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1 **Versatile and multiplexed mass spectrometry-based absolute quantification with**
2 **cell-free-synthesized internal standard peptides**
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23 **Abstract**
24 Preparation of stable isotope-labeled internal standard peptides is crucial for mass
25 spectrometry (MS)-based targeted proteomics. Herein, we developed versatile and
26 multiplexed absolute protein quantification method using MS. A previously developed
27 method based on the cell-free peptide synthesis system, termed MS-based quantification
28 by isotope-labeled cell-free products (MS-QBiC), was improved for multiple peptide
29 synthesis in one-pot reaction. We pluralized the quantification tags used for the
30 quantification of synthesized peptides and thus, made it possible to use cell-free
31 synthesized isotope-labeled peptides as mixtures for the absolute quantification. The
32 improved multiplexed MS-QBiC method was proved to be applied to clarify ribosomal
33 proteins stoichiometry in the ribosomal subunit, one of the largest cellular complexes.
34 The study demonstrates that the developed method enables the preparation of several
35 dozens and even several hundreds of internal standard peptides within a few days for
36 quantification of multiple proteins with only a single-run of MS analysis.
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39 **Introduction**

40 In terms of both high sensitivity and accuracy, mass spectrometry (MS) is becoming one
41 of the most dominant approaches for protein identification and quantification. A key
42 technique for the protein quantification is the use of a stable isotope-labeled (SIL)
43 peptide as an internal standard. We cannot simply quantify the amount of peptides based
44 on their signal intensity values since the ionization efficiency of a peptide differs
45 according to their physical properties. The quantification is achieved by utilizing a SIL
46 peptide at a known concentration, with the identical molecular property to a target
47 peptide. The target peptide (a light peptide) can be quantified by comparing the signal
48 intensities of the target peptide with those of an internal standard peptide (a heavy
49 peptide).

50 Various methods are available for preparation of SIL peptides. Chemical labeling
51 such as dimethyl labeling [1] is a useful technique for comparative quantification by
52 labeling samples with both light and heavy tags. Another type of chemical labeling,
53 using isobaric tags, including TMT [2], iTRAQ [3], and mTRAQ [4], are commercially
54 available and exploited for a variety of multiplexed quantification [5].

55 Direct synthesis of SIL peptides is also common in the quantitative proteomics field.
56 Chemical synthesis termed AQUA [6] is the most intuitive approach for the absolute
57 quantification. Preparation of internal standards can be achieved in a manner with high
58 yield and high purity, without unnecessary additional sequences. The use of
59 non-canonical amino acids in the chemical reactions enables preparation of peptides
60 with post-translational modifications such as phosphorylation, methylation, acetylation,
61 and amidation. Application of a cellular protein synthesis system, such as SILAC [7, 8],
62 is suitable for the preparation of peptides or proteins that are difficult to be chemically
63 synthesized, which is an alternative way for the direct synthesis of the SIL peptides.
64 However, these approaches have some drawbacks such as high cost using expensive
65 labeled amino acids and the need to control its metabolism, which result in difficulties
66 in applying them to a large scale studies.

67 As an alternative and effective method for the preparation of internal standards, a
68 cell-free protein synthesis system has been getting popular in recent years [9]. The
69 history of the cell-free protein synthesis system is so long, dating back to a report in
70 1954 which showed that rat liver extract has an amino acid polymerization ability [10].
71 Presently, various cell-free systems are available, originating from *Escherichia coli*,
72 archaea, protozoan, yeast, wheat germ, tobacco, insects, and mammals [11]. Compared
73 to chemical or cellular synthesis methods, the cell-free system can be performed in

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74 micro-litter (pico-mol yield) scale, hence it is easy to minimize the use of expensive SIL
75 amino acids.
76 Various forms of cell-free systems have been proposed, of which the reconstituted
77 system, composed of only factors related to the translation and transcription systems,
78 has some features applicable to the quantitative proteomics studies. The system, termed
79 Protein synthesis Using Recombinant Elements (PURE) system [12], does not include
80 nucleases and therefore, short and linear DNA templates such as PCR-amplified
81 products could be utilized. The system also does not contain proteases nor metabolic
82 enzymes, and therefore, unexpected degradation of synthesized peptides and stable
83 isotope scrambling [13] could be avoidable. These features are valuable for ensuring
84 quality of synthesized internal standards and resultantly precise quantification.
85 By utilizing the PURE system, we have previously established a cost-effective
86 absolute protein quantification method, termed MS-based Quantification By
87 isotope-labeled Cell-free products (MS-QBiC) [14]. The method uses a unique design of
88 an internal standard peptide composed of a FLAG tag, a spacer peptide, a quantification
89 tag, and any target peptide, which we term the MS-QBiC peptide (**Fig. 1**). The sequence
90 of the quantification tag "LVTDLTK" is a tryptic peptide derived from bovine serum
91 albumin (BSA) that is easily available from the public resources. Therefore, it is easy to
92 estimate the amounts of MS-QBiC peptides by adding a known amount of BSA. After
93 tryptic digestion of mixtures containing a sample and the MS-QBiC peptide, the ratio of
94 light and heavy peptide is analyzed using MS. Based on the known amount of light
95 quantification tag, the amount of the target peptide derived from the sample can be
96 quantified. Since this quantification scheme is based on the single intermediary
97 quantification tag (LVTDLTK), there is a difficulty to apply the MS-QBiC method for
98 the multiplexed quantification. Such a drawback can be improved by increasing the
99 variation of the quantification tag.
100 In the present study, we improved the MS-QBiC method by designing new
101 quantification tags. First, we designed more than 100 candidates of LVXXLTK, where
102 X is variable amino acid, and picked up 34 tags which showed good solubility and
103 signal intensity on MS analyses. Then, we evaluated the new 34 tags and demonstrated
104 multiplexed MS-QBiC method using two samples. One is the commercially available
105 tryptic peptides mixture derived from six proteins in which the quality is guaranteed.
106 Another is the 30S ribosomal subunit from *Escherichia coli*, one of the largest cellular
107 complexes composed of one RNA (16S rRNA) and 21 ribosomal proteins. We
108 demonstrate that all quantified amount of proteins were within the same order of

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109 magnitude as the theoretical values, indicating usefulness of the developed method for
110 multiplexed quantitative proteomics studies.
111
112 **Results**
113 ***Selection of new quantification tags***
114 Based on the original quantification tag (LVTDLTK), we made a list of 110 candidates
115 of single and double point mutants, LVXXLTK, by replacing third Thr and fourth Asp
116 with 11 amino acids, including Ala, Asp, Glu, Phe, Gly, Leu, Pro, Ser, Thr, Val, and Tyr
117 (**Table S1**). We did not use other amino acids for variable reasons. Lys and Arg were
118 excluded to avoid internal tryptic digestion. Met, Cys, and Trp were not used because
119 they can be oxidized. Asn and Gln were amino acids potentially be deamidated. We also
120 avoided His that tends to show week MS intensity [15]. Ile was excluded because it is a
121 structural isomer of Leu.
122 Resultant 110 candidates were arranged in the order of m/z ($z = 2$) and the number
123 was reduced to 42 to keep proper intervals at least more than 0.2 m/z (**Table S1**). The
124 selection was performed to avoid extracted-ion chromatograms (XICs) become
125 complicated. We note that m/z of $^{13}C_6$ Lys labeled peptides (heavy peptides) were also
126 considered in this selection scheme. Next, the selected 42 candidates were chemically
127 synthesized by Fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis.
128 Hydrophobic peptides tended to be problematic, where some peptides, such as
129 LVLLLTK, LVLFLTK, and LVFFLTK, resulted in low yield and some peptides, such as
130 LVVLLTK, LVVFLTK, LVVYLTK, LVYLLTK, and LVFYLTK, resulted in insoluble
131 in water. After the selection based on the characteristics of the actually synthesized
132 peptides, we obtained 34 quantification tags (**Table S2**).
133 Using a series of chemically synthesized peptides, we prepared a quantification tag
134 mixture containing 100 μM each of 35 peptides including original LVTDLTK and
135 newly designed 34 tags. Concentration of each peptide was measured by amino group
136 determination [16]. According to the designed 34 tags, we also prepared 34 plasmids
137 encoding each new quantification tag by site-directed mutagenesis using the original
138 plasmid encoding LVTDLTK.
139
140 ***Multiplexed cell-free peptide synthesis and quantification***
141 To validate the newly designed tags, we tried to measure protein mixtures with known
142 concentration using the MS-QBiC method based on 34 quantification tags. As a protein
143 mixture, we used a commercially available equimolar tryptic peptide mixture from six
144 proteins including bovine cytochrome C (12 kDa), chicken lysozyme C (16 kDa), yeast

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145 alcohol dehydrogenase 1 (37 kDa), bovine serum albumin (69 kDa), bovine
146 serotransferrin (78 kDa), and *E. coli* beta-galactosidase (117 kDa) (Pierce 6 Protein
147 Digest, equimolar, Thermo Scientific). A total of 34 target peptides was selected for 6
148 proteins quantification (**Data S1** and **Table S3**). For the selection of the target peptides,
149 we focused on those composed of 6 to 20 amino acids without Met and Cys. Also, the
150 peptide sequences that may cause miss-cleavages, such as KP, RP, KK, and RR were
151 avoided. We note that the original quantification tag, LVTDLTK, was not used for this
152 study because it was derived from bovine serum albumin, a component of the target
153 protein mixture.

154 A mixture of 34 MS-QBiC peptides were synthesized in a single PURE system
155 reaction. DNA templates for each peptide synthesis were amplified in separate PCR
156 reactions, and then, all 34 templates were added to the PURE system and peptides were
157 synthesized in a one pot reaction. The resultant mixture of 34 MS-QBiC peptides was
158 mixed with chemically synthesized non-labeled quantification tags and six proteins
159 mixture (total 1 pmol, 167 fmol each) and then further processed for MS analysis
160 including tryptic digestion.

161 The yield of each MS-QBiC peptide was measured by LC-MS analysis using
162 Orbitrap mass spectrometer in full-scan mode (**Fig. 2a**). The measurement was based on
163 the light/heavy ratio of the quantification tags and it showed that all 34 MS-QBiC
164 peptides were successfully synthesized. The yields from 5 μl PURE reaction mixture
165 ranged between 11 and 126 fmol and the total amounts were 2 pmol. They were within
166 the range for the MS-based quantification, although some peptides, such as
167 YVVDTSK*, AWSVAR*, LVNELTEFAK*, YYGYTGAFR*, and LWSAEIPNLYR*,
168 showed low yield (asterisks represent labeled lysine or arginine). We note that
169 additional synthesis of peptides can compensate for the low yield of specific peptides.
170 When YVVDTSK*, LVNELTEFAK*, and LWSAEIPNLYR* were synthesized
171 separately, the yield from 1 μl of the PURE reaction were 200, 270, and 120 fmol,
172 respectively. Therefore, supplementation of the separately synthesized peptides can be
173 performed if necessary.

174 Subsequently, target peptides in a six proteins mixture were quantified based on the
175 light/heavy ratio of the target peptides by LC-MS analysis (**Fig. 2b**, red). We also
176 performed selected reaction monitoring (SRM) using triple quadrupole mass
177 spectrometer and found that the quantification results were almost similar with those
178 with LC-MS analysis (**Fig. 2b**, orange). Further validation was performed by shuffling
179 the combination of the quantification tags and target peptides by reversing the order of
180 the quantification tags (**Table S4**) and target peptides were quantified with both LC-MS

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181 and SRM. We found that the quantification results did not vary according to the used
182 quantification tags (**Fig. 2b**, blue and green).
183 It was possible that quantitative values of 34 target peptides can vary due to various
184 factors such as miss-cleavage, non-specific modification, hydrophobicity, and
185 detectability. Detailed analysis of the ion chromatograms showed the miss-cleavage
186 product of YIPGTK (**Fig. S1a**). Also deamidated products originated from NYQEAK
187 and QQDDFGK were found (**Fig. S1b, c**). NYQEAK also showed early retention time,
188 suggesting it is very hydrophilic and not sufficiently retained by the column (**Fig. S1b**).
189 As a result, these peptides were quantified at relatively low values (**Fig. 2b**). After
190 excluding the values of these three peptides, average of quantification values of
191 cytochrome C, lysozyme C, alcohol dehydrogenase 1, serum albumin, serotransferrin,
192 and beta-galactosidase were 62, 102, 103, 113, 95, and 86 fmol, respectively, which
193 were within the same order of magnitude as the amounts of added proteins (167 fmol).
194
195 ***Quantification of ribosomal 30S subunit***
196 Further verification of the developed method was performed by analyzing the ribosomal
197 proteins stoichiometry in *E. coli* ribosomal 30S subunit composed of 21 ribosomal
198 proteins. Quantifying ribosome composition is crucial for understanding its biogenesis
199 [17, 18]. Also it might be important because the ribosome stoichiometry is suggested to
200 be variable depending on tissue types, physiological conditions, and aging, which might
201 cause phenotypic changes in eukaryotes [19-21].
202 A total of 68 peptides were designed (**Data S2** and **Table S5**) and two sets of the
203 PURE reaction were performed where 34 peptides were synthesized in each reaction.
204 Synthesized peptides were quantified with chemically synthesized non-labeled
205 quantification tags and then mixed as a solution for 30S subunit quantification
206 containing 68 MS-QBiC peptides.
207 *E. coli* ribosomal 30S subunit (100 fmol), in which the concentration was determined
208 with UV absorbance, was mixed with the cell-free synthesized peptide solution and
209 each peptide was quantified. As with the result of six proteins quantification, most of
210 the peptides were within the same order of magnitude as the amount of added 30S
211 subunit (**Fig. 3a**). Five peptides marked with asterisks (ISELSEGQIDTLR from uS13,
212 AIISDVNASDEDR from uS14, WNAVLK from uS14, GPFIDLHLLK from S19, and
213 ENEPFDVALR from S21) were less than 30% compared to the added 30S subunit
214 amount. Detailed analysis of the ion chromatograms again showed the miss-cleavage of
215 these peptides (**Fig. S2**), suggesting these peptides are not suitable for the quantification.
216 After excluding the values of these five peptides, protein amounts were calculated as the

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217 average of quantification values of individual peptides (**Fig. 3b**). The data showed that
218 all proteins were within the same orders of magnitude, suggesting equimolar amount of
219 proteins are included in the prepared 30S subunit.
220

221 **Discussion**

222 Mass spectrometric methods have outstanding advantages in sensitivity and accuracy
223 for protein quantification. However, a variety of preliminary surveys are required for
224 analyzing the samples of interest. In order to obtain an accurate quantification result, it
225 is indispensable to select appropriate target peptides, which are effectively ionized and
226 detectable on MS ("proteotypic") and quantitatively reliable ("quantotypic") [22, 23].
227 Quantotypic peptides are those that do not have chemical and/or post-translational
228 modification sites and also do not have sequences which are susceptible to incomplete
229 tryptic digestion. Generally, screening of quantotypic peptides is performed
230 computationally and experimentally that takes much time and effort [24, 25], and then,
231 their SIL counterparts are synthesized. When SRM method is applied, further practical
232 screening of reliable transitions of target peptides are necessary. There could be a case
233 that preparing all target peptides as a SIL form is difficult for financial reasons.
234 Furthermore, there is a problem that the practical screening is not always applicable
235 when acquiring standard proteins are difficult.

236 By using the approach presented here, it is possible to prepare internal standard
237 peptides without limiting the number of peptides to be synthesized, which may result in
238 more practical screening of quantotypic peptides. By increasing the variation of the
239 quantification tag, the throughput of both the internal peptides preparation and the
240 sample quantification can be highly improved because multiplexed quantification tags
241 make it possible to synthesize at most 35 peptides in one pot reaction. Preparation steps
242 including PCR amplification, cell-free peptide synthesis, FLAG affinity purification,
243 and tryptic digestion are finished only within two days.

244 Two quantification steps are required to quantify the samples of interest, in that
245 synthesized MS-QBiC peptides are quantified first and then the samples are quantified
246 with the MS-QBiC peptides as references. It is noteworthy that these multi-step
247 quantification can be completed by a single run of LC-MS analysis. We used the
248 orbitrap mass analyzer at full scan mode which covers whole information of target
249 peptides and miss-cleaved peptides. Target peaks are easily identified by contrasting
250 light peptides and heavy peptides eluting at the same retention time, not even need
251 MS/MS characterization. Kumar *et al.* called such an approach, MS western, originating
252 from a western blotting [26]. MS/MS-free method does not waste time acquiring MS2

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253 and therefore enables short and high-throughput analysis [27]. After processing the peak
254 data and calculation, some peptides show outliers due to miss-cleavages, noisy peaks, or
255 missing peaks. Those discordances can be corroborated by analyzing back the full scan
256 data and the outliers will be eliminated accordingly. Whole processes will be completed
257 within a weekday: Designing and acquisition of reverse primers encoding target
258 peptides (2 days including a shipping time), PCR amplification of DNA template for the
259 PURE system (3 hours, for the first time only), synthesis of SIL peptides using the
260 PURE system (2 hours including a purification time), tryptic digestion of samples and
261 MS-QBiC peptides (overnight), and MS analysis (a few hours per sample) (**Figure 4**).
262 By establishing new 34 quantification tags, now we can basically perform the 35-plex
263 quantification by a single run of MS analysis including both steps of quantification of
264 cell-free synthetic heavy peptides and quantification of target peptides in samples.
265 Furthermore, we showed that it is possible to increase the multiplexity by using multiple
266 sets of MS-QBiC peptides if the quantification of the MS-QBiC peptides and samples
267 are separated. For the quantification of the ribosomal proteins in 30S subunit, we
268 designed and prepared 68 peptides as two sets of 34 MS-QBiC peptides (**Table S6,**
269 **Data S2**). Then, in the first step, each set of MS-QBiC peptides was quantified with
270 chemically synthesized quantification tags. Next, two sets of 34 MS-QBiC peptides
271 were mixed and 68 MS-QBiC peptides mixture was obtained. Finally, the mixture
272 containing known amount of 68 MS-QBiC peptides was spiked into the sample and
273 analyzed to quantify the endogenous target peptides. Using this approach, increasing the
274 multiplexity is possible for quantification of the multi-protein complex such as the
275 ribosome as shown here and/or measuring a systems-level dynamics of biological
276 systems, *e.g.*, signal transduction networks.
277

278 **Materials and Methods**

279 **Preparation of plasmids encoding quantification tags**

280 Preparation of plasmid encoding an original quantification tag (LVTDLTK) was already
281 reported [14]. Briefly, a DNA sequence
282 (5′-ATGGACTACAAGGACGACGACGACAAGCTGCTGCTGCTGAAGCTGGTTA
283 CTGACCTGACTAAG-3′) that encodes tandemly arranged FLAG tag, spacer peptide,
284 and a quantification tag (MDYKDDDDK-LLLLK-LVTDLTK) was cloned into a
285 pURE1 vector (BioComber) containing a T7 promoter sequence and the
286 ribosome-binding site. Using the original plasmid as a template, 34 plasmids encoding
287 new quantification tag (LVXXLTK) were amplified using PfuUltra II Fusion HS DNA
288 Polymerase (Agilent Technologies) using appropriate DNA primers (**Table S7**). After

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289 amplification, restriction enzyme Dpn I (Takara Bio) was added to cut and remove the
290 original template plasmid at 37 °C overnight. Using the remaining PCR products, *E.*
291 *coli* JM109 competent cells (Toyobo) were transformed and cultured on LB agar plate
292 containing ampicillin at 37 °C overnight. Formed colonies were picked up and
293 re-cultured in LB medium containing ampicillin at 37 °C overnight. The resultant
294 plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen).
295
296 **Preparation of non-labeled peptides by chemical synthesis**
297 Non-labeled quantification tags were synthesized by Fluorenylmethyloxycarbonyl
298 (Fmoc) chemistry using a peptide synthesizer SyroWave (Biotage, Uppsala, Sweden).
299 Fmoc amino acids were purchased from Watanabe Chemical Industries and contained
300 the following side chain protecting groups: Asp(OtBu), Glu(OtBu), Ser(tBu), Thr(tBu),
301 and Tyr(tBu). Fmoc-Lys(Boc)-TrtA-PEG resin (loading 0.21 mmol/g),
302 1-[Bis(dimethylamino)methyliumyl]-1H-benzotriazole-3-oxide hexa fluorophosphate
303 (HBTU), and *N,N*-diisopropylethylamine (DIEA) were also obtained from Watanabe
304 Chemical Industries. Piperidine was purchased from Nacalai Tesque (Kyoto, Japan).
305 Fmoc deprotection was performed using 40% piperidine/DMF for 3 + 12 min. Coupling
306 was performed using Fmoc amino acids/HBTU/DIPEA (equivalents = 5:5:10) for 60
307 min. After the synthesis, the products were washed with dichloromethane and dried.
308 The peptides were cleaved from the resin with reagent K (82.5% TFA, 5% phenol, 5%
309 thioanisol, 5% H_2O, and 2.5% 1,2-ethanedithiol) for 3 h at room temperature. Cleaved
310 peptides were precipitated and washed with pre-cooled diethyl ether, and dried. The
311 product was purified by reverse phase high-performance liquid chromatography
312 (RP-HPLC) using a YMC-Pack Pro C18 column (10 × 150 mm; YMC, Kyoto, Japan)
313 with a linear gradient of acetonitrile containing 0.1% TFA with a flow rate of 1.0
314 ml/min. The elution was monitored at 220 nm. Collected peak fraction was evaporated
315 and lyophilized.
316 Lyophilized peptides were dissolved in 2% acetonitrile containing 0.1% TFA. The
317 concentrations were measured by an amino group determination method [16]. Then,
318 aliquot of them were mixed to make a stock mixture at a concentration of 100 µM and
319 stored at −80 °C until use.
320
321 **Preparation of heavy-labeled peptides by cell-free synthesis**
322 Using the plasmids encoding each quantification tag as templates, DNA templates for
323 MS-QBiC peptide synthesis were amplified by PCR using Taq DNA polymerase (NEB)
324 with a T7 promoter primer (5′-GGGCCTAATACGACTCACTATAG-3′) as a forward

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325 primer and appropriate reverse primers listed in **Table S4 and Table S6**. PCR product
326 mixtures (2.5 μL) were directly added to yield 50 μL PURE system reaction mixtures
327 [28] containing $^{13}C_6$ $^{15}N_4$ L-Arginine (R*) and $^{13}C_6$ L-Lysine (K*) (Thermo Scientific)
328 as a substitute for non-labeled L-Arginine and L-Lysine, respectively. The mixtures
329 were incubated at 37 °C for 60 min. The synthesized peptides were purified using 10 μL
330 slurry of anti-FLAG M2 Magnetic Beads (Sigma Aldrich) according to the
331 manufacturer's instruction. The peptides were eluted with 20 μL of 0.1% TFA and dried
332 with SpeedVac.
333
334 **Reduction, alkylation, and tryptic digestion**
335 Enzymatic digestion was performed basically according to a phase transfer surfactant
336 (PTS)-aided protocol [29]. MS-QBiC peptides or ribosomal 30S subunit, prepared
337 according to the previous report [18], were dissolved in 10 μl of PTS buffer (10 mM
338 sodium deoxycholate, 10 mM sodium N-lauroylsarcosinate, and 50 mM NH_4HCO_3),
339 reduced with 10 mM TCEP at 37 °C for 30 min, alkylated with 20 mM iodoacetamide at
340 37 °C for 30 min, and quenched with 20 mM L-cysteine residues. Digestion was
341 performed by adding 100 ng of trypsin (Thermo Scientific) and 100 ng of Lys-C
342 (Thermo Scientific) at 37 °C overnight. After the digestion, 1 μl of 10% TFA was added
343 to the sample to precipitate the detergents. After the sample was centrifuged at 15,000 ×
344 g for 5 min at 4 °C, supernatant was desalted by using self-prepared stage tips [30] and
345 dried with SpeedVac. The dried peptides were dissolved in 5% acetonitrile containing
346 0.1% TFA and stored at −80 °C until use. Commercially available tryptic peptides
347 mixture (Pierce 6 Protein Digest, equimolar, Thermo Scientific) was just dissolved in
348 H_2O at a final concentration of 1 pmol/μl and stored at −80 °C until use.
349
350 **Mass spectrometric analysis**
351 Two types of mass spectrometers were used. One was an Orbitrap mass spectrometer
352 (positive mode, scan range of 200–1,500 m/z, 60,000 FWHM resolution, LTQ Orbitrap
353 Velos Pro, Thermo Scientific), which was used for a high-resolution full-scan analysis.
354 The other was a triple quadrupole mass spectrometer (positive mode, Q1 and Q3
355 resolutions of 0.7 FWHM, a cycle time of 1 sec, a gas pressure of 1.0 mTorr, TSQ
356 Vantage, Thermo Scientific), which was used for SRM analyses. Both of them were
357 equipped with a nanospray ion source (Nanospray Flex, Thermo Scientific) and a
358 nano-LC system (UltiMate 3000, Thermo Scientific). Peptides were concentrated using
359 a trap column (0.075 × 20 mm, 3 μm, Acclaim PepMap 100 C18, Thermo Scientific)
360 and then separated using a nano capillary column (0.1 × 150 mm, 3 μm, C18, Nikkyo

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361 Technos) at a flow rate of 500 nL/min using two mobile phases A (0.1% formic acid)
362 and B (acetonitrile and 0.1% formic acid). For the analysis of tryptic six protein mix, we
363 used a gradient of 5% B for 5 min, 5–35% B in 65 min, 35–90% B in 1 min, and 90% B
364 in 4 min. For the analysis of tryptic ribosome 30S subunit, we used a gradient of 5% B
365 for 5 min, 5–45% B in 40 min, 45–90% B in 1 min, and 90% B in 4 min. Elution was
366 directly electrosprayed (2.2 kV) into the MS. Data analysis was performed using
367 Skyline (v4.2.0.18305) (MacCoss Lab Software) [31]. For the full scan analyses, peak
368 area was calculated with setting MS1 filter to a count of three (M, M+1, and M+2). For
369 the SRM analysis, firstly primary SRM transitions and collision energies (CE) was
370 calculated using Skyline. In principle, *m/z* values of doubly charged precursor ions and
371 *m/z* values of singly charged fragment ions which are greater than precursor ions were
372 used. Then, optimal SRM transitions were developed by LC-SRM analysis of tryptic
373 MS-QBiC peptide using primary SRM transitions and by screening of transitions with
374 strong intensities. A total of 226 SRM transitions (452 transitions for light and heavy
375 peptides) was obtained for 34 quantification tags and 34 target peptides (**Table S8**).
376

377 **CRediT authorship contribution statement**

378 Conceptualization: KM, KK, RN, and YS; Methodology: KM; Investigation: KM
379 Resources: KM, KK, RN, and MS; Writing – Original Draft: KM; Writing – review &
380 editing: YS; Visualization: KM and YS; Supervision: YS; Funding acquisition: KM and
381 YS.
382

383 **Declaration of Competing Interest**

384 The authors declare no conflict of interest.
385

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393

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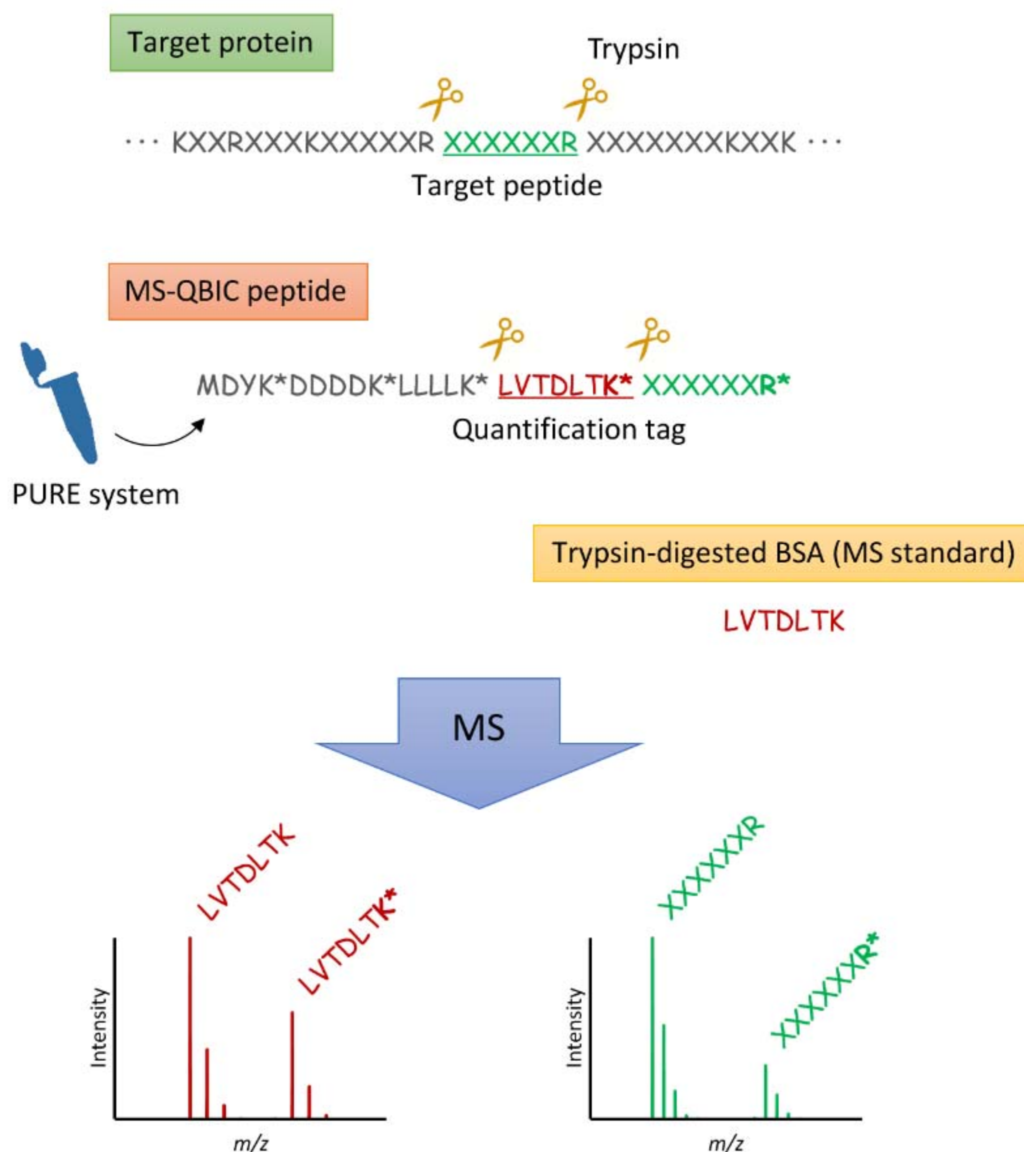
510 **Figures and legends**



511

512 **Fig. 1. MS-QBiC method.** Tryptic digestion of the MS-QBiC peptide, synthesized with
513 the PURE system, generates a quantification tag (red) and a target peptide (green)
514 containing stable isotope labeled arginine (R*) or lysine (K*). The target peptide
515 derived from a target protein by tryptic digestion is analyzed with MS by comparing
516 peak intensities of light and heavy peptides. The quantification tag is analyzed with MS
517 by comparing peak intensities of light (derived from BSA or chemically synthesized)
518 and heavy peptides to obtain the amount of synthesized MS-QBiC peptide.

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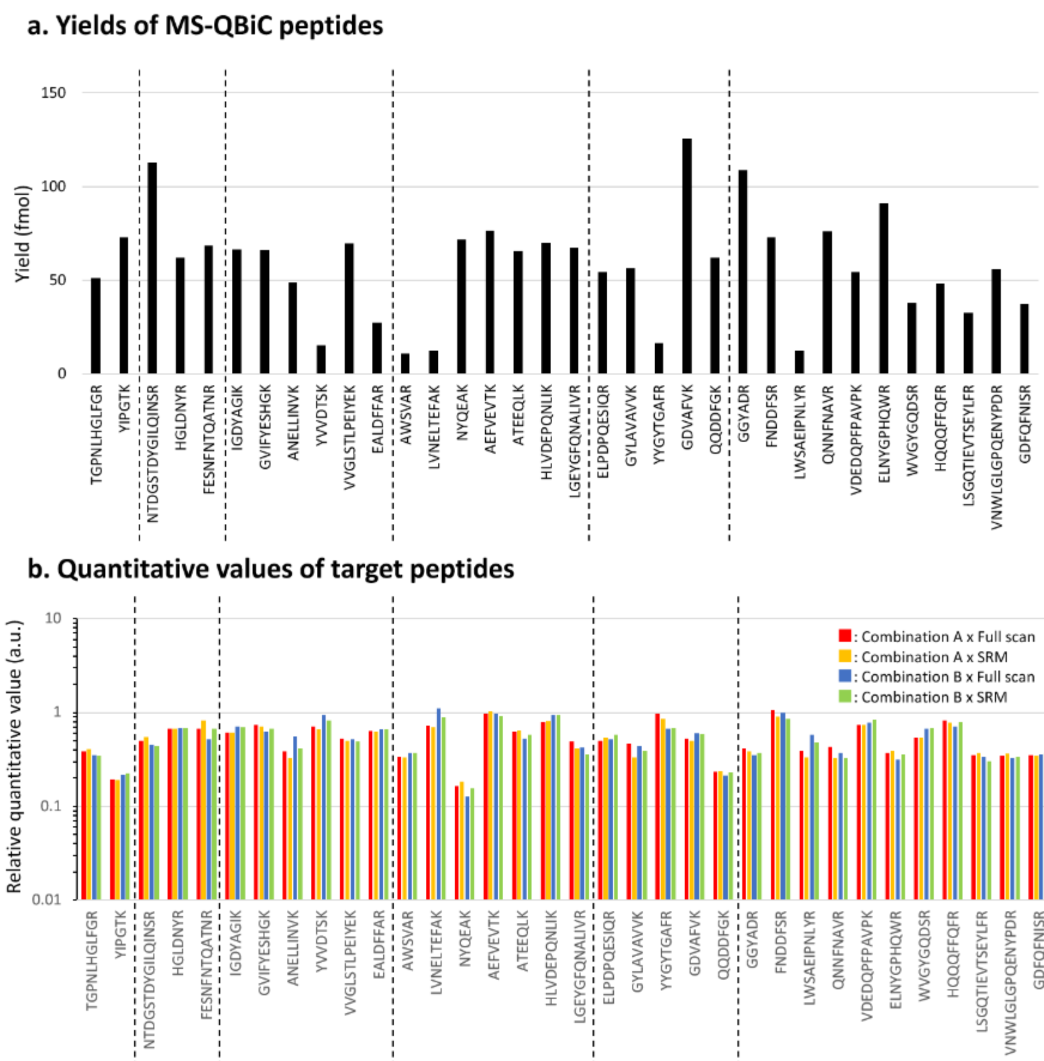

519

520 **Fig. 2. Multiplexed MS-QBiC method using newly designed 34 quantification tags.**
521 (a) Yields of MS-QBiC peptides. A total of 34 MS-QBiC peptides were synthesized in a
522 single PURE reaction and the yields of each peptide were quantified according to the
523 light/heavy ratio of the quantification tags. Yields from 5 μL PURE reaction mixture are
524 shown. (b) Quantitative values of target peptides. Ratios of the calculated quantitative
525 value to the input protein amount are shown as relative quantitative values. The results
526 obtained with LC-MS analysis (red and blue) and SRM analysis (yellow and green)
527 using MS-QBiC peptides in combination A (**Table S4**) (red and yellow) and those using
528 combination B (blue and green) are shown.
529

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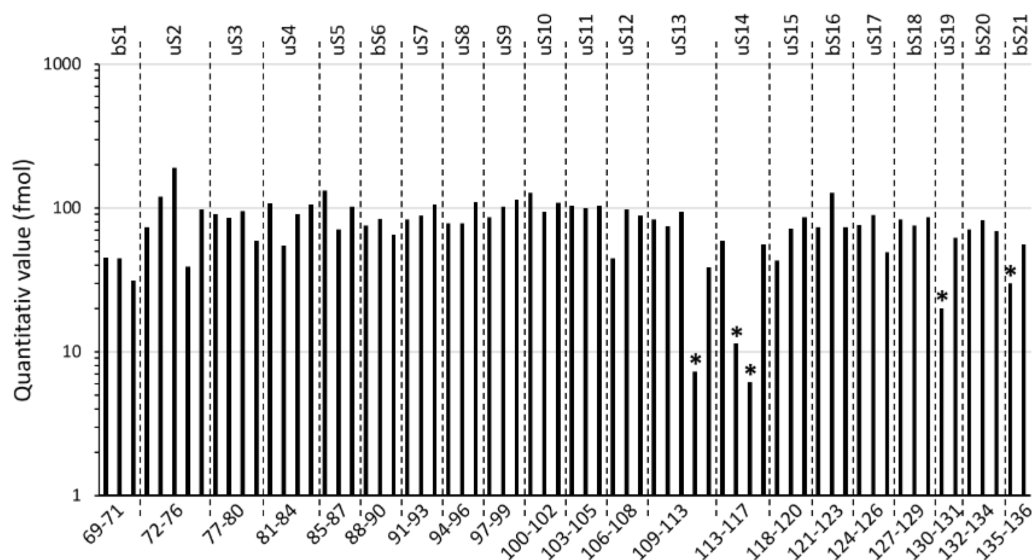

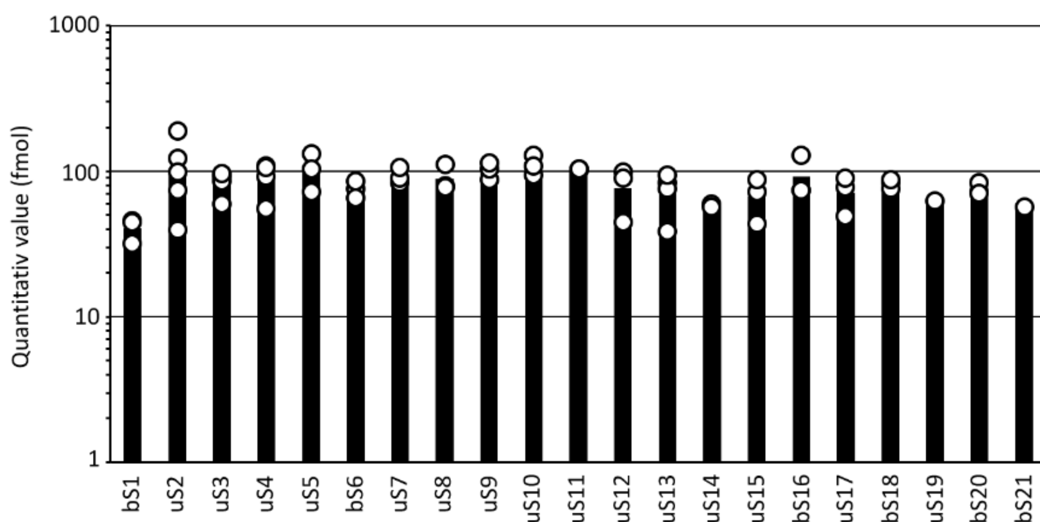

Fig. 3. Quantification of ribosomal 30S subunit. (a) Quantitative values of each peptide. Target peptides derived from 21 ribosomal proteins in 100 fmol ribosomal 30S subunit was quantified with 68 MS-QBiC peptides. Asterisks represent peptides less han 30% compared to the added 30S subunit. (b) Quantitative values of each protein. Protein amounts calculated as the average of quantitative values of individual peptides are shown. The peptides marked with asterisks in (a) were not included in the calculation because of the miss-cleavage issues. Each dot represents the quantitative value of individual peptides.

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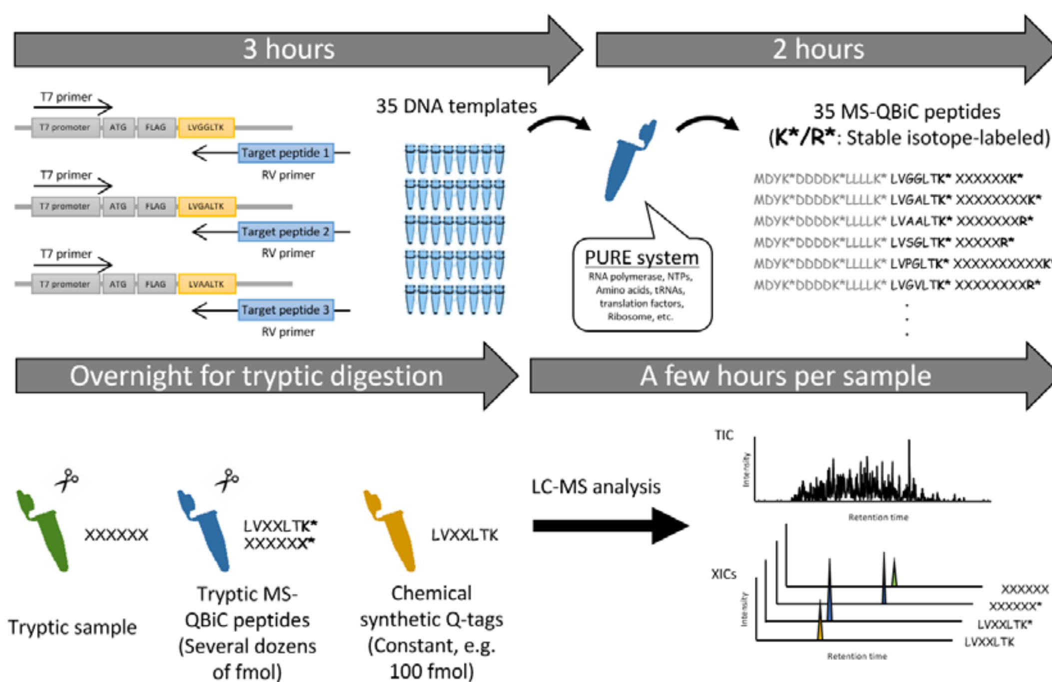

540

541 **Fig. 4. Workflow of multiplexed MS-QBiC method.** A total of 35 MS-QBiC peptides
542 can be obtained in 5 hours using only one PURE reaction mixture. After tryptic
543 digestion with sample proteins overnight, quantification results based on the LC-MS
544 analysis can be obtained in the next day.

545

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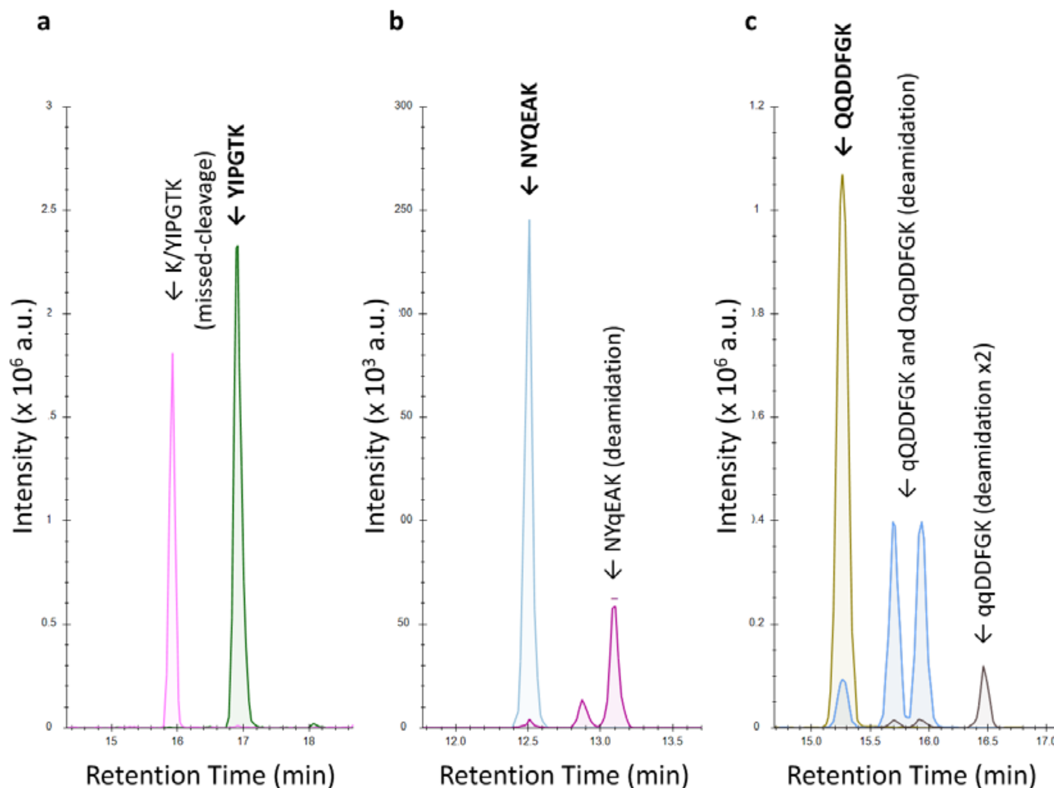
546 **Supplementary Figures**



547

548 **Fig. S1. Extracted ion chromatograms of peptides problematic for quantification of**
549 **six test proteins.** Extracted ion chromatograms of (a) KYPGTK and YPGTK, (b)
550 NYQEAK and NYqEAK (q represents deamidated glutamine), and (c) QQDDFGK,
551 qQDDFGK/QqDDFGK, and qqDDFGK are shown using Skyline software [31].

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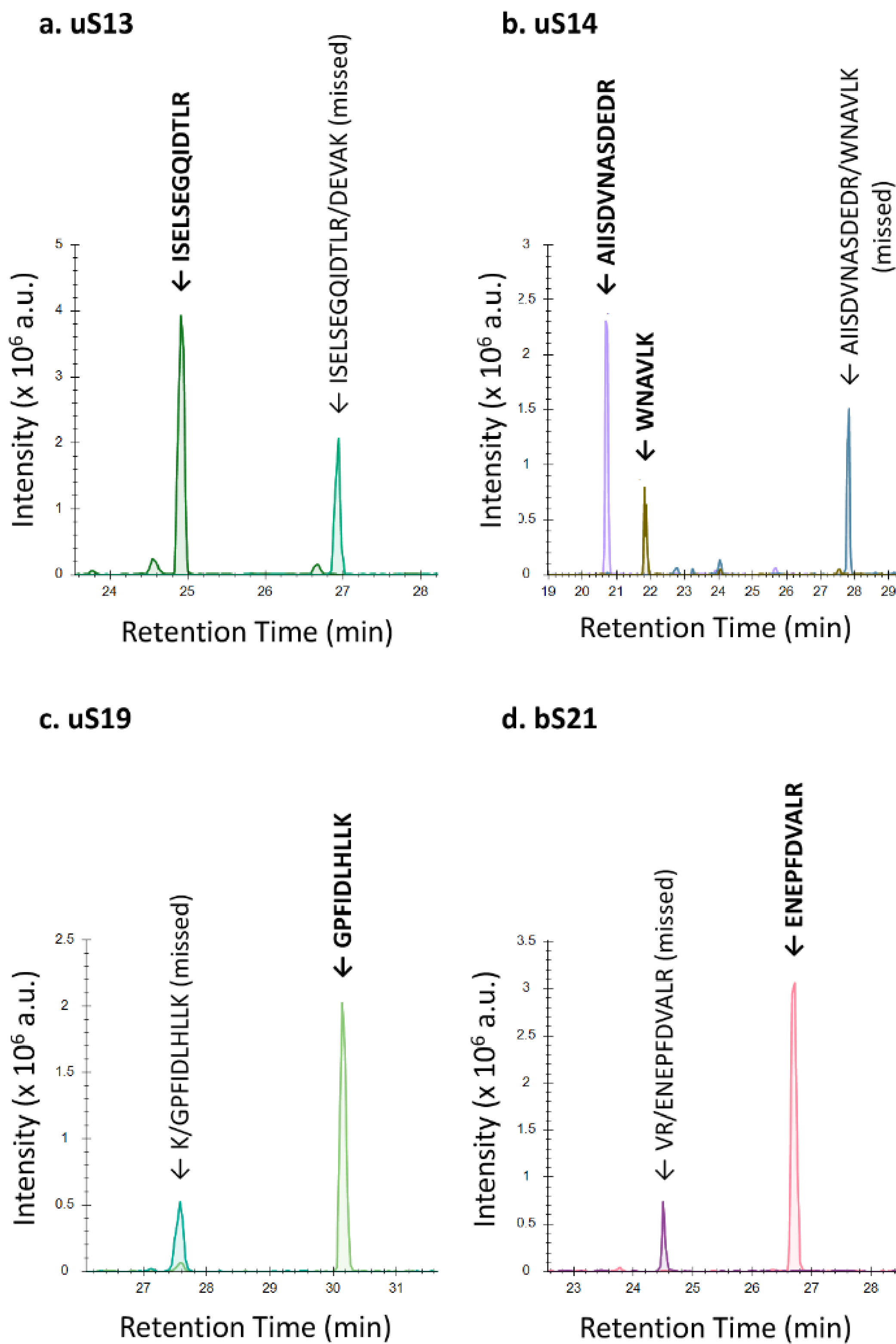

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554 **Fig. S2. Extracted ion chromatograms of peptides problematic for quantification of**
555 **ribosomal proteins.** Extracted ion chromatograms of (a) ISELSEGQIDTLR and
556 ISELSEGQIDTLRDEVAK from uS13, (b) AIISDVNASDEDR, WNAVLK, and
557 AIISDVNASDEDRWNAVLK from uS14, (c) KGPFIDLHLLK and GPFIDLHLLK
558 from uS19, and VRENEPFDVALR and ENEPFDVALR from bS21 are shown using
559 Skyline software [31].
560

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561 **Supplementary Tables**

562 **Table S1. A list of 110 candidates of single and double point mutants (LVXXLTK)**
563 **of original quantification tag (LVTDLTK).** A total of 110 candidates and the original
564 tag are listed in ascending order according to their *m/z*.
565
566 **Table S2. A list of 34 newly designed quantification tags.** A total of 34 tags are listed
567 in ascending order according to their *m/z*.
568
569 **Table S3. A list of target peptides for quantification of six test proteins.** Designed
570 target peptides for quantification of bovine cytochrome C, chicken lysozyme C, yeast
571 alcohol dehydrogenase 1, bovine serum albumin, bovine serotransferrin, and *E. coli*
572 beta-galactosidase included in Pierce 6 Protein Digest, equimolar purchased from
573 Thermo Scientific (**Data S1**) are listed.
574
575 **Table S4. A corresponding table of quantification tags and target peptides for six**
576 **test proteins.** Two combinations of quantification tags and target peptides, as well as
577 reverse primers to synthesize corresponding peptides in a cell-free reaction, are listed.
578
579 **Table S5. A list of target peptides for quantification of 21 ribosomal proteins.**
580 Designed target peptides for quantification of ribosomal proteins of *E. coli* ribosomal
581 30S subunit (bS1-bS21) (**Data S2**) are listed.
582
583 **Table S6. A corresponding table of quantification tags and target peptides for**
584 **ribosomal proteins.** A combination of quantification tags and target peptides, as well as
585 reverse primers to synthesize corresponding peptides in a cell-free reaction, are listed.
586
587 **Table S7. A list of primer sets for preparation of plasmids encoding newly designed**
588 **quantification tags.**
589
590 **Table S8. A list of SRM transitions of peptides analyzed in this study.**
591

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592 **Supplementary Data**

593 **Data S1. Amino acid sequences of six test proteins.** Amino acid sequences of bovine
594 cytochrome C, chicken lysozyme C, yeast alcohol dehydrogenase 1, bovine serum
595 albumin, bovine serotransferrin, and *E. coli* beta-galactosidase included in Pierce 6
596 Protein Digest, equimolar purchased from Thermo Scientific are shown. Target peptides
597 are shown with underlined bold characters.
598
599 **Data S2. Amino acid sequences of 21 ribosomal proteins.** Amino acid sequences of
600 ribosomal proteins of *E. coli* ribosomal 30S subunit (bS1-bS21) are shown. Target
601 peptides are shown with underlined bold characters.
602