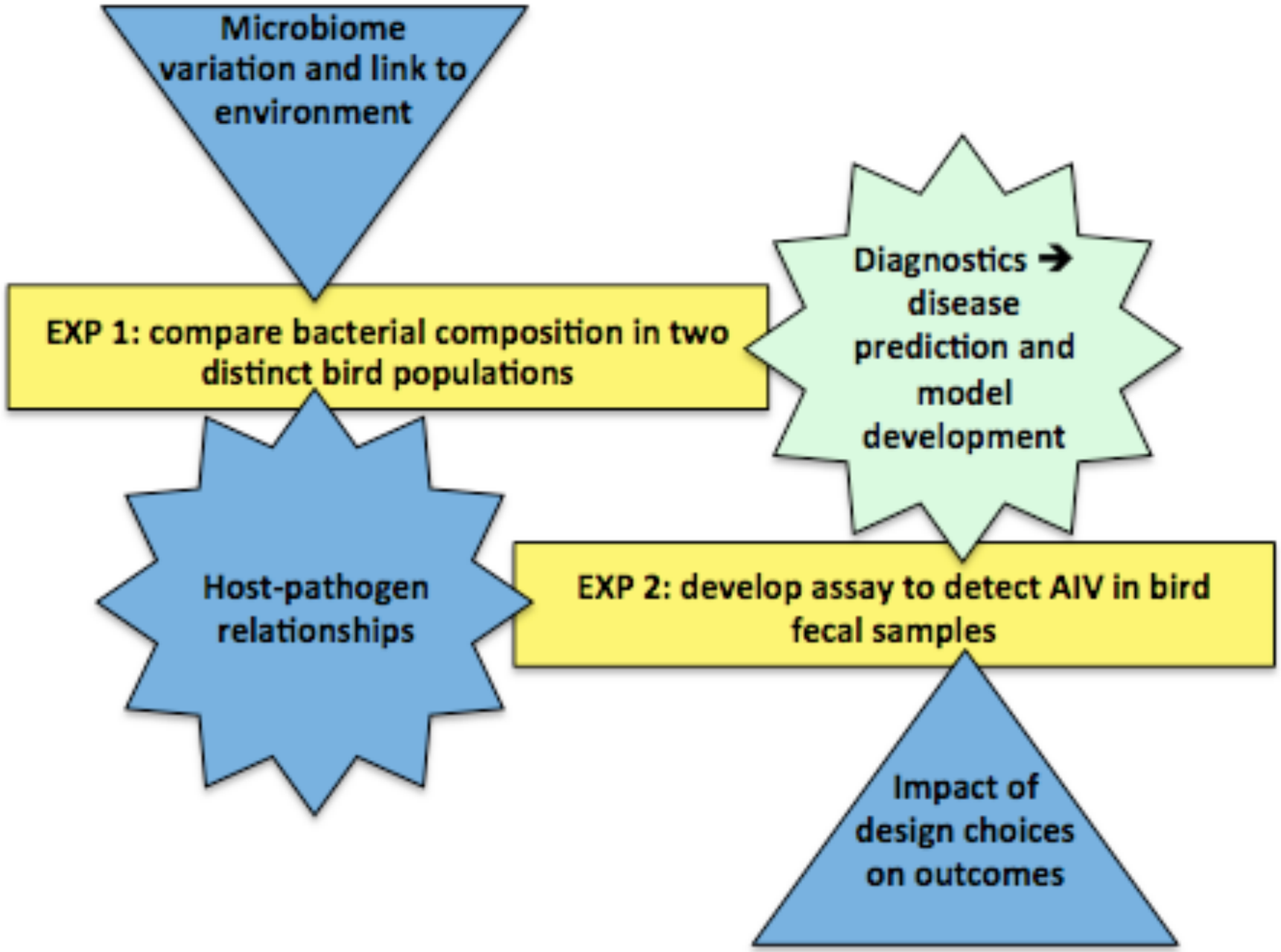
M1D2: Diagnostic Primer Design

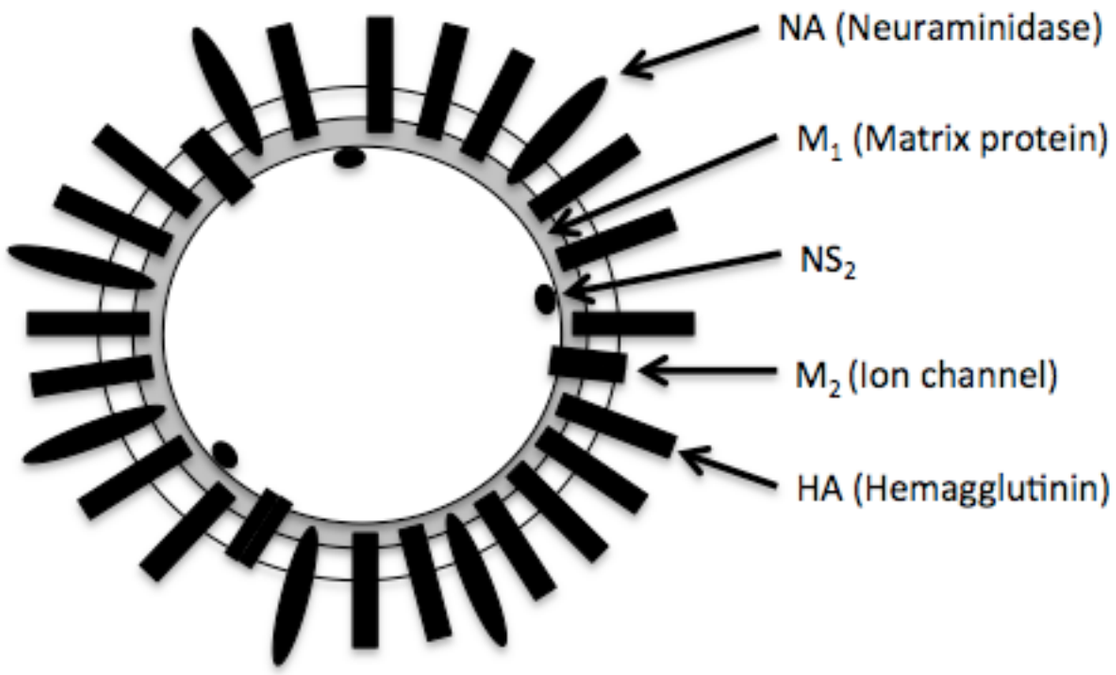
2/10/15

Announcements

1. *Expanded office hours for this week:*
    - Wednesday, 3-5pm in 16-319
    - Friday, 3-5pm in 16-319
    - Sunday, 3-5pm in 16-319
2. *Weekly office hours (starting week of Feb. 16th):*
    - Monday, 4-5pm in 16-319
    - Friday, 3-4pm in 16-319
    - If we fill up my office I'll put up a sign where to find us (likely places include the 3rd floor lunch room and 56-302)
3. *Don't miss the homework at the bottom of the M1D2:*
    - Q1 and Q2 are due on **M1D4** (versus M1D3)
    - Q3 will happen next time (paper + slide preparation)
    - Q4 is something to start thinking about
4. ***Before you move on please read the M1D2 Introduction for some more background on the AIV matrix gene***

Module 1 Conceptual Overview





Structure of AIV

Goal: Improve the detection limit of avian influenza virus (AIV) detection in gulls.

Detection technique?

Quantitative Polymerase Chain Reaction
(aka qPCR, aka real time PCR)

How?

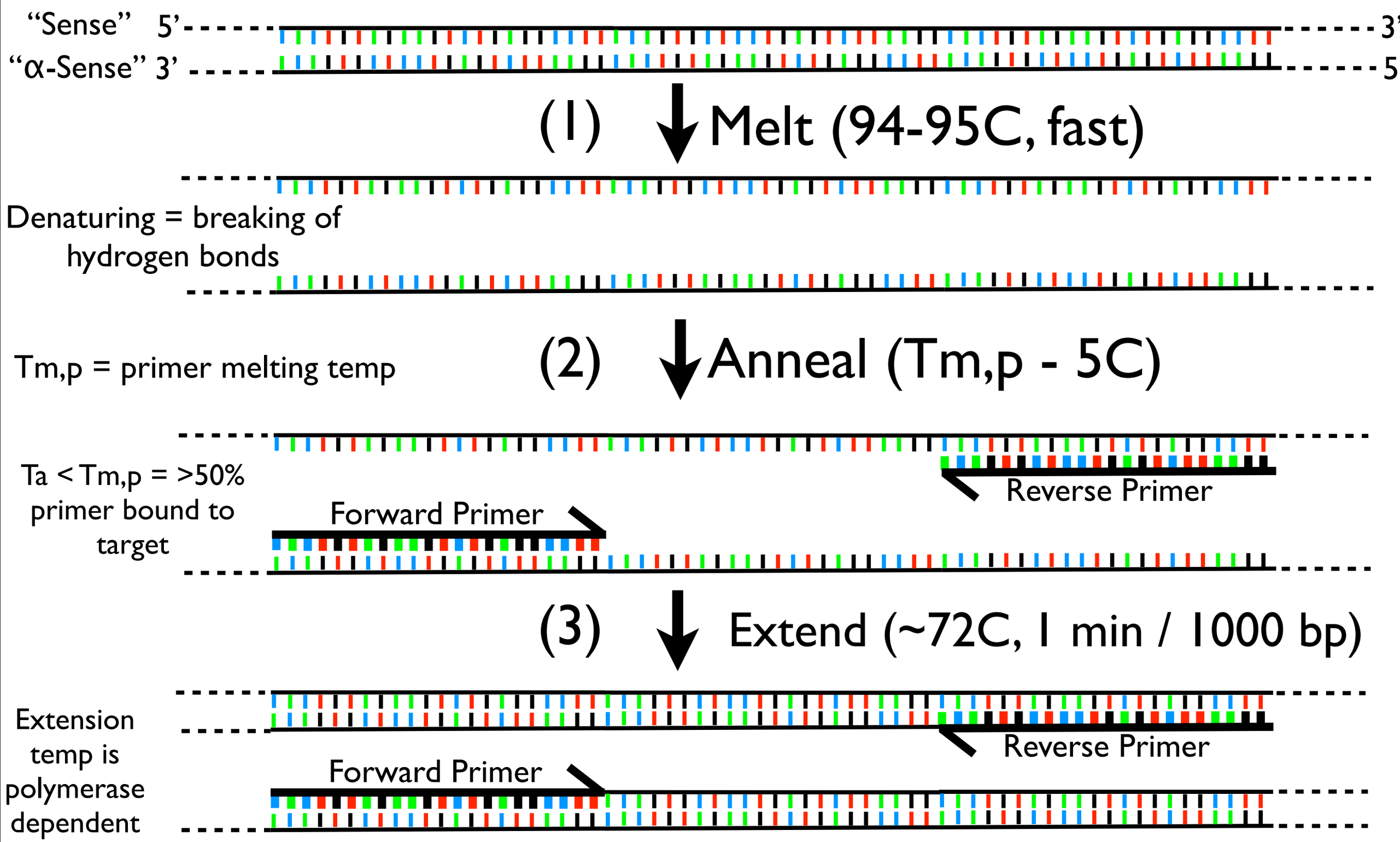
Re-engineer the primers used for

Important design considerations:

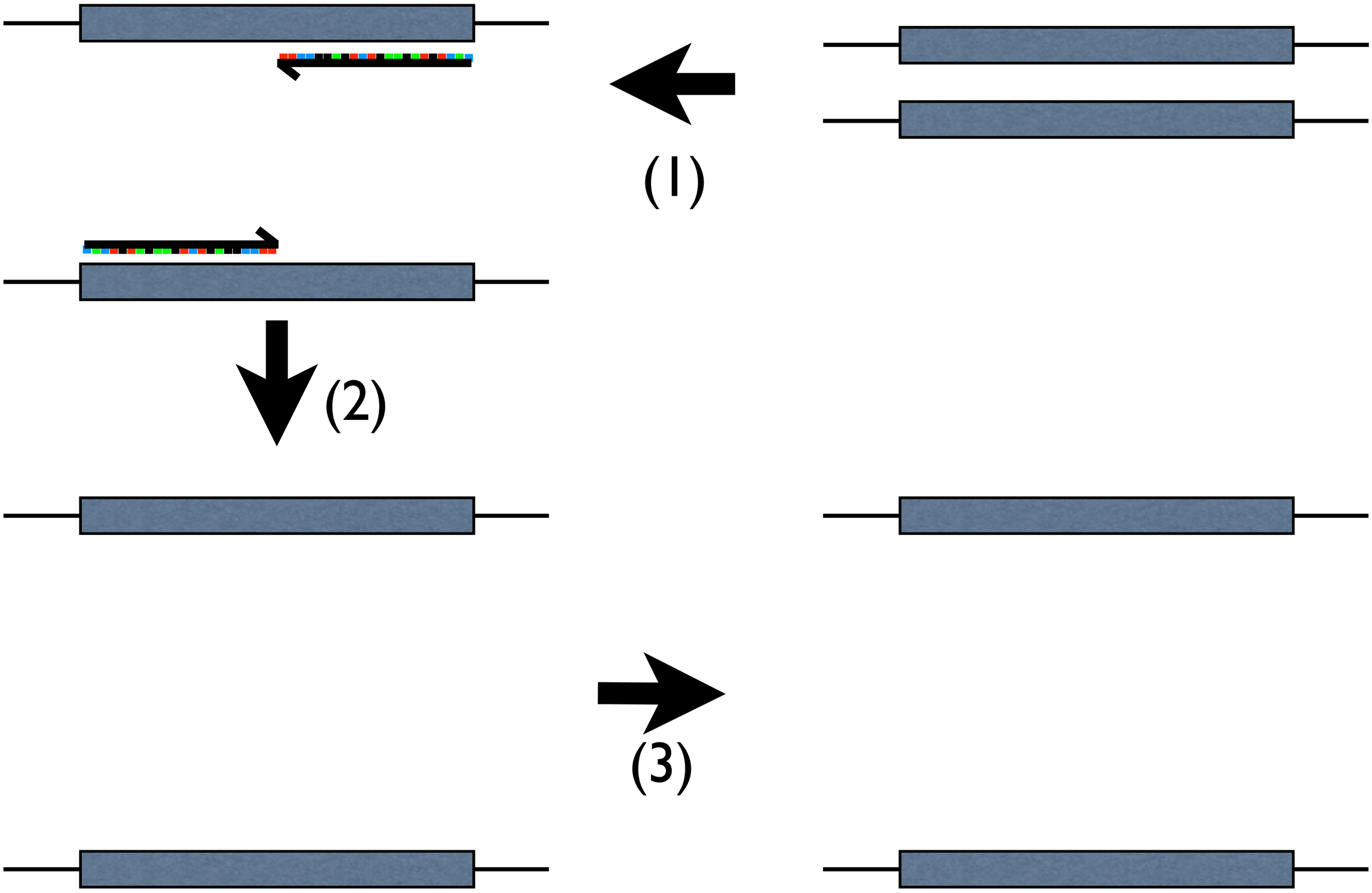
(1) This qPCR assay is the *first* step of detection — you want to capture all instance of flu virus so your primers should target a highly conserved gene. Would the HA or NA genes be good choices?

(2) The goal is to maximize *sensitivity* — some false positives could be acceptable

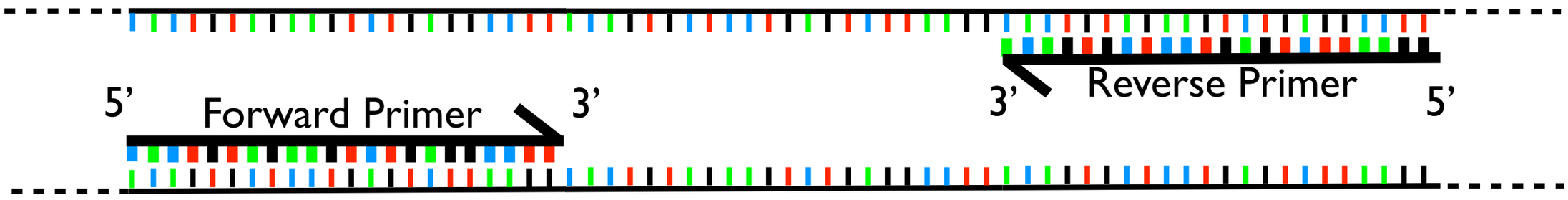
(1) How does Polymerase Chain Reaction (PCR) work?



First three rounds of PCR: no final product is formed until cycle #3
Try to draw out the reaction — we will review on Thursday



(2) Design primers to increase sensitivity of AIV detection: Primer Design

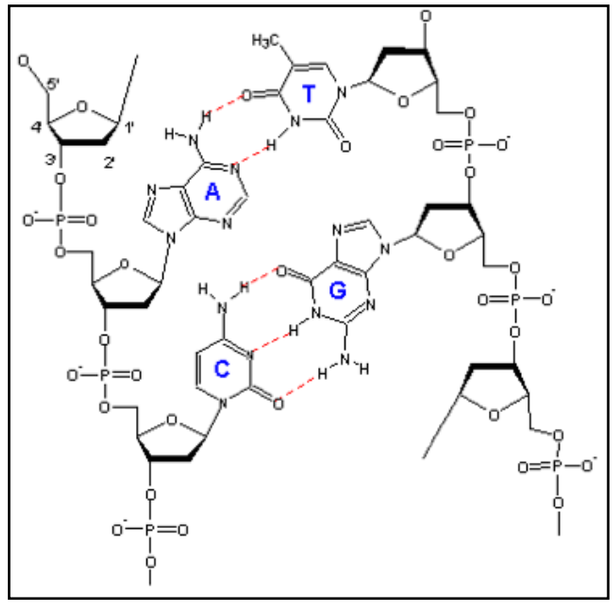


1. The <u>forward primer binds to the α-sense strand</u> (or the 'Template') and 'reads' in an intuitive direction from 5' to 3'. Look at the reverse primer and consider it's orientation.
2. Primer <u>length is important</u> to decrease the chances of off-target binding:
   - Consider that the human genome is ~ 3x10^9 bp. If we designed primers that were only 10 bp long, we might expect to find that 10bp sequence once in every 4^10 ≈ 10^6 bp -- a very risky gamble for off target binding.
   - The optimal primer length is > 17 bp for specificity. Think about why.

3. Primer <u>melting temperature should optimal</u>ly be kept between 55-60 C.
   - Tm,p is the temp ~50% of the primer is double vs. single stranded.
   - The melting temperature will be higher with increased G/C content.

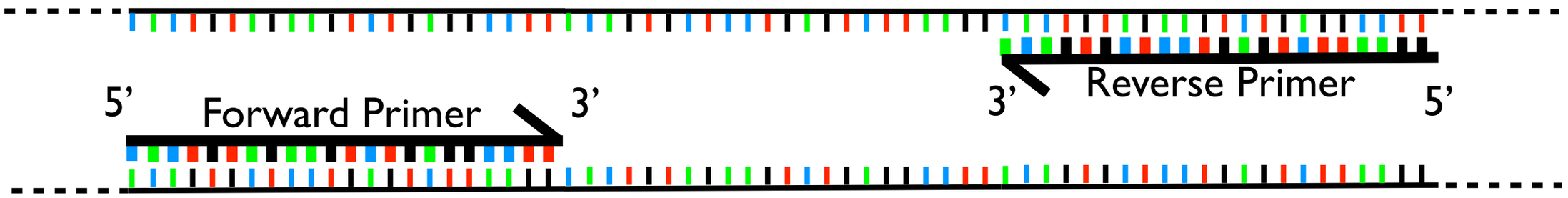
   Why? Look at the diagram of bp hydrogen bonding to the right -- which pair requires more energy to denature? *Also explains why optimal primer design calls for only 40-50% of the bp to be G/C.*

   The Tm,p is kept between 55-60C so that the annealing (hybridization) step is optimally efficient. ****You will target 58C****



Thermodynamics of DNA Duplex, New Mexico State University

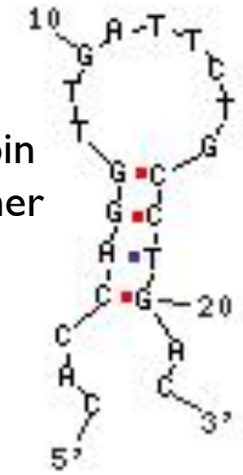
(2) Design primers to increase sensitivity of AIV detection: Primer Design



4. Avoid long repeats of one type of bp (ex. ATATATA) or one bp individually -- especially TTTT -- remember the polyA tail on pre-mRNA? This can lead to non-specific priming.

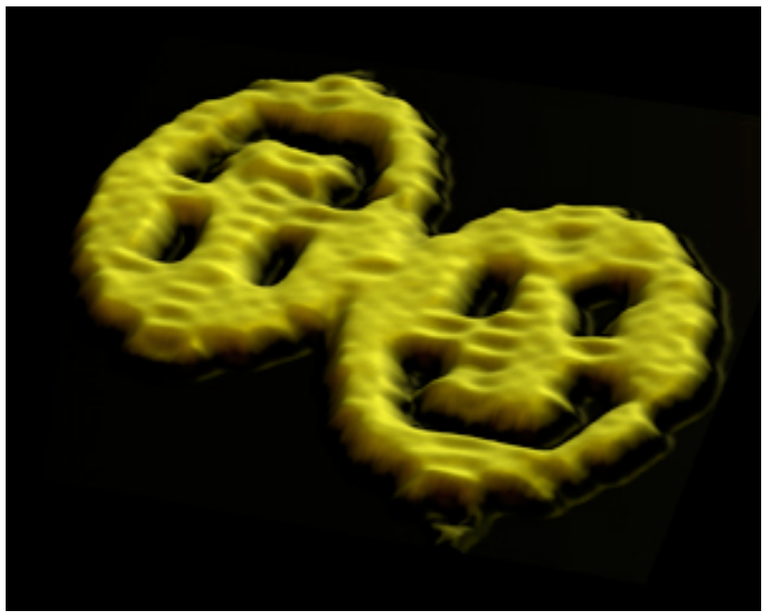
5. Consider secondary structure of your primer.
   - Does the primer have an internal sequence that can bind itself?

If so, you can end up with a hairpin structure that will prefer (energetically speaking) to bind to itself and not your target sequence.



Making a hairpin with your primer is bad.

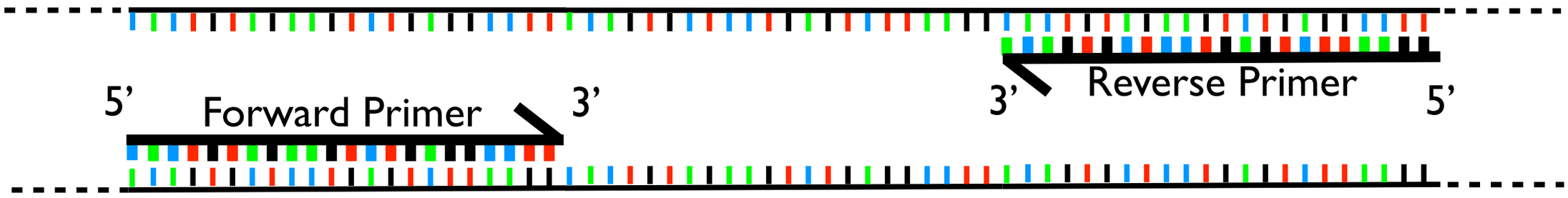
In fact, this behavior of DNA has been harvested to make higher order structures: DNA Origami is an active area of research.



DNA Origami is cool.

Image from: http://www.dna.caltech.edu/~pwkr/

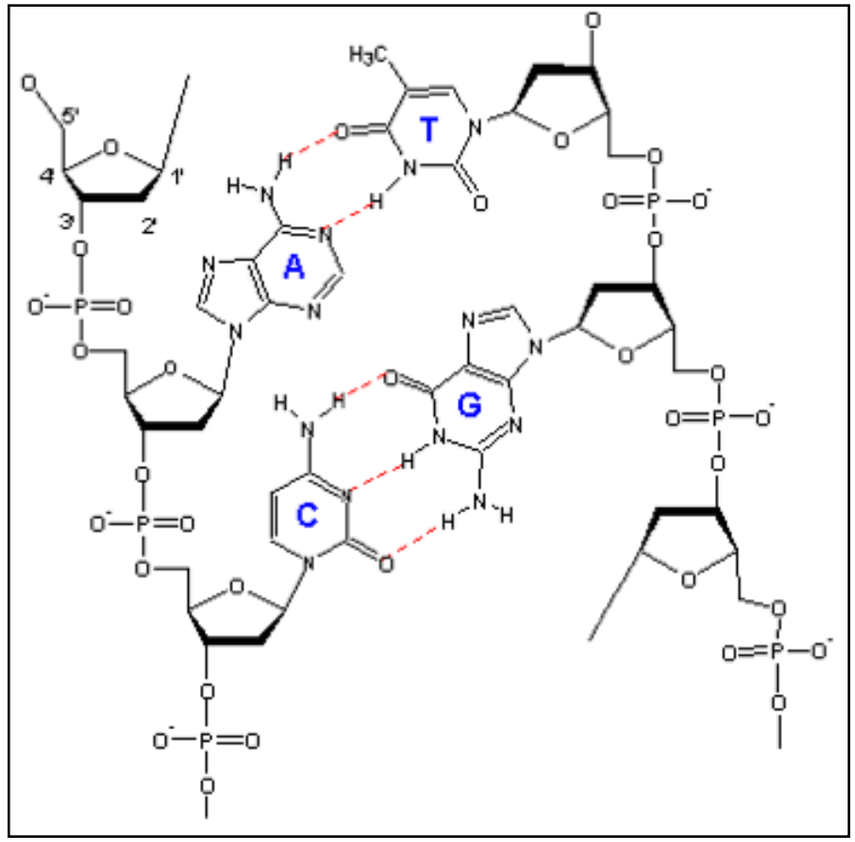
(2) Design primers to increase sensitivity of AIV detection: Primer Design



6. Tip the deck in your favor: Add a GC clamp to the 3’ end if possible.
   - Consider again the image below. G/C binding is more stable and can help to increase efficiency of polymerase binding at the 3’ end to promote extension.
   - But don’t go overboard! > 5 G/C pairs won’t help you.

I have also found this website useful: http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html

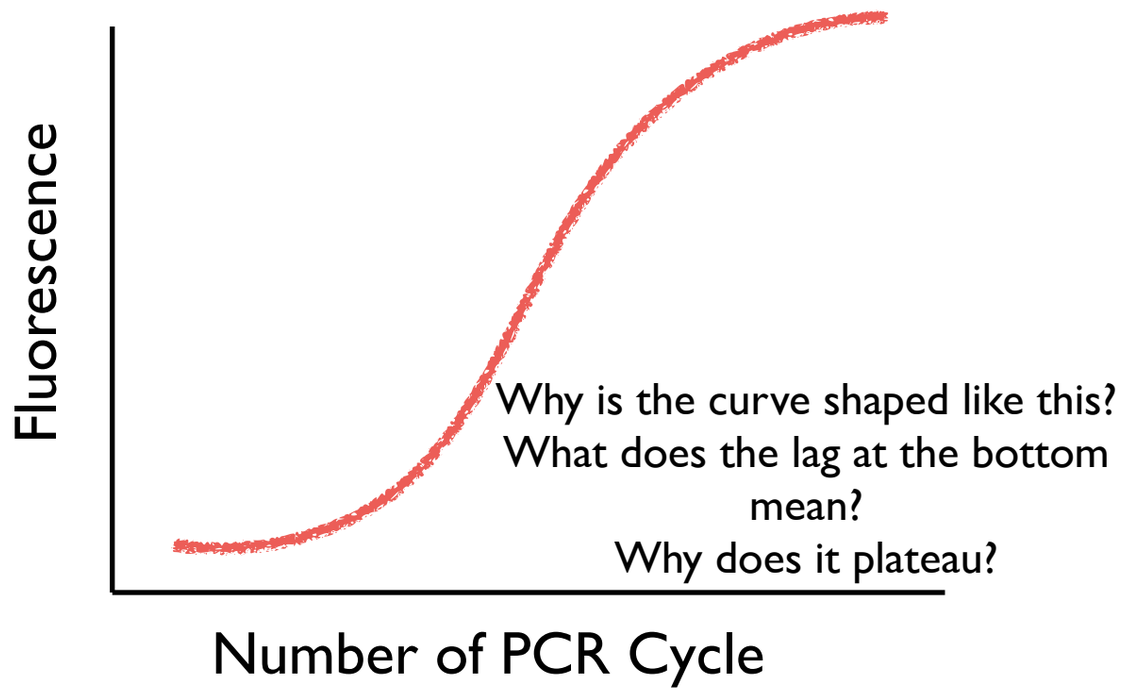
7. There are some qPCR-specific design considerations:
   - Amplicon (your product) length should be between 100 and 200 bp long.
   - Design amplicon around an exon-exon boundary to decrease contamination from genomic DNA (we don’t have to worry about this — we’ll discuss later!).



Thermodynamics of DNA Duplex, New Mexico State University

(2) Design primers to increase sensitivity of AIV detection: What we'll do next

You design —> We order —> You prep primers and set-up qPCR (D5) —>We perform qPCR in BioMicro Center —>You analyze the data (D8)



We will spend more time discussing the mechanism behind qPCR, but for now keep the following in mind:

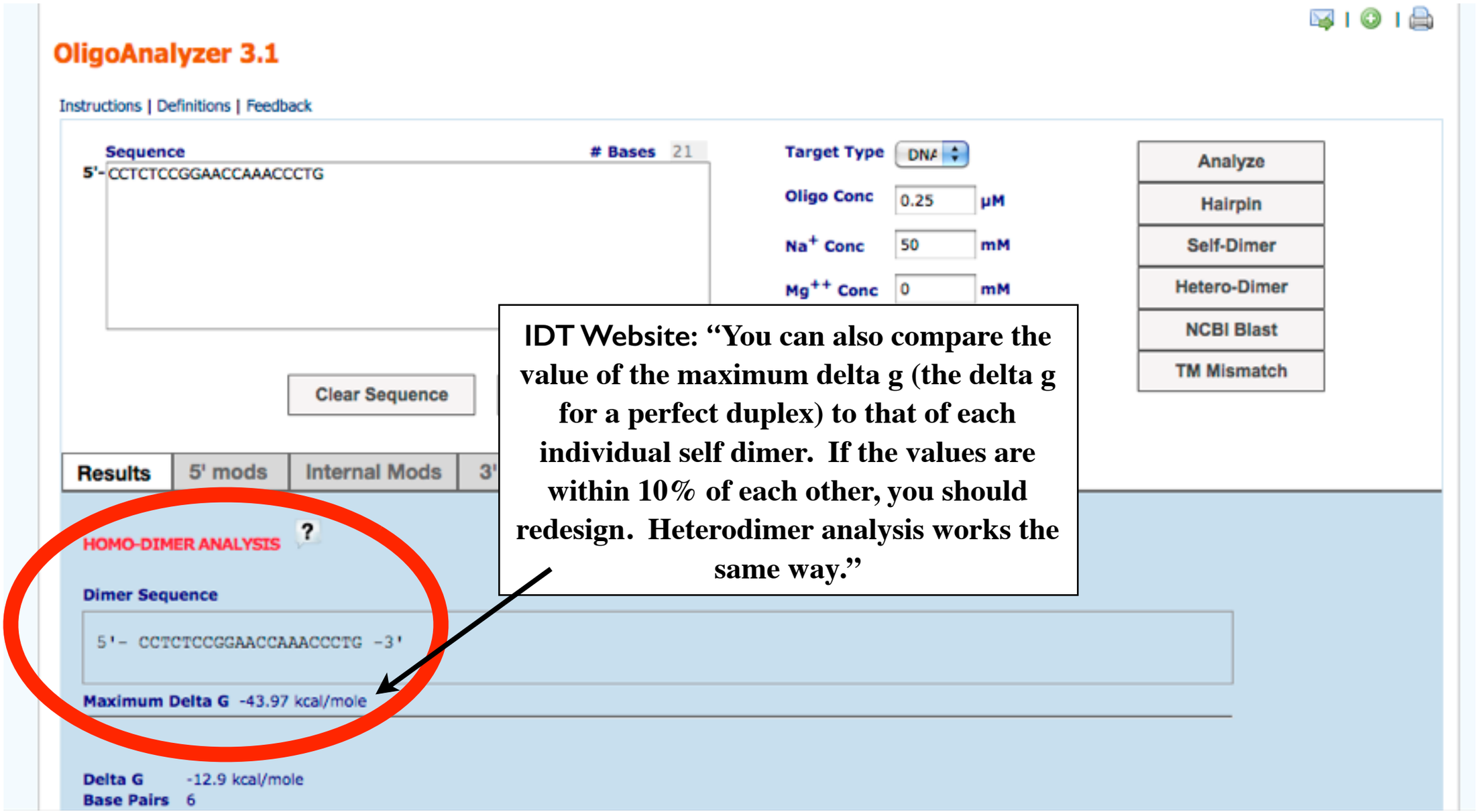
(1) A dye (Sybr Green) is used to detect double stranded DNA product (the product of your PCR reaction!).

(2) There isn't enough Sybr Green in solution to detect, but when the dye is localized within double stranded DNA the signal is brighter — and can be detected.

(3) Therefore, the amount of fluorescent signal is proportional to the amount of PCR product that is formed.

(4) Fluorescence is 'read' once per PCR cycle to quantify the amount of product formed

An explanation that might help along the way:



Lab Quizzes — Lab quiz next time!

- Purpose: Continuity and accountability

  1. 10 points
  2. 10 min
  3. Start at 1:05pm

- First quiz covers M1D1 lecture and M1D1 & M1D2 lab content

- See wiki for information on quiz schedule

Today in Lab

- Explore existing AIV matrix gene primers
- Design new primers
  - Post your primer designs to the M1D2 Talk page
- Make sure to start keeping your notebook today!
  - You should add your design criteria and results to your lab notebook
- Primer design information will be used to prepare a *Memo* as part of the written assignment for Mod1 (5% of total grade)

Next time on M1D3: Koenig et al PNAS paper discussion + slide preparation/presentation practice + WRAP visit

We will review a recent paper about the development of the human intestinal microbiome. Each team will be assigned a figure in the paper (see M1D3 wiki for assignment) — please prepare 1-2 slides that best present the important information from that figure (you may need to include some background information!).