



# Application of synthetic DNA-standards for the quantitative screening of different genetically modified rapeseed lines via real-time PCR



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# Outline

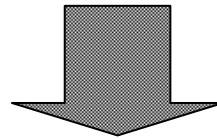
- 1. Regulatory framework regarding the use of GMO-derived material for the production of foods and feeds**
- 2. Analytical strategies for the development of methods for purposes of surveillance testing**
- 3. Development and validation of real-time PCR assays and synthetic DNA standards for the quantitative screening of GM rapeseed**

# Regulatory framework

**Authorization, traceability and labeling of genetically modified foods and feeds**

**Regulation (EG) Nr. 1829/2003**

**Regulation (EG) Nr. 1830/2003**



**Labeling is mandatory except for products showing adventitious or technical unavoidable contaminations:**

**authorized GMO: 0.9%**

**non-authorized GMO: 0.5%**

**(favorable risk assessment)**

# Control of compliance with labeling provisions

- **Inspection of traceability documentation**
  - ➔ Documentation acquired within the traceability system
  - ➔ Measures to avoid contaminations with GM-material
  - ➔ Certificates, IP systems, quality control
- **Surveillance testing of foods and feeds**
  - ➔ Applicant: provide event-specific assays (authorization)
  - ➔ CRL, ENGL: responsible for the method validation
  - ➔ IRMM: development of reference materials

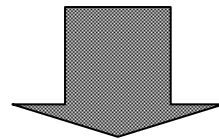
# Challenges for the surveillance testing

- **The steadily growing number of GMO; authorized or non-authorized in the EU**
- **Considerable number of event-specific assays; delivered by the applicant and validated by the CRL**
- **Hesitant supply of reference materials**
  - ➔ Not available for every authorized GMO
  - ➔ Not available for non-authorized GMO

# Method development

## Quantitative screening approaches

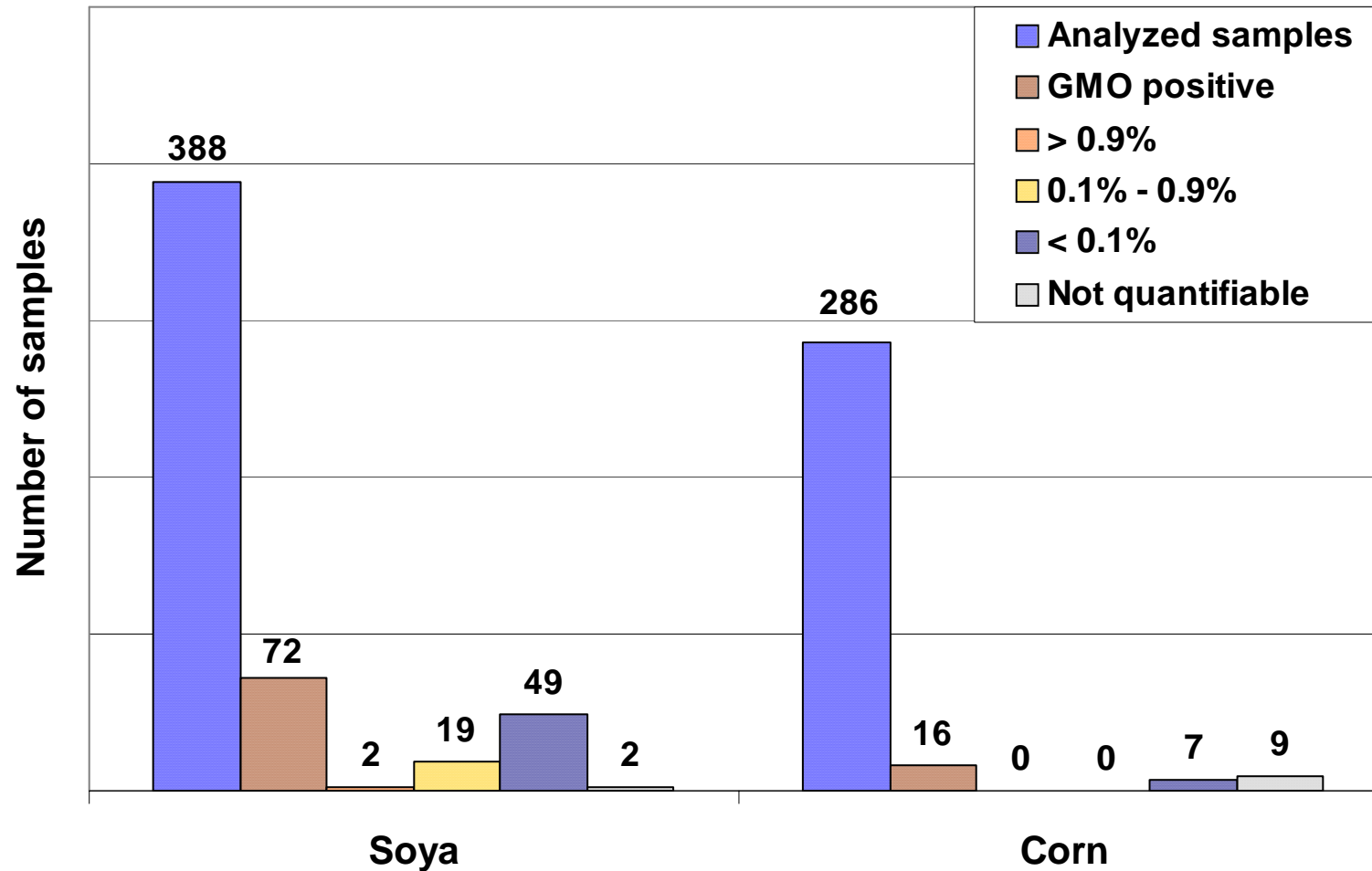
as a tool for the pre-selection of positive samples



**Allowing the detection of a broad spectrum of GMO  
at a moderate level of specificity**

**Delivering valuable information about the  
composition of any sample**

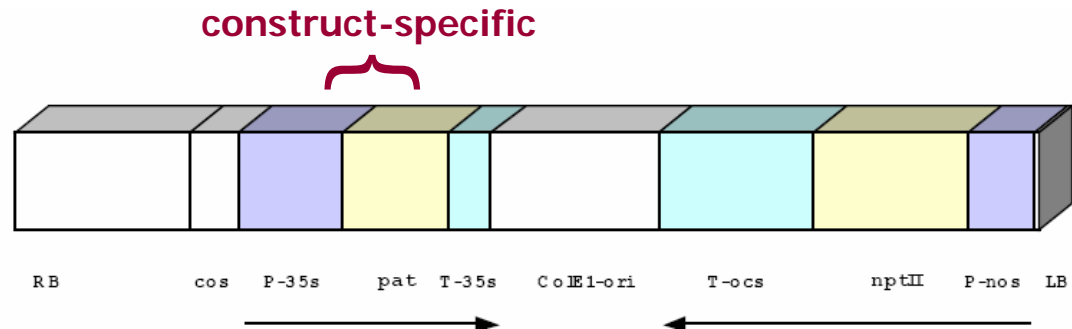
# Surveillance of GM foods (Bavaria, 2004)



# Quantitative screening of GM rapeseed

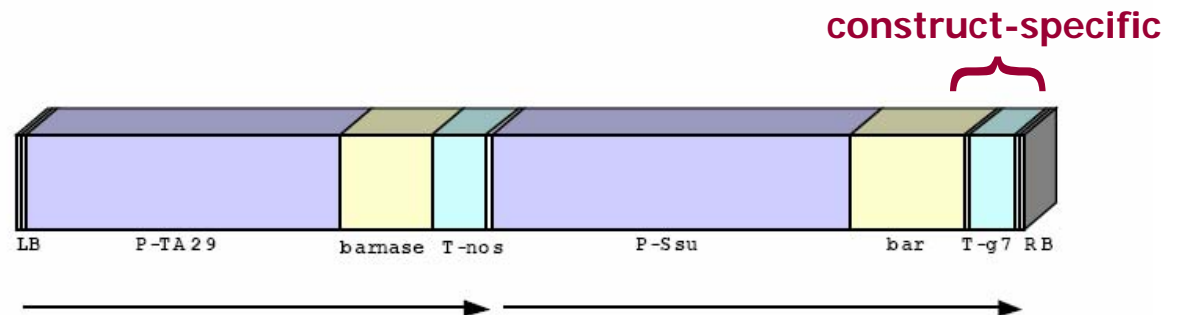
## ■ LibertyLink lines

- Falcon GS/40/90
- HCN10, HCN92
- Liberator L62
- T45



## ■ SeedLink lines

- MS1, RF1, RF2, MS1xRF1, MS1xRF2
- MS8, RF3, MS8xRF3
- PHY14, PHY35, PHY23, PHY36



Source: BATS Report, 2003

# Rapeseed-specific *BnACCg8* gene

## ENTRANSFOOD WG 4

### **BnACCg8 – Lambada** (conventional line)

GAGAATGAGGAGGACCAAGCTCAAGAAAGAGTGGAGAAAATTCTCAAAGAGGAAGAAGTTA  
GTTTCGAGCCTGTGTTCCGCAGGTGTGGGTGTGGTGTGAGCTGTATAATCGAGCGAGATGAAG  
GACGAACACCTATTAGACATTCGTTCCATTGGTCRATGGAGAAACAGTACTATGCAGAAGA  
GCCGATGCTGCGCC

### **BnACCg8 – MS8xRF3**

GAGAATGAGGAGGACCAAGCTCAAGAAAGAGTGGAGAAAATTCTCAAAGAGGAAGAAGTTA  
GTTTCGAGCCTGTGTTCCYGCAGGTGTGGGTGTGGTGTGAGCTGTATAATCGAGCGAGATGAAG  
GACGAACACCTATTAGACATTCGTTCCATTGGTCRATGGAGAAACAGTACTATGCAGAAGA  
GCCGATGCTGCGCC

### **BnACCg8 – Falcon GS 40/90**

GAGAATGAGGAGGACCAAGCTCAAGAAAGAGTGGAGAAAATTCTCAAAGAGGAAGAAGTTA  
GTTTCGAGCCTGTGTTCCGCAGGTGTGGGTGTGGTGTGAGCTGTATAATCGAGCGAGATGAAG  
GACGAACACCTATTAGACATTCGTTCCATTGGTCGATGGAGAAACAGTACTATGCAGAAGA  
GCCGATGCTGCGCC

### **BnACCg8 – Liberty Link** (GeneScan cat. nr. 5211501601)

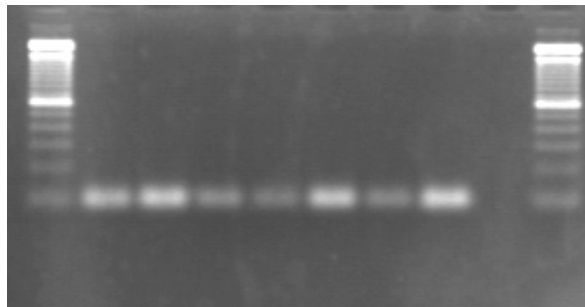
GAGAATGAGGAGGACCAAGCTCAAGAAAGAGTGGAGAAAATTCTCAAAGAGGAAGAAGTTA  
GTTTCGAGCCTGTGTTCTGCAGGTGTGGGTGTGGTGTGAGCTGTATAATCGAGCGAGATGAAG  
GACGAACACCTATTAGACATTCGTTCCATTGGTCRATGGAGAAACAGTACTATGCAGAAGA  
GCCGATGCTGCGCC

# Specificity testing: reference gene

Plant species	Chloroplast-specific PCR	<i>BnACCG8</i> - gene PCR
<i>Brassica oleracea ssp. botrytis</i>	+	-
<i>Brassica oleracea ssp. gemmifera</i>	+	-
<i>Brassica oleracea ssp. italica</i>	+	-
<i>Brassica alba (Sinapis alba)</i>	+	-
<i>Glycine max</i>	+	-
<i>Gossypium arboreum</i>	+	-
<i>Helianthus annuus</i>	+	-
<i>Lycopersicon esculentum</i>	+	-
<i>Oryza sativa</i>	+	-
<i>Panicum miliaceum</i>	+	-
<i>Secale cereale</i>	+	-
<i>Solanum tuberosum</i>	+	-
<i>Sorghum bicolor</i>	+	-
<i>Triticale</i>	+	-
<i>Triticum aestivum</i>	+	-
<i>Vicia faba</i>	+	-
<i>Zea Mays</i>	+	-

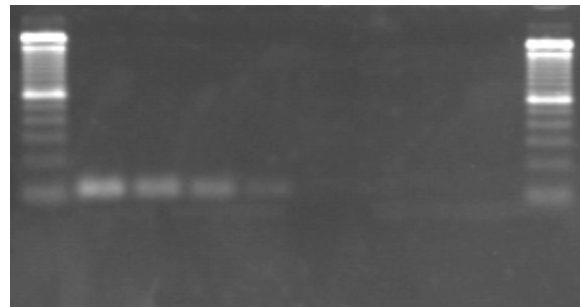
# Specificity testing: screening assays

*BnACCg8*- gene



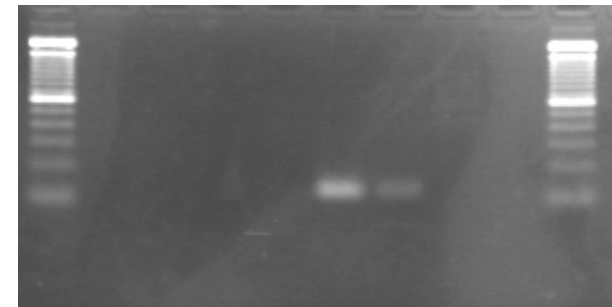
Falcon  
T45  
LibertyLink  
Liberator L62  
MS1xRF1  
MS8xRF3  
Conv. rapeseed  
Negative control

*pat* - cassette



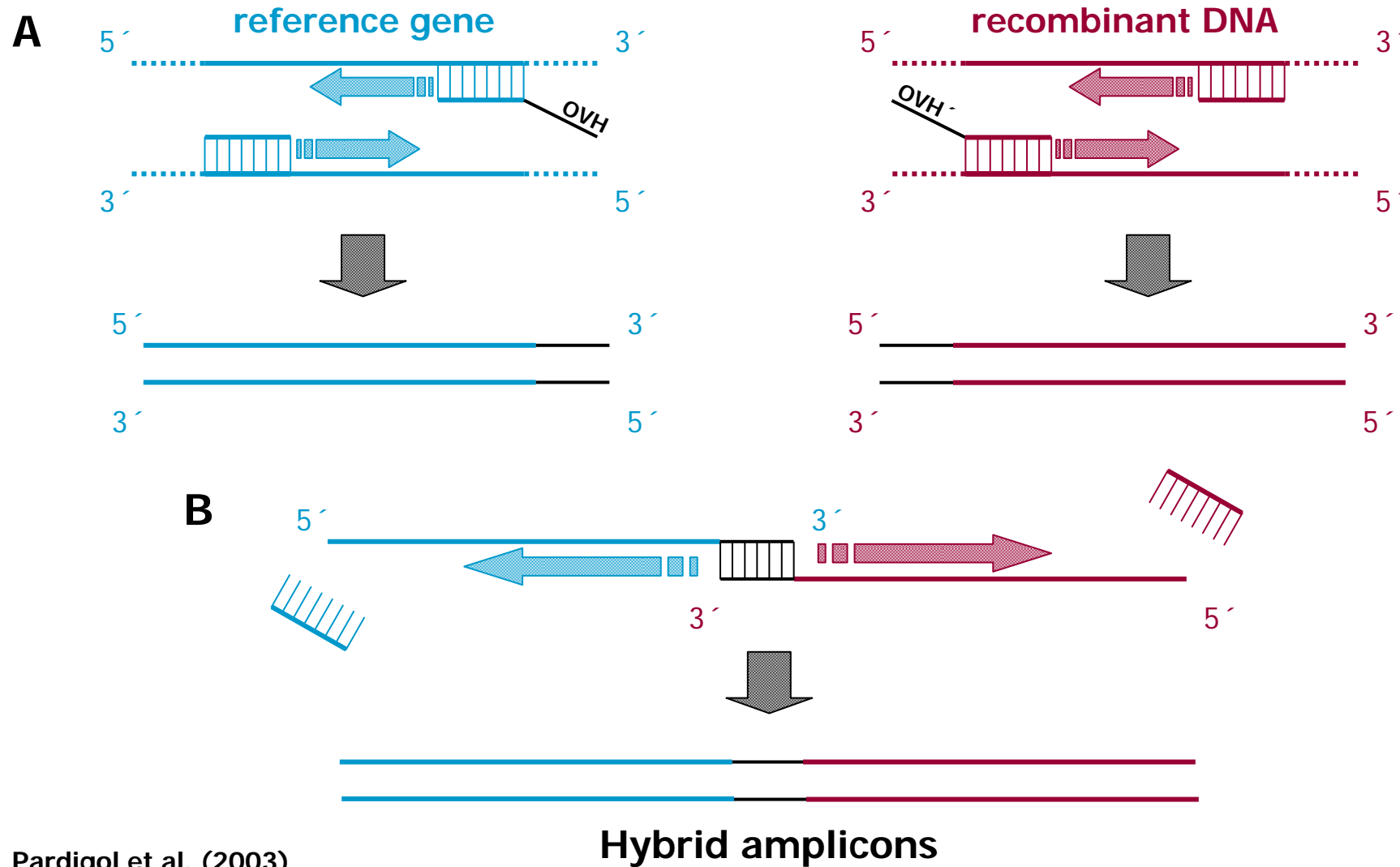
Falcon  
T45  
LibertyLink  
Liberator  
MS1xRF1  
MS8xRF3  
Conv. rapeseed  
Negative control

*bar* - cassette



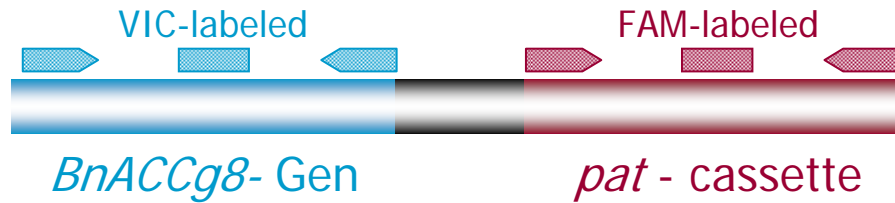
Falcon  
T45  
LibertyLink  
Liberator  
MS1xRF1  
MS8xRF3  
Conv. rapeseed  
Negative control

# Synthetic quantification standards

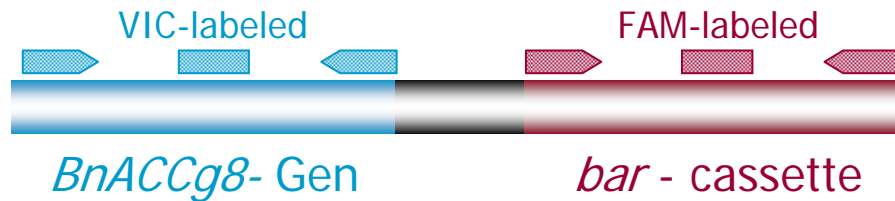


Pardigol et al. (2003)

# Quantitative screening systems

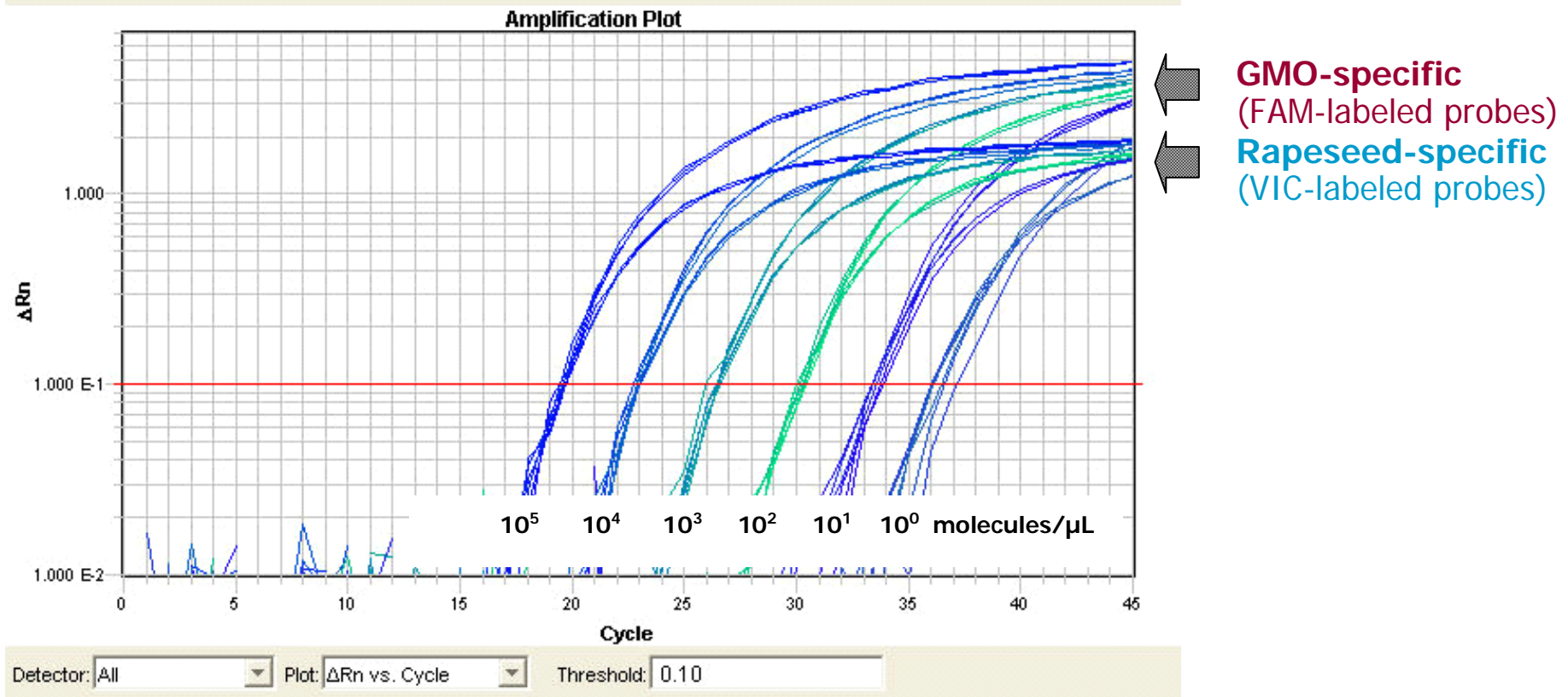


- Falcon GS/40/90
- HCN10, HCN92
- Liberator L62
- T45



- MS1, RF1, RF2, MS1xRF1, MS1xRF2
- MS8, RF3, MS8xRF3
- PHY14, PHY35
- PHY23
- PHY36

# Synthetic standards: performance in duplex



# Synthetic standards: calibration curves

## LibertyLink assay:

$$\text{Log (Qty recDNA)} = -3.414 * C_t + 38.914$$

$$\text{Log (Qty reference)} = -3.432 * C_t + 39.335$$

## SeedLink assay:

$$\text{Log (Qty recDNA)} = -3.492 * C_t + 39.387$$

$$\text{Log (Qty reference)} = -3.353 * C_t + 38.874$$

→  **$R^2 > 0.99$**

# Validation results: LibertyLink assay

Expected GMO content [%]	1st experiment *			2nd experiment *		
	GMO [%]	Recovery [%]	RSD [%]	GMO [%]	Recovery [%]	RSD [%]
<b>1.8</b>	1.9	106.7	9.7	1.8	98.1	4.3
<b>0.9</b>	0.9	99.7	11.1	0.8	90.2	13.1
<b>0.09</b>	0.06	68.8	11.0	0.06	62.8	11.3
<b>0.045</b>	False negatives = 0%			False negatives = 0%		

\* n = 10

# Validation results: SeedLink assay

Expected GMO content [%]	1st experiment *			2nd experiment *		
	GMO [%]	Recovery [%]	RSD [%]	GMO [%]	Recovery [%]	RSD [%]
<b>1.8</b>	2.5	140.1	13.2	2.1	117.8	14.5
<b>0.9</b>	1.0	114.5	10.8	0.9	99.9	20.3
<b>0.09</b>	0.04	46.8	9.6	0.06	64.1	8.8
<b>0.045</b>	False negatives = 0%			False negatives = 0%		

\* n = 10

# Summary

- Real-time PCR assays for the quantitative screening of genetically modified rapeseeds were developed and validated
- Synthetic DNA standards for relative quantifications were obtained under application of a technique that allows the generation of hybrid amplicons
  - ➔ Linkage of recombinant and reference target sequences
  - ➔ Dynamic range:  $5.0 \times 10^5$  – 5.0 copies/reaction
  - ➔ Targeted design permitted the performance of duplex reactions
- Performance of quantitative screening was tested using mixtures of genomic DNA from recombinant and conventional rapeseeds
  - ➔ Range of quantification: 1.8 – 0.09% GMO
  - ➔ Limits of detection < 0.045% GMO
  - ➔ Reliable screening of recombinant DNA and discrimination of non-quantifiable samples

**Thank you for your attention!**

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