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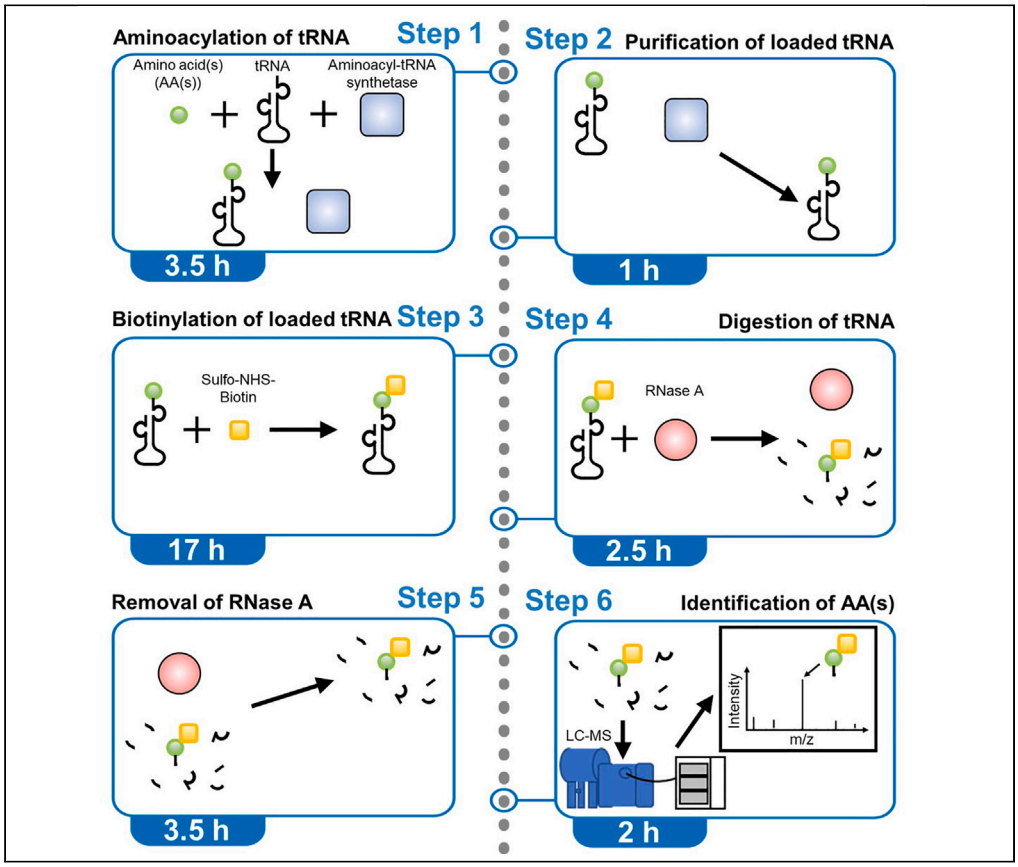
Protocol to identify amino acids bound to tRNA by aminoacylation using mass spectrometry



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Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Identification of tRNA-loaded amino acids

Characterize molecular recognition fidelity of aminoacyl-tRNA-synthetases

Mass spectrometry avoids dealing with radioactive labels

Finkler et al., STAR Protocols 4, 102504
September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102504

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Protocol to identify amino acids bound to tRNA by aminoacylation using mass spectrometry

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SUMMARY

tRNA-bound amino acids often need to be identified, for instance, in cases where different amino acids compete for binding to the same tRNA. Here, we present a mass-spectrometry-based protocol to determine the amino acids bound to tRNA by aminoacylation. We detail how to perform the aminoacylation reaction, the preparation of the aminoacyl-tRNA for measurement, and the mass spectrometric analysis. We use arginine acylation as an example; however, this protocol can be applied to any other amino acid.

BEFORE YOU BEGIN

The protocol below first shows how to prepare the components of the aminoacylation reaction: aminoacyl-tRNA synthetase (AaRS) and tRNA. Here we focus on arginyl-tRNA synthetase (ArgRS) mediated aminoacylation of arginine tRNA ($tRNA^{Arg}$) with L-arginine (Arg). However, since sample preparation and mass spectrometric analysis are not specific to the aminoacylated $tRNA^{Arg}$, the here presented method applies to different tRNA/AaRS pairs.

Note: Be aware that the tRNA and AaRS must originate from the same species!

Note: In the following you will have to follow manuals from different manufacturers. A note will explicit deviations from a manual or give alternative procedures that are required.!

ArgRS production and purification

Timing: 5 days + delivery time of the plasmid

1. Order a plasmid containing all elements for bacterial expression of His-tagged AaRS. For *E. coli* ArgRS, order pET16b-His-ArgRS, a gift from Sebastian Maerkl & Takuya Ueda (Addgene plasmid # 124104 ; http://n2t.net/addgene:124104; (last accessed on April 20th, 2023); RRID:Addgene_124104; Addgene).[1]
2. Since the plasmid pET16b-His-ArgRS will be delivered in bacteria as agar stab, grow the bacteria at 37°C for 12–14 h in LB-medium supplemented with ampicillin (c = 100 μg/mL).

3. Pellet the culture and isolate the plasmid DNA (ZymoPURE Plasmid Miniprep Kit; Zymo Research) according to the supplied manual (https://files.zymoresearch.com/protocols/_d4208t_d4209_d4210_d4211_d4212_zymopure_plasmid_miniprep.pdf; last accessed on April 20th, 2023).

   Note: Use ultrapure water for elution.

4. Transform competent cells (BL21(DE3) Competent *E. coli*; New England Biolabs) with the purified pET16b-His-ArgRS plasmid according to the supplied manual (https://international.neb.com/protocols/0001/01/01/transformation-protocol-for-bl21-de3-competent-cells-c2527; last accessed on April 20th, 2023).

   Note: Let the transformed cells grow on a LB-agar plate with ampicillin (c = 100 μg/mL) at 37°C for 12–14 h.

5. Pick a single colony from the LB-agar plate to inoculate 50 mL LB-medium supplemented with ampicillin (c = 100 μg/mL). Incubate this preculture at 37°C for 12–14 h.

   Note: The following steps concerning the production and purification of ArgRS are performed according to[1] with minor modifications.

6. Inoculate 2 L LB-medium containing ampicillin (c = 100 μg/mL) with 20 mL of the preculture.
7. Let the culture grow at 37°C to an OD_{600} of 0.6–0.8.
8. Induce recombinant ArgRS production by adding IPTG to a final concentration of 0.1 mM.
9. Incubate the induced culture at 170 rpm for 4 h at 37°C.
10. Pellet the cells by centrifugation at 3,400 × *g* for 15 min at 4°C.
11. Resuspend the cells in 100 mL resuspension buffer (50 mM HEPES-KOH, pH 7.6, 1 M NH_4Cl, 10 mM $MgCl_2$, 0.3 mg/mL lysozyme, 0.1% Triton X-100, 0.2 mM PMSF, 7 mM 2-Mercaptoethanol)
12. Sonicate the cells (period: 60 s, duty cycle: 0.5, amplitude: 12%, time: 20 min).
13. Ultracentrifuge the lysate at 100,000 × *g* for 1 h at 4°C to remove cell debris.

   Note: For the following steps of purification, use a fast protein liquid chromatography (FPLC) system (NGC Quest 10 Plus Chromatography System, Bio-Rad).

   Note: While centrifuging, equilibrate a column suitable for His-tag-purification (HisTrap High Performance (HP) column, Sigma-Aldrich) with 10 column volumes resuspension buffer using the FPLC. For equilibration as well as for all following steps using the FPLC, set a flow rate of 3 mL/min and keep the temperature at 7°C.

14. Apply the supernatant to the equilibrated column.
15. Wash the column with 100 mL HT buffer (50 mM HEPES-KOH, pH 7.6, 1 M NH_4Cl, 10 mM $MgCl_2$, 7 mM 2-Mercaptoethanol) containing 10 mM imidazole.
16. Elute with 150 mL HT buffer with a linear gradient ranging from 10 mM to 400 mM imidazole over 50 min and collect fractions.
17. Dialyze the ArgRS containing fractions against stock buffer (50 mM HEPES-KOH, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 30% glycerol, 7 mM 2-Mercaptoethanol) three times.
    a. First dialysis for 2 h.
    b. Second dialysis for 4 h.
    c. Third dialysis for 12–14 h.

   Note: This protocol uses a Molecular weight cut-off (MWCO) of 12,000–14,000 Da for retaining ArgRS (SERVAPOR dialysis tubing, MWCO 12 000–14 000, Serva).

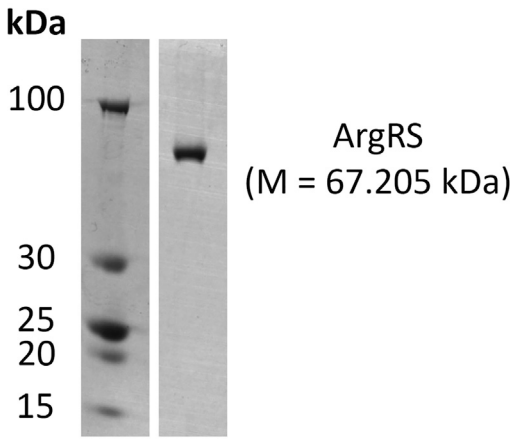

Figure 1. SDS-PAGE of purified ArgRS
The figure shows two lanes of the same gel stained with Coomassie Blue G250. The first lane represents the molecular weight marker (PageRuler Unstained Low Range Protein Ladder), the second lane 2 μg of the purified protein. The band around 67 kDa suggests the presence of the purified protein, ArgRS ofmass M = 67,205.56 Da.

18. Dilute the ArgRS with stock buffer to a concentration of 10 mg/mL.
19. Aliquot (3 μL).
20. Freeze with liquid nitrogen and store at −80°C.

Note: The presence of ArgRS can be verified by standard methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see Figure 1).

$tRNA^{Arg}$ preparation

Timing: 1.5 h + delivery time of the RNA oligonucleotide

21. Get the sequence of a tRNA corresponding to the selected AaRS (here *E. coli* $tRNA^{Arg}$ for ArgRS). Sequences can be found in the Genomic tRNA Database (http://gtrnadb.ucsc.edu/; last accessed on April 20th, 2023).[2] For *E. coli* ArgRS choose the sequence of *E. coli* $tRNA^{Arg}$ recognizing the codon CGU.

Note: Here we chose the sequence tRNA-Arg-ACG-1-1 (RNAcentral: URS0000349C05_511145) (http://gtrnadb.ucsc.edu/genomes/bacteria/Esch_coli_K_12_MG1655/genes/tRNA-Arg-ACG-1-1.html; last accessed on April 20th, 2023).

22. Order a custom synthesized RNA oligonucleotide with the sequence of interest (e.g., from Metabion).
23. Dissolve the RNA oligonucleotide in DEPC-treated water containing 10 mM $MgCl_2$ (promoting the stability of the correct tRNA conformation[3]) at a final concentration of 211.6 μM.
    a. Vortex the solution for 30 s.
    b. Let the solution rest at 18°C–22°C for 1 h.
24. As described in,[3] to ensure a correct folding of the tRNA, subject the dissolved tRNA oligonucleotide (30 μL aliquots) to thermal treatment in a thermocycler (2 min at 95°C, 3 min at 21°C, 5 min at 37°C; lid temperature: 105°C).
25. Aliquot (6 μL).
26. Freeze with liquid nitrogen and store at −80°C.

Note: The handling of RNA is difficult because of the frequent presence of RNases. Take precautions to prevent RNase contamination resulting in RNA degradation (see troubleshooting, problem 5).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
BL21(DE3) Competent *E. coli*	New England Biolabs	C2527
Chemicals, peptides, and recombinant proteins		
Acetonitrile gradient grade for HPLC (min. 99.9%) (HPLC grade ACN)	Th. Geyer	2653
Adenosine 5-triphosphate disodium salt trihydrate (ATP)	Sigma-Aldrich	ATPD-RO
Agar	Sigma-Aldrich	05039
Ammonium acetate	Grüssing	101311000
Ampicillin sodium salt	Sigma-Aldrich	A9518
Coomassie Blue G250	National Diagnostics	HS-605
DL-Dithiothreitol (DTT)	Sigma-Aldrich	D9163
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	EDS
EZ-Link Sulfo-NHS-Biotin	Thermo Fisher Scientific	21217
Formic acid 98%–100% for HPLC LiChropur (HPLC grade FA)	Sigma-Aldrich	5.43804
GelRed Nucleic Acid Stain 10000× Water (GelRed)	Sigma-Aldrich	SCT123
Glycerol	Grüssing	110122500
HCl	Fisher Scientific	H/1200/PB17
Hexakis(2,2-difluoroethoxy)phosphazene	Apollo Scientific	PC1165
N-2-Hydroxyethylpiperazine-N′-2-ethane sulphonic acid (HEPES)	Carl Roth	9105.4
Water, HPLC for gradient analysis, Fisher Chemical (HPLC grade water)	Fisher Scientific	W/0106
Imidazole	Carbolution Chemicals	CC03002
Isopropyl-β-D-thiogalactoside (IPTG)	Sigma-Aldrich	I6758
2-Propanol	Sigma-Aldrich	34863
KCl	Grüssing	120081000
KOH	Fisher Scientific	P/5640/60
L-Arginine (Arginine, Arg) buffered solution (as an ingredient of RTS Amino Acid Sampler)	Biotechrabbit	BR1401801
LB Broth (Lennox)	Sigma-Aldrich	L3022
L-Canavanine sulfate salt (Canavanine)	Sigma-Aldrich	C9758
Lysozyme from chicken egg white (Lysozyme)	Sigma-Aldrich	L6876
2-Mercaptoethanol	Sigma-Aldrich	M3148
Methyl stearate analytical standard	Sigma-Aldrich	85769
$MgCl_2$ hexahydrate	Grüssing	120881000
$MgCl_2$ solution	New England Biolabs	B9021S
NH_4Cl	VWR	21235.297
OmniPur Water, DEPC Treated, Autoclaved, Nuclease-Free (DEPC-treated water)	Sigma-Aldrich	9601-OP
PageRuler Unstained Low Range Protein Ladder	Thermo Fisher Scientific	26632
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	PMSF-RO
RNase A from bovine pancreas (RNase A)	Sigma-Aldrich	RNASEA-RO
Sodium trifluoroacetate (NaTFA)	Sigma-Aldrich	132101
Streptavidin	Thermo Fisher Scientific	21122
Triton X-100	Sigma-Aldrich	X100
Trizma base (Tris)	Sigma-Aldrich	T1503
Ultrapure water	Stakpure	OmniaTap
Critical commercial assays		
PureLink miRNA Isolation Kit	Thermo Fisher Scientific	K157001
ZymoPURE Plasmid Miniprep Kit	Zymo Research	D4209
Oligonucleotides		
tRNA-Arg-ACG-1-1: 5′-GCAUCCGUAGCUCAGC UGGAUAGAGUACUCGGCUACGAACCGAGCG GUCGGAGGUUCGAAUCCUCCCGGAUGCACCA-3′	Metabion	Custom synthesis
Recombinant DNA		
pET16b-His-ArgRS	Addgene; Maerkl & Ueda[1]	Cat# 124104

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Compass DataAnalysis	Bruker	Version 5.0 SR1
Compass HyStar	Bruker	Version 4.1 SR1
Genomic tRNA Database	Chan & Lowe[2]	http://gtrnadb.ucsc.edu/
Other		
7T solariX (Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS)) with electrospray ionization (ESI)	Bruker	NI 0204 G 002
Aeris 3.6 µm WIDEPORE XB-C8 200 Å, LC Column 150 × 2.1 mm	Phenomenex	00F-4481-AN
HisTrap High Performance (HP) column, 5 mL	Sigma-Aldrich	GE17-5248-01
ND9 vial 1.5 mL 32 × 11.6 mm amber label (HPLC vials)	VWR	548–0030
Inserts for vials with wide opening, with plastic spring, 0.1 mL (100 µL inserts)	VWR	MANA702818
NGC Quest 10 Plus Chromatography System	Bio-Rad	7880003
Pierce Concentrator, PES, 3k MWCO, 0.5 mL	Thermo Fisher Scientific	88512
Prominence UFLC XR System	Shimadzu	Controller: 228-45012-28
SERVAPOR dialysis tubing, MWCO 12 000–14 000	Serva	44146.01

Note: All consumables like microcentrifuge tubes, pipette tips, etc., must be classified as "RNase-free"!

MATERIALS AND EQUIPMENT

LB-medium with ampicillin		
Reagent	Final concentration	Amount
LB Broth (Lennox)	20 g/L	60 g
Ampicillin (add before usage)	100 µg/mL	Dependent on the used volume
Ultrapure water	N/A	Up to 3 L
Total	**N/A**	**3 L**

Autoclave at 121°C for 20 min and store at 18°C–22°C. Maximum storage time is one month.

Note: Add ampicillin right before usage of the medium. For this purpose, use ampicillin stock solution (c = 100 mg/mL) to get a final concentration of 100 µg/mL.

LB-agar plate with ampicillin		
Reagent	Final concentration	Amount
LB Broth (Lennox)	20 g/L	10 g
Agar	15 g/L	7.5 g
Ampicillin (add after autoclaving and cooling down to approximately 60°C)	100 µg/mL	500 µL of an ampicillin stock solution (c = 100 mg/mL)
Ultrapure water	N/A	Up to 500 mL
Total	**N/A**	**500 mL**

Autoclave at 121°C for 20 min, let the solution cool down to approximately 60°C, add 500 µL of ampicillin stock solution (c = 100 mg/mL) to a final concentration of 100 µg/mL, pour the medium into petri dishes, let them cool down and store upside-down at 4°C. Maximum storage time is one month.

Resuspension buffer		
Reagent	Final concentration	Amount
HEPES (adjust pH to 7.6 with KOH)	50 mM	1.787 g
NH_4Cl	1 M	8.023 g
$MgCl_2$ hexahydrate	10 mM	304.9 mg
Lysozyme	0.3 mg/mL	45 mg
Triton X-100	0.1%	150 µL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
PMSF	0.2 mM	Dependent on the used volume
2-Mercaptoethanol (add before usage)	7 mM	Dependent on the used volume
Ultrapure water	N/A	Up to 150 mL
Total	**N/A**	**150 mL**

Store at 4°C. Maximum storage time is two weeks.

Note: Add PMSF and 2-Mercaptoethanol right before usage of the buffer. For PMSF, use a PMSF stock solution (c = 200 mM) to get a final concentration of 0.2 mM.

HT buffer		
Reagent	Final concentration	Amount
HEPES (adjust pH to 7.6 with KOH)	50 mM	11.915 g
NH_4Cl	1 M	53.49 g
$MgCl_2$ hexahydrate	10 mM	2.033 g
2-Mercaptoethanol (add before usage)	7 mM	Dependent on the volume
Ultrapure water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Split the buffer into 3 bottles (1 × 100 mL, 2 × 450 mL). Add imidazole to one 450 mL bottle to get a final imidazole-concentration of 10 mM. Add imidazole to the other 450 mL bottle to get a final imidazole-concentration of 400 mM. Store at 18°C–22°C. Maximum storage time is one month.

Note: Add 2-Mercaptoethanol right before usage of the buffer.

Stock buffer		
Reagent	Final concentration	Amount
HEPES (adjust pH to 7.6 with KOH)	50 mM	35.746 g
KCl	100 mM	22.365 g
$MgCl_2$ hexahydrate	10 mM	6.099 g
Glycerol	30%	900 mL
2-Mercaptoethanol (add before usage)	7 mM	Dependent on the volume
Ultrapure water	N/A	Up to 3 L
Total	**N/A**	**3 L**

Store at 18°C–22°C. Maximum storage time is one month.

Note: Add 2-Mercaptoethanol right before usage of the buffer.

10 x reaction buffer		
Reagent	Final concentration	Amount
HEPES	500 mM	5.958 g
KCl	250 mM	931.9 mg
$MgCl_2$ hexahydrate	150 mM	1.525 g
DTT (add before usage)	1 mM	Dependent on the volume
DEPC-treated water	N/A	Up to 50 mL
Total	**N/A**	**50 mL**

Adjust pH to 7.5 with KOH. Store at 4°C. Maximum storage time is one month.

Note: Add DTT right before usage of the buffer. For this purpose, use a DTT stock solution (c = 1 M) to get a final concentration of 1 mM.

HEPES buffer		
Reagent	Final concentration	Amount
HEPES	120 mM	1.43 g
Ultrapure water	N/A	Up to 50 mL
Total	**N/A**	**50 mL**

Adjust pH to 8.0 with KOH. Store at 18°C–22°C. Maximum storage time is one month.

RNase A stock solution		
Reagent	Final concentration	Amount
Tris (adjust pH to 7.5 with HCl)	10 mM	
EDTA	1 mM	
Glycerol	50%	15.625 mL
RNase A (add at last)	4 U/100 μL	25 mg
Ultrapure water	N/A	Up to 31.25 mL
Total	**N/A**	**31.25 mL**

Store at −20°C. Maximum storage time is 6 months.

Note: Prepare the solution first without RNase A. Subsequently dissolve the RNase A powder in the solution.

- DEPC-treated water containing 10 mM MgCl2: add 1 mL $MgCl_2$ solution (c = 25 mM) to 1.5 mL DEPC-treated water.

Store at −20°C. Maximum storage time is 6 months.

- Ampicillin stock solution (c = 100 mg/mL): add 5 g Ampicillin sodium salt in 50 mL ultrapure water.

Aliquot (1 mL). Store at −20°C. Maximum storage time is 6 months.

- PMSF stock solution (c = 200 mM): add 34.8 mg PMSF in 1 mL 2-Propanol.

Store at −20°C. Maximum storage time is 6 months.

- DTT stock solution (c = 1 M): add 771.2 mg DTT in 5 mL ultrapure water.

Aliquot (1 mL). Store at −20°C. Maximum storage time is 6 months.

- Arginine stock solution (c = 10 mM): add 10 μL L-Arginine (Arginine, Arg) buffered solution (c = 168 mM) to 158 μL DEPC-treated water.

Store at −20°C. Maximum storage time is 6 months.

- Arginine working solution (c = 18.3 μM): add 1.83 μL Arginine stock solution to 998.17 mL DEPC-treated water.

Store at −20°C. Maximum storage time is 6 months.

- Eluent A: add 1 mL HPLC grade FA to 999 mL HPLC grade water.

Store at 18°C–22°C. Maximum storage time is one month.

- Eluent B: add 1 mL HPLC grade FA to 999 mL HPLC grade ACN.

Store at 18°C–22°C. Maximum storage time is one month.

- Methyl stearate solution (c = 5 mg/ mL): add 5 mg methyl stearate analytical standard in 1 mL HPLC grade water.

Store at 4°C. Maximum storage time is one month.

- Hexakis(2,2-difluoroethoxy)phosphazene solution (c = 1 mg/mL): add 1 mg hexakis(2,2-difluoroethoxy)phosphazene in 1 mL HPLC grade water.

Store at 4°C. Maximum storage time is one month.

- NaTFA solution (c = 0.1 mg/mL): add 10 mg NaTFA in 100 mL HPLC grade water.

Store at 4°C. Maximum storage time is one month.

⚠ CRITICAL: Most natural amino acids can be ordered readily in solution. However, the amino acid stock solutions can also be prepared in the lab. This may be required for amino acids that are available in solid form only. Be aware that some amino acids may not dissolve well in water upon addition of acid/base or special salts.[4,5] Therefore, check optimal conditions for each amino acid to be dissolved!

⚠ CRITICAL: When working with liquid nitrogen, wear face shield, Cryo-gloves and lab coat. Liquid nitrogen may cause severe burns!

⚠ CRITICAL: Some of the chemicals/reagents may pose a health hazard. Always check against the corresponding material safety datasheet!

The following chemicals/reagents exhibit acute toxicity: ACN, Canavanine, DTT, EZ-Link sulfo-NHS biotin, imidazole, 2-Mercaptoethanol, NH_4Cl, PMSF.

The following chemicals can cause severe eye damage and skin burns: HCl, KOH, Triton X-100.

The following chemicals/reagents cause severe irritation: Ampicillin sodium salt, EDTA, hexakis(2,2-difluoroethoxy)phosphazene, 2-Propanol.

It is recommended to wear nitrile gloves and safety goggles while working. Carry-over of contaminations must be prevented!

The work should be carried out in a well-ventilated room. It is strongly recommended to work under a fume hood while using ACN, 2-Mercaptoethanol and HCl. These substances release hazardous vapors. ACN can form explosive mixtures with air!

Alternatives: This protocol uses the NGC Quest 10 Plus Chromatography System and a HisTrap High Performance (HP) column for purification of ArgRS. Any other FPLC system and a compatible immobilized metal affinity chromatography (IMAC) column for His-tag recombinant protein purification can be used. For chromatography and mass spectrometry this protocol uses an LC-MS (liquid chromatography with mass spectrometry) system consisting of a high-performance liquid chromatography (HPLC) system (Prominence UFLC XR System, Shimadzu) and a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) (7T solariX, Bruker) with electrospray ionization (ESI). Other systems with

Table 1. Reagents of an aminoacylation reaction mix

Reagent	Final concentration	Amount
Arginine working solution (18.3 μM)	14 μM	115 μL
10 x reaction buffer (500 mM HEPES, 250 mM KCl, 150 mM $MgCl_2$, 1 mM DTT, pH 7.5)	1×	15 μL
DEPC-treated water	N/A	7.48 μL
ATP solution (100 mM)	3.3 mM	4.95 μL
$tRNA^{Arg}$ (211.6 μM)	8 μM	5.67 μL
ArgRS (10 mg/mL)	126 μg/mL	1.9 μL

The table lists the reagents and their amounts to obtain the given, final concentrations.

comparable sensitivity and resolution can be used. However, the ionization of the analyte might be affected by another ion source! The HPLC column (Aeris 3.6 μm WIDEPORE XB-C8 200 Å, LC Column 150 × 2.1 mm, Phenomenex) can be replaced by any other comparable HPLC column. The dialysis tubing (SERVAPOR dialysis tubing, MWCO 12 000–14 000, Serva) can be replaced by alternative dialyzing equipment.

Alternatives: This protocol uses a ZymoPURE Plasmid Miniprep Kit for plasmid purification. Any other plasmid purification kit will work as well. For production of ArgRS, the plasmid pET16b-His-ArgRS is transferred into "BL21(DE3) Competent *E. coli*". The plasmid is expressed well in this strain. A different ArgRS synthesis mediating plasmid may require changing the strain or organism for expression as well. Any company in the field will be able to provide $tRNA^{Arg}$ custom synthesis. However, the quality of the oligonucleotide may vary. For separation of RNase A and the digestion product, the protocol uses Pierce Concentrator, PES, 3k MWCO, 0.5 mL. Any other separator will do. The PureLink miRNA Isolation Kit may be essential for RNA purification. Although purification through phenol-chloroform extraction and alcohol precipitation as described in[3] will also work, the kit avoids the use of toxic/harmful substances. Moreover, it is faster and easier to perform. Other RNA purification kits may not be as suitable as our recommendation here since they may or may not remove remaining free amino acids from the reaction mix. Insufficient separation between $tRNA^{Arg}$ bound and remaining free amino acids will cause problems later during mass spectrometry since it will remain unclear if prior to measurement the amino acids were bound to $tRNA^{Arg}$ or not.

STEP-BY-STEP METHOD DETAILS

The protocol below is the main part of this publication. It describes the aminoacylation reaction, the sample preparation, and the mass spectrometric analysis.

Aminoacylation reaction

Timing: 3.5 h

The aminoacylation reaction is performed according to[3] with minor modifications.

1. Prepare a fresh ATP solution (c = 100 mM). For this purpose, dissolve 6.0519 mg ATP in 100 μL DEPC-treated water.
2. Mix the reagents from Table 1 in the listed order to obtain a reaction mix.
3. As a reference, prepare a second reaction mix without ArgRS (use DEPC-treated water as a substitute).
4. Incubate at 37°C for 3 h. During this time, arginine is attached to the 2′ OH-group of the 3′-terminal adenosine of $tRNA^{Arg}$.[6]

Sample preparation

Timing: 1 day

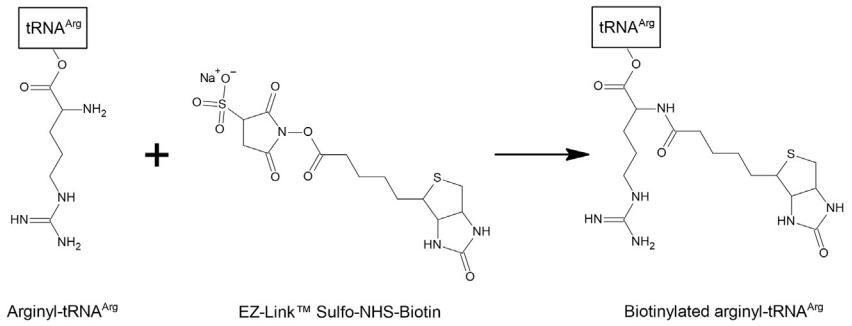

Figure 2. Biotinylation of arginyl-tRNA^{Arg}

The figure shows the reaction of arginyl-tRNA^{Arg} with EZ-Link Sulfo-NHS-Biotin. EZ-Link Sulfo-NHS-Biotin reacts with primary amines[7,9] while the guanidine group will be unaffected.[8] The resulting molecule is biotinylated arginyl-tRNA^{Arg}.

At this stage the other ingredients in the reaction mix besides the tRNA^{Arg} of interest preclude mass spectrometric detection. Detection of aminoacylated tRNA^{Arg} in the presence of remaining free tRNA^{Arg} is a challenge. Accordingly, the following steps describes purification and digestion of the (aminoacylated) tRNA^{Arg}. Treat each reaction mix similarly.

5. For purification of tRNA^{Arg} (non-aminoacylated or aminoacylated with arginine), use a RNA purification kit (PureLink miRNA Isolation Kit; Invitrogen) and follow the supplied manual (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fpurelink_microRNA_man.pdf; last accessed on April 20th, 2023).

   Note: Use one column per reaction mix, start from step 5 in the section "Prepare bacterial lysates" of the manual mentioned above. Use a final elution volume of 50 µL.

6. Biotinylate aminoacylated tRNA^{Arg} according to[7] with minor modifications. Treat the reference as well.
   a. Dissolve 6.6 mg EZ-Link Sulfo-NHS-Biotin in 100 µL HEPES buffer (120 mM HEPES, pH 8.0).
   b. Add 50 µL of the EZ-Link Sulfo-NHS-Biotin solution to each purified tRNA^{Arg} sample.
   c. Incubate at 4°C for 12–14 h.

   Note: Figure 2 shows the reaction for arginyl-tRNA^{Arg}. In solution the NHS esters do not target the guanidine group which remains non-biotinylated.[8]

   Note: Biotinylation stabilizes the acyl bond between the tRNA and the amino acid and prevents deacylation that might occur at physiological pH.[7] The free tRNA^{Arg} in the sample for reference remains non-biotinylated. In addition, biotin acts as a label for mass spectrometry.[9,10]

   Note: At this stage, aminoacylation can be checked for. The method uses toxic/harmful chemicals. How to detect biotinylated aminoacylated tRNA using streptavidin and SDS-PAGE is presented in.[7] This method can be modified by first staining the gel for nucleic acid (see Figure 3A), and subsequently for protein (see Figure 3B). Taken together this shows the presence of biotinylated, aminoacylated tRNA attached to streptavidin, corresponding to a successful aminoacylation reaction. However, it is not possible to determine which amino acids were attached to the tRNA. In addition, Moreover, it is hard to make any statements about the amounts.

7. Digest tRNA^{Arg}.

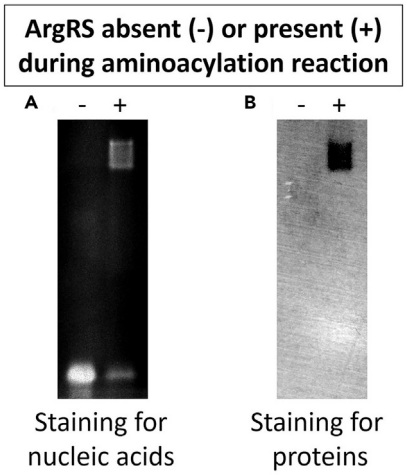

Figure 3. SDS-PAGE of tRNA^{Arg} and biotinylated arginyl-tRNA^{Arg} with streptavidin attached

The gel was stained with GelRed to visualize nucleic acids (tRNA^{Arg}, A) and Coomassie Blue G250 to visualize protein (streptavidin, B). The two lanes represent an aminoacylation reaction mix where ArgRS was absent (-) or present (+). Without ArgRS, tRNA^{Arg} is not aminoacylated so that no biotinylation will occur and streptavidin cannot bind. In the presence of ArgRS arginyl-tRNA^{Arg} will be biotinylated. Streptavidin can then bind and increase the mass of the construct substantially. Nucleic acid staining shows a strong band of tRNA^{Arg} (see lane A-). This band is also present in lane A+, but with much lower intensity (remaining free tRNA^{Arg}). In lane A+, an additional band is present. Using protein stain only, this band is still observed (see lane B+) while the bands of tRNA^{Arg} are not (see lane B- and B+). Double staining reveals the presence of nucleic acids (tRNA^{Arg}) as well as proteins (streptavidin). This shows the presence of streptavidin-bound biotinylated arginyl-tRNA^{Arg}.

a. Add 25 μL RNase A stock solution (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50% Glycerol, 4 U/100 μL RNase A) and 5 μL of ammonium acetate solution (c = 220 mM) to each sample.
b. Incubate at 37°C for 2 h.

Note: Mass spectrometric assessments of heterogenous samples with complex ingredients in small quantities is challenging.[11] Here, the digestion of tRNA enables direct detection of tRNA^{Arg} fragments[12] (with and without attached biotinylated amino acid). No further processing of the data (e.g., deconvolution) is required so that peaks can be directly interpreted. .

Note: The molecule of interest resulting from the digestion of biotinylated aminoacylated tRNA^{Arg} is the tRNA^{Arg} fragment with attached biotinylated amino acid, biotinylated arginyl-adenosine (see Figure 4). In the sample for reference, this molecule is missing.

8. Use protein concentrators (Pierce Concentrator, PES, 3k MWCO, 0.5 mL; Thermo Fisher Scientific) to separate digested tRNA^{Arg} and RNase A.

Note: Use one concentrator per sample.

a. Add 500 μL ultrapure water to pre-rinse the concentrator.
b. Centrifuge at 15,000 × *g* for 5 min.
c. Remove ultrapure water from both chambers.
d. Add the sample to the concentrator.
e. Centrifuge at 15,000 × *g* for 3 h. 3 h are required because of the glycerol in the RNase stock solution.
f. Transfer filtrated fraction to a new vial and store at 4°C.

Note: The concentrators retain RNase A. The filtrated fraction contains molecules smaller than 3 kDa and therefore the tRNA^{Arg} fragments (with and without attached biotinylated amino acid) as obtained from digestion.

Mass spectrometry

Timing: 2 h

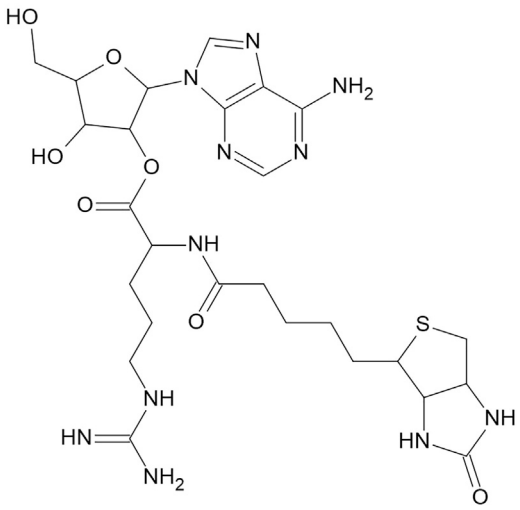

Figure 4. Molecule of interest obtained from RNA digestion of biotinylated arginyl-tRNA^{Arg}

RNase A will cleave the tRNA^{Arg} after each C and U resulting in Cp or Up at the 3′ end of the fragment.[11] During the aminoacylation of tRNA^{Arg}, the amino acid links to the CCA sequence at the 3′ end of the tRNA^{Arg}.[13] Accordingly, the last cleavage site in front of the attached amino acid will be after the last C. The resulting molecule is biotinylated arginyl-adenosine. This is the molecule of interest since it will show which amino acid was attached to the tRNA^{Arg} during aminoacylation.

The final step describes the mass spectrometric method identifying the amino acid of the aminoacylated tRNA^{Arg}. Detection of the tRNA^{Arg} fragment with attached biotinylated amino acid requires a LC-MS system. Here we use a HPLC system (Prominence UFLC XR System, Shimadzu) coupled with a FT-ICR-MS (7T solariX, Bruker) with ESI. Both instruments communicate via a plug-in (Compass HyStar, Bruker). The HPLC system uses a HPLC column suitable for separation of the different tRNA^{Arg} fragments (Aeris 3.6 μm WIDEPORE XB-C8 200 Å, LC Column 150 × 2.1 mm, Phenomenex).

9. Use two eluents (A and B).
    a. Eluent A (HPLC grade water with 0.1% HPLC grade FA).
    b. Eluent B (HPLC grade ACN with 0.1% HPLC grade FA).
10. Define a separation method for the HPLC system (Prominence UFLC XR System, Shimadzu).
    a. Set the column oven temperature to 60°C.
    b. Set the autosampler temperature to 7°C.
    c. Equilibrate the column with 10% of eluent B at a flow rate of 0.5 mL/min until start of the measurement.

    Note: In this step and the following, only the percentage of eluent B is mentioned. The lacking percentage corresponds to eluent A.

    Note: The equilibration takes 10 min. It should be performed prior to calibration of the mass spectrometer.

    d. Use 20 μL of the sample for the measurement.
    e. Use a linear gradient program with a constant flow rate of 0.5 mL/min for separation of the molecules in the sample.
        i. Increase the percentage of eluent B from 10% to 30% over 10 min.
        ii. Increase the percentage of eluent B from 30% to 65% over 10 min.
        iii. Hold 65% of eluent B for 3 min.
        iv. Decrease the percentage of eluent B from 65% to 10% over 2 min.
        v. Hold 10% of eluent B for 10 min.

Note: The last step will equilibrate the column for the next measurement.

Note: HPLC parameters may vary for other columns.

11. Define a method for the mass spectrometer (7T solariX, Bruker).
    a. Use the following parameters.
        i. Use positive mode.

    Note: m/z-values represent $[M + H]^+$.

        ii. Use a mass range from 100 to 1000 Da.
        iii. Set the capillary voltage of the ESI to 4500 V with an End Plate Offset of −500 V.
        iv. Set the Nebulizer Gas pressure to 3.0 bar.
        v. For the Dry Gas, use a flow rate of 8 L/min and a temperature of 250°C.
        vi. Acquire single scans with a scan time of 50 ms.

    Note: Parameters regarding ESI and the mass spectrometer may vary for other types of mass spectrometers.

    b. To prevent mass drift over longer measurement periods and to maintain a high accuracy use two lock masses during measurement.
        i. Use methyl stearate (299.294457 m/z). For the 7T solariX, apply 50 µL of a methyl stearate solution (c = 5 mg/mL) to the ceramic slides of the lock mass reservoir.
        ii. Use hexakis(2,2-difluoroethoxy)phosphazene (622.028960 m/z). For the 7T solariX, apply 50 µL of a hexakis(2,2-difluoroethoxy)phosphazene solution (c = 1 mg/mL) to the ceramic slides of the lock mass reservoir.

    Note: The masses of the analytes should always be in between the two lock masses.

12. Calibrate the mass spectrometer.
    a. Use a NaTFA solution (c = 0.1 mg/mL) for calibration.
    b. Inject the solution with the syringe pump of the mass spectrometer setting a flow rate of 2 µL/min.
    c. Acquire 64 scans with the parameters listed in 11 a.

    Note: The scans must be accumulated (single scans are insufficient!).

    d. Calibrate the mass spectrometer with the obtained spectrum.

    Note: Calibration should achieve an accuracy of 1 ppm. If the error is higher, repeat the steps 12 b–12 d.

    e. Save the calibration.
13. Change the 6-port diverter valve of the 7T solariX back to HPLC.
14. Set the number of scans to 1.
15. Select serial mode.
16. Prepare the 2 samples (digested $tRNA^{Arg}$; free and aminoacylated with arginine).
    a. Use 90 µL of the filtrated fraction from step 8 f and add 10 µL of HPLC grade ACN to obtain a final ACN concentration of 10% in the sample. Add 0.1 µL of HPLC grade FA.
    b. Vortex the sample for 10 s.
    c. Briefly centrifuge for 10 s.
    d. Transfer the sample to 100 µL inserts placed in HPLC vials.
    e. Put the HPLC vials into the autosampler (pre-chilled at 7°C).

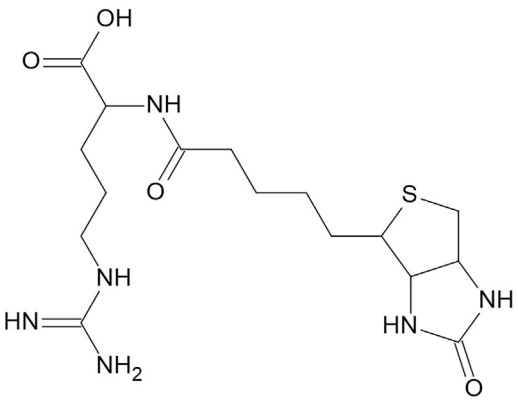

Figure 5. Molecule detected by mass spectrometry

The molecule of interest (biotinylated arginyl-adenosine) could only be detected with low intensity, however, mass spectrometry revealed the presence of biotinylated arginine exhibiting a much stronger peak. This molecule is missing in the sample for reference, showing that it originates from the purified aminoacylated $tRNA^{Arg}$ – and not from the aminoacylation reaction mix (containing free amino acids).

Biotinylated arginine

17. Create a sample list in Compass HyStar and select the written separation and measurement method as well as the saved calibration.
18. Perform a measurement on the 2 samples.
19. Use a suitable program (Compass DataAnalysis, Bruker) for the evaluation of the obtained data.
20. Recalibrate the data using the lock masses.
21. Extract the ion chromatogram of the analyte. The selected m/z depends on the amino acid that was attached to the $tRNA^{Arg}$ via the aminoacylation reaction.

Note: In the mass spectrum the expected analyte, biotinylated arginyl-adenosine (650.28274 m/z), is identified as well as biotinylated arginine (see Figure 5). Since biotinylated arginine was absent from the sample for reference, it must originate from biotinylated arginyl-adenosine, that is, from the aminoacylated $tRNA^{Arg}$. The removal of adenosine is likely due to acidic hydrolysis caused by FA in the solvent.

Note: A standard is required to confirm the m/z value of the analyte. Since a standard of biotinylated arginine is easily obtained (see step 24), we decided to focus on this analyte. However, for quantitative analysis the presence of both compounds must be considered. Here, for qualitative analysis, the analyte of interest is biotinylated arginine. Extract the ion chromatogram of biotinylated arginine (401.19655 m/z).

Note: Theoretical m/z used for ion chromatogram extraction are calculated with the IsotopePattern tool in Compass DataAnalysis (Bruker). The deviation of the measured m/z from the theoretical m/z is determined by the SmartFormula tool in Compass DataAnalysis (Bruker). It must not exceed the error of the calibration of the instrument (here 1 ppm).

22. Analyze the extracted peaks.
23. Examine the compound spectrum (The m/z deviation must not be higher than 1 ppm).
24. Verify that the analyzed molecule is biotinylated arginine by measurement with a suitable standard.
    a. Dissolve 3.3 mg EZ-Link Sulfo-NHS-Biotin in 50 μL HEPES buffer (120 mM HEPES, pH 8.0).
    b. Add 50 μL of arginine working solution.
    c. Incubate at 4°C for 12–14 h.
    d. Measure the sample without further purification with the mass spectrometer as described in this section.

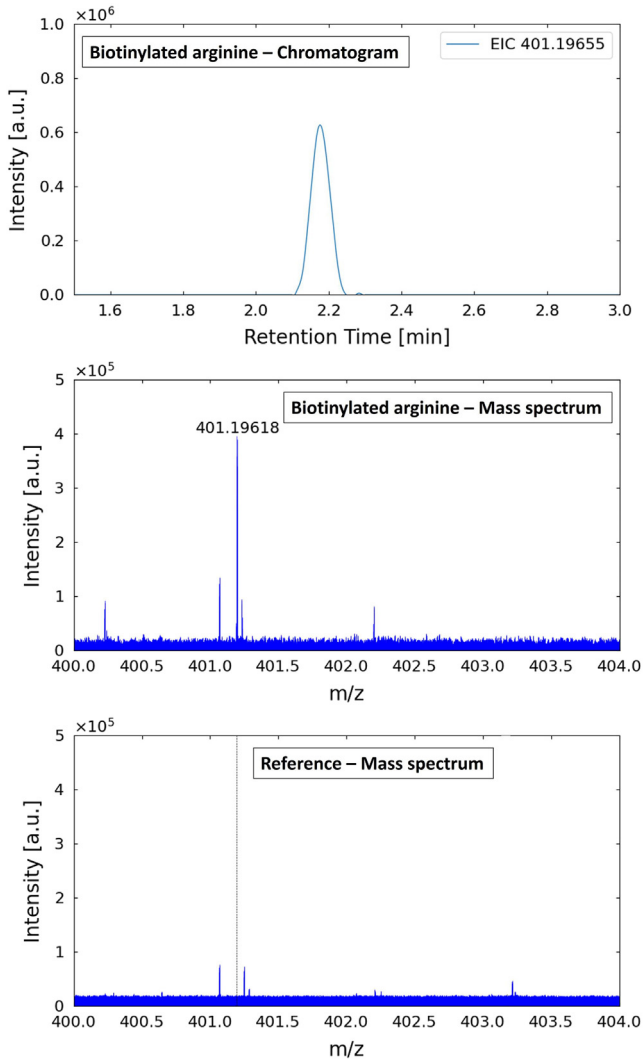

Figure 6. Chromatogram (top) and mass spectrum (middle) of biotinylated arginine

The measured m/z (401.19618 m/z) is within the range of the theoretical m/z (401.19655 m/z), showing that the detected molecule can be attributed to the prediction. Moreover, the chromatogram of the reference (no ArgRS during aminoacylation reaction) exhibits no peak at the retention time of interest (data not shown). The mass spectrum at this retention time (bottom) shows no signal of biotinylated arginine at all (dotted vertical line). Accordingly, the biotinylated arginine was already bound to $tRNA^{Arg}$ prior to measurement.

Note: The retention time and the m/z of the analyzed molecule must not significantly deviate from the standard.

EXPECTED OUTCOMES

Ion chromatogram extraction will create a chromatogram showing the intensity of the selected m/z over time (see Figure 6, top). Analysis of the chromatogram-peaks will deliver mass spectra showing the measured m/z (see Figure 6, middle). The m/z-peaks must not diverge more than 1 ppm from the theoretical m/z. Moreover, any related signals must be absent in the reference sample (no ArgRS during aminoacylation reaction) (see Figure 6, bottom).

The results presented above show that this protocol works well to investigate the aminoacylation of $tRNA^{Arg}$ with arginine. Since the method only depends on the presence of aminoacylated tRNA,

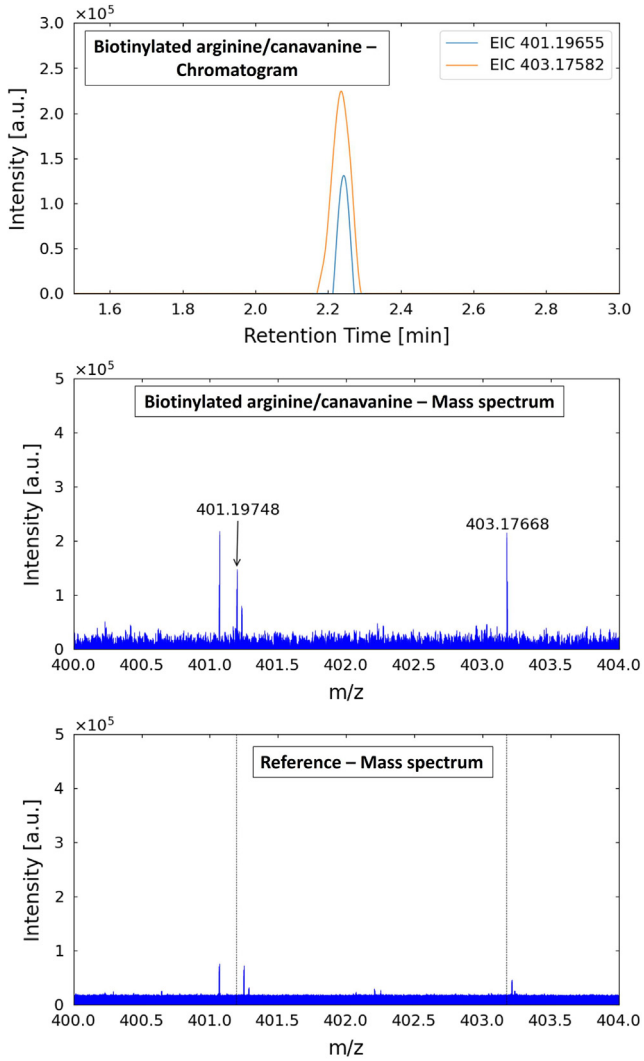

Figure 7. Chromatogram (top) and mass spectrum (middle) of biotinylated arginine/canavanine

For the presented data, canavanine and arginine were simultaneously present in an aminoacylation reaction mix (c(arginine) = 1.8 μM, c(canavanine) = 12.2 μM). The measured m/z (401.19748 m/z for biotinylated arginine and 403.17668 m/z for biotinylated canavanine) is within the range of the theoretical m/z (401.19655 m/z for biotinylated arginine and 403.17582 m/z for biotinylated canavanine). The mass spectrum of the reference (no ArgRS during aminoacylation reaction; bottom) shows no signal of biotinylated arginine/canavanine at all (dotted vertical lines). Since biotinylated arginine as well as biotinylated canavanine is detected, the presented method can give an idea on how much of each amino acid was bound to $tRNA^{Arg}$. We expect that the assessment can be made quantitative as described at the end of this section.

independently of the kind of amino acid considered, the method is also suitable for the analysis of other amino acids bound to tRNA. Further, quantification experiments determining the amount of tRNA-bound amino acids may be performed as described at the end of this paragraph. In this context, the presented method can be used to study the error rates in aminoacylation, since different amino acids can simultaneously be present in the aminoacylation reaction and quantities of the bound amino acids can be determined. Figure 7 shows an example of such an experiment where arginine as well as the arginine analog canavanine were present during the aminoacylation reaction.

To increase our confidence, measurements with standards (free amino acids that were biotinylated prior to the measurement) are taken (see Figure 8).

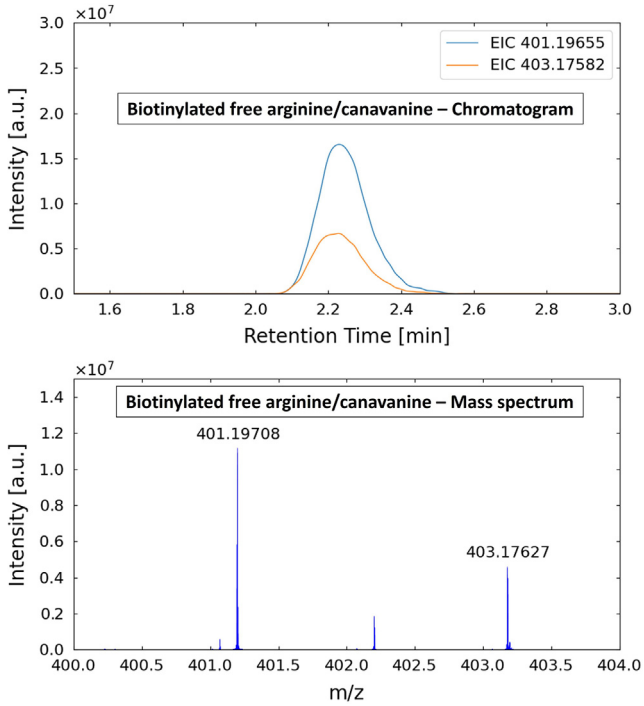

Figure 8. Chromatogram (top) and mass spectrum (bottom) of biotinylated arginine/canavanine (standard)

The reaction was performed with free amino acids and with EZ-Link Sulfo-NHS-Biotin. The resulting peaks correspond to biotinylated amino acids. Canavanine and arginine were present at the same concentration (c = 9.2 μM) in the reaction solution. We obtain a retention time of around 2.2 min for both species and a m/z of 401.19708 for biotinylated arginine and 403.17627 for biotinylated canavanine.

Comparing Figures 6 and 7 with Figure 8, similar retention times of around 2.2 min can be observed. The measured m/z differ by about 0.9 ppm from the theoretical m/z, within the accuracy of the instrument.

> *Note:* In contrast to arginine having a single reaction site for NHS esters,[8] the oxoguanidine group of canavanine can be biotinylated as well, resulting in doubly biotinylated canavanine. Although singly as well as doubly biotinylated canavanine is present simultaneously (data not shown), here we limited ourselves to the singly biotinylated species.

The method can be used for any aminoacylation reaction using different tRNA/AaRS pairs. Table 2 shows the expected m/z values for biotinylated canonical amino acids.

Besides the listed canonical amino acids, the method can also be used for non-canonical ones. However, the masses of the amino acids need to be known, since the molecule specific ion chromatograms must be extracted. Be aware that amino acids with additional primary amine groups like lysine may become biotinylated multiple times.[8,10] This must be considered for ion chromatogram extraction.

The method can be used to confirm the identity of non-canonical amino acids bound to tRNAs. AaRS engineering is one field that may benefit.[5] In addition, the method can be applied to determine the amino acid of self-aminoacylating ribozymes.[16]

The here presented method gives a first hint regarding the amount of tRNA bound amino acids. This can be improved further to achieve quantitative results. However, biotinylated aminoacyl-adenosine as well as biotinylated amino acids are present in the sample (both originating from aminoacyl-tRNA).

Table 2. Expected m/z values for biotinylated canonical amino acids

Amino acid	Expected m/z of biotinylated amino acid
Alanine	316.13255 m/z
Arginine	401.19655 m/z
Asparagine	359.13837 m/z
Aspartic acid	360.12238 m/z
Cysteine	348.10463 m/z
Glutamine	373.15402 m/z
Glutamic acid	374.13803 m/z
Glycine	302.1169 m/z
Histidine	382.15435 m/z
Isoleucine	358.1795 m/z
Leucine	358.1795 m/z
Lysine	373.1904 m/z + 600.27583 m/z (doubly biotinylated)
Methionine	376.13593 m/z
Phenylalanine	392.16385 m/z
Proline	342.1482 m/z
Serine	332.12747 m/z
Threonine	346.14312 m/z
Tryptophan	431.17475 m/z
Tyrosine	408.15877 m/z
Valine	344.16385 m/z

The method cannot distinguish between leucine and isoleucine since both have the same m/z. Amino or hydroxyl groups in the side chain of an amino acid may also be reactive to NHS esters[8,10,14,15] resulting in doubly biotinylated amino acids. However, such reactions are not occurring in aqueous condition, except for lysine.[8,10,14,15]

Since only a standard for biotinylated amino acids can be easily obtained, the sample should be pretreated before the LC-MS measurement to achieve a complete conversion of biotinylated aminoacyl-adenosine to biotinylated amino acids. For quantification, a calibration curve must be established by measuring the standard (biotinylated amino acid of interest) at different, known concentrations. The curve will give the relationship between concentration and peak area. For all measurements (calibration curve and samples) a different, internal standard must be added at a specific concentration prior to LC-MS. The mass spectra are then normalized with the peak area of the internal standard. With the peak area of the biotinylated amino acid in the sample and the calibration curve, the amount of bound amino acids can be determined quantitatively. Moreover, the quantitative analysis applies as well to determine how much of the amino acid and its corresponding analog are bound to tRNA if both were present simultaneously during the aminoacylation reaction.

LIMITATIONS

The here described method applies to any aminoacylation reaction, however, it cannot be used if the masses of the amino acids are unknown. This might be the case for a pool of unknown amino acids with the aim to find out which amino acid is bound to the tRNA.

TROUBLESHOOTING

Problem 1

No peak in the extracted ion chromatogram (section "step-by-step method details", step 21).

Potential solution

- Check the expected amino acid for multiple primary amine groups or possible reactions during biotinylation. You can make measurements with a standard (as described in section "step-by-step method details", step 24) to check which molecule results from biotinylation.

- Repeat the experiment and check if tRNA was aminoacylated by the reaction. To do so, make an assay according to the third note following step 6 in the section "step-by-step method details".

Problem 2

Measured m/z diverges from the theoretical m/z more than the calibration suggests (section "step-by-step method details", step 23).

Potential solution

- Make a measurement with a standard (as described in section "step-by-step method details", step 24) to check if the molecule resulting from this biotinylation leads to a peak of the same m/z.
- Perform MS/MS.

Problem 3

No or low aminoacylation during aminoacylation reaction (section "step-by-step method details", step 4).

Potential solution

- Prepare the solutions freshly and repeat the reaction.
- Check for protein degradation by comparing the current concentration with the initial concentration.
- Check for tRNA degradation by comparing the current concentration with the initial concentration.
- Check if step 24 in the section "before you begin" was performed. This step is crucial since aminoacylation may not occur on misfolded tRNA.

Problem 4

Low AaRS levels in the AaRS aliquots (section "step-by-step method details", step 2).

Potential solution

- If the protein level is lower than initially, produce fresh protein (as described in the section "before you begin - ArgRS production and purification"). Add protease inhibitors to prevent protein degradation right after the AaRS production and purification.
- Adjust the aliquot volume to the protein concentration to prevent multiple freeze-thaw cycles.

Problem 5

Low tRNA levels in the tRNA aliquots (section "step-by-step method details", step 2) or at a later stage (section "step-by-step method details", until step 7).

Potential solution

- If the tRNA level is lower than initially, prepare fresh tRNA (as described in section "before you begin - tRNAArg preparation"). However, be aware that RNases may be present. This makes handling RNA difficult. Therefore, the following steps are crucial.
- Wash the workspace with solutions that remove RNases.
- Check that all consumables like microcentrifuge tubes, pipette tips, etc., are classified as "RNase-free".
- Check all substances for RNase activity.
- Ensure that DEPC-treated water was used during all steps until digestion (section "step-by-step method details", step 7).
- Adjust the aliquot volume to the tRNA concentration to prevent multiple freeze-thaw cycles.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Albrecht Ott (albrecht.ott@physik.uni-saarland.de).

Materials availability

No new materials were generated in this study.

Data and code availability

No datasets or codes were generated in this study.

ACKNOWLEDGMENTS

This work was supported by the collaborative research center SFB 1027 funded by the Deutsche Forschungsgemeinschaft (DFG) and (in part) by the Human Frontier Science Program (HFSP, RGP0037/2015).

We thank Klaus Hollemeyer and Björn Finkler for help and fruitful discussions.

AUTHOR CONTRIBUTIONS

A.O. and M.F. designed the research. M.F. performed the research with the help of J.B., T.B., and F.G. J.B. performed the mass spectrometry with help from M.F. M.F. and J.B. analyzed data with help from A.O., T.B., and P.H. M.F. and A.O. wrote the paper with help from J.B. A.O. supervised.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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