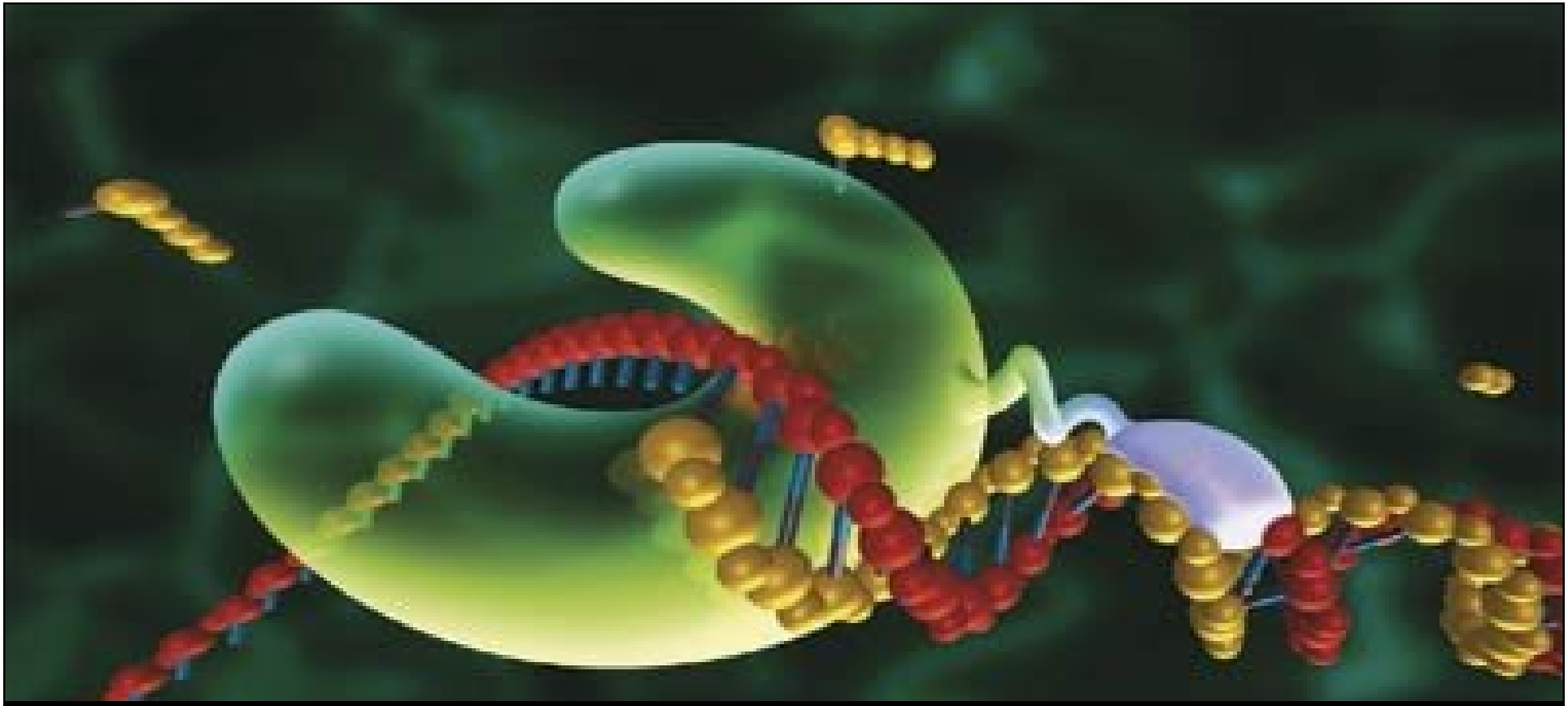
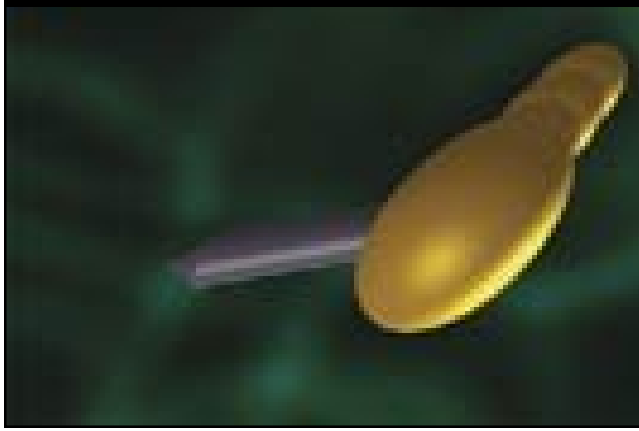


# Fundamentals of Real Time PCR




**Mohamed Abdel Fattah**

*Senior Technical Specialist*

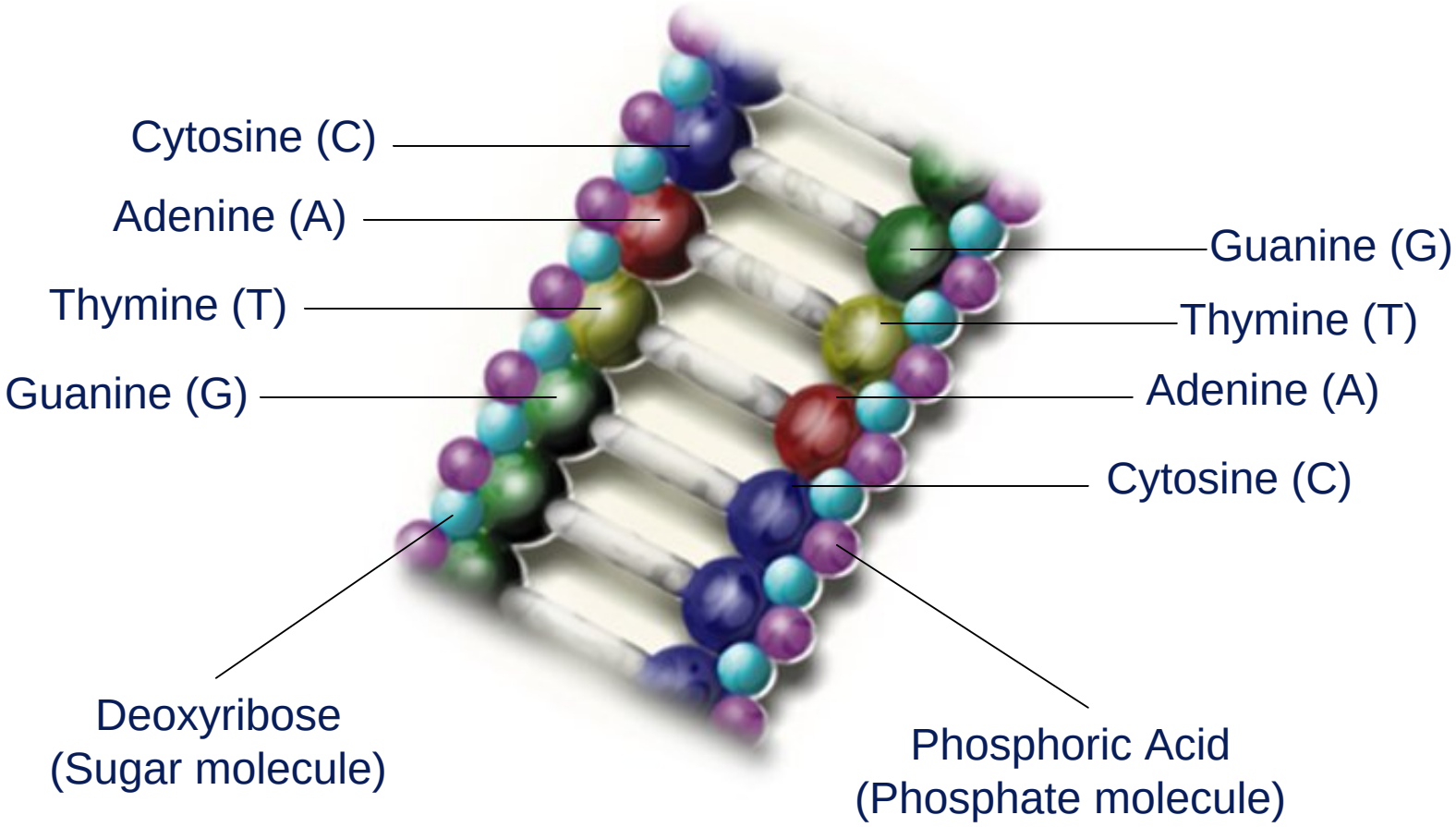
*Scientific Support - Molecular Biology Dept.*

*AnalysisAB Co.*

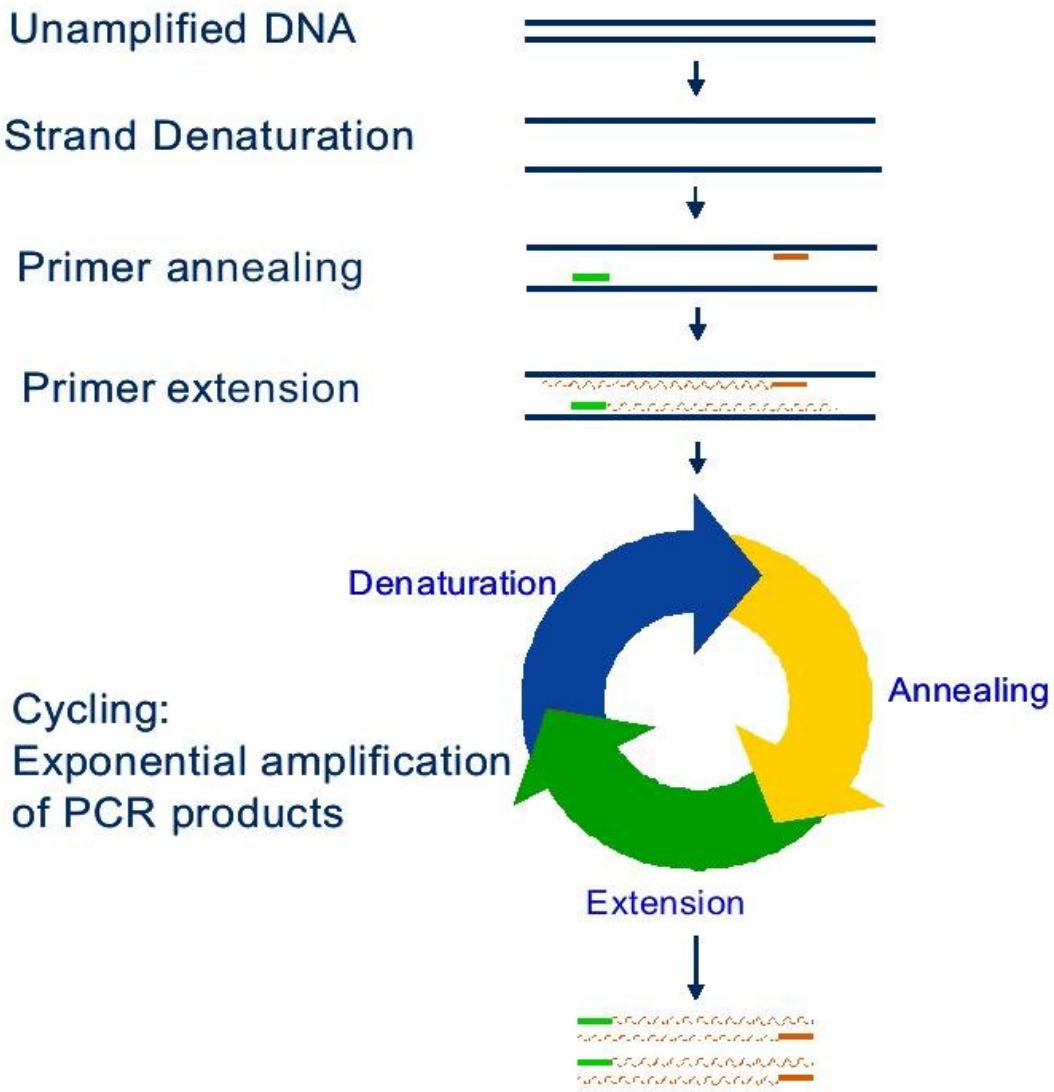
Mobile: 012 27 906 74

E-mail: analysis@analysis-ab.com

# What is that DNA ?

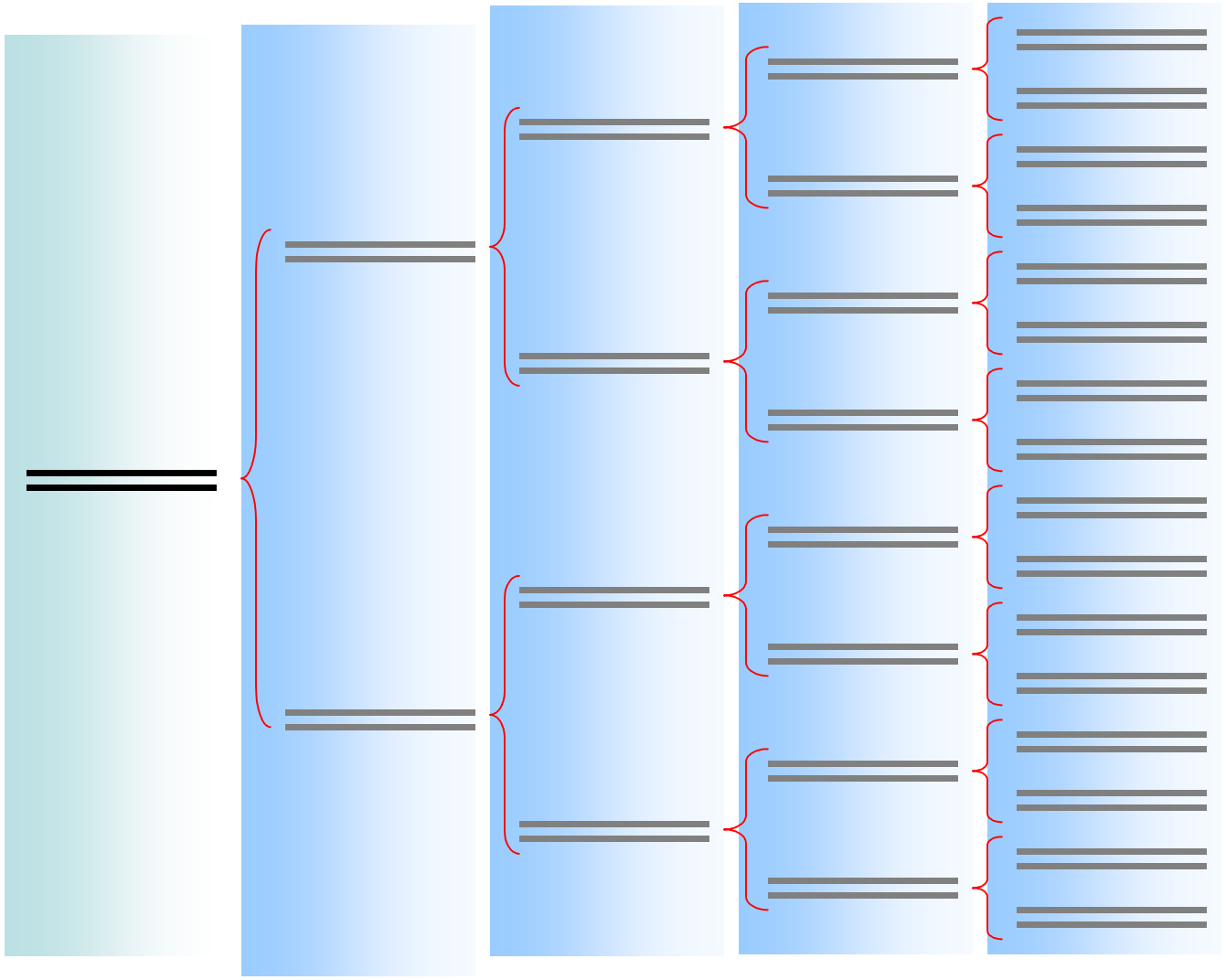


# Polymerase Chain Reaction (PCR)




Cycling:
Exponential amplification
of PCR products

# PCR As Exponential Amplification



**25** cycles

$2^{25}$ copies

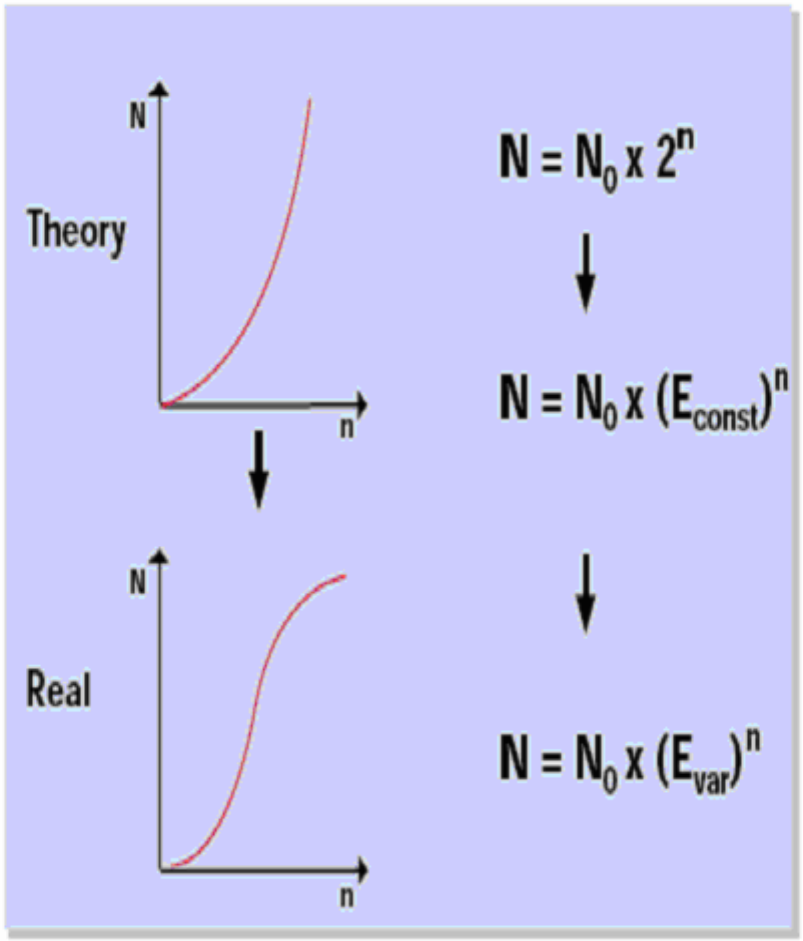
*(33,554,432)*

# PCR In Theoretical Aspect

$$N = N_0 \times 2^n$$

| | |
|---|---|
| N: | number of amplified molecules |
| $N_{0:}$ | initial number of molecules |
| n: | number of amplification cycles |

# PCR In Graphical Aspect




**N:** number of amplified molecules; $N_0$: initial number of molecules; **n:** number of amplification cycles; **E:** amplification efficiency

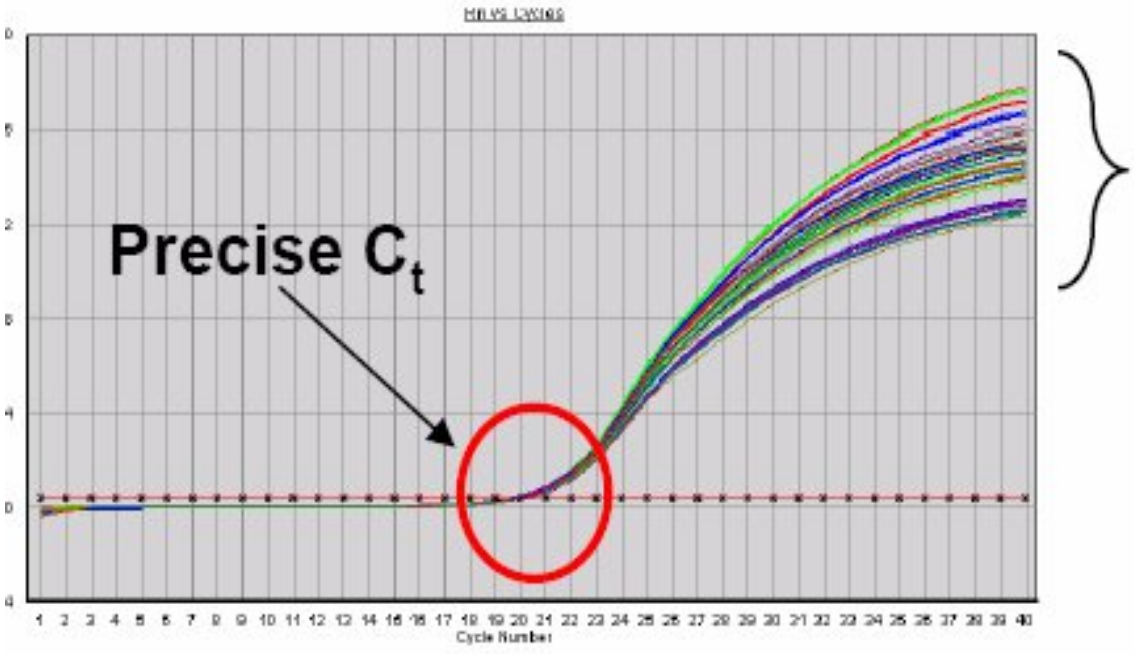
# Limitations of PCR Efficiency, *Plateau Effect*

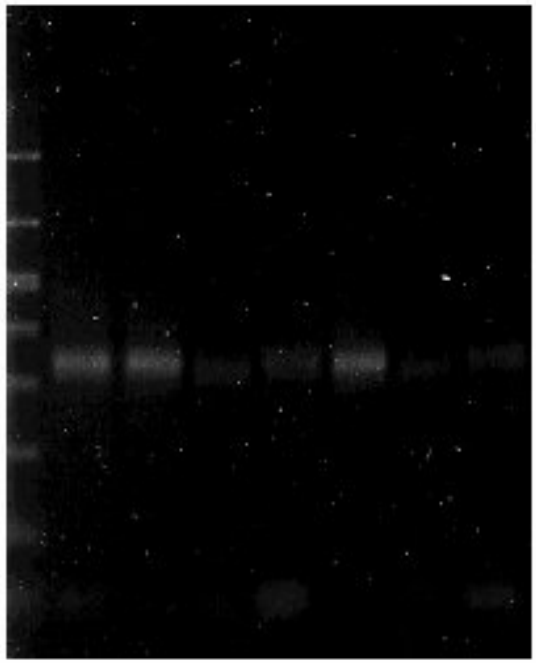
The Plateau Effect is caused by:

- Enzyme instability.
- Reagents consumption.
- Product inhibition. e.g. pyrophosphate accumulation.
- Competition of re-annealing amplification product with primers.

# Limitations of PCR Efficiency

Variable PCR Plateau

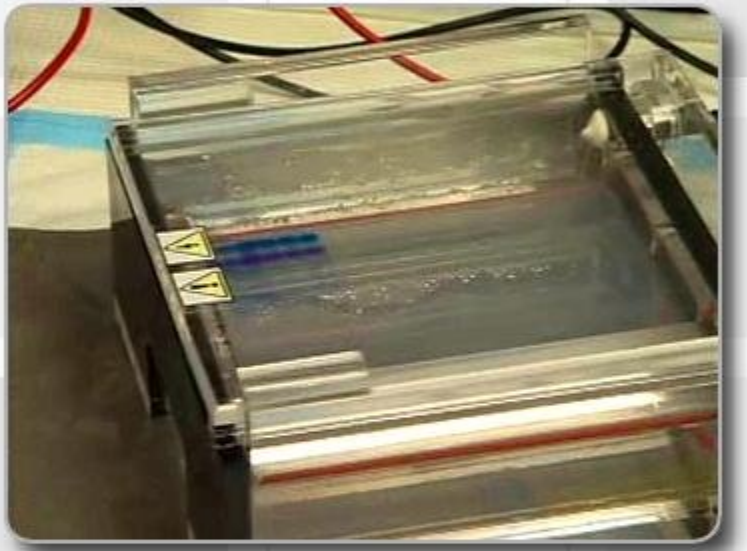


Amplification Plot of 96 Sample Replicates

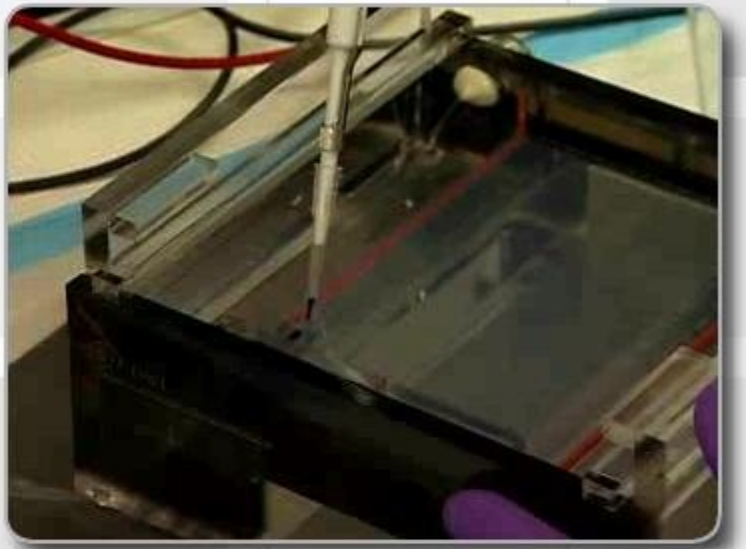
Real-Time PCR System

time
consuming



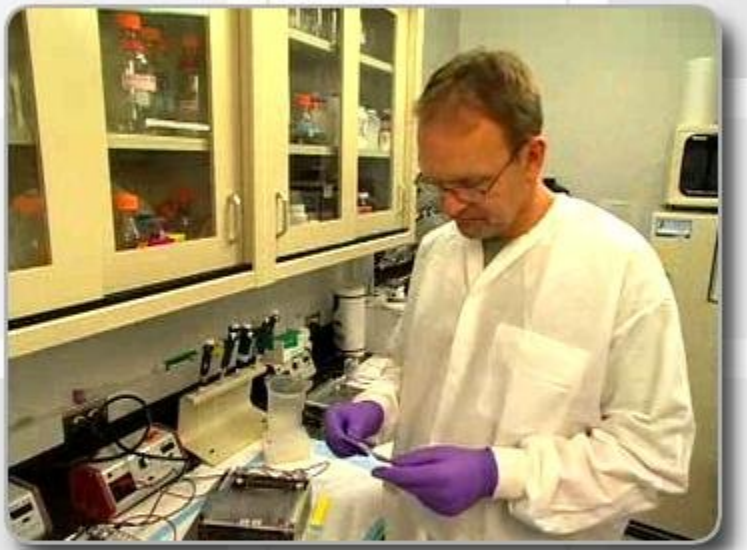
messy

Real-Time PCR System




waiting

Real-Time PCR System



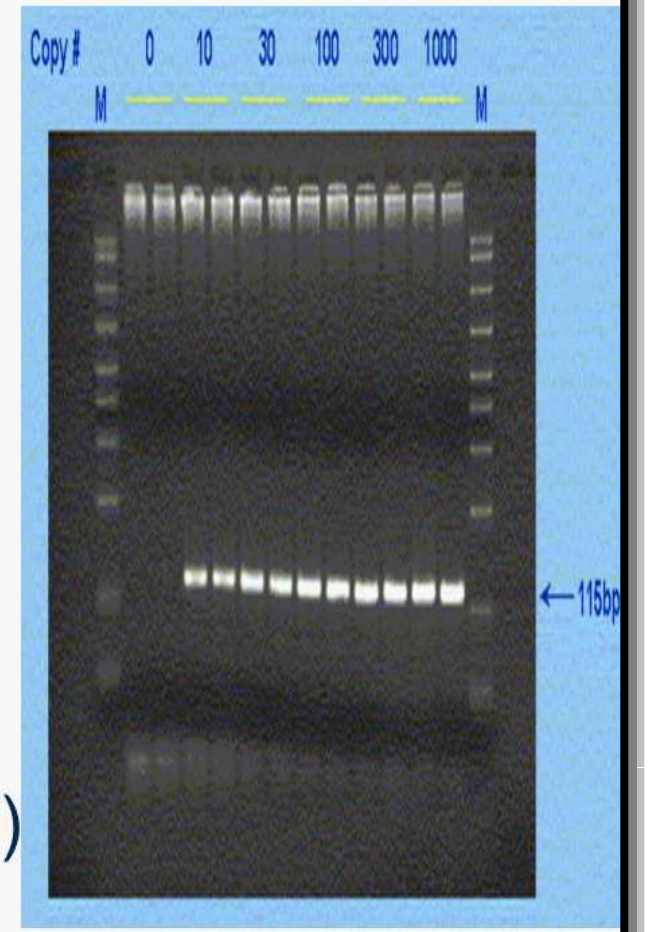
result A

result B

result C

# Limitations of Traditional End-Point PCR

- Low sensitivity
- Poor precision
- Results are not expressed as numbers
- Ethidium bromide staining is not quantitative
- Post-PCR processing required
- Narrow dynamic range (<2 logs)



# From ***PCR*** to ***Real-Time PCR***

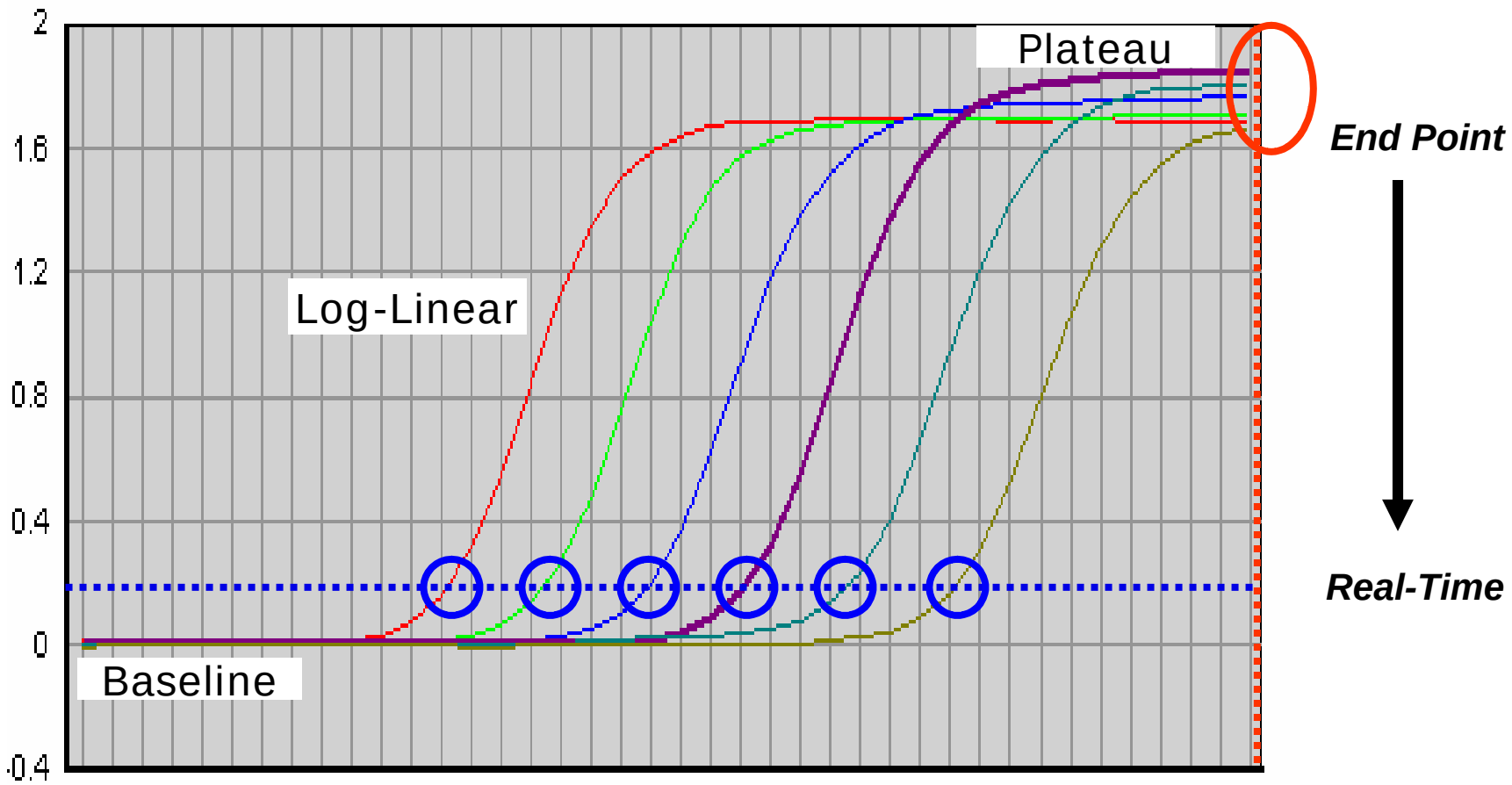
Real-Time PCR turns the question

*HOW MUCH?*

Into the question

*WHEN?*

# From *PCR* to *Real-Time PCR*



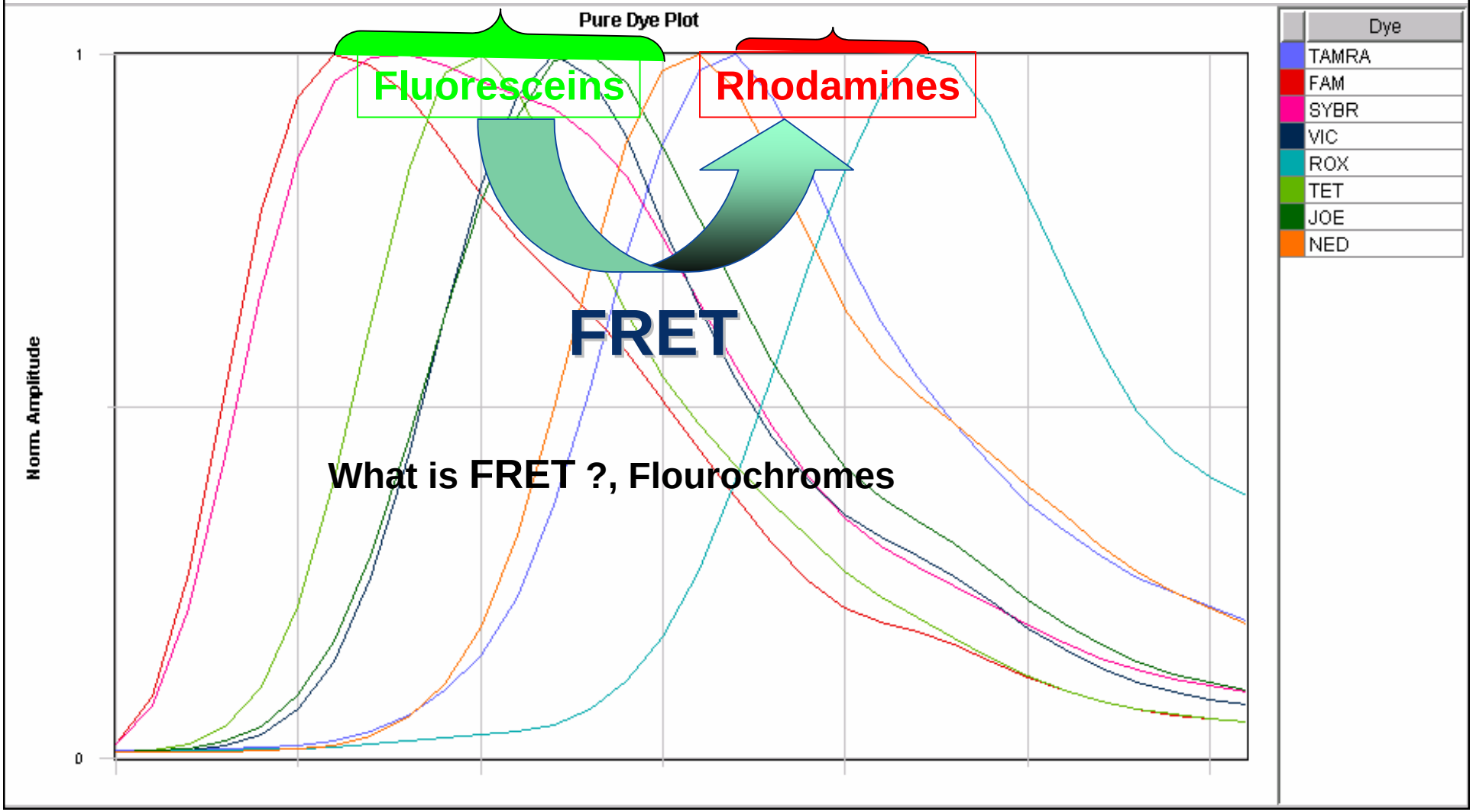
# Towards ***Real-Time PCR***

**1st Point**

Detection of Specific PCR Product by utilizing the 5’ to 3’ Exonuclease Activity of Thermus Aquaticus DNA Polymerase

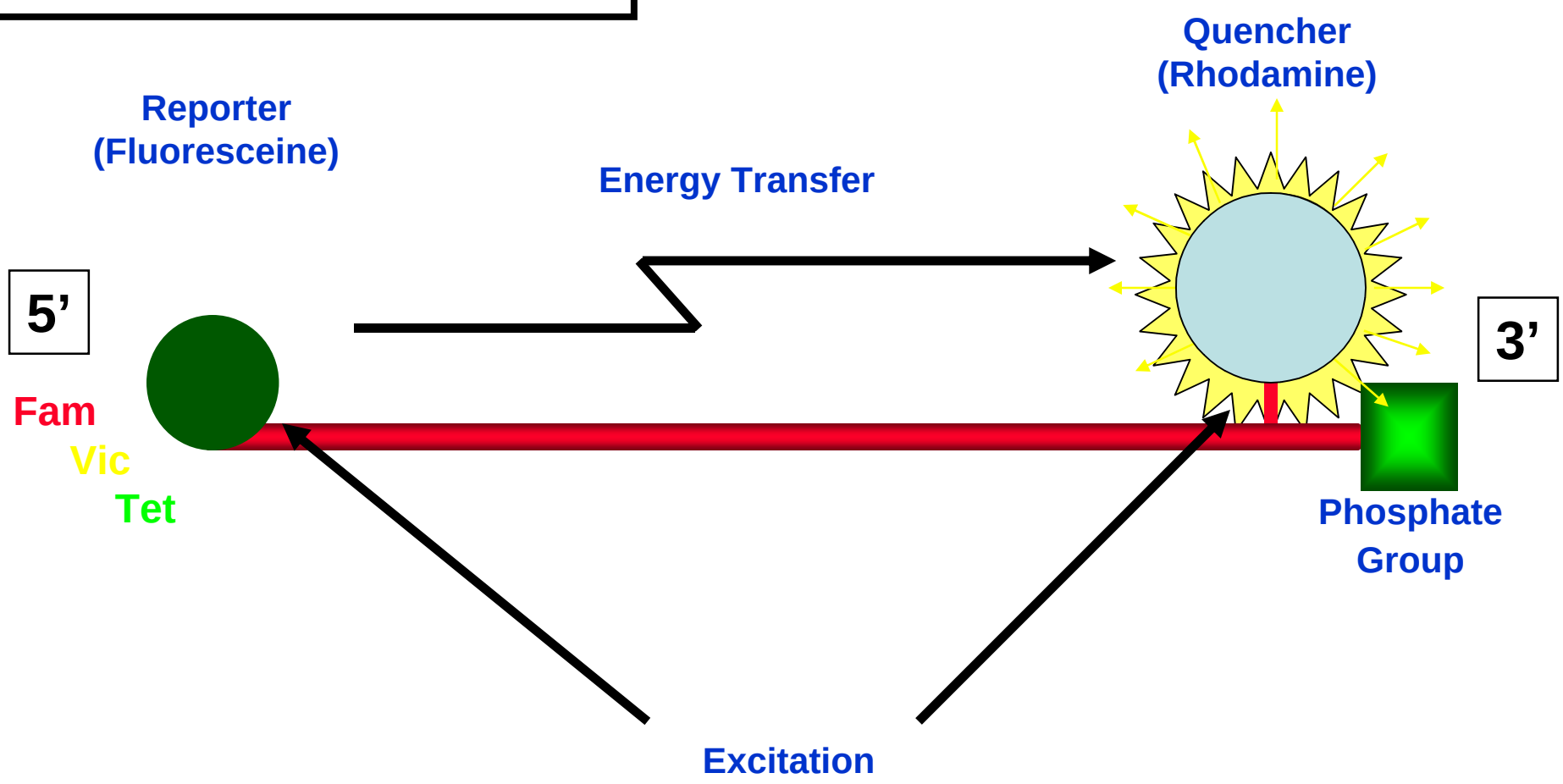
# Towards ***Real-Time PCR***

**2nd Point**



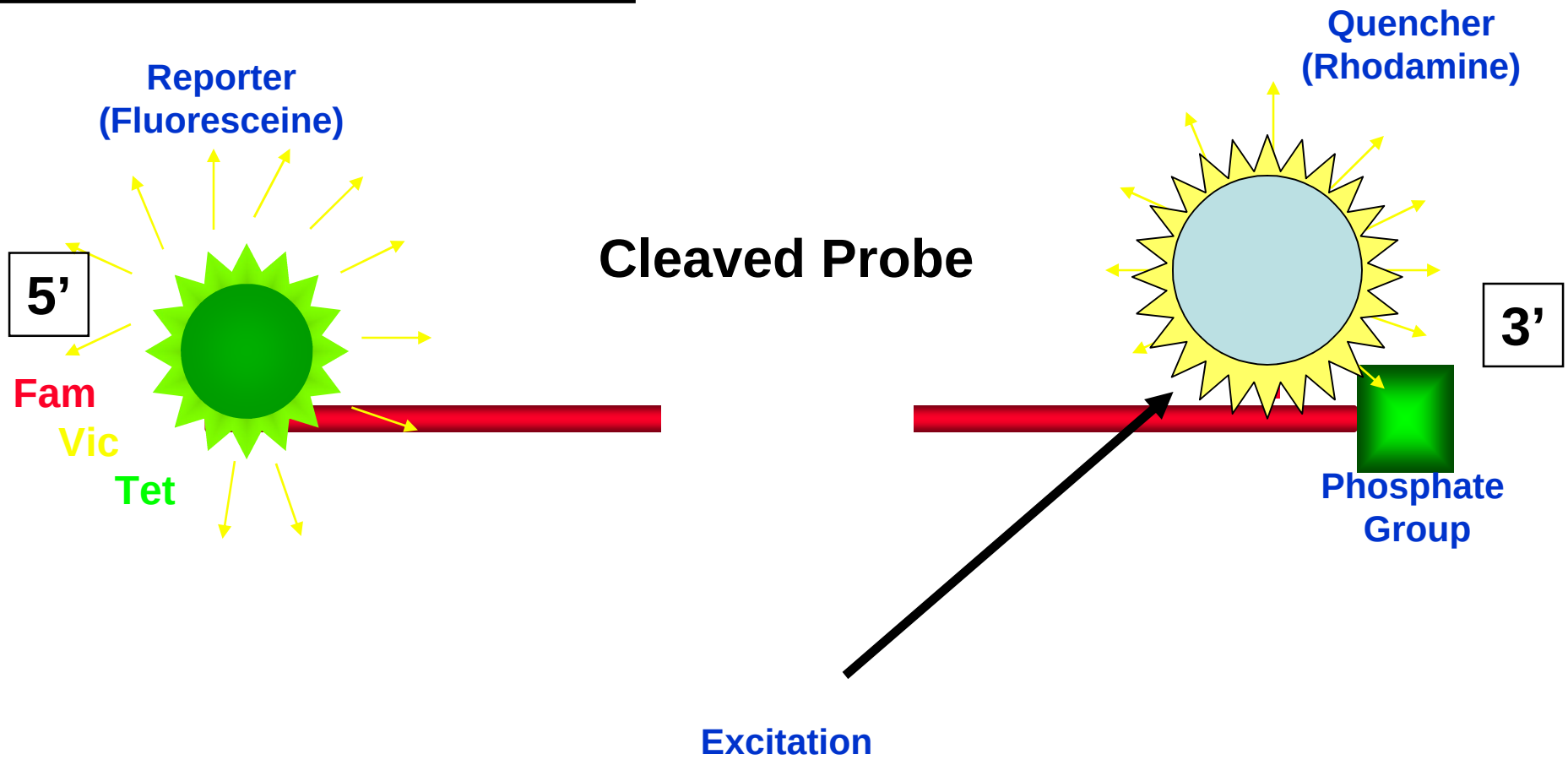
# Towards ***Real-Time PCR***

**What is FRET ?, Flourochromes**



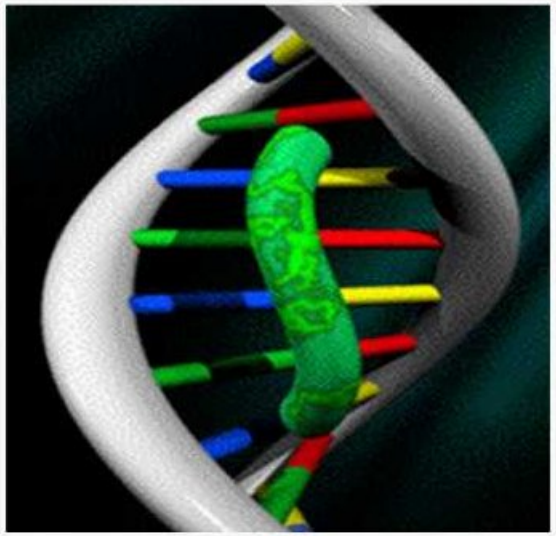
# Towards *Real-Time PCR*

**What is FRET ?, Flourochromes**



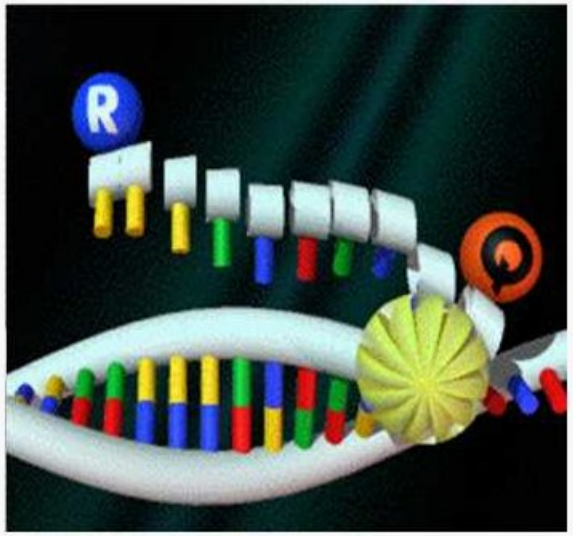
# Real-Time PCR Chemistries

## SYBR® Green I dye



Binds double stranded DNA

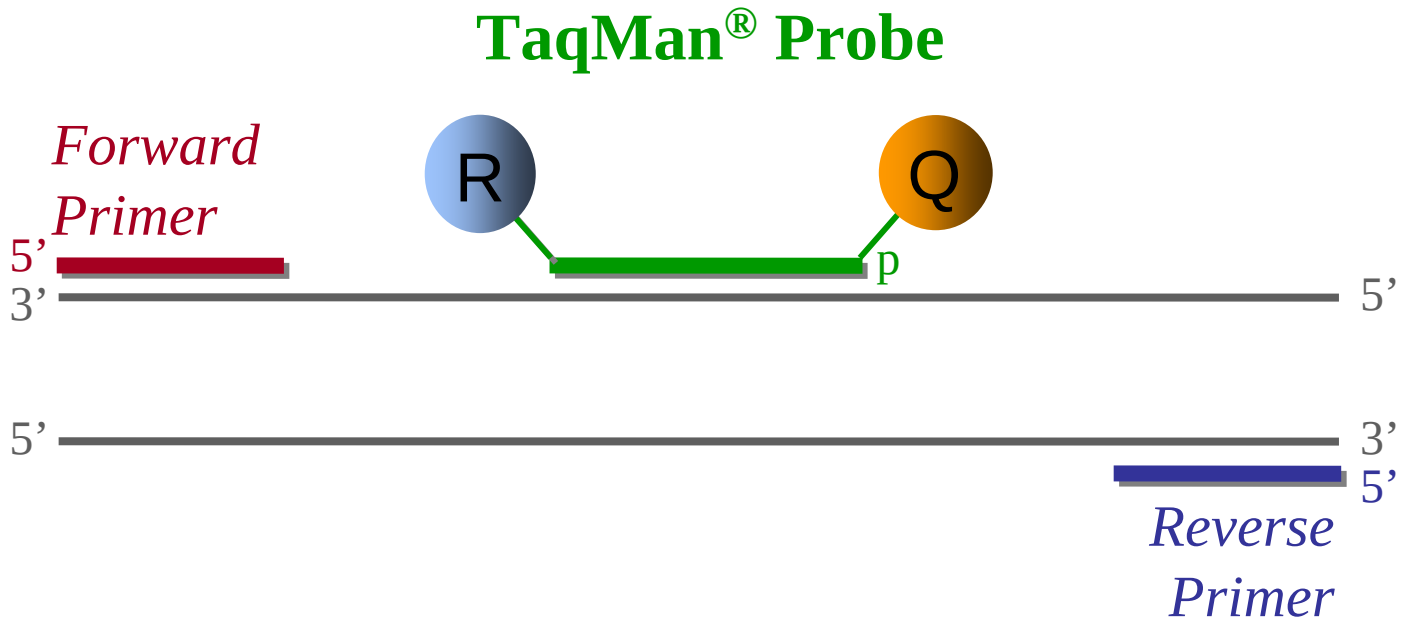
## Fluorogenic 5' Nuclease Assay




Uses a TaqMan® probe

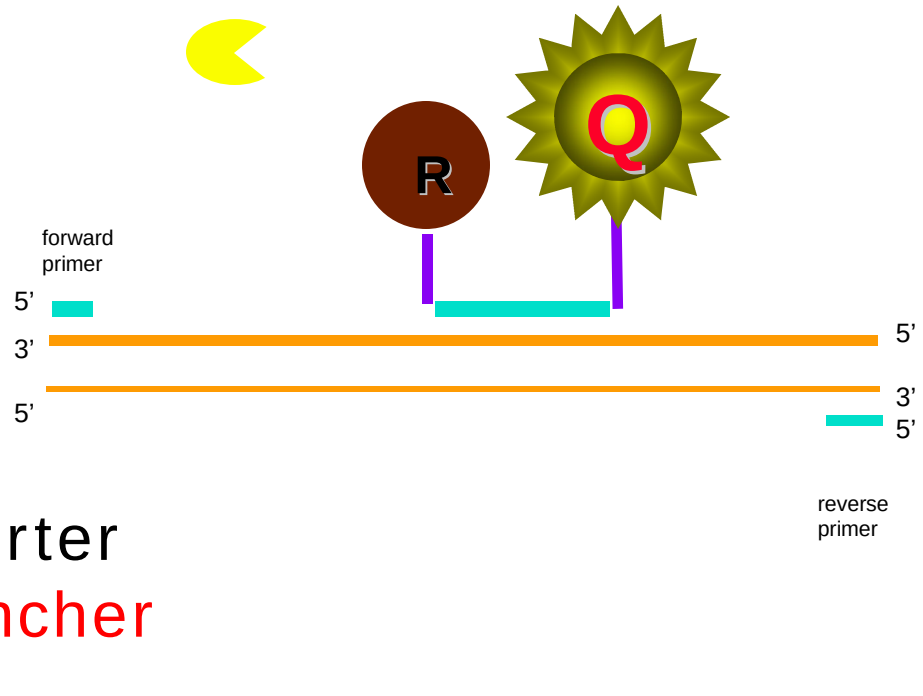
# Real-Time PCR Chemistries

## TaqMan Probe-Based Chemistery



# *TaqMan Probe-Based Chemistery*

**Denaturation** → **Annealing** → **Polymerization**

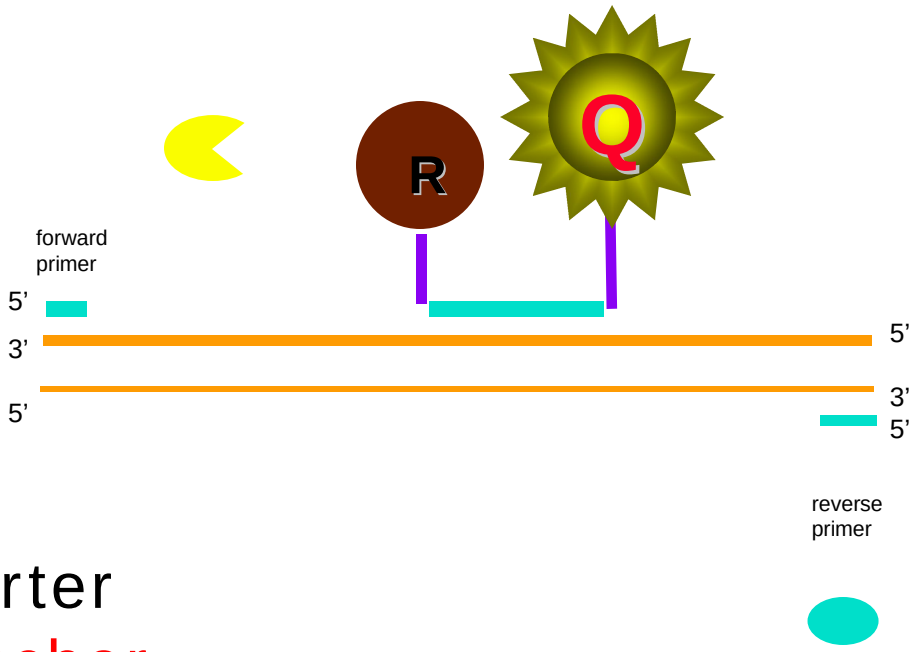



R----- Reporter

Q----- Quencher

# *TaqMan Probe-Based Chemistery*

**Denaturation** → **Annealing** → **Polymerization**




R----- Reporter

Q----- Quencher

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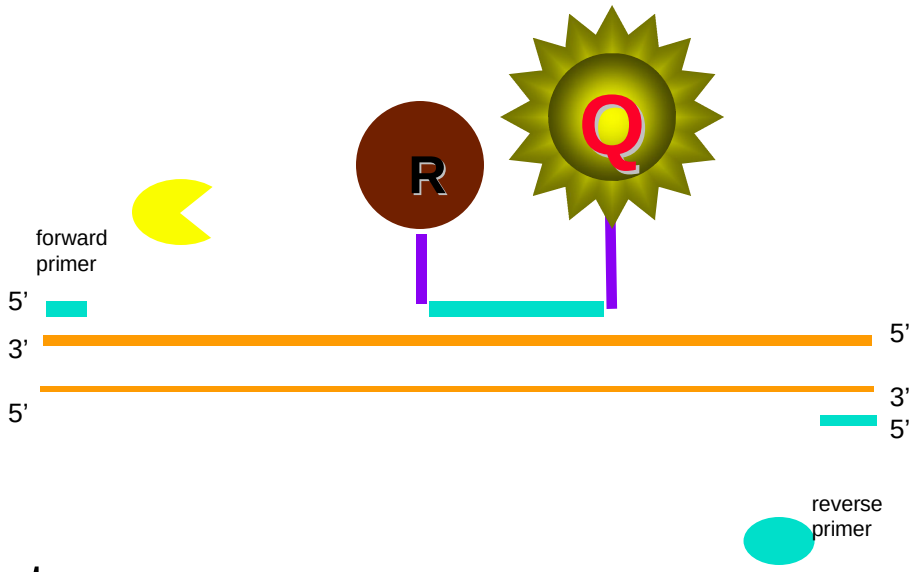


R----- Reporter

Q----- Quencher

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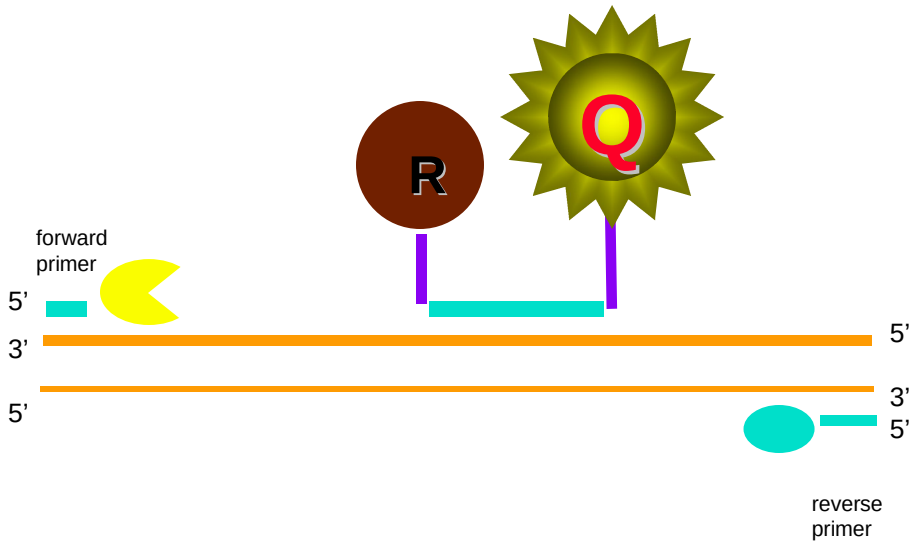
**Denaturation** → **Annealing** → **Polymerization**




R----- Reporter

Q----- Quencher

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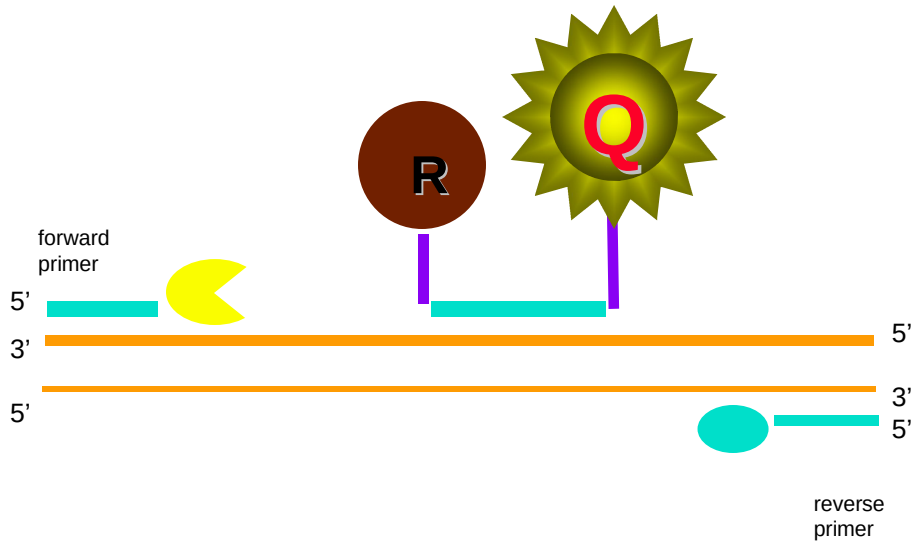
**Denaturation** → **Annealing** → **Polymerization**




R----- Reporter

Q----- Quencher

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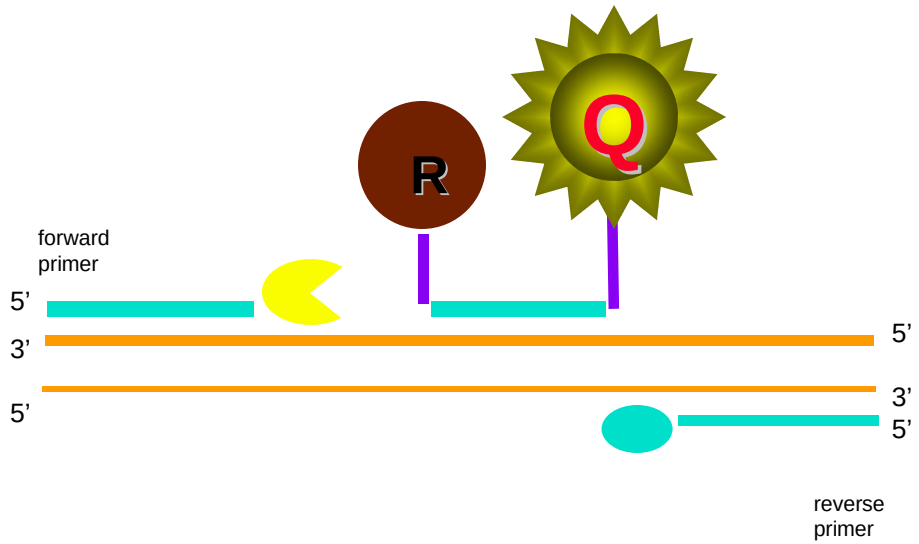
Denaturation → Annealing → **Polymerization**

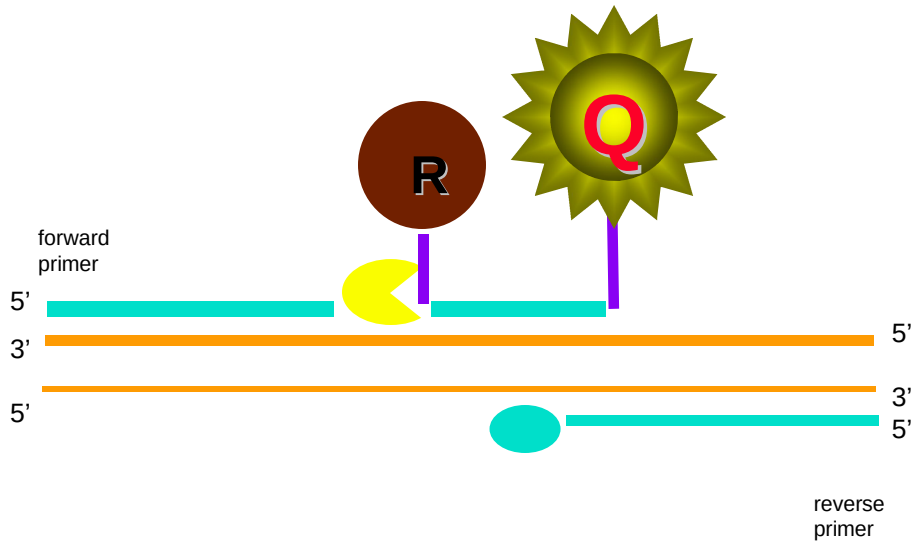



R----- Reporter

Q----- Quencher

# *TaqMan Probe-Based Chemistery*

Denaturation → Annealing → **Polymerization**

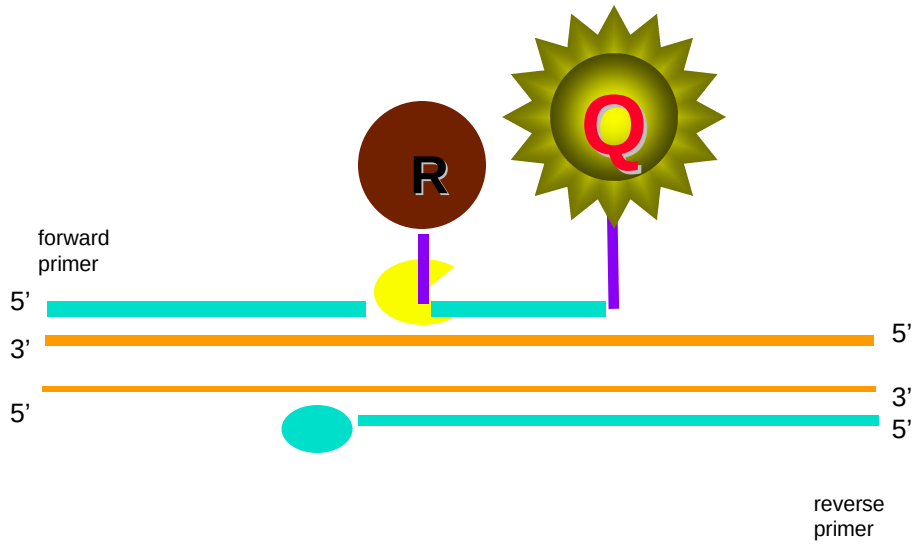



R----- Reporter

Q----- Quencher

# *TaqMan Probe-Based Chemistery*

Denaturation → Annealing → **Polymerization**



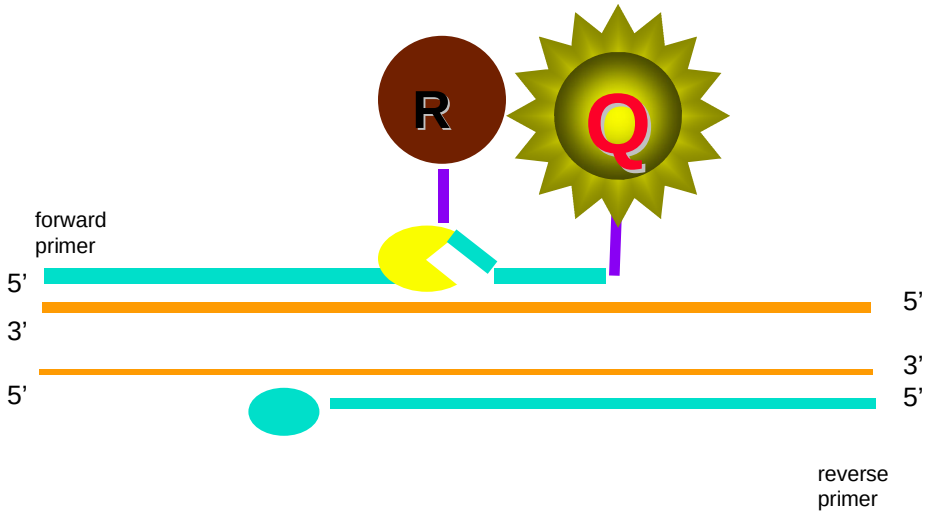

R----- Reporter

Q----- Quencher

# *TaqMan Probe-Based Chemistery*

Denaturation → Annealing → **Polymerization**

. *Strand Displacement*



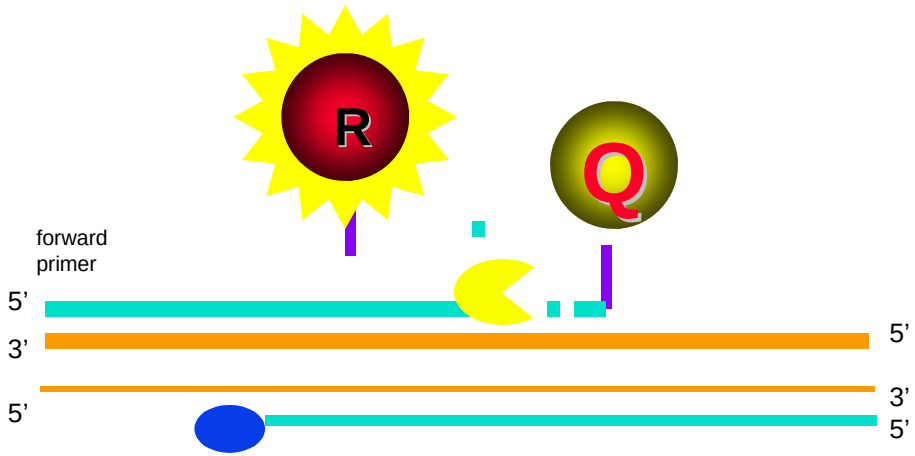

R----- Reporter

Q----- Quencher

# *TaqMan Probe-Based Chemistery*

Denaturation → Annealing → **Polymerization**

. *Cleavage*



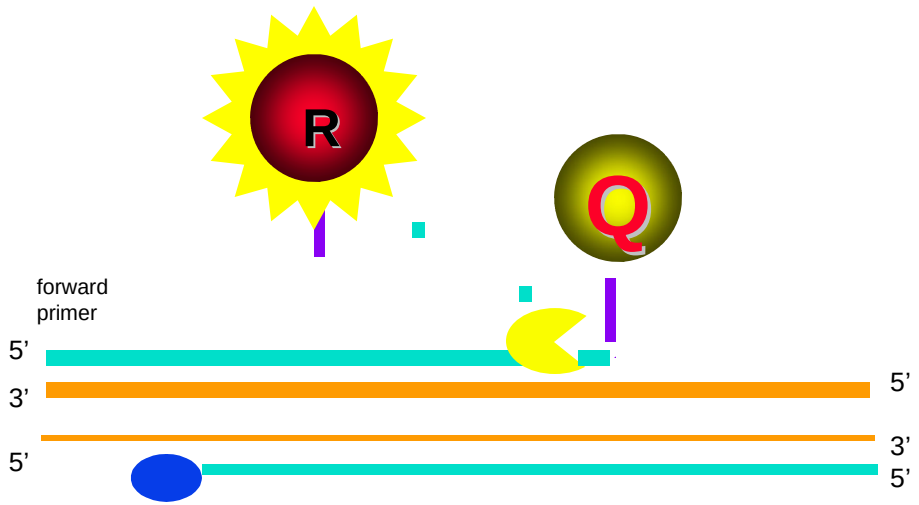

R----- Reporter

Q----- Quencher

# *TaqMan Probe-Based Chemistery*

Denaturation → Annealing → **Polymerization**

. *Cleavage*




R----- Reporter

Q----- Quencher

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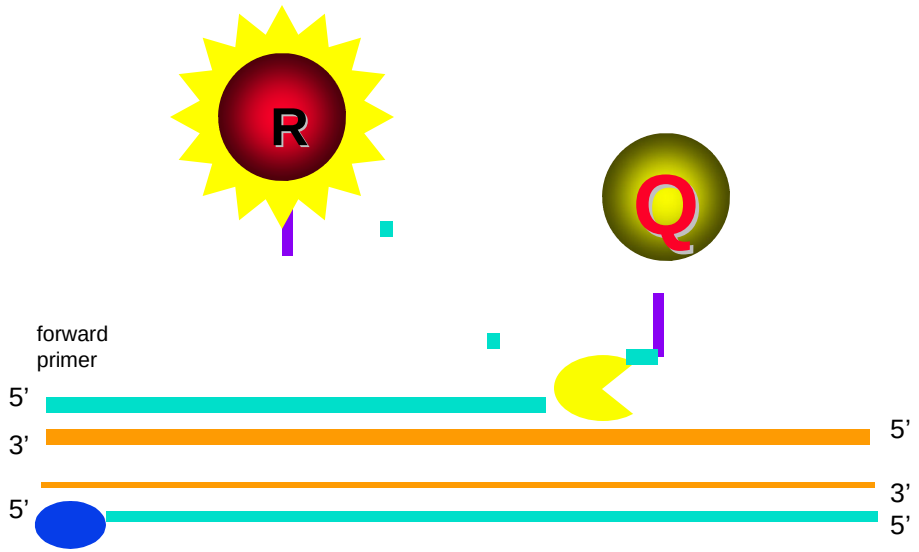
Denaturation → Annealing → **Polymerization**

. *Cleavage*



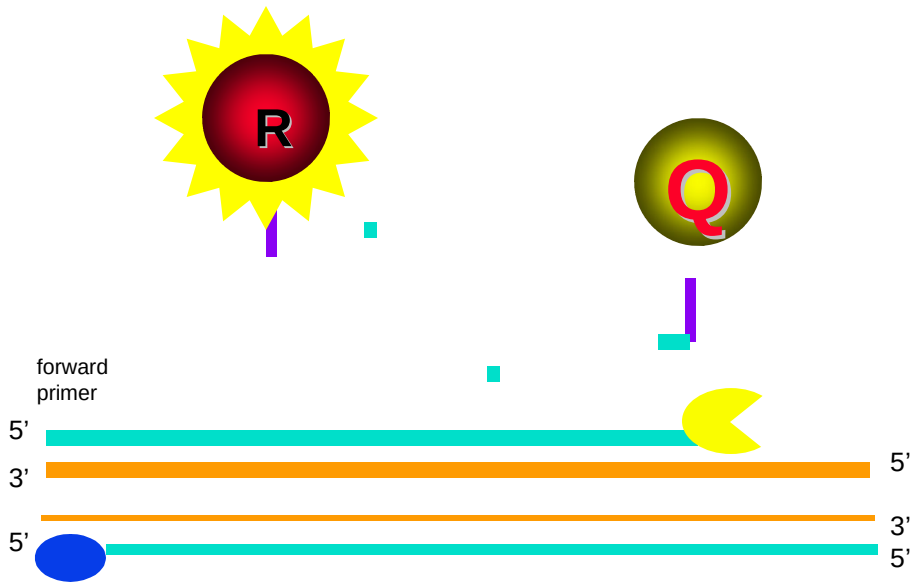

R----- Reporter

Q----- Quencher

# *TaqMan Probe-Based Chemistery*

Denaturation → Annealing → **Polymerization**

. *Cleavage*



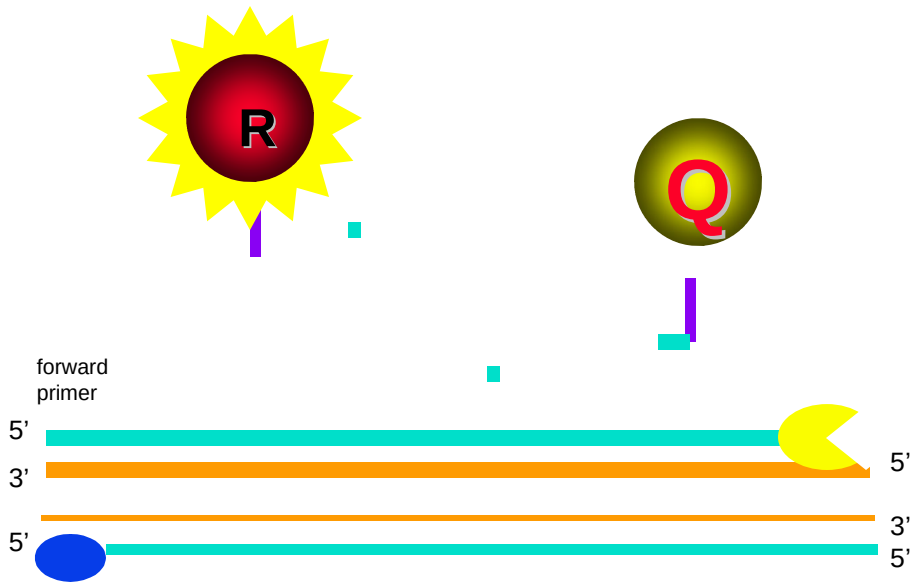

R----- Reporter

Q----- Quencher

# *TaqMan Probe-Based Chemistery*

Denaturation → Annealing → **Polymerization**

. *Polymerization Completed*




R----- Reporter

Q----- Quencher

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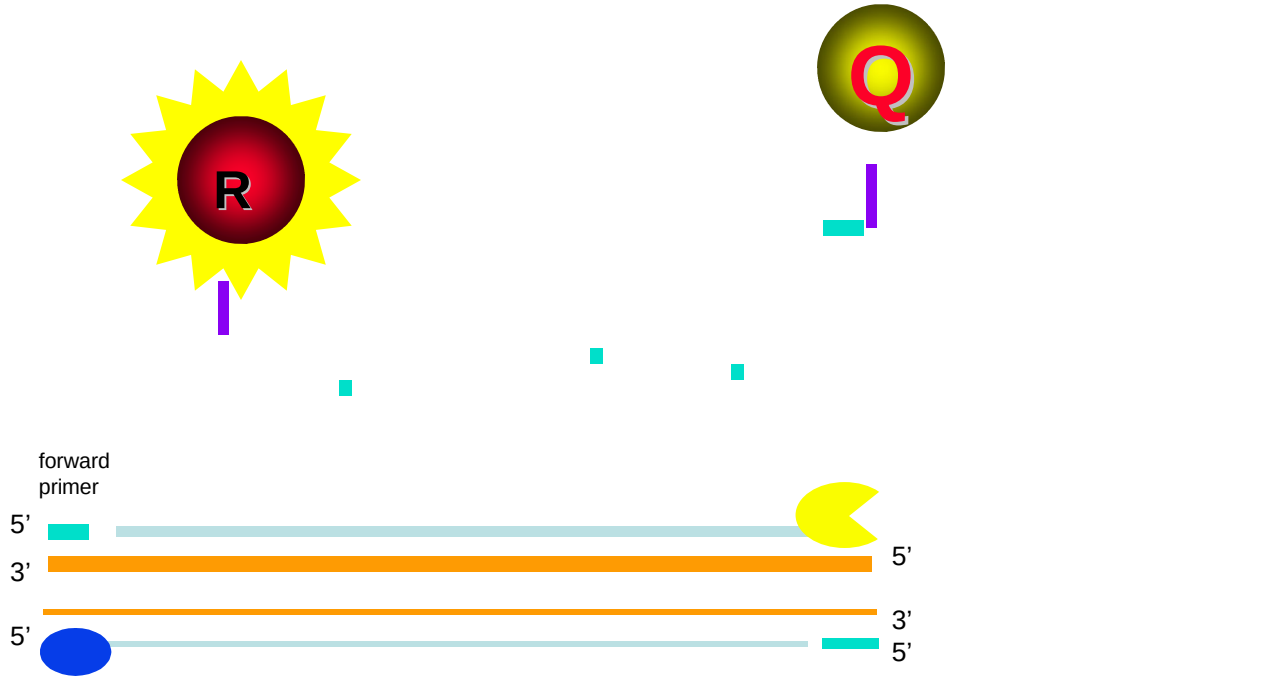
Denaturation → Annealing → **Polymerization**

. *Polymerization Completed*

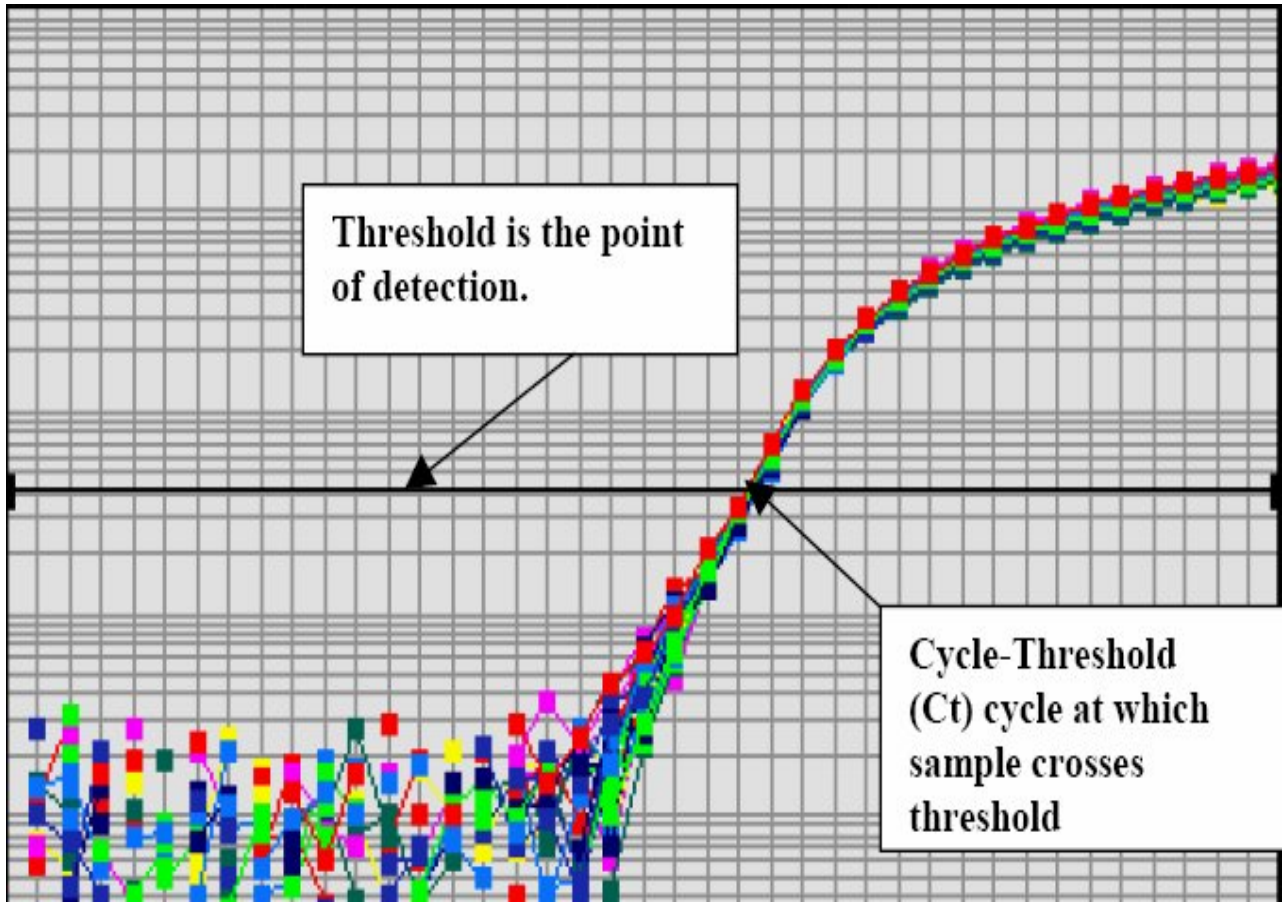



R----- Reporter

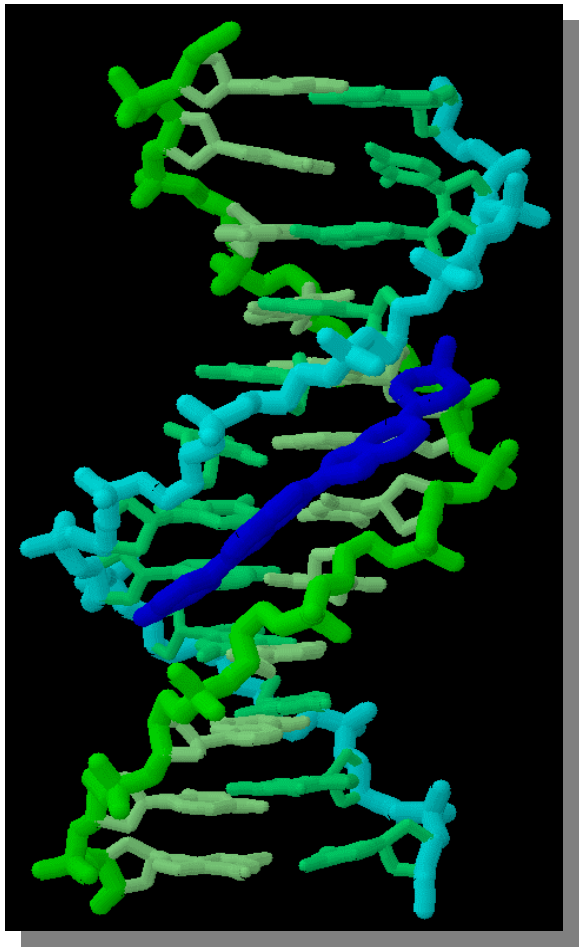
Q----- Quencher

# Real-Time PCR Principles

## *Real-Time Amplification Plots*

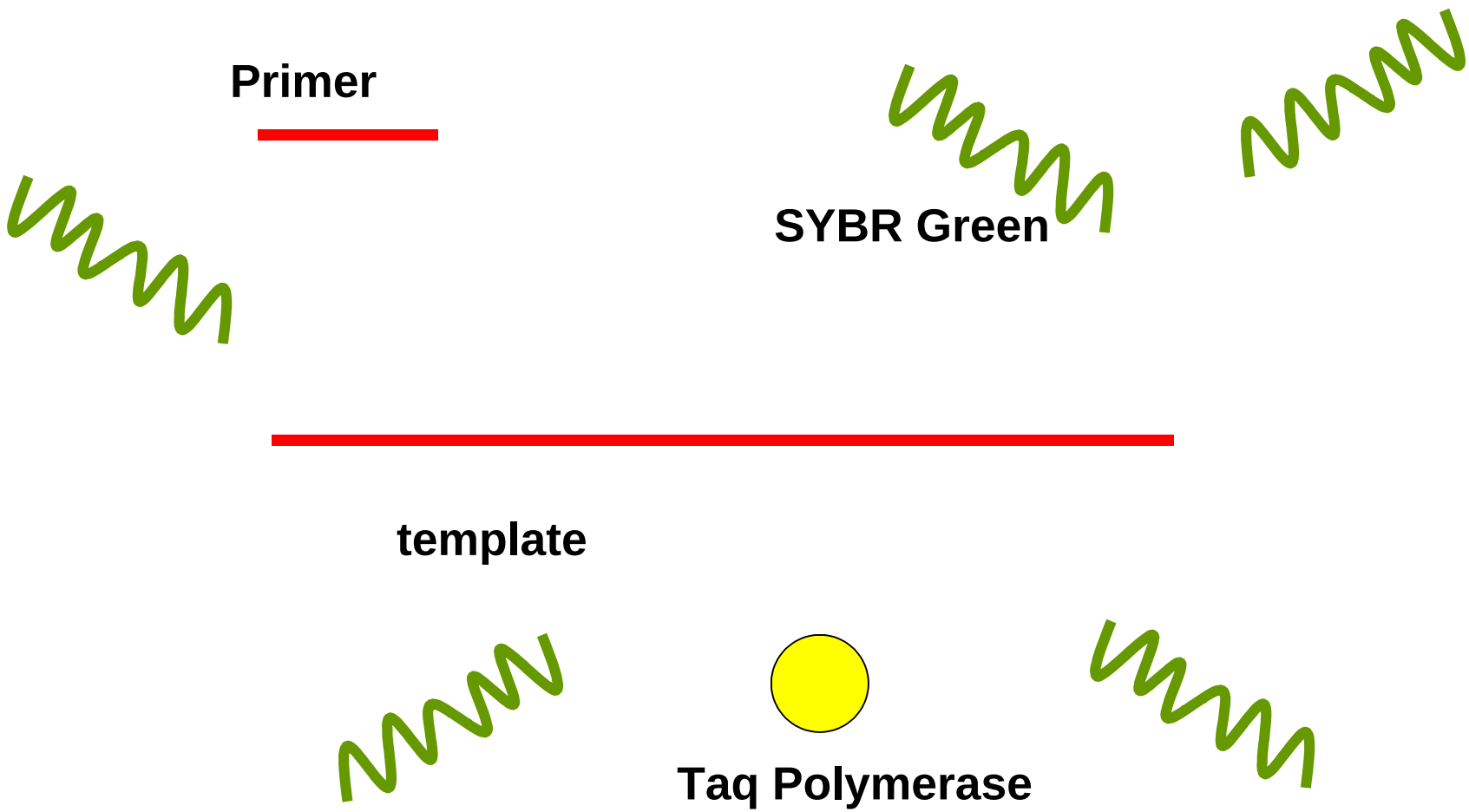


# SYBR® green chemistry

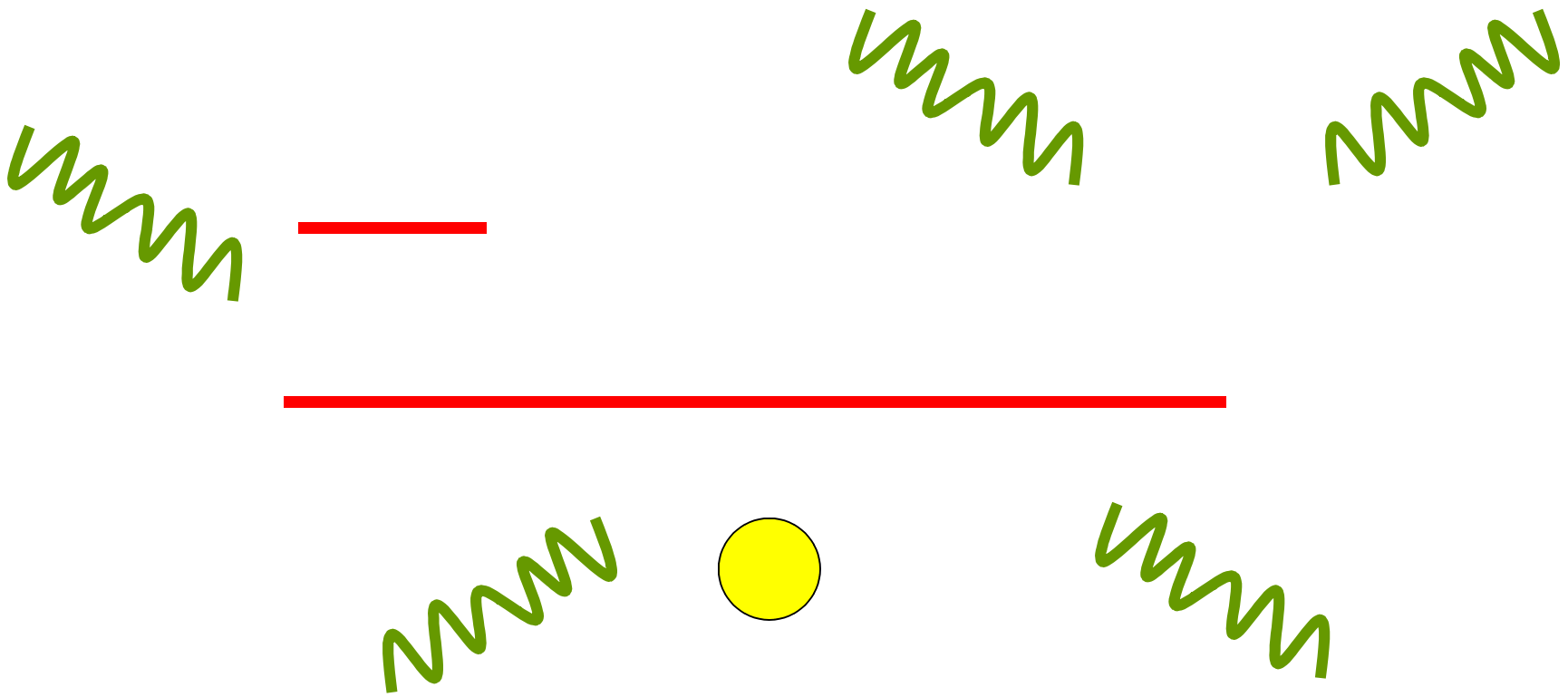


**Double stranded DNA Binding Dye**

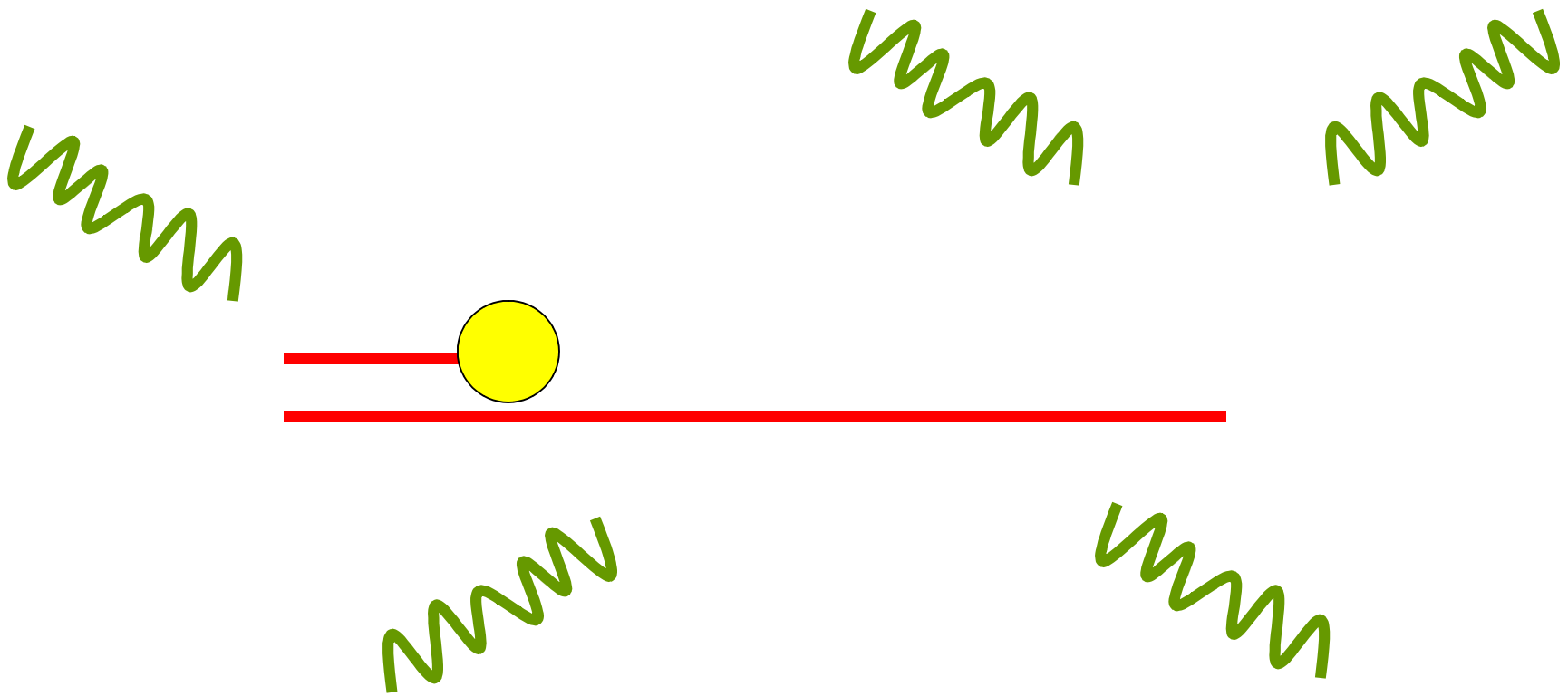
# SYBR Green detection



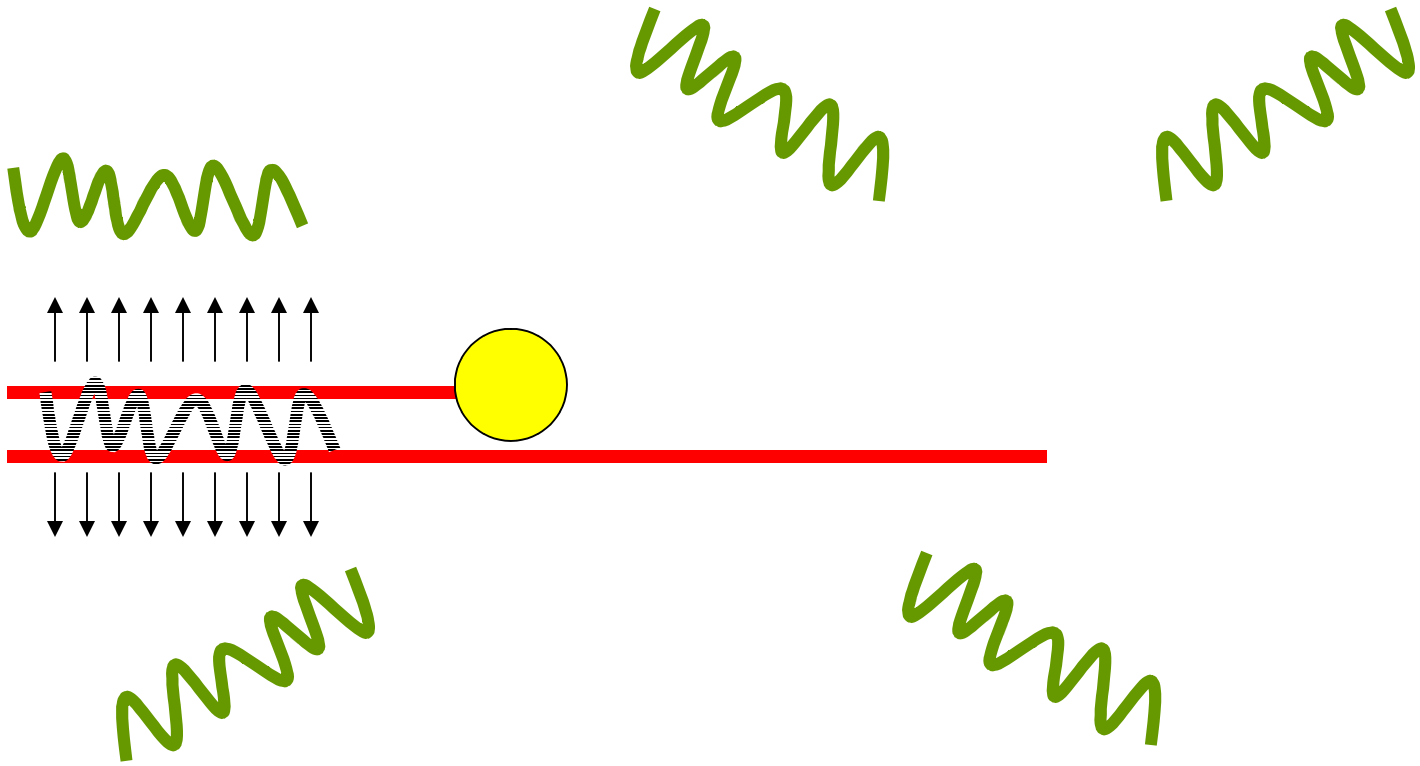
# SYBR Green detection



# SYBR Green detection



# SYBR Green detection



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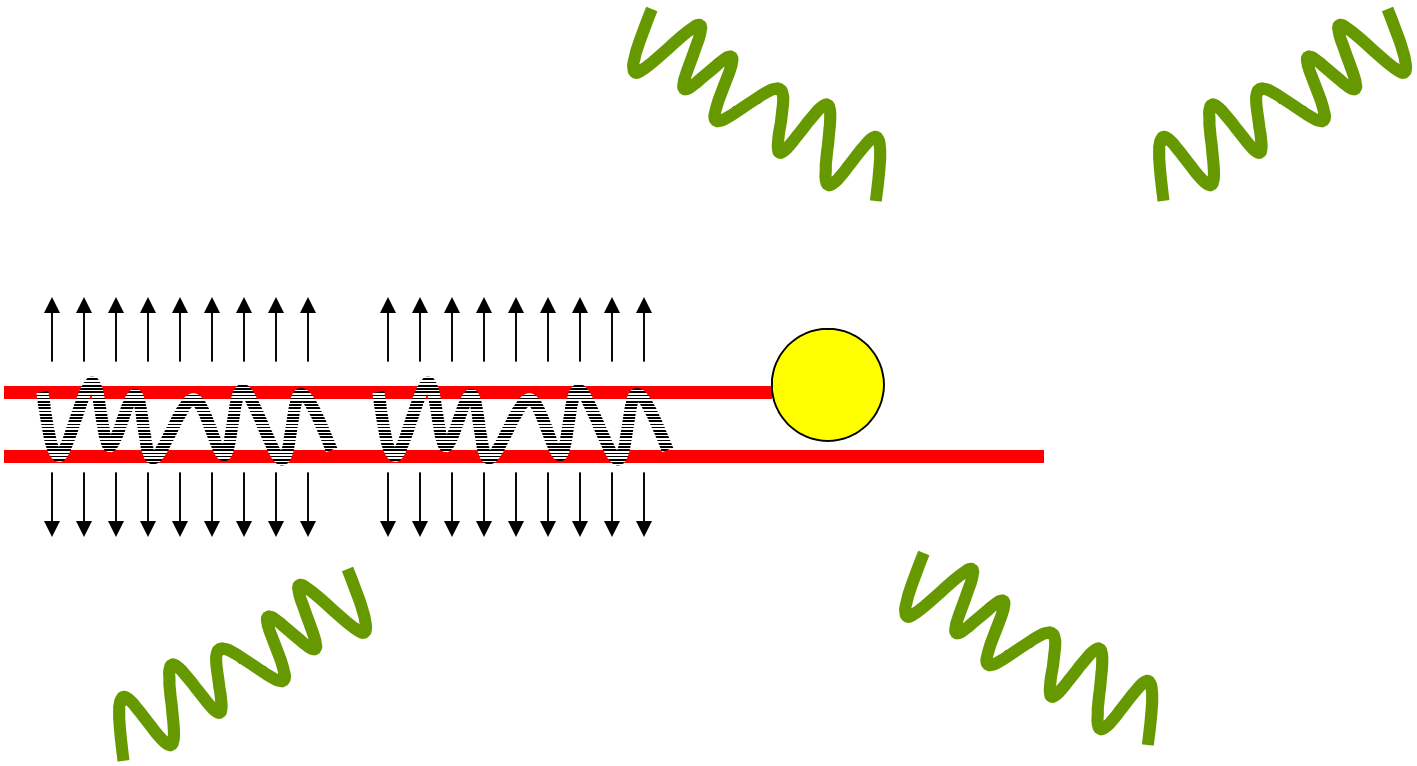

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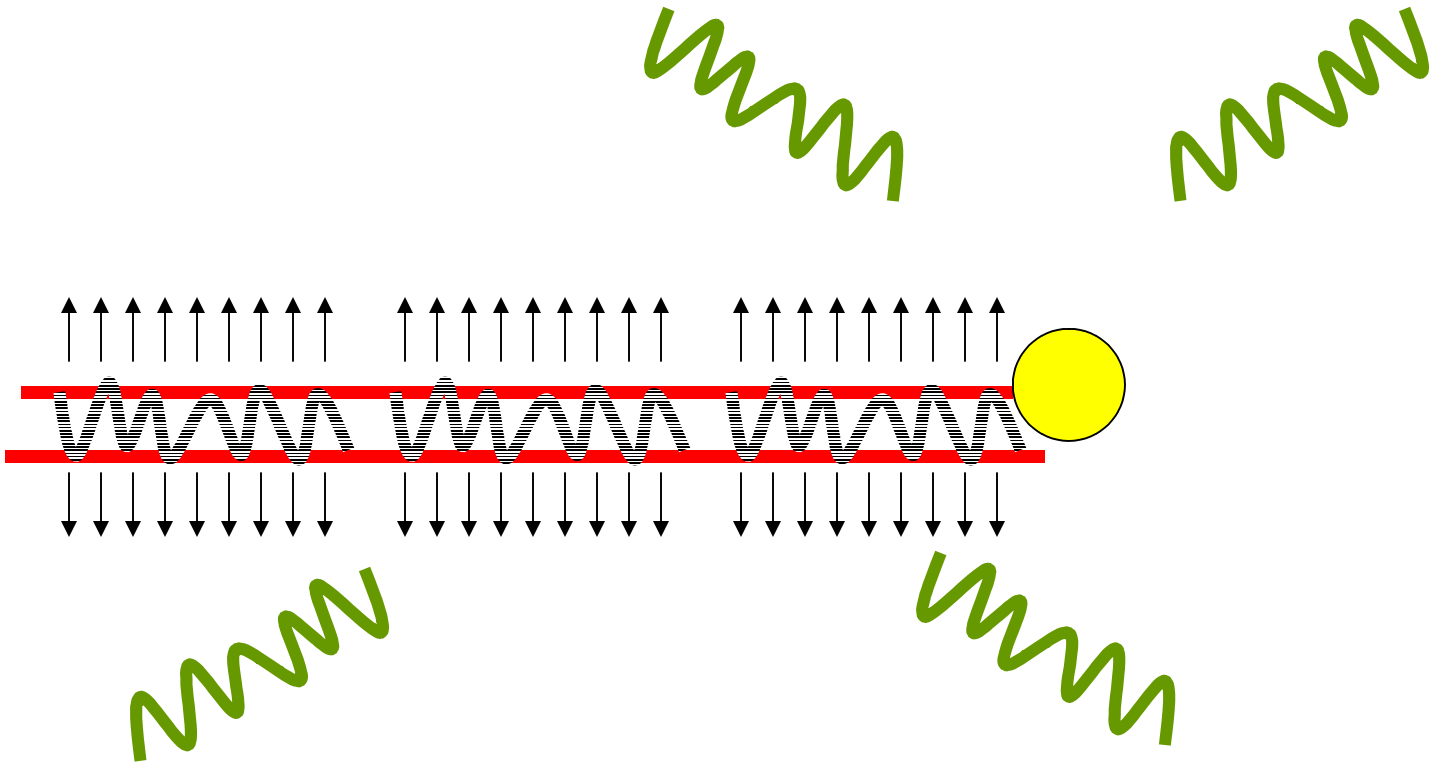
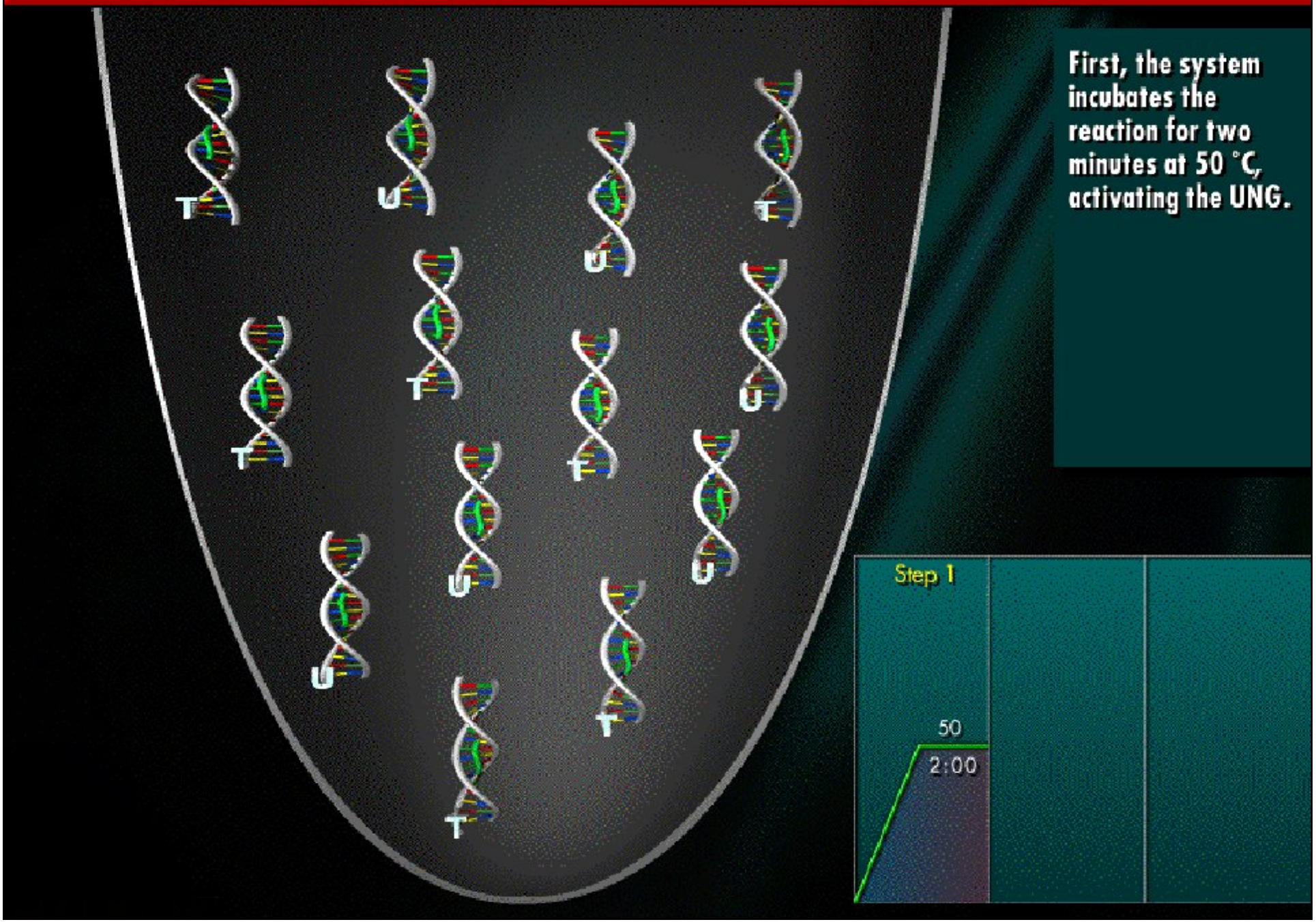
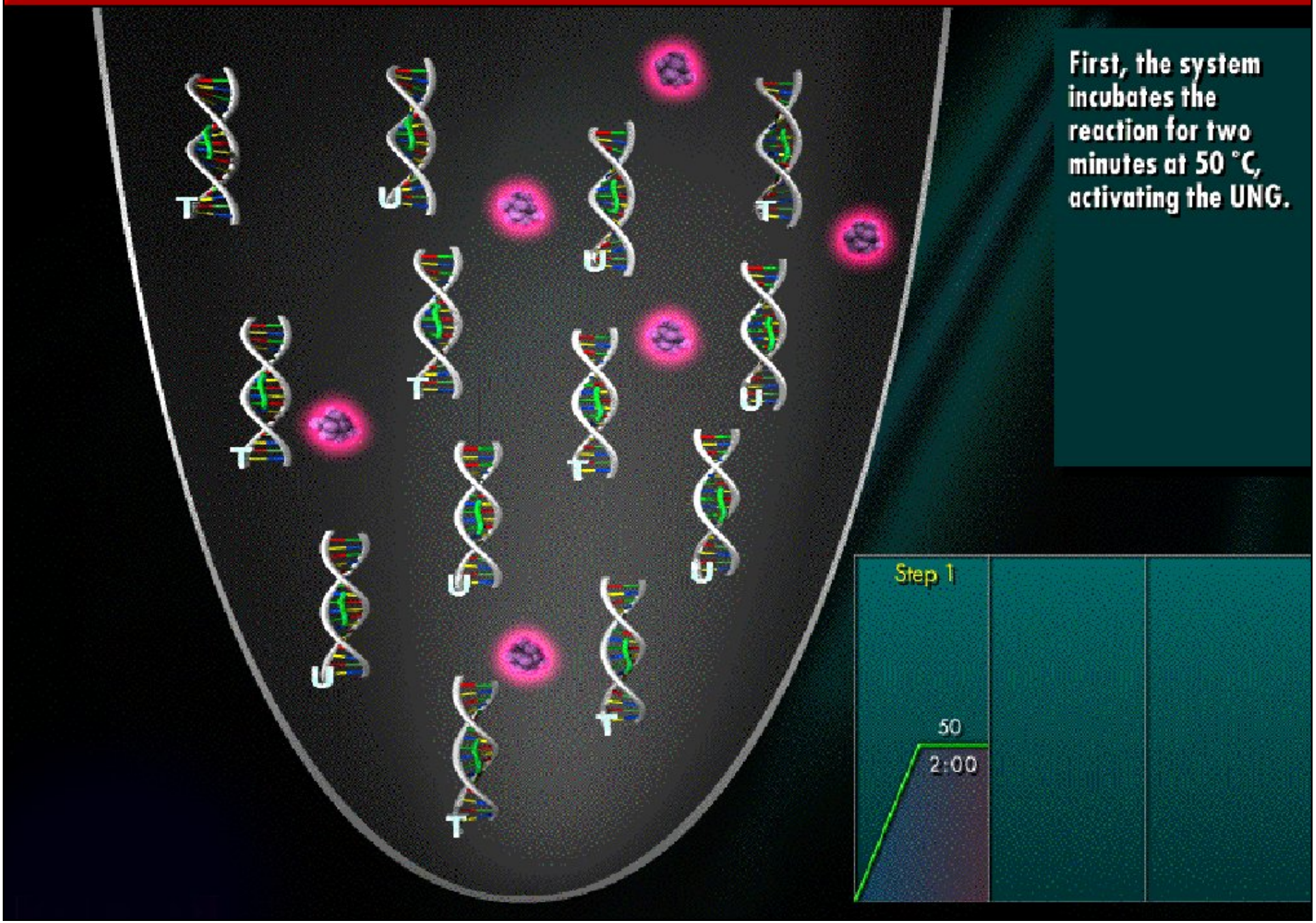


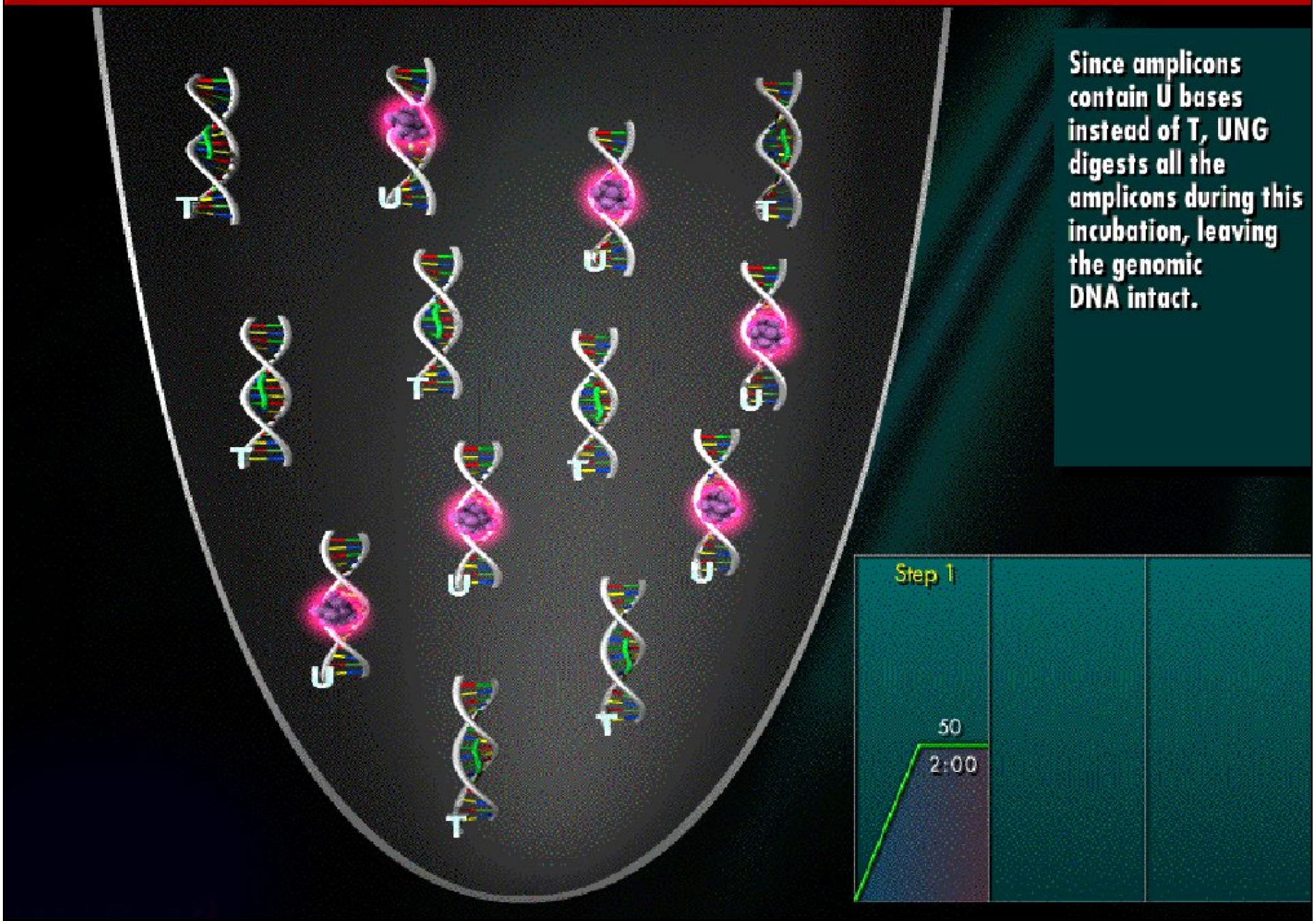
# CARRY-OVER PREVENTION WITH UNG



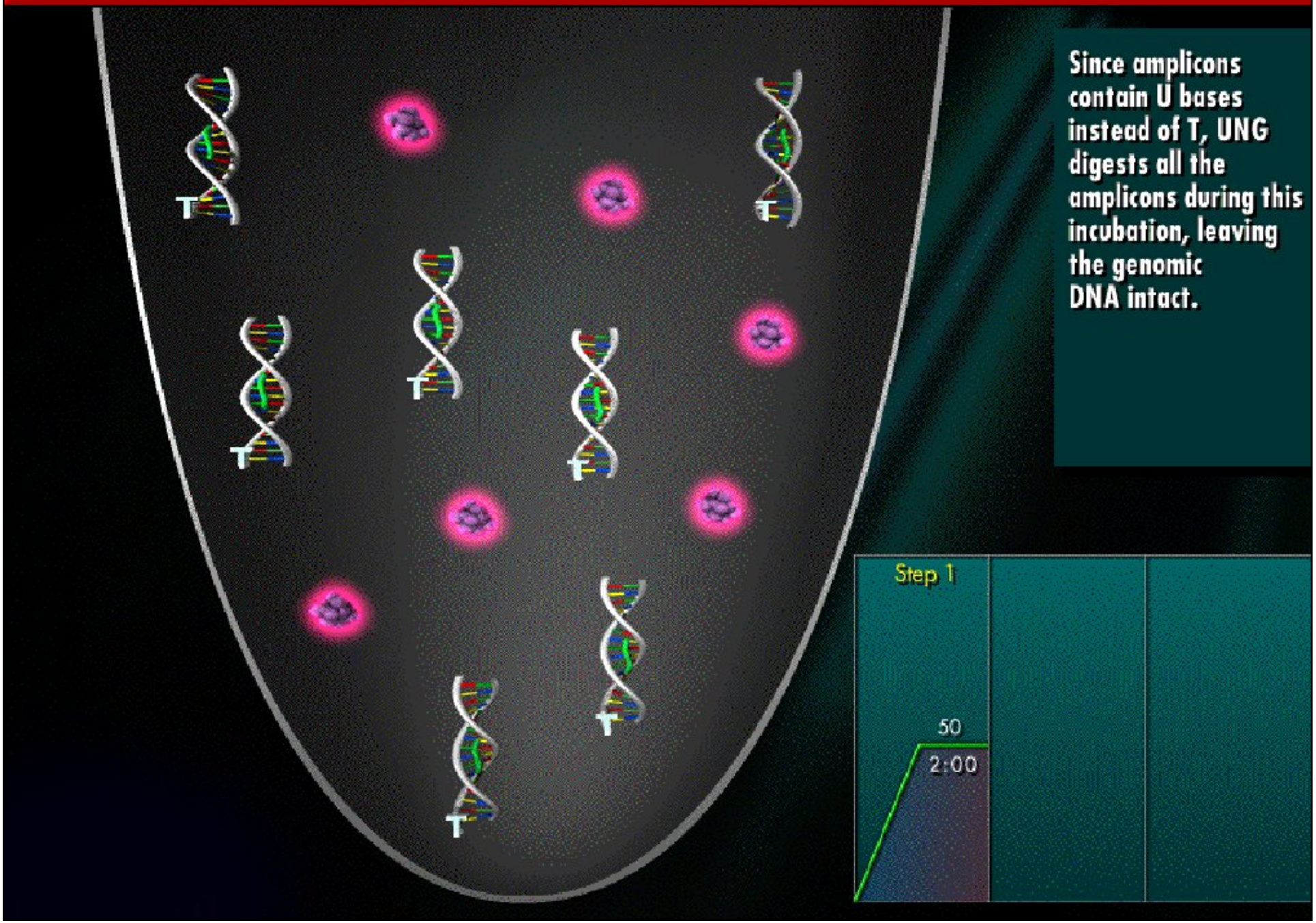
# CARRY-OVER PREVENTION WITH UNG



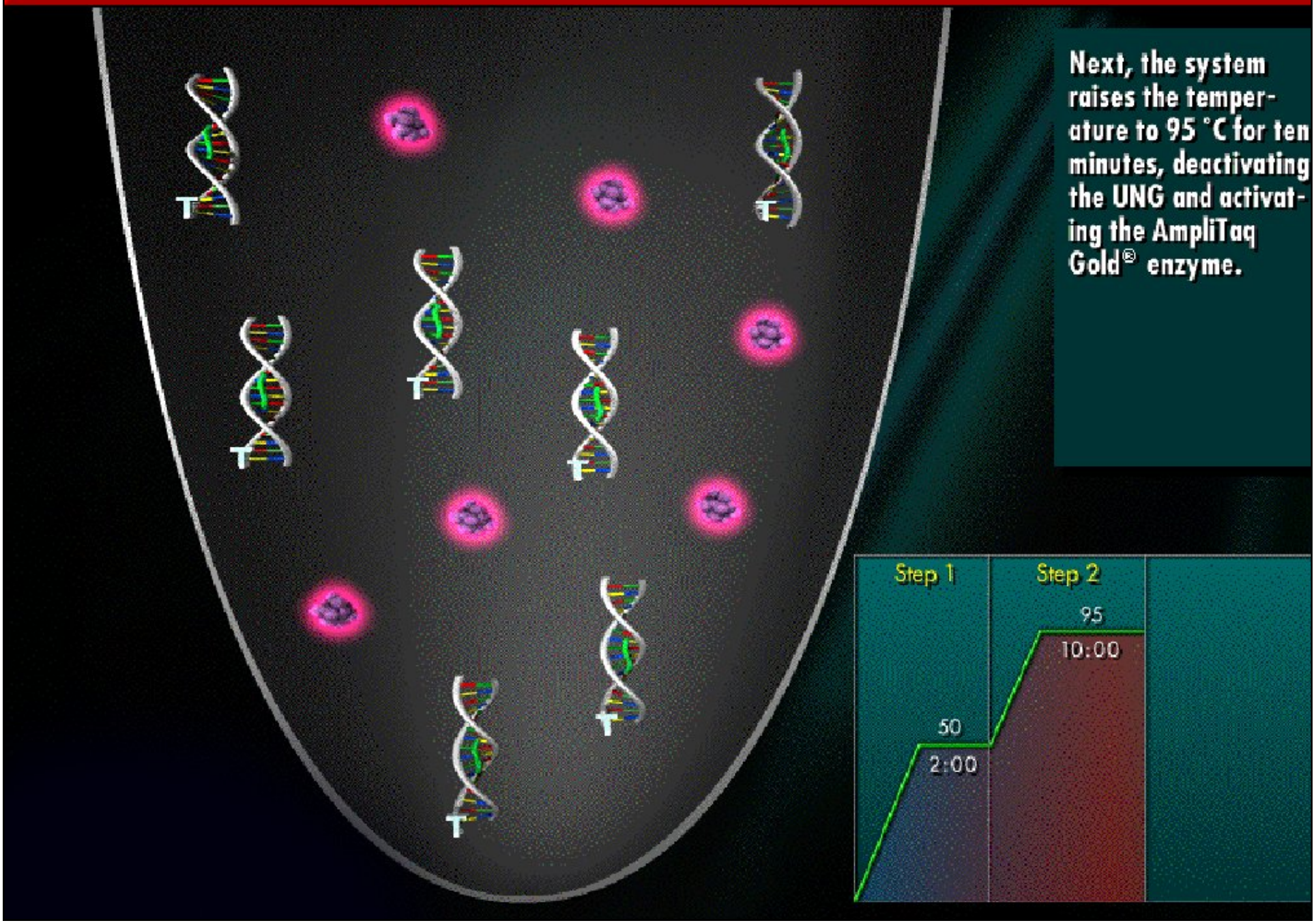
# CARRY-OVER PREVENTION WITH UNG



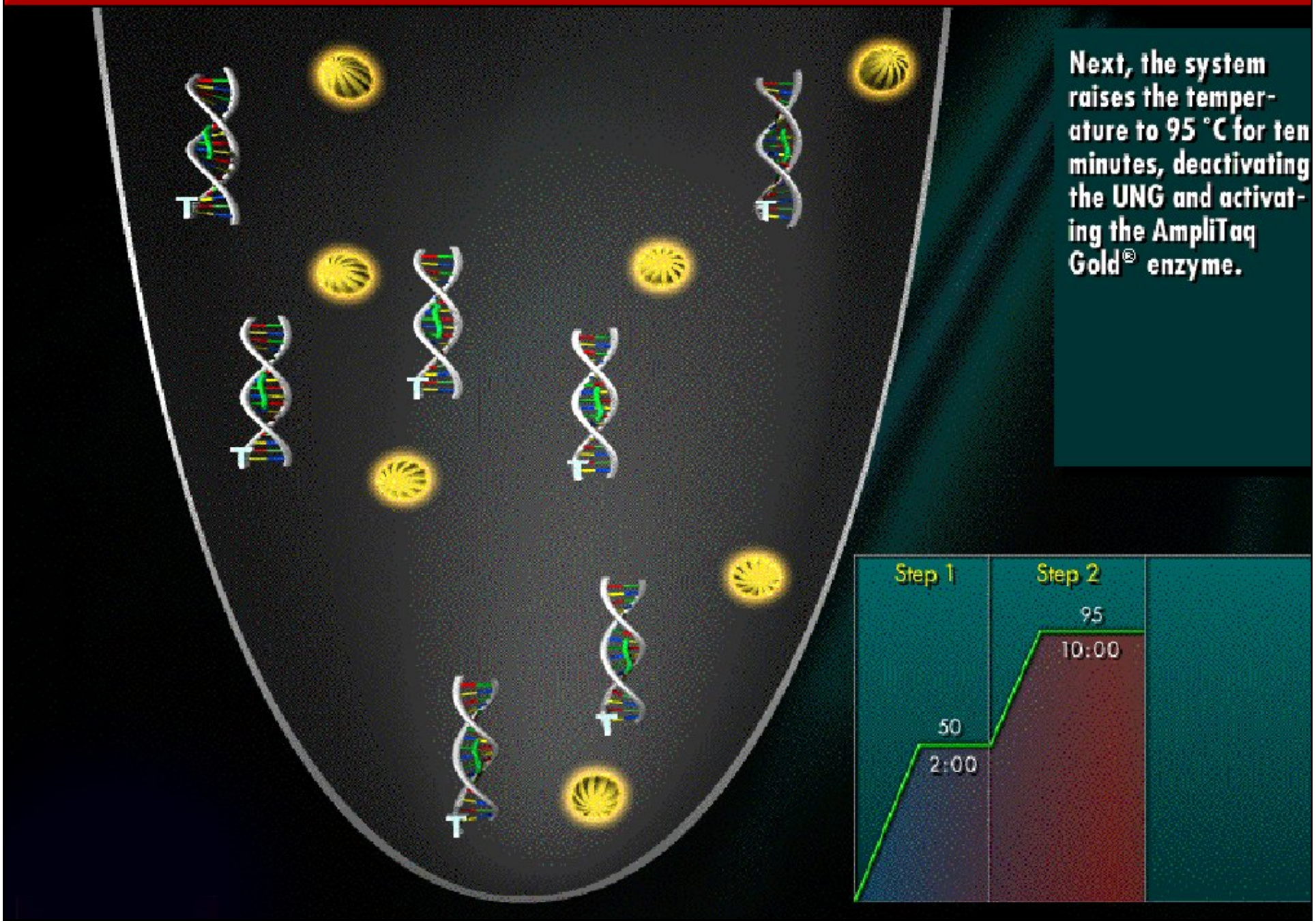
# CARRY-OVER PREVENTION WITH UNG



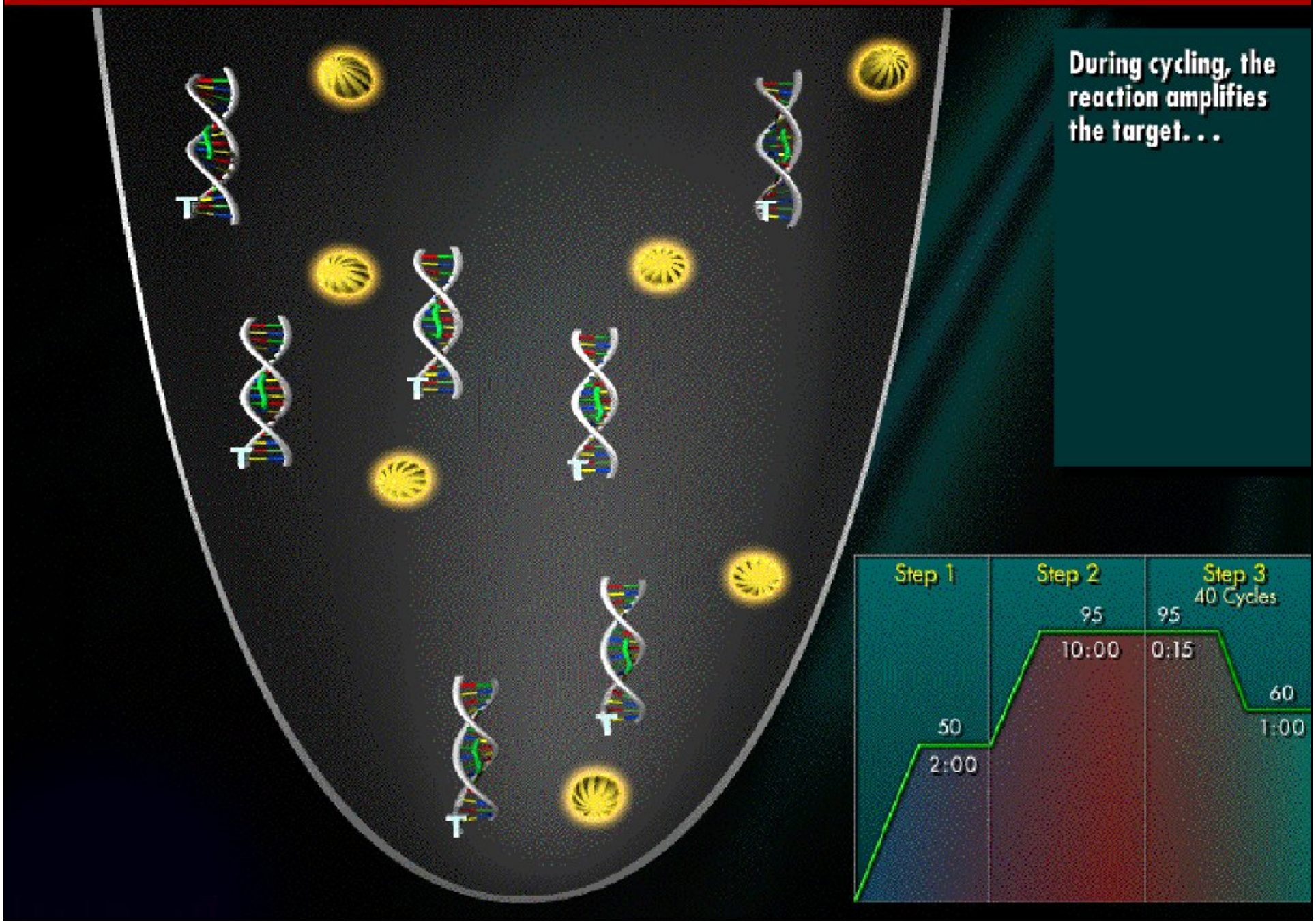
# CARRY-OVER PREVENTION WITH UNG



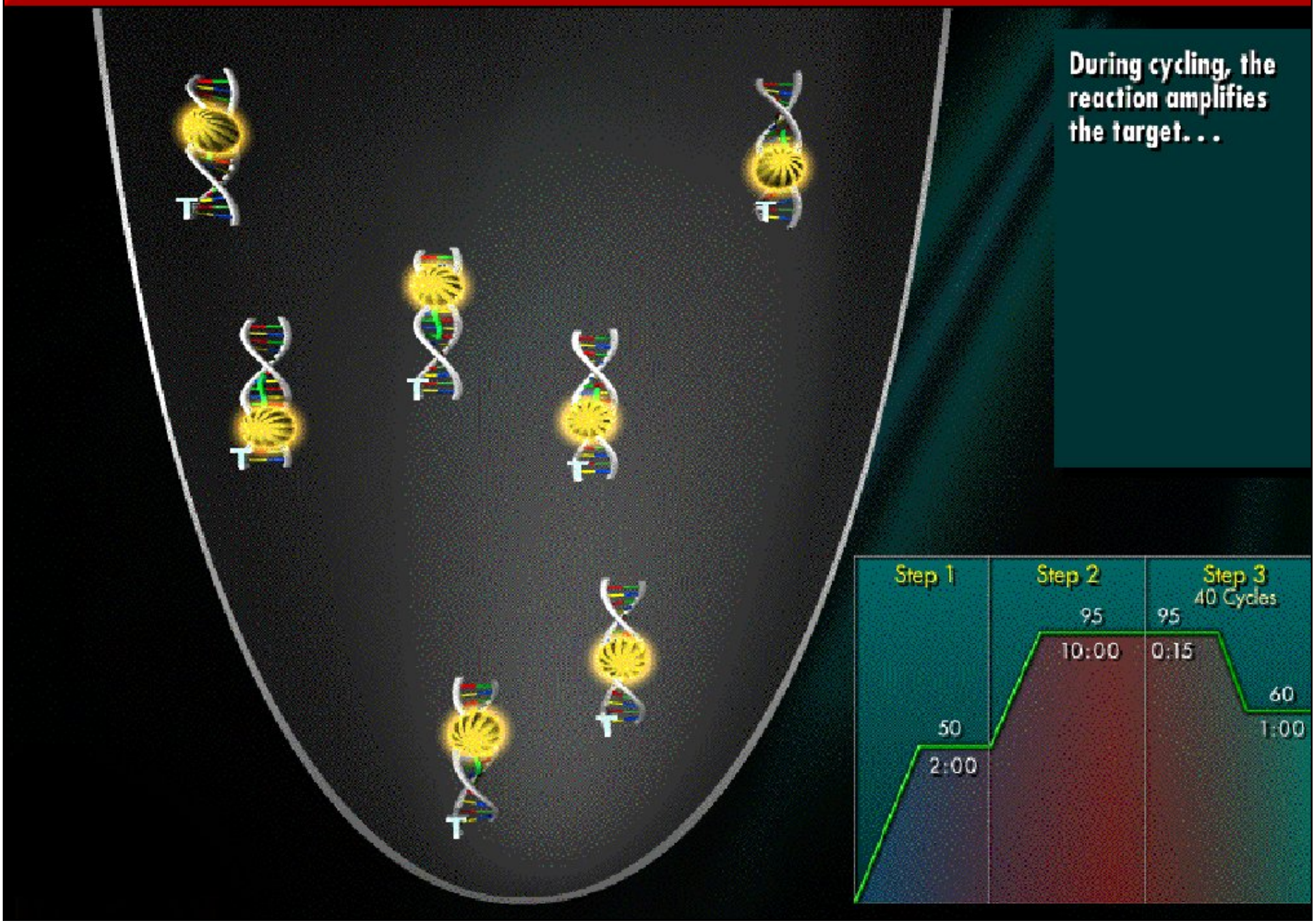
# CARRY-OVER PREVENTION WITH UNG / AmpliTaq Gold™ ACTIVATION



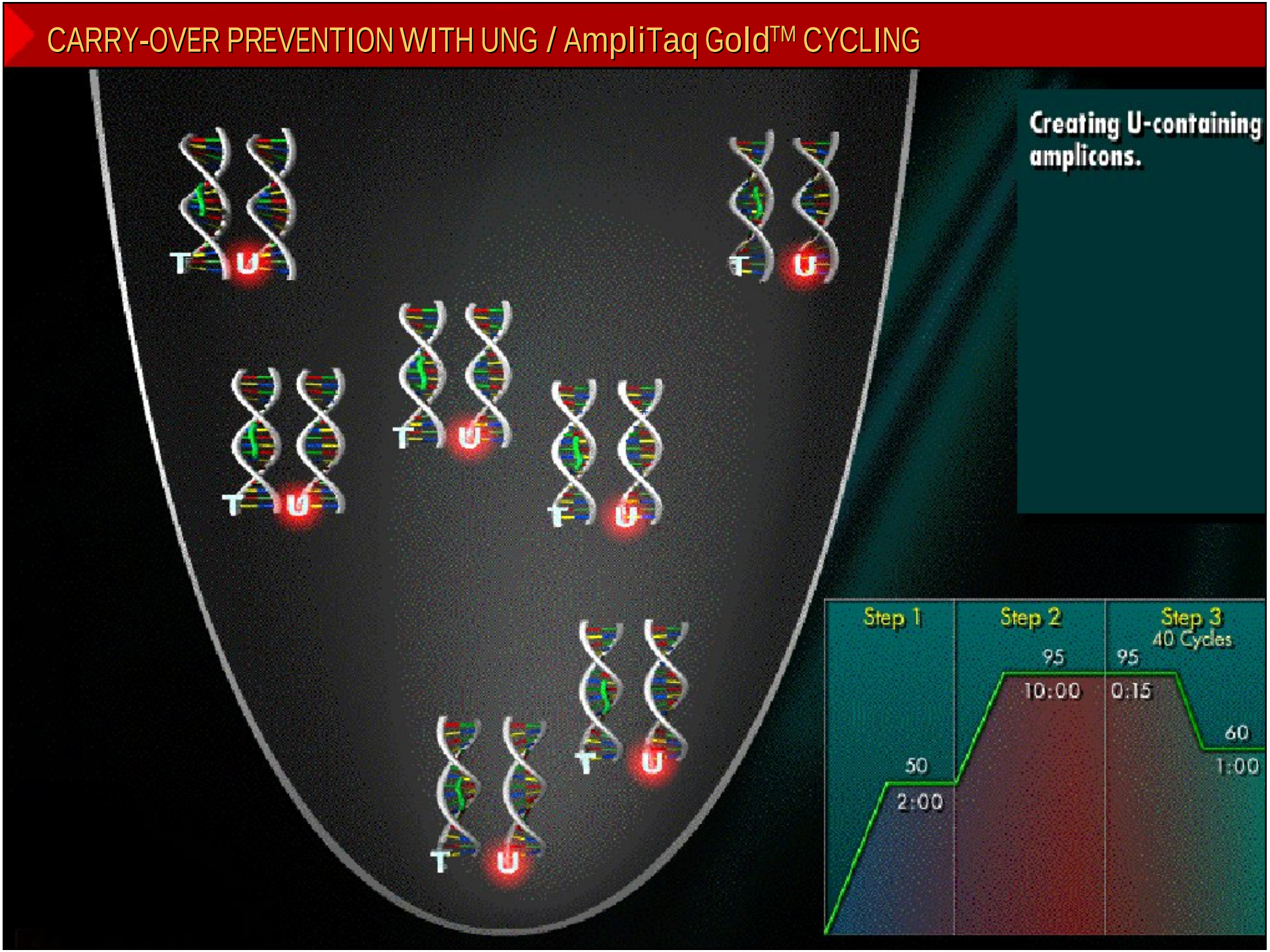
# CARRY-OVER PREVENTION WITH UNG / AmpliTaq Gold™ CYCLING



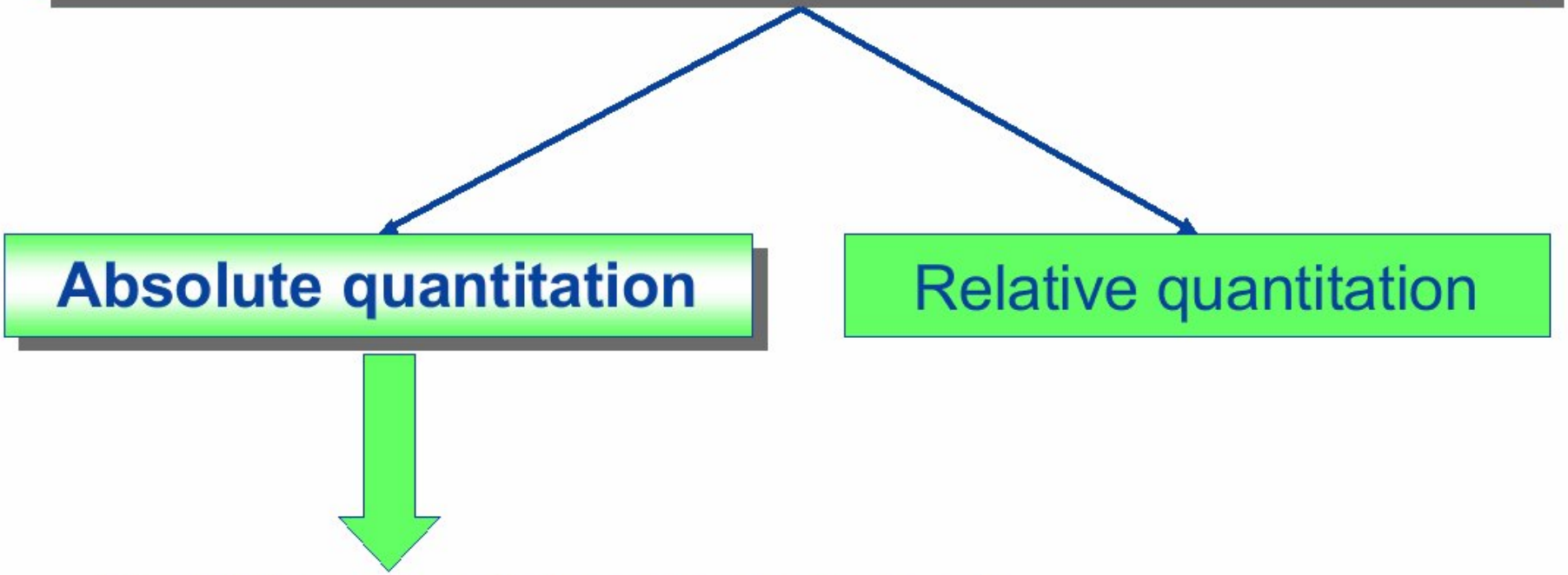
# CARRY-OVER PREVENTION WITH UNG / AmpliTaq Gold™ CYCLING



# CARRY-OVER PREVENTION WITH UNG / AmpliTaq Gold™ CYCLING



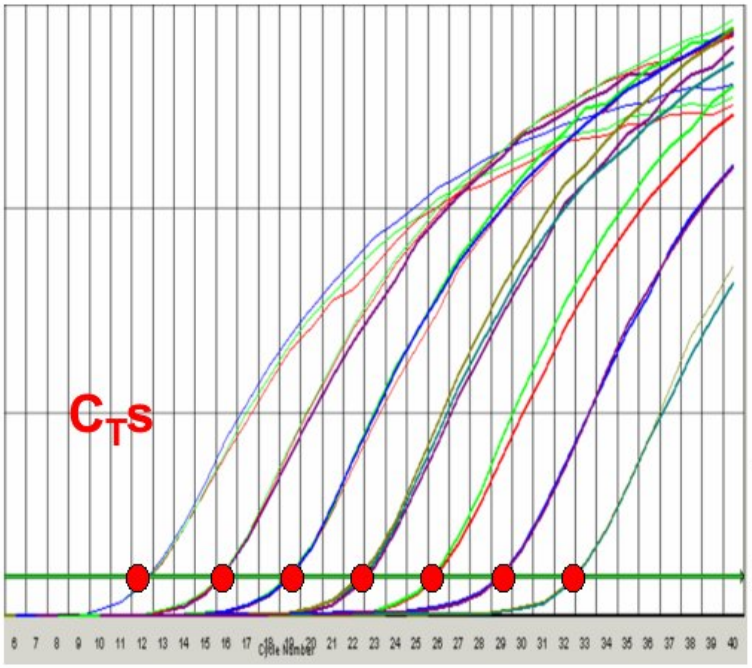
# Types of Quantitation Assays

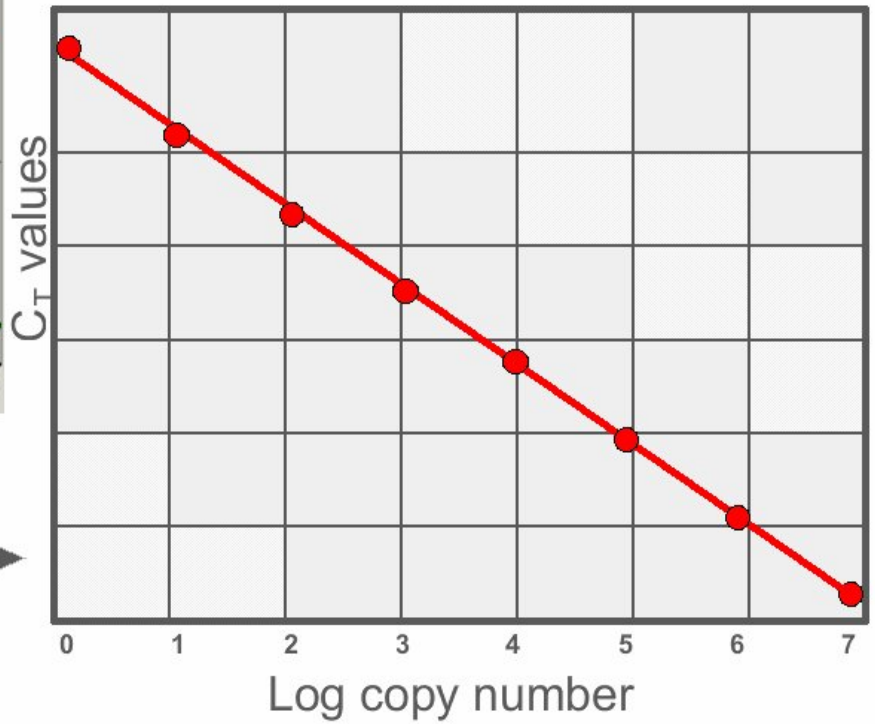



Provides absolute measurement of starting copy number

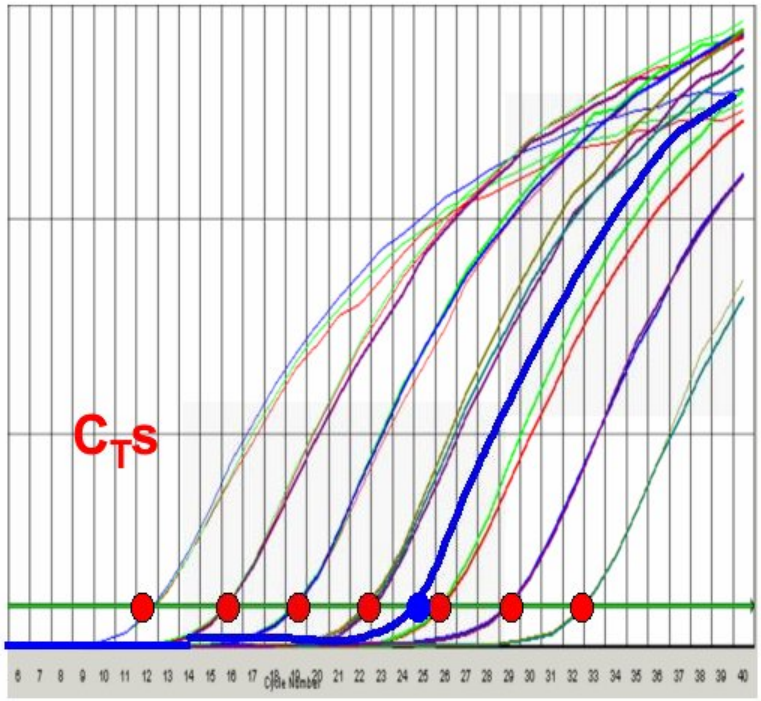
- –Requires standards of known quantity
- –e.g. Detecting DNA copy number for forensics purposes

# From Fluorescence to Copy Number



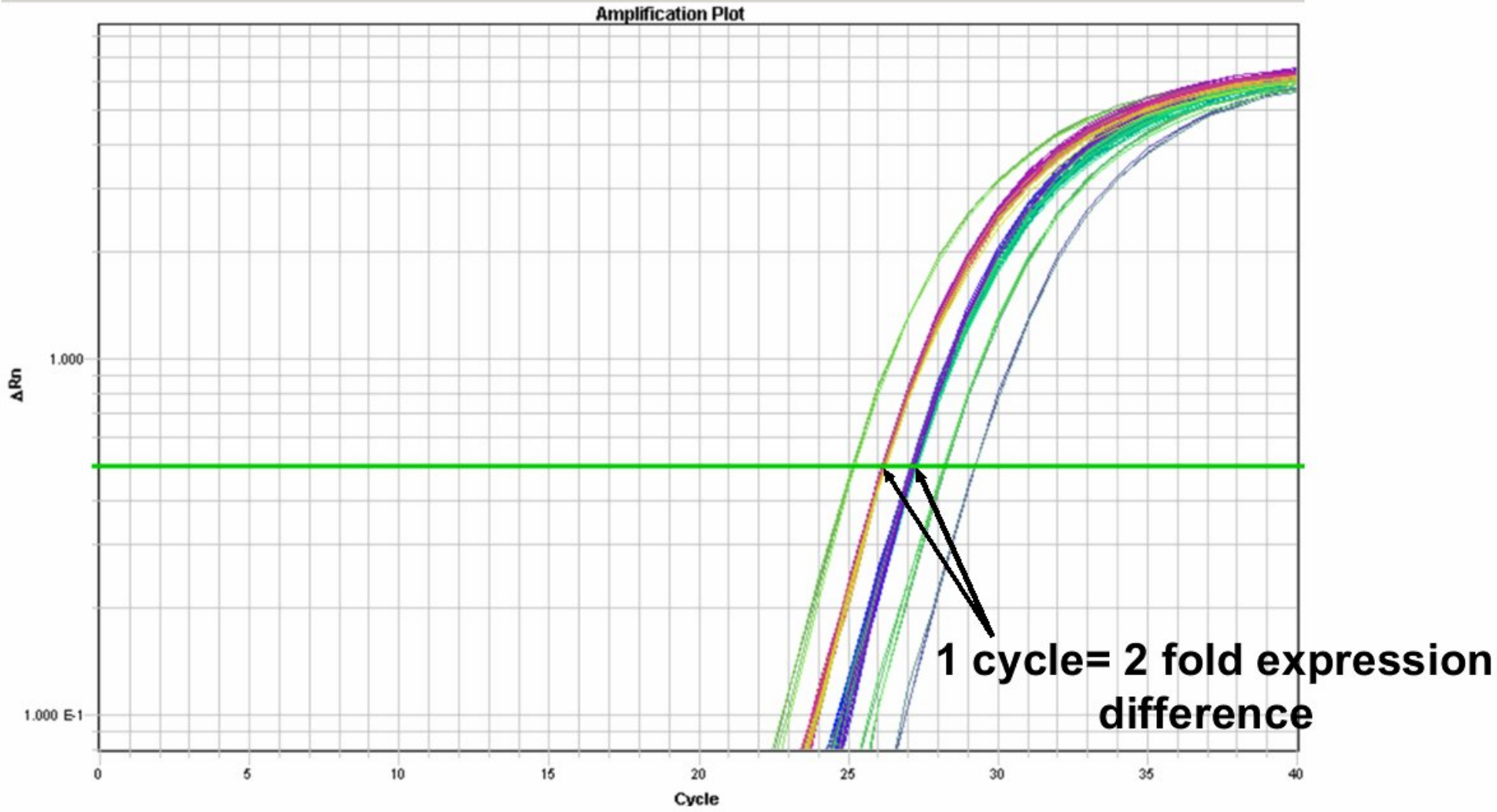
# From Fluorescence to Copy Number



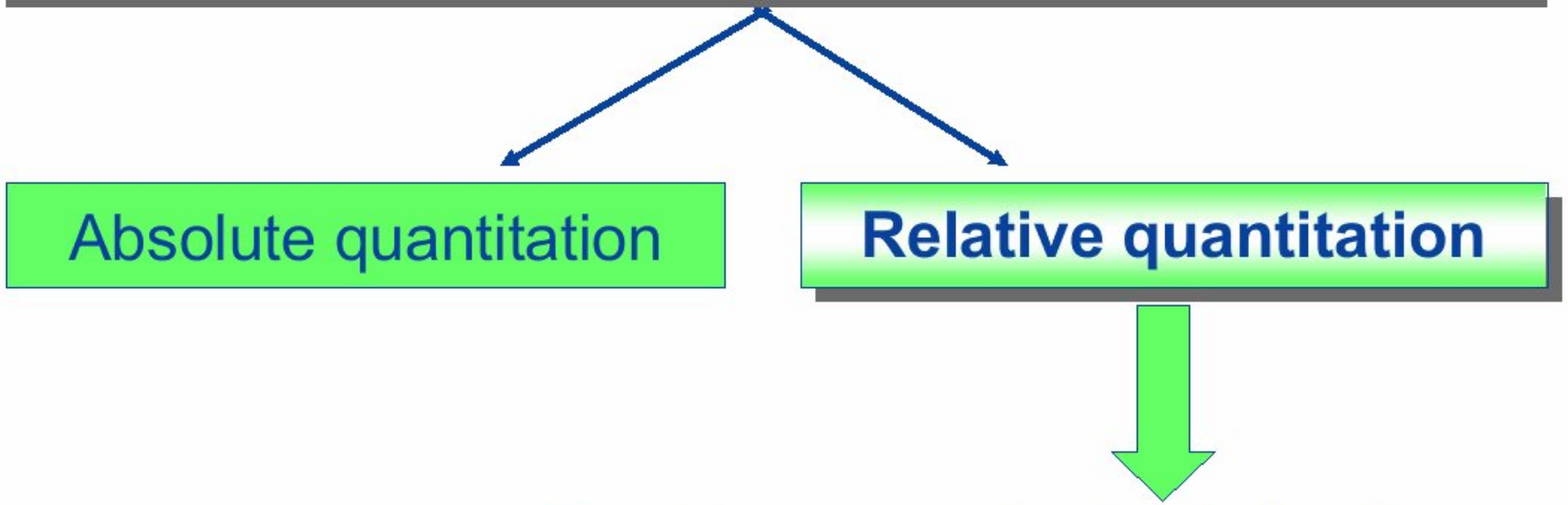


$C_T$ is directly proportional to log of amount of input template

# High (100%) Amplification Efficiency



# Types of Quantitation Assays



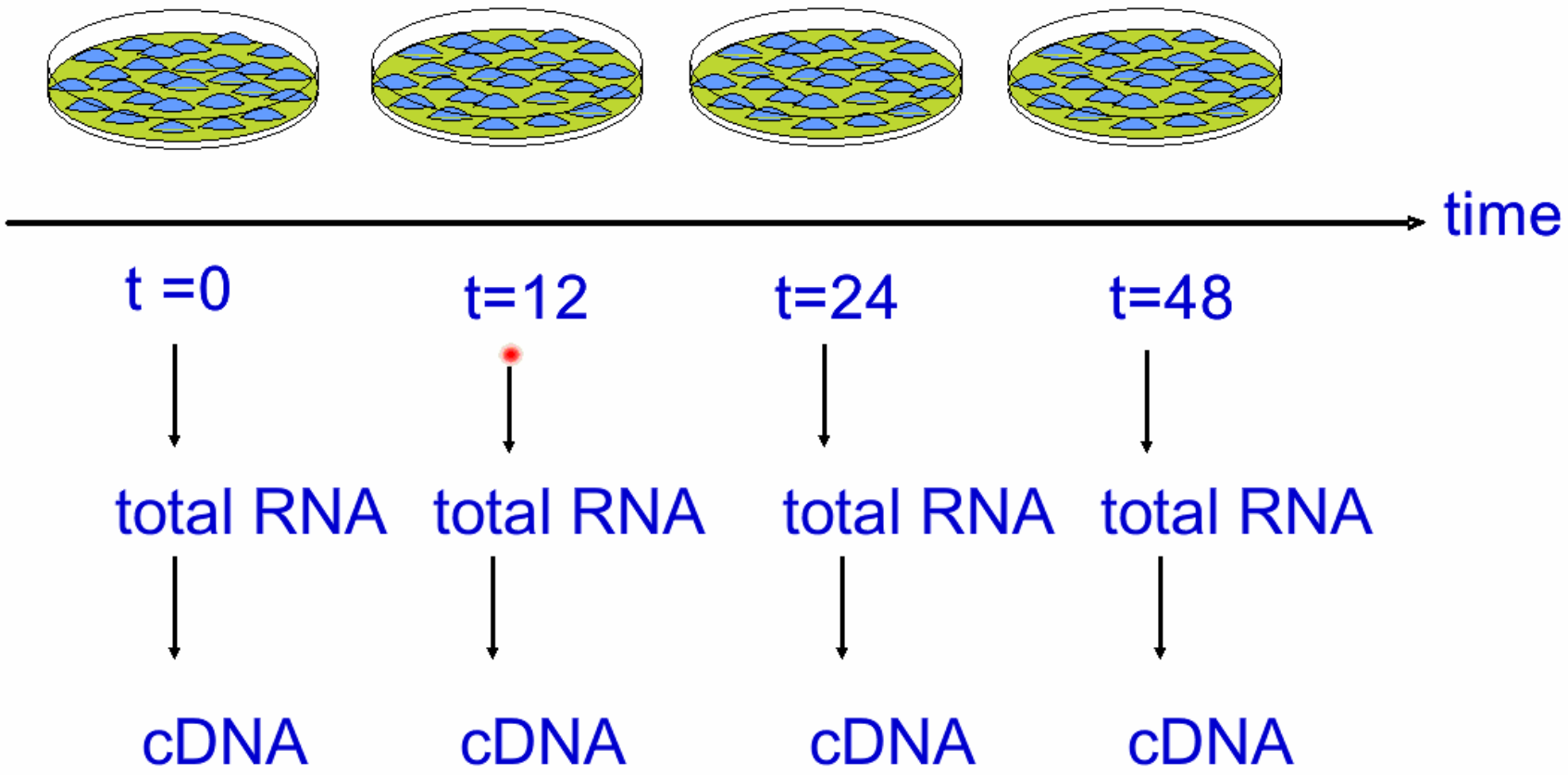

Provides accurate discrimination between relative amounts of starting material

–e.g. Comparing expression levels of wildtype vs. mutated alleles

–e.g. Comparing expression levels of a gene across different tissues or between different biological conditions

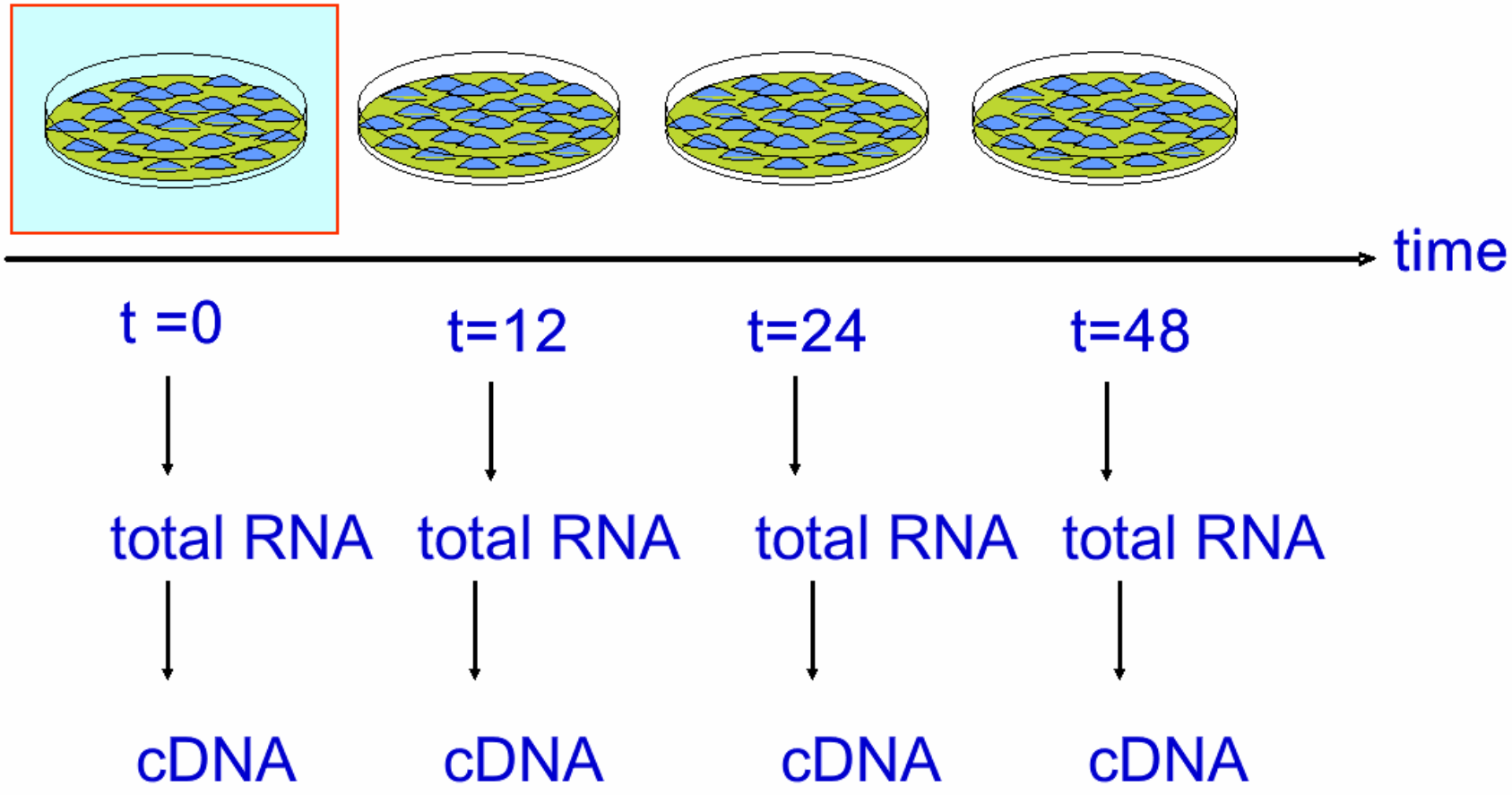
–e.g. Validating array results

# Relative Quantitation



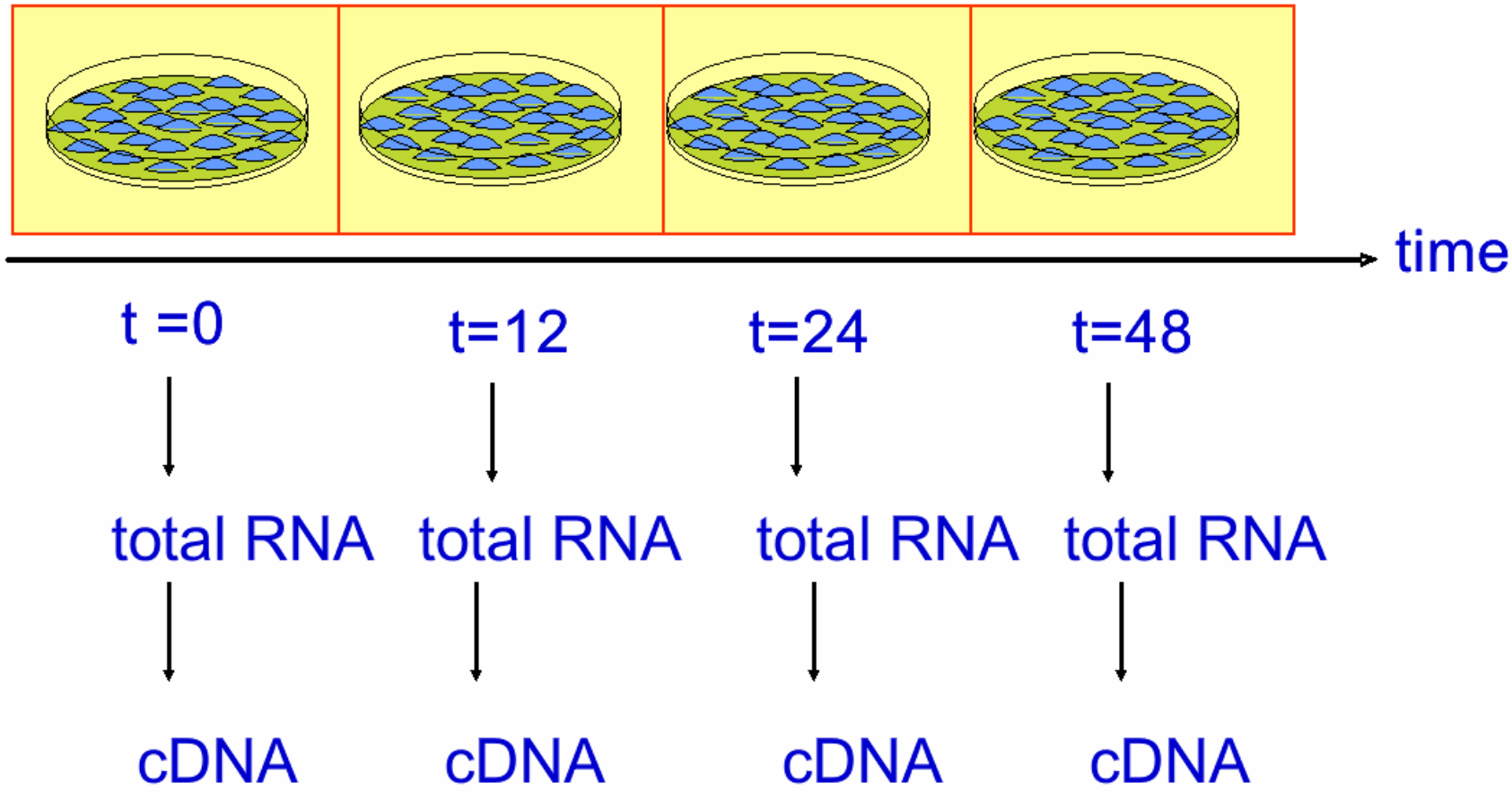
# Relative Quantitation

**Calibrator** = The sample used as the basis for comparative results

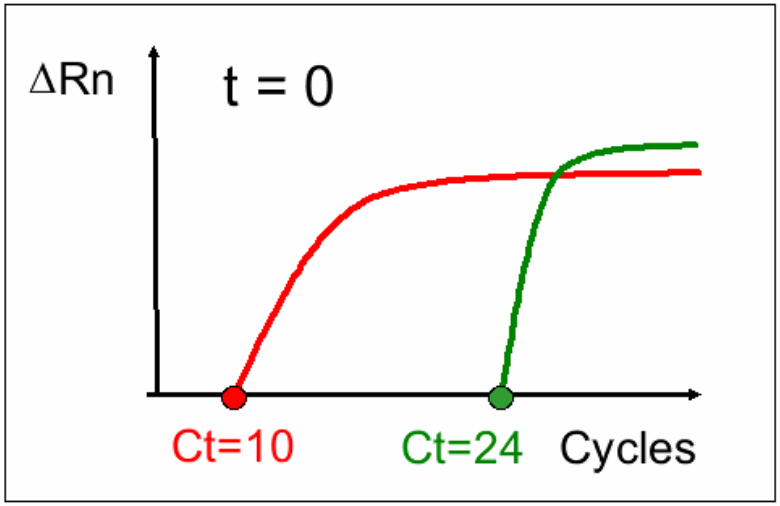


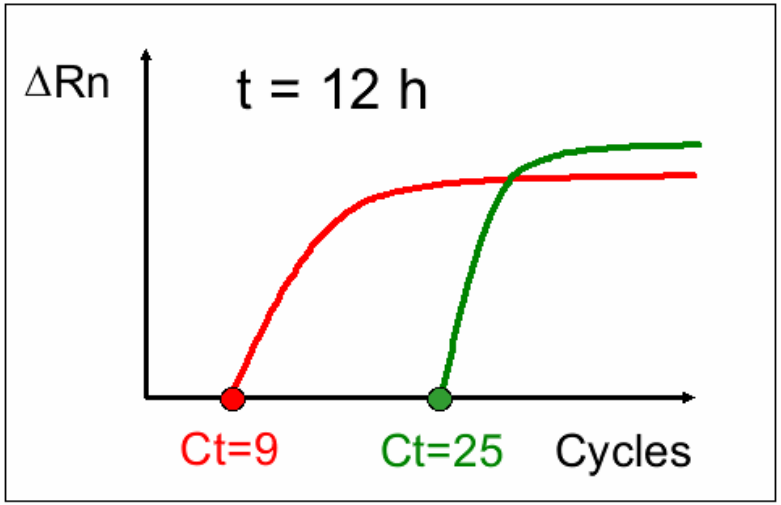
# Relative Quantitation

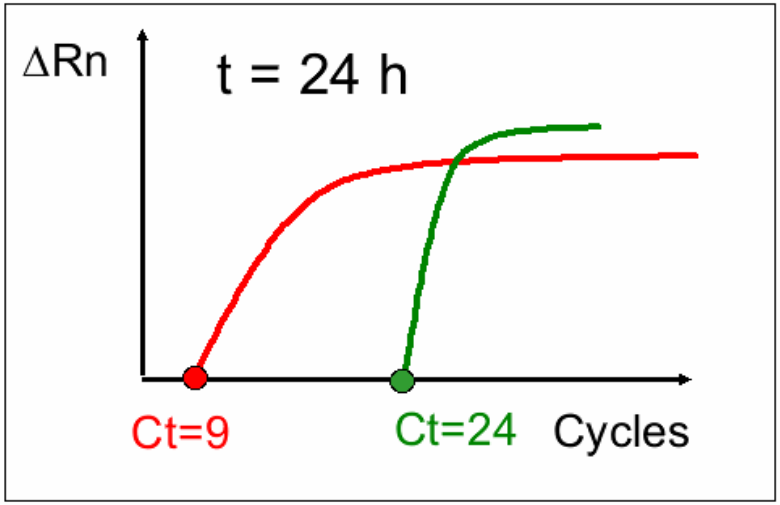
**Endogenous Control** = Target used to normalize for sample handling (e.g. 18S rRNA, GAPDH, β-actin)

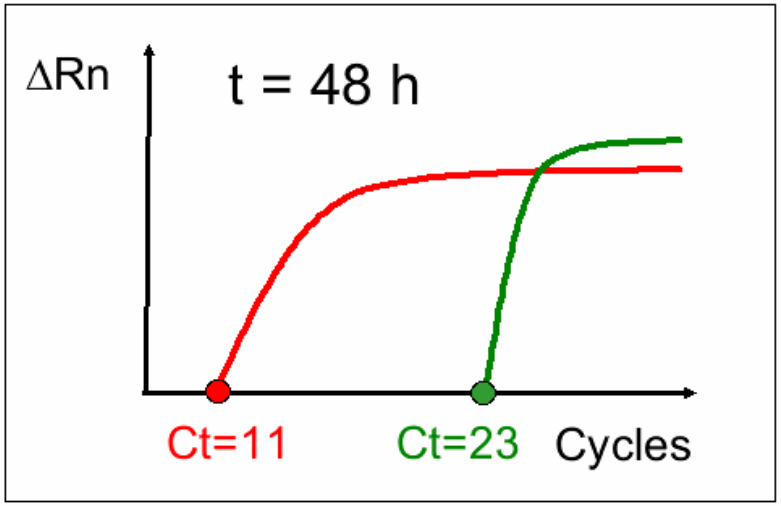


# Comparative $C_T$ Method




**Endogenous control** **Target gene**

# Comparative $C_T$ Method Calculation:

***Normalized to endogenous control:***

$C_{T\ 48hrs}$ (23) $- C_{T\ Endo.\ Control}$ (11) $= \Delta C_{T\ 48hrs}$ (12)

$C_{T\ 0hrs,Calibrator}$ (24) $- C_{T\ Endo.\ Control}$ (10) $= \Delta C_{T\ 0hrs}$ (14)

***Normalized to calibrator sample:***

$\Delta C_{T\ 48hrs}$ (12) $- \Delta C_{T\ 0hrs,Calibrator}$ (14) $= \Delta\Delta C_T$ (-2)

***Relative fold change:***

$2^{(-\Delta\Delta C_T)} = 2^{(2)} =$ (4)

**There is a 4-fold overexpression of my gene at T=48h compared T=0h...48hrs after drug treatment!**

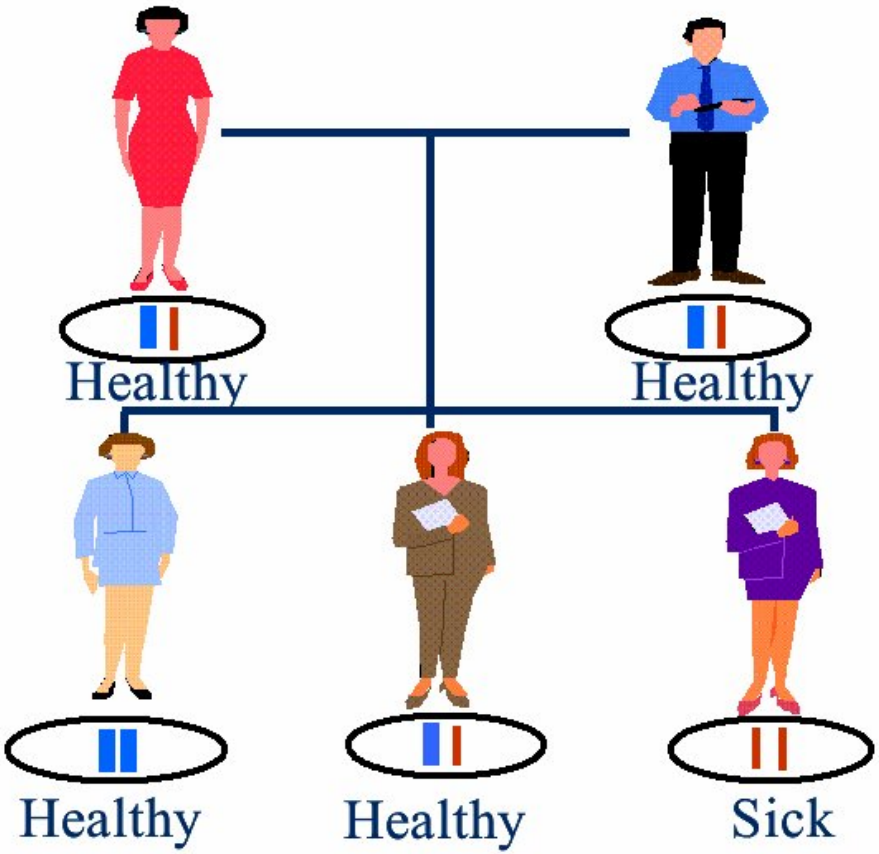
# Applications

- Real-Time Detection
  - Absolute Quantitation
  - Relative Quantitation

- End Point Detection
  - Allelic Discrimination (SNP)
  - +/- Assay (IPC)
    - Pathogen Detection

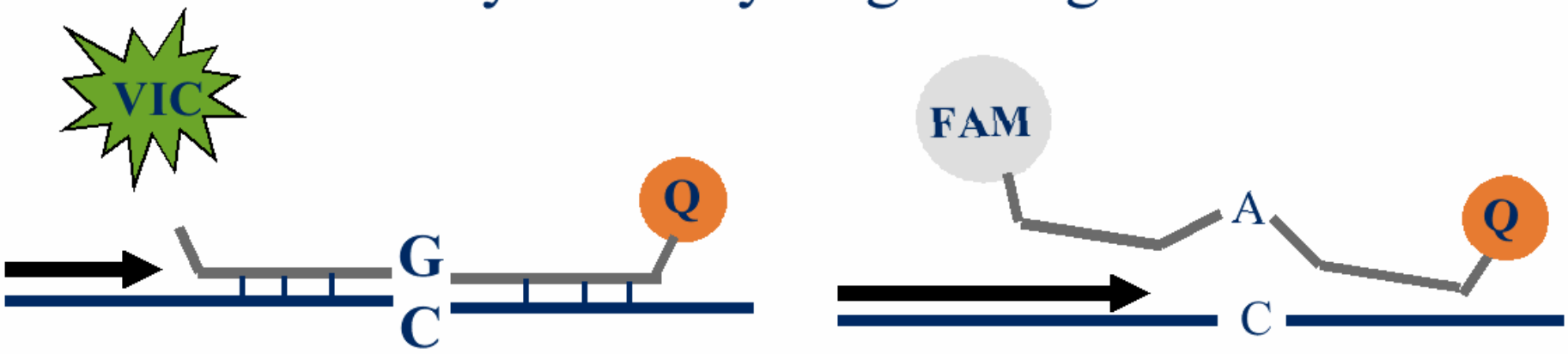
# Allelic Discrimination (SNP)

- Determines the genotype of samples
  - Possible to differentiate a single nucleotide polymorphism (SNP)

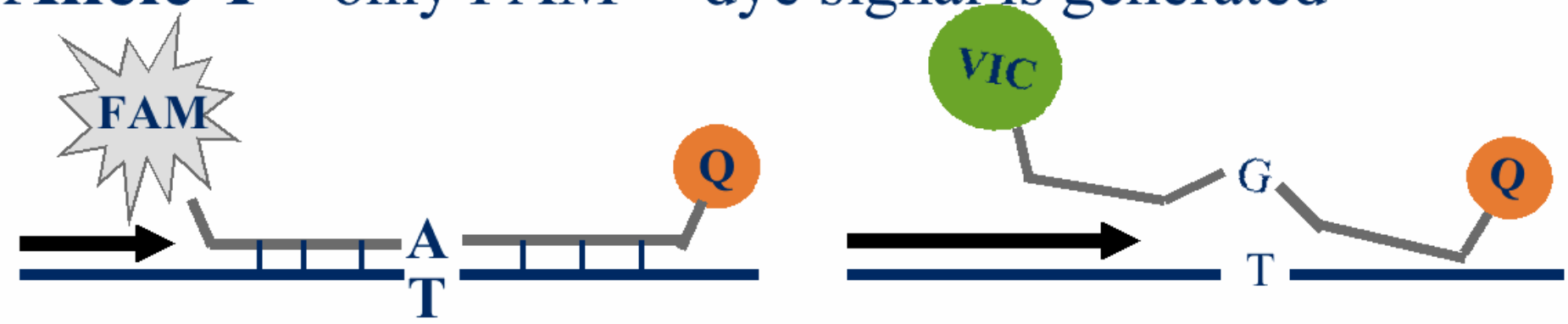


# Allelic Discrimination Assay

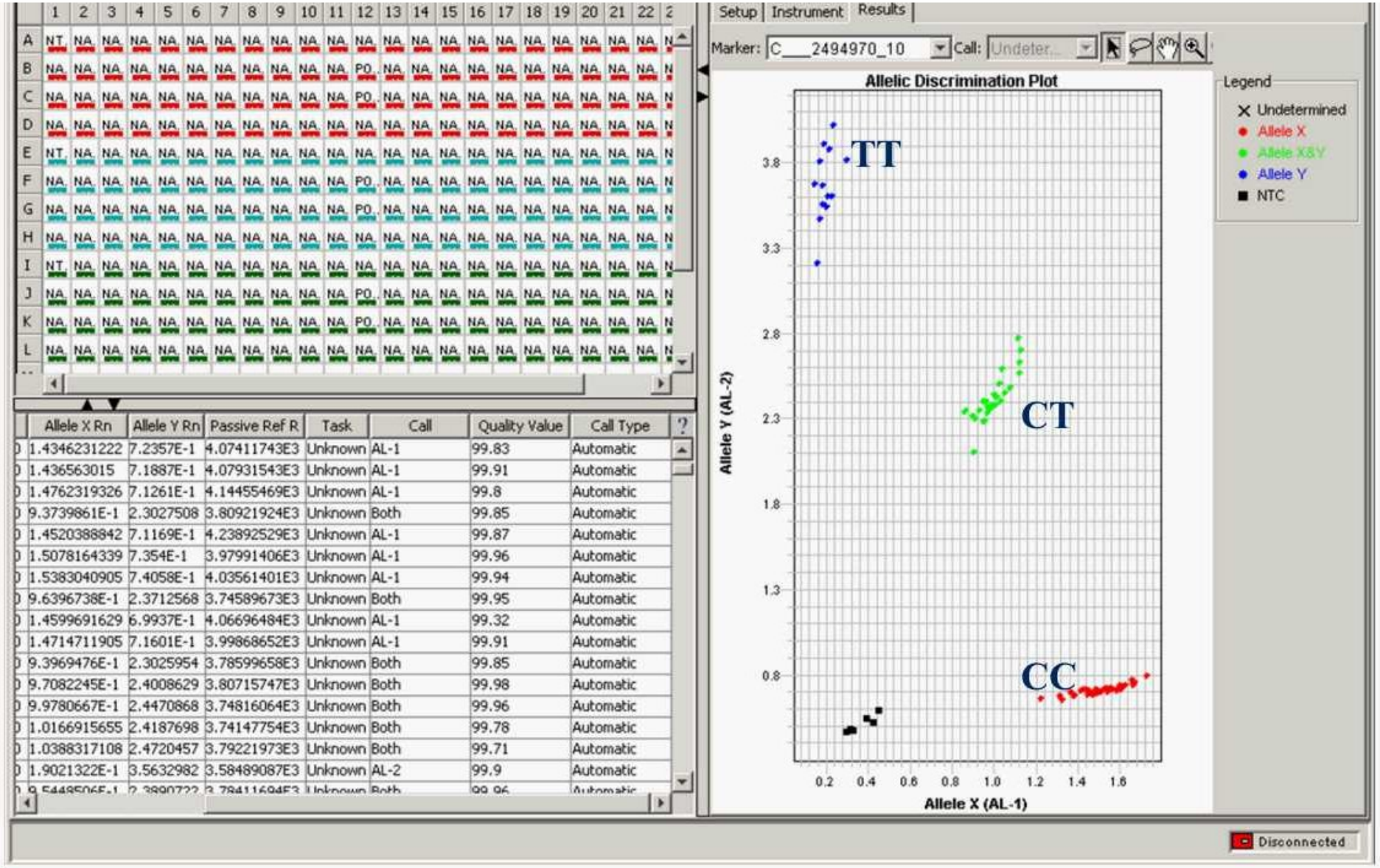
**Allele C -** only VIC$^{R}$ dye signal is generated




**Allele T -** only FAM™ dye signal is generated



# Allelic Discrimination (SNP)




| Allele X Rn | Allele Y Rn | Passive Ref R | Task | Call | Quality Value | Call Type |
|---|---|---|---|---|---|---|
| 1.4346231222 | 7.2357E-1 | 4.07411743E3 | Unknown | AL-1 | 99.83 | Automatic |
| 1.436563015 | 7.1887E-1 | 4.07931543E3 | Unknown | AL-1 | 99.91 | Automatic |
| 1.4762319326 | 7.1261E-1 | 4.14455469E3 | Unknown | AL-1 | 99.8 | Automatic |
| 9.3739861E-1 | 2.3027508 | 3.80921924E3 | Unknown | Both | 99.85 | Automatic |
| 1.4520388842 | 7.1169E-1 | 4.23892529E3 | Unknown | AL-1 | 99.87 | Automatic |
| 1.5078164339 | 7.354E-1 | 3.97991406E3 | Unknown | AL-1 | 99.96 | Automatic |
| 1.5383040905 | 7.4058E-1 | 4.03561401E3 | Unknown | AL-1 | 99.94 | Automatic |
| 9.6396738E-1 | 2.3712568 | 3.74589673E3 | Unknown | Both | 99.95 | Automatic |
| 1.4599691629 | 6.9937E-1 | 4.06696484E3 | Unknown | AL-1 | 99.32 | Automatic |
| 1.4714711905 | 7.1601E-1 | 3.99868652E3 | Unknown | AL-1 | 99.91 | Automatic |
| 9.3969476E-1 | 2.3025954 | 3.78599658E3 | Unknown | Both | 99.85 | Automatic |
| 9.7082245E-1 | 2.4008629 | 3.80715747E3 | Unknown | Both | 99.98 | Automatic |
| 9.9780667E-1 | 2.4470868 | 3.74816064E3 | Unknown | Both | 99.96 | Automatic |
| 1.0166915655 | 2.4187698 | 3.74147754E3 | Unknown | Both | 99.78 | Automatic |
| 1.0388317108 | 2.4720457 | 3.79221973E3 | Unknown | Both | 99.71 | Automatic |
| 1.9021322E-1 | 3.5632982 | 3.58489087E3 | Unknown | AL-2 | 99.9 | Automatic |

# Applications

- Real Time Detection
  - Absolute Quantitation
  - Relative Quantitation
- End Point Detection
  - Allele Detection (SNP)
  - +/- Assay (IPC)
    - Pathogen Detection

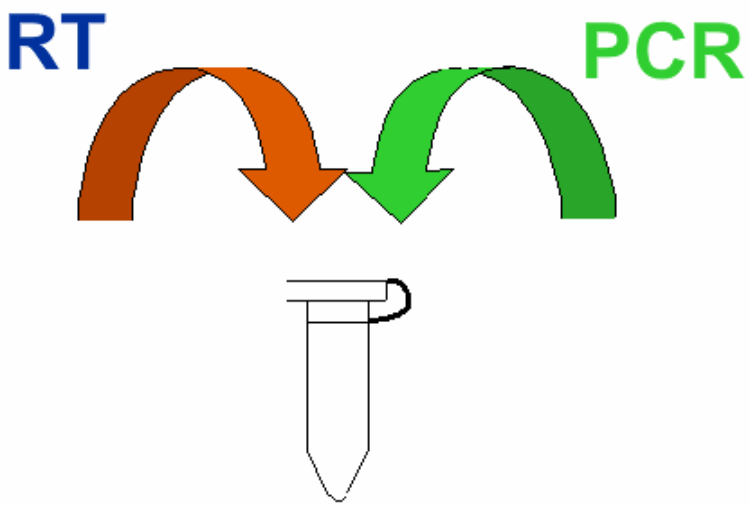
# Internal Positive Control (IPC)

- Distinguish true target negative from PCR inhibition
- Co-amplified with target DNA without compromising amplification of the target sequence

# Plus/Minus assay with IPC

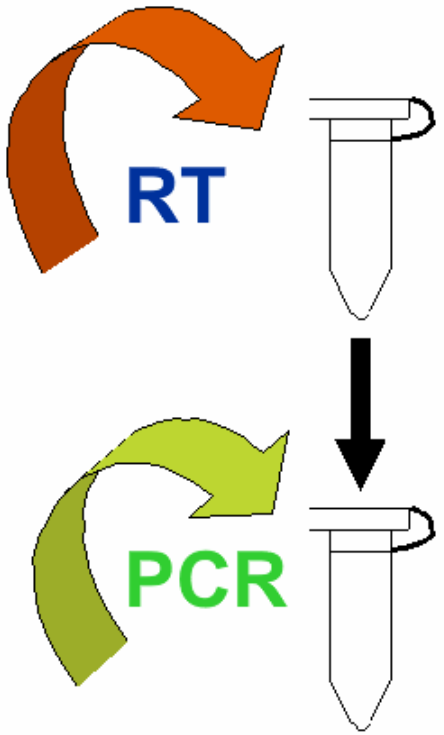
# RNA to Amplified cDNA: 1-Step vs. 2-Step

## 1-STEP




- Closed tube (no contamination)
- Easy-to-use

## 2-STEP

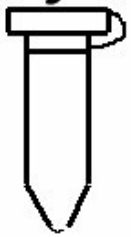



- Archive-ready sample

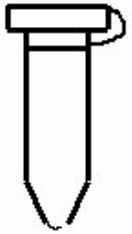
# Formats: Master Mix vs. Core Reagent

Core reagents allow flexibility and optimization

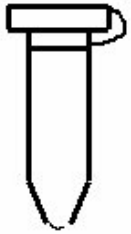
*AmpliTaq Gold® DNA Polymerase*



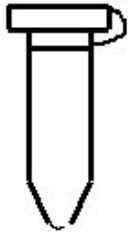
*AmpErase® UNG*



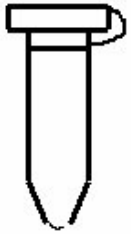
*10X Buffer*



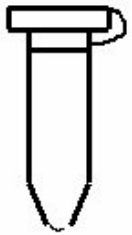
*dNTPs*



*$MgCl_2$*



## Master mixes are easy-to-use and convenient



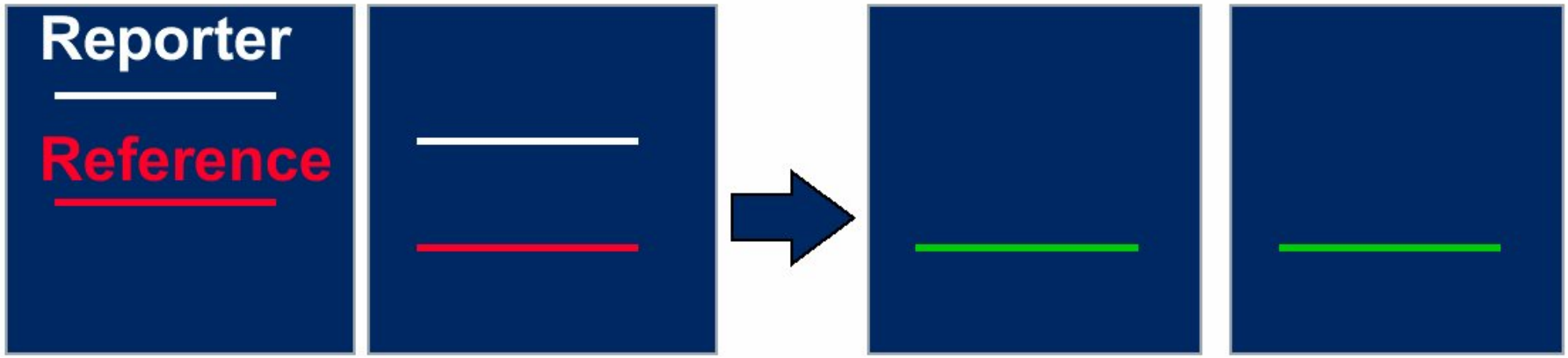
*All components in one tube!*

# Advantage of Using a ROX™ Dye Normalizer

## Improves precision

Compensates for small fluorescent fluctuations that can occur from well-to-well

## Reporter / Reference



please wait for Q&A

# *Thank you*


**Mohamed Abdel Fattah**

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