Answers to Problem Sets

EXPERIMENT 1

1. Calculate the mean, range, and standard deviation of each data set (10 µl, 100 µl, 1 mL) from the Table 3.
   Assuming the density of water is 1 g/ml the mass of each sample of water in the order 10 uL, 100 uL and 1 mL is expected to be approximately 0.01g, 0.1 g and 1g respectively. Depending on the sensitivity of the scale or balance used the range is likely to be from 0.007-0.013 for the smallest aliquots and similarly 0.07-0.13 in the middle case. In the largest aliquot it will likely vary from 0.9-1.1 or less. The mean will be obtained by summing up the three independent mass measurements obtained and dividing by 3 (the n or number of samples weighed). The standard deviation (SD or sigma σ) is simply the sum of all the individual data points subtracted from the mean squared divided by the n number of samples which in our case is 3. So for example for three hypothetical data points for the 100 uL sample we have 0.10g, 0.09g and 0.12g with a mean of 0.103 so $(0.1 – 0.103)^2 + (0.09 – 0.103)^2 + (0.12 – 0.103)^2$ and the sum is 0.00001 + 0.026 + 0.00029 = 0.0263/3 = 0.009
2. Perform a student's t test on each data set to calculate the confidence limit of 95%. n/a
3. What is the weight percent (% mass/volume) of the sodium chloride?
   A saturated solution of sodium chloride (NaCl) is approximately 5M (moles/liter). A 1 mL sample would therefore be 0.001L * 5 moles/liter = 0.005 moles. The formula or molecular weight of sodim chloride is 23 g/mol (Na) + 35.4 g/mol (Cl) = 58.4 g/mol. 0.005 moles * 58.4 g/mol = 0.292 g. 0.292g/1 ml is equivalent to 29.2 g per 100 mL. Therefore the mass percent volume (M/V%) is equal to 29.2
4. What number of NaCl moles are dissolved in 1 ml? What is the molarity (M)? Convert this into mMoles and mM.
   1 mmoles of NaCl is dissolved in 1 ml of water. The molarity is 1 M, it can be converted to 1000 mM.
5. Indicate the correct experiment "tools" to transfer the following volumes of liquid:
   2 µl (2 – 20 uL pipetteman)
   5 ml (5 ml graduated pipette)
   350 µl (200 – 1 mL pipetteman)
   2 L (2 L graduated cylinder or volumetric flask or 2 x 1 L of either)

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800 ml (1 L graduated cylinder)
15 µl (2 – 20 uL pipetteman)
50 µl (20 – 200 uL pipetteman)
15 ml (50 graduated cylinder)

EXPERIMENT 2

1. Calculate the number of moles you dissolved of every individual salt and component in each buffer (TAE and PBS).
2. Calculate the molarity of each of these buffer components in units of molar (M).
3. Convert these into mass/volume (wt%)

Buffers	pH range	Components	Final Conc.	moles	Molarity (M)	% (W/V)
100 ml of TAE (10X)	8.0	Tris-Base	400 mM	40 mmoles	0.4 M	2.4%
		Acetic acid (99%)	200 mM	20 mmoles	0.2 M	1.2%
		EDTA (0.5 M)	10 mM	1 mmoles	0.01 M	0.29%
100 ml of PBS (1X)	7.2	NaCl		13.7 mmoles	0.137 M	0.8%
		Na_2HPO_4		0.85 mmoles	0.0085 M	0.12%
		KH_2PO_4		0.15 mmoles	0.0015 M	0.02%
		KCl		0.27 mmoles	0.0027 M	0.02%

4. How many types of ions existing in your PBS buffer? What are they?
   7 types and they are Na^+, K^+, H^+, Cl^-, OH^-, $HPO4^{2-}$, $H_2PO_4^-$
5. Using Henderson-Hasselbach for Tris and for phosphate and using the final pH and known values for pKa calculate the [A-]/[HA] which refers to the dissociation into ions.
   Tris: 1, phosphate: 1
6. If a student mistakenly used 0.5M EDTA instead of Tris what ramifications would this have for the buffer?
   The TAE buffer won't be able to function as a buffer to absorb protons and maintain pH around 8.0
7. If a student mistakenly forgot to include EDTA in his/her TAE buffer what would be the impact of this on their buffer and its ability to protect biological molecules?
   EDTA is a chelating reagent that can absorb ions such as Mg2+, Ca2+. Those ions are cofactors of DNA degrading enzymes, so without EDTA the dissolved DNA sample maybe degraded very soon.

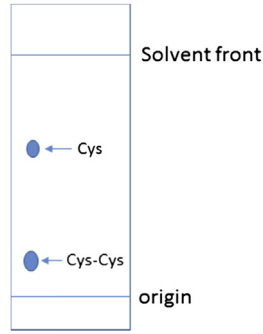
EXPERIMENT 3

1. Based upon your Rf values and the pattern observed for a representative non-polar, polar and ionic amino acid, what would you predict the R_f for another ionic amino acid such as Aspartate might be?
   The Rf of a given type of amino acid depends upon the bead type and coating of the TLC plate primarily and the polarity/hydrophobicity of the solvent. Both the amino acid and the solvent interact with the beads. In our case the solvent involves a mixture of ethanol and acetic acid which is relatively polar and the deriviatized silica which tends to be relatively non-polar. The differential in terms of the amino acid interacting with the bead and the solvent is what will determine how far up the plate the amino acid moves. An amino acid that is relatively polar such as aspartate where there is little interaction with the bead and where a more polar solvent is able to disperse or compete with the amino acid:bead interaction would be expected to travel up the plate all other things being equal. Since the amino acids are approximately the same size from a molecular weight perspective the relative size of the amino acid has little bearing on how far it travels. Rather this distance more reasonably depends again on the polarity of the amino acid relative to the solvent and bead type and hence if there is any interaction enabled by the side chain of amino acid with the bead and whether the solvent can dissociate that interaction. If the solvent cannot the amino acid won't travel very far. Therefore the pattern observed on the TLC plate for this representative set of amino acids should correlate with the polarity/non-polarity of the amino acids, aspartate being quite polar and ionic.
2. Was the color for any of the amino acids in your test set different upon staining with ninhydrin? How might this reflect the difference in its chemical reaction?
   The ninhydrin reaction with primary amino groups has long been known and it has been the basis for many bio-assays of biological molecules and other compounds possessing a primary amino group. Students should research the mechanism providing at least the end product structure of this reaction. Essentially amino acids that turn a different color on reaction with ninhydrin therefore generate a different reaction product which in the case of proline is because its amino group is part of a ring structure and in the case of cysteine likely involves product(s) invoking the sulfydryl (-SH) group. In the case of Pro this is the reason for the yellow color which develops and in the case of Cys for the brownish-red product observed instead of the typical purple.
3. Bioconjugate Chemistry is a relatively new branch of science whereby biological macromolecules, sometimes even from different

classes are connected by a covalent bond or linkage. For example, a lipoprotein or a glycolipid in which part of the molecule is a lipid and part protein, or part carbohydrate and part protein can be considered bioconjugates, and there are many other examples. After a bioconjugation synthesis, typically medicinal chemists will use TLC to determine the extent of their reaction where it can be used to separate the starting reactants from the product (the bioconjugate). For example consider the reaction of two cysteine amino acids together in the formation of a disulfide bond and the biological compound cystine (Eq. 2):

$$\textbf{Cys-SH+Cys-SH} \rightarrow \textbf{Cys-S-S-Cys}\,(\textbf{Cystine}) \qquad \text{(Eq. 2)}$$

Draw or sketch what the TLC pattern might look like where the starting material is separated by the disulfide bond linked dipeptide. The conjugate will run up the TLC plate more slowly and be closer to the origin than the free amino acid (Cysteine). Unless the reaction goes to 100% completion some starting material (Cysteine) will be visible as will the end product (Cystine). See a sketch below:



4. TLC is still useful in the experiment, but is being replaced by automated chromatographic methods. For example, TLC, FPLC, and HPLC are now automated processes and computers can control various parameters, such as flow rate, blends of solvent mixtures, etc. Can you think of how the ability to manipulate the parameters of these automated chromatographic methods (sample size, column plate length, flow rate, solvent gradients, etc.) might accentuate or improve an investigators ability to separate amino acids or other biomolecules? Being able to better computer control parameters such as flow rate, gradient etc., will make the chromatographic separation more reproducible and likely improve the separation.
5. Since proteins are made up of these four, plus the other 16, how might you take advantage of these properties of the amino acids in the

chromatography of a larger protein? How about a complex mixture of proteins?
Proteins can be separated by chromatography since they will also vary in their polarity and hydrophobicity. Typically larger proteins are not separated or isolated by hydrophobic interaction chromatography as discussed above but are still separated by flow through beads which are often functionalized with an ionic compound allowing for anion or cation exchange chromatography. Sometimes the beads can be functionalized with for example and antibody which will specifically bind to a certain type of protein, and this is called "affinity chromatography".

EXPERIMENT 4

1. Is their evidence of having successfully purified Luciferase?
If students loaded a lane of their crude sample onto the analysis gel and a lane of the semi-purified protein, the total number of observable bands of stained proteins should decrease the further along in the purification. A completely pure protein would be expected to show up as one band only on the gel. In this experiment given the brief time we have to purify the protein and the small number of steps involved which might otherwise get rid of impurities, it is likely that while a band will be present at the correct molecular weight for Luciferase (55-65 kDa) other proteins will be present as well. However the activity assay should show students that there are significant relative light units (10^4-10^5 RLU) contained within a sample of the purified protein demonstrating the Luciferase enzyme activity has been maintained and is present.
2. How do you argue or prove that? How do you demonstrate that?
One way is to calculate the specific activity, the amount of RLU per milligram or per microgram of protein and to compare this to literature values reported for Luciferase. Other confirmation comes from the gel analysis and whether the protein purified migrates on the gel in comparison to a molecular weight standards or ladder as it should (see below).
3. If you see a band in the tail sample on the stained gel is it at the correct molecular weight based on what is in the literature for Luciferase? Is there such a band in the head sample?
In addition to the protein band present in your tail sample being in the correct molecular weight range on the gel and to it having activity in terms of RLU another check is to see if that same protein is present in the head sample used as a negative control. This assumes that Luciferase is confined to the specialized tail organ of the firefly and

that a protein present in large amount in the head in that molecular weight range does not exist.

4. How many units of activity are present in the tail sample? The head?
   As given above a good purification will have 10,000 to 100,000 RLU present in the aliquot sacrificed to test the activity or more. Generally the background is from 1,000 to 5,000 RLU so activities more than 10X this amount are required to demonstrate significant Luciferase present.
5. Calculate the specific activity (RLU/ug protein). What assumptions did you make here?
   While the exact answer may vary, it is likely that in excess of 10^5 RLU/ug protein would be present for a highly purified Luciferase if not higher so students calculations should ultimately yield something close to this. One assumption is that the majority of the tail is comprised of Luciferase or that a very percentage of the original 0.5 mg tail is as it is very difficult to weight with any precision or accuracy the gross amount of Luciferase yielded from such a small amount of sample.
6. Now that you have a purified sample of Luciferase what other kinds of studies or characterization if time were available could you perform on it?
   The ultimate proof that a student has isolated Luciferase would come from actually sequencing the isolated and purified protein. Today this is routinely accomplished by liquid chromatography-mass spectrometry (LC-MS). However given the time and cost of such analysis it is unlikely most students can get the protein they isolated sequenced since this would require extraction from the gel and some clean up and for their samples to be sent out for analysis. As a convenient alternative, the instructor may have a standard or control sample of Luciferase protein enzyme and this can be run on a lane of the gel to check that the test sample protein band co-migrates with the standard. Alternatively antibodies to Luciferase are available and a western blot could be conducted to confirm identity.

EXPERIMENT 5

1. What if 1 mole of glucose can generate 2 moles of NADH? How would that affect our calculation?
   The glucose concentration will be calculated withNADH concentration divided by 2.
2. A student measured the glucose concentration of the cell culture media without any dilution. How would you expect the result? Do you think the measure is accurate?

The glucose concentration maybe too high for the measurement, because there maybe not enough reagents in the well to catalyze the amount, and the absorbance may be higher than the highest point on the standard curve.

EXPERIMENT 7

1. Find the sequence for protamine and determine how many arginine's it has?
Protamine is generally isolated from a cold water fish such as Herring, Tuna, etc. Unless great care is taken to isolate a single type of protamine protein or polypeptide chain there can be several forms present (this is a good lesson in molecular biology as this is the case for some, if not many proteins. In general however most forms of protamine have from 4 to 6 separate tracts of arginine (Arg) and these can contain as few as 4 to as many as 8 or 9 arginines in each tract. Depending on the citation or source for the protamine sequence obtained from the literature or the various searchable protein databases this can be as few as 16 arginines to as many as 54 arginines in the entire protein sequence as well as one or two random arginines present in other regions of the sequence. Suffice it to say though that accordingly, given the arginine-rich nature of protamine, it is one of the most basic or highly positively charged proteins in nature at physiological pH.

2. Based on the predicted sequence for protamine what is its overall charge expected to be at neutral pH?
At physiological pH each arginine (Arg) contributes a +1 charge. So with a short low arg protamine as above it would have an overall +16 charge. With a longer protamine protein containing 54 arginines, the overall charge would be +54.
If there are other positively charged amino acids such as lysine (Lys) or histidine (His) present within the sequence these would also contribute positive charge to the overall charge of the protamine. If there are negatively charged amino acids present within the sequence the student chooses to use, such as aspartate (Asp) or glutamate (Glu) these would each contribute one negative charge (−1). Thus depending on how many Asp or Glu amino acids present within the sequence there are each would correspond to a subtraction of −1 from the overall positive charge of the protamine.

3. At the amounts of protamine added in each tube and based on a 2mg/ml stock what was the number of moles and the molarity in units of molar (M) and millimolar (mM) of protamine in each tube?
The protamine stock used is 2 mg/ml. 1, 2, 5 and 10 microliters of this stock are added and the final volume of the solution or binding mixture is 15 microliters (15 ul). The molecular weight of protamine may vary as above depending on the source used but as an example calculation let's assume the molecular weight of a typical protamine is about 6 kDa or 6,000 g/mol:

$$\left(2\times10^{-3}\text{g/ml}\right) * \left(1,2,5 \text{ or } 10\times10^{-3}\text{ ml}\right)$$
$$= \left(1\times10^{-6}\text{g}\right) * 1 \text{ mol}/6000 \text{ g}$$
$$= \mathbf{2\times10^{-10}mol}$$

$$\left(2\times10^{-10}\text{mol}\right)/15\times10^{-6}\text{L} = \mathbf{1\times10^{-5}M;}$$
$$2\times10^{-10}\text{mol} * 1000 \text{ millimoles/mol}$$
$$= 2\times10-7 \text{ mmol}/15\times10-6 \text{ L}$$
$$= \mathbf{1\times10^{-2}mM}$$

icroliters of pDNA. One sample should be a DNA alone control. The rest of them should have progressively more protamine in them, for example of.

4. Based on the GFP plasmid used in this experiment and assuming an equal ratio of A,T, G and C) and that the plasmid is 5757 base pairs, to achieve charge neutrality calculate what number of protamine molecules are bound to each DNA molecule.
Let's assume that we are using the protamine containing 54 arginines or at least that the overall charge per protamine molecule is about +54. If the plasmid has 5757 base pairs, for every nucleotide the phosphodiester backbone to which it is attached in the long DNA biopolymer strand would mean that the overall plasmid DNA as a negative charge of –5757. 5757/54 = 106.6 which rounds to 107 protamine molecules are bound to each plasmid in order to completely neutralize its charge to zero.

5. Based on this molar ratio what is the overall expected charge of the complexes assuming one negative charge is provided per base pair of the plasmid.
A typical plasmid is provided at as little as a few micrograms per ml to upwards of 1 mg/ml or more. For ease of calculation let's assume a mid-range concentration of 100 ug/ml. Assuming 2 microliters was provided to the solution containing protamine which we've previously calculated above this would mean 100 x 10^{-6} g/ml * 2 x 10^{-3} ml = 2 x 10^{-7} g plasmid. The plasmid has 5757 base pairs and each

dNTP nucleotide is approximately 500 g so the total molecular weight Mw (plasmid) = 2.9 X 10^6 g/mol

$$2\times10^{-7}\text{g} * 1\text{ mol plasmid}/2.9\times10^{6}\text{g}$$
$$=7\times10^{-14}\text{mol plasmid}\times5757\ -\text{ve charges per mole}$$
$$=\mathbf{4\times10^{-10}mole\ -ve\ charge}$$

From #3 above, the number of moles of protamine is in the range of 10^{-10} moles with between +16 to +54 +ve charges per mole. Clearly the plasmid:protamine complexes are at least neutral if not slightly positive.

6. Can you think of another technique that could be used to probe these complexes that would be more insightful and/or give you additional information about them?
Circular dichroism (CD) spectroscopy can be used to detect protein:nucleic acid complexes as can other forms of spectroscopy. Essentially some physical-chemical property of either the nucleic acid or protein (absorbance, nuclear or electronic signature) will change or shift upon complexation. Being able to crystalize the complex and examining it by X-Ray diffraction would be the ultimate means to observe such molecular species.

EXPERIMENT 8

1. Which enzyme is more active?
Your answer here may well depend on the results of your experiment. If done appropriately, it should be possible to directly compare both enzymes. The enzyme that degrades the RNA the fastest or at least at the lowest ratio of enzyme to RNA may well be described as most active.

2. Was the RNA degraded more over time with RNase A or B?
In the authors experiment it appears all other things being equal that RNase A actually degrades RNA more rapidly than does RNase B. However caution is warranted here as the gel assay here utilizes indirect relative fluorescence of the stained band and is not a direct measure of the kinetics of the reaction per se. In many experiments we conducted comparing RNase A and B, version A appeared to degrade RNA more completely.

3. Since the two enzymes only differ in having a/an (un)glycosylated amino acid residue, what can be inferred about how the glycosylation affects enzymatic activity?
Again this depends on students results. But if as above RNase B which is glycosylated shows less activity in comparison to A, it could be inferred that glycosylation reduces or inhibits enzyme activity.

4. From a biomolecular interactions perspective come up with some plausible explanations for this?
   The obvious hypothesis is that glycosylation occurs in or near the active site and therefore limits the ability to access the RNA substrate, bind it or cleave it in some way. However the glycosylation could also cause a conformational change in the RNase and the way it folds which could also effect the activity of the enzyme, albeit indirectly.
5. How would you test these hypotheses experimentally?
   As in the protein:nucleic acid gel shift assay, it may be possible to compare the binding of RNase A and B to RNA by gel shift and if a binding curve was established under stoichiometric or a dose-response conditions determine that the binding of RNA by RNase A or compared to B was more efficient, thus supporting the glycosylation influences substrate access to the active site or interaction in the active site. There may be other types of simple experiments students could think of to determine how glycosylation influences RNA cleavage activity.

EXPERIMENT 9

In writing up your discussion you might reflect on some of the questions below and incorporate them into your discussion:

1. What are the CAS (Chemical Abstract Service) numbers for the lipids used today?
   CAS # 555-44-2 (Tripalmitin)
   CAS # 57-88-5 (Cholesterol)
2. What is the mole to mole ratio of the lipids used including the fluorescently labeled cholesterol?
   Answer: 10 mg of cholesterol (386.65) and 10 mg of tripalmitin (807.32). $(10 \times 10^{-3}\ g) \times (1\ mol/3.8 \times 10^{2}\ g) = 2.6 \times 10^{-5}$ moles Cholesterol, $(10 \times 10^{-3}\ g) \times (1\ mol/8.1 \times 10^{2}\ g) = 1.2 \times 10^{-5}$ moles Tripalmitin. Assuming about 1% incorporation of the fluorescently derivatized cholesterol with a larger molecular weight, fluor-cholesterol molarity will be approximately 100-1000x less than underivitized cholesterol.
3. Design an experiment the goal or end result of which is to confirm or test the mole to mole ratio of lipids within a given liposome test batch?
   Answer: Incorporation of lipids into the liposome will be less than 100%. One straightforward approach would be to extract the liposomal suspension with an organic solvent such as chloroform or hexane and to evaporate the solvent to dryness. At this point one would need to confirm the ratio of tripalmitin:cholesterol. This is

probably best accomplished with the technology available today by GC or LC-MS, where the resulting area underneath the peak (AUP) for cholesterol versus tripalmitin when the mixture is analyzed would give an estimate of their mol:mol ratio.

4. Discuss how you would expect the temperature or other conditions/parameters during the liposome preparation stage to effect the outcome of your experiment?
   Answer: Increasing the temperature during the evaporation stage or afterwards too much beyond 40-45 °C would not be recommended, because the tripalmitin could approach its melting point. Increasing the temperature above room temperature to physiological temperature may somewhat improve incorporation efficiency of the lipids into the liposome but could also increase aggregation as more liposomes would be moving around interacting together and potentially fuse to form larger structures. Liposomes are often stored for short periods of time at temperatures between 4 to 10 °C, but just as milk should not be frozen, as it will cause the lipids and proteins present in it to fall out of solution, so too storage temperatures for each different type of liposome require optimization and temperatures which are too low can cause the liposomes to become unstable and fall out of the suspension.
5. Suppose in your research you are interested in using a putative liposome to deliver a drug to a particular tissue (e.g. liver or lung). What additional or different components would the liposome possess and how would you incorporate them or associate them into the liposome?
   Answer: The idea here is to incorporate into the liposome a lipid or a protein indigenous or specific to the type of tissue (lung, liver) one is trying to target. One classic example that has been used is a protein or peptide able to bind specifically to a particular receptor on the tissue or cell of which there are many examples in the literature. While this seems straightforward it actually isn't all that easy because especially with proteins, exposure to an organic solvent can often denature the protein even if it has a portion of its sequence that is normally hydrophobic and membrane bound, the organic solvent causing the protein to unfold irreparably. So the trick is to associate the protein into the liposome in such a way as to retain the structure-function of that peptide or protein such that it can interact with the receptor or otherwise. One of the methods used for this is to simply associate the protein to the surface primarily by electrostatic interaction after the liposome is formed where the protein will insert its hydrophobic domain into the membrane. This avoids exposure to organic solvent. Other techniques such as reverse-emulsion have also been developed for this.

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