5-Day Theoretical and Practical Training Workshop on Laboratory Identification of Species, Screening of Living Modified Organisms and Detection of Plant Pathogens

Quantitative determination of living modified organisms, including new types of LMOs. Calculation and measurement uncertainty - theoretical training

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Outline

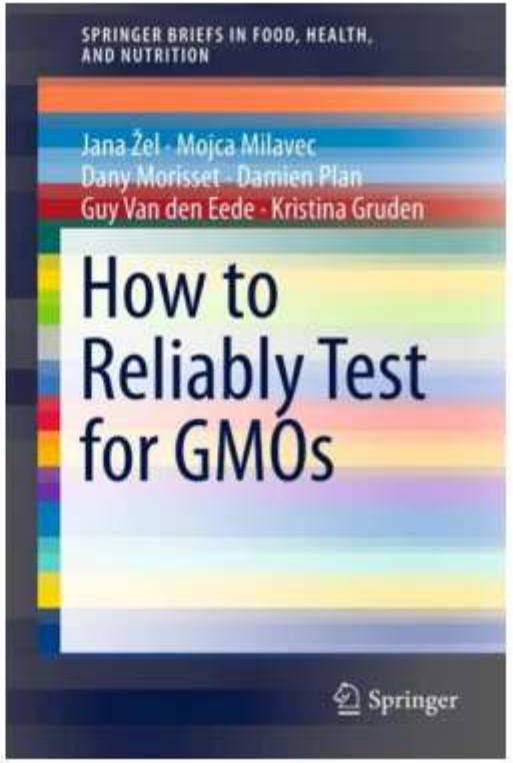
- Quantitative determination of LMOs
- Measurement uncertainty
- Detection of new types of LMOs

Quantitative determination of LMOs

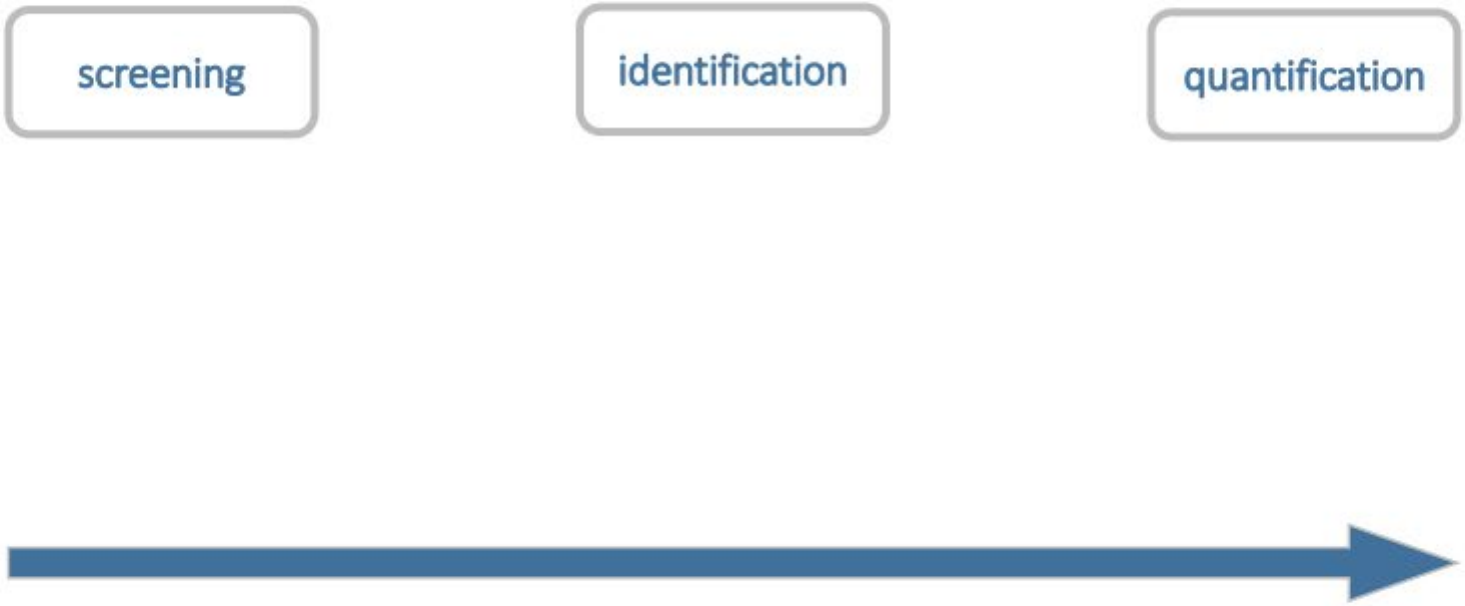
In some jurisdictions labeling of products composed of or containing GMOs is required.

Table 2 Labeling requirements in different countries adapted from Gruere and Rao (2007)

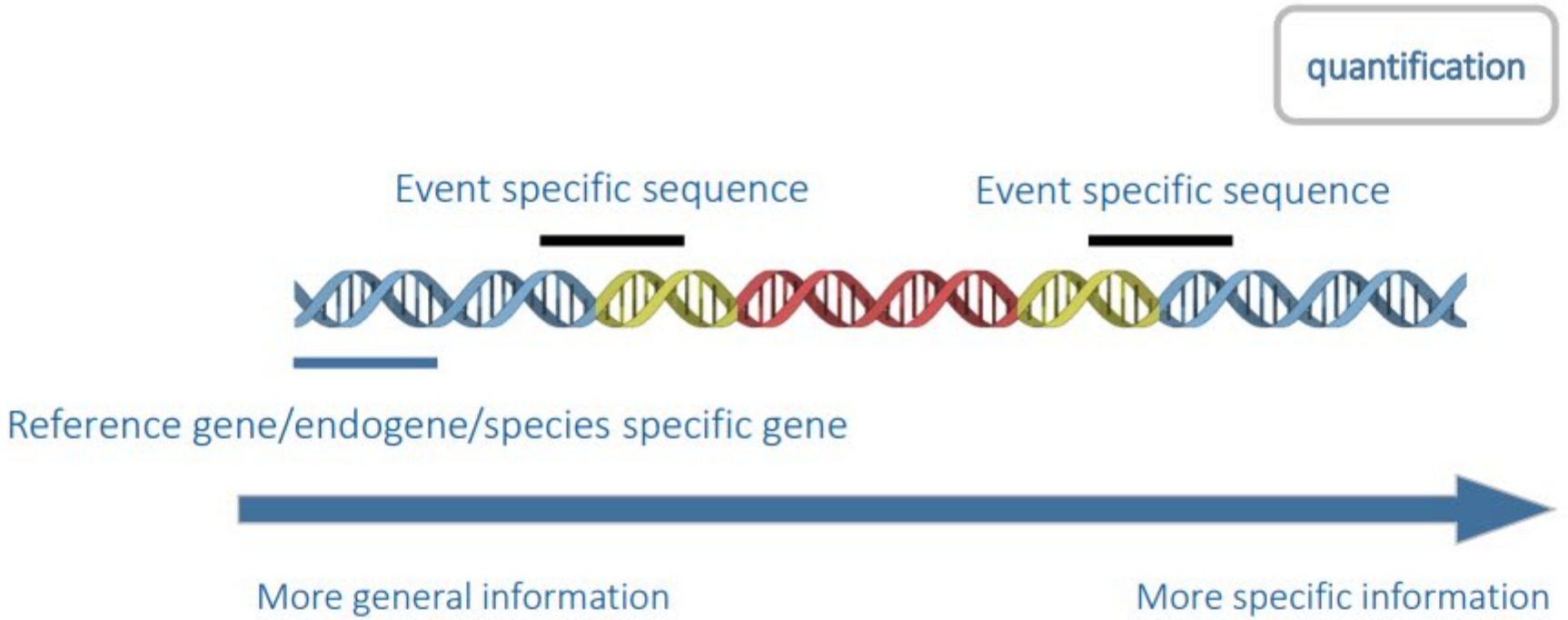
Country	Mandatory Vs. Voluntary labeling	Product Vs. Process labeling	Threshold level (%)
EU	Mandatory	Process	0.9
China	Mandatory	Process	0
Australia, N.Z.	Mandatory	Product	1
Japan	Mandatory	Product	5
Canada	Voluntary	Product	5
U.S.A.	Voluntary	Product	n/a



Three partite PCR-based testing scheme



Three partite PCR-based testing scheme



Quality control

For PCR (targeted element and reference gene):

- Positive control as dilution series (CRM, if possible)
- Negative control (water as no-template control, NTC)

An example for quantification - plan

Experiment ID	.ixo
Analyst:	

Pipets used	
DNA 1 A	
manual 0.1uL-2.5uL	□
manual 0.5uL-10uL	□
multistep 0.2uL-10uL	□

DNA 1 B	
manual (0.5uL-10uL)	□
manual (10uL-100uL)	□
multistep (0.2uL-10uL)	□
multistep (2.0uL-20uL)	□
multistep (5.0uL-120uL)	□

Plazmids C	
manual (0.5uL-10uL)	□
manual (2.0uL-20uL)	□
manual (10uL-100uL)	□
manual (20uL-200uL)	□
manual (100uL-1000uL)	□

TARGET:		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	A																								
	B																								
	C																								
Lektin	D	NTC1		CV127 1x		CV127 10x		CV127 100x		CV127 400x		CV127 1600x		CV127 6400x			ID299 10x		ID299 40x		ID464 10x		ID464 40x		
Lektin	E			G338/22 -1 3x		G338/22 -1 12x		G338/22 -1 48x		G338/22 -1 192x		G338/22 -2 3x		G338/22 -2 12x		G338/22 -2 48x		G338/22 -2 192x							NTC2
	F																								
CV127	G	NTC1		CV127 1x		CV127 10x		CV127 100x		CV127 400x		CV127 1600x		CV127 6400x			ID299 10x		ID299 30x		ID464 10x		ID464 40x		
CV127	H			G338/22 -1 3x		G338/22 -1 12x		G338/22 -1 48x		G338/22 -1 192x		G338/22 -2 3x		G338/22 -2 12x		G338/22 -2 48x		G338/22 -2 192x							NTC2

384 plate, 20 µL reaction

An example for quantification - results

Reference gene - lectin

Well	Sample Name	Ct	Comment
D1	NTC1	Undetermined	NTC OK.
E24	NTC2	Undetermined	NTC OK.

Fill yellow fields by pasting results from raw data sheet.

Fill blue fields by entering values or comment.

Well	Sample Name	Cq	Quantity	Log Quant	quant. endogene	Average PCR paralels	St. dev endogene	Cv endogene	Log copy no.
D3	ID672 1x	20,01375981	100	2	109,352239	102,60158	9,546873998	0,093048022	4,94694327
D4	ID672 1x	20,21079902	100	2	95,8509203				4,94694327
D5	ID672 10x	23,59530732	10	1	9,96649875	9,80223502	0,23230399	0,023699084	3,94694327
D6	ID672 10x	23,64542487	10	1	9,6379713				3,94694327
D7	ID672 100x	26,9867312	1	0	1,03152621	1,03693413	0,007647967	0,007375557	2,94694327
D8	ID672 100x	26,97113509	1	0	1,04234206				2,94694327
D9	ID672 400x	29,30471546	0,25	-0,60206	0,21887987	0,22500819	0,00866675	0,038517487	2,34488328
D10	ID672 400x	29,22324822	0,25	-0,60206	0,23113651				2,34488328
D11	ID672 1600x	31,16387517	0,0625	-1,20412	0,06312482	0,0644378	0,001856838	0,028815975	1,74282329
D12	ID672 1600x	31,10293396	0,0625	-1,20412	0,06575078				1,74282329
D13	ID672 6400x	33,27553271	0,015625	-1,80618	0,0153764	0,01621483	0,001185714	0,07312532	1,1407633
D14	ID672 6400x	33,12076764	0,015625	-1,80618	0,01705325				1,1407633

k1	-3,441287373	Slope OK.	
R12	0,999545544	R12 OK.	
k2	-0,290456866		
n2	7,851961622		
n3	10,79890489		
	Cv umeritvene	OK!	

$k_1 = SLOPE(Cq; \log_{10}value)$

$R_1^2 = RSQ(Cq; \log_{10}value)$

$k_2 = SLOPE\ (\log_{10}value; Cq)$

$n_2 = INTERCEPT\ (\log_{10}value; Cq)$

$n_3 = INTERCEPT\ (\log_{10}\ copy\ no.; Cq)$

For log copy number: transfer spacies data to J6!

4,5638369	maize	4,60206	cotton
	soy	4,7242759	potato
5,3222193	rice	4,90309	sugar beet
4,9393195	oilseed rape		

Log of genome copy number (2n) in 100 ng of DNA

CRM (100% CV127)

Well	Sample Name	Cq	Rel. quant.	Log rel. quant.	Slope	Difference in slopes	Dilutions used for calculation	quant. endogene	Average PCR paralels	St. dev endogene	Cv endogene	DNA copy number	Relative LOD	Relative LOQ	relative LOD - 5plex
E3	G338/22 -1 3x	22,20308398	16	1,20412	-3,3058	0,1355	3x12x48x	25,28852183	25,074048	0,303312	0,0120966	22380,34182	0,01	0,22	0,07
E4	G338/22 -1 3x	22,22866354	16	1,20412	-3,3273	0,1140	3x12x	24,85957409	3x			22000,72307	0,01	0,23	0,07
E5	G338/22 -1 12x	24,24285814	4	0,60206	-3,2844	0,1569	12x48x	6,463340477	6,56774021	0,147644	0,0224801	5720,056322	0,03	0,87	0,26
E6	G338/22 -1 12x	24,19531885	4	0,60206				6,672139934	12x			5904,843842	0,03	0,85	0,25
E7	G338/22 -1 48x	26,17753251	1	0				1,772219781	1,75004022	0,031367	0,0179234	1568,414506	0,13	3,19	0,96
E8	G338/22 -1 48x	26,2154344	1	0				1,727860655	48x			1529,15668	0,13	3,27	0,98
E11	G338/22 -2 3x	21,93105635	16	1,20412	-3,3694	0,0719	3x12x48x	30,33442284	28,7640923	2,220783	0,0772068	26845,96422	0,01	0,19	0,06
E12	G338/22 -2 3x	22,0944763	16	1,20412	-3,2544	0,1869	3x12x	27,19376178	3x			24066,47918	0,01	0,21	0,06
E13	G338/22 -2 12x	23,89932212	4	0,60206	-3,4844	0,0431	12x48x	8,132787617	7,75555394	0,533489	0,068788	7197,517041	0,03	0,69	0,21
E14	G338/22 -2 12x	24,04489254	4	0,60206				7,378320271	12x			6529,81344	0,03	0,77	0,23
E15	G338/22 -2 48x	26,00238542	1	0				1,992462954	1,9064412	0,121653	0,0638116	1763,329714	0,11	2,84	0,85
E16	G338/22 -2 48x	26,13740992	1	0				1,820419437	48x			1611,071201	0,12	3,10	0,93

Sample

An example for quantification - results

Targeted event – CV127

Well	Sample Name	Ct	Comment
K1	NTC1	Undetermine	NTC OK.
L24	NTC2	Undetermine	NTC OK.

Fill yellow fields by pasting results from raw data sheet.

Fill blue fields by entering values or comment.

Correct the formula if not the same dilutions were used for trangene than for endogene!

Well	Sample Name	Cq	Quantity	Log Quant	GMO quant.	Average PCR paralels GMO	St. dev GMO	Cv GMO
K3	ID672 1x	20,4734985	100	2	105,3556	101,61523	5,28961558	0,0520553
K4	ID672 1x	20,5841553	100	2	97,87491			
K5	ID672 10x	23,9682705	10	1	10,29132	10,189632	0,14380316	0,0141127
K6	ID672 10x	23,9982581	10	1	10,08795			
K7	ID672 100x	27,3723357	1	0	1,067834	1,0139766	0,07616622	0,0751163
K8	ID672 100x	27,5320927	1	0	0,960119			
K9	ID672 400x	29,6243273	0,25	-0,60206	0,238534	0,2171876	0,03018894	0,1389993
K10	ID672 400x	29,9206287	0,25	-0,60206	0,195841			
K11	ID672 1600x	31,5601875	0,0625	-1,20412	0,065762	0,0641808	0,00223687	0,0348526
K12	ID672 1600x	31,634257	0,0625	-1,20412	0,062599			
K13	ID672 6400x	33,8075842	0,0156	-1,80618	0,014735	0,0169497	0,00313196	0,1847791
K14	ID672 6400x	33,4127095	0,0156	-1,80618	0,019164			

k1	-3,4556	Slope OK.
R12	0,99887	R12 OK.
k2	-0,2891	
n2	7,94065	
CV of calibration curve		OK!

CRM

Measurement uncertainty calculated according to G0002/20 (<0.445% - 45%, =>0.445% - 30% for all plants and lines). Round MU to 2 "significant" digits, then to the same % GMO.

Well	Sample Name	Cq	Rel. quant.	Log rel. quant.	Slope	Difference in slopes	Dilutions used for calculation	GMO quant.	Average PCR paralels GMO	St. dev GMO	Cv GMO	Average PCR paralels endogene	St. dev endogene	Cv endogene	% GMO	Cv3	Average % GMO	Measurement uncertainty
L3	G338/22 -1 3x	28,8484654	16	1,20412	-3,5372	0,0816	3x12x48x	0,3997781	0,3684775	0,0443	0,12013	25,074	0,303311856	0,0121	1,47	0,04	1,43	0,43
L4	G338/22 -1 3x	29,1043368	16	1,20412	-3,4327	0,0229	3x12x	0,3371768	3x			3x						
L5	G338/22 -1 12x	30,9592601	4	0,60206	-3,6417	0,1861	12x48x	0,0981025	0,0929215	0,0073	0,07885	6,56774	0,147643512	0,02248	1,41	0,02	1,40	0,42
L6	G338/22 -1 12x	31,1269776	4	0,60206				0,0877405	12x			12x						
L7	G338/22 -1 48x	33,2591044	1	0				0,0212273	0,0215636	0,0005	0,02206	1,75004	0,031366639	0,01792	1,23	0,16	1,39	0,42
L8	G338/22 -1 48x	33,2122359	1	0				0,0218999	48x			48x						
L11	G338/22 -2 3x	28,8665907	16	1,20412	-3,2516	0,2041	3x12x48x	0,3949842	0,3971261	0,003	0,00763	28,7641	2,220782734	0,07721	1,38			
L12	G338/22 -2 3x	28,8503842	16	1,20412	-3,2815	0,1741	3x12x	0,3992679	3x			3x						
L13	G338/22 -2 12x	30,7525432	4	0,60206	-3,2216	0,2340	12x48x	0,1125727	0,1067773	0,0082	0,07676	7,75555	0,533488977	0,06879	1,38			
L14	G338/22 -2 12x	30,9157973	4	0,60206				0,1009819	12x			12x						
L15	G338/22 -2 48x	32,6803308	1	0				0,0312028	0,0293783	0,0026	0,08783	1,90644	0,121653138	0,06381	1,54			
L16	G338/22 -2 48x	32,8671883	1	0				0,0275538	48x			48x						

Sample

Measurement uncertainty

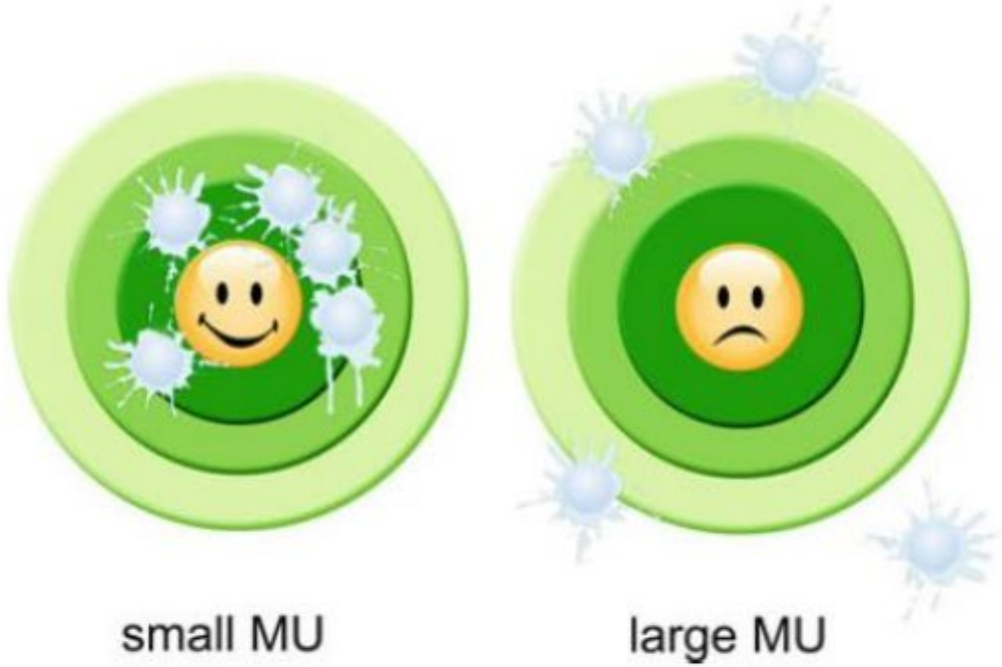


Fig. 17 Results of repeated measurements of the same sample can give very distributed values around target value, representing large MU, whereas results concentrated near the target value represent small MU

Bias

Fig. 18 Presentation of bias. All the measurement results are concentrated around one virtual point that is not the target concentration. The difference between this virtual point and the real target value is bias

Interpretation of GMO testing result

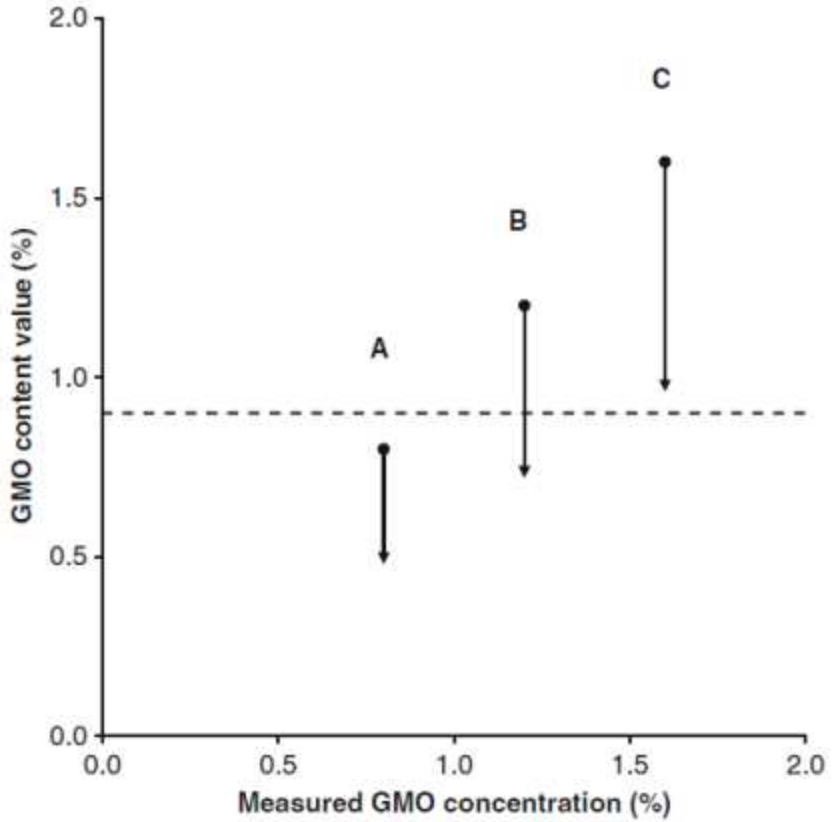


Fig. 20 Interpretation of GMO testing result for labeling compliance within EU legislation. GMO content value (*arrowhead*) obtained by subtracting the expanded uncertainty (*vertical line*, calculated using 40% as relative MU) from the measured GMO concentration (*circle*) is used to assess compliance with the EU legislation (*dashed line*). Samples with GMO content value (*arrowhead*) above the threshold value (*dashed line*) should be labeled, in this case only sample C

Only sample C should be labeled in line with EU legislation.

Two approaches for determination of MU

Bottom-up approach: calculates all the individual factors influencing the final result.

Top-down approach: practical approach for GMO detection. In this approach, data from collaborative trials and sample analyses, including all the factors influencing the MU during the analytical procedure, are used as a source for estimation of MU.

Bottom-up approach

Used for measurements in the frame of NIB as a holder of national standard for nucleic acid analysis.

NIB is designated institute for amount of substance/Bioanalysis of NucleicAcids/GMOs and Microorganisms (from 05 March 2010)

Example for bottom-up approach

Oilseed rape GT73 measured using dPCR

- Repeatability and intermediate precision for GT73 ($uC_{precGT73}$) and Fat (A) ($uC_{precFat(A)}$)
- Pipetting/preparation of dilutions (calculated based on previous experiments with gravimetric preparation of dilutions) (uD)
- Volumen of partitions (Bogožalec Košir et al., 2017) (uV_d)
- Homogeneity of material (uH)

Standard uncertainty:

$$u_C = \sqrt{\left(\frac{uC_{precGT73}}{\bar{C}_{GT73}}\right)^2 + \left(\frac{uC_{precFat(A)}}{\bar{C}_{Fat(A)}}\right)^2 + \left(\frac{uD}{\bar{D}}\right)^2 + \left(\frac{uV_d}{\bar{V}_d}\right)^2 + uH^2}$$

Measurement results

	Fat(A)	Technical replicate		
	Sample	1	2	3
Day1	T2-1 10x	60262	60786	59658
	T2-2 10x	64650	65970	67166
	T2-3 10x	52287	52655	53474
Day 2	T2-1 10x	61731	64297	58620
	T2-2 10x	63719	64129	64854
	T2-3 10x	47656	48247	53002

	GT73	Technical replicate		
	Sample	1	2	3
Day1	T2-1 1x	2732	2739	2813
	T2-2 1x	3077	2916	2957
	T2-3 1x	2465	2467	2455
Day 2	T2-1 1x	2784	2750	2781
	T2-2 1x	2937	2912	2893
	T2-3 1x	2426	2505	2440

Calculations of measurement uncertainty

Anova: Single Factor						
Fat(A)						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Row 1	3	180706,493	60235,5	318896,3		
Row 2	3	197786,19	65928,73	1584150		
Row 3	3	158416,027	52805,34	369061,7		
Row 4	3	184648,128	61549,38	8081884		
Row 5	3	192700,848	64233,62	330279,7		
Row 6	3	148905,642	49635,21	8590049		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6,28E+08	5	1,26E+08	39,12451	5,06E-07	3,105875
Within Groups	38548642	12	3212387			
Total	6,67E+08	17				

Anova: Single Factor						
GT73						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Row 1	3	8284,33663	2761,446	2034,83		
Row 2	3	8949,57718	2983,192	6973,832		
Row 3	3	7386,80938	2462,27	36,86668		
Row 4	3	8315,33626	2771,779	360,2333		
Row 5	3	8742,09273	2914,031	480,4944		
Row 6	3	7371,97021	2457,323	1780,963		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	739771,3	5	147954,3	76,08717	1,15E-08	3,105875
Within Groups	23334,44	12	1944,536			
Total	763105,8	17				

$$u_r = \frac{\sqrt{MS_{within}}}{\sqrt{n}} \qquad u_{ipl} = \sqrt{\frac{MS_{between} - MS_{within}}{n \times N}}$$

Measurements/experiment = 9

Number of ekspeiments = 2

Calculation of MU

	u_{rel} (%)	Distribution	Divisor	Standard uncertainty (%)
Repeatability and intermediate precision for GT73	0.7	normal	1	0.7
Repeatability and intermediate precision for Fat(A)	8.9	normal	1	8.9
Partition volume	2.9	normal	2.1	1.4
Pipetting	3.1	normal	1	3.1
Homogeneity	2.8	normal	1	2.8
			u_c	9.95
			k	2
		Expanded measurement uncertainty (U)		**19.90**

Top-down approach – used at NIB

Used for measurements in routine GMO diagnostics.

An example of calculation of MU using data from routine samples (modified from Žel et al. 2009 and Trapmann et al. 2009). Two measurement are done for every sample.

For G018/05:

$$Mean\ c = (0.155 + 0.146) / 2 = 0.150$$

The absolute difference (d) between c_1 and c_2 is calculated:

$$d = | c_1 - c_2 | \qquad d = |0.155 - 0.146| = 0.009$$

The relative difference between c_1 and c_2 is calculated as

$$rad = (d / Meanc) * 100 \qquad rad = (0.009 / 0.150) * 100 = 5.707$$

From a set of calculated differences (d) and relative differences (rad), the average difference (Meand) and average relative difference (Meanrad) are calculated.

Table 20 Measurement results obtained on routine samples ($n = 2$) and calculation of the relative difference

Analysis number	GMO concentration c_1	GMO concentration c_2	Mean GMO concentration Mean c	Differenced	Relative difference rad
			[%]	[%]	[%]
G018/05	0.155	0.146	0.150	0.009	5.707
G199/06	0.143	0.178	0.161	0.035	21.737
G128/09	0.200	0.210	0.205	0.010	4.878
G098/05	0.282	0.339	0.311	0.057	18.381
G142/05	0.344	0.285	0.314	0.059	18.613
G126/08	0.415	0.292	0.354	0.123	34.795
G162/08	0.456	0.556	0.506	0.100	19.763
G101/05	0.440	0.638	0.539	0.198	36.729
G252/06	0.738	0.651	0.695	0.087	12.468
G121/05	0.669	0.890	0.780	0.221	28.282
G230/06	1.082	1.490	1.286	0.407	31.680
G133/06	1.262	1.511	1.386	0.249	17.976
G013/05	1.948	1.250	1.599	0.698	43.681
G115/05	1.582	1.677	1.630	0.094	5.793
G237/06	2.117	2.141	2.129	0.024	1.126
G175/05	2.174	2.226	2.200	0.052	2.356
G176/05	2.371	2.157	2.264	0.215	9.492
G015/05	2.322	2.456	2.389	0.134	5.606
G100/05	3.332	1.922	2.627	1.410	53.682
G247/06	2.891	2.548	2.719	0.344	12.639
G104/05	4.321	3.856	4.088	0.465	11.362
G103/04	4.901	4.776	4.839	0.125	2.581
G117/05	5.234	5.464	5.349	0.230	4.300
G118/06	12.514	14.418	13.466	1.904	14.143
G116/05	17.462	17.889	17.675	0.427	2.416
G119/05	19.902	19.589	19.745	0.313	1.586
G021/05	23.143	20.127	21.635	3.016	13.942
G159/08	21.675	22.608	22.142	0.933	4.214
G099/05	24.448	22.302	23.375	2.146	9.179
G102/05	36.473	41.474	38.973	5.001	12.832
G072/08	46.502	44.409	45.456	2.093	4.605
G124/08	48.186	49.881	49.034	1.695	3.457
G131/08	51.450	57.181	54.316	5.731	10.551
G133/05	63.665	54.036	58.850	9.629	16.362
G101/08	61.346	61.294	61.320	0.052	0.085
G135/05	56.809	71.972	64.391	15.163	23.549
G112/05	65.804	66.282	66.043	0.477	0.723
G174/08	68.788	75.231	72.010	6.443	8.947
G093/08	86.590	85.354	85.972	1.236	1.438
G158/08	114.636	81.385	98.011	33.251	33.926
Mean			**21.273**	**2.371**	**14.140**

The within laboratory standard deviation (s_R) is calculated from Meand divided by factor d_2 (1.13 in the case of two independent measurements):

$$s_R = Meand / d_2 \qquad s_R = 2.731 / 1.13 = 2.417\%$$

Within laboratory reproducibility relative standard deviation (RSD_R) is calculated using Meanrad and d_2:

$$RSD_R = Meanrad / d_2 \qquad RSD_R = 14.140 / 1.13 = 12.513\%$$

The expanded uncertainty U , corresponding to a confidence level of approximately 95%, is obtained by multiplication of RSD_R by coverage factor k = 2.

$$U = RSD_R * 2 \qquad U = 12.513 * 2 = 25.026\%$$

Table 20 Measurement results obtained on routine samples ($n = 2$) and calculation of the relative difference

Analysis number	GMO concentration c_1	GMO concentration c_2	Mean GMO concentration Mean c	Differenced	Relative difference rad
			[%]	[%]	[%]
G018/05	0.155	0.146	0.150	0.009	5.707
G199/06	0.143	0.178	0.161	0.035	21.737
G128/09	0.200	0.210	0.205	0.010	4.878
G098/05	0.282	0.339	0.311	0.057	18.381
G142/05	0.344	0.285	0.314	0.059	18.613
G126/08	0.415	0.292	0.354	0.123	34.795
G162/08	0.456	0.556	0.506	0.100	19.763
G101/05	0.440	0.638	0.539	0.198	36.729
G252/06	0.738	0.651	0.695	0.087	12.468
G121/05	0.669	0.890	0.780	0.221	28.282
G230/06	1.082	1.490	1.286	0.407	31.680
G133/06	1.262	1.511	1.386	0.249	17.976
G013/05	1.948	1.250	1.599	0.698	43.681
G115/05	1.582	1.677	1.630	0.094	5.793
G237/06	2.117	2.141	2.129	0.024	1.126
G175/05	2.174	2.226	2.200	0.052	2.356
G176/05	2.371	2.157	2.264	0.215	9.492
G015/05	2.322	2.456	2.389	0.134	5.606
G100/05	3.332	1.922	2.627	1.410	53.682
G247/06	2.891	2.548	2.719	0.344	12.639
G104/05	4.321	3.856	4.088	0.465	11.362
G103/04	4.901	4.776	4.839	0.125	2.581
G117/05	5.234	5.464	5.349	0.230	4.300
G118/06	12.514	14.418	13.466	1.904	14.143
G116/05	17.462	17.889	17.675	0.427	2.416
G119/05	19.902	19.589	19.745	0.313	1.586
G021/05	23.143	20.127	21.635	3.016	13.942
G159/08	21.675	22.608	22.142	0.933	4.214
G099/05	24.448	22.302	23.375	2.146	9.179
G102/05	36.473	41.474	38.973	5.001	12.832
G072/08	46.502	44.409	45.456	2.093	4.605
G124/08	48.186	49.881	49.034	1.695	3.457
G131/08	51.450	57.181	54.316	5.731	10.551
G133/05	63.665	54.036	58.850	9.629	16.362
G101/08	61.346	61.294	61.320	0.052	0.085
G135/05	56.809	71.972	64.391	15.163	23.549
G112/05	65.804	66.282	66.043	0.477	0.723
G174/08	68.788	75.231	72.010	6.443	8.947
G093/08	86.590	85.354	85.972	1.236	1.438
G158/08	114.636	81.385	98.011	33.251	33.926
Mean			**21.273**	**2.371**	**14.140**

An example for quantification - results

Targeted event – CV127

Well	Sample Name	Ct	Comment
K1	NTC1	Undetermine	NTC OK.
L24	NTC2	Undetermine	NTC OK.

Fill yellow fields by pasting results from raw data sheet.

Fill blue fields by entering values or comment.

Correct the formula if not the same dilutions were used for trangene than for endogene!

Well	Sample Name	Cq	Quantity	Log Quant	GMO quant.	Average PCR paralels GMO	St. dev GMO	Cv GMO
K3	ID672 1x	20,4734985	100	2	105,3556	101,61523	5,28961558	0,0520553
K4	ID672 1x	20,5841553	100	2	97,87491			
K5	ID672 10x	23,9682705	10	1	10,29132	10,189632	0,14380316	0,0141127
K6	ID672 10x	23,9982581	10	1	10,08795			
K7	ID672 100x	27,3723357	1	0	1,067834	1,0139766	0,07616622	0,0751163
K8	ID672 100x	27,5320927	1	0	0,960119			
K9	ID672 400x	29,6243273	0,25	-0,60206	0,238534	0,2171876	0,03018894	0,1389993
K10	ID672 400x	29,9206287	0,25	-0,60206	0,195841			
K11	ID672 1600x	31,5601875	0,0625	-1,20412	0,065762	0,0641808	0,00223687	0,0348526
K12	ID672 1600x	31,634257	0,0625	-1,20412	0,062599			
K13	ID672 6400x	33,8075842	0,0156	-1,80618	0,014735	0,0169497	0,00313196	0,1847791
K14	ID672 6400x	33,4127095	0,0156	-1,80618	0,019164			

k1	-3,4556	Slope OK.
R12	0,99887	R12 OK.
k2	-0,2891	
n2	7,94065	

CRM

CV of calibration curve: OK!

Measurement uncertainty calculated according to G0002/20 (<0.445% - 45%, =>0.445% - 30% for all plants and lines). Round MU to 2 "significant" digits, then to the same % GMO.

Well	Sample Name	Cq	Rel. quant.	Log rel. quant.	Slope	Difference in slopes	Dilutions used for calculation	GMO quant.	Average PCR paralels GMO	St. dev GMO	Cv GMO	Average PCR paralels endogene	St. dev endogene	Cv endogene	% GMO	Cv3	Average % GMO	Measurement uncertainty
L3	G338/22 -1 3x	28,8484654	16	1,20412	-3,5372	0,0816	3x12x48x	0,3997781	0,3684775	0,0443	0,12013	25,074	0,303311856	0,0121	1,47	0,04	1,43	0,43
L4	G338/22 -1 3x	29,1043368	16	1,20412	-3,4327	0,0229	3x12x	0,3371768	3x			3x						
L5	G338/22 -1 12x	30,9592601	4	0,60206	-3,6417	0,1861	12x48x	0,0981025	0,0929215	0,0073	0,07885	6,56774	0,147643512	0,02248	1,41	0,02	1,40	0,42
L6	G338/22 -1 12x	31,1269776	4	0,60206				0,0877405	12x			12x						
L7	G338/22 -1 48x	33,2591044	1	0				0,0212273	0,0215636	0,0005	0,02206	1,75004	0,031366639	0,01792	1,23	0,16	1,39	0,42
L8	G338/22 -1 48x	33,2122359	1	0				0,0218999	48x			48x						
L11	G338/22 -2 3x	28,8665907	16	1,20412	-3,2516	0,2041	3x12x48x	0,3949842	0,3971261	0,003	0,00763	28,7641	2,220782734	0,07721	1,38			
L12	G338/22 -2 3x	28,8503842	16	1,20412	-3,2815	0,1741	3x12x	0,3992679	3x			3x						
L13	G338/22 -2 12x	30,7525432	4	0,60206	-3,2216	0,2340	12x48x	0,1125727	0,1067773	0,0082	0,07676	7,75555	0,533488977	0,06879	1,38			
L14	G338/22 -2 12x	30,9157973	4	0,60206				0,1009819	12x			12x						
L15	G338/22 -2 48x	32,6803308	1	0				0,0312028	0,0293783	0,0026	0,08783	1,90644	0,121653138	0,06381	1,54			
L16	G338/22 -2 48x	32,8671883	1	0				0,0275538	48x			48x						

Sample

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New types of LMOs – new breeding techniques

New breeding techniques (NBTs) encompass a suite of methods designed to modify DNA and create novel traits.

Their objective is to accelerate the development of improved crops (compared to ‘classic’ GMO techniques).

More info:

https://www.eu-sage.eu/genome-search

http://plantcrispr.org/cgi-bin/crispr/index.cgi

https://www.europarl.europa.eu/doceo/document/TA-9-2024-0067_EN.html - current version of EU legislation

Methods involved:

Genome Editing: Precisely modify the genome by targeting specific locations within plant genes. Tools like **Zinc finger nucleases**, **TALENs**, and **CRISPR/Cas** are used.

Oligonucleotide-Directed Mutagenesis (ODM): Introduce changes to just a few base pairs.

Cisgenesis/Intragenesis: Transfer genes from closely related species or incorporate reshuffled regulatory instructions from the same species.

RNA-Dependent DNA Methylation (RdDM): Alter gene activity without changing the DNA itself.

Grafting on GM Rootstock: Combine unaltered plants with genetically modified rootstock.

Benefits and Challenges

Advantages:

- Faster trait development.
- Precision in modifying specific genes.
- Potential for disease resistance and improved yield.

Challenges:

- Regulatory debates: Should NBTs be treated like genetically modified organisms (GMOs)?
- Lack of consistent global policies for NBT-derived products.
- Detection is possible, identification for some product is not possible, consequently control is difficult.

Key types of changes achieved through NBTs:

Precision Genome Editing:

- Single-base modifications
- Short insertions/deletions
- Gene knockouts
- Gene insertions

Modification of Regulatory Elements:

- Promoters and Enhancers

Epigenetic Changes:

- Methylation patterns

Methods for detection

Detection of all changes is possible. Approaches are differing depending on the change.

Methods for SNVs, short deletions and insertions are described:

Two examples described in Bulletin 6407 VerB (Bio-Rad)

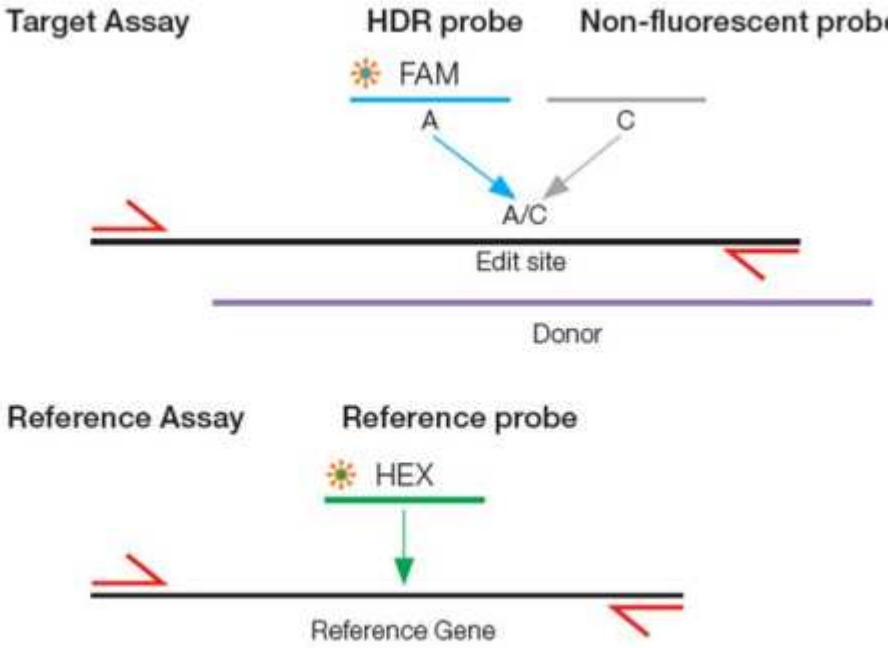

Fig. 7.4. HDR assay design.

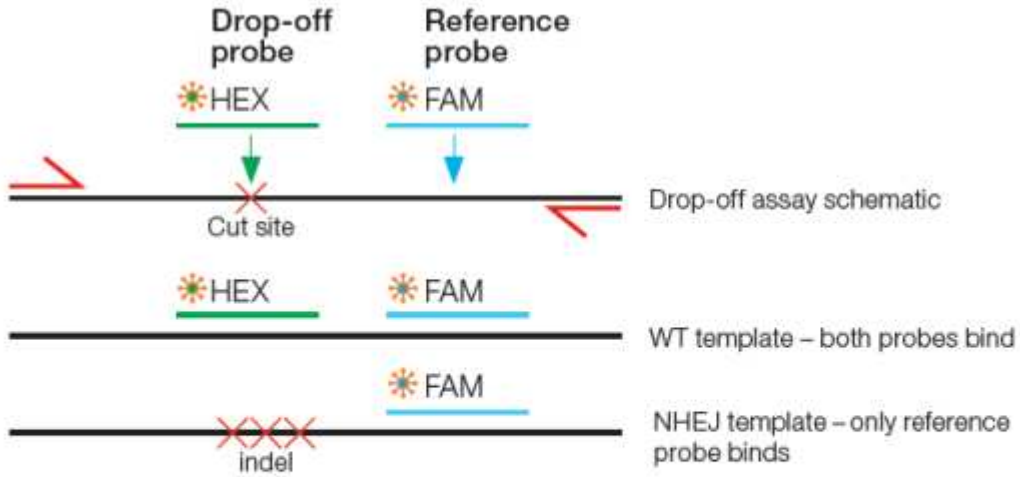

Fig. 7.6. NHEJ assay design.

Methods Mol Biol. 2018 ; 1768: 349–362. doi:10.1007/978-1-4939-7778-9_20.

Detection and Quantification of HDR and NHEJ Induced by Genome Editing at Endogenous Gene Loci Using Droplet Digital PCR

Yuichiro Miyaoka, **Steven J. Mayerl**, **Amanda H. Chan**, and **Bruce R. Conklin**

Abstract

Genome editing holds great promise for experimental biology and potential clinical use. To successfully utilize genome editing, it is critical to sensitively detect and quantify its outcomes: homology-directed repair (HDR) and nonhomologous end joining (NHEJ). This has been difficult at endogenous gene loci and instead is frequently done using artificial reporter systems. Here, we describe a droplet digital PCR (ddPCR)-based method to simultaneously measure HDR and NHEJ at endogenous gene loci. This highly sensitive and quantitative method may significantly contribute to a better understanding of DNA repair mechanisms underlying genome editing and to the improvement of genome editing technology by allowing for efficient and systematic testing of many genome editing conditions in parallel.

Keywords

Genome editing; TALEN; CRISPR/Cas9; HDR; NHEJ; ddPCR

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Report

Detection of Deleterious On-Target Effects after HDR-Mediated CRISPR Editing

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REVIEW

GMO quantification: valuable experience and insights for the future

Mojca Milavec · David Dobnik · Litao Yang · Dabing Zhang · Kristina Gruden · Jana Žel

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Article

Fast and Accurate Multiplex Identification and Quantification of Seven Genetically Modified Soybean Lines Using Six-Color Digital PCR

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Digital PCR as an effective tool for GMO quantification in complex matrices

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Thank you!

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