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FACTSHEET PCR

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"His (note: Kary Mullis) invention is highly original and significant, virtually dividing biology into the two epochs of before P.C.R. and after P.C.R." [1]

HISTORY - **P**olymerase **C**hain **R**eaction (PCR) allows rapid and exponential amplification of millions to billions of copies from an extremely small amount of any DNA sequence of interest. Based on the concept of "repair replication [2]", **Kary Mullis** (December 28, 1944 - August 7, 2019) extended and improved the technique by applying repeated thermocycling. In 1993, he was awarded the Nobel Prize in Chemistry, together with Michael Smith. Later, a heat stable DNA polymerase was incorporated into the process.

"With PCR, if you do it well, you can find almost anything in anybody." [3]

PRINCIPLE - PCR comprises a sequence of **30-35 DNA amplification cycles**, each of which consists of the following three steps:

1. **Denaturation** (94-98 °C): Separation of the double-stranded DNA template into two single DNA strands.
2. **Annealing** (60-65 °C): Binding of a specific primer pair representing short single-stranded DNA starter sequences located at both ends of the DNA sequence of interest and thus defining the "target" to be amplified.
3. **Extension** (72 °C): Elongation of the primer starter sequences by DNA polymerase, the enzyme that copies DNA in cells, finally resulting in the DNA target sequence complementary to that of the DNA template sequence.

The DNA product of each cycle serves as starting material for the following cycle, thus enabling exponential amplification of the initial target sequence. Due to its specific size, the PCR end product can be analyzed on an agarose or a polyacrylamide gel.

In the **COVID-19 PCR tests**, **real-time PCR** is used for the detection of specific SARS-CoV-2 sequences, which involves a third primer. This so-called "probe" is located between the flanking primer pair and labelled with an inactive fluorescent dye. During each extension step, DNA polymerase destroys the "probe", resulting in the release of a measurable fluorescent signal.

- The **Cycle threshold (Ct)** is the cycle number after which the fluorescence linked to the amplified PCR product can be specifically detected above the background signal. The Ct-value is inversely related to the initial amount of the target, so **the lower the Ct-value, the more target material was initially present**.
- For a valid **quantitative PCR (qPCR)**, an external standard with a known quantity of target DNA, with which the Ct-value of the sample can be compared, is an absolute requirement.
- For the analysis of single-stranded RNA (e.g. in coronaviruses), RNA must first be reversely transcribed (RT) into DNA before it can be subjected to PCR. This is known as **RT-PCR**.
- Due to the high sensitivity of any type of PCR, it is of crucial importance to avoid any kind of contamination, as **contaminating DNA will be amplified as effectively as the target DNA**. In

a worst-case scenario, a new PCR sample may already be contaminated with amplified targets from a previous PCR.

Importantly, its high sensitivity makes PCR an excellent technique for analyzing extremely small amounts of DNA (e.g. in forensics), however, it also opens the door for multiple errors and outright deception if the method is not properly used (i.e. unspecific testing of healthy individuals), or if its results are not properly interpreted (i.e. irrational summation of PCR-positive "cases").

"To date, no diagnostic tests exist that reliably determine the presence of infectious virus." [4]

It is claimed that a PCR test-positive "case" represents an infectious individual, but this is NOT TRUE

→ **Here are the FACTS**

- **Sample preparation** prior to any type of PCR **requires complete break-up of biological structures** to separate nucleic acids from proteins, lipids and cell debris. To this end, the samples are treated with a mixture of highly toxic chemicals [5], completely destroying any complex organism.
- Irrespective of any protocol design, **PCR can exclusively test for the presence of the target sequence(s)** selected by specific primers, **which represent(s) only a small part of the entire viral genome**. Even when performed properly, PCR can by no means prove that an infectious virus with an intact genome is present in an analyzed sample.
- **Any positive PCR test result considered on its own cannot prove the presence of a replication-competent, infectious virus.** It solely represents a molecular indicator that confirms or rejects an initial suspicion, but requires further differential diagnosis by a clinician within the context of the patient´s symptoms - as is the case with any other laboratory assay!
- Importantly, **symptoms of respiratory tract infections may be caused by a variety of different viruses** and even bacteria, fungi or protozoa, alone or in combination. In general, differential diagnosis is based on clinical symptoms involving additional laboratory assays, e.g. the growth of a causative bacterium in culture. **Testing for virus subtypes** may be of scientific interest, but **has no relevance in a routine clinical setting**, as anti-virus therapies are mostly symptomatic only, independent of virus subtypes or combined viruses. This is clearly different to bacteria (i.e., antibiotics), fungi (i.e., antimycotics) or protozoa (i.e., antibiotics).
- Consequently, **multiplex testing for a broad range of pathogens is mandatory to differentiate between distinct pulmonary infections manifested in similar clinical symptoms** and also to differentiate between different families of pathogens or, for the sake of surveillance, to evaluate the currently circulating pathogen(s) [6]. Due to the high sensitivity of PCR, it is possible to detect RNA/DNA sequences of an under-represented pathogen within a complex pathogen mixture, as was in fact reported for two of the first five COVID-19 patients [7]. However, it is also possible to miss a pathogen entirely when specific PCR probes for this pathogen are not included in the test.
- Finally, there remains the risk of false-positive and false-negative results that can arise from both technical and clinical errors. **PCR-positive individuals do not necessarily transmit or even carry an intact virus.** This discrepancy becomes obvious among the PCR positive-tested, but asymptomatic healthy individuals displaying low initial target numbers and high Ct-values in their test readout. The vast majority of such individuals will neither carry the pathogen nor be infectious. The low amount of viral nucleic acids detected may either constitute remnants of a

past infection or, more likely, constitute a false-positive test result due to improper sample handling and contamination.

It is claimed that PCR represents the "new gold standard" for testing infectiousness, but this is NOT TRUE → Here are the FACTS

- **The gold standard for determining the infectious viral load is represented by the reproducibility of the virus of interest in a proper cell culture** [4]. Since the handling of infectious viruses requires special safety precautions, such tests may only be carried out in specialized laboratories and therefore cannot be used in routine diagnostics.
- In routine diagnostics, **PCR can be performed as a substitute** to provide the doctor with a decision-making molecular marker for the best possible further treatment of the patient. Importantly, **the PCR test result must not be understood as a simple yes-or-no answer, but requires the specification of a Ct "cut-off" value** that must be determined in advance by a specialized laboratory (comparing PCR results with results from cell culture) and, subsequently, must be adjusted by the laboratory performing the PCR test (comparing PCR results with results from external standards containing inactivated virus with known concentrations).
- While clinical testing aims at high sensitivity to confirm or rule out a suspected infection in a symptomatic individual, **during an epidemic specificity is however more important than sensitivity** [8], in order to avoid sending healthy people with false-positive test results into unnecessary quarantine.
- High sensitivity goes hand in hand with a severe bottleneck in the performance of PCR. Even in the case of a 100% test specificity, which means that there are no false-positives, this only means that no sequences other than the selected target sequence(s) will be amplified. However, in real world testing, **contaminants and handling errors will unavoidably result in the generation of some false-positive test results**.
- As already indicated above, absolute quantification of a defined viral load present in a specific sample requires qPCR, involving a dilution series of known quantities of inactivated virus. Subsequently, the Ct-value of an unknown sample can be correlated with the Ct-values of the dilution series and the quantity of virus particles can be estimated to determine the viral load. **A positive PCR signal as such does not permit us to make inferences about a possible infectious viral load if no Ct-value is provided**, and if the result is not specifically related to a defined standard curve [9].
- Since reverse transcription, priming conditions and secondary structures at the primer binding sites represent stochastic processes, **the Ct-value may vary in a small range between different PCR approaches and different laboratories**. Therefore, reference genes of defined amounts are mandatory to measure relative quantification between various laboratories.
- A possible replication activity of a virus within a tested individual may be proven by a RT-PCR assay that is based on the detection of subgenomic RNA (sgRNA), which will solely be generated during virus replication in infected cells. However, as sgRNA can be detected even days and weeks after infection, absence of sgRNA indicates absence of viral replication, while presence of sgRNA does not necessarily indicate infectiousness [10].

It is claimed that PCR mass tests represent a suitable tool for surveilling viral dissemination and population health, but this is NOT TRUE → Here are the FACTS

- As PCR is not able to detect or predict whether a positively tested individual carries or can transmit an intact virus, **PCR-based laboratory assays should never be used for surveilling an asymptomatic healthy population with the aim of simply detecting nucleic acid sequences of any pathogen**.

- **Any laboratory assay**, even when exhibiting both high specificity and high sensitivity, **will generate false-positives that may even outnumber true-positives when prevalence (number of really infected persons) is low**, as is exactly the case when performing mass testing of asymptomatic individuals [11].

- **Positive-tested, healthy individuals typically present low initial target numbers** associated with high Ct-values. Even in the case that the test readout is correct, **these individuals will not be infectious, but represent clinical false-positives** comprising either recovered individuals, who still show viral remnants, or immune individuals, who are not contagious due to a low viral load [12].

- Hardly discussed is the fact that **high throughput (due to unspecific mass testing)** of always the same PCR in a laboratory will increase the risk of handling errors and generate huge amounts of aerosol [13], which may contaminate the subsequent PCR approach and **result in increased false-positive test results**, which indeed was found to be the case in COVID-19 testing.

- The only approach that would drive false-positives to zero requires performance of **Sanger sequencing** [14], which is in fact **recommended by the WHO** [15]. But even this **would not eliminate false-positives due to contamination**.

It is claimed that new virus subtypes generally represent increased infectiousness and lethality, but this is NOT TRUE → Here are the FACTS

- New virus subtypes are characterized by small changes in their nucleic acid sequence, which are NOT necessarily associated with increased infectiousness. Additionally, increased infectiousness is NOT necessarily associated with increased lethality. By contrast, **evolutionarily successful viruses tend to become more infectious, but less lethal with each successive mutation**.

- **New virus subtypes may NOT be new to our immune system**, as previous contact(s) with viruses of the same virus family may have already generated cross-immunity. This was indeed demonstrated for SARS-CoV-2 in stored blood donor samples from the pre-COVID-19 era [16,17,18].

References [1] https://www.nytimes.com/1998/09/15/science/scientist-at-work-kary-mullis-after-the-eureka-a-nobelist-drops-out.html, [2] https://doi.org/10.1016/0022-2836(71)90469-4, [3] https://www.facebook.com/nico.davinci.56/videos/1749227998568399/, [4] https://doi.org/10.1038/s41579-022-00822-w, [5] https://doi.org/10.1006/abio.1987.9999, [6] https://doi.org/10.1002/14651858.CD013665, [7] https://doi.org/10.1097/CM9.0000000000000722, [8] https://doi.org/10.1007/s10441-020-09393-w, [9] https://doi.org/10.1016/j.jviromet.2021.114102, [10] https://doi.org/10.15252/emmm.202115290, [11] https://doi.org/10.1016/j.cmi.2020.10.003, [12] https://doi.org/10.1136/bmj.m3862, [13] https://pubmed.ncbi.nlm.nih.gov/15648778/, [14] https://publichealthpolicyjournal.com/volume/v4-2019-2024/, [15] https://www.who.int/news/item/20-01-2021-who-information-notice-for-ivd-users-2020-05, [16] https://doi.org/10.1038/s41586-020-2598-9, [17] https://doi.org/10.1016/j.cell.2020.05.015, [18] https://doi.org/10.1126/science.abh1823. For detailed information on PCR, refer to the following review https://doi.org/10.56098/ijvtpr.v3i1.71.

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