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Single-Turnover Kinetics of Methyl Transfer to tRNA by Methyltransferases

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Summary

Methyl transfer from *S*-adenosyl methionine (abbreviated as AdoMet) to biologically active molecules such as mRNAs and tRNAs is one of the most fundamental and widespread reactions in nature, occurring in all three domains of life. The measurement of kinetic constants of AdoMet-dependent methyl transfer is therefore important for understanding the reaction mechanism in the context of biology. When kinetic constants of methyl transfer are measured in steady state over multiple rounds of turnover, the meaning of these constants is difficult to define and is often limited by non-chemical steps of the reaction, such as product release after each turnover. Here the measurement of kinetic constants of methyl transfer by tRNA methyltransferases in rapid equilibrium binding condition for one methyl transfer is described. The advantage of such a measurement is that the meaning of kinetic constants can be directly assigned to the steps associated with the chemistry of methyl transfer, including the substrate binding affinity to the methyl transferase, the pre-chemistry re-arrangement of the active site, and the chemical step of methyl transfer. An additional advantage is that kinetic constants measured for one methyl transfer can be correlated with structural information of the methyl transferase to gain direct insight into its reaction mechanism.

Keywords

AdoMet-dependent methyl transfer; rapid equilibrium binding

1. Introduction

Methyl transfer contributes to a broad spectrum of biological reactions in all living organisms, being involved in biosynthesis, metabolism, detoxification, signal transduction, protein sorting and repair, and nucleic acid processing and expression. The products of methyl transfer therefore play major roles from fetal development to brain function. For example, methylation of DNA is required to regulate gene expression, while methylation of mRNA is implicated to control the stability of message [1]. Methylation of hormones, neurotransmitters, and signal transduction pathways helps to regulate the action of each, while methylation of phospholipids keeps membranes fluid and receptors mobile. Methylation of the cobalamin cofactor of methionine synthase provides a repair mechanism

for this enzyme [2]. The most common form of methyl transfer uses AdoMet (Figure 1A, also known as SAM) as the methyl donor, which is synthesized by condensation of methionine with ATP by methionine adenosyl transferase (or SAM synthetase) [3], and the product of methyl transfer is *S*-adenosyl homocysteine (AdoHcy). The ratio of AdoMet *versus* AdoHcy is known as the methylation potential and the level of this methylation potential can regulate the circadian length in mammals [4], indicating an even broader scope of regulation by AdoMet-dependent methyl transfer reactions. AdoMet is the second most widely used enzyme substrate [5], following ATP. The selective preference for AdoMet over other methyl donors, such as folate, reflects the favorable energetics resulting from nucleophilic attack on the positively charged methyl group of the sulfonium center (Figure 1A). The energy release upon methyl transfer from AdoMet is ~17 kcal/mol [5], more than twice of the energy release upon hydrolysis of ATP to ADP and Pi. The target atoms of methyl transfer from AdoMet are also broadly ranged, including nitrogen, oxygen, carbon, and sulfur.

The widespread importance of AdoMet in biology has led to the discovery of at least 5 classes (class I-V) of structurally distinct methyl transferases [6]. The distinction among the 5 classes is in the topological fold of each class that binds AdoMet. This diversity is paralleled only by the diversification of multiple classes of ATP-dependent protein kinases and phosphoryl transferases. Why nature evolved 5 classes of AdoMet-fold is difficult to address, but the identification of different AdoMet conformations among these folds suggests mechanistic distinctions. For example, the class I fold is the most common of the 5 classes and it includes the greatest majority of AdoMet-dependent methyl transferases. The class I fold binds AdoMet in the open space of a dinucleotide-fold that is similar in structure to the NAD(P)-binding fold of the Rossmann-fold [6]. A major feature of the class I fold is that AdoMet adopts a straight conformation, where the adenosine and methionine moieties are extended in opposite direction from each other [6]. In contrast, other classes are much less common and they are characterized by positioning AdoMet in a bent conformation, where the two component moieties can face each other [6]. Of these, class IV stands out, because it binds AdoMet to the bottom of a topological knotted protein fold in a deep cleft. Proteins with a knotted fold are rare in the protein data bank and the class IV fold is distinguished because it is made up of three passages of the protein backbone in and out of a loop in a structure known as the trefoil-knot fold [7,8]. Notably, the bent conformation of AdoMet in the trefoil-knot fold is the most pronounced, where the two component moieties are spatially facing each other almost at a right angle [6]. This is in a striking contrast from the straight conformation of AdoMet in class I methyl transferases.

An example of two different conformations of AdoMet leading to two distinct kinetic mechanisms of methyl transfer is found in the pair of the TrmD and Trm5 methyl transferases. These two enzymes are analogous to each other, because while they catalyze the same methyl transfer reaction, they do not use homologous motifs but instead unrelated motifs [9–13]. Specifically, TrmD is broadly conserved in the bacterial domain [14–16], and Trm5 is conserved in the eukaryotic and archaeal domain [9,17], while both catalyzing methyl transfer from AdoMet to the N¹ atom of G37 on the 3' side of the tRNA anticodon [9] (Figure 1B). Because the synthesized m¹G37-tRNA is a critical determinant to prevent ribosomes from shifting into the +1-frame [16,18], both TrmD and Trm5 are essential for

cell growth [14,18]. However, despite their functional similarity, TrmD is a member of class IV and uses the trefoil-knot fold to bind AdoMet to the interface of an obligated dimer structure [10,11], whereas Trm5 is a member of class I and uses the Rossmann dinucleotide-fold to bind AdoMet to an active monomeric structure [12,13]. Their structural distinction confers mechanistic distinction in virtually all aspects of the methyl transfer reaction. For recognition of AdoMet, TrmD binds it in the signature bent conformation using a lock-and-key mode, whereas Trm5 binds it in the characteristic straight conformation using an induced-fit mode [19]. For methyl transfer to N¹ of G37-tRNA, TrmD stabilizes only the anticodon stem-loop of the tRNA [20] but it carefully discriminates among all three major functional groups of the base with high stringency [21]. In contrast, Trm5 requires the entire tRNA L-shaped structure as the substrate [20] but it examines without high stringency only two of the three functional groups of the base [21]. Most importantly, TrmD catalyzes methyl transfer in a kinetic mechanism limited by the chemistry-associated steps, whereas Trm5 catalyzes methyl transfer in a mechanism limited by the non-chemical step involving release of the m¹G37-tRNA product [22]. Together, these observations show that the structural distinction between different conformations of AdoMet in TrmD and Trm5 has resulted in the kinetic distinction of methyl transfer of these two enzymes. This emphasizes the importance to perform kinetic analysis to correlate with structure as the basis to understand the mechanism of each methyl transfer.

For decades, kinetic analysis of methyl transfer, particularly to RNA substrates, has been dominated by steady state assays. In these assays, the enzyme is in catalytic amounts, the RNA substrate is in excess, and the reaction proceeds over multiple rounds of turnover. Fitting the initial rate of methyl transfer (V_0) as a function of the RNA substrate to the Michaelis-Menten equation yields the catalytic turnover k_{cat} and the Michaelis constant K_m . However, because each parameter is a composite term of multiple turnovers, k_{cat} does not mean the rate constant of methyl transfer and K_m does not mean the binding affinity of the substrate to the enzyme, thus limiting insight into the reaction mechanism. The recent development of pre-steady-state assays has overcome this barrier [23]. In pre-steady-state assays, the enzyme is in excess, the RNA substrate is limiting, and the reaction proceeds only once. This affords two advantages. First, because the rate constant (k_{obs}) directly reports the kinetics of one methyl transfer, the extrapolation from the plot of k_{obs} vs. concentration leads to determination of kinetic parameters intrinsically associated with the methyl transfer. Second, because the enzyme is saturating relative to the RNA substrate, the enzyme-substrate affinity is measured under the condition of rapid binding equilibrium, leading to the determination of a true equilibrium binding affinity. It is with such pre-steady-state assays that TrmD and Trm5 are distinguished in their kinetic mechanism of methyl transfer [22].

This chapter describes the procedures to perform pre-steady-state assays to measure AdoMet-dependent methyl transfer to synthesize m¹G37-tRNA in one turnover by TrmD or Trm5. The representative enzymes are the bacterial *E. coli* TrmD (*Ec*TrmD) and the archaeal *Methanococcus jannaschii* Trm5 (*Mj*Trm5), each of which has been well characterized in our lab and has a high-resolution crystal structure in complex with AdoMet or the product AdoHcy [11–13]. These assays have also been applied to study human Trm5 (*Homo sapiens* Trm5, *Hs*Trm5) to reveal similar kinetic parameters [17] to those of *Mj*Trm5, demonstrating

the reliability of these assays for different members of the same methyl transferase family. The chosen assay temperature for each enzyme is 37 °C for *Ec*TrmD and *Hs*Trm5 and 55 °C for *Mj*Trm5. The G37-tRNA substrate for each enzyme is *Ec*tRNA^{Leu}, *Hs*tRNA^{Cys}, and *Mj*tRNA^{Cys}, respectively.

Two key features of these assays are worthy of mentioning. First, these assays use radioactive [³H-methyl]-AdoMet as the methyl donor (where the methyl group is ³H-labeled). Upon methyl transfer, the ³H-methyl group is transferred to the tRNA substrate and becomes acid precipitable as an integral part of the tRNA, whereas the unincorporated ³H-methyl group is not acid precipitated and is washed away. The product m¹G37-tRNA carrying the ³H-methyl in m¹G37 is then quantified using scintillation counting and the fractional conversion from the substrate G37-tRNA is calculated. An alternative and also quantitative approach [24] is to prepare the G37-tRNA substrate with a site-specifically placed ³²P at the 5' end of G37. After methyl transfer, both the G37 and m¹G37-tRNA are digested to single 5'-monophosphate nucleotides, which are resolved by one-dimensional TLC (thin layer chromatography). Due to their different chromatography mobility, m¹G37 is separated from G37 and their amounts can be quantified by analysis on a phosphorimager screen. Compared to the alternative method, the method using ³H-AdoMet is simpler, at least with respect to the substrate G37-tRNA preparation. Second, for methyl transfer that proceeds on the time scale around 0.1 s⁻¹ or faster, such as the *Ec*TrmD ($k_{\text{obs}} = 0.09 \pm 0.01$ s⁻¹) or *Mj*Trm5 ($k_{\text{obs}} = 0.12 \pm 0.03$ s⁻¹) reaction [22], meaning that one turnover occurs in less than 10–11 sec, the measurement is carried out on a rapid mixing and quench instrument. Our lab uses the KinTek RQF-3 model (KinTek Corp, Texas), which operates with a computer-controlled panel for rapid mixing the contents of two syringes and rapid quenching of the reaction over the time course of methyl transfer. For methyl transfer that proceeds on a slower time scale, the mixing and sampling can be performed without the instrument. We have found that data obtained from the same protocol performed with or without the instrument are reproducible, with the standard deviation being less than 20%.

2. Materials

2.1. Preparation of the DNA template for synthesis of the G37-tRNA substrate

All three enzymes, *Ec*TrmD, *Mj*Trm5, and *Hs*Trm5, can use the transcript of G37-tRNA, made by *in vitro* transcription and devoid of any post-transcriptional modification, as the substrate for AdoMet-dependent methyl transfer. In the ³H-based assay, the transcript is made according to a DNA template, using analytical grade reagents and autoclaved double deionized water (ddH₂O).

1. Sequenase, a T7 DNA polymerase lacking the editing domain. This enzyme can be purchased or purified from an over-producer strain [25].
2. DNA oligonucleotides for construction of the template sequence are synthesized by a commercial supplier and used directly without further purification.
3. Sequenase buffer (5×): 12.5 mM DTT, 250 mM NaCl, 100 mM MgCl₂, 200 mM Tris-HCl, pH 7.5
4. dNTPs: 25 mM solution of each dNTP

5. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA
6. 3 M ammonium acetate
7. Ethanol, absolute and 70%
8. Speedvac system

2.2. Synthesis of G37-tRNA by *in vitro* transcription and purification

1. DNA template from section 2.1 or prepared as a linearized plasmid.
2. T7 RNA polymerase, commercially available or purified from an over-expression clone such as pAT1219 in BL21(DE3) [26]
3. 1 M Tris-HCl, pH 8.0
4. 1 M MgCl₂
5. 0.1 M spermidine
6. 0.5 M DTT
7. 0.5% Triton-X100
8. 50 mM each of ATP, CTP, GTP, and UTP, pH 8.0
9. 200 mM GMP, pH 8.0
10. 0.5 M EDTA, pH 8.0
11. Items 5–7 from section 2.1
12. RNA loading dye solution: 7 M urea, 0.05% xylene cyanol and 0.05% bromophenol blue in 1× TBE buffer
13. A hand-held UV lamp

2.3. Denaturing polyacrylamide gel analysis

1. 20× TBE buffer: dissolve 486.6 g of Tris-base, 37.2 g of EDTA (disodium salt) and 220 g of boric acid in water to a final volume of 2 L.
2. Stock solution of 12% acrylamide gel solution with 7 M urea (12% PAGE/7 M urea): mix 200 g of acrylamide:bis-acrylamide (29:1), 700 g of urea, 83.25 mL 20× TBE, and 1655 mL of water. Stir to dissolve all components at room temperature overnight. Adjust the final volume to 2 L with additional water, filter the solution through a 0.22 µm filtering unit and store the solution in a dark brown bottle at room temperature.
3. 1× TBE buffer: 89 mM Tris-HCl, pH 8.0, 89 mM boric acid, and 2 mM EDTA. Dilute 20× TBE 19:1 with water.
4. Mini gel electrophoresis system (e.g., from BioRad)
5. Preparative gel electrophoresis system with plates of dimensions ~400 × 200 mm and 2.0 mm spacers and comb. Depending on your system, heavy packaging tape and metal binder clips may be required to seal the plates when pouring the gel.

6. 10% ammonium persulfate (APS)
7. N,N,N',N'-tetramethyl- ethane-1,2-diamine (TEMED)

2.4. Methyl transfer to G37-tRNA to synthesize m¹G37-tRNA

1. The substrate G37-tRNA synthesized by *in vitro* transcription and purified by denaturing 12% PAGE/7 M urea
2. Recombinant methyl transferases *Ec*TrmD (purified from an over-producer *E. coli* clone) [27], *Mj*Trm5 (purified from an over-producer *E. coli* clone) [9], and *Hs*Trm5 (purified from an over-producer *E. coli* clone) [17]. Each enzyme is expressed as a fusion to a His-tag and is purified from *E. coli* cells using a metal affinity resin (the cobalt resin Talon of Clontech or the nickel resin of Promega). Preferably, each enzyme should be stored at ~400 μM in 1× methyl transfer buffer and 40% glycerol.
3. ³H-AdoMet commercial solution (purchased from Perkin Elmer, NET155H, 60 Ci/mmol, 6.6 μM, 0.55 μCi/μL)
4. Unlabeled AdoMet (1 mM): dissolve 0.57 mg of AdoMet in 1.0 mL water with 1 μL concentrated H₂SO₄ (final concentration of the acid = 12 mM). Store the solution at -20 °C.
5. A working stock of AdoMet: mix 200 μL ³H-AdoMet commercial solution (step 3) with 90 μL unlabeled AdoMet (1 mM, step 4) to give a final concentration of ~300 μM AdoMet with a specific activity of 2,650 dpm/pmol (see Note 1).
6. TE buffer
7. 1 M Tris-HCl, pH 8.0
8. 3 M NH₄Cl
9. 2 M KCl
10. 1 M MgCl₂
11. 1.0 M DTT
12. 50 mM EDTA
13. 10 mg/ml bovine serum albumin (BSA)
14. Heat-cool buffer: 10 mM Tris-HCl, pH 8.0, and 20 mM MgCl₂

¹Calculation of the specific activity of the working stock of AdoMet:

The working stock consists of 200 μL of the commercial ³H-AdoMet (6.6 μM, 0.55 μCi/μL) with 90 μL of unlabeled AdoMet (1 mM)

Total concentration of the commercial ³H-AdoMet in the mixture: 4.55 μM

Total concentration of unlabeled AdoMet in the mixture: 310.34 μM

Combined concentration of AdoMet: 314.89 μM

Total μCi in the mixture: 0.55 μCi/ μL×200 μL = 110 μCi

Specific activity in dpm/pmol: [110 μCi×(2.2 × 10⁶ dpm/ μCi)] / [314.89 pmol/ μL×290 μL] = 2,650 dpm/pmol.

15. Whatman 3MM filter pads
16. 5% (w/v) trichloroacetic acid (TCA)
17. 95% ethanol
18. Ether
19. A liquid scintillation counter
20. Centri-Spin-20 spin columns (Princeton separations)

3. Methods

3.1 Preparation of DNA template for transcription of G37-tRNA

1. Design two DNA oligonucleotide primers with an overlapping sequence, such that upon hybridization the hybrid encodes the promoter sequence of T7 RNA polymerase and the coding sequence of a G37-tRNA substrate. For example, based on the sequence of *EctRNA*^{Leu}, the forward primer encodes a 5' anchor of 3 nucleotides (5'-GCT), followed by the T7 RNA polymerase promoter sequence (underlined by red), and by the sequence for nucleotides G1 to U46, while the reverse primer encodes the sequence complements for nucleotides A76 to U33, followed by a 3' anchor of 3 nucleotides (AGC-3'). In this design, the 5'-terminal two nucleotides of the reverse primer contain a 2'-*O*-methyl ribose, which help to terminate transcription by T7 RNA polymerase without non-template nucleotide addition [28] (Fig. 1B). The hybrid of the two primers provides the template sequence for synthesis of the transcript of *EctRNA*^{Leu} (Fig. 1C)
2. Mix the two primers at a final concentration of 4 μ M each in a final volume of 1 mL. Add 200 μ L of a 5 $\times$ Sequenase buffer and water to bring the volume to 970 μ L.
3. Divide the solution containing the two primers into two equal volumes. Incubate 2.5 min at 80 $^{\circ}$ C to denature any secondary structures of the primers. Remember to secure the top of each tube with a plastic clamp.
4. Spin the tubes briefly in a microfuge and place them in an ice bath to allow the two primers to anneal. Reserve one aliquot of 3.0 μ L as a control for subsequent electrophoretic analysis of the Sequenase reaction.
5. Add 10 μ L of 25 mM dNTPs and 2.5–10 μ L of Sequenase to each solution at 37 $^{\circ}$ C. The amount of Sequenase to use is determined empirically by adding gradual amounts of the enzyme to each 100 μ L reaction, followed by analysis for product formation on a mini-size 12% PAGE/7 M urea gel for 30–40 min at 200 V in 1 $\times$ TBE. The extension of primers by Sequenase will synthesize a double-stranded DNA that migrates slower than either of the two starting primers.
6. Incubate the rest of the Sequenase reaction overnight at 37 $^{\circ}$ C. Check for completion of the reaction by analysis of a 3 μ L aliquot of one of the reactions on a mini-size 12% PAGE/7M urea gel (see Note 2).
7. Divide the total 1 mL reaction into 3 $\times$ 333 μ L aliquots in separate tubes.

8. Add 33 μL of 3 M ammonium acetate (1/10 volume) and cold 1.0 mL ethanol (3 volumes) to each tube. Keep the solution at $-20\text{ }^{\circ}\text{C}$ for 30 min and then spin for 15 min at maximum speed in a refrigerated microfuge. Pour off the ethanol supernatant and wash the pellets with 1.0 mL cold 70% ethanol. Dry pellets for 5 min in a Speedvac.
9. Dissolve each set of pellets in 100 μL of TE buffer.
10. Determine the concentration of the DNA by measuring the absorbance at 260 nm (1 OD unit = 50 $\mu\text{g}/\text{mL}$), which is usually in the range of 25 μM .
11. Store the DNA template at $-20\text{ }^{\circ}\text{C}$, which should be stable for several months. For each transcription reaction, a 40 μL aliquot of the synthesized template is used for 1 mL of T7 transcription reaction.

3.2. Synthesis of G37-tRNA by in vitro transcription

1. In a 1 mL T7 RNA polymerase transcription reaction, add the following reagents in order:

	Stock concentration	Volume	Final concentration
DNA template	25 μM	40 μL	1.0 μM
Tris-HCl, pH 8.0	1 M	40 μL	40.0 mM
MgCl_2	1 M	24 μL	24.0 mM
Spermidine	0.1 M	10 μL	1.0 mM
DTT	0.5 M	10 μL	5.0 mM
Triton X-100	0.5 %	10 μL	0.005%
ATP	50 mM	150 μL	7.5 mM
CTP	50 mM	150 μL	7.5 mM
GTP	50 mM	150 μL	7.5 mM
UTP	50 mM	150 μL	7.5 mM
GMP (Note 3)	200 mM	150 μL	30.0 mM
T7 RNA polymerase (see Note 3)	10 μL		
ddH ₂ O	to 1000 μL		

2. Allow the transcription to continue for 3–5 hours at $37\text{ }^{\circ}\text{C}$.
3. Spin down pyrophosphates that have formed for 10 min in a microfuge at the maximum speed. Transfer the supernatant containing the tRNA transcript to a new tube.

²In the Sequenase reaction, some of the overlapping primers are extended to completion in only 2–3 hours while others take longer time. Overnight incubation should assure complete reaction.

³In the tRNA transcription reaction, GMP (5'-monophosphate guanosine nucleoside) is added in excess of GTP to promote initiation of transcription with GMP, so

³In the tRNA transcription reaction, GMP (5'-monophosphate guanosine nucleoside) is added in excess of GTP to promote initiation of transcription with GMP, so that the transcript will have a 5'-monophosphate, rather than a 5'-triphosphate. If T7 RNA polymerase is purified from an overproducer strain [25], it should be titrated to the level where 10 μL of the enzyme would give visible precipitation of pyrophosphate that forms aggregates with Mg^{+2} in less than one hour at $37\text{ }^{\circ}\text{C}$. The pyrophosphate is released from NTP due to incorporation of NMP during active transcription. The observation of such precipitation is usually a good indication of strong transcription. If no precipitation is observed in more than one hour, then add more T7 RNA polymerase to the transcription reaction.

4. Adjust the solution by adding EDTA to a final concentration of 0.1 M and ammonium acetate to a final concentration of 0.3 M. Add two volumes of cold ethanol and precipitate the tRNA transcript at -20°C for 15 min.
5. Spin the tRNA in a microfuge at the maximum speed for 15 min, 4°C . Resuspend the pellet in 100 μL TE at 37°C for 10 min. Not all pyrophosphate precipitation goes into solution.
6. Add 100 μL RNA loading dye to the resuspended pellet. The sample is ready for gel isolation of the tRNA transcript.

3.3. Isolation of tRNA transcript from the transcription reaction

1. Make a large preparative 12% PAGE/7M urea gel by assembling two glass plates and spacers.
2. Seal the short-edge side of the glass plates with heavy packaging tape, and clamp along the two long-edge sides with metal binder clips.
3. Mix a 10 mL solution of 12% PAGE/7 M urea with 120 μL of 10% APS and 6 μL of TEMED and pour the solution to the bottom of the assembled glass plates.
4. Upon fast polymerization, the 10 mL gel serves as a seal at the bottom.
5. Mix 160 mL of 12% PAGE/7 M urea with 960 μL of 10% APS and 48 μL of TEMED and pour the gel solution to fill the rest of the assembled glass plates. Insert combs or spacers to form the sample-loading wells.
6. After 10–20 min, the gel should be polymerized and ready to use. Remove the combs, clamps, and tape from the glass plates, and place the gel in an appropriate electrophoresis apparatus. Fill both the top and bottom chambers with $1\times$ TBE buffer.
7. Wash each well on the 12% PAGE/7 M urea gel with $1\times$ TBE buffer several times. When all wells are cleared of the gel material, immediately load the 200 μL solution of tRNA in RNA loading dye solution (from section 3.2, step 6). Run the gel at 700 V for 15 hours until xylene cyanol is about 8 cm above the bottom of the gel.
8. Remove the glass plates from the apparatus and transfer the gel to a saran wrap and place the gel and saran wrap on top of a silica gel fluorescent TLC plate.
9. Use a hand-held UV lamp to localize a UV shadow, indicating the site of migration of the tRNA transcript. Cut off the portion of the gel that exhibits UV shadow and crush the gel into pieces using a sterile glass rod.
10. Add 5 mL TE to the crushed gel pieces and shake the suspension on a rotator shaker at room temperature for 4–6 hours to elute the tRNA.
11. Spin down gel pieces using a tabletop centrifuge. Remove the supernatant to a clean tube.

12. Add more TE back to the gel solution and continue elution for another 4–6 hours. Again, spin and keep the supernatant.
13. Combine the two supernatants and precipitate the tRNA transcript by adding 1/10 volume of 3 M ammonium acetate and 3 volumes of ethanol.
14. Spin down the tRNA transcript from ethanol precipitation and wash the pellet two times with 70% ethanol.
15. Dry the pellet and resuspend the tRNA in 100 μ L of TE buffer. Determine the tRNA concentration by measuring the absorbance at 260 nm (1 OD unit = 40 μ g/mL).

3.4. Assay for methyl transfer in one turnover

1. Take an aliquot of the unmodified transcript of G37-tRNA (200 pmole) and adjust the volume with TE to 15 μ L.
2. Heat the tRNA solution for 3 min at 80 $^{\circ}$ C, a temperature that is above the estimated melting temperature of the tRNA transcript.
3. Quickly spin the solution and add 5 μ L of the heat-cool buffer. Anneal the tRNA at 37 $^{\circ}$ C for 15 min. The resulting stock concentration of tRNA is 100 μ M.
4. Prepare a 5 $\times$ *Ec*TrmD buffer as an example (see Note 4):

<i>Ec</i> TrmD Component	Volume	5 $\times$ Concentration	1 $\times$ Concentration
1 M Tris-HCl, pH 8.0	500.0 μ L	0.5 M	0.1 M
3 M NH_4Cl_2	40.0 μ L	0.12 M	0.024 M
1 M MgCl_2	30.0 μ L	30.0 mM	6.0 mM
1 M DTT	20.0 μ L	20.0 mM	4.0 mM
50 mM EDTA	10.0 μ L	0.5 mM	0.1 mM
10 mg/mL BSA	12.0 μ L	0.12 mg/mL	0.024 mg/mL
ddH ₂ O	<u>388.0 μL</u>		

⁴The 5 $\times$ for *Mj*Trm5 and *Hs*Trm5 buffer is as follows:

Trm5 Component	Volume	5 $\times$ Concentration	1 $\times$ Concentration
1 M Tris-HCl, pH 8.0	500.0 μ L	0.5 M	0.1 M
2 M NH_4Cl_2	250.0 μ L	0.5 M	0.1 M
1 M MgCl_2	30.0 μ L	30.0 mM	6.0 mM
1 M DTT	20.0 μ L	20.0 mM	4.0 mM
50 mM EDTA	10.0 μ L	0.5 mM	0.1 mM
10 mg/mL BSA	12.0 μ L	0.12 mg/mL	0.024 mg/mL
ddH ₂ O	<u>178.0 μL</u>		
Total	1000.0 μ L		

<i>Ec</i> TrmD Component	Volume	5× Concentration	1× Concentration
Total	1000.0 µL		

5. Prepare the tRNA solution for syringe #1 of the RQF-3 instrument (see Note 5):

Syringe 1	Volume	2× Concentration	1× Concentration
G37-tRNA transcript (100 µM)	1.5 µL	0.5 µM	0.25 µM
ddH ₂ O	209.5 µL		
5× buffer	60.0 µL		1×
Working stock of AdoMet (315 µM)	<u>29.0 µL</u>	30.0 µM	15.0 µM
Total	300.0 µL		

Mix G37-tRNA and ddH₂O first and denature the tRNA by heating at 85 °C for 3 min. Briefly spin down the heated solution and then add the 5× buffer to allow annealing of the tRNA at 37 °C for 15 min. Add the working stock of AdoMet and place the solution on ice until loading onto syringe #1.

6. Prepare a series of *Ec*TrmD solutions (ranging from 2 to 32 µM as the 2×) for syringe #2 of the RQF-3 instrument (see Note 6). For each concentration, prepare a 300 µL solution. A total of 6 concentrations are prepared.

	1	2	3	4	5	6
2× <i>Ec</i> TrmD	2 µM	4 µM	8 µM	16 µM	24 µM	32 µM
1× <i>Ec</i> TrmD	1 µM	2 µM	4 µM	8 µM	12 µM	16 µM
<i>Ec</i> TrmD stock (400 µM)	1.5 µL	3.0 µL	6 µL	12 µL	18 µL	24 µL
5× buffer	60 µL	60 µL	60 µL	60 µL	60 µL	60 µL
ddH ₂ O	238.5 µL	237 µL	234 µL	228 µL	222 µL	216 µL
Total	300 µL	300 µL	300 µL	300 µL	300 µL	300 µL

Mix ddH₂O and the 5× buffer, add an appropriate amount of the enzyme. Place on ice until loading onto syringe #2.

7. On the RQF-3 instrument, fill the large syringe on each side with 1× buffer and the middle syringe with 5% TCA. Fill syringe #1 with 300 µL of the tRNA solution to one sample loop and syringe #2 with 300 µL of an enzyme solution to the second sample loop. Enter time points on the control panel. Upon hitting the start button, the 1× buffer pushes 15 µL from syringe #1 and 15 µL from syringe #2 into the reaction loop. After the specified time lapse, the reaction is quenched with 54 µL of

⁵When measurements are performed on the RQF-3 instruments, the tRNA and AdoMet concentration in syringe #1 should be the 2× of the final, while the enzyme concentration in syringe #2 should be 2× of the final. Both syringe #1 and syringe #2 solutions are made in 1× buffer so that, after mixing at an equal volume of each, the final buffer remains at 1×.

⁶For single-turnover assays of methyl transfer, the reaction rate is driven by the enzyme concentration, not by the tRNA concentration [29]. Therefore, a series of reactions are designed, with increasing concentration of the enzyme. The 6 reactions designed here are to provide an initial evaluation of the data points. More reactions with additional concentrations of the enzyme can be designed to obtain more data points for better curve fitting.

5% TCA. Repeat the reaction with a different time point up to 17 time points (e.g., 0, 1, 3, 5, 7, 9, 12, 15, 20, 25, 30, 40, 50, 60, 80, 100, and 120 sec).

8. Collect the quenched solution of each time point in an Eppendorf tube. Spot 20 μL of the quenched solution onto a 1 cm^2 Whatman 3 MM paper pad, which should be labeled for the reaction by a #2 HB pencil.
9. Drop all filter pads into a beaker containing 100–200 mL cold 5% TCA. Estimate the volume of TCA as 5 mL per filter pad.
10. After all pads are in TCA, shake the solution for 10 min at 4 °C to wash off unincorporated AdoMet, while allowing the synthesized $\text{m}^1\text{G37-tRNA}$ to precipitate in TCA. Decant and repeat the 5% TCA wash.
11. Wash all filter pads with 95% ethanol by shaking for 10 min at 4 °C in the beaker. Repeat the ethanol wash one more time.
12. Wash all filter pads with ether. Agitate gently by hand and let the ether solution sit at room temperature for 5 min under a fume hood. Decant ether and dry the filter pads under the fume hood for 15 min.
13. Transfer each filter pad to a scintillation solution in a vial and measure the amount of radioactivity using a liquid scintillation counter.
14. Calculate $\text{m}^1\text{G37-tRNA}$ synthesis based on the specific activity of AdoMet (see Note 1).
15. Correct the ^3H counting by measuring the quenching factor using the following procedure: Take a 5 μL aliquot at the final time point of a reaction and pass it through a quick spin column (see Section 2.4, item 20) to remove unincorporated $^3\text{H-AdoMet}$, which stays with the column. Directly transfer the eluate 5 μL (which contains counts only associated with the methylated tRNA) into the liquid scintillation fluid and measure the counts. The ratio of the direct measurement of this count over the count on the TCA precipitated filter pad at the same time point reveals the quenching factor, which should be used to correct the fraction of methylation. For the protocol described here, the quenching factor is usually 4.

3.5. Data analysis

1. Data points for each time course are fit to the single exponential equation:

$$y = y_0 + A \times (1 - e^{-k_{\text{app}} \times t}) \quad (\text{equation 1})$$

where y_0 is the y intercept, A is the scaling constant, k_{app} is the apparent rate constant, and t is the time in seconds to determine k_{obs} [29,30] (Note 7). The data of k_{obs} vs. enzyme concentration for single turnover analysis of $\text{m}^1\text{G37-tRNA}$ synthesis are fit to the hyperbolic equation:

⁷Control experiments should be performed to confirm that the mixing order of enzyme, tRNA, and AdoMet does not affect the rate of methyl transfer. This confirmation establishes rapid equilibrium binding.

$$y = k_{\text{chem}} \times E_o / (E_o + K_d) \quad (\text{equation 2})$$

where k_{chem} is the rate constant for the steps associated with the methyl transfer chemistry (see notes 8–9), K_d is the enzyme affinity for the tRNA substrate (K_d (tRNA)), and E_o is the enzyme concentration [29,30].

2. The procedure can be repeated with the wild-type enzyme to obtain the K_d (AdoMet) or repeated with a mutant enzyme to obtain all three parameters K_d (tRNA), K_d (AdoMet), and k_{chem} to investigate the enzyme structure-function relationship.

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⁸In rapid chemical quench experiments, the k_{obs} is a composite term that includes all of the steps leading to the chemistry of methyl transfer. Under the condition of rapid equilibrium binding, the formation of enzyme complexes ([E-AdoMet], [E-tRNA], and [E-AdoMet-tRNA]) is fast. The k_{chem} in this condition therefore refers to either the pre-chemistry enzyme re-arrangement of the active site or the chemical step of methyl transfer involving the nucleophilic attack of the N¹ atom of G37 on the methyl group of AdoMet.

⁹The experiments can be repeated to obtain the K_d of the enzyme affinity for the AdoMet substrate (K_d (AdoMet)). For this purpose, the enzyme concentration should be in excess of the AdoMet substrate, while the tRNA concentration is saturating. Repeat Section 3.4, with the modification of step 5 as follows (Note 10).

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Figure 1.

AdoMet-dependent methyl transfer to G37-tRNA to synthesize m¹G37-tRNA. (A) The chemical structure of AdoMet. (B) Design of the former and reverse primers for synthesis of the template for transcription of *EctRNA^{Leu}*. The T7 promoter sequence is underlined in red, and the two 5'-terminal nucleotides in the reverse primer, which are 2'-O-methylated, are underlined in red as well. (C) The sequence and cloverleaf structure of *EctRNA^{Leu}*, showing the target base G37 (circled in red) and its chemical structure and the product m¹G37. The methyl transfer reaction is catalyzed by *EcTrmD*, using AdoMet as the methyl donor and an

Mg²⁺ ion as the catalyst [31]. The folding of the cloverleaf structure is driven by sequence complementarity among regions of the tRNA. The numbering of nucleotide sequence of *EctRNA*^{Leu} is based on the standard 76-nucleotide framework of tRNA [32].