3 PCR, Sequencing, Cell Structure, and Localization

11/29: 1. PCR.

a) Can you describe a way to amplify a strand of RNA (i.e., make a large number of [DNA or RNA] copies so that you can submit it for sequencing, for example)? But your challenge is to achieve this using any enzyme (or set of enzymes) except a DNA polymerase. (4 pts)

Answer. Given 50-100 copies of the RNA starting material, mix in RNA replicase, NTPs, and 20-30 bp primers. Stick the system into a thermal cycler, allowing repeated annealing, extension, and denaturation steps to take place until the desired quantity of RNA has been synthesized. ☐

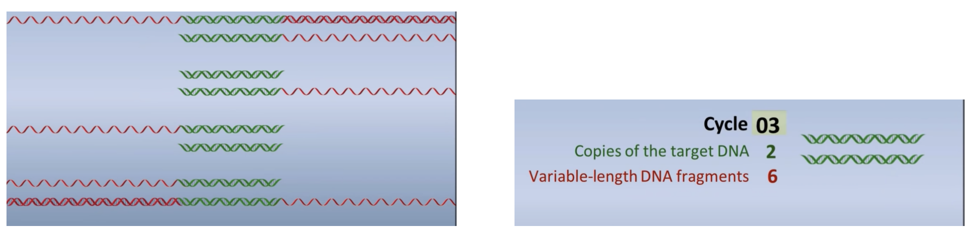
b) Professor Tang has invented a new DNA polymerase that produces 3 DNA strands every time it copies a template strand. After 10 cycles of PCR, on a target segment she amplified, how many target DNA strands will she have in the mixture? How many variable length strands will she have in the mixture? 2 bonus points for a mathematical rationale. $(4 + 2 \text{ pts})$

Answer. To answer this question, we must make several assumptions:

i. Before the first cycle, there exists a single segment of dsDNA containing the target region as well as some additional base pairs on the sides. It looks like this:

Figure 1: Initial DNA segment.

ii. Every cycle has three steps.



(a) DNA after cycle 3. (b) Counting DNA after cycle 3.

Figure 2: What qualifies as a "target DNA strand?"

They are denaturation, then annealing, then extension. We count the number of target DNA strands and variable length strands *after* the extension step and *before* the next denaturation step. Thus, two target dsDNA strands (four target ssDNA strands) count as TWO "target DNA strands." All of the composite dsDNA/ssDNA strands count as variable length strands. This convention is taken directly from the video used in class (see Figure 2).

iii. On the action of the new DNA polymerase.

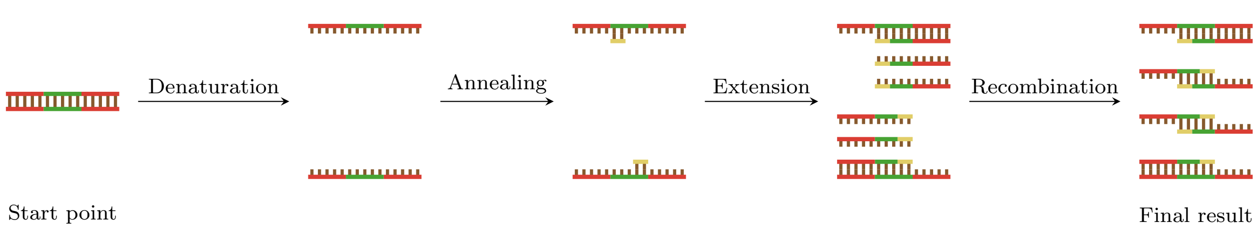


Figure 3: Counting procedure for the first cycle.

We assume that whereas a typical DNA polymerase synthesizes one new complementary DNA (cDNA) strand at a time and binds it to the template strand, the new DNA polymerase still does this but also synthesizes an *additional* two identical cDNA strands that it releases into solution. We postulate the existence of a fourth **recombination** step directly following extension, in which these additional unbound ssDNA strands bond to complementary ones in solution "for thermodynamic reasons" (we make further assumptions concerning what binds to what below). Modifying assumption (ii), we count target DNA and variable length strands *after* this new recombination step and *before* the next denaturation step in the next cycle. For example, we would count four variable length DNA strands after the first cycle (see Figure 3).

At this point, we are ready to begin the mathematical derivation. First we enumerate the different types of DNA that can be produced according to our model. Now we investigate what is produced

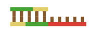
Type	**Presentation**
1	
2	
3	
4	
5	
6	
7	
8	
9	

Table 1: Types of DNA.

during a cycle when each of the above types is present. Let's begin.

Type 1 DNA: As we can see in Figure 3, a single segment of Type 1 DNA will lead to the generation of one Type 2 segment, one Type 3 segment, and two Type 4 segments. We will not explicitly draw out the counting procedure for the other types as we did in Figure 3, but we will do our best to rationalize with words.

Type 2 DNA: From the top strand, we get another Type 2 and two single strands for recombination. We will say that each of these single strands joins with an analogous one from Type 3 to form a Type 4. Thus, we say that from the top strand, we get one Type 2 and one Type 4 on average. From the bottom strand, we get a Type 5 and two other single strands for recombination. Again, we will pair these with the analogous ones from Type 3 to form Type 9s. Thus, we say that from the bottom strand, we get one Type 5 and one Type 9.

Type 3 DNA: Similarly to Type 2, we get one Type 3, one Type 4, one Type 6, and one Type 9 on average.

Type 4 DNA: We get one Type 5, one Type 6, and two Type 9s on average.

Type 5 DNA: We get one Type 5 and three Type 9s on average.

Type 6 DNA: We get one Type 6 and three Type 9s on average.

Type 7 DNA: We get one Type 7 and three Type 9s on average.

Type 8 DNA: We get one Type 8 and three Type 9s on average.

Type 9 DNA: We get four Type 9s.

A natural way to represent all of this data is using matrices. Indeed, the following matrix M summarizes all of the above insights, and acts on the initial condition vector $x_0 \in \mathbb{R}^9$ defined by $x_0 = (1,0,0,0,0,0,0,0,0)^T$.

$$M = \begin{pmatrix} 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 2 & 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 1 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 1 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 1 & 1 & 2 & 3 & 3 & 3 & 3 & 4 \end{pmatrix}$$

Notice that we have, for example, $Mx_0 = (0,1,1,2,0,0,0,0,0)^T$, as expected: The physical interpretation of the vector Mx_0 is that after cycle 1, we should expect one Type 2 segment, one Type 3 segment, and two Type 4 segments, as depicted in Figure 3.

To compute the number of strands of each type after 10 cycles, we compute

$$M^{10}x_0 = \begin{pmatrix} 0 \\ 1 \\ 1 \\ 2 \\ 27 \\ 27 \\ 0 \\ 0 \\ 1\,048\,518 \end{pmatrix}$$

The bottommost number in the above vector corresponds to the number of Type 10 DNA segments (target DNA strands) after 10 cycles. The rest of the numbers correspond to variable length strands of the various types; their sum is the total number of variable length strands in the mixture. Therefore, there are approximately

$$\boxed{1\,048\,518 \text{ target DNA strands}}$$

and

$$\boxed{58 \text{ variable length strands}}$$

in the mixture.

Note that as per the 11/29 Canvas announcement on this subject, it appears that we may want to count the pairs of *all* target DNA strands produced, including those I counted as bound up in Types 5, 6, 7, and 8 DNA segments at the conclusion of the 10 cycles. If this is the proper assumption, then since there are $27 + 27 + 0 + 0 = 54$ variable length strands of these types (containing 54 ssDNA strands which pair to $54/2 = 27$ additional target DNA strands and 27 fewer variable length strands) we adjust our counts to

$$1\,048\,518 + 27 = 1\,048\,545 \qquad\qquad 58 - 27 = 31$$

An alternate way to get this latter answer would be to make the following two observations. First, notice that the *total* number of strands is 4^n following n cycles. Second, notice that the only ssDNA strands that can produce new variable length ssDNA are the original two. Together, these account for the production of six new non-target (hence variable) ssDNA strands per cycle, or three new variable length dsDNA strands per cycle. Thus, the number of variable length strands is $1 + 3n$ following n-cycles. It follows that after 10 cycles, we would have $4^{10} = 1\,048\,576$ total strands and $1 + 3(10) = 31$ variable length strands. Therefore, since target = total − variable, we have

$$1\,048\,576 - 31 = 1\,048\,545 \qquad\qquad 31$$

target DNA and variable length strands, respectively, in agreement with the above. □

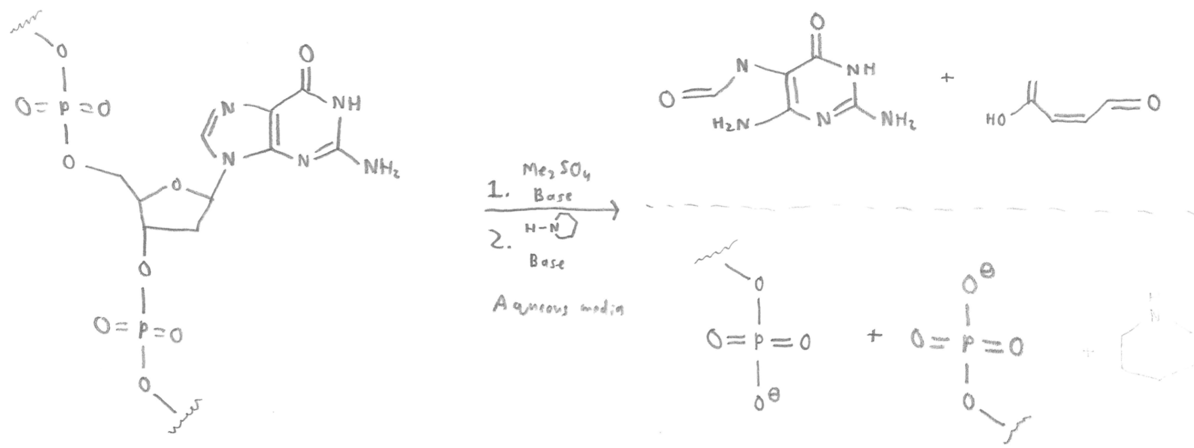
2. Sequencing.

   In Maxam-Gilbert sequencing, the 4 nucleotides A, T, G, and C react in 4 specific reactions, leading to the strand being cleaved when it is treated with hot piperidine. For each nucleobase, write below the chemical reaction that occurs, and how the strand gets cleaved. Hefty bonus points for the reaction mechanism. (4×1 pt for the reaction; 4×2 pts for the reaction mechanism)

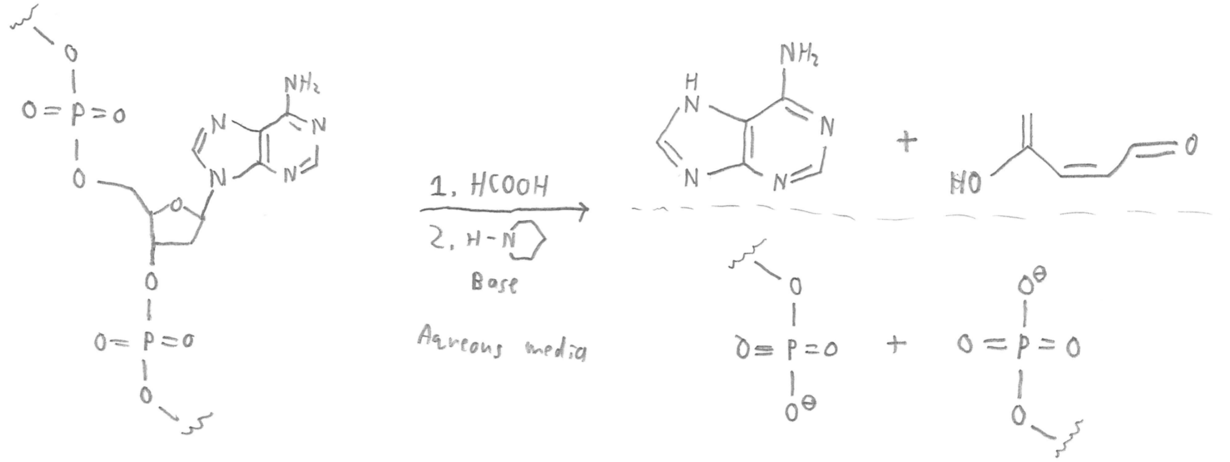
   Answer. Each strand gets cleaved *at* the targeted nucleotide; in other words, the reagents we use heavily disfigure the targeted nucleotide and eventually cleave it from both the 5′ and 3′ sides. In Maxam-Gilbert sequencing, the 5′ side, minus the modified/disfigured nucleotide and the rest of the chain that follows it to the 3′ end, will go on to show up in the final radiograph.

   Below are the net reactions — and mechanisms — for the reaction of guanine with Me_2SO_4 followed by hot piperidine, adenine and formic acid followed by hot piperidine (there is also a guanine variant that will not be shown), cytosine and hydrazine/sodium chloride followed by hot piperidine, and thymine and hydrazine followed by hot piperidine (there is also a cytosine variant that will not be shown). All reactions occur in aqueous (often basic) media. Some parts of later reaction mechanisms are left off because of complete homology to preceding mechanisms; a note is always made when this is the case.

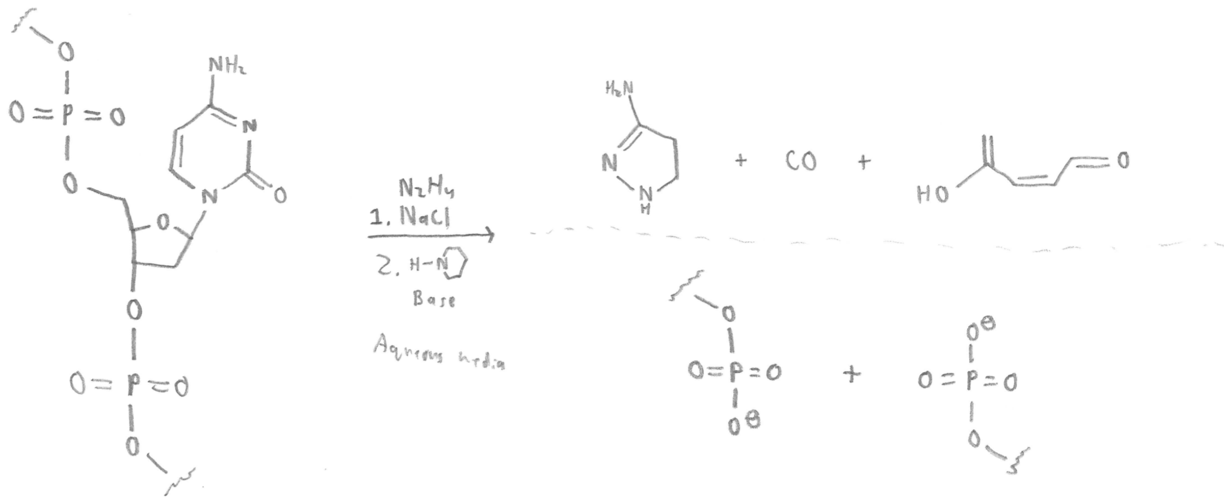
   Without further ado, here are the results.



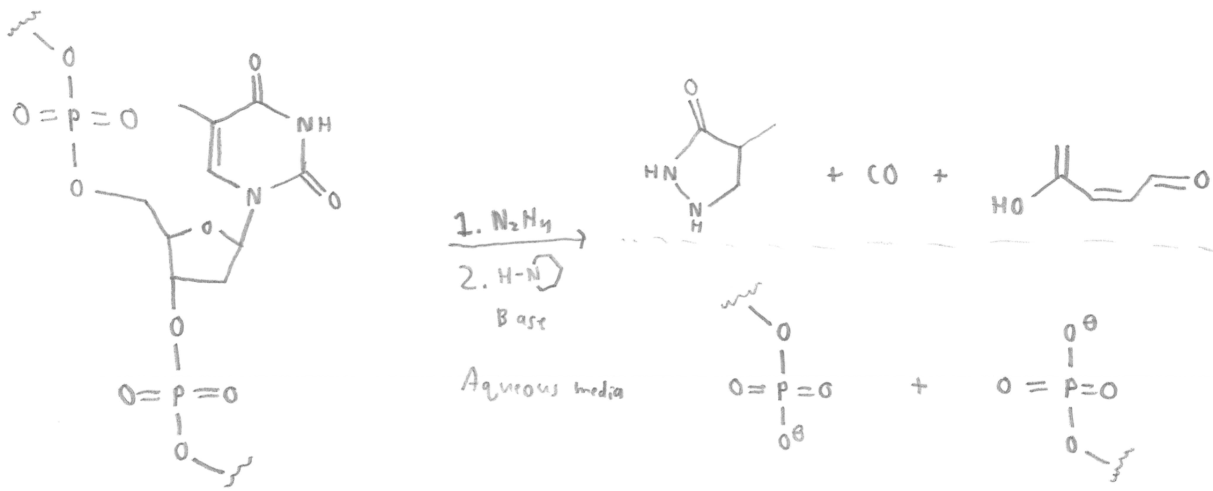
(a) G.



(b) A.

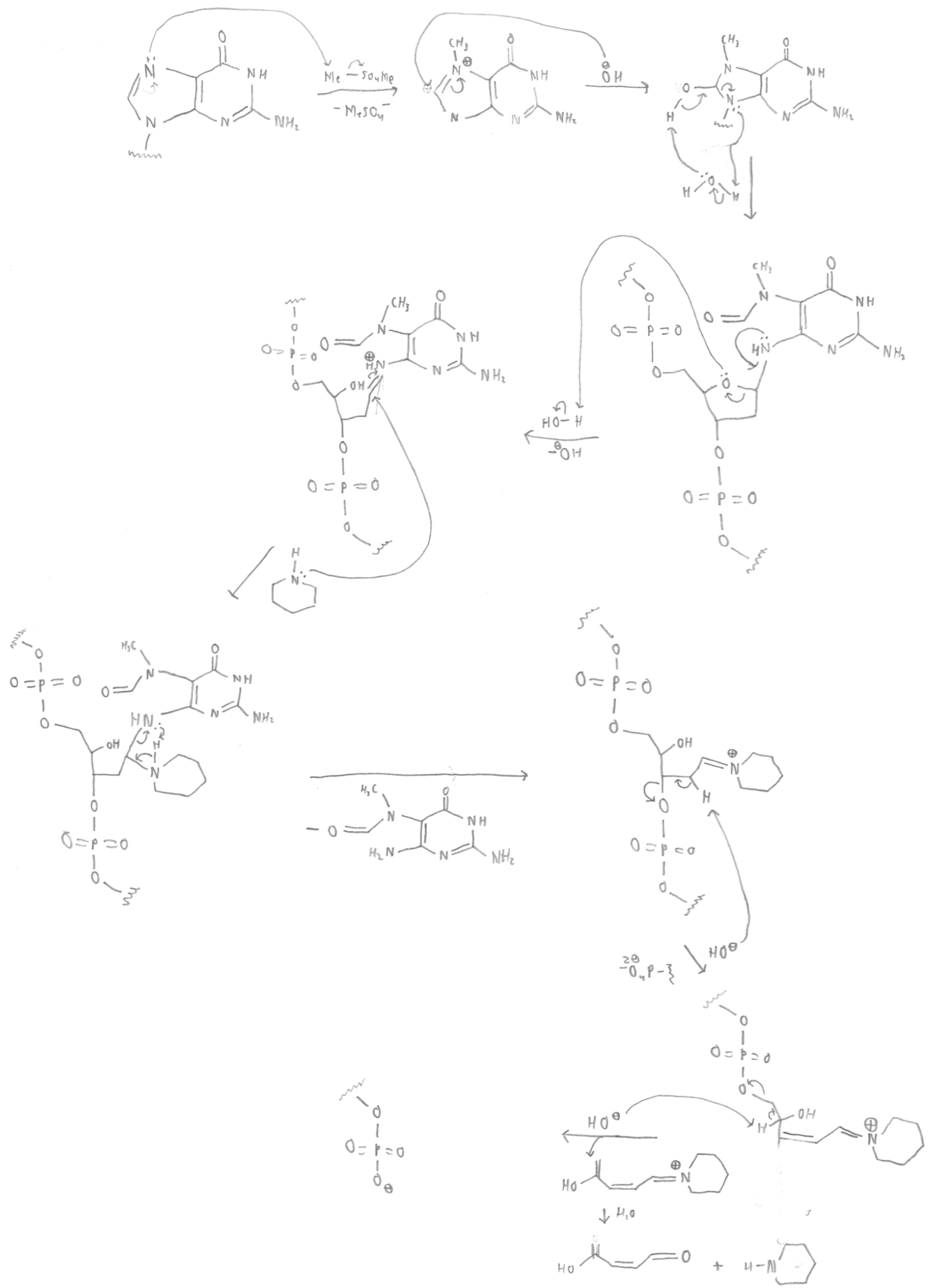


(c) C.

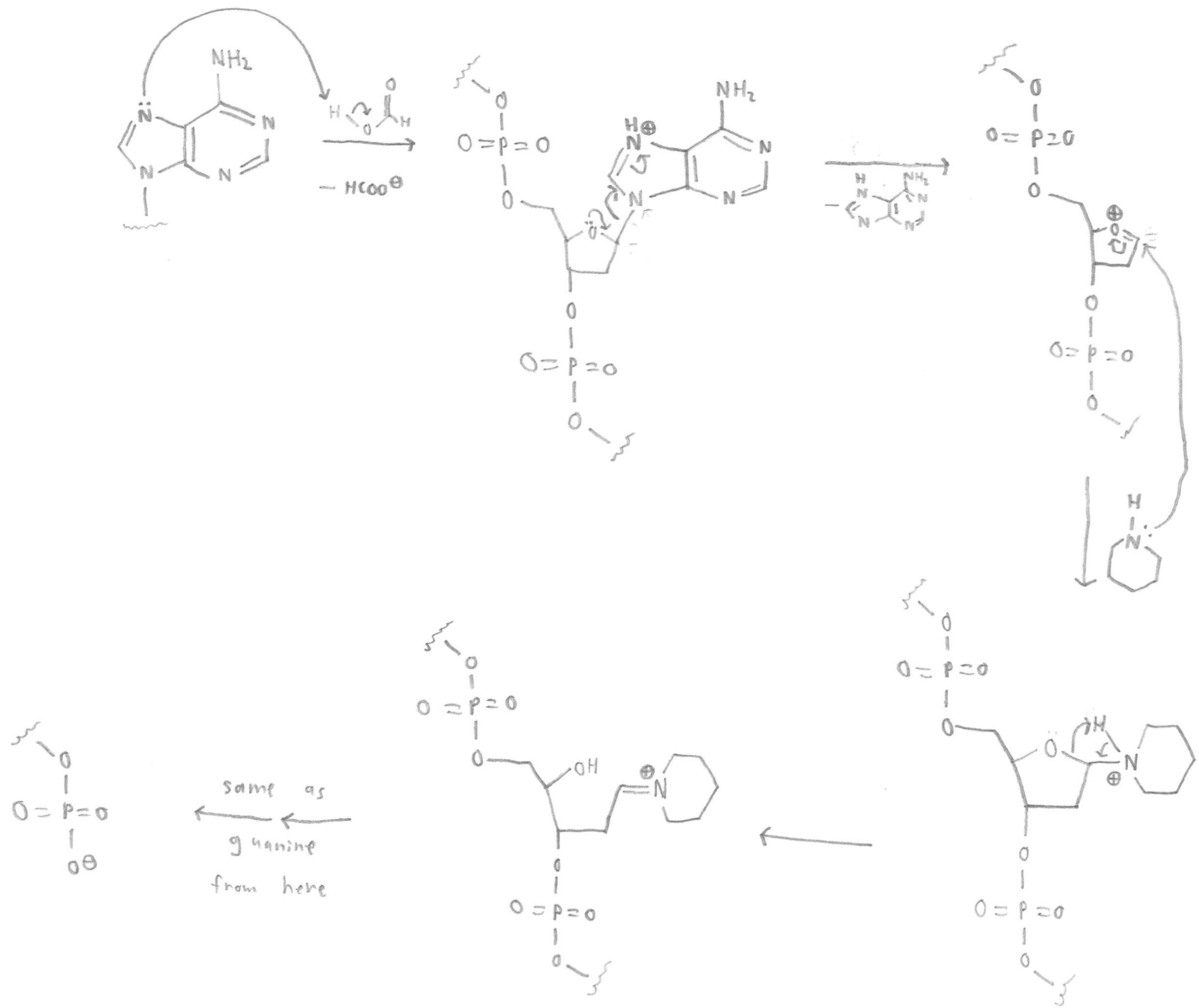


(d) T.

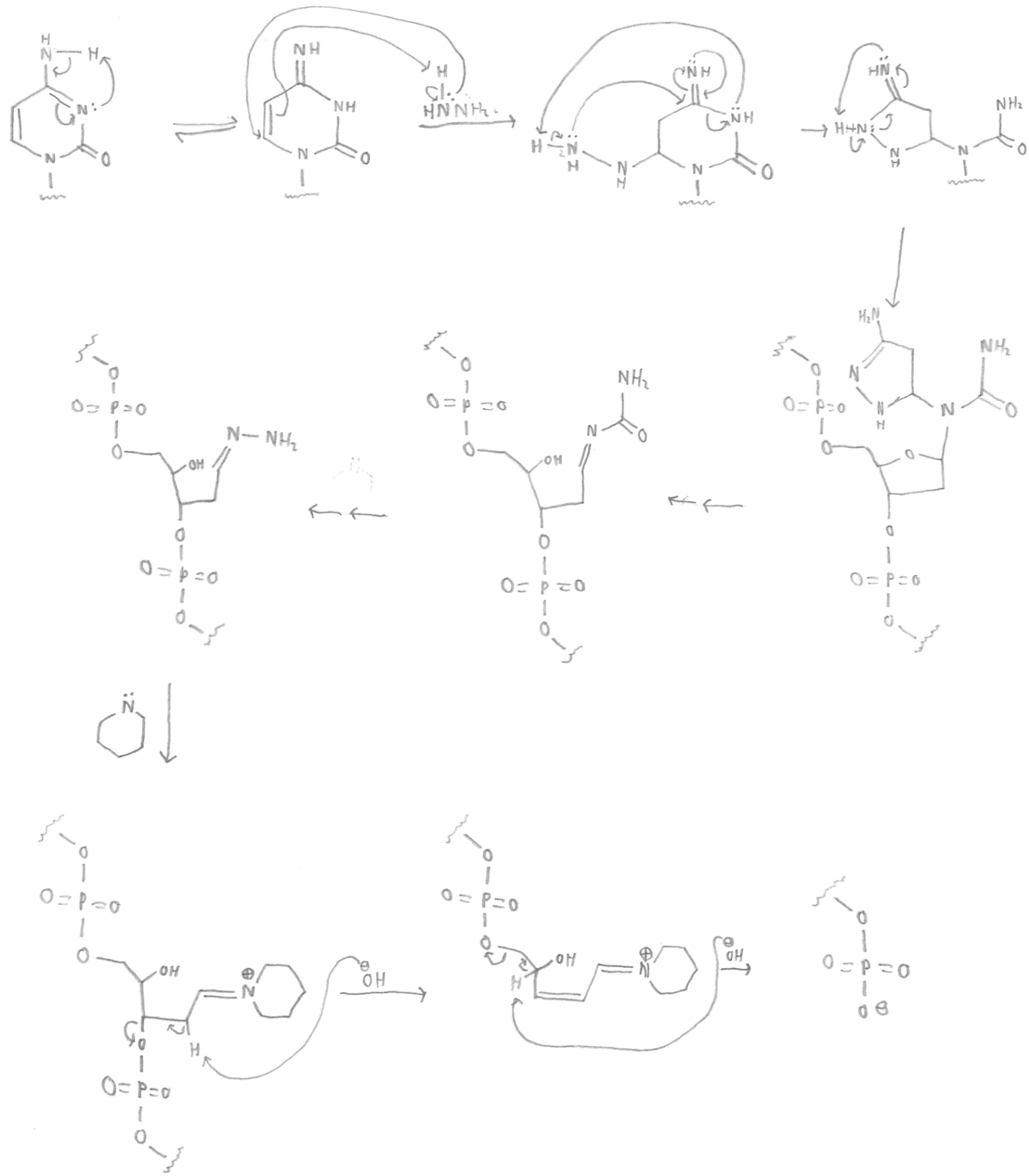
Figure 4: Net reactions.



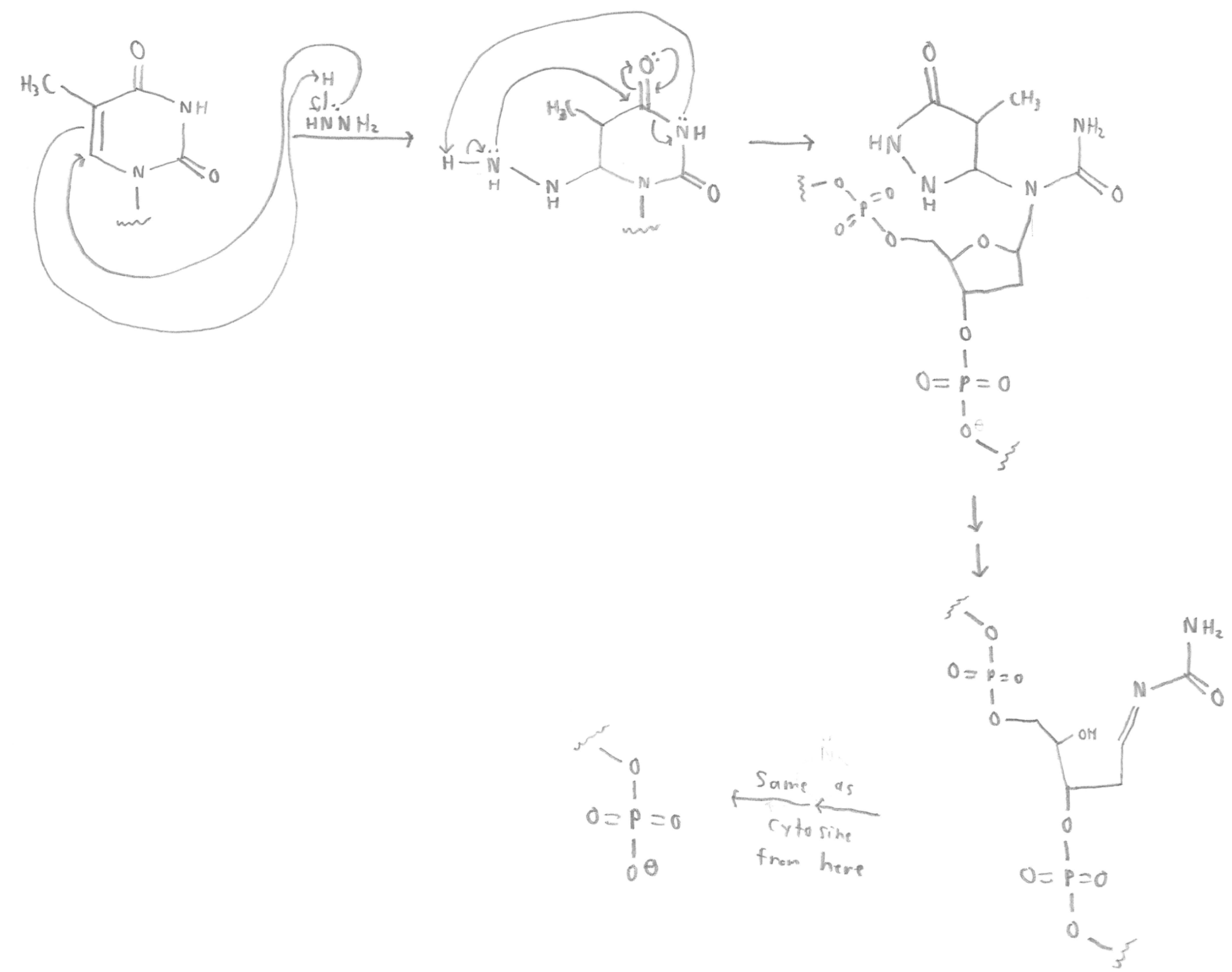
(a) G.



(b) A.



(c) C.



(d) T.

Figure 5: Mechanisms.

□

3. Sequencing primer.

   Assuming the sequence of the human genome is completely random (it is not!), what is the minimum length of DNA primer you would need for it to bind only to a single site in the human genome? The number alone is invalid, unless accompanied by a calculation. (4 pts)

   Answer. The human genome contains 3×10^9 base pairs.

   A completely *random* genome means that every sequence has an equal probability of occurring. Thus, for example, the 3 base pair sequence TGA has a $1/4^3$ chance of occurring (4 possible base pairs in each slot). Therefore, TGA will be present $3 \times 10^9 \cdot 4^{-3}$ times in the theoretical random human genome.

   We want to find the smallest n such that a sequence of length n occurs at most once. Thus, mathematically, we want to find the smallest $n \in \mathbb{N}$ such that

$$3 \times 10^9 \cdot \frac{1}{4^n} \leq 1 \quad \Longleftrightarrow \quad \log_4(3 \times 10^9) \approx 15.74 \leq n$$

   Therefore,

$$\boxed{n = 16}$$

□

4. Plasma membrane and cell structure.

   a) When a lipid bilayer snaps or breaks, why can it not repair itself by forming a hemi-micelle cap as shown in the figure below? (4 pts)

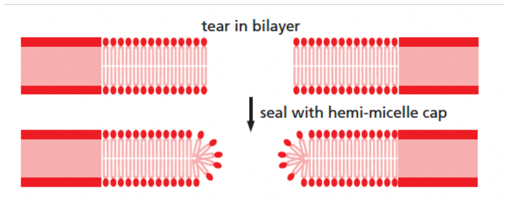


Figure 6: Hypothetical hemi-micelle membrane repair.

Answer. Membrane puckering is induced by large head groups (e.g., glycolipids). Thus, to see such extreme bending, we would need a plethora of very large head groups in the very small area of the hemi-micelle. Energetically, this would be extremely unfavorable, and such groups would likely move away from each other very rapidly if ever brought so close together, returning the tear to its original form. Indeed, it will be far more energetically favorable for the two hydrophobic regions to just reunite into one continuous region. □

   b) You are studying the binding of proteins to the cytoplasmic face of tumor associated macrophages for which you need a pure population of "inside-out vesicles" made from the plasma membrane. You have devised a method that gives a good yield of inside-out vesicles from the plasma membrane, but your preps are still contaminated with varying amounts of right-side-out vesicles. A senior grad student in the lab suggests that you pass your vesicles over an affinity column made of lectin coupled to solid beads. What is the rationale underlying her suggestion? (3 pts)

Answer. If the population of vesicles is made from the plasma membrane of tumor associated macrophages, then any vesicles with right-side-out proteins will likely have sugars that can bind to lectin among them. Thus, when we pass the vesicles through an affinity column made of lectin coupled to solid beads, the lectin beads bind all right-side-out vesicles. All inside-out vesicles will pass through and not get stuck. Thus, such an affinity column is a valid method of purification. □

5. Protein export and import.

   Describe a strategy to position a fluorescent protein, say GFP, anchored to the inner mitochondrial membrane, but projecting into the intra-lumenal space. What are the steps that lie between the mRNA of this protein being translated, up until the protein is positioned as above? (7 pts)

Answer. Herein, I will describe the strategy that makes use of the mitochondrial OXA complex.

We assume that GFP already contains the correct primary and secondary localization/signal sequences (likely a translocation sequence on the C-terminus). If it does not, we should be able to add it either pre-translationally (by modifying the GFP-encoding mRNA) or post-translationally (a nuclear localization sequence can be fused with GFP per Lecture 6.1, so a mitochondrial localization sequence should be able to be attached, too). We also assume that the protein has made its way into the cytosol and can navigate (or be directed) to the mitochondrial outer membrane.

Upon arrival at the mitochondria, the primary GFP localization sequence attaches to the receptor protein of the TOM complex and begins to be unwound and pulled in toward the intra-lumenal space. Since we are supposing an OXA pathway is used, the TOM and TIM complexes lock together, allowing translocation to happen all at once from the cytosol to the mitochondrial matrix. Successive ATP physphorylations of the complexes pull the protein through a few peptides at a time. Once the translocation sequence enters the matrix, a signal peptidase cleaves it off, exposing the secondary localization sequence. Once GFP is completely inside the matrix, TOM and TIM separate.

Now inside the matrix, the secondary signal sequence sends GFP to the OXA complex. The OXA complex flips the protein so that the tag is in the inner membrane and the bulk of the protein is in the intra-lumenal space. □