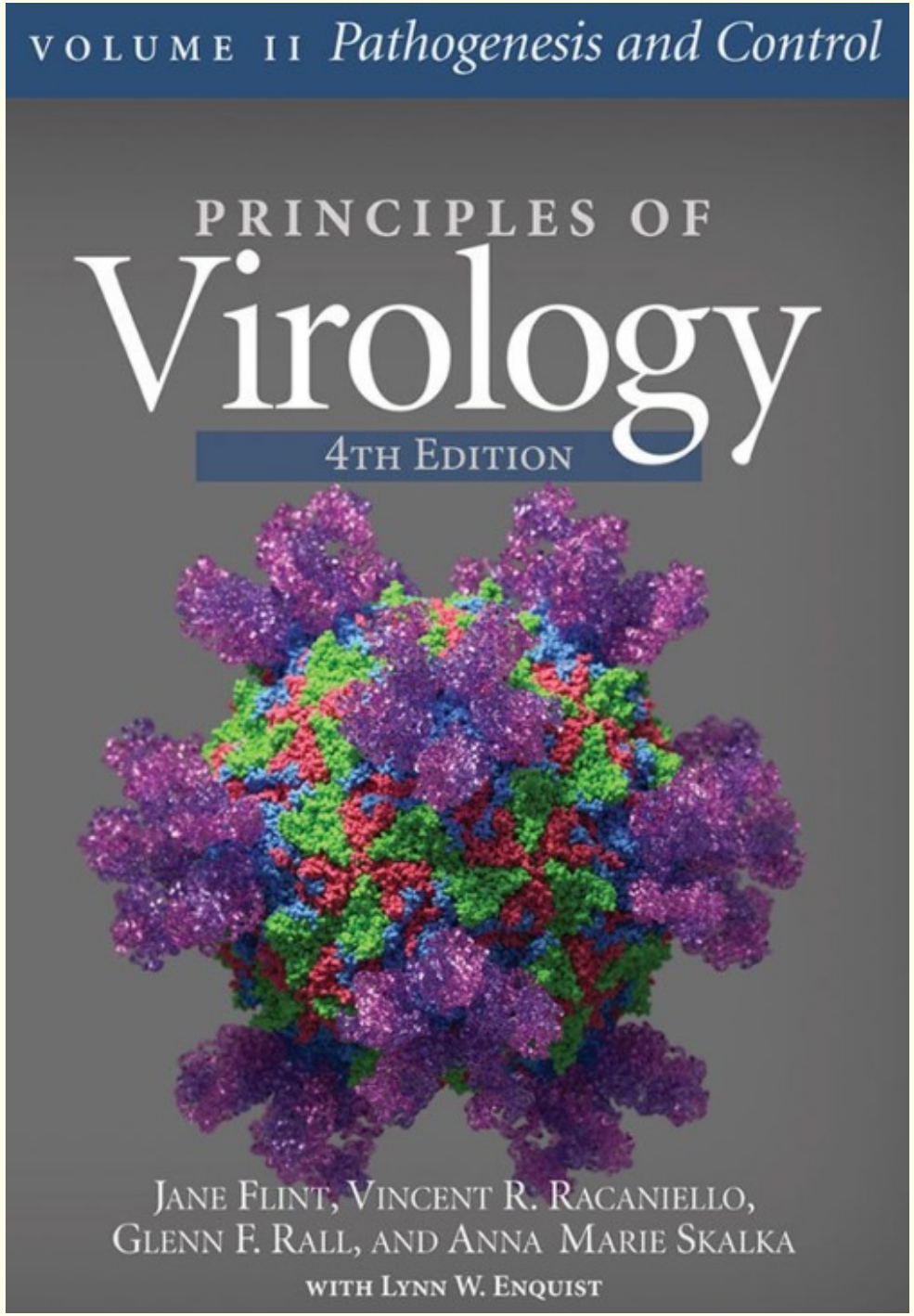
Polymerase Chain Reaction (PCR)

How does it work?

Jerneja Tomsic, PhD



BOX 1.1

DISCUSSION

Why viruses may not fulfill Koch's postulates

Although Koch's postulates provided a framework to identify a pathogen unambiguously as an agent of a particular disease, some infectious agents, including viruses, cause disease but do not adhere to all of the postulates. In fact, it has been argued that the rigid application of these criteria to viral agents may have impeded early progress in the field of virology. Koch himself became aware of the limitations of his postulates upon discovery that *Vibrio cholerae*, the agent of cholera, could be isolated from both sick and healthy individuals.

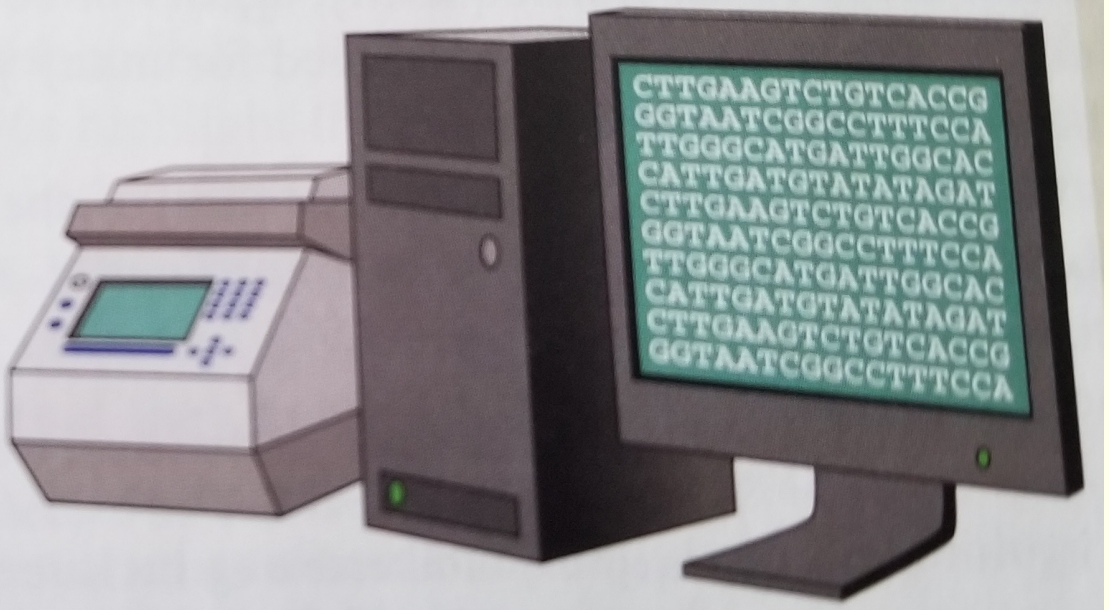
Application of these criteria to viruses can be particularly problematic. For example, the first postulate, which states that the microorganism must be "regularly associated" with the disease, does not hold true for many animal reservoirs, such as bats, in which the virus actively reproduces but

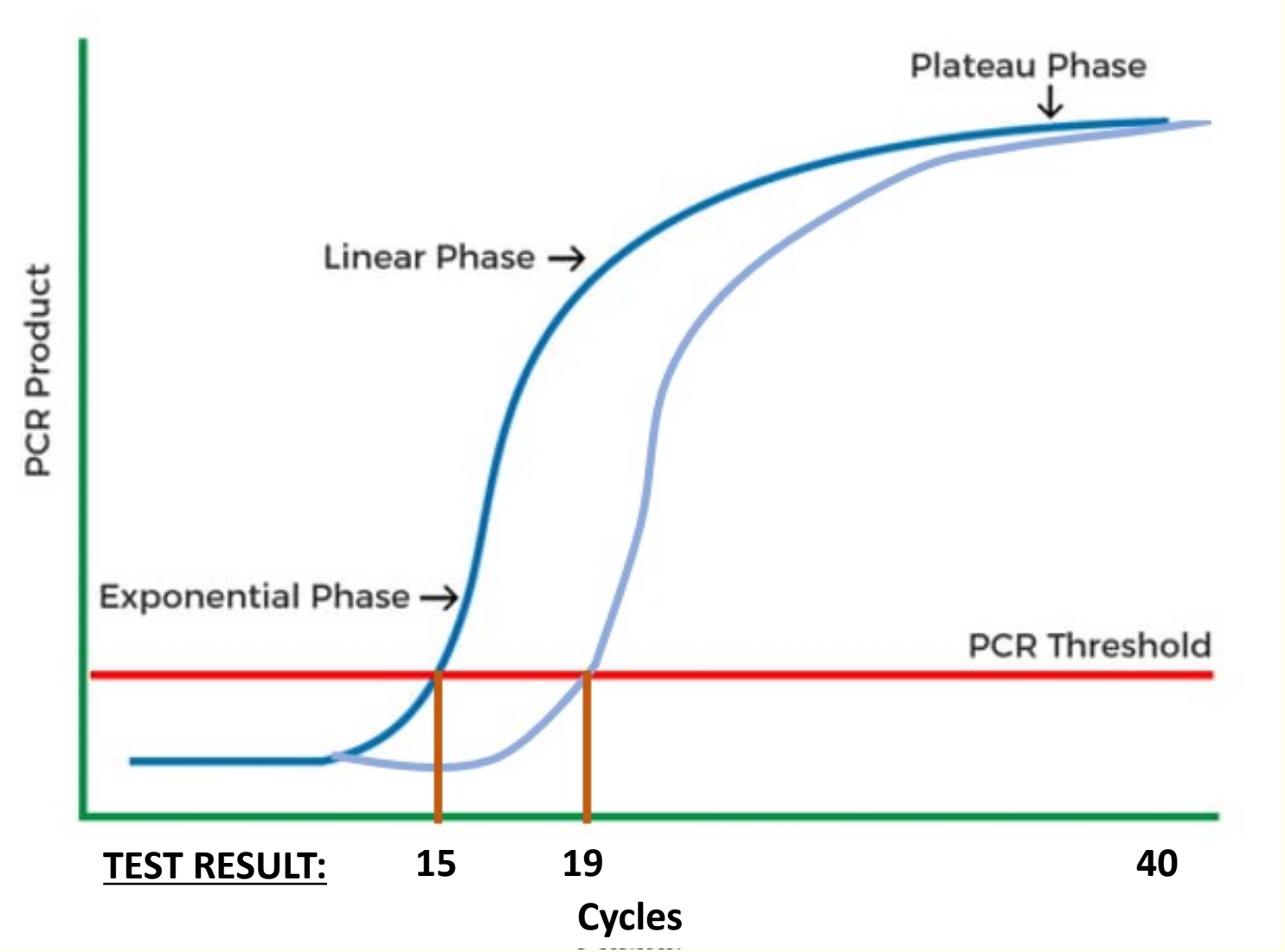
More-sensitive technologies, including DNA sequencing, have triggered a reconsideration of Koch's postulates.

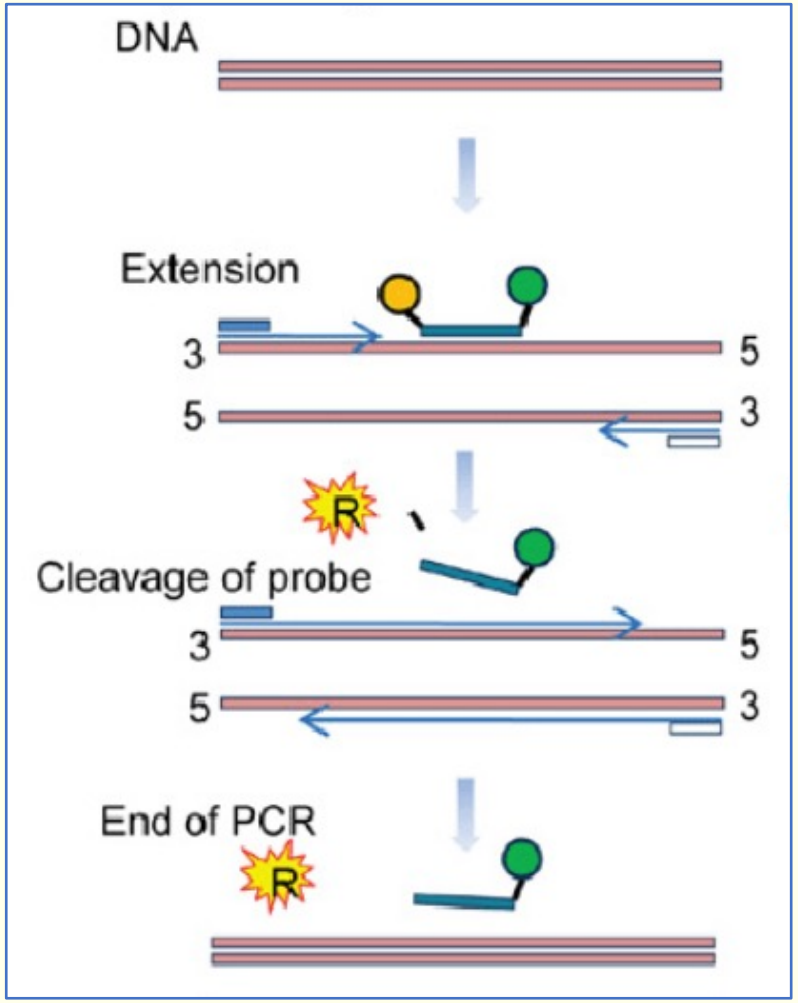
Standard Techniques

VS.

New Technologies







Threshold: an **arbitrary level of fluorescence** chosen on the basis of the baseline variability. A signal detected above the threshold is considered **a real signal** that can be used to define the threshold cycle (Ct). Threshold can be adjusted for each experiment so that it is in the region of exponential amplification across all plots.

Polymerase Chain Reaction – PCR (Chemical Reaction)

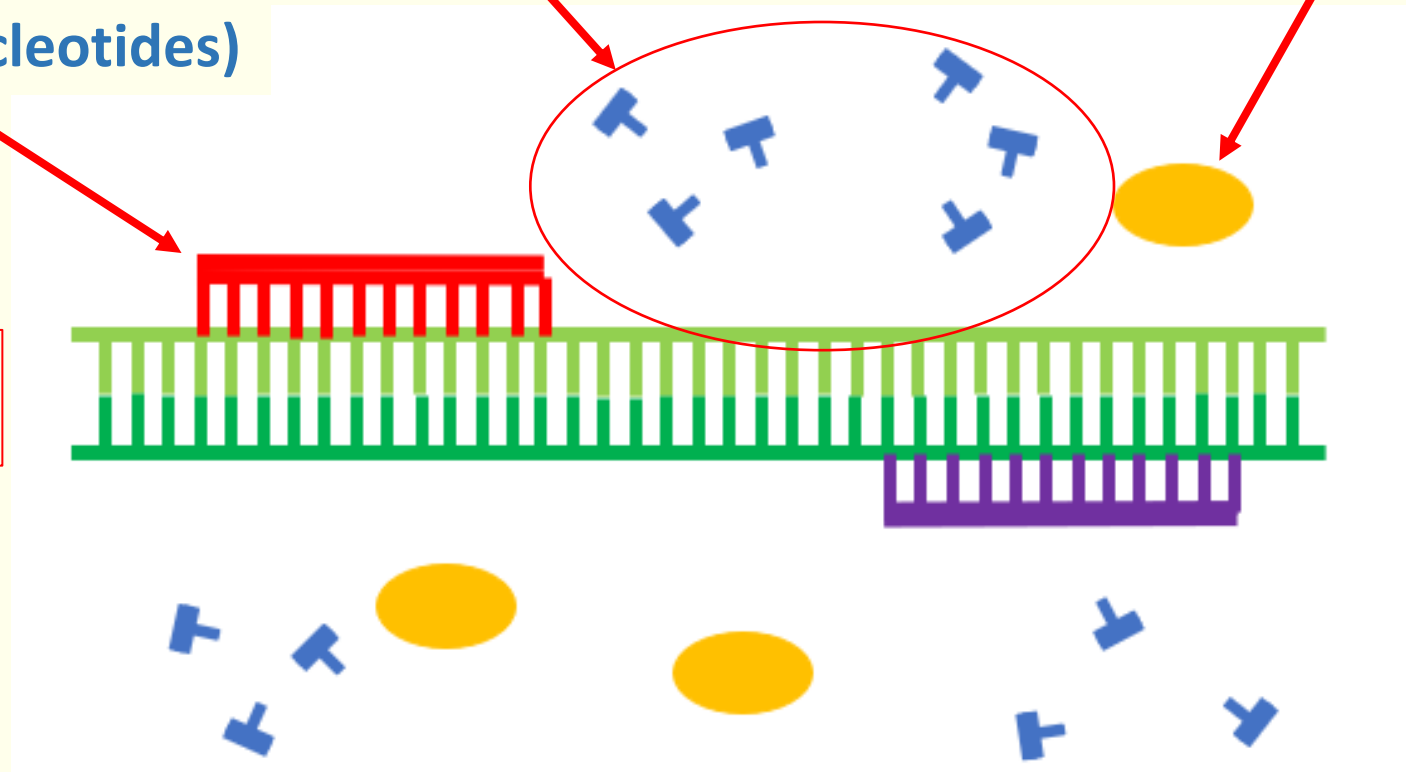
Nucelotides ("building blocks" of DNA):

G – C

A – T

Polymerase ("bricklayer")

primer (18-22 nucleotides)



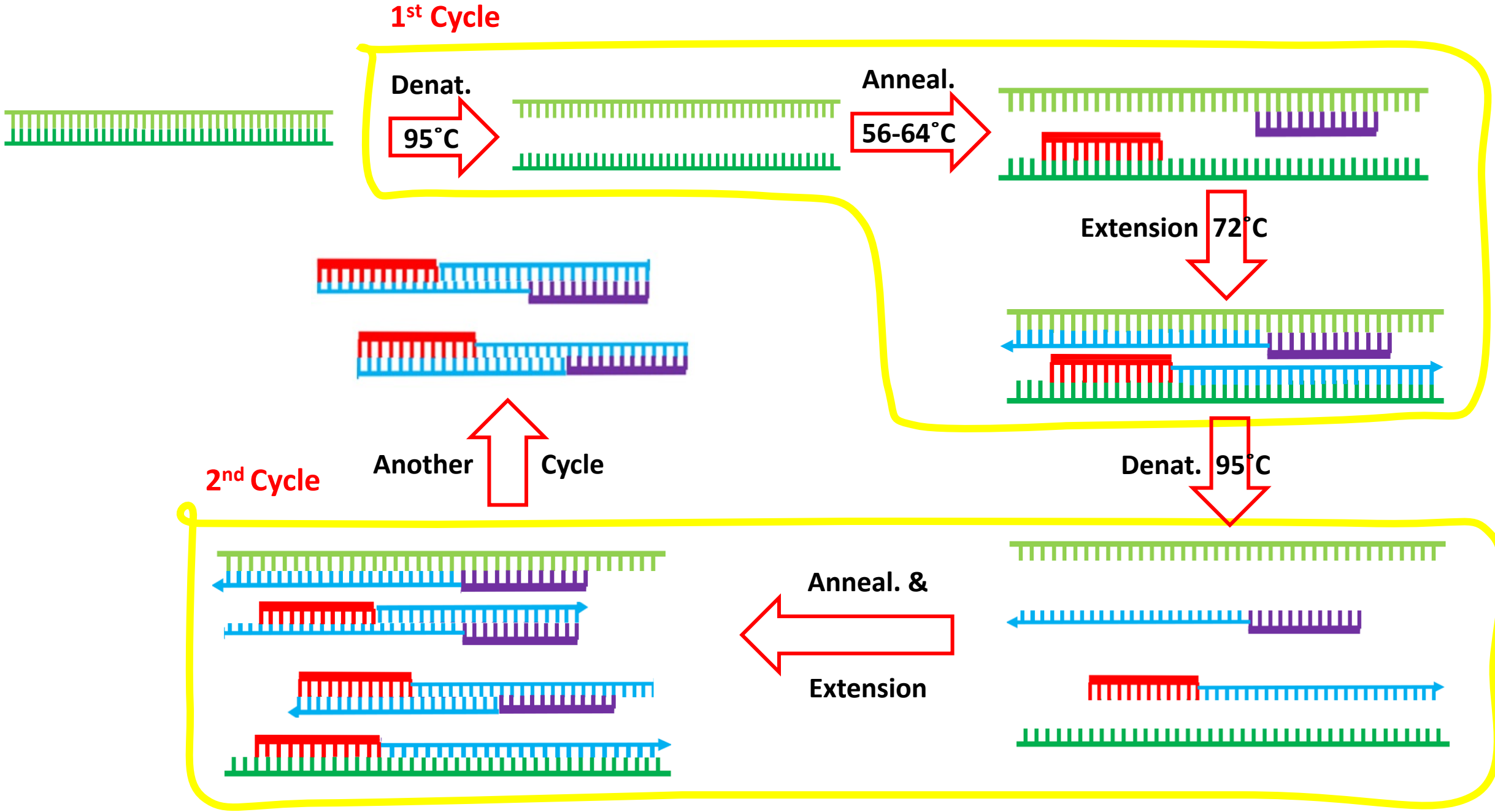
Example:

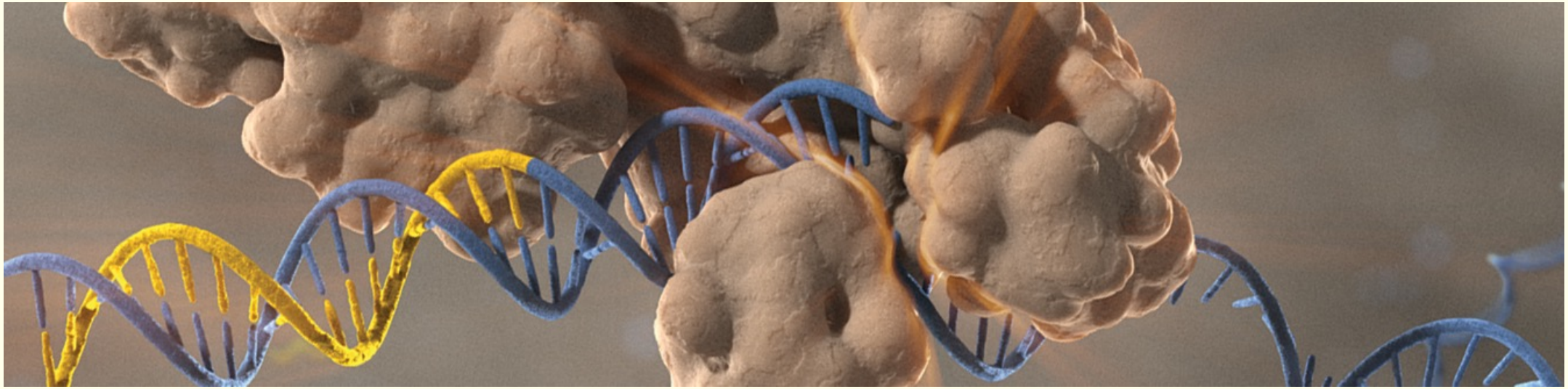
G A T C
C T A G

Cycle (temperature change): 95˚C – opening of DNA

56-64˚C – binding of primer to complementary sequence

72˚C – polymerase adds nucleotides to build new double strand

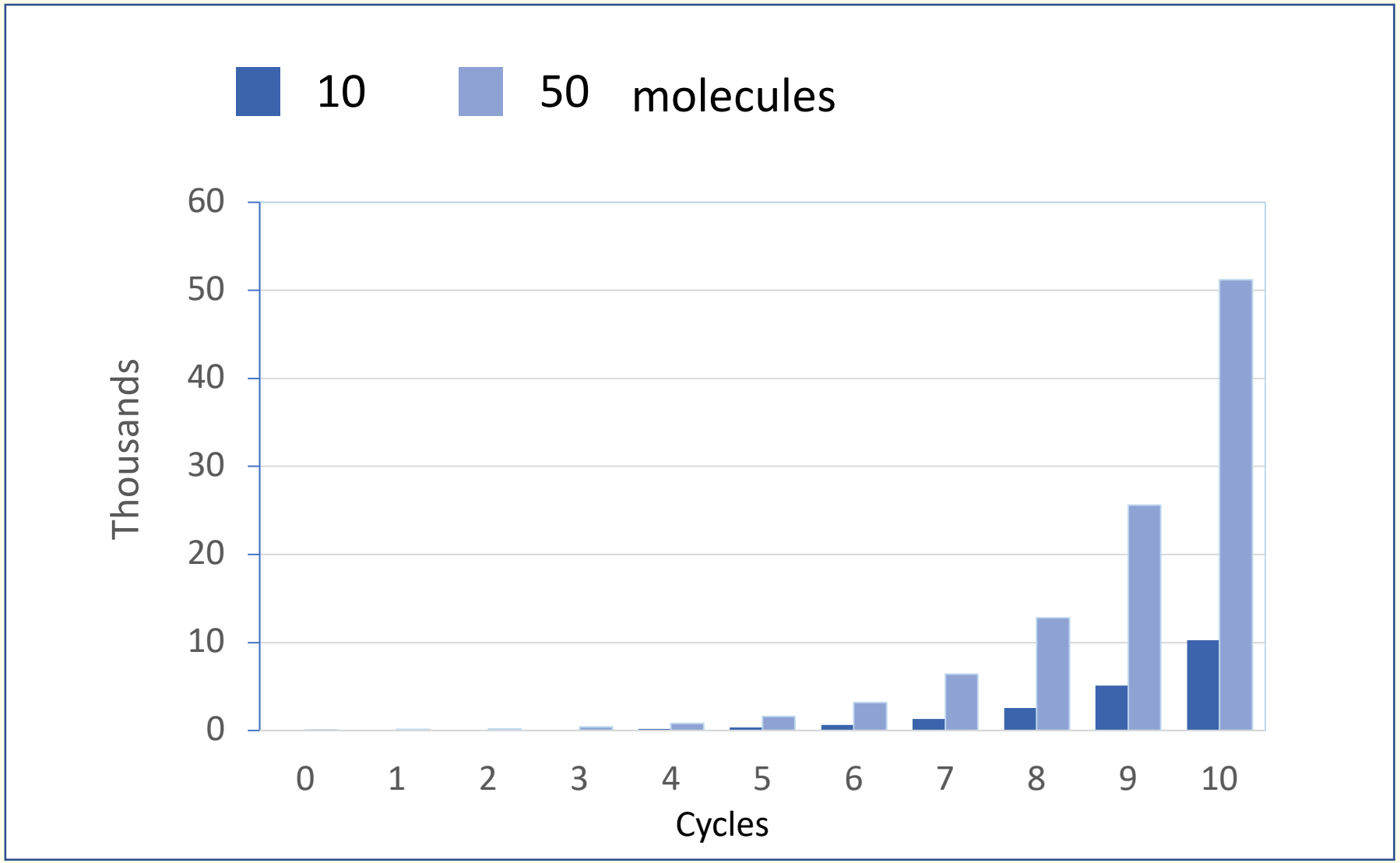

1st Cycle
Denat.
95˚C
Anneal.
56-64˚C
Extension 72˚C
Denat. 95˚C
Anneal. &
Extension
Another Cycle
2nd Cycle

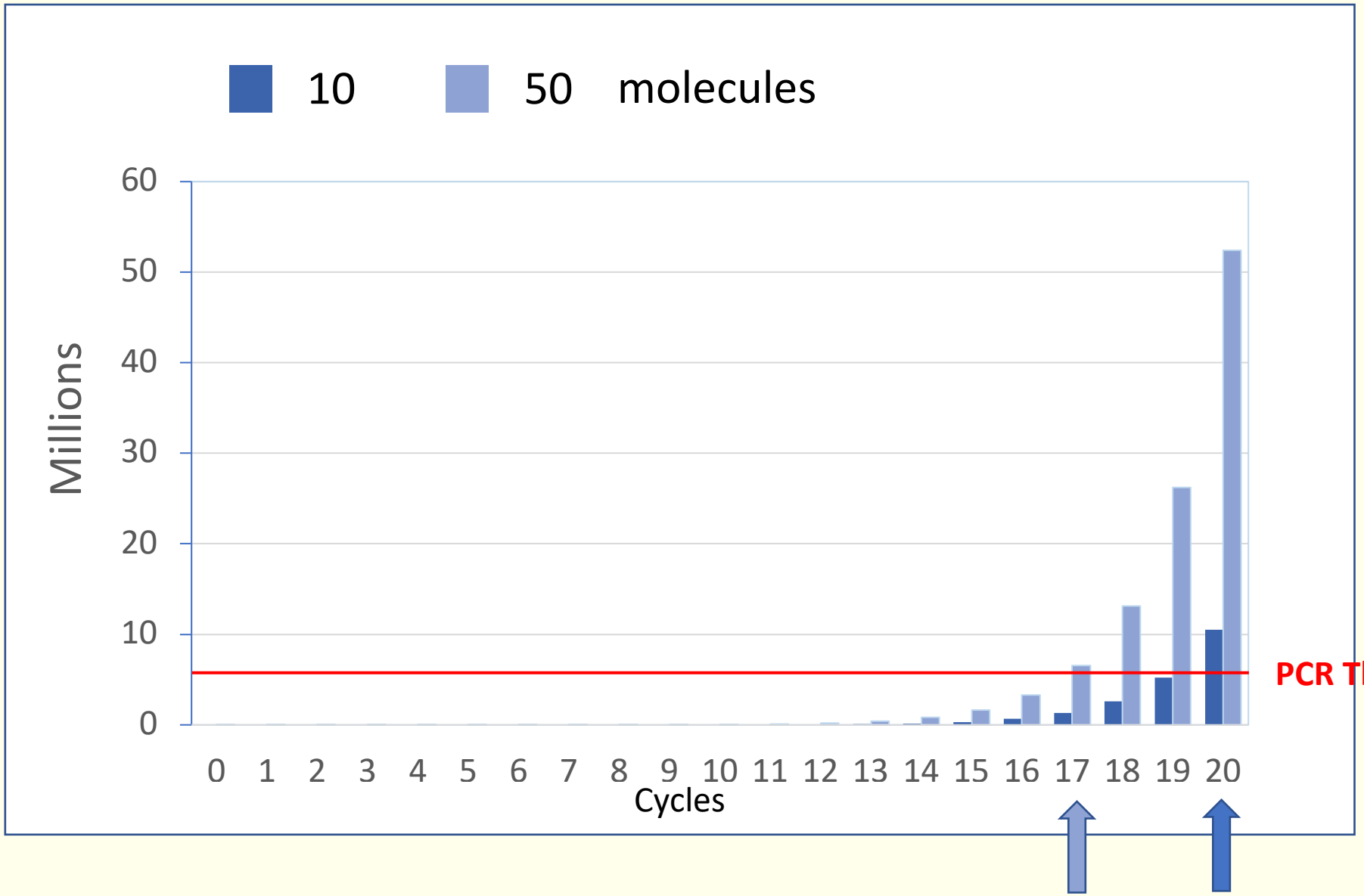


When is the fidelity of a DNA polymerase important?

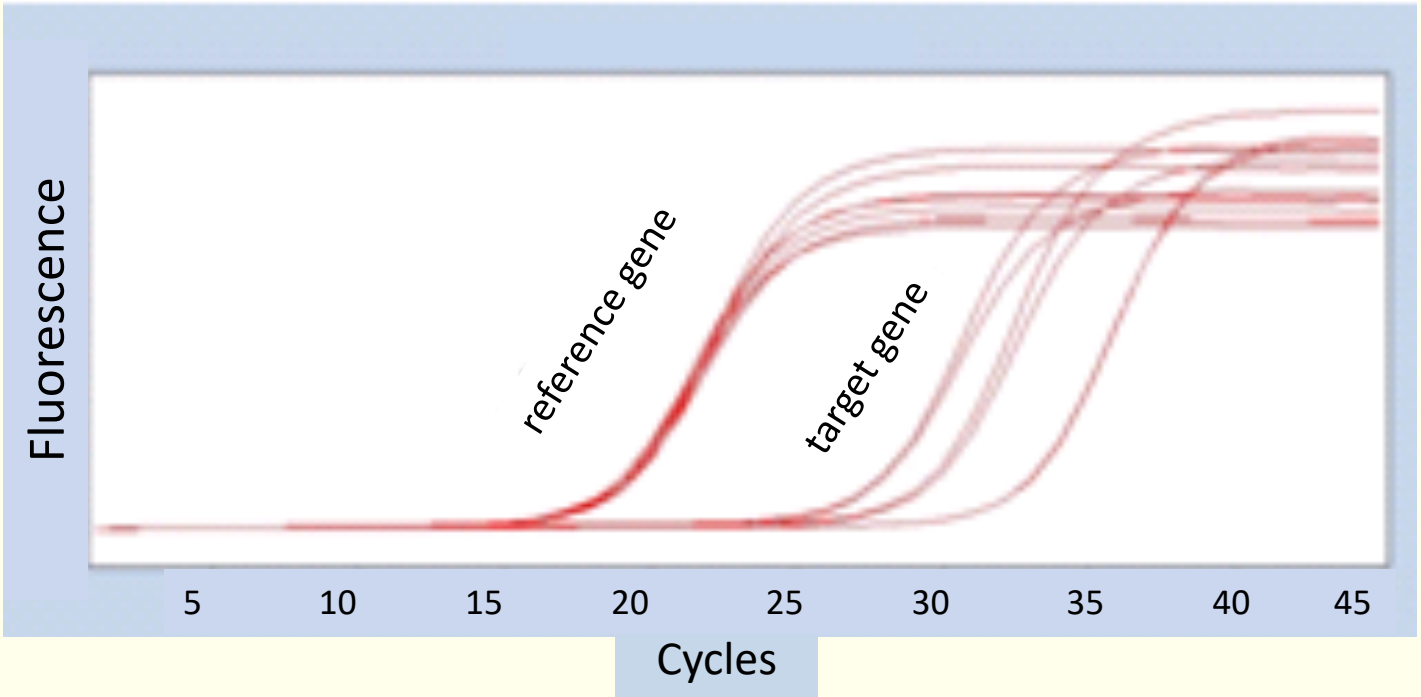
Fidelity is important for applications in which the DNA sequence must be correct after amplification. Common examples include cloning/subcloning DNA for protein expression, SNP analysis and next generation sequencing applications. Fidelity is less important for many diagnostic applications where the read-out is simply the presence or absence of a product.

Taq DNA Polymerase – Error Rate = 1/3,500 nucleotides


10
50
molecules
60
50
40
30
20
10
0
Thousands
0
1
2
3
4
5
6
7
8
9
10
Cycles


10
50
molecules
60
50
40
30
20
10
0
Millions
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Cycles
PCR Threshold

In tests there is no reference gene (reference sequence)!



Several problems with PCR:

- Polymerase makes mistakes (during cDNA synthesis and PCR amplification)
- Often even clean water amplifies when using high number of cycles (above 35)
- In a research lab we never run more than 40 amplification cycles
- A fluorescent signal DOES NOT MEAN the targeted sequence was amplified

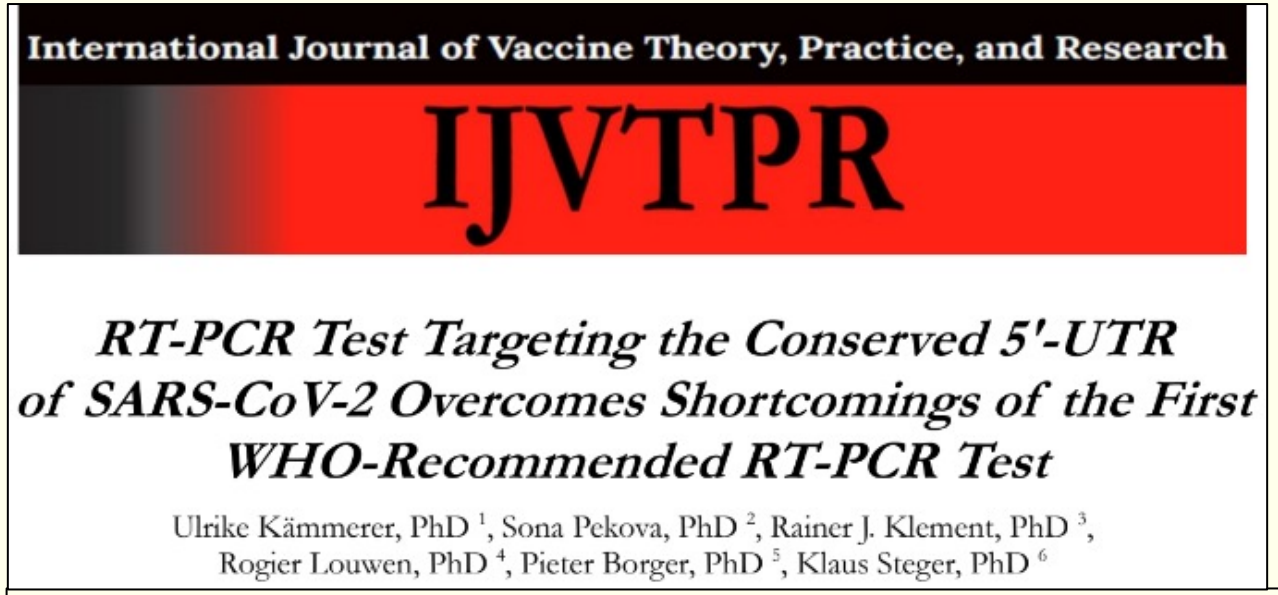
Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR

Victor M Corman[1], Olfert Landt[2], Marco Kaiser[3], Richard Molenkamp[4], Adam Meijer[5], Daniel KW Chu[6], Tobias Bleicker[1], Sebastian Brünink[1], Julia Schneider[1], Marie Luisa Schmidt[1], Daphne GJC Mulders[4], Bart L Haagmans[4], Bas van der Veer[5], Sharon van den Brink[5], Lisa Wijsman[5], Gabriel Goderski[5], Jean-Louis Romette[7], Joanna Ellis[8], Maria Zambon[8], Malik Peiris[6], Herman Goossens[9], Chantal Reusken[5], Marion PG Koopmans[4], Christian Drosten[1]

Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 nM per reaction
	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV. Use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs. Use 100 nM per reaction and mix with P2
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nM per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nM per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nM per reaction
N gene	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nM per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nM per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nM per reaction

[a] W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.



Box 1: No diagnostic value of RT-PCR for the proof of an infectious virus

Of utmost importance and irrespective of any protocol design, RT-PCR solely detects the reverse-transcribed and amplified RNA target(s) selected by applied primers and, therefore, can by no means prove that a replication-competent, infectious virus is **actually present in a given sample**. **Of note, due to the high sensitivity of RT-PCR, residual, non-infectious viral RNA remains detectable even in the absence of infectious viruses.** When applying external standards with defined viral RNA copy numbers, RNA viral loads can be correlated with Ct-values obtained by RT-qPCR. However, neither a certain RNA copy number, nor a specific Ct-value used as a threshold can enable any secure conclusion even concerning whether the viral load is increasing or decreasing.