Hyal-EGF Domain Essential for PH20 Enzymatic Activity

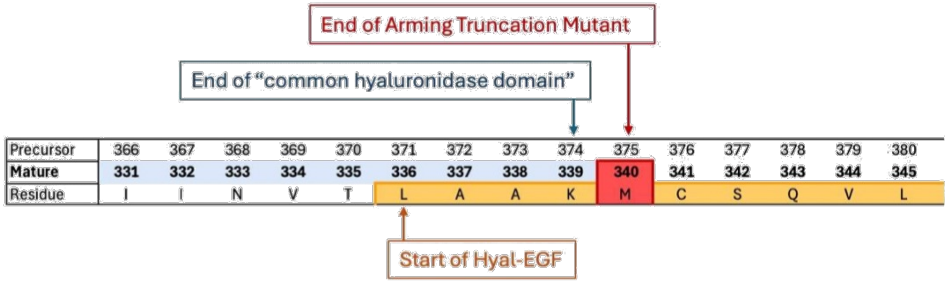
84. A notable finding in Chao was its identification of the "Hyal-EGF" domain in the C-terminal region of human hyaluronidases. Chao observed that the C-terminal region of mammalian hyaluronidases does not exhibit significant homology but does contain a cysteine-rich pattern of residues recognized by sequence analysis tools as an epidermal growth factor (EGF)-like motif (below). Chao identified the pattern of sequences that generates this Hyal-EGF structural motif (*i.e.*, "x4Cx0-48Cx3-12Cx1-70Cx1-6Cx2Gax0-21Gx2C, where "a" denotes a hydrophobic residue, "x" denotes any residue, and the gaps between cysteine residues vary in length as indicated by the subscripts.").[41] This is a good

EX1003 (Hecht), ¶ 84; Pet., 12, 38; Reply, 13

Deletion of the EGF-like Domain Abolishes Hyal1 Activity Independently of Asparagine 350—The C-terminal EGF-like domain is not found in the bacterial or bee venom hyaluronidases, so previous models of Hyal1 using coordinates for the bee venom ortholog depicted this region as an independently folded domain with minimal contacts to the active site containing domain. 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 (Fig. 5*C*). Western blot showed the N350tr lysates contained a major band of ≈38 kDa, the predicted molecular mass for the polypeptide, whereas the major band in L356tr lysates was ≈42 kDa, consistent with the 4-kDa shift observed for the fully glycosylated wild-type enzyme. The lysates showed ≈2.5 and ≈6.3% of wild-type activity for N350tr and L356tr, respectively, at the 125 μM HA amount. Thus, loss of this domain is deleterious to both secretion and activity of Hyal1, although the residual activity suggests the effect is indirect.

EX1010 (Zhang), 9439; Pet., 12; Reply, 14, 18

Disclosure's Common Hyaluronidase Domain Does Not Identify Hyal-EGF Domain



Precursor	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380
Mature	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345
Residue	I	I	N	V	T	L	A	A	K	M	C	S	Q	V	L

Reply, 19

among PH20 hyaluronidase polypeptides. PH20 hyaluronidases contain a common core hyaluronidase domain region of about 340 amino acids in length that corresponds to amino acid residues 38-374 of the precursor human PH20 sequence set forth in SEQ ID NO:6. 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 of SEQ ID NO:6 (*e.g.*, amino acid residues corresponding to positions 36-464 of the amino acid sequence set forth in SEQ ID NO:6) is the minimal sequence required for hyaluronidase activity (see *e.g.*, U.S. Patent Application No. 10/795,095,which is issued as U.S. Patent No. 7,767,429; see also U.S. Publication No. US20100143457).

EX1026 ('731 App.), 79:22-31; Reply, 18

161. If Dr. Petsko is suggesting that positions 3 to 339 correspond to the α/β barrel motif in PH20, that would be misleading. That is because several loops in PH20 are much more extensive and contain additional and elongated secondary structural elements when compared to the canonical α/β barrel (which comprises 248 residues in 1TIM). Some examples are the strand loop strand (Tyr 64 – Ser 84), a helix (Lys126-Gln140), a sixteen residue longer helix (Leu146-Leu175), a longer loop (Pro187-Asn205), and a twenty residue longer helix (Met313 – Leu345). Similar observations were made by Chao (EX1006) in the paragraph discussed in ¶ 141.[182]

EX1133 (Naismith), ¶ 161; Reply 18

162. The "common core hyaluronidase domain" of PH20 referred to in the common disclosure (quoted by Dr. Petsko), when produced, has no enzymatic activity as Dr. Hecht noted.[183] The model of PH20 also shows that position 339 is part of an elongated alpha helix. The "common core hyaluronidase domain" also excludes the EGF domain, an indispensable feature of the structure-function relationship of PH20.

EX1133 (Naismith), ¶ 162; Reply 17-19

Hyal-EGF Domain Not Characterized in Disclosure

Finally, Petitioner makes much of a "hyal-EGF" domain," which Petitioner acknowledges is common structural feature shared by actives. Petitioner contends the hyal-EGF domain is not identified by name in the specification. But the patent need not identify common structural features using Petitioner's preferred terminology (particularly given the Hyal-EGF is a misnomer). The patent expressly reports the import of this region ("amino acid residues corresponding to positions 36-464… is the minimal sequence required for hyaluronidase activity") and incorporates by reference Arming, which Petitioner itself says "proves" the region was essential based on its teaching that a C-terminal truncation mutant ending at position 340 was not secreted or active. EX1001, 69:67-70:15.

PO Sur-Reply, 22-23

96. The data in the patent (particularly Tables 3 and 5 for "active mutants" and "inactive mutants," respectively) shows that making single amino acid substitutions in the region from 337-409 resulted in both active mutants and inactive mutants for most of the range of positions. See Appendix A-9. In the aggregate, there appear to be somewhat more single mutations that result in inactive mutants than those that result in active mutants, but that is a qualitative assessment. Unsurprisingly, mutations that changed the cysteine residues (which are also highly conserved) were uniformly inactive. The varying effects of changing residues in the Hyal-EGF region of PH20 show that a skilled artisan's belief that changes in this region would be unpredictable were warranted and would be more so if multiple changes were made concurrently.

EX1003 (Hecht), ¶ 96; Reply, 34

Lack of Enablement

- **The disclosure must enable the full scope of the claims**
  - The claims encompass multi-modified PH20 polypeptides that are not predictable from the disclosure
- **Neither iterative mutagenesis nor homology modeling can enable the full scope of the claims**
  - Scale of experimentation is impossible
  - Dr. Petsko's tolerance analysis is flawed
- **Halozyme's claim construction encompasses mutants with no credible utility and inoperative species**

See, e.g., Pet., 66-76, 79-83; Reply, 22-30, 33-35, 38-43

Limitations of Homology Models

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Drs. Hecht, Park, and Naismith Explained Limits of Homology Models

158. The effects of these myriad sets of combinations of multiple substitutions within PH20 could not have been predicted by a skilled artisan in the 2011 timeframe using the tools that were available then. For example, while the PH20 protein structure models Dr. Park used provided reliable insights when modeling the change of a single residue at a position where the model was, they cannot provide reliable insights when the modeled sequence incorporates many (*e.g.*, more than ~5) substitutions not found in a naturally occurring protein. That is because (i) if the modeled sequence incorporates multiple changes, it no longer has validity as a naturally occurring sequence, and (ii) the changes significantly diminish the reliability of other positions of the model used to assess the change because they are no longer based on the structural positioning of residues within the template structure used to generate the model. Thus, a skilled artisan would have had to discover which combinations of substitutions to the PH20 protein would result in mutants that do exhibit hyaluronidase activity by making and testing all of them, an impossibly large undertaking.

EX1003 (Hecht), ¶ 158; Pet. 69, 74; Reply, 7, 9-10, 21, 23-24

127. Computational tools available in the 2011-2012 timeframe also were not capable of identifying and predicting the myriad types of subtle disruptions to a protein structure that collectively determine if a particular mutated sequence will properly fold and remain stably folded.[142]

EX1133 (Naismith), ¶ 127; Reply, 8-9

167. Protein structure models generated by SWISS-MODEL and similar tools in the 2011 time frame would have been reliable for evaluating single amino acid substitutions that are in regions of the PH20 model where there is a sufficiently reliable local QMEAN score. This is possible because only one residue is being changed and the remainder of the protein is still based on a reliable experimentally-determined structure (here, HYAL1).

168. In 2011, a second or third substitution could possibly be considered, but modeling multiple substitutions is more challenging and predictions can quickly become unreliable. This is especially true if the mutated residues are close to each other, either in sequence or in space, so that they interact with each other. That is because one needs to model interaction between substituted residues as well as interaction between a substituted residue with constant neighbors. Because this is difficult to visualize, it becomes necessary to model individual combinations through homology modeling, which becomes combinatorial intractable.

169. Consequently, a skilled artisan in 2011 could not have used SWISS-MODEL to reliably predict the effects on the structure of a modified PH20 polypeptide of many substitutions (*e.g.*, 5 or more) in the PH20 sequence, if each position is allowed to vary independently to one of several amino acids. In addition, a skilled artisan could not have used a SWISS-MODEL of PH20 to reliably predict the effects of substitutions within portions of the PH20 sequence where the model is not reliable (*i.e.*, outside of the PH20 sequence between positions 7 and 403).

EX1004 (Park), ¶¶ 167-169; Pet., 14, 75; Reply, 8-9, 35

Homology Models Could Not Accurately Predict Structure from Sequence in 2011-2012

132. The 2024 Chemistry Nobel Price Background paper that Dr. Hecht references in his declaration provides an accurate historical perspective on the capabilities of computational tools to accurately predict a protein's tertiary structure from its amino acid sequence. As that paper shows, those computational tools did not reach predictive accuracy levels comparable to experimental determination of structure until around 2019 (below).[151] While experimental techniques were known by 2011-2012 that could investigate the preservation of secondary structures and generally characterize the folded state of proteins, it would not have been possible for a POSA to use them to assess even a tiny fraction of the sequence space defined by the claim parameters that include modified PH20 polypeptides with 5, 10, 15 or 20 changes.

EX1133 (Naismith), ¶ 132 (citing EX1027 (Nobel Chemistry Background), 7-8, Figure 6); Reply, 8-9

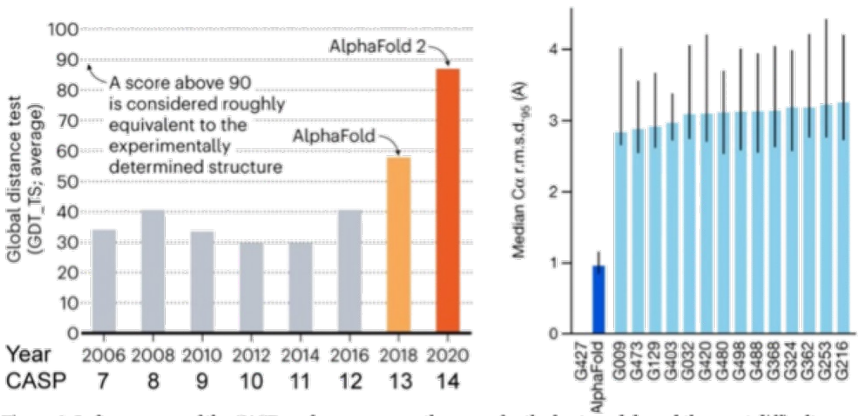


Figure 6. Left: progress of the CASP performance over the years for the best models and the most difficult targets.[38] Right: performance of AlphaFold2 relative to the top 15 entries by other groups in CASP14. Data are the median coordinate error and the 95% confidence interval of the median, estimated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

EX1027 (Nobel Chemistry Background), 8, Figure 6; Reply, 8-9

However, the reliability of contact prediction remained low for many years, until CASP12 in 2016, when the accuracy suddenly increased dramatically. … Despite the progress outlined above, the average GDT score for the best *ab initio* predictions was stuck below 40% up until and including CASP12 in 2016.

EX1027 (Nobel Chemistry Background), 7; Reply, 8-9

Homology Model Cannot Model C-Terminal Region of PH20 Past Position 403

Additionally, all parties agree that an accurate homology model of PH20's structure, showing the distorted PH20 alpha-beta barrel fold and location of each amino acid in the structure, was available to a POSA. Pet., 11-15, 74, 100-109;

PO Sur-Reply, 5

159. Portions of a structural model become less reliable in regions where there are differences in the target-template alignment.[124] The HYAL1 and PH20 sequences have different lengths: PH20 has 474 residues in its mature sequence, while HYAL1 has only 414. Also, the HYAL1 template structure cannot provide structural information for PH20 amino acids past position 403, because the sequence is no longer homologous after position 421 in HYAL.

EX1004 (Park), ¶ 159; PO Sur-Reply, 5-6, 22



EX2070 (Petsko), ¶ 131; PO Sur-Reply, 5

```
SEQ ID NO: 3          moltype = AA  length = 447
FEATURE               Location/Qualifiers
REGION                1..447
                      note = Mature PH20 36-482
source                1..447
                      mol_type = protein
                      organism = Homo sapiens
SEQUENCE: 3
LNFRAPPVIP NVPFLWAWNA PSEFCLGKFD EPLDMSLFSF IGSPRINATG QGVTIFYVDR  60
LGYYPYIDSI TGVTVNGGIP QKISLQDHLD KAKKDITFYM PVDNLGMAVI DWEEWRPTWA  120
RNWKPKDVYK NRSIELVQQQ NVQLSLTEAT EKAKQEFEKA GKDFLVETIK LGKLLRPNHL  180
WGYYLFPDCY NHHYKKPGYN GSCFNVEIKR NDDLSWLWNE STALYPSIYL NTQQSPVAAT  240
LYVRNRVREA IRVSKIPDAK SPLPVFAYTR IVFTDQVLKF LSQDELVYTF GETVALGASG  300
IVIWGTLSIM RSMKSCLLLD NYMETILNPY IINVTLAAKM CSQVLCQEQG VCIRKNWNSS  360
DYLHLNPDNF AIQLEKGGKF TVRGKPTLED LEQFSEKFYC SCYSTLSCKE KADVKDTDAV  420
DVCIADGVCI DAFLKPPMET EEPQIFY                                      447
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EX1026 ('731 App.), SEQ ID NO:3

Prior Art PH20 Model Did Not Accurately Describe PH20 Structure

Additionally, all parties agree that an accurate homology model of PH20's structure, showing the distorted PH20 alpha-beta barrel fold and location of each amino acid in the structure, was available to a POSA. Pet., 11-15, 74, 100-109;

PO Sur-Reply, 5

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.

EX1006 (Chao), 6913; Pet., 103

7.1. Description of the Model Structures

For all five human Hyal structure models, the major catalytic domain (Figures 4 and 5) closely resembles the $(\beta/\alpha)_8$ TIM barrel of the parent BVHyal structure. The number of sheets constituting the central part of the TIM barrel varies, however, but is similar to those of various forms of the BVHyal structures. The wider end of the TIM barrel assumes a conformation that creates a large and elongated cleft. Its sides are built primarily from aa residues of loops formed between sheets and helices of the TIM barrel. Based on similarity to BVHyal[11] and mutation analysis of HPH-20,[89] this region is responsible for binding the HA substrate and for catalysis, and it is large enough to bind at least an octasaccharide segment of HA. The extreme N-terminal segment of the catalytic domain, ranging in length from 28 aa for human Hyal-1 to 41 aa for HPH-20, always assumes a helical conformation. It takes on a structure of a single helix for Hyal-1 and Hyal-2 (the Hyals with the shortest segment at their N-terminus) or a set of two helices connected by a short loop for Hyal-3, Hyal-4, and HPH-20. Hyal-4 and HPH-20 have the longest aa segments at their N-termini (Figure 3). In all five cases, however, the helices are located on the outside of the TIM barrel core structure, are not incorporated into the distorted TIM barrel fold, and are primarily associated with the catalytic domain only; the interactions with the C-terminal domain are minimal, if any (Figures 4 and 5). The sequence similarity in this region of all human Hyals is very low, compared to the remaining portion of these Hyals, and therefore allows for some variations in structure.

EX1008 (Stern), 829; Reply, 13-14, 16

Regions of PH20 Have Insufficient Quality in the PH20 Homology Model

Additionally, all parties agree that an accurate homology model of PH20's structure, showing the distorted PH20 alpha-beta barrel fold and location of each amino acid in the structure, was available to a POSA. Pet., 11-15, 74, 101-107;

PO Sur-Reply, 5

Position	Local QMEAN Score from	Position	Local QMEAN Score from	Position	Local QMEAN Score from	Position	Local QMEAN Score from	Position	Local QMEAN Score from	Position	Local QMEAN Score from	Position	Local QMEAN Score from	Position	Local QMEAN Score from	Position	Local QMEAN Score from
1		51	0.58	101	0.77	151	0.74	201	0.83	251	0.74	301	0.83	351	0.77	401	0.66
2		52	0.69	102	0.73	152	0.79	202	0.81	252	0.71	302	0.84	352	0.77	402	0.59
3	0.37	53	0.68	103	0.79	153	0.86	203	0.81	253	0.78	303	0.83	353	0.76	403	0.65
4	0.47	54	0.66	104	0.55	154	0.82	204	0.71	254	0.75	304	0.79	354	0.74	404	0.63
5	0.50	55	0.80	105	0.59	155	0.78	205	0.58	255	0.69	305	0.71	355	0.72	405	0.51
6	0.63	56	0.82	106	0.72	156	0.80	206	0.84	256	0.55	306	0.64	356	0.74	406	0.52
7	0.68	57	0.87	107	0.73	157	0.84	207	0.83	257	0.54	307	0.66	357	0.36	407	0.62
8	0.67	58	0.84	108	0.76	158	0.80	208	0.84	258	0.47	308	0.79	358	0.52	408	0.59
9	0.71	59	0.74	109	0.84	159	0.81	209	0.80	259	0.49	309	0.66	359	0.66	409	0.61
10	0.75	60	0.70	110	0.87	160	0.88	210	0.76	260	0.44	310	0.78	360	0.70	410	0.63
11	0.57	61	0.84	111	0.91	161	0.87	211	0.83	261	0.52	311	0.67	361	0.74	411	0.62
12	0.68	62	0.88	112	0.88	162	0.81	212	0.78	262	0.58	312	0.73	362	0.78	412	0.64
13	0.67	63	0.87	113	0.81	163	0.84	213	0.74	263	0.78	313	0.66	363	0.81	413	0.63
14	0.73	64	0.89	114	0.89	164	0.83	214	0.72	264	0.73	314	0.68	364	0.81	414	0.64
15	0.76	65	0.91	115	0.82	165	0.84	215	0.77	265	0.76	315	0.73	365	0.84	415	0.59
16	0.75	66	0.83	116	0.81	166	0.86	216	0.71	266	0.76	316	0.83	366	0.83	416	
17	0.81	67	0.85	117	0.88	167	0.82	217	0.76	267	0.80	317	0.78	367	0.83	417	
18	0.70	68	0.76	118	0.84	168	0.82	218	0.73	268	0.82	318	0.74	368	0.84	418	
19	0.70	69	0.79	119	0.76	169	0.81	219	0.70	269	0.84	319	0.72	369	0.75	419	
20	0.67	70	0.71	120	0.78	170	0.76	220	0.74	270	0.74	320	0.64	370	0.86	420	
21	0.62	71	0.67	121	0.77	171	0.82	221	0.77	271	0.80	321	0.74	371	0.90	421	
22	0.65	72	0.72	122	0.76	172	0.79	222	0.76	272	0.71	322	0.81	372	0.85	422	
23	0.61	73	0.76	123	0.69	173	0.70	223	0.82	273	0.65	323	0.73	373	0.80	423	
24	0.54	74	0.78	124	0.77	174	0.70	224	0.81	274	0.59	324	0.71	374	0.79	424	
25	0.67	75	0.88	125	0.74	175	0.67	225	0.85	275	0.54	325	0.84	375	0.73	425	
26	0.64	76	0.86	126	0.74	176	0.69	226	0.86	276	0.58	326	0.83	376	0.74	426	
27	0.51	77	0.87	127	0.82	177	0.79	227	0.85	277	0.51	327	0.81	377	0.59	427	
28	0.46	78	0.85	128	0.83	178	0.74	228	0.84	278	0.51	328	0.76	378	0.61	428	
29	0.53	79	0.84	129	0.82	179	0.72	229	0.78	279	0.71	329	0.82	379	0.66	429	
30	0.54	80	0.88	130	0.83	180	0.73	230	0.74	280	0.78	330	0.82	380	0.73	430	
31	0.48	81	0.80	131	0.82	181	0.82	231	0.68	281	0.82	331	0.83	381	0.79	431	
32	0.52	82	0.70	132	0.71	182	0.88	232	0.81	282	0.81	332	0.81	382	0.85	432	
33	0.52	83	0.75	133	0.82	183	0.89	233	0.81	283	0.82	333	0.81	383	0.83	433	
34	0.57	84	0.79	134	0.84	184	0.87	234	0.49	284	0.78	334	0.84	384	0.90	434	
35	0.56	85	0.78	135	0.74	185	0.87	235	0.45	285	0.74	335	0.77	385	0.85	435	
36	0.64	86	0.79	136	0.78	186	0.84	236	0.48	286	0.80	336	0.81	386	0.85	436	
37	0.58	87	0.81	137	0.84	187	0.87	237	0.57	287	0.83	337	0.84	387	0.83	437	
38	0.66	88	0.82	138	0.81	188	0.79	238	0.75	288	0.82	338	0.83	388	0.86	438	
39	0.77	89	0.84	139	0.79	189	0.75	239	0.75	289	0.81	339	0.74	389	0.83	439	
40	0.67	90	0.84	140	0.81	190	0.79	240	0.74	290	0.84	340	0.81	390	0.80	440	
41	0.70	91	0.83	141	0.84	191	0.77	241	0.76	291	0.88	341	0.82	391	0.85	441	
42	0.68	92	0.87	142	0.78	192	0.57	242	0.77	292	0.88	342	0.77	392	0.89	442	
43	0.60	93	0.77	143	0.79	193	0.67	243	0.83	293	0.82	343	0.72	393	0.77	443	
44	0.50	94	0.79	144	0.81	194	0.67	244	0.74	294	0.81	344	0.78	394	0.80	444	
45	0.55	95	0.82	145	0.80	195	0.66	245	0.82	295	0.83	345	0.79	395	0.84	445	
46	0.50	96	0.76	146	0.79	196	0.65	246	0.79	296	0.79	346	0.73	396	0.80	446	
47	0.48	97	0.73	147	0.78	197	0.73	247	0.82	297	0.72	347	0.72	397	0.72	447	
48	0.58	98	0.77	148	0.78	198	0.65	248	0.77	298	0.75	348	0.79	398	0.79		
49	0.61	99	0.70	149	0.88	199	0.64	249	0.81	299	0.76	349	0.61	399	0.77		
50	0.62	100	0.65	150	0.81	200	0.79	250	0.82	300	0.81	350	0.77	400	0.77		

Red - QMEAN score < 0.6
Blue - QMEAN score > 0.6

EX1133 (Naismith), App'x B; Reply, 34

312. A POSA can classify residues as buried or surface residues using information in a PH20 homology model file ("pdb" file) that measures the solvent-accessible surface area ("SASA") for each modeled residue. For PH20, that information is only available for positions 4-403 because that is the portion of the PH20 protein that is supported by the Hyal-1 template structure. A POSA would not rely on portions of a PH20 homology model that fall outside of the template structure used to generate it, or for which insufficient quality in that part of the model exists. A POSA thus would not have included residues 1-3 and 404-447 in the set of positions Dr. Petsko labeled "generally tolerant" because there is no structural context available for those positions. Likewise, the POSA would have considered positions 3–5, 27–35, 44–48, 104–105, 234–237, 256–262, 274–278, 357–358, 405–406 to have unreliable local QMEAN values.

EX1133 (Naismith), ¶ 312; Reply, 34

No Mutant Data Beyond Position 447, No Homology Modeling Past Position 403

sequence.[86] They also are not representative—they do not reflect the types or physicochemical diversity of modified PH20s claimed, including truncations and mutants with changes beyond position 447 (for which no data is provided).[87]

Reply, 21

118. I do not believe a POSA would consider the set of 21 hypothetical mutants Dr. Petsko discussed are at all representative of the diversity of enzymatically active modified PH20 polypeptides being claimed. That person also would not believe those 21 are in any way representative of the sequences within the claim parameter sequence space. They do not meaningfully probe physicochemical changes within the PH20 protein structure or mutants with truncations or changes beyond position 447, for which no data is provided. Other

EX1133 (Naismith), ¶ 118; Reply, 21

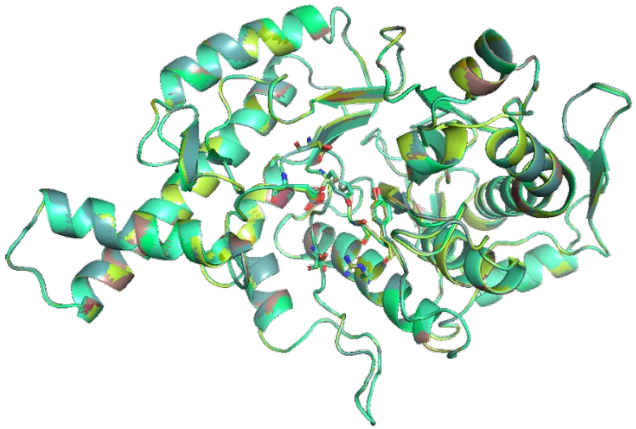
159. Portions of a structural model become less reliable in regions where there are differences in the target-template alignment.[124] The HYAL1 and PH20 sequences have different lengths: PH20 has 474 residues in its mature sequence, while HYAL1 has only 414. Also, the HYAL1 template structure cannot provide structural information for PH20 amino acids past position 403, because the sequence is no longer homologous after position 421 in HYAL.

EX1004 (Park), ¶ 159; Pet., 82, 107; PO Sur-reply, 5-6, 22

Homology Models Do Not Identify Structural Differences of Functionally Distinct Proteins

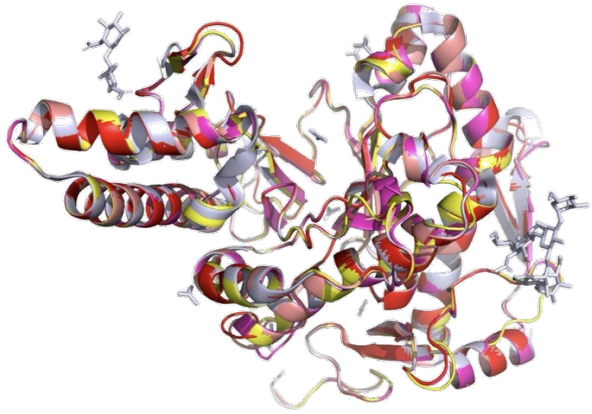
431. Finally, in the figures below, the human L307S, chimpanzee L307S, gibbon L307S, orangutan L307S, N47A/N131A/L307S, N47A/N219A/L307S, N131A/N219A/L307S and N47A/N131A/N219A/L307S variant models are shown on top of each other. The figures shows that the key active region residues I described above are predicted to adopt essentially the same position in all variant models, illustrating how the variants adopt a common structure in the active site.

EX2070 (Petsko), ¶ 431; POR, 4-5



183. When modeled on the human Hyal-1 structure as the template, the overlaid structures have only subtle visual differences (below).[203] This is a similar overlay of the homology models Dr. Petsko used in his declaration.[204] Using Dr. Petsko's high level perspective, all appear have the same "alpha-beta barrel" motif. Despite this similarity, the five proteins exhibit subtle but meaningfully different substrate preferences and activity.

EX1133 (Naismith), ¶ 183; Reply, 22



Homology Models Cannot Predict Effects of Many Changes

Position	WT	V	S	T	W
33	L	-	0.48	0.45	1.58
165	L	1.22	1.31	-	1.14
175	L	0.94	-	1.43	

EX1133 (Naismith), ¶ 272; Reply, 30

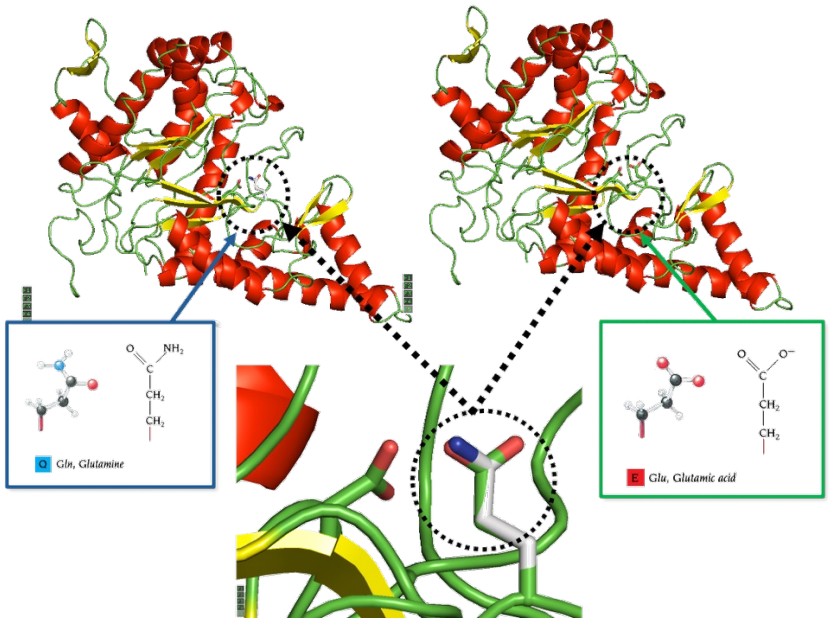
Position	WT	V	A	L	S	T	F
47	N	-	0.48	ND	0.85	1.49	1.32
291	G	1.63	-	ND	0.45	-	-
315	S	-	0.85	0.42		0.97	ND

EX1133 (Naismith), ¶ 273; Reply, 30

272. Much of the missing substitution data in the common disclosure cannot be reliably predicted using a homology model. This is because the disclosure reported different effects depending on sequence position, even where there are barely perceptible spatial differences in atoms. For example, Val and Thr are similarly sized residues; the difference is in Thr there is an OH in the side chain rather than a CH3. When compared to Ser which also has an OH, Thr has an additional CH3. As reflected in the table below, substitutions among these closely related residues at L175, L165, and L33—each located in hydrophobic environments—produce inconsistent and position-specific effects on activity, revealing no clear structural or chemical pattern that a POSA could have reliably predicted based on residue size or chemistry alone. The common disclosure does not explain this pattern of results, which a POSA in 2011-2012 could not have predicted from homology modeling or residue size and chemistry alone. (Grey denotes not reported, purple inactive, green active).

EX1133 (Naismith), ¶ 272; Reply, 30

Homology Models Cannot Identify Effects of Many Changes



281. Second, depending on the degree of sequence identity, the accuracy of a model generated by Swiss-Model can vary significantly from reality. If the modeled protein has at least 50% sequence identity to the protein used as the template, the positioning of the backbone α-carbon atoms typically deviate by ~1 Å RMSD from an experimentally determined structure, with side chains and loop structures deviating more.[307] PH20 and Hyal-1 have approximately ~36% overall sequence identity, with about 41% sequence identity in the overlapping portion of the PH20 sequence. This suggests that structural deviations less than around 1-2 Å will not be reliably detectable in the homology model. Epistasis (in multiple mutants) and changes in amino acids can alter the positioning of atoms by less than 1-2 Å. Yet enzyme catalysis and substrate recognition are exquisitely sensitive to much smaller positional and electrostatic differences. Indeed, as shown in the literature, the conservative Glu131→Gln substitution abolishes activity.[308] This is because the Glu residue is essential for catalysis, emphasizing that it is the precise structure-function relationship that is key to enzyme activity; not simply the overall fold of the protein.

EX1133 (Naismith), ¶ 281; Reply, 22, 30

Methods Require Impossible Scale of Experimentation

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Iterative Mutagenesis Method in the ’731 Application

347. A POSA seeking to make a modified PH20 with the ’590 patent in hand would not make a randomly generated combinatorial library of billions of sequences that happen to be at least 91% identical to SEQ ID NO: 3 and then screen it for activity, because it makes no sense to do so. It is difficult to imagine why anyone would do such a thing. If the sequences truly are random, then it is likely that a substantial number would include substantial modifications to residues known to be invariant because they are involved in catalysis, the active site, or in protein structure. A POSA would not make a library with billions of sequences that the POSA expects are not going to fold into PH20 proteins because it makes no sense to do that, and, thanks to the ’590 patent, the POSA has a much more sensible strategy.

EX2070 (Petsko), ¶ 347; Reply, 36

A. I did not go sequence by sequence and do that.
I guess maybe I need to say that no POSA would start with the theoretical maximum number of potential sequence variants as a starting point for making a comparative analysis of what amino acids would retain activity in a PH20 polypeptide.

EX1130, 93:18-24; Reply, 23

4. Iterative Methods

The method provided herein also is iterative. In one example, after the method is performed, any modified hyaluronan-degrading enzymes identified as exhibiting stability, such as increased stability, under a denaturation condition can be modified or further modified to increase or optimize the stability. A secondary library can be created by introducing additional modifications in a first identified modified hyaluronan-degrading enzyme. For example, modifications that were identified as conferring stability, such as increasing stability, can be combined to generate a combinatorial library. The secondary library can be tested using the assays and methods described herein.

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.

EX1026 (’731 App.), 150:1-26; Reply, 36

Limits of Screening Assays Well Below Scale Required

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.

EX1133 (Naismith), ¶ 123 (citing EX1147 (Van Rossum), 2991, Table 1); Reply, 8-9

Total Change	Total Sequences	#
1	1	1.00E+00
2	8,152	8.15E+03
3	33,150,118	3.32E+07
4	89,660,073,504	8.97E+10
5	181,449,739,545,075	1.81E+14
6	293,077,978,086,132,000	2.93E+17
7	393,555,483,371,437,000,000	3.94E+20
8	451,914,977,218,566,000,000,000	4.52E+23
9	452,989,259,911,317,000,000,000,000	4.53E+26
10	402,658,125,051,895,000,000,000,000,000	4.03E+29
11	321,362,356,487,055,000,000,000,000,000,000	3.21E+32
12	232,608,649,507,388,000,000,000,000,000,000,000	2.33E+35
13	153,968,078,195,545,000,000,000,000,000,000,000,000	1.54E+38
14	93,849,823,863,830,800,000,000,000,000,000,000,000,000	9.38E+40
15	52,991,852,975,982,400,000,000,000,000,000,000,000,000,000	5.30E+43
16	27,859,708,843,273,000,000,000,000,000,000,000,000,000,000,000	2.79E+46
17	13,698,336,949,198,000,000,000,000,000,000,000,000,000,000,000,000	1.37E+49
18	6,323,829,801,952,950,000,000,000,000,000,000,000,000,000,000,000,000	6.32E+51
19	2,750,529,891,360,250,000,000,000,000,000,000,000,000,000,000,000,000,000	2.75E+54
20	1,130,619,221,191,820,000,000,000,000,000,000,000,000,000,000,000,000,000,000	1.13E+57
21	440,435,474,482,722,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000	4.40E+59

EX1133 (Naismith), ¶ 126; Reply, 7-8

Limited Investigation Still Implicates Impossible Scale of Effort

Dr. Petsko described a similar methodology. In ¶ 359 of his declaration (below), he provides an example where the POSA inspects positions that tolerated between 60% and 100% of the tested changes using a homology model and selects sets of 22 substitutions from those positions. Dr. Petsko also suggests that "[t]he POSA seeking to make multiple mutations could generate a model using, for example, Swiss Model to evaluate the results of the anticipated modifications."[148] He also frequently mentions targeting surface residues. A POSA also would need to use a homology model to determine whether residues are surface residues or partially buried.[149] He outlines his proposed methodology in several other paragraphs.

359. A POSA could select 23 residues to change and reasonably expect to obtain an active PH20 as those changes were based on the data in the patent. For example, there are 40 positions that tolerated all tested substitutions, 87 that tolerated 90% or more and 158 that tolerated 60% or more. A POSA could select sets of 22 residues within this set based on the highlighted homology model that I described above and reasonably anticipate that most of the resulting proteins will be active.

EX1133 (Naismith), ¶ 130 (citing EX2070 (Petsko), ¶ 359); Reply, 8-9, 23

131. A POSA could not have performed this homology model-guided selection and positional assessment methodology for even the tiniest fraction of the claim parameter sequence space. Assume that one worked with the shortest reference sequence (430 residues), allowed 1 change at position 307, avoided changing the 57 essential residues identified in the common disclosure (*see* ¶¶ 303-304) and only made changes at Dr. Petsko's "≥60% tolerated positions". Also assume that only 5 additional changes were made, and the options were restricted to the tested active mutants at those ≥60% positions (an average of 11.6 alternatives). For that "simple" investigation of only one change at position 307 and 5 additional substitutions per polypeptide, a POSA would have to individually model and assess 86,548,299,246,165 distinct sequences. Spending only 1 minute per assessment would translate into 60 billion days of working 24 hours a day. That is plainly impossible. As Dr. Hecht explained, in the 2011-2012 timeframe, computational tools for modeling protein structure were not capable of accurately predicting the effect of 5 or more mutations on a native protein sequence and its corresponding structure.[150]

EX1133 (Naismith), ¶ 131; Reply, 8-9, 23

Problems with Dr. Petsko's Positional Tolerance Assessment

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Dr. Petsko's Positional Tolerance Table

Position	WT	30	31	32	61	62	63	92	93	94	123	124	125	154	155	156	185	186	187	216	217	218	247	248	249	278	279	280	309	310	311	340	341	342	371	372	373	402	403	404	433	434	435	436
1	L	D	E	P	L	G	Y	A	K	K	W	K	P	K	Q	E	L	F	P	W	L	W	V	R	E	L	K	I	M	I	M	R	M	S	A	I	Q	C	Y	S	F	L	K	P
2	N	L	D	K	Y	D	Y	L	D	D	K	D	K	F	K	K	D	D	D	N	L	N	A	K	A	L	S	L	S	M	R	S	C	S	I	Q	L	T	S	T	L	K	P
3	F	D	G	Y	P	K	K	D	I	K	D	V	P	E	K	E	K	C	P	W	L	W	I	R	I	L	Q	K	L	K	S	Q	S	T	Q	L	T	L	S	D	P	M	P
4	R	M	K	K	Y	K	Y	P	T	K	V	K	K	K	L	K	Y	K	K	E	L	T	I	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
5	A	S	K	P	P	K	Y	I	T	K	D	V	P	K	L	K	N	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
6	P	L	D	K	Y	D	Y	F	K	K	Y	K	P	A	G	E	K	C	P	A	L	W	I	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
7	P	F	S	P	P	K	Y	M	T	K	K	V	P	K	L	K	N	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
8	V	S	I	P	P	K	Y	P	T	K	D	V	P	K	L	K	N	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
9	I	F	T	P	P	K	Y	V	T	K	S	V	P	F	L	K	K	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
10	P	I	G	P	P	K	Y	D	T	K	I	V	P	L	L	K	K	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
11	N	G	V	P	P	K	Y	N	T	K	E	V	P	V	L	K	P	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
12	V	S	T	P	P	K	Y	L	T	K	L	V	P	E	L	K	G	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
13	P	P	V	P	P	K	Y	G	T	K	V	V	P	T	L	K	Y	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
14	F	R	N	P	P	K	Y	M	T	K	Q	V	P	I	L	K	N	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
15	L	I	G	P	P	K	Y	A	T	K	Q	V	P	K	L	K	G	K	K	T	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
16	W	N	G	P	P	K	Y	V	T	K	Q	V	P	K	L	K	L	K	K	Q	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
17	A	A	I	P	P	K	Y	I	T	K	N	V	P	K	L	K	G	K	K	Q	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
18	W	T	P	P	P	K	Y	D	T	K	V	V	P	K	L	K	F	K	K	F	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
19	N	G	Q	P	P	K	Y	W	T	K	Q	V	P	L	L	K	N	K	K	V	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
20	A	Q	K	P	P	K	Y	E	T	K	L	V	P	L	L	K	V	K	K	V	V	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
21	P	G	I	P	P	K	Y	E	T	K	S	V	P	R	L	K	E	K	K	V	A	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
22	S	V	S	P	P	K	Y	W	T	K	L	V	P	L	L	K	P	K	K	I	A	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
23	E	T	L	P	P	K	Y	R	T	K	T	V	P	N	L	K	P	K	K	I	K	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
24	F	I	Q	P	P	K	Y	P	T	K	E	V	P	H	L	K	R	K	K	R	L	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
25	C	F	D	P	P	K	Y	T	T	K	A	V	P	L	L	K	N	K	K	N	Y	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
26	L	Y	H	P	P	K	Y	W	T	K	T	V	P	W	L	K	D	K	K	D	V	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
27	G	V	L	P	P	K	Y	A	T	K	E	V	P	G	L	K	D	K	K	D	R	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
28	K	D	D	P	P	K	Y	R	T	K	K	V	P	Y	L	K	L	K	K	Y	N	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P
29	F	R	K	P	P	K	Y	N	T	K	A	V	P	A	L	K	S	K	K	S	R	A	V	R	I	L	Q	D	K	L	K	L	S	Q	K	L	T	L	S	E	P	M	P

EX2070 (Petsko), ¶ 274; Reply, 38

Inactive Mutant Results Cannot Provide Insights on PH20 Structure

245. Dr. Hecht expressed concerns over use of the mutants classified as inactive to draw conclusions about PH20 folding,[278] and I share those concerns. The primary reason for this conclusion is that the common disclosure does not provide information that permits a POSA to determine ***why*** any particular single amino acid substitution in the $PH20_{1-447}$ wild type sequence exhibited reduced (< 20 %) hyaluronidase activity. A POSA cannot tell from what is reported in the common 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.

EX1133 (Naismith), ¶ 245; Reply, 33-34

246. The possible outcomes of a single substitution in $PH20_{1-447}$ wild-type protein (*see* ¶ 48) have distinct implications for folding and positional tolerance. Outcomes (i)–(v) reflect defects in expression, secretion, correct folding, stability, or aggregation. It is not possible from the data provided in the common 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.

EX1133 (Naismith), ¶ 246; Reply, 33-34

Disclosure's Experimental Assay Had Unusually High Variability

Experimental design flaws and incomplete reporting of results independently render Dr. Petsko's ratings unreliable:[115]

- inadequate numerical, structural context, and physicochemical interrogation of positions prevent reliable ratings of tolerance;
- no use of positive/negative controls in the primary assay, and no evidence of proteins in supernatants or their folded state;
- no activities reported for inactives, precluding differentiation of non-secreted mutants from those with residual activity;
- averaging of activities conceals inter-assay variability;
- unusually high (~40%) assay variability (ordinarily ~10-15%) renders classifications unreliable.

Reply, 34

The results are reported in the table below:

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%

EX1133 (Naismith), ¶ 293; Reply, 34

[0271] In another example, hyaluronidase activity is measured using a microtiter assay in which residual biotinylated hyaluronic acid is measured following incubation with hyaluronidase (see e.g. Frost and Stern (1997) *Anal. Biochem.* 251:263-269, U.S. Patent Publication No. 20050260186). In Example 4, the hyaluronidase activity of truncated human PH20 hyaluronidase is determined using biotinylated hyaluronic acid. The free carboxyl groups on the glucuronic acid residues of hyaluronic acid are biotinylated, and the biotinylated hyaluronic acid substrate is covalently coupled to a microtiter plate. Following incubation with hyaluronidase, the residual biotinylated hyaluronic acid substrate is detected using an avidin-peroxidase reaction, and compared to that obtained following reaction with hyaluronidase standards of known activity. As the substrate is covalently bound to the microtiter plate, artifacts such as pH-dependent displacement of the biotinylated substrate does not occur. The sensitivity permits rapid measurement of hyaluronidase activity from cultured cells and biological samples with an inter-assay variation of less than 10%.

EX1133 (Naismith), ¶ 296 (citing EX2165 (US-457), ¶ 0271); Reply, 34

Dr. Petsko's Tolerance Table Conflicts with Guidance in Disclosure

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 7, 16, 17, 18, 19, 21, 25, 53, 55, 56, 57, 62, 64, 76, 78, 80, 88, 95, 100, 101, 109, 111, 112, 113, 115, 116, 121, 123, 126, 129, 185, 187, 188, 189, 190, 191, 194, 199, 201, 203, 207, 210, 223, 225, 227, 228, 229, 241, 243, 244, 246, 249, 250, 252, 254, 262, 268, 282, 295, 296, 298, 299, 303, 319, 322, 329, 330, 332, 333, 336, 337, 340, 341, 344, 345, 346, 350, 352, 354, 355, 362, 363, 364, 365, 366, 370, 372, 382, 384, 386, 390, 400, 402, 408, 423, 424, 429, 430, 431, with reference to

EX1026 ('731 App.), 91:30-37; Reply, 38

Q. So if I understand your answer, you're saying that a POSA might change the residue of position 299, despite it being marked in red in the table?
A. I'm saying that a POSA might change that residue if their objective to -- is to retain hyaluronidase activity in careful examination with what additional residues might need to be also concurrently mutagenized to compensate for that, the effects of that mutation.
I'm saying a POSA could predict that by examining the area around that residue and considering what they were seeking to substitute it with.

EX1130 (Simpson Dep.), 195:12-25; Reply, 38

Contraceptive Utility Issues

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Extent of Disclosure on Modified PH20s as Contraceptive Vaccines

C. MODIFIED PH20 POLYPEPTIDES

Provided herein are modified or variant PH20 polypeptides. The modified PH20 polypeptides provided herein exhibit altered activities or properties compared to a wildtype, native or reference PH20 polypeptide. Included among the modified PH20 polypeptides provided herein are PH20 polypeptide that are active mutants, whereby the polypeptides exhibit at least 40% of the hyaluronidase activity of the corresponding PH20 polypeptide not containing the amino acid modification (*e.g.*, amino acid replacement). In particular, provided herein are PH20 polypeptides that exhibit hyaluronidase activity and that exhibit increased stability compared to the PH20 not containing the amino acid modification. Also provided are modified PH20 polypeptides that are inactive, and that can be used, for example, as antigens in contraception vaccines.

EX1026, 99:13-22; Pet. 24 (cited as EX1001 ('590 Patent), 72:61-63)

Modified PH20 polypeptides provided herein can be used as vaccines in contraceptive applications. PH20 is present in the male reproductive tract, and is expressed in both the testis and epididymis and is present in sperm. PH20 plays a role in fertilization by facilitating entry of the sperm through the cumulus layer surrounding the unfertilized egg. PH20 also is able to bind to hyaluronic acid (HA) on the zona pellucida during early phases of fertilization. This binding also initiates intracellular signaling that aids in the acrosome reaction. Immunization with PH20 has been show 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. The polypeptides can be administered directly or can be administered as a recombinant virus to deliver the antigen.

EX1026 ('731 App.), 213:1-13; Pet., 24 (cited as EX1001 ('590 Patent), 194:48-195:67)

No Evidence of Any Modified PH20 Polypeptide with Contraceptive Effect

Q. And you are not aware of studies before 2012 of successful contraception in humans using modified PH20 polypeptides as a vaccine?
A. I'm not aware, no.
Q. Are you aware of any evidence published after 2012 of successful use of a modified PH20 polypeptide as a vaccine?
MR. POWERS: Object to form.
THE DEPONENT: No, I am not.
Q. (By Ms. Wang)In humans or in any other mammals?
A. No, I am not.
Q. So is fair to say there's no evidence of modified PH20 polypeptide being used successfully for contraception in humans?
MR. POWERS: Objection. Form.
THE DEPONENT: Not that I'm aware of.

EX1129 (Cherr Dep.), 101:18–102:9; Reply, 41

Q. (By Ms. Wang) Are you aware of any evidence published before 2012 investigating the effect of human PH20 on fertility in nonhuman mammals?
MR. POWERS: Objection. Form.
THE DEPONENT: No, I am not aware.
Q.(By Ms. Wang) Are you aware of any evidence published before 2012 of using modified human PH20 polypeptide to see its effect on fertility in nonhuman mammal?
MR. POWERS: Same objection.
THE DEPONENT: No, I am not.

EX1129 (Cherr Dep.), 104:9-20; Reply, 41

Prior Art: PH-20 Is Not a Useful Antigen for Immunocontraceptive Vaccines

Table 3
SPAM1 and Immunocontraception

Species	Immunization			Fertility	Response	Reference
	M	F	Form	Reduction		
Guinea pigs[a]	+	+	Aff. Purif.	+	(1) Caudal sperm depletion (2) Autoimmune orchitis	Primakoff et al. (1988), Tung et al. (1997)
Rabbit	+	+	rSPAM1	–	Restricted entry of IgG in reproductive tracts	Pomering et al. (2002)
Macaque	+	+	rSPAM1	–	”	Deng et al. (2002)
Mouse	+	+	rSpam1	–	”	Hardy et al. (2004)

M: male, F: female, Aff. Purif.: affinity purified, rSPAM1/Spam1: recombinant SPAM1.
[a] The guinea pig may be fundamentally different from other species in its autoimmune response.

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.

EX1189 (Martin-DeLeon), 119; Reply 41

Useful Contraceptive Activity According to Halozyme's Expert, Dr. Cherr

Q. And I am trying to understand how do you define "useful"?
A. Yes, so -- I define "useful" -- I mean, there's -- in the literature with experimental animals reducing litter size, people still use the term as an effective contraception. But in my mind, when it comes to human biology, it needs to be close to 100 percent.
Q. So from modified PH20 polypeptide to be a useful contraceptive in humans, it would need to cause 100 percent infertility?
MR. POWERS: Object to form.
THE DEPONENT: Or close to 100 percent, yes.

Q.(By Ms. Wang) For a modified PH20 polypeptide to be a useful contraceptive in nonhuman mammals, how much or what percentage of reduction in fertility would need to be achieved?
MR. POWERS: Objection. Form.
THE DEPONENT: It would be similar ideally.
Q. (By Ms. Wang) It would need to be 100 percent or close to it?
A. Similar to what hormone contraceptives are.

EX1129 (Cherr Dep.), 57:16-58:15; Reply 40

Extraordinarily Unlikely That Every Modified PH20 Polypeptide Exhibits Contraceptive Activity

52. Dr. Cherr stated that a modified PH20 polypeptide would need to cause close to 100 percent infertility to be useful as a contraceptive in humans. He also believes that a modified PH20 polypeptide would need to cause similar infertility to hormone contraceptives (which is typically near 100%) to be useful in nonhuman mammals.[67]

(EX1129 (Cherr Dep.), 57:16–58:15.). Dr. Cherr's opinions regarding the expected usefulness of modified PH20 polypeptides are irreconcilable with this high threshold, particularly in view of the experimental evidence available by the relevant timeframe. A POSA would have viewed it as extraordinarily unlikely that every possible modified PH20 polypeptide could elicit antibody responses that would provide near-100% effective contraception. To claim otherwise flouts well-established immunological principles.

EX1134 (Kelsoe) ¶ 52; *See also* Reply 40

Claimed AA Substitution Has Nothing to Do with Contraceptive Function

Q. (By Ms. Wang) So your opinion doesn't change as between a required modification at position 30 versus a required modification at position 313?
A. Right.

Q. And if a modified PH20 polypeptide had 95 percent sequence similarity to Sequence ID No. 3 or 32 through 66, but did not have a mutation at position 320, the opinions in your declaration would not change, would they?
MR. POWERS: Objection. Form.
THE DEPONENT: Yes, that's correct.

EX1129 (Cherr Dep.), 41:10-14, 42:8-14; Reply, 16, 41

Immunogens Must Retain Antigenic Determinants Relevant to Reproduction

105. A POSA would have expected that meaningful contraception (and certainly the near-100% contraceptive activity Dr. Cherr stated would be necessary for modified PH20 polypeptides to be useful antigens) could only be achieved with antibody responses that are specific to native epitopes implicated in reproductive function and that are sufficiently strong to carry out immune function.[164] A POSA would have expected that only a subset of modified PH20 polypeptides (if any) could elicit such a meaningful immune response. Determining which of the many possible modified PH20 polypeptides could reliably elicit contraceptive antibody responses could only be determined empirically.[165]

EX1134 (Kelsoe), ¶ 105; Reply 42-43

Modified PH20s Lacking Antigenic Determinants Relevant to Contraception are Unlikely to Exhibit Contraceptive Activity

93. I understand the modified PH20 polypeptides I've been asked to consider in my opinion can contain multiple amino acid modifications, including substitutions, insertions, and/or deletions, in addition to the required change at position 307. At least some of the possible multiple modifications would be expected to denature the native protein structure. This sort of structural change often results in misfolded and/or insoluble proteins that cannot transit the endoplasmic reticulum (ER) and are targeted by ubiquitin for destruction in the proteosome.[143] A POSA would know methods of producing mammalian proteins in prokaryotes to bypass proteosomal degradation, but these proteins are recovered as insoluble inclusion bodies requiring (to the extent possible in view of modifications to the amino acid sequence) solubilization and refolding.[144] These methods are inefficient and often produce non-native protein structures. Moreover, prokaryotic systems do not typically support glycosylation, which was known to be important to PH20 structure and function.[145] Thus, a POSA would have expected that it was highly likely that some, and perhaps a great many, of the possible modified PH20 polypeptides would be misfolded forms of the native structure, or would be unstable, causing them to unfold or denature before injection. Such forms would lack the epitopes present on native PH20, including those relevant to contraception. Immunization with such misfolded modified PH20 polypeptides would not be expected to elicit a quality polyclonal response against native PH20 because the modified antigen would be expected to have different structures from the native.

EX1134 (Kelsoe) ¶ 93; Reply 42

Prior Art Directed POSA to Focus on Relevant Epitopes in PH20 for Contraceptive Effect

106. Contrary to Dr. Cherr's suggestion that binding location does not matter, researchers in 1999 sought to understand the nature of the immune response in guinea pigs that caused contraception.[166] These researchers recognized that "[m]ammalian gamate [sic] fusion involves multiple steps and is in most cases species specific," that PH20 has more than one functionality, and that "multiple isoforms of the protein exist in the sperms of a given mammalian species."[167] Though they sought to find linear epitopes on guinea pig PH20 (gpPH-20) the researchers reported results that "indicated the epitope sequence is conformation sensitive and likely to be discontinuous."[168] They also found two immunodominant and surface accessible regions that they hypothesized served different functions.[169] Importantly, they acknowledged that further research was required to dissect gpPH20 functions and that such experiments "are important to the ultimate definition of PH-20 ***structure*** as it relates to the development of a peptide-based contraceptive vaccine."[170]

EX1134 (Kelsoe), ¶ 106; Reply, 42

In summary, we have defined two regions of sequences on gpPH-20 that are immunodominant and surface accessible in the native state. Since the anti-peptide sera are mono-specific with defined epitopes, they are valuable tools for future dissection of the mode of action of gpPH-20 in enzymatic function as well as the mediation of sperm-egg interaction. These experiments, together with the selection of mimitopes of conformation-dependent epitopes using phage-display technology, are important in the ultimate definition of PH-20 structure as it relates to the development of a peptide-based contraceptive vaccine.

EX2120 (Chan 1999), 1999; EX1134 (Kelsoe), ¶ 106; Reply, 42

Prior Art Directed POSA to Focus on Specificity and Relevant Epitopes in PH20 for Contraceptive Effect

Like anti-PH-20 mAbs[10], the anti-PH-20 polyclonal sera from the infertile females blocked sperm binding to the egg *zona pellucida in vitro*. The relative inhibitory potency of the antiserum provided a second, perhaps more physiologically significant, measure of antiserum titres. In the *in vitro* sperm-*zona* binding assay[10], antisera from the infertile females inhibited sperm-*zona* binding by 90–94% at a 10^{-2} or 10^{-3} dilution. The extent of inhibition decreased at 10^{-4} dilution, but remained substantial even at 10^{-5} dilution (Table 2).

EX2010 (Primakoff 1988), 544; Reply, 41

Several factors may have contributed to the inability of the rMCMV-mPH20 or MBP-mPH20 to cause infertility in mice. In particular, native PH20 proteins may have antigenic epitopes with immunocontraceptive potential not present in either of the recombinant forms used in this study. For example, native PH20 is proteolytically cleaved late in sperm maturation during passage in the epididymis in rodents such as guinea pigs (Phelps *et al.* 1990), rats (Seaton *et al.* 2000) and mice (Deng *et al.* 1999). In addition, modifications to N-linked oligosaccharides on native mPH20 have also been reported during the passage of mouse sperm through the epididymis (Deng *et al.* 1999). Neither of these modifications is likely to have occurred to the recombinant proteins. It is therefore possible that such epididymis-specific modifications to PH20 are required to provide B-cell epitopes associated with the immunocontraceptive effects seen in guinea pigs with native gpPH20 isolated from sperm (Primakoff *et al.* 1988, 1997, Tung *et al.* 1997). In this respect, there is evidence

EX1019 (Hardy 2004), 332; Pet., 81, Reply, 41

Current research on defining the antigenic specificity of ASA and distinguishing those ASA that induce infertility from those that do not

From the preceding sections, it should be clear that ASA are highly variable both in terms of their antigenic specificity and their biological effects. Most techniques used in clinical practice to detect ASA rely on detecting gross binding of antibodies to whole sperm or extracts of whole sperm, either directly or indirectly. Although clinical laboratories have developed considerable expertise in using these assays and judging the value of their results, even in the best of settings these existing tests fall short of being able to distinguish reliably between ASA that contribute to infertility and those that do not. A significant step forward in this field would be the identification of specific antigens that when bound by ASA would specifically affect fertility.

EX2116 (Chamley 2007), 177; Reply, 42

Non-Specific Binding to PH20 Would Not Have Been Expected to Cause Infertility

109. I disagree that a POSA would have expected such non-PH20-specific mechanisms to play a significant role in causing infertility. None of the papers Dr. Cherr cited for these mechanisms relate to PH20—they address anti-sperm antibody.[175] Anti-sperm antibodies are not necessarily specific to a particular sperm-protein (such as PH20) and may bind multiple and distinct targets on sperm, including in locations where PH20 is not expressed. Thus, the effects of anti-sperm antibodies are highly variable, context dependent, and often non-determinative, as confirmed in the literature Dr. Cherr cited. Dr. Cherr did not explain why a POSA would have expected anti-PH20 antibodies to cause contraception through the same mechanisms as these non-specific interactions and did not identify any experimental evidence justifying such extrapolation.

EX1134 (Kelsoe), ¶¶ 109, 113; Reply 42 ; PO Sur-Reply 28

Immune Tolerance Applies to Misfolded Proteins

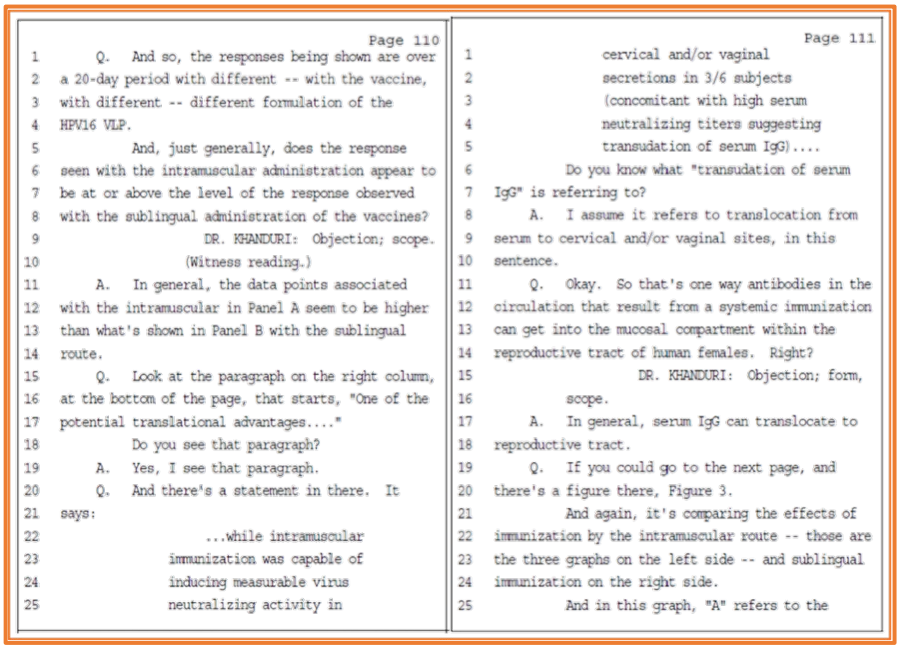
98. Finally, a POSA would have expected the issues of immunodominance and immune tolerance, as discussed above in Sections IV.B–C. and Paragraphs 85–91, to play a role in a polyclonal response to denatured or misfolded modified PH20 polypeptides as well. B cells reactive to such forms, even with limited T-cell help, would have been less likely to produce effective antibodies reactive with the native PH20 protein.

EX1134 (Kelsoe), ¶ 98; Reply 42

Non-Mucosal Routes of Administration Result in Mucosal Antibodies

43. Although the systemic and mucosal immune systems are different compartments, serum antibodies can transit into mucosal surfaces via transudation.[49] For example, it was known by the late 1990s that "systemic immunization with sperm-specific antigens in macaques and mice…, or non-gamete antigens in a variety of species …, results in significant levels of predominantly IgG antibodies in oviductal fluids (up to 27% of those found in serum) which are derived from serum."[50]

46. At his deposition, Dr. Moon confirmed that the intramuscular administration of Gardisil4 resulted in a greater frequency of antibody secreting cells than the sublingual administration.[56] After reviewing the article's suggestion that these results were due to transudation of serum IgG, Dr. Moon further confirmed that "[i]n general, serum IgG can translocate to [the] reproductive tract."[57] I agree, and this would have been known to a POSA by the relevant timeframe.[58]



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Q. And so, the responses being shown are over a 20-day period with different -- with the vaccine, with different -- different formulation of the HPV16 VLP.
And, just generally, does the response seen with the intramuscular administration appear to be at or above the level of the response observed with the sublingual administration of the vaccines?
DR. KHANDURI: Objection; scope.
(Witness reading.)
A. In general, the data points associated with the intramuscular in Panel A seem to be higher than what's shown in Panel B with the sublingual route.
Q. Look at the paragraph on the right column, at the bottom of the page, that starts, "One of the potential translational advantages...."
Do you see that paragraph?
A. Yes, I see that paragraph.
Q. And there's a statement in there. It says:
"...while intramuscular immunization was capable of inducing measurable virus neutralizing activity in

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cervical and/or vaginal secretions in 3/6 subjects (concomitant with high serum neutralizing titers suggesting transudation of serum IgG)...."
Do you know what "transudation of serum IgG" is referring to?
A. I assume it refers to translocation from serum to cervical and/or vaginal sites, in this sentence.
Q. Okay. So that's one way antibodies in the circulation that result from a systemic immunization can get into the mucosal compartment within the reproductive tract of human females. Right?
DR. KHANDURI: Objection; form, scope.
A. In general, serum IgG can translocate to reproductive tract.
Q. If you could go to the next page, and there's a figure there, Figure 3.
And again, it's comparing the effects of immunization by the intramuscular route -- those are the three graphs on the left side -- and sublingual immunization on the right side.
And in this graph, "A" refers to the

EX1134 (Kelsoe), ¶¶ 46 (citing EX1132, 110-111), 43; Reply, 41

No Evidence of Contraceptive Effect from Mucosal Administration of PH20

ducts. Studies are presently in progress to determine if immunization methods that elicit mucosal immune responses may be more effective at inducing immuno-contraception. Because rabbits are known to have at

EX1020 (Pomering), 181; Pet., 81; Reply, 41

[83] EX1020 (Pomering 2002), 181. They further observed that "[s]tudies are presently in progress to determine if immunization methods that elicit mucosal immune responses may be more effective at inducing immunocontraception." *Id.* Dr. Cherr did not identify any published results from these studies, and I am not aware of any such results.

EX1134 (Kelsoe), ¶ 59 n.83; Reply 40

?

No Published Outcome

Eliciting Antibody Production is Not Contraceptive Activity

…the underlying published study cited for the macaque model—Deng (2002)—reports that immunization produced "significant immune responses," including antibodies that successfully recognized sperm-surface PH20. EX1189, 119. Petitioner's reliance on an unsupported anecdote cannot overcome published evidence of contraceptive activity in macaques. EX1189, 119.

PO Sur-Reply, 28

In a primate model, *Cynomolgus macaques*, combinations of adjuvant and antigens derived from synthesized and recombinant proteins all produced significant immune responses in females, with circulating antibodies recognizing macaque sperm surface PH-20 (Deng et al., 2002). However, there was a lack of sterility following immunization in this model also (Deng, personal communication). Finally, in a study designed to assess

ceptive vaccines, recombinant mouse PH-20 was studied. Mice immunized with either recombinant murine cytomegalovirus expressing PH-20 or directly with the purified recombinant PH-20 developed circulating antibodies, but failed to produce a significant reduction in fertility in males or females when limitations of the delivery systems were unlikely (Hardy et al., 2004).

vaccine in other species have produced different results. When bacterially produced recombinant rabbit PH-20 was used for immunization, there was a lack of contraceptive effect in rabbits (Holland et al., 1997). The lack of sterility resulted from insuffi-

EX1189 (Martin-DeLeon), 119 (referencing EX1019 (Hardy), 331 and EX1020 (Pomering), 180); EX1134 (Kelsoe), ¶¶ 66, 57; Reply 40-41

Modified PH20s with Immunogenic Properties Capable of Eliciting Antibodies Are Research Intermediates

And Petitioner's argument that anti-PH20 antibodies are unclaimed misses the point: the claims are directed to modified PH20 polypeptides possessing antigenic and immunogenic properties capable of eliciting such contraceptive antibodies.

PO Sur-Reply, 28-29

***In re Fisher*, 421 F.3d 1365, 1371-73 (Fed. Cir. 2005)**

Essentially, the claimed ESTs **act as no more than research intermediates that may help scientists to isolate the particular underlying protein-encoding genes and conduct further experimentation on those genes**. The overall goal of such experimentation is presumably to understand the maize genome — the functions of the underlying genes, the identity of the encoded proteins, the role those proteins play during anthesis, whether polymorphisms exist, the identity of promoters that trigger protein expression, whether protein expression may be controlled, etc. Accordingly, **the claimed ESTs are, in words of the Supreme Court, mere "object [s] of use-testing," to wit, objects upon which scientific research could be performed with no assurance that anything useful will be discovered in the end**. *Brenner*, 383 U.S. at 535, 86 S. Ct. 1033.

Unless and until a process is refined and developed to this point—where **specific benefit exists in currently available form**—there is **insufficient justification** for permitting an applicant **to engross what may prove to be a broad field**.

Sur-Reply – Dr. Kelsoe's Testimony on Claims

The claimed modified PH20 polypeptides are defined by their structure—an L307G, L307K, L307N, L307Q, L307S, L307T, L307V, L307W, or L307Y mutation and 91% sequence identity—not by their function. Petitioner's expert, Dr. Kelsoe, admitted as much…

PO Sur-Reply, 1

15. I understand that the '590 Patent at issue in these proceedings concerns "modified PH20 polypeptides" with certain required characteristics. I have been asked to develop my opinions based upon the following understanding of "modified PH20 polypeptides" provided by counsel: polypeptides comprising an amino acid sequence that is at least 91% identical to the amino acid sequence of any one of SEQ ID NO: 3, 7, and 32-66 in the '590 Patent, and includes, at least, a replacement at position 307 such that L is replaced with G, K, N, Q, S, T, V, W, or R.

EX1134 (Kelsoe), ¶ 15; Reply, 41

Q. And did you previously review the Claims of this patent?
A. Only those claims related to the use of PH20 variants as a immunocontraceptive.
Q. Did you review claim 2 of this patent?
A. If that – I don't remember what Claim 1 is, but if I – whatever claim I reviewed Only dealt with the contraceptive function of the Potential contraceptive function of PH20 immunogen

EX2302 (Kelsoe Dep.), 40:5-14

Q. Okay. And you reviewed this patent in Association with preparation of your declarations, right?
A. In the aspects that were relevant to my testimony, yes.

EX2302 (Kelsoe Dep.), 46:7-11; PO Sur-Reply, 1

Obviousness Grounds

Obviousness

- **Halozyme's '429 patent motivates a POSA to make (and claims) single-substitution $PH20_{1-447}$ mutants in the scope of the claims**
- **Dr. Park used techniques that were conventional in 2011-2012**
- **Dr. Park's multi-sequence alignment identified positions and amino acids to consider as single-substitutions in non-essential regions of $PH20_{1-447}$ without hindsight**
- **Dr. Park's assessment of individual substitutions suggested by the multi-sequence alignment was objective and unbiased**

See e.g., Pet., 84-109; Reply, 43-48

’429 Patent Suggests Single Amino Acid Substitution in Non-Essential Regions of PH20

Thus, reference, for example, to “sHASEGP” encompasses all glycoproteins encoded by the sHASEGP gene family, including but not limited to: Human sHASEGP, mouse sHASEGP, or an equivalent molecule obtained from any other source or that has been prepared synthetically or that exhibits the same activity. Sequences of encoding nucleic acid molecules and the encoded amino acid sequences of exemplary sHASEGP’s and/or domains thereof are set forth, for example in SEQ ID NO: 4. The term also encompasses sHASEGP with amino acid substitutions that do not substantially alter activity of each member and also encompasses splice variants thereof. Suitable substitutions, including, although not necessarily, conservative substitutions of amino acids, are known to those of skill in this art and can be made without eliminating the biological activity, such as the catalytic activity, of the resulting molecule.

EX1005 (’429 Patent), 9:65-10:13; Pet. , 86-87

Suitable conservative substitutions of amino acids are known to those of skill in this art and can be made generally without altering the biological activity, for example enzymatic activity, of the resulting molecule. 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 (see, e.g., Watson et al. Molecular

Nv Other substitutions are also permissible and can be determined empirically or in accord with known conservative substitutions.

EX1005 (’429 Patent), 16:14-20, 16:34-36; Pet., 95-96; Reply, 43

As used herein equivalent, when referring to two sequences of nucleic acids means that the two sequences in question encode the same sequence of amino acids or equivalent proteins. When equivalent is used in referring to two proteins or peptides, it means that the two proteins or peptides have substantially the same amino acid sequence with only amino acid substitutions (such, as but not limited to, conservative changes such as those set forth in Table 1, above) that do not substantially alter the activity or function of the protein or peptide. When equivalent refers to a property, the property

EX1005 (’429 Patent), 18:64-19:6; Pet., 86- 87

'429 Patent Claims Modified $PH20_{1-447}$ with ≥1 Substitution

(10) **Patent No.: US 7,767,429 B2**
(45) **Date of Patent: Aug. 3, 2010**

(54) **SOLUBLE HYALURONIDASE GLYCOPROTEIN (SHASEGP), PROCESS FOR PREPARING THE SAME, USES AND PHARMACEUTICAL COMPOSITIONS COMPRISING THEREOF**

(75) Inventors: **Louis H. Bookbinder**, San Diego, CA (US); **Anirban Kundu**, San Diego, CA (US); **Gregory I. Frost**, Del Mar, CA (US)

(73) Assignee: **Halozyme, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1297 days.

(21) Appl. No.: **10/795,095**

(22) Filed: **Mar. 5, 2004**

1. A substantially purified hyaluronidase glycoprotein that consists of the sequence of amino acids set forth as amino acids 1-477, 36-477, 1-478, 36-478, 1-479, 36-479, 1-480, 36-480, 1-481, 36-481, 1-482, 36-482, 1-483 or 36-483 of SEQ ID NO: 1, or contains amino acid substitutions in the sequence of amino acids set forth as amino acids 1-477, 36-477, 1-478, 36-478, 1-479, 36-479, 1-480, 36-480, 1-481, 36-481, 1-482, 36-482, 1-483 or 36-483 of SEQ ID NO: 1, whereby the amino-acid substituted hyaluronidase glycoprotein consists of a sequence of amino acids that has at least 95% amino acid sequence identity with the sequence of amino acids set forth as amino acids 1-477, 36-477, 1-478, 36-478, 1-479, 36-479, 1-480, 36-480, 1-481, 36-481, 1-482, 36-482, 1-483 or 36-483 of SEQ ID NO: 1, wherein the glycoprotein contains at least one sugar moiety that is covalently attached to an asparagine (N) residue of the polypeptide.

EX1005 ('429 Patent), Claim 1; Pet. 98-99; Reply, 43-44

Dr. Park Identified Non-Essential Regions of PH20 Using MSA

25. In general, amino acids that do not vary at the same aligned positions within a set of structurally-related proteins are essential to the structure and functions of the protein.[6] When a residue does not vary in so many naturally occurring proteins, it is strong evidence that it is essential to the protein's structure and function and needs to be preserved.[7]

26. I investigated essential residues in hyaluronidase enzymes in two ways. First, I identified invariant residues by analyzing a multiple sequence alignment of a set of published hyaluronidase sequences that was available in December of 2011. Second, I reviewed scientific literature that identified important residues in hyaluronidase proteins or which reported experimental results showing that modifying single residues impaired or eliminated activity of the enzymes.[8] These two overlapped significantly and many residues that were experimentally shown to be essential for activity proved to be highly conserved based on sequence alignment. Below I address the first investigation.

EX1004 (Park), ¶¶ 25-26; Pet., 10, 13-14, 92; Reply, 44-45

2. Essential Residues in PH20

30. Using the alignment, I identified 68 largely invariant residues that a skilled artisan would have deemed "essential residues" in $PH20_{1-447}$ based on the sequence alignment (table below).[15] These are positions where the amino acid was conserved 95% of the time or more and non-identical amino acids appear in less than ~5% of the proteins in the data set[16].

3. Non-Essential Residues in PH20

31. The 379 positions in $PH20_{1-447}$ other than the 68 essential residues are positions at which there is evolutionary variation among the 88 protein sequences that I analyzed. The existence of evolutionary variation at these positions indicates that the homologous proteins have tolerated different amino acids at those positions. Amino acids at these positions in the different hyaluronidase proteins would be considered "non-essential" residues because the proteins presumably still exhibit hyaluronidase activity when they are mutated. The 379 non-essential positions in $PH20_{1-447}$ are compiled in Appendix D-2.

EX1004 (Park), ¶¶ 30-31, Pet., 92; Reply, 44-45

Dr. Park Identified Non-Essential Regions of PH20 Using MSA

2. Essential Residues in PH20

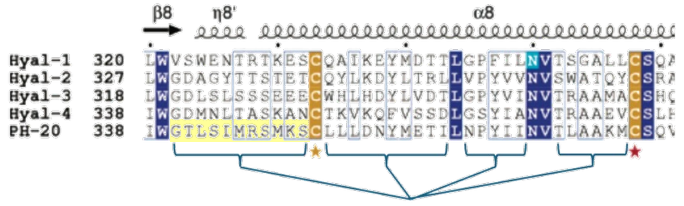
Residue #	Mature Residue #	PH20 Residue	Residue %		Residue #	Mature Residue #	PH20 Residue	Residue %
49	14	F	95.5		222	187	P	100
53	18	W	100		224	189	C	100
60	25	C	100		226	191	N	98.9
91	56	F	98.9		234	199	Y	98.9
158	123	W	96.6		335	300	G	98.9
164	129	Y	100		339	304	W	98.9
168	133	S	100		351	316	C	100
188	153	A	100		362	327	L	96.6
192	157	F	100		368	333	N	97.7
203	168	T	98.9		369	334	V	96.6
211	176	R	95.5		376	341	C	100
212	177	P	97.7		377	342	S	96.6
215	180	L	95.5		381	346	C	100
216	181	W						
217	182	G						
219	184	Y						

EX1004 (Park), ¶ 30; Pet., 92; Reply, 44

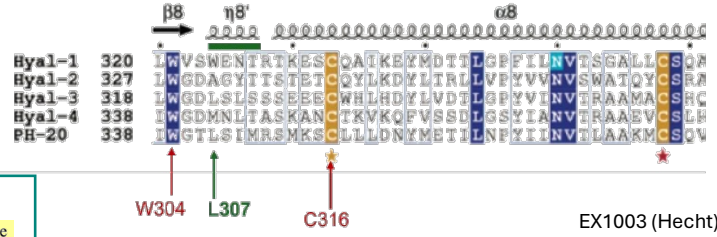
3. Non-Essential Residues in PH20

31. The 379 positions in PH20$_{1-447}$ other than the 68 essential residues are positions at which there is evolutionary variation among the 88 protein sequences that I analyzed. The existence of evolutionary variation at these positions indicates that the homologous proteins have tolerated different amino acids at those positions. Amino acids at these positions in the different hyaluronidase proteins would be considered "non-essential" residues because the proteins presumably still exhibit hyaluronidase activity when they are mutated. The 379 non-essential positions in PH20$_{1-447}$ are compiled in Appendix D-2.

EX1004 (Park), ¶ 31; Pet., 92; Reply, 44



EX1004 (Park), ¶ 32; Pet., 92-93; Reply, 44



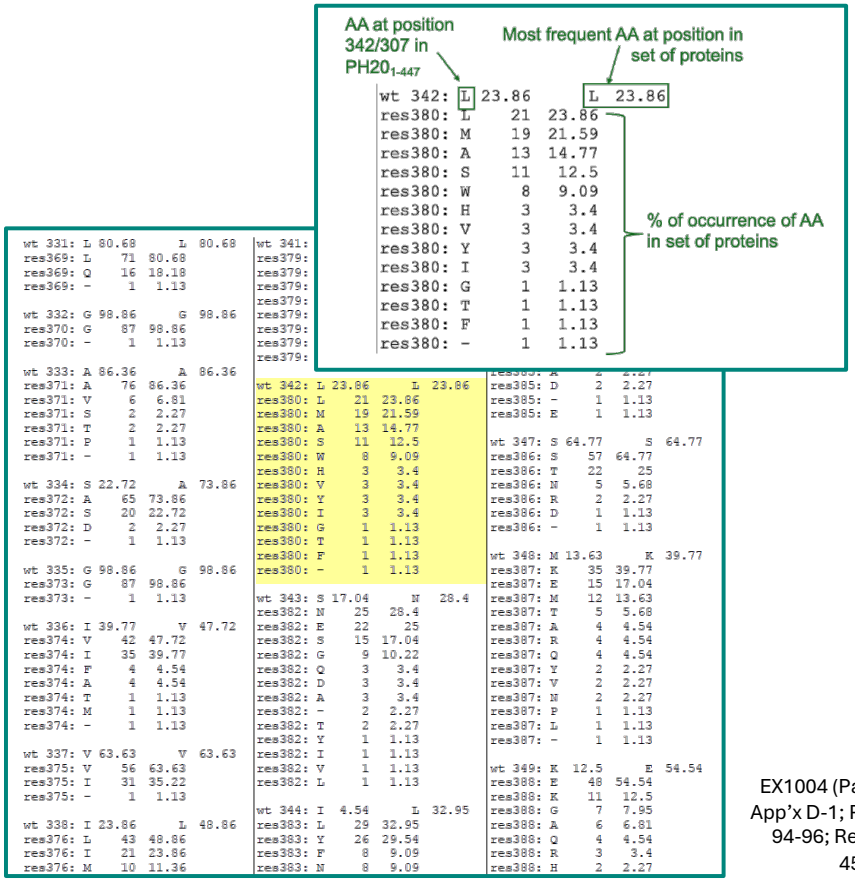
EX1003 (Hecht), ¶ 217; Pet., 94

Dr. Park Identified Substitutions to Assess Using MSA

42. Within the 88 homologous hyaluronidase proteins that I analyzed, there were varying numbers of amino acids that occurred at each non-essential position.[35] A higher frequency for one amino acid within a set of amino acids at a particular position indicates that a larger number of naturally occurring hyaluronidase proteins contain that amino acid at that position. For example, a 5% frequency of occurrence of an amino acid in a set of 88 related proteins means that ~4 naturally occurring hyaluronidase proteins have that amino acid at that position. In essence, evolution has "sampled" those substitutions and found they are tolerated in at least 4 isoforms of PH20.

43. An example of a set of amino acids from the MSA based on 88 sequences for position 307 (342 with the signal sequence) of $PH20_{1-447}$ is provided below.

EX1004 (Park), ¶¶ 42-43, App'x D-1; Pet., 94-96; Reply, 45-46



EX1004 (Park), App'x D-1; Pet., 94-96; Reply, 45-46

Dr. Park's Ratings for Amino Acid Substitutions

85. In my evaluation of whether single amino acid substitutions may be tolerated at a position, I considered all of the factors discussed above.[57] I balanced the type of impact of the substitutions based on the magnitude of each interaction may have on the protein's structure.

86. Based on my assessment, I assigned each substitution a score of 1, 2, or 3.

- A score of 3 indicates that I would expect the substitution to improve the overall stability of the protein.
- A score of 1 indicates that the mutation would likely reduce the protein stability and may even result in some loss of protein function.
- A score of 2 indicates that the substitution would have either no effect on the structure of the protein, or a slightly positive or slightly negative effect on the structure and function.

EX1004 (Park), ¶¶ 85-86; Reply, 46

87. Substitutions that I scored as a 2 or 3 would be expected to be tolerated by the PH20 protein. These types of changes would either be inconsequential to the protein structure or would cause a change that would likely be beneficial to the stability of the protein. The substitutions that I scored as a 1 would likely not be tolerated by the PH20 protein. These types of changes often significantly disrupt the protein structure at the location of the substitution, which significantly increases the probability that the substitution will adversely affect folding of the protein or structural features of the PH20 protein that are important to its biological activity (*e.g.*, catalysis, ligand binding). My scoring table is provided below.

Score	Expected Impact	Expected Toleration
1	Significantly Destabilized	Likely Not Tolerated
2	Neutral or Minor Impacts	Tolerated
3	Improved Stability	Tolerated

EX1004 (Park), ¶ 87; Reply, 46

Dr. Park Ensured His Methodology Was Unbiased

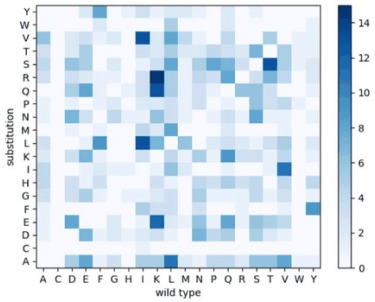
4. Review of the Methodology Demonstrates Unbiased Evaluation

102. To develop an unbiased scoring system, it is necessary to evaluate a lot of different types of substitutions. Therefore, it was important to me to evaluate and assign scores to many different substitutions because, through this process, I developed a consistent methodology to evaluate potential substitutions. As I went through the analysis, I would revisit the scores for each position multiple times to make sure that the way I assigned scores was consistent. Because I considered a broad range of situations, I believe my methodology provided an objective and unbiased evaluation of substitutions throughout the protein.

EX1004 (Park), ¶ 102; Pet., 101; Reply, 46

103. I conducted my analysis in a manner that did not focus on any particular position. For instance, I considered some substitutions involving similar amino acids (*e.g.*, both amino acids were hydrophobic, charged or polar) as well as those with dissimilar amino acids (*e.g.*, hydrophobic vs. charged, polar vs. hydrophobic, large vs. small). I also considered substitutions at buried positions as well as substitutions at solvent exposed positions. In this declaration, I have been asked by counsel to report my conclusions with respect to position 307.

EX1004 (Park), ¶ 103; Pet., 101; Reply, 46



EX2176 (Park Spreadsheet); Reply, 47

Q. Why did you prepare this heat map?
A. One of the things that I was aiming for as I was going through the substitution analysis was to develop a robust system, and I needed a way of knowing how robust my system was, and that I believed involved looking at many different kinds of substitutions; and therefore, I wanted to keep track of what sort of substitutions I was looking at.

Q. Okay. So you wanted to make sure that you were evaluating substitutions across the spectrum of – of the 20 amino acids?
A. That's correct.

EX2078 (Park Dep.), 300:2-10, 300:21-301:2; Reply, 47

Dr. Park Recorded Observations and Later Explained Them in His Declaration

118. Replacing the leucine at position 307 with tryptophan would eliminate the predicted hydrophobic contact between L307 and GlcNAc at position -3 of the HA substrate. However, the lost hydrophobic interaction may be compensated for by new interactions involving tryptophan's indole nitrogen at position 307, which should have a stabilizing effect on the interaction of HA with PH20 via a hydrogen bond to the glucuronic acid at position -2 of HA.

124. Replacing leucine with serine at position 307 would eliminate the predicted hydrophobic contact between L307 and HA. However, serine's hydroxyl moiety at position 307 may form a hydrogen bond with the carboxyl group of glucuronic acid at position -2 or the N-acetyl of GlcNAc at position -3 of the HA, which may compensate for the lost hydrophobic contacts from L307.

130. Since threonine's side chain is shorter than leucine's, the predicted hydrophobic contact between L307 and HA would be lost if leucine is substituted with threonine. However, I expect the hydroxyl side chain of T307 in PH20 would participate in favorable hydrogen bond interactions with HA (below left, "3.0" and "4.1"). The serine at the corresponding position in bee venom hyaluronidase has those types of favorable hydrogen bonding interactions with HA (below right, "3.0" and "3.4"), despite having a different orientation relative to the orientation of the hydroxyl group in the L307T substitution in PH20.

EX1004 (Park), ¶¶ 118, 124,130; Pet., 104-106

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1												Factors		
2	Hyal1 Residue#	Hyal1 Residue	PH20 Residue #	PH20 Mature Residue #	PH20 Residue	Alternative Residue	Residue %	Rating (1-3)	Comments	#neigh	fSASA	Hydrophobicity	Secondary Structure	Interactions
3	324	W	342	307	L	-	23.86			4	0.63			
4	324		342	307	-	M	21.59	2	neutral: both contact the sugar face	0	0.69			
5	324		342	307	-	A	14.77	2	loss: lost contacts to ligand	0	0.64			1
6	324		342	307	-	S	12.5	2	neutral: lost van der Waals to ligand,gained hbond, seen in bvh	2	0.66		1	1
7	324		342	307	-	W	9.09	2	neutral: hydrophobic residue solvent exposed, possible hbond, seen in hyal	3	0.69	1		1
8	324		342	307	-	H	3.4	2	loss: lost hydrophobic contacts to ligand, new hbond, lower H propensity	2	0.66		1	1
9	324		342	307	-	V	3.4	2	neutral:both contact the sugar face	3	0.6			
10	324		342	307	-	Y	3.4	2	loss: lost hydrophobic contacts to ligand	6	0.46			1
11	324		342	307	-	I	3.4	2	neutral	3	0.65			
12	324		342	307	-	G	1.13	1	loss: lost van der Waals, lower H propensity	0	0.57		1	
13	324		342	307	-	T	1.13	2	neutral	3	0.65			
14	324		342	307	-	F	1.13	2	loss: lost van der Waals to ligand	6	0.47			1

EX1004 (Park), App'x C; Pet., 104

10 Q. So why did you evaluate those
11 substitutions?
12 A. By that time, I had already completed all
13 my analysis of all of positions, and provided a
14 spreadsheet to counsel, who then asked me to
15 elaborate on certain positions, including 320,
16 317, and so on. And since I'm being asked to
17 provide detailed description of the substitutions
18 and evaluation at those positions, I went back and
19 finished the analysis with all the substitutions
20 that were available at those positions.

EX2078 (Park Dep.),320:10-20; Reply, 46

The Positions and Numbers of Substitutions Dr. Park Evaluated

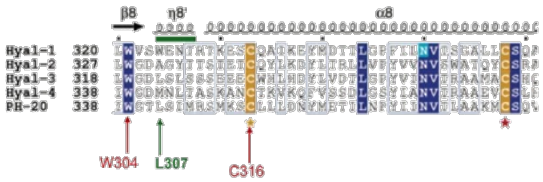
Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT	Position	WT
4	R	42	G	80	P	118	T	156	E	194	Y	232	T	270	R	308	S	346	C	384	G
5	A	43	S	81	Q	119	W	157	F	195	K	233	Q	271	I	309	I	347	Q	385	K
6	P	44	P	82	K	120	A	158	E	196	K	234	Q	272	V	310	M	348	E	386	P
7	P	45	R	83	I	121	R	159	K	197	P	235	S	273	F	311	R	349	Q	387	T
8	V	46	I	84	S	122	N	160	A	198	G	236	P	274	T	312	S	350	G	388	L
9	I	47	N	85	L	123	W	161	G	199	Y	237	V	275	D	313	M	351	V	389	E
10	P	48	A	86	Q	124	K	162	K	200	N	238	A	276	Q	314	K	352	C	390	D
11	N	49	T	87	D	125	P	163	D	201	G	239	A	277	V	315	S	353	I	391	L
12	V	50	G	88	H	126	K	164	F	202	S	240	T	278	L	316	C	354	R	392	E
13	P	51	Q	89	L	127	D	165	L	203	C	241	L	279	K	317	L	355	K	393	Q
14	F	52	G	90	D	128	V	166	V	204	F	242	Y	280	F	318	L	356	N	394	F
15	L	53	V	91	K	129	Y	167	E	205	N	243	V	281	L	319	L	357	W	395	S
16	W	54	T	92	A	130	K	168	T	206	V	244	R	282	S	320	D	358	N	396	E
17	A	55	I	93	K	131	N	169	I	207	E	245	N	283	Q	321	N	359	S	397	K
18	W	56	F	94	K	132	R	170	K	208	I	246	R	284	D	322	Y	360	S	398	F
19	N	57	Y	95	D	133	S	171	L	209	K	247	V	285	E	323	M	361	D	399	Y
20	A	58	V	96	I	134	I	172	G	210	R	248	R	286	L	324	E	362	Y	400	C
21	P	59	D	97	T	135	E	173	K	211	N	249	E	287	V	325	T	363	L	401	S
22	S	60	R	98	F	136	L	174	L	212	D	250	A	288	Y	326	I	364	H	402	C
23	E	61	L	99	Y	137	V	175	L	213	D	251	I	289	T	327	L	365	L	403	Y
24	F	62	G	100	M	138	Q	176	R	214	L	252	R	290	F	328	N	366	N		
25	C	63	Y	101	P	139	Q	177	P	215	S	253	V	291	G	329	P	367	P		
26	L	64	Y	102	V	140	Q	178	N	216	W	254	S	292	E	330	Y	368	D		
27	G	65	P	103	D	141	N	179	H	217	L	255	K	293	T	331	I	369	N		
28	K	66	Y	104	N	142	V	180	L	218	W	256	I	294	V	332	I	370	F		
29	F	67	I	105	L	143	Q	181	W	219	N	257	P	295	A	333	N	371	A		
30	D	68	D	106	G	144	L	182	G	220	E	258	D	296	L	334	V	372	I		
31	E	69	S	107	M	145	S	183	Y	221	S	259	A	297	G	335	T	373	Q		
32	P	70	I	108	A	146	L	184	Y	222	T	260	K	298	A	336	L	374	L		
33	L	71	T	109	V	147	T	185	L	223	A	261	S	299	S	337	A	375	E		
34	D	72	G	110	I	148	E	186	F	224	L	262	P	300	G	338	A	376	K		
35	M	73	V	111	D	149	A	187	P	225	Y	263	L	301	I	339	K	377	G		
36	S	74	T	112	W	150	T	188	D	226	P	264	P	302	V	340	M	378	G		
37	L	75	V	113	E	151	E	189	C	227	S	265	V	303	I	341	C	379	K		
38	F	76	N	114	E	152	K	190	Y	228	I	266	F	304	W	342	S	380	F		
39	S	77	G	115	W	153	A	191	N	229	Y	267	A	305	G	343	Q	381	T		
40	F	78	G	116	R	154	K	192	H	230	L	268	Y	306	T	344	V	382	V		
41	I	79	I	117	P	155	Q	193	H	231	N	269	T	307	L	345	L	383	R		

Note :
Red - essential residues
Dark Green - ≥ 66% MSA Options assessed
Green - 33% to 66% MSA options assessed
Light Green - < 33% MSA options assessed
Unshaded - No MSA options assessed

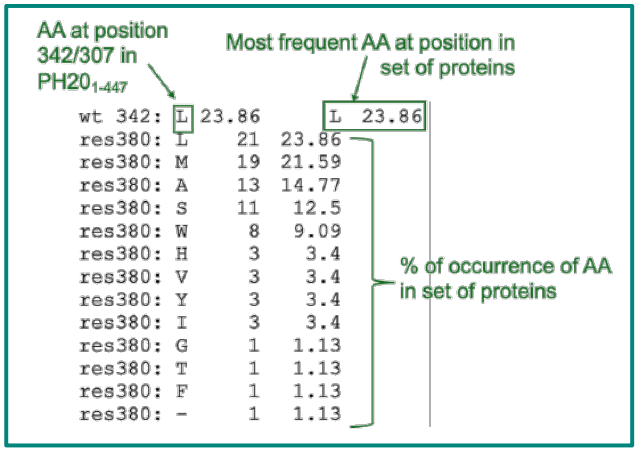
EX1133 (Naismith), ¶ 339 (citing EX2176 (Park Spreadsheet)), Reply, 47-48

Petition Explained Motivation to Assess L307W, L307T, and L307S

The skilled artisan also would have selected tryptophan (W), threonine (T), and serine (S) as obvious choices for such a single substitution at position 307 in $PH20_{1-447}$.[270] While leucine is the most prevalent amino acid found at positions corresponding to 307 in PH20, many different amino acids are tolerated at this position in homologous proteins, as shown by Chao and Dr. Park's multiple-sequence alignment, including tryptophan, threonine and serine.[271] Tryptophan occurs at a position corresponding to 307 in PH20 in 11 naturally occurring hyaluronidase enzymes, including human HYAL1, while threonine occurs at that position in 8 such proteins, and serine occurs at that position in the bee venom hyaluronidase protein.[272] Consequently, a skilled artisan would have considered each of tryptophan, threonine, and serine to have been obvious candidates to substitute for leucine at position 307 of position 307 in $PH20_{1-447}$ pursuant to the guidance in the '429 Patent.[273]



Pet., 96-98



EX1004 (Park), ¶ 43; Pet., 95-96

Halozyme's Representations in the '429 Patent Must Be Credited

Replacing the leucine (L) at position 307 with tryptophan (W), threonine (T), or serine (S) yields a $PH20_{1-447}$ with a single amino acid substitution in a non-essential region of the polypeptide.[274] In its '429 Patent, Patentee stated:

> 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.[275]

Patentee also secured claims in the '429 patent to modified $PH20_{1-447}$ proteins with at least one substitution (*e.g.*, claim 1), despite not providing examples of PH20 proteins with any substitutions. Patentee, thus, made and relied on its statements that a skilled artisan would have expected ***any*** single amino acid substitution in ***any*** non-essential position of $PH20_{1-447}$ to not substantially affect the activity of the enzyme. Patentee should not be permitted to now contend a skilled artisan would not have reasonably expected that the L307W, L307T, or L307S substitutions in $PH20_{1-447}$ would yield an enzyme with substantially the same activity as unmodified $PH20_{1-447}$.

Pet., 98-99

Drs. Park and Hecht Reasonably Expected L307W, L307T, and L307S to be Tolerated

Dr. Park assigned a score of 2 for each of the L307W, L307T, and L307S substitutions in $PH20_{1-447}$, indicating each would be expected to be tolerated.[289]

Dr. Park consequently found that the L307W substitution in PH20 would be neutral.[297]

Dr. Park also evaluated the L307S and L307T substitutions and concluded each would be tolerated by PH20.[298] Both the L307S and L307T substitutions will introduce a hydrophilic residue (serine or threonine) into the hydrophilic solvent environment of position 307 in PH20.[299] The L307S substitution in PH20 also yields a conformation similar to the corresponding residue in bee venom hyaluronidase, which is serine.[300] And each of the L307S and L307T substitutions in PH20 can form new favorable interactions with the HA substrate (below, left and right, respectively).[301]

Pet., 102, 106

Dr. Hecht reviewed Dr. Park's analysis and conclusions and for the same reasons agreed that the L307W, L307T, and L307S single substitutions would likely have been tolerated in $PH20_{1-447}$.[304] For example, because leucine is tolerated at position 307 in PH20, a skilled artisan would expect other hydrophobic residues like tryptophan to be tolerated there.[305] The L307S and L307T substitutions, conversely, introduce hydrophilic residues into the compatible solvent accessible environment of position 307 in PH20.[306] And all three substitutions would yield $PH20_{1-447}$ mutants having favorable interactions with the HA substrate that could offset any lost favorable interactions between L307 and HA.[307]

Pet., 108

Other Topics

DEMONSTRATIVE EXHIBIT – NOT EVIDENCE

Rescue Mutations Are Not Described or Predictable

209. In ¶¶ 374–383, Dr. Petsko suggests that a POSA could use the data in the common disclosure and a PH20 homology model to identify "rescue" mutations that restore activity lost from deleterious substitutions, and that examining structural interactions would allow a POSA to predictably design such activity restoring combinations. This is not described in the common disclosure. There also is no technique or passage in the common disclosure that could provide a basis for Dr. Petsko's theory that a POSA could reliably identify rescue mutations to restore activity in inactive modified PH20 mutants. Instead, it only proposes methods that discover multiply-modified PH20 variants after performing iterative rounds of mutagenesis and experimental testing, in which libraries of PH20 variants are generated, assayed for activity, and used to guide further mutation.[236]

210. Dr. Petsko's rescue mutation proposal would not have yielded predictable results based on what was known as of 2011. Although the general concept of "rescue mutations" was recognized, there was no reliable way to predict in advance which, if any, additional mutations would restore activity in a given protein, or PH20. Available approaches—including (i) mutagenesis-and-screening methodologies, (ii) computational or in silico modeling, and (iii) rational or semi-rational design—were inherently uncertain. Dr. Petsko's focus on local structural interactions would likely miss many—if not most—potential rescue mutations for a given "inactive" mutant, as such mutations are often distributed throughout a protein and act through indirect effects on stability or dynamics, rather than through obvious local structural corrections. The rational or semi-rational approaches that Dr. Petsko proposes also were known to succeed only in a fraction of cases and still required experimental testing to confirm restoration of function.[237]

EX1133 (Naismith), ¶¶ 209-210; Reply, 40

Halozyme Sur-Reply: What A POSA "Could" Or "Would" Do

As Dr. Petsko testified, "[t]aken together, these figures show that there are many positions that are very tolerant of substitution on the surface of PH20 that can be targeted by a POSA seeking to generate a modified PH20 that retains hyaluronidase activity." EX2070, ¶322. As he testified at deposition based on the patent data:

> Q. So your opinion is that a POSA could use the data from testing single mutants in the patent to select 15 or 20 substitutions in the PH20 sequence, and you would be able to predict whether that mutated 15 or 20 residue-mutated PH20 will exhibit the activity that you are hoping to get?
>
> **A. My opinion is that you could do so**….
>
> ***

PO Sur-Reply, 9

9 Q. So your opinion is that a POSA could use
10 the data from testing single mutants in the
11 patent to select 15 or 20 substitutions in the
12 PH20 sequence, and you would be able to predict
13 whether that mutated 15 or 20 residue-mutated
14 PH20 will exhibit the activity that you are
15 hoping to get?
16 A. My opinion is that you could do so. My
17 opinion is also that you would almost never do
18 so. You would probably rarely, if ever, make
19 that many substitutions. You wouldn't need to.

EX1131 (Petsko Dep.), 111:9-19; PO Sur-Reply, 9

13 Q. Did you perform that analysis you just
14 described of comparing the possible sequences to
15 some smaller number of them that match the structure
16 of the model of PH20?
17 MS. MARTIN:· Object to the form.
18 A. I did not go sequence by sequence and do
19 that.
20 I guess maybe I need to say that no POSA
21 would start with the theoretical maximum number of
22 potential sequence variants as a starting point for
23 making a comparative analysis of what amino acids
24 would retain activity in a PH20 polypeptide.

EX1130 (Simpson Dep.), 93:13-24; Reply, 5, 23

Merck Correctly Represented the Disclosure

Petitioner contends that the patent mandates a random screening approach to making multiply mutated PH20s (Reply, 35-36), and thus that Drs. Petsko and Simpson contradict the common disclosure by using the same "prevalent" method used by Petitioner's experts. Pet., 100-109; Reply, 7-10. But Petitioner misunderstands the specification. The experiment that Petitioner identifies involves testing for stability—not activity— and that approach is called out as exemplary only. EX1026, 47:11-18, 141:7-33; 150:1-26. The patent in no way mandates it.

PO Sur-Reply, 13

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

EX1026, ('731 App.), 141:30-33; EX1003, ¶ 174; Pet., 80

In any of the methods provided herein for identifying or selecting a modified hyaluronan-degrading enzyme that exhibits stability, the method is performed *in vitro*. Also provided are any of the methods that are iterative, whereby the steps of the method are repeated a plurality of times, wherein in each repetition, further modified hyaluronan-degrading enzymes of a selected modified hyaluronan-degrading enzyme are generated and tested, whereby the modified hyaluronan-degrading enzyme is evolved to exhibit increased stability under a denaturation condition. Also provided herein is a modified hyaluronan-degrading enzyme identified by any of the methods provided herein.

EX1026 ('731 App.), 47:11-18; PO Sur-Reply 13

D. METHODS FOR IDENTIFYING MODIFIED HYALURONAN-DEGRADING ENZYMES WITH ALTERED PROPERTIES OR ACTIVITIES

Provided herein are methods for identifying a modified or variant hyaluronan-degrading enzyme, such as a modified hyaluronidase or modified PH20 polypeptide, that exhibits an altered activity or property compared to an unmodified hyaluronan-degrading enzyme. In particular, the methods provided herein can be used to screen for one or more

EX1026 ('731 App.), 140:15-20; PO Sur-Reply 13

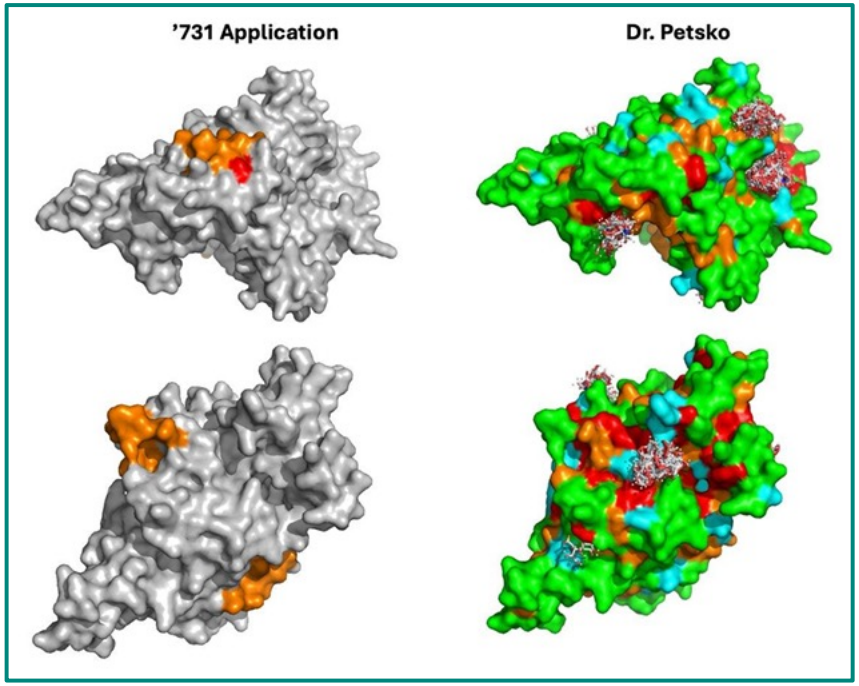
The Data for the Single Substitution Mutants Does Not Reflect Structural Features

But as Dr. Petsko explains, the intolerant positions (and the tolerant positions) are not randomly scattered throughout the protein but rather are clustered, reflecting structural features rather than random epistastic effects. EX1131, 157:5-25; EX2070, ¶¶334-342.

PO Sur-Reply, 15-16

229. This part of Dr. Petsko's analysis relies on potential additional HA binding sites that differ from those identified in the common disclosure and on information not disclosed in any published report prior to 2011-2012. I find, as of the date of this report, no experimental identification of such additional extended HA sites on PH20. The common disclosure does not reference the AutoDock Vina tool or a comparable computational tool for identifying HA binding sites. The hypothesis he offers is one for future experimental work, not guidance a POSA would have derived from the common disclosure.

EX1133 (Naismith), ¶ 229; Reply, 39



EX1133 (Naismith), ¶ 228; Reply, 39

As Dr. Petsko testified: "the POSA would not start that way at all. They would start with a much more focused program making use of the data in the patent," as set out above. EX1131, 106:21-25, 113:20-114:24. That is because POSA could "reliably predict" actives and practice the claims without "undue" experimentation.

PO Sur-Reply, 18-19

Q. And is -- is your opinion that the POSA would believe that any amino acid substitution tolerated at a particular position, for example, in the dark green ones, would be tolerated in any PH20 mutant regardless of how many changes or where those changes were in the protein?
A. Most of the time, yes. If the other changes were in adjacent residues, residues in contact with that one, then you would want to look at the structure.
Q. Okay. So that's something you'd have to kind of do on a case-by-case basis?
A. You would do that on a case-by-case basis when you were trying to make the mutants that had the purposes that you intended.

EX1131 (Petsko Dep.), 160:22-161:11

Q. -- is it your view that a protein that had those 15 mutations in them would fold, regardless of which amino acids were put in each position?
A. That would depend on which mutations you picked. If you picked a mutation that only had -- a residue that was only mutated to one or two others, you might be hesitant to make that prediction without first checking with the structure.

EX1131 (Petsko Dep.), 161:15-24

*He testified that "properly" folded meant "stably" folded, which he said meant exhibiting activity at **8 hours**. EX2301, 138:6-141:19, 180:2-184:24, 190:9-19, 200:9-16, 250:17-25. Of course, the patent's activity data was measured after 90 minutes. EX1001, Example 3.*

PO Sur-Reply, 4, 22

Q. Okay. So how can you tell if the folded enzyme is sufficiently stable to retain its folded state?
A. You would look at different time points. A protein which – in the assay they talk about overnight for example. That could have been one of the things you could do to see if the protein was stable overnight or for a few hours.

EX2301 (Naismith Dep.), 137:7-14

Q. Okay. I think in the Examples 3 activity assay they were using an incubation time of 90 minutes?
A. Yes.
Q. Is that what you would consider?
A. Yes, but they had isolated the protein too. So there is more to it than just, you know, they purified it. Sorry, they took the extract I should say, not "purified it". They took the extract. So protein has a bit of time, not just 90 minutes.

EX2301 (Naismith Dep.), 137:15-25

Moreover, Dr. Naismith admitted at deposition that epistasis simply refers to the fact that multiple mutations will not necessarily be "additive" (EX2301, 129:17-130:18)—that is, that a POSA could not necessarily predict the precise activity level when combining mutations by looking at the effect on activity from individual mutations. But an exact prediction of activity level is not at issue here—particularly because Petitioner's 40% activity construction must be rejected.

PO Sur-Reply, 15

23 Q. Okay.
24 A. And those are very large, and
25 Because of that a POSA would look at that and

2 realize that the difference between a mutation,
3 particular introduction, that makes the protein
4 2.5 times more active and one that makes a protein
5 half as active, roughly, right, I'm not going to
6 get into the precision here. That is a really
7 interesting issue about what is happening to the
8 protein active site or binding or whatever, that

9 is quite interesting. So it speaks to this
10 problem that this mutation is doing something to
11 the protein that we don't understand. We can see
12 their outcome. So therefore if you combine that
13 mutation which has done something to the active
14 site in some way, because we can see it in the
15 assay, which another mutation, that is where we
16 start to worry, really would worry about epistasis
17 because we know these are affecting at a distance
18 in some way the active site because you can see it
19 in the assay, and that is a big difference.

EX2301, (Naismith Dep.), 293:23-294:19

When he learned at deposition the data was for every position in PH20, Dr. Hecht admitted that the patent data would in fact be useful in predicting changes in PH20. EX2076, 262:7-22, 253:9-254:12.

Q. So is it your testimony that the data in the patent provide no information whatsoever that is useful to a POSA that is seeking to make, for example, a PH20 with 10 mutations, compared to wild-type?

A Once again, I'll say it's the continuum. Having zero data gives you zero insight; having more data gives you more insight.

That's not the same as saying that having X amount of data allows you to predict something accurately.

EX2076 (Hecht Dep.), 262:15-262:22, 263:1-3; PO Sur-Reply, 19