CHAPTER SIXTEEN

Investigating the consequences of mRNA modifications on protein synthesis using *in vitro* translation assays

Jeremy G. Monroe[†], **Tyler J. Smith**[†], **and Kristin S. Koutmou***
Department of Chemistry, University of Michigan, Ann Arbor, MI, United States
*Corresponding author: e-mail address: kkoutmou@umich.edu

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[†] Authors contributed equally.

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Abstract

The ribosome translates the information stored in the genetic code into functional proteins. In this process messenger RNAs (mRNAs) serve as templates for the ribosome, ensuring that amino acids are linked together in the correct order. Chemical modifications to mRNA nucleosides have the potential to influence the rate and accuracy of protein synthesis. Here, we present an in vitro *Escherichia coli* translation system utilizing highly purified components to directly investigate the impact of mRNA modifications on the speed and accuracy of the ribosome. This system can be used to gain insights into how individual chemical modifications influence translation on the molecular level. While the fully reconstituted system described in this chapter requires a lengthy time investment to prepare experimental materials, it is highly verstaile and enables the systematic assessment of how single variables influence protein synthesis by the ribosome.

1. Introduction

Translation of the genetic code into protein molecules is accomplished by the ribosome. The ribosome uses messenger RNAs (mRNAs) as molecular blueprints to direct the rapid and accurate synthesis of proteins. The ability of the cell to faithfully express its genetic code is essential for cellular survival. However, the speed and fidelity of the ribosome are not uniform. Even in healthy cells, amino acid addition rates vary between codons, and amino acids not specified by the mRNA are erroneously incorporated into growing polypeptide chains every 1000–10,000 codons (Gromadski & Rodnina, 2004). While most miscoding events are inconsequential for protein function, reductions in translational fidelity can have biological consequences—both perturbing and promoting cellular health. Increases in amino acid substitution levels are deleterious to cellular health and to linked a variety of neurological disorders (Kapur & Ackerman, 2018; Steiner & Ibba, 2019). However, under some conditions temporary, modest increases in miscoding enhance cellular fitness under environmental stress (Drummond & Wilke, 2009; Garrett & Rosenthal, 2012; Pan, 2013).

Many factors, including the availability of aminoacyl-tRNAs and the post-transcriptional modification status of RNAs in the translational machinery, influence the how quickly and accurately ribosomes decode mRNAs (Dunkle & Dunham, 2015; Eyler et al., 2019; Hudson & Zaher, 2014; Hoernes et al., 2018; Jackman & Alfonzo, 2013). Evaluating the impact of individual RNA modifications on translational speed and fidelity is challenging in the context of a cell because modifications are often incorporated into multiple RNAs important for protein synthesis (tRNAs,

rRNAs and mRNAs) by the same enzyme. This makes it difficult to deplete RNA modifying enzymes and confidently assign observed changes in protein output to a distinct RNA species. Determining the influence of mRNA modifications on translation is becoming an important question with the discovery of modifications in mRNA codons, and the incorporation of modified nucleosides into mRNAs used in emerging mRNA-based vaccine and therapeutic platforms (Andries et al., 2015; Jones, Monroe, & Koutmou, 2020; Karikó et al., 2008; Pardi, Hogan, Porter, & Weissman, 2018; Sahin, Karikó, & Türeci, 2014).

The use of in vitro and cell free systems to study translation dates back to the 1960s when they were used to reveal the triplet codon pattern of the genetic code (Nirenberg & Matthaei, 1961). Here we discuss the application of a fully reconstituted *E. coli* in vitro translation system to investigate how chemically modified mRNA codons impact ribosome speed and accuracy at the molecular level. This approach has long been used by researchers to discover how the ribosome decodes mRNA codons (Dever, Dinman, & Green, 2018; Pape, Wintermeyer, & Rodnina, 1998; Rodnina & Wintermeyer, 2001). We present how to purify the individual components required for translation (ribosomes, mRNAs, tRNAs and translation factors), reconstitute active translation complexes from purified components, and perform single turnover assays to assess amino acid incorporation (and misincorporation) by the ribosome (Fig. 1). While these experiments focus on investigating modifications in mRNAs, the approaches we discuss can also be applied to studying other aspects of translation.

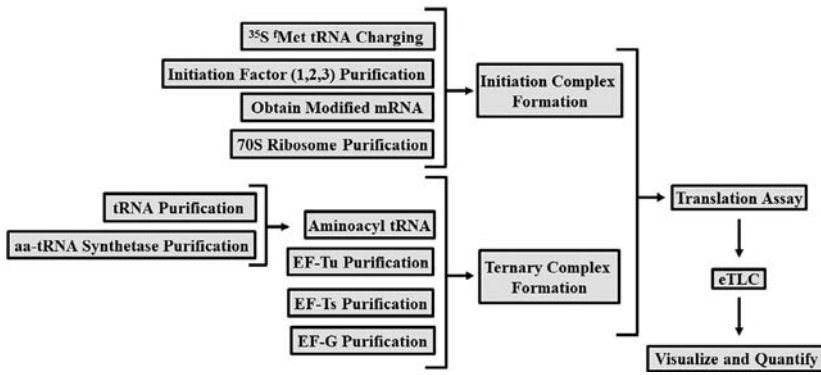

Fig. 1 Experimental flowchart for in vitro translation assays.

1.1 In vitro system chemicals and equipment

Chemicals	Equipment
Tris Base	HPLC (+ Fraction collector)
Ampicillin	FPLC (+ Fraction collector) Fume hood Phosphorimager
Acetic Acid	Waters XBridge BEH C18 OBD Prep wide pore HPLC column
Lactose	HisTrap FF column, 5 mL
Sucrose	Resource Q Column, 5 mL
Glycerol	Beckman Coulter Optima XE-90 Ultracentrifuge with zonal setting
GTP	Ti-15 Zonal Rotor
Iron (III) Chloride ($FeCl_3$)	Type 45 Ti Fixed-Angle Titanium Rotor
	Ti45 Polycarbonate Ultracentrifuge tubes
Magnesium Chloride ($MgCl_2$)	Beckman TLA 100 Benchtop Ultracentrifuge
Ammonium Chloride (NH_4Cl_2)	TLA-100 Rotor
Phosphoenol pyruvate (PEP)	5 mL polycarbonate Ultracentrifuge tubes
Magnesium Acetate ($MgOAc_2$)	Teledyne ISCO UA-6 UV/Vis Detector and Type 6 Optical Unit Attachment
Magnesium Sulfate (Mg_2SO_4)	French press, microfluidizer or sonicator
Sodium Chloride (NaCl)	Large Shaking Incubator
Ammonium Acetate (NH_4OAc)	Cell Density Meter Electrophoresis gel rig and glass plates
Potassium Hydroxide (KOH)	4 L Erlenmeyer flasks Vacuum Filtering Flask
β-mercaptoethanol (BME)	50 mL conical tubes Scintillation vials
Ethylenediaminetetraacetic acid (EDTA)	15 mL conical tubes

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Chemicals	Equipment
Trichloroacetic acid (TCA) Methylene blue	
Urea 29:1 acrylamide Tris/Borate/EDTA (TBE) buffer TEMED Ammonium per sulfate (APS) Pyridine ScintiVerse BD Cocktail	15 mL Amicon Ultra centrifugal filters (10 K MWCO) Ultracentrifuge tubes
Stoddard Solvent Formamide RNA loading dye	Bio-Rad P6 Spin Columns 0.22 μM PES filter paper
Ethanol (EtOH)	Micropipettes and filter tips (2, 20, 200, and 1000 μL)
Liquid Nitrogen	Cellulose TLC plates (20 × 20 cm) 500 mL Bottle Top filter Whatman filter paper Phosphorscreens and cassettes Heat gun
Chloroform ($CHCl_3$—HPLC grade, ≥99.9%)	Beckman Coulter Avanti J-E Floor Centrifuge
Phenol (pH 4.6)	Beckman Coulter Avanti J-20 I Floor Centrifuge
Glyco-blue	Polypropylene centrifuge bottles (1 L)
Carrier DNA (calf thymus DNA)	Heat block(s) capable of a range of temperatures (25–95 °C)
Phenylalanine amino acid	JA-10 Rotor
Complete amino acid mixture	JA-20 Rotor
MilliQ/RNase Free water (H_2O)	JLA-8.100 Rotor
Biological components	
E. coli strain MRE600	
HB101 *E. coli* cells	

Continued

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pUC57 plasmids containing tRNA sequences of interest
S100 Extract
Purified total tRNA
Plasmids to express the following proteins:
Initiation factor 1 (IF-1)
Initiation factor 2 (IF-2)
Initiation fator 3 (IF-3)
Elongation factor Tu (EF-Tu)
Elongation factor Ts (EF-Ts)
Elongation factor G (EF-G)
Pyruvate Kinase

1.2 Ribosome purification buffers

Media
Luria-Base (LB) Broth
Luria-Base (LB) Agar
Terrific Base (TB) Broth

Buffer R-A		Buffer R-D		Buffer R-O (10×)	
Reagent	Final concentration	Reagent	Final concentration	Reagent	Final concentration
Tris HCl, pH 7.5	20 mM	Tris HCl, pH 7.5	20 mM	Tris HCl, pH 7.5	200 mM
NH_4Cl	100 mM	NH_4Cl	500 mM	NH_4Cl	600 mM
$MgCl_2$	10 mM	$MgCl_2$	10 mM	$MgCl_2$	52.5 mM
EDTA	0.5 mM	EDTA	0.5 mM	EDTA	2.5 mM
BME	6 mM	Sucrose	1.1 M		

—cont'd

Buffer R-10: 1× Buffer R-O		Buffer R-40		Buffer R-50	
Reagent	**Final concentration**	**Reagent**	**Final concentration**	**Reagent**	**Final concentration**
Buffer R-O (10×)	1×	Buffer R-O (10×)	1×	Buffer R-O (10×)	1×
Sucrose	10% (w/v)	Sucrose	40% (w/v)	Sucrose	50% (w/v)
BME	3 mM	BME	3 mM	BME	3 mM

Buffer R-60	
Reagent	**Final concentration**
Buffer R-O (10×)	1×
Sucrose	60% (w/v)
BME	3 mM

1.3 tRNA purification buffers

tRNA growth media		tRNA extraction buffer, pH 7	
Reagent	**Concentration**	**Reagent**	**Concentration**
Terrific Broth (TB)	25 g/L	Tris-HCl	20 mM
Glycerol	5 g/L	$Mg(OAC)_2$	20 mM
NH_4Cl	50 mM		
$MgSO_4$	2 mM		
$FeCl_3$	0.1 mM		
Glucose	0.05% (w/v)		
Lactose (if using autoinduction)	0.2% (w/v)		

Buffer A (IEX)		Buffer B (IEX)	
Regent	**Final concentration**	**Regent**	**Final concentration**
NH_4OAc	50 mM	NH_4OAc	50 mM
NaCl	300 mM	NaCl	800 mM
$MgCl_2$	10 mM	$MgCl_2$	10 mM

HPLC Buffer A, pH 5		HPLC Buffer B, pH 5	
Reagent	**Concentration**	**Reagent**	**Concentration**
NH_4OAc	20 mM	NH_4OAc	20 mM
$MgCl_2$	10 mM	$MgCl_2$	10 mM
NaCl	400 mM	NaCl	400 mM
		Methanol	60%

1.4 Reaction buffers

5× Charging buffer, pH 7.6		10× Translation buffer	
Reagent	**Final concentration**	**Reagent**	**Final concentration**
HEPES	0.5 M	Tris-Cl pH 7.5	0.5 M
KCl	0.05 M	NH_4Cl	0.7 M
$MgCl_2$	0.1 M	KCl	0.3 M
H_2O	to 1 mL	$MgCl_2$	0.07 M
		BME	0.05 M
		H_2O	To 1 mL

1.5 eTLC separation buffers

eTLC buffers

pH	Contents	Volume in 3 L (final volume)
1.9	Formic acid/acetic acid	60 mL/240 mL
2	Formic acid	80 mL
2.5	Acetic acid	200 mL
2.8	Pyridine/acetic acid	15 mL/600 mL
3.5	Pyridine/acetic acid	6.6 mL/66 mL
3.6	Ammonium formate/formic acid	6.3 g/10 ml

—cont'd
eTLC buffers

pH	Contents	Volume in 3 L (final volume)
3.7	2 M citric acid/1 N sodium hydroxide	40 mL/67 mL
4.5	Acetic acid/sodium acetate/EDTA	9.4 mL/10.3 g/1.3 g
5.3	Pyridine/acetic acid	20 mL/8 mL
6	Acetic acid/pyridine	14 mL/136 mL
6.4	Pyridine/acetic acid	200 mL/8 mL
8.6	Barbitone/sodium diethylbarbitone	3.3 g/25.5 g
9.2	Sodium borate	38 g

2. Ribosome purification

Zonal centrifugation of crude *E. coli* cell lysate yields fractions of 30S and 50S ribosomal subunits, 70S ribosomes and polysomes. We use a linear sucrose gradient to purify coupled 70S ribosomes and separate the 30S and 50S subunits. In our experience, standard double pelleting ribosomes yields materials of insufficient purity for our assays (Rivera, Maguire, & Lake, 2015).

2.1 Protocol

2.1.1 Day 1

1. Streak MRE 600 cells onto an LB-agar plate without antibiotic and incubate overnight at 37 °C. *E. coli* MRE 600 is the strain of choice ribosome growth because it lacks Ribonuclease I and has negligible nuclease activity (Cammack & Wade, 1965).

2.1.2 Day 2

2. Inoculate 50 mL of LB media with a single MRE600 colony. Grow overnight (~16 hrs) in a shaker-incubator at 37 °C, 220 rpm.

2.1.3 Day 3

3. Prewarm 6 × 4 L flasks containing 1 L of LB media. Add 5 mL of the MRE600 overnight culture to each flask. Shake and incubate at 37 °C, 220 rpm.

4. While the cultures grow, prepare an ice bath for the 4 L flasks.
5. Once the cultures reach an OD600 of 0.6, transfer them to the ice bath for 20 min.
6. Spin the chilled cultures at in a JLA-8.100 rotor at 4000 rpm, 4 °C for 15 min. Combine the cell pellets in a 50 mL conical tube. Either store the pellet at −80 °C, or continue to step 7.
7. Resuspend pellet in ~50 mL of cold Buffer R-A. Lyse cells by microfluidizer or French-Press.
8. Clarify lysate by centrifugation. Spin in a JA-20 rotor at 16,000 rpm, 4 °C for 30 min.
9. While the lysate spins, prepare sucrose cushions by filling 4–6 Ti45 ultracentrifuge tubes with 35 mL of Buffer R-D. Chill on ice.
10. Once the spin in step 8 is finished, collect the supernatant and discard the pellet. Filter supernatant through a 0.22 μM PES syringe filter.
11. Add cold Buffer R-A to bring the filtered supernatant volume to 100 mL.
12. Slowly pour (layer) 25 mL of the supernatant onto each of the pre-chilled Buffer R-D in Ti45 ultracentrifuge tubes. Balance centrifuge tubes with Buffer R-A.
13. Centrifuge tubes in a Ti45 Ultracentrifuge rotor at 37,000 × rpm, 4 °C for 18 hrs. Use the slowest acceleration and deceleration setting available on the centrifuge.

2.1.4 Day 4

14. Gently remove supernatant and rinse each pellet with ~50 μL of cold Buffer R-A.
15. Add 400 μL of Buffer R-A and resuspend pellets in the centrifuge bottles by orbital shaking at 120 rpm, 4 °C for 2 hrs.
16. Prepare, filter (0.22 μM PES) and chill (store at 4 °C) Buffer R-O, Buffer R-5, Buffer R-10, Buffer R-40, Buffer R-50, and Buffer R-60. Add BME only after filtering.

**Note*: make enough R-10 and R-40 to fill your zonal rotor (~1.85 L for the Ti-15 zonal rotor used here).

17. While the pellets resuspend, generate a sucrose gradient in the chilled Ti-15 zonal rotor. Using a gradient maker, begin slowly loading Buffer R-10 via the loading/unloading device (this device is rotor specific). A peristaltic pump can be used to automate buffer loading. After adding ~250 mL of Buffer R-10, begin slowly adding Buffer R-40 into Buffer R-10 to create a 10–40% sucrose gradient. Make sure to keep stirring

the buffers in the gradient maker to ensure proper mixing of sucrose to form desired gradient. After R-40 is loaded, add Buffer R-50 (~100 mL) until sucrose solution begins coming out of the top of the rotor or loading device to ensure rotor is completely filled. Keep the rotor and centrifuge chilled at 4 °C and spinning at 3000 rpm.

18. Clarify resuspended ribosomes (from step 15) by centrifuging in a benchtop microfuge at 14,800 rpm for 1 min.
19. Combine the clarified ribosome-containing supernatants (from step 18) in a 50 mL conical tube on ice. Make a 1:1000 dilution of the supernatant and measure (in triplicate) the absorbance readings at 260 nm.
20. Dilute supernatant to ~30 mL in cold Buffer R-5. Load onto the top of the sucrose gradient in the zonal rotor (step 17) via the loading/unloading device. For best results, load using a 50 mL syringe.
21. Use a 50 mL syringe to slowly add 30 mL of cold Buffer R-O to the top of the gradient in the zonal rotor via the loading/unloading device. This will fully displace the ribosome suspension onto the sucrose gradient.
22. Spin the zonal rotor at 28,000 rpm, 4 °C for 19 hrs. Following the 19 hr spin, the centrifuge should not stop, but be programmed to transition to spin at 3000 rpm, 4 °C.

Note: check if your zonal rotor has different cap components for loading/unloading and higher speed centrifugation, as these caps may need to be switched out.

2.1.5 Day 5

23. Attach a UA-6 UV spectrophotometer to the loading/unloading device for the zonal rotor to follow ribosome unloading at 260 nM.
24. Slowly unload the rotor by adding Buffer R-50 (as described in step 17). After adding ~250 mL, begin mixing Buffer R-60. Collect 15–50 mL fractions in conical tubes when UV peaks at 250 nM are observed. Label tubes with fraction number, place on ice.
25. Remove 100 µL from each fraction of interest (containing ribosomes) and dilute to 500 µL with Buffer RA. Phenol-chloroform extract the solution and ethanol precipitate the top aqueous phase of the samples with 2.5 volumes of ethanol for at least 15 min on ice. The samples can be precipitated longer at − 20 °C if desired.
26. Centrifuge ethanol precipitated samples for 15 min in a benchtop microfuge at maximum speed. Remove supernatant and wash pellets with 70% ethanol. Resuspend pelleted fractions in 25 µL of MilliQ H_2O.

27. Mix up to 2 μg from extracted samples with 2 × formamide RNA loading dye and heat denature at 95 °C for 10 min. Run samples on a 5% denaturing PAGE gel and visualize by UV shadowing or methylene blue staining. Fractions containing 70S ribosomes will have two predominant bands corresponding to the 16S and 23S rRNA. While the PAGE gel is running, chill Ti-45 centrifuge tubes on ice.
28. Pool the fractions (from step 24) that contain 70S ribosomes. Measure and record the absorbance of the pooled sample at 260 nm.
29. Divide the 70S ribosomes into chilled Ti-45 centrifuge tubes. Balance tubes with Buffer R-A and spin at 37,000 × rpm, 4 °C for 18 hrs. Use the slowest possible acceleration and deceleration settings.

2.1.6 Day 6

30. Slowly, and gently remove the supernatant from Ti-45 tubes, taking care because the ribosome pellets are glassy and do not attached strongly to the sides of the tube. Gently resuspend the pellets using a total of 2–5 mL of Buffer R-A. Do not pull the ribosome pellet up into the pipette tip, instead repeatedly (~50 times) rinse over the pellet buffer until resuspended. Avoid the creation of bubbles during resuspension. This procedure is commonly conducted in a cold room (4 °C).
31. Measure the absorbance of the pooled ribosomes at 260 nm and calculate the concentration ($\varepsilon = 4 \times 10^7\,M^{-1}\,cm^{-1}$). Aliquot (50–100 μL) ribosomes, flash freeze in liquid nitrogen and store at −80 °C.

3. Translation factor purification

A single round of translation involves a host of translation factor proteins. At a minimum, initiation factors 1, 2, and 3 (IF1, IF2, IF3), ^{f}methionyl-tRNA formyltransferase (MTF), aminoacyl-tRNA synthetases (AA-RSs) and elongation factor-Tu (EF-Tu) are required for the reconstituted bacterial translation system to function. Additional protein factors, including elongation factor-Ts (EF-Ts) and elongation factor-G (EF-G), are needed if more than one round of amino acid addition is desired. We purify His-tagged translation factor proteins from *E. coli* using plasmids available from AddGene. Multiple expression and purification protocols for translation factors can be found in the literature, and are therefore not included here (Dahlquist & Puglisi, 2000; Eyler et al., 2019; Rodnina & Wintermeyer, 1995; Shimizu et al., 2001; Soffientini et al., 1994; Studer & Joseph, 2007).

4. Purification of natively modified tRNA

Transfer RNAs (tRNAs) contain multiple post-transcriptional modifications important for their function. While T7 transcribed tRNAs can be used for reconstituted translation assays, these tRNAs often exhibit reduced speeds and accuracy in translation reactions compared to their natively modified counterparts (Dunkle & Dunham, 2015; Jackman & Alfonzo, 2013). As such, we typically use natively modified tRNAs in our translation assays. Below we describe the large-scale expression and purification of individual natively modified *E. coli* tRNAs.

4.1 Protocol

4.1.1 Day 1—Transform tRNA plasmid

1. Transform a pUC57 plasmid containing an *E. coli* tRNA sequence of interest (e.g., $tRNA^{Phe}$) into HB101 cells. Grow overnight on an LB-ampicillin agar plate at 37 °C.

4.1.2 Day 2—Overnight starter culture

2. Inoculate 5 mL of LB-ampicillin media (final ampicillin concentration = 100 μg/mL) with a single tRNA-expressing colony. Shake for ~16 hrs at 37 °C and 220 rpm.

4.1.3 Day 3—Large scale expression of tRNA

3. Add 400 μg/mL ampicillin to 1 L of enriched TB media. Innoculate TB media with 5 mL of the starter culture from Day 2 (for details see Studier, 2005).
4. Grow cells ~16–18 hrs in a shaker incubator at 37 °C and 220 rpm.

4.1.4 Day 4—Isolate tRNA from cells

5. Harvest cells by spinning in a JLA-8.100 rotor at 4000 rpm, 4 °C for 30 min.
6. Pour off supernatant and weigh the cell pellet(s). Pellets can be stored at −80 °C, or extracted as described below.
7. For each cell pellet gather 2 × 250 mL Teflon centrifuge bottles with ETFE O-rings. Label the half of the centrifuge bottles A, and half of the centrifuge bottles B.
8. Resuspend each cell pellet in tRNA extraction buffer (200 mL buffer/25 g cells).

9. Place resuspended cells in Teflon centrifuge bottle A.
10. Add a 1:1 volume ratio of RNase free acid phenol: chloroform (5:1), pH 4.3.
11. Tape centrifuge bottle A horizontally in a shaker-incubator. Shake at 4 °C and 200 rpm for 1 hr.
12. Remove the cells from the shaker-incubator. Separate the aqueous and phenol layer by centrifuging the bottles in an A-4-44 swinging bucket rotor with 250 mL bottle adaptors at 5000 rpm for 1 hr, 4 °C. There will be three layers: brown (bottom, cell debris), thin white (middle, lipids), and transparent (top, aqueous).
13. Use a 25 mL glass pipette to transfer the aqueous layer from each centrifuge bottle A to a centrifuge bottle labeled B, avoiding the lipid layer. Add 1:1 ratio of chloroform to centrifuge bottle B.
14. Add 100 mL of tRNA extraction buffer to solution remaining in tube A. Shake the tubes for 30 s in a fume hood.
15. Spin tubes A and B at A-4-44 swinging bucket rotor at 5000 rpm, 4 °C for 1 hr.
16. Transfer the top layer from tube B to a collection tube. Move the top layer of tube A to tube B.
17. Shake tube B for 30 s by hand in a fume hood, then spin in a A-4-44 swinging bucket rotor at 5000 rpm, 4 °C for 1 hr.
18. Collect the top layer from tube B and combine with the top layer from tube A in a 500 mL JA-10 centrifuge tube.
19. To the contents of the JA-10 tube, add NaOAc, pH 5.2 to 0.3 M (final) and 100% Isopropanol to 20% (final volume). Shake by hand for 30 sec.
20. Centrifuge in a JA-10 rotor at 9000 rpm, 4 °C for 1 hr. A small DNA pellet will be visible following centrifugation.
21. Transfer the supernatant to another 500 mL JA-10 tube. Increase the amount of isopropanol in the solution from 20% to 60% (final). Mix by shaking.
22. Precipitate tRNA at −20 °C for at least 2 hrs.

4.1.5 Day 4—Deacylate tRNA

23. Centrifuge the tRNA precipitant solution in a JA-10 rotor at 9000 rpm, 4 °C for 1 hr.
24. Discard the supernatant and resuspend the pellet in 10 mL of 200 mM Tris-Acetate, pH 8.0.
25. De-acylate tRNA by taping centrifuge bottles horizontally in a shaker-incubator at 37 °C and 220 rpm for at least 30 min.

26. Adding NaOAc to 0.3 M (final concentration) and 2.1 volumes of 100% ethanol to the deacylated tRNA. Precipitate by placing at −20 °C overnight.

4.1.6 Day 5—FPLC purification

27. Spin precipitated deacylated tRNA in a JA-10 rotor at 9000 rpm, 4 °C for 1 hr.
28. Wash the pellets with 70% ethanol. Resuspend tRNA in 5 mL of MilliQ H_2O.
29. Filter tRNA with a 0.22 μm syringe filter.
30. Load the filtered tRNA onto 5 mL ResourceQ ion exchange column equilibrated with Buffer A (IEX) on an FPLC.
31. Monitor column flow through at multiple absorbance readings (A260, A280 and A230) if possible because the tRNA may saturate the detector. Wash the column with Buffer A (IEX) until the A260 reading returns to zero.
32. Elute over a linear gradient to 100% Buffer B (IEX) with >15 column volumes, collecting 1.5 mL fractions.
33. Ethanol precipitate the fractions of interest (identified by absorbance readings) overnight at −20 °C by adding 1 μL glycoblue/1.5 mL of fraction, 0.3 M NaOAc (final) and 2.1 V 100% ethanol.

4.1.7 Day 6—Selecting tRNA fraction

34. Spin down the ethanol precipitated tRNA fractions at maximum speed in a refrigerated micro-centrifuge set at 4 °C for 45 min.
35. Wash each pellets with 70% ethanol and resuspend washed pellets in ~20–50 μL MilliQ H_2O.
36. Estimate the tRNA concentration via absorbance at 260 nm ($\varepsilon = 76{,}000\ M^{-1}\ cm^{-1}$).
37. Since tRNA can distributed throughout the peak, the ability of each fraction to be aminoacylated with the amino acid of interest should be evaluated. An example test aminoacylation reaction is given in Table 1 for $tRNA^{Phe}$. A control (null) aminoacylation reaction with no tRNA included should be performed in parallel. Aminoacylation reactions should be run for 30 min at 37 °C.
38. While the aminoacylation reaction is running, chill 50 mL of 10% TCA and 50 mL of 100% ethanol for 30 min.

Table 1 $tRNA^{Phe}$ aminoacylation reaction.

Reagent	Final concentration
H_2O	to 20 μL
Charging Buffer	1 ×
DTT	1 mM
ATP	10 mM
^{14}C-Phe	19.7 μM
Phe	80 μM
tRNA in fraction (estimated from A260)	5 μM
Phe-RS	1 ×

39. After the aminoacylation reactions are complete, remove 1 μL from each reaction and spot on a piece of Whatman paper. Measure the input cpms by scintillation counting.
40. To the reaction mixtures, add 5 μL of heat denatured 10 mg/μL carrier DNA (e.g., calf thymus DNA).
41. Add 500 μL of chilled 10% TCA to each mixture and pipette to mix. Place the TCA/reaction mixture on ice for 10 min.
42. While the TCA/aminoacyl-tRNA reaction mixtures incubate, set up a vacuum flask apparatus with microfiber glass filter paper.
43. After 10 min pre-wet the filter with 1 mL of cold 10% TCA and pull the liquid through with a vacuum. Next add the TCA/aminoacyl-tRNA reaction mixture to the filter, and again apply the vacuum.
44. Wash the precipitated aminoacyl-tRNA on the filter twice by adding 1.5 mL cold 10% TCA and pulling the liquid through with the vacuum.
45. Finally, rinse the filter with 2 mL of cold 100% ethanol, again pulling the liquid through the filter by vacuum.
46. Dry the filter by running the vacuum and wash the edges of the filter paper with ethanol to remove any residual contaminates.
47. Remove the filter from the vacuum flask apparatus and measure the output counts (cpms) in a scintillation counter.
48. Use the input cpms and the concentration of unlabeled amino acid (e.g., Phe) added to the reaction to calculate the cpm/pmol for each fraction.

$$\frac{cpm}{pmol} = \frac{Input\ cpm}{Input\ amino\ acid\ concentration\ (\mu M)}$$

49. Next determine the pmols of Phe in the output.

$$pmols\ amino\ acid\ output = \frac{(Output\ cpm - Null\ output\ cpm)}{\left(cpm/_{pmol}\right)}$$

50. Calculate the concentration of amino acid output by:

$$amino\ acid\ output\ (\mu M) : \frac{pmol\ amino\ acid\ output}{reaction\ volume\ (19\ \mu L)}$$

51. Determine the percent charging:

$$\frac{Output\ amino\ acid\ concentration\ (\mu M)}{Input\ tRNA\ concentration\ (\mu M)} \times 100\%$$

52. Fractions with greater than 50% charging should be pooled and further purified. At this point, fractions can either stored in the −80 °C freezer, or moved to directly to the HPLC.

4.1.8 Day 7—HPLC purification

53. Pre-equilibrate a Waters XBridge BEH C18 OBD Prep wide pore column with HPLC Buffer A.

54. Inject the pooled tRNA samples from step 52 onto the column on an HPLC.

55. Elute purified tRNA by setting the HPLC to run the program below. Monitor tRNA elution at 260 and 280 nm. Set the fraction collector to collect peaks (peak defined as a change of 50 mAU).
- **(a)** Flow rate: 3.75 mL/min
- **(b)** Inject
- **(c)** Linear gradient to 35% HPLC Buffer B over 35 min
- **(d)** Linear gradient to 100% B over 5 min
- **(e)** Hold 100% HPLC Buffer B for 10 min
- **(f)** Linear gradient to 0% HPLC Buffer B over 1 min

56. Pool the fractions of interest and buffer exchange into water with a 15 mL Amicon Ultra centrifugal filter (10 K MWCO).

57. Ethanol precipitate pooled fractions of interest.

58. Estimate concentration via absorbance at A260 ($\varepsilon = 76{,}000\ M^{-1}\ cm^{-1}$). Concentrate tRNA to ~100 μM by spinning in a 15 mL Amicon Ultra centrifugal filter (10 K MWCO) if necessary.

59. Measure the absorbance of 1 μL of purified tRNA at 260 nm—this the A260/μL value required later for the calculation of acceptor activity.

4.1.9 Day 8—Calculating tRNA acceptor activity

60. Prepare three reactions (Null, S100, and appropriate purified aminoacyl-tRNA synthetase (AA-RS)) in triplicate to determine the purified tRNA acceptor activity (Table 2). An acceptor activity greater than 1000 pmols/A260 unit is desired. An A260 unit is the amount of nucleic acid contained in 1 mL and producing an OD of 1 at 260 nm.

Follow the same reaction steps and calculation as a test charging reaction (steps 37–51).

61. Determine aminoacylation acceptor activity for the S100 and RS reactions.

$$Acceptor\ Activity = \frac{Average\ pmols\ of\ Phe\ output}{\left(\mu L\ of\ tRNA\ used\ in\ reaction \times \frac{A260}{1\ \mu L\ of\ tRNA}\right)} \times 1000$$

62. Compare extent of the aminoacylation in the RS and S100 reaction. Use the higher value of acceptor activity as the measure of tRNA purity.

5. Preparing aminoacylated tRNAs and mRNA

Aminoacylated-tRNAs are prepared using purified aminoacyl-tRNA synthetases (AA-RS) as previously described (Walker & Fredrick, 2008). mRNAs used in these studies have the following sequence: 5′–GGUGUC

Table 2 tRNA acceptor activity assays.

RS Charging reaction		S100 Charging reaction		Null charging reaction	
Reagent	**Final concentration**	**Reagent**	**Final concentration**	**Reagent**	**Final concentration**
H_2O	to 20 μL	H_2O	to 20 μL	H_2O	to 20 μL
Charging Buffer	1 ×	Charging Buffer	1 ×	Charging Buffer	1 ×
DTT	1 mM	DTT	1 mM	DTT	1 mM
ATP	10 mM	ATP	10 mM	ATP	10 mM
^{14}C-Phe	19.7 μM	^{14}C-Phe	19.7 μM	^{14}C-Phe	19.7 μM
Phe	80 μM	Phe	80 μM	Phe	80 μM
Phe tRNA fraction	5 μM	Phe tRNA fraction	5 μM	Phe tRNA fraction	0 μM
Phe RS	1 ×	S100	1 ×	Phe RS	1 ×

UUGCGAGGAUAAGUGCAUUAUGXXXUAAGCCCUUCUGUA-GCCA–3′ with XXX denoting the codons positioned in the ribosome A site. Unmodified mRNAs are generated by transcription with T7 polymerase. Chemically modified mRNA can be purchased from Dharmacon, Keck and IDT, or prepared by ligation as previously described (Keedy, Thomas, & Zaher, 2018). UHPLC MS/MS can be used to verify the stoichiometry of mRNA modification incorporation in commercially prepared mRNAs (Eyler et al., 2019).

6. Initiation complex formation and amino acid addition reactions

The first step in assembling active in vitro translating ribosomes is to form initiation complexes (ICs). ICs consist of 70S ribosomes bound to mRNA with ^{35}S-^{f}Met-tRNA^{fMet} in the P site and can be stored at −80 °C for ∼3–6 months. To perform amino acid addition reactions, the ICs are mixed with ternary complexes (TCs) assembled immediately before the translation reactions are started. Reactions can be performed either by hand, or on a rapid quenching device (quench-flow) depending on the time-frame of the experiment. When planning experiments note that different quenching methods consume varying amounts material per timepoint (i.e., ∼1 μL of IC/TC mixture per timepoint for benchtop assays vs ∼15 μL per timepoint for quench-flow).

6.1 Protocol

Before forming ICs and carrying out translation reactions, have following components available: 1 M KOH, 10 × translation buffer, 10 mM GTP, 70S ribosomes, ^{35}S-^{f}Met-tRNA^{fMet}, aminoacyl-tRNA of interest, IF-1, IF-2, IF-3, EF-Tu, EF-G and mRNAs (see Sections 2–5).

6.1.1 Part 1—Assemble 70S E. coli Initiation Complexes (ICs)

1. Prepare a 10 × mix of Initiation Factors (IFs) containing 20 μM (each) of IF-1, IF-2, and IF-3 in 1 × Translation buffer. Place the IF mixture on ice.
2. Assemble ICs by mixing components and *gently* pipetting up and down: 1 × Translation Buffer, 1 mM GTP, 1 × IF mixture, 2 μM mRNA, 1 μM 70S Ribosomes and 2.5 μM ^{35}S-methionine-tRNA^{f}Met. Add ^{35}S-^{f}Met-tRNA^{fMet} and 70S ribosomes to the tube last.

3. Incubate IC mixture at 37 °C for 30 min. Pellet ICs for higher concentrations as described below and store at −80 °C, or proceed directly to Part 3—Ternary Complex Formation. Pelleting removes unbound ^{35}S-^{f}Met-tRNA^{fMet} and is recommended.
4. If pelleting ICs, pre-chill TLA 100.3 rotor and benchtop ultracentrifuge (such as a TLA-100) to 4 °C. Additionally, add 1 mL cold Buffer R-D to 5 mL polycarbonate ultracentrifuge tubes and chill on ice.

6.1.2 Part 2 (recommended optional step)—Pellet ICs

5. After IC formation, remove 1 μL of IC and dilute in 9 μL of H_2O. Spot 1 μL of the dilution onto Whatman filter paper. Save this 'spotted' filter to measure the ^{35}S counts (cpm) by scintillation counting. This measurement is needed to eventually calculate the final concentration of the pelleted, resuspended ICs.
6. Stabilize ICs for pelleting by raising the final concentration of Mg^{2+} to 12 mM using $MgCl_2$. Remember that 1 × Translation Buffer already contains 7 mM Mg^{2+}.
7. Layer (by slowly and smoothly pipetting) the IC mixture onto chilled Buffer R-D prepared in step 4. Place tubes in a cold TLA 100.3 rotor and spin at 69,000 × rpm, 4 °C for 2 hrs in a benchtop ultracentrifuge.
8. Immediately following centrifugation, place the tubes on ice.
9. Gently remove the supernatant with a pipette. The IC pellet is glassy, fragile and often poorly attached to the tube.
10. Resuspend each pellet in the minimum amount of 1 × Translation Buffer possible (~20–100 μL). For best results, resuspend pellet by *gently* pipetting the Translation Buffer up and down slowly (up to 50 times). Avoid making the ribosome suspension bubbly.
11. Spot 1 μL of the resuspended IC onto Whatman filter paper and measure the ^{35}S counts (cpm) by scintillation counting. Aliquot the remaining pelleted IC into 5–50 μL samples, freeze in liquid N_2 and store at −80 °C.
12. Calculate percent yield for IC formation and pelleting the equation below. A good efficiency to aim for is ≥60%.

$$Percent\ Yield = \frac{\left(\frac{cpm\ of\ resuspended\ pellet}{vol.\ of\ pellet\ counted\ by\ scintillation}\right) * vol.\ of\ resuspended\ pellet}{\left(\frac{cpm\ of\ IC\ formation\ reaction}{vol.\ of\ IC\ counted\ by\ scintillation}\right) * vol.\ used\ in\ IC\ formation}$$

13. Calculate the IC concentration using the following equation:

$$[IC] = \frac{[70S\ Ribosomes]}{\left(\frac{cpm\ of\ IC\ formation\ reaction}{vol.\ of\ IC\ counted\ by\ scintillation}\right)} * \left(\frac{cpm\ of\ resuspended\ pellet}{vol.\ of\ pellet\ counted\ by\ resuspension}\right)$$

6.1.3 Part 3—Ternary complex (TC) formation

In contrast to ICs, TCs cannot be preassembled and frozen. The previously prepared protein and nucleic acid component of TCs (EF-Tu, EF-G and aminoacylated tRNAs) should be thawed on ice.

14. Prepare an "EF-Tu mix" containing final concentrations of 1 × translation buffer, 10 mM GTP and 20 μM EF-Tu. Incubate EF-Tu mix at 37 °C for 15 min. The volume of EF-Tu mix required will vary depending on the scale of the experiment and should be ~1/3 of the total volume of the planned translation assay.

15. While the EF-Tu mix is incubating, prepare a "tRNA mix" containing final concentrations of 1 × translation buffer, 10 mM GTP, 10–20 μM aminoacyl-tRNA (aa-tRNA^{aa}). If your investigations involve the formation of more than a single peptide bond, include 24 μM EF-G in the tRNA mix to enable translocation. Keep tRNA mix on ice for 10–15 min. The volume of tRNA mix required will vary depending on the scale of the experiment and should be ~1/3 of the total volume of the planned translation assay.

16. Form ternary complexes (TCs) by combining equal volumes of the EF-Tu mix and tRNA mixes and incubating at 37 °C for 10 min.

6.1.4 Part 4—Amino acid addition time courses

17. Before running reactions decide on a set of 8–12 timepoints. If your reaction is slow enough to stop timepoints by hand (first time point ≥3 sec) prepare a series of quench tubes containing 1 μL KOH prior to beginning assays. If the timepoints are fast enough (< 3 sec) to need a quench-flow apparatus (e.g., KinTek Model RQF-3) load 1 M KOH as the quench.

18. If using frozen ICs, thaw on ice. Make a 160–180 nM solution of ICs in 1 × translation buffer for use in your reaction. Immediately freeze any remaining thawed IC.

19. Initiate translation reactions by mixing equal volumes of ICs and TCs. For reactions performed on the benchtop that are quenched by hand, this usually means mixing 4–6 μL of ICs with TCs to make a 8–12 μL reaction. Much larger volumes (>120 μL of IC and TC) are required for experiments conducted on the quench flow. Reactions are typically carried out at either room temperature (22–25 °C) or 37 °C.
20. For slower reactions performed on the bench-top, transfer 1 μL of translation reaction to a KOH quench tube prepared in step 17 at each of the pre-selected time points. Review your quench-flow manual for information about how to quench time points 3 sec or faster.
21. Quenched timepoints can be stored at −20 °C or visualized as described in Section 8.

Note: If storing samples for extended periods of time, consider neutralizing timepoints with acetic acid.

7. Miscoding screening assays

Miscoding screening assays use the endpoint level of overall miscoded dipeptide product to evaluate if a modification alters the fidelity of amino acid incorporation. Screening assays are much like the general translation assays described in Section 6, only TCs are formed with a mixture of aminoacylated total-tRNA. Several controls should be run concurrently to confidently identify the miscoded dipeptide products. Specifically, make sure to include: (1) a null reaction performed with TCs formed without any aminoacyl-total tRNA, (2) a positive control with the correctly charged aminoacyl-tRNA, and (3) a reaction with ICs formed on mRNA with an unmodified codon. These assays only provide qualitative insights and observations should be followed by the careful kinetic studies presented in Section 8.

7.1 Protocol

7.1.1 Part 1—Aminoacylate total tRNA

1. Aminoacylate total tRNA by combining, in order, the following reagents (final concentrations given) on ice: MilliQ H_2O, 1 × Charging buffer, 0.1 mM amino acid mixture (each amino acid is present at 0.1 mM), 3 mM ATP, 8 mM total RNA, 1 × S100. Pipette to mix.
2. Incubate at 37 °C for 20 min.
3. Add NaOAc pH 5.2 to a final concentration of 0.3 M.

4. Perform two sequential acid phenol extractions (1:1 volume phenol: aminoacylation reaction, collect top aqueous phase), followed by a chloroform wash (1:1 chloroform:aminoacylation reaction, collect top aqueous phase).
5. Desalt the final aqueous layer using a Bio-Rad P6 spin column or equivalent.
6. To precipitate aminoacyl-total tRNA (aa-total tRNA), add NaOAc to 0.3 M final and 2.3 volumes of 100% ethanol for at least 2 hrs at −20 °C.
7. Spin the ethanol precipitation at maximum speed in a refrigerated microfuge for 30 min at 4 °C. Remove the supernatant and resuspend pellet in ~20 μL 20 mM KOAc, pH 5.2.
8. Approximate the overall concentration of aa-total tRNA by absorbance at 260 nm ($\varepsilon = 76{,}000\,M^{-1}\,cm^{-1}$).

Note: This is an *estimated* concentration, there is no way to determine the charging efficiency of the S100 or acceptor activity for each aa-$tRNA^{aa}$ with this method.

9. Aliquot aminoacyl-total tRNA and store at −80 °C.

Note: Select aliquot sizes keeping in mind that aa-total tRNA samples can become significantly deacylated after three or more freeze/thaw cycles.

7.1.2 Part 2—Perform miscoding screening assay

10. Assemble total-TCs by combining, in order, the following reagents (final concentrations given) on ice: water, 1 × translation buffer, 8 mM GTP, 4 μM aa-total tRNA, 30 μM EF-Tu. Incubate total-TC reaction at 37 °C for 15 min.
11. While the total-TCs incubate, prepare ICs as in Section 6. If using frozen ICs, thaw on ice. Make a 200 nM solution of ICs in 1 × translation buffer for use in your reaction. Immediately freeze any remaining thawed IC in liquid N_2.
12. Initiate the miscoding screening reaction by mixing equal volumes of total-TCs with ICs (final concentration 100 nM ribosomes, 2 μM aa-total tRNA). Typically, small volumes (~1–2 μL of total-TC and IC) are used in these reactions. Incubate at 37 °C for 15 min. The control reactions discussed above should be set up in parallel.
13. Quench each reaction by adding 1 μL of 1 M KOH. Visualize the resulting peptide products by eTLC as described in Section 9.

8. Measuring rate constants for miscoding

To develop an understanding of how different modifications impact miscoding, single turnover kinetic assays should be employed. Due to the ribosome's stringent proofreading mechanisms an energy regeneration mix is used to produce multiple rounds of accommodation, while remaining single turnover with respect to peptidyl transfer, thus producing measurable amounts of miscoded dipeptide product. The energy regeneration mix consists of the ternary complex with the addition of EFTs, pyruvate kinase (PK) and phosphoenolpyruvate (PEP). This protocol is adapted from previous work (Rodnina & Wintermeyer, 2001). Before starting this miscoding assay, it is important to ensure saturating levels of aminoacyl-tRNA are being used (typically 5–10 μM). These reactions have a $t_{1/2}$ of ~2 min, permitting reactions to be manually quenched.

8.1 Protocol

1. As in Section 6, determine a time-course and set-up a series of tubes containing 5 μL 1 M KOH.
2. Prepare 1 μM ICs in 1 ×-translation buffer as described in Section 6.
3. Assemble the EF-Tu/Ts mixture by combining the reagents listed in Table 3 on ice:
4. Incubate the EF-Tu/Ts mixture at 37 °C for 15 min.
5. Add 1.11 μM of the aa-tRNA^{aa} of interest. Incubate at 37 °C for 10 min to form miscoding ternary complexes (MC-TCs).

Table 3 EF-Tu/Ts mixture for miscoding kinetic assays

Reagent	Concentration in mixture	Final reaction concentration
Water	–	to 500 μL
10 × Translation Buffer	1.1 ×	1 ×
GTP	1.11 mM	1 mM
$MgCl_2$	7.77 mM	7 mM
EF-Tu	44.44 μM	40 μM
EF-Ts	11.11 μM	10 μM
PEP	3.33 mM	3 mM
PK	0.11 mM	0.1 mM

6. Initiate translation reactions by adding the MC-TC to IC in a 10:1 ratio at room temperature.
7. For each timepoint, transfer 1 μL of the reaction to a tube containing 1 μL 1 M KOH.
8. After approximately 5 min, add 2 μL of 5 M acetic acid to each quenched tube.
9. Visualize reaction products on a cellulose TLC plate as described in Section 9.

9. Quantification and kinetic analysis

Electrophoretic thin layer chromatography (eTLC) separates small charged species by size and charge, similar to isoelectric focusing. Below we describe how to use this method to visualize the unreacted ^{35}S-^{f}Met and small peptide products from the translation reactions in Sections 6–8. Following separation by eTLC, the different ^{35}S-labeled species can be detected via scintillation counting or phosphorescence (the later is used in this protocol). Volatile buffers are used for separation so that the TLC plates are dry prior to exposure to phosphorscreens. Different peptide compositions and charge states affect separation and resolution. Consider the pI of potential peptides to be synthesized and choose an appropriate pH and composition for your eTLC buffer (see Section 1.5). An example set-up with buffer system is shown in Fig. 2A.

9.1 Protocol

1. Use a pipette to spot 1.0 μL of each timepoint onto a cellulose TLC plate, leaving ~1 cm between spots.
2. Evenly wet TLC plate with the selected buffer (most commonly pyridine acetate buffer, pH 2.8). Take care to ensure that the buffer does not pool.
3. Run TLC for 10–50 min in an electrophoresic TLC tank at 1200–1400V with an appropriate eTLC buffer (e.g., pyridine acetate) in the cathode and anode reservoirs, and an organic, nonpolar solvent—such as Stoddard Solvent—as a liquid stationary phase (Fig. 2A). Peptide charge state and pI affect separation in this system and longer times may be needed for full separation and resolution of peptide products.
4. Remove TLCs from the eTLC tank and dry completely (typically with a heat gun).

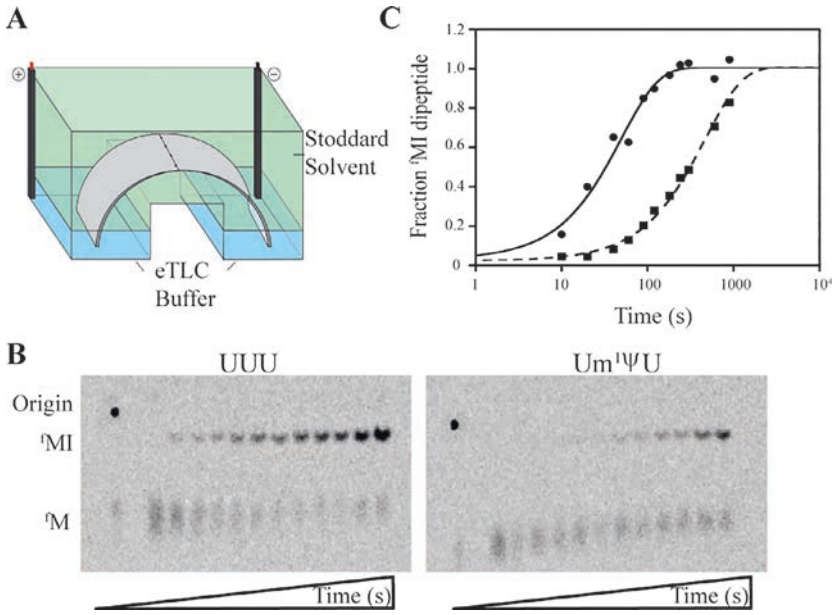

Fig. 2 Visualizing translation products by electrophoretic thin-layer chromatography (eTLC). (A) TLC electrophoresis tank used in this protocol. (B) Example eTLC plate after separation and phosphorimaging. Timecourses of miscoding product formed when Ile-tRNA^{Ile} TCs are mixed with ICs programmed with phenylalanine codons (UUU and Um$^{1}\Psi$U) in the A site. (C) Representative curves fits of MI dipeptide formed during experiment shown in (B). Data from Ile added on UUU [circle] or Um$^{1}\Psi$U [square] are fit with the equation $[MI]_{Eq} = [M]_0 * e^{-(k_{obs})t}$ to determine the observed rate constants (k_{obs}).

5. Once dry, wrap TLCs in plastic wrap and expose them against a phosphorscreen for 1–48 hrs. The specific activity of the radiolabel and dilution state of samples will dictate exposure time.
6. Scan phosphorscreen in an instrument capable of imaging in a phosphorescence mode, scanning at a voltage of 4000 PMT and a resolution of 100 μm.
7. Using the image analysis software of your choice (e.g., ImageQuant or ImageJ) quantify phosphorescence signals to obtain percent and volume of peptide species at each time point in the assay.

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