

tRNA Background

RNA is fundamental to life and plays essential roles in both transcription and translation. During transcription RNA is synthesized from DNA, and during translation proteins are synthesized from RNA. The process of translation involves the interactions of three different forms of RNA: (a) messenger RNA (mRNA) carries the genetic code, (b) transfer RNA (tRNA) transports the amino acids, and (c) ribosomal RNA (rRNA) catalyzes the protein synthesis reaction. Transfer RNA (tRNA) is one of the most well studied RNA molecules, mainly because it is small, abundant, and easily purified. Whereas DNA is found almost exclusively in the double helix form, tRNA is rich in structural features including stems, loops, and bulges, as well as more complex tertiary folding interactions.¹ Before our first tRNA laboratory session, please read Chapter 15 in Saenger and the Kirk/Tor research article (refs. 1 and 4).

As you will see from the Saenger reading, different regions of the folded tRNA molecule serve specific functions, including anti-codon/codon binding, amino acid attachment, double-helical stabilization, etc. By altering tRNA in various ways, we can study how structure affects function.

Each of the 20 amino acids has one or more different tRNA molecules to transport it. tRNA^{Phe} was the first tRNA molecule to be crystallized² and will be the focus of this course. The structure of tRNA^{Phe} can be visualized online. Although each of the tRNA molecules has a unique primary sequence of nucleotide bases, their general secondary and tertiary three-dimensional structures are quite similar. Thus conclusions drawn regarding tRNA^{Phe} structure and function can often be generalized to all tRNAs.

RNA is made up of the nucleic acid bases guanine, cytosine, adenine, and uracil (G, C, A and U). These bases are bonded to ribose moieties which are attached via phosphodiester bonds; each base contributes one anionic (P-O)⁻ to the phosphate backbone. In order for RNA to fold properly, there must be positively charged ions around (e.g., Na⁺, K⁺, Mg²⁺) to neutralize the negatively charged phosphate backbone. Both small molecule cations and metal cation ligands have been found to stabilize the active structure of tRNA.¹

This semester we will investigate several small molecule cationic ligands that bind to tRNA (spermine, spermidine, neomycin B, kanamycin, etc.), as well as a variety of metal ions. Both spermine and spermidine are biological polyamines found in eukaryotic cells. Spermine is required in order to form well-ordered tRNA^{Phe} crystals and is known to bind at two tRNA binding sites.¹ Neomycin B (NeoB) and kanamycin belong to the aminoglycoside antibiotic family. Neomycin B has been shown to interfere with prokaryotic protein synthesis via specific binding to the 16S ribosomal RNA.³ NeoB has also been shown to bind tRNA^{Phe} at two distinct binding sites.⁴ Please read ref. 4 in its entirety.

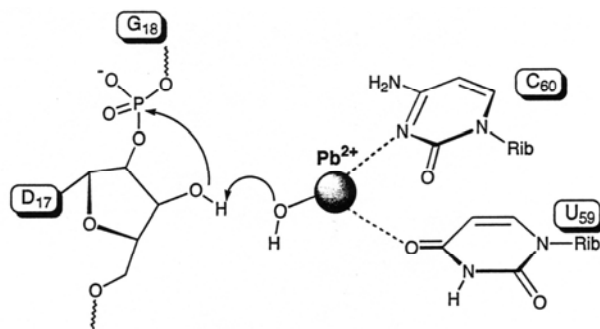
PRE-LABORATORY QUESTIONS (place answers in your laboratory notebook)

1. Look up the structures for NeoB, kanamycin, paromomycin, spermine, and spermidine. Use ChemDraw to draw each of these molecules in the neutral (deprotonated) form, and paste your ChemDraw structures into your lab notebook.
2. How many protonatable amines are in each ligand molecule?
3. All amines in these ligand molecules have $pK_{as} > 7.5$, except for one in NeoB (pK_a 5.7), paromomycin and kanamycin. Estimate the approximate net charge of each ligand molecule at pH 7. Use ChemDraw to draw each of these molecules in the protonated form prevalent at pH 7, and paste your ChemDraw structures into your lab notebook.
4. Qualitatively, how would the charge change if the pH is raised above 7?

At low concentrations, metal ions are necessary to stabilize electrostatic repulsion in the polynucleotide backbone as it folds. However, at higher concentrations many metals have been shown to catalyze hydrolysis of the phosphodiester bonds holding the backbone together. For example, at low concentrations Mg^{2+} stabilizes folded tRNA, but at high concentrations Mg^{2+} catalyzes the hydrolysis of tRNA.⁵ RNA, unlike DNA, has a 2' hydroxyl group that can be deprotonated (by base catalysis) to initiate a nucleophilic attack on the phosphoryl P, as shown in the scheme below. Please read ref. 6 in its entirety.

Scheme 1 below shows how aqueous Pb^{2+} (with its hydroxide ligand) base-catalyzes phosphoester hydrolysis.⁶ In order for lead-catalyzed cleavage to occur, the tRNA^{Phe} must be in

the correctly folded three-dimensional structure. As such, cleavage by lead is a sensitive assay for tRNA^{Phe} tertiary structure. We will study the effects of our chosen small molecule cationic ligands



Scheme 1. Lead catalyzed cleavage of tRNA^{Phe}.

on the structure of tRNA^{Phe} and gain information regarding tRNA·ligand binding equilibria. We will also study the effects of a variety of metal ions on the cleavage of tRNA^{Phe}.

Aqueous metal ions have structures more complex than can be indicated by the simple designation “Mⁿ⁺(aq)”. Metal ions in water form aquo complexes, where the number of bound water molecules typically varies from four to nine. Due to their partially-filled *d* orbitals, transition metals form almost exclusively octahedral, square planar, or tetrahedral complexes. In the case of cobalt(III), exactly six water molecules form covalent bonds between a lone pair on each water molecule and a lobe of a *d* orbital on the central metal, resulting in the octahedral complex ion Co(H₂O)₆³⁺(aq), see **Figure 1**. Metal ions such as Mg²⁺, Pb²⁺, and Eu³⁺ (which have no *d* electrons or whose *d* orbitals are filled) are surrounded by a less predictable number of water molecules, depending on the size and charge of the metal ion.

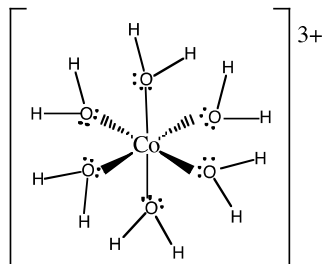
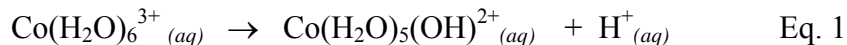


Figure 1. The octahedral cobalt(III) aquo complex, Co(H₂O)₆³⁺.

Metal aquo complexes in neutral or basic solution undergo a reaction that inorganic chemists refer to as *hydrolysis*, in which a hydrogen ion is released from one of the waters of hydration, resulting in a hydroxo complex. Hydrolysis of $\text{Co}(\text{H}_2\text{O})_6^{3+}$ is shown in Eq. 1:



In fact, it is a $\text{Pb}(\text{OH})^+{}_{(aq)}$ complex that has been identified as the active species that catalyzes tRNA^{Phe} cleavage (Scheme 1).

5. What would you expect for the pH dependence of Pb^{2+} catalyzed cleavage of tRNA^{Phe} ?

A direct assay of binding can be obtained by probing a modified Y base (see Figure 2 below) found at position 37 in tRNA^{Phe} . Locate the position of Y^{37} in the tRNA structure in ref. 3. Y^{37} has an additional imidazole ring (see structure below) which gives it fluorescent properties. It fluoresces to a great degree when it is π stacked in between other nucleotide base pairs and shielded from the polar aqueous solvent. Once a ligand binds to tRNA^{Phe} , the structure may change in such a way as to force the Y base out of the π -stack and increase its exposure to water, thus causing a decrease in fluorescence. (This phenomenon is known as “solvent quenching.”) Conversely, ligand binding to tRNA^{Phe} could stabilize the π -stacked Y base, thus increasing its fluorescence.⁷

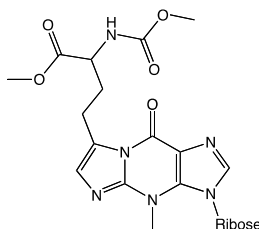


Figure 2. Y base nucleotide.

In addition to Y base intrinsic fluorescence, the pyrimidine and purine nucleotide bases all have aromatic rings attached to ribose. These rings afford nucleotides an intrinsic UV absorbance, with $\lambda_{\text{max}} \approx 260$ nm. When the bases are π -stacked and protected from aqueous solvent, they have a lower UV absorbance (i.e., lower ϵ_{260}) than when they are unstacked and

exposed to water. Thus A_{260} is lower for folded tRNA than for unfolded tRNA, and changes in A_{260} can be used to follow tRNA folding and unfolding.

Ethidium bromide (EtBr) is a small fluorescent molecule (see structure below) that binds to DNA and RNA in two ways: Its aromatic rings intercalate into the central π stacked rings, and also, its quaternary ammonium cation salt bridges to the anionic phosphate backbone. When complexed to tRNA, EtBr fluorescence is enhanced greatly over that of its aqueous form.⁸ In the laboratory, you will observe how binding of EtBr affects Y base fluorescence, and how the subsequent addition of a small molecule ligand affects the fluorescence of both the tRNA Y base and the tRNA-bound EtBr.

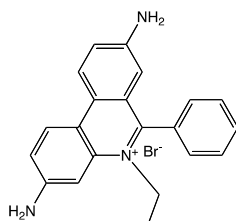


Figure 3. Ethidium Bromide (EtBr).

- Please address all of the questions from the background reading in your notebook.

REFERENCES

- 1 W. Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, 1984, pp. 331-349.
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- 3 D. Moazed and H.F. Noller (1987), *Nature* **327**, 389.
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- 8 J. -G. Delcros, M.C. Sturkenboom, H.S. Baso, R.H. Shafer, J. Szöllösi, B.G. Feuerstein, and L. Marton (1993), *Biochem. J.* **291**, 269.

Overview of tRNA Laboratory Projects

In addition to our initial review of common laboratory skills like pipetting, generating calibration curves, etc., this semester's laboratory will expose you to three important types of instrumentation in the modern biochemistry laboratory: (A) polyacrylamide gel electrophoresis (PAGE), (B) UV-Vis, and (C) fluorescence spectrophotometry.

A. You will use PAGE to separate cleaved fragments of tRNA^{Phe} from uncleaved tRNA^{Phe}. By quantifying the amount of tRNA^{Phe} in the cleaved and uncleaved bands, you will be able to calculate the percentage of cleaved and uncleaved tRNA^{Phe} under various experimental conditions. The basic idea behind PAGE is fairly simple: because tRNA is a polyanion, it will move toward the positive electrode in an applied electric field. If the field is applied across a viscous cross-linked gel, molecules will separate as they move through the gel, with the smallest moving fastest. For a brief review of this technique, see your biochemistry text, *Lehninger's Principles of Biochemistry*, by Nelson and Cox.

PRE-LABORATORY QUESTIONS

6. Lead-catalyzed hydrolysis of tRNA^{Phe} yields a large fragment and a small fragment. Of the three molecules (large and small fragment, and uncleaved tRNA) which molecule will move fastest into the gel and which the slowest?

In the PAGE project, you will incubate tRNA^{Phe} under various conditions, then electrophorese your samples through a gel, separating tRNA^{Phe} molecules and fragments by size. Active hydrolysis will result in more tRNA^{Phe} fragments and less uncleaved tRNA^{Phe}. Inhibited hydrolysis will have the opposite profile. You will analyze your results, either qualitatively by eye, or quantitatively using the Gel Doc System in Olin 301. In the latter case, you will plot your quantitative results, and model them based on equilibrium binding models.

B. You will use UV-Vis spectrophotometry to determine concentrations of biological molecules (using Beer's Law, see *Lehninger*), and also to monitor their three-dimensional conformation. Many of the purine and pyrimidine bases in tRNA feature a UV absorbance maximum near 260

nm, hence tRNA itself has a fairly strong UV λ_{\max} , with $\epsilon_{\lambda_{\max}} = 371,000 \text{ M}^{-1}\text{cm}^{-1}$. You will use this value of ϵ to convert absorbance of tRNA^{Phe} solutions at 260 nm to [tRNA] in units of mol/L (or mM, μM , etc.).

It turns out that tRNA's UV absorbance is in fact a fairly sensitive function of environment. That is, purine and pyrimidine bases exposed to water absorb more strongly than those stacked among non-polar aromatic rings. Hence as tRNA^{Phe} unfolds either due to heat or chemical denaturants in a process called "melting", the bases will destack and increase their exposure to water; this causes absorbance to rise. By the same token, tRNA^{Phe} refolding causes absorbance to decline to its original level. By following absorbance as a function of temperature, we can determine T_{melt} , the temperature at which the folded and unfolded forms of tRNA are in a 50/50 equilibrium.

7. Would you expect tRNA made from poly-GC to have a higher or lower T_{melt} than poly-AU? Explain.
8. Upon addition of polycations like spermine, NeoB, and Mg^{2+} , will T_{melt} for tRNA^{Phe} increase or decrease? Explain.

C. A UV-Vis spectrophotometer measures the incident light absorbed by chromophores in solution, whereas a fluorimeter measures the light emitted by fluorophores in solution. To refresh your memory regarding fluorescence spectroscopy, please review your I & E text, Skoog, Holler, and Nieman's *Principles of Instrumental Analysis*. Even more so than absorbance, fluorescence is exquisitely sensitive to the local environment surrounding the fluorophore. In tRNA^{Phe}, the Y base can exist in two different conformations: (a) folded into the central π stack where the environment is hydrophobic and fluorescence is high; or (b) flipped out toward solvent where the base is exposed to polar solvent and fluorescence is quenched.

Small ligands that bind to tRNA tend to favor one of the Y base's two conformations. Most polycations (e.g., NeoB, spermine) bind to the major groove in tRNA^{Phe} and stabilize the stacked-in conformation of the Y base. The aromatic monocation ethidium bromide (EtBr) binds to tRNA by intercalating into its central π stack. When EtBr binds to tRNA^{Phe}, it tends to

stabilize the flipped-out conformation for tRNA's Y base. Furthermore, EtBr itself is fluorescent and can be used to independently monitor binding to tRNA^{Phe}. When bound within the π stack of tRNA^{Phe}, EtBr fluorescence is higher than it is in water.

9. How would you expect Y base fluorescence to change as you titrate spermine or EtBr into a tRNA^{Phe} solution? Based on this, would you expect spermine and EtBr to bind competitively at the same site on tRNA^{Phe}, or are they more likely to occupy different sites?

You will titrate small molecule ligands (e.g., NeoB, spermine, EtBr) into a tRNA^{Phe} solution, and follow changes in Y base fluorescence. These changes should saturate with ligand concentration; fitting the fluorescence titration data to a hyperbolic saturation curve will allow you to determine K_d , the equilibrium dissociation constant for the tRNA•ligand complex, as discussed in the handout entitled “Ligand Binding Equilibrium”.

Once you form a saturated solution of the tRNA^{Phe}•EtBr complex, further titration with other small ligands may displace EtBr from its tRNA^{Phe} binding sites. This would cause changes in both the Y base fluorescence of the tRNA^{Phe}, and also in the EtBr fluorescence as it is released into solution.

10. As you add NeoB to a solution of tRNA^{Phe}•EtBr, would you expect the Y base fluorescence to increase or decrease? Why?
11. Would you expect the EtBr fluorescence to increase or decrease? Why?

- Please address all of the questions from the overview in your notebook.

tRNA 1: tRNA^{Phe} Structure Tutorial

You will use the computers in Olin 326 such that you can access the following websites to visualize important features of tRNA^{Phe}:

<http://www.infection.bham.ac.uk/Teaching/minchin/jmol/Tutorials/tRNA/tRNA.html>

http://www.biochem.umd.edu/biochem/kahn/teach_res/trna_tutorial/jmol-trna-big.html

1. Observe the different folding in the secondary and tertiary structures:
Secondary Structure: cloverleaf
Tertiary Structure: L-shaped
2. Rotate and explore the different features of the molecule.
3. Print out figures of both the 2° and 3° structures.
4. Identify and label the following features on both figures:
 - a. Structural regions,
AA Stem, the amino acid acceptor stem
D Arm, the dihydrouridine hairpin
AC Arm, the anticodon hairpin
V Loop, the variable loop
T Arm, the TΨC hairpin
 - b. Relevant bases,
Lead cleavage: **D¹⁷, G¹⁸, U⁵⁹, C⁶⁰**
Fluorescence Spectroscopy: **Y³⁷**
 - c. Mg²⁺ binding sites.
5. Based on the Saenger reading, where would you expect spermine to bind to tRNA^{Phe}?

PAGE 1: Lead Catalyzed Cleavage of tRNA^{Phe}

GOAL

In the following experiment, you will observe the effects of concentration and time on lead catalyzed cleavage of tRNA^{Phe}.

PROCEDURE

Set up 11 Eppendorf tubes (0.6 or 1.5 mL tubes) in a tray and label the tops 1 – 11.

Using 1.00 M stock solutions of Tris (pH 7.5), NaCl, and MgCl₂, prepare 2 mL of a stock solution of 5× Reaction Buffer with the following concentrations: 250 mM Tris•HCl (pH 7.5), 500 mM NaCl, and 50 mM MgCl₂. (You will eventually dilute this to 1x buffer: 2 μL → 10 μL)

You will start with a *fresh*¹ 400 mM stock solution of Pb(OAc)₂.

You must dilute 4 μL of a Pb(OAc)₂ *intermediate* stock solution to a final volume of 10 μL to give a final [Pb²⁺] = 1.6 mM. What is the Pb(OAc)₂ concentration of this *intermediate* stock soln?

Determine the volumes of this and one or more other Pb(OAc)₂ *intermediate* stock solutions needed to reach final Pb(OAc)₂ concentrations of 0.8, 0.4, 0.2, and 0.1 mM. Make sure that all aliquot volumes are between 1–4 μL.²

In your lab notebook, make a table similar to the one below. Write your calculated Pb(OAc)₂ aliquot volumes for reactions 1-6 (i.e., 0-1.6 mM Pb²⁺) in the appropriate cells in rows 4, 5, and 6 (if necessary); fill in the Pb(OAc)₂ *intermediate* stock concentration in the leftmost column.

	1	2	3	4	5	6	7	8	9	10	11
Total [Pb ²⁺] (mM)	0	0.1	.2	0.4	0.8	1.6					
Cleavage Time (min.)	60	60	60	60	60	60	0	15	30	45	60
___ mM Pb(OAc) ₂ , μL											
___ mM Pb(OAc) ₂ , μL											
___ mM Pb(OAc) ₂ , μL											
40 μM tRNA ^{Phe} , μL											
5× Rxn Buffer, μL											
di H ₂ O, μL											

Tube 1 is your tRNA-only (0 Pb²⁺) control. Tubes 2–6 have increasing concentrations of Pb²⁺ (0.1 - 1.6 mM); tubes 1-6 are quenched after 60 min of incubation. Tubes 7–11 will be quenched after the incubation time specified in row 3 (0-60 min). Tubes 7-11 should all contain the concentration of Pb²⁺ listed for your group. Group A: 0.2 mM; Group B: 0.4 mM; Group C: 0.6 mM; Group D: 0.8 mM

¹ Be certain that the 400 mM Pb(OAc)₂ stock solution you use has today's date on it.

² If your smallest pipettor is 1-10 μL, your smallest aliquot is 1 μL; otherwise, it's 1.5 μL.

All tubes, 1-11, will have a final buffer concentration of 1× (starting with 5× stock buffer), a final [tRNA] of 16 μM (starting with a 40 μM stock), various concentrations of Pb(OAc)₂, and sufficient deionized water to give a final volume of 10 μL.

Specify all aliquot volumes in the table in your notebook (similar to the sample table above). Have the instructor check before you continue. All samples must add to 10 μL total volume.

Once you have determined all aliquot volumes and made all of your stock solutions, add the appropriate volume of diH₂O, tRNA^{Phe}, and 5× reaction buffer to all tubes. Vortex and centrifuge.

Add the specified aliquots of Pb(OAc)₂ *intermediate* stock solutions when you are ready to initiate cleavage, and vortex followed by spin. Incubate with Pb²⁺ for the times specified below (as well as in row 3 of the table above).

Tubes 2–6: incubate 60 min. before quenching	Tube 7 quench immediately
Tube 8 quench after 15 minutes	Tube 9 quench after 30 minutes
Tube 10 quench after 45 minutes	Tube 11 quench after 60 minutes

For convenience, you may wish to add the Pb²⁺ such that all tubes can be quenched at the same time. For example, initiate tube 10 fifteen minutes after tube 11.

Reactions are quenched by adding 30 μL of the loading dye (0.1 mg/mL Bromophenol blue dissolved in 8 M Urea and 1× TBE), vortexing, and freezing at –20 °C.

Pour polyacrylamide gel for following laboratory session (see next page).

The following laboratory session:

Pre-electrophorese polyacrylamide gel in 1× TBE buffer (see next page) for at least 30 minutes.

Heat all sample tubes for 30 seconds at 90 °C in the heat blocks. Vortex, centrifuge, and load one tube per gel lane, leaving lane 1 blank. (See detailed instructions in the next section.)

Instructor will train you to use the power supply. Stop gel before the tracking dye reaches 2 cm from the bottom.

Following the detailed “Staining” instructions at the end of the next section, disassemble gel plates, carefully lift the gel off of the plate, stain it in EtBr for 30 minutes and rinse. Make a digital image with the Gel Doc System (see Data Analysis section).

Wrap the gel in Saran wrap, label with Group and date, and store in the refrigerator.

POST-LABORATORY QUESTIONS

1. Use your results from tubes 2–6 to estimate the concentration of Pb(OAc)₂ that will cleave ≈ 50 % of tRNA^{Phe} after 60 minutes.

2. Use your results from tubes 7–11 to estimate the length of time needed to cleave $\approx 50\%$ of tRNA^{Phe}, at your group's chosen $[\text{Pb}^{2+}]$.

store it in a carboy. The 1× TBE buffer can be reused once (from a separate carboy), then discarded in the sink.

1. Fill the bottom well of the V16-2 chamber with 1× TBE buffer to ~1 cm above the top of the bottom risers.
2. Red and black male plugs on the inner shell chamber must be on opposite sides.
3. Remove the comb (if you haven't already) and the bottom spacer from the gel. Clean the front (small) plate with water.
4. Place the gel(s) in the apparatus with the small plate side facing inward by lowering it slowly at an angle to remove all bubbles from the dead space at the bottom of the gel. Set the gel on the small riser, orange sponges against the black sponges. Clamp each side with two clamps at the sponge-sponge interface.
5. Pour 1× TBE buffer into central chamber until buffer level is ~1 cm higher than the small plate. Check for leaks and adjust plate(s)/clamp(s) as needed.
6. Place the outer shell on top and plug red plugs and black plugs into their respective outlets. Insert the red plug to the positive pole \oplus and the black plug to the negative pole \ominus on the Power Supply.
7. Turn on the Power Supply once all chambers have been connected (black switch on lower left). Run/ Modify/ Set to: 0500V, 030mA (per gel), 300W/ Exit/ Run. Choose an appropriate current based on the number of gels that will be run. For example, running four gels requires a current of 120mA. Actual voltage and wattage will be significantly lower than those settings; it is the current that should be fixed.
8. Once the power supply is on and electrophoresis has begun, check that the electrical circuit is closed by watching for the evolution of gas bubbles from the wires inside the chamber. Also check every once in a while that the gel is not getting hot (warm is OK).
9. Pre-electrophorese the gels for a minimum of 30 minutes before running samples. This might be a good time to remove your samples from the freezer and give them time to thaw. If you must pre-electrophorese for more than an hour, lower the current to 5 mA.
10. When the pre-electrophoresing step is complete, turn off power, disconnect from Power Supply, and remove the outer shell.
11. Prewash all lanes: use a pipettor to squirt copious amounts of 1× buffer (taken from the top chamber) into each lane.
12. Attach a special gel loading pipet tip (long thin) to a 100 or 200 μ L pipettor. Adjust the pipettor so that it sucks up all of the sample in each Eppendorf tube. This should be about 40-42 μ L. Use the pipettor to load the entire volume of each incubated sample slowly, placing one sample in each lane. Use a new pipet tip for each sample.
13. Reconnect to Power Supply and run under the same settings for about 1.5 – 2 hours, until the tracking dye nears the bottom of the gel (about 2 cm from bottom).

14. Turn off power, disconnect from Power Supply, and remove gel from the apparatus.

Staining

The EtBr staining solution is 1.0 $\mu\text{g/mL}$, in deionized water. EtBr is carcinogenic, so handle carefully and dispose appropriately. Pour about 500 mLs of the EtBr stain solution into a 19 \times 19 cm casserole destaining dish, to a depth of 1–2 cm. Remove spacers, and disassemble gel plates by rotating them in opposite directions. Using both hands (gloved, of course), carefully pry and lift the gel off of the plate and place the gel in the EtBr staining dish. The stain is light sensitive, so cover and set in a dark place such as a closed cabinet. Let it stain for 30 – 40 minutes, agitating the gel once in while.

When staining is complete, hold the gel in the staining dish while pouring the EtBr staining solution into the “used once” bottle (if it was fresh), or into the “EtBr waste” bottle (if it was already used once). Rinse the gel twice with tap water. The gel is then wrapped carefully in Saran Wrap (try to smooth out creases) and analyzed with Gel Doc. Even if you do not have time to do the full analysis with the Gel Doc program, take your gel down to Olin 205 to capture and save the image immediately after staining. You may carry the Saran-wrapped gel on top of a transparency for convenience, but you must place the gel directly on the UV window in the light box (the transparency polymer absorbs UV light).

PAGE 2: Pb^{2+} -Catalyzed Cleavage of tRNA^{Phe} in the Presence of Small Molecule Ligands

GOAL

To observe the effects of your small molecule ligand on lead catalyzed cleavage of tRNA^{Phe} .

PROCEDURE

Set up 12 Eppendorf tubes (0.6 or 1.75 mL tubes) in a tray and label the tops 1 – 12. To each tube you will add 4 μL of 40 μM tRNA^{Phe} stock, 2 μL of 5 \times Reaction Buffer, a predetermined quantity of $\text{Pb}(\text{OAc})_2$, varying quantities of small molecule ligands, and sufficient dH_2O to give a final volume of 10 μL .

Using as a model the table that you prepared at the end of the “Preparing Solutions and Designing Experimental Tables” section, design an experiment to test the effect of increasing concentrations of your small molecule ligand on lead-catalyzed hydrolysis of tRNA^{Phe} . First make 2 mL of the initial stock solution of your group’s small molecule ligand specified in the table below; the concentration of this initial stock solution is in parentheses, e.g., 0.25 M neomycin B. To make this initial stock solution, obtain an autoclaved, graduated 2.2 mL Eppendorf tube. Add the weighed solid ligand to the tube, then add autoclaved water to the 2.0 mL mark and vortex. You will need to make subsequent dilutions of this initial stock in order to make samples 3-12 in the table below. Any leftover ligand stock solutions should be stored in the freezer for later use.

Create a logarithmic concentration gradient using the concentration range specified below for your small molecule ligand. As in the previous table, your two controls will be: Tube 1, tRNA -only (0 Pb^{2+}), and Tube 2, $\text{tRNA} + \text{Pb}^{2+}$ (0 ligand). For each group, Tube 3 will contain $\text{tRNA} + \text{Pb}^{2+} + 0.10$ mM ligand.

Group	Small Molecule Ligand	Range
A	neomycin B (0.25 M)	0 – 50 mM
B	spermine (1 M)	0 – 100 mM
C	kanamycin A (0.25 M)	0 – 75 mM
D	spermidine (1 M)	0 – 200 mM

From your answers to Post-Lab Questions 1 and 2, determine the ideal concentration of $\text{Pb}(\text{OAc})_2$ and cleavage time, and prepare the appropriate $\text{Pb}(\text{OAc})_2$ stock solution.

Set up an experimental table in your lab notebook similar to the one below. Have the instructor check your calculations before you continue.

	1	2	3	4	5	6	7	8	9	10	11	12
[Ligand] (mM)	tRNA	0	0.10									
Stock Concentrations	_____ mM											
	_____ mM											
	_____ mM											
	_____ mM											
	_____ mM											
	_____ mM											
	_____ mM											
	_____ mM											
	_____ mM											
	40 μ M tRNA ^{Phe}											
5 \times Rxn Buffer												
_____ mM Pb(OAc) ₂												
di H ₂ O												

Add the appropriate volume of diH₂O, tRNA^{Phe}, reaction buffer, and small molecule ligand to all tubes. Vortex and centrifuge.

Heat tubes for 30 seconds at 90 °C, remove, and let sit at room temperature for 15 minutes before initiating cleavage.

Add Pb²⁺ when you are ready to initiate cleavage and incubate for the predetermined time.

Reactions are quenched by adding 30 μ L of the loading dye (0.1 mg/mL Bromophenol blue dissolved in 8 M Urea and 1 \times TBE) and freezing at -20 °C.

Pour gel to be used in the next laboratory session.

The following laboratory session:

Pre-electrophorese gel in 1 \times TBE for at least 30 minutes prior to loading gel.

Heat all sample tubes for 30 seconds at 90 °C in the heat blocks. Vortex, centrifuge, and load on gel. Stop gel before the tracking dye is 1 inch from the bottom.

Disassemble gel plates and stain gel with EtBr stain for 30 minutes. Make a digital image with the Gel Doc System (see Data Analysis section).

Store gel enclosed in Saran wrap, labeled with Group and date in the refrigerator.

POST-LABORATORY QUESTIONS

3. Does the presence of your small molecule enhance or decrease Pb^{2+} mediated cleavage? Explain this effect in the context of RNA structure.
4. Go to the final section of this lab manual, "Ligand Binding Equilibrium," to learn about hyperbolic curve fitting. Complete the exercises therein.
5. Use Kaleidagraph to plot the background-corrected volume vs. [ligand] for each important gel band. Determine the K_d of your small molecule by using the equation for hyperbolic saturation to fit a curve to your band volume vs. [ligand] data points.
6. Tabulate K_d s with associated errors for all three small molecule ligands.
7. Which ligand has the lowest K_d ? Is this the most effective or least effective ligand?
8. Does ligand net charge correlate with K_d ? Why would you expect this to be the case?

PAGE 3: Metal Ion Catalyzed Cleavage of tRNA^{Phe}

GOAL

In the following experiment, you will observe the cleavage of tRNA^{Phe} catalyzed by a variety of metal ions at varying concentrations.

PROCEDURE

Set up 20 Eppendorf tubes (0.6 or 1.75 mL tubes) in a tray and label the tops 1 – 20. To each tube you will add 4 μL of 40 μM tRNA^{Phe} stock, 2 μL of 5 \times Reaction Buffer, varying concentrations of metals, and sufficient diH₂O to give a final volume of 10 μL .

Design an experiment to test the effect of increasing concentrations of two different metal cations on tRNA^{Phe} (see table below). As previously, you will have two control samples: tRNA-only (in Tubes 1 and 19), and tRNA + Pb²⁺ (Tubes 2 and 20). Tubes 3–10 will contain 8 different concentrations of your first metal, and Tubes 11–18 will contain 8 different concentrations of your second metal. Create a logarithmic concentration gradient using the suggested range for your metals in the table below.

Group	Metal Ion	Salt	Range
A	Eu ³⁺	EuCl ₃ ·6H ₂ O	2 – 500 μM
	Cu ²⁺	CuSO ₄ ·5H ₂ O	5 – 200 mM
B	Sm ³⁺	Sm(NO ₃) ₃ ·6H ₂ O	2 – 1500 μM
	Mn ²⁺	MnSO ₄ ·1H ₂ O	0.1 – 40 mM
C	Gd ³⁺	GdCl ₃ ·6H ₂ O	2 – 1000 μM
	Sn ²⁺	SnBr ₂	50 – 3000 μM
D	Fe ³⁺	Fe(NO ₃) ₃ ·9H ₂ O	0.5 – 15 mM
	Mg ²⁺	MgCl ₂ ·6H ₂ O	5 – 800 mM

Use the same concentration of Pb(OAc)₂ and cleavage time as in the previous small molecule ligand experiment.

Set up an experimental table in your notebook specifying appropriate aliquots and stock solutions for each of your 20 samples. Have instructor check before you continue. Make a gel with the appropriate number of lanes (choose the correct comb!).

Add the appropriate volume of diH₂O, tRNA^{Phe}, and reaction buffer to all tubes. Vortex and centrifuge.

Add metal ions when you are ready to initiate cleavage.

Quench only the tube with Pb²⁺ after the appropriate amount of time by adding 30 μL of the loading dye (0.1 mg/mL Bromophenol blue dissolved in 8 M Urea and 1 \times TBE) and freezing at -20 °C. Allow all other metal-containing tubes to continue cleaving at room temperature for 16-20 hours. Quench the following day by adding 30 μL of the loading dye and freezing.

The following week:

Pre-electrophorese gel in $1\times$ TBE for at least 30 minutes prior to loading gel.

Heat all sample tubes for 30 seconds at $90\text{ }^{\circ}\text{C}$ in the heat blocks. Vortex, centrifuge, and load on gel. Stop gel before the dye is 1 inch from the bottom.

Disassemble gel plates and stain with EtBr stain for 30 minutes. Make a digital image with the Gel Doc System (see Data Analysis section).

Store gel enclosed in Saran wrap, labeled with Group and date in the refrigerator.

POST-LABORATORY QUESTIONS

9. Briefly summarize the observed cleavage pattern caused by each of the metals you studied and qualitatively compare the results with those from $\text{Pb}(\text{OAc})_2$. Calculate the metal:tRNA ratio and compare the ranges of metal concentration necessary to promote cleavage.
10. For each of your two metals, speculate on the reasons for the cleavage pattern that you observed. Consider relevant topics from Inorganic Chemistry, including Lewis acidity, electronic configuration, charge-to-radius ratio, structure of the primary coordination sphere, etc. Be specific: If Lewis acidity is important specify pK_a s; if charge-to-radius ratios are important, calculate them; if coordination sphere is important, specify coordination numbers, etc.

TM 1: tRNA^{Phe} Thermal Melting Curves in the Presence of Ligands

GOAL

In this project you will use the Cary 3 spectrophotometer to determine T_{melt} for tRNA^{Phe} bound to various small ligands. You will determine whether each ligand stabilizes the folded tRNA and prevents unfolding (thereby increasing T_{melt}), or destabilizes the folded tRNA and causes unfolding (thereby decreasing T_{melt}).

PROCEDURE

You will use six cuvettes:

- cuvette 1: tRNA-only control;
- cuvette 2-5: tRNA^{Phe} + Mg²⁺ or EtBr;
- cuvette 6: tRNA^{Phe} + small molecule ligand

In the second part of this project (see next section) you will determine T_{melt} for the tRNA^{Phe}·ligand complex at five more concentrations of your small molecule ligand. Finally, you will plot T_{melt} vs. [ligand] and analyze your results appropriately.

Table:

	Cell #1 [tRNA ^{Phe}]	Cell #2 [MgCl ₂]	Cell #3 [MgCl ₂]	Cell #4 [EtBr]	Cell #5 [EtBr]	Cell #6 [ligand]
Group A (Neomycin B)	1 μM	0.1 mM	2 mM	0.5 μM	10 μM	100 μM
Group B (Spermine)	1 μM	0.4 mM	5 mM	2 μM	25 μM	1 mM
Group C (Kanamycin A)	1 μM	1 mM	10 mM	5 μM	50 μM	5 mM
Group D (Spermidine)	1 μM	0.2 mM	4 mM	1 μM	4 μM	5 mM

Obtain 6 stoppered semi-micro quartz UV-Vis cuvettes for your tRNA sample solutions.¹ The buffer for this experiment will be 100 mM NaCl *plus* 50 mM Tris, pH 7.5 and no Mg²⁺. You will make this buffer from aliquots of 1.0 M stock solutions of NaCl and Tris, already prepared for you. All six sample cells will contain 1 μM tRNA^{Phe} in buffer, and the final volume will be 1.6 mL. Based on the concentrations of your available stock solutions and the desired final concentrations specified in the table above, calculate appropriate stock solution aliquots for each of the six sample solutions. Prepare each solution in an Eppendorf tube (1.75 or 2.2 mL) and dilute to 1.6 mL using deionized water. When all additions have been made to the sample cells, mix, heat to 90 °C for 30 sec. and cool to room temperature over 15 min.

¹ When working with the thermal melt cuvettes, and when returning them, pls. keep the quartet (numbered 1-4 on the bottom) together, and separate from the pair (numbered 5-6 on bottom). This is important because, believe it or not, the top hole and stoppers are bigger for the quartet than for the pair.

With a Pasteur pipet, quantitatively transfer all cooled samples into semi-micro cuvettes, stopper and load them in order into the Cary 3 sample cell holder. Align the cuvettes in the cell holder correctly. No reference cells are necessary for this experiment. The cuvettes must be stoppered in order to avoid solvent evaporation at high temperature.

Turn on the Cary 3 instrument and its attached temperature control unit. To run the thermal melting curves, open the Cary 3 program entitled 'Thermal'. From the 'file' menu, choose 'Open Method'. In the upper left, under 'Look in', find the 'CaryMethods' folder. (This can be found at: Desktop/My Computer/Local Disk(C:)/Documents & Settings/All Users/Shared Documents/Cary Methods.) Choose the method file 'thermaltRNA.mtm' and load the method ('OK'). This method has several features, which you should check by clicking the 'setup' box at the upper left.

'Cary' tab: wavelength = 260 nm

'Collect Temps' tab: start = 20°C; return to 20°C; # stages =4; T monitor = Block;

Stage 1: data interval = 1.0 °C; rate = 0.5 °C/min; end= 90.0 °C; hold = 1 min

Stage 2: data interval = 1.0 °C; rate = 0.5 °C/min; end= 20.0 °C; hold = 1 min

Stage 3: data interval = 1.0 °C; rate = 0.5 °C/min; end= 90.0 °C; hold = 1 min

Stage 4: data interval = 1.0 °C; rate = 0.5 °C/min; end= 20.0 °C; hold = 1 min

'Options' tab: UV lamp only

'Accessories' tab: check 'use cell changer box'; select cells 1-6; temp display = Block;
check the 'show status display' box

'Analyze' tab: derivative

'Reports' tab: check boxes for results, graph, and user data form

'AutoStore' tab: storage on (prompt at start)

When all six of your cells are loaded in the cell compartment, and all of the settings in your 'Thermal' methods file are correct, press 'Start'. Name your file (e.g., "TMtRNA_date_your initials") and your four samples (e.g., tRNA, Mg, NeoB, EtBr) appropriately. Leave a sign on the Cary stating "Thermal Melts in Progress. Please leave the instrument on. Samples will be retrieved at 10AM." Include on this sign your name and a phone number that you can be reached at. Leave the instrument to ramp through the temperatures overnight. Be sure to return the next morning by 10 AM as others will need to use the spectrophotometer.

When you return in the morning, click on the lower left 'recalculate' box. The software will then find the T_{melt} for each ramp, i.e., the inflection point of each absorbance vs. temperature curve. You will then be able to print out a report (on the color printer) that includes 24 T_{melt} values, four for each sample. For example, for sample 1 you will have four T_{melt} values, one for ramp 1 (initial heating 20 → 90°C), one for ramp 2 (cooling from 90 back down to 20°C), one for ramp 3 (second heating from 20 → 90 °C), and one for ramp 4 (second cooling back to 20°C). If any of the T_{melt} values seem like outliers, examine the actual A vs. T curves to see if the instrument-chosen value is due to noise in the curve. For all reliable replicate values of T_{melt} , calculate the average and standard deviation. Report these values, to the correct number of significant figures.

After you have saved all files, shut down the spectrophotometer, carefully rinse out all cuvettes, and return them to your instructor. Again, these are very expensive cuvettes, so please treat them with utmost care and responsibility.

POST-LABORATORY QUESTIONS

1. From class data, you have T_{melt} vs. $[\text{Mg}^{2+}]$ (0.1-10 mM) and vs. $[\text{EtBr}]$ (0.5-50 μM). Use appropriate plots to determine K_d for Mg^{2+} and EtBr binding to tRNA by fitting the curves to the equation for hyperbolic saturation.
2. Make a table of your results.
3. Do your ligands stabilize or destabilize the folded structure of tRNA^{Phe}? Explain this effect.
4. Which of the ligands stabilizes tRNA^{Phe} the most? Would you predict this? Explain.

TM 2: UV Thermal Melting Curves of tRNA^{Phe} in the Presence of Increasing Concentrations of a Small Molecule Ligand

GOAL

In this project you will use the Cary 3 spectrophotometer to determine T_{melt} for tRNA^{Phe} bound to various concentrations of your small ligand, and develop a binding curve.

PROCEDURE

You will determine T_{melt} for the tRNA^{Phe}·ligand complex of your small molecule ligand at five more concentrations, after which you will plot T_{melt} vs. [ligand] and analyze your results appropriately. Follow the laboratory procedure from the previous thermal melt experiment.

For your chosen ligand, determine T_{melt} for tRNA^{Phe} alone and in the presence of five different ligand concentrations.

Table:

	Cell #1 [tRNA ^{Phe}]	Cell #2 [ligand]	Cell #3 [ligand]	Cell #4 [ligand]	Cell #5 [ligand]	Cell #6 [ligand]
Group A (Neo B)	1 μM	1 μM	2 μM	5 μM	10 μM	50 μM
Group B (Spermine)	1 μM	100 μM	250 μM	500 μM	2 mM	10 mM
Group C (Kanamycin)	1 μM	100 μM	500 μM	1 mM	2 mM	10 mM
Group D (Spermidine)	1 μM	500 μM	1 mM	2 mM	10 mM	20 mM

POST-LABORATORY QUESTIONS

- Plot T_{melt} vs. [ligand] using all seven concentrations of your small molecule ligand: five concentrations from this experiment, and two from the previous one. Determine the K_d of your small molecule by fitting the curve to the equation for hyperbolic saturation.
- Tabulate K_d 's with associated errors for all four small molecule ligands and draw appropriate conclusions.

Fluorimetry

BACKGROUND

The tRNA^{Phe} Y base and EtBr are both fluorophores, i.e., groups that absorb light and emit photons of somewhat lower energy (longer wavelength). Fluorescence is dependent on environment; within the tRNA molecule, fluorescence is affected by whether a fluorophore is turned outward and exposed to solvent, or turned inward and surrounded by base pair aromatic rings.

In your fluorescence titrations you will be adding small molecule ligands to tRNA^{Phe} and measuring the resultant changes in fluorescence. Using a specific excitation wavelength (λ_{ex}) and scanning a narrow emission region, you will determine $\lambda_{\text{max,em}}$ and the fluorescence intensity at the $\lambda_{\text{max,em}}$ after each aliquot of Mg²⁺ or small molecule ligand is added. The values of λ_{ex} and the emission region depend on whether you are monitoring the fluorescence of the Y base in tRNA^{Phe} or ethidium bromide, a fluorescent molecule that can intercalate into the base pairs of tRNA^{Phe}. In certain samples you will excite and monitor *both* fluorescent markers for a single sample cuvette in two consecutive steps.

PROCEDURE

General Fluorimeter instructions:

Turn on the Cary Eclipse fluorescence spectrophotometer and start up the Scan program: Start/Programs/Cary Eclipse/Scan. Find the Chem431 folder on the C: drive: (Desktop/My Computer/Local Disk (C:)/Varian/CaryEclipseWinFL/samples/CHEM431). Open either the method called 'trna' or 'EtBr', depending on which fluorophore you are probing for your experiment. The parameters within each method are as follows:

Method name	Fluorescence Marker	λ_{ex}	λ_{em} region	Slit widths (ex/em)	Scan Speed
trna	tRNA ^{Phe} Y base	318 nm	380-460 nm	20nm/20nm	medium
EtBr	Ethidium Bromide	546 nm	580-620 nm	10nm/10nm	medium

Under Options: The PMT Detector Voltage should be set as manual (600) and the CAT mode at 3 scans

Under Accessories: check multicell holder #4 and set temperature at 25 °C, display block

Familiarize yourself with the above parameters and be confident that you understand why, for example, the λ_{em} region is at longer wavelengths than the λ_{ex} .

For each titration series you will first collect a background scan by adding an aliquot of the titrant to the buffer-only cuvette. If this background fluorescence is zero, you may begin the titration experiment by simply adding titrant aliquots to the sample cuvette, mixing, and scanning the emission range. If background fluorescence is not zero, then

you must collect a background scan for every titration point that you do; that is, each addition of titrant must be scanned in both the buffer-only cuvette (background) and the tRNA cuvette (sample). You will save these scans, and ultimately subtract the background fluorescence from the sample fluorescence, in order to obtain a corrected value for the tRNA fluorescence only.

Note the directionality of the asymmetric cuvette within the instrument, as per the picture on the sample holder. The 1.00 cm path length in the cuvette must face the excitation light beam, as shown in **Figure 1** below. Note also that there are four cuvette slots, only one of which is in the light path; be sure that the cuvette is in the slot that is exposed to the light beam.

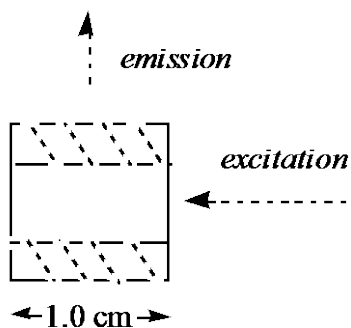


Figure 1. Semi-micro fluorescence cuvette, viewed from the top.

Fluor 1: Fluorescence Spectroscopy of tRNA^{Phe} Y base in the Presence of Mg²⁺ and a Small Molecule Ligand

GOAL

In this experiment you will use fluorescence spectrophotometry to probe structural changes within the tRNA^{Phe} molecule by observing the Y base as Mg²⁺ and small molecules are titrated into a solution of tRNA^{Phe}. Please read Project D1 (previous 2 pages) before proceeding further.

PROCEDURE

You will need four semi-micro fluorescence cuvettes¹ for this experiment. Two cuvettes initially contain buffer only; #1 will be used to zero the fluorimeter and also as a reference for the 260 nm UV absorbance measurement; the second buffer-only cuvette (#2) will be used to collect background fluorescence emission scans. The other two cuvettes (#3 and 4) will contain 1.00 mL of 2 μ M tRNA^{Phe} dissolved in the following buffer: 100 mM NaCl + 50 mM Tris•HCl buffer, pH 7.5. This buffer MUST be Mg²⁺-free. You will make a sufficient volume of this buffer from 1.0 M stock solutions of NaCl and Tris already prepared for you.

Prepare enough tRNA^{Phe} solution to do two experiments. Heat this initial tRNA^{Phe} solution to 90°C for 30 seconds, then cool to room temperature over 15 minutes.

Before starting the day's experiment, check the concentration of tRNA^{Phe} by measuring the UV absorbance of one of the tRNA cuvettes at ~260 nm using the Cary UV-Vis spectrophotometer. Collect a spectrum over the 220-300 nm range the first time through, and thereafter you need only measure the absorbance value at 260 nm. Given $\epsilon_{260} = 371,000 \text{ M}^{-1}\text{cm}^{-1}$ for tRNA^{Phe}, calculate [tRNA^{Phe}] and record it in your lab notebook; if the concentration is not close to 2 μ M, remake the solution.

In part A of this experiment you will *titrate* Mg²⁺ into the tRNA^{Phe} solution in cuvet #3 and monitor the Y base fluorescence. In part B you will *titrate* your small molecule (e.g., spermine, Neomycin B) into tRNA in cuvet #4 and monitor Y base fluorescence. In both parts A and B, use the Cary Eclipse method 'trna'. Note that in a *titration*, you have a single sample cuvette (#3 or 4) to which you add successive aliquots of titrant solution, measuring after each addition.

Part A will be done by all groups. [Mg²⁺] range: 0 – 4 mM. In the 0 – 0.04 mM range, collect data every 0.01 mM. In the 0.04 – 0.12 mM range, collect data every 0.02 mM. In the 0.12 – 4 mM range, collect five or six more data points.

Part B will be divided such that each group titrates one of the following small molecules into the tRNA^{Phe} solution (still no Mg²⁺ present).

¹ Fluorescence cuvettes have four transparent sides; UV-Vis cuvettes which have only two transparent sides. Semi-micro cuvettes hold a maximum of 1.75 mL; standard rectangular cuvettes which hold a maximum of 3.75 mL.

[spermine] range: 0–1200 μM . In the 0 – 200 μM range, collect data every 50 μM . In the 200 – 1200 μM range, collect data every 200 μM .

[neomycin B] range: 0 – 300 μM . In the 0 – 100 μM range, collect data every 20 μM . In the 100 – 300 μM range, collect data every 50 μM .

[kanamycin A] range: 0–2000 μM . In the 0 – 200 μM range, collect data every 50 μM . In the 200 – 2000 μM range, collect data every 200 μM .

[spermidine] range: 0–2000 μM . In the 0 – 200 μM range, collect data every 50 μM . In the 200 – 2000 μM range, collect data every 200 μM .

The concentration ranges given above for parts A and B are general guidelines. You may wish to use slightly different concentrations depending on your available pipet volumes and stock solutions. Given that you wish each titrant aliquot to be small (1-4 μL , depending on your smallest pipettor), calculate what stock solution(s) you will need to deliver the appropriate titrant aliquots, and make this stock solution(s).

EXAMPLE: If your first aliquot addition is 2.0 μL of an unknown stock solution of your small molecule ligand, to be added to 2.00 mLs of sample to give a final [ligand] = 49.95 μM and final volume = 2.002 mLs. Then

$$V_d M_d = V_c M_c \rightarrow 2002 \mu\text{L} \times 49.95 \mu\text{M} = 2.0 \mu\text{L} \times \text{unk} \mu\text{M}$$
$$\rightarrow \text{unk. Stock [ligand]} = 2002 \mu\text{L} \times 49.95 \mu\text{M} / 2.0 \mu\text{L} = 50,000 \mu\text{M} =$$

50 mM

1. Thus, adding 2.0 μL of a 50 mM ligand stock solution to 2.00 mLs of sample solution

In a fluorescence cuvet gives a final solution of 49.95 μM ligand, volume = 2.002 mL

2. Addition of a second 2.0 μL aliquot of the same 50 mM stock solution to the same sample cuvet gives a final solution that is

$$4.0 \mu\text{L} \times 50.0 \text{ mM} / 2004 \mu\text{L} = \underline{\underline{99.8 \mu\text{M}}}$$

ligand.

3. A third 2.0 μL aliquot of the 50 mM stock solution added to the sample cuvet gives:

$$6.0 \mu\text{L} \times 50.0 \text{ mM} / 2006 \mu\text{L} = \underline{\underline{149.55 \mu\text{M}}}$$

ligand.

4. A fourth aliquot that is twice as large, 4.0 μL , of the 50 mM stock solution added to the sample cuvet gives:

$$10.0 \mu\text{L} \times 50.0 \text{ mM} / 2010 \mu\text{L} = \underline{\underline{248.76 \mu\text{M}}}$$

ligand.

5. Subsequent 4.0 μL aliquot additions to the sample cuvet give ligand concentrations of
347.56 μM , 446.3 μM , 544.0 μM , and 641.66 μM .
6. A final aliquot addition of 6.5 μL of the 50 mM stock solution to the sample cuvet gives
 $32.5 \mu\text{L} \times 50.0 \text{ mM} / 2032.5 \mu\text{L} = \underline{\underline{799.5 \mu\text{M ligand}}}$.

For each titration point, start by zeroing the fluorimeter with the first buffer-only cuvette. Then check for background fluorescence by adding the appropriate aliquot of titrant stock solution to the second buffer-only cuvette. If background fluorescence is close to zero, then you need only perform this background fluorescence check once for each new titrant stock solution that you use. Use one of the tRNA/buffer cuvettes for your Mg^{2+} /tRNA titration (Part A), and the other for your small molecule ligand/tRNA titration (Part B).

If background fluorescence is not zero, then for each titration point, add the appropriate aliquot of titrant to the second buffer-only cuvette and measure and record the fluorescence of both the background (buffer-only + titrant), as well as the sample (tRNA + titrant). Subtract the background fluorescence from the sample fluorescence to obtain the intensity due to tRNA alone.

When you finish both titrations (parts A and B), shut down the fluorimeter, carefully rinse out all cuvettes, and return them to your instructor. Again, these are very expensive cuvettes, so please treat them with utmost care and responsibility.

POST-LABORATORY QUESTIONS

1. How does the addition of Mg^{2+} alter the Y base fluorescence? What does this suggest about changes to the RNA structure?
2. How does the addition of your small molecule ligand alter the Y base fluorescence? What does this suggest about changes to the RNA structure?
3. Determine the K_d for the binding of Mg^{2+} and your small molecule to tRNA^{Phe} by fitting fluorescence vs. concentration curves to the equation for hyperbolic saturation.
4. Tabulate K_d 's with associated errors for all four small molecule ligands.
5. Which ligand has the lowest K_d ? Does this ligand bind most tightly or loosely?
6. Given the structures of magnesium and the small molecule ligands, three types of attractive interactions control tRNA binding: (a) charge-charge (ionic bonding or salt bridging); (b) dipole-dipole (hydrogen bonding between polar groups); and (c) charge-dipole. Judging from your K_d s, discuss which of these are most important.

Fluor 2: Fluorescence Spectroscopy of tRNA^{Phe} Y base in the Presence of Ethidium bromide and a Small Molecule Ligand

GOAL

In this experiment you will use fluorescence spectrophotometry to probe structural changes within the tRNA^{Phe} molecule. You will monitor the Y base as EtBr is titrated into solution, and then monitor changes in both the Y base and the EtBr upon addition of a small molecule ligand.

PROCEDURE

You will need three semi-micro fluorescence cuvettes (four-sided) for this experiment. As in the previous fluorescence titration experiments, two cuvettes initially contain buffer only: #1 will be used to zero the fluorimeter and also as a reference for the UV absorbance measurement; the second buffer-only cuvette (#2) will be used to collect background fluorescence emission scans. The third cuvette (#3) will contain 1.00 mL of 2 μM tRNA^{Phe} dissolved in buffer (100 mM NaCl + 50 mM Tris•HCl, pH 7.5). This buffer MUST be Mg²⁺-free. Heat this initial tRNA^{Phe} solution to 90°C for 30 seconds, then cool to room temperature over 15 minutes.

Before starting the experiment, check the concentration of tRNA^{Phe} by measuring the UV absorbance of the tRNA cuvette at ~260 nm. Calculate [tRNA^{Phe}] (given $\epsilon_{260} = 371,000 \text{ M}^{-1}\text{cm}^{-1}$) and record it in your lab notebook; if the concentration is not close to 2 μM , remake the solution. Also check to make sure that both filters on the fluorimeter are set to auto.

Although you will carry out two experiments, they are done in the same cuvette (#3). In part A of this experiment you will titrate EtBr into tRNA^{Phe} and monitor how the Y base fluorescence is affected; as in the previous fluorescence titration, you will monitor Y base fluorescence using the 'trna' scan method. Once you have finished part A and added 50 μM EtBr to the tRNA solution in cuvet #3, you will start part B: titrate a small molecule ligand (e.g., spermine, neomycin B) into the tRNA^{Phe}/ethidium bromide mixture in cuvet #3. For **EACH** titration point in part B, you will monitor **BOTH** the Y base fluorescence (using the 'trna' scan method) and the ethidium bromide fluorescence (using the 'EtBr' scan method).

Part A will be done by all groups. [EtBr] range: 0–50 μM . In the 0–10 μM range, collect data every 2 μM . In the 10–50 μM range, collect data every 10 μM .

Part B: Each group titrates only one small molecule into the tRNA^{Phe}/ethidium bromide mixture. Fluorescence measurements for this part of the experiment will be made by switching back and forth between the 'trna' and the 'EtBr' methods for each titration point.

[spermine] range: 0 – 2000 μM . In the 0 – 200 μM range, collect data every 50 μM . In the 200 – 2000 μM range, collect data every 200 μM .

[neomycin B] range: 0 – 10 μM . In the 0 – 2 μM range, collect data every 0.1 μM . In the 2 – 10 μM range, collect data every 2 μM .

[kanamycin A] range: 0–2000 μM . In the 0 – 200 μM range, collect data every 50 μM . In the 200 – 2000 μM range, collect data every 200 μM .

[spermidine] range: 0–2000 μM . In the 0 – 200 μM range, collect data every 50 μM . In the 200 – 2000 μM range, collect data every 200 μM .

When you finish both titrations (parts A and B), shut down the fluorimeter, carefully rinse out all cuvettes, and return them to your instructor. Again, these are very expensive cuvettes, so please treat them with utmost care and responsibility.

POST-LABORATORY QUESTIONS

7. Propose conformational changes that could occur structurally as ethidium bromide is added to tRNA (consult the Saenger reading). Explain how these structural changes would affect the fluorescence of the Y base.
8. Determine the K_d ethidium bromide binding to tRNA^{Phe} by fitting your fluorescence vs. concentration curves to the equation for hyperbolic saturation.
9. How does addition of each of the small molecules affect the Y base and EtBr fluorescence? What does this suggest about structural changes that occur with the addition of the small molecule ligand?
10. More than one equilibrium is probably occurring; propose what they might be.
11. Share results for your small molecule ligand with your classmates.