PCR

Polymerase chain reaction

Revolution in the work with DNA

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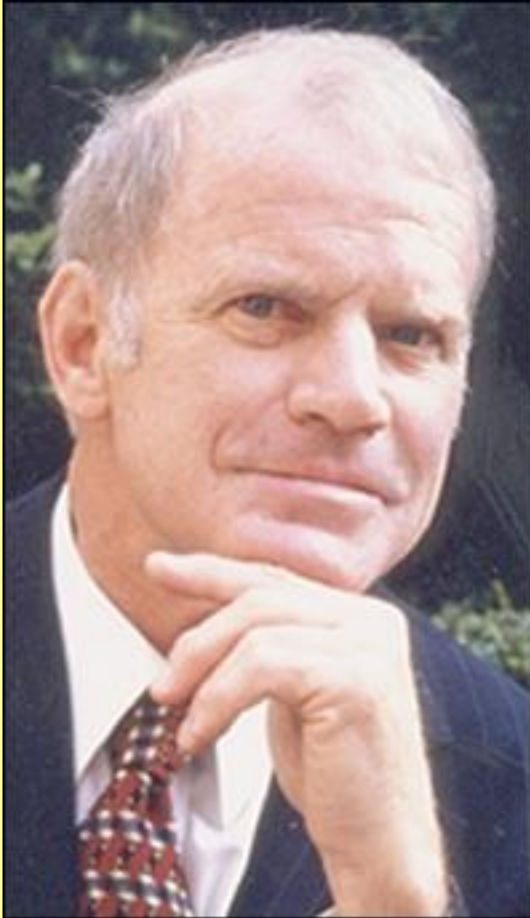
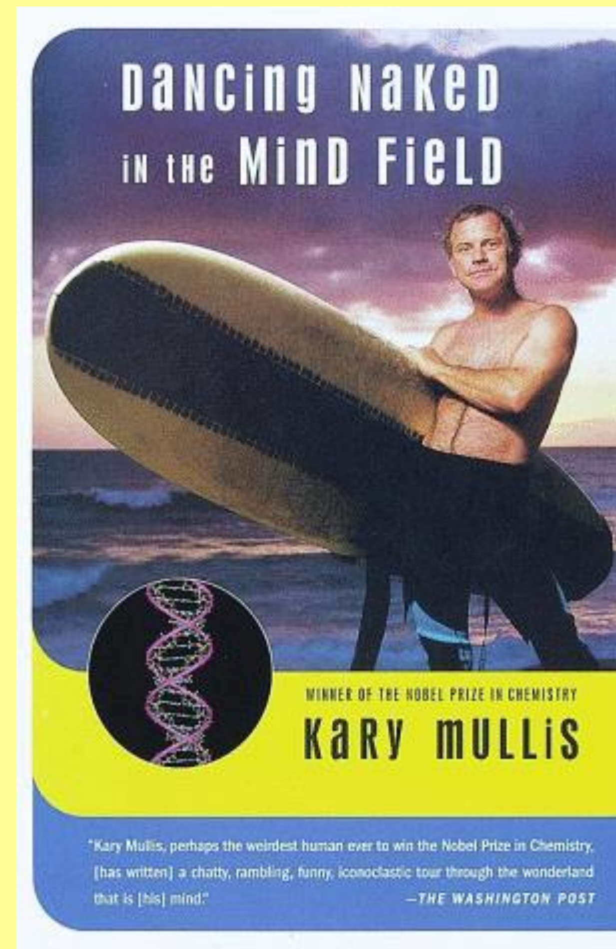
Who is responsible?



Kary Mullis 1985

Nobel prize in 1993



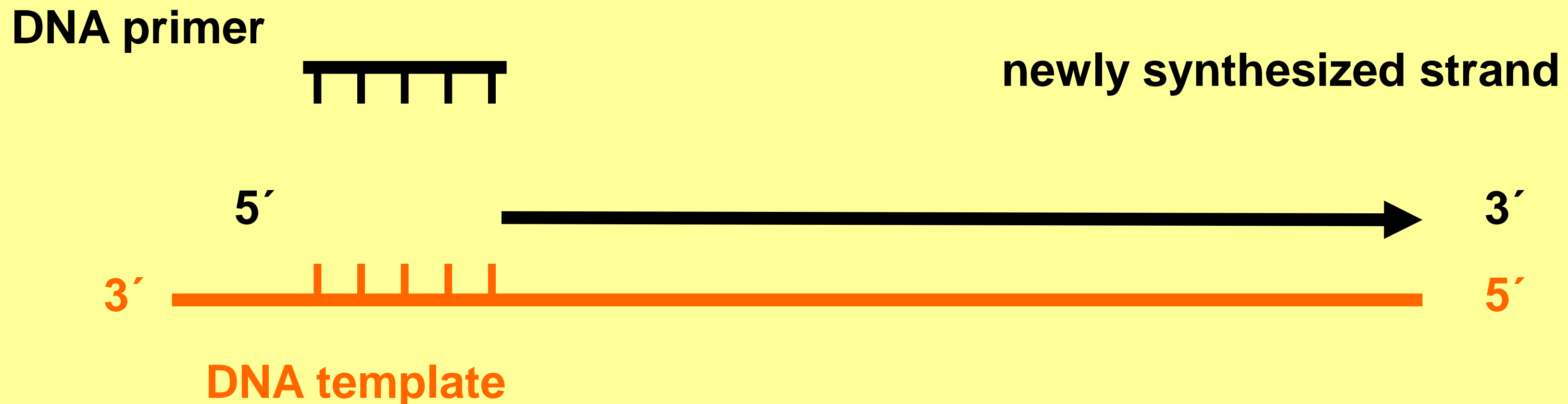
The origin of PCR by Kary Mullis

- “Sometimes a good idea comes to you when you are not looking for it. Through an improbable combination of coincidences, naïveté, and luck mistakes, such a revelation came to me one Friday night in April, 1983, as I gripped the steering wheel of my car and snaked along a moonlit mountain road into northern California’s redwood country.” Sci. Am. 1990 262:56-61, 64-5.

Principle of PCR

Polymerase chain reaction (PCR) enables selective amplification of specific region of DNA *in vitro*;

by the process which resembles DNA synthesis in vivo



Basic prerequisites of amplification

Template DNA

Amplification proceeds only on a DNA sequence - template

Primers

Represent beginning of amplification

Complementarity

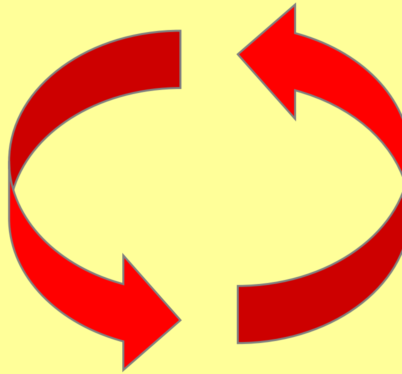
Growing of DNA chains are performed according the rules of base pairing on the level primer/template

Direction of polymerisation

New nucleotides are done only in the 5´- 3´ direction

PCR = cyclic changes of temperature in the reaction mixture

Denaturation



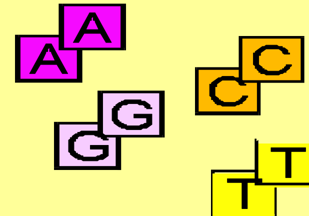
Annealing **Extension**

The components of PCR

Thermus aquaticus, Thermococcus, Thermophilus, Pyrococcus

TAQ

buffer
$MgCl_2$



DNA primers

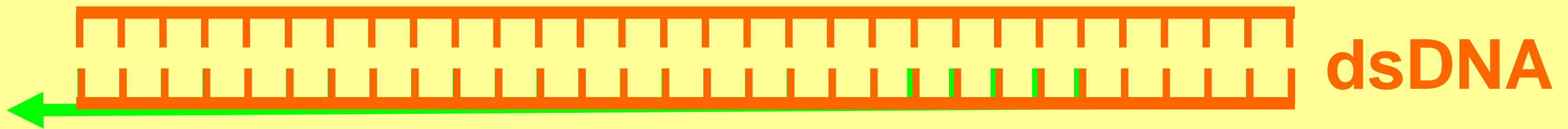
artificial synthesis

ssDNA

Denaturation 92-96°C

The 1st PCR cycle

1. denaturation (92-96°C)

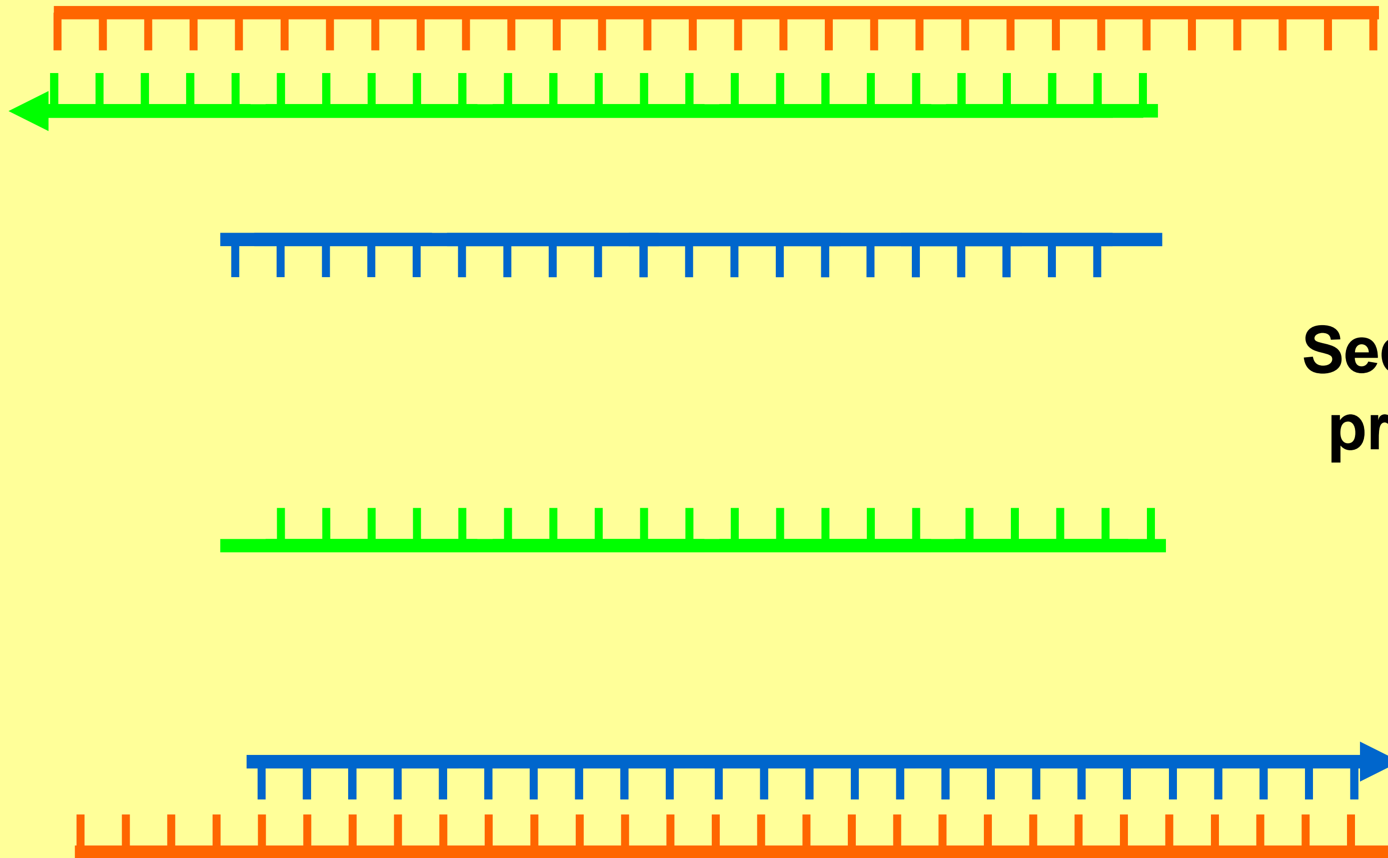


2. annealing (45-72°C)

Primary products

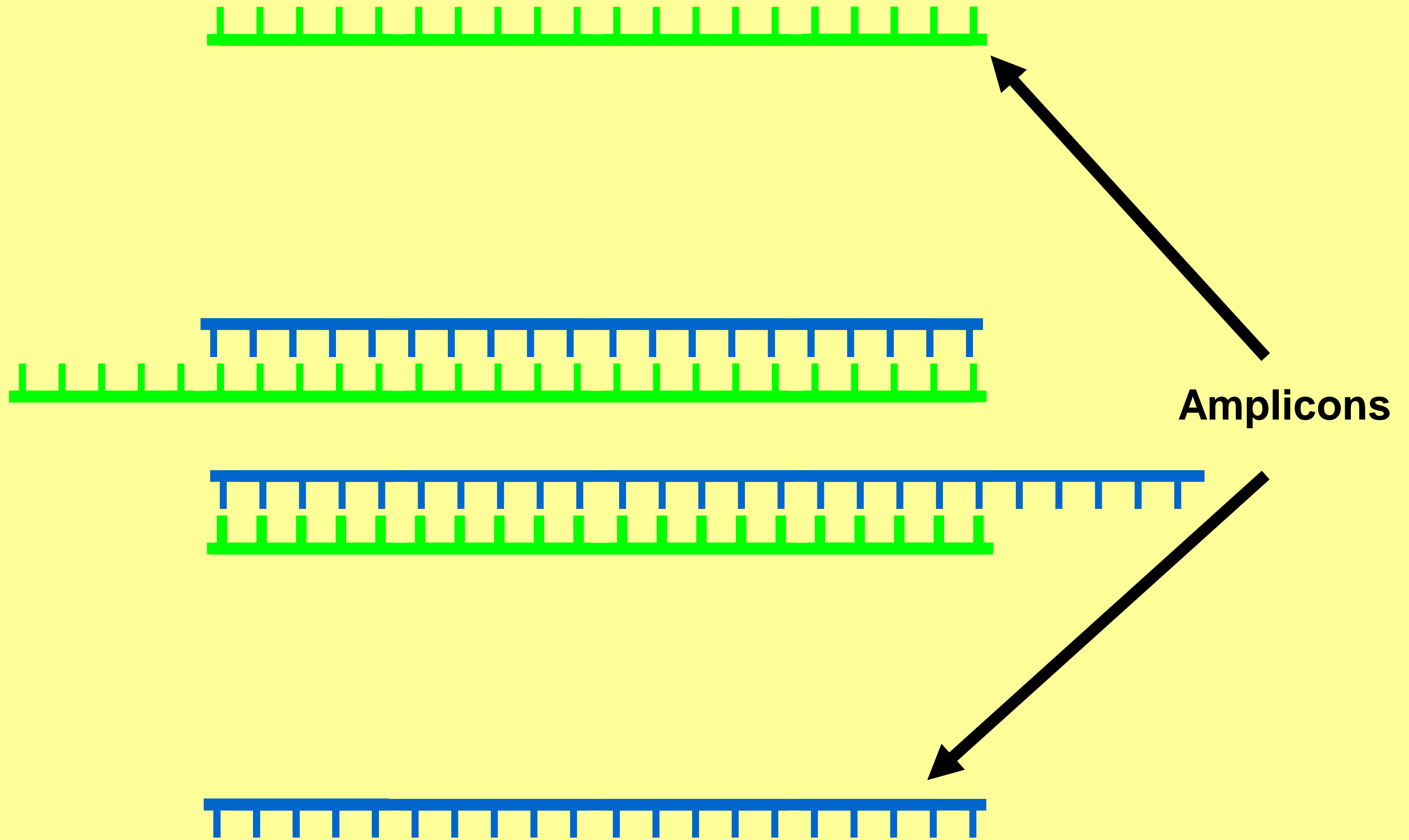
3. extension (72°C)

The 2nd PCR cycle

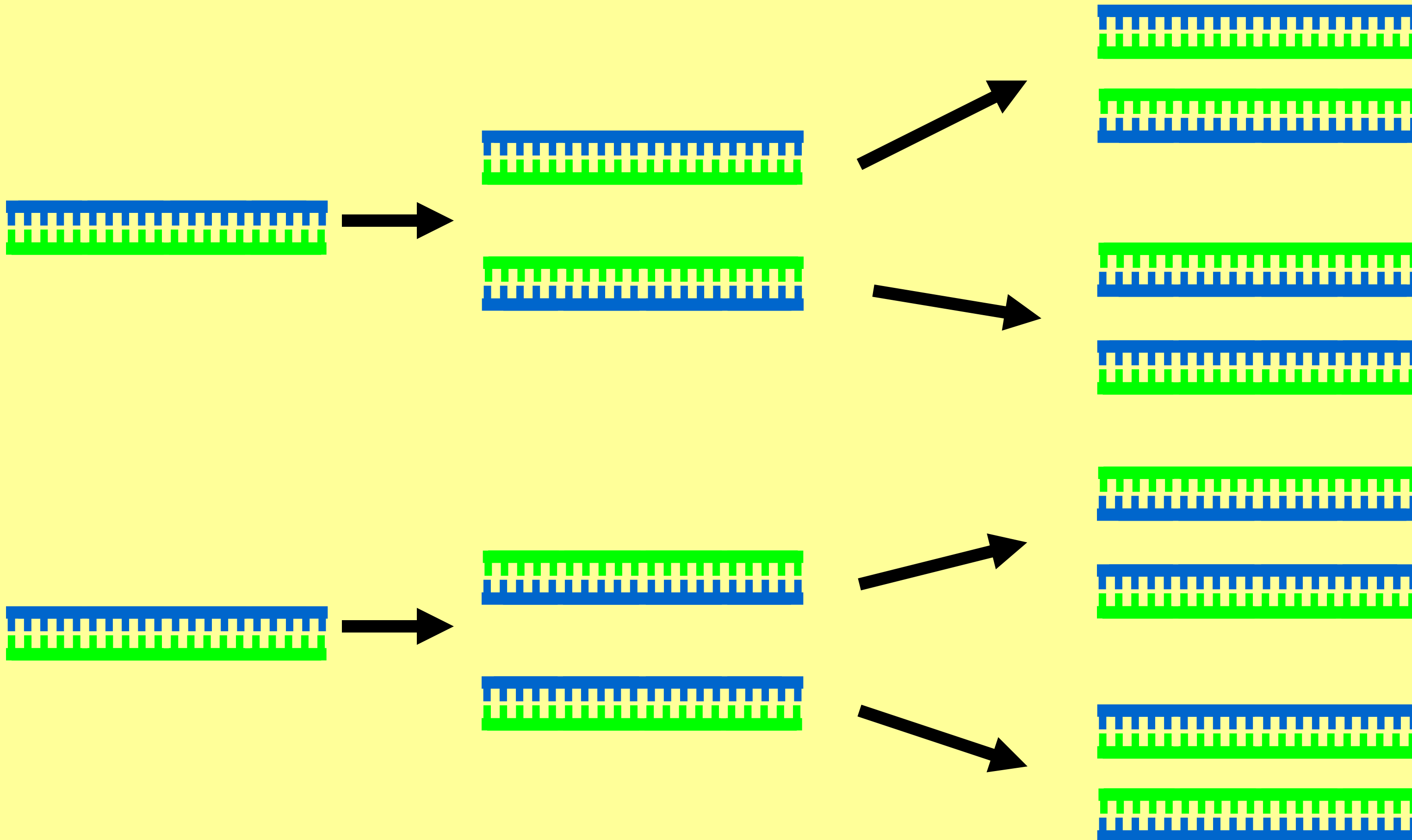


Secondary products

The 3rd PCR cycle

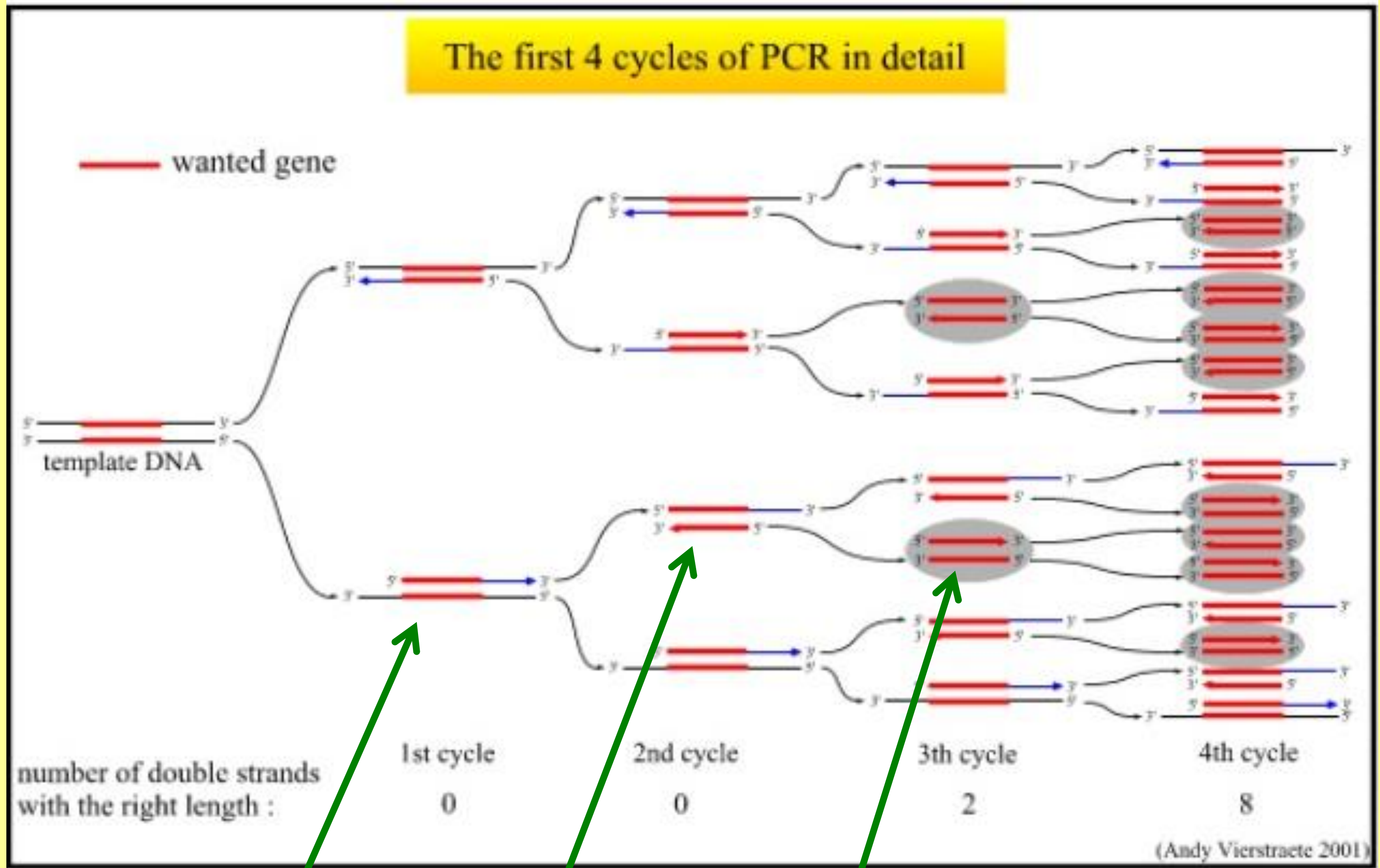


The other cycles



Number of amplicons is growing geometrically

PCR - cycles



Primary product

Secondary product

Amplicon

Amounts and types of the PCR products

Cycle No.	Primary	Secondary	Amplicons	Total (for X =1)
0	0	0	0	1
1	2	0	0	2
2	2	2	0	4
3	2	4	2	8
4	2	6	8	16
5	2	8	22	32
Generally	2x	x(2n-2)	$(2^n-2n)x$	$(2^n)x$

X = number of template at the beginning, n = number of cycles

Yield and numbers of the PCR cycles

Cycle No.	Primary	Secondary	Amplicons	Total
10	2	18	1 004	1 024
20	2	38	10 485 438	$1\ 024^2$
30	2	58	$\sim 1.1 \times 10^9$	$1\ 024^3$
40	2	78	$\sim 1.1 \times 10^{12}$	$1\ 024^4$
50	2	98	$\sim 1.1 \times 10^{15}$	$1\ 024^5$

Conclusions

- **Each amplicon is formed from two strands = two templates that are doubled in each PCR cycle**
- **The number of amplicons is extremely growing in comparison with primary and secondary products**
- **After several cycles the amplicons absolutely dominate as products of the PCR reaction**
- **Primary and secondary products form only minor part of the resulting products**

Parameters of PCR

Quality of PCR reaction and result of amplification are influenced by several predictable factors which are

Chemical factors

Physical factors

Chemical parameters of PCR

1) **Amount of Taq polymerase**
2) **Reaction buffer**
3) **Amount of dNTPs**
4) **Primers**
5) **Volume of the PCR reaction**
6) **Quality of DNA**

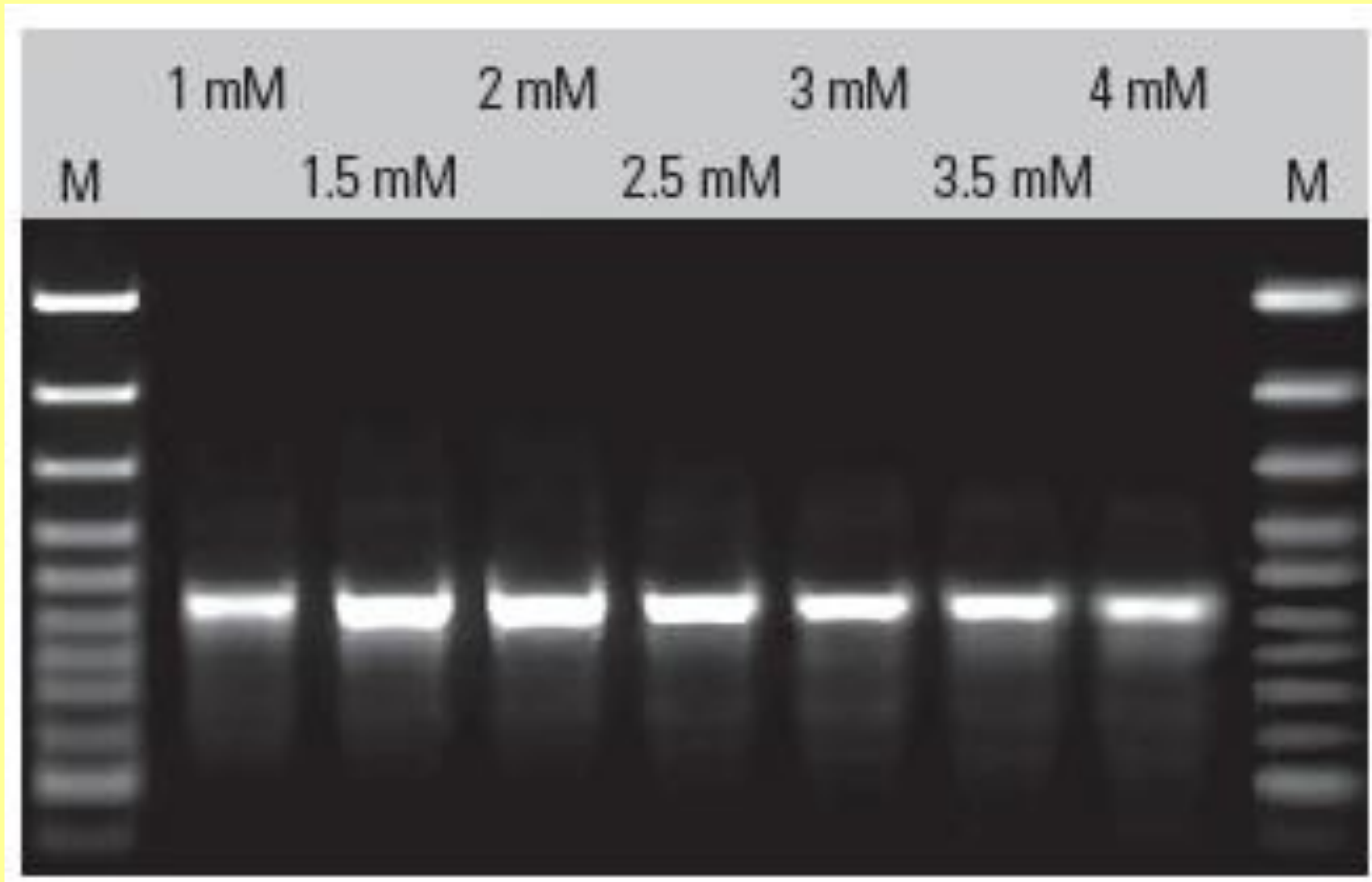
Amount of Taq polymerase

- **0,5-2,5u correspond to 25-125 fmol of enzyme**
- **A lot of different Taq polymerases and their mixtures is available at the present time**
- **High termostability and accuracy to include the correct nucleotide to the DNA strand is called fidelity**

Reaction buffer

- **It contains a cofactor of Taq polymerase - Mg^{2+} ions in the form of $MgCl_2$ or $MgSO_4$**
- **Concentration of Mg^{2+} varies between 0,5-5,0 mM (1,5 mM)**
- **The ions influence activity of the enzyme, increase Ta of dsDNA, and form soluble complexes with nucleotides, which is necessary to incorporation of nucleotides to DNA**

Influence of Mg^{2+} ions on PCR



http://www.thermoscientificbio.com/

Amount of dNTP

- ***In vitro*** **the nucleotides are sufficiently incorporated to the DNA structure in concentrations about 10 µM, that are lower than the concentrations used in PCR (100-200 µM)**

The correct concentration (amount) of dNTPs depends on:

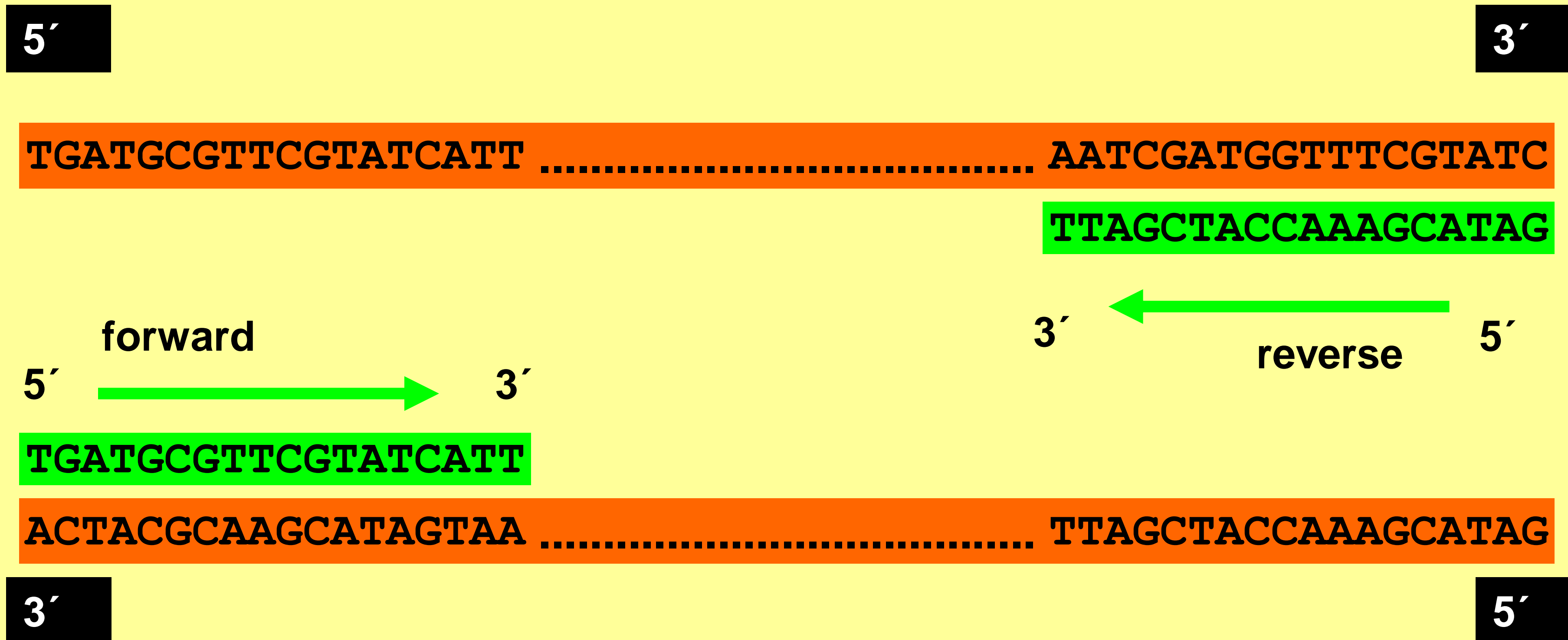
- **Length of the products of amplification**
- **Concentration of Mg^{2+}**
- **Concentration of the primers**
- **Temperature profile of the reaction**

Primers

- **Responsible for the specificity of PCR**
- **Long from 14 to 40 nucleotides**
- **Content of G+C from 40% to 75%**
- **3´- end of one of the primers should avoid complementarity to prevent „primer dimer“ formation**
- **Avoid imbalanced distribution of G/C- and A/T- rich domains. But oligo (dT) and poly (dC) work well.**
- **5´- end is less sensitive to mutations presented on the template DNA**
- **Concentration in the range of 0,1 - 1,0 μM**

Primers

The length and specificity of PCR products are done by the position of primers on target sequences of the DNA template



Design of primers - example

Design the primers sequences that could amplify the selected part of dsDNA

- You know the sequence of only one DNA strand
- Write orientation in 5´- 3´ direction

```
5´- TGA TGC AAA GTT CGC TCA GGT ACG ATT CCC
    AAA TGT GGA GCT TAG TCG ATG ATG GGC AAA
    TCT GTG ATT ATC CGA CGT CCC ATG TGC GTC
    AAA TGC CGT AGG ACC CTA TTT TGA CGT CCT
    GCT GGT ACG CAT CAT CCC TGG TGA CGT CCT
    ACG TGC TGC GCT CGC ACG ATG CGT ACG AAC
    GCT CGT CGG - 3´
```

How long will be the resulting amplicons ?

Design of primers - resolution

Primer forward

5´- AAA GTT CGC TCA GGT ACG – 3´

Primer reverse

5´- CGT ACG CAT CGT GCG AGC – 3´

Amplicon will be 171 bp long

Volume of the PCR reaction

- **Influence the result less than the previously described factors**
- **May influence sensitivity of reaction (detection limit of rare template)**
- **Reaction volumes are usually 20-100 µl**
- **PCR in capillaries – reaction volume slow down to 10 µl**

DNA quality

- **One of the most important factor to receive good results**
- **Purity and quality of DNA influence especially sensitivity of PCR reaction ⇒ Rubbish in, rubbish out.**
- **PCR is completely inhibited by compounds such are heparin, porphins and similar; and $HxPO_4^{n-}$ ions**

Physical parameters of PCR

1) Initial denaturation
2) Primer annealing
3) Primer extension
4) Cycle number
5) Final extension

Initial denaturation

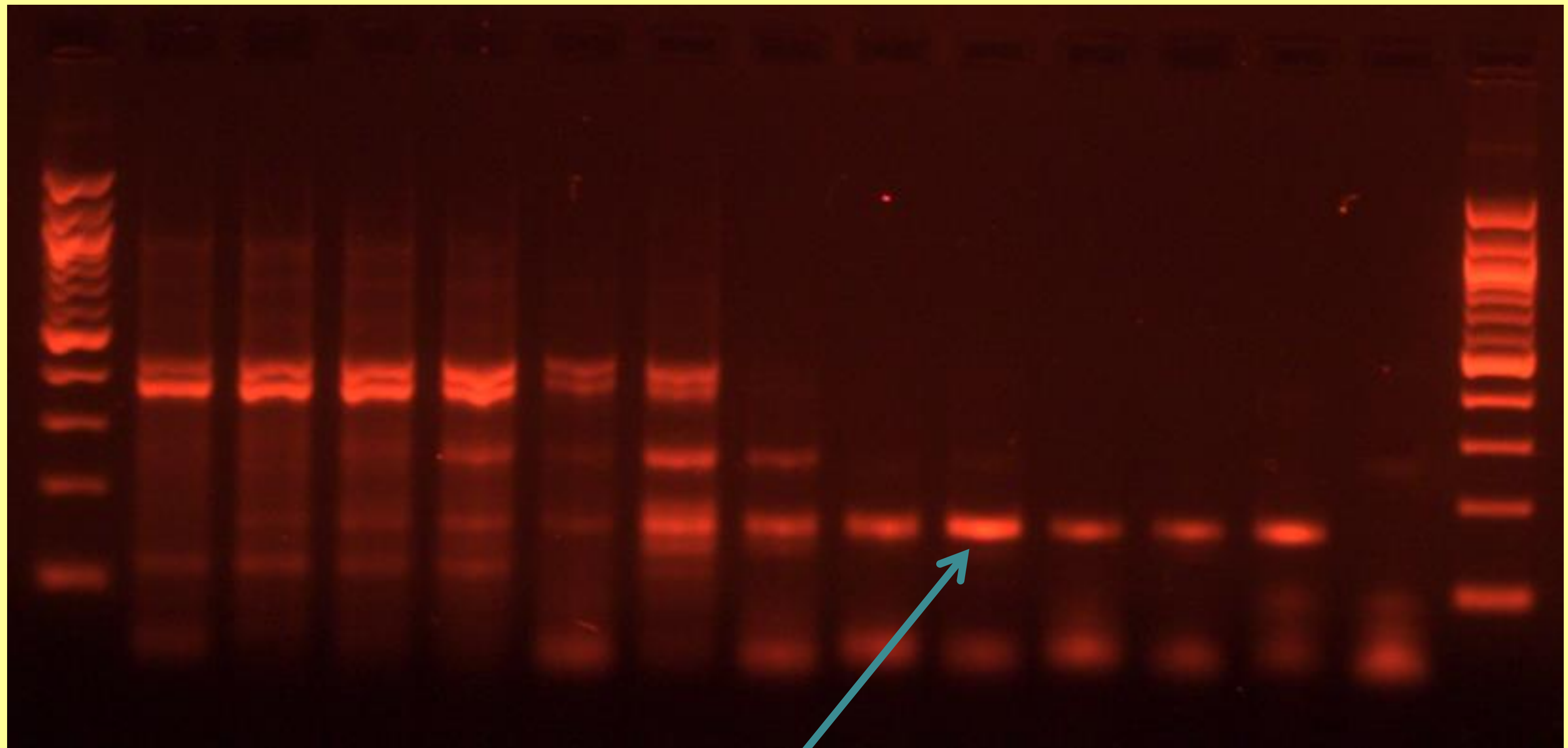
- **Initial heating of the PCR mixture for 3 to 5 min at 94-96°C**
- **It is enough to complete denaturing complex genomic DNA so that primers can anneal to their target sequences after cooling**
- **It has not be very long – heat damages DNA strands and Taq polymerase**

Primer annealing

- **Critical for specificity of PCR reaction**
- **Very high temperature = primers are not able to anneal**
- **Very low temperature = non specific products are formed**

Influence of T_a on PCR

Temperature



Optimal temperature

Temperature for primer annealing

Tm (melting temperature)

Temperature during which about 50% primers will bind to template

Tm = (number of G+C) x 4 + (number of A+T) x 2

Ta (annealing temperature)

Temperature during which the most of the primers will bind to template

Ta = Tm – (3-5ºC)

Calculation of Ta – example

What are the Tm and Ta of the following primers?

Primer forward

5´- AAA GTT CGC TCA GGT ACG – 3´

Primer reverse

5´- CGT ACG CAT CGT GCG AGC – 3´

Calculation of Ta – resolution

Primer forward

5´- AAA GTT CGC TCA GGT ACG – 3´

Tm = 54ºC, Ta = 50ºC

Primer reverse

5´- CGT ACG CAT CGT GCG AGC – 3´

Tm = 60ºC, Ta = 56ºC

Optimal Ta for the both of the primers will be about 50ºC

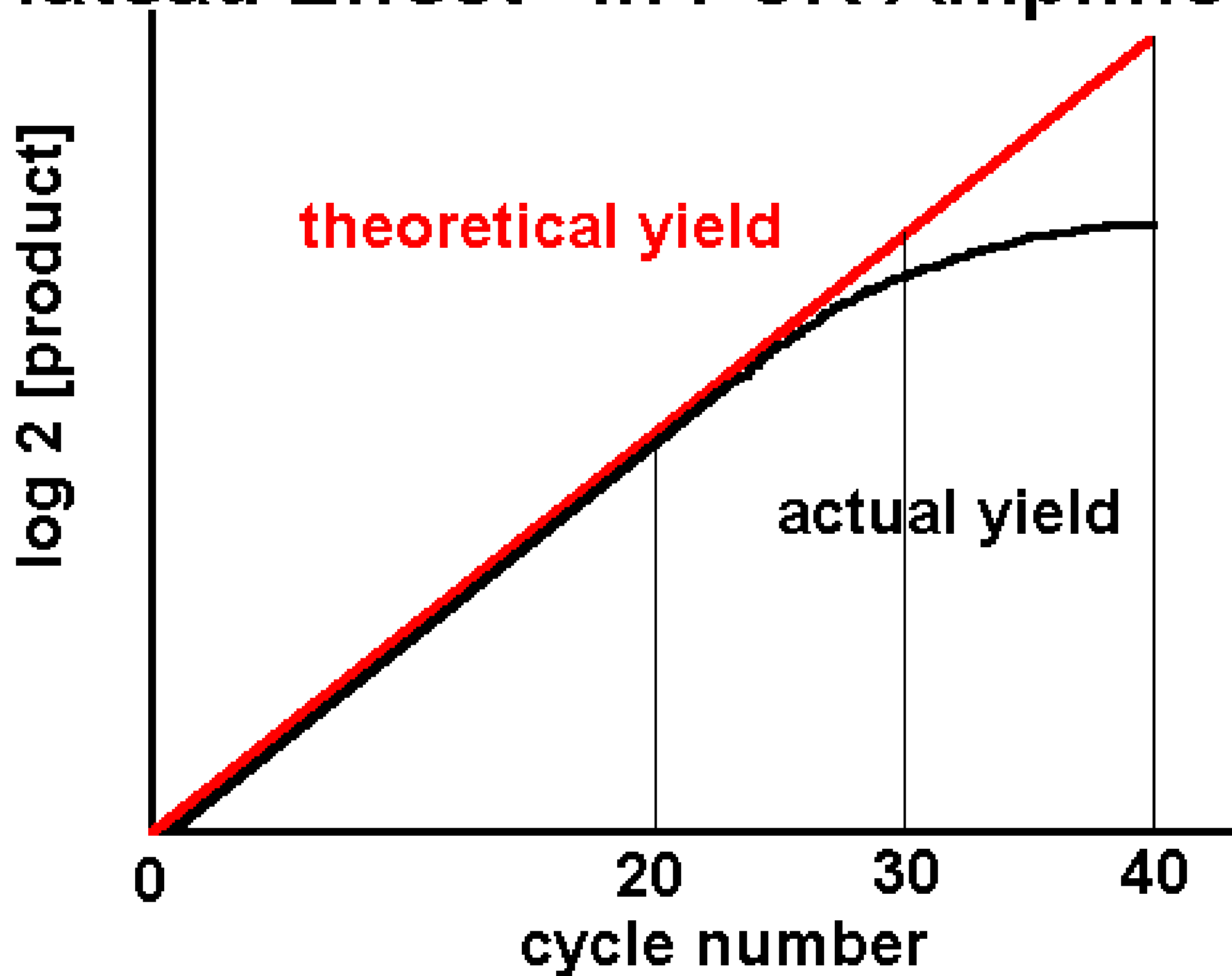
Primer extension

- Synthesis of new DNA strands
- Proceeds in 72°C
- It is recommended not longer than 20 s for amplicons to 500 bp long
- For fragments to 1,2 kbp about 40s
- Taq polymerase synthesizes about 150 nucleotides /s

Cycle number

- **Analytical PCR – no more than 40 cycles**
- **Mostly 25 to 35 cycles**
- **Higher cycle number = formation of nonspecific products**
  - **exhausting of reaction compounds**
  - **polymerase and/or DNA degradation**

Cycle number



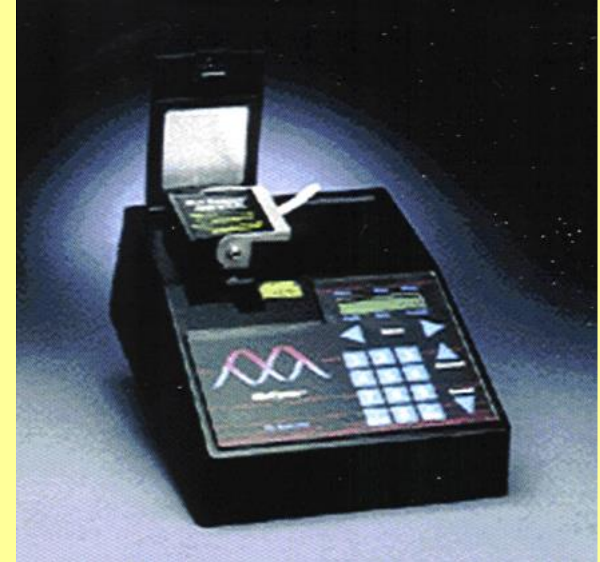
http://www.mcb.uct.ac.za/

Final extension

- **To promote completion of partial extension products and complete annealing of ss complementary products**
- **It is hold usually, after the last cycle, for 5-15 min. in 72°C**
- **After PCR completion, the tube with reaction can be stored at -20°C until needed for product analysis**

Technical performance of PCR - termocyclers

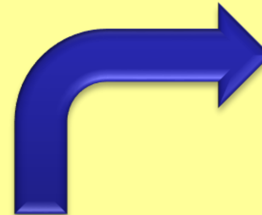
By microprocessor controlled equipment which contains metal reaction block heated and cooled by semiconductors (Peltier pump), water, air or microwaves



Termocyclers quickly change temperature in reaction blocks between the three temperatures of the PCR cycle

Thermocycler evolution

Far far ago…



Now

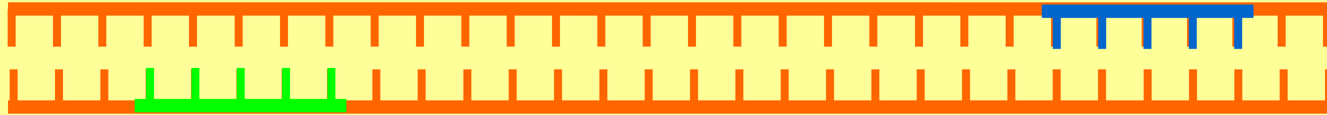
"Baby Blue", a 1986 prototype machine for doing PCR

http://en.wikipedia.org/

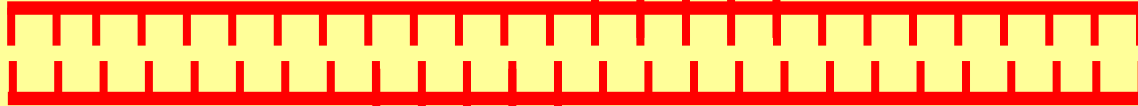
Types of PCR

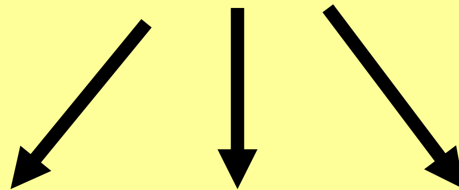
- **PCR-REA, PRA, PCR-RFLP**
- **nested PCR**
- **multiplex PCR**
- **competitive PCR**
- **RT-PCR**
- **real-time PCR**

PCR-REA, PRA, PCR-RFLP

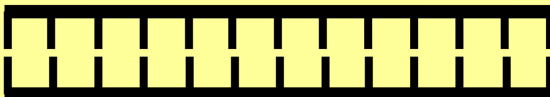


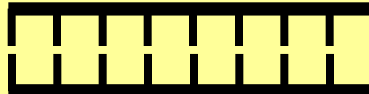
amplification

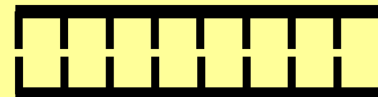




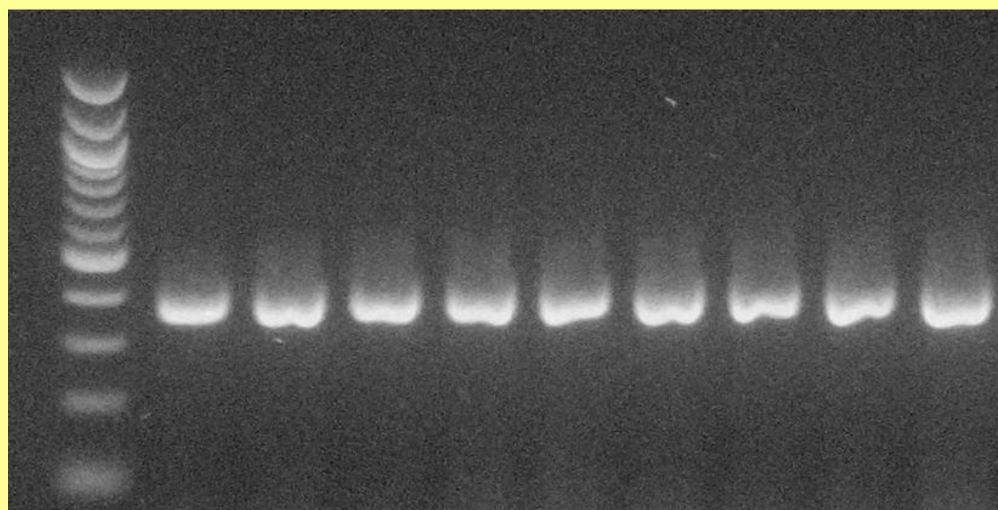
restriction digestion





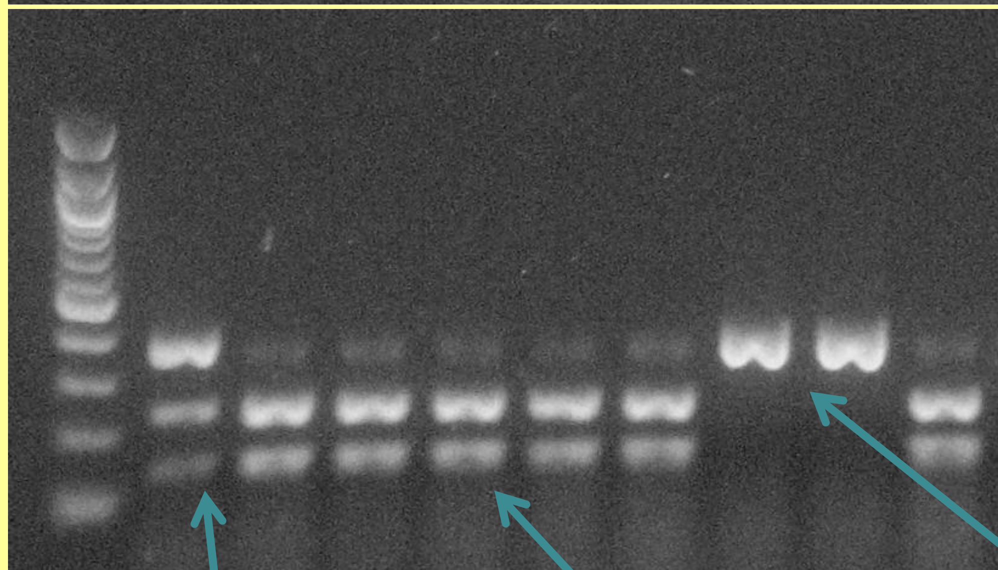


PCR-REA, PRA, PCR-RFLP - example



Uncut

Detection of polymorphism G908R in NOD2 gene



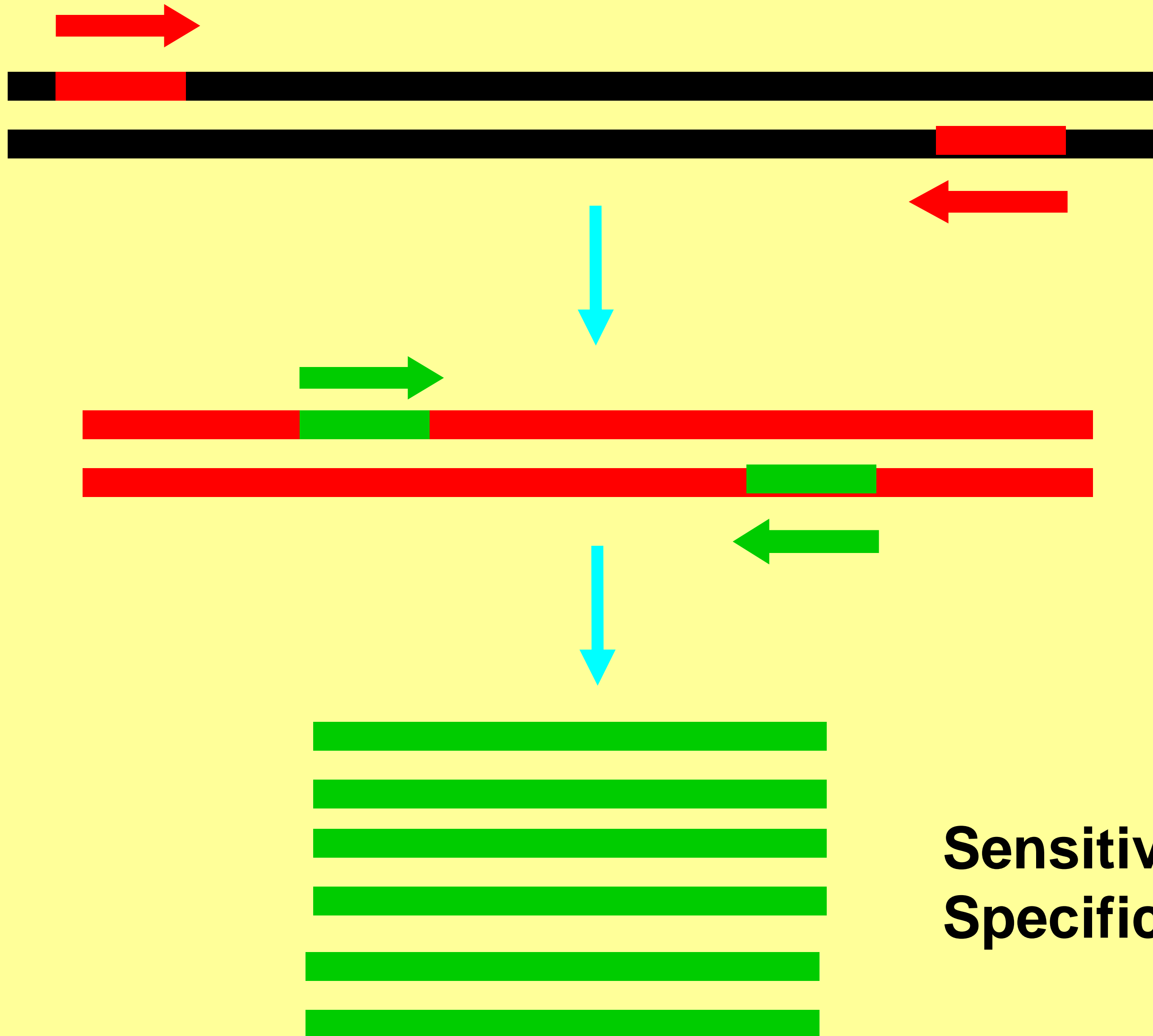
Cut by restriction endonuclease

Heterozygot

Homozygot 1

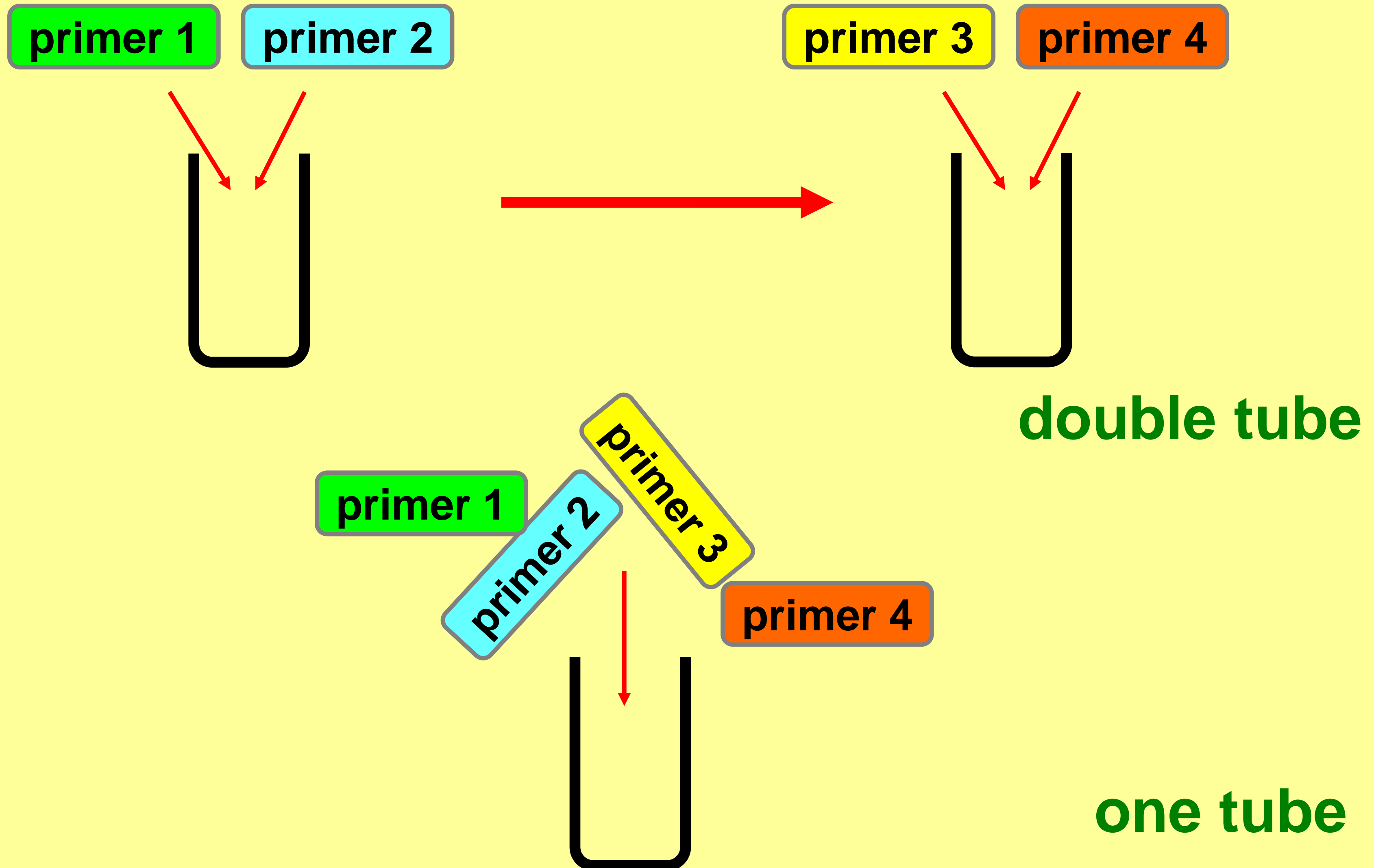
Homozygot 2

Nested PCR

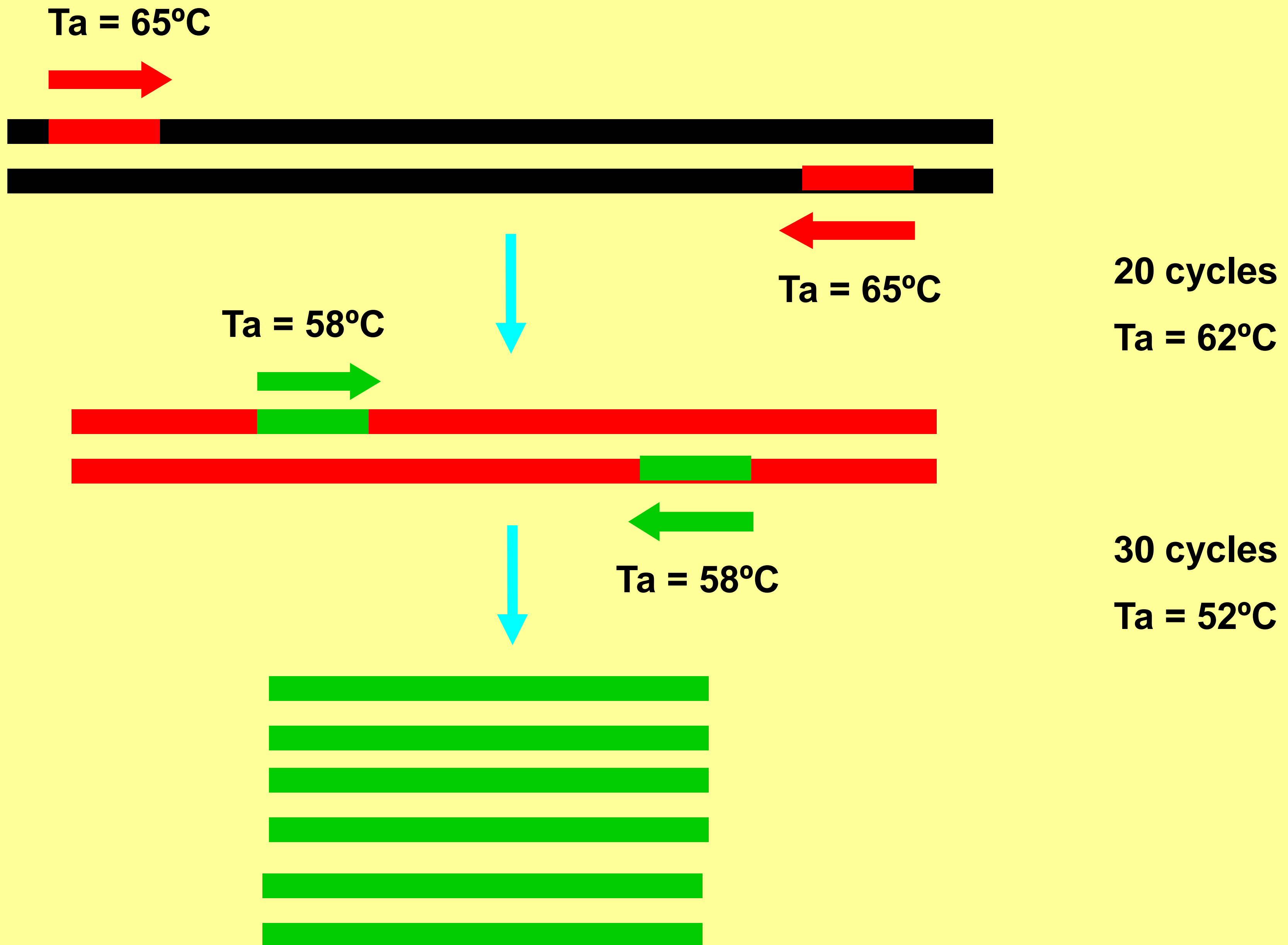


Sensitivity
Specificity

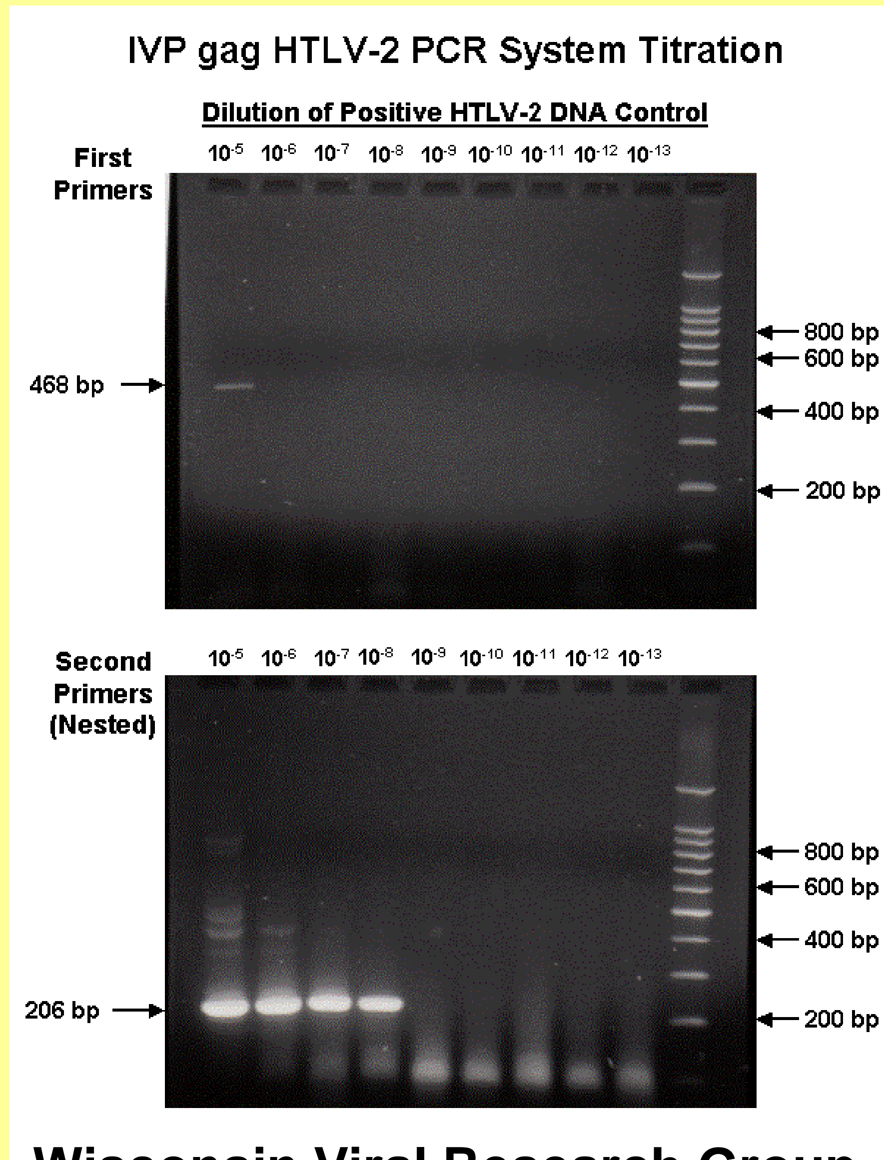
Arrangement of nested PCR



One tube nested PCR



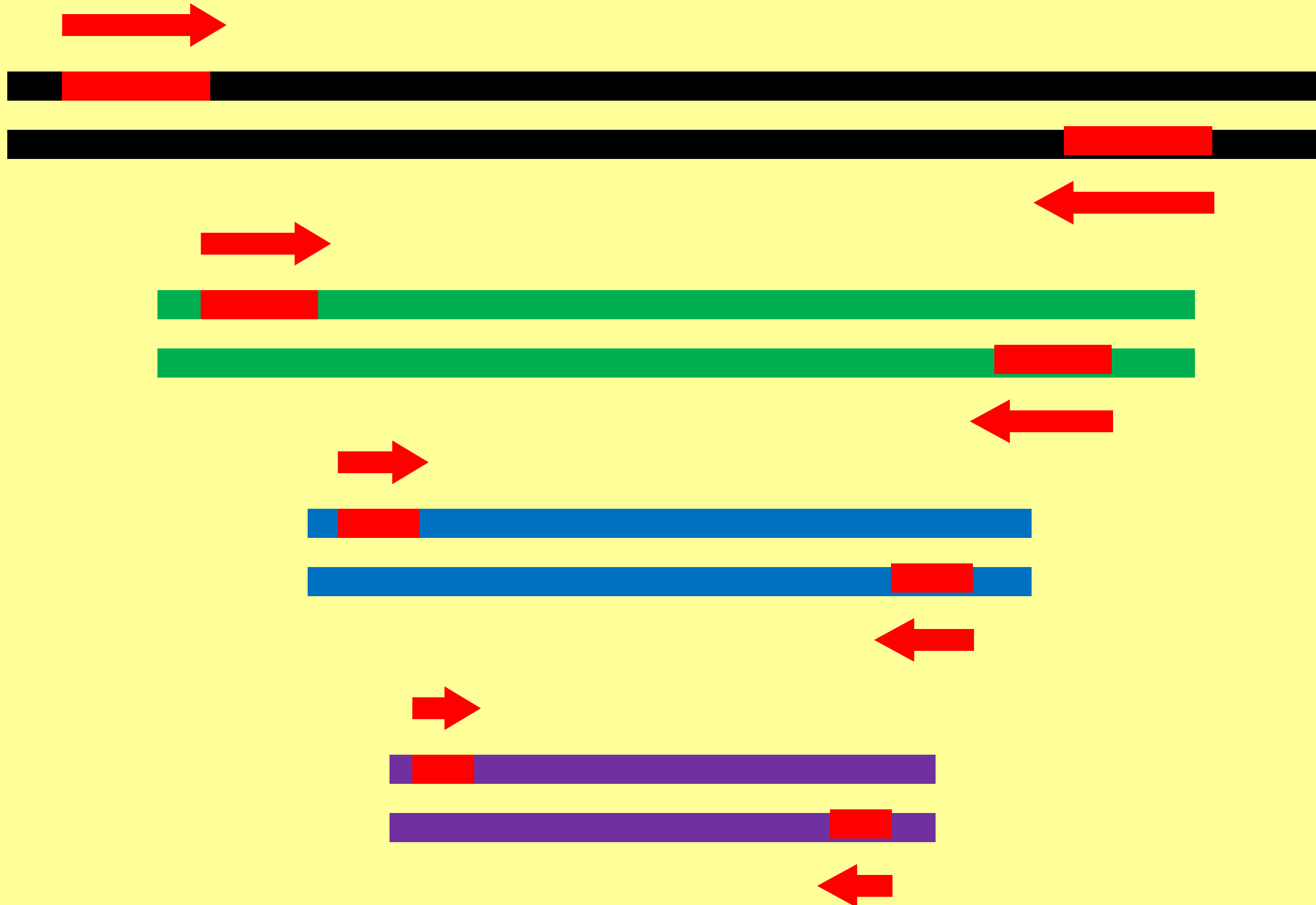
Nested PCR/PCR - example



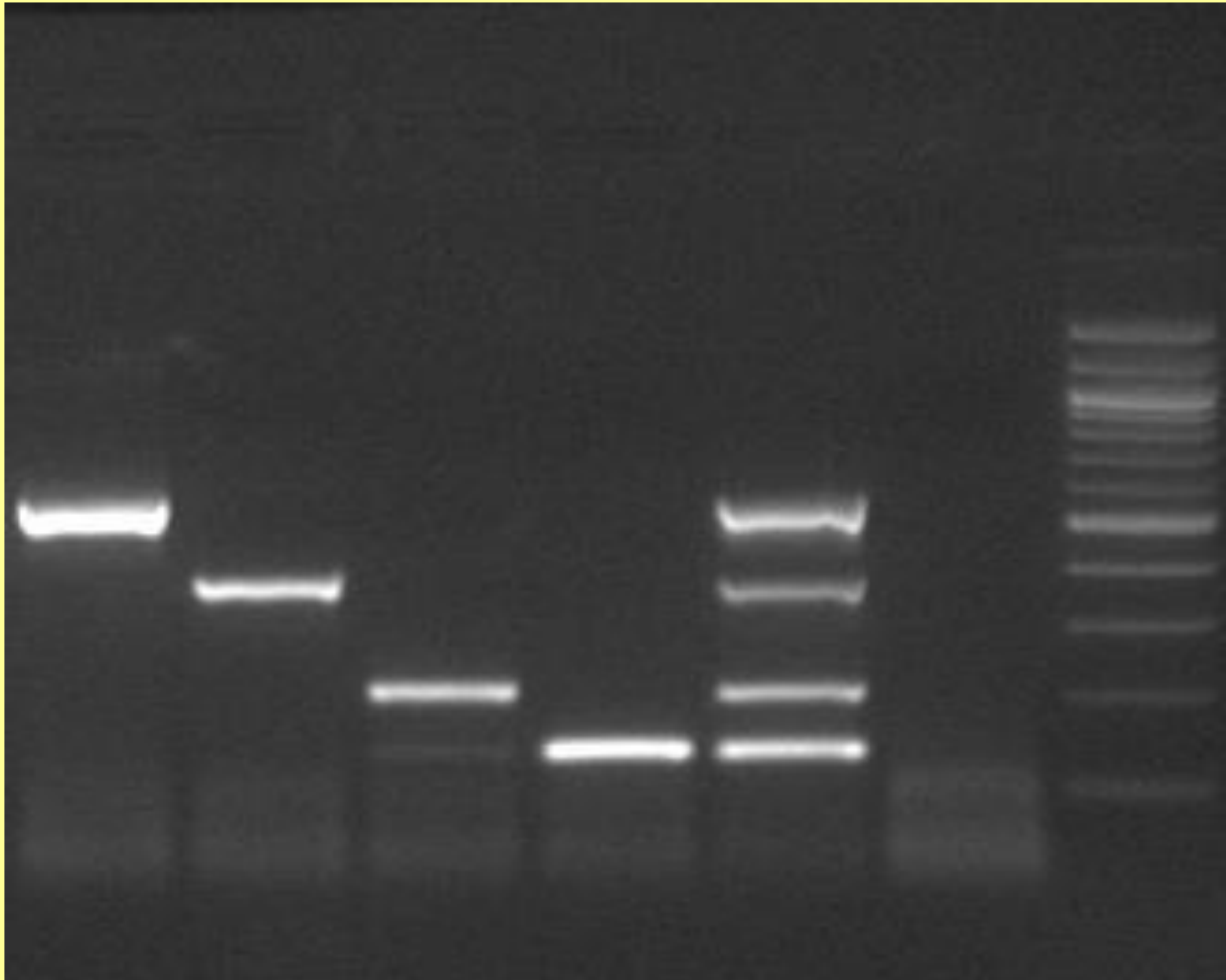
Wisconsin Viral Research Group, Ltd.
Testing for HHV-6 and EBV by nested PCR

Multiplex PCR

Simultaneous amplification of several loci in one PCR reaction

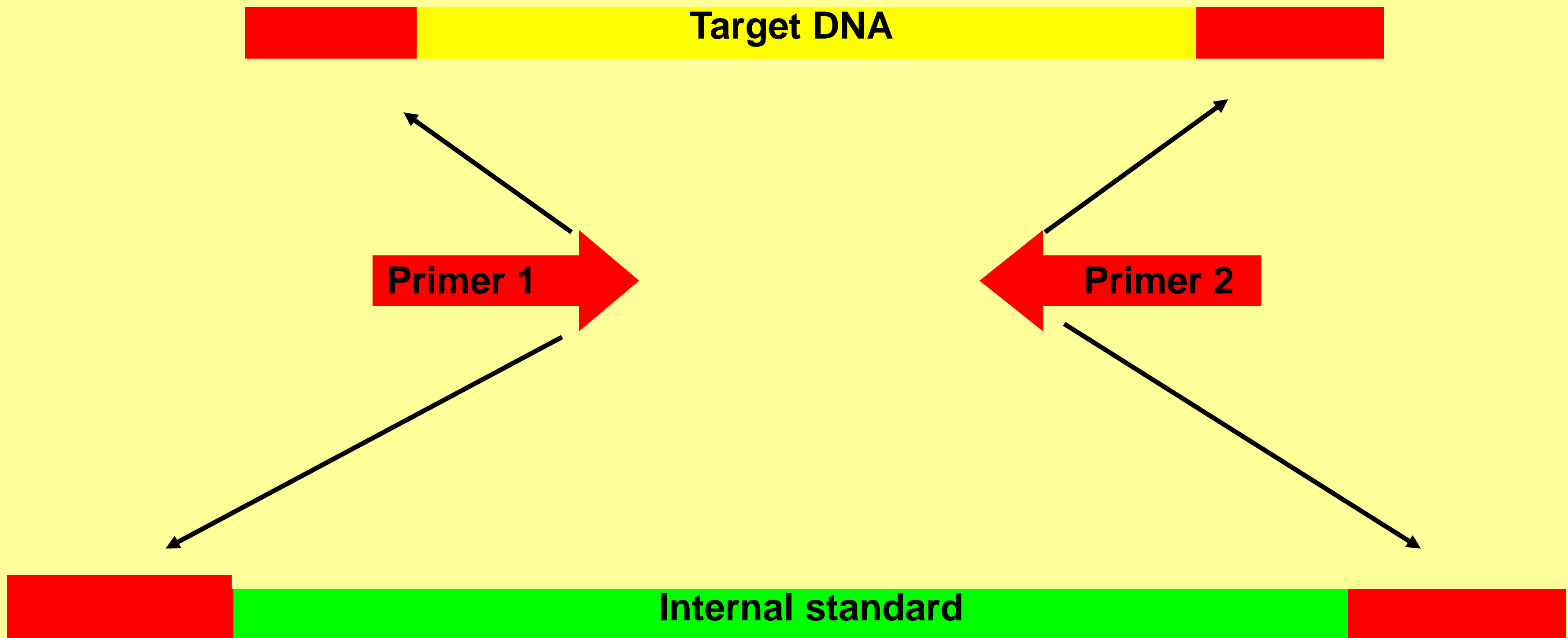


Multiplex PCR



Strategy for the detection and differentiation of *Mycobacterium avium* species in isolates and heavily infected tissues.
Moravkova M, Hlozek P, Beran V, Pavlik I, Preziuso S, Cuteri V, Bartos M

Competitive PCR



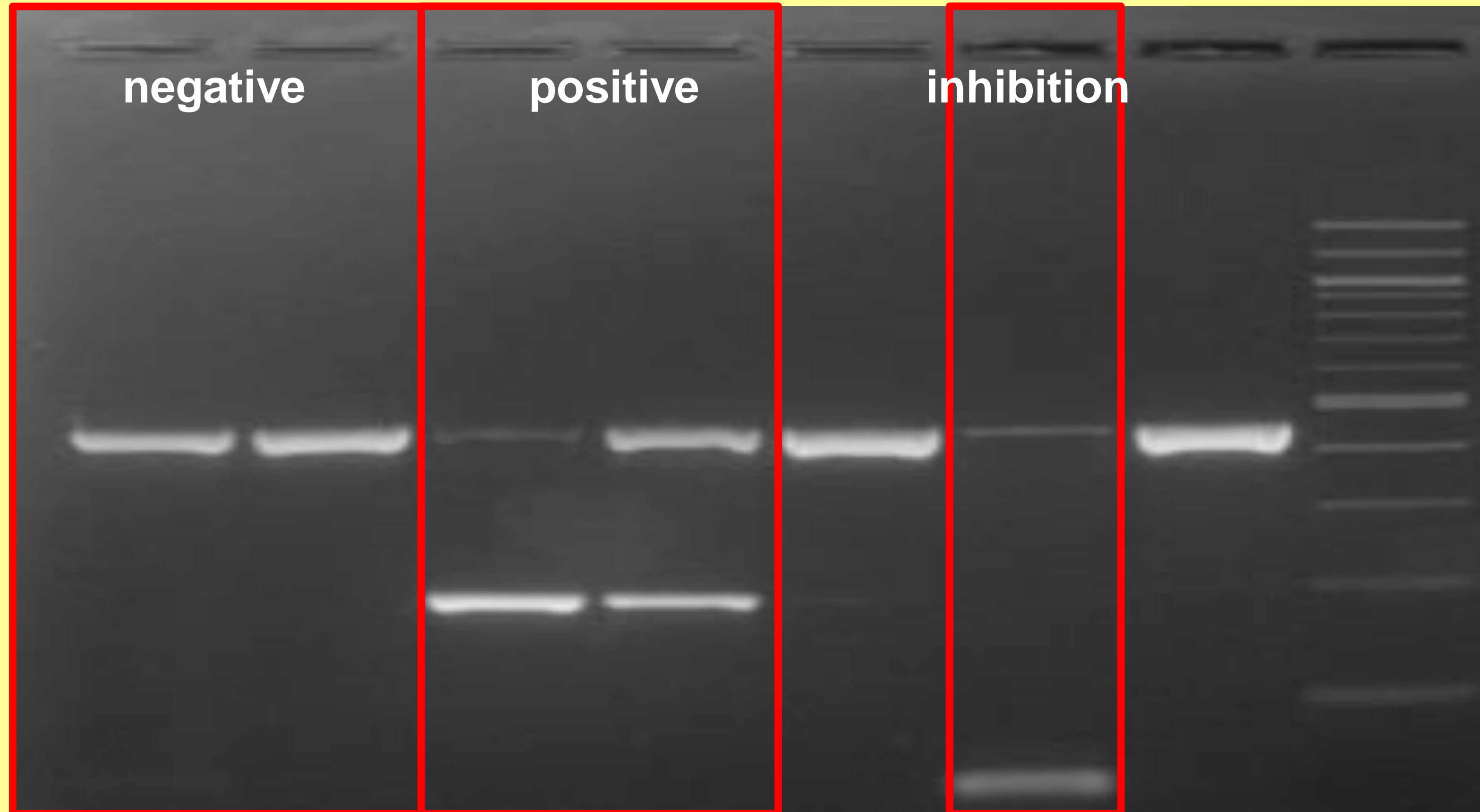
Competitive PCR

Positive | **Negative** | **Inhibition**

Target DNA amplification

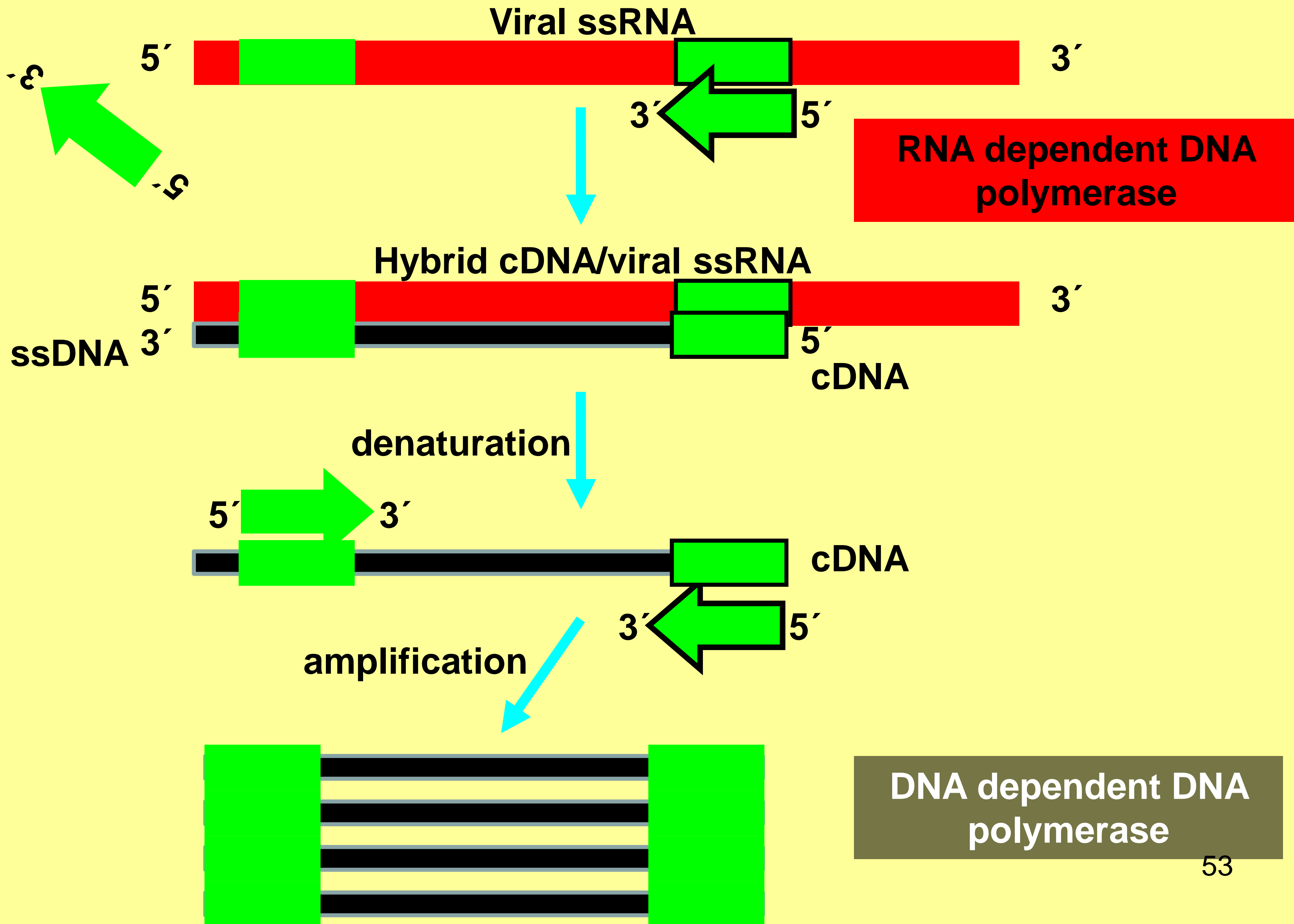
Internal standard amplification

Competitive PCR - example



Mycobacterium tuberculosis **PCR Kit**

Reverse transcription PCR



Thank you for your attention