Lecture 5 – Polymerase Chain Reaction

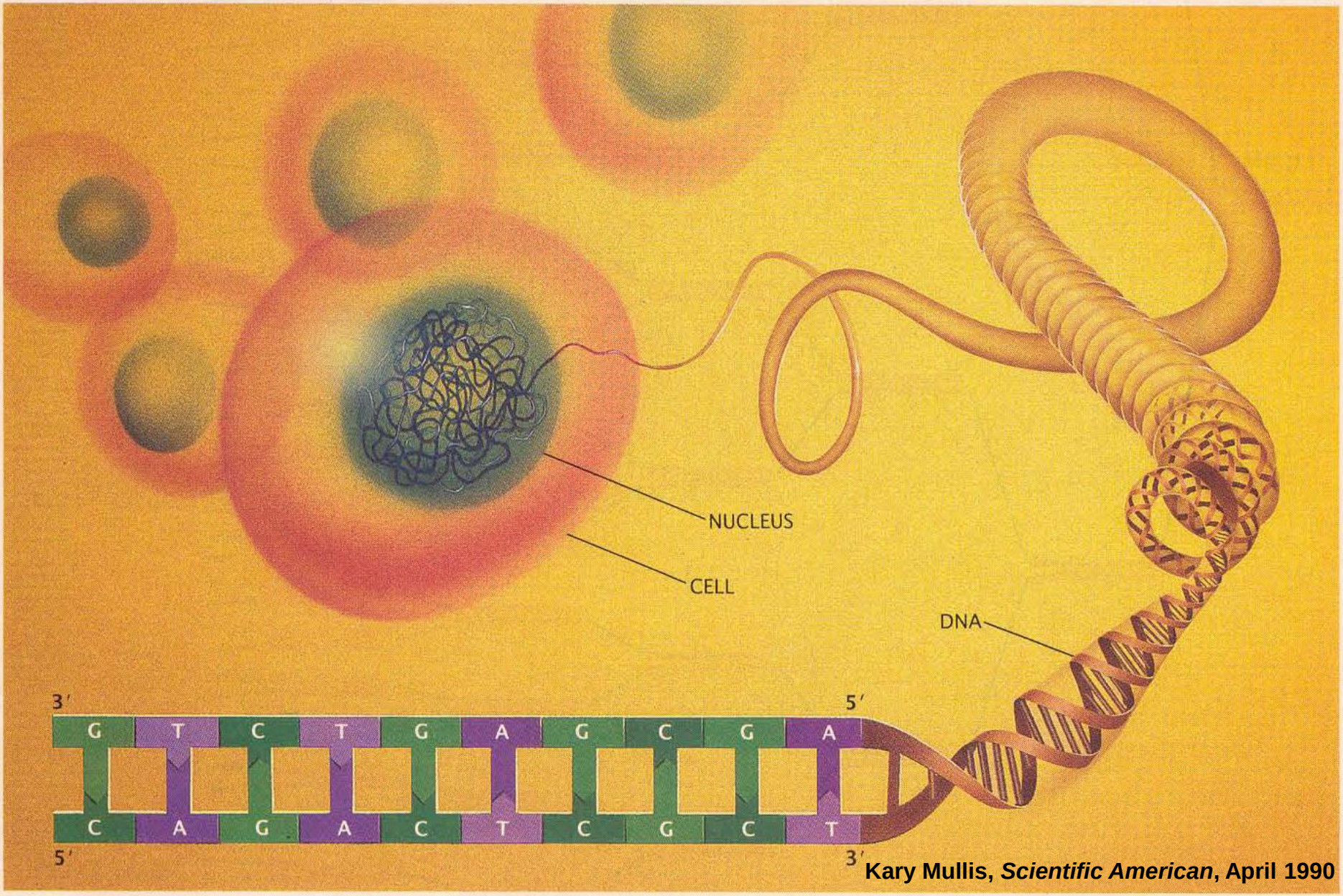
The Unusual Origin of the Polymerase Chain Reaction

A surprisingly simple method for making unlimited copies of DNA fragments was conceived under unlikely circumstances—during a moonlit drive through the mountains of California

by Kary B. Mullis

Scientific American, April 1990

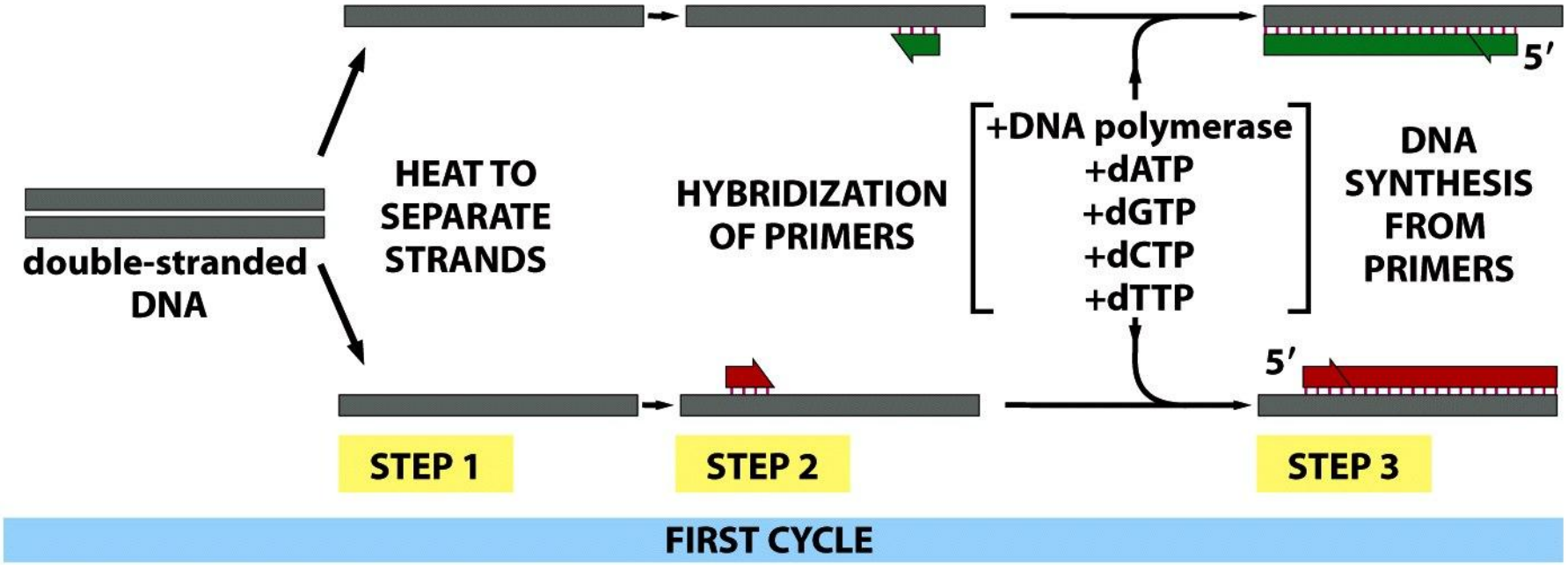
The polymerase chain reaction makes life much easier for molecular biologists: it gives them as much of a particular DNA as they want. Casual discussions of DNA molecules sometimes make them sound like easily obtained objects. The truth is that in practice it is difficult to get a well-defined molecule of natural DNA from any organism except extremely simple viruses.



Kary Mullis, *Scientific American*, April 1990

DNA consists of two strands of linked nucleotides: deoxyadenylates (*A*'s), deoxythymidylates (*T*'s), deoxyguanylates (*G*'s) and deoxycytidylates (*C*'s). The sequence of nucleotides in one strand is complementary to that in the other strand—the *A*'s are always opposite *T*'s, and the *G*'s are opposite *C*'s—and this complementarity binds the strands together. Each strand has a three-prime and a five-prime end. Because their orientations oppose one another, the strands are said to be antiparallel.

Amplification of DNA by PCR



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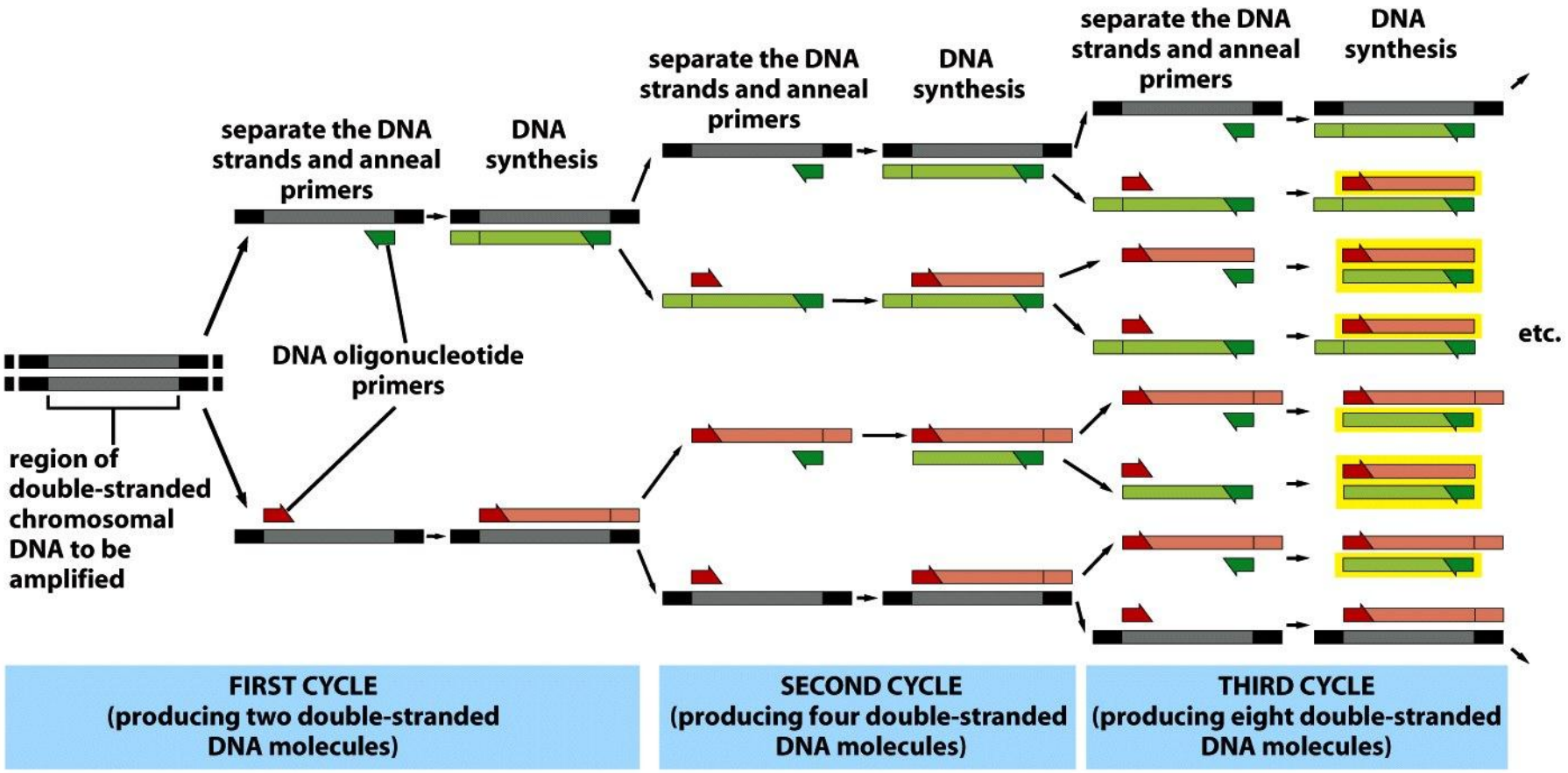
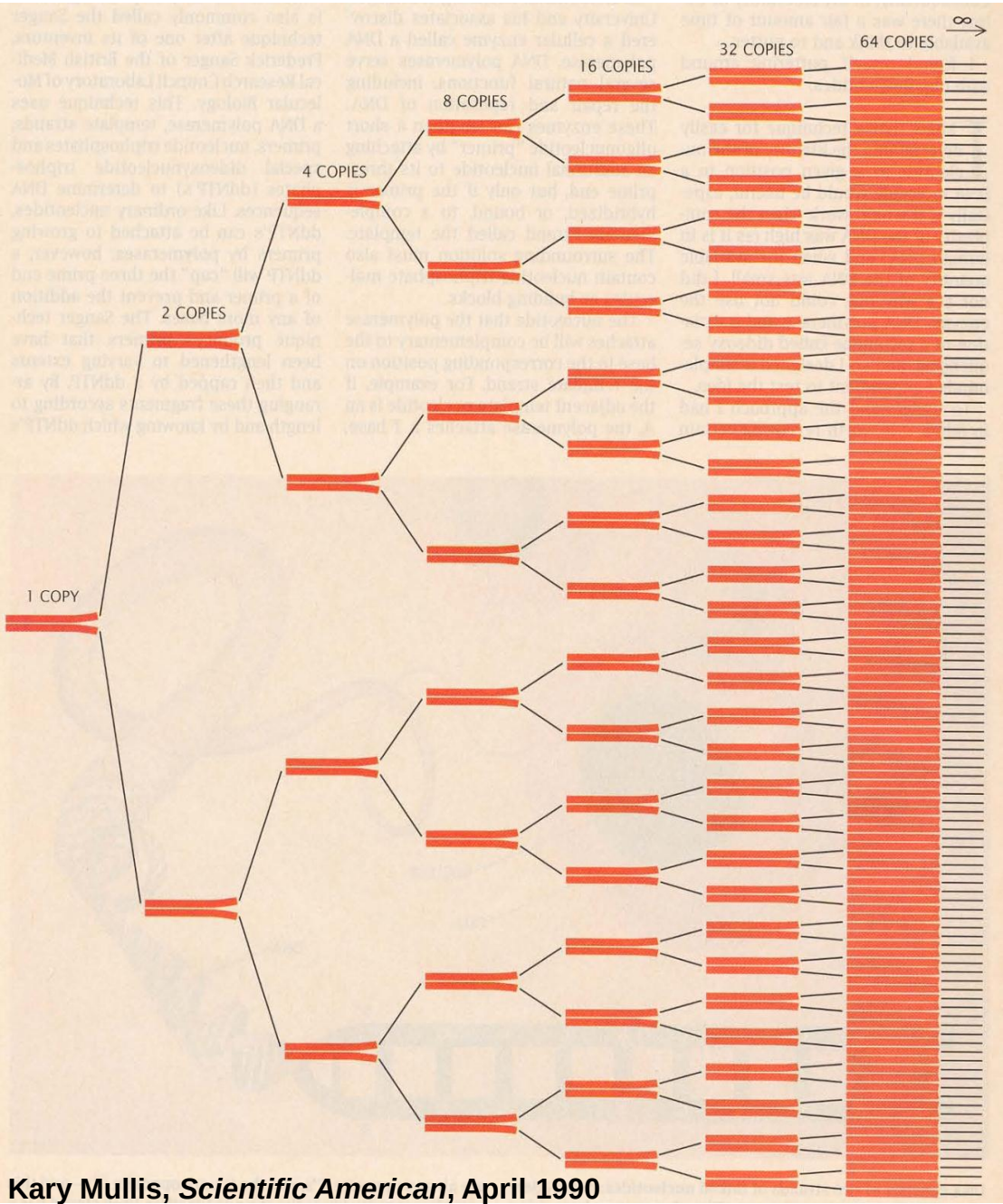


Theoretical Yield of Correctly Formed dsDNA Molecules During a PCR Experiment

Cycle Number	Total Double-stranded DNA Molecules Produced
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824



Kary Mullis, *Scientific American*, April 1990

Three Steps of Polymerase Chain Reaction (PCR)

Denaturation	**DNA template is denatured with high heat to separate strands. 94°C – 95°C for 5-10 minutes**
Annealing	**Each DNA primer anneals, binding to its complementary sequence on the template DNA 55°C-65°C for 30-45 seconds**
Extension	**DNA polymerase creates a new strand of DNA complementary to the template DNA starting from the primer. 68°C-72°C, 1 kilo-base/minute**

- **Multiple rounds (25-40) of denaturation-annealing-extension (cycle) are performed to create many copies of the template DNA between the two primer sequences.**
- **To generate a 500-base pair product will take ~ 1.5 hours.**

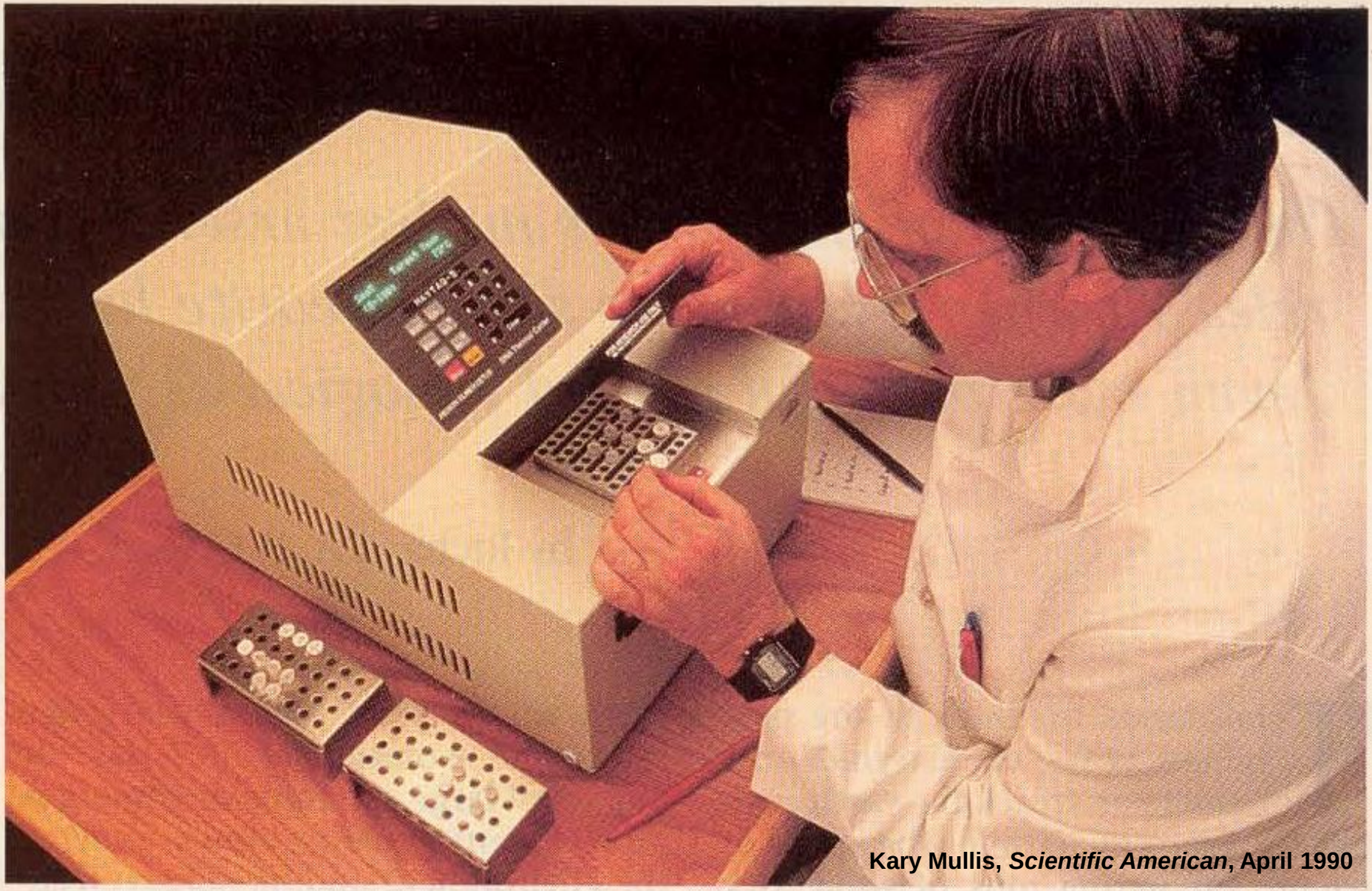
Components in a PCR reaction

Multiple repetitions of DNA replication are performed in a test tube, thus producing large amount of DNA from very small amount of sample.

Mix in test tube:

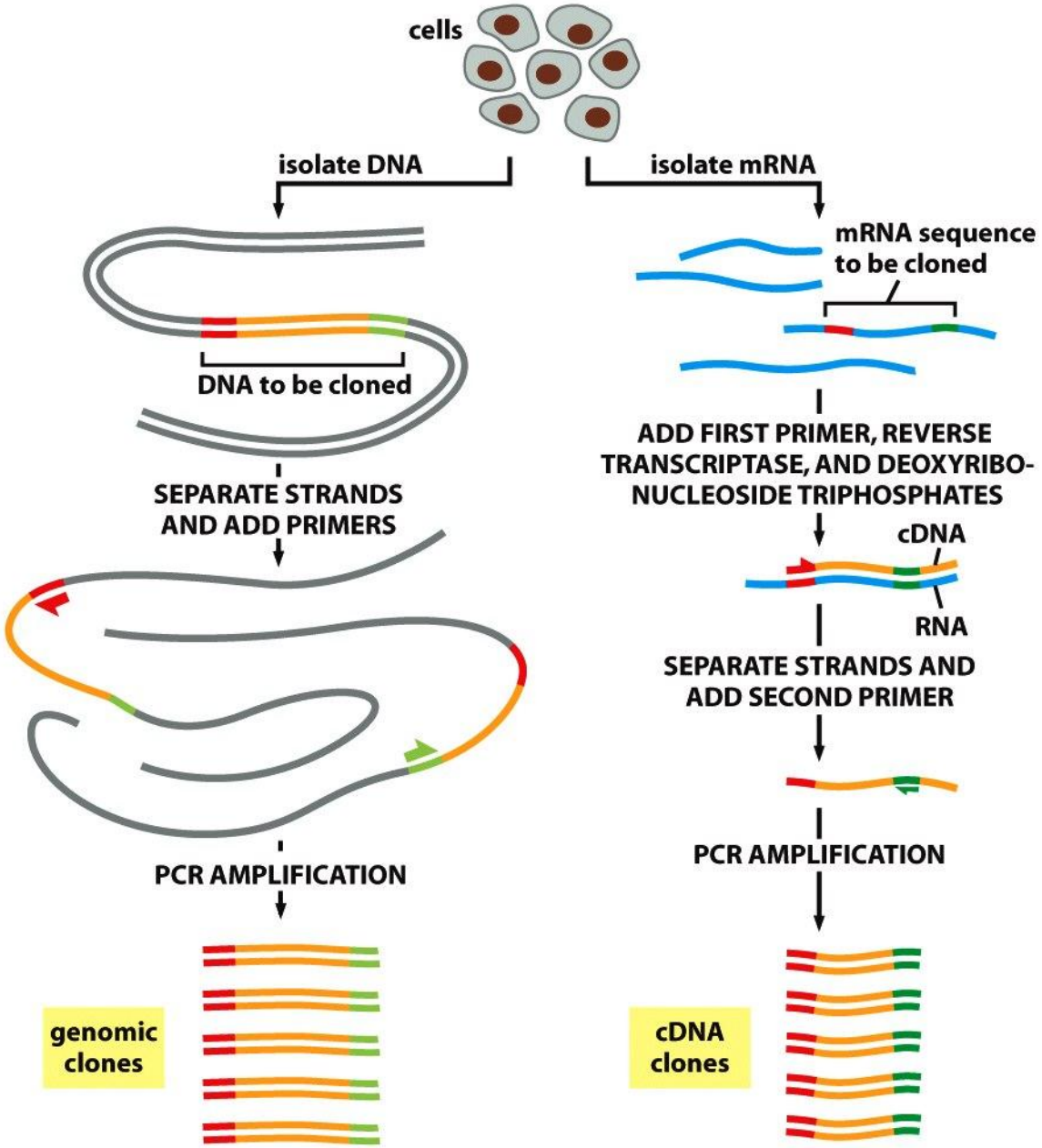
Items	Function	Amount
DNA template	DNA to be amplified	≤ 1 μg
Primers	one complementary to each strand	0.1-0.5 μM
Nucleotides	dA, dG, dC and dT (dNTPs)	20-200 μM
DNA polymerase	heat-stable form from thermophilic bacteria	1-2.5 units
$MgCl_2$	Co-factor for DNA polymerase	0.5-2.5 mM
Buffer	Maintain optimal pH	pH 8.3-8.8

Kary Mullis, *Scientific American*, April 1990

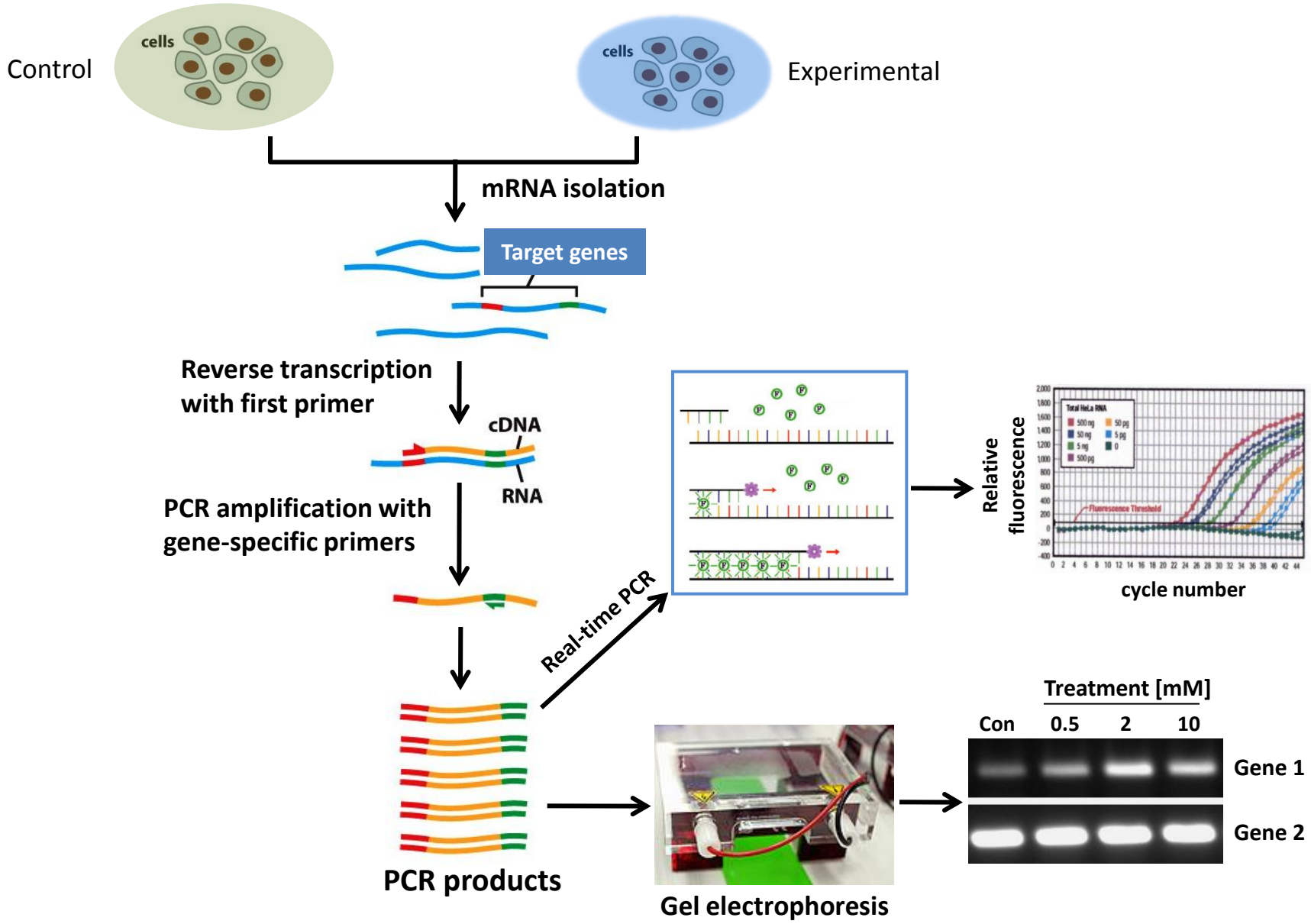


MACHINE that performs the polymerase chain reaction is shown being loaded with samples of DNA. Such devices are rapidly becoming common fixtures in laboratories.

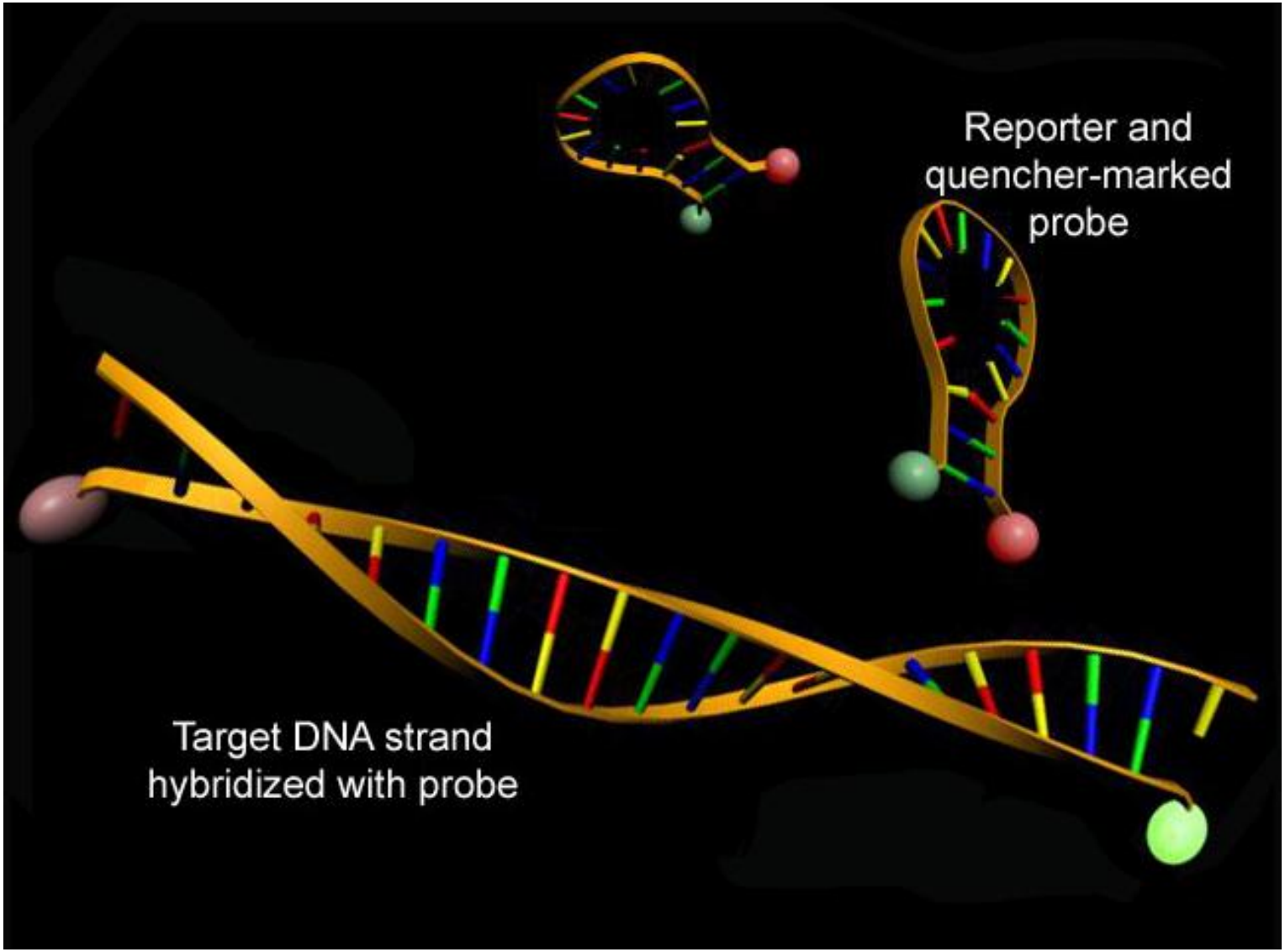
Use of PCR to Obtain a Genomic or cDNA Clone



Use of PCR in Studying Gene Expression



Real-time quantitative PCR uses fluorophores to detect levels of gene expression.



Quantitative PCR

- A quantitative polymerase chain reaction (qPCR), also called real-time polymerase chain reaction, is based on standard PCR, and is used to amplify and simultaneously quantify a targeted DNA molecule.
- For one or more specific sequences in a DNA sample, qPCR enables both detection and quantification.
- Its key feature is that the amplified DNA is detected as the reaction progresses in "real time".
- Two common methods for the detection of products in quantitative PCR are:
  - ✓non-specific fluorescent dyes that intercalate with any double-stranded DNA
  - ✓sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence to quantify mRNA and non-coding RNA in cells or tissues.
- qPCR is the abbreviation used for quantitative PCR (real-time PCR).
- Real-time reverse-transcription PCR is often denoted as qRT-PCR.
- The acronym "RT-PCR" commonly denotes reverse transcription polymerase chain reaction and not real-time PCR, but not all authors adhere to this convention.

Advantages of Real-time qPCR Over Traditional PCR

- **qPCR is a closed tube system requiring no post-PCR processing.**
- **qPCR has higher precision, increased sensitivity (down to 1 copy), increased dynamic range (greater than 8 logs) and high resolution (less than 2-fold differences).**

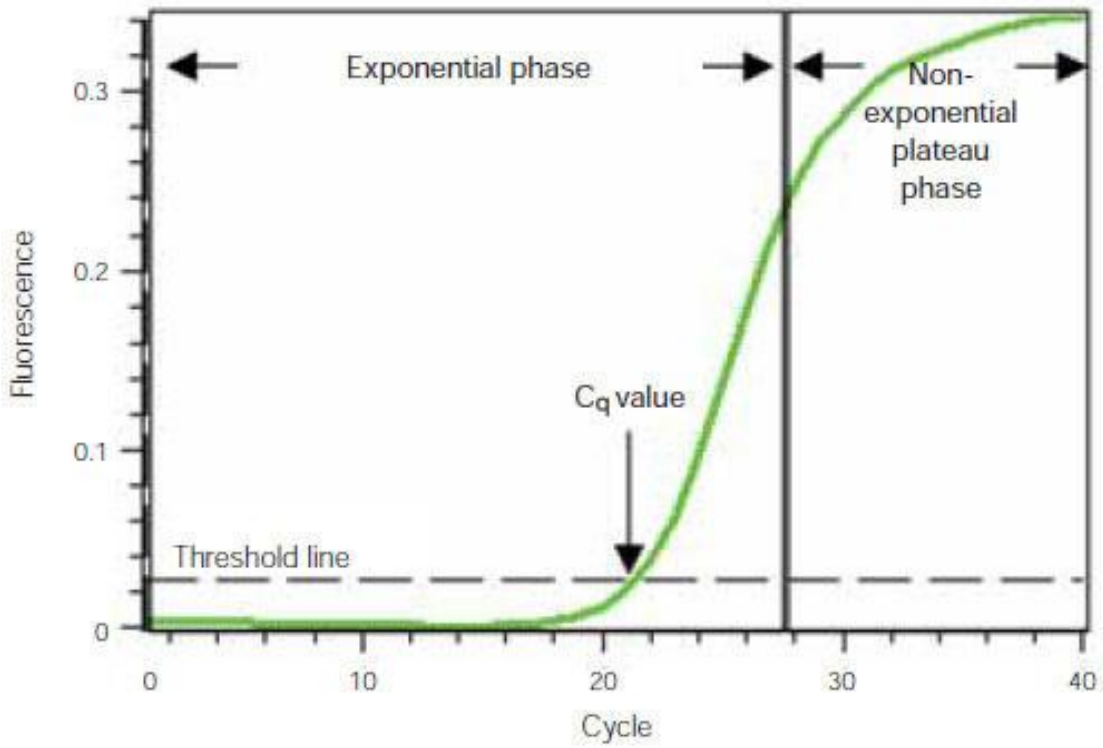
Uses of Real-Time qPCR

- **Fluorescence-based detection of amplification products through the use of a DNA-binding dye or hybridization probe**
- **Quantification of input nucleic acid by measuring the number of cycles required to reach a set level of product**
- **Amplification of DNA with end-point analysis to distinguish products, in contrast to traditional PCR**

Basic qPCR Terms

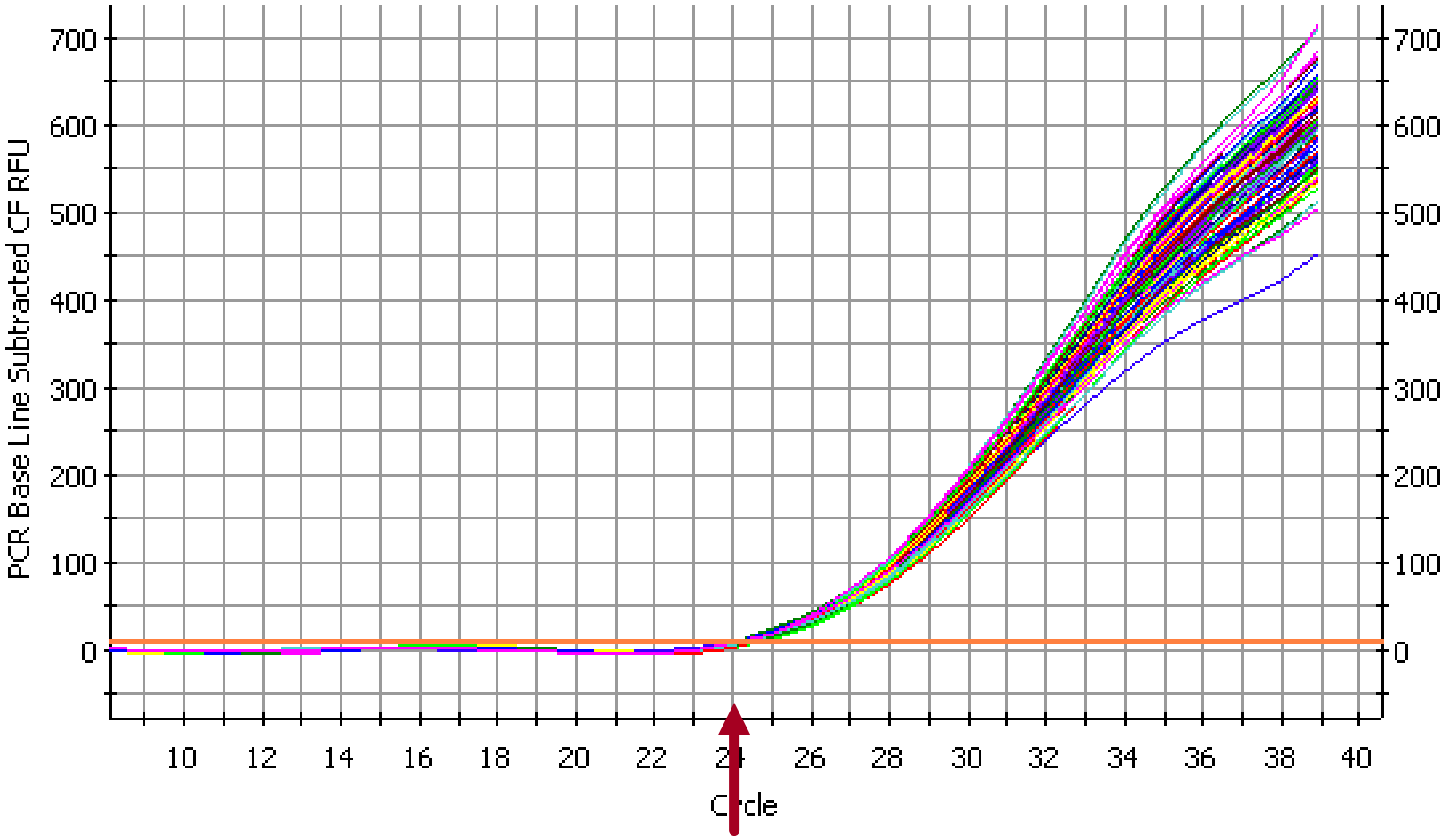
- **Amplification plot – plot of fluorescent signal versus cycle number**
- **Amplicon – fragment of DNA or RNA that is the source and/or product of natural or artificial amplification or replication events**
- **Baseline – the initial cycles of PCR where there is little to no change in fluorescence**
- **Threshold – the arbitrary level of fluorescence used for Cq determination; should be set above the baseline and within the exponential growth phase of the amplification plot**
- **Cq – quantification cycle, the fractional cycle number where fluorescence increases above the threshold; also referred to as Ct (threshold cycle) or Cp (quantification cycle)**
- **R – reporter signal**
- **Rn – normalized reporter signal**
- **ΔRn – baseline-subtracted normalized reporter signal**
- **Slope – Used to determine efficiency of reaction; with 10-fold dilutions, a slope of -3.32 indicates a perfect doubling of product per cycle (100% PCR efficiency)**
- **R2 – Reports linearity of standard curve**

Amplification Plot



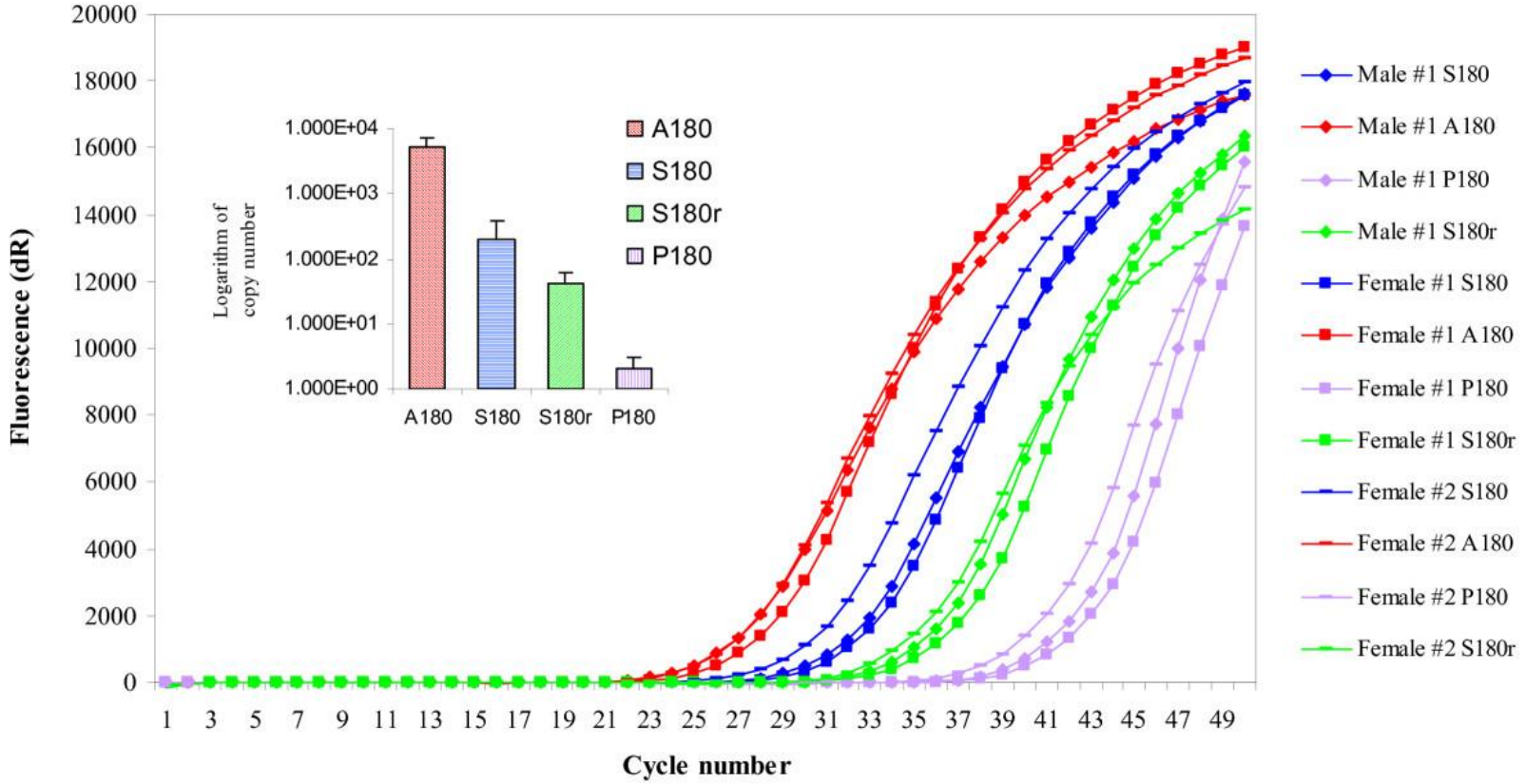
- **The amplification plot shows two phases, an exponential phase followed by a non-exponential plateau phase.**
- **During the exponential phase, the amount of PCR product approximately doubles in each cycle.**
- **As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting.**
- **At this point, the reaction slows and enters the plateau phase.**

Threshold Cycle, C_q



96 identical reactions will have almost identical C_q values

Quantitative PCR Amplification Plot and Logarithm Histogram of Original Copy Number



- **Four transcripts from cDNA of three adult guppies were quantified using qPCR with the amplification plot shown.**
- **Cq (dR) values are indicated on the X axis. Rn (normalized reporter signal) is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. An amplification plot shows the variation of log (ΔRn) with PCR cycle number.**
- **A histogram of the averaged logarithmic value of original transcript copy number is shown (with standard error bars included).**

qPCR relies on rapid heating and cooling and fluorophores.

- Quantitative PCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by the excited fluorochrome.
- The thermal cycler is able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and DNA polymerase.

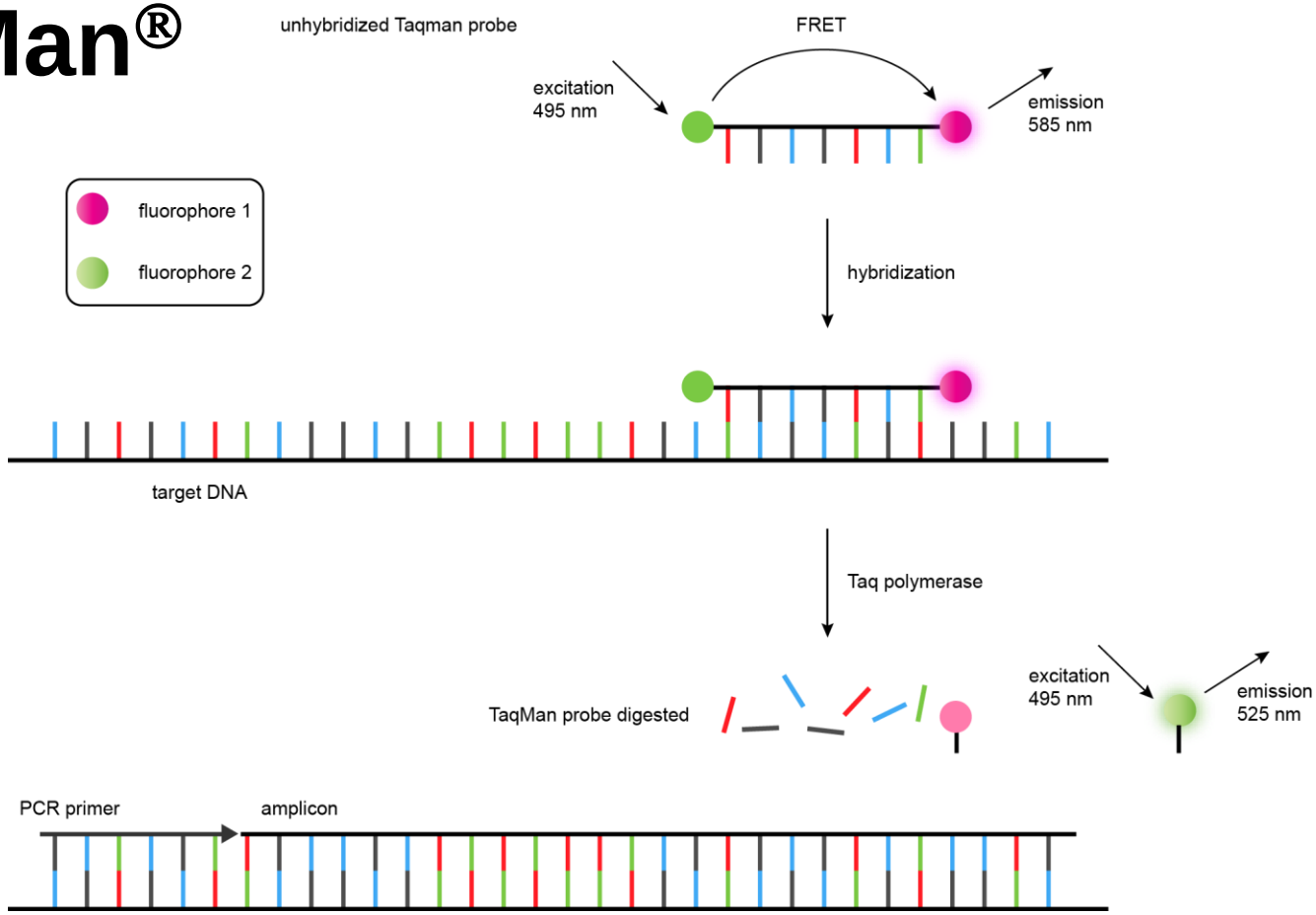
Chemistries Used in Real-time PCR

- **Hybridization Probes**
  - ✓**TaqMan – most commonly used probe-based chemistry**
  - ✓**Molecular Beacons – sequence-specific, fluorescently labeled oligonucleotide (25-40 nt)**
  - ✓**Scorpion primer assays – employ two primers, one of which serves as a probe and contains a stem-loop structure with a 5′-fluorescent reporter and a 3′-quencher**
  - ✓**LUX primers – employ two primers, one of which is a hairpin-shaped primer with a fluorescent report attached near the 3′-end**
- **Intercalation Dyes**
  - ✓**SYBR Green I – most commonly used DNA-binding dye**

TaqMan® and SYBR® Green 1

- **TaqMan probe-based chemistry, also known as the fluorogenic 5′ nuclease assay, uses an oligonucleotide probe that is designed to anneal to a specific sequence downstream to one of the PCR primers.**
  - ✓**The oligonucleotide is labeled with a fluorescent reporter dye at the 5′ end and a quencher dye at the 3′.**
  - ✓**When the probe is intact, the reporter is in close proximity to the quencher and the fluorescent signal is low as the energy from the reporter will be transferred to the quencher through Fluorescent Resonant Energy Transfer (FRET).**
  - ✓**During PCR, as Taq DNA polymerase extends from the primers, the 5′-exonuclease activity of the enzyme will cleave the annealed probe separating the reporter dye from the quencher dye, increasing the fluorescent signal.**
- **SYBR Green I is a dye that binds only to double-stranded DNA (dsDNA) and its fluorescent signal increases only when bound to dsDNA. During PCR, as more dsDNA amplicon is being produced, the fluorescent signal of SYBR Green I will increase.**

TaqMan®

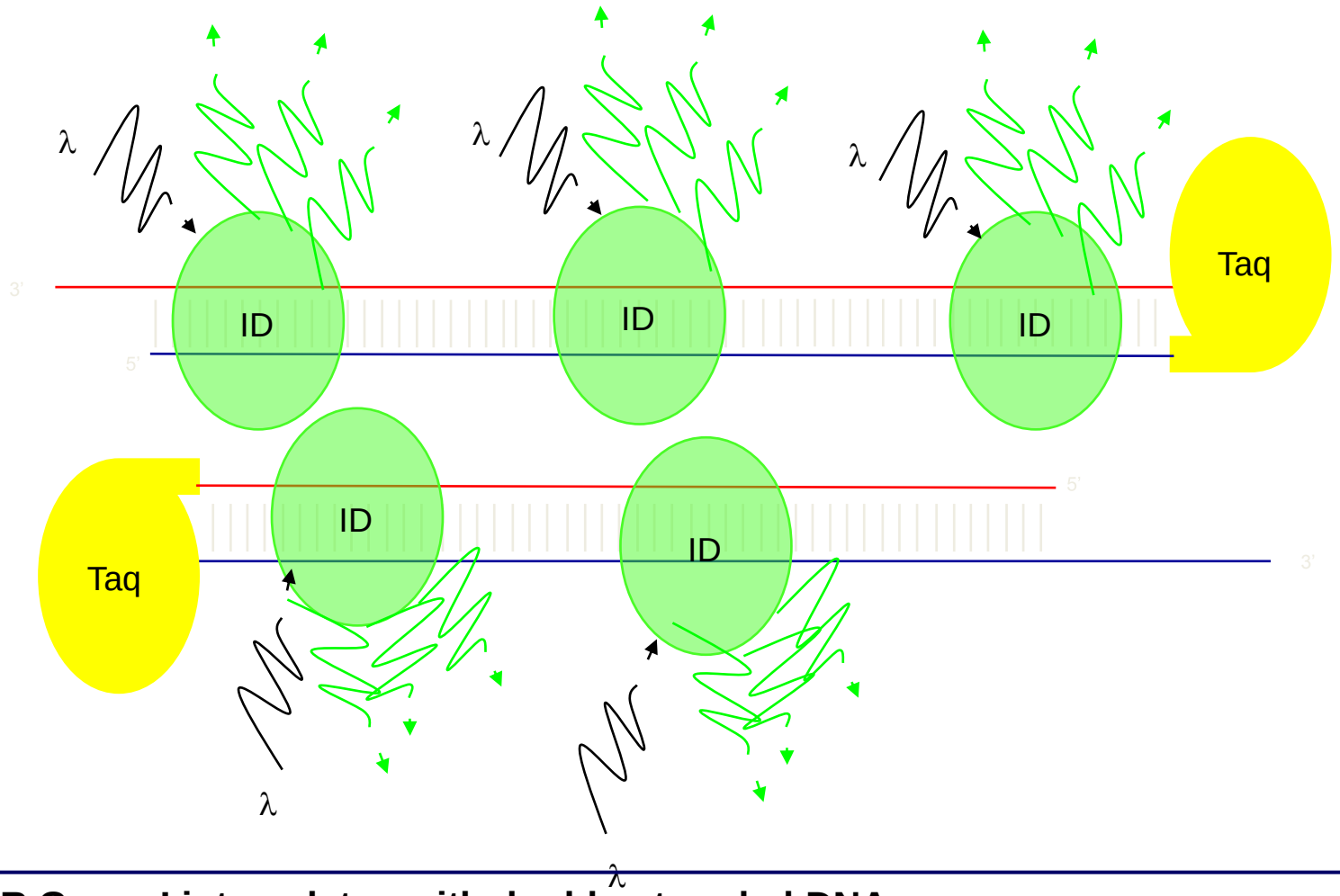


- **In each cooling cycle, prior to the extension phase, the TaqMan probe hybridizes to its complementary sequence in the PCR amplicon.**
- **When Taq polymerase encounters the TaqMan probe during polymerization, the 5' to 3' exonuclease activity of the enzyme leads to digestion of the probe.**
- **This separates the fluorescence donor (green) from the acceptor (red) and excitation at 495 nm now leads to fluorescence emission by donor at 525 nm.**
- **No energy transfer from donor to acceptor can now take place because the two fluorescent dyes are too far apart. Therefore, the acceptor dye does not fluoresce when the TaqMan probe has been digested.**
- **Overall, an increase in fluorescence emission at 525 nm and a decrease at 495 nm is indicative of a positive PCR reaction, and, importantly, proves the presence of the correct amplicon.**

TaqMan: Cleavage-based Assay

- **Advantages**
  - ✓**Target-specific fluorescence**
  - ✓**Multiplexing**
- **Disadvantages**
  - ✓**High initial cost**
  - ✓**Assay design not trivial**

SYBR Green I: Intercalation Dye



- **SYBR Green I intercalates with double-stranded DNA.**
- **In the bound state, SYBR Green I exhibits 1000-fold more fluorescence than the unbound state.**
- **The fluorescence signal increases in proportion with the increase in amplified DNA.**

SYBR Green I: Intercalation Dye

- **Advantages**
  - ✓**Experiment requires primers only**
- **Disadvantages**
  - ✓**Potential contribution to fluorescence from non-specific products (primer-dimers)**
  - ✓**No multiplexing**

Real-time quantitative PCR uses a thermal cycler/detection system.

The Bio-Rad CFX96 Real-Time PCR Detection System is equipped with a sensitive camera that monitors the fluorescence in each well of a 96-well plate at frequent intervals during the PCR reaction.

Real-time PCR Data Markup Language (RDML)

- The RDML is a structured and universal data standard for exchanging qPCR data.
- The data standard should contain sufficient information to understand the experimental setup, re-analyze the data and interpret the results.
- The data standard is a compressed text file in Extensible Markup Language (XML) and enables transparent exchange of annotated qPCR data between instrument software and third-party data analysis packages, between colleagues and collaborators, and between authors, peer reviewers, journals and readers.
- To support the public acceptance of this standard, both an on-line RDML file generator is available for end users, as well as RDML software libraries to be used by software developers, enabling import and export of RDML data files.

MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

- **The RDML file format is a recommended element in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Busten et al., *Clinical Chemistry*, 2009). *See MIQE Guidelines in Supplemental Readings.**
- **The aim of MIQE, coordinated by a group of research-active scientists and coordinated under the umbrella of MIBBI (Minimum Information for Biological and Biomedical Investigations) is to provide authors, reviewers and editors specifications for the minimum information that must be reported for a qPCR experiment in order to ensure its relevance, accuracy, correct interpretation and repeatability.**
- **A checklist, which should be submitted along with the paper, is available for authors in preparing a manuscript employing qPCR. *See MIQE checklist in Supplemental Readings.**