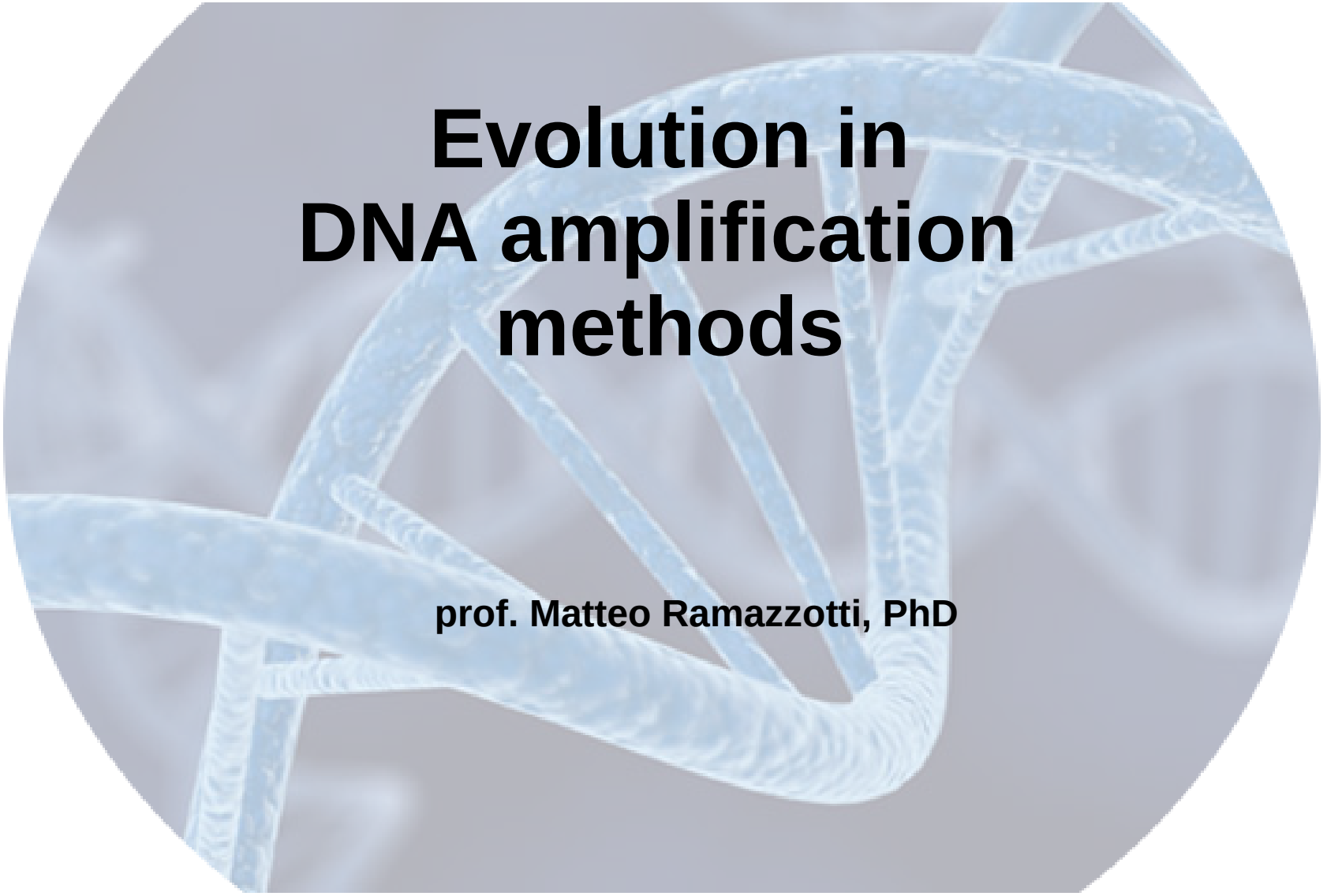
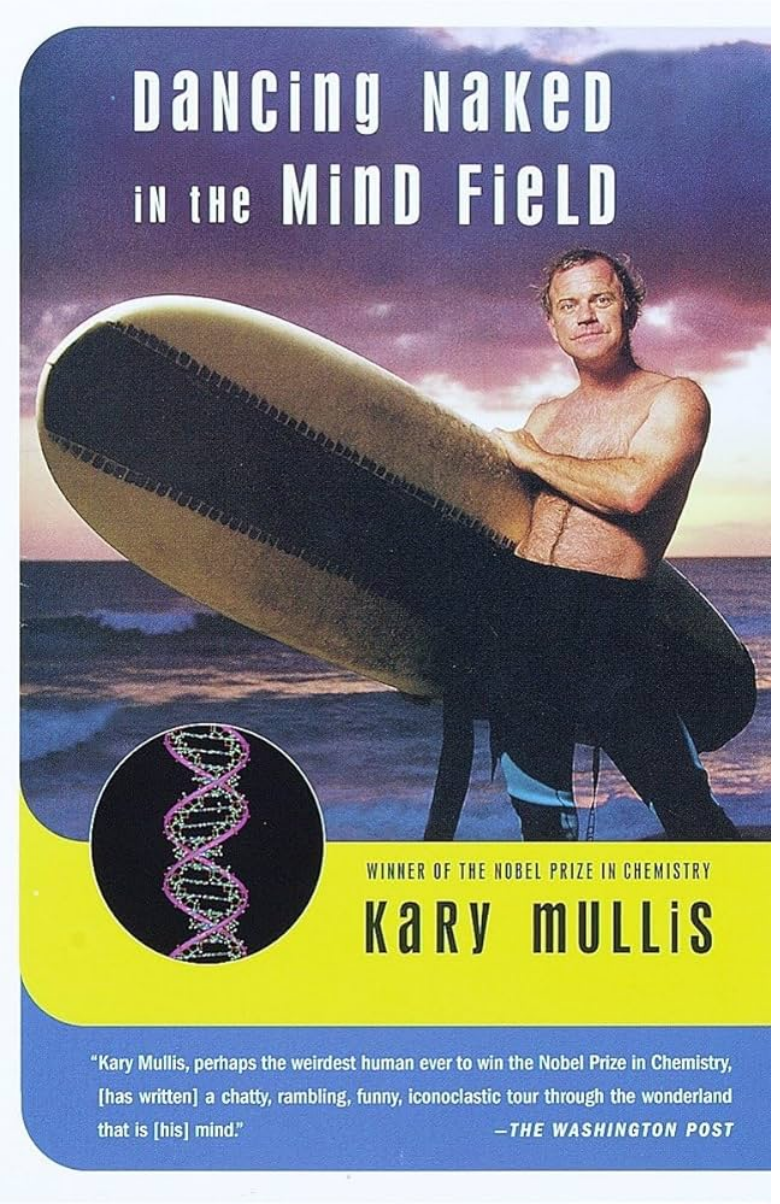


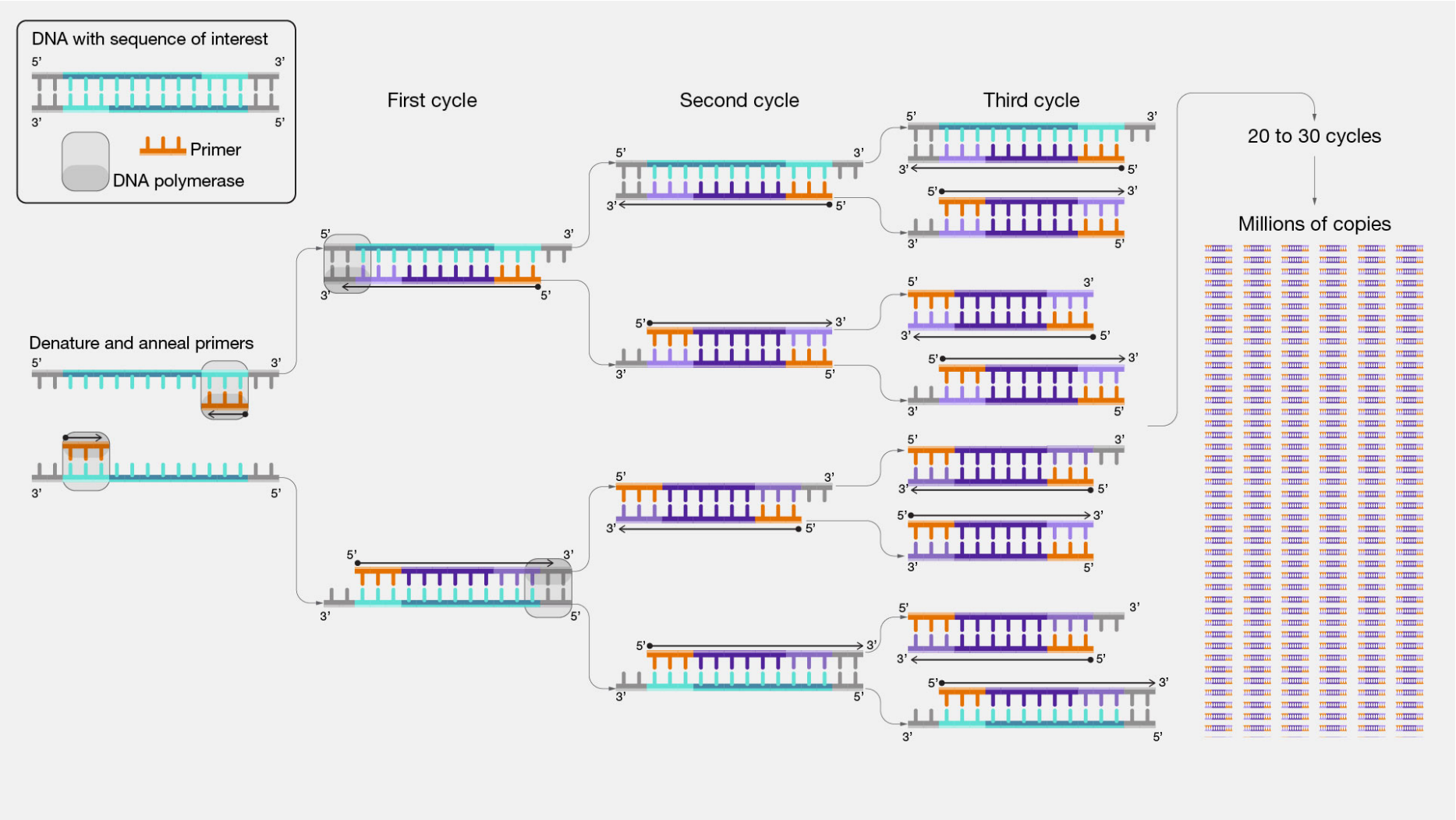
Evolution in DNA amplification methods

prof. Matteo Ramazzotti, PhD

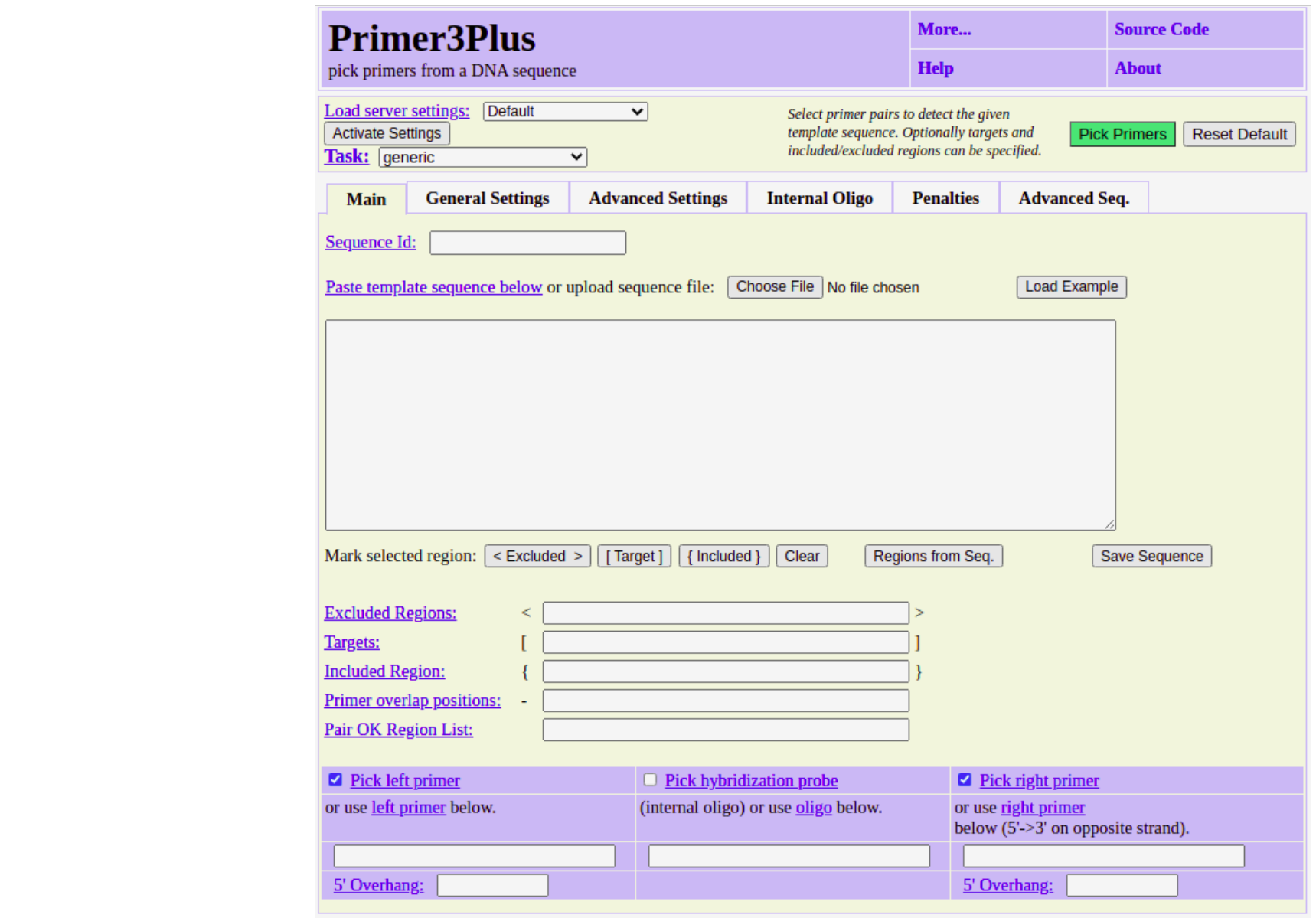
Kari Mullis 1986 invention (1993 Nobel Prize)



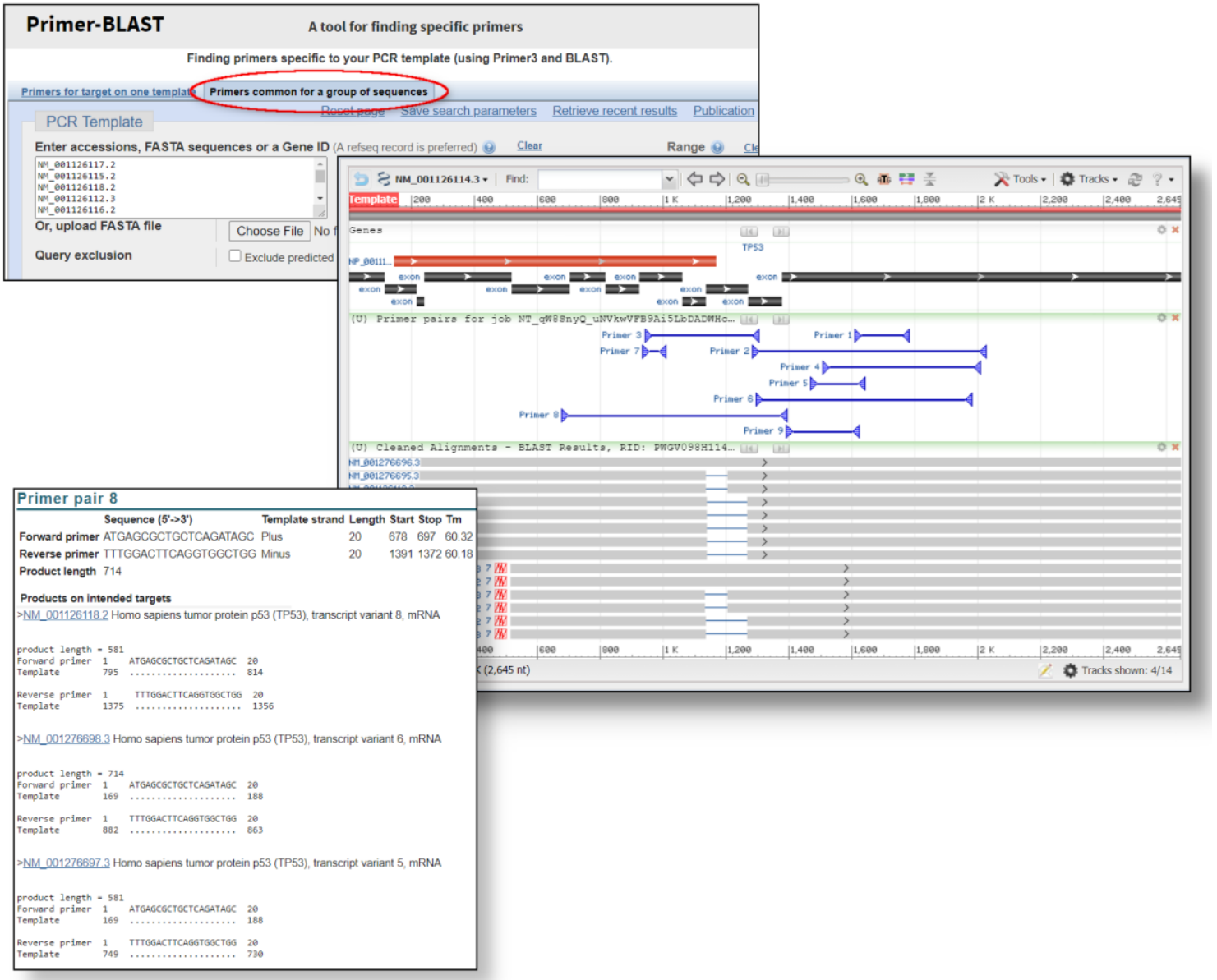
PCR the Polymerase Chain Reaction



Primer design – Primer 3



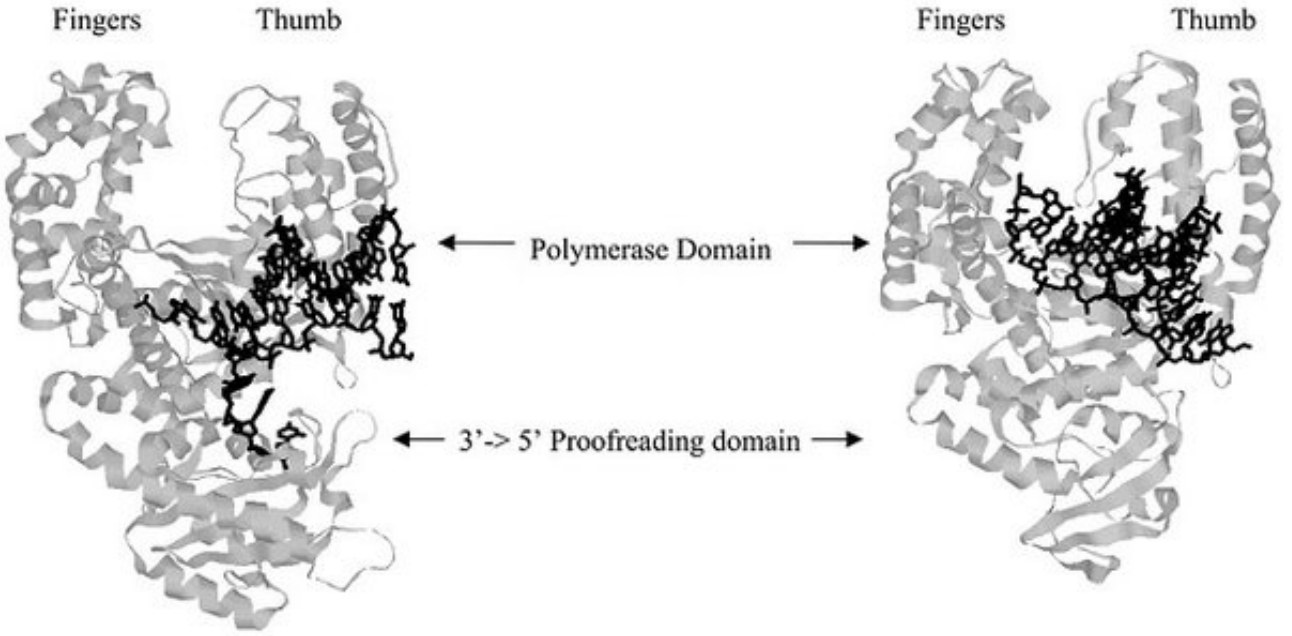
Primer design – Primer BLAST



Thermostable polymerases

Chien et al in **1976** first isolated Taq Polymerase from Thermus aquaticus (Yellowston Park), able to polimerize DNA up to 80°C and "survive" at 95°C.

After **Mullis** invented the polymerase chain reaction in **1983**, **Saiki** and others in **1988** implemented Taq as the active polymerizing enzyme in PCR, giving birth to modern molecular biology and biotechnology.



E. coli DNA polymerase **Taq DNA polymerase**

World's leading manufacturers in DNA Polymerases industry

TaKaRa

NEW ENGLAND BioLabs Inc.

Roche

Agilent

ThermoFisher SCIENTIFIC

illumina

MERCK

QIAGEN

BD

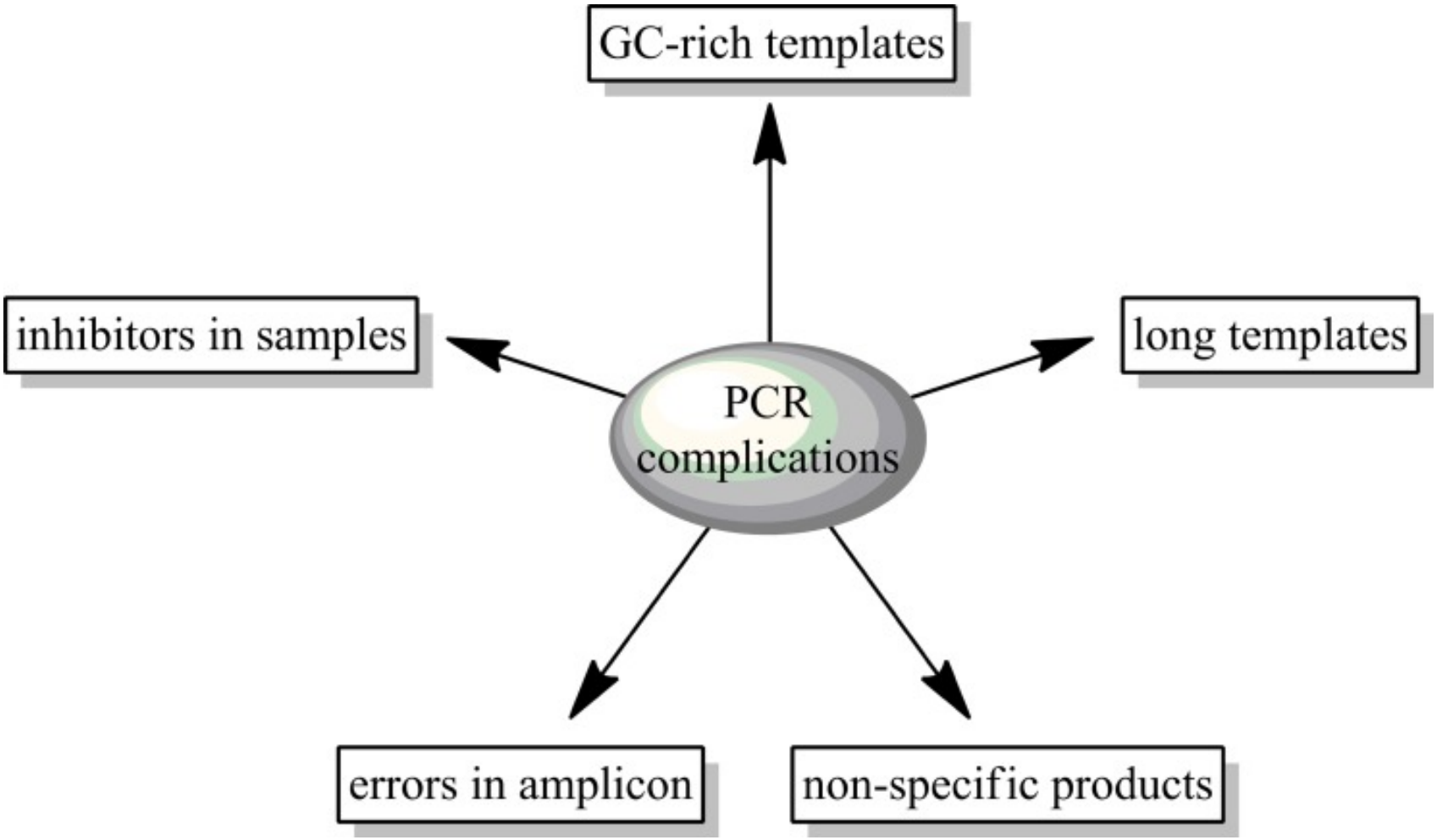
Promega

Polymerase features

PCR Polymerases	3′–>5′ Exonuclease	Fidelity	5′–>3′ Exonuclease	Strand Displacement	Nick Translation	Extend RNA Primer	Extension from Nick	dU Tolerance	Resulting Ends
Taq DNA Polymerase	no	1x	Yes	no	Yes	No	Yes	Yes	3'A
High Fidelity PCR									
Q5® High-Fidelity DNA Polymerase	++++	280x	No	no	No	No	No	No	Blunt
Q5U® Hot Start High-Fidelity DNA Polymerase	++++		No	no	No	No	No	Yes	Blunt
Polymerase*	++++	50x	No	no	No	No	No	No	Blunt
Routine PCR									
OneTaq® DNA Polymerase	++	2x	Yes	no	Yes	No	Yes	Yes	3'A/Blunt
Specialty PCR									
LongAmp® Taq DNA Polymerase	++	2x	Yes	no	Yes	No	Yes	Yes	3'A/Blunt
Hemo KlenTaq	no		No	+	No	No	No	Yes	3'A
Epimark® Hot Start Taq DNA Polymerase	no		Yes	no	Yes	No	Yes	Yes	3'A
Isothermal Amplification and Strand Displacement		**Error / Mb**							
Bst DNA Polymerase, Full Length	no		Yes	no	Yes	Yes	Yes	Yes	3'A
Bst DNA Polymerase, Large Fragment	no		No	++++	No	Yes	Yes	Yes	3'A
Bst 2.0 DNA Polymerase	no	62	No	++++	No	Yes	Yes	Yes	3'A
Bst 3.0 DNA Polymerase	no	70	No	++++	No	Yes	Yes	Yes	3'A
Bsu DNA Polymerase, Large Fragment	no		No	+	No	Yes	Yes	Yes	3'A
phi29 DNA Polymerase	++++	5	No	++++	No	Yes	Yes	Yes	Blunt
phi29-XT DNA Polymerase	++++	5	No	++++	No	Yes	Yes	Yes	Blunt
Polymerases for DNA Manipulation		**Error / Mb**							
T7 DNA Polymerase (unmodified)	++++	1.5	No	no	No	Yes	No		Blunt
Sulfolobus DNA Polymerase IV	no		No	no	No				3'A
Therminator™ DNA Polymerase	no		No	+	No	Yes	Yes	Yes	3'A
DNA Polymerase I (E. coli)	++	0.1	Yes	no	Yes	Yes	Yes	Yes	Blunt
DNA Polymerase I, Large (Klenow) Fragment'	++	18	No	+	No	Yes	Yes	Yes	Blunt
Klenow Fragment (3′→5′ exo-)	no	100	No	+++	No	Yes	Yes	Yes	3'A
T4 DNA Polymerase	++++	<1	No	no	No	Yes	No		Blunt

elaborated from NEB polymerase chart (link)

Issues with amplifications



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5243913/

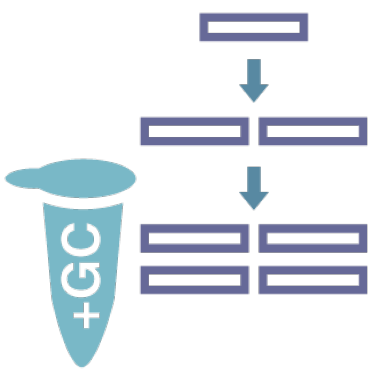
Inhibitors of amplification

Depending on the origin of the sample, **inhibitors** may be present:

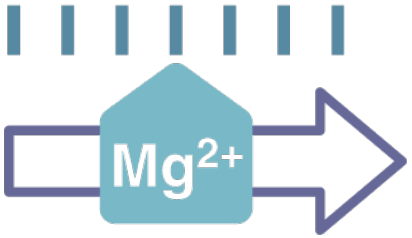
Inhibitors include various kinds of	- organic and - inorganic compounds
that can interact with	- the nucleic acid template - DNA polymerase active centers - cofactors (e.g. magnesium ions)
Examples of PCR inhibitors include	- bile salts in feces - heme In blood - urea in urine - heparin - formalin - charcoal

https//www.ncbi.nlm.nih.gov/pmc/articles/PMC5243913/

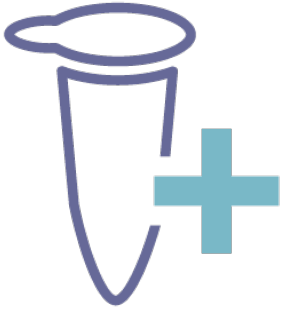
Tools to tweak ineffective PCR



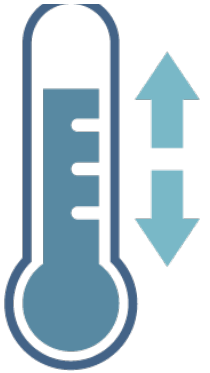
Polymerase choice



Mg^{2+} concentration gradient



Effects of additives



Annealing temperature range

Amplification of long / GC-rich DNA

A PCR may involve a region with

- GC pairs above 60%
  - ~3% of human genome
  - often found in the promoters
  - of housekeeping and tumor suppressor genes
- a length >10Kb

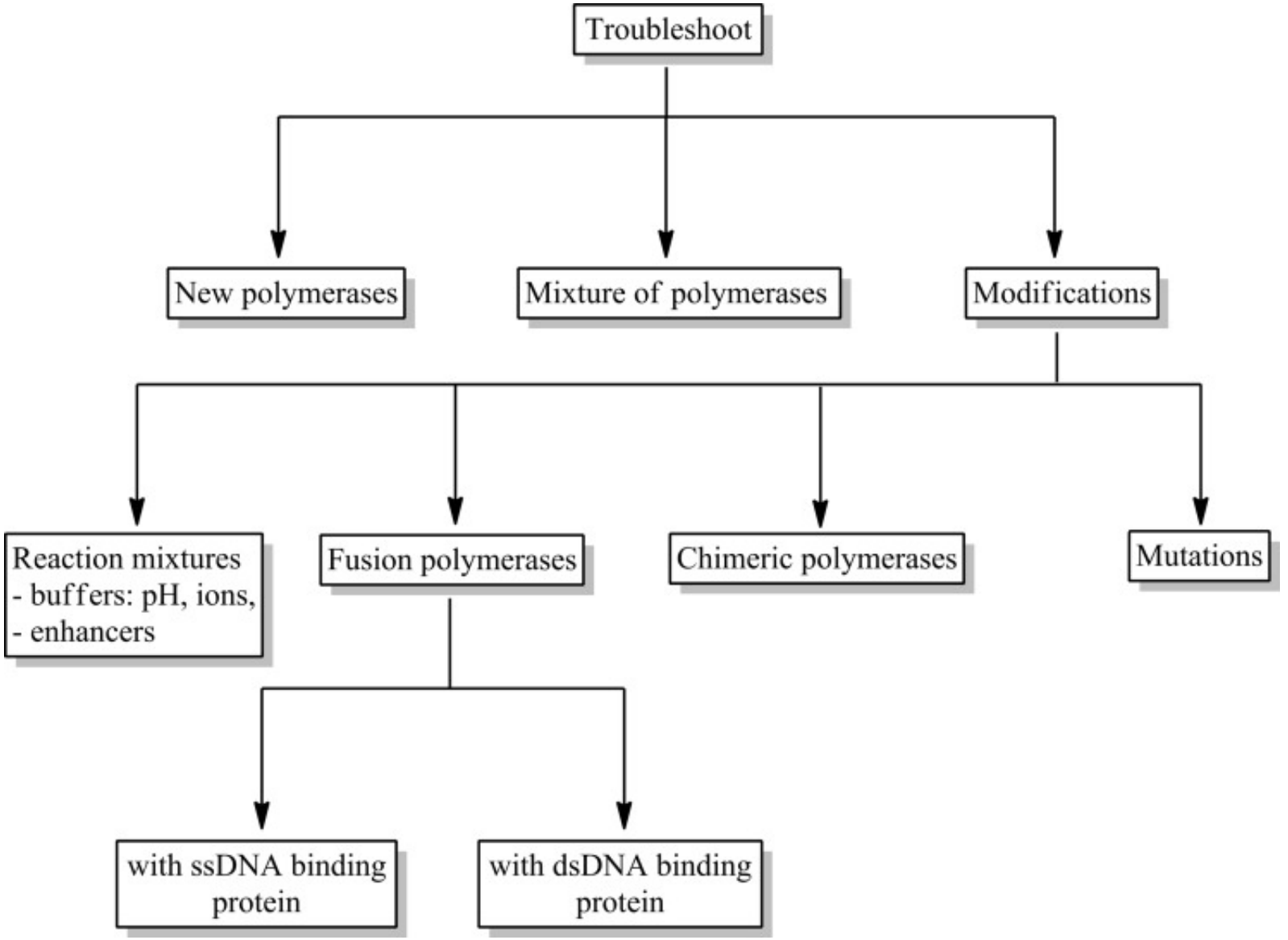
GC-rich regions are - intrinsically harder to open
- prone to form secondary structures

Such issues may be surpasses by **tweaking Pol buffer** with

- glycerol / DMSO: to reduce DNA secondary structure
- tetramethylammnium: to strengthen primer binding

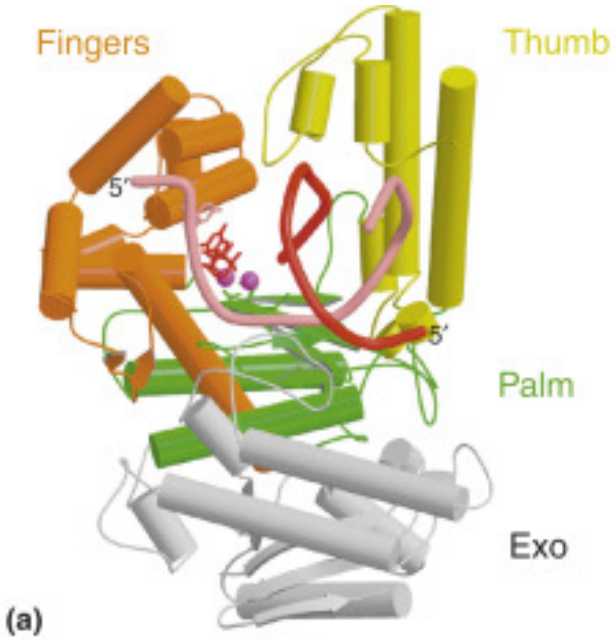
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5243913/

Troubleshooting failed PCR



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5243913/

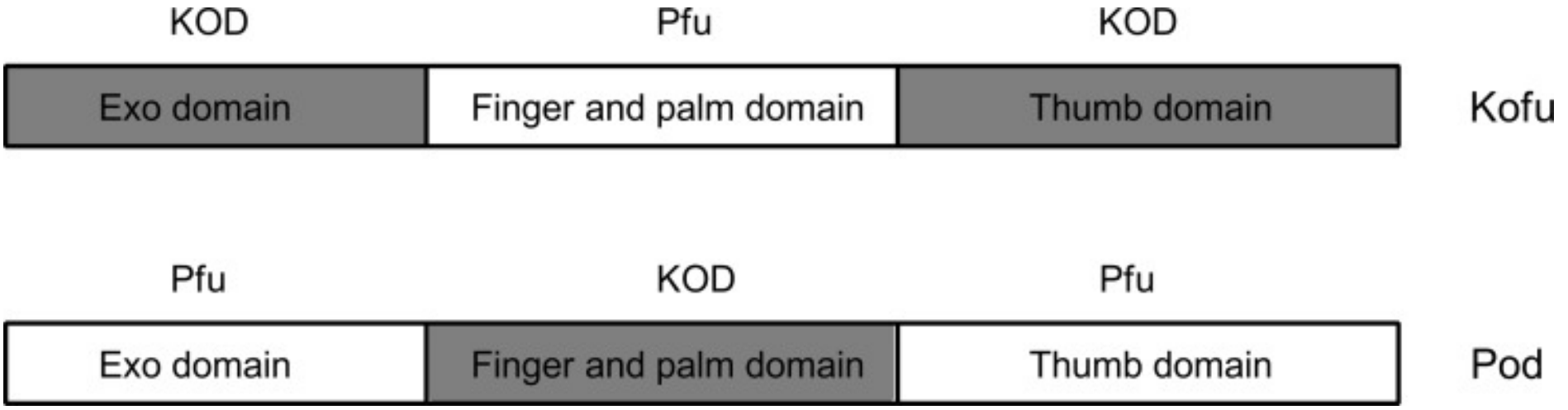
Polymerase domains



Name	Family	
Pol β	X	HTH · thumb · palm^{L} · palm^{S} · fingers
Pol X*	X	HTH · thumb · palm^{L} · palm^{S} · fingers · PHP
Pol C	C	OB · PH · 3-5exo · P · palm^{L} · thumb · $\beta_2\alpha$ · palm^{S} · fingers
Pol III	C	3-5exo + PHP · palm^{L} · thumb · $\beta_2\alpha$ · palm^{S} · fingers · OB
Pol I	A	5-3exo · 3-5exo · thumb · $\beta_2\alpha$ · palm^{S} · fingers · palm^{L}
Pol II	B	xxx** · 3-5exo · palm^{S} · fingers · palm^{L} · thumb
Pol IV	Y	palm^{S} · fingers · palm^{L} · thumb · pad ***

Chimeric polymerases

Given the conserved structure, polymerase domains can be switched in order to modulate binding and efficiency.

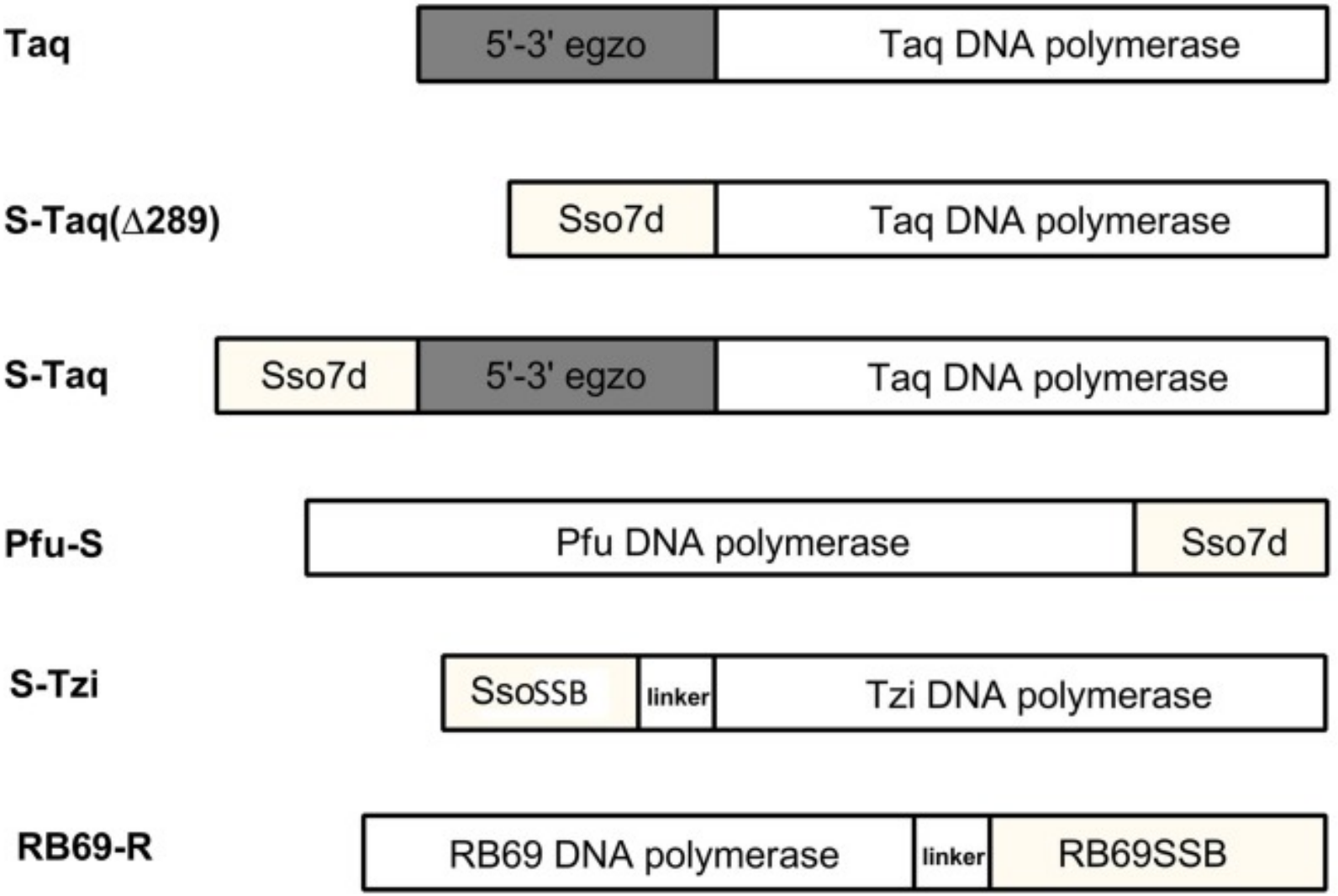


KOD: domain derived from Thermococcus kodakarensis polymerase;

Pfu: domain derived from Pyrococcus furiosus polymerase

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5243913/

Fusion polymerases



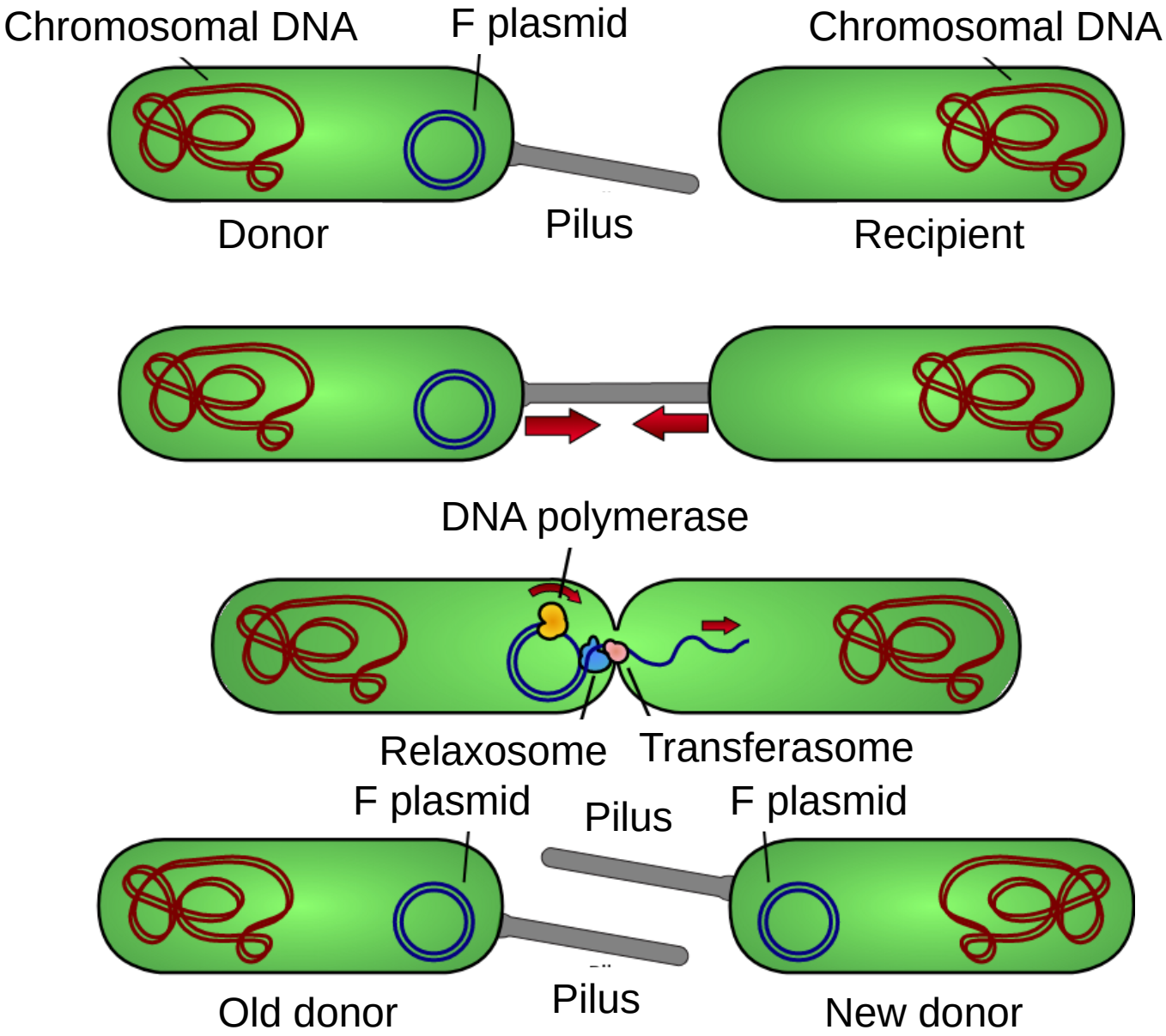
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5243913/

Commercially available polymerase

Trade name	Structure	Effect
Phusion High-Fidelity (Thermo Scientific)	*Sso7d* + *Pfu*	Increased fidelity and processivity, amplification of longer DNA fragments
Hercules II Fusion (Agilent Technologies)	*Sso7d* + *Pfu*	Amplification of matrixes that are rich in GC, high sensitivity, increased processivity
Phusion (NEB)	*Sso7d* + *Pfu*	Greater fidelity, rate, and specificity, amplification of matrixes that are rich in GC
iProof ™ High-Fidelity DNA Polymerase (Bio-Rad)	*Sso7d* + *Pfu*	Amplification of longer DNA fragments, DNA processivity and fidelity

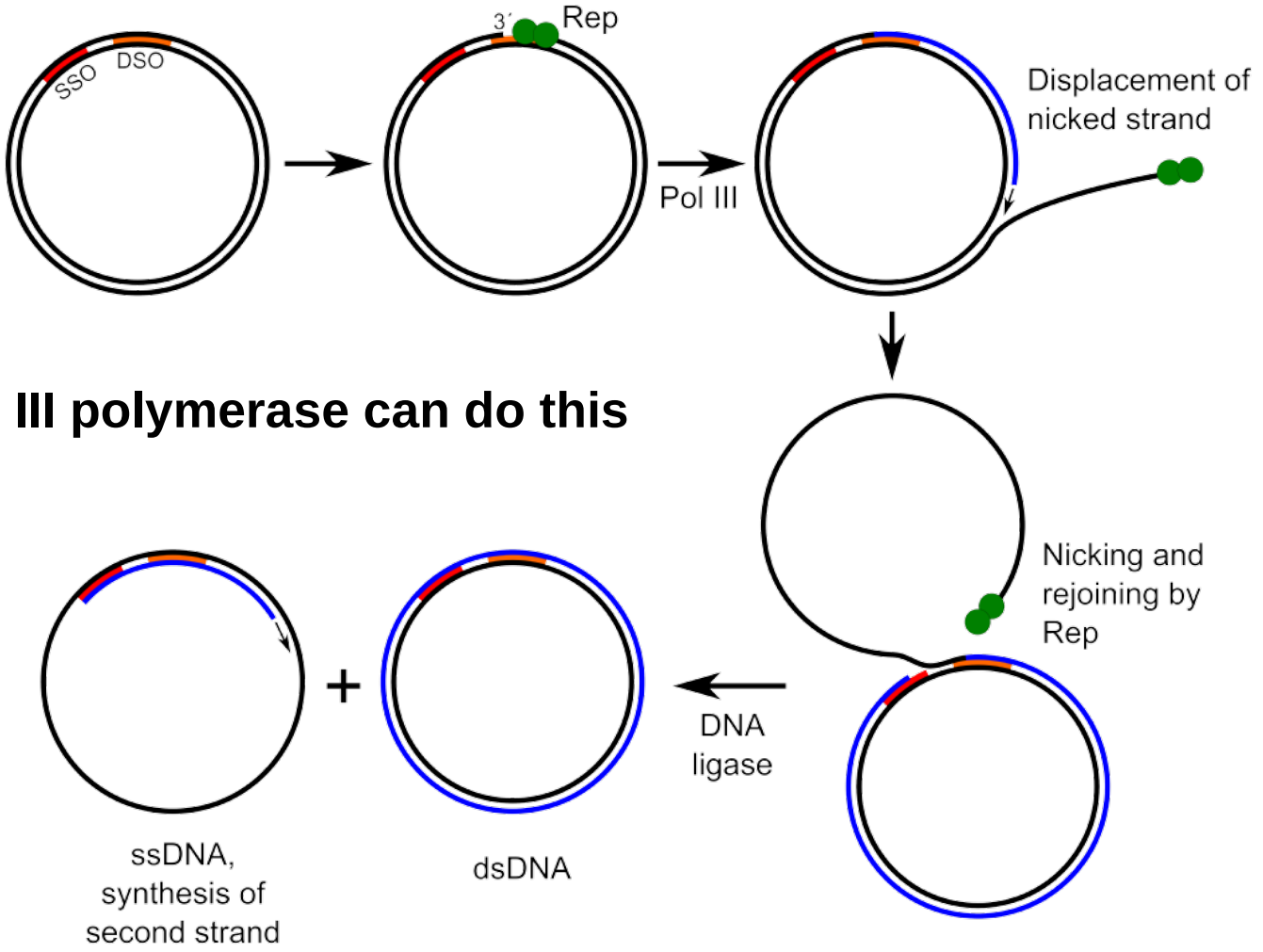
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5243913/

Plasmid transmission is about DNA polymerization



Rolling circle DNA duplication

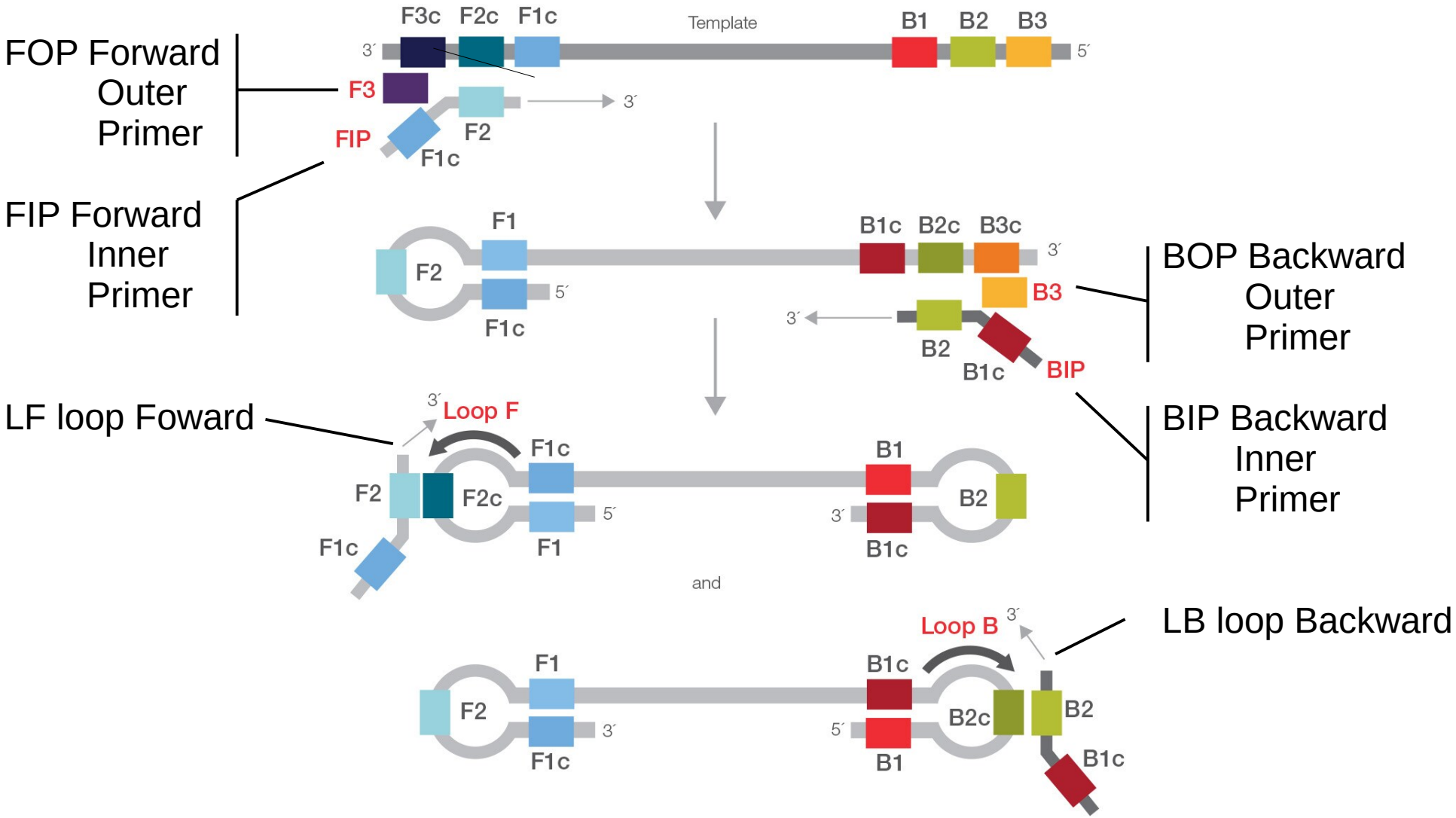
Rolling Circle Replication (RCR) is the unidirectional nucleic acid **replication** that can rapidly synthesize multiple copies of **circular** molecules of DNA (or RNA).



Tye III polymerase can do this

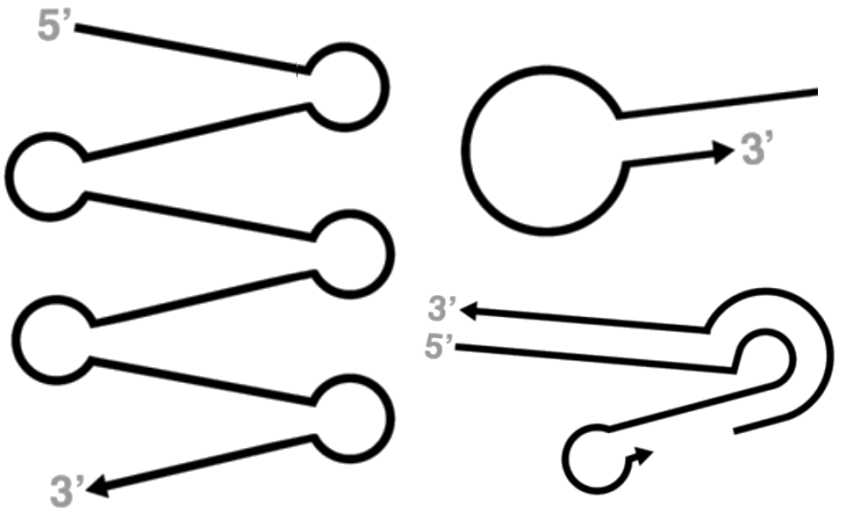
Loop-mediated isothermal amplification (LAMP)

Type III polymerases can be use with appropriate priming strategies for DNA amplification



Loop-mediated isothermal amplification (LAMP)

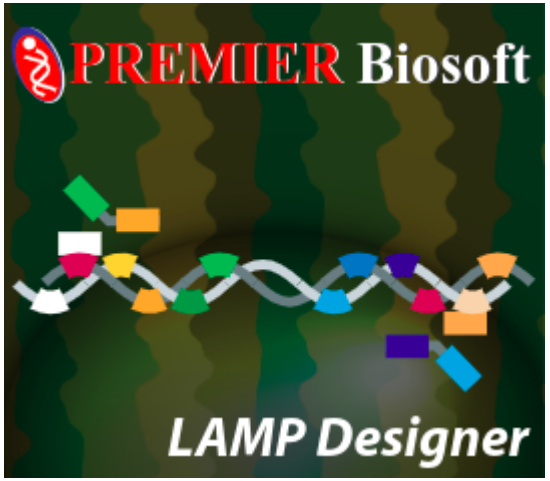
Keep at 65°C **isothermal**



many different forms of the same fragment can be observed

an amplification anyway

Software for LAMP primer design



1) Avoid Cross Homologies: by BLAST

2) Verification of specificity: by PirimerBLAST

3) Export Result in CSV form.

1) Can design
   - 4 primers based on
   - 6 regions required by the LAMP method

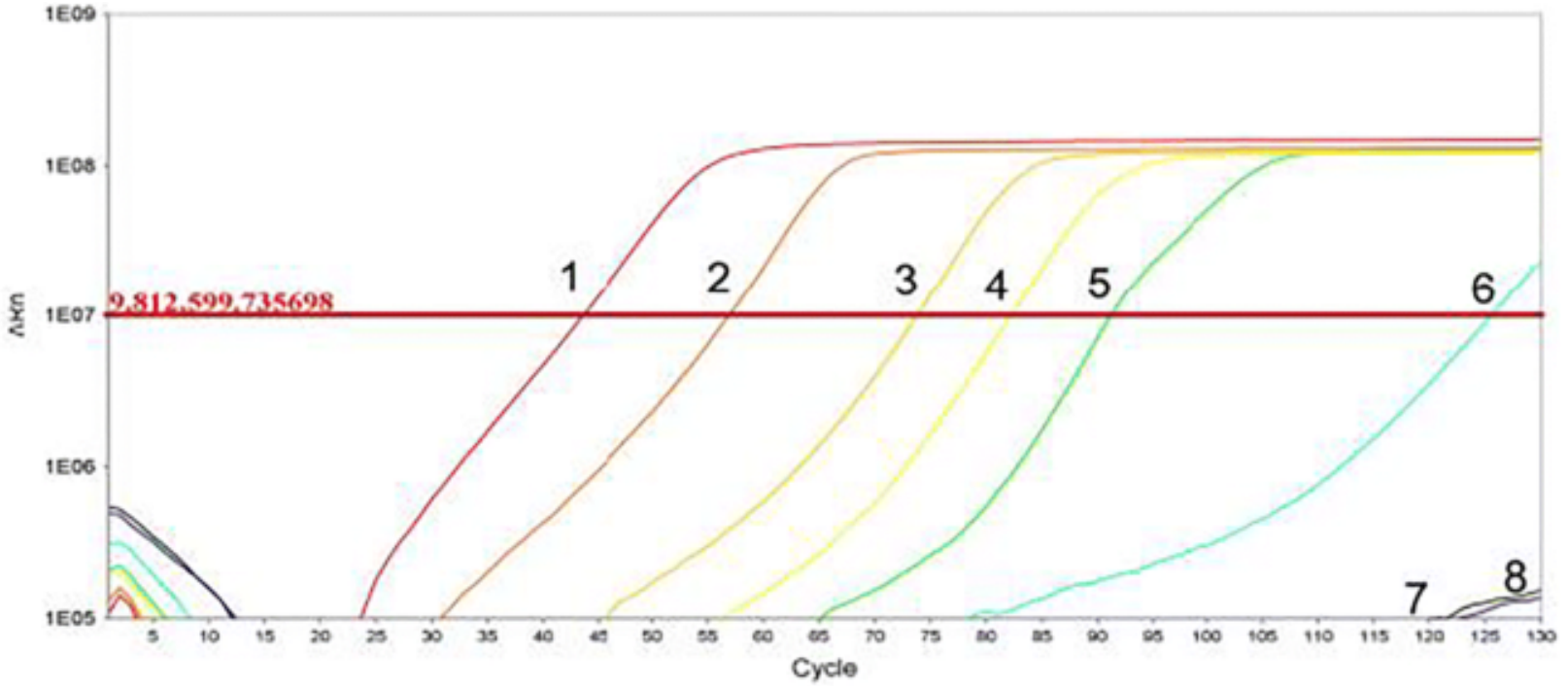
2) Can also design loop primers

3) Use the "nearest neighbor" method for Tm calculation

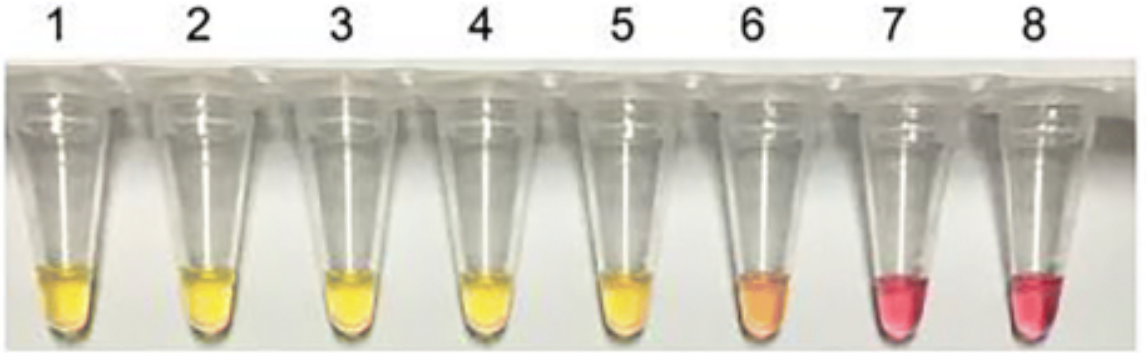
4) Web usage

Loop-mediated isothermal amplification (LAMP)

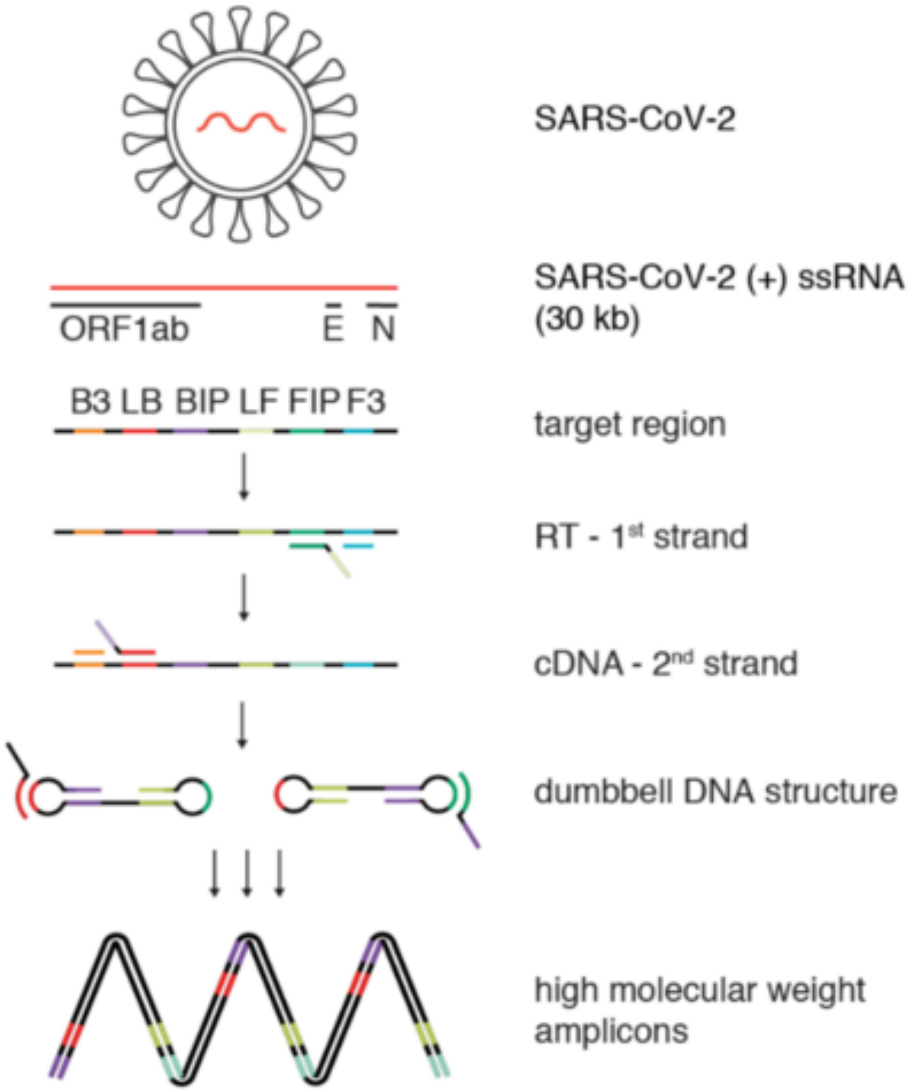
a



b



RT-LAMP for SARS-Cov2



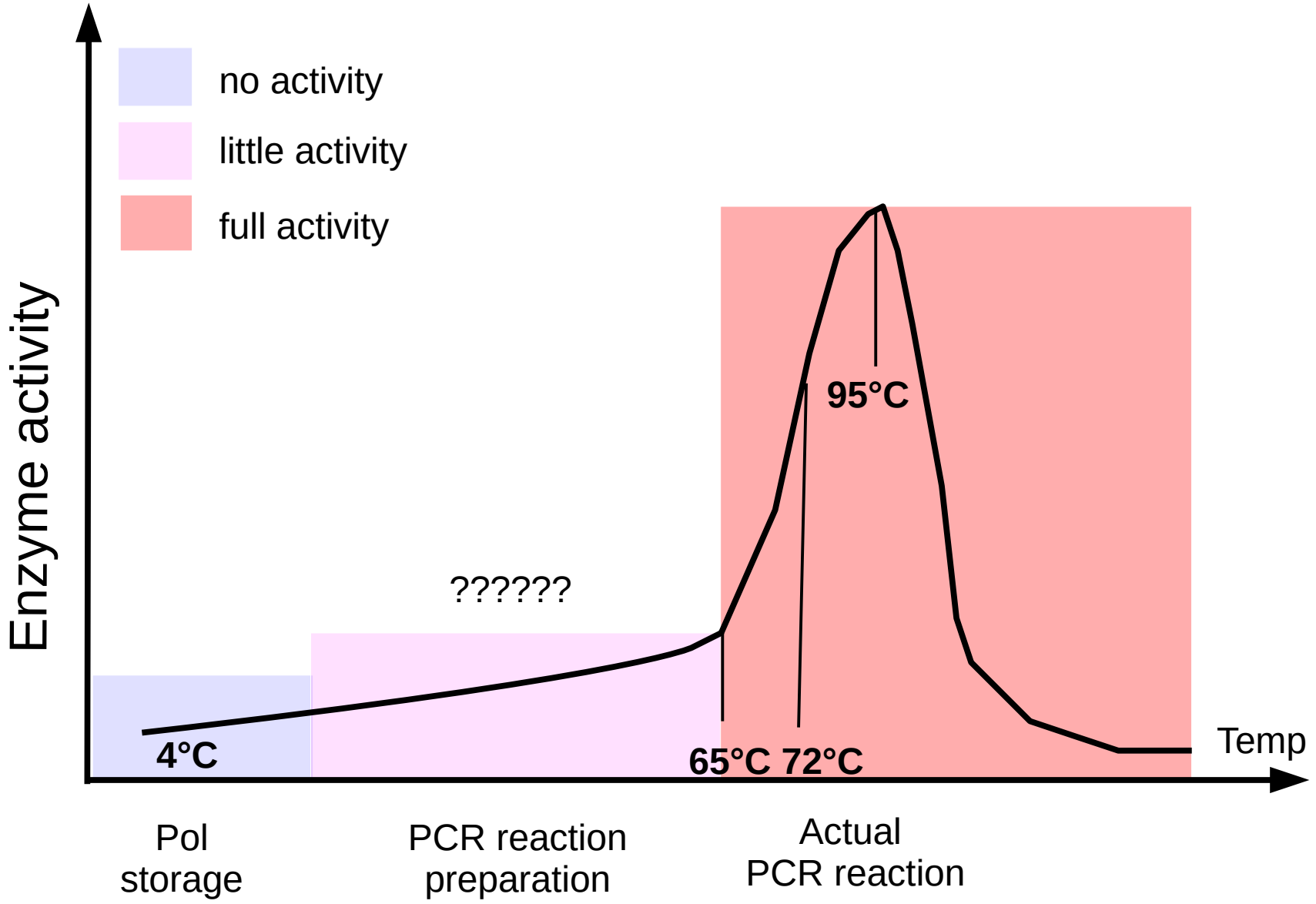
The tests can be done at home

(a) colorimetric RT-LAMP

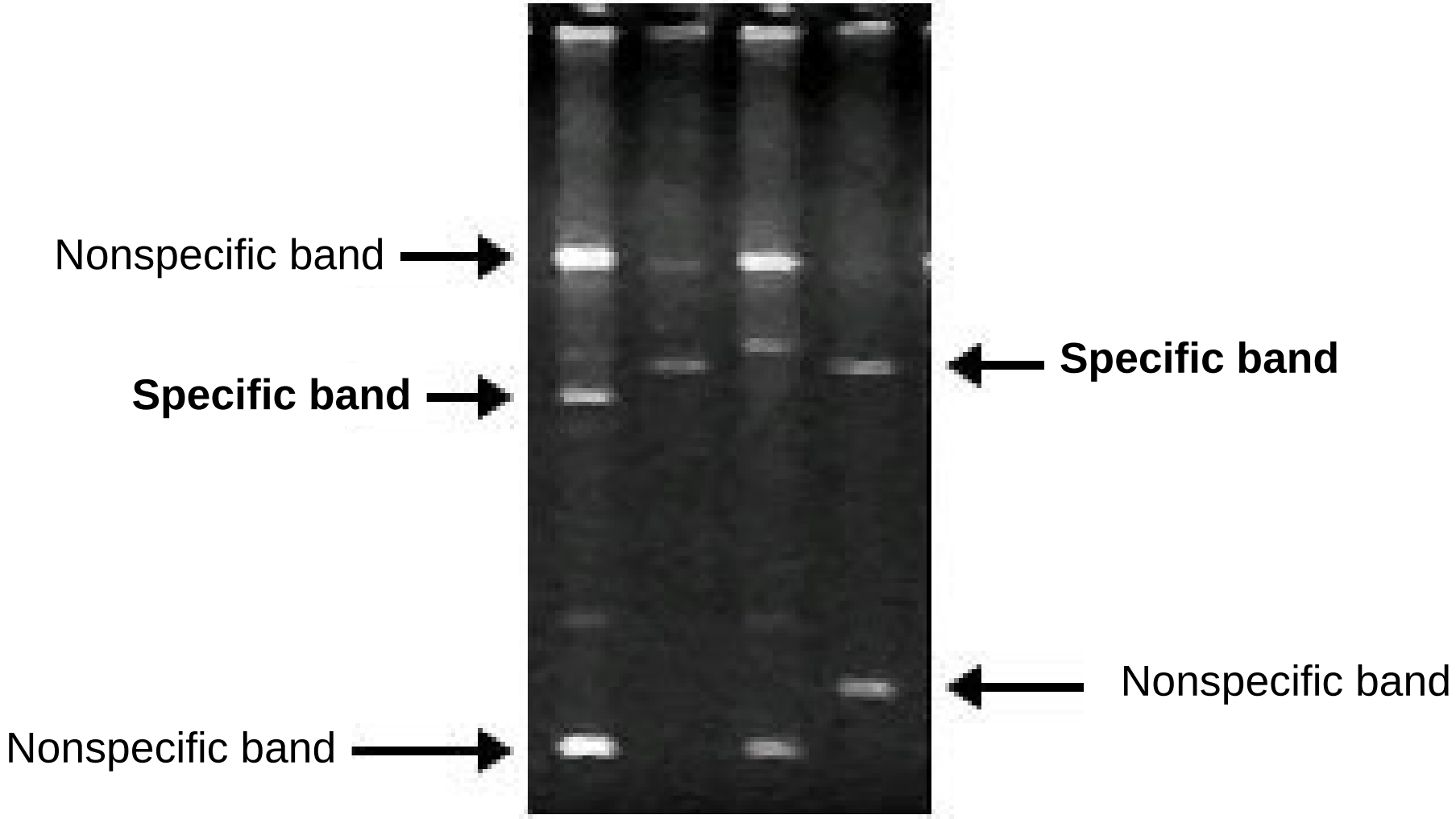
(b) self-collected saliva specimens

(c) direct testing on crude saliva samples without RNA extraction.

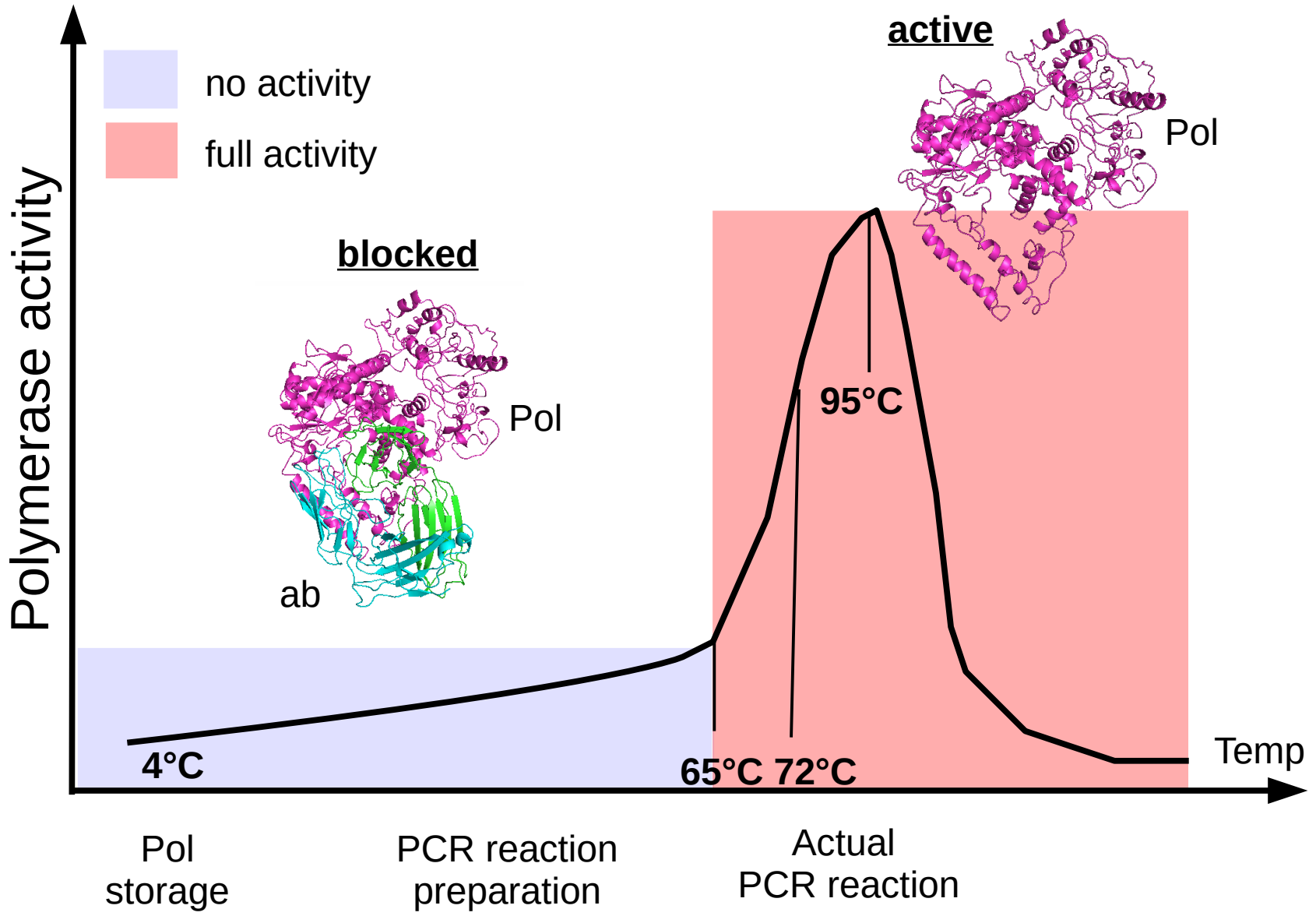
The DNA polymerase perspective



A PCR may be nonspecific

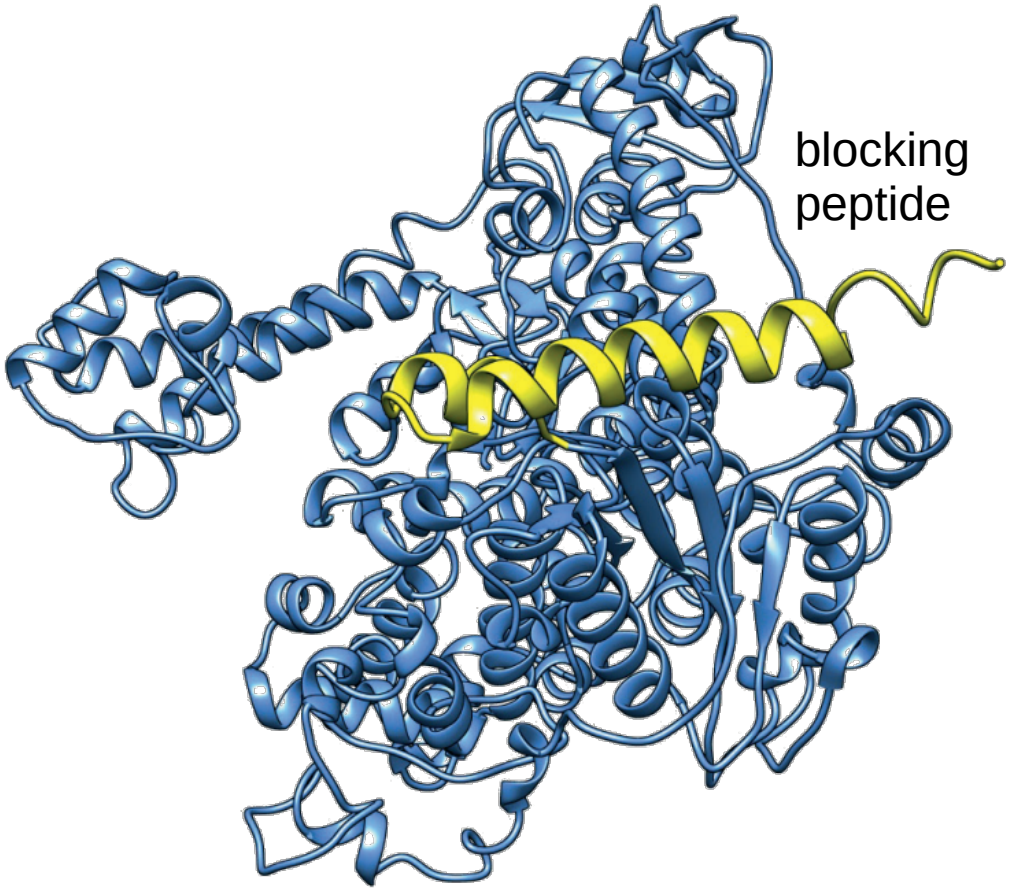


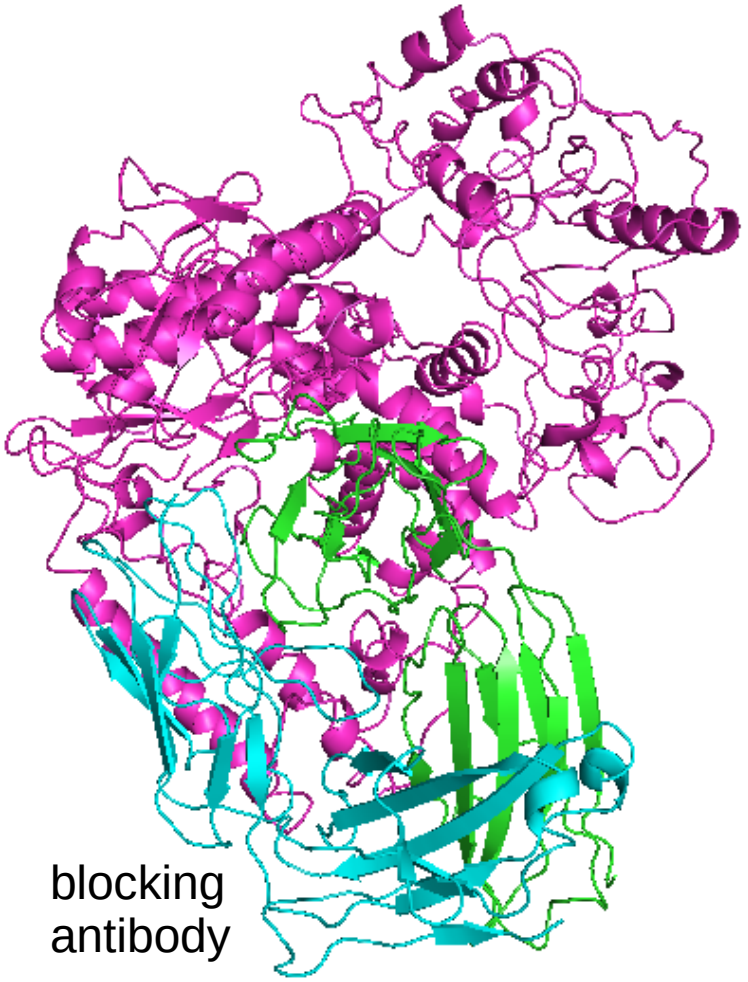
The DNA polymerase perspective



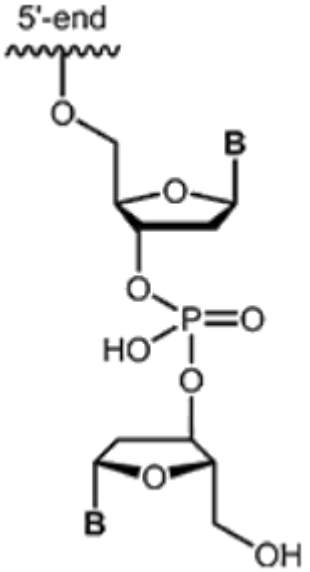
Stabilized polymerases for Hot/Warm-Start

Mutations | Stabilizers | Aptamers | Chemicals

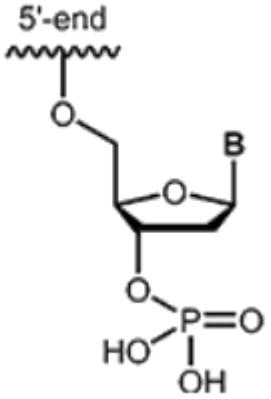




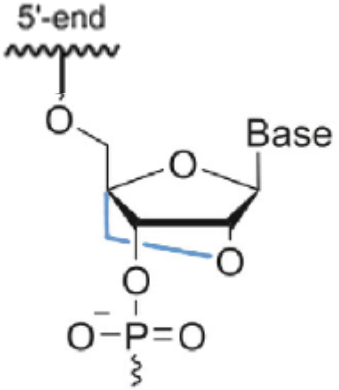
Blocking unwanted amplification



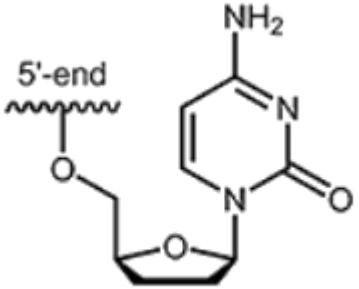
3'-Inverted End



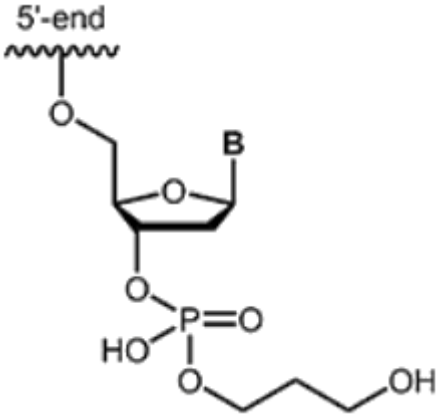
3'-Phosphate



Locked NA

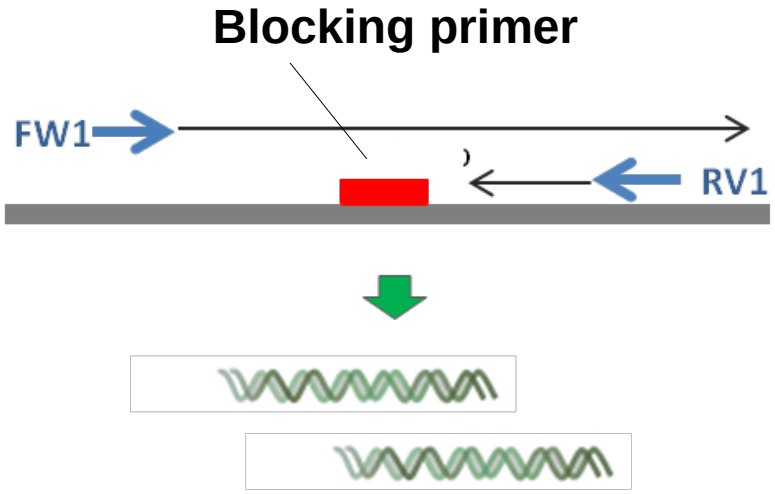


3'-ddC

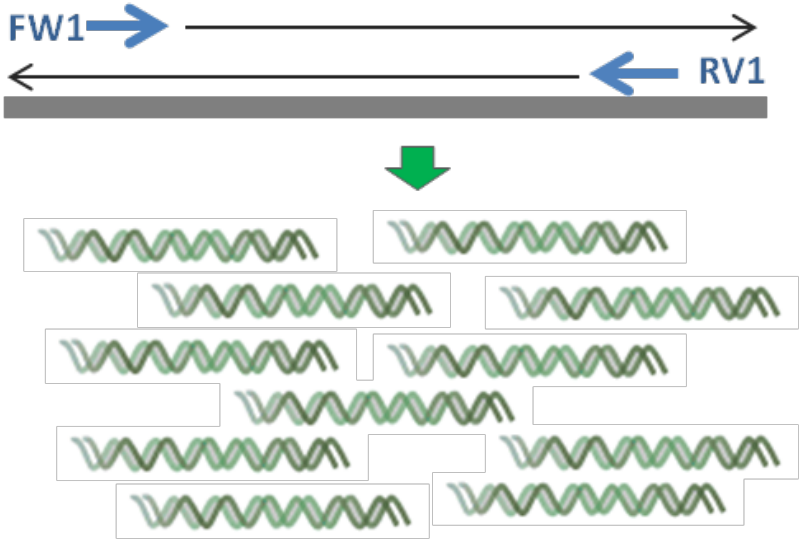


3'-Spacer C3

Blocking primers in action

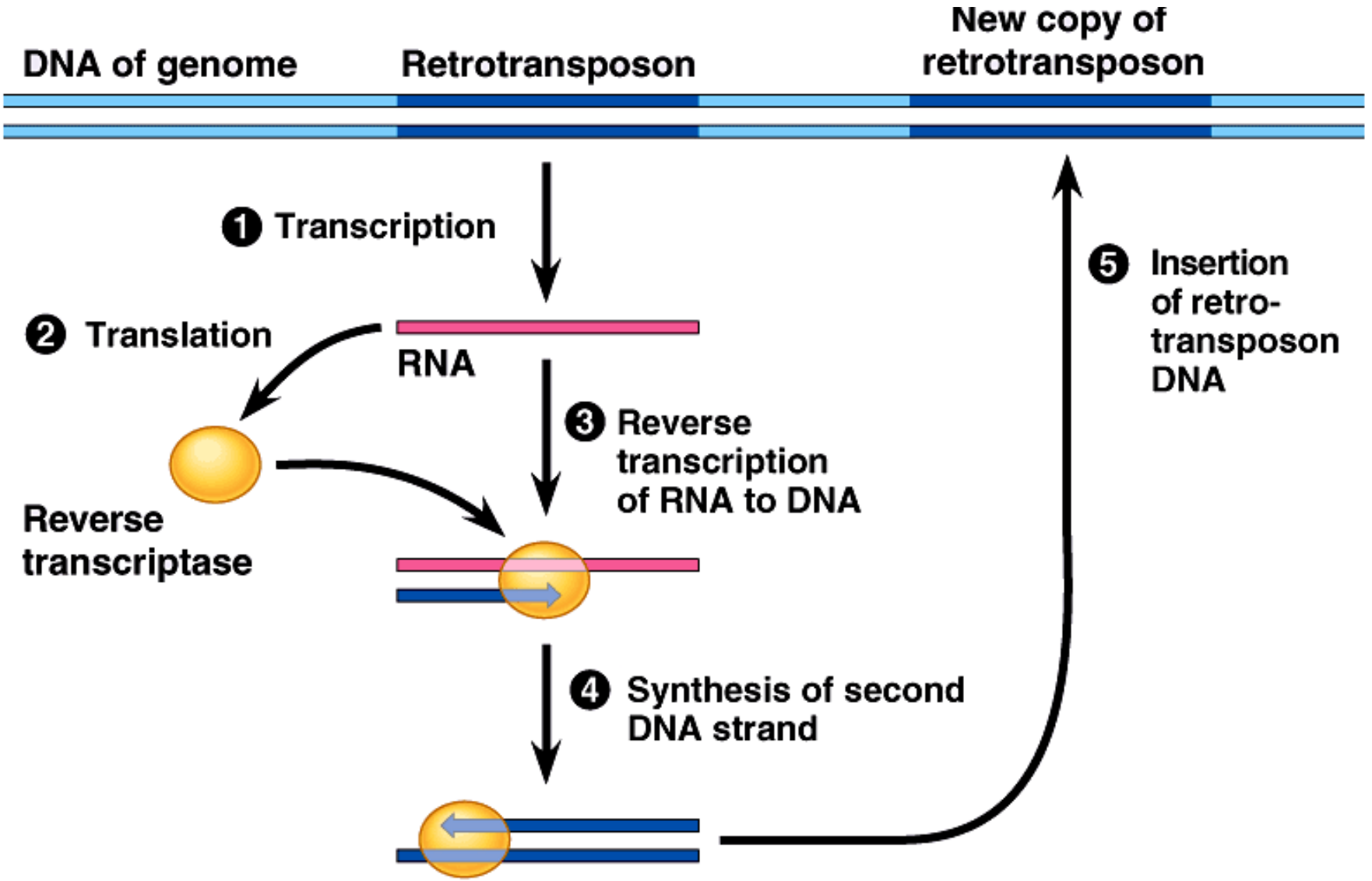


No amplification



Amplification

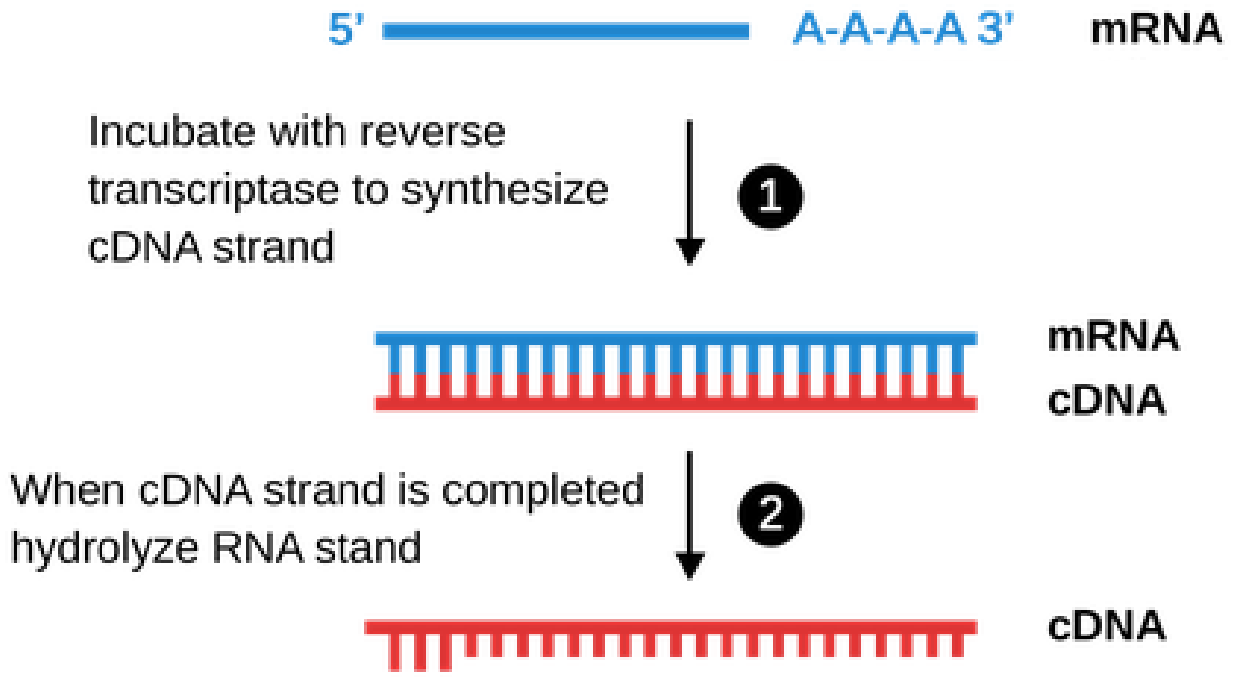
Reverse transcriptase: a tool to move



Reverse transcriptase (RT)

Retroviral RT has three sequential biochemical activities:

- RNA-dependent DNA polymerase
- ribonuclease H (RNase H)
- DNA-dependent DNA polymerase activity.

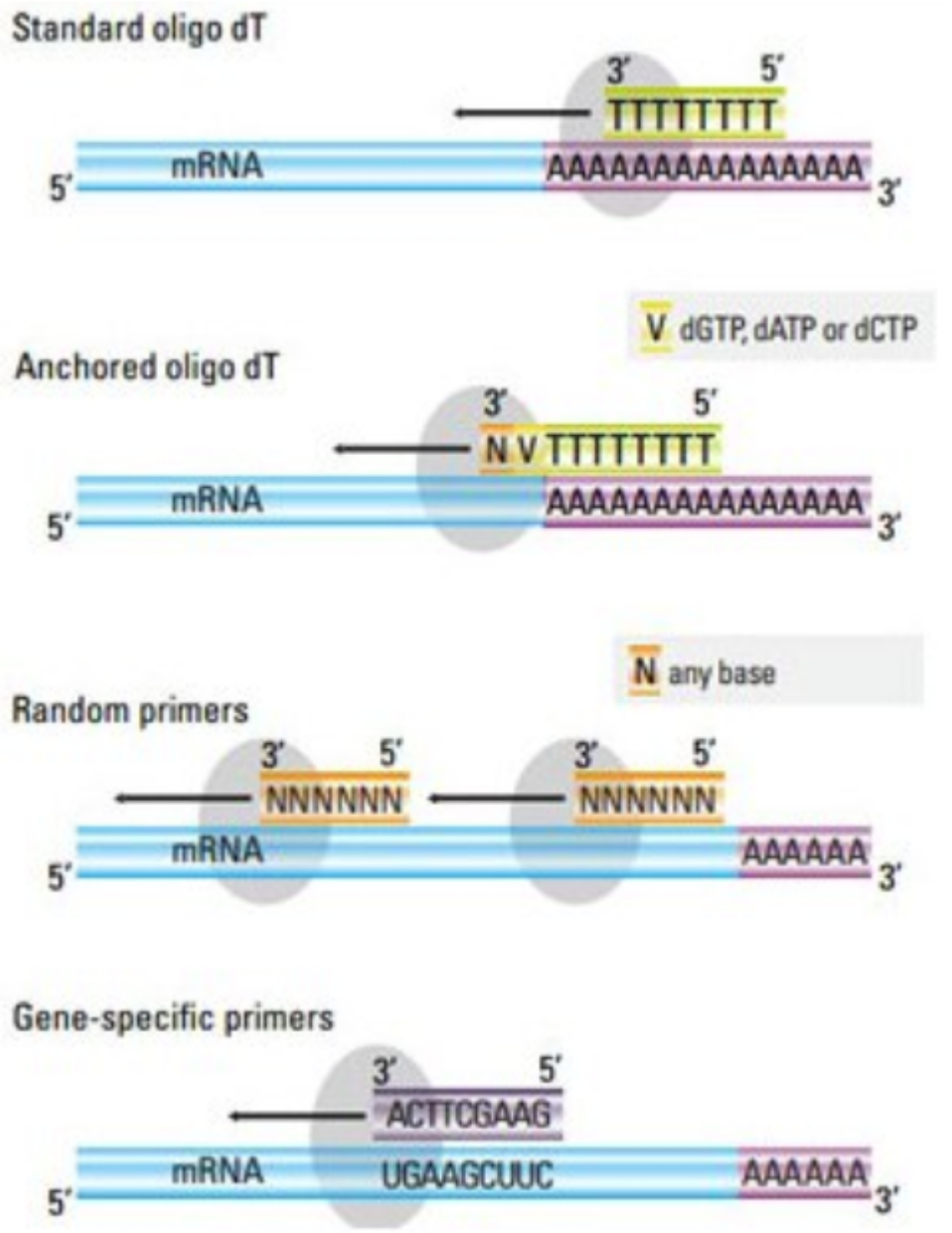


Priming the Reverse Transcriptase

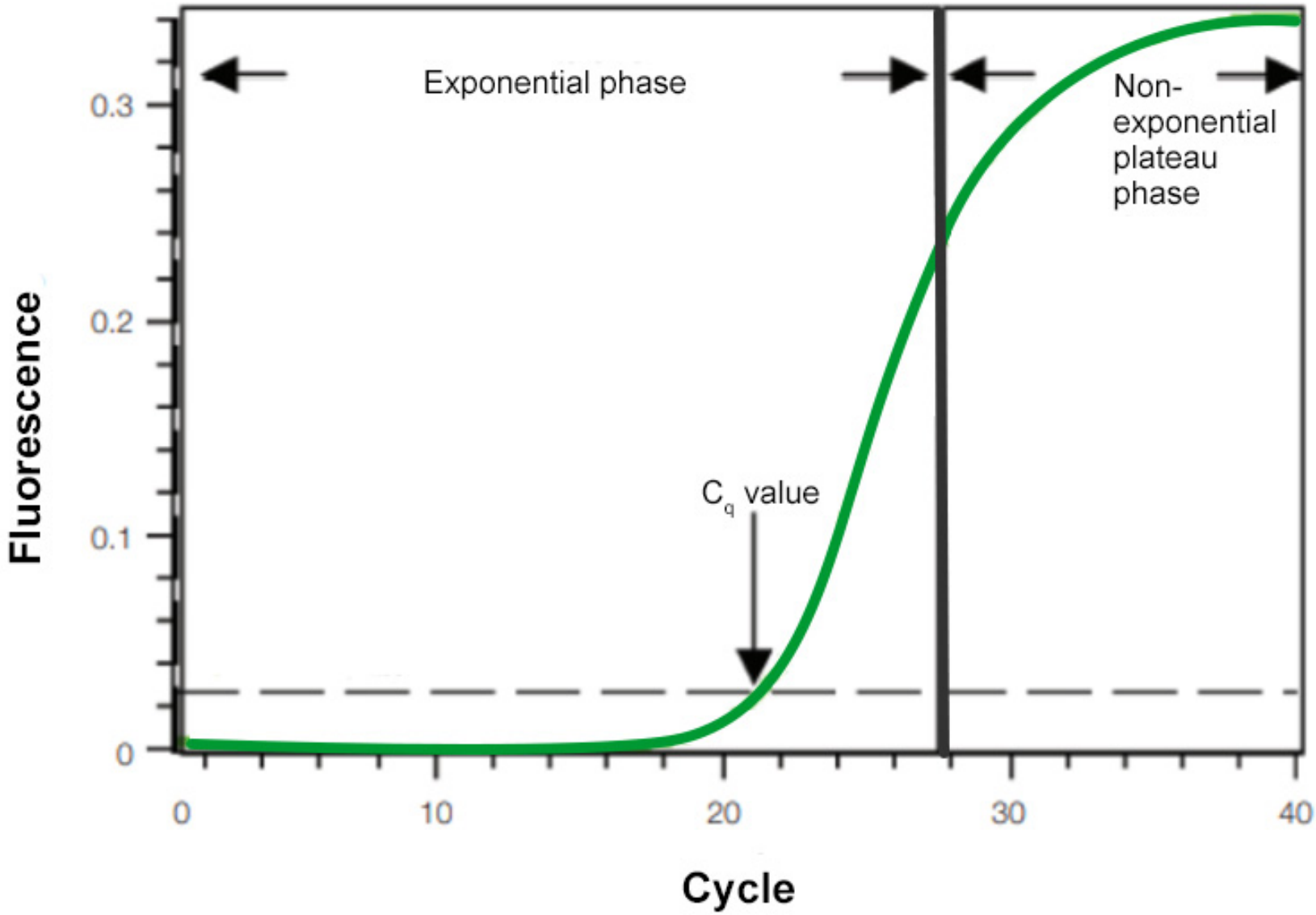
Valid if polyA is present

Maximal aspecificity

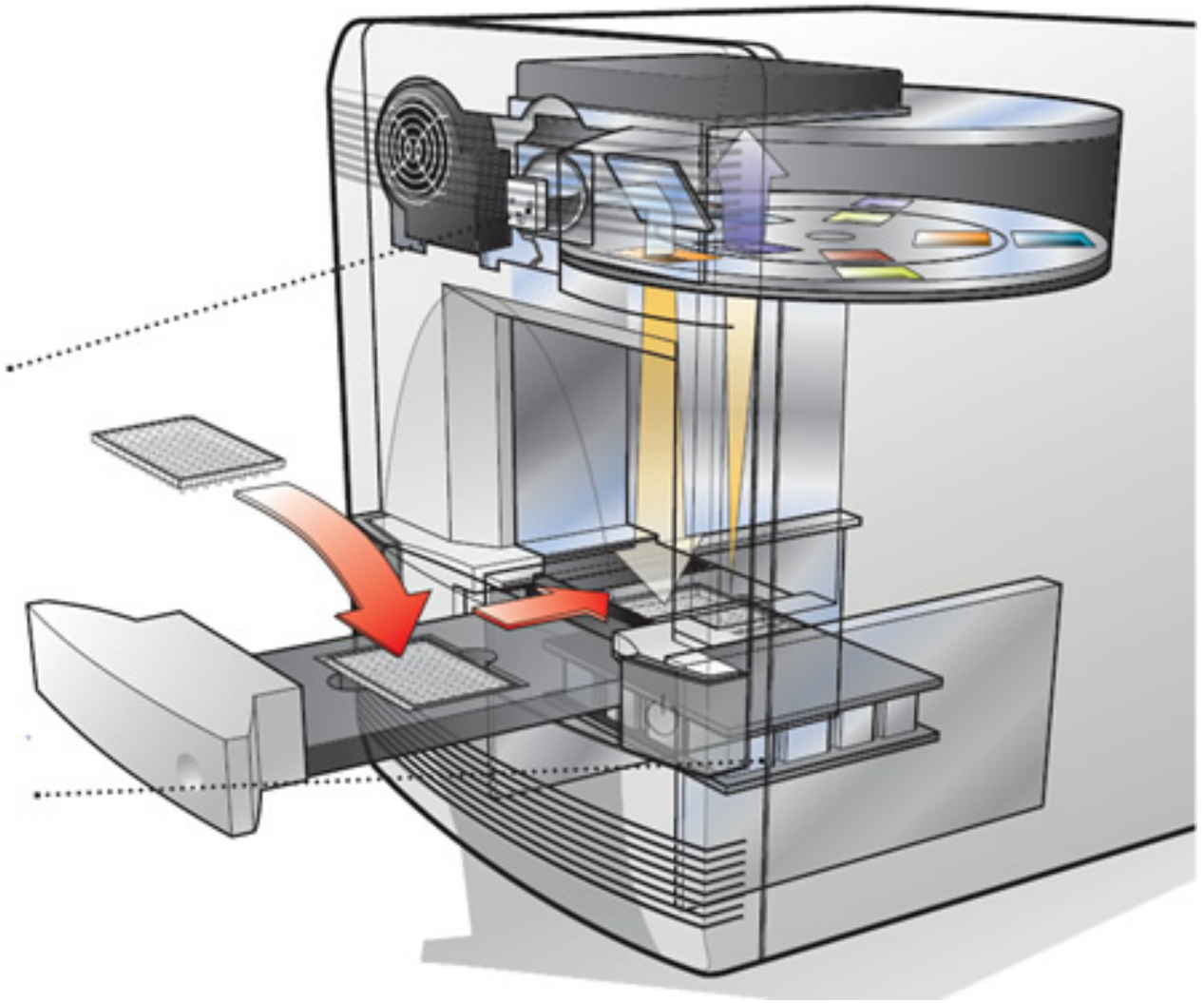
Minimal aspecificity



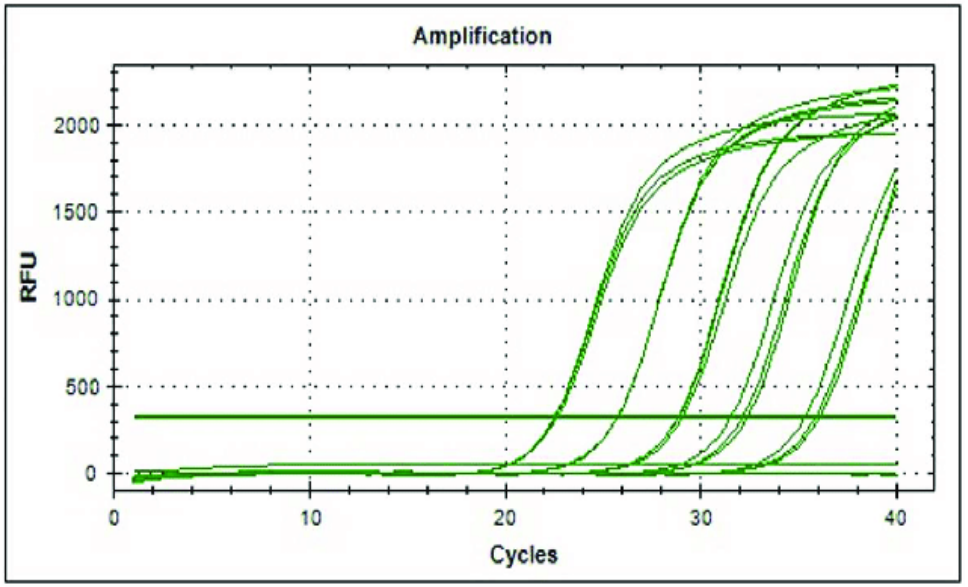
Real Time Quantitative PCR (RT-qPCR)

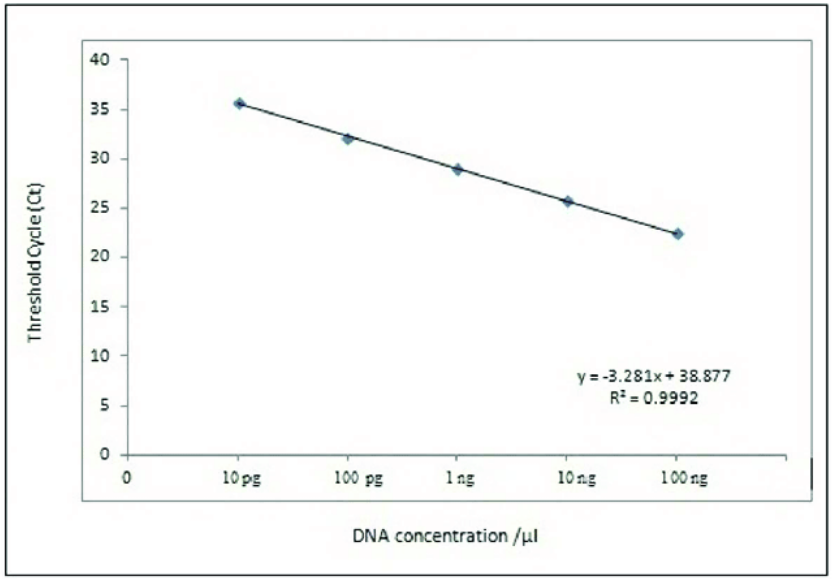


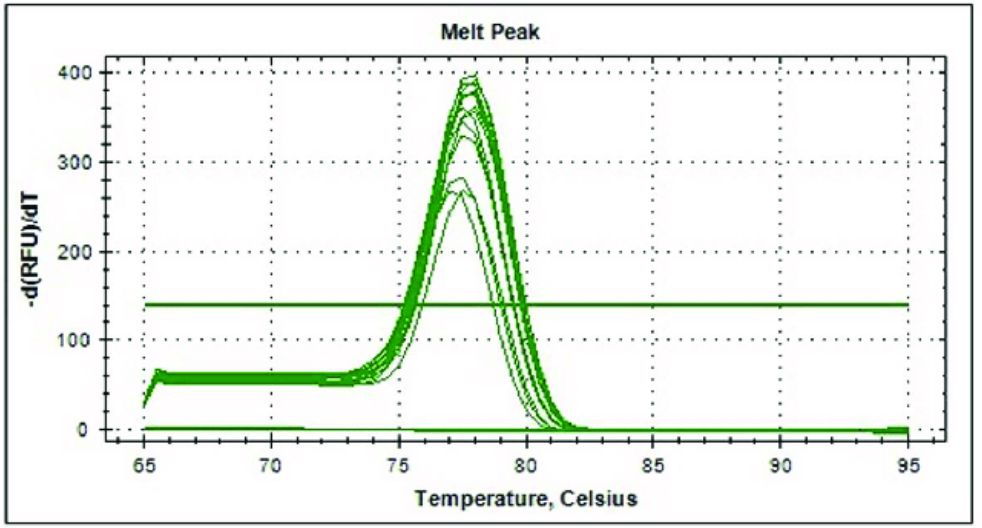
Real Time Quantitative PCR (RT-qPCR)



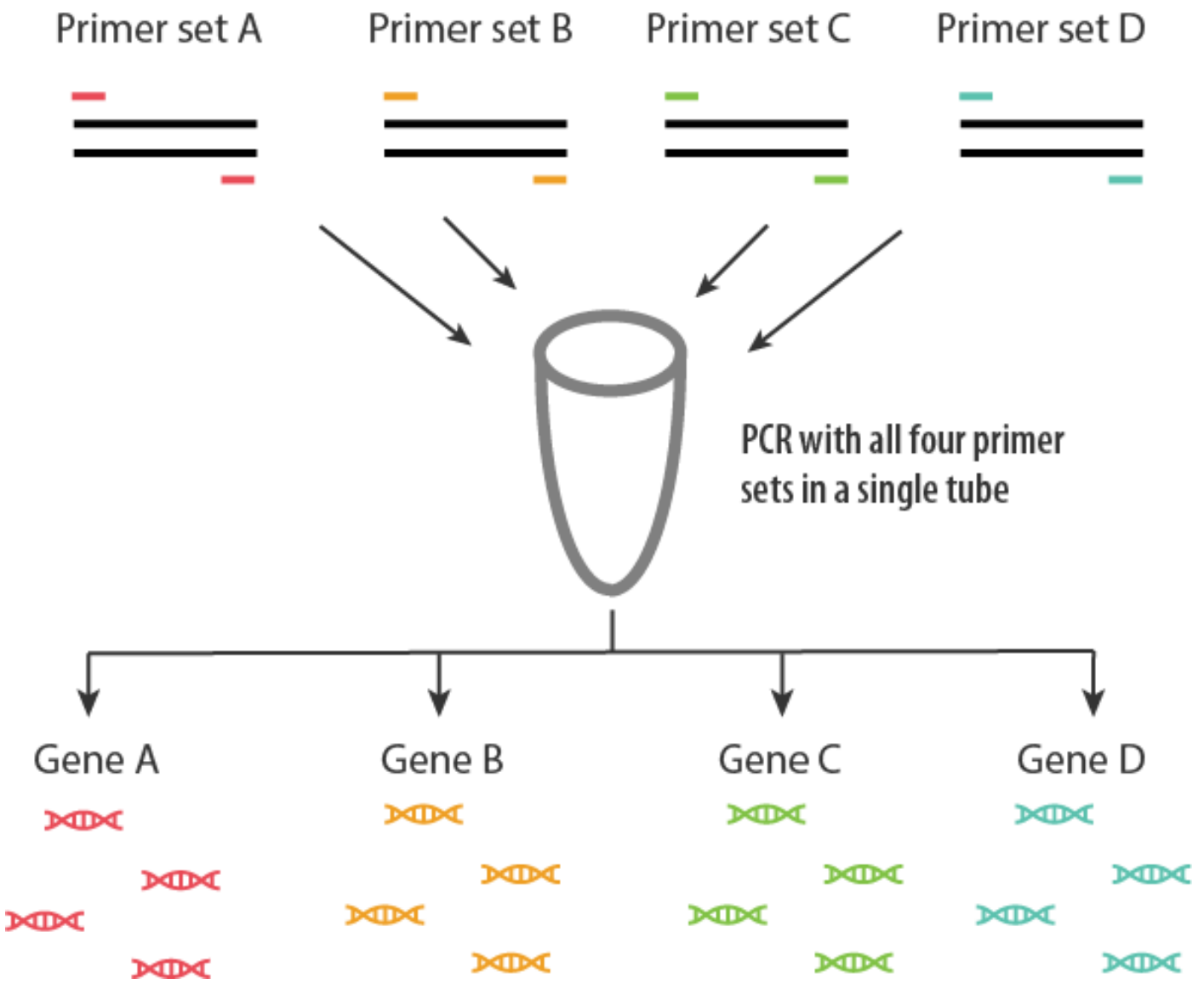
Real Time PCR – different perspectives



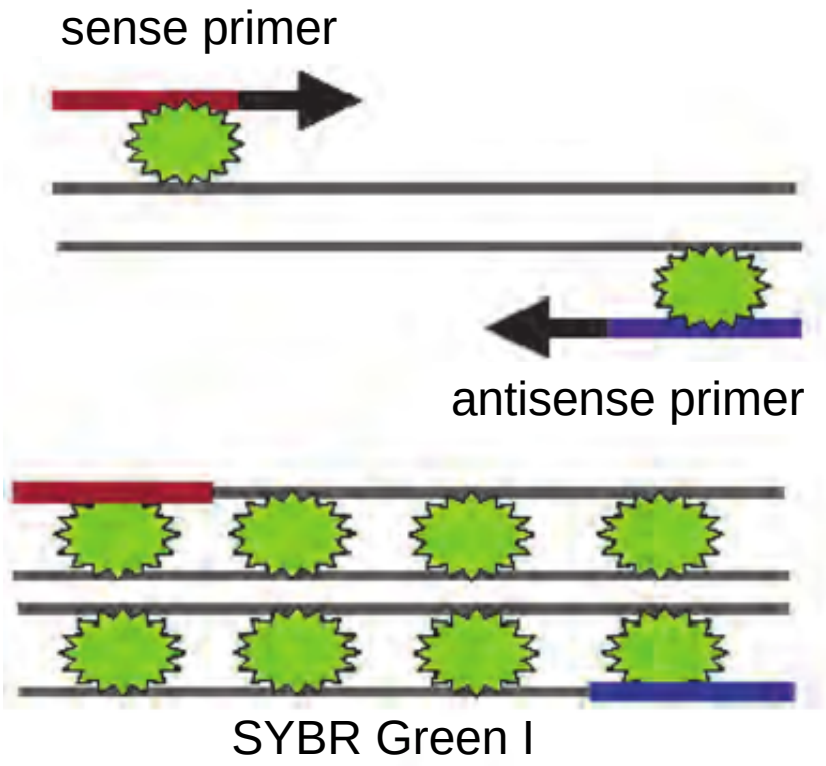


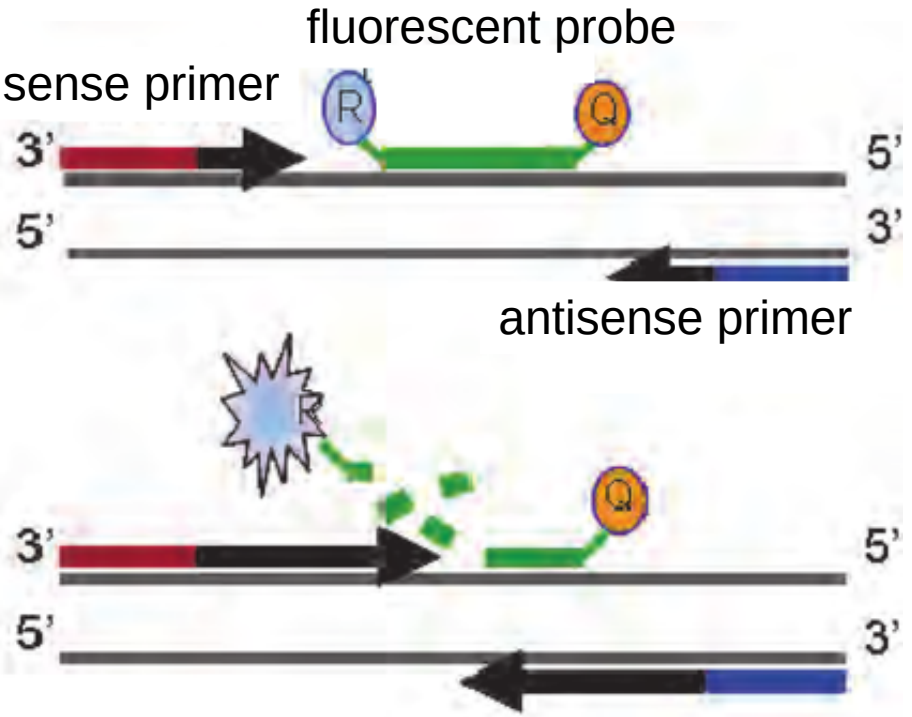


Real Time PCR – multiplex (or multicolor)

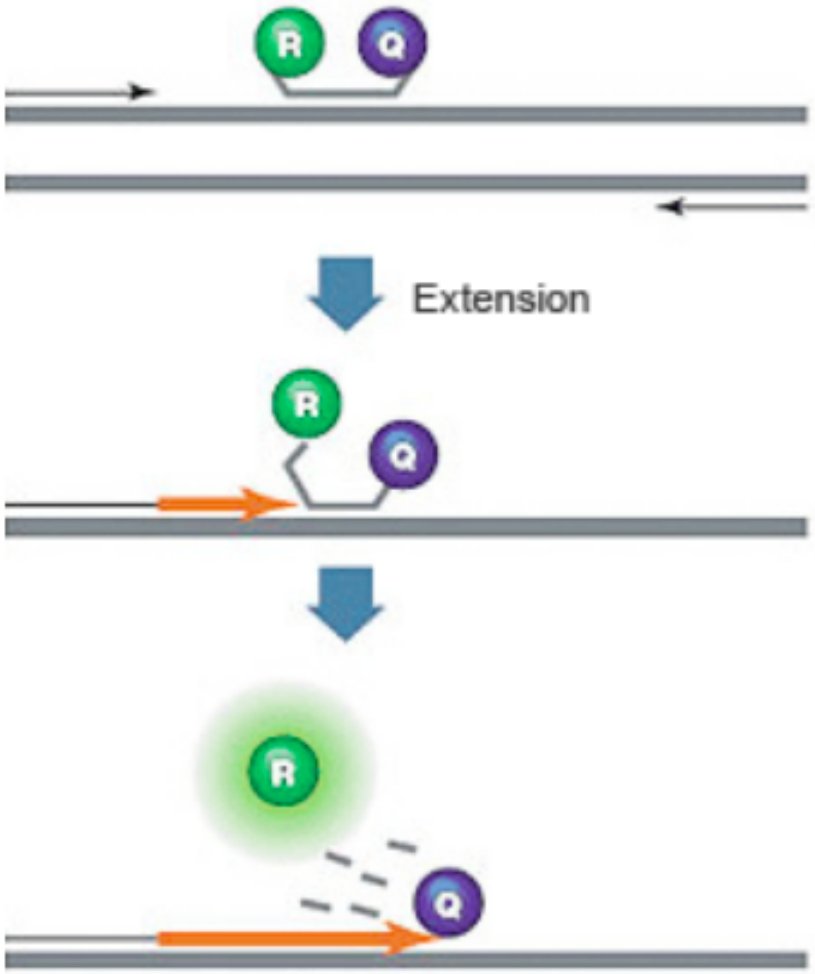


SYBR Green vs Probes





SYBR Green vs Probes



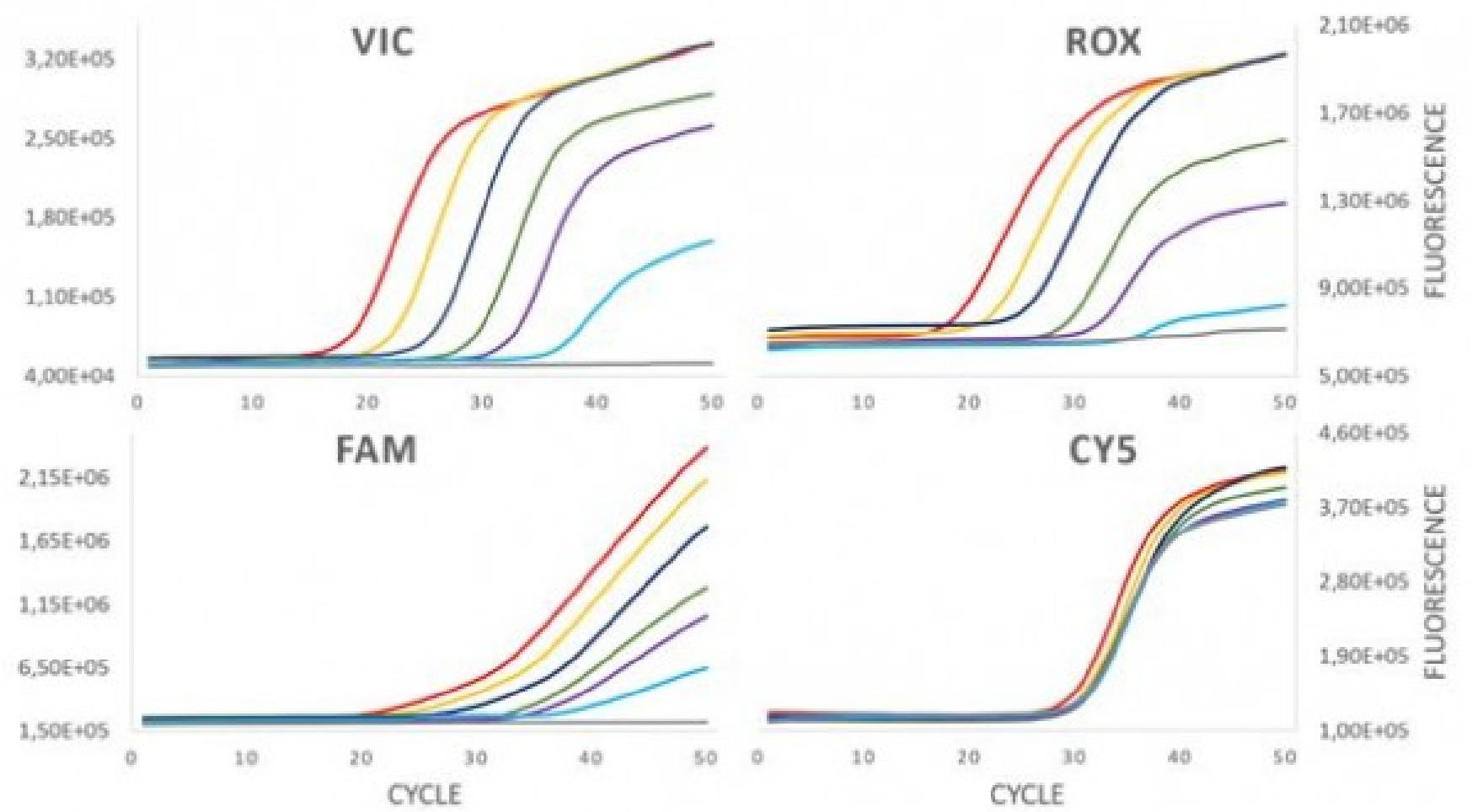
During annealing, the hydrolysis probe binds to the target sequence

During extension, the probe is partially displaced and the reporter is cleaved. The free reporter fluoresces

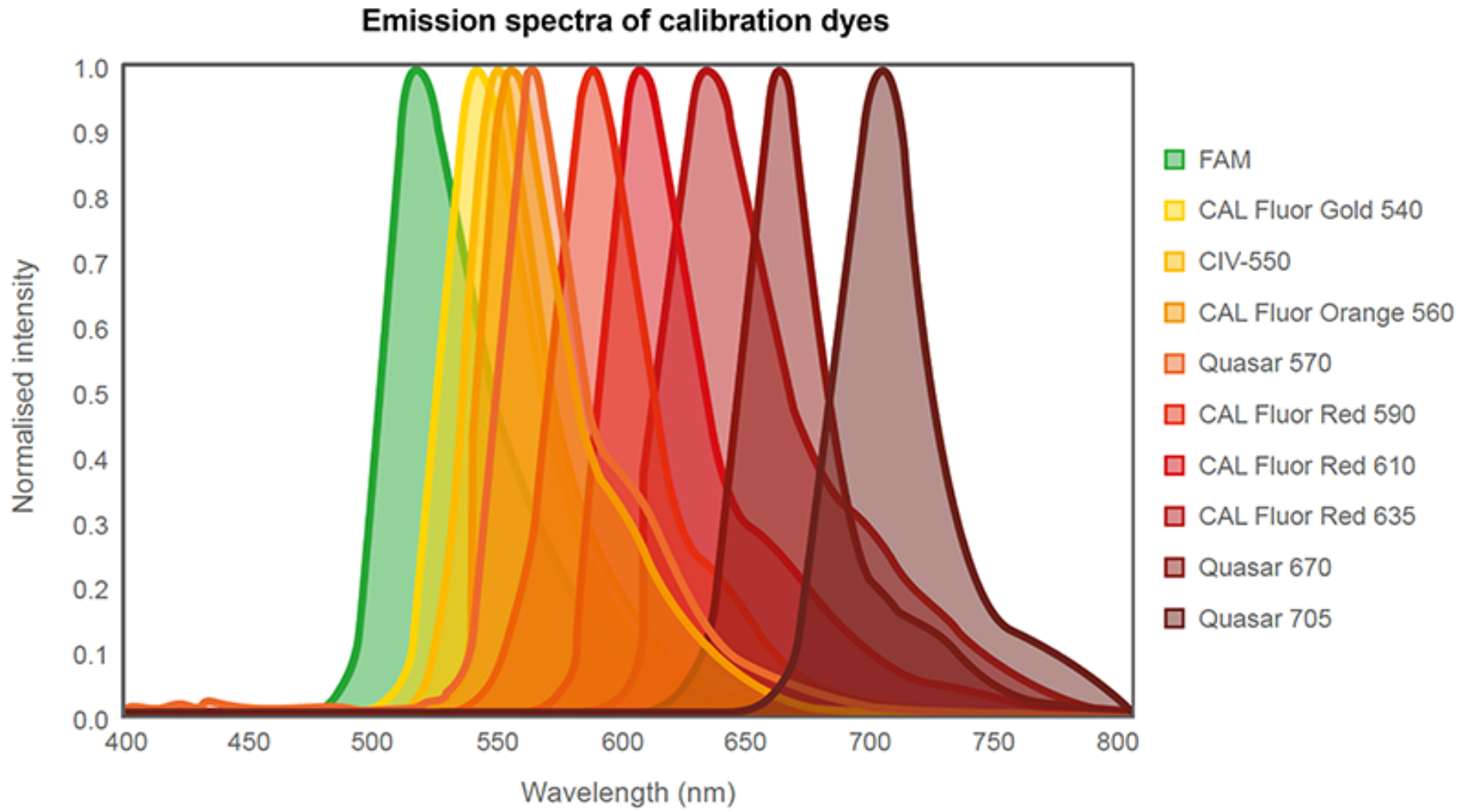
R Reporter

Q Quencher

Real Time PCR – multiplex (or multicolor)



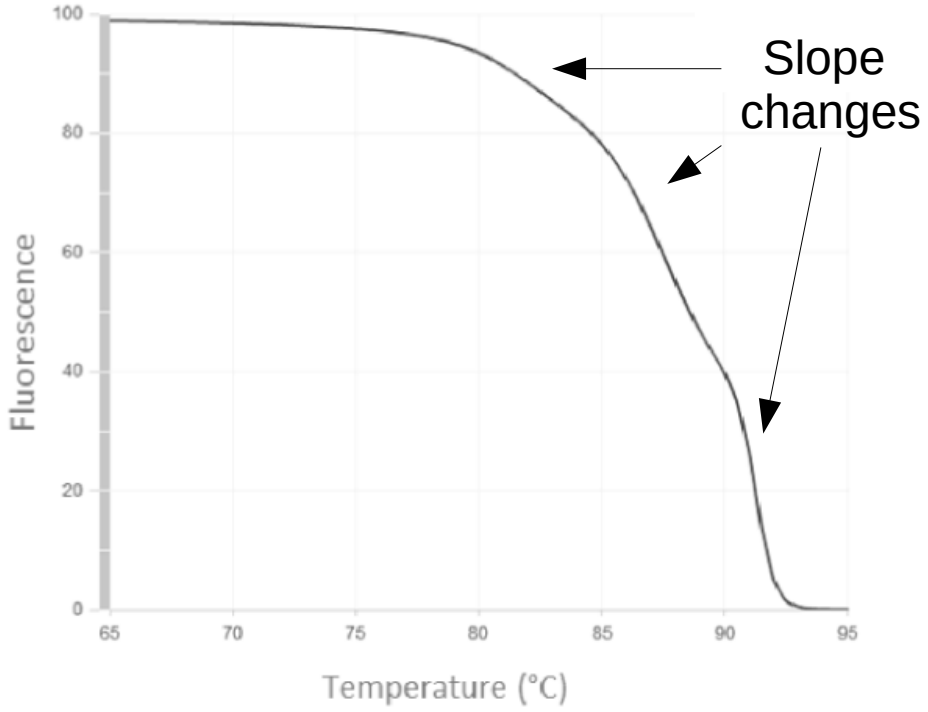
Spectral issues in qPCR



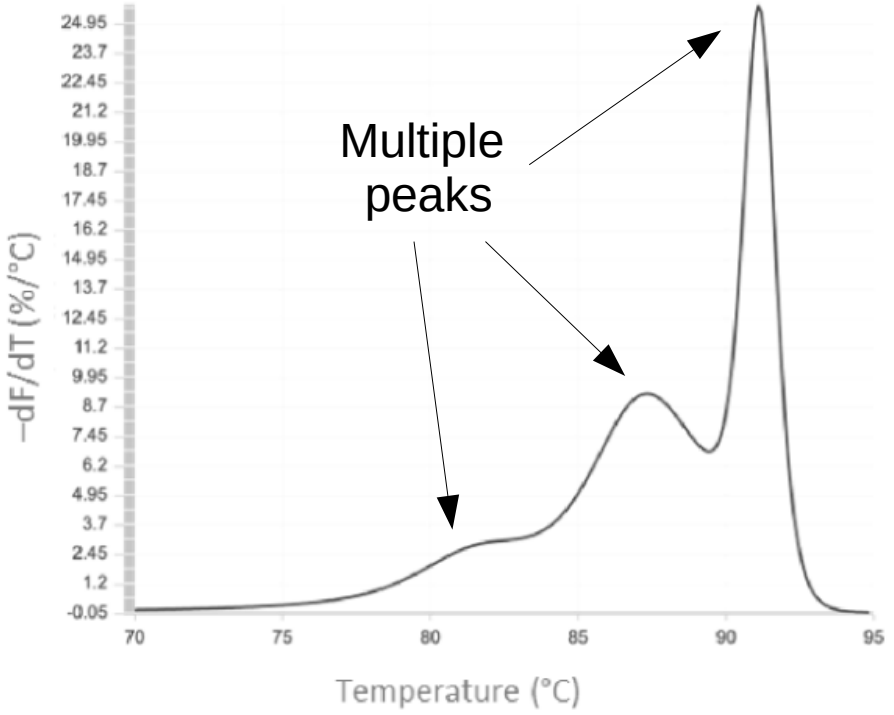
Real Time PCR – the melting curves

After PCR run, the temperature is increased to denature all products. In presence of fluorescent dyes (intercalating or) the temperature starts decreasing: a temperature dependent dissociation of DNA strands can be measured revealing possible multiple amplicons with different sizes.

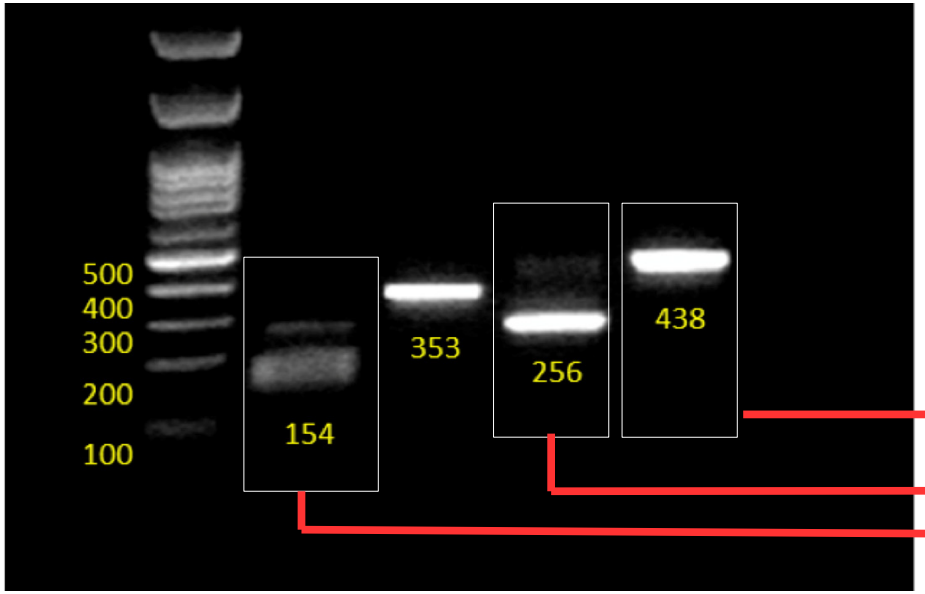
Dissociation curve



Melting curve



Real Time PCR – interpreting amplicons

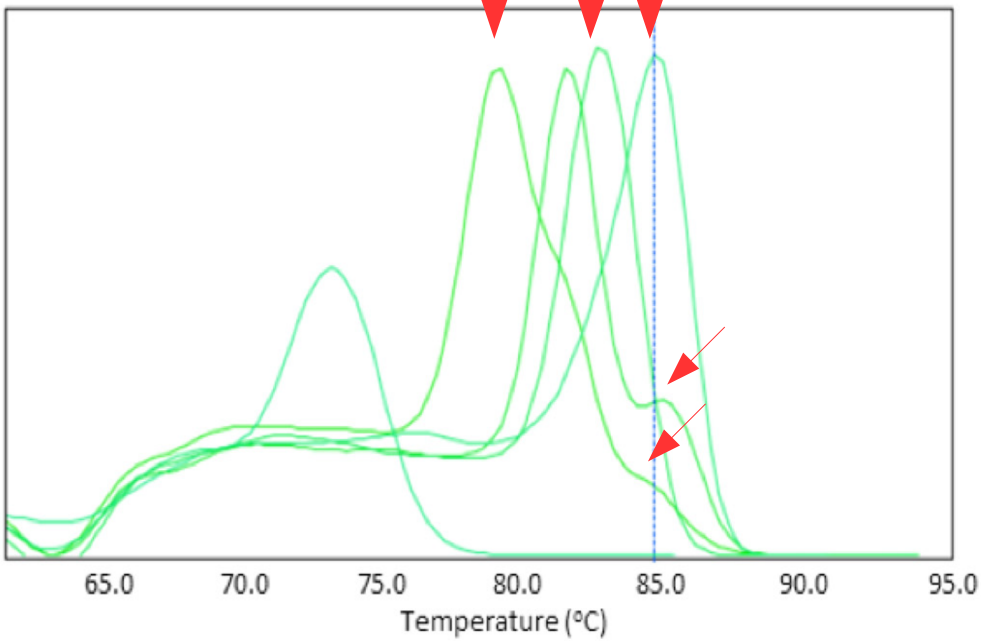


Agarose gel shows amplicon sizes and the bands show intensity that depends on DNA amount

In case of by-products, signals may be faint and hard to detect.

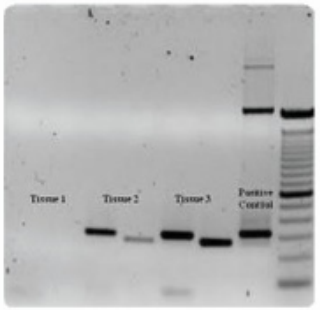
Melting curves show amplicon sizes as well (peaks), but reveals unwanted spurious bands with high precision.

In fact, shoulders appear in the curves while agarose show faint, hard to detect signals.



Digital PCR

Ordinary PCR

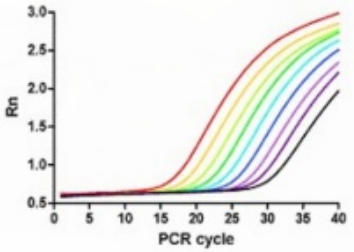


Amplify Target DNA

Qualitative research

1st

qPCR



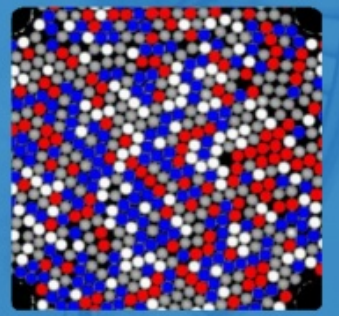
Real-time with standard curves

Ubiquitously spread method

Relative quantification

2nd

dPCR



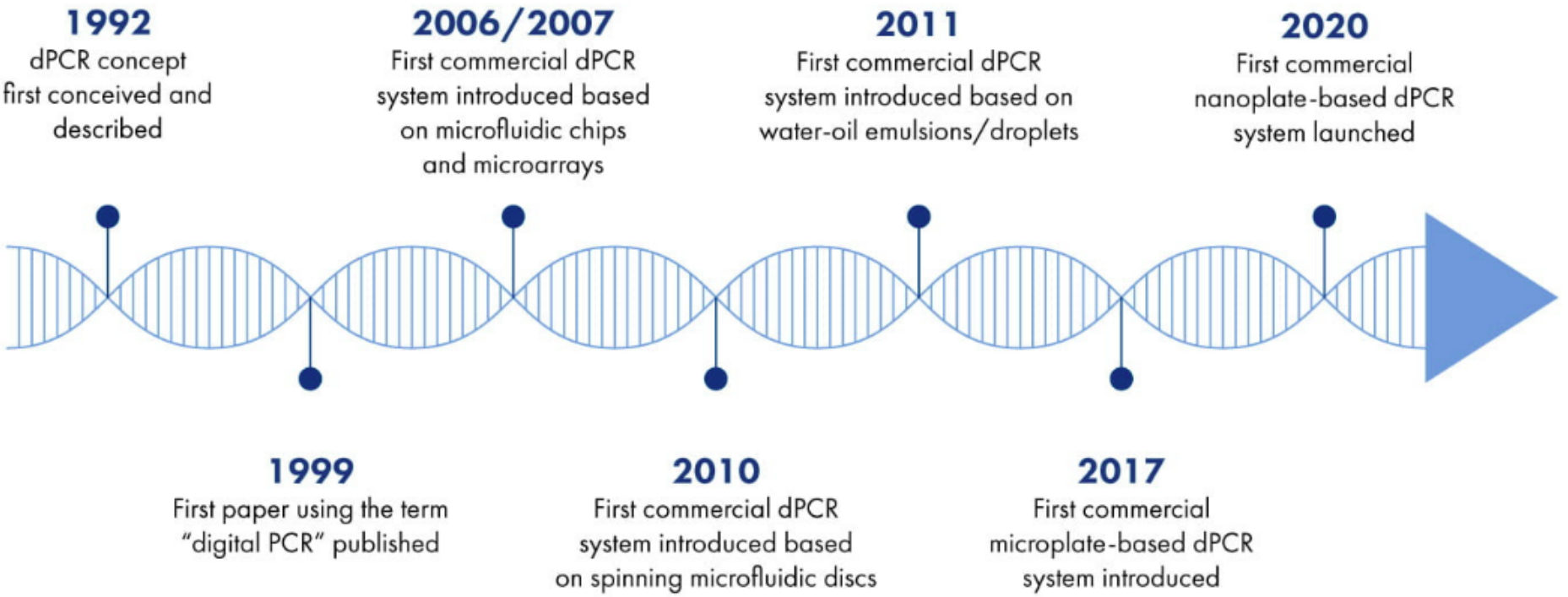
No standard curve (endpoint PCR)

Increased sensitivity

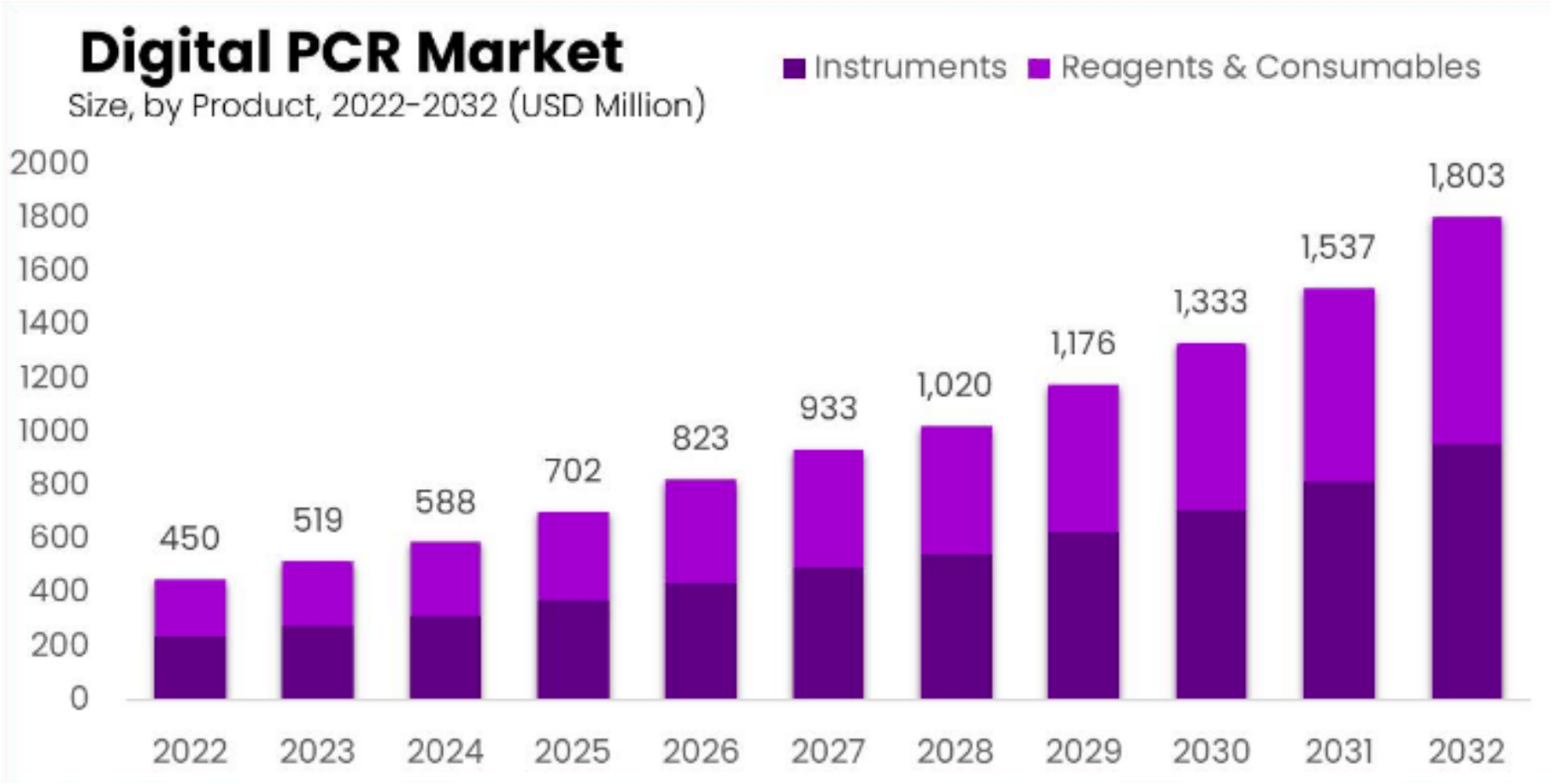
Absolute quantification

3rd

Digital PCR

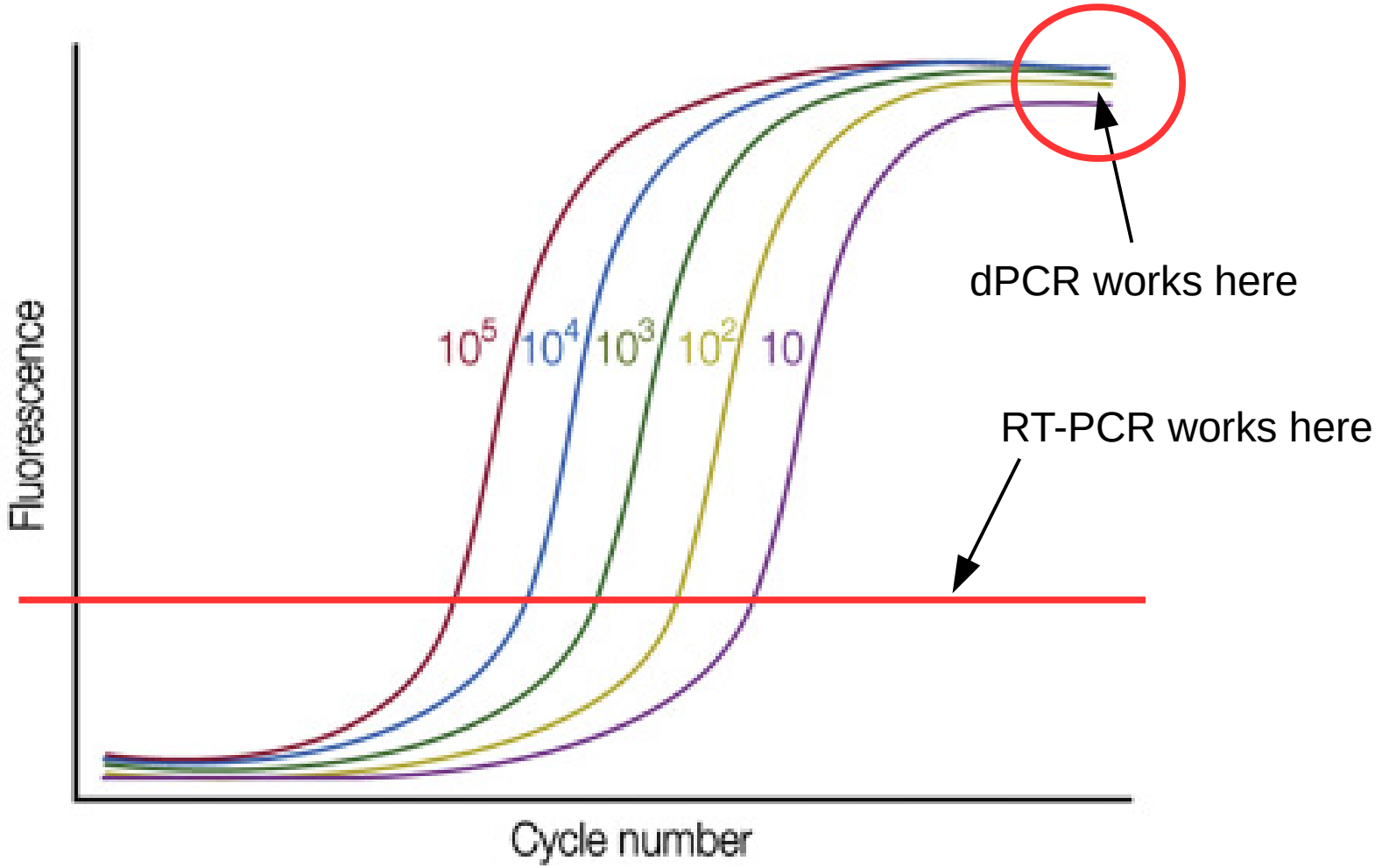

1992
dPCR concept first conceived and described
1999
First paper using the term "digital PCR" published
2006/2007
First commercial dPCR system introduced based on microfluidic chips and microarrays
2010
First commercial dPCR system introduced based on spinning microfluidic discs
2011
First commercial dPCR system introduced based on water-oil emulsions/droplets
2017
First commercial microplate-based dPCR system introduced
2020
First commercial nanoplate-based dPCR system launched

Digital PCR


Digital PCR Market
Size, by Product, 2022-2032 (USD Million)
Instruments
Reagents & Consumables
2000
1800
1600
1400
1200
1000
800
600
400
200
0
450
519
588
702
823
933
1,020
1,176
1,333
1,537
1,803
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032

Digital PCR – Sensitivity and absence of inhibition

PCR and RT-PCR inhibitors seem to be everywhere:: They lie dormant in your starting material and can co-purify with the template of interest, and they can be introduced during sample handling or reaction setup. The effects of these inhibitors range from partial inhibition and underestimation of the target nucleic acid amount to complete amplification failure. What a scientist to do?



Digital PCR

BioRad QX200/QX600

Thermo QuantStudio™ Absolute Q™

Qiagen QIAcuity Digital PCR

Stilla Crystal Digital PCR

Digital PCR – Partitioning

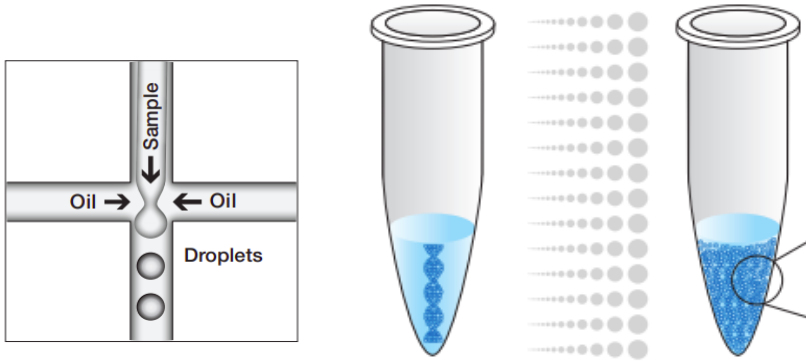
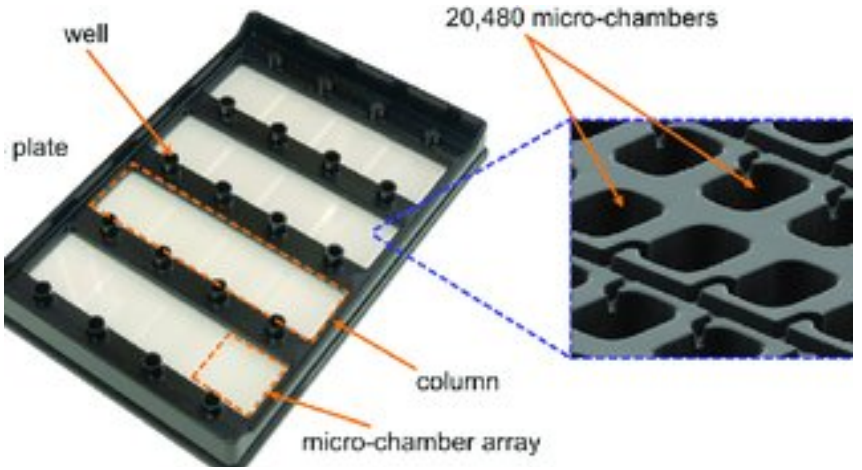
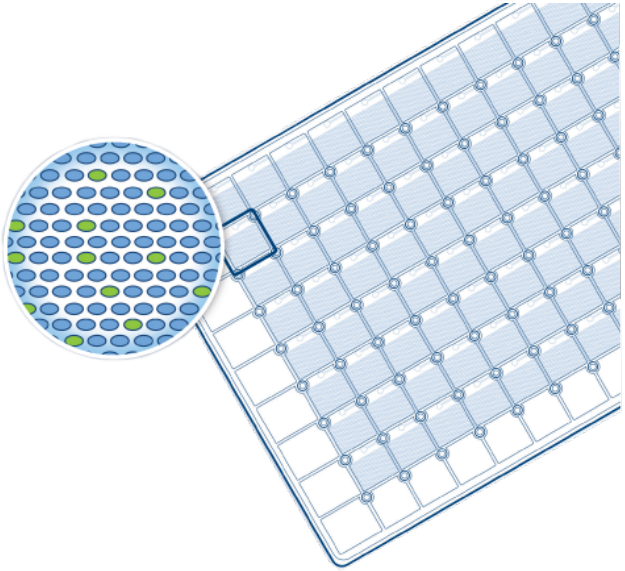


Fig. 1.3. In ddPCR, a single PCR sampl

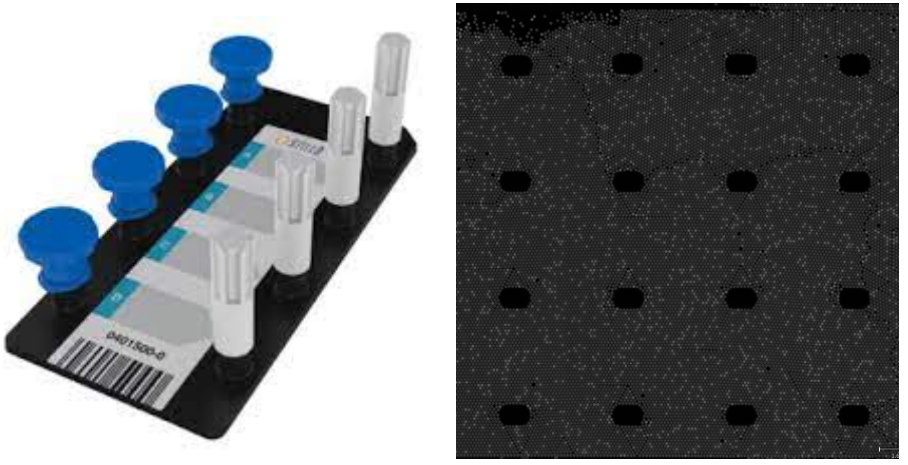
BioRad QX200/QX600



Thermo QuantStudio™ Absolute Q™



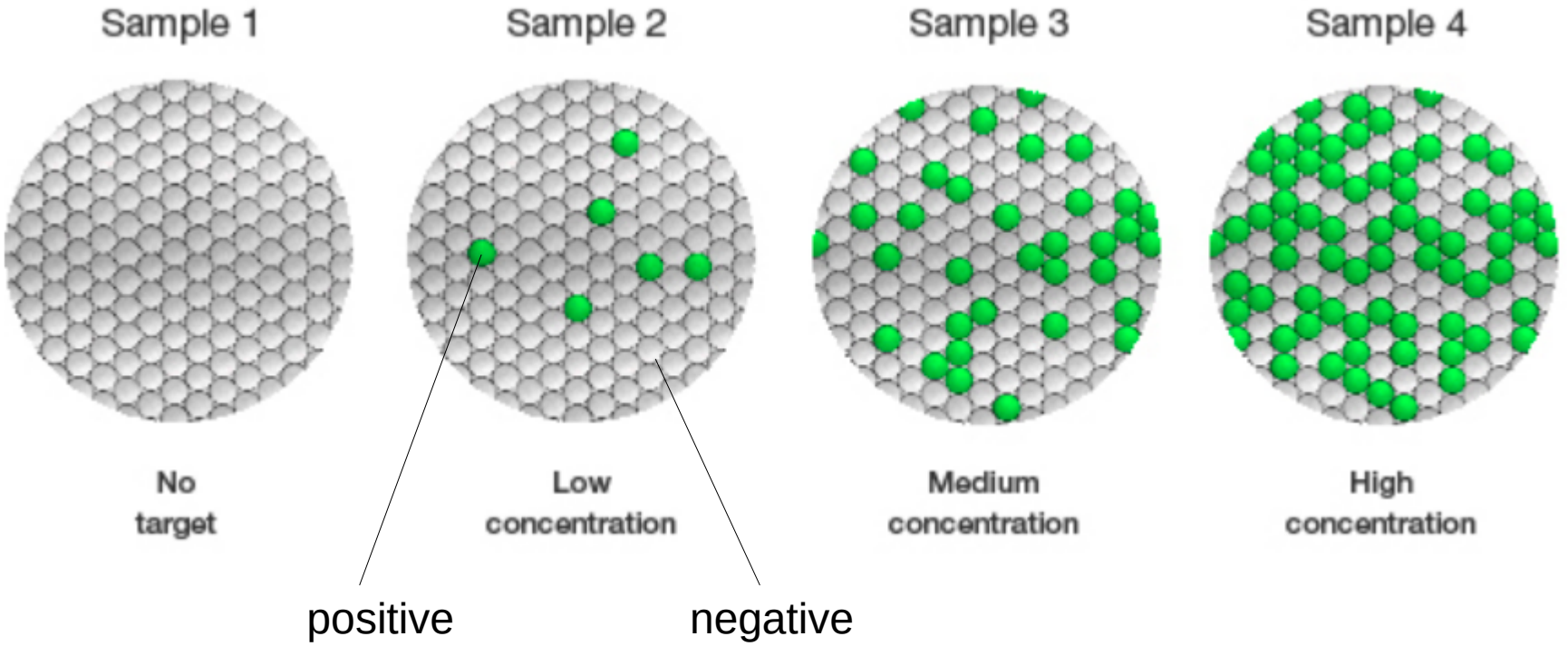
Qiagen QIAcuity Digital PCR



Stilla Crystal Digital PCR

Digital PCR digitalization

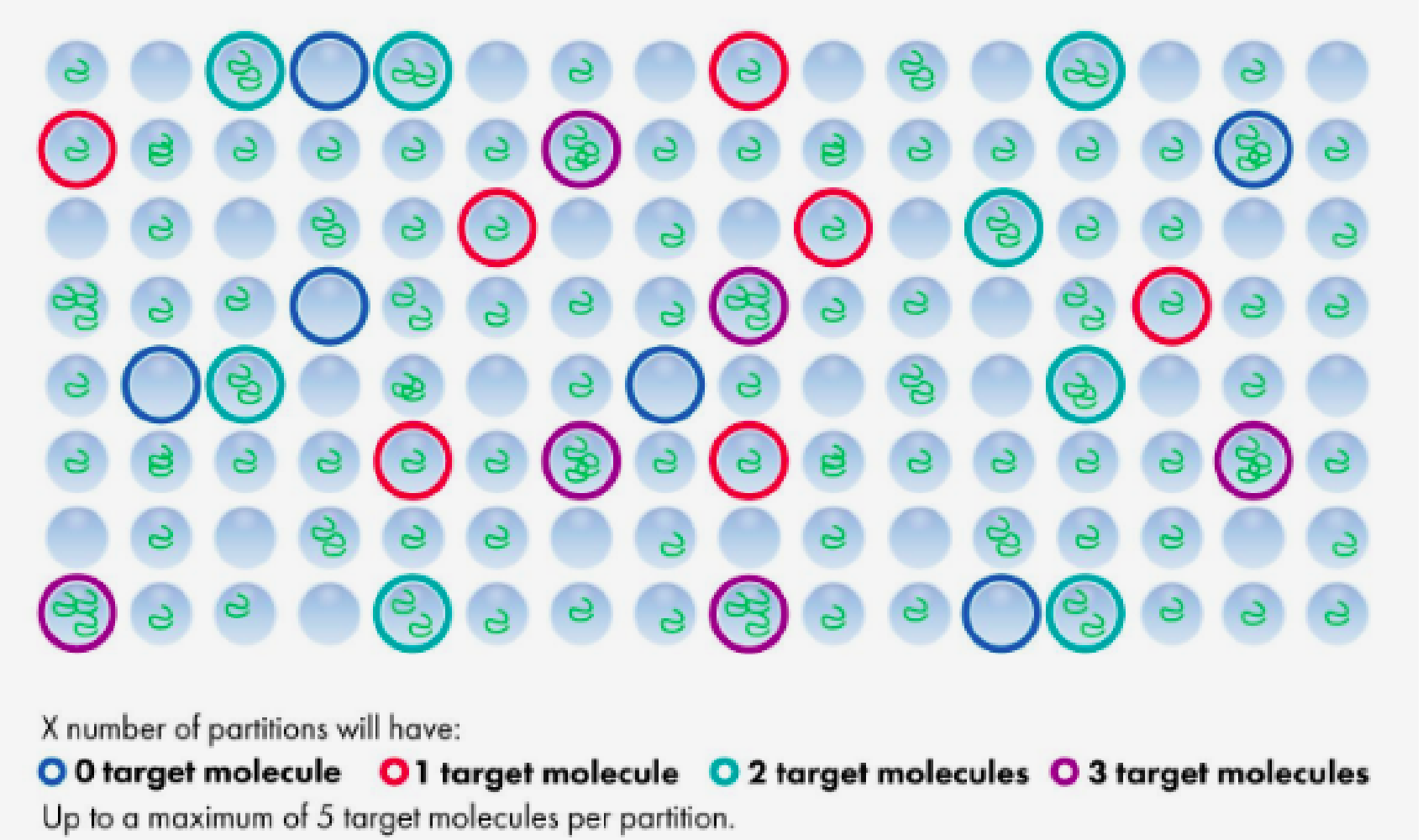
During partitioning, template molecules are distributed randomly in drops/wells



Some droplets contain no template, some contain one or more template molecule. After PCR, partitions may be fluorescent (+) or not (-).

Due to the random nature of the partitioning, the fluorescence data after amplification are well **fit by a Poisson distribution**.

Poisson's law copes with multiple targets in a partition



Concentration Calculation

The software uses the formula and variables shown below.

$$c = -\ln\left(\frac{N_{neg}}{N}\right) / V_{droplet}$$

must be well determined !!!

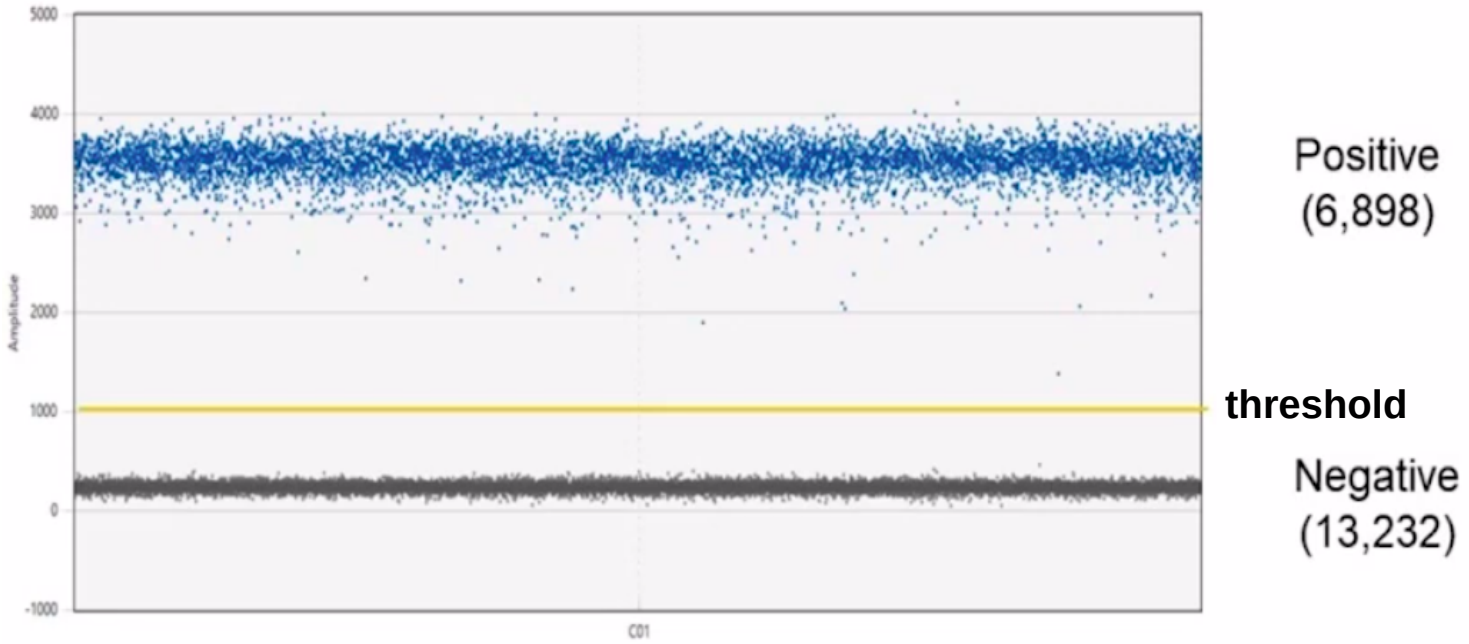
Where:

-ln	Negative natural logarithm
$\mathbf{V_{droplet}}$	Volume of droplet
$\mathbf{N_{neg}}$	Number of negative droplets
N	Total number of droplets

must be well determined !!!

Siméon Denis Poisson
(1781-1840)

Digital PCR output counting negatives and positives



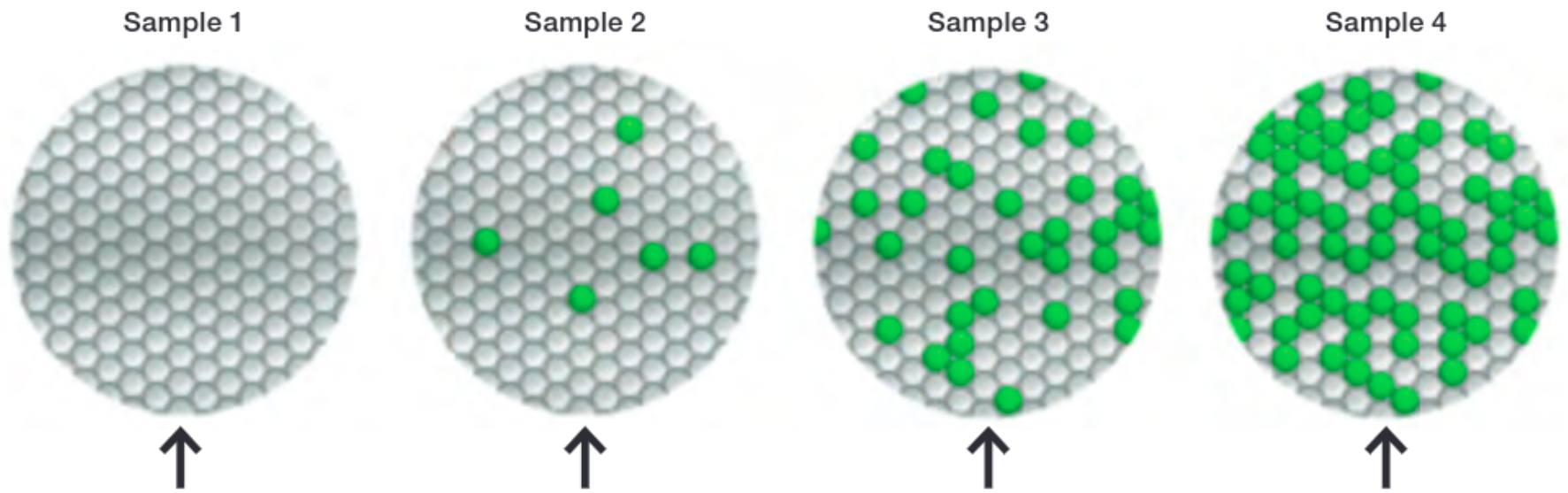
$$\lambda = -\ln \frac{\text{negatives}}{\text{valid}} = -\ln \frac{13323}{20000} = \mathbf{0{,}406}\ \text{copies/}\mathbf{partition}$$

V_d (µl) = ~ 0.85 nL $N_{\mathbf{part}}$ = 20000 V_{tot} (µl) = ~ 20000 x 0.85 = 17000 nl = **17 ul**

$$\text{Conc} = \frac{\lambda}{V\ (\mu l)} = \frac{\mathbf{0.406}}{\mathbf{17}} = 0.024\ \text{copies} / \mu l$$

final absolute concentration

Poisson observed and expected



$p = \frac{0 \text{ positive}}{143 \text{ total}}$

$p = \frac{6}{143}$

Poisson corrected 6.2/143

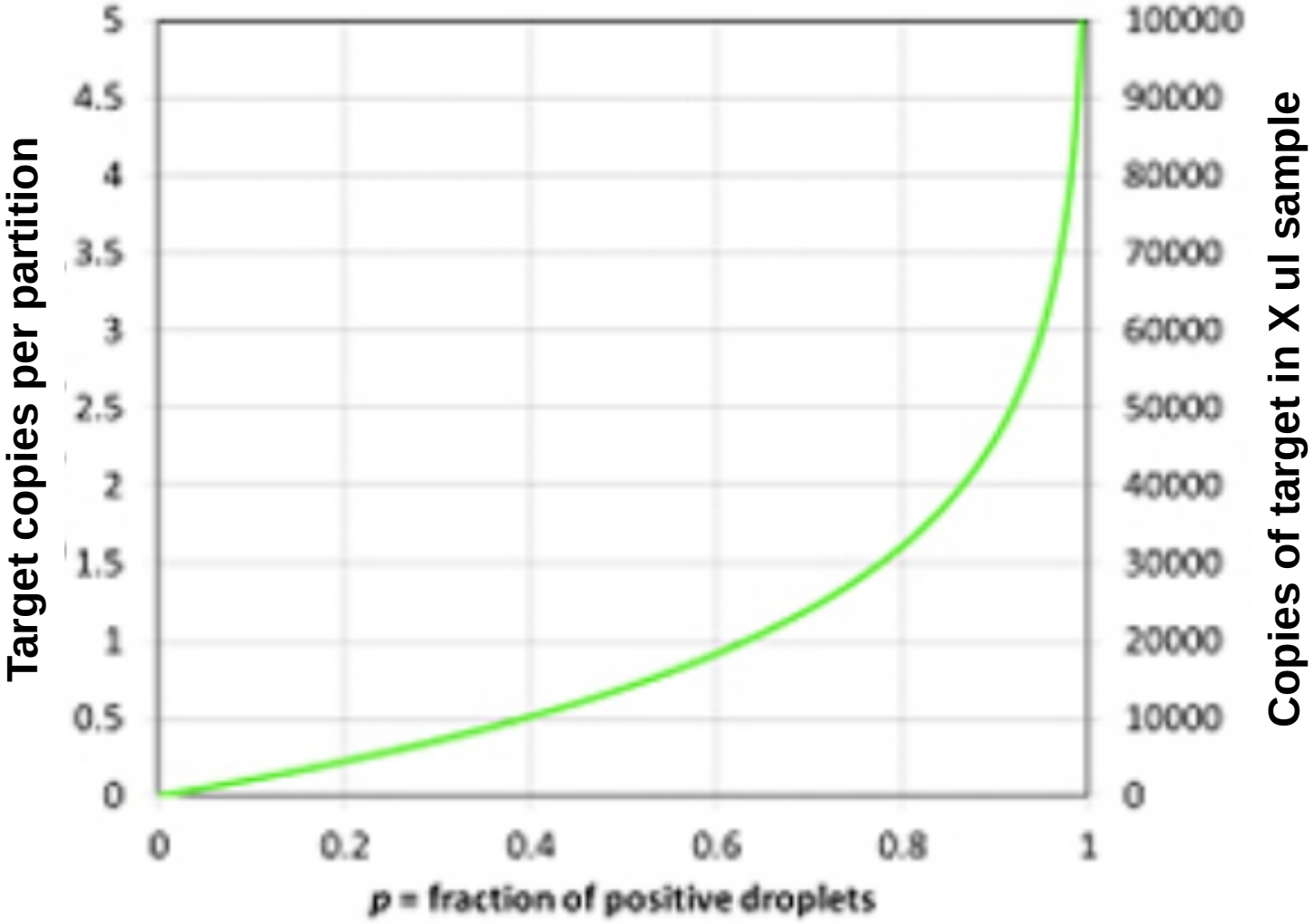
$p = \frac{34}{143}$

Poisson corrected 38/143

$p = \frac{70}{143}$

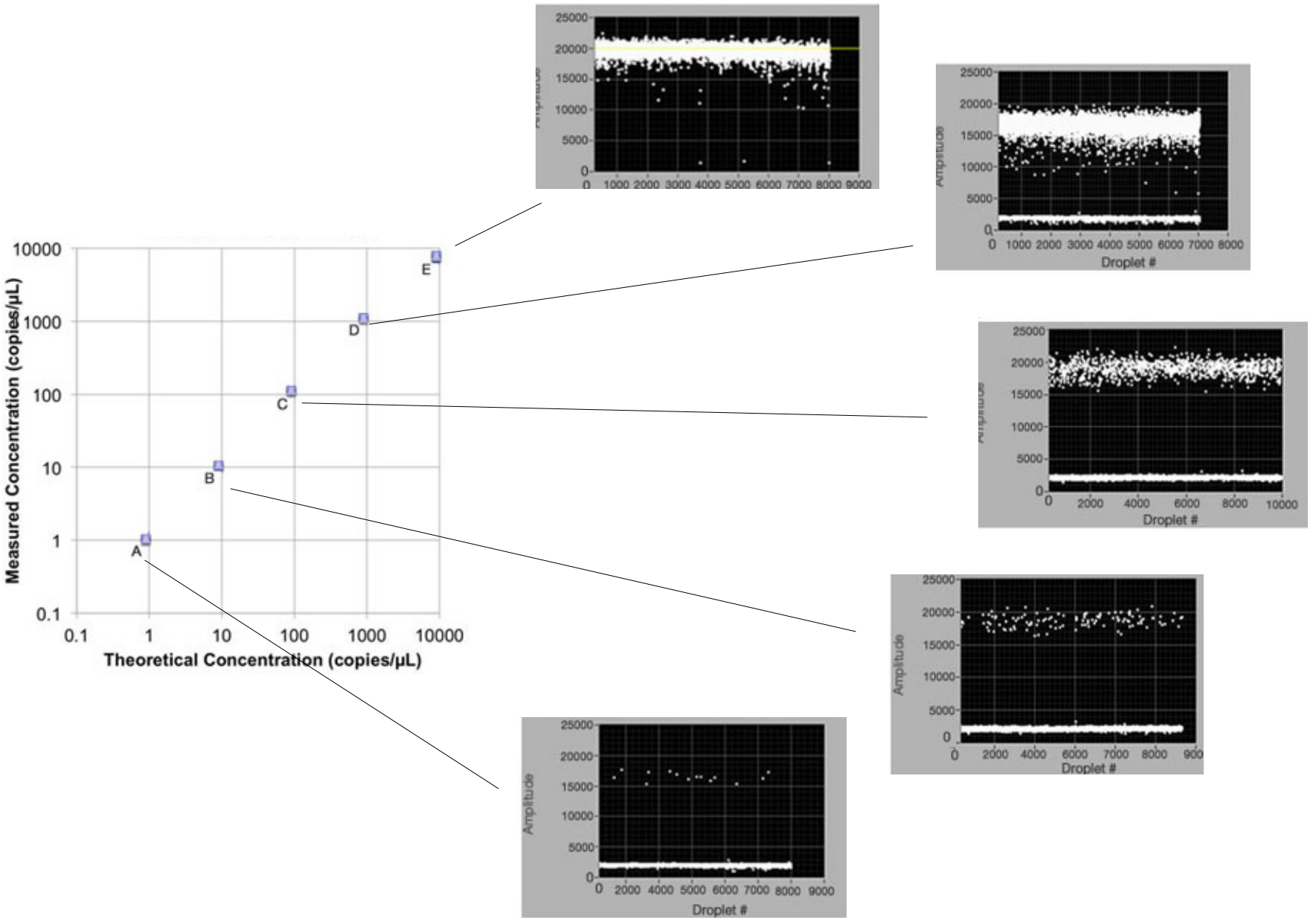
Poisson corrected 96/143

Digital PCR Poisson statistics comes in hand



Curve representing the relationships among the fraction of positive droplets, the number of target copies per droplet, and the number of copies of target in a X µl of sample.

Positive and negative signals (and the "rain")



Digital PCR usage

Top 5 benefits

Absolute target quantification
No need for references or standard curves

High tolerance to inhibitors
Due to partitioning and endpoint measurement

Superior precision
Detect very small fold change differences

Increased sensitivity
Detect rare mutations and low abundance targets

High reproducibility
Eliminate amplification efficiency bias

Top 5 applications

Copy number variation

Rare mutation detection

NGS library quantification

Viral load detection

Gene expression analysis