Cloned plasmid DNA molecules as a tool for GMO analysis

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Overview

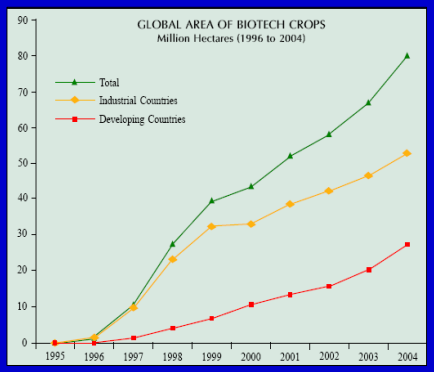
- *European context on GMOs*
- *Basics of GMO quantification*
- *Concept of plasmid markers*
- *Experimental testing*
- *Implementation in practice*

Overview

European context on GMOs

European context on GMOs

Worldwide
increasing area of transgenic crops



James, 2004

Europe
opposition from consumer groups and environmentalists

1998-2004
moratorium for cultivation and marketing approvals

> April 2004
new, strict and comprehensive regulatory framework

European context on GMOs

Contained use — Deliberate release into the environment — Labelling and traceability

Field trials — *Pre-market*

Marketing — *Post-market*






European context on GMOs

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98/81/EC





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European context on GMOs

Contained use — Deliberate release into the environment — Labelling and traceability

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98/81/EC

2001/18/EC Part B





European context on GMOs

Contained use — Deliberate release into the environment — Labelling and traceability

Field trials — *Pre-market*

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98/81/EC

2001/18/EC Part B

2001/18/EC Part C

1829/2003

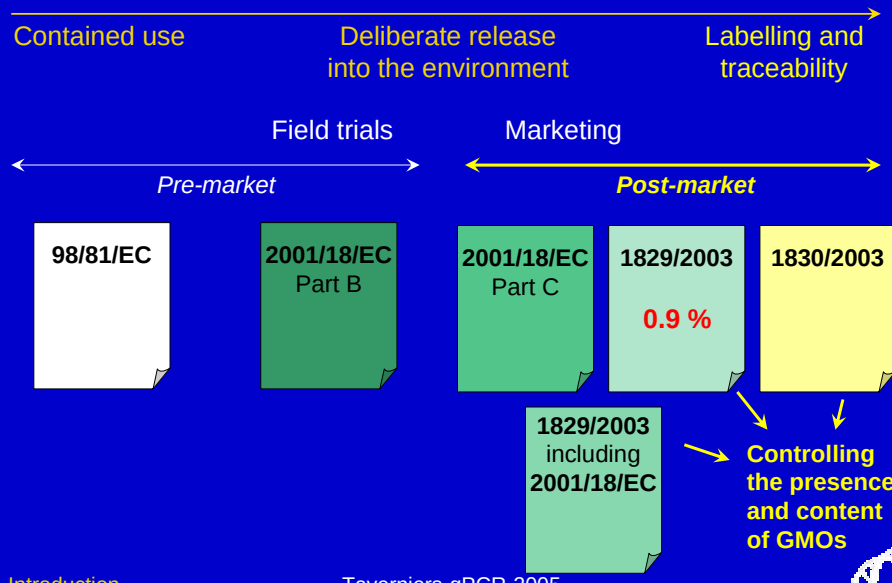
0.9 %

1830/2003

1829/2003 including **2001/18/EC**

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European context on GMOs



European context on GMOs

- **Reg. (EC) 1829/2003** on food and feed: Labelling is mandatory above a certain threshold value
- Quantification on ingredient level (single species)
- **Rec. (EC) 2004/787**: Haploid genome basis for measuring and expressing GMO contents
  100% x (# copies GMO target/# copies plant target)
- Authorized GM food & feed products: **0.9 %**
- Non-authorized GMOs:
  - with positive risk evaluation: **0.5 %**
  - no evaluation: **0.1 %**
- GM seeds:
  - cross-pollinators *(rapeseed)*: **0.3 %**
  - self-pollinators + exception cross-pollinator *(maize)*: **0.5 %**
  - exception self-pollinator *(soybean)*: **0.7 %**

Overview

European context on GMOs

Basics of GMO quantification

Overview

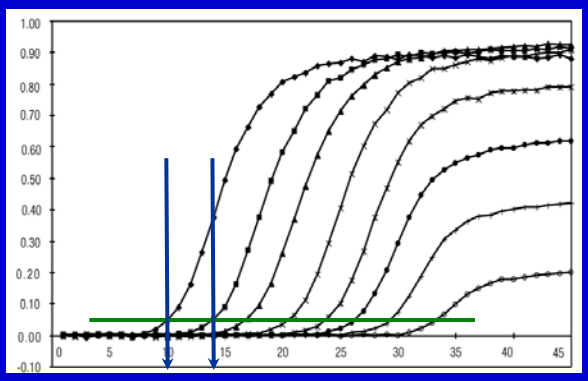
Basics of GMO quantification

- Real-time PCR technique
- Units of measurement and expression
- Calibrators for GMO quantification

DNA quantification with real-time PCR

- Exponential amplification of a DNA target molecule gives a measured fluorescent signal

F signal (ΔR_n)



Cycle number

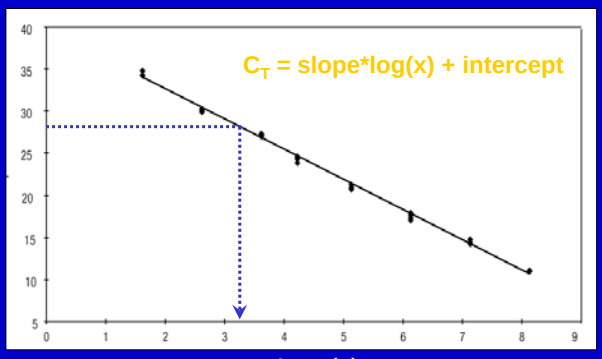
Threshold fluorescence value

Calculated C_T value

DNA quantification with real-time PCR

- Relationship between calculated C_T value and initial target DNA concentration is basis for quantification

C_T value



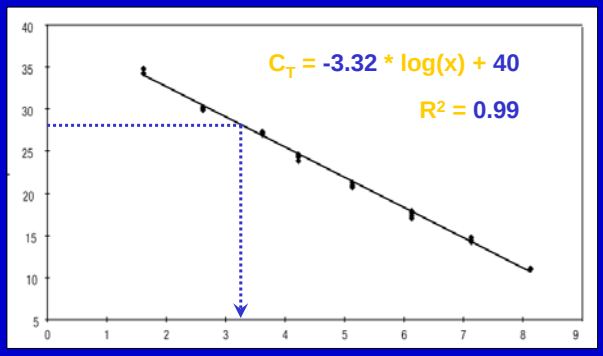
Log (x)

Slope ~ efficiency of amplification: $E = 10^{(-1/slope)}$

DNA quantification with real-time PCR

- Relationship between calculated C_T value and initial target DNA concentration is basis for quantification

C_T value



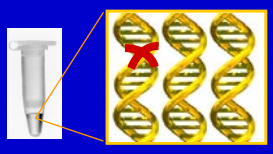
Log (x)

Slope of -3.32 ~ Maximum efficiency of amplification: E = 2

Expression of "x" ~ used calibrators

Units for measurement and expression

0.9 % *of what ?*

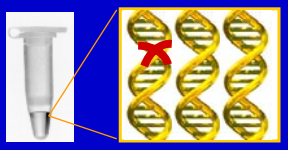
Seeds	Food and feed	DNA samples
		
number of seeds	**mass of material**	**number of genomes**
% GM maize	**% GM maize**	**% GM maize**
$100 \times \frac{\#\text{ GM seeds}}{\#\text{ total seeds}}$	$100 \times \frac{\text{GM maize mass}}{\text{total maize mass}}$	$100 \times \frac{\#\text{ GMO-specific DNA copies}}{\#\text{ plant-specific DNA copies}}$

Calibrators for GMO quantification



matrix RM

mass %



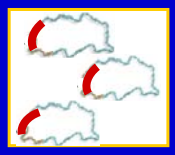
pure DNA RM

DNA copy n° %

genomic DNA



plasmid DNA



Calibrators for GMO quantification

- Matrix RMs:

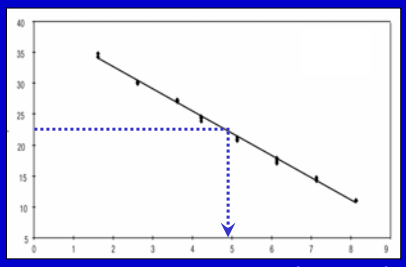
  CRMs (EC-IRMM, Fluka); 0-5 % GMO w/w
- Pure DNA RMs:
  - genomic DNA (EC-IRMM, Fluka); 0-5 % GMO w/w
  - plasmid DNA fragments
    - single target plasmids - STP *(Taverniers et al., 2001; 2004; others;* BNGL & ENGL plasmid database*)*
    - duplo target plasmids – DTP or 'tandem-marker plasmids' *(Mattarucchi et al., 2005; others;* BNGL & ENGL database*)*
    - multiple target plasmids - MTP *(Kuribara et al., 2002; others;* Nippon Gene Japan, Diagenode Belgium*)*
  - PCR amplicon fragments
    - single PCR amplicons *(Holck et al., 2002; others)*
    - hybrid amplicons *(Pardigol et al., 2003; others)*

Approaches for GMO quantification

- Two means of deriving a relative % result

Relative quantification
Single calibration curve

ΔC_T

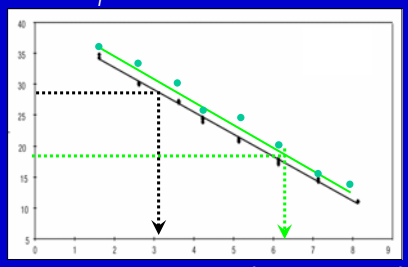


Log (% GMO)

Calibrators in %

Absolute quantification
Double calibration curves

Absolute C_T



Log (GMO amount)

Calibrators in amounts

Overview

European context on GMOs

↓

Basics of GMO quantification

↓

Concept of plasmid markers

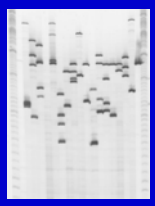
Plasmid DNA calibrators

1. Generation of the target DNA sequences

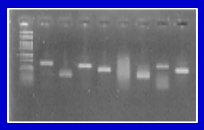
Selection and DNA preparation of transgenic plants



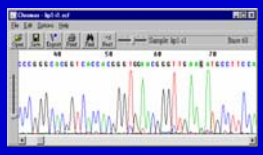
Anchor PCR



Excision and reamplification of event-specific fragments



Sequencing



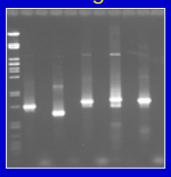
Characterization of event-specific fragments



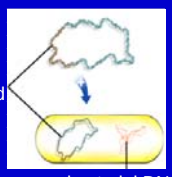
Plasmid DNA calibrators

2. Cloning of DNA fragments into plasmid vectors

Amplification of DNA fragments



Cloning and transformation



Preparation of pure plasmid DNA



Calculation of DNA copy numbers

nanogram
↓
copies

Dilution series in number of copies



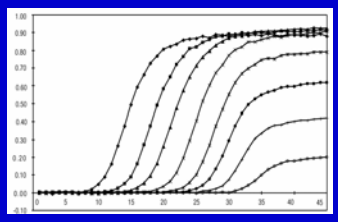
Plasmid DNA calibrators

3. Use of plasmid DNA series in real-time PCR

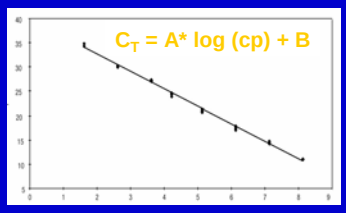
Real-time PCR



Setting up of calibration curves



C_T



Log (**# copies**)

Overview

European context on GMOs

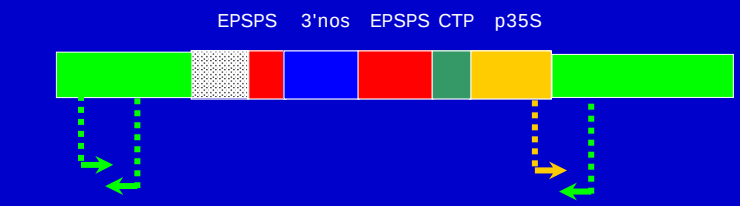
Basics of GMO quantification

Concept of plasmid markers

Experimental testing

Case study: RR soybean quantification

- Construction of plasmids



Lectin (*Le1*) gene

event-specific junction



pAS106 (118 bp)

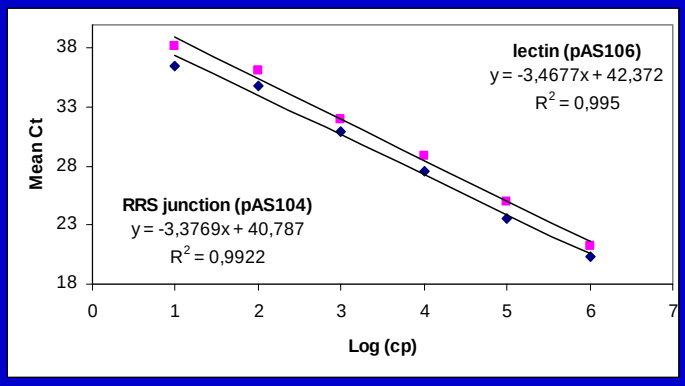


pAS104 (359 bp)

Case study: RR soybean quantification

- Absolute simplex PCR quantification

2 STP series (10^6-10 cp) - 2 standard curves (C_T)



Case study: RR soybean quantification

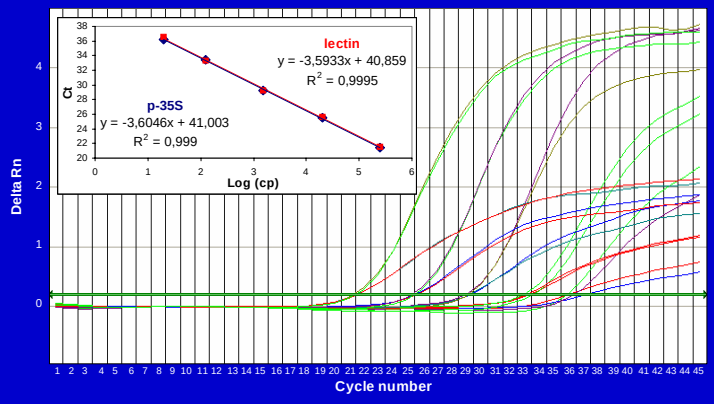


Sample	Cp junction	Mean cp junction	Cp lectin	Mean cp lectin	Conc (%)	Mean conc (%)	SD	%RSD	% error
NTC		NTC		NTC					
10		10		10					
100		100		100					
1000		1000		1000					
10000		10000		10000					
100000		100000		100000					
1E+06		1000000		1000000					
0	19,76 8,54	**14,15**	31595 19594	**25594,1**	0,063 0,044	***0,054***	0,013	24,07	5,4
0,1	1194,23 1317,9	**1256,07**	1E+06 1E+06	**1185553**	0,099 0,11	***0,105***	0,008	7,62	5
0,5	2163,36 1949,07	**2056,22**	521604 472012	**496808**	0,41 0,41	***0,41***	0	0	18
1	5863,98 7074,68	**6469,33**	683686 511536	**597611**	0,86 1,38	***1,12***	0,37	33,04	12
2	7743,99 11515,2	**9629,59**	306760 330960	**318860**	2,52 3,48	***3***	0,68	22,67	50

Case study: RR soybean quantification

- Absolute simplex PCR quantification
- Absolute duplex PCR quantification

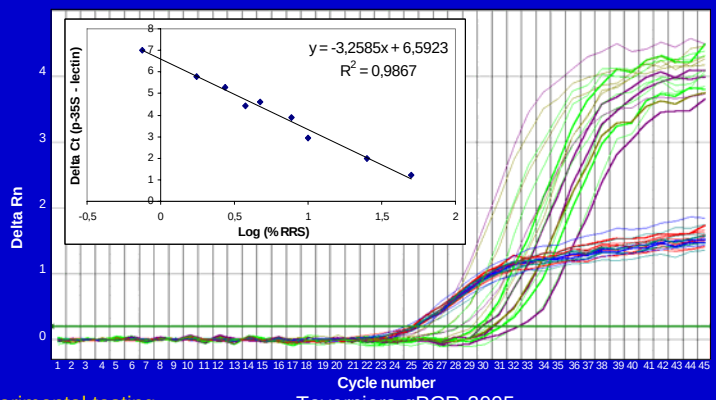
1 MTP series (10^6-10 cp) - 2 standard curves (C_T)



Case study: RR soybean quantification

- Absolute simplex PCR quantification
- Absolute duplex PCR quantification
- Relative duplex PCR quantification

1 STP series in gDNA (%) - 1 standard curve (ΔC_T)

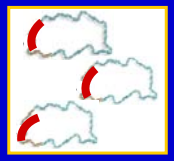


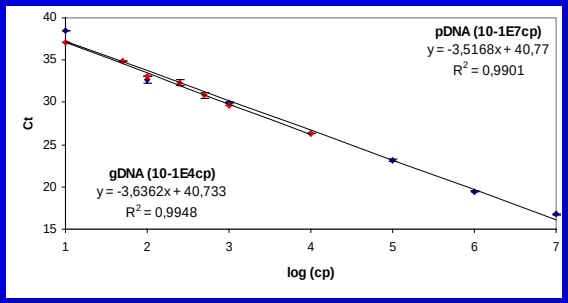
Comparability between gDNA and pDNA

genomic DNA



plasmid DNA





Bt176 maize, event-specific method

Comparable behaviour in the PCR

Overview

European context on GMOs

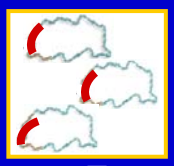
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Concept of plasmid markers

Experimental testing

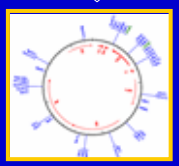
Implementation in practice

Implementation of plasmid system



In-house construction and testing of plasmids

pAS104, pAS106, pCM1, pCM2, …

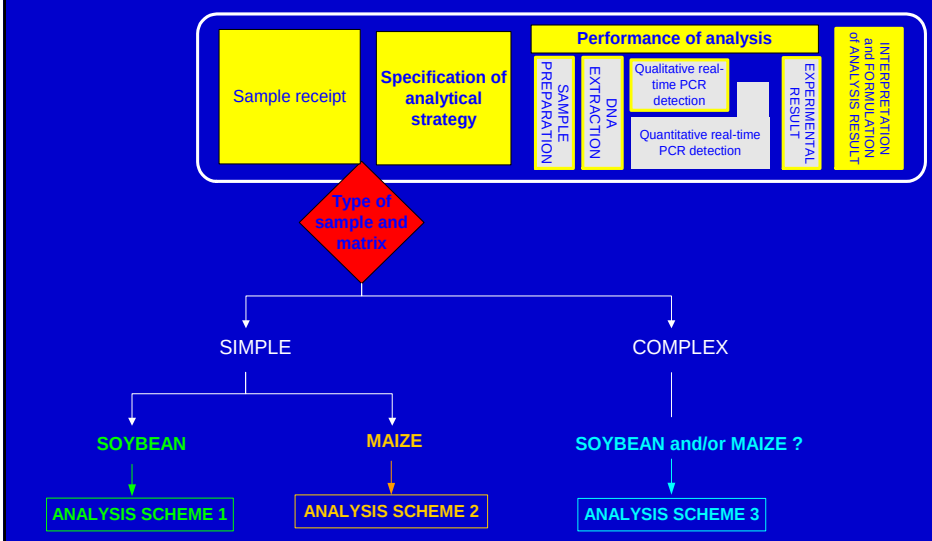


Subcloning to pUC18

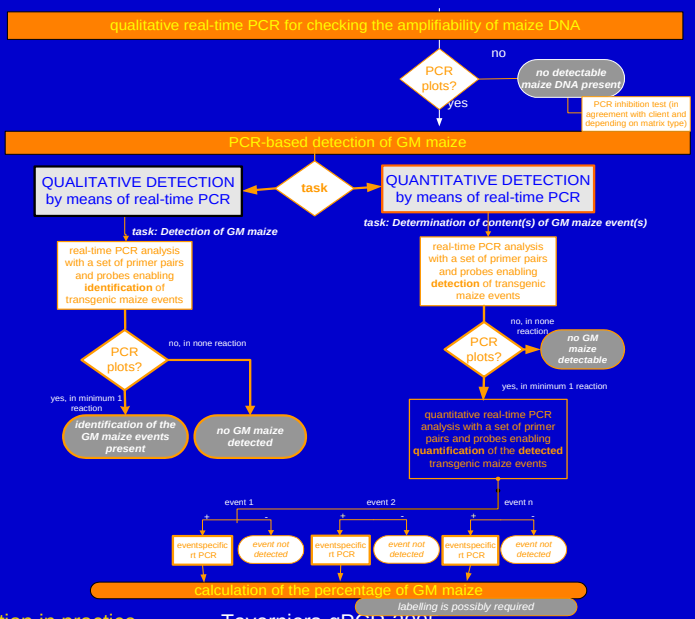
pENGL1, pENGL2, pENGL3,…

Officialization and standardization: ENGL database

Use of plasmids in routine practice



Use of plasmids in routine practice



Implementation of plasmid system

- Copy number-based DNA calibrators are in compliance with Comm. Rec. **2004/787/EC**, Reg. (EC) **1829/2003** and Reg. (EC) **1830/2003**
- Plasmid DNA markers are easy in production, storage, distribution and wide in applicability
- Plasmid DNA and genomic DNA calibrators show perfectly comparable behaviour (commutability)
- Reduction of analytical errors and improvement of efficiency with:
  - duplo-target plasmids (DTP) or multiple-target plasmids (MTP)
  - duplexing GM-specific and plant-specific target amplification
- Future challenges:
  - certification of copy number-based reference materials?
  - multiplexing?

Cloned plasmid DNA molecules as a tool for GMO analysis



Questions ?