

Supporting Information

Suppression of Formylation Provides an Alternative Approach to Vacant Codon Creation in Bacterial In Vitro Translation

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1.	GENERAL INFORMATION.....	2
2.	MODEL TEMPLATES.....	2
3.	AMINOACYLATION OF NON-CANONICAL AMINO ACIDS.....	3
4.	<i>IN VITRO</i> TRANSLATION AND UPLC-MS METHODS.....	4
5.	UPLC-MS RESULTS.....	7
6.	SYNTHESIS OF MOLECULES.....	28
7.	ACID PAGE FOR AMINOACYLATION TESTING	30
8.	FLUORESCENCE AND SILVER STAIN IMAGES OF SDS-PAGE GELS.....	31
9.	NMR SPECTRA	35
10.	SUPPLEMENTARY REFERENCES.....	36

1. General Information

Chemicals were purchased from Sigma Aldrich, Alfa Aesar and used without further purification. PURExpress (E6840S) was purchased from New England Biolabs.

UPLC-MS experiments were performed on an Agilent 6560 Ion Mobility Q-TOF LC/MS with an Agilent 1290 Infinity LC system. Experiments were performed using an Agilent Eclipse Plus C18 RRHD 1.8 μm , 2.1 X 50 mm column. A seven-minute time program was used with a 10-70% acetonitrile gradient. All the HPLC-grade solvents were purchased from Biosolve BV Netherlands. Gel pictures were taken on Amersham Imager 600 or Bio-Rad ChemiDoc™ Touch Gel Imager. Ac-Phe-CME,^[1] Biotin-Phe-CME,^[2] AMB-Phe-CME,^[3] and ClAc-Tyr-CME^[4] were prepared as previously reported.

2. Model templates

Peptide templates were prepared by multi-step assembly PCR using synthetic DNA primers (IDT europe, Belgium) and isolated by precipitation with 70% ethanol in 0.3 M NaCl, with final DNA and resulting peptide sequences detailed below. The linear DNA template for nanobody translation was prepared by two-step tailed PCR using the primers listed below and plasmid DNA as template. DNA products were quantified by A₂₆₀.

Template 1

Peptide sequence: MWSHPQFEKTEYEYLDYDFLPEMEPLGSGSGS

DNA sequence:

TAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGTGGAGCCAT
CCGCAGTTTGAGAAGACCGAATACGAATACCTGGATTACGATTTTCTGCCGAAATG
GAACCGCTGGGCAGCGGCAGCGGCAGCTAGGACGGGGGGCGGAAA

Template 2

Peptide sequence: MCGSGCSGAMSRYEVDWRGRGSAMG

DNA sequence:

TAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGTGCGGCAGC
GGCTGCAGCGGCGCGATGAGCCGCTATGAAGTGGATTGGCGCGGCCGCGGCAGC
GCGATGGGCTAGGACGGGGGGCGGAAA

Template 3

Peptide sequence: MTEYEYLDYDFLPEWEPLGAGAGA

DNA sequence:

TAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGACCGAATAC
GAATACTTAGATTACGATTTTTTACCTGAAUGGGAACCTTTAGGTGCAGGCCGAGGC
GCATAGGACGGGGGGCGGAAA

Primers for nanobody DNA preparation

Forward primer 1 (T7 promotor insertion)

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG

Forward primer 2

TTTAAGAAGGAGATATACATATGGAAGTTCAGCTGGTTGAATCTG

Reverse primer 1 (T7 terminator insertion)

TTTCCGCCCCCGTCTTAAGCTTCCGGTTCGTAATC

3. Aminoacylation of non-canonical amino acids

Testing acylation yield

Aminoacylation reactions (5 μ L) were carried out with the following standard conditions: A mixture of 3 μ L containing 125 pmol of μ helix and 125 pmol eFx in 0.25 M HEPES-KOH buffer (pH 7.5) was heated at 95 °C for 2 minutes and subsequently allowed to cool to room temperature for 5 minutes. Then, 1 μ L 3 M MgCl₂ solution was added, followed by an incubation at room temperature for another 5 minutes. The mixture was then cooled on ice, followed by the addition of 1 μ L 25 mM amino acid solution in DMSO. The reaction was left to incubate on ice for a varied amount of time (as detailed in the figures below). The reaction was quenched with 20 μ L 0.3 M NaOAc buffer (pH 5.2). This was followed by the addition of 50 μ L Ethanol to precipitate the product. This mixture was then centrifuged at 13000 rpm for 15 minutes to form a pellet of the precipitated product. The supernatant was removed and the pellet was washed with 30 μ L 70% EtOH, after which it was again centrifuged for 5 minutes at 13000 rpm. The supernatant was removed and the pellet was allowed to air dry for 5 minutes, after which the pellet was dissolved in 16 μ L RNA Loading buffer. All aminoacylation samples were loaded onto 20% acid PAGE gel and run for 150 minutes at 120 V in sodium acetate (pH 5.2) buffer. Gels were subsequently incubated with TBE and then stained with SYBR Green II (Thermo-Fisher, USA). Product ratios were determined by densitometry in ImageJ.

Aminoacylation of tRNA^{Met}

Aminoacylation reactions (5 μ L) were carried out with the following standard conditions: A mixture of 3 μ L of 125 pmol of tRNA^{Met} and 125 pmol eFx in 0.25 M HEPES-KOH buffer (pH 7.5) was heated at 95 °C for 2 min followed by an incubation at room temperature for 5 minutes. Then, 1 μ L MgCl₂ solution (3 M) was added and the mixture was left to incubate another 5 min at room temperature. The mixture was then cooled on ice, followed by the addition of 1 μ L amino acid solution in DMSO (25 mM). The reaction was left to incubate on ice for 2 hours. The reaction was quenched with 20 μ L NaOAc buffer (0.3 M, pH 5.2). This was followed by the addition of 50 μ L Ethanol to precipitate the product. This mixture was then centrifuged at 13000 rpm for 15 min to form a pellet of the precipitated product. The supernatant was removed and the pellet was vortexed with 40 μ L NaOAc buffer (0.1 M, pH 5.2) in 70% EtOH then centrifuged for 10 minutes at 13000 rpm, and this process was repeated for two washes. A final wash was then performed (without vortexing) using 30 μ L 70% ethanol in water and followed by a 3 min centrifugation at 13000 rpm. Following removal of the supernatant, the pellet was left to air dry for 5 minutes and stored at -20 °C.

4. *In vitro* translation and UPLC-MS methods

Translation using PURExpress Solution A Δ (aa, tRNA)

In vitro translation reactions were prepared on ice with the following composition:

- 20% (v/v) PURExpress solution A Δ (aa, tRNA)
- 30% (v/v) PURExpress solution B (NEB, USA)
- 1 $\mu\text{g}\cdot\mu\text{L}^{-1}$ E. coli tRNA
- 25 μM or 50 μM acylated tRNA^{fMet}
- 10 $\text{ng}\cdot\mu\text{L}^{-1}$ DNA template or plasmid
- 0.5 mM 20 amino acid mix (-Methionine, + Hpg (or Aha))
- 0, 100 or 250 μM Methotrexate

Volume was adjusted to a final volume of 5 μL with milliQ water and incubated at 37 °C for 30 minutes (or 1 hour in the case of some protein translations, as detailed below). Samples were prepared for analysis depending on the product.

For peptides: After translation, 3 volumes of acetonitrile were added and the sample was centrifuged at 13000 rpm for 5 minutes. Samples were analyzed using UPLC-MS.

For proteins: After translation, 5 μL glycine buffer (0.5 M, pH 10) was added and the solution was incubated for another hour to degrade remaining biotin-Phe tRNA. A Vivaspin 500 centrifugal concentrator (10 kDa) was then used to remove excess amino acids. DBCO-Sulfo-Cy3 (Jena Bioscience) was subsequently added to a final concentration of 100 μM and the solution incubated for 3 hours at 37 °C under mild denaturing conditions (4 M urea, 150 mM NaCl). The mixture was then incubated with Dynabeads M-280 Streptavidin beads (Invitrogen, USA) for 20 minutes, followed by two washes of the beads with PBS-T. Proteins were eluted off the beads by incubating at 95 °C for 5 minutes with SDS loading buffer. Samples were then analyzed by running on a SDS-PAGE gel, and where relevant bands were quantified by densitometry after silver staining using ImageJ and a BSA standard curve.

Translation using custom energy solution (SolAFD-)

In vitro translation reactions were prepared on ice with the following composition:

- 14% (vol%) SolAFD-^[5]
- 30% (vol%) PURExpress solution B (NEB, USA)
- 25 μM or 50 μM acylated tRNA^{fMet}
- 10 $\text{ng}\cdot\mu\text{L}^{-1}$ DNA template
- 0.5 mM 20 amino acid mix (-Methionine, + Hpg (or Aha))
- 0, 100 or 250 μM Methotrexate

Volume was adjusted to a final volume of 5 μL with milliQ water and incubated at 37 °C for

30 min. After translation, 3 volumes of acetonitrile were added and the sample was centrifuged at 13000 rpm for 5 minutes. Samples were analyzed using UPLC-MS.

Click reaction after translation (Based on 5 μ L translation volume)

A premixed solution of 0.5 μ L CuSO_4 (20 mM) and 1 μ L THPTA (50 mM) was prepared, which should change color to light blue immediately. This mixture was then added to the 5 μ L translation mixture, followed by addition of 1 μ L aminoguanidine hydrochloride solution (100 mM) and 1 μ L sodium ascorbate solution (100mM). The tube was then sealed and the reaction was allowed to incubate overnight at 37 °C. After the incubation 3 volumes of acetonitrile were added and the samples were centrifuged at 13000 rpm for 5 minutes. Samples were analyzed using UPLC-MS.

TCEP reduction

After the click reaction, 1 μ L TCEP solution (350 mM) was added and the solution incubated at 37 °C for 1 hour. After the incubation 3 volumes of acetonitrile were added and the samples were centrifuged at 13000rpm for 5 minutes. Samples were analyzed using UPLC-MS.

Mass extraction

Extracted ion chromatograms were generated by adding all product mass peaks identified for a given calculated mass from 2+ through 5+ ionization states (not all observed in all cases, depending on sequence) and including cation adducts where relevant (Na, K, NH_4) with a 20 ppm error range around the observed mass, summed across all isotopes with significant signal above baseline noise. Calculated and observed masses are for most abundant isotope peaks in all cases to avoid issues with low signal to noise for minor side product monoisotopic peaks. Peak areas were calculated using Agilent Masshunter.

Table S1. Summary of product ratios and reaction conditions for reprogrammed translation with various initiating amino acid and methionine analogue combinations.

Entry	Initiator ^[a]	Template	Conditions	AUG Elongator ^[a]	Fully re-programmed product	N-formyl methionine (analogue) initiated product	α -amino methionine (analogue) initiated product	initiator-truncated product	Main product peak area (normalized counts) ^[b]
1	Ac-Phe	1	SolA Δ (aa, tRNA) 25 μ M aa-tRNA ^{Met}	Hpg	69	31	0 ^[c]	0	249 552
2	Ac-Phe	1	SolAFD- 25 μ M aa-tRNA ^{Met}	Hpg	92	8	0	0	982 853
3	Ac-Phe	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Hpg	>99	0	0	0	365 599
4	Ac-Phe	1	SolA Δ (aa, tRNA) 100 μ M Methotrexate 25 μ M aa-tRNA ^{Met}	Hpg	83	17	0	0	314 535
5	Ac-Phe	1	SolA Δ (aa, tRNA) 250 μ M Methotrexate 50 μ M aa-tRNA ^{Met}	Hpg	93	7	0	0	326 663
6	-	3	SolAFD-	Hpg	0	25	63	12	111 990/ 2 762 220/ 54 120
7	-	3	SolAFD- 50 μ M tRNA ^{Met}	Hpg	0	0	0	>99	261 492
8	Ac-Phe	2	SolAFD- 50 μ M aa-tRNA ^{Met}	Aha	>99	0	0	0	241 186
9	ClAc-Tyr	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Hpg	>99	0	0	0	272 852
10	ClAc-Tyr	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Aha	>99	0	0	0	504 470
11	AMB-Phe	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Hpg	77	4	15	5	517 738
12	AMB-Phe	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Aha	65	5	14	16	144 270
13	(5/6)FAM-Phe	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Hpg	78	4	9	9	265 586
14	(5/6)FAM-Phe	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Aha	72	7	11	10	340 194
15	Biotin-Phe	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Hpg	>99	0	0	0	492 636
16	Biotin-Phe	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Aha	>99	0	0	0	688 105
17	Ac-Phe	1	SolAFD- 50 μ M aa-tRNA ^{Met}	Met	>99	0	0	0	73 264
18	Pyn-Phe	1	SolAFD- 50 μ M aa-tRNA ^{Met} then Cu ⁺ click	Aha	>99	0	0	0	269 960

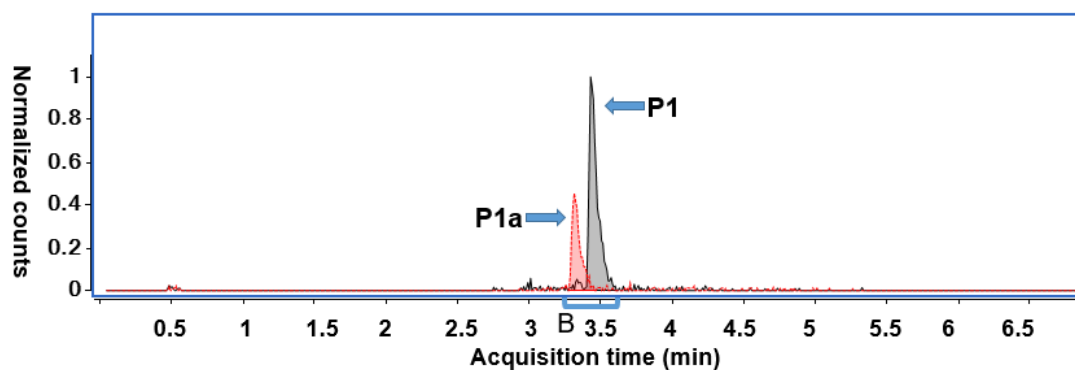
[a] Aha: azidohomoalanine, Hpg: homopropargylglycine, AMB: 4-aminomethylbenzoic acid, ClAc: chloroacetyl, FAM: carboxyfluorescein, Pyn: 4-pentynoic acid.

[b] Area under peak without reprogramming (template 1); 370 546.

[c] none detected.

5. UPLC-MS results

A.



B.

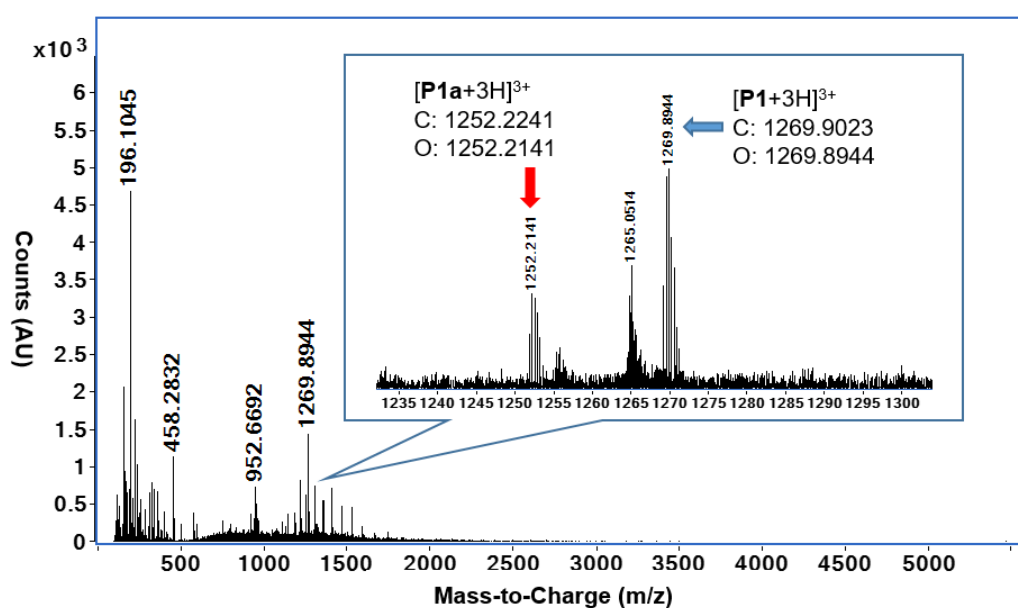


Figure S1. The EIC and mass spectrum for Table S1, entry 1: translation of peptide sequence 'MWSHPQFEKTEYEYLDYDFLPEMEPLGSGSGS' using NEB SolIA Δ (aa, tRNA) supplemented with 25 μ M Ac-Phe-tRNA^{fMet} (initiation) and homopropargylglycine (elongation). A. The EIC for **P1** and **P1a**. B. The mass spectrum (3.27-3.59 min, 35 scans) of **P1** and **P1a**

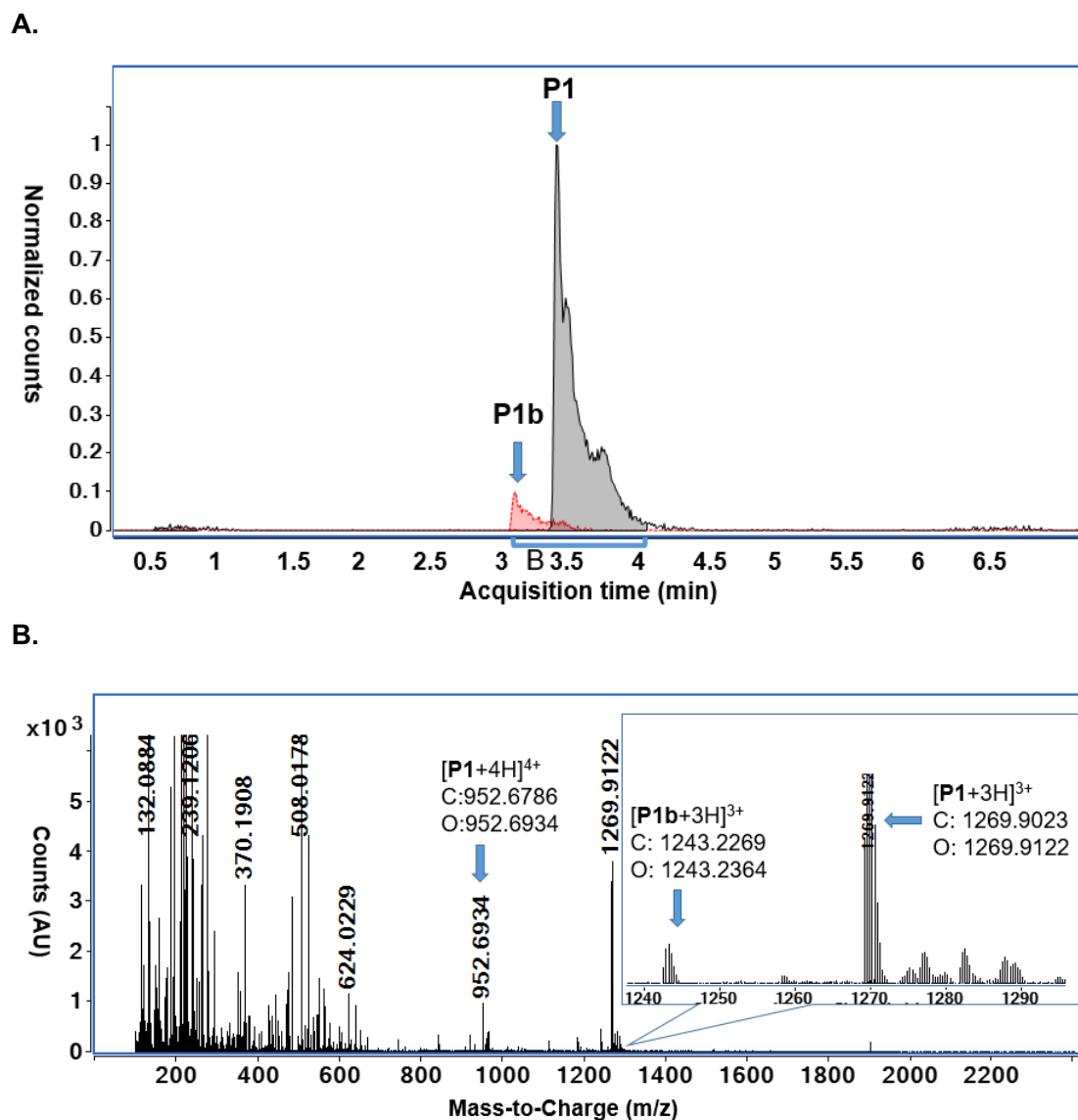
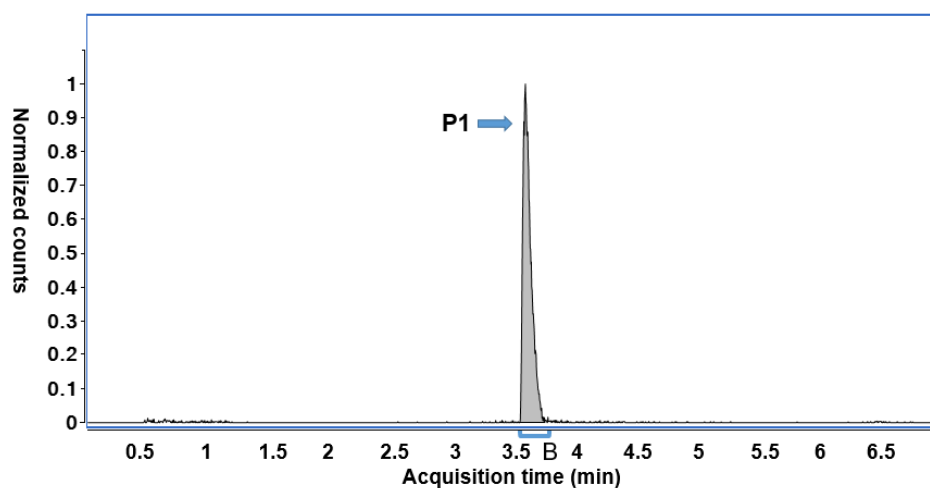


Figure S2. The EIC and mass spectrum for Table S1, entry 2: translation of peptide sequence 'MWSHPQFEKTEYEYLDYDFLPEMEPLGSGSGS' using SolAFD-supplemented with 25 μ M Ac-Phe-tRNA^{fMet} (initiation) and homopropargylglycine (elongation). A. The EIC for **P1** and **P1b**. B. The mass spectrum (2.87-3.82 min, 101 scans) of **P1** and **P1b**

A.



B.

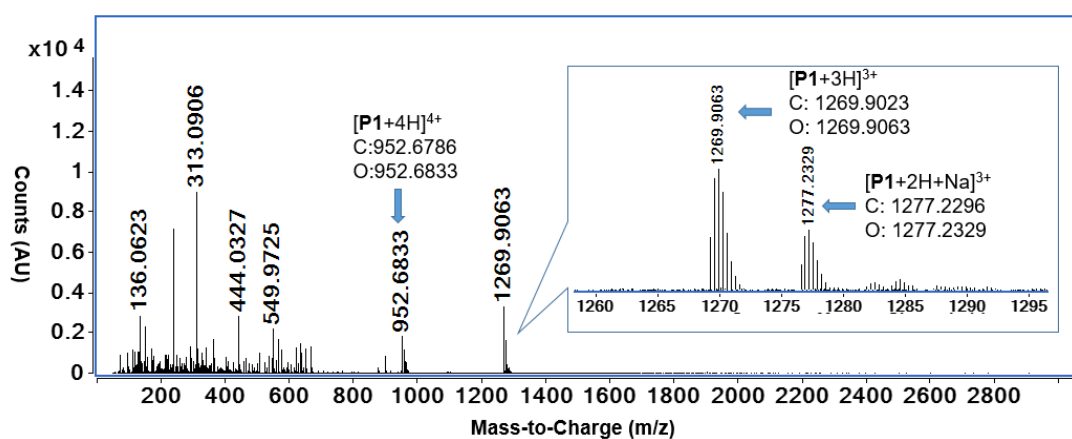


Figure S3. The EIC and mass spectrum for Table S1, entry 3: translation of peptide sequence 'MWSHPQFEKTEYELYLDYDFLPEMEPLGSGSGS' using SolAFD-supplemented with 50 μ M Ac-Phe-tRNA^{fMet} (initiation) and homopropargylglycine (elongation). A. The EIC for P1. B. The mass spectrum (3.50-3.81 min, 34 scans) of P1.

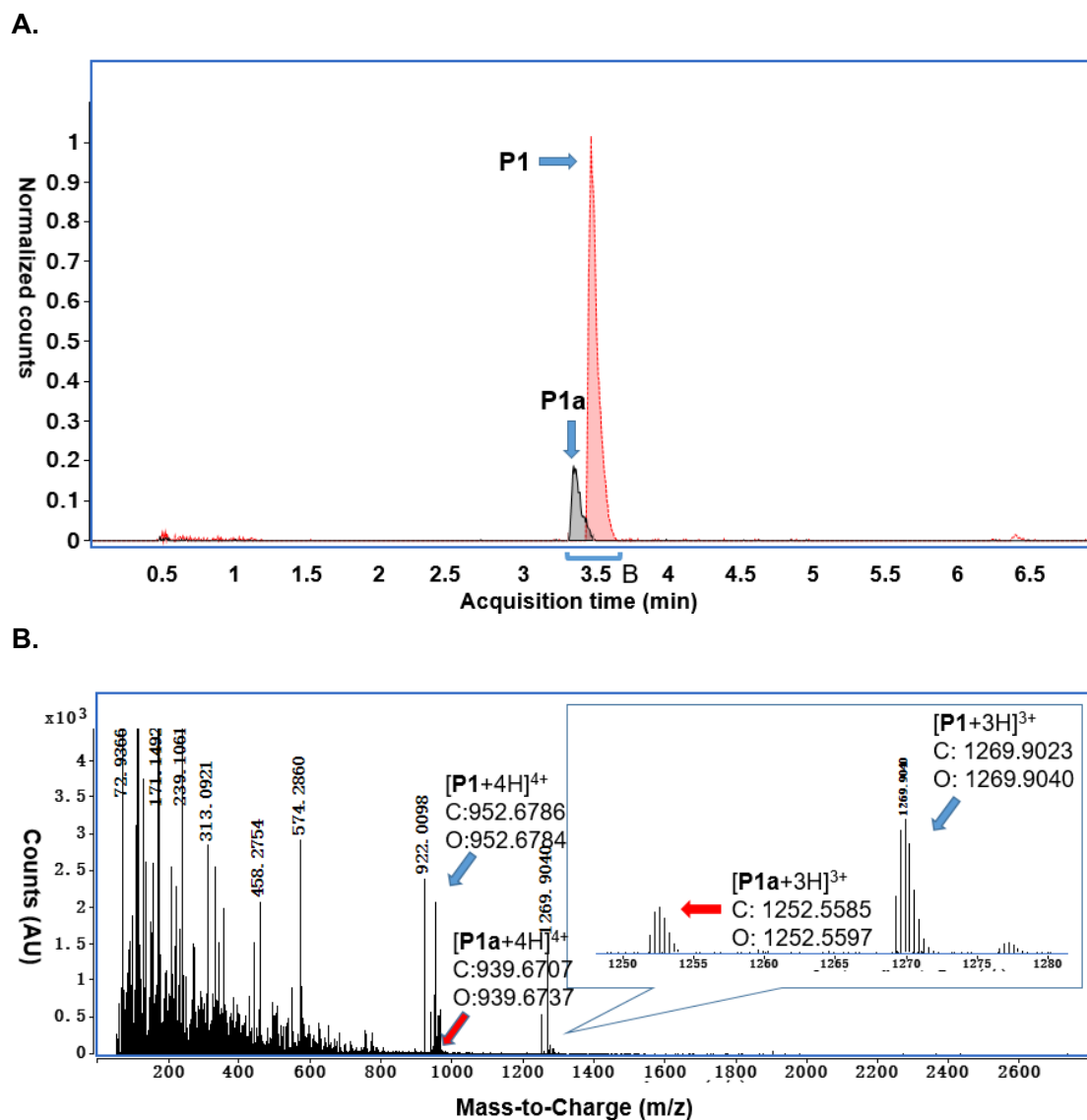


Figure S4. The EIC and mass spectrum for Table S1, entry 4: translation of peptide sequence 'MWSHPQFEKTEYEYLDYDFLPEMEPLGSGSGS' using NEB SolIA Δ (aa, tRNA) supplemented with 25 μ M Ac-Phe-tRNA^{Met} (initiation), 100 μ M Methotrexate and homopropargylglycine (elongation). A. The EIC for Peptide **P1** and **P1a**. B. The mass spectrum (3.34-3.68 min, 57 scans) of peptide.

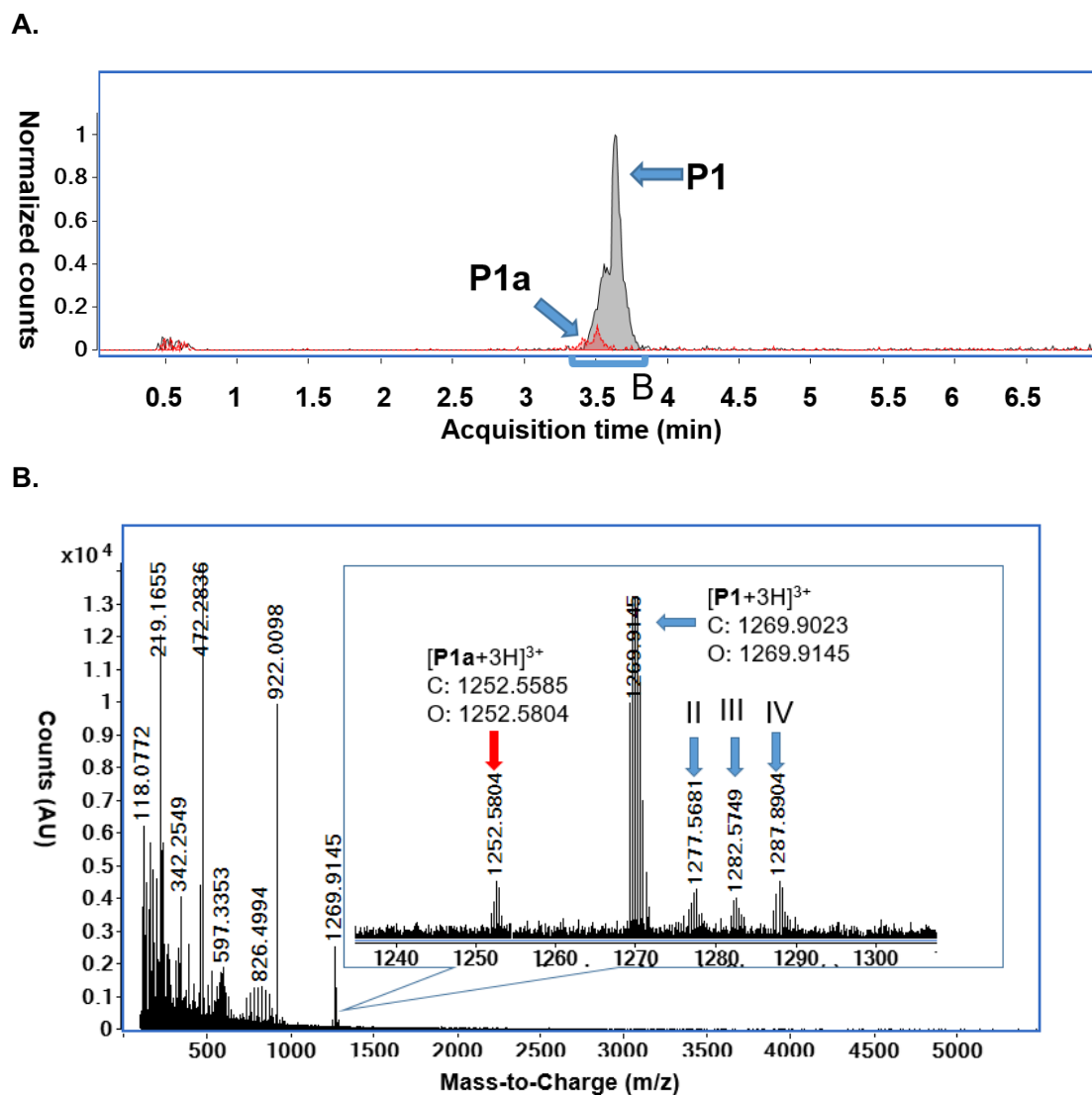
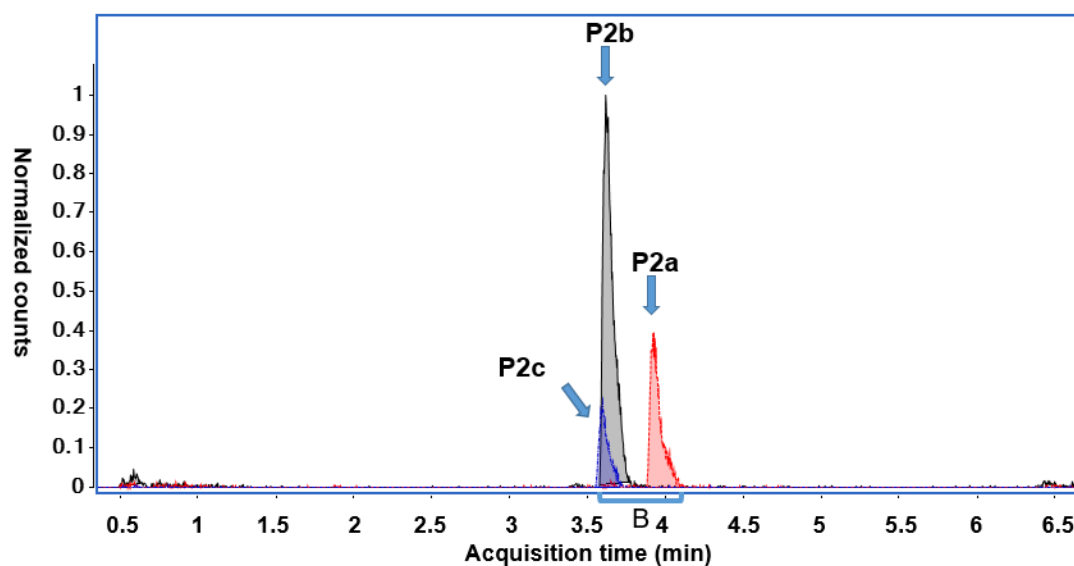


Figure S5. The EIC and mass spectrum for Table S1, entry 5: translation of peptide sequence 'MWSHPQFEKTEYEYLDYDFLPEMEPLGSGSGS' using NEB *SoIA* Δ (aa, tRNA) supplemented with 50 μ M Ac-Phe-tRNA^{fMet} (initiation), 250 μ M Methotrexate and homopropargylglycine (elongation). A. The EIC for Peptide **P1** and **P1a**. B. The mass spectrum (3.43-3.85 min, 45 scans) of peptide. II, [P1+2H+Na]³⁺. C:1277.5640. O: 1277.5681. III, [P1+2H+K]³⁺. C:1282.5543. O: 1282.5749. IV, [P1+H+NH₄+K]³⁺. C:1287.8953 O: 1287.8904.

A.



B.

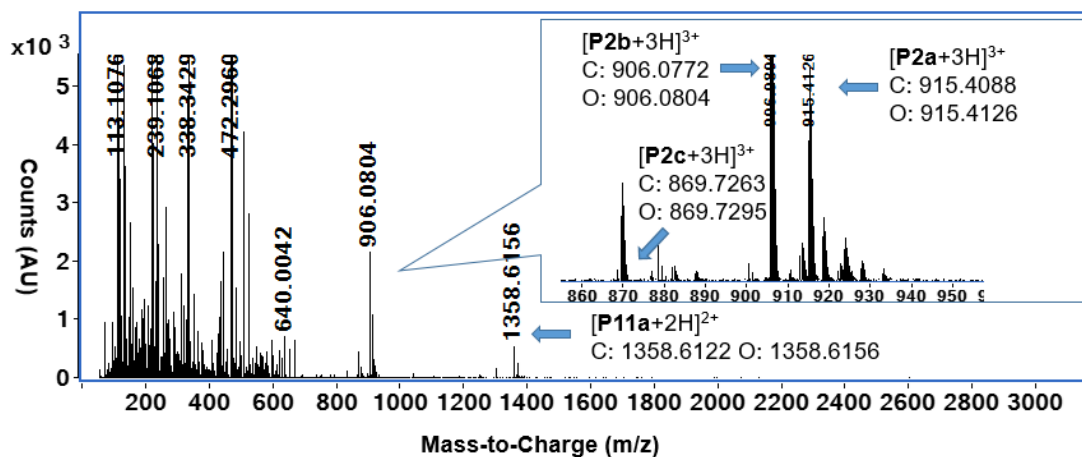


Figure S6. The EIC and mass spectrum for Table S1, entry 6: translation of peptide sequence 'MTEYEYLDYDFLPEWEPLGAGAGA' using SolAFD- supplemented with homopropargylglycine (initiation and elongation). A. The EIC for Peptide and byproduct. B. The mass spectrum (3.56-4.07 min, 157 scans) of peptide.

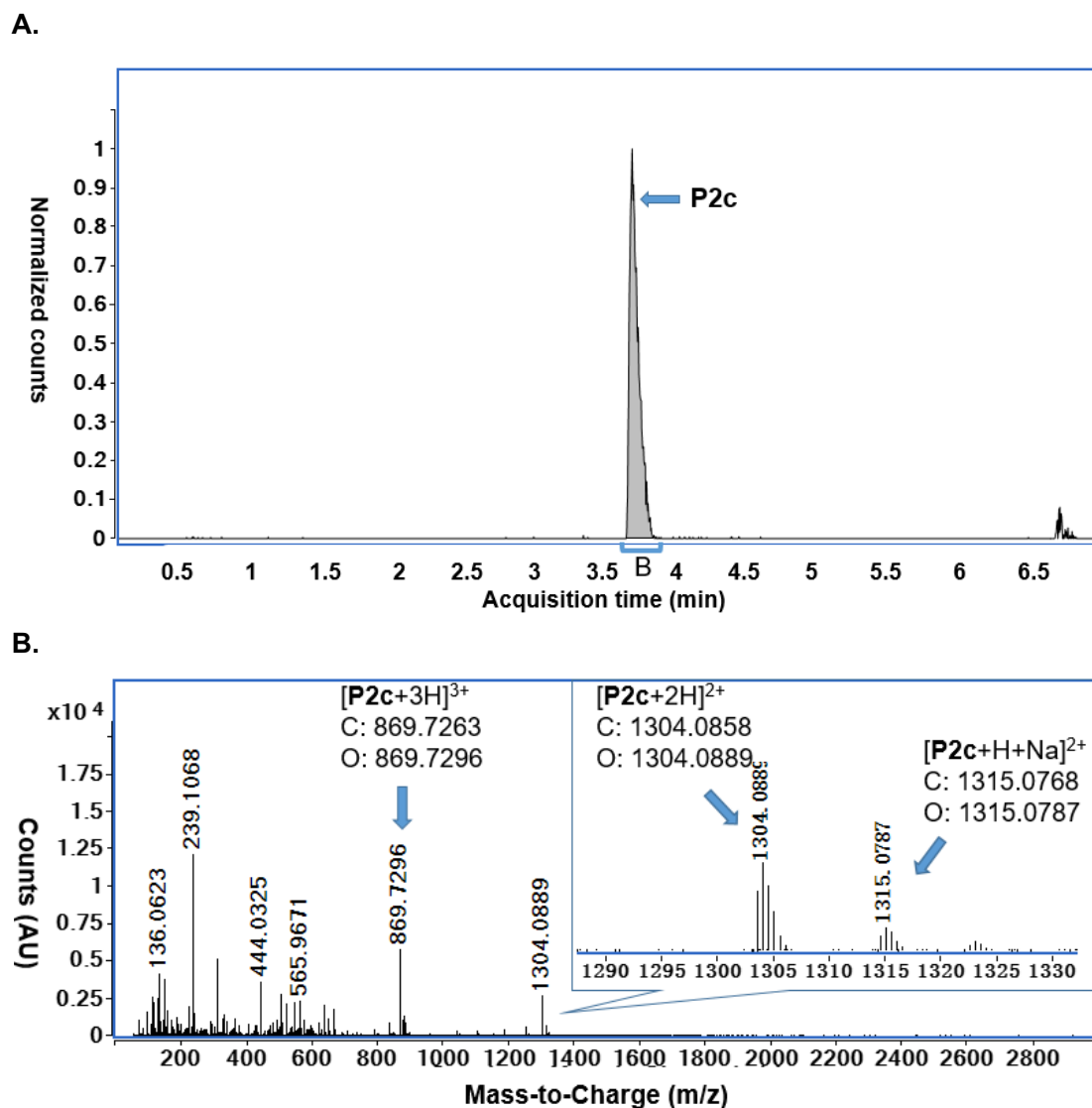


Figure S7. The EIC and mass spectrum for Table S1, entry 7: translation of peptide sequence 'MTEY EYLDYDFLPEWEPLGAGAGA' using SolAFD- supplemented with 50 μ M tRNA^{fMet} (uncharged initiator) and homopropargylglycine (elongation). A. The EIC for Peptide and byproduct. B. The mass spectrum (3.65-3.84 min, 62 scans) of peptide.

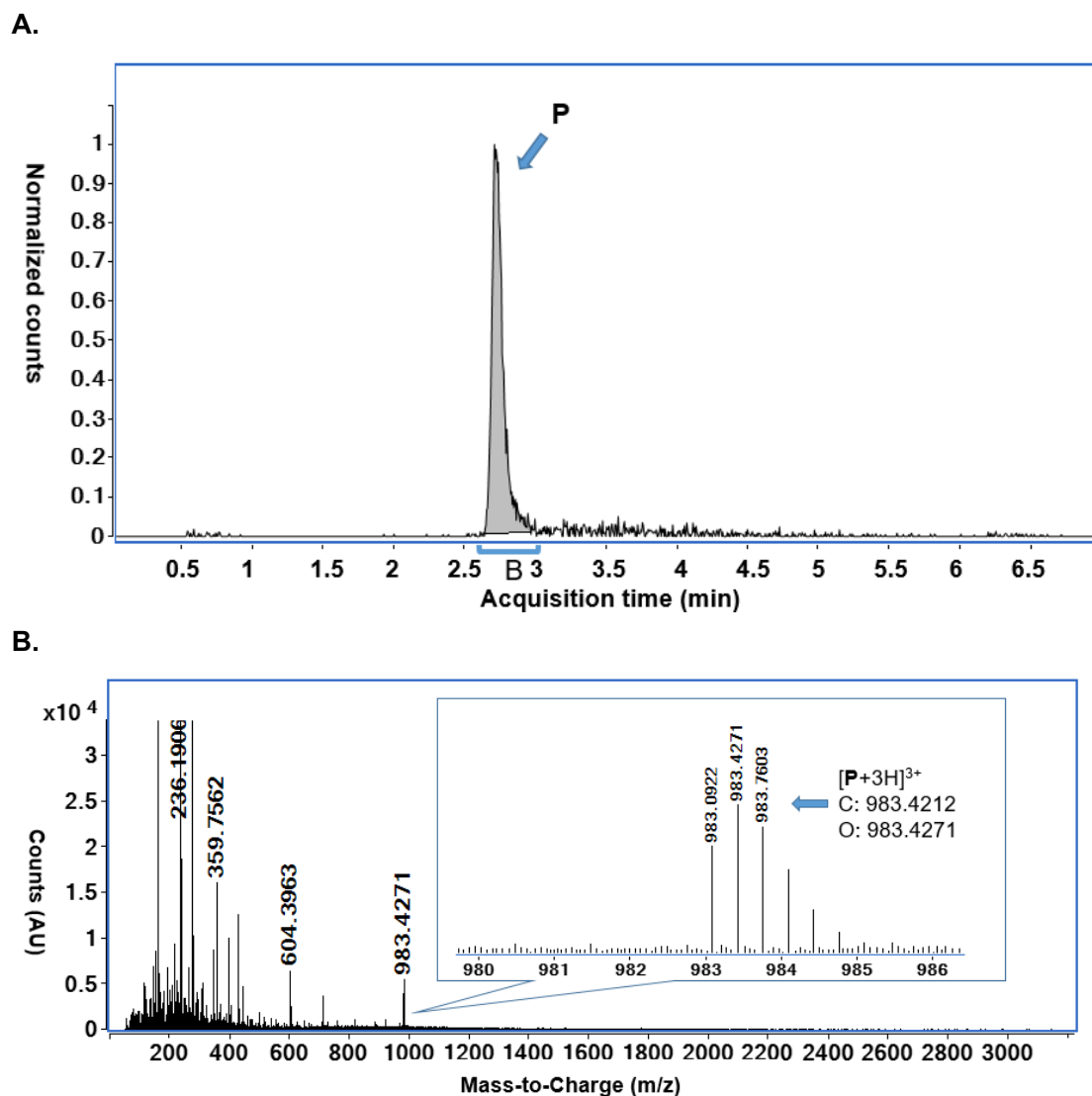


Figure S8. The EIC and mass spectrum for Table S1, entry 8: translation of peptide sequence ‘MFCGSGCAMSRYEVDWRGRGSAMGSGSGS’ using SolAFD- supplemented with 50 μM Ac-Phe-tRNA^{Met} (initiation) and azidohomoalanine (elongation). A. The EIC for Peptide. B. The mass spectrum (2.62-2.92 min, 55 scans) of peptide.

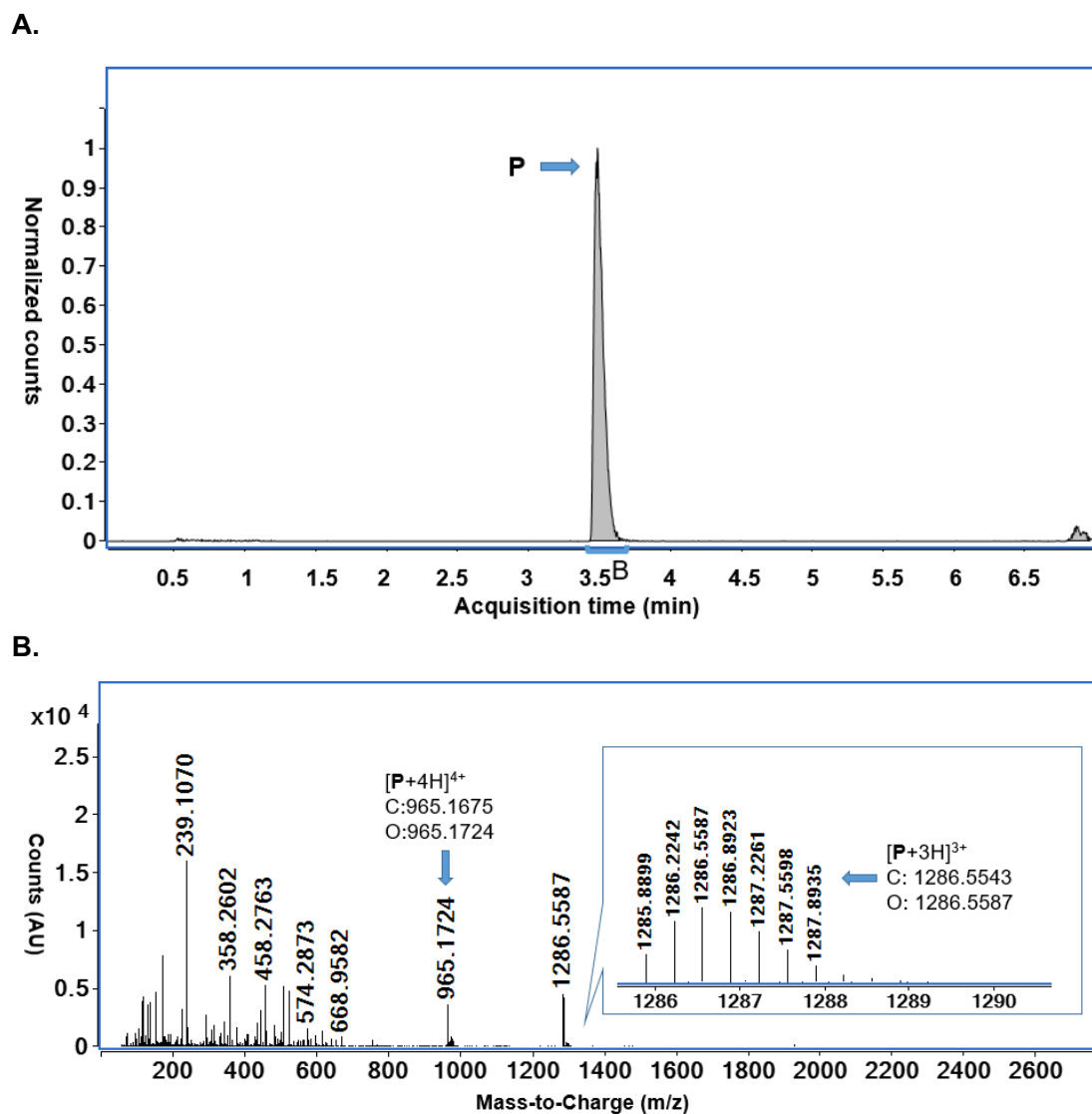
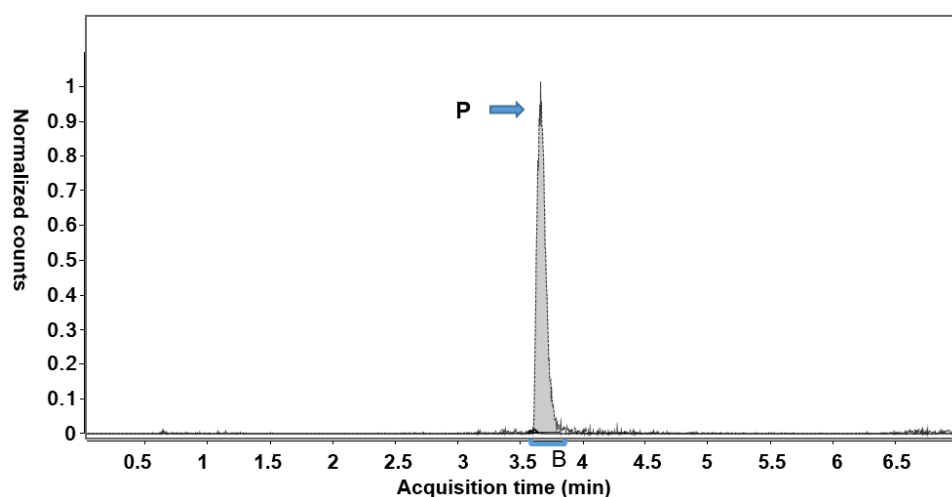


Figure S9. The EIC and mass spectrum for Table S1, entry 9: translation of peptide sequence 'MWSHPQFEKTEY EYLDYDFLP EMEPLGSGSGS' using SolAFD-supplemented with 50 μ M ClAc-Tyr-tRNA^{Met} (initiation) and homopropargylglycine (elongation). A. The EIC for Peptide and possible byproduct. B. The mass spectrum (3.44-3.63 min, 62 scans) of peptide.

A.



B.

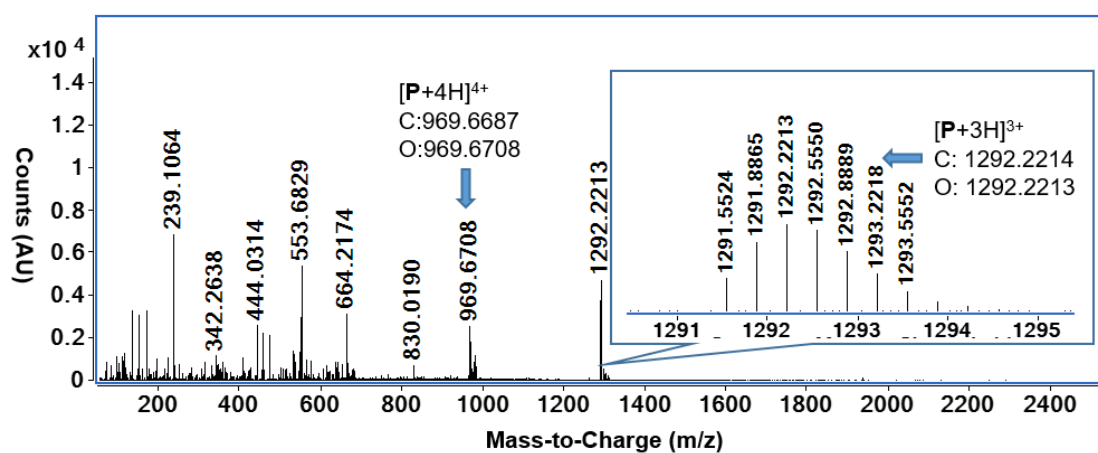


Figure S10. The EIC and mass spectrum for Table S1, entry 10: translation of peptide sequence 'MWSHPQFEKTEY EYLDYDFLPEMEPLGSGSGS' using SolAFD-supplemented with 50 μ M ClAc-Tyr-tRNA^{fMet} (initiation) and azidohomoalanine (elongation). A. The EIC for Peptide and possible byproduct. B. The mass spectrum (3.59-3.81 min, 70 scans) of peptide.

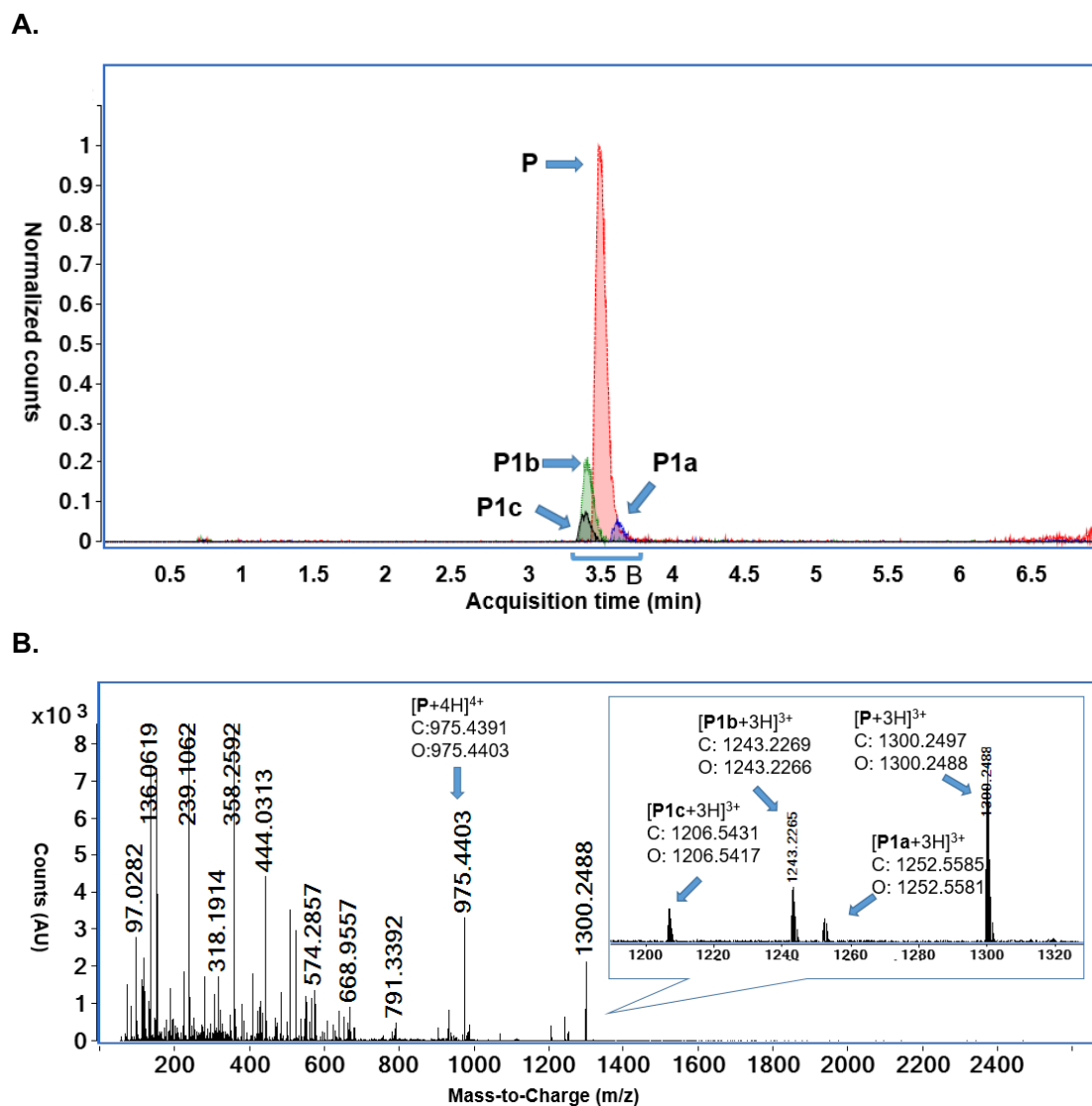


Figure S11. The EIC and mass spectrum for Table S1, entry 11: translation of peptide sequence ‘MWSHPQFEKTEY EYLDYDFLPEMEPLGSGSGS’ using SolAFD-supplemented with 50 μ M AMB-Phe-tRNA^{Met} (initiation) and homopropargylglycine (elongation). A. The EIC for Peptide and byproduct. B. The mass spectrum (3.35-3.76 min, 127 scans) of peptide.

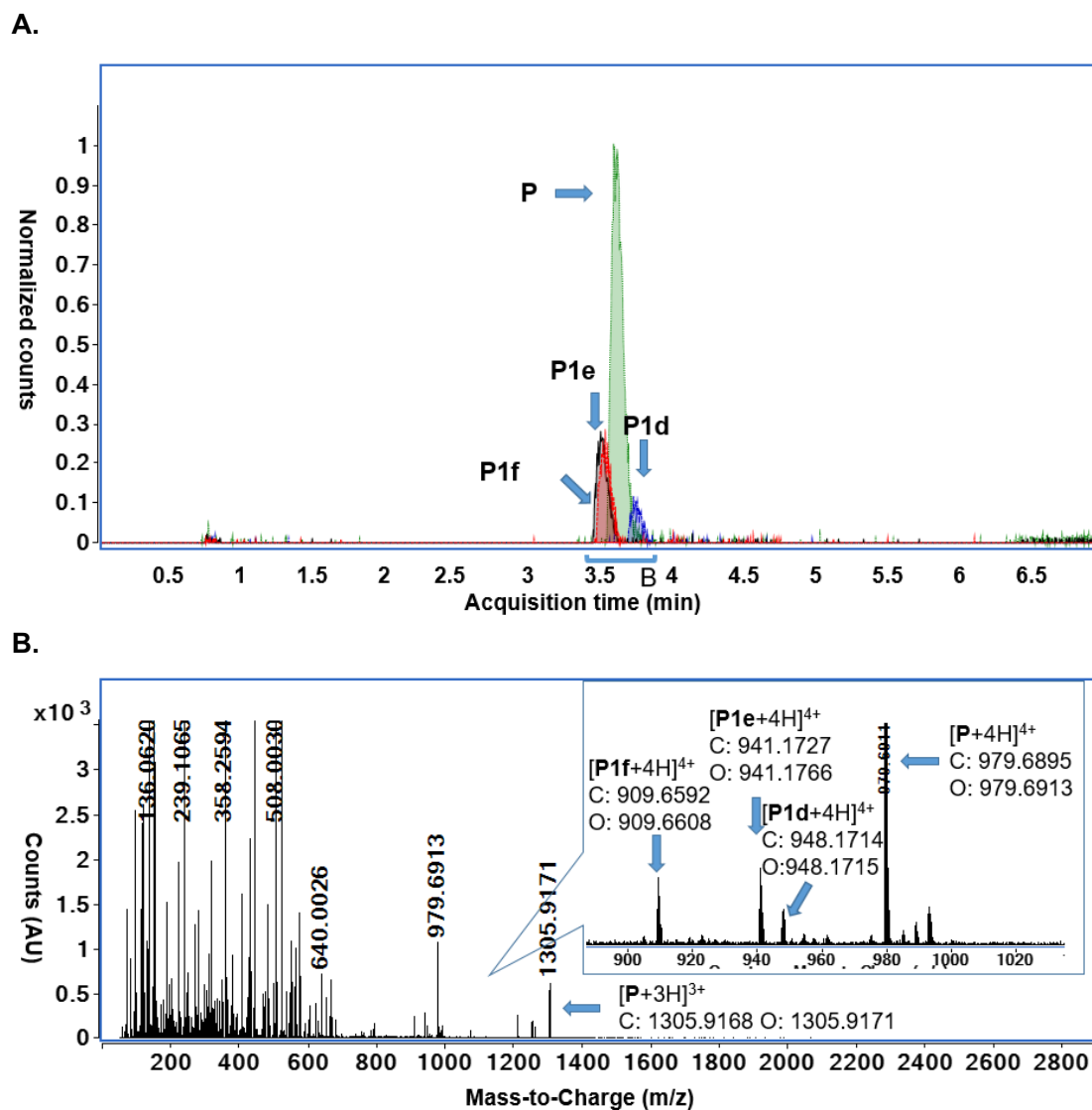
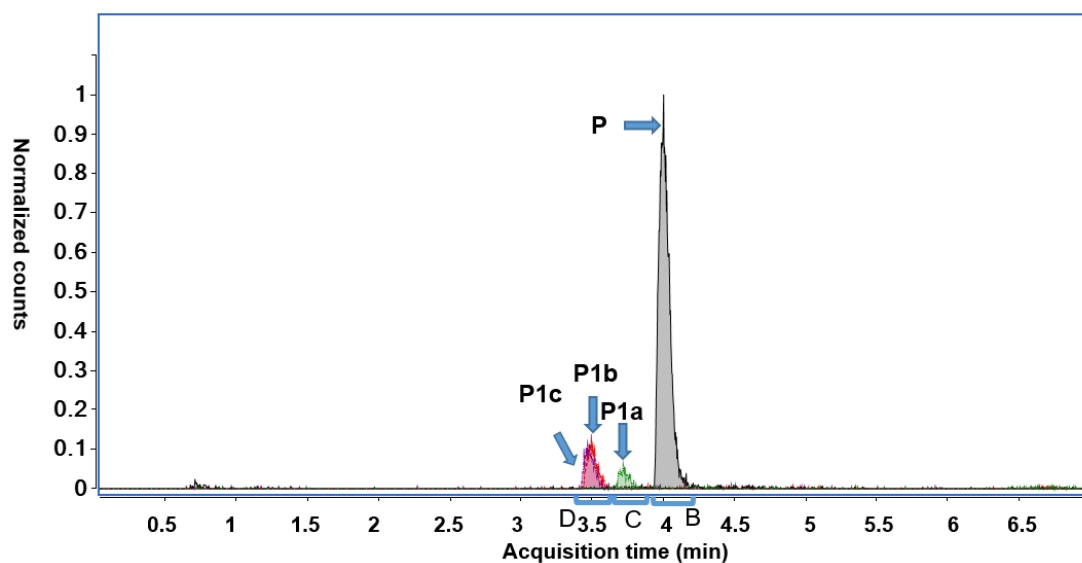
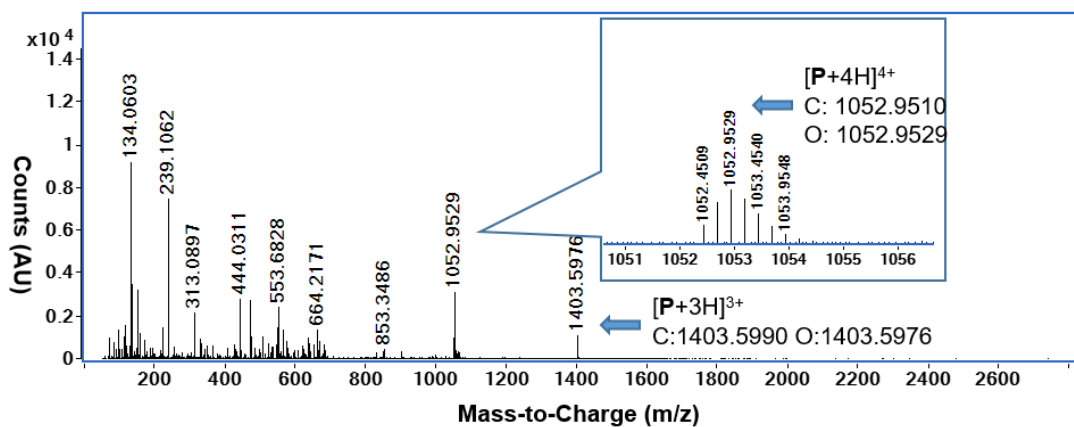


Figure S12. The EIC and mass spectrum for Table S1, entry 12: translation of peptide sequence ‘MWSHPQFEKTEY EYLDYDFLP EMEPLGSGSGS’ using SolAFD-supplemented with 50 μ M AMB-Phe-tRNA^{Met} (initiation) and azidohomoalanine (elongation). A. The EIC for Peptide and byproduct. Red, **P1f**; Grey, **P1e**. B. The mass spectrum (3.48-3.88 min, 122 scans) of peptide.

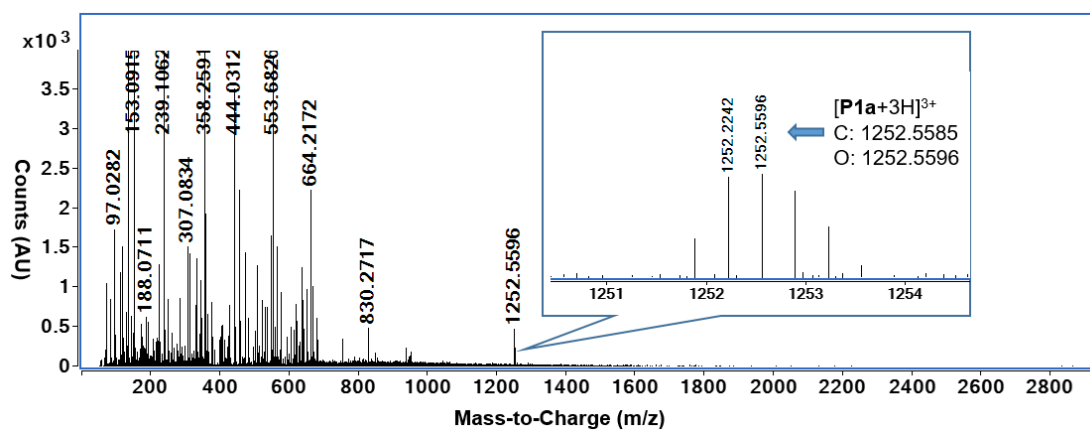
A.



B.



C.



D.

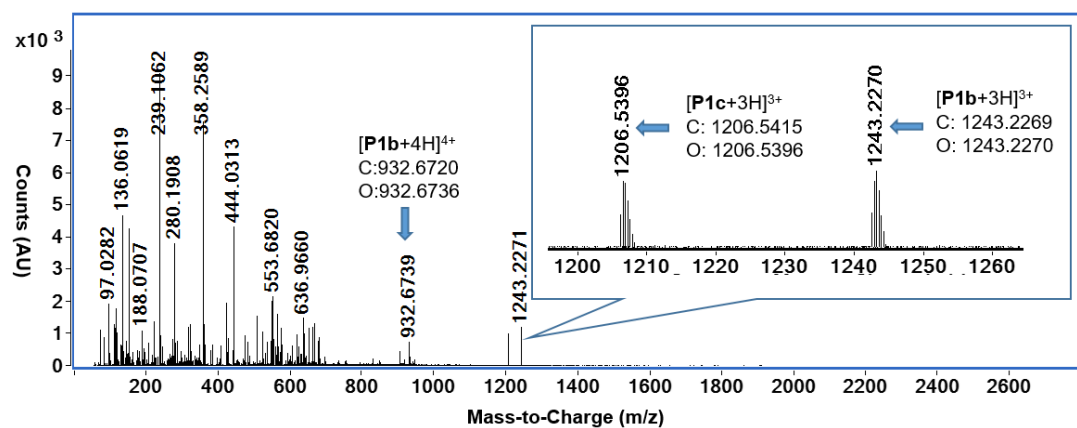
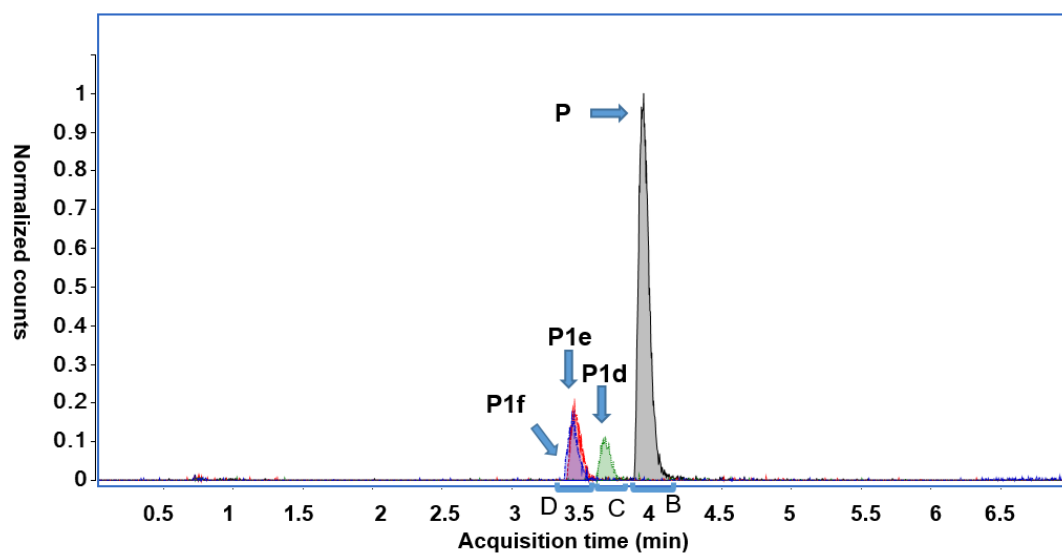
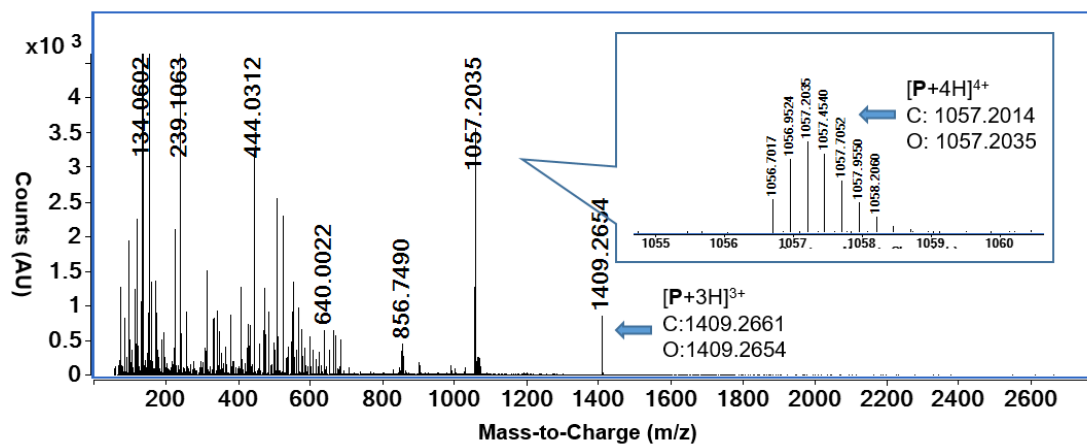


Figure S13. The EIC and mass spectrum for Table S1, entry 13: translation of peptide sequence 'MWSHPQFEKTEYEYLDYDFLPEMEPLGSGSGS' using SolAFD-supplemented with 50 μ M (5/6)FAM-Phe-tRNA^{fMet} (initiation) and homopropargylglycine (elongation). **A.** The EIC for Peptide and byproduct. Red, **P1b**; Blue, **P1c** **B.** The mass spectrum (3.93-4.17 min, 77 scans) of peptide. **C.** The mass spectrum (3.65-3.83 min, 57 scans) for **P1a**. **D.** The mass spectrum (3.44-3.61 min, 53 scans) for **P1b** and **P1c**

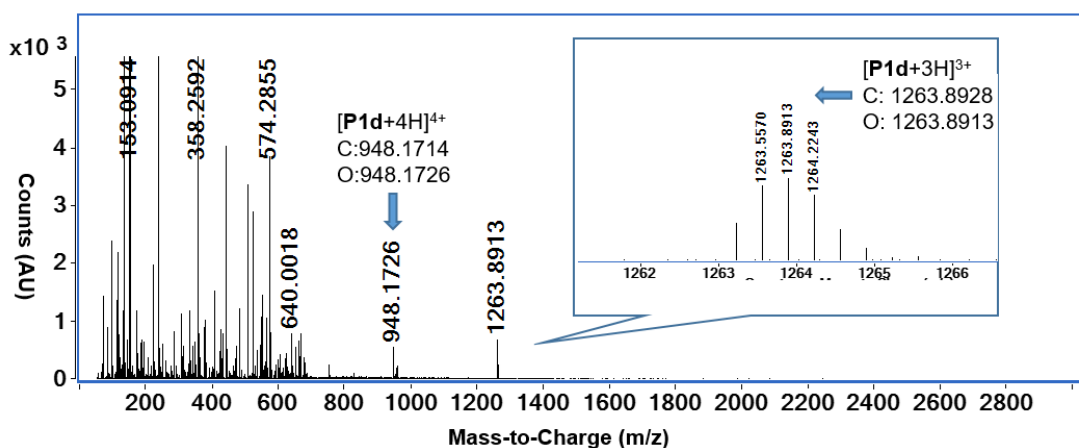
A.



B.



C.



D.

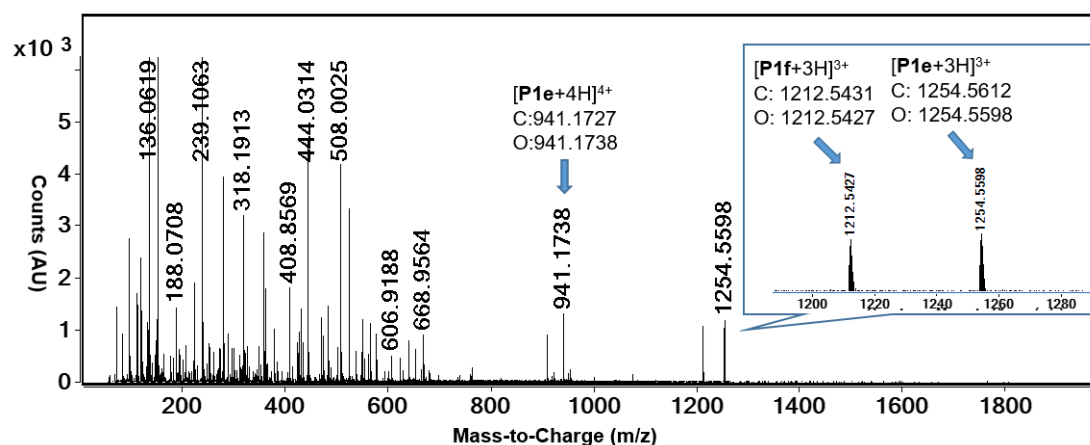


Figure S14. The EIC and mass spectrum for Table S1, entry 14: translation of peptide sequence 'MWSHPQFEKTEYEYLDYDFLPEMEPLGSGSGS' using SolAFD-supplemented with 50 μ M (5/6)FAM-Phe-tRNA^{Met} (initiation) and azidohomoalanine (elongation). A. The EIC for Peptide and byproduct. Red, **P1e**; Blue, **P1f** B. The mass spectrum (3.85-4.11 min, 77 scans) of peptide. C. The mass spectrum (3.60-3.73 min, 40 scans) for **P1d**. D. The mass spectrum (3.39-3.52 min, 40 scans) for **P1e** and **P1f**

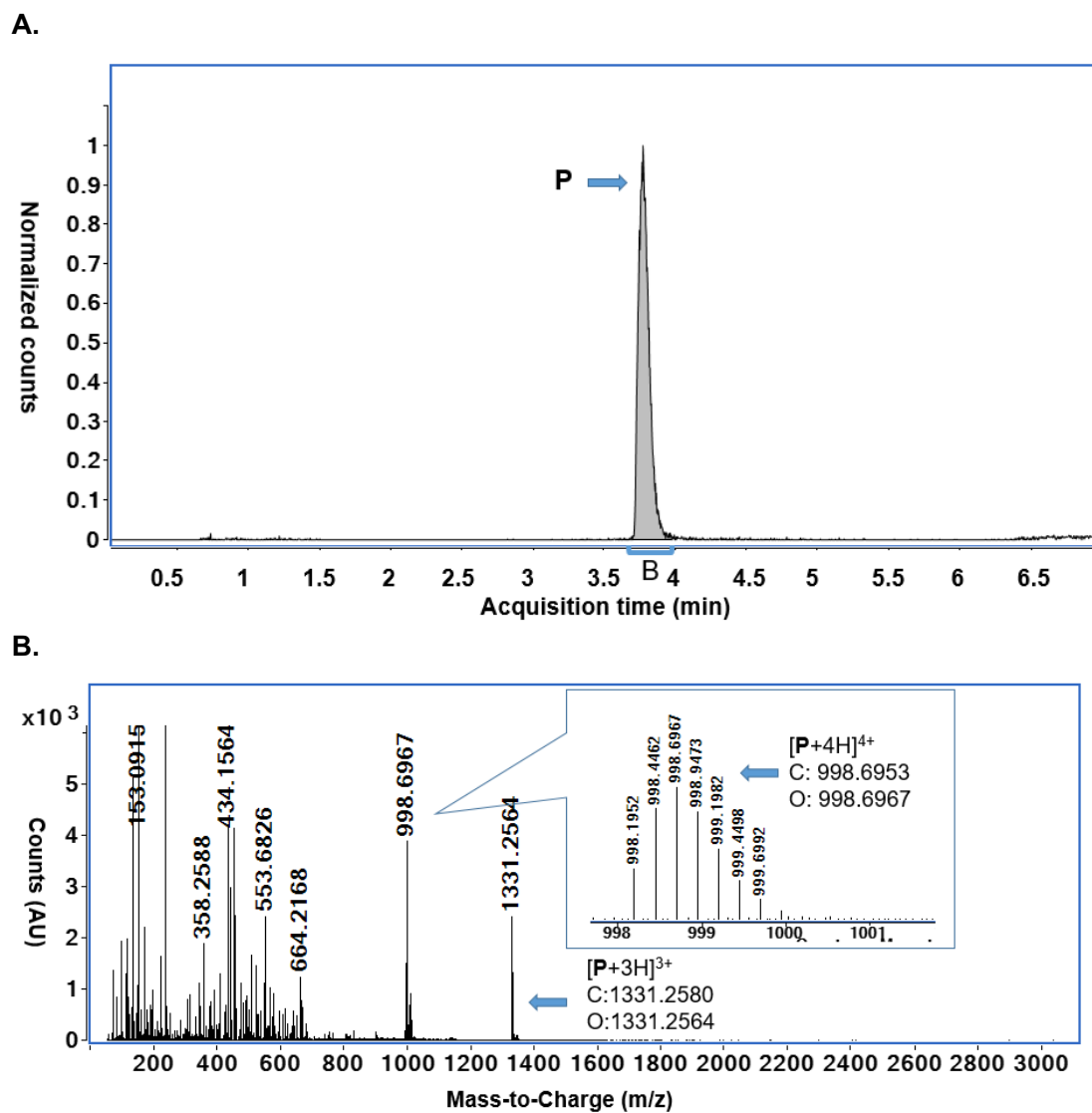


Figure S15. The EIC and mass spectrum for Table S1, entry 15: translation of peptide sequence ‘MWSHPQFEKTEY EYLDYDFLPEMEPLGSGSGS’ using SolAFD-supplemented with 50 μ M Biotin-Phe-tRNA^{fMet} (initiation) and homopropargylglycine (elongation). A. The EIC for Peptide and possible byproduct. B. The mass spectrum (3.70-3.93 min, 70 scans) of peptide.

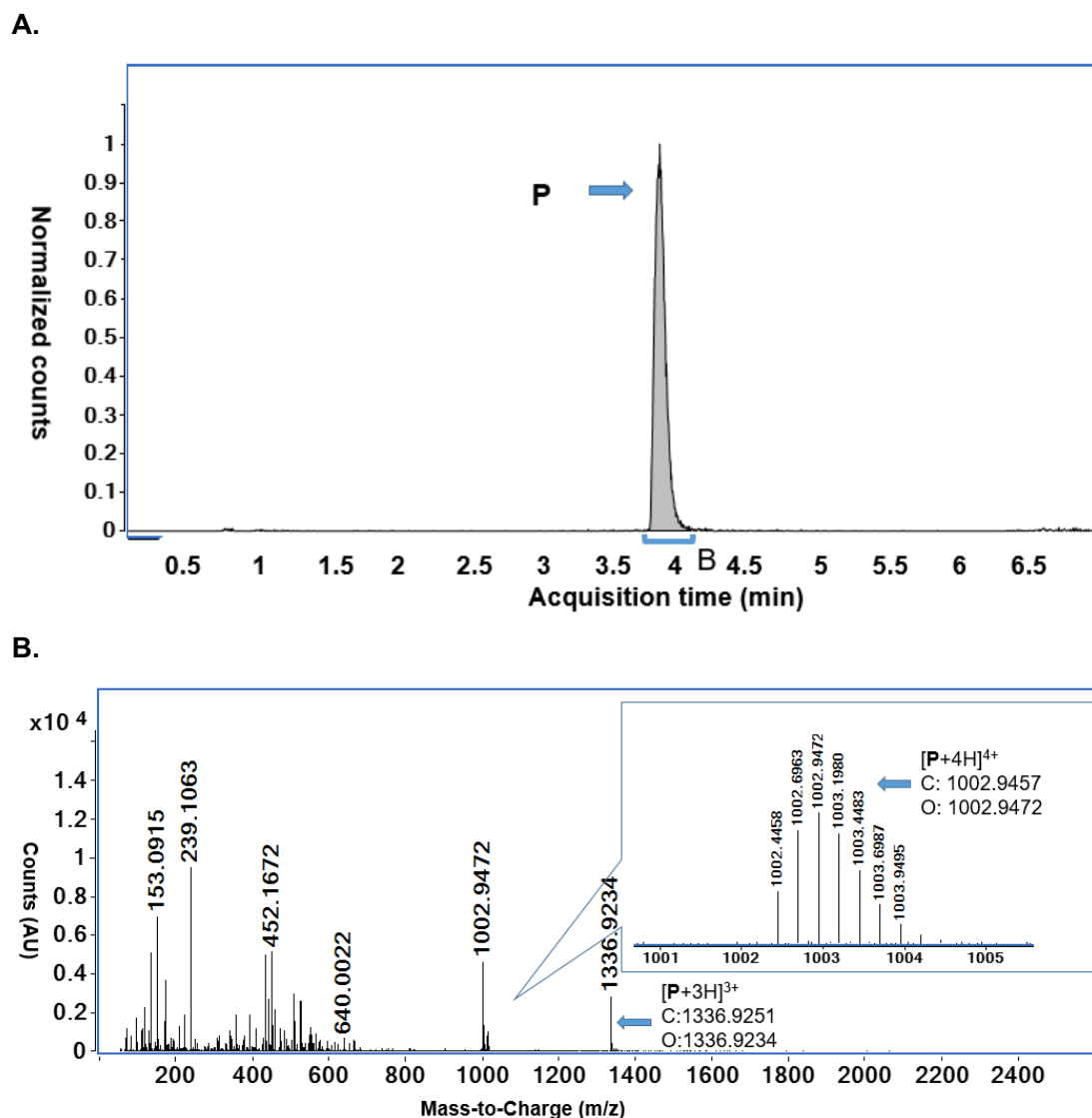
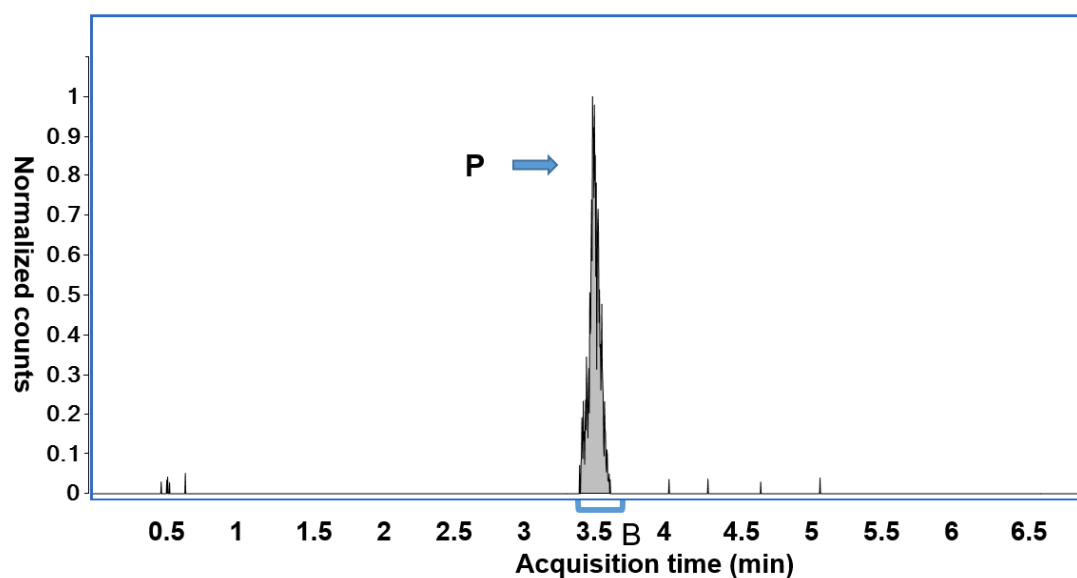


Figure S16. The EIC and mass spectrum for Table S1, entry 16: translation of peptide sequence ‘MWSHPQFEKTEY EYLDYDFLP EMEPLGSGSGS’ using SolAFD-supplemented with 50 μ M Biotin-Phe-tRNA^{Met} (initiation) and azidohomoalanine (elongation). A. The EIC for Peptide and byproduct. B. The mass spectrum (3.75-4.03 min, 87 scans) of peptide.

A.



B.

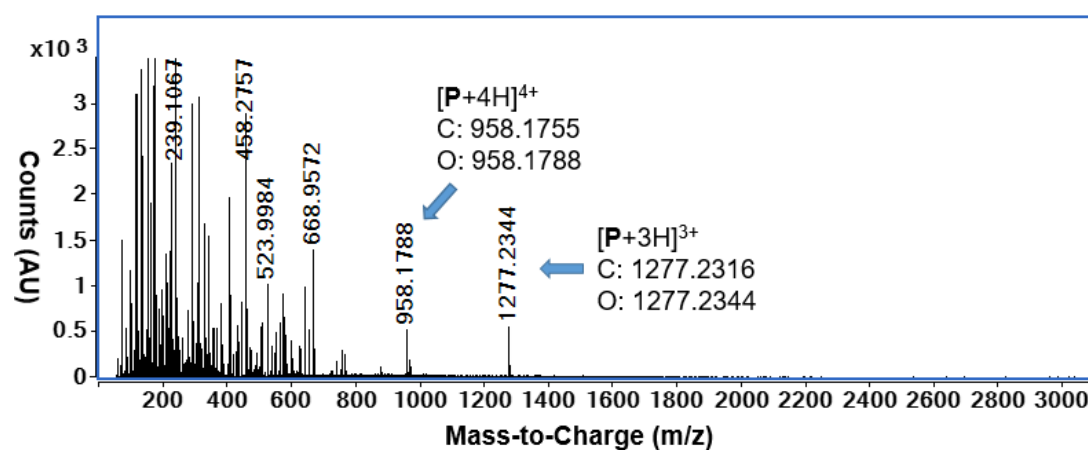
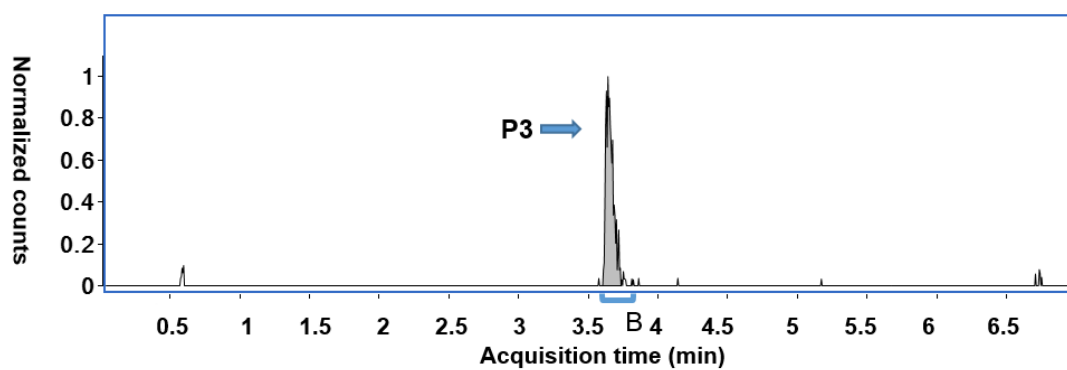
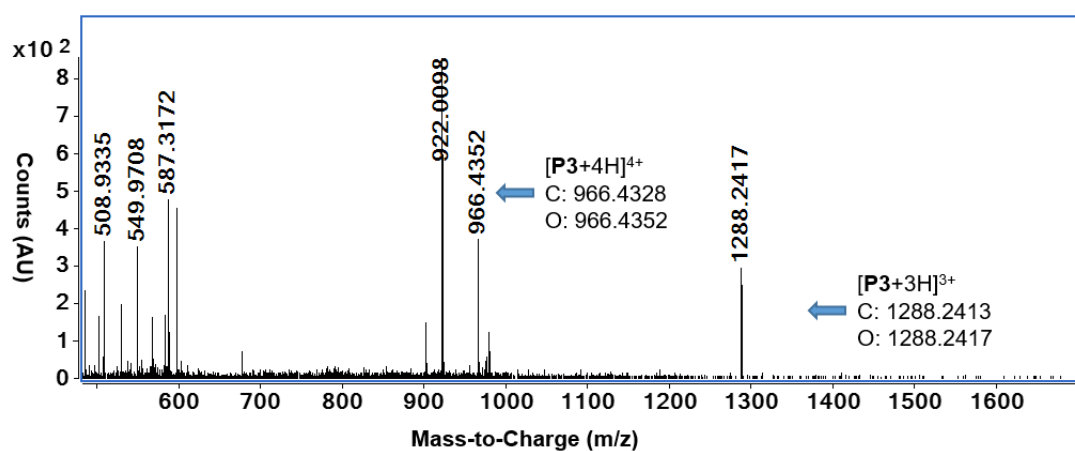


Figure S17. The EIC and mass spectrum for Table S1, entry 17: translation of peptide sequence 'MWSHPQFEKTEYEYLDYDFLPEMEPLGSGSGS' using SolAFD-supplemented with 50 μ M Ac-Phe-tRNA^{Met} (initiation) and methionine (elongation). A. The EIC for Peptide. B. The mass spectrum (3.43-3.65 min, 70 scans) of peptide.

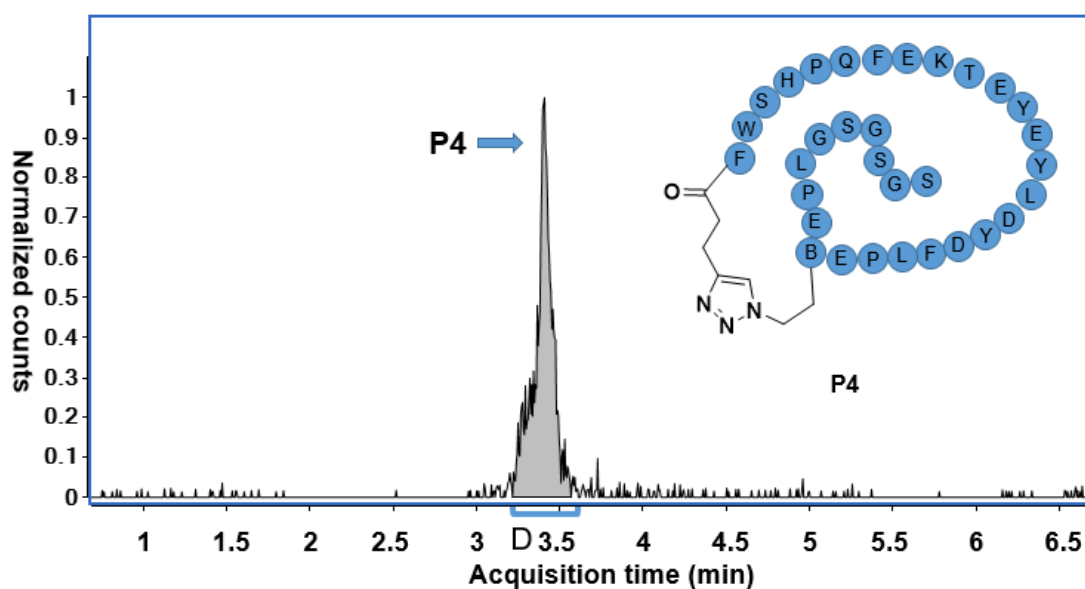
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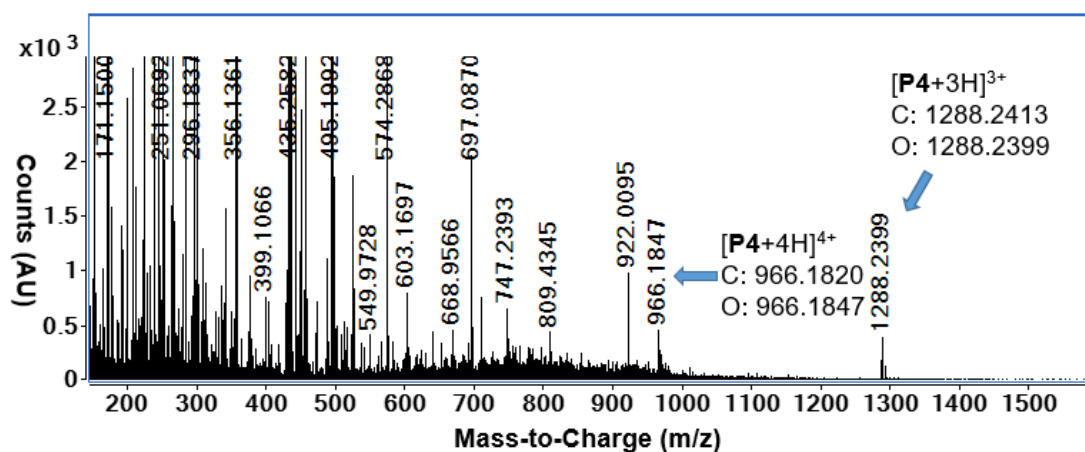
B.



C.



D.



E.

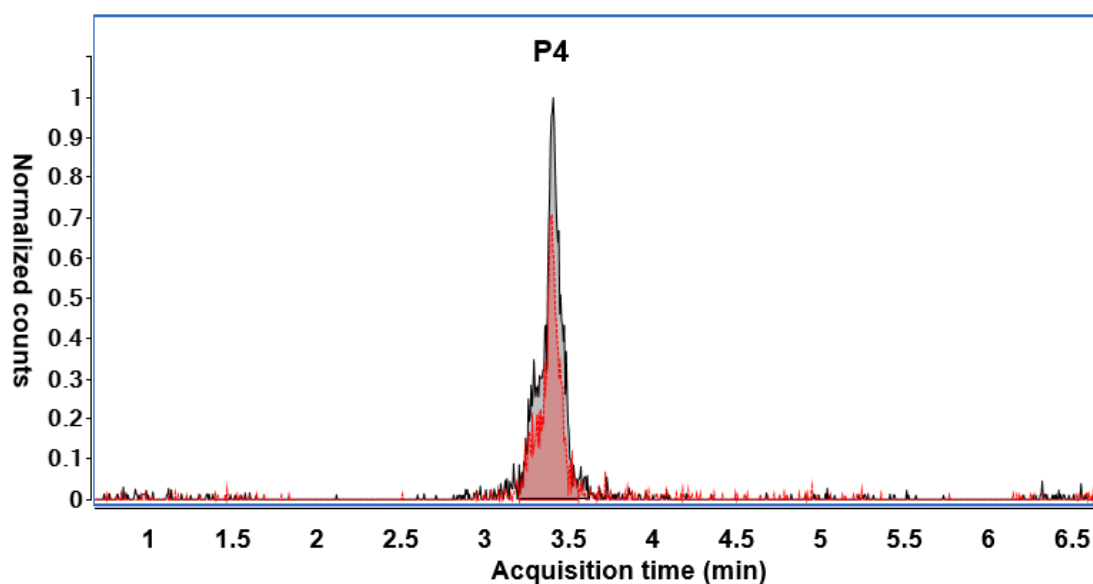
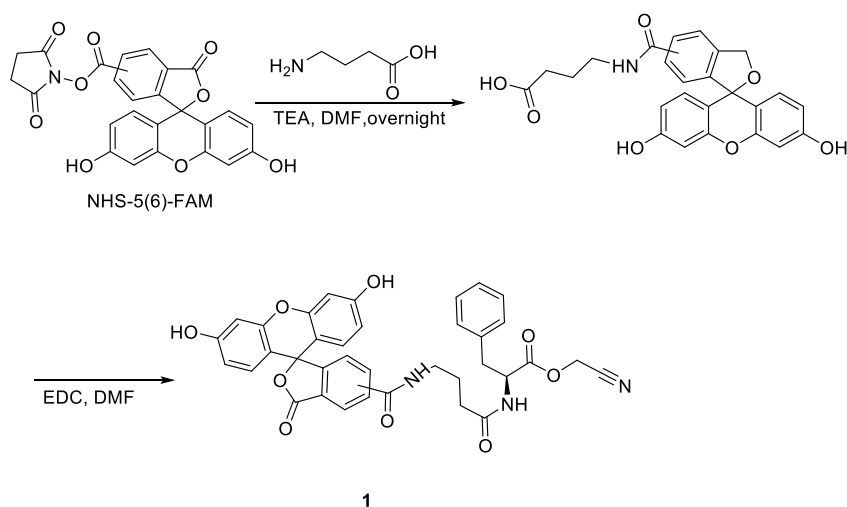
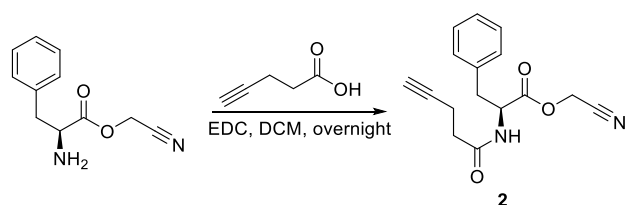


Figure S18. The EIC and mass spectrum for Table S1, entry 18: translation of peptide sequence 'MWSHPQFEKTEY EYLDYDFLPEMEPLGSGSGS' using SolAFD-supplemented with 50 μM Pyn-Phe-tRNA^{Met} (initiation) and azidohomoalanine (elongation). A. The EIC for Peptide **P3**. B. The mass spectrum (3.59-3.76 min, 31 scans) of peptide. C. The EIC for Peptide **P4**. D. The mass spectrum (3.26-3.54 min, 52 scans) for **P4**. E. Comparison EIC before TCEP (**P4**, grey) and after TCEP (**P4**, red) reaction.

6. Synthesis of molecules



N-(*N*-(5/6-Carboxyfluoresceinamide)-4-aminobutyric)-phenylalanine cyanomethyl ester
 5/6-FAM (378 mg, 1 mmol) was dissolved in 5 mL DMF, followed by addition of EDC (286 mg, 1.5 mmol) and NHS (126 mg, 1.2 mmol). The reaction mixture was then stirred at room temperature for 2 hours. This was then followed by the addition of 4-aminobutyric acid (103 mg, 1 mmol) and 3 equiv of TEA (420 μ L, 303 mg, 3 mmol). The reaction mixture was stirred at room temperature overnight, followed by the addition of Phe-CME (204 mg, 1 mmol), *N,N'*-diisopropylcarbodiimide (626 μ L, 4 mmol,) and ethyl isonitrosocyanacetate (566 mg, 4 mmol). The reaction mixture was then stirred at room temperature overnight again. The solution was then poured into 100 mL brine and extracted with 100mL ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by silica column chromatography (DCM/MeOH/CH₃COOH = 10:1:0.01, R_f = 0.5) to obtain 100 mg (16%) compound **1** as a yellow foam. ¹H NMR (400 MHz, CD₃OD) δ : 8.42 (s, 1H, -NH-Ph-H), 8.21 – 8.04 (m, 2H, -NH-Ph-H), 7.61 (m, 0.5H, -NH-Ph-H), 7.37 – 7.04 (m, 5H, Ph-H), 6.78 – 6.44 (m, 6H, HO-Ph-H), 4.85 (s, 2H, -CH₂-CN), 4.51 – 4.27 (m, 1H, -NH-CH-CO-), 3.69 – 3.37 (m, 2H, -NH-CH₂-), 3.23 – 2.95 (m, 2H, Ph-CH₂-), 2.54 – 2.14 (m, 2H, -NH-CH₂-CH₂-), 2.00 – 1.67 (m, 2H, -CO-CH₂-CH₂-) ppm. Calculated for C₃₆H₂₉N₃O₉ [M+H]⁺: 648.1977, found: 648.1970. Note: this product is a mixture, attaching to positions 5 and 6 of the fluorescein.



N-(4-pentynoic)-Phenylalanine cyanomethyl ester

Phenylalanine cyanomethyl ester (134 mg, 0.65 mmol, 1 equiv) was dissolved in anhydrous DCM, followed by addition of 4-pentynoic acid (95 mg, 0.98 mmol, 1.5 equiv) and EDC (186 mg, 0.98 mmol, 1.5 equiv), then the reaction mixture stirred at room temperature overnight. The solvent was subsequently removed under vacuum and the residue was purified by silica column chromatography (PE/EA = 1:1, R_f = 0.3) to obtain 180

mg (65%) compound **2** as colorless oil. ^1H NMR (400 MHz, CDCl_3) δ : 7.37 – 7.27 (m, 3H, Ph-H), 7.18 – 7.13 (m, 2H, Ph-H), 6.08 (d, $J = 7.6$ Hz, 1H, -NH-CO-), 4.94 (dt, $J = 7.7, 6.2$ Hz, 1H, -NH-CH-), 4.79 (d, $J = 15.7$ Hz, 1H, -CH₂-CN), 4.69 (d, $J = 15.7$ Hz, 1H, -CH₂-CN), 3.23 – 3.09 (m, 2H, Ph-CH₂-), 2.53 – 2.44 (m, 2H, CH \equiv C-CH₂-), 2.44 – 2.38 (m, 2H, -CO-CH₂-CH₂) 1.96 (t, $J = 2.6$ Hz, 1H, CH \equiv C-) ppm. Calculated for $\text{C}_{16}\text{H}_{16}\text{N}_2\text{O}_3$ $[\text{M}+\text{H}]^+$: 285.1234, found: 285.1227.

7. Acid PAGE for aminoacylation testing

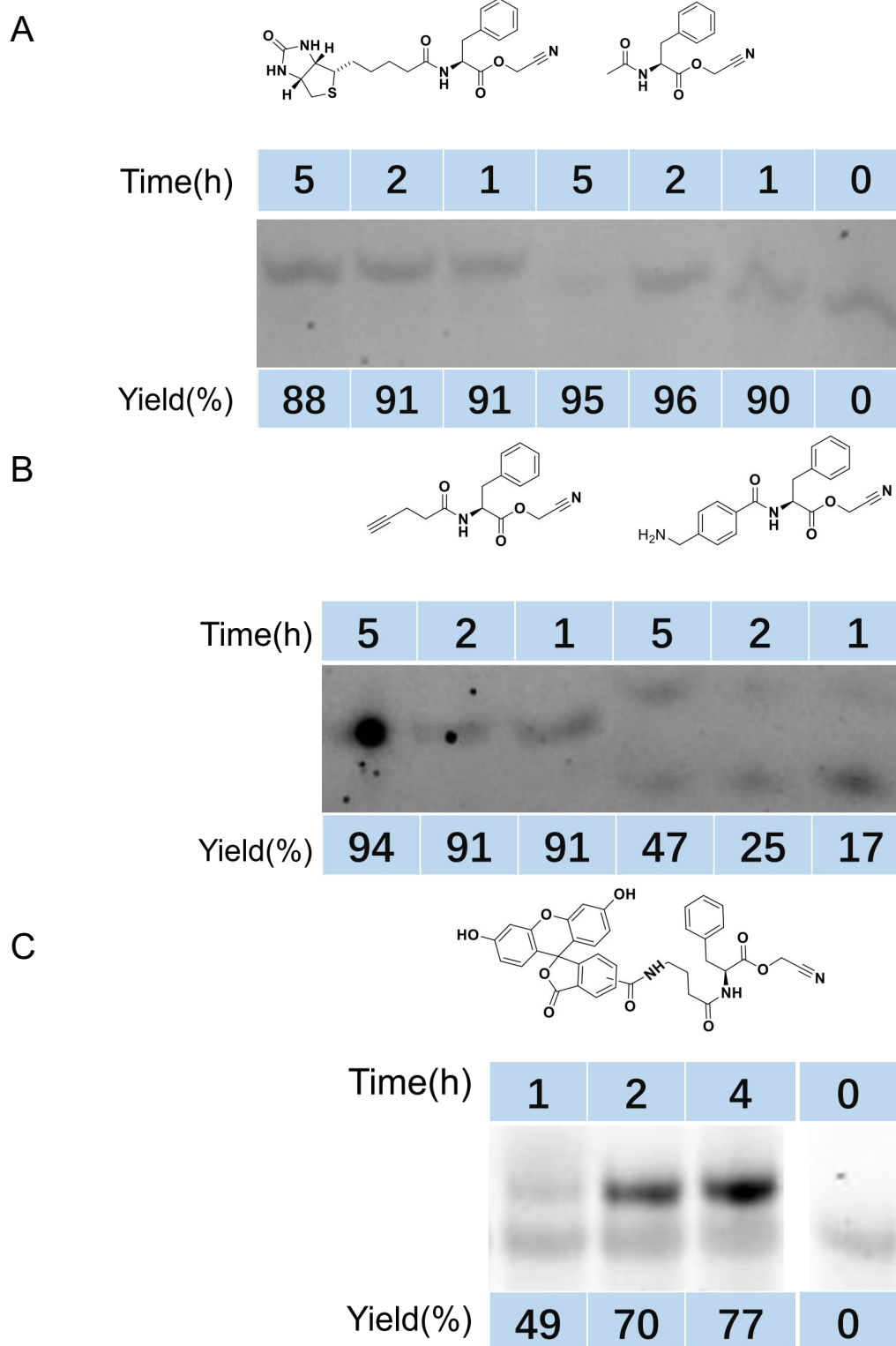
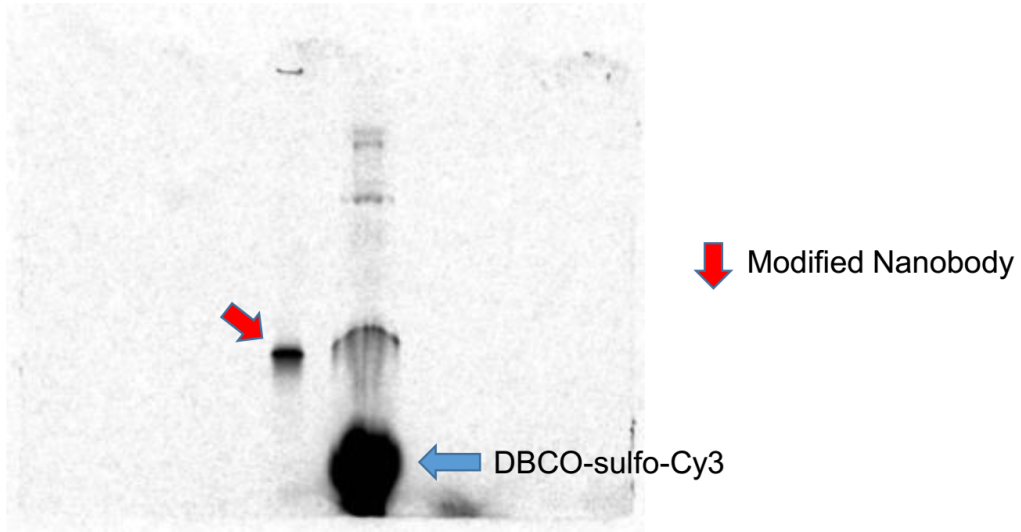


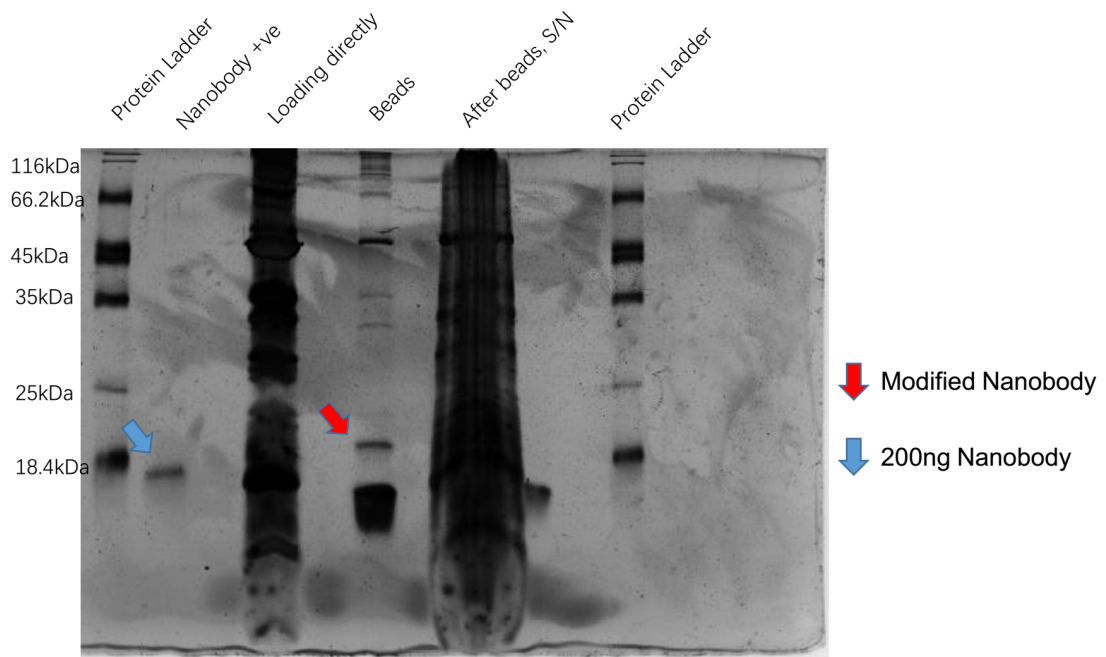
Figure S19. Aminoacylation test for different amino acids. **(A)** The aminoacylation test for *Biotin*-L-Phe-CME. **(B)** The aminoacylation *Pyn*-L-Phe-CME and *AMB*-L-Phe-CME. **(C)** The aminoacylation for *FAM*-Phe-CME. Note that *FAM*-Phe-CME has inherent fluorescence that likely interferes with this assay, and so quantification of its efficiency is not accurate but product can be confirmed to be present.

8. Fluorescence and silver stain images of SDS-PAGE gels.

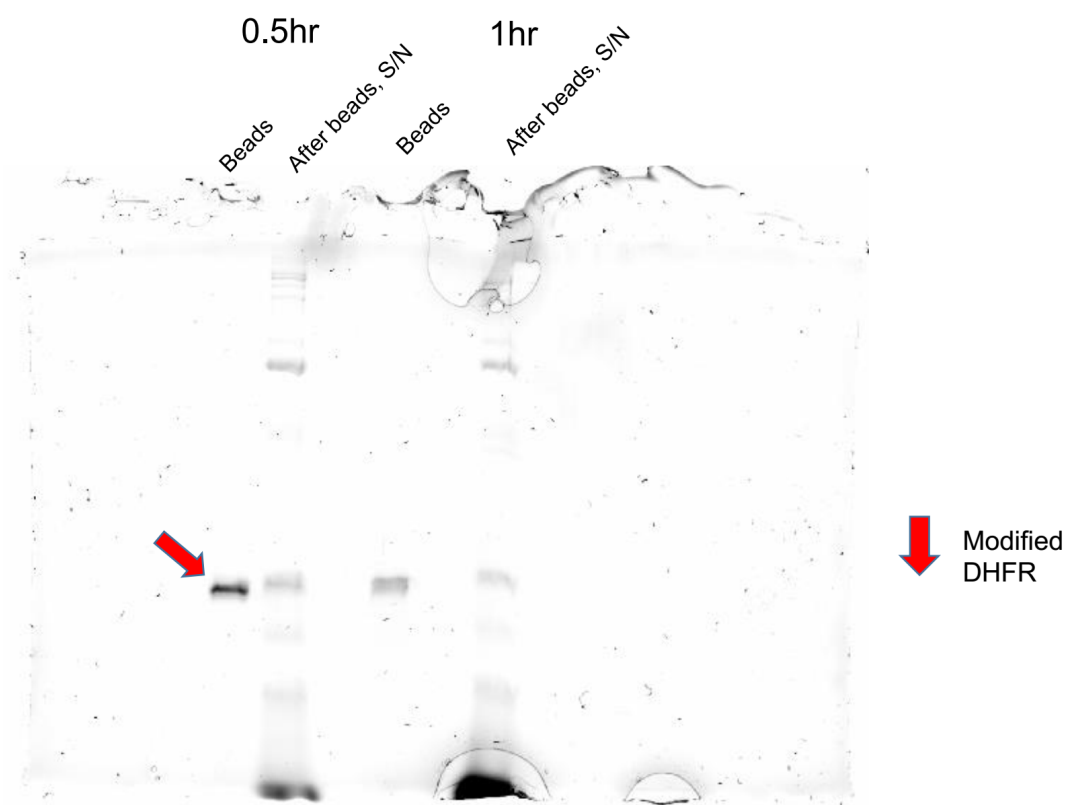
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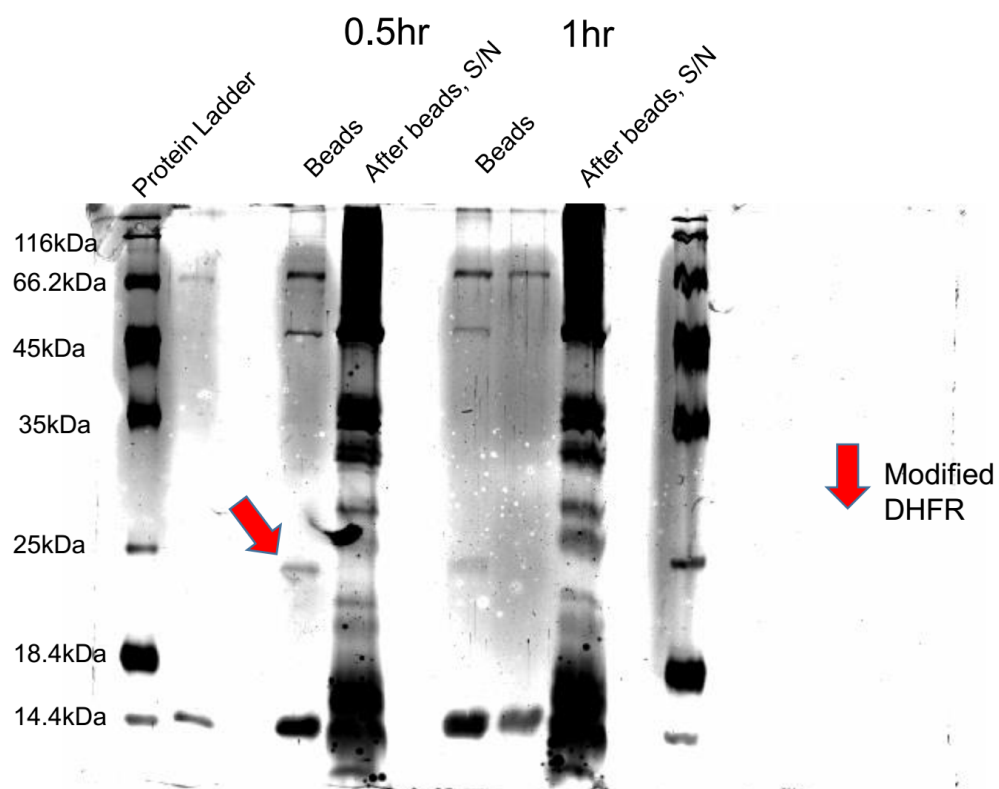
B.



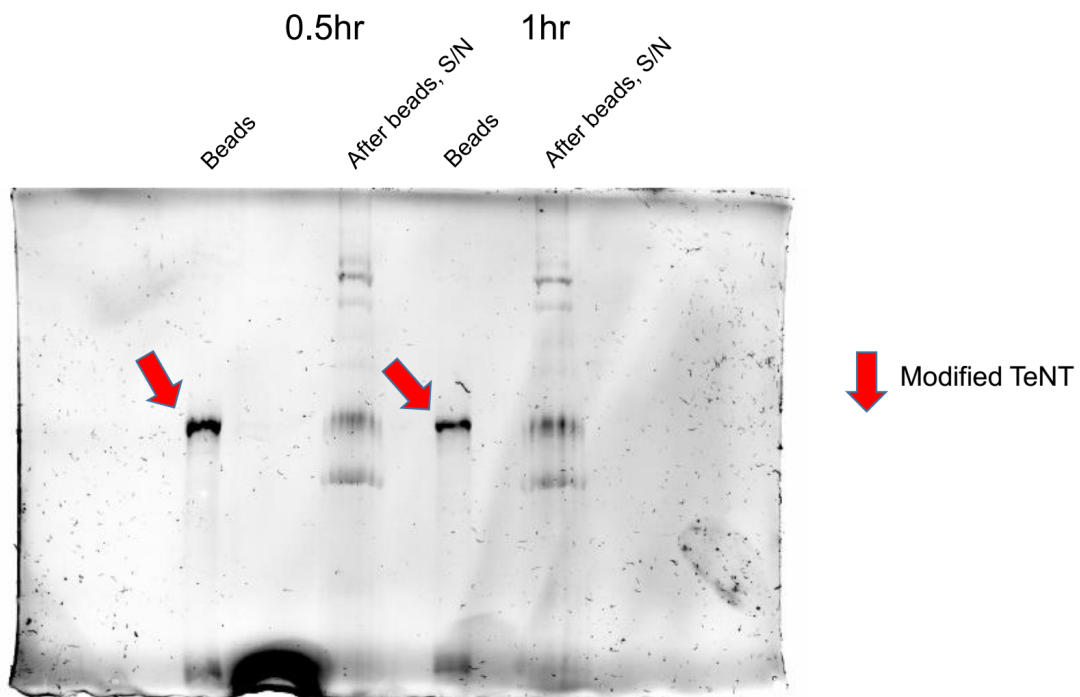
C.



D.



E.



F.

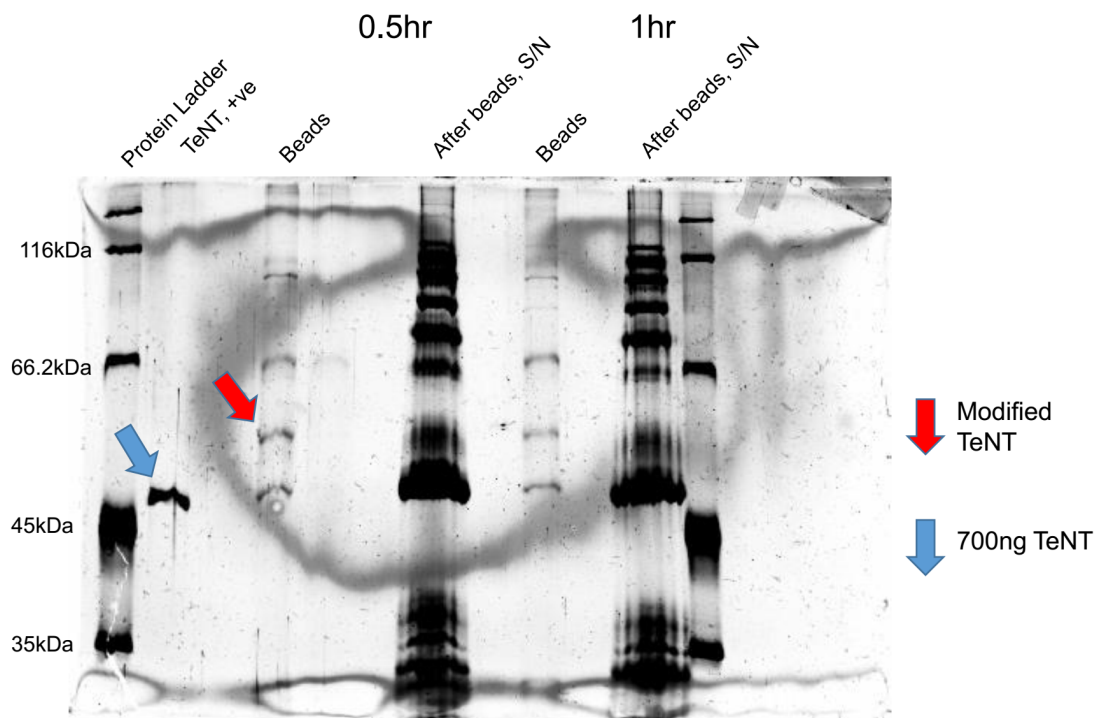
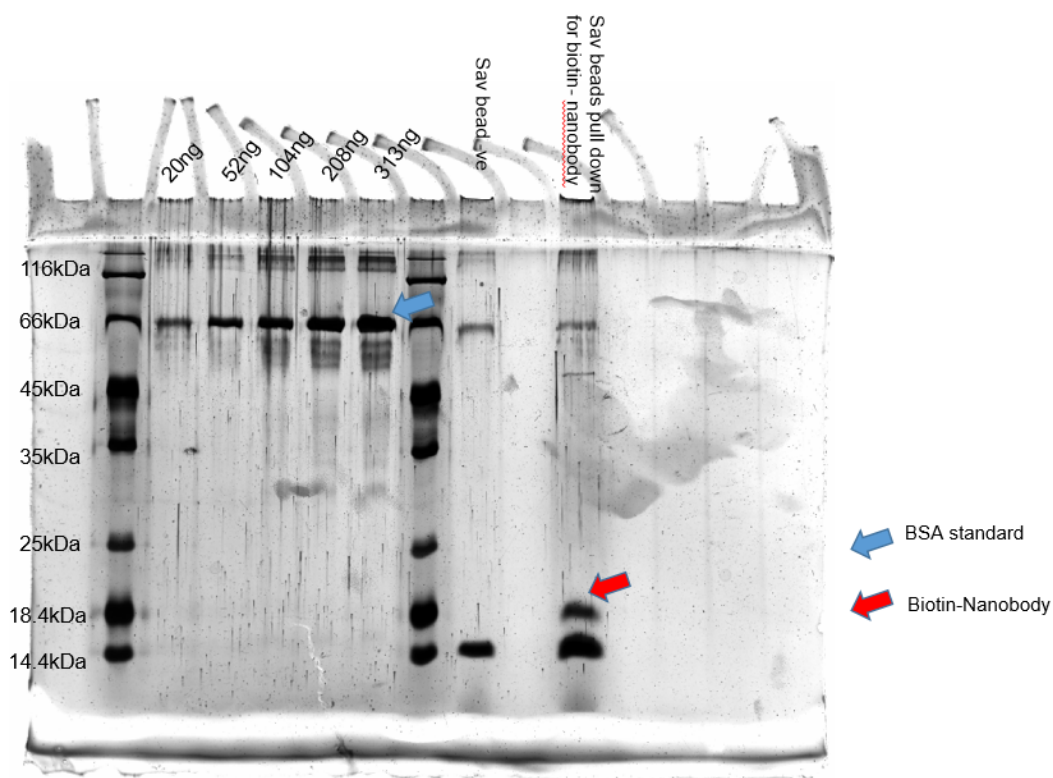


Figure S20. Initiation reprogramming in protein expression (translated following the general method with 250 μ M methotrexate and 50 μ M acyl-tRNA). (A) Fluorescence gel image for Nanobody. (B) Silver stain gel image for Nanobody (same gel as A). (C) Fluorescence gel image for DHFR. (D) Silver stain gel image for DHFR (same gel as C). (E) Fluorescence gel image for TeNT. (F) Silver stain gel image for TeNT (same gel as E). S/N = supernatant, Beads = streptavidin-bound fraction, +ve = native protein.

A.



B.

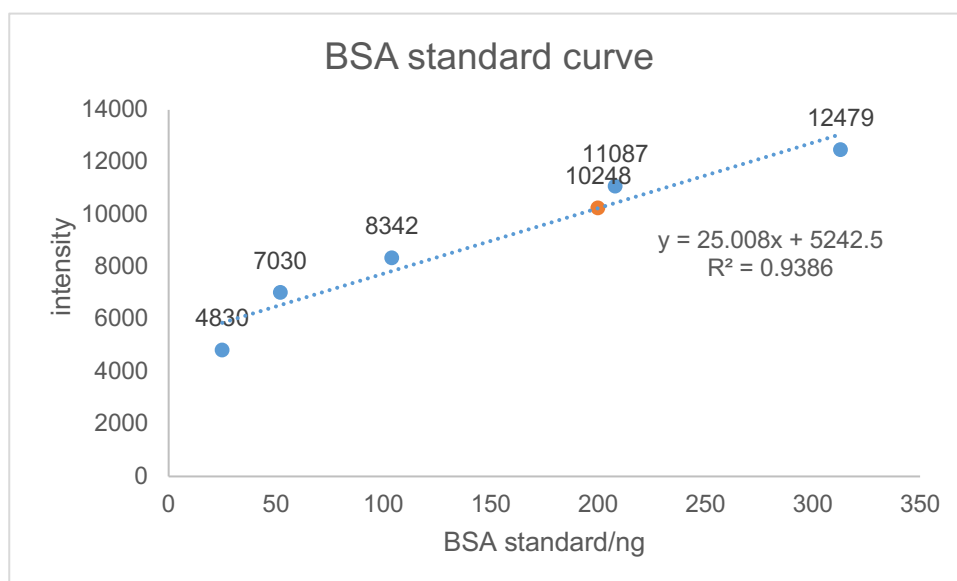


Figure S21. Initiation reprogramming in protein expression (translated following the general method with 250 μ M methotrexate and 50 μ M acyl-tRNA). **(A)** Protein yield quantification by BSA standard curve for nanobody reprogrammed translation (no DBCO-Sulfo-Cy3 modification). **(B)** BSA standard curve (blue), with biotin-nanobody band intensity (orange) giving an estimated yield of 200 ng nanobody from 5 μ L translation (40 μ g/mL, *cf.* manufacturer's estimated expected yield of 10-200 μ g/mL).

10. Supplementary references

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- [4] Y. Goto, A. Ohta, Y. Sako, Y. Yamagishi, H. Murakami, H. Suga, *ACS Chem. Biol.* **2008**, 3, 120–129.
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