442Q		74%	A259R		72%	A259Q		70%
R004A		73%	I271F		72%	M310V		70%
P010R		73%	S308T		72%	L318I		70%
I041V		73%	I309D		72%	Q349M	649	70%
I046F		73%	R311K		72%	E389T		70%
L061M		73%	S315G		72%	L406A		70%
E114G		73%	E392T		72%	E410K		70%
R132V		73%	Q393N		72%	A425M		70%
E135F		73%	S407N		72%	P443F	828	70%
L136A		73%	K409S		72%	I009K		69%
Q139K		73%	P436H		72%	N011H		69%
Q140G		73%	M438E	804	72%	F024A		69%
Q143N		73%	F446Q		72%	L033M		69%
E148I		73%	K028R	107	71%	N047Q		69%
Q155V		73%	S036L		71%	R060K		69%
K159N		73%	V075A		71%	G072E		69%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
I096D		69%	V237N		67%	L105R		65%
V102C		69%	R248Y		67%	L136I		65%
N104A		69%	M313T	597	67%	Q138C		65%
E114S		69%	N328A		67%	Q139G		65%
R132Q		69%	E348D		67%	K152I		65%
Q138A		69%	W357C		67%	A160Q		65%
E156T		69%	K376R	687	67%	K196L		65%
E167D	429	69%	F380L		67%	V277A	524	65%
P197Q		69%	E410N		67%	Q283T		65%
S215V		69%	D416G		67%	S308P		65%
Q233L		69%	E441D		67%	S359V		65%
P257L		69%	Q444Y		67%	Q373A		65%
P257N		69%	R004T		66%	E389L	711	65%
R270S		69%	P010E		66%	K409D		65%
K376Q	686	69%	I041W		66%	E410V		65%
E389Q	713	69%	I046N		66%	A412L		65%
A419W		69%	A048R		66%	E441H		65%
F433C		69%	T054V		66%	E442V		65%
1445W	843	69%	D087V	284	66%	F014D		64%
F003H		68%	K094M		66%	S036G		64%
V012I	87	68%	R132H		66%	G042A		64%
P013S		68%	Q140F		66%	I046S		64%
K028T		68%	E148C		66%	A048I		64%
T071Q		68%	K159M		66%	K082G		64%
V073M		68%	L165Y		66%	K094A		64%
I096V		68%	L175Y		66%	K159A		64%
V128C		68%	S235T		66%	K162P		64%
R132A		68%	K260A		66%	L171V		64%
Q138M		68%	K260S		66%	L180M		64%
H193G		68%	S261T		66%	T222L		64%
K196S		68%	V277Y		66%	I256L		64%
K209R	458	68%	E292A		66%	L307W		64%
A259G		68%	N358C		66%	N321T		64%
N328R		68%	S359K		66%	M323F		64%
S342A		68%	L374Y		66%	D368L	665	64%
Q373R		68%	E392K		66%	G378N		64%
K028M		67%	E392R	719	66%	E396D		64%
P032M		67%	A412Y		66%	S404P		64%
I041A		67%	A412R	752	66%	T405P		64%
N047K		67%	D421Y		66%	K409H		64%
K091R		67%	E441T		66%	K435G		64%
V102G		67%	Q444F		66%	P443W		64%
M107I		67%	N002V		65%	Q444W		64%
T118N		67%	V012K	88	65%	L037P		63%
N141E		67%	L015A		65%	N047W	163	63%
K152Y		67%	V058W		65%	Y063A		63%
K159S		67%	G072R		65%	V075Y		63%
A160L		67%	Q086I		65%	L089M		63%
R176K		67%	F098Q		65%	P125R		63%

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
N131F		63%	K385G		62%	Q081P		60%
E135W		63%	Y403F		62%	F098A		60%
H179M		63%	K409E		62%	N131C		60%
F204P	449	63%	E410Y		62%	R132F		60%
T222S		63%	D421G		62%	I134T		60%
A259S		63%	D426E		62%	Q138Y		60%
Q283E		63%	V428T		62%	Q155C		60%
M313E		63%	D431L	784	62%	E167G		60%
S315M		63%	P437D		62%	S215L		60%
L318T		63%	E439W		62%	N219I	467	60%
A338Q		63%	N002A		61%	A238H	489	60%
S359M		63%	G027F		61%	A238K		60%
S404A	737	63%	G027T		61%	K260L		60%
K409T		63%	L033R		61%	P264H		60%
K411T		63%	K093H		61%	D284G		60%
D416I		63%	N104M		61%	L307Y		60%
P436L		63%	L105S		61%	I309V	586	60%
M438C		63%	R132T		61%	L374A		60%
L001G	76	62%	L136Q		61%	L388R		60%
P010D		62%	Q138N		61%	E389M		60%
F024L		62%	Q140V		61%	T405R		60%
K028D		62%	N141L		61%	A432E		60%
D030W		62%	V142C		61%	L434M		60%
L037I		62%	K152F		61%	E442Y		60%
V075H		62%	F157W		61%	F003L		59%
K082S		62%	A160C		61%	P010W		59%
T097P		62%	L224I		61%	D034Q		59%
A120T		62%	P257Q		61%	P044E		59%
K124R		62%	L307Q		61%	L061V		59%
Q143G	358	62%	K314S	599	61%	K094F		59%
L146G		62%	K314T		61%	A120W		59%
E156S		62%	L317D		61%	Q138L	341	59%
K159L		62%	K339M		61%	Q140D		59%
K162H		62%	Q347M		61%	Q140W		59%
L174V		62%	R383M		61%	L165R		59%
H179E		62%	E396R		61%	E167T		59%
L180G		62%	E396S	729	61%	L180F		59%
P197H		62%	L406I		61%	F186Y		59%
I208A		62%	E410R		61%	V206T		59%
I208S		62%	D426N		61%	S221C		59%
S215D		62%	A432S		61%	A238E	488	59%
E220S		62%	P436M		61%	Q283R		59%
T274S		62%	E442T		61%	E285Q		59%
K314Q		62%	R004S		60%	M310Q	589	59%
S315K		62%	F024N		60%	E324A		59%
E324S		62%	M035H		60%	N328T		59%
T325W		62%	A048V		60%	G377E	692	59%
W357T		62%	Q051N		60%	D416V		59%
Q373F		62%	G072D		60%	P436T		59%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
P006H		58%	L214Q		57%	L001E	75	55%
N011K		58%	W218F		57%	P010G	83	55%
K028N		58%	G297A		57%	P010N		55%
D034E		58%	K314N		57%	P010S		55%
G050Y		58%	P367K	660	57%	L026P		55%
S069T	206	58%	A371L	674	57%	K028I		55%
K082M		58%	A371M		57%	I046T		55%
Q086T	275	58%	K379H		57%	V058C	177	55%
D087M	280	58%	D416H		57%	I067Y		55%
F098C		58%	P437S		57%	D087L	279	55%
F098L		58%	M438W		57%	V128Q		55%
R132C		58%	P006R		56%	E167Y		55%
S145A		58%	V012A		56%	K196W		55%
S145N		58%	S039R		56%	G198Q		55%
K162Q		58%	S039Y		56%	A259W		55%
E167P		58%	T054F		56%	M323I		55%
K170V		58%	K082T		56%	M323L		55%
H193Y		58%	F098S		56%	Q347R		55%
K195T	444	58%	P117D		56%	E348G		55%
K196R	447	58%	N122M		56%	P367A	658	55%
P197D		58%	D127V		56%	N369R	670	55%
P197R		58%	R132I		56%	D431G	780	55%
G198R		58%	E135N		56%	N002P		54%
N205M		58%	L136C		56%	R004I		54%
I251L		58%	Q139H		56%	N011D		54%
K260E		58%	Q143L		56%	K028E		54%
K260Q		58%	S145E		56%	P032W		54%
V265I		58%	S145H		56%	D034K		54%
F266Y		58%	S145M		56%	S036H	147	54%
D284A		58%	E158A		56%	V058G		54%
N358T		58%	A160N		56%	Q086F		54%
L374S		58%	K162G		56%	L105A		54%
K376V	689	58%	P197L		56%	P125S		54%
E392A	715	58%	D213L		56%	S133I		54%
E439C	809	58%	T240P		56%	Q138I		54%
Q444I		58%	S261M		56%	E158H		54%
P007M		57%	D284M		56%	K162L		54%
S022H		57%	M313G	592	56%	I169R		54%
D030T		57%	N328G	639	56%	N205R		54%
L033G	143	57%	L374V		56%	S282G		54%
L037V		57%	F380I		56%	R311G		54%
V058I		57%	F394L		56%	K314I		54%
P065R		57%	S395H	724	56%	N369S		54%
K082V		57%	T405A		56%	E375M		54%
I083F		57%	S407V		56%	E392F	716	54%
S084P		57%	A419L		56%	A412W		54%
Q143V		57%	D431W		56%	G427I		54%
L146N		57%	A432V		56%	F433G		54%
L175H		57%	P437A		56%	E441V		54%

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
V008L		53%	R248W		52%	T274A		51%
I009R		53%	K255N		52%	Q276I		51%
L026W		53%	R270N		52%	D284H		51%
I041C		53%	E285G		52%	V287I		51%
R045K		53%	T306E		52%	L318F		51%
G052R	174	53%	R311T		52%	Q343V		51%
D087I		53%	L318N		52%	T381N		51%
T097C	305	53%	D320N		52%	R383A		51%
T097W		53%	L327M		52%	R383E		51%
Y099R		53%	P367S	661	52%	L388T		51%
T118K		53%	A371F	671	52%	K409I		51%
T118M		53%	K379G		52%	A432N		51%
W119F		53%	E392S		52%	P437L		51%
V128A		53%	E396A	726	52%	E441Y		51%
V128S		53%	Y399N		52%	E442G	824	51%
Q138R		53%	D413E		52%	F003Y		50%
Q139F		53%	G427K		52%	V012R		50%
K209A		53%	A432G		52%	A020S	93	50%
Q233F		53%	P013Y		51%	D068P	194	50%
S235K		53%	L026I		51%	T071D		50%
A259L		53%	K028L		51%	K082E		50%
L286R		53%	K028W		51%	K093F		50%
R311G		53%	S036T		51%	V128W		50%
K314D		53%	L037R		51%	K130I		50%
K376T	688	53%	I041E		51%	E135Y		50%
A412V	753	53%	Y066R		51%	Q140C		50%
V414M		53%	G072S		51%	N141V		50%
A419N		53%	L105G		51%	L146E		50%
K435C		53%	L105T		51%	K162A		50%
I445Y		53%	I110V		51%	H192T		50%
L026V		52%	D127R	327	51%	K195I		50%
L026Y		52%	V137T		51%	V206H		50%
M035L		52%	Q140L		51%	K209N		50%
T074M	246	52%	A149K		51%	K209S		50%
S084E	265	52%	K152A		51%	K209T		50%
K094L		52%	A153L		51%	Q233Y		50%
F098I		52%	E156I		51%	N245H		50%
L146A		52%	E158F		51%	D284L		50%
A149G		52%	K162E	405	51%	V287T	548	50%
T150I		52%	H179S		51%	M310R		50%
K152C		52%	K195A		51%	S315Y	603	50%
K159D		52%	N211W		51%	E324M		50%
K162R		52%	L217M		51%	S360T		50%
K162V		52%	P226W		51%	A371I		50%
P197E		52%	I256H		51%	K385H		50%
N205F		52%	I256V		51%	K409G		50%
S215W		52%	P257G	496	51%	D413R		50%
V237W		52%	A259T		51%	K435Y		50%
V247M		52%	A259Y		51%	P437G		50%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
S022M		49%	F273T		48%	V075C		46%
G052T	176	49%	Q276L		48%	I096L		46%
V058N	184	49%	T289S	549	48%	F098V		46%
V128G		49%	L307V		48%	K154V		46%
E156G		49%	R311H		48%	T168H		46%
E156W		49%	T381H		48%	N200D		46%
K159E		49%	T405M		48%	K209E		46%
G161E		49%	K409P		48%	S221M		46%
H192S		49%	K409V		48%	Q233G	477	46%
K195W		49%	K411A		48%	P236H		46%
G198E		49%	V420P		48%	A259I		46%
N205V		49%	V008M	81	47%	R270M		46%
D213W		49%	F014M		47%	L286S		46%
D213Y		49%	G050M	171	47%	V302W		46%
T222G	475	49%	Y066H		47%	L318D	614	46%
A238R		49%	F098D		47%	V334P		46%
A259N		49%	A108G		47%	N356S	654	46%
L263T		49%	T118H		47%	P367R		46%
Y288W		49%	L136D		47%	E375L		46%
S312G		49%	T147C		47%	K376M		46%
L318G		49%	A160H		47%	K379S		46%
Q343T		49%	K162S		47%	K385T		46%
Q349T		49%	V166N		47%	Y399C		46%
D368G		49%	D213V		47%	S401E	736	46%
V422T		49%	S235A	481	47%	D416R		46%
A425R		49%	F280G		47%	A425N		46%
P436Y		49%	E285F		47%	L434V		46%
S022T	94	48%	I303V		47%	N011G		45%
F029W		48%	T335S	645	47%	L015M	92	45%
L033S		48%	Q349A		47%	S022Y		45%
N047A		48%	P367G	659	47%	L033Q		45%
T054N		48%	L374T		47%	L033T		45%
N104G		48%	F380P		47%	S036A		45%
L136N		48%	L388H		47%	N047Y		45%
V137A		48%	E396H	727	47%	T049V		45%
Q138S		48%	L406S		47%	I067L		45%
Q139D		48%	E410D		47%	G072A		45%
N141M		48%	A425S		47%	I083R		45%
S145D		48%	L434G		47%	L089K		45%
G161R		48%	N002L		46%	N141T		45%
L165A		48%	V012N		46%	E156V		45%
P197F		48%	P013H		46%	K195G		45%
G198L		48%	F014V	90	46%	K195H		45%
I208C		48%	G027P		46%	K195L		45%
W216Y		48%	K028S		46%	D258R		45%
N219W		48%	D030V		46%	A267M		45%
T240A	491	48%	D034W	144	46%	G291R		45%
T240M		48%	Q051S		46%	V302I		45%
S261V		48%	G072H		46%	K314Y	600	45%

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
L318H	615	45%	P125H		43%	S401N		42%
S359D		45%	E148W		43%	E410M		42%
Q393D		45%	E156L		43%	K411P		42%
D418G		45%	E156R		43%	D413K		42%
D418G		45%	A160I		43%	K415S		42%
A419E		45%	L175E		43%	K415W		42%
R0451		45%	H179T		43%	A419G		42%
N002G		44%	K196E	446	43%	V422I		42%
A005H		44%	V206M		43%	D426Q		42%
V012L		44%	N219C		43%	G427S		42%
K082H		44%	T222D		43%	V428M		42%
F098E		44%	T222F		43%	P437Y		42%
E114M		44%	R248A	494	43%	L001F		41%
D127W		44%	I251M		43%	G027I		41%
N131E		44%	K255S		43%	D034H		41%
Q139C		44%	P264A		43%	G050C		41%
S145C		44%	F280Q		43%	D090G		41%
S145L		44%	E285M		43%	N104C		41%
E158L		44%	A298G	568	43%	Q138W		41%
G161C		44%	L307K		43%	Q140Y		41%
K173N		44%	R311Q		43%	K159V		41%
H179P		44%	L374N		43%	K196G		41%
L180K		44%	L374P	679	43%	P197T		41%
K209G		44%	E375R	683	43%	G198T		41%
S235H		44%	K385V	708	43%	N205W		41%
A238T		44%	D426Y		43%	N211L		41%
S261W		44%	P436W		43%	V237F		41%
L278V		44%	F003E		42%	A259V		41%
K279H	544	44%	F014I		42%	K260D		41%
E348S		44%	T049I		42%	V277C		41%
Q349N		44%	L061I		42%	S282D		41%
S359H	656	44%	L085V		42%	S282Q		41%
T405Y		44%	Q086P		42%	F290I		41%
A425I		44%	A120P		42%	G291S	550	41%
K435V		44%	L146C		42%	E292G		41%
Q1431		44%	E148F		42%	V294M		41%
P010H	84	43%	G161V		42%	A298I		41%
L037K		43%	G161V		42%	S315E		41%
S043T		43%	H179V		42%	C316D		41%
I046E		43%	H193S		42%	N356A		41%
G050L		43%	K195S		42%	W357S		41%
G052P		43%	I256A		42%	N358G		41%
T054A		43%	I271S		42%	D368V		41%
Y063V		43%	E292R	556	42%	K385N		41%
D068L		43%	G300R		42%	Q393T		41%
G072L		43%	S315L		42%	F394W		41%
K091Q		43%	I353T		42%	S407W		41%
Y099S		43%	E375A	680	42%	D413T		41%
G106V		43%	K376Y	690	42%	D416F		41%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
L434F		41%	V334T		39%	D284Y		37%
N002C		40%	Q347L		39%	D320L		37%
P006N		40%	F380V		39%	D361H		37%
I009Q	82	40%	F380T		39%	K376L		37%
K028P		40%	S395T		39%	S404T		37%
P032C	138	40%	E396L		39%	Q444M		37%
I041N		40%	S401Q		39%	L037W		36%
D059Q		40%	A412H		39%	Q051R		36%
I083H		40%	D413S		39%	L105H		36%
D127M		40%	K415V		39%	W119P		36%
K170A		40%	K415Y		39%	P125A		36%
K170Q		40%	V421I		39%	V137S		36%
L174G	434	40%	A425Y		39%	P177V		36%
R176L		40%	G427Q	776	39%	K195V		36%
S202M		40%	P436C		39%	E220M		36%
N205Y		40%	D034A		38%	P257C		36%
K209F		40%	S036N	148	38%	L263H		36%
T222I		40%	L089P		38%	Q276G		36%
Q234L		40%	K093P		38%	G305N		36%
R248H		40%	K154I		38%	V351I		36%
K255A		40%	K159G		38%	W357K		36%
R270T		40%	D163W		38%	E375I		36%
D275V		40%	L174H		38%	T405F		36%
D284N		40%	L180I		38%	V414L		36%
E285N		40%	H193F		38%	D426S		36%
D320W		40%	K209L		38%	G072Y		35%
N321Y		40%	W218M		38%	N104I		35%
K379T		40%	L286W		38%	K124H		35%
S395W		40%	M310Y		38%	E156C		35%
S407L		40%	S312K		38%	S221I		35%
K415G		40%	S312L		38%	V237C	483	35%
D416Y		40%	N358L		38%	V287N		35%
E442L		40%	A371G		38%	D320V		35%
N011S	85	39%	S401G		38%	V351C		35%
D103N		39%	A413Q		38%	T381E		35%
A160W		39%	D413A		38%	F398L		35%
D163F		39%	E442W		38%	A412Q	751	35%
Y184W		39%	N002I		37%	G427H		35%
K196N		39%	M035Q		37%	G427T	777	35%
K196Y		39%	M035V	146	37%	S043N		34%
P197W		39%	M035Q	145	37%	Q051A		34%
F204W		39%	I046C		37%	L089R		34%
N205L	452	39%	D068G		37%	L105W		34%
E220D		39%	V137C		37%	K124L		34%
K255G		39%	K152W	396	37%	A153S		34%
D258G		39%	E158G		37%	E285A		34%
V272E		39%	N200T		37%	K297R		34%
D284T		39%	I251Y		37%	I303D		34%
I326C		39%	K260G		37%	I331E		34%

KEY
Coloration of Percent (%) Activity Values
> 200%
120% - 200%
80% - 119%
40% - 79%
< 40%

Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity	Mutant	SEQ ID	Activity
V351Q		34%						
L374W		34%						
A432H		34%						
V008P		33%						
K094C		33%						
Y099A		33%						
L105C		33%						
H193R		33%						
V206Q		33%						
N328W		33%						
K411H		33%						
S036D		32%						
G077H		32%						
G077K		32%						
K082Y		32%						
K173Q		32%						
Y183E		32%						
A239N		32%						
L307G	570	32%						
D087P		31%						
E167F		31%						
V272M		31%						
E392W		31%						
D413H		31%						
G050V		30%						
L061F		30%						
I083K		30%						
S084Y		30%						
M310F		30%						
V414K		30%						
W181K		29%						
G198W		29%						
Q143C		28%						
W218V		28%						
L165C		27%						
K279A		27%						
G291C		27%						
I331C		27%						
L089W		26%						
L144R	360	26%						
D426K		26%						
I067R		24%						
K093V		24%						
D275L		24%						
A092H		22%						
V137L		21%						

APPENDIX A-6

List of Active Mutant Activity Data Under Temperature and Phenophilic Conditions

Source(s) – Table 12

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
L001A	117.908	8.13	9.59	127.997	9.179	11.75
L001E	107.231	13.14	14.09	125.207	10.727	13.43
L001G	171.264	9.23	15.8	115.952	4.586	5.32
L001Q	119.435	10.13	12.09	88.763	11.121	9.87
L001R	117.366	6.96	8.17	160.41	5.623	9.02
P006A	137.875	9.88	13.63	108.946	12.446	13.56
V008M	134.884	0	0	119.772	0.477	0.57
I009Q	104.922	6.61	6.94	124.934	6.303	7.87
P010G	109.772	15	16.47	121.986	16.57	20.21
P010H	131.924	0	0	112.99	0	0
N011S	152.32	7.16	10.9	131.289	5.067	6.65
V012E	48.208	14.18	6.83	100.163	16.896	16.92
V012I	128.745	2.94	3.78	170.812	1.371	2.34
V012K	146.6	13.31	19.52	114.264	14.311	16.35
F014V	154.167	1.35	2.08	146.667	0	0
L015M	113.747	1.66	1.88	83.97	1.887	1.58
A020S	188.889	14.64	27.64	118.821	15.153	18
S022T	111.203	20.27	22.54	86.404	22.854	19.75
L026M	136.775	11.05	15.11	138.989	10.446	14.52
K028R	110.487	10.74	11.86	109.467	10.27	11.24
F029R	154.644	7.58	11.72	121.707	7.613	9.27
F029S	118.119	7.01	8.28	97.4	8.037	7.83
F029T	126.74	11.96	15.16	120.619	10.266	12.38
P032C	128.649	1.26	1.62	127.446	7.491	9.55
L033G	121.201	0.15	0.18	89.571	4.147	3.71
D034W	146.765	15.23	22.35	146.729	14.65	21.5
M035V	81.285	16.09	13.08	102.034	3.528	3.6
S036H	106.222	9.93	10.55	150.931	2.291	3.46
S036N	112.045	19.15	21.46	92.069	30.268	27.87
L037M	79.268	10.77	8.54	87.376	9.065	7.92
F040L	135.036	7.88	10.64	105.252	8.703	9.16
I046L	132.507	12.79	16.95	112.944	16.667	18.82
N047D	115.797	1.24	1.44	111.869	0.796	0.89

KEY
Coloration of Percent (%) Activity Values
nc
>120
between 100 and 120
between 80 and 100
between 40 and 80
between 20 and 40
between 10 and 20
between 0 and 10

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
N047W	104.703	0	0	109.88	3.728	4.1
A048N	114.954	5.59	6.43	51.931	3.778	1.96
T049R	122.704	5.81	7.13	90.76	5.363	4.87
G050D	93.824	7.85	7.36	95.934	8.742	8.39
G050M	157.686	8.99	14.18	139.048	12.115	16.85
G052N	96.148	15.98	15.37	142.502	7.748	11.04
G052T	116.407	21.23	24.71	117.075	32.31	37.83
G052S	98.513	23.49	23.14	98.199	28.833	28.31
V058C	92.507	16.05	14.85	99.162	16.141	16.01
V058K	217.914	38.66	84.24	217.914	38.655	84.24
V058R	96.905	56.55	54.8	102.858	65.305	67.17
V058N	129.167	12.9	16.67	129.787	11.475	14.89
V058Y	102.981	36.23	37.31	141.299	41.728	58.96
V058Q	154.383	8.49	13.11	293.51	4.804	14.1
V058P	83.304	21.15	17.62	173.652	17.262	29.98
V058H	200.264	10.88	21.79	181.75	13.067	23.75
D068P	99.07	0.47	0.47	83.721	102.222	85.58
S069T	138.609	10.82	15	122.579	8.985	11.01
I070P	101.713	0.77	0.78	99.749	2.014	2.01
I070V	170.462	13.97	23.82	136.849	10.885	14.9
V073Q	121.337	6.51	7.9	107.094	8.186	8.77
V073R	137.931	2.5	3.45	100.125	7.338	7.35
T074E	133.241	17.22	22.94	100.965	16.172	16.33
T074M	115.29	12.04	13.88	103.629	10.765	11.16
T074N	91.87	10.96	10.06	157.218	6.811	10.71
T074P	108.323	12.24	13.26	166.227	10.008	16.64
T074R	80.681	7.44	6.01	130	2.158	2.8
T074V	115.093	7.4	8.52	114.063	5.479	6.25
V075M	134.46	0.24	0.33	121.527	2.12	2.58
K082L	114.758	20.79	23.86	251.869	10.721	27
K082N	106.059	23.32	24.73	95.104	26.541	25.24
I083V	140.151	29.88	41.88	137.296	28.133	38.63
I083Q	112.163	27.02	30.3	188.798	13.881	26.21

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
I083S	104.637	26.7	27.94	95.351	26.667	25.43
I083G	106.239	22.54	23.95	76.381	32.208	24.6
S084E	124.762	6.27	7.82	113.41	6.833	7.75
S084F	83.291	2.55	2.12	91.007	0	0
S084N	144.922	18.27	26.47	131.144	22.092	28.97
S084R	119.873	10.92	13.09	203.099	4.977	10.11
Q086A	136.516	14.24	19.43	156.132	9.728	15.19
Q086H	102.612	7.14	7.33	129.6	5.015	6.5
Q086K	99.213	25.4	25.2	65.455	31.944	20.91
Q086S	100.435	6.81	6.84	103.218	11.215	11.58
Q086T	93.837	10.24	9.61	179.465	8.9	15.97
D087G	81.742	1.51	1.23	90.579	6.19	5.61
D087L	106.039	14.76	15.65	101.493	12.938	13.13
D087M	110.964	7.61	8.44	87.656	16.438	14.41
D087S	134.031	8.15	10.92	139.728	6.445	9.01
D087V	114.107	9.14	10.43	87.023	15.922	13.86
D090E	92.91	14.26	13.25	161.281	6.221	10.03
D090N	111.06	12.14	13.48	98.631	10.596	10.45
K093Q	91.008	5.82	5.3	95.448	6.646	6.34
K093R	103.617	11.7	12.12	99.301	16.362	16.25
K094D	86.544	6.52	5.64	102.107	9.897	10.11
K094R	125.373	8.96	11.23	108.69	9.905	10.77
T097C	165.152	8.07	13.33	81.715	17.228	14.08
T097D	123.654	8.55	10.58	117.522	10.994	12.92
T097E	127.19	15.57	19.8	115.106	16.143	18.58
T097L	118.465	23.1	27.36	103.589	24.174	25.04
N104R	114.673	9.7	11.12	118.421	8.53	10.1
A120H	94.107	8.28	7.8	113.015	6.903	7.8
D127R	56.439	70.47	39.77	58.702	34.171	20.06
V128I	113.654	10.97	12.47	102.656	14.819	15.21
N131M	177	1.86	3.29	76.888	2.811	2.16
N131R	94.253	21.3	20.07	95.93	19.376	18.59
N131V	137.681	10.22	14.07	104.92	10.907	11.44

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
R132L	98.578	10.34	10.19	91.685	14.498	13.29
Q138L	107.831	25.45	27.44	91.627	22.814	20.9
Q140K	176.6	10.93	19.3	109.815	12.522	13.75
N141R	103.411	4.35	4.5	115.682	2.292	2.65
N141S	131.758	4.66	6.13	109.527	5.529	6.06
N141W	130.644	5.19	6.78	104.783	6.391	6.7
V142D	114.185	4.39	5.02	146.066	2.098	3.06
V142G	117.686	13.21	15.55	90.256	13.51	12.19
V142K	109.485	14.77	16.17	154.599	15.621	24.15
V142N	155.556	15.33	23.84	103.88	14.771	15.34
V142P	166.998	13.91	23.23	97.338	15.397	14.99
V142Q	149.666	8.9	13.32	99.957	9.83	9.83
V142R	149.441	12.38	18.5	103.622	12.272	12.72
V142S	170.778	8.73	14.92	117.035	16.9	19.78
V142T	223.936	11.48	25.7	123.65	11.709	14.48
Q143G	143.6	13.88	19.94	98.837	16.096	15.91
Q143K	200.468	14.32	28.7	136.421	20.747	28.3
L144R	136.247	10.71	14.59	111.482	10.182	11.35
L144T	129.746	14.68	19.05	108.923	11.961	13.03
L146P	116.626	1.15	1.34	115.601	3.429	3.96
T147S	142.175	3.93	5.59	130.287	2.605	3.39
T150N	140.724	6.27	8.82	116.923	6.725	7.86
T150S	107.327	6.4	6.87	142	6.087	8.64
E151A	103.31	12.11	12.51	126.047	11.783	14.85
E151L	132.125	4.9	6.48	121.83	6.264	7.63
E151S	115.423	6.2	7.15	136.397	4.695	6.4
E151T	128.337	0	0	110.3	0	0
E151V	111.531	7.31	8.15	99.647	7.42	7.39
E151W	158.415	1.15	1.83	94.919	0.895	0.85
K152T	149.169	5.57	8.31	136.747	3.558	4.87
K152W	122.313	2.47	3.02	134.039	2.868	3.84
E158S	133.038	0	0	102.519	0	0
K162E	67.857	3.51	2.38	41.026	30	12.31

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
L165F	106.283	11.82	12.57	96.667	14.286	13.81
V166Q	155.975	13.35	20.82	117.99	10.953	12.92
V166T	183.384	12.69	23.26	136.882	13.056	17.87
E167D	136.745	10.01	13.69	162.637	3.784	6.15
I169L	140.177	13.19	18.49	122.272	15.528	18.99
K170R	160.71	8.24	13.24	97.128	10.075	9.79
G172A	167.554	7.51	12.59	133.735	7.207	9.64
K173R	106.771	9.8	10.46	134.3	7.489	10.06
L174G	114.13	12.38	14.13	264.368	13.478	35.63
L174N	154.332	13.27	20.48	126.186	18.907	23.86
L174T	124.819	13.06	16.3	144.876	6.098	8.83
N178K	166.871	5.27	8.8	103.154	8.021	8.27
N178R	199.596	4.08	8.15	144.957	3.943	5.72
H193Q	213.585	15.28	32.64	138.113	18.326	25.31
K195T	126.161	22.48	28.36	237.097	15.28	36.23
K195N	130.253	22.38	29.15	96.381	25.487	24.57
K196E	90.574	36.8	33.33	154.091	23.5	36.21
K196R	106.1	13.22	14.02	95.142	17.663	16.81
F204P	83.571	84.62	70.71	82.418	126	103.85
N205A	139.223	21.34	29.71	102.031	18.735	19.12
N205E	160.93	19.3	31.06	93.313	18.503	17.27
N205L	107.472	10.56	11.35	0		8.55
N205T	145.085	10.05	14.58	110.627	13.054	14.44
V206I	189.274	13.17	24.92	111.22	15.575	17.32
K209R	119.794	11.9	14.26	79.535	3.947	3.14
D212N	112.626	2.66	3	132.249	5.352	7.08
D212S	122.899	8.35	10.27	147.936	6.841	10.12
D213A	183.83	26.85	49.36	154.77	13.699	21.2
D213M	159.255	6.83	10.88	98.365	6.94	6.83
S215H	109.069	10.04	10.95	78.992	5.758	4.55
S215M	174.883	4.2	7.35	74.943	8.957	6.71
N219I	254.438	8.84	22.49	291.2	11.264	32.8
E220V	131.985	7.43	9.81	113.61	5.909	6.71

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
T222G	153.033	0.61	0.94	105.454	0.793	0.84
T232F	132.839	12.43	16.51	62.882	19.59	12.32
Q233G	280.488	0	0	127.368	0	0
Q234M	95.605	22.31	21.33	80.766	20.283	16.38
S235A	129.818	11.06	14.36	120.916	12.026	14.54
V237C	138.042	0	0	116.384	0	0
V237H	122.112	12.43	15.18	145.253	7.407	10.76
V237T	167.105	21.26	35.53	126.02	21.457	27.04
A238E	94.878	8.17	7.76	142.167	6.682	9.5
A238H	59.585	26.09	15.54	204.683	8.345	17.08
T240A	141.283	9.14	12.92	144.667	9.063	13.11
T240Q	162.763	14.76	24.02	120.98	13.776	16.67
R248A	113.237	1.05	1.19	124.65	2.408	3
E249V	142.752	15.29	21.83	111.068	16.462	18.28
P257G	125.22	0.78	0.98	112.803	0.677	0.76
K260M	116.69	8.58	10.01	97.396	7.273	7.08
S261A	57.547	67.52	38.86	86.173	54.021	46.55
S261K	161.931	16.05	26	116.159	22.82	26.51
S261N	142.901	10.46	14.95	35.529	13.403	4.76
A267T	196.154	35.29	69.23	111.579	38.679	43.16
F273H	122.647	6	7.35	119.037	5.973	7.11
F273Y	119.713	7.78	9.32	102.772	9.634	9.9
Q276H	74.908	8.93	6.69	106.393	10.065	10.71
Q276M	98.323	5.64	5.55	104.948	0	0
Q276R	121.431	10.93	13.27	150.18	8.778	13.18
Q276S	110.643	9.95	11.01	138.696	8.745	12.13
V277A	140.765	6.97	9.82	129.58	5.167	6.7
V277E	175.779	3.75	6.6	195.598	5.222	10.21
V277H	129.434	3.16	4.09	137.684	7.014	9.66
V277K	375.721	13.21	49.63	373.799	12.029	44.96
V277M	137.138	15.05	20.64	112.084	14.851	16.65
V277N	89.645	7.29	6.54	273.386	6.762	18.49
V277Q	119.93	5.7	6.83	116.151	7.772	9.03

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
V277R	96.071	15.57	14.96	171.465	9.801	16.81
V277S	66.26	7.65	5.07	144.916	4.731	6.86
V277T	101.01	7.99	8.07	143.311	7.788	11.16
L278E	75.408	5.11	3.85	100.179	7.214	7.23
L278G	122.274	6.5	7.94	104.077	7.887	8.21
K279H	138.964	14.99	20.83	123.183	20.09	24.75
V287T	145.345	16.49	23.97	124.738	12.019	14.99
T289S	104.598	0.98	1.02	98.234	0.699	0.69
G291S	184.581	12.17	22.47	119.565	4.156	4.97
G291V	112.807	19.87	22.42	151.039	12.609	19.05
E292C	127.307	8.07	10.27	101.126	8.905	9.01
E292F	137.93	6.17	8.52	132.34	5.84	7.73
E292H	170.153	8.73	14.85	115.501	11.775	13.6
E292R	112.278	12.61	14.16	129.89	11.983	15.56
E292V	163.075	13.28	21.66	133.274	11.847	15.79
T293A	128.197	3.38	4.33	57.524	4.248	2.44
A298G	212.422	8.77	18.63	86.131	9.322	8.03
L307G	117.857	0	0	91.528	0	0
S308D	127.652	4.15	5.3	105.846	2.907	3.08
S308K	126.882	1.33	1.69	99.169	0	0
S308N	170.413	5.67	9.66	139.083	5.907	8.22
I309E	123.847	16.25	20.12	129.94	14.414	18.73
I309G	102.601	7.37	7.56	114.091	6.458	7.37
I309L	153.681	9.58	14.72	106.948	10.905	11.66
I309M	123.425	8.02	9.9	136.797	7.065	9.66
I309N	111.901	6.98	7.81	97.361	6.47	6.3
I309S	169.951	4.11	6.98	551.493	0.862	4.75
I309T	97.936	7.63	7.47	148.864	5.542	8.25
I309V	113.138	1.5	1.7	138.313	3.47	4.8
M310G	167.656	11.44	19.18	110.739	12.916	14.3
M310Q	107.237	27.81	29.82	106.323	28.254	30.04
M313G	138.095	9.77	13.49	109.231	10.141	11.08
M313H	271.914	3.71	10.09	197.024	3.886	7.66

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
M313K	118.882	0.86	1.02	107.111	0.555	0.59
M313P	103.654	4.98	5.16	132.802	4.516	6
M313R	157.272	4.62	7.27	32.845	8.296	2.72
M313T	162.074	7.04	11.4	99.844	7.007	7
M313Y	120.038	7.52	9.03	103.011	6.846	7.05
K314S	141.924	9.67	13.73	132.112	9.066	11.98
K314Y	243.011	5.75	13.98	138.931	10.989	15.27
S315A	91.372	16.51	15.08	137.153	9.973	13.68
S315H	151.244	12.06	18.24	105.305	10.867	11.44
S315Y	170.968	30.61	52.33	57.827	39.503	22.84
L317A	123.51	6.97	8.6	132.724	8.395	11.14
L317I	187.477	12.72	23.84	110.696	10.67	11.81
L317K	96.199	3.45	3.31	134.204	3.534	4.74
L317N	127.382	12.02	15.31	121.233	14.528	17.61
L317R	238.501	3.87	9.22	99.467	5.673	5.64
L317S	90.929	15.54	14.13	85.81	6.423	5.51
L317T	145.964	6.96	10.16	154.334	1.087	1.68
L317W	163.704	11.92	19.51	147.606	10.27	15.16
L318D	105.543	17.43	18.4	97.97	16.684	16.35
L318H	99.907	4.29	4.29	124.69	7.363	9.18
L318R	160.469	5.63	9.03	120.872	6.21	7.51
N321R	164.842	9.53	15.71	112.18	8.613	9.66
N321S	102.489	8.29	8.49	108.732	4.534	4.93
E324N	104.618	7.72	8.08	131.265	9.124	11.98
T325E	124.837	14.44	18.02	106.457	10.577	11.26
N328G	197.098	4.15	8.18	109.722	7.233	7.94
N328Y	180.981	10.3	18.64	100	10.5	10.5
T335S	107.956	11.57	12.49	125.286	6.288	7.88
Q347A	101.395	10.89	11.04	96.213	11.001	10.58
Q347G	222.459	8.37	18.63	207.054	9.013	18.66
Q349M	99.531	11.98	11.92	108.042	12.33	13.32
Q349R	147.007	11.76	17.29	104.545	13.211	13.81
V351S	130.819	0	0	100.857	0	0

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
I353V	132.334	10.45	13.83	138.025	11.902	16.43
N356H	100	8.54	8.54	130.377	3.912	5.1
N356S	51.908	0	0	125.692	2.516	3.16
S359E	135.589	10.77	14.6	135.104	9.354	12.64
S359H	110.422	0	0	100.809	0	0
P367A	167.03	12.94	21.62	127.366	13.153	16.75
P367G	115.683	0	0	122.642	0	0
P367K	125.884	5.06	6.36	66.884	10.136	6.78
P367S	74.263	14.39	10.69	88.355	16.433	14.52
D368A	121.623	1.45	1.76	81.646	2.111	1.72
D368E	166.628	9.18	15.3	97.937	11.462	11.23
D368L	108.977	0	0	109.364	0.969	1.06
D368M	119.744	2.72	3.25	103.662	2.536	2.63
D368R	164.735	10.16	16.74	118.14	11.805	13.95
D368T	107.122	2.87	3.07	126.693	3.366	4.26
N369R	161.693	6.39	10.34	74.366	6.182	4.6
A371F	180.217	6.19	11.16	76.436	5.578	4.26
A371H	957.055	1.81	17.32	89.541	1.697	1.52
A371H	111.143	0	0	95.589	8.61	8.23
A371K	136.514	12.84	17.53	114.354	12.454	14.24
A371L	695.108	1.51	10.52	107.003	2.215	2.37
A371L	104.327	0	0	60.232	1.205	0.73
A371R	nc	nc	11.03	nc	nc	14.06
A371R	121.162	0	0	97.97	2.587	2.53
A371S	147.672	8.38	12.38	131.555	16.938	22.28
L374P	392.038	5.77	22.63	123.033	7.365	9.06
E375A	88.836	0	0	134.714	2.05	2.76
E375G	126.88	10.32	13.1	139.03	14.673	20.4
E375R	163.18	13.15	21.45	116.431	19.727	22.97
K376D	113.1	12.36	13.97	165.064	5.049	8.33
K376E	100	13.55	13.55	153.016	10.394	15.9
K376Q	125.172	12.75	15.96	90	12.057	10.85
K376R	81.687	31.63	25.84	199.112	10.372	20.65

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
K376T	121.133	14.91	18.06	113.387	5.639	6.39
K376V	124.221	3.19	3.96	118.583	2.547	3.02
K376Y	102.812	9.24	9.5	96.139	12.985	12.48
G377D	110.871	15.72	17.43	132.357	10.55	13.96
G377E	130.445	8.04	10.49	128.402	7.401	9.5
G377H	146.855	8.34	12.25	104.837	10.117	10.61
G377K	185.922	4.42	8.21	119.751	4.989	5.97
G377R	119.708	5.87	7.03	94.749	7.137	6.76
G377S	108.609	6.91	7.51	101.106	7.877	7.96
G377T	112.557	17.14	19.29	109.036	18.279	19.93
F380W	147.077	9.97	14.67	104.881	9.253	9.7
T381S	135.827	13.41	18.21	112.559	10.315	11.61
R383I	527.82	6.33	33.44	98.328	7.522	7.4
R383S	132.894	10.5	13.96	119.466	10.545	12.6
K385A	126.096	4.64	5.85	112.706	0	0
K385Q	137.629	9.03	12.43	124.892	7.512	9.38
K385V	112.581	5.12	5.76	80.571	2.979	2.4
E389A	306.767	2.13	6.53	224.872	1.824	4.1
E389G	113.253	2.13	2.41	139.901	0	0
E389L	143.219	14.24	20.4	112.185	12.609	14.15
E389Q	135.807	11.88	16.14	99.738	12.767	12.73
E389S	165.62	0	0	93.03	0.285	0.27
E392A	112.465	7.27	8.18	155.693	6.376	9.93
E392F	115.619	3.9	4.51	143.781	3.905	5.61
E392Q	112.993	10.53	11.89	93.789	16.705	15.67
E392R	129.528	3.69	4.79	123.407	2.947	3.64
E392V	124.365	7.73	9.61	154.768	6.404	9.91
Q393F	139.966	10.59	14.82	101.647	10.171	10.34
Q393M	139.696	1.6	2.24	86.966	3.086	2.68
S395A	208.246	12.98	27.04	112.714	12.395	13.97
S395H	159.975	12.55	20.07	113.401	10.452	11.85
E396A	131.894	8.42	11.1	128.716	9.777	12.58
E396H	210.364	9.19	19.33	128.571	3.216	4.14

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
E396Q	122.977	10.06	12.37	95.938	10.263	9.85
E396S	156.267	2.77	4.33	111.753	2.022	2.26
Y399T	130.536	0	0	122.738	0.05	0.06
Y399V	110.592	15.98	17.68	116.018	17.801	20.65
Y399W	122.5	13.76	16.86	103.346	11.973	12.37
S401A	122.003	13.9	16.96	99.275	13.024	12.93
S401E	125.223	16.3	20.42	128.67	15	19.3
S404A	149.379	0	0	105.443	1.102	1.16
L406F	122.805	0	0	146.122	0	0
L406N	152.836	6.36	9.72	131.321	6.705	8.81
S407A	141.351	11.33	16.02	110.376	16.836	18.58
S407D	241.053	11.29	27.22	98.135	10.12	9.93
S407P	143.308	6.85	9.81	121.898	11.088	13.52
A412Q	146.177	9.54	13.94	99.452	8.511	8.46
A412R	140.07	8.92	12.49	123.675	9.39	11.61
A412V	146.804	4.99	7.32	101.739	5.383	5.48
D416L	120.82	17.64	21.31	127.662	15.34	19.58
D418R	117.749	7.59	8.94	112.193	10.721	12.03
A419H	241.224	8.82	21.27	188.179	5.999	11.29
A419K	191.165	10.42	19.91	2022.616	1.523	30.81
D421A	102.111	12.49	12.75	301.584	4.51	13.6
D421H	333.471	10.18	33.95	67.652	86.552	58.55
D421K	124.19	7.62	9.46	102.316	13.562	13.88
D421N	110.806	14.96	16.58	100.116	16.449	16.47
D421Q	104.37	10.72	11.18	143.63	12.4	17.81
D421R	138.783	8.85	12.28	137.964	9.778	13.49
D421S	142.171	11	15.64	166.162	8.564	14.23
A425G	74.81	10.61	7.94	120.947	11.137	13.47
G427Q	133.135	2.31	3.08	98.243	8.618	8.47
G427T	125.113	4.81	6.02	119.058	3.956	4.71
V428L	137.044	1.81	2.48	109.39	0.99	1.08
D431E	70.178	26.32	18.47	95.135	20.739	19.73
D431H	186.49	7.32	13.65	95.071	9.941	9.45

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
D431K	240.835	11.61	27.97	68.277	20.207	13.8
D431L	129.149	10.49	13.54	119.177	11.74	13.99
D431N	138.404	9.79	13.55	125.433	9.246	11.6
D431Q	232.96	10.83	25.23	109.483	10.716	11.73
D431S	78.069	7.52	5.87	88.796	9.135	8.11
F433A	147.286	9.78	14.4	99.486	12.798	12.73
F433H	140.196	13.48	18.9	87.943	16.888	14.85
F433I	108.569	11.3	12.27	86.984	16.616	14.45
F433K	91.159	11.12	10.14	342.29	3.608	12.35
F433R	128.958	10.53	13.58	133.353	9.565	12.75
F433T	161.799	13.66	22.1	134.977	19.229	25.96
F433V	1412.071	1.61	22.69	112.033	17.307	19.39
F433W	149.049	10.46	15.59	113.585	7.53	8.55
P437I	148.88	2.39	3.56	107.028	1.782	1.91
M438A	106.463	10.07	10.72	135.705	10.194	13.83
M438D	105.37	10.16	10.71	113.283	2.59	2.93
M438E	115.061	8	9.21	113.782	9.12	10.38
M438L	65.794	10.06	6.62	214.958	6.526	14.03
M438N	130.428	8.06	10.52	100.669	11.889	11.97
M438T	104.058	13.39	13.93	103.691	12.16	12.61
E439A	137.279	11.63	15.97	95.555	14.073	13.45
E439A	154.14	4.72	7.28	147.295	7.415	10.92
E439C	193.243	14.69	28.38	111.719	15.734	17.58
E439K	124.464	13.28	16.52	104.762	10.552	11.05
E439P	118.34	15.59	18.44	87.446	14.998	13.12
E439Q	101.589	10.67	10.84	127.358	10.648	13.56
E439T	110.891	14.36	15.93	122.975	11.322	13.92
T440D	118.877	11.52	13.69	79.518	18.426	14.65
T440H	142.296	4.46	6.34	130.928	7.553	9.89
T440M	84.722	8.83	7.48	86.929	12.774	11.1
T440P	111.931	13.54	15.16	91.205	17.272	15.75
T440S	100.436	11.17	11.22	131.174	9.81	12.87
E441F	129.315	11.25	14.55	110.874	11.41	12.65

Mutant	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol	% Activity at 37°C+mcr/4°C
E442G	111.216	10.24	11.39	100.21	10.965	10.99
P443E	94.377	5.14	4.85	130.704	6.789	8.87
P443F	146.612	11.22	16.45	97.932	12.322	12.07
P443G	239.171	8.56	20.48	157.96	16.385	25.88
Q444E	81.997	8.54	7.01	160.917	9.561	15.38
Q444H	150.301	8.46	12.71	119.665	10.892	13.03
Q444V	129.822	13.49	17.51	122.591	10.995	13.48
I445M	85.09	17.25	14.68	101.149	15.393	15.57
I445N	106.43	13.89	14.79	87.351	12.945	11.31
I445W	117.213	11.7	13.72	100.037	10.983	10.99
Y447E	99.579	16.55	16.48	108.969	12.849	14
Y447G	143.704	13.77	19.79	103.624	11.563	11.98
Y447P	139.152	13.78	19.17	107.737	12.282	13.23
positive control (OHO)	94.998	5.23	4.97	96.871	8.456	8.19
	105.798	4.48	4.74	108.066	5.246	5.67
	100	3.33	3.33	82.778	3.759	4.59
	94.762	19.07	18.07	109.539	16.529	18.11
	142.024	4.48	6.36	130.947	5.595	7.33
	45.115	20.77	9.37	68.017	11.035	7.51
	53.324	21.95	11.71	74.253	9.96	7.4
	59.581	25.24	15.04	75.872	16.231	12.31
	91.844	19.05	17.5	80.371	13.977	11.23
	93.828	13.47	12.63	96.63	19.454	18.8
	57.773	17.04	9.85	83.536	17.573	14.68
	100	18.56	18.56	148.226	16.239	24.07
	74.325	18.29	13.6	61.119	9.286	5.68
	98.132	8.48	8.32	87.677	10.006	8.77
	93.817	9.62	9.02	102.223	9.745	9.96
	96.922	8.56	8.3	87.993	9.064	7.98
	96.648	9.91	9.58	86.891	9.938	8.63

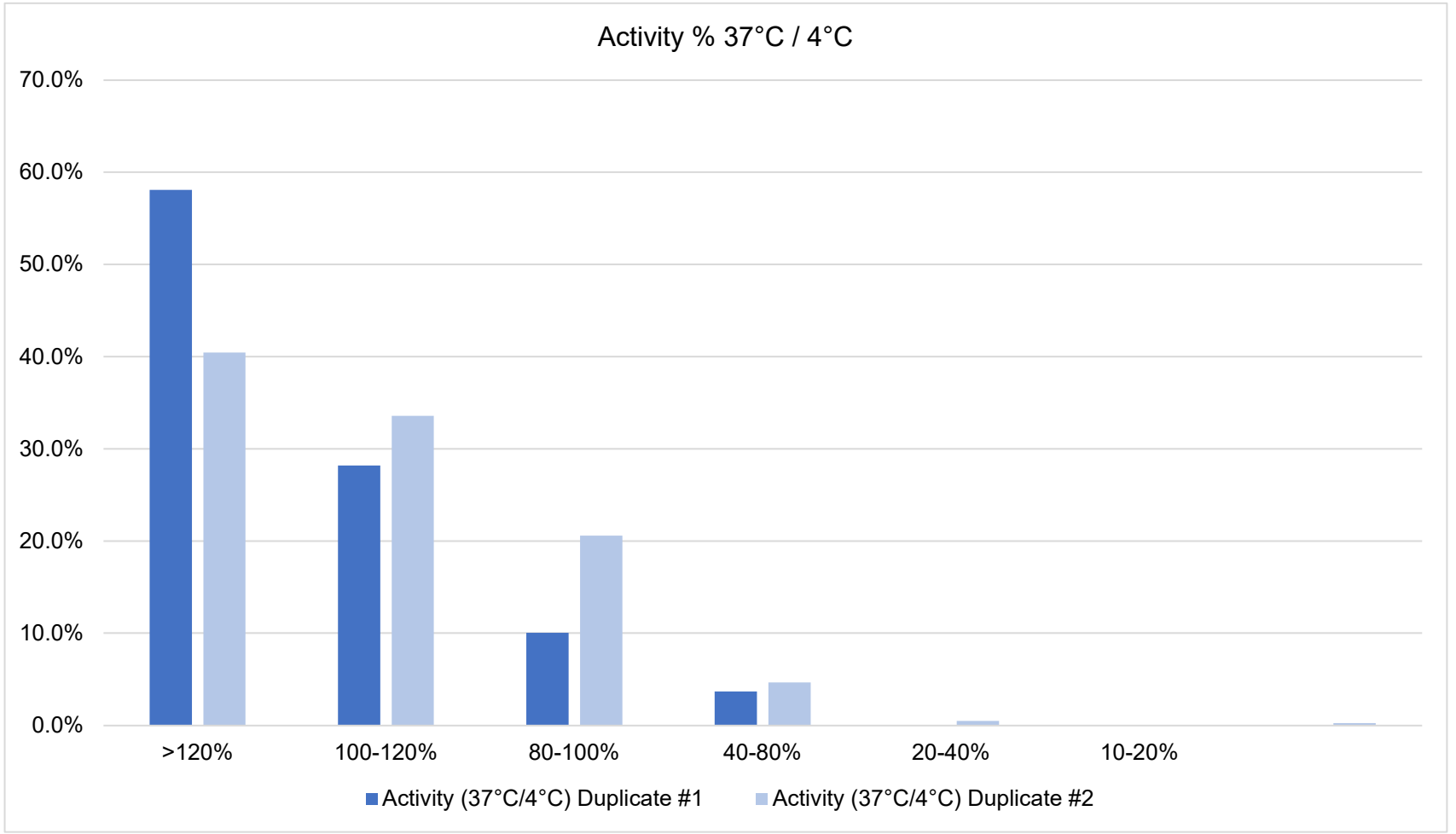
APPENDIX A-7

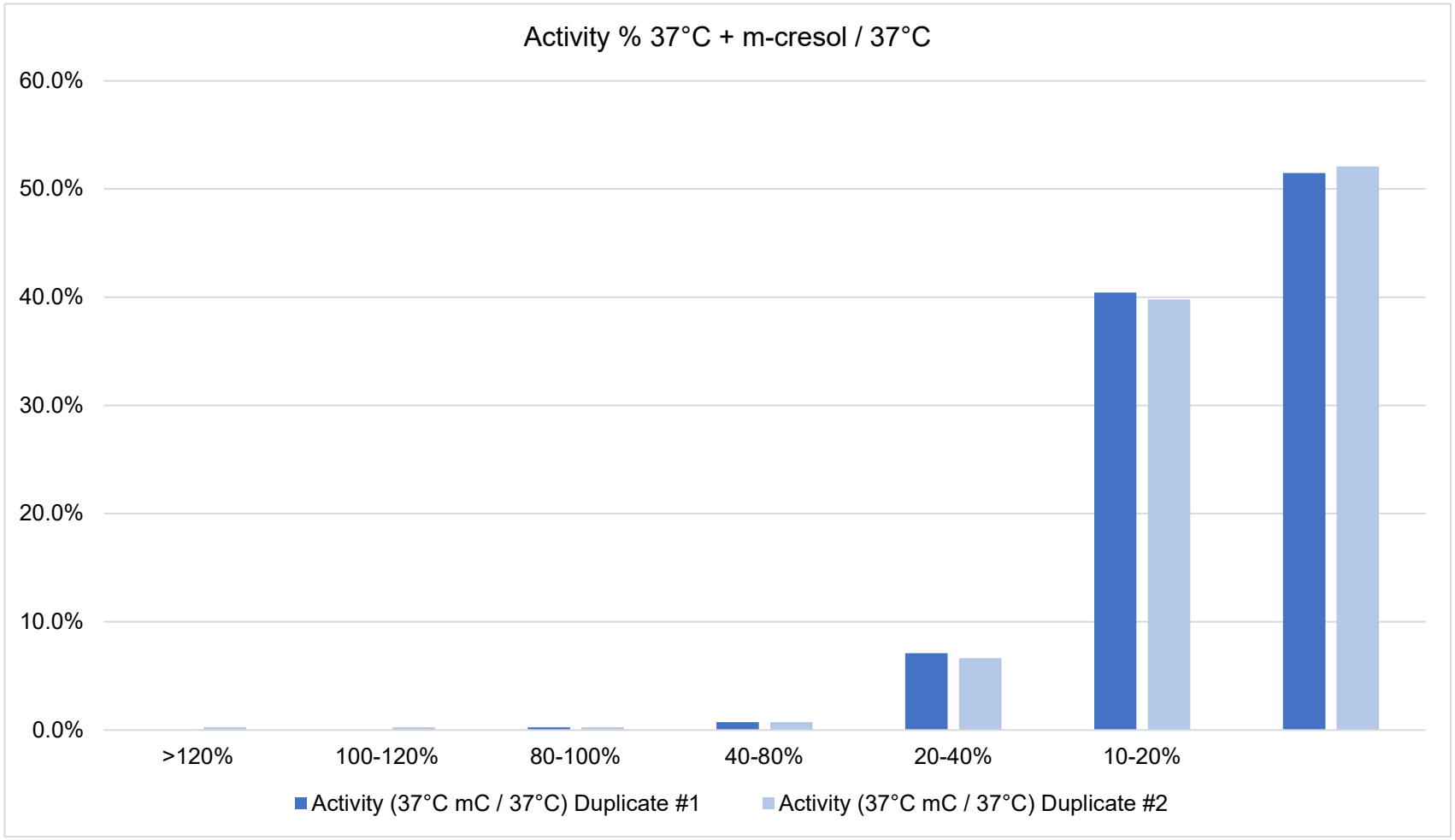
Composite Table and Plots of Active Mutants Grouped by Activity Under Temperature and Phenophilic Conditions

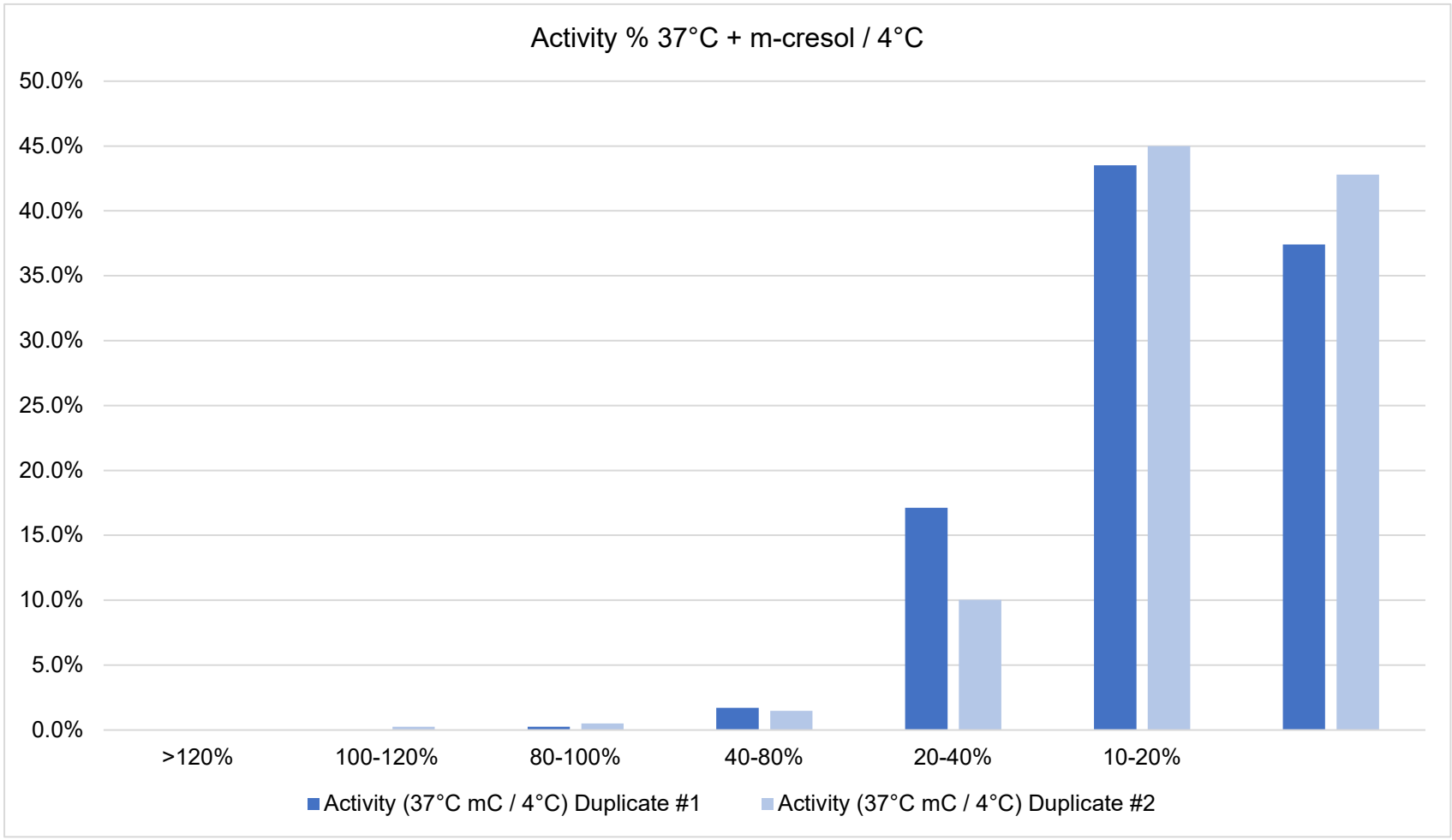
Source(s) – Table 12

	Activity (37°C/4°C)			Activity (37°C mC / 37°C)			Activity (37°C mc / 4°C)	
	Duplicate #1	Duplicate #2		Duplicate #1	Duplicate #2		Duplicate #1	Duplicate #2
>120%	237	165	>120%	0	1	>120%	0	0
100-120%	115	137	100-120%	0	1	100-120%	0	1
80-100%	41	84	80-100%	1	1	80-100%	1	2
40-80%	15	19	40-80%	3	3	40-80%	7	6
20-40%	0	2	20-40%	29	27	20-40%	70	41
10-20%	0	0	10-20%	165	162	10-20%	178	184
0-10%	0	1	0-10%	210	212	0-10%	153	175
Totals	408	408		408	407		409	409

	Activity (37°C/4°C)			Activity (37°C mC / 37°C)			Activity (37°C mC / 4°C)	
	Duplicate #1	Duplicate #2		Duplicate #1	Duplicate #2		Duplicate #1	Duplicate #2
>120%	58.1%	40.4%	>120%	0.0%	0.2%	>120%	0.0%	0.0%
100-120%	28.2%	33.6%	100-120%	0.0%	0.2%	100-120%	0.0%	0.2%
80-100%	10.0%	20.6%	80-100%	0.2%	0.2%	80-100%	0.2%	0.5%
40-80%	3.7%	4.7%	40-80%	0.7%	0.7%	40-80%	1.7%	1.5%
20-40%	0.0%	0.5%	20-40%	7.1%	6.6%	20-40%	17.1%	10.0%
10-20%	0.0%	0.0%	10-20%	40.4%	39.8%	10-20%	43.5%	45.0%
0-10%	0.0%	0.2%	0-10%	51.5%	52.1%	0-10%	37.4%	42.8%


Activity % 37°C / 4°C
70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
>120%
100-120%
80-100%
40-80%
20-40%
10-20%
Activity (37°C/4°C) Duplicate #1
Activity (37°C/4°C) Duplicate #2


Activity % 37°C + m-cresol / 37°C
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
>120%
100-120%
80-100%
40-80%
20-40%
10-20%
Activity (37°C mC / 37°C) Duplicate #1
Activity (37°C mC / 37°C) Duplicate #2


Activity % 37°C + m-cresol / 4°C
50.0%
45.0%
40.0%
35.0%
30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
>120%
100-120%
80-100%
40-80%
20-40%
10-20%
Activity (37°C mC / 4°C) Duplicate #1
Activity (37°C mC / 4°C) Duplicate #2

APPENDIX A-8

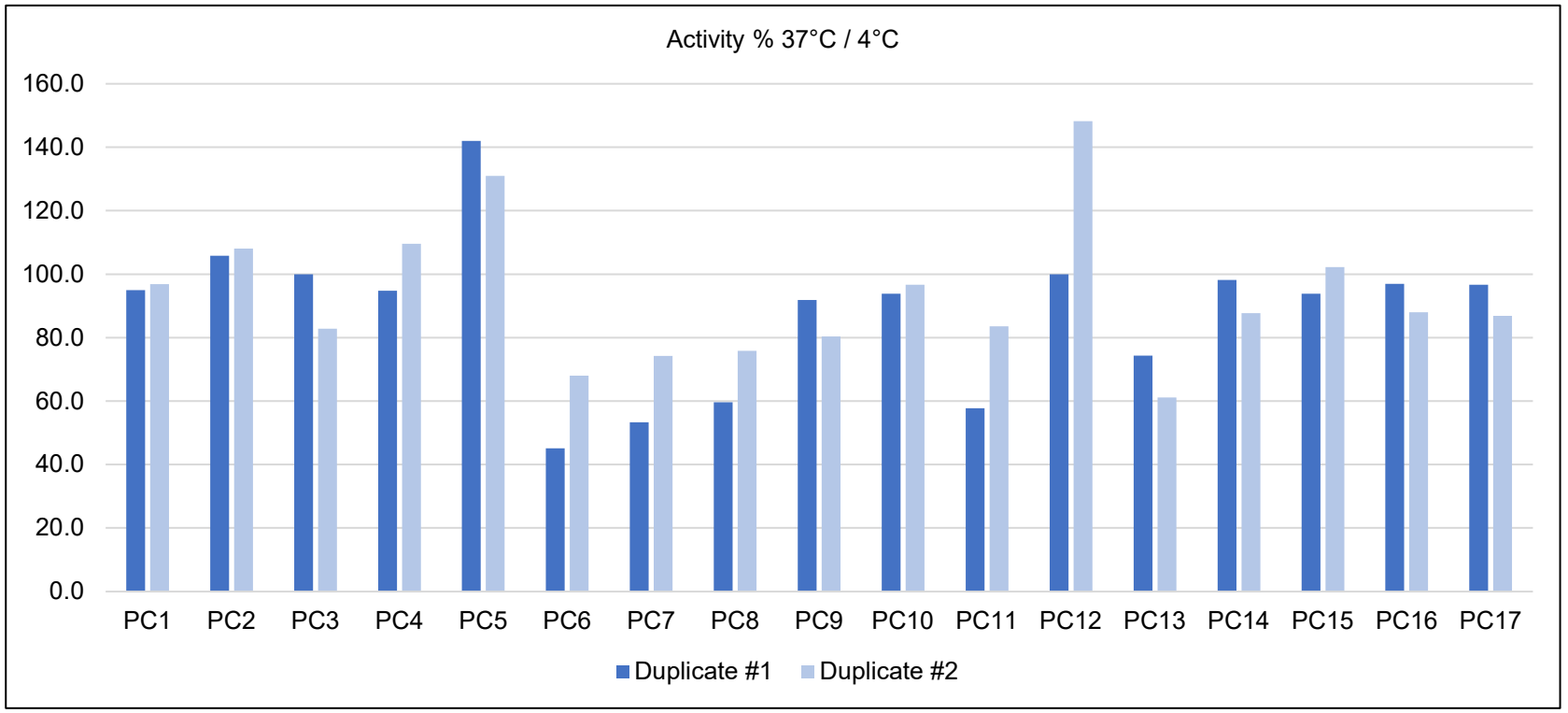
Table and Plots of Positive Control Activity Data Under Temperature and Phenophilic Conditions

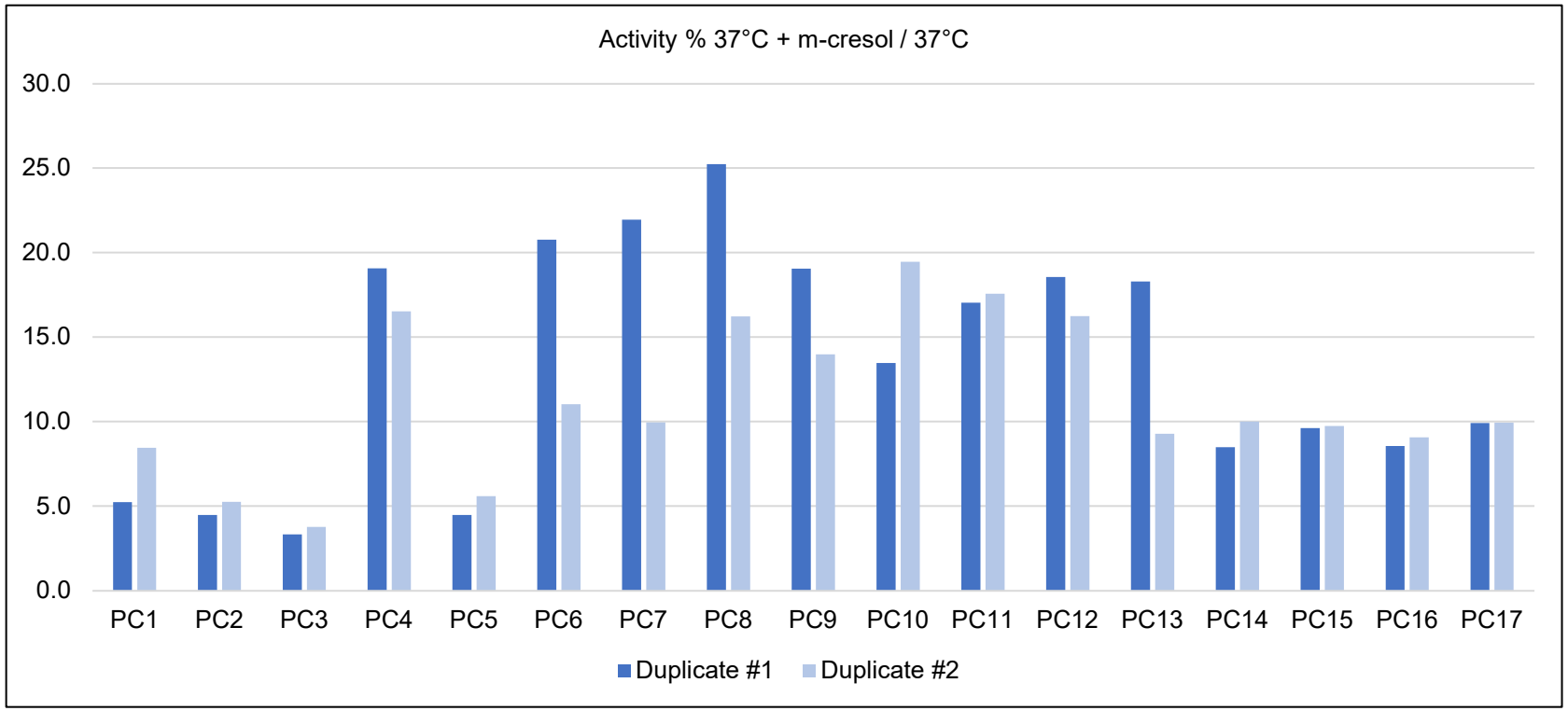
Source(s) – Table 12

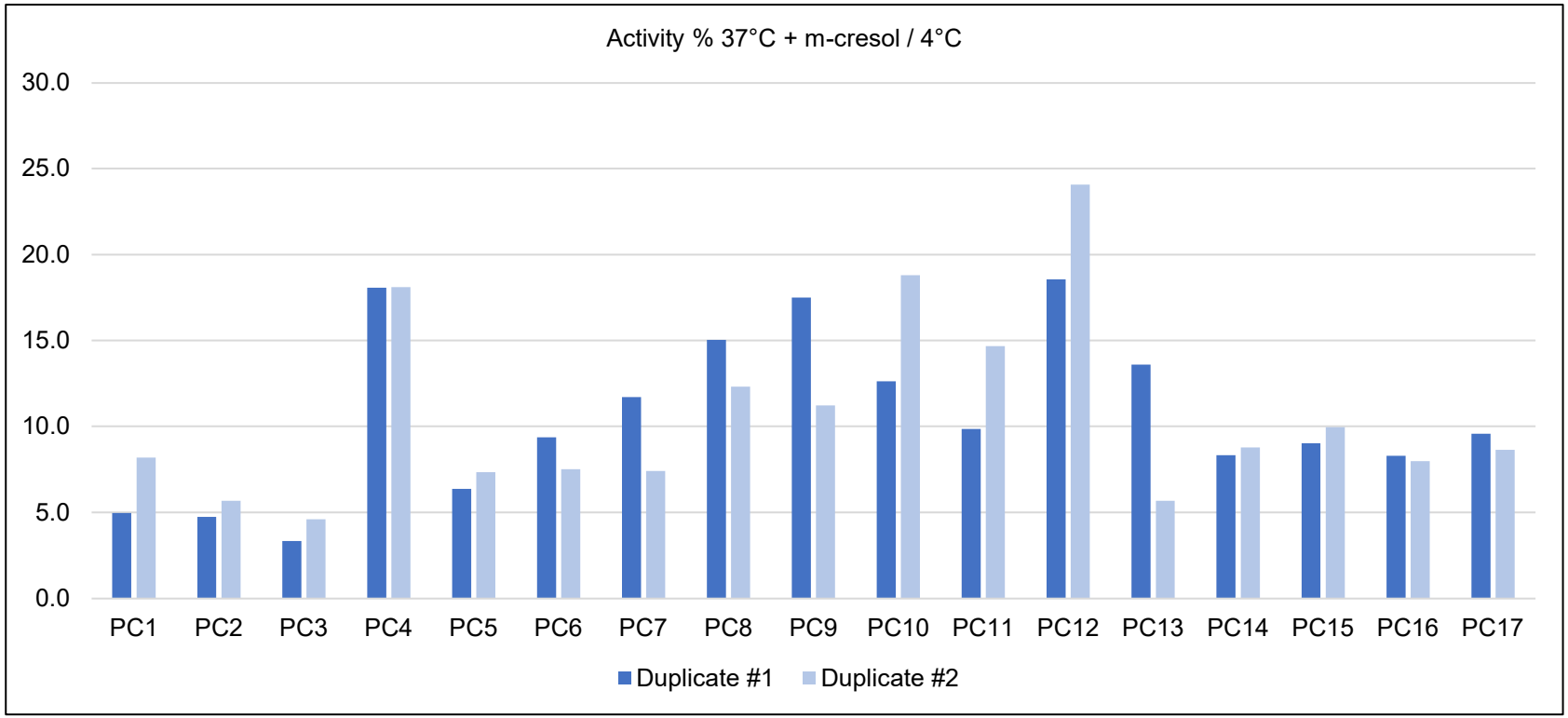
Positive Control ("PC") (OHO)	Duplicate #1			Duplicate #2		
	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol / 37°C	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C+m-cresol / 37°C	% Activity at 37°C+mcr/4°C
PC1	94.998	5.230	4.970	96.871	8.456	8.190
PC2	105.798	4.480	4.740	108.066	5.246	5.670
PC3	100.000	3.330	3.330	82.778	3.759	4.590
PC4	94.762	19.070	18.070	109.539	16.529	18.110
PC5	142.024	4.480	6.360	130.947	5.595	7.330
PC6	45.115	20.770	9.370	68.017	11.035	7.510
PC7	53.324	21.950	11.710	74.253	9.960	7.400
PC8	59.581	25.240	15.040	75.872	16.231	12.310
PC9	91.844	19.050	17.500	80.371	13.977	11.230
PC10	93.828	13.470	12.630	96.630	19.454	18.800
PC11	57.773	17.040	9.850	83.536	17.573	14.680
PC12	100.000	18.560	18.560	148.226	16.239	24.070
PC13	74.325	18.290	13.600	61.119	9.286	5.680
PC14	98.132	8.480	8.320	87.677	10.006	8.770
PC15	93.817	9.620	9.020	102.223	9.745	9.960
PC16	96.922	8.560	8.300	87.993	9.064	7.980
PC17	96.648	9.910	9.580	86.891	9.938	8.630

KEY
Coloration of Percent (%) Activity Values
n/a
>120
between 100 and 120
between 80 and 100
between 40 and 80
between 20 and 40
between 10 and 20
between 0 and < 10

*Data taken from '731 App., at 302-303 (and confirmed against T12 in '520 Patent)


Activity % 37°C / 4°C
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
PC1
PC2
PC3
PC4
PC5
PC6
PC7
PC8
PC9
PC10
PC11
PC12
PC13
PC14
PC15
PC16
PC17
Duplicate #1
Duplicate #2


Activity % 37°C + m-cresol / 37°C
30.0
25.0
20.0
15.0
10.0
5.0
0.0
PC1
PC2
PC3
PC4
PC5
PC6
PC7
PC8
PC9
PC10
PC11
PC12
PC13
PC14
PC15
PC16
PC17
Duplicate #1
Duplicate #2


Activity % 37°C + m-cresol / 4°C
30.0
25.0
20.0
15.0
10.0
5.0
0.0
PC1
PC2
PC3
PC4
PC5
PC6
PC7
PC8
PC9
PC10
PC11
PC12
PC13
PC14
PC15
PC16
PC17
Duplicate #1
Duplicate #2

APPENDIX A-9

Table of Amino Acid Residues Comprising the Hyal-EGF Region and Impact of Residue Substitution on Activity

Source(s) – Tables 3, 5

		Active (Table 3)															Inactive (Table 5)													
Pos	WT																													
337	A																C	F	G	I	K	L	M	R	T	W				
338	A	Q															C	D	E	F	G	H	I	K	L	P	R	T	V	
339	K	M															D	E	F	G	H	L	N	P	S	T	V	W	Y	
340	M																A	C	D	E	F	G	H	K	P	R	S	T	V	W
341	C																A	E	G	H	K	L	M	N	Q	R	S	T	V	Y
342	S	A															D	E	F	H	K	L	M	P	Q	R	T	Y		
343	Q	T	V														C	D	F	I	P	W								
344	V																F	G	H	L	M	N	P	Q	R	S	T	W	Y	
345	L																A	C	E	H	K	N	Q	R	T	V	Y			
346	C																A	D	F	G	I	K	L	M	P	R	S	T	V	W
347	Q	A	E	G	L	M	R	S									C	F	I	P	T	V	W							
348	E	D	G	S													C	H	I	L	P	Q	R	T	V	W	Y			
349	Q	A	E	K	M	N	R	T									D	F	G	P	V	W	Y							
350	G																A	D	E	F	H	K	L	M	N	P	R	S	T	V	Y
351	V	A	C	I	Q	S											C	D	E	F	H	N	R	W	Y					
352	C																A	D	E	F	G	K	M	P	Q	R	S	T	V	W	Y
353	I	T	V														C	F	G	H	K	L	M	Q	R	S	W			
354	R																C	D	E	G	H	I	K	L	M	P	Q	S	V	W	Y
355	K																D	F	G	H	L	M	N	P	Q	R	S	T	V	W	Y
356	N	A	D	H	S												C	G	K	L	P	R	T	V	W					
357	W	A	C	K	S	T											D	E	F	G	L	M	Q	R						
358	N	C	G	L	T												E	H	I	K	P	Q	R	W						
359	S	D	E	H	K	M	T	V									A	F	G	L	P	W								
360	S	T															A	C	E	F	G	I	K	L	M	P	Q	R	V	
361	D	H															A	C	E	G	M	N	P	Q	R	S	V	W		
362	Y																A	C	E	G	H	K	L	M	N	P	R	S	T	V	W
363	L																A	C	D	E	F	G	H	I	P	Q	R	S	T	V	W
364	H																A	C	D	E	F	G	K	L	M	P	R	S	T	V	Y
365	L																A	C	D	E	G	M	N	P	Q	R	S	T	W	Y	
366	N																A	C	E	F	G	K	M	P	Q	R	T	W		
367	P	A	C	G	K	R	S										E	F	I	L	M	Q	V							
368	D	A	E	G	H	K	L	M	R	S	T	V	H	R	S		C	P	W											
369	N																C	E	F	I	K	L	P	Q	V	W				
370	F																A	D	E	G	H	K	L	N	P	Q	R	S	V	Y
371	A	E	F	G	H	I	K	L	M	R	S	V					P													
372	I																A	D	E	F	G	H	K	L	N	P	R	S	T	V	W
373	Q	A	E	F	K	L	M	R	S	V							C	P	W											
374	L	A	H	I	M	N	P	R	S	T	V	W	Y				D	E												
375	E	A	G	I	K	L	M	N	R	S	T						C	F	P	V	Y									
376	K	A	D	E	L	M	Q	R	S	T	V	Y					I	P	W											
377	G	D	E	H	K	P	R	S	T								C	I	L	V										
378	G	K	N	R													D	E	F	I	L	M	Q	T	W	Y				
379	K	G	H	R	S	T											A	C	E	F	I	L	M	W						
380	F	I	L	P	T	V	W	Y									C	D	E	G	Q	R	S							
381	T	E	H	K	N	Q	R	S	V								G	L	P	W	Y									
382	V																E	G	H	K	L	M	N	P	Q	R	S	T	W	Y
383	R	A	E	H	I	K	L	M	N	S	T	V					G	P												
384	G																C	F	M	Q	S	T								
385	K	A	G	H	Q	R	S	T	V								C	L	M	P	W	Y								
386	P																A	C	F	G	H	I	L	M	N	Q	R	S	T	V	Y
387	T	S															C	E	F	G	H	I	L	M	N	V	W	Y		
388	L	F	H	I	M	R	T	V	W	Y							C	G	P	Q	S									
389	E	A	G	H	K	L	M	P	Q	R	S	T	Y				F	V												
390	D																A	C	E	F	G	H	L	N	P	R	S	T	V	W	Y
391	L	C															A	D	G	H	K	N	P	Q	R	S	T	V	W	Y	
392	E	A	G	K	L	M	R	Q	S	T	V	W	Y	F			C	P												
393	Q	A	D	F	H	K	L	M	N	R	S	T					C	P												
394	F	L	W														A	D	E	G	I	K	N	P	Q	R	S	T	V	
395	S	A	G	H	K	R	T	W									C													
396	E	A	D	H	L	Q	R	S	T								C	F	G	I	P	Y								
397	K	R															A	C	E	F	G	I	L	M	P	Q	T	V		
398	F	L															A	C	E	G	H	I	L	N	P	R	S	T	V	W	Y
399	Y	A	C	E	K	M	N	Q	R	S	T	V	W				D	P												
400	C																A	D	E	F	G	I	L	M	P	Q	R	S	T	V	Y
401	S	A	E	G	Q	N											C	F	H	K	R	W	Y							
402	C																A	D	E	F	L	M	P	Q	R	S	T	V	W	Y	
403	Y	F															A	C	E	G	H	K	L	M	N	P	Q	R	T		
404	S	A	P	T													C	D	F	G	H	L	M	N	R	V	W	Y			
405	T	A	F	G	K	M	P	Q	R	S	W	Y					C	I	V											
406	L	A	C	E	F	G	I	N	Q	S	T	V	Y				P	R												
407	S	A	D	E	F	G	H	L	M	N	P	Q	R	V	W															
408	C																A	E	F	G	I	K	L	P	R	S	T	V	W	Y	
409	K																													
410	E																W													
411	K																D	E	F	G										
412	A																E	H												

Key:
Orange Fill - Hyal-EGF Cysteine ("C") Residue
Green Fill - Hyal-EGF Glycine ("G") Residue

APPENDIX B

C.V. of Michael Hecht, Ph.D

MICHAEL H. HECHT, PH.D.

Department of Chemistry, Princeton University, Princeton, NJ 08544-1009
PHONE: 609-258-2901 FAX: 609-258-6746 EMAIL: hecht@princeton.edu WEB: https://hecht.princeton.edu

EDUCATION

CORNELL UNIVERSITY, B. A. *Summa cum laude* in Chemistry 1977
Mentor: Prof. Harold A. Scheraga
Thesis: Studies of the α-helical Propensities of Amino Acids in Synthetic Copolymers.

MIT, Department of Biology, Ph.D. 1984
Mentor: Prof. Robert T. Sauer:
Thesis: The Effect of Amino Acid Replacement on the Structure and Stability
of the N-terminal Domain of λ-Repressor

POST-DOCTORAL

DUKE UNIVERSITY, Department of Biochemistry 1986–1989
Mentors: Professors David and Jane Richardson
Research: Design of Novel Proteins.

FACULTY POSITIONS

PRINCETON UNIVERSITY - Department of Chemistry	- Assistant Professor	1990–1996
	- Associate Professor	1996–2003
	- Professor	2003–
	- Associate Chair of Chemistry Department	2004–2007
	- Director of Undergraduate Studies	2001–2008
PRINCETON UNIVERSITY – Forbes College	- Master (Head) of Forbes College	2010-2018

HONORS AND AWARDS

- College Scholar, CORNELL UNIVERSITY 1973–1977
- *Summa cum laude* with honors in Chemistry, CORNELL UNIVERSITY 1977
- National Science Foundation Graduate Fellow 1979–1983
- Life Sciences Research Foundation Burroughs-Wellcome Post-doctoral Fellow 1986–1989
- Whitaker Foundation Young Investigator Fellowship 1992
- Beckman Young Investigator Award 1993
- Protein Society - Kaiser Award 2003
- Japan Society for the Promotion of Science – Visiting Fellow 2019

RESEARCH INTERESTS

- Synthetic Biology: Artificial proteomes and genomes
- Protein Engineering
- *De novo* protein design
- Origin of life
- Astrobiology
- Protein folding and stability
- Combinatorial methods
- Alzheimer's disease, Amyloid, protein misfolding and aggregation

SERVICE – CHEMISTRY DEPARTMENT & PRINCETON UNIVERSITY

- Faculty Advisor for Undergraduates, Forbes College 1992-2010
- Council of the Princeton University Community (& Executive Committee) 2002-2005
- Faculty Advisory Committee on Policy 2002-2005
- McGraw Center for Teaching and Learning, Advisory Committee 2003-2006
- Director of Undergraduate Studies, Department of Chemistry 2001-2008
- Associate Chair, Department of Chemistry 2004-2007
- University Council on Science & Technology 2005-2008
- Institutional Biosafety Committee 2008-2009
- Executive Committee, Program in Quantitative and Computational Biology 2006-2014
- Center for Jewish Life, Board of Directors 2011-2014
- Presidential Task Force on the Residential Colleges at Princeton 2014-2015
- Task Force Subcommittee on Community Engagement, Chair 2014-2015
- Master (Head) of Forbes College 2010-2018
- Council on Science and Technology, Executive Committee 2022-
- Policy Committee on Athletics and Campus Recreation 2023-

SERVICE – SCIENTIFIC COMMUNITY

- Co-Organizer of Biannual Conference in Crete on Self-Assembling Peptides in Biology, Medicine & Engineering 1999, 2001, 2003, 2005, 2007
- Organizer of Conference in Jerusalem on Protein Design 2005
- Science & Technology Steering Committee, Brookhaven National Laboratory 2000-2005
- Editorial Advisory Board – *Protein Science* 2003-
- Editorial Advisory Board – *Protein Engineering, Design & Selection* 2003-
- Editorial Advisory Board – *Biopolymers* 2006-
- Biopolymers Gordon Conference Associate Chair (2008) Chair (2010)
- Organized NSF Workshop on the Future of Protein Engineering & Design 2014

TEACHING

- CHM 201: General Chemistry Fall Semesters
  → Typically 250-350 students matriculate, making CHM 201 the largest science course at Princeton
- CHM 542: Principles of Macromolecular Structure: Protein Structure, Folding & Design Spring Semesters
  → Taught from the scientific literature. Taken by graduate students & upper-level undergrads

TRAINING OF STUDENTS

POST DOCTORAL RESEARCHERS

- Joel Ybe 1991-1995
- James Beaseley 1995-1997
- Tun Liu 1997-1999
- Christine Wurth 2000-2002
- Peter Thumfort 2001-2005
- Luke Bradley 2001-2006
- Michael Ackerman 2003-2005
- Ryoichi Arai 2006-2007
- Izhack Cherny 2007-2011
- Betsy Smith 2010-2014

- Grant Murphy 2012-2016
- Joshua Mancini 2017-2018
- Shlomo Zharzitsky 2015-2020
- Sarangan Chari 2010- *(Visiting scientist / Departmental guest)*
- John Sakizadeh 2023-

GRADUATE STUDENTS

• Huayu Xiong	Chemistry	Ph.D	1995
• Satwik Kamtekar	Chemistry	Ph.D	1995
• Adam Brunet	Chemistry	Ph.D	1996
• Brian Johnson	Chemistry	Ph.D	1996
• Michael West	Chemistry	Ph.D	1996
• Felicia Messing	Molecular Biology	M.S	1996
• Nina Rojas	Chemistry	Ph.D	1997
• Sushmita Roy	Chemistry	Ph.D	1998
• Maria Nedwidek	Molecular Biology	Ph.D	1999
• Weixun Wang	Chemistry	Ph.D	2001
• Dave Moffet	Chemistry	Ph.D	2002
• Yinan Wei	Chemistry	Ph.D.	2003
• Aditi Das	Chemistry	Ph.D.	2005
• Emily Breneman	Chemistry	M.S	2005
• Woojin Kim	Chemistry	Ph.D.	2006
• Abi Go	Chemistry	Ph.D.	2008
• Shona Patel	Chemical Engineering	Ph.D.	2008
• Jermont Chen	Chemistry	Ph.D.	2008
• Michael Fisher	Molecular Biology	Ph.D.	2009
• Angela Fortner	Chemistry	Ph.D.	2011
• Siyi Wang	Chemistry	M.S	2012
• Maria Korolev	Chemistry	Ph.D.	2013
• Nettie Pyne	Molecular Biology	M.S	2014
• Scott Mellon	Molecular Biology	M.S	2015
• Kenric Hoegler	Molecular Biology	Ph.D.	2016
• Ann Mularz	Chemistry	Ph.D.	2016
• Katie Digianantonio	Chemistry	Ph.D.	2016
• Christina Karas	Molecular Biology	Ph.D.	2019
• Sha Tao	Visiting PhD Student	2019 - 2020	
• Michael Wang	Chemistry	Ph.D.	2022
• DaBin Jeon	Visiting PhD Student	2023	
• Yueyu Yao	Chemistry	Ph.D.	2024
• Guanyu Liao	Chemistry	Current	
• Brendan Sperling	Chemistry	Current	
• Jingyun (Chloe) Wu	Chemistry	Current	
• Nora Hubbard	Chemistry	Current	

UNDERGRADUATE (SENIOR THESIS) STUDENTS

• Robert Weltman	Chemistry	AB	1991
• Laura Lanier	Chemistry	AB	1991
• Jarad Schiffer	Molecular Biology	AB	1991
• Aaron Cypess	Chemistry	AB	1992
• Enoch Huang	Molecular Biology	AB	1992
• Rodgers Palmer	Molecular Biology	AB	1992
• Alexandra Van Geel	Molecular Biology	AB	1993

• Jonathan Loeb	Molecular Biology	AB	1993
• Mary Elizabeth Huffine	Molecular Biology	AB	1994
• Eugene Kim	Chemistry	AB	1994
• Jennifer Babik	Molecular Biology	AB	1995
• Frank Raia	Chemistry	AB	1995
• Kate Wesseling	Chemistry	AB	1995
• Cyrena Torrey Simons	Chemistry	AB	1996
• Jeremy Mclean	Molecular Biology	AB	1997
• Kim Helmer	Chemistry	AB	1997
• Adam Kessel	Chemistry	AB	1998
• Jennifer Patterson	Chemical Engineering	BSE	1998
• Dan Rosenbaum	Chemistry	AB	1999
• Bede Broome	Chemistry	AB	1999
• Allison Smith	Chemistry	AB	1999
• Joe Mancias	Chemistry	AB	2000
• Laura Certain	Chemistry	AB	2000
• Christina Brown	Chemistry	AB	2001
• Steve Sazinsky	Chemistry	AB	2001
• Jennifer Foley	Molecular Biology	AB	2001
• Nathalie Guimard	Chemistry	AB	2001
• Emily Hung	Chemistry	AB	2002
• Jeff Clough	Molecular Biology	AB	2002
• Diana Lee	Chemistry	AB	2003
• Dominic Notario	Chemistry	AB	2003
• Jonathan Goldwasser	Chemistry	AB	2004
• Jonathan Chou	Molecular Biology	AB	2004
• Ralph Kleiner	Chemistry	AB	2005
• Christine Henry	Chemistry	AB	2005
• Danielle Shin	Molecular Biology	AB	2005
• Anna Wang	Chemistry	AB	2006
• Jesse Platt	Chemistry	AB	2007
• Ellen Duncan	Chemistry	AB	2007
• Debbie Chen	Molecular Biology	AB	2007
• Sayuri Jinadasa	Chemistry	AB	2008
• Anne Armstrong	Chemistry	AB	2008
• Sara Viola	Molecular Biology	AB	2008
• Steve Sasson	Chemistry	AB	2009
• Sam Leachman	Chemistry	AB	2009
• Beverly Hon	Molecular Biology	AB	2009
• Jessica Langholtz	Chemistry	AB	2009
• Atrish Bagchi	Chemistry	AB	2010
• Kara McKinley	Molecular Biology	AB	2010
• David Canner	Chemistry	AB	2011
• Dan Echelman	Chemistry	AB	2011
• Charlotte Rajasingh	Chemistry	AB	2011
• Roselyn Kellen	Molecular Biology	AB	2011
• Maria Aristova	Chemical Engineering	BSE	2012
• Laura Bock	Molecular Biology	AB	2012
• Richard Hildreth	Chemistry	AB	2013
• Jack Greisman	Molecular Biology	AB	2014
• Eliza Hompe	Chemistry	AB	2014
• Kelly Ivins-O'Keefe	Chemistry	AB	2014

• Harry Cape	Chemistry	AB	2015
• Alankrita Raghavan	Molecular Biology	AB	2015
• James Agolia	Chemistry	AB	2016
• Bennett McIntosh	Chemistry	AB	2016
• Matthew Volpe	Chem. & Biol. Engin.	AB	2016
• Taylor Myers	Chemistry	AB	2017
• Elizabeth Stanley	Chem. & Biol. Engin.	BSE	2018
• Emily Schneider	Chem. & Biol. Engin.	BSE	2018
• Colin Yost	Chemistry	AB	2019
• Esther Choi	Chemistry	AB	2019
• Jessi Dessau	Chemistry	AB	2020
• Alex Jiang	Molecular Biology	AB	2020
• Natalie Bahrami	Chemistry	AB	2021
• Shanaz Deen	Chemistry	AB	2021
• Ananya Vinayak	Chemistry	AB	2022
• Daniel Strayer	Chemistry	AB	2022
• Kaelix Johnson	Molecular Biology	AB	2022
• Kevin Yeung	Chem. & Biol. Engin.	BSE	2023
• Obinna Uzosike	Molecular Biology	AB	2024
• Lily Kronenberg	Chemistry	AB	2024
• Emely Fernandez	Chemistry	Class of 2025	
• Osose Egbase	Chemistry	Class of 2025	
• Yejin Bann	Chemistry	Class of 2025	
• Jacob Davis	Chemistry	Class of 2026	
• Daniel Choi	Chemistry	Class of 2027	

MICHAEL H. HECHT, PH.D. – PUBLICATIONS

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- Hecht MH, Richardson JS, Richardson DC & Ogden RC (1990) *De Novo* Design, Expression, and Characterization of Felix: A Four-Helix Bundle Protein of Native-Like Sequence. ***Science*** 249, 884-891. DOI: 10.1126/science.2392678

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- Kamtekar S, Schiffer JM, Xiong H, Babik JM & Hecht MH (1993) Protein Design by Binary Patterning of Polar and Non-Polar Amino Acids. ***Science*** 262, 1680-1685. DOI: 10.1126/science.8259512

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- Wang MS, Hoegler KJ, Hecht MH (2019) Unevolved *De Novo* Proteins Have Innate Tendencies to Bind Transition Metals. ***Life*** 9(1), 8; doi: 10.3390/life9010008

- Kimura N, Mochizuki K, Umezawa K, Hecht MH, Arai R (2020) Hyperstable *De Novo* Proteins with a Dimeric Bisecting Topology ***ACS Synthetic Biology*** 9 254–259. doi.org/10.1021/acssynbio.9b00501

- Karas C, Hecht MH (2020) A Strategy for Combinatorial Cavity Design in *De Novo* Proteins**.** ***Life*** 10, 9; doi: 10.3390/life10020009

- Zarzhitsky S, Jiang A, Stanley E, Hecht MH (2020) Harnessing Synthetic Biology to Enhance Heterologous Protein Expression ***Protein Science*** 29, 1698-1706 *DOI: 10.1002/pro.3907*

- Mancini J, Pike D, Tyryshkin A, Haramaty L, Wang M, Poudel S, Hecht MH, Nanda V. (2020) Design of a Fe_4S_4 Cluster into the Core of a *De Novo* 4-Helix Bundle. ***Biotechnology and Applied Biochemistry.*** 67, 574-585 *https://doi.org/10.1002/bab.2003*

- Wang MS, Hecht MH (2020) A Completely De Novo ATPase from Combinatorial Protein Design ***J. Am. Chem. Soc.*** 142, 36, 15230–15234. *https://dx.doi.org/10.1021/jacs.0c02954*

- Markwalter CE, Uralcan B, Pelczer I, Zarzhitsky S, Hecht MH, Prud'homme RK, Debenedetti PG (2020) Stability of Protein Structure During Nanocarrier Encapsulation: Insights on Solvent Effects From Simulations and Spectroscopic Analysis. ***ACS Nano*** *https://doi.org/10.1021/acsnano.0c06056*

- Spangler LC, Yao Y, Cheng G, Yao N, Chari SL, Scholes GD, Hecht MH (2022) A *de novo* protein catalyzes the synthesis of semiconductor quantum dots. ***Proc. Natl Acad. Sci.(USA)*** 119, e2204050119 https://doi.org/10.1073/pnas.2204050119

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- Schnettler JD, Wang MS, Gantz M, Bunzel HA, Karas C, Hollfelder F, Hecht MH (2024). *Nature Chemistry.* Selection of a Promiscuous Minimalist cAMP Phosphodiesterase from a Library of *De Novo* Designed Proteins. ***Nature Chemistry*** 16, 1200–1208. https://doi.org/10.1038/s41557-024-01490-4

MICHAEL H. HECHT, PH.D. – INVITED LECTURES

- Berkeley Structural Biology Symposium, University of California, Berkeley, CA, January 10-12, 1990
- Bristol-Myers Squibb, Princeton, NJ, March 21, 1991
- The Peptide/Protein Bridge Conference, Toronto, Canada, June 10-14, 1991
- Rutgers University Molecular Biophysics Program, Piscataway, NJ, November 26, 1991
- FEBS (Federation of European Biochemical Societies) - 21st Annual meeting, Dublin, Ireland, August 9-14, 1992
- Duke University, Department of Biochemistry, Durham, NC, October 23, 1992
- NIH, Laboratory of Mathematical Biology - Molecular Structure Section, Bethesda, MD, January 12, 1993
- University of Pennsylvania, Department of Chemistry, Philadelphia, PA, February 11, 1993
- American Cyanamid Corporation, Princeton, NJ, February 24, 1993
- University of Delaware, Department of Chemistry and Biochemistry, Newark, DE, May 17, 1993
- Gordon Research Conference on Proteins, Tilton, NH, June 13-19, 1993
- Protein Society Annual Symposium, San Diego, CA, July 24-28, 1993
- University of California - Berkeley, Structural Biology Program, Berkeley, CA, October 18, 1993
- University of California - San Francisco, Department of Biophysics, San Francisco, CA, October 19, 1993
- Symposium on Molecular Recognition, CABM, Rutgers University, Piscataway, NJ, October 21-22, 1993
- Indiana University, Division of Biochemistry - Dept. of Chemistry, Bloomington, IN, November 19, 1993
- University of Toronto, Department of Biochemistry, Toronto, Canada, November 22, 1993
- University of Rochester, Department of Biochemistry, Rochester, NY, December 2, 1993
- Rutgers University, Department of Biochemistry, Piscataway, NJ, December 10, 1993
- City College of New York, Department of Chemistry, New York, NY, February 1, 1994
- National Institutes of Health, Laboratory of Chemical Physics, Bethesda, MD, February 10, 1994
- Gordon Research Conference on the Chemistry and Biology of Peptides, Ventura, CA, February 13-18, 1994
- International Congress on the Design and Modification of Biomolecular Structure, Logan, UT, March 2-4, 1994
- University of Massachusetts Medical School, Department of Pharmacology, Worcester, MA, April 11, 1994
- Bristol-Myers Squibb Research Institute, Dept. of Macromolecular Modeling, Princeton, NJ, April 25, 1994
- University of California - San Francisco, Department of Biochemistry, San Francisco, CA, May 3, 1994
- Stanford University, Departments of Cell Biology & Biochemistry, Stanford, CA, May 4, 1994
- Albert Einstein College of Medicine, Department of Physiology and Biophysics, Bronx, NY, May 12, 1994
- American Chemical Society, Middle Atlantic Regional Meeting, Univ. of Maryland (Baltimore), May 27, 1994
- University of Toronto / Ontario Cancer Institute, Toronto, Canada, June 23, 1994

- Indiana University, Department of Chemistry, Bloomington, IN, September 1, 1994
- IBC Conference on Artificial Antibodies and Enzymes, San Diego, CA, September 19-20, 1994
- Columbia University, Dept. of Biochemistry and Molecular Biophysics, New York, NY, September 30, 1994
- Office of Naval Research: "Biomolecular Recognition at ONR", Berkeley Springs, WV, October 26-30, 1994
- 39th Annual Meeting of the Biophysical Society, San Francisco, CA, February 12-16, 1995
- NEC Corporation, Princeton, NJ, March 7, 1995
- Texas A&M University, Department of Biochemistry and Biophysics, College Station, TX, May 3, 1995
- The Protein Society - First European Symposium, Davos, Switzerland, May 28 - June 1, 1995
- Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, NJ, June 7, 1995
- The Karolinska Institute. Center for Structural Biochemistry, Stockholm, Sweden, September 12, 1995
- Pharmacia Pharmaceuticals Inc., Division of Structural Biochemistry, Stockholm, Sweden, September 13, 1995
- Annual Meeting of the Swedish Society for Biochemistry and Molecular Biology (Plenary address), Linkoping, Sweden, September 14-15, 1995
- Symposium on Protein Folding and Design, University of Minnesota, Minneapolis, MN, October 19-20, 1995
- Tulane University, Department of Biochemistry, New Orleans, LA, October 23, 1995
- Advances in Protein Science Symposium, Mount Sinai Medical School, New York, NY, November 8, 1995
- University of Texas - Austin, Department of Chemistry and Biochemistry, Austin, TX, November 10, 1995
- Brandeis University, Department of Biochemistry, Waltham, MA, November 15, 1995
- Cornell Univ. Medical School, Dept. of Biochemistry and Structural Biology, New York, NY, December 4, 1995
- University of Washington, Department of Biochemistry, Seattle, WA, December 14, 1995
- Pacifichem International Chemical Congress, Honolulu, HA, December 17-22, 1995
- University of California - San Diego, Department of Chemistry and Biochemistry, La Jolla, CA, January 23, 1996
- Scripps Research Institute, La Jolla, CA, January 25, 1996
- Haverford College, Department of Biology, Haverford, PA, February 1, 1996
- Fox Chase Cancer Research Institute, Philadelphia, PA, February 6, 1996
- University of Rochester, Department of Biochemistry, Rochester, NY, March 6, 1996
- Weizmann Institute of Science, Department of Biochemistry, Rehovot, Israel, March 19, 1996
- University of Maryland (CARB) and National Institutes of Standards &Technology, Rockville, MD, April 16, 1996
- International Conference on Protein Folding & Design, Fogerty Center - NIH, Bethesda, MD, April 23-26, 1996
- Washington University, Department of Chemistry, St. Louis, MO, May 9, 1996
- Beckman Institute Symposium: Beyond Protein Structure, Univ. of Illinois, Urbana-Champaign, IL, June 6-9, 1996
- NEC Symposium in Biophysics, NEC Corporation, Princeton, NJ, June 23-27, 1996

- Washington University Medical School, Department of Biochemistry, St. Louis, MO, July 22, 1996
- FASEB Summer Research Conference on Protein Folding & Assembly, Saxtons River, VT, July 27-August 1, 1996
- University of Alberta, Department of Biochemistry, Edmonton, Alberta, Canada, October 10, 1996
- University of Michigan, Department of Chemistry, Ann Arbor, MI, October 23, 1996
- 76th Statistical Mechanics Conference, Rutgers University, New Brunswick, NJ, December 15-17, 1996
- Nature Biotechnology Symposium on Biomolecular Design, Form & Function. Miami, FL, February 1-5, 1997
- Wistar Institute, University of Pennsylvania, Philadelphia, PA, February 18, 1998
- DIMACS Workshop on Molecular Selection, Princeton University, Princeton, NJ, March 15-17, 1998
- Sigma Xi Lecture, Princeton, NJ , March 30, 1998
- Symposium on Structural Biology, University of Texas Medical School, Galveston, TX, April 3-5, 1998
- Symposium on Computational Chemistry & the Living World: From Sequence to Function, Chambery, France, April 20-24, 1998
- Gordon Research Conference on Biopolymers, Salve Regina University Newport, RI, June 14 - 18, 1998
- University of Virginia, Department of Chemistry, Charlottesville, VA, September 11, 1998
- Symposium on Principles of Protein Design: Theory, Experiments & Applications, Durham, NC, Nov. 19-21, 1998
- Rutgers University, Department of Chemistry, Newark NJ, January 11, 1999
- Weizmann Institute of Science, Department of Biological Chemistry, Rehovot, Israel, February 23, 1999
- Weizmann Institute of Science, Department of Chemistry, Rehovot, Israel, March 8, 1999
- American Chemical Society National Meeting, Anaheim, California, March 23, 1999
- Second Israeli Symposium on Computational Aspects of Molecular Biology, The Hebrew University, Jerusalem, Israel, April 28, 1999
- Tel Aviv University, Structural Biology Program, Ramat Aviv, Israel, June 22, 1999
- Symposium on Self-assembling Peptides in Biology, Engineering & Medicine, Crete, Greece, July 1-6, 1999
- New Jersey Center for Biomaterials 3rd Annual Retreat, Rutgers University, Piscataway, NJ , October 13, 1999
- American Physical Society National Meeting, Minneapolis, MN, March 20-24, 2000
- Naval Research Laboratory - Center for Bio/Molecular Science and Engineering, Washington, DC, May 5, 2000
- American Chemical Society, Mid-Atlantic Regional Meeting, University of Delaware, May 17, 2000
- Duke University, Department of Biochemistry, Durham, NC, May 16, 2000
- National Cancer Institute Laboratory of Experimental & Computational Biology. Frederick, MD June 13, 2000
- DARPA Focus 2000 Conference on the Intersection of Bio:Info:Physical Systems, Chantilly, VA, June 29-30, 2000
- Gordon Research Conference on Tetrapyrroles, Salve Regina University, Newport, RI, July 16-21, 2000
- Plenary Lecture - Annual Meeting of the German Society for Biochemistry & Molecular Biology, Munich, Germany - October 10-13, 2000 (Unable to attend due to family illness)

- Swiss/US Forum on NanoBioSciences, Princeton NJ, December 14-15, 2000
- University of Lausanne, Switzerland (Troisieme Cycle 2001 - 3 Lecture Series) Feb. 12 – Feb. 14, 2001
- University of Bern, Switzerland (Troisieme Cycle 2001 - 2 Lecture Series), Feb. 15 – Feb. 16, 2001
- MIT, Department of Chemistry, Cambridge, MA, March 8, 2001
- NEC Corporation, Princeton, NJ, March 30, 2001
- Wesleyan University, Department of Chemistry, Middletown, CT, May 4, 2001
- Hyseq Inc., Sunnyvale, CA , May 16, 2001
- Genencor Inc., Palo Alto, CA, May 17, 2001
- University of California, Santa Barbara, Program in Bioengineering, Santa Barbara, CA, May 18, 2001
- Symposium on Disorders of Protein Misfolding and Aggregation, Helsinki, Finland, June 6-10, 2001
- Dupont Corporation, Wilmington, DE, June 27, 2001
- Second Symposium on Self-assembling Peptides in Biology, Engineering & Medicine, Crete, July 13-17, 2001
- IBM, Blue Gene Deep Computing Project, IBM Watson Research Center, Yorktown Heights, NY, Sept. 6, 2001
- DARPA Workshop on Applications of Biologically Based Nanostructures. Arlington, VA, October 16, 2001
- Carnegie Institute, Baltimore, MD, December 17, 2001
- AstraZeneca Pharmaceuticals Inc, Wilmington, DE, June 18, 2002
- Brookhaven National Laboratory, Department of Biology, Brookhaven NY, July 11, 2002
- Wyeth Corporation, Princeton, NJ January 20, 2003
- Emory University, Department of Chemistry, Atlanta, GA, March 10, 2003
- University of Washington, Department of Chemistry, Seattle, WA, April 4, 2003
- Hunter College, City University of New York, Department of Chemistry, New York, NY, April 11, 2003
- American Chemical Society, Middle Atlantic Regional Meeting, Princeton, June 10, 2003
- 3rd Peptide Engineering Meeting. (cancelled because of SARS concerns) Toronto, Canada, July 16-18, 2003
- Protein Society Annual Symposium – Kaiser Award Lecture. Boston, MA, July 26-30, 2003
- 3rd Symposium on Self-Assembling Peptides in Biology, Engineering & Medicine, Crete, Greece, Aug. 1-5, 2003
- American Chemical Society National Meeting, New York, NY, Sept. 7-11, 2003
- DARPA Workshop on Protein Design Processes, Seattle, WA, Oct. 29, 2003
- Rutgers University, Department of Chemistry, Piscataway, NJ, Feb. 3, 2004.
- Symposium on "Protein Misaggregation: From Biomolecules to Neurodegeneration" Boston MA, Feb. 9-11, 2004
- City College of New York, Department of Chemistry. New York, NY, March 10, 2004
- Georgia Institute of Technology (GA Tech), Department of Chemistry. Atlanta, GA, March 23, 2004
- Materials Research Society, National Meeting, San Francisco, CA, April 16, 2004

- Foundations of Nanoscience Conference, Snowbird Utah, April 21-23, 2004
- University of Illinois, Department of Microbiology, Champaign, IL, May 4, 2004
- St Jude Research Hospital, Department of Structural Biology, Memphis, TN, May 11, 2004
- Symposium on 'Proteins: Structure, Folding and Disease', University of Toronto, Canada, June 3-4, 2004
- Bioorganic Gordon Conference, Proctor Academy, NH, June 13-18, 2004
- Symposium on Proteins: Folding, *De novo* Design and Interactions - CUNY Institute for Macromolecular Assemblies, New York, NY, November 9, 2004.
- New York NanoScience Discussion Group, Department of Chemistry, New York University, February 8, 2005.
- Symposium on Protein Design - University of Pennsylvania, Department of Chemistry, and Department of Biochemistry and Biophysics, Philadelphia, PA, March 10, 2005.
- Invitrogen – Molecule Probes Inc., Eugene, Oregon, March 15, 2005
- University of North Carolina, Department of Biochemistry, Chapel Hill, NC, April 5, 2005
- Ben Gurion University, Department of Biotechnology Engineering, Beer-Sheva, Israel, May 2, 2005
- Symposium on The Design, Engineering, and Selection of Novel Proteins, Institute for Advanced Studies, Hebrew University, Jerusalem, Israel, May 5-10, 2005
- Princeton University, Department of Chemistry. Alumni Reunions, Keynote Speaker. May 27, 2005.
- 4th Symposium on Self-Assembling Peptides in Biology, Engineering & Medicine, Crete, Greece, June 25-28, 2005
- Bristol-Myers Squibb Research Institute, Lawrenceville, NJ, July 28, 2005.
- Cornell University, Department of Chemistry Chemical & Biomolecular Engineering, August 29, 2005.
- Swarthmore College, Department of Chemistry and Biochemistry, Swarthmore, PA, February 9, 2006
- Keystone Conference on Protein Misfolding Diseases, Breckenridge, Colorado, February 21-26, 2006.
- Johns Hopkins University, Department of Chemical & Biomolecular Engineering, March 16, 2006.
- Hebrew University, Department of Biological Chemistry, Jerusalem, Israel, April 2, 2006.
- Weizmann Institute of Science, Department of Biological Chemistry, Rehovot, Israel, April 4, 2006.
- Tel Aviv University, Department of Molecular Microbiology and Biotechnology, Tel Aviv, Israel, April 5, 2006.
- Faculty of Medicine, Hebrew Univ., Dept. of Molec. Genetics & Biotechnology, Jerusalem, Israel, May 11, 2006.
- Symposium in Honor of Ephraim Katzir's 90th Birthday, Weizmann Institute, Rehovot, Israel, May14-15, 2006
- American Chemical Society Mid-Atlantic Regional Meeting, Hershey, PA, June 4-7 2006,
- Biopolymers Gordon Research Conference, Salve Regina University, Newport, RI, June 11-16 2006.
- Centocor, Inc. Radnor, PA, August 3, 2006.
- *Biotech 2006* Symposium, Philadelphia, PA "*Therapeutic Interventions for Alzeheimer's Disease"* Oct. 17, 2006.
- University of Massachusetts, Department of Chemistry, Amherst, MA, November 2, 2006.
- University of Pennsylvania, Department of Anesthesiology, Philadelphia, PA, January 11, 2007.

- City University of New York (CUNY), Department of Chemistry & Biochemistry, February 9, 2007.
- American Chemical Society National Meeting, Chicago, IL, March 25-29, 2007
- Polytechnic University, Department of Chemical and Biological Sciences, Brooklyn, NY, May 22, 2007.
- ExSAR Inc., Monmouth Junction, NJ, June 7, 2007.
- University of Cambridge, Department of Chemistry, Cambridge, UK July 5, 2007.
- Symposium on Protein Assembly in Materials, Biology, and Medicine, Crete, Greece, July 8-11, 2007.
- National Institutes of Health, Translational Research on Alzheimer's Disease. Bethesda, MD. Sept. 17-18, 2007.
- Rutgers University, Department of Chemistry and Chemical Biology. Piscataway, NJ, October 30, 2007.
- International Conference on Alzheimer's Disease, Chicago, IL, July 26-31, 2008.
- Arizona State University, Department of Chemistry, Tempe, AZ, October 31, 2008.
- Duke University, Department of Chemistry, Durham, NC, December 16, 2008.
- University of Toronto, Mississauga Campus, Department of Chemistry, Toronto, Canada, January 22, 2009.
- University of Toronto, Downtown Campus, Department of Chemistry Toronto, Canada, January 23, 2009.
- AD/PD Conference on Alzheimer's Disease & Parkinson's Disease, Prague, Czech Republic, March 11-15 2009.
- Columbia University, Department of Chemistry, "Grandpierre Memorial Lecture" New York, NY, March 26, 2009.
- University of Kentucky, Department of Chemistry, Naff Symposium Lecture, Lexington, KY, April 24, 2009.
- University of Maryland, Department of Chemistry & Biochemistry, College Park, MD, May 5, 2009.
- Workshop on Protein Misfolding in the Test Tube and in Disease, Hagoshrim, Israel, May 13-15, 2009.
- New York University, Department of Chemistry, New York, NY, September 11, 2009.
- Princeton University, The Lewis-Sigler Institute for Integrative Genomics, Princeton, NJ, December 1, 2009.
- American Chemical Society Central NJ Section, Princeton, NJ, February 24, 2010.
- Quest University, Squamish British Columbia, Canada, March 18, 2010
- Rockefeller University, Center for Studies in Physics and Biology, New York, NY, May 11, 2010.
- Synthetic Biology Workshop, St Anne's College, University of Oxford, United Kingdom, July 12-14 2010.
- Pacifichem International Chemical Congress, Honolulu, HA, December 15-20, 2010.
- Los Alamos Nat'l Laboratory: "Synthetic Biology: From Protein Design to Artificial Genomes" March 16, 2011.
- Los Alamos Nat'l Lab: "Alzheimer's disease: Molecular Underpinnings & Search for Therapeutics" Mar. 17, 2011.
- ECI Conference on Biochemical & Molecular Engineering, Seattle, Washington, June 26-30, 2011.
- Massey University, Auckland, New Zealand, August 16, 2011.
- BioInteractions Conference, Crown Plaza Hotel, Queenstown, New Zealand, Aug 28, 2011.
- Queenstown Molecular Biology Conference, Queenstown, New Zealand, Aug 29-31, 2011.
- Origin of Life Gordon Research Conference, Galveston, TX, January 8-13, 2012.

- NASA Headquarters, Astrobiology Institute Director's Seminar, Washington, DC, March 5, 2012.
- AbSciCon 2012 NASA Astrobiology Conference, Atlanta, GA, April 16-20.
- DARPA workshop on Protein Synthesis, Arlington, VA, May 17, 2012,
- DuPont De Nemours Experimental Station, Central Research and Development, Wilmington, DE, June 22, 2012.
- Q-Bio (Quantitative Biology) Conference, Santa Fe, NM, August 8-12, 2012.
- University of Minnesota, Dept. of Biochemistry, Molecular Biology & Biophysics, St. Paul, MN, Sept. 5, 2012.
- UCLA, Molecular Biology Institute, Los Angeles, CA, January 10, 2012.
- Princeton Origin of Life Conference, Center for Theoretical Science, Princeton, NJ, January 21-24, 2013.
- University of Missouri, Department of Biochemistry, Columbia, MO, March 1, 2013.
- The Helix Center for Interdisciplinary Investigation. New York, NY, May 11, 2013.
- Conference on Emergence in Chemical System. Univ. of Alaska, Anchorage, AK, June 17-20, 2013.
- Conference on Proteomics and BioInformatics, Philadelphia, PA. July 15-17, 2013.
- NSF Workshop on Design, Engineering, & Selection of Novel Proteins (Organizer), Arlington VA, May 12-13, 2014.
- Protein Society Annual Symposium, San Diego, CA, July 27-30, 2014.
- American Chemical Society Central NJ Section, Princeton, NJ, November 19, 2014.
- Bristol Myers Squibb / Princeton University Joint research Symposium, Princeton, NJ, December 4, 2014.
- University of Pennsylvania, Department of Biochemistry & Biophysics, Philadelphia, PA, February 19, 2015.
- Yale University, Department of Genetics, New Haven, CT, May 26, 2015.
- International Workshop: Frontiers in Protein Folding, Evolution & Function, Oaxaca, Mexico, November 3-7, 2015
- Lawrence Berkeley National Laboratory, Berkeley CA, January 19, 2016.
- TEDx, Princeton NJ. March 26, 2016.
- Mosbacher Kolloquium: Protein Design. Mosbach, Germany, March 31 - April 2, 2016.
- Protein Engineering Conference, Ottawa, Canada, June 17-19, 2016.
- Protein Society Annual Symposium, Baltimore, MD, July 16-19, 2016.
- Rutgers University, Center for Integrative Proteomics Research and Institute for Quantitative Biomedicine, Piscatawy, NJ. January 25, 2017.
- Symposium: Life Together? Examining Our Assumptions. Stellenbosch Institute for Advanced Studies, South Africa, February 13-15, 2017.
- Linderstrøm-Lang Center for Protein Science, Department of Biology, University of Copenhagen, Denmark. February 20, 2017.
- AbSciCon 2017 (NASA Astrobiology Conference), Mesa AZ, April 24-28, 2017.
- University of California at Davis, Department of Chemistry, Davis, CA, May 2, 2017.
- IGEM Meeting, Ludwig-Maximilian University, Munich, Germany, July 20-22, 2018.

- Synthetic Biology III, Landshut, Germany, July 23-25, 2018.
- Society for Industrial Microbiology Annual Meeting, Chicago, IL, August 12-14, 2018.
- Syracuse University, Department of Chemistry, Syracuse, NY, November 27, 2018.
- Earth-Life Science Institute (ELSI), Tokyo Institute of Technology, Tokyo, Japan, March 7, 2019.
- Symposium on BioDesign and BioEngineering (*Keynote speaker*), Tokyo, Japan, March 8, 2019.
- Symposium on New Frontiers in Chemistry and Synthetic Biology (*Keynote speaker*), Shinshu University, Ueda, Japan, March 13, 2019.
- Symposium on New Frontiers in Protein Design and Engineering (*Keynote speaker*), Institute of Molecular Science, Okazaki, Japan, March 15-16, 2019.
- Nara Institute of Science and Technology (NAIST), Nara, Japan, March 20, 2019.
- Conference on Computational Design of Protein-Cofactor Complexes, Galilion, Israel, March 25 -28 2019.
- Hebrew University, Institute of Life Sciences, Jerusalem, Israel, April 1, 2019.
- Ben Gurion University of the Negev, Department of Life Sciences, , Beer-Sheva, Israel, April 8, 2019.
- Weizmann Institute of Science, Department of Biomolecular Sciences, Rehovot, Israel, April 15, 2019.
- ETH Zürich, Department of Chemistry and Applied Biosciences, Zürich, Switzerland, April 30, 2019.
- Uppsala University, Department of Cell and Molecular Biology, Uppsala, Sweden, May 3, 2019.
- University of Cambridge, Department of Chemistry, Cambridge, UK, May 8, 2019.
- Oxford Global SynGen Symposium on Synthetic Biology, Boston, MA, May 14-15, 2019.
- Gordon Research Conference on Proteins, Holderness, NH, June 16-21, 2019.
- Rutgers University, Department of Physics, Piscataway, NJ, September 18, 2019.
- Georgia Tech University, Astrobiology Program, Atlanta, GA, February 21, 2020.
- Defense Threat Reduction Agency, Fort Belvoir, VA, (via Zoom), November 2, 2020.
- Life in the Universe: Breakthrough Initiatives Program. Cape Town, South Africa (via Zoom), November 4-5, 2020
- Truman State University, Department of Chemistry. Kirksville, MO, (via Zoom), February 11, 2022
- Rutgers University, ENIGMA Program in Astrobiology, Piscataway, NJ, December 12, 2022.
- University of Colorado, Dept. of Molecular, Cellular & Developmental Biology, Boulder, CO, March 16, 2023.
- MIT, Retirement Symposium for Prof. Robert T. Sauer, Cambridge, MA, June 1-2, 2024.
- Protein Society Annual Symposium, Vancouver, BC, Canada, July 23-26, 2024.
- Wake Forest University, Department of Physics, Winston-Salem, NC, August 29, 2024