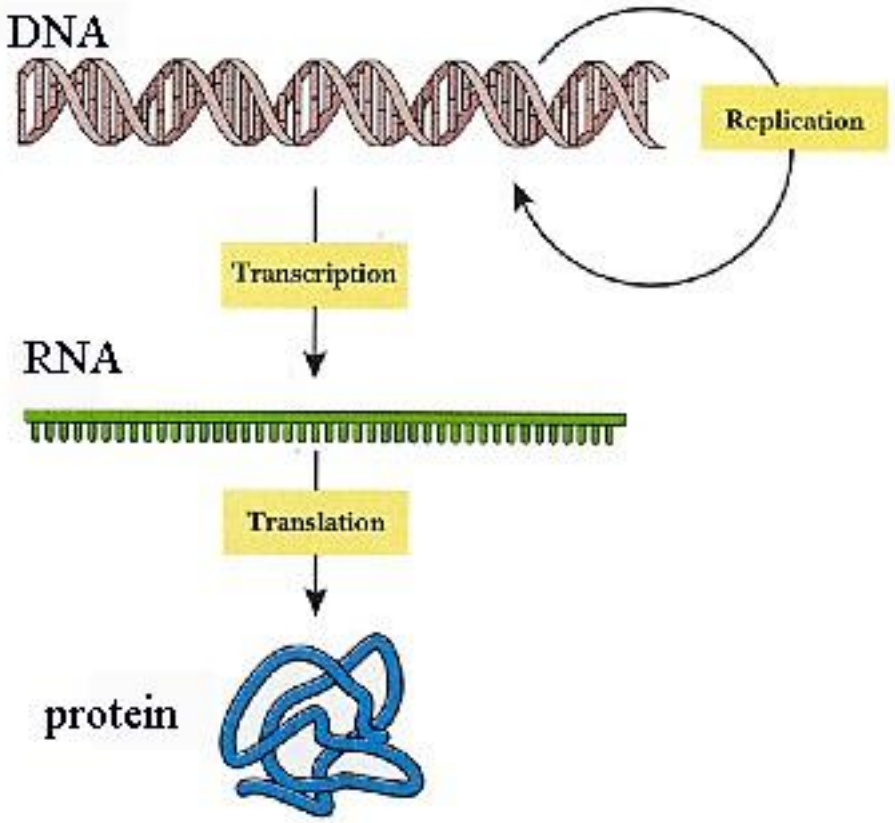
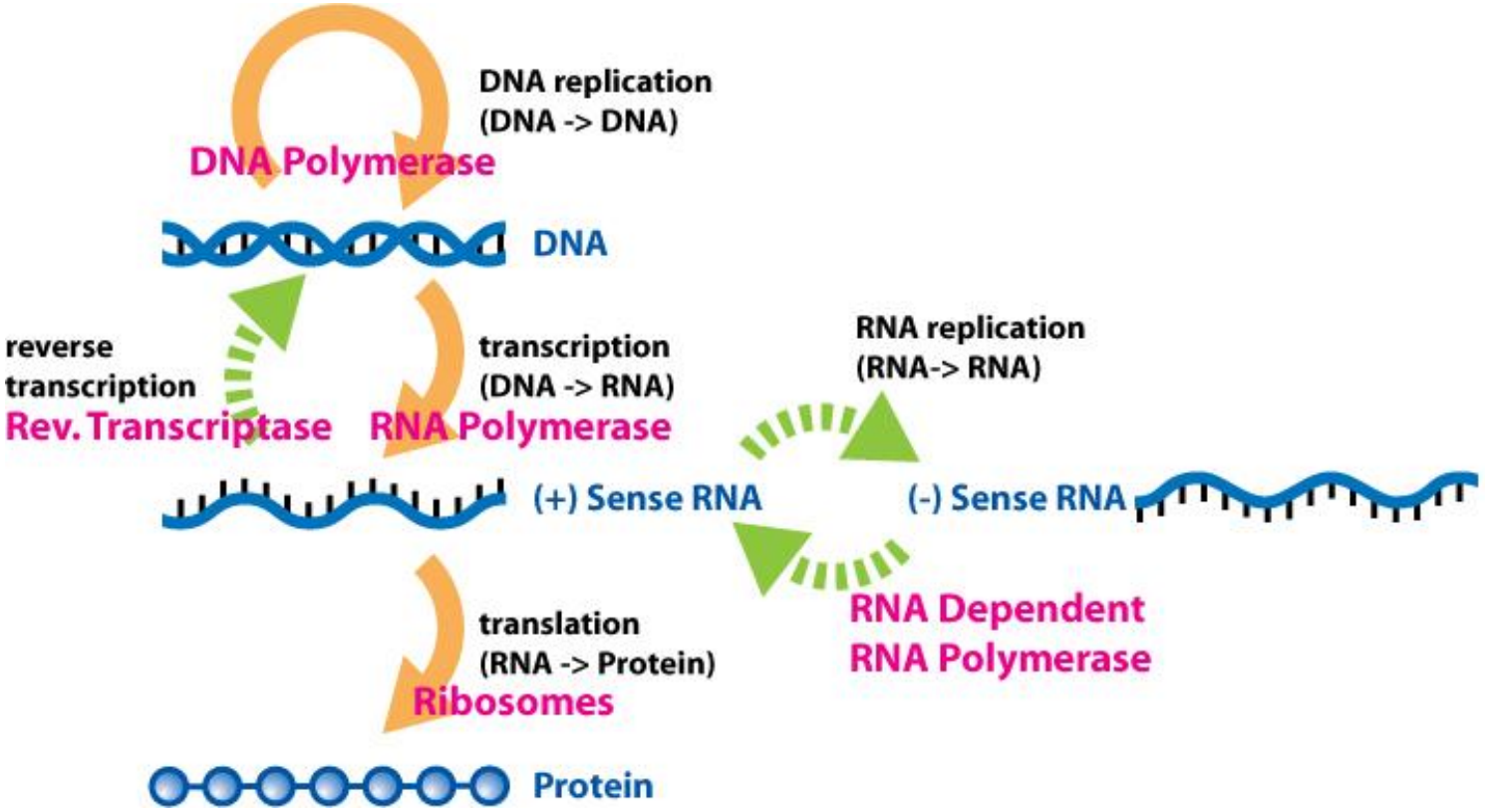


Introduction to qPCR

Central Dogma of Molecular Biology



Central Dogma of Molecular Biology

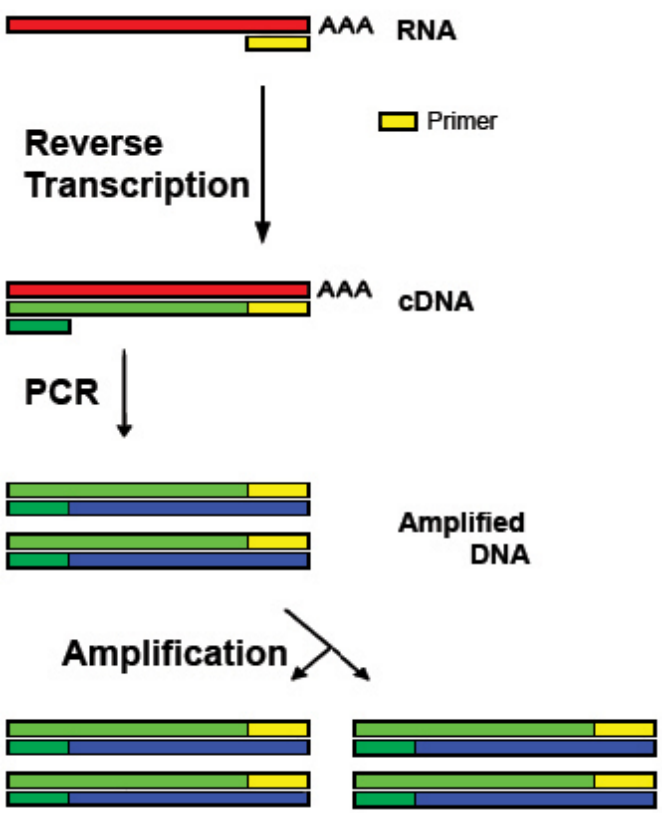


qRT-PCR Overview

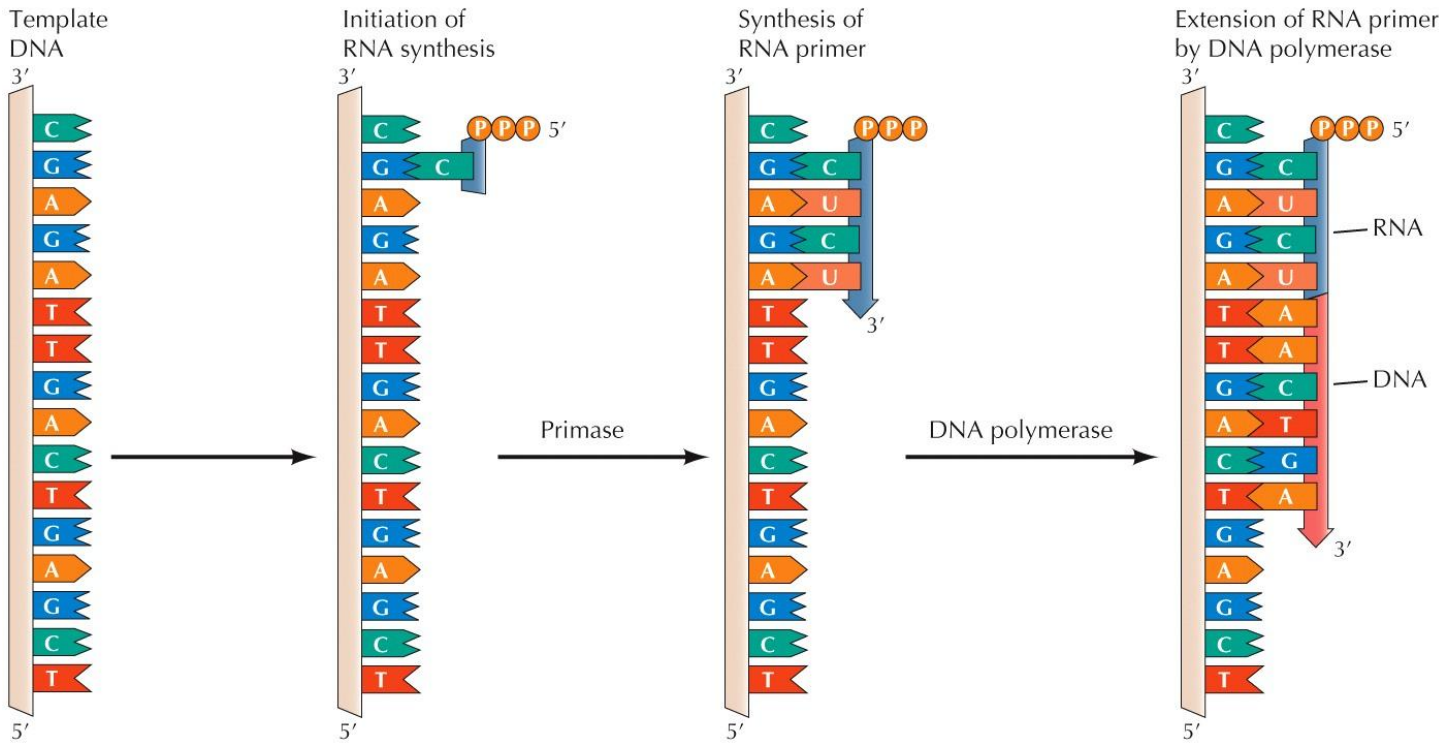
Step 1: Isolate mRNA from cells (control and experimental)

Step 2: Convert mRNA into cDNA

Step 3: Amplify cDNA and quantify (relative) amount of starting mRNA in sample using qPCR (quantitiative/real-time polymerase chain reaction)



RNA Primers and DNA Polymerase in DNA Synthesis

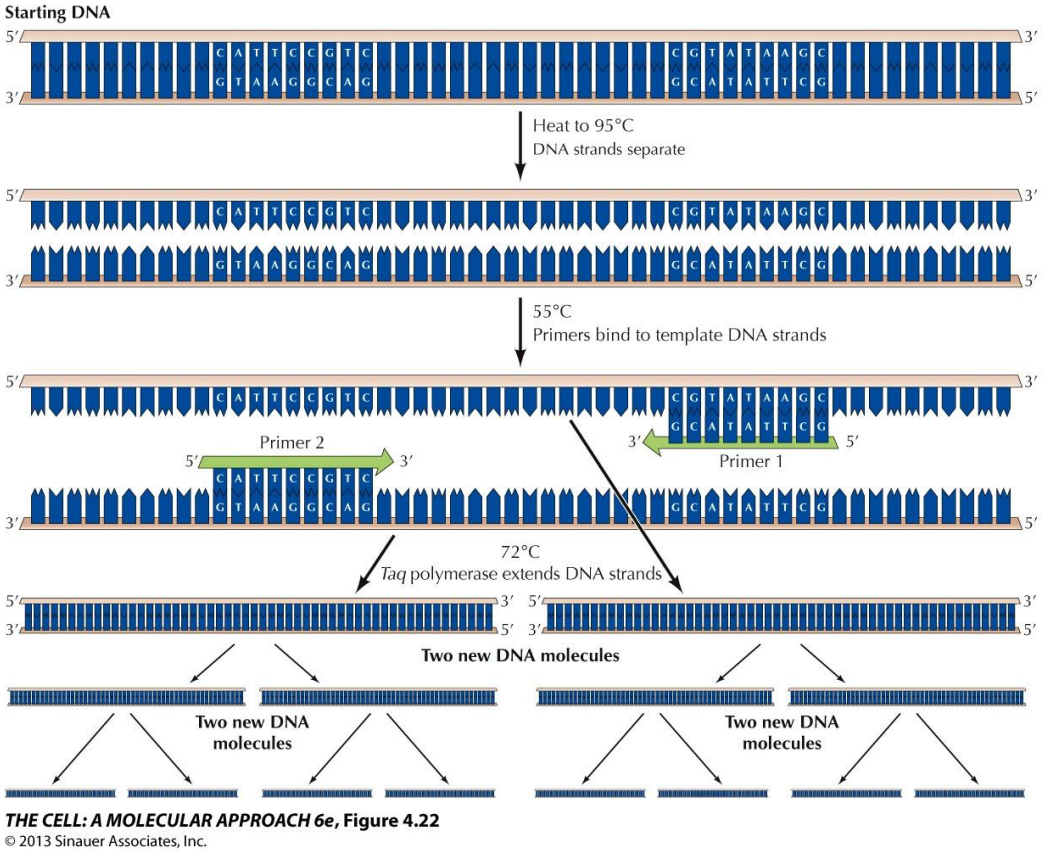


***THE CELL: A MOLECULAR APPROACH 6e*, Figure 6.4**

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Remind yourself… what is PCR??

PCR (polymerase chain reaction) allows the production of millions of copies of a DNA fragment from just one starting DNA molecule.



***THE CELL: A MOLECULAR APPROACH 6e*, Figure 4.22**

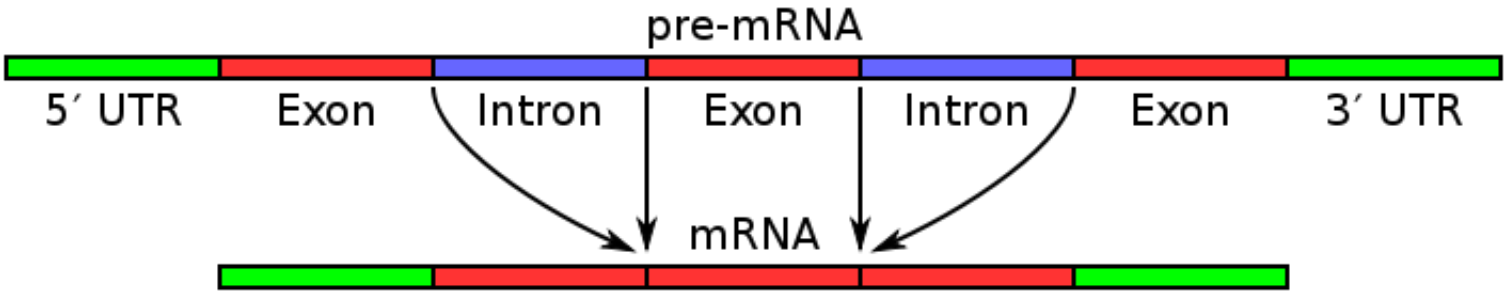
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http://learn.genetics.utah.edu/content/labs/pcr/

Central Dogma of Molecular Biology

RNA splicing

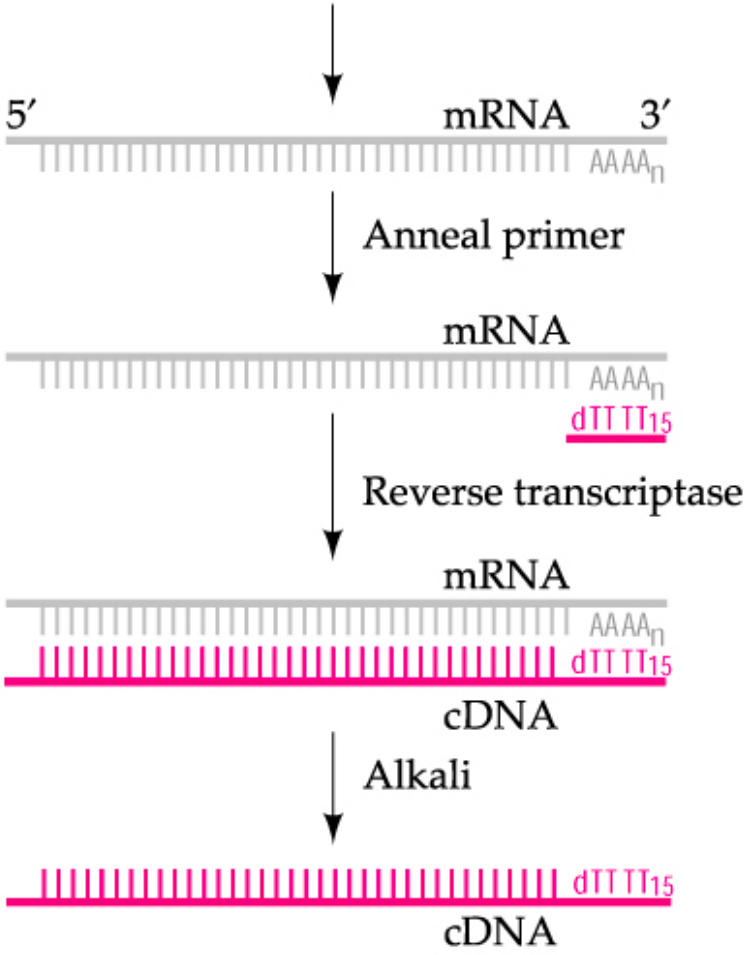
• generally catalyzed by spliceosome (RNA-protein complex composed of 5 snRNPs)



http://en.wikipedia.org/wiki/RNA_splicing

PCR can also be used to amplify RNA…

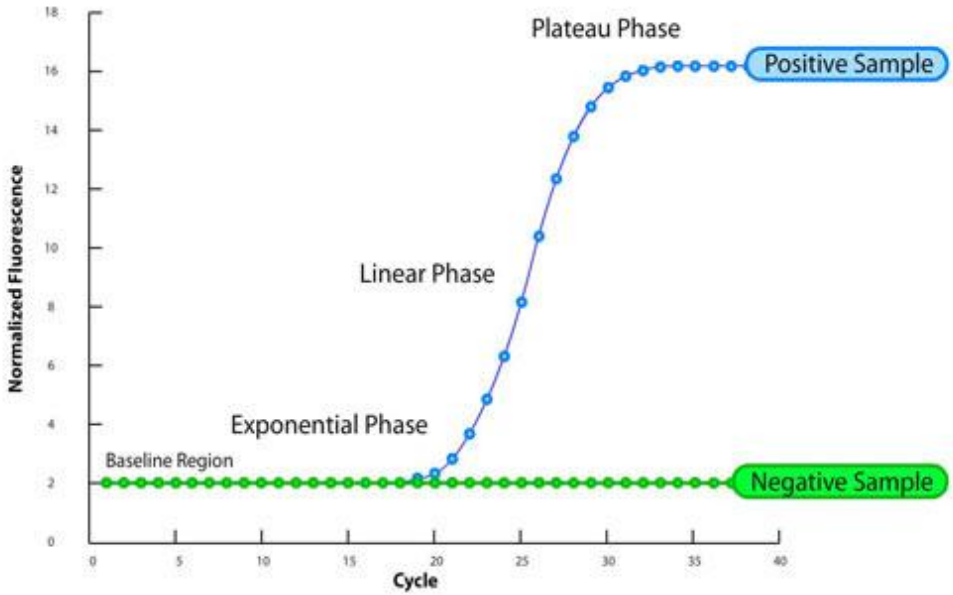
reverse transcription PCR (RT-PCR)



Quantitative PCR (qPCR)

Quantitative (or real-time) PCR allows the researcher to not only amplify DNA (or cDNA) but also measures the amount of DNA (or cDNA) present in the sample.

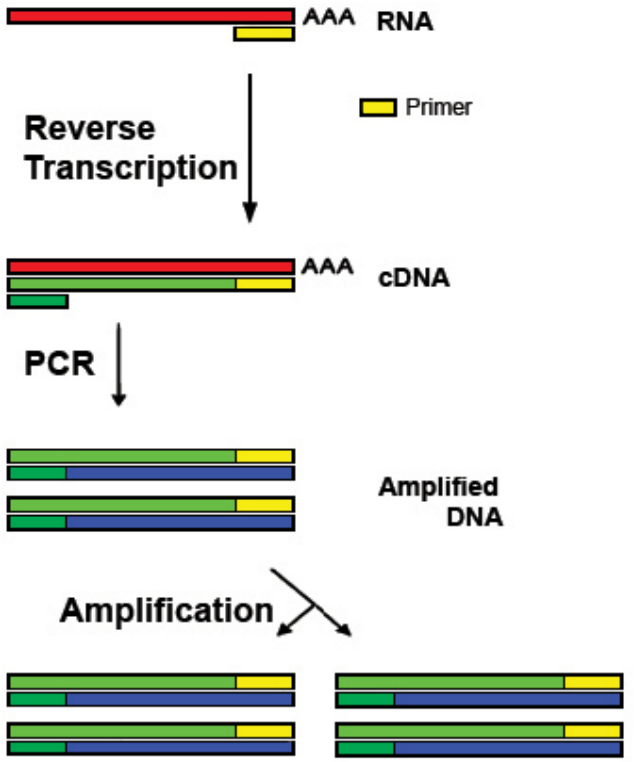
This is possible through the use of fluorescent reporters (commonly TaqMan or SYBR Green) along with controlled thermocycling.



Reverse transcription/Quantitative PCR Combined Technique

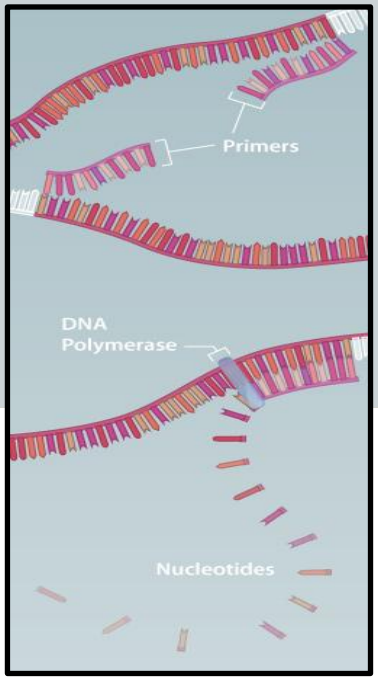
qRT-PCR

By combining the techniques of reverse transcription and quantitative PCR, the researcher can measure the amount of mRNA of interest in a sample.

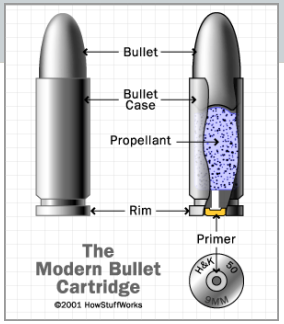












Primer Design for qPCR

Generating Primers

Step 1. Identify the Gene you want and save the FASTA format of its sequence

https://www.ncbi.nlm.nih.gov/
*Search Nucleotide, by selecting from the pull down menu of All Databases

Step 2. Generate a primer to the sequence you want to amplify. You will need to set the criteria for this algorithm to those that are required for our PCR kit.

https://www.ncbi.nlm.nih.gov/tools/primer-blast/

Step 3. Analyze your primer pairs for secondary structure and free energy. Determine if they are good or bad primers.

https://www.idtdna.com/calc/analyzer
Login 4140 Password CellBio4140

Primer Design Criteria for Final Project

Primer-BLAST settings

- primer T_m nearest 65° (min 60, max 70)
- amplicon between 50-150 bp (max 200)
- primer size between 18 and 24 bp (ideally, 20 bp)
- $MgCl_2$ 2.5mM
- max poly-X: 3
- max 3' self-complementarity: 1
- unique amongst transcripts (if possible)
- Targeting the last 1000 bp
- Ignore targets that have 2 or more mismatches to the primer (increase if necessary)

Selecting the best primer pair

- T_m of primers should be within 1° of eachother (max 2°)
- G/C content of ~50% or more
- Spans an exon-exon junction
- Minimize self complementarity and 3' complementarity

Primer Design Criteria for Final Project

IDT DNA OligoAnalyzer

- Combines the functions of Biosoft and UNAfold
- Check for self dimers, hetero dimers, and hairpins between primers
- Check for secondary structures (hairpins) formed by the amplicon

Criteria for Secondary Structure

- Avoid primers that form dimers where the 3' end has 3 bp matches (unless ΔG is greater than -2 kcal/mol)
- If self-dimers or cross dimers are unavoidable, choose primers with the highest $-\Delta G$
- Discard primers with ΔGs more negative than -6 kcal/mol
- If hairpins cannot be avoided, avoid hairpins that involve the 3' end or hairpin structures with T_m greater than 60°C
- Secondary structures of amplicons should have a T_m less than the annealing temperature (60°C)

$\Delta G > 0$: the reaction is unlikely to occur

$\Delta G < 0$: the reaction is likely to occur

Good Primers, Energy, Temp

- We want the primers to bind our mRNA, not the other primers in the mix
  - Cross-Dimers, Self-Dimers, Hairpins
- ΔG is the predicted free energy change that would allow for a particular structure to form
  - $\Delta G > 0$: the reaction is unlikely to occur
  - $\Delta G < 0$: the reaction is likely to occur
- Temperature
  - If you can't get away from secondary structure, check at what temperature that structure forms.
  - It may be far enough away from the temperatures that you will putting the sequences in.