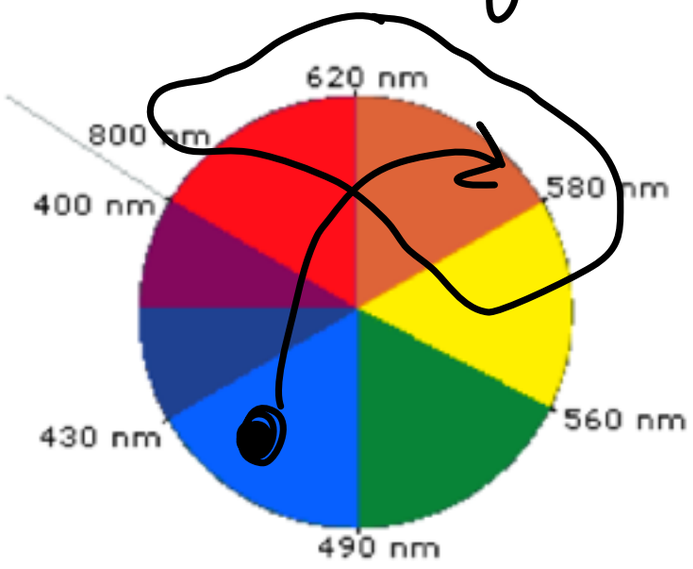
M1D2: Diagnostic Primer Design

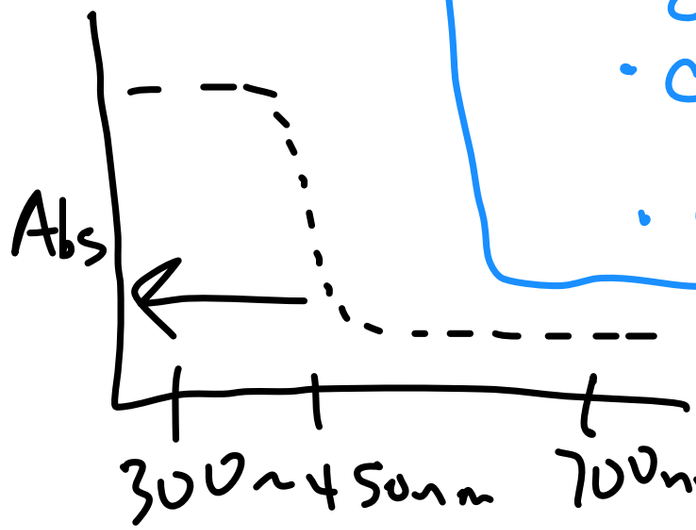
2/11/14

Announcements

ROY
GBV



orange/red/yellow



Why coacd samples?
- more info about
- degradation bird
- contamination
- concentration

$V_T = 100mL$

2% SDS

0.5 M NaOH

want. 0.2% SDS / 200mM NaOH

$C_1V_1 = C_2V_2$

$(2\%)(X) = (0.2\%)(100mL)$

$X = 10mL$ SDS

$C_3V_3 = C_2V_2$

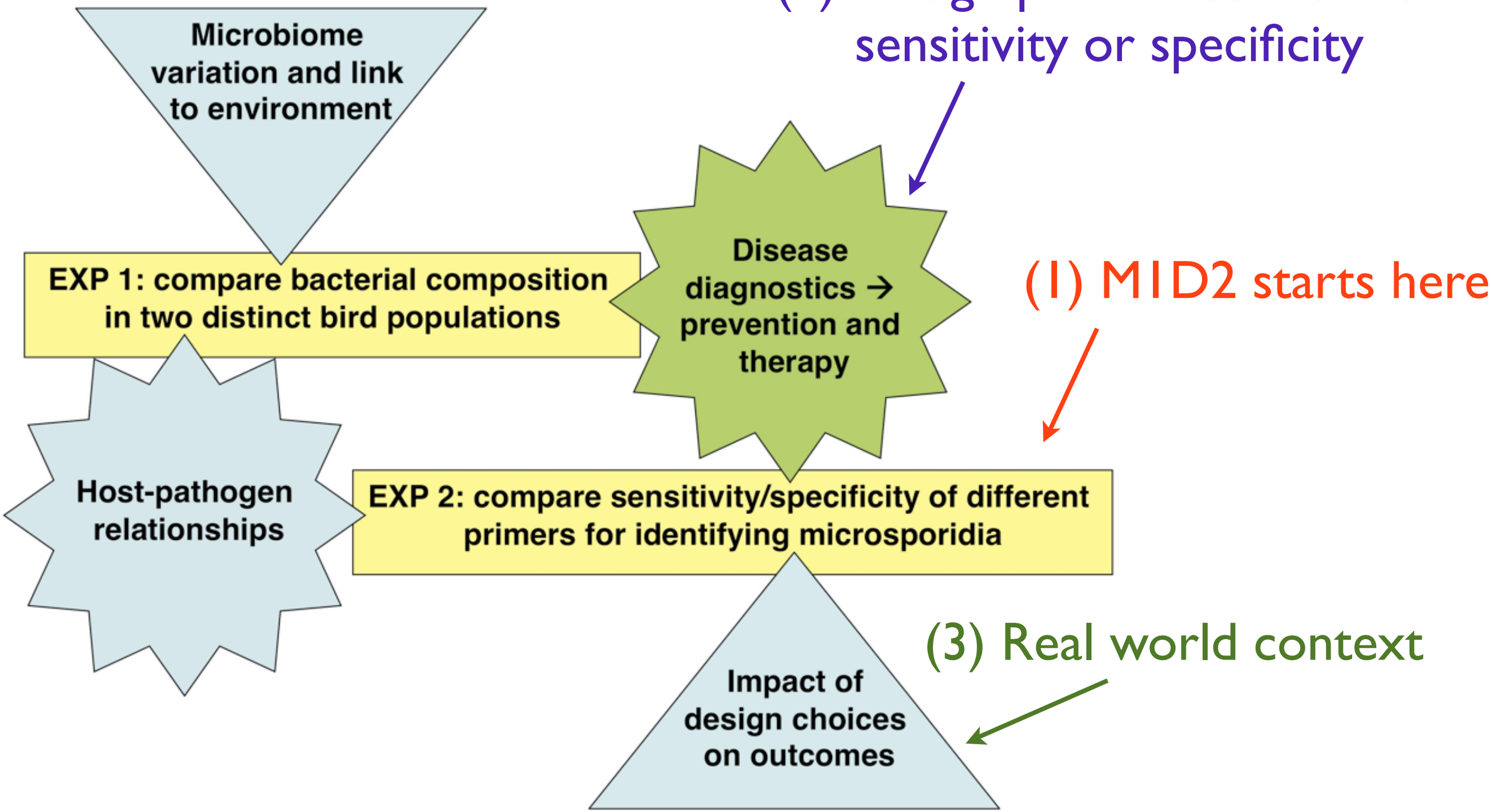
$(0.5M)(Y) = (0.2M)(100mL)$

$y = 40mL$ NaOH

50 mL H_2O

$(100 - 40 - 10)$

Module 1 Overview & Outline for lab today



(1) MID2 Starts Here

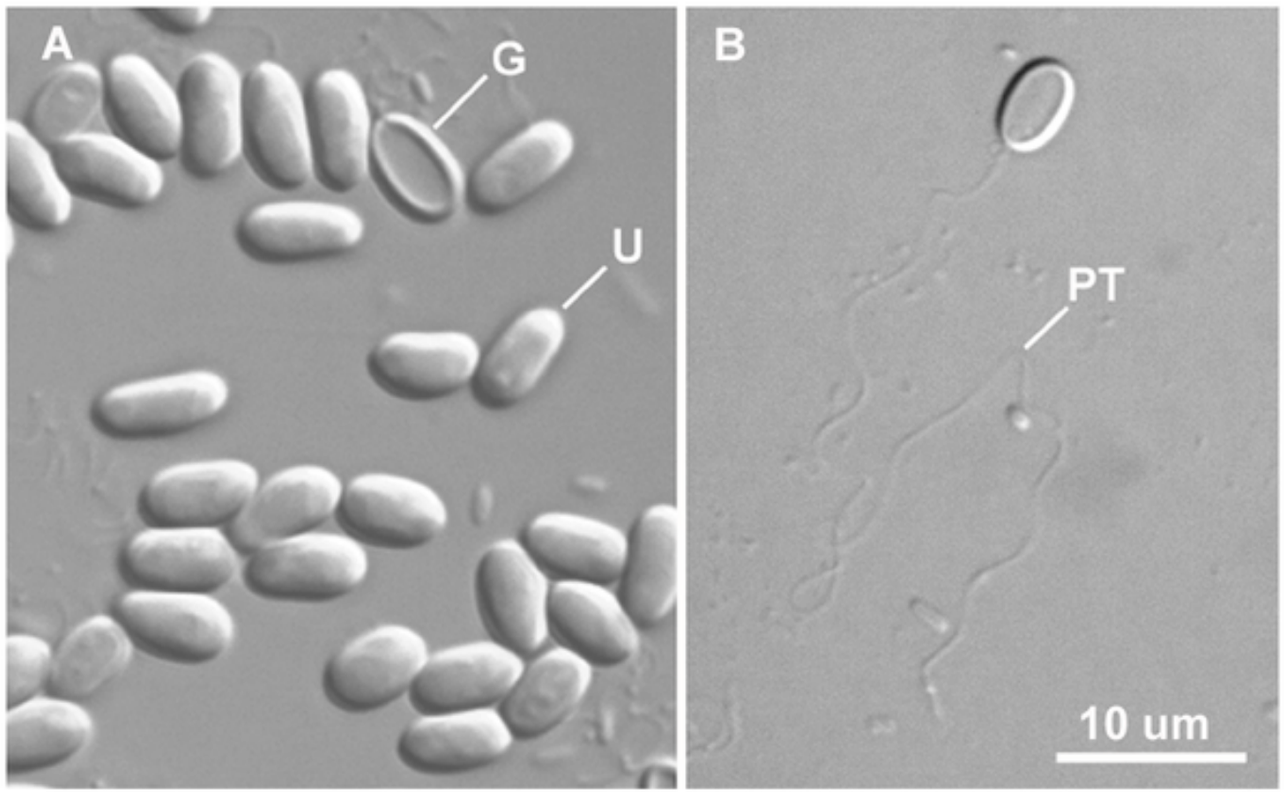
1957

No mitoch. | No nuc!

<3 Mbp

~2000 proteins

Microsporidia



G = germinated spore
U = ungerminated spore
PT = polar tube

Image from: http://www.plospathogens.org/article/info%3Adoi%2F10.1371%2Fjournal.ppat.1000489

~2001 (genome seq.)

This is a free resource that has great information about microsporidia in a friendly format.

microsomes
Hsp70
* Fungus

We will work with DNA from two species of microsporidia:
Encephalitozoon hellem (or *E. hellem*)
Vittaforma corneae (or *V. corneae*)

(1) MID2 Starts Here

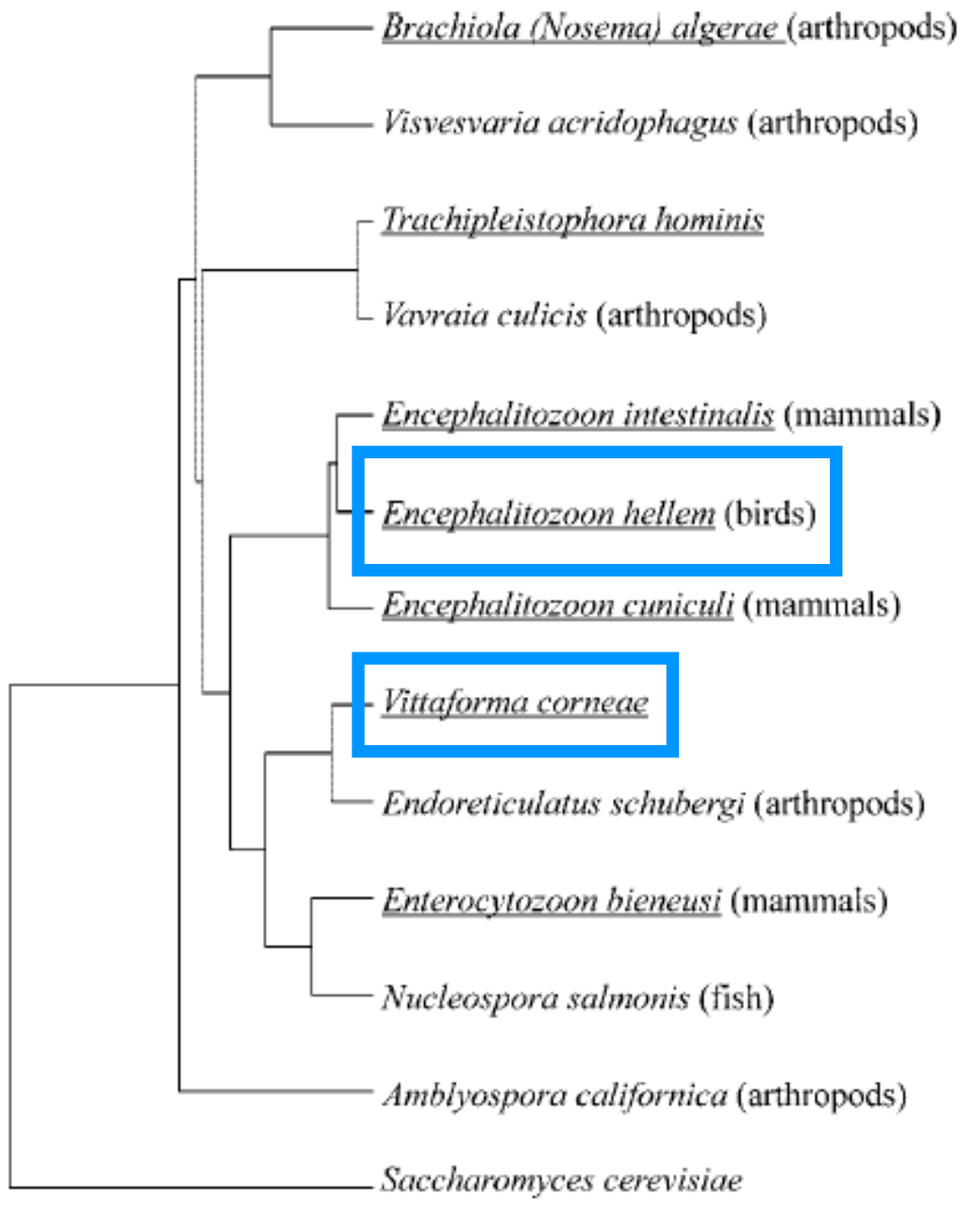


FIG. 2. Dendrogram generated from the small subunit ribosomal RNA (ssrRNA) gene of microsporidian species identified in humans (underlined) and selected other species (Kimura's distance, unweighted pair group method of analysis). Known animal hosts are indicated in brackets; the brewer's yeast *Saccharomyces cerevisiae* serves as an outgroup. No corresponding gene sequences are known for the human-infecting microsporidian species *Pleistophora ronneafiei*, *Trachipleistophora anthropophthera*, *Brachiola* (formerly *Nosema*) *connori*, *B. vesicularum*, *Nosema ocularum*, *Microsporidium ceylonensis*, and *M. africanum* (Table 1).

- μsporidia are most closely related to fungus.
- μsporidia are obligate intracellular -- meaning they require a host for survival.
- That requirement allowed: simplify their genome organisms
- What hosts?
  - See tree to the left -- common hosts for μsporidia species are in brackets.
  - Those identified in human infections are underlined.

Dendogram generated from the ssrRNA gene of microsporidian species

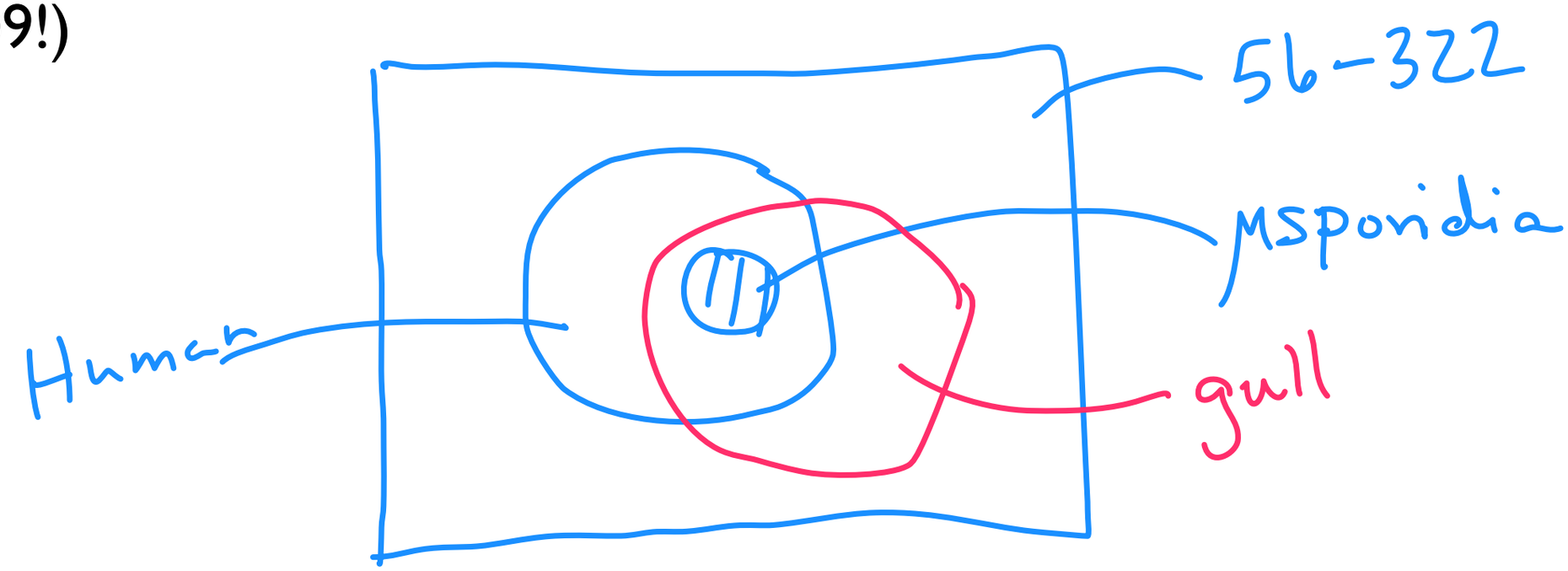
Figure from Mathis et al., *Clinical Microbiology Reviews*, July 2005

(3) Real World Context

- A couple more things about μsporidia:

1. Humans infected with μsporidia share a common trait -- weak immune systems
   - HIV or other immunodeficiency diseases (lupus, RA, etc)
   - Cancer patients undergoing treatment

2. μsporidia are tricky to isolate -- the isolation has been done for you and you will work with purified DNA (this makes the analysis doable for 109!)



(2) Design primers to increase sensitivity or specificity

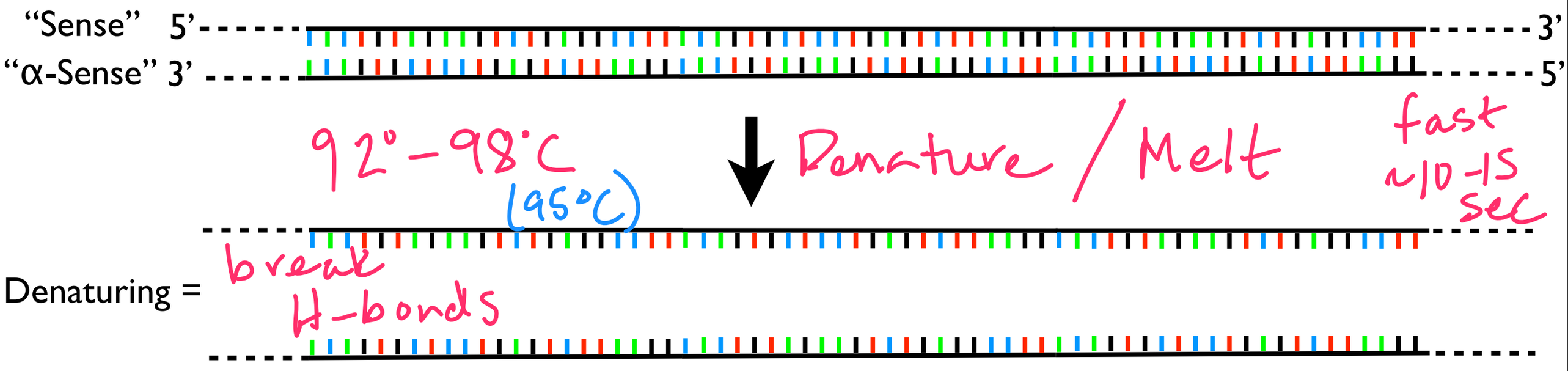
There are two design challenges for this part of Module 1

1. Selectivity
   - ✦ Design PCR primers that can differentiate *E. hellum* and *V. corneae*

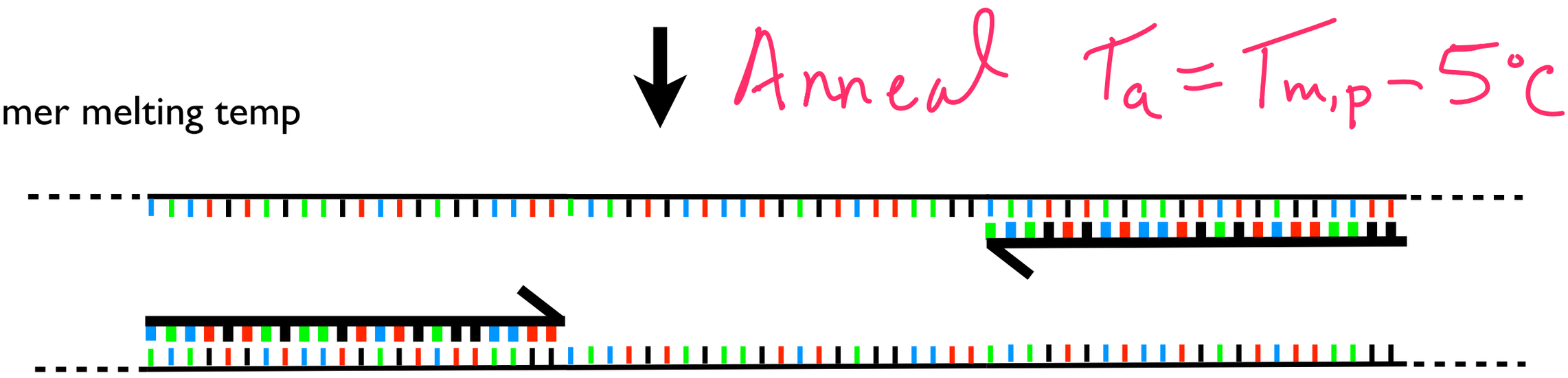
2. Sensitivity
   - ✦ Design PCR primers that can improve detection.
     * detect both species

Three teams per challenge. Sign up on the 'Talk' page of M1D2.

(2) Design primers to increase sensitivity or specificity

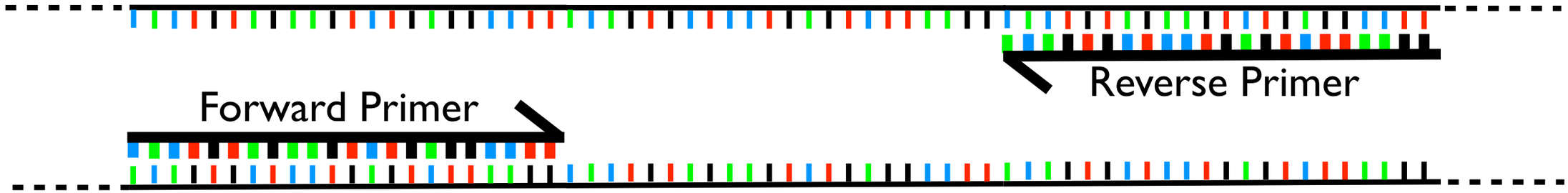


Tm,p = primer melting temp

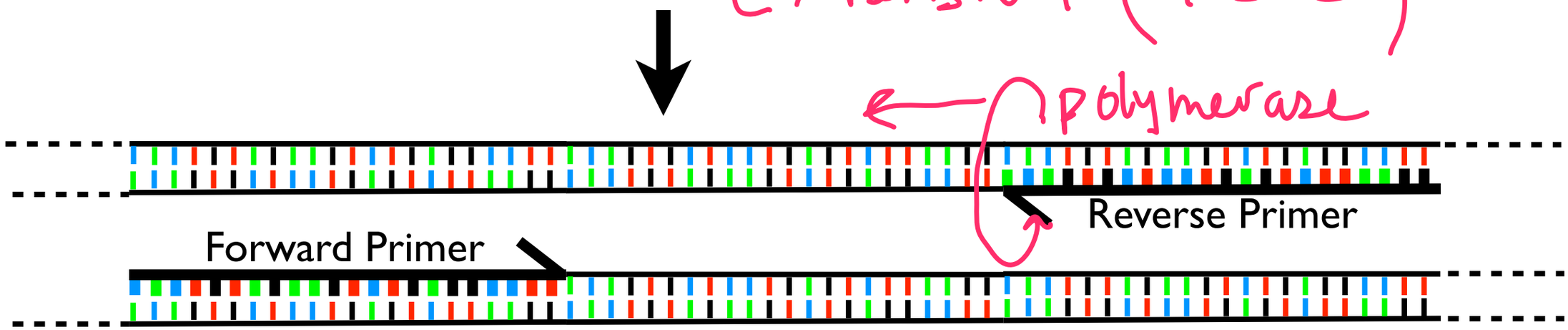


~ >50% primer sticks @ Ta

Ta < Tm,p
Why?



Extension (72°C)

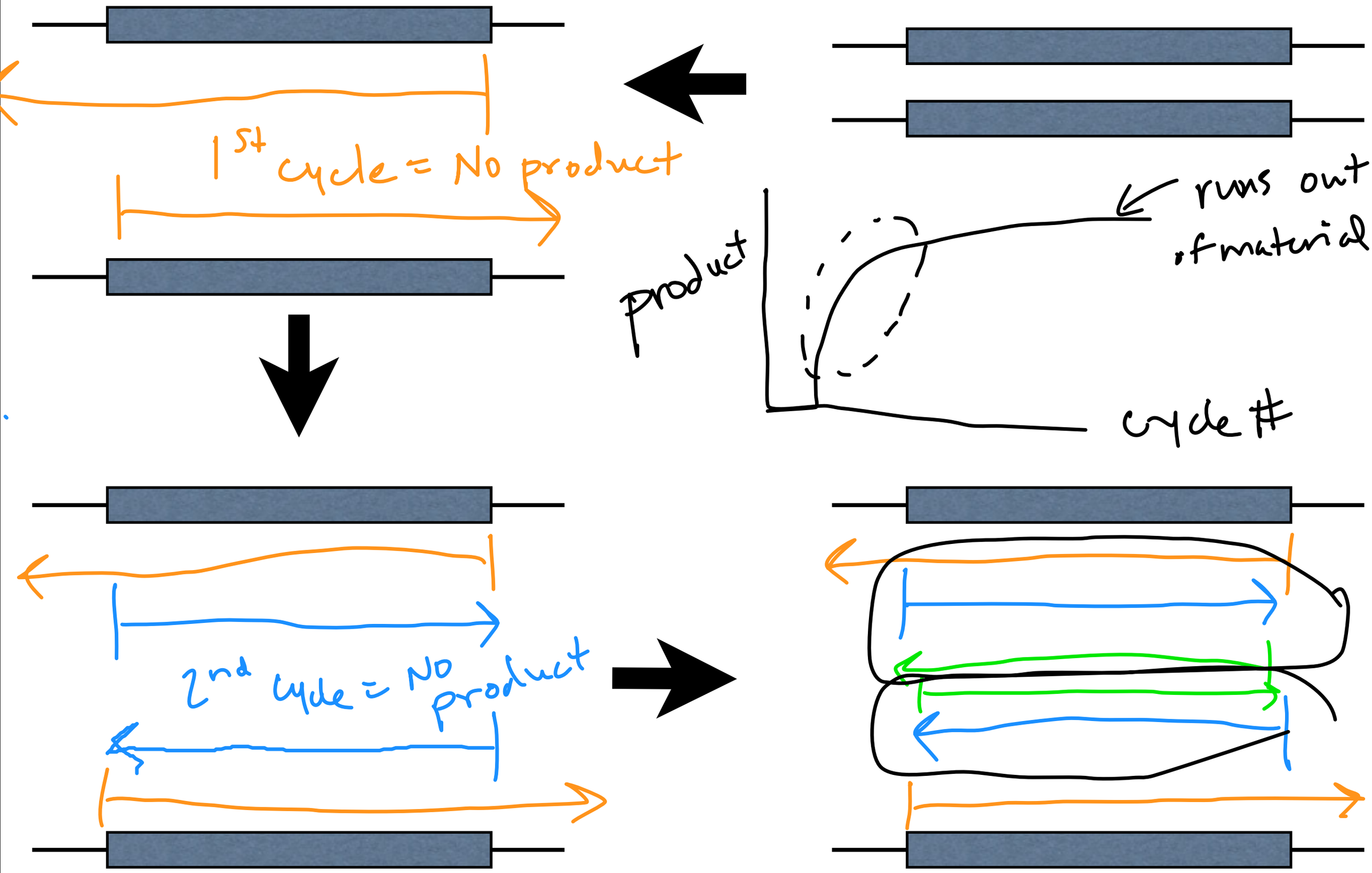


Extension
temp is:

1 min/1000bp

< 500bp

(2) Design primers to increase sensitivity or specificity: First three rounds of PCR



(2) Design primers to increase sensitivity or specificity: Primer Design Basics

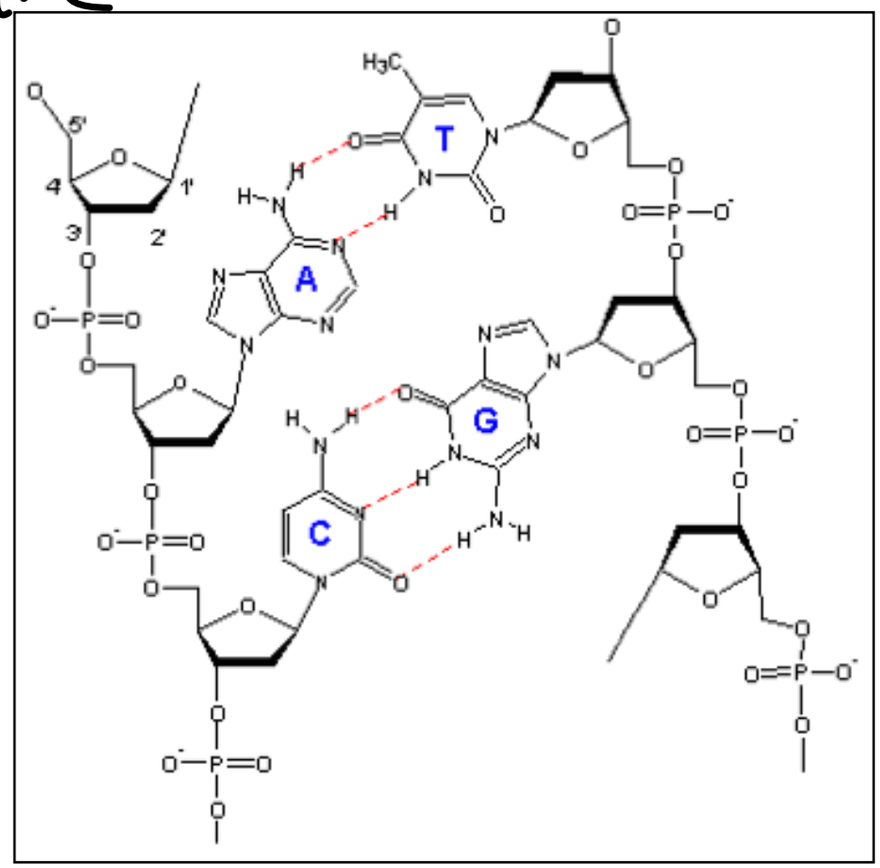
- Length (17-28 bp)

~ 3×10^9 bp

primer 10 bp

~ $4^{10} \sim 10^6$ bp

- Melting Temp — G/C content
  - thermodynamics
- TATATATA
  - secondary structure
  - low M_T
  - "off-target" priming
- CAGT ~~~ ACTG
  - hairpin
- G/C clamp 3'
  - → promote polymerization

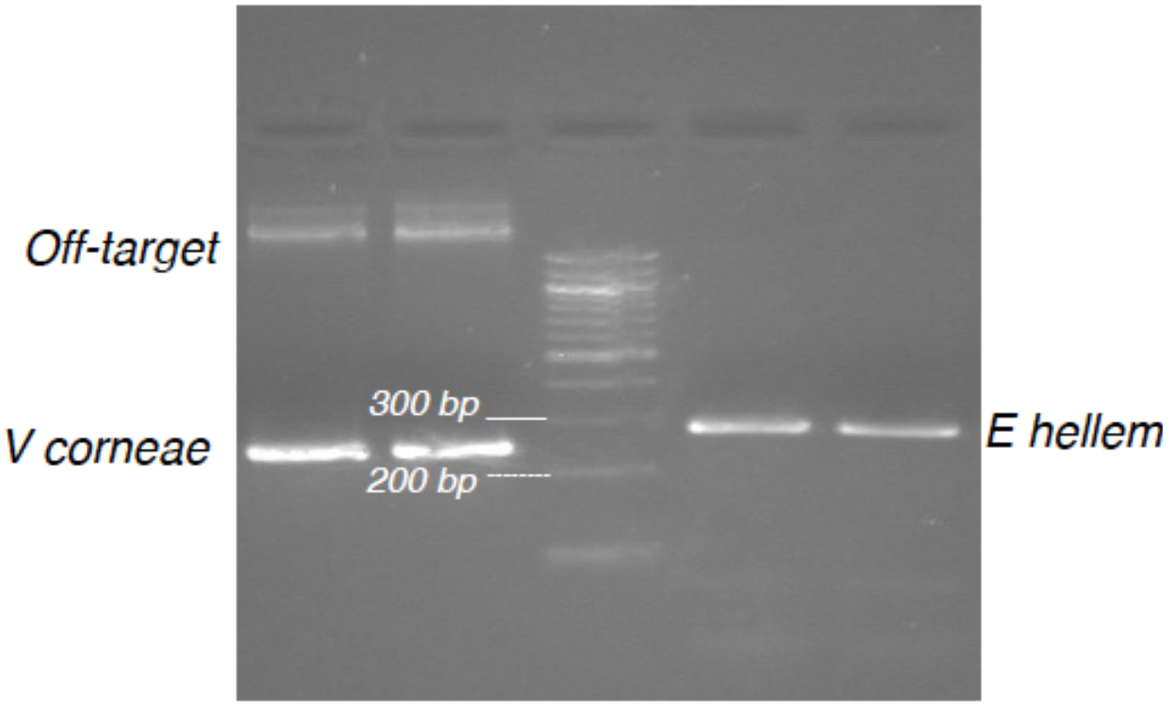


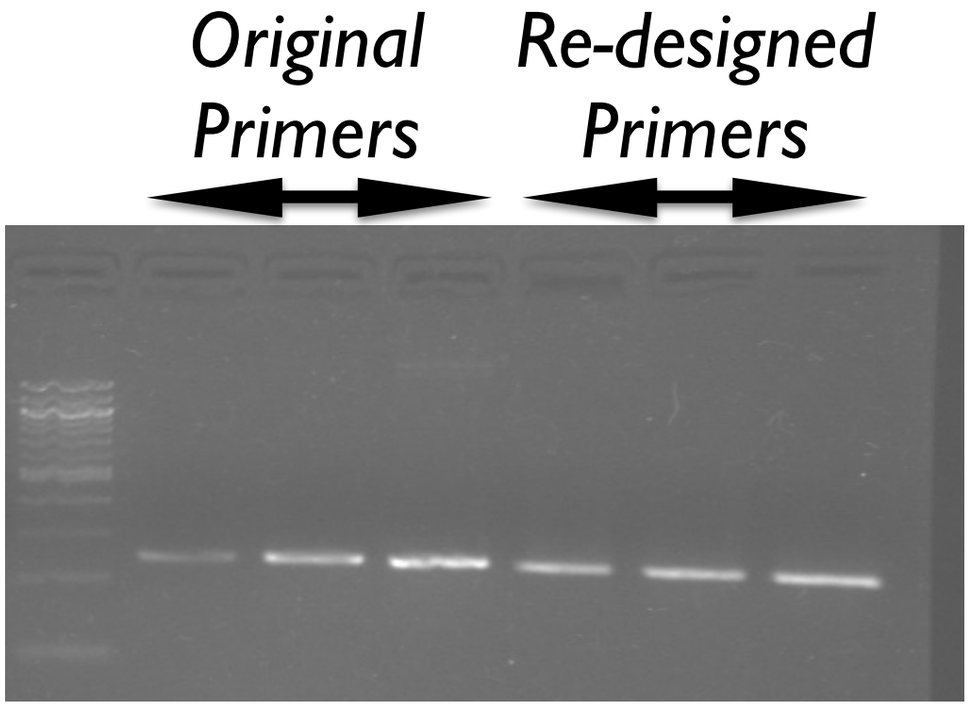
Thermodynamics of DNA Duplex, New Mexico State University

(2) Design primers to increase sensitivity or specificity: What we'll do next

You design —> We order —> You prep primers (D5) —> We run PCR —> You analyze via electrophoresis (D7)

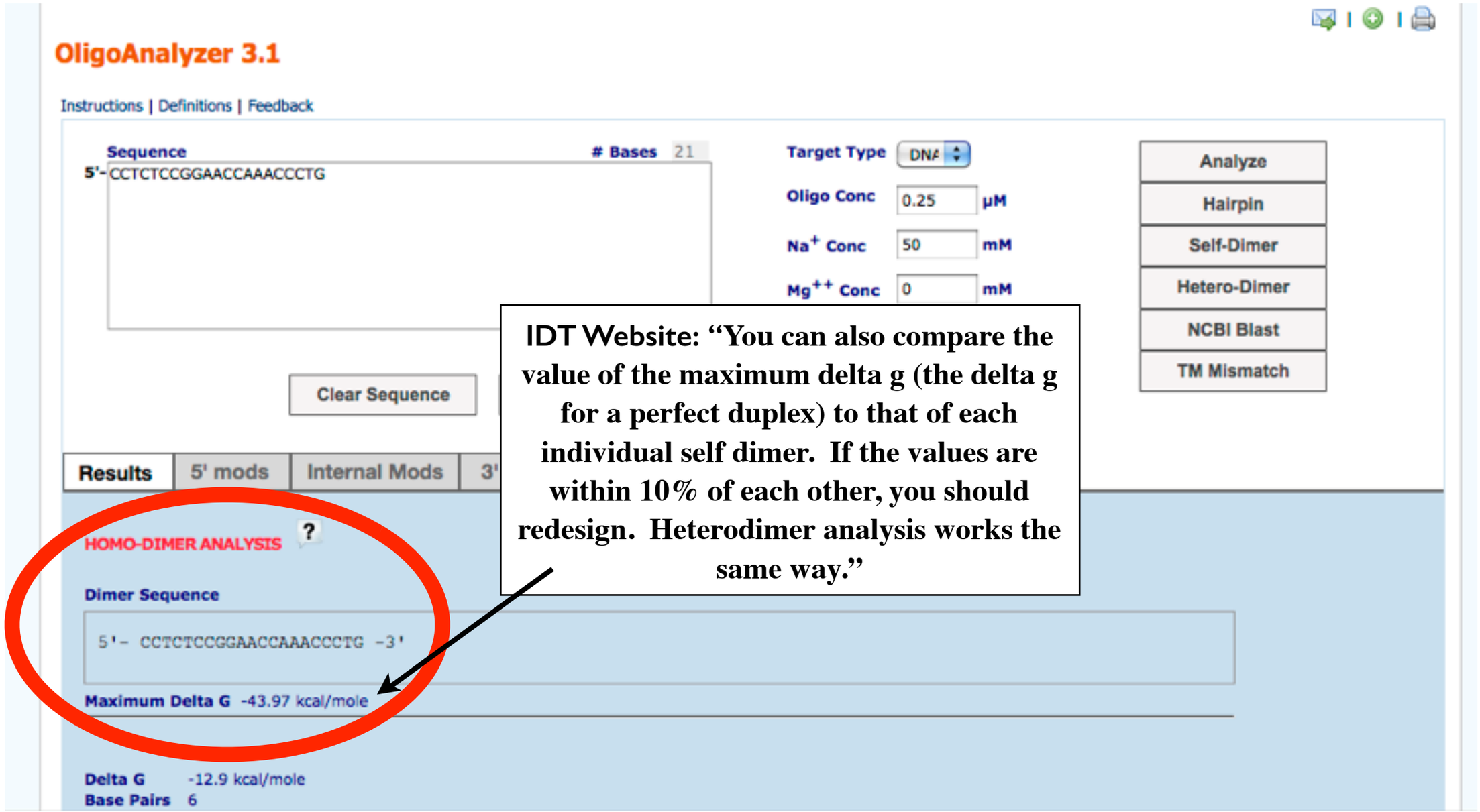
current primer set





*Note: Sensitivity challenge must detect both species

M1D2 Part 1 explanation



Lab Quizzes

- Purpose: Continuity and accountability

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

- First quiz covers M1D1 & 2 lecture and lab content

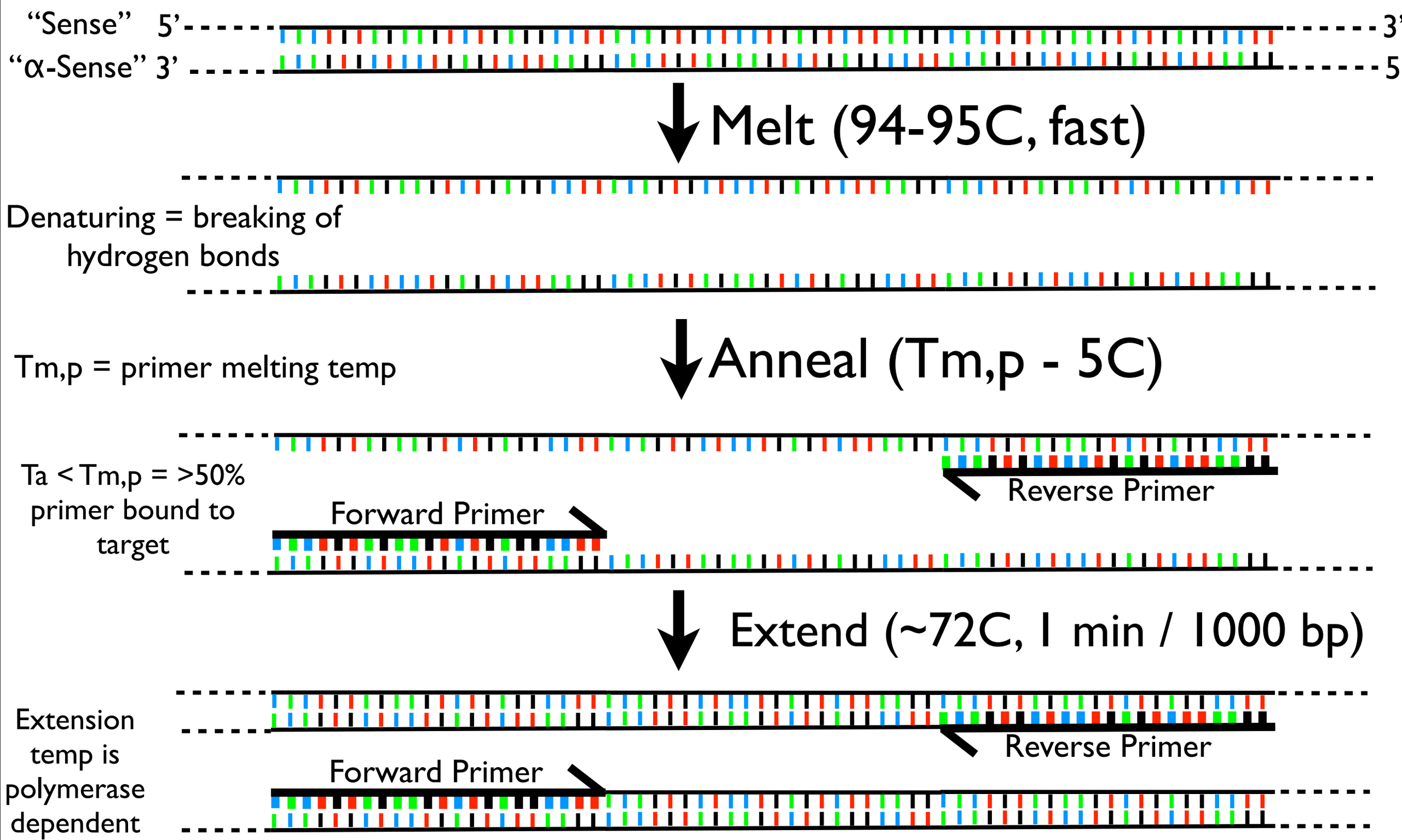
- See wiki for information on quiz schedule

Today in Lab

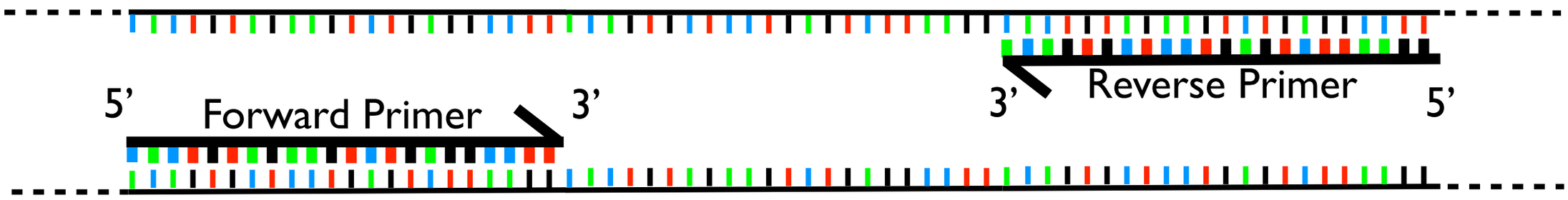
- ✦ Explore existing microsporidia primers
- ✦ Design new primers
  - ✦ Specificity or Sensitivity challenge — sign up on Talk page
- ✦ Start keeping your notebook today!
  - ✦ You should add your design criteria and results to your lab notebook
  - ✦ Primer design information will be used to prepare a *Memo* as part of the written assignment for Mod1 (5% of total grade)

For Next Time: Koenig et al PNAS paper discussion + slide preparation/presentation practice + WAC visit

(2) Design primers to increase sensitivity or specificity: A PCR Cycle



(2) Design primers to increase sensitivity or specificity: Primer Design



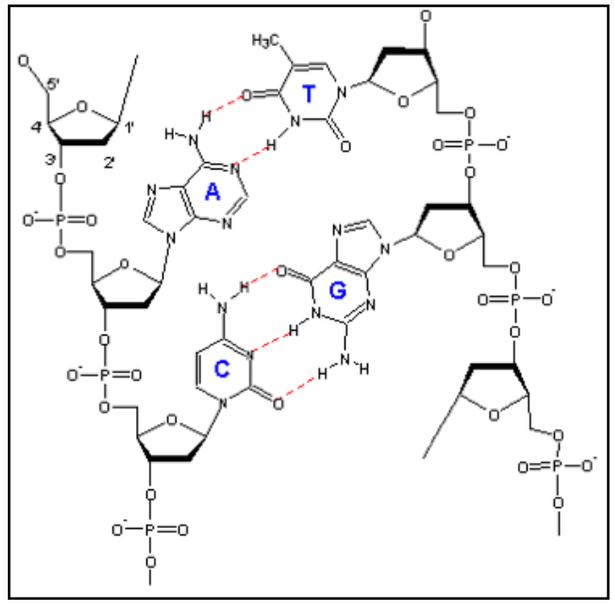
1. The <u>forward primer binds to the α-sense strand</u> (or the 'Template') and 'reads' in an intuitive direction from 5' to 3'. Look at the reverse primer and consider it's orientation.

3. 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 > 16 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.

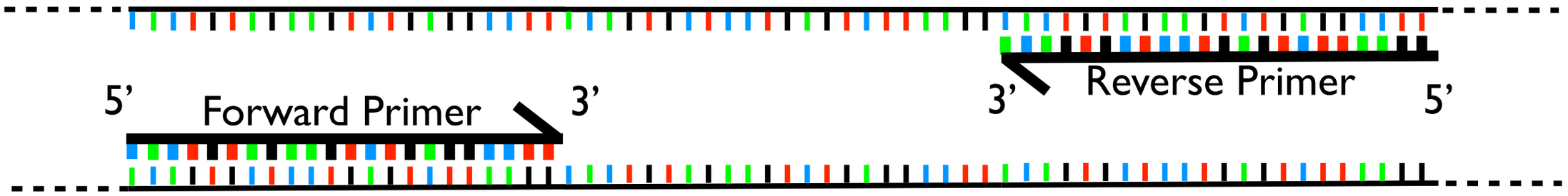
Why? Look at the diagram of bp hydrogen bonding to the right -- which pair requires more energy to denature? *Also explains why optimal primer design calls for only 40-50% of the bp to be G/C.*

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



Thermodynamics of DNA Duplex, New Mexico State University

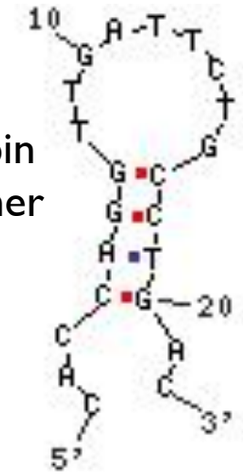
(2) Design primers to increase sensitivity or specificity: Primer Design



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.

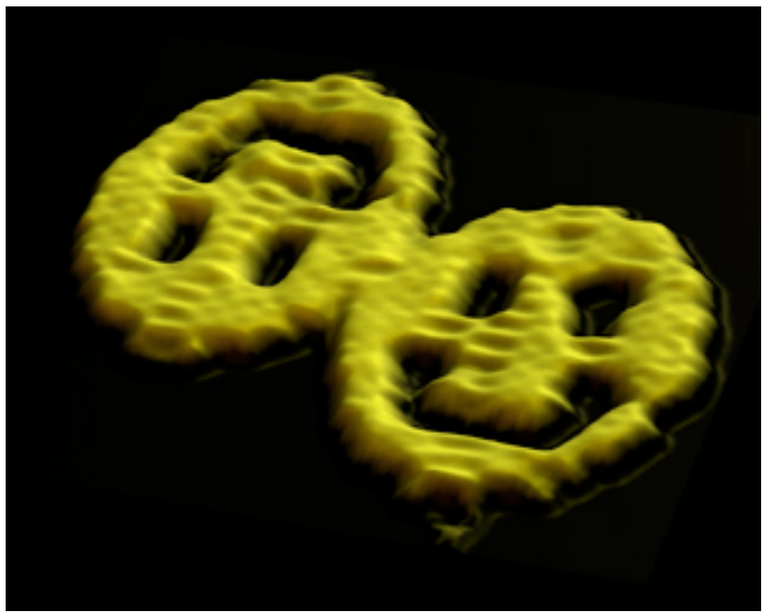
5. Consider secondary structure of your primer.
   - Does the primer have an internal sequence that can bind itself?

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.

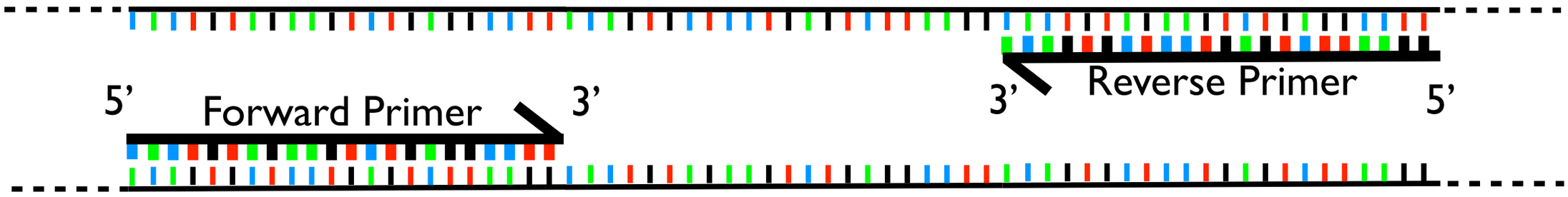
In fact, this behavior of DNA has been harvested to make higher order structures: DNA Origami is an active area of research.



DNA Origami is cool.

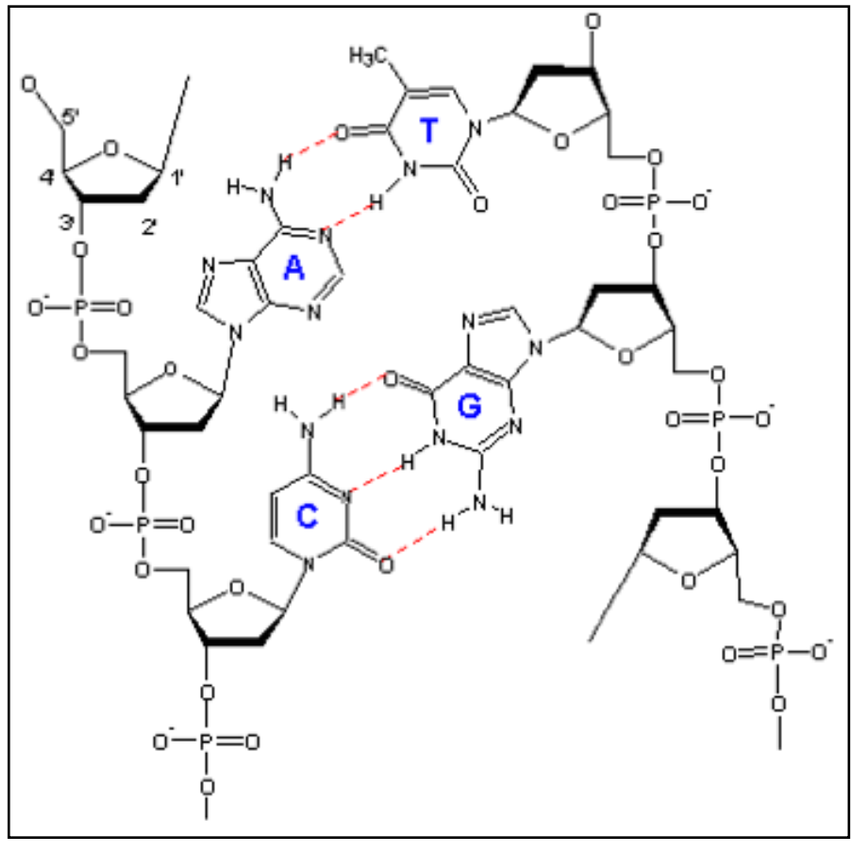
Image from: http://www.dna.caltech.edu/~pwkr/

(2) Design primers to increase sensitivity or specificity: 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



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