An Improved Method for Screening and MALDI-TOF MS Sequencing of Encoded Combinatorial Libraries

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Supporting Information

Optimization of MALDI-TOF MS Methods

We examined four conditions of sample preparation for MALDI-TOF MS sequencing of a single bead: **a.** a bead was loaded on a sample position of a standard MALDI-TOF MS sample plate, followed by addition of matrix solution onto the bead and irradiation at 365 nm for one hr; **b.** a bead was loaded on a sample position of the sample plate, followed by irradiation at 365 nm in acetonitrile-water (1:1) (2 μl) for one hr and then addition of matrix solution onto the bead; **c.** a bead was placed into a micro tube containing acetonitrile-water (1:1) and irradiated at 365 nm for one hr, from which an aliquot of liquid was mixed with matrix solution in 1:1 ratio, and loaded onto a sample position of the sample plate; **d.** a bead was loaded on a sample position of the sample plate, followed by addition of matrix solution onto the bead. After the samples were air-dried, MALDI-TOF MS analysis indicated that the sequence of the peptide can be obtained from the spectra at all conditions. Among them, the spectra from conditions a and c had a noisy baseline and the spectrum from condition d had weak signal intensities, while the spectrum from condition b had the highest peak signals and lowest baseline noise. It was subsequently determined that a 15 min-irradiation at 365 nm was sufficient to obtain a mass spectrum of good quality.

TGase Library Screening Using a Fluorescent Gln Substrate

In our first attempt to perform the on-resin TGase enzyme assay directly using a fluorescent TGase substrate, we conjugated the known glutamine substrate peptide Gln-Gln-Ile-Val (Parameswaran, K. N.; Velasco, P. T.; Wilson, J.; Lorand, L. *Proc. Natl. Acad. Sci. U.S.A* **1990**, *87*, 8472-8475.) to a fluorescent dansyl label (dansyl-ε-aminocaproyl-Gln-Gln-Ile-Val (DAG)), for study of Lys peptide substrates. The on-resin assay involved TGase-catalyzed incorporation of DAG onto a resin-bound Lys peptide.

As a result, resin-bound peptides were expected to be fluorescently labeled to an extent that reflected the quality of the peptide as a substrate for TGase enzyme. However, the native fluorescence of PEGA resin beads at the excitation and emission bands of dansyl, and nonspecific staining of resin beads by DAG used in the enzyme reaction, precluded screening using this method.

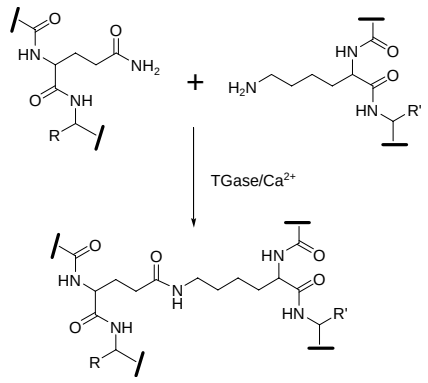
High concentration of the fluorescent glutamine peptide DAG used in the assay solution caused nonspecific fluorescent labeling of all beads, resulting in that all beads were evenly and equally fluorescent under a fluorescence microscope. Therefore, we developed a new two-stage screening procedure: 1. TGase-catalyzed incorporation of a desthiobiotinylated glutamine peptide, or cadaverine onto the resin-bound Lys peptides, or Gln peptides, and magnetic separation of the desthiobiotinylated resin-peptides; 2. removal of magnetic labeling, and relabeling of the desthiobiotinylated resin-peptides with low concentration of rhodamine B-streptavidin and fluorescent separation.

Streptavidin-Magnetic Particles of First-Stage Magnetic Screening

PEGA resin and biotin-PEGA resin were incubated with streptavidin-magnetic particles with different sizes for 30 min, respectively.
Magnetic separation using a magnet showed that streptavidin microbeads (50 nm, Miltenyi Biotec) nonspecifically labeled both of the resin beads, MagnaBind streptavidin beads (4 μm, Pierce) labeled biotin-PEGA resin and partially labeled PEGA resin, and streptavidin magnetic particles (8.0-9.9 μm, Spherotech) specifically labeled biotin-PEGA resin. These results indicated the size of the magnetic particles was a key factor to the specificity of magnetic labeling. It seemed that smaller or nano magnetic particles could be entrapped and adhered inside the resin beads to magnetically label any beads. Therefore, we chose to use the streptavidin magnetic particles (8.0-9.9 μm) for our magnetic labeling.

On-resin Cross-linking Enzyme Assay of Lysine library

The first on-resin TGase-catalyzed cross-linking assay was performed by incubating the synthesized lysine library (770,000-1,270,000 beads) in a buffer solution of 100 mM MOPS, pH 7.2, 1 mM DOQ, 10 mM CaCl2, 10 mM DTT, and 1 mM EDTA with TGase (0.25 U/ml) at room temperature for 10 min. After exhausted wash, the library was incubated with 2 ml of streptavidin-magnetic particles (8.8-9.0 μm) in 30 ml of PBS, pH 7.2, 0.1% BSA for 2 hrs. Magnetic separation by a magnet showed that none of beads were magnetically labeled. After wash, the on-resin TGase-catalyzed cross-linking assay was performed again by incubating the library at the same condition as above for 30 min. The library was treated as above again with the streptavidin-magnetic particles (8.8-9.0 μm). Magnetic separation by a magnet showed that a tenth of beads (v/v) were magnetically labeled and selected. It was obvious that the selected beads had a darker color than the sorted-out ones. At the same conditions with different reaction time, we can conclude that the magnetic labeling of resin-bound peptide library was desthiobiotin-specific from the assay results.



Scheme S1. The reaction catalyzed by TGase.

Table S1. Protected amino acids used for Gln-Gln-Ile-Val ladder synthesis

Amino acid	Primary tag	MW*	Secondary tag	MW*	PD†
Fmoc-Ahx-OH	Boc-Abu-OH	85.05			
Fmoc-Val-OH	Boc-Val-OH	99.07			
Fmoc-Ile-OH	Boc-Ile-OH	113.08	Boc-Abu-OH	85.05	-28
Fmoc-Gln(Trt)-OH	Boc-Gln-OH	128.06			

*: Residual molecular weight; †: Molecular weight difference from primary tag.
Ahx: 6-aminohexanoic acid; Abu: 4-aminobutyric acid.

Table S2. Protected amino acids used for combinatorial library by ladder synthesis

Amino acid	Primary tag	MW*	Secondary tag	MW*
Fmoc-Ala-OH	Boc-Ala-OH	71.04		
Fmoc-Arg(Pbf)-OH	Boc-Arg(Mtr)-OH	156.10		
Fmoc-Asn(Trt)-OH	Boc-Asn(Trt)-OH	114.04		
Fmoc-Asp(OBu)-OH	Boc-Asp(OBu)-OH	115.03	Ac-Asp(OBu)-OH	157.05
Fmoc-Gln(Trt)-OH	Boc-Gln(Trt)-OH	128.06	Ac-Gln(Trt)-OH	170.08
Fmoc-Glu(OBu)-OH	Boc-Glu(OBu)-OH	129.04		
Fmoc-Gly-OH	Boc-Gly-OH	57.02		
Fmoc-His(Trt)-OH	Boc-His(Trt)-OH	137.06		
Fmoc-Ile-OH	Boc-Ile-OH	113.08	Boc-Me-Ile-OH	127.08
Fmoc-Leu-OH	Boc-Leu-OH	113.08	Boc-α-Abu-OH	85.05
Fmoc-Lys(Boc)-OH	Boc-Lys(Boc)-OH	128.09	Ac-Dab(Boc)-OH	142.08
Fmoc-Met-OH	Boc-Met-OH	131.04		
Fmoc-Phe-OH	Boc-Phe-OH	147.07		
Fmoc-Pro-OH	Boc-Pro-OH	97.05		
Fmoc-Ser(tBu)-OH	Boc-Ser(tBu)-OH	87.03		
Fmoc-Thr(tBu)-OH	Boc-Thr(tBu)-OH	101.05		
Fmoc-Trp(Boc)-OH	Boc-Trp-OH	186.08		
Fmoc-Tyr(tBu)-OH	Boc-Tyr(tBu)-OH	163.06		
Fmoc-Val-OH	Boc-Val-OH	99.07	Boc-Ile-OH	113.08

* Residual molecular weight; Abu: 4-aminobutyric acid; Dab: diaminobutyric acid.

Bead Sorting Experiment

Materials and methods

The peptides on resin beads were labeled with rhodamine (Molecular Probes, cat# S871, wavelengths are Abs at 570 nm and Em at 590 nm).
Instrument: The COPAS Plus was used for the experiment. Performance of the instrument was verified with control particles before the experiments. The following optical filters were used: excitation 514 nm, emission 585 nm.
Sheath solution: 1X PBS with 0.1% Tween-20.
Sample solution: 1X PBS with 0.1% Tween-20 and 0.3% BSA.

Results

The COPAS instrument measures five parameters of an object: TOF (Time of Flight, equivalent to the length of the object), EXT (EXTinction, equivalent to the optical density), Green, Yellow and Red fluorescence intensities. Data are displayed in real time (dot-plot) with the COPAS software. This enables the user to define a region for analyzing and sorting samples based on size or fluorescence intensity properties.

To achieve the optimal sorting result, the following settings were performed:

(1) Sorting based on global fluorescence intensity level of a bead. At this setting, beads with the highest overall fluorescence intensity levels were collected (Figure S2).
(2) Sorting based on fluorescence peak height. Profiler, an add-on module of the COPAS instrument, continuously monitors the fluorescence intensity change of a bead as it enters and exits the laser beam. The result is displayed as a stack of fluorescence "slices", representing fluorescence intensity levels at different locations of the bead. Peak height is the level of the "highest" fluorescence intensity slice (see figure S1). At this setting, beads with the highest "peak height" levels were collected (Figure S3).

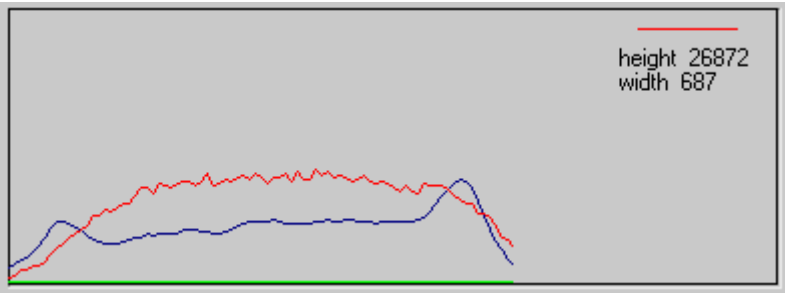


Figure S1. Profiles of a single bead. Blue trace: extinction; Red trace: red fluorescence.

(3) Sorting based on TOF normalized global fluorescence intensity. To compensate for the larger surface area of bigger beads and thus the higher measured fluorescence intensity levels, the measured fluorescence intensity is normalized by dividing it with the bead's TOF value. At this setting, beads with the highest normalized fluorescence intensity levels were collected (Figure S4).

Individual beads were collected into 96-well microtiter plates at one bead per well. The corresponding measured parameters were recorded for all of the analyzed beads. Downstream experiments will be performed to analyze the protein affinity of peptides attached to these beads.

Row	Column	TOF	Red
A	1	957	346
A	2	809	440
A	3	683	296
A	4	813	280
A	5	746	262
A	6	935	264
A	7	888	284
A	8	1032	306
A	9	899	292
A	10	704	277
A	11	786	332
A	12	935	278

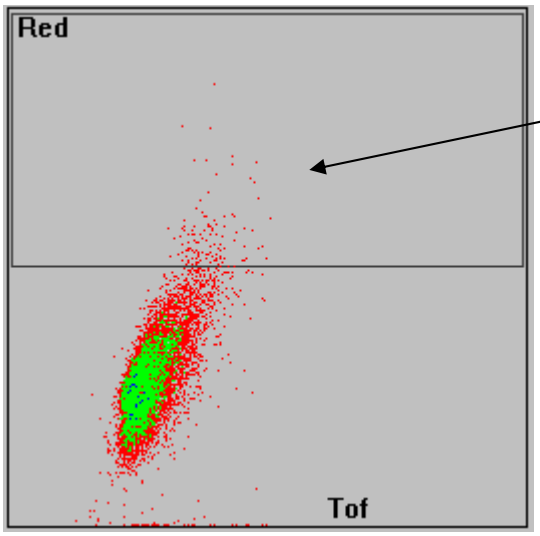


Figure S2. Sorting based on global fluorescence intensity of individual beads. Sorting region represents the top 1% brightest beads. Average TOF: 558, average Red: 145; Sorted TOF: 786, Sorted Red: 291. The table shows a sample of collected beads with their dispensed locations and corresponding measured values.

Row	Column	TOF	Red	Red peak height/100
A	1	792	717	170
A	2	552	361	218
A	3	640	512	345
A	4	880	558	296
A	5	760	567	307
A	6	808	622	328
A	7	632	527	346
A	8	736	501	279
A	9	600	423	245
A	10	656	506	304
A	11	504	321	227
A	12	536	427	241

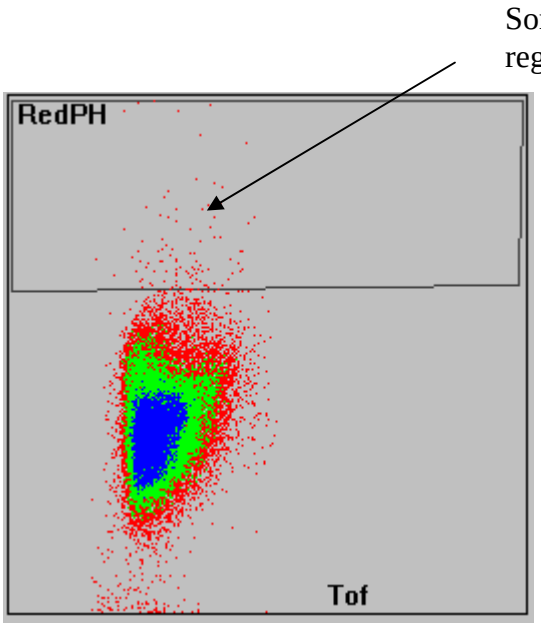


Figure S3. Sorting based on red fluorescence peak height of individual beads. Sorting region represents the top 0.2 % brightest beads. Average TOF: 592, average Red: 250, average Red peak height, 22200; Sorted TOF: 692, Sorted Red: 507, Sorted Red peak height 27623. The table shows a sample of collected beads with their dispensed locations and corresponding measured values.

Row	Column	TOF	Red	Normalized Red
A	1	744	595	80
A	2	720	596	83
A	3	704	563	80
A	4	680	545	80
A	5	712	563	79
A	6	648	514	79
A	7	616	550	89
A	8	736	589	80
A	9	760	617	81
A	10	688	574	83
A	11	776	652	84
A	12	720	582	81

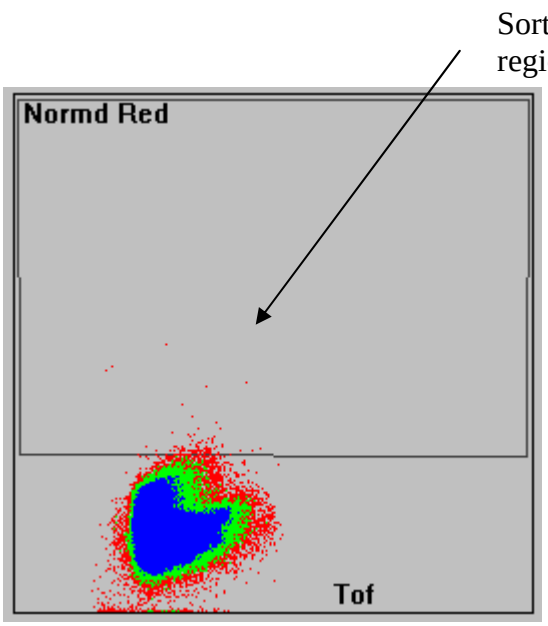


Figure S4. Sorting based on normalized fluorescence intensity of individual beads. Sorting region represents the top 0.1 % brightest beads. Average TOF: 592, average Red: 228, average normalized red, 38; Sorted TOF: 695, Sorted Red: 592, Sorted normalized red, 85. Normalized red = 100 * red/TOF. The table shows a sample of collected beads with their dispensed locations and corresponding measured values.

Table S3. Lysine Peptide Sequences of TGase substrate

No		Sequence	TR	NTR
1	1B11	Met-Ile-Lys-Gln-Asp	230	396
2	1B4	Lys-Asn-Lys-Val-Gly	283	404
3	1B1	Gly-Thr-Lys-Ala-Ala	273	416
4	1C1	Leu-Pro-Lys-Met-Tyr	238	428
5	1C6	Leu-Lys-Lys-Leu-Met	229	400
6	1C7	Thr-Arg-Lys-Tyr-Pro	299	385
7	1D11	Asp-Leu-Lys-Met-Arg	237	395
8	1D7	Met-Arg-Lys-Thr-Asp	247	393
9	1E6	Arg-Asn-Lys-Val-Pro	331	449
10	1E7	Arg-Arg-Lys-Tyr-Met	293	398
11	1F9	Ala-Tyr-Lys-Gln-Ile	263	432
12	1F3	Val-Thr-Lys-Gly-Gly	264	395
13	1G1	Ile-Thr-Lys-Trp-Phe	212	421
14	1G5	Leu-Val-Lys-Gly-Arg	307	404
15	1G7	Phe-Ala-Lys-Met-Pro	253	395
16	1H6	His-Arg-Lys-Glu-Gly	309	422
17	2A3	Tyr-Asn-Lys-Ala-Ala	294	408
18	2A4	Lys-His-Lys-Lys-Tyr	328	310
19	2A5	Gly-Lys-Lys-Asp-Tyr	299	406
20	2A9	Asp-Ala-Lys-Arg-Thr	313	355
21	2A11	Leu-Phe-Lys-Ile-Gln	357	429
22	2B9	Phe-Met-Lys-Tyr-Ile	330	412
23	2B4	Thr-Asp-Lys-Asp-Thr	306	382
24	2B2	Arg-Phe-Lys-Leu-His	293	398
25	2C1	Tyr-Ile-Lys-Phe-Pro	295	428
26	2C2	Gln-Arg-Lys-Gln-Ile	289	451
27	2C3	Phe-Met-Lys-Arg-Phe	292	434
28	2C6	Pro-Ser-Lys-Phe-Met	329	447
29	2C7	Asp-Leu-Lys-His-Arg	312	433
30	2C8	Val-His-Lys-Val-Asn	334	444
31	2D12	Gln-Ile-Lys-Gly-Leu	322	428
32	2D10	Thr-Gly-Lys-Gly-Arg	317	421
33	2D9	Asn-Ser-Lys-Gly-Phe	346	424
34	2D7	Arg-Arg-Lys-Ser-Tyr	302	439
35	2D6	Met-Lys-Lys-Gly-Phe	461	600
36	2D1	Ala-His-Lys-Ile-Leu	312	398
37	2E1	Arg-Ser-Lys-Thr-Leu	318	382
38	2E2	Asn-Ala-Lys-Pro-Phe	316	395
39	2E5	Ile-Leu-Lys-His-Gly	306	335
40	2E9	Ile-Met-Lys-Tyr-Asp	319	406
41	2E10	Val-Ser-Lys-Tyr-Met	292	405
42	2F7	His-Pro-Lys-Arg-Gln	328	402
43	2F3	Ser-Val-Lys-Leu-Lys	314	417
44	2G1	Arg-Asn-Lys-Phe-Asn	340	337
45	2G8	Ile-Val-Lys-His-Gly	359	448
46	2G10	Arg-Leu-Lys-His-Ala	319	433
47	2H9	Gln-Arg-Lys-Ser-Asn	397	477
48	2H7	Glu-Pro-Lys-Phe-Pro	347	394

49	2H4	Phe-Gln-Lys-Thr-Pro	383	488
50	3A2	Phe-Thr-Lys-Phe-Ile	298	445
51	3A4	Val-Ser-Lys-Lys-Phe	312	452
52	3A5	Leu-Thr-Lys-Ala-Ser	303	447
53	3A3	Ile-Met-Lys-Phe-Gly	304	397
54	3A7	Gln-Ala-Lys-His-Ala	299	428
55	3A8	Arg-Gly-Lys-Ser-Tyr	286	436
56	3A10	Met-Val-Lys-Asn-Lys	302	443
57	3A12	Phe-Val-Lys-Phe-His	306	439
58	3B9	Asn-Phe-Lys-Met-Pro	321	382
59	5A3	Met-Pro-Lys-Thr-Thr	296	433
60	5C6	Phe-Glu-Lys-Gly-His	305	375
61	5C10	Arg-His-Lys-His-Phe	309	395
62	5C11	Ala-Phe-Lys-Tyr-His	365	467
63	5C12	Arg-Val-Lys-Ser-Tyr	281	413
64	5D11	Ala-Ser-Lys-Arg-Asp	282	400
65	5D8	Phe-Leu-Lys-Gly-Phe	313	411
66	5D7	Val-Ile-Lys-Tyr-Gln	290	403
67	5D5	Leu-Gln-Lys-Ile-Asn	325	430
68	5D3	Phe-Gln-Lys-Phe-Ile	271	407
69	5D2	Thr-Gly-Lys-Arg-Phe	396	496
70	5E5	Ala-Ala-Lys-His-Gly	300	430
71	5E7	Met-Arg-Lys-Tyr-Asn	295	382
72	5E10	Gln-Met-Lys-Arg-Leu	351	426
73	5F7	Tyr-Gln-Lys-His-His	296	368
74	5G2	Val-Trp-Lys-Gly-Tyr	309	346
75	5G5	Thr-Arg-Lys-Met-Lys	321	520
76	5H6	Asn-Phe-Lys-Leu-Arg	288	427
77	5H5	Pro-Arg-Lys-Val-Phe	305	441
78	4A1	Ser-Val-Lys-Gln-Ala	285	442
79	4A2	Asn-Leu-Lys-His-Arg	255	418
80	4A5	Tyr-Ser-Lys-Pro-Lys	305	421
81	4A11	Asp-Ile-Lys-Gln-Ile	265	437
82	4B11	Val-Ser-Lys-Val-Val	263	427
83	4B10	Tyr-Val-Lys-Ala-Arg	310	411
84	4B7	Leu-Met-Lys-Gly-Leu	295	414
85	4B2	Gly-Val-Lys-Ile-Ile	260	434
86	4C1	Thr-Phe-Lys-Tyr-Leu	293	426
87	4C2	Phe-Thr-Lys-His-Asn	259	426
88	4C8	Arg-Gln-Lys-Glu-Phe	273	425
89	4C10	Met-Arg-Lys-Ile-Asn	314	394
90	4C11	Asn-Ser-Lys-Ile-Met	317	409
91	4D7	Thr-Pro-Lys-Arg-Met	303	402
92	4D6	Tyr-Thr-Lys-Arg-Asn	301	436
93	4D5	Thr-Ala-Lys-Met-Gly	254	416
94	4E4	Ile-Lys-Lys-Leu-His	331	373
95	4E6	Ile-Thr-Lys-Glu-Ile	321	391
96	4E11	Gly-Tyr-Lys-Arg-Ala	275	411
97	4F4	Arg-Met-Lys-His-Asp	336	400
98	4G1	Glu-Lys-Lys-Phe-Gln	313	377
99	4G2	His-Leu-Lys-Thr-Phe	308	357

100	4G3	Phe-Thr-Lys-His-Thr	288	437
101	4G6	Phe-Ser-Lys-Thr-Gln	302	396
102	4G12	Phe-Ala-Lys-Thr-Asn	252	423
103	4H11	Tyr-Ala-Lys-His-Leu	273	412
104	4H7	Gln-Tyr-Lys-Val-Leu	303	386
105	4H4	Val-Asn-Lys-Met-Ala	362	466
106	4H3	Val-Asn-Lys-Gly-Thr	301	445
107	Plate 4	Arg-Leu-Lys-Leu-Pro		
108		Tyr-Gly-Lys-Ser-Leu		
109		Lys-Ile-Lys-Asn-Gln		
110		Gln-Arg-Lys-Thr-Ala		
111		Tyr-Met-Lys-Tyr-Leu		
112		Arg-His-Lys-Gly-Pro		
113		Arg-Thr-Lys-Gln-Asp		
114		Ser-Val-Lys-Pro-Arg		
115		Asn-Gly-Lys-Arg-Met		
116		His-Gly-Lys-Val-Lys		
117		Leu-Thr-Lys-Tyr-Ala		
118		Gly-Ser-Lys-His-Gly		
119		Met-Thr-Lys-Gln-Met		
120		Met-Gln-Lys-Gln-Arg		
121	Plate 5	Phe-Thr-Lys-Arg-Gln		
122		Ser-Gly-Lys-Thr-Glu		
123		Ser-Leu-Lys-Thr-His		
124		Arg-His-Lys-Ile-Val		
125		Asn-Ser-Lys-Arg-Pro		
126		Leu-Ser-Lys-Thr-Val		
127		Thr-Leu-Lys-Val-His		
128		Lys-Leu-Lys-Gly-Tyr		
129		Asn-Ser-Lys-Leu-Arg		
130		Pro-Arg-Lys-Asp-Ala		
131		His-Ser-Lys-Arg-Thr		
132		Pro-Met-Lys-Met-Asn		
133	Plate 7	Val-Pro-Lys-Phe-Gln		
134	7A2	Asn-Arg-Lys-His-Phe	436	680
135	7A5	Glu-Gly-Lys-Lys-Leu	500	608
136	7A11	Phe-Ser-Lys-Gln-Pro	504	578
137	7B3	Lys-Tyr-Lys-Tyr-Ser	441	634
138	7B9	Asn-Pro-Lys-Leu-His	491	590
139	7B10	Val-Glu-Lys-Ser-Pro	425	690
140	7B11	Arg-Val-Lys-Gln-Ser	516	586
141	7C1	Thr-Ala-Lys-Phe-Pro	441	506
142	7C2	Tyr-Tyr-Lys-Gln-His	461	559

143	7C5	Thr-Leu-Lys-Met-Ile	497	580
144	7C8	Tyr-Ser-Lys-Lys-Asp	467	687
145	7C9	Ile-Lys-Lys-Ser-Ser	520	677
146	7D4	Val-Leu-Lys-His-Pro	475	560
147	7D7	Arg-Val-Lys-Thr-Phe	448	683
148	7E4	Phe-Lys-Lys-Lys-Glu	273	487
149	7E6	Asn-Phe-Lys-Met-Phe	471	613
150	7E12	Ile-Ser-Lys-Tyr-Ser	529	655
151	7F5	Ala-Thr-Lys-Gln-Leu	512	566
152	7F8	Asn-Gly-Lys-Ala-Arg	264	492
153	7F11	Phe-Arg-Lys-Leu-Pro	431	544
154	7G1	Asn-Ile-Lys-Ser-His	489	541
155	7G10	Tyr-Leu-Lys-Gln-Gly	524	697
156	7G12	Val-Lys-Lys-Tyr-Ser	426	656
157	7H1	Gly-Phe-Lys-Ile-Asp	460	491
158	7H4	Val-Leu-Lys-Tyr-Pro	698	739
159	7H7	His-Phe-Lys-Ala-Pro	427	550
160	7H8	Val-His-Lys-Phe-Tyr	691	738
161	7H10	Ala-Pro-Lys-Pro-Arg	402	653
162	7H11	Lys-Thr-Lys-Thr-Gly	498	556
163	8A2	Gly-Tyr-Lys-His-Arg	405	684
164	8A5	Leu-Thr-Lys-Tyr-Leu	295	595
165	8A9	Arg-Tyr-Lys-Gln-Thr	279	554
166	8A12	Asp-Phe-Lys-His-Leu	502	668
167	8B12	Gln-Ile-Lys-Arg-Phe	592	755
168	8B11	Ser-Pro-Lys-Phe-Ile	386	603
169	8B2	Arg-Ile-Lys-Thr-Asp	499	600
170	8B1	Val-Val-Lys-Gly-Gly	525	637
171	8C1	Arg-Met-Lys-Pro-Thr	469	598
172	8C7	Arg-Arg-Lys-Gln-Phe	322	592
173	8C12	Phe-Ile-Lys-Ser-Glu	567	738
174	8D4	Arg-Leu-Lys-Asp-Gln	322	610
175	8E5	Glu-Phe-Lys-Arg-Gln	327	576
176	8E11	Val-Arg-Lys-Ile-Ala	399	616
177	8F12	Tyr-Val-Lys-Gln-Phe	448	629
178	8F6	Ile-Val-Lys-Gly-Asp	461	588
179	8F5	Val-Ile-Lys-Trp-His	314	654
180	8G7	Arg-Ser-Lys-His-Asp	412	606
181	8G9	Val-Ile-Lys-Arg-Val	403	600
182	8H9	Asn-Leu-Lys-Arg-Gln	341	592
183	9A1	Ser-Gly-Lys-Arg-Phe	464	617
184	9A2	Asp-Thr-Lys-Leu-Ile	422	651
185	9A8	Gly-Ser-Lys-Pro-Gly	495	695
186	9A11	Tyr-Tyr-Lys-His-Leu	467	671
187	9B10	Glu-Thr-Lys-Val-Pro	414	681
188	9B7	Pro-Tyr-Lys-Phe-Leu	548	745
189	9B5	Tyr-Tyr-Lys-His-Leu	419	680
190	9C3	Thr-Val-Lys-Leu-Arg	396	688
191	9C10	Gly-Leu-Lys-Phe-Ala	428	660
192	9C12	Tyr-Phe-Lys-Val-His	378	656
193	9D8	Arg-Thr-Lys-Ile-Val	393	692

194	9D6	Arg-Val-Lys-Thr-Lys	433	677
195	9D5	Tyr-Asn-Lys-Ser-His	579	762
196	9D3	Phe-Leu-Lys-Leu-Ala	412	678
197	9E4	Lys-Phe-Lys-Ala-Ala	437	635
198	9F1	Ala-Tyr-Lys-Lys-Phe	446	648
199	9F2	Arg-Ile-Lys-Ser-Ala	504	724
200	9F9	Gln-Val-Lys-Tyr-His	474	581
201	9G11	Glu-Phe-Lys-Tyr-Lys	352	579
202	9G10	His-Thr-Lys-Leu-His	425	670
203	9G9	Ile-Lys-Lys-Gln-Tyr	451	705
204	9G6	Val-Pro-Lys-Asn-Gln	527	708
205	9G4	Thr-Arg-Lys-Val-Ala	429	670
206	9G2	Thr-Asn-Lys-Gln-Arg	491	563
207	9H5	Tyr-Ala-Lys-Arg-Ala	391	660
208	9H6	Val-Arg-Lys-Pro-Phe	415	674
209	9H12	Glu-Lys-Lys-Tyr-Thr	441	656
210		Gln-Thr-Lys-Met-Gly		
211	10A4	Val-Val-Lys-Arg-Glu	438	684
212	10A5	Lys-Thr-Lys-Phe-His	509	699
213	10B12	Pro-Pro-Lys-Gly-Phe		
214	10B8	Ile-Phe-Lys-Gln-His	477	557
215	10C1	Arg-Pro-Lys-Thr-Thr	508	722
216	10C3	Phe-His-Lys-Arg-Val	462	621
217	10C7	Val-Ser-Lys-Gly-Tyr	621	776
218	10C10	Leu-Phe-Lys-Lys-Val		
219	10D2	Gln-Ala-Lys-Arg-Ile	455	729
220	10D4	Asn-Val-Lys-Met-Gln	497	668
221	10E3	Pro-Val-Lys-Met-Tyr	471	727
222	10F3	Phe-Phe-Lys-Asn-Leu	473	610
223	10G2	Gln-Lys-Lys-Ala-Leu	382	682
224	10G6	Lys-Pro-Lys-Ala-Arg	967	1246
225	10G7	Asn-Arg-Lys-Asn-Ile	537	738
226	10G12	Ile-Ile-Lys-Gly-Asp		
227	10H11	Ala-Ala-Lys-Phe-Met		
228	10H3	His-His-Lys-Lys-Ala	282	511
229	10H2	Val-Gly-Lys-Met-Tyr	463	715
230	11A3	Asn-Met-Lys-Lys-Ser	422	723
231	11A4	Leu-Tyr-Lys-Ile-Asn	433	712
232	11A10	Met-Tyr-Lys-His-Met		
233	11A11	Ala-Tyr-Lys-Val-Thr		
234	11A12	Gly-Tyr-Lys-His-Pro		
235	11B11	Tyr-Lys-Lys-Ala-Gly		
236	11B10	Val-Lys-Lys-His-Asn		
237	11B9	Arg-Thr-Lys-Ile-Met		
238	11B6	Met-Gln-Lys-His-Phe	449	624
239	11B5	Ser-Val-Lys-Gln-Met	389	760
240	11C5	Thr-Arg-Lys-Ile-Gly	475	660
241	11C6	Val-Tyr-Lys-Gln-Ile	480	594
242	11C7	Ala-Gly-Lys-Ser-Tyr	259	506
243	11C9	Lys-Thr-Lys-Met-Phe	503	629
244	11C12	Asn-Phe-Lys-Val-Tyr		

245	11D9	Gln-Ser-Lys-Tyr-Phe	445	592
246	11D8	Val-Arg-Lys-Ser-Asp	539	642
247	11D4	Leu-Ala-Lys-Pro-Leu	403	637
248	11D2	Arg-Thr-Lys-Pro-Arg	424	688
249	11D1	Met-Asp-Lys-Arg-His	456	679
250	11E6	Asp-Lys-Lys-Thr-Lys	581	757
251	11F10	Phe-His-Lys-Phe-Ala		
252	11F4	Arg-Val-Lys-His-Ala	502	609
253	11F3	Glu-Ser-Lys-Ile-Tyr	641	880
254	11F2	Val-Lys-Lys-Gln-Ala	573	884
255		His-Ser-Lys-Thr-His		
256	11G3	Leu-Gly-Lys-Tyr-Ser	551	669
257	11G7	Met-Glu-Lys-Pro-Arg	577	714
258	11G10	Thr-Arg-Lys-Gln-Tyr		
259	11G11	Ser-Glu-Lys-Arg-Gly		
260	11H11	Ala-Arg-Lys-Val-Ser		
261	11H8	Asn-Gly-Lys-Gly-Tyr	603	819
262	11H7	Gly-Trp-Lys-Ser-Leu	224	438
263	11H5	Pro-Val-Lys-Val-Arg	520	747
264	11H2	Phe-Gln-Lys-Arg-Phe	362	696
265	12A3	Arg-Pro-Lys-Asp-His	512	800
266	12A6	Val-Arg-Lys-Pro-Asp	622	770
267	12A7	Ala-Leu-Lys-Phe-Tyr	527	834
268	12A12	Val-Gln-Lys-Pro-Gly		
269	12B10	Phe-Tyr-Lys-His-Pro		
270	12B7	Asn-Pro-Lys-Phe-Gln	679	738
271	12B5	Leu-Tyr-Lys-Met-Met	422	723
271	12B3	Gly-Leu-Lys-Pro-Thr	340	567
273	12B1	Ala-Lys-Lys-Leu-Lys	680	1024
274	12D10	Ala-Gly-Lys-Gly-Ala		
275	12D8	Leu-Phe-Lys-Glu-Pro		
276		Ala-Arg-Lys-Thr-Arg		
277	12D7	Phe-Leu-Lys-Asn-Met	650	903
278	12D3	Glu-Asn-Lys-Lys-Met	692	883
279	12D2	Gln-Arg-Lys-Ile-Ile	609	674
280	12E1	Asp-Val-Lys-Arg-Ile	701	835
281	12E6	Leu-Tyr-Lys-Asn-Phe	599	841
282	12E7	Val-Trp-Lys-Val-Ser	754	951
283	12E10	Asn-Phe-Lys-His-Tyr		
284	12F2	Gly-Ile-Lys-Val-Glu	643	699
285	12F5	Val-Gly-Lys-Ile-Phe	717	887
286	12F6	Ser-Phe-Lys-Thr-Phe	638	831
287	12F7	Val-Arg-Lys-Gly-Gly	625	831
288	13A7	Gln-Tyr-Lys-Tyr-Lys	569	837
289	13A8	Arg-Tyr-Lys-Val-His	628	863
290	13A12	Ser-Lys-Lys-Ala-Lys		
291	13B12	Ala-Phe-Lys-Val-Lys		
292	13B8	Thr-Ser-Lys-Thr-Phe	637	653
293	13B6	Thr-Tyr-Lys-Tyr-Thr	494	870
294	13B3	Asn-Asn-Lys-Arg-Ile	526	843
295	13C5	Ala-Ser-Lys-Thr-Gln	574	920

296	13D8	Phe-Arg-Lys-Val-Met	654	861
297	13D6	Gly-Arg-Lys-Ile-Ser	697	880
298	13E2	Phe-Phe-Lys-Asn-Met	631	822
299	13E3	Ile-Arg-Lys-Gly-Arg	590	819
300	13E5	Ser-Phe-Lys-Arg-Phe	632	806
301	13E6	Ile-Phe-Lys-Gly-Gly	622	854
302	13E7	Tyr-Val-Lys-Phe-Thr	611	878
303	13E9	Ile-Arg-Lys-Gly-Met	617	820
304	13E12	Phe-Arg-Lys-Val-Phe		
305	13F7	Val-Trp-Lys-Ser-Arg	626	775
306	13F4	Pro-Tyr-Lys-His-Met	649	863
307	13F1	Gly-Ile-Lys-Phe-Tyr	863	1112
308	13G9	Phe-His-Lys-Gly-Phe	632	832
309	13G12	Tyr-Leu-Lys-Asn-Ser		
310	13H12	Ala-Arg-Lys-Val-Asn		
311	13H7	Gly-Val-Lys-Leu-Phe	636	864
312	13H6	Gly-Ile-Lys-Gln-Gln	587	781
313	13H5	Val-Ser-Lys-Asn-Tyr	622	810
314	13H2	Tyr-Arg-Lys-Thr-Met	608	809
315	p14_42	Thr-Ser-Lys-Lys-Ser		
316	43	Phe-Gly-Lys-His-Ile		
317	44	Val-Ala-Lys-Met-Lys		
318	45	His-Trp-Lys-Pro-Ile		
319	46	Val-Ala-Lys-Tyr-Met		
320	48	Glu-Lys-Lys-Pro-His		
321	52	Val-Phe-Lys-Ala-Ser		
322	54	Ile-Gly-Lys-Lys-His		
323	55	Val-Ile-Lys-Arg-His		
324	56	Ser-Gly-Lys-Arg-Asp		
325	57	Gly-Pro-Lys-Lys-Thr		
326	58	Lys-Val-Lys-Gln-Arg		
327	15A11	Ile-Phe-Lys-Gly-Phe		
328	15B11	Leu-Ser-Lys-Phe-Phe		
329	15C3	Ala-His-Lys-Gln-Met	679	980
330	15C9	Ile-Val-Lys-Lys-Gln	669	820
331	15C10	Met-Leu-Lys-Lys-Ala		
332	15D9	Tyr-Ser-Lys-Leu-Arg	636	850
333	15D5	Arg-Thr-Lys-Lys-Glu	622	830
334	15D4	Ile-Ala-Lys-Asn-Arg	589	860
335	15D1	Met-Tyr-Lys-Leu-Gln	618	810
336	15E2	Phe-Ser-Lys-Gly-Met	681	830
337	15E4	Ser-Met-Lys-Leu-His	636	810
338	15E7	Leu-Val-Lys-Arg-Glu	655	860
339	15E8	Gly-Val-Lys-Ala-Asn	652	820
340	15F9	Asn-Phe-Lys-Val-Arg	688	880
341	15F4	His-Val-Lys-Leu-Arg	751	800
342	15F1	His-Lys-Lys-Asn-His	745	940
343	15G4	Met-Thr-Lys-Phe-Asn	624	890
344		Gly-Ser-Lys-His-Ala		
345		Ser-Ile-Lys-Lys-Pro		

TR: global total red fluorescence; NTR: normalized global total red fluorescence.

Supporting Information for Glutamine Peptide Library

Experimental Section

Described below is the synthesis, two-stage screening, MALDI-TOF MS sequencing and enzymatic study of an encoded glutamine hexapeptide library with the general sequence of $X_1X_2QQX_5X_6$.

Materials and Methods

Materials.

PEGA amine was purchased from Polymer Laboratories, Inc. (Amherst, MA). Fmoc-photocleavable linker (Fmoc-PCL), Fmoc-amino acids, Boc-amino acids, 1, 5-diaminopentane trityl resin, Fmoc-PEG600 acid, Fmoc-Wang resin and 2-chlortrityl resin were obtained from Novabiochem (La Jolla, CA). Fmoc-ε-Ahx and Boc-ε-Ahx were purchased from Advanced Chemtech (Louisville, KY). MULTIBLOCK manual library synthesizer was purchased from CSPS Pharmaceuticals (CA). Desthiobiotin, dansyl-cadaverine, Substance P peptide was obtained from Sigma (St. Louis, MO). SPHERO^{TM} streptavidin-magnetic particles were purchased from Spherotech Inc. (Libertyville, IL). Rhodamine B-streptavidin was purchased from Molecular Probes (Eugene, OR).

Methods

Library Synthesis. An overview of the synthesis of the peptides on the beads is outlined in **Scheme S2**. PEGA amine resin (300-500 μm, 1 g, 0.2 mmol, 770,000-1,270,000 beads) was washed with methanol (MeOH) and *N*-methyl-2-pyrrolidinone (NMP), three times each. Fmoc-photocleavable linker (Fmoc-PCL) (312 mg, 0.6 mmol, 3 equiv), benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate

(BOP) (265 mg, 0.6 mmol), and *N*-hydroxybenzotriazole (HOBt) (92 mg, 0.6 mmol) were dissolved in NMP (7 mL), followed by addition of diisopropylethylamine (DIEA) (105 mL, 0.6 mmol). After 10 min at room temperature with occasional stirring, the solution was added into the resin and incubated overnight with rocking. Completion of the coupling reaction was confirmed by a negative Kaiser test. After removal of Fmoc in 20% piperidine in NMP for 20 min, NH_2-Pro-Tyr(tBu)-Phe-Arg(Pbf)-Val-PCL-PEGA (MSS-PCL-PEGA) resin was synthesized by Fmoc solid-phase peptide synthesis strategy. Each coupling reaction was performed for 30 min with a 10-min pre-activation of five equivalents of Fmoc-amino acid-BOP-HOBt-DIEA (1:1:1:1) at room temperature. Fmoc was removed by 20% piperidine in NMP for 20 min. After Fmoc removal, Fmoc-ε-Ahx/Boc-ε-Ahx (9:1, 318 mg/23.1 mg) were coupled to the resin using the above coupling protocol.

The resin was suspended in methanol and dispensed evenly into 19 syringe reaction vessels of a MULTIBLOCK manual library synthesizer (CSPS Pharmaceuticals, CA). The resin was washed with methanol and NMP, and Fmoc was removed. For the synthesis of position X_6, each Fmoc amino acid (0.18 mmol, 20 equiv) (totaling 19, cysteine not included) and its counterpart Boc-amino acid (0.02 mmol), or Boc-Lys(Boc)-OH (0.01 mmol) and Boc-Lys(Ac)-OH (0.01 mmol) as well as Boc-Leu-OH (0.01 mmol) and Boc-γ-Abu-OH (0.01 mmol) for the binary tag of Lys and Leu residues, were dissolved in NMP (0.5 mL), mixed with BOP (0.2 mmol) and HOBt (0.2 mmol) in NMP (0.5 mL), and followed by addition of DIEA (0.2 mmol) in NMP (0.5 mL). After 10 min, each solution was added to one syringe reaction vessel (totaling 19) and shaken for 2 h. The randomization step was carried out using the apparatus in methanol by

mixing the resins from all 19 syringe vessels and redistributing them uniformly into 19 syringe vessels. For the syntheses of positions X_1, X_2, and X_5, all above procedures for the synthesis of position X_6 were repeated. For the synthesis of two glutamine residues, the procedures were the same as above except Fmoc-glutamine-OH was used and reaction time was 3 h for all reaction vessels. The randomization step was omitted.

After removal of N-terminal Fmoc, the peptide resins were pooled and treated with trifluoroacetic acid (TFA)-triisopropylsilane (TIS)-H_2O (95:2.5:2.5) for 3 h and followed by treatment with TFA-thioanisole-H_2O-EDT-TIS (86.5:5:5:2.5:1) for 24 h. The beads were washed with NMP, dichloromethane (DCM), MeOH, and acetonitrile (ACN)-H_2O (1:1). Some beads were randomly selected for MALDI-TOF MS analysis.

Synthesis of Desthiobiotin-PEG600-Cadaverine (DPC). The solid-phase synthesis of this conjugate was carried out manually on a 1,5-diaminopentane trityl resin (0.87 mmol/g) (0.3 g, 0.87 mmol/g, 0.26 mmol) by Fmoc strategy. Desthiobiotin and Fmoc-PEG600 acid were treated as Fmoc-amino acid. Fmoc-PEG600-OH (291 mg, 0.347 mmol), BOP (154 mg, 0.347 mmol), and HOBt (53 mg, 0.347 mmol) were dissolved in DCM-NMP (1:1) (4 mL), and followed by addition of DIEA (60 μL, 0.347 mmol). After 10 min, the solution was added to a reaction vessel containing 0.3 g of 1,5 diaminopentane trityl resin (0.26 mmol) and rocked overnight, followed by washing with NMP, DCM, and NMP again, three times each. Fmoc was removed by treatment with 20% piperidine in NMP for 20 min and washed with NMP four times. Desthiobiotin (167 mg, 0.78 mmol) and BOP (344 mg, 0.78 mmol) were dissolved in DCM (4 mL) and NMP (2 mL), followed by addition of DIEA (136 μL). The solution was added to the resin and rocked for 60 min, followed by washing with NMP, DCM, and 2-propanol

(IPA), three times each. Then, the resin was treated with 20% TFA and 2.5% TIS in DCM (50 mL) for 15 min four times. The crude product was obtained by concentration of the TFA solution and addition of diethyl ether. The crude product was purified using a semi-preparative C-18 RP-HPLC column (250 × 22 mm, 10 μm, Vydac) with an acetonitrile gradient of 14%-38% in 120 min. The fractions containing the product were pooled and lyophilized. ESI-MS (LCQ-LC-MS system, Finnigan, Thermoquest, CA) of the purified product gave peaks at m/z 898.4.

On-Resin Enzymatic Reaction. The library beads were washed with glacial acetic acid, H_2O, and 0.1 M MOPS buffer (pH 7.2), three times each and drained. Ten milliliters of 0.1 M MOPS buffer solution (pH 7.2) containing 20 mM $CaCl_2$, 4 mM DPC were added to the resin bead and rocked for 10 min, followed by addition of 10 mL of 0.1 M MOPS buffer solution (pH 7.2) containing 2 mM EDTA, 20 mM DTT, and tTG (6 units). The reaction mixture was rocked at room temperature for 30 min, and then drained. The beads were washed with 0.1 M MOPS buffer solution (pH 7.2), H_2O, 0.1% TWEEN-20 in H_2O, H_2O, PBS (pH 7.2), three times each.

Two-Stage Library Screening. A diagram demonstrating the screening methods is shown in Scheme S3. The beads were washed with PBS (pH 7.2) three times and drained. PBS (pH 7.2) (20 mL) was added to the resin beads. SPHERO^{TM} streptavidin-magnetic particles (2 mL) were added to the beads, and the mixture was incubated at room temperature for 2 h. PBS (pH 7.2) containing 0.5% BSA was added to dilute the resin beads and separated using a magnet ×3 times.

The magnetically selected beads (ca. 1 mL) were treated with 50 mM biotin in PBS (pH 7.2) for 2 h, and washed with H_2O, NMP, H_2O, PBS (pH 7.2), 0.3% BSA in

PBS (pH 7.2), 0.1% Tween-20 in H_2O, H_2O, PBS (pH 7.2), three times each and drained. Then, 0.3% BSA and 0.1% Tween-20 in PBS (pH 7.2) (3 mL) were added to the beads, and incubated after addition of 0.1 mg of rhodamine B-streptavidin at room temperature for 30 min. The beads were washed with PBS (pH 7.2), 0.3% BSA in PBS, and 0.1% Tween-20 in PBS, three times each. The beads were diluted with 0.1% Tween-20 and 0.3% BSA in PBS, and selected under a fluorescence microscope using a micropipette.

MALDI-TOF MS Analysis of Single Beads. Individual beads selected according to their fluorescence intensity were washed with H_2O. MALDI-TOF mass spectra were obtained by using the following procedures: (1) Prepare the matrix solution by dissolving 10 mg of α-cyano-4-hydroxycinnamic acid (CHCA) in 100 μL of 3% TFA in H_2O, 300 μL of H_2O, and 600 μL of acetonitrile. (2) Irradiate a single bead with 2 μL of ACN-H_2O (1:1) added in the MALDI-TOF sample plate for 15 min at UV 365 nm. (3) Apply 1 μL of the matrix solution onto the sample plate with the bead on the sample plate, and air-dry the liquid. (4) Perform MALDI-TOF MS analysis on a Voyager-DE^{TM}Pro mass spectrometer (PerSpective Biosystems). The amino acid sequences of the 267 selected glutamine peptides are shown in Table S4.

MALDI-TOF PSD MS Evaluation. MALDI-TOF PSD MS analysis of the product peaks revealed that the enzyme reacted with the first fixed Gln for most of the peptides. Examples of the MALDI-TOF PSD mass spectra are shown in **Figure S8**. The bold Gln in **Table 2** indicate the residues that were involved in the tTG cross-linking as determined by PSD. Peptides 5 and 10 also involved the Gln in the X_2 position. Six peptides had both fixed Gln residues involved in the tTG reaction. The cross-linking was

found to include two Gln if two different fragment peaks containing DC were identifiable in the spectra (**Figure S8B**).

Additional Discussion of Glutamine Peptide Library

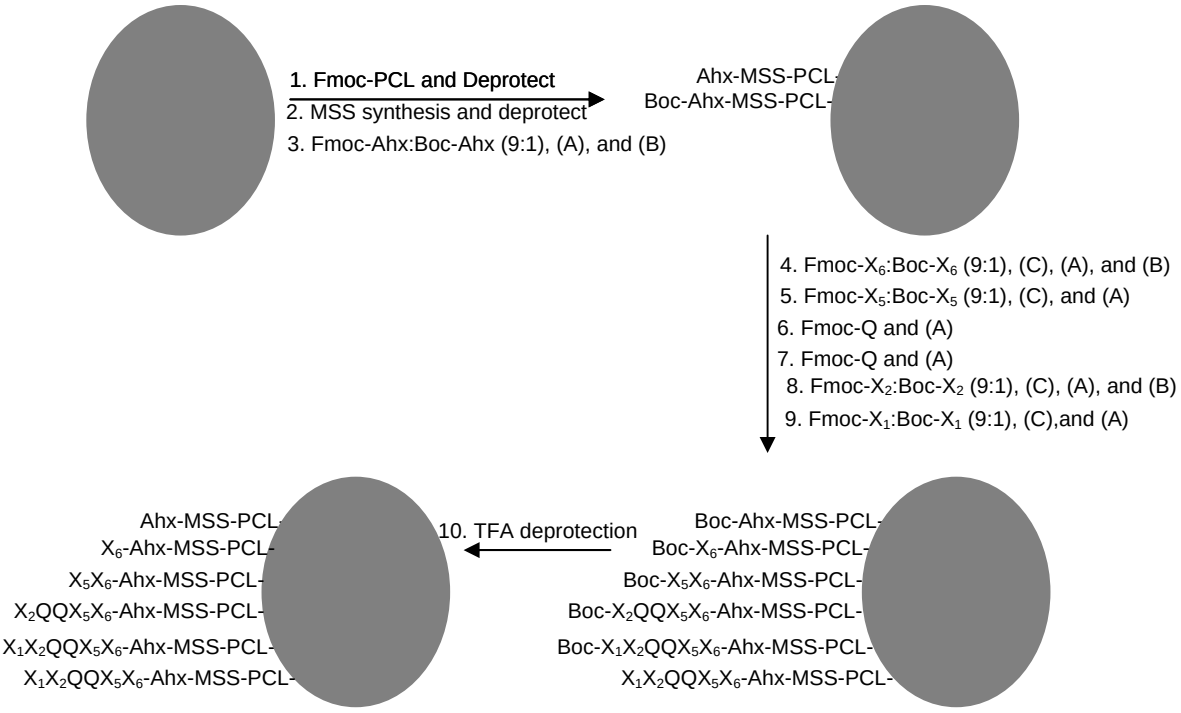
This library has peptides with a general sequence of $X_1X_2QQX_5X_6$, which was designed anticipating that an increase in the numbers of Gln translated into an increase in reactivity.[1, 2] In addition, there are a number of known substrates where the reactive Gln is usually found adjacent to another Gln.[3-5]

After the DPC had been conjugated to the reactive peptides and the on-bead enzymatic reactions were quenched, the beads were screened first by magnetic separation and then by fluorescence intensity. The first stage of screening was accomplished using streptavidin-conjugated magnetic particles. The beads with the most magnetic particles, which corresponded to the beads with the most reactive peptides, were isolated magnetically. The second screening was completed by first using biotin to wash out the magnetic streptavidin and then conjugating the beads with rhodamine-labeled streptavidin. The brightest beads (267 total) were selected visually using a fluorescent microscope, which has its associated subjectivity.

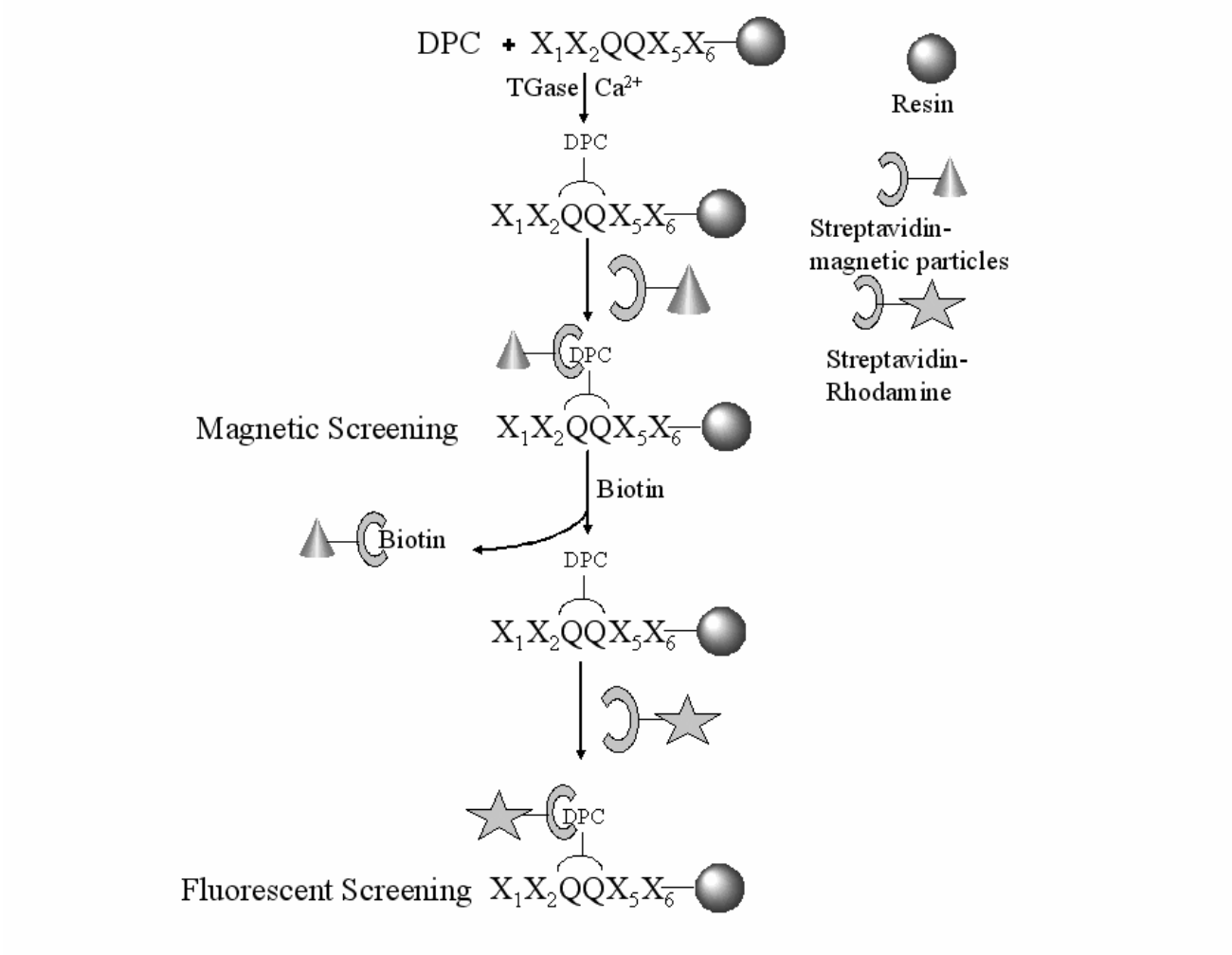
In the present study, the first invariant Gln was usually involved in the isopeptide bond formation (see **Table 2**). This is similar to observations of Substance P by Pastor et al. where the first Gln retained 94% of the reactivity when the second was replaced by Asn.[3] Parameswaran et al. suggested that in the AEQQIV peptide, the first Gln was the primary acyl donor in the tTG reaction because Boc-QIV was unreactive[6]. This was also observed in half of the peptides containing 2 adjacent Gln in a phage-display library.[7] In six of the peptides, both invariant Gln were reactive and in two others, the Gln in X_2

served as a second reactive Gln. This is likely due to other features of the peptide that facilitate either binding to the enzyme and/or reaction with tTG.

Of the 267 peptides identified in the library, peptides with five or six glutamines were not found. There were 33 peptides that contained 3 or 4 contiguous Gln. Ten of these peptides were synthesized and their enzyme kinetic values were evaluated (one was insoluble). While a general examination of their kinetic values do not indicate superior properties over the peptides containing 2 Gln, individual comparisons demonstrate that a substitution of Gln at X_2 or X_5 improves the K_m. (Compare peptides nos 5 and 6, nos 7 and 11 and nos 10 and 13). This is congruous with studies by Gorman and Folk which demonstrated that an increase from 1 to 2 Gln lead to a decrease in K_m[2]. In previously reported studies, an increase in number of contiguous Gln (1, 3, 5, 8 and 12) lead to an increase in the rate and quantity of amine donor incorporated[1]. However, the differences in the amount of contiguous Gln between peptides (3 and 4) in this study have not been observed.



Scheme S2. Overview of the glutamine library synthesis. (A) Deprotect Fmoc using piperidine. (B) Split the resin into 19 syringes and perform amino acid coupling. (C) Combine resin. Amino acid (Fmoc-X:Boc-X, 9:1) coupling was performed using BOP and DIPEA. When Lys and Leu are coupled, the Fmoc-X:Boc-X:Secondary tag ratio was 9:0.5:0.5.



Scheme S3. Summary of the two-stage screening of glutamine substrate peptide library.

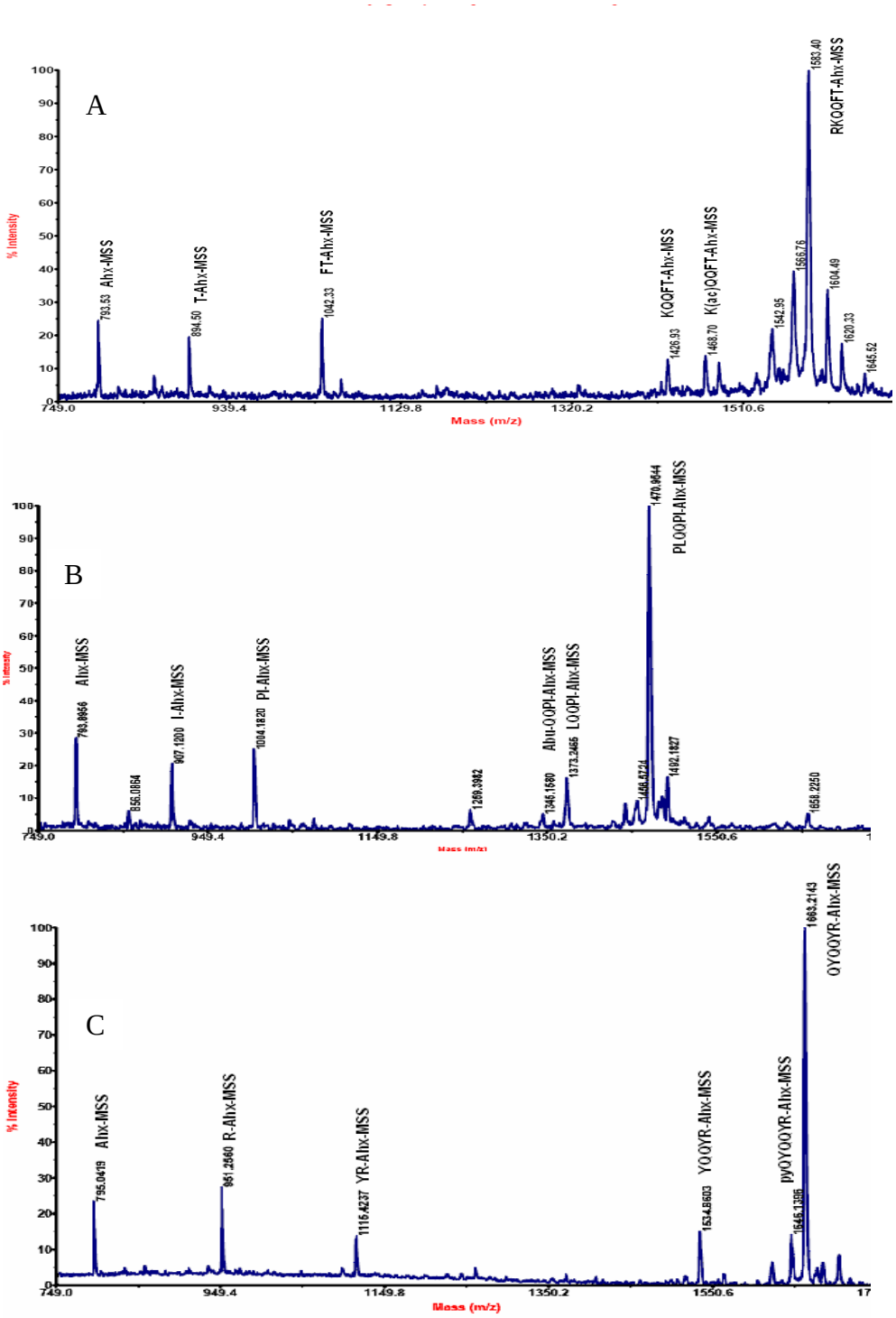


Figure S5. MALDI-TOF spectra of peptide sequence on the resin bead. Each peak in the spectra represents a peptide tag. The difference between adjacent peaks is the MW of the amino acid residue difference between them. (A) PLQQPI. (B)RKQQFT. (C) QYQQYR.

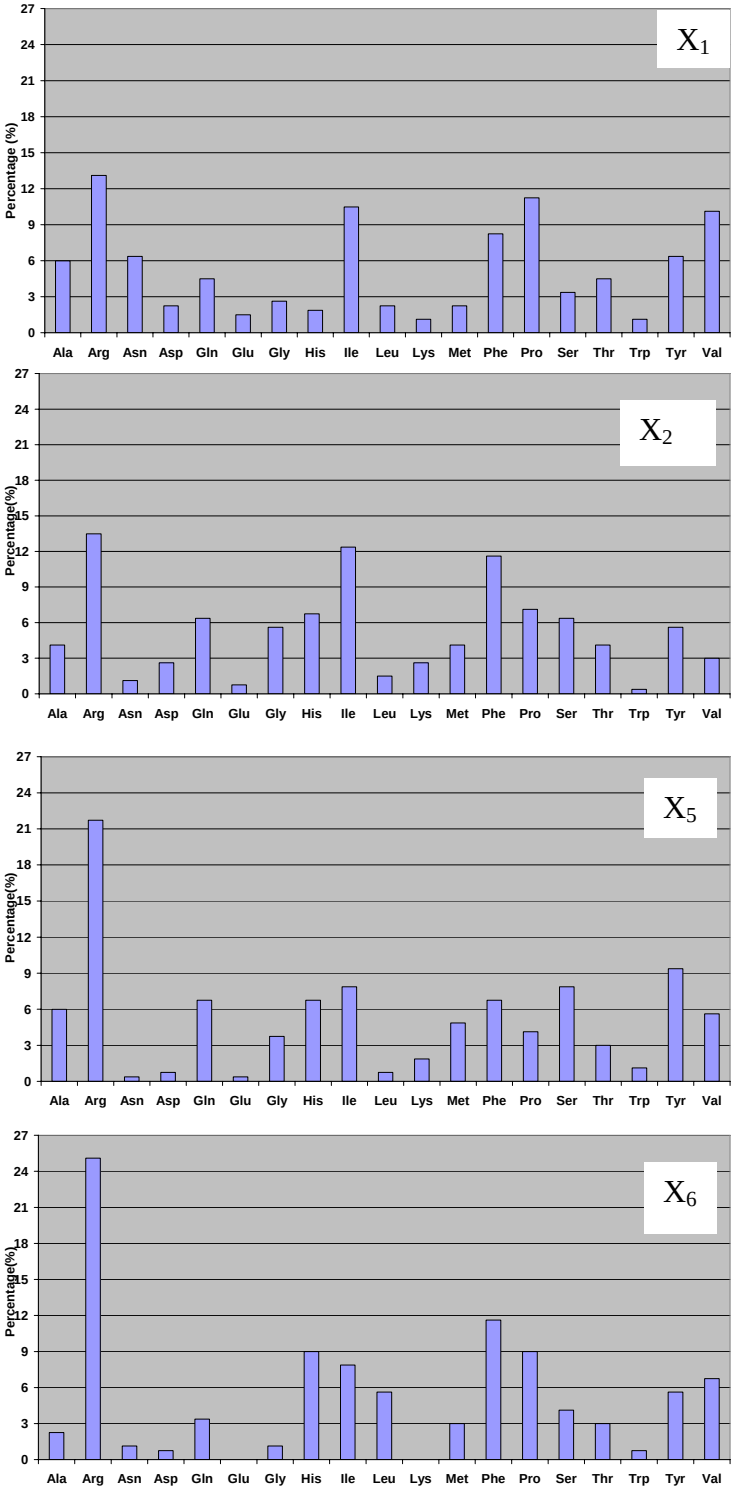


Figure S6. Amino acid frequency in the randomized positions in the peptide $X_1X_2QQX_5X_6$.

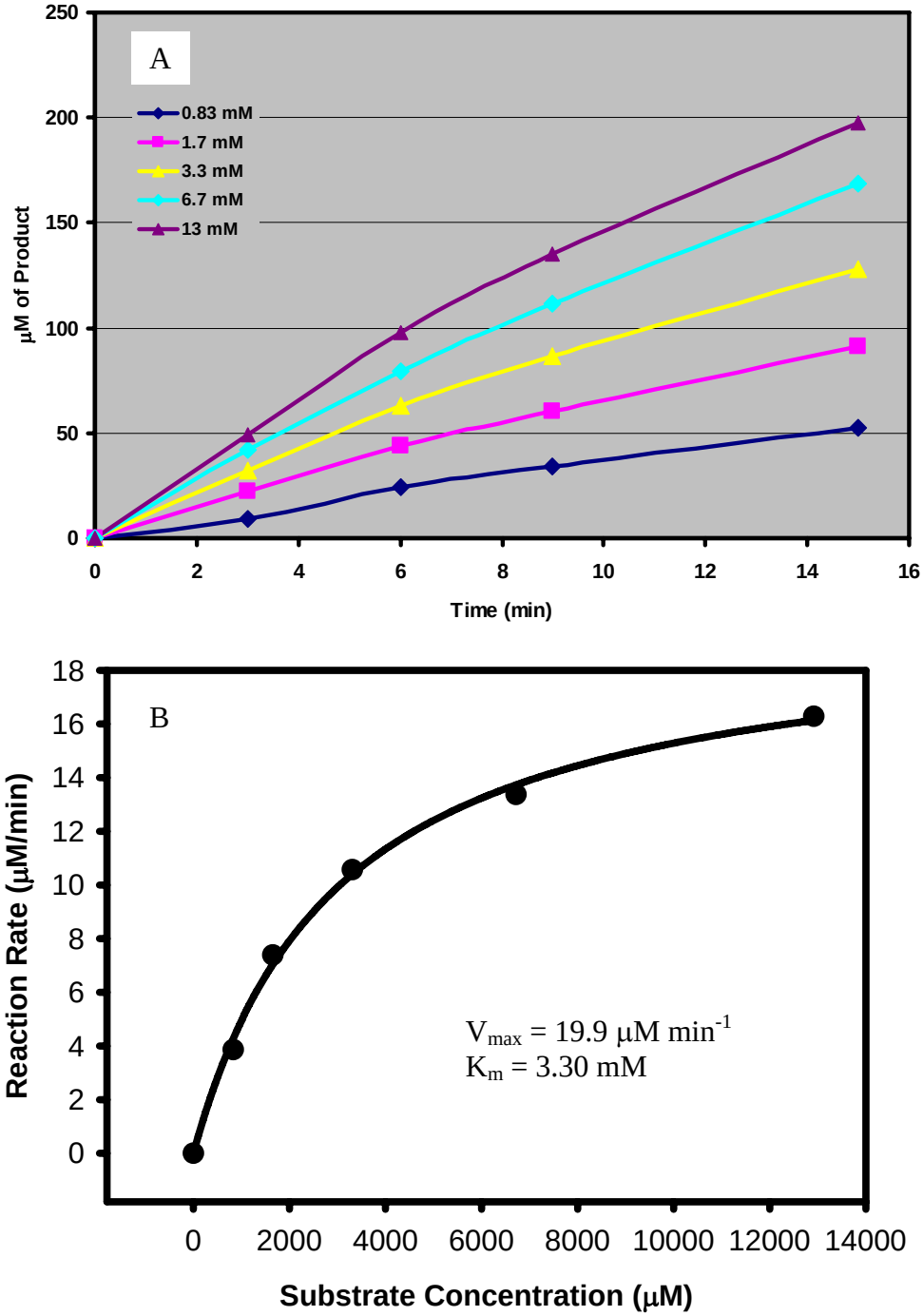


Figure S7. Sample of enzymatic reactions and curve fitting for Val-His-Gln-Gln-Gln-Arg. (A) Reaction progression curves. (B) Michaelis-Menten plot with fitted V_{max} and K_m.

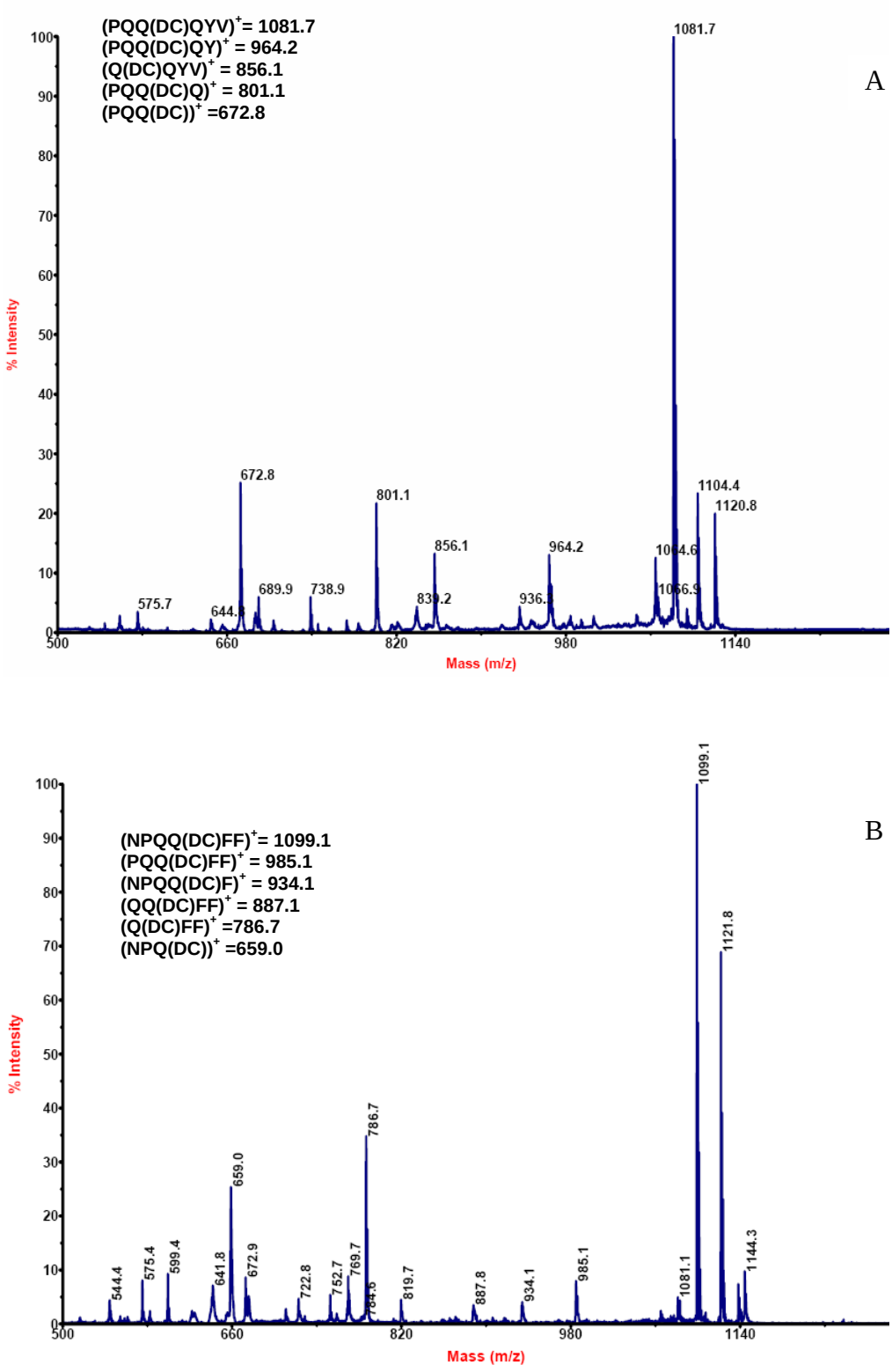


Fig. S8. MALDI PSD evaluation of peptide products. (A) Pro-Gln-Gln-Gln-Tyr-Val; (B) Asn-Pro-Gln-Gln-Phe-Phe.

Table S4 Glutamine Peptide Sequences of TGase substrate

Pro	Phe	Gln	Gln	Pro	Ile
Ile	Arg	Gln	Gln	Arg	Phe
Val	Arg	Gln	Gln	Pro	Phe
Pro	Asp	Gln	Gln	Arg	Val
Ile	Asn	Gln	Gln	Val	Arg
Arg	Met	Gln	Gln	Ile	Tyr
Pro	His	Gln	Gln	Gln	Phe
Tyr	Asp	Gln	Gln	Gly	Phe
Thr	Ala	Gln	Gln	His	Ile
Pro	Ile	Gln	Gln	Tyr	Pro
Arg	Phe	Gln	Gln	Ser	Met
Asp	Gly	Gln	Gln	Arg	Phe
Lys	Phe	Gln	Gln	Arg	Asp
Thr	Ala	Gln	Gln	Arg	Tyr
Arg	Asp	Gln	Gln	Met	Pro
Gln	Tyr	Gln	Gln	Tyr	Arg
Asp	Trp	Gln	Gln	Arg	Val
Arg	His	Gln	Gln	Ala	Phe
Phe	Gln	Gln	Gln	Met	His
Pro	Gly	Gln	Gln	Phe	Arg
Arg	Ile	Gln	Gln	Gly	Arg
Pro	Leu	Gln	Gln	Pro	Ile
Ala	Gly	Gln	Gln	Ile	Arg
Ile	Phe	Gln	Gln	Arg	Ile
Asn	Ile	Gln	Gln	Arg	Tyr
Val	Arg	Gln	Gln	Gly	Ile
Ile	Ile	Gln	Gln	Ile	Arg
Thr	Phe	Gln	Gln	Arg	Arg
Asn	Arg	Gln	Gln	Arg	His
Val	Arg	Gln	Gln	Phe	Ile
Gly	Pro	Gln	Gln	Val	Leu
Val	Ile	Gln	Gln	Thr	Arg
Arg	Gly	Gln	Gln	Pro	Arg
Arg	Lys	Gln	Gln	Phe	Thr
Arg	Asp	Gln	Gln	Arg	Pro
Asn	Gly	Gln	Gln	His	Phe
Phe	Ser	Gln	Gln	Arg	Met
Pro	Tyr	Gln	Gln	Glu	Leu
Asp	Gln	Gln	Gln	Ala	Met
Ser	Arg	Gln	Gln	Val	Val
Ser	Pro	Gln	Gln	Tyr	Ile
Val	Arg	Gln	Gln	Met	Phe
Asn	Phe	Gln	Gln	Tyr	Arg
His	Val	Gln	Gln	His	Pro
Ile	Gly	Gln	Gln	Arg	Met
Arg	Pro	Gln	Gln	Arg	Arg
Arg	Arg	Gln	Gln	Lys	Pro

Leu	Arg	Gln	Gln	Ser	Leu
Ile	Ile	Gln	Gln	Ser	His
Pro	Gln	Gln	Gln	Tyr	Val
Phe	Ile	Gln	Gln	Gln	Phe
Pro	Gln	Gln	Gln	Tyr	Val
Pro	Gln	Gln	Gln	Gln	Met
Tyr	Lys	Gln	Gln	Tyr	Arg
Phe	Met	Gln	Gln	Tyr	Arg
Glu	Ile	Gln	Gln	Val	Ile
Thr	Ile	Gln	Gln	Ser	Pro
Leu	Phe	Gln	Gln	Arg	Ser
Ser	Pro	Gln	Gln	Thr	Leu
Leu	His	Gln	Gln	His	Arg
Pro	Phe	Gln	Gln	Tyr	Arg
Pro	Met	Gln	Gln	Phe	Phe
Ala	Phe	Gln	Gln	Met	His
Phe	Arg	Gln	Gln	Phe	Pro
Ala	Ala	Gln	Gln	Gln	Arg
Tyr	Gln	Gln	Gln	Met	His
Arg	His	Gln	Gln	Gln	Ser
Phe	Tyr	Gln	Gln	Gln	Gln
Glu	His	Gln	Gln	Arg	Pro
Phe	Phe	Gln	Gln	Ile	Thr
Met	Phe	Gln	Gln	Ser	His
Ala	Phe	Gln	Gln	Ile	His
Phe	Ile	Gln	Gln	Gly	His
Pro	Leu	Gln	Gln	His	Gln
		Gln	Gln	Phe	His
Val	His	Gln	Gln	Pro	Thr
Ile	Tyr	Gln	Gln	Val	Tyr
Asn	Ala	Gln	Gln	Arg	Ile
Phe	Ile	Gln	Gln	His	His
Ile	Phe	Gln	Gln	Tyr	Arg
Phe	Ser	Gln	Gln	Arg	Asp
Asn	Arg	Gln	Gln	Val	Val
His	Ala	Gln	Gln	His	Arg
Gly	Phe	Gln	Gln	Thr	Val
Arg	Arg	Gln	Gln	Ala	Pro
Phe	Val	Gln	Gln	Gly	Arg
Ala	Ile	Gln	Gln	Met	Ile
Pro	Thr	Gln	Gln	Arg	Ser
Asn	Thr	Gln	Gln	Arg	Met
Gln	Tyr	Gln	Gln	Ala	Arg
Val	Gly	Gln	Gln	Leu	Val
Arg	Thr	Gln	Gln	Arg	Val
Ile	Phe	Gln	Gln	Thr	Arg
Gln	Leu	Gln	Gln	Arg	Pro
Pro	Gln	Gln	Gln	Phe	Phe
Gln	Tyr	Gln	Gln	Arg	Trp
Pro	Ile	Gln	Gln	Val	His

Phe	Ala	Gln	Gln	Pro	Pro
Ala	Met	Gln	Gln	Ile	Phe
Ile	Ser	Gln	Gln	Pro	Val
Asn	Pro	Gln	Gln	Phe	Phe
Pro	Pro	Gln	Gln	Ile	Leu
Ala	Ser	Gln	Gln	Tyr	Phe
Tyr	Asp	Gln	Gln	Ala	Phe
Val	Tyr	Gln	Gln	Met	Val
Ser	Arg	Gln	Gln	Tyr	Val
Arg	His	Gln	Gln	Phe	Pro
Asn	Arg	Gln	Gln	Ser	Gln
Asn	Ile	Gln	Gln	Arg	Tyr
Arg	Ser	Gln	Gln	Phe	Ile
Arg	Ser	Gln	Gln	Ser	Ser
Gln	Phe	Gln	Gln	Pro	Phe
Lys	Thr	Gln	Gln	Gln	Arg
Asn	Pro	Gln	Gln	Met	Arg
Arg	Glu	Gln	Gln	Tyr	Arg
Ile	Pro	Gln	Gln	Tyr	Arg
Val	His	Gln	Gln	Gln	Arg
Asn	His	Gln	Gln	Trp	Ser
Ile	Met	Gln	Gln	Ile	Arg
Tyr	Asn	Gln	Gln	Arg	Leu
Pro	Ile	Gln	Gln	Asn	Arg
	Met	Gln	Gln	Arg	Val
Pro	Gln	Gln	Gln	Tyr	Arg
Asp	Phe	Gln	Gln	Arg	Phe
Pro	His	Gln	Gln	His	Ile
Arg	Pro	Gln	Gln	Tyr	Pro
Pro	Phe	Gln	Gln	Arg	Arg
Ala	Leu	Gln	Gln	Ala	Arg
Ile	Gly	Gln	Gln	Ser	Tyr
Gln	Pro	Gln	Gln	Arg	Arg
Tyr	Lys	Gln	Gln	Arg	Phe
Tyr	Arg	Gln	Gln	Pro	Tyr
Gln	Gly	Gln	Gln	Val	Arg
Val	Phe	Gln	Gln	Arg	Gln
Met	Ile	Gln	Gln	His	Ile
Val	Thr	Gln	Gln	Met	Arg
Val	Ile	Gln	Gln	Gln	Tyr
Tyr	Arg	Gln	Gln	Ile	Pro
Ile	Val	Gln	Gln	Ile	His
Gln	Asp	Gln	Gln	Val	Arg
Ala	Asp	Gln	Gln	Arg	Val
Arg	Tyr	Gln	Gln	Val	Ala
Val	Ile	Gln	Gln	Tyr	Arg
Ile	Thr	Gln	Gln	Arg	Arg
Thr	Arg	Gln	Gln	His	Pro
Gln	Ile	Gln	Gln	Lys	Tyr
Ile	Thr	Gln	Gln	Trp	Arg

Phe	Thr	Gln	Gln	Arg	Gln
Ile	Tyr	Gln	Gln	Arg	Phe
Arg	Ile	Gln	Gln	Ala	Asn
Asn	Ser	Gln	Gln	Arg	Ala
Arg	Arg	Gln	Gln	Phe	Met
Pro	Ala	Gln	Gln	Ile	Asn
Ile	Ser	Gln	Gln	Arg	Tyr
Tyr	Ile	Gln	Gln	Tyr	Pro
Met	His	Gln	Gln	Phe	Tyr
Val	His	Gln	Gln	Gly	Leu
Tyr	Thr	Gln	Gln	Arg	Gly
Arg	Ile	Gln	Gln	Phe	Ser
Val	Arg	Gln	Gln	Lys	Pro
Val	Gln	Gln	Gln	Gln	Asn
Arg	Ile	Gln	Gln	Tyr	Gln
His	Arg	Gln	Gln	Phe	Arg
Val	Phe	Gln	Gln	Ser	Ile
Pro	Phe	Gln	Gln	Arg	Val
Thr	Ile	Gln	Gln	Phe	Arg
Phe	Gly	Gln	Gln	Gln	Pro
Ile	Phe	Gln	Gln	His	Ser
Ile	Val	Gln	Gln	Ser	His
Glu	Gln	Gln	Gln	Ile	Trp
Phe	Ile	Gln	Gln	Ile	Arg
Arg	Glu	Gln	Gln	Ala	Ala
Phe	Phe	Gln	Gln	Asn	Thr
Val	Ile	Gln	Gln	Val	Gln
Val	Ile	Gln	Gln	Ser	Gln
Tyr	Gln	Gln	Gln	Ile	Leu
Arg	Gln	Gln	Gln	Ala	Ala
Val	Met	Gln	Gln	Thr	His
Phe	Ser	Gln	Gln	Val	Arg
Val	Met	Gln	Gln	Thr	Ile
Gln	Gly	Gln	Gln	Ala	His
Gly	Phe	Gln	Gln	Thr	Leu
Pro	Phe	Gln	Gln	Ile	His
Ser	Gln	Gln	Gln	His	Ile
Ile	Arg	Gln	Gln	Trp	Thr
Ile	Phe	Gln	Gln	Met	His
Phe	Arg	Gln	Gln	Ser	Phe
Phe	Pro	Gln	Gln	Ser	Ile
Ala	His	Gln	Gln	Ala	His
Ile	Arg	Gln	Gln	Ile	Ala
Val	His	Gln	Gln	His	Val
Ser	Gly	Gln	Gln	Arg	Ile
Tyr	Ala	Gln	Gln	Arg	Ser
Leu	Ser	Gln	Gln	His	Arg
Ile	Gln	Gln	Gln	His	Ile
Ala	Phe	Gln	Gln	Leu	Thr
Gln	Phe	Gln	Gln	Arg	Gly

Tyr	Pro	Gln	Gln	Gln	Pro
Trp	Arg	Gln	Gln	Asp	His
Ser	His	Gln	Gln	Arg	Val
Tyr	Thr	Gln	Gln	Arg	Pro
Trp	Arg	Gln	Gln	Arg	Pro
Tyr	Pro	Gln	Gln	Gln	Pro
Trp	Arg	Gln	Gln	Asp	His
Thr	Ala	Gln	Gln	Val	Tyr
Thr	His	Gln	Gln	Arg	Val
Asn	Arg	Gln	Gln	Ile	Gly
Ile	Tyr	Gln	Gln	Gln	Leu
Arg	Ser	Gln	Gln	Gln	Phe
Val	Ile	Gln	Gln	Met	Arg
Asn	Arg	Gln	Gln	Arg	Leu
Lys	Arg	Gln	Gln	Pro	Phe
Tyr	Lys	Gln	Gln	Gly	Leu
His	Lys	Gln	Gln	Met	Phe
Leu	Pro	Gln	Gln	Tyr	Phe
Pro	Met	Gln	Gln	His	Phe
Arg	Ile	Gln	Gln	Ile	Pro
Leu	Pro	Gln	Gln	Ile	Arg
Val	Ser	Gln	Gln	Ala	Tyr
Ala	Arg	Gln	Gln	Ser	Arg
Pro	Tyr	Gln	Gln	Arg	His
Arg	Arg	Gln	Gln	Gly	Ala
Ile	Phe	Gln	Gln	Val	Ser
Thr	Phe	Gln	Gln	Arg	Phe
Asp	Met	Gln	Gln	Arg	Ile
Met	Gln	Gln	Gln	Ser	Leu
Tyr	Arg	Gln	Gln	Ala	His
Asn	Arg	Gln	Gln	Arg	Pro
Thr	Val	Gln	Gln	Gln	Arg
Thr	His	Gln	Gln	Arg	Tyr
Arg	Ile	Gln	Gln	Tyr	Ser
Ala	Ala	Gln	Gln	Arg	Ser
Met	Arg	Gln	Gln	Tyr	Arg
Ala	Val	Gln	Gln	Phe	His
Phe	Tyr	Gln	Gln	Ser	Leu
Ile	Pro	Gln	Gln	Met	Arg
Val	Lys	Gln	Gln	Lys	Arg
Arg	Tyr	Gln	Gln	Tyr	Gln
Gly	His	Gln	Gln	Phe	Arg
Pro	Gly	Gln	Gln	Ser	Phe
Arg	Ile	Gln	Gln	Gln	Arg
Ala	Val	Gln	Gln	Gly	Phe
Gln	Ser	Gln	Gln	Arg	Arg
Arg	Tyr	Gln	Gln	His	Arg
Val	Ser	Gln	Gln	Tyr	Arg
Ser	Tyr	Gln	Gln	Ala	His
His	Met	Gln	Gln	Pro	Arg

Pro	Gly	Gln	Gln	Ile	Phe
Gly	Pro	Gln	Gln	Ala	Arg
Pro	Phe	Gln	Gln	Gly	Phe
Arg	Ile	Gln	Gln	Ala	Thr
Gly	Arg	Gln	Gln	Arg	Thr
Phe	Val	Gln	Gln	Val	Arg
Met	Gln	Gln	Gln	Ser	Leu
Phe	Gly	Gln	Gln	Tyr	Ile
Glu	Ser	Gln	Gln	Phe	Tyr
Thr	Ser	Gln	Gln	Arg	Arg
Asn	Pro	Gln	Gln	Arg	Arg
Val	Asn	Gln	Gln	Ile	Arg
Ile	Ala	Gln	Gln	Ser	Arg
Arg	Thr	Gln	Gln	Thr	Arg
Ala	Arg	Gln	Gln	Ser	Arg
Asp	Ile	Gln	Gln	His	Arg
Gly	Pro	Gln	Gln	Gln	Phe
Pro	Phe	Gln	Gln	Pro	Ile
Ile	Arg	Gln	Gln	Arg	Phe
Val	Arg	Gln	Gln	Pro	Phe

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