ECO Workshop on "GMOs Biosafety & Regulation"

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Methods for Detection of Genetically Modified Organisms (GMOs)

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A GMO can be defined as an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination

GM crops around the world (1996 - 2020)

1996	**1.7 Mha**
1998	**31.0 Mha**
2003	**67.7 Mha**
2020	**192.0 Mha**

113 X Increase!

Source: ISAAA, 2019

Table 1. Global Area of Biotech Crops in 2019: by Country (Million Hectares)**

Rank	Country	Area (Million Hectares)	Biotech Crops
1	USA*	71.5	Maize, soybeans, cotton, alfalfa, canola, sugar beets, potatoes, papaya, squash, apples
2	Brazil*	52.8	Soybeans, maize, cotton, sugarcane
3	Argentina*	24.0	Soybeans, maize, cotton, alfalfa
4	Canada*	12.5	Canola, soybeans, maize, sugar beets, alfalfa, potatoes
5	India*	11.9	Cotton
6	Paraguay*	4.1	Soybeans, maize, cotton
7	China*	3.2	Cotton, papaya
8	South Africa*	2.7	Maize, soybeans, cotton
9	Pakistan*	2.5	Cotton
10	Bolivia*	1.4	Soybeans
11	Uruguay*	1.2	Soybeans, maize
12	Philippines*	0.9	Maize
13	Australia*	0.6	Coton, canola, safflower
14	Myanmar*	0.3	Cotton
15	Sudan*	0.2	Cotton
16	Mexico*	0.2	Cotton
17	Spain*	0.1	Maize
18	Colombia*	0.1	Maize, cotton
19	Vietnam*	0.1	Maize
20	Honduras*	<0.1	Maize
21	Chile	<0.1	Maize, canola
22	Malawi	<0.1	Cotton
23	Portugal	<0.1	Maize
24	Indonesia	<0.1	Sugarcane
25	Bangladesh	<0.1	Brinjal/Eggplant
26	Nigeria	<0.1	Cotton
27	Eswatini	<0.1	Cotton
28	Ethiopia	<0.1	Cotton
29	Costa Rica	<0.1	Cotton, pineapple
	Total	**190.4**	

*19 biotech mega-countries growing 50,000 hectares, or more, of biotech crops
**Rounded-off to the nearest hundred thousand.

Source: ISAAA, 2019

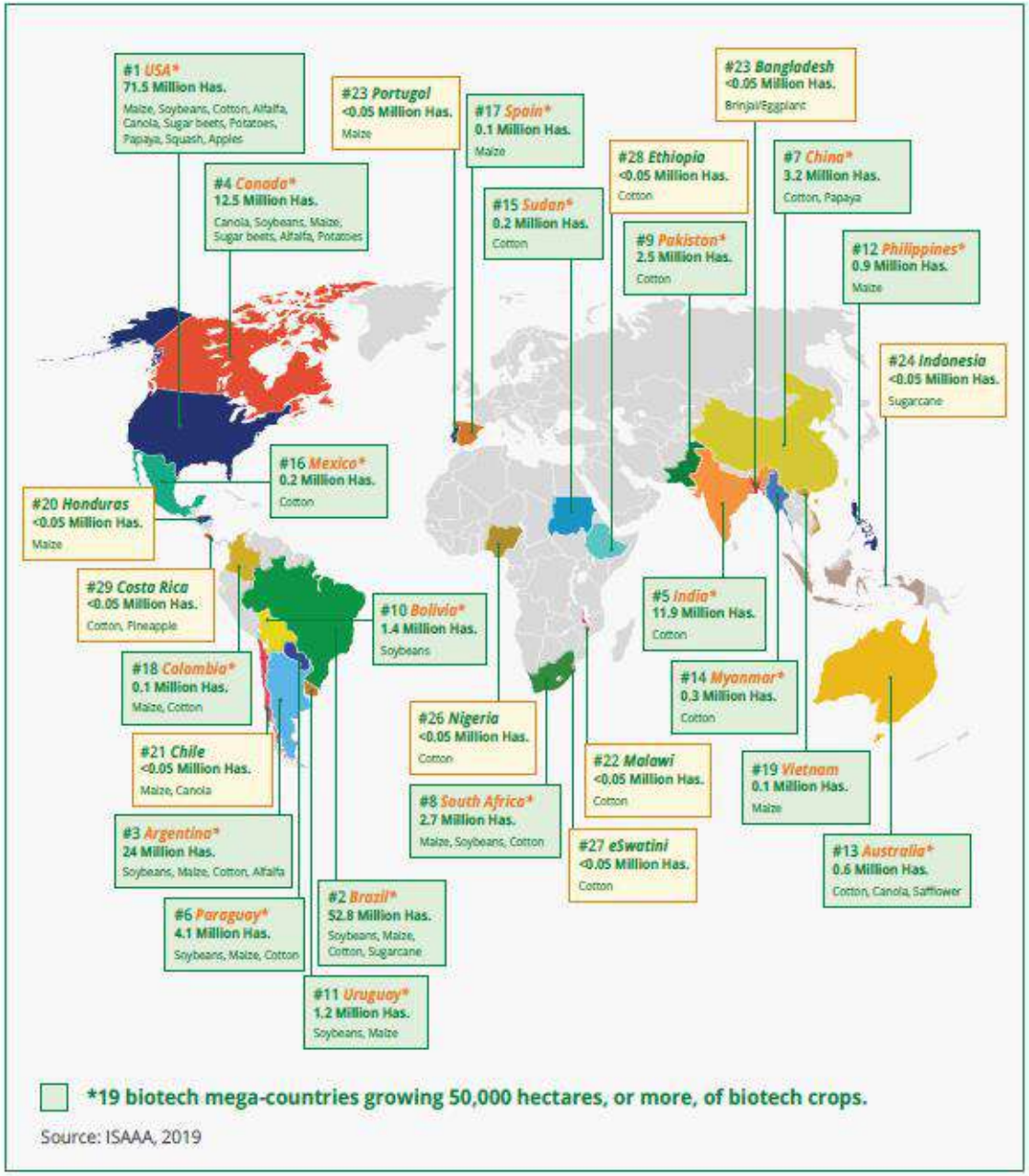
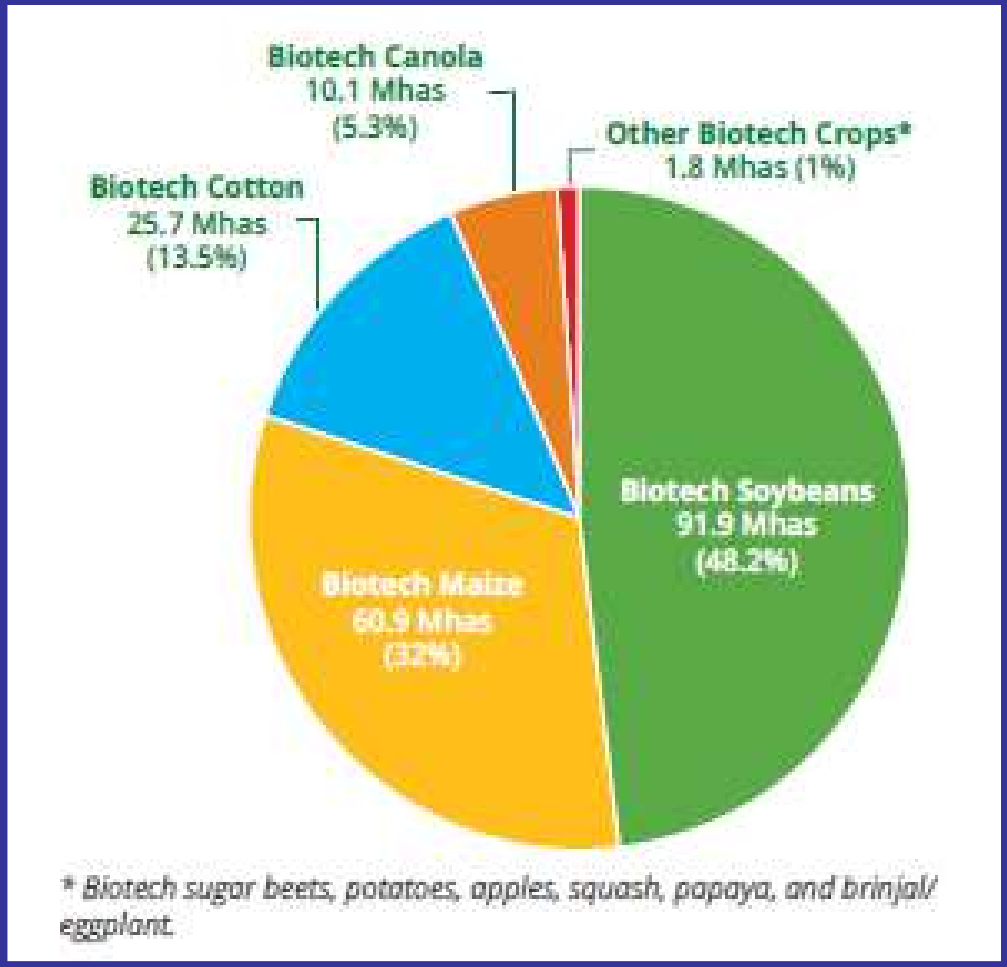


Figure 1. Global Map of Biotech Crop Countries and Mega-Countries in 2019

Biotech Crops in 2019



Source: ISAAA, 2019

Contribution of Biotech Crops to Food Security, Sustainability, and Climate Change Solutions

INCREASE CROP PRODUCTIVITY

US$225 BILLION

FARM INCOME GAINS IN 1996-2018 GENERATED GLOBALLY BY

BIOTECH CROPS

CONSERVE BIODIVERSITY

IN 1996-2018, PRODUCTIVITY GAINED THROUGH BIOTECHNOLOGY SAVED

231 MILLION HECTARES

OF LAND FROM PLOWING AND CULTIVATION

PROVIDE A BETTER ENVIRONMENT

DECREASED USE OF CROP PROTECTION PRODUCTS BY

776 MILLION KGS

A GLOBAL REDUCTION OF 8.6% IN 1996-2018

REDUCE CO2 EMISSIONS

SAVED **23 BILLION KGS CO2** EQUIVALENT TO REMOVING

15.3 MILLION CARS

OFF THE ROAD FOR 1 YEAR

HELP ALLEVIATE POVERTY AND HUNGER

BIOTECH CROPS UPLIFTED THE LIVES OF

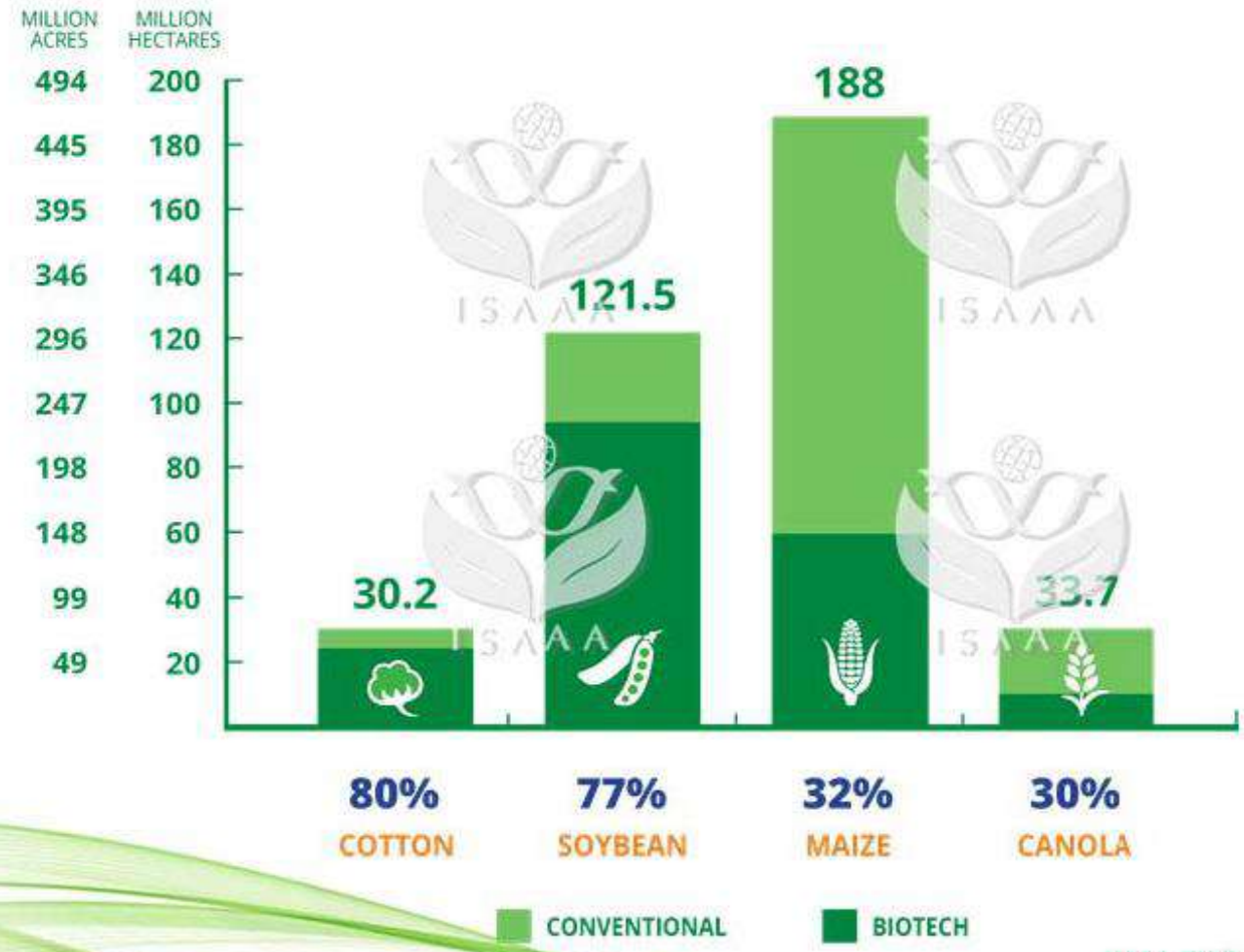
17 MILLION FARMERS

AND THEIR FAMILIES TOTALING

>65 MILLION PEOPLE

Source: Graham Brookes, 2020

Global Adoption Rates (%) for Principal Biotech Crops (Million Hectares, Million Acres), 2017



ISAAA, 2017

Necessity for GMOs Detection?

Every country import some part of its food

- Seeds (mostly for animal feed):
  Maize, Soybean, Canola,…

- Foods:
  Edible Oil (90%), Sugar (30%, 1 Mt), Cereal-based Foods, Soybean Foods,…

1. Screening: Is the sample considered a GM or non-GM?
2. Identification: If yes, is the GM-Event among the approved ones?

Labeling

~ If the sample is determined as GM or exceeds the Thresholds (for the maximum adventitious presence of GMO in conventional seed lots)

0.9% EU

3.0% South Korea and Malaysia

5.0% Japan, Canada

0.0% Iran, China

0.0% in general for non-authorized events

When the threshold is exceeded, the seed lot has to Labeled, more than….% GMO.

Safety Aspects of GM Crops

1. Agronomy
2. Food and Feed
3. Environment

The assessment of GMO content in seed lots

OBJECTIVES

Detection

- Does a lot contain GMOs ?

Identification

- If it is positive, is/are the GMO(s) approved or not ?

Quantification

- Does it comply with threshold ?

Who needs detection methods and why?

- **GMO producers**
  . To assure purity and segregation of products
  . To be able to trace genetic modification in breeding

- **Food and feed industry, seed companies**
  . To assure purity and segregation of products
  . To assure compliance with legislation

- **Component (enforcement) authorities**
  . Product control compliance with legislation
  . To be able to retrieve specific products e.g. if marketing permission is withdrawn

- **Laboratories**
  . To provide services to society (inclu. above)

Transgenic plants

A transgenic plant contains genetic material that has been modified or foreign gene (transgene) tat has been transferred from an unrelated organism,

Genetic Modification

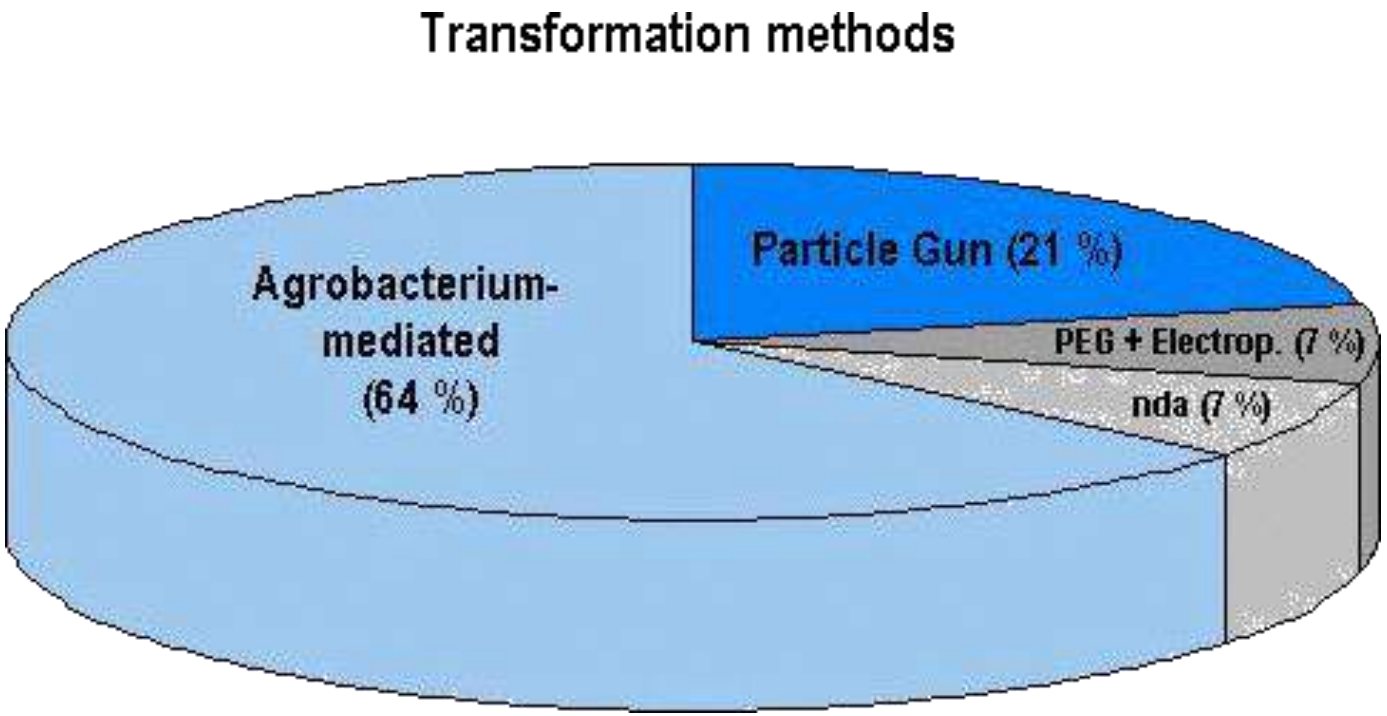
Alteration of genetic makeup of living organism in a way that does not occur naturally.

The insertion in plant genome occurs at random

The progeny of a transformed cell is called Event

The genetic modification(s) characteristics of an event are introduced in many varieties by conventional crossing

Transformation Methods



What Genes are Transferred ?

CONTROL SEQUENCES

- **Promoters**
- **Terminators**
- **Introns**

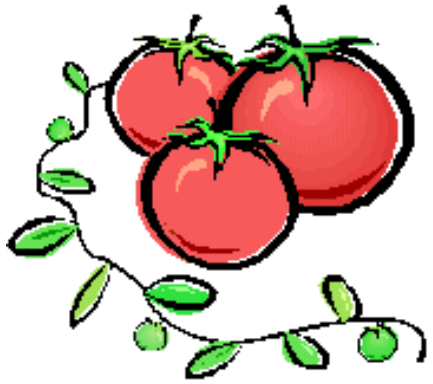
STRUCTURAL GENES (New traits)

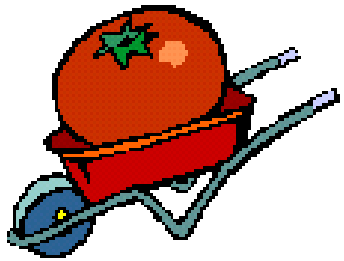
- **Enzymes**
  - **herbicide tolerance: *epsps, bar, pat***
  - **herbicidedetoification: *gox***
  - **new metabolic pathway (fat and oils)**
- **Other products**
  - **resistance to pests: Bt endotoxins, viral coat**
  - **protein, etc.**
- **Antisense constructs**
  - **anti-polygalacturonase (FLAVR SAVR ™ tomato)**
  - **reduced synthesis or increased degradation of ethylene, etc.**

GENES FOR SELECTABLE MARKERS

- **Resistance to antibiotics (*nptII*)**
- **Resistance to herbicide (*bar, pat*)**

Falvr Savr

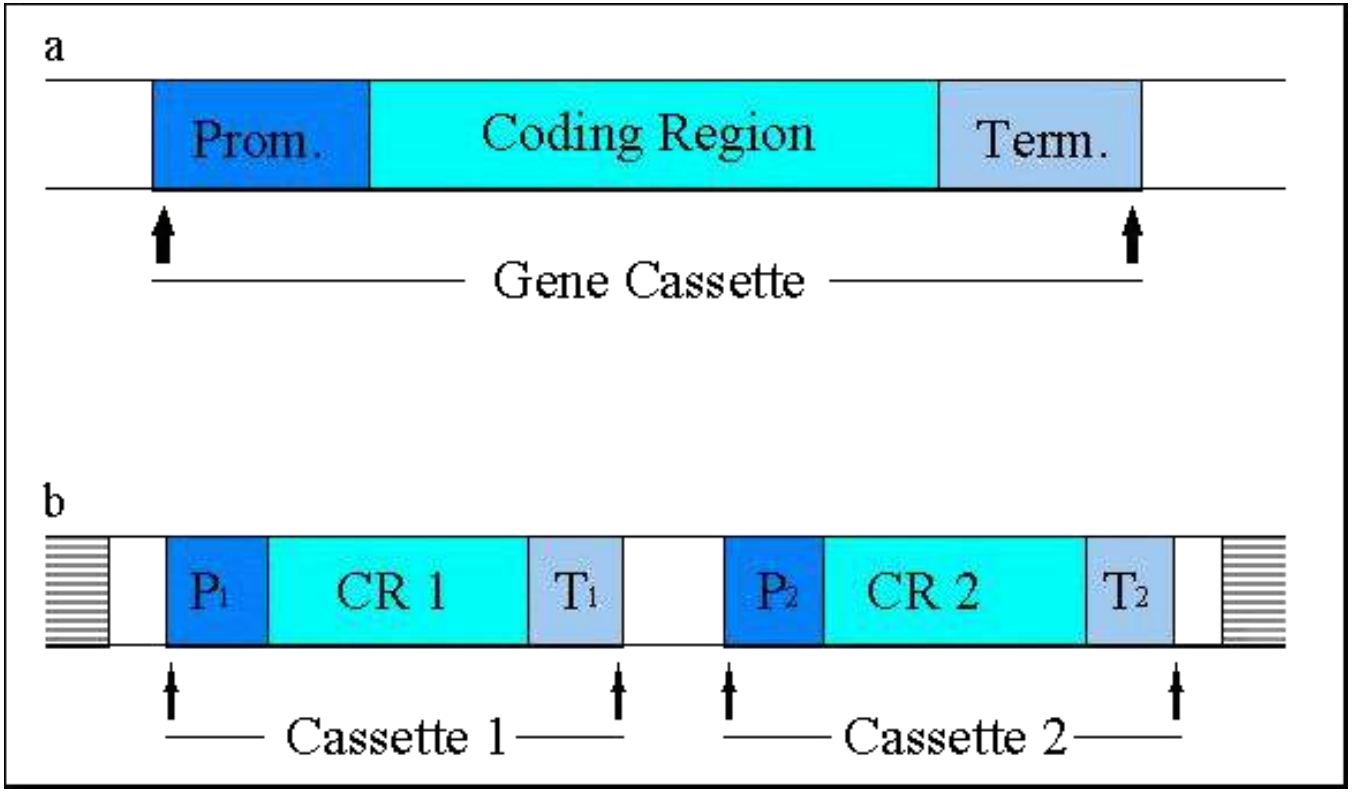




Traditional

ETHYLENE

Representation of gene constructs (gene cassettes) made of promoters (P), a structural gene (coding region) and terminator



Methods for GMO Detection

1. Analysis of the phenotype
1.1. Bioassay
1.2. Protein-based methods (ELISA, lateral flow strip)

2. Analysis of the genotype
. PCR-based methods
2.1. Qualitative PCR
2.2. Competitive-quantitative PCR
2.3. Real-time PCR

Phenotypic Characterization (Bioassay)

. Allows detection of the presence or absence of a specific trait

. So far, only tests for traits as tolerance to herbicides are available

. They consist on germination tests on solid media in the presence of a specific herbicide

. Negative trait and positive trait seeds should be included as controls with every sample seed

. Samples tested positive should be exposed to subsequent tests for confirmation

. Bioassay are available for:

Roundup Ready Soybean, Maize, Cotton, and Canola

Typically, a test of 400 seeds/seed lot is carried out (can be increased to 1,000 to 2,000 seed, if the germination is low)

Immunoassays

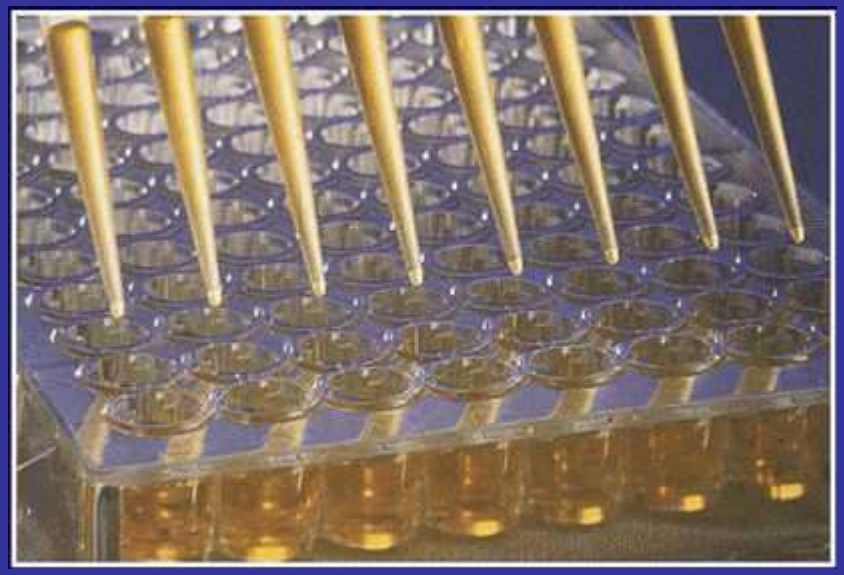
. The crucial component is the antibody with high specificity for the target molecule (Ag)

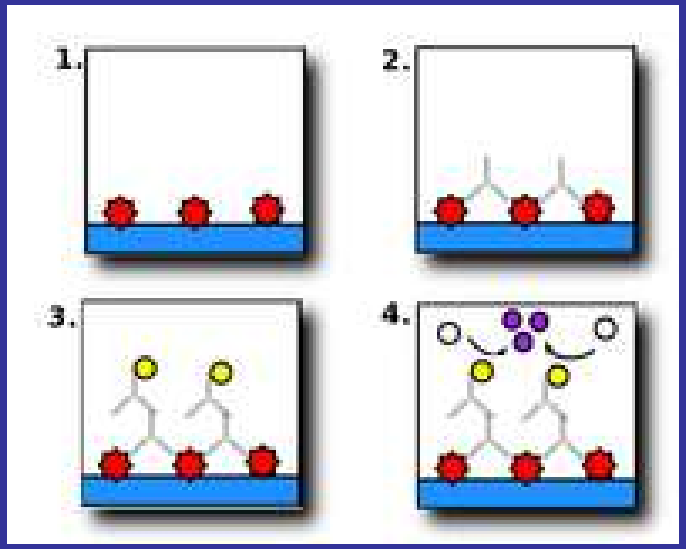
. Antibodies can be polyclonal (raised in animals) or monoclonal (from cell culture)

. The Ag/Ab reaction is detected through a second Ab that carries a label that generate a signal

. Immunoassay are available for both field as well as for well-equipped laboratories

ELISA Test





Immunoassays

Advantages

. Can be highly specific

.Samples often need only a simple preparation

. Can be used qualitatively or quantitatively over a wide range of concentrations

. Results in a few hours

Disadvantages

. Development of a Ab might be a difficult task

. Proteins can be proprietary and not commonly available

. Require separate tests for each trait in question

Lateral Flow Strips or Immuno Strips

Application for:

Seed Industry:

. Determine presence of a certain level of a specific protein in a sample

. May be used by research, seed production and sales team member

Grain Handlers:

Determine presence of a certain level of a specific protein in grain samples

Lateral Flow Strips are available in different types:

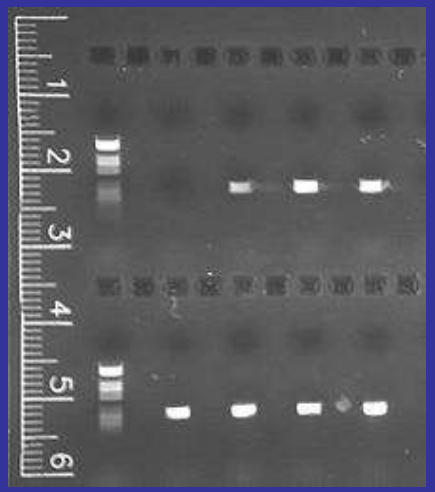
Single analyte strip, Multi analyte strip and Multi-strip comb

Lateral Flow Strips (Immunoassay)

PCR-based Analysis of Seed Samples

- Sampling according to ISTA rules
- Choice of testing plan
- Preparation of working sample (thousand seed weight)
- Grinding
- Sample the flour
- DNA extraction
- DNA quantification
- PCR - qualitative (electrophoresis)

  - quantitative (e.g. *real time* detection of fluorescence)
- Analysis and reporting of results



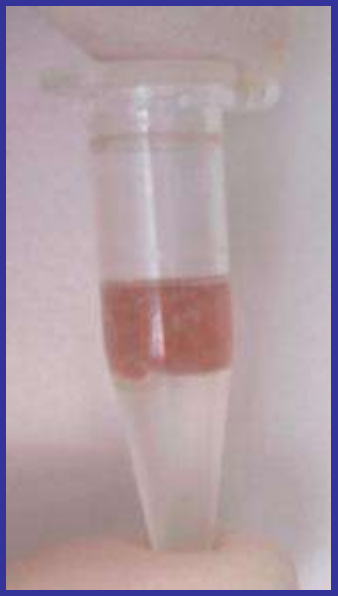
Sample Grinding

- Grinding is a critical step for subsequent analysis
- Fine grinding limits the sampling error
- Fine grinding of samples yield more DNA

Recommendations:

- Avoid dust during grinding
- The grinding apparatus and container must be accurately cleaned or washed after each use (e.g. with sodium hypochlorite)

DNA Extraction



Isolate and purify DNA from other plant components that can interfere with further manipulation

Steps:

1. Lysis of cell walls and membranes (detergents)
2. Elimination of proteins and polysaccharides (chloroform)
3. DNA precipitation (alcohol)
4. DNA resuspension (in water or buffer)

PCR-based methods for GMO Detection

1. Screening methods

. Useful as a first tool for analysis of unknown samples

. Based on detection of genetic elements common to many GMO events

. These elements should not be present in conventional crop

. Primers should allow to detect the presence of most variants of the element

Example: P35S promoter, NOS terminator, *nptII* gene

PCR-based methods for GMO Detection

2. Specific methods (Identification methods)

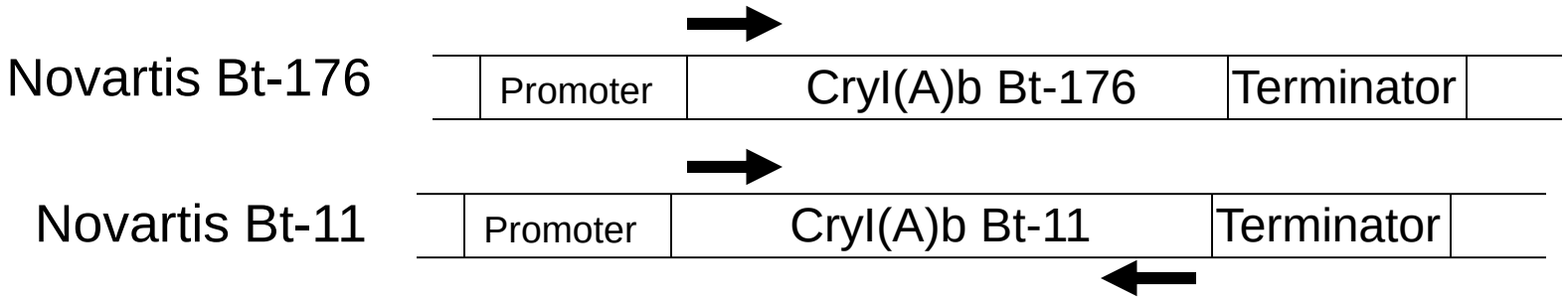
. Useful for confirming positive results

. Necessary for distinction between approved and unapproved events (different thresholds)

. Necessary for trait confirmation (purity of GM lots)

. Allow tracking events during development, production and trade

Gene Specific Methods
Specific gene contained in a given event

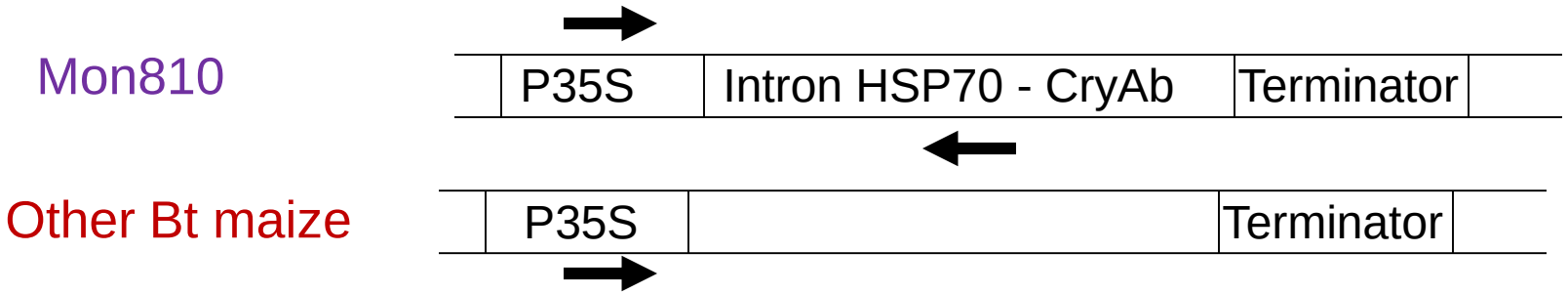


But the same gene can be present in different events

Construct-specific Method

Based on specific detection of cross border sequences between genetic elements in different events, e.g. between promoter and structural gene

In this way, the assay become more selective

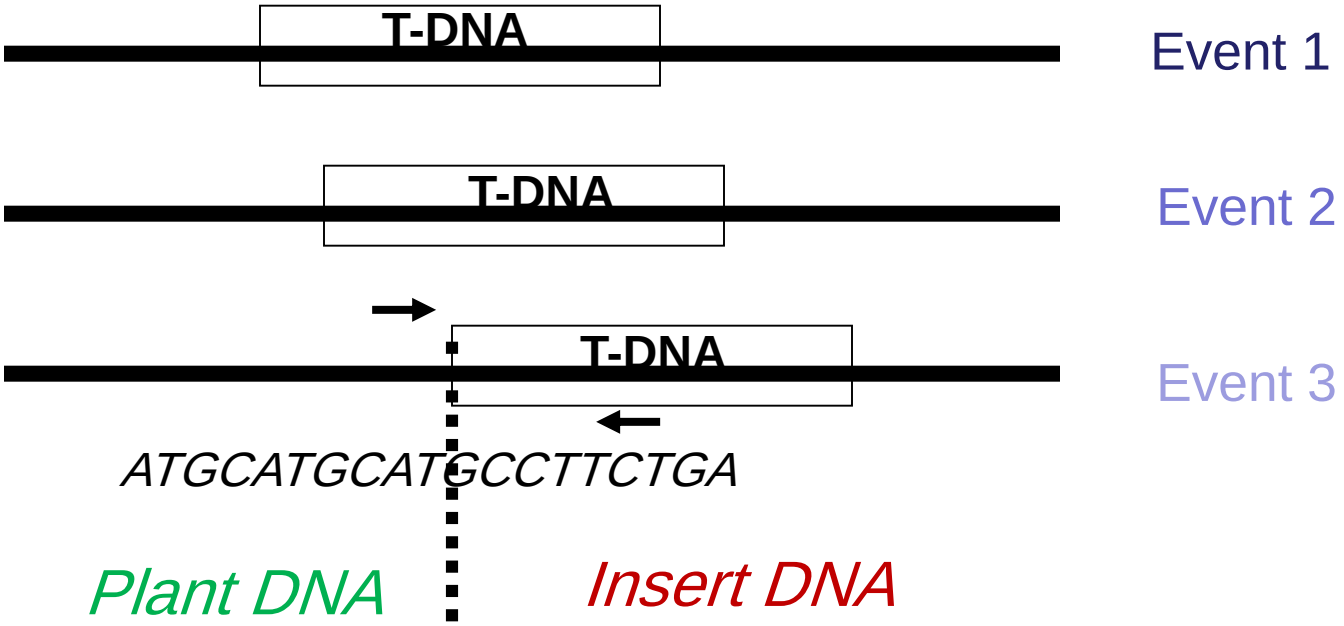


But the same construct can be present in different events !

Event – specific Methods

A transformation event is defined by T-DNA/Plant DNA junction

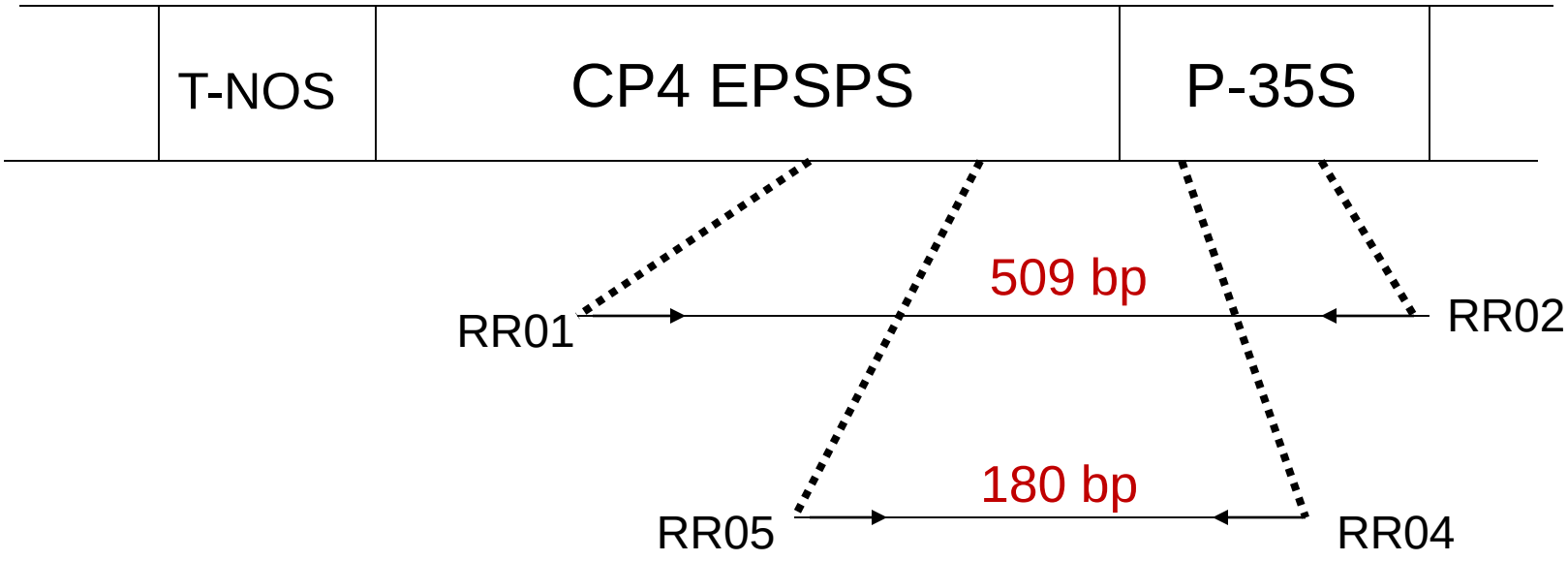
(This information is not directly available)



Nested PCR

PCR “nested” for RR Soybean

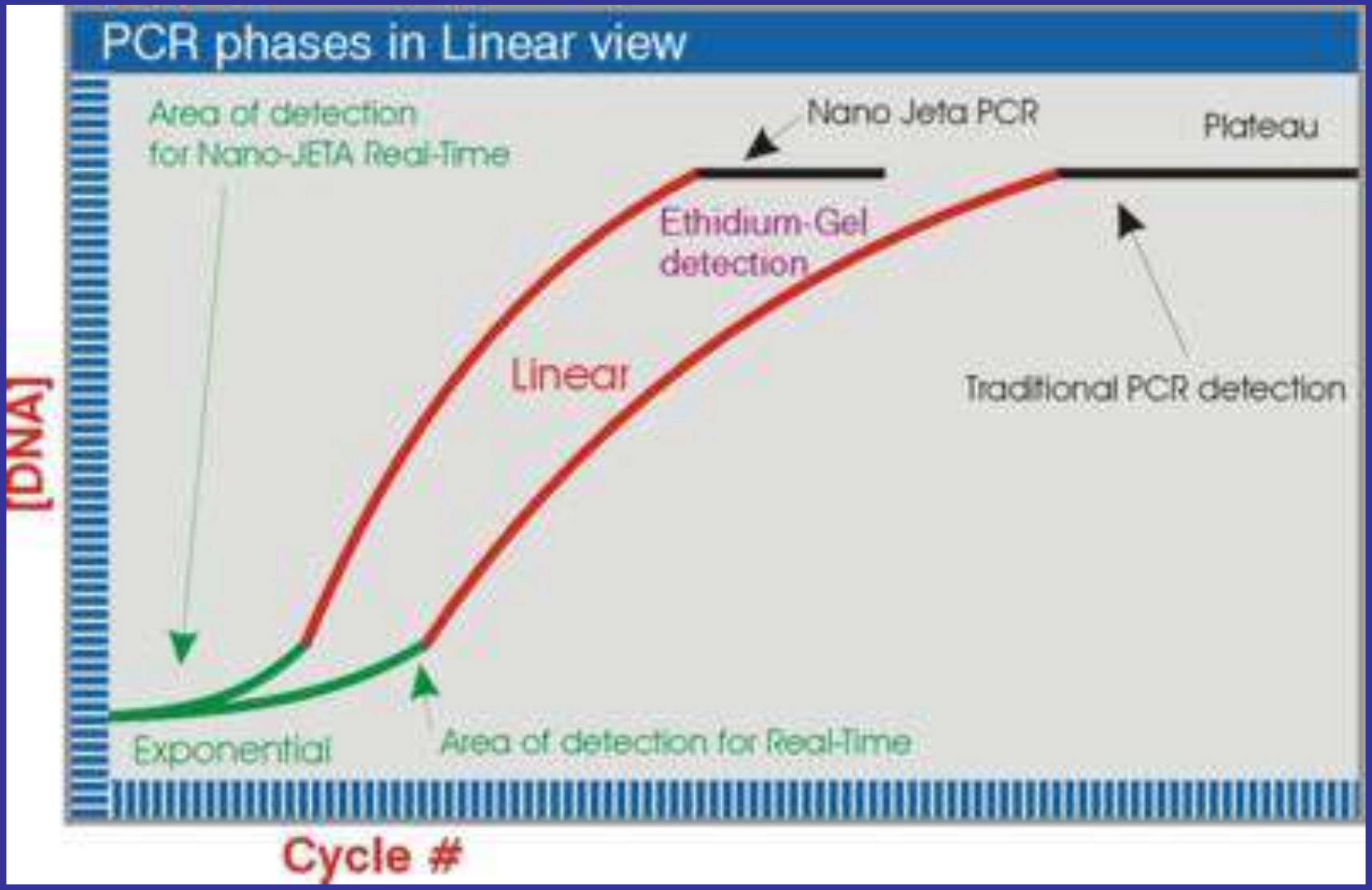
(Koppel et al., 1997)



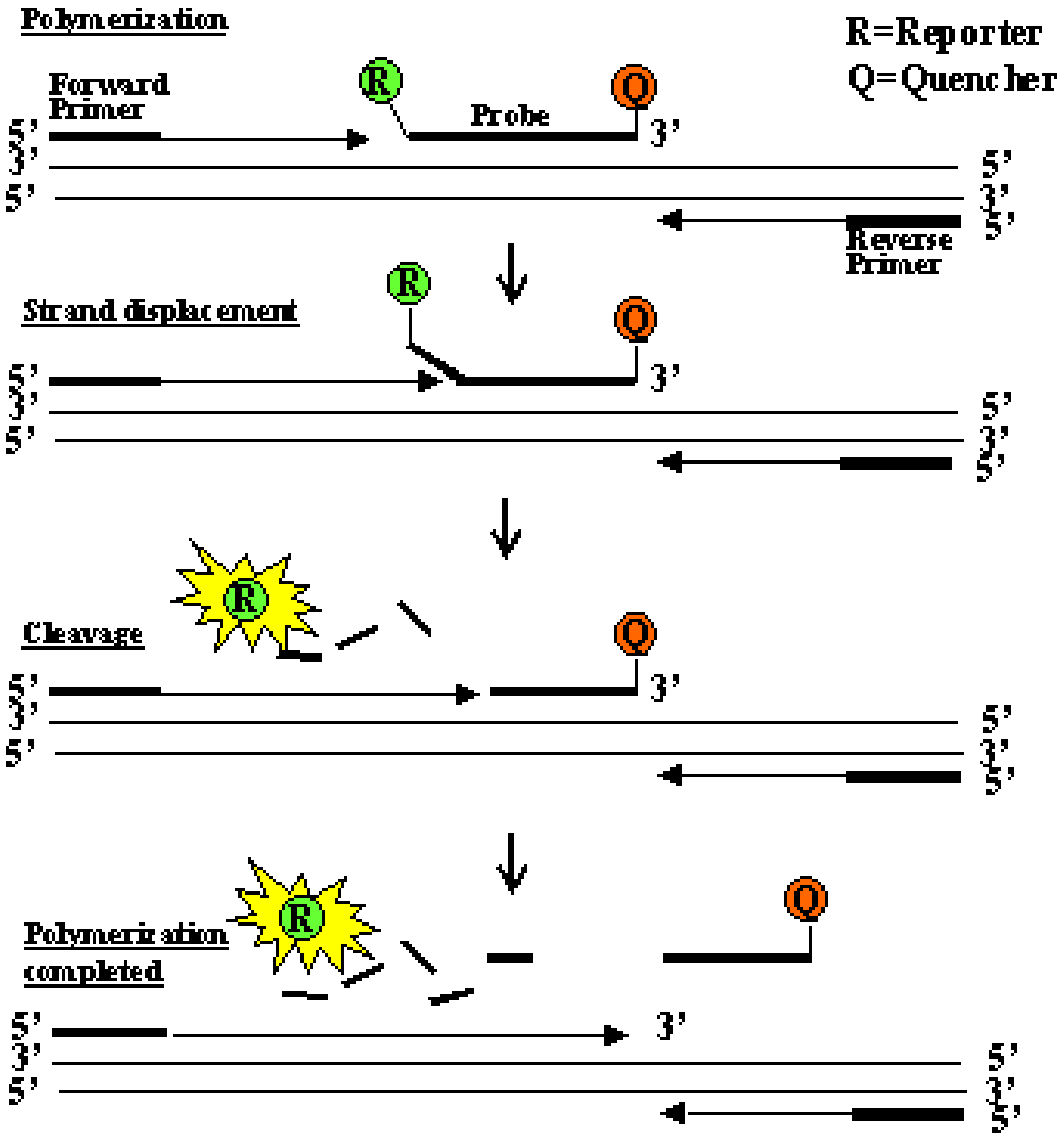
Nested PCR protocols represent also a means to increase sensitivity in detection, on the other hand, the risk of false positive results is decreased

Limitations of End-point PCR

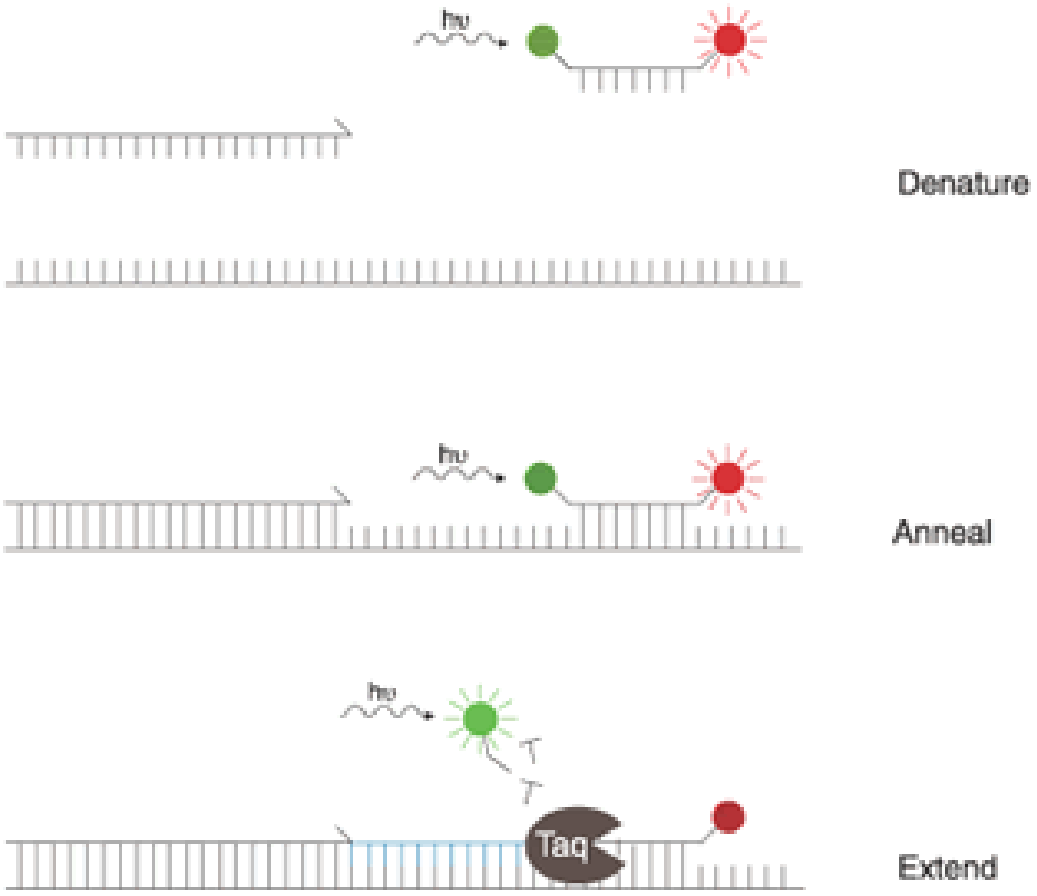
- **Pour precision to quantify**
- **Low sensitivity**
- **Short dynamic range < 2 logs**
- **Low resolution**
- **Non-automated**
- **Size-based discrimination only**
- **Results are not expressed as numbers**
- **Ethidium bromide for staining is not very quantitative**
- **Post PCR processing**
- **More susceptible to cross-contamination due to PCR product transfer from the tube to the detection step**
- **Detecting PCR product in plateau phase**


PCR phases in Linear view
Area of detection for Nano-JETA Real-Time
Nano Jeta PCR
Plateau
Ethidium-Gel detection
Linear
Traditional PCR detection
[DNA]
Exponential
Area of detection for Real-Time
Cycle #

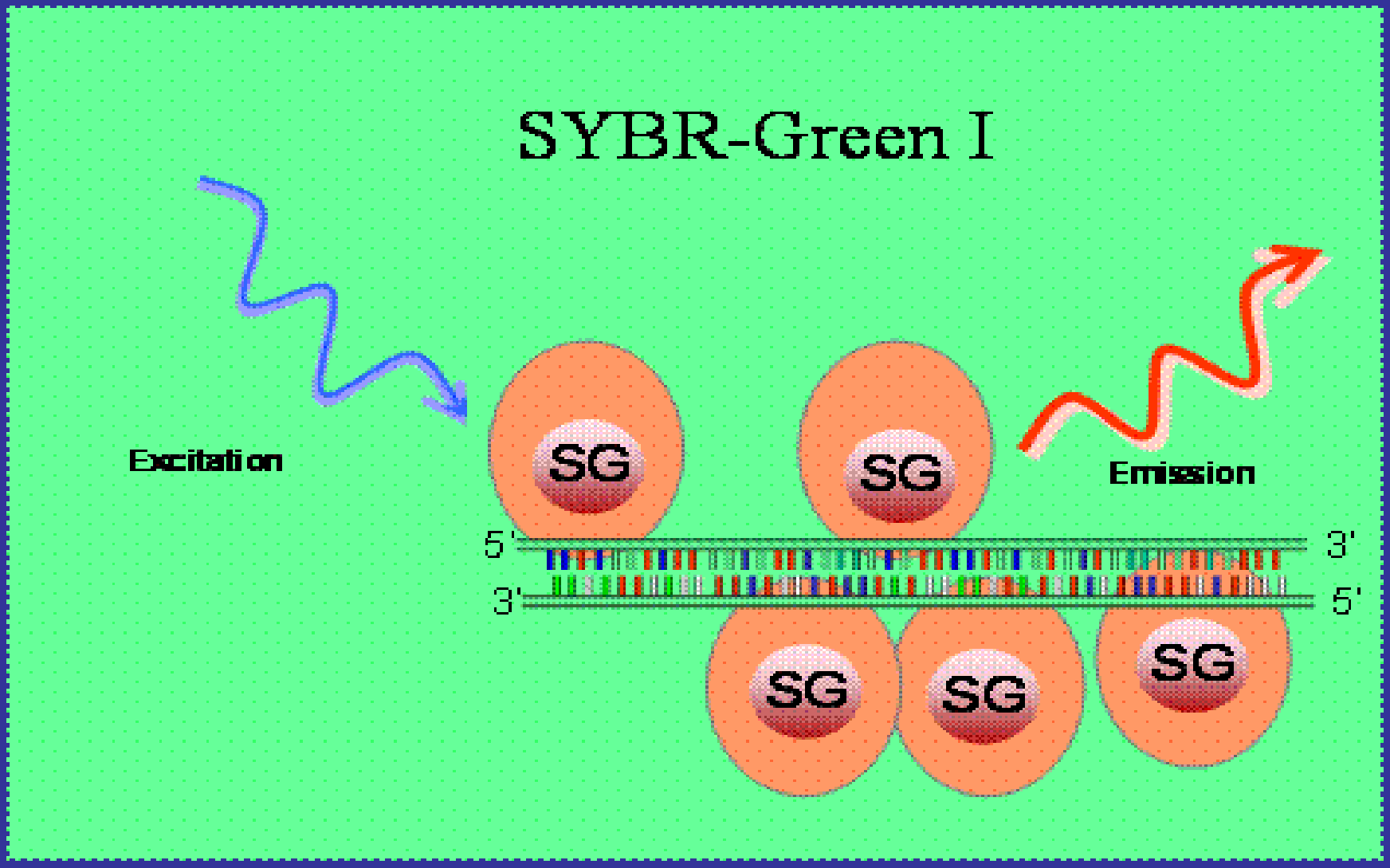
Real Time-PCR



TaqMan® Probe Chemistry

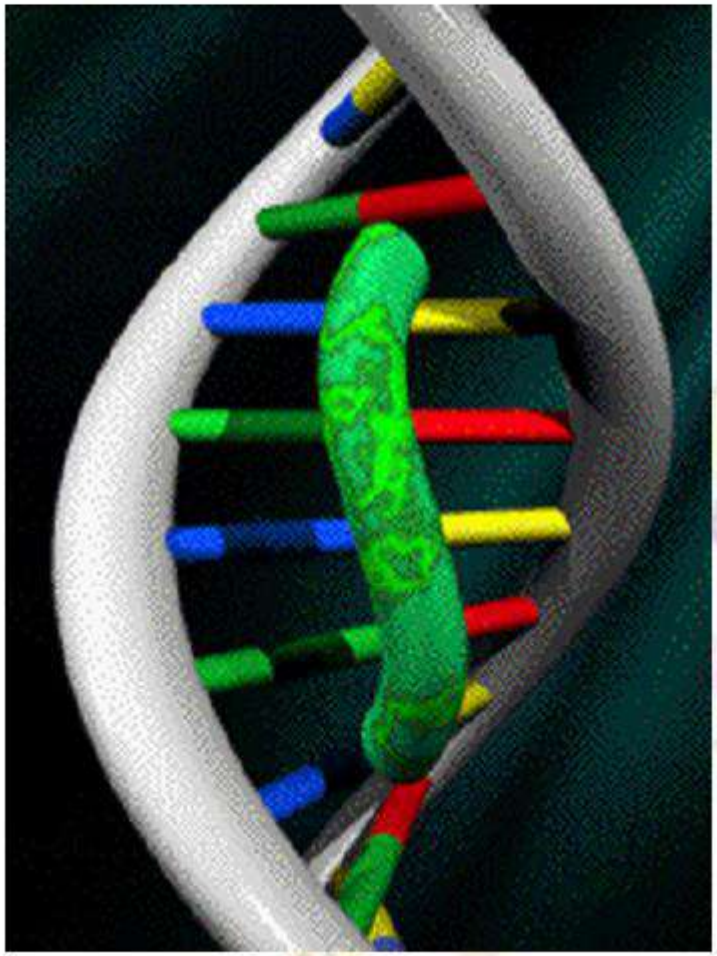


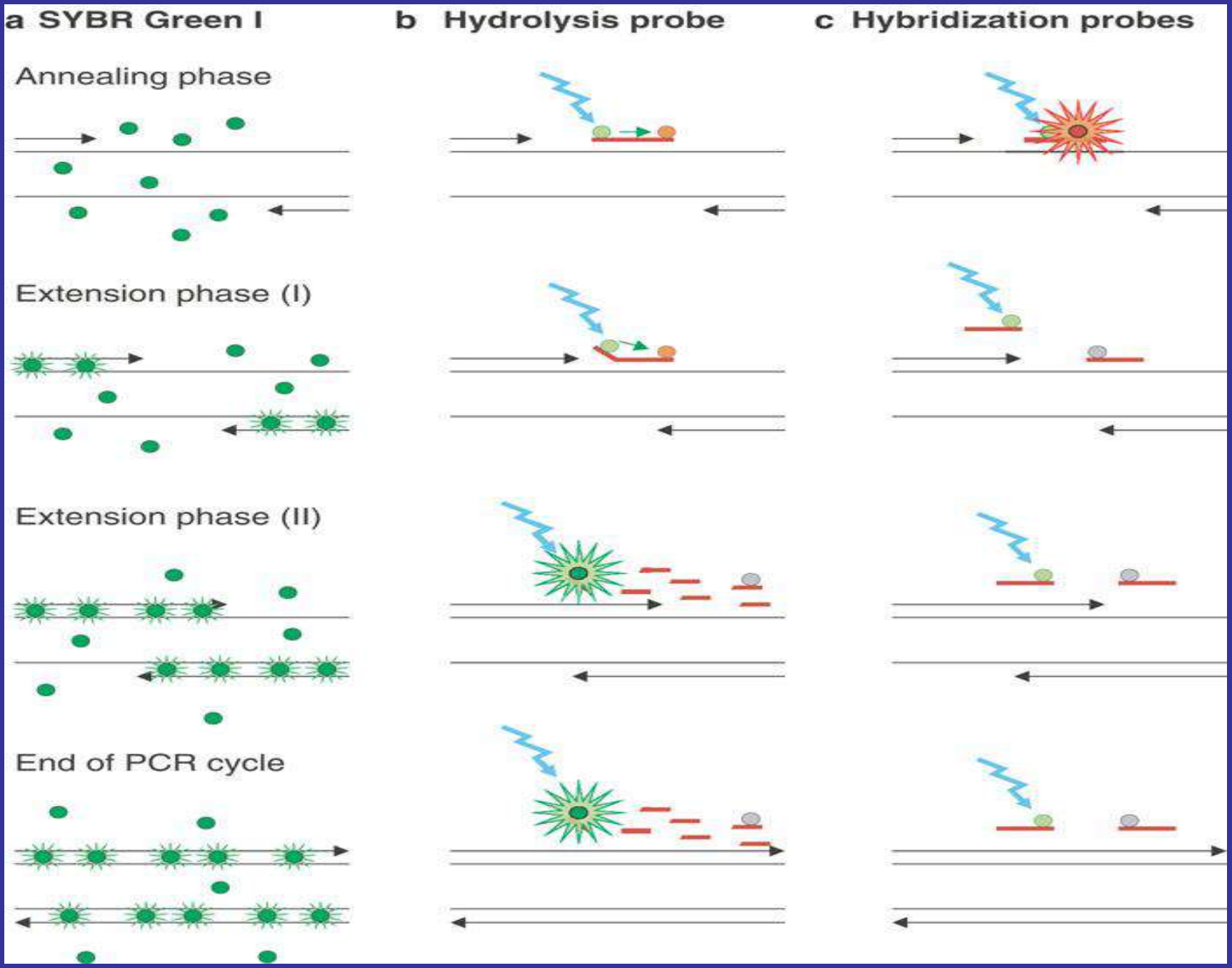
Example of Sybr Green-1 Binding to dsDNA (DNA-binding agents)

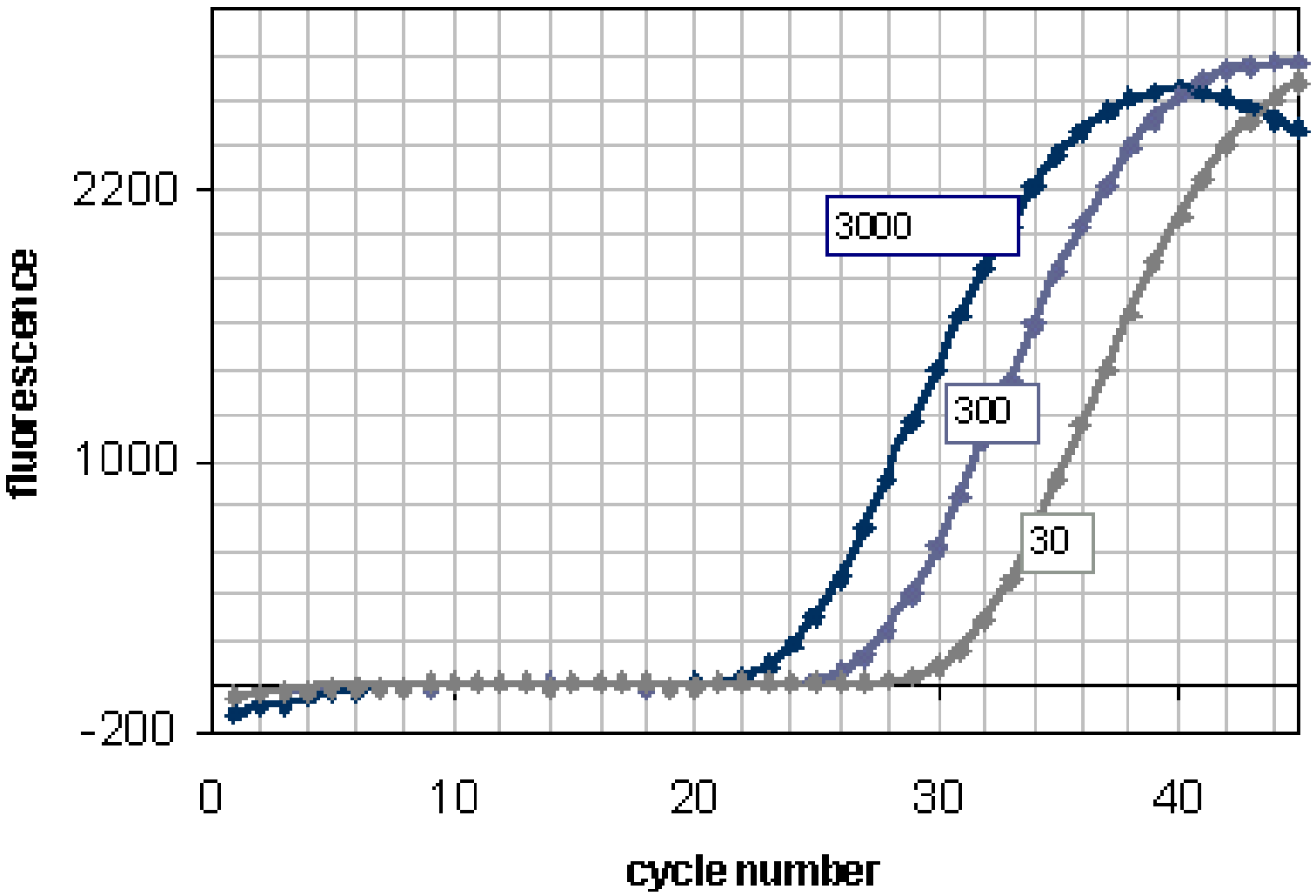


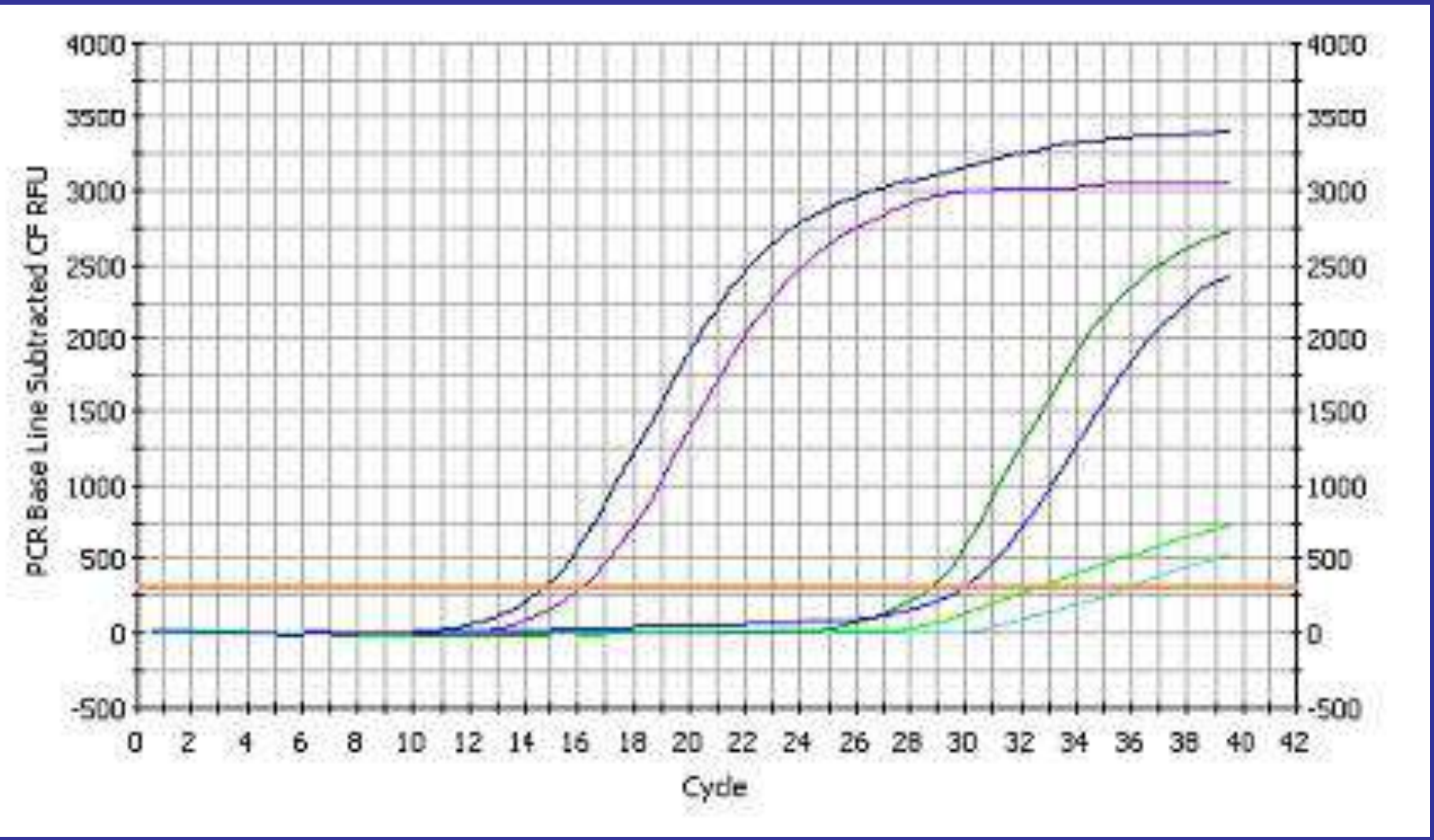
Thermal Cycling
SYBR Double-Stranded DNA binding Dye

- SYBR® green 1 DYE
  - SYBR dye fluoresces upon binding to double stranded DNA
  - Any double stranded DNA → NON-specific
  - Used for target identification (screening) assays
  - Low cost
  - Drawback: Primer dimers or non-specific amplicons also fluoresce

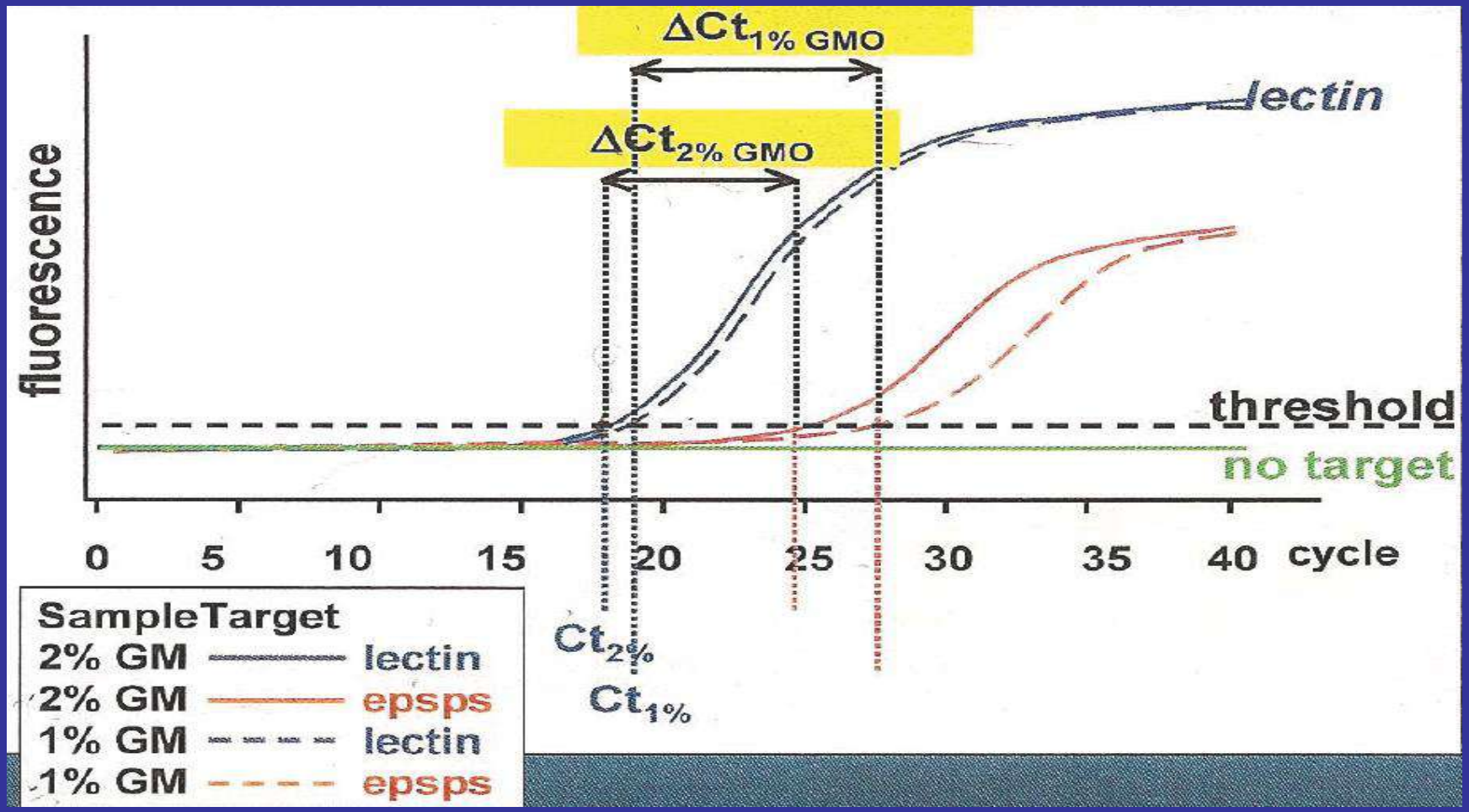



a SYBR Green I
b Hydrolysis probe
c Hybridization probes
Annealing phase
Extension phase (I)
Extension phase (II)
End of PCR cycle


2200
1000
-200
fluorescence
0
10
20
30
40
cycle number
3000
300
30


PCR Base Line Subtracted CF RFU
4000
3500
3000
2500
2000
1500
1000
500
0
-500
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Cycle

Real Time PCR for *EPSPS* Gene
(The lower the ΔCt, the higher is the % GMO In the sample)



Advantages of Using Real-time PCR

- Collect data in the exponential growth phase
- The increase of the reporter fluorescence is directly proportional to the number of amplicons generated
- The cleaved probe provides a permanent record amplification of the amplicon
- Increase dynamic range of detection
- No post-PCR processing
- Faster, since less handling procedure
- Higher sample throughput
- No toxic chemicals

Drawbacks of Using rt - PCT

- More expensive devices and chemicals (Probes)
- Result evaluation is more sophisticated
- Higher cognitive requirement for technicians

ISTA (International Seed Testing Association) GMO Task Force Objectives and Progress, 2001-2005

1. Chapter in ISTA rules for detection, identification and quantification of GMO in conventional seed lots
2. Implementation of laboratory accreditation under performance based approaches
3. Organizing proficiency tests on detection and quantification of GMO in conventional seed
4. Establishing a system for *Performance Data Evaluation for the presence of Seeds with Specific Trait(s) in Seed Lots*
5. Providing technical help for the laboratories (training courses, workshops,…)

New Methods of GMO Detection

- **Biosensors**

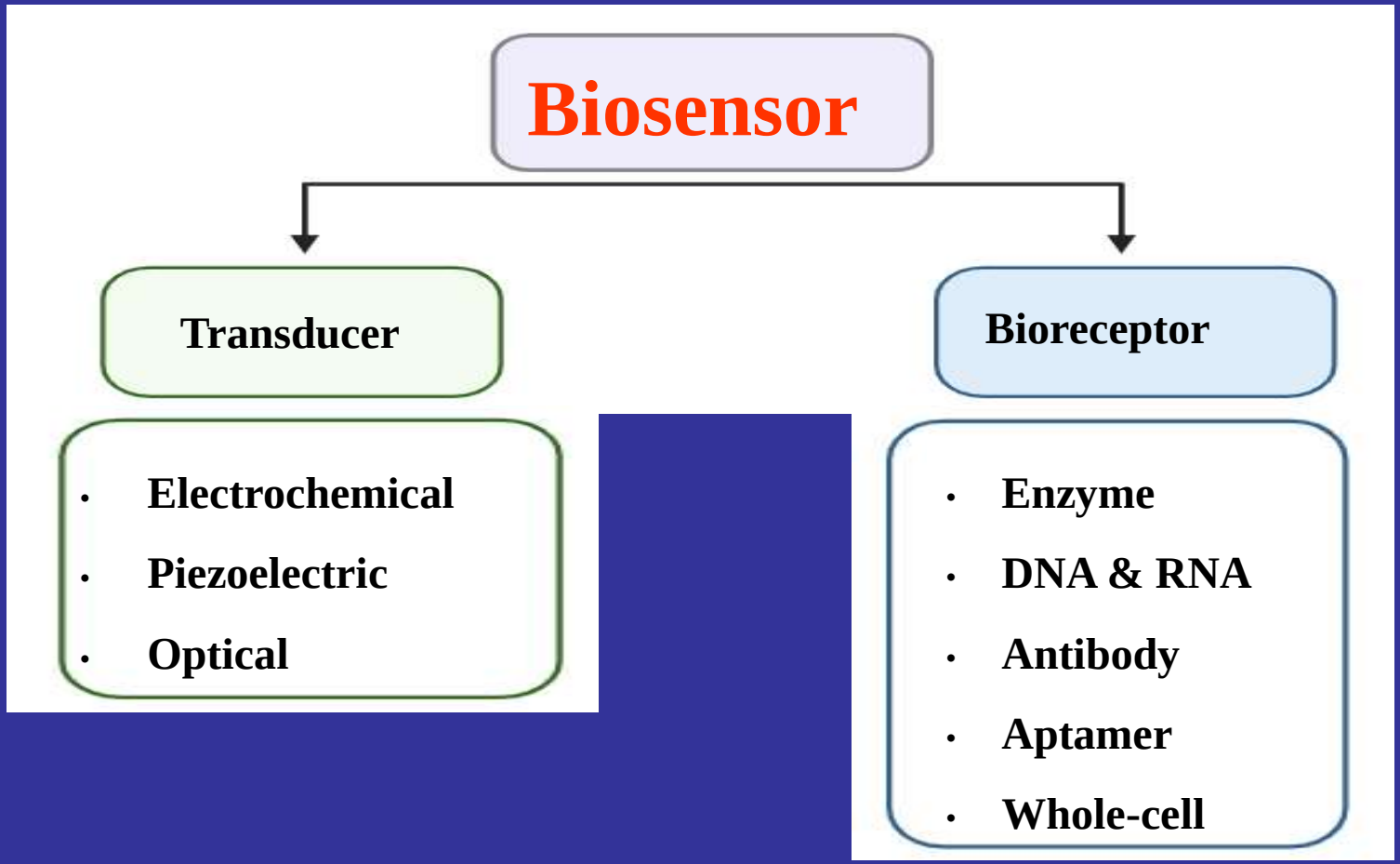
IUPAC definition:

"A device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals."

Biosensor Applications

- Medical Diagnosis (Clinical and Laboratory)
- Food and Agricultural Processes (Quality and Safety)
- Environmental (Air, Soil, & Water) Monitoring
- Industry (Detecting Pathogens in Fresh Meat, Poultry or Fish)
- Detection Systems (DNA, Protein, Hormone)

Components of a Biosensor



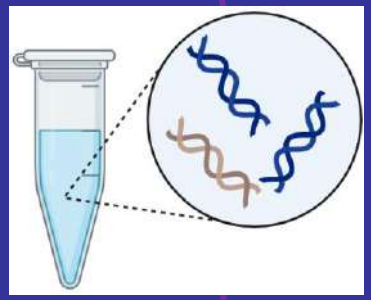
A Biosensor consists of three main elements:

1. Bioreceptor

1. Transducer

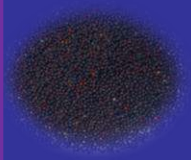
1. Signal Processing System.

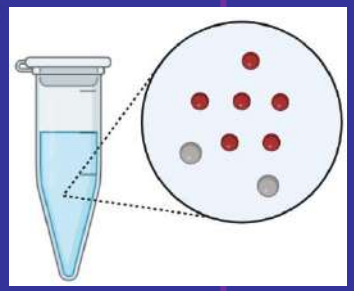
GM or non-GM?



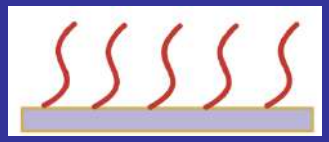
DNA extraction

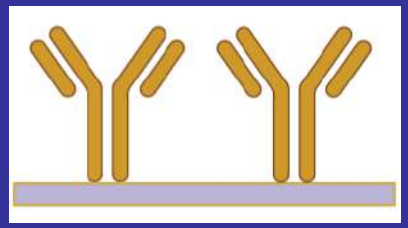
Protein extraction



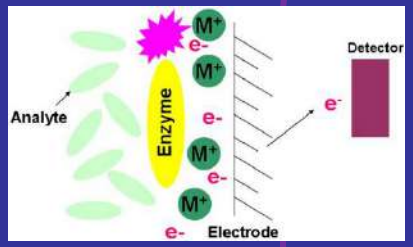


Material

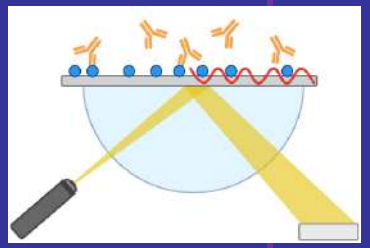




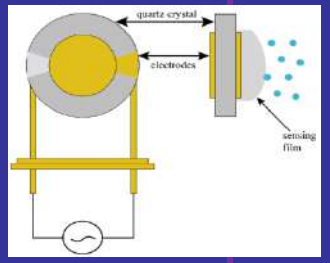
Biorecognition Elements



Electrochemical

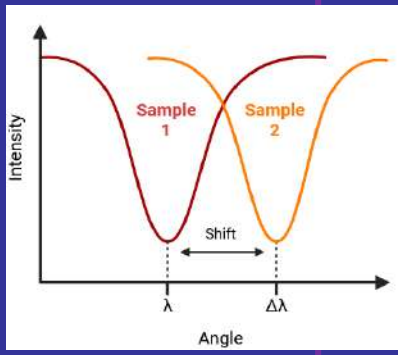


Optical



Piezoelectric

Transducer



Signal Processing

ISTA focuses its activity on developing a system targeting the:

uniformity in GMO testing results, not primarily by the standardization of methods to test GMOs, but by the combination of the proficiency tests and a performance-based approach

Thank You for Your Attention !