Bavarian Health and Food
Safety Authority

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



Dr. Francisco X. Moreano

Outline

1. **Regulatory framework regarding the use of GMO-derived material for the production of foods and feeds**

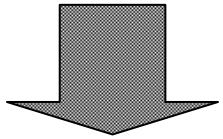
2. **Analytical strategies for the development of methods for purposes of surveillance testing**

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

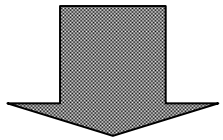
Challenges for the surveillance testing

- **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

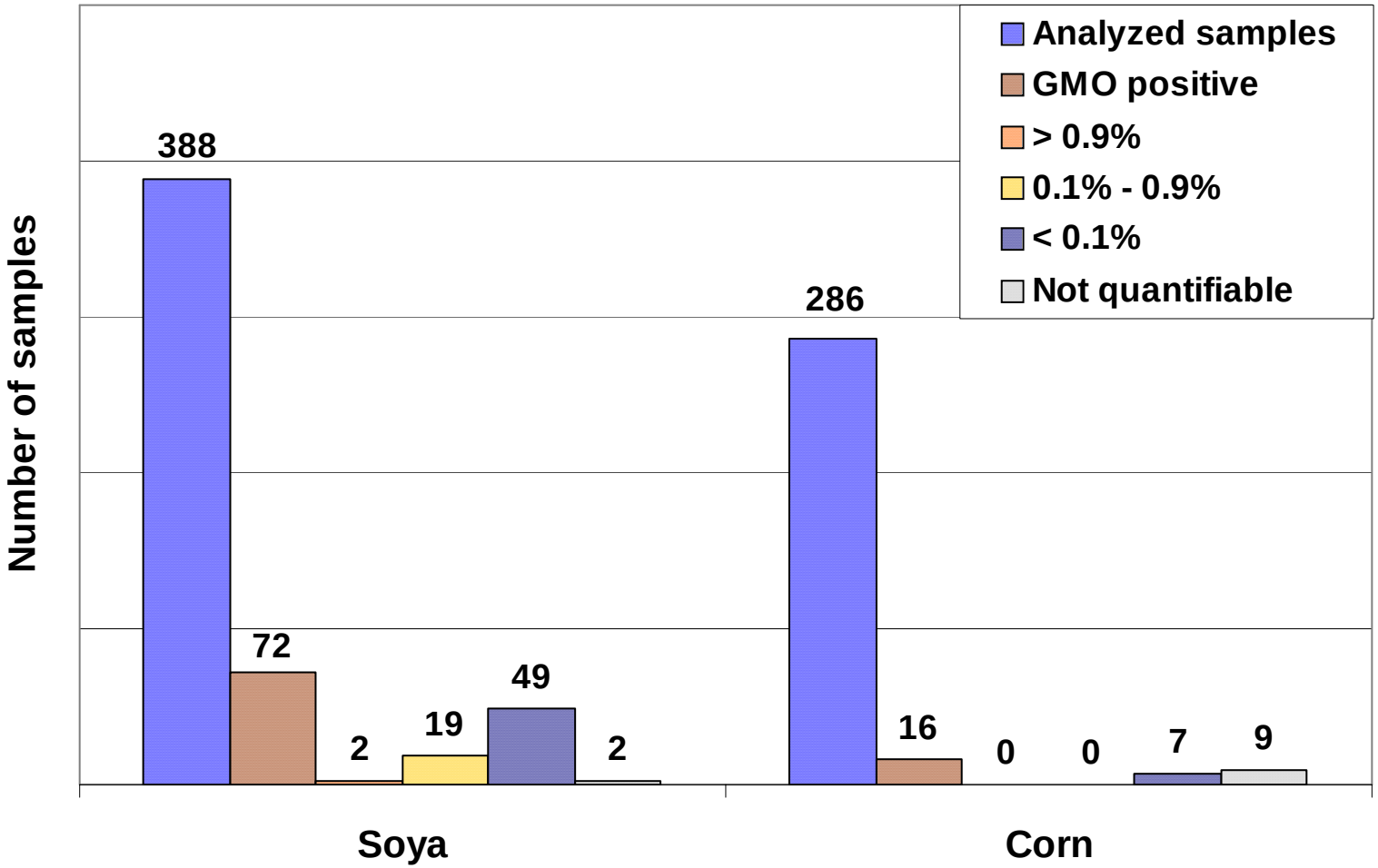
as a tool for the pre-selection of positive samples



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

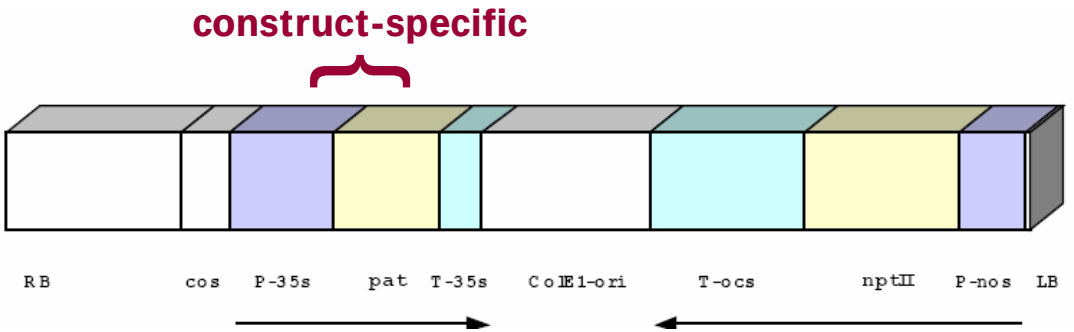
Delivering valuable information about the composition of any sample

Surveillance of GM foods (Bavaria, 2004)

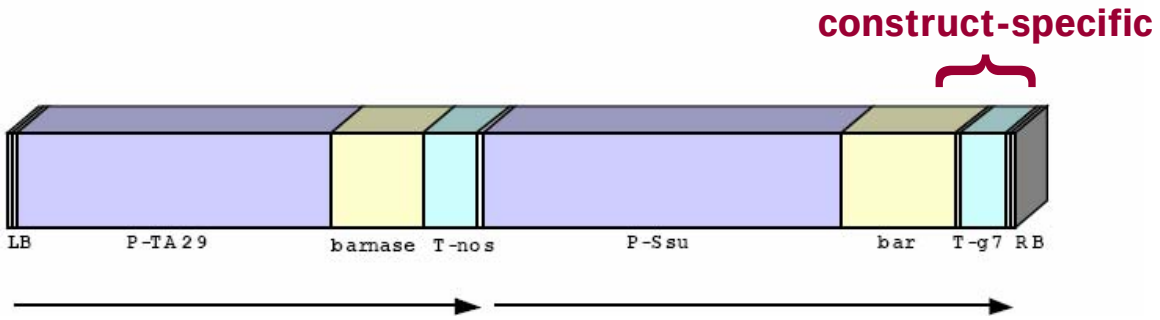


Quantitative screening of GM rapeseed

- **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
GTTCGAGCCTGTGTTC**C**GCAGGTGTGGGTGTGGTGAGCTGTATAATCGAGCGAGATGAAG
GACGAACACCTATTAGACATTCGTTCCATTGGTC**R**ATGGAGAAACAGTACTATGCAGAAGA
GCCGATGCTGCGCC

BnACCg8 – MS8xRF3

GAGAATGAGGAGGACCAAGCTCAAGAAAGAGTGGAGAAAATTCTCAAAGAGGAAGAAGTTA
GTTCGAGCCTGTGTTC**Y**GCAGGTGTGGGTGTGGTGAGCTGTATAATCGAGCGAGATGAAG
GACGAACACCTATTAGACATTCGTTCCATTGGTC**R**ATGGAGAAACAGTACTATGCAGAAGA
GCCGATGCTGCGCC

BnACCg8 – Falcon GS 40/90

GAGAATGAGGAGGACCAAGCTCAAGAAAGAGTGGAGAAAATTCTCAAAGAGGAAGAAGTTA
GTTCGAGCCTGTGTTC**C**GCAGGTGTGGGTGTGGTGAGCTGTATAATCGAGCGAGATGAAG
GACGAACACCTATTAGACATTCGTTCCATTGGTC**G**ATGGAGAAACAGTACTATGCAGAAGA
GCCGATGCTGCGCC

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

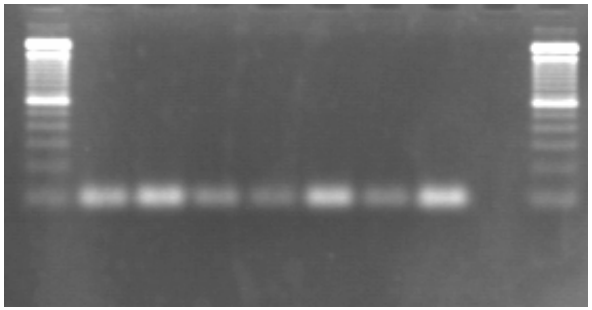
GAGAATGAGGAGGACCAAGCTCAAGAAAGAGTGGAGAAAATTCTCAAAGAGGAAGAAGTTA
GTTCGAGCCTGTGTTC**T**GCAGGTGTGGGTGTGGTGAGCTGTATAATCGAGCGAGATGAAG
GACGAACACCTATTAGACATTCGTTCCATTGGTC**A**ATGGAGAAACAGTACTATGCAGAAGA
GCCGATGCTGCGCC

Specificity testing: reference gene

Plant species	Chloroplast-specific PCR	*BnACCg8*- gene PCR
Brassica oleracea ssp. botyritis	+	-
Brassica oleracea ssp. gemmifera	+	-
Brassica oleracea ssp. italica	+	-
Brassica alba (*Sinapis alba*)	+	-
Glycine max	+	-
Gossypium arboreum	+	-
Helianthus annuus	+	-
Lycopersicon esculentum	+	-
Oryza sativa	+	-
Panicum miliaceum	+	-
Secale cereale	+	-
Solanum tuberosum	+	-
Sorghum bicolor	+	-
Triticale	+	-
Triticum aestivum	+	-
Vicia faba	+	-
Zea Mays	+	-

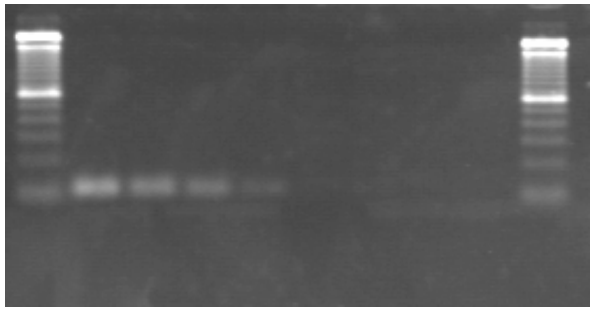
Specificity testing: screening assays

BnACCg8- gene



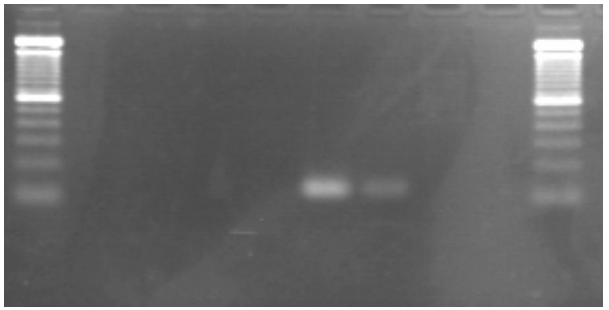
Falcon
T45
LibertyLink
Liberator L62
MS1xRF1
MS8xRF3
Conv. rapeseed
Negative control

pat - cassette



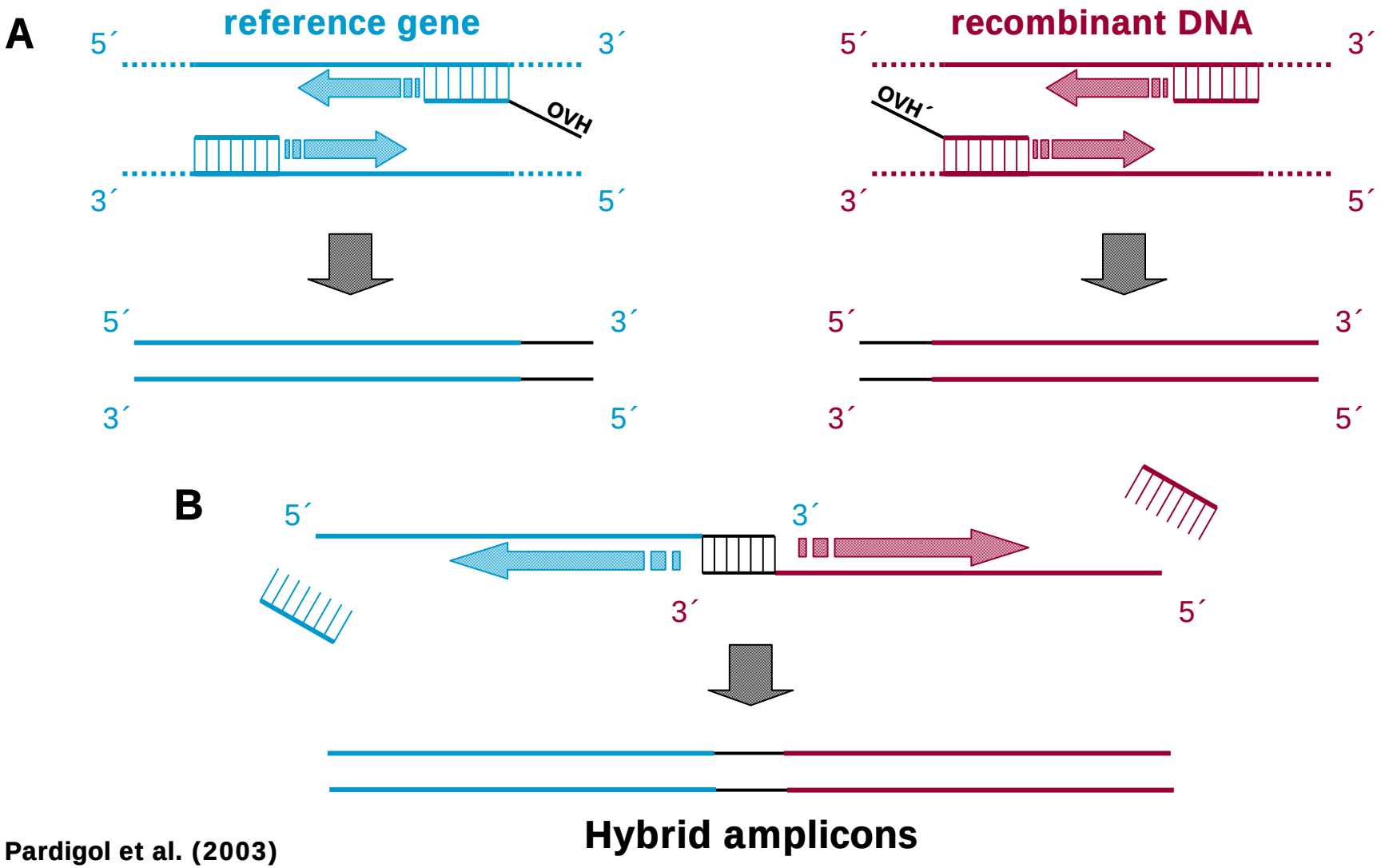
Falcon
T45
LibertyLink
Liberator
MS1xRF1
MS8xRF3
Conv. rapeseed
Negative control

bar - cassette



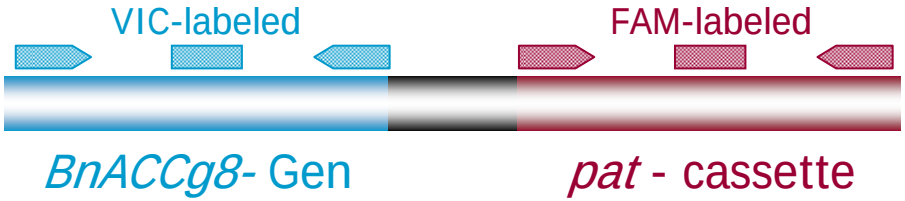
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MS1xRF1
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Synthetic quantification standards

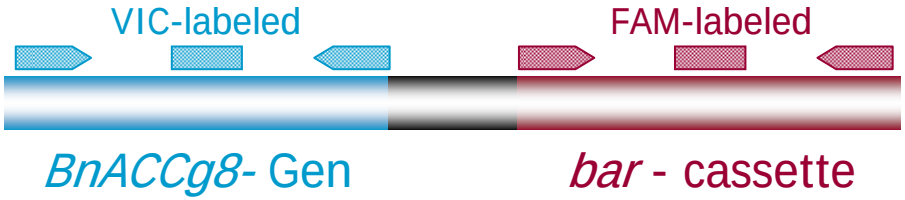


Pardigol et al. (2003)

Quantitative screening systems

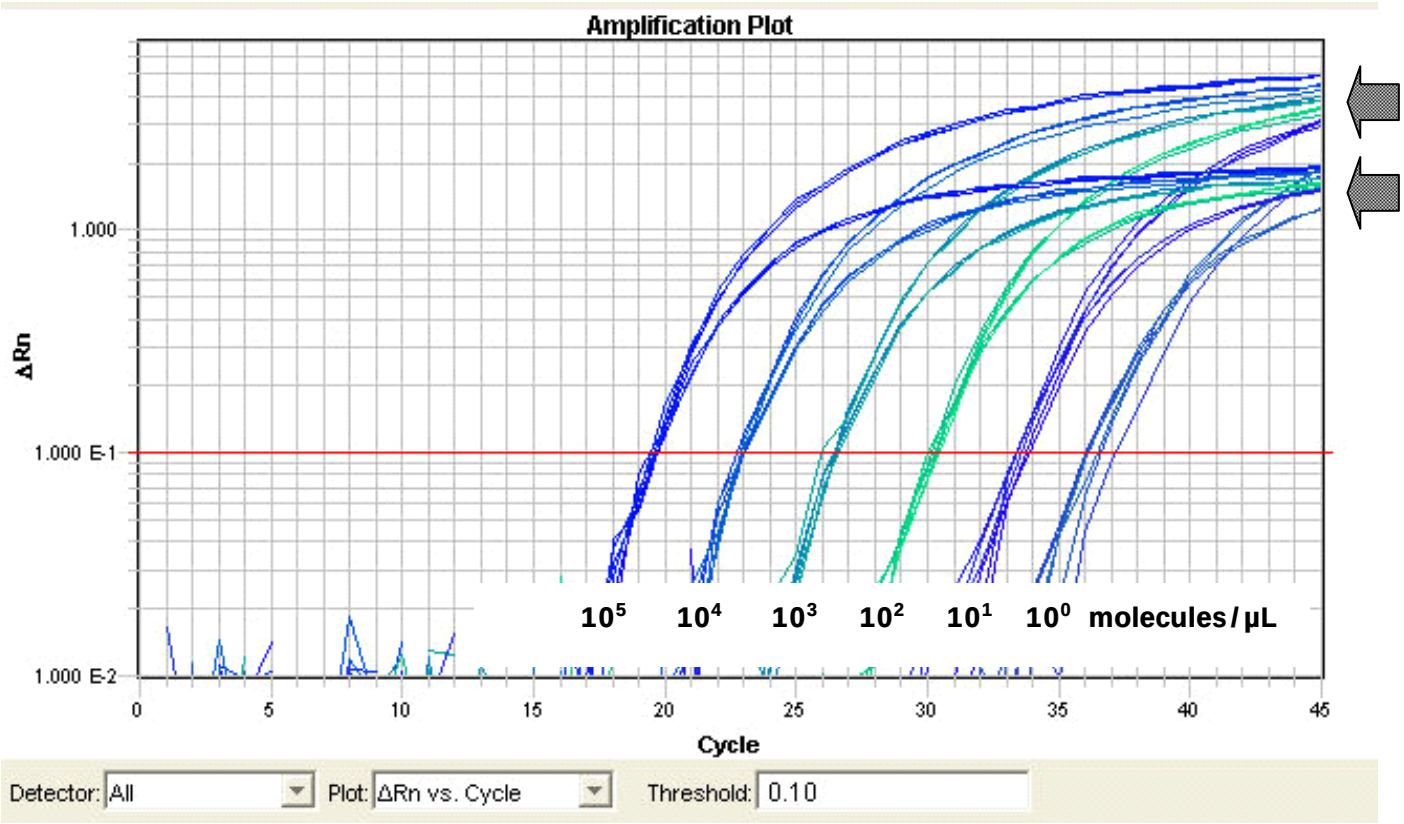


- Falcon GS/40/90
- HCN10, HCN92
- Liberator L62
- T45



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

Synthetic standards: performance in duplex



GMO-specific
(FAM-labeled probes)
Rapeseed-specific
(VIC-labeled probes)

Synthetic standards: calibration curves

LibertyLink assay:

Log (Qty recDNA) = -3.414 * C_t + 38.914

Log (Qty reference) = -3.432 * C_t + 39.335

SeedLink assay:

Log (Qty recDNA) = -3.492 * C_t + 39.387

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 [%]
1.8	1.9	106.7	9.7	1.8	98.1	4.3
0.9	0.9	99.7	11.1	0.8	90.2	13.1
0.09	0.06	68.8	11.0	0.06	62.8	11.3
0.045	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 [%]
1.8	2.5	140.1	13.2	2.1	117.8	14.5
0.9	1.0	114.5	10.8	0.9	99.9	20.3
0.09	0.04	46.8	9.6	0.06	64.1	8.8
0.045	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: 5.0×10^5 – 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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