



Bayer CropScience

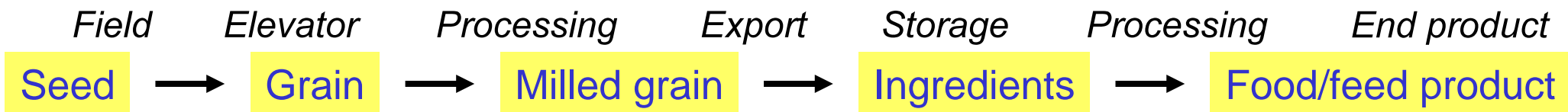
BioScience

GM product labeling in Europe:

Development of Quantitative Detection Methods

for 11 Bayer GM events

Food / feed supply chain



Some current issues

- Co-existence of GM & nonGM products
- Regulations (global, continental, national, ...)
- Detection methods (qualitative, quantitative, protein, DNA)
- Traceability
- Labeling

Regulations demanding GM product testing

	AP unauthorized GM events		Threshold authorized GM events
	Food	Feed	Food labeling
USA / Canada	0%	0%	none
Japan	0%	1%*	5%
Korea	0%	?	3%
China	0%	?	0.9% or 5% (TBD)
Taiwan	0%	?	5%
EU	0% / 0.5%**	0% / 0.5%**	0.9%

*If authorized in another OECD country;

** 0.5% for events with positive safety evaluation; temporary measure

Organizations involved with GM product testing

Seed companies

- Seed testing:
 - Purity testing
 - Adventitious presence of **undesired** GM events

Grain handlers, food/feed companies

- Food / feed regulations: - Adventitious presence of **unauthorized** GM events
- Labeling regulations: - Adventitious presence of **authorized** GM events
- Traceability regulations: - Identity of **authorized** GM events

Commercial testing laboratories

- Seed & food/feed testing
(Commercial testing tools: primarily protein tests)

Government agencies, enforcement laboratories

- Test compliance to regulations
- National or regional (e.g. ENGL, ISTA)
- Seed & food/feed testing

Testing tools for detection of GM events

Protein detection methods: Immunological

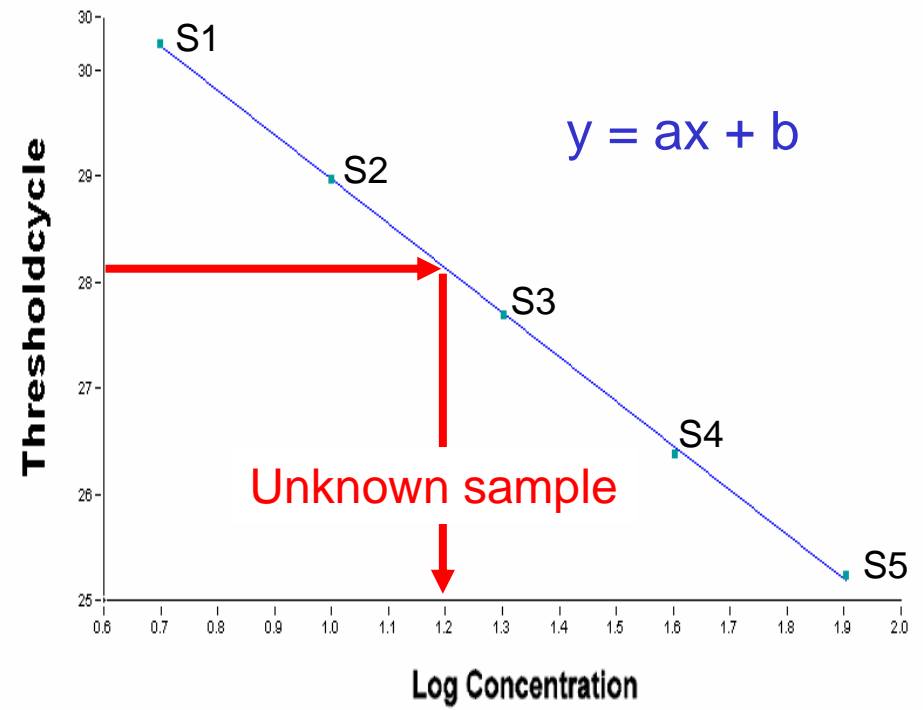
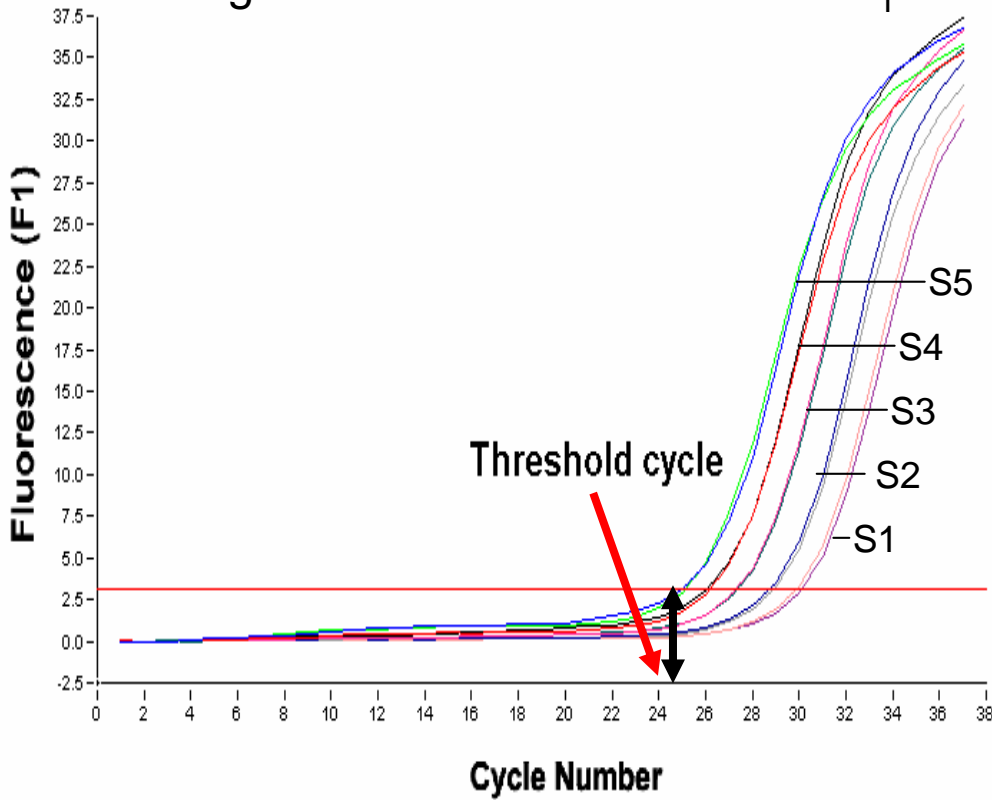
- Enzyme Linked Immunosorbent Assay (ELISA)
- Lateral flow strip

DNA detection methods: Polymerase Chain Reaction (PCR)

- Qualitative methods: End-point PCR
- Semi-quantitative methods (grain): Sub-sampling
- Quantitative methods: Real-time PCR

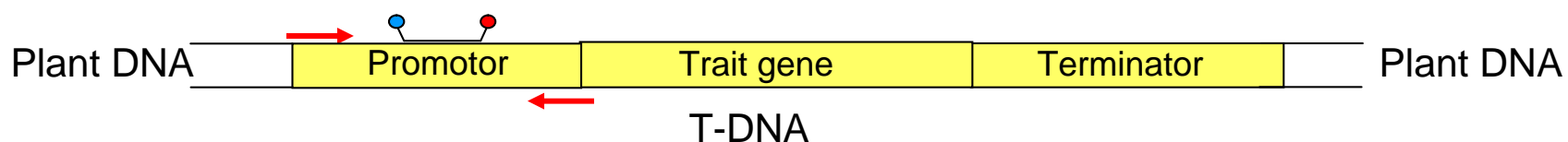
Real-time PCR: standard curve

Target DNA concentration $\approx 1 / C_T$

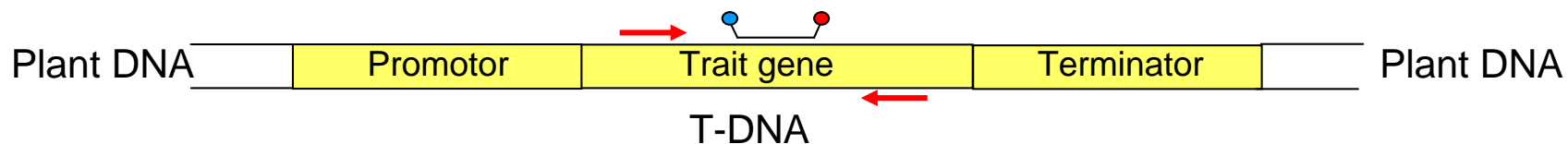


GM event PCR tools (end-point / Real-time)

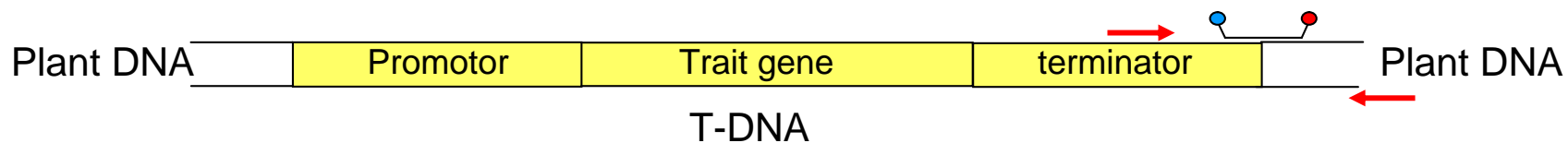
Generic methods



Trait- or construct-specific methods



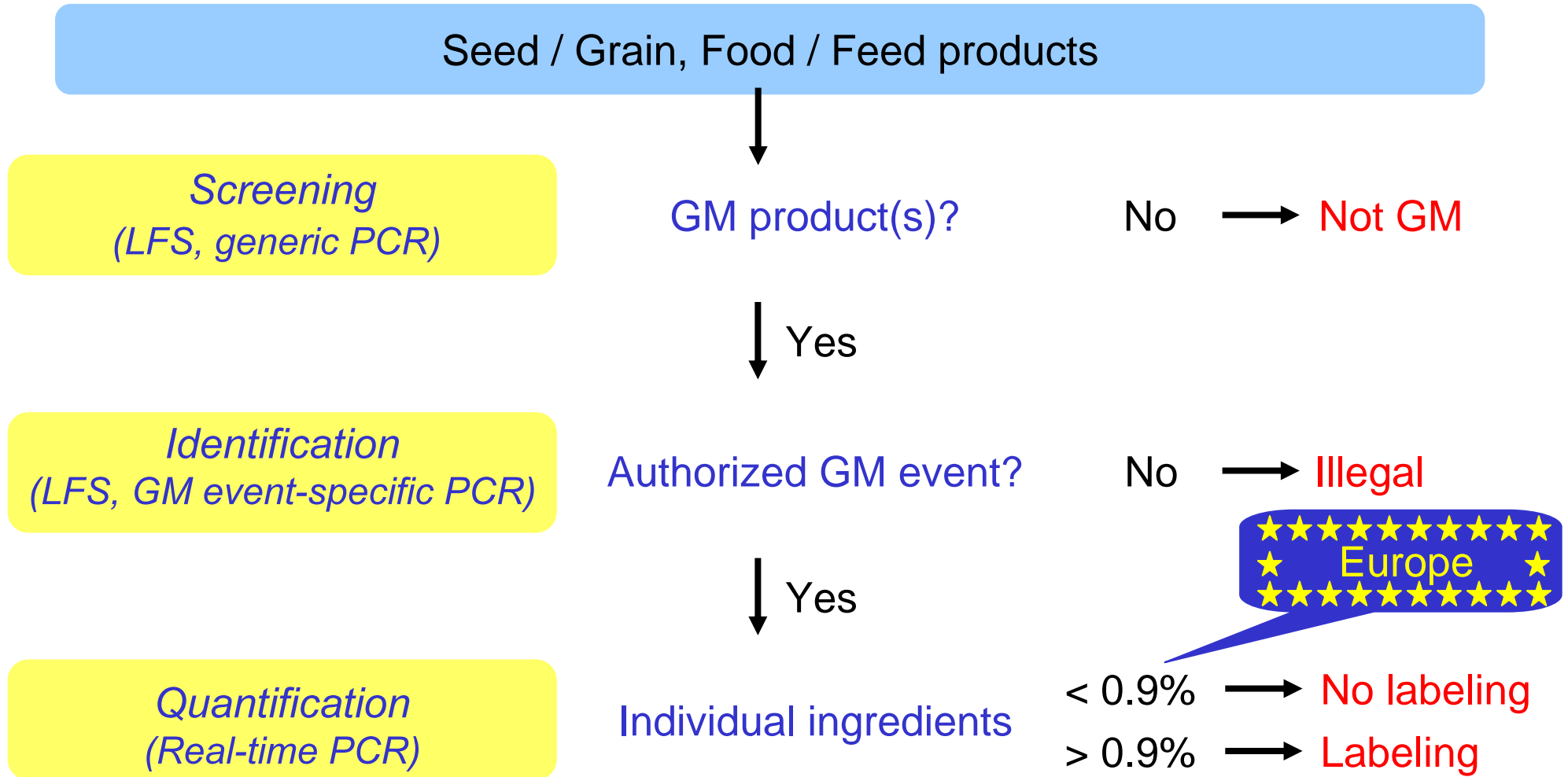
GM event-specific methods



Application of GM detection tools

	Seed / Grain	Food / Feed
1. Screening	<ol style="list-style-type: none"> 1. LFS 2. Generic PCR (P35S,Tnos) 3. Trait-specific PCR 	<ol style="list-style-type: none"> 1. LFS? 2. Generic PCR (P35S,Tnos) 3. Trait-specific PCR
2. Identification	<ol style="list-style-type: none"> 1. LFS 2. GM event-specific PCR 	<ol style="list-style-type: none"> 1. LFS? 2. GM event-specific PCR
3. Quantification	Sub-sampling with LFS or Event-specific PCR (semi-quantitative)	Not applicable
	GM event-specific Real-time PCR	GM event-specific Real-time PCR

Summary GM product testing



Individual testing laboratories

Regulatory clarity and harmonization of testing criteria

- Interpretation of regulations
 - ⇒ Inconsistent results communicated to clients

Officially validated testing methods

- Development of in-house methods based on 'public' information
 - ⇒ Incorrect service to clients (incorrect results; cost increase)

Certified reference materials and proper working standards

- In-house working standards without comparison to reference material
 - ⇒ Inconsistent results between laboratories

Interaction with technology developers

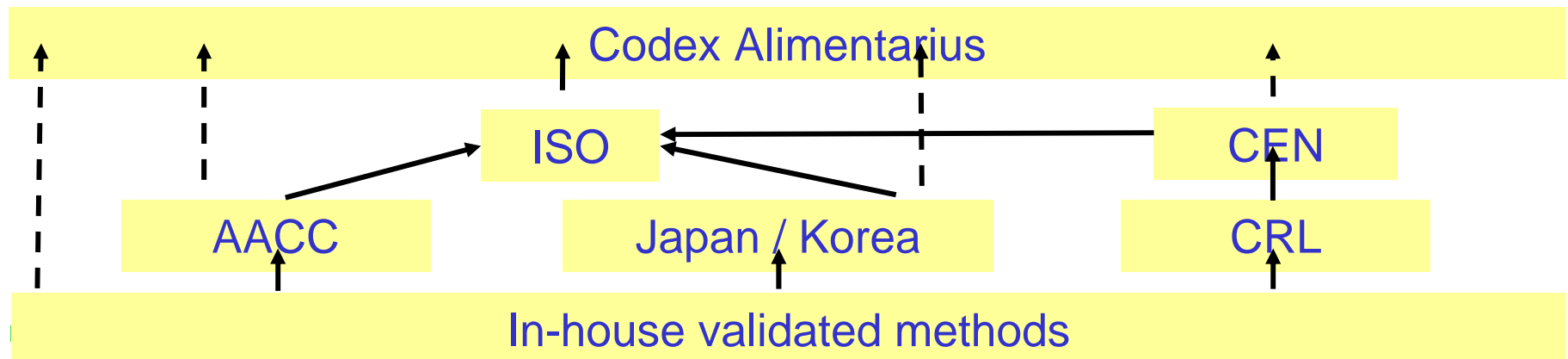
Global harmonization of detection tools

Officially validated GM detection methods

- Provide consistent results throughout the food/feed supply chain
- Allow organized regulatory compliance throughout the food/feed supply chain
- Ensure global uniformity of units of measurement
- Reduce costs of repeated validation

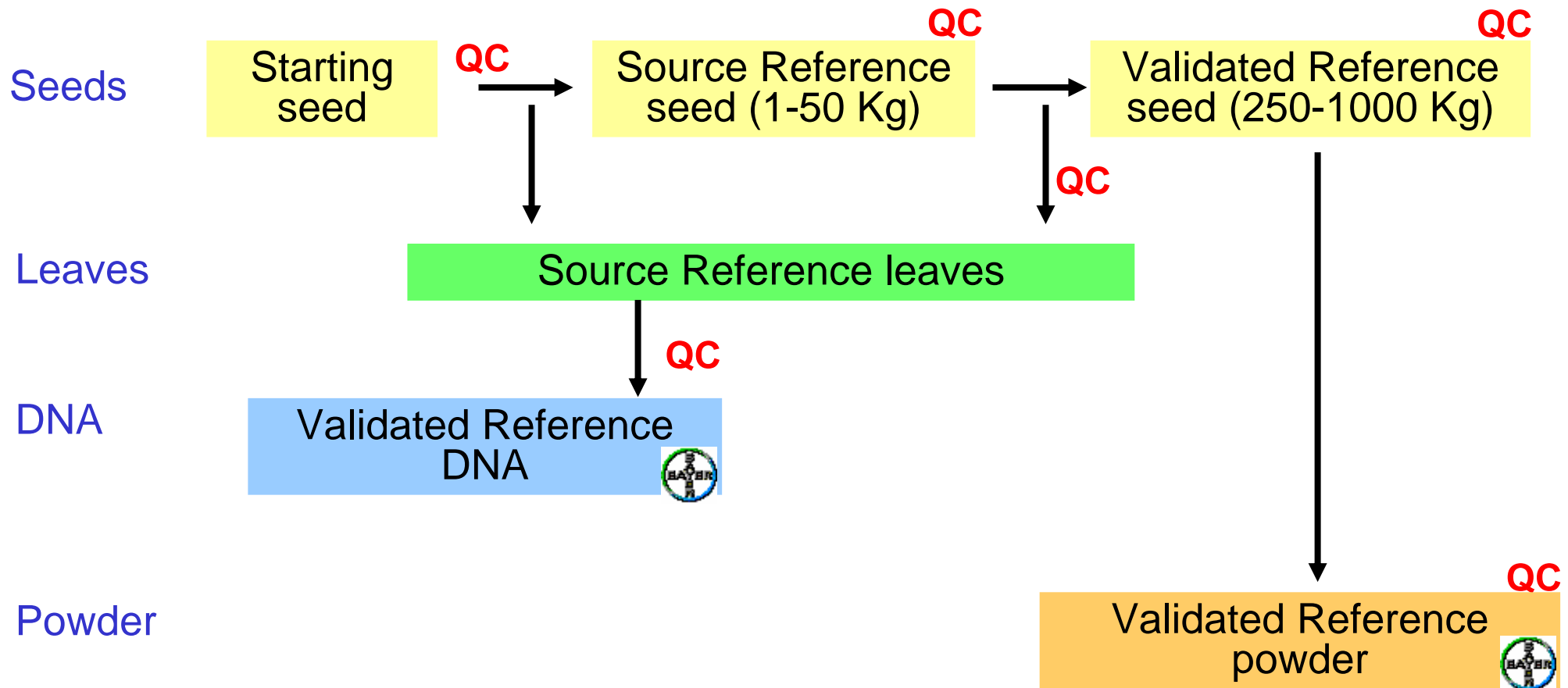
GM testing laboratories

- Accreditation to ISO Standard 17025
- Proficiency testing
- Internal quality control (traceability)
- Auditing by clients



Progress

Bayer Reference material



Bayer quality standards for Reference material

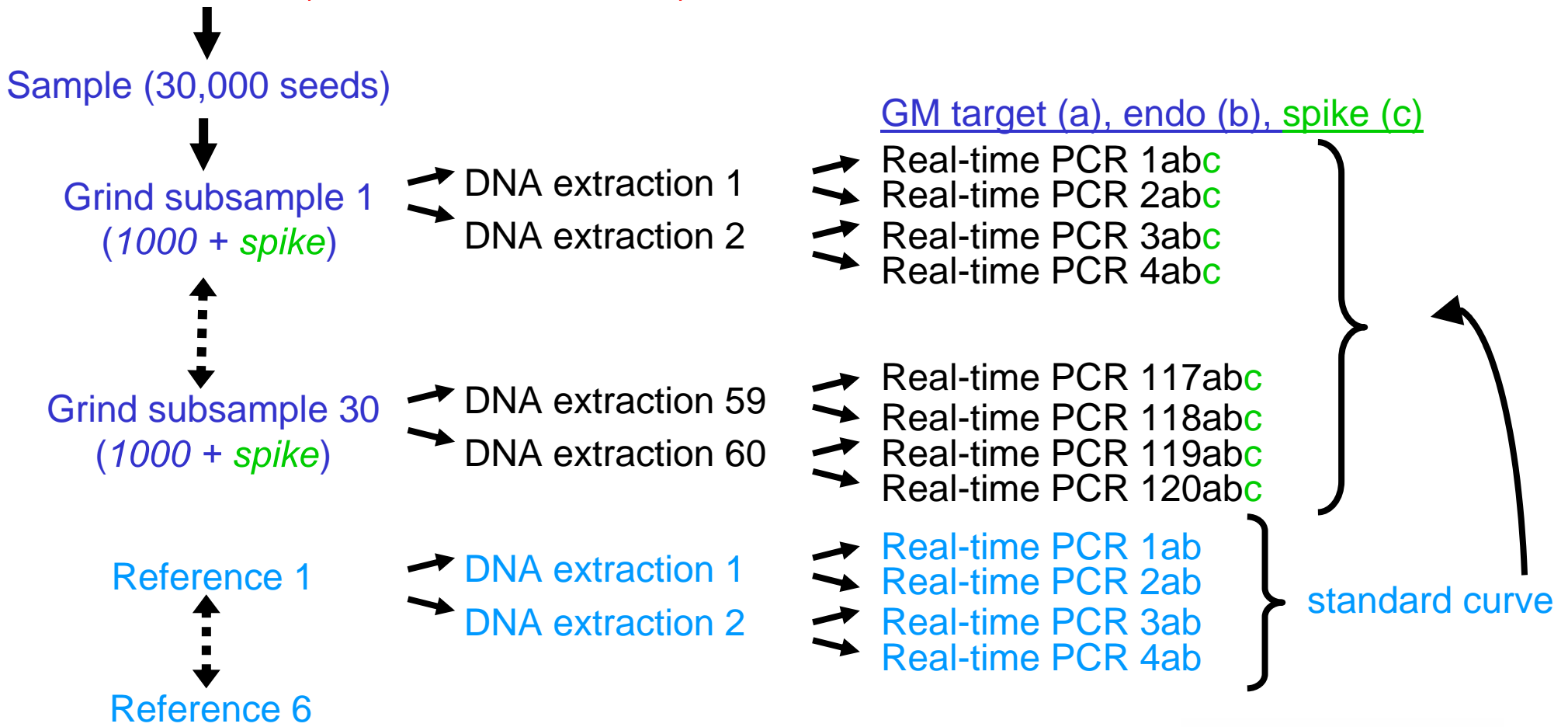
<i>Quality standard</i>	GM event	non-GM Isoline
GM event purity	> 99%	not applicable
Adventitious presence	< 0.01%	< 0.01%
GM event homozygosity	> 99%	not applicable
Genetic purity	> 95%	> 95%
Genetic background	Identical (> 95%)	

Procedures for GM seed testing



Overview semi-quantitative GM seed detection

Grain / seedlot (AP <0.01%, P=95%)



EC regulations

European Commission

EC Regulation 1829/2003

- Regulates the placing on the market of GM food / feed products
- **Methods for sampling, GM detection and identification, control samples**
- Applied from April 18, 2004

Community Reference Laboratory (CRL)

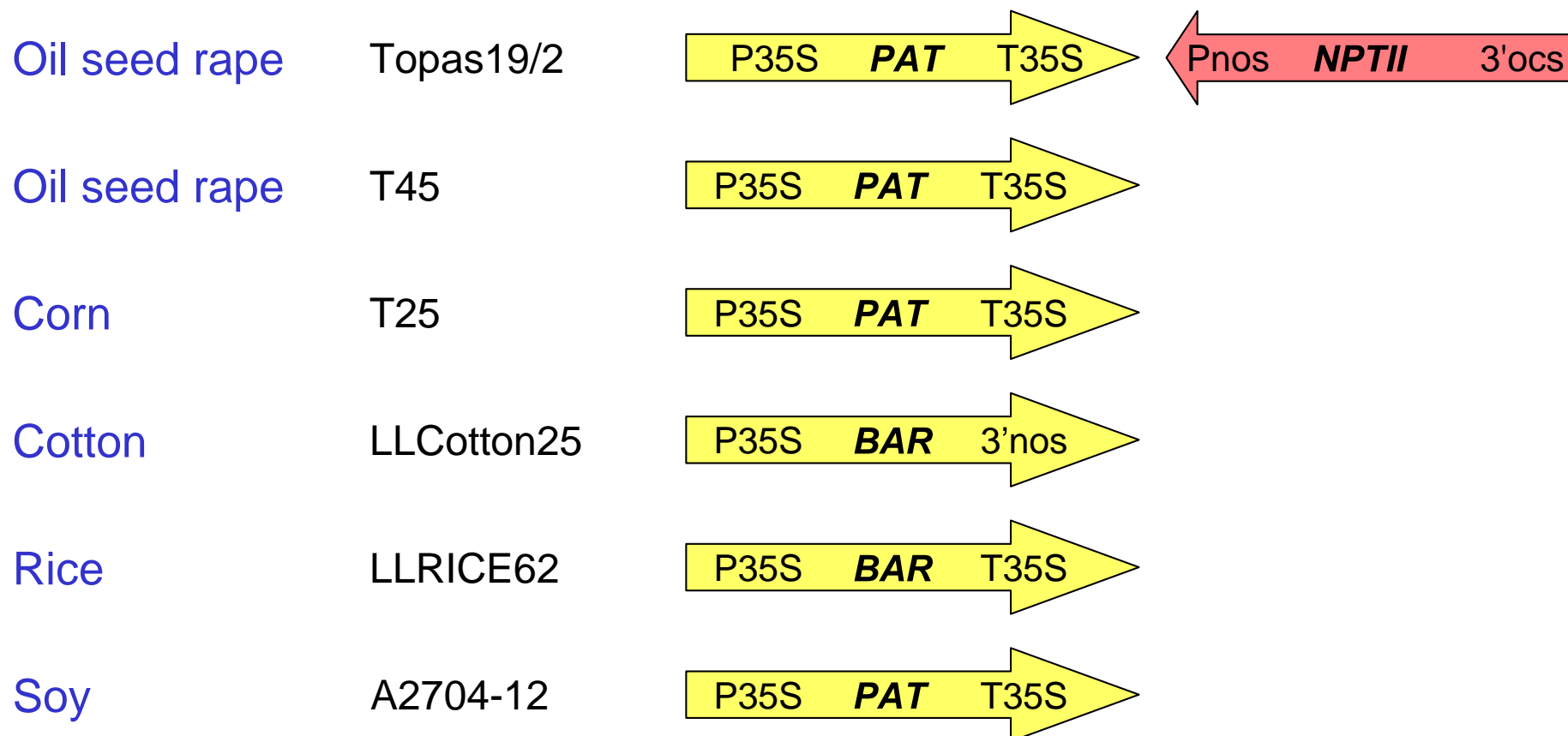
European Network of GMO laboratories (ENGL)

European criteria for quantitative GM detection

1. GM event-specific method
2. Modular method development approach
 - Separate DNA extraction & detection methods
3. Working range for European threshold level
 - 0.9% for approved events
4. Strict method performance & acceptance criteria
 - External pre-validation of the method (2 - 4 labs)
 - Independent validation of the method (8 - 12 ENGLabs)
5. Control samples
 - 200 µg GM DNA & 4,000 µg non-GM DNA
 - 1% GM seeds (4 Kg)

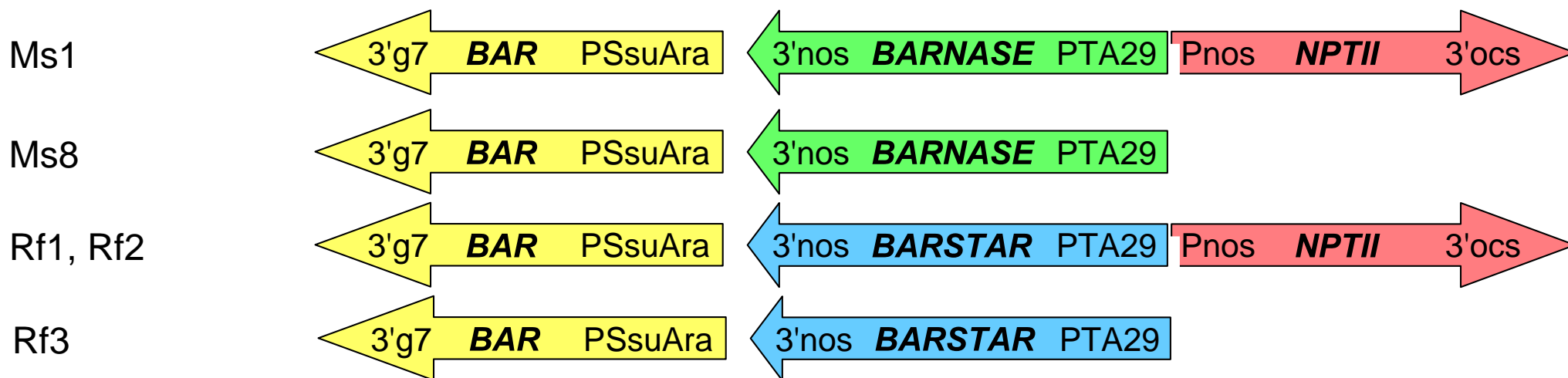
EC regulations

LibertyLink™ products



EC regulations

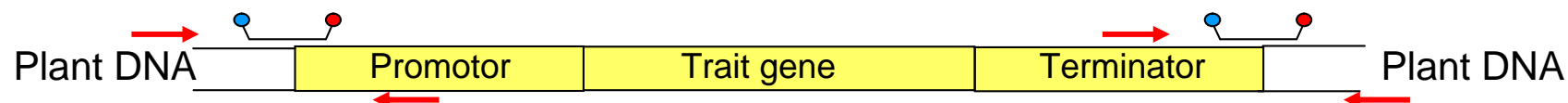
Oil seed rape *SeedLink™* products



Method development parameters

1. Applicability
 2. Practicability
 3. Robustness
 4. Specificity
 5. Stability
 6. Detection limit
 7. Quantification limit
 8. Dynamic range
 9. Sensitivity
 10. Accuracy: trueness, precision
 11. Repeatability
 12. Reproducibility
- Single laboratory validation
- Multiple laboratory validation
-
- The diagram shows a list of 12 parameters. A bracket on the right side of the list groups parameters 1 through 7 under the label 'Single laboratory validation'. A second bracket on the right side groups parameters 8 through 12 under the label 'Multiple laboratory validation'.

Method specificity



		Ms1	Ms8	Rf1	Rf2	Rf3	T45	To19/2	T25	LL62	LL25	A2704-12	MON810	Bt11	Bt176	GA21	NK603	CBH351
OSR	Ms1	+
OSR	Ms8	.	+
OSR	Rf1	.	.	+
OSR	Rf2	.	.	.	+
OSR	Rf3	+
OSR	T45	+
OSR	Topas19/2	+
Corn	T25	+
Rice	LLRICE62	+
Cotton	LLCotton25	+
Soy	A2704-12	+

Method stability testing: $\Delta\Delta\text{Ct}$ -method

Stability of method in different commercial backgrounds

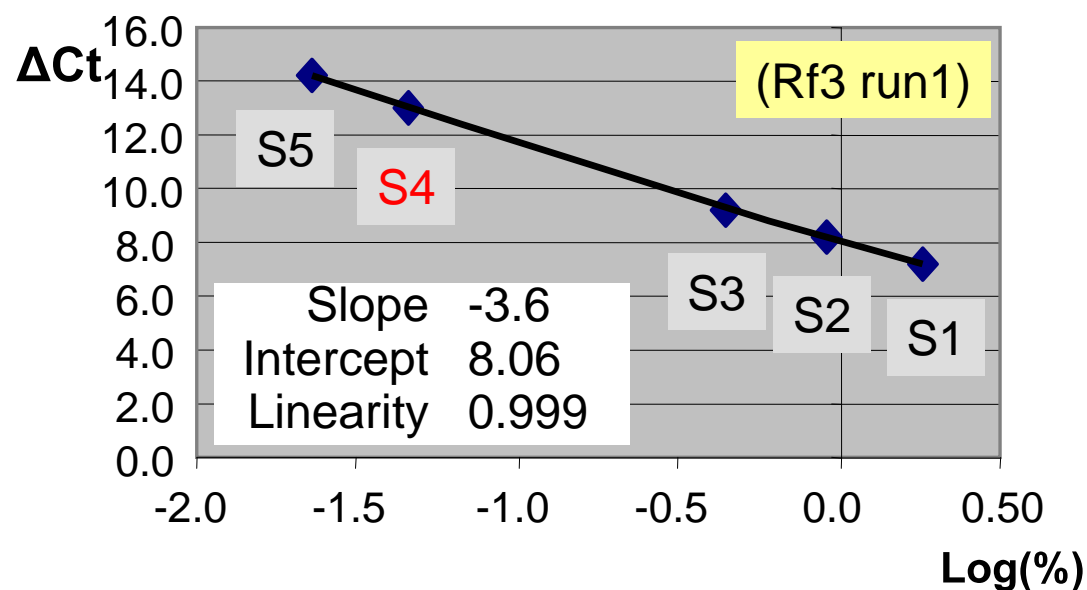
- Equal copynumber endogenous control gene (e.g. corn *Adh1*)

T25 variety	Variety descriptor	Copy ($\Delta\Delta\text{-Ct}$)
TR3026LL x TR1968	Dent x Flint early maturity hybrid	1.00
TR3026 x TR1957	Stiff stalk x Flint / Iodent ultra-early maturity hybrid	1.06
TR3026LL x TR1984	Stiff stalk x unrelated non-stiff stalk early maturity hybrid	0.88
TR3621LL x TR1017	Iodent/OH43 x Stiff stalk early maturity hybrid	1.03
SGI822LL x TR4563	Stiff stalk / Lancaster x Mo17 / Iodent / OH43 hybrid	0.89
FR1064LL1.2 x FR2108	Stiff stalk x OH43 mid-early maturity hybrid	0.93
FR1064LL1.2 x FR9661	Stiff stalk x Unrelated mid-early maturity hybrid	0.79
TR7245 x TR3622LL	Stiff stalk x OH43 early maturity hybrid	0.88
TR4006LL x FR4901	Stiff stalk x Unrelated Stiff Stalk mid maturity hybrid	0.99

EC regulations

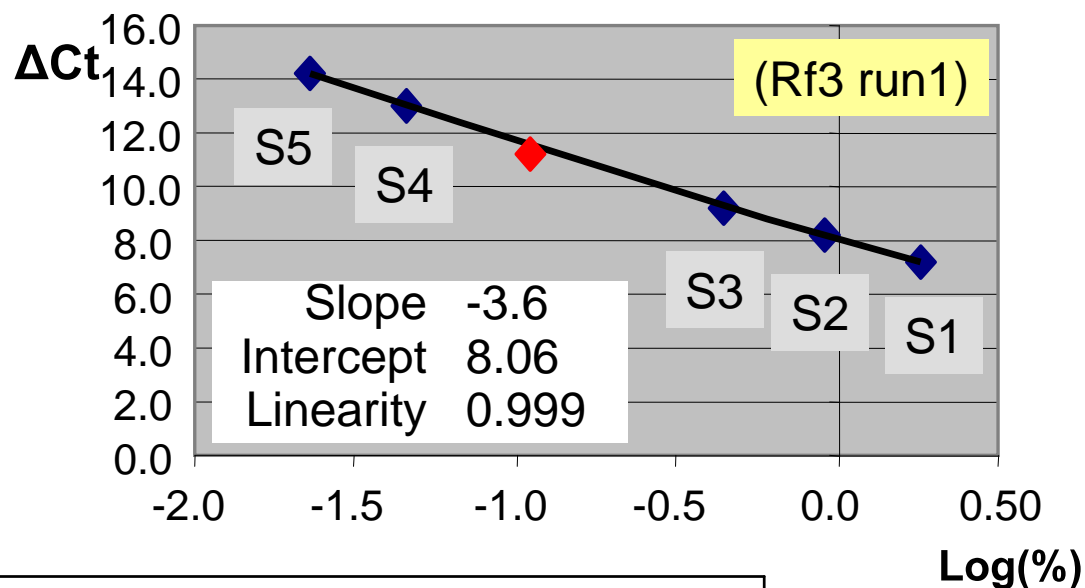
Detection limit: $< 1/20^{\text{th}}$ of 0.9% value = 0.045%

Sample	%	Log(%)	ΔCt
S1	1.80	0.25	7.18
S2	0.90	-0.04	8.22
S3	0.45	-0.34	9.24
S4	0.045	-1.34	13.05
S5	0.023	-1.64	14.17



Quantification limit: $< 1/10^{\text{th}}$ of 0.9% value = 0.09%

Sample	%	Log(%)	ΔCt
S1	1.80	0.25	7.18
S2	0.90	-0.04	8.22
S3	0.45	-0.34	9.24
S4	0.045	-1.34	13.05
S5	0.023	-1.64	14.17



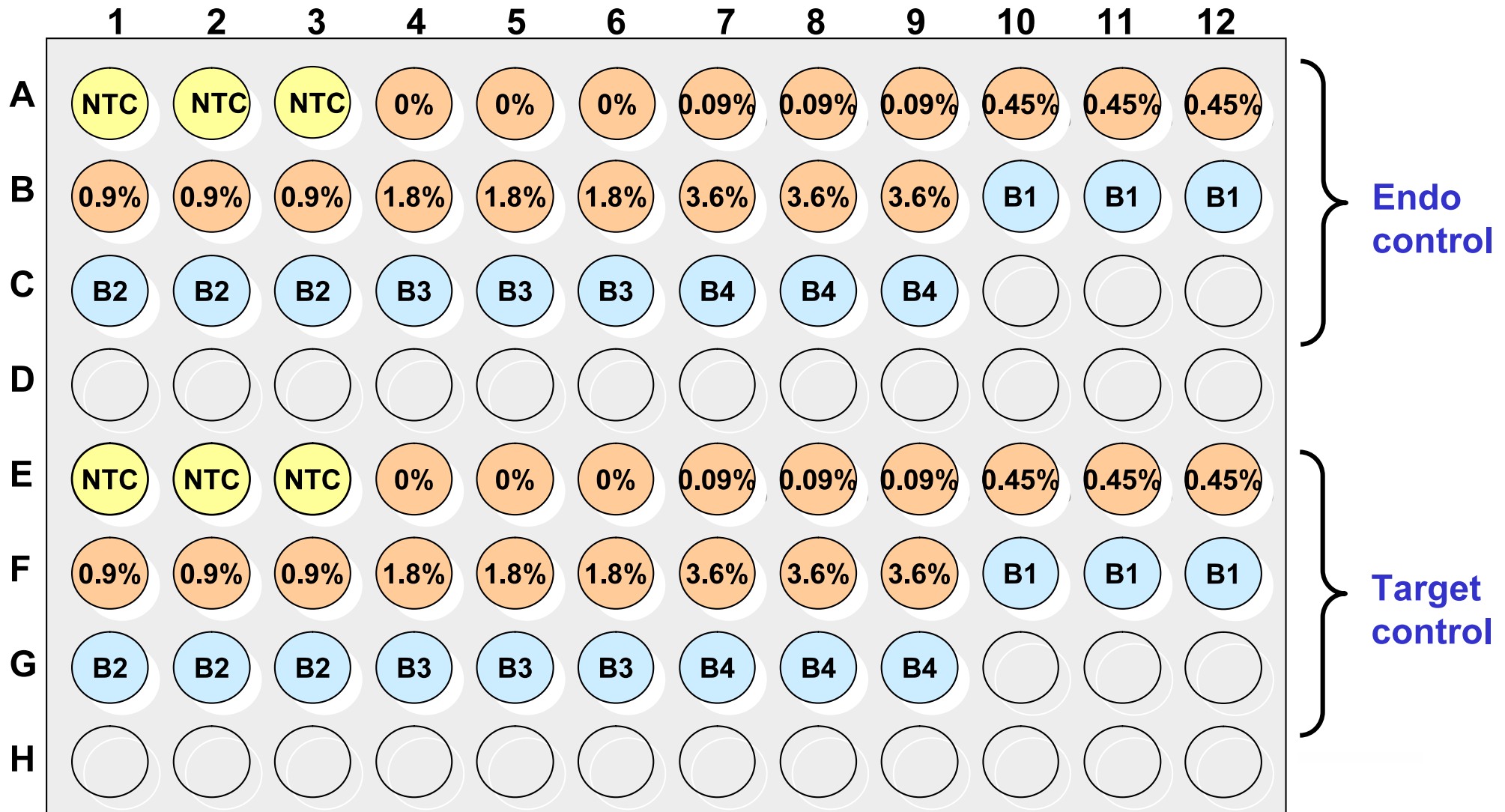
0.09% Blind sample					
			Precision		Trueness
ΔCt	Estimated %	Mean %	SD	%SD	%Error
11.90	0.091	0.105	0.013	12.17	17.0
11.51	0.117				
11.65	0.107				

External method pre-validation

Materials send to 2-4 external laboratories:

1. Six Reference samples: standard curve
 - 0%, 0.09%, 0.45%, 0.9%, 1.8%, 3.6% GM in non-GM (constant 200 ng)
2. Four blind samples
 - 0.3%, 0.6%, 1.2%, 2.4% GM in non-GM (constant 200 ng)
3. Reagents: primers, probes,
4. Complete protocol, calculation template (spreadsheet)

Pre-validation: Microtiter plate lay out for RT-PCR

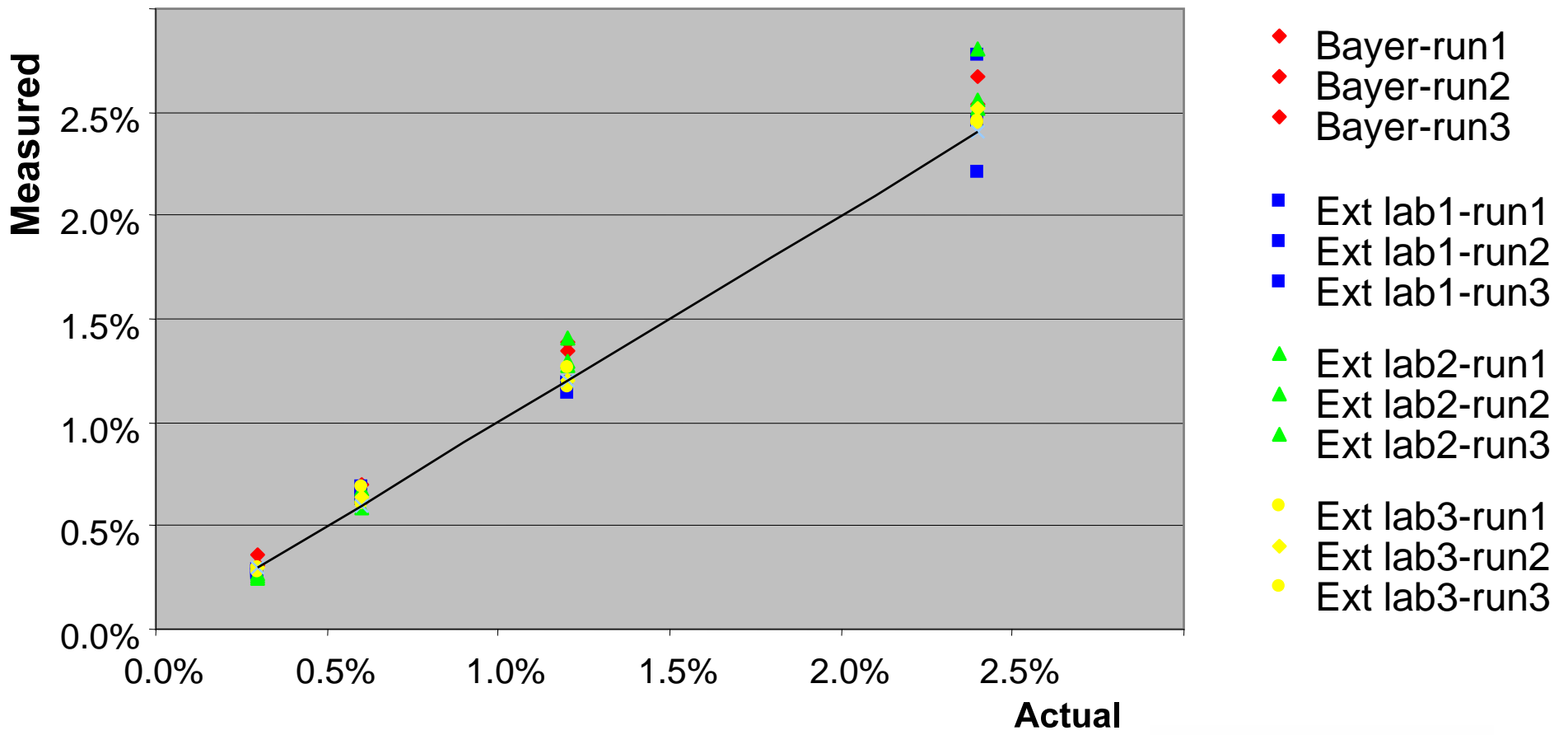


External pre-validation result spreadsheets

Reference Samples Blind Samples					Target			Control			Normalized						
#	Well	Sample	True %	log(%)	Ct	Average ± Ct	SD	Ct	Average ± Ct	SD	dCt	Normalized					
1	1	NTC	H ₂ O		45.00	45.00	0.00	45.00	45.00	0.00	0.00		<p>Slope (a) -3.35</p> <p>Intercept (b) 5.8</p> <p>Linearity (R²) 0.9946</p>	<p>Estimated</p> <p>Mean</p> <p>Precision</p> <p>Trueness</p>			
	2	NTC			45.00			23.29			21.71				<p>SD</p> <p>±SD</p> <p>±Error</p>		
	3	NTC			45.00			23.26			9.23						
2	4	Ref0.0%	0.00		45.00	45.00	0.00	23.33	23.33	0.04	6.21						
	5	Ref0.0%	0.00		45.00	45.00	0.00	23.31	23.33	0.03	4.82						
	6	Ref0.0%	0.00		32.41	32.56	0.14	23.36	23.35	0.02	3.89						
3	7	Ref0.09%	0.09	-1.05	32.59	32.56	0.14	23.32	23.35	0.03	7.57	0.299	<p>0.273</p> <p>0.534</p> <p>1.334</p> <p>2.664</p>	<p>0.024</p> <p>0.049</p> <p>0.062</p> <p>0.096</p>	<p>8.73</p> <p>9.24</p> <p>11.19</p> <p>11.00</p>		
	8	Ref0.09%	0.09		32.68	32.56	0.14	23.36	23.35	0.02	7.81	0.254					
	9	Ref0.09%	0.09		30.21	30.35	0.13	23.29	23.33	0.04	7.75	0.265					
4	10	Ref0.45%	0.45	-0.35	30.36	30.35	0.13	23.29	23.33	0.04	6.88	0.482				<p>0.024</p> <p>0.049</p> <p>0.062</p> <p>0.096</p>	<p>8.73</p> <p>9.24</p> <p>11.19</p> <p>11.00</p>
	11	Ref0.45%	0.45		30.36	30.35	0.13	23.33	23.33	0.04	6.72	0.538					
	12	Ref0.45%	0.45		30.47	30.35	0.13	23.36	23.35	0.02	6.61	0.581					
5	13	Ref0.9%	0.90	-0.05	29.79	29.55	0.21	23.35	23.34	0.02	5.47	1.265	<p>0.024</p> <p>0.049</p> <p>0.062</p> <p>0.096</p>	<p>8.73</p> <p>9.24</p> <p>11.19</p> <p>11.00</p>			
	14	Ref0.9%	0.90		29.44	29.55	0.21	23.31	23.35	0.01	5.34	1.383					
	15	Ref0.9%	0.90		29.42	29.55	0.21	23.35	23.35	0.01	5.37	1.355					
6	16	Ref1.8%	1.80	0.26	28.19	28.17	0.02	23.35	23.35	0.01	4.35	2.737			<p>0.024</p> <p>0.049</p> <p>0.062</p> <p>0.096</p>	<p>8.73</p> <p>9.24</p> <p>11.19</p> <p>11.00</p>	
	17	Ref1.8%	1.80		28.16	28.17	0.02	23.35	23.35	0.01	4.45	2.555					
	18	Ref1.8%	1.80		28.15	28.17	0.02	23.34	23.35	0.01	4.37	2.700					
7	19	Ref3.6%	3.60	0.56	27.18	27.16	0.02	23.28	23.27	0.02	7.57	0.299	<p>0.024</p> <p>0.049</p> <p>0.062</p> <p>0.096</p>	<p>8.73</p> <p>9.24</p> <p>11.19</p> <p>11.00</p>			
	20	Ref3.6%	3.60		27.15	27.16	0.02	23.28	23.27	0.02	7.81	0.254					
	21	Ref3.6%	3.60		27.16	27.16	0.02	23.25	23.27	0.02	7.75	0.265					
8	22	Bl-1	0.3	-0.52	30.88	31.02	0.12	23.33	23.31	0.02	6.21	0.581			<p>0.024</p> <p>0.049</p> <p>0.062</p> <p>0.096</p>	<p>8.73</p> <p>9.24</p> <p>11.19</p> <p>11.00</p>	
	23	Bl-1	0.3		31.12	31.02	0.12	23.29	23.31	0.02	5.47	1.265					
	24	Bl-1	0.3		31.06	31.02	0.12	23.31	23.35	0.01	5.34	1.383					
9	25	Bl-2	0.6	-0.22	30.18	30.04	0.14	23.27	23.30	0.03	4.35	2.737	<p>0.024</p> <p>0.049</p> <p>0.062</p> <p>0.096</p>	<p>8.73</p> <p>9.24</p> <p>11.19</p> <p>11.00</p>			
	26	Bl-2	0.6		30.02	30.04	0.14	23.32	23.30	0.03	4.45	2.555					
	27	Bl-2	0.6		29.91	30.04	0.14	23.32	23.35	0.01	4.37	2.700					
10	28	Bl-3	1.2	0.08	28.76	28.68	0.07	23.27	23.29	0.03	7.57	0.299			<p>0.024</p> <p>0.049</p> <p>0.062</p> <p>0.096</p>	<p>8.73</p> <p>9.24</p> <p>11.19</p> <p>11.00</p>	
	29	Bl-3	1.2		28.63	28.68	0.07	23.27	23.27	0.03	7.81	0.254					
	30	Bl-3	1.2		28.66	28.68	0.07	23.32	23.35	0.01	7.75	0.265					
11	31	Bl-4	2.4	0.38	27.56	27.60	0.05	23.27	23.21	0.11	6.21	0.581	<p>0.024</p> <p>0.049</p> <p>0.062</p> <p>0.096</p>	<p>8.73</p> <p>9.24</p> <p>11.19</p> <p>11.00</p>			
	32	Bl-4	2.4		27.66	27.60	0.05	23.28	23.21	0.11	5.47	1.265					
	33	Bl-4	2.4		27.58	27.60	0.05	23.08	23.21	0.11	5.34	1.383					

Pre-validation: Accuracy

Measured vs Actual Rf3 DNA amounts



Repeatability (RSD_r <30%) & Reproducibility (RSD_R <33%)

	RT-PCR Method	Repeatability (RSD _r)	Reproducibility (RSD _R)
OSR	Ms1	3.8	5.4
OSR	Ms8	6.2	6.7
OSR	Rf1	3.7	4.9
OSR	Rf2	3.6	5.0
OSR	Rf3	3.5	6.2
OSR	T45	5.5	8.0
OSR	Topas19/2	11.7	7.4
Corn	T25	3.8	6.5
Rice	LLRICE62	5.2	6.0
Cotton	LLCotton25	2.9	3.2
Soy	A2704-12		

Concluding remarks...

Successfully developed, optimized, characterized, and pre-validated
11 GM event-specific quantitative Real-time PCR methods in 5 crops

- Next step: CRL full validation through ENGLabs....

EC 1829/2003, EC1830/2003, EC 641/2004, & CRL guidance documents

- Provide guidelines for method development, validation, submission
- Modular validation approach (separating DNA extraction and PCR)
- Guidance documents allow some interpretation and flexibility
- Validated GM event-specific methods will become available
- Further experience will improve the technical implementation of regulations



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Thank you for your attention !