Appendix A: Worksheet

Primer design for RT-qPCR simulation: An online lab exercise using a SARS-CoV-2 model

Introduction

PCR is a powerful method used to amplify DNA. It can also detect RNA (RT-PCR) when the enzyme reverse transcriptase (RT) is first used to convert RNA into DNA. Quantitative PCR (qPCR) uses a DNA-binding dye to monitor the accumulation of amplified DNA during the PCR reaction. The core components of a PCR reaction include the DNA template, primers, nucleotides, DNA polymerase and buffer. Primers are short DNA fragments that bind specifically to the DNA region of interest, directing the DNA polymerase to begin building a new strand of DNA. Using appropriate and specific primers, PCR diagnostic tests can be extremely sensitive and specific.

SARS-CoV-2, the virus that causes COVID-19 infection, has an RNA genome, so reverse transcription (RT) must be performed prior to performing quantitative PCR (qPCR). The enzyme reverse transcriptase is used to copy the RNA into DNA that can be used in the reaction. For RT-qPCR to amplify SARS-CoV-2 specifically, scientists must choose a pair of primers that bind to a unique site in the SARS-CoV-2 genome. These primers should not bind to other related coronavirus sequences – this would generate false positive results. To avoid false negatives, the primers must recognize and amplify all variations (variants) of the virus. The CDC has published a set of recommended primers for testing patients for COVID-19 infection. This diagram summarizes how the RT-qPCR tests for the presence of SARS-CoV-2.

The National Center for Biotechnology Information (NCBI) site serves as a database for DNA, RNA and protein sequences generated around the world. Information is stored in the “FASTA” file format, in which nucleotides or amino acids are represented by single letter codes, and allows for a first descriptive line beginning with a “>”.

For example, the FASTA format for a fragment of SARS OC43 is shown below.

```
>SARSoc43
cgtgcacccc gttcactga tctttttta gatctttttg taatctaaac tttataaaaa catccactcc
```

In this lab, you will use NCBI FASTA sequences and the tool NCBI Primer-BLAST to generate primers specific for PCR amplification of SARS-CoV-2. You will then complete virtual PCR reactions to assess the specificity of the CDC’s PCR primer set and the PCR primer set you generated for amplification of different coronavirus strains.
Part 1: Finding primers for SARS-CoV-2 RT-qPCR

Primer properties (best practice)*

• Length of 18-24 bases
• 40-60% G/C content
• Start and end with 1-2 G/C pairs (G/C pairs have a stronger bond than A/T pairs)
• Avoid runs of 4 or more of one base, or dinucleotide repeats (for example, ACCCC or ATATATAT) as this can cause primer mispriming (binding at non-specific sites).

*Note: sometimes compromises must be made if the rules cannot be satisfied exactly.

1. You will find primers using the Wuhan sequence of SARS-CoV-2, which is often used as the reference. Start by clicking on the following RefSeq number that will take you directly to the FASTA sequence: NC_045512.2.

2. On the menu at the right-hand side, click on “Pick Primers”. This will open the Primer-BLAST site.

3. Most sections of the Primer-BLAST site will be auto-populated. Note that the first section, “PCR Template”, has the NC_045512.2 sequence already listed.

4. On the fourth block titled “Primer Pair Specificity Checking Parameters,” you will need to make 2 changes:
   a. Change the Database to “RefSeq RNA (refseq_rna)”. You need to change the database because we are examining viral sequences which are listed in this database not the default database.
   b. Change the organism number to “2697049”, which is the unique NCBI Taxonomy ID for the SARS-CoV-2 virus.

5. Finally, click on “Get Primers”, the button at the bottom of the page.

   The Primer-BLAST database will process for a few moments, then report out a graphical view of the primers and a detailed report of each of the primer pairs identified.

   Why do G/C pairs have a stronger bond than A/T pairs?

6. Spend some time looking at the primer information that is reported. Using the primer properties described above, choose one primer pair (a set of two primers) that you would like to use for your amplification. (If you don’t see a primer pair where each sequence ends in a G or C, you may trim off a nucleotide) Write the sequences of the two primers you chose here:

   Forward Primer:

   Reverse Primer:

7. What nucleotide positions in the genome did your primers come from? (hint: look under the headings Start and Stop, or after “Template” in the primer alignment)

   Forward Primer:

   Reverse Primer:
8. Go back to the top of your Primer-BLAST results page. **Click on the RefSeq number** next to ‘Input PCR template’. This should open a new tab which takes you back to GenBank. As you scroll down, you will see **Features** listed along the left-hand side of the screen. These features are genes and other regions of the genome and are listed along with their position within the genome, as shown below.

| gene       | SFYEDFLEYHDVRVVLDFI |
|------------|---------------------|
| /gene      | "N"                 |
| /locus_tag | "GU280_gpl0"        |
| /db_xref   | "GeneID:43740575"   |

Scroll down to find in which feature/gene your primers are located. (See the nucleotide position in question 7, then find which gene this location corresponds to.) The example above shows the SARS-C0V-2 gene, “N”, which encodes the nucleocapsid protein that forms the capsid “shell” around the RNA genome.

Consider the region covered by your primers.

**Does the primer fall into a coding or non-coding region?** (Does GenBank tell you the region codes for a gene or protein or is it an untranslated region?)

If it is a coding region, what does this part of the genome code for?

What is the function, if any, of the coded product?

9. Compare your Primer-BLAST results to the CDC primer set listed below. **Do the CDC primers match any of your results?** (Yes or No) Note: you can use the Find feature in Google Docs or on NCBI to look for matches!

**Part 2: Evaluating primer accuracy**

1. The CDC primer set that you will be testing is below:

   **Forward:** 5’ - ATGAGCTTAGTCTGTTG - 3’
   **Reverse:** 5’ - CTCCCTTTGTGTGGTTGTG - 3’

2. Enter the primer set that you identified earlier (in question 6 from part 1):

   **Forward (plus):**

   **Reverse (minus):**

3. Test your primers using [this virtual PCR tool](https://www.bioinformatics.org/sms2/pcr_products.html).
Steps for using the Virtual PCR tool:

a. In the box labeled “Paste the raw sequence…”, look at how the information in here is formatted. You will copy and paste the different genomic sequences linked in the table below to analyze each of these as templates for your PCR.
   i. Click on one of the genomic sequences in the table below which will take you to its GenBank page.
   ii. Click on the FASTA link and copy this entire sequence
   iii. Go to the virtual PCR tool and replace the demonstration sequence with this sequence.

b. Enter your primer information in the boxes under “Enter the name of the first primer…”.
   i. In the first box you will replace the demonstration text with a name for your primer (such as CDC Forward).
   ii. In the second box you will replace the demonstration sequence with your primer sequence. (such as the base sequence for your CDC reverse primer from above). Note that they only have the sequence information, not the 5’ and 3’ notations.

c. Next, you will do the same for the boxes under “Enter the name of the second primer…”. This time using the second primer for the primer set you are examining (reverse primer)

d. Click the “submit” button.

e. You will either get an amplified sequence (PCR product, also called an amplicon) or not. Record this in your table. If you got an amplicon, record the amplicon size in the table.

4. To check your other set of primers on the same sequence, close the results window and replace the CDC primers with your set of primers on the virtual PCR tool.

5. To check the other coronavirus genomes in the table below, remove the sequence from the “raw sequence” box, obtain another FASTA sequence by following the link from the table, and paste the new sequence into the “raw sequence” box.

6. Test each of the Coronavirus sequences in Table 1 for PCR amplification by both the CDC primer set (above) and your primer set (above). Record the results including your PCR product (amplicon) length in bp in the table below.

   a. The first 4 sequences in the table are controls to test for false positive amplification by the primer sets. A false positive is when samples should be negative, but they test as positive. These genomes are not for SARS-CoV-2 variants, but are other coronaviruses that are known to infect humans, such as common cold coronaviruses (OC43, HKU1), MERS, and SARS. If the primer set you are using is specific for SARS-CoV-2, you should NOT get amplification products from any of these other sequences. Record your results in the table below (sequences 1-4)

   b. Sequences 5-9 in the table are SARS-CoV-2 variants. For your primer pairs to be successful as a PCR assay to detect SARS-CoV-2, all of these variants need to be amplified when using your primer set. If the variant is not amplified, then this is a false negative result. A false negative is when samples are positive, but they test negative. Record your results in the table below (sequences 5-9)

   c. For sequence 10, you will find and test your own sequence. Find a different SARS-CoV-2 variant using a sequence from the SARS-CoV-2 and Lineages of Concern website. From the Overview tab, find a variant of interest and click on “Representative Strain”. From this page, scroll down and click
on the “Genbank Sequence Accession” ID which will take you to GenBank. Here you can copy the FASTA sequence as you did previously and analyze both the CDC and your primer sets for PCR amplification. Record your sequence name, GenBank ID, and results in the table below (sequence 10)

Table 1. Virtual PCR Results summary.

| Sequence | Coronavirus | Link to genomic sequence | CDC Primer Pair Results (Y/N, bp) | Your Primer Pair Results (Y/N, bp) |
|----------|-------------|--------------------------|-----------------------------------|-----------------------------------|
| 1.       | OC43        | Human coronavirus OC43 accession strain KF530099.1 |                                |                                  |
| 2.       | HKU1        | Human coronavirus HKU1 accession strain KF430201.1 |                                |                                  |
| 3.       | MERS        | Middle East respiratory syndrome-related coronavirus accession NC_019843.3 |                                |                                  |
| 4.       | SARS        | SARS coronavirus Tor2 accession NC_004718.3 |                                |                                  |
| 5.       | SARS-CoV-2 (Wuhan China) | SARS-CoV-2 isolate Wuhan-Hu-1 accession NC_045512.2 |                                |                                  |
| 6.       | SARS-CoV-2 (Minnesota) | SARS-CoV-2 USA/MN1-MDH1/2020 MT188341.1 |                                |                                  |
| 7.       | SARS-CoV-2 (Delta variant) | SARS-CoV-2 B.1.617.2 OL336792 |                                |                                  |
| 8.       | SARS-CoV-2 (Omicron variant) | SARS-CoV-2 OM095411 “omicron-1” |                                |                                  |
| 9.       | SARS-CoV-2 (Omicron stealth) | SARS-CoV-2 OM371884 |                                |                                  |
| 10.      |             |                          |                                    |                                  |
Part 3: Analyze the PCR amplification data

1. Confirm that you have completed Table 1 above with your results.
   a. **Did you get amplification of the sequence with the CDC primer set or with your primer set?** Indicate Yes or No in those columns. If YES, write the number of base pairs of the amplicon.
   b. **Did you remember to choose a new variant for sequence 10?** Make sure you fill out the name of this coronavirus variant, the GenBank ID, whether the primers amplified the sequence, and the number of base pairs in the amplicon if it was amplified.

2. Examine your results for the CDC primer set.
   a. **Did you have any negative PCR results?** Were these results expected or unexpected?
   b. **Did you have any false positives or false negative results?** If so, were they expected or unexpected? What do these results mean?
   c. **Is this CDC primer set suitable for detection of SARS-CoV-2 by RT-qPCR?** Explain.

3. Examine your results for YOUR primer set.
   a. **Did you have any negative PCR results?** Were these results expected or unexpected?
   b. **Did you have any false positives or false negative results?** Were they expected or unexpected? What do these results mean?
   c. **Is your primer set suitable for detection of SARS-CoV-2 by RT-qPCR?** Explain.

4. **What does PCR product (amplicon) size indicate?** For a review, read the Introduction or watch this short video on PCR: [https://youtu.be/2KoLnIwoZKU](https://youtu.be/2KoLnIwoZKU)

5. Consider a choice between 2 sets of SARS-CoV-2 diagnostic primers: one set has an amplicon of 850bp in length, and the other has an amplicon of 125bp. **Which primer pair would be the best choice? Why?** (Use literature search to find the answer)
Appendix B: Worksheet Key

Primer design for RT-qPCR simulation: An online lab exercise using a SARS-CoV-2 model

Introduction

PCR is a powerful method used to amplify DNA. It can also detect RNA (RT-PCR) when the enzyme reverse transcriptase (RT) is first used to convert RNA into DNA. Quantitative PCR (qPCR) uses a DNA-binding dye to monitor the accumulation of amplified DNA during the PCR reaction. The core components of a PCR reaction include the DNA template, primers, nucleotides, DNA polymerase and buffer. Primers are short DNA fragments that bind specifically to the DNA region of interest, directing the DNA polymerase to begin building a new strand of DNA. Using appropriate and specific primers, PCR diagnostic tests can be extremely sensitive and specific.

SARS-CoV-2, the virus that causes COVID-19 infection, has an RNA genome, so reverse transcription (RT) must be performed prior to performing quantitative PCR (qPCR). The enzyme reverse transcriptase is used to copy the RNA into DNA that can be used in the reaction. For RT-qPCR to amplify SARS-CoV-2 specifically, scientists must choose a pair of primers that bind to a unique site in the SARS-CoV-2 genome. These primers should not bind to other related coronavirus sequences – this would generate false positive results. To avoid false negatives, the primers must recognize and amplify all variations (variants) of the virus. The CDC has published a set of recommended primers for testing patients for COVID-19 infection. This diagram summarizes how the RT-qPCR tests for the presence of SARS-CoV-2.

The National Center for Biotechnology Information (NCBI) site serves as a database for DNA, RNA and protein sequences generated around the world. Information is stored in the “FASTA” file format, in which nucleotides or amino acids are represented by single letter codes, and allows for a first descriptive line beginning with a “>”.

For example, the FASTA format for a fragment of SARS OC43 is shown below.

> SARSoc43
cgtgcatacc gctttactga tctctcttgta gatcttttta tatctaatc tacatctaca cattttttttt catccactcc

In this lab, you will use NCBI FASTA sequences and the tool NCBI Primer-BLAST to generate primers specific for PCR amplification of SARS-CoV-2. You will then complete virtual PCR reactions to assess the specificity of the CDC’s PCR primer set and the PCR primer set you generated for amplification of different coronavirus strains.
Part 1: Finding primers for SARS-CoV-2 RT-qPCR

Primer properties (best practice)*

- Length of 18-24 bases
- 40-60% G/C content
- Start and end with 1-2 G/C pairs (G/C pairs have a stronger bond than A/T pairs)
- Avoid runs of 4 or more of one base, or dinucleotide repeats (for example, ACCCC or ATATATAT) as this can cause primer mispriming (binding at non-specific sites).

*Note: sometimes compromises must be made if the rules cannot be satisfied exactly.

1. You will find primers using the Wuhan sequence of SARS-CoV-2, which is often used as the reference. Start by clicking on the RefSeq number below that will take you directly to the FASTA sequence: NC_045512.2.

2. On the menu at the right-hand side, click on “Pick Primers”. This will open the Primer-BLAST site.

3. Most sections of the Primer-BLAST site will be auto-populated. Note that the first section, “PCR Template”, has the NC_045512.2 sequence already listed.

4. On the fourth block titled “Primer Pair Specificity Checking Parameters,” you will need to make 2 changes:
   a. Change the Database to “RefSeq RNA (refseq_rna)”. You need to change the database because we are examining viral sequences which are listed in this database not the default database.
   b. Change the organism number to “2697049”, which is the unique NCBI Taxonomy ID for the SARS-CoV-2 virus.

5. Finally, click on “Get Primers”, the button at the bottom of the page. The Primer-BLAST database will process for a few moments, then report out a graphical view of the primers and a detailed report of each of the primer pairs identified.

   Why do G/C pairs have a stronger bond than A/T pairs?

   Answer: G/C has 3 hydrogen bonds while A/T has two hydrogen bonds. This makes G/C more stable and therefore harder to undergo denaturation.

6. Spend some time looking at the primer information that is reported. Using the primer properties described above, choose one primer pair (a set of two primers) that you would like to use for your amplification. (If you don’t see a primer pair where each ends in a G or C, you may trim off a nucleotide) Write the sequences of the two primers you chose here:

   These example primers were chosen from ten possible pairs generated by Primer-BLAST.

   Forward Primer: CGGATGGCTTATTGTTGGCG
   Reverse Primer: TTGTGCTTACAAAGGCACGC

7. What nucleotide positions in the genome did your primers come from? (hint: look under the headings Start and Stop, or after “Template” in the primer alignment)
Example:  
**Forward Primer:** 25521 - 25540  
**Reverse Primer:** 26226 - 26226

8.  
Go back to the top of your Primer-BLAST results page. **Click on the RefSeq number** next to ‘Input PCR template’. This should open a new tab which takes you back to GenBank. As you scroll down, you will see **Features** listed along the left-hand side of the screen. These features are genes and other regions of the genome and are listed along with their position within the genome, as shown below:

```
| gene       | SFYEDFLEYHDVRVVLDFI |
|------------|----------------------|
| 28274..29533 | /gene="N"           |
| /locus_tag="GU280_gp10" |                  |
| /db_xref="GeneID:43740575" |
```

Scroll down to find in which feature/gene your primers are located. (See the nucleotide position in question 7, then find which gene this location corresponds to.) The example above shows the SARS-CoV-2 gene, “N”, which encodes the nucleocapsid protein that forms the capsid “shell” around the RNA genome.

**Example data:**

```
25393..26220
/gene="ORF3a"
/locus_tag="GU280_gp03"
/codon_start=1
/product="ORF3a protein"
/protein_id="YP_009724391.1"
/db_xref="GeneID:43740569"
```

Consider the region covered by your primers. **Does the primer fall into a coding or non-coding region?** (Does GenBank tell you the region codes for a gene or protein or is it an untranslated region?) . **If it is a coding region, what does this part of the genome code for?**

**Example Answer:** Codes for the ORF3a protein

**Students can also be asked to find the function if any, of the coded product:**

**Example Answer:** Function; Unique to SARS-CoV-2, a viroporin that is said to play a role during viral entry (endocytosis), transcription and during release (exocytosis). It is also immunogenic and can induce cytokine storm. ([https://doi.org/10.3389/fmicb.2022.854567](https://doi.org/10.3389/fmicb.2022.854567))

9.  
Compare your Primer-BLAST results to the CDC primer set listed below. **Do the CDC primers match any of your results?** (Yes or No) **Note:** you can use the Find feature in Google Docs or on NCBI to look for matches!

**Example Answer:** No
Part 2: Evaluating primer accuracy

1. The CDC primer set that you will be testing is below:
   
   **Forward:**  
   `5’ - ATGAGCTTAGTCCTGTTG - 3’`
   
   **Reverse:**  
   `5’ - CTCCCTTTGTTGTGTTGT - 3’`

2. **Enter the primer set that you identified earlier** (in question 6 from part 1):
   
   *Example primers from Question 6 in Part I are listed below.*

   **Forward (plus):**  
   `CGGATGGCTTATTGTTGGCG`

   **Reverse (minus):**  
   `TTGTGCTTACAAAGGCACGC`

3. Test your primers using [this virtual PCR tool](https://www.bioinformatics.org/sms2/pcr_products.html).

   **Steps for using the Virtual PCR tool:**
   
   a. In the box labeled “Paste the raw sequence…”, look at how the information in here is formatted. You will copy and paste the different genomic sequences linked in the table below to analyze each of these as templates for your PCR.
      
      i. Click on one of the genomic sequences in the table below which will take you to its GenBank page.
      
      ii. Click on the FASTA link and copy this entire sequence
      
      iii. Go to the virtual PCR tool and replace the demonstration sequence with this sequence.
   
   b. Enter your primer information in the boxes under “Enter the name of the first primer…” we will start with the CDC primer then your primer. You will enter results in the table below.
      
      i. In the first box you will replace the demonstration text with a name for your primer (such as CDC Forward).
      
      ii. In the second box you will replace the demonstration sequence with your primer sequence, (such as the base sequence for your CDC forward primer from above). *Note that they only have the sequence information, not the 5’ and 3’ notations.*
   
   c. Next, you will do the same for the boxes under “Enter the name of the second primer…”. This time using the second primer for the primer set you are examining (reverse primer)
   
   d. Click the “submit” button.
      
   e. You will either get an amplified sequence (PCR product, also called an amplicon) or not. Record this in your table. If you got an amplicon, record the amplicon size in the table.
   
4. To check your other set of primers on the same sequence, close the results window and replace the CDC primers with your set of primers on the virtual PCR tool.
5. To check the other coronavirus genomes in the table below, remove the sequence from the “raw sequence” box, obtain another FASTA sequence by following the link from the table, and paste the new sequence into the “raw sequence” box.

6. Test each of the Coronavirus sequences in Table 1 for PCR amplification by both the CDC primer set (above) and your primer set (above). Record the results including your PCR product (amplicon) length in bp in the table below.

   a. The first 4 sequences in the table are controls to test for false positive amplification by the primer sets. A false positive is when samples should be negative, but they test as positive. These genomes are not for SARS-CoV-2 variants, but are other coronaviruses that are known to infect humans, such as common cold coronaviruses (OC43, HKU1), MERS, and SARS. If the primer set you are using is specific for SARS-CoV-2, you should NOT get amplification products from any of these other sequences. Record your results in the table below (sequences 1-4)

   b. Sequences 5-9 in the table are SARS-CoV-2 variants. For your primer pairs to be successful as a PCR assay to detect SARS-CoV-2, all of these variants need to be amplified when using your primer set. If the variant is not amplified, then this is a false negative result. A false negative is when samples are positive, but they test negative. Record your results in the table below (sequences 5-9)

   c. For sequence 10, you will find and test your own sequence. Find a different SARS-CoV-2 variant using a sequence from the SARS-CoV-2 and Lineages of Concern website. From the Overview tab, find a variant of interest and click on “Representative Strain”. From this page, scroll down and click on the “Genbank Sequence Accession” ID which will take you to GenBank. Here you can copy the FASTA sequence as you did previously and analyze both the CDC and your primer sets for PCR amplification. Record your sequence name, GenBank ID, and results in the table below (sequence 10)

   (The South Africa Beta variant was used as an example here.)
### Table 1. Virtual PCR Results summary.

| Sequence | Coronavirus | Link to genomic sequence | CDC Primer Pair Results (Y/N, bp) | Your Primer Pair Results (Y/N, bp) |
|----------|-------------|----------------------------|-----------------------------------|-----------------------------------|
| 1.       | OC43        | **Human coronavirus OC43 accession strain KF530099.1** | N                                  | N                                  |
| 2.       | HKU1        | **Human coronavirus HKU1 accession strain KF430201.1** | N                                  | N                                  |
| 3.       | MERS        | **Middle East respiratory syndrome-related coronavirus accession NC_019843.3** | N                                  | N                                  |
| 4.       | SARS        | **SARS coronavirus Tor2 accession NC_004718.3** | N                                  | N                                  |
| 5.       | SARS-CoV-2 (Wuhan China) | **SARS-CoV-2 isolate Wuhan-Hu-1 accession NC_045512.2** | 108 bp product | 706 bp product |
| 6.       | SARS-CoV-2 (Minnesota) | **SARS-CoV-2 USA/MN1-MDH1/2020 MT188341.1** | 108 bp product | 706 bp product |
| 7.       | SARS-CoV-2 (Delta variant) | **SARS-CoV-2 B.1.617.2 OL336792** | 108 bp product | 706 bp product |
| 8.       | SARS-CoV-2 (Omicron variant) | **SARS-CoV-2 OM095411 “omicron-1”** | 108 bp product | 706 bp product |
| 9.       | SARS-CoV-2 (Omicron stealth) | **SARS-CoV-2 OM371884** | 108 bp product | 706 bp product |
| 10.      | **SARS-CoV-2 (Beta V2 South Africa)** | **SARS-CoV-2 OM371884** | 108 bp product | 706 bp product |

### Part 3: Analyze the PCR amplification data

1. Confirm that you have completed Table 1 above with your results.

   a. **Did you get amplification of the sequence with the CDC primer set or with your primer set?** Indicate Yes or No in those columns. If YES, write the number of base pairs of the amplicon.

      *Expected answers are shown in the table.*

   b. **Did you remember to choose a new variant for sequence 10?** Make sure you fill out the name of this coronavirus variant, the GenBank ID, whether the primers amplified the sequence, and the number of base pairs in the amplicon if it was amplified.

      *Expected answers are shown in the table.*
2. Examine your results for the CDC primer set.
   
a. **Did you have any negative PCR results?** Were these results expected or unexpected?  
   
   Answer:  
   
   [Students should note negative PCR results for sequences 1-4. These are expected to fail using primers specific to SARS-CoV-2.]  
   
   Yes, all the other corona viruses tested negative. This was expected as the primers are meant to be specific to SARS-CoV-2.
   
   b. **Did you have any false positives or false negative results?** If so, were they expected or unexpected? What do these results mean?  
   
   Answer:  
   
   [Students should not find negative PCR results for sequences 5-9. These are expected to amplify using primers specific to SARS-CoV-2, and any failure is a false negative. Any amplification from sequences 1-4 is considered a false positive.]  
   
   No, all the SARS-CoV-2 variants were positive while all the others were negative.
   
   c. **Is this CDC primer set suitable for detection of SARS-CoV-2 by RT-qPCR? Explain.**
   
   Answer:  
   
   [Students should hopefully find this primer set to be a great choice for detection of SARS-CoV-2 by RT-qPCR.]

3. Examine your results for YOUR primer set.
   
a. **Did you have any negative PCR results?** Were these results expected or unexpected?  
   
   Answer:  
   
   [Students should note negative PCR results for sequences 1-4. These are expected to fail using primers specific to SARS-CoV-2.]  
   
   Yes, all the other corona viruses tested negative. This was expected as the primers are meant to be specific to SARS-CoV-2.
   
   b. **Did you have any false positives or false negative results?** Were they expected or unexpected? What do these results mean?  
   
   Answer:  
   
   Students should not find negative PCR results for sequences 5-9. These are expected to amplify using primers specific to SARS-CoV-2, and any failure is a false negative. Any amplification from sequences 1-4 is considered a false positive.]  
   
   No, all the SARS-CoV-2 variants were positive while all the others were negative.
   
   c. **Is your primer set suitable for detection of SARS-CoV-2 by RT-qPCR? Explain.**

   Answer:
Yes, the primer set was able to detect all the variants.

4. **What does PCR product (amplicon) size indicate?** For a review, read the Introduction or watch this short video on PCR: [https://youtu.be/2KoLnlwoZKU](https://youtu.be/2KoLnlwoZKU)

   **Answer:**

   [Amplicon size indicates the size of the DNA fragment amplified by PCR using your primers. The presence of an amplicon indicates the target DNA (in this case, from SARS-CoV-2) is present in the sample.]

5. Consider a choice between 2 sets of SARS-CoV-2 diagnostic primers: one set has an amplicon of 850bp in length, and the other has an amplicon of 125bp. **Which primer pair would be the best choice?** Why?

   **Answer:**

   [An amplicon with a longer length requires a much longer extension time, resulting in a non-rapid diagnostic result. A shorter amplicon allows for speedy processing of SARS-CoV-2 samples and rapid diagnosis.]