Update and validation of real-time PCR array method for comprehensive GMO detection

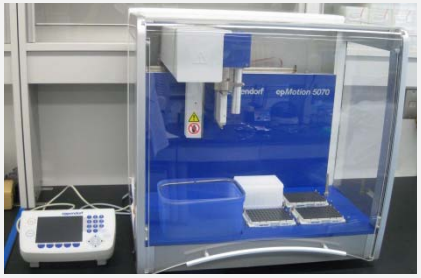
Junichi MANO and Kazumi KITTA
National Food Research Institute, Japan

Overview of the real-time PCR array analysis

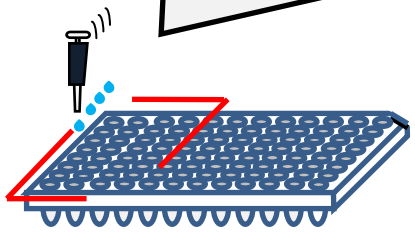
Preparation of 96 well PCR plates which have primer probe mixtures for detecting individual DNA regions in the respective wells

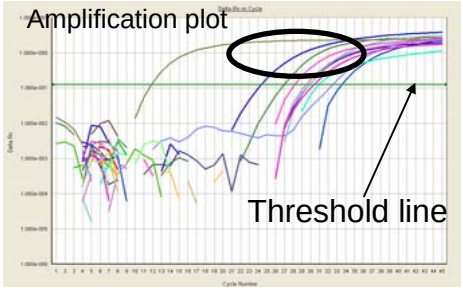
	1	2	3	4
A	Bt11	MIR604	NOS ter.	SSIIb (maize)
B	E176	MON88017	Rice Actin int.	Le1 (soy)
C	GA21	DAS59122	NPTII	SPS (rice)
D	MON810	RRS	PAT	HMG (canola)
E	MON863	A2704	BAR	18SrRNA
F	NK603	A5547	GOX	CaMV
G	T25	35S pro.	EPSPS1	NTC
H	TC1507	FMV pro.	EPSPS2	NTC

: GM maize event
: GM soybean event
: Recombinant DNA segment
: Taxon specific
: Donor organism specific
: Negative control



We manufacture the plates by epmotion 5070 system (Eppendorf).





Step 1

DNA extract (20 ng/ml) and TaqMan Universal PCR Master Mix are mixed and dispenced into each wells.
The reaction volume was set as 10 μl.

Step 2

The thermal cycling was performed by ABI real-time PCR instruments 7500 or 7900HT.

Step 3

Reactions whose amplification plot crossed with the threshold line was determined as positive.

Mano J. et al. *J. Agric. Food Chem.* 57, 1, 26-37 (2009).

Validation scheme of the real-time PCR array

1. Validation of DNA extraction methods

-Evaluation of DNA quantity

-Evaluation of fragmentation of extracted DNA

-Evaluation of PCR inhibition

2. Validation of component PCR reactions

-Evaluation of specificity

-Evaluation of limit of detection (LOD)

3. Participation of proficiency testing

-GeMMA program

* All validation schemes are implemented in house.

*For each validation step, criteria for acceptance were set reffering to the ISO24276 and the EURL-GMFF guidance document.

Validation of DNA extraction method

-Evaluation of DNA quantity

Our criteria:

The average of DNA quantity is more than 20 ng/μl and its RSD is below 25%.

The results of evaluation

Sample	DNA extraction method	DNA concentration	
		Average (ng/μl)	RSD (%)
Maize seed flour	DNeasy Plant Maxi kit (Qiagen)	674	18
	DNeasy Plant Mini kit (Qiagen)	131	7.2
	GM quicker (Nippon gene)	71	4.9
Soybean flour	DNeasy Plant Maxi kit (Qiagen)	262	20
	DNeasy Plant Mini kit (Qiagen)	57.1	14
	GM quicker (Nippon gene)	61.0	11

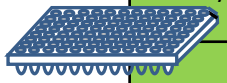
Validation of component PCR assays

-Evaluation of specificity

Our criteria: An authentic DNA sample gives the same results in triplicate assay.

Type of detection	Assay name	Sample																			
		Maize												Soybean				Rice		Canola	
		Bt11	E176	GA21	M810	M863	NK603	T25	TC1507	D59122	M88017	MIR604	NG maize	RRS	A2704	A5547	NG soy	LLRice62	NG rice	RT73	NG canola
GM event detection	Bt11	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E176	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	GA21	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M810	-	-	-	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M863	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	NK603	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T25	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	TC1507	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	D59122	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	M88017	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	MIR604	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	RRS	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	A2704	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	A5547	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Recombinent DNA Segment detection	P35S	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	-
	TNOS	+	-	+	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-
	PFMV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	AINT	-	-	+	-	+	+	-	-	-	+	-	-	-	-	-	-	+	+	-	-
	NPTII	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PAT	+	-	-	-	-	-	+	+	+	-	-	-	-	+	+	-	-	-	-	-
	BAR	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	GOX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	EPSPS1	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-
	EPSPS2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Taxon specific detection	SSIIb	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	Le1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
	SPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
	HMG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	18SrRNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

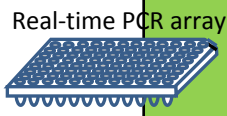
Real-time PCR array



Validation of component PCR assays

-Evaluation of limit of detection

Our criteria:
We determine the lowest concentration of analyte at which more than 20 times were positive in 21 analyses in both the 7900HT and 7500 real-time PCR systems. (False negative rate should be less than 5%.)



Type of detection	Assay name	Limit of detection
GM event detection	Bt11	0.10%
	E176	0.05%
	GA21	0.05%
	M810	0.25%
	M863	0.25%
	NK603	0.25%
	T25	0.25%
	TC1507	0.25%
	D59122	0.25%
	M88017	0.25%
	MIR604	0.25%
	RRS	0.05%
	A2704	0.05%
	A5547	0.05%
Recombinant DNA segment detection	P35S	0.25% for MON810, 0.05% for RRS
	TNOS	0.25% for MON863, 0.05% for RRS
	PFMV	0.01% for RT73
	AINT	0.25% for MON863
	NPTII	0.10% for MON863
	PAT	0.25% for D59122
	BAR	0.25% for E176
	GOX	0.01% for RT73
	EPSPS1	0.05% for RRS
	EPSPS2	0.01% for RT73

Update of the real-time PCR array system

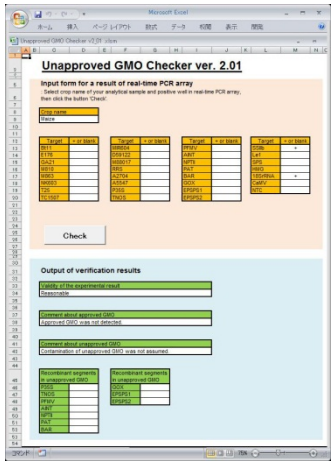
We added the new PCR assays as the 2nd series.
Now we are proceeding the validation process.

GM event: (maize) LY038, MON89034, 3272
(soybean) MON89788, DP305423, DP356043
(canola) RT73, T45
(sugar beet) H7-1

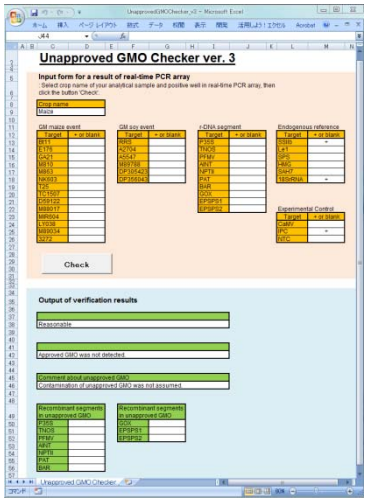
Taxon specific: (cotton) SAH7
(potato) UGPase
(sugar beet) GS

The real-time PCR array system enables us to assume the contamination of unapproved GMO(s) from the results. The excel spread sheet for the assumption, Unapproved GMO cheker, is also updated as increase of the number of GM events.

Ver 2.01



Ver 3



The comparison of the activities in EU and Japan

	A ready-to-use multi-target analytical system(JRC, EC)	Real-time PCR array system (NFRI, Japan)
Target	Approved and unapproved events taxon specific	Approved events, r-DNA segments, taxon specific, donor organisms
PCR Reagent	TaqMan Universal Master mix	TaqMan Universal Master mix
Reaction volume	50 μL/well	10 μL/well
DNA amount	100 ng/well	20 ng/well
Thermal cycle	95°C 10min→ (95°C 15 s→60°C 60 s) x 45 cycles	95°C 10min→ (95°C 15 s→60°C 60 s) x 45 cycles
Data analysis	Thresold line 0.2	Thresold line 0.256
Validation	Interlaboratory study	In-house evaluation
Limit of detection	< 0.045%	< 0.25%

Conclusive remarks

-Real-time PCR array (and also European system) is a powerful tool for comprehensive GMO detection in the light of updatability, flexibility and accuracy of detection and user friendliness. Howerver, ABI's licence for manufacturing testing kit costs a lot of money. So, we have not commercially distributed the real-time PCR array plates in Japan.

- Japanese and European system is fortuitously similar except for the running cost and LOD. Japanese system doesn't request high sensitivity (we think that 0.25% LOD is enough for Japanese regulation). So, the reaction volume in Japanese system was 5 times smaller and the running cost was cheaper than European system.

-Real-time PCR array system (also European system) is good platform for international cooperation. For example, if someone develops and validates a new PCR assay with the same reaction condition of the already existing system, everyone can use the assay on the common real-time PCR array platform without modification.

-We hope the ISO standard of real-time PCR array analysis in order to clarify tasks and criteria for method validation.

Thank you for your attention.

NARO NFRI Food