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Making sense of PCR (paper-based)

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EMBL Science Education and Public Engagement

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EMBL's Science Education and Public Engagement (SEPE) office leads and coordinates the institute's science education programmes and public engagement efforts.

Formerly known as European Learning Laboratory for the Life Sciences, ELLS, we are building on EMBL's long history of science education and public engagement, and support EMBL's commitment of sharing and discussing our research with young learners, teachers and diverse publics.

Our science education activities share the scientific discoveries of EMBL through inspiring teaching and learning experiences with school science
teachers and young people of all backgrounds aged between 10 and 19 years.

Our programmes convey complex, cutting-edge topics in life science research in an exciting and insightful way, fostering the discovery of current research trends, the scientific method, and scientific career paths. Our activities are developed and run in close collaboration with EMBL scientists.

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EMBL Science Education and Public Engagement

Teacher's guide

The aim of this paper-based, hands-on activity is to design a PCR experiment. The students are tasked with designing primers and a PCR programme in order to detect a viral gene. To achieve this goal, the students use a cut-out paper mask to find potential primers in a cDNA sequence. The activity workflow represents authentic, practical considerations researchers in a molecular biology laboratory face on a daily basis.

Step by step, the activity worksheet takes the students through the complex process of designing a PCR experiment providing them with key instructions to help them master the task. The activity can be used for teaching in the classroom or remotely. It can be conducted without internet or computer access.

Learning outcomes

The students will

- design PCR primers
- gain in-depth knowledge on PCR
- understand why this basic method is still indispensable
- solve an authentic problem
- learn how to interpret PCR results

Prerequisites and supplementary materials

This activity is intended for students aged 16-20 years. Students should be familiar with the basic concepts of DNA, RNA and cDNA. It will also be beneficial if they already know some basics of PCR.

To introduce the students to new topics and equip them with the information they need to complete the exercises, Fact Sheets are provided. The teachers have the opportunity and freedom to choose which ones to hand out to their students. As an optional variation, teachers could also ask more advanced students to prepare topics they are already familiar with and share them with their classmates in order to prepare their own Fact Sheets for example about DNA or even PCR.

The resources provided with the activity also include a data sheet for the ELLS Taq Polymerase, a cut-out mask for the detection of primers and a fraction of the cDNA sequence of the SARS-CoV2 RNA dependent RNA Polymerase.

To find potential primers in a DNA sequence, students will cut out the discover-your-primer mask, as well as the white window within the mask. Within this window, the students will look for primer sequences that fit the characteristics mentioned on the mask while moving over the DNA sequence. Please note that the cut-out mask contains several deliberate limitations to the primer characteristics (e.g. the length of the primers). Those were chosen to limit the number of potential primers that can be found and to make the activity manageable for the students.

Structure of the activity

The activity worksheet is designed to guide the students through the different steps that have to be taken to reach the goal and it gives the students the opportunity to note down their results. The whole activity will take 60-90 minutes to complete.

In the **first exercise**, the students will design primers using the discover-your-primer mask provided. Please note that the students can potentially find different primers, they just have to meet the criteria provided in the worksheet and on the mask.

In the **second exercise**, the students will design a PCR programme using the specifications of their primers and the polymerase data sheet.

Finally, in the **third exercise**, they will be asked to interpret an agarose gel picture to identify individuals with positive results.

Optional nucleotide puzzle

As optional, additional material, to help students visualise the sequence of the reverse primer, a nucleotide puzzle is provided. Teachers can print out the puzzle (make sure to print enough copies to have enough of all nucleotides) and cut out the pieces. When laminated, the pieces can be re-used with several classes. The students can use the puzzle to piece together the sequence they have identified as potential primers. They can then add the complementary nucleotides and use this to read the reverse primer sequence.

Curriculum links

Curriculum	Topic
Advanced Placement (AP)	6.8
A-Level	3.8
International Baccalaureate (IB)	2.7, 3.5, B4

Fact Sheets

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Making sense of PCR (paper-based)

Fact Sheets: Table of Contents

Polymerase Chain Reaction (PCR)

PCR is a technique that allows the exponential amplification of very few or even single DNA molecules. To achieve this, the DNA molecules are subjected to several cycles of denaturation, primer annealing and DNA strand elongation/extension.

The reaction mixture for a PCR requires the following reagents:

- The DNA to be amplified (template DNA)
- Primers complementary to the template DNA
- An enzyme which synthesises the new DNA strand (DNA polymerase)
- Free nucleotides (dNTPs) the DNA polymerase uses to extend the new DNA strand
- A buffer providing the optimal conditions for the reaction

The amplification cycle comprises three different steps:

1. During the **denaturation** step the double stranded template DNA is separated into the two single strands. The temperature during this step, therefore, has to be high enough to break the hydrogen bonds between the complementary DNA strands. However, the polymerases needed for the amplification of DNA are enzymes and most enzymes are very sensitive to high temperatures. Fortunately, polymerases from bacteria living under extreme conditions have been isolated and found to be able to tolerate the high temperatures needed for denaturation. Therefore, the temperature has to be adapted depending on the polymerase used (commonly 94-98°C).

2. During the annealing step, the temperature of the reaction is lowered to allow specifically designed primers to bind (anneal) to the single stranded DNA by complementary base pairing. The primers used for standard PCR applications typically contain around 20 nucleotides (18-24) and a GC-content of 40-60%.

   The temperature in this step is dependent on the melting temperature (Tm) of the primers. The primer Tm is defined as the temperature at which 50% of the primers dissociate from the template DNA while the other 50% still form double strands with the DNA. Lowering the annealing temperature (Ta) will increase the percentage of primers bound to the specific binding sites but can also increase binding to nonspecific sites. Increasing the temperature lowers total primer binding but increases the specificity. Ta is generally chosen to be 3°C to 5°C below the Tm of the primer pair, although it is also dependent on the polymerase used. For most reactions it lies between 55°C and 65°C. The (theoretically) optimal Ta can be calculated using a mathematical formula, but even after carefully calculating the Ta for the PCR, scientists often have to determine the optimal Ta by performing a temperature gradient PCR experiment in which they can test different annealing temperatures in parallel.

PCR continued

3. After the primers have annealed to the DNA strands, the temperature is raised to the optimal working temperature of the polymerase at 68°C-72°C. During this extension/elongation step, the polymerase synthesises the new DNA strand, that was started by the primers, by sequentially adding free dNTPs as predefined by the template DNA.

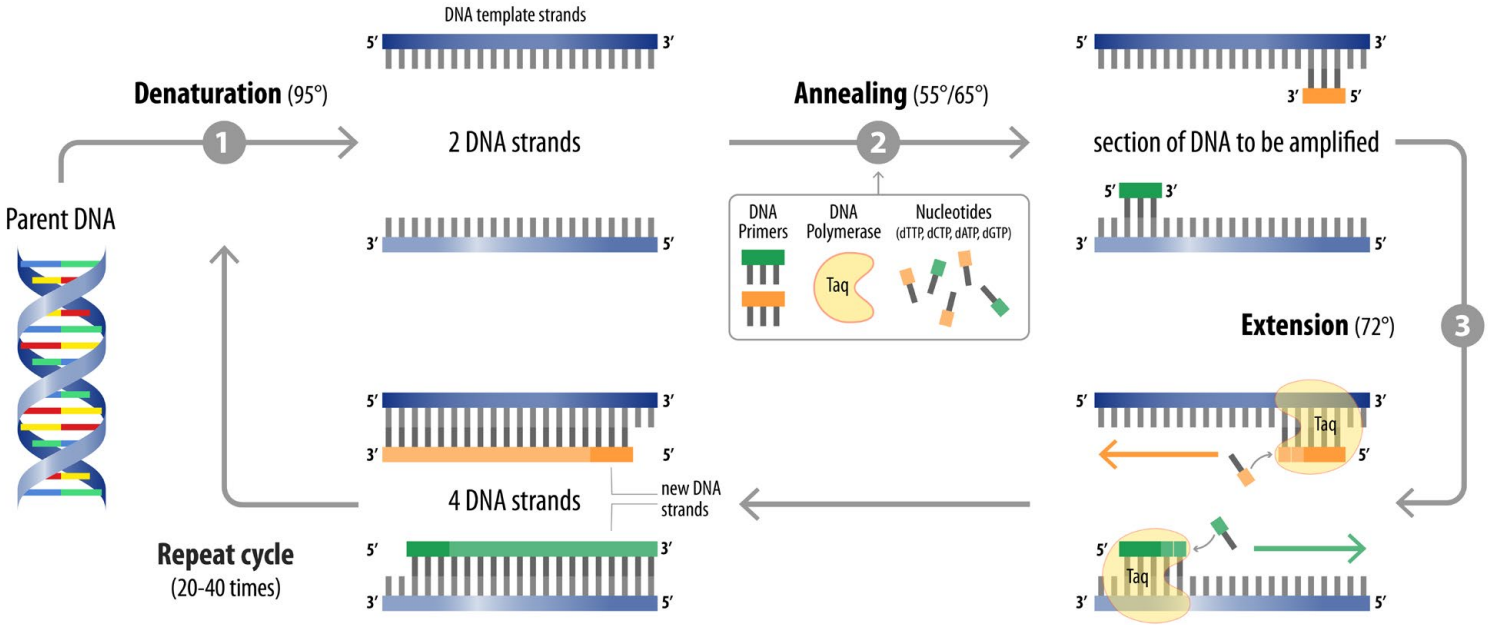


Figure 1: The PCR process.
The parent DNA is denatured (1) allowing the primers to anneal to their complementary sequences (2) before the new DNA strands are extended by the DNA polymerase (3). By repeating these steps, the DNA is amplified further. Please note that the temperatures given in this figure are those optimal for the use of Taq *polymerase. Other polymerases might require different temperatures.*

The sequence of denaturation, annealing and extension/elongation is then repeated several times and each time the amount of DNA is doubled, leading to an exponential increase of the amount of the desired PCR product. Unfortunately, polymerases are not perfect and can make mistakes like adding a wrong nucleotide. As a consequence, during every elongation step, there is the possibility of introducing mutations into the DNA. In deciding on the number of cycles, one therefore always has to find a balance between producing enough DNA and minimising the danger of introducing unwanted mutations, especially when the DNA is to be used in cloning experiments. Normally, the number of repetitions lies between 20 and 35 cycles.

To start and end the sequence of PCR cycles, they are flanked by an initial denaturation of 30 sec to 5 min at the denaturation temperature and a final extension of 5-10 min at the extension temperature (both dependent on the polymerase).

Resource

Video: "Polymerase Chain Reaction (PCR)", AK LECTURES; A 13-minute video that gives a detailed overview of the PCR process, https://www.youtube.com/watch?v=HmkNzMrhIFQ.

Primers

Primers are short nucleotide sequences that are complementary to an already existing DNA strand and serve as the starting point for DNA synthesis.

During DNA replication in cells, an enzyme, the so-called primase, synthesizes short RNA primers which are complementary to a piece of single-stranded template DNA. These primers are essential for the DNA polymerases to start synthesizing the complementary DNA strand since these enzymes can only add new nucleotides to an already existing strand of nucleotides. Before completion of DNA replication, the RNA primers are removed and the gaps are filled with DNA nucleotides.

In PCR reactions, primers are also essential for the DNA polymerases to start synthesizing a DNA strand. In contrast to primers synthesized by the primase in cells, the primers used in PCR experiments consist of DNA nucleotides. In order to amplify a certain DNA sequence, e.g. a gene, primer pairs that flank said sequence are used. The two primers are often referred to as the forward and the reverse primer. It is the standard in science to write down the nucleotide sequence of each primer starting from the 5' end.

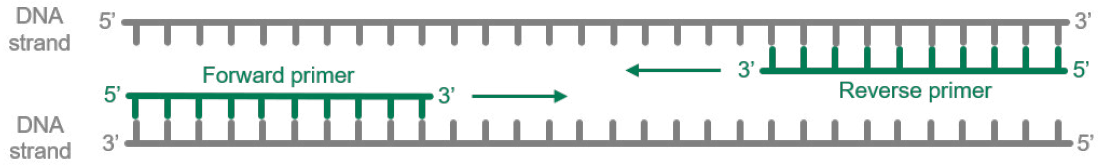


Figure 1: Primer pair bound to DNA.
Depicted are the two complementary DNA strands (grey) and the two primers bound to the strands (green). The arrow depicts the direction of amplification.

Within one single primer there should not be sequences that are complementary to each other, and the two primers used in one PCR reaction should also not be able to anneal to each other. This would create internal secondary structures ("hairpin" structures) and primer dimers, respectively, and could impede or disrupt the amplification process.

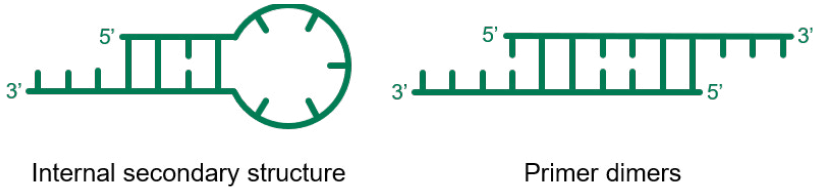
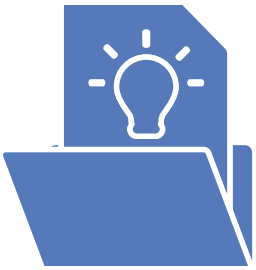


Figure 2: Secondary structures formed by PCR primers.
Similar sequences within single primers may cause internal secondary structures, also called hairpin structures. Similar sequences in the two primers of a primer pair might cause them to anneal with each other and form primer dimers. Both secondary structures can impede or disrupt the amplification.



Primer design

PCR primers strongly influence the performance of the PCR. Therefore, during primer design, it is very important to keep a close eye on properties like primer length or nucleotide composition.

PCR primers are complementary to the DNA flanking the sequence which is to be amplified and usually 18 to 24 nucleotides in length (optimally 20). They should contain 40-60% guanine and cytosine bases (GC content), since this content affects their melting temperature (T_m). The T_m of the primers is defined as the temperature at which 50% of the primers dissociate from the template DNA while the other 50% still form double strands with the DNA. The T_m of a primer is dependent on its length and nucleotide composition, because A and T form two hydrogen bonds with the template DNA, while G and C form three of them. Therefore, the energy it takes to dissolve the hydrogen bonds between A and T is lower than the one it takes to dissociate G-C bonds. The T_m of the primers should lie between 56°C and 65°C and to ensure that both the forward and the reverse primer perform comparably in a PCR reaction, the T_m of the pair should lie within 2°C of each other.

One frequently used formula to calculate the primer T_m is the following:

$$T_m = 4(wG+xC) + 2(yA+zT)$$

where w, x, y and z are the number of the respective bases within the primer.

NOTE: This formula is accurate only for sequences shorter than 14 nucleotides, but it is nevertheless often used to roughly estimate the T_m of primers longer than that.

Since calculating the exact T_m is more difficult and primers should possess a few important properties, scientists often use online tools to help them with picking optimal primers. One such example is the free online tool Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi).

Visualising DNA fragments using gel electrophoresis

To check the outcome of a PCR, gel electrophoresis is used.

At the end of the PCR, the reaction mix can be loaded on an agarose gel. These gels contain differently sized pores through which smaller DNA fragments can move faster than larger ones. Since DNA molecules are negatively charged due to the phosphate groups in the backbone, they will move towards the positively charged end when an electric field is established. Thereby, with these gels, DNA fragments can be separated based on their size.

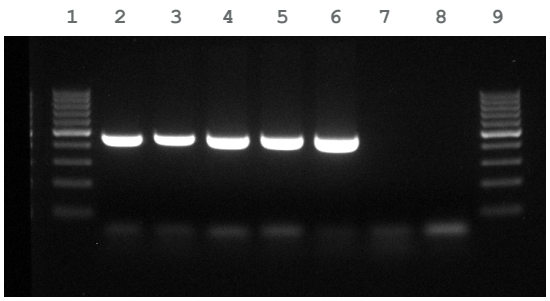


Figure 1: Image of an agarose gel to which PCR samples have been loaded
The image shows a gel with 9 lanes. Lanes 1 and 9 show bands of a DNA ladder with bands of known molecular weights (and thereby sizes); the ladder helps to determine the size of the bands visible in lanes where samples have been loaded. In lanes 7 and 8, the PCR results of reaction mixes in which no template DNA (lane 7) or no polymerase enzyme were added (lane 8). Consequently, no DNA bands can be observed in these lanes (negative controls).Lanes 2-6 show the result that was obtained after performing a PCR with 5 different template DNAs. The DNA products appear as bands on the gel. In all 5 samples, the PCR was successful. The faint bands at the bottom of the gel result from the PCR primers that had been added in excess and were not needed for amplification.

Additional resources

Video: "Restriction Map and Gel Electrophoresis", AK Lectures; An 8-minute video that explains the principles of gel electrophoresis and how differently sized DNA fragments will be visible on a gel, https://www.youtube.com/watch?v=afdP3eWFj3k.

Video: "Gel Electrophoresis", Amoeba Sisters; An 8-minute video explaining gel electrophoresis also giving examples for its usages. It also explains the use of DNA ladders as size markers, https://www.youtube.com/watch?v=ZDZUAleWX78.

Applications of PCR

Invented in the 1980s, PCR has become indispensable in research laboratories worldwide but also has important applications in clinical diagnostics, forensics and agriculture.

Examples for the most important applications of PCR include:

1. Clinical diagnostics

   - Testing for infectious diseases

     Viruses, bacteria and other pathogens contain DNA or RNA that can be detected using PCR. Currently, the most prominent example is testing for SARS-CoV-2 but it can be used to detect all kinds of pathogens.

   - Genetic testing
     - Detection of oncogenic mutations that cause cancer

       Cancer results from mutations in different genes, for example genes involved in cell growth or signalling. These mutations can then lead to uncontrolled growth and proliferation of the affected cells. Using PCR, cancer cells can be analysed to determine which mutations they have acquired. This has become an important step as the treatment of cancers gets more and more tailored to the exact set of mutations found in them (so-called personalised medicine). PCR also allows to detect tiny amounts of cancer cells or even free DNA of cancer cells in the blood that would go unnoticed by other diagnostic techniques.

     - Testing for inherited disorders

       Some diseases have their origin in DNA mutations that can be inherited. Examples of such diseases include for example b-thalassaemia or cystic fibrosis. To detect such disorders even before birth (prenatal diagnostics), PCR is used to amplify areas in the genome that are known to carry mutations responsible for these diseases. Also Chromosomal disorders like Trisomies can be detected and confirmed using PCR-based methods. In recent years, PCR even allowed the development of non-invasive prenatal tests that detect foetal DNA in the mother's blood.

2. Forensics

   Analysis of DNA samples that have for example been collected at the scene of a crime. They mostly rely on the amplification of unique short tandem repeats (STRs) to differentiate individuals. Similar to what is done in a paternity test.

3. Agriculture

   PCR plays an integral role in food pathogen detection, plant genotyping for breeding, and GMO testing.

Student Worksheet

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Making sense of PCR (paper-based)

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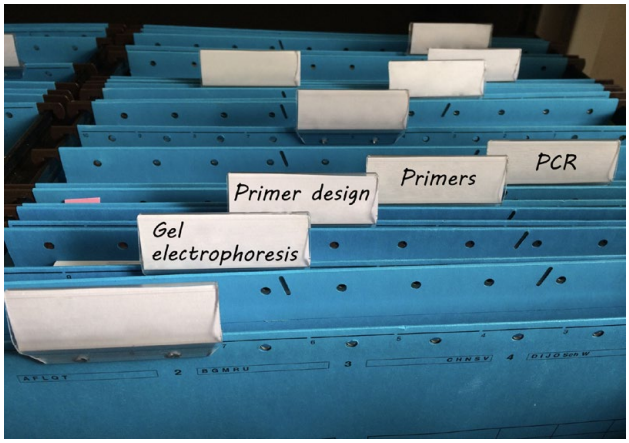
Making sense of PCR (paper-based)

In the laboratory, scientists are often faced with the task of amplifying a certain gene or stretch of DNA. This is for example the case, if they would like to detect a certain gene or the presence of its RNA transcript. In this activity, you will design a Polymerase Chain Reaction (PCR) experiment with the aim to amplify a viral gene from cDNA. This will help you identify people who have been infected with said virus.

If you wish to brush-up and increase your knowledge on everything related to the task, some Fact Sheets are at your disposal.



Materials

To execute the activity, you will need print-outs of the following materials:

- Discover-your-primer mask (see Appendix page 21)
- A data sheet for the ELLS ***Taq*** Polymerase (see on page 22)
- cDNA sequence of your gene of interest (RNA-dependent RNA polymerase of the SARS-CoV-2 virus[1]) (see on page 17)

1 Please note that only the sequence between base pairs 601 and 1500 are shown for simplicity. Length of the entire sequence of the gene: 2469 bp. Link to the entire SARS-CoV-2 sequence: https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2?report=fasta

Partial cDNA[1] sequence: RNA-dependent RNA polymerase

The sequence of the "upper" DNA strand is shown 5'→3'

```
601  TGTGCAAACTTTAATGTTTTATTCTCTACAGTGTTCCCACCTACAAGTTT  650
651  TGGACCACTAGTGAGAAAAATATTTGTTGATGGTGTTCCATTTGTAGTTT  700
701  CAACTGGATACCACTTCAGAGAGCTAGGTGTTGTACATAATCAGGATGTA  750
751  AACTTACATAGCTCTAGACTTAGTTTTAAGGAATTACTTGTGTATGCTGC  800
801  TGACCCTGCTATGCACGCTGCTTCTGGTAATCTATTACTAGATAAACGCA  850
851  CTACGTGCTTTTCAGTAGCTGCACTTACTAACAATGTTGCTTTTCAAACT  900
901  GTCAAACCCGGTAATTTTAACAAAGACTTCTATGACTTTGCTGTGTCTAA  950
951  GGGTTTCTTTAAGGAAGGAAGTTCTGTTGAATTAAAACACTTCTTCTTTG 1000
1001 CTCAGGATGGTAATGCTGCTATCAGCGATTATGACTACTATCGTTATAAT 1050
1051 CTACCAACAATGTGTGATATCAGACAACTACTATTTGTAGTTGAAGTTGT 1100
1101 TGATAAGTACTTTGATTGTTACGATGGTGGCTGTATTAATGCTAACCAAG 1150
1151 TCATCGTCAACAACCTAGACAAATCAGCTGGTTTTCCATTTAATAAATGG 1200
1201 GGTAAGGCTAGACTTTATTATGATTCAATGAGTTATGAGGATCAAGATGC 1250
1251 ACTTTTCGCATATACAAAACGTAATGTCATCCCTACTATAACTCAAATGA 1300
1301 ATCTTAAGTATGCCATTAGTGCAAAGAATAGAGCTCGCACCGTAGCTGGT 1350
1351 GTCTCTATCTGTAGTACTATGACCAATAGACAGTTTCATCAAAAATTATT 1400
1401 GAAATCAATAGCCGCCACTAGAGGAGCTACTGTAGTAATTGGAACAAGCA 1450
1451 AATTCTATGGTGGTTGGCACAACATGTTAAAAACTGTTTATAGTGATGTA 1500
```

Double stranded cDNA[1] sequence where you should look for the **forward primer**:

```
5'  TGACCCTGCTATGCACGCTGCTTCTGGTAATCTATTACTAGATAAACGCA  3'
3'  ACTGGGACGATACGTGCGACGAAGACCATTAGATAATGATCTATTTGCGT  5'
```

Double stranded cDNA[1] sequence where you should look for the **reverse primer**:

```
5'  ATCTTAAGTATGCCATTAGTGCAAAGAATAGAGCTCGCACCGTAGCTGGT  3'
3'  TAGAATTCATACGGTAATCACGTTTCTTATCTCGAGCGTGGCATCGACCA  5'
```

[1] cDNA stands for complementary DNA. To get cDNA, RNA sequences are copied using DNA nucleotides. This process is reminiscent of a reversed transcription, where RNA nucleotides are used to copy DNA, no wonder the enzyme capable of doing this is called Reverse transcriptase. The reason why scientists create cDNA is that it gives them a means to analyse the RNA sequence without actually having to work with the unstable RNA itself but they can instead work with the much more stable cDNA.

EXERCISE 1 Design PCR primers

First, you have to design primers for the PCR.

To find optimal primers, cut out the discover-your-primer mask on page 13. With this mask, move over the highlighted passages (in yellow) in the cDNA sequence on page 9 until you find a sequence that fulfils the requirements mentioned on the mask. You can also use the given double-stranded cDNA sequences to look for your primer sequence.

Always write down the primer sequence in 5’ to 3’ direction.

For more information on primers, refer to the “Primers” and “Primer Design” Fact Sheets.

A

What is the sequence of your forward primer?

Sequence forward primer (5’ to 3’)	

B

What is the sequence of your reverse primer?

Keep in mind that this primer has to bind to the other strand of the cDNA and you should note down the primer sequence from 5' to 3'. Here, looking for the primer in the double-stranded cDNA sequences might be helpful.

Sequence reverse primer (5’ to 3’)	

C

What is the melting temperature (Tm) of your primers?

Calculate the rough estimate using the formula Tm = 4(wG+xC) + 2(yA+zT).

T_m *forward primer*	°C
T_m *reverse primer*	°C

Based on the T_m of your primers, what would be the annealing temperature (T_a) you have to use in the PCR?

Annealing temperature (T_α)	°C

Can you find information on the recommended annealing temperature in the polymerase data sheet (page 14)?

D

How long will the amplified sequence (amplicon) be?

Length of your amplicon in base pairs (bp): ..... bp

EXERCISE 2 Design PCR programme

A

With this information and the polymerase data sheet, you can now design the PCR programme for the amplification.

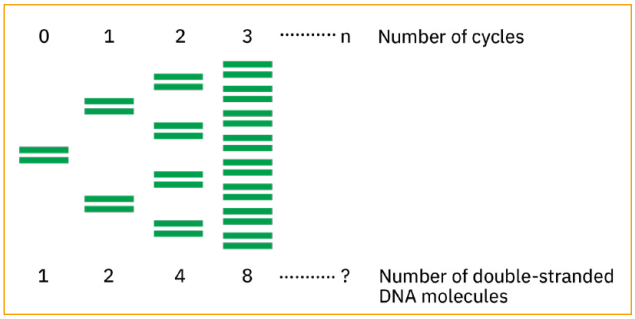
#	*Step name*	*Temperature in °C*	*Time in seconds*	*Number of cycle repetitions (if applicable)*
1	*Initial denaturation*			
2				
3				
4				
5	*Final elongation*			

B

Based on the specifications of your PCR programme, how long would it take for the PCR to run (in hours/minutes/seconds)?

C

The number of double stranded DNA molecules that are present after PCR and resulted from the amplification of one specific template DNA can be calculated.
When looking at the image below, can you figure out how the mathematical formula for n cycles looks like?



After the number of cycles you chose to use, how many double-stranded molecules will you get that resulted from one specific double-stranded cDNA molecule?

EXERCISE 3 Detect a viral infection

Now that you have designed your primers and PCR programme, you are ready to determine whether within a cohort of 6 individuals, someone has contracted the virus.

A

For every sample, you prepare a reaction mixture and run the PCR programme. You include one positive control (+) and two negative controls (-RT and -Taq).

The positive control contains a sample where you know that the gene is present. The -RT negative control contains a sample in which the RNA was not reverse transcribed into cDNA because no Reverse Transcriptase (RT) was added, while to the -Taq negative control cDNA was added but no *Taq* Polymerase. Therefore, if the PCR was successful, in the positive control the gene will be amplified and you should see a band on the gel, while there should be no amplification occurring in the negative controls.

Can you think of reasons why scientists always include negative controls?

.....

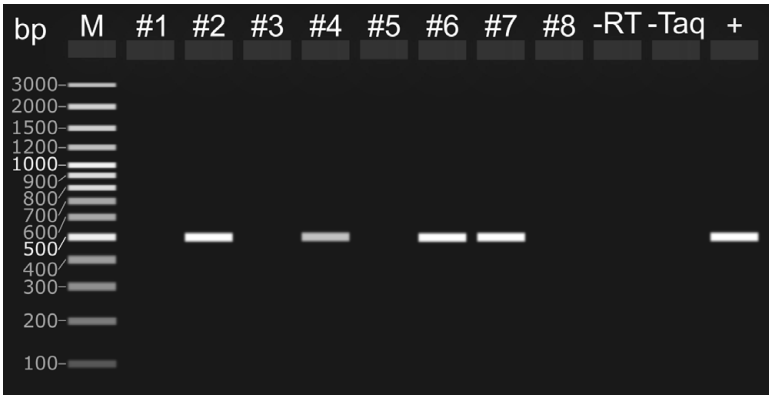
.....

.....

B

To determine whether the PCR result of an individual is positive (i.e. that the viral gene is present) or negative (i.e. that the viral gene is not present), you visualise the results of the PCR by running the mixture containing the PCR product on an agarose gel (see also the fact sheet "Visualizing DNA fragments using gel electrophoresis").

Image of the agarose gel:



M= *Marker (DNA ladder with differently sized DNA fragments). Fragment size is shown in base pairs (bp);*
#1-#8 *depict the samples taken from individual #1-#8;* **-RT** *= negative control without RT;* **-Taq** *= negative control without Taq Polymerase;* **+** *= positive control*

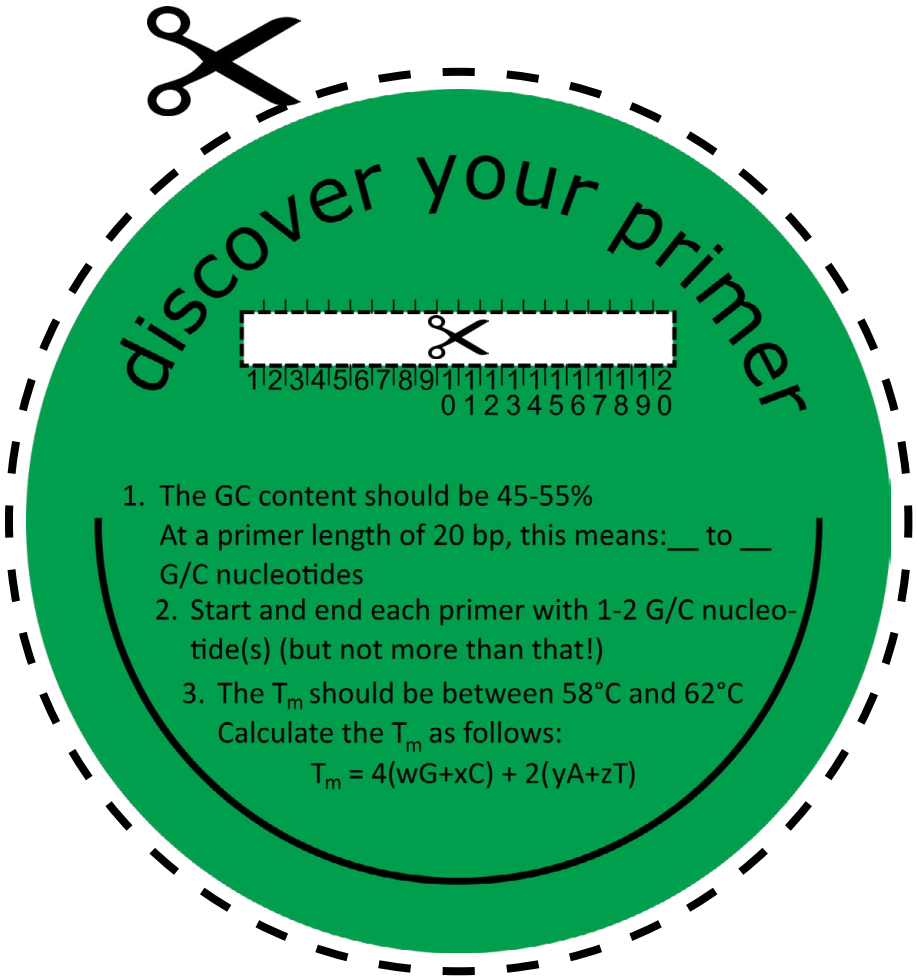
Judging from the outcome of the PCR, who has contracted the virus?

Individuals # .....

ANNEX

Discover-your-primer mask

Cut out the mask and the white window along the dashed lines.



ELLS *Taq* Polymerase

Description: *Taq* polymerase was the first temperature stable DNA polymerase isolated from bacteria living under extreme conditions (1). It has been found to be able to tolerate the high temperatures needed for denaturation of the DNA without denaturing itself and is often used in PCR (1,2,3). It has a 5'→3' polymerase activity and a 3'→5' exonuclease activity without proofreading ability (4).

Origin: *Thermus aquaticus*, a bacterium that lives in hot springs at 65-70°C. It can survive temperatures up to 80°C.

PCR

Polymerase Chain Reaction (PCR) allows the exponential amplification of very few or even single DNA molecules. To achieve this, the DNA molecules are subjected to several cycles of denaturation, primer annealing and DNA strand elongation/ extension. Taq polymerase is widely used in PCR.

Adhere to the following guidelines to ensure successful PCR using the ELLS Taq Polymerase.

Amplification of templates with high GC content, high secondary structure, low template concentrations, or amplicons greater than 5 kb may require further optimisation.

Thermocycling conditions for PCR:

STEP	TEMP	TIME
Initial Denaturation	*95°C*	*30 sec*
30 cycles	*95°C*	*15-30 sec*
	52-65°C	*15-60 sec*
	72°C	*1 min/kb*
Final Extension	*72°C*	*5 min*

General Guidelines:

1. Denaturation: For most amplicons, an initial denaturation of 30 seconds at 95°C is sufficient. During thermocycling, a 15–30 second denaturation at 95°C is recommended.

2. Annealing: The length of the annealing step is typically 15–60 seconds. The annealing temperature (Ta) is based on the Tm of the primer pair. Typically, it is chosen to be 5°C lower than the Tm. Annealing temperatures can be optimized by doing a temperature gradient PCR. You can also use tools like for example the NEB Tm Calculator, (https://tmcalculator.neb.com) to determine the appropriate annealing temperature for PCR.

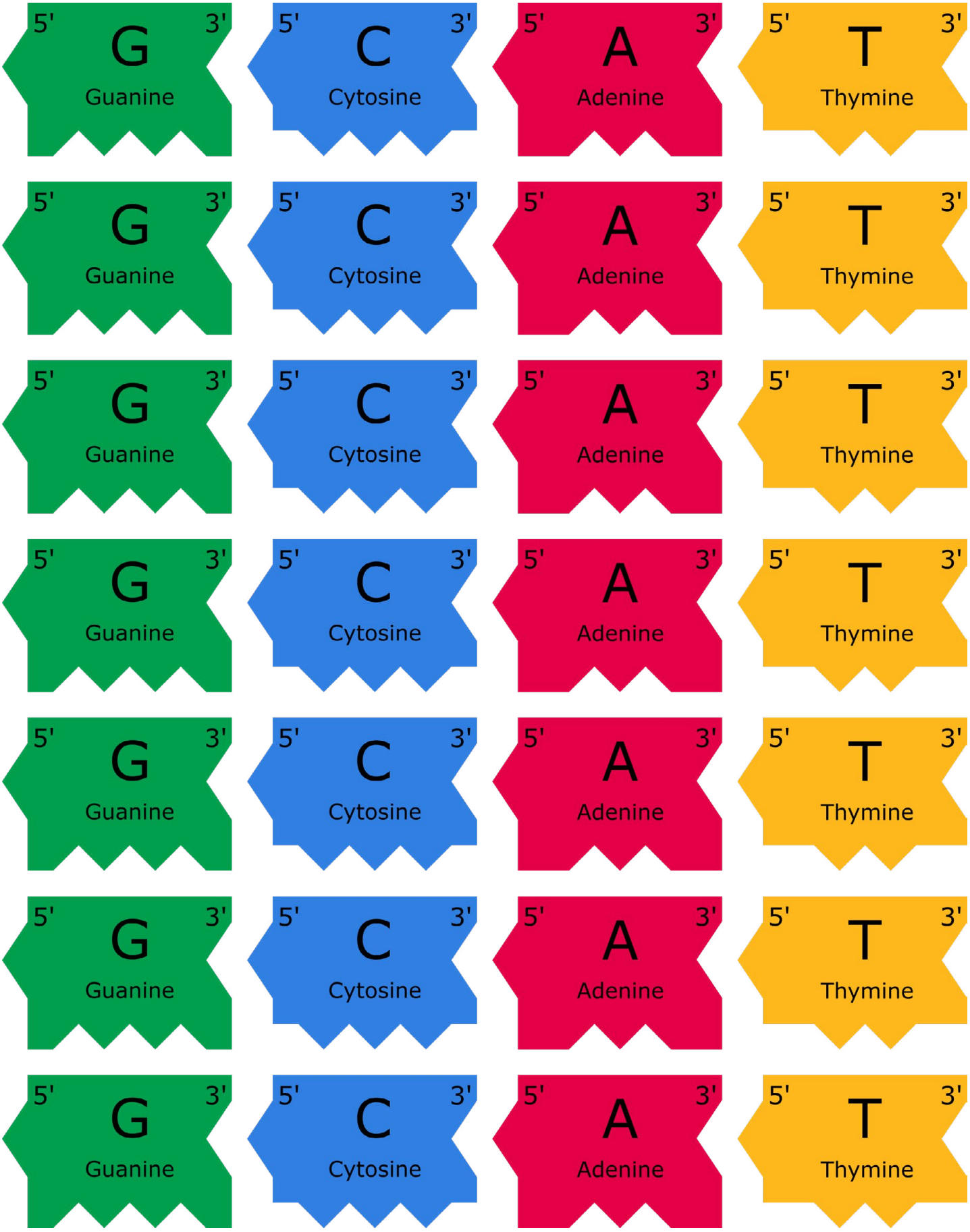
3. Extension: An extension temperature of 72°C is recommended. Extension times are generally 1 minute per kilo base pairs (kb) (=1000 base pairs). A final extension of 5 minutes at 72°C has to be included in the PCR programme.

4. Cycle number: Generally, 25–35 cycles yield sufficient product.

References:

1. Chien, A., Edgar, D.B. and Trela, J.M. (1976) J. Bact., 127, 1550–1557.
2. Lawyer, F.C. et al. (1993) PCR Methods and Appl., 2, 275–287.
3. Saiki, R.K. et al. (1988) Science, 239, 487-491.
4. Park, Y. et al. (1997), Mol. Cells, 7(3), 419-424.

NUCLEOTIDE PUZZLE



Answer Sheet

Copy that!

Making sense of PCR (paper-based)

Partial cDNA[1] sequence: RNA-dependent RNA polymerase

The sequence of the "upper" DNA strand is shown 5'→3'

```
601  TGTGCAAACTTTAATGTTTTATTCTCTACAGTGTTCCCACCTACAAGTTT  650
651  TGGACCACTAGTGAGAAAAATATTTGTTGATGGTGTTCCATTTGTAGTTT  700
701  CAACTGGATACCACTTCAGAGAGCTAGGTGTTGTACATAATCAGGATGTA  750
751  AACTTACATAGCTCTAGACTTAGTTTTAAGGAATTACTTGTGTATGCTGC  800
801  TGACCCTGCTATGCACGCTGCTTCTGGTAATCTATTACTAGATAAACGCA  850
851  CTACGTGCTTTTCAGTAGCTGCACTTACTAACAATGTTGCTTTTCAAACT  900
901  GTCAAACCCGGTAATTTTAACAAAGACTTCTATGACTTTGCTGTGTCTAA  950
951  GGGTTTCTTTAAGGAAGGAAGTTCTGTTGAATTAAAACACTTCTTCTTTG 1000
1001 CTCAGGATGGTAATGCTGCTATCAGCGATTATGACTACTATCGTTATAAT 1050
1051 CTACCAACAATGTGTGATATCAGACAACTACTATTTGTAGTTGAAGTTGT 1100
1101 TGATAAGTACTTTGATTGTTACGATGGTGGCTGTATTAATGCTAACCAAG 1150
1151 TCATCGTCAACAACCTAGACAAATCAGCTGGTTTTCCATTTAATAAATGG 1200
1201 GGTAAGGCTAGACTTTATTATGATTCAATGAGTTATGAGGATCAAGATGC 1250
1251 ACTTTTCGCATATACAAAACGTAATGTCATCCCTACTATAACTCAAATGA 1300
1301 ATCTTAAGTATGCCATTAGTGCAAAGAATAGAGCTCGCACCGTAGCTGGT 1350
1351 GTCTCTATCTGTAGTACTATGACCAATAGACAGTTTCATCAAAAATTATT 1400
1401 GAAATCAATAGCCGCCACTAGAGGAGCTACTGTAGTAATTGGAACAAGCA 1450
1451 AATTCTATGGTGGTTGGCACAACATGTTAAAAACTGTTTATAGTGATGTA 1500
```

Double stranded cDNA[1] sequence where you should look for the **forward primer**:

```
5'  TGACCCTGCTATGCACGCTGCTTCTGGTAATCTATTACTAGATAAACGCA  3'
3'  ACTGGGACGATACGTGCGACGAAGACCATTAGATAATGATCTATTTGCGT  5'
```

Double stranded cDNA[1] sequence where you should look for the **reverse primer**:

```
5'  ATCTTAAGTATGCCATTAGTGCAAAGAATAGAGCTCGCACCGTAGCTGGT  3'
3'  TAGAATTCATACGGTAATCACGTTTCTTATCTCGAGCGTGGCATCGACCA  5'
```

Highlighted in green: the area where the primers are located

EXERCISE 1 Design PCR primers

A

What is the sequence of your forward primer?

Sequence forward primer (5' to 3')	**GCACGCTGCTTCTGGTAATC**

B

What is the sequence of your reverse primer?

Sequence reverse primer (5' to 3')	**GTGCGAGCTCTATTCTTTGC ;** **GGTGCGAGCTCTATTCTTTG ;** **CTACGGTGCGAGCTCTATTC ;**

C

What is the melting temperature (Tm) of your primers?

T_m *forward primer*	**62**	*°C*
T_m *reverse primer*	**60-62**	*°C*

Based on the T_m of your primers, what would be the annealing temperature (T_a) you have to use in the PCR?

Annealing temperature (T_a)	**55-57 °C (use lower T_m to calculate)**

D

How long will the amplified sequence (amplicon) be?

Length of your amplicon in base pairs (bp): **526-533 bp**
(depending on the reverse primer)

EXERCISE 2 Design PCR programme

A

With this information and the polymerase data sheet, you can now design the PCR programme for the amplification.

#	Step name	Temperature in °C	Time in seconds	Number of cycle repetitions (if applicable)
1	*Initial denaturation*	95°C	30 sec	
2	Denaturation	95°C	15 sec*	30§
3	Annealing	55°C#	30 sec&	
4	Extension/ elongation	72°C	30 sec$	
5	*Final elongation*	72°C	300 sec	

*: 15-30 sec; &: 15-60 sec; #: 5°C below the T_m of the primers (always use the lower T_m as reference); $: 1 min/kb → 31 sec/523bp; 32sec/533bp; 30 sec is fine as well!; §: the whole sequence of denaturation/annealing/extension is repeated 30 times

B

Based on the specifications of your PCR programme, how long would it take for the PCR to run (in hours/minutes/seconds)?

In this example: 2580 sec = 43 min

(depends on the times the students choose)

C

The number of double stranded DNA molecules that are present after PCR and resulted from the amplification of one specific template DNA can be calculated.
When looking at the image below, can you figure out how the mathematical formula for n cycles looks like?

The formula for all double stranded molecules is $x=2^n$.

To calculate only those that are only made up of newly copied strands, it is $x=2^n-2n$.

After the number of cycles you chose to use, how many double-stranded molecules will you get that resulted from one specific double-stranded cDNA molecule?

The number of cycles is n=30.

→ $x = 2^n = 2^{30} = 1.073.741.824$ double-stranded molecules

EXERCISE 3 Detect a viral infection

A

The positive control contains a sample where you know that the gene is present. The -RT negative control contains a sample in which the RNA was not reverse transcribed into cDNA because no Reverse Transcriptase (RT) was added, while to the -Taq negative control cDNA was added but no *Taq* Polymerase. Therefore, if the PCR was successful, in the positive control the gene will be amplified and you should see a band on the gel, while there should be no amplification occurring in the negative controls.

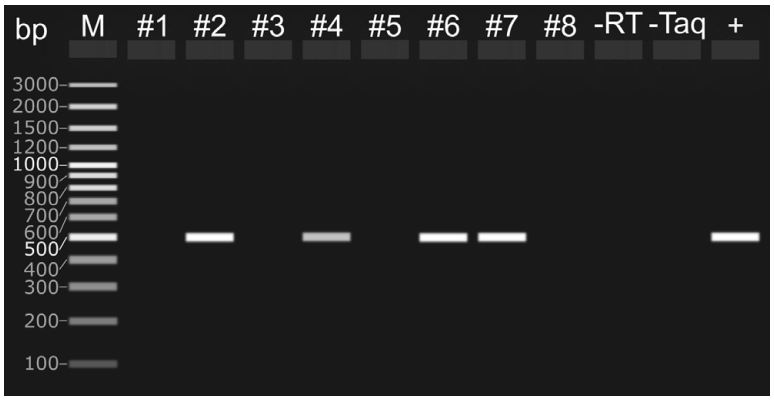
Can you think of reasons why scientists always include negative controls?

Generally, negative controls are immensely important because they show researchers that when they get a positive result for a sample, this is not due to contaminations of any sort.
-RT control: to test whether the RNA and thereby later the cDNA was contaminated by genomic DNA
-Taq control: to test whether there is any polymerase activity or contamination with DNA in the components used for the PCR reaction mixture.

B

To determine whether the PCR result of an individual is positive (i.e. that the viral gene is present) or negative (i.e. that the viral gene is not present), you visualise the results of the PCR by running the mixture containing the PCR product on an agarose gel (see also the fact sheet "Visualizing DNA fragments using gel electrophoresis").

Image of the agarose gel:



Judging from the outcome of the PCR, who has contracted the virus?

Individuals # **2, 4, 6, 7**

NOTES

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