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Analysis of aminoacyl- and peptidyl-tRNAs by gel electrophoresis

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Abstract

During protein synthesis, the ribosome translates the genetic information encoded within messenger RNAs into defined amino acid sequences. Transfer RNAs (tRNAs) are crucial adaptor molecules in this process, delivering amino acid residues to the ribosome and holding the nascent peptide chain as it is assembled. Here, we present methods for the analysis of aminoacyl- and peptidyl-tRNA species isolated from *Escherichia coli*. These approaches utilize denaturing gel electrophoresis at acidic pH to preserve the labile ester bonds that link amino acids to tRNA. Specific aminoacyl- and peptidyl-tRNAs are detected by Northern blot hybridization using probes for tRNA isoacceptors. Small peptidyl-tRNAs can be differentiated from aminoacyl-tRNA through selective deacylation of the latter with copper sulfate. Additionally, peptidyl-tRNAs can be detected through metabolic labeling of the nascent peptide. This latter approach is amenable to pulse-chase analysis to examine peptidyl-tRNA turnover *in vivo*. We have applied these methods to study programmed translational arrests and the kinetics of paused ribosome turnover.

Keywords

aminoacyl-tRNA; gel electrophoresis; Northern blot hybridization; peptidyl-tRNA; peptidyl-tRNA hydrolase (Pth); pulse-chase analysis; ribosome pausing; translation

1. Introduction

Ribosomes use aminoacyl-tRNAs as precursors for the assembly of protein chains during translation. This strategy serves at least two purposes. First, activation of amino acids as acyl esters helps to promote peptide bond formation on the ribosome. Second, the covalently linked tRNAs are essential nucleic acid adaptors that decode the genetic information within mRNAs. Apart from translation initiation, all aminoacyl-tRNAs are delivered to the ribosome A site, where the base-pairing interactions between codon and anticodon are monitored. Cognate codon-anticodon interactions promote full accommodation of aminoacyl-tRNA into the A site for transpeptidation onto the nascent peptidyl-tRNA, which is bound within the ribosome P site. As a result, the P-site tRNA is deacylated and the nascent peptide chain transferred to the A-site tRNA. Subsequent ribosome translocation repositions the deacylated and peptidyl-tRNAs into the E and P sites (respectively) and presents a new codon in the A site for decoding. Thus, the ribosome iteratively adds new amino acid residues to the C-terminus of the nascent polypeptide, which remains linked to tRNA continuously. This elongation cycle is repeated until a stop codon is presented in the

A site. Protein release factors bind to A-site stop codons and promote hydrolysis of P-site peptidyl-tRNA to release newly synthesized proteins from the ribosome. Although aminoacyl-tRNAs play a central role in protein synthesis, they also perform other extra-ribosomal functions. In some bacteria, aminoacyl-tRNAs are used to modify membrane lipids and the peptidoglycan cell wall, and can also be involved in the synthesis of antibiotics (1).

Cellular aminoacyl-tRNA levels are largely determined by the relative rates of synthesis by aminoacyl-tRNA synthetases and consumption by the ribosome. In *E. coli* and other bacteria, aminoacyl-tRNA synthesis is often limited by amino acid availability. Acute amino acid starvation leads to an abrupt decrease in tRNA aminoacylation and an attendant halt to protein synthesis. The resulting deacylated tRNA is a potent signal of nutritional stress in bacteria and plays a critical role in redirecting gene expression to adapt to amino acid starvation (2, 3). Deacylation of specific tRNAs can also occur during the overproduction of heterologous proteins in *E. coli*, resulting in translational pausing and poor protein yields. This phenomenon is often associated with non-preferred or “rare” codons, which are typically decoded by low-abundance tRNA isoacceptors. Although protein expression can usually be increased through over-expression of the limiting isoacceptor, the underlying deficit is due to selective aminoacylation rather than the absolute level of tRNA (4–6). The over-expressed tRNAs compete more effectively with other isoacceptors for aminoacylation, and thereby provide sufficient aminoacyl-tRNA to support translation.

The first method to analyze aminoacyl-tRNAs in complex biological samples was described by Ho & Kan (7). Their method utilizes acid-urea polyacrylamide gel electrophoresis followed by Northern blot hybridization to detect specific aminoacyl-tRNAs. This approach exploits a small gel mobility shift to resolve aminoacylated tRNA from deacylated tRNA. Although the mass difference between aminoacyl- and deacylated tRNAs is generally small (< 1%), acid-urea gel electrophoresis has been used successfully with several tRNAs and is capable of differentiating *N*-formyl-methionyl-tRNA_i^{Met} from deformylated methionyl-tRNA_i^{Met} (5, 8–10). More recently, Tao Pan, Måns Ehrenberg and their colleagues have developed a microarray approach to quantitatively monitor the aminoacylation of all tRNA isoacceptors (4). This elegant approach gives a comprehensive view of tRNA aminoacylation, but requires specialized reagents and equipment that may not be available to many researchers.

Acid-urea gel electrophoresis can also be used to detect and quantify peptidyl-tRNAs in biological samples. As outlined above, peptidyl-tRNAs are intermediates of protein synthesis and are generally bound stably to the ribosome P site. However, in some instances peptidyl-tRNA dissociates from the ribosome in process termed “drop-off” (11–15). Drop-off occurs frequently with small peptidyl-tRNAs (three to seven amino acid residues), but these abortive translation products are rapidly broken down into constituent peptide and tRNA by peptidyl-tRNA hydrolase (Pth). Pth is an essential enzyme in bacteria, but peptidyl-tRNA drop-off products can be detected and studied in conditional *pth* mutants (12, 16, 17). Pth is unable to hydrolyze ribosome-bound peptidyl-tRNA (18, 19), and therefore peptidyl-tRNAs isolated from *pth*⁺ cells most likely occupy the ribosome P site at the time of extraction. Thus, in principle, it is possible to infer the positions of ribosomes on a given mRNA by identifying the associated peptidyl-tRNAs. In practice, it is difficult to detect individual peptidyl-tRNA species even during gratuitous protein overproduction (20). Presumably, this difficulty reflects the asynchrony of protein synthesis, in which each of the many nascent peptidyl-tRNA intermediates is present at a low level. However, specific peptidyl-tRNAs accumulate to relatively high levels during translational pauses (20–24). In these instances, the position of the arrested ribosome can be determined by identifying the tRNA isoacceptor that is linked to the nascent chain.

Here, we apply acid-urea gel electrophoresis and Northern blot hybridization to detect and differentiate aminoacyl- and peptidyl-tRNA isolated from *E. coli* cells. Peptidyl-tRNAs carrying nascent peptides of 10 or more amino acid residues are readily resolved from aminoacyl-tRNA on acid-urea gels, but it is often difficult to unambiguously identify smaller peptidyl-tRNAs. In these instances, aminoacyl-tRNAs can be specifically deacylated using copper sulfate treatment. We employ this strategy to detect small peptidyl-tRNAs that dissociate from the ribosome in response to erythromycin treatment. Finally, we present a pulse-chase protocol to examine the turnover of specific peptidyl-tRNAs *in vivo*.

2. Materials

All solutions should be prepared using NANOpure water (Thermo-Barnstead) or equivalent source of 18 M Ω -cm resistivity water. All chemical and radioactive wastes should be disposed of according to the appropriate institutional safety protocols.

2.1 Alkali treatment

1. AcE storage buffer: 8 M urea, 10 mM sodium acetate (pH 5.0), 1 mM EDTA
2. 1 M Tris-HCl (pH 8.9)
3. 10 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0)
4. 3 M sodium acetate (pH 5.0)
5. Ice bucket
6. Microcentrifuge
7. 95% ethanol
8. 75% ethanol

2.2 Copper sulfate treatment

1. AcE storage buffer: 8 M urea, 10 mM sodium acetate (pH 5.0), 1 mM EDTA
2. 100 mM CuSO₄
3. microcentrifuge
4. 95% ethanol
5. 75% ethanol
6. AcE loading buffer: 8 M urea, 10 mM sodium acetate (pH 5.0), 1 mM EDTA, 0.01% xylene cyanol, 0.01% bromophenol blue

2.3 Acid-urea polyacrylamide gel electrophoresis

1. 3 M sodium acetate (pH 5.0)
2. 500 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0)
3. AcE gel loading buffer: 8 M urea, 10 mM sodium acetate pH 5.0, 1 mM EDTA, 0.01% xylene cyanol, 0.01% bromophenol blue
4. Urea (98% or greater purity)
5. 30% acrylamide: *bis*-acrylamide (29:1) solution
6. 10% ammonium persulfate (APS), prepare every few weeks and store at 4 °C.
7. *N,N,N',N'*-tetramethylethane-1,2-diamine (TEMED)

8. Prepare gel solution in 15 mL conical tube: add 3 g of urea, 0.6 mL of 1 M sodium acetate (pH 5.0), 12.5 μ L of 500 mM EDTA (pH 8.0) and 1.2 mL of 30% acrylamide solution (29:1 acrylamide:*bis*-acrylamide). For 12% gels, add 2.4 mL of 30% acrylamide solution. Add water to a final volume of 6 mL and mix solution on a rotisserie at room temperature until the urea is completely dissolved.
9. Sodium acetate electrophoresis running buffer: 100 mM sodium acetate (pH 5.0), 1 mM EDTA. Prepare in pre-chilled (4 °C) water.
10. Mini-Protein 3 (Bio-Rad) or equivalent mini-gel electrophoresis system

2.4 Northern blot

1. 10 $\times$ TBE: 0.89 M Tris-HCl (108 g/L), 0.87 M boric acid (54 g/L), 6.4 mM EDTA (1.86 g/L)
2. 20 $\times$ SSC: 3 M sodium chloride (175.32 g/L), 0.3 M sodium citrate (88.23 g/L), adjust to pH 7.0 with 1 N HCl and filter.
3. 10% sodium dodecyl sulfate (SDS)
4. 100 $\times$ Denhardt's solution (250 mL): 5 g Ficoll 400, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin (fraction V), freeze 50 mL aliquots at -20 °C for long-term storage.
5. Hybridization solution (50 mL): to 29 mL of water, add 5 mL 100 $\times$ Denhardt's solution, 15 mL 20 $\times$ SSC and 0.5 mL of 10% SDS.
6. Whatman 3MM CHR filter paper: cut four pieces (70 mm $\times$ 90 mm) for each gel to be transferred.
7. Nylon membrane (0.45 μ m pore, Nytran[®] SPC, Whatman)
8. Semi-dry electrotransfer apparatus
9. Power supply
10. Adenosine 5'-triphosphate [γ -³²P]-labeled (3,000 Ci/mmol)
11. Bacteriophage T4 polynucleotide kinase and 10 $\times$ reaction buffer
12. Oligonucleotide probes: *E. coli* tRNA₂^{Pro} probe, (5' - CAC CCC ATG ACG GTG CG); *E. coli* tRNA₃^{Gly} probe, (5' - CTT GGC AAG GTC GTG CT); and *E. coli* tRNA₂^{Arg} probe, (5' - CCT CCG ACC GCT CGG TTC G).
13. Sephadex G-25 spin columns equilibrated in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (see Note 1)
14. Bambino II rotisserie hybridization oven
15. Plastic food wrap
16. 8 $\times$ 10-inch phosphorimaging screen
17. Phosphorimager

¹Pre-packed, disposable Sephadex G-25 gel filtration spin columns are available from GE Healthcare and a number of other commercial vendors. We prepare our own spin columns using recycled silica-based mini-prep spin columns (e.g. Qiagen or Promega). Used mini-prep columns are loaded with up to 750 μ L of Sephadex G-25 slurry equilibrated in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. Centrifuge the column at 3,000 $\times$ g for 2 min to remove the void volume. Pipette the labeling reaction (40 – 50 μ L) onto the resin and spin at 3,000 $\times$ g for 2 min to collect the [³²P]-labeled oligonucleotide probe. Single-stranded oligonucleotides bind poorly to the silica resin and are therefore recovered efficiently.

2.5 Pulse-chase

1. MOPS-tricine-NaCl solution: 0.5 M 3-morpholinopropane-1-sulfonic acid (MOPS), 0.05 M tricine, 0.625 M NaCl (pH 7.4)
2. FeCl₂/trace metal solution (for 100 mL): 5 g FeCl₂•4H₂O, 184 mg CaCl₂•2H₂O, 64 mg H₃BO₃, 40 mg MnCl₂•4H₂O, 18 mg CoCl₂•6H₂O, 4 mg CuCl₂•2H₂O, 340 mg ZnCl₂, 605 mg Na₂MoO₄•2H₂O, 8 mL concentrated HCl, q/s to 100 mL
3. O solution (500 mL): 28.8 g MgCl₂•6H₂O, 10 mL of FeCl₂/trace metal solution, q/s to 500 mL and filter to sterilize.
4. S solution: 275 mM K₂SO₄ (4.8 g/100 mL)
5. N solution: 3.74 M NH₄Cl (20 g/100 mL)
6. 0.5 M K₂HPO₄ (dibasic)
7. 1 mg/mL thiamine-HCl
8. 40% D-glucose
9. MOPS defined media (10 mL): To 8.97 mL of sterile water add 20 µL of O solution, 800 µL of MOPS-tricine-NaCl solution, 10 µL of S solution, 50 µL of N solution, 26.4 µL of 0.5 M K₂HPO₄, 100 µL of 40% D-glucose, 10 µL of 1 mg/mL thiamine-HCl. Supplement with appropriate antibiotics and all amino acids (final concentration 20 µg/mL) except L-methionine and L-cysteine. MOPS defined media is based on the protocol of Neidhardt *et al.* (25).
10. 125 mL Erlenmeyer flask with cap
11. Ice bucket
12. 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG)
13. Radiolabeling solution: [³⁵S]-L-methionine and L-cysteine (1175 Ci/mmol, MP Biomedicals)
14. Chase solution: unlabeled L-methionine and L-cysteine, 25 mg/mL of each
15. 50% trichloroacetic acid (TCA)
16. Microcentrifuge
17. Cell wash buffer: 50 mM Tris-acetate (pH 7.0), 1 mM EDTA
18. Cell lysis buffer: 1% sodium dodecyl sulfate (SDS), 50 mM Tris-acetate (pH 7.0), and 1 mM EDTA.
19. 2% cetyltriethylammonium bromide (CTABr)
20. 0.5 M sodium acetate (pH 5.0)
21. 100% acetone
22. AcE storage buffer: 8 M urea, 10 mM sodium acetate (pH 5.0), 1 mM EDTA
23. AcE gel loading buffer: 8 M urea, 10 mM sodium acetate (pH 5.0), 1 mM EDTA, 0.01% xylene cyanol, 0.01% bromophenol blue
24. 30% methanol
25. 15 × 15 cm Tupperware® or equivalent plastic container
26. 3MM Chr filter paper (Whatman)

27. Plastic food wrap
28. Gel dryer and vacuum pump
29. 8 × 10-inch phosphorimaging screen
30. Phosphorimager

3. Methods

The methods outlined in sections 3.1, 3.2 and 3.3 are designed to analyze total cellular RNA prepared by the one-step guanidinium isothiocyanate (GITC)-phenol extraction method of Chomczynski & Sacchi (26, 27). Commercially available extraction reagents (e.g. TRIzol[®] or TRI Reagent[®]) yield the same results at a significantly higher cost. GITC-phenol extraction performs well for the isolation of peptidyl-tRNAs carrying nascent chains up to 80 residues in length (Fig. 1), but is not suitable for the recovery of peptidyl-tRNAs with longer nascent chains. Presumably, larger peptidyl-tRNAs partition to the organic phenol phase during extraction due to their higher protein content (see Note 2 for analysis of large peptidyl-tRNAs). GITC-phenol reagents are typically buffered with acetate to pH ~ 5, so the labile ester bonds within aminoacyl- and peptidyl-tRNAs are stabilized during extraction. Once isolated, RNA samples must be stored at acidic pH to preserve aminoacyl-tRNA. We use dilute sodium acetate (10 mM, pH 5.0) supplemented with 1 mM EDTA for long-term storage of RNAs. In contrast to the original report by Ho & Kan (7), we have observed no appreciable tRNA deacylation in samples that have been subjected to multiple freeze-thaw cycles.

3.1 Alkaline pH treatment of RNA

1. Pipette 50 µg of total *E. coli* RNA into a microfuge tube (e.g. 25 µL of a 2 mg/mL RNA solution). Add water to 40 µL, then 5 µL of 10 mM EDTA (pH 8.0) and 5 µL of 1 M Tris-HCl (pH 8.9). Spot 2 µL of the sample onto pH indicator paper to confirm pH > 8.0.
2. Incubate at 37 °C for 60 min.
3. Add 3 M sodium acetate (pH 5.0) to a final concentration of 0.3 M.
4. Add 2.5-volumes of ice-cold 95% ethanol and incubate on ice for 10 min.
5. Precipitate RNA by centrifugation in a microcentrifuge at 13,000 × *g* for 10 min at 4 °C.
6. Remove the supernatant and wash once with 70% ethanol followed by centrifugation at 13,000 × *g* at 4 °C.
7. Remove supernatant and dissolve the RNA pellet in 10 – 20 µL of AcE storage buffer for gel electrophoresis. We typically load 2.5 to 10 µg of total RNA per lane on a 1.0 mm thick mini-gel. Therefore, the concentration of the re-dissolved RNA should be at least 0.5 µg/mL to minimize the volume of the loaded sample.
8. Perform Northern blot analysis as described in Section 3.3. Representative results of this procedure are presented in Figure 2.

²Although larger peptidyl-tRNAs are difficult to isolate with GITC-phenol, they can be precipitated from whole-cell SDS lysates using CTABr. Follow the procedures in Section 3.5 steps 11 through 16, and analyze by Northern blot hybridization. This procedure has been used successfully to isolate tRNAs carrying up to 166 nascent chain residues (20, 30, 31).

3.2 Copper sulfate treatment of RNA

1. Pipette 30 μg of total RNA into a fresh 1.5 mL microfuge tube (e.g. 15 μL of a 2 mg/mL RNA solution), then add AcE storage buffer to 27 μL .
2. Add 3 μL of 100 mM CuSO_4 to the RNA solution (10 mM CuSO_4 final concentration).
3. Incubate at 37 °C for 1 h.
4. The deacylated samples may be analyzed directly. Add 5 μL of AcE gel loading buffer to 5 μL of CuSO_4 -treated RNA and perform acid-urea gel electrophoresis and Northern blot hybridization (Section 3.3). Figure 3 shows an application of this procedure to detect peptidyl-tRNA drop-off products induced by erythromycin. Alternatively, deacylated samples may be precipitated for long-term storage following steps 5 through 10 below.
5. Add 1 mL 95% ethanol, invert to mix and incubate at -80 °C for 1 h.
6. Centrifuge at $13,000 \times g$ for 15 min at 4 °C.
7. Remove supernatant and add 1 mL 75% ethanol.
8. Centrifuge at $13,000 \times g$ for 10 min at 4 °C.
9. Carefully remove supernatant with a pipette and allow the RNA pellet to air dry.
10. Dissolve RNA pellet in AcE storage buffer.

3.3 Acid-urea polyacrylamide gel electrophoresis

The following procedures exploit gel mobility shifts to separate deacylated tRNA from aminoacyl- and peptidyl-tRNAs. We routinely use 12% polyacrylamide mini-gels (70 mm $\times$ 90 mm) to analyze aminoacyl-tRNAs. This system can resolve tRNAs carrying large (e.g. Trp) and basic (Arg, Lys and His) amino acid residues from the corresponding deacylated tRNAs (8, 28). However, larger gel systems are required to separate most other aminoacyl-tRNAs from their deacylated counterparts. A portion of each RNA sample should be incubated at alkaline pH to hydrolyze aminoacyl- and peptidyl-tRNA esters. These base-treated samples should be included in each experiment to determine the gel migration position of deacylated tRNA. In general, peptidyl-tRNAs are easily resolved from deacylated and aminoacyl-tRNAs, and therefore 6% polyacrylamide gels and shorter periods of electrophoresis may be used for their analysis. Small peptidyl-tRNAs (two to four amino acid residues) can be differentiated from aminoacyl-tRNA by treating samples with 10 mM CuSO_4 (29). Cu^{2+} ions promote the deacylation of aminoacyl-tRNA, but have little effect on peptidyl-tRNA. Therefore, direct comparison of untreated and CuSO_4 -treated samples can reveal peptidyl-tRNAs that migrate near aminoacyl-tRNA.

1. Prepare the acid-urea polyacrylamide gel solution in a graduated 15 mL conical polypropylene tube.
2. Assemble the Mini-Protein 3 gel-casting stand according to manufacturer's instructions.
3. Add 70 – 100 μL of 10% APS to the gel solution and invert gently to mix.
4. Add 7 – 10 μL of TEMED to initiate gel polymerization. Invert gently to mix and pour (or pipette) the solution into the gel cast. Insert a 10-well comb and ensure that no bubbles are trapped beneath the comb. Polymerization is typically complete within 20 – 30 min at room temperature (see Note 4).

5. After polymerization is complete, carefully remove the comb from the gel and assemble into the BioRad Mini-Protean 3 apparatus.
6. Add cold sodium acetate-EDTA running buffer to the upper and lower electrophoresis chambers. Use a needle and syringe to carefully wash the unpolymerized acrylamide and urea from the wells. Because urea continually diffuses from the gel into the sample wells, it is important to remove the urea with a needle and syringe *immediately* prior to sample loading. Failure to do so results in diffuse samples and significantly reduces resolution during electrophoresis.
7. Load the gel with 2.5 – 10 μ g of total RNA. Samples may be denatured at 95 °C for 3 min if desired (see Note 5).
8. Perform electrophoresis at 100 V (constant voltage) at 4 °C in a cold room (see Note 6). Run 6% polyacrylamide gels for 105 min to analyze peptidyl-tRNAs, and 12% gels for up to 6 hr to analyze aminoacyl-tRNAs.

3.4 Northern blot analysis

1. After electrophoresis, remove the gel sandwich from the apparatus. Carefully remove the gel and soak in $0.5 \times$ TBE in a small plastic container.
2. Soak the nylon membrane in $0.5 \times$ TBE buffer in a separate container.
3. While the gel and membrane are soaking, assemble the transfer apparatus: wet a piece of Whatman filter paper in $0.5 \times$ TBE and place on the transfer apparatus; roll a 15×100 mm glass test tube (or equivalent) over the wetted filter paper to remove air bubbles; pour a small amount (1 to 2 mL) of $0.5 \times$ TBE buffer onto the filter paper; carefully hold the gel in the Tupperware container and pour out all excess buffer.
4. Carefully place a dry piece of filter paper onto the gel and lift. Place the gel (filter-paper side down) onto the first wetted filter paper. Roll carefully to remove air bubbles. Place the wetted nylon membrane directly onto the gel, followed by the remaining two wetted filter papers. Roll carefully to remove air bubbles. Use a large Kim-wipe to remove excess transfer buffer.
5. Place the top electrode (anode) onto the sandwich and transfer at 650 mA (constant current) for 30 min.
6. Disassemble the transfer apparatus. Remove the nylon membrane and incubate in $5 \times$ SSC briefly (1 – 2 min).
7. Cross-link the RNA to the nylon membrane using a Stratalinker or other UV light source for 2 min.

⁴Deprotonated TEMED is required to initiate acrylamide/*bis*-acrylamide polymerization. Therefore, acid-urea polyacrylamide gels polymerize more slowly than Tris-buffered gels. Riboflavin/visible light and other initiator strategies are sometimes used to initiate polymerization at acidic pH (32, 33). However, we find that higher concentrations of both APS (100 μ L of 10% solution per 6 mL gel) and TEMED (10 – 15 μ L per 6 mL gel) are sufficient to promote polymerization at acidic pH. If polymerization requires more than 20 min, prepare a fresh 10% APS solution.

⁵Nucleic acid samples are usually heat denatured prior to polyacrylamide gel electrophoresis. In principle, high temperatures could promote deacylation, but we have seen no difference between unheated samples and those incubated at 95 °C for 3 min. Peptidyl-tRNAs are typically more stable than aminoacyl-tRNAs and can be heated without degradation. The acid-urea gels described here possess no stacking mechanism and therefore it is critical to minimize the loaded sample volume. For optimal resolution of aminoacyl-tRNA from deacylated tRNA, we recommend a maximum volume of 10 μ L (5 μ L sample + 5 μ L AcE loading buffer). If using larger gels, then sample volumes may be increased accordingly.

⁶Polyacrylamide gels buffered with 100 mM sodium acetate draw high currents during electrophoresis. This results in substantial Joule heating, which can readily fracture glass plates. We conduct electrophoresis in a 4 °C environmental room using currents ranging from 17 to 22 mA (corresponds to 100 V for the BioRad Mini-Protean 3 apparatus). Although the Mini-Protean 3 and other electrophoresis apparatuses can run multiple gels simultaneously, we recommend running individual gels to reduce Joule heating.

8. Wash the blotted membrane with 10 – 15 mL of $0.1 \times \text{SSC}$, 0.1% SDS at 65 °C in a rotisserie hybridization oven for 30 min. We perform hybridizations in disposable 50 mL polypropylene conical tubes.
9. Remove the wash solution and add 10 – 15 mL of pre-hybridization solution.
10. Incubate for 1 hour at the hybridization temperature. The hybridization temperature is 15 °C below the calculated melting temperature (T_m) of the oligonucleotide probe.
11. Label the oligonucleotide probe with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and T4 polynucleotide kinase (PNK). In a microfuge tube add: 33 μL water, 4 μL $10 \times \text{PNK}$ buffer, 2 μL oligonucleotide (10 μM) and 1 μL $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$. Add 1 μL of polynucleotide kinase and mix gently by pipette. Incubate at 37 °C for 40 min. Stop the reaction by incubating at 70 °C in a water bath for 10 min, and collect reaction by centrifugation. Pipette the reaction onto a microspin G-25 gel filtration column to remove free radiolabeled ATP.
12. Add the radiolabeled oligonucleotide probe to the hybridization solution and incubate in the rotisserie oven overnight.
13. Decant the hybridization solution into a 50 mL polypropylene conical tube. Hybridization solutions can be frozen and re-used several times.
14. Wash the membrane with 10 – 15 mL of $6 \times \text{SSC}$, 0.1% SDS for 5 min at room temperature. Repeat this wash step two more times. Decant the wash solutions into the appropriate ^{32}P -waste container.
15. Wash the membrane with 10 – 15 mL of $6 \times \text{SSC}$, 0.1% SDS for 20 min at 10 °C below the calculated T_m for the oligonucleotide probe.
16. Remove the membrane with tweezers and blot onto a KimWipe to remove excess fluid. Wrap in plastic food wrap and expose to a phosphorimager screen (2 – 48 hr) or X-ray film (1 – 3 days).
17. Visualize by phosphorimager or film developer.

3.5 Pulse-chase protocol

The following pulse-chase approach is used to monitor peptidyl-tRNA turnover *in vivo*. *E. coli* cells are grown in defined media and pulsed with ^{35}S -L-methionine/L-cysteine to radiolabel nascent peptide chains. The decay of ^{35}S -labeled peptidyl-tRNA is monitored following the addition of excess unlabeled L-methionine/L-cysteine. For this analysis, peptidyl-tRNAs are purified from bulk cellular proteins using CTABr precipitation. Once isolated, the peptidyl-tRNAs can be hydrolyzed and specific nascent chains immunoprecipitated for subsequent SDS-PAGE analysis as described (30, 31).

Alternatively, plasmid-borne over-expression systems can be used to increase the level of peptidyl-tRNAs of interest. We have used this approach to study specific peptidyl-tRNAs in unfractionated CTABr precipitates (20). If multiple peptidyl-tRNAs are observed in ^{35}S -labeled samples, then the species of interest can be identified by Northern blot hybridization (see Note 3).

³Multiple peptidyl-tRNA species may be observed in ^{35}S -labeled CTABr precipitates (see Fig. 4). Northern blot analysis can be used to unambiguously identify these radiolabeled peptidyl-tRNAs through the correlation of gel migration positions (20). Briefly, peptidyl-tRNA is isolated from ^{35}S -labeled and unlabeled cells. The labeled and unlabeled samples are run in adjacent lanes on an acid-urea polyacrylamide gel, followed by electrotransfer to nylon membrane. The membrane is then cut between the two lanes, and the unlabeled section is subjected to Northern blot hybridization with a ^{32}P -labeled oligonucleotide probe. After hybridization and washing, the two membranes are carefully realigned with one another and exposed to a phosphorimager screen.

1. Grow *E. coli* cells overnight at 37 °C in MOPS-glucose defined media supplemented with the appropriate antibiotics (see Note 7).
2. Resuspend the overnight culture in 10 mL of pre-warmed MOPS media to an optical density at 600 nm (OD₆₀₀) of 0.05.
3. Place flask in a rotary shaker and incubate at 37 °C with shaking (250 – 300 rpm) until the culture grows to OD₆₀₀ ~ 0.5.
4. Prepare pre-chilled microfuge tubes containing 20 µL of 50% TCA. Prepare one tube for each time point in your experiment. Label the tubes and place on ice for use in step 7 below.
5. Add 15 µL of 1 M IPTG to induce expression of the plasmid construct. Incubate culture for 15 – 30 min.
6. Add 20 µL of [³⁵S]-L-methionine/L-cysteine solution (final concentration of 20 µCi/mL) to the culture.
7. Incubate for 2 min with shaking.
8. Add 80 µL of chase solution (final concentration 0.2 mg/mL, see Note 8) to the flask and rapidly mix by swirling.
9. Remove 1 mL of the culture and rapidly transfer into a microfuge tube containing 20 µL of 50% TCA on ice. Mix briefly, cap tube and place into ice.
10. Continue to swirl culture by hand or on shaker. Remove 1 mL aliquots every 15 s for the first minute, then at 30 – 60 s intervals for the next 2 min.
11. After all culture aliquots have been removed, centrifuge all the sample tubes at 13,000 × *g* for 2 min in a microcentrifuge to collect cells (see Note 9).
12. Carefully remove the supernatant by pipette and dispense into a [³⁵S]-waste container.
13. Add 1 mL of cell wash buffer to each tube – do not resuspend the cell pellet.
14. Centrifuge at 13,000 × *g* for 5 min and remove the supernatant.
15. Resuspend the cell pellet in 100 µL of cell lysis buffer, and allow cells to lyse at room temperature for at least 20 min. Cell lysates can be frozen at this point and the protocol can be resumed at step 16 at a later time.
16. Remove cell debris by centrifugation at 13,000 × *g* for 10 min. Carefully remove supernatant and transfer to a fresh microfuge tube.
17. Remove 45 µL of each cell lysate and place in a fresh microfuge tube, freeze the remaining 55 µL of cell lysate as a backup sample. Add 0.5 mL of 2% CTABr solution and 0.5 mL of 0.5 M sodium acetate (pH 5.0) solution. Cap and invert tube to mix.
18. Place in an ice-water bath for 20 min.

⁷*E. coli* cells undergo growth arrest for several hours after transfer from rich broth into defined MOPS-glucose medium. Therefore, we recommend overnight culture in MOPS-glucose medium prior to running the experiment. Most K-12-derived *E. coli* strains will reach mid-log phase within 4 to 5 hr if seeded from an overnight MOPS-glucose culture.

⁸In our experience, L-methionine/L-cysteine chase solutions lose their effectiveness within hours after preparation. Treatment of the chase solution with reducing agents (e.g. dithiothreitol and β-mercaptoethanol) exacerbates the problem. Therefore, we recommend preparing fresh L-methionine/L-cysteine chase solution immediately prior to each pulse-chase experiment.

⁹Radioactive contamination of the rotor and interior surfaces of the microcentrifuge commonly occurs during this procedure. To minimize contamination, stretch a small piece of Parafilm® M around the microcentrifuge tub caps prior to centrifugation.

19. Thaw precipitates at 30 °C for 10 min.
20. Centrifuge at $13,000 \times g$ for 30 min.
21. Carefully remove supernatant and add 1 mL of 100% acetone.
22. Centrifuge $13,000 \times g$ for 10 min. Carefully remove supernatants and allow pellet to air-dry until the acetone evaporates.
23. Resuspend pellets in 25 μ L of AcE gel-loading buffer (see Note 10).
24. Determine the absorbance at 260 nm (A_{260}) of each reaction. Load equivalent A_{260} units onto the acid-urea polyacrylamide gel.
25. Load 10 μ L of each sample onto an acid-urea polyacrylamide gel and perform electrophoresis (100 V constant voltage) at 4 °C for 105 min.
26. Disassemble the Mini-Protean 3 apparatus and carefully separate the glass plates.
27. Submerge the glass plate to which the gel is attached in 30% methanol.
28. Gently shake the gel for 10 min at room temperature to remove urea.
29. Decant the methanol into an [35 S]-waste container.
30. Place a piece of dry filter paper onto the gel and carefully lift the gel from the glass plate.
31. Cover the gel with plastic food wrap and place in a gel dryer at 75 °C under vacuum until the gel is completely dehydrated.
32. Expose the dried gel to a phosphorimaging screen for 8 – 16 hr.
33. Image the screen and quantify the appropriate peptidyl-tRNA species using phosphorimager software. Fit exponential decay equations to the experimental data using DeltaGraph (RedRock Software) or equivalent software package:

$$\begin{aligned} \text{single-exponential decay: } N(t) &= N_0 e^{-\lambda t} \\ \text{single-exponential half-life: } t_{1/2} &= \ln 2 / \lambda \end{aligned}$$

$$\begin{aligned} \text{double-exponential decay: } N(t) &= N_0 e^{-(\lambda a + \lambda b)t} \\ \text{double-exponential half-life: } T_{1/2} &= \ln 2 / (\lambda a + \lambda b) \end{aligned}$$

where N_0 = peptidyl-tRNA at $t = 0$ s, λ is the decay constant, t is time, λa and λb are constants for two independent decay processes. Multiple exponential decay equations generally yield better fits, but should only be used if the decay process is known to occur through multiple independent pathways. Typical results from these procedures are presented in Figure 4.

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¹⁰CTABr precipitates can be diffusely distributed over the interior surfaces of microfuge tubes. To dissolve the entire sample, take care to pipette gel-loading buffer onto all surfaces that may have collected diffuse precipitate. Adherence to this procedure will significantly increase the tRNA concentration in the samples.

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Figure 1. Analysis of peptidyl-tRNAs by Northern blot hybridization

Total RNA was extracted from *E. coli* cells expressing peptides containing a C-terminal Pro-Pro motif. The Pro-Pro nascent peptide interferes with translation termination, causing the accumulation of peptidyl prolyl-tRNA₂^{Pro} on paused ribosomes (20, 34). RNA samples were resolved on an acid-urea 6%-polyacrylamide gel followed by Northern blot hybridization with a radiolabeled oligonucleotide probe specific for *E. coli* tRNA₂^{Pro}. The number of nascent chain residues encoded by each expression construct is indicated above the lanes. Peptidyl prolyl-tRNA₂^{Pro} is not detected in the uninduced sample (no IPTG).

Figure 2. Northern blot analysis of aminoacyl- and peptidyl-tRNAs

Total RNA from *E. coli* cells carrying an inducible *secM* mini-gene construct was extracted and analyzed by acid-urea gel electrophoresis. Subsequent Northern blot hybridization was conducted with probes for tRNA₃^{Gly} and tRNA₂^{Arg}. The SecM nascent peptide induces a site-specific translational arrest with the codon corresponding to Gly¹⁶⁵ in the ribosome P site (21, 35). The resulting peptidyl glycyl-tRNA₃^{Gly} is detected by Northern blot upon induction with IPTG. Treatment at alkaline pH (+ **base**) promotes the deacylation of both peptidyl glycyl-tRNA₃^{Gly} and arginyl-tRNA₂^{Arg}.

Figure 3. Deacylation of aminoacyl-tRNA with copper sulfate

Total RNA was isolated from *E. coli pth(ts)* cells grown at the permissive temperature (30 °C) with and without 100 µg/mL of erythromycin. Erythromycin induces drop-off of small peptidyl-tRNAs from the ribosome (36). These drop-off products are rapidly hydrolyzed in *pth*⁺ cells, but can be detected in *pth(ts)* cells even at the permissive temperature. RNA samples were treated with copper sulfate (CuSO₄) as described in Section 3.3 and run directly on an acid-urea 12% polyacrylamide gel. Northern blot hybridization was performed with a probe for tRNA^{Arg}. The migration positions of deacylated tRNA^{Arg}, arginyl-tRNA^{Arg} and peptidyl arginyl-tRNA^{Arg} are indicated.

Figure 4. Pulse-chase analysis of peptidyl-tRNA turnover

A) Autoradiogram illustrating peptidyl-tRNA turnover using [^{35}S] pulse-labeling and acid-urea gel electrophoresis. Samples were taken at the indicated time points for tRNA isolation as described in Section 3.4. Radiolabeled species corresponding to methionyl-/cysteinyl-tRNAs and peptidyl-tRNAs are indicated. **B)** Fitting of exponential decay equations. Peptidyl-tRNA signal intensities in panel **A** were quantified using Quantity One software (BioRad) and the data fitted to a double-exponential decay equation to calculate composite half-life ($T_{1/2}$) as described in Section 3.4.