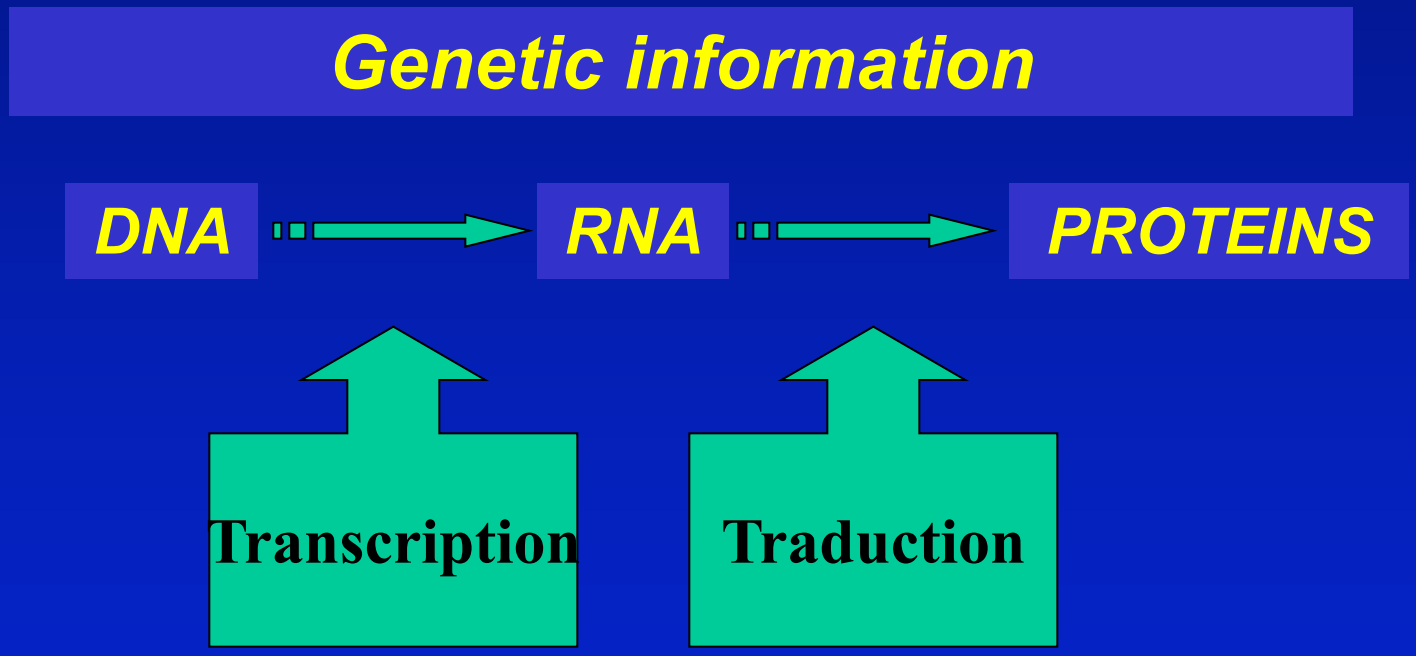
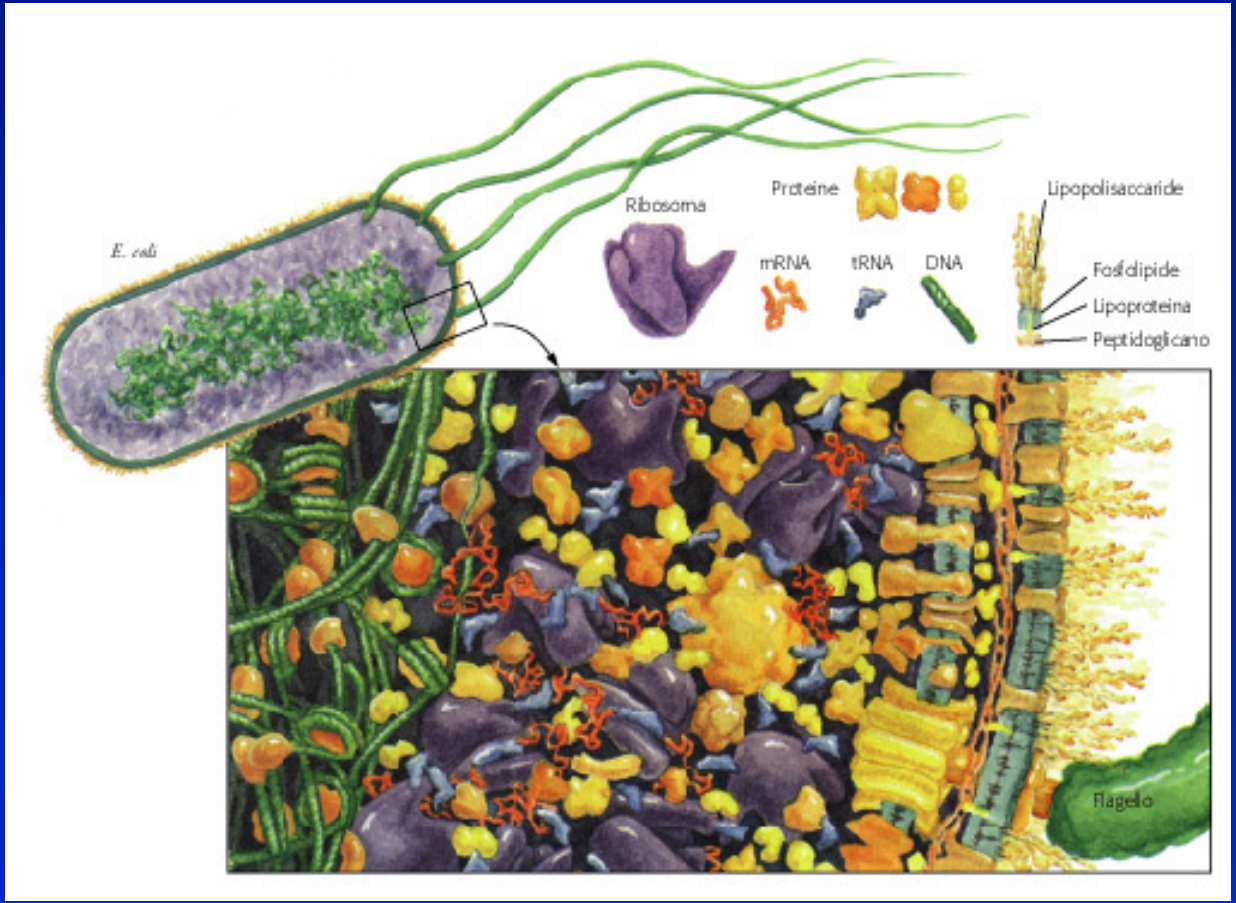
The universal genetic code

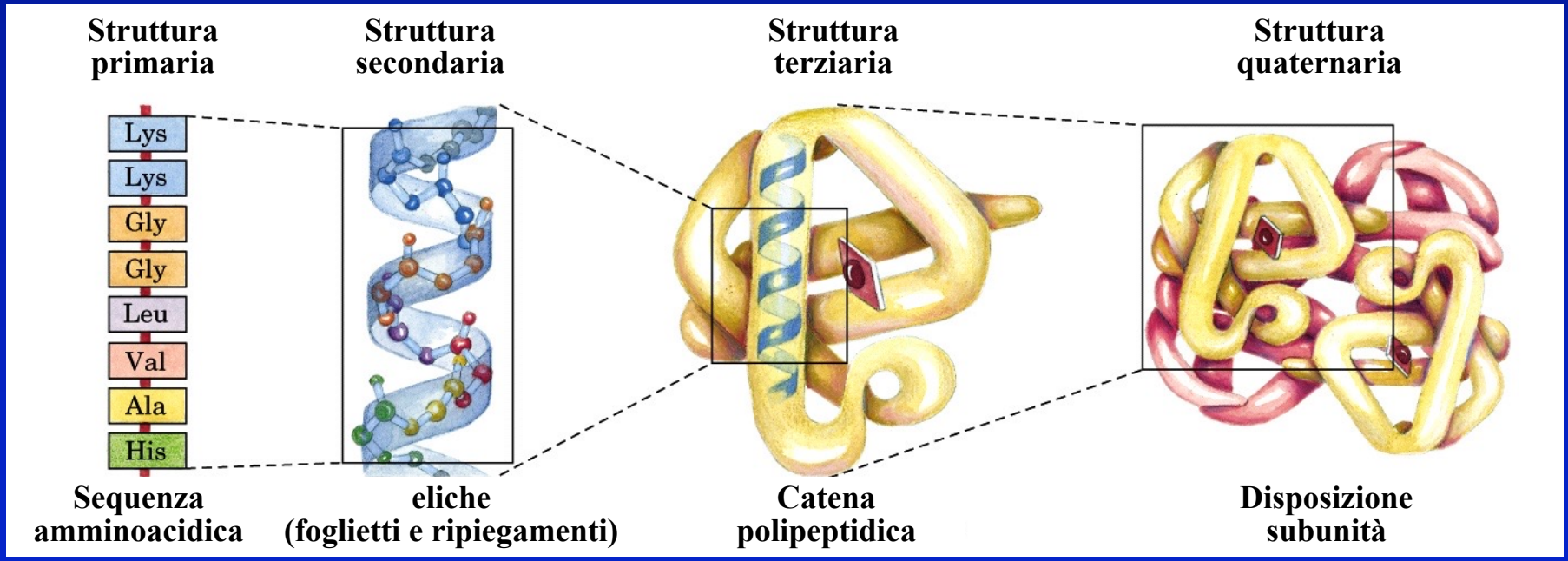
From genes to proteins



Proteins are amazingly versatile molecules

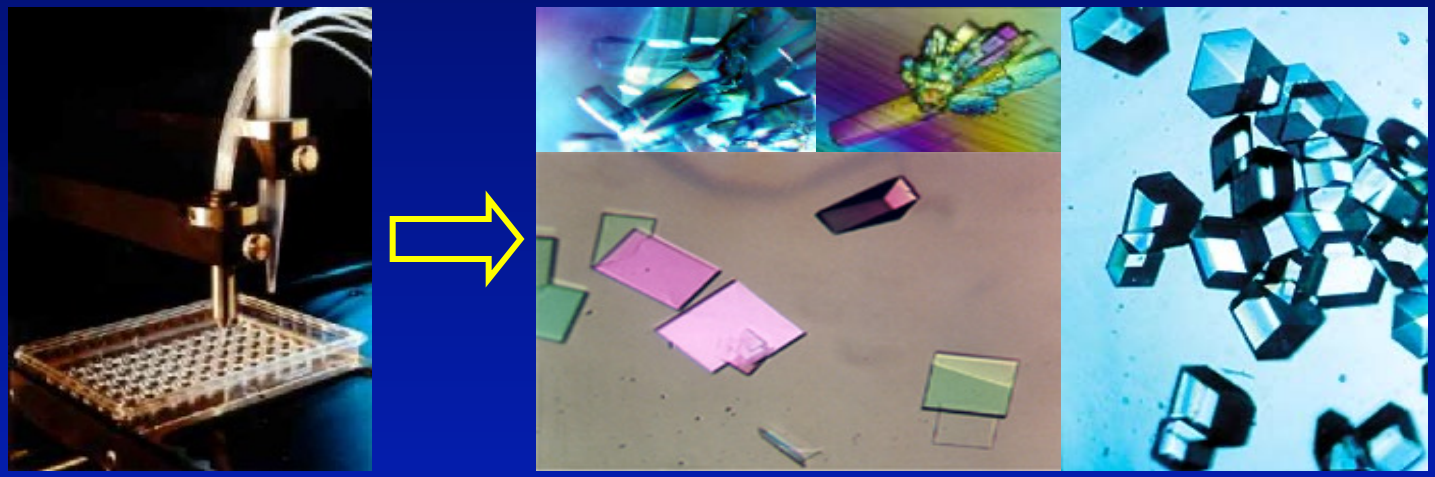


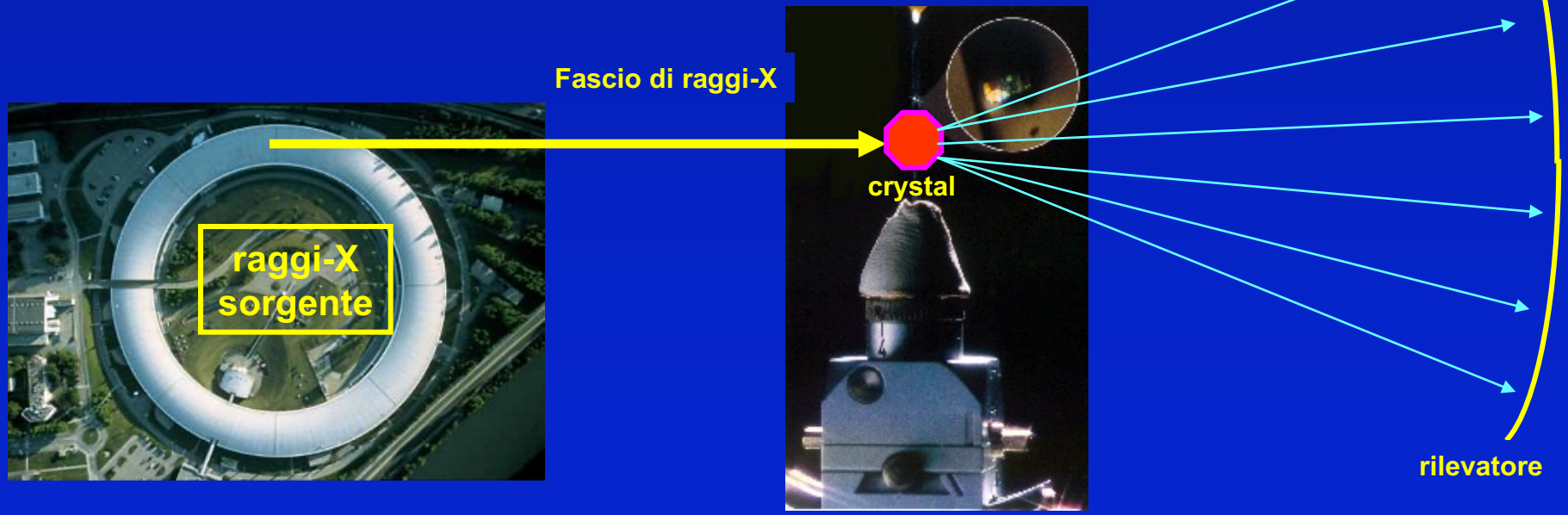
Protein structure



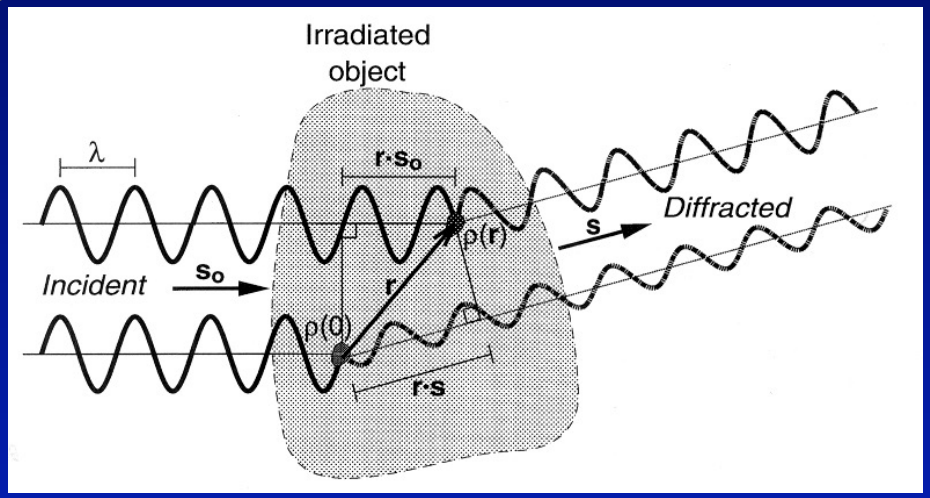
Protein folding

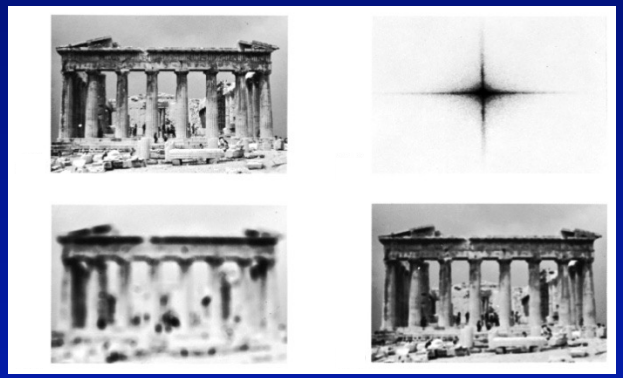
Structural studies by X-ray crystallography

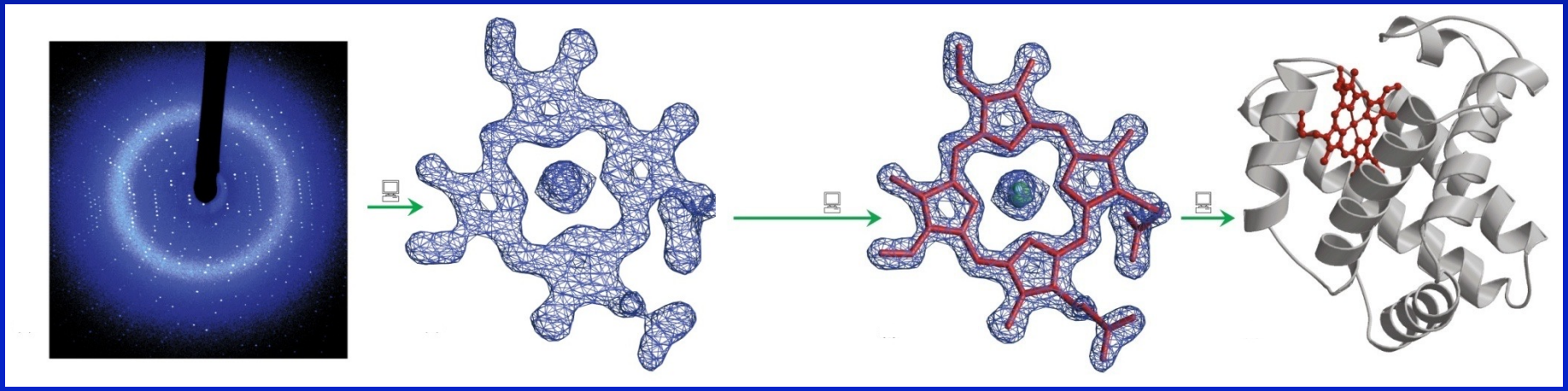




Structural studies by X-ray crystallography



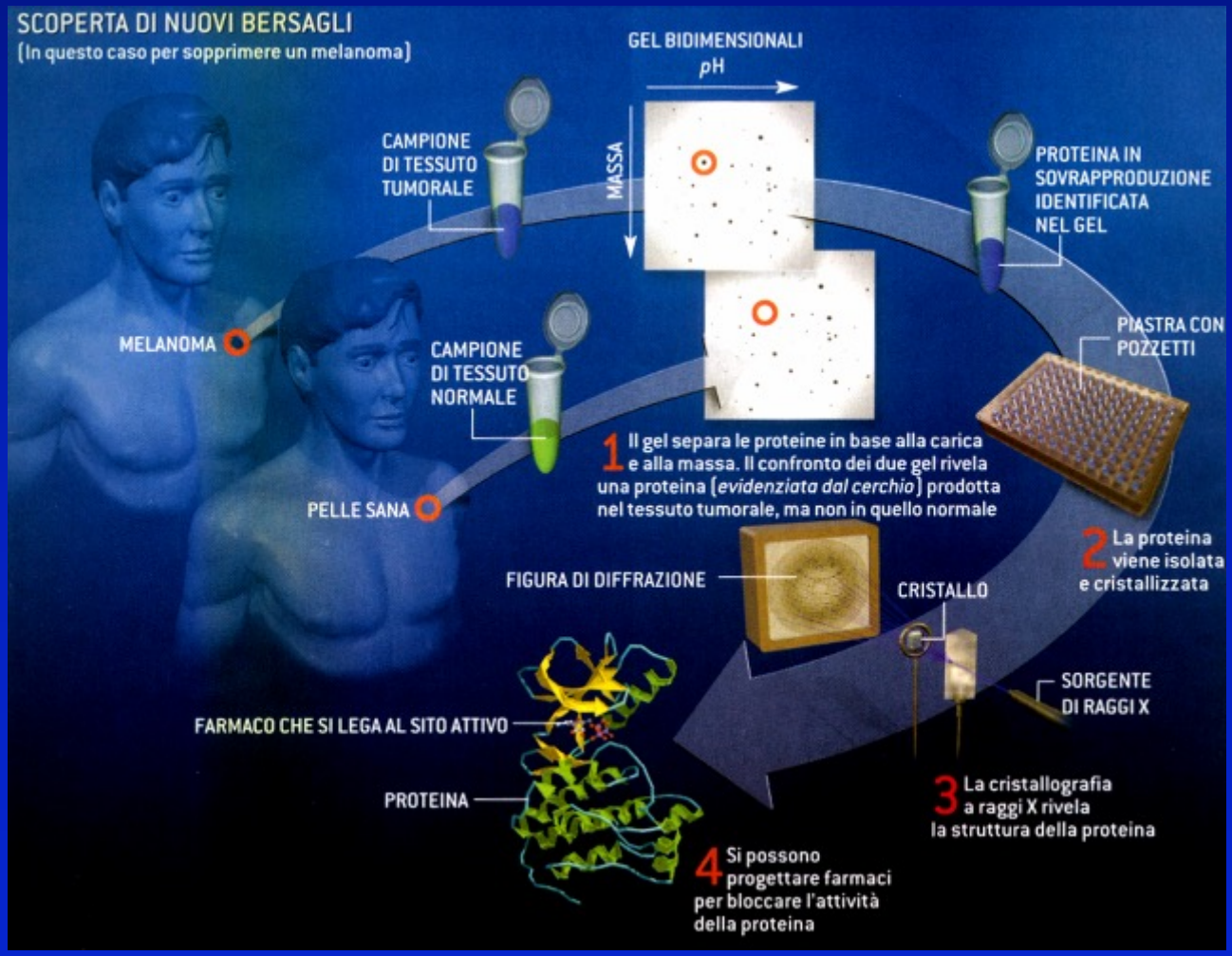




Rilevatore a raggi-X | Mappa della densità elettronica | Modello atomico preliminare | Modello atomico finale

Protein Data Bank: *http://www.rcsb.org/pdb/*

Biotechnological applications



Biotechnologies applied to genetically modified food control must take into account the evolving regulations

All the regulations provide for a proper screening program

What to look for?

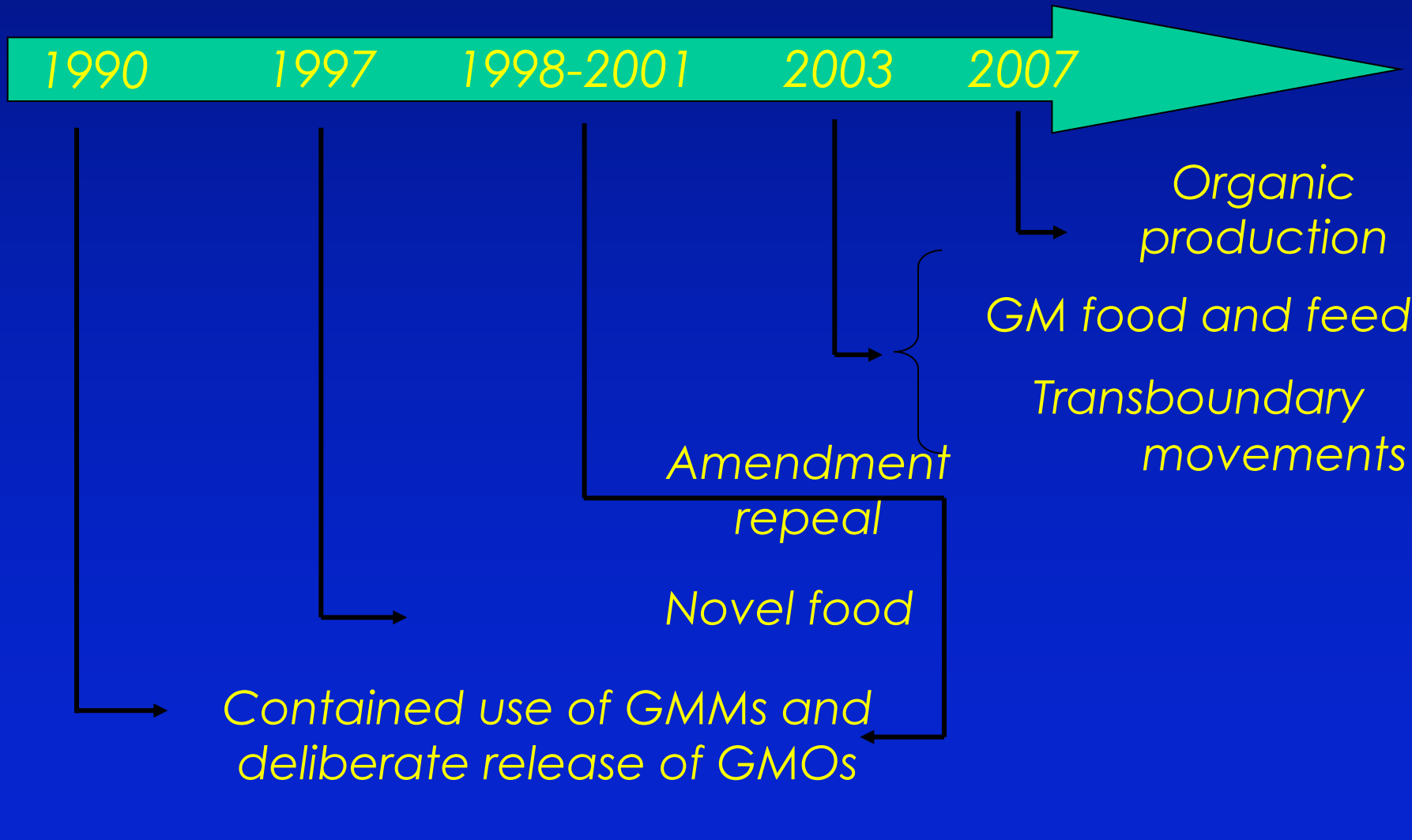
GMOs

How to search?

National Reference Laboratory for GMO analysis, Italy

Overview of the EU legislation on GMOs

Historical evolution



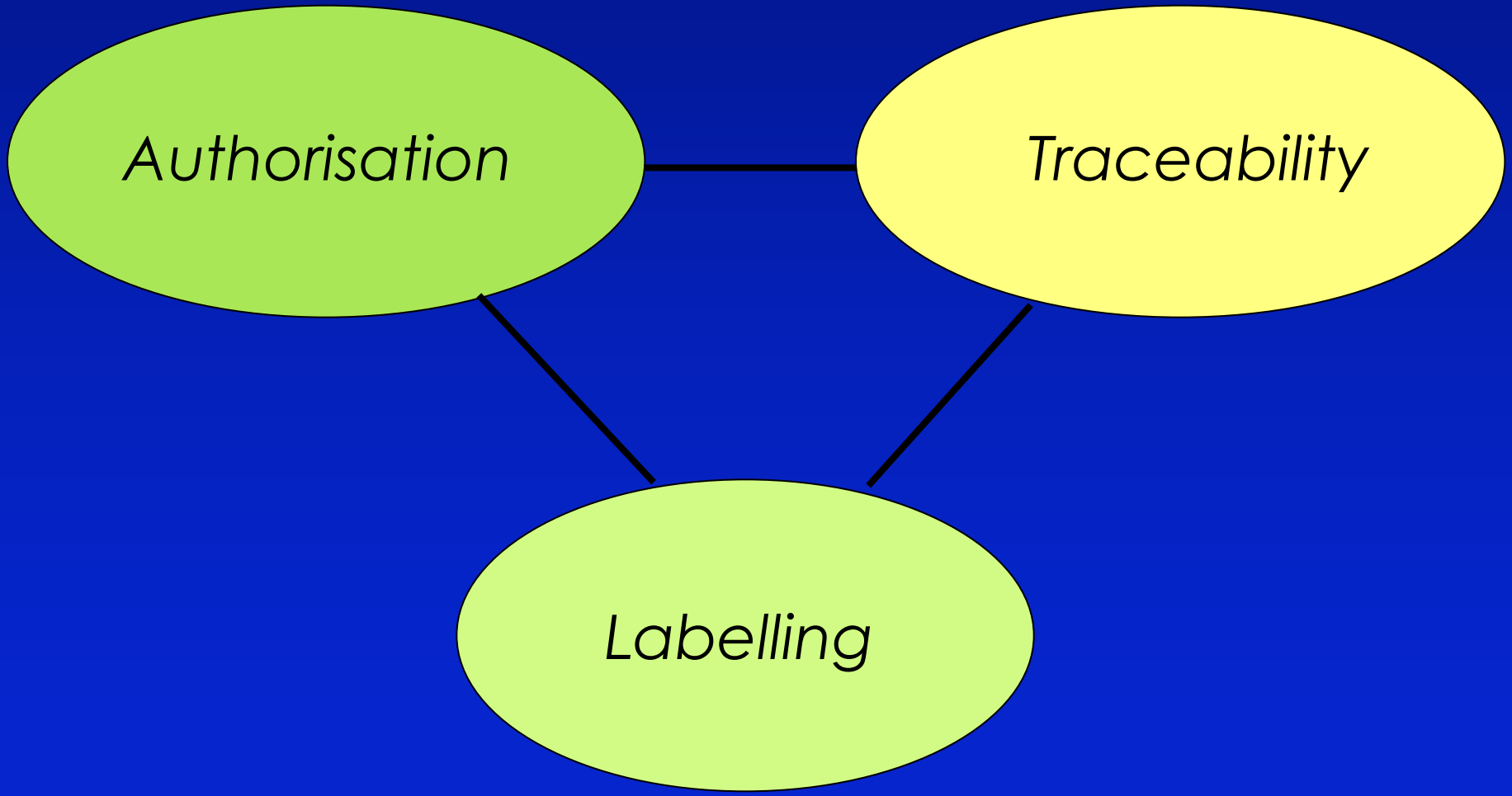
Overview

- *Directive 2001/18 on the deliberate release into the environment of GMOs*
- *Regulation (EC) No 1829/2003 of the European Parliament and of the Council on GM food and feed*
- *Regulation (EC) No 1830/2003 of the European Parliament and of the Council concerning the traceability and labelling of GMOs and the traceability of food and feed products produced from GMOs and amending Directive 2001/18/EC*
- *Commission Regulation (EC) No 641/2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new GM food and feed, the notification of existing products and adventitious or technically unavoidable presence of GM material which has benefited from a favourable risk evaluation*
- *Commission Regulation (EC) No 65/2004 establishing a system for the development and assignment of unique identifiers for GMOs*
- *Regulation (EC) 1946/2003 on transboundary movements of GMOs*
- *Recommendation 2004/787/EC on technical guidance for sampling and detection of GMOs and material produced from GMOs as or in products in the context of Regulation (EC) No 1830/2003*
- *Recommendation 2003/556/EC on guidelines for the development of national strategies and best practices to ensure the coexistence of GM crops with conventional and organic farming*

Main objective of EU legislation on GMOs

- protection of human life and health, animal health and welfare, environment and consumer interests in relation to GMOs
- ensuring the effective functioning of the internal market

EU legislation on GMOs



Directive 2001/18/EC on the deliberate release of GMOs into the environment

- Experimental release of GMOs into the environment (part B)
- Placing on the market of GMOs: e.g. cultivation, import, transformation (part C)

Regulation (EC) No 1829/2003

On genetically modified food and feed

Authorisation

Labelling

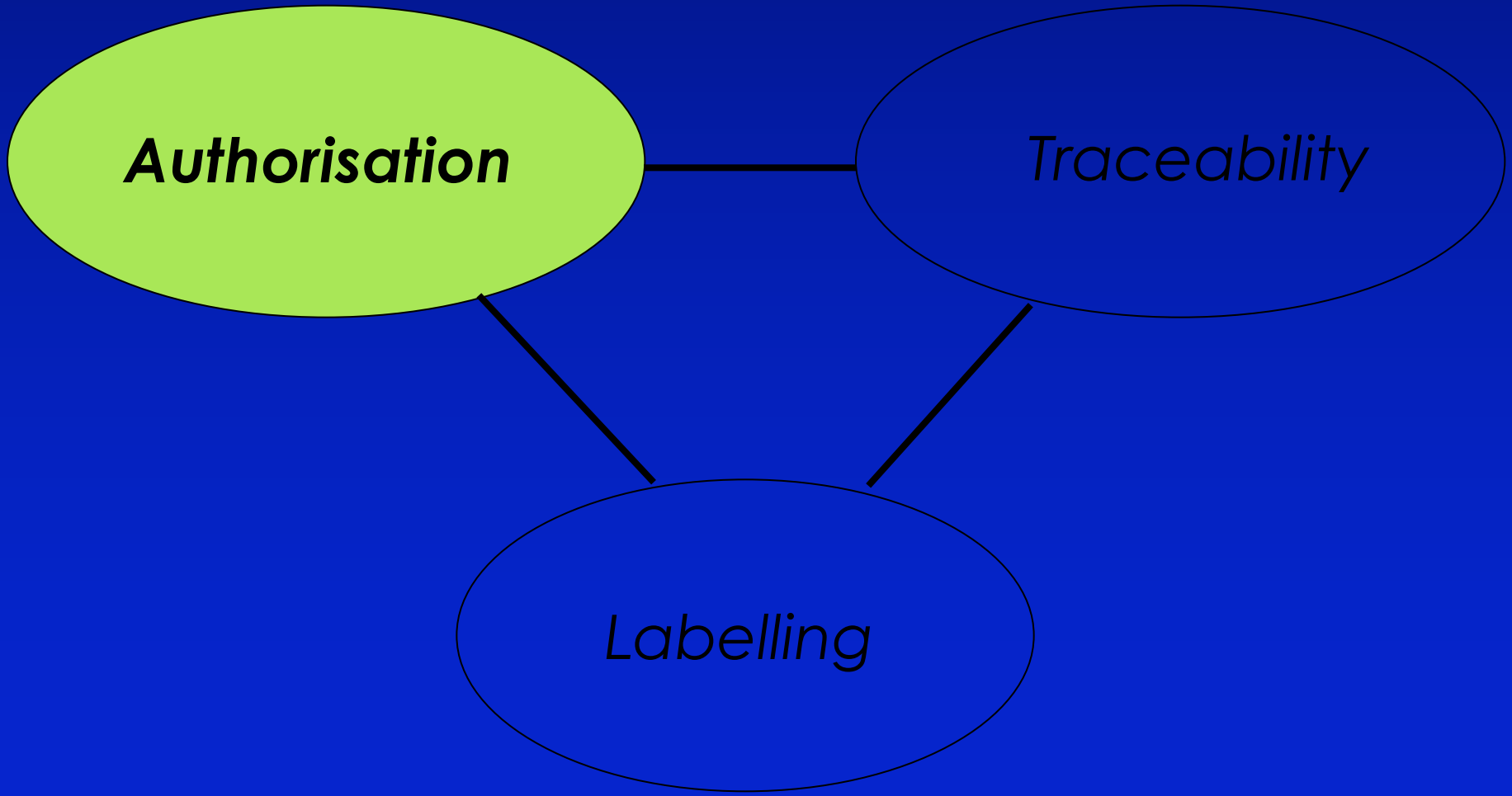
Regulation (EC) No 1830/2003

on the traceability and labelling of GMOs and the traceability of GM food and feed

Traceability

Labelling

EU legislation on GMOs



EU legislation on the placing on the market of GMOs

Principles

- Authorisation (10-year validity)
- Risk assessment

Environmental

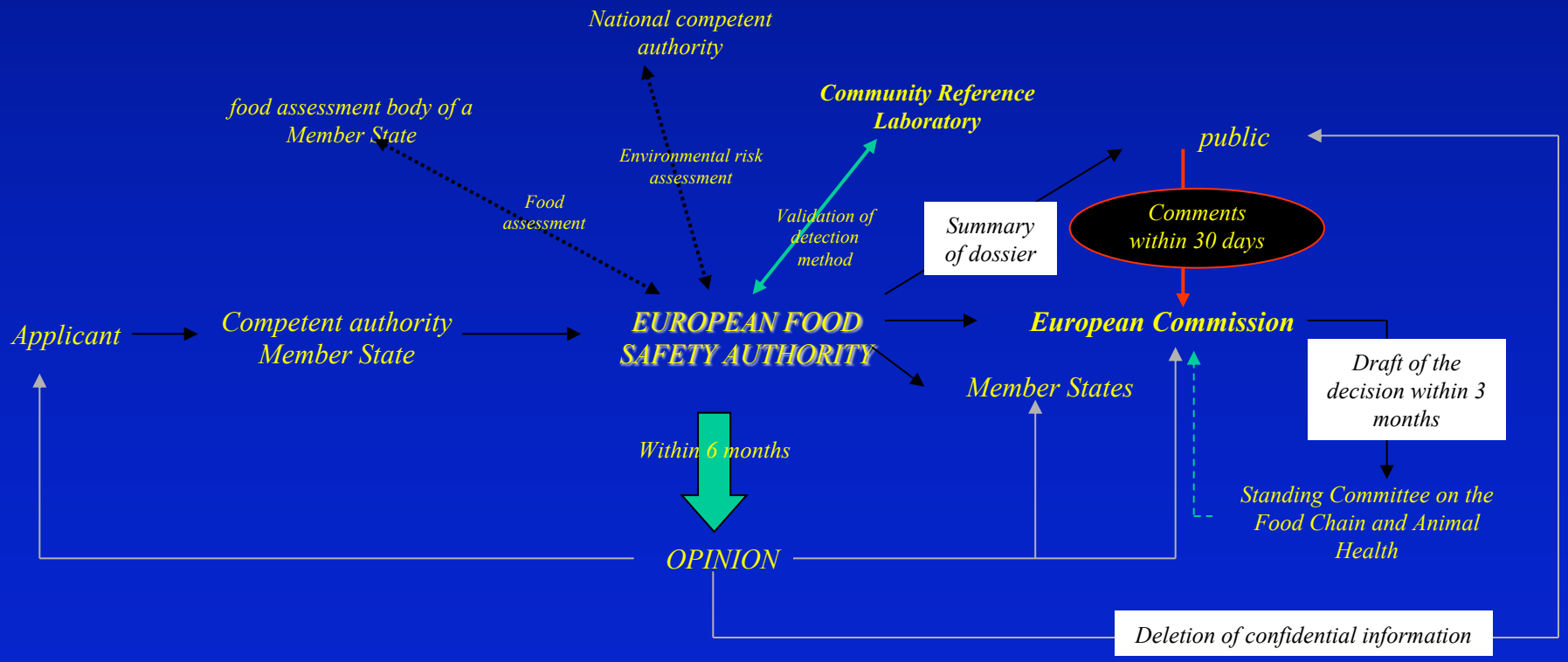
Food/feed

- Consultation of scientific committees
- Labelling and traceability
- Information and material for GMOs identification and detection
- Monitoring plan in order to identify effects of the GMO(s) on human health or the environment
- Safeguard clause
- Consultation of and information to the public

Reg. (EC) 1829/2003
Novelties

- One door, one key principle ⇒ same application procedure for different uses
- Authorisation granted for food and feed uses when both uses are expected

Reg. (EC) No 1829/2003
Authorisation procedure



Reg. (EC) No 1829/2003
Application for authorisation

Accompanied by the following:

- …omissis
- studies and any other available material to demonstrate that the food does not have adverse effects
- demonstration that the characteristics of the food are not different from those of its conventional counterpart or a proposal for labelling
- a reasoned statement that the food does not give rise to ethical or religious concerns or a proposal for labelling
- methods for detection, sampling and identification of the transformation event
- samples of the food and their control samples, and information as to the place where the reference material can be accessed

Reg. (EC) No 1829/2003
Authorisation

- valid throughout the EU for 10 years
- renewable for 10-year periods
- The authorised food shall be entered in the Community register of genetically modified food and feed available to the public (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm)

- …. the unique identifier attributed to the GMO (as defined in Regulation (EC) No 65/2004)

http://www.gmo-compass.org/eng/gmo/db/

Community Register of GM food and feed

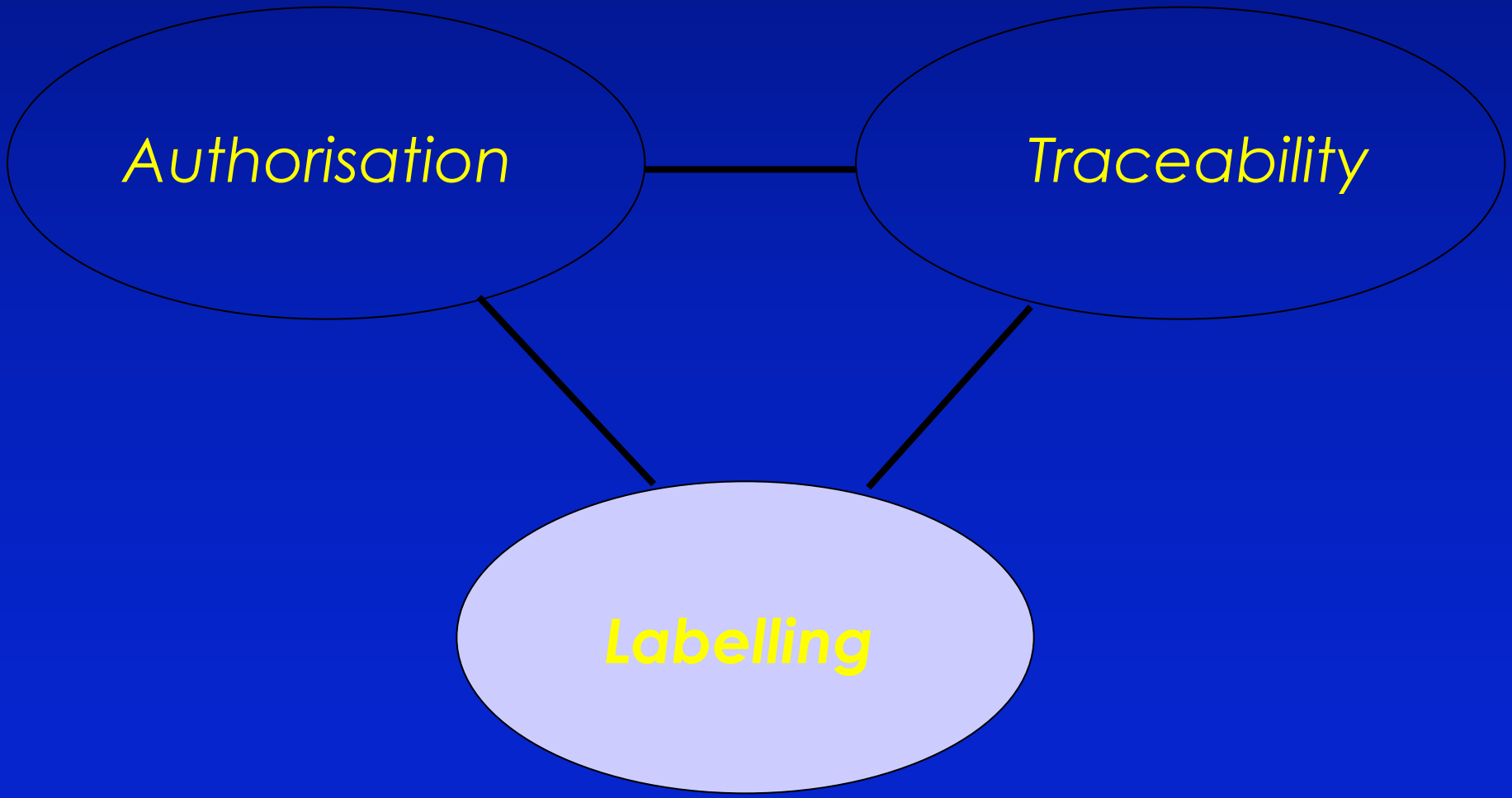
Status of authorisation of GM food and feed in EU:

authorised events

Community Register of GM food and feed

http://ec.europa.eu/food/dyna/gm_register/index_en.cfm

EU legislation on GMOs



EU legislation on GMOs Labelling

- Specific labelling requirements when GM material > 0.9% of the food/feed ingredient
- Labelling not compulsory when GM material < 0.9%, provided that this presence is adventitious or technically unavoidable

  ⇨ operators must be in a position to supply evidence to satisfy the competent authorities that they have taken appropriate steps to avoid the presence of such material

EU legislation on GMOs

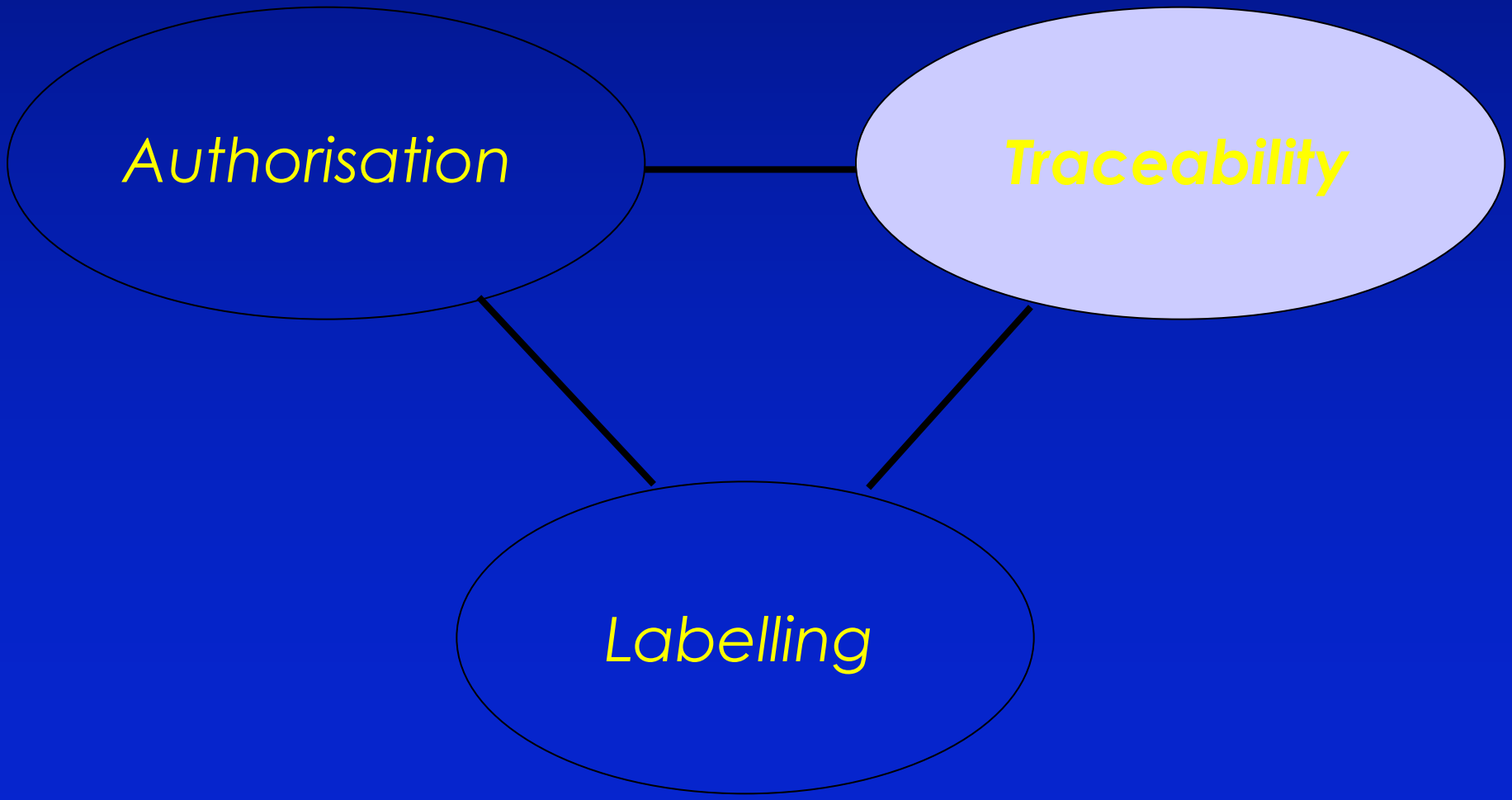
Labelling

~~CONTAINS GMO~~

CONTAINS
GM "NAME OF THE INGREDIENT"

e.g. CONTAINS GM MAIZE FLOUR

EU legislation on GMOs



EU legislation on GMOs
Traceability

ability to trace GMOs and products produced from GMOs at all stages of their placing on the market through the production and distribution chains

- traceability of GMOs
- traceability of food and feed products produced from GMOs

Reg. (EC) No 1830/2003
Traceability

<u>WHAT</u> information shall be transmitted:

above the 0,9% threshold

- That the product/ingredient consists of or contains or is produced from GMOs
- For products containing or consisting of GMOs, the **Unique Identifier** shall be provided

TO WHOM information shall be transmitted:

Biotech & Breeding
(Institutions/Companies)

Agriculture

Food Processors

Food Retailers

Consumer

At all subsequent stages of the placing on the market, information received is transmitted to the operators receiving the products

At the first stage of the placing on the market, information is transmitted to the operator receiving the product

Traceability

- Operators shall have in place systems and standardised procedures to allow:
  - ✓ the holding of information
  - ✓ the identification, for a period of five years from each transaction of the operator by whom and the operator to whom the products have been made available

Traceability and labelling requirements do not apply to:

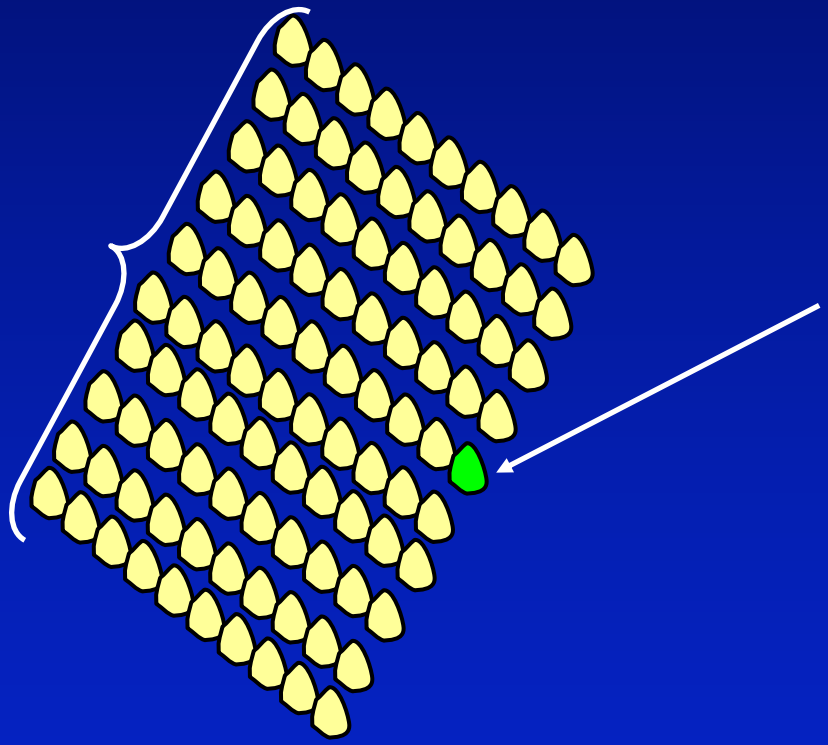
- GM processing aids used only during the production process
- animals fed with GM feed

Reg. (EC) 834/2007

on organic production and labelling of organic products (repealing Regulation (EEC) 2092/91)

- Prohibition of use of GMOs and products produced from or by GMOs
- 0.9% - tolerance threshold

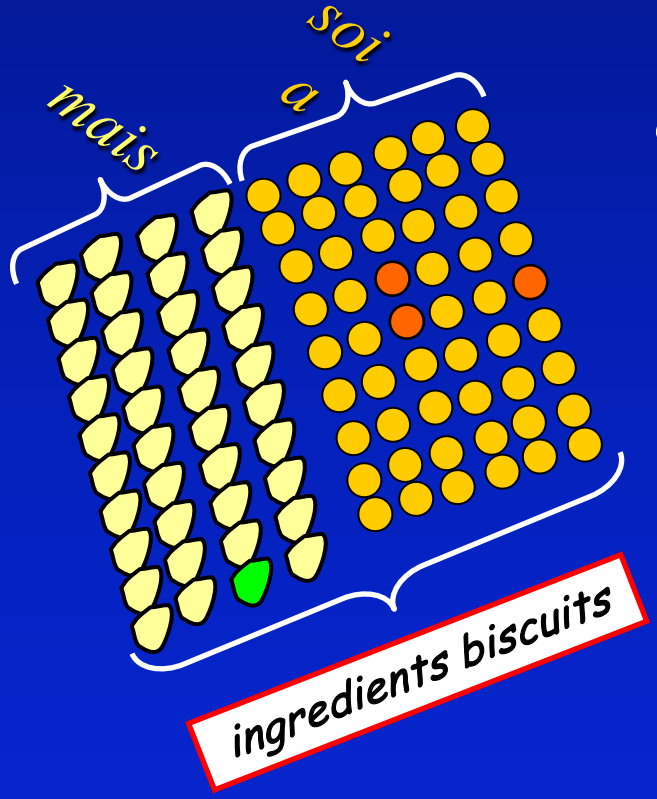
(CE) N° 1829/2003
Food labelling (artt. 12 e 24)



If a package of corn (for popcorn) contains 100 grains including one GM the percentage is 1% and is greater than the threshold (0.9%), therefore, the label must indicate:

contains MAIS GM

IF YOU ARE ANALYZING A FOOD CONSTITUTED BY MORE THAN ONE INGREDIENT?

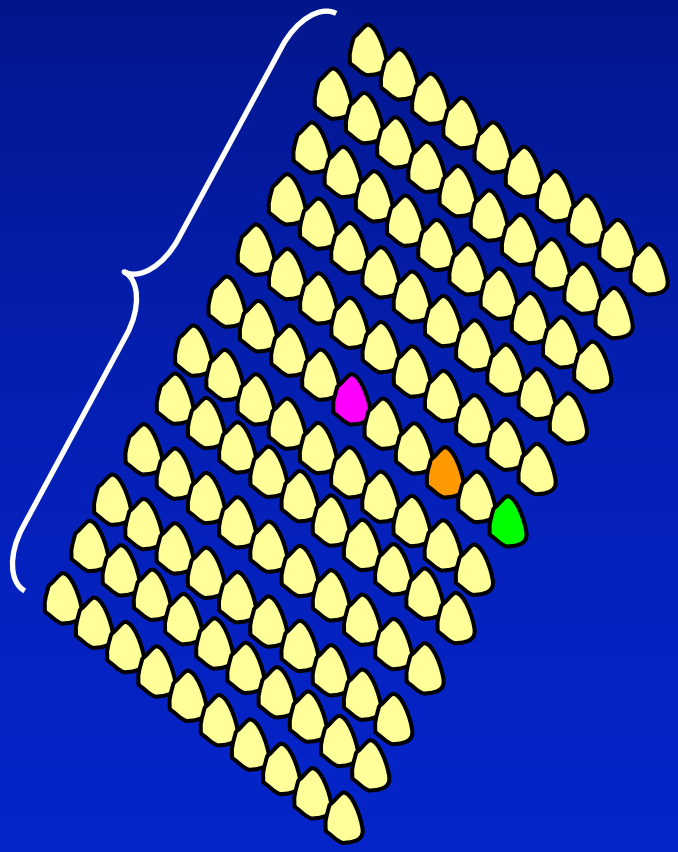


If you are analyzing biscuits containing both corn flour and soy, it should be evaluated for each ingredient (corn and soybeans) the presence of DNA produced from GMOs, checking for each species if the percentage of transgenic is present (respectively GM maize and GM soybeans) less than the threshold of 0.9%;

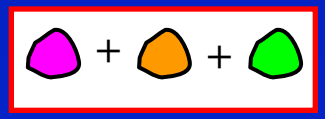
Suppose that GM soy is 1.3% and GM maize is 0.7%, only in the first case is the threshold, so you have to write on the label:

Contains GM soy

AND IF FOR AN INGREDIENT (eg. Zea mays) EXIST DIFFERENT TYPES (EVENTS) OF GMO?



The individual GMO, if any, are identified by searching the sequences (transformation events) affecting them; the% of GM maize will be the sum of individual contributions



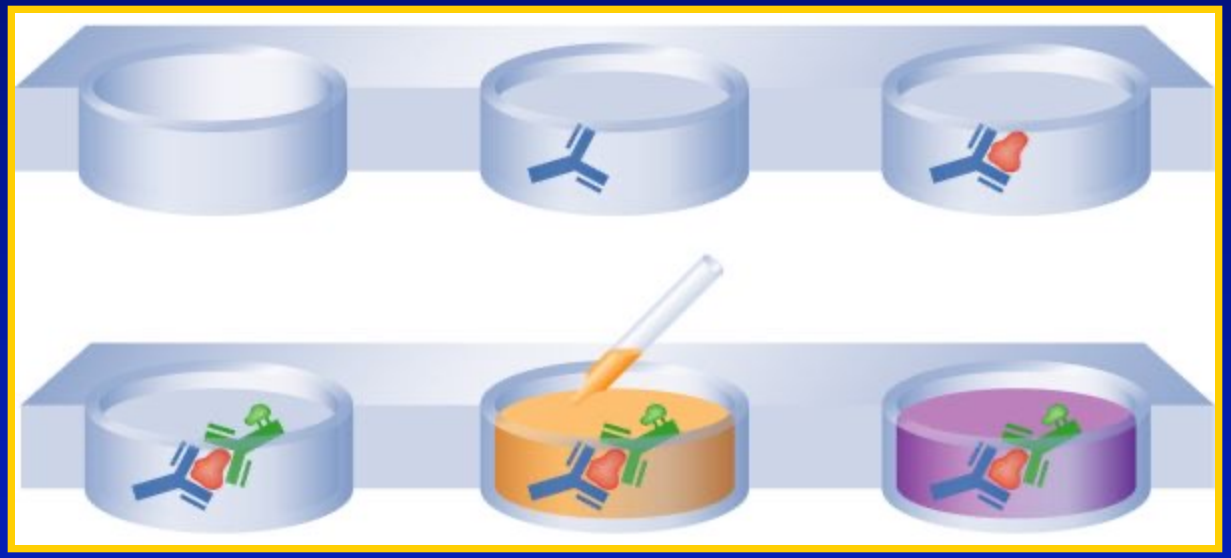
METHODS USED FOR OFFICIAL CONTROL OF GMOs IN FOOD INDUSTRY

RESEARCH OF GMOs IN FOOD CAN BE DONE THROUGH:

ANALYSIS OF PROTEINS

DNA analysis

METHODS FOR THE ANALYSIS OF PROTEINS: ELISA



PRO and AGAINST

Very easy execution

rapid

inexpensive

quantitative

sensitivity is not high

rarely applicable on transformed food

METHODS FOR DNA ANALYSIS: the PCR

PRO e *AGAINST*

- *high sensitivity and specificity*
- *applicable to transformed food*
- *quantitative*
- *expensive*
- *requires adequate laboratory facilities*

DIFFERENT STEPS BY PCR

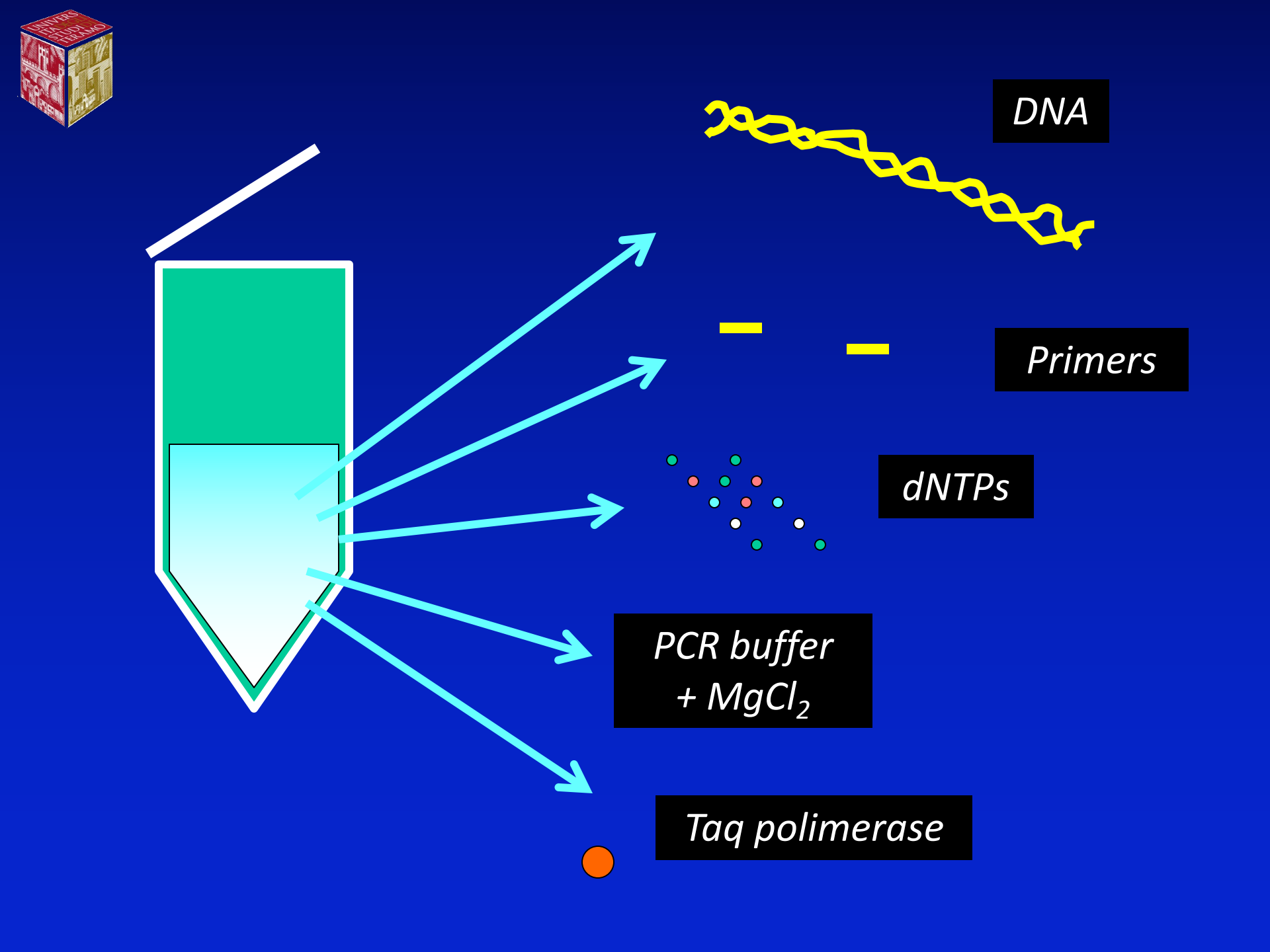
1. PCR to research the plant species corresponding to the ingredient ●
2. PCR Screening GM on single genetic elements ● ●
3. Quantitative real-time PCR { *event specific PCR* / *on the specific transgene* }

OGM Identification ⟹ *OGM Quantification*

PCR

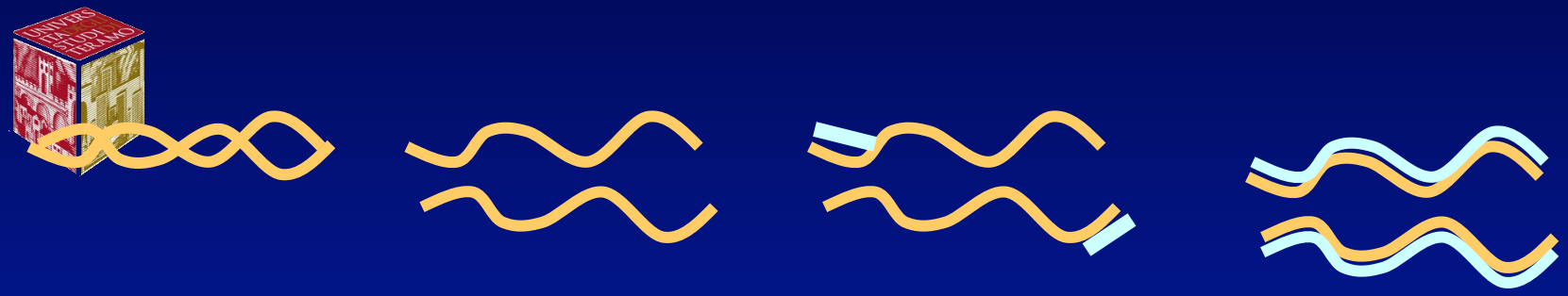
Polymerase Chain Reaction

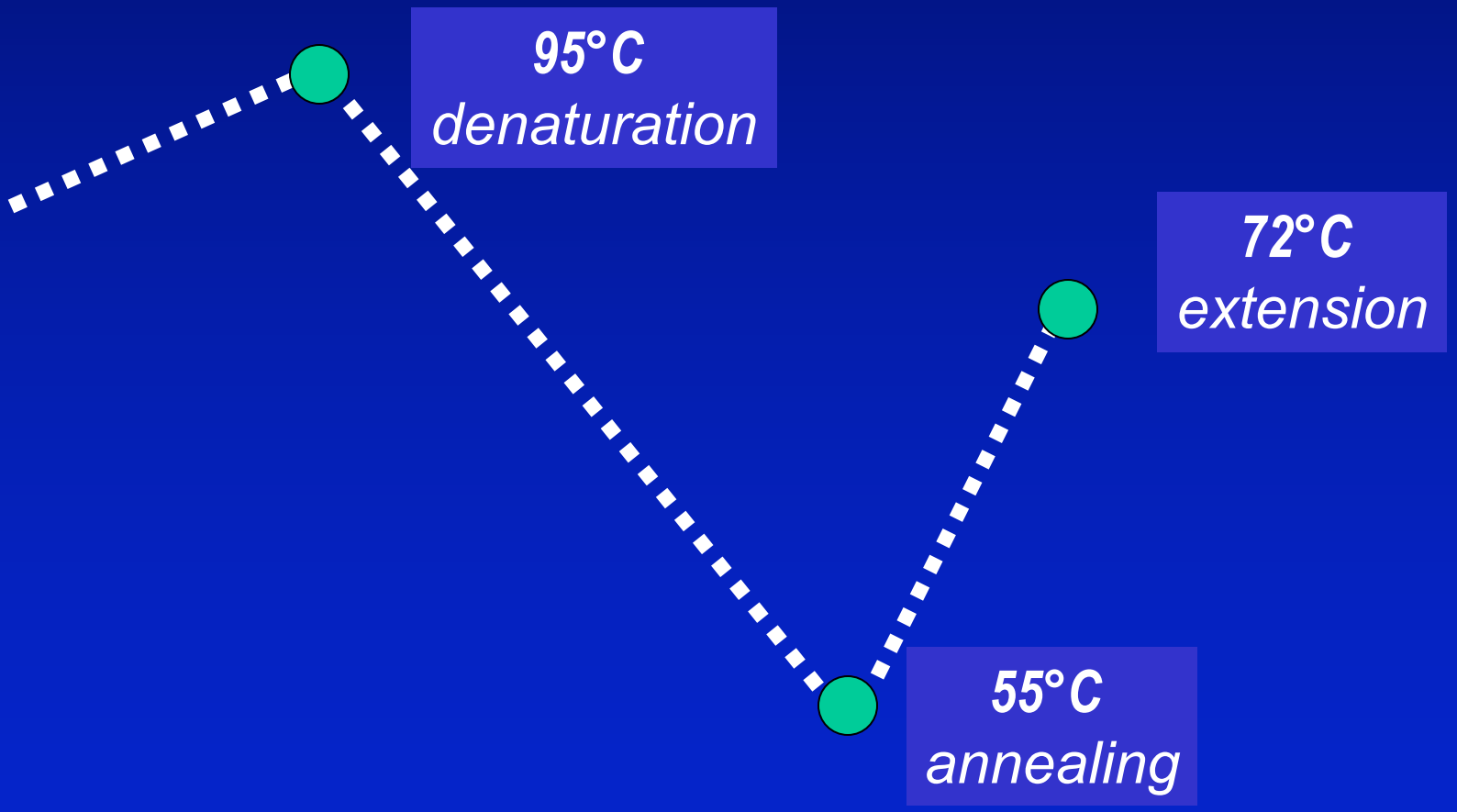
Saiki R et al, Science 1985


DNA
Primers
dNTPs
PCR buffer
+ MgCl2
Taq polimerase

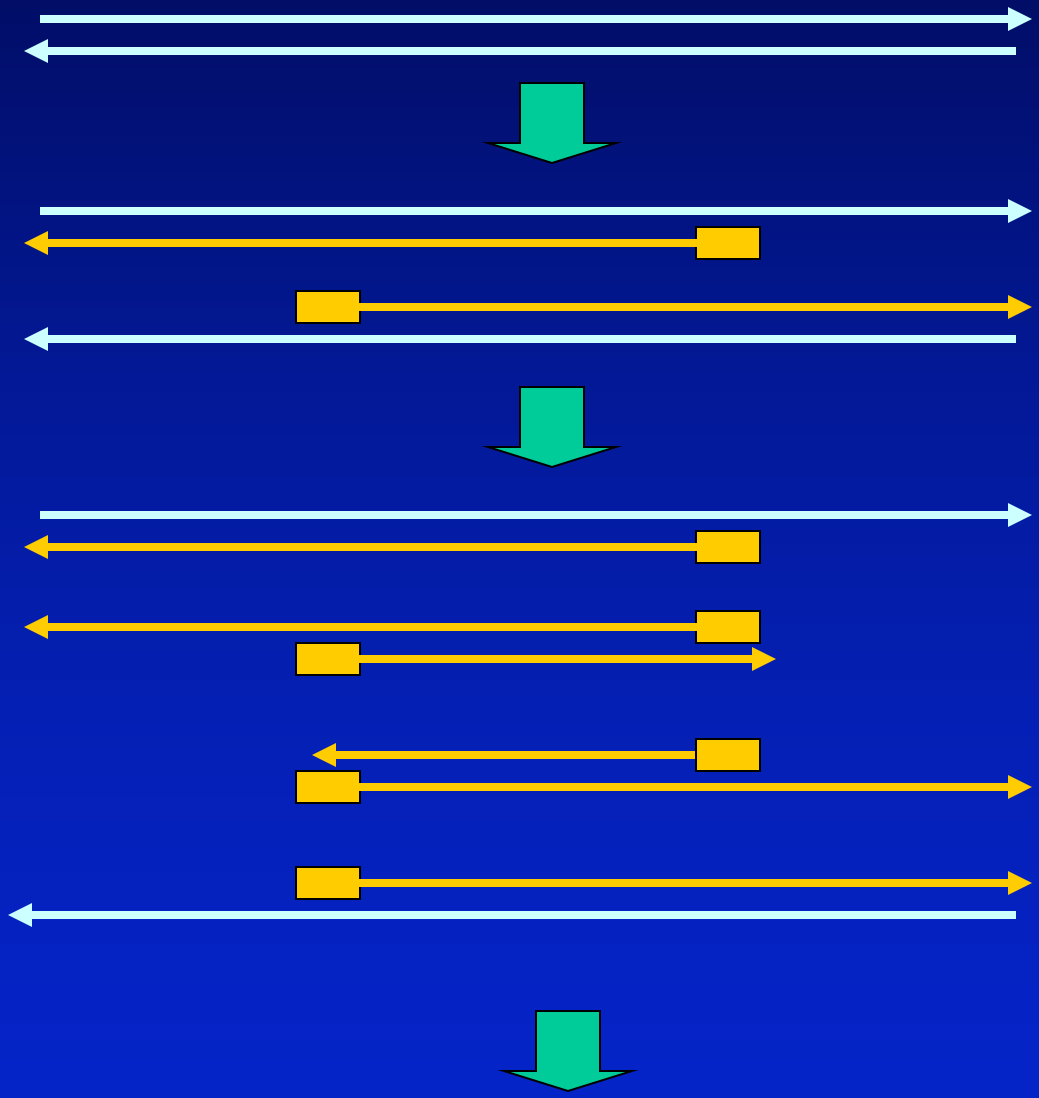
Taq polimerase *From T. aquaticus*

DNA-dependent DNA polymerase
Thermostable even at temperatures> 90 ° C
Optimum activity around 72 ° C
Optimum pH range: 8.2-9.0 in 10 mM Tris (25 ° C)



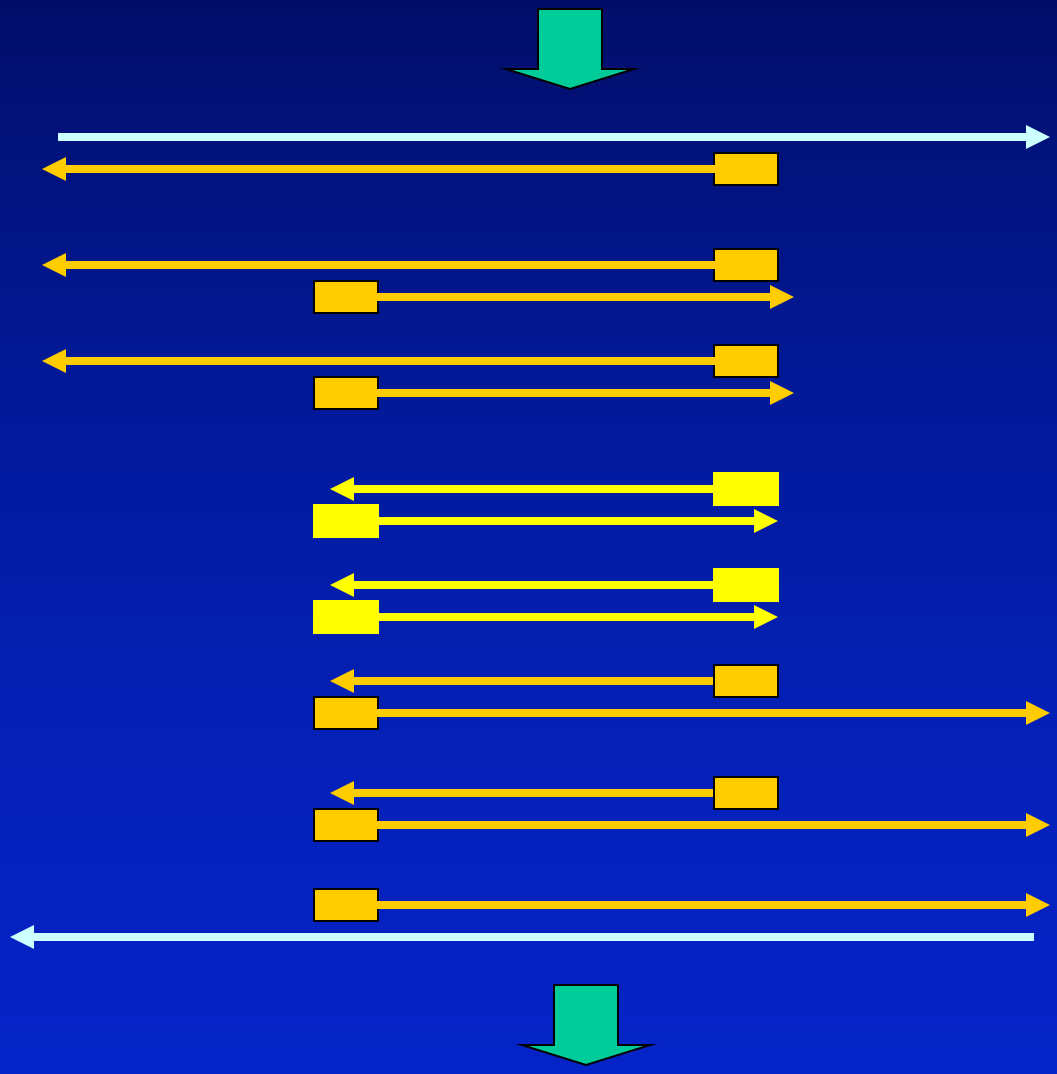

95°C
denaturation
72°C
extension
55°C
annealing

DNA ds



Cycle 1

Cycle 2



Cycle 3

further amplifications

$$N = N_O (1+E)^n$$

N = *final number of molecules of DNA*

N_O = *initial number of molecules of DNA*

E = *efficiency of the reaction (from 0 to 1)*

n = *number of PCR cycles*

e.g.: $E = 0.85$

$n = 30$ $\quad N = N_o (1+0.85)^{30}$

$= N_o \times 103.550.000$

Checking the results of the PCR

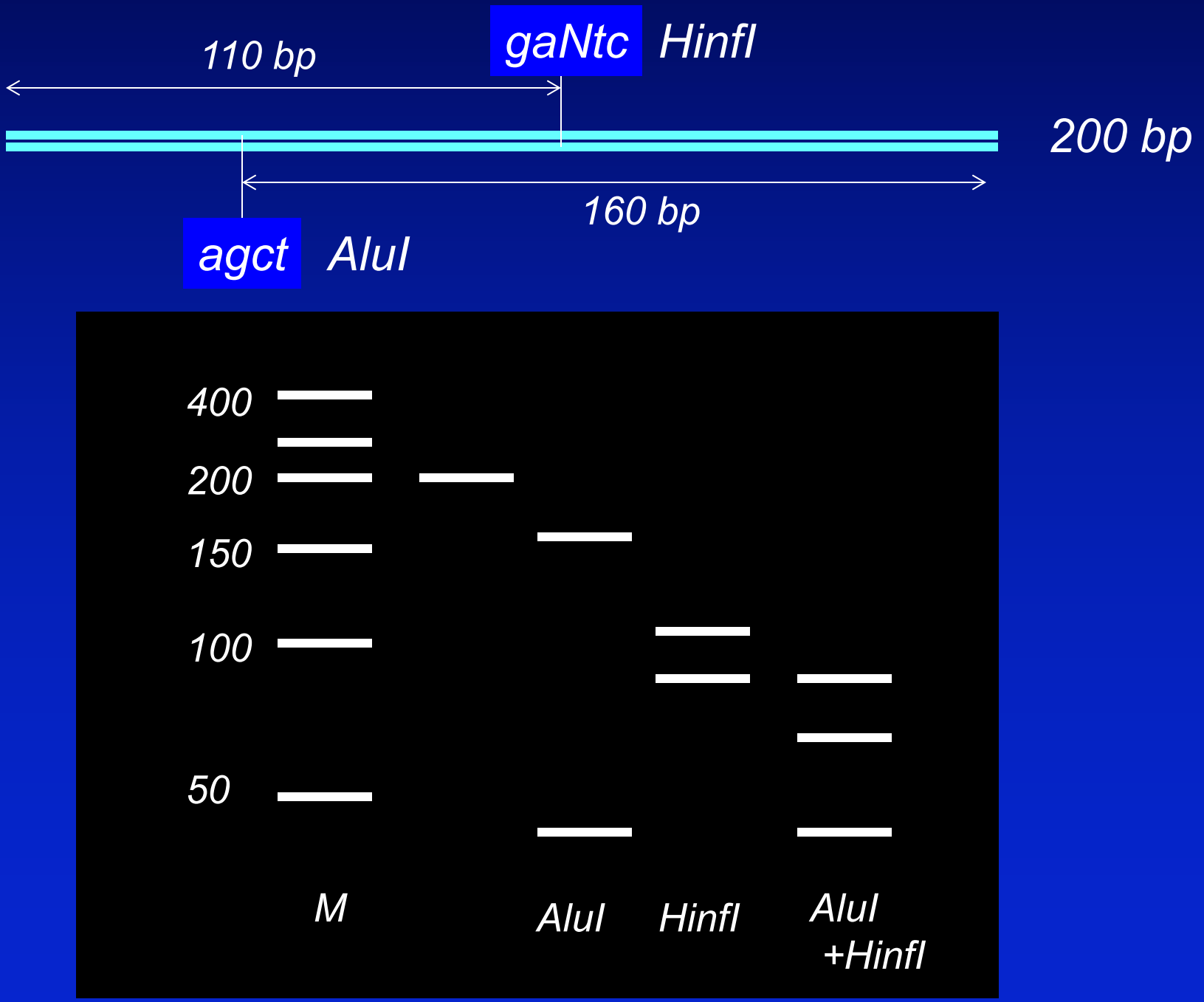
Analysis of the length of amplified fragment by gel electrophoresis and staining with bromide ethidium

Restriction analysis with specific endonucleases

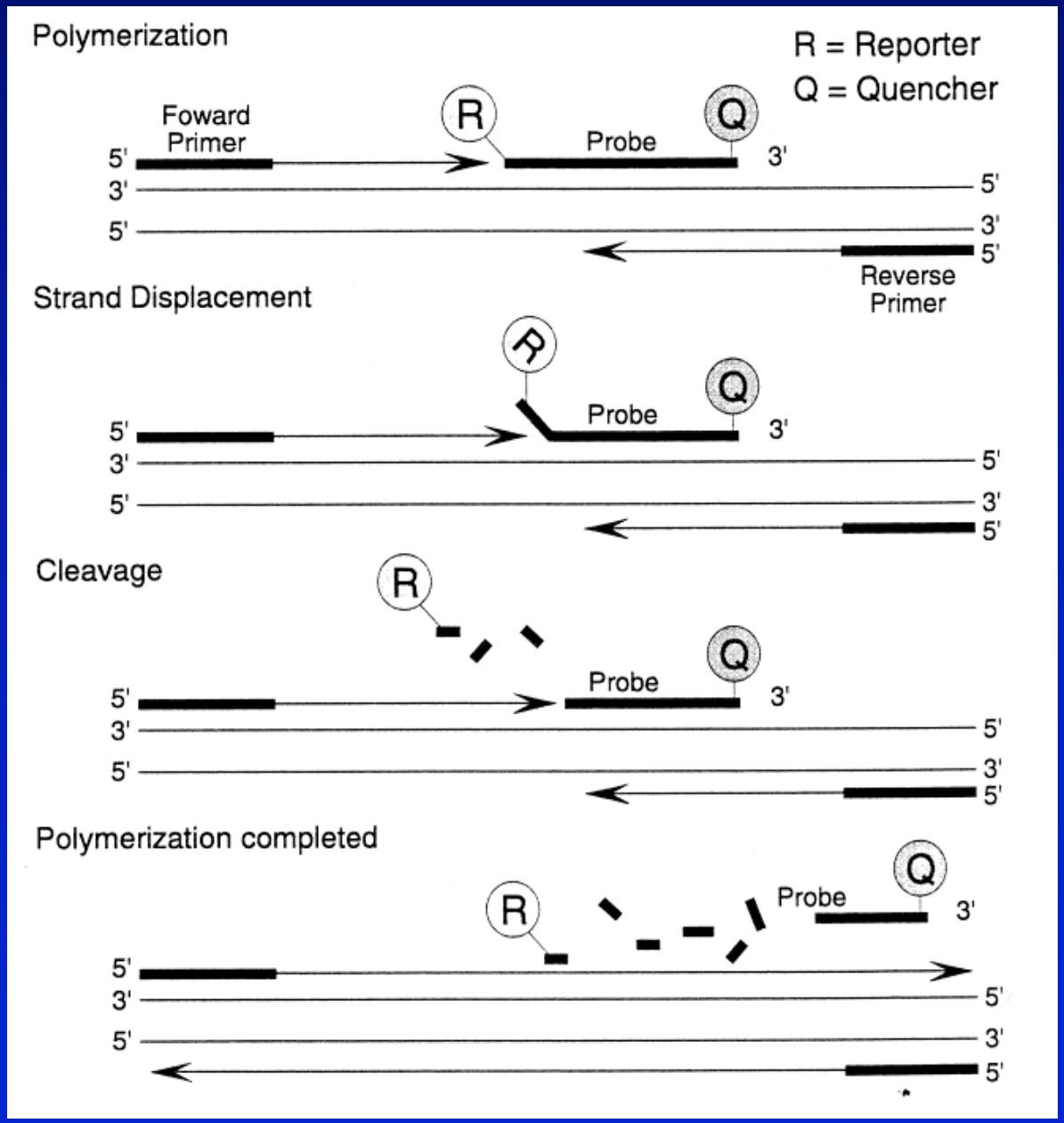
Hybridization with labeled probes (Southern or Dot blotting)

Immuno-PCR ELISA

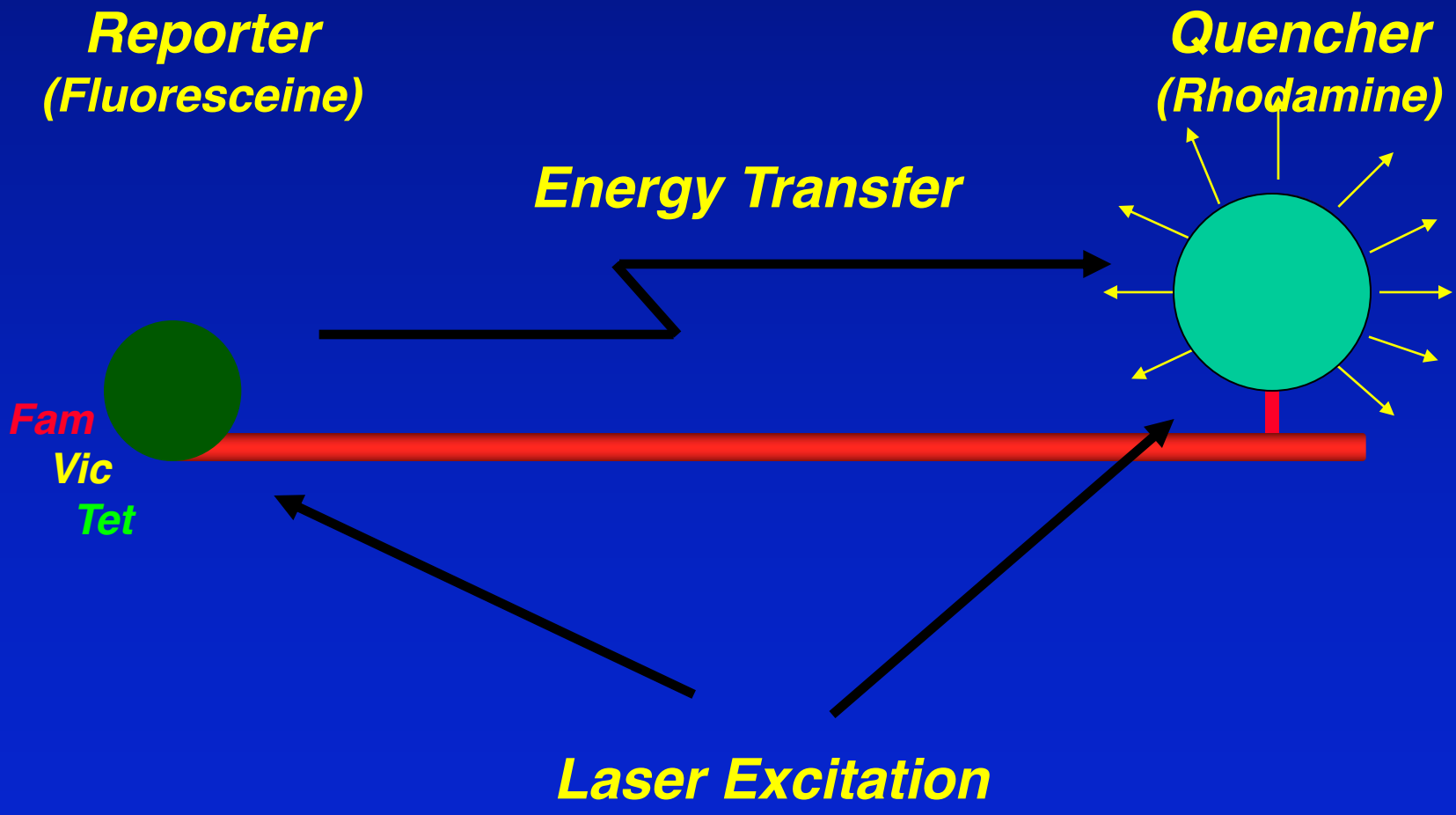
Direct sequencing


110 bp
gaNtc
HinfI
200 bp
160 bp
agct
AluI
400
200
150
100
50
M
AluI
HinfI
AluI
+HinfI

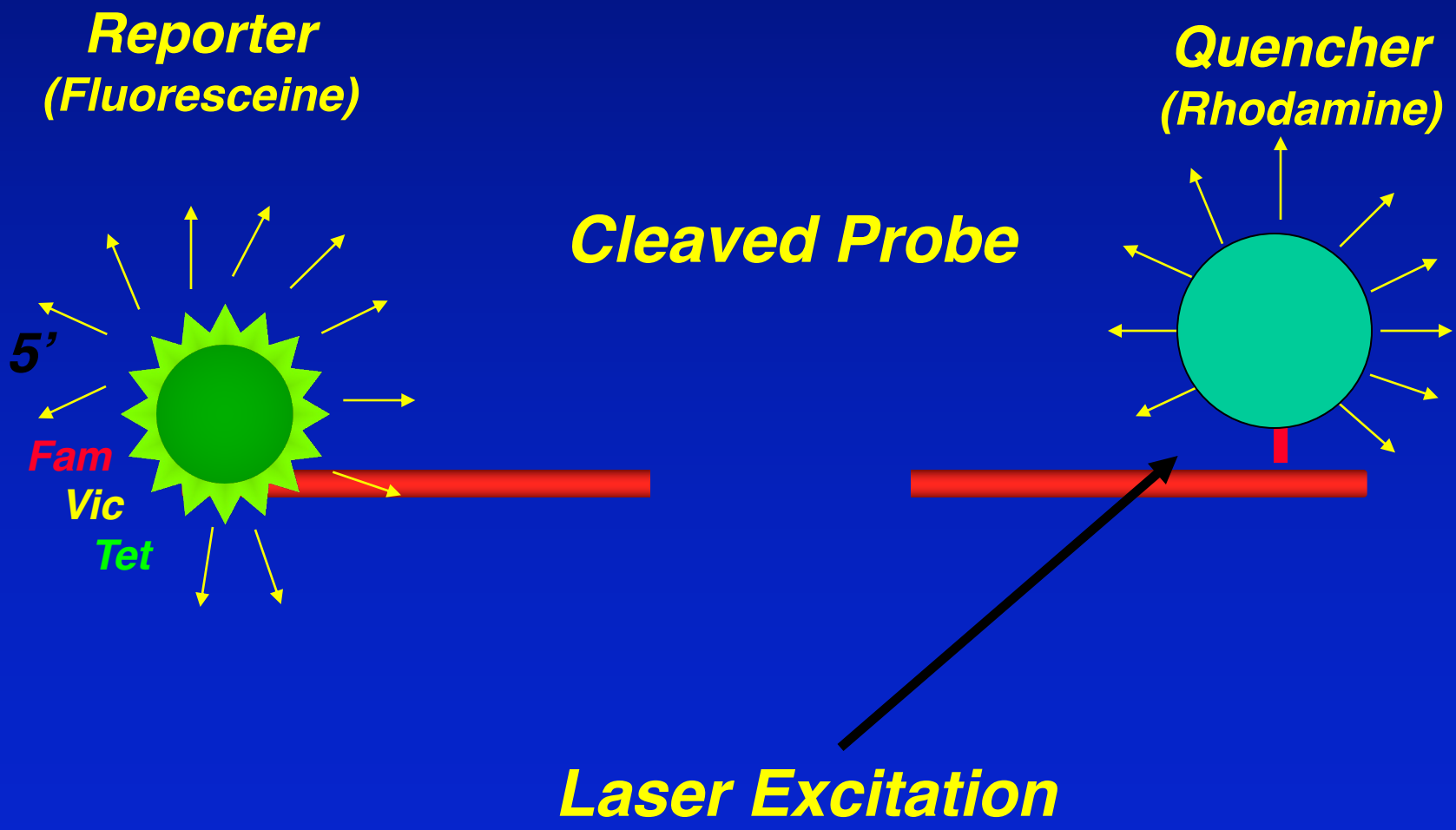
Quantitative analysis by REAL-TIME PCR



TaqMan™ Fluorogenic Probe



TaqMan™ Fluorogenic Probe



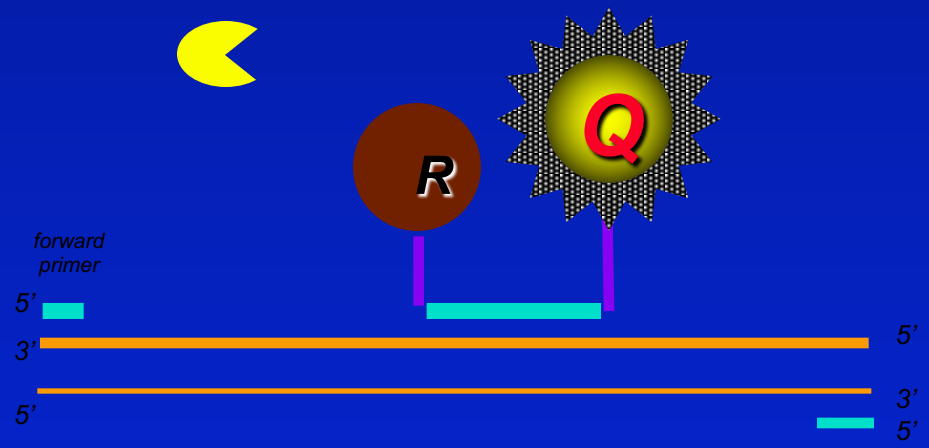
Taqman PCR Chemistry

Denaturation → *Annealing*

R = Reporter

Q = Quencher

- *Polymerization*



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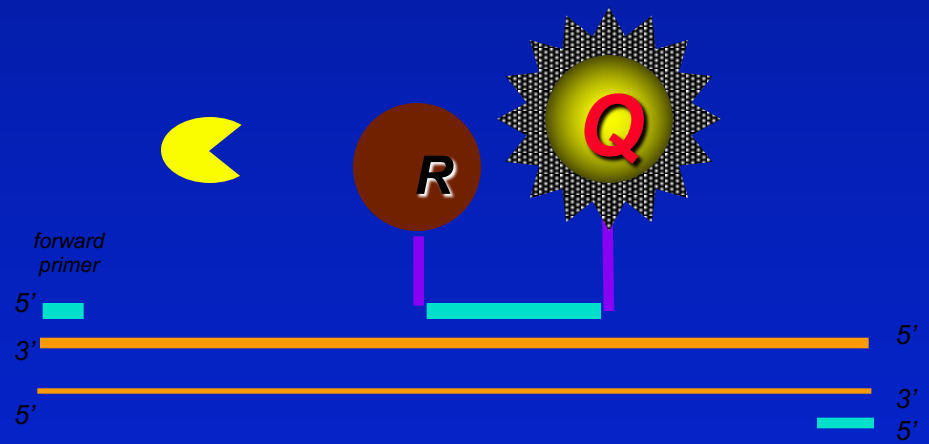

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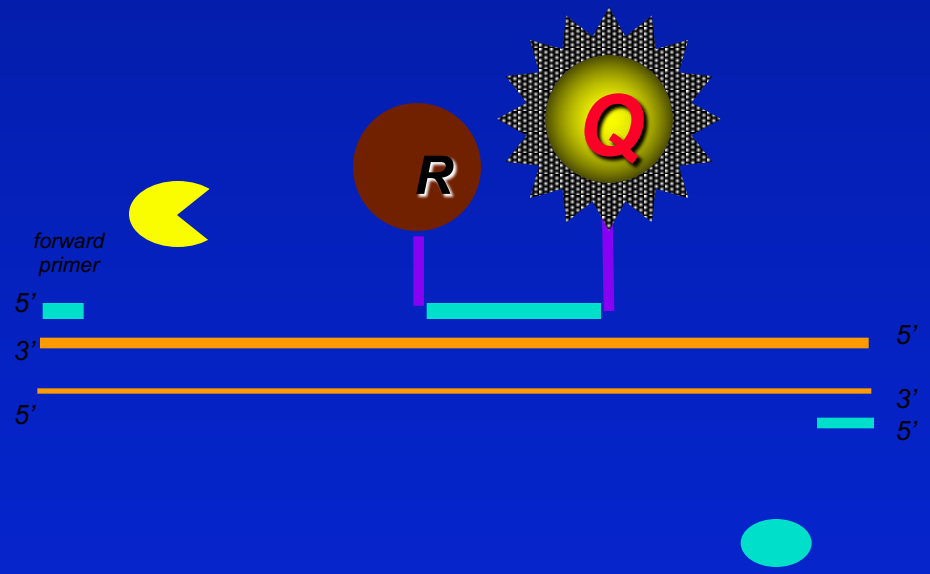

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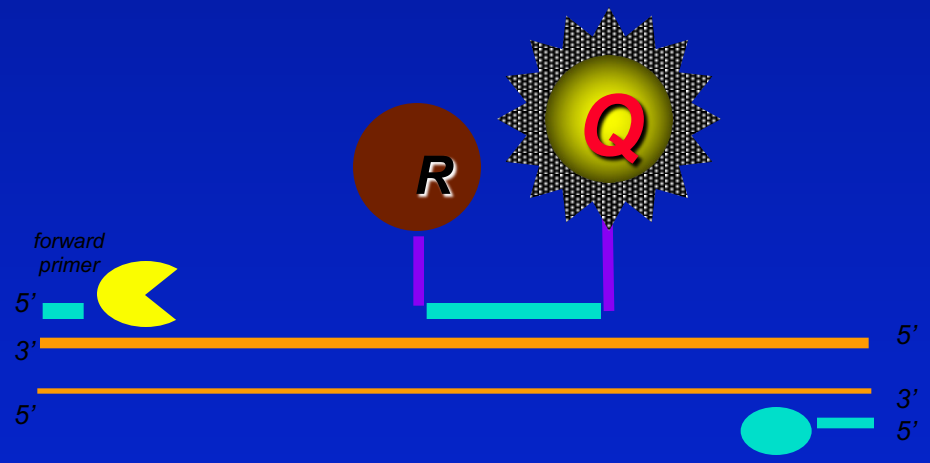

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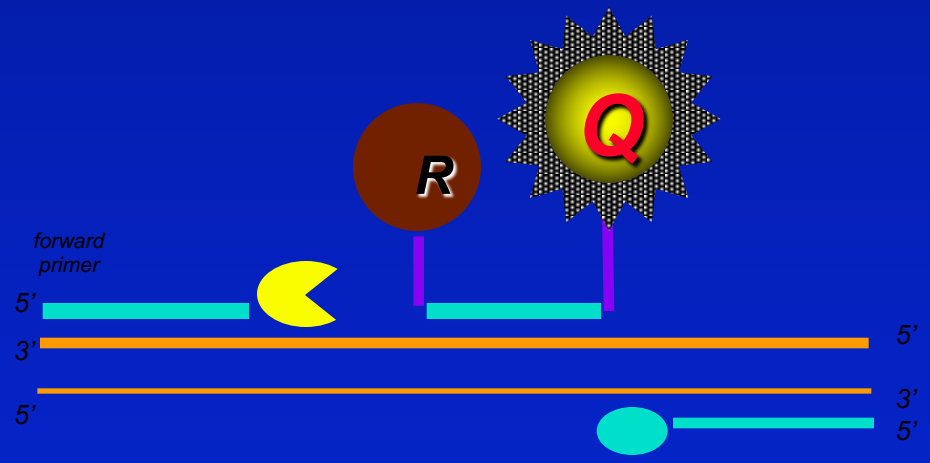

Taqman PCR Chemistry

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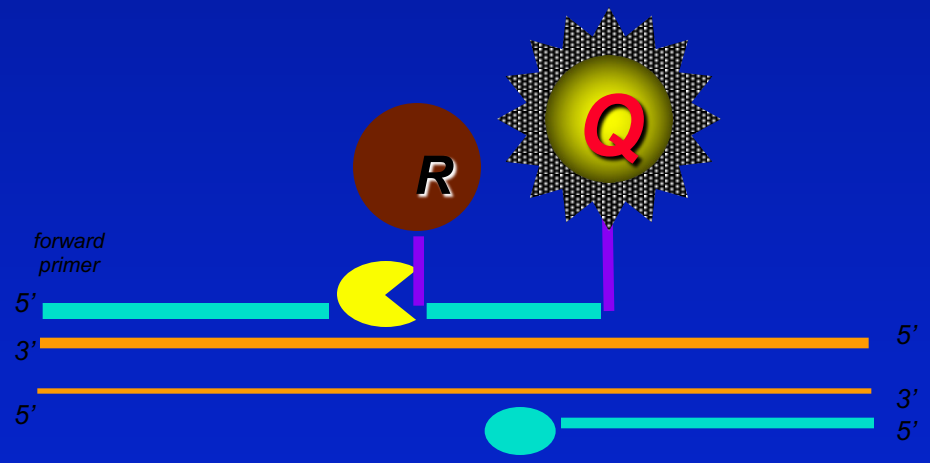
Q = Quencher



Taqman PCR Chemistry

Denaturation → *Annealing*

↓

- *Polymerization*

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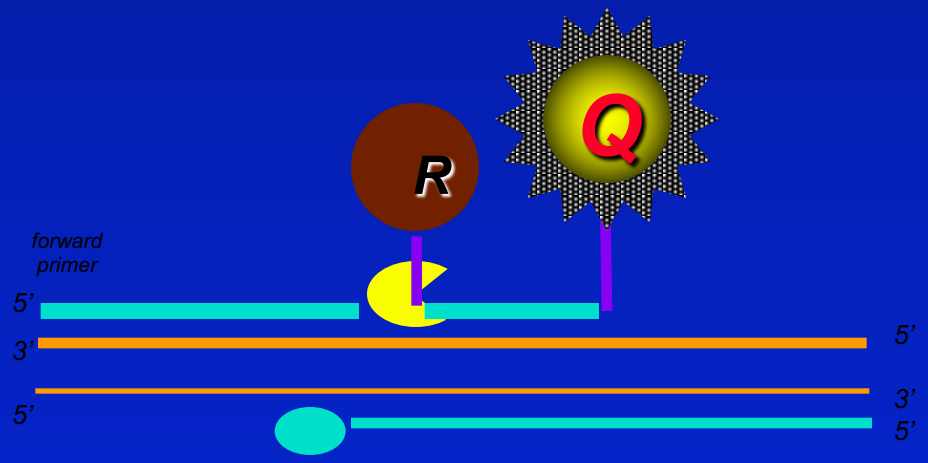
Q = Quencher

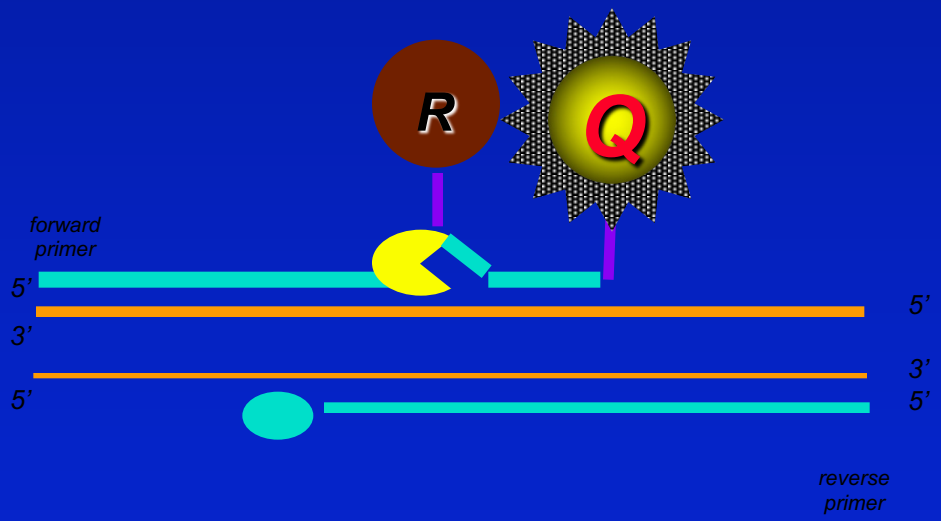


Taqman PCR Chemistry

- Strand displacement

R = Reporter

Q = Quencher

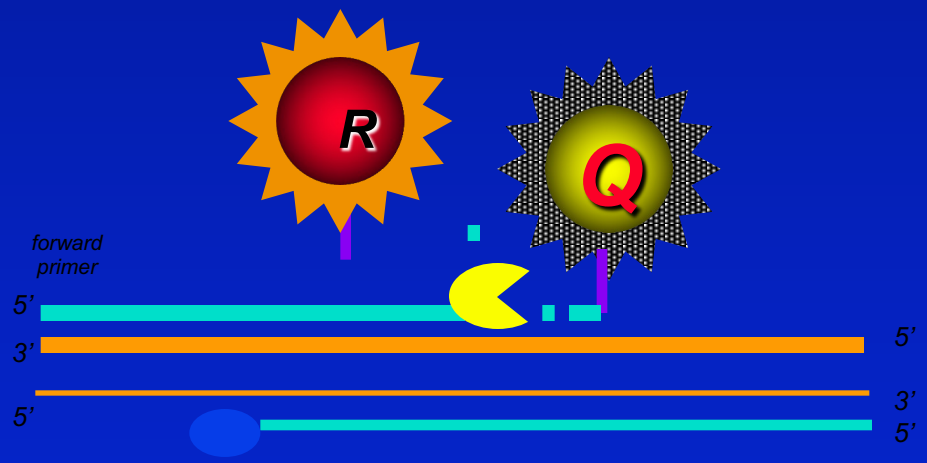


Taqman PCR Chemistry

- *Cleavage*

R = Reporter

Q = Quencher

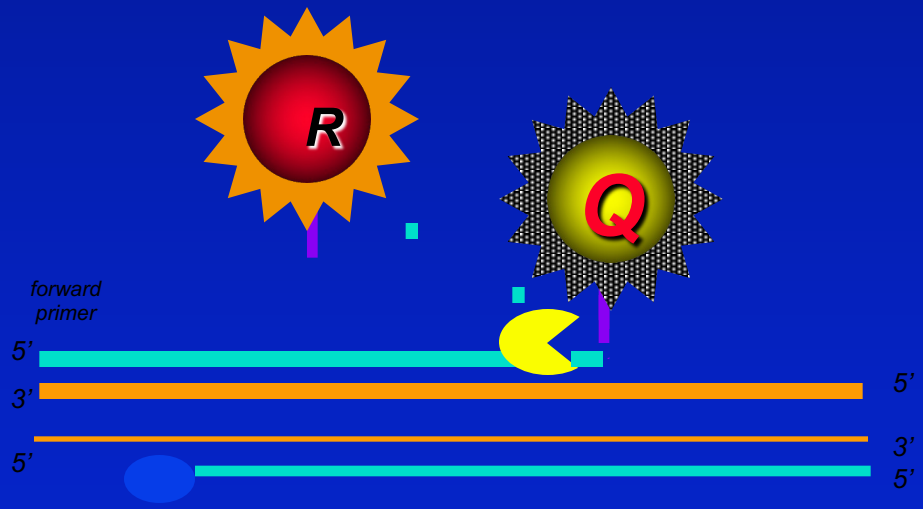


Taqman PCR Chemistry

- Cleavage

R = Reporter

Q = Quencher

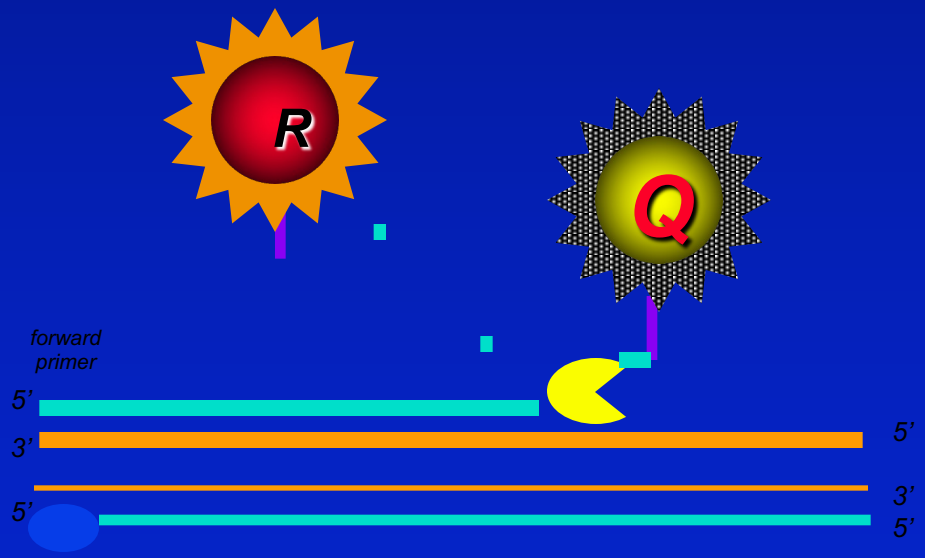


Taqman PCR Chemistry

- *Cleavage*

R = Reporter

Q = Quencher

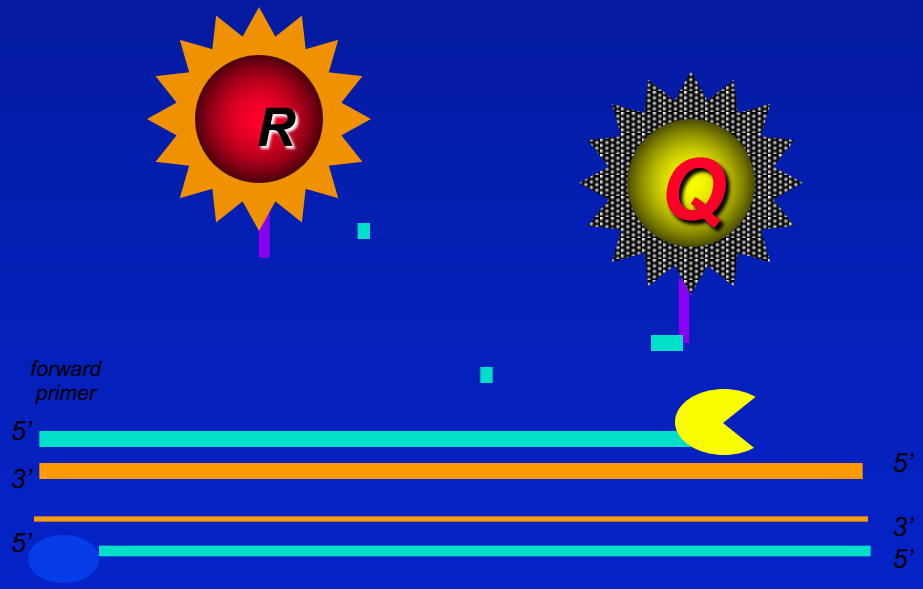


Taqman PCR Chemistry

- *Cleavage*

R = Reporter

Q = Quencher

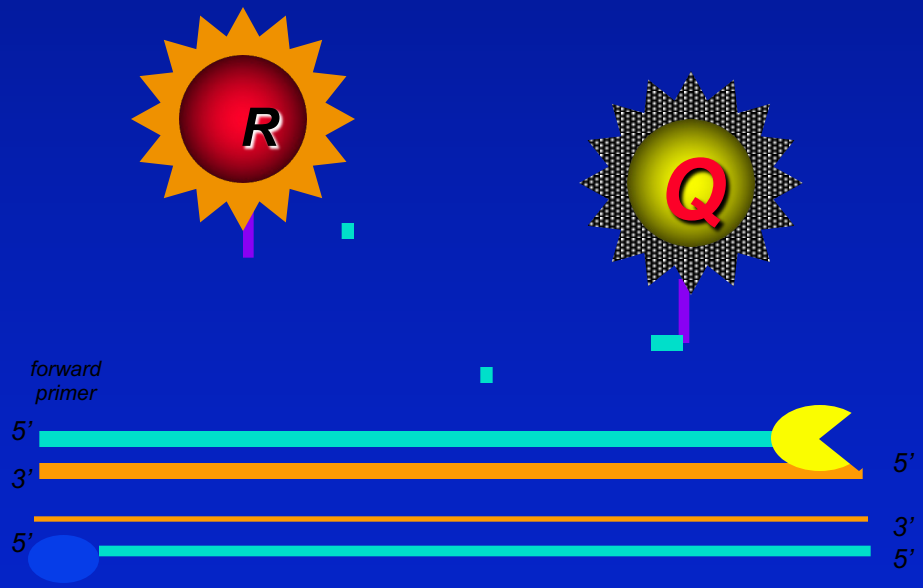


Taqman PCR Chemistry

- *Polymerization completed*

R = Reporter

Q = Quencher

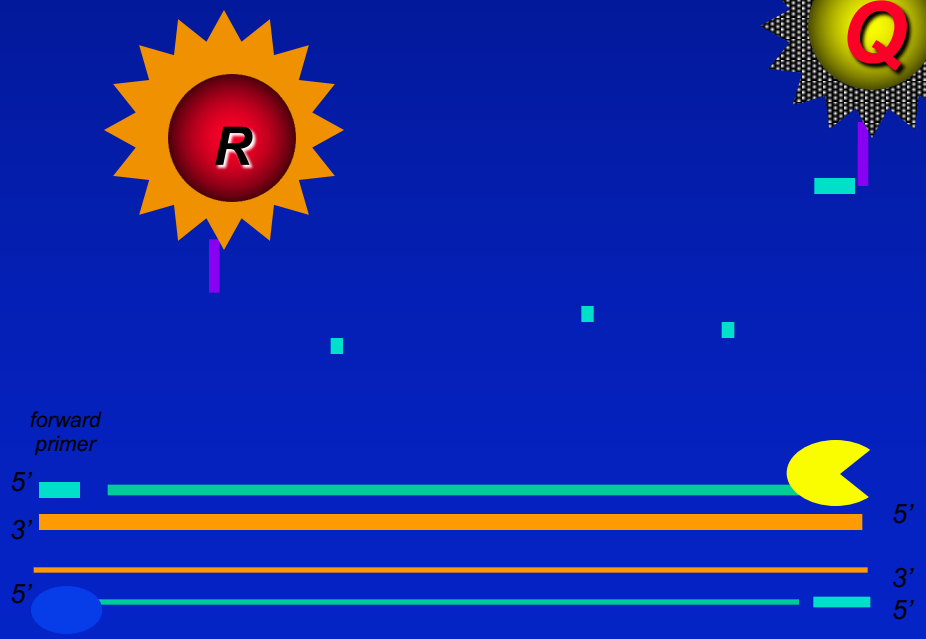


Taqman PCR Chemistry

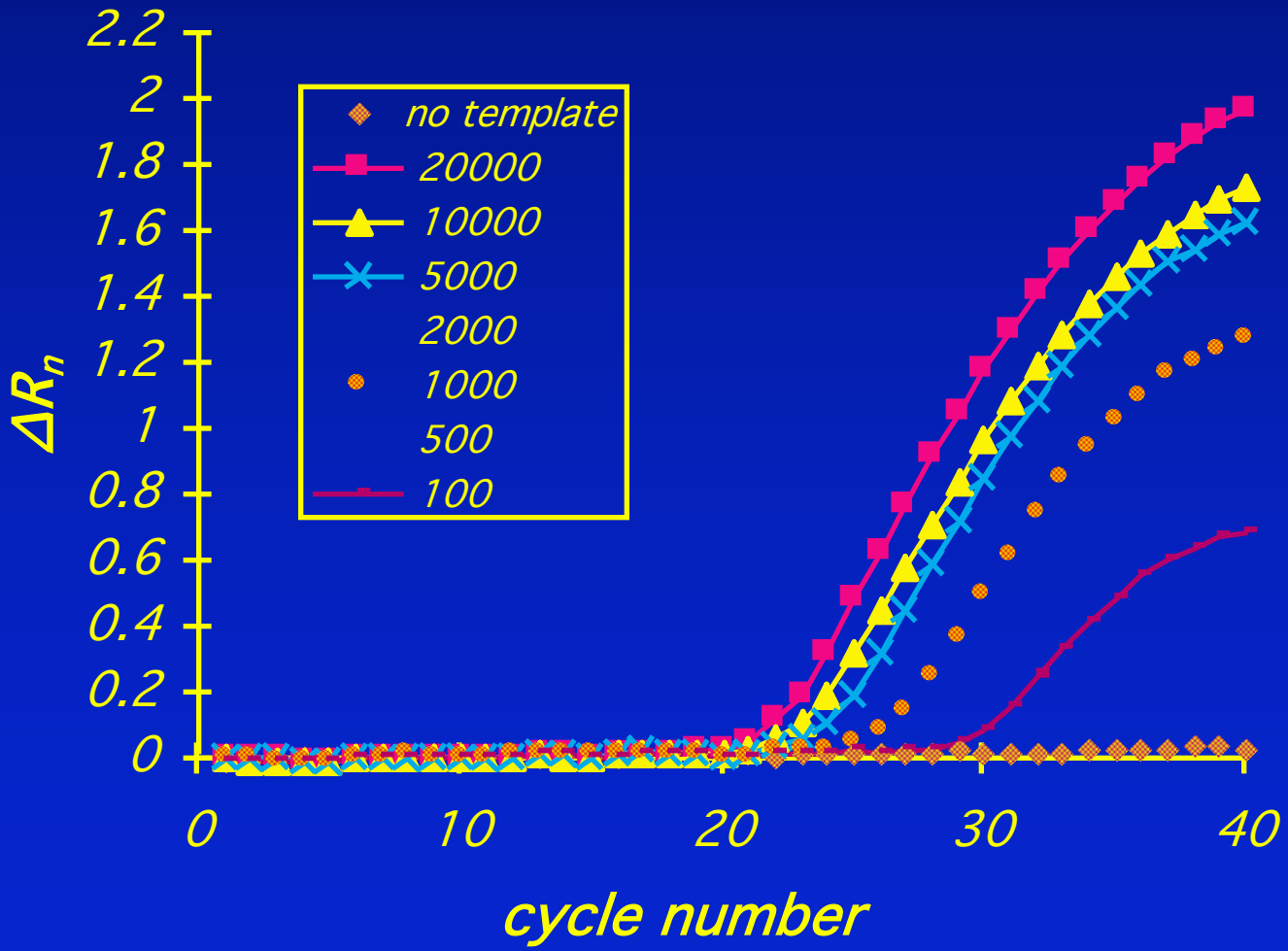
- **Polymerization completed**

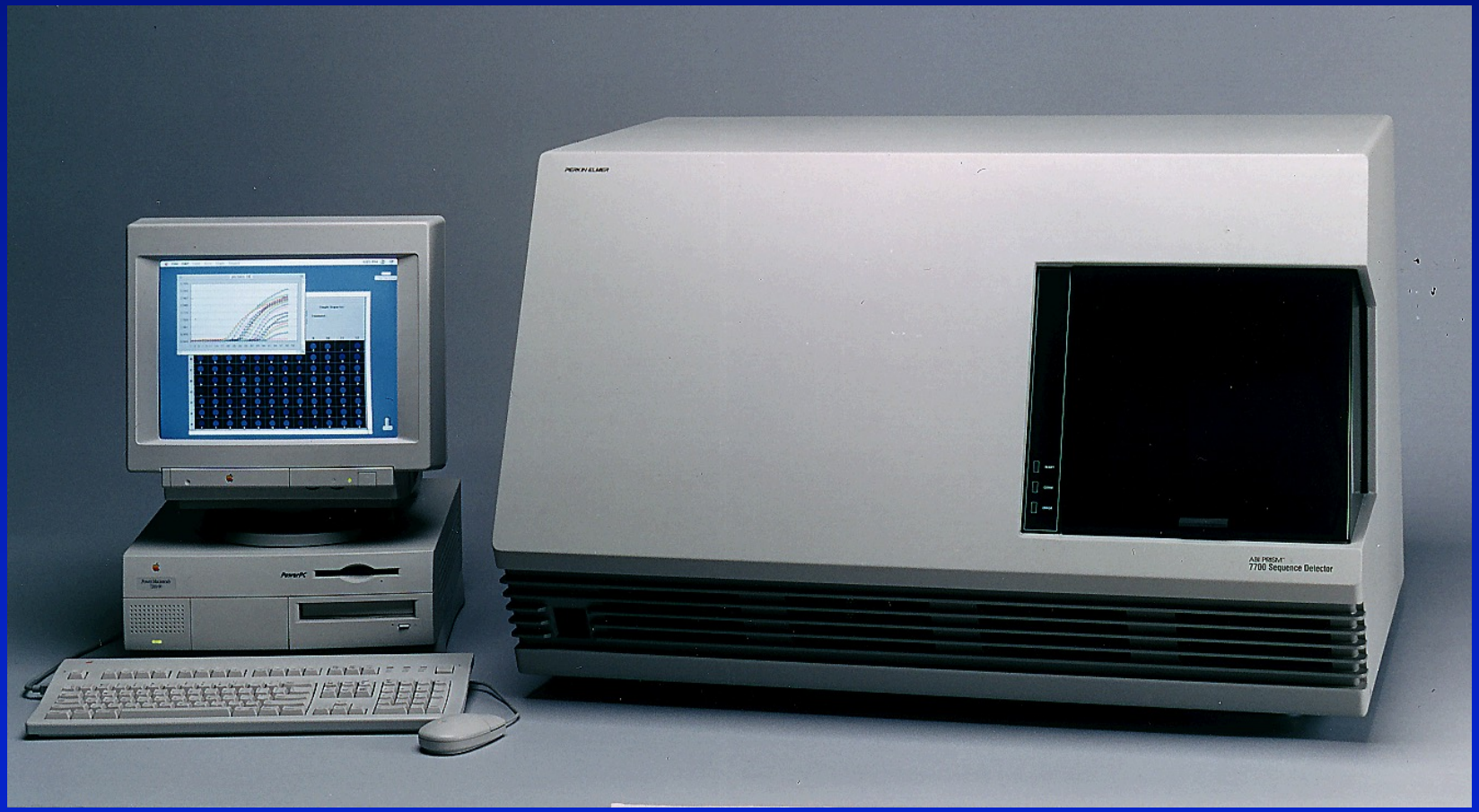
R = Reporter

Q = Quencher

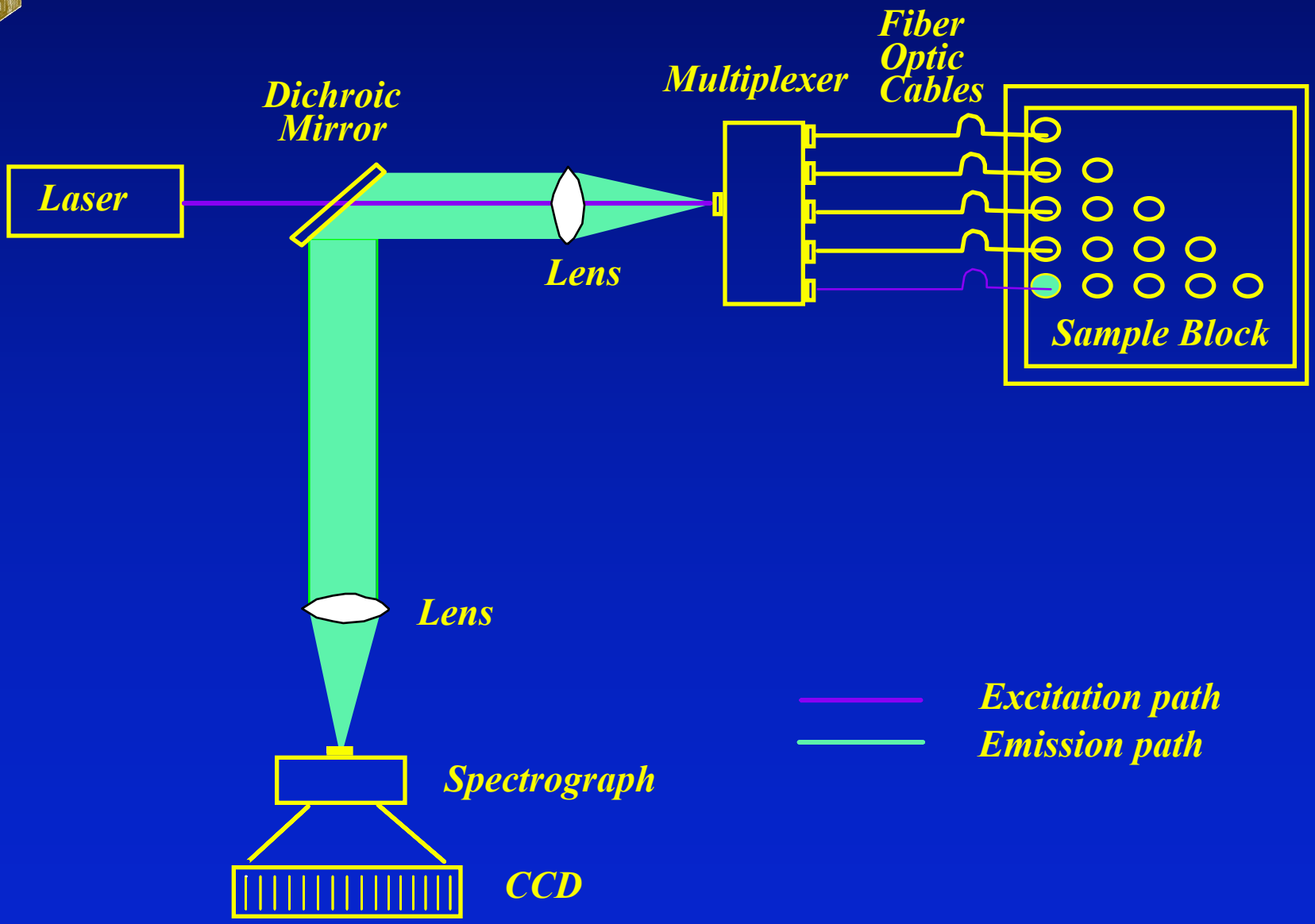


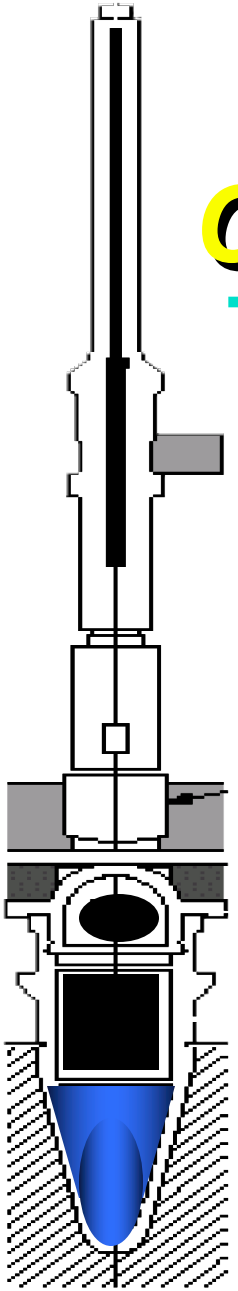
ß-Actin
R_n vs Cycle Number




PERKIN ELMER
PowerPC
7700 Sequence Detector

INSTRUMENT DESIGN





Fiber Close Tube Detection

CONTAMINATION CONTROL

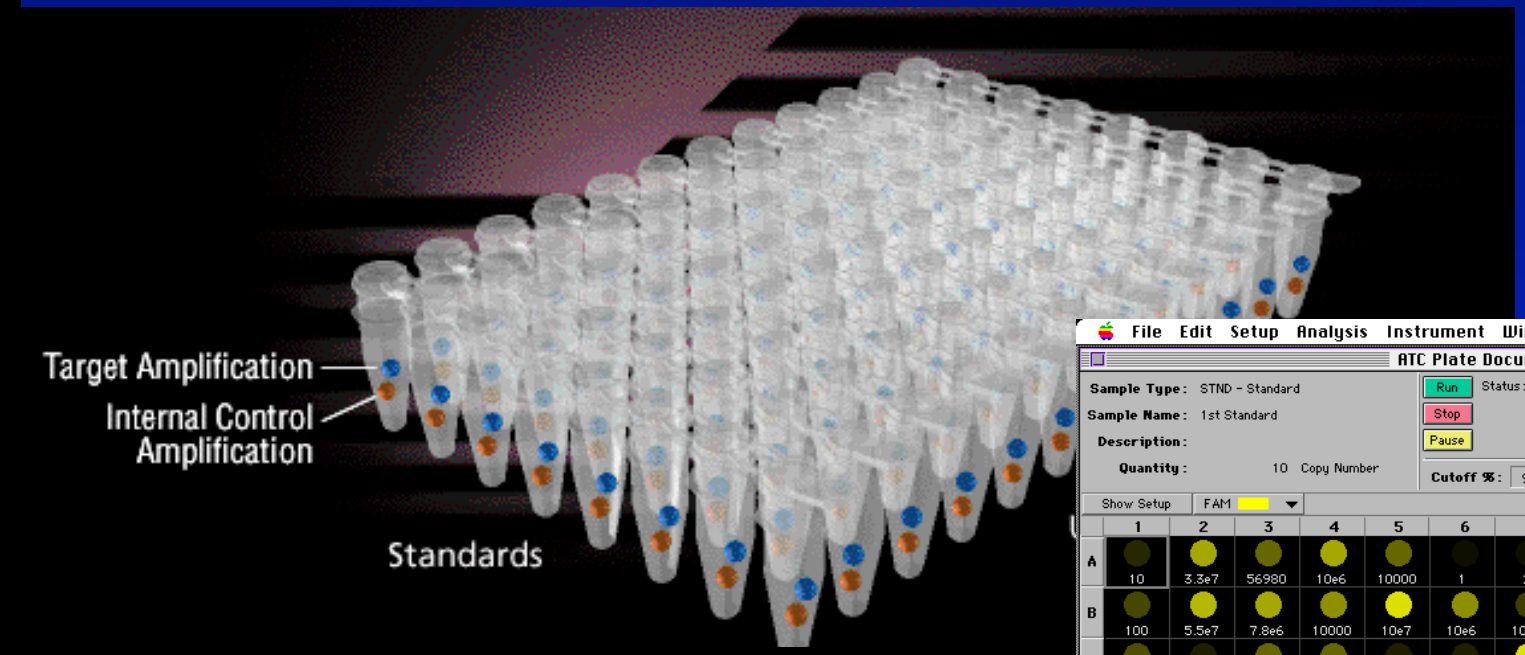
Lens

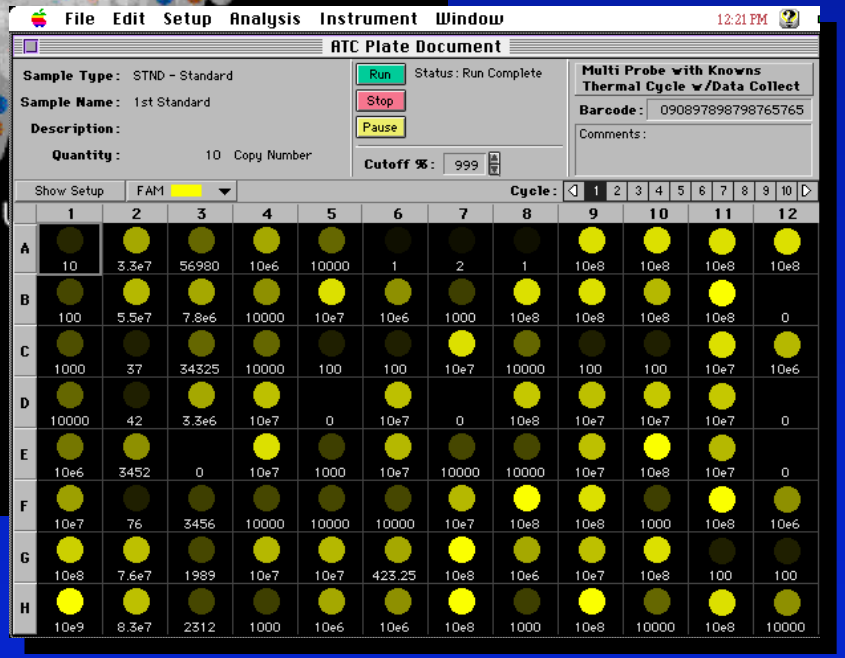
Cap

Tube

Thermal Cycler base

RTD Carryover Control: Closed Tube Assay

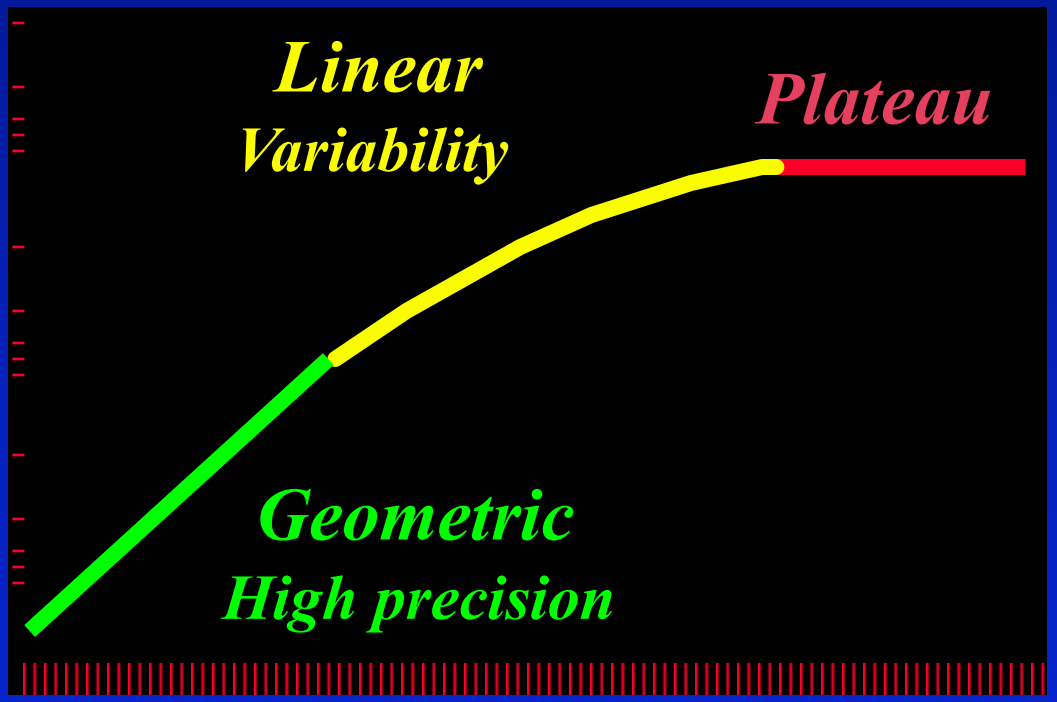




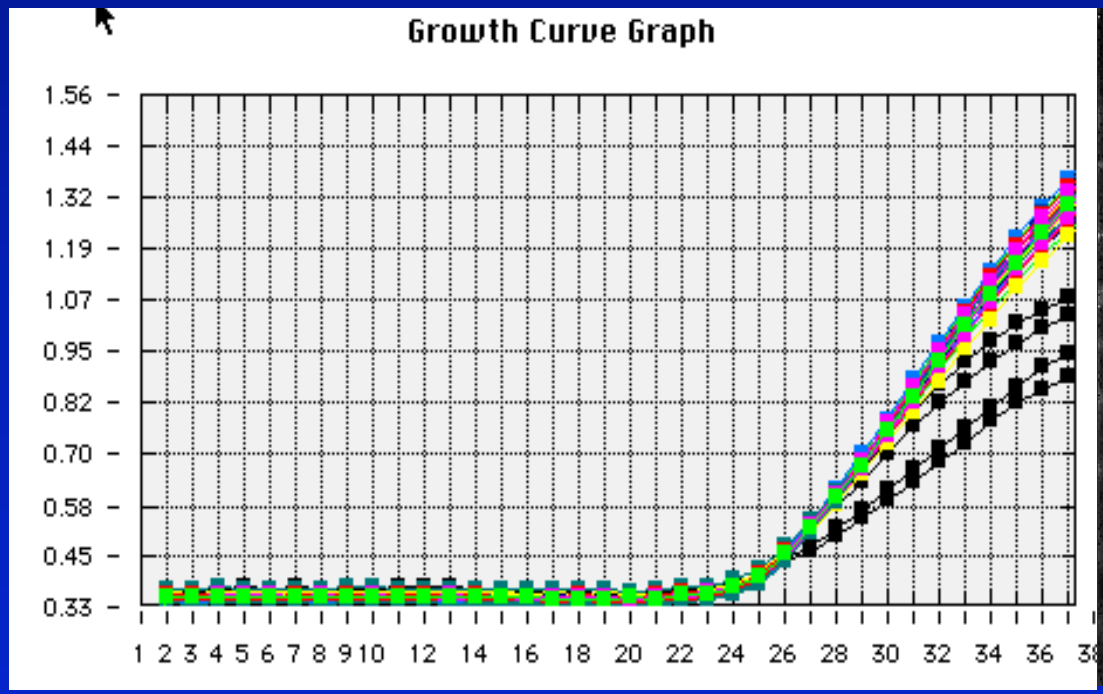
Tubes are never opened
At end of run, all data are in the computer.

Why Real Time PCR?

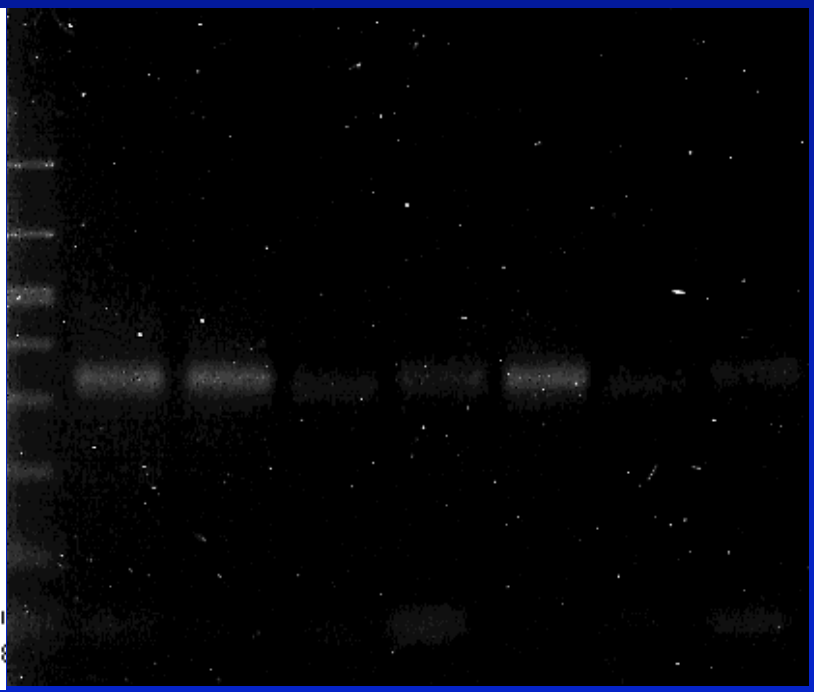
PCR Phases


Linear
Variability
Plateau
Geometric
High precision
Log [DNA]
Cycle #

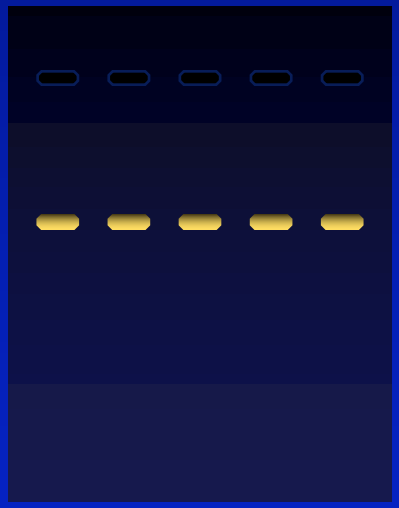
Real Time Vs End Point



9,048 9,498 10,180 9,238 9,111 12,885 10,539



Ethidium Bromide Challenges



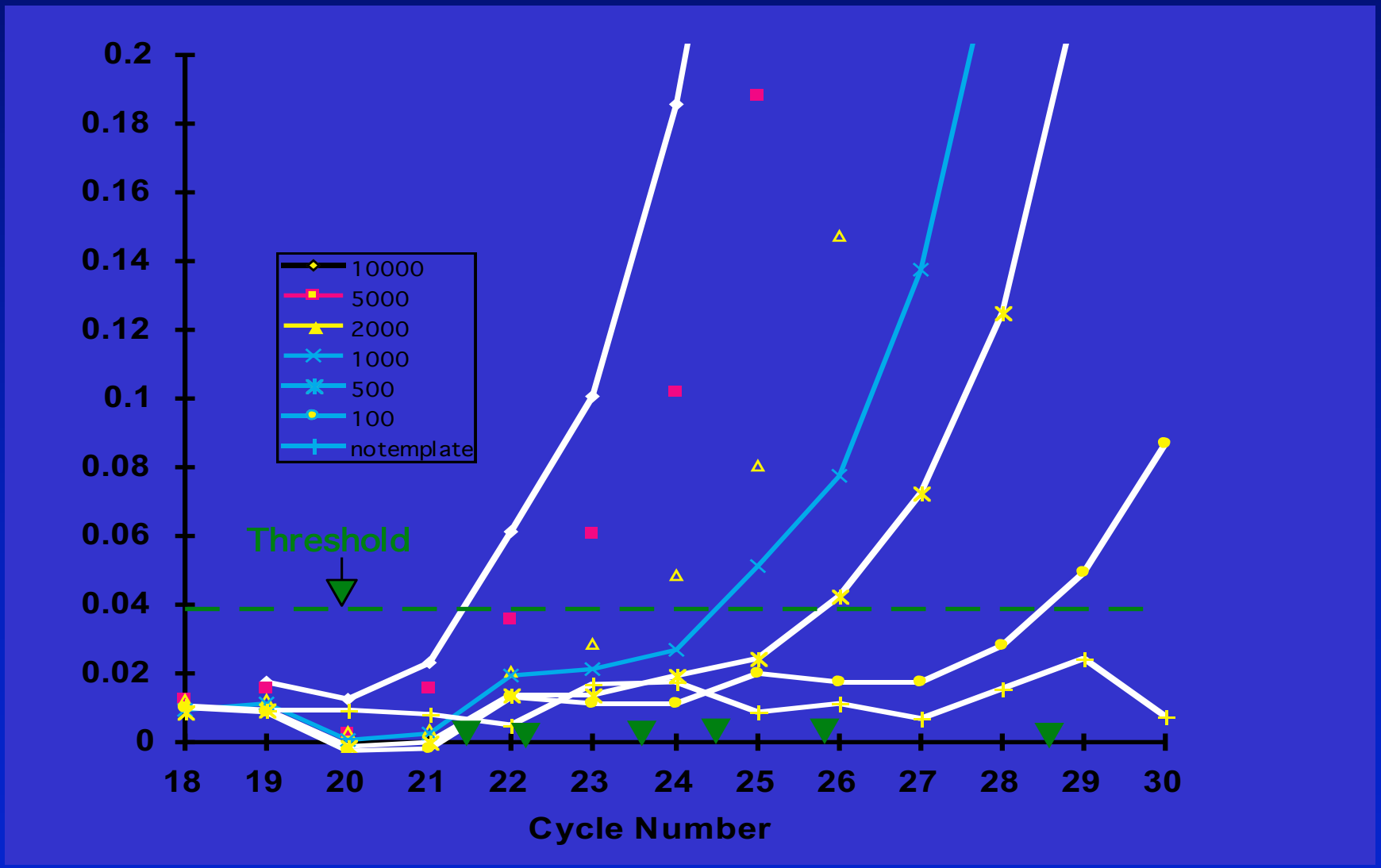
Signal not very sensitive.

Cannot detect geometric phase.

Low precision - adds about 30% error.

Narrow dynamic range

Determination of Threshold Cycle (C_T)



Quantitative PCR Applications

***Gene Expression*: changes in mRNA levels.**

***Drug Therapy*: effect of drugs on mRNA.**

***Transgenics*: adding genes to the germ line.**

***DNA Damage*: effect of harsh chemicals or radiation on DNA integrity.**

***Quality Control*: detecting unwanted biologicals.**

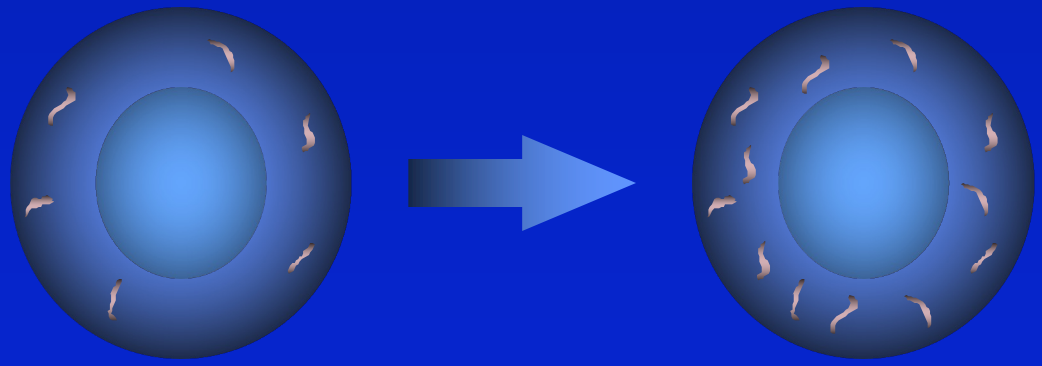
***Pathogen quantitation*: detecting viruses, bacteria**

***GMOs quantitation*: detecting transgenic sequences**

Two Types of Quantitation

Absolute: ***Determining exact numbers of molecules.***

Relative: ***Making comparisons of quantity.***



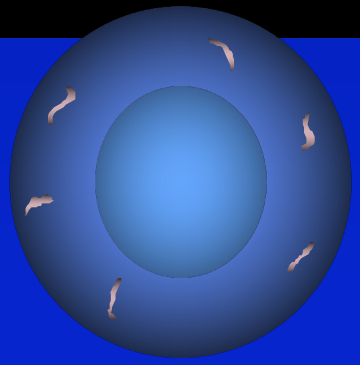
- Absolute
  - Requires Standard whose Concentration is Known Absolutely
  - Use Standard Curve
  - Unnecessary for Most Studies
- Relative
  - Gene Expression
  - Active Reference

Absolute Quantitation

Determine exact number of target nucleic acid molecules.

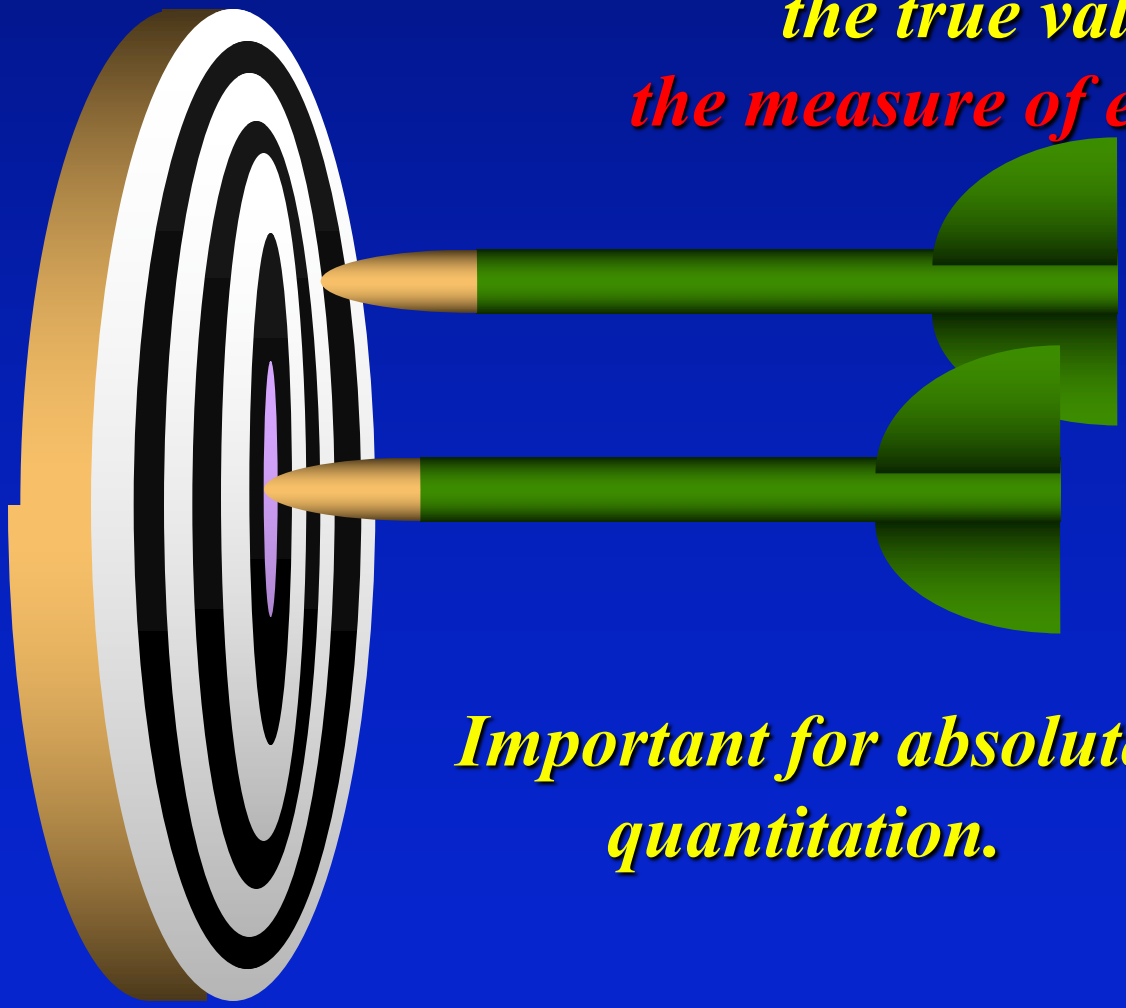
Big Issue: Accuracy

Virus quantitation
Transgenics
Gene therapy



Accuracy

How well a measured sample matches the true value = the measure of exactness.

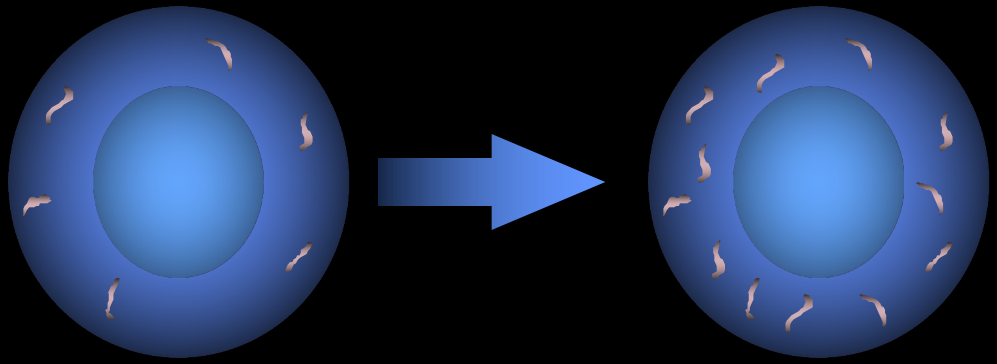


Important for absolute quantitation.

Relative Quantitation

Make quantitative comparisons of a target nucleic acid.

Big Issue: Precision

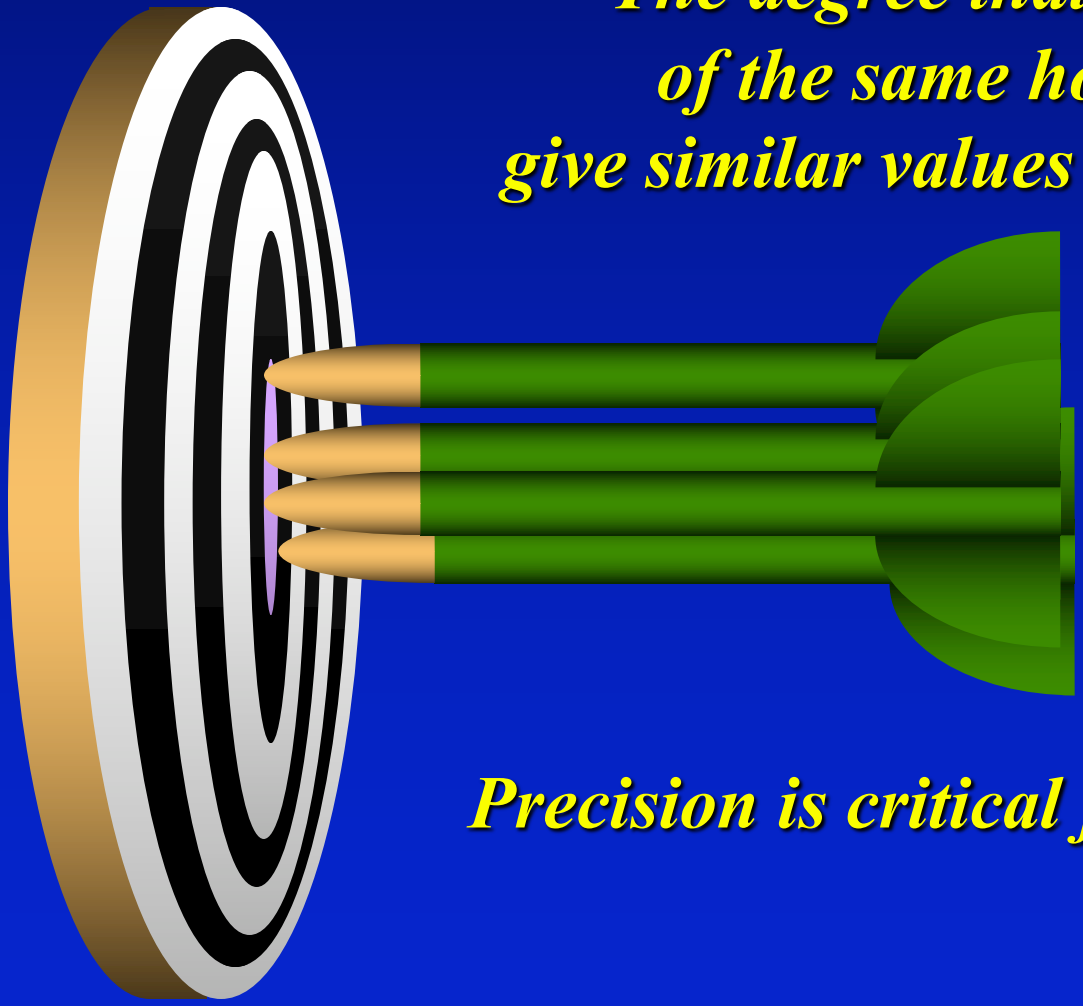


Gene expression

Drug therapy

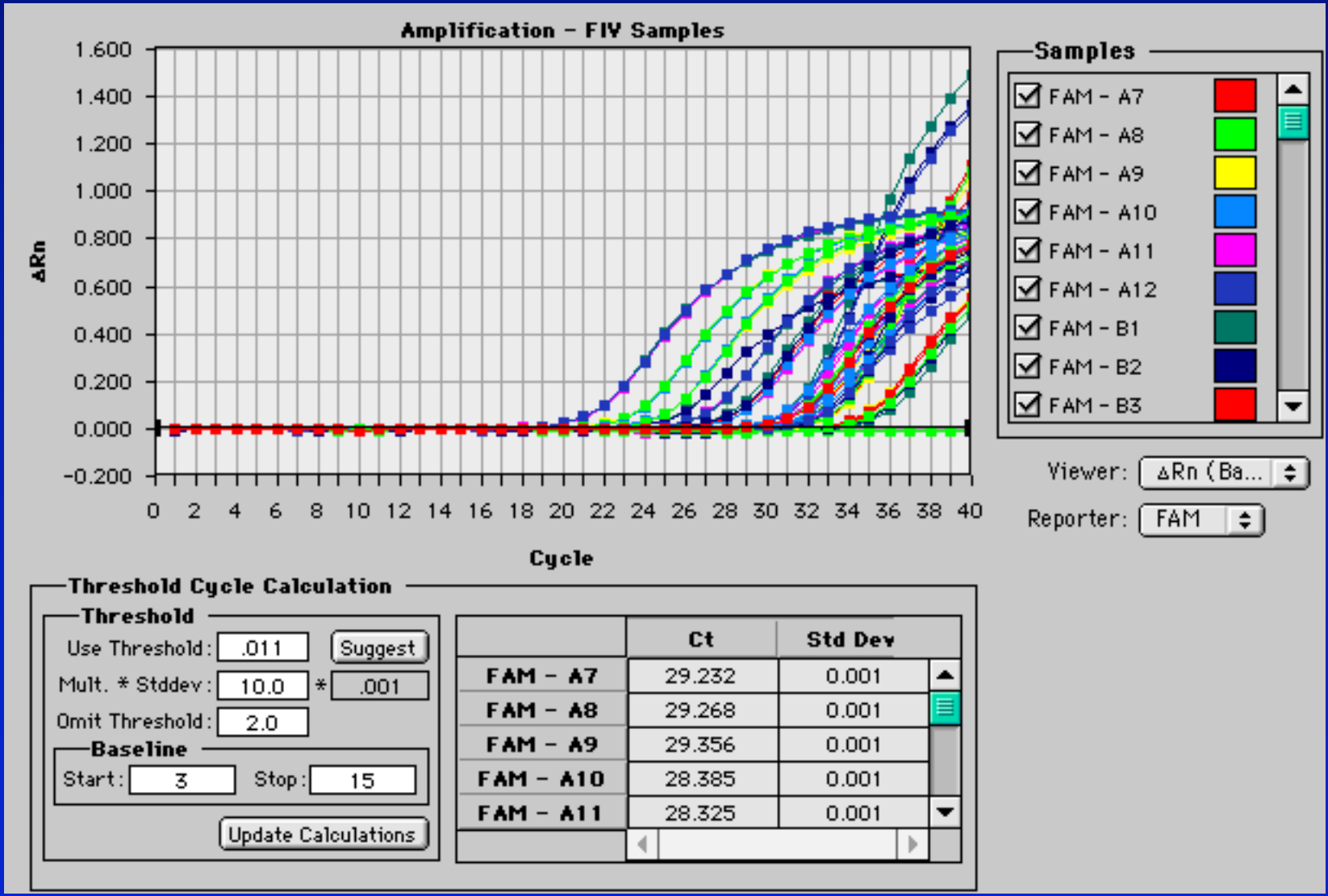
Precision

The degree that multiple samplings of the same homogenous source give similar values = degree of agreement.



Precision is critical for quantification.

FIV Results: Amplification Plot



End point Detection and quantitation

$$Y = X(1 + E)^{n}$$

RTD allows use of mathematics

$$Y = X(1+E)^{n}$$

Products get visible in all wells at the same quantity level

RTD allows use of mathematics

$$K = X(1+E)^{n}$$

Products get visible in all wells at the same quantity level

RTD allows use of mathematics

$$K = X(1+E)^{n}$$

Efficiency before Ct is really = 100%

RTD allows use of mathematics

$$K = X \quad 2^{n}$$

Good Bye Competitor!

Efficiency before Ct is really = 100%

RTD allows use of mathematics

$$K = X \quad 2^{n}$$

The value "n"
Is given by the instrument
(Ct)

RTD allows use of mathematics

$$K = X \quad 2^{Ct}$$

The value "n"
Is given by the instrument
(Ct)

RTD allows use of mathematics

$$K = X \; 2^{Ct}$$

The only variable left
Is X

Optimization?

- Primer and Probe Design
- Primer and probe concentration

Applications

HCV RNA (primers e probe nominate VEC; marcata FAM)
HIV RNA marcature FAM
HIV DNA
HIVDNA p1
HIVDNA p2

Clamydia pneumoniae
CpneuF
CpneuR
Cpneu (FAM)

Clamydia tracomatis
CtraF
CraR
Ctra (FAM)

Helicobacter pylori
HPF
HPR
HP probes 1-2-3
(FAM da ordinare tutte per le tre mutazioni principali)

H. influenzae
CapF
CapR
CapProbe FAM

Legionella spp
5S LEGIONELLA
LEGIOP (FAM/MGB)

Legionella pneumophila
Legionella MIP

Listeria monocitogenes
LM bgla1F
LM bgla1 R
LM bgla1 (FAM)

Mycobacteriun tubercolosis
MTF
MTR
MTp (FAM)

Neisseria meningitis
CapTF
CapTR
CapTProbe (FAM)

Streptococcus pneumoniae
PLYF
PLYR
PLY (FAM)

Parvovirus B19
b19F
b19R
b19p FAM

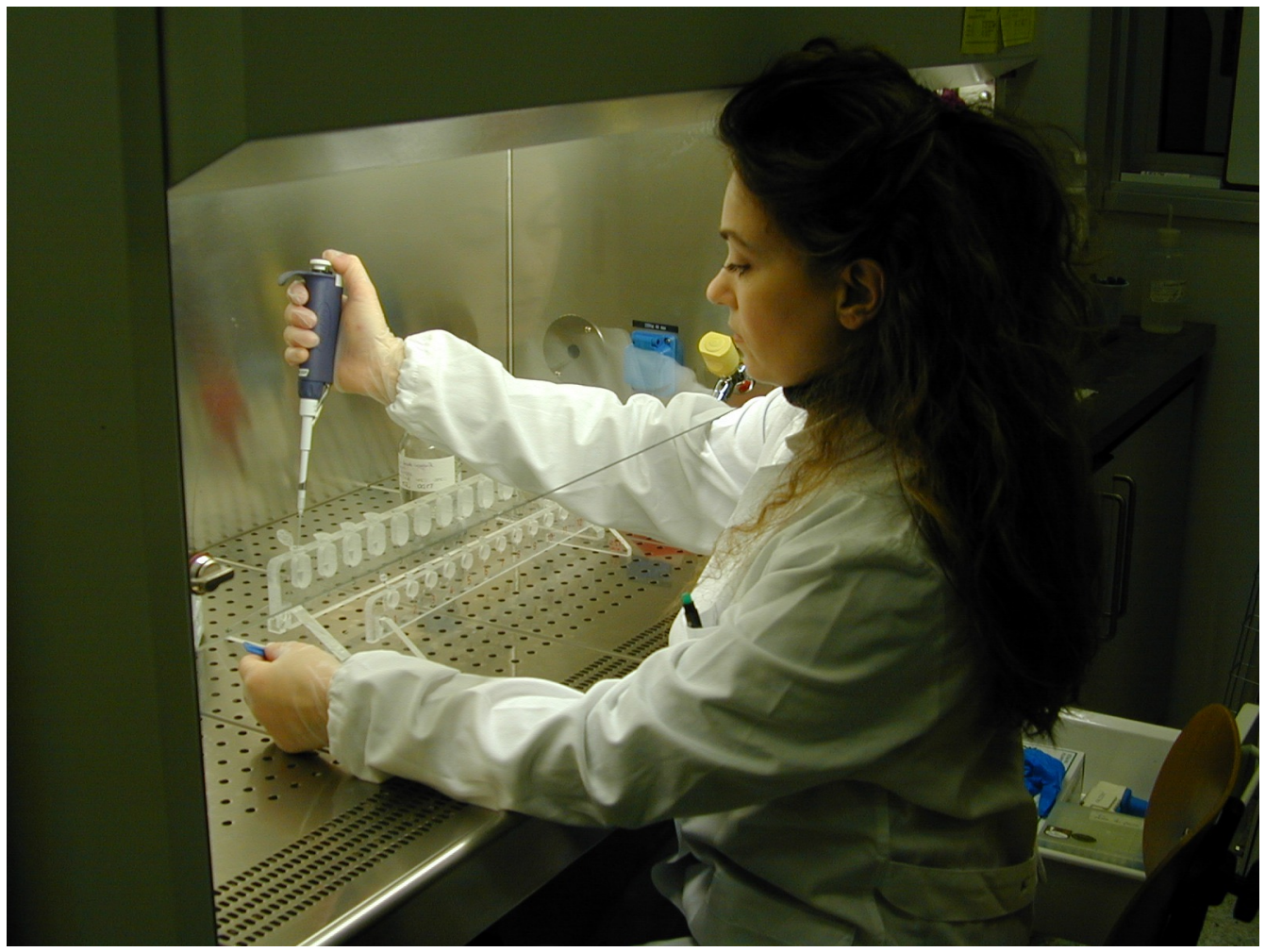
Bacillus anthracis
BAX

EPSTAIN BARR
EB For EB Rev EB Probe

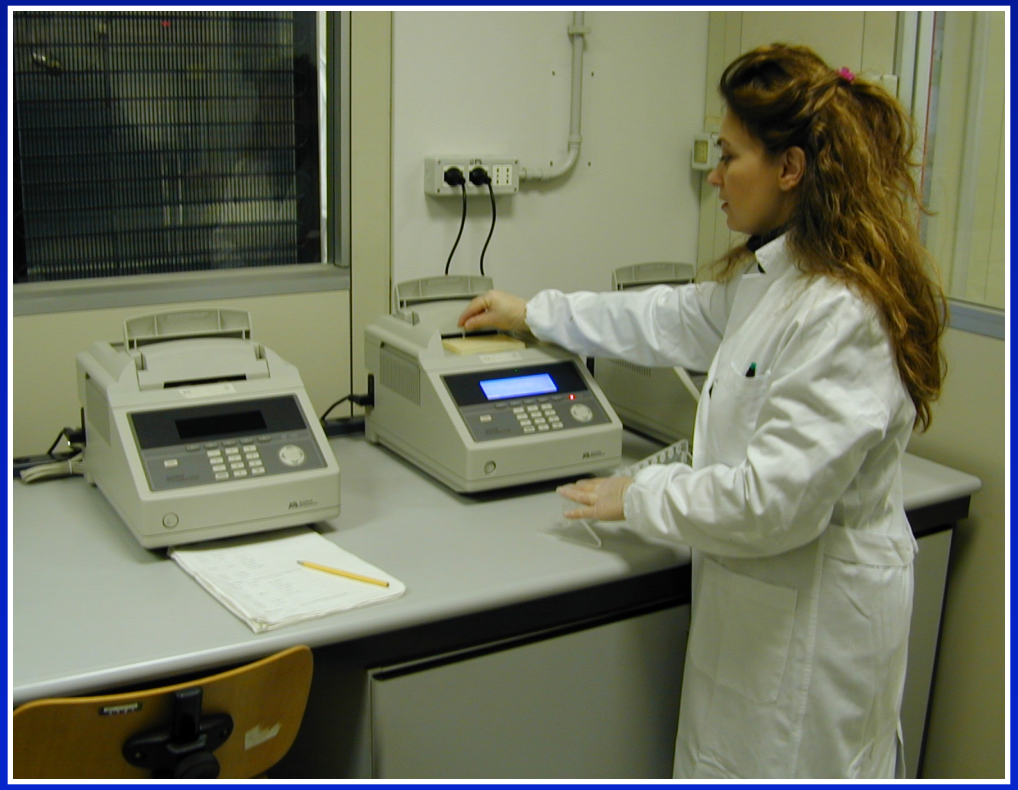
Chlamydia trachomatis
Ctrach Clo F Ctrach Clo R
Clo Probe

Chlamydia pneumoniae
CpneumoniaeClo F
CpneumoniaeClo R
CpneumoniaeClo Probe

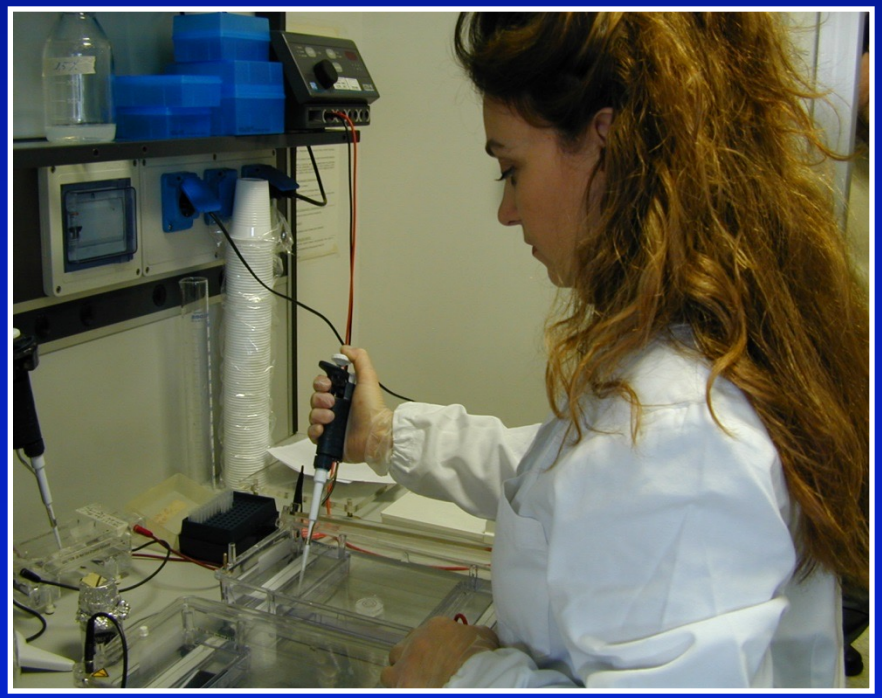
ALLESTIMENTO DELLA PCR

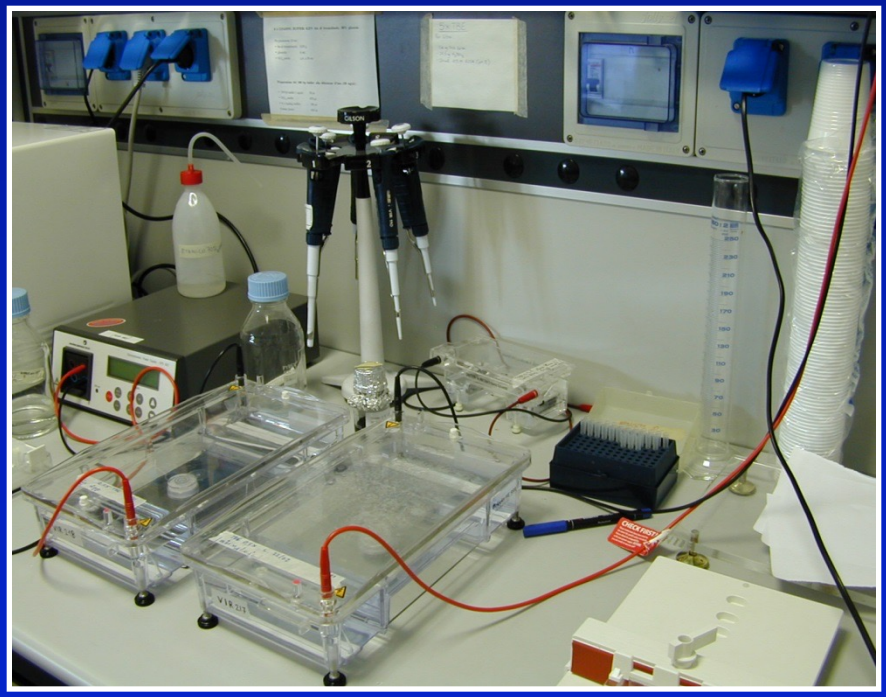


AMPLIFICAZIONE DEL DNA BERSAGLIO

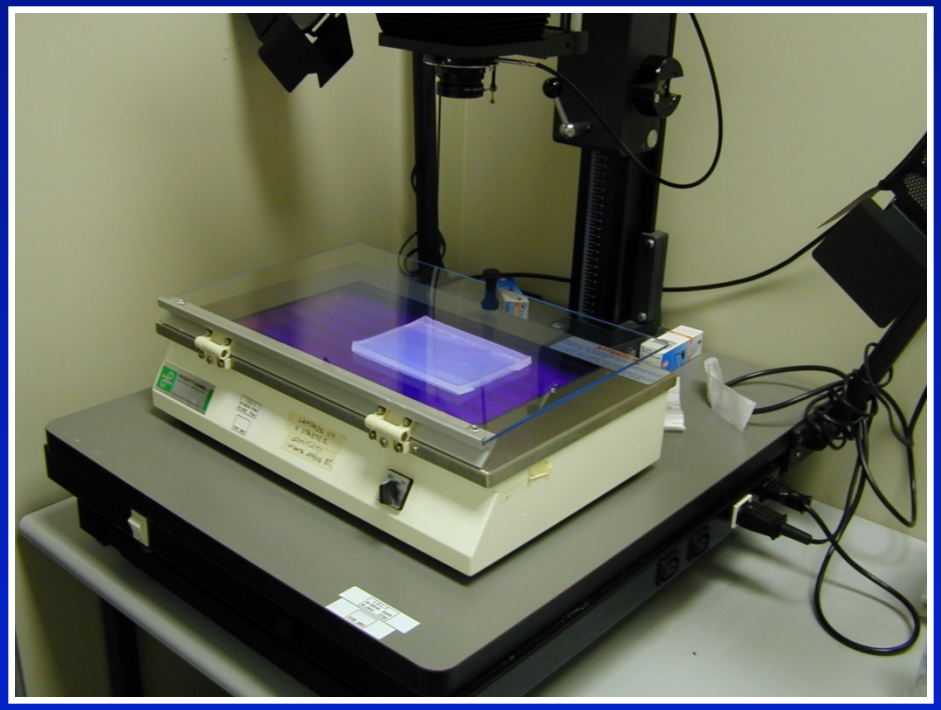


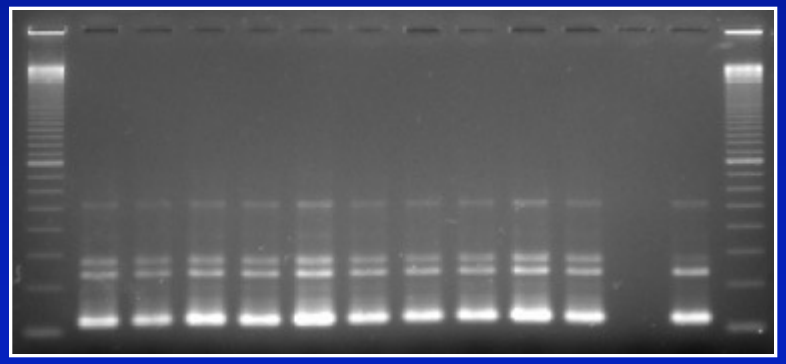
ELETTROFORESI DELL'AMPLIFICATO





RIVELAZIONE DELL'AMPLIFICATO





Estrazione e purificazione di DNA da alimenti

Metodi impiegati

CTAB
esadecil-trimetil-ammonio-bromuro

Wizard® Miniprep
resina

DNAeasy® mini plant kit
resina

Fattori di degradazione del DNA negli alimenti

1. Idrolisi del DNA in seguito a prolungato trattamento termico
2. Degradazione enzimatica da parte di nucleasi
3. Effetti del pH sulla depurinazione e idrolisi del DNA

Lunghezza media dei frammenti di DNA in alimenti processati

Carne fresca	*30.000 bp*
Carne a 100°C per 10 min	*1.100 bp*
Carne a 120°C per 30 min	*300 bp*
Salame	*100-15.000 bp*
Patè	*100-1.500 bp*
Prodotti della soia	***100-400 bp***
Prodotti a base di pomodoro	*<400 bp*

Qualità del DNA negli alimenti

Lunghezza media dei frammenti di DNA

↓

La lunghezza dei tratti amplificati con la PCR deve essere inferiore alla lunghezza media dei frammenti di DNA presenti nel campione

Esempi di amplificazioni PCR nell'analisi degli OGM

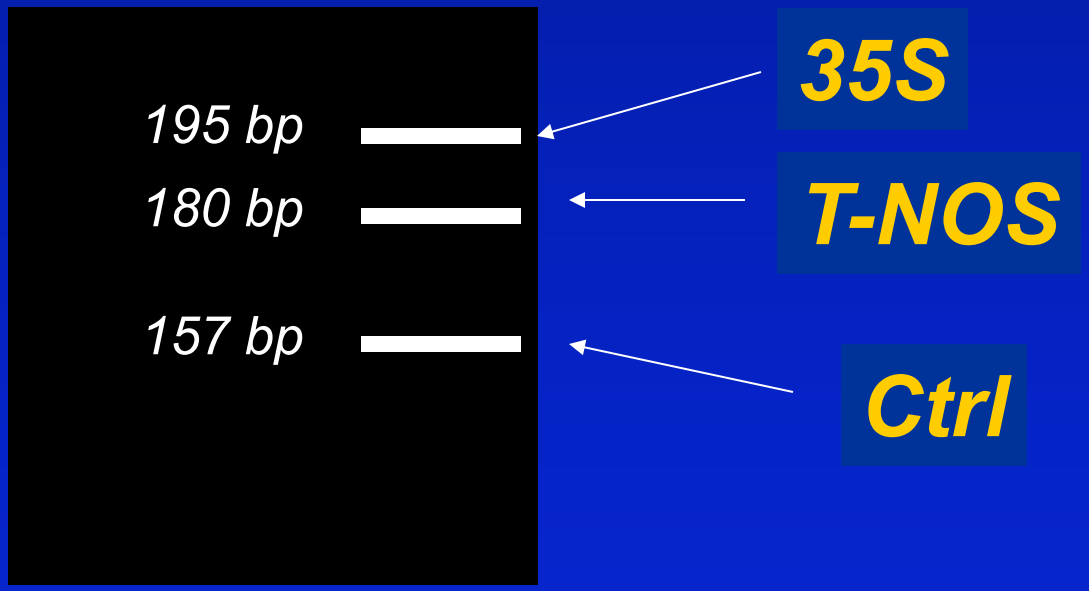
Target	Lunghezza amplicon	Applicazione
All'interno di sequenze regolatrici		
P-35S	195 bp	Pomodoro Soia Mais
nos 3'	180 bp	Soia Patata
Tra sequenze regolatrici		
P-35S nos 3'	890 bp	Pomodoro

Esempi di amplificazioni PCR nell'analisi degli OGM

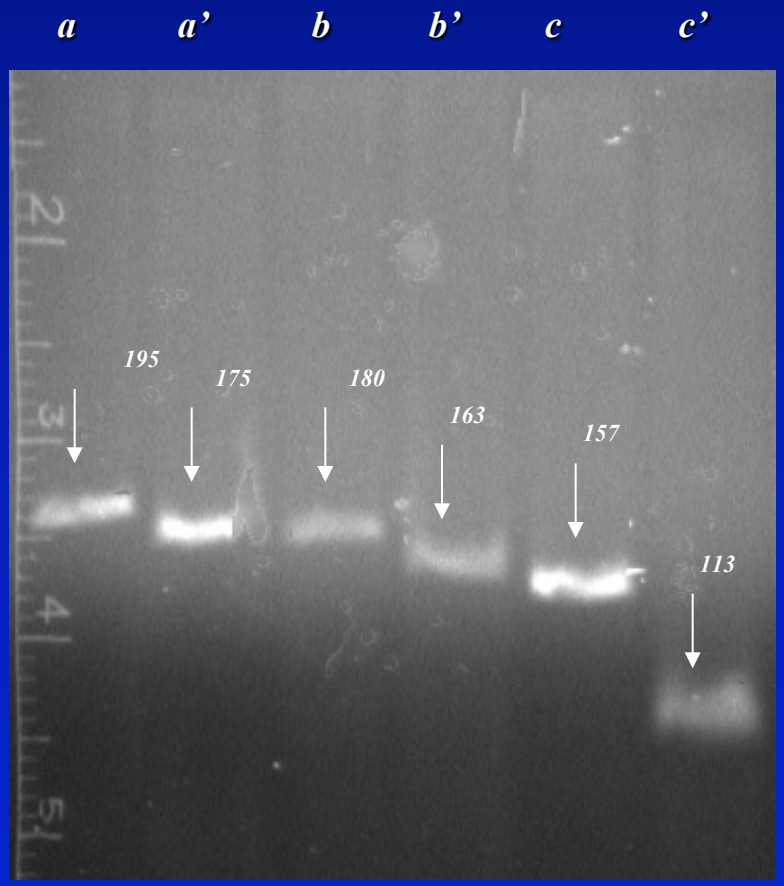
Target	Lunghezza amplicon	Applicazione
All'interno di geni strutturali		
nptII	172 bp	Pomodoro Patata
PG	180 bp	Pomodoro
Tra sequenze regolatrici e geni strutturali		
Tra geni strutturali		

PCR multiplex

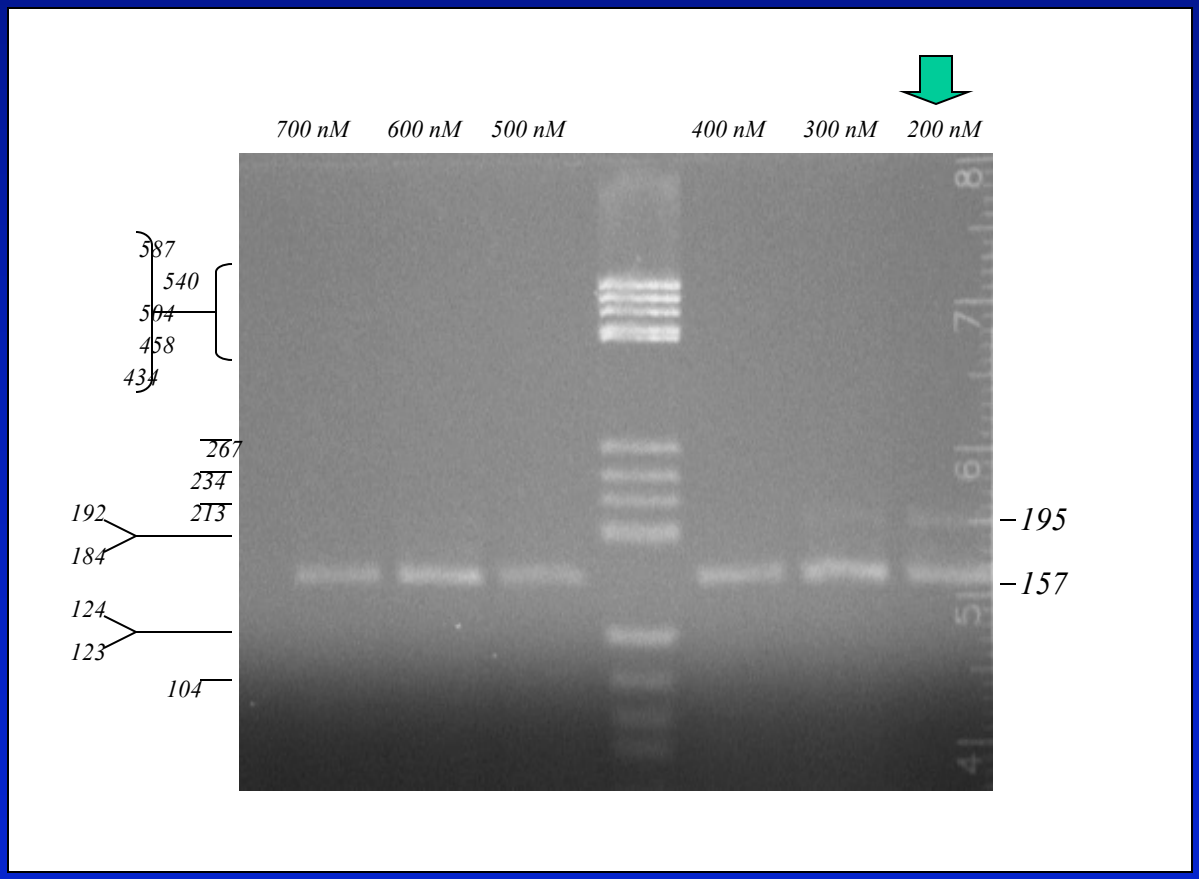
Analisi simultanea di più sequenze bersaglio in una singola reazione di amplificazione



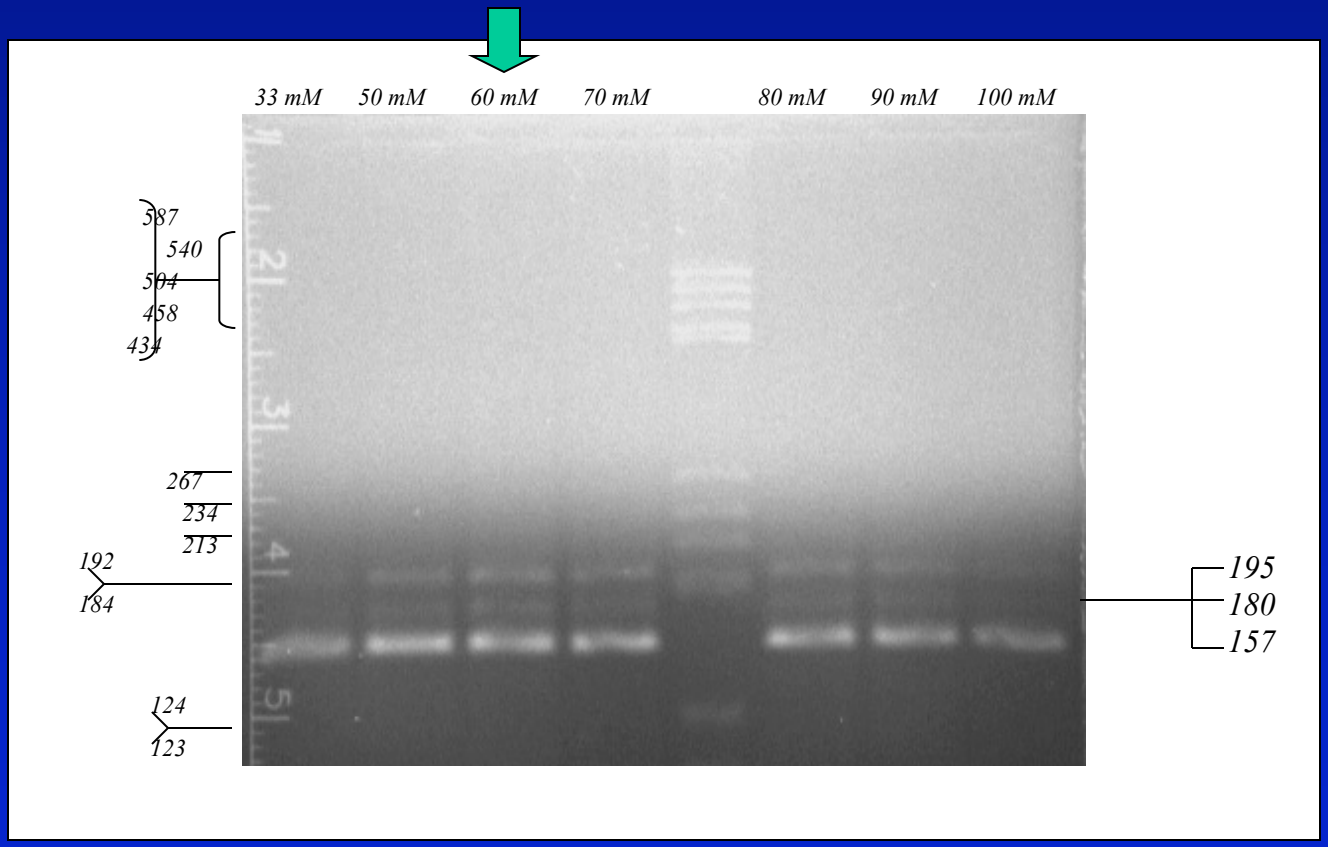
Analisi di restrizione mediante MwoI degli amplificati di 35S, NOS-3' e lectina



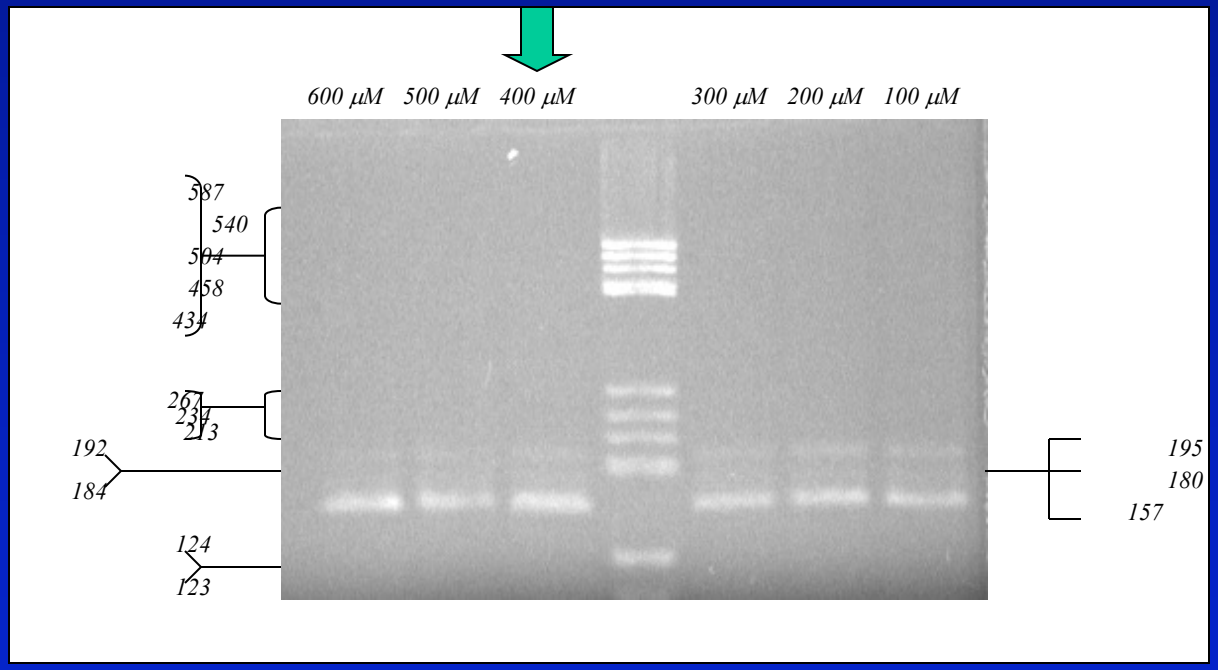
Effetto della concentrazione dei primers sul risultato della amplificazione simultanea di 35S, NOS-3' e lectina.



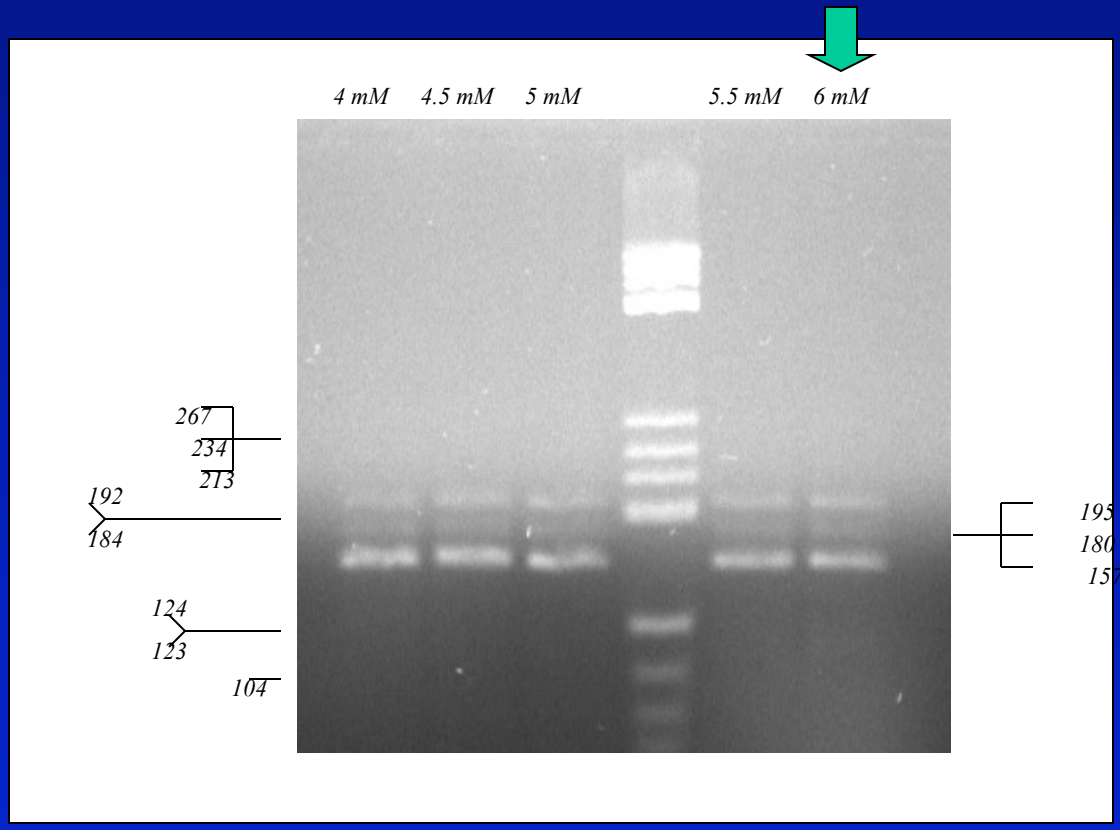
Effetto della concentrazione del KCl



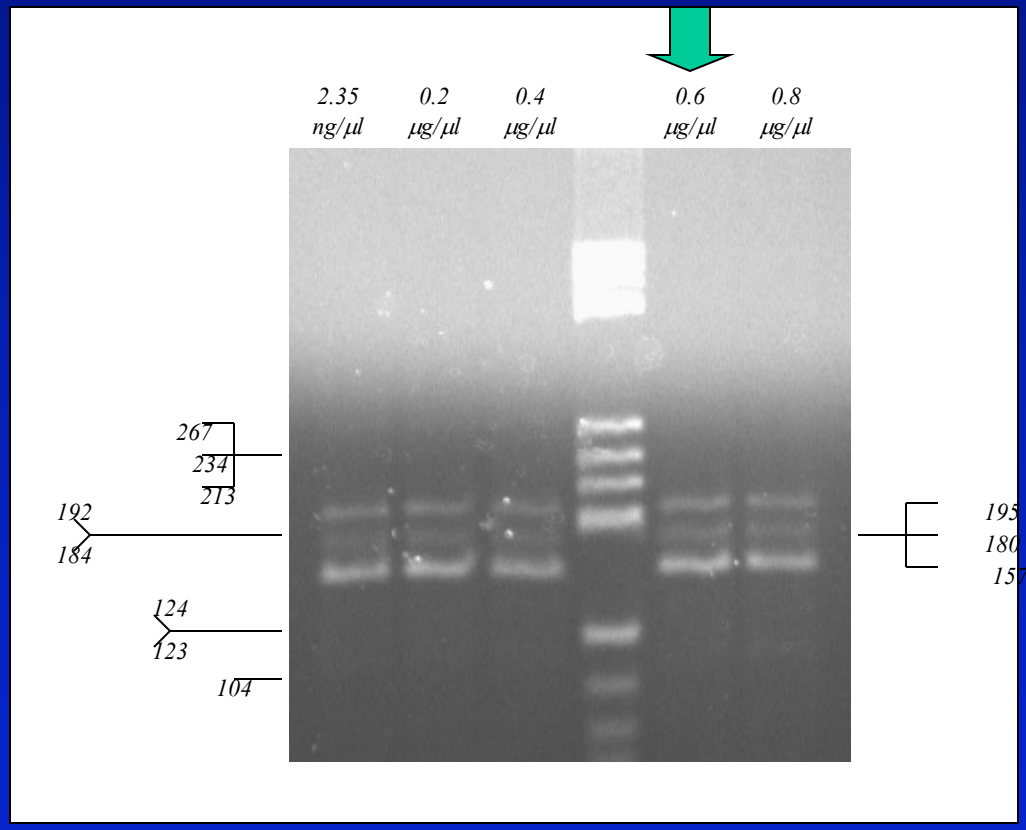
Effetto della concentrazione dei dNTP



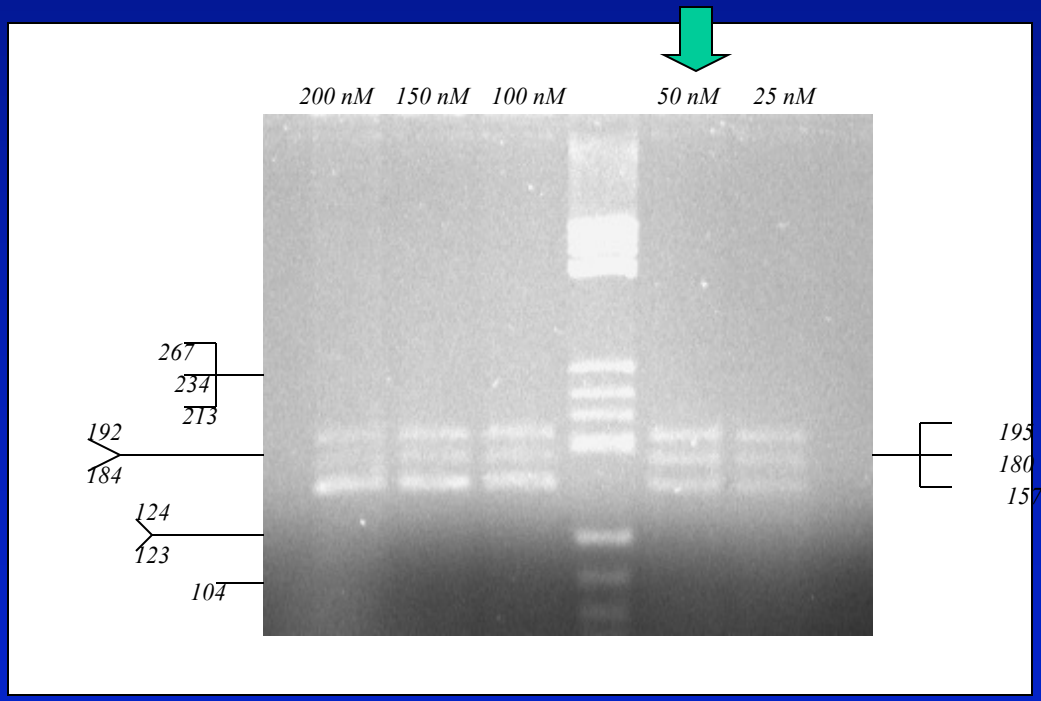
Effetto della concentrazione del MgCl2



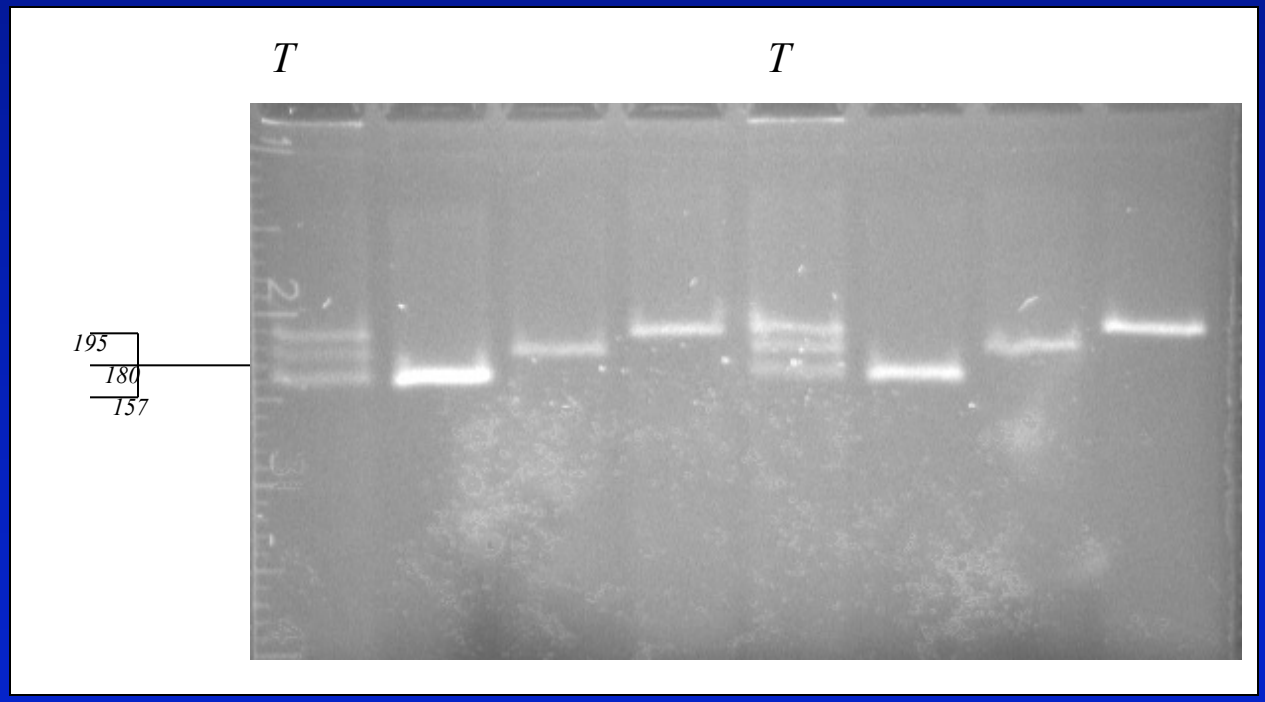
Effetto della concentrazione della BSA



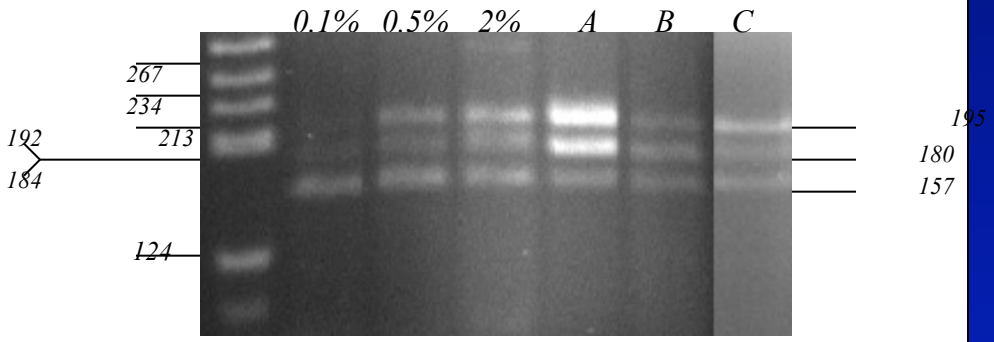
Effetto della diminuzione della concentrazione dei primers del gene della lectina sul risultato della amplificazione del 35S e del NOS-3'

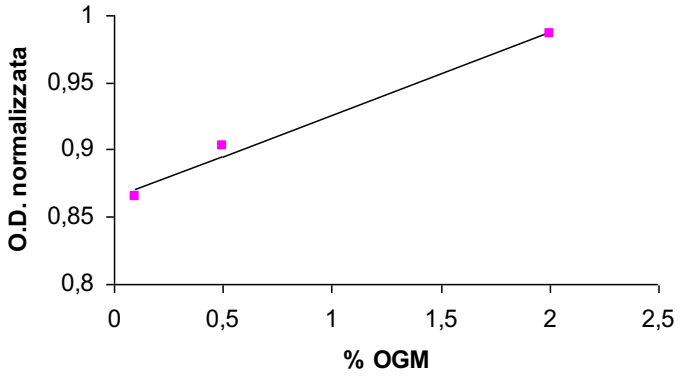


TRIPLEX PCR

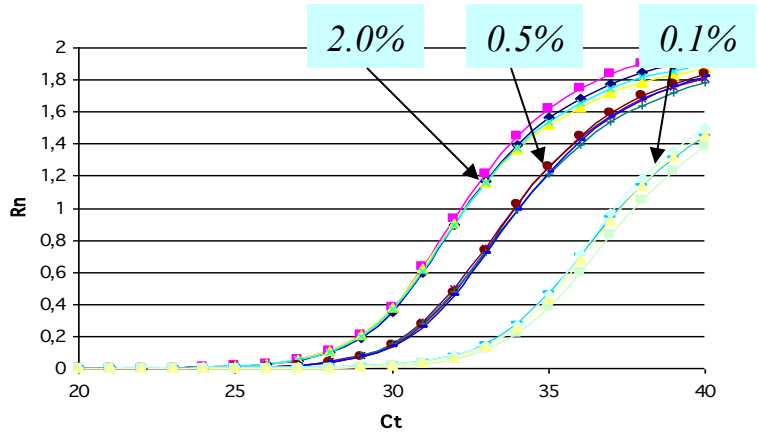


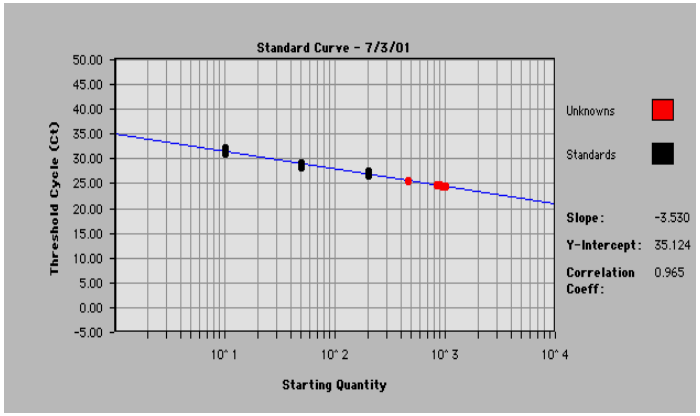
Analisi semi-quantitativa campioni reali mediante PCR-triplex





Analisi quantitativa mediante real-time PCR





Tipo di analisi	Campione tipo A	Campione tipo B	Campione tipo C
Semi-quantitativa (Triplex-PCR)	9.2 ± 2 %	0.9 ± 0.4 %	2.9 ± 0.9 %
Quantitativa (Real-time PCR)	20.5 ± 0.2 %	1.5 ± 0.05 %	3.2 ± 0.1 %

Criteri per un programma di screening su OGM tramite PCR

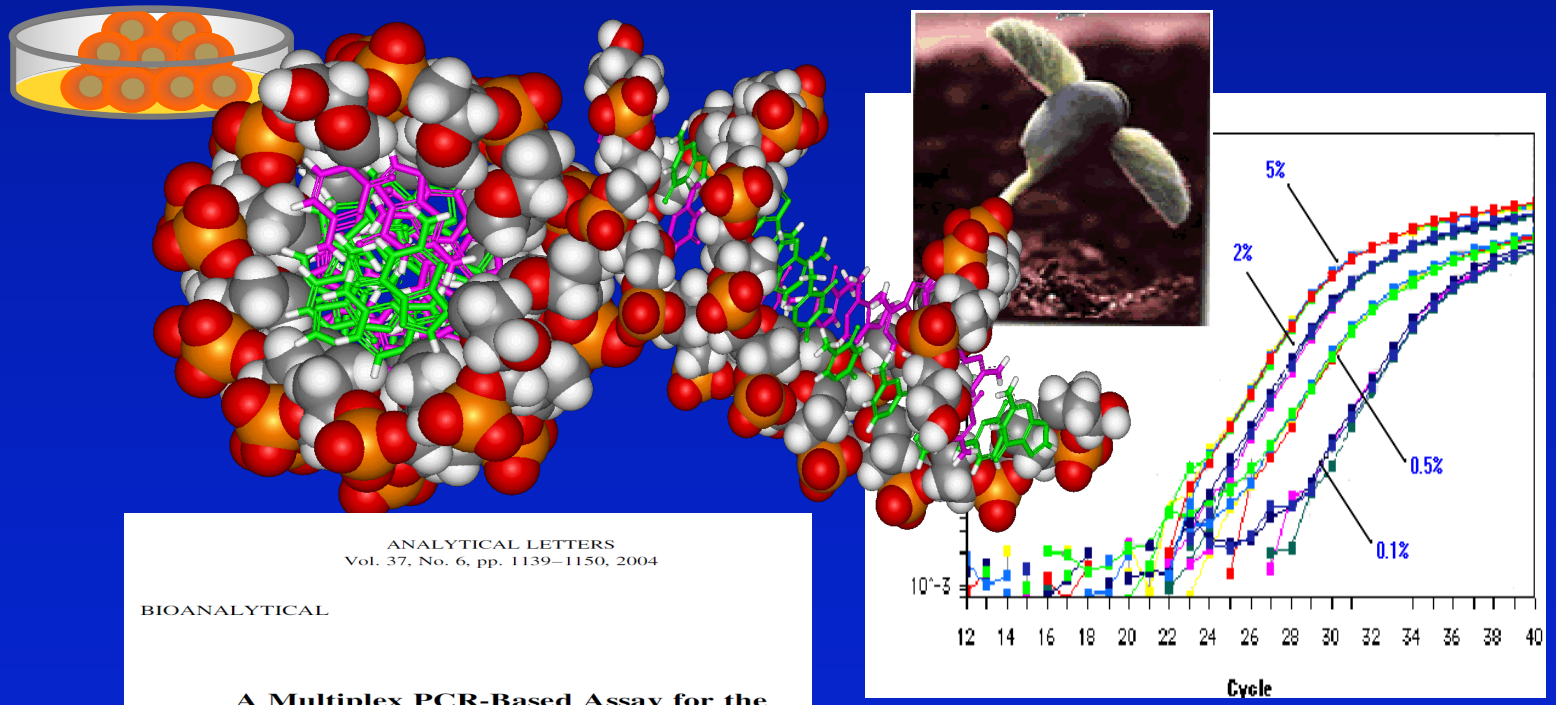
1- Scelta di primers che consentano l'identificazione specifica di quante più varianti di ogni singolo elemento

2- L'amplificazione simultanea mediante opportuni protocolli di PCR di tipo multiplex di più sequenze bersaglio

3- Alla luce della continua evoluzione delle normative che regolano il controllo e la commercializzazione degli alimenti contenenti OGM lo sviluppo di metodiche di PCR di tipo multiplex potrà rispondere alle necessità di abbattere tempi e costi nella fase iniziale di screening di un numero elevato di campioni.

Development of new analytical methods for the screening of genetically modified organisms (GMO) in food

Multiplex and real-time PCR-based assays for the detection of genetically modified soybean.



ANALYTICAL LETTERS
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BIOANALYTICAL

A Multiplex PCR-Based Assay for the Detection of Genetically Modified Soybean†

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A Multiplex PCR-Based Assay for the Detection of Genetically Modified Soybean[†]

A2704-12	ACS-GM005-3		E	European Commission (2009)
A5547-127	ACS-GM006-4		E	European Commission (2009)
DP-305423-1	DP-305423-1		E	European Commission (2009)
DP-356043-5	DP-356043-5			European Commission (2009)
GTS40-3-2	MON-04032-6	RoundupReady	MG (QL)	Dainese et al. (2004)
			C	Kim et al. (2004), Lerat et al. (2005) Liu et al. (2006),Pan and Shih (2003),Peano et al. (2005b), Tani et al. (2005), Vaïtilingom et al. (1999), Vollenhofer et al. (1999),Wang and Fang (2005), Zhang et al. (2007),Zhou et al. (2007)
			DC	Foti et al. (2006)
			MC	Germini et al. (2004), Hernandez et al. (2003b), Peano et al. (2005a)
			E	Berdal and Holst-Jensen (2001), Burns et al. (2003), European Commission (2009),Huang and Pan (2005), Moreano et al. (2006),Pang et al. (2007),Tavernier et al. (2001),Terry and Harris (2001)
			ME (QL)	Xu et al. (2007)
MON89788	MON-89788-1		E	European Commission (2009)

[†]This paper is dedicated to Prof. Corradino Motti sorrowfully missed on May 1, 2001.

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Research review paper

Testing for genetically modified organisms (GMOs): Past, present and future perspectives

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ABSTRACT

This paper presents an overview of GMO testing methodologies and how these have evolved and may evolve in the next decade. Challenges and limitations for the application of the test methods as well as to the interpretation of results produced with the methods are highlighted and discussed, bearing in mind the various interests and competences of the involved stakeholders. To better understand the suitability and limitations of detection methodologies the evolution of transformation processes for creation of GMOs is briefly reviewed.

Soybean	A2704-12	ACS-GM005-3		E	European Commission (2009)
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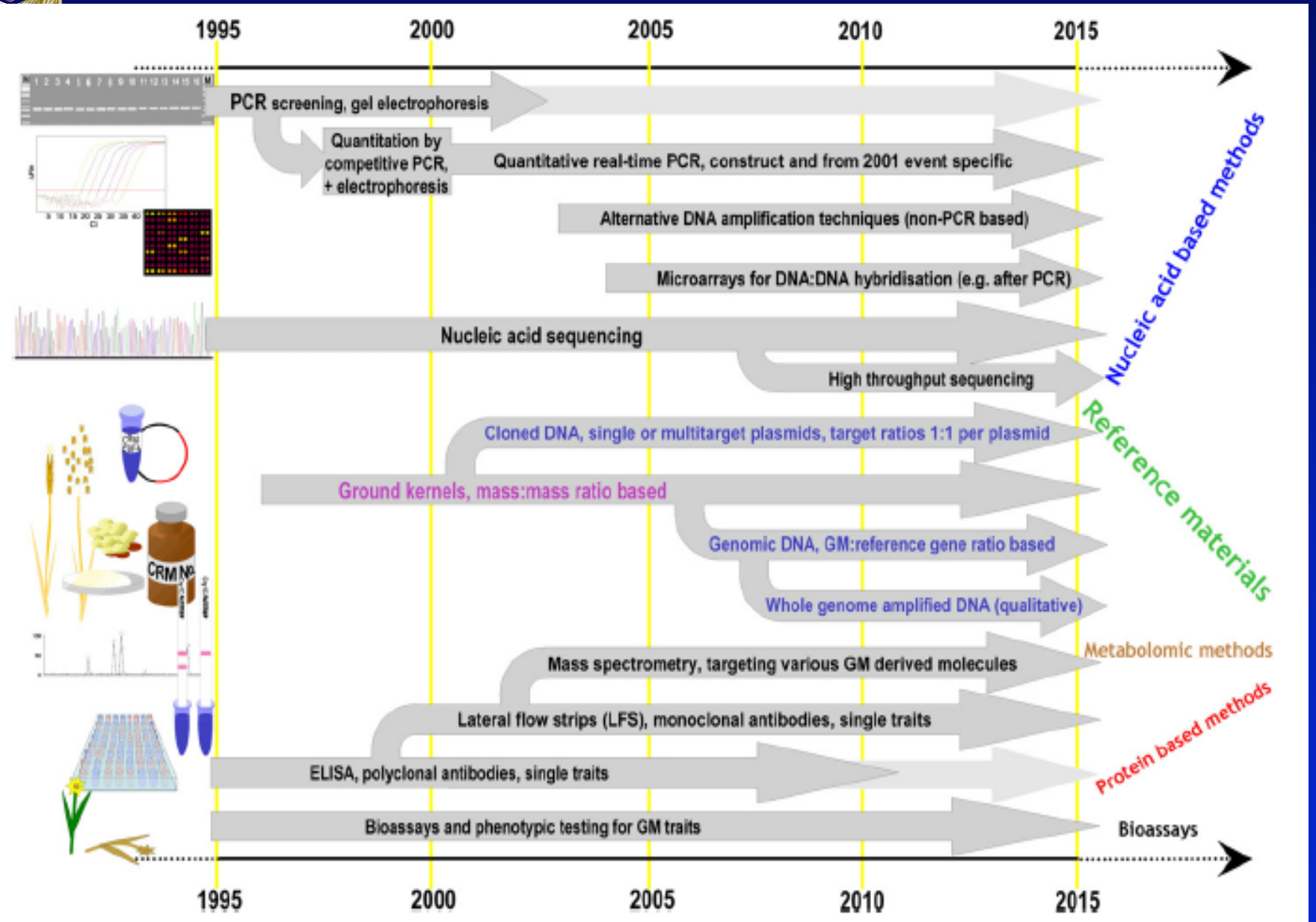

Fig 1. Evolution of GMO detection methods and associated reference materials.