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. IPR2026-00312
U.S. Patent No. 10,865,400

Declaration of James Naismith, Ph.D.

Halozyme EX2305
Merck v. Halozyme
PGR2025-00050

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C. C.V. of Dr. James H. Naismith, 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 (Appendix C), which provides a full and accurate description of my educational background, professional experience, appointments, honors, and publications.

2. I received my Bachelor of Science degree with First Class Honours in Chemistry from the University of Edinburgh in 1989. I received my Ph.D. in Chemistry in 1992 from the University of Manchester for research involving the structural study of concanavalin A and a zinc-dependent aldolase, using X-ray crystallography and enzymological analysis, for which I was awarded the Hibbert Prize. I completed two years postdoctoral training as a research fellow at the Howard Hughes Medical Institute University of Texas Southwestern Medical Center Dallas, where I continued work in structural biology.

3. I started my own laboratory in structural biology and enzymology at the University of St Andrews in 1995. I earned a Doctor of Science (D.Sc.) degree from the University of St. Andrews in 2016 for sustained contributions to chemical and structural studies of proteins.

4. I currently serve as Professor of Structural Biology in the Nuffield Department of Medicine at the University of Oxford and as Head of the Division of Mathematical, Physical and Life Sciences at Oxford. I am also a member of the University Council of the University of Oxford. From 2017 to 2023, I led the establishment of the Rosalind Franklin Institute as its founding Director and Chief Executive Officer, where I was responsible for scientific vision, faculty recruitment, infrastructure development, and delivery of the Institute's research program. I currently serve as the non-clinical Vice-President of the Academy of Medical Sciences.

5. Prior to my appointment at Oxford, I served as a faculty member at the University of St. Andrews from 1995 to 2017, progressing from Lecturer to Professor and later Bishop Wardlaw Professor of Chemical Biology. During that period, I led the Biomedical Sciences Research Complex, including its design, funding, construction, and scientific direction, and I held senior leadership roles within the University.

6. Throughout my career, I have taught and supervised courses in enzyme kinetics, structural biology, chemistry in biology, and drug discovery, and I have taught undergraduate and graduate-level biochemistry and chemistry for more than two decades. I have supervised over thirty Ph.D. students who have gone on to graduate, and over twenty post-doctoral associates.

7. I have over 30 years of research experience in protein structure and enzymology. My laboratory has investigated the structural and chemical biology of enzymes and molecular recognition complexes, including detailed studies of enzyme mechanisms, substrate specificity, protein–ligand interactions, and the structural basis of catalysis. My work has addressed diverse biological systems, including natural product biosynthesis, carbohydrate-processing enzymes, and mechanisms relevant to antimicrobial resistance.

8. My research contributions have been recognized by election to several scientific academies, including Fellow of the Royal Society of Edinburgh (2005), Fellow of the Academy of Medical Sciences (2012), Fellow of the Royal Society (2014), Fellow of the American Association for the Advancement of Science (2016), Membership of European Molecular Biology Organization (2009), and Membership of Academia Europa (2018). I am a Fellow of the Royal Society of Chemistry, Royal Society of Biology, Institute of Physics, and a Member of the American Chemical Society.

9. I have received numerous awards for research excellence, including the Royal Society Wolfson Research Merit Award (2014), the Tilden Prize (2018), and the Horizon Prize (2022) from the Royal Society of Chemistry. I am the only individual to have received both the Corday-Morgan Medal (2004) for

contributions to experimental chemistry and the Colworth Medal (2004) for research in biochemistry, reflecting the interdisciplinary nature of my work.

10. I have co-authored just under 300 peer-reviewed scientific publications in the areas of protein structure, enzymology, chemical biology, and membrane proteins. A complete list of my publications is included in Appendix C. Google Scholar shows the work has over 28,000 citations and an h-factor of 90.

B. Compensation

11. I am being compensated for my time at the rate of $850 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

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.

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" or "POSA") 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. Before and after the 2011-2012 timeframe, I have had at least the qualifications I outline above for a POSA. The opinions I provide in this declaration are provided from the perspective of a POSA during the 2011-2012 timeframe as I have described it above.

15. I have understood and presently understand that the perspective of a POSA is to be assessed at the time the application or applications resulting in the patent were filed. I understand that December 29, 2011 is the earliest filing date to which U.S. Patent No. 10,865,400 (the '400 Patent) and U.S. Application No. 13/694,731 (the '731 Application) claim priority. I also understand that I am to assess whether the '731 Application provides sufficient written description and

enablement for the claims of the ’400 Patent as of December 29, 2011 (as this is the date of the filing of the earliest provisional application related to the ’731 Application) as well as of the date that the ’731 Application was actually filed (*i.e.*, December 28, 2012). I considered both dates in forming my opinions in this declaration. While I am not aware of any developments in that timeframe that would impact my opinions, if any of my opinions would have varied within this period I will note that variance.

D. Terminology Used in this Declaration; Patent Documents

16. Unless otherwise noted, 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. 10,865,400.

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

(b) When I refer to a "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 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.

17. I was asked to assess several patent documents. One is an issued U.S. Patent, which has the number 10,865,400, which I refer to as the '400 Patent (EX1001). Another is U.S. Patent Application having the number 13/694,731, which I will refer to as the '731 Application (EX1026). I have also considered each of the documents incorporated-by-reference in the '731 Application,

including each of the provisional applications (61/631,313, filed November 1, 2012 and 61/796,208, filed December 30, 2011) to which the '731 Application claims priority, and U.S. Patent No. 7,767,429 ("the '429 Patent") (EX1005) and U.S. Publication No. 2010/0143457 ("US-457") (EX1105). Finally, I have considered United States Patent Publication No. 2013/0302275 ("US-275") (EX1004), which I understand is the version of the '731 Application that published on November 14, 2013.

18. 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). When I use the term "patent disclosure" or "disclosure," I am using it with this understanding. I understand that patents end with claims that define the invention.

19. I reviewed the claims of the '400 Patent, as well as the disclosure of the '731 Application and its incorporated-by-reference materials. I also reviewed a redline comparison of the specifications of the '731 Application to the '400 Patent (EX1045) and US-275 (EX1208). Based on that review, the specifications appear to be substantively the same, with only minor differences that do not impact my opinions. In this declaration, I will refer to the '731 Application and its

incorporated-by-reference materials together as the "disclosure." Citations will be to the disclosure in the '731 Application. I also note that the '731 Application alters several passages of the two incorporated-by-reference provisional disclosures, adds new examples and tested mutants, and makes other changes, and therefore all of the deficiencies I note herein regarding the '731 Application also apply to the disclosures of the provisional applications.[2]

E. Materials Considered

20. 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. Finally, I have reviewed the declarations and testimony submitted by Michael Hecht, Ph.D. and Sheldon Park, Ph.D. that I understand were submitted in several PGR proceedings between Petitioner and Halozyme that concern patents that are related to the '400 Patent through their relationship to the '731 Application and share substantially the same disclosure. For example, I provided a reply declaration in PGR2025-00003, PGR2025-00004, PGR2025-

[2] EX1026, 37:19-51:11, 140:15-150:26, 311-21, 6:25-26; EX1051 ('313 App.); EX1052 ('208 App.).

00006, and PGR2025-00009, and reviewed Dr. Hecht and Dr. Park's declarations and testimony in connection with preparing my opinions in those proceedings. I have also reviewed the declarations and testimony of Patent Owner's experts, Dr. Gregory Petsko and Dr. Melanie Simpson, in connection with those related proceedings.

F. Legal Principles

21. 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, which I used to form my opinions in this declaration. Some of these legal explanations are summarized below.

22. I have been informed that at the end of the patent specification are a series of claims, which define the invention being patented. I have been informed that interpreting patent claim terms is an issue of law to be resolved by the court (or in this case, the Patent Trial and Appeal Board ("PTAB")). I also have been informed that when interpreting patent claims, a court or the PTAB will follow certain principles, including that: (i) the words used in the claim are generally given their plain and ordinary meaning as understood by the POSA at the time of the invention, (ii) the context of how the words in the claim are being used within the patent claim offers insight into their meaning, and (iii) the patent claims are to be read in view of the disclosure of the patent.

23. I have been informed that terms used in the claims should be given their ordinary meaning, unless the terms have been expressly defined to have a particular meaning by the patent disclosure.

24. I have been informed that a patent claim defining an invention is to be interpreted in a manner that is consistent with the description of the claimed invention in the patent disclosure (*i.e.*, the specification, drawings and sequence listings).

25. I have been informed that what a claim defines must be determined by evaluating the claim as a whole, including all of the language used in the claim. I understand that a claim may use combinations of terms or use terms in conjunction with additional language to yield an overall meaning for a claim that is narrower than what a single defined term might alone suggest.

26. 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.

27. 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."

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

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

30. 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 POSA are factors, among others, that are considered in assessing the question of enablement.

31. I have been informed that for a patent to claim the benefit of the earlier filing date of a related patent application, that related patent application must provide written description and enablement support for the claims. If the claims lack written description and enablement support in the earlier-filed applications, then the patent cannot have a priority date earlier than the filing date of the application from which it issued.

32. I have been informed that a separate patentability requirement is that an invention must not have been anticipated by the prior art that existed before the effective filling date of the patent (*i.e.*, the filing date to which the patent application is entitled). I have been informed that for an invention to be anticipated, at least one embodiment falling within the scope of the claims must have been disclosed—expressly or inherently—within the four corners of a publication that constitutes prior art before the filing date to which the claims of the patent are entitled.

II. Scientific Principles

33. The disclosure of the ’731 Application addresses modified forms of a human hyaluronidase enzyme known as PH20. PH20 is part of a family of five related hyaluronidase enzymes that have been identified in humans.

34. As discussed in more detail below (§ IV), the patent claims refer to modified PH20 polypeptides that include at least one amino acid substitution at one of several listed positions (*e.g.*, 85 possible positions in claim 1). The claims further permit, but do not require, the presence of additional amino acid changes—ranging from 1 to 22 (or 9) substitutions—at other locations relative to the wild-type sequence. Depending on the specific claim, the referenced PH20 polypeptides comprise between 430 and 465 (or 474) amino acid residues, before accounting for possible deletions or additions permitted by the claims. These attributes define a “sequence space” within which any claimed modified PH20 polypeptides must reside. Notably, all such claimed modified PH20 polypeptides are required to exhibit increased hyaluronidase activity that is at least 120% of the hyaluronidase activity of a recited reference unmodified PH20 polypeptide.

35. The subject matter of the patent falls within the scientific disciplines of protein structure and engineering. Foundational principles in these areas are well established in the literature and are summarized in three standard textbooks,

including: (i) Branden & Tooze ("Branden," EX1014) (chapters 1-6 and 17), (ii) Alberts (EX1039) (chapter 3), and Petsko & Ringe (EX1209).

A. Protein Structure Basics

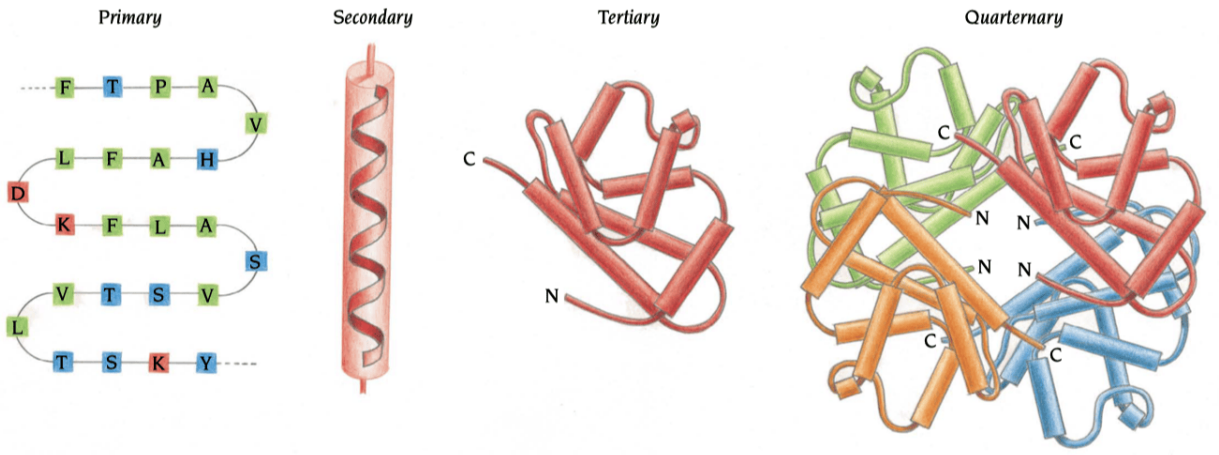
36. Proteins are comprised of amino acids arranged in order—referred to as its "primary structure" or "sequence." To exhibit its unique biological function(s), the amino acid sequence of a protein folds to adopt a precise and stable three-dimensional structure (the "native fold" or "native state" of the protein).[3] Each protein's structure – function relationship has been shaped by selective pressure applied during evolution.

> The proteins we observe in nature have evolved, through selective pressure, to perform specific functions. The functional properties of proteins depend upon their three-dimensional structures. The three-dimensional structure arises because particular sequences of amino acids in polypeptide chains fold to generate, from linear chains, compact domains with specific three-dimensional structures (Figure 1.1). The folded domains can serve as modules for building up large assemblies such as virus particles or muscle fibers, or they can provide specific catalytic or binding sites, as found in enzymes or proteins that carry oxygen or that regulate the function of DNA.

37. Regions of amino acid within the protein's amino acid sequence form regions of characteristic "secondary structures" (*e.g.*, alpha-helices, beta-strands, loop regions). Secondary structural elements associate to form structural motifs, which together with other regions of the polypeptide chain, assemble into compact globular assemblies known as domains. A protein may contain one domain or

[3] EX1014 (Branden), 3.

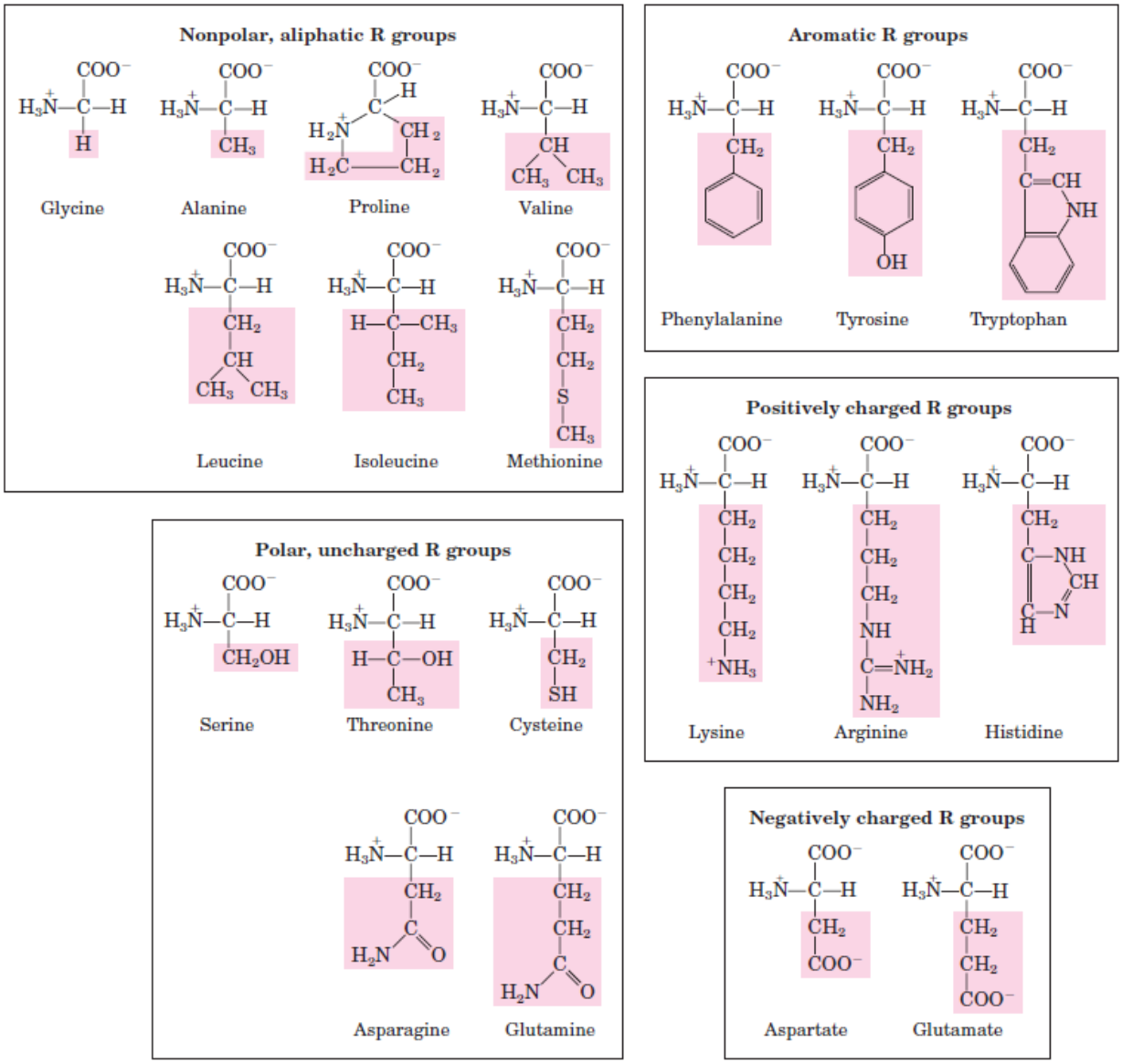
multiple domains. The overall three-dimensional structure of the protein is called its "tertiary structure." Multiple polypeptides can also form complexes, referred to as the "quaternary structure" of the protein. PH20 is a globular protein, and has a single, compact active fold; it does not have a quaternary structure.[4]



38. Amino acids are commonly grouped according to their side chains, which govern their chemical attributes and their interactions within a protein. These classifications can be described at varying levels of generality. For instance, amino acids may be categorized into distinct physiochemical classes—including (i)

[4] EX1131 (Petsko Dep.), 37:4-11; EX1039 (Alberts), 130 ("As a result of all of these interactions, most proteins have a particular three-dimensional structure, which is determined by the order of the amino acids in its chain. The final folded structure, or conformation, of any polypeptide chain is generally the one that minimizes its free energy.").

negatively charged residues; (ii) positively charged residues; (iii) polar uncharged residues, (iv) hydrophobic residues, and (v) large hydrophobic, aromatic residues, which impose different structural and energetic constraints on a protein:[5]



[5] EX1210 (Lehninger), 75 (Figure 3-5).

39. Although amino acids may be grouped according to general characteristics (*e.g.*, polar vs. non-polar), each amino acid has distinct combinations of physiochemical attributes and properties. Certain amino acids exhibit a combination of distinct chemical properties. Lysine (K) and arginine (R) contain terminal amine groups that are hydrophilic and positively charged, while also possessing extended aliphatic chains that contribute a hydrophobic character.[6] Other amino acids, including tryptophan (W), phenylalanine (F), tyrosine (Y), histidine (H), proline (P), incorporate rigid ring structures, and apart from proline are aromatic. Cysteine (C) is unique in its ability to form covalent disulfide bonds with other cysteine residues, a feature that can impart substantial structural stability to specific regions of a protein. Collectively, the wide range of side chain chemistries underlies the remarkable structural diversity observed in proteins. Due to the unique physical and chemical characteristics of each amino acid, substituting one or more amino acids within a sequence alters the local chemical environment, and can disrupt secondary or higher-order protein structures.[7]

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[6] EX1014 (Branden), 6-7.

[7] EX1039 (Alberts), 147.

40. In their natively folded state, soluble proteins (as opposed to integral membrane proteins) are typically organized with hydrophobic residues buried in the interior and hydrophilic residues exposed at the surface.

41. Alterations to a protein's structure—whether arising from environmental conditions or chemical reactions after folding or from changes to the amino acid sequence that promote unfolding or misfolding—can disrupt this native structure and expose hydrophobic regions that are normally sequestered within the protein core to the aqueous environment. Exposure of these hydrophobic patches to water promotes aggregation, either through interactions with separate protein molecules or through inappropriate intramolecular contacts between now-exposed hydrophobic residues or structures within a single protein molecule.[8] When protein molecules form aggregates, they become insoluble.

[8] EX1014 (Branden), 99 ("Before protein molecules attain their native folded state they may expose hydrophobic patches to the solvent. Isolated purified proteins will aggregate during folding even at relatively low protein concentrations. Inside cells, where there are high concentrations of many different proteins, aggregation could therefore occur during the folding process."); 118 ("The cytoplasm of all cells contains folded proteins and folding polypeptides at high concentrations. Unfolded proteins with exposed

PH20 protein molecules that form aggregates will not exhibit hyaluronidase activity.

42. Secondary structure elements generally fall into two categories: (i) the alpha-helix (α-helix), and (ii) the beta-strands (β-strands).[9] β-strands can combine to form beta sheets, an energetically favorable arrangement stabilized by hydrogen

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hydrophobic patches aggregate easily by non-specific hydrophobic interactions.").

[9] EX1014 (Branden), 32 ("The interiors of protein molecules contain mainly hydrophobic side chains. The main chain in the interior is arranged in secondary structures to neutralize its polar atoms through hydrogen bonds. There are two main types of secondary structure, a helices and b sheets. Beta sheets can have their strands parallel, antiparallel, or mixed. Protein structures are built up by combinations of secondary structural elements, α-helices, and β-strands. These form the core regions—the interior of the molecule—and they are connected by loop regions at the surface. Schematic and simple topological diagrams where these secondary structure elements are highlighted are very useful and are frequently used. Alpha helices or β-strands that are adjacent in the amino acid sequence are also usually adjacent in the three-dimensional structure.").

bonds between backbone amide (NH) and carbonyl (C'=O) groups. α helices are stabilized by hydrogen bonds between backbone amide N–H and C=O groups within the helix itself.

43. Secondary structures pack together to form larger motifs and higher order arrangements, both of which further stabilize protein structure (illustrated below).[10]

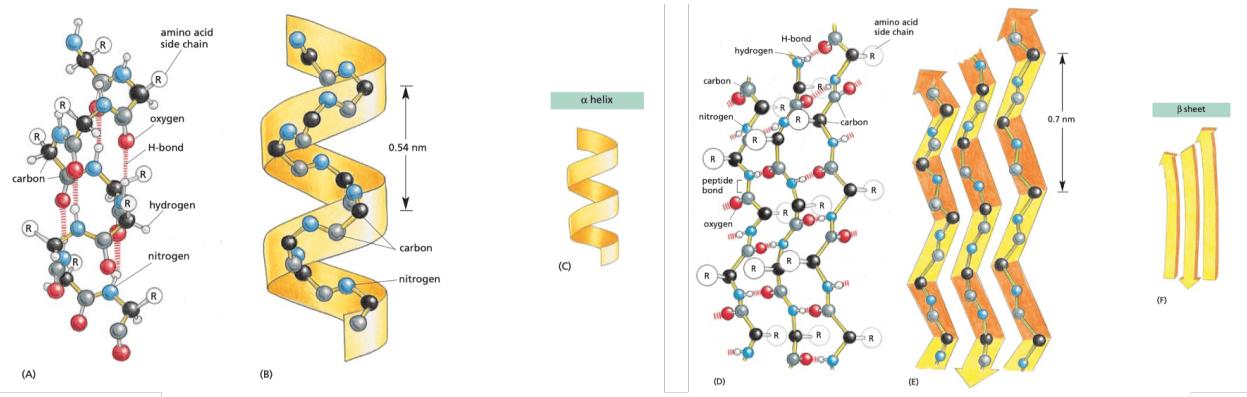


Figure 3–7 The regular conformation of the polypeptide backbone in the α helix and the β sheet. <GTAG> <TGCT> (A, B, and C) The α helix. The N–H of every peptide bond is hydrogen-bonded to the C=O of a neighboring peptide bond located four peptide bonds away in the same chain. Note that all of the N–H 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).

44. Each general category of secondary structure encompasses numerous variants, each characterized by particular amino acid patterns, spacing and residue types within the polypeptide sequence. The stability and formation of these structures depend on the identity of the amino acids and their precise positioning

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

along the sequence.[11] Certain amino acids, such as leucine, are commonly associated with and tend to stabilize helical conformations, whereas other amino acids can disrupt or disfavor secondary structures when introduced into a sequence that would otherwise form an α-helix or β-sheet.[12]

45. In an α-helix or β-sheet secondary structure, the amino acid side chains of the participating residues will either be directed to the interior of the secondary structure or directed to the 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 on the protein generally.

46. Structural motifs arise from specific combinations and spatial arrangements of secondary structures. These motifs subsequently assemble to produce the higher order structure that defines a protein domain. The formation and stabilization of such motifs are influenced by a variety of factors. For example, sequences that form secondary structures are often separated by stretches

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11 *See generally*, EX1014 (Branden), 16-20; EX1039 (Alberts), 131-35.

12 EX1014 (Branden), 353-54.

of amino acids of varying lengths, which are often referred to as "loops" or "loop regions" which are often much more flexible and mobile. 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 figure below, which shows schematically how different lengths of intervening sequences between β-sheet secondary structures influence the packing of pairs of β-sheets and then the structural motifs created by those pairs into a higher-order structure.[13] Specific types of amino acids in specific positions of an amino acid sequence thus play a critical role in inducing the proper folding of the protein into its destined structure, as well as in the protein's ability to maintain that structure under physiological conditions.[14]

[13] EX1014 (Branden), 77-78.

[14] EX1039 (Alberts), 125-30.

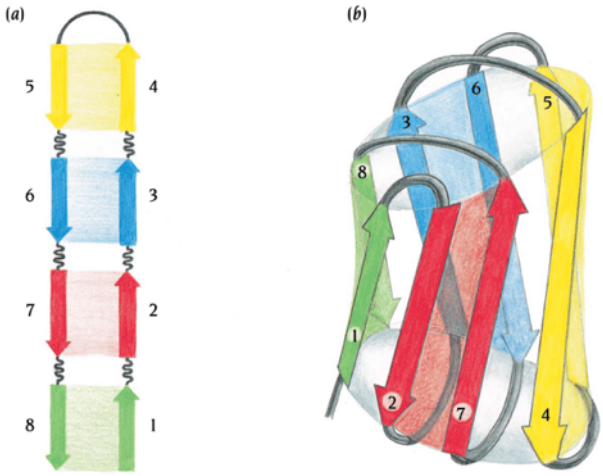


Figure 5.17 A simple illustration of the way eight β strands are arranged in a jelly roll motif. (a) The eight β strands are drawn as arrows along two edges of a strip of paper. The strands are arranged such that strand 1 is opposite strand 8, etc. The β strands are separated by loop regions. (b) The 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.

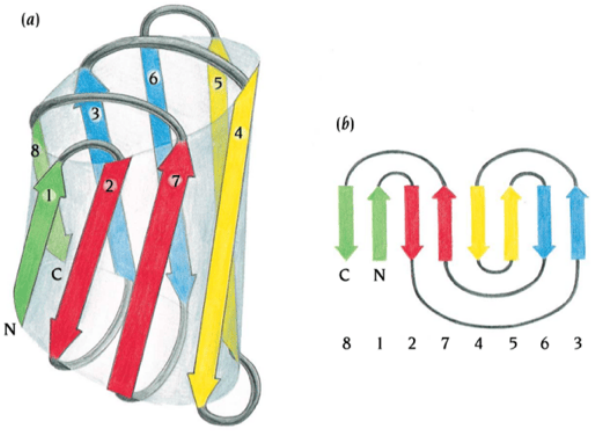


Figure 5.18 Topological diagrams of the jelly roll structure. The same color scheme is used as in Figure 5.17.

47. The formation and retention of a protein's properly folded, tertiary structure is dependent on thousands of non-covalent interactions that occur within the protein structure. Examples of non-covalent interactions that occur between residues within a protein structure are listed below:[15]

[15] EX1039 (Alberts), 125-31.

(a) Hydrogen bonds: These occur between a hydrogen atom and an electronegative atom like oxygen or nitrogen and are energetically favorable. They have both distance and orientation requirements.

(b) Ionic interactions: Often referred to as salt bridges, these interactions arise between oppositely charged side chains and contribute favorably to protein stability. In contrast, side chains have the same charge repel one another, making such interactions energetically unfavorable.

(c) Van der Waals forces: These interactions are relatively weak and occur between all atoms when they are in close proximity but have no orientation requirement. Although individually small, van der Waals interactions are generally energetically favorable and contribute to protein stability. Nonpolar side chains tend to associate with one another and are driven toward the interior of the protein to minimize water exposure. Interactions between two hydrophobic residues are energetically favorable, whereas interactions between a hydrophobic residue and a polar residue are energetically less favorable.

(d) Pi-pi interactions: These interactions occur between aromatic side chains, including phenylalanine, tyrosine, and tryptophan, and are energetically favorable, due to stacking or edge-to-face arrangements of the aromatic rings.

(e) Cation-pi interactions: These arise between positively charged side chains and aromatic side chains and are energetically favorable, contributing to the stabilization of protein structure.

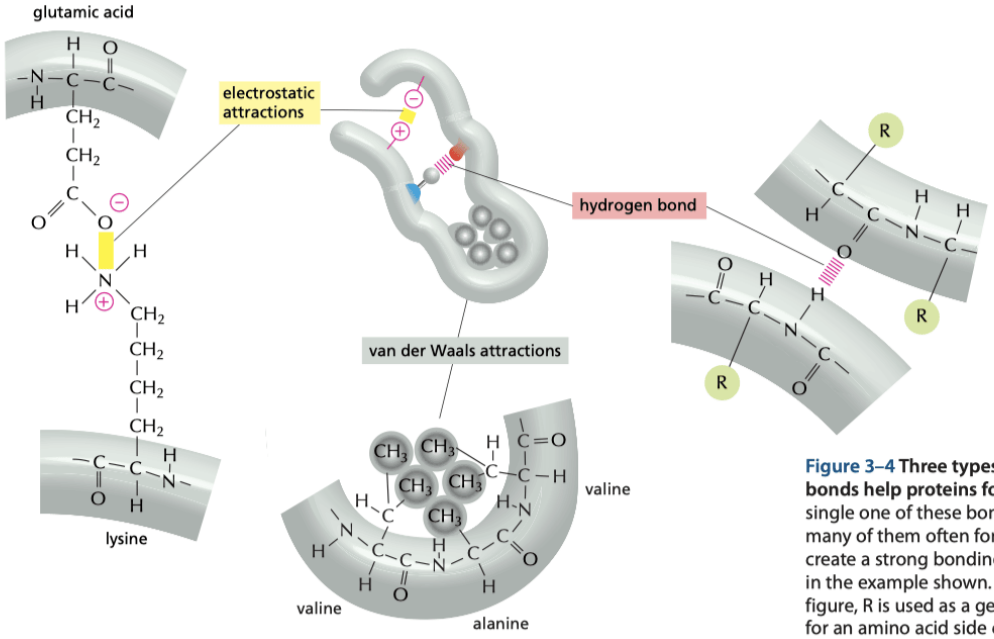


Figure 3–4 Three types of noncovalent bonds help proteins fold. Although a single one of these bonds is quite weak, many of them often form together to create a strong bonding arrangement, as in the example shown. As in the previous figure, R is used as a general designation for an amino acid side chain.

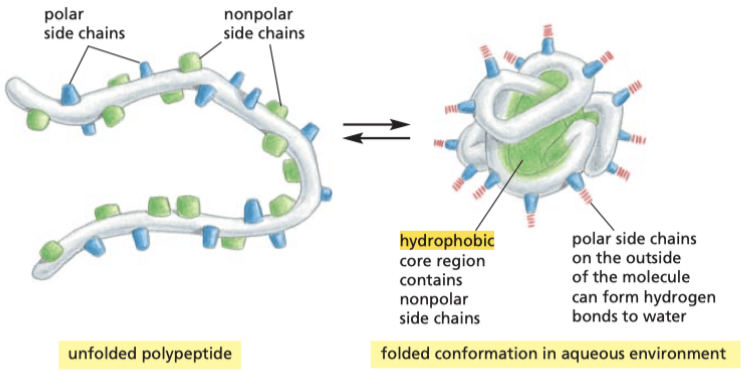


Figure 3–5 How a protein folds into a compact conformation. The polar amino acid side chains tend to gather on the outside of the protein, where they can interact with water; the nonpolar amino acid side chains are buried on the inside to form a tightly packed hydrophobic core of atoms that are hidden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

B. Protein Stability

48. The PH20 enzyme must adopt and maintain a stable tertiary structure at physiological conditions to exhibit its ability to bind to the HA substrate and

catalyze its hydrolysis.[16] However, globular proteins like PH20 are only marginally stable, and changes in pH or in temperature can convert a properly folded and active protein like PH20 into an unfolded and inactive form (figure below).[17]

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[16] EX1209 (Petsko Textbook), 3 ("For a polypeptide to function as a protein, it must usually be able to form a ***stable*** tertiary structure (or *fold*) under physiological conditions.") (emphasis added); EX1131 (Petsko Dep.), 36:7-37:30-37:20 (for PH20 to "function as a protein, it must be able to form a stable tertiary structure under physiological conditions…"); EX1131 (Petsko Dep.), 36:22-25 ("Q. Is the human PH20 protein a protein that forms a stable tertiary structure under physiological conditions? A. Fortunately, yes.").

[17] EX1014 (Branden), 90 ("Every biochemist or molecular biologist who has worked with proteins knows by experience that they are unstable. Slight changes in pH or temperature can convert a solution of biologically active protein molecules in their native state to a biologically inactive denatured state. The energy difference between these two states in physiological conditions is quite small, about 5–15 kcal/mol, not much more than the energy contribution of a single hydrogen bond, which is of the order of 2–5 kcal/mol.").

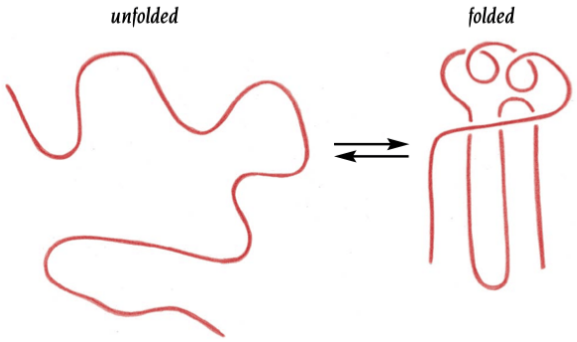


Figure 6.1 A polypeptide chain is extended and flexible in the unfolded, denatured state whereas it is globular and compact in the folded, native state.

49. The stability of a protein can be represented by the amount of energy required to induce a transition from an unfolded state (an ensemble of disordered chains) to the folded state.[18] The free energy difference between a protein's folded and unfolded states comprises two components: enthalpy and entropy. Entropy reflects the second law of thermodynamics, under which the formation of ordered structures requires an energetic cost. Enthalpy represents the stabilizing energetic contribution that offsets this cost and arises from the collective effect of thousands of non-covalent interactions that occur between the residues within the folded form of the protein.[19] Each of those non-covalent interactions can be energetically favorable or unfavorable, and can vary in magnitude. These non-covalent interactions also are dynamic rather than fixed, reflecting the continuous motion of

[18] EX1014 (Branden), 90; EX1039 (Alberts), 126.

[19] EX1014 (Branden), 90.

residues with proteins and their sensitivity to environmental conditions such as pH, temperature, and ionic strength.[20]

50. The "net free energy of stabilization" represents the net difference in energy resulting from the decrease in enthalpy that occurs during the formation of hydrogen bonds and through van der Waals interactions in the protein's folded state (below).[21] For many proteins, this net free energy difference between the two states is small (*e.g.*, 5-15 kcal/mol), which is not much greater than the energy contribution of a handful of hydrogen bonds or hydrophobic contacts.[22]

> Finally, even though many hundreds of hydrogen bonds and van der Waals interactions occur in a folded protein, the net free energy of stabilization of most folded proteins—the difference in free energy between the folded and unfolded states—is actually rather small, about 21–42 kJ/mole, or only about 10 times the average thermal energy available at physiological temperature. Most folded proteins are marginally stable because the free energy released when hundreds of weak interactions form is almost exactly counterbalanced by the enormous loss of conformational flexibility (loss of entropy) that occurs when the unfolded chain folds into a compact, ordered structure. A folded protein is a thermodynamic compromise.

51. The "free energy" difference between folded and unfolded states of a protein can be experimentally determined. In the 2011-2012 timeframe, POSAs could use various methods that involve subjecting the protein to changing conditions—such as increasing concentrations of a denaturing agent (*e.g.*, urea,

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20 EX1039 (Alberts), 126-31; EX1014 (Branden), 13-14.

21 EX1209 (Petsko Textbook), 26.

22 EX1014 (Branden), 90; EX1209 (Petsko Textbook), 26.

guanidine HCL) or elevated temperatures—and monitoring the point at which the protein transitions from a folded to an unfolded state using appropriate instrumentation (illustrated below).[23] For example, differential scanning calorimetry (DSC) measures changes in heat capacity as the protein is heated and enables determination of both the melting temperature and the enthalpy change associated with unfolding. Other known techniques commonly used to monitor folding–unfolding transitions include circular dichroism, fluorescence or nuclear magnetic resonance (NMR) spectrometers.[24]

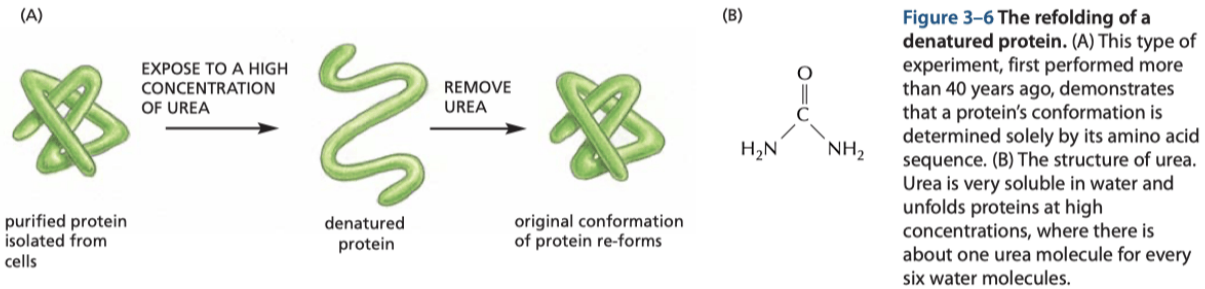


Figure 3–6 The refolding of a denatured protein. (A) This type of experiment, first performed more than 40 years ago, demonstrates that a protein's conformation is determined solely by its amino acid sequence. (B) The structure of urea. Urea is very soluble in water and unfolds proteins at high concentrations,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

52. Mutations introduced into an amino acid sequence can (and usually will) alter the net free energy of stabilization of the protein because they will alter the non-covalent interactions that are responsible for the protein's stability. If the changes decrease the net free energy of stabilization of the protein, the mutated

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23 EX1039 (Alberts), 130-31.

24 EX1136 (Dobson 2004a), 5, 7, Table 1 (illustrating experimental techniques used to investigate protein folding, structural features, and aggregation).

protein may not fold, or if it does fold, may be more prone to partial or complete unfolding at physiological conditions. Changes that cause even modest reductions in the net free energy of stabilization of a modified PH20 polypeptide can slow or prevent folding or result in rapid unfolding of the protein after it has folded and/or been secreted. Mutations can thus destabilize a modified PH20 protein, which will cause it to partially or completely unfold, resulting in a loss of its ability to bind the HA substrate and/or hydrolyze it.[25]

53. In the 2011-2012 timeframe, a POSA could not have predicted the effect of a set of mutations on the net free energy of stabilization of the PH20 protein.[26] Doing that would have required the POSA to determine the collective

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[25] EX1209 (Petsko Textbook), 3 ("For a polypeptide to function as a protein, it must usually be able to form a ***stable*** tertiary structure (or *fold*) under physiological conditions.") (emphasis added); EX1131 (Petsko Dep.), 36:7-37:3 (for PH20 to "function as a protein, it must be able to form a stable tertiary structure under physiological conditions…").

[26] EX1131 (Petsko Dep.), 102:1-9 ("Q. So your testimony is that a person of ordinary skill in the art in the 2011-2012 timeframe would be able to predict the change to a protein's net free energy of stabilization associated with a set of 10 or 15 or 20 mutations? A. No. They would not be able to predict the

net energetic effect of thousands of non-static interactions that occur within the PH20 protein in its folded state.

54. Dr. Petsko's textbook provides an illustration of the potential impact of even a single mutation to the structure and stability of a globular protein (*e.g.*, PH20).[27] In this example, the single mutation does not prevent secretion of the globular protein but disrupts its stability to a degree that causes it to partially or completely unfold in the extracellular environment. After that occurs, the sequence can refold partially or completely. However, as Dr. Petsko's textbook explains, "the partially unfolded form is prone to aggregation, which results in the formation of fibrils [] and other aggregates that accumulate in the extracellular space."[28]

number. They would be able to get a sense of whether those were likely to be destabilizing or not.").

[27] EX1209 (Petsko Textbook), 160 (Figure 4-53); *see also* EX1140 (Dobson 1999), 329 (Figure 1).

[28] EX1209 (Petsko Textbook), 160 (Figure 4-53).

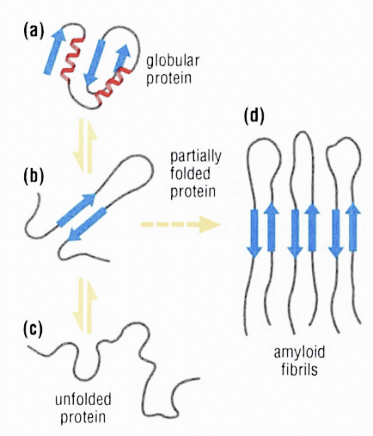


Figure 4-53 A possible mechanism for the formation of amyloid fibrils by a globular protein The correctly folded protein **(a)** is secreted from the cell. Under certain conditions, or because it contains a mutation, the protein unfolds partially **(b)** or completely **(c)**; the unfolded forms can also refold partially or completely. The partially unfolded form is prone to aggregation, which results in the formation of fibrils **(d)** and other aggregates that accumulate in the extracellular space.

55. Aggregation can occur when hydrophobic patches on a protein that are ordinarily buried in the protein interior become exposed to the hydrophilic aqueous environment. Rather than remain exposed in the aqueous environment, the hydrophobic patches will strongly favor binding to other hydrophobic patches available in the environment (*e.g.*, different hydrophobic patches within the same protein, or in other modified proteins). This phenomenon is explained in textbooks, like Branden & Tooze (below).[29]

[29] EX1014 (Branden), 99-100, 118.

Proteins can fold or unfold inside chaperonins

Before protein molecules attain their native folded state they may expose hydrophobic patches to the solvent. Isolated purified proteins will aggregate during folding even at relatively low protein concentrations. Inside cells, where there are high concentrations of many different proteins, aggregation could therefore occur during the folding process. This is prevented by

molecular chaperones, ubiquitous and abundant families of proteins that assist the folding of both nascent polypeptides still attached to ribosomes and released completed polypeptide chains. Several chaperones are induced by heat shock, because protein unfolding and aggregation is increased at elevated temperatures; and consequently chaperones were first classified as **heat-shock proteins** (Hsps). Several of these heat-shock proteins, such as the 70 kDa protein **DnaK** (**Hsp 70**) are also expressed normally and participate in various other cellular processes including assembly and disassembly of multimeric proteins and membrane translocation of secreted proteins. The Hsp

The cytoplasm of all cells contains folded proteins and folding polypeptides at high concentrations. Unfolded proteins with exposed hydrophobic patches aggregate easily by non-specific hydrophobic interactions. To circumvent this problem a class of proteins called chaperones have evolved to sequester unfolded polypeptides. The complex structure of one class of multimeric chaperones, the chaperonins GroEL and GroES, has been elucidated and has shed light on how chaperones function. These chaperonins are short cylinders which, because they have hydrophobic residues in the interior, can bind to any unfolded polypeptide that has large exposed hydrophobic patches, regardless of its amino acid sequence. Once the polypeptide chain is shielded inside the chaperonins it is protected from aggregation with other protein molecules. During folding some polypeptide chains go through many cycles of binding and release from the chaperonins.

56. Aggregation is a phenomenon that occurs commonly when a protein is not folded into its native state, which can occur inside or outside the cell, and is one of "[t]he most common obstacles to correct folding"[30] (below).

[30] EX1014 (Branden), 91-92, 99-100, 118.

Such a folding mechanism raises several important questions that are difficult to examine experimentally, since the possible intermediates have a very short lifetime. If kinetic factors are important for the folding process it is possible that the observed folded conformation is not the one with the lowest free energy but rather the most stable of those conformations that are kinetically accessible. The protein might be kinetically trapped in a local low energy state with a high energy barrier that prevents it from reaching the global energy minimum which might have a different fold. In such a case structure prediction by energy calculations would give the wrong structure even if such calculations could be made with great accuracy. One important question therefore is how a living cell can prevent the folding pathway from becoming blocked at an intermediate stage. The most common obstacles to correct folding seem to be (1) aggregation of the intermediates through exposed hydrophobic groups, (2) formation of incorrect disulfide bonds, and (3) isomerization of proline residues. To circumvent these three obstacles cells produce special proteins that assist the folding process, as we shall discuss later in this chapter.

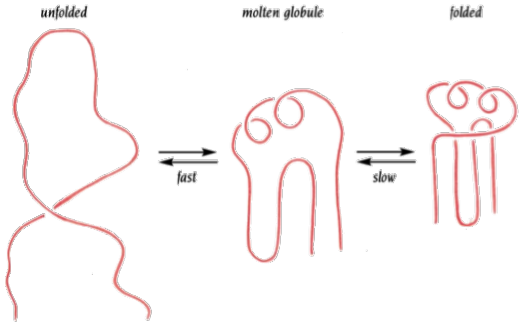


Figure 6.2 The molten globule state is an important intermediate in the folding pathway when a polypeptide chain converts from an unfolded to a folded state. The molten globule has most of the secondary structure of the native state but it is less compact and the proper packing interactions in the interior of the protein have not been formed.

57. Aggregation is a "fundamental problem in biotechnology"[31] and a "key challenge in the development of protein biotherapeutics."[32] For example,

[31] EX1155 (Calloni), 10607.

[32] EX1158 (Wang), 1, Abstract; EX1026 ('731 App.), 118:15-119:6 ("In particular, increasing or high amounts of preservatives can negatively impact the stability of a protein, including effects on physical stability (aggregation or precipitation) that can impact protein activity."); *see also* EX1152 (Ringe &

"the *de novo* design of novel proteins or the rational modification of existing proteins, is frequently frustrated by the fact that the polypeptide chains aggregate into large assemblies."[33] A protein's propensity to aggregate is not "restricted to a relatively few amino acid sequences associated with specific diseases but is a generic phenomenon of polypeptide changes."[34]

58. Individual mutations can directly impact the likelihood of protein aggregation by, for example, increasing "the hydrophobicity of the polypeptide chain", increasing "its propensity to convert from α-helical to β-sheet structure" or decreasing "the overall net charge on the protein molecule."[35] Critically, the effects of ***multiple*** mutations on aggregation can be cumulative, arising from combined perturbations to fundamental physicochemical properties of the polypeptide chain.[36] As a result, substitutions that do not individually produce

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Petsko), 80.1, 80.3 (acknowledging that mis-folded proteins can aggregate with "deleterious consequences").

[33] EX1155 (Calloni), 10607.

[34] EX1156 (Chiti 2002), 16419.

[35] EX1155 (Calloni), 10607.

[36] EX1157 (Chiti), 808.

detectable aggregation can, when combined, increase the rate or likelihood of aggregation sufficiently to yield an aggregated protein.[37]

C. Protein Engineering

59. Protein engineering involves the deliberate modification or design of proteins to impart properties that are not present in naturally occurring proteins. At a conceptual level, when starting from reference proteins found in nature, there are two broad approaches for creating engineered proteins, referred to as "rational design" and "directed evolution."[38]

60. A concise summary of the two alternative approaches—rational design on the one hand and directed evolution on the other—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

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37 EX1157 (Chiti), 808.

38 EX1059 (Leisola), 1225-1227; EX1018 (Chica), 378-79; EX1017 (Green), 223.

> 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.[39]

61. I discuss these approaches and their limitations in the 2011-2012 timeframe below.

1. Rational Design

62. "Rational design" refers to an approach in which a protein's structural characteristics are analyzed to understand how particular features contribute to its stability and function. That analysis, combined with established principles of protein structure, is then used to plan specific changes to the protein sequence (*e.g.*, substitutions, deletions, or insertions) intended to modify structural features in a controlled way. The objective of these sequence modifications is to produce a protein that exhibits targeted properties, for example improved stability or enhanced activity.[40]

63. Rational protein design commonly relies on a range of computational techniques. One such technique involves examining evolutionary-related, homologous proteins. By generating sequence alignments of these homologous proteins, conserved residues can be identified, as well as positions at which amino

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[39] EX1059 (Leisola), 1226, Figure 1.

[40] EX1018 (Chica), 378.

acid variation occurs and therefore tolerated.[41] The other important tool is the structural model of the protein, which can be generated using automated procedures, such as the SWISS-MODEL server. A protein engineer conducting a study using rational design would use these models to visualize parts of the protein structure, and consider spatial interactions between the different residues.[42]

64. At the time point relevant to my opinions here (2011-2012), the application of rational design methods was subject to practical limitations.[43] Introducing a single amino acid substitution into a sequence region not essential to the protein's function was generally accepted to be predictable in outcome (*i.e.*, whether the change would have a positive, negative, or neutral effect on the structure of the protein in that region). In such cases, the effects of the substitution could be evaluated by considering interactions between the introduced residue and

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41 EX1017 (Green), 224-28.

42 EX1017 (Green), 227-30.

43 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), 1225-26.

its surroundings. However, a POSA's confidence in the ability to predict structural outcomes would decrease rapidly as additional substitutions are introduced. While evaluating combinations of two or three substitutions might have been considered a manageable and reasonable undertaking, by the time one reaches five substitutions, the combinatorial complexity and interdependence among residues make reliable prediction effectively impossible. Each substitution alters the local environment of the others, resulting in non-linear and context-dependent effects that cannot be robustly anticipated through rational design. As the number of changes increases, the required experimental effort escalates correspondingly. I discuss these issues further below. *See* §§ II.D-II.E.

2. Directed Evolution

65. Directed evolution developed in response to the practical limitations of rational design.[44] Like rational design, its objective is to generate proteins with altered amino acid sequences that confer desired properties. Rather than attempting to predict favorable sequence changes in advance, directed evolution

[44] EX1059 (Leisola), 1225-26 ("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.").

instead relies on iterative experimentation. In this approach, large libraries of mutant proteins containing randomly introduced sequence variations are generated using genetic techniques and then screened to identify variants exhibiting the target properties. Mutants displaying improved characteristics are then isolated and further analyzed, and the amino acid sequences associated with those enhanced properties are ultimately identified and recorded.

66. The challenge with directed evolution is scale.[45] Identifying a successful variant requires selecting a functional mutant from an enormous number of possible sequence variants, which presents its own set of challenges. In practice, successful directed evolution efforts depend on the availability of

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

advanced methodologies for introducing mutations with a reasonable likelihood of producing beneficial effects, as well as screening approaches that are rapid, accurate and capable of distinguishing variants which have desired properties from those without. Efficient techniques for recovery and characterization of the desired mutants are also required. In addition, the approach is constrained by fundamental scientific considerations: the targeted function must be physically achievable in the protein, and the protein must be capable of accommodating mutations that confer 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 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.[46]

[46] EX1059 (Leisola), 1226-27.

67. Even the largest scale screening techniques available in 2011-2012 could not screen more than around ~10^{12} mutants, which represents an extremely small fraction of the sets of sequences captured by the sequence identity parameters of the claims (discussed below in § IV).[47] A June 2013 review of large scale screening technologies used with enzymes identified a practical upper limit of about 10^9 sequences:[48]

Table 1. Characteristics of general *in vivo* screening and selection strategies. It is the product of the enzymatic reaction or the enzymatic conversion itself that needs to be detected, making these strategies reaction dependent (in contrast to most reporter-based strategies). Requirements encompass everything except basic microbial techniques/reagents and substrate. m, metagenomic library; ev, enzyme variant library. Adapted from [3].

Strategy	Library type	Library size	Requirements	Advantages	Disadvantages
Selection	m/ev	~ 10^9	None	Only desired variants	Low activity variants might be missed
				Simple to operate	Difficult to quantify
					Higher false positive risk
Agar plate screening	m/ev	~ 10^5	Reagents[a]	Simple to operate	Low dynamic range
			Detection device[a]		Difficult to quantify
Microtiter plate screening	m/ev	~ 10^4	Detection device	Multiple analytical detection methods possible	
				Increased sensitivity by cell lysis	
				Large dynamic range	
Cell-in-droplets (screening)	m/ev	~ 10^9	Detection device	Pico/femto-litre scale (not much reagent needed)	Product should stay in the droplet
			Cell sorter		Technically challenging
Cells as micro-reactors (screening)	m/ev	~ 10^9	Detection device	Femto-litre scale (not much reagent needed)	Product should stay in the cell
			Cell sorter	No compartmentalization needed	Technically challenging
Cell surface display (screening)	ev	~ 10^9	Detection device	Substrate can stay extracellular	Product needs to attach to cell surface
			Cell sorter		Technically challenging
					Careful selection of host and anchoring motif needed
					Fusion can alter enzyme structure/activity

[a] In some cases.

47 EX1141 (Goldsmith), 409 (describing limit of "ultrahigh throughput" screening techniques being 10^6 to 10^{12} mutants).

48 EX1147 (van Rossum), 2991 (Table 1).

68. There also were significant challenges in designing and carrying out multiple mutagenesis experiments in the 2011-2012 timeframe. These challenges include the epistatic effect of combining mutations and were summarized concisely in a 2012 review by Goldsmith (EX1141) noting that a "target change in activity depends on highly epistatic mutations that demand the simultaneous exploration of more than one position."[49] The '731 Application's disclosure whilst suggesting an iterative mutagenesis strategy to discover multi-mutated PH20s provides no guidance or insight as to how these well-known (in the relevant timeframe) challenges could be addressed.

> **To screen or select?**
> The main dilemma facing those designing directed enzyme evolution experiments is whether to apply a high, or even ultrahigh throughput selection methodology (10^6 to $>10^{12}$ variants processed in parallel), or a screen that might be of much lower throughput (10^2–10^4 variants tested individually). Ultra or high throughput screens or selections become advantageous with libraries of variants generated by extensive random mutagenesis or by simultaneous saturation mutagenesis of many positions. Such libraries need to be applied when: firstly, the available phylogenetic and/or structural information is insufficient for the design of small and focused libraries. Secondly, the identity and position of the mutations required to induce the desired property are hard to predict. Thirdly, the target change in activity depends on highly epistatic mutations that demand the simultaneous exploration of more than one position. In these, and possibly in other cases, large libraries might be the method of choice.

[49] EX1141 (Goldsmith), 409.

69. The '731 Application proposes to target positions identified in a first library that were identified as being deleterious in a first library (below).[50] But because of epistasis (*see* § II.D), combinations generated in a second or subsequent library may alter the effect of the first mutation.

> 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. A further library of modified proteins can be generated with amino acid mutations targeted at or near to the identified critical amino acid residues, such as adjacent to the identified critical amino acid residues. In some examples, the mutations can be amino acid replacement to any other of up to 19 other amino acid residues. The secondary library can be tested using the assays and methods described herein.

70. The number of possible sequences encompassed by the challenged claims (*i.e.,* the sequence space) is extraordinarily large—far beyond what a person of ordinary skill in the art could realistically explore or test.[51] This is evident from the parameters in the claims (illustrated with claim 4 below).

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50 EX1026 ('731 App.), 150:14-26.

51 I provide a more detailed assessment of the "sequence space" defined by each claim in § IV.A.

a. Claim 4 requires at least one amino acid replacement that confers increased hyaluronidase activity selected from the 96 specific substitutions at one of 37 positions in PH20 as shown (below).

Position	Amino Acid Replacement(s)	Position	Amino Acid Replacement(s)
12	E	261	N
26	E, K	277	R, T
46	R	309	L, N, Q, R, T
52	Q	310	A, G
58	Q	315	H
68	H, Q	317	I, K, R
70	A, C, F, G, H, K, L, N, P, R, S, T	318	M
74	A, C, G, N, P, R, S, V, W	347	G, S
84	G, R	399	M, T, W
97	G	407	D, Q
127	H, N	419	F, I, R, S
131	G, V	421	K, N, Q, R, S
142	R	431	A, H, K, Q, R, S, V
166	A, F, H, L, R, Y	433	L, R
174	K	439	V
206	K	440	H, R
213	M, N	443	A, M
237	E	445	M, P
238	S		

b. Claim 4 then permits the amino acid sequence to be 95% identical to one of the recited "unmodified" PH20 sequences (*e.g.*, SEQ ID NOS: 3, 7, and 32-66). As discussed in Section

IV, below, if SEQ ID NO: 3 is the reference sequence, this permits the sequence to have an additional 22 substitutions. Even under a highly conservative assumption, the "sequence space" covered by these parameters is astronomical. The table below illustrates the growth in the number of sequences possible with each additional mutation after a first, assuming that SEQ ID NO: 3 ($PH20_{1-447}$) is used as the reference sequence and just two alternatives are permitted at the one required substitution (which can be at one of the 37 positions):

Additional Mutations	Sequences		% Total
1	16,950	1.70E+04	0.00%
2	71,664,620	7.17E+07	0.00%
3	201,544,912,660	2.02E+11	0.00%
4	424,151,627,100,830	4.24E+14	0.00%
5	712,490,709,670,351,000	7.12E+17	0.00%
6	995,113,439,017,070,000,000	9.95E+20	0.00%
7	1,188,593,960,274,710,000,000,000	1.19E+24	0.00%
8	1,239,408,841,642,340,000,000,000,000	1.24E+27	0.00%
9	1,146,180,397,864,990,000,000,000,000,000	1.15E+30	0.00%
10	951,790,683,583,952,000,000,000,000,000,000	9.52E+32	0.00%
11	716,873,523,810,270,000,000,000,000,000,000,000	7.17E+35	0.00%
12	493,807,967,214,815,000,000,000,000,000,000,000,000	4.94E+38	0.00%
13	313,265,281,703,832,000,000,000,000,000,000,000,000,000	3.13E+41	0.00%
14	184,111,187,741,072,000,000,000,000,000,000,000,000,000,000	1.84E+44	0.00%
15	100,758,334,357,507,000,000,000,000,000,000,000,000,000,000,000	1.01E+47	0.00%
16	51,575,902,930,433,400,000,000,000,000,000,000,000,000,000,000,000	5.16E+49	0.00%
17	24,789,924,805,868,100,000,000,000,000,000,000,000,000,000,000,000,000	2.48E+52	0.00%
18	11,227,138,919,686,200,000,000,000,000,000,000,000,000,000,000,000,000,000	1.12E+55	0.00%
19	4,805,832,508,728,440,000,000,000,000,000,000,000,000,000,000,000,000,000,000	4.81E+57	0.00%
20	1,949,737,500,771,650,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000	1.95E+60	0.00%
21	751,581,971,925,538,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000	7.52E+62	0.27%
22	275,900,686,198,289,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000	2.76E+65	99.73%

c. With those same constraints, but allowing up to 10 total modifications per $PH20_{1–447}$ sequence (as recited by Claim 16), the number of possible sequences expands similarly.

Additional Mutations	Sequences	#	% Total
1	16,950	1.70E+04	0.00%
2	71,664,620	7.17E+07	0.00%
3	201,544,912,660	2.02E+11	0.00%
4	424,151,627,100,830	4.24E+14	0.00%
5	712,490,709,670,351,000	7.12E+17	0.00%
6	995,113,439,017,070,000,000	9.95E+20	0.00%
7	1,188,593,960,274,710,000,000,000	1.19E+24	0.00%
8	1,239,408,841,642,340,000,000,000,000	1.24E+27	0.11%
9	1,146,180,397,864,990,000,000,000,000,000	1.15E+30	99.89%

71. The production and screening of this number of PH20 mutants would be impossible given the astronomical size of the sequence space at issue and the limits of screening technologies existing in 2011-2012. The size of the libraries would rapidly exceed the capacity of available screening technologies in the 2011-2012 time frame to screen them for hyaluronidase activity.

Additional Mutations	Sequences	#
1	16,950	1.70E+04
2	71,664,620	7.17E+07
3	201,544,912,660	2.02E+11
4	424,151,627,100,830	4.24E+14
5	712,490,709,670,351,000	7.12E+17
6	995,113,439,017,070,000,000	9.95E+20
7	1,188,593,960,274,710,000,000,000	1.19E+24
8	1,239,408,841,642,340,000,000,000,000	1.24E+27
9	1,146,180,397,864,990,000,000,000,000,000	1.15E+30



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

72. 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 (for example, if a residue is involved in catalysis carried out by the protein)

or is an amino acid essential to the folding or stability. A single amino acid substitution in a protein that is made at a position that is fully solvent exposed (*i.e.*, interacts primarily with water molecules rather than other residues of the protein) and varies extensively within a set of homologous, naturally occurring variants of the protein is less likely to be impactful to the folding, stability or function of the protein. A POSA, however, would need to assess each position and each proposed mutation on a case-by-case basis by considering the characteristics of the wild-type and substituted amino acids and the environment within the protein where the change is proposed to be made (*e.g.,* whether it is a buried or solvent-exposed residue, whether it is in a secondary structure, loop or other region).

73. Introducing multiple mutations—whether substitutions, insertions, or deletions—into a protein sequence presents a markedly different challenge. Many secondary structural elements depend on specific amino acid patterns, including precise spacing and residue characteristics at defined positions. When more than one amino acid is altered at the same time, those patterns can be disrupted, increasing the risk that the original secondary structure will fail to form. Loss or alteration of a secondary structural element can, in turn, interfere with proper folding and significantly destabilize to the overall protein structure.[52]

[52] EX1046 (Beasley), 2033-34; EX1047 (Xiong), 6349, 6352-53.

74. Introducing multiple substitutions or other types of modifications (*e.g.*, insertions, deletions) 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 that are possible, with each depending on the pair of amino acids being considered (*see* ¶ 47).[53] Any single modification may impact numerous interactions, potentially with competing effects (*e.g.*, some beneficial and some adverse to the protein's stability).

75. Within any given region of a protein, numerous interactions occur among neighboring residues, and together these interactions define the local structure. The combined effect of these interactions governs the stability of that region and its arrangement in the overall protein structure. A substitution, insertion, or deletion introduced into a particular region of a protein will alter, even if only subtly, interactions with neighboring residues that favor local or overall stability. As substitutions accumulate, their effects become increasingly interdependent and context-specific, such that changes that might appear beneficial in isolation can destabilize the structure when combined (*see* ¶ 64). Combining multiple modifications may destabilize a structure, despite any stabilizing

[53] EX1039 (Alberts), 126-27, 130.

contribution from the first single substitution. These issues are especially likely where the additional changes are introduced in the same region.

76. Introducing multiple mutations into a protein can cause unpredictable effects due to the well-known phenomenon termed "epistasis." This can occur when an individual mutation has no detectable deleterious effect on folding or activity of the protein. Epistasis is commonly categorized into three types.[54] *Magnitude epistasis* occurs when the combined effect of two mutations differs from the effect predicted by simple additivity, even though the direction (beneficial or deleterious) of each mutation remains the same. *Sign epistasis* occurs when a mutation that is beneficial in one sequence background becomes deleterious in the presence of another mutation. *Reciprocal sign epistasis* occurs when two mutations that are each individually beneficial become jointly deleterious when combined, yielding a protein that is less active than the native form. These principles apply not only to enzymatic activity, but also to protein stability and folding, reflecting the fundamentally non-linear effects of combining mutations on protein properties. Quantifying epistatic interactions is highly complex area of research, and in the 2011–2012 timeframe such effects could not be predicted in a general or reliable manner.

[54] EX1160 (Poelwijk), 383-85.

77. The observed impact of an additional mutation —whether beneficial, neutral, or deleterious—depends on the other mutations already present in the protein sequence. This context-dependence is the central reason why single-mutant data, which measures each mutation only in the wild-type context, has limited predictive power for combining mutations in a single polypeptide. Epistasis progressively limits POSAs confidence in their ability to predict the functional impact of additional mutations and strongly hinders rationally protein design strategies where multiple mutations are introduced (*see* ¶ 64). As each additional mutation is introduced, the potential for epistasis grows.

78. Deleterious epistatic interactions can arise when individually tolerated mutations are combined, disrupting proper folding. Such efforts may be local and long-range, reflecting interactions between otherwise independent changes.[55] Consequences of these deleterious epistatic interactions include:

- Disruption of successful folding of a nascent polypeptide chain within the cell by disrupting co-translational folding kinetics or creating aggregation-prone folding intermediates. For example,

[55] EX1131 (Petsko Dep.), 270:9-17 ("And charged residues can exert long-range electrostatic effects, for example, that have nothing to do with direct binding.").

the combination may alter local or long-range intramolecular interactions not caused by any single substitution;

- Unpredictable changes in protein stability, where independently neutral substitutions may collectively disrupt packing interactions, hydrogen-bonding networks, electrostatic complementarity, or dynamic motions that are only critical when multiple sites are altered simultaneously. Impacts on stability can also occur via long-range electrostatic or allosteric effects associated with the individual changes; and
- Disruption of different functional effects in combination that are not observed as individual mutations (*e.g.*, altering the spatial positioning of catalytic residues, impairing substrate binding, causing long-range electrostatic effects on either).

79. Systematic scanning mutagenesis studies published before and around the 2011-2012 timeframe illustrated epistasis by directly examining the effects of large numbers of single, double, and higher-order substitutions. These studies demonstrated that positional tolerance cannot be inferred from a small or "representative" subset of substitutions, and that increasing numbers of combined mutations rapidly lead to deleterious outcomes. For example, Pal and colleagues performed comprehensive combinatorial scanning of a protein–protein interaction

interface and showed that mutational tolerance is highly context-dependent and cannot be predicted from limited sampling or from chemically "conservative" substitutions alone.[56] Similarly, Fowler and colleagues applied deep mutational scanning to map protein sequence–function relationships at high resolution and explicitly quantified the effects of single, double, and triple mutants across a large mutational space. Although many individual single substitutions were tolerated, the overwhelming majority of higher-order variants were deleterious relative to wild type. In that study, while hundreds of single substitutions retained activity, approximately 97.2% of the variants observed—including the vast majority of double and triple mutants—were deleterious.[57] These results illustrate that effects observed for individual substitutions do not translate into predictability for combinations of substitutions.

80. The formation of secondary and higher order structures often depends on the precise spatial and temporal positioning of segments of the polypeptide chain during the folding process.[58] When multiple substitutions are introduced into a sequence, those changes can disrupt the assembly of secondary structural

[56] *See, e.g.*, EX1146 (Pal), 22381–82.

[57] EX1151 (Fowler), 743-44.

[58] EX1209 (Petsko Textbook), 52-53.

elements in into higher-order structures. Introducing several amino acid changes at the same time may therefore interfere with the formation of secondary structures and structural motifs or destabilize those structures even if they are initially formed.

81. A series of articles by Dr. Christopher Dobson at Cambridge University before 2011 provide a useful overview of the complexity and challenges of protein folding, and the diversity of conformations an amino acid sequence can adopt. Below are two of his figures. The first (Figure 5 from EX1136) illustrates the various events that can occur after synthesis of an amino acid sequence that can prevent a sequence from folding into the tertiary structure found in the native state of the wild-type protein (designated "native" in the figure).[59] It is perfectly possible that proportions of a single sequence will end up as the different species shown in Figure 5 (some folded, some aggregated etc.).

[59] EX1136 (Dobson 2004a), 11 (Figure 5); *see also* EX1137 (Dobson 2003), 888 (Figure 4); EX1138 (Dobson 2004b), 11 (Figure 2); EX1131 (Petsko Dep.), 38:18–39:23 ("Q. Okay. So when a protein like PH20 folds, does it proceed through intermediate states or structures before it reaches its final folded tertiary state? A. It's thought to for all proteins that fold…").

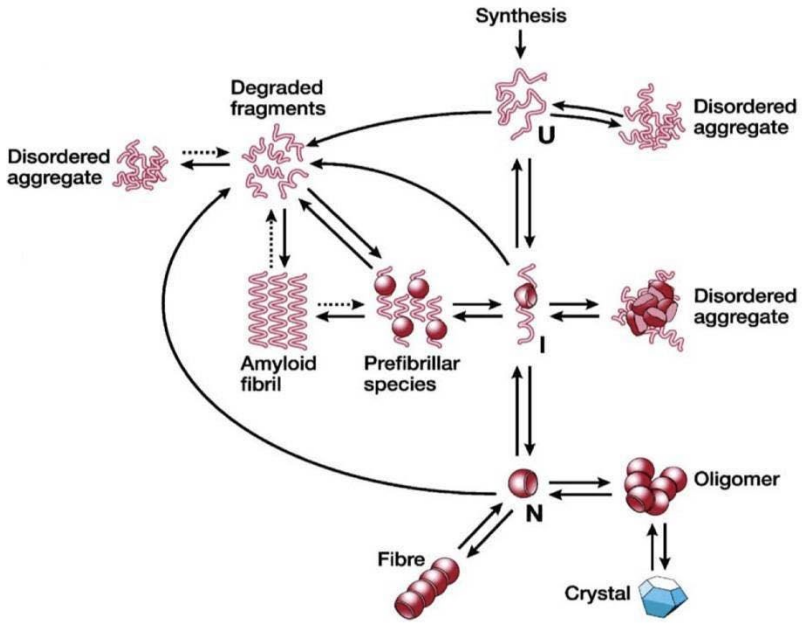


Fig. 5. Schematic representation of some of the states accessible to a polypeptide chain following its synthesis on a ribosome. The relative populations of the different states depend on the kinetics and thermodynamics of the various equilibria shown in the diagram. In living systems the fate of a given protein molecule is closely regulated by molecular chaperones and other quality control mechanisms, rather as metabolic pathways in cells are controlled by enzymes and associated molecules such as cofactors. From [36].

82. The second figure (Figure 2 of EX1139) identifies various conformational states that amino acid sequences can adopt, and how those conformation states can transition from one to the other. As the caption to Figure 2 explains, the figure provides a "…representation of some of the many conformational states that can be adopted by polypeptide chains and of the means by which they can be interconverted." [60]

[60] EX1139 (Chiti & Dobson 2006), 351 (Figure 2).

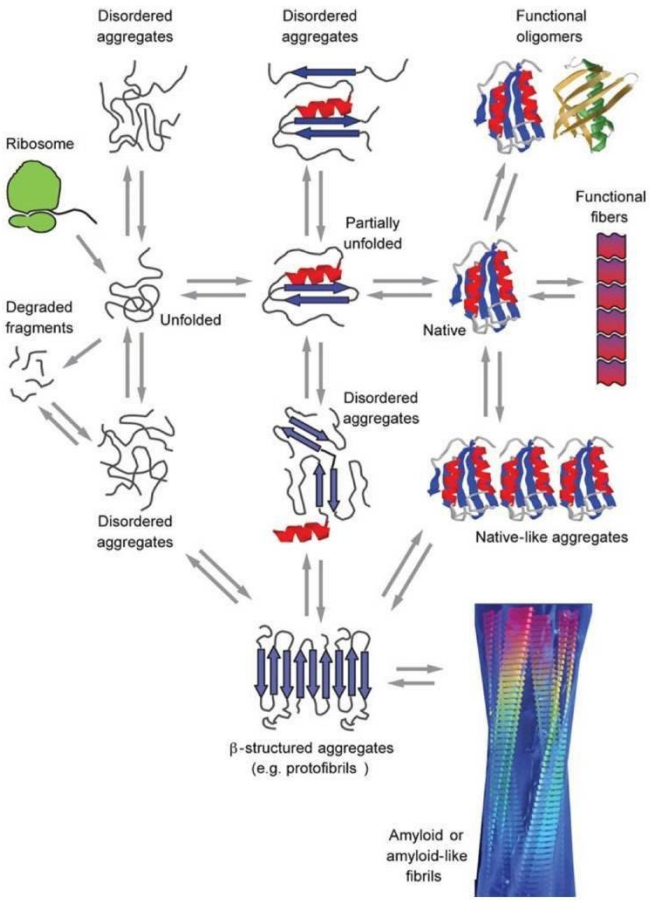


Figure 2

A schematic representation of some of the many conformational states that can be adopted by polypeptide chains and of the means by which they can be interconverted. The transition from β-structured aggregates to amyloid fibrils can occur by addition of either monomers or protofibrils (depending on protein) to preformed β-aggregates. All of these different conformational states and their interconversions are carefully regulated in the biological environment, much as enzymes regulate all the chemistry in cells, by using machinery such as molecular chaperones, degradatory systems, and quality control processes. Many of the various states of proteins are utilized functionally by biology, including unfolded proteins and amyloid fibrils, but conformational diseases will occur when such regulatory systems fail, just as metabolic diseases occur when the regulation of chemical processes becomes impaired.

83. As Dr. Dobson's figures illustrate, in order to fold stably into the native three-dimensional tertiary structure, the naturally occurring amino acid sequence must navigate a complex pathway with various branches at key steps. Some of the key steps where the protein (or portions of it) can come off the track that leads to a native three-dimensional structure are:

a. The cell may not complete the synthesis of the encoded amino acid sequence.

b. The unfolded nascent polypeptide chain may form disordered aggregates or be degraded into fragments.

c. Partially folded forms of the sequence may lead to disordered or ordered aggregates.

d. The natively folded proteins may form various types of aggregates, or may unfold and be degraded within the extracellular environment.[61]

84. To successfully fold into the native folded state of the protein, an amino acid sequence must follow the folding pathway to the natively folded structure. As shown in the figure below, an unfolded protein can adopt a tremendous number of possible conformations. The numbers of possible conformations that sequence could have will decrease during the folding process of the sequence until it achieves a final native state, typically a single lowest energy conformation. This is illustrated as a "[s]chematic energy landscape for protein folding" in Dr. Dobson's papers. For example, Figure 1 of Dobson 2004a models the energy landscape for a simple 27-residue protein.[62] Even this short sequence has 10^{16} possible starting configurations. The figure illustrates three possible

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61 EX1136 (Dobson 2004a), 11 (Figure 5); EX1139 (Chiti & Dobson 2006), 351 (Figure 2).

62 EX1136 (Dobson 2004a), 4 (Figure 1).

folding pathways (red, yellow and green) within a landscape of possibilities. The polypeptide chain follows a particular sequence of conformations in each folding pathway. The sequence will adopt various conformations until it arrives at the conformation with the lowest free energy state that is the native state of the protein.

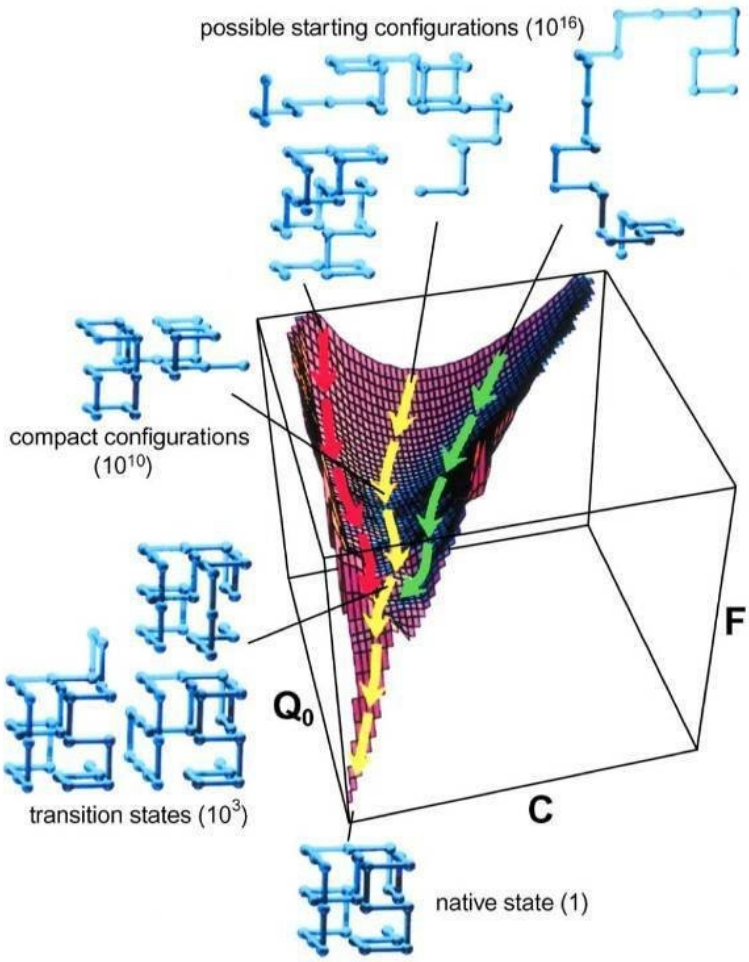


Fig. 1. Schematic energy landscape for protein folding. This surface is derived from a computer simulation of the folding of a highly simplified 27-residue protein. The free energy (F) of the system is shown as a function of the total number of contacts between residues (C) and the number of contacts that correspond to those of the most stable structure, denoted the native state (Q_0). The simulation shows that the surface serves to "funnel" the multitude of denatured conformations to the unique native state. From [9].

85. Sequences stochastically explore conformations during the folding process based on their ability to adopt those different conformations. The Branden

textbook illustrates that for a hypothetical 150 residue polypeptide, it would take 10^{48} years to stochastically search through all of its possible conformations:[63]

> Intuitively one might imagine that all protein molecules search through all possible conformations in a random fashion until they are frozen at the lowest energy in the conformation of native state. The biophysicist Cyrus Levinthal showed in 1968 by a simple calculation that this is impossible. Assume as a gross simplification that each peptide group has only three possible conformations, the allowed regions α, β and L in the Ramachandran diagram (see Figure 1.7), and that it converts one conformation into another in the shortest possible time, one picosecond (10^{-12} seconds). A polypeptide chain of 150 residues would then have $3^{150} = 10^{68}$ possible conformations. To search all these conformations would require 10^{48} years (10^{56} seconds)—an astronomical number compared with the actual folding time, which is between 0.1 and 1000 seconds both *in vivo* and *in vitro*. To occur on this short time scale, the folding process must be directed in some way through a kinetic pathway of unstable intermediates to escape sampling a large number of irrelevant conformations.

86. As Dobson 2004a explains, native, unmutated sequences have been specifically selected by evolution to fold. Only these "special" evolutionarily selected amino acid sequences will induce the "correct (native-like) interactions between different residues [that] are on average more stable than the incorrect (nonnative) ones."[64] Mutated sequences do not benefit from this evolutionarily conferred advantage, which is why so many of them cannot find the pathway to the native state of the folded protein.

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63 EX1014 (Branden), 91.

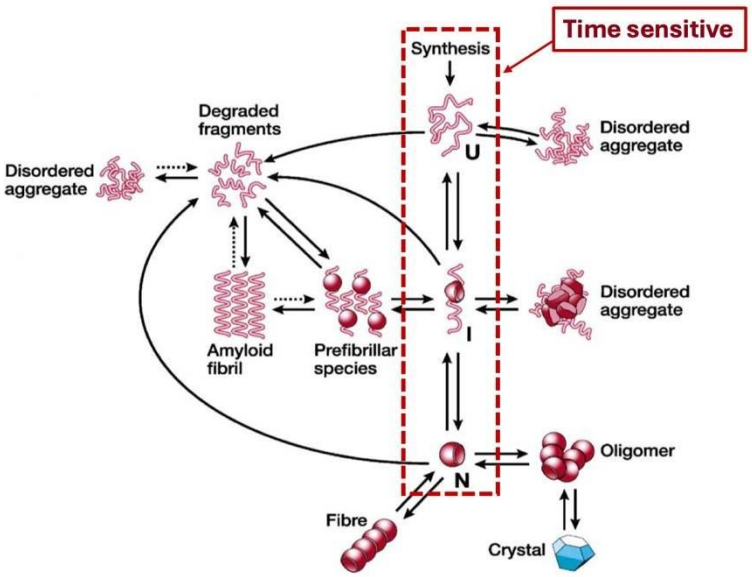
64 EX1136 (Dobson 2004a), 4.

> It is now evident that the folding process does not involve a series of mandatory steps between specific partially folded states, but rather a stochastic search of the many conformations accessible to a polypeptide chain [5–8]. The conceptual basis of such a mechanism is shown in Fig. 1. In essence, the inherent fluctuations in the conformation of an incompletely folded polypeptide chain enable even residues at very different positions in the amino acid sequence to come into contact with one other. Because the correct (native-like) interactions between different residues are on average more stable than the incorrect (nonnative) ones, such a search mechanism is in principle able to find the lowest energy structure [9]. It is evident that this process is extremely efficient for those special sequences that have been selected during evolution to fold to globular structures, and indeed only a very small number of all possible conformations needs be sampled during the search process.

87. In the annotated figure below, the dashed box shows the path an amino acid sequence must follow to efficiently and successfully fold into the natively folded, three-dimensional structure of the protein. The path is also time-sensitive, meaning the amino acid sequence must complete its folding pathway within a relatively short period of time in order to avoid degradation either within or outside of the cell.[65]

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[65] EX1131 (Petsko Dep.), 43:15-23 ("Proteins have to fold within a defined timeframe, otherwise a partially folded protein might accidentally get degraded while it hasn't got enough time to complete the folding."); *see also*



88. Figure 2 of Dobson 2004a illustrates a more complex energy landscape for a larger protein with two domains. This landscape has "hills" and "valleys" representing conformations of partially folded forms of the sequence that have higher or lower level energy barriers that must be correctly navigated for the sequence to adopt the native conformation. It also illustrates two folding pathways. The yellow path is a "fast track" in which the two domains fold concurrently. The red path involves sequential folding of the two domains and produces an intermediate that becomes trapped in an energy "valley." As it explains, "[f]urther folding from this intermediate involves either a transition over

98:10–13 (explaining that an intermediate unfolded form can gain stability relative to the folded form).

a higher barrier, or partial unfolding to enable the remainder of the folding to take place along a fast track."[66]

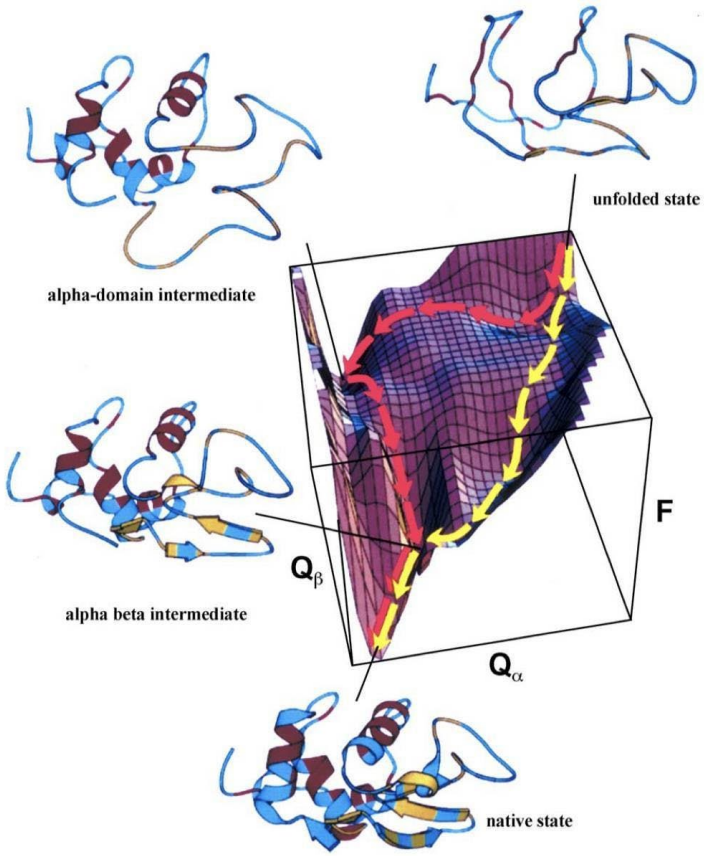


Fig. 2. Schematic energy landscape for the folding of lysozyme. This protein has 129 residues and its structure consists of two domains, denoted α and β. The free energy (F) is shown as a function of the number of native contacts (Q_α and Q_β) in each domain. The yellow trajectory represents a "fast track" in which the two domains fold concurrently and populate an intermediate (labelled alpha/beta) only briefly. The red trajectory represents a "slow track" in which the chain becomes trapped in a long-lived intermediate state with persistent structure only in the alpha domain. Further folding from this intermediate involves either a transition over a higher barrier, or partial unfolding to enable the remainder of the folding to take place along a fast track. The structures of the various species were generated by constraining molecular dynamics simulations with experimental data characterising each species, derived from NMR experiments. From [9].

89. Dr. Dobson's illustrations depict deviations from the folding pathway as being reversible, but as a practical matter they often are irreversible. Aggregated proteins do not normally refold spontaneously in biology. A sequence may adopt an off-path intermediate conformation that is energetically more stable

66 EX1136 (Dobson 2004a), 6 (Figure 2).

than the on-path intermediate conformation.[67] The protein in this incorrect but more energetically stable conformation cannot continue toward folding to the native state and will likely degrade if it remains in that form for too long.[68]

90. Amino acid sequences that differ from the evolutionarily selected wild-type sequence are at a greater risk of diversion from the folding pathway that must be followed to yield the natively folded protein structure. For example, the wild-type amino acid sequence of PH20 has resulted from evolutionary pressure that would have removed it from the human genome if it could not efficiently fold

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[67] EX1131 (Petsko Dep.), 98:4-20 ("The intrinsic -- … intrinsic stability of a protein is the difference in free energy between the folded form of the protein and the unfolded form of the protein. All right? So that can change not only because you can lose stability of the folded form, but you can -- ***that can change because you can gain stability of the unfolded form***.") (emphasis added).

[68] EX1131 (Petsko Dep.), 43:15-23 ("Proteins have to fold within a defined timeframe, otherwise a partially folded protein might accidentally get degraded while it hasn't got enough time to complete the folding.").

to adopt its native fold.[69] That reflects the ability of the wild-type sequence to be synthesized, to traverse the folding pathways to the folded native state of the PH20 protein fast enough to avoid degradation and to stably retain its native fold. Non-naturally occurring sequences, such as those with introduced mutations, have not been shaped by and survived the evolutionary pressure that removes sequences that cannot be synthesized by cells, cannot fold, or if they do fold, are not adequately stable.[70] I discuss impacts of mutations on stability further below. *See* § II.F.

91. Ochoa-Leyva *et al.* (EX1144) is a 2009 paper that illustrates the unpredictable effects amino acid substitutions can have on protein folding. It reports experiments investigating the effects of replacing loops within phosphoribosylanthranilate isomerase (PRAI) enzymes, an enzyme with a canonical α/β barrel motif. Importantly, the study investigated substitutions of 4 to 19 amino acids in a single protein and their effects on protein folding, directly by

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[69] EX1136 (Dobson 2004a), 4 (the folding "process is extremely efficient for those special sequences that have been selected during evolution to fold to globular structures, and indeed only a very small number of all possible conformations needs be sampled during the search process.")

[70] *See* EX1137 (Dobson 2003), 887 ("[M]any such disorders are familial because the probability of misfolding is often greater in mutational variants.").

addressing the tolerance of α/β structures to substantial sequence changes. The study examined a highly constrained set of changes: substitutions were limited to loop sequences already explored and optimized by natural evolution and known to be tolerated in naturally occurring α-β barrel enzymes with the same fold, increasing the likelihood that the resulting proteins would remain folded and screenable for function.[71]

Table 1. Characteristics of the exchanged loops in the PRAI-LoxP scaffold

Enzyme (EC number)	Position of the exchanged loop[a]	Sequence of the loop	Lengths
MR (EC 5.1.2.2)	β/α loop 2	GYPAL	5
FBPA (EC 4.1.2.13)	β/α loop 2	SNGGASFIAGKGVKSDVPQ	19
Ure (EC 3.5.1.5)	β/α loop 2	GGTGPAAGTHATTCTPG	17
PRAI WT (5.3.1.24)	β/α loop 2	VATSPRCVN[b]	9
DHDPS (EC 4.2.1.52)	β/α loop 4	PYYNRPS	7
TPS (EC 2.5.1.3)	β/α loop 4	LGQEDLH	7
MR (EC 5.1.2.2)	β/α loop 4	EPTLEHD	7
FBPA (EC 4.1.2.13)	β/α loop 4	DLSEES	6
αTS (EC 4.2.1.20)	β/α loop 4	DVPVQQS	7
Ure (EC 3.5.1.5)	β/α loop 4	EDWGAT	6
ADA (EC 3.5.4.4)	β/α loop 4	GDELGFPGSLF	11
PBGS (EC 4.2.1.24)	β/α loop 4	AAMDG	5
PRAI WT (5.3.1.24)	β/α loop 4	GNEE[b]	4
DHDPS (EC 4.2.1.52)	β/α loop 6	TGNL	4
PBGS (EC 4.2.1.24)	β/α loop 6	PAGAY	5
αTS (EC 4.2.1.20)	β/α loop 6	SRAGVTGAENRAALP	15
PRAI WT (5.3.1.24)	β/α loop 6	NGQGGSGQRFD[b]	11

[a] Structural position in which the WT loop was replaced for the new loop in the PRAI-LoxP scaffold.
[b] Sequence corresponding to the WT loops from PRAI-LoxP.

92. The locations for modification were further constrained to three loops "where there is higher participation of their residues in substrate binding and catalysis."[72] Figure 1 annotates these loop replacement sites within the α-β barrel sequence.

71 EX1144 (Ochoa-Leyva), 950 ("The exchanged loops belong to eight different proteins sharing the same fold but have diverse functions.").

72 EX1144 (Ochoa-Leyva), 950-51.

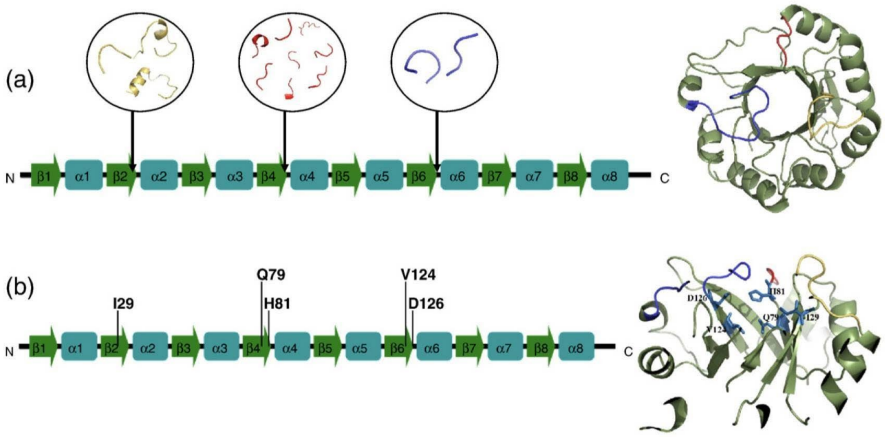
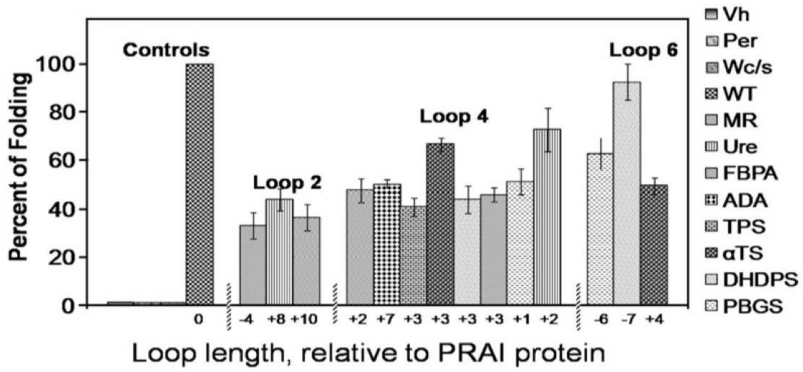


Fig. 1. Secondary-structure elements and tertiary structure (PDB code 1pii) of WT PRAI (ribbon diagram showing a view from the top of the central β-barrel). (a) The loops 2, 4, and 6 are shown in yellow, red, and blue, respectively. The circular insets show the loops to exchange in the PRAI-LoxP scaffold at the three structural positions (β/α 2, β/α 4, and β/α 6). (b) The mutated positions are indicated and a side-view slice of the TIM barrel structure of the PRAI scaffold is shown on the right.

93. The paper reported that 545 folded mutants were identified using a folding reporter gene, with frequencies varying by loop: approximately 30-40% for loop 2, 40-70% for loop 4 and 50 to 90% for loop 6) (below, and Figure 4).[73]

> The percentage of folded proteins for the three different libraries generated in loop 2 had a very similar value, around 30–40%, and in fact they were the lowest values found in the whole set of constructions. Exchanges at loop 4, where more libraries were generated, afforded a higher viability, from 40% to approximately 70% of folded peptides, depending on the incoming guest loop. Finally, in the case of loop 6, for which only three libraries were constructed, we observed the highest viability, ranging from 50% to 90%.

[73] EX1144 (Ochoa-Leyva), 953-54.



94. The authors found it "somewhat surprising" and observed that "considering the drastic changes introduced in the PRAI-LoxP sequence, the relatively high percentages of folded proteins that were obtained according to the Cm resistance are somewhat surprising."[74] They found that the insertion location mattered more than the inserted sequence, consistent with the active-site end of the TIM barrel, which is more tolerant to variability. They also observed that selective pressure used in the experiments influenced these successful outcomes.[75]

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[74] EX1144 (Ochoa-Leyva), 955-56.

[75] EX1144 (Ochoa-Leyva), 958 ("It is clear from the data that the introduction of variability at the hinges connecting the loops with the scaffold has a noticeable effect for obtaining folded proteins. The distribution of amino acids found in randomized positions without selective pressure of the analyzed samples shows only the normal bias inherent to the combinations NNG/C and NNT in the oligonucleotides used for mutagenesis. Thus, the amino acid

95. The Ochoa-Leyva paper illustrates the challenges to folding of an enzyme when it incorporates sets of changes, even when the changes are to other sequences found in homologous proteins and are under highly constrained conditions. When 4 to 19 changes were introduced into the enzymes, up to 70% of the mutated protein sequences did not fold. Ochoa-Leyva's results also demonstrate that when one changes the length of the loops in an α/β barrel motif enzyme, it also can have surprising effects on the ability of the protein to fold. The experimental results in the Ochoa-Leyva paper reinforces that even highly constrained and conservative changes to enzymes with particular structural motifs, such as a canonical α/β barrel motif, can have unpredictable effects on protein folding.

96. 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

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preferences observed with the introduction of each particular loop are indicative of the importance of the position adjacent to the loop for conformational fitness.").

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.[76]

97. A PH20 containing a single amino acid substitution that exhibits increased enzymatic activity does not establish that the same enzyme will retain that activity after the introduction of several additional changes. *See* §§ II.C.1, II.D. The inclusion of nine further modifications, for example, may individually or collectively disrupt the enzyme's structure and folding in a manner that reduces its activity or eliminates it all together. Such effects may arise independently of the initial substitution, or the added changes may alter the local structural environment in a way that counteracts the benefit conferred by the first substitution. As a result, the functional consequences of a single amino acid substitution in a protein such as PH20 cannot be used to predict the behavior of variants incorporating multiple additional modifications whether five, ten, fifteen or more. Accordingly, a POSA would not consider a PH20 variant containing only one substitution to be representative of a broader set of modified PH20 proteins that include that substitution together with numerous additional changes.

[76] EX1014 (Branden), 368-70.

98. Solubility is another characteristic of a protein that that becomes unpredictable as increasing numbers and type of changes are made to a PH20. For example, the wild-type human PH20 becomes soluble when the C-terminus is truncated below position 455 in SEQ ID NO: 3.[77] Many single substitution mutants also exhibited at least 100% activity in the $PH20_{1-447}$ truncation mutant, but many other single substitution mutants were deleterious. Given the uncertainty, a POSA could not know whether a $PH20_{1-447}$ polypeptide with 5, 10, 15, or 20 modifications would remain soluble until it is made and tested. Variants incorporating numerous changes, such as multiple substitutions or deletions of one or more residues may alter protein structure in ways that are difficult to predict. Such alterations include the exposure of hydrophobic residues or structural

[77] EX1026 ('731 App.), 82:26-31 ("Thus, the literature reports that a GPI-anchor attachment signal sequence of human PH20 is located at amino acid positions 491-509 of the precursor polypeptide set forth in SEQ ID NO:6, and the m-site is amino acid position 490. Thus, in this modeling of human PH20, amino acids 491-509 are cleaved following transport to the ER and a GPI anchor is covalently attached to the serine residue at position 490."). The positions in SEQ ID NO: 6 include the 35-residue signal sequence (*i.e.*, 490 in SEQ ID NO: 6 = 455 in SEQ ID NO: 3).

elements to the surrounding aqueous environment, thereby increasing the risk that extensively modified PH20 polypeptides will form aggregates and become insoluble at physiological conditions.

E. Using Protein Models to Visually Assess Modifications

99. During the 2011-2012 timeframe, a POSA seeking to evaluate whether a proposed single amino acid substitution at a specific position would be tolerated would typically construct and examine a structural model of the protein. Such visualization enables assessment of the interactions formed by the native residue with surrounding amino acids at that location, and comparison of those interactions with the interactions expected for a substituted residue. By evaluating the differences between the interaction profile of the wild-type residue and that at the proposed substitution, one could predict whether the change was likely to be favorable, neutral, or detrimental to the protein's structure-function relationship.

100. In the 2011-2012 period, it was common for a POSA to visually evaluate an amino acid substitution within a protein structural model. By inspecting the local structural environment surrounding a substitution site, a POSA could assess the potential impact of that change. For example, as one group reported:

> Many protein-engineering applications involve the creation of a small number of mutations to a naturally occurring protein so as to enhance its function in a well-defined manner. In these cases,

> a structural biologist's intuition is often an important tool in the design of the desired variants, an approach that may be termed structure-based protein design to borrow a term from the drug design field. Visualization of the known reference structure is a key component of this. For example, visualization can identify unsatisfied hydrogen bond donors or acceptors that may be mutated to increase stability or affinity. Similarly, visualizing steric interactions can help engineer interactions to discriminate among several potential binding targets.[78]

101. Additional illustrative work was carried out by a group led by Dr. Moult at UMBI. That group evaluated the effects of single amino acid substitutions arising from single nucleotide polymorphisms by visually inspecting protein structural models. Their analyses and conclusions based on those visual inspections were reported in peer review journals.[79]

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[78] EX1017 (Green), 228-29.

[79] EX1031 (Yue), 459 (reporting effects of single amino acid substitutions "relied primarily on visual inspection of an amino acid substitution on protein structure and function."); EX1032 (Wang), 265-66 (analyzing homology models for single amino acid substitutions to study mechanisms of hereditary disease).

102. The structural environment surrounding a proposed amino acid substitution can be examined by visualizing the relevant region of the protein using a structural model. Although the experimentally determined structure of human PH20 was not available in 2011-2012,[80] that would not preclude the use of structure-based modeling methods.[81] At the time, protein structures were commonly approximated using a process called "homology modeling."[82] Homology modeling was well established by 2011-2012 and provided an "accurate computational method to generate reliable structural models."[83]

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80 The structure of human PH20 was not experimentally determined until 2024. EX1126 (Im), 1067.

81 EX1017 (Green), 229 ("In many cases, protein engineering targets a protein whose structure has not been solved. This does not preclude the use of structure-based methods…").

82 EX1017 (Green), 229 ("[T]he known structures of related proteins can be used to create model structures through the process of homology modeling."), 230 ("Homology building can allow a model structure to be built from the structure of a related sequence.").

83 EX1012 (Bordoli), 1 ("Homology modeling is currently the most accurate computational method to generate reliable structural models and is routinely

103. Protein homology models are generated using an experimentally determined structure of a protein with a highly homologous sequence.[84] A model can be generated because "[h]omologous proteins have similar three-dimensional structures."[85] To build a homology model, the "backbone of homologous residues from a protein of unknown structure" is mapped "onto a known structure."[86] In many cases, however, "there are regions of nonhomologous sequence, even in

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used in many biological applications"), (homology modeling is "the method of choice to build reliable" models); EX1014 (Branden), 348 ("This model can serve as an excellent basis for identifying amino acid residues involved in the active site…").

84 EX1017 (Green), 229; EX1012 (Bordoli), 1 ("Homology modeling aims to build three-dimensional protein structure models using experimentally determined structures of related family members as templates"); EX1014 (Branden), 348 ("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.").

85 EX1014 (Branden), 370.

86 EX1017 (Green), 229.

highly homologous proteins" in which a "new backbone…must be constructed for these regions."[87]

104. By 2011-2012, a variety of homology modeling tools were available, including the SWISS-MODEL program.[88] SWISS-MODEL identifies appropriate template structures by searching databases experimentally determined protein structures and then constructs a structural model for that protein based on the sequence alignment of the target protein and the template structure.[89] Protein structure models generated by SWISS-MODEL and similar tools in the 2011-2012 timeframe would have been reliable for evaluating single amino acid substitutions where there is a sufficiently reliable QMEAN score and the model is supported by the aligned portion of the template structure. The modelled protein is itself a native protein and thus has been shaped by evolution to fold. Notably only one residue is being changed whilst the remainder of the protein is unchanged from the

87 EX1017 (Green), 229.

88 EX1017 (Green), 229, Table 10.2; *see also* EX1012 (Bordoli), 2.

89 EX1012 (Bordoli), 1; EX1066 (SWISS-MODEL), 3 ("Building a homology model comprises four main steps: identification of structural template(s), alignment of target sequence and template structure(s), model building, and model quality evaluation.").

reliably homology-modelled native state; these are the key features that make this a reliable approach for single substitutions.

105. In 2011-2012, homology models produced by SWISS-MODEL and comparable tools could not be used to reliably model the effects of more than a few amino acid substitutions upon PH20. One reason is that multiple changes to the modeled sequence negates the presumption that the sequence will fold because it is no longer similar enough to the wild-type sequence that was evolutionarily selected and is properly folded and active. The additional changes also will cause the model to deviate more substantially from the experimentally determined structure used as the template (*e.g.*, Hyal-1). Each change after the first influences the positioning of neighbors of the first changed residue and cause more subtle long-distance effects on other residues. Homology models in the 2011-2012 timeframe could not predict epistatic effects associated with combinations of mutations to a protein's structure.[90]

106. In the 2011-2012 timeframe, SWISS-MODEL homology model of PH20 could not reliably differentiate structural deviations of 1-2 Å. Mutations that cause atoms to shift less than 1 Å, however, can cause significant effects on a protein's structure, stability and function. For example, shifts of that scale can

[90] *Supra* § II.D.

alter the positioning of critical catalytic atoms of an enzymes' active site (*e.g.*, PH20). Homology models also would not be able to predict the effects of electrostatic effects associated with non-additive combinations of changes (epistasis). Structural models on their own can be uninformative. For example, a Glu131→Gln substitution results in essentially identical structures. However, it is known that Glu131 is a key catalytic residue and its mutation abolishes activity in Hyal-1.[91] Thus, while visual assessment of a modification in a protein homology model could provide insights into the effects on the local structure at the site of a modification, experimental investigations were needed to determine the intricate networks of amino acid interactions, as substitutions may lead to rearrangements in protein cores and binding interfaces that depend on three-dimensional, geometric packing of native residues.[92] Moreover, at that time, no experimentally determined three-dimensional structure of PH20 was available.[93] Instead, any modeling efforts necessarily relied on structurally related templates such as bee venom hyaluronidase or Hyal-1. However, those proteins differ in sequence and structural features from PH20, limiting the accuracy of any derived model. Without an

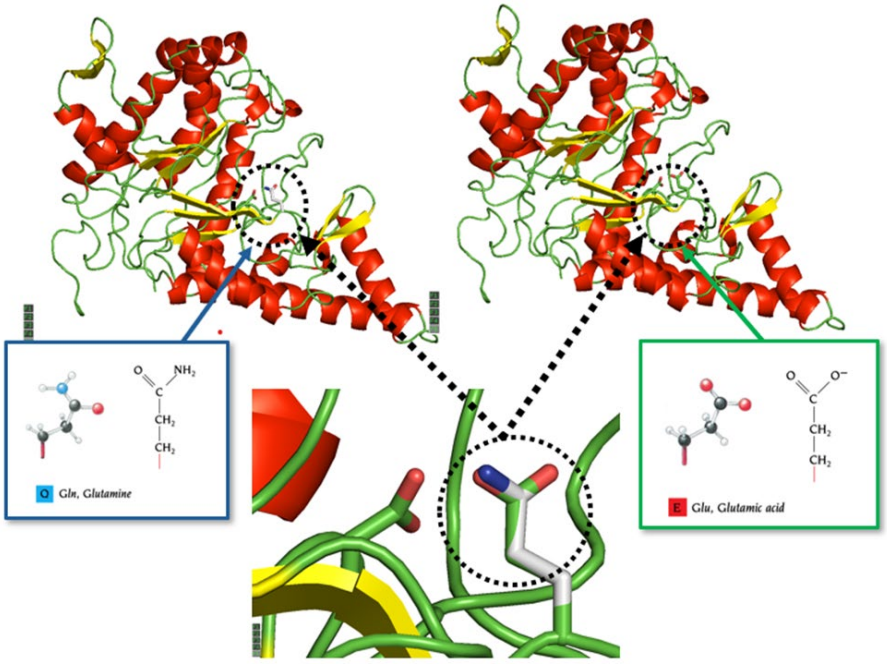
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91 EX1010 (Zhang), Abstract, 9437 (Figure 2).

92 EX1017 (Green), 234.

93 EX1126 (Im), 1067.

experimentally determined PH20 structure, one could not definitively identify structural elements critical to proper catalytic activity, substrate binding, or stability.



107. It was not until around 2020 that computer-based structure predictions from an amino acid sequence began to approach the accuracy of experimentally-determined protein structures, illustrated in Figure 6 from the 2024 Chemistry Nobel Prize Background paper, below.[94]

94 *See, e.g.*, EX1027 (2024 Chemistry Nobel Prize Background), 6-11, Figure 6.

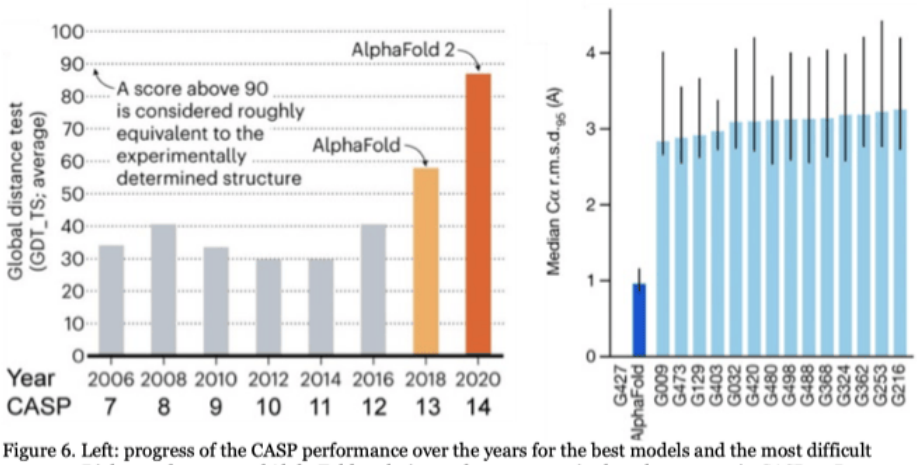


Figure 6. Left: progress of the CASP performance over the years for the best models and the most difficult targets.[38] Right: performance of AlphaFold2 relative to the top 15 entries by other groups in CASP14. Data are the median coordinate error and the 95% confidence interval of the median, estimated from 10 000 bootstrap samples.[41]

108. Consequently, a POSA in 2011-2012 would have had diminishing confidence in using SWISS-MODEL–generated PH20 homology models to predict the effects of multiple substitutions on the ability of a modified PH20 polypeptide to fold, adopt the native structure of the human PH20 enzyme, or remain properly folded. While limited insight might be drawn from single substitutions, that confidence would erode as additional mutations were combined, and by the time approximately five changes were introduced, reliable structural or functional predictability would no longer have been possible using the tools then available. This is particularly true for the claimed modified PH20 polypeptides, which place no restrictions on the locations or nature of the amino acid changes.

109. There is no methodology described in the ’731 Application where a PH20 homology model is used together with mutagenesis data to identify combinations of substitutions to make in modified PH20 polypeptides, particularly

ones that will retain hyaluronidase activity. It also does not reference or discuss experimentally-determined hyaluronidase protein structures, particularly the Hyal-1 structure reported in Chao (EX1006) or the bee venom hyaluronidase structure reported in Marković-Housley (EX1033). *See* § II.G.

F. Activity Is Not Synonymous with Stability

110. While a single measurement showing a modified PH20 polypeptide possessed enzymatic activity would confirm that the PH20 protein had folded, there would need to be additional evidence to justify a conclusion that it had stably folded. For example, a POSA would not believe an enzyme had properly folded if it did not maintain activity over a meaningful period (*e.g.*, at least 8-12 hours after expression) or if it formed aggregates within a short period. Both of those experimental results are indicative of the structure of the modified PH20 polypeptide being unstable, even if it did originally fold and was secreted.

111. Observing an increased relative activity of an enzyme in a single measurement shortly after expression cannot establish that the mutated protein is more stable than the unmodified form of the enzyme. Determining whether a mutation affects protein stability requires use of appropriate experimental techniques that compare the energetic requirements for folding or unfolding transitions in wild-type and mutant proteins. In the absence of evidence establishing such a connection, a POSA would not equate an increase in biological

activity of an enzyme as proof that a mutation increased the stability of the enzyme.

112. The disclosure conflates the observed increase in the activity of mutants of the $PH20_{1-447}$ enzyme with a general assumption of increased stability of these mutants relative to the unmodified $PH20_{1-447}$ enzyme.[95] The data reported in Example 5 address preservation of activity for 409 mutants at 4 ℃ and 37 ℃, and in the presence of m-cresol (phenophile).[96] Table 11 reports measured hyaluronidase activities of the mutants. 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).

113. With the exception of a single mutant (F204P), the ’731 Application provides no direct experimental evidence from which a skilled artisan could conclude that any of the 409 single substitution mutants exhibit increased protein stability. Nor does the disclosure distinguish whether any observed increase in activity is attributable to stability as opposed to other factors such as enhanced

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95 EX1026 (‘731 App.), 10:17-12:15.

96 EX1026 (’731 App.), 282:6-303:1, Tables 11, 12; *see also* Appendices B-4, B-5.

catalytic efficiency. Moreover, the data in Example 5 also show that a majority of the mutants exhibited decreased activity in the presence of m-cresol, with most retaining less than 20% activity (the disclosure's classification as inactive) under those conditions. This further undermines the assertion that the activity data support a broad conclusion regarding enhanced structural stability.

114. There is no explanation in the '731 Application of how any specific mutation affected any structural features of the PH20 protein, such as the catalytic site, substrate binding site, or other structurally important portions of the enzyme. There also are no explanations whether or how any particular substitutions altered secondary structures or higher-order structural motifs within PH20, or how any such structural changes influence protein stability or hyaluronidase activity.

115. The disclosure implies that 37°C constitutes a denaturing temperature for PH20.[97] However, 37°C is the normal human physiological temperature, and is the temperature at which PH20 naturally exists and functions in vivo. Denaturation, in a biochemical context, refers to loss of native tertiary structure, typically observed near or above a protein's melting temperature (T_m) which is reported for unmodified $PH20_{1-447}$ as 44 °C.[98] Moreover, it is a well-established

[97] EX1026 ('731 App.), 193:1-7.

[98] EX1026 ('731 App.), 119:17-20.

principle of kinetics that reaction rates generally increase with temperature. It is therefore unsurprising that many single-replacement $PH20_{1-447}$ polypeptides showed higher activity at 37°C than at 4°C. The graphic below demonstrates, for each single-replacement PH20 mutant, the percentage of activity at 37°C relative to 4°C. Green indicates variants exhibiting more activity at 37°C compared to 4°C.

Mutant	Duplicate 1 % Activity at 37°C/4°C	Duplicate 2 % Activity at 37°C/4°C															
L001A	117.908	127.997	D090N	111.06	98.631	T232F	132.839	62.882	T289S	104.598	98.234	A371S	147.672	131.555	F433K	91.159	342.29
L001E	107.231	125.207	K098Q	91.008	95.448	Q233G	280.488	127.368	G291S	184.581	119.565	L374P	392.038	123.033	F433R	128.958	133.353
L001G	171.264	115.952	K093R	103.617	99.301	Q234M	95.605	80.766	G291V	112.807	151.039	E375A	88.836	134.714	F433T	161.799	134.977
L001Q	119.435	88.763	K094D	86.544	102.107	S235A	129.818	120.916	E292C	127.307	101.126	E375G	126.88	139.03	F433V	1412.071	112.033
L001R	117.366	160.41	K094R	125.373	108.69	V237C	138.042	116.384	E292F	137.93	132.34	E375R	163.18	116.431	F433W	149.049	113.585
P006A	137.875	108.946	T097C	165.152	81.715	V237H	122.112	145.253	E292H	170.153	115.501	K376D	113.1	165.064	P437I	148.88	107.028
V008M	134.884	119.772	T097D	123.654	117.522	V237T	167.105	126.02	E292R	112.278	129.89	K376E	100	153.016	M438A	106.463	135.705
I009Q	104.922	124.934	T097E	127.19	115.106	A238E	94.878	142.167	E292V	163.075	133.274	K376Q	125.172	90	M438D	105.37	113.283
P010G	109.772	121.986	T097L	118.465	103.589	A238H	59.585	204.683	T293A	128.197	57.524	K376R	81.687	199.112	M438E	115.061	113.782
P010H	131.924	112.99	N104R	114.673	118.421	T240A	141.283	144.667	A298G	212.422	86.131	K376T	121.133	113.387	M438L	65.794	214.958
N011S	152.32	131.289	A120H	94.107	113.015	T240Q	162.763	120.98	L307G	117.857	91.528	K376V	124.221	118.583	M438N	130.428	100.669
V012E	48.208	100.163	D127R	56.439	58.702	R248A	113.237	124.65	S308D	127.652	105.846	K376Y	102.812	96.139	M438T	104.058	103.691
V012I	128.745	170.812	V128I	113.654	102.656	E249V	142.752	111.068	S308K	126.882	99.169	G377D	110.871	132.357	E439A	137.279	95.555
V012K	146.6	114.264	N131M	177	76.888	P257G	125.22	112.803	S308N	170.413	139.083	G377E	130.445	128.402	E439A	154.14	147.295
F014V	154.167	146.667	N131R	94.253	95.93	K260M	116.69	97.396	I309E	123.847	129.94	G377H	146.855	104.837	E439C	193.243	111.719
L015M	113.747	83.97	N131V	137.681	104.92	S261A	57.547	86.173	I309G	102.601	114.091	G377K	185.922	119.751	E439K	124.464	104.762
A020S	188.889	118.821	R132L	98.578	91.685	S261K	161.931	116.159	I309L	153.681	106.948	G377R	119.708	94.749	E439P	118.34	87.446
S022T	111.203	86.404	Q138L	107.831	91.627	S261N	142.901	35.529	I309M	123.425	136.797	G377S	108.609	101.106	E439Q	101.589	127.358
L026M	136.775	138.989	Q140K	176.6	109.815	A267T	196.154	111.579	I309N	111.901	97.361	G377T	112.557	109.036	E439T	110.891	122.975
K028R	110.487	109.467	N141R	103.411	115.682	F273H	122.647	119.037	I309S	169.951	551.493	F380W	147.077	104.881	T440D	118.877	79.518
F029R	154.644	121.707	N141S	131.758	109.527	F273Y	119.713	102.772	I309T	97.936	148.864	T381S	135.827	112.559	T440H	142.296	130.928
F029S	118.119	97.4	N141W	130.644	104.783	Q276H	74.908	106.393	I309V	113.138	138.313	R383I	527.82	98.328	T440M	84.722	86.929
F029T	126.74	120.619	V142D	114.185	146.066	Q276M	98.323	104.948	M310G	167.656	110.739	R383S	132.894	119.466	T440P	111.931	91.205
P032C	128.649	127.446	V142G	117.686	90.256	Q276R	121.431	150.18	M310Q	107.237	106.323	K385A	126.096	112.706	T440S	100.436	131.174
L033G	121.201	89.571	V142K	109.485	154.599	Q276S	110.643	138.696	M313G	138.095	109.231	K385Q	137.629	124.892	E441F	129.315	110.874
D034W	146.765	146.729	V142N	155.556	103.88	V277A	140.765	129.58	M313H	271.914	197.024	K385V	112.581	80.571	E442G	111.216	100.21
M035V	81.285	102.034	V142P	166.998	97.338	V277E	175.779	195.598	M313K	118.882	107.111	E389A	306.767	224.872	P443E	94.377	130.704
S036H	106.222	150.931	V142Q	149.666	99.957	V277H	129.434	137.684	M313P	103.654	132.802	E389G	113.253	139.901	P443F	146.612	97.932
S036N	112.045	92.069	V142R	149.441	103.622	V277K	375.721	373.799	M313R	157.272	32.845	E389L	143.219	112.185	P443G	239.171	157.96
L037M	79.268	87.376	V142S	170.778	117.035	V277M	137.138	112.084	M313T	162.074	99.844	E389Q	135.807	99.738	Q444E	81.997	160.917
F040L	135.036	105.252	V142T	223.936	123.65	V277N	89.645	273.386	M313V	120.038	103.011	E389S	165.62	93.03	Q444H	150.301	119.665
I046L	132.507	112.944	Q143G	143.6	98.837	V277Q	119.93	116.151	K314S	141.924	132.112	E392A	112.465	155.693	Q444V	129.822	122.591
N047D	115.797	111.869	Q143K	200.468	136.421	V277R	96.071	171.465	K314Y	243.011	138.931	E392F	115.619	143.781	I445M	85.09	101.149
N047W	104.703	109.88	L144R	136.247	111.482	V277S	66.26	144.916	S315A	91.372	137.153	E392Q	112.993	93.789	I445N	106.43	87.351
A048N	114.954	51.931	L144T	129.746	108.923	V277T	101.01	143.311	S315H	151.244	105.305	E392R	129.528	123.407	I445W	117.213	100.037
T049R	122.704	90.76	L146P	116.626	115.601	L278E	75.408	100.179	S315Y	170.968	57.827	E392V	124.365	154.768	Y447E	99.579	108.969
G050D	93.824	95.934	T147S	142.175	130.287	L278G	122.274	104.077	L317A	123.51	132.724	Q393F	139.966	101.647	Y447G	143.704	103.624
G050M	157.686	139.048	T150N	140.724	116.923	K279H	138.964	123.183	L317I	187.477	110.696	Q393M	139.696	86.966	Y447P	139.152	107.737
G052N	96.148	142.502	T150S	107.327	142	V287T	145.345	124.738	L317K	96.199	134.204	S395A	208.246	112.714			
G052T	116.407	117.075	E151A	103.31	126.047	T289S	104.598	98.234	L317N	127.382	121.233	S395H	159.975	113.401			
G052S	98.513	98.199	E151L	132.125	121.83	G291S	184.581	119.565	L317R	238.501	99.467	E396A	131.894	128.716			
V058C	92.507	99.162	E151S	115.423	136.397	G291V	112.807	151.039	L317S	90.929	85.81	E396H	210.564	128.571			
V058K	217.914	217.914	E151T	128.337	110.3	E292C	127.307	101.126	L317T	145.964	154.334	E396Q	122.977	95.938			
V058R	96.905	102.858	E151V	111.531	99.647	E292F	137.93	132.34	L317W	163.704	147.606	E396S	156.267	111.753			
V058N	129.167	129.787	E151W	158.415	94.919	E292H	170.153	115.501	L318D	105.543	97.97	Y399T	130.536	122.738			
V058Y	102.981	141.299	K152T	149.169	136.747	E292R	112.278	129.89	L318H	99.907	124.69	Y399V	110.592	116.018			
V058Q	154.383	293.51	K152W	122.313	134.039	E292V	163.075	133.274	L318R	160.469	120.872	Y399W	122.5	103.346	Green =	value > 100% for activity at 37°C/4°C	
V058P	83.304	173.652	E158S	133.038	102.519	T293A	128.197	57.524	N321R	164.842	112.18	S401A	122.003	99.275			
V058H	200.264	181.75	K162E	67.857	41.026	A298G	212.422	86.131	N321S	102.489	108.732	S401E	125.223	128.67			
D068P	99.07	83.721	L165F	106.283	96.667	L307G	117.857	91.528	E324N	104.618	131.265	S404A	149.379	105.443			
S069T	138.609	122.579	V166Q	155.975	117.99	S308D	127.652	105.846	T325E	124.837	106.457	L406F	122.805	146.122			
I070P	101.713	99.749	V166T	183.384	136.882	S308K	126.882	99.169	N328G	197.098	109.722	L406N	152.836	131.321			
I070V	170.462	136.849	E167D	136.745	162.637	S308N	170.413	139.083	N328Y	180.981	100	S407A	141.351	110.376			
V073Q	121.337	107.094	I169L	140.177	122.272	I309E	123.847	129.94	T335S	107.956	125.286	S407D	241.053	98.135			
V073R	137.931	100.125	K170R	160.71	97.128	T240Q	162.763	120.98	Q347A	101.395	96.213	S407P	143.308	121.898			
T074E	133.241	100.965	G172A	167.554	133.735	R248A	113.237	124.65	Q347G	222.459	207.054	A412Q	146.177	99.452			
T074M	115.29	103.629	K173R	106.771	134.3	E249V	142.752	111.068	Q349M	99.531	108.042	A412R	140.07	123.675			
T074N	91.87	157.218	L174G	114.13	264.368	P257G	125.22	112.803	Q349R	147.007	104.545	A412V	146.804	101.739			
T074P	108.323	166.227	L174N	154.332	126.186	K260M	116.69	97.396	V351S	130.819	100.857	D416L	120.82	127.662			
T074R	80.681	130	L174T	124.819	144.876	S261A	57.547	86.173	I353V	132.334	138.025	D418R	117.749	112.193			
T074V	115.093	114.063	N178K	166.871	103.154	S261K	161.931	116.159	N356H	100	130.377	A419H	241.224	188.179			
V075M	134.46	121.527	N178R	199.596	144.957	S261N	142.901	35.529	N356S	51.908	125.692	A419K	191.165	2022.616			
K082L	114.758	251.869	H193Q	213.585	138.113	A267T	196.154	111.579	S359E	135.589	135.104	D421A	102.111	301.584			
K082N	106.059	95.104	K195T	126.161	237.097	F273H	122.647	119.037	S359H	110.422	100.809	D421H	333.471	67.652			
I083V	140.151	137.296	K195N	130.253	96.381	F273Y	119.713	102.772	P367A	167.03	127.366	D421K	124.19	102.316			
I083Q	112.163	188.798	K196E	90.574	154.091	Q276H	74.908	106.393	P367G	115.683	122.642	D421N	110.806	100.116			
I083S	104.637	95.351	K196R	106.1	95.142	Q276M	98.323	104.948	P367K	125.884	66.884	D421Q	104.37	143.63			
I083G	106.239	76.381	F204P	83.571	82.418	Q276R	121.431	150.18	P367S	74.263	88.355	D421R	138.783	137.964			
S084E	124.762	113.41	N205A	139.223	102.031	Q276S	110.643	138.696	D368A	121.623	81.646	D421S	142.171	166.162			
S084F	83.291	91.007	N205E	160.93	93.313	V277A	140.765	129.58	D368E	166.628	97.937	A425G	74.81	120.947			
S084N	144.922	131.144	N205L	107.472	0	V277E	175.779	195.598	D368L	108.977	109.364	G427Q	133.135	98.243			
S084R	119.873	203.099	N205T	145.085	110.627	V277H	129.434	137.684	D368M	119.744	103.662	G427T	125.113	119.058			
Q086A	136.516	156.132	V206I	189.274	111.22	V277K	375.721	373.799	D368R	164.735	118.14	V428L	137.044	109.39			
Q086H	102.612	129.6	K209R	119.794	79.535	V277M	137.138	112.084	D368T	107.122	126.693	D431E	70.178	95.135			
Q086K	99.213	65.455	D212N	112.626	132.249	V277N	89.645	273.386	N369R	161.693	74.366	D431H	186.49	95.071			
Q086S	100.435	103.218	D212S	122.899	147.936	V277Q	119.93	116.151	A371F	180.217	76.436	D431K	240.835	68.277			
Q086T	93.837	179.465	D213A	183.83	154.77	V277R	96.071	171.465	A371H	957.055	89.541	D431L	129.149	119.177			
D087G	81.742	90.579	D213M	159.255	98.365	V277S	66.26	144.916	A371H	111.143	95.589	D431N	138.404	125.433			
D087L	106.039	101.493	S215H	109.069	78.992	V277T	101.01	143.311	A371K	136.514	114.354	D431Q	232.96	109.483			
D087M	110.964	87.656	S215M	174.883	74.943	L278E	75.408	100.179	A371L	695.108	107.003	D431S	78.069	88.796			
D087S	134.031	139.728	N219I	254.438	291.2	L278G	122.274	104.077	A371L	104.327	60.232	F433A	147.286	99.486			
D087V	114.107	87.023	E220V	131.985	113.61	K279H	138.964	123.183	A371R			F433H	140.196	87.943			
D090E	92.91	161.281	T222G	153.033	105.454	V287T	145.345	124.738	A371R	121.162	97.97	F433I	108.569	86.984			

Increased activity at 37°C, standing alone, does not indicate increased stability; it is consistent with ordinary temperature-dependent kinetic effects. The control data do not evidence that 37°C meaningfully reduces PH20 activity. Among the positive control measurements, 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 a measurable decrease in activity for the wild-type $PH20_{1-447}$ polypeptide at 37°C vs 4°C.

116. Amongst the 409 mutants evaluated in Example 5, the disclosure identified two (F204P and V58R) that retained greater than 70% of their hyaluronidase activity in the presence of m-cresol preservative in both duplicate measurements. In Example 9 (Table 15), the F204P variant is shown to maintain activity for a longer duration than the wild-type in the presence of m-cresol, remaining active over a period of four weeks to six days. Example 11 (Table 22) similarly reports that V58R preserved higher activity over time with m-cresol compared to the unmodified control. However, these follow-up stability studies were limited to just these two single-substitution variants. The disclosure does not provide comparable long-term preservative stability data for the remaining screened single-substitution mutants. The isolated thermal stability observations for F204P and V58R cannot be generalized to the full set of 409 single-substitution PH20 variants.

117. The one direct evaluation of protein stability reported in the disclosure appears in Example 13 and involves the F204P $PH20_{1-447}$ mutant. It reports a melting temperature (Tm) for F204P $PH20_{1-447}$ that was 9°C higher than unmodified $PH20_{1-447.}$ This result shows that the F204P variant is more thermally stable than unmodified $PH20_{1-447}$. The data offers no insight into the basis for this

increased thermal stability, and thus no guidance as to how to generalize the observation to other single-substitution mutations. Additional substitutions made to the F204P $PH20_{1-447}$ variant are susceptible to epistasis and thus the effects of combinations are unpredictable. In such a multiply modified protein the stabilizing effect of F204P cannot be reliably assumed.

118. Taken as a whole, the results reported for the 409 mutants reported in Tables 11 and 12 do not provide any meaningful guidance to a POSA about which types of mutations would enhance the stability of PH20 polypeptides, whether in the $PH20_{1-447}$ form or otherwise. Nor do these data shed light on the structural consequences of introducing multiple substitutions in combination with one of the tested changes, as none of the evaluated mutants contain more than a single amino acid change. For many individual mutants, the results of the two duplicate experiments are inconsistent, and relatively few variants exhibit activity values outside the experimental variation observed for the positive control. Where increased activity is reported, it is limited to m-cresol inactivation rather than wider insights to stability. Accordingly, the single-substitution data does not meaningfully inform a POSA how to design a multiply-modified PH20 polypeptide that maintain or exhibit increased stability.

G. Hyaluronidases and PH20

119. PH20 belongs to a family of five human hyaluronidase enzymes and is similar to hyaluronidase enzymes found across a broad range of organisms (*e.g.*, bacterial, fungal, insect, mammalian).[99] Functionally, PH20 selectively catalyzes the hydrolysis of β1,4 glycosidic bonds in hyaluronan (also called "HA" or hyaluronic acid) (below).[100] In contrast, PH20 does not cleave β1,3 glycosidic bonds in HA.

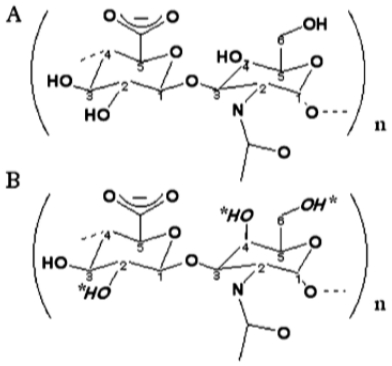


Figure 1. Chemical structures of (A) hyaluronan and (B) unsulfated chondroitin and chondroitin sulfates. The substrate structures differ only in the anomericity at the C4 position of the *N*-acetyl-D-glycosamine, *N*-acetyl-D-glucosamine in hyaluronan, and *N*-acetyl-D-galactosamine in chondroitin. Potential 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.

99 EX1008 (Stern), 819.

100 EX1008 (Stern), 820 (Figure 1).

1. Characterization of Hyaluronidases

120. An earlier publication by Arming (EX1011) identified several residues that are conserved between human PH20 and bee venom hyaluronidase ("bvH"). In that study, Arming further reported the effects of mutating five such residues and observed a pronounced reduction in enzymatic activity. As Arming explained "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."[101]

121. Arming further identified several conserved cysteine residues in the PH20 protein, as shown in Figure 1 of that publication.[102] Those same conserved cysteines were later highlighted in an alignment of the human hyaluronidases reported in Chao.[103] Together, the Arming and Chao studies established that these cysteine residues play a critical structural role, as they participate in disulfide bond formation required to maintain the proper structure of PH20 and related hyaluronidases.

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101 EX1011 (Arming), 813.

102 EX1011 (Arming), 811 (Figure 1).

103 EX1006 (Chao), 6916 (Figure 3).

122. The first experimentally resolved hyaluronidase structure was that of bvH, which was reported in 2007 both in isolation and in complex with HA. Marković-Housley identified the enzyme's catalytic site and residues responsible for catalytic activity using this structure.[104] That work also included a comparative sequence alignment of PH20, human Hyal-1, human Hyal-2 and bvH which highlighted residues conserved across these proteins.[105]

2. Chao Reports Structure of Human Hyal-1 and the Hyal-EGF Domain Highlighting Differences in Alpha-Beta Motifs

123. A structure for human Hyal-1 was published by Chao and Herzberg in 2007 ("Chao") (EX1006). In that work, the authors compared the alpha-beta motif from human Hyal-1 structure with that previously reported for the bvH structure and found that the two motifs share some similarities, as illustrated in Figure 2C, Chao (below).[106] In the paper they also drew attention to important structural differences between the alpha-beta motifs between Hyal-1 and bvH.[107] In

[104] EX1033 (Marković-Housley), 1028-31, 1035 (PDB identifiers: 1FCU, 1FCQ).

[105] EX1033 (Marković-Housley), 1026 (Figure 1).

[106] EX1006 (Chao), 6912-13, 6915 (Figure 2C).

[107] EX1006 (Chao), 6913-16.

comparing the structures, the authors noted they omitted the EGF domain of Hyal-1.[108]

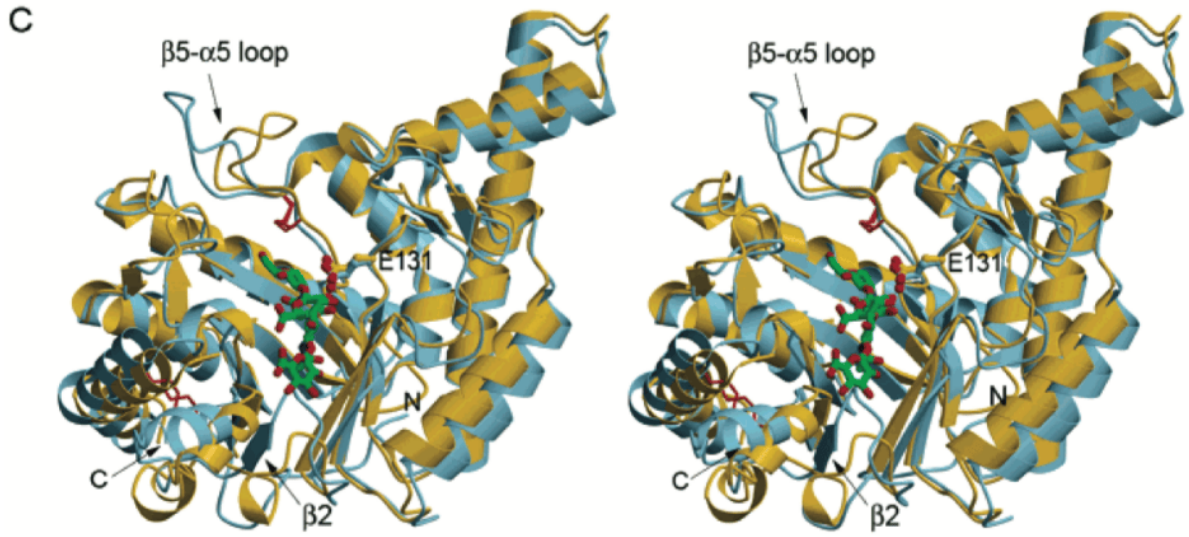


FIGURE 2: Structure of hHyal-1. (C) Stereoscopic representation of the catalytic domain (colored light blue) superimposed on the structures bvHyal in complex with $(GlcUA\text{-}GlcNAc)_2$ (colored gold; (*22*), PDB entry code 1FCV). The tetrasaccharide is colored with an atomic color scheme: green, carbon; red, oxygen; blue, nitrogen.

124. Chao also compared the catalytic sites of the two proteins, and found that they were similar[109]:

[108] EX1006 (Chao), 6913, 6915 (Figure 2C).

[109] EX1006 (Chao), 6917 (Figure 4A).

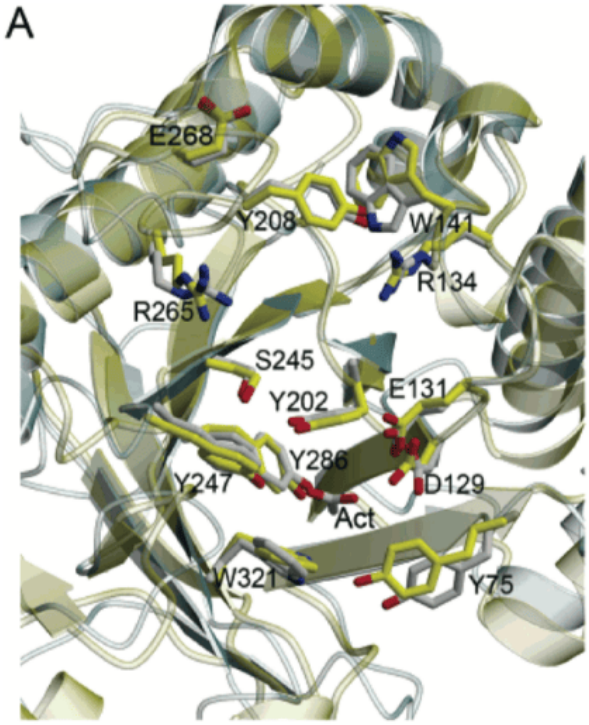


FIGURE 4: (A) Stereoscopic representation of the active site region of hHyal-1 (gray ribbon) superimposed on that of bvHyal (yellow ribbon; (*22*)). Selected amino acids are colored in the atomic color scheme: red, oxygen; blue, nitrogen; gray (hHyal-1) and yellow (bvHyal), carbon.

125. Chao also presented an annotated sequence alignment of the five human hyaluronidase enzymes that highlighted residues conserved across those proteins, identified cysteine residues participating in disulfide bonds, and marked secondary structural elements within the proteins (*i.e.*, α-helices indicated by coiled illustration, and β-strands by an arrow).[110]

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

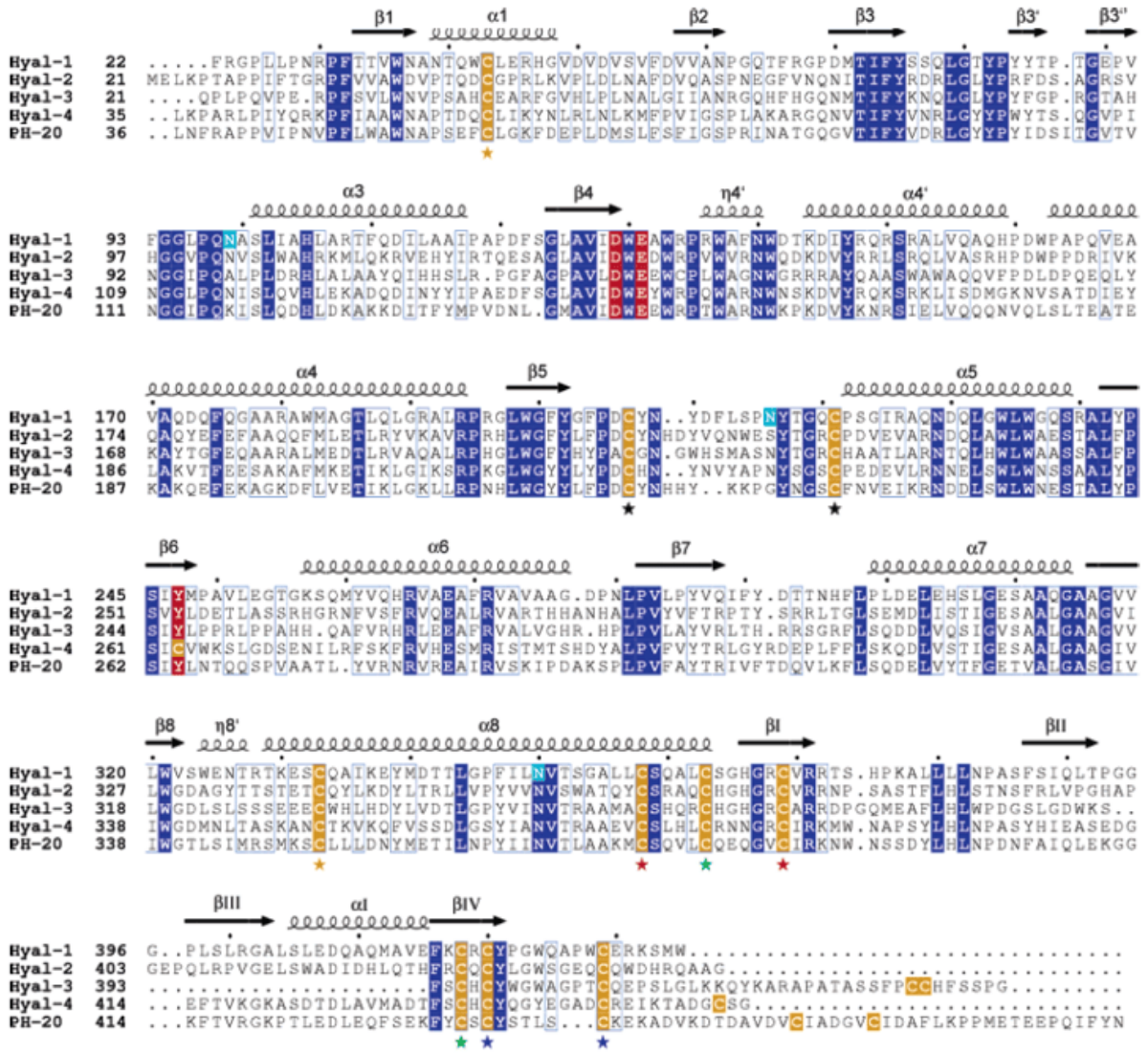


FIGURE 3: Structure-based sequence alignment of human hyaluronidases. Invariant residues are shown in blue except for three key catalytic residues that are colored red. Cysteine residues are colored yellow. The hHyal-1 N-glycosylated asparagines residues are colored turquoise. Residues exhibiting conservative 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.

126. A significant contribution of the Chao study was the identification and first experimental characterization of the "Hyal-EGF" domain in the C-terminal region of human hyaluronidases, and identification of the residue patterns responsible for it. Despite being recognized by those in the field as a "huge

breakthrough," the disclosure of the '731 Application does not discuss this structural motif at all.[111]

127. Based on experimentally determined structural data, Chao demonstrated that an earlier "*ab initio*" structural model—derived from the bee venom hyaluronidase—was inaccurate, particularly in the C-terminal region containing the Hyal-EGF domain.[112]

> The HyalEGF-like fold does not resemble the Hyal-1 C-terminal domain fold predicted by ab initio approaches (*33*). The close interaction between the two domains is also very different from the earlier prediction that proposed a flexible linker and lack of interactions between the two domains. Moreover, the modeling of the five human hyaluronidases C-terminal domains yielded a different fold in each case, whereas we expect that these domains adopt the HyalEGF-like fold because the amino acid sequences contain the characteristic disulfide bond pattern.

128. Chao further reported that, although C-terminal regions of mammalian hyaluronidases do not share strong overall sequence homology, they do contain a

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[111] EX1130 (Simpson Dep.), 115:10-116:9 ("That is among the things that this paper showed for us. Yes, it was a huge breakthrough in the field.").

[112] EX1006 (Chao), 6913. Chao cites EX1009 (Jedzrejas). The Stern review also addresses this *ab initio* modeling work. *See* EX1008 (Stern), 828-31, 833 ("The lack of experimental data and of sequence/structural homologue(s) for the C-terminus makes functional studies of this human Hyal domain purely speculative.").

conserved, cysteine-rich pattern that sequence analysis tools recognize as an epidermal growth factor (EGF)-like motif (below).[113] Chao defined the amino acid pattern underlying the Hyal-EGF structural motif as "$x_4Cx_{0-48}Cx_{3-12}Cx_{1-70}Cx_{1-6}Cx_2Gax_{0-21}Gx_2C$, where 'a' denotes a hydrophobic residue, 'x' denotes any residue, and the gaps between cysteine residues vary in length as indicated by the subscripts."[114]

Figure 4B

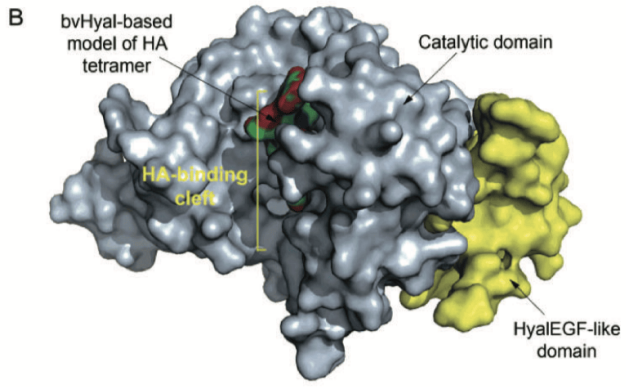


FIGURE 4: (B) Molecular surface of the catalytic domain (light blue) and HyalEGF-like (yellow) domains of hHyal-1, illustrating the separation between the HyalEGF-like domain and the active site. A docked tetrasaccharide, inferred from the structure of bvHyal, is shown as a space filling model.

Figure 2A

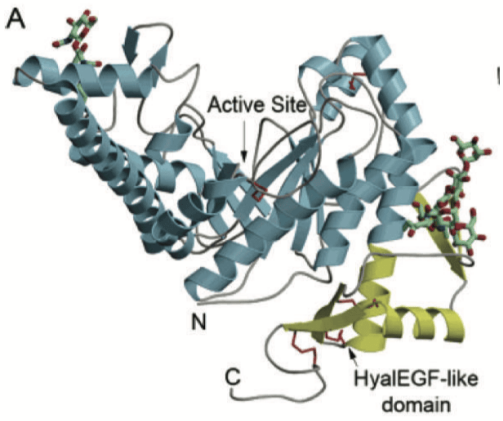


FIGURE 2: Structure of hHyal-1. (A) Stereoscopic representation of a side view. The catalytic and the HyalEGF-like domains are colored light blue and yellow, respectively. Disulfide bonds are shown in red. The N-linked oligosaccharides are shown as stick models with the atomic color scheme: gray, carbon; red, oxygen; blue, nitrogen.

129. The Chao publication proposed that the Hyal-EGF domain may mediate interactions with other proteins given its positioning away from the active

113 EX1006 (Chao), 6915 (Figure 2A), 6917 (Figure 4B).

114 EX1006 (Chao), 6912.

site of the molecule and the characteristics of the Hyal-EGF domain. Chao noted that the Hyal-EGF domain "is elongated and has extensive solvent accessibility, consistent with mediation of protein-protein interactions" and that "[t]he β-hairpin unit (βII-βIII) appears suitable for that role because its flexibility implies potential to adjust in response to a protein partner."[115] Additionally, Chao observed that the positioning of Hyal-EGF domain meant it was "not likely to be involved in substrate recognition."[116] Chao also observed that because this region "varies significantly within the hyaluronidase family," the "partner identity or affinity may vary for each enzyme."[117]

130. Chao noted that epidermal growth factor (EGF) domains were already understood to play roles in cell-adhesion and cell-cell communication.[118] Chao then referenced a 1996 paper by Hunnicutt as providing "[t]he most direct evidence for such interaction pertain[ing] to mammalian PH-20."[119] The Hunnicutt paper reported that "PH-20 has two distinct activities, a hyaluronidase activity and

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115 EX1006 (Chao), 6917.

116 EX1006 (Chao), 6916.

117 EX1006 (Chao), 6917.

118 EX1006 (Chao), 6916.

119 EX1006 (Chao), 6916 (citing EX1079 (Hunnicutt)).

a different activity required in secondary adhesion."[120] Hunnicutt associated the hyaluronidase activity of PH20 with the N-terminal domain, and suggested "the active site involved in secondary sperm-zona binding is in the C-terminal region (27 kDa on SDS-PAGE, residues 312-468) or at the interface between the two domains."[121] Chao subsequently attributed this secondary binding function in PH20 to the Hyal-EGF domain, and suggested it was the type of protein-protein interaction observed for other EGF domains:

> The exact role of the EGF domains is yet to be determined in many cases, but they are thought to mediate protein-protein interactions often associated with regulation of growth and development. Studies have shown involvement of protein partners with hyaluronidases, although the sites of interactions have not been established. The most direct evidence for such interaction pertains to mammalian PH-20, where it was demonstrated that the C-terminal domain encompassing the HyalEGF-like region is responsible for the secondary binding of

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120 EX1079 (Hunnicutt), 84. The Hunnicutt authors demonstrated that an anti-PH20 that inhibited the sperm-zona binding had no effect on hyaluronidase activity, and that a hyaluronidase inhibitor blocked 93% of PH-20 activity without inhibiting sperm-zona binding. EX1079 (Hunnicutt), 83.

121 EX1079 (Hunnicutt), 84.

> the acrosome-reacted sperm to the zona pellucida (a glycoprotein membrane) of oocyte during fertilization.[122]

131. The excerpt reproduced below[123] from the Chao sequence alignment highlights the region corresponding to the Hyal-EGF domain, which spans positions 337-409 in PH20.[124] Within this region, the constituent cysteines of the pattern are at positions 341, 346, 352, 400, 402, and 408, and the associated glycine residues are located at positions 350, 377, 378, and 384. Appendix B-13 further identifies the amino acid substitutions within positions 337-409 (as well as positions 410-412) that were designated as "active" or "inactive" mutants in Tables 3 and 5 of the '731 Application's disclosure.

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[122] EX1006 (Chao), 6916.

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

[124] The terminus of the sequence can plausibly be located at either position 409 or position 416 in human PH20. Chao included part of the last helix arranged around the barrel as part of the definition of the Hyal-EGF, which would put the terminus at position 416. Extending the definition of the Hyal-EGF to position 416 would not alter my opinions.

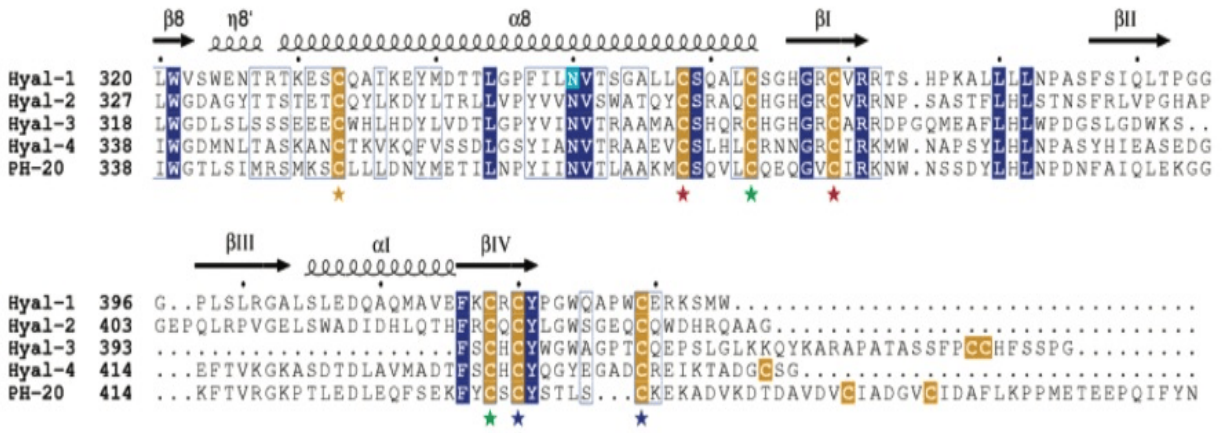


FIGURE 3: Structure-based sequence alignment of human hyaluronidases. Invariant residues are shown in blue except for three key catalytic residues that are colored red. Cysteine residues are colored yellow. The hHyal-1 N-glycosylated asparagines residues are colored turquoise. Residues exhibiting conservative 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.

132. The Chao paper provided important and highly relevant information for evaluating structural features of PH20, particularly from the perspective of an individual interested in modifying the structure of PH20s. Notably, Chao reported the first experimentally determined structure of a human hyaluronidase (Hyal-1), which offered significant insights into human hyaluronidases. Among those insights were the identification of the Hyal-EGF domain, and the availability of a reliable structural template for more accurate modeling of PH20. Chao's sequence alignment further annotated secondary structural elements and identified conserved amino acid residues shared between PH20 and Hyal-1. Chao also emphasized the structural significance of and the relative positioning of the six conserved cysteine residues in human hyaluronidases, including C341, C346, C352, C400, C402, C408, C423 and C429 in human PH20, noting their role in forming disulfide bonds within the Hyal-EGF domain. A POSA would have understood that maintaining

the positions of these cysteine residues is critical, as alterations disrupt or prevent the formation of disulfide bonds within the Hyal-EGF domain of human PH20.[125] I did not find any discussion of Chao in the disclosure of the '731 application, even though Chao was published before the application and its associated provisional applications were first filed in late 2011.

133. In the 2011-2012 timeframe, SWISS-MODEL would generate models that would be very closely based on the Hyal-1 (2PE4) structure provided by Chao, with an overall RMSD deviation of 0.34 Å across 410 aligned residues used in the superposition of the two structures (positions 404-447 in PH20). The human Hyal-1 structure was determined by X-ray crystallography to a resolution of 2.0 Å.[126] This is considered medium high resolution and accurately positions atoms, although some side chains in some loops are less well defined by the experimental electron density. The non-aligned regions are at the N and C-termini and the loop at 376. Although the models would be broadly correct, a POSA would have expected an experimental structure of PH20 determined to equivalent resolution in 2011-2012 to show differences in loops and other subtle changes. For example, portions of the PH20 sequence that lie outside the Hyal-1 template (*i.e.*, positions

[125] EX1006 (Chao), 6912-13.

[126] EX1006 (Chao), 6913 (Table 1) (reporting 2PE4 structure).

1-3 and 404-447) are not based on a structure and thus are not reliable, and other regions have unreliable QMEAN values below 0.6 (PH20 positions 3-5, 27-35, 44-48, 104-105, 234-237, 256-262, 274-278, 357-358, 405-406).

134. A 2009 publication by Zhang et al. (EX1010) reported that deletion of the Hyal-EGF domain abolishes Hyal-1 activity ("Deletion of the EGF-like Domain Abolishes Hyal1 Activity Independently of Asparagine 350.").[127] Zhang also reported that "loss of this domain is deleterious to both secretion and activity" with the impact described as "indirect."[128] I note the level of "residual" activity of these constructs, and their lack of secretion, would classify them as "inactive mutants" as that classification was used in the disclosure of the '731 Application.

135. The study also examined the effects of mutations at residues located near the HA substrate within the active site. To identify residues involved in ligand-interactions, Zhang overlaid the Hyal-1 structure with the previously reported bee venom hyaluronidase (bvH) structure in complex with HA ligand, and measured distances between HA and nearby residues. This structural comparison

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127 EX1010 (Zhang), Abstract, 9439 ("Deletion of the C-terminal epidermal growth factor-like domain, in which Asn^{350} is located, also eliminated activity, irrespective of glycosylation.").

128 EX1010 (Zhang), 9439.

was feasible because the active sites of hyaluronidases are conserved. Zhang further demonstrated that single substitution mutants at each of these identified positions rendered Hyal-1 inactive or markedly reduced its hyaluronidase activity (summarized in Table 1, below).[129]

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.

136. Zhang further reported that the loss of significant enzymatic activity in deletion mutants of Hyal-1 that eliminated the entire Hyal-EGF domain or a C-terminal segment containing an essential asparagine at position 350 suggests that the Hyal-EGF could be involved in proper folding and secretion of the protein and

[129] EX1010 (Zhang), 9435-38, Table 1.

in stabilizing the portion of the protein containing the active site.[130] These observations led Zhang to suggest that the Hyal-EGF domain may play a role in proper folding and secretion of the protein, as well as in stabilizing regions associated with the active site. Zhang also observed that any possible protein-protein interactions mediated by the Hyal-EGF domain would have to occur with the solvent-exposed surface of the domain, given that the deletion mutants affected the enzymatic activity of the protein.[131] Zhang's findings regarding the Hyal-EGF

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[130] EX1010 (Zhang), 9439 ("Inspection of the human Hyal1 model reveals a single overall folded domain, with extensive contacts between the EGF-like region, the sugar moieties of the Asn^{350} glycosyl adduct housed within that region, and the remainder of the protein. We introduced stop codons at position 350 (N350tr), which deleted 86 amino acids, including the Asn^{350} glycosylation site, and at position 356 (L356tr), which deleted the 80-amino acid EGF-like domain but retained Asn^{350}. Neither deletion mutant was secreted to the extracellular space but both were expressed well in the soluble lysate.").

[131] EX1010 (Zhang), 9441 ("It has been proposed that the C-terminal EGF-like domain, found in several reported splice variants of Hyal1, may be the site of as yet undefined protein-protein interactions []. The results presented here

domain were also notable given that bee venom hyaluronidase was known to exhibit activity despite the absence of a Hyal-EGF in its known structure.

137. Zhang also found that the Hyal-1 structure has a "single overall folded domain" with extensive contacts between the EGF-like region … and the remainder of the protein"[132] and that "because the inactivity of the truncated mutants and their inability to be secreted imply that this region does not function as an independently folded domain."[133] Zhang thus would have led a POSA to believe that the truncation disrupted the wild-type protein's native structural arrangement required for substrate binding and catalysis.

138. Zhang illustrates the limits of using homology models to predict effects of single amino acid substitutions, which I described in § II.E. Zhang reports that a Hyal-1 model was used to predict likely catalytic residues, then tested those predictions experimentally, with mixed results. For example, mutating

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suggest that such interactions would have to occur specifically with the solvent-exposed surface of the domain, because the inactivity of the truncated mutants and their inability to be secreted imply that this region does not function as an independently folded domain.").

[132] EX1010 (Zhang), 9439.

[133] EX1010 (Zhang), 9441.

Glu131 abolished activity, but mutating Asp129 did not.[134] This reflects the understanding of a POSA in 2011-2012 that one could make limited predictions using a homology or structural model but experimental confirmations are typically used to test the accuracy of those predictions.

139. Thus, before 2011, a number of residues within the region of the catalytic site in PH20 or Hyal-1 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 Hyal-1 corresponding to D111, E113, D184, S227, Y229, R246, W304, and N333 in PH20, as well as the Hyal-EGF domain from 337-409 in PH20 identified by Chao.

140. Based on Chao and Zhang, for example, a POSA would have understood the Hyal-EGF domain to be necessary for proper folding and enzymatic activity of PH20. Yet the disclosure of the ’731 Application does not cite Chao or Zhang, or discuss their findings. For example, it does not identify the specific portion of the C-terminal sequence of PH20 corresponding to the Hyal-EGF domain as being associated with a particular domain within PH20 or other human hyaluronidase proteins. It does not explain the relationship of the Hyal-EGF

[134] EX1130 (Simpson Dep.), 130:9-16; EX1010 (Zhang), 9436-37.

domain with the catalytic domain, which are reported in Chao and Zhang. While the disclosure refers to a "common core hyaluronidase domain" this is only defined in sequence terms; starting at residue 3 to residue 339 of the mature PH20 protein, it does not associate that region of the protein to any particular domain or structural motif(s).[135]

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

141. A patent filed by Halozyme in 2004 and issued in 2010 (the '429 Patent) (EX1005) 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).[136] The truncation removes the membrane anchor and yielded a soluble version of the wild-type protein that was enzymatically active at neutral pH.

142. Data in the '429 Patent and a 2007 paper by Frost (EX1013) identified seven C-terminally truncated human PH20 proteins that retained >40% activity of the 1-447 PH20 sequence (*i.e.*, $PH20_{1-442}$, $PH20_{1-443}$, $PH20_{1-444}$, $PH20_{1-445}$, $PH20_{1-}$

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[135] EX1026 ('731 App.), 79:20-80:10.

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

$_{446}$, PH20$_{1-447}$, PH20$_{1-448}$). Additional truncations of varying length at the C-terminus of PH20 caused significant variations in hyaluronidase activity (below).[137] For example, the '429 Patent observed that when the PH20 protein was terminated at position 432 (mature protein), it only had 10% of the hyaluronidase activity as mutants that terminate at positions 443 or 448.[138] Also, Frost reported that PH20 had to terminate after 442 and 448 to recover "soluble hyaluronidase activity"[139] 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.[140] Figure 2 from Frost is shown below.[141]

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137 EX1013 (Frost), 432 (Figure 2); *see also* EX1005 ('429 Patent), 87:52-88:24.

138 EX1005 ('429 Patent), 88:15–17 ("The results also showed that deletions beyond A467 eliminated any secreted activity from the A467 clones decreased to approximately 10% of that found P4778 or N483 clones.").

139 EX1013 (Frost), 432.

140 EX1005 ('429 Patent), 88:15-24.

141 EX1013 (Frost), 432 (Figure 2).

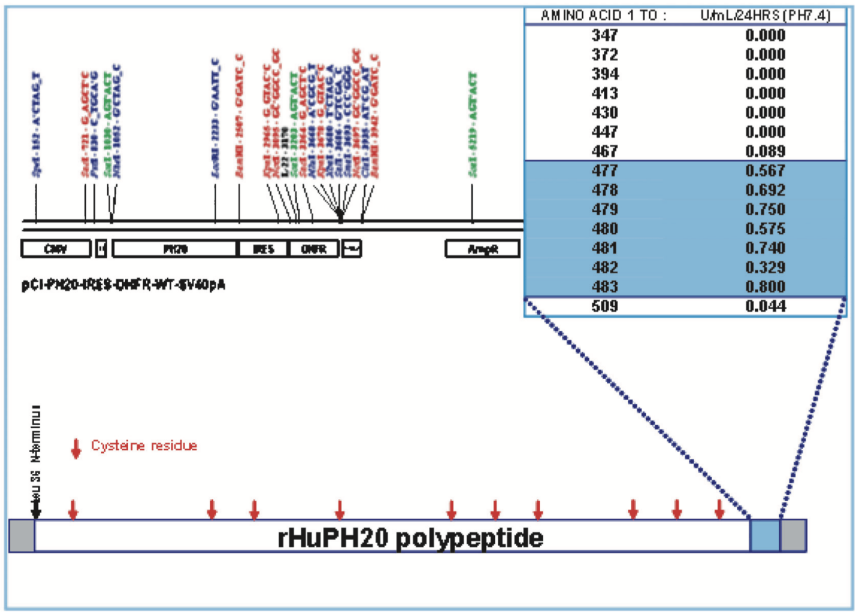
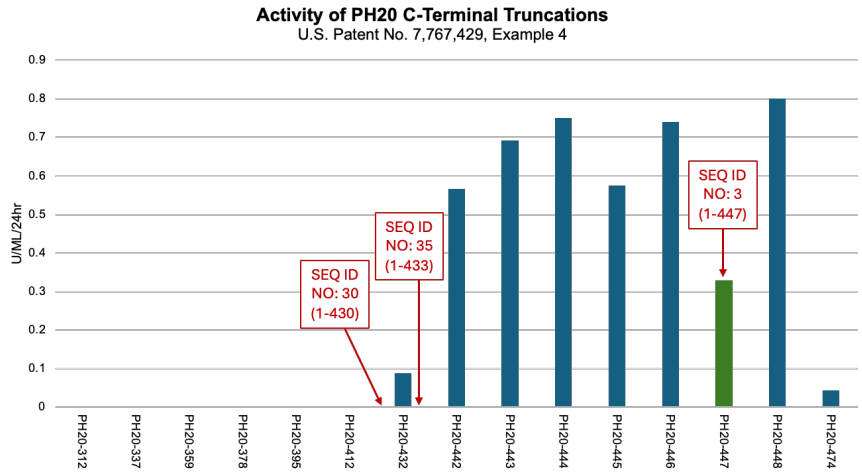


Figure 2. Structural diversity of the hyaluronidase family and development of a soluble recombinant human PH20 enzyme (continued). **C.** Deletions in human PH20 gene within the carboxy terminus from amino acids 477 – 483 result in a secreted active enzyme at neutral pH.
rHuPH20: Human recombinant DNA-derived hyaluronidase enzyme.

143. The data reported in the ’429 Patent and Frost show that C-terminal truncations of varying length had an unusual effect on activity (below). From what these documents report, C-terminal wild-type mutants that differ by only one residue have widely varying activities. For example, the $PH20_{1-446}$ and $PH20_{1-448}$ mutants have twice the activity as $PH20_{1-447}$, even though both differ from the 447-residue mutant by only one amino acid. The terminal residues of these mutants are phenylalanine (F446), tyrosine (Y447), and asparagine (N448). No rationalizing insight is provided in the ’429 Patent for the variation in the data reported for these three mutants.



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

Figure: Measured hyaluronidase activity of 15 C-terminally truncated PH20 variants (source: EX1005, 87:52-88:24 (Example 4)) with values normalized to $PH20_{1-447}$ (100%). Activity data (right) reported by the '429 Patent is shown (1) in U/mL/24 hr and (2) normalized to the reported $PH20_{1-447}$ activity as 100%.

144. Another Halozyme patent application, U.S. 2010/0143457 ("US-457") (EX1105) reported testing of a larger number of C-terminally truncated human wild-type PH20 mutants. US-457 reported that C-terminal truncation mutants terminating before position 430 (*i.e.*, 415-429) were inactive, but those terminating between 430 and at least position 465 were all enzymatically active.[142] The $PH20_{1-}$

[142] EX1105 (US-457), ¶ 0362 (Table 6) ("Human PH20 hyaluronidase carboxy-terminus deletion mutants shorter than I430, corresponding to SPAMl-GDVC to SPAMl-ADVK (SEQ ID NOS:88-95), are inactive. … All other human PH20 hyaluronidase carboxy-terminus deletion mutants (SEQ ID NOS:55-57 and 60-87) are active hyaluronidases at both pH 7.4 and pH 5.5."). In US-

$_{430}$ and $PH20_{1-431}$ mutants are among those described in US-457 as being active.[143] In addition, US-457 reports data showing that activities of C-terminally truncated wild-type PH20s exhibited fluctuations in activity, including those that differ by a single terminal amino acid (see figure and table below).

a. Similar to what was reported in the '429 Patent, US-457 reported that $PH20_{1-446}$ exhibited 103% and $PH20_{1-448}$ exhibited 140% of the hyaluronidase activity of $PH20_{1-447}$.

b. C-terminal truncation mutants terminating between positions 430 and 440 (*i.e.,* $PH20_{1-430}$ and $PH20_{1-440}$) showed widely varying activities. For example, C-terminal truncation mutants terminating at positions 434, 435, and 436 had reported activities between ~300% to ~600% of neighboring C-terminal truncation mutants having one more (437) or one less (433) residue. The activity difference between the 437 and 436 length forms of wild-type PH20 was ~136%.

457, SEQ ID NO: 87 corresponds to $PH20_{1-430}$, while SEQ ID NO: 86 corresponds to $PH20_{1-431}$.

143 EX1105 (US-457), ¶ 0362 (Table 6).

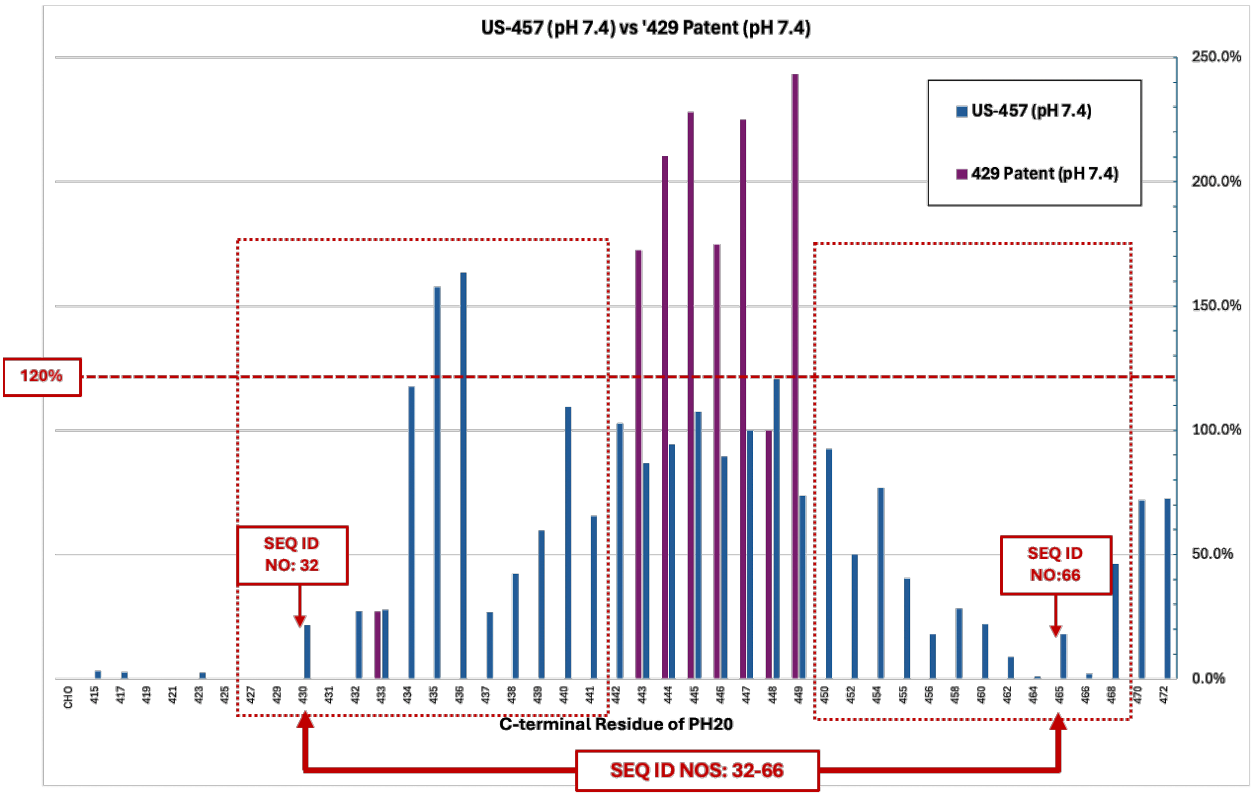


Figure: Measured activity of C-terminal truncation mutants reported US-457 (blue) (EX1105, ¶ 0362, Table 6) and in the '429 Patent (purple) (EX1005, 87:52-88:24 (Example 4). Activities have been normalized to the $PH20_{1-447}$ value in each series (values shown in table below). *See* Appendix B-8.

Deletion Mutant	Precursor	Mature	US-457 (pH 7.4)	429 Patent (pH 7.4)
SPAM1-SSVA	507	472	72.4%	
SPAM1-IISS	505	470	71.8%	
SPAM1-FLII	503	468	46.3%	
SPAM1-ILFL	501	466	2.0%	
SPAM1-VSIL	499	464	1.0%	
SPAM1-FIVS	497	462	8.6%	
SPAM1-TMFI	495	460	22.1%	
SPAM1-SATM	493	458	28.1%	
SPAM1-TLSA	491	456	17.9%	
SPAM1-STLS	490	455	40.3%	
SPAM1-PSTL	489	454	76.6%	
SPAM1-ASPS	487	452	50.1%	
SPAM1-YNAS	485	450	92.4%	
SPAM1-FYNA	484	449	73.8%	
SPAM1-IFYN	483	448	120.6%	243.2%
SPAM1-QIFY	482	447	100.0%	100.0%
SPAM1-PQIF	481	446	89.5%	224.9%
SPAM1-EPQI	480	445	107.4%	174.8%
SPAM1-EEPQ	479	444	94.4%	228.0%
SPAM1-TEEP	478	443	86.8%	210.3%
SPAM1-ETEE	477	442	102.8%	172.3%
SPAM1-METE	476	441	65.6%	
SPAM1-PMET	475	440	109.4%	
SPAM1-PPME	474	439	59.6%	
SPAM1-KPPM	473	438	42.3%	
SPAM1-LKPP	472	437	26.8%	
SPAM1-FLKP	471	436	163.5%	
SPAM1-AFLK	470	435	157.7%	
SPAM1-DAFL	469	434	117.6%	
SPAM1-IDAF	468	433	27.7%	
SPAM1-CIDA	467	432	27.2%	27.1%
SPAM1-VCID	466	431	0.0%	
SPAM1-GVCI	465	430	21.7%	
SPAM1-DGVC	464	429	0.0%	
SPAM1-IADG	462	427	0.0%	
SPAM1-VCIA	460	425	0.0%	
SPAM1-VDVC	458	423	2.4%	
SPAM1-DAVD	456	421	0.0%	
SPAM1-DTDA	454	419	0.0%	
SPAM1-VKDT	452	417	2.7%	
SPAM1-ADVK	450	415	3.1%	
CHO-S	CHO	CHO	0.0%	

TABLE 6

Deletion Mutant	Precursor (AA)	Mature (AA)	Hyaluronidase Activity: pH 7.4 Activity (Units/ml)	Hyaluronidase Activity: pH 5.5 Activity (Units/ml)
SPAM1-AFLK (SEQ ID NO: 82)	470	435	3.204	2.925
SPAM1-DAFL (SEQ ID NO: 83)	469	434	2.3895	2.2365
SPAM1-IDAF (SEQ ID NO: 84)	468	433	0.5625	0.62775
SPAM1-CIDA (SEQ ID NO: 85)	467	432	0.5535	0.4725
SPAM1-VCID (SEQ ID NO: 86)	466	431	0	0.2115
SPAM1-GVCI (SEQ ID NO: 87)	465	430	0.441	0.468

145. The ’429 Patent and Frost study reported that several PH20 truncation mutants before position 430 lacked detectable hyaluronidase activity. A number of these truncations fall within the region later identified by Chao as encompassing the Hyal-EGF domain, which corresponds to positions 337-409 in the mature PH20 sequence. One truncation mutant, $PH20_{1-412}$, terminates only three residues beyond the C-terminal boundary of the Hyal-EGF domain, as shown in the table below.[144] The truncation variants within the Hyal-EGF region terminate at positions 312, 337, 359, 378, and 395. Consistent with these observations, Zhang

[144] EX1005 (’429 Patent), 87:55-67.

reported that deletion of a segment just upstream of the Hyal-EGF domain in Hyal-1 "abolishes HYAL1 activity."[145]

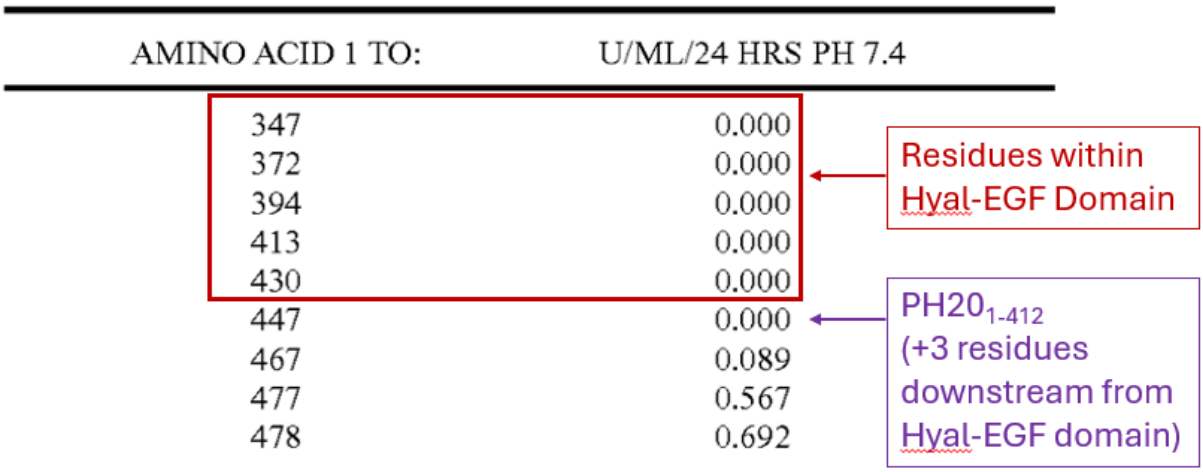


Figure: C-terminal truncation PH20 mutant activity data reported by the '429 Patent. Overlayed markers indicate which positions and corresponding data fall within the Hyal-EGF domain ("red"), and which do not ("purple"). The numbers in the Table include the N-terminal signal peptide, the corresponding number in the mature protein is obtained by subtracting 35.

146. The 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:

[145] EX1010 (Zhang), 9439 ("Deletion of the EGF-like Domain Abolishes Hyal1 Activity Independently of Asparagine 350"; "The lysates showed ~2.5 and ~6.3% of wild-type activity for N350tr and L356tr… Thus, loss of this domain is deleterious to both secretion and activity of Hyal1, although the residual activity suggests the effect is indirect.").

> 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 EX1005 ('429 Patent)].[146]

147. The disclosure refers to the wild-type PH20 truncation mutants of PH20 discussed in the '429 Patent, Frost paper, and US-457 but does not discuss the notable variations in the activities of those truncation mutants.[147] Neither the scientific literature existing by 2011 nor the disclosure provides an explanation why these PH20 truncation mutations that differ by one residue (*e.g.*, $PH20_{1-446}$ vs. $PH20_{1-447}$ vs. $PH20_{1-448}$) exhibit such variability in their activity. The illustration below compiles some of the findings in the scientific literature and the '731

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[146] EX1026 ('731 App.), 79:25-30.

[147] EX1026 ('731 App.), 84:21-25 ("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…).").

Application regarding truncations in C-terminal region of the PH20 and Hyal-1 proteins.

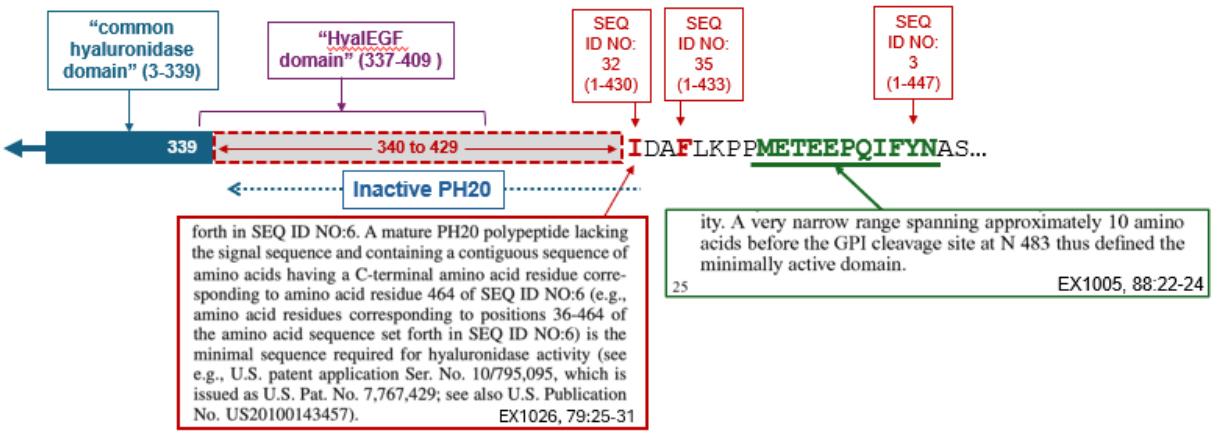


Figure: (1) "Green" region is the sequence identified in '429 Patent as the "minimally active domain" of PH20 extending further into the C-terminus of PH20. (2) "Red-dashed" box is the C-terminal portion of the "minimal sequence" identified by disclosure as being necessary for hyaluronidase activity. (3) "Red" SEQ ID boxes designate identified terminal residues of SEQ ID NOS: 32 (1-430), 35 (1-433) and 3 (1-447). (4) "Purple" box identifies the Hyal-EGF domain identified by Chao. (5) "Blue" box identifies the "common hyaluronidase domain" referred to in the disclosure of the '731 Application.

148. Before 2011-2012, a POSA would have connected the data reported in the '429 Patent and US-457 with the Chao report of the existence of the Hyal-EGF domain. The POSA 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 Hyal-1. The Hunnicutt publication also reported that the functions of the catalytic and Hyal-EGF domains

in PH20 could be independently inhibited.[148] Overall, the experimental evidence suggests that making multiple amino acid substitutions within the Hyal-EGF domain of PH20 that result in changes to that domain structure could impair the enzymatic activity of PH20, even if the PH20 protein possessed an otherwise intact catalytic site.

149. The experimental results reported in the disclosure portray single amino acid substitutions within the 337-409 region of PH20 as producing both active mutants and inactive mutants across most of that region. *See* Appendix B-13. Substitutions at cysteine residues—which are highly conserved—almost uniformly resulted in inactive mutants. The range of outcomes observed for single-residue changes within the Hyal-EGF region underscores the unpredictability associated with modifying this portion of PH20. That unpredictability would reasonably be expected to increase further if multiple changes were made concurrently.

150. The variations in activities observed for the C-terminal truncation wild-type PH20 mutants with even single amino acid differences indicate that PH20 is likely to be influenced by epistasis when multiple modifications are introduced. Epistasis can produce unpredictable, non-local effects. *See* § II.D.

[148] EX1079 (Hunnicutt), 80. *See* § II.G.2.

Given the unexplained variability in the C-terminal truncation mutants and the likely epistatic effects of making additional mutations, POSAs would be unable to reliably predict the outcome of mutating multiple residues in this region.

151. There are no examples in the disclosure of a PH20 polypeptide truncated to a position below position 447 (*e.g.*, 433, 430) with one or more substitutions (or any other type of modification). Likewise, there are no examples in the disclosure of single-substitutions in PH20 mutants at positions above 447. Given the varying activities seen with different C-terminal truncation mutants in the wild-type PH20 polypeptide and the likelihood of epistatic effects in PH20, a POSA would not have considered the effects observed from making single amino acid substitutions in the $PH20_{1\text{-}447}$ protein to be predictive of making the same changes (alone or with other changes) in PH20 proteins truncated before or after position 447, even if the changes are remote from the catalytic site of the PH20 enzyme.

III. The Disclosure's Classification of Mutants

A. The Disclosure Describes Two Distinct, Non-Overlapping Categories of Modified PH20 Polypeptides

152. The disclosure identifies two non-overlapping forms of modified PH20 polypeptides. The disclosure distinguishes these forms by reference to hyaluronidase activity. One group, characterized as "active mutants" is defined as

retaining at least 40% hyaluronidase activity of the unmodified form of PH20.[149] The other group, referred to as "inactive mutants," is described as lacking meaningful hyaluronidase activity, which the disclosure equates to activity levels of 20% or less relative to unmodified PH20.[150]

153. The disclosure discusses these two categories of mutants in separate portions of the document. The section devoted to "active mutants" spans from page 90, line 32 through page 130, line 28. That section includes Table 3, which summarizes the "active mutants" that were generated, each of which contains a single amino acid replacement in the $PH20_{1-447}$ wild-type sequence. In addition, the disclosure identifies single-replacement $PH20_{1-447}$ "active mutants" in Table 9, along with measurements of their relative activity to unmodified $PH20_{1-447}$.

154. For "active" mutants, a threshold of 40% of the hyaluronidase activity of the corresponding unmodified PH20 is expressly stated in connection with Table 3, which identifies "active mutants" as those modified PH20 polypeptides that exhibit at least 40% of the hyaluronidase activity of PH20 polypeptide lacking the amino acid substitution.[151] The disclosure similarly explains in connection with

[149] EX1026 ('731 App.), 90:33-91:6.

[150] EX1026 ('731 App.), 130:29-35.

[151] EX1026 ('731 App.), 90:32-93:3.

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."[152] The values in Table 9, however, reflect ***an average of two assay results***. Some mutants in Table 9 are reported as having activity between 20% and 40% activity of unmodified $PH20_{1-447}$.[153] This means in at least some cases this is an average of "active" result (> 40%) and "inactive" result (< 20 %). This large variation in duplicates would be apparent to a POSA and lead them to question the reliability of the assay as executed in the disclosure.

155. "Inactive mutants" are discussed from page 130, line 29 to page 132, line 13 in the 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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[152] EX1026 ('731 App.), 251:2-3 (emphasis added).

[153] EX1026 ('731 App.), 251:6-9.

> such as less than 10%, of the hyaluronidase activity of a wildtype or reference PH20 polypeptide…[154]

156. Example 4 explains that mutants showing less than 20% of wild-type PH20 hyaluronidase activity of wild-type PH20 in at least one of the duplicate assays were "rescreened to confirm that the dead mutants are inactive."[155] The same biotinylated HA assay was used in Example 3 but the samples were incubated overnight before measurement.[156] As described in the disclosure:

> 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.[157]

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[154] EX1026 ('731 App.), 130:30-35.

[155] EX1026 ('731 App.), 272:2-8, 272:20-22, Table 10.

[156] An "overnight" incubation period indicates these samples would have been measured between 8 and 12 hours after the first assay was performed.

[157] EX1026 ('731 App.), 272:5-8.

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

157. Table 8 of the disclosure lists 6,753 $PH20_{1-447}$ mutants that were produced from a library "created by mutagenesis of a PH20 template."[158] That template was generated by cloning DNA into a plasmid that was used to transfect individual CHO cells. The plasmid encoded two proteins: (i) a mutagenized $PH20_{1-447}$ sequence and (ii) secreted alkaline phosphatase (SEAP); both sequences were under the control of "a single CMV promotor that drives expression of PH20 and SEAP in the construct."[159] The library was "made to generate encoded variant proteins wherein each of residues …1-447 of SEQ ID NO:3 … was changed to one of about 15 amino acids, such that each member contained a single amino acid change."[160] This yielded a library of CHO cells that each were capable of expressing the different singly-substituted $PH20_{1-447}$ mutants in the library.

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158 EX1026 ('731 App.), 220:27-28, 220:17-20 ("The resulting library contained 6753 variant members, each containing a single amino acid mutation compared to residues 23-469 of SEQ ID NO: 2 (corresponding to residues 1-447 of SEQ ID NO:3 …)").

159 EX1026 ('731 App.), 219:31-220:9.

160 EX1026 ('731 App.), 220:14-17.

1. The Experimental Protocol Classified Mutants Based On Screening of Supernatants for Hyaluronidase Activity

158. The disclosure indicates that "each member" of the mutant library of ~6,800 mutants "was expressed and screened for hyaluronidase activity."[161] High throughput screening was performed on supernatants obtained from the transfected cells.[162] The supernatants were tested for their ability to hydrolyze a biotinylated 1.2MDa hyaluronic acid substrate[163] as well as for alkaline phosphatase (SEAP) activity.[164] Duplicate assays were performed for each supernatant, and readouts were obtained for both SEAP and hyaluronidase activity.[165] Hyaluronidase activity for each supernatant was then normalized to the SEAP activity measured for that supernatant and a relative activity to an unmodified control $PH20_{1-447}$ was

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[161] EX1026 ('731 App.), 220:27-28; *also* EX1026 ('731 App.), 250:34-37 ("Each generated variant was screened for hyaluronidase activity.").

[162] EX1026 ('731 App.), 248:5-6 ("Activities of the supernatants were screened as described in the following examples."), 248:9-250:31.

[163] EX1026 ('731 App.), 248:16-249:3 (describing the bHA activity assay).

[164] EX1026 ('731 App.), 250:21-31.

[165] EX1026 ('731 App.), 249:4-250:19.

calculated.[166] The disclosure also indicates that it used "criteria for the high throughput (HTP) screening" that included >0 SEAP activity, and that the control PH20 (*i.e.*, rHuPH20$_{1-447}$) wild type sequence had >1 U/ml activity.[167]

159. Approximately ~5,866 of the ~6,800 PH20$_{1-447}$ variants were classified as "active mutants" or "inactive mutants" based on the measured hyaluronidase activity in supernatants from each transfected cell in the library.[168]

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[166] EX1026 ('731 App.), 248:9-250:36, 250:34-37 ("Each generated variant was screened for hyaluronidase activity as described in Example 3. The SEAP expression was used to normalize PH20 activity of each variant to the PH20 wildtype.").

[167] EX1026 ('731 App.), 250:26-28 ("The criteria for the high throughput (HTP) screening were that the transfected supernatant resulted in a SEAP signal of ≥0.1 and the signal for the rHuPH20 wildtype control produced a signal of ≥1 U/mL.").

[168] EX1026 ('731 App.), 250:34-37 ("Each generated variant was screened for hyaluronidase activity as described in Example 3. The SEAP expression was used to normalize PH20 activity of each variant to the PH20 wildtype. Mutants were identified that exhibited altered hyaluronidase activity compared to wildtype.").

PH20 mutants were included in Table 9 if at least one of two duplicate assays reported ≥0.4 (≥40%) of $PH20_{1-447}$ activity.[169] The two measured hyaluronidase values for these mutants, however, are not reported in Table 9; what is reported instead is an average of the two values.[170] Mutants were included in Table 10 if they exhibited activity <0.2 (20%) of $PH20_{1-447}$.[171] There are no reported hyaluronidase activity measurements in Table 10, so it is possible a mutant classified as "inactive" could have measured 19.4 % activity.

160. The '731 Application explains that the supernatant of transfected cells was generally collected 96 hours after transfection and stored at -20°C.[172] It also

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169 EX1026 ('731 App.), 251:1-3 ("Active mutants were selected whereby at least one duplicate sample exhibited greater than 40% of wildtype activity when normalized to SEAP activity.").

170 EX1026 ('731 App.), 251:6-9 ("The Table also sets forth the average hyaluronidase activity of tested duplicates normalized by SEAP values compared to average of wildtype PH20 activities in each plate, which were also normalized by their own SEAP values.").

171 EX1026 ('731 App.), 251:2-9, 272:3-8.

172 EX1026 ('731 App.), 247:2-248:6.

suggests that supernatants "***containing*** the variant PH20 protein" were screened using the described assays.[173] This is inaccurate. While screened supernatants exhibiting hyaluronidase activity contain some quantity of a mutant PH20 protein, the same cannot be said for assayed supernatants which were classified as inactive.[174] That is because the absence of measurable hyaluronidase activity in the supernatant cannot differentiate between situations where (i) the transfected cell did not secrete any PH20 mutant into the supernatant and (ii) the PH20 polypeptide was secreted into the supernatant, but either (a) did not fold or maintain a folded state in the supernatant or (b) was properly folded but exhibited hyaluronidase activity below the 20% activity cutoff used for classification of mutants.

161. The '731 Application does not describe any step that investigated whether supernatants classified as "inactive" contained any amount of a mutant PH20 protein. Instead, it indicates that supernatants that reported less than 20% hyaluronidase activity "were rescreened to confirm that the dead mutants are inactive" by testing them for activity over a longer period (*i.e.*, a modified form of the screening assay was used that incorporated "an overnight 37° C substrate-

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[173] EX1026 ('731 App.), 247:2-248:6 (emphasis added); 248:11-15.

[174] EX1026 ('731 App.), 248:9-250:37.

sample incubation step prior to measurement of enzymatic activity" explaining this "modified assay is intended to detect PH20 activities below 0.2 U/ml.").[175] The experimental design did test for SEAP activity in the transfected cells, and SEAP activity allows one to infer that a host cell was successfully transfected and that the plasmid was expressed.[176] The presence of SEAP activity, however, does not demonstrate that the intended $PH20_{1-447}$ variant was successfully expressed, was properly folded, was stable, or was secreted from the transfected cell.[177]

162. Among the flaws I identified in the hyaluronidase assay design and reporting of results for inactive mutants were the following:[178]

a. No steps taken to confirm that each inactive mutant PH20 was produced or results from such investigations;

b. No steps taken to confirm secretion or quantify levels of inactive mutants in the supernatants, or report such results;

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175 EX1026 ('731 App.), 272:2-5.

176 EX1026 ('731 App.), 250:21-31.

177 EX1131 (Petsko Dep.), 241:3-16, 242:24-243:10, 247:1-248:11.

178 EX1026 ('731 App.), 272:3-22.

c. No steps taken to detect aggregation of inactive mutants in supernatants, or results from such investigations;[179]

d. No steps taken (*e.g.*, circular dichroism) to detect whether any mutants classified as "inactive" had folded, or results from such investigations;

e. No values are reported for the activity measurements of mutants classified as "inactive" despite being measured at two time points[180] (*i.e.*, mutants are simply listed in Tables 5 and 10 without activity readings);

f. No evidence of controls regarding cryopreservation methods of −20°C freezing followed by freeze/thaw prior to activity screening. This protocol could plausibly promote uncontrolled variability in the measured sample activity;

g. No turbidity measurements or other assessments of sample clarity to detect aggregation;

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[179] EX1131 (Petsko Dep.), 239:12-23 ("Q. It could be that the protein was secreted but then aggregated to the point where it had no activity? A. Less likely. You'd probably pick that up as turbidity. Q. Right. There is no measurement of turbidity in these - A. You just notice it, if you were careful. Q. Just from what we have in the patent, we don't know if it is one possible outcome is -- it is an aggregated outcome, right? A. Um-hmm.").

[180] EX1026 ('731 App.), 248:1-5, 272:3-19.

h. No disclosure of the standard curve, positive-control data, or statistical analysis needed to determine whether "inactive" classifications reflect true loss of activity or assay variability; and

i. No protein-level evidence that PH20 was expressed or secreted for mutants classified as "inactive," and no validation that SEAP normalization sufficiently controlled for experimental variability.

2. Observations Regarding the Mutant Activity Classifications

163. I reviewed the mutant classifications reported in the disclosure, particularly Tables 9 and 10. However, it is also important to consider the data in the context of the single-substitution $PH20_{1-447}$ mutants that were made but not classified (as identified in Table 8), as well as in view of the single-substitution $PH20_{1-447}$ mutants that are not listed in Table 8. Viewed in this way, as a POSA would recognize:[181]

a. Approximately 2,357 supernatants exhibited at least 40% relative activity and were classified as "active mutants";

b. Approximately 3,330 supernatants were classified as being "inactive mutants";

[181] EX1135 (Dr. Naismith Analysis Spreadsheet); *see also* Appendix B-1.

c. Approximately 127 of the mutants classified as active had measured activities between 20 and 40%;

d. There are approximately 50 errors in the classifications of the mutants (*e.g.*, due to double counting, errors in counts, etc.);

e. Approximately 897 of the mutants made (Table 8) were not classified as an "active" or "inactive" mutant. No reason is provided for the lack of classification of these mutants;

f. Approximately 1,740 single-substitutions that could have been made were not made;

g. The majority of single-substitution $PH20_{1-447}$ variants that are classified in the disclosure displayed lower activity than the unmodified $PH20_{1-447.}$ Specifically, 56.3% of the tested mutants were classified as inactive, and an additional 28.5% were reported as exhibiting average activity levels below that of the unmodified protein (<100%);

h. Approximately 13.5% of the single-substitution $PH20_{1-447}$ mutants are reported as exhibiting increased activity relative to unmodified $PH20_{1-447}$, with 9% of that group exhibiting activity >120%;

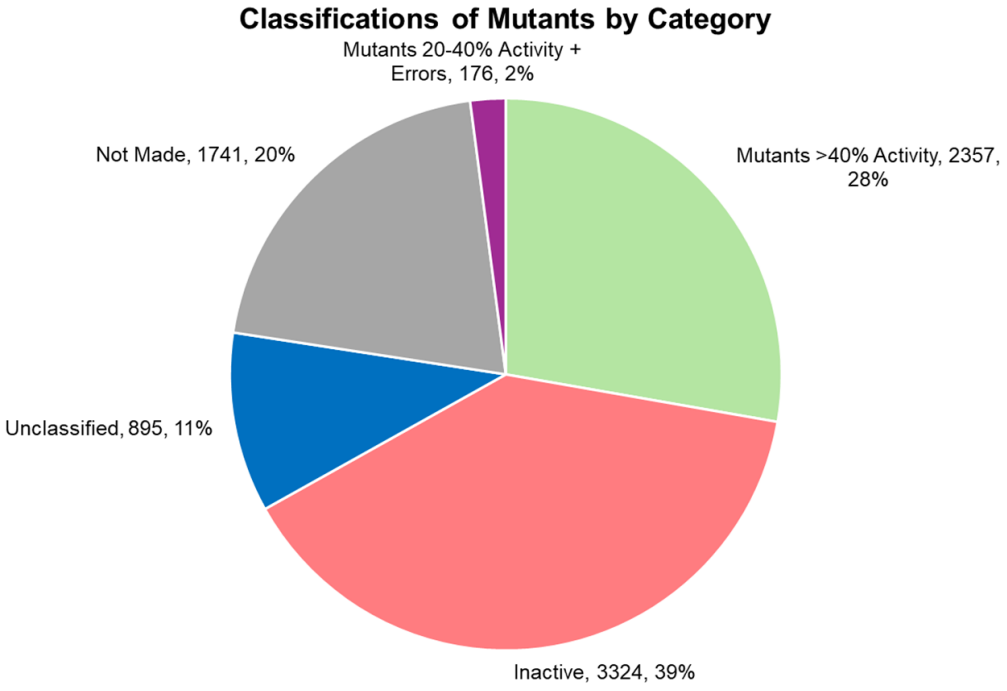
i. Of those single-substitution mutants with ≥120% relative activity to $PH20_{1-447}$, ~290 have a substitution that falls within the scope of independent claim 1, while ~96 fall within the scope of independent claim 4.[182] I further note that the disclosure does not contain any position 209 mutants with ≥120% activity,[183] and the E151L substitution recited in claim 2 is described as having only 103% activity (*i.e.*, <120%).[184]

Activity vs. Unmodified PH20	**Number**	**% of Tested (5,916)**
Active Mutants (Table 9)		
>120%	532	9.0%
100%-120%	265	4.5%
40%-100%	1,562	26.4%
Inactive Mutants (Table 9)		
20-40%	127	2.1%
Inactive Mutants (Table 10)		
Number	3,330	56.3%
Double Counted Mutants		
Tables 9 & 10	50 (100)	1.7%

182 EX1200 (US-275 Activity Data and '400 Patent Claims Comparison Spreadsheet), 1, 3; *see also* Appendix B-12.

183 EX1026 ('731 App.), 262, 272 (Table 9).

184 EX1026 ('731 App.), 255 (Table 9).


Classifications of Mutants by Category
Mutants 20-40% Activity + Errors, 176, 2%
Not Made, 1741, 20%
Mutants >40% Activity, 2357, 28%
Unclassified, 895, 11%
Inactive, 3324, 39%

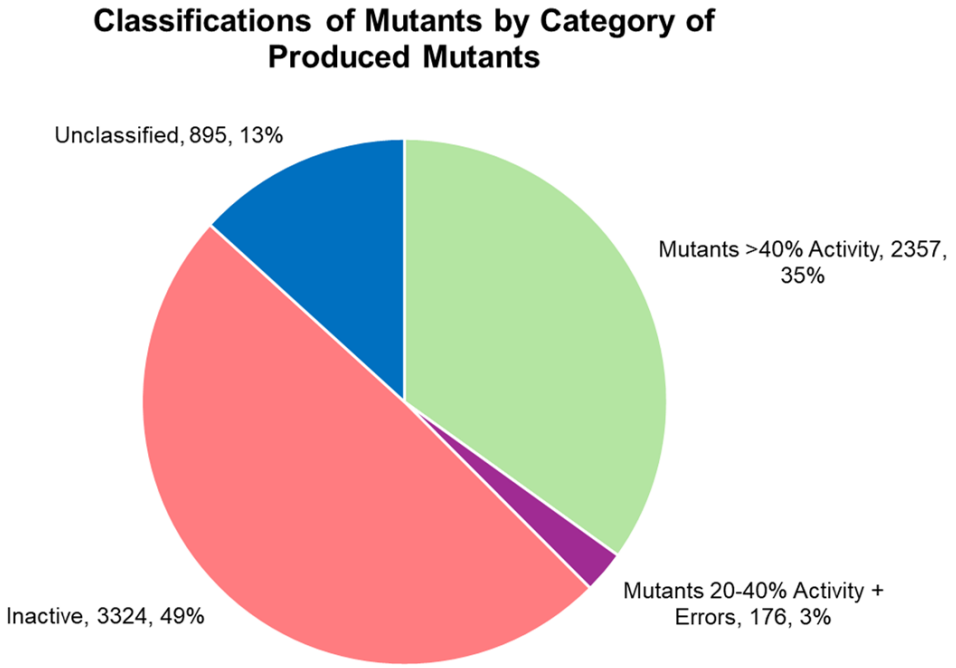

Classifications of Mutants by Category of Produced Mutants
Unclassified, 895, 13%
Mutants >40% Activity, 2357, 35%
Inactive, 3324, 49%
Mutants 20-40% Activity + Errors, 176, 3%

TABLE 9: ACTIVE MUTANTS

mutant	SEQ ID NO	AvgNorm Act.
E151K	384	2.05
E151L	385	1.03
E151M	386	1.26

K209A		0.53
K209E		0.46
K209G		0.44
K209N		0.50
F398L		0.35
S404T		0.37
K209R	458	0.68
K209S		0.50
K209T		0.50

164. There are also errors in the classifications reported by the disclosure. A substantial number of individual mutants (50) were double-counted as both "active" and as "inactive" mutants in the disclosure (*e.g.*, mutants listed in both Tables 9 and 10), including: L061F, P065R, I067R, D068G, D068L, D068P, S069T, G072H, Q081P, K082Y, I083K, S084Y, Q086P, D087P, L089W, D090G, A092H, L105C, P117D, W119P, Q143C, L165C, P177V, W181K, Y183E, K195S, G198W, N211W, W218V, P226W, D275L, D275V, K279A, V287N, T289S, G291C, I303D, W304G, W304I, M310F, D320V, I331E, I331C, V351C, F398L, A412H, D413K, D413H, V414K and A425Y.[185] These double-counted mutants create an ambiguity that cannot be resolved using the information in the disclosure. The disclosure, for example, does not report measured hyaluronidase

[185] EX1026 ('731 App.), Tables 9, 10; EX1135 (Dr. Naismith Analysis Spreadsheet); *see also* Appendix B-1.

activity values for the inactive mutants. A POSA would have found the classifications of these mutants to be unreliable.[186]

165. Significantly, Tables 9 and 10 account for 5,917 of the 6,753 mutants in Table 8, leaving 897 unclassified single-substitution $PH20_{1-447}$ mutants not discussed in the disclosure (about 13%).[187] The '731 Application does not explain why data from the characterization of these ~900 mutants were omitted and it does not classify any of these ~900 mutants as an "active mutant" or an "inactive mutant." Given these points, a POSA could not have determined from the disclosure and could not have known whether any of these ~900 unclassified mutants were "active mutants" or "inactive mutants" (mutants that were incapable of folding, could not be recovered from the transfected cells, and/or were secreted but remained unstable and/or unfolded polypeptides).

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[186] Tables 9 and 10 also contain other errors, for example: E249V has no reported activity value but is listed in Table 9 (EX1026 ('731 App.), 267); Table 9 lists mutant K297R even though G297 is wildtype (EX1026 ('731 App.), 262); R311G is listed twice in Table 9 with two different activities (EX1026 ('731 App.), 253); and Table 10 lists mutants G378D, G378E, G378F, and G378I twice (EX1026 ('731 App.), 272-273, 282).

[187] For simplicity, I am referring to these as the "~900 unclassified mutants."

166. For each position in PH20, there are 19 possible amino acid substitutions that can be substituted for the wild-type residue. The 19 alternatives will span several physiochemical classes of amino acids—including (i) charged residues (positively or negatively charged), (ii) polar uncharged residues, (iii) hydrophobic residues, and (iv) large hydrophobic, aromatic residues—that impose different structural and energetic constraints on a protein. Because substitutions from different classes of amino acids can introduce different types of physical or chemical changes in the environment of a substitution, it is important to assess whether a particular position has been substituted with a sufficiently diverse set of physicochemical interrogations associated with the 19 alternative amino acids. But as explained in ¶ 163, nearly 20% of the possible substitutions were not made and another 11% were made but not classified by their effects. For positions that were tested, the numbers of substitutions varied between 2 and 17 per position, with many positions not being fully interrogated from a physicochemical perspective.[188] These shortcomings limit the insights that can be obtained for particular positions in PH20 from the single-substitution mutant data in the disclosure.

167. The relative activities reported in the disclosure for "active" mutants do not provide measured values for the activity of the unmodified $PH20_{1-447}$ used

[188] EX1135 (Dr. Naismith Analysis Spreadsheet); *see also* Appendix B-1.

as the reference for percentages reported in Table 9. There is no statistical analysis of the data provided. Given the activity values of the positive control reported in Table 11 and 12 varied so extensively from run to run this lack of information would give concern to the POSA as regards the reliability of the thresholds used for classification. For example, if the measured activity of the control (the unmodified $PH20_{1-447}$) used as the reference assay exhibited a variation of +/- 25% or more in its replicates, it would be difficult to differentiate mutants exhibiting 125% vs 75% from the unmodified PH20.

168. The disclosure does not report any analysis of intra-assay or inter-assay variability data for the activity measurements. Example 5 reports 17 measurements of the activity in duplicate of samples of the wild-type $PH20_{1-447}$ protein used as the positive control. It also includes one set of measured values at the same temperature (37°C) used in the primary screen of the mutant library. The measured replicate hyaluronidase activity values for these positive controls are in Table 11.[189] One can therefore use the reported wild-type $PH20_{1-447}$ positive control values in Example 5 to estimate both the intra-plate (between duplicates) and inter-plate (between plates) variability of the biotinylated hyaluronan substrate

[189] EX1026 ('731 App.), 284:26–36, 293 (Table 11), 302-03 (Table 12).

assay used in Examples 2-4.[190] To estimate intra-plate and inter-plate variability using the Example 5 data, one can analyze the individual wild-type PH20 activity values reported for each of the three assay conditions.[191] As noted in the table below, many of the duplicate measurements showed large intra-plate variability (below):

[190] Example 5 reports activity values for 17 wild-type PH20 control measurements under each condition but does not explicitly state whether those measurements were obtained on separate plates or in separate assay runs. I conservatively assume that these measurements reflect results obtained across multiple plates or runs because the values are presented individually rather than as averaged replicates, and because Example 5 describes rescreening and repeated testing under distinct conditions. Even if some measurements were obtained on the same plate, the observed dispersion among wild-type values would still reflect assay-level variability relevant to interpretation of high-throughput screening results.

[191] EX1026 (’731 App.), 293, Tables 11–12.

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

Positive Control #	Absolute Hylarudonidase Activity														
	No Incubation (4°C)					37°C (no cresol)					37°C (with m-cresol)				
	Dup. 1	Dup. 2	Mean	STDEV	CV	Dup. 1	Dup. 2	Mean	STDEV	CV	Dup. 1	Dup. 2	Mean	STDEV	CV
1	2.919	2.173	2.546	0.527502	20.7%	2.773	2.105	2.439	0.472347	19.4%	0.145	0.178	0.1615	0.023335	14.4%
2	3.984	4.463	4.2235	0.338704	8.0%	4.215	4.823	4.519	0.429921	9.5%	0.189	0.253	0.221	0.045255	20.5%
3	3	2.725	2.8625	0.194454	6.8%	3	3.325	3.1625	0.22981	7.3%	0.1	0.125	0.1125	0.017678	15.7%
4	2.501	2.883	2.692	0.270115	10.0%	2.37	3.158	2.764	0.5572	20.2%	0.452	0.522	0.487	0.049497	10.2%
5	7.629	2.989	5.309	3.280975	61.8%	10.835	3.914	7.3745	4.893886	66.4%	0.485	0.219	0.352	0.18809	53.4%
6	5.783	5.356	5.5695	0.301935	5.4%	2.609	3.643	3.126	0.731148	23.4%	0.542	0.402	0.472	0.098995	21.0%
7	5.279	5.422	5.3505	0.101116	1.9%	2.815	4.026	3.4205	0.856306	25.0%	0.618	0.401	0.5095	0.153442	30.1%
8	4.775	4.385	4.58	0.275772	6.0%	2.845	3.327	3.086	0.340825	11.0%	0.718	0.54	0.629	0.125865	20.0%
9	3.617	4.264	3.9405	0.457498	11.6%	3.322	3.427	3.3745	0.074246	2.2%	0.633	0.479	0.556	0.108894	19.6%
10	5.881	4.511	5.196	0.968736	18.6%	5.518	4.359	4.9385	0.819537	16.6%	0.743	0.848	0.7955	0.074246	9.3%
11	6.754	4.932	5.843	1.288349	22.0%	3.902	4.12	4.011	0.154149	3.8%	0.665	0.724	0.6945	0.041719	6.0%
12	3.911	3.494	3.7025	0.294864	8.0%	3.911	5.179	4.545	0.896611	19.7%	0.726	0.841	0.7835	0.081317	10.4%
13	5.406	7.559	6.4825	1.522401	23.5%	4.018	4.62	4.319	0.425678	9.9%	0.735	0.429	0.582	0.216375	37.2%
14	4.015	3.887	3.951	0.09051	2.3%	3.94	3.408	3.674	0.376181	10.2%	0.334	0.341	0.3375	0.00495	1.5%
15	2.604	2.339	2.4715	0.187383	7.6%	2.443	2.391	2.417	0.03677	1.5%	0.235	0.233	0.234	0.001414	0.6%
16	3.736	3.473	3.6045	0.185969	5.2%	3.621	3.056	3.3385	0.399515	12.0%	0.31	0.277	0.2935	0.023335	8.0%
17	3.759	3.509	3.634	0.176777	4.9%	3.633	3.049	3.341	0.41295	12.4%	0.36	0.303	0.3315	0.040305	12.2%
				Std Dev	13.9%				Std Dev	14.4%				Std Dev	12.9%

169. Inter-plate variability for the WT replicates also varied significantly: between 33% and 49% for some conditions (below). For each condition, reported wild-type measurements are treated as independent observations and calculated

basic descriptive statistics, including the mean activity (μ), standard deviation (σ), and coefficient of variation (CV)[192]:

$$\mathrm{CV} = \frac{\sigma}{\mu}$$

Assay Condition	Number of WT Measurements (n)	Mean WT Activity (μ)	Standard Deviation (σ)	Coefficient of Variation (CV = σ/μ)
37 °C, No Preservative	17	3.756	1.488	40%
37 °C, + Preservative (m-cresol)	17	0.444	0.222	49%
Control Condition (4 °C baseline)	17	4.233	1.407	33%

170. The magnitude of variability observed in Example 5 is substantially higher than what a POSA would consider acceptable for reliable quantitative interpretation of a high-throughput screening assay. It is also significantly higher than the inter-assay (inter-plate) variability benchmarks reported for properly

[192] EX1148 (Reed), 1235 ("In many laboratories, the variability of the ELISA and other methods of chemical assay that produce continuous-type values is summarized not by the standard deviation (SD) but by the CV, which is defined as the SD divided by the mean, with the result often reported as a percentage.").

characterized biotinylated hyaluronan microtiter assays. For example, Halozyme's US-457 publication reported that the typical inter-assay variation of the biotinylated hyaluronan microtiter assay was less than 10%.[193] The significant variability observed in Example 5 indicates that the disclosure's measured activity values are subject to uncontrolled experimental variability. The estimated coefficients of variation for the 37° C assay run without m-cresol was ~40%, and large differences (≥20 %) are reported for some duplicates. Classification of mutants (inactive, active, ≥ 120 %) could in several cases therefore reflect uncontrolled experimental variability rather than actual differences in enzymatic activity. As a result, the POSA could not rely on the precise 20% and 40% thresholds to classify mutants as "active" or "inactive" because of the variability observed in the same biotinylated-HAS assay when run under comparable conditions (*i.e.*, 37°C, no added m-cresol).

[193] EX1105 (US-457), ¶ 0271 ("The sensitivity permits rapid measurement of hyaluronidase activity from cultured cells and biological samples with an inter-assay variation of less than 10%"). A POSA would expect the intra-plate variability (variability between duplicates) to be lower than the measured inter-plate (inter-assay) variability.

171. I therefore believe a POSA would only have confidence in the disclosure's data for single-substitution "active mutants" with reported activities above 40%.

3. Not all Mutant Variants Can Be Produced

172. The extraordinarily large number of variants encompassed by the sequence identity parameters of the claims—on the order of more than 10^{29}-10^{66} distinct PH20 polypeptides (*see* § IV.B)—also makes it highly likely that an appreciable, though indeterminant subset of these variants cannot be successfully produced or isolated. This expectation is consistent with well-established phenomena observed for naturally occurring proteins that incorporate one or more amino acid substitutions, many of which are associated with a loss of stability of the mutated protein. For example, mutations that reduce stability can slow the protein's folding kinetics, which, when occurring intracellularly, "can target the mutant for recycling by the quality control machinery in the endoplasmic reticulum and elsewhere."[194] Mutations can also result in altered structures that expose

[194] EX1031 (Yue), 467 (noting ~40% of mutants that affect stability also affect folding rate and that those mutants "folding may be slowed so much that most molecules are targeted for recycling by the quality control machinery in the endoplasmic reticulum and elsewhere."); EX1081 (Goldberg), 895-96 (20% of

hydrophobic residues that are normally buried, which can result in aggregation that prevents the protein from being secreted and/or cause it to be degraded within the cell.[195] Reduced stability and increased aggregation propensity can also cause

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hemoglobin point mutations are rapidly degraded), 896-97; EX1032 (Wang), 266-68 (reporting that approximately 90% of single-missense mutations resulted in decreased stability and that proteins that traffic through the endoplasmic reticulum may be eliminated by mechanisms that detect excessive fluctuations); EX1014 (Branden), 90-92; EX1087 (Vembar), 944 ("Proteins that pass [endoplasmic reticulum quality control] criteria traffic to their final destinations through the secretory pathway, whereas non-native and unassembled subunits of multimeric proteins are degraded by the ER-associated degradation (ERAD) pathway.").

[195] EX1081 (Goldberg), 897 ("A characteristic feature of denatured proteins is that they aggregate and leave solution (because of the tendency of normally buried hydrophobic domains to associate with one another). Similarly, when the production of misfolded proteins exceeds the cell's degradative capacity, these polypeptides often form intracellular aggregates before their rapid degradation.").

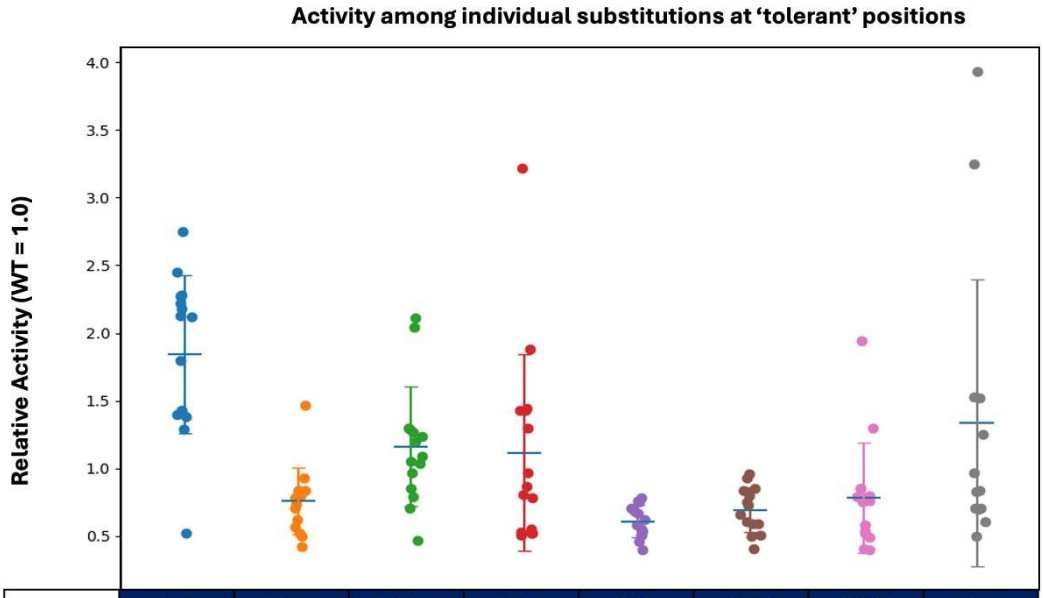
problems after the polypeptide is secreted, with increased unfolding.[196] Any of these types of problems can result in modified PH20 polypeptides that are neither "active mutants" exhibiting hyaluronidase activity nor "inactive mutants" that are properly folded forms of PH20 that do not exhibit hyaluronidase activity, but are instead unfolded and/or aggregated polypeptides that have no use (*e.g.*, they are neither enzymatically active nor resemble the native protein structure of PH20).

4. Observations Regarding the "Active" Single-Substitution Mutant Data

173. The experimental data reported in the disclosure illustrates substantial variation at a single position depending on the substituted amino acid (below). This is evidence that even for a single substitution the physicochemical character of the substitute residue influences the structure-activity relationship. The large changes in observed activity of the tested mutants were not analyzed.

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[196] EX1031 (Yue), 467 ("The second way in which non-synonymous base changes may affect in vivo activity is by reduction of the concentration of protein. This may come about through less successful folding, or an increase in the fraction of unfolded protein, caused by a reduction in stability.").



	T74	L146	T147	L26	K28	Q140	N205	K93
Mean activity	1.84	0.76	1.16	1.12	0.61	0.70	0.78	1.33
Std. dev.	0.58	0.25	0.44	0.73	0.12	0.17	0.41	1.06
CV	32%	33%	38%	65%	20%	24%	52%	80%
Tested	15	15	15	15	14	14	14	13
Active (>40%)	15	15	15	15	14	14	14	13

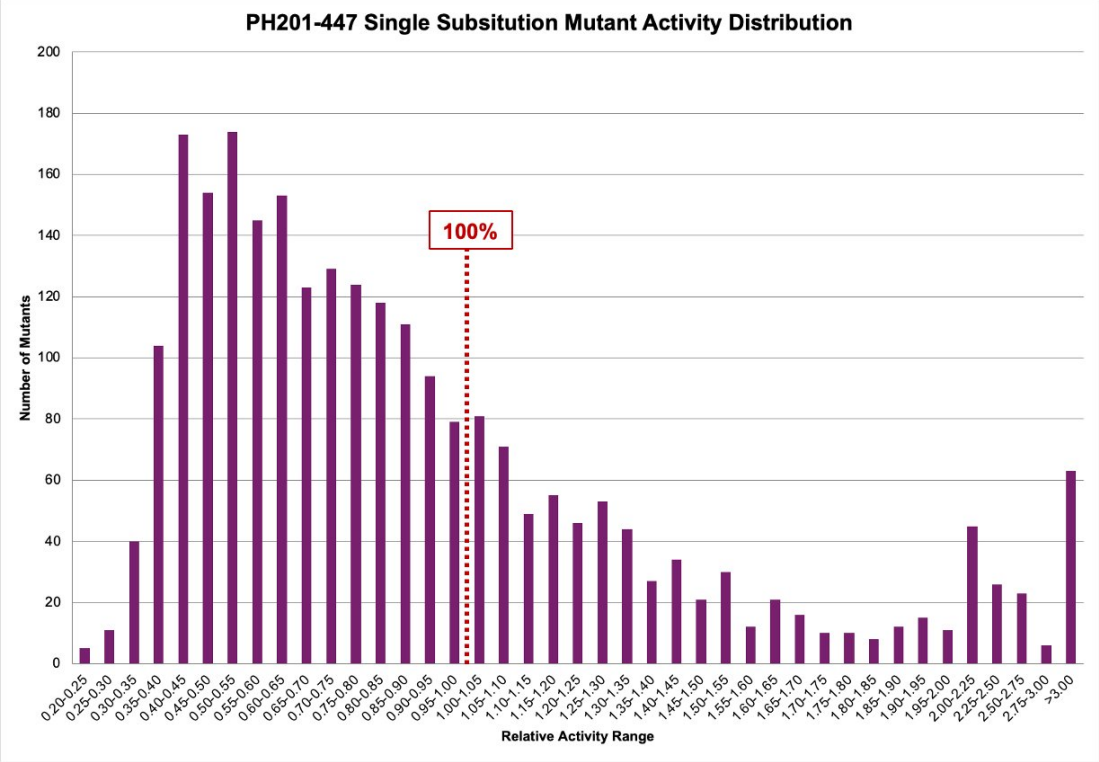
174. Mutants at these positions show wide activity ranges, with coefficients of variation from ~24% to 80%, demonstrating the existence of substitution- and position-specific differences in effects on the structure-function relationship of the PH20 protein. For example, T74M roughly halves activity, whilst T74F increases it by roughly half. T74 is on the surface of the protein and points away from the substrate binding and catalytic sites, yet different changes at this position have significant and entirely opposite effects on the structure-function relationship. Thus, there is no consistent functional outcome that could be mapped to a defined structural role of mutations at such positions, and no detailed structure–function

correlation can be drawn. Because these effects were not predictable in 2012, and given the data demonstrating that such context-dependent effects occurred in PH20, a POSA would have had diminishing confidence in the ability to reliably predict the structural or functional consequences as additional mutations were combined.

175. The data reported in the disclosure also indicates the structure-function relationship of human PH20 is sensitive to the majority of single-substitutions.[197] The data shows that most single amino acid substitutions in PH20 were deleterious, with another 13% uncharacterized. Even among the "active mutants" many substitutions clustered at the boundary of the 40% cutoff (histogram below, Table 9), indicating substantial functional impact from these substitutions. Only approximately 7% of mutants retained near-native activity (*i.e.*, between 95-105% activity), thus indicating that most single site mutations perturb the structure function relationship of $PH20_{1-447}$. The disclosure does not address this observation at all. A POSA would conclude from the disclosure itself that even single mutants distant from the active site alter the structure and function

[197] Declaration of Gregory A. Petsko, PGR2025-00003 (U.S. Patent No. 11,952,600), EX2070, ¶ 292 (Sept. 22, 2025) ("But the data in the '600 patent shows that it is not.").

of $PH20_{1-447}$. The vast majority of these changes in the structure are likely too small to reliably identify using tools in 2011-2012. *See* § II.E. This experimental data demonstrates a high degree of sensitivity of the structure-activity relationship for single mutants, which in turn further highlights the difficulty of combining multiple mutations in a predictable manner.



176. As the histogram above shows, there is a significant portion of mutants below the 40% activity cutoff (histogram above, Table 9). A POSA reviewing the data in the patent would find that PH20 is not tolerant to single mutations.[198] Given the protocol (at least one sample > 40% activity), average

[198] *See* Appendix B-3 (Histogram of $PH20_{1-447}$ Activity Data).

values can be based on very different individual measurements. For example, for an average of 0.35 the smallest difference between the two measurements is 0.12 equivalent to ~ 30 % (0.41 and 0.29). The disclosure does not comment on whether such large discrepancies between duplicates were common.

177. The disclosure shows that different substitutions at the same position in $PH20_{1-447}$ yielded active and inactive mutants.[199]

Position	Active	Inactive	Unclassified
45	I, K	A, D, F, G, P, W	H, M, Q, S, T, V, Y
110	V	F, K, L, M, P, W	A, C, D, G, H, N, R, S
124	H, L, R	C, D, E, F, N	A, G, I, P, S, T, V, W
290	I, M	D, Q, Y	A, C, G, H, K, L, R, S, T, V
343	T, V	C, D, F, I, P, W	E, G, L, M, R, S, Y

178. The experimental data associated with the disclosure's "active mutant" classifications would alert a POSA to the high potential for epistasis when multiple mutations are introduced. *See* § II.D. A position with no influence on PH20 activity would show no activity change across substitutions. The data discussed above instead show that different mutations at the same position—even far from the active site—produce different activity outcomes, demonstrating sensitivity to distal perturbations. And as the activity distribution chart directly

[199] EX1026 ('731 App.), Tables 8, 9, 10.

above shows, only ~160 substitutions retain near-native activity (0.95 to 1.05), and in no position do all changes result in native activity. Seeing such a complex picture of structure-function effects for even single mutants, would also alert a POSA to the likelihood of epistasis. When multiple such mutations are combined particularly outside the active site, a POSA in 2011-2012 could not predict how their long-range effects would interact to alter activity.

IV. The Claims' Sequence Identity Parameters Encompass a Vast and Diverse "Sequence Space" of Distinct PH20 Polypeptides

A. The Claims Define Modified PH20 Polypeptides Having ≥120% Activity

179. I reviewed the claims of the '400 Patent. Claims 1-2, 4-6, 12, 14, and 16 each define slightly different sets of modified PH20 polypeptides using different sequence identity parameters. Other dependent claims reference the sequence identity requirements of these claims. In general, the claims require the presence of at least one amino acid substitution at one or more identified positions, allow for additional substitutions at other positions within the polypeptide, and require that the resulting modified PH20 polypeptide "exhibits increased hyaluronidase activity that is at least 120% of the hyaluronidase activity compared to the unmodified PH20 polypeptide not containing the amino acid replacement." Claim 1 is reproduced below.

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

the unmodified PH20 polypeptide consists of the amino acid sequence selected from the group consisting of SEQ ID NOS: 3, 7 and 32-66;

the modified PH20 polypeptide exhibits increased hyaluronidase activity that is at least 120% of the hyaluronidase activity compared to the unmodified PH20 polypeptide not containing the amino acid replacement(s); wherein:

the activity of the unmodified PH20 polypeptide not containing the replacement(s) is 100%;

hyaluronidase activity of the modified PH20 polypeptide and hyaluronidase activity of the unmodified PH20 polypeptide are measured under identical conditions;

the amino acid replacement(s) confer(s) the increased hyaluronidase activity;

at least one amino acid replacement confers the increased hyaluronidase activity, and is at a position corresponding to a position selected from 1, 15, 24, 27, 29, 30, 31, 32, 33, 37, 39, 48, 63, 67, 69, 71, 72, 73, 75, 87, 92, 93, 94, 118, 120, 135, 141, 147, 148, 150, 151, 152, 155, 156, 163, 164, 165, 170, 198, 209, 212, 215, 219, 233, 236, 247, 257, 259, 260, 263, 269, 271, 272, 276, 278, 282, 293, 305, 308, 313, 320, 324,

325, 326, 328, 353, 359, 371, 377, 380, 389, 392, 395, 405, 409, 410, 418, 425, 436, 437, 438, 441, 442, 446 and 447, with reference to amino acid positions set forth in SEQ ID NO:3;

corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide having the sequence of amino acids set forth as SEQ ID NO:3, with the proviso that if the modified PH20 polypeptide includes only a single amino acid replacement, the replacement does not correspond to amino acid replacement N219A with reference to amino acid positions set forth in SEQ ID NO:3; and

the modified PH20 polypeptide has at least 95% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 7 and 32-66.

Included below is a table summarizing the positions recited in Claim 1, and the amino acid replacements at those positions recited in Claim 2:

Positions Recited in Claim 1	Single Amino Acid Replacement(s) Identified in Claim 2	Positions Recited in Claim 1	Single Amino Acid Replacement(s) Identified in Claim 2	Positions Recited in Claim 1	Single Amino Acid Replacement(s) Identified in Claim 2
1	Q	150	G	308	G, N
15	V	151	K, M, Q, R	313	K, R
24	H, E	152	R	320	H, K, R
27	K, R	155	G, K	324	R
29	E, I, L, M, P, S, V	156	D	325	A, D, E, G, H, K, M, N, Q, S
30	G, H, K, M, R, S	163	A, E, K, R	326	V
31	A, C, H, I, K, L, P, R, S, T, V	164	M, V	328	I, K, L, S, Y
32	F, G, H	165	D, N	353	V
33	W	170	R	359	T
37	F	198	D	371	R, P, T
39	N, T	209	–	380	W, Y
48	F, H, N	212	N	389	K
63	W	215	M	392	M
67	V	219	S	395	R
69	A, C, F, G, I, L, M, P, R, W, Y	233	K, R	405	G
71	R, S	236	R	409	A, Q
72	M, Q	247	I	410	T
73	H, L, W	257	T	418	P
75	F, L, R, T	259	P	425	K
87	C, T, Y	260	Y	436	K
92	C	263	K, R	437	I, M
93	I, L, R, T	269	A	438	T
94	R	271	L, M	441	F
118	Q	272	T	442	R
120	F, V, Y	276	D, S, Y	446	A
135	D, G, R	278	H, K, N, R, S, T, Y	447	D, N, Q
141	H, Y	282	M		
147	R, V	293	A, C, F, M, P, Q, V		
148	K	305	E		

The positions and amino acid replacements recited in independent Claim 4 are likewise summarized below:

Position Recited in Claim 4	Amino Acid Replacement(s) Identified in Claim 4	Position Recited in Claim 4	Amino Acid Replacement(s) Identified in Claim 4
12	E	261	N
26	E, K	277	R, T
46	R	309	L, N, Q, R, T
52	Q	310	A, G
58	Q	315	H
68	H, Q	317	I, K, R
70	A, C, F, G, H, K, L, N, P, R, S, T	318	M
74	A, C, G, N, P, R, S, V, W	347	G, S
84	G, R	399	M, T, W
97	G	407	D, Q
127	H, N	419	F, I, R, S
131	G, V	421	K, N, Q, R, S
142	R	431	A, H, K, Q, R, S, V
166	A, F, H, L, R, Y	433	L, R
174	K	439	V
206	K	440	H, R
213	M, N	443	A, M
237	E	445	M, P
238	S		

180. The claims specify three characteristics for each modified PH20 polypeptide within the scope of the claims:

- First, they require that the wild-type residue at one of 85 positions (in the case of Claim 1) or 37 positions (in the case of Claim 4) be changed to another amino acid.

- Second, they permit (but do not require) the modified PH20 polypeptide to contain additional changes besides the substitution at one of the recited positions. The number of additional changes permitted are set by the sequence identity parameters of each claim, but in all cases permit between 9 and 22 additional changes.
- Third, they require that the modified PH20 polypeptide exhibits at least 120% activity relative to the unmodified PH20 polypeptide that does not contain the amino acid replacement. I note that the disclosure reports activity data only for mutants generated from SEQ ID NO. 3, whereas the claims extend this requirement to unmodified PH20 sequences (*e.g.*, SEQ ID NOS: 7 and 32-66) that were not tested for activity with any substitutions or other modifications.

181. The discussion below demonstrates how the sequence identity requirement in the claims governs the number of additional substitutions or other modifications that may be included in a modified PH20 polypeptide that may fall within the scope of the claims (the "sequence space"). For example:

a. Claim 1 requires the modified PH20 polypeptide to have at least 95% sequence identity to SEQ ID NOS: 3, 7, or 32-66, which have a length of between 430 (SEQ ID NO: 32) and 474 (SEQ ID NO: 7) residues. The maximum number of changes

each polypeptide can have is equal to 5% of the number of amino acids in the unmodified PH20 (*i.e.*, 5% of 474 = 23).

b. Claim 3 maintains the sequence identity requirement of 95% as compared to the same unmodified PH20 sequences, but removes the SEQ ID NO: 7, which means the longest permitted reference sequence is SEQ ID NO: 66 (465 residues). However, the maximum number of changes each polypeptide can have remains the same (*i.e.*, 5% of 465 = 23).

c. Claims 5 and 14 require 96% sequence identity to various SEQ ID NOS, each of which contain 447 amino acids, meaning they permit up to 17 amino acid modifications (*i.e.*, 4% of 447 = 17).

d. Claims 12 and 16 recite that the modified PH20 polypeptide comprises up to 10 total amino acid modifications selected from amino acid replacements, insertions, and deletions.

182. The claims allow modifications other than the one required substitution at a recited position to be made in any positions or regions of the sequence. The sequence identity parameters do not restrict the location(s) or type(s) of amino acid changes permitted, other than the substitution required at the

recited position.[200] This is made very clear by explanations in the disclosure (below):[201]

> Therefore, as used herein, the term "identity" represents a comparison or alignment between a test and a reference polypeptide or polynucleotide. In one non-limiting example, "at least 90% identical to" refers to percent identities from 90 to 100% relative to the reference polypeptide or polynucleotide. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polypeptide or polynucleotide length of 100 amino acids or nucleotides are compared, no more than 10% (*i.e.*, 10 out of 100) of amino acids or nucleotides in the test polypeptide or polynucleotide differs from that of the reference polypeptides. Similar comparisons can be made between a test and reference polynucleotides. 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 also can be due to deletions or truncations of amino acid residues. Differences are defined as nucleic acid or amino acid substitutions, insertions or deletions. Depending on the length of the compared sequences, at the level of homologies or identities above about 85-90%, the result can be independent of the program and gap parameters set; such high levels of identity can be assessed readily, often without relying on software.
>
> 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. Modifications also can include post-translational modifications or other changes to the molecule that can occur due to conjugation or linkage, directly or indirectly, to another moiety. Methods of modifying a polypeptide are routine to those of skill in the art, such as by

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200 EX1026 ('731 App.), 68:26-34, 86:23-31; EX1130 (Simpson Dep.), 54:2-11, 90:25-91:12; EX1131 (Petsko Dep.), 55:22-56:23.

201 EX1026 ('731 App.), 68:26-30; 53:1-3, 53:10-11; 144:9-14; 46:19-26.

As used herein, a "modified hyaluronan-degrading enzyme" refers to a hyaluronan-degrading enzyme that contains a modification compared to a reference or unmodified hyaluronan-degrading enzyme. The modification can be an amino acid replacement (substitution), insertion (addition) or deletion of one or more amino acid residues. The amino acid residue can be a natural or non-natural amino acid. In some cases, the modification can be a post-translational modification. A modified hyaluronan-degrading enzyme can have up

The amino acids selected to replace the target positions on the particular protein being optimized can be either all of the remaining 19 amino acids, or a more restricted group containing only selected amino acids. In some methods provided herein, each amino acid that is replaced is independently replaced by 19 of the remaining amino acids or by less than 19 of the remaining amino acids, such as 10, 11, 12, 13, 14, 15, 16, 17 or 18 of the remaining amino acids.

collection is tested in each of a) and/or b). In the collection or library, each modified hyaluronan-degrading enzyme contains a single amino acid replacement compared to the unmodified form of the hyaluronan-degrading enzyme, such that the 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, whereby each modified hyaluronan-degrading enzyme contains a different amino acid replacement, and every amino acid along the length of the hyaluronan-degrading enzyme, or a selected portion thereof, is replaced.

183. Based on the points in ¶ 182, and provided that the entire amino acid sequence of the claimed PH20 polypeptide satisfies the numerical sequence identity threshold and has one of the required position substitutions, each claimed modified PH20 polypeptide can include any (or all) of the following types of changes:

(i) changes at any position in the amino acid sequence (*e.g.*, at highly conserved positions or positions known to be necessary for activity);

(ii) changes can replace any wild-type amino acid with one of the 19 alternative amino acids; and

(iii) changes can delete or add one or several amino acids, and those deletions or additions can occur anywhere within the amino acid sequence.

B. The Claims Identify an Immense Sequence Space that Contains an Unknown Number of ≥120% Active Multiply-Modified PH20 Polypeptides

184. The claims' sequence identity parameters define an astronomical number of distinct polypeptide sequences. Under Claim 1, the modified PH20 polypeptide must have at least 95% sequence identity to one of the recited reference sequences (SEQ ID NOS: 3, 7, and 32-66) and must include at least one amino acid replacement at one or more of the specifically enumerated positions. Claims 2 and 4 further identify particular substitutions that may be made at certain of those enumerated positions. Apart from the requirement that at least one substitution occur at a listed position (and, in dependent Claims 2 and 4, that certain specific substitutions be used), the claims do not otherwise restrict the identity of additional substitutions, provided the overall 95% sequence-identity threshold is satisfied. Because an amino acid at a substituted position may be replaced with any of the other 19 naturally occurring amino acids, each permitted substitution position allows up to 19 alternatives. For example, SEQ ID NO: 7

contains 474 amino acids; a 95% sequence-identity requirement permits up to approximately 23 amino acid differences relative to that reference sequence (including the required substitution at an enumerated position). Thus, even with the 95% identity limitation, the claims encompass polypeptides containing up to 23 substitutions distributed across the sequence, yielding an astronomically large number of possible distinct amino acid sequences.

185. The Alberts textbook provides an illustration of the magnitude of sequence diversity encompassed by the claims' sequence identity language:

> 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 an enormous number that to produce just one molecule of each kind would require many more atoms than exist in the universe.[202]

186. I have conducted a simplified illustrative analysis aid in the visualization of the number of distinct PH20 polypeptides that meet the sequence

[202] EX1039 (Alberts), 136-137.

identity parameters in the claims. Similar to the illustration in Alberts (above), the calculation permits 19 choices at up to 22 positions in the protein (in order to meet the 95 percent sequence identity limitation) and accounts for the fact that the 22 positional changes can be at any of the up to 474 positions (SEQ ID NO: 7) in the reference protein, depending on the claim parameters. It also accounts for the fact that one change must be at a position recited in the claim, and, in some cases, must be to one of the recited alternative amino acids. I have simplified this illustration by selecting only the first recited position for the required replacement in each claim. Additionally, I assumed all the changes arise from replacements of amino acids in the sequence and for simplicity did not account for insertions or deletions. These assumptions cause my illustrative calculation to underestimate the number of distinct sequences that meet the sequence identity parameters of each claim.[203] The sequence identity claim parameters define astronomical "sequence spaces" based on the different sets of parameters used in the claims (below), all of which are dominated by species with multiple changes.

[203] For example, claim 1 requires a replacement at one of 85 positions to any of 19 amino acids, which means there are 85 x 19 = 1,615 possible starting sequences with a required change that can then be further modified. My calculation accounts for only 19 of these possibilities.

Claims	Max Length	Seq. Id. %	Max Total Position Changes	First Recited Position Change	Amino Acid Choices @ First Recited Position	# of Distinct Polypeptides
1, 6-11, 13	474	95	23	1	19	9.85×10^{66}
2	474	95	23	1	2	1.04×10^{66}
3	465	95	23	1	2	6.73×10^{65}
4, 15, 17	474	95	23	1	1	5.18×10^{65}
5	447	96	17	1	2	2.48×10^{52}
12	474	-	10	1	19	1.85×10^{31}
14	447	96	17	1	2	2.61×10^{52}
16	474	-	10	1	1	9.75×10^{29}
18	465	95	23	1	1	3.36×10^{65}

187. One way to put the scale of these sequence spaces into perspective is to consider the aggregate weight of one set of these mutants from the '400 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 ~8.94×10^{-20} grams.[204] For

[204] 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 '731 Application as 53870.95 Daltons. The weight of one molecule of

simplicity, assume that all the modified PH20 polypeptides have the same weight. With that assumption, the aggregate weight of one molecule of just the portion of Claim 1's sequence space that each contains the first recited change (position 1) would be $9.85 \times 10^{66} \times 8.94 \times 10^{-20} = 8.81 \times 10^{47}$ kg. The weight of Earth is ~5.97×10^{24} kg.[205]

188. Others have performed similar calculations to confirm the number of polypeptides that are possible when substitutions freely permitted in a sequence:

> [F]or a relatively short sequence of 100 residues composed of the 20 naturally occurring amino acid, there are 20^{100} possibilities. 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.[206]

189. The calculations above illustrate that, conservatively, at least between ~10^{29} and ~10^{66} sequences make up the sequence space. An unknown portion of

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that polypeptide is determined by multiplying 53870.95 D by $1.66063906660 \times 10^{-24}$ g/D, or approximately 8.94×10^{-20} grams.

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

206 EX1046 (Beasley), 2031.

that sequence space will be sequences that will properly fold and exhibit ≥120% hyaluronidase activity relative to an unmodified form of the sequence. It was not possible for a POSA to calculate and it was not feasible to experimentally determine what that portion of the sequence space is in 2011-2012.

190. The POSA would understand that the sequence identity requirement is not a "structural feature" of the claimed modified PH20 polypeptides. Rather it establishes a mathematical boundary. Sequence identity is determined by counting the number of differences between two amino acid sequences and then dividing that number by the total number of amino acids in the reference PH20 sequence.[207] This calculation does not define any particular physical characteristic of a modified PH20 molecule; instead it sets a numerical threshold. The sequence identity limitation only delineates the outer bounds of the sequence space within which the claimed modified PH20 polypeptides with at least 120% activity are found.

191. By definition, the primary structure of the modified PH20 polypeptides within any particular portion of the claim parameter sequence space (*i.e.*, the complete amino acid sequence of each of those polypeptides) cannot be a

[207] EX1026 ('731 App.), 66:32-34 ("As used herein, 'sequence identity' refers to the number of identical or similar amino acids or nucleotide bases in a comparison between a test and a reference polypeptide or polynucleotide.").

"common structural feature" of all of the claimed PH20 polypeptides in each claim. That is because every one of those modified PH20 polypeptides will have a ***unique*** amino acid sequence (primary structure), each with differences relative to one of the reference wild-type PH20 sequences (*i.e.*, SEQ ID NOS: 3, 7, and 32-66).[208]

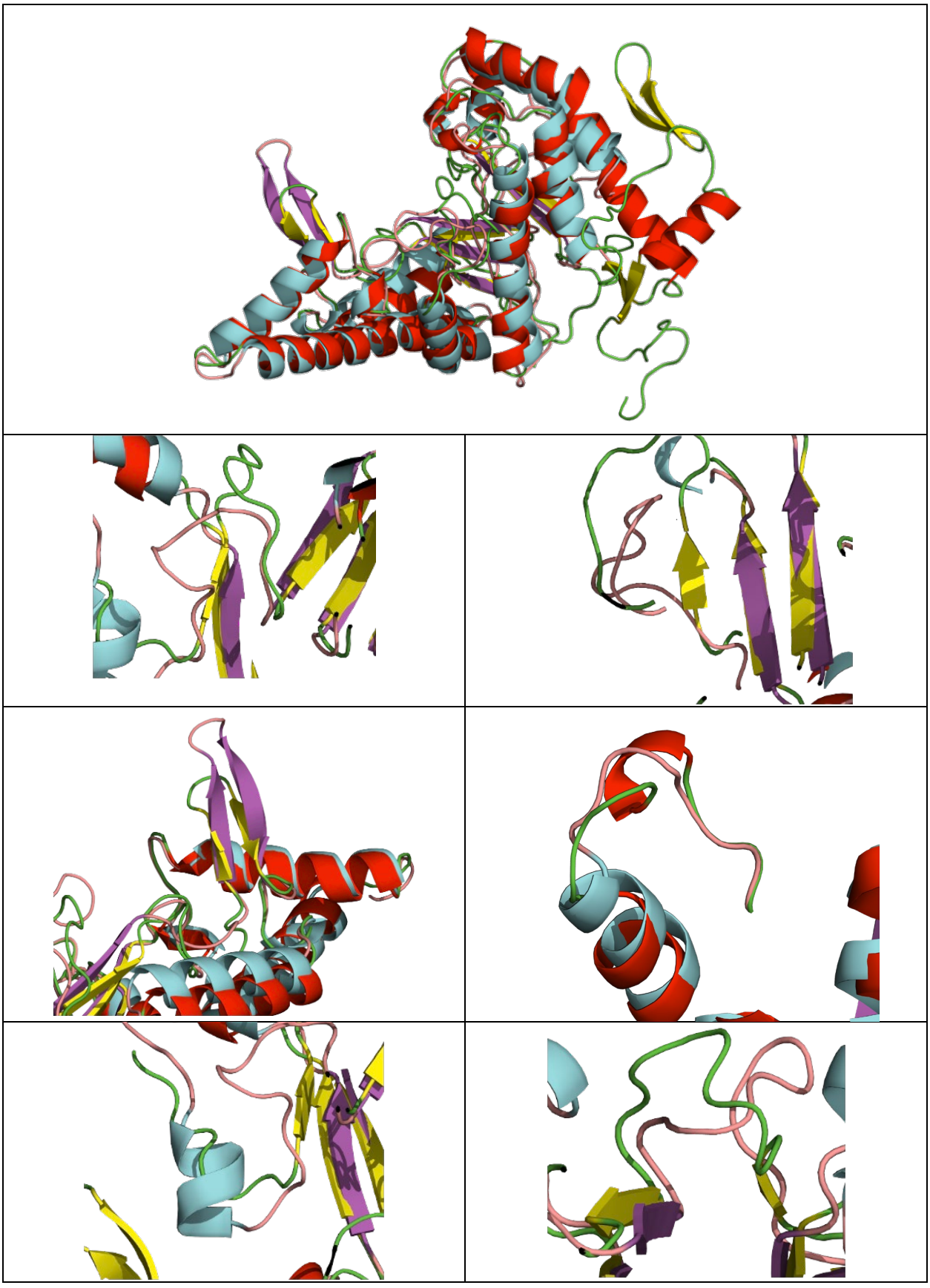
192. A POSA would consider "structural features" of a protein to be the physical characteristics of the protein structure. Structural features of folded proteins are discrete three-dimensional structures within the folded proteins: secondary structures, higher order motifs (*e.g.*, a catalytic site or substrate binding site), a domain, or the protein's overall tertiary structure. Although proteins are sometimes described as sharing a common "fold", a fold refers to a general architectural arrangement of secondary structural elements—an overall scaffold or framework. Proteins that share the same fold may nevertheless differ in their

[208] The "primary structure" does not describe the three-dimensional structure of a protein. It is only a description of the arrangement of amino acids along a linear polypeptide chain. *See, e.g.*, EX1014 (Branden), 29 ("Primary structure is the amino acid sequence, or, in other words, the arrangement of amino acids along a linear polypeptide chain."); EX1209 (Petsko Textbook), 6 ("[P]rimary structure: the amino-acid sequence of a polypeptide chain.").

specific three-dimensional structural features, including the precise arrangement of secondary elements and the spatial positioning of functional residues, and those differences can be structurally and functionally significant.[209] For example, in the illustrations below, a number of significant structural differences can be seen between the bee venom and human Hyal-1 hyaluronidase proteins when the two structures are overlaid and inspected in PyMOL, even though the α/β barrel motif in both proteins share the same "fold." The phrase "structure of a protein" would, unless prefixed by "primary" or "secondary," be taken by a POSA to refer only to the three-dimensional structure of a protein.

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[209] EX1008 (Stern), 824; EX1033 (Marković-Housley), 1025; EX1006 (Chao), 6913 ("The catalytic domain adapts a distorted $(\beta/\alpha)_8$-barrel fold similar to that of the bvHyal structure (22) but with some variations (Figure 2C): The hHyal-1 β2-strand is missing in bvHyal. However, this β2 does not form β-sheet interactions with β3, hence the β-barrel is open despite the presence of all eight strands. The barrel imperfection extends to the α-helix following β2, which is absent in hHyal-1. A number of secondary structure units are inserted in loops on the C-terminal side of the β-barrel: an α-helix (labeled α4′ following the numbering of the primary β/α motifs), two 3_{10} helices (η4′ and η8′), and a β-hairpin (β3′-3″) (Figures 2B and 3).")



193. Finally, the requirement for 95% sequence identity to a reference sequence does not mean that all sequences within the claimed set differ from one another by only 5%. For example, if two sequences (A and B) are each 95% identical to the same reference sequence (*e.g.*, SEQ ID NO: 3), but share no common substitutions, the A and B sequences may differ from one another at up to 10% of their positions.

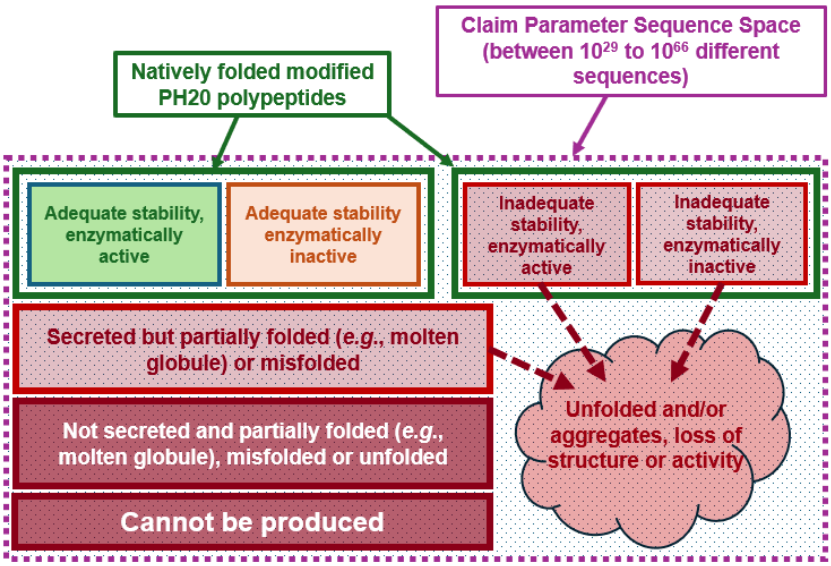
C. Illustration of the Structural Diversity of the Claim Parameter Sequence Space

194. In the 2011-2012 timeframe, a POSA would not view the sequence space defined by the sequence identity claim parameters as being structurally homogeneous. That is because it contains many unfolded sequences that can adopt an immense number of conformational states, all of which are different from the native fold. Sequences can also become trapped in these intermediate conformational states.[210]

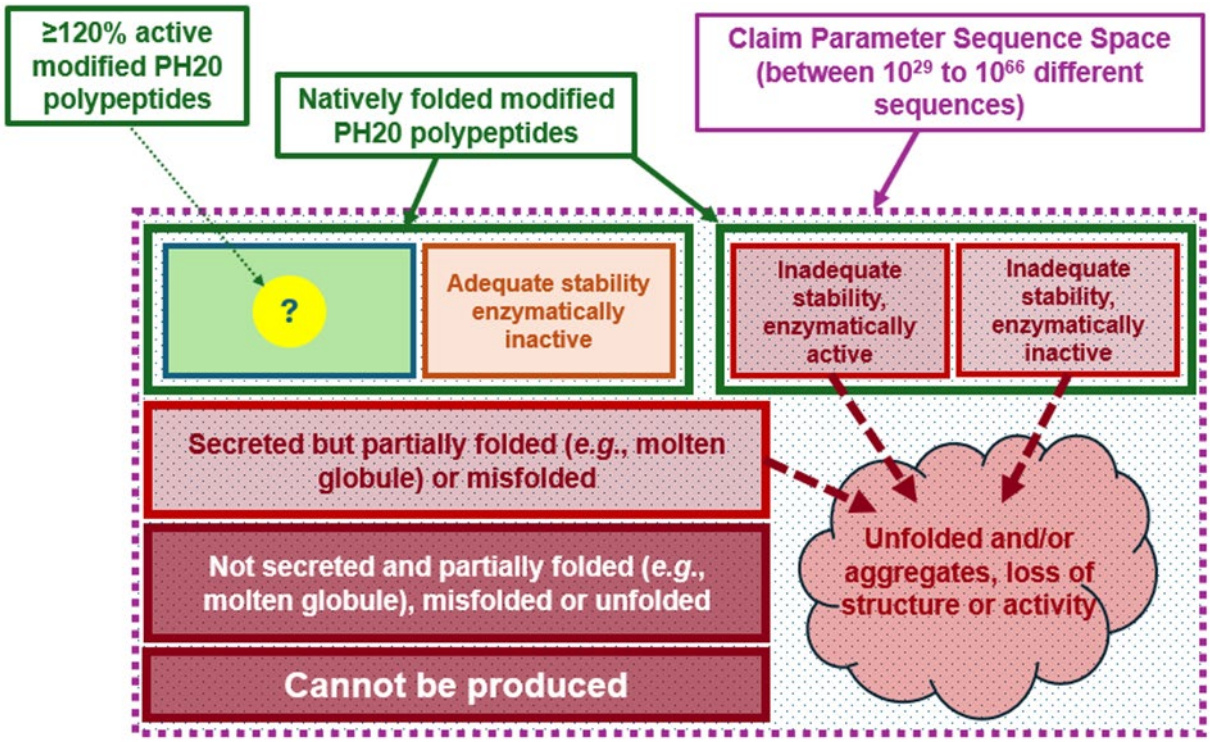
195. The subset of polypeptides within the sequence space that fold will include (1) proteins with a properly folded structure which possess activity (a subset of which may exhibit increased activity), (2) proteins with a folded structure similar to the native which do not possess activity, (3) proteins with an initial

[210] EX1136 (Dobson 2004a), 6 (Figure 2); EX1131 (Petsko Dep.), 98:4-25.

properly folded structure similar to the native which possess activity but are unstable, causing them to unfold and/or aggregate resulting in loss of structure and activity. Other portions of the sequence space may exist as partially folded or misfolded sequences, along with higher order aggregates (*e.g.*, disordered or ordered). Still others will not be secreted by the cell or cannot be produced by the cell at all. A conceptual illustration of these distinct sets of sequences within the claim parameter sequence space (including the distinct structures for those that are secreted) is provided in the figure below:



196. An unknown subset of the natively-folded, stable, and enzymatically active portion of the sequence space will be those that exhibit increased activity (or ≥120% activity) relative to the unmodified polypeptide, as shown below:



197. The disclosure provides no guidance in predicting the conformational state of a protein that meets the claims' mathematical sequence identity requirement. The 10^{29} to 10^{66} different amino acid sequences encompass highly diverse sets of modified PH20 polypeptides.[211] Large numbers of amino acid

[211] EX1131 (Petsko Dep.), 61:19-62:8 ("Q. In the genus of polypeptides that are defined by claim 1, would you agree that there are enzymatically active, properly folded, modified PH20 polypeptides? A. Yes. Q. Okay. And some number of those 10[^49]-plus polypeptide sequences will be properly folded PH20 proteins that are enzymatically active? A. Yes. Q. Do you know how

sequences within the claim parameter sequence space will exist in an unfolded state and exhibit a wide variety of three-dimensional conformations, while other sequences will adopt a diverse array of misfolded or partially folded structures (*e.g.*, molten globules).[212] Many of these unfolded, misfolded or incompletely folded amino acid sequences also will exist as aggregates rather than as properly folded monomeric proteins. The overall sequence space would further include multiple structurally distinct subsets of stably folded and enzymatically active proteins. In 2011-2012, it would not have been possible for a POSA to calculate or experimentally determine which of the 10^{29}+ sequences meeting the claims' sequence parameters would properly fold and be active (or exhibit increased activity above any particular threshold).[213]

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many? A. No. Q. Do you have an approximation that you can come up with of how many might be? A. Not an approximation, no.").

212 EX1014 (Branden), 91-92 ("The first observable event in the folding pathway of at least some proteins is a collapse of the flexible disordered unfolded polypeptide chain into a partly organized globular state, which is called the molten globule…"); *see also* 92 (Figure 6.2).

213 EX1131 (Petsko Dep.), 63:19-64:17.

198. I also note that not all modified PH20 polypeptides that fold will adopt the same tertiary structure of the wild-type PH20 protein. A modified PH20 polypeptide may adopt a folded structure that is stable but has differences notable to a POSA in its structure relative to the structure within the wild-type PH20 protein. Making multiple changes to an amino acid sequence can alter secondary structures, the positioning of secondary structures, the formation of higher order structural motifs or domains and other features of the protein's three-dimensional shape, and the folding pattern of the protein. *See* § II.D, II.G. Consequently, the changes permitted by the claims may introduce meaningful distinctions into the structure of the protein relative to the structure of the wild-type PH20 protein, including changes that impair the enzymatic activity or stability of the protein. Those that do adopt the native fold may exhibit significantly reduced enzymatic activity due to subtle changes in the substrate binding or catalytic sites, and some that natively fold and are active may unfold or degrade to less than 40% of activity on relevant timescales, for example, within 8 to 12 hours.

199. A POSA thus would believe claim parameter sequence space of 10^{29} to 10^{66}+ distinct amino acid sequences as having a vast amount of structural diversity within it. A POSA would view each set of modified PH20 polypeptides to be highly heterogeneous from a structural perspective.

D. The Claims Require Folded PH20 Modified Polypeptides with Increased Hyaluronidase Activity

200. Each of the claims requires each set of modified polypeptides to exhibit "increased hyaluronidase activity" that is at least 120% or greater relative to an unmodified PH20 polypeptide measured under identical conditions.

201. The disclosure 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."[214] The 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.[215]

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[214] EX1026 ('731 App.), 109:9-11.

[215] EX1026 ('731 App.), 109:9-17. As I noted previously, the disclosure, provides activity data only for single-substitution mutants derived from SEQ ID NO: 3, while the claims contemplate comparative activity assessments to other unmodified PH20 sequences of various lengths, for which no mutant activity data are reported. *See* § IV.A.

202. The disclosure states several times that changes made to a starting PH20 polypeptide after a first one caused the mutant to be an "active mutant" should be chosen to preserve enzymatic activity of the protein:

> 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 replacements, ***so long as the resulting modified PH20 polypeptide exhibits hyaluronidase activity***.[216]
>
> 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.[217]
>
> 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

[216] EX1026 ('731 App.), 54:5-8 (emphasis added).

[217] EX1026 ('731 App.), 86:32-34.

> hyaluronidase activity and is secreted from cells (*e.g.*, into the media) when expressed.[218]
>
> 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%, […] 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.[219]

203. A POSA would understand from the description above that a "modified PH20 polypeptide" is a natively folded form of a PH20 polypeptide. A modified PH20 must adopt the same fold as the wild-type human PH20 protein in order to exhibit enzymatic activity. The POSA would understand that additional changes to the modified PH20 polypeptide beyond a first one must not impair its

[218] EX1026 ('731 App.), 87:35-88:3.

[219] EX1026 ('731 App.), 92:28-93:3 (emphasis added).

ability to stably fold, which is a prerequisite to the modified PH20 polypeptide retaining hyaluronidase activity.

204. The disclosure explicitly defines the word "PH20" to mean "a type of hyaluronidase that occurs in sperm and is neutral-active."[220] A POSA would know that proteins found on the surface of sperm and that are neutral-active are folded forms of PH20.[221] That person would thus understand that a "modified PH20 polypeptide" must be a mutated form of PH20 that has stably folded to adopt the

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[220] EX1026 ('731 App.), 50:31-32. The disclosure defines "neutral active" as "the ability of a PH20 polypeptide to enzymatically catalyze the cleavage of hyaluronic acid at neutral pH, such as at a pH between or about between pH 6.0 to pH 7.8." EX1026 ('731 App.), 56:21-23.

[221] *See, e.g.*, Declaration of Gregory A. Petsko, PGR2025-00003 (U.S. Patent No. 11,952,600), EX2070, ¶ 53 (Sept. 22, 2025) ("Enzymatically active modified PH20s within the scope of the claims share a common mechanism of action in that they cleave hyaluronan ("HA") through a substrate-assisted hydrolysis reaction. All share the same active site."), ¶ 128 ("And enzymatically active 95% identical PH20 variants share a common tertiary structure (the PH20 alpha-beta barrel fold) as well as other common structural features…").

native state (the tertiary structure) of the PH20 protein.[222] The paragraphs containing the definitions of "PH20" and "modified PH20 polypeptide" are reproduced below:[223]

> As used herein, PH20 refers to a type of hyaluronidase that occurs in sperm and is neutral-active. PH-20 occurs on the sperm surface, and in the lysosome-derived acrosome, where it is bound to the inner acrosomal membrane. PH20 includes those of any origin including, but not limited to, human, chimpanzee, Cynomolgus monkey, Rhesus monkey, murine, bovine, ovine, guinea pig, rabbit and rat origin. Exemplary PH20 polypeptides, including precursor and mature forms, include those from human (SEQ ID NO:6 and 7), chimpanzee (SEQ ID NO:8, 9, 10, 869 and 870), Rhesus monkey (SEQ ID NO:11 and 12), Cynomolgus monkey (SEQ ID NO:13 and 14), cow (*e.g.*, SEQ ID NOS:15-18); mouse (SEQ ID NO:19 and 20); rat (SEQ ID NO:21 and 22); rabbit (SEQ ID NO:23 and 24); sheep (SEQ ID NOS:25-27), guinea pig (SEQ ID NO:28 and 29); fox (SEQ ID NO: 30 and 31); Gibbon (SEQ ID NO:856 and 857), Marmoset (SEQ ID NO:858 and 859) and orangutan (SEQ ID NO:860 and 861) . Reference to PH20 includes precursor PH20 polypeptides and mature PH20 polypeptides (such as those in which a signal sequence has been removed), truncated forms thereof that have activity, and includes allelic variants and species variants, variants encoded by splice variants, and other variants, including polypeptides that have at least 40%, 45%, 50%, 55%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the precursor polypeptides set forth in SEQ ID NO:7, or the mature forms thereof. PH20 polypeptides also include those that contain chemical or

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[222] Declaration of Gregory A. Petsko, PGR2025-00003 (U.S. Patent No. 11,952,600), EX2070, ¶ 345 (Sept. 22, 2025) ("POSAs think of PH20 as a folded protein.").

[223] EX1026 ('731 App.), 50:31-51:13 (upper quote), 54:5-14 (lower quote).

> 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 replacements, so long as the resulting modified PH20 polypeptide exhibits hyaluronidase activity. Typically, a modified PH20 polypeptide contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acid replacements. It is understood that a modified PH20 polypeptide also can include any one or more other modifications, in addition to at least one amino acid replacement as described herein.

205. The disclosure also uses the term "PH20" to refer to naturally occurring PH20 proteins from different species, which the POSA would understand are folded proteins.[224] The disclosure also uses the term "PH20" to refer to C-terminally truncated forms of the wild-type PH20 protein sequence that have retained activity. That again is using PH20 to indicate that the truncated wild-type sequence has folded.[225] Stable folding is what differentiates a "PH20 polypeptide" from being just a "(poly)peptide" (a sequence of amino acids of defined length that has not folded into a compact structure). A POSA would understand that the words "PH20" and "polypeptide" used together mean that the

[224] EX1026 ('731 App.), 50:35-51:5 (listing "[e]xemplary PH20 polypeptides" from various species).

[225] EX1026 ('731 App.), 51:5-7 ("Reference to PH20 includes… truncated forms thereof that have activity…").

amino acid sequence of the polypeptide has folded to adopt a stable tertiary structure.

206. The definition of the phrase "unmodified PH20 polypeptide" in the disclosure also supports reading "modified PH20 polypeptide" to mean a stably folded protein (below).[226] That explanation points out that a starting unmodified PH20 polypeptide can either be "a naturally-occurring, wild-type form of a polypeptide" or an "altered or mutated" PH20 that "differs from a native wildtype isoform" but has "been modified to have a desired increase or decrease in a particular activity or property."[227] Because both types of starting, unmodified PH20 polypeptides exhibit some hyaluronidase activity, a POSA would have believed both types were stably folded proteins.

> As used herein, an unmodified PH20 polypeptide refers to a starting PH20 polypeptide that is selected for modification as provided herein. The starting polypeptide can be a naturally-occurring, wild-type form of a polypeptide. In addition, the starting polypeptide can be altered or mutated, such that it differs from a native wild type isoform but is nonetheless referred to herein as a starting unmodified polypeptide relative to the subsequently modified polypeptides produced herein. Thus, existing proteins known in the art that have been modified to have a desired increase or decrease in a particular activity or property compared to an unmodified reference protein can be selected and used as the starting unmodified polypeptide. For example, a protein that has been modified from its native form

[226] EX1026 ('731 App.), 54:15-23.

[227] EX1026 ('731 App.), 54:15-27.

207. Therefore, a POSA would understand that all claims require folded "active mutant" modified PH20 polypeptides that meet the claims' sequence identity parameters and exhibit at least 120% of the hyaluronidase activity of unmodified PH20 polypeptides without the required amino acid replacement. It is my opinion that the disclosure does not describe all of the "active mutant" modified PH20 polypeptides that meet the sequence identity and other parameters of the claims and exhibit increased hyaluronidase activity (or >120% activity) that are being claimed in the '400 Patent. For similar reasons, the disclosure does not enable a POSA to identify out of the 10^{29}-10^{66}+ PH20 mutants meeting the claims' sequence identity parameters which are "inactive mutants" or which ones cannot be produced without performing an impossibly large number of experiments.

V. Observations on the Disclosure

A. The Claims' Sequence Identity Parameters 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

1. The Data from Testing Single Replacement $PH20_{1-447}$ Mutants Does Not Identify a Correlation Between PH20 Polypeptides with 2-23 Substitutions and PH20 Proteins Having > 40% Hyaluronidase Activity or Increased Activity

208. The disclosure reports the results of a mutagenesis experiment that produced ~6,800 mutants, each with a single substitution made across the human $PH20_{1-447}$ sequence, and classified them based on a biotinylated hyaluronic acid

activity assay.[228] *See* § III.B.1. The ~6,800 tested mutants (and the ~2,500 single-replacement $PH20_{1-447}$ polypeptides reported in the patent as retaining hyaluronidase activity) represent an infinitesimally small fraction of the number of sequences encompassed by the parameters used in the claims (10^{29}-10^{66}).

209. This disclosure's suggested approach to making multiple mutants aligns with what would be generally characterized as a directed evolution approach to making and testing single-mutated proteins. While mutagenesis experiments can yield useful empirical observations, they are typically followed by additional analyses aimed at understanding the basis for the observed results and identifying which changes affect specific structures features of the protein. With the exception of the limited discussion relating to two individual mutants (F204P and V58R) (*see* § II.F), no such follow on analysis is documented in the disclosure. For example, the disclosure does not describe or analyze how individual mutations influenced the structure activity relationship of the PH20 protein. Nor does it provide guidance regarding additional mutations that might be introduced to further enhance hyaluronidase activity or otherwise modify the properties of these mutants.

[228] EX1026 ('731 App.), 249:4-251:20 (Examples 3-4).

210. The disclosure does not provide any information that a POSA could use to predict the effect of incorporating into a PH20 protein the myriad different sets of between 2 and 23 substitutions drawn from the thousands of individual mutations in $PH20_{1\text{-}447}$ listed in the disclosure. The consequences on the structure activity relationship of combining multiple mutations would very rapidly become unpredictable due to epistasis even in situations where the mutants are dispersed in the structure. *See* § II.D. A POSA's confidence in modeling tools would diminish as additional mutations were introduced, and once more than approximately five changes were combined, reliable extrapolation from the disclosure's multi-mutant data would no longer have been reasonably achievable. *See* §§ II.C.1, II.D. Moreover many multiply-modified mutants—which constitute nearly the entirety of the claims' sequence space—will have multiple changes in the same local region, which particularly can result in directly energetically disfavored interactions not present in the native PH20 protein, disrupt patterns necessary for secondary structure formation and stability, induce changes in positioning of secondary structures and structural motifs, impede folding of structurally significant regions of the protein, render the protein insoluble, or any of a large number of other consequences that will disrupt and destabilize the protein's structure. *See* §§ II.D, IV.C.

211. The disclosure does not provide any guidance as to how one of the substitutions that caused the $PH20_{1-447}$ mutant to exhibit increased activity can be combined with other mutations in a way that ensures that the resulting multiply-modified PH20 protein will have a similarly increased activity (or even be active or folded), regardless of the number, location, or identity of the additional modifications. There was no scientific approach a POSA could have used in 2011-2012 to predict the effects of making the myriad of possible sets of 2-22 additional changes contemplated by the claims, so the POSA would be forced into trial-and-error experimentation. *See* §§ II.D, II.E.

212. Including additional modifications can adversely impact the structure function relationship which is already modified from native by the first substitution. *See* § II.D. The homology modeling techniques available in the relevant timeframe could not reliably identify or describe perturbations in the structure-activity relationship. The disclosure has no examples of any PH20 with multiple substitutions and does not characterize the effects of the single-mutations on the PH20 protein structure (let alone identify any common structural features shared by all or even sets "active" single mutants of $PH20_{1-447}$, *see* §§ III.B.1, III.B.4). Therefore a POSA could not use the disclosure to predict whether an effect observed in a modified PH20 polypeptide with only that single substitution would be observed in any of the other $>10^{29}$-10^{66} possible modified PH20

polypeptides that incorporate a recited positional mutation plus between 1-22 additional substitutions, insertions, or deletions.

213. The particular positions within the modified PH20 polypeptide sequence at which substitutions or other modifications (*i.e.,* insertions or deletions) are made, and the identity of the amino acids being replaced, inserted, or deleted at those positions, can dramatically influence the structure-function relationship of the PH20 protein. For example, multiple substitutions can disrupt secondary structure elements and/or conformations of loops, while insertions or deletions can alter or completely remove interactions with other residues by shifting the residue positioning. There can also be unpredictable effects on the folding pathways for the protein. *See* § II.D. These types of effects can impair expression of the protein, its stability, and/or eliminate functional characteristics of the protein.

214. The results of the disclosure's mutagenesis study (discussed in § III.B.2) are compiled, not analyzed. Those results by themselves do not identify any correlation between the examples of singly-substituted $PH20_{1-447}$ polypeptides and enzymatically active or inactive modified PH20 polypeptides having sets of 2 to 23 amino acid substitutions and PH20 polypeptides that retain hyaluronidase activity, or that exhibit >100% or ≥120% activity. As the data confirms, single amino acid substitutions demonstrated significant variability in activity even among mutations made at the same position. § III.B.4.

215. While the single-substitution $PH20_{1-447}$ mutants that were classified as "active" (≥40% of wild-type PH20 activity) represent a positive experimental result confirming proper folding, the classification of mutants as "inactive" provide no information to a POSA. As I discussed in ¶¶ 160-162, the disclosure did not investigate independently why any particular single amino acid substitution resulted in a modified $PH20_{1-447}$ sequence with hyaluronidase activity <20 % of unmodified $PH20_{1-447}$. A POSA cannot tell from what is reported in the disclosure whether an inactive mutant was classified as such because (i) it was not successfully synthesized by the cell, (ii) it was not secreted, (iii) it did not fold within the cell, (iv) it properly folded and was secreted but unfolded after secretion due to instability, (v) it was secreted in a misfolded or partially folded state, or (vi) it was properly folded but exhibited impaired or reduced enzymatic activity. Outcomes (i)–(v) reflect defects in expression, secretion, correct folding, stability, or aggregation. It is not possible from the data provided in the disclosure to ascertain which of these reasons (or experimental errors) caused the mutant to be reported having activity below the 20% threshold. Grouping these disparate outcomes under a single "inactive" classification obscures the cause of inactivity and prevents a POSA from drawing a meaningful structure-function inference about positional tolerance in PH20.

216. The disclosure also does not reference any experimentally determined hyaluronidase structures published before 2012, or any homology models depicting the structure or overall fold of PH20 (§ II.G.2):

a. The disclosure does not reference or discuss the findings in Marković-Housley (EX1033) about the unusual structural motifs of the bee venom hyaluronidase protein.

b. The disclosure does not reference or discuss the findings in Marković-Housley (EX1033) regarding the structure of the hyaluronic acid complex.

c. The disclosure does not reference or discuss the published reports of the human Hyal-1 structure reported in Chao (EX1006) nor the contrasts drawn by Chao et al with the bee venom hyaluronidase structure.

d. The disclosure does not reference or discuss the experimental studies of the human Hyal-1 protein reported in Zhang (EX1010). These omissions are particularly significant because Chao and Zhang analyze the homologous human hyaluronidase.

e. While the disclosure references earlier work with C-terminally truncated forms of wild-type PH20 and the presence of a glycosyl phosphatidylinositol (GPI) anchor sequence within it, it does not correlate any of those findings to the Hyal-EGF domain structure or the characteristic pattern of residues associated with it that was published in 2007 in Chao (EX1006) and discussed in Zhang (EX1010).

217. The disclosure also does not identify any three-dimensional structural feature within the tertiary structure in PH20 as being a common structural feature of all modified PH20 polypeptides that exhibit enzymatic activity (or increased activity). While it does reference the active site residues and substrate binding residues, it does not describe their structural context.[229] It also cites three publications: (i) Arming (EX1011), (ii) Ten Have (EX1149), and (iii) Chowpongpang (EX1150). None of those publications contain an experimentally determined structure or a homology model of a hyaluronidase enzyme.

218. Even if the disclosure had referenced a structural model, the effects of making combinations of substitutions or other modifications on a PH20 polypeptide could not have been accurately predicted by a POSA in the 2011-2012 timeframe using the tools that were available. *See* § II.E. As additional modifications were combined, confidence in predictive accuracy would have diminished due to increasingly interdependent and context-specific structural effects—at five modifications, for example, prediction would have been impossible. Moreover, the only structural template available for generating a PH20 homology model at the time was the Hyal-1 crystal structure. However, Hyal-1 is shorter than PH20: the mature PH20 protein contains 474 residues,

[229] EX1026 (’731 App.), 80:11-18.

whereas Hyal-1 contains only 414 residues. The experimentally resolved Hyal-1 structure does not cover the full sequence of PH20 and provides reliable structural coordinates only through approximately residue 403 of PH20 (*e.g.*, the local QMEAN values between position 403 and 414 are approximately 0.6). Consequently, the C-terminal portion of PH20—comprising more than 50 residues—could not have been modeled with confidence using Hyal-1 as a template. There also are no examples of single mutations in PH20 beyond position 447, even though the claims extend out to at least position 465 of human PH20 (*i.e.,* SEQ ID NO: 66).

219. Therefore, the disclosure's examples of single-replacement $PH20_{1\text{-}447}$ mutants are not representative of (and do not provide insight into) the diversity of possible modified PH20 polypeptides having different sets of 1 to 22 *additional* substitutions that encompassed by the sequence identity parameters of the claims. *See* § IV.C. Nor are those single-replacement $PH20_{1\text{-}447}$ mutants representative of the diversity of modified PH20 polypeptides having varying lengths that are encompassed by the claims' sequence identity parameters, particularly given the widely-varying activity and/or inactivity observed for such truncated forms of PH20 in the prior art. *See* §§ II.G.3, V.A.4-V.A.5. Thus, a POSA would have had to discover which combinations of substitutions and truncations to PH20 would

result in mutants that exhibit activity by making and screening all of them, which is an impossibly large undertaking.

2. The Disclosure Does Not Identify Any Specific PH20 Polypeptides Having 2-23 Substitutions that Retain >40% Hyaluronidase Activity or Exhibit Increased Hyaluronidase Activity

220. With a handful of exceptions,[230] the disclosure does not describe any examples of a modified PH20 polypeptide with more than one amino acid substitution. It does not identify any specific sets of 2 to 23 single amino acid substitutions that will, in combination, confer improved stability or activity on PH20 proteins, even with respect to the $PH20_{1\text{-}447}$ polypeptide.

221. There are no mutants with a first mutation that led to its classification as an "active mutant" where any further mutation has been introduced. As a result there are no examples of "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. As a result a POSA has no examples or other descriptions that show or explain how

[230] The disclosure lists only a few examples of combinations to not include in a multiply modified PH20 polypeptide (*i.e.*, P13A/L464W, N47A/N131A, N47A/N219A, N131A/N219A or N333A/N358A). EX1026 ('731 App.), 88:29-89:1.

combinations of mutations in $PH20_{1-447}$ will affect the structure-activity relationship.

222. There is no discussion of how any of the observed changes in activity of single-substituted $PH20_{1-447}$ proteins correlate with changes at the active site or other structural features of the PH20 protein. The disclosure does not describe how single amino acid substitutions affected PH20's native structure or affect any structure-function relationship, let alone the effect of multiple substitutions to the native protein.

223. The 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 NOS: 32-66). 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.[231] Stating the parameters that enclose possible sets of PH20 polypeptides does not identify any specific PH20 polypeptides, much less identify those that retain the >40% activity to meet the disclosure's classification as active nor those with increased (> 100%) hyaluronidase activity. The disclosure does not discuss or identify any common structure-function relationship among them. Instead sequence identity

[231] EX1026 ('731 App.), 7:20-26.

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. *See* § IV.C.

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

224. The disclosure says that substitutions that resulted in $PH20_{1-447}$ being classified as 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.[232]

225. A number of the single substitutions that the 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.[233]

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[232] EX1026 ('731 App.), 91:30-92:20 (emphasis added).

[233] EX1026 ('731 App.), 80:19-26. The 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.

226. A POSA 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.[234] The disclosure, however, does not provide any explanation for substitutions classified as inactive at positions outside of these highly conserved residues. The disclosure also contains unclear or contradictory observations regarding whether conserved amino acid positions should or should not be changed. For example, the disclosure indicates that

[234] EX1006 (Chao), 6914 (explaining that mutation to a conserved residue in all human hyaluronidases results in loss of activity); EX1011 (Arming), 813 (observing that mutation of conserved residues between mammalian and hymenopteran hyaluronidase yields enzymes without detectable activity); EX1010 (Zhang), 9437 (observing that essential residue mutagenesis significantly reduces hyaluronidase, Hyal-1, activity); EX1005 ('429 Patent), 16:4-21 ("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.").

positions 176 and 316 when modified result in a PH20 that is "not active (*e.g.*, it is not expressed or is inactive when expressed, see e.g., Tables 5 and 10").[235]

227. The lack of any explanation why single amino acid substitutions at non-conserved residues resulted in modified $PH20_{1-447}$ being classified as inactive significantly limits the usefulness of the disclosure. *See* § III.B.1.

228. The value of these data are even more limited when considering PH20 variants that incorporate multiple substitutions. For example, the disclosure says to simply not include any of the substitutions in Tables 5 and 10 that resulted in $PH20_{1-447}$ being classified as inactive in multiply-modified PH20 polypeptides that are intended to have activity, regardless of the length of the PH20 polypeptide or the number of additional amino acid replacements. It does not provide explanations beyond this simple instruction.

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[235] *See, e.g.,* EX1026 ('731 App.), 80:19-24 (describing 18 positions—including positions 176 and 316—that when modified result in a PH20 enzyme that is "not active" (*e.g.*, "not expressed or is inactive when expressed") and also reporting modified PH20 polypeptides having replacements at positions 176 and 316 that retained hyaluronidase activity), 91:30-92:22 (listing positions 176 and 316 as positions that can be changed), pages 94-95 (Table 3, reciting modifications to positions 176 and 316 as producing "Active Mutants").

229. The disclosure does not provide any examples of a modified PH20 polypeptide in which one amino acid substitution that conferred increased hyaluronidase activity in $PH20_{1–447}$ is combined with additional substitutions, including substitutions that rendered $PH20_{1–447}$ inactive when present alone. Nevertheless, the claims' sequence-identity parameters encompass such combinations of substitutions. The disclosure, however, provides no guidance regarding how these substitutions would interact when combined—whether the inactivating substitution would abolish activity, whether the activating substitution might somehow offset that effect, or whether the combined variant would retain, lose, or unpredictably alter hyaluronidase activity.

4. The Prior Art and the Disclosure Show that C-Terminal Truncations Can Inactivate PH20 Polypeptides, Yet the Claims' Sequence Space Captures Such Mutants

230. The disclosure cautions against C-terminal truncations that reduce the PH20 sequence with fewer than 429 residues, explaining that such truncations result in inactive proteins:

> 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***.[236]

231. As discussed above (*see* § III.B.2), the disclosure only reports data from a single-substitution $PH20_{1-447}$ mutagenesis experiment.[237] The disclosure, for example, does not report any activity data or other experimental characterization of single-substitutions in PH20 polypeptides that terminate ***prior to or after*** residue 447; nor does it explain how mutagenesis results obtained for $PH20_{1-447}$ should be applied or extrapolated to different-length C-terminally truncated polypeptides within the sequence space of the claims, particularly given the variability in activity observed by the prior art for such truncated mutants. *See* § II.G.3. From the disclosure, a POSA would not have been able to reliably predict the effect of introducing one or more amino acid substitutions in a PH20 that was truncated at or before position 447. Consistent with this uncertainty, Table 5 of the disclosure identifies several $PH20_{1-447}$ mutants having single-substitutions in the C-terminal region between positions 430 and 443 as "inactive" (below).[238] These

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236 EX1026 ('731 App.), 79:25-31 (emphasis added).

237 EX1026 ('731 App.), 251:2-9, 272:5-8.

238 EX1026 ('731 App.), p. 136 (Table 5).

results indicate that even a single amino acid change within this C-terminal region can unpredictably impact $PH20_{1-447}$ activity.

TABLE 5-continued

Inactive Mutants

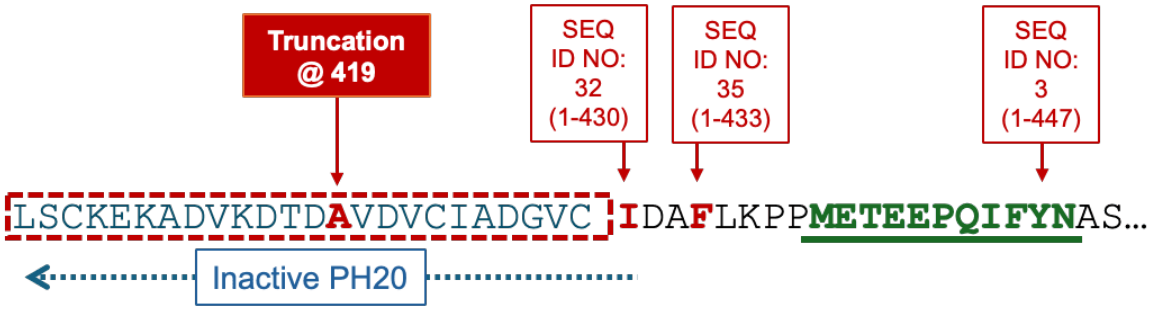
Corresponding Position	Replacement	Corresponding Position	Replacement	Corresponding Position	Replacement
430	ADELMNSTV	431	P	432	CFIKLMPY
434	HKPQRW	437	T	438	Y
439	NR	440	Q	441	R
442	MNS	443	D		

232. A POSA would have understood that alterations in the C-terminal region of PH20 perturbed activity. *See* § II.G.3. Published data showed that truncations near the position 430 boundary result in fluctuations in activity between variants differing by a single residue, and that truncations extending further into the C-terminus often eliminated activity altogether. *See* § II.G.3.[239]

[239] For example, C-terminal deletions within the Hyal-EGF region abolished hyaluronidase activity in PH20, while Zhang reported that mutants that deleted all or part of the Hyal-EGF domain in Hyal-1 prevented their secretion from host cells and reduced activity to ~2.5% or ~6.3% of unmodified Hyal-1. EX1010 (Zhang), 9439 ("Thus, loss of this domain is deleterious to both

Nevertheless, the claims' sequence identity language encompasses multiply mutated PH20 polypeptides derived from shorter reference sequences such as SEQ ID NO: 32 (430 residues) and SEQ ID NO: 35 (433 residues), as well as additional truncation variants satisfying the 95% identity threshold. The patent provides no guidance explaining how truncations within this region (where activity is highly variable depending on truncation)—alone or in combination with additional substitutions—could reliably yield modified PH20 polypeptides meeting the claimed increased activity (≥120%) requirement.

233. To illustrate this, consider a hypothetical modified PH20 polypeptide truncated to position 419 of PH20 (below), which would require 14 changes relative to SEQ ID NO: 35 (433 residues) and 11 changes relative to SEQ ID NO: 32 (430 residues). Both are permitted by claims 1 and 4's 95% identity requirement to SEQ ID NOS: 32 and 35, which permits 21 total changes.



secretion and activity of Hyal1, although the residual activity suggests the effect is indirect"), 9441; *see also* EX1026 ('731 App.), 79:25-31.

234. The disclosure does not report any examples of singly- or multiply-substituted "active mutant" PH20 polypeptides with fewer than 447 residues, and provides no guidance about making such enzymatically active (or increased activity) mutants. For example, the disclosure reports no examples of a $PH20_{1-433}$ polypeptide modified to have one or more amino acid substitutions or the effects of making such modifications on that particular protein.[240] It also did not report any experimental results showing that introduction of any particular mutation into an inactive C-terminally truncated PH20 polypeptide (*e.g.*, $PH20_{1-419}$ or other inactive truncation mutants) would restore hyaluronidase activity to that mutant. The POSA could not have predicted whether combinations of substitutions could be made in the $PH20_{1-419}$ sequence (or comparably truncated sequences) that would restore hyaluronidase activity to an inactive mutant.

235. The disclosure does not guide the POSA as to how to identify which of the 10^{29}-10^{66} possible PH20 polypeptides of varying length with 2 to 23 substitutions have increased hyaluronidase activity. There are likely to be variants with 23 substitutions that are active, however, a POSA cannot estimate how many

[240] EX1013 (Frost), 432, Figure 2; EX1005 ('429 Patent), 87:57-88:10; EX1105 (US-457), ¶¶ 0360-62, Table 6.

exist, and cannot reliably predict which combinations of substitutions result in active proteins.

5. The Claims' Sequence Space Captures Multiply-Modified Proteins with Changes in the Hyal-EGF Domain

236. There was uncertainty in the scientific literature regarding the function(s) of the Hyal-EGF domain in PH20, but it was believed to play a role in protein-protein interactions.[241] *See* §§ II.G.2-II.G.3. Given the historical role of EGF-domains in mediating protein-protein interactions, it is possible that the Hyal-EGF domain plays a role that influences the ability of PH20 to degrade the extracellular matrix, such as by presenting PH20 within clinically relevant portions of the extracellular space or for a duration that causes sufficient hyaluronan degradation to enable spreading (one of the utilities identified for active mutants in the disclosure).[242] The disclosure and other publications reported that

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[241] EX1006 (Chao), 6916; EX1079 (Hunnicutt), 84.

[242] EX1026 ('731 App.), 83:24-29 (citing EX1080 (Bookbinder)). *See, e.g.*, EX1080 (Bookbinder), 230-31 ("Spreading agents derived from animal testes extracts containing interstitial matrix-degrading enzymes have been used clinically for over 50 years to facilitate the dispersion and absorption of drugs [7]. The active agent in these testes extracts was later associated with hyaluronidase activity [8].").

modifications in or near the Hyal-EGF domain can be disruptive in PH20 and Hyal-1 (*see* §§ II.G.2-II.G.3). For example, C-terminal deletions within the Hyal-EGF region abolished hyaluronidase activity, while Zhang reported that mutants that deleted all or part of the Hyal-EGF domain (N350tr and L356tr) in Hyal-1 prevented their secretion from host cells and resulted "residual" activity of 6.3 % ~2.5% (comparable to D129N) of unmodified Hyal1.[243]

237. The disclosure indicates that modified PH20 polypeptides should be tested for their ability to cause spreading using an *in vivo* mouse assay, where the effects of the PH20 on spreading are assessed *in situ* in a mouse model.[244] The

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[243] EX1010 (Zhang), 9439 ("Thus, loss of this domain is deleterious to both secretion and activity of Hyal1, although the residual activity suggests the effect is indirect.").

[244] EX1026 ('731 App.), 194:35-195:3 ("The ability of a PH20 polypeptide, including a modified PH20 polypeptide provided herein, to act as a spreading or diffusing agent also can be assessed. For example, trypan blue dye can be injected subcutaneously with or without a PH20 polypeptide into the lateral skin on each side of nude mice. The dye area is then measured, such as with a microcaliper, to determine the ability of the PH20 polypeptide to act as a spreading agent [].").

disclosure provides no examples of PH20 polypeptides with multiple substitutions within the Hyal-EGF domain, and does not otherwise provide guidance about modifying this domain in a way that preserves the ability of a modified PH20 to cause spreading. Accordingly, a POSA would be left to experiment within the astronomical sequence space to determine whether any such multiply substituted mutants retain spreading activity.

B. A POSA Would Have to Engage in an Impossible Scale of Experimentation to Make and Identify the Full Scope of Multiply-Modified PH20 Polypeptides with Increased Hyaluronidase Activity Within the Sequence Identity Parameters of the Claims

238. Making and identifying all of the multiply-modified PH20 polypeptides exhibiting ≥120% activity that are within the immense sequence space of the claims (over 10^{29}-10^{66} distinct mutants) would require an amount of experimentation that is not only undue, but impossible.

239. The disclosure 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). *See* §§ III.B, II.F. None of this information, or the prior art available at the time, provides meaningful guidance that a POSA could use to determine which of the 10^{29}-10^{66}+ multiply-modified PH20 polypeptides within the claims' sequence space are enzymatically active. This would also require an impossible amount of experimentation.

240. The remainder of the disclosure defines an outcome (*i.e.*, active proteins, ≥120% activity) without providing guidance about how to achieve it other than by experimentation (except in the case of the single substitutions reported for $PH20_{1-447}$). It describes possible PH20 polypeptides that have one of the tested single substitutions but then might have numerous additional changes. It neither identifies nor describes any examples of PH20 polypeptides with multiple amino acid substitutions (*i.e.*, specific amino acids being inserted at two or more different positions of the same PH20 polypeptide), including any that were enzymatically active proteins. It provides no information about which amino acids (if any) can be deleted from or added to regions within the PH20 polypeptide sequence without impairing enzymatic activity. The disclosure does not report the making or testing of any multiply-modified PH20 polypeptides. That is consistent with the disclosure's description of theoretical ways of making multiply-substituted PH20 polypeptides. The disclosure is essentially a research plan that one might use to discover multiply-modified PH20 polypeptides that retain or increase hyaluronidase activity (through extensive time and effort).

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

a) Overview of the Research Plan

241. The disclosure's description of how to make modified PH20 polypeptides, including variants containing more than one amino acid substitution, is found in a section of the patent titled "Methods for Identifying Modified Hyaluronan-Degrading Enzymes with Altered Properties or Activities."[245] This section is describing a directed evolution type experiment. Most of the discussion sets forth, at a general level, well-known mutagenesis and screening techniques for generating and evaluating libraries of mutated proteins.[246] Rather than providing specific guidance for producing particular multiply substituted PH20 variants, the section outlines a research plan—describing in general terms, the steps one might take to conduct a mutagenesis and screening effort.

242. The part of this research plan that might create multiply-modified polypeptides involves an iterative process of mutagenesis and screening steps.

> 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

[245] EX1026 ('731 App.), 48.

[246] EX1026 ('731 App.), 140:36-141:29, 141:36-143:34, 144:16-149:37.

> the method is performed, any identified modified hyaluronan-degrading enzyme can be modified or further modified to increase or optimize the activity.[247]

243. The disclosure's mutagenesis methodology 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."[248] It says these techniques can be configured to introduce any of 10-19 alternative amino acids as single mutations.[249]

b) Screening Selects Mutants that Retain Hyaluronidase Activity

244. The disclosure's description of screening is clearly geared to finding active modified PH20 polypeptides. For example, the 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

[247] EX1026 ('731 App.), 141:30-33.

[248] EX1026 ('731 App.), 144:2-6.

[249] EX1026 ('731 App.), 143:35-144:14.

as a measure of its resistance to denaturation."[250] It also 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."[251]

c) Two General Approaches

245. The disclosure provides two general approaches 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 the first approach, 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

[250] EX1026 ('731 App.), 147:36-148:2. *Also* EX1026 ('731 App.), 148:9-12 ("In examples of the methods herein, the activity of the modified hyaluronan 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.").

[251] EX1026 ('731 App.), 148:23-32.

hyaluronan-degrading enzyme" and then testing that secondary library "using the assays and methods described herein."[252]

246. 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."[253] The targeting of these further mutations is described as follows:[254]

[252] EX1026 ('731 App.), 150:1-9.

[253] EX1026 ('731 App.), 150:10-26.

[254] EX1026 ('731 App.), 150:14-21.

> In another example of an iterative aspect of the method, modified hyaluronan-degrading enzymes 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 the a denaturation condition), can be further modified and retested for stability under a denaturation condition. 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. A further library of modified proteins can be generated with amino acid mutations targeted at or near to the identified critical amino acid residues, such as adjacent to the identified critical amino acid residues. In some examples, the mutations can be amino acid replacement to any other of up to 19 other amino acid residues. The secondary library can be tested using the assays and methods described herein.

247. This conceptual, trial-and-error research plan assumes that "ablated or reduced" enzymatic activity identifies critical residues "associated with activity and/or stability of the molecule."[255] This assumption is not warranted. A change in activity is not a direct measure of stability, as activity differences may be caused by mutations that influence the rate of catalysis without altering the overall stability of the protein (*e.g.*, influencing presentation or catalysis of the substrate), while changes that do affect the stability of the protein may not materially impair activity of the catalytic active site. *See* § II.F. In addition, for those mutants classified as inactive, there is no data or information in the patent that would allow

[255] EX1026 ('731 App.), 150:10-21.

a POSA to attribute any of those mutations to arise from changes in stability of the protein.

248. The disclosure's second theoretical protocol is of similarly limited practical value. It proposes to introduce modifications "near particular regions (*e.g.*, particular amino acid residues) associated with activity and/or stability of the molecule."[256] These "particular amino acid residues" are "associated with activity and/or stability of the molecule" and "generally are critical residues that are involved in structural folding or other activities of the molecule."[257] The plan proposes that "[a] further library of modified proteins can be generated with amino acid mutations targeted at or near to the identified critical amino acid residues, such as adjacent to the identified critical amino acid residues."[258] Again, for those mutants classified as inactive, there is no data or information in the patent that would allow a POSA to attribute any of those mutations to arise from changes that affected stability or folding of the singly-modified PH20 polypeptide.

249. As it is depicted in the disclosure, the second theoretical protocol is not targeting the first or successive rounds of mutagenesis to any particular region

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256 EX1026 ('731 App.), 150:14-17.

257 EX1026 ('731 App.), 150:15-17.

258 EX1026 ('731 App.), 150:18-24.

of the PH20 polypeptide amino acid sequence. Instead, it says to target sites "near the identified critical amino acid residues" (*e.g.*, adjacent to them). There are two possible sources of such "critical residues."

a. One source of such "critical residues" are positions reported in Tables 5 and 10, which report 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.[259] Using the Table 5+10 "inactive mutant" list of positions, and following the logic of the disclosure's research plan, the POSA would "target" mutations at positions "near" 90% of the PH20 sequence (*i.e.*, effectively the entirety of the PH20 sequence).

b. The alternative source of "critical residues" would be the conserved residues reported in the prior art (*i.e.*, the positions reported in the papers discussed in the disclosure or in papers before 2011 that were not discussed, such as Chao (EX1006), Stern (EX1008) and Zhang (EX1010)).

[259] EX1026 ('731 App.), Tables 5, 10.

Thus, the research plan provides little to no guidance on how to design and/or choose mutations throughout the PH20 amino acid sequence.

d) The Disclosure's Unfocused Mutagenesis and Screening Protocols Would Require an Impossible Amount of Experimentation to Produce Every Multiply Modified PH20 Polypeptide Meeting the Claims' Parameters

250. The major challenge in using directed evolution techniques in protein engineering is scale. *See* § II.C.2.

251. The number of mutagenesis experiments can be reduced somewhat if focused on regions likely to produce productive mutations. The disclosure however leaves the entire protein open as a target for mutagenesis.

252. When the mutagenesis approach 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 disclosure's assays do not do that. They instead say the mutants "can be tested using the assays and methods described herein."[260] The only example in the disclosure showing a screening assay being applied to a library of mutants is in Examples 3 and 4 (*see* § III.B.1).[261] This assay is based on a hyaluronidase

[260] EX1026 ('731 App.), 150:25-26.

[261] EX1026 ('731 App.), 248:8-250:31 (Example 3), 250:33-251:20 (Example 4).

activity screen, which classified active mutants if they show at least 40% of the hyaluronidase activity of a control. As I pointed out previously (§ III.B.2), the controls 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.

253. A scientist following this iterative mutagenesis and screening research plan cannot know in advance of conducting multiple rounds of experiments whether multiply-modified PH20 polypeptides will be produced that retain sufficient activity and thus will be selected for the next round of the process. That is because directed evolution is the quintessential "make and test" trial-and-error technique. 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 POSA cannot know ***which*** combinations of amino acids in ***which*** positions will be in the sequence of a modified PH20 polypeptide that has increased activity until that mutant is actually made, tested, isolated and characterized.

254. Likewise, until multiply-mutated PH20 polypeptides are actually made, isolated, and tested, the POSA could not know which of them would yield modified PH20 polypeptides with particular functional profiles, such as exhibiting

more than 40%, 100%, or 120% of the activity of the unmodified PH20 polypeptides. There is no guidance in the disclosure that helps the POSA predict which particular combinations of 2 to 23 amino acid substitutions will yield modified PH20 polypeptides with these attributes. To produce a set of multiply-modified PH20 polypeptides with up to 23 substitutions as claimed, the disclosure teaches using "iterative mutagenesis"—sequentially introducing single additional mutations, screening for activity, and repeating the cycle as many as 22 times to accumulate 23 substitutions in a single PH20 variant. At the end of this iterative process, there may be one, none or some unidentified number of mutants having up to 23 single substitutions that exhibits increased activity (or ≥120% activity). After completing between 2 and 22 rounds of this iterative mutate–screen–select process, the experimenter would likely still not obtain all the multiply-substituted PH20 polypeptides with increased activity that exist in the sequence space. Instead, they would have only those variants that happened to survive each successive screening step. This is because each round narrows the pool of candidates, many other possible multi-mutant combinations within the claim scope would never be generated or tested. To identify all multi-mutant PH20 variants exhibiting increased activity would require restarting the process repeatedly to explore different mutational paths. Given the astronomically large number of possible

sequence combinations encompassed by the claims, exhaustively identifying all such variants is not practically achievable.

255. A POSA could not realistically carry out this iterative mutate/screen/select process across the scope of the claims. Even under extremely conservative assumptions—using the shortest reference sequence (430 residues), excluding essential residues, limiting substitutions to relatively tolerated positions, restricting substitutions to variants previously shown to retain activity, and introducing only five substitutions per polypeptide—the number of distinct five-substitution combinations would exceed 3.5×10^{14} sequences. Each of those variants would need to be individually constructed, expressed, isolated, and tested for activity. Even if one assumed, unrealistically, that the entire process for each variant could be completed in a single minute, working continuously 24 hours per day without interruption, evaluating 3.5×10^{14} variants would require approximately 240 billion days. That amount of time vastly exceeds any practical human timescale. And this simplified five-substitution example represents only a minute fraction of the claim-defined sequence space, which permits up to 23 substitutions. The full scope of the claims therefore could not have been explored through iterative experimental testing. *See* §§ II.C.1; II.E.

256. A POSA would also face significant uncertainty about making C-terminal truncation mutants and/or multiple modifications to the Hyal-EGF domain

sequence in PH20. Papers published in 2007 and 2009 observed that the role and functions of the Hyal-EGF domain were poorly understood.[262] *See* §§ II.G.2-II.G.3. It was, however, recognized that while the Hyal-EGF domain was a functionally important domain within hyaluronidases, deleting it abolished the hyaluronidase activity of a PH20 protein.[263] Likewise, C-terminal truncations exhibited widely varying effects on the activity of PH20. § II.G.3. A POSA would thus have to produce and screen modified PH20 proteins that incorporate multiple substitutions within and/or deletions from the Hyal-EGF domain sequence in PH20 (*i.e.*, positions 337-409 of PH20) and truncations to the C-terminus to determine which would, for example, increase activity.

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[262] EX1006 (Chao), 6916, 6917 ("Because the sequence of the β-hairpin region varies significantly within the hyaluronidase family (Figure 3), the partner identity or. affinity may vary for each enzyme. Now that the 3D structure of hHyal-1 has revealed the presence of a novel HyalEGF-like domain, a search for partners and characterization of their interactions are timely."); EX1010, Zhang, 9441 ("It has been proposed that the C-terminal EGF-like domain, found in several reported splice variants of Hyal1, may be the site of as yet undefined protein-protein interactions [].").

[263] EX1010 (Zhang), 9439, 9441.

C. Soluble Human PH20 Polypeptides

257. The disclosure identifies two attributes of PH20 polypeptides that make them "soluble PH20" polypeptides (annotated with [i] and [ii] below):

> As used herein, soluble human PH20 (sHuPH20) includes human PH20 polypeptides that
>
> [i] lack a contiguous sequence of amino acids from the C-terminus of human PH20 that includes all or a portion of the glycosylphosphatidylinositol (GPI) anchor sequence (C-terminally truncated PH20 polypeptides) such that
>
> [ii] upon expression, the polypeptides are soluble under physiological conditions.[264]

258. In the mature full-length native human PH20 polypeptide (SEQ ID NO: 7), the GPI anchor sequence is an 18-residue sequence located at positions 456 to 474 (ATMFIVSILFLIISSVAS), while the glycosylphosphatidylinositol (GPI) anchor residue is one residue upstream from that sequence (*i.e.*, S455). [265]

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[264] EX1026 ('731 App.), 51:18-21, 52:1-8.

[265] In the precursor human PH20 polypeptide with the signal sequence (SEQ ID NO: 6), the GPI anchor sequence is located at positions 491 to 509 and the GPI anchor residue is S490. *See* EX1026 ('731 App.), 82:26-31 ("…a GPI-anchor attachment signal sequence of human PH20 is located at amino acid

The GPI anchor sequence is highly hydrophobic, which is compatible with its role in anchoring the C-terminus of the native human PH20 protein in the cell membrane.

259. The disclosure explains that a "soluble PH20" polypeptide lacks "all or a portion of the glycosylphosphatidylinositol (GPI) anchor sequence."[266] US-457 reported that various C-terminal truncation PH20 variants terminating between positions 415 and 474 may be soluble, while others remained insoluble or had low expression.[267] The disclosure, however, did not report results from testing modified PH20 polypeptides containing 1 to 17 or 22 additional substitutions,

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positions 491-509 of the precursor polypeptide set forth in SEQ ID NO:6, and the on-site is amino acid position 490").

[266] EX1026 ('731 App.), 52:1-8.

[267] EX1105 (US-457), ¶ 0356 (Table 4) (reporting solubility of C-terminally truncated human PH20 polypeptides through detection of PH20 polypeptides expressed in cell culture media.); *see also* EX1105 (US-457), ¶ 0356 did not report media expression (*i.e.,* solubility) data for fifteen C-terminally truncated PH20 mutants terminating at positions 416, 418, 420, 422, 424, 426, 428, 451, 453, 457, 459, 461, 469, 471, 473.

insertions, or deletions.[268] Consequently, it is not possible to know whether the multiply-modified mutants contemplated by the disclosure will yield soluble or insoluble proteins, or more crucially, whether the variants will be expressed.[269] Demonstrating that the unmodified PH20 can be made soluble (*i.e.*, non-membrane bound) by deleting a C-terminal sequence that includes the GPI anchor sequence (456-474) does not establish that every multiply-modified PH20 polypeptide lacking that sequence will be soluble (defined below in ¶ 261).

260. Before 2011, there were reports that larger C-terminal truncations that deleted the entirety of the GPI anchor sequence did yield soluble forms of the human PH20 protein, but as the size of those truncations increased, activity was eventually lost. The data shows wide variations in these truncation mutants. For example, the '429 Patent reported that PH20 mutants terminating between positions 432 and 448 had widely varying activities.[270]

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[268] EX1026 ('731 App.), 220:14-28.

[269] EX1105 (US-457), ¶ 0356 (Table 4) (reporting "weak" protein expression for C-terminally truncated PH20 variants terminating at positions 463 to 466).

[270] EX1005 ('429 Patent), 87:52-88:24 ($PH20_{1-442}$ activity "decreased to approximately 10%"); EX1013 (Frost), Figure 2, 430-32 ("[l]ess than 10%

261. The disclosure states that soluble PH20 polypeptides are "soluble under physiological conditions."[271] Physiological conditions preclude use of solubilizing agents such as detergents and denaturants. The disclosure then defines "solubility" as meaning that the protein is homogeneous in an aqueous solution, which it explains as follows:[272]

> As used herein, "solubility" with reference to a protein refers to a protein that is homogenous in an aqueous solution, whereby protein molecules diffuse and do not sediment spontaneously. Hence a soluble protein solution is one in which there is an absence of a visible or discrete particle in a solution containing the protein, such that the particles cannot be easily filtered. Generally, a protein is soluble if there are no visible or discrete particles in the solution. For example, a protein is soluble if it contains no or few particles that can be removed by a filter with a pore size of 0.22 μm.

262. The disclosure states in multiple places that the solubility of a human PH20 polypeptide is determined experimentally. It explains, for example, that "the solubility of a PH20 polypeptide can be determined by any method known to one of the skill in the art."[273] It also describes an experimental procedure for testing

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activity was recovered when constructs terminated after amino acid 467 [432] or when using the full-length PH20 cDNA").

271 EX1026 ('731 App.), 52:1-8.

272 EX1026 ('731 App.), 56:31-35.

273 EX1026 ('731 App.), 195:13-14.

whether proteins like PH20 protein are soluble (*i.e.,* not membrane bound) that was published in 1981 by Bordier.[274] As it explains:[275]

> The solubility of a PH20 polypeptide can be determined by any method known to one of the skill in the art. One method for determining solubility is detergent partitioning. For example, a soluble PH20 polypeptide can be distinguished, for example, by its partitioning into the aqueous phase of a Triton® X-114 solution at 37 °C (Bordier et al., (1981) *J. Biol. Chem.*, 256:1604-1607). Membrane-anchored polypeptides, such as lipid-anchored hyaluronidases, including GPI-anchored hyaluronidases, will partition into the detergent-rich phase, but will partition into the detergent-poor or aqueous phase following treatment with Phospholipase C. Phospholipase C is an enzyme that cleaves the phospho-glycerol bond found in GPI-anchored proteins. Treatment with PLC will cause release of GPI-linked proteins from the outer cell membrane.
>
> into the media when expressed from cells, and thereby can exist in soluble form. Soluble PH20 can be detected and discriminated from insoluble, membrane-bound PH20 using methods well known in the art, including, but not limited to, those using a Triton® X-114 assay. In this assay, soluble PH20 hyaluronidases partition into the aqueous phase of a Triton® X-114 solution warmed to 37 °C (Bordier *et al.*, (1981) *J. Biol. Chem.*, 256:1604-7) while membrane-anchored PH20 hyaluronidases partition into the detergent rich phase. Thus, in addition to using algorithms to assess whether a PH20 polypeptide is naturally GPI-anchored and hence membrane-bound, solubility experiments also can be performed.
>
> portion of a glycophosphatidyl anchor (GPI) attachment sequence. Hence, upon expression from a cell, a soluble PH20 is secreted into the medium. Soluble PH20 proteins can be distinguished, for example, by its partitioning into the aqueous phase of a Triton X-114 solution warmed to 37 °C (Bordier et al., (1981) *J. Biol. Chem.*, 256:1604-7). Membrane-anchored, such as lipid anchored hyaluronidases, will partition into the detergent rich phase, but will partition into the detergent-poor or aqueous phase following treatment with Phospholipase-C. Included among soluble PH20 hyaluronidases are membrane anchored

[274] EX1028 (Bordier), at 1604-07.

[275] EX1026 ('731 App.), 195:14-20; 84:6-13; 51:23-29.

263. Following the Bordier protocol, a PH20 protein such as modified human PH20 polypeptide, is placed in a biphasic solution at 0°C, and then the solution is heated (*e.g.*, to 30-37°C). At low temperature, Triton X-114 forms a homogeneous solution, but upon warming above its cloud point, the solution separates into distinct aqueous and detergent phases.[276] In a Triton® X-114 partitioning assay, hydrophilic proteins remain in the aqueous phase, whereas hydrophobic or amphipathic proteins partition into the detergent rich-phase.[277] Migration into the detergent phase therefore indicates the protein has significant hydrophobic character and is membrane-associated.[278] Other assays exist to assess solubility in the more general sense, such as centrifugation-based separation of insoluble aggregates from soluble protein.

264. In 2011-2012, a POSA would reasonably expect a wild-type PH20 truncated to remove the GPI anchor sequence and that exhibited enzymatic (hyaluronidase) activity would be "soluble" as that term is being used in the

[276] EX1028 (Bordier), 1604.

[277] EX1026 ('731 App.), 84:9-13; EX1028 (Bordier), 1604.

[278] EX1028 (Bordier), 1604-05.

disclosure.[279] A POSA would also reasonably expect that a modified $PH20_{1-447}$ polypeptide with a single substitution that exhibits increased hyaluronidase activity (*e.g.*, >120%) relative to unmodified $PH20_{1-447}$ (which is reported in the disclosure as being soluble[280]) would be soluble to some degree. However, a POSA could not have predicted whether a multiply-modified PH20 that incorporates multiple replacements, deletions, and/or insertions would be soluble, because any confidence derived from single-mutation data would diminish as additional modifications were introduced and their structural effects became increasingly interdependent and context-specific. (*See* §§ II.C.1, II.D.) For example, single or

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[279] EX1026 ('731 App.), 34:9-11 ("The modified PH20 polypeptides provided herein can be substantially purified or isolated, can exhibit catalytic activity at neutral pH, can be secreted upon expression from cells and are soluble in the supernatant,…"); 87:30-33 ("…provided herein are modified soluble PH20 polypeptides that are PH20 polypeptides containing a modification provided herein, and that when expressed from cells are secreted into the media as a soluble protein."); 84:5-6 ("PH20 can exist in membrane-bound or membrane-associated form, or can be secreted into the media when expressed from cells, and thereby can exist in soluble form.")

[280] EX1026 ('731 App.), 7:18-20.

multiple mutations can cause hydrophobic patches to become exposed to the aqueous environment, which induces aggregation and resulting insolubility (*see* ¶¶ 41, 54-58). Whether they do or do not could not have been predicted given the limited information in the disclosure about mutations and their structural impact in PH20 proteins, along with the general unpredictability of the phenomenon of aggregation. Given the scale of modified PH20 polypeptides within the claims' sequence identity parameters (*i.e.*, greater than ~10^{29}-10^{66} distinct sequences), testing all of the mutants for solubility would require an impossible amount of experimental work.

265. I also note that substantially larger quantities of each protein would have been required for the types of characterization described in Bordier protocol than would have been available from the library of transfected cells used to screen for hyaluronidase activity. Bordier describes using a 0.2 to 1 mg/mL in its assays.[281] By contrast, the amounts of mutant protein produced and secreted by individual CHO cells in the screening library would have been at the picogram level. Indeed, when particular mutants were subjected to more extensive characterization in the examples of the disclosure, each clone was produced in a

[281] EX1028 (Bordier), 1604.

greater quantity.[282] Scaling production to obtain sufficient material for solubility testing of 10^{29}-10^{66}+ distinct mutant PH20 polypeptides would have been impossible.

VI. US-275 Discloses Many Single-Replacement $PH20_{1-447}$ Mutants That Meet Each Limitations the Claims of the '400 Patent

266. I also was asked to opine whether US-275's disclosure of single-substitution $PH20_{1-447}$ mutants having >120% hyaluronidase activity compared to unmodified $PH20_{1-447}$ meets the requirements of the claims of the '400 Patent. As I explain below, US-275's disclosure of such mutants does meet all requirements of the claims. For example, the following mutants anticipate each claim:

Claims	Illustrative Anticipatory $PH20_{1-447}$ Mutants
1-3, 5-14	I271L, M313K, D320K
4, 15-18	V166L, I309N

267. As noted at ¶ 19, the '731 Application and US-275 contain substantially identical disclosures.

268. US-275's mutagenesis experiment—certain of the results of which are depicted in Table 9—discloses a class of ~530 single amino acid substitutions of

[282] EX1026 ('731 App.), 284:14-32 (Example 5, rescreening of mutants), 304:6-305:31 (Example 6, production and isolation of particular mutant).

$PH20_{1-447}$ that resulted in mutants exhibiting ***at least 120%*** of the activity of unmodified $PH20_{1-447}$ (SEQ ID NO: 3).[283] These mutants include, *e.g.*, V166L, I271L, I309N, M313K, and D320K $PH20_{1-447}$.[284] Example 4 of US-275 reports that the hyaluronidase activity of SEQ ID NO: 3 and each of these single-substitution mutants were measured under identical conditions.[285] As the only difference in each $PH20_{1-447}$ mutant relative to unmodified $PH20_{1-447}$ is the single identified substitution, each substitution would be understood to "confer[] the increased hyaluronidase activity" reported in Example 4 of US-275.

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283 *See, e.g.*, EX1004 (US-275), pp. 120-138 (Table 9); [0626] ("For example, about 536 mutants exhibit 120% or greater than 120% of the hyaluronidase activity of wildtype PH20…"); [0011] ("In some instances, among the PH20 polypeptides provided herein, the modified PH20 polypeptide exhibits at least 120%, 130%...").

284 EX1004 (US-275), [0626] (identifying increased activity of "mutant I271L is about 10-fold"); [0019]; Table 9 (pp. 128, 131, 133) (identifying V166L as having 438% relative activity, I271L as having 1,062% relative activity, I309N as having 311% relative activity, M313K as having 285% relative activity, and D320K as having 642% relative activity).

285 EX1004 (US-275), [0623]-[0626].

269. Each of those mutants have 1 amino acid replacement relative to SEQ ID NO: 3 (with no deletions or insertions), and therefore have 99.8% sequence identity to that reference sequence (446/447).

270. Regarding claim 1, I271L-PH20$_{1-447}$, M313K-PH20$_{1-447}$ and D320K-PH20$_{1-447}$ each contain a required substitution at a position recited in the claim, are 99.8% identical to SEQ ID NO: 3, and have >120% activity relative to SEQ ID NO: 3. Each mutant thus satisfies all limitations of claim 1.

271. Each mutant also satisfies all limitations of claim 2, which depends from claim 1. Specifically, I271L-PH20$_{1-447}$, M313K-PH20$_{1-447}$ and D320K-PH20$_{1-447}$ each contain a specific amino acid replacement at a position recited in claim 2.

272. Claim 3 is directed to soluble PH20 polypeptides. Because the disclosure indicates that PH20$_{1-447}$—due to its truncation at position 447—is a soluble PH20 polypeptide,[286] a POSA would reasonably conclude that each of the I271L-PH20$_{1-447}$, M313K-PH20$_{1-447}$ and D320K-PH20$_{1-447}$ single-substitution PH20$_{1-447}$ mutants, which exhibit >120% relative hyaluronidase activity to PH20$_{1-}$

[286] *See e.g.*, EX1004 (US-275), [0015] ("For example, the unmodified PH20 polypeptide is a soluble PH20 polypeptide that has the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66.").

$_{447}$, are also likely to be soluble PH20 polypeptides. The combination of only a single substitution and retention of ≥100% activity of the wild-type would lead a POSA to believe those single mutants did retain the soluble nature of the wild type $PH20_{1-447}$ protein. Each of these mutants meet the disclosure's articulation that "soluble PH20 polypeptides include any that has the sequence of amino acids set forth in SEQ ID NOS: 3 or 32-66 or a sequence of amino acids that exhibits at least 85% sequence identity… to the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66."[287] As noted, all are 99.8% identical to SEQ ID NO: 3, and therefore a POSA would reasonably believe that these single-substitution mutants of $PH20_{1-447}$ are soluble. Thus, all three mutants meet all requirements of claim 3.

273. Claims 5 and 14 require at least 96% sequence identity to one of a long list of SEQ ID NOS. Each of those SEQ ID NOS is associated with a $PH20_{1-447}$ mutant that has a single amino acid replacement.[288] As a result, I271L $PH20_{1-447}$, M313K $PH20_{1-447}$ and D320K $PH20_{1-447}$ each have 2 amino acid differences to the SEQ ID NOS recited in claims 5 and 14, which equates to 99.6% (445/447)

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[287] EX1004 (US-275), [0250]; *see also id.*, [0016], [0017], [0020], [0116], [0238], [0256].

[288] *See generally* EX1004 (US-275), Table 9.

sequence identity >96% sequence identity to the recited SEQ ID NOS, thus meeting all requirements of claims 5 and 14.

274. I271L $PH20_{1-447}$, M313K $PH20_{1-447}$, and D320K $PH20_{1-447}$ are also reported to exhibit greater than 2.0-fold (*i.e.*, 200%) activity compared to unmodified $PH20_{1-447}$ (*i.e.*, 10.62, 2.85 and 6.42, respectively).[289] Thus I271L $PH20_{1-447}$, M313K $PH20_{1-447}$, and D320K $PH20_{1-447}$ each meet all requirements of claim 6.

275. US-275 further discloses that modified PH20 polypeptides can be modified through glycosylation, sialation, albumination, farnesylation, carboxylation, hydroxylation, or phosphorylation.[290] A POSA would understand that this discussion applies to each mutant that falls within the scope of claim 1, as each of these fall within the disclosure's classification of modified PH20 polypeptides. Each of the recited single-substitution $PH20_{1-447}$ mutants recited in the disclosure (I271L $PH20_{1-447}$, M313K $PH20_{1-447}$, and D320K $PH20_{1-447}$) was

[289] EX1200 (US-275 Activity Data and '400 Patent Claims Comparison Spreadsheet), 4; EX1004 (US-275), Table 9; *see also* Appendix B-12.

[290] EX1004 (US-275), [0015], [0033], [0115], [0118], [0318], [0383].

produced in CHO cells, which is known to glycosylate the $PH20_{1-447}$ sequence.[291] Therefore, all requirements of claim 7 are met.

276. US-275 also discloses that modified PH20 polypeptides can comprise at least one N-acetylglucosamine moiety linked to each of at least three asparagine (N) residues.[292] It further discloses that the three asparagine (N) residues can correspond to amino acid residues 200, 333 and 358 of SEQ ID NO: 3.[293] This disclosure would apply to I271L $PH20_{1-447}$, M313K $PH20_{1-447}$, and D320K $PH20_{1-447}$, as none of those mutants replace the asparagine residues of SEQ ID NO: 3, and because each is produced in CHO cells, each of those positions will be glycosylated.[294] Thus, the requirements of claims 8 and 9 are met.

277. US-275 further discloses that modified PH20 polypeptides (which again would be understood by a POSA to include I271L $PH20_{1-447}$, M313K $PH20_{1-447}$

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291 EX1004 (US-275), [0608] .

292 EX1004 (US-275), [0015], [0033].

293 *Id.*

294 EX1004 (US-275), [0015], [0033], [0608].

$_{447}$ and D320K PH20$_{1-447}$ mutants) can be conjugated to a dextran or PEG polymer.[295] Thus, all requirements of claims 10 and 11 are met.

278. US-275's I271L-PH20$_{1-447}$, M313K-PH20$_{1-447}$ and D320K-PH20$_{1-447}$ mutants anticipate claim 12 because, each contains only 1 amino acid replacement relative to SEQ ID NO: 3, which is less than the 10 modifications recited by the claim.

279. US-275 also discloses that pharmaceutical compositions can contain modified PH20 polypeptides (which again would include all of the increased-activity mutants that meet the parameters of claim 1, including the I271L-PH20$_{1-447}$, M313K-PH20$_{1-447}$ and D320K-PH20$_{1-447}$ mutants).[296] I note that the claim imposes no requirements on the pharmaceutical compositions–*i.e.*, any can satisfy its requirements. Therefore, US-275 discloses all requirements of claim 13.

280. US-275 also discloses 96 single-substitution mutants having >120% activity relative to SEQ ID NO: 3 and with an amino acid replacement that is

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295 EX1004 (US-275), [0015], [0033], [0042], [0192], [0261], [0326]-[0332], [0421]-[0434].

296 EX1004 (US-275), [0036], [0040], [0045], [0435]-[0544].

recited in claim 4.[297] These mutants include, *e.g.*, V166L and I309N $PH20_{1-447}$, which each have a single amino acid change (corresponding to 99.8% (446/447) sequence identity) relative to SEQ ID NO: 3 and are reported exhibit 438% and 311% activity relative to SEQ ID NO: 3, respectively, when measured under identical conditions.[298] These mutants thus meet every requirement of claim 4.

281. Each of the V166L $PH20_{1-447}$ and I309N $PH20_{1-447}$ mutants would be reasonably understood and expected by a POSA to be soluble, as they exhibit increased activity and meet the definition's description of soluble human PH20s (*see* discussion of claim 3 above). US-275 therefore discloses mutants meeting all requirements of claim 18.

282. The disclosure's discussion regarding use of modified PH20 polypeptides in pharmaceutical compositions, and conjugation of such PH20 polypeptides to a polymer, would be understood by the POSA would also apply to the V166L $PH20_{1-447}$ and I309N $PH20_{1-447}$ mutants, thus meeting all requirements of claims 15 and 17.

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297 EX1200 (US-275 Activity Data and '400 Patent Claims Comparison Spreadsheet), 3; EX1004 (US-275), Table 9; *see also* Appendix B-12.

298 EX1004 (US-275), pp. 128, 133 (Table 9), [0007], [0051], [0119], [0120], [0176], [0253], [0255]-[0256], [0276], [0283], [0284].

283. Finally, the V166L $PH20_{1-447}$ and I309N $PH20_{1-447}$ mutants each contain only 1 amino acid replacement relative to SEQ ID NO: 3, which is less than the 10 modifications permitted by claim 16. US-275 therefore discloses all limitations of claim 16.

284. I have also included a summary of the disclosures in US-275 corresponding to each element of the claims of the ’400 Patent in Appendix B-14. Therefore, it is my opinion that US-275 discloses mutants that meet all parameters of all claims of the ’400 Patent.

VII. The Only Identified Use for Inactive Mutants Is as the Antigen in a Contraceptive Vaccine

285. Pages 197-213 of the ’731 Application’s disclosure describe therapeutic applications that depend on the ability of modified PH20 polypeptides to degrade hyaluronan (HA). Those applications necessarily require meaningful hyaluronidase activity and are therefore associated with “active mutant” modified PH20 polypeptides that have at least 40% of the activity of the unmodified PH20 (*e.g.*, $PH20_{1-447}$).

286. By contrast, the only utility proposed for an inactive modified PH20 polypeptide is the context of a contraceptive vaccines (below):

> 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. …[299]

287. The disclosure references two publications examining whether immunization of guinea pigs with guinea pig PH20 could cause contraceptive effects.[300] I am aware of later published papers reporting negative results in similar efforts to induce contraception in mammals including rabbits and mice, using PH20 as an immunogen.[301] For example, Hardy reported that "recombinant

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[299] EX1026 ('731 App.), 213:1-13.

[300] EX1026 ('731 App.), 212:36-213:13 ("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)."); *see also* EX1112, 544-546; EX1023, 1133; EX1022, 1144-1145.

[301] EX1019 (Hardy), 325; EX1020 (Pomering), 181 ("[I]mmunization [of rabbits] with reproductive antigens expressed only in the reproductive tract using routes which induce predominantly plasma IgG are unlikely to result in

mPH20 is not a useful antigen for inclusion in immunocontraceptive vaccines that target mice" even though it induced production of anti-mouse PH20 antibodies in the mice. As it explained:

> Mice treated with either vaccine formulation developed serum antibodies that cross-reacted to a protein band of 55 kDa corresponding to mPH20 in Western blots of mouse sperm. However, there was no significant reduction in the fertility of males or females compared with control animals with either formulation.[302]

288. Scientists in the field had reported before 2012 that even native PH20 from various non-human mammals was not a viable antigen for use in contraceptive vaccines. In a 2006 paper, Martin-DeLeon surveyed experimental results testing wild-type mammalian PH20 proteins for contraceptive effects and concluded that available evidence showed non-human wild-type PH20 "is not a

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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…").

[302] EX1019 (Hardy), 325, 330-331, 332 (observing that "native PH20 proteins may have antigenic epitopes with immunocontraceptive potential not present in either of the recombinant forms used in this study.").

useful antigen for inclusion in immunocontraceptive vaccines (below).[303] This assessment, based on the experimental results referenced in the common disclosure as well as additional pre-2011 studies, reflects that researchers did not view even native non-human PH20s as suitable vaccines candidates. Moreover, no experimental investigations of modified human PH20 for use in contraceptive vaccines had been reported before 2011.

> Taken together, the data from non-primate and primate models (where there are no known redundant reproductive hyases) suggest that PH-20 is not a useful antigen for inclusion in immunocontraceptive vaccines. The absence of critical epitopes in recombinant PH-20 has been considered as an explanation for a lack of sterility after immunization in mice (Hardy et al., 2004). However, it is possible that the strong immunocontraceptive effect reported for affinity purified guinea pig PH-20 could reflect a fundamental difference in the autoimmune response in guinea pigs compared to other species.

289. I also reviewed publications describing the human clinical studies involving Hylenex® (wild-type $PH20_{1-447}$).[304] One study, published in 2018,

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303 EX1189 (Martin-DeLeon), 119.

304 EX1206 (Hylenex Medical Review), 2, 29. I note that Hylenex is the unmodified PH201-447, corresponding to the SEQ ID NO: 3 reported in the disclosure. Hylenex's formulation comprises various stabilizers, to protect

reported that "approximately 5% of the US adult population test positive for recombinant human PH20 (rHuPH20)-reactive antibodies in the absence of exposure to rHuPH20" but despite that "no evidence for a negative effect on fertility in these individuals was found."[305] A separate publication from 2015 reported that a subset of subjects administered Hylenex developed detectable antibody responses, with reported incidence rates ranging from 2.5% to 18%.[306] That study specifically considered whether such antibodies might interact with reproductive tissues and impair fertility. The authors—including researchers 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

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against thermal and/or chemical instability (sodium phosphate dibasic dihydrate, sodium hydroxide, albumin (human), calcium chloride dihydrate, sodium chloride, edetate disodium dihydrate, and water). *See also* EX1206, 12, §3.1; EX1211 (Hylenex Label), 8.

[305] EX1024 (Rosengren-2018), 83 ("Key points"), 87 (5.2% of males and 2.6% of females test positive for anti-rHuPH20 antibodies), ("…no evidence of a negative effect on fertility in either sex … was demonstrated").

[306] EX1061 (Rosengren 2015), 1146, 1147 (Table II reporting antibody production).

> treatment-induced antibody to an endogenous protein involved in aspects of reproduction and are further supported by published reports in 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).[307]

290. Reports describing the absence of contraceptive effects associated with PH20 proteins in mice and rabbits were published prior to December 2011, whereas publications reporting clinical testing of Hylenex appeared later, in 2015 and 2018.[308] Taken together, these studies indicate that wild-type human $PH20_{1-447}$ protein, as well as PH20 polypeptides derived from other species, can elicit

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[307] EX1061 (Rosengren 2015), 1154; *see 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.").

[308] EX1020 (Pomering 2002); EX1019 (Hardy 2004); EX1061 (Rosengren 2015); EX1024 (Rosengren 2018).

antibody responses, but not antibodies that impair fertility in humans, mice, rabbits, or macaques. In light of these experimental findings, the disclosure's brief suggestion that inactive PH20 proteins could serve as immunogens for a useful human contraceptive vaccine lacks credible scientific support.

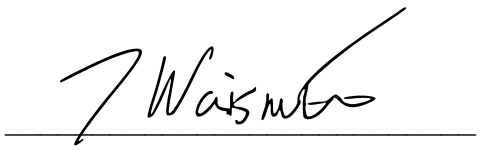
291. I further observe that the disclosure does not identify any ***mutated*** PH20 polypeptides that have been shown to be effective as contraceptive vaccines. Published studies demonstrate that antibodies lacking contraceptive effects can arise naturally in humans or following administration of recombinantly produced active $PH20_{1\text{-}447}$.[309] These findings indicate that the wild-type human PH20 contains antigenic features that are capable of eliciting antibody responses without impairing fertility. The disclosure, however, does not distinguish between such non-contraceptive antigenic features and any structures that might induce antibodies with contraceptive effect; nor does it address whether any such structures are retained or altered in specific modified PH20 polypeptides. Moreover, the disclosure provides no guidance that would allow a POSA to determine whether any active or inactive mutants would be suitable for use as contraceptive vaccines—for example by identifying common structural or

[309] EX1061 (Rosengren 2015), Abstract, 1149, 1154; EX1024 (Rosengren 2018), 87.

functional characteristics shared by those inactive mutants)—without making and testing the immense number of mutants within the sequence space of each claim.

292. A substantial portion of the single substitution mutants—approximately 13%—are not characterized at all in the disclosure and an additional ~49% are classified as "inactive." The patent provides no analysis of the reasons for this inactivity. Some of these variants may have failed to fold properly, may not have been secreted, may have misfolded, aggregated, or unfolded or may reflect combinations of these defects, but the disclosure does not investigate or distinguish among these possibilities. Moreover, the "active" single-substitution mutants that are described represent only a minute subset sequence space encompassed by the claims (6.753×10^3 vs. over 10^{29} to 10^{66}).

I, James Naismith, 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.



Executed on: **22 March 2026**

Appendix A – Exhibit List

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

EXHIBIT #	REFERENCE NAME
1001	U.S. Patent No. 10,865,400
1004	United States Patent Publication No. 2013/0302275
1005	U.S. Patent No. 7,767,429
1006	Chao et al., "Structure of Human Hyaluronidase-1, a Hyaluronan Hydrolyzing Enzyme Involved in Tumor Growth and Angiogenesis," Biochemistry, 46:6911-6920 (2007)
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Appendix B

Tables

Appendix B-1

Counts and Classifications of $PH20_{1-447}$ Mutants

Source(s): EX1026 ('731 App.), at Tables 9 and 10

Position	Wild Type (WT)	Active Mutants	Mutants 20-40% Activity	Active Mutants (>40%, No errors)	Mutants 20-40% Activity (no errors)	Inactive Mutants	Inactive Mutants (no errors)	Unclassified	Not Made	Errors	Mutants w/ 20-40% Activity + Errors
1	L	ACEFGHKNPQRSTVW		ACEFGHKNPQRSTVW				D	IMY		
2	N	IACGLPQSTV	I	ACGLPQSTV	I	HKWY	HKWY	F	DEMR		I
3	F	EHLY		EHLY		AGKPTV	AGKPTV	IMNRS	CDQW		
4	R	AISTV		AISTV		DEFGLPWY	DEFGLPWY	MN	CHKQ		
5	A	H		H		DGILMNPQRTVWY	DGILMNPQRTVWY	S	CEFK		
6	P	AHKLNQR		AHKLNQR		EFTVY	EFTVY	DGSW	CIM		
7	P	M		M		CDFGHIKLQRSTWY	CDFGHIKLQRSTWY	AV	EN		
8	V	PILM	P	ILM	P	DEGHNRSW	DEGHNRSW	AQT	CFKY		P
9	I	KLQRSV		KLQRSV		CDEGNP	CDEGNP	AHT	FMWY		
10	P	DEGHNQRSW		DEGHNQRSW		FILMY	FILMY	T	ACKV		
11	N	SDGHK	S	DGHK	S	ACFILPTWY	ACFILPTWY	E	MQRV		S
12	V	AEIKLNRST		AEIKLNRST		GHW	GHW	DMP	CFQY		
13	P	HSTY		HSTY		EGILMV	EGILMV	AFQRW	CDKN		
14	F	DIMV		DIMV		AEGHKNPQW	AEGHKNPQW	RT	CLSY		
15	L	AMV		AMV		EFGKNPQRSY	EFGKNPQRSY	TW	CDHI		
16	W					ACDEFGHKMPRSTY	ACDEFGHKMPRSTY	L	INQV		
17	A					DEGHILNPQRSTVWY	DEGHILNPQRSTVWY		CFKM		
18	W					CDFGHILMPQSTVY	CDFGHILMPQSTVY	R	AEKN		
19	N					ACFGHILMPQRSVWY	ACFGHILMPQRSVWY		DEKT		
20	A	S		S		DEFHKLNPRTVY	DEFHKLNPRTVY	GQ	CIMW		
21	P					ACDEGHILMRSTVW	ACDEGHILMRSTVW	K	FNQY		
22	S	HMTY		HMTY		CEGKP	CEGKP	ADLNRV	FIQW		
23	E	D		D		AFLMNPRSTV	AFLMNPRSTV	GHQW	CIKY		
24	F	AEGHIKLMNRTVY		AEGHIKLMNRTVY				CP	DQSW		
25	C					DEFGHIKLNPRSTVY	DEFGHIKLNPRSTVY		AMQW		
26	L	AEGHIKMPQRSTVWY		AEGHIKMPQRSTVWY					CDFN		
27	G	ADEFHIKLPQRSTW		ADEFHIKLPQRSTW		C	C		MNYV		
28	K	ADEFILMNPRSTVW		ADEFILMNPRSTVW				G	CHQY		
29	F	AEGHIKLMPRSTVW		AEGHIKLMPRSTVW				C	DNQY		
30	D	AFGHKLMPQRSTVW		AFGHKLMPQRSTVW				E	CINY		
31	E	ACGHIKLPRSTVWY		ACGHIKLPRSTVWY				N	DFMQ		
32	P	ACFGHKLMNQRSTVWY		ACFGHKLMNQRSTVWY					DEI		
33	L	GMPQRSTW		GMPQRSTW		CDHNVY	CDHNVY	I	AEFK		
34	D	AEHKQRW	A	EHKQRW	A	ILNSTV	ILNSTV	GP	CFMY		A
35	M	QVFHLTY	QV	FHLTY	QV	ADGPRS	ADGPRS	IN	CEKW		QV
36	S	DNAGHKLRT	DN	AGHKLRT	DN	CFVWY	CFVWY	P	EIMQ		DN
37	L	WFIKMPRV	W	FIKMPRV	W	CEGNS	CEGNS	AT	DHQY		W
38	F	Y		Y		EGKLNQRTW	EGKLNQRTW	ACMPS	DHIV		
39	S	ALNQRTY		ALNQRTY		CDFW	CDFW	GMPV	EHIK		
40	F	LW		LW		ADEGKNRSTV	ADEGKNRSTV	IQY	CHMP		
41	I	ACDEGHNTVW		ACDEGHNTVW		Q	Q	FPRS	KLMY		
42	G	A		A		DEHIKLMPQRSTV	DEHIKLMPQRSTV	C	FNWY		
43	S	NT	N	T	N	AEFGIKLQRV	AEFGIKLQRV	DHP	CMWY		N
44	P	E		E		ACFGHILNQRSTWY	ACFGHILNQRSTWY		DKMV		
45	R	IK		IK		ADFGPW	ADFGPW	HMNQSTVY	CEL		
46	I	CAEFHLMNRSTVY	C	AEFHLMNRSTVY	C	PW	PW		DGKQ		C
47	N	ADFGHKMQRSTWY		ADFGHKMQRSTWY		V	V	ILP	CE		
48	A	FGHIKMNQRSVY		FGHIKMNQRSVY		P	P	CELW	DT		
49	T	IKRSV		IKRSV		CDGHP	CDGHP	AFLNW	EMQY		
50	G	VACDEHLMQRSY	V	ACDEHLMQRSY	V			FPW	IKNT		V
51	Q	ARNS	AR	NS	AR	CFIMPTWY	CFIMPTWY	DHK	EGLV		AR
52	G	NPQRST		NPQRST		CEFWY	CEFWY	AHKL	DIMV		
53	V					ACDEGHLNPQRSTWY	ACDEGHLNPQRSTWY		FIKM		
54	T	AFNQSV		AFNQSV		DEGPRY	DEGPRY	HIM	CKLW		
55	I					ADGHNPQRTVY	ADGHNPQRTVY	CFLS	EKMW		
56	F					ACEGHIKLPRSTVW	ACEGHIKLPRSTVW	N	DMQY		
57	Y					ADFGILMPQRVW	ADFGILMPQRVW	EST	CHKN		
58	V	CGHIKLNPQRSWY		CGHIKLNPQRSWY		A	A	D	EFMT		
59	D	QN		QN		AEILMPRTVWY	AEILMPRTVWY	GH	CFKS		
60	R	K		K		ADFGHILNPQSTVY	ADFGHILNPQSTVY		CEMW		
61	L	FIMV	F	IMV		AEGHNPQRTWYF	AEGHNPQRTWY		CDKS	F	F
62	G					ACDFIKLMPQRSTVY	ACDFIKLMPQRSTVY		EHNW		
63	Y	AHIKLMNRSTVW		AHIKLMNRSTVW		CGP	CGP		DEFQ		
64	Y					ACDEFGHIKLPQRSTVW	ACDEFGHIKLPQRSTVW		MN		
65	P	R				ACDGHIKNSTVWYR	ACDGHIKNSTVWY	F	ELMQ	R	R
66	Y	HR		HR		ACDEGIKLNPSTV	ACDEGIKLNPSTV		FMQW		
67	I	RFLVY	R	FLVY		DEGPTWR	DEGPTW	CHNQ	AKMS	R	R
68	D	GEHKLPQRST	G	EHKQRST		ACIVYGLP	ACIVY		FMNW	GLP	GLP
69	S	ACEFGILMPRTWY		ACEFGILMPRWY		NT	N	V	DHKQ	T	T
70	I	ACFGHKLNPRSTVY		ACFGHKLNPRSTVY		Q	Q		DEMW		
71	T	ADGHLMNQRS		ADGHLMNQRS		P	P	CEVY	FIKW		
72	G	YADEHKLMQRS	Y	ADEKLMQRS	Y	CFIPVWH	CFIPVW	T	N	H	YH
73	V	ACDGHKLMQRSTW		ACDGHKLMQRSTW		P	P	F	EINY		
74	T	ACEFGHKLMNPRSVW		ACEFGHKLMNPRSVW					DIQY		
75	V	ACFHLMNQRSTY		ACFHLMNQRSTY		DGP	DGP	W	EIK		
76	N					ACFGIKLPQRSTVW	ACFGIKLPQRSTVW	D	EHMY		
77	G	HK	HK		HK	DELPQRTV	DELPQRTV	FMNSY	ACIW		HK
78	G					ADIMPTY	ADIMPTY	CHKLQRSV	EFNW		
79	I	LTV		LTV		ADFGHKNPSWY	ADFGHKNPSWY	R	CEMQ		
80	P					ADEFGIKLMNRSTVY	ADEFGIKLMNRSTVY		CHQW		
81	Q	P				ACEGHLNSVWYP	ACEGHLNSVWY	FMR	DIKT	P	P
82	K	YAEGHILMNQRSTV	Y	AEGHILMNQRSTV		WY	W	P	CDF	Y	Y
83	I	KFGHLNQRSTV	K	FGHLNQRSTV		EK	E	PY	ACDMW	K	K
84	S	YDEFGHILMNPQRTW	Y	DEFGHILMNPQRTW		Y			ACKV	Y	Y
85	L	V		V		ACDEFGHNQST	ACDEFGHNQST	KPR	IMWY		
86	Q	ADEFGHIKLMNPRSTVW		ADEFGHIKLMNRSTVW		CP	C		Y	P	P
87	D	PACEGHILMQRSTVY	P	ACEGHILMQRSTVY		P			FKNW	P	P
88	H					ACEFGIKLMPRSTVY	ACEFGIKLMPRSTVY		DNQW		

Position	Wild Type (WT)	Active Mutants	Mutants 20-40% Activity	Active Mutants (>40%, No errors)	Mutants 20-40% Activity (no errors)	Inactive Mutants	Inactive Mutants (no errors)	Unclassified	Not Made	Errors	Mutants w/ 20-40% Activity + Errors
89	L	RPWCKM	RPW	CKM	RP	ADEGQSTYW	ADEGQSTY	N	FHIV	W	RPW
90	D	AEGHIKLNQRSTW		AEHIKLNQRSTW		CG	C	P	FMVY	G	G
91	K	AQR		AQR		DEFGHILNT	DEFGHILNT	SY	CMPVW		
92	A	HCLMTV	H	CLMTV		EFKPQRWYH	EFKPQRWY	G	DINS	H	H
93	K	PVDEFGHILMNQRST	PV	DEFGHILMNQRST	PV				ACWY		PV
94	K	CADEFHLMNQRST	C	ADEFHLMNQRST	C	GP	GP		IVWY		C
95	D					ACEFGHKLMPQSVWY	ACEFGHKLMPQSVWY		INRT		
96	I	DLV		DLV		ACGHPRSTW	ACGHPRSTW	EFN	KMQY		
97	T	ACDEFGILNPQRSWY		ACDEFGILNPQRSWY					HKMV		
98	F	ACDEHILMQRSVW		ACDEHILMQRSVW		P	P	G	KNTY		
99	Y	ARS	A	RS	A	CGINPVWE	CGINPVWE	FLQT	HKMD		A
100	M					CEFGNPRSTWY	CEFGNPRSTWY	KLQV	ADHI		
101	P					ACFHIKLMNQRST	ACFHIKLMNQRST	GY	DEVW		
102	V	ACEGHKLMNQRSTW		ACEGHKLMNQRSTW		P	P		DFIY		
103	D	N	N		N	AEFGHILQRTVWY	AEFGHILQRTVWY	S	CKMP		N
104	N	IACGKMRST	I	ACGKMRST	I	FPW	FPW	HLV	DEQY		I
105	L	WCHAGIPQRSTV	WCH	AGIPQRSTV	WH	MNC	MN	DE	FKY	C	WCH
106	G	V		V		ACDFHLMNPSWY	ACDFHLMNPSWY	EI	KQRT		
107	M	FIL		FIL		ACHKPQSVW	ACHKPQSVW	DGR	ENTY		
108	A	G		G		DEFKLMPQTVY	DEFKLMPQTVY	HNRS	CIW		
109	V					CDELMRTW	CDELMRTW	AFGHPQY	IKNS		
110	I	V		V		FKLMPW	FKLMPW	ACDGHNRS	EQTY		
111	D					HIQ	HIQ	CEGKLMPRSTVWY	AFN		
112	W					CEGHLNPS	CEGHLNPS	DFIQRVY	AKMT		
113	E					RV	RV	ACDFGHLPQSTWY	IKMN		
114	E	AGHMS		AGHMS		ILPTV	ILPTV	CDRWY	FKNQ		
115	W					ACDFGHIKLMRSVY	ACDFGHIKLMRSVY	P	ENQT		
116	R					ACDEGHILNPQSVW	ACDEGHILNPQSVW	T	FKMY		
117	P	D				GIKNQRSVWD	GIKNQRSVW	EFHTY	ACLM	D	D
118	T	HKLMNQV		HKLMNQV		CDEGPRWY	CDEGPRWY		AFIS		
119	W	PFQY	P	FQY		AIKLNRP	AIKLNR	DEGSV	CHMT	P	P
120	A	DFGHILNPRSTVWY		DFGHILNPRSTVWY				C	EKMQ		
121	R					ACEFGHKLMPWY	ACEFGHKLMPWY	DSTV	INQ		
122	N	M		M		ACEFIKQRSTV	ACEFIKQRSTV	LPW	DGHY		
123	W					ACDEHLMPQRSTVY	ACDEHLMPQRSTVY	G	FIKN		
124	K	LHR	LH	R	LH	CDEFN	CDEFN	AGIPSTVW	MQY		LH
125	P	AHRS	A	HRS	A	CDGLNW	CDGLNW	QTVY	EFIKM		A
126	K					FHILNPY	FHILNPY	ADEGMQRSTVW	C		
127	D	AEGHLMNQRSTVW		AEGHLMNQRSTVW		K	K	F	CIPY		
128	V	ACGIKLQRSW		ACGIKLQRSW		EP	EP	FHY	DMNT		
129	Y					ACDEGHLPQSTVW	ACDEGHLPQSTVW	MR	FIKN		
130	K	IR		IR		CDGHLNSTWY	CDGHLNSTWY	EQV	AFMP		
131	N	CEFGHILMQRSTVY		CEFGHILMQRSTVY		P	P		ADKW		
132	R	ACEFHIKLNQSTVY		ACEFHIKLNQSTVY		P	P		DGMW		
133	S	I		I		DEFGHLMNPRTVW	DEFGHLMNPRTVW	A	CKQY		
134	I	LTV		LTV		ACDFGHKPQRSW	ACDFGHKPQRSW		EMNY		
135	E	ACDFGHKLNQRSWY		ACDFGHKLNQRSWY		P	P		IMTV		
136	L	ACDFHIMNQRSTW		ACDFHIMNQRSTW		P	P	G	EKVY		
137	V	CSLAIT	CSL	AIT	CSL	FGHNPRWY	FGHNPRWY	EQ	DKM		CSL
138	Q	ACHILMNRSWY		ACHILMNRSWY		V	V	EFG	DKPT		
139	Q	ACDEFGHKLMRSTV		ACDEFGHKLMRSTV		P	P		INWY		
140	Q	ACDFGHIKLMRVWY		ACDFGHIKLMRVWY				S	ENPT		
141	N	ADEFGHLMQRSTVWY		ADEFGHLMQRSTVWY				P	CIK		
142	V	CDEGHIKLMNPQRST		CDEGHIKLMNPQRST					AFWY		
143	Q	CEGIKLNV	C	EGIKLNV		HPRSTC	HPRST	FMY	ADW	C	C
144	L	RTW	R	TW	R	AEFIKPQSVY	AEFIKPQSVY	GN	CDHM		R
145	S	ACDEGHLMNPR		ACDEGHLMNPR		TW	TW	FV	IKQY		
146	L	ACEGHIKNPQRSTVY		ACEGHIKNPQRSTVY					DFMW		
147	T	ACDFGILMPQRSVWY		ACDFGILMPQRSVWY					EHKN		
148	E	CFGHIKLQRSTVWY		CFGHIKLQRSTVWY				P	ADMN		
149	A	CGKLMQRSTV		CGKLMQRSTV		EP	EP	FWY	DHIN		
150	T	ACDEFGILNPRSWY		ACDEFGILNPRSWY		V	V		HKMQ		
151	E	ACGHKLMNQRSTVWY		ACGHKLMNQRSTVWY					DFIP		
152	K	WACFIMRTVY	W	ACFIMRTVY	W	L	L	GNPS	DEHQ		W
153	A	SIL	S	IL	S	EFMPRTV	EFMPRTV	CGHKQW	DNY		S
154	K	IRTV	I	RTV	I	DEGPSWY	DEGPSWY	ACHLQ	FMN		I
155	Q	ACDFGHKLMRSTVW		ACDFGHKLMRSTVW		PY	PY		EIN		
156	E	CADGILMQRSTVW	C	ADGILMQRSTVW	C	P	P	K	FHNY		C
157	F	W		W		ACDEGHIKLMPQRSTV	ACDEGHIKLMPQRSTV		NY		
158	E	GAFHLQS	G	AFHLQS	G	DKPRY	DKPRY	CNV	IMTW		G
159	K	GADEHLMNQRSV	G	ADEHLMNQRSV	G	WY	WY	F	CIPT		G
160	A	WCFGHIKLMNQRSVY	W	CFGHIKLMNQRSVY	W				DEPT		W
161	G	ACDERSV		ACDERSV		W	W	HIKLMQT	FNPY		
162	K	ADEGHLMPQRSVWY		ADEGHLMPQRSVWY				F	CINT		
163	D	WFAEGKLQRSTV	WF	AEGKLQRSTV	WF	CP	CP	H	IMNY		WF
164	F	LMVW		LMVW		ACDEGHNPQR	ACDEGHNPQR	S	IKTY		
165	L	CADFNRSVWY	C	ADFNRSVWY		HPTC	HPT	GQ	EIKM	C	C
166	V	ACEFGHLNQRTWY		ACEFGHLNQRTWY		D	D	P	IKMS		
167	E	FADGHKMNPRSTY	F	ADGHKMNPRSTY	F	V	V	L	CIQW		F
168	T	H		H		ACDEFGKLPRSVWY	ACDEFGKLPRSVWY		IMNQ		
169	I	LRV		LRV		ADFGHKNPQSTY	ADFGHKNPQSTY		CEMW		
170	K	AQNRV		AQNRV		CDEGMPWY	CDEGMPWY	IL	FHST		
171	L	IV		IV		CDHMNRSWY	CDHMNRSWY	AFKPQ	EGT		
172	G	AC		AC		DEILPQTVWY	DEILPQTVWY	MRS	FHKN		
173	K	QNR	Q	NR	Q	DEGHILMPSVWY	DEGHILMPSVWY		ACFT		Q
174	L	HAGKMNQRSTVWY	H	AGKMNQRSTVWY	H	P	P	C	DEFI		H
175	L	EHTVY		EHTVY		CDGKPRS	CDGKPRS	FNW	AIMQ		
176	R	KL		KL		ACEFGHIPQSTVW	ACEFGHIPQSTVW		DMNY		

Position	Wild Type (WT)	Active Mutants	Mutants 20-40% Activity	Active Mutants (>40%, No errors)	Mutants 20-40% Activity (no errors)	Inactive Mutants	Inactive Mutants (no errors)	Unclassified	Not Made	Errors	Mutants w/ 20-40% Activity + Errors
177	P	V	V			ACDFGHLMQRSTWV	ACDFGHLMQRSTW	Y	EIKN	V	V
178	N	GKMR		GKMR		EILVWY	EILVWY	ADPST	CFHQ		
179	H	ACEGIKLMNPRSTV		ACEGIKLMNPRSTV		W	W		DFQY		
180	L	IFGKM	I	FGKM	I	ACEPRS	ACEPRS	HNTW	DQVY		I
181	W	KMQ	K	MQ		ACDEFHILRSVK	ACDEFHILRSV	N	GPTY	K	K
182	G	L		L		ACDEHNPQRSTVY	ACDEHNPQRSTVY	M	FIKW		
183	Y	EL	E	L		CDGIKNPQRSVE	CDGIKNPQRSV	AW	FHMT	E	E
184	Y	W	W		W	ACDEFGHKLMPRSV	ACDEFGHKLMPRSV		INQT		W
185	L					ADEFGIKPRSTVWY	ADEFGIKPRSTVWY	N	CHMQ		
186	F	Y		Y		ADGHIKLNPQRSVW	ADGHIKLNPQRSVW		CEMT		
187	P					AFGHILMNQRSTVWY	AFGHILMNQRSTVWY		CDEK		
188	D					ACFGHLMNPQRSTVW	ACFGHLMNPQRSTVW		EIKY		
189	C					AEGHKLMNPRSTVWY	AEGHKLMNPRSTVWY		DFIQ		
190	Y					CEFGHKLNQRSTVW	CEFGHKLNQRSTVW	P	ADIM		
191	N					AEFGKLMPQRSTVWY	AEFGKLMPQRSTVWY		CDHI		
192	H	ST		ST		CFGKLMNPQRVWY	CFGKLMNPQRVWY		ADEI		
193	H	RFGQSY	RF	GQSY	RF	ADKLMPV	ADKLMPV	CT	EINW		RF
194	Y					ACILPSTV	ACILPSTV	EFGNQRW	DHKM		
195	K	VAGHILNQRSTW	V	AGHILNQRTW	V	S		EFY	CDMP	S	VS
196	K	NYEGLRSTW	NY	EGLRSTW	NY			ACDIPV	FHMQ		NY
197	P	WADEFGHKLMQRST	W	ADEFGHKLMQRST	W	C	C		INVY		W
198	G	WADEHLNQRSTY	W	ADEHLNQRSTY		VW	V	CP	FIKM	W	W
199	Y					EGHIKLPRSW	EGHIKLPRSW	ACNQT	DFMV		
200	N	TD	T	D	T	AFGHKLMPQRSWY	AFGHKLMPQRSWY	V	CEI		T
201	G					AFLMNPRSTVW	AFLMNPRSTVW	EHKQ	CDIY		
202	S	M		M		AEFGHKNPQRVWY	AEFGHKNPQRVWY	T	CDIL		
203	C					ADEGHLMNQRSTV	ADEGHLMNQRSTV	PW	FIKY		
204	F	WP	W	P	W	ACEGHIKQRST	ACEGHIKQRST	LMV	DNY		W
205	N	ADEFGKMPRSTVWLY	L	ADEFGKMPRSTVWY	L				HQCI		L
206	V	QHIKLMRST	Q	HIKLMRST	Q	CDFGPY	CDFGPY		AENW		Q
207	E					AFGMPQRSTVW	AFGMPQRSTVW	HIKL	CDNY		
208	I	ACKLMQRSTV		ACKLMQRSTV		DGPW	DGPW	E	FHNY		
209	K	LAEFGNRST	L	AEFGNRST	L	CP	CP	DVWY	HIMQ		L
210	R					ACDEGKMNPSTVWY	ACDEGKMNPSTVWY	L	FHIQ		
211	N	LW		L		CFGHIKMPRSTVW	CFGHIKMPRSTV	A	DEQY	W	W
212	D	NST		NST		AGHIKLMPVW	AGHIKLMPVW	EQ	CFRY		
213	D	AEGHKLMNQRVWY		AEGHKLMNQRVWY		PS	PS		CFIT		
214	L	Q		Q		ACDEGHKNPRSTY	ACDEGHKNPRSTY	V	FIMW		
215	S	ADEGHKLMQRTVW		ADEGHKLMQRTVW		CP	CP		FINY		
216	W	Y		Y		DEGHIKLMNPQRTV	DEGHIKLMNPQRTV		ACFS		
217	L	M		M		ACGHPQSTVW	ACGHPQSTVW	EIRY	DFKN		
218	W	MVF	MV	F	M	AIKLPSV	AIKLPS	DGHQRT	CENY	V	MV
219	N	ACDEHIKLMRSTW		ACDEHIKLMRSTW		P	P	G	FQVY		
220	E	DMAHILSTV	DM	AHILSTV	DM	GKNPRW	GKNPRW		CFQY		DM
221	S	IACMQTV	I	ACMQTV	I	DEHKPR	DEHKPR	GL	FNWY		I
222	T	DFGIKLNRSV		DFGIKLNRSV		PY	PY	AEW	CHMQ		
223	A					CDEGHKLPQRSTVWY	CDEGHKLPQRSTVWY		FIMN		
224	L	I		I		ADEFGMPQRSTWY	ADEFGMPQRSTWY	V	CHKN		
225	Y					ADEGHKPQRTVW	ADEGHKPQRTVW	LS	CFIMN		
226	P	W				ACDEFGLNQRSTVYW	ACDEFGLNQRSTVY		HIKM	W	W
227	S					AFGHIKLMPQRTVWY	AFGHIKLMPQRTVWY		CDEN		
228	I					AEFGHLMNPRSTW	AEFGHLMNPRSTW	KQ	CDVY		
229	Y					EFGKLPQTVW	EFGKLPQTVW	HINRS	ACDM		
230	L	I		I		AEGHKMNPRSTVWY	AEGHKMNPRSTVWY		CDFQ		
231	N	T		T		ACDFGHIKLPQRSV	ACDFGHIKLPQRSV		EMWY		
232	T	FS		FS		CGHKLNPQVY	CGHKLNPQVY	AR	DEIMW		
233	Q	AFGKLRY		AFGKLRY		DIPST	DIPST	CVW	EHMN		
234	Q	LM		LM		ADEGHNPSTVW	ADEGHNPSTVW	CR	FIKY		
235	S	AEGHKT		AEGHKT		FLMRWY	FLMRWY	PQV	CDIN		
236	P	AGHKRS		AGHKRS		CILNQTY	CILNQTY	EW	DFMV		
237	V	CAEFHLNQRSTW	C	AEFHLNQRSTW	C			GPY	DIKM		C
238	A	DEHKQRST		DEHKQRST		FGLPVWY	FGLPVWY		CIMN		
239	A	N	N		N	CFGHILPRSTVWY	CFGHILPRSTVWY	K	DEMQ		N
240	T	KAMPQRSV		KAMPQRSV		EFGNWY	EFGNWY	L	CDHI		
241	L					ACDEGIPRSTVW	ACDEGIPRSTVW	FKQ	HMNY		
242	Y	F		F		ACDGILMPRSTVW	ACDGILMPRSTVW	K	EHNQ		
243	V					CDFGHLMPQRSWY	CDFGHLMPQRSWY	AT	EIKN		
244	R					ADGIVY	ADGIVY	HKMNPQSTW	CEFL		
245	N	H		H		ACFLPQRSTV	ACFLPQRSTV	GIKW	DEMY		
246	R					ACDEGHIKLMPSTVW	ACDEGHIKLMPSTVW		FNQY		
247	V	ILM		ILM		ACFHNPQRSTWY	ACFHNPQRSTWY		DEGK		
248	R	AHWY		AHWY		CDEGIMPT	CDEGIMPT	LSV	FKNQ		
249	E	V		V		AGHIKMQSY	AGHIKMQSY	LPRTW	CDFN		
250	A					CFGHKLMNPQRSTVW	CFGHKLMNPQRSTVW		DEIY		
251	I	YLM	Y	LM	Y	DFGHKPSTW	DFGHKPSTW	CQV	AENR		Y
252	R					ADEFGHIKLNPSTY	ADEFGHIKLNPSTY	V	CMQW		
253	V	I		I		ADEGHLMNQRSW	ADEGHLMNQRSW	PT	CFKY		
254	S					CDEGIKLPQRTVWY	CDEGIKLPQRTVWY	N	AFHM		
255	K	GANQRS	G	ANQRS	G	CDLPVW	CDLPVW	HTY	EFIM		G
256	I	AHLV		AHLV		CDEGP	CDEGP	MNQRTW	FKSY		
257	P	CAGIKLMNQRTV	C	AGIKLMNQRTV	C	D	D	SW	EFHY		C
258	D	GHNRS	G	HNRS	G	LPVW	LPVW	AEIQTY	CFKM		G
259	A	EGIKLNPQRSTVWY		EGIKLNPQRSTVWY				M	CDFH		
260	K	GADEHLMQRSY	G	ADEHLMQRSY	G	CP	CP	VW	FINT		G
261	S	AFKMNQRTVW		AFKMNQRTVW		P	P	EGIL	CDHY		
262	P					ADEFGHIKQRSTVWY	ADEFGHIKQRSTVWY		CLMN		
263	L	HAKMRTV	H	AKMRTV	H	EFPQW	EFPQW	GNS	CDIY		H
264	P	AH		AH		DEFGLMRTVWY	DEFGLMRTVWY	NS	CIKQ		

Position	Wild Type (WT)	Active Mutants	Mutants 20-40% Activity	Active Mutants (>40%, No errors)	Mutants 20-40% Activity (no errors)	Inactive Mutants	Inactive Mutants (no errors)	Unclassified	Not Made	Errors	Mutants w/ 20-40% Activity + Errors
265	V	I		I		ADFGHKLMNQRS	ADFGHKLMNQRS	CEPWY	T		
266	F	Y		Y		ACGHMPQRSTVW	ACGHMPQRSTVW	DL	EIKN		
267	A	MT		MT		DGHIKNRSW	DGHIKNRSW	ELPV	CFQY		
268	Y					ACFGHKLNPQSTVW	ACFGHKLNPQSTVW	R	DEIM		
269	T	ACDS		ACDS		EKLMNPQR	EKLMNPQR	GVY	FHIW		
270	R	MNST		MNST		ACEFGHIPY	ACEFGHIPY	DQV	KLW		
271	I	FGLMSV		FGLMSV		ADEHKTW	ADEHKTW	PR	CNQY		
272	V	EMDRST	EM	DRST	EM	AHLNPW	AHLNPW	CGK	FIQY		EM
273	F	HTY		HTY		ACDGILPQSVW	ACDGILPQSVW	R	EKMN		
274	T	AFS		AFS		CEGHNQWY	CEGHNQWY	LPRV	DIKM		
275	D	LV	L			AFGIKMQTWLV	AFGIKMQTW	CERS	HNPY	LV	LV
276	Q	GCDEHILMRSY	G	CDEHILMRSY	G	FPW	FPW	V	AKNT		G
277	V	ACDEGHKMNQRSTY		ACDEGHKMNQRSTY				L	FIPW		
278	L	AEFGHIKNRSTVY		AEFGHIKNRSTVY		MP	MP		CDQW		
279	K	AHQRT	A	HQRT		CFGLWYA	CFGLWY	DPSV	EIMN	A	A
280	F	GQ		GQ		DILMNRSTVW	DILMNRSTVW	EHP	ACKY		
281	L					ADGHIKNPQRSVW	ADGHIKNPQRSVW	FY	CEMT		
282	S	DGMQ		DGMQ		FLVWY	FLVWY	ACEPRT	HIKN		
283	Q	EPRST		EPRST		ACDFW	ACDFW	GHLNY	IKMV		
284	D	TYAEGHLMNQS	TY	AEGHLMNQS	TY	CIP	CIP	V	FKRW		TY
285	E	AFGHMNQY	A	FGHMNQY	A	KPRTV	KPRTV	SW	CDIL		A
286	L	WRS	W	RS	W	ACDFHKMPTY	ACDFHKMPTY	EG	INQV		W
287	V	NIT	N	IT		ACDEGKLPQRSN	ACDEGKLPQRS	F	HMWY	N	N
288	Y	LW		LW		DEFGHIKPRT	DEFGHIKPRT	QSV	ACMN		
289	T	KS		K		ACEGHLPQRYS	ACEGHLPQRY	MNV	DFIW	S	S
290	F	IM		IM		DQY	DQY	ACGHKLRSTV	ENPW		
291	G	CQRSV	C	QRSV		ADEFMNTWYC	ADEFMNTWY	HLP	IK	C	C
292	E	ACFGHKNPRVW		ACFGHKNPRVW		ILT	ILT	Q	DMSY		
293	T	ACDFGKLMPQSVY		ACDFGKLMPQSVY		EN	EN		HIRW		
294	V	M		M		AEGHKLNPQRSTW	AEGHKLNPQRSTW	C	DFIY		
295	A					CGHILNPTVY	CGHILNPTVY	DEFQS	KMRW		
296	L					CFGIKMQRSTVWY	CFGIKMQRSTVWY	AP	DEHN		
297	G	RA	R	A	R	CEHLNPQRSTY	CEHLNPQSTY	IVW	DFKM		R
298	A	GI		GI		CELMNPQSTWY	CELMNPQSTWY	RV	DFHK		
299	S					ACDFGHLMPQT	ACDFGHLMPQT	EIRY	KNVW		
300	G	R		R		ACDEFLMNPQSTVW	ACDEFLMNPQSTVW		HIKY		
301	I	AV		AV		EGHKMNPQRSWY	EGHKMNPQRSWY	L	CDFT		
302	V	IW		IW		CDEFGHLMPRSTY	CDEFGHLMPRSTY		AKNQ		
303	I	DV	D	V		ACEFGKLMRWYD	ACEFGKLMRWY	PS	HNQT	D	D
304	W	GI				ACDMNPQSTVYGI	ACDMNPQSTVY	LR	EFHK	GI	GI
305	G	NDE	N	DE	N	LPQRSTVY	LPQRSTVY	CFHK	AIMW		N
306	T	DES		DES		ACHILVWY	ACHILVWY	FGPR	KMNQ		
307	L	GKNQSTVWY	G	KNQSTVWY	G	CIP	CIP	EFR	ADHM		G
308	S	DGHKNPRT		DGHKNPRT		CFLMVWY	CFLMVWY		AEIQ		
309	I	DEGHKLMNQRSTVW		DEGHKLMNQRSTVW				Y	ACFP		
310	M	FYAGQRSV	FY	AGQRSV	Y	CEKLF	CEKL	NPW	DHIT	F	FY
311	R	GHKQST		GHKQST		CEFILPVW	CEFILPVW	A	DMNY		
312	S	KLGNT	KL	GNT	KL	CEMVW	CEMVW	AFHPQR	DIY		KL
313	M	AEGHKLPRSTVY		AEGHKLPRSTVY		C	C	DF	INQW		
314	K	ADHINQRSTY		ADHINQRSTY		CLW	CLW	PV	EFGM		
315	S	AEGHKLMRTY		AEGHKLMRTY		CIV	CIV	PW	DFNQ		
316	C	D		D		EGIKLMPRSTVWY	EGIKLMPRSTVWY	A	FHNQ		
317	L	ADHIKMNQRSTW		ADHIKMNQRSTW		GP	GP	C	EFVY		
318	L	DFGHIKMNQRST		DFGHIKMNQRST		CPW	CPW		AEVY		
319	L					CEFGHIKMPQRSVWY	CEFGHIKMPQRSVWY		ADNT		
320	D	LVEGHIKMNRSWY	LV	EGHIKMNRSWY	L	CPV	CP	F	AQT	V	LV
321	N	ADHKRSTY		ADHKRSTY		EMP	EMP	GILV	CFQW		
322	Y					CDEGILNPRSTVW	CDEGILNPRSTVW	FH	AKMQ		
323	M	FIL		FIL		ACEGHKNRSTV	ACEGHKNRSTV	P	DQWY		
324	E	ADHMNRS		ADHMNRS		CFPVWY	CFPVWY	GL	IKQT		
325	T	ADEGHKMNQSVW		ADEGHKMNQSVW		CR	CR	I	FLPY		
326	I	CKLSVY	C	KLSVY	C	EGHNW	EGHNW	ADPR	FMQT		C
327	L	M		M		AEFGHNQRSTVWY	AEFGHNQRSTVWY	D	CIKP		
328	N	WACGHIKLQRSTVY	W	ACGHIKLQRSTVY	W			D	EFMP		W
329	P					CFGHIKLNQRSTVWY	CFGHIKLNQRSTVWY		ADEM		
330	Y					ACDEGILMNPRSVW	ACDEGILMNPRSVW	F	HKQT		
331	I	CEV	CE	V		ADFHKQRSTWYCE	ADFHKQRSTWY	L	GMNP	CE	CE
332	I					ACDEFGHKLNPRSTY	ACDEFGHKLNPRSTY		MQVW		
333	N					GHIKPRSTWY	GHIKPRSTWY	AELMV	CDFQ		
334	V	TP	T	P	T	ACDEGMNRS	ACDEGMNRS	HLQY	FIKW		T
335	T	S		S		FGHIKLPVWY	FGHIKLPVWY	ACNQ	DEMR		
336	L					AEFGKNPRSTVWY	AEFGKNPRSTVWY	HM	CDIQ		
337	A					CFGIKLMRTW	CFGIKLMRTW	HNPSV	DEQY		
338	A	Q		Q		CDEFGHIKLPRTV	CDEFGHIKLPRTV	S	MNWY		
339	K	M		M		DEFGHLNPSTVWY	DEFGHLNPSTVWY	R	ACIQ		
340	M					ACDEFGHKPRSTVW	ACDEFGHKPRSTVW	L	INQY		
341	C					AEGHKLMNQRSTVY	AEGHKLMNQRSTVY	W	DFIP		
342	S	A		A		DEFHKLMPQRTY	DEFHKLMPQRTY	GI	CNVW		
343	Q	TV		TV		CDFIPW	CDFIPW	EGLMRSY	AHKN		
344	V					FGHLMNPQRSTWY	FGHLMNPQRSTWY	EI	ACDK		
345	L					ACEHKNQRTVY	ACEHKNQRTVY	DGPW	FIMS		
346	C					ADFGIKLMPRSTVW	ADFGIKLMPRSTVW	Q	EHNY		
347	Q	LAEGMRS	L	AEGMRS	L	CFIPTVW	CFIPTVW	Y	DHKN		L
348	E	DGS		DGS		CHILPQRTVWY	CHILPQRTVWY	M	AFKN		
349	Q	AEKMNRT		AEKMNRT		DFGPVWY	DFGPVWY	HLS	CI		
350	G					ADEFHKLMNPRSTVY	ADEFHKLMNPRSTVY		CIQW		
351	V	CIQAS	CIQ	AS	IQ	DEFHNRWYC	DEFHNRWY	GL	KMPT	C	CIQ
352	C					ADEFGKMPQRSTVWY	ADEFGKMPQRSTVWY		HILN		

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353	I	TV		TV		CFGHKLMQRSW	CFGHKLMQRSW	AE	DNPY		
354	R					CDEGHIKLMPQSVWY	CDEGHIKLMPQSVWY		AFNT		
355	K					DFGHLMNPQRSTVWY	DFGHLMNPQRSTVWY		ACEI		
356	N	ADHS		ADHS		CGKLPRTVW	CGKLPRTVW	FQ	EIMY		
357	W	KACST	K	ACST	K	DEFGLMQR	DEFGLMQR	PV	HINY		K
358	N	LCGT	L	CGT	L	EHIKPQRW	EHIKPQRW	DV	AFMSY		L
359	S	DEHKMTV		DEHKMTV		AFGLPW	AFGLPW	CR	INQY		
360	S	T		T		ACEFGIKLMPQRV	ACEFGIKLMPQRV	N	DHWY		
361	D	H	H		H	ACEGMNPQRSVW	ACEGMNPQRSVW	LY	FIKT		H
362	Y					ACEGHKLMNPRSTVW	ACEGHKLMNPRSTVW		DFIQ		
363	L					ACDEFGHIPQRSTVW	ACDEFGHIPQRSTVW		KMNY		
364	H					ACDEFGKLMPRSTVY	ACDEFGKLMPRSTVY		INQW		
365	L					ACDEGMNPQRSTWY	ACDEGMNPQRSTWY	IV	FHK		
366	N					ACEFGKMPQRTW	ACEFGKMPQRTW	LSV	DHIY		
367	P	ACGKRS		ACGKRS		EFILMQV	EFILMQV	HW	DNTY		
368	D	AEGHKLMRSTV		AEGHKLMRSTV		CPW	CPW	Y	FINQ		
369	N	HRS		HRS		CEFIKLPQVW	CEFIKLPQVW	AT	DGMY		
370	F					ADEGHKLNPQRSVY	ADEGHKLNPQRSVY	I	CMTW		
371	A	GEFHIKLMRSV	G	EFHIKLMRSV	G	PW	PW	CT	DNQY		G
372	I					ADEFGHKLNPRSTVW	ADEFGHKLNPRSTVW		CMQY		
373	Q	AEFKLMRSV		AEFKLMRSV		CPW	CPW	GHNT	DIY		
374	L	WAHIMNPRSTVY	W	AHIMNPRSTVY	W	DE	DE	G	CFKQ		W
375	E	IAGKLMNRST	I	AGKLMNRST	I	CFPVY	CFPVY		DHQW		I
376	K	LADEMQRSTVY	L	ADEMQRSTVY	L	IPW	IPW	G	CFHN		L
377	G	DEHKPRST		DEHKPRST		CILV	CILV	FMY	ANQW		
378	G	KNR		KNR		DEFILMQTWY	DEFILMQTWY	SV	ACHP		
379	K	GHRST		GHRST		ACEFILMW	ACEFILMW	NV	DPQY		
380	F	VTILPWY	VT	ILPWY	VT	CDEGRSQ	CDEGRSQ	A	HKMN		VT
381	T	EHKNQRSV	E	HKNQRSV	E	GLPWY	GLPWY	AF	CDIM		E
382	V					EGHKLMNPQRSTWY	EGHKLMNPQRSTWY	I	ACDF		
383	R	AEHIKLMNSTV		AEHIKLMNSTV		GP	GP	FW	CDQY		
384	G					CFMQST	CFMQST	ADEHIKLPR	NVWY		
385	K	AGHNQRSTV		AGHNQRSTV		CLMPWY	CLMPWY		DEFI		
386	P					ACFGHILMNQRSTVY	ACFGHILMNQRSTVY		DEKW		
387	T	S		S		CEFGHILMNVWY	CEFGHILMNVWY	KQ	ADPR		
388	L	FHIMRTVWY		FHIMRTVWY		CGPQS	CGPQS	A	DEKN		
389	D	AGHKLMPQRSTY		AGHKLMPQRSTY		FV	FV	I	CENW		
390	D					ACEFGHLNPRSTVWY	ACEFGHLNPRSTVWY		IKMQ		
391	L	C		C		ADGHKNPQRSTVWY	ADGHKNPQRSTVWY		EFIM		
392	E	WAFGKLMQRSTVY	W	AFGKLMQRSTVY	W	CP	CP		DHIN		W
393	Q	ADFHKLMNRST		ADFHKLMNRST		CP	CP	GI	EVWY		
394	F	LW		LW		ADEGIKNPQRSTV	ADEGIKNPQRSTV		CHMY		
395	S	TAGHKRW	T	AGHKRW	T	CLMP	CLMP	DEVY	FINQ		T
396	E	LADHQRST	L	ADHQRST	L	CFGIPY	CFGIPY	V	KMNW		L
397	K					ACEFGILMPQTV	ACEFGILMPQTV	NRS	DHWY		
398	F	L	L			ACEGHINPRSTVWYL	ACEGHINPRSTVWY		DKMQ	L	L
399	Y	ACEKMNQRSTVW		ACEKMNQRSTVW		DP	DP	G	FHIL		
400	C					ADEFGILMPQRSTVY	ADEFGILMPQRSTVY		HKNW		
401	S	GQAEN	GQ	AEN	GQ	CFHKRWY	CFHKRWY	DLT	IMPV		GQ
402	C					ADEFLMPQRSTVWY	ADEFLMPQRSTVWY	G	HIKN		
403	Y	F		F		ACEGHKLMNPQRT	ACEGHKLMNPQRT	S	DIVW		
404	S	TAP	T	AP	T	CDFGHLMNRVWY	CDFGHLMNRVWY		EIKQ		T
405	T	FAGKMPQRSWY	F	AGKMPQRSWY	F	CIV	CIV	L	DEHN		F
406	L	ACEFGINQSTVY		ACEFGINQSTVY		PR	PR	D	HKMW		
407	S	ADEFGHLMNPQRVW		ADEFGHLMNPQRVW				T	CIKY		
408	C					AEFGIKLPRSTVWY	AEFGIKLPRSTVWY	N	DHMQ		
409	K	ADEGHIPQRSTV		ADEGHIPQRSTV				CLW	FMNY		
410	E	DKMNPQRSTVY		DKMNPQRSTVY		W	W	GIL	ACFH		
411	K	HANPRSTV	H	ANPRSTV	H	DEFG	DEFG	ILW	CMQY		H
412	A	HQDGILNPRSVWY	HQ	DGILNPRSVWY	Q	EH	E		CFKMT	H	HQ
413	D	AHSEKNRTQ	AHSQ	ENRT	ASQ	ILPHK	ILP	FGW	CMVY	HK	AHSKQ
414	V	KLIM	KL	IM	L	ADEGHRSTK	ADEGHRST	FQY	CNPW	K	KL
415	K	YVGSW	YV	GSW	YV	CDEP	CDEP	LMQRT	AFHIN		YV
416	D	FGHIKLNQRTVY		FGHIKLNQRTVY		CS	CS	W	AEMP		
417	T	I		I		ADEFGHKMPQR	ADEFGHKMPQR	LSW	CNVY		
418	D	AEFGILMNPQRSVY		AEFGILMNPQRSVY				C	HKTW		
419	A	EFGHIKLNRSWY		EFGHIKLNRSWY		DP	DP	T	CMQV		
420	V	IP		IP		ADFGHKLNRSTWY	ADFGHKLNRSTWY		CEMQ		
421	D	AEGHKLMNQRSTYI	I	AEGHKLMNQRSTY	I			W	CFPV		I
422	V	IT		IT		CDGHLMNQRSY	CDGHLMNQRSY	AEPW	FK		
423	C					ADEFGHLMPQRSTVW	ADEFGHLMPQRSTVW		IKNY		
424	I					ACEGHNQRSWY	ACEGHNQRSWY	KLTV	DFMP		
425	A	YGIKMNRS	Y	GIKMNRS		ELPWY	ELPW	CDV	FHQT	Y	Y
426	D	KSEGNPQY	KS	EGNPQY	KS	CFMR	CFMR	AIL	HTVW		KS
427	G	HTQIKS	HTQ	IKS	HTQ	ACFLPVWY	ACFLPVWY	R	DEMN		HTQ
428	V	LMPT		LMPT		ACDEGHNRSY	ACDEGHNRSY	F	IKQW		
429	C					ADKLNPSTVWY	ADKLNPSTVWY	GIMR	EFHQ		
430	I					ADELMNSTV	ADELMNSTV	GHKPRW	CFQY		
431	D	AEGHIKLNQRSVWY		AEGHIKLNQRSVWY		P	P		CFMT		
432	A	HEGNSV	H	EGNSV	H	CFIKLMPY	CFIKLMPY	R	DQTW		H
433	F	ACDEGHIKLPRSTVW		ACDEGHIKLPRSTVW					MNQY		
434	L	FGIMV		FGIMV		HKPQRW	HKPQRW	NST	ACDEY		
435	K	ACEGHRSTVY		ACEGHRSTVY				FILPW	DMNQ		
436	P	CDEGHIKLMQRSTWY	C	DEGHIKLMQRSTWY	C				AFNV		C
437	P	ADGHIKLMQRSY		ADGHIKLMQRSY		T	T	FW	CENV		
438	M	ACDEGLNPQRSTVW		ACDEGLNPQRSTVW		Y	Y		FHIK		
439	E	ACFGHKLPQSTVW		ACFGHKLPQSTVW		NR	NR		DIMY		
440	T	ADEFGHILMPRSVY		ADEFGHILMPRSVY		Q	Q		CKNW		

Position	Wild Type (WT)	Active Mutants	Mutants 20-40% Activity	Active Mutants (>40%, No errors)	Mutants 20-40% Activity (no errors)	Inactive Mutants	Inactive Mutants (no errors)	Unclassified	Not Made	Errors	Mutants w/ 20-40% Activity + Errors
441	E	ADFGHKLNQSTVY		ADFGHKLNQSTVY		R	R	C	IMPW		
442	E	WCGHKLPQRTVY	W	CGHKLPQRTVY	W	MNS	MNS		ADFI		W
443	P	AEFGHLMNQRSTW		AEFGHLMNQRSTW		D	D	I	CKVY		
444	Q	MDEFGHIKNRVWY	M	DEFGHIKNRVWY	M			CL	APST		M
445	I	AGHLMNPQRSTVWY		AGHLMNPQRSTVWY				CDFK	E		
446	F	ACDEGHIKLMQRTVW		ACDEGHIKLMQRTVW					NPSY		
447	Y	DEFGILMNPQRTVW		DEFGILMNPQRTVW				K	ACHS		

Position #	Wild Type (WT)	Count Active	Count Active (>40%, no errors)	Count Inactive	Count Inactive (no errors)	Count Unclassified	Count Not Made	Count Mutants 20-40% Activity	Count Mutants 20-40% Activity (no errors)	Count Errors	Count Mutants 20-40% Activity + Errors	Tested (w/o errors)	Total
1	L	15	15	0	0	1	3	0	0	0	0	15	19
2	N	10	9	4	4	1	4	1	1	0	1	13	19
3	F	4	4	6	6	5	4	0	0	0	0	10	19
4	R	5	5	8	8	2	4	0	0	0	0	13	19
5	A	1	1	13	13	1	4	0	0	0	0	14	19
6	P	7	7	5	5	4	3	0	0	0	0	12	19
7	P	1	1	14	14	2	2	0	0	0	0	15	19
8	V	4	3	8	8	3	4	1	1	0	1	11	19
9	I	6	6	6	6	3	4	0	0	0	0	12	19
10	P	9	9	5	5	1	4	0	0	0	0	14	19
11	N	5	4	9	9	1	4	1	1	0	1	13	19
12	V	9	9	3	3	3	4	0	0	0	0	12	19
13	P	4	4	6	6	5	4	0	0	0	0	10	19
14	F	4	4	9	9	2	4	0	0	0	0	13	19
15	L	3	3	10	10	2	4	0	0	0	0	13	19
16	W	0	0	14	14	1	4	0	0	0	0	14	19
17	A	0	0	15	15	0	4	0	0	0	0	15	19
18	W	0	0	14	14	1	4	0	0	0	0	14	19
19	N	0	0	15	15	0	4	0	0	0	0	15	19
20	A	1	1	12	12	2	4	0	0	0	0	13	19
21	P	0	0	14	14	1	4	0	0	0	0	14	19
22	S	4	4	5	5	6	4	0	0	0	0	9	19
23	E	1	1	10	10	4	4	0	0	0	0	11	19
24	F	13	13	0	0	2	4	0	0	0	0	13	19
25	C	0	0	15	15	0	4	0	0	0	0	15	19
26	L	15	15	0	0	0	4	0	0	0	0	15	19
27	G	14	14	1	1	0	4	0	0	0	0	15	19
28	K	14	14	0	0	1	4	0	0	0	0	14	19
29	F	14	14	0	0	1	4	0	0	0	0	14	19
30	D	14	14	0	0	1	4	0	0	0	0	14	19
31	E	14	14	0	0	1	4	0	0	0	0	14	19
32	P	16	16	0	0	0	3	0	0	0	0	16	19
33	L	8	8	6	6	1	4	0	0	0	0	14	19
34	D	7	6	6	6	2	4	1	1	0	1	12	19
35	M	7	5	6	6	2	4	2	2	0	2	11	19
36	S	9	7	5	5	1	4	2	2	0	2	12	19
37	L	8	7	5	5	2	4	1	1	0	1	12	19
38	F	1	1	9	9	5	4	0	0	0	0	10	19
39	S	7	7	4	4	4	4	0	0	0	0	11	19
40	F	2	2	10	10	3	4	0	0	0	0	12	19
41	I	10	10	1	1	4	4	0	0	0	0	11	19
42	G	1	1	13	13	1	4	0	0	0	0	14	19
43	S	2	1	10	10	3	4	1	1	0	1	11	19
44	P	1	1	14	14	0	4	0	0	0	0	15	19
45	R	2	2	6	6	8	3	0	0	0	0	8	19
46	I	13	12	2	2	0	4	1	1	0	1	14	19
47	N	13	13	1	1	3	2	0	0	0	0	14	19
48	A	12	12	1	1	4	2	0	0	0	0	13	19
49	T	5	5	5	5	5	4	0	0	0	0	10	19
50	G	12	11	0	0	3	4	1	1	0	1	11	19
51	Q	4	2	8	8	3	4	2	2	0	2	10	19
52	G	6	6	5	5	4	4	0	0	0	0	11	19
53	V	0	0	15	15	0	4	0	0	0	0	15	19
54	T	6	6	6	6	3	4	0	0	0	0	12	19
55	I	0	0	11	11	4	4	0	0	0	0	11	19
56	F	0	0	14	14	1	4	0	0	0	0	14	19
57	Y	0	0	12	12	3	4	0	0	0	0	12	19
58	V	13	13	1	1	1	4	0	0	0	0	14	19
59	D	2	2	11	11	2	4	0	0	0	0	13	19
60	R	1	1	14	14	0	4	0	0	0	0	15	19
61	L	4	3	12	11	0	4	1	0	1	1	14	19
62	G	0	0	15	15	0	4	0	0	0	0	15	19
63	Y	12	12	3	3	0	4	0	0	0	0	15	19
64	Y	0	0	17	17	0	2	0	0	0	0	17	19
65	P	1	0	14	13	1	4	0	0	1	1	13	19
66	Y	2	2	13	13	0	4	0	0	0	0	15	19
67	I	5	4	7	6	4	4	1	0	1	1	10	19
68	D	10	7	8	5	0	4	1	0	3	3	12	19
69	S	13	12	2	1	1	4	0	0	1	1	13	19
70	I	14	14	1	1	0	4	0	0	0	0	15	19
71	T	10	10	1	1	4	4	0	0	0	0	11	19
72	G	11	9	7	6	1	1	1	1	1	2	15	19
73	V	13	13	1	1	1	4	0	0	0	0	14	19
74	T	15	15	0	0	0	4	0	0	0	0	15	19
75	V	12	12	3	3	1	3	0	0	0	0	15	19
76	N	0	0	14	14	1	4	0	0	0	0	14	19
77	G	2	0	8	8	5	4	2	2	0	2	8	19
78	G	0	0	7	7	8	4	0	0	0	0	7	19
79	I	3	3	11	11	1	4	0	0	0	0	14	19
80	P	0	0	15	15	0	4	0	0	0	0	15	19
81	Q	1	0	12	11	3	4	0	0	1	1	11	19
82	K	14	13	2	1	1	3	1	0	1	1	14	19
83	I	11	10	2	1	2	5	1	0	1	1	11	19
84	S	15	14	1	0	0	4	1	0	1	1	14	19
85	L	1	1	11	11	3	4	0	0	0	0	12	19
86	Q	17	16	2	1	0	1	0	0	1	1	17	19
87	D	15	14	1	0	0	4	1	0	1	1	14	19

Position #	Wild Type (WT)	Count Active	Count Active (>40%, no errors)	Count Inactive	Count Inactive (no errors)	Count Unclassified	Count Not Made	Count Mutants 20-40% Activity	Count Mutants 20-40% Activity (no errors)	Count Errors	Count Mutants 20-40% Activity + Errors	Tested (w/o errors)	Total
88	H	0	0	15	15	0	4	0	0	0	0	15	19
89	L	6	3	9	8	1	4	3	2	1	3	11	19
90	D	13	12	2	1	1	4	0	0	1	1	13	19
91	K	3	3	9	9	2	5	0	0	0	0	12	19
92	A	6	5	9	8	1	4	1	0	1	1	13	19
93	K	15	13	0	0	0	4	2	2	0	2	13	19
94	K	13	12	2	2	0	4	1	1	0	1	14	19
95	D	0	0	15	15	0	4	0	0	0	0	15	19
96	I	3	3	9	9	3	4	0	0	0	0	12	19
97	T	15	15	0	0	0	4	0	0	0	0	15	19
98	F	13	13	1	1	1	4	0	0	0	0	14	19
99	Y	3	2	8	8	4	4	1	1	0	1	10	19
100	M	0	0	11	11	4	4	0	0	0	0	11	19
101	P	0	0	13	13	2	4	0	0	0	0	13	19
102	V	14	14	1	1	0	4	0	0	0	0	15	19
103	D	1	0	13	13	1	4	1	1	0	1	13	19
104	N	9	8	3	3	3	4	1	1	0	1	11	19
105	L	12	9	3	2	2	3	3	2	1	3	11	19
106	G	1	1	12	12	2	4	0	0	0	0	13	19
107	M	3	3	9	9	3	4	0	0	0	0	12	19
108	A	1	1	11	11	4	3	0	0	0	0	12	19
109	V	0	0	8	8	7	4	0	0	0	0	8	19
110	I	1	1	6	6	8	4	0	0	0	0	7	19
111	D	0	0	3	3	13	3	0	0	0	0	3	19
112	W	0	0	8	8	7	4	0	0	0	0	8	19
113	E	0	0	2	2	13	4	0	0	0	0	2	19
114	E	5	5	5	5	5	4	0	0	0	0	10	19
115	W	0	0	14	14	1	4	0	0	0	0	14	19
116	R	0	0	14	14	1	4	0	0	0	0	14	19
117	P	1	0	10	9	5	4	0	0	1	1	9	19
118	T	7	7	8	8	0	4	0	0	0	0	15	19
119	W	4	3	7	6	5	4	1	0	1	1	9	19
120	A	14	14	0	0	1	4	0	0	0	0	14	19
121	R	0	0	12	12	4	3	0	0	0	0	12	19
122	N	1	1	11	11	3	4	0	0	0	0	12	19
123	W	0	0	14	14	1	4	0	0	0	0	14	19
124	K	3	1	5	5	8	3	2	2	0	2	6	19
125	P	4	3	6	6	4	5	1	1	0	1	9	19
126	K	0	0	7	7	11	1	0	0	0	0	7	19
127	D	13	13	1	1	1	4	0	0	0	0	14	19
128	V	10	10	2	2	3	4	0	0	0	0	12	19
129	Y	0	0	13	13	2	4	0	0	0	0	13	19
130	K	2	2	10	10	3	4	0	0	0	0	12	19
131	N	14	14	1	1	0	4	0	0	0	0	15	19
132	R	14	14	1	1	0	4	0	0	0	0	15	19
133	S	1	1	13	13	1	4	0	0	0	0	14	19
134	I	3	3	12	12	0	4	0	0	0	0	15	19
135	E	14	14	1	1	0	4	0	0	0	0	15	19
136	L	13	13	1	1	1	4	0	0	0	0	14	19
137	V	6	3	8	8	2	3	3	3	0	3	11	19
138	Q	11	11	1	1	3	4	0	0	0	0	12	19
139	Q	14	14	1	1	0	4	0	0	0	0	15	19
140	Q	14	14	0	0	1	4	0	0	0	0	14	19
141	N	15	15	0	0	1	3	0	0	0	0	15	19
142	V	15	15	0	0	0	4	0	0	0	0	15	19
143	Q	8	7	6	5	3	3	1	0	1	1	12	19
144	L	3	2	10	10	2	4	1	1	0	1	12	19
145	S	11	11	2	2	2	4	0	0	0	0	13	19
146	L	15	15	0	0	0	4	0	0	0	0	15	19
147	T	15	15	0	0	0	4	0	0	0	0	15	19
148	E	14	14	0	0	1	4	0	0	0	0	14	19
149	A	10	10	2	2	3	4	0	0	0	0	12	19
150	T	14	14	1	1	0	4	0	0	0	0	15	19
151	E	15	15	0	0	0	4	0	0	0	0	15	19
152	K	10	9	1	1	4	4	1	1	0	1	10	19
153	A	3	2	7	7	6	3	1	1	0	1	9	19
154	K	4	3	7	7	5	3	1	1	0	1	10	19
155	Q	14	14	2	2	0	3	0	0	0	0	16	19
156	E	13	12	1	1	1	4	1	1	0	1	13	19
157	F	1	1	16	16	0	2	0	0	0	0	17	19
158	E	7	6	5	5	3	4	1	1	0	1	11	19
159	K	12	11	2	2	1	4	1	1	0	1	13	19
160	A	15	14	0	0	0	4	1	1	0	1	14	19
161	G	7	7	1	1	7	4	0	0	0	0	8	19
162	K	14	14	0	0	1	4	0	0	0	0	14	19
163	D	12	10	2	2	1	4	2	2	0	2	12	19
164	F	4	4	10	10	1	4	0	0	0	0	14	19
165	L	10	9	4	3	2	4	1	0	1	1	12	19
166	V	13	13	1	1	1	4	0	0	0	0	14	19
167	E	13	12	1	1	1	4	1	1	0	1	13	19
168	T	1	1	14	14	0	4	0	0	0	0	15	19
169	I	3	3	12	12	0	4	0	0	0	0	15	19
170	K	5	5	8	8	2	4	0	0	0	0	13	19
171	L	2	2	9	9	5	3	0	0	0	0	11	19
172	G	2	2	10	10	3	4	0	0	0	0	12	19
173	K	3	2	12	12	0	4	1	1	0	1	14	19
174	L	13	12	1	1	1	4	1	1	0	1	13	19

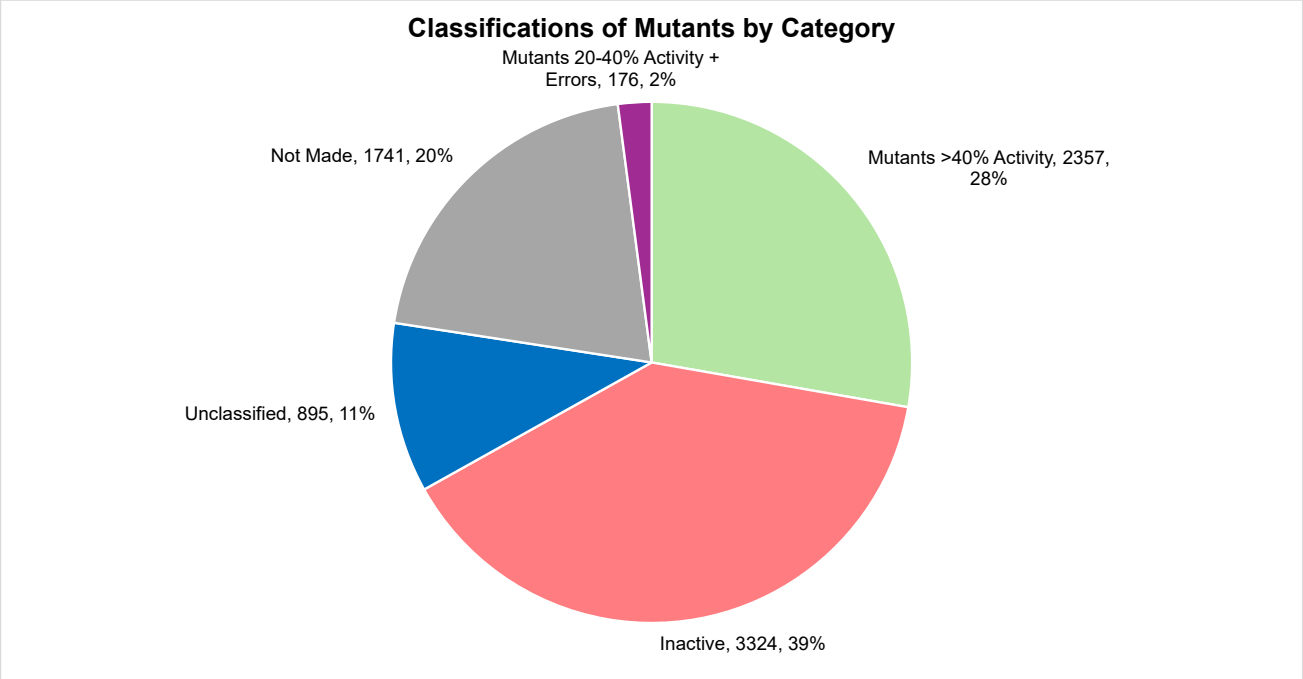
Position #	Wild Type (WT)	Count Active	Count Active (>40%, no errors)	Count Inactive	Count Inactive (no errors)	Count Unclassified	Count Not Made	Count Mutants 20-40% Activity	Count Mutants 20-40% Activity (no errors)	Count Errors	Count Mutants 20-40% Activity + Errors	Tested (w/o errors)	Total
175	L	5	5	7	7	3	4	0	0	0	0	12	19
176	R	2	2	13	13	0	4	0	0	0	0	15	19
177	P	1	0	14	13	1	4	1	0	1	1	13	19
178	N	4	4	6	6	5	4	0	0	0	0	10	19
179	H	14	14	1	1	0	4	0	0	0	0	15	19
180	L	5	4	6	6	4	4	1	1	0	1	10	19
181	W	3	2	12	11	1	4	1	0	1	1	13	19
182	G	1	1	13	13	1	4	0	0	0	0	14	19
183	Y	2	1	12	11	2	4	1	0	1	1	12	19
184	Y	1	0	14	14	0	4	1	1	0	1	14	19
185	L	0	0	14	14	1	4	0	0	0	0	14	19
186	F	1	1	14	14	0	4	0	0	0	0	15	19
187	P	0	0	15	15	0	4	0	0	0	0	15	19
188	D	0	0	15	15	0	4	0	0	0	0	15	19
189	C	0	0	15	15	0	4	0	0	0	0	15	19
190	Y	0	0	14	14	1	4	0	0	0	0	14	19
191	N	0	0	15	15	0	4	0	0	0	0	15	19
192	H	2	2	13	13	0	4	0	0	0	0	15	19
193	H	6	4	7	7	2	4	2	2	0	2	11	19
194	Y	0	0	8	8	7	4	0	0	0	0	8	19
195	K	12	10	1	0	3	4	1	1	1	2	10	19
196	K	9	7	0	0	6	4	2	2	0	2	7	19
197	P	14	13	1	1	0	4	1	1	0	1	14	19
198	G	12	11	2	1	2	4	1	0	1	1	12	19
199	Y	0	0	10	10	5	4	0	0	0	0	10	19
200	N	2	1	13	13	1	3	1	1	0	1	14	19
201	G	0	0	11	11	4	4	0	0	0	0	11	19
202	S	1	1	13	13	1	4	0	0	0	0	14	19
203	C	0	0	13	13	2	4	0	0	0	0	13	19
204	F	2	1	11	11	3	3	1	1	0	1	12	19
205	N	15	14	0	0	0	4	1	1	0	1	14	19
206	V	9	8	6	6	0	4	1	1	0	1	14	19
207	E	0	0	11	11	4	4	0	0	0	0	11	19
208	I	10	10	4	4	1	4	0	0	0	0	14	19
209	K	9	8	2	2	4	4	1	1	0	1	10	19
210	R	0	0	14	14	1	4	0	0	0	0	14	19
211	N	2	1	13	12	1	4	0	0	1	1	13	19
212	D	3	3	10	10	2	4	0	0	0	0	13	19
213	D	13	13	2	2	0	4	0	0	0	0	15	19
214	L	1	1	13	13	1	4	0	0	0	0	14	19
215	S	13	13	2	2	0	4	0	0	0	0	15	19
216	W	1	1	14	14	0	4	0	0	0	0	15	19
217	L	1	1	10	10	4	4	0	0	0	0	11	19
218	W	3	1	7	6	6	4	2	1	1	2	7	19
219	N	13	13	1	1	1	4	0	0	0	0	14	19
220	E	9	7	6	6	0	4	2	2	0	2	13	19
221	S	7	6	6	6	2	4	1	1	0	1	12	19
222	T	10	10	2	2	3	4	0	0	0	0	12	19
223	A	0	0	15	15	0	4	0	0	0	0	15	19
224	L	1	1	13	13	1	4	0	0	0	0	14	19
225	Y	0	0	12	12	2	5	0	0	0	0	12	19
226	P	1	0	15	14	0	4	0	0	1	1	14	19
227	S	0	0	15	15	0	4	0	0	0	0	15	19
228	I	0	0	13	13	2	4	0	0	0	0	13	19
229	Y	0	0	10	10	5	4	0	0	0	0	10	19
230	L	1	1	14	14	0	4	0	0	0	0	15	19
231	N	1	1	14	14	0	4	0	0	0	0	15	19
232	T	2	2	10	10	2	5	0	0	0	0	12	19
233	Q	7	7	5	5	3	4	0	0	0	0	12	19
234	Q	2	2	11	11	2	4	0	0	0	0	13	19
235	S	6	6	6	6	3	4	0	0	0	0	12	19
236	P	6	6	7	7	2	4	0	0	0	0	13	19
237	V	12	11	0	0	3	4	1	1	0	1	11	19
238	A	8	8	7	7	0	4	0	0	0	0	15	19
239	A	1	0	13	13	1	4	1	1	0	1	13	19
240	T	8	8	6	6	1	4	0	0	0	0	14	19
241	L	0	0	12	12	3	4	0	0	0	0	12	19
242	Y	1	1	13	13	1	4	0	0	0	0	14	19
243	V	0	0	13	13	2	4	0	0	0	0	13	19
244	R	0	0	6	6	9	4	0	0	0	0	6	19
245	N	1	1	10	10	4	4	0	0	0	0	11	19
246	R	0	0	15	15	0	4	0	0	0	0	15	19
247	V	3	3	12	12	0	4	0	0	0	0	15	19
248	R	4	4	8	8	3	4	0	0	0	0	12	19
249	E	1	1	9	9	5	4	0	0	0	0	10	19
250	A	0	0	15	15	0	4	0	0	0	0	15	19
251	I	3	2	9	9	3	4	1	1	0	1	11	19
252	R	0	0	14	14	1	4	0	0	0	0	14	19
253	V	1	1	12	12	2	4	0	0	0	0	13	19
254	S	0	0	14	14	1	4	0	0	0	0	14	19
255	K	6	5	6	6	3	4	1	1	0	1	11	19
256	I	4	4	5	5	6	4	0	0	0	0	9	19
257	P	12	11	1	1	2	4	1	1	0	1	12	19
258	D	5	4	4	4	6	4	1	1	0	1	8	19
259	A	14	14	0	0	1	4	0	0	0	0	14	19
260	K	11	10	2	2	2	4	1	1	0	1	12	19
261	S	10	10	1	1	4	4	0	0	0	0	11	19

Position #	Wild Type (WT)	Count Active	Count Active (>40%, no errors)	Count Inactive	Count Inactive (no errors)	Count Unclassified	Count Not Made	Count Mutants 20-40% Activity	Count Mutants 20-40% Activity (no errors)	Count Errors	Count Mutants 20-40% Activity + Errors	Tested (w/o errors)	Total
262	P	0	0	15	15	0	4	0	0	0	0	15	19
263	L	7	6	5	5	3	4	1	1	0	1	11	19
264	P	2	2	11	11	2	4	0	0	0	0	13	19
265	V	1	1	12	12	5	1	0	0	0	0	13	19
266	F	1	1	12	12	2	4	0	0	0	0	13	19
267	A	2	2	9	9	4	4	0	0	0	0	11	19
268	Y	0	0	14	14	1	4	0	0	0	0	14	19
269	T	4	4	8	8	3	4	0	0	0	0	12	19
270	R	4	4	9	9	3	3	0	0	0	0	13	19
271	I	6	6	7	7	2	4	0	0	0	0	13	19
272	V	6	4	6	6	3	4	2	2	0	2	10	19
273	F	3	3	11	11	1	4	0	0	0	0	14	19
274	T	3	3	8	8	4	4	0	0	0	0	11	19
275	D	2	0	11	9	4	4	1	0	2	2	9	19
276	Q	11	10	3	3	1	4	1	1	0	1	13	19
277	V	14	14	0	0	1	4	0	0	0	0	14	19
278	L	13	13	2	2	0	4	0	0	0	0	15	19
279	K	5	4	7	6	4	4	1	0	1	1	10	19
280	F	2	2	10	10	3	4	0	0	0	0	12	19
281	L	0	0	13	13	2	4	0	0	0	0	13	19
282	S	4	4	5	5	6	4	0	0	0	0	9	19
283	Q	5	5	5	5	5	4	0	0	0	0	10	19
284	D	11	9	3	3	1	4	2	2	0	2	12	19
285	E	8	7	5	5	2	4	1	1	0	1	12	19
286	L	3	2	10	10	2	4	1	1	0	1	12	19
287	V	3	2	12	11	1	4	1	0	1	1	13	19
288	Y	2	2	10	10	3	4	0	0	0	0	12	19
289	T	2	1	11	10	3	4	0	0	1	1	11	19
290	F	2	2	3	3	10	4	0	0	0	0	5	19
291	G	5	4	10	9	3	2	1	0	1	1	13	19
292	E	11	11	3	3	1	4	0	0	0	0	14	19
293	T	13	13	2	2	0	4	0	0	0	0	15	19
294	V	1	1	13	13	1	4	0	0	0	0	14	19
295	A	0	0	10	10	5	4	0	0	0	0	10	19
296	L	0	0	13	13	2	4	0	0	0	0	13	19
297	G	2	1	11	10	3	4	1	1	0	1	11	19
298	A	2	2	11	11	2	4	0	0	0	0	13	19
299	S	0	0	11	11	4	4	0	0	0	0	11	19
300	G	1	1	14	14	0	4	0	0	0	0	15	19
301	I	2	2	12	12	1	4	0	0	0	0	14	19
302	V	2	2	13	13	0	4	0	0	0	0	15	19
303	I	2	1	12	11	2	4	1	0	1	1	12	19
304	W	2	0	13	11	2	4	0	0	2	2	11	19
305	G	3	2	8	8	4	4	1	1	0	1	10	19
306	T	3	3	8	8	4	4	0	0	0	0	11	19
307	L	9	8	3	3	3	4	1	1	0	1	11	19
308	S	8	8	7	7	0	4	0	0	0	0	15	19
309	I	14	14	0	0	1	4	0	0	0	0	14	19
310	M	8	6	5	4	3	4	2	1	1	2	10	19
311	R	6	6	8	8	1	4	0	0	0	0	14	19
312	S	5	3	5	5	6	3	2	2	0	2	8	19
313	M	12	12	1	1	2	4	0	0	0	0	13	19
314	K	10	10	3	3	2	4	0	0	0	0	13	19
315	S	10	10	3	3	2	4	0	0	0	0	13	19
316	C	1	1	13	13	1	4	0	0	0	0	14	19
317	L	12	12	2	2	1	4	0	0	0	0	14	19
318	L	12	12	3	3	0	4	0	0	0	0	15	19
319	L	0	0	15	15	0	4	0	0	0	0	15	19
320	D	13	11	3	2	1	3	2	1	1	2	13	19
321	N	8	8	3	3	4	4	0	0	0	0	11	19
322	Y	0	0	13	13	2	4	0	0	0	0	13	19
323	M	3	3	11	11	1	4	0	0	0	0	14	19
324	E	7	7	6	6	2	4	0	0	0	0	13	19
325	T	12	12	2	2	1	4	0	0	0	0	14	19
326	I	6	5	5	5	4	4	1	1	0	1	10	19
327	L	1	1	13	13	1	4	0	0	0	0	14	19
328	N	14	13	0	0	1	4	1	1	0	1	13	19
329	P	0	0	15	15	0	4	0	0	0	0	15	19
330	Y	0	0	14	14	1	4	0	0	0	0	14	19
331	I	3	1	13	11	1	4	2	0	2	2	12	19
332	I	0	0	15	15	0	4	0	0	0	0	15	19
333	N	0	0	10	10	5	4	0	0	0	0	10	19
334	V	2	1	9	9	4	4	1	1	0	1	10	19
335	T	1	1	10	10	4	4	0	0	0	0	11	19
336	L	0	0	13	13	2	4	0	0	0	0	13	19
337	A	0	0	10	10	5	4	0	0	0	0	10	19
338	A	1	1	13	13	1	4	0	0	0	0	14	19
339	K	1	1	13	13	1	4	0	0	0	0	14	19
340	M	0	0	14	14	1	4	0	0	0	0	14	19
341	C	0	0	14	14	1	4	0	0	0	0	14	19
342	S	1	1	12	12	2	4	0	0	0	0	13	19
343	Q	2	2	6	6	7	4	0	0	0	0	8	19
344	V	0	0	13	13	2	4	0	0	0	0	13	19
345	L	0	0	11	11	4	4	0	0	0	0	11	19
346	C	0	0	14	14	1	4	0	0	0	0	14	19
347	Q	7	6	7	7	1	4	1	1	0	1	13	19
348	E	3	3	11	11	1	4	0	0	0	0	14	19

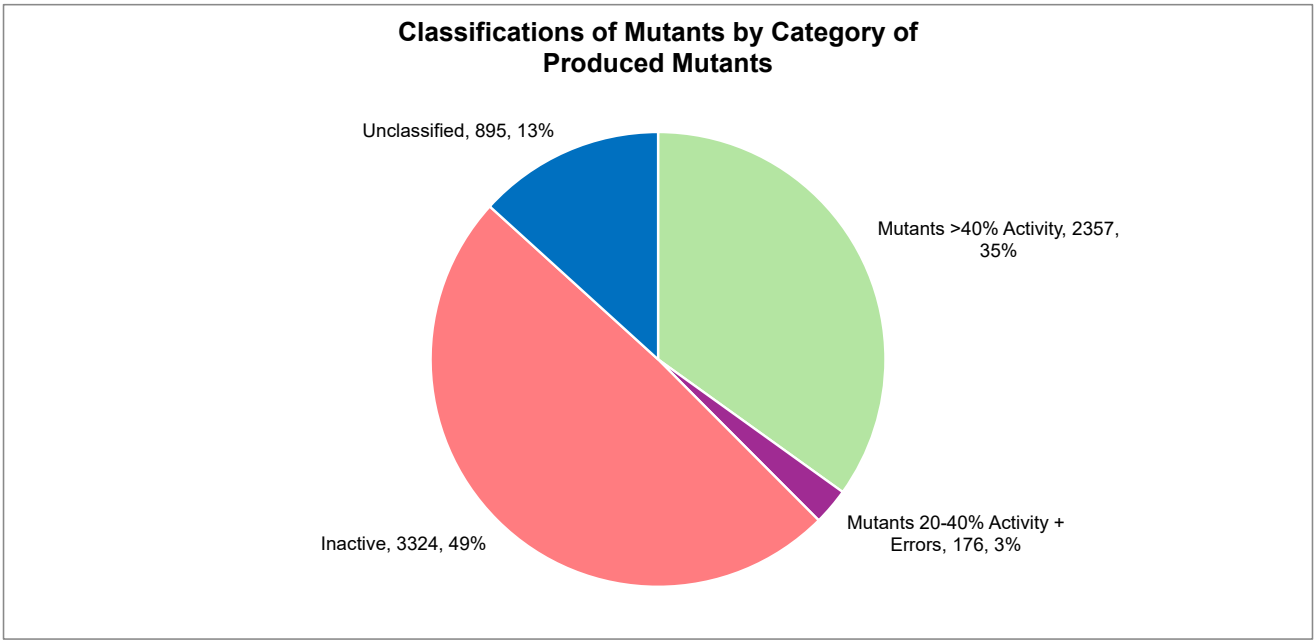
Position #	Wild Type (WT)	Count Active	Count Active (>40%, no errors)	Count Inactive	Count Inactive (no errors)	Count Unclassified	Count Not Made	Count Mutants 20-40% Activity	Count Mutants 20-40% Activity (no errors)	Count Errors	Count Mutants 20-40% Activity + Errors	Tested (w/o errors)	Total
349	Q	7	7	7	7	3	2	0	0	0	0	14	19
350	G	0	0	15	15	0	4	0	0	0	0	15	19
351	V	5	2	9	8	2	4	3	2	1	3	10	19
352	C	0	0	15	15	0	4	0	0	0	0	15	19
353	I	2	2	11	11	2	4	0	0	0	0	13	19
354	R	0	0	15	15	0	4	0	0	0	0	15	19
355	K	0	0	15	15	0	4	0	0	0	0	15	19
356	N	4	4	9	9	2	4	0	0	0	0	13	19
357	W	5	4	8	8	2	4	1	1	0	1	12	19
358	N	4	3	8	8	2	5	1	1	0	1	11	19
359	S	7	7	6	6	2	4	0	0	0	0	13	19
360	S	1	1	13	13	1	4	0	0	0	0	14	19
361	D	1	0	12	12	2	4	1	1	0	1	12	19
362	Y	0	0	15	15	0	4	0	0	0	0	15	19
363	L	0	0	15	15	0	4	0	0	0	0	15	19
364	H	0	0	15	15	0	4	0	0	0	0	15	19
365	L	0	0	14	14	2	3	0	0	0	0	14	19
366	N	0	0	12	12	3	4	0	0	0	0	12	19
367	P	6	6	7	7	2	4	0	0	0	0	13	19
368	D	11	11	3	3	1	4	0	0	0	0	14	19
369	N	3	3	10	10	2	4	0	0	0	0	13	19
370	F	0	0	14	14	1	4	0	0	0	0	14	19
371	A	11	10	2	2	2	4	1	1	0	1	12	19
372	I	0	0	15	15	0	4	0	0	0	0	15	19
373	Q	9	9	3	3	4	3	0	0	0	0	12	19
374	L	12	11	2	2	1	4	1	1	0	1	13	19
375	E	10	9	5	5	0	4	1	1	0	1	14	19
376	K	11	10	3	3	1	4	1	1	0	1	13	19
377	G	8	8	4	4	3	4	0	0	0	0	12	19
378	G	3	3	10	10	2	4	0	0	0	0	13	19
379	K	5	5	8	8	2	4	0	0	0	0	13	19
380	F	7	5	7	7	1	4	2	2	0	2	12	19
381	T	8	7	5	5	2	4	1	1	0	1	12	19
382	V	0	0	14	14	1	4	0	0	0	0	14	19
383	R	11	11	2	2	2	4	0	0	0	0	13	19
384	G	0	0	6	6	9	4	0	0	0	0	6	19
385	K	9	9	6	6	0	4	0	0	0	0	15	19
386	P	0	0	15	15	0	4	0	0	0	0	15	19
387	T	1	1	12	12	2	4	0	0	0	0	13	19
388	L	9	9	5	5	1	4	0	0	0	0	14	19
389	D	12	12	2	2	1	4	0	0	0	0	14	19
390	D	0	0	15	15	0	4	0	0	0	0	15	19
391	L	1	1	14	14	0	4	0	0	0	0	15	19
392	E	13	12	2	2	0	4	1	1	0	1	14	19
393	Q	11	11	2	2	2	4	0	0	0	0	13	19
394	F	2	2	13	13	0	4	0	0	0	0	15	19
395	S	7	6	4	4	4	4	1	1	0	1	10	19
396	E	8	7	6	6	1	4	1	1	0	1	13	19
397	K	0	0	12	12	3	4	0	0	0	0	12	19
398	F	1	0	15	14	0	4	1	0	1	1	14	19
399	Y	12	12	2	2	1	4	0	0	0	0	14	19
400	C	0	0	15	15	0	4	0	0	0	0	15	19
401	S	5	3	7	7	3	4	2	2	0	2	10	19
402	C	0	0	14	14	1	4	0	0	0	0	14	19
403	Y	1	1	13	13	1	4	0	0	0	0	14	19
404	S	3	2	12	12	0	4	1	1	0	1	14	19
405	T	11	10	3	3	1	4	1	1	0	1	13	19
406	L	12	12	2	2	1	4	0	0	0	0	14	19
407	S	14	14	0	0	1	4	0	0	0	0	14	19
408	C	0	0	14	14	1	4	0	0	0	0	14	19
409	K	12	12	0	0	3	4	0	0	0	0	12	19
410	E	11	11	1	1	3	4	0	0	0	0	12	19
411	K	8	7	4	4	3	4	1	1	0	1	11	19
412	A	13	11	2	1	0	5	2	1	1	2	12	19
413	D	9	4	5	3	3	4	4	3	2	5	7	19
414	V	4	2	9	8	3	4	2	1	1	2	10	19
415	K	5	3	4	4	5	5	2	2	0	2	7	19
416	D	12	12	2	2	1	4	0	0	0	0	14	19
417	T	1	1	11	11	3	4	0	0	0	0	12	19
418	D	14	14	0	0	1	4	0	0	0	0	14	19
419	A	12	12	2	2	1	4	0	0	0	0	14	19
420	V	2	2	13	13	0	4	0	0	0	0	15	19
421	D	14	13	0	0	1	4	1	1	0	1	13	19
422	V	2	2	11	11	4	2	0	0	0	0	13	19
423	C	0	0	15	15	0	4	0	0	0	0	15	19
424	I	0	0	11	11	4	4	0	0	0	0	11	19
425	A	8	7	5	4	3	4	1	0	1	1	11	19
426	D	8	6	4	4	3	4	2	2	0	2	10	19
427	G	6	3	8	8	1	4	3	3	0	3	11	19
428	V	4	4	10	10	1	4	0	0	0	0	14	19
429	C	0	0	11	11	4	4	0	0	0	0	11	19
430	I	0	0	9	9	6	4	0	0	0	0	9	19
431	D	14	14	1	1	0	4	0	0	0	0	15	19
432	A	6	5	8	8	1	4	1	1	0	1	13	19
433	F	15	15	0	0	0	4	0	0	0	0	15	19
434	L	5	5	6	6	3	5	0	0	0	0	11	19
435	K	10	10	0	0	5	4	0	0	0	0	10	19

Position #	Wild Type (WT)	Count Active	Count Active (>40%, no errors)	Count Inactive	Count Inactive (no errors)	Count Unclassified	Count Not Made	Count Mutants 20-40% Activity	Count Mutants 20-40% Activity (no errors)	Count Errors	Count Mutants 20-40% Activity + Errors	Tested (w/o errors)	Total
436	P	15	14	0	0	0	4	1	1	0	1	14	19
437	P	12	12	1	1	2	4	0	0	0	0	13	19
438	M	14	14	1	1	0	4	0	0	0	0	15	19
439	E	13	13	2	2	0	4	0	0	0	0	15	19
440	T	14	14	1	1	0	4	0	0	0	0	15	19
441	E	13	13	1	1	1	4	0	0	0	0	14	19
442	E	12	11	3	3	0	4	1	1	0	1	14	19
443	P	13	13	1	1	1	4	0	0	0	0	14	19
444	Q	13	12	0	0	2	4	1	1	0	1	12	19
445	I	14	14	0	0	4	1	0	0	0	0	14	19
446	F	15	15	0	0	0	4	0	0	0	0	15	19
447	Y	14	14	0	0	1	4	0	0	0	0	14	19

Mutants >40% Activity	Inactive	Unclassified	Not Made	Mutants 20-40% Activity + Errors	Errors	Total
2357	3324	895	1741	176	50	8493



Mutants >40% Activity	Mutants 20-40% Activity + Errors	Inactive	Unclassified
2357	176	3324	895



Appendix B-2

$PH20_{1-447}$ Physiochemical and Activity Data

Source(s): EX1026 ('731 App.), at Tables 9 and 10

Position	Wildtype (WT)	G	A	P	V	L	I	M	C	T	S	N	Q	D	E	H	K	R	Y	F	W
1	L	0.62	0.95	0.92	1.00				0.89	0.99	0.74	0.87	3.27		0.55	1.90	1.39	0.72		0.41	0.88
2	N	0.44	0.61	0.54	0.65	0.46			0.40	1.05	0.78		0.84								
3	F					0.59									0.42	0.68			0.50		
4	R		0.73		1.09		0.54			0.66	0.60										
5	A															0.44					
6	P		0.78			0.76						0.40	0.89			0.58	0.80	0.56			
7	P							0.57													
8	V					0.53	1.17	0.47													
9	I				0.84	1.08					0.98		0.40				0.69	0.53			
10	P	0.55									0.55	0.55	0.89	0.62	0.66	0.43		0.73			0.59
11	N	0.45												0.54		0.69	0.58				
12	V		0.56			0.44	0.68			1.50	0.75	0.46			1.86		0.65	0.50			
13	P									0.90	0.68					0.46			0.51		
14	F				0.46		0.42	0.47						0.64							
15	L		0.65		2.20			0.45													
16	W																				
17	A																				
18	W																				
19	N																				
20	A										0.50										
21	P																				
22	S							0.49		0.48						0.57			0.45		
23	E													0.97							
24	F	0.75	0.69		1.15	0.62	0.70	0.85		1.18		0.60			3.99	2.07	0.96	1.22	0.90		
25	C																				
26	L	0.81	1.30	0.55	0.52		0.51	1.43		0.87	0.78		1.44		3.22	0.97	1.88	1.43	0.52		0.53
27	G		0.79	0.46		0.76	0.41			0.61	0.94		1.12	1.22	1.18	1.11	2.71	1.88		0.61	0.76
28	K		0.78	0.40	0.76	0.51	0.55	0.67		0.68	0.46	0.58		0.62	0.54			0.71		0.75	0.51
29	F	1.05	0.90	3.79	1.65	2.36	1.53	2.08		0.85	2.21				4.03	0.82	1.34	1.24			0.48
30	D	2.02	1.12	1.19	0.46	1.32		1.85		0.57	1.62		0.84			1.69	2.63	1.82		0.84	0.62
31	E	1.27	2.05	1.51	4.57	2.62	3.89		2.95	3.96	1.70					2.74	3.13	2.27	1.13		1.26
32	P	1.60	0.92		0.81	0.82		0.67	0.40	0.77	1.01	0.70	1.11			2.08	1.04	1.17	1.01	2.71	0.54
33	L	0.57		0.87				0.69		0.45	0.48		0.45					0.61			1.58
34	D												0.59		0.58	0.41	0.54	1.17			0.46
35	M					0.52				0.83						0.60			0.78	0.87	
36	S	0.64	0.45			0.71				0.51						0.54	0.83	1.09			
37	L			0.63	0.57		0.62	1.46									0.43	0.51		3.33	
38	F																		1.29		
39	S		1.06			0.80				1.57		2.32	1.10					0.56	0.56		
40	F					0.92															1.11
41	I	0.76	0.67		0.73				0.53	1.47		0.40		0.78	0.51	0.77					0.66
42	G		0.64																		
43	S									0.43											
44	P														0.59						
45	R						0.45										0.53				
46	I		1.04		1.01	1.08		1.00		0.55	0.64	0.66			0.43	0.82		2.29	0.76	0.73	
47	N	0.82	0.48					0.77		1.49	0.85		0.69	0.82		1.16	0.67	0.84	0.45	1.32	0.63
48	A	0.83			0.60		0.64	0.76			1.06	4.25	1.05			1.99	1.28	0.66	0.81	2.51	
49	T				0.45		0.42				0.92						0.85	1.41			
50	G		0.93			0.43		0.47	0.41		1.24		0.86	1.37	0.78	0.74		0.86	0.58		
51	Q										0.46	0.60									
52	G			0.43						0.49	1.32	0.89	3.71					0.53			
53	V																				
54	T		0.43		0.66						0.70	0.48	0.91							0.56	
55	I																				
56	F																				
57	Y																				
58	V	0.54		0.90		1.54	0.57		0.55		0.83	0.49	4.54			1.09	4.08	1.92	1.07		0.65
59	D											1.27	0.40								
60	R																0.69				
61	L				0.59		0.42	0.73													
62	G																				
63	Y		0.63		0.43	1.33	1.03	1.32		1.07	1.00	0.96				1.07	1.36	1.40			1.53
64	Y																				
65	P																	0.57			
66	Y															0.47		0.51			
67	I				1.80	0.45													0.55	1.00	
68	D									0.75	0.81		1.67		0.72	2.06	1.08	0.70			
69	S	6.06	22.06	8.14		3.44	3.12	2.67	1.97						1.48			14.06	2.71	8.75	2.18
70	I	6.22	27.00	3.03	6.34	3.05			2.57	5.43	3.63	6.19				9.09	14.64	13.95	1.26	5.69	
71	T	1.41	0.86			1.09		0.89			1.54	1.21	0.68	0.50		0.93		2.17			
72	G		0.45			0.43		3.11			0.51		2.33	0.60	0.69		1.39	0.65			
73	V	1.17	1.38			1.59		0.68	0.84	1.34	0.86		0.96	0.94		1.54	1.42	0.72			1.91
74	T	2.75	2.28	2.45	2.27	1.43		0.52	2.18		1.80	2.12			1.38	1.40	1.29	2.22		1.43	2.13
75	V		0.71			5.22		1.16	0.46	4.34	0.76	0.81	1.51			0.62		3.02	0.63	2.00	
76	N																				
77	G																				
78	G																				

Position	Wildtype (WT)	G	A	P	V	L	I	M	C	T	S	N	Q	D	E	H	K	R	Y	F	W
79	I				1.01	1.44				0.79											
80	P																				
81	Q																				
82	K	0.64	0.94		0.57	0.87	1.01	0.58		0.56	0.62	0.96	0.76		0.50	0.44		0.85			
83	I	1.05			0.99	0.93				0.95	0.79	0.82	1.07			0.40		0.45		0.57	
84	S	8.68		0.57		0.92	0.90	0.77		0.82		0.89	0.86	0.98	0.52	0.96		1.89		0.72	0.86
85	L				0.42																
86	Q	1.02	2.70		0.97	0.92	0.65	1.06		0.58	0.85	1.28		0.88	1.18	1.70	0.97	0.93		0.54	1.21
87	D	1.00	1.00		0.66	0.55	0.53	0.58	1.77	1.70	0.99		1.05		0.86	0.72		1.28	2.72		
88	H																				
89	L							0.63	1.46								0.45				
90	D		1.48			1.15	1.10			1.02	1.15	1.18	1.11		1.15	1.24	1.36	1.49			0.81
91	K		0.89										0.43					0.67			
92	A				1.09	1.29		0.86	1.97	0.70											
93	K	0.97				1.53	3.25	0.70		3.93	1.25	0.71	0.84	0.71	0.83	0.61		1.52		0.50	
94	K		0.64			0.52		0.66		1.14	0.94	0.99	1.22	0.93	0.79	0.72		3.94		0.59	
95	D																				
96	I				0.68	0.46								0.69							
97	T	4.84	1.25	0.62		1.22	0.85		0.53		1.21	1.10	1.17	1.31	1.19			0.95	0.74	0.75	0.53
98	F		0.60		0.46	0.58	0.52	0.87	0.58		0.56		0.65	0.47	0.44	1.06		0.72			0.81
99	Y										0.43							0.53			
100	M																				
101	P																				
102	V	0.67	0.83			0.71		0.77	0.69	1.26	1.41	1.02	1.03		0.90	0.88	1.03	0.94			0.76
103	D																				
104	N	0.48	0.69					0.61	0.41	0.71	1.03						0.88	1.25			
105	L	0.51	0.54	0.84	0.99		0.94			0.51	0.61		0.90					0.65			
106	G				0.43																
107	M					1.32	0.67													0.91	
108	A	0.47																			
109	V																				
110	I				0.51																
111	D																				
112	W																				
113	E																				
114	E	0.73	1.44					0.44			0.69					0.75					
115	W																				
116	R																				
117	P													0.56							
118	T				0.79	1.09		0.53				0.67	3.37			0.47	0.53				
119	W												0.72						1.08	0.53	
120	A	1.03		0.42	1.53	1.25	1.33			0.62	1.21	0.81		0.76		1.11		0.82	1.95	2.62	0.59
121	R																				
122	N							0.56													
123	W																				
124	K																	0.62			
125	P										0.54					0.43		0.63			
126	K																				
127	D	0.97	0.89		0.56	0.84		0.40		1.11	0.77	1.69	1.21		1.31	2.33		0.51			0.44
128	V	0.49	0.53			0.95	1.25		0.68		0.53		0.55				1.16	0.74			0.50
129	Y																				
130	K						0.50											1.42			
131	N	2.47			2.08	0.82	1.40	0.99	0.60	1.02	0.76		1.24		0.44	0.80		2.81	0.85	0.63	
132	R		0.68		0.73	0.76	0.56		0.58	0.61	0.79	1.28	0.69		0.70	0.66	1.05		0.78	0.60	
133	S						0.54														
134	I				1.08	1.04				0.60											
135	E	2.79	0.99			0.82			0.77		1.13	0.56	1.59	2.68		0.79	1.15	2.08	0.50	0.73	0.63
136	L		0.73				0.65	1.05	0.56	0.72	0.80	0.48	0.61	0.47		1.00		0.74		0.96	1.11
137	V		0.48				1.01			0.51											
138	Q		0.69			0.59	0.54	0.68	0.65		0.48	0.61				0.71		0.53	0.60		0.41
139	Q	0.65	0.92		0.77	0.70		0.95	0.44	1.31	0.81			0.48	0.94	0.56	0.73	0.79		0.53	
140	Q	0.73	0.96		0.61	0.51	0.75	0.80	0.50					0.59		0.84	0.93	0.85	0.41	0.66	0.59
141	N	1.15	1.12		0.50	0.61		0.48		0.45	0.72		1.16	1.09	0.67	2.03		1.40	1.55	0.81	0.83
142	V	0.98		0.88		0.75	0.81	0.76	0.61	1.19	0.93	0.98	1.04	0.71	0.87	1.11	1.40	1.53			
143	Q	0.62			0.57	0.56	0.44					0.73			0.77		1.30				
144	L									1.02											0.79
145	S	0.94	0.58	1.04		0.44		0.56	0.44			0.58		0.48	0.56	0.56		0.97			
146	L	0.62	0.52	0.93	0.84		0.82		0.42	0.74	0.71	0.57	0.84		0.50	0.78	0.84	1.47	0.80		
147	T	1.05	1.20	1.09	2.04	1.30	0.85	0.79	0.47		1.27		1.29	0.71				2.11	1.04	1.24	0.97
148	E	1.05			0.99	0.85	0.73		0.66	0.82	1.15		1.44			1.24	1.63	0.97	0.95	0.42	0.43
149	A	0.52			1.34	0.88		0.88	1.15	1.24	1.08		1.15				0.51	1.02			
150	T	2.19	1.21	0.88		0.70	0.52		0.70		0.92	0.91		1.24	1.05			0.90	1.36	0.71	1.25
151	E	1.06	1.27		1.38	1.03		1.26	1.00	1.21	1.28	0.95	2.01			1.34	2.05	1.61	1.31		1.31
152	K		0.51		0.82		0.65	0.75	0.52	1.20								1.85	0.67	0.61	
153	A					0.51	0.93														
154	K				0.46					0.83								0.86			
155	Q	1.61	0.91		0.73	0.86		0.97	0.60	0.76	0.77			1.49		1.03	1.57	1.27		0.70	0.91
156	E	0.49	0.79		0.45	0.43	0.51	0.87		0.69	0.62		0.84	1.95				0.43			0.49

Position	Wildtype (WT)	G	A	P	V	L	I	M	C	T	S	N	Q	D	E	H	K	R	Y	F	W
157	F																				0.61
158	E		0.56			0.44					0.95		1.25			0.54				0.51	
159	K		0.64		0.41	0.62		0.66			0.67	0.73	0.92	0.52	0.49	0.74		0.88			
160	A	0.75			0.73	0.67	0.43	0.77	0.61		1.35	0.56	0.65			0.47	0.91	0.89	1.07	0.79	
161	G		0.99		0.42				0.44		0.77			0.86	0.49			0.48			
162	K	0.56	0.50	0.64	0.52	0.54		1.04			0.47		0.58	0.77	0.51	0.62		0.52	0.72		1.01
163	D	1.15	1.52		0.76	1.18				1.13	1.34		1.40		1.63		1.90	1.80			
164	F				1.23	1.13		1.66													0.72
165	L		0.48		1.22						1.31	2.19		5.79				0.59	0.66	1.23	1.14
166	V	1.11	2.85			4.38			1.16	4.26		0.47	3.61		1.28	1.74		5.56	2.08	1.67	1.26
167	E	0.60	0.84	0.58				0.87		0.59	1.17	0.83		0.69		0.89	0.91	1.02	0.55		
168	T															0.46					
169	I				0.74	2.08												0.54			
170	K		0.40		0.58							0.72	0.40					2.58			
171	L				0.64		0.73														
172	G		1.20						1.03												
173	K											0.44						0.82			
174	L	0.40	1.20		0.62			0.79		1.12	0.85	1.36	0.99				2.39	1.50	1.06		0.78
175	L				0.94					1.43					0.43	0.57			0.66		
176	R					0.40											0.67				
177	P																				
178	N	0.85						0.88									0.85	1.10			
179	H	0.86	1.06	0.44	0.42	0.73	0.90	0.63	0.94	0.43	0.51	0.96			0.62		1.39	0.96			
180	L	0.62						0.64									0.44			0.59	
181	W							0.88					0.88								
182	G					0.90															
183	Y					0.70															
184	Y																				
185	L																				
186	F																		0.59		
187	P																				
188	D																				
189	C																				
190	Y																				
191	N																				
192	H									0.50	0.49										
193	H	0.68									0.42		0.82						0.58		
194	Y																				
195	K	0.45	0.51			0.45	0.50			0.58		0.74	0.71			0.45		0.85			0.49
196	K	0.41				0.65				1.18	0.68				0.43			0.58			0.55
197	P	0.75	0.81			0.56		1.03		0.41	0.70		0.69	0.58	0.52	0.62	0.99	0.58		0.48	
198	G		0.80			0.48				0.41	0.76	0.80	0.55	1.99	0.49	0.84		0.58	0.81		
199	Y																				
200	N													0.46							
201	G																				
202	S							0.40													
203	C																				
204	F			0.63																	
205	N	0.79	1.30	0.75	0.49			0.58		0.85	0.80			0.85	1.94		0.76	0.54	0.40	0.52	0.41
206	V					1.57	0.94	0.43		0.59	0.72					0.50	1.75	1.30			
207	E																				
208	I		0.62		1.07	0.84		0.88	0.48	1.01	0.62		0.77				0.91	1.14			
209	K	0.44	0.53							0.50	0.50	0.50			0.46			0.68		0.40	
210	R																				
211	N					0.41															
212	D									0.76	0.93	1.52									
213	D	0.81	0.85		0.47	0.56		1.56				1.53	1.04		0.79	0.75	0.82	0.92	0.49		0.49
214	L												0.57								
215	S	0.88	0.74		0.69	0.60		1.77		0.80			0.79	0.62	0.74	0.91	0.99	0.71			0.52
216	W																		0.48		
217	L							0.51													
218	W																			0.57	
219	N		1.29			0.72	0.60	1.02	0.43	0.82	2.48			0.75	0.95	0.97	1.45	1.10			0.48
220	E		0.75		1.35	1.45	1.34			0.91	0.62					1.40					
221	S		0.72		1.04			0.46	0.59	0.94			1.37								
222	T	0.49			0.79	0.64	0.40				0.63	0.80		0.43			0.75	0.75		0.43	
223	A																				
224	L						0.61														
225	Y																				
226	P																				
227	S																				
228	I																				
229	Y																				
230	L						0.87														
231	N									1.10											
232	T										0.76									0.73	
233	Q	0.46	0.71			0.69											1.69	1.50	0.50	0.53	
234	Q					0.40		1.65													

Position	Wildtype (WT)	G	A	P	V	L	I	M	C	T	S	N	Q	D	E	H	K	R	Y	F	W
235	S	0.95	0.47							0.66					1.00	0.44	0.53				
236	P	1.09	1.07								0.91					0.46	0.71	3.09			
237	V		0.90			1.12				1.01	1.03	0.67	1.46		1.93	0.75		0.71		0.41	0.52
238	A									0.44	2.62		1.02	0.75	0.59	0.60	0.60	0.49			
239	A																				
240	T		0.48	0.56	0.77			0.48			0.74		0.75				1.13	0.91			
241	L																				
242	Y																			1.08	
243	V																				
244	R																				
245	N															0.50					
246	R																				
247	V					0.83	2.01	0.52													
248	R		0.43													0.40			0.67		0.52
249	E																				
250	A																				
251	I					0.58		0.43													
252	R																				
253	V						0.76														
254	S																				
255	K		0.40								0.43	0.52	0.91					0.71			
256	I		0.42		0.51	0.64										0.51					
257	P	0.51	0.82		0.88	0.69	1.07	0.90		2.04		0.69	0.61				0.92	1.38			
258	D										1.44	1.44				0.84		0.45			
259	A	0.68		1.54	0.41	0.53	0.46			0.51	0.63	0.49	0.70		0.85		0.76	0.72	0.51		0.55
260	K		0.66			0.60		0.85			0.66		0.58	0.41	0.58	0.87		0.83	1.73		
261	S		0.74		0.48			0.56		0.66		1.98	0.76				2.54	1.19		0.73	0.44
262	P																				
263	L		0.76		0.75			0.89		0.49							2.73	1.63			
264	P		0.43													0.60					
265	V						0.58														
266	F																		0.58		
267	A							0.45		1.34											
268	Y																				
269	T		1.63						0.75		1.01			0.76							
270	R							0.46		0.40	0.69	0.52									
271	I	1.29			1.05	10.62		3.24			0.42									0.72	
272	V									1.61	0.96			1.36				0.74			
273	F									0.48						1.41			0.90		
274	T		0.51								0.62									1.28	
275	D																				
276	Q					0.48	0.51	1.14	0.88		1.63			1.69	1.05	1.20		1.30	1.94		
277	V	1.18	0.65					0.94	0.41	1.94	0.83	1.15	0.82	0.79	1.02	1.09	1.51	1.63	0.66		
278	L	1.33	1.13		0.44		0.93			1.66	1.67	1.74			1.03	4.50	1.75	5.87	1.51	1.26	
279	K									0.86			0.84			0.44		1.10			
280	F	0.47											0.43								
281	L																				
282	S	0.54						2.64					0.41	0.41							
283	Q			1.18						0.65	1.73				0.63			0.59			
284	D	0.60	0.58			0.50		0.56			0.99	0.40	0.95		1.21	0.51					
285	E	0.52						0.43				0.40	0.59			1.30			0.99	0.47	
286	L										0.46							0.53			
287	V						0.51			0.50											
288	Y					0.79															0.49
289	T																0.75				
290	F						0.41	1.03													
291	G				1.63						0.45		0.80					0.45			
292	E	0.41	0.66	1.05	1.28				0.71			0.99				1.26	1.27	0.42		0.90	0.83
293	T	1.00	1.90	1.64	2.15	1.00		2.29	1.67		0.89		1.83	1.46			1.35		1.49	1.94	
294	V							0.41													
295	A																				
296	L																				
297	G		0.57																		
298	A	0.43					0.41														
299	S																				
300	G																	0.42			
301	I		0.88		0.88																
302	V						0.45														0.46
303	I				0.47																
304	W																				
305	G													1.00	1.62						
306	T										1.02			0.76	0.52						
307	L				0.48					1.08	0.86	0.76	0.61				0.43		0.60		0.64
308	S	1.73		0.65						0.72		2.33		0.92		1.15	1.33	1.34			
309	I	1.44			0.60	1.72		1.47		2.09	1.16	3.11	1.64	0.72	1.99	1.30	0.98	2.27			0.88
310	M	2.73	1.50		0.70						1.61		0.59					1.61			
311	R									0.52	0.84		0.43			0.48	0.72				
312	S	0.49								0.75		1.26									

Position	Wildtype (WT)	G	A	P	V	L	I	M	C	T	S	N	Q	D	E	H	K	R	Y	F	W
313	M	0.56	1.34	1.11	0.99	1.05				0.67	0.88				0.63	1.23	2.85	2.30	1.12		
314	K		0.82				0.54			0.61	0.61	0.57	0.62	0.53		1.10		0.95	0.45		
315	S	0.72	0.85			0.42		0.63		0.97					0.41	2.04	0.62	1.04	0.50		
316	C													0.41							
317	L		1.27				1.76	1.20		0.93	1.03	0.73	1.67	0.61		1.05	5.11	2.41			0.84
318	L	0.49					0.70	1.68		0.63	0.71	0.52	0.71	0.46		0.45	1.36	1.34		0.51	
319	L																				
320	D	0.83					1.00	0.79			1.19	0.52			0.78	1.75	6.42	3.19	0.86		0.40
321	N		1.01							0.64	1.26			1.25		0.92	1.29	1.23	0.40		
322	Y																				
323	M					0.55	0.55													0.64	
324	E		0.59					0.50			0.62	1.01		1.15		0.79		2.28			
325	T	4.21	1.87		1.24			2.11			3.19	4.64	5.08	1.78	4.03	3.45	4.37				0.62
326	I				6.29	1.50					0.95						0.95		0.77		
327	L							0.52													
328	N	0.56	0.67		1.16	2.01	1.85		1.25	0.59	2.22		1.13			0.88	2.12	0.68	1.66		
329	P																				
330	Y																				
331	I				0.94																
332	I																				
333	N																				
334	V			0.46																	
335	T										0.47										
336	L																				
337	A																				
338	A												0.63								
339	K							0.61													
340	M																				
341	C																				
342	S		0.68																		
343	Q				0.51					0.49											
344	V																				
345	L																				
346	C																				
347	Q	2.68	0.78					0.61			2.38				0.78			0.55			
348	E	0.55									0.44			0.67							
349	Q		0.47					0.70		0.49		0.44			0.83		0.93	0.73			
350	G																				
351	V		1.14								0.92										
352	C																				
353	I				1.61					0.42											
354	R																				
355	K																				
356	N		0.41								0.46			0.79		0.82					
357	W		0.80						0.67	0.62	0.41										
358	N	0.41							0.66	0.58											
359	S				0.65			0.63		2.11				0.45	1.05	0.44	0.66				
360	S									0.50											
361	D																				
362	Y																				
363	L																				
364	H																				
365	L																				
366	N																				
367	P	0.47	0.55						0.83		0.52						0.57	0.46			
368	D	0.49	1.34		0.41	0.64		0.78		0.80	0.93				1.28	0.96	1.31	1.31			
369	N										0.54					1.33		0.55			
370	F																				
371	A				0.94	0.57	0.50	0.57			1.45				1.05	1.20	1.76	1.51		0.52	
372	I																				
373	Q		0.65		1.05	0.84		1.43			0.87				0.81		0.73	0.68		0.62	
374	L		0.60	0.43	0.56		0.80	1.11		0.47	0.58	0.43				1.42		0.83	0.66		
375	E	0.90	0.42			0.46		0.54		1.17	0.77	0.81					1.49	0.43			
376	K		0.95		0.58			0.46		0.53	0.80		0.69	0.78	0.88			0.67	0.42		
377	G			2.30						3.83	1.80			1.35	0.59	1.49	1.50	1.28			
378	G											0.64					1.22	1.03			
379	K	0.52								0.40	0.46					0.57		0.74			
380	F			0.47		0.67	0.56												1.50		2.15
381	T				0.89						0.87	0.51	0.84			0.48	1.06	0.87			
382	V																				
383	R		0.61		1.05	1.31	0.71	0.61		0.98	0.87	0.77			0.51	0.71	1.30				
384	G																				
385	K	0.62	1.12		0.43					0.46	1.05	0.41	0.73			0.50		0.94			
386	P																				
387	T										0.93										
388	L				0.78		0.98	0.79		0.51						0.47		0.60	1.18	0.92	0.77
389	E	0.91	1.14	0.75		0.65		0.60		0.70	1.08		0.69			1.17	1.91	0.94	0.77		
390	D																				

Position	Wildtype (WT)	G	A	P	V	L	I	M	C	T	S	N	Q	D	E	H	K	R	Y	F	W
391	L								0.90												
392	E	1.00	0.58		1.27	0.80		1.54		0.72	0.52		1.01				0.66	0.66	0.92	0.54	
393	Q		1.26			0.91		0.80		0.41	1.15	0.72		0.45		1.05	0.80	0.74		1.23	
394	F					0.56															0.41
395	S	0.77	1.10													0.56	0.96	1.98			0.40
396	E		0.52							0.89	0.61		0.73	0.64		0.47		0.61			
397	K																				
398	F																				
399	Y		1.01		1.44			2.70	0.46	2.40	1.01	0.52	1.18		1.49		1.94	1.20			1.92
400	C																				
401	S		0.82									0.42			0.46						
402	C																				
403	Y																			0.62	
404	S		0.63	0.64																	
405	T	2.32	0.56	0.64				0.48			0.94		0.75				0.74	0.60	0.44		0.73
406	L	1.00	0.70		0.87		0.61		0.98	0.83	0.47	0.76	0.93		0.73				0.74	1.42	
407	S	0.75	1.16	0.94	0.56	0.40		0.74				0.72	1.71	1.52	1.38	1.34		1.04		1.42	0.41
408	C																				
409	K	0.50	2.18	0.48	0.48		0.51			0.63	0.72		3.33	0.65	0.62	0.64		0.84			
410	E			0.73	0.65			0.42		1.54	0.81	0.67	0.85	0.47			0.70	0.61	0.62		
411	K		0.48	0.42	0.99					0.63	1.21	1.02						0.97			
412	A	0.80		0.77	0.53	0.65	0.81				0.86	0.86		0.74				0.66	0.66		0.54
413	D									0.41		0.94			0.52			0.50			
414	V						1.12	0.53													
415	K	0.40									0.42										0.42
416	D	0.67			0.59	0.75	0.63			0.85		0.73	0.83			0.57	0.76	0.46	0.40	0.41	
417	T						1.22														
418	D	0.45	0.92	2.11	1.43	1.28	0.99	1.09			0.78	0.91	1.05		1.31			1.18	0.97	0.81	
419	A	0.42				0.56	1.64				2.65	0.53			0.45	1.21	1.88	1.81	1.44	2.17	0.69
420	V			0.48			1.04														
421	D	0.62	1.28			0.73		0.94		0.80	2.12	1.89	1.54		0.81	1.98	2.42	2.21	0.66		
422	V						0.42			0.49											
423	C																				
424	I																				
425	A	1.20					0.44	0.70			0.47	0.46					1.75	0.49			
426	D	0.85		1.03								0.61	0.42		0.62				0.43		
427	G						0.54				0.42						0.52				
428	V			0.82		1.25		0.42		0.62											
429	C																				
430	I																				
431	D	0.55	2.42		1.52	0.62	1.05				1.91	1.30	2.16		1.27	3.13	1.83	2.20	0.85		0.56
432	A	0.52			0.56						0.61	0.51			0.60						
433	F	0.54	0.97	0.95	1.63	1.87	1.06		0.69	1.86	0.86			0.95	0.82	0.83	1.36	1.63			1.28
434	L	0.47			0.46		0.89	0.60												0.41	
435	K	0.64	1.08		0.44				0.53	0.73	1.03				0.78	1.05		1.01	0.50		
436	P	1.19				0.63	0.84	0.61		0.59	0.92		0.86	1.19	0.74	0.72	2.05	1.00	0.49		0.43
437	P	0.50	0.56			0.51	2.46	2.55			0.57		0.96	0.62		1.11	0.83	0.85	0.42		
438	M	0.83	0.75	0.81	0.85	0.86			0.63	3.99	0.83	1.08	0.85	0.87	0.72			0.99			0.57
439	E	1.22	1.20	1.16	1.57	0.88			0.58	1.15	1.02		1.32			0.74	1.20			1.00	0.62
440	T	0.86	1.22	0.88	1.02	0.97	1.04	1.08			1.17			1.03	1.00	3.00		1.77	1.11	0.85	
441	E	0.87	1.47		0.54	0.82				0.66	0.79	0.82	0.81	0.67		0.65	0.80		0.51	3.91	
442	E	0.51		0.91	0.65	0.40			1.38	0.61			0.74			0.76	0.73	3.94	0.60		
443	P	1.12	1.63			1.19		1.99		0.87	0.99	1.25	0.96		1.07	1.08		1.04		0.70	0.64
444	Q	0.93			1.12		0.58					1.01		0.97	1.19	0.97	1.03	0.85	0.67	0.66	0.64
445	I	0.98	0.97	1.67	1.25	1.06		1.57		1.38	1.21	1.24	1.26			1.35		1.08	0.53		0.69
446	F	1.12	1.58		0.91	0.93	1.06	1.31	0.75	0.89			0.72	1.18	1.10	1.28	0.94	0.89			1.40
447	Y	0.92		1.46	1.38	1.09	1.36	0.90		1.90		1.58	2.37	3.25	1.36			1.12		1.41	1.07

Subset of Surface Residues	Wildtype (WT)	Local QMEAN Score from
1	L	
2	N	
3	F	0.37
4	R	0.47
5	A	0.50
6	P	0.63
7	P	0.68
8	V	0.67
9	I	0.71
10	P	0.75
11	N	0.57
12	V	0.68
13	P	0.67
14	F	0.73
15	L	0.76
16	W	0.75
17	A	0.81
18	W	0.70
19	N	0.70
20	A	0.67
21	P	0.62
22	S	0.65
23	E	0.61
24	F	0.54
25	C	0.63
26	L	0.64
27	G	0.51
28	K	0.46
29	F	0.53
30	D	0.54
31	E	0.48
32	P	0.52
33	L	0.52
34	D	0.57
35	M	0.58
36	S	0.64
37	L	0.58
38	F	0.66
39	S	0.77
40	F	0.67
41	I	0.70
42	G	0.68
43	S	0.60
44	P	0.50
45	R	0.55
46	I	0.50
47	N	0.48
48	A	0.58
49	T	0.61
50	G	0.62
51	Q	0.58
52	G	0.69
53	V	0.68
54	T	0.68
55	I	0.80
56	F	0.82
57	Y	0.87
58	V	0.84
59	D	0.74
60	R	0.70
61	L	0.84
62	G	0.88
63	Y	0.87
64	Y	0.89
65	P	0.91
66	Y	0.83
67	I	0.85
68	D	0.76
69	S	0.79
70	I	0.71
71	T	0.67
72	G	0.72
73	V	0.76
74	T	0.78
75	V	0.88
76	N	0.86
77	G	0.87
78	G	0.85
79	I	0.84
80	P	0.88
81	Q	0.80
82	K	0.70
83	I	0.75
84	S	0.79
85	L	0.78
86	Q	0.79
87	D	0.81
88	H	0.82
89	L	0.84
90	D	0.84
91	K	0.83
92	A	0.87
93	K	0.77
94	K	0.79
95	D	0.82
96	I	0.76
97	T	0.73
98	F	0.77
99	Y	0.70
100	M	0.65
101	P	0.77
102	V	0.73
103	D	0.79
104	N	0.55
105	L	0.59
106	G	0.72

Note:
Local QMEAN scores (col. Z) were obtained from the Swiss-Model stores in the b-factor column of the pdb file values obtained from "model_01.pdb.cif" file, provided by Halozyme.

Red - QMEAN score < 0.6

Blue - QMEAN score > 0.6

Position	Wildtype (WT)	Small Hydrophobes (7)	Polar Uncharged (5)	Polar Negative Charge (2)	Polar Positive Charge (3)	Large Hydrophobes, Aromatics (3)	Count Active (>40%, no errors)	Tested (w/o errors)
1	L	4	5	1	3	2	15	15
2	N	5	4	0	0	0	9	13
3	F	1	0	1	1	1	4	10
4	R	3	2	0	0	0	5	13
5	A	0	0	0	1	0	1	14
6	P	2	2	0	3	0	7	12
7	P	1	0	0	0	0	1	15
8	V	3	0	0	0	0	3	11
9	I	2	2	0	2	0	6	12
10	P	1	3	2	2	1	9	14
11	N	1	0	1	2	0	4	13
12	V	3	3	1	2	0	9	12
13	P	0	2	0	1	1	4	10
14	F	3	0	1	0	0	4	13
15	L	3	0	0	0	0	3	13
16	W	0	0	0	0	0	0	14
17	A	0	0	0	0	0	0	15
18	W	0	0	0	0	0	0	14
19	N	0	0	0	0	0	0	15
20	A	0	1	0	0	0	1	13
21	P	0	0	0	0	0	0	14
22	S	1	1	0	1	1	4	9
23	E	0	0	1	0	0	1	11
24	F	6	2	1	3	1	13	13
25	C	0	0	0	0	0	0	15
26	L	6	3	1	3	2	15	15
27	G	4	3	2	3	2	14	15
28	K	6	3	2	1	2	14	14
29	F	7	2	1	3	1	14	14
30	D	6	3	x	3	2	14	14
31	E	6	3	x	3	2	14	14
32	P	5	5	0	3	3	16	16
33	L	3	3	0	1	1	8	14
34	D	0	1	1	3	1	6	12
35	M	1	1	0	1	2	5	11
36	S	3	1	0	3	0	7	12
37	L	4	0	0	2	1	7	12
38	F	0	0	0	0	1	1	10
39	S	2	3	0	1	1	7	11
40	F	1	0	0	0	1	2	12
41	I	3	3	2	1	1	10	11
42	G	1	0	0	0	0	1	14
43	S	0	1	0	0	0	1	11
44	P	0	0	1	0	0	1	15
45	R	1	0	0	1	0	2	8
46	I	4	3	1	2	2	12	14
47	N	3	3	1	3	3	13	14
48	A	4	3	0	3	2	12	13
49	T	2	1	0	2	0	5	10
50	G	3	3	2	2	1	11	11
51	Q	0	2	0	0	0	2	10
52	G	1	4	0	1	0	6	11
53	V	0	0	0	0	0	0	15
54	T	2	3	0	0	1	6	12
55	I	0	0	0	0	0	0	11
56	F	0	0	0	0	0	0	14
57	Y	0	0	0	0	0	0	12
58	V	4	4	0	3	2	13	14
59	D	0	2	x	0	0	2	13
60	R	0	0	0	1	0	1	15
61	L	3	0	0	0	0	3	14
62	G	0	0	0	0	0	0	15
63	Y	5	3	0	3	1	12	15
64	Y	0	0	0	0	0	0	17
65	P	0	0	0	1	0	0	13
66	Y	0	0	0	2	0	2	15
67	I	2	0	0	0	2	4	10
68	D	0	3	1	3	0	7	12
69	S	6	1	1	1	3	12	13
70	I	5	4	0	3	2	14	15
71	T	4	3	1	2	0	10	11
72	G	3	2	2	2	0	9	15
73	V	4	4	1	3	1	13	14
74	T	6	3	1	3	2	15	15
75	V	3	5	0	2	2	12	15
76	N	0	0	0	0	0	0	14
77	G	0	0	0	0	0	0	8
78	G	0	0	0	0	0	0	7
79	I	2	1	0	0	0	3	14
80	P	0	0	0	0	0	0	15
81	Q	0	0	0	0	0	0	11
82	K	6	4	1	2	0	13	14
83	I	3	4	0	2	1	10	11
84	S	5	3	2	2	2	14	14
85	L	1	0	0	0	0	1	12
86	Q	6	3	2	3	2	16	17
87	D	6	4	1	2	1	14	14
88	H	0	0	0	0	0	0	15
89	L	1	1	0	1	0	3	11
90	D	3	4	1	3	1	12	13
91	K	1	1	0	1	0	3	12
92	A	3	2	0	0	0	5	13
93	K	4	4	2	2	1	13	13
94	K	3	4	2	2	1	12	14
95	D	0	0	x	0	0	0	15
96	I	2	0	1	0	0	3	12
97	T	5	4	2	1	3	15	15
98	F	5	3	2	2	1	13	14
99	Y	0	1	0	1	0	2	10
100	M	0	0	0	0	0	0	11
101	P	0	0	0	0	0	0	13
102	V	4	5	1	3	1	14	15
103	D	0	0	x	0	0	0	13
104	N	3	3	0	2	0	8	11
105	L	5	3	0	1	0	9	11
106	G	1	0	0	0	0	1	13

Note:
Column AE ("Position") and Column AF ("Wildtype (WT)") are shaded according to the sufficiecy or insufficiency of testing of alternate residues in each physicochemical group: Small Hydrophobes (G, A, P, V, L, I, M), Polar Uncharged (C, T, S, N, Q), Polar Negative Charge (D, E), Polar Positive Charge (H, K, R), Large Hydrophobes, Aromatics (Y, F, W). An 'x' is placed at positions in the Polar Negative class where the other polar negative residue, either D or E, was not tested, yet the WT residue was one of D or E.

Red - insufficient testing of each physicochemical group.

Yellow - 0 members of the physichochemical class were tested at the position reported in the disclosure

Subset of Surface Residues	Wildtype (WT)	Local QMEAN Score from
107	M	0.73
108	A	0.76
109	V	0.84
110	I	0.87
111	D	0.90
112	W	0.88
113	E	0.81
114	E	0.80
115	W	0.82
116	R	0.81
117	P	0.88
118	T	0.84
119	W	0.76
120	A	0.78
121	R	0.77
122	N	0.76
123	W	0.69
124	K	0.77
125	P	0.74
126	K	0.74
127	D	0.82
128	V	0.83
129	Y	0.82
130	K	0.80
131	N	0.82
132	R	0.71
133	S	0.82
134	I	0.84
135	E	0.74
136	L	0.79
137	V	0.84
138	Q	0.81
139	Q	0.79
140	Q	0.81
141	N	0.84
142	V	0.76
143	Q	0.79
144	L	0.81
145	S	0.80
146	L	0.79
147	T	0.78
148	E	0.78
149	A	0.88
150	T	0.81
151	E	0.74
152	K	0.79
153	A	0.86
154	K	0.82
155	Q	0.78
156	E	0.80
157	F	0.84
158	E	0.80
159	K	0.81
160	A	0.88
161	G	0.87
162	K	0.81
163	D	0.84
164	F	0.83
165	L	0.84
166	V	0.86
167	E	0.82
168	T	0.82
169	I	0.81
170	K	0.76
171	L	0.82
172	G	0.79
173	K	0.70
174	L	0.70
175	L	0.67
176	R	0.66
177	P	0.79
178	N	0.74
179	H	0.72
180	L	0.73
181	W	0.82
182	G	0.88
183	Y	0.89
184	Y	0.87
185	L	0.87
186	F	0.84
187	P	0.87
188	D	0.79
189	C	0.75
190	Y	0.79
191	N	0.77
192	H	0.57
193	H	0.67
194	Y	0.67
195	K	0.66
196	K	0.65
197	P	0.73
198	G	0.65
199	Y	0.64
200	N	0.75
201	G	0.83
202	S	0.81
203	C	0.81
204	F	0.71
205	N	0.56
206	V	0.84
207	E	0.83
208	I	0.84
209	K	0.80
210	R	0.76
211	N	0.83
212	D	0.78

Position	Wildtype (WT)	Small Hydrophobes (7)	Polar Uncharged (5)	Polar Negative Charge (2)	Polar Positive Charge (3)	Large Hydrophobes, Aromatics (3)	Count Active (>40%, no errors)	Tested (w/o errors)
107	M	2	0	0	0	1	3	12
108	A	1	0	0	0	0	1	12
109	V	0	0	0	0	0	0	8
110	I	1	0	0	0	0	1	7
111	D	0	0	x	0	0	0	3
112	W	0	0	0	0	0	0	8
113	E	0	0	x	0	0	0	2
114	E	3	1	x	1	0	5	10
115	W	0	0	0	0	0	0	14
116	R	0	0	0	0	0	0	14
117	P	0	0	1	0	0	0	9
118	T	3	2	0	2	0	7	15
119	W	0	1	0	0	2	3	9
120	A	5	3	1	2	3	14	14
121	R	0	0	0	0	0	0	12
122	N	1	0	0	0	0	1	12
123	W	0	0	0	0	0	0	14
124	K	0	0	0	1	0	1	6
125	P	0	1	0	2	0	3	9
126	K	0	0	0	0	0	0	7
127	D	5	4	1	2	1	13	14
128	V	4	3	0	2	1	10	12
129	Y	0	0	0	0	0	0	13
130	K	1	0	0	1	0	2	12
131	N	5	4	1	2	2	14	15
132	R	4	5	1	2	2	14	15
133	S	1	0	0	0	0	1	14
134	I	2	1	0	0	0	3	15
135	E	3	4	1	3	3	14	15
136	L	3	5	1	2	2	13	14
137	V	2	1	0	0	0	3	11
138	Q	4	3	0	2	2	11	12
139	Q	5	3	2	3	1	14	15
140	Q	6	1	1	3	3	14	14
141	N	5	3	2	2	3	15	15
142	V	5	5	2	3	0	15	15
143	Q	4	1	1	1	0	7	12
144	L	0	1	0	0	1	2	12
145	S	5	2	2	2	0	11	13
146	L	5	5	1	3	1	15	15
147	T	7	3	1	1	3	15	15
148	E	4	4	x	3	3	14	14
149	A	4	4	0	2	0	10	12
150	T	5	3	2	1	3	14	15
151	E	5	5	x	3	2	15	15
152	K	4	2	0	1	2	9	10
153	A	2	0	0	0	0	2	9
154	K	1	1	0	1	0	3	10
155	Q	5	3	1	3	2	14	16
156	E	6	3	1	1	1	12	13
157	F	0	0	0	0	1	1	17
158	E	2	2	x	1	1	6	11
159	K	4	3	2	2	0	11	13
160	A	5	4	0	3	2	14	14
161	G	2	2	2	1	0	7	8
162	K	6	2	2	2	2	14	14
163	D	4	3	1	2	0	10	12
164	F	3	0	0	0	1	4	14
165	L	2	2	1	1	3	9	12
166	V	3	4	1	2	3	13	14
167	E	4	3	1	3	1	12	13
168	T	0	0	0	1	0	1	15
169	I	2	0	0	1	0	3	15
170	K	2	2	0	1	0	5	13
171	L	2	0	0	0	0	2	11
172	G	1	1	0	0	0	2	12
173	K	0	1	0	1	0	2	14
174	L	4	4	0	2	2	12	13
175	L	1	1	1	1	1	5	12
176	R	1	0	0	1	0	2	15
177	P	0	0	0	0	0	0	13
178	N	2	0	0	2	0	4	10
179	H	7	4	1	2	0	14	15
180	L	2	0	0	1	1	4	10
181	W	1	1	0	0	0	2	13
182	G	1	0	0	0	0	1	14
183	Y	1	0	0	0	0	1	12
184	Y	0	0	0	0	0	0	14
185	L	0	0	0	0	0	0	14
186	F	0	0	0	0	1	1	15
187	P	0	0	0	0	0	0	15
188	D	0	0	x	0	0	0	15
189	C	0	0	0	0	0	0	15
190	Y	0	0	0	0	0	0	14
191	N	0	0	0	0	0	0	15
192	H	0	2	0	0	0	2	15
193	H	1	2	0	0	1	4	11
194	Y	0	0	0	0	0	0	8
195	K	4	3	0	2	1	10	10
196	K	2	2	1	1	1	7	7
197	P	4	3	2	3	1	13	14
198	G	2	4	2	2	1	11	12
199	Y	0	0	0	0	0	0	10
200	N	0	0	1	0	0	1	14
201	G	0	0	0	0	0	0	11
202	S	1	0	0	0	0	1	14
203	C	0	0	0	0	0	0	13
204	F	1	0	0	0	0	1	12
205	N	5	2	2	2	3	14	14
206	V	3	2	0	3	0	8	14
207	E	0	0	0	0	0	0	11
208	I	4	4	0	2	0	10	14
209	K	2	3	1	1	1	8	10
210	R	0	0	0	0	0	0	14
211	N	1	0	0	0	0	1	13
212	D	0	3	x	0	0	3	13

Subset of Surface Residues	Wildtype (WT)	Local QMEAN Score from
213	D	0.74
214	L	0.72
215	S	0.77
216	W	0.71
217	L	0.76
218	W	0.73
219	N	0.70
220	E	0.74
221	S	0.77
222	T	0.76
223	A	0.82
224	L	0.81
225	Y	0.85
226	P	0.86
227	S	0.85
228	I	0.84
229	Y	0.78
230	L	0.74
231	N	0.58
232	T	0.61
233	Q	0.61
234	Q	0.49
235	S	0.45
236	P	0.49
237	V	0.57
238	A	0.75
239	A	0.75
240	T	0.74
241	L	0.76
242	Y	0.77
243	V	0.83
244	R	0.74
245	N	0.82
246	R	0.79
247	V	0.87
248	R	0.77
249	E	0.81
250	A	0.82
251	I	0.74
252	R	0.71
253	V	0.79
254	S	0.75
255	K	0.69
256	I	0.55
257	P	0.54
258	D	0.47
259	A	0.49
260	K	0.44
261	S	0.52
262	P	0.58
263	L	0.70
264	P	0.73
265	V	0.76
266	F	0.76
267	A	0.80
268	Y	0.82
269	T	0.84
270	R	0.74
271	I	0.80
272	V	0.71
273	F	0.65
274	T	0.59
275	D	0.54
276	Q	0.56
277	V	0.51
278	L	0.51
279	K	0.71
280	F	0.78
281	L	0.82
282	S	0.81
283	Q	0.82
284	D	0.78
285	E	0.74
286	L	0.80
287	V	0.83
288	Y	0.82
289	T	0.81
290	F	0.84
291	G	0.88
292	E	0.80
293	T	0.82
294	V	0.81
295	A	0.83
296	L	0.79
297	G	0.72
298	A	0.75
299	S	0.75
300	G	0.81
301	I	0.83
302	V	0.84
303	I	0.80
304	W	0.79
305	G	0.71
306	T	0.64
307	L	0.66
308	S	0.70
309	I	0.66
310	M	0.70
311	R	0.67
312	S	0.73
313	M	0.66
314	K	0.68
315	S	0.73
316	C	0.63
317	L	0.70
318	L	0.74

Position	Wildtype (WT)	Small Hydrophobes (7)	Polar Uncharged (5)	Polar Negative Charge (2)	Polar Positive Charge (3)	Large Hydrophobes, Aromatics (3)	Count Active (>40%, no errors)	Tested (w/o errors)
213	D	5	2	1	3	2	13	15
214	L	0	1	0	0	0	1	14
215	S	5	2	2	3	1	13	15
216	W	0	0	0	0	1	1	15
217	L	1	0	0	0	0	1	11
218	W	0	0	0	0	1	1	7
219	N	4	3	2	3	1	13	14
220	E	4	2	x	1	0	7	13
221	S	3	3	0	0	0	6	12
222	T	4	2	1	2	1	10	12
223	A	0	0	0	0	0	0	15
224	L	1	0	0	0	0	1	14
225	Y	0	0	0	0	0	0	12
226	P	0	0	0	0	0	0	14
227	S	0	0	0	0	0	0	15
228	I	0	0	0	0	0	0	13
229	Y	0	0	0	0	0	0	10
230	L	1	0	0	0	0	1	15
231	N	0	1	0	0	0	1	15
232	T	0	1	0	0	1	2	12
233	Q	3	0	0	2	2	7	12
234	Q	2	0	0	0	0	2	13
235	S	2	1	1	2	0	6	12
236	P	2	1	0	3	0	6	13
237	V	2	4	1	2	2	11	11
238	A	0	3	2	3	0	8	15
239	A	0	0	0	0	0	0	13
240	T	4	2	0	2	0	8	14
241	L	0	0	0	0	0	0	12
242	Y	0	0	0	0	1	1	14
243	V	0	0	0	0	0	0	13
244	R	0	0	0	0	0	0	6
245	N	0	0	0	1	0	1	11
246	R	0	0	0	0	0	0	15
247	V	3	0	0	0	0	3	15
248	R	1	0	0	1	2	4	12
249	E	0	0	x	0	0	1	10
250	A	0	0	0	0	0	0	15
251	I	2	0	0	0	0	2	11
252	R	0	0	0	0	0	0	14
253	V	1	0	0	0	0	1	13
254	S	0	0	0	0	0	0	14
255	K	1	3	0	1	0	5	11
256	I	3	0	0	1	0	4	9
257	P	6	3	0	2	0	11	12
258	D	0	2	x	2	0	4	8
259	A	5	4	1	2	2	14	14
260	K	3	2	2	2	1	10	12
261	S	3	3	0	2	2	10	11
262	P	0	0	0	0	0	0	15
263	L	3	1	0	2	0	6	11
264	P	1	0	0	1	0	2	13
265	V	1	0	0	0	0	1	13
266	F	0	0	0	0	1	1	13
267	A	1	1	0	0	0	2	11
268	Y	0	0	0	0	0	0	14
269	T	1	2	1	0	0	4	12
270	R	1	3	0	0	0	4	13
271	I	4	1	0	0	1	6	13
272	V	0	2	1	1	0	4	10
273	F	0	1	0	1	1	3	14
274	T	1	1	0	0	1	3	11
275	D	0	0	x	0	0	0	9
276	Q	3	2	2	2	1	10	13
277	V	3	5	2	3	1	14	14
278	L	4	3	1	3	2	13	15
279	K	0	2	0	2	0	4	10
280	F	1	1	0	0	0	2	12
281	L	0	0	0	0	0	0	13
282	S	2	1	1	0	0	4	9
283	Q	1	2	1	1	0	5	10
284	D	4	3	1	1	0	9	12
285	E	2	2	x	1	2	7	12
286	L	0	1	0	1	0	2	12
287	V	1	1	0	0	0	2	13
288	Y	1	0	0	0	1	2	12
289	T	0	0	0	1	0	1	11
290	F	2	0	0	0	0	2	5
291	G	1	2	0	1	0	4	13
292	E	4	2	x	3	2	11	14
293	T	6	3	1	1	2	13	15
294	V	1	0	0	0	0	1	14
295	A	0	0	0	0	0	0	10
296	L	0	0	0	0	0	0	13
297	G	1	0	0	0	0	1	11
298	A	2	0	0	0	0	2	13
299	S	0	0	0	0	0	0	11
300	G	0	0	0	1	0	1	15
301	I	2	0	0	0	0	2	14
302	V	1	0	0	0	1	2	15
303	I	1	0	0	0	0	1	12
304	W	0	0	0	0	0	0	11
305	G	0	0	2	0	0	2	10
306	T	0	1	2	0	0	3	11
307	L	1	4	0	1	2	8	11
308	S	2	2	1	3	0	8	15
309	I	4	4	2	3	1	14	14
310	M	3	2	0	1	0	6	10
311	R	0	3	0	2	0	6	14
312	S	1	2	0	0	0	3	8
313	M	5	2	1	3	1	12	13
314	K	2	4	1	2	1	10	13
315	S	4	1	1	3	1	10	13
316	C	0	0	1	0	0	1	14
317	L	3	4	1	3	1	12	14
318	L	3	4	1	3	1	12	15

Subset of Surface Residues	Wildtype (WT)	Local QMEAN Score from
319	L	0.72
320	D	0.64
321	N	0.74
322	Y	0.81
323	M	0.73
324	E	0.71
325	T	0.84
326	I	0.85
327	L	0.81
328	N	0.76
329	P	0.82
330	Y	0.82
331	I	0.81
332	I	0.81
333	N	0.81
334	V	0.84
335	T	0.77
336	L	0.83
337	A	0.84
338	A	0.80
339	K	0.74
340	M	0.81
341	C	0.82
342	S	0.77
343	Q	0.72
344	V	0.75
345	L	0.79
346	C	0.73
347	Q	0.72
348	E	0.70
349	Q	0.61
350	G	0.77
351	V	0.77
352	C	0.77
353	I	0.76
354	R	0.74
355	K	0.72
356	N	0.74
357	W	0.36
358	N	0.52
359	S	0.68
360	S	0.70
361	D	0.74
362	Y	0.78
363	L	0.81
364	H	0.81
365	L	0.84
366	N	0.83
367	P	0.83
368	D	0.84
369	N	0.75
370	F	0.86
371	A	0.90
372	I	0.85
373	Q	0.80
374	L	0.75
375	E	0.73
376	K	0.74
377	G	0.59
378	G	0.61
379	K	0.66
380	F	0.71
381	T	0.79
382	V	0.85
383	R	0.83
384	G	0.90
385	K	0.85
386	P	0.85
387	T	0.83
388	L	0.86
389	E	0.80
390	D	0.80
391	L	0.85
392	E	0.80
393	Q	0.77
394	F	0.80
395	S	0.84
396	E	0.80
397	K	0.72
398	F	0.79
399	Y	0.77
400	C	0.77
401	S	0.66
402	C	0.59
403	Y	0.65
404	S	0.61
405	T	0.51
406	L	0.52
407	S	0.62
408	C	0.59
409	K	0.61
410	E	0.63
411	K	0.62
412	A	0.64
413	D	0.63
414	V	0.64
415	K	0.59
416	D	
417	T	
418	D	
419	A	
420	V	
421	D	
422	V	
423	C	
424	I	

Position	Wildtype (WT)	Small Hydrophobes (7)	Polar Uncharged (5)	Polar Negative Charge (2)	Polar Positive Charge (3)	Large Hydrophobes, Aromatics (3)	Count Active (>40%, no errors)	Tested (w/o errors)
319	L	0	0	0	0	0	0	15
320	D	3	2	1	3	2	11	13
321	N	1	2	1	3	1	8	11
322	Y	0	0	0	0	0	0	13
323	M	2	0	0	0	1	3	14
324	E	2	2	1	2	0	7	13
325	T	4	3	2	2	1	12	14
326	I	2	1	0	1	1	5	10
327	L	1	0	0	0	0	1	14
328	N	5	4	0	3	1	13	13
329	P	0	0	0	0	0	0	15
330	Y	0	0	0	0	0	0	14
331	I	1	0	0	0	0	1	12
332	I	0	0	0	0	0	0	15
333	N	0	0	0	0	0	0	10
334	V	1	0	0	0	0	1	10
335	T	0	1	0	0	0	1	11
336	L	0	0	0	0	0	0	13
337	A	0	0	0	0	0	0	10
338	A	0	1	0	0	0	1	14
339	K	1	0	0	0	0	1	14
340	M	0	0	0	0	0	0	14
341	C	0	0	0	0	0	0	14
342	S	1	0	0	0	0	1	13
343	Q	1	1	0	0	0	2	8
344	V	0	0	0	0	0	0	13
345	L	0	0	0	0	0	0	11
346	C	0	0	0	0	0	0	14
347	Q	3	1	1	1	0	6	13
348	E	1	1	1	0	0	3	14
349	Q	2	2	1	2	0	7	14
350	G	0	0	0	0	0	0	15
351	V	1	1	0	0	0	2	10
352	C	0	0	0	0	0	0	15
353	I	1	1	0	0	0	2	13
354	R	0	0	0	0	0	0	15
355	K	0	0	0	0	0	0	15
356	N	1	1	1	1	0	4	13
357	W	1	3	0	0	0	4	12
358	N	1	2	0	0	0	3	11
359	S	2	1	2	2	0	7	13
360	S	0	1	0	0	0	1	14
361	D	0	0	x	0	0	0	12
362	Y	0	0	0	0	0	0	15
363	L	0	0	0	0	0	0	15
364	H	0	0	0	0	0	0	15
365	L	0	0	0	0	0	0	14
366	N	0	0	0	0	0	0	12
367	P	2	2	0	2	0	6	13
368	D	5	2	1	3	0	11	14
369	N	0	1	0	2	0	3	13
370	F	0	0	0	0	0	0	14
371	A	4	1	1	3	1	10	12
372	I	0	0	0	0	0	0	15
373	Q	4	1	1	2	1	9	12
374	L	5	3	0	2	1	11	13
375	E	4	3	x	2	0	9	14
376	K	3	3	2	1	1	10	13
377	G	1	2	2	3	0	8	12
378	G	0	1	0	2	0	3	13
379	K	1	2	0	2	0	5	13
380	F	3	0	0	0	2	5	12
381	T	1	3	0	3	0	7	12
382	V	0	0	0	0	0	0	14
383	R	5	3	1	2	0	11	13
384	G	0	0	0	0	0	0	6
385	K	3	4	0	2	0	9	15
386	P	0	0	0	0	0	0	15
387	T	0	1	0	0	0	1	13
388	L	3	1	0	2	3	9	14
389	E	5	3	x	3	1	12	14
390	D	0	0	x	0	0	0	15
391	L	0	1	0	0	0	1	15
392	E	5	3	x	2	2	12	14
393	Q	3	3	1	3	1	11	13
394	F	1	0	0	0	1	2	15
395	S	2	0	0	3	1	6	10
396	E	1	3	1	2	0	7	13
397	K	0	0	0	0	0	0	12
398	F	0	0	0	0	0	0	14
399	Y	3	5	1	2	1	12	14
400	C	0	0	0	0	0	0	15
401	S	1	1	1	0	0	3	10
402	C	0	0	0	0	0	0	14
403	Y	0	0	0	0	1	1	14
404	S	2	0	0	0	0	2	14
405	T	4	2	0	2	2	10	13
406	L	4	5	1	0	2	12	14
407	S	6	2	2	2	2	14	14
408	C	0	0	0	0	0	0	14
409	K	5	3	2	2	0	12	12
410	E	3	4	1	2	1	11	12
411	K	3	3	0	1	0	7	11
412	A	5	2	1	1	2	11	12
413	D	0	2	1	1	0	4	7
414	V	2	0	0	0	0	2	10
415	K	1	1	0	0	1	3	7
416	D	4	3	x	3	2	12	14
417	T	1	0	0	0	0	1	12
418	D	7	3	1	1	2	14	14
419	A	3	2	1	3	3	12	14
420	V	2	0	0	0	0	2	15
421	D	4	4	1	3	1	13	13
422	V	1	1	0	0	0	2	13
423	C	0	0	0	0	0	0	15
424	I	0	0	0	0	0	0	11

Subset of Surface Residues	Wildtype (WT)	Local QMEAN Score from
425	A	
426	D	
427	G	
428	V	
429	C	
430	I	
431	D	
432	A	
433	F	
434	L	
435	K	
436	P	
437	P	
438	M	
439	E	
440	T	
441	E	
442	E	
443	P	
444	Q	
445	I	
446	F	
447	Y	

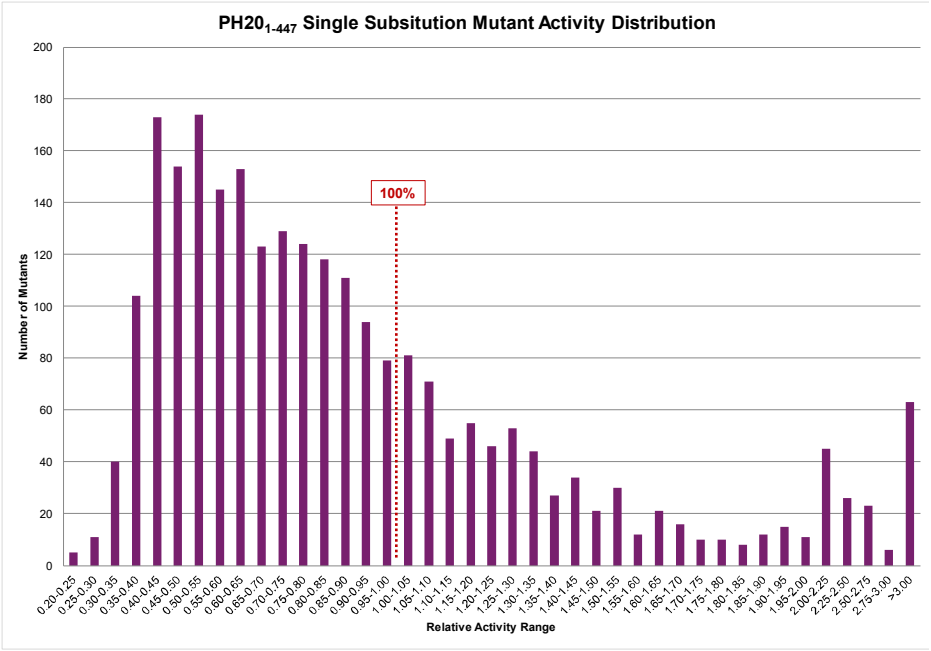
Position	Wildtype (WT)	Small Hydrophobes (7)	Polar Uncharged (5)	Polar Negative Charge (2)	Polar Positive Charge (3)	Large Hydrophobes, Aromatics (3)	Count Active (>40%, no errors)	Tested (w/o errors)
425	A	3	2	0	2	0	7	11
426	D	2	2	1	0	1	6	10
427	G	1	1	0	1	0	3	11
428	V	3	1	0	0	0	4	14
429	C	0	0	0	0	0	0	11
430	I	0	0	0	0	0	0	9
431	D	5	3	1	3	2	14	15
432	A	2	2	1	0	0	5	13
433	F	6	3	2	3	1	15	15
434	L	4	0	0	0	1	5	11
435	K	3	3	1	2	1	10	10
436	P	4	3	2	3	2	14	14
437	P	5	2	1	3	1	12	13
438	M	5	5	2	1	1	14	15
439	E	5	4	x	2	2	13	15
440	T	7	1	2	2	2	14	15
441	E	4	4	1	2	2	13	14
442	E	4	3	x	3	1	11	14
443	P	4	4	1	2	2	13	14
444	Q	3	1	2	3	3	12	12
445	I	6	4	0	2	2	14	14
446	F	6	3	2	3	1	15	15
447	Y	6	3	2	1	2	14	14

Appendix B-3

Histogram of $PH20_{1-447}$ Activity Data

Source(s): EX1026 ('731 App.), Table 9

Activity Range	Count
0.20-0.25	5
0.25-0.30	11
0.30-0.35	40
0.35-0.40	104
0.40-0.45	173
0.45-0.50	154
0.50-0.55	174
0.55-0.60	145
0.60-0.65	153
0.65-0.70	123
0.70-0.75	129
0.75-0.80	124
0.80-0.85	118
0.85-0.90	111
0.90-0.95	94
0.95-1.00	79
1.00-1.05	81
1.05-1.10	71
1.10-1.15	49
1.15-1.20	55
1.20-1.25	46
1.25-1.30	53
1.30-1.35	44
1.35-1.40	27
1.40-1.45	34
1.45-1.50	21
1.50-1.55	30
1.55-1.60	12
1.60-1.65	21
1.65-1.70	16
1.70-1.75	10
1.75-1.80	10
1.80-1.85	8
1.85-1.90	12
1.90-1.95	15
1.95-2.00	11
2.00-2.25	45
2.25-2.50	26
2.50-2.75	23
2.75-3.00	6
>3.00	63
TOTAL	**2526**



Note:

Information in this spreadsheet was obtained from EX1026 ('731 App.), Table 9.

Appendix B-4

Activity Data of Table 11 $PH20_{1-447}$ Mutants

Source(s): EX1026 ('731 App.), Table 11

Mutant	Absolute Hylarudonidase Activity					
	Duplicate #1			Duplicate #2		
	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)
L001A	2.993	3.529	0.287	2.511	3.214	0.295
L001E	2.669	2.862	0.376	2.539	3.179	0.341
L001G	0.348	0.596	0.055	0.583	0.676	0.031
L001Q	5.135	6.133	0.621	6.443	5.719	0.636
L001R	5.603	6.576	0.458	4.39	7.042	0.396
P006A	2.965	4.088	0.404	3.208	3.495	0.435
V008M	1.376	1.856	0	1.401	1.678	0.008
I009Q	0.447	0.469	0.031	0.381	0.476	0.03
P010G	0.747	0.82	0.123	0.564	0.688	0.114
P010H	0.473	0.624	0	0.485	0.548	0
N011S	0.862	1.313	0.094	0.962	1.263	0.064
V012E	11.019	5.312	0.753	5.519	5.528	0.934
V012I	2.804	3.61	0.106	3.844	6.566	0.09
V012K	1.691	2.479	0.33	1.963	2.243	0.321
F014V	0.144	0.222	0.003	0.165	0.242	0
L015M	0.902	1.026	0.017	1.073	0.901	0.017
A020S	1.494	2.822	0.413	2.205	2.62	0.397
S022T	3.035	3.375	0.684	3.788	3.273	0.748
L026M	1.482	2.027	0.224	1.226	1.704	0.178
K028R	0.944	1.043	0.112	0.845	0.925	0.095
F029R	1.195	1.848	0.14	1.511	1.839	0.14
F029S	3.019	3.566	0.25	3.615	3.521	0.283
F029T	1.451	1.839	0.22	1.712	2.065	0.212
P032C	0.37	0.476	0.006	0.419	0.534	0.04
L033G	0.566	0.686	0.001	0.7	0.627	0.026
D034W	0.34	0.499	0.076	0.321	0.471	0.069
M035V	0.887	0.721	0.116	0.639	0.652	0.023
S036H	1.109	1.178	0.117	0.752	1.135	0.026
S036N	0.797	0.893	0.171	0.933	0.859	0.26
L037M	0.574	0.455	0.049	0.404	0.353	0.032
F040L	2.603	3.515	0.277	3.941	4.148	0.361
I046L	3.027	4.011	0.513	2.959	3.342	0.557
N047D	2.222	2.573	0.032	2.359	2.639	0.021
N047W	0.404	0.423	0	0.415	0.456	0.017
A048N	12.398	14.252	0.797	45.971	23.873	0.902
T049R	7.893	9.685	0.563	13.334	12.102	0.649
G050D	3.287	3.084	0.242	3.148	3.02	0.264
G050M	1.763	2.78	0.25	2.333	3.244	0.393
G052N	7.217	6.939	1.109	9.809	13.978	1.083
G052T	1.542	1.795	0.381	1.224	1.433	0.463
G052S	2.152	2.12	0.498	1.999	1.963	0.566
V058C	1.428	1.321	0.212	1.312	1.301	0.21
V058K	28	61.016	23.586	28	61.016	23.586
V058R	5.719	5.542	3.134	4.688	4.822	3.149
V058N	1.2	1.55	0.2	1.175	1.525	0.175
V058Y	1.04	1.071	0.388	0.77	1.088	0.454
V058Q	11.956	18.458	1.567	15.363	45.092	2.166
V058P	3.36	2.799	0.592	2.949	5.121	0.884
V058H	3.79	7.59	0.826	5.074	9.222	1.205
D068P	0.215	0.213	0.001	0.215	0.18	0.184
S069T	1.927	2.671	0.289	2.179	2.671	0.24
I070P	1.284	1.306	0.01	1.593	1.589	0.032
I070V	1.818	3.099	0.433	2.437	3.335	0.363
V073Q	4.846	5.88	0.383	5.441	5.827	0.477
V073R	0.522	0.72	0.018	0.803	0.804	0.059
T074E	2.903	3.868	0.666	3.834	3.871	0.626
T074M	0.569	0.656	0.079	0.744	0.771	0.083
T074N	2.792	2.565	0.281	1.905	2.995	0.204
T074P	2.331	2.525	0.309	1.593	2.648	0.265
T074R	0.999	0.806	0.06	0.82	1.066	0.023
T074V	1.186	1.365	0.101	1.28	1.46	0.08
V075M	0.917	1.233	0.003	1.087	1.321	0.028
K082L	1.362	1.563	0.325	1.311	3.302	0.354
K082N	3.202	3.396	0.792	3.411	3.244	0.861
I083V	3.706	5.194	1.552	2.633	3.615	1.017
I083Q	2.376	2.665	0.72	1.946	3.674	0.51
I083S	0.841	0.88	0.235	1.054	1.005	0.268
I083G	2.276	2.418	0.545	2.443	1.866	0.601
S084E	1.47	1.834	0.115	1.484	1.683	0.115
S084F	1.179	0.982	0.025	1.212	1.103	0
S084N	2.255	3.268	0.597	1.888	2.476	0.547

KEY
Coloration of Percent (%) Activity Values
n/a
> 14
between 10 and 14
between 8 and 10
between 6 and 8
between 4 and 6
between 2 and 4
between 0 and 2

Mutant	Absolute Hylarudonidase Activity					
	Duplicate #1			Duplicate #2		
	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)
S084R	8.534	10.23	1.117	14.779	30.016	1.494
Q086A	2.084	2.845	0.405	2.12	3.31	0.322
Q086H	1.187	1.218	0.087	1	1.296	0.065
Q086K	0.127	0.126	0.032	0.11	0.072	0.023
Q086S	2.528	2.539	0.173	2.082	2.149	0.241
Q086T	3.018	2.832	0.29	2.542	4.562	0.406
D087G	2.755	2.252	0.034	2.176	1.971	0.122
D087L	2.07	2.195	0.324	2.277	2.311	0.299
D087M	2.262	2.51	0.191	2.325	2.038	0.335
D087S	5.21	6.983	0.569	10.305	14.399	0.928
D087V	1.361	1.553	0.142	1.364	1.187	0.189
D090E	8.251	7.666	1.093	12.299	19.836	1.234
D090N	2.812	3.123	0.379	2.775	2.737	0.29
K093Q	2.491	2.267	0.132	2.065	1.971	0.131
K093R	2.986	3.094	0.362	2.862	2.842	0.465
K094D	2.393	2.071	0.135	2.088	2.132	0.211
K094R	1.407	1.764	0.158	1.542	1.676	0.166
T097C	0.33	0.545	0.044	0.618	0.505	0.087
T097D	0.52	0.643	0.055	0.565	0.664	0.073
T097E	1.096	1.394	0.217	1.41	1.623	0.262
T097L	0.899	1.065	0.246	1.198	1.241	0.3
N104R	2.508	2.876	0.279	2.356	2.79	0.238
A120H	2.155	2.028	0.168	2.551	2.883	0.199
D127R	0.264	0.149	0.105	0.339	0.199	0.068
V128I	3.12	3.546	0.389	3.313	3.401	0.504
N131M	15.335	27.143	0.505	20.678	15.899	0.447
N131R	8.195	7.724	1.645	8.748	8.392	1.626
N131V	1.656	2.28	0.233	1.87	1.962	0.214
R132L	3.306	3.259	0.337	3.235	2.966	0.43
Q138L	1.494	1.611	0.41	1.66	1.521	0.347
Q140K	2.829	4.996	0.546	4.065	4.464	0.559
N141R	1.29	1.334	0.058	1.32	1.527	0.035
N141S	2.201	2.9	0.135	2.708	2.966	0.164
N141W	1.475	1.927	0.1	1.568	1.643	0.105
V142D	2.552	2.914	0.128	2.186	3.193	0.067
V142G	1.357	1.597	0.211	1.796	1.621	0.219
V142K	3.532	3.867	0.571	2.381	3.681	0.575
V142N	0.432	0.672	0.103	0.567	0.589	0.087
V142P	4.624	7.722	1.074	7.213	7.021	1.081
V142Q	5.09	7.618	0.678	6.9	6.897	0.678
V142R	1.968	2.941	0.364	2.595	2.689	0.33
V142S	2.789	4.763	0.416	2.988	3.497	0.591
V142T	1.926	4.313	0.495	3.26	4.031	0.472
Q143G	3.922	5.632	0.782	4.903	4.846	0.78
Q143K	3.634	7.285	1.043	3.671	5.008	1.039
L144R	3.81	5.191	0.556	4.581	5.107	0.52
L144T	1.496	1.941	0.285	1.681	1.831	0.219
L146P	0.818	0.954	0.011	0.782	0.904	0.031
T147S	0.984	1.399	0.055	1.149	1.497	0.039
T150N	0.442	0.622	0.039	0.585	0.684	0.046
T150S	1.747	1.875	0.12	1.4	1.988	0.121
E151A	2.87	2.965	0.359	2.269	2.86	0.337
E151L	3.365	4.446	0.218	3.289	4.007	0.251
E151S	5.187	5.987	0.371	4.591	6.262	0.294
E151T	2.442	3.134	0	3	3.309	0
E151V	3.998	4.459	0.326	4.247	4.232	0.314
E151W	7.166	11.352	0.131	14.248	13.524	0.121
K152T	1.204	1.796	0.1	1.377	1.883	0.067
K152W	2.084	2.549	0.063	1.795	2.406	0.069
E158S	0.339	0.451	0	0.397	0.407	0
K162E	0.168	0.114	0.004	0.195	0.08	0.024
L165F	4.775	5.075	0.6	5.25	5.075	0.725
V166Q	1.883	2.937	0.392	2.507	2.958	0.324
V166T	0.993	1.821	0.231	1.315	1.8	0.235
E167D	0.811	1.109	0.111	0.91	1.48	0.056
I169L	1.812	2.54	0.335	1.796	2.196	0.341
K170R	1.578	2.536	0.209	2.054	1.995	0.201
G172A	0.413	0.692	0.052	0.581	0.777	0.056
K173R	1.654	1.766	0.173	1.551	2.083	0.156
L174G	0.184	0.21	0.026	0.087	0.23	0.031
L174N	1.616	2.494	0.331	2.276	2.872	0.543

Mutant	Absolute Hylarudonidase Activity					
	Duplicate #1			Duplicate #2		
	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)
L174T	0.552	0.689	0.09	0.566	0.82	0.05
N178K	2.931	4.891	0.258	4.375	4.513	0.362
N178R	8.16	16.287	0.665	13.82	20.033	0.79
H193Q	1.06	2.264	0.346	1.367	1.888	0.346
K195T	1.227	1.548	0.348	0.806	1.911	0.292
K195N	1.266	1.649	0.369	1.437	1.385	0.353
K196E	0.732	0.663	0.244	0.66	1.017	0.239
K196R	2.246	2.383	0.315	2.285	2.174	0.384
F204P	3.5	2.925	2.475	4.55	3.75	4.725
N205A	0.515	0.717	0.153	0.837	0.854	0.16
N205E	1.011	1.627	0.314	2.004	1.87	0.346
N205L	1.084	1.165	0.123	1.029	0	0.088
N205T	0.295	0.428	0.043	0.367	0.406	0.053
V206I	0.317	0.6	0.079	0.508	0.565	0.088
K209R	2.041	2.445	0.291	2.453	1.951	0.077
D212N	5.568	6.271	0.167	4.549	6.016	0.322
D212S	1.987	2.442	0.204	1.502	2.222	0.152
D213A	0.235	0.432	0.116	0.283	0.438	0.06
D213M	1.664	2.65	0.181	2.08	2.046	0.142
S215H	2.448	2.67	0.268	3.056	2.414	0.139
S215M	1.497	2.618	0.11	2.175	1.63	0.146
N219I	0.338	0.86	0.076	0.25	0.728	0.082
E220V	3.783	4.993	0.371	3.828	4.349	0.257
T222G	3.528	5.399	0.033	5.262	5.549	0.044
T232F	0.539	0.716	0.089	1.242	0.781	0.153
Q233G	0.041	0.115	0	0.095	0.121	0
Q234M	6.029	5.764	1.286	6.031	4.871	0.988
S235A	0.55	0.714	0.079	0.502	0.607	0.073
V237C	0.623	0.86	0	0.708	0.824	0
V237H	0.303	0.37	0.046	0.316	0.459	0.034
V237T	0.152	0.254	0.054	0.196	0.247	0.053
A238E	2.05	1.945	0.159	1.8	2.559	0.171
A238H	0.579	0.345	0.09	0.363	0.743	0.062
T240A	1.107	1.564	0.143	0.9	1.302	0.118
T240Q	0.333	0.542	0.08	0.51	0.617	0.085
R248A	2.274	2.575	0.027	2.499	3.115	0.075
E249V	3.001	4.284	0.655	3.894	4.325	0.712
P257G	3.981	4.985	0.039	4.452	5.022	0.034
K260M	0.719	0.839	0.072	0.96	0.935	0.068
S261A	3.253	1.872	1.264	3.117	2.686	1.451
S261K	6.089	9.86	1.583	5.421	6.297	1.437
S261N	14.149	20.219	2.115	40.257	14.303	1.917
A267T	0.052	0.102	0.036	0.095	0.106	0.041
F273H	0.34	0.417	0.025	0.436	0.519	0.031
F273Y	0.558	0.668	0.052	0.505	0.519	0.05
Q276H	2.706	2.027	0.181	1.877	1.997	0.201
Q276M	0.775	0.762	0.043	0.768	0.806	0
Q276R	6.08	7.383	0.807	9.717	14.593	1.281
Q276S	1.353	1.497	0.149	1.212	1.681	0.147
V277A	1.202	1.692	0.118	1.643	2.129	0.11
V277E	2.44	4.289	0.161	2.34	4.577	0.239
V277H	5.548	7.181	0.227	5.302	7.3	0.512
V277K	8.95	33.627	4.442	8.996	33.627	4.045
V277M	1.279	1.754	0.264	1.622	1.818	0.27
V277N	14.351	12.865	0.938	4.306	11.772	0.796
V277Q	5.459	6.547	0.373	5.461	6.343	0.493
V277R	18.3	17.581	2.737	12.038	20.641	2.023
V277S	14.351	9.509	0.727	10.444	15.135	0.716
V277T	8.412	8.497	0.679	7.804	11.184	0.871
L278E	4.416	3.33	0.17	2.795	2.8	0.202
L278G	7.502	9.173	0.596	7.456	7.76	0.612
K279H	0.888	1.234	0.185	1.087	1.339	0.269
V287T	0.58	0.843	0.139	0.667	0.832	0.1
T289S	0.783	0.819	0.008	1.019	1.001	0.007
G291S	0.227	0.419	0.051	0.322	0.385	0.016
G291V	3.662	4.131	0.821	3.707	5.599	0.706
E292C	1.344	1.711	0.138	1.599	1.617	0.144
E292F	6.106	8.422	0.52	4.697	6.216	0.363
E292H	2.62	4.458	0.389	3.316	3.83	0.451
E292R	2.81	3.155	0.398	2.178	2.829	0.339
E292V	0.891	1.453	0.193	1.121	1.494	0.177

Mutant	Absolute Hylarudonidase Activity					
	Duplicate #1			Duplicate #2		
	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)
T293A	1.986	2.546	0.086	3.11	1.789	0.076
A298G	0.161	0.342	0.03	0.274	0.236	0.022
L307G	0.616	0.726	0	0.661	0.605	0
S308D	0.264	0.337	0.014	0.325	0.344	0.01
S308K	0.651	0.826	0.011	0.722	0.716	0
S308N	3.995	6.808	0.386	4.406	6.128	0.362
I309E	3.166	3.921	0.637	2.819	3.663	0.528
I309G	6.651	6.824	0.503	5.429	6.194	0.4
I309L	0.326	0.501	0.048	0.403	0.431	0.047
I309M	2.809	3.467	0.278	2.473	3.383	0.239
I309N	4.865	5.444	0.38	5.191	5.054	0.327
I309S	10.719	18.217	0.748	28.759	158.604	1.367
I309T	3.052	2.989	0.228	2.509	3.735	0.207
I309V	1.705	1.929	0.029	1.292	1.787	0.062
M310G	4.514	7.568	0.866	6.397	7.084	0.915
M310Q	3.648	3.912	1.088	3.179	3.38	0.955
M313G	0.252	0.348	0.034	0.325	0.355	0.036
M313H	3.767	10.243	0.38	5.276	10.395	0.404
M313K	12.689	15.085	0.129	12.122	12.984	0.072
M313P	4.05	4.198	0.209	2.951	3.919	0.177
M313R	4.634	7.288	0.337	10.863	3.568	0.296
M313T	2.903	4.705	0.331	4.474	4.467	0.313
M313Y	1.063	1.276	0.096	1.262	1.3	0.089
K314S	2.848	4.042	0.391	4.45	5.879	0.533
K314Y	0.093	0.226	0.013	0.131	0.182	0.02
S315A	1.472	1.345	0.222	1.082	1.484	0.148
S315H	2.412	3.648	0.44	3.242	3.414	0.371
S315Y	0.279	0.477	0.146	0.626	0.362	0.143
L317A	3.254	4.019	0.28	2.845	3.776	0.317
L317I	1.078	2.021	0.257	1.524	1.687	0.18
L317K	12.129	11.668	0.402	9.382	12.591	0.445
L317N	2.907	3.703	0.445	3.066	3.717	0.54
L317R	8.631	20.585	0.796	15.187	15.106	0.857
L317S	11.586	10.535	1.637	29.267	25.114	1.613
L317T	1.338	1.953	0.136	1.073	1.656	0.018
L317W	0.81	1.326	0.158	1.128	1.665	0.171
L318D	1.75	1.847	0.322	1.97	1.93	0.322
L318H	1.073	1.072	0.046	0.806	1.005	0.074
L318R	2.856	4.583	0.258	3.464	4.187	0.26
N321R	3.069	5.059	0.482	4.409	4.946	0.426
N321S	0.683	0.7	0.058	0.71	0.772	0.035
E324N	4.309	4.508	0.348	2.53	3.321	0.303
T325E	1.071	1.337	0.193	1.27	1.352	0.143
N328G	0.379	0.747	0.031	0.504	0.553	0.04
N328Y	2.629	4.758	0.49	4.543	4.543	0.477
T335S	0.905	0.977	0.113	0.787	0.986	0.062
Q347A	8.316	8.432	0.918	11.961	11.508	1.266
Q347G	1.358	3.021	0.253	1.12	2.319	0.209
Q349M	1.493	1.486	0.178	1.629	1.76	0.217
Q349R	0.451	0.663	0.078	0.572	0.598	0.079
V351S	1.379	1.804	0	1.633	1.647	0
I353V	2.335	3.09	0.323	1.954	2.697	0.321
N356H	0.445	0.445	0.038	0.451	0.588	0.023
N356S	0.262	0.136	0	0.253	0.318	0.008
S359E	2.616	3.547	0.382	2.635	3.56	0.333
S359H	0.403	0.445	0	0.371	0.374	0
P367A	0.643	1.074	0.139	0.782	0.996	0.131
P367G	0.593	0.686	0	0.53	0.65	0
P367K	0.707	0.89	0.045	0.767	0.513	0.052
P367S	3.967	2.946	0.424	3.478	3.073	0.505
D368A	1.762	2.143	0.031	2.321	1.895	0.04
D368E	3.464	5.772	0.53	4.944	4.842	0.555
D368L	0.557	0.607	0	0.566	0.619	0.006
D368M	0.861	1.031	0.028	1.065	1.104	0.028
D368R	4.503	7.418	0.754	5.27	6.226	0.735
D368T	2.345	2.512	0.072	1.993	2.525	0.085
N369R	1.548	2.503	0.16	2.719	2.022	0.125
A371F	2.76	4.974	0.308	5.207	3.98	0.222
A371H	8.101	77.531	1.403	86.587	77.531	1.316
A371H	3.509	3.9	0	4.058	3.879	0.334
A371K	2.903	3.963	0.509	3.546	4.055	0.505

Mutant	Absolute Hylarudonidase Activity					
	Duplicate #1			Duplicate #2		
	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)
A371L	11.018	76.587	1.159	40.668	43.516	0.964
A371L	3.328	3.472	0	3.445	2.075	0.025
A371R	25.855	n/a	2.851	25.855	n/a	3.634
A371R	6.592	7.987	0	7.733	7.576	0.196
A371S	3.329	4.916	0.412	3.505	4.611	0.781
L374P	2.939	11.522	0.665	7.129	8.771	0.646
E375A	0.627	0.557	0	0.507	0.683	0.014
E375G	1.596	2.025	0.209	1.299	1.806	0.265
E375R	0.937	1.529	0.201	1.132	1.318	0.26
K376D	0.458	0.518	0.064	0.312	0.515	0.026
K376E	1.572	1.572	0.213	1.094	1.674	0.174
K376Q	0.727	0.91	0.116	0.94	0.846	0.102
K376R	2.086	1.704	0.539	1.351	2.69	0.279
K376T	0.847	1.026	0.153	1.001	1.135	0.064
K376V	0.834	1.036	0.033	0.861	1.021	0.026
K376Y	1.316	1.353	0.125	0.777	0.747	0.097
G377D	1.159	1.285	0.202	1.332	1.763	0.186
G377E	0.877	1.144	0.092	0.926	1.189	0.088
G377H	3.037	4.46	0.372	3.432	3.598	0.364
G377K	3.445	6.405	0.283	4.101	4.911	0.245
G377R	1.096	1.312	0.077	1.257	1.191	0.085
G377S	0.453	0.492	0.034	0.452	0.457	0.036
G377T	2.198	2.474	0.424	2.313	2.522	0.461
F380W	17.497	25.734	2.566	27.987	29.353	2.716
T381S	2.861	3.886	0.521	3.161	3.558	0.367
R383I	1.959	10.34	0.655	6.936	6.82	0.513
R383S	2.429	3.228	0.339	2.548	3.044	0.321
K385A	0.479	0.604	0.028	0.669	0.754	0
K385Q	1.746	2.403	0.217	2.089	2.609	0.196
K385V	1.232	1.387	0.071	1.75	1.41	0.042
E389A	6.872	21.081	0.449	10.944	24.61	0.449
E389G	0.166	0.188	0.004	0.203	0.284	0
E389L	1.814	2.598	0.37	2.142	2.403	0.303
E389Q	2.547	3.459	0.411	3.432	3.423	0.437
E389S	1.847	3.059	0	2.64	2.456	0.007
E392A	1.797	2.021	0.147	1.37	2.133	0.136
E392F	1.575	1.821	0.071	1.407	2.023	0.079
E392Q	5.826	6.583	0.693	4.653	4.364	0.729
E392R	4.555	5.9	0.218	5.306	6.548	0.193
E392V	3.817	4.747	0.367	2.936	4.544	0.291
Q393F	1.754	2.455	0.26	2.186	2.222	0.226
Q393M	1.252	1.749	0.028	1.826	1.588	0.049
S395A	4.22	8.788	1.141	6.127	6.906	0.856
S395H	1.609	2.574	0.323	2.261	2.564	0.268
E396A	1.135	1.497	0.126	1.184	1.524	0.149
E396H	0.357	0.751	0.069	0.532	0.684	0.022
E396Q	1.31	1.611	0.162	1.625	1.559	0.16
E396S	3.375	5.274	0.146	5.709	6.38	0.129
Y399T	2.538	3.313	0	3.25	3.989	0.002
Y399V	2.738	3.028	0.484	2.697	3.129	0.557
Y399W	1.4	1.715	0.236	1.883	1.946	0.233
S401A	2.636	3.216	0.447	3.171	3.148	0.41
S401E	1.685	2.11	0.344	1.601	2.06	0.309
S404A	1.288	1.924	0	1.635	1.724	0.019
L406F	0.706	0.867	0	0.49	0.716	0
L406N	0.617	0.943	0.06	0.795	1.044	0.07
S407A	2.428	3.432	0.389	2.949	3.255	0.548
S407D	2.09	5.038	0.569	5.79	5.682	0.575
S407P	2.66	3.812	0.261	2.708	3.301	0.366
A412Q	2.001	2.925	0.279	2.918	2.902	0.247
A412R	4.562	6.39	0.57	5.132	6.347	0.596
A412V	2.581	3.789	0.189	3.451	3.511	0.189
D416L	0.61	0.737	0.13	0.817	1.043	0.16
D418R	4.541	5.347	0.406	4.847	5.438	0.583
A419H	10.409	25.109	2.214	20.311	38.221	2.293
A419K	12.835	24.536	2.556	10.298	208.289	3.173
D421A	5.968	6.094	0.761	5.617	16.94	0.764
D421H	48.012	160.106	16.3	48.012	32.481	28.113
D421K	5.527	6.864	0.523	5.225	5.346	0.725
D421N	9.06	10.039	1.502	8.635	8.645	1.422
D421Q	7.529	7.858	0.842	5.581	8.016	0.994

Mutant	Absolute Hylarudonidase Activity					
	Duplicate #1			Duplicate #2		
	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)	No Incubation (4°C)	37°C (no cresol)	37°C (with m-cresol)
D421R	6.637	9.211	0.815	5.463	7.537	0.737
D421S	5.556	7.899	0.869	5.355	8.898	0.762
A425G	10.421	7.796	0.827	8.827	10.676	1.189
G427Q	1.008	1.342	0.031	1.252	1.23	0.106
G427T	1.33	1.664	0.08	1.38	1.643	0.065
V428L	2.138	2.93	0.053	2.769	3.029	0.03
D431E	2.81	1.972	0.519	2.22	2.112	0.438
D431H	2.154	4.017	0.294	3.185	3.028	0.301
D431K	8.123	19.563	2.272	16.953	11.575	2.339
D431L	1.211	1.564	0.164	1.215	1.448	0.17
D431N	11.819	16.358	1.601	12.063	15.131	1.399
D431Q	6.077	14.157	1.533	9.828	10.76	1.153
D431S	14.523	11.338	0.853	10.22	9.075	0.829
F433A	4.035	5.943	0.581	4.673	4.649	0.595
F433H	1.836	2.574	0.347	2.397	2.108	0.356
F433I	2.754	2.99	0.338	2.643	2.299	0.382
F433K	17.815	16.24	1.806	14.495	49.615	1.79
F433R	8.198	10.572	1.113	6.719	8.96	0.857
F433T	6.005	9.716	1.327	5.941	8.019	1.542
F433V	10.645	150.315	2.415	7.762	8.696	1.505
F433W	0.526	0.784	0.082	0.795	0.903	0.068
P437I	0.759	1.13	0.027	0.996	1.066	0.019
M438A	1.996	2.125	0.214	1.518	2.06	0.21
M438D	2.849	3.002	0.305	2.522	2.857	0.074
M438E	4.681	5.386	0.431	4.992	5.68	0.518
M438L	10.127	6.663	0.67	5.268	11.324	0.739
M438N	6.172	8.05	0.649	5.531	5.568	0.662
M438T	2.218	2.308	0.309	2.411	2.5	0.304
E439A	3.557	4.883	0.568	4.432	4.235	0.596
E439A	1.099	1.694	0.08	0.998	1.47	0.109
E439C	0.148	0.286	0.042	0.256	0.286	0.045
E439K	0.466	0.58	0.077	0.588	0.616	0.065
E439P	2.868	3.394	0.529	3.736	3.267	0.49
E439Q	1.07	1.087	0.116	0.848	1.08	0.115
E439T	1.965	2.179	0.313	1.889	2.323	0.263
T440D	4.148	4.931	0.568	4.443	3.533	0.651
T440H	2.317	3.297	0.147	1.982	2.595	0.196
T440M	3.397	2.878	0.254	3.305	2.873	0.367
T440P	3.562	3.987	0.54	3.593	3.277	0.566
T440S	2.522	2.533	0.283	2.207	2.895	0.284
E441F	1.402	1.813	0.204	1.407	1.56	0.178
E442G	2.871	3.193	0.327	3.34	3.347	0.367
P443E	0.907	0.856	0.044	0.71	0.928	0.063
P443F	1.83	2.683	0.301	2.37	2.321	0.286
P443G	4.077	9.751	0.835	2.921	4.614	0.756
Q444E	8.293	6.8	0.581	3.861	6.213	0.594
Q444H	3.823	5.746	0.486	3.936	4.71	0.513
Q444V	2.193	2.847	0.384	2.107	2.583	0.284
I445M	5.265	4.48	0.773	4.438	4.489	0.691
I445N	3.375	3.592	0.499	4.024	3.515	0.455
I445W	2.289	2.683	0.314	2.694	2.695	0.296
Y447E	2.373	2.363	0.391	2.464	2.685	0.345
Y447G	0.945	1.358	0.187	1.352	1.401	0.162
Y447P	0.991	1.379	0.19	1.383	1.49	0.183
Positive Control (OHO)	0.264	0.337	0.014	2.173	2.105	0.178
	0.262	0.136	0	4.463	4.823	0.253
	0.252	0.348	0.034	2.725	3.325	0.125
	0.235	0.432	0.116	2.883	3.158	0.522
	0.227	0.419	0.051	2.989	3.914	0.219
	0.215	0.213	0.001	5.356	3.643	0.402
	0.184	0.21	0.026	5.422	4.026	0.401
	0.168	0.114	0.004	4.385	3.327	0.54
	0.166	0.188	0.004	4.264	3.427	0.479
	0.161	0.342	0.03	4.511	4.359	0.848
	0.152	0.254	0.054	4.932	4.12	0.724
	0.148	0.286	0.042	3.494	5.179	0.841
	0.144	0.222	0.003	7.559	4.62	0.429
	0.127	0.126	0.032	3.887	3.408	0.341
	0.093	0.226	0.013	2.339	2.391	0.233
	0.052	0.102	0.036	3.473	3.056	0.277
	0.041	0.115	0	3.509	3.049	0.303

Appendix B-5

Activity Data of Table 12 $PH20_{1-447}$ Mutants

Source(s): EX1026 ('731 App.), Table 12

Mutant	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% 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
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
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

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

Mutant	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C
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
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
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

Mutant	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C
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
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
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

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

Mutant	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C
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
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
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

Mutant	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C
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	#VALUE!	#VALUE!	11.03	#VALUE!	#VALUE!	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
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
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

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

Mutant	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C	% Activity at 37°C/4°C	% Activity at 37°C + m-cresol/4°C	% Activity at 37°C + mcr/4°C
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
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
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
positive control (OHO)	105.798	4.48	4.74	108.066	5.246	5.67
positive control (OHO)	100	3.33	3.33	82.778	3.759	4.59
positive control (OHO)	94.762	19.07	18.07	109.539	16.529	18.11
positive control (OHO)	142.024	4.48	6.36	130.947	5.595	7.33
positive control (OHO)	45.115	20.77	9.37	68.017	11.035	7.51
positive control (OHO)	53.324	21.95	11.71	74.253	9.96	7.4
positive control (OHO)	59.581	25.24	15.04	75.872	16.231	12.31
positive control (OHO)	91.844	19.05	17.5	80.371	13.977	11.23
positive control (OHO)	93.828	13.47	12.63	96.63	19.454	18.8
positive control (OHO)	57.773	17.04	9.85	83.536	17.573	14.68
positive control (OHO)	100	18.56	18.56	148.226	16.239	24.07
positive control (OHO)	74.325	18.29	13.6	61.119	9.286	5.68
positive control (OHO)	98.132	8.48	8.32	87.677	10.006	8.77
positive control (OHO)	93.817	9.62	9.02	102.223	9.745	9.96
positive control (OHO)	96.922	8.56	8.3	87.993	9.064	7.98
positive control (OHO)	96.648	9.91	9.58	86.891	9.938	8.63

	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%	127	95	>120%	0	1	>120%	0	0
100-120%	58	68	100-120%	0	1	100-120%	0	1
80-100%	20	39	80-100%	1	1	80-100%	1	2
40-80%	7	8	40-80%	2	3	40-80%	6	6
20-40%	0	1	20-40%	22	17	20-40%	48	29
10-20%	0	0	10-20%	80	76	10-20%	78	78
0-10%	0	1	0-10%	107	112	0-10%	80	97
Totals	212	212		212	211		213	213

	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%	0.5990566	0.4481132	>120%	0	0.0047393	>120%	0	0
100-120%	0.2735849	0.3207547	100-120%	0	0.0047393	100-120%	0	0.0046948
80-100%	0.0943396	0.1839623	80-100%	0.004717	0.0047393	80-100%	0.0046948	0.0093897
40-80%	0.0330189	0.0377358	40-80%	0.009434	0.014218	40-80%	0.028169	0.028169
20-40%	0	0.004717	20-40%	0.1037736	0.0805687	20-40%	0.2253521	0.1361502
10-20%	0	0	10-20%	0.3773585	0.3601896	10-20%	0.3661972	0.3661972
0-10%	0	0.004717	0-10%	0.504717	0.5308057	0-10%	0.3755869	0.4553991

Appendix B-6

Selected Activity Data of Table 12 $PH20_{1-447}$ Mutants at 37°C/4°C

Source(s): EX1026 ('731 App.), Table 12

Mutant	Duplicate 1 % Activity at 37°C/4°C	Duplicate 2 % Activity at 37°C/4°C																
L001A	117.908	127.997	D090N	111.06	98.631	T232F	132.839	62.882	T289S	104.598	98.234	A371S	147.672	131.555	F433K	91.159	342.29	
L001E	107.231	125.207	K093Q	91.008	95.448	Q233G	280.488	127.368	G291S	184.581	119.565	L374P	392.038	123.033	F433R	128.958	133.353	
L001G	171.264	115.952	K093R	103.617	99.301	Q234M	95.605	80.766	G291V	112.807	151.039	E375A	88.836	134.714	F433T	161.799	134.977	
L001Q	119.435	88.763	K094D	86.544	102.107	S235A	129.818	120.916	E292C	127.307	101.126	E375G	126.88	139.03	F433V	1412.071	112.033	
L001R	117.366	160.41	K094R	125.373	108.69	V237C	138.042	116.384	E292F	137.93	132.34	E375R	163.18	116.431	F433W	149.049	113.585	
P006A	137.875	108.946	T097C	165.152	81.715	V237H	122.112	145.253	E292H	170.153	115.501	K376D	113.1	165.064	P437I	148.88	107.028	
V008M	134.884	119.772	T097D	123.654	117.522	V237T	167.105	126.02	E292R	112.278	129.89	K376E	100	153.016	M438A	106.463	135.705	
I009Q	104.922	124.934	T097E	127.19	115.106	A238E	94.878	142.167	E292V	163.075	133.274	K376Q	125.172	90	M438D	105.37	113.283	
P010G	109.772	121.986	T097L	118.465	103.589	A238H	59.585	204.683	T293A	128.197	57.524	K376R	81.687	199.112	M438E	115.061	113.782	
P010H	131.924	112.99	N104R	114.673	118.421	T240A	141.283	144.667	A298G	212.422	86.131	K376T	121.133	113.387	M438L	65.794	214.958	
N011S	152.32	131.289	A120H	94.107	113.015	T240Q	162.763	120.98	L307G	117.857	91.528	K376V	124.221	118.583	M438N	130.428	100.669	
V012E	48.208	100.163	D127R	56.439	58.702	R248A	113.237	124.65	S308D	127.652	105.846	K376Y	102.812	96.139	M438T	104.058	103.691	
V012I	128.745	170.812	V128I	113.654	102.656	E249V	142.752	111.068	S308K	126.882	99.169	G377D	110.871	132.357	E439A	137.279	95.555	
V012K	146.6	114.264	N131M	177	76.888	P257G	125.22	112.803	S308N	170.413	139.083	G377E	130.445	128.402	E439A	154.14	147.295	
F014V	154.167	146.667	N131R	94.253	95.93	K260M	116.69	97.396	I309E	123.847	129.94	G377H	146.855	104.837	E439C	193.243	111.719	
L015M	113.747	83.97	N131V	137.681	104.92	S261A	57.547	86.173	I309G	102.601	114.091	G377K	185.922	119.751	E439K	124.464	104.762	
A020S	188.889	118.821	R132L	98.578	91.685	S261K	161.931	116.159	I309L	153.681	106.948	G377R	119.708	94.749	E439P	118.34	87.446	
S022T	111.203	86.404	Q138L	107.831	91.627	S261N	142.901	35.529	I309M	123.425	136.797	G377S	108.609	101.106	E439Q	101.589	127.358	
L026M	136.775	138.989	Q140K	176.6	109.815	A267T	196.154	111.579	I309N	111.901	97.361	G377T	112.557	109.036	E439T	110.891	122.975	
K028R	110.487	109.467	N141R	103.411	115.682	F273H	122.647	119.037	I309S	169.951	551.493	F380W	147.077	104.881	T440D	118.877	79.518	
F029R	154.644	121.707	N141S	131.758	109.527	F273Y	119.713	102.772	I309T	97.936	148.864	T381S	135.827	112.559	T440H	142.296	130.928	
F029S	118.119	97.4	N141W	130.644	104.783	Q276H	74.908	106.393	I309V	113.138	138.313	R383I	527.82	98.328	T440M	84.722	86.929	
F029T	126.74	120.619	V142D	114.185	146.066	Q276M	98.323	104.948	M310G	167.656	110.739	R383S	132.894	119.466	T440P	111.931	91.205	
P032C	128.649	127.446	V142G	117.686	90.256	Q276R	121.431	150.18	M310Q	107.237	106.323	K385A	126.096	112.706	T440S	100.436	131.174	
L033G	121.201	89.571	V142K	109.485	154.599	Q276S	110.643	138.696	M313G	138.095	109.231	K385Q	137.629	124.892	E441F	129.315	110.874	
D034W	146.765	146.729	V142N	155.556	103.88	V277A	140.765	129.58	M313H	271.914	197.024	K385V	112.581	80.571	E442G	111.216	100.21	
M035V	81.285	102.034	V142P	166.998	97.338	V277E	175.779	195.598	M313K	118.882	107.111	E389A	306.767	224.872	P443E	94.377	130.704	
S036H	106.222	150.931	V142Q	149.666	99.957	V277H	129.434	137.684	M313P	103.654	132.802	E389G	113.253	139.901	P443F	146.612	97.932	
S036N	112.045	92.069	V142R	149.441	103.622	V277K	375.721	373.799	M313R	157.272	32.845	E389L	143.219	112.185	P443G	239.171	157.96	
L037M	79.268	87.376	V142S	170.778	117.035	V277M	137.138	112.084	M313T	162.074	99.844	E389Q	135.807	99.738	Q444E	81.997	160.917	
F040L	135.036	105.252	V142T	223.936	123.65	V277N	89.645	273.386	M313Y	120.038	103.011	E389S	165.62	93.03	Q444H	150.301	119.665	
I046L	132.507	112.944	Q143G	143.6	98.837	V277Q	119.93	116.151	K314S	141.924	132.112	E392A	112.465	155.693	Q444V	129.822	122.591	
N047D	115.797	111.869	Q143K	200.468	136.421	V277R	96.071	171.465	K314Y	243.011	138.931	E392F	115.619	143.781	I445M	85.09	101.149	
N047W	104.703	109.88	L144R	136.247	111.482	V277S	66.26	144.916	S315A	91.372	137.153	E392Q	112.993	93.789	I445N	106.43	87.351	
A048N	114.954	51.931	L144T	129.746	108.923	V277T	101.01	143.311	S315H	151.244	105.305	E392R	129.528	123.407	I445W	117.213	100.037	
T049R	122.704	90.76	L146P	116.626	115.601	L278E	75.408	100.179	S315Y	170.968	57.827	E392V	124.365	154.768	Y447E	99.579	108.969	
G050D	93.824	95.934	T147S	142.175	130.287	L278G	122.274	104.077	L317A	123.51	132.724	Q393F	139.966	101.647	Y447G	143.704	103.624	
G050M	157.686	139.048	T150N	140.724	116.923	K279H	138.964	123.183	L317I	187.477	110.696	Q393M	139.696	86.966	Y447P	139.152	107.737	
G052N	96.148	142.502	T150S	107.327	142	V287T	145.345	124.738	L317K	96.199	134.204	S395A	208.246	112.714				
G052T	116.407	117.075	E151A	103.31	126.047	T289S	104.598	98.234	L317N	127.382	121.233	S395H	159.975	113.401				
G052S	98.513	98.199	E151L	132.125	121.83	G291S	184.581	119.565	L317R	238.501	99.467	E396A	131.894	128.716				
V058C	92.507	99.162	E151S	115.423	136.397	G291V	112.807	151.039	L317S	90.929	85.81	E396H	210.364	128.571				
V058K	217.914	217.914	E151T	128.337	110.3	E292C	127.307	101.126	L317T	145.964	154.334	E396Q	122.977	95.938				
V058R	96.905	102.858	E151V	111.531	99.647	E292F	137.93	132.34	L317W	163.704	147.606	E396S	156.267	111.753				
V058N	129.167	129.787	E151W	158.415	94.919	E292H	170.153	115.501	L318D	105.543	97.97	Y399T	130.536	122.738				
V058Y	102.981	141.299	K152T	149.169	136.747	E292R	112.278	129.89	L318H	99.907	124.69	Y399V	110.592	116.018				
V058Q	154.383	293.51	K152W	122.313	134.039	E292V	163.075	133.274	L318R	160.469	120.872	Y399W	122.5	103.346				
V058P	83.304	173.652	E158S	133.038	102.519	T293A	128.197	57.524	N321R	164.842	112.18	S401A	122.003	99.275				
V058H	200.264	181.75	K162E	67.857	41.026	A298G	212.422	86.131	N321S	102.489	108.732	S401E	125.223	128.67				
D068P	99.07	83.721	L165F	106.283	96.667	L307G	117.857	91.528	E324N	104.618	131.265	S404A	149.379	105.443				
S069T	138.609	122.579	V166Q	155.975	117.99	S308D	127.652	105.846	T325E	124.837	106.457	L406F	122.805	146.122				
I070P	101.713	99.749	V166T	183.384	136.882	S308K	126.882	99.169	N328G	197.098	109.722	L406N	152.836	131.321				
I070V	170.462	136.849	E167D	136.745	162.637	S308N	170.413	139.083	N328Y	180.981	100	S407A	141.351	110.376				
V073Q	121.337	107.094	I169L	140.177	122.272	I309E	123.847	129.94	T335S	107.956	125.286	S407D	241.053	98.135				
V073R	137.931	100.125	K170R	160.71	97.128	T240Q	162.763	120.98	Q347A	101.395	96.213	S407P	143.308	121.898				
T074E	133.241	100.965	G172A	167.554	133.735	R248A	113.237	124.65	Q347G	222.459	207.054	A412Q	146.177	99.452				
T074M	115.29	103.629	K173R	106.771	134.3	E249V	142.752	111.068	Q349M	99.531	108.042	A412R	140.07	123.675				
T074N	91.87	157.218	L174G	114.13	264.368	P257G	125.22	112.803	Q349R	147.007	104.545	A412V	146.804	101.739				
T074P	108.323	166.227	L174N	154.332	126.186	K260M	116.69	97.396	V351S	130.819	100.857	D416L	120.82	127.662				
T074R	80.681	130	L174T	124.819	144.876	S261A	57.547	86.173	I353V	132.334	138.025	D418R	117.749	112.193				
T074V	115.093	114.063	N178K	166.871	103.154	S261K	161.931	116.159	N356H	100	130.377	A419H	241.224	188.179				
V075M	134.46	121.527	N178R	199.596	144.957	S261N	142.901	35.529	N356S	51.908	125.692	A419K	191.165	2022.616				
K082L	114.758	251.869	H193Q	213.585	138.113	A267T	196.154	111.579	S359E	135.589	135.104	D421A	102.111	301.584				
K082N	106.059	95.104	K195T	126.161	237.097	F273H	122.647	119.037	S359H	110.422	100.809	D421H	333.471	67.652				
I083V	140.151	137.296	K195N	130.253	96.381	F273Y	119.713	102.772	P367A	167.03	127.366	D421K	124.19	102.316				
I083Q	112.163	188.798	K196E	90.574	154.091	Q276H	74.908	106.393	P367G	115.683	122.642	D421N	110.806	100.116				
I083S	104.637	95.351	K196R	106.1	95.142	Q276M	98.323	104.948	P367K	125.884	66.884	D421Q	104.37	143.63				
I083G	106.239	76.381	F204P	83.571	82.418	Q276R	121.431	150.18	P367S	74.263	88.355	D421R	138.783	137.964				
S084E	124.762	113.41	N205A	139.223	102.031	Q276S	110.643	138.696	D368A	121.623	81.646	D421S	142.171	166.162				
S084F	83.291	91.007	N205E	160.93	93.313	V277A	140.765	129.58	D368E	166.628	97.937	A425G	74.81	120.947				
S084N	144.922	131.144	N205L	107.472	0	V277E	175.779	195.598	D368L	108.977	109.364	G427Q	133.135	98.243				
S084R	119.873	203.099	N205T	145.085	110.627	V277H	129.434	137.684	D368M	119.744	103.662	G427T	125.113	119.058				
Q086A	136.516	156.132	V206I	189.274	111.22	V277K	375.721	373.799	D368R	164.735	118.14	V428L	137.044	109.39				
Q086H	102.612	129.6	K209R	119.794	79.535	V277M	137.138	112.084	D368T	107.122	126.693	D431E	70.178	95.135				
Q086K	99.213	65.455	D212N	112.626	132.249	V277N	89.645	273.386	N369R	161.693	74.366	D431H	186.49	95.071				
Q086S	100.435	103.218	D212S	122.899	147.936	V277Q	119.93	116.151	A371F	180.217	76.436	D431K	240.835	68.277				
Q086T	93.837	179.465	D213A	183.83	154.77	V277R	96.071	171.465	A371H	957.055	89.541	D431L	129.149	119.177				
D087G	81.742	90.579	D213M	159.255	98.365	V277S	66.26	144.916	A371H	111.143	95.589	D431N	138.404	125.433				
D087L	106.039	101.493	S215H	109.069	78.992	V277T	101.01	143.311	A371K	136.514	114.354	D431Q	232.96	109.483				
D087M	110.964	87.656	S215M	174.883	74.943	L278E	75.408	100.179	A371L	695.108	107.003	D431S	78.069	88.796				
D087S	134.031	139.728	N219I	254.438	291.2	L278G	122.274	104.077	A371L	104.327	60.232	F433A	147.286	99.486				
D087V	114.107	87.023	E220V	131.985	113.61	K279H	138.964	123.183	A371R			F433H	140.196	87.943				
D090E	92.91	161.281	T222G	153.033	105.454	V287T	145.345	124.738	A371R	121.162	97.97	F433I	108.569	86.984				

Green = value > 100% for activity at 37°C/4°C

Note: Information in this tab was obtained from EX1026 ('731 App.), 293-303 (Table 12)

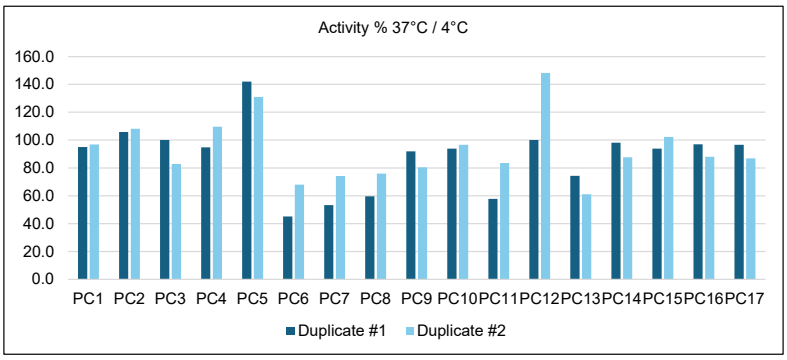
Appendix B-7

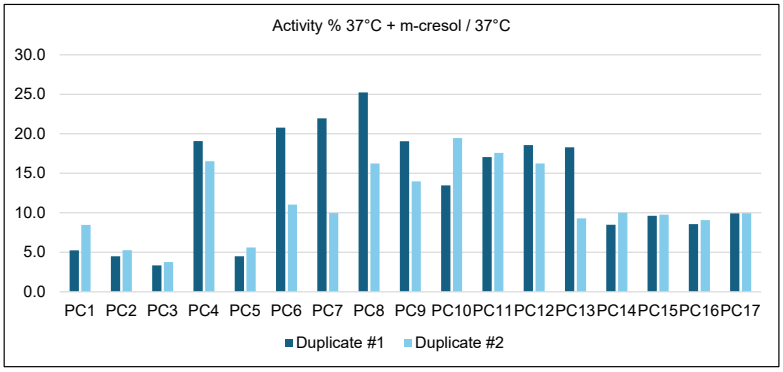
Positive Control $PH20_{1-447}$ Activity Data and Plots

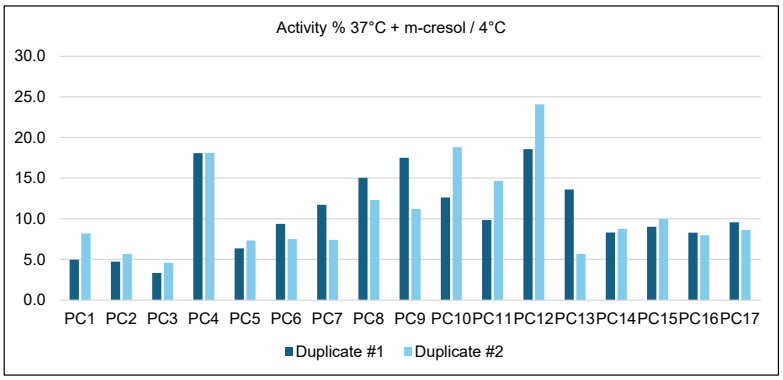
Source(s): EX1026 ('731 App.), Table 12

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





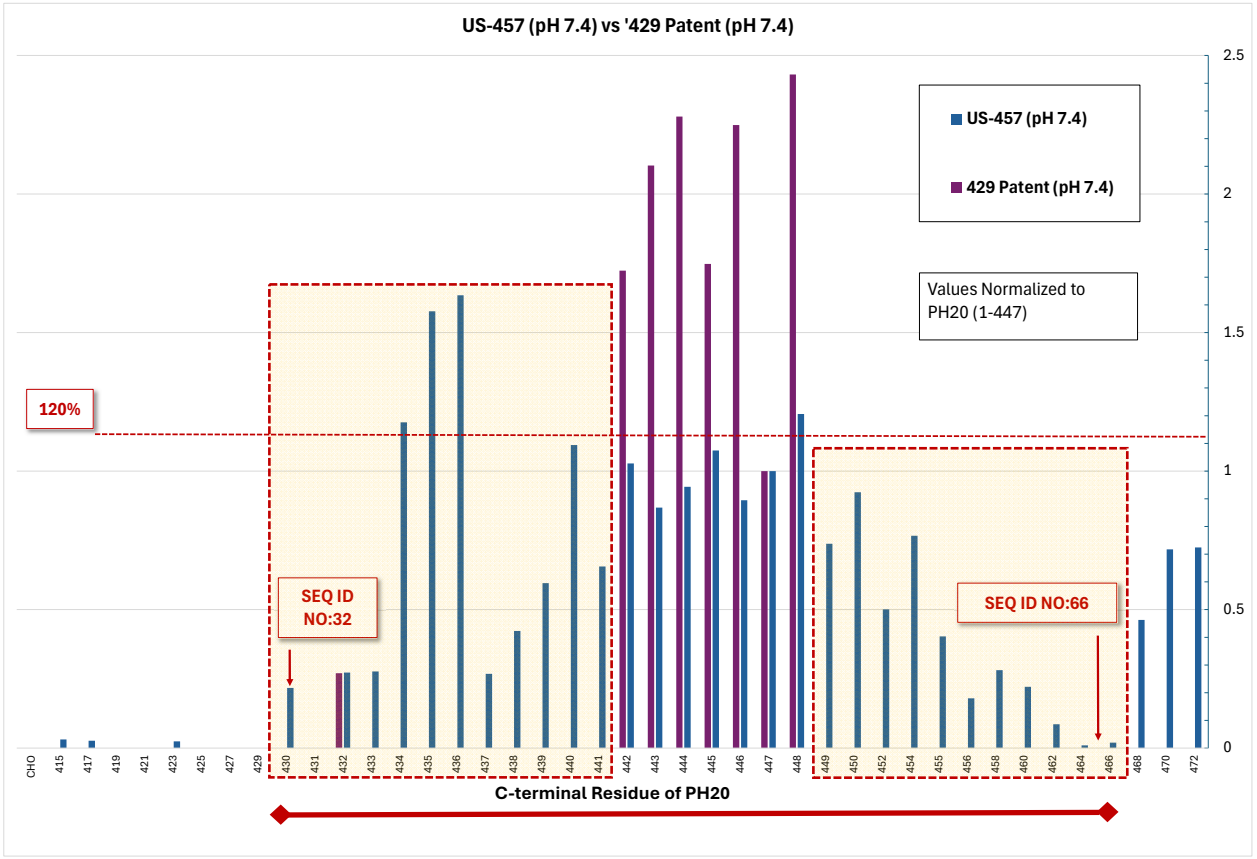


Appendix B-8

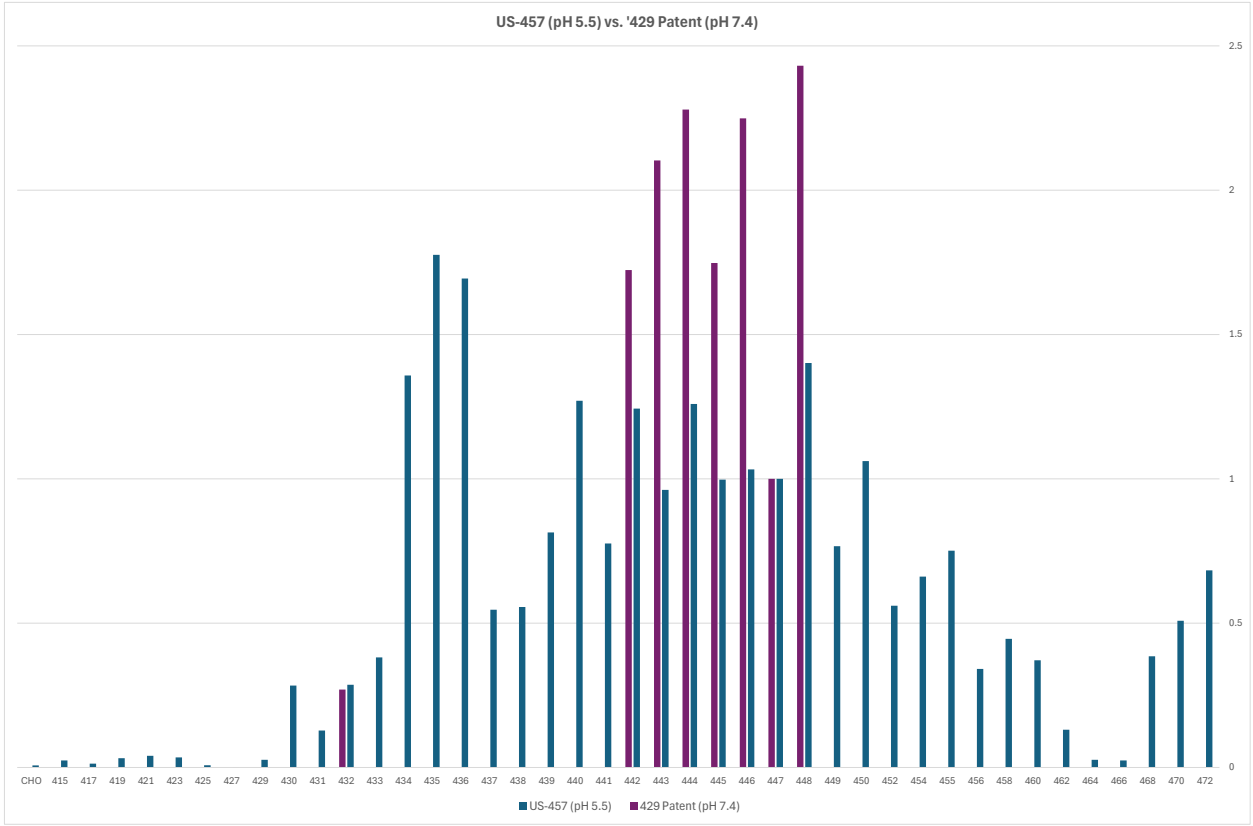
Activity Data Plot of C-terminal Truncation PH20 Mutant from US-457 and the '429 Patent

Source(s): EX1105 (US-457), EX1005 ('429 Patent)

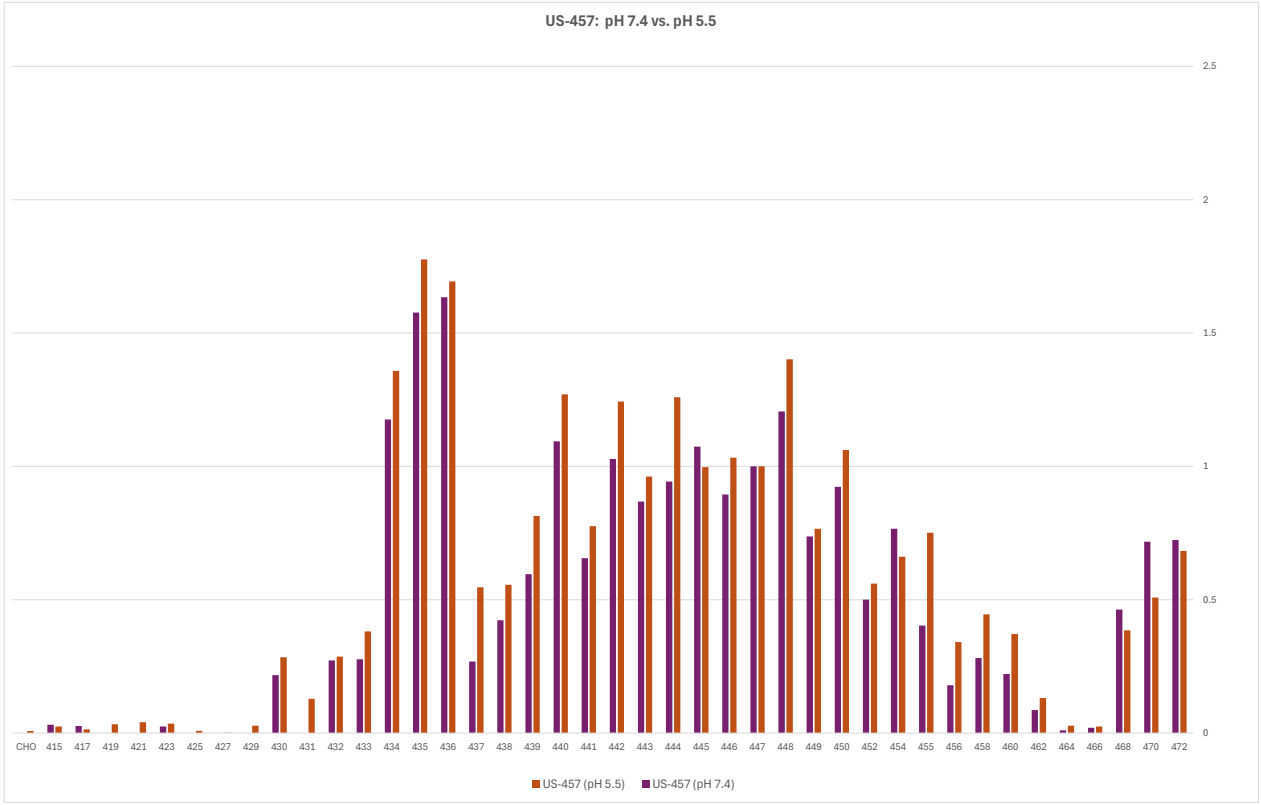
Deletion Mutant	Precursor	Mature	US-457 (pH 7.4)	429 Patent (pH 7.4)	US-457 pH7.4 (Units/ml)
SPAM1-SSVA	507	472	72.4%		1.4715
SPAM1-IISS	505	470	71.8%		1.458
SPAM1-FLII	503	468	46.3%		0.9405
SPAM1-ILFL	501	466	2.0%		0.0405
SPAM1-VSIL	499	464	1.0%		0.02025
SPAM1-FIVS	497	462	8.6%		0.1755
SPAM1-TMFI	495	460	22.1%		0.45
SPAM1-SATM	493	458	28.1%		0.5715
SPAM1-TLSA	491	456	17.9%		0.3645
SPAM1-STLS	490	455	40.3%		0.819
SPAM1-PSTL	489	454	76.6%		1.557
SPAM1-ASPS	487	452	50.1%		1.017
SPAM1-YNAS	485	450	92.4%		1.8765
SPAM1-FYNA	484	449	73.8%		1.4985
SPAM1-IFYN	483	448	120.6%	243.2%	2.45025
SPAM1-QIFY	482	447	100.0%	100.0%	2.03175
SPAM1-PQIF	481	446	89.5%	224.9%	1.818
SPAM1-EPQI	480	445	107.4%	174.8%	2.1825
SPAM1-EEPQ	479	444	94.4%	228.0%	1.917
SPAM1-TEEP	478	443	86.8%	210.3%	1.764
SPAM1-ETEE	477	442	102.8%	172.3%	2.088
SPAM1-METE	476	441	65.6%		1.332
SPAM1-PMET	475	440	109.4%		2.223
SPAM1-PPME	474	439	59.6%		1.2105
SPAM1-KPPM	473	438	42.3%		0.8595
SPAM1-LKPP	472	437	26.8%		0.5445
SPAM1-FLKP	471	436	163.5%		3.321
SPAM1-AFLK	470	435	157.7%		3.204
SPAM1-DAFL	469	434	117.6%		2.3895
SPAM1-IDAF	468	433	27.7%		0.5625
SPAM1-CIDA	467	432	27.2%	27.1%	0.5535
SPAM1-VCID	466	431	0.0%		0
SPAM1-GVCI	465	430	21.7%		0.441
SPAM1-DGVC	464	429	0.0%		0
SPAM1-IADG	462	427	0.0%		0
SPAM1-VCIA	460	425	0.0%		0
SPAM1-VDVC	458	423	2.4%		0.0495
SPAM1-DAVD	456	421	0.0%		0
SPAM1-DTDA	454	419	0.0%		0
SPAM1-VKDT	452	417	2.7%		0.054
SPAM1-ADVK	450	415	3.1%		0.063
CHO-S	CHO	CHO	0.0%		0



Deletion Mutant	Precursor	Mature	US-457 (pH 5.5)	429 Patent (pH 7.4)	US-457 (pH 5.5)(Units/ml)
SPAM1-SSVA	507	472	68.3%		1.125
SPAM1-IISS	505	470	50.8%		0.837
SPAM1-FLII	503	468	38.5%		0.6345
SPAM1-ILFL	501	466	2.5%		0.0405
SPAM1-VSIL	499	464	2.7%		0.045
SPAM1-FIVS	497	462	13.1%		0.216
SPAM1-TMFI	495	460	37.2%		0.612
SPAM1-SATM	493	458	44.5%		0.7335
SPAM1-TLSA	491	456	34.2%		0.5625
SPAM1-STLS	490	455	75.1%		1.2375
SPAM1-PSTL	489	454	66.1%		1.089
SPAM1-ASPS	487	452	56.0%		0.9225
SPAM1-YNAS	485	450	106.1%		1.74825
SPAM1-FYNA	484	449	76.6%		1.26225
SPAM1-IFYN	483	448	140.2%	243.2%	2.3085
SPAM1-QIFY	482	447	100.0%	100.0%	1.647
SPAM1-PQIF	481	446	103.3%	224.9%	1.701
SPAM1-EPQI	480	445	99.7%	174.8%	1.6425
SPAM1-EEPQ	479	444	126.0%	228.0%	2.0745
SPAM1-TEEP	478	443	96.2%	210.3%	1.584
SPAM1-ETEE	477	442	124.3%	172.3%	2.0475
SPAM1-METE	476	441	77.6%		1.278
SPAM1-PMET	475	440	127.0%		2.0925
SPAM1-PPME	474	439	81.4%		1.341
SPAM1-KPPM	473	438	55.6%		0.91575
SPAM1-LKPP	472	437	54.6%		0.9
SPAM1-FLKP	471	436	169.4%		2.79
SPAM1-AFLK	470	435	177.6%		2.925
SPAM1-DAFL	469	434	135.8%		2.2365
SPAM1-IDAF	468	433	38.1%		0.62775
SPAM1-CIDA	467	432	28.7%	27.1%	0.4725
SPAM1-VCID	466	431	12.8%		0.2115
SPAM1-GVCI	465	430	28.4%		0.468
SPAM1-DGVC	464	429	2.7%		0.045
SPAM1-IADG	462	427	0.1%		0.00225
SPAM1-VCIA	460	425	0.8%		0.0135
SPAM1-VDVC	458	423	3.6%		0.0585
SPAM1-DAVD	456	421	4.1%		0.0675
SPAM1-DTDA	454	419	3.3%		0.054
SPAM1-VKDT	452	417	1.4%		0.0225
SPAM1-ADVK	450	415	2.5%		0.0405
CHO-S	CHO	CHO	0.8%		0.012375



Deletion Mutant	Precursor	Mature	US-457 (pH 5.5)	US-457 (pH 7.4)	US 457 - 7.4 vs. 5.5
SPAM1-SSVA	507	472	68.3%	72.4%	130.8%
SPAM1-IISS	505	470	50.8%	71.8%	174.2%
SPAM1-FLII	503	468	38.5%	46.3%	148.2%
SPAM1-ILFL	501	466	2.5%	2.0%	100.0%
SPAM1-VSIL	499	464	2.7%	1.0%	45.0%
SPAM1-FIVS	497	462	13.1%	8.6%	81.3%
SPAM1-TMFI	495	460	37.2%	22.1%	73.5%
SPAM1-SATM	493	458	44.5%	28.1%	77.9%
SPAM1-TLSA	491	456	34.2%	17.9%	64.8%
SPAM1-STLS	490	455	75.1%	40.3%	66.2%
SPAM1-PSTL	489	454	66.1%	76.6%	143.0%
SPAM1-ASPS	487	452	56.0%	50.1%	110.2%
SPAM1-YNAS	485	450	106.1%	92.4%	107.3%
SPAM1-FYNA	484	449	76.6%	73.8%	118.7%
SPAM1-IFYN	483	448	140.2%	120.6%	106.1%
SPAM1-QIFY	482	447	100.0%	100.0%	123.4%
SPAM1-PQIF	481	446	103.3%	89.5%	106.9%
SPAM1-EPQI	480	445	99.7%	107.4%	132.9%
SPAM1-EEPQ	479	444	126.0%	94.4%	92.4%
SPAM1-TEEP	478	443	96.2%	86.8%	111.4%
SPAM1-ETEE	477	442	124.3%	102.8%	102.0%
SPAM1-METE	476	441	77.6%	65.6%	104.2%
SPAM1-PMET	475	440	127.0%	109.4%	106.2%
SPAM1-PPME	474	439	81.4%	59.6%	90.3%
SPAM1-KPPM	473	438	55.6%	42.3%	93.9%
SPAM1-LKPP	472	437	54.6%	26.8%	60.5%
SPAM1-FLKP	471	436	169.4%	163.5%	119.0%
SPAM1-AFLK	470	435	177.6%	157.7%	109.5%
SPAM1-DAFL	469	434	135.8%	117.6%	106.8%
SPAM1-IDAF	468	433	38.1%	27.7%	89.6%
SPAM1-CIDA	467	432	28.7%	27.2%	117.1%
SPAM1-VCID	466	431	12.8%	0.0%	0.0%
SPAM1-GVCI	465	430	28.4%	21.7%	94.2%
SPAM1-DGVC	464	429	2.7%	0.0%	0.0%
SPAM1-IADG	462	427	0.1%	0.0%	0.0%
SPAM1-VCIA	460	425	0.8%	0.0%	0.0%
SPAM1-VDVC	458	423	3.6%	2.4%	84.6%
SPAM1-DAVD	456	421	4.1%	0.0%	0.0%
SPAM1-DTDA	454	419	3.3%	0.0%	0.0%
SPAM1-VKDT	452	417	1.4%	2.7%	240.0%
SPAM1-ADVK	450	415	2.5%	3.1%	155.6%
CHO-S	CHO	CHO	0.8%	0.0%	0.0%



Note:
The information in this sheet was obtained from Published U.S. Patent Application 2010/0143457 ("US-457"), Example 5, ¶¶ 360-362 (Table 6).

Petitioner Merck
EX1003, p. 289

Appendix B-9

Activity Classification of US-457 C-terminal Truncation PH20 Mutants

Source(s): EX1105 (US-457), EX1005 ('429 Patent)

Terminus (precursor)	Terminus (mature)	pH 7.4	pH 5.5	Normalized Activity (pH 7.4)	Normalized Activity (pH 5.5)	US-457 Classification	Table
482	**447**	**2.03175**	**1.647**	**100%**	**100%**	**Active**	**6**
465	**430**	0.441	0.468	22%	28%	Active	6
466	**431**	0.000	0.212	0%	13%	Active	6
467	**432**	0.545	0.900	27%	55%	Active	6
468	**433**	0.860	0.916	42%	56%	Active	6
472	**437**	0.545	0.900	27%	55%	Active	6
490	**455**	0.819	1.238	40%	75%	Active	6
491	**456**	0.365	0.563	18%	34%	Active	6
491	**456**	0.851	0.729	42%	44%	Active	6A
492	**457**	n/t	n/t	n/a	n/a	-	none
493	**458**	0.689	0.851	34%	52%	Active	6
494	**459**	n/t	n/t	n/a	n/a	-	none
495	**460**	0.689	0.770	34%	47%	Active	6
496	**461**	n/t	n/t	n/a	n/a	-	none
497	**462**	0.176	0.216	9%	13%	Active	6
497	**462**	0.365	0.162	18%	10%	Active	6A
498	**463**	0.527	0.203	26%	12%	Low expression	6A
499	**464**	0.020	0.045	1%	3%	Low expression	6
499	**464**	0.486	0.122	24%	7%	Low expression	6A
500	**465**	0.365	0.162	18%	10%	Low expression	6A
501	**466**	0.446	0.122	22%	7%	Active	6
502	**467**	0.608	0.324	30%	20%	Active	6
503	**468**	1.094	0.648	54%	39%	Active	6

Note:
The information in this sheet is from Published U.S. Patent Application 2010/0143457 ("US-457"), Example 5, ¶¶ 360-362 (Table 6).

Appendix B-10

PH20 Positional Alignment: Domains, Motifs, and Prior Art

Source(s): EX1026 (’731 App.), EX1006 (Chao), EX1011 (Arming)

Precursor	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
Mature	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
Residue	L	N	F	R	A	P	P	V	I	P	N	V	P	F	L	W	A	W	N	A	P	S	E	F	C	L	G	K	F	D

Precursor	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95
Mature	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
Residue	E	P	L	D	M	S	L	F	S	F	I	G	S	P	R	I	N	A	T	G	Q	G	V	T	I	F	Y	V	D	R

Precursor	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
Mature	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**	**85**	**86**	**87**	**88**	**89**	**90**
Residue	L	G	Y	Y	P	Y	I	D	S	I	T	G	V	T	V	N	G	G	I	P	Q	K	I	S	L	Q	D	H	L	D

Precursor	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155
Mature	**91**	**92**	**93**	**94**	**95**	**96**	**97**	**98**	**99**	**100**	**101**	**102**	**103**	**104**	**105**	**106**	**107**	**108**	**109**	**110**	**111**	**112**	**113**	**114**	**115**	**116**	**117**	**118**	**119**	**120**
Residue	K	A	K	K	D	I	T	F	Y	M	P	V	D	N	L	G	M	A	V	I	D	W	E	E	W	R	P	T	W	A

Precursor	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185
Mature	**121**	**122**	**123**	**124**	**125**	**126**	**127**	**128**	**129**	**130**	**131**	**132**	**133**	**134**	**135**	**136**	**137**	**138**	**139**	**140**	**141**	**142**	**143**	**144**	**145**	**146**	**147**	**148**	**149**	**150**
Residue	R	N	W	K	P	K	D	V	Y	K	N	R	S	I	E	L	V	Q	Q	Q	N	V	Q	L	S	L	T	E	A	T

Precursor	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215
Mature	**151**	**152**	**153**	**154**	**155**	**156**	**157**	**158**	**159**	**160**	**161**	**162**	**163**	**164**	**165**	**166**	**167**	**168**	**169**	**170**	**171**	**172**	**173**	**174**	**175**	**176**	**177**	**178**	**179**	**180**
Residue	E	K	A	K	Q	E	F	E	K	A	G	K	D	F	L	V	E	T	I	K	L	G	K	L	L	R	P	N	H	L

Precursor	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245
Mature	**181**	**182**	**183**	**184**	**185**	**186**	**187**	**188**	**189**	**190**	**191**	**192**	**193**	**194**	**195**	**196**	**197**	**198**	**199**	**200**	**201**	**202**	**203**	**204**	**205**	**206**	**207**	**208**	**209**	**210**
Residue	W	G	Y	Y	L	F	P	D	C	Y	N	H	H	Y	K	K	P	G	Y	N	G	S	C	F	N	V	E	I	K	R

Precursor	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275
Mature	**211**	**212**	**213**	**214**	**215**	**216**	**217**	**218**	**219**	**220**	**221**	**222**	**223**	**224**	**225**	**226**	**227**	**228**	**229**	**230**	**231**	**232**	**233**	**234**	**235**	**236**	**237**	**238**	**239**	**240**
Residue	N	D	D	L	S	W	L	W	N	E	S	T	A	L	Y	P	S	I	Y	L	N	T	Q	Q	S	P	V	A	A	T

Precursor	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305
Mature	**241**	**242**	**243**	**244**	**245**	**246**	**247**	**248**	**249**	**250**	**251**	**252**	**253**	**254**	**255**	**256**	**257**	**258**	**259**	**260**	**261**	**262**	**263**	**264**	**265**	**266**	**267**	**268**	**269**	**270**
Residue	L	Y	V	R	N	R	V	R	E	A	I	R	V	S	K	I	P	D	A	K	S	P	L	P	V	F	A	Y	T	R

Precursor	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335
Mature	**271**	**272**	**273**	**274**	**275**	**276**	**277**	**278**	**279**	**280**	**281**	**282**	**283**	**284**	**285**	**286**	**287**	**288**	**289**	**290**	**291**	**292**	**293**	**294**	**295**	**296**	**297**	**298**	**299**	**300**
Residue	I	V	F	T	D	Q	V	L	K	F	L	S	Q	D	E	L	V	Y	T	F	G	E	T	V	A	L	G	A	S	G

Precursor	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365
Mature	**301**	**302**	**303**	**304**	**305**	**306**	**307**	**308**	**309**	**310**	**311**	**312**	**313**	**314**	**315**	**316**	**317**	**318**	**319**	**320**	**321**	**322**	**323**	**324**	**325**	**326**	**327**	**328**	**329**	**330**
Residue	I	V	I	W	G	T	L	S	I	M	R	S	M	K	S	C	L	L	L	D	N	Y	M	E	T	I	L	N	P	Y

Precursor	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395
Mature	**331**	**332**	**333**	**334**	**335**	**336**	**337**	**338**	**339**	**340**	**341**	**342**	**343**	**344**	**345**	**346**	**347**	**348**	**349**	**350**	**351**	**352**	**353**	**354**	**355**	**356**	**357**	**358**	**359**	**360**
Residue	I	I	N	V	T	L	A	A	K	M	C	S	Q	V	L	C	Q	E	Q	G	V	C	I	R	K	N	W	N	S	S

Precursor	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425
Mature	**361**	**362**	**363**	**364**	**365**	**366**	**367**	**368**	**369**	**370**	**371**	**372**	**373**	**374**	**375**	**376**	**377**	**378**	**379**	**380**	**381**	**382**	**383**	**384**	**385**	**386**	**387**	**388**	**389**	**390**
Residue	D	Y	L	H	L	N	P	D	N	F	A	I	Q	L	E	K	G	G	K	F	T	V	R	G	K	P	T	L	E	D

Precursor	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455
Mature	**391**	**392**	**393**	**394**	**395**	**396**	**397**	**398**	**399**	**400**	**401**	**402**	**403**	**404**	**405**	**406**	**407**	**408**	**409**	**410**	**411**	**412**	**413**	**414**	**415**	**416**	**417**	**418**	**419**	**420**
Residue	L	E	Q	F	S	E	K	F	Y	C	S	C	Y	S	T	L	S	C	K	E	K	A	D	V	K	D	T	D	A	V

Precursor	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482
Mature	**421**	**422**	**423**	**424**	**425**	**426**	**427**	**428**	**429**	**430**	**431**	**432**	**433**	**434**	**435**	**436**	**437**	**438**	**439**	**440**	**441**	**442**	**443**	**444**	**445**	**446**	**447**
Residue	D	V	C	I	A	D	G	V	C	I	D	A	F	L	K	P	P	M	E	T	E	E	P	Q	I	F	Y

Note

Dark Yellow - Hyal-EGF Domain (L336-D416)

Red - site of truncation reported in EX1011 (Arming), 813.

Light Blue (position numbers) - common hyaluronidase domain (3-339) (EX1026 ('731 App.), 79:20-31)

Purple - peptide 2 binding domain (170-200) (EX1026 ('731 App.), 81:7-14)

Appendix B-11

Essential Residues in $PH20_{1-447}$ Identified in the Prior Art

Source(s): EX1026 ('731 App.)

Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT
1	L	51	Q	101	P	151	E	201	G	251	I	301	I	351	V	401	S
2	N	52	G	102	V	152	K	202	S	252	R	302	V	352	C	402	C
3	F	53	V	103	D	153	A	203	C	253	V	303	I	353	I	403	Y
4	R	54	T	104	N	154	K	204	F	254	S	304	W	354	R	404	S
5	A	55	I	105	L	155	Q	205	N	255	K	305	G	355	K	405	T
6	P	56	F	106	G	156	E	206	V	256	I	306	T	356	N	406	L
7	P	57	Y	107	M	157	F	207	E	257	P	307	L	357	W	407	S
8	V	58	V	108	A	158	E	208	I	258	D	308	S	358	N	408	C
9	I	59	D	109	V	159	K	209	K	259	A	309	I	359	S	409	K
10	P	60	R	110	I	160	A	210	R	260	K	310	M	360	S	410	E
11	N	61	L	111	D	161	G	211	N	261	S	311	R	361	D	411	K
12	V	62	G	112	W	162	K	212	D	262	P	312	S	362	Y	412	A
13	P	63	Y	113	E	163	D	213	D	263	L	313	M	363	L	413	D
14	F	64	Y	114	E	164	F	214	L	264	P	314	K	364	H	414	V
15	L	65	P	115	W	165	L	215	S	265	V	315	S	365	L	415	K
16	W	66	Y	116	R	166	V	216	W	266	F	316	C	366	N	416	D
17	A	67	I	117	P	167	E	217	L	267	A	317	L	367	P	417	T
18	W	68	D	118	T	168	T	218	W	268	Y	318	L	368	D	418	D
19	N	69	S	119	W	169	I	219	N	269	T	319	L	369	N	419	A
20	A	70	I	120	A	170	K	220	E	270	R	320	D	370	F	420	V
21	P	71	T	121	R	171	L	221	S	271	I	321	N	371	A	421	D
22	S	72	G	122	N	172	G	222	T	272	V	322	Y	372	I	422	V
23	E	73	V	123	W	173	K	223	A	273	F	323	M	373	Q	423	C
24	F	74	T	124	K	174	L	224	L	274	T	324	E	374	L	424	I
25	C	75	V	125	P	175	L	225	Y	275	D	325	T	375	E	425	A
26	L	76	N	126	K	176	R	226	P	276	Q	326	I	376	K	426	D
27	G	77	G	127	D	177	P	227	S	277	V	327	L	377	G	427	G
28	K	78	G	128	V	178	N	228	I	278	L	328	N	378	G	428	V
29	F	79	I	129	Y	179	H	229	Y	279	K	329	P	379	K	429	C
30	D	80	P	130	K	180	L	230	L	280	F	330	Y	380	F	430	I
31	E	81	Q	131	N	181	W	231	N	281	L	331	I	381	T	431	D
32	P	82	K	132	R	182	G	232	T	282	S	332	I	382	V	432	A
33	L	83	I	133	S	183	Y	233	Q	283	Q	333	N	383	R	433	F
34	D	84	S	134	I	184	Y	234	Q	284	D	334	V	384	G	434	L
35	M	85	L	135	E	185	L	235	S	285	E	335	T	385	K	435	K
36	S	86	Q	136	L	186	F	236	P	286	L	336	L	386	P	436	P
37	L	87	D	137	V	187	P	237	V	287	V	337	A	387	T	437	P
38	F	88	H	138	Q	188	D	238	A	288	Y	338	A	388	L	438	M
39	S	89	L	139	Q	189	C	239	A	289	T	339	K	389	E	439	E
40	F	90	D	140	Q	190	Y	240	T	290	F	340	M	390	D	440	T
41	I	91	K	141	N	191	N	241	L	291	G	341	C	391	L	441	E
42	G	92	A	142	V	192	H	242	Y	292	E	342	S	392	E	442	E
43	S	93	K	143	Q	193	H	243	V	293	T	343	Q	393	Q	443	P
44	P	94	K	144	L	194	Y	244	R	294	V	344	V	394	F	444	Q
45	R	95	D	145	S	195	K	245	N	295	A	345	L	395	S	445	I
46	I	96	I	146	L	196	K	246	R	296	L	346	C	396	E	446	F
47	N	97	T	147	T	197	P	247	V	297	G	347	Q	397	K	447	Y
48	A	98	F	148	E	198	G	248	R	298	A	348	E	398	F		
49	T	99	Y	149	A	199	Y	249	E	299	S	349	Q	399	Y		
50	G	100	M	150	T	200	N	250	A	300	G	350	G	400	C		

Note:
Yellow shading denotes positions identified in the prior art / confirmed essential by the disclosure (EX1026 ('731 App.), 80:19-35).

Appendix B-12

Comparison of Disclosed US-275 PH20 Mutant Data to the '400 Patent

Source(s): EX1001 ('400 Patent), EX1004 (US-275)

Position	US-275 Table 9 - Claim 1 Single Substitution $PH20_{1-447}$ Mutants Reported Having 120% or Greater Activity													Sum
1	H	K	Q											3
15	V													1
24	H	R	E											3
27	D	K	R											3
29	E	I	K	L	M	P	R	S	V					9
30	G	H	K	L	M	R	S							7
31	A	C	G	H	I	K	L	P	R	S	T	V	W	13
32	F	G	H											3
33	W													1
37	F	M												2
39	N	T												2
48	F	H	K	N										4
63	K	L	M	R	W									5
67	V													1
69	A	C	E	F	G	I	L	M	P	R	W	Y		12
71	G	N	R	S										4
72	K	M	Q											3
73	A	H	K	L	T	W								6
75	F	L	Q	R	T									5
87	C	R	T	Y										4
92	C	L												2
93	I	L	R	S	T									5
94	Q	R												2
118	Q													1
120	F	I	L	S	V	Y								6
135	D	G	Q	R										4
141	H	R	Y											3
147	A	F	L	Q	R	S	V							7
148	H	K	Q											3
150	A	D	G	W	Y									5
151	A	H	K	M	Q	R	S	T	V	W	Y			11
152	R	T												2
155	D	G	K	R										4
156	D													1
163	A	E	K	Q	R	S								6
164	M	V												2
165	D	F	N	S	V									5
170	R													1
198	D													1
209														0
212	N													1
215	M													1
219	A	K	S											3
233	K	R												2
236	R													1
247	I													1
257	R	T												2
259	P													1
260	Y													1
263	K	R												2
269	A													1
271	G	L	M											3
272	D	T												2
276	D	H	R	S	Y									5
278	F	G	H	K	N	R	S	T	Y					9
282	M													1
293	A	C	D	F	K	M	P	Q	V	Y				10
305	E													1
308	G	K	N	R										4
313	A	H	K	R										4
320	H	K	R											3
324	R													1
325	A	D	E	G	H	K	M	N	Q	S	V			11
326	L	V												2
328	C	I	K	L	S	Y								6
353	V													1
359	T													1
371	H	K	R	S										4
377	D	H	K	P	R	S	T							7
380	W	Y												2
389	K													1
392	M	V												2
395	R													1
405	G													1
409	A	Q												2
410	T													1
418	E	L	P	V										4
425	G	K												2
436	K													1
437	I	M												2
438	T													1
441	A	F												2
442	C	R												2
446	A	H	M	W										4
447	D	E	F	I	N	P	Q	T	V					9
													Total	290

Note: Information in this sheet was compiled from US-275 [0626] (Table 9)

Yellow shading designates single substitution mutant replacements that are also recited in claim 2 of the '400 Patent.

Position	US-275 Table 9 - Claim 2 Single Substitution PH20 $_{1-447}$ Mutants Reported Having 120% or Greater Activity													Sum
1			Q											1
15	V													1
24	H		E											2
27		K	R											2
29	E	I		L	M	P		S	V					7
30	G	H	K		M	R	S							6
31	A	C		H	I	K	L	P	R	S	T	V		11
32	F	G	H											3
33	W													1
37	F													1
39	N	T												2
48	F	H		N										3
63					W									1
67	V													1
69	A	C		F	G	I	L	M	P	R	W	Y		11
71			R	S										2
72		M	Q											2
73		H		L		W								3
75	F	L		R	T									4
87	C		T	Y										3
92	C													1
93	I	L	R		T									4
94		R												1
118	Q													1
120	F				V	Y								3
135	D	G		R										3
141	H		Y											2
147					R		V							2
148		K												1
150			G											1
151			K	M	Q	R								4
152	R													1
155		G	K											2
156	D													1
163	A	E	K		R									4
164	M	V												2
165	D		N											2
170	R													1
198	D													1
209														0
212	N													1
215	M													1
219			S											1
233	K	R												2
236	R													1
247	I													1
257		T												1
259	P													1
260	Y													1
263	K	R												2
269	A													1
271		L	M											2
272		T												1
276	D			S	Y									3
278			H	K	N	R	S	T	Y					7
282	M													1
293	A	C		F		M	P	Q	V					7
305	E													1
308	G		N											2
313			K	R										2
320	H	K	R											3
324	R													1
325	A	D	E	G	H	K	M	N	Q	S				10
326		V												1
328		I	K	L	S	Y								5
353	V													1
359	T													1
371			R											1
377				P			T							2
380	W	Y												2
389	K													1
392	M													1
395	R													1
405	G													1
409	A	Q												2
410	T													1
418			P											1
425		K												1
436	K													1
437	I	M												2
438	T													1
441		F												1
442		R												1
446	A													1
447	D				N		Q							3
													Total	189

Note: Information in this sheet was compiled from US-275 [0626] (Table 9)

Position	US-275 Table 9 - Claim 4 Single Substitution $PH20_{1-447}$ Mutants Reported Having 120% or Greater Activity													Sum
12	E													1
26	E	K												2
46	R													1
52	Q													1
58	Q													1
68	H	Q												2
70	A	C	F	G	H	K	L	N	P	R	S	T		12
74	A	C	G	N	P	R	S	V	W					9
84	G	R												2
97	G													1
127	H	N												2
131	G	V												2
142	R													1
166	A	F	H	L	R	Y								6
174	K													1
206	K													1
213	M	N												2
237	E													1
238	S													1
261	N													1
277	R	T												2
309	L	N	Q	R	T									5
310	A	G												2
315	H													1
317	I	K	R											3
318	M													1
347	G	S												2
399	M	T	W											3
407	D	Q												2
419	F	I	R	S										4
421	K	N	Q	R	S									5
431	A	H	K	Q	R	S	V							7
433	L	R												2
439	V													1
440	H	R												2
443	A	M												2
445	M	P												2
													Total	**96**

Note: Information in this sheet was compiled from US-275 [0626] (Table 9)

Position	US-275 Table 9 - Claim 6 Single Substitution PH20 1-447 Mutants Reported Having 200% or Greater Activity													Sum
1			Q											1
15	V													1
24	H		E											2
27		K												1
29	E			L	M	P		S						5
30	G		K											2
31	A	C		H	I	K	L		R		T	V		9
32	F		H											2
33														0
37	F													1
39	N													1
48	F			N										2
63														0
67														0
69	A			F	G	I	L	M	P	R	W	Y		10
71			R											1
72		M	Q											2
73														0
75	F	L		R	T									4
87				Y										1
92														0
93	I				T									2
94		R												1
118	Q													1
120	F													1
135	D	G		R										3
141	H													1
147					R		V							2
148														0
150			G											1
151			K		Q									2
152														0
155														0
156														0
163														0
164														0
165	D		N											2
170	R													1
198														0
209														0
212														0
215														0
219			S											1
233														0
236	R													1
247	I													1
257		T												1
259														0
260														0
263	K													1
269														0
271		L	M											2
272														0
276														0
278			H			R								2
282	M													1
293						M			V					2
305														0
308			N											1
313			K	R										2
320		K	R											2
324	R													1
325			E	G	H	K	M	N	Q	S				8
326		V												1
328			K	L	S									3
353														0
359	T													1
371														0
377				P			T							2
380	W													1
389														0
392														0
395														0
405	G													1
409	A	Q												2
410														0
418			P											1
425														0
436	K													1
437	I	M												2
438	T													1
441		F												1
442		R												1
446														0
447	D						Q							2
													Total	108

Note: Information in this sheet was compiled from US-275 [0626] (Table 9)

Appendix B-13

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

Source(s): EX1026 ('731 App.)

		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-14

Summary of Illustrative Disclosures in US-275 of ’400 Patent Claim Limitations

Source(s): EX1001 (’400 Patent), EX1004 (US-275)

Appendix B-14
Summary of Illustrative Disclosures in US-275 of '400 Patent Claim Limitations

U.S. Patent No. 10,865,400	
Claim 1	
1. A modified PH20 polypeptide, comprising one or more amino acid replacements in an unmodified PH20 polypeptide, wherein: the unmodified PH20 polypeptide consists of the amino acid sequence selected from the group consisting of SEQ ID NOS: 3, 7 and 32-66;	US-275 discloses modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants, which each contain a single amino acid replacement relative to SEQ ID NO:3 (the "unmodified PH20 polypeptide"). *See* EX1004, [0606], Table 9 ("The first library was made to generate encoded variant proteins wherein each of residues … 1-447 of SEQ ID NO:3 [] was changed to ***one*** of about 15 amino acid residues, such that each member contained ***a single amino change***.") (emphasis added). Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of the preamble of claim 1.
1(a). the modified PH20 polypeptide exhibits increased hyaluronidase activity that is at least 120% of the hyaluronidase activity compared to the unmodified PH20 polypeptide not containing the amino acid replacements); wherein:	US-275 discloses modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants, which each contain a single amino acid replacement and exhibit 120% or greater hyaluronidase activity compared to the unmodified PH20 polypeptide that does not contain the amino acid replacement. EX1004, Table 9, [0626] ("For example, about 536 mutants exhibit 120% or greater than 120% of the hyaluronidase activity of wildtype PH20…"), [0011] ("In some instances, among the PH20 polypeptides provided herein, the modified PH20 polypeptide exhibits at least 120%, 130%..."). *See e.g.*, EX1004, [0626] (identifying increased activity of "mutant I271L is about 10-fold"); [0019]; Table 9 (p. 128, 131, 133) (identifying I271L as having 1,062% relative activity, M313K as having 285% relative activity, and D320K as having 642% relative activity).

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	Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 1(a).
1(b). the activity of the unmodified PH20 polypeptide not containing the replacement(s) is 100%; hyaluronidase activity of the modified PH20 polypeptide and hyaluronidase activity of the unmodified PH20 polypeptide are measured under identical conditions;	US-275 discloses modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants. *See* EX1004, [0606], Table 9. The activity value for each of these modified PH20 polypeptides is provided as a percent of the hyaluronidase activity of the modified PH20 polypeptide compared to that of the wildtype PH20, which lacks the amino acid replacement. *See* EX1004, [0623]-[0626]. The activity of the wildtype PH20 is 100%. *See* EX1004, [0625] ("For example, a value of 0.40 indicates that the variant exhibits 40% of the hyaluronidase activity of wildtype PH20, a value of 1 indicates that the variant exhibits a similar hyaluronidase activity of wildtype…"). The activity levels of these modified PH20 polypeptides were measured under identical conditions. *See* EX1004, [0623]-[0626] ("Each generated variant was screened for hyaluonidase [sic] activity as described in Example 3."); *see generally* EX1004, Examples 3 and 4. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 4(b).
1(c). the amino acid replacement(s) confer(s) the increased hyaluronidase activity;	US-275 discloses modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants, which each contain a single amino acid replacement. *See* EX1004, [0606], Table 9. As the only difference in each $PH20_{1-447}$ mutant relative to unmodified $PH20_{1-447}$ is the single identified replacement, each replacement is understood to confer the increased hyaluronidase activity reported in Example 4 of US-275. *See id*. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 1(c).

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1(d). at least one amino acid replacement confers the increased hyaluronidase activity, and is at a position corresponding to a position selected from 1, 15, 24, 27, 29, 30, 31, 32, 33, 37, 39, 48, 63, 67, 69, 71, 72, 73, 75, 87, 92, 93, 94, 118, 120, 135, 141, 147, 148, 150, 151, 152, 155, 156, 163, 164, 165, 170, 198, 209, 212, 215, 219, 233, 236, 247, 257, 259, 260, 263, 269, 271, 272, 276, 278, 282, 293, 305, 308, 313, 320, 324, 325, 326, 328, 353, 359, 371, 377, 380, 389, 392, 395, 405, 409, 410, 418, 425, 436, 437, 438, 441, 442, 446 and 447, with reference to amino acid positions set forth in SEQ ID NO:3;	US-275 discloses modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants, which each contain a single amino acid replacement at one of the recited positions relative to SEQ ID NO:3 (the "unmodified PH20 polypeptide"). *See* EX1004, [0606], Table 9. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 1(d).
1(e). corresponding amino acid positions are identified by alignment of the PH20 polypeptide with the polypeptide having the sequence of amino acids set forth as SEQ ID NO:3, with the proviso that if the modified PH20 polypeptide includes only a single amino acid replacement, the replacement does not correspond to amino acid replacement N219A with reference to amino acid positions set forth in SEQ ID NO:3; and	US-275 discloses modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants, which each contain only a single amino acid replacement relative to SEQ ID NO:3 (the "unmodified PH20 polypeptide"). *See* EX1004, [0606], Table 9 ("The first library was made to generate encoded variant proteins wherein each of residues … 1-447 of SEQ ID NO:3 [] was changed to ***one*** of about 15 amino acid residues, such that each member contained ***a single amino change***.") (emphasis added). None of these modified PH20 polypeptides contain additional replacements or the amino acid replacement N219A relative to SEQ ID NO:3. *See id*. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 1(e).
1(f). the modified PH20 polypeptide has at least 95% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 7 and 32-66.	US-275 discloses modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants, which each contain a single amino acid replacement relative to SEQ ID NO:3 (the "unmodified PH20 polypeptide"). *See* EX1004, [0606], Table 9. Thus, each of these modified PH20 polypeptides are 99.8% identical to SEQ ID NO:3. *See* EX1004, [0172], [0176].

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	Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 1(f).
Claim 2	
2. The modified PH20 polypeptide of claim 1, comprising at least one amino acid replacement selected from among replacement with: at a position corresponding to position 1; Q at a position corresponding to position 1; V at a position corresponding to position 15; E at a position corresponding to position 24; H at a position corresponding to position 24; K at a position corresponding to position 27; R at a position corresponding to position 27; E at a position corresponding to position 29; I at a position corresponding to position 29; L at a position corresponding to position 29; M at a position corresponding to position 29; P at a position corresponding to position 29; S at a position corresponding to position 29; V at a position corresponding to position 29; G at a position corresponding to position 30; H at a position corresponding to position 30; K at a position corresponding to position 30; M at a position corresponding to position 30; R at a position corresponding to position 30; S at a position corresponding to position 30; A at a position corresponding to position 31; C at a position corresponding to position 31; H at a position corresponding to position 31; I at a position corresponding to position 31; K at a position corresponding to position 31; L at a position corresponding to position 31; P at a position corresponding to position 31; R at a position corresponding to position 31; S at a position corresponding to position 31; T at a position corresponding to position 31; V at a position corresponding to position 31; F at a position corresponding to position 32; G at a position corresponding to position 32; H at a position corresponding to position 32; W at a position corresponding to position 33; F at a position corresponding to	Claim 2 is a dependent claim, and therefore inherits the limitations of claim 1. The analysis of the modified PH20 polypeptide(s) of claim 1 also applies to claim 2. US-275 discloses a class of ~530 single amino acid substitutions of $PH20_{1-447}$ that resulted in mutants exhibiting ***at least 120%*** of the activity of unmodified $PH20_{1-447}$ (SEQ ID NO:3). EX1004, [0606], [0626], [0019]; Table 9 (p. 128, 131, 133). These mutants include I271L, M313K, and D320K $PH20_{1-447,}$ each of which is a mutant that contains one of the recited amino acid substitutions. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 2.

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position 37; N at a position corresponding to position 39; T at a position corresponding to position 39; F at a position corresponding to position 48; H at a position corresponding to position 48; N at a position corresponding to position 48; W at a position corresponding to position 63; V at a position corresponding to position 67; A at a position corresponding to position 69; C at a position corresponding to position 69; F at a position corresponding to position 69; G at a position corresponding to position 69; I at a position corresponding to position 69; L at a position corresponding to position 69; M at a position corresponding to position 69; P at a position corresponding to position 69; R at a position corresponding to position 69; W at a position corresponding to position 69; Y at a position corresponding to position 69; R at a position corresponding to position 71; S at a position corresponding to position 71; M at a position corresponding to position 72; Q at a position corresponding to position 72; H at a position corresponding to position 73; L at a position corresponding to position 73; W at a position corresponding to position 73; F at a position corresponding to position 75; L at a position corresponding to position 75; R at a position corresponding to position 75; T at a position corresponding to position 75; C at a position corresponding to position 87; T at a position corresponding to position 87; Y at a position corresponding to position 87; C at a position corresponding to position 92; I at a position corresponding to position 93; L at a position corresponding to position 93; R at a position corresponding to position 93; T at a position corresponding to position 93; R at a position corresponding to position 94; Q at a position corresponding to position 118; F at a position corresponding to position 120; V at a position corresponding to position 120; Y at a position corresponding to position 120; D at a position	

corresponding to position 135; G at a position corresponding to position 135; R at a position corresponding to position 135; H at a position corresponding to position 141; Y at a position corresponding to position 141; R at a position corresponding to position 147; V at a position corresponding to position 147; K at a position corresponding to position 148; G at a position corresponding to position 150; K at a position corresponding to position 151; L at a position corresponding to position 151; M at a position corresponding to position 151; Q at a position corresponding to position 151; Rat a position corresponding to position 151; Rat a position corresponding to position 152; G at a position corresponding to position 155; K at a position corresponding to position 155; D at a position corresponding to position 156; A at a position corresponding to position 163; E at a position corresponding to position 163; K at a position corresponding to position 163; R at a position corresponding to position 163; M at a position corresponding to position 164; D at a position corresponding to position 165; N at a position corresponding to position 165; R at a position corresponding to position 170; D at a position corresponding to position 198; N at a position corresponding to position 212; M at a position corresponding to position 215; S at a position corresponding to position 219; K at a position corresponding to position 233; R at a position corresponding to position 233; R at a position corresponding to position 236; I at a position corresponding to position 247; T at a position corresponding to position 257; P at a position corresponding to position 259; Y at a position corresponding to position 260; K at a position corresponding to position 263; R at a position corresponding to position 263; A at a position corresponding to position 269; L at a position corresponding to position 271; M at a position corresponding to position 271; T at a position corresponding to position 272; D at	

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a position corresponding to position 276; S at a position corresponding to position 276; Y at a position corresponding to position 276; H at a position corresponding to position 278; K at a position corresponding to position 278; N at a position corresponding to position 278; R at a position corresponding to position 278; S at a position corresponding to position 278; T at a position corresponding to position 278; Y at a position corresponding to position 278; M at a position corresponding to position 282; A at a position corresponding to position 293; C at a position corresponding to position 293; F at a position corresponding to position 293; M at a position corresponding to position 293; P at a position corresponding to position 293; Q at a position corresponding to position 293; V at a position corresponding to position 293; E at a position corresponding to position 305; G at a position corresponding to position 308; N at a position corresponding to position 308; K at a position corresponding to position 313; R at a position corresponding to position 313; H at a position corresponding to position 320; K at a position corresponding to position 320; R at a position corresponding to position 320; R at a position corresponding to position 324; A at a position corresponding to position 325; D at a position corresponding to position 325; E at a position corresponding to position 325; G at a position corresponding to position 325; H at a position corresponding to position 325; K at a position corresponding to position 325; M at a position corresponding to position 325; N at a position corresponding to position 325; Q at a position corresponding to position 325; S at a position corresponding to position 325; V at a position corresponding to position 326; I at a position corresponding to position 328; K at a position corresponding to position 328; L at a position corresponding to position 328; S at a position corresponding to position 328; Y at a position corresponding to	

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position 328; V at a position corresponding to position 353; T at a position corresponding to position 359; R at a position corresponding to position 371; P at a position corresponding to position 377; T at a position corresponding to position 377; W at a position corresponding to position 380; Y at a position corresponding to position 380; K at a position corresponding to position 389; M at a position corresponding to position 392; R at a position corresponding to position 395; G at a position corresponding to position 405; A at a position corresponding to position 409; Q at a position corresponding to position 409; T at a position corresponding to position 410; P at a position corresponding to position 418; K at a position corresponding to position 425; K at a position corresponding to position 436; I at a position corresponding to position 437; M at a position corresponding to position 437; T at a position corresponding to position 438; F at a position corresponding to position 441; R at a position corresponding to position 442; A at a position corresponding to position 446; D at a position corresponding to position 447; N at a position corresponding to position 447; and/or Q at a position corresponding to position 447, with reference to amino acid positions set forth in SEQ ID NO:3.	
Claim 3	
3. The modified PH20 polypeptide of claim 2, wherein [M] the modified PH20 polypeptide is a soluble PH20 polypeptide; and the unmodified polypeptide consists of the amino acid sequence set forth in any of SEQ ID NOs: 3 or 32-66.	Claim 3 depends from claim 2, which depends from claim 1. Claim 3 therefore inherits the limitations of claims 1 and 2. The analysis of the modified PH20 polypeptide(s) of claims 1 and 2 also applies to claim 3. US-275 discloses that $PH20_{1-447}$ is a soluble PH20 polypeptide due to its truncation to position 447. *See* EX1004, [0015]. US-275 also discloses that "soluble PH20 polypeptides include any that has the sequence of amino acids set forth in SEQ ID NOS: 3 or 32-66 or a sequence of amino acids that exhibits at least 85%

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	sequence identity… to the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66." EX1004, [0250]; *see also id.*, [0016], [0017], [0020], [0116], [0238], [0256]. The modified PH20 polypeptides I271L, M313K, and D320K $PH20_{1-447}$ each contain a single amino acid mutation compared to the unmodified polypeptides consisting of residues 1-447 of SEQ ID NO 3. *See* US-275, [0606], Table 9. Thus, each of these three mutants is 99.8% identical to SEQ ID NO:3 and reasonably believed to be soluble. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 3.

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Claim 4	
4. A modified PH20 polypeptide, comprising one or more amino acid replacements in an unmodified PH20 polypeptide, wherein: the unmodified PH20 polypeptide consists of the amino acid sequence selected from the group consisting of SEQ ID NOS: 3, 7 and 32-66;	US-275 discloses modified PH20 polypeptides, including V166L and I309N $PH20_{1-447}$ mutants, which each contain a single amino acid replacement relative to SEQ ID NO:3 (the "unmodified PH20 polypeptide"). *See* EX1004, [0606], Table 9 ("The first library was made to generate encoded variant proteins wherein each of residues … 1-447 of SEQ ID NO:3 [] was changed to ***one*** of about 15 amino acid residues, such that each member contained ***a single amino change***.") (emphasis added). Therefore, at least the modified $PH20_{1-447}$ polypeptides of V166L and I309N meet the limitations of the preamble of claim 4.
4(a). the modified PH20 polypeptide exhibits increased hyaluronidase activity that is at least 120% of the hyaluronidase activity compared to the unmodified PH20 polypeptide not containing the amino acid replacements);	US-275 discloses modified PH20 polypeptides, including V166L and I309N $PH20_{1-447}$ mutants, which each contain a single amino acid replacement and exhibit 120% or greater hyaluronidase activity compared to the unmodified PH20 polypeptide that does not contain the amino acid replacement. EX1004, [0019]; Table 9 (p. 128, 131, 133) (identifying V166L as having 438% relative activity and I309N as having 311% relative activity). Therefore, at least the modified $PH20_{1-447}$ polypeptides of V166L and I309N meet the limitations of claim 4(a).

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4(b). wherein: the activity of the unmodified PH20 polypeptide not containing the replacement(s) is 100%; hyaluronidase activity of the modified PH20 polypeptide and hyaluronidase activity of the unmodified PH20 polypeptide are measured under identical conditions:	US-275 discloses modified PH20 including V166L and I309N $PH20_{1-447}$ mutants. *See* EX1004, [0606], Table 9. The activity value for each of these modified PH20 polypeptides is provided as a percent of the hyaluronidase activity of the modified PH20 polypeptide compared to that of the wildtype PH20, which lacks the amino acid replacement. *See* EX1004, [0623]-[0626]. The activity of the wildtype PH20 is 100%. *See* EX1004, [0625] ("For example, a value of 0.40 indicates that the variant exhibits 40% of the hyaluronidase activity of wildtype PH20, a value of 1 indicates that the variant exhibits a similar hyaluronidase activity of wildtype…"). The activity levels of these modified PH20 polypeptides were measured under identical conditions. *See* EX1004, [0623]-[0626] ("Each generated variant was screened for hyaluonidase [sic] activity as described in Example 3."); *see generally* EX1004, Examples 3 and 4. Therefore, at least the modified $PH20_{1-447}$ polypeptides of V166L and I309N meet the limitations of claim 4(b).

4(c). at least one amino acid replacement confers the increased hyaluronidase activity, and is selected from E at a position corresponding to position 12; E at a position corresponding to position 26; K at a position corresponding to position 26; R at a position corresponding to position 46; Q at a position corresponding to position 52; Q at a position corresponding to position 58; H at a position corresponding to position 68; Q at a position corresponding to position 68; A at a position corresponding to position 70; C at a position corresponding to position 70; F at a position corresponding to position 70; G at a position corresponding to position 70; H at a position corresponding to position 70; K at a position corresponding to position 70; L at a position corresponding to position 70; N at a position corresponding to position 70; P at a position corresponding to position 70; R at a position corresponding to position 70; S at a position corresponding to position 70; T at a position corresponding to position 70; A at a position corresponding to position 74; C at a position corresponding to position 74; G at a position corresponding to position 74; N at a position corresponding to position 74; P at a position corresponding to position 74; R at a position corresponding to position 74; S at a position corresponding to position 74; V at a position corresponding to position 74; W at a position corresponding to position 74; G at a position corresponding to position 84; R at a position corresponding to position 84; G at a position corresponding to position 97; H at a position corresponding to position 127; N at a position corresponding to position 127; G at a position corresponding to position 131; V at a position corresponding to position 131; R at a position corresponding to position 142; A at a position corresponding to position 166; F at a position corresponding to position 166; H at a position corresponding to position 166; L at a position corresponding to position 166; R at a position corresponding to	US-275 discloses modified PH20 polypeptides, including V166L and I309N $PH20_{1-447}$ mutants, which each contain one of the recited single amino acid replacements relative to SEQ ID NO:3 (the "unmodified PH20 polypeptide"). *See* EX1004, [0606], Table 9. Therefore, at least the modified $PH20_{1-447}$ polypeptides of V166L and I309N meet the limitations of claim 4(c).

position 166; Y at a position corresponding to position 166; K at a position corresponding to position 174; K at a position corresponding to position 206; M at a position corresponding to position 213; N at a position corresponding to position 213; E at a position corresponding to position 237; S at a position corresponding to position 238; N at a position corresponding to position 261; R at a position corresponding to position 277; T at a position corresponding to position 277; L at a position corresponding to position 309; N at a position corresponding to position 309; Q at a position corresponding to position 309; R at a position corresponding to position 309; T at a position corresponding to position 309; A at a position corresponding to position 310; G at a position corresponding to position 310; H at a position corresponding to position 315; I at a position corresponding to position 317; K at a position corresponding to position 317; R at a position corresponding to position 317; M at a position corresponding to position 318; G at a position corresponding to position 347; S at a position corresponding to position 347; M at a position corresponding to position 399; T at a position corresponding to position 399; W at a position corresponding to position 399; D at a position corresponding to position 407; Q at a position corresponding to position 407; F at a position corresponding to position 419; I at a position corresponding to position 419; R at a position corresponding to position 419; S at a position corresponding to position 419; K at a position corresponding to position 421; N at a position corresponding to position 421; Q at a position corresponding to position 421; R at a position corresponding to position 421; S at a position corresponding to position 421; A at a position corresponding to position 431; H at a position corresponding to position 431; K at a position corresponding to position 431; Q at a position corresponding to position 431; R at a position corresponding to position 431; S at a position corresponding to	

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position 431; V at a position corresponding to position 431; L at a position corresponding to position 433; R at a position corresponding to position 433; V at a position corresponding to position 439; H at a position corresponding to position 440; R at a position corresponding to position 440; A at a position corresponding to position 443; M at a position corresponding to position 443; M at a position corresponding to position 445, and/or P at a position corresponding to position 445, with reference to amino acid positions set forth in SEQ ID NO:3;	
4(d). and the modified PH20 polypeptide has at least 95% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 7, and 32-66.	US-275 discloses modified PH20 polypeptides, including V166L and I309N PH20$_{1-447}$ mutants, which each contain a single amino acid replacement relative to SEQ ID NO:3 (the "unmodified PH20 polypeptide"). *See* EX1004, [0606], Table 9. Thus, each of these modified PH20 polypeptides are 99.8% identical to SEQ ID NO:3. *See* EX1004, [0172], [0176]. Therefore, at least the modified PH20$_{1-447}$ polypeptides of V166L and I309N meet the limitations of claim 4(d).
Claim 5	
5. The modified PH20 polypeptide of claim 3 comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 86, 99, 100, 159, 173, 181, 193, 195, 209-220, 238, 239, 242, 247-252, 267, 269, 308, 324, 325, 330, 335, 355, 419, 421-423, 425, 428, 435, 455, 462, 463, 484, 490, 506, 531, 533, 579, 581-583, 585, 587, 588, 602, 605, 606, 609, 613, 648, 731, 732, 734, 742, 746, 760, 762, 764, 765, 769-773, 779, 782, 783, 786-789, 794, 795, 814, 816, 819, 826, 830, 836, and 838, or an amino acid sequence that has at least 96% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NOs: 86, 99, 100, 159, 173, 181, 193, 195, 209-220, 238, 239, 242, 247-252, 267, 269, 308, 324, 325, 330, 335, 355, 419, 421-423, 425, 428, 435, 455, 462, 463, 484, 490, 506, 531,	Claim 5 depends from claim 3, which depends from claims 1 and 2. Claim 5 therefore inherits the limitations of claims 1, 2, and 3. The analysis of the modified PH20 polypeptide(s) of claims 1, 2 also applies to claim 5. US-275 discloses that the I271L-PH20$_{1-447}$, M313K-PH20$_{1-447}$ and D320K-PH20$_{1-447}$ modified PH20 polypeptides have 2 differences relative to the claimed SEQ IDs in the second set recited in claim 5. *See generally* EX1004, Table 9. Thus, each of the modified PH20 polypeptides is 99.6% identical to each of those listed SEQ IDs, which is greater than the recited limitation of at least 96% sequence identity.

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533, 579, 581-583, 585, 587, 588, 602, 605, 606, 609, 613, 648, 731, 732, 734, 742, 746, 760, 762, 764, 765, 769-773, 779, 782, 783, 786-789, 794, 795, 814, 816, 819, 826, 830, 836 and 838, and that contains the amino acid replacement as compared to corresponding positions in a polypeptide of any of SEQ ID NOs:3, 7, and 32-66.	Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 5.
Claim 6	
6. The modified PH20 polypeptide of claim 1 that exhibits at least 2.0-fold of the hyaluronidase activity compared to the PH20 polypeptide not containing the amino acid replacement(s).	Claim 6 is a dependent claim, and therefore inherits the limitations of claim 1. The analysis of the modified PH20 polypeptide(s) of claim 1 also applies to claim 6. US-275 discloses that I271L $PH20_{1-447}$, M313K $PH20_{1-447}$, and D320K $PH20_{1-447}$ are also reported to exhibit greater than 2.0-fold (*i.e.*, 200%) activity compared to unmodified $PH20_{1-447}$. EX1004, [0626] (identifying increased activity of "mutant I271L is about 10-fold"); [0019]; Table 9 (p. 128, 131, 133) (identifying I271L as having 1,062% relative activity, M313K as having 285% relative activity, and D320K as having 642% relative activity). Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 6.
Claim 7	
7. The modified PH20 polypeptide of claim 1 that comprises one or more of a modification selected from among glycosylation, sialation, albumination, farnesylation, carboxylation, hydroxylation and phosphorylation.	Claim 7 is a dependent claim, and therefore inherits the limitations of claim 1. The analysis of the modified PH20 polypeptide(s) of claim 1 also applies to claim 7. US-275 discloses that "modified PH20 polypeptides" can contain amino acids that are further modified. These modifications include glycosylation, sialation, albumination, farnysylation, carboxylation, hydroxylation, and phosphorylation. *See, e.g.*, EX1004, [0115], [0118], [0033].

	Each of the single-substitution $PH20_{1-447}$ mutants recited in the disclosure were produced in mammalian CHO cells, including $PH20_{1-447}$ mutants I271L, M313K, and D320K, *See* EX1004, [0608]. US-275 discloses that glycosylation is also required for PH20 hyaluronidase activity. *See* EX1004, [0236]. A POSA would also understand that mammalian CHO cells are known to glycosylate the $PH20_{1-447}$ sequence. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 7.
Claim 8	
8. The modified PH20 polypeptide of claim 1 that comprises at least an N-acetylglucosamine moiety linked to each of at least three asparagine (N) residues.	Claim 8 is a dependent claim, and therefore inherits the limitations of claim 1. The analysis of the modified PH20 polypeptide(s) of claim 1 also applies to claim 8. A POSA would also understand that mammalian CHO cells are known to glycosylate the $PH20_{1-447}$ sequence. US-275 discloses that glycosylation is required for PH20 hyaluronidase activity, and that $PH20_{1-447}$ corresponding to SEQ ID NO:3 contains 6 N-linked glycosylation sites. *See* EX1004, [0236]. Three of which, N200, N333, and N358, are required for secretion and/or activity of PH20. *See* EX1004, [0236]. Modified PH20 polypeptides, I271L $PH20_{1-447}$, M313K $PH20_{1-447}$, and D320K $PH20_{1-447}$, each are unmodified at positions N200, N333, and N358, and therefore retain the necessary N-linked glycosylation sites. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 8.
Claim 9	
9. The modified PH20 polypeptide of claim 8, wherein the three asparagine residues correspond to amino acid residues 200, 333 and 358 of SEQ ID NO:3.	Claim 9 is a dependent claim, and therefore inherits the limitations of claim 1. The analysis of the modified PH20 polypeptide(s) of claim 1 also applies to claim 9.

	US-275 discloses that glycosylation is required for PH20 hyaluronidase activity, and that $PH20_{1-447}$ corresponding to SEQ ID NO:3 contains 6 N-linked glycosylation sites. *See* EX1004, [0236]. Three of which, N200, N333, and N358, are required for secretion and/or activity of PH20. *See* EX1004, [0236]. Modified PH20 polypeptides, I271L $PH20_{1-447}$, M313K $PH20_{1-447}$, and D320K $PH20_{1-447}$, each are unmodified at positions N200, N333, and N358. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 9.
Claim 10	
10. The modified PH20 polypeptide of claim 1 that is conjugated to a polymer.	Claim 10 is a dependent claim, and therefore inherits the limitations of claim 1. The analysis of the modified PH20 polypeptide(s) of claim 1 also applies to claim 9. US-275 discloses that modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants, can contain amino acids that are further modified. *See* EX1004, [0033]. These modifications include conjugation to a polymer, such as PEGylation or conjugation to dextran. *See* EX1004, [0033]. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 10.
Claim 11	
11. The modified PH20 polypeptide of claim 10, wherein the polymer is dextran or PEG.	Claim 11 depends from claim 10, which depends from claim 1. Claim 11 therefore inherits the limitations of claims 1 and 10. The analysis of the modified PH20 polypeptide(s) of claims 1 and 10 also applies to claim 11.

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	US-275 discloses that modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants, can contain amino acids that are further modified. *See* EX1004, [0033]. These modifications include conjugation to a polymer, such as PEGylation or conjugation to dextran. *See* EX1004, [0033]. Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 11.
Claim 12	
12. The modified PH20 polypeptide of claim 1, comprising up to 10 amino acid modifications selected from among amino acid replacements, insertions and deletions.	Claim 12 is a dependent claim, and therefore inherits the limitations of claim 1. The analysis of the modified PH20 polypeptide(s) of claim 1 also applies to claim 12. US-275 discloses modified PH20 polypeptides, including I271L, M313K, and D320K $PH20_{1-447}$ mutants, which each contain only one modification (*i.e.*, substitution) relative to SEQ ID NO:3 (unmodified $PH20_{1-447}$). *See* EX1004, [0606] ("The first library was made to generate encoded variant proteins wherein each of residues … 1-447 of SEQ ID NO:3 [] was changed to ***one*** of about 15 amino acid residues, such that each member contained ***a single amino change***.") (emphasis added). Therefore, at least the modified $PH20_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 12.
Claim 13	
13. A pharmaceutical composition, comprising a modified PH20 polypeptide of claim 1.	Claim 13 is a dependent claim, and therefore inherits the limitations of claim 1. The analysis of the modified PH20 polypeptide(s) of claim 1 also applies to claim 13. US-275 discloses that pharmaceutical compositions can contain modified PH20 polypeptides, which would include the increased

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	activity mutants of I271L-PH20$_{1-447}$, M313K-PH20$_{1-447}$ and D320K-PH20$_{1-447}$). *See, e.g.*, EX1004, [0036], [0435]. Therefore, at least the modified PH20$_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 13.
Claim 14	
14. The modified PH20 polypeptide of claim 2, comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 73, 78, 91, 95, 96, 105, 106, 108, 109, 111, 112, 113, 115, 117, 118, 119, 120, 123-126, 128-136, 139-141, 149, 154, 155, 164, 165, 167, 191, 192, 196, 197, 199-205, 207, 208, 225, 226, 228, 229, 231, 233, 237, 253, 254, 256, 257, 277, 283, 293, 295, 296, 298, 300, 303, 316, 318, 321, 322, 338-340, 344, 348, 367, 369, 371, 377, 384-388, 394, 398, 399, 401, 406-408, 410, 412, 414, 416, 431, 448, 459, 465, 469, 478, 479, 482, 493, 497, 501, 503, 507, 508, 510-512, 514, 518, 522, 523, 537-543, 545, 558, 559, 561, 563-566, 569, 572, 574, 594, 596, 618-620, 624-634, 637, 640-644, 652, 657, 675, 695, 698, 699, 700, 712, 717, 725, 738, 748-750, 757, 775, 799-801, 807, 822, 825, 844, 847, 85C and 853, or an amino acid sequence that has at least 96% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NOs: 73, 78, 91, 95, 96, 105, 106, 108, 109, 111, 112, 113, 115, 117, 118, 119, 120, 123-126, 128-136, 139-141, 149, 154, 155, 164, 165, 167, 191, 192, 196, 197, 199-205, 207, 208, 225, 226, 228, 229, 231, 233, 237, 253, 254, 256, 257, 277, 283, 293, 295, 296, 298, 300, 303, 316, 318, 321, 322, 338-340, 344, 348, 367, 369, 371, 377, 384-388, 394, 398, 399, 401, 406-408, 410, 412, 414, 416, 431, 448, 459, 465, 469, 478, 479, 482, 493, 497, 501, 503, 507, 508, 510-512, 514, 518, 522, 523, 537-543, 545, 558, 559, 561, 563-566, 569, 572, 574, 594, 596, 618-620, 624-634, 637, 640-644, 652, 657, 675, 695, 698, 699, 700, 712, 717, 725, 738, 748-750, 757, 775, 799-801,	Claim 14 depends from claim 2, which depends from claim 1. Claim 14 therefore inherits the limitations of claims 1 and 2. The analysis of the modified PH20 polypeptide(s) of claims 1 and 2 also applies to claim 9. Each of the SEQ ID NOs recited in claim 14 correspond to a PH20$_{1-447}$ mutant that contains a single amino acid replacement. *See* EX1004, [0626] (Table 9). PH20$_{1-447}$ mutants I271L, M313K, and D320K, each have 2 amino acid differences to the SEQ IDs recited in claim 14, which equates to 99.6% (445/447) sequence identity. Therefore, at least the modified PH20$_{1-447}$ polypeptides of I271L, M313K, and D320K meet the limitations of claim 14.

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807, 822, 825, 844, 847, 851 and 853, and that contains the amino acid replacement as compared to corresponding positions in a polypeptide of any of SEQ ID NOs:3, 7, and 32-66.	
Claim 15	
15. The modified PH20 polypeptide of claim 4 that is conjugated to a polymer.	Claim 15 is a dependent claim, and therefore inherits the limitations of claim 4. The analysis of the modified PH20 polypeptide(s) of claim 4 also applies to claim 15. US-275 discloses that modified PH20 polypeptides, including V166L and I309N $PH20_{1-447}$ mutants, can contain amino acids that are further modified. *See* EX1004, [0033]. These modifications include conjugation to a polymer, such as PEGylation or conjugation to dextran. *See* EX1004, [0033]. Therefore, at least the modified $PH20_{1-447}$ polypeptides of V166L and I309N meet the limitations of claim 15.
Claim 16	
16. The modified PH20 polypeptide of claim 4, comprising up to 10 amino acid modifications selected from among amino acid replacements, insertions and deletions.	Claim 16 is a dependent claim, and therefore inherits the limitations of claim 4. The analysis of the modified PH20 polypeptide(s) of claim 4 also applies to claim 16. US-275 discloses modified PH20 polypeptides, including V166L and I309N $PH20_{1-447}$ mutants, which each contain only one modification (*i.e.*, substitution) relative to SEQ ID NO:3 (unmodified $PH20_{1-447}$). *See* EX1004, [0606] ("The first library was made to generate encoded variant proteins wherein each of residues … 1-447 of SEQ ID NO:3 [] was changed to ***one*** of about 15 amino acid residues, such that each member contained ***a single amino change***.") (emphasis added).

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	Therefore, at least the modified $PH20_{1-447}$ polypeptides I271L, M313K, and D320K meet the limitations of claim 16.
Claim 17	
17. A pharmaceutical composition, comprising a modified PH20 polypeptide of claim 4.	Claim 17 is a dependent claim, and therefore inherits the limitations of claim 4. The analysis of the modified PH20 polypeptide(s) of claim 4 also applies to claim 17. US-275 discloses that pharmaceutical compositions can contain modified PH20 polypeptides, which would include the increased activity mutants of V166L and I309N. *See, e.g.*, EX1004, [0036], [0435]. Therefore, at least the modified $PH20_{1-447}$ polypeptides of V166L and I309N meet the limitations of claim 17.
Claim 18	
18. The modified PH20 polypeptide of claim 4, wherein the modified PH20 polypeptide is a soluble PH20 polypeptide; and the unmodified polypeptide consists of the amino acid sequence set forth in any of SEQ ID NOs: 3 or 32-66.	Claim 18 is a dependent claim, and therefore inherits the limitations of claim 4. The analysis of the modified PH20 polypeptide(s) of claim 4 also applies to claim 18. US-275 discloses that $PH20_{1-447}$ is a soluble PH20 polypeptide due to its truncation to position 447. *See* EX1004, [0015]. US-275 also discloses that "soluble PH20 polypeptides include any that has the sequence of amino acids set forth in SEQ ID NOS: 3 or 32-66 or a sequence of amino acids that exhibits at least 85% sequence identity… to the sequence of amino acids set forth in any of SEQ ID NOS: 3 or 32-66." EX1004, [0250]; *see also id.*, [0016], [0017], [0020], [0116], [0238], [0256]. The modified PH20 polypeptides V166L and I309N $PH20_{1-447}$ each contain a single amino acid mutation compared to the unmodified polypeptides consisting of residues 1-447 of SEQ ID NO 3. *See* US-275, [0606], Table 9. Thus, each of these three mutants is

U.S. Patent No. 10,865,400	
	99.8% identical to SEQ ID NO:3 and reasonably believed to be soluble. Therefore, at least the modified $PH20_{1-447}$ polypeptides V166L and I309N meet the limitations of claim 18.

Appendix C

CV

JAMES HENDERSON NAISMITH
BSc, PhD, DSc, FRS, FRSE, FMedSci, MAE, FRSC, FinstP, FRSB, Member of EMBO, Fellow American Association Advancement of Science (AAAS).

Date of birth: 26th July 1968 Hamilton, Scotland, UK

Current role (from Oct 2023): Head of MPLS Division, Oxford University
As Head of Division I am responsible for teaching, research and administration across all science (except medicine) and engineering. I hold the budget of around £420 M pa, I agree with heads of the departments' academic strategy, I chair annual promotion panels, set the internal tone within Division and representing the division within the University. MPLS is ranked both in research and teaching as the leading Division in Oxford and the world.
Specific achievements include:

- Partnership with Ellison Institute around AI
- Partnered with Govt on AI & engineering initiatives
- Progress on broadening curriculum from ahistorical exclusive western lens
- Managing finances ahead of budget target
- Estates Strategy for science and engineering

Education
(1) Undergraduate Degree
University of Edinburgh 1985-1989 First Class Honours Bachelor of Science Degree in Chemistry
Awards: Crum Brown Medal 1986 (1st year Chemistry); Class Medal 1988 (3rd Year Chemistry); MacFarlane-Smith Prize 1989 (Honours Chemistry Medal); Stephenson Prize 1989 (joint) (Honours Project); Gillies Prize 1988 (Faculty of Science); SmithKline French Prize 1988 (joint) (U.K. Chemistry); Faculty of Science Scholarship 1988-1989 (joint); Dawson Trust Bursary 1986-1989; Carnegie Summer Scholarship 1988; I.C.I. Summer Studentship 1988; Class Medal Industrial Management 1988
(2) Postgraduate Degree (Carnegie Scholarship)
University of Manchester 1989-1992 Ph.D. "Structural studies of concanavalin A and a zinc aldolase"
Supervisors: Prof. J.R. Helliwell, Prof. C.D. Garner & Dr. W.N. Hunter (principal supervisor).
Award: Hibbert Prize 1992 (joint) (Thesis Prize).
University of Oxford, MA, Degree by Resolution 2023
(3) Higher Degree
University of St Andrews 2016 DSc "Chemical and structural studies upon proteins"
(4) Degree by resolution
University of Oxford 2023 MA

Previous Posts
(1) Post-doctoral, 2 years, 1993-1994
Howard Hughes Medical Institute, Dallas, (Postdoctoral Fellow)
Appointed SERC/NATO fellow (UK competition), in the laboratory of Professor Stephen R. Sprang.
(2) Principal Investigator St Andrews, 1995 - 2017
Biomedical Sciences Research Complex, University of St. Andrews (Lecturer - Professor), 1995-2017
Appointed Lecturer; Promoted Lecturer Grade B, October 1996; Reader, October 1998; Professor June 2001-17, Bishop Wardlaw Professor of Chemical Biology 2014-17
(3) Director of Research Complex Harwell 2017-2019
Appointed to turn around struggling institute, successfully lead the quinquennial review
(4) Founding Director and CEO Rosalind Franklin Institute 2017-2023

Specific achievements include:

- Operationalisation of a fully functioning independent research institute, during the covid19 pandemic (budgeting, charitable status, shareholder, governance)
- Establishment of an outstanding scientific and operational team, that was able to respond with resilience to the Covid pandemic and ensure delivery of the institutional objectives and provide a high level of support for all students and staff.
- An open diverse results-oriented culture, with career structures and initiatives that are designed to remove institutional barriers to groups that are often under-represented in UK science / research organisations.
- Delivery of a new building (of 5,500 m^2) 18 months faster than other comparable public sector buildings. The building secured a design award, is "Excellent" in terms of sustainability & >10 % more efficient in usable to gross space ratio than the next best public sector lab.
- A unique UK wide PhD program that will create a new generation of cross-disciplinary biomedical scientists (10 per year).
- Delivery of the national flagship program Electrifying Life Science, a £60 M revolution in electron cryo tomography
- New therapeutic agents which are progressing towards the clinic for treatment of covid19
- Establishing a unique cross disciplinary scientific environment

(5) Principal Investigator Oxford, 2017-

From June 2017 Professor Nuffield Department of Medicine with cross appointment to Chemistry, University of Oxford,

Previous Leadership

Deputy of Biomolecular Science Research Complex (BSRC) (then known as BMS) 2001-2009
Director of BSRC 2010-2017
I drove the vision, wrote the grant, secured University and Wellcome Trust funding for the BSRC and oversaw its design, construction and delivery.
Elected member of University Court St Andrews, 2012 - 2016

Major research recognition

1. Carbohydrate Chemistry Prize 2000, The Royal Society of Chemistry (Prize for most significant carbohydrate chemistry awarded to early career researcher open to UK citizen based in any country or UK based scientist from any country)
2. BBSRC Career Development Fellowship 200-2005
3. Leverhulme Trust Molecular Biology Prize 2001, The Leverhulme Trust (UK wide prize for most significant contribution of a person under 40 to molecular biology)
4. Corday-Morgan Medal 2004, The Royal Society of Chemistry (UK citizen from any country or UK based scientist, aged 36 or under, who has made the most significant contribution to chemistry)
5. Colworth Medal 2004, The Biochemical Society (UK based scientist identified by published independent work as the most promising Biochemist in the UK aged 36 or under)
6. Jeremy Knowles Medal 2009, The Royal Society of Chemistry (UK scientist under 55 who has made most meritorious contribution to Chemical Biology)
7. Rita & John Cornforth Award 2012, The Royal Society of Chemistry (Awarded to a team (CCP4), that has made the most significant contribution to life science)
8. Royal Society Wolfson Research Merit Award 2014
9. 1000 Talent Scholar (Chinese Academy of Science) 2015-2017
10. The Tilden Prize 2018, The Royal Society of Chemistry (Awarded to the scientist who has done the most to advance chemistry)
11. Horizon Prize 2022, The Royal Society of Chemistry (The nanobody team).

Elections to learned societies – research recognition

1. Fellowship of the Royal Society of Edinburgh (2005) (Scotland's 230 year old National Academy of Arts, Business & Science)
2. Membership of EMBO (2009) (European wide academy of Life Science)
3. Fellowship of the Academy Medical Sciences (2012) (The Academy is one the four UK National Academies)
4. Fellowship of the Royal Society (2014) (The UK's 350+ year old National Academy of Science)
5. Fellow of American Association for Advancement of Science 2016 (AAAS)
6. Member of Academia Europa 2022 (The European Academy of Arts and Science).

Professional societies

1. Fellow of the Royal Society of Chemistry (FRSC)
2. Fellow of the Royal Society of Biology (FRSB)
3. Fellow of the Institute of Physics (FInstP)
4. Member American Chemical Society

Positions of Trust

1. Non-executive Director of Diamond Light Source PLC 2008 - 2016
2. Member of Court (the University governing and corporate body) and Trustee of the University of St Andrews 2012-2016
3. Selection panel Vice-Chancellor of the University of St Andrews 2014
4. Director of the Rosalind Franklin Institute, (Charity Trustee), 2018-
5. UK government nominee on the Council of the European X-ray Free Electron Laser (equivalent to non-Exec Director) 2019-2022
6. Non Executive Director GyreOx, Spin out company from my research, 2020-
7. Council of the Royal Society, Nov 2020 – Nov 2022 (Charity Trustee)
8. Committee for nomination of the Vice-Chancellor Oxford 2021-22
9. Elected Vice-Chair of Council (*super partes*) of the European X-ray Free Electron Laser, 2022-2024
10. Council of Academy of Medical Sciences (Charity Trustee) Dec 2022 -2025
11. Vice-President (non-clinical) Academy of Medical Sciences Dec 2022 -2025

Other significant Responsibilities

1. Chair of SC6 at the Royal Society (2016-2018) (two cycles)
2. Served on Fellowship election committees at the Royal Society, Royal Society Edinburgh and Academy of Medical Science
3. Grant panels Wellcome, RCUK and Royal Society
4. Review panels for major Institutes
5. Royal Society group on Covid evidence assessment
6. Chair of financial sustainability working group on European X-ray Free Electron Laser

Consultancies

Hoescht Marrion Roussel 2000-2 complete
Limatech 2017 complete
Sidley Austin LLP 2017 complete, Expert Witness in Immunex vs Sandoz

Publication & metrics

Full list https://scholar.google.com/citations?user=fLmKKQMAAAAJ&hl=en

Outreach

I have lectured at both general science events (British Association for Advancement of Science) and in schools. I have carried out practical demonstrations for primary school children involving them in hands on experiments (Iodine clock, flame colours, splitting of water). During covid19 I have provided over 100 written comments on the progress of the pandemic through the Science Media Centre. https://www.sciencemediacentre.org/?s=naismith&cat=. Many of these comments were quoted by international (USA, Canada, Brazil, Australia, Germany, France, Japan, South Korea) & UK newspapers (Times, Telegraphy, Mail, Guardian, Financial Times, Sun, Express); often I have extensive background information for the articles (including for the Marr show). I have been interviewed on national TV (Sky News, BBC News, ITV news) and interviewed on international (USA, Australia) & UK radio (BBC R4, BBC Scotland, BBC Oxford, BBC West Midlands). I wrote a commentary for the Guardian and was given a small profile on Radio 5.

Service to community

For over 12 years, 8 years as Chairman I was heavily involved in running CCP4 (finishing in 2010). CCP4 is the *de facto* standard software for protein crystallography. I twice wrote and successfully renewed funding for the project. I managed both the strategic and operational aspects of the entire suite. I organised and taught on specific workshops (refinement) in addition to the regular every two years graduate course at St Andrews. I have led the UK structural biology into the European X-FEL. I was the elected head of the International User Consortium that oversaw the construction of the SFX/SPB beamline from outset to completion.

Undergraduate teaching

At St Andrews I have taught undergraduate chemistry and biochemistry every year since 1995. My teaching load in terms of lectures and tutorials has been on average been in the top 1/3 of academics. In 2017 I gave lectures on the origin of life, enzyme kinetics, chemistry in biology, new medicines and structural biology. I organise the final year module on structural biology. I have supervised labs for second year students. I was voted Chemistry teacher of the year.

Career progression of PhD students supervised to date

1. **Davina Moothoo** 1995-1998 BBSRC. "Protein carbohydrate interactions". Now medical doctor.
2. **Stephan McMahon** 1995-1998 BBSRC. "Protein carbohydrate recognition". Postdoc at UCSD, currently PDRA in Naismith lab.
3. **Louise Buchanan** 1997-2000 BBSRC. "Structural investigations of E. Coli KDPG aldolase a carbohydrate synthesising protein". Took up post developing and managing Unix computing for Ford UK.
4. **Jennifer Harrison** 1995-1998 BBSRC. "Investigation of the substrate specificity of recombinant Trypanosoma cruzi trans-sialidase". Joint supervision with Rob Field (main supervisor). Post-doc in Dundee.
5. **Simon Allard** 1998-2001 BBSRC. "Carbohydrate processing pathogenic bacteria". Post-doc in Madison Wisconsin then moved into scientific publishing.
6. **Michael Tatham** 1998-2001 BBSRC. "Conjugation and deconjugation of the small ubiquitin-like modifiers SUMO-1, SUMO-2 and SUMO-3". Joint with Ron Hay (main supervisor). Now senior researcher in Hay lab.
7. **Changjiang Dong** 1999-2002 ORS "RmlC structure, mechanism and inhibitor design". PDRA with JHN, now a WT career development fellow and then Professor at UEA
8. **Iain Kerr** 1999-2003 "Molecular recognition in proteins". PDRA at St Judes, facility manager at UCSF, now Biotech Salt lake City.
9. **Stephen Fullerton** 1999-2003 "Structural and biochemical studies of carbohydrate binding proteins". PDRA in Leicester, then staff scientist at EMBL. Steve is now working in the biotech sector in Germany.
10. **Kostas Beis** 2001-2004 BBSRC "Structural studies of carbohydrate biosynthesis and translocation across membranes". PDRA in Scripps, now a RCUK lecturer in MPL (Diamond / Imperial)
11. **Alexandra Merkel** 2001-2004 "Enzyme-catalysed carbohydrate biosynthesis and modification ". Now director of a German technology company.
12. **Andrew McEwan** 2002-2005 BBSRC "Structures and function of 5'-fluorodeoxyadenosine synthase". Andy took a post-doc in Aberdeen where he is now a senior researcher.
13. **Walter De Laurentis** 2003-2005 BBSRC "PyrH and PrnB crystal structures ". Returned to Italy, then into grant management in Spain, now post-doc in USA.
14. **Gareth Williams** 2001-2004 Wellcome Trust "Structural studies of ArnA, a bifunctional enzyme from E. Coli and an archaeal heterotrimeric sliding clamp from Sl sulfataricus". Post-doc in LBL, Berkley USA. Gareth is a senior scientist in at LBL synchrotron in the Tainer lab.
15. **Thomas Kowatz** 2005-2009 SFC "Mechanisms of silicate polymerisation, carbohydrate epimerisation and metalloprotease inhibition". Thomas is working as a PDRA in the USA.
16. **Stefan Schmelz** 2003-2006. SFC "Adenylate forming enzymes involved in NRPR-independent siderophore biosynthesis". Stefan has a post-doc in Hemlholz Insitute for infection.
17. **Hubing Lou** SFC 2006-2010. "Structural and functional studies of bacterial outer membrane proteins". Postdoc in Beijing University and is now a PDRA in the USA
18. **Xuan Yan** (self funded and University) 2007-2011. "Structural analysis of the potential therapeutic targets from specific genes in methicillin resistant Staphylococcus aureus" Xuan has a prosperous career in Biotech China.
19. **Wassila Abdelli Boulkeroura** (Algerian scholarship) 2009-2013. "The application of the fragment-based screening approach to RmlA protein and PA1645 structure" Wassila is now a senior pharmacist in the NHS (Milton Keynes Trust)
20. **Emma Brannigan** (BBSRC) "Spin labeling of MscS" PDRA in Dundee in Ron Hay's lab

21. **Judith A Reekes** (BBSRC), 2009-2013 "Structural Studies of CRISPR-associated proteins" PDRA in Newcastle with Martin Noble.
22. **Hexian Huang** (Self funded & part time) 2008 – 2013 "Regulation of export and chain length of extracellular bacterial polysaccharides" PDRA in Naismith lab.
23. **Gregor Wallat (University)** 2010-2014 I took over Gregor's supervision when his supervisor left St Andrews. "Structure determination of the MOMO from C. jejuni & structural and functional studies of the endonuclease from lassa virus" Works for a company in Germany
24. **Andrew F Bent (University)** 2011-2015 "Structural biology of patellamide biosynthesis" PDRA in RIPPTIDE PHARMA; deceased.
25. **Greg Mann (St And)** 2012-2016 "Cyanobactins: structural and chemical " PDRA Switzerland
26. **Laura Woodward (BBSRC)** 2013-2017 "Structural and Biochemical Characterisation of Enzymes Required for Gram-Negative Capsule Biosynthesis" PDRA Germany, now editor of Angewantie
27. **Luana Ferrara (EU)** 2013-2017 "Structural and functional studies of porins from pathogenic bacteria" Post-doc Italy
28. **Ying Ge (ERC)** 2014-2018 "Mechanistic structural biology" PDRA Imperial College
29. **Hannes Ludwig (BBSRC)** 2016-2020 "Structural and functional characterisation of enzymes promising biocatalytic prowess" Post-doc USA
30. **Sisi Gao (ERC)** 2016-2020 "Enzyme mechanism" PDRA LM-MRC
31. **Micah Lee (Self)** 2018-2021 "Carbohydrate processing" Legal profession
32. **Wenbo Li (Self)** 2020-2023 "C-C bond forming enzymes" PDRA
33. **Helena Watson (EPRSC)** 2021-2024 "Structural Biology and CryoET" PDRA
34. **William Bowles (BBSRC)** 2021-2024 "Electron Damage to biological samples" PDRA

*One student under my supervision (2001-2006) Michael Hutchings did not submit a thesis.

Destination of post-docs

1. Marie-France Giraud (Faculty position CNRS)
2. Wulf Blankenfelt (Professor Germany)
3. David Sanders (Faculty position Canada)
4. Haitham Idriss (Medical Student)
5. Miryam Asuncion (Permanent job in Industry)
6. Adam Staines (post-doc Dundee, now BBSRC)
7. Lesley Thompson (Permanent job in NHS)
8. Genevive Temple (Permanent job in NHS)
9. Irina Grishkovska (post-doc in Germany)
10. Vicki Bamford (Lectureship Reading University)
11. Changjiang Dong (Independent Wellcome Fellowship, Professor UEA)
12. Lester G. Carter (Post-doc in Paul Adams lab, California)
13. Ken Johnson (Scientist, Tromso Sweden)
14. Kerstin Steiner (Faculty position Graz, Austria)
15. Muse Oke (scientist, KAUST; now Faculty University of Lagos)
16. Gregor Hageluken (Faculty position, Bonn)
17. Simon Bushell (PDRA Oxford)
18. Jesko Koehnke (Faculty position Saarbruken)
19. Christos Pliotas (Faculty St Andrews)
20. Haigang Song (Senior Post-doc Oxford)
21. Lucile Moynie (Junior Investigator Franklin)
22. Jiwei Liu (1000 Talent Scholar & Investigator Sichuan University)

23. Yun Yang (Lecturer Sichuan University)

First destination of technicians & MSc students

1. Didier Danillon (permanent job in Industry)
2. Helen McMiken (permanent job in Industry)
3. Amanda Crawford (emigrated to Australia)
4. Helen Powers (resigned from employment to raise family)
5. Mark Dorward (permanent post Dundee)
6. Helen Falconer (post-doc at Edinburgh)
7. Michal Zawadski (job with Syngenta)
8. Fraser Duthie (science job in Bonn)
9. Phil Ward (retired reaching normal retirement age)

Naismith publications

2025 (6 publications)

292. Velazco, A., T. Glen, S. Klumpe, A. Pennington, J. Zhang, J. L. R. Smith, C. Glynn, W. Bowles, M. Kobylynska, R. A. Fleck, J.H. Naismith, J. S. Kim, M. C. Darrow, M. Grange, A. I Kirkland, M. Dumoux 2025 Reduction of SEM charging artefacts in native cryogenic biological samples *Nat Comm* 16, 5204 (doi 10.1038/s41467-025-60545-3)
291. Matabaro, E., H. Song, L. Sonderegger, F. Gherlone, A. M. Giltrap, S. Liver, A. D. Gossert, M. Künzler, J. H. Naismith 2025 Macrocyclization of backbone N-methylated peptides by a prolyl oligopeptidase with a distinctive substrate recognition mechanism *Chem Sci* 16, 14196-14206 (doi 10.1039/D5SC03723A)
290. Yang, Y., M. Batista, B. R. Clarke, M. R. Agyare-Tabbi, H. Song, N. M. Kuehfuss, A. Le Bas, C. V. Robinson, C. Whitfield, J. H. Naismith, J. Liu 2025 Molecular basis for the phosphorylation of bacterial tyrosine kinase Wzc. *Nat Comm* 16, 3437 (doi 10.1038/s41467-025-58693-7)
289. Berger, C., H. Watson, J.H. Naismith, M. Dumoux, M. Grange 2025 Xenon plasma focused ion beam lamella fabrication on high-pressure frozen specimens for structural cell biology *Nat Comm* 16, 2286 (doi 10.1038/s41467-025-57493-3)
288. Hussain, N., H. Mikolajek, P. J. Harrison, N. Paterson, M. W. Akhtar, S. Sadaf, J. H. Naismith 2025 Structural and functional snapshots of a broad-specificity endoglucanase from Thermogutta terrifontis for biomass saccharification Arch Bioch Biophy, 764, 110274 (doi 0.1016/j.abb.2024.110274)
287. Le Bas A, et al 2025 Structure of WzxE the lipid III flippase for Enterobacterial Common Antigen polysaccharide *Open Biol*15, 240310 (doi 10.1098/rsob.240310)

2024 (6 publications)

286. Cornish, K. et al. 2024 Structural and functional characterization of nanobodies that neutralize Omicron variants of SARS-CoV-2 *Open Biol* 14, 230252 (doi 10.1098/rsob.230252)
285. Richards, N. G. J., J. H Naismith 2024 The chemistry of Formycin biosynthesis *Front. Chem. Biol*. 3, 1428646 (doi 10.3389/fchbi.2024.1428646)
284. Deimel, L.P., et al. 2024 Covalent penicillin-protein conjugates elicit anti-drug antibodies that are clonally and functionally restricted. *Nat Comm* 15, 6851 (2024). (doi 10.1038/s41467-024-51138-7
283. Parkhurst, J. M., T. Varslot, M. Dumoux, C.A. Siebert, M. Darrow, M. Basham, A. Kirkland, M. Grange, G. Evans, J.H. Naismith 2024 Pillar data-acquisition strategies for cryo-electron tomography of beam-sensitive biological samples Acta Cryst D80, 421-438 (doi 10.1107/S2059798324004546)
282. Parkhurst, J. M., A. Cavalleri, M. Dumoux, D. Clare, C.A. Siebert, G. Evans, J.H. Naismith, A. Kirkland, J. W. Essex 2024 Computational models of amorphous ice for accurate simulation of cryo-EM images of biological samples Ultramicroscopy, 256, 113882 (doi 10.1016/j.ultramic.2023.113882)
281. Couturier, C. 2024. et al. Studies of antibacterial activity (in vitro and in vivo) and mode of action for des-acyl tridecaptins (DATs) *Eur. J. Med. Chem* 266, 116087 (doi 10.1016/j.ejmech.2023.116097)

2023 (8 publications)

280. Madhusudanan, A., C. Iddon, M. Cevik, J. H. Naismith, S. Fitzgerald 2023 Non-pharmaceutical interventions for COVID-19: a systematic review on environmental control measures *Phil Trans R Soc* A381 20230130 (doi 10.1098/rsta.2023.0130)
279. Gao, X., J. W. Thrush, J. Gor, J. H. Naismith, R. J. Owens, S. J. Perkins 2023 The solution structure of the heavy chain–only C5-Fc nanobody reveals exposed variable regions that are optimal for COVID-19 antigen interactions *J. Biol. Chem*. 299, 105337 (doi 10.1016/j.jbc.2023.105337)

278. Parkhurst, J. M. et al. 2023 Investigation of the milling characteristics of different focused-ion-beam sources assessed by three-dimensional electron diffraction from crystal lamellae. *IUCrJ* (doi 10.1107/S2052252523001902).
278. Dumoux, M., J. L. R. Smith, T. Glen, M. Grange, M. C. Darrow, & J. H. Naismith. 2023 A protocol for cryogenic volumetric imaging using serial plasma FIB/SEM. In *Methods in Cell Biology* (eds. pp. Elsevier) (doi: 10.1016/bs.mcb.2023.01.015)
277. Weckener, M., L. S. Woodward, B.R. Clarke, H. Liu, P. N. Ward, A. Le Bas, D. Bhella, C. Whitfield, J.H. Naismith 2023 The lipid linked oligosaccharide polymerase Wzy and its regulating co-polymerase, Wzz, from enterobacterial common antigen biosynthesis form a complex *Roy Soc Open Bio* (doi 10.1098/rsob.220373)
276. Berger, C., M. Dumoux, T. Glen, N. B. Yee, J. M. Mitchels, Z. Patáková, M. C. Darrow, J. H. Naismith, & M. Grange. 2023 Plasma FIB milling for the determination of structures *in situ*. *Nat Commun* **14**: 629 (doi 10.1038/s41467-023-36372-9).
275. Li, W., G. C. Girt, A. Radadiya, J. J. P. Stewart, N. G. J. Richards, & J. H. Naismith. 2023 Experimental and computational snapshots of C-C bond formation in a C-nucleoside synthase. *Open Biol* **13**: 220287 (doi 10.1098/rsob.220287).
274. Dumoux, M. et al. 2023 Cryo-plasma FIB/SEM volume imaging of biological specimens. *Elife* **12**: e83623 (doi 10.7554/eLife.83623).

2022 (6 publications)

273. Mikolajek, H. M. Weckener, Z.F. Brotzakis, J. Huo, E.V Dalietou, A. Le Bas, P. Sormanni, P.J. Harrison, P.N. Ward, S. Truong, L. Moynie, D. Clare, M. Dumoux, J. Dormon, C. Norman, N. Hussain, V. Vogirala, R. J. Owens, M. Vendruscolo & J. H. Naismith (2022) Correlation between the binding affinity and the conformational entropy of nanobodies targeting the SARS-CoV-2 spike protein. Proc Natl Acad Sci USA 118, e2205412119 (doi 10.1073/pnas.2205412119)
272. Le Bas, A., H. Mikolajek, J. Huo, C. Norman, J. Dormon, J. H. Naismith & R. J. Owens Production and Crystallization of Nanobodies in Complex with the Receptor Binding Domain of the SARS-CoV-2 Spike Protein 12, 10.21769/BioProtoc.4406
271. Buchanan, C.J., B. Gaunt, P. J. Harrison, Y. Yang, J. Liu, A. Khan, A. M. Giltrap, A. Le Bas, P. N. Ward, K. Gupta, M. Dumoux, T. K. Tan, L. Schimaski, S. Daga, N. Picchiotti, M. Baldassarri, E. Benetti, C. Fallerini, F. Fava, A. Giliberti, P. I. Koukos, M. J. Davy, A. Lakshminarayanan, X. Xue, G. Papadakis, L. P. Deimel, V. Casablancas-Antràs, T. D. W. Claridge, A. M .J .J. Bonvin, Q. J. Sattentau, S.Furini, M. Gori, J. Huo, R. J. Owens, C. Schaffitzel, I. Berger, A. Renieri, GEN-COVID Multicenter Study, Naismith, J.H., Baldwin, A.J. & B. G. Davis (2022) Cryptic pathogen-sugar interactions revealed by universal saturation transfer analysis. Science, 337, 385-401 (doi 10.1126/science.abm3125)
270. Levitz, T.S, Weckener, M. I. Fong, Naismith, J. H., Drennan, C. L., Brignole, E.J., Clare, D.K, & Darrow, M.C. (2022) Approaches to using the chameleon: robust, automated, fast-plunge cryoEM specimen preparation. Frontiers Molecular Biosciences. (doi 10.3389/fmolb.2022.903148)
269. Moynié, L., Hoegy, F., Milenkovic, S., Munier, M., Paulen, A., Gasser, V., Faucon, A.L., Zill, N., Naismith, J.H., Ceccarelli, M., Schalk, I.J., Mislin, G.L.A. (2022) Hijacking of the enterobactin pathway by a synthetic catechol vectors designed for antibiotic delivery in pseudomonas aeruginosa. ACS Infectious Disease (doi 10.1021/acsinfecdis.2c00202).
268. Le Bas, A., H. Mikolajek, J. Huo, C. Norman, J. Dormon, J. H. Naismith & R. J. Owens Production and Crystallization of Nanobodies in Complex with the Receptor Binding Domain of the SARS-CoVCoV-2 Spike Protein. Bio-protocol 2022 May 5; 12(09): e4406. (doi: 10.21769/BioProtoc.4406)

2021 (14 publications)

267. Tang, Q., R. J. Owens, & J. H. Naismith. 2021 Structural Biology of Nanobodies against the Spike Protein of SARS-CoV-2. *Viruses* **13**: 2214 (doi 10.3390/v13112214).

266. Zhang, J. et al. 2021 Catalytic flexibility of rice glycosyltransferase OsUGT91C1 for the production of palatable steviol glycosides. *Nat Commun* **12**: 7030 (doi 10.1038/s41467-021-27144-4).
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