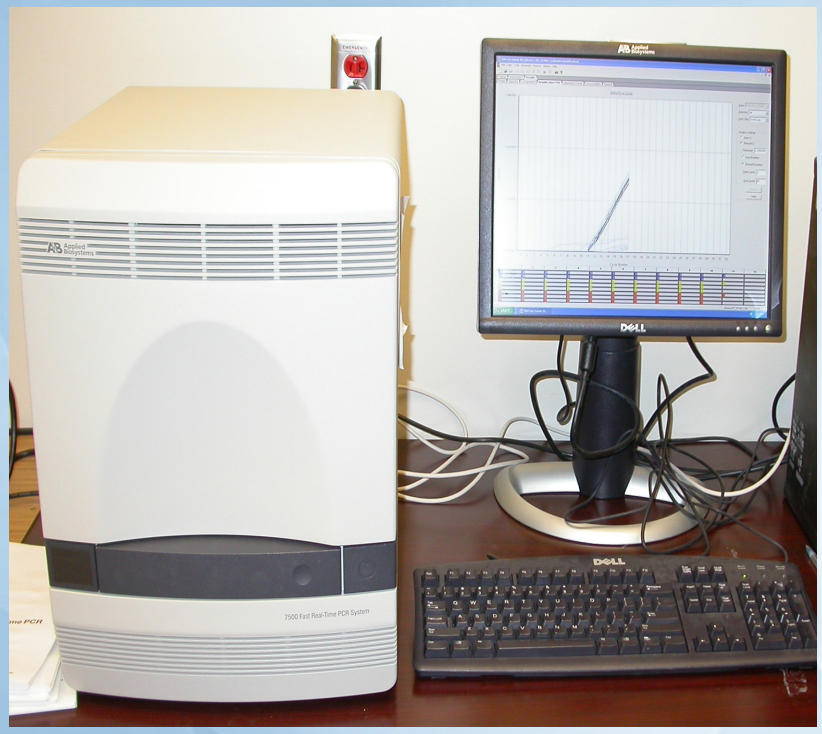
Different types of PCR and principles of Real Time PCR.

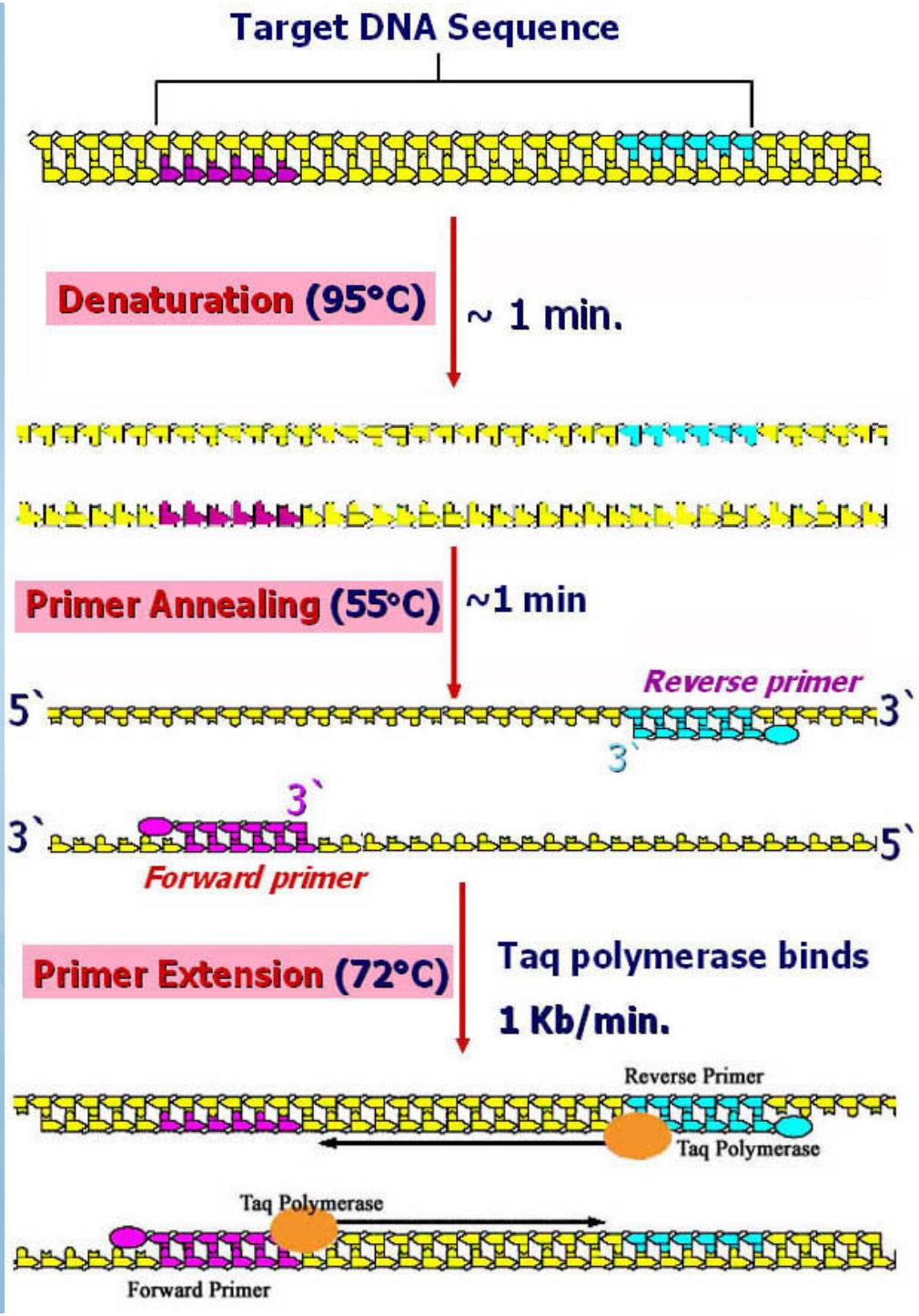


Prof. Dr. Hamdy M. El-Aref

Assiut University, Faculty of Agriculture

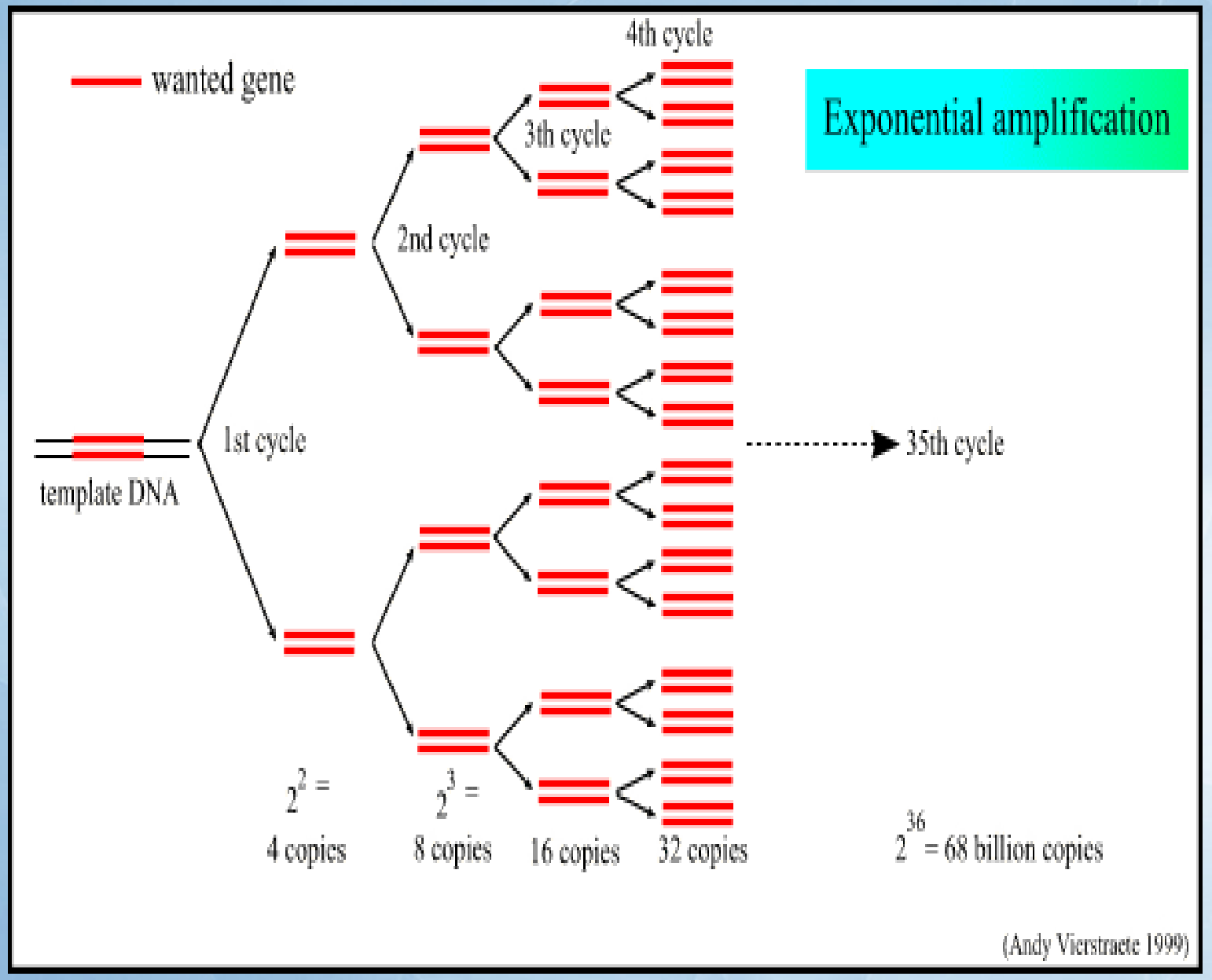
Genetics Department

INTRODUCTION



PCR Cycle (round)

INTRODUCTION



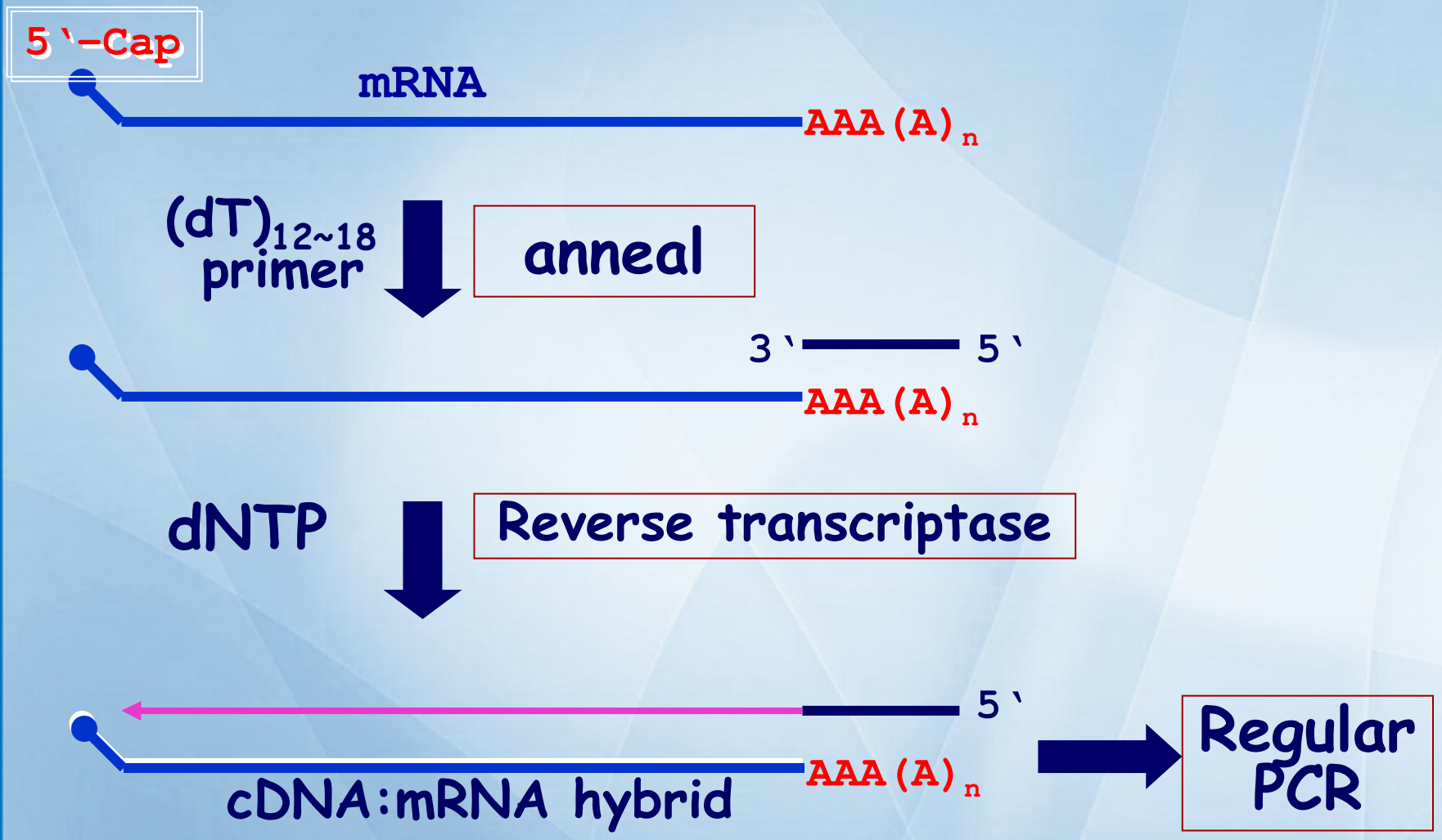
Types of PCR

- **Standard PCR, DNA**
- **RT-PCR, RNA**
- **Real-Time PCR – RTQ-PCR (DNA or RNA)**

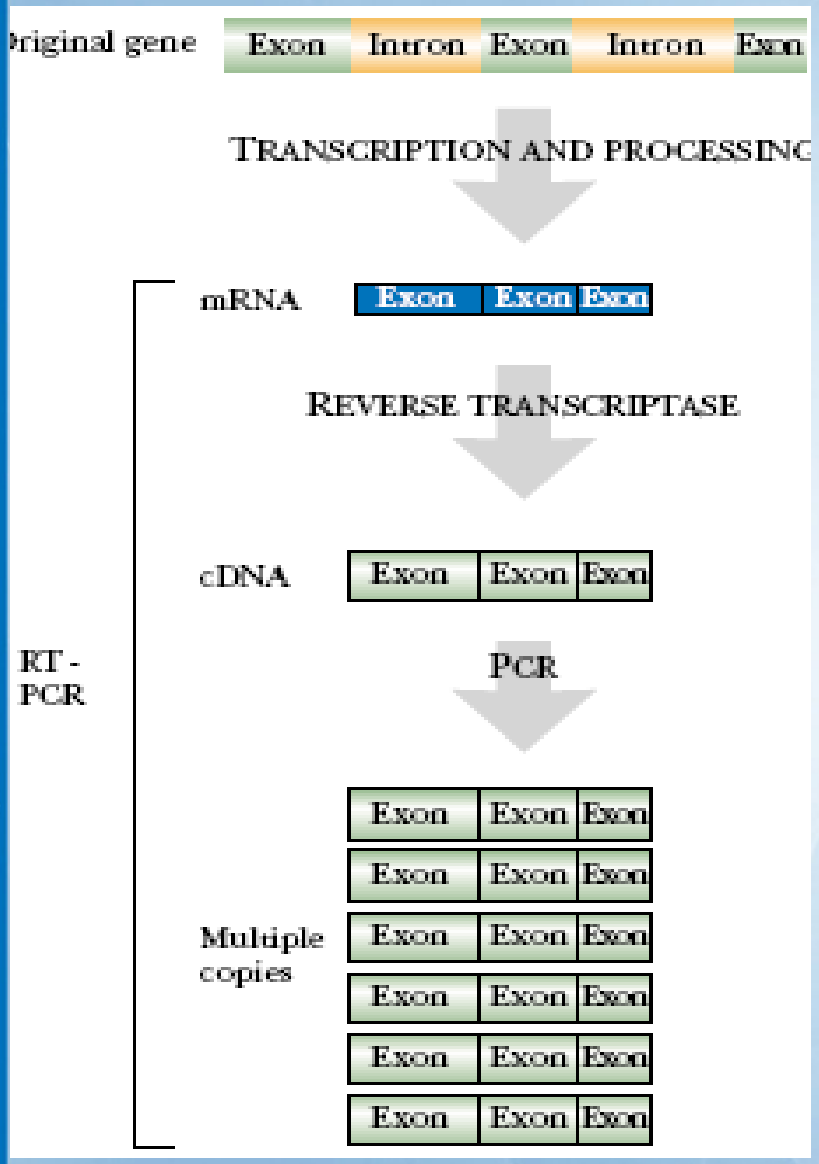
Reverse-transcriptase-polymerase chain reaction
RT-PCR

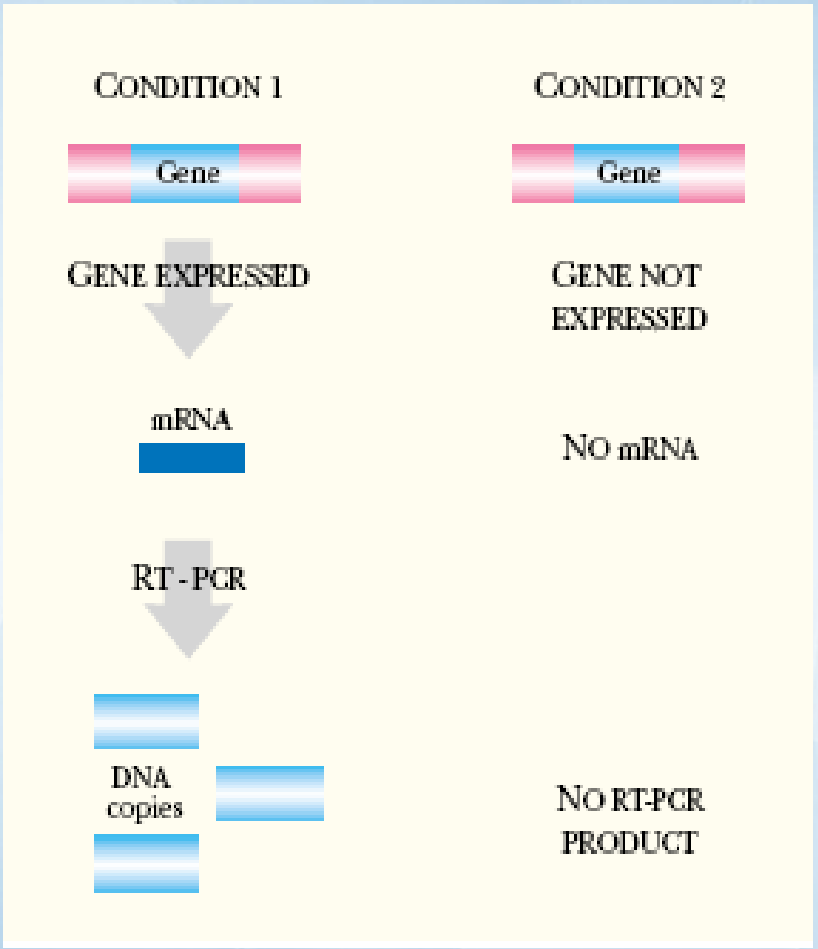
- In RT-PCR, specific mRNA could to be detected and quantified.
- In RT-PCR, reverse transcriptase (RT) is used to copy all of the mRNAs in an RNA sample into cDNA.
- This single stranded cDNA can then be amplified by PCR using primers that anneal to a specific cDNA (vis. mRNA).

RT-PCR


5 '-Cap
mRNA
AAA(A)n
(dT)12~18 primer
anneal
3 '
5 '
AAA(A)n
dNTP
Reverse transcriptase
5 '
AAA(A)n
cDNA:mRNA hybrid
Regular PCR

RT-PCR for Gene expression





- One step RT`-PCR
- Two steps RT and PCR

What is Real-time PCR?

- **detection and the quantification of a fluorescent transmitter during the process of amplification.**
- **Can view PCR cycles in 'real time'**
- **DNA is quantified after each cycle of PCR**
  - **Therefore can simultaneously quantify and amplify**
- **Can detect the number of copies in the sample.**
- **no post-PCR processing of products.**

  (No gel-based analysis at the end of the PCR reaction)

How to measure the PCR product?

- **Two general principles for the quantitative detection of amplicons:**

1- agents (dye) binding to the double-stranded-DNA (SybrGreen I).

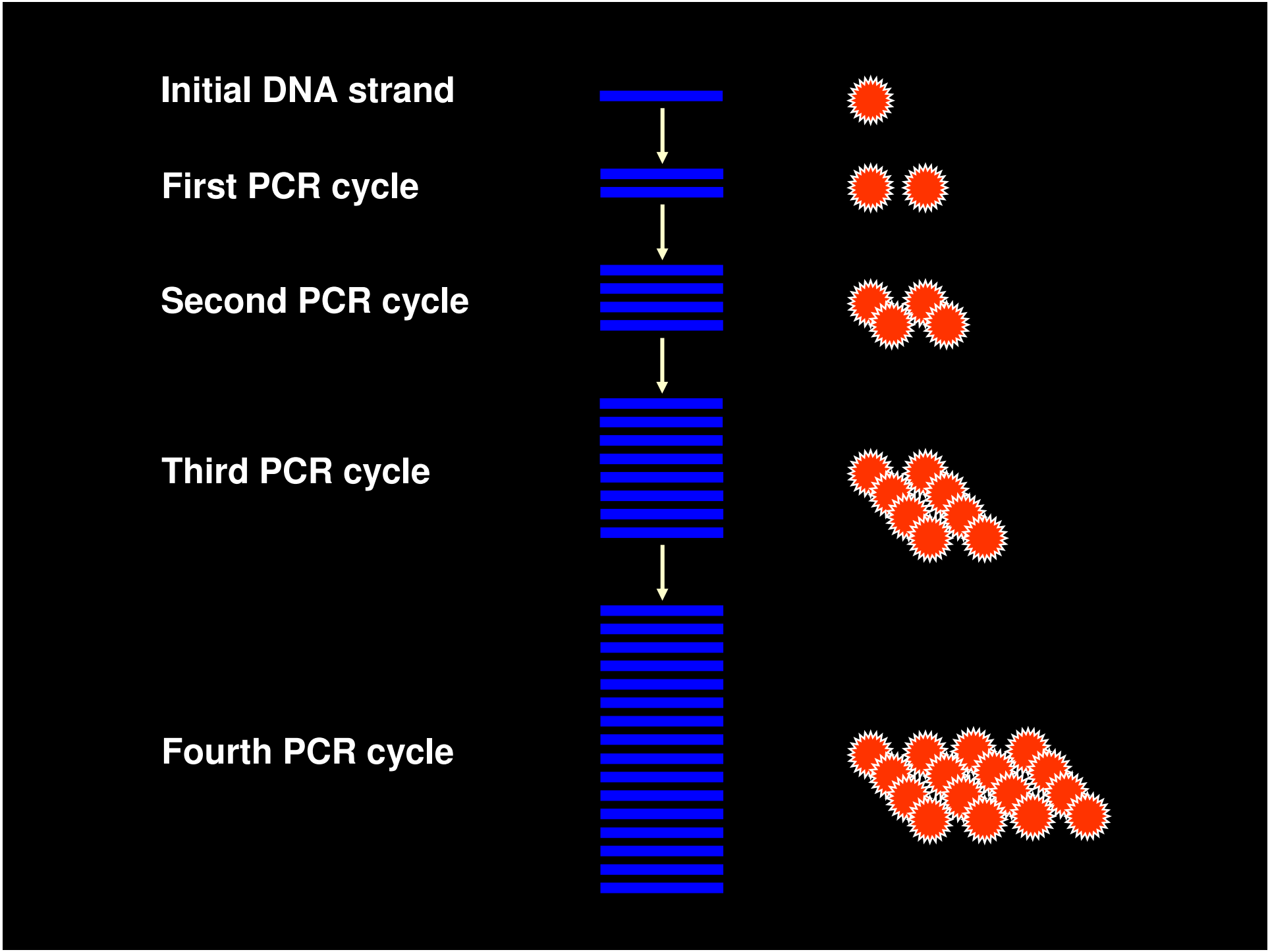
2- fluorescent probes (FAM, TAMRA, JOE, ROX,)

*** TagMan® analysis (Hydrolysis probes)**

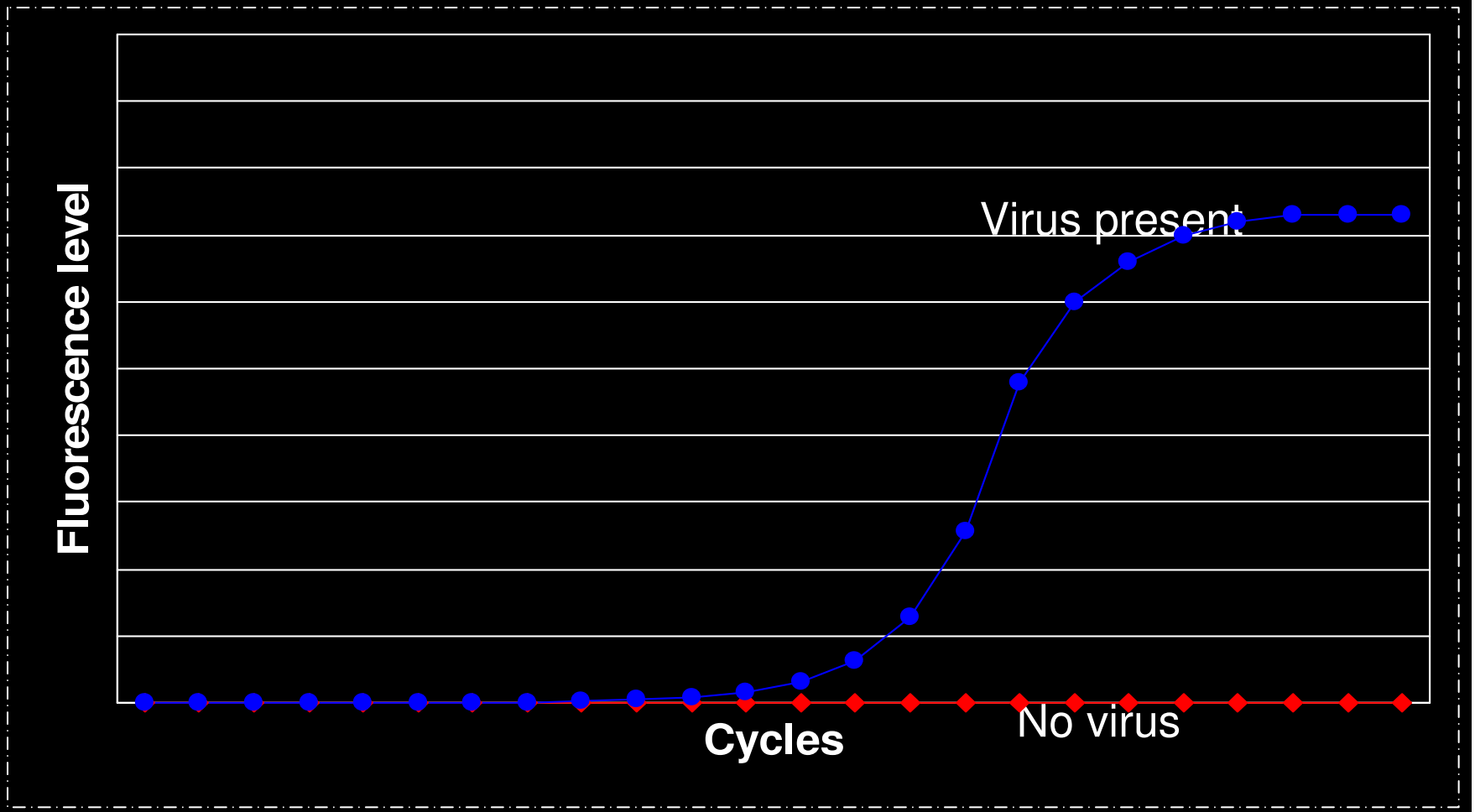
*** Molecular Beacons,**

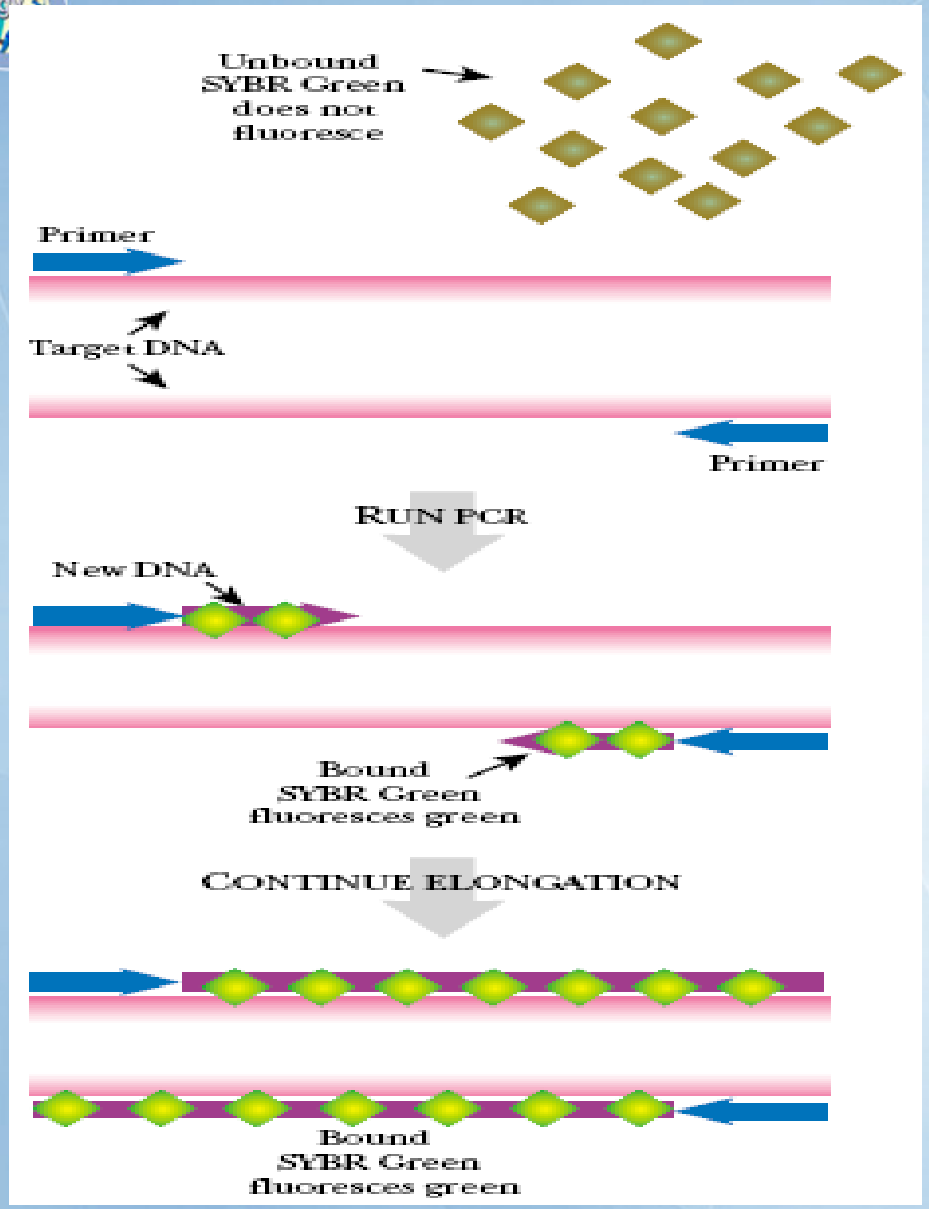
*** Scorpions™**

... other probes


Initial DNA strand
First PCR cycle
Second PCR cycle
Third PCR cycle
Fourth PCR cycle

Any increase in fluorescence level can be plotted onto a graph and easily interpreted

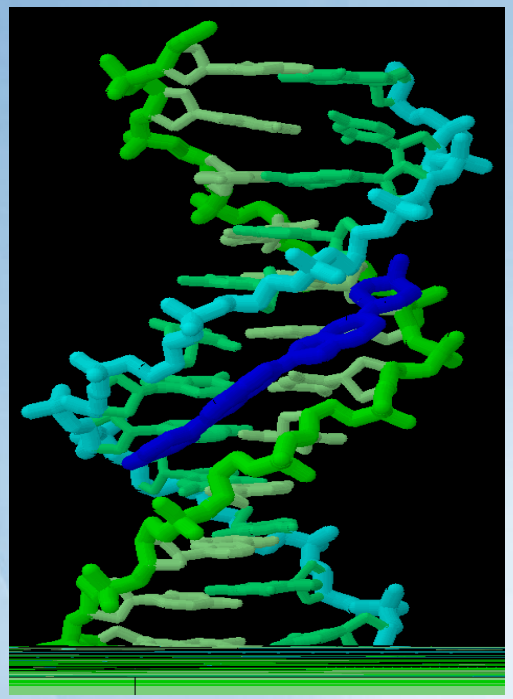


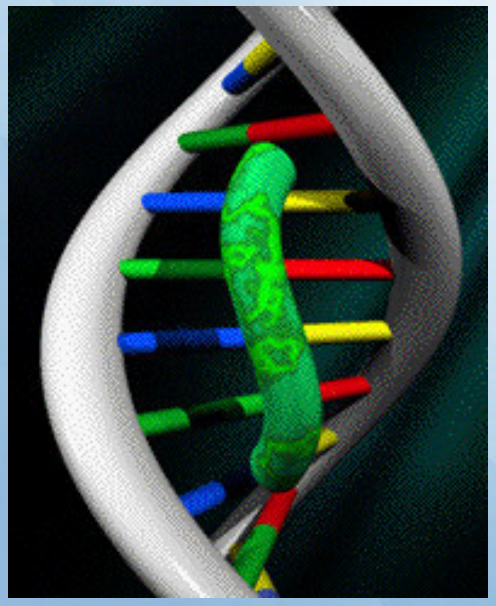


Realtime Fluorescent PCR with

SYBR Green

When the fluorescent probe SYBR® green is present during a PCR reaction, it binds to the doublestranded PCR product and emits light at 520 nm. The SYBR® Green dye only fluoresces when bound to DNA. Hence, the amount of fluorescence correlates with the amount of PCR product produced. This allows the accumulation of PCR product to be followed through many cycles.





SYBR Green I

- No probe; lower reagent cost than TaqMan.
- No more than 0.5 µg of DNA in sample
- Believed to be a minor groove binding dye.

The SYBR Green: advantages

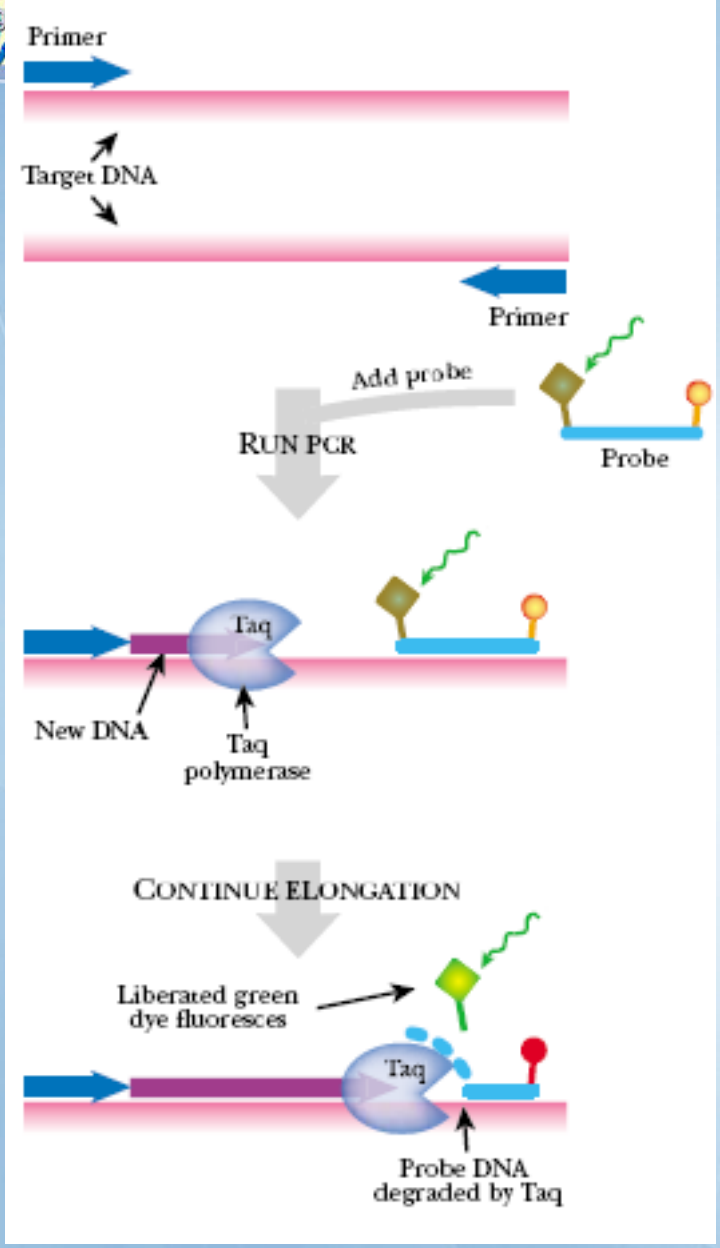
- ✓ - Economic
- ✓ - Easy to use
- ✓ - Has more sensibility than the ethidium bromide (another intercalating agent)
- ✓ - Does not inhibit the reaction of amplification
- ✓ - Does not require any fluorescent probe, thus does not require any particular expertise for the design of the probes
- ✓ - Is not affected by mutations in the target DNA

The SYBR Green disadvantages

- Impossible to make sure of specificity of amplicons
- Bad pairing can lead to positive forgeries or an over-estimate of the quantification
- Still unspecified mutagen capacity

Realtime Fluorescent PCR with TaqMan® Probe

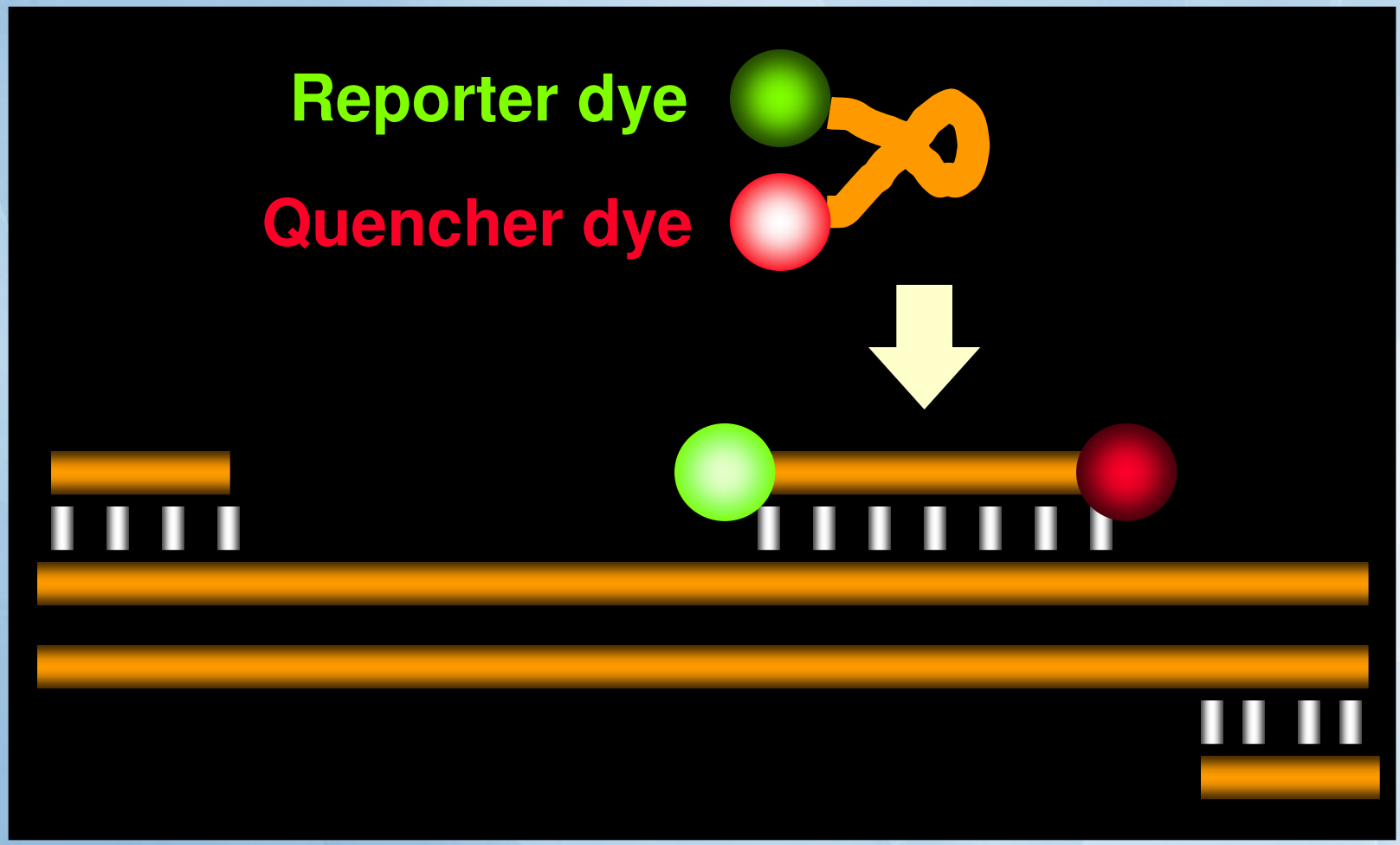
(probe hydrolysis)



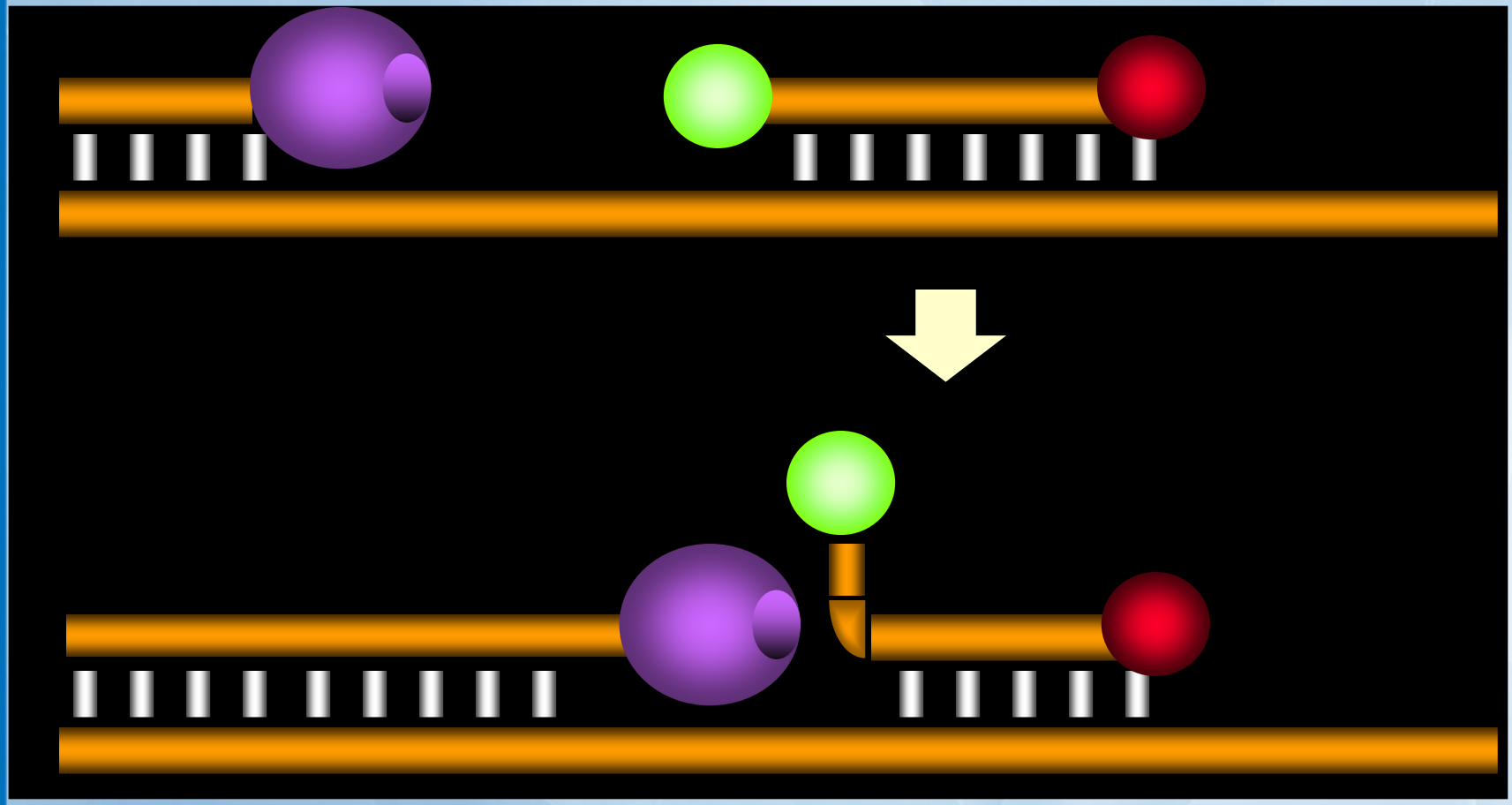
The TaqMan® probe has three elements: a short-wavelength fluorophore on one end (diamond), a sequence that is specific for the target DNA (blue), and alongwavelength fluorophore at the other end (circle). The two fluorophores are so close that fluorescence is quenched and no green light is emitted. This probe is designed to anneal to the center of the target DNA. When Taq polymerase elongates the second strand during PCR, its nuclease activity cuts the probe into single nucleotides. This releases the two fluorophores from contact and abolishes quenching. The short-wavelength fluorophore can now fluoresce and a signal will be detected that is proportional to the number of new strands synthesized.

quencher Molecule that prevents fluorescence by binding to the fluorophore and absorbing its activation

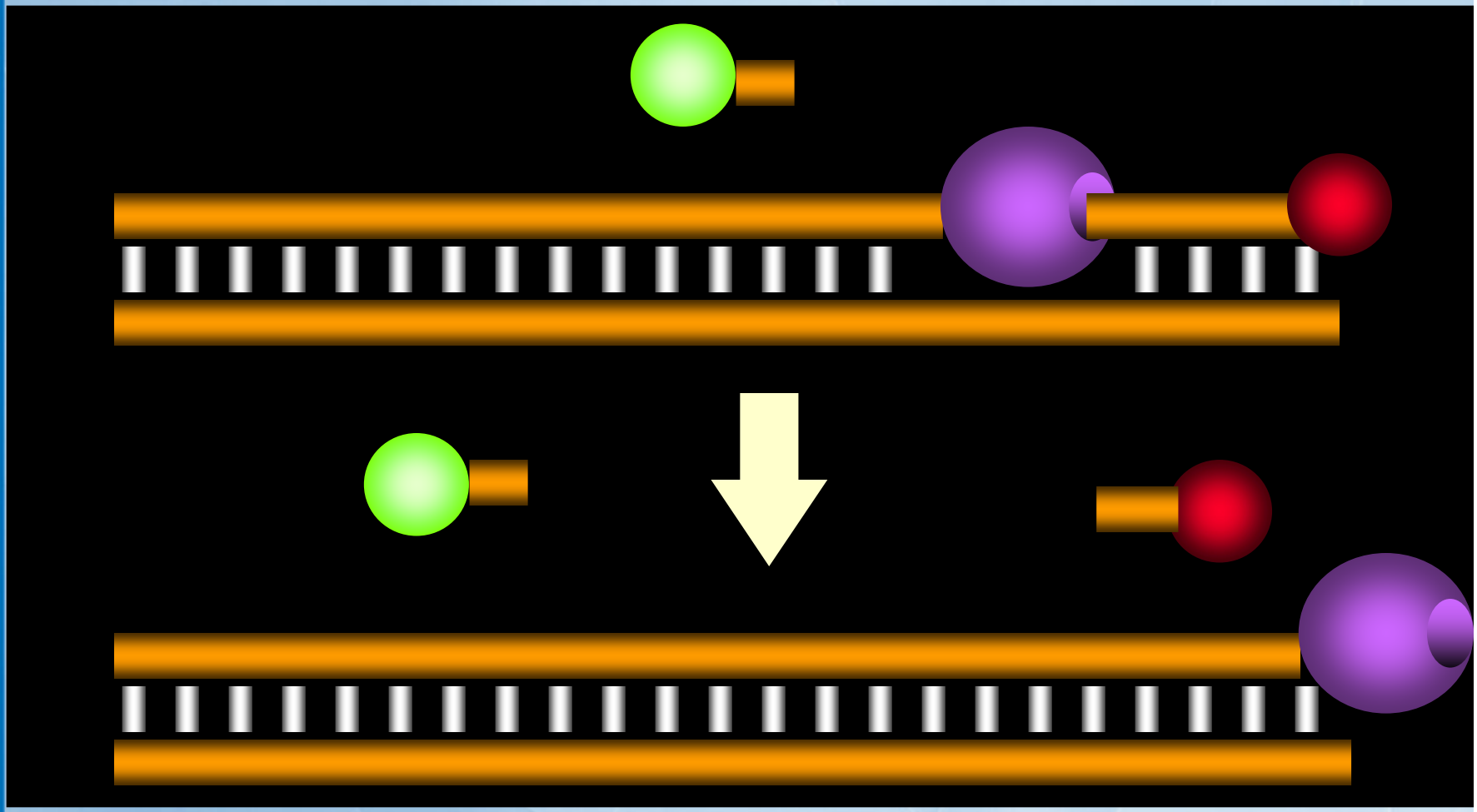
TaqMan Uses a Fluorescent Probe



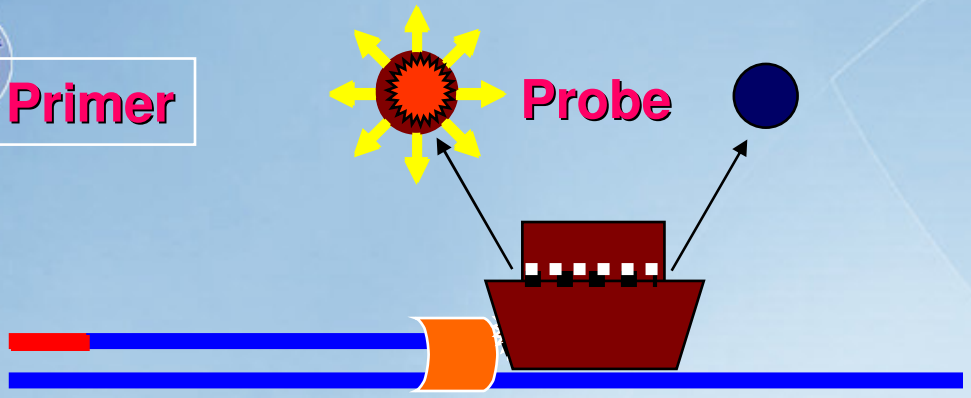
Taq Extends from Upstream Primer



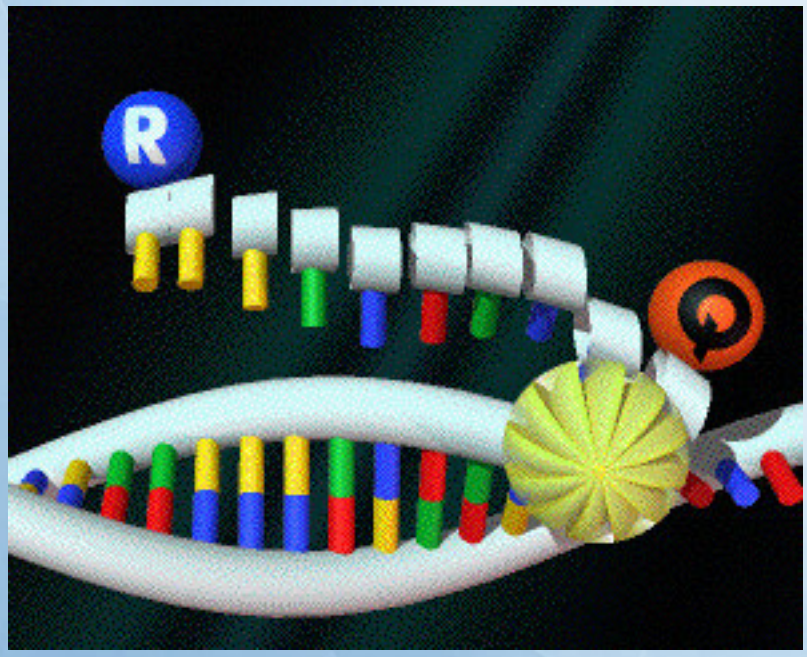
5′-Nuclease Activity Digests Probe



Primer

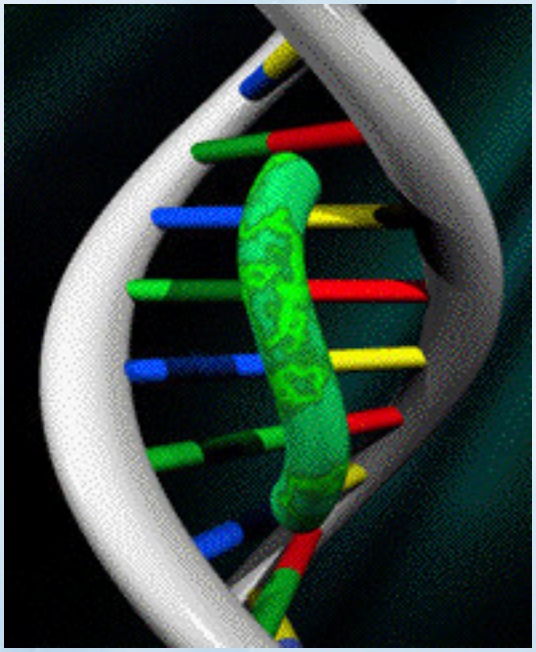


TaqMan



probe hydrolysis

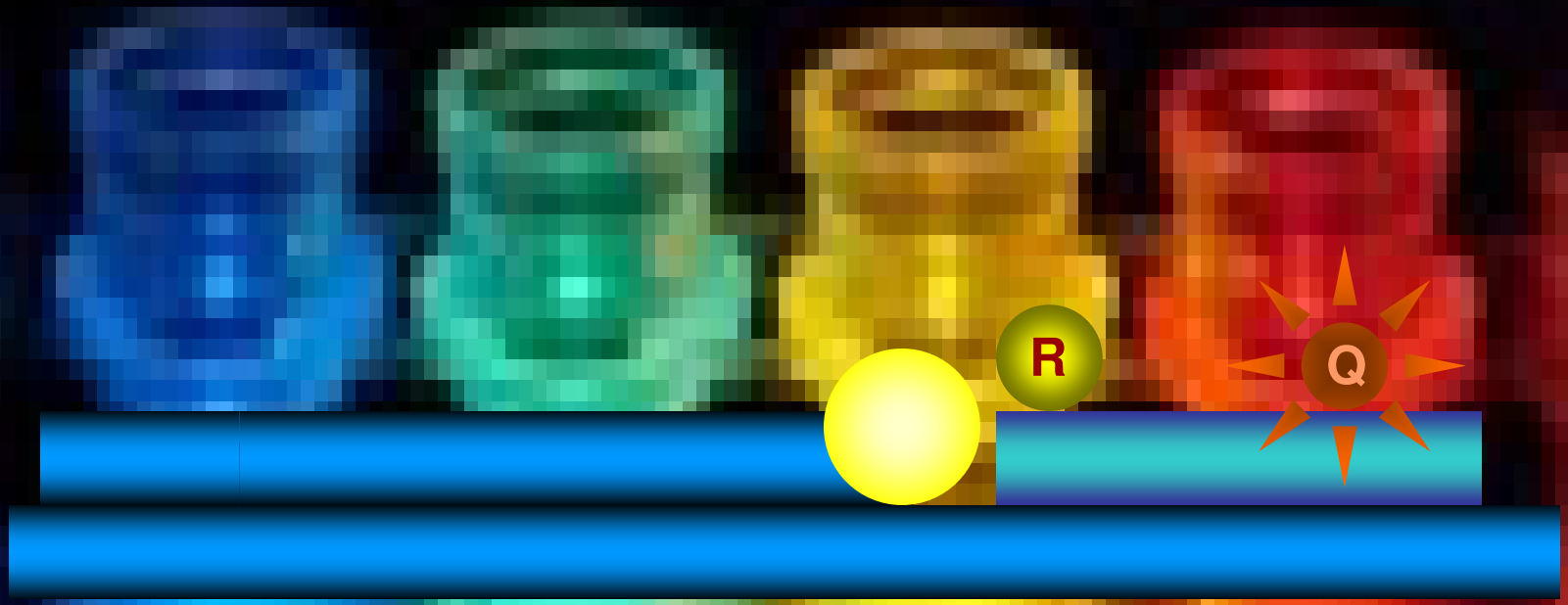
SYBR Green



agents binding
No probe

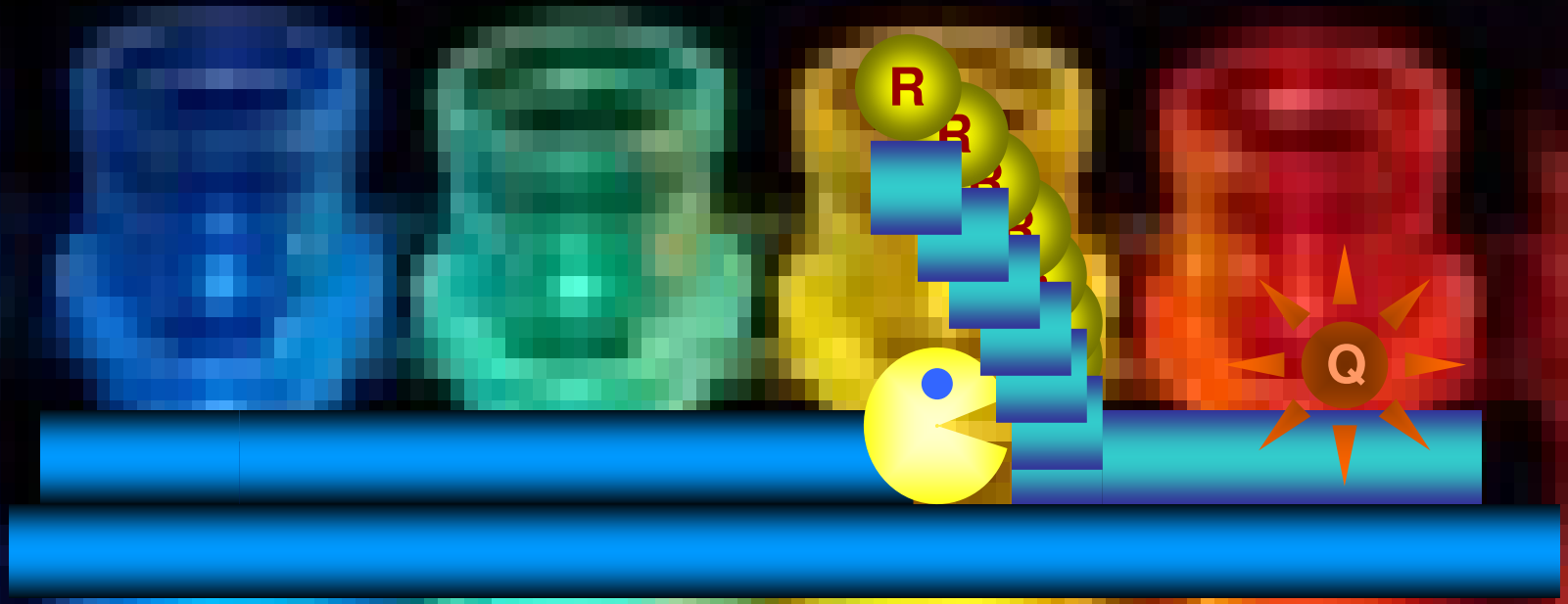
2- Theory of Real-time PCR ?

TaqMan Chemistry



2- Theory of Real-time PCR ?

TaqMan Chemistry

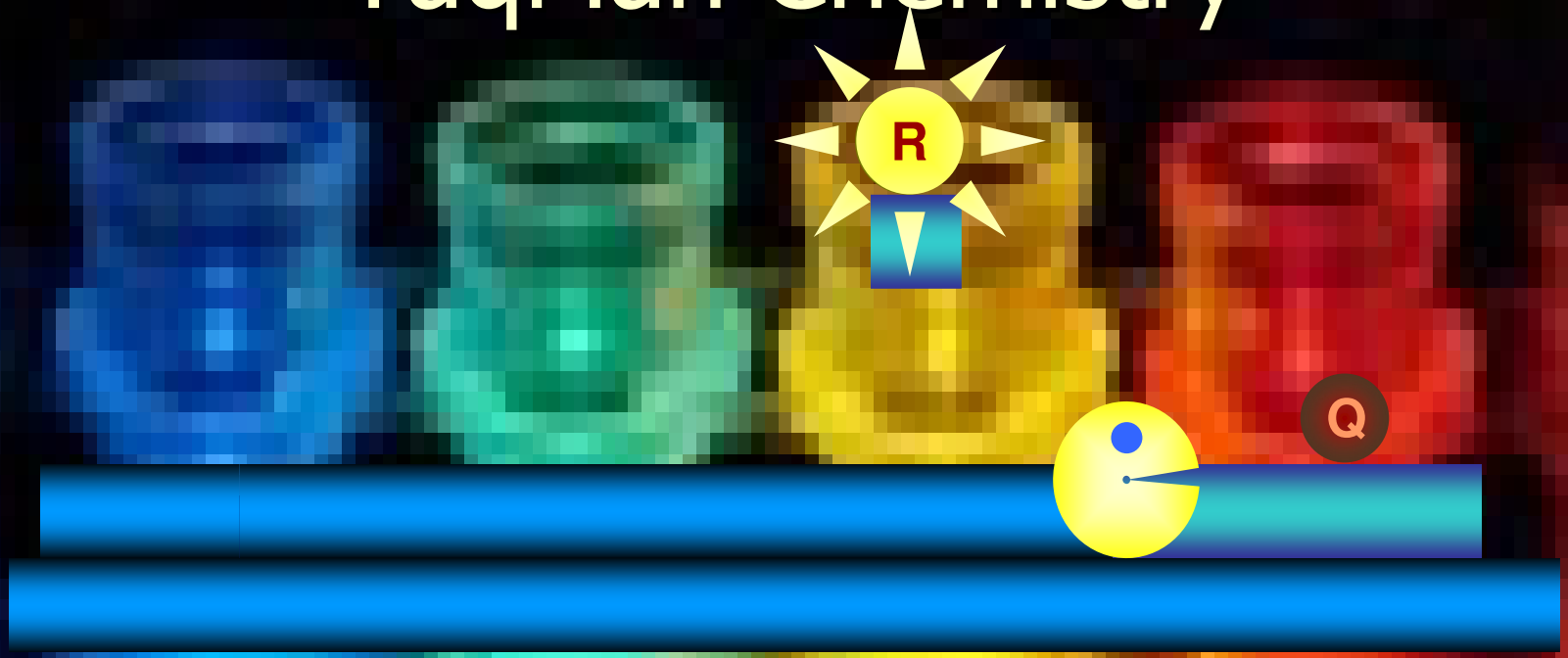


2- Theory of Real-time PCR ?

TaqMan Chemistry

2- Theory of Real-time PCR ?

TaqMan Chemistry

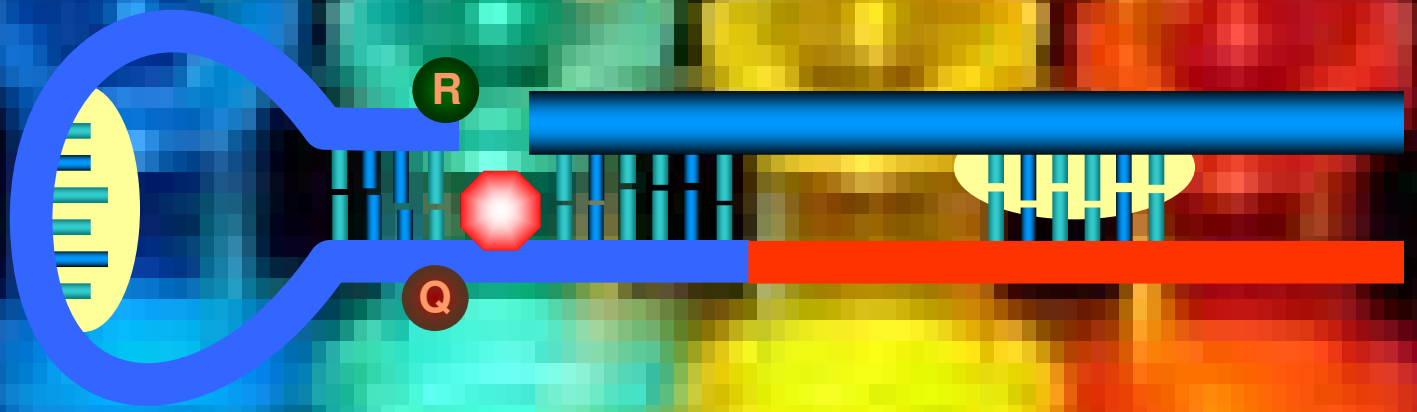


2- Theory of Real-time PCR ?

Molecular Beacons

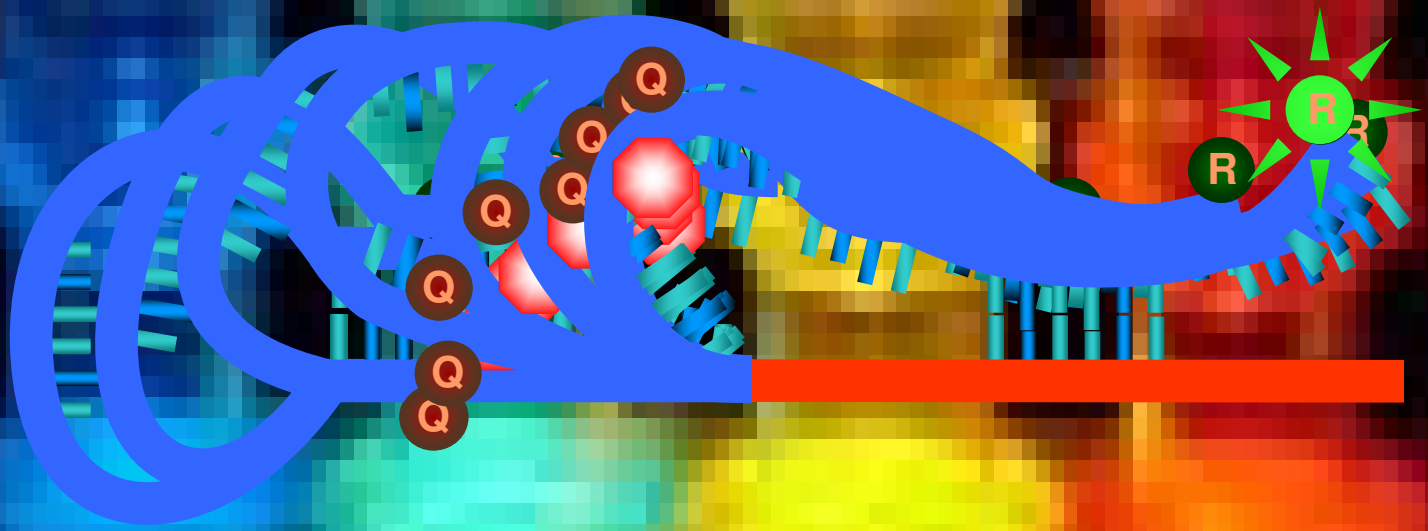
2- Theory of Real-time PCR ?

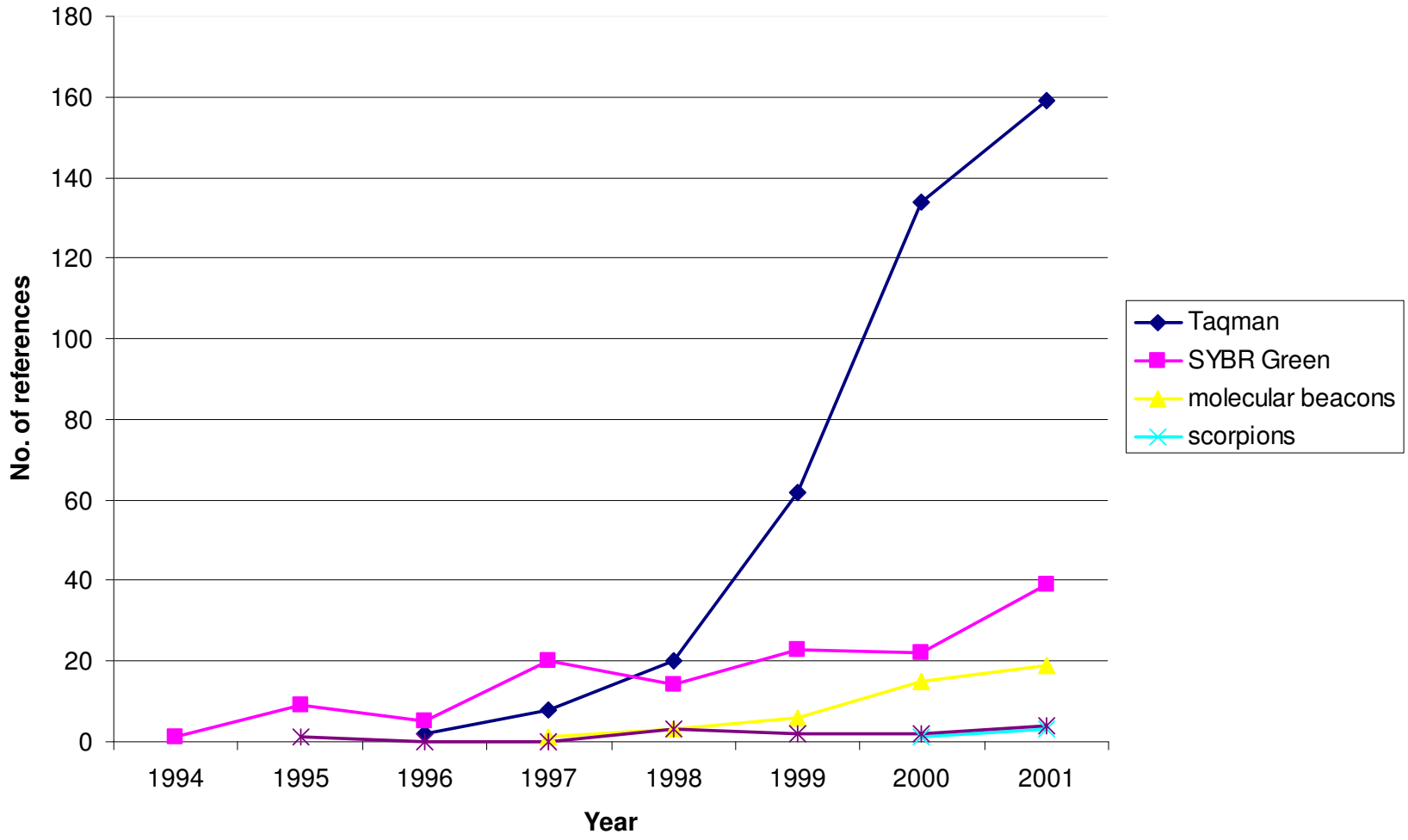
SCORPIONS



2- Theory of Real-time PCR ?

SCORPIONS




Published References for real-time PCR Fluorescent Chemistries
No. of references
180
160
140
120
100
80
60
40
20
0
1994
1995
1996
1997
1998
1999
2000
2001
Year
Taqman
SYBR Green
molecular beacons
scorpions

Advantages and Disadvantages

Advantages:

- Fast PCR *eg 30 cycles in 20 minutes*
- Quick = faster results = faster treatment
- Increased specificity
- Qualitative and Quantitative
- Easy interpretation of results
- Small sample volume

Disadvantages:

- To respect the principles of design of the probes
- Expensive

Probe design

- 30 bases in length
- G-C content of around 50 %
- The probes should not overlap with, or have sequence complementarity with either of the primers
- Probes should not contain a G at their 5’ ends, because such an arrangement quenches reporter fluorescence, even after cleavage
- A Tm of 5 to 10°C greater than that of the primers

Q RT-PCR Applications

- Gene expression.
- DNA target quantification (nuclear, mitochondrial DNA).
- SNP detection.
- Viral load assays, pathogen & GMO detection.
- Clinical Diagnostics (Cancer, Therapy Response)
- cDNA cloning (cDNA Library).

References

- **Micklos, David, Greg Freyer and David Crotty. DNA Science a First Course. New York:Cold Spring Habor Laboratory Press, 2003.**
- **Purves, Sadava, Orians, Heller. "Life." 6th ed. Sinauer Associates, 2001.**
- **Demidov.V, Broude. N(2004). DNA Amplification: Current Technologies and Applications**
- **Websites:**
- http://faculty.plattsburgh.edu/donald.slish/PCRmov.html (Animation)
- http://www.accessexcellence.org/RC/AB/IE/PCR_Xeroxing_DNA.html
- http://www.people.virginia.edu/~rjh9u/pcranim.html (PCR Animation)
- http://homepages.strath.ac.uk/~dfs99109/BB211/RecombDNAtechlect4.html
- http://www.escience.ws/b572/L3/L3.htm
- http://allserv.rug.ac.be/~avierstr/principles/pcrani.html


Thank you
Prof. Dr. Hamdy El-Aref