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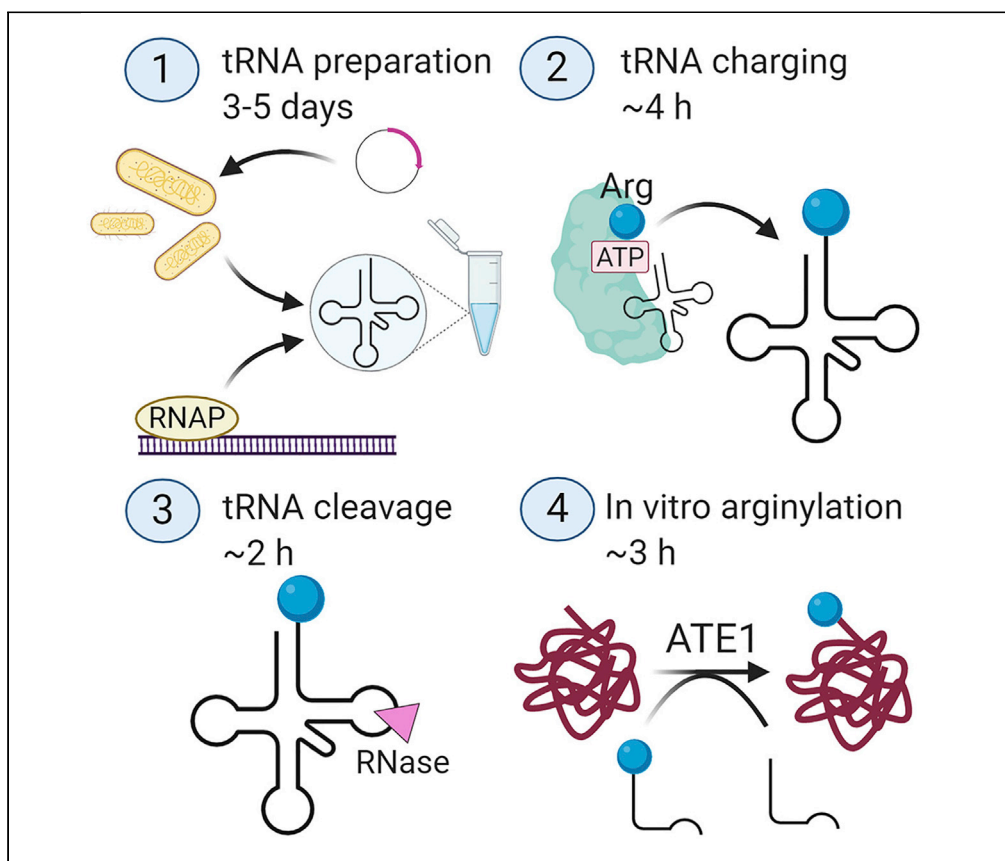
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Protocol

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Post-translational addition of amino acids to proteins by enzymes using aminoacyl-tRNA is an emerging regulatory mechanism. Examples include Arg transfer in eukaryotes, Leu/Phe transfer in bacteria, and tRNA-synthetase-mediated addition of amino acids to Lys side chains. Here, we present a method of purification and use of tRNA for such reactions, focusing on tRNA^{Arg} and its use for arginylation. This method can also be used for other tRNA-mediated reactions.

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HIGHLIGHTS

Preparing specific
tRNAs by *in vitro*
transcription and
enriched bacterial
expression

tRNA aminoacylation
and preparation of
charged aa-tRNA for
enzymatic reactions

Preparation of aa-
tRNA-derived
fragments (aa-tRF)
using *in vitro*
cleavage with RNase
T2

Using Arg-tRNA and
Arg-tRF for *in vitro*
arginylation

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Protocol

Purification and Use of tRNA for Enzymatic Post-translational Addition of Amino Acids to Proteins

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SUMMARY

Post-translational addition of amino acids to proteins by enzymes using aminoacyl-tRNA is an emerging regulatory mechanism. Examples include Arg transfer in eukaryotes, Leu/Phe transfer in bacteria, and tRNA-synthetase-mediated addition of amino acids to Lys side chains. Here, we present a method of purification and use of tRNA for such reactions, focusing on tRNA^{Arg} and its use for arginylation. This method can also be used for other tRNA-mediated reactions. For complete details on the use and execution of this protocol, please refer to Avcilar-Kucukgoze et al. (2020)

BEFORE YOU BEGIN

Design tDNA Oligonucleotides for tRNA Transcription

⌚ Timing: 2–3 h

1. Design tDNA oligonucleotides with the T7 promoter.

Note: Two single-stranded tDNA oligonucleotides are designed to cover the entire sequence of the tRNA of interest. One of the two tDNA oligonucleotides encodes the 5'-end sequence of the sense strand, while the other encodes the 3'-end sequence of the anti-sense strand. The two oligonucleotides of the opposite polarity overlap in the middle of the tRNA sequence for ~25 nucleotides, which, upon annealing, provide the template for synthesis of the double-stranded tRNA sequence that can be transcribed by T7 RNA polymerase (RNAP). The oligonucleotide encoding the 5'-end sequence of the sense strand includes an upstream T7 promoter sequence, which has the minimal sequence of 5'-TAATACGACTCACTATAG-3'. The underlined G is the first nucleotide to be incorporated into the transcribed tRNA, which should be followed by another G for high efficiency of transcription. The sequence of tRNA genes is obtained from Genomic tRNA Database (<http://gtmadb.ucsc.edu/>) (Chan and Lowe, 2016). The oligonucleotide encoding the 3'-end sequence of the anti-sense strand should include the 3' CCA tail, which is not encoded in eukaryotic tRNA gene.

2. An example of tDNA oligonucleotides for mouse tRNA^{Arg}ACG:

- a) Full-length tRNA^{Arg}ACG: 5'-GGGCCAGTGGCGCAATGGATAACCGTCTGACTACGGATCAGAAGATTCCAGGTTTCGACTCCTGGCTGGCTCGcca-3', where the anticodon ACG is underlined



- b) + T7 promoter: TAATACGACTCACTATAGGGCCAGTGGCGCAATGGATAACGCGTCTGA
CTACGGATCAGAAGATTCCAGGTTCCGACTCCTGGCTGGCTCGcca
- c) Forward tDNA oligonucleotide: 5'-TAATACGACTCACTATAGGGCCAGTGGCGCAATGGA
TAACGCGTCTGACTACGGATCA-3'
- d) Reverse tDNA oligonucleotide: 5'-TGGCGAGCCAGCCAGGAGTCGAACCTGGAATCTTCT
GATCCGTAGTCAGACGCGTTATCC-3'

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
<i>E. coli</i> JM109	Promega	Cat# L2005
Chemicals, Peptides, and Recombinant Proteins		
Tris base	Fisher Scientific	Cat# BP152-1
dNTP mix (10 mM)	Invitrogen	Cat# 18427088
DNA polymerase I, Large (Klenow) Fragment	New England Biolabs	Cat# M0210S
10× NEB Buffer 2	New England Biolabs	Cat# B7002S
KOAc potassium acetate	Fisher Scientific	Cat# BP364-500
KCl	Fisher Scientific	Cat# BP366-500
Phenol/chloroform/isoamyl alcohol	Fisher Scientific	Cat# BP1752I-100
Ampicillin	Fisher Scientific	Cat# BP1760-25
LB, Lennox	BD Biosciences	Cat# 240230
IPTG	LabScientific	Cat# I-555
EDTA	Fisher Scientific	Cat# BP120-500
NaOAc	Sigma-Aldrich	Cat# S-2889
MgOAc	Fisher Scientific	Cat# BP215-500
Phenol, saturated, pH 4.5 (acidic phenol)	VWR Scientific	Cat# 97064-716
NaCl	Sigma-Aldrich	Cat# S6191
L-[2,3,4- ³ H]-Arg	PerkinElmer	Cat# NET1123001MC
L-[¹⁴ C(U)]-Arg	Moravek Biochemicals	Cat# MC137
L-[¹³ C, ¹⁵ N]-Arg	Pierce Chemical	Cat# 89990
ATP	Fisher Scientific	Cat# BP413-25
HEPES	Fisher Scientific	Cat# BP310-100
MgCl ₂ hexahydrate	Fluka	Cat# 63068
DTT	Fisher Scientific	Cat# BP172-5
Chloroform	Fisher Scientific	Cat# BP1145-1
Acetonitrile	Sigma-Aldrich	Cat# 34998
TFA	Sigma-Aldrich	Cat# T6508
Sybr Gold	Invitrogen	Cat# S11494
Ethanol	Decon Laboratories	Cat# 2716
Isopropanol	Sigma-Aldrich	Cat# I9030
Aminoacyl-tRNA synthetase mix	Sigma-Aldrich	Cat# A3646
Recombinant mouse ATE1	Wang et al., 2019	N/A
Recombinant human RARS	Wang et al., 2019	N/A
Recombinant human RNase T2	Thorn et al., 2012	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Angiotensin II (DRVYIHPF) (Custom synthesized peptide can be also used)	Sigma-Aldrich	Cat# A9525
DEPC	Sigma-Aldrich	Cat# D5758
Low range ssRNA ladder	New England Biolabs	Cat# N0364S
Critical Commercial Assays		
Megashortscript Kit	Invitrogen	Cat# AM1354
Monarch RNA Cleanup Kit	New England Biolabs	Cat# T2040S
Oligonucleotides		
Forward tDNA oligonucleotide: 5'-TAATACGACTCACTATAGGGCCAG TGGCGCAATGGATAACGCGTCTGAC TACGGATCA-3'	This paper	N/A
Reverse tDNA oligonucleotide: 5'-TGGCGAGCCAGCCAGGAGTCGAA CCTGGAATCTTCTGATCCGTAGTC AGACGCGTTATCC-3'	This paper	N/A
Recombinant DNA		
pKK223-3 tRNA ^{Arg} ACG plasmid	Gamper et al., 2015	N/A
Software and Algorithms		
BioRender	BioRender	https://biorender.com
Genomic tRNA Database	Chan and Lowe, 2016	http://gtrnadb.ucsc.edu/
Other		
UreaGel System	National Diagnostics	Cat# EC-833
Ecoscint scintillation counter	National Diagnostics	Cat# LS-271
Autofluor	National Diagnostics	Cat# LS-315
C18 spin column	The Nest Group	Cat# HUM S18V
0.22 μm syringe filter	Millipore	Cat# SLGS0250S
RNaseZap	Ambion	Cat# AM9780

MATERIALS AND EQUIPMENT

Crush and Soak Buffer, pH 7.5

Reagent	Final Concentration	Amount
Potassium acetate (KOAc)	50 mM	0.98 g
KCl	200 mM	2.98 g
DEPC-treated water		Up to 200 mL

Prepare the solution under RNase-free conditions. Adjust pH to 7.5. Aliquot to avoid RNase contamination and store at -20°C for up to a year.

10× Buffer, pH 7.5

Reagent	Final Concentration	Amount
HEPES	500 mM	11.91 g
KCl	250 mM	1.86 g
MgCl ₂ hexahydrate	150 mM	3.05 g
DTT (added freshly from stock solution on the day of experiment).	1 mM	1 mL of 100 mM stock solution, stored at -20°C for up to a year.
DEPC-treated water		Up to 100 mL

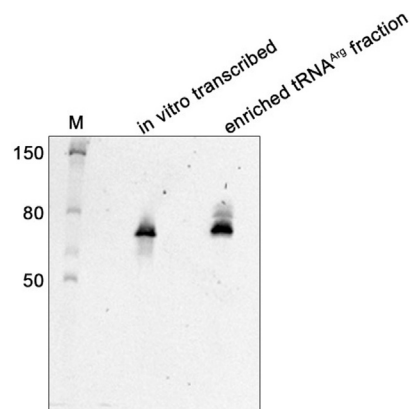


Figure 1. tRNA^{Arg} Preparations Obtained by the Methods Described in Steps 1–30
300 ng of RNA samples was separated on 12% PAGE/7.5 M urea.

Mix all the components except DTT under RNase-free conditions. Adjust pH to 7.5. Aliquot to avoid RNase contamination and store at 20°C–22°C for up to a month. Add DTT freshly from a frozen stock to the working aliquot on the day of experiment.

STEP-BY-STEP METHOD DETAILS

tRNA Preparation

Here we present two different methods for tRNA preparation. The first method involves *in vitro* transcription by T7 RNAP, which efficiently synthesizes the tRNA of interest as a transcript, but lacking all natural post-transcriptional modifications (Hall et al., 1989; Sampson and Uhlenbeck, 1988; Samuelsson et al., 1988). It allows to produce micrograms of an individual tRNA (see Figure 1, left lane, for an example). The second method involves the expression of the tRNA of interest in bacteria and isolation of total tRNA mixture enriched for the expressed tRNA (see Figure 1, right lane, for an example). This method produces milligrams of total tRNA mixture, containing a major proportion of the overexpressed tRNA (an estimated 40%–50%). While this purity is sufficient for many applications, the *in vitro* transcribed tRNA should be used in cases where the use of highly homogeneous tRNA preparation is important, regardless of the post-transcriptional modifications. Both preparations work well in the case of arginylation.

In Vitro Transcription of Mouse tRNA^{Arg}

⌚ Timing: 2–3 days

1. To anneal the two single-stranded tDNA oligonucleotides, mix the following: 10 μ L of the forward oligonucleotide (100 μ M), 10 μ L of the reverse oligonucleotide (100 μ M), 4 μ L of 200 mM Tris-HCl (pH 7.5), 16 μ L of nuclease-free water.

Reagent	Final Concentration	Amount
Forward oligonucleotide (100 μ M)	25 μ M	10 μ L
Reverse oligonucleotide (100 μ M)	25 μ M	10 μ L
200 mM Tris-HCl (pH 7.5)	20 mM	4 μ L
Nuclease-free water	n/a	16 μ L

2. Unfold the oligonucleotides by heating for 2 min at 95°C and anneal the overlapping oligonucleotides for 3 min at 20°C–22°C.
3. To fill-in the 5' overhangs and form blunt ends, combine the following, in this order: 20 µL of 10× buffer (NEB Buffer 2), 6.6 µL of 1 mM dNTP mix, 4 µL of DNA polymerase I, Large (Klenow) Fragment (5 U/µL), 129.4 µL of nuclease-free water and 40 µL of the tDNA oligonucleotide mix (from step 1).

Reagent	Amount
10× buffer (NEB Buffer 2)	20 µL
1 mM dNTP mix	6.6 µL
DNA polymerase I, Large (Klenow) Fragment (5 U/µL)	4 µL
Nuclease-free water	129.4 µL
tDNA oligonucleotide mix (from step 1)	40 µL

4. Incubate for 15 min at 25°C.
5. Add 1 volume of the crush and soak buffer (50 mM potassium acetate (KOAc), 200 mM KCl, pH 7.5) and 2 volumes of phenol/chloroform/isoamyl alcohol (25:24:1). Vortex 30 s and centrifuge at 21,000 × g for 5 min at 4°C.
6. Precipitate the aqueous phase with 2.7 volumes of ethanol (100%). Incubate for 30 min at –80°C, followed by centrifugation at 21,000 × g for 30 min at 4°C.
7. Air dry the pellet for 5–10 min at 20°C–22°C. Dissolve it into 50 µL of nuclease-free water. Determine the concentration by Nanodrop, and the integrity by 2% agarose gel. Store the synthesized double-stranded DNA template of the tRNA at –20°C.
8. For *in vitro* transcription of the tRNA, use Megashortscript Kit with 1 µg of the double-stranded template DNA according to the manufacturer's instructions (<https://www.thermofisher.com/order/catalog/product/AM1354>). Incubate for 16 h at 37°C.

△ CRITICAL: The reagents, chemicals, tips, and tubes should be RNase-free. All buffers should be prepared with DEPC-treated water in RNase-free conditions. Reused plastics or glassware should be washed with RNaseZap followed by pure water.

9. To digest the DNA template, add 1 µL of TURBO DNase provided by Megashortscript Kit to the reaction and mix well. Incubate at 37°C for 15 min.
10. Separate the transcription reaction using 12% PAGE/7.5 M urea. The dimension of the gel should be 14 by 14 by 0.1 cm. Visualize the synthesized tRNA transcript by Sybr Gold under UV light and excise the tRNA band. Place the excised gel pieces into a 0.2 mL tube with holes at the bottom. Holes can be made by a needle. Put the 0.2 mL tube into 1.5 mL tube and centrifuge at 16,100 × g for 1 min at 20°C–22°C. Remove the 0.2 mL tube. Add 2 volumes of crush and soak buffer (50 mM KOAc, 200 mM KCl, pH 7.5) to the gel pieces and elute tRNA transcript by shaking the tube on a rotator for 16 h at 4°C.

Note: tRNAs can be cleaned by Monarch RNA Cleanup Kit according to the manufacturer's instructions (<https://www.neb.com/protocols/2018/06/28/monarch-rna-cleanup-kit-protocol>). It should be noted that T7 RNAP tends to add one or more nontemplated nucleotides at the 3' terminus of the transcribed RNA (Krupp, 1988; Milligan et al., 1987). Recovering tRNA from a gel effectively removes these longer tRNA transcripts, although it is more time-consuming with a reduction in the yield. To enhance the rate of the correct size transcribed tRNA in the reaction, modified DNA template with methoxy moieties at the

ribose C2' position of the penultimate nucleotide of the complementary strand could be used (Kao et al., 1999).

11. To separate the gel pieces from the buffer containing transcribed tRNA, filter the sample through a 0.22 μm syringe filter. Add 1 volume of isopropanol and precipitate tRNA for 30 min at -80°C .
12. Pellet tRNA by centrifugation at $21,000 \times g$ for 30 min at 4°C . Wash the tRNA pellet with 70% ethanol twice. Dissolve it into DEPC-treated water. Determine the concentration by Nanodrop. The A_{260}/A_{280} ratio should be ≥ 2 .
13. To ensure correct tRNA folding, unfold the tRNA for 2 min at 95°C , followed by 3 min at 20°C – 22°C . Place the sample for further 5 min at 37°C . Store it at -80°C .

Note: (1) Step 13 should be performed only once. The tRNA sample should be aliquoted and stored at -80°C to prevent damage during multiple freeze/thaw cycles. (2) Since divalent cations, especially Mg^{2+} , are very effective at stabilizing the tertiary structures of most RNAs (Hall et al., 1989; Klein et al., 2004; Serebrov et al., 1998), addition of Mg^{2+} at step 13 may promote the stability of the correct conformation of tRNA.

Preparation of an Enriched tRNA^{Arg} Fraction by *E. coli* Expression

⌚ Timing: 4–5 days

This procedure involves expression of a tRNA-encoding plasmid in *E. coli*, followed by isolation of total tRNA. The resulting total *E. coli* tRNA mixture is greatly enriched for the expressed tRNA (tRNA^{Arg} in the case of arginylation, which constitutes an estimated 40%–50% of the total tRNA in the preparation). Aminoacylation of this mixture with purified arginyl-tRNA synthetase (RARS) can be used to estimate the exact fraction of tRNA^{Arg} in the mixture. This procedure, described below, yields an enriched preparation of tRNA^{Arg} which is suitable for arginylation reaction regardless of the presence of other tRNA species. Because of its high yield compared to *in vitro* transcription, this preparation is recommended for routine arginylation experiments.

14. We generate an expression plasmid for tRNA^{Arg} in the pKK223-3 vector, driven by the strong pTac promoter. Transform *E. coli* JM109 cells with the plasmid that harbors *E. coli* tRNA^{Arg}ACG following the manufacturer's protocol for *E. coli* JM109 transformation (https://www.promega.com/products/cloning-and-dna-markers/bacterial-strains-and-competent-cells/pro-5_alpha_-jm109-and-hb101-competent-cells/?catNum=L2005).
15. Select transformants for the presence of ampicillin-resistance marker by plating the bacteria on Luria broth (LB) agar plates containing 100 $\mu\text{g}/\text{mL}$ of ampicillin. Pick a single colony and grow it into 20 mL of LB containing 100 $\mu\text{g}/\text{mL}$ of ampicillin for 16 h as the pre-culture.
16. Dilute the pre-culture 1:100 into 1 L LB medium supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin.
17. Continually grow the culture with shaking at 37°C until OD_{600} reaches 0.4–0.6.
18. Induce tRNA^{Arg}ACG expression by adding 0.3 mM IPTG. Incubate on a shaker for approximately 10 h at 37°C .

Note: If the tRNA of interest is eukaryotic, it should be noted that expression of a eukaryotic tRNA in *E. coli* generates the post-transcriptional modifications provided by *E. coli* enzymes, which may not reproduce those found in a eukaryotic host.

19. Collect the cells by centrifuging at $5,000 \times g$ for 25 min at 4°C . Discard the supernatant.
20. Resuspend the cell pellet into 30 mL of 0.9% NaCl.
21. Collect the cells by centrifuging at $5,000 \times g$ for 15 min at 4°C . Discard the supernatant.

Optional: Weight the pellet. Typical yield is 3.5–5 g of wet cells for 1 L of culture.

22. **First Extraction:** Resuspend the pellet into 18 mL of 50 mM sodium acetate (NaOAc), 10 mM MgOAc, pH 5.0 and add 17.2 mL of commercial acidic phenol, pH 4.5. Transfer the emulsion into a 125 mL flask and shake it at 215 rpm for 30 min at 20°C–37°C. Centrifuge at 5,000 × g for 15 min at 4°C. Collect the upper aqueous phase by gentle aspiration with a pipette and transfer it to a new tube. Be careful not to take any of the interface.

△ **CRITICAL:** The reagents, chemicals, tips, and tubes should be RNase-free. All buffers should be prepared with DEPC-treated water in RNase-free conditions. Reused plastics or glassware should be washed with RNaseZap followed by pure water.

23. **Second Extraction:** Add 14 mL of 50 mM NaOAc, 10 mM MgOAc, pH 5.0 to phenol/inter-phase, and transfer back into the 125 mL flask. Shake it for 15 min as described in step 22. Repeat the centrifugation and recovery of the aqueous phase. Combine aqueous phases (approx. 36 mL).

24. **Precipitation of total nucleic acids:** Add 1.5 mL of 5 M NaCl to the aqueous phases collected in steps 22 and 23 to the final concentration of 0.2 M NaCl. Add 1 volume of isopropanol. Store briefly at 20°C–22°C and centrifuge at 14,500 × g for 15 min at 20°C–22°C. Wash the nucleic acid pellet with cold 70% ethanol, and air dry for 5–10 min.

25. **Removal of rRNA by precipitation (needed because at this step the preparation contains ribosomal RNA):** Resuspend the pellet into 10 mL of cold 1 M NaCl by vortexing and/or pipetting. This process is facilitated by letting the pellet hydrate in 1 M NaCl prior to resuspension. Centrifuge the sample at 9,500 × g for 20 min at 4°C. Collect the supernatant. Resuspend the pellet into 5 mL of cold 1 M NaCl and centrifuge it again at 9,500 × g for 20 min at 4°C. Collect the supernatant and combine it with the first supernatant (total 15 mL).

Optional: The second extraction is optional. The pellet can be resuspended into 15 mL of cold 1 M NaCl.

26. **Precipitation of remaining nucleic acids (including DNA and tRNA):** Add 30 mL (2 volumes) of cold ethanol to the supernatant collected in step 25. Incubate for 30 min at –20°C. Centrifuge at 14,500 × g for 5 min at 4°C. Wash the pellet with 70% ethanol and air dry for 5–10 min.

27. **Removal of DNA by precipitation:** Dissolve the pellet into 6 mL of 0.3 M NaOAc, pH 5.0. This may require heating and swirling at 60°C along with pipetting. Add 3.4 mL of isopropanol (0.56 volume) to the nucleic acid solution and incubate it for 10 min at 20°C–22°C. Centrifuge at 14,500 × g for 5 min at 20°C–22°C. Collect the supernatant.

Note: (1) Dispersion is easier if the pellet is in the NaOAc buffer up to 16 h. (2) If the yield is low, the pellets from multiple 1-liter cultures can be pooled into 6 mL of 0.3 M NaOAc pH 5.0.

28. **Final precipitation of tRNAs:** Add 2.3 mL of isopropanol to the supernatant (water:isopropanol is 1.00:0.95) and incubate it for 30 min at –20°C. Centrifuge the suspension at 14,500 × g for 15 min at 4°C. The pellet should appear white. Wash the pellet with 70% ethanol and air dry for 5–10 min. Dissolve the pellet into 500 µL of DEPC-treated water. Do not over-dry the pellet. Over-dried pellet looks transparent, but not white.

29. **Deacylation:** Add 35 µL of 1.5 M Tris pH 9.0 and incubate it for 45 min at 37°C. Add 53.5 µL (0.1 volume) of 3 M NaOAc, pH 5.0 and add 1.6 mL (2.7 volumes) of ethanol (100%). Incubate for 30 min at –80°C. Centrifuge the suspension at 16,100 × g for 25 min at 4°C. Wash the pellet with 70% ethanol and air dry. Dissolve the pellet into 250–300 µL of DEPC-treated water.

30. **Quantification:** Determine the concentration by Nanodrop. The A_{260}/A_{280} ratio should be ≥ 2 . Typical yield of total tRNA is 5–20 mg. The tRNA sample at this stage should be aliquoted and stored at –80°C to prevent damage during multiple freeze/thaw cycles.

Aminoacylation of tRNA^{Arg}

⌚ Timing: 3–4 h

This procedure is performed similarly for both the *in vitro* transcribed tRNA^{Arg} and the bacterially expressed preparation enriched in tRNA^{Arg}. Since the enzyme used in this reaction (RARS) is specific to tRNA^{Arg}, the charging should be independent of the presence of other tRNA species in the mix; however, the expected yield of the charged tRNA^{Arg} species would depend on the amount of tRNA^{Arg}. Thus, the procedure below does not distinguish between the tRNA prepared by these two methods, which are referred to, in both cases, as tRNA^{Arg}.

Note: In our experience, while the *in vitro* transcribed tRNA needs to be refolded prior to charging with RARS, the tRNA preparation from *E. coli* is already folded correctly, thus we do not use a refolding step with this preparation, and have encountered no problems with RARS charging or arginylation.

31. Prepare a 150 μL reaction by mixing the following: 5.67 μL of tRNA^{Arg} (5,200 ng/ μL ; 211.6 μM ; 8 μM final concentration), 4.95 μL of ATP (100 mM; 3.3 mM final concentration), 115 μL of L-[2,3,4-³H]-Arg (18.3 μM ; 14 μM final concentration), 9.38 μL of purified human arginyl-tRNA synthetase (RARS) (1,800 $\mu\text{g}/\text{mL}$; 24 μM ; 1.5 μM final concentration), 15 μL of 10 \times buffer (500 mM HEPES, 250 mM KCl, 150 mM MgCl₂, 1 mM DTT, pH 7.5). At the same time, set up the control reaction containing above components, but no RARS.

Reagent	Final Concentration	Amount
tRNA ^{Arg} (5,200 ng/ μL ; 211.6 μM)	8 μM	5.67 μL
ATP (100 mM)	3.3 mM	4.95 μL
L-[2,3,4- ³ H]-Arg (18.3 μM)	14 μM	115 μL
Purified human arginyl-tRNA synthetase (RARS) (1,800 $\mu\text{g}/\text{mL}$; 24 μM)	1.5 μM	9.38 μL
10 \times buffer	1x	15 μL

⚠ CRITICAL: Since the protocol requires to use a radioactive isotope, the sample should be handled in a hot lab with a proper personal protective equipment, e.g., lab coat, goggles etc, after step 31.

Note: (1) If cognate aminoacyl-tRNA synthetase is not available, a mixture of cell lysates of the organism of interest, or a commercial *E. coli* aminoacyl-tRNA synthetase mix, can be used in its place. It is best, if possible, to use cognate aminoacyl-tRNA synthetase mix, however, the only alternative that is currently commercially available is a mix of *E. coli* aminoacyl-tRNA synthetases. Our tests indicate that, despite considerations related to organism specificity (Liu et al., 2011), *E. coli* and human aminoacyl-tRNA-synthetases work equally well with both mouse and *E. coli* tRNA^{Arg} (Avcilar-Kucukgoze et al., 2020). Thus, the commercial alternative appears reasonable in the case of tRNA^{Arg}. (2) Purification of recombinant human RARS expressed in *E. coli* can be found in (Wang et al., 2019). (3) For arginylation detection by methods other than scintillation counting, L-[¹⁴C(U)]-Arg or L-[¹³C, ¹⁵N]-Arg should be used in place of L-[2,3,4-³H]-Arg for autoradiography and mass spectrometry, respectively. Note that the U in L-[¹⁴C(U)]-Arg indicates that all carbons are uniformly labeled.

32. Incubate the reaction at 37°C for 2 h. Add a total of 250 μL of a solution containing 100 mM NaOAc, 1 mM EDTA, pH 4.8 then mix it with 400 μL of freshly prepared acidic phenol/chloroform (5:1). Vortex 30 s and centrifuge at 21,000 $\times g$ for 5 min at 4°C.

Note: It is crucial to keep the aminoacyl-tRNAs (aa-tRNA) in acidic conditions, because the ester bond between the amino acid and the tRNA is easily hydrolyzed at the physiological pH.

33. Add 0.1 volume of 3 M NaOAc, pH 5.0 to the aqueous phase and precipitate it with 1 volume of isopropanol. Incubate the precipitation reaction for 30 min at -80°C , followed by centrifugation at 21,000 $\times g$ for 30 min at 4°C.

34. Wash the aa-tRNA pellet with cold 70% ethanol 2–3 times. Air dry the pellet and dissolve it into 23.5 μL of 10 mM NaOAc, 1 mM EDTA, pH 4.8 to preserve the aminoacyl moiety. The final concentration of aa-tRNA is 1 $\mu\text{g}/\mu\text{L}$, assuming 80% recovery after phenol/chloroform extraction. The level of aminoacylation should be directly determined by scintillation counter. Typical yields of aminoacylation are 20%–30% of tRNA^{Arg}. Ideally, use a freshly prepared aa-tRNA for further steps.

Note: Charging level of tRNA is calculated as follows: (1) Measure the cpm value of 1 μL stock L-[2,3,4-³H]-Arg. (2) Measure the cpm value of 1 μL of Arg-tRNA (1 $\mu\text{g}/\mu\text{L}$) at step 34. (3) Calculate the moles of L-[2,3,4-³H]-Arg for 1 μg (40.70 pmol) of Arg-tRNA. (4) Calculate the ratio of L-[2,3,4-³H]-Arg:tRNA in moles.

Note: An example of calculating the aminoacylation level of tRNA^{Arg}:

Stock L-[2,3,4-³H]-Arg: 54.5 Ci/mmol, 1 mCi/mL (1 $\mu\text{Ci}/\mu\text{L}$)

1 μL of stock L-[2,3,4-³H]-Arg gives 825,008 cpm.

1 μCi	825,008 cpm
X	527,503 cpm (for 1 μg of Arg-tRNA measured by a scintillation counter)

$$x = 0.63 \mu\text{Ci}$$

54.5 μCi	1 nmol
0.63 μCi	X

$$x = 0.012 \text{ nmol} = 12 \text{ pmol}$$

1 μg of Arg-tRNA (40.70 pmol) contains 12 pmol of L-[2,3,4-³H]-Arg, thereby aminoacylation level is 29.5% (=12/40.70).

Note: Charged tRNA at this step can be used directly in the arginylation reaction (see below, step 39). Alternatively, charged tRNA can be further processed by RNase T2 to produce Arg-charged tRNA^{Arg}-derived fragments (Arg-tRF^{Arg}) for the arginylation reaction.

Cleavage of Arg-tRNA^{Arg} by RNase T2

⌚ Timing: 1–2 h

Since RNase T2 generates tRF from all tRNAs and is not specific to tRNA^{Arg}, both *in vitro* transcribed and *E. coli* expressed preparations can in principle be used in this step. However, *in vitro* transcribed tRNA is recommended, given its purity that enables fully controlled conditions of the reaction.

35. Prepare a 200 μL reaction by mixing the following: 20 μL of Arg-tRNA^{Arg} (1 $\mu\text{g}/\mu\text{L}$; 4 μM final concentration), 20 μL of purified recombinant form of human RNase T2 (2.4 $\text{ng}/\mu\text{L}$; 0.24 $\text{ng}/\mu\text{L}$ final concentration), 50 μL of 800 mM NaOAc, pH 4.5, 200 mM NaCl and 110 μL DEPC-treated water.

Reagent	Amount	Final Concentration
Arg-tRNA ^{Arg} (1 $\mu\text{g}/\mu\text{L}$; 40.70 μM)	20 μL	4 μM
Purified Recombinant form of human RNase T2 (2.4 $\text{ng}/\mu\text{L}$)	20 μL	0.24 $\text{ng}/\mu\text{L}$
NaOAc (800 mM)+ NaCl (200 mM)	50 μL	200 mM NaOAc + 50 mM NaCl
DEPC-treated water	110 μL	n/a

Note: It is crucial to keep the reaction in acidic conditions to preserve the aminoacyl group on the tRNA. Notably, RNase T2 used in this protocol acts better in an acidic pH (Campomenosi et al., 2006). The detailed purification protocol of recombinant human RNase T2 can be found in (Thorn et al., 2012).

36. Incubate the reaction for 30 min at 37°C. Add a total of 200 μL of a solution containing 10 mM NaOAc, 1 mM EDTA, pH 4.8 then mix the reaction with 400 μL of freshly prepared acidic phenol/chloroform (5:1). Vortex the mixture for 30 s and centrifuge at 21,000 $\times g$ for 5 min at 4°C.

Note: To analyze Arg-tRNA^{Arg}-derived fragments (Arg-tRF^{Arg}) on a gel, autoradiography can be used if aminoacylation is performed with L-[¹⁴C(U)]-Arg. After incubation, mix the reaction directly with an acidic 2 \times RNA loading dye and separate the Arg-tRF^{Arg} from Arg-tRNA^{Arg} by an 8% acidic denaturing PAGE/7.5 M urea (8% polyacrylamide, 7.5 M urea, 0.1 M NaOAc/AcOH pH 5.0). Run the gel at 100–150 V (~12 V/cm) in a cold room. Treat the gel with AutoFluor according to the manufacturer's protocol (https://www.nationaldiagnostics.com/sites/default/files/autofluor_protocol0812_0.pdf). Dry the gel under a vacuum for 2 h at 80°C. The gel can be exposed to X-ray films or phosphor screens. Because L-[¹⁴C(U)]-Arg is highly stable, the gel can be stored and re-exposed multiple times without decomposition of the label (see Figure 2 for an example).

37. Add 0.1 volume of 3 M NaOAc, pH 5.0 to the aqueous phase and precipitate it with 1 volume of isopropanol. Incubate at –80°C for 30 min. Pellet Arg-tRF^{Arg} by centrifugation at 21,000 $\times g$ for 30 min at 4°C.
38. Wash the pellet with cold 70% ethanol 2–3 times. Air dry the pellet and dissolve it into 16 μL of 10 mM NaOAc, 1 mM EDTA, pH 4.8. The final concentration of Arg-tRF^{Arg} is 40.70 μM , assuming 80% recovery after phenol/chloroform extraction. Ideally, use freshly prepared Arg-tRF^{Arg} for enzymatic reactions.

Using Arg-tRNA^{Arg} or Arg-tRF^{Arg} in an ATE1-Mediated Reaction with a Peptide Substrate

⌚ Timing: 2–3 h

This protocol utilizes Arg-tRNA^{Arg} or Arg-tRF^{Arg} prepared in the steps above, which can both serve as Arg donors for arginyltransferase ATE1. The procedure is the same regardless of the source and

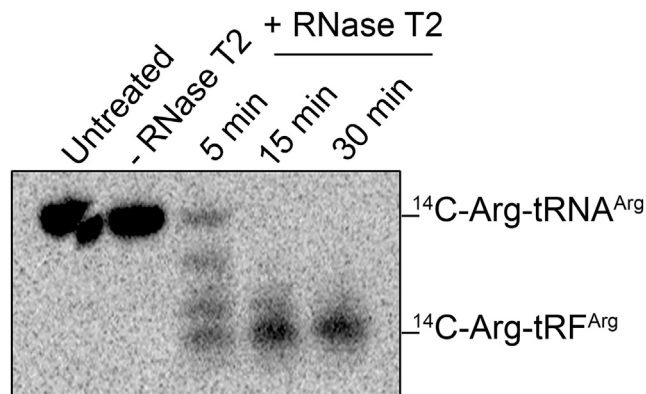


Figure 2. Autoradiography of ^{14}C -Arg-tRNA^{Arg} Before (Untreated) and After Treatment with RNase T2 under Acidic Conditions to Produce ^{14}C -Arg-tRNA^{Arg}-Derived Fragments (Arg-tRF^{Arg})

Time intervals after RNase T2 addition are shown on top (the lane marked -RNase T2 represents 0 time point). Arg charging is retained by the tRFs after RNase T2 cleavage. Arg-tRF^{Arg} were separated on 8% acidic denaturing PAGE/7.5 M urea. Reprinted with permission from [Avcilar-Kucukgoze et al. \(2020\)](#).

purity of tRNA, and thus in the protocol below we denote both the *in vitro* transcribed and bacterially expressed preparations as Arg-tRNA^{Arg}. Arginylation activity is analyzed by detecting the labeled L-[2,3,4-³H]-Arg, which is transferred from Arg-tRNA^{Arg} or Arg-tRF^{Arg} onto the peptide substrate by ATE1, using scintillation counting. Alternatively, arginylation can be detected by mass spectrometry; in this case L-[¹³C, ¹⁵N]-Arg should be used, to avoid contamination of the instrument with the radioactive label. For protein substrates, arginylation can also be detected by gel autoradiography; in this case, L-[¹⁴C(U)]-Arg is recommended, as it tends to have stronger and more stable autoradiography signal. Notably, the arginylation reaction with full-length tRNA^{Arg} can also be performed in the presence of RARS, which should provide ongoing supply of newly re-charged Arg-tRNA^{Arg} and thus may improve the efficiency of the reaction (see [Wang et al., 2019](#)).

39. Prepare a 50 μL reaction by mixing the following: 6.25 μL of Arg-tRNA^{Arg} or Arg-tRF^{Arg} (40.70 μM ; 5 μM final concentration), 2.5 μL of angiotensin II (DRVYIHPF) (300 μM ; 15 μM final concentration), 6.81 μL ATE1 (1,320 $\mu\text{g}/\text{mL}$; 22 μM ; 3 μM final concentration), 5 μL of 10 \times buffer (500 mM HEPES, 250 mM KCl, 150 mM MgCl₂, 1 mM DTT, pH 7.5) and 29.44 μL DEPC-treated water. In parallel, set up the negative control reaction with the above components, but no ATE1.

Reagent	Final Concentration	Amount
Arg-tRNA ^{Arg} or Arg-tRF ^{Arg} (40.70 μM)	5 μM	6.25 μL
Angiotensin II (DRVYIHPF) (300 μM)	15 μM	2.5 μL
ATE1 (1,320 $\mu\text{g}/\text{mL}$; 22 μM)	3 μM	6.81 μL
10 \times buffer (500 mM HEPES, 250 mM KCl, 150 mM MgCl ₂ , 1 mM DTT, pH 7.5)	1 \times	5 μL
DEPC-treated water	n/a	29.44 μL

Note: The detailed purification protocol of recombinant mouse ATE1 can be found in ([Wang et al., 2019](#)).

40. Incubate the reactions for 5 min at 37°C.

41. Heat the reactions at 95°C for 15 min to terminate the reactions and hydrolyze the intact Arg-tRNA^{Arg} or Arg-tRF^{Arg}. Note that this heating step also denatures and precipitates proteins in the mixture, including the enzymes.

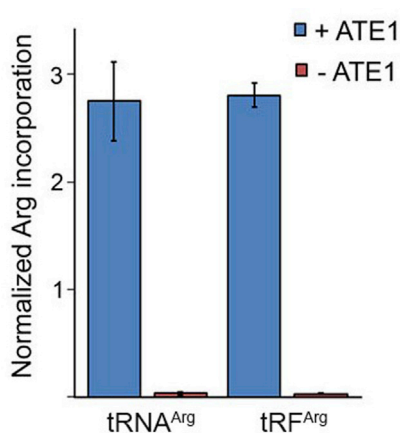


Figure 3. Normalized Incorporation of ³H-Arg into Angiotensin II Performed with ³H-Arg-tRNA^{Arg} and ³H-Arg-tRF^{Arg}

Data are means ± SEM of three biological replicates. Reprinted with permission from [Avcilar-Kucukgoze et al. \(2020\)](#).

42. Keep the tubes on ice for 20 min, then spin each at 17,000 × g for 15 min at 20°C–22°C to pellet the precipitated proteins. The peptides will remain in the supernatant at this step.
43. Load each supernatant onto a C18 spin column, pre-washed with 100% acetonitrile and water, by applying the supernatant on top of the column and centrifuging the column at 110 × g for 1 min. The peptides will bind to the column at this step. Discard the collection tube, containing free Arg not utilized in the reaction, into radioactive waste disposal.
44. Wash the columns with 150 μL of peptide wash buffer, 0.1% (v/v) trifluoroacetic acid (TFA) in water, by centrifugation at 110 × g for 1 min into a new collection tube. Repeat the washing step one more time. Both collection tubes should be discarded as radioactive waste.
45. Elute the column-bound peptide from each reaction with 150 μL of peptide elution buffer, 60% (v/v) acetonitrile, 0.1% (v/v) TFA in water, by centrifugation at 110 × g for 1 min into a new collection tube. Repeat the elution step one more time.
46. Transfer the eluted peptide from each reaction into a scintillation vial filled with Ecoscint scintillation solution and analyze on a liquid scintillation counter.

△ CRITICAL: For detection of arginylation by mass spectrometry, the eluate is concentrated by a Speedvac until 10–20 μL liquid remains. The concentrated arginylated peptide can be directly analyzed by MALDI-TOF or LC-MS/MS.

EXPECTED OUTCOMES

Examples of tRNA preparation and generation of Arg-charged tRNA and tRF are shown in [Figures 1](#) and [2](#), respectively. Outcomes of an arginylation reaction detected by scintillation counting and MALDI-TOF mass spectrometry are shown in [Figures 3](#) and [4](#), respectively. These figures illustrate arginylation of angiotensin II – a highly efficient peptide substrate. In the case of protein substrates, autoradiography can also be used.

In our protocol, we present two different methods for preparation of tRNA^{Arg}. While *in vitro* transcribed tRNA can be efficiently used in the case of arginylation, bacterially expressed tRNAs that carry endogenous *E. coli*-specific post-transcriptional modifications may be more favorable for other enzymatic reactions. In this protocol, we use RNase T2 to generate Arg-tRF^{Arg}. The efficiency of *in vitro* arginylation reactions tend to vary, depending on the aminoacylation state of the tRNA (which tends to deteriorate with storage), and can be calculated using a formula presented below. Arginylation can be qualitatively detected by MALDI-TOF, but this method should not be used for quantification ([Szajli et al., 2008](#)). The yield of arginylation can be enhanced by increasing the level of Arg-tRF^{Arg}/Arg-tRNA^{Arg} in the arginylation reaction (step 39). Another method to increase efficiency involves combining the RARS-mediated tRNA charging and arginylation step into a single two-enzyme reaction, where RARS continuously re-charges the Arg-tRNA^{Arg} present in the mix and

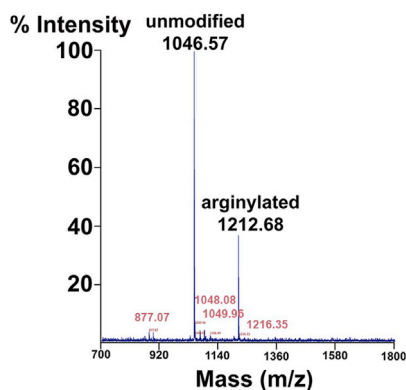


Figure 4. MALDI-TOF Chromatogram of Angiotensin II Arginylated Using Purified Pre-charged $^{13}\text{C}^{15}\text{N}$ -Arg-tRNA^{Arg} or $^{13}\text{C}^{15}\text{N}$ -Arg-tRF^{Arg}

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thus effectively increases its availability for arginylation. The yields in such 2-enzyme reaction can be substantially higher, and in some cases approach 100% (tested for angiotensin II arginylation ([Wang et al., 2014](#))). In the reaction using Arg-tRF^{Arg}, or pre-purified Arg-tRNA^{Arg}, the efficiency is lower, likely due to the ongoing hydrolysis of the bond between Arg and tRNA/tRF, which leads to eventual depletion of Arg-tRNA/tRF from the reaction.

An example of calculating the arginylation level:

Stock L-[2,3,4-³H]-Arg: 54.5 Ci/mmol, 1 mCi/mL (1 $\mu\text{Ci}/\mu\text{L}$)

1 μL of stock L-[2,3,4-³H]-Arg gives 825,008 cpm.

1 μCi	825,008 cpm
X	1,547,265 cpm (for 750 pmol angiotensin measured by a scintillation counter)
x = 1.875 μCi	

54.5 μCi	1 nmol
1.875 μCi	X
x = 0.0344 nmol = 34.4 pmol	

750 pmol angiotensin contains 34.4 pmol of L-[2,3,4-³H]-Arg, thereby arginylation level is 4.6% (=34.4/750).

LIMITATIONS

Although the *in vitro* T7 transcription reaction provides an efficient method to synthesize a specific tRNA, it has limitations. In order for T7 RNAP to initiate transcription, the promoter region needs to be double-stranded, therefore filling-in the 5' overhangs of the tDNA oligonucleotides is critical. As T7 RNAP is best to initiate with a G nucleotide (G+1), RNA transcripts starting with a nucleotide other than G can be transcribed with lower efficiency. The two Gs following G + 1 significantly increase the transcription efficiency. Since the ester bond between tRNA and amino acid is pH sensitive, it is crucial to keep the aa-tRNA in acidic conditions at all time. Therefore, an RNase which is catalytically active in acidic pH should be used to generate aa-tRF. Otherwise, aa-tRNA may lose its aminoacyl moiety.

As scintillation counting usually has a background noise, even if no arginylation occurs in control samples (the reaction without ATE1), it is difficult to detect the arginylation by scintillation counting for an inefficient substrate. Any impurities in the individual components may affect the outcome of the reaction. We also find that different substrates have different maximum efficiency in the arginylation reaction, with peptides and small proteins (e.g., α -synuclein) being much more efficient than larger proteins (e.g., BSA). While we do not fully understand the reasons for these differences, we speculate that they could be due, at least in part, to structural or sterical constraints that limit the accessibility of the arginylation sites in a folded polypeptide chain.

TROUBLESHOOTING

Problem

The obtained cpm value is lower than expected (step 46).

Potential Solution

Purified ATE1 protein loses its enzymatic activity if it is stored at -20°C for a week. Another aliquot of the purified ATE1 protein from -80°C should be tested. The second concern would be degradation of tRNA due to contamination in buffer. The integrity of tRNA should be analyzed by 10% denaturing PAGE/7.5 M urea before use.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Anna Kashina (akashina@upenn.edu).

Materials Availability

All materials used for this protocol are commercially available.

Data and Code Availability

This protocol does not include any datasets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

I.A.-K. and A.K. wrote the manuscript. H.G. and Y.-M.H. edited the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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