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# Supplementary Information

## High-throughput production of a stable isotope-labeled peptide library for targeted proteomics using a wheat germ cell-free synthesis system

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## Supplementary Methods

Cell-free protein synthesis and purification

Artificial genes encoding self-designed QconCAT sequences (Table S3) were synthesized by commercial gene synthesis services (Biomatik USA, Wilmington, DE, USA). A split-primer PCR[30] was performed to produce DNA templates for *in vitro* transcription. The first PCR was performed using a 10-nM primer set consisting of the gene-specific sense primer (Table S6) and an antisense primer (5'- GCGGCCAACTTACTTCTGAC-3'). Subsequently, the template DNA for *in vitro* transcription was amplified by the next PCR using the second primer mix containing 100 nM of the SPu primer (5'-GCGTAGCATTTAGGTGACACT-3'), 1 nM of the deSP6E02 primer (5'-GGTGACACTATAGAACTCACCTATCTCTCTACACAAAACATTTCCCTACATACAACTTTCAACTTCCTATTATGGGCCATCACCATCACCATCATCTCGAGGCTGCACGTGGCTATTCCTTTACCACCACCGCCGAGAAG-3'), and 100 nM of the antisense primer.

Cell-free synthesis of His-tagged QconCAT proteins was performed using the bilayer reaction method with a wheat germ extract WEPRO8240H (CellFree Sciences, Matsuyama, Japan) as described previously[9,31]. After *in vitro* translation, QconCAT proteins were purified using a Ni-Sepharose High Performance resin (GE Healthcare, Pittsburgh, PA, USA). First, the synthesized protein solution (total 220 µL) was mixed with 400 µL of equilibration buffer (20 mM phosphate buffer, 0.3 M NaCl, 10 mM imidazole, 6 M urea, pH 7.5) and then incubated with 10 µL of resin (50% slurry) for 1 h at room temperature. The resin was washed twice with 400 µL of wash buffer (20 mM phosphate buffer, 0.3 M NaCl, 50 mM imidazole, 6 M urea, pH 7.5). The binding protein was eluted with 30 µL of elution buffer (20 mM phosphate buffer, 0.3 M NaCl, 500 mM imidazole, 6M urea, pH 7.5). Purified QconCAT solution (0.7 µL) was solubilized with 10 µL of the LDS lysis buffer containing 50 mM DTT and separated by NuPAGE Bis-Tris gels (Life Technologies, Carlsbad, CA, USA). After gel electrophoresis, protein bands were visualized with Bio-Safe CBB staining (Bio-Rad, Hercules, CA, USA).

In gel tryptic digestion of synthesized QconCAT proteins

Protein bands (~ 500 ng total protein) were excised from the gel, and each gel piece was destained with 50% (v/v) acetonitrile/100 mM $NH_4HCO_3$ (pH 8.9). Synthesized proteins in the gel pieces were reduced by soaking with 10 µL of 40 mM DTT solution for 1.5 h at 37$^{o}$C and were subsequently alkylated by soaking with 10 µL of 250 mM acrylamide solution for 30 min at room temperature. Individual gel pieces were dehydrated with 1 mL of acetonitrile and then rehydrated with 2 µL of 0.1 µg/µL sequencing-grade modified trypsin (Promega, Madison, WI, USA), followed by

incubation in 50 µL of 100 mM $NH_4HCO_3$ at 37ºC for 16 h. Each peptide was extracted from the gel piece three times with 50 µL of 50% (v/v) acetonitrile/5% (v/v) trifluoroacetic acid. Peptide samples were concentrated using a centrifugal vacuum concentrator, and then reconstituted with 10 µL of 0.1% (v/v) TFA solution. For MS/MS analysis, 1 µL of peptide solution was injected onto LC.

In-solution digestion of human serum proteins

Human serum samples were obtained from three healthy donors after obtaining informed consent in accordance with the procedures approved by the human ethics committee of the Ehime University. Major serum proteins were depleted using a MARS spin cartridges Human-14 (Agilent Technologies, Santa Clara, CA, USA). Immunodepleted serum samples (90 µg total protein) in 120 µL lysis buffer (8 M Urea in 150 mM Tris-HCl buffer, pH 8.8) were digested with Lys-C and trypsin as described previously.[32] After peptide purification with a C18 STAGE tip, the isolated peptides were completely dried using a vacuum centrifuge. The dried samples were resuspended in 180 µL of 0.1% TFA and dispensed into 1 µL aliquots. The obtained aliquots were mixed with an internal standard (1 µL) containing tryptic digests of SI-labeled QconCAT and subjected to SRM analysis.

Mass spectrometry (MS/MS and SRM)

All MS analyses were performed using a QTRAP 5500 hybrid triple quadrupole/linear ion trap MS (AB SCIEX, Framingham, MA, USA) coupled with an Eksigent nanoLC system via a cHiPLC-nanoflex module (AB SCIEX). The peptides were ionized by a nano-ESI source using PicoTip emitter with an internal diameter of 10 µm (New Objective, Woburn, MA, USA). The ion spray voltage was maintained at 2,300 V, and the positive ion mode was used for all experiments. The temperature of the interface heater was held at 150°C. For MS/MS analysis, we used information-dependent acquisition workflow. Each acquisition cycle started with an enhanced mass spectrum (EMS) scan, followed by an enhanced resolution scan to determine the ion charge states and enhanced product ion (EPI) scans for 1–3 of the most intense peaks (charge states: +2 to +4) detected from the EMS scan. The mass range was set to m/z 400–1,000 for EMS and m/z 100–1,000 for EPI. The scan rate was set to 10,000 Da/s for EMS and EPI. The LC parameters used for each MS analysis are summarized in Table S7.

MS/MS data analysis and protein identification were performed using the Analyst ver.1.5 and the ProteinPilot ver.4.0 (AB SCIEX), respectively. The database search was performed against the IPI human database version 20100624 using the following

parameters: sample type, SILAC (Lys +8, Arg +10); cys alkylation, acrylamide; digestion, trypsin; and search effort, rapid ID. The Skyline software[33] was used to build SRM assays for the targeted peptides. The MS/MS search results from the ProteinPilot program were used to generate a spectral library with a peptide probability cut-off of 0.9. Up to five of the most intense fragment ions (y or b ions) for doubly or triply charged precursor ions were selected based on library data, and a set of transitions with a dot product score >0.8 was then chosen for inclusion in the final assay. At least three transitions were selected per targeted peptide. The resolution at quadrupoles 1 and 3 was set to unit mass and the dwell time was set to 20 msec. We manually checked for the presence of interference in the selected transitions in the case of human serum analysis. The presence of interferences in each transition was evaluated on the basis of comparison of the SRM chromatograms for human serum and reference peptides. A set of at least three transitions with no apparent interference (dot product score > 0.8) was finally selected.

**References**


30. T. Sawasaki, T. Ogasawara, R. Morishita and Y. Endo, *Proc Natl Acad Sci U S A*, 2002, **99**, 14652-14657.
31. T. Sawasaki, Y. Hasegawa, M. Tsuchimochi, N. Kamura, T. Ogasawara, T. Kuroita and Y. Endo, *FEBS Lett*, 2002, **514**, 102-105.
32. N. Takemori, A. Takemori, J. Ishizaki and H. Hasegawa, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2014, **967**, 36-40.
33. B. MacLean, D. M. Tomazela, N. Shulman, M. Chambers, G. L. Finney, B. Frewen, R. Kern, D. L. Tabb, D. C. Liebler and M. J. MacCoss, *Bioinformatics*, 2010, **26**, 966-968.

**Figure S1.** A workflow diagram for the high-throughput synthesis of QconCATs. PCR amplification of cDNA encoding for a targeted QconCAT was performed using a split-primer PCR method.[30] PCR products were used as the templates for in vitro protein synthesis using WG-CFS. QT, a peptide tag sequence for the quantitation of synthesized QconCATs. His, his-tag sequence.

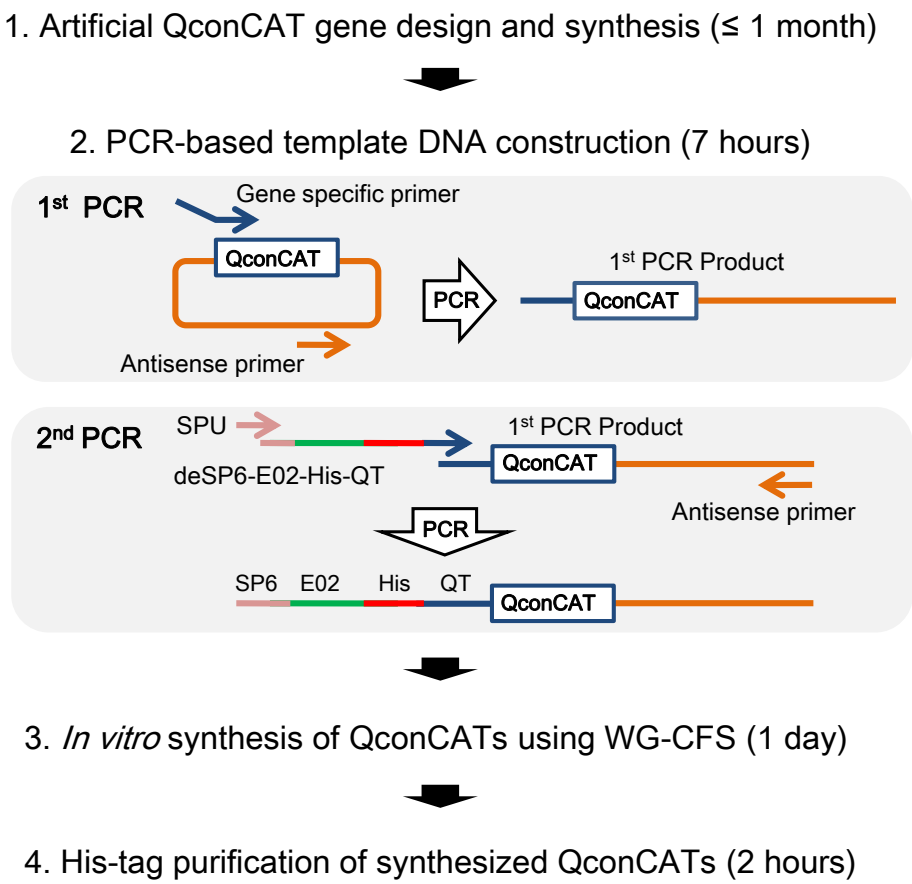
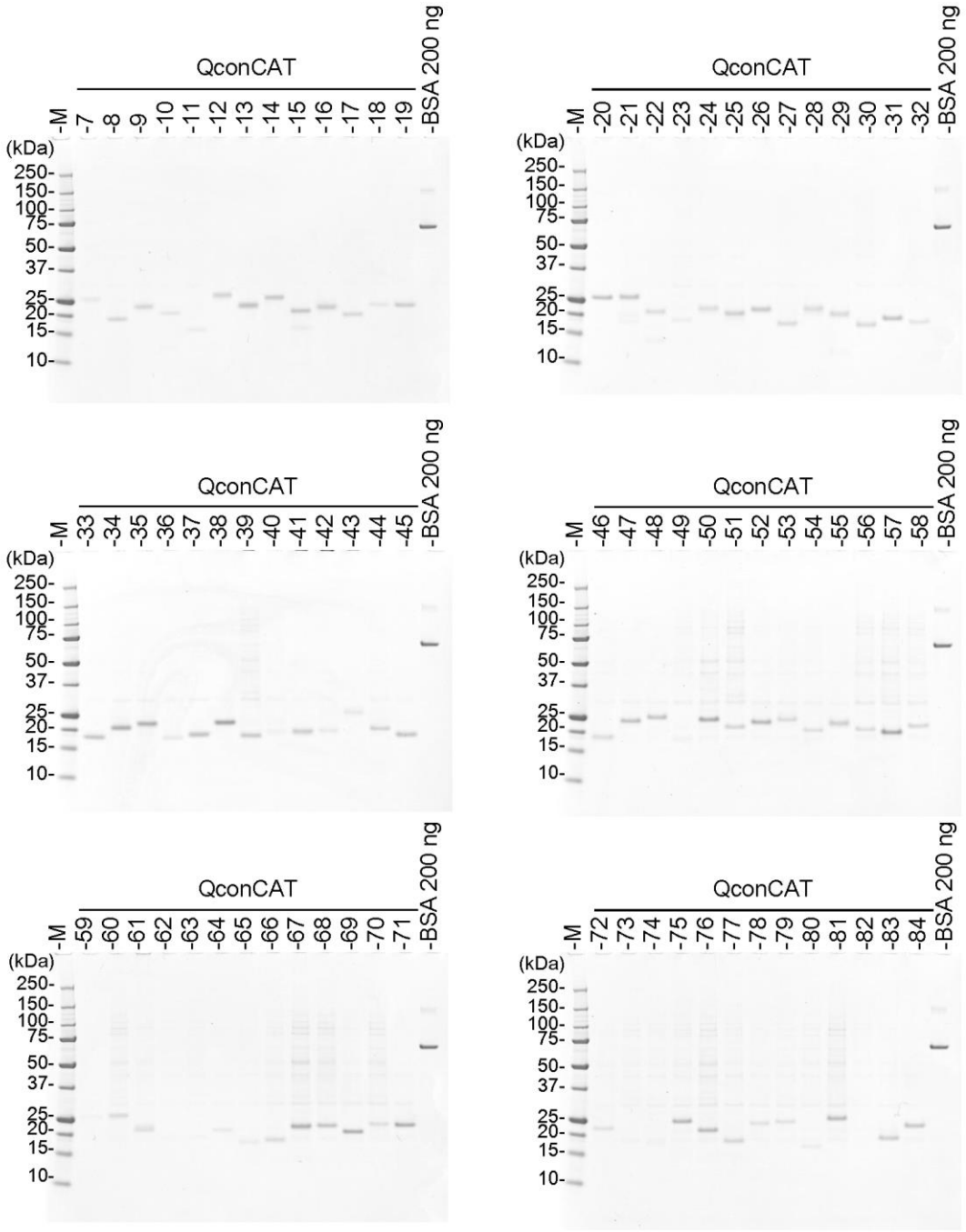
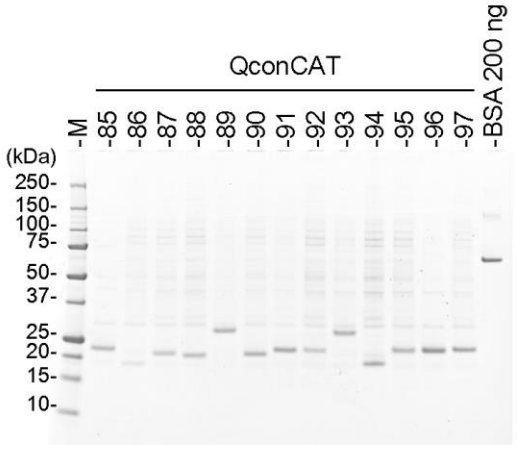


**Figure S2.** Representative SDS-PAGE images of synthesized QconCATs. Synthesized QconCATs (ID: 7~162) were separated on acrylamide gels (NuPAGE 4-12% Bis-Tris gel) and visualized with CBB. Lane M: Molecular weight marker (BioRad Precision Plus Protein™ Unstained standards). Lane number: ID numbers of QconCATs.

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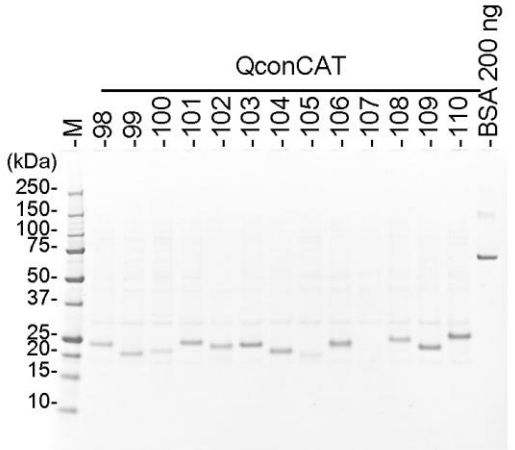
-M
-85
-86
-87
-88
-89
-90
-91
-92
-93
-94
-95
-96
-97
-BSA 200 ng
(kDa)
250-
150-
100-
75-
50-
37-
25-
20-
15-
10-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-98
-99
-100
-101
-102
-103
-104
-105
-106
-107
-108
-109
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(kDa)
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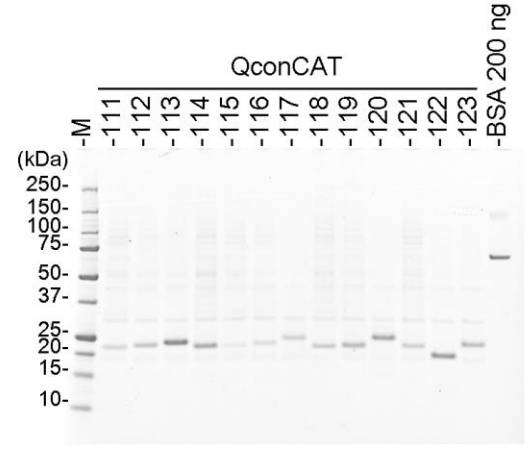
150-
100-
75-
50-
37-
25-
20-
15-
10-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-112
-113
-114
-115
-116
-117
-118
-119
-120
-121
-122
-123
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(kDa)
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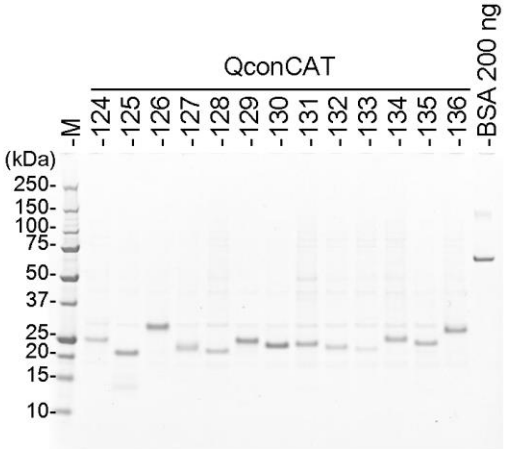
150-
100-
75-
50-
37-
25-
20-
15-
10-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-126
-127
-128
-129
-130
-131
-132
-133
-134
-135
-136
-BSA 200 ng
(kDa)
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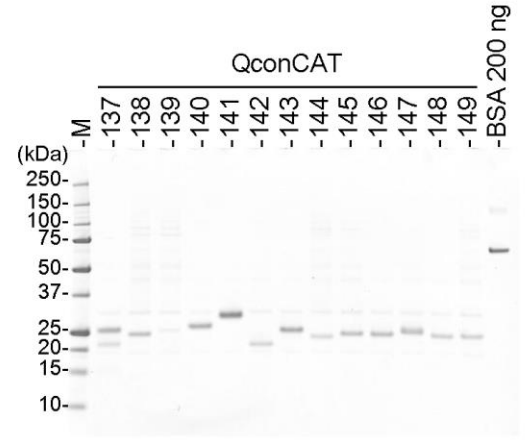
150-
100-
75-
50-
37-
25-
20-
15-
10-


QconCAT
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-137
-138
-139
-140
-141
-142
-143
-144
-145
-146
-147
-148
-149
-BSA 200 ng
(kDa)
250-
150-
100-
75-
50-
37-
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20-
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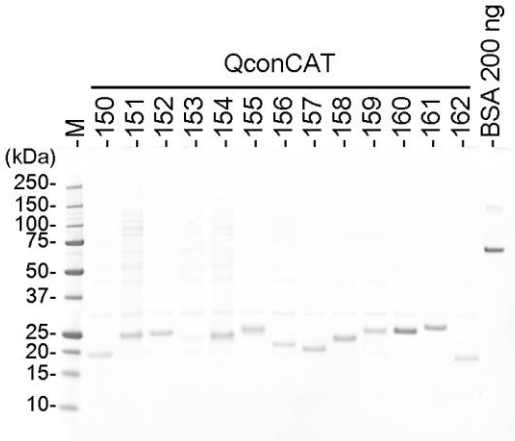
QconCAT
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-150
-151
-152
-153
-154
-155
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-157
-158
-159
-160
-161
-162
-BSA 200 ng
(kDa)
250-
150-
100-
75-
50-
37-
25-
20-
15-
10-

**Figure S3.** SDS-PAGE analysis of aggregated QconCAT proteins during *in vitro* translation. Aggregated QconCAT proteins were precipitated by centrifugation and solubilized with SDS lysis buffer. Resolubilized proteins were separated on a self-made 15% bis-acrylamide gel and visualized with CBB. Lane M: Molecular weight marker (BioRad Precision Plus Protein™ Unstained standards).