Report on the application of P-35S, T-nos, CryIAb/Ac and rice PLD methods for the detection of Genetically Modified Rice originating from China Using Real-Time PCR

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Joint Research Centre
Institute for Health and Consumer Protection
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Executive Summary

In support to the repeal of Commission Decision 2008/289/EC of 3 April 2008, the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, was requested to carry out an in-house verification study to assess the performance of PLD-rice, P-35S, T-nos and CryIAb/Ac methods as a screening approach for the detection of genetically modified rice in food products. For this, the four SYBR®Green Real-time PCR methods developed by the Scientific Institute of Public Health (Brussels, Belgium) were assessed by the EURL-GMFF for their performance in detecting these markers in rice material.

The present verification report documents the results of this EURL-GMFF in-house verification study and confirms that all four SYBR®Green Real-time PCR methods can effectively detect the respective targets in control materials of the Bt63, Kefeng6 and KMD1 genetically modified rice events.

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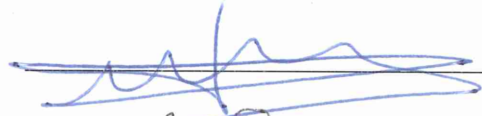

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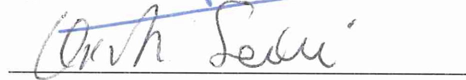

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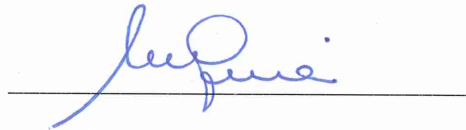

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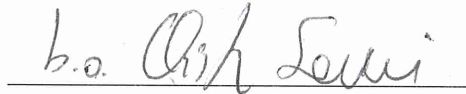

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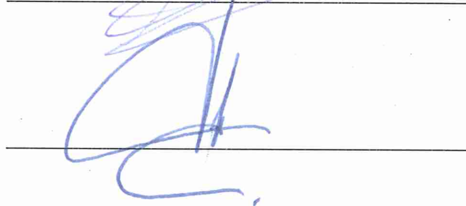

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1. Introduction

In support to the repeal of Commission Decision 2008/289/EC of 3 April 2008, the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the fitness of the P-35S, T-nos and CryIAb/Ac SYBR®Green Real-time PCR methods for the detection of genetically modified (GM) rice in food products. The methods have been developed by the Scientific Institute of Public Health (Brussels, Belgium) and tested on a broad range of crops (including rice) and in a broad range of matrices. The respective methods were assessed at the EURL-GMFF for their specificity and sensitivity in detecting these GM markers in genomic DNA isolated from GM rice materials originating from China.

2. Materials and methods

2.1 Materials

The EURL-GMFF tested the following control materials in this study:
- Genomic DNA from Bt63 GM rice (source: EURL-GMFF)
- Genomic DNA from Kefeng6 GM rice (source: EURL-GMFF)
- Genomic DNA from KMD1 GM rice (source: EURL-GMFF)
- Genomic DNA from Minghui63 non-transgenic rice (source: EURL-GMFF)
- Genomic DNA from LL62 GM rice (source: AOCS)
- Genomic DNA from LL601 GM rice (source: EURL-GMFF)
- Genomic DNA from Bt11 GM maize (source: EURL-GMFF)
- Genomic DNA from MON 810 GM maize (source: EURL-GMFF)

All materials were obtained as genomic DNA except Bt11 and MON 810 GM maize materials which were control materials. Genomic DNA was extracted from the latter using the NucleoSpin® Plant II Maxi kit (Machery-Nagel, Germany).

2.2 Methods

DNA quantification
Total DNA concentration was determined by fluorescence detection using the PicoGreen® dsDNA Quantification kit (Catalogue Number P7589, Molecular Probes). Suitable dilutions of each genomic DNA sample were prepared in 3 replicates and mixed with the PicoGreen® reagent. The DNA concentration was determined on the basis of a five-point standard curve ranging from 0 to 500 ng/mL using a Bio-Rad VersaFluor Fluorometer as fluorescence detector.

Agarose Gel-electrophoresis
Intactness of the genomic DNA was assessed by 1.0% agarose gel electrophoresis and the DNA content was visualised by Ethidium Bromide staining.

PCR primers and reagents
The primers for the rice phospholipase D gene (PLD-rice Fwd & Rev), the 35S promoter from Cauliflower Mosaic Virus (P-35S Fwd & Rev), the nopaline synthase terminator (T-nos Fwd & Rev) from *Agrobacterium tumefaciens* and the CryIAb/Ac toxin from *Bacillus thuringiensis* (CryIAb/Ac Fwd & Rev) were purchased from Microsynth at desalted grade (See table 1 for specifications).

In all Real-time PCR reactions, *Power* SYBR®Green PCR Mastermix (Part Number 4367659; Applied Biosystems) was used.

Real-time PCR analysis
All PCR analyses were performed according to Barbau-Piednoir *et al.* (2010) with minor adaptations. For all PCR analyses, a standard 20 μL reaction volume was applied containing 5 μL of template DNA, 1X *Power* SYBR®Green PCR Mastermix, and 250 nM of each primer. The thermal program consisted of a single cycle of DNA polymerase activation for 10 min at 95°C, followed by 40 amplification cycles of 15 sec at 95°C (denaturing step) and 1 min at 60°C (annealing-extension step). After completion of the run, a melting curve analysis was performed by stepwise temperature increase (± 1.75°C/min) from 60°C to 95°C. The threshold cycle (C_t) for each sample was calculated in automatic mode according to the manufacturer's specifications. If considered necessary (e.g. due to highly diverging baseline values close to the exponential phase), the threshold and baseline were adjusted manually.

3. Experimental design

3.1 Specificity testing of the SYBR®Green methods for GM rice originating from China

As to date no official DNA sequence data are available on the complete GM inserts of any GM rice events originating from China; the presence of a particular target in a consignment was defined as follows:

1. A target is considered as being present when detected by a particular method meaning that both the measured T_m-value and the C_t-value fall within the criteria of a positive result. In all other cases (thus, when T_m & C_t values are either pos-neg, or neg-pos, or neg-neg), the target is considered as not detected by the applied method.

2. For the T_m-values, a positive result is obtained when the measured T_m-value corresponds with the T_m (± 1°C) of the corresponding target in the positive control, while a negative result is obtained when the measured T_m-value differs more than ± 1°C from the T_m-value of the corresponding target in the positive control.

3. For the C_t-values, a measurement is positive when an (exponential) amplification is obtained. In all other cases, the measurement is considered negative.

For the verification of the specificity of the respective SYBR®Green Real-time PCR methods, all tests were performed in triplicate using: the phospholipase D (PLD-rice) for rice, the 35S promoter (P-35S) from Cauliflower Mosaic Virus, the nopaline synthase terminator (T-nos) from *Agrobacterium tumefaciens* and the CryIAb/Ac toxin from *Bacillus thuringiensis* (CryIAb/Ac) as targets.

3.2 Sensitivity testing of the SYBR®Green methods on GM rice originating from China

No Limit of Detection (LOD) value was determined for the detection of these targets in the GM rice material as neither the exact copy number of the respective targets in the control samples nor the purity of these control samples were known to the EURL-GMFF.

All methods were tested for their sensitivity using 0.05, 2.5, 5, 10 and 20 pg genomic DNA of control samples from Bt63 and Kefeng6 as template DNA respectively. Taking the rice diploid C-value equalling 0.97 (Arumuganathan *et al*, 1991), the corresponding haploid genome equivalents (HGE) would be 0.1, 5, 10, 20 and 40 HGE. All measurements were performed using six replicates.

4. Method

A description of the use of the respective SYBR®Green Real-time PCR methods in a screening analysis for the presence of GMO in rice consignments at the EURL-GMFF and the interpretation of the obtained results is given below. A schematic representation of all steps in the analysis is shown in figure 1.

- A laboratory sample of 2.5 kg is to be provided to the analysing laboratory.

Note: For the purpose of Article 11(5) of Regulation (EC) No 882/2004 (2), a second laboratory sample shall be constituted.

- From the homogenised laboratory sample four analytical samples of 240 grams (equivalent to 10,000 rice grains) are collected.

- The four analytical samples are ground and further analysed separately.

- Total DNA is extracted from each analytical sample and the total DNA content is then determined by PicoGreen® as described above.

- From the extracted DNA, a suitable amount (preferentially 20 ng/reaction) is analysed in duplicate by SYBR®Green Real-time PCR.

- All PCR analyses are performed as described above. For the detection of GM rice originating from China. in food and feed products, PCR methods for the following targets are included: the phospholipase D (PLD-rice) for rice, the 35S promoter (P-35S) from Cauliflower Mosaic Virus, the nopaline synthase terminator (T-nos) from

Agrobacterium tumefaciens and the CryIAb/Ac toxin from *Bacillus thuringiensis* (CryIAb/Ac)

Note: In addition to the above PCR targets, a CaMV donor-specific marker can be included to exclude false positives of the p35S target. Also, inclusion of PCR methods to detect soybean, maize, rapeseed and cotton can be included for confirmation of absence of these species as an ingredient in the rice consignment (Mbongolo Mbella et al., 2011)

- The T_m- and C_t-values obtained from each target are then independently scored applying the beforehand established acceptance ranges for both the T_m- and the C_t-value of each method (see above).

Interpretation of the PCR results per subsample per target:

As in SYBR®Green Real-time PCR analysis two parameters, the T_m and the C_t-value, determine the presence of a target in a sample, in total 8 combinations are possible when taking a decision on the basis of 2 repetitions (2 x 2^2 combinations) (see also Van den Bulcke *et al.*, 2010):

Target	*Case 1*		*Case 2*		*Case 3*		*Case 4*		*Case 5*		*Case 6*		*Case 7*		*Case 8*	
	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t
Rep 1	+	+	-	-	-	+	+	-	+	-	+	+	-	+	+	+
Rep 2	+	+	-	-	-	+	+	-	+	+	+	-	+	+	-	+
Interpretation	Present		Not detectable		Not detectable		Not detectable		Subsample 1 to be repeated		Subsample 2 to be repeated		Subsample 1 to be repeated		Subsample 2 to be repeated	

In all cases where one parameter of a target does not provide the same outcome in the two repetitions (cases 5-8), a third repetition for that particular target is to be performed. When this re-testing is negative, the target is to be considered as 'Not detectable', when this re-testing is positive, the target is to be considered 'Present'.

Decision:

In absence of any other detectable ingredients in the sample and in the absence of evidence for the presence of donor-organisms, the detection of any of the three GM targets in any of the four subsamples of a rice product renders this product as containing GM rice material.

Three examples of possible outcomes and the conclusion are shown in figure 1.

5. Deviations reported

No deviations have been reported during the course of these analyses.

6. Summary of results

6.1 Testing of the specificity of the respective SYBR®Green methods on GM rice samples

All four SYBR®Green Real-time PCR methods were tested for their specificity on non-GM rice, the LibertyLink GM rice events LL601 and LL62 and on the GM events Bt63, Kefeng6 and KMD1 originating from China. The results of the analysis are shown in table 2.

All methods are shown to detect the expected targets in each of the GM events and do not give background signals with non-GM rice.

In separate studies, these methods were shown not to recognize any non-specific targets in genomic DNA from non-GM soya, maize, cotton, rapeseed, potato and sugar beet (Barbau-Piednoir *et al*., 2010; Mbongollo Mbella *et al*., 2011).

6.2 Testing of the sensitivity of the respective SYBR®Green methods on GM rice samples

The integrity of the respective genomic DNA samples of all tested materials was verified by agarose gel-electrophoresis. All materials contained high-molecular weight genomic DNA and no visual traces of degradation could be observed (see figure 2).

As the exact insert(s) present in the respective GM events originating from China has not been officially reported to the EURL-GMFF, the exact targets in terms of expected haploid genome copies could not be determined. For this, a minimal performance sensitivity test was performed to estimate the sensitivity of the methods for detection of GM rice Bt63 and Kefeng6.

For this, a dilution series ranging from an estimated 40 to 0.1 HGE was analysed in six repetitions with all three GMO screening methods (see table 3).

All methods are shown to detect reproducibly (6/6 positives) at least 5 haploid genome copies in both GM events. For all methods, the 0.1 HGE level still resulted in relatively frequent numbers of positive results (up to 3/6 positives). Considering that the no-template controls and the wild-type rice controls do not give any positive signal, this higher frequency is presumably due to the presence of multiple target copies at different chromosomal locations in both GM events.

7. Conclusions

The four SYBR®Green Real-time PCR methods targeting the phospholipase D (PLD-rice) in rice, the 35S promoter (P-35S) from Cauliflower Mosaic Virus, the nopaline synthase terminator (T-nos) from *Agrobacterium tumefaciens* and the CryIAb/Ac toxin from *Bacillus thuringiensis* (CryIAb/Ac) allowed detecting the respective targets in

control samples of GM rice originating from China. All methods are capable to detect at least five haploid genome copies of the GM rice Bt63 and Kefeng6 control DNAs.

These four SYBR®Green Real-time PCR methods can thus be used to screen rice consignments originating from China for the presence of GM materials.

8. Quality assurance

The EURL-GMFF carries out all operations according to ISO 9001:2008 (certificate number: CH-32232) and all technical activities regarding method validation under REg. EC1981/2006 according to ISO 17025:2005 (certificate number: ACCREDIA 1172, [Flexible Scope for DNA extraction and qualitative /quantitative PCR] - Reference Methods available at the Accredia WebSite under: http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7).

9. References

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Barbau-Piednoir E, Lievens A, Mbongollo Mbella EG, Leunda-Casi A, Roosens N, Sneyers M, and Van den Bulcke M. (in press) "SYBR®Green qPCR screening methods for the detection of Roundup Ready®, LibertyLink® and CryIAb traits in genetically modified materials"

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Table 1: *Targets, corresponding primer sequences, and predicted molecular weight of the resulting PCR amplicons as used in this study.*

Target (method)	Primer name	Primer sequence	Amplicon [bp]
PLD–rice[1]	PLD-Fwd	GCTTAGGGAACAGGGAAGTAAAGTT	*80*
	PLD-Rev	CTTAGCATAGTCTGTGCCATCCA	
P-35S[2]	35S-Fwd	AAAGCAAGTGGATTGATGTGATA	*75*
	35S-Rev	GGGTCTTGCGAAGGATAGTG	
T-nos[2]	Tnos-Fwd	GATTAGAGTCCCGCAATTATACATTTAA	*69*
	Tnos-Rev	TTATCCTAGKTTGCGCGCTATATTT	
CryIAb/Ac[3]	CryIAb/Ac- Fwd	ACCGGTTACACTCCCATCGA	*73*
	CryIAb/Ac- Rev	CAGCACCTGGCACGAACTC	

1: Mbongolo Mbella et al., 2011; 2: Barbau-Piednoir et al, 2009; 3: Barbau-Piednoir et al, submitted

Table 2: *Specificity assessment of the four SYBR®Green Real-time PCR methods using genomic DNA from relevant GM rice events.*
For the T_m- and the C_t-values the average value of the three replicates is indicated for any "+" measurement, while a "-" means that for this parameter the measured value was outside the acceptance interval (for the T_m-measurement) or not detected (for the C_t measurement).

Sample Name	**Species**	**GM % m/m**	**Origin**	**PLD-rice**			**CryIAb/Ac**			**P-35S**			**T-Nos**		
				Expected presence	C_t	T_m	Expected presence	C_t	T_m	Expected presence	C_t	T_m	Expected presence	C_t	T_m
Bt11	Maize	100	EURL-GMFF	No	-	-	Yes	25.52	**78.2**	Yes	27.43	**76.3**	Yes	25.52	**71.3**
Mon810	Maize	100	EURL-GMFF	No	-	-	Yes	26.18	79.5	Yes	30.72	**76.1**	No	-	-
Wt Minghui86 Rice	Rice	0	China	Yes	+	**77.2**	No	-	-	No	-	-	No	-	-
LL62	Rice	100	AOCS	Yes	+	**77.2**	No	-	-	Yes	27.41	**75.9**	No	-	-
LL601	Rice	100	EURL-GMFF	Yes	+	**77.0**	No	-	-	Yes	29.56	**75.9**	No	-	-
Bt63	Rice	Not reported	China	Yes	+	**77.2**	Yes	23.39	**78.8**	No	-	-	Yes	26.61	**71.3**
Kefeng6	Rice	Not reported	China	Yes	+	**77.2**	Yes	23.19	**78.6**	Yes	26.66	**75.9**	Yes	27.21	**71.3**
KMD1	Rice	Not reported	China	Yes	+	**77.2**	Yes	27.4	**78.2**	Yes	31.46	**75.9**	Yes	30.21	**71.1**
				SYBR®Green Real-time PCR											

Abbreviations: "PLD-rice": phospholipase D of rice (*Oryza sativa*); "P-35S": 35S promoter from Cauliflower Mosaic Virus, "T-nos": nopaline synthase terminator from *Agrobacterium tumefaciens*; "CryIAb/Ac": CryIAb or CryIAc toxin (or a combination) from *Bacillus thuringiensis.*

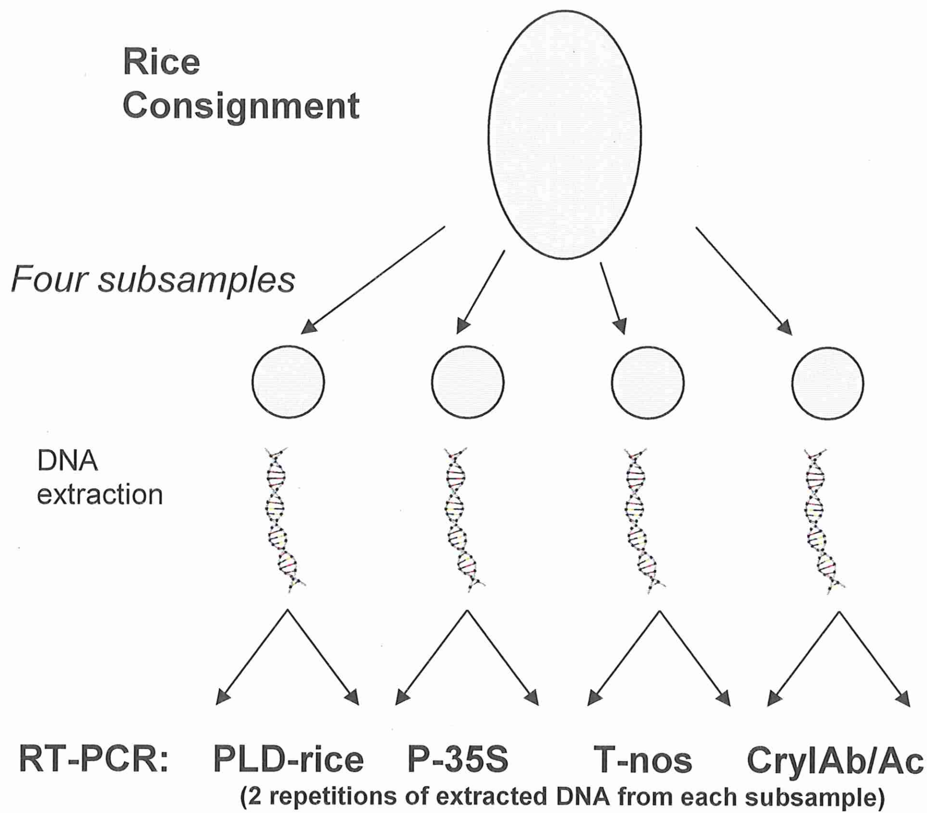
Table 3: *Sensitivity testing of the P-35S, T-nos and CryIAb/Ac SYBR®Green Real-time PCR methods on a dilution series of genomic DNA from the GM rice events Kefeng6 and Bt63*. The indicated values of the T_m and C_t are the average of all positives.

Kefeng6 rice

	P-35S			T-nos			CryIAb/Ac		
estimated copies	# pos. (n=6)	C_t	T_m	# pos. (n=6)	C_t	T_m	# pos. (n=6)	C_t	T_m
40	6	30,15	76,25	6	28,12	71,72	6	27,17	79,40
20	6	31,09	76,08	6	29,16	72,10	6	28,18	79,13
10	6	31,95	76,35	6	30,08	71,77	6	29,24	79,35
5	6	33,12	76,30	6	31,17	71,43	6	30,10	79,10
1	6	35,23	76,05	6	33,19	71,5	6	31,94	79,15
0,1	3	33,12	76,30	3	36,44	71,47	1	34,60	78,80
0,02	0	-	-	1	36,79	72	1	34,18	78,9
0,002	0	-	-	0	-	-	0	-	-

Bt63 rice

	T-nos			CryIAb/Ac		
estimated copies	# pos. (n=6)	C_t	T_m	# pos. (n=6)	C_t	T_m
40	6	29,87	71,98	6	27,97	79,10
20	6	30,95	72,15	6	28,57	78,92
10	6	32,01	71,87	6	29,91	78,55
5	6	32,81	71,62	6	30,75	79,23
1	5	35,33	71,98	5	32,87	78,9
0,1	3	36,61	71,47	2	34,53	79,2
0,02	0	-	-	1	34,26	78,5
0,002	0	-	-	0	-	-



Outcome of PCR screening (3 examples)

Conclusion:

Product A	Subsample 1	Subsample 2	Subsample 3	Subsample 4
PLD-rice	+/+	+/+	+/+	+/+
P-35S	-/-	-/-	-/-	-/-
T-nos	-/-	-/-	-/-	-/-
CryIAb/Ac	-/-	-/-	-/-	-/-

GM negative
Accept

Product B	Subsample 1	Subsample 2	Subsample 3	Subsample 4
PLD-rice	+/+	+/+	+/+	+/+
P-35S	-/-	-/-	-/-	-/-
T-nos	-/-	+/+	-/-	-/-
CryIAb/Ac	-/-	+/+	-/-	-/-

GM positive
Reject

Product C	Subsample 1	Subsample 2	Subsample 3	Subsample 4
PLD-rice	+/+	+/+	+/+	+/+
P-35S	+/+	+/+	-/-	+/+
T-nos	+/+	+/+	-/-	+/+
CryIAb/Ac	+/+	+/+	-/-	+/+

GM positive
Reject

Figure 1*: Analytical scheme of the PCR screening procedure for the presence of GM rice materials in rice consignments* ***originating from China*** (detailed description is given in the text).

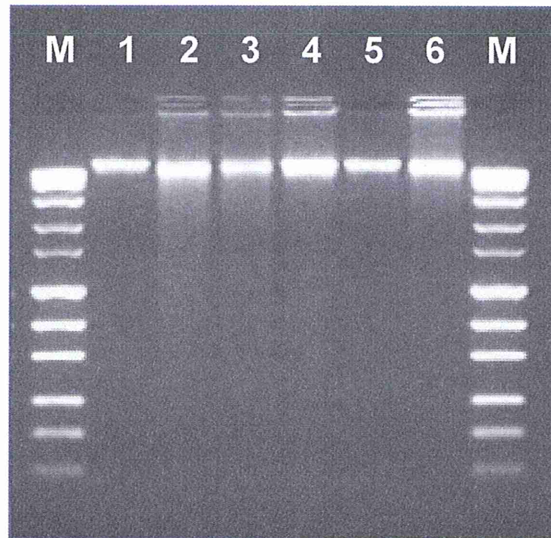


Figure 2: *Agarose gel-electrophoresis of genomic DNA of control samples of GM rice applied in this study*. In all assays, 100ng of total genomic DNA was loaded. Sample order: M (= Molecular weight marker); 1: LL62; 2: Bt63; 3: Kefeng6; 4: LL62; 5:LL601; 6: MingHui.