Uncertainties and certainties in GMO analytics using qPCR

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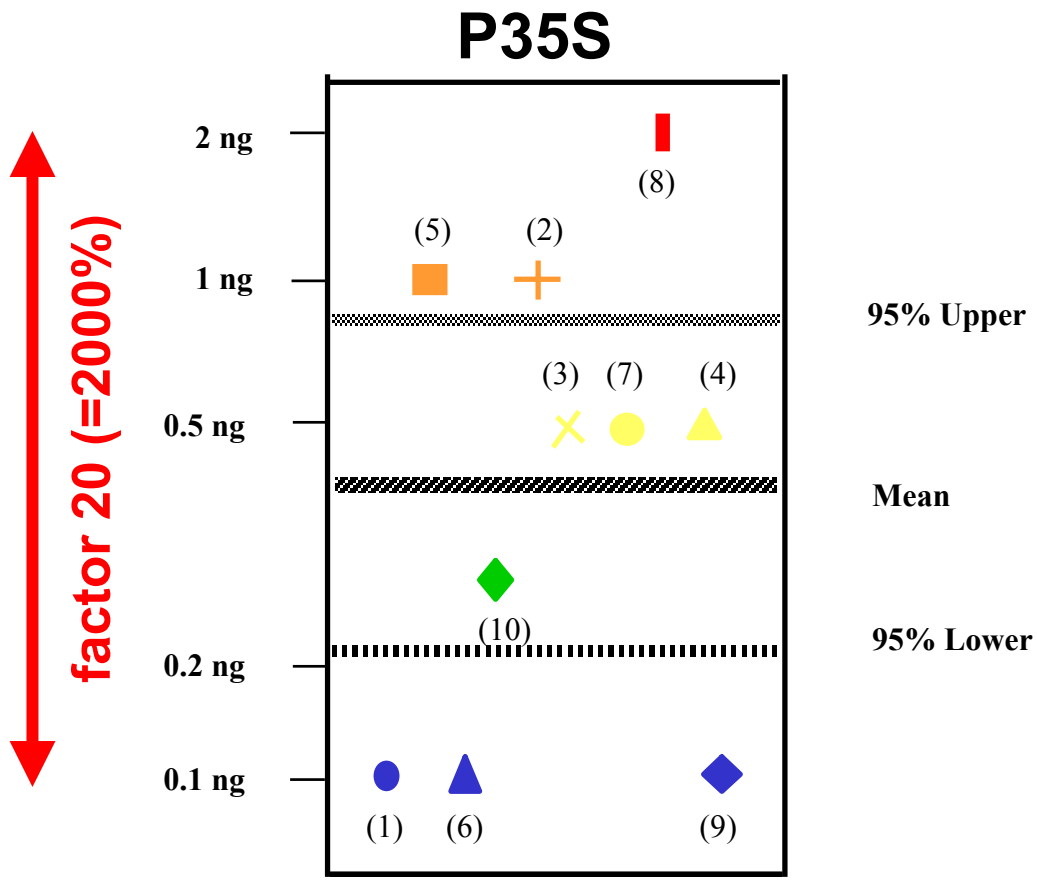
(Un-)certainties in GMO analytics using qPCR

- „historical“ review
- Short crash course in validation
  - Accuracy
  - Limits of detection and quantitation
- Error calculation
- Identity Tag for Quantitation
- Conclusion

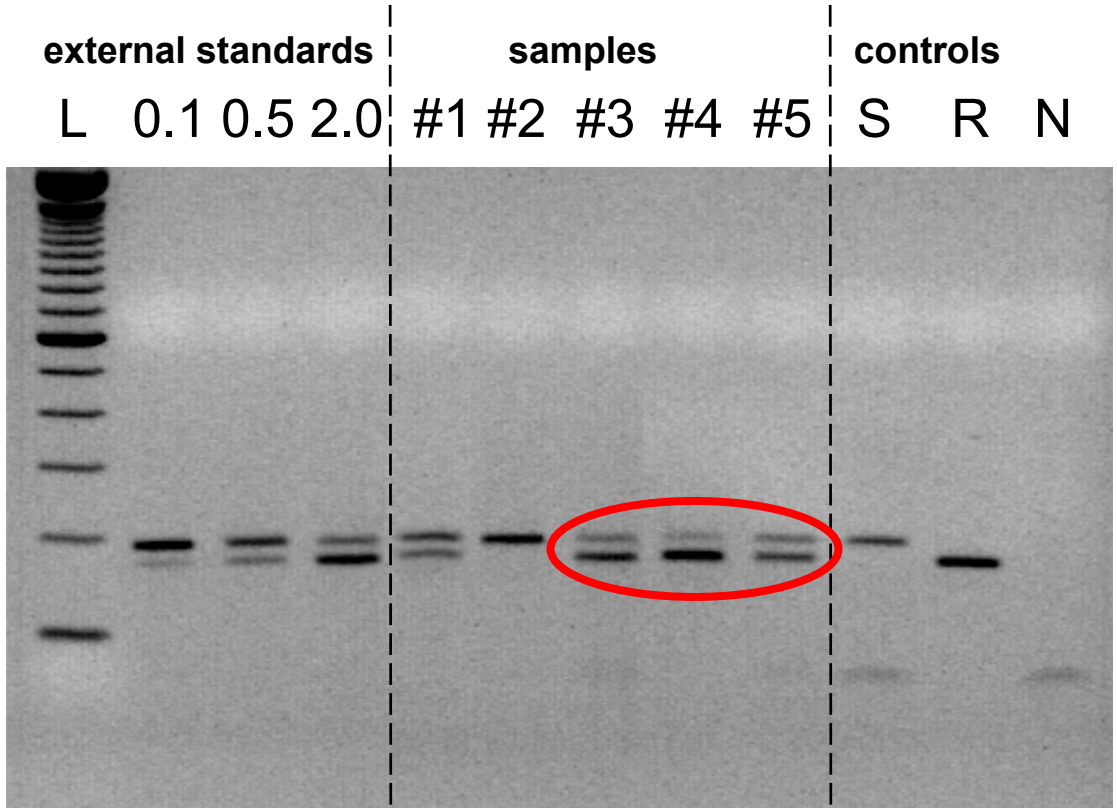
Limit of detection of 35S-PCR

GMO labeling depended on PCR testing with reference method targeting 35S promotor



Implementation of Quantitative Competitive PCR

Co-amplification of 500 ng target DNA with a defined amount of standard DNA (equivalent to 1 % GMO)



#1: Lecithine 1%

#2: Flour < 0.1%

#3: Protein 2%

#4: Grist > 5%

#5: Grist 1 - 2%

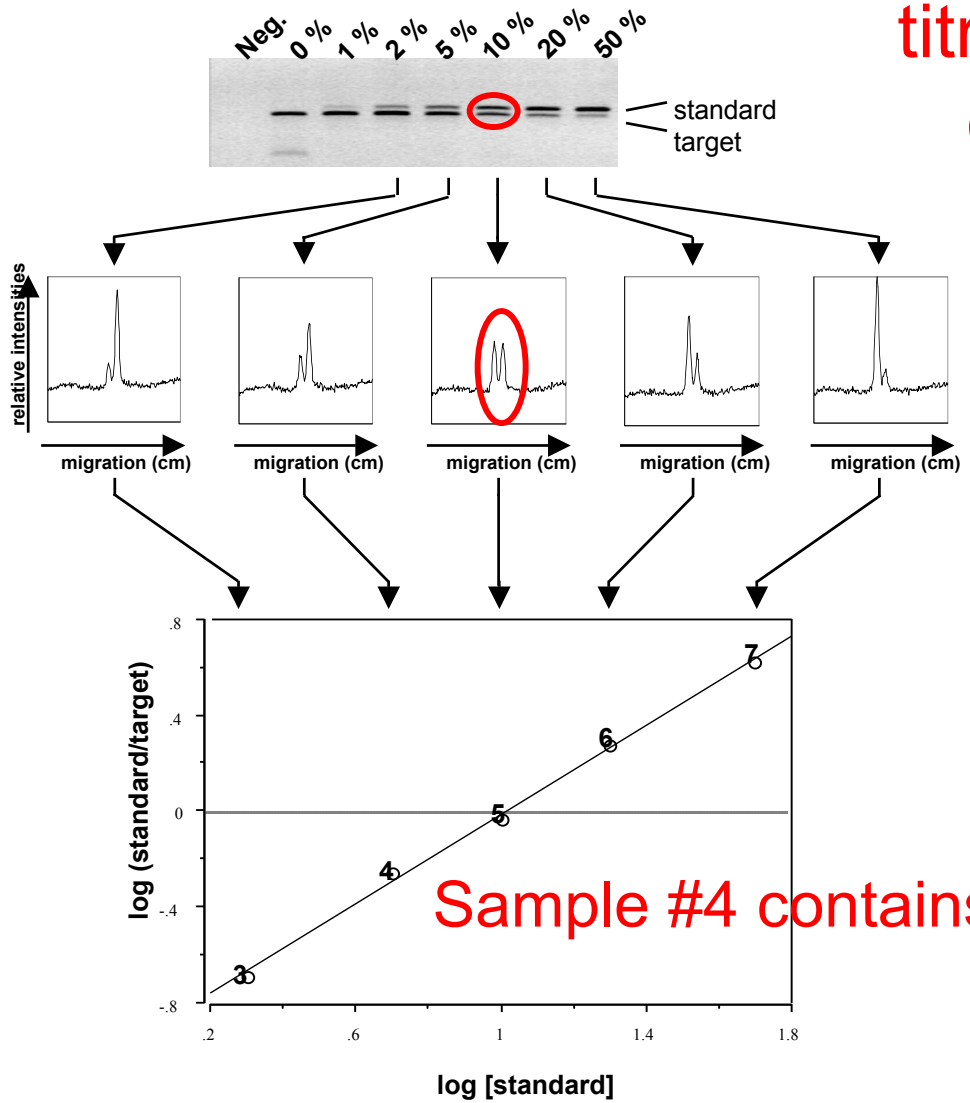
S: Standard DNA

R: Roundup Ready soybean DNA

N: PCR negative control (H_2O)

QC-PCR for the Determination of the GMO content

Example: Sample #4 grist



titration with internal competitor DNA

$y = -0.949 + 0.933 \cdot x$, $r^2 = 0.998$

for $y = 0$, $x = 1.02$

$10^x = 10^{1.02} = 10.5$

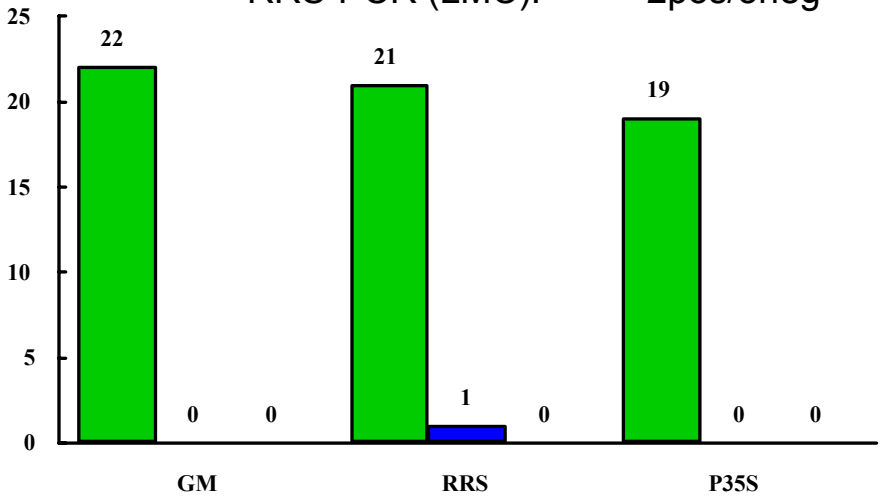
Sample #4 contains 10% GMO

Results of inter-laboratory study 1997

12 participants, mainly Swiss labs

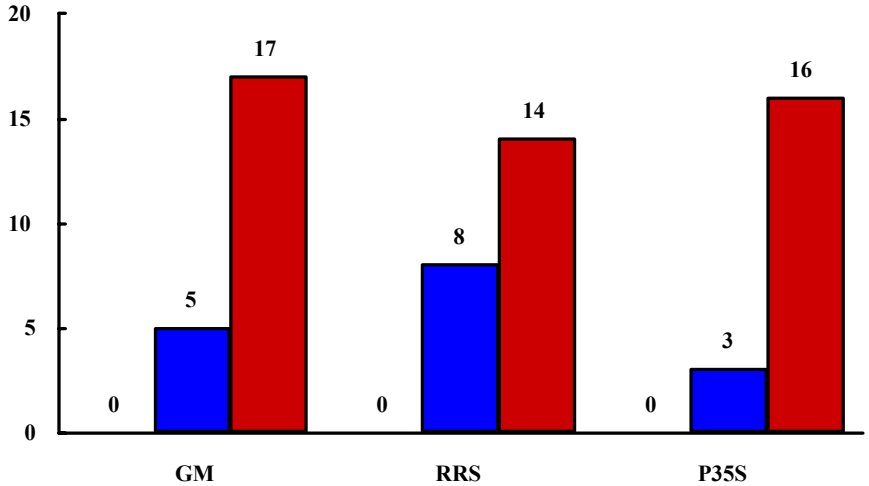
sample #6 **flour LMC137**

QC-PCR GM (LMC):	<0.1%
35S-PCR (RAP;LMC):	neg
RRS-PCR (RAP):	neg
RRS-PCR (LMC):	2pos/3neg

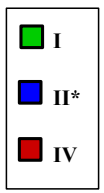


sample #7 **flour LMC107**

QC-PCR GM (LMC):	6%
35S-PCR (RAP):	pos
RRS-PCR (RAP;LMC):	pos



GMO content



x < 0.5%
0.5% < x < 2%
x > 2%

Categories depended on available reference material -> semiquantitative results

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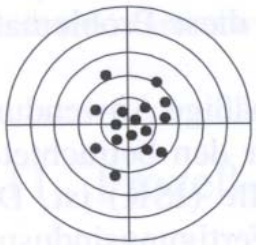
Which validation parameter are important?

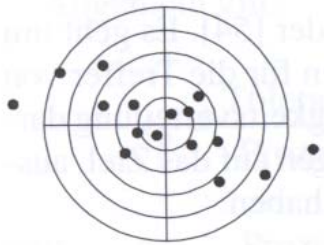
- we would like to measure accurately:
  - trueness and precision
- we need a robust method which gives comparable results in other laboratories

Accuracy: trueness and precision



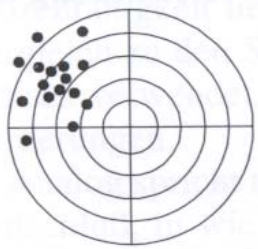
Bogenschütze 1

high trueness
high precision



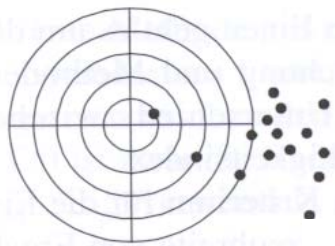
Bogenschütze 3

high trueness
low precision



Bogenschütze 2

low trueness
high precision



Bogenschütze 4

low trueness
low precision

what is needed for validation?

- for determination of relative trueness:
  - certified reference material*
  - samples material which can be traced back to CRMs
  - interlaboratory studies or proficiency testing schemes (e.g. FAPAS)

  * commercial availability is restricted to view events

what is needed for validation? (2)

- for determination of precision:
  - replicates of sample extraction and rt-PCR are informative for the precision of the whole procedure
  - whereas replicates of extracted DNA are only informative for the rt-PCR part of the procedure
  - reproducibility can be assessed by replicate measurements in different labs

Accuracy: Trueness and precision

Table 5 Quantitative estimates with DNA [illegible] ixed and processed foodstuffs. Each foodstuff [illegible] mes (A–D)

Food sample	RRS %			Mean	SD	RSD_r	Bias (%)
Biscuit 1		[illegible]76	12.41	12.9	0.94	7.3	29.0
Biscuit 2		15.07	12.07	12.66	4.28	34	26.6
Acidi[illegible]	128.3	107.2	–	111.8	14.7	13	11.8
[illegible]	[illegible]7.4	111.0	109.4	109.4	99.3	21.3	21	0.7

RSD_r varied from 7% to 34%

Bias varied from 1% to 29%

Berdal and Holst-Jensen (2001)

Accuracy (trueness and precision)

- should be determined at the „legal“ limit (i.e. 0.9 % GMO)
- RSDs in the range of 25% to 35% have been achieved in ring trials
- FAPAS indicates a target standard deviation of 0.2 $\log_{10}$. This means that the lower 95% confidence intervall is at 40% of the mean, the upper limit at 250% of the mean (z-score:=2)
  - Example mean:= 10%; 95% confidence intervall: 4% - 25%
- 0.2 $\log_{10}$ is equivalent to 0.66 $\log_2$. This means ΔC_t-values up to 0.33 are acceptable by FAPAS

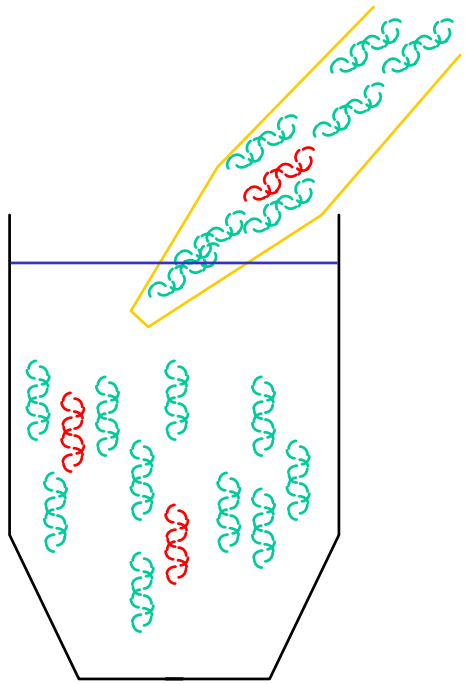
Limit of Detection (LOD)
Limit of Quantification (LOQ)

Definitions (IUPAC)

- The limit of quantification of an analytical procedure is the lowest amount or concentration of analyte in a sample which can be quantitatively determined with an acceptable level of precision and accuracy
- the limit of detection is the smallest amount or concentration of analyte in the test sample, that can be reliably distinguished with stated significance, from the background or blank level.

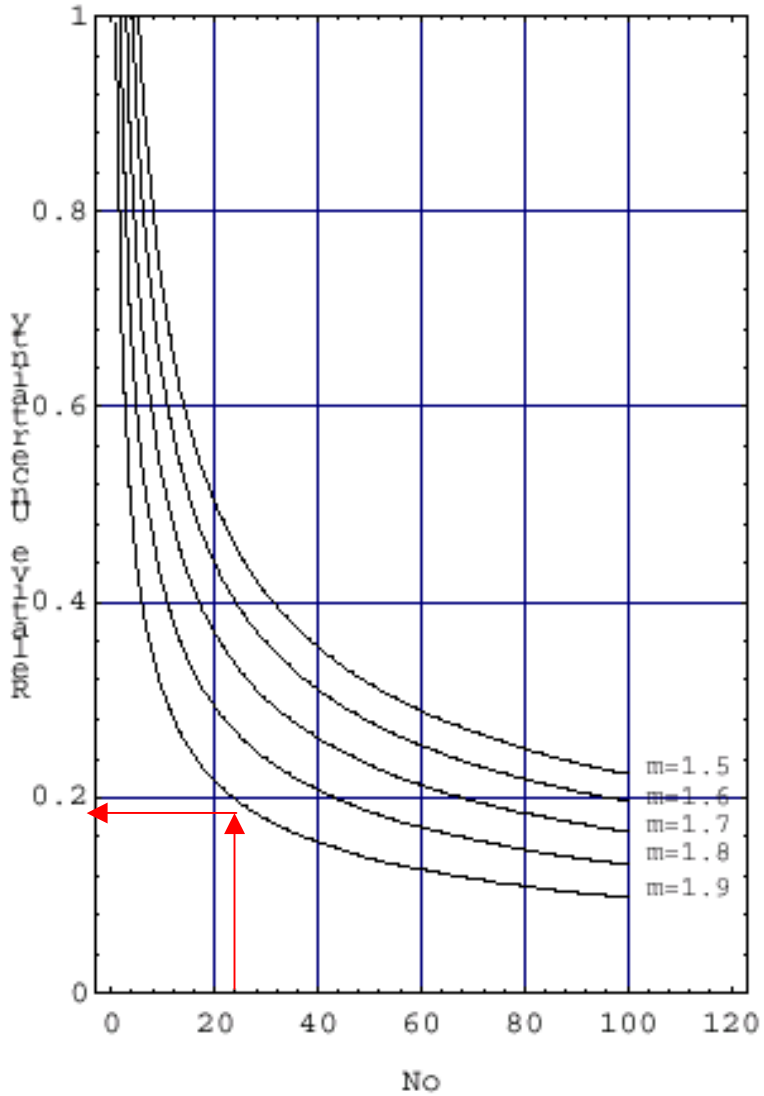
Pipetting of target molecules

mean	StDev	RSD (%)
μ	$\sqrt{\mu}$	
100	10.0	10.0
80	8.9	11.2
60	7.7	12.9
40	6.3	15.8
30	5.5	18.3
25	5.0	20.0
20	4.5	22.4
15	**3.9**	**25.8**
10	3.2	31.6
9	**3.0**	**33.3**
8	2.8	35.4
7	2.6	37.8
5	2.2	44.7
3	1.7	57.7
2	1.4	70.7
1	1.0	100.0



Codex alimentarius:
LOQ is at RSD = 25%
LOD is at RSD = 33%

Theoretical uncertainty of measurements using quantitative PCR Jean Peccoud and Christine Jacob 1996



2 σ = 0.19

σ = 0.095

realtime PCR: estimation of LOQ and LOD

	α=0.95	pipetting	PCR efficiency	CRM	
mean	**x>μ**	**RSD**	**RSD**	**RSD**	**RSD**
μ		%	m=1.9		overall
100		10.0	4	10	15
80		11.2	5	10	16
60		12.9	6.5	10	18
40	52	15.8	7.5	10	20
30	41	18.3	8	10	22
25	**36**	**20.0**	**9.5**	**10**	**24**
20	29	22.4	11	10	27
15	23	25.8	13	10	31
10	**17**	**31.6**	**15**	**10**	**36**
9	16	33.3	16	10	38
8	14	35.4	17.5	10	41
7	13	37.8	20	10	44
5	11	44.7	22	10	51
3	8	57.7	26	10	64
2	6	70.7	30	10	77
1	5	100.0	50	10	112

LOQ

LOD

Codex alimentarius:
LOQ is at RSD = 25%
LOD is at RSD = 33%

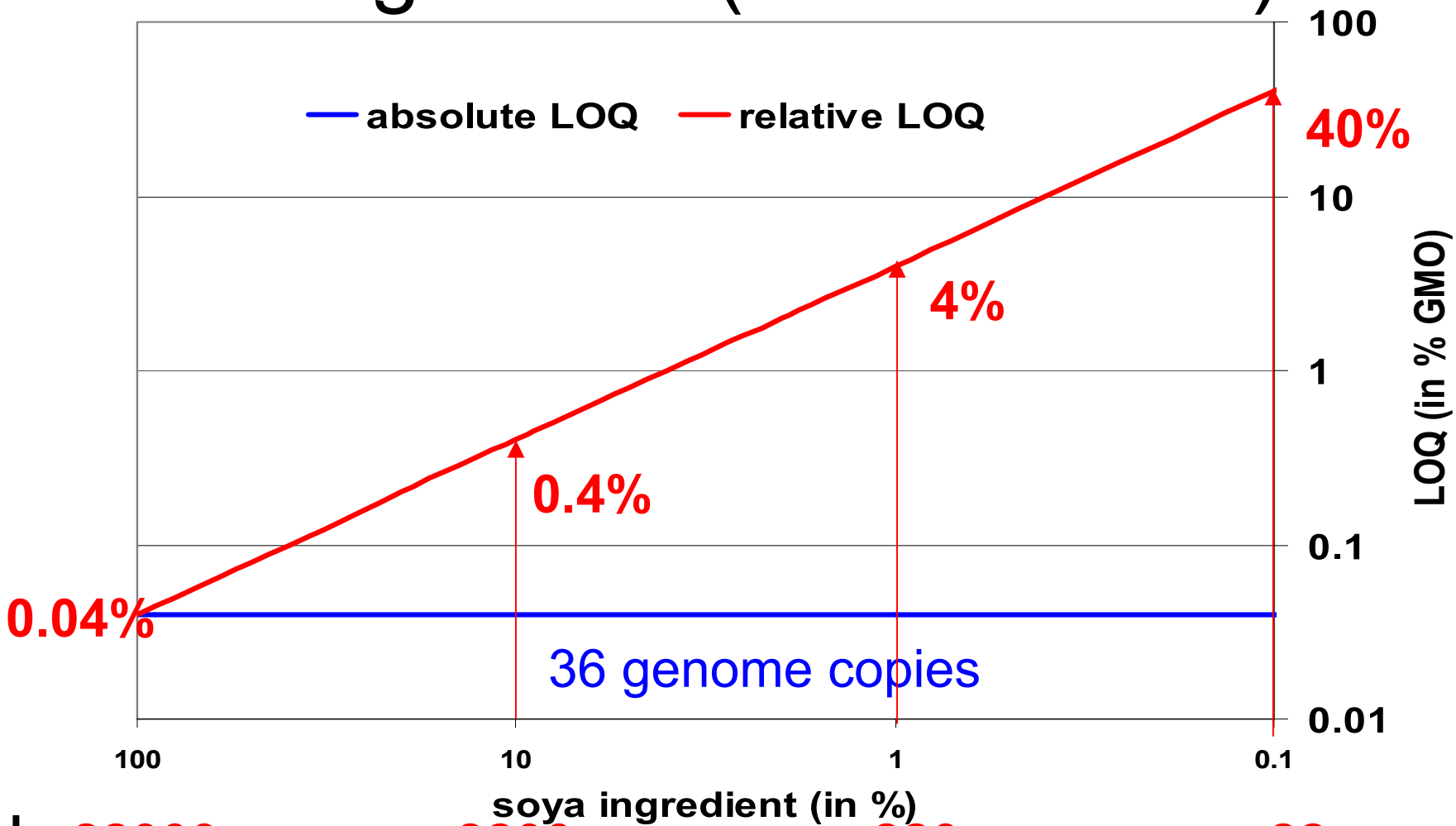
Influence of plant genome size on LOQ

common name	scientific name	genome size[a] [in Mia bp]	genome copies [per 200 ng]	1%	LOQ[b]
corn	***Zea mays***	5.0	36‘000	360	**0.1 %**
rice	*Oryza sativa*	0.9	210‘000	2100	0.02 %
soybean	***Glycine max***	2.2	82‘000	820	**0.04 %**
wheat	*Triticum aestivum*	31.9	6‘000	60	0.6 %

[a] published genome sizes (per 2C) were taken from Arumuganathan et al .

[b] the theoretical limit of quantitation is expressed as the fraction (in %) of **36 copies** divided by the number of copies of the corresponding plant species within 200 ng DNA

The LOQ depends on the amount of the ingredient (matrix match)



82000 8200 820 82 copies

Limit of Detection (LOD)
Limit of Quantification (LOQ)

- the sensitivity of the whole procedure can only be determined by analysing CRMs with low GMO contents in the range of 0.1% to 0.01%; at present CRMs with 0.1% GMO can be tested, all published methods reach this sensitivity
- the sensitivity of the rt-PCR part of the procedure can be assessed by measuring replicates of diluted GMO-DNA test samples. Attention: this sensitivity does not reflect the sensitivity of the whole procedure!
  - **LOQ: 30 to 50 gene copies**
  - **LOD: 10 to 20 gene copies**

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- General Quantitation Marker
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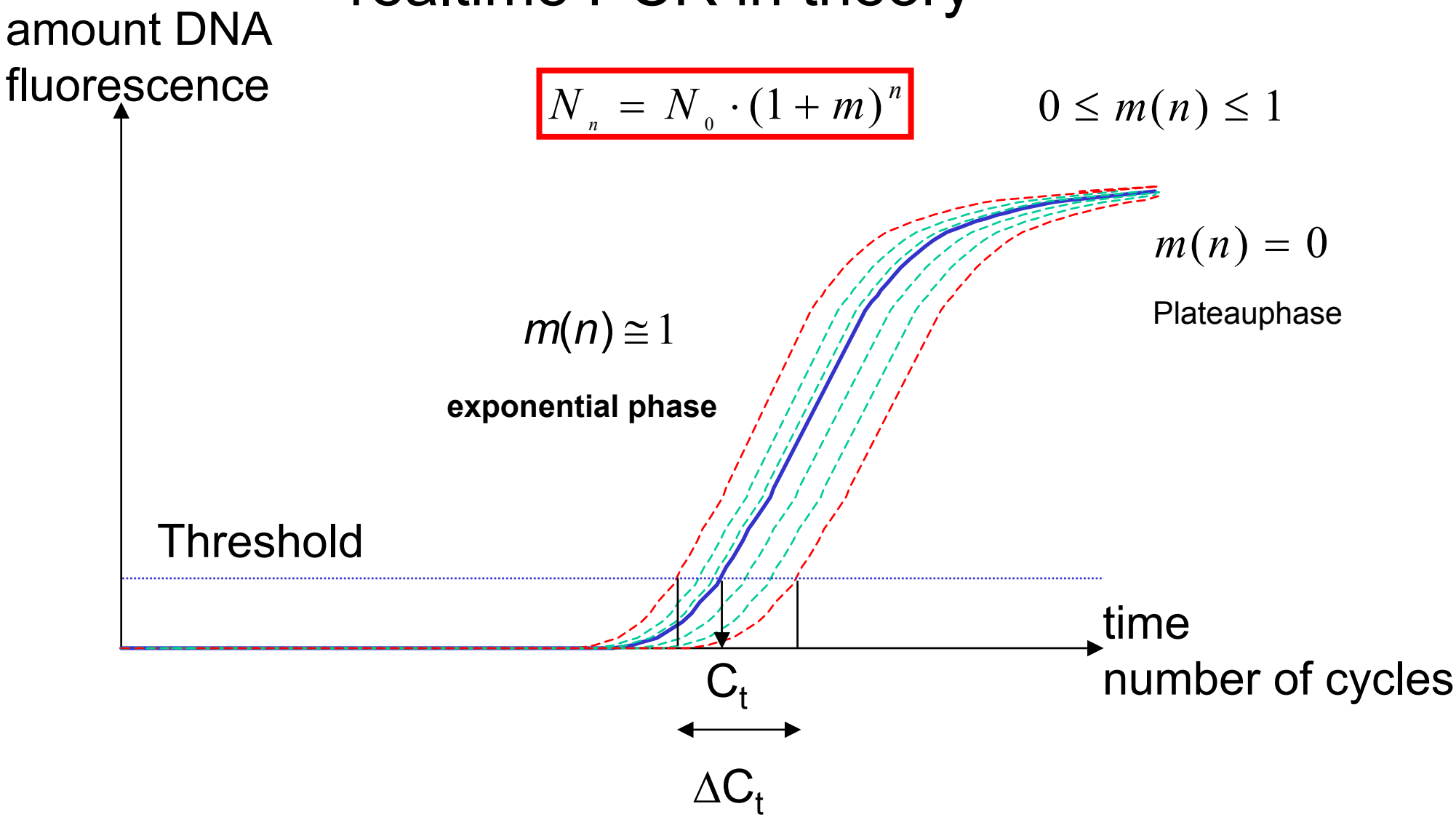
realtime PCR in theory



error calculus of realtime PCR

Which parameter has the greatest influence on the measured Ct-value (n)?

- PCR-efficiency m ("Biochemistry")
- pipetting error ΔN_0
- error of fluorescence measurement ΔN_n

law of error propagation by Gauss:

$$\Delta f(x) = \frac{\partial f(x)}{\partial x} \cdot \Delta x$$

(1) PCR-efficiency m (“biochemistry")

$$n = \frac{\ln(N_n / N_0)}{\ln(1+m)} \qquad \Delta n = \frac{\partial f(m)}{\partial m} \cdot \Delta m$$

$$\Delta n = \frac{n}{(1+m)} \cdot \frac{1}{\ln(1+m)} \cdot \Delta m$$

Δn (ΔC_t) is proportional to Δm and to the number of cycles n and inverse proportional to the mean PCR efficiency (1+m)

(2) pipetting error ΔN_0

$$n = \frac{\ln(N_n / N_0)}{\ln(1+m)} \qquad \Delta n = \frac{\partial f(N_0)}{\partial N_0} \cdot \Delta N_0$$

$$\Delta n = -\frac{1}{\ln(1+m)} \cdot \frac{\Delta N_0}{N_0}$$

Δn (ΔC_t) is proportional to the relative pipetting error $\Delta N_0/N_0$

(3) error of fluorescence determination ΔN_n

$$n = \frac{\ln(N_n / N_0)}{\ln(1+m)} \qquad \Delta n = \frac{\partial f(N_n)}{\partial N_n} \cdot \Delta N_n$$

$$\Delta n = \frac{1}{\ln(1+m)} \cdot \frac{\Delta N_n}{N_n}$$

Δn (ΔC_t) is proportional to the relative error of the fluorescence measurement $\Delta N_n / N_n$

what are the contributions of the different terms?

$\frac{\Delta N_0}{N_0}$ relative pipetting error: 2.5%

$\frac{\Delta N_n}{N_n}$ relative error of fluorescence measurement:
1 to 10% (depends on used apparatus)

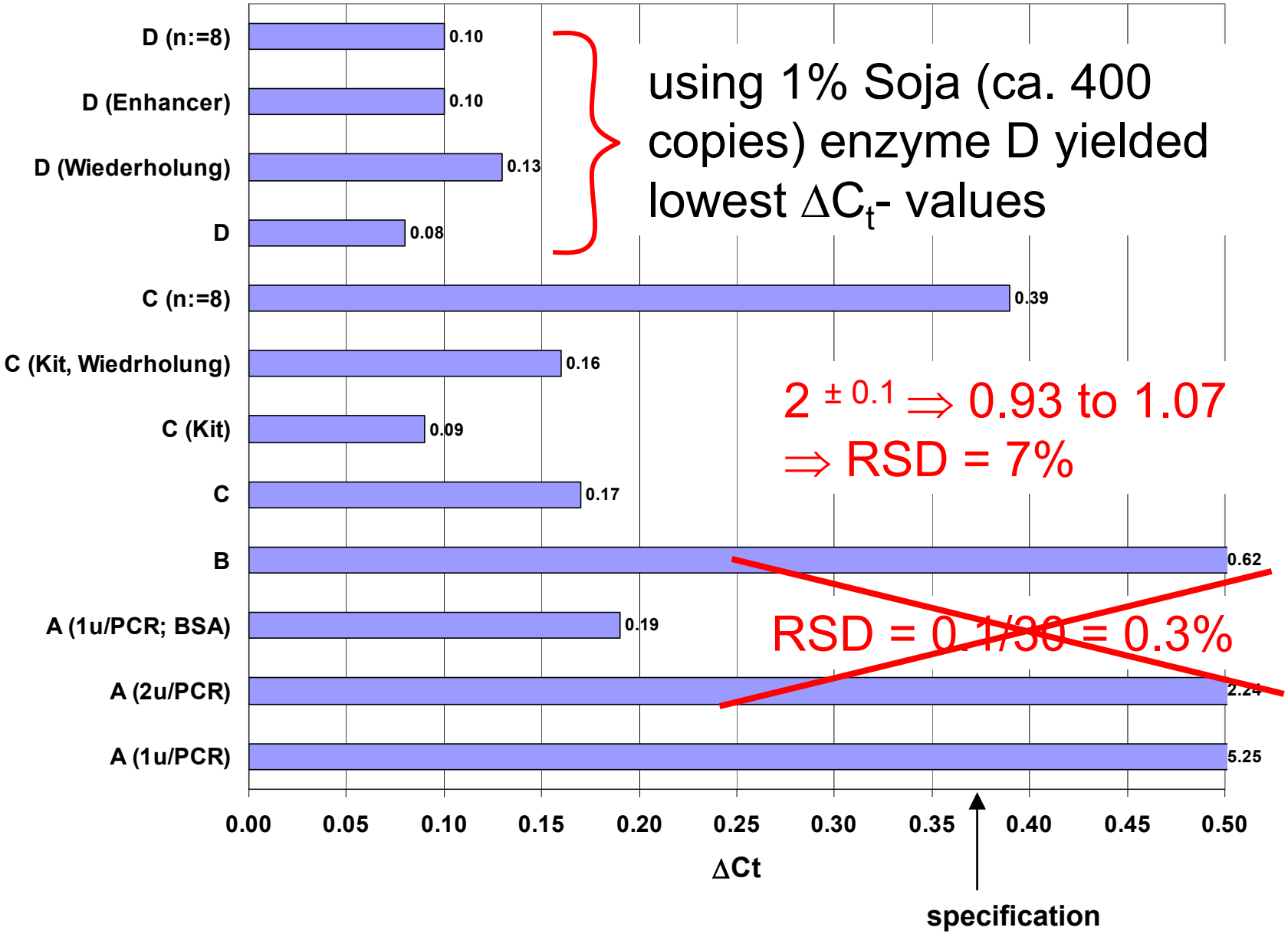
$\Delta n_{exp(specification)}$: 0.16 to 0.387

Δn_1	Δn_2	Δn_3	**1 PCR efficiency** **2 pipetting** **3 Fluorometer**
0.1 to 0.2	0.04	0.02 to 0.14	

$\Delta n_1 \geq \Delta n_3 > \Delta n_2$

The precision of the measurement is mainly determined by the factors „biochemistry“ and fluorescence measurement

Influence of Taq DNA-Polymerases on ΔCt



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Unique identifiers for authorized GMOs
Commission Regulation (EC) No 65/2004

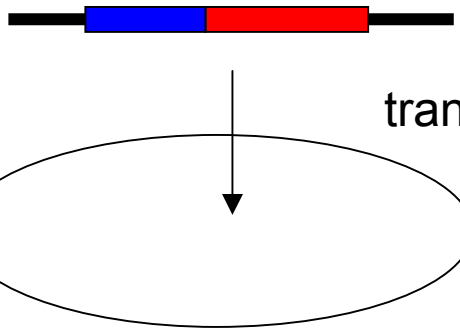
Applicants shall, in accordance with the formats set out in the Annex, develop the unique identifier for each GMO concerned, ...

- Standardised code containing 9 alphanumerical signs
- 3 components
  - A. Identification of applicant (2-3 signs)
  - B. Definition of event (5-6 signs)
  - C. Control of code (1 sign)

Example: Roundup Ready Soja **MON-04032-6**

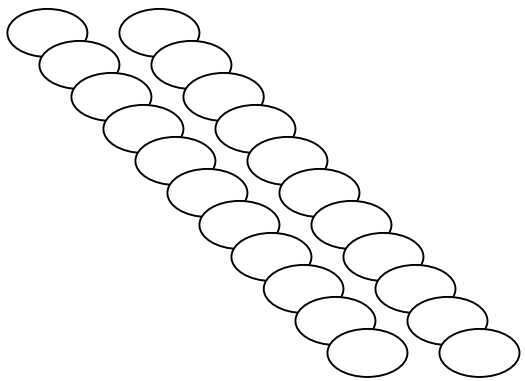
A B C

Detection of *Saccharomyces cerevisiae* carrying ID-tags



transformation of yeast

cultivation of GM-yeast



baking bread

Isolation of DNA

realtime PCR

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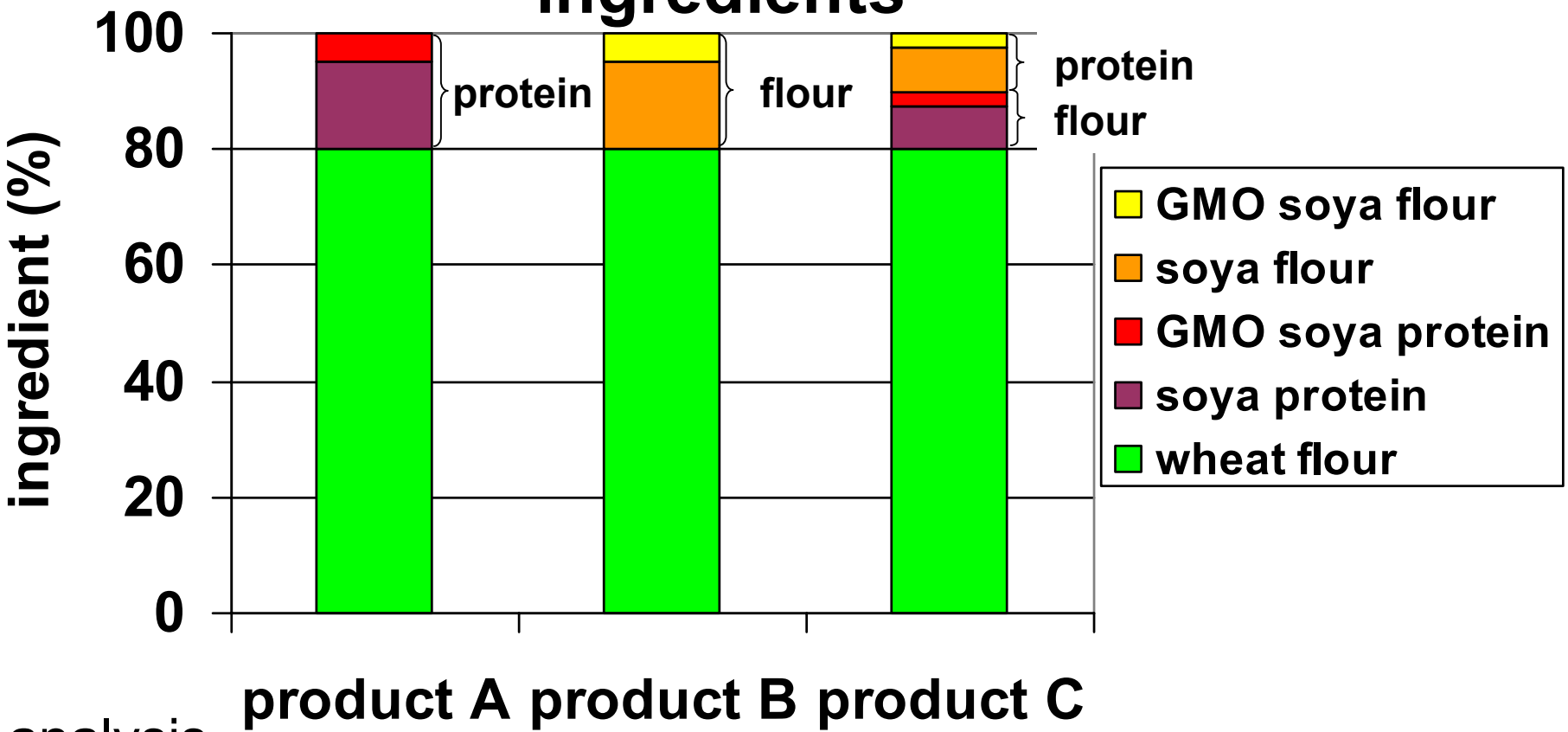
Limitation: composed food with several ingredients



	product A	product B	product C
GMO analysis per ingredient	possible	possible	not possible

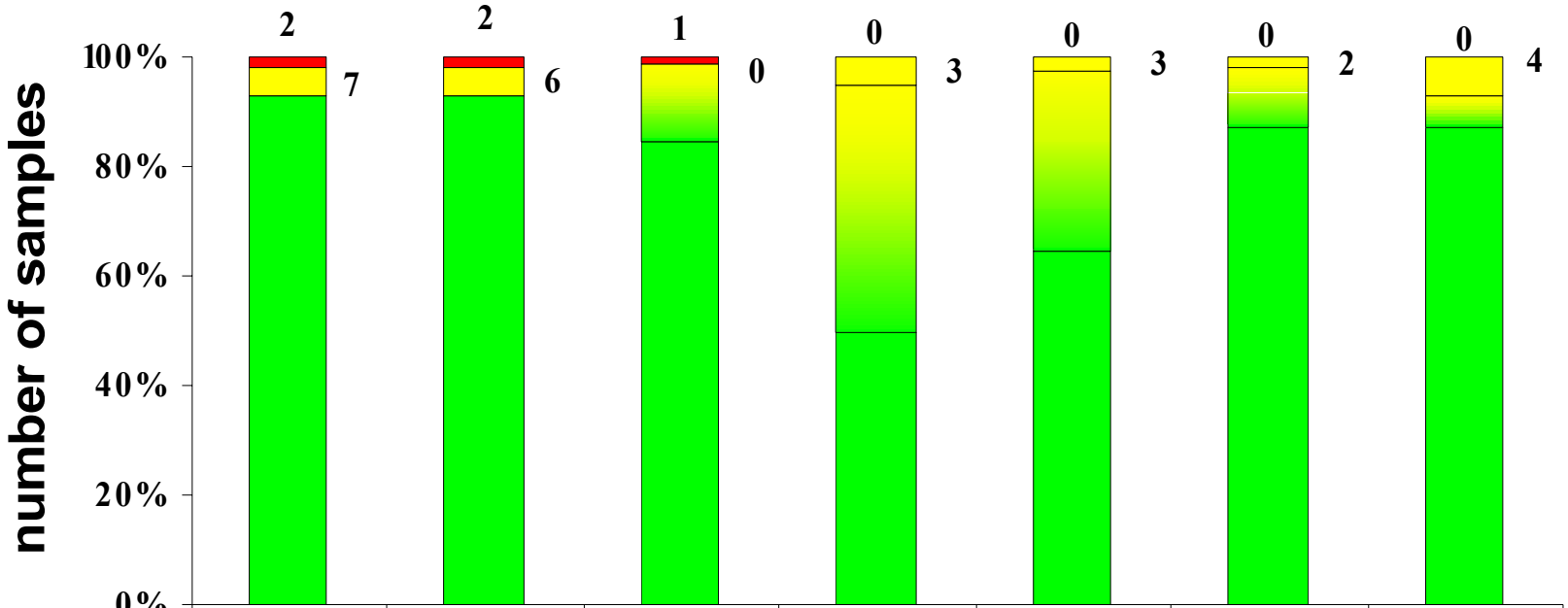
Limits of GMO analytics

- Availability of reference materials (quantitative and qualitative)
- sample matched LOQ (practical LOQ)
- Products with several ingredients derived from corn or soya
- Purified („DNA-free“) ingredients or products

GMO-ID-Tag

- fast and reliable screening, identification and quantitation of GMOs
- no cross reactivity
- Analysis without reference material gets possible
- amplifiable yeast-DNA could be extracted from bread

GMO-Analyses at KL BS



	1998 n = 122	1999 n = 111	2000 n = 77	2001 n = 56	2002 n = 119	2003 n = 91	2004 n = 54
positive (>1%)	2	2	1	0	0	0	0
positive (0.1%<x<1%)	7	6	0	3	3	2	4
positive (<0.1%)			11	25	39	10	3
negative	113	103	65	28	77	79	47