Making a transgenic plant

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Why make transgenic plants?

Improvement of crop plants by introduction of new genes

Studies on promoter expression

Find out the function of a gene by overexpressing it or by reducing its expression etc.

1.1. Creation of transgenic plants

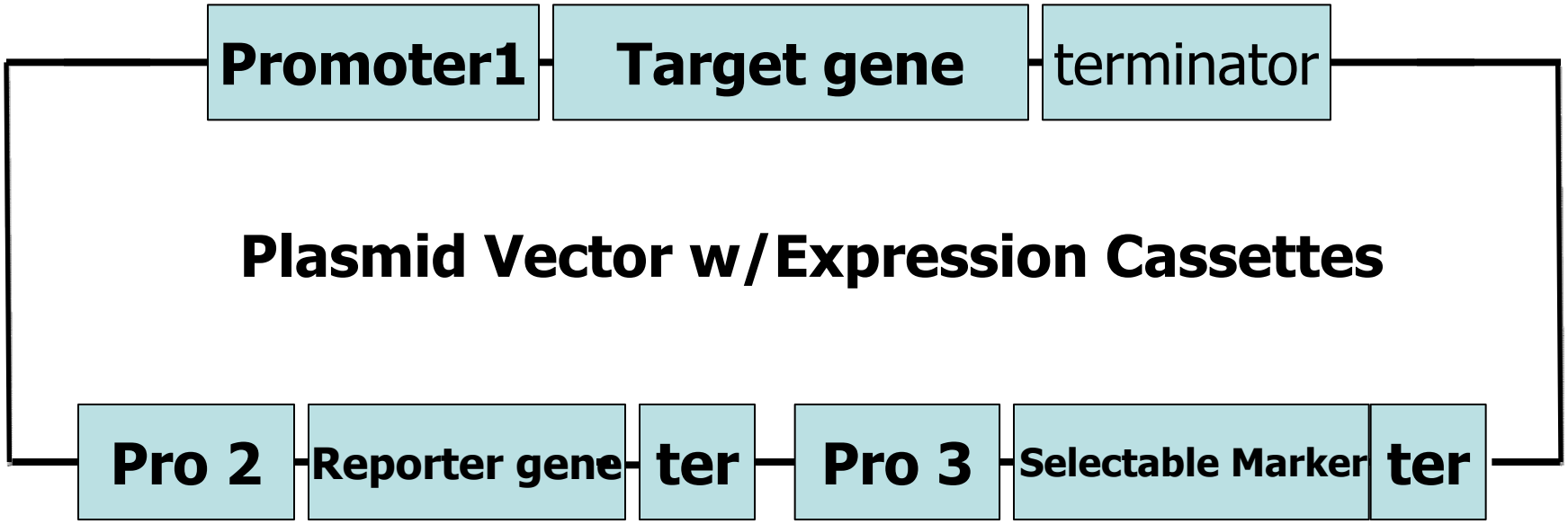
It is now time to cover the development of transgenic crops in greater depth. The three major steps are creating a transformation cassette that contains the ***gene of interest****, then* ***successfully introducing the cassette into the plant*** *and, finally,* ***the regeneration of the transformed tissue****.*

4.1.1.Create transformation cassette

All of these components of the transformation cassette contain multiple components. In addition to the coding region that encodes the protein product, the gene of interest region also contains two important controlling regions (target gene).

The promoter region resides just before the coding region and determines when, where, and to what degree the gene of interest will be expressed.

Multiple expression cassette (e.g. selectable marker, reporter and target genes) are constructed into a plasmid vector



In general, this leads to a relatively high level of gene expression. The most often used constitutive promoter controls the expression of the cauliflower mosaic virus (CaMV) 35S promoter. Other promoters direct a very specific expression pattern. For example, the glutelin 1 promoter directs the expression of the glutelin storage protein at a specific time of seed development. It also ensures the protein is only expressed in the rice endosperm. If the gene of interest is preceded by the CaMV35S promoter, it will be expressed in all tissues at all times.

Conversely, the expression of the target gene could be limited to the endosperm if it is controlled by the glutelin 1 promoter.

CaMV 35S Promoter

from cauliflower mosaic virus

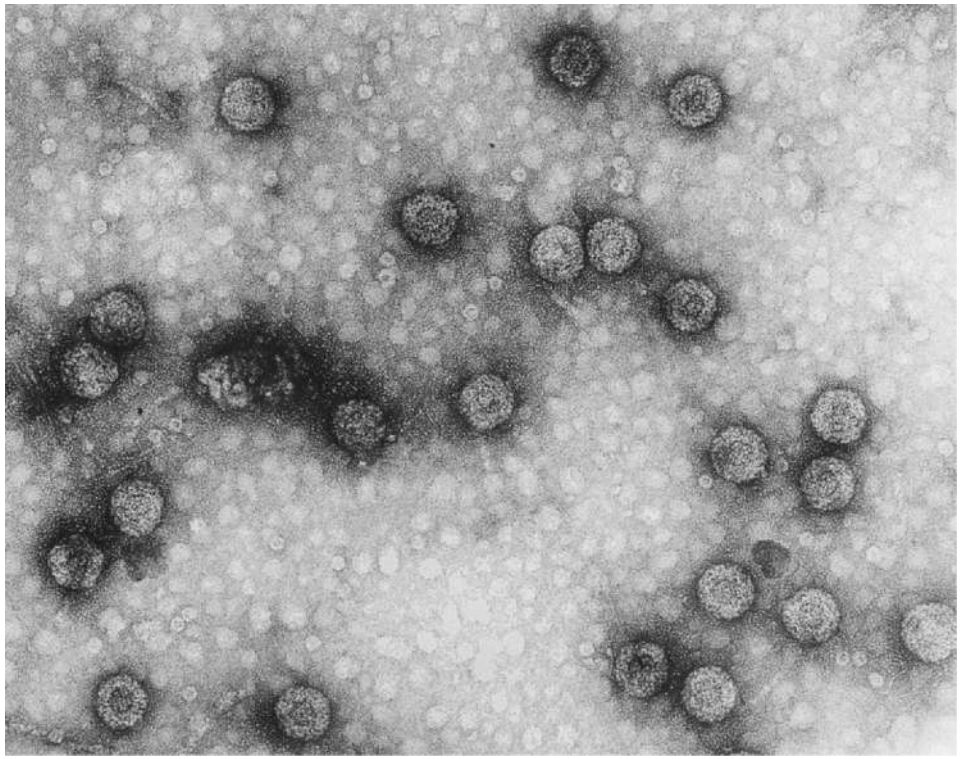
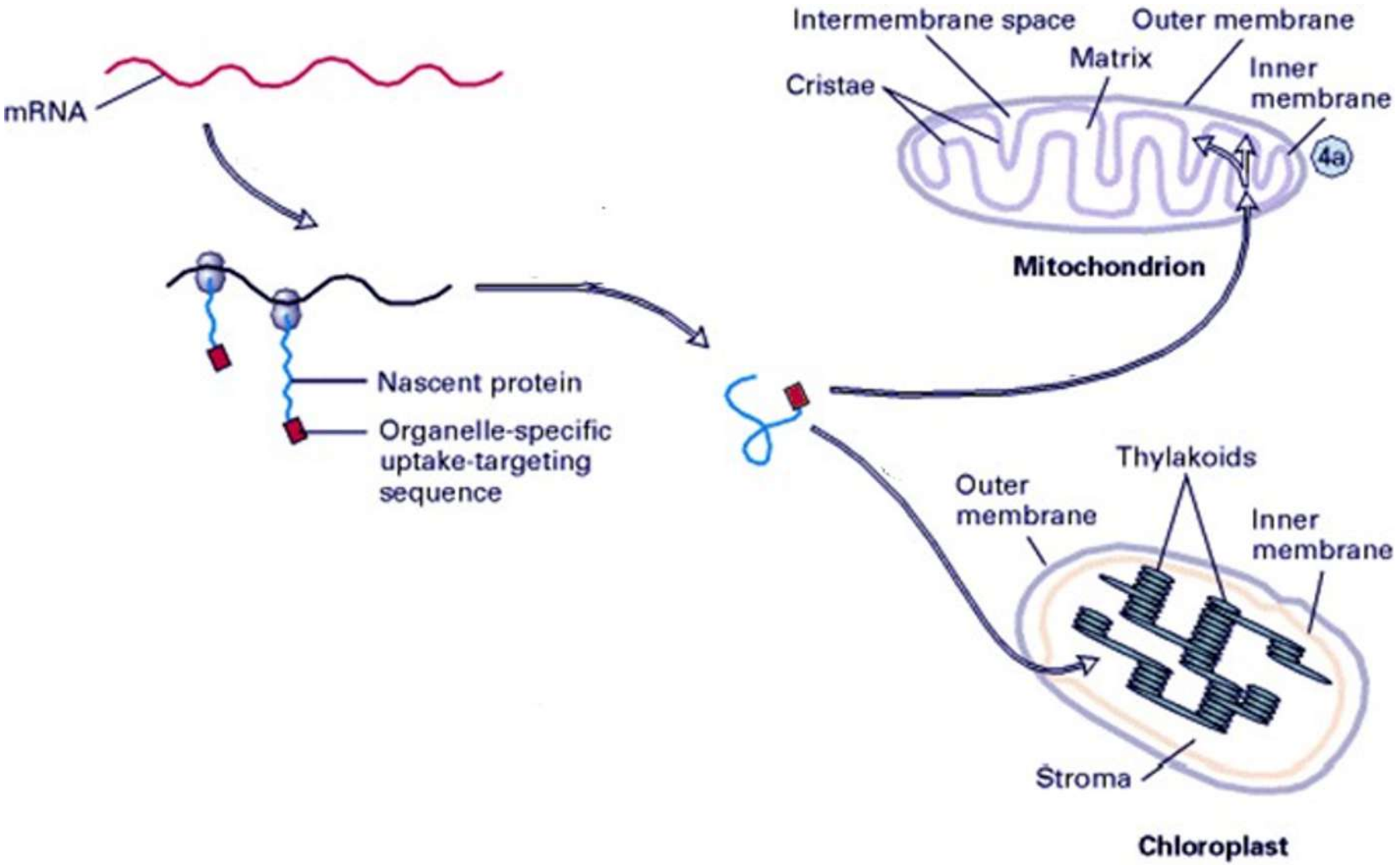
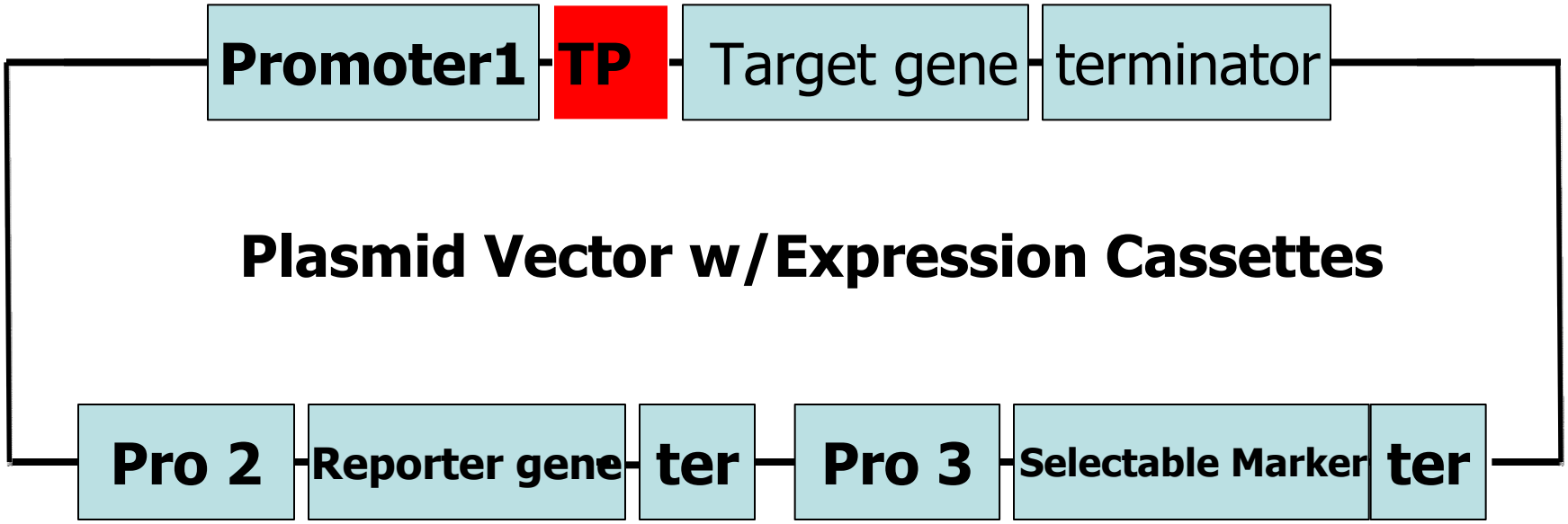


Photo source:
www.ncbi.nlm.nih.gov

Some, but not all genes, encode protein that functions in the plant organelles. These organelles are the chloroplast and the mitochondria. For example, photosynthesis, and part of the carbon and lipid metabolism pathways are carried out in the organelles. To ensure these proteins are delivered to the appropriate organelle, an organelle-specific uptake-targeting sequence or transit peptide (TP) is required. This is a short amino acid sequence that is found directly before the coding region. This sequence is recognized by proteins in the outer membranes of the appropriate organelle. This recognition process leads to the import of the protein into the organelle. Therefore, for genes function in the organelle, an appropriate transit peptide must be included in the transformation cassette.


mRNA
Nascent protein
Organelle-specific uptake-targeting sequence
Intermembrane space
Outer membrane
Matrix
Cristae
Inner membrane
4a
Mitochondrion
Thylakoids
Outer membrane
Inner membrane
Stroma
Chloroplast

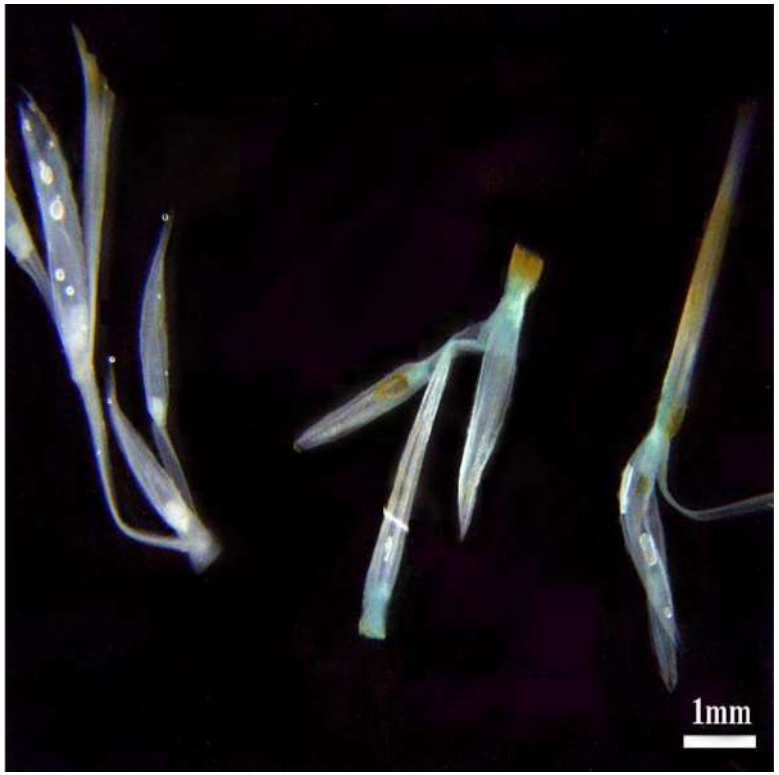
The use of transit peptid (TP) for transformation in chloroplast or in the mitochondria

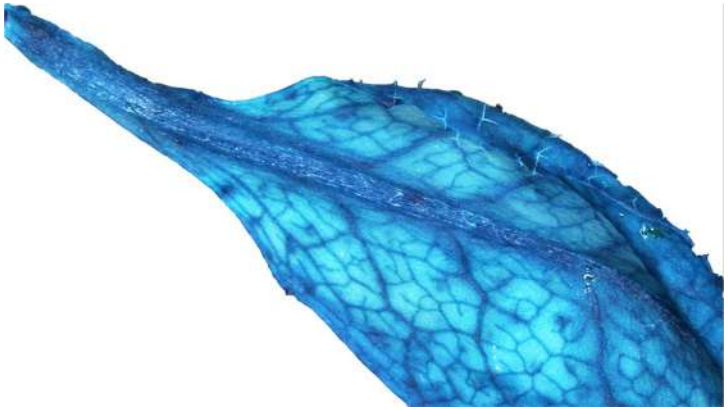


The selectable marker/reporter gene is a gene that encodes a protein product. For it to be expressed, it also needs a promoter region. It is typical to use the constitutive CaMV35S promoter.

- *Reporter Genes code for a gene product that has an easily detectable phenotype (GUS gene).*
- *The selectable marker it controls encodes a protein that enables a transformed plant to survive in the presence of a normally toxic compound. The most often used selective agents are kanamycin and hygromycin, two bacterial antibiotics, and the herbicide glufosinate. The protein encoded by the selectable marker genes generally renders these selective agents harmless to the transgenic plant.*

GUS Activity in Vetiver inflorescence Explants

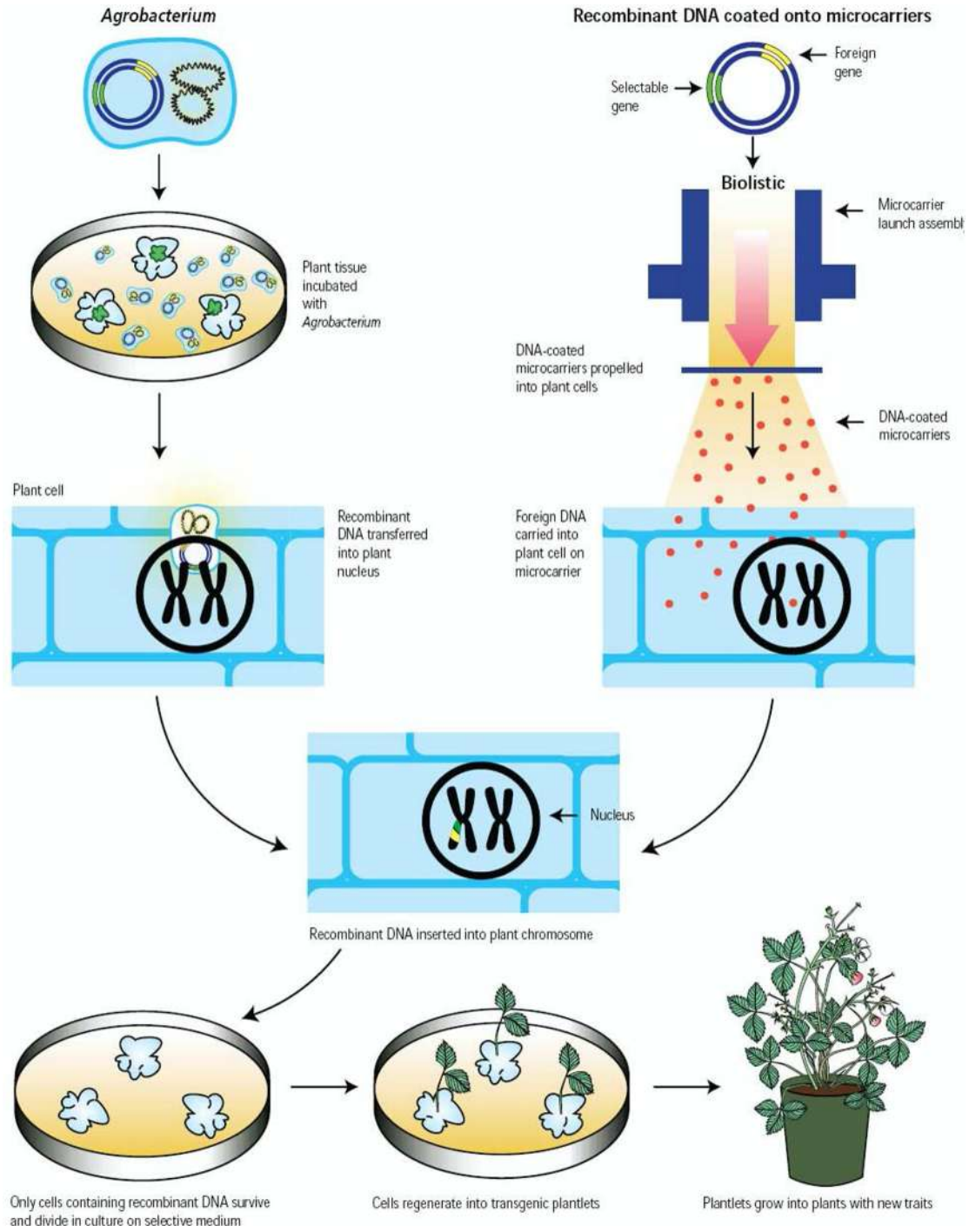


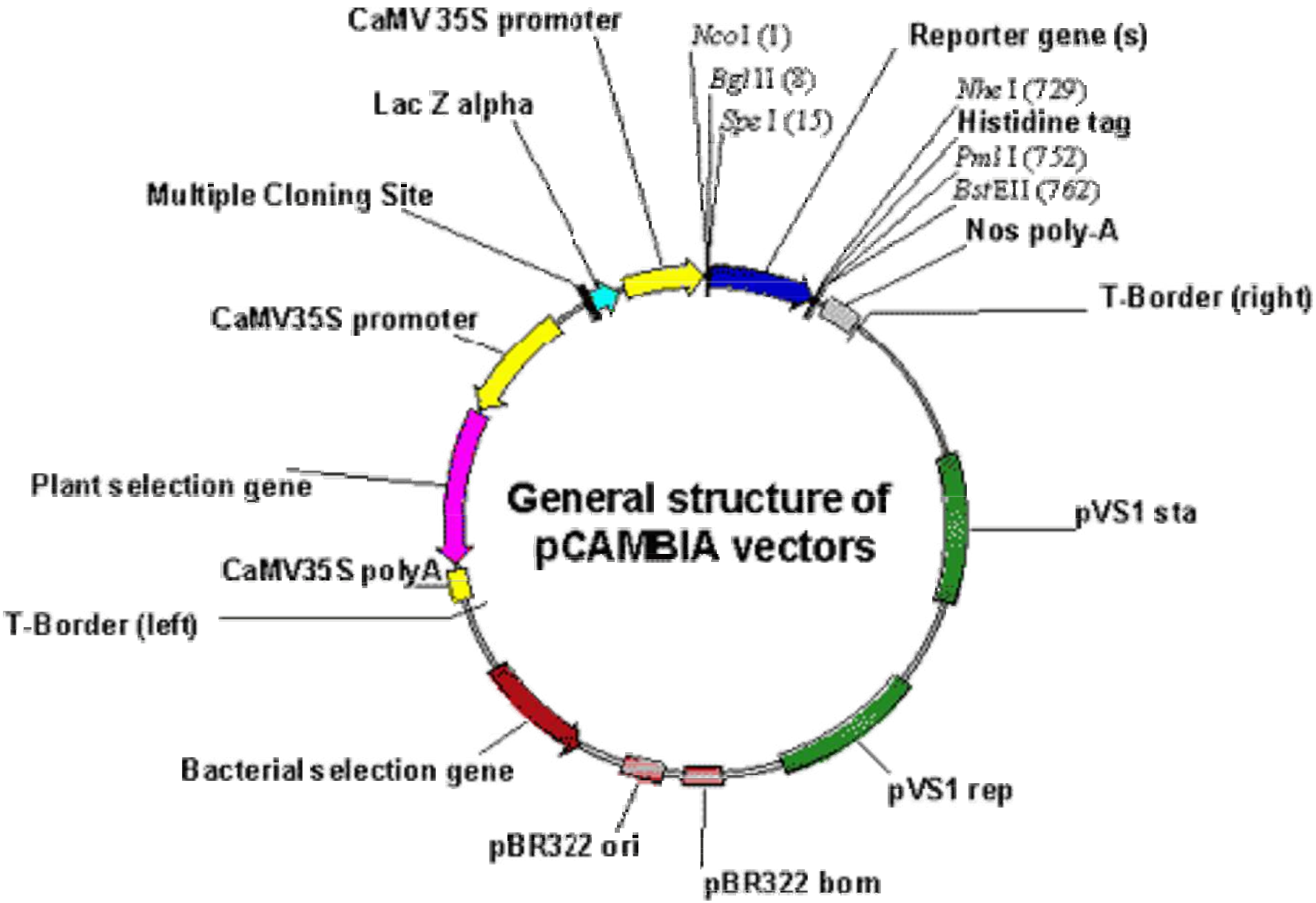


1.2. Introduce and select for transformants

Two techniques are used to deliver DNA found in the transformation cassette into plant tissues during the plant transformation process. One is a biological system based on the plant pathogen Agrobacterium tumefaciens. The second is a mechanical method where the DNA is "shot" into plant cells using a gene gun.

The most common method to introduce the transformation cassette is by using the plant pathogen Agrobacterium. For this system to work it is necessary that the cassette contain insertion sequences that are used by the bacteria (LB and RB).

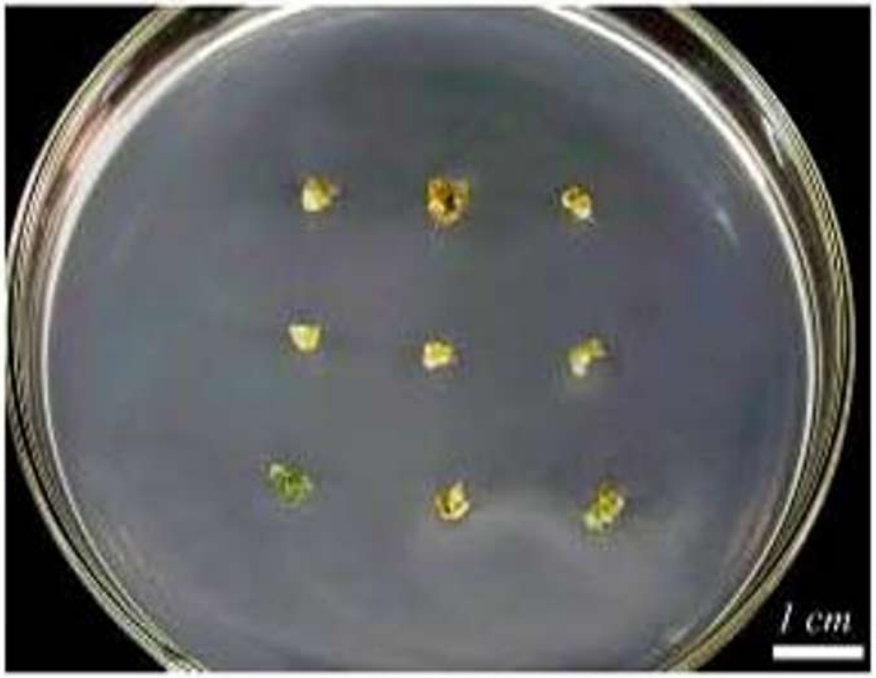

Agrobacterium
Plant tissue incubated with Agrobacterium
Plant cell
Recombinant DNA transferred into plant nucleus
Recombinant DNA coated onto microcarriers
Selectable gene
Foreign gene
Biolistic
Microcarrier launch assembl
DNA-coated microcarriers propelled into plant cells
DNA-coated microcarriers
Foreign DNA carried into plant cell on microcarrier
Nucleus
Recombinant DNA inserted into plant chromosome
Only cells containing recombinant DNA survive and divide in culture on selective medium
Cells regenerate into transgenic plantlets
Plantlets grow into plants with new traits


CaMV 35S promoter
Lac Z alpha
Multiple Cloning Site
CaMV35S promoter
Plant selection gene
CaMV35S polyA
T-Border (left)
Bacterial selection gene
pBR322 ori
pBR322 bom
pVS1 rep
pVS1 sta
Nco I (1)
Bgl II (8)
Spe I (15)
Reporter gene (s)
Nhe I (729)
Histidine tag
Pml I (752)
BstEII (762)
Nos poly-A
T-Border (right)
General structure of
pCAMBIA vectors

All transformation cassettes contain three regions. The "gene of interest" region contains the actual gene that is being introduced into the plant. This is the gene that provides the new function to the plant.

Many plant tissues are treated with the transformation cassette during the transformation step. Not all of these tissues actually receive the cassette. To distinguish those that contain the gene from those that don't, it is necessary to use a selection process. The selectable marker is a gene that provides the ability to distinguish transformed from non-transformed plants.

The kanamycine used as selective agents to distinguish transformed from non-transformed plants (e.g. cassava explants)



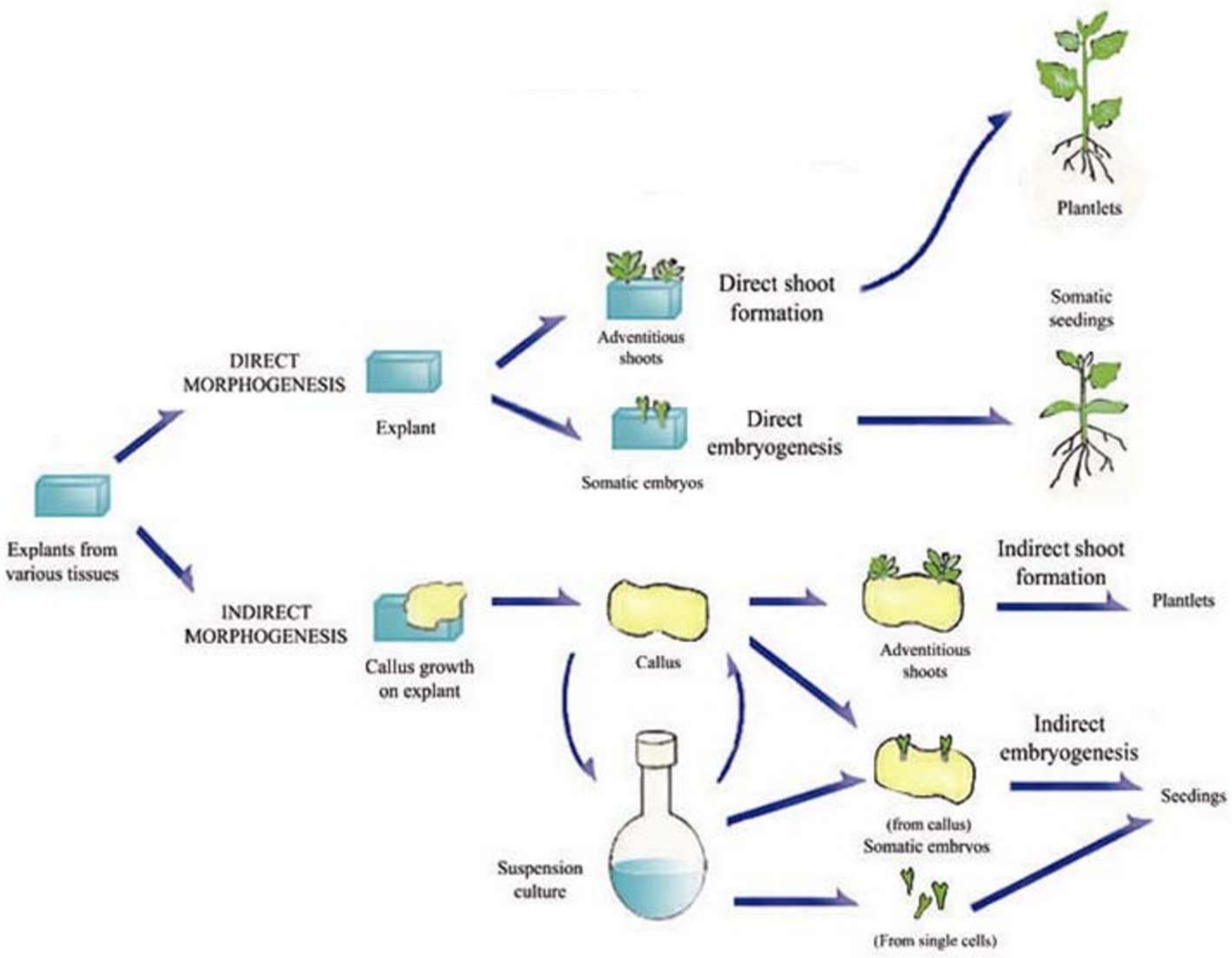
Explants non-transformed
(Kanamycine 50 mg/l)

Explants transformed
(Kanamycine 50 mg/l)

The insertion sequences straddle the gene-of-interest coding region and the selectable marker. These are use by Agrobacteria to create a DNA molecule that is sent out of the bacteria into the plant where it is eventually inserted into the nucleus of a cell in the recipient plant tissue. If the cell follows the proper developmental pathway that leads to a new plant, every cell in that plant will contain the sequences in between the insertion sequences.

1.3. Regeneration of the transformed tissue

The insertion sequences straddle the gene-of-interest coding region and the selectable marker. These are use by Agrobacteria to create a DNA molecule that is sent out of the bacteria into the plant where it is eventually inserted into the nucleus of a cell in the recipient plant tissue. If the cell follows the proper developmental pathway that leads to a new plant, every cell in that plant will contain the sequences in between the insertion sequences.



Modified from Edwin F. Geoge. Plant propagation by tissue culture 3rd Ed. Springer publisher (2008).

Some plant part is placed on a defined culture media. That media induces the tissue to develop callus. Callus is an undifferentiated mass of cells. These cells then grow into plant shoots, which are later rooted. The small seedling will then grow into a mature, seed-producing plant. When developing transgenic plants, the transformation cassette is introduced into that plant part that can be induced to grow new plants.

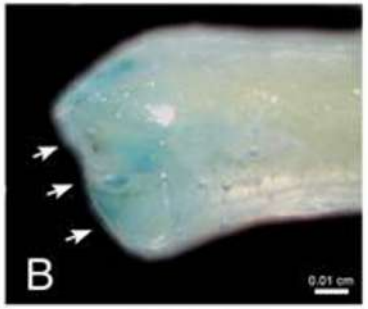
Sometime adventitious shoot formation and somatic embryogenesis protocols is directly applied for regeneration of transgenic plants.

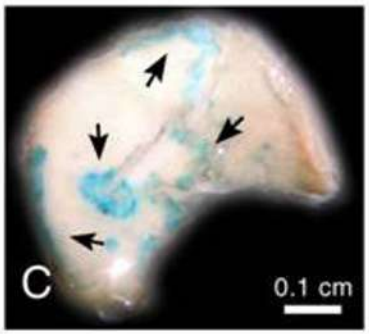
Regeneration of the tobacco transformed tissue

Original sample
(using leaf disks)

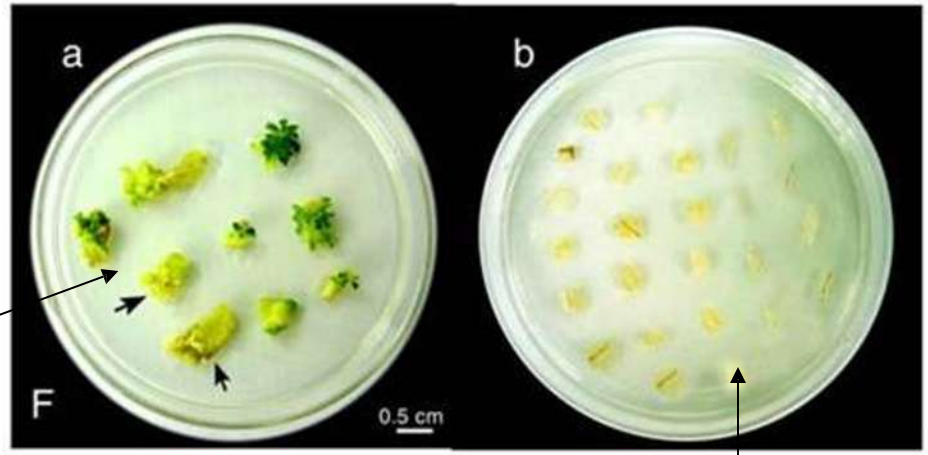
A. tumefaciens

C58pGV2260 with
pBIN19GUSintron





GUS
essay



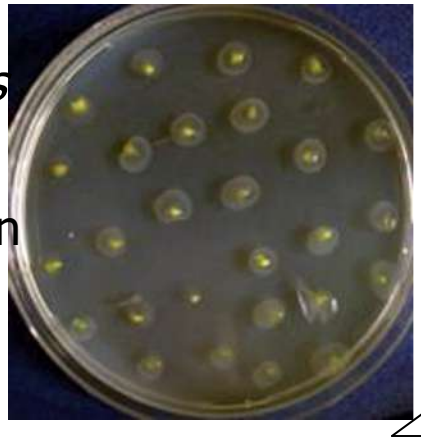
Regenerated shoot
grow on MS medium

Control sample

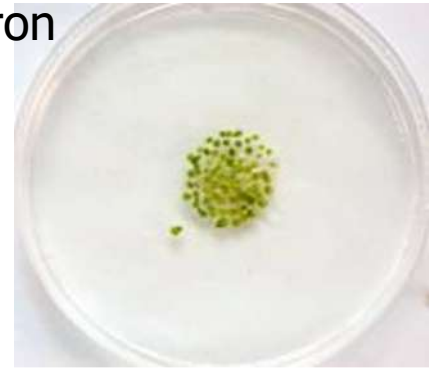
Regeneration of the citrus transformed tissue

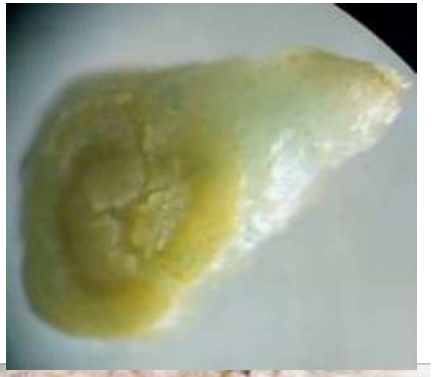
Original sample (using thin stem cell layer)

A. tumefaciens AGL1 with pBIN19GUSintron

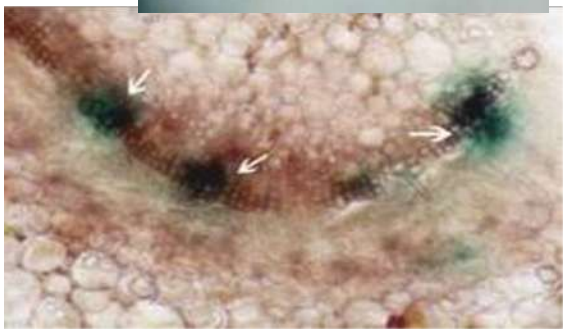


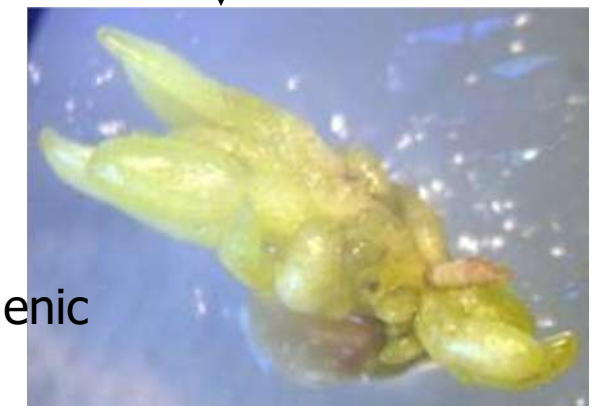
Biolistics p35SGUSintron





GUS essay



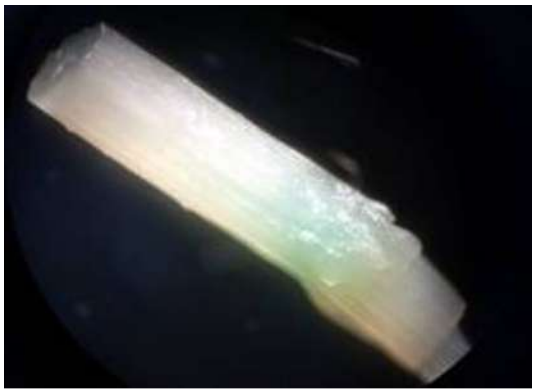


Regenerated transgenic shoot

Regeneration of the Vetiver transformed tissue

Original sample
(using inflorescence)

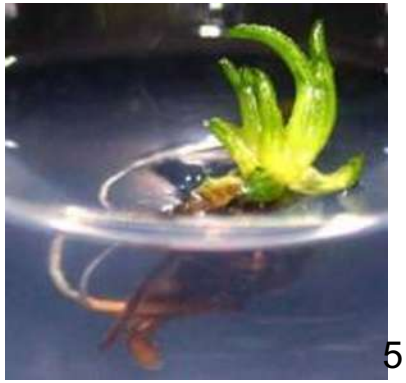
A. tumefaciens
AGL1 with
pBIN19GUSintron



GUS
essay

Biolistics
pBARGUS

GUS
assay



Regenerated transgenic shoot

2. Selection a transgenic plant

Many transgenic events need to be tested to find ones that are agronomically suitable

Dozens to hundreds tested prior to commercial use

Stable gene and trait expression (look for Mendelian inheritance like native gene)

Single gene insertion for stability and simple breeding

Desired level and pattern of expression (position effects)

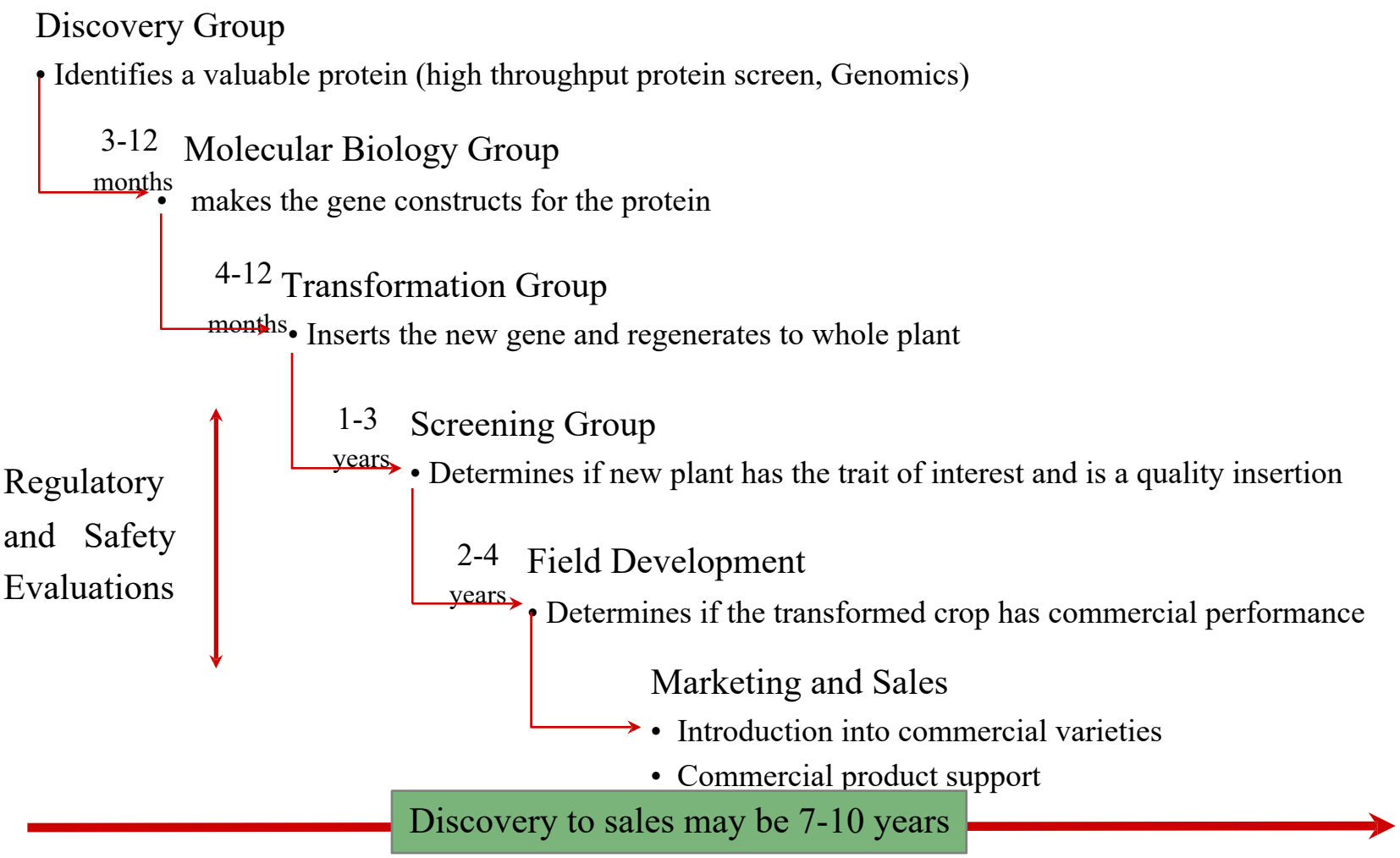
Many transgenic events need to be tested to find ones that are agronomically suitable

No deleterious effects on plant health/nearby genes (i.e. lack of somaclonal variation = unintended mutations)

. Introgression or insertion into other varieties for commercial use

Regulation considerations: Plant biochemistry, novel protein safety. allergenic potential. Environmental impacts.

Developing Transgenic Crops



Photos source:
http://www.usda.gov

3. Methods for detection of transgenic plants

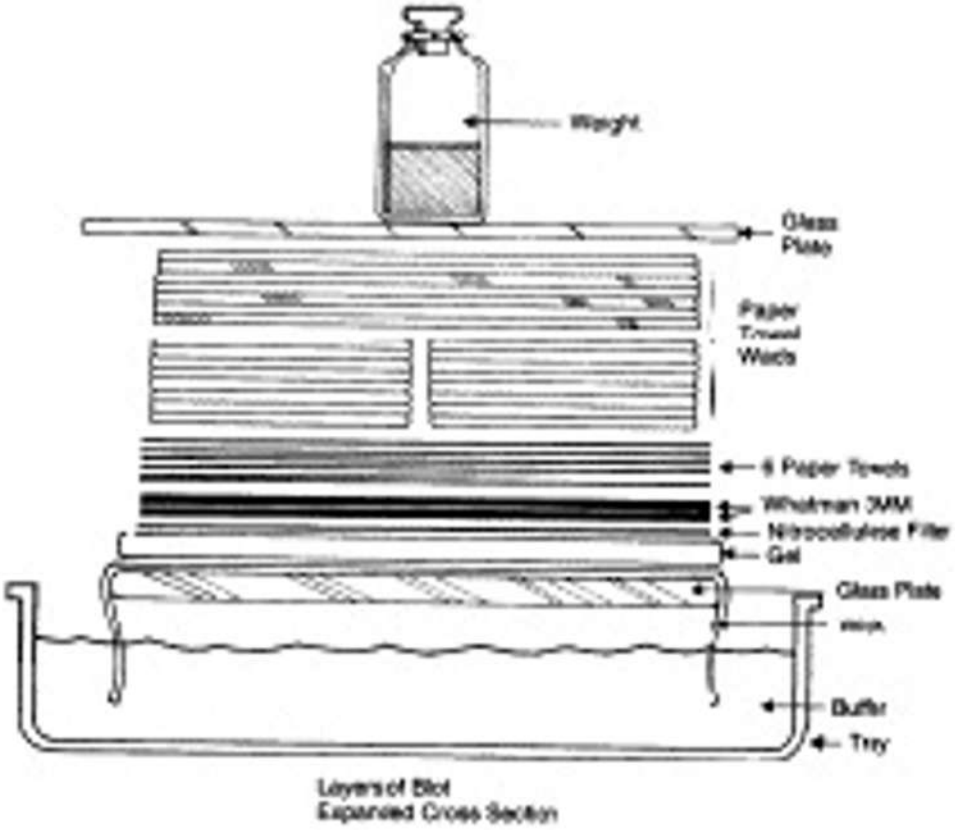
The need to monitor and verify the presence of target gene(s) in transgenic plants and in products derived thereof has generated a demand for analytical methods capable of detecting, identifying and quantifying either the DNA introduced or the protein(s) expressed in transgenic plants, because these components are considered as the fundamental constituents.

Detection of transgenic plants

Several methods either based on foreign DNA detection using Southern blot analysis, the polymerase chain reaction (PCR) technique, or based on protein detection using enzyme linked immunosorbent assays (ELISA) , Southern blot analysis and Lateral flow strip assay … Several other analytical technologies that can provide solutions to current technical issues in GM plant analysis are Reverse transcriptase PCR (RT-PCR) , Quantitative Competitive PCR (QC-PCR) or Real- time PCR (QT-PCR).

DNA detection using Southern blot

Professor Sir Ed Southern, Whitley Professor of Biochemistry at the University of Oxford



Layers of Blot
Expanded Cross Section

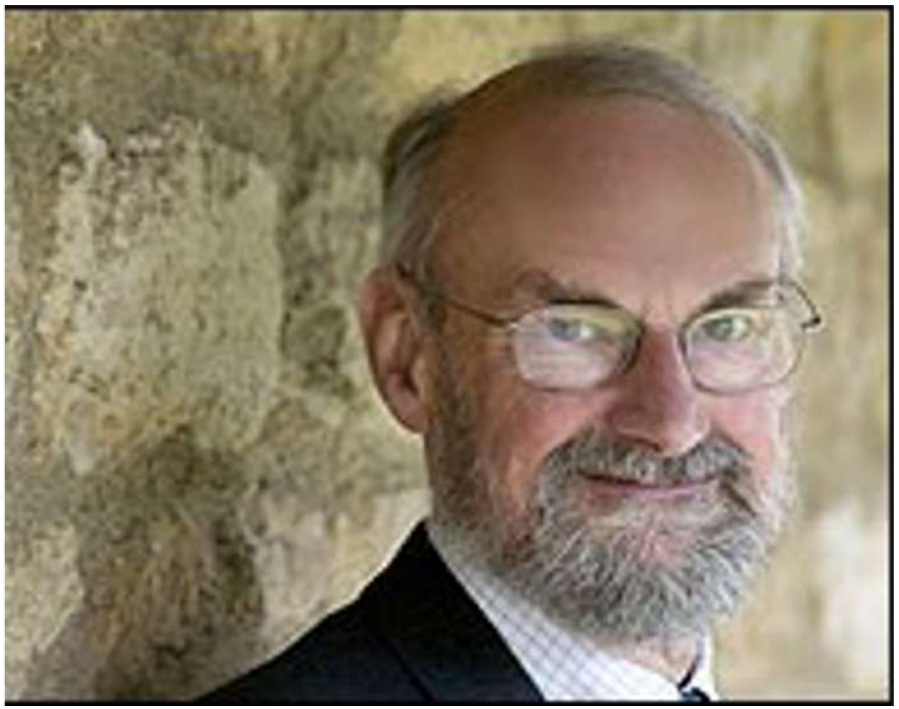


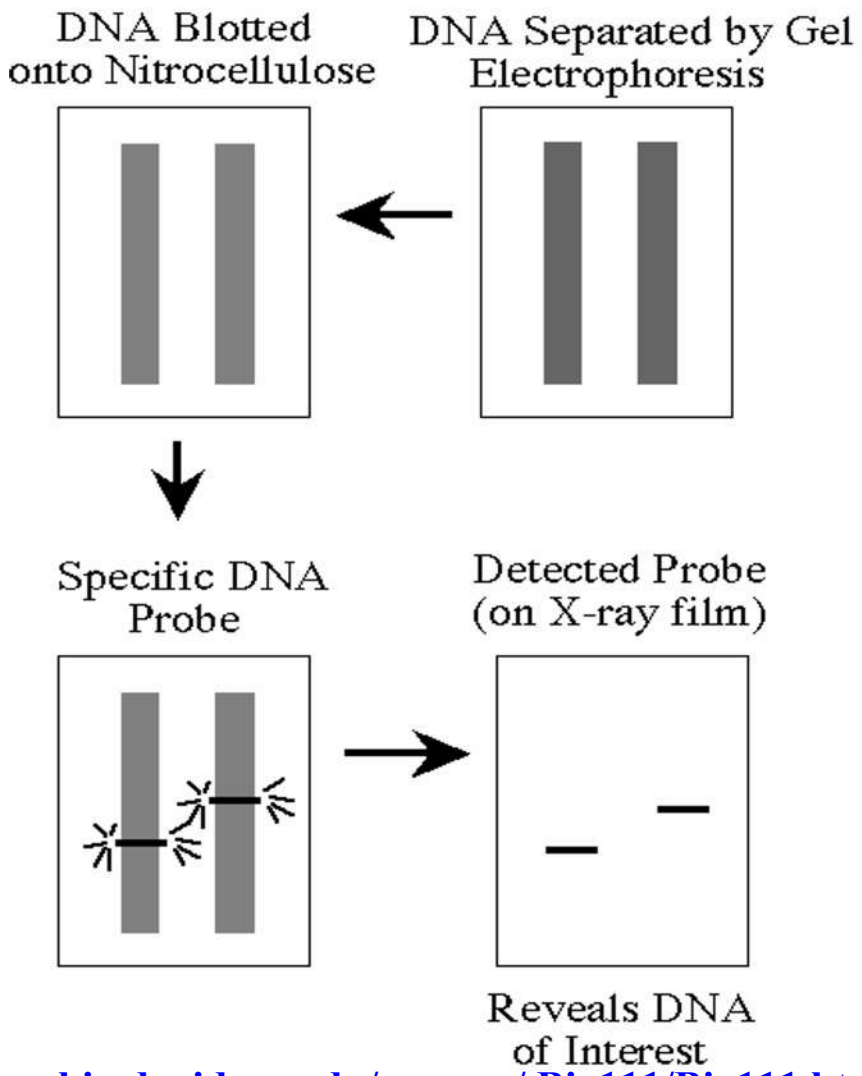
Photo source:
www.bbc.co.uk

A. Invented by Edward Southern in the mid-1970s.

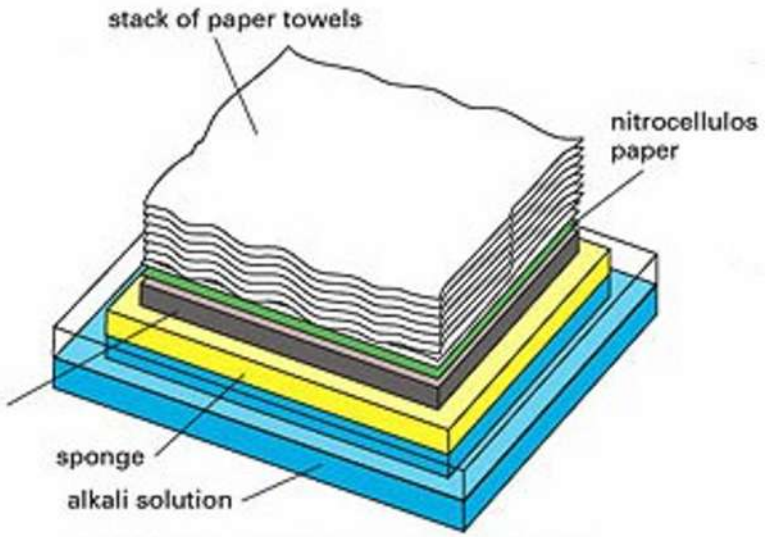
B. Created in the following fashion:

1. *DNA fragments in a gel are denatured by alkaline buffer.*
2. *DNA fragments are transferred to a nylon or nitrocellulose membrane.*
3. *DNA probes are hybridized to the membrane, and the membrane will be exposed to show a band representing where the probe successfully hybridized.*

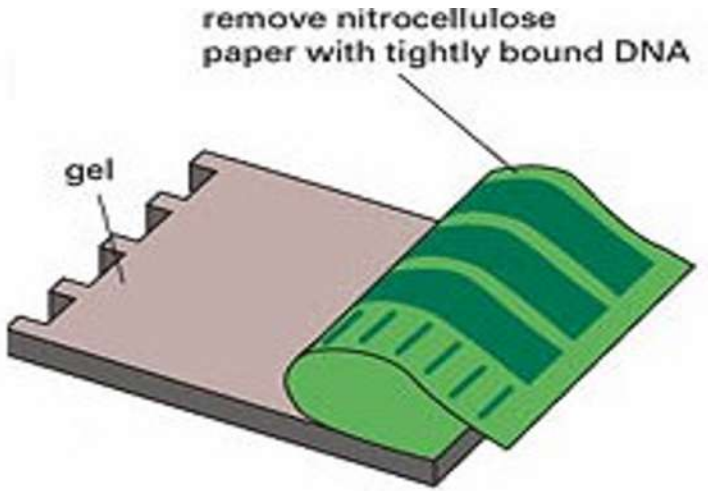
Visualization of radioactive hybridization (photo film)



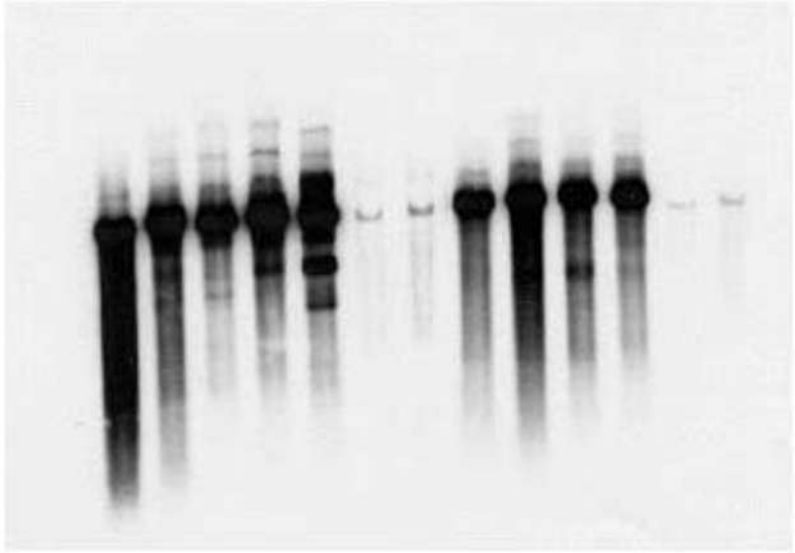
www.bio.davidson.edu/courses/ Bio111/Bio111.html

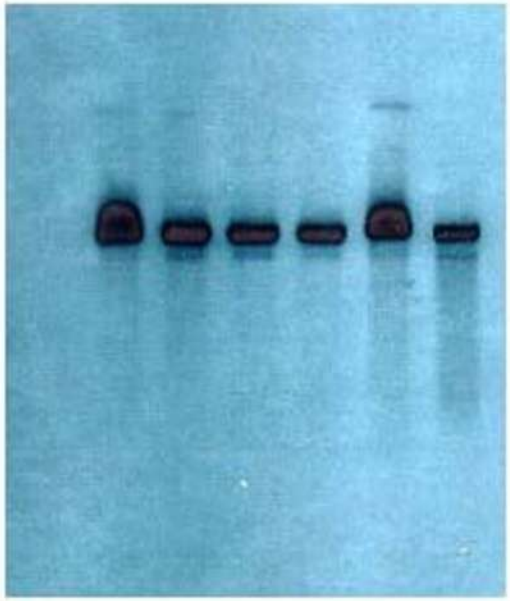


SEPARATED DNA FRAGMENTS BLOTTED ONTO NITROCELLULOSE PAPER

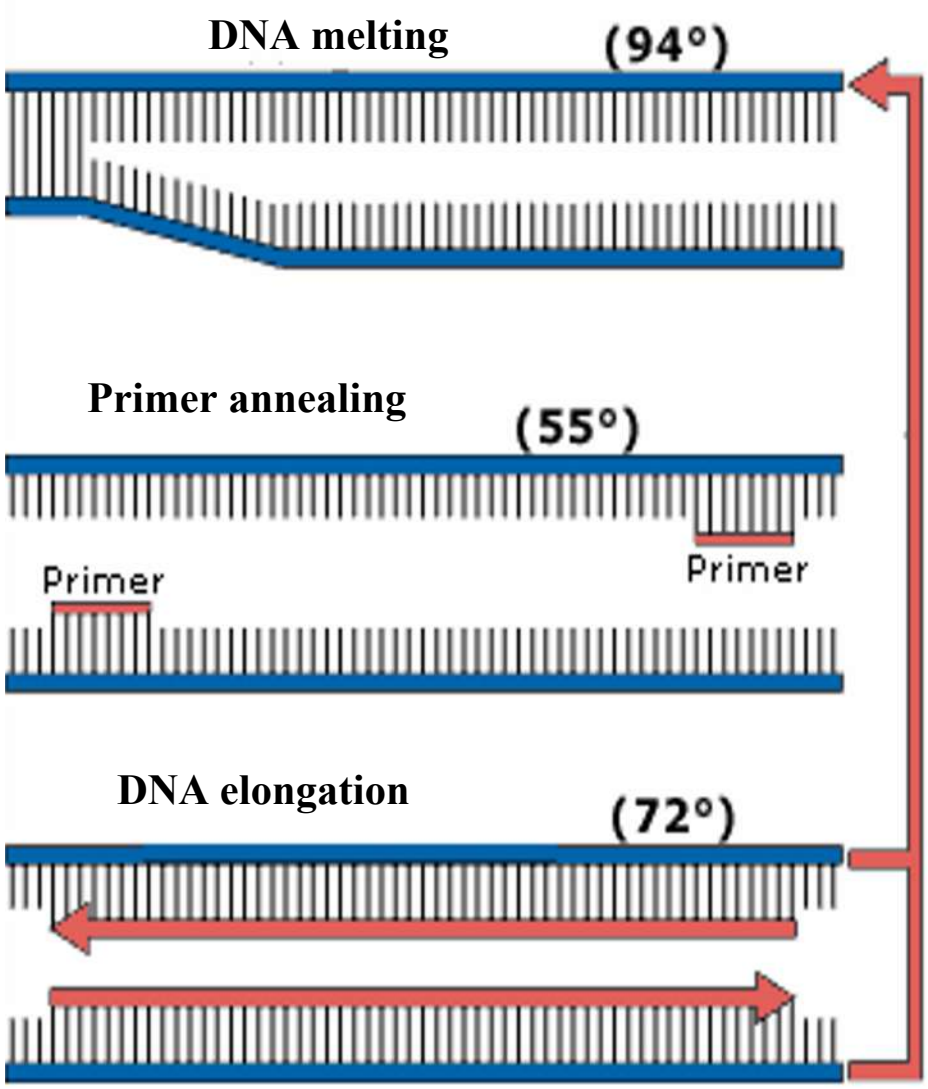


Southern blot analysis of transformed plants





DNA detection using the Polymerase chain reaction technique



Biochemistry from U.C. Berkeley

Nobel Prize in Chemistry 1993, at age 48

Kary Mullis

(invented PCR in 1983)

PhD

"The Cosmological Significance of Time Reversal,"

Photo source: www.mcgill.ca

Polymerase chain reaction (PCR)

Selective amplification of a chosen region of DNA molecule

The only requirement is that the sequence at the borders of the selected DNA region must be known so that two short oligonucleotides can anneal to the target DNA molecule for amplification

DNA polymerase I from *Thermus aquaticus, a* bacterium that lives in hot springs = Taq DNA polymerase

Polymerase chain reaction (PCR)

1. Denaturation of a template DNA duplex by heating at 94oC

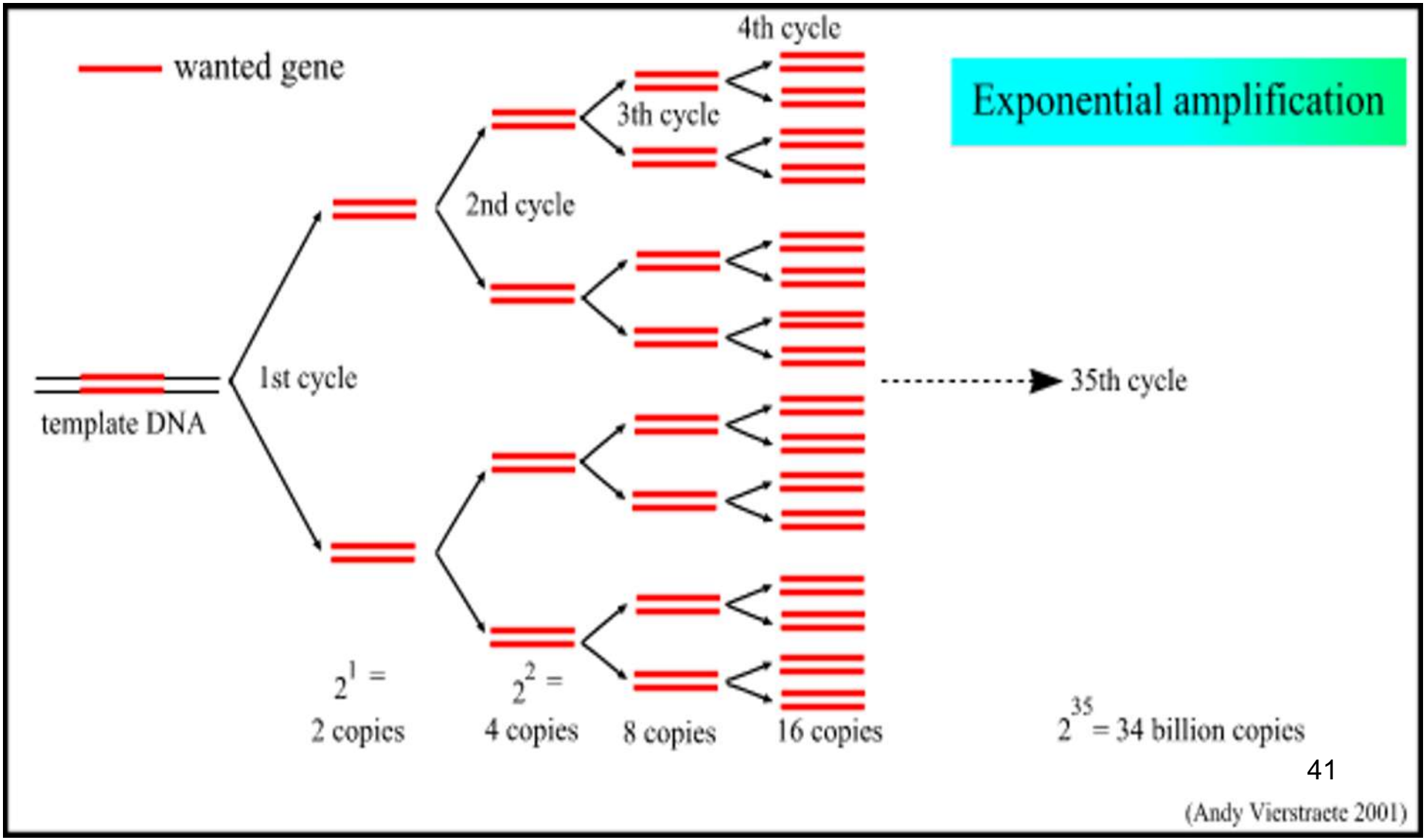
2. Annealing of oligonucleotide primers to the target sequences of separated DNA strands at 55-65oC

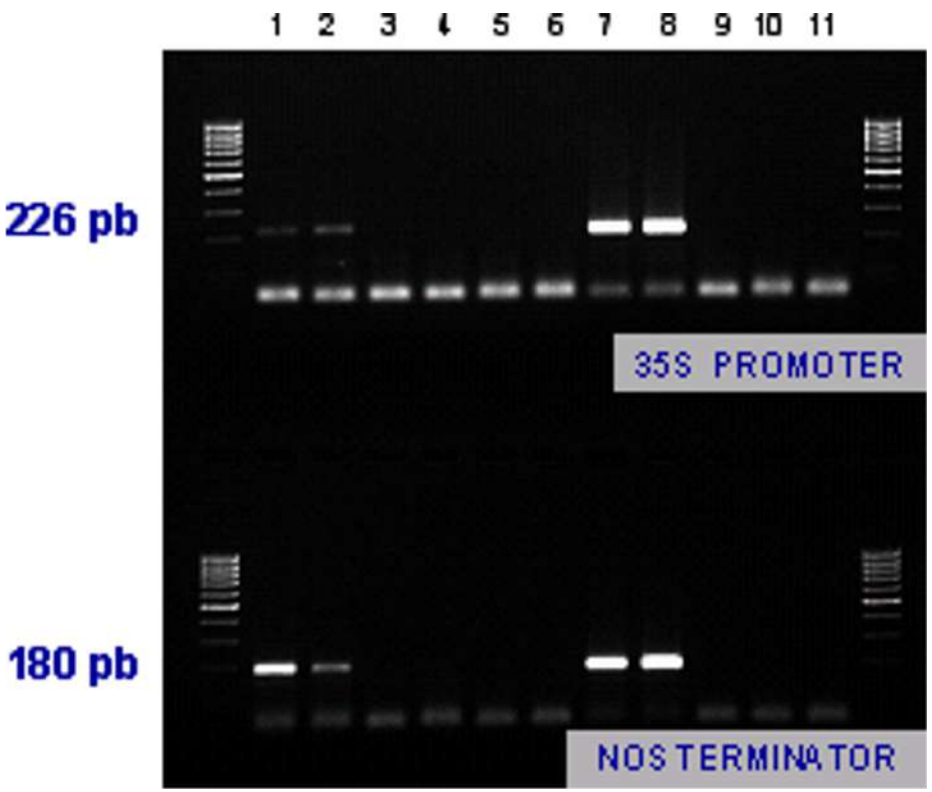
3. DNA synthesis from the 3′ -OH end of each primer by DNA polymerase at 72oC

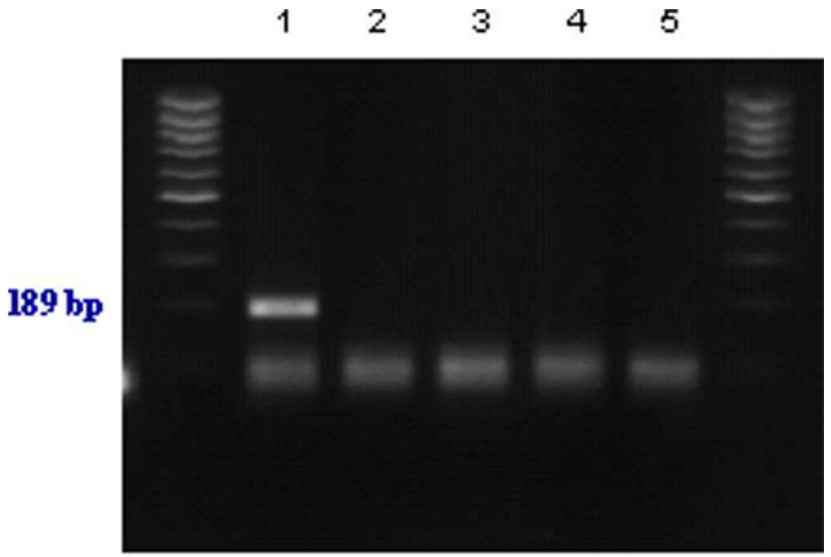
In theory, each amplification cycle should double the number of target molecules, resulting in an exponential increase in the PCR product

However, even before substrate or enzyme becomes limiting, the efficiency of exponential amplification is less than 100% due to suboptimal DNA polymerase activity, poor primer annealing, and incomplete denaturation of the templates

Exponential nature of PCR amplification

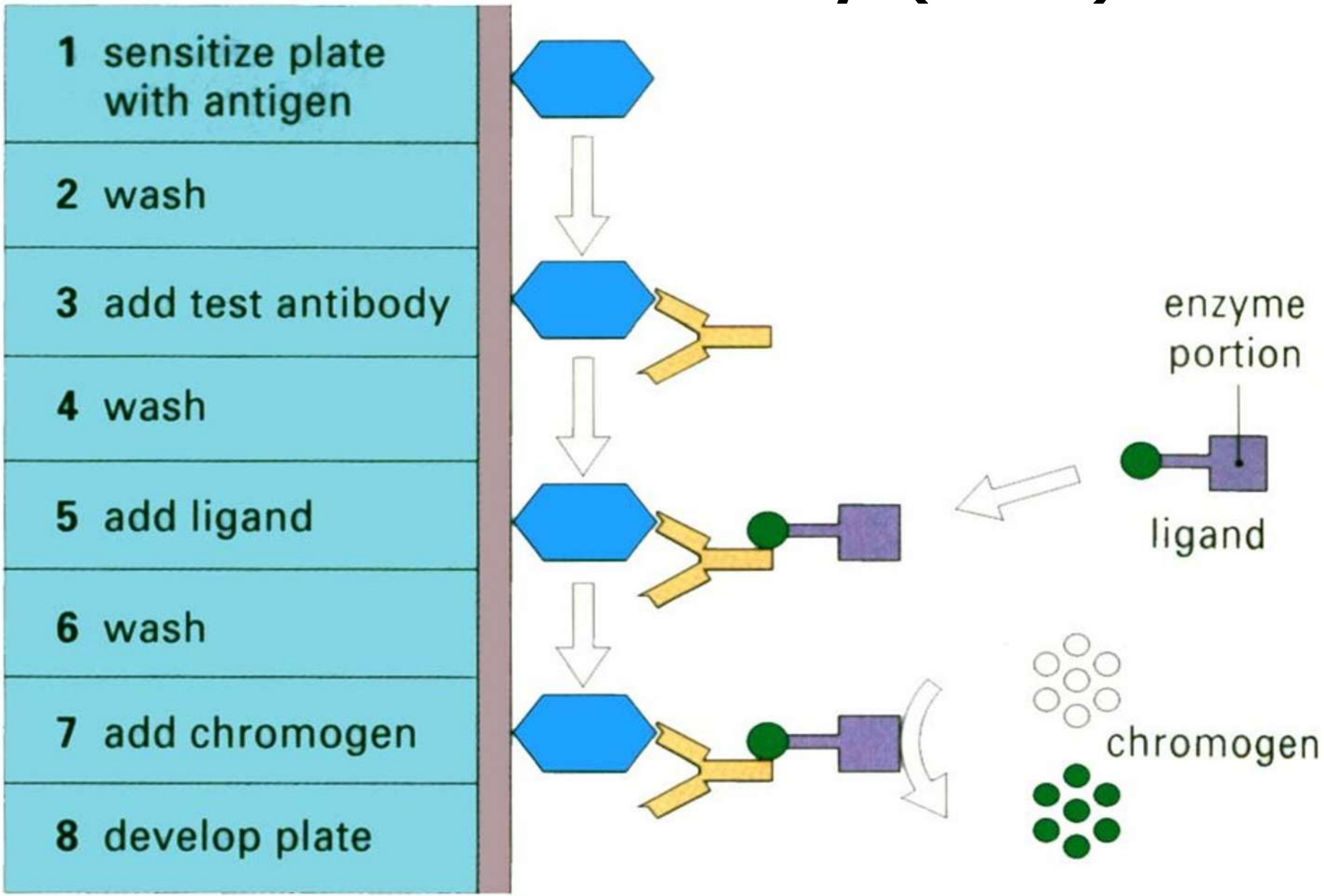






Lanes:
1. Bt-11 Maize (present at 1%)
2. Bt-176 Maize (not detected)
3. MON810 Maize (not detected)
4. Extraction Negative Control
5. PCR Negative Control

Protein detection using enzyme linked immunosorbent assays (ELISA)



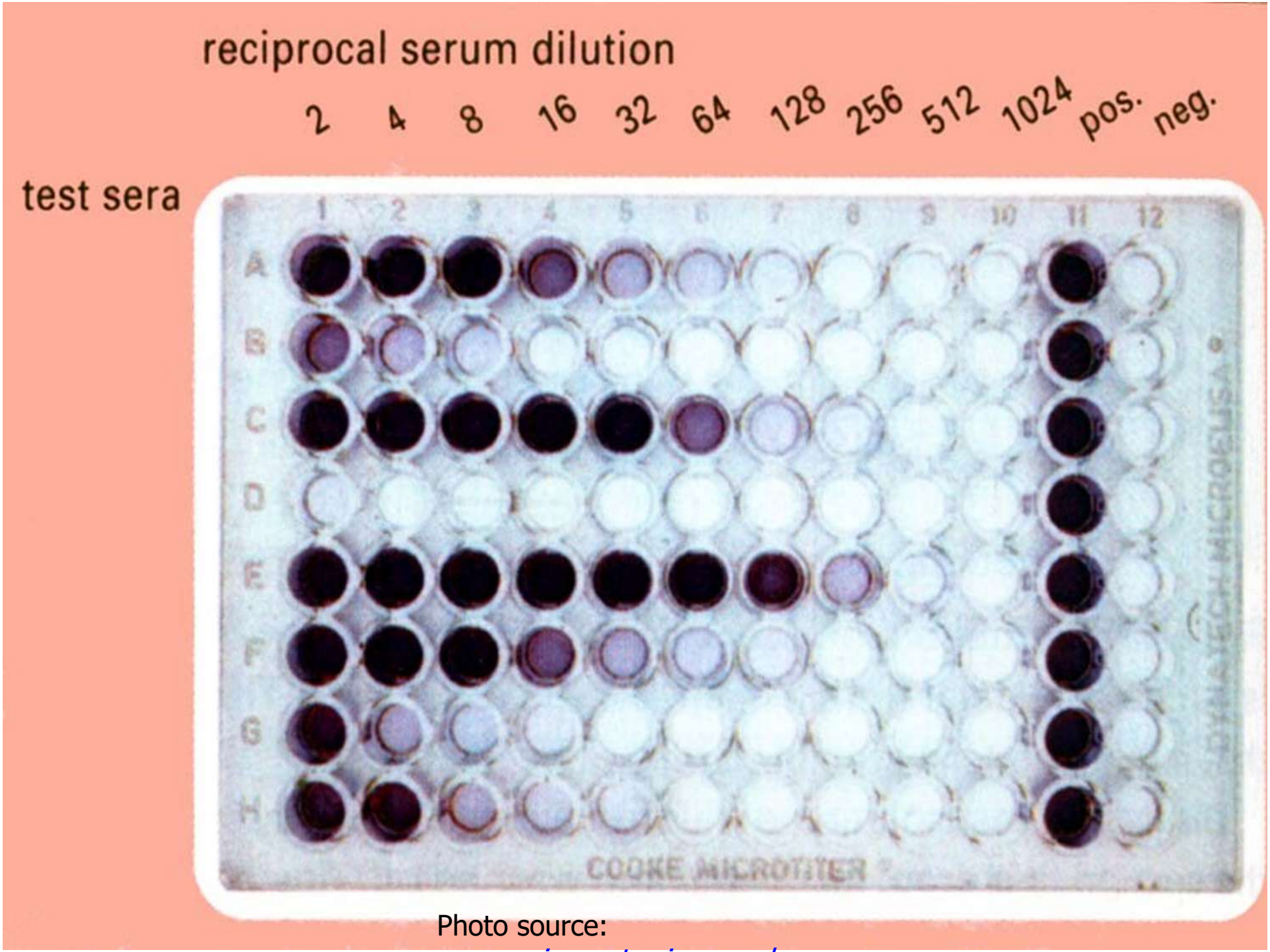
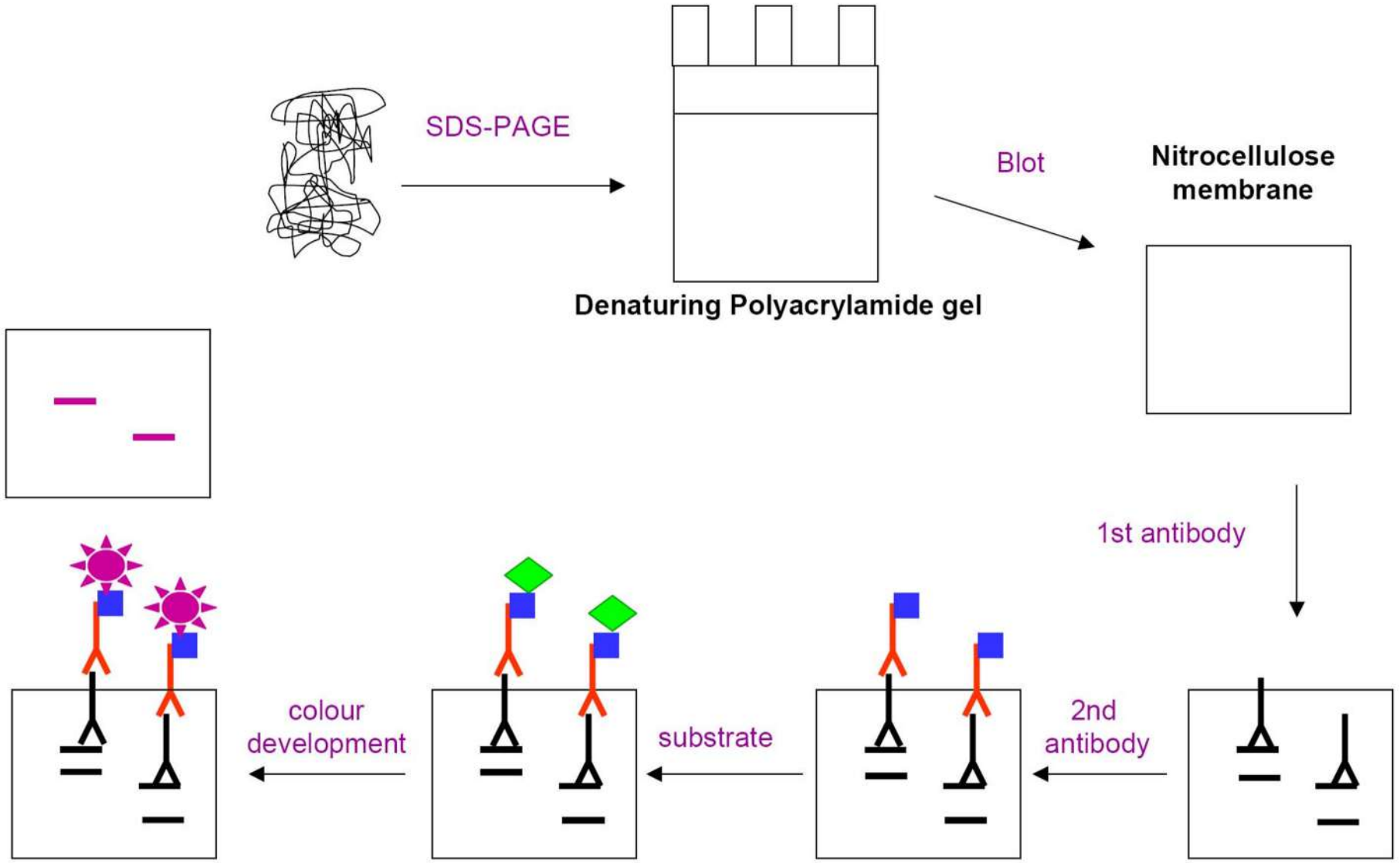
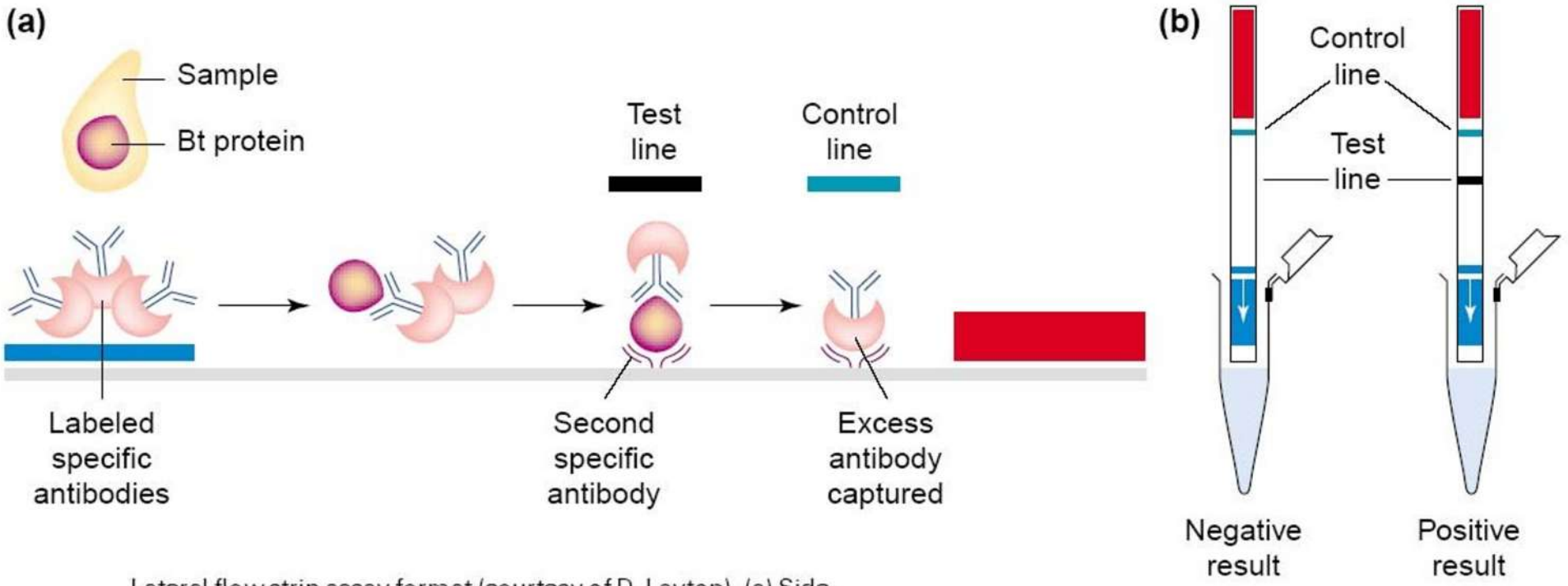


Photo source:
www.microvet.arizona.edu

Protein detection using western blot analysis



Protein detection using lateral flow strip assay



Lateral flow strip assay format (courtesy of D. Layton). (a) Side view illustrating principles of the immunological test, and relative location of control and test lines on a nitrocellulose strip. (b) Vertical view of test strips dipped in an eppendorf containing genetically modified material, and showing both negative and positive test results. Abbreviation: Bt, *Bacillus thuringiensis*.

TRENDS in Biotechnology Vol.20 No.5 May 2002

Source: *Trends in Biotechnology Vol.20, No. 5 May, 2002*

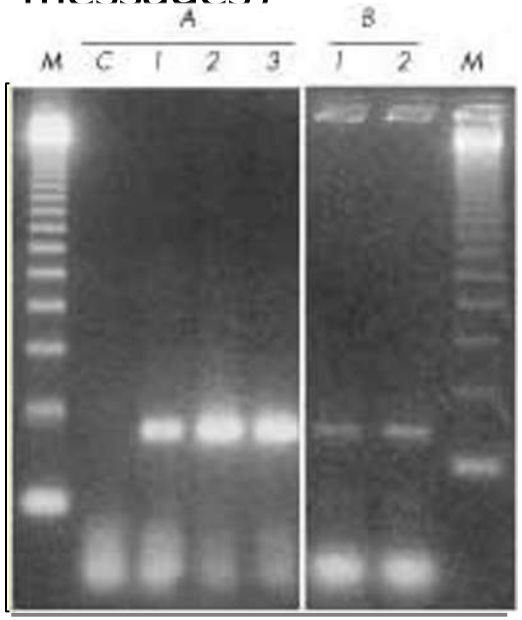
Reverse transcriptase (RT-) PCR detection

total mRNA (low abundance of individual messages) → RT → cDNA mixture

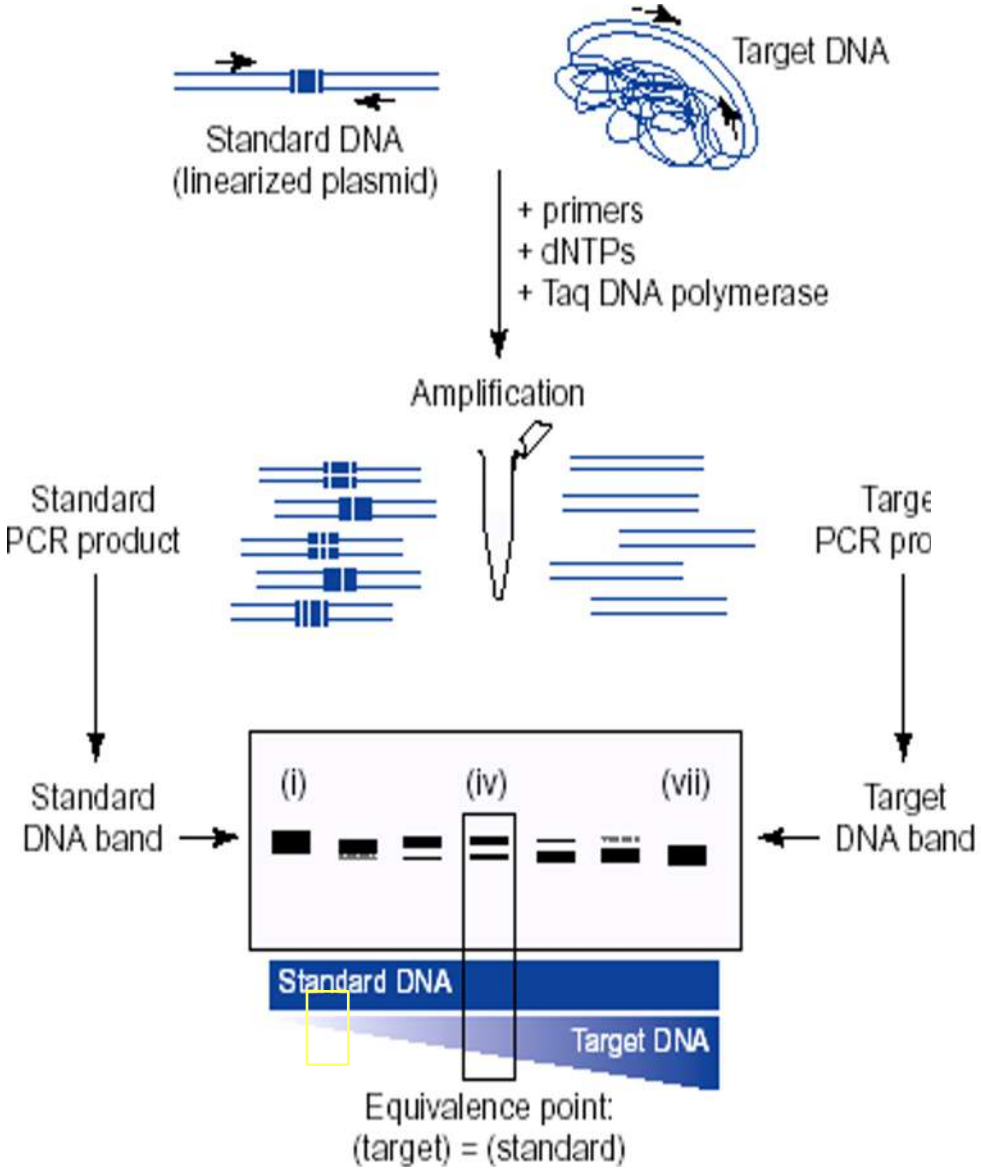
Gene-specific oligonucleotide primers + dNTPs + Taq polymerase

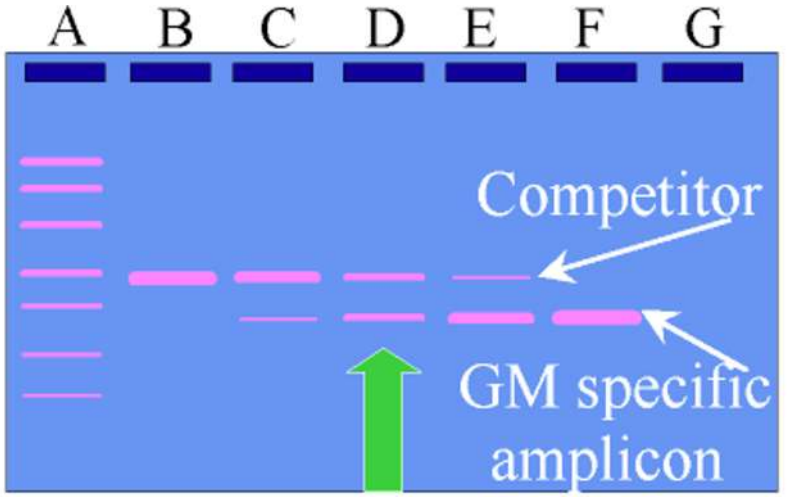
Amplification of specific region of target gene

Electrophoresis of amplicon DNA and visualization



Quantitative Competitive (QC-) PCR detection



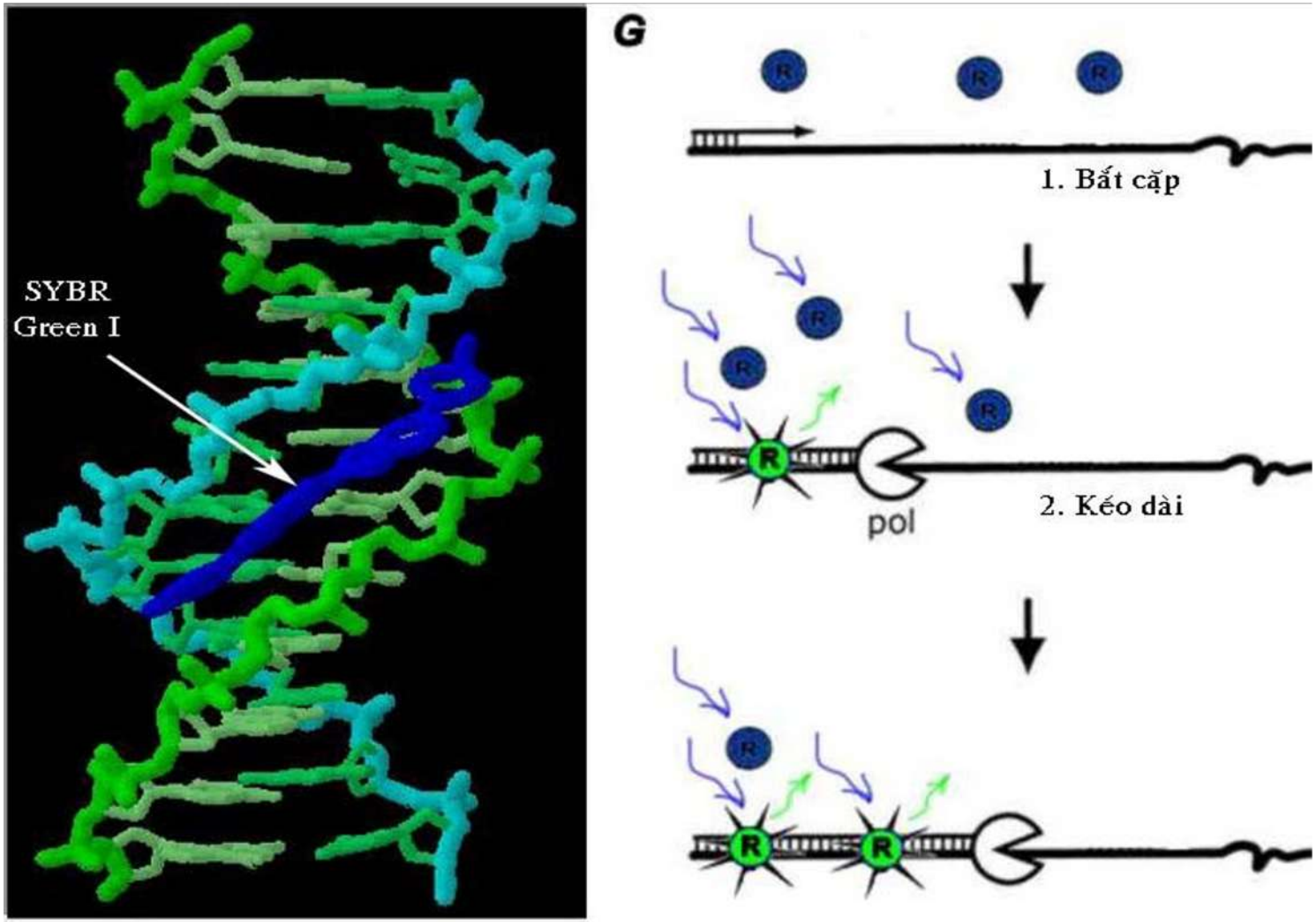


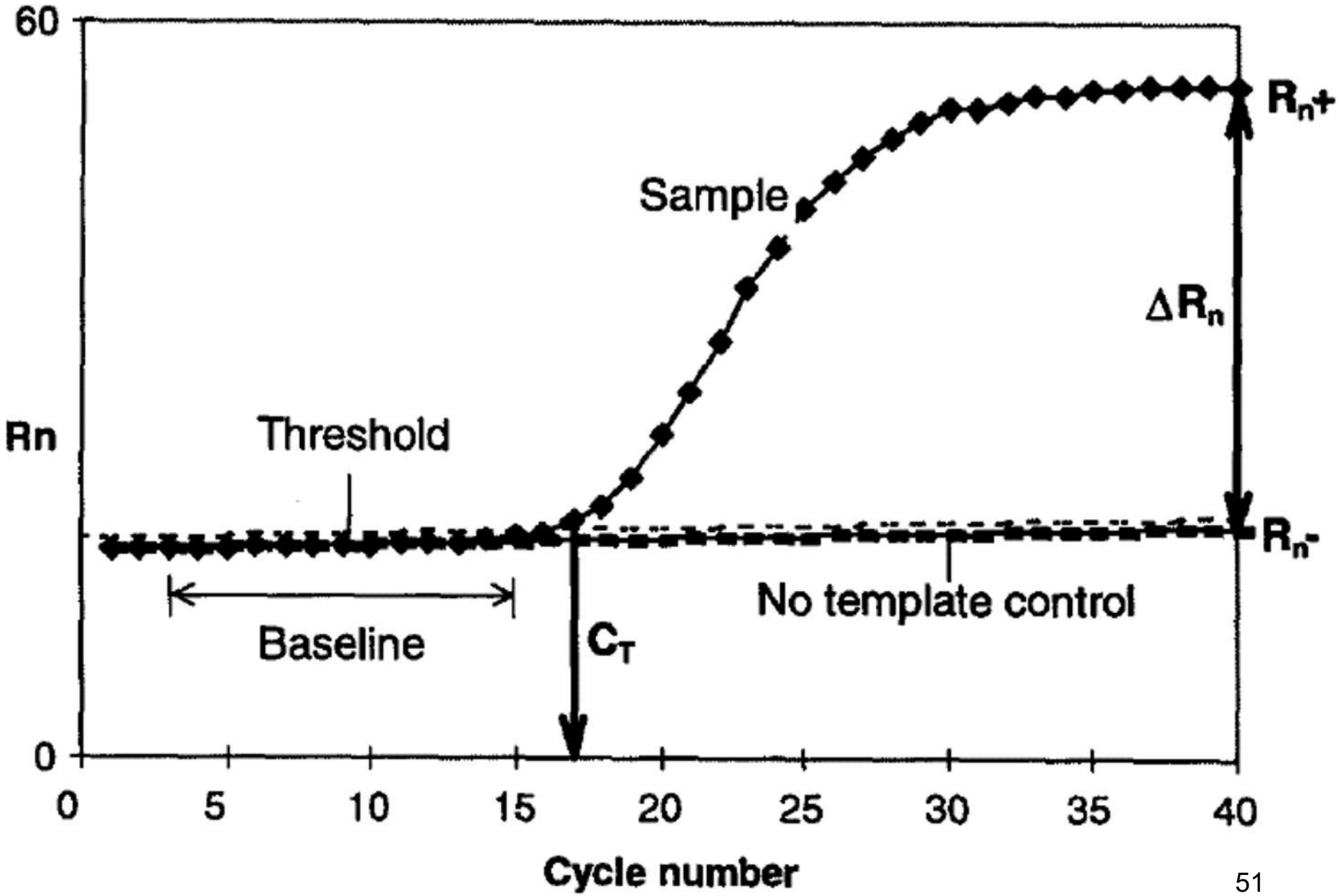
Ratio of GM : competitor = 1 : 1

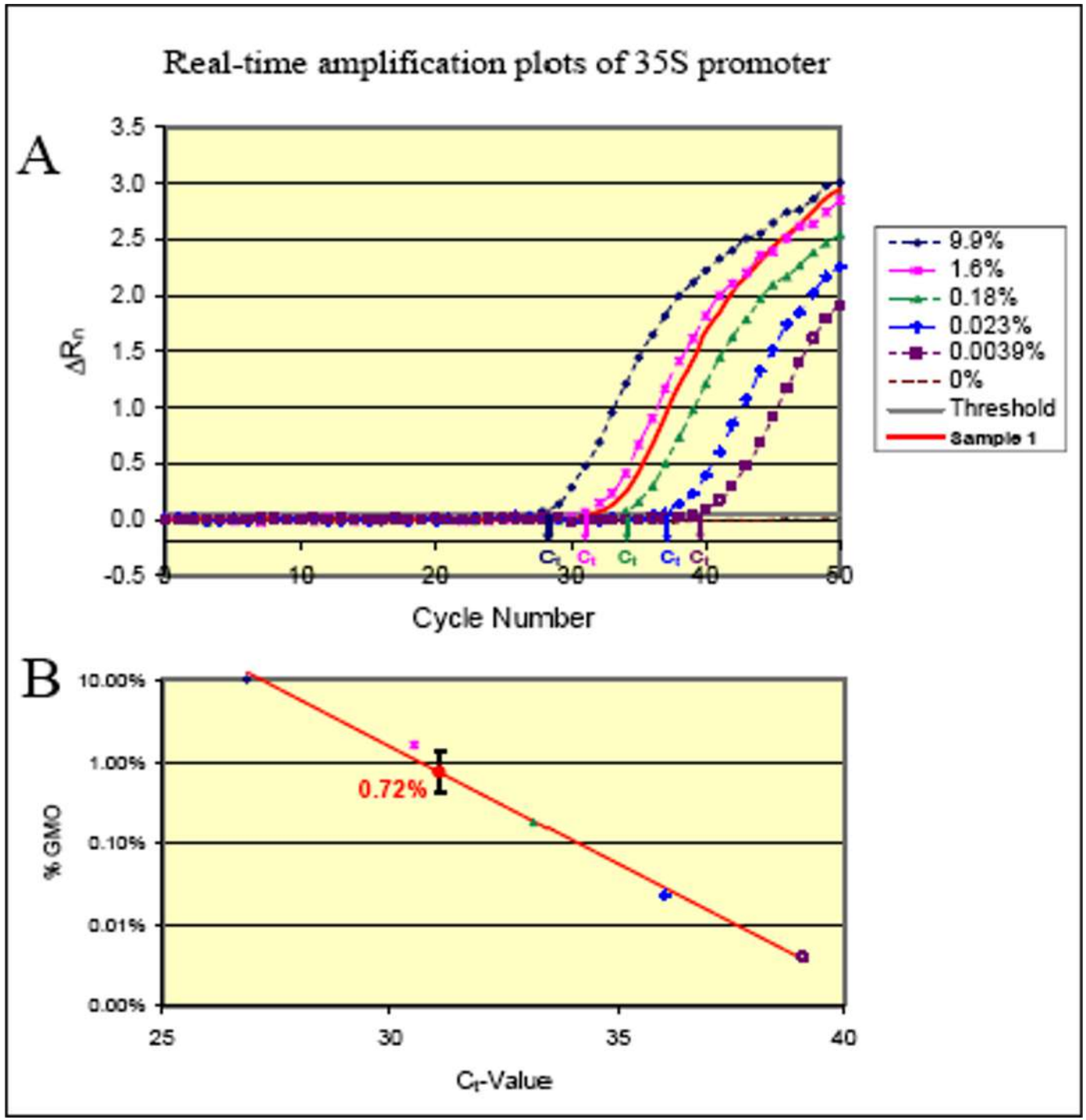
Real-time PCR detection

- Two types of detection systems
  - Detection of ANY dsDNA generated during PCR
    - SYBR Green
  - Detection of specific dsDNA fragments
    - Specific probes
      - TaqMan probes
      - Molecular Beacons

SYBR Green I




60
Rn
0
0
5
10
15
20
25
30
35
40
Cycle number
Sample
Threshold
Baseline
C_T
No template control
R_n+
R_n-
ΔR_n


Real-time amplification plots of 35S promoter
A
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
ΔRn
0
10
20
30
40
50
Cycle Number
9.9%
1.6%
0.18%
0.023%
0.0039%
0%
Threshold
Sample 1
B
10.00%
1.00%
0.10%
0.01%
0.00%
% GMO
0.72%
25
30
35
40
Ct-Value

Comparison of the different methods

Parameter	Western blot	ELISA	Lateral flow strip	Southern blot	Qualitative PCR	QC-PCR	RT-PCR
Ease of use	Difficult	moderate	Simple	Difficult	Difficult	Difficult	Difficult
Special equipment needed	Yes	Yes	No	Yes	Yes	Yes	Yes
Sensitivity	High	High	High	Moderate	Very High	High	High
Duration	2d	30 - 90 min	10 min	6h	1.5d	2d	1d
Cost/sample	150	5	2	150	250	350	450
Quantitative	No	Yes	No	No	No	Yes	Yes
Field application	No	Yes	Yes	No	No	No	No
Where applied	Academic lab	Test facility	Field Testing	Academic lab	Test facility	Test facility	Test facility

To date, several methods for different food matrices have been submitted to validation trials. Those include mass spectrometry, chromatography, near infrared spectroscopy, micro fabricated devices, DNA chip technology and nanoscale GM plant analysis. However, in combination with different levels of cost, complexity and speed, the methods vary in their reliability, robustness and reproducibility. Moreover, there is no one method that is applicable to all circumstances.