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Review Article

Current Detection of Viral Genomes and their Variants

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Abstract

Detection and determination of some viruses and the knowledge of their genome is inextricably linked to a reaction carried out by a thermostable enzyme: *Polymerase Chain Reaction* (PCR) that was visualized during the 1980s in the United States and its invention has been so important that some separated in time in reference to advances in Medicine/Biology as before and after the PCR. The foregoing, without a doubt, constitutes an immeasurable advance in Human and Animal Medicine. It would be enough to turn on some audiovisual news media or read a written medium to find out how the real epidemic caused by SARS-CoV-2 called COVID19 has developed and the detection of the viral agent or how we have found out about the current omicron variant.

Keywords: Nobel Prize, PCR, viral genomes, COVID19

Introduction

Currently, those who do not know the PCR developed by Kary Mullis are considered illiterate, since it is a molecular technique that came to stay just like COVID 19. Unlike the current disease, PCR is a molecular tool that allows a DNA fragment of interest to be amplified to a level of at least one billion copies (some refer to it as a photocopier...!).

This molecular technique is so magnificent that the Swedish Academy of Sciences had no choice but to award Kary Mullis the Nobel Prize in 1993. Dr. Mullis had several detractors, because he was an atypical scientist and in his youth he used LSD and he even once mentioned that the idea of PCR was advised to him by a green raccoon… (LSD: psychedelic substance that produces special effects).

He was also in love with surfing, as can be seen in his book Dancing Naked In The Mind Field [2] and according to his indications, a small amount of DNA from a problem sample is enough to amplify a gene or part of a gene present in the sample (Figure 1).

The product, double-stranded DNA, is easily detected and today is the technique of choice for the detection of pathogens of veterinary and/or human interest.

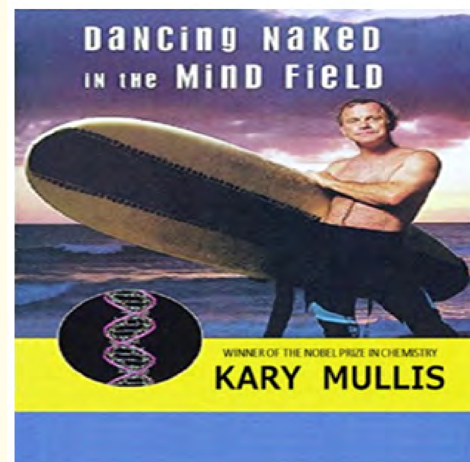

Figure 1. Book of Kary Mullis

It required?

Following his brilliant idea and summarizing, it is necessary to include in a test tube (today Eppendor tubes, 0.2 mL) the sample (DNA), the four nucleotides (A,T,C,G), a thermostable Polymerase (originally Taq Polymerase), an enzyme cofactor (Mg^{+2}) and the primers of the PCR reaction [3].

As can be seen, Dr. Mullis's idea was always to emulate the duplication of DNA that occurs in the cell of a living organism, but without the participation of the notorious enzymatic system involved. For Mr. Mullis, everything can be done with changes in temperature. Thus, to denature double-stranded DNA, apply heat (94°C), then allow primer alignment (X°C) and finally elongation (72°C). So far a cycle has been completed. The genius of Kary Mullis indicates that the process should be repeated about 30 times [3]. Thus, the product is amplified a billion times, according to the formula $P = 2^n$ (Figure 2).

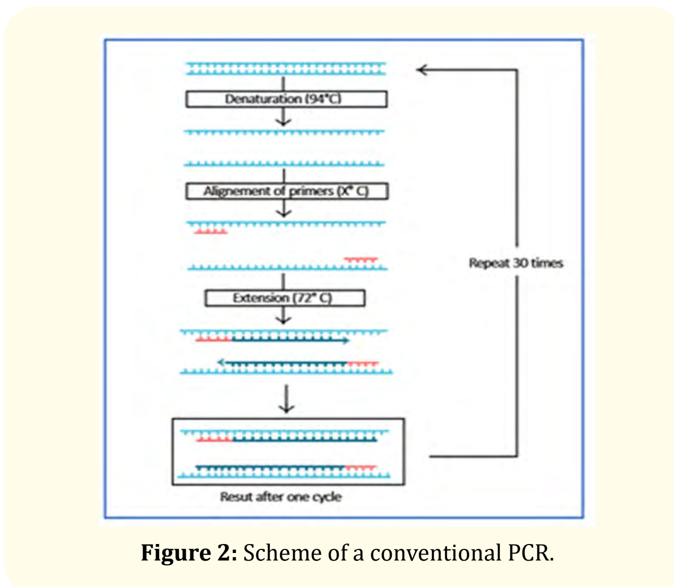

Figure 2: Scheme of a conventional PCR.

The detection of positive samples can be performed by nucleic acid electrophoresis, that is, by the migration of biomolecules (DNA fragments) in an electric field and subsequent "revealing" of the gel by means of a chemical agent that binds to the DNA and "fluorescent" when hit by ultraviolet light. Another of the current facilities to carry out a PCR is the existence of a programmable machine to carry out the proposed temperature changes: the thermocycler. Both the temperature (X°C) and the nucleotide sequence of the primers are specific for the pathogen to be detected.

Primers? What's that? A primer is an oligonucleotide, that is, it corresponds to a chemical compound consisting of about 20 nucleotides and the characteristics of an optimal primer will be described later, but it should be mentioned that its sequence must be complementary to that of the sample DNA [4,5]. There are variants of this technique, such as nested PCR, which corresponds to two serial PCR reactions, and multiplex PCR, which considers the use of two or more pairs of primers in a single reaction. Additionally, this technique allows detecting RNA if the conditions are previously granted so that, by means of a reverse transcriptase, DNA can be synthesized from RNA. This technique is called RT-PCR and is mainly used to detect viral pathogens whose genome is RNA, such as canine distemper virus or SARS-CoV-2 [6].

Conditions for preparing the PCR primers

What are the primers? First, let us remember that primers are chemical reagents that correspond to oligonucleotides, around twenty nucleotides (nt) linked, whose main characteristic is to be complementary (A-T, C-G) to a defined area in the genome of the pathogen to be detected.

In other words, its nucleotide sequence was designed with that main characteristic, but it must meet others as the optimal primer to be used in a PCR (or RT-PCR) reaction [4,5].

In each choice of primers, the % of GC that each one has must be considered, as well as the melting temperature (Tm). In the case of the % of GC, it should be close to 50% and the Tm should not vary by more than 3°C. Consider the following example (Table 1).

Primer	Sequence	Size (nt)	%GC	Tm (°C)
P1	ATCCCTCGGCATCTATTACCG	21	52	60.05
P2	TCCAGGCTTATACGTCTATC	20	45	61.05
P3	TCTGAGTCAATTGGCCGTAG	20	50	60.05
P4	GATGCCGGTTAACTGAGTCT	20	50	61.06

Table 1: Four sequences of *primers* from software

Under these conditions, the optimal pair would be P3 and P4 due to a GC% closer to 50%, which defines the case, since the Tm are similar and do not have a major influence.

To perform a PCR, primers must be available and there are two possibilities

Choose primers from a previous article, published in a scientific journal or design them ourselves. In both cases, what is indicated should be fulfilled, with the difference that in the article the sequence is named and the rest is omitted. When the sequence is available, it is copied in word format and sent to a commercial

company whose business is that, to synthesize oligonucleotides on request. In our country, after 15 days, they are available, paying in advance to a man who brings them by motorcycle, that is, at room temperature (Figure 3).

Figure 3: Arrival of *primers*

They arrive in an envelope that contains two tubes, in solid state (lyophilized) and with a label that indicates the amount contained in each tube. In this example, 33.1 nmoles (the other label probably says something else). What is done now? After paying, the content of each tube must be solubilized by adding nuclease-free water (NFW), considering that value measured in nmoles.

Figure 4: *Primers* concentration

Recipe

The amount indicated in nmols (in this case 33.1; Figure 4) must be multiplied by ten, which results in 331. Then, that value, measured in microliters (uL) of NFW, must be added to the tube. It should be shaken gently. The tube now contains the primer in a soluble state, but at a concentration of 100 uM (stock solution), very concentrated for a PCR, therefore another more diluted solution must be prepared as follows: in another Eppendorff-type tube (2 mL), 900 uL of NFW are added and 10 uL are taken from the stock solution, thus obtaining a working solution for PCR at a concentration of 1 uM, suitable for a PCR reaction. Trust me for now. The stock solution (100 uM) is stored at -20°C until further notice and the working solution (1 uM) at 4°C [7].

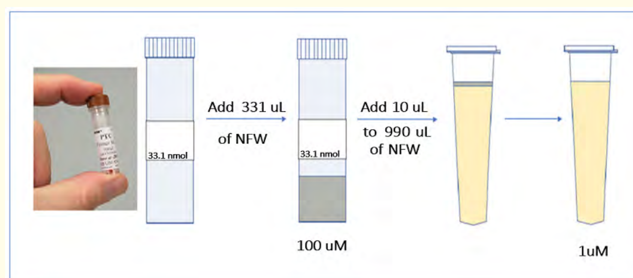

Figure 5: *Primers reconstitution*

Summary

You have 321 uL of a stock solution (frozen) and 1000 uL of working solution at 4°C. Oh.! The tube containing the other primer follows the same sequence described, but with the corresponding value in nmoles. A typical PCR reaction involves placing in the mixing tube: 5 uL of sample (DNA), 15 uL of Master Mix, 5 uL of each primer, generating a final volume of 30 uL.

The Master Mix comes from the factory at double (2X) concentration of what is needed in the PCR reaction. Therefore, the amount mentioned above is correct, since 15 uL are added for a final value of 30 uL.

Questions

If 5 uL of each primer are used… In theory, for how many PCR reactions is the content of the working tube sufficient? According to what has been described, what concentration of the primer is reached in the reaction tube?

Sample identification

The genome is generally DNA except for one type of virus: the Ribovirus whose genome is RNA (existing single-stranded and double-stranded). Knowing the foregoing and the existence of the Genbank® database [8], today there are no excuses for the detection of any pathogen or for identifying which genotype would belong (for example).

The identification of the genome begins with its detection, using the incredible occurrence of Kary Mullis, obtaining double-stranded DNA fragments by means of the appropriate PCR (RT-PCR in the case of most RNA viruses). Once these fragments have been obtained, it is necessary to know their nucleotide sequence and due to the inevitable errors inherent in the laboratory, it is preferred to send each sample to be sequenced in triplicate (minimum). Thus, for each sample three (at least) sequences are received. So, it is necessary to know the "average" sequence known as the consensus sequence and for that the Clustal Omega software can be used [9]. When entering the sequences into the program, an alignment is obtained, which will indicate by means of a "*" where there is 100% nucleotide identity for each nucleotide position, for example:

```
s2        TTAATTATGCCAGAAAGTGAAAATTTTAAAAGAGTTGTTGTAAATAATATGGATAAAACT    60
s1        TTAAATATGCCTGAAAGTGAAAATTATTAAAGAGTAGTTGTAAATAATATGGATTAAACT    60
s3        TTAAATATGCCAGAAAGTGAAAATTATAAAAGAGTAGTTGTAAATAATATGGATAAAACT    60
          **** ****** ************* * ******* ***************** *****
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Figure 6. Clustal Omega results

Here, S1, S2 and S3 correspond to the sequences of the same sample and the number of asterisks show that there is "a great resemblance" between the sequences (obviously, asterisks should appear in all positions: it is the same sample). We must build the consensus sequence by assigning a nucleotide in the place where the "*" does not appear, considering the one that is repeated the most in that position, in this case it corresponds to:

"Consensussequence"

TGAAAATTATAAAAGAGTAGTTGTAAATAATATGGATAAAACT

This program (Clustal Omega) can align several sequences at once and each sequence can be of variable length. The constructed consensus sequence represents the work done in the laboratory, but.... What does it correspond to? Does it correspond to the genome we want to detect? ...

The next stage contemplates the use of another free online software up to now: BLAST, a very powerful tool that indicates the percentage of nucleotide identity (PIN) of the entered sequence with respect to ALL the available database [10]. That is, it tells us with which genome it has the greatest "coincidence". In this output, the program shows the first 100 alignments and each nucleotide identity percentage (NIP). For example, in this case, inputting the newly constructed consensus sequence to BLAST yields the following output.

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — New Select columns — Show 100

select all 100 sequences selected — GenBank — Graphics — Distance tree of results — New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Feline panleukopenia virus isolate Giant panda/CD/2018 capsid protein (VP2) gene, complete cds	Feline panleukopenia virus	106	106	100%	2e-19	98.36%	1755	MZ322607.1
Feline panleukopenia virus isolate 18Q234-1 capsid protein (VP2) gene, partial cds	Feline panleukopenia virus	106	106	100%	2e-19	98.36%	1752	MW035310.1
Feline panleukopenia virus isolate 19SP_CK-8 capsid protein (VP2) gene, partial cds	Feline panleukopenia virus	106	106	100%	2e-19	98.36%	1752	MW035309.1

Figure 7: BLAST results

That is, the consensus sequence has a NIP = 98.36% with respect to the Feline Panleukopenia virus, referring to several viral isolates and strains described in the Genbank® database. Only for reasons of space, the remaining 90 results are not shown, but the last 10 results indicate a PIN of 98.36%. Undoubtedly... it is the virus already mentioned...!!!

Feline panleukopenia virus isolate SH-121 capsid protein VP2 gene, complete cds	Feline panleukopenia virus	106	106	100%	2e-19	98.36%	1755	MW017627.1
Feline panleukopenia virus isolate SH-120 capsid protein VP2 gene, complete cds	Feline panleukopenia virus	106	106	100%	2e-19	98.36%	1755	MW017626.1
Feline panleukopenia virus isolate SH-118 capsid protein VP2 gene, complete cds	Feline panleukopenia virus	106	106	100%	2e-19	98.36%	1755	MW017625.1

Figure 8: Results corroboration

And what does a NIP = 98.36% mean? In this case that of 100 nucleotides of the sequence, that 98 are the same and are in the same place in the sequence as the official Genbank® data. Thus, in our country we have implemented diagnostic methods for viruses that affect small animals [11-14] and it is the same methodology that is applied to pathogens that affect humans

Conclusion

Although the brilliant idea of Kary Mullis has divided biology into before and after PCR, it also opened the real possibility of a faster, more specific and sensitive diagnostic method for viral, bacterial or parasitic pathogens, and that together with some software -even free- have generously contributed to their identification.

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