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Grain testing method for detection of Rice GM events containing P35S::bar sequences using RT-PCR protocols PGS0494 and PGS0476

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Document approval

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Remarks

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General information

This method describes a *Trait-specific* Real-time semi-quantitative TaqMan® PCR procedure for the determination of the presence of *P35S::bar* sequence-containing DNA in Rice species DNA extracted from Rice grain samples. For each Laboratory sample under examination, three representative "Test samples" of 30 grams are taken. On each individual Test sample one single DNA extraction is performed followed by two replicate Real-time PCR reactions.

The method has been optimized for use by an ABI Prism® 7500 sequence detection system. Other systems may be used, but thermal cycling conditions must be verified. The use of 200 ng template DNA per reaction is recommended.

For specific detection of *P35S::bar* DNA, a fragment of the *P35S::bar* sequence is amplified using two specific oligonucleotide primers (protocol PGS0494). The exact size of the amplicon in base pairs depends on the specific *P35S::bar* construct or vector that is amplified. PCR products are measured during each cycle (real-time) by means of a Target-specific oligonucleotide probe labeled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and MGBNFQ as a quencher at its 3′ end (MGB Taqman probe).

For *relative* quantitation of *P35S::bar* containing DNA in total Rice species DNA, a Rice-specific Reference system (protocol PGS0476) amplifies a 68-bp fragment of *phospholipase D*, a Rice endogenous sequence, using a pair of sequence-specific primers and a sequence-specific probe labeled with VIC as reporter dye at its 5′ end and TAMRA as a quencher at its 3′ end (TAMRA Taqman probe).

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantitation of the amount of *Target* DNA in a Test sample the ΔΔCt method is applied. This method is a comparative method that uses arithmetic formulas to achieve relative quantification. In this method the amount of Target DNA is expressed relative to a reference sample (i.e. the Positive Sample Control [PSC] bulk sample).

For all Test samples, the Target quantity is determined and divided by the Target quantity of the *reference* sample. Thus, the reference sample becomes the 1X sample; all other quantities are expressed as an n-fold difference relative to the reference sample. This relative amount of Target DNA is used to determine the presence level of the Target sequence in the test DNA sample.

Validation Status

The development of this method was carried out according to Bayer CropScience standard operating procedures including the method acceptance criteria and the method performance requirements. A brief summary is provided under “Performance Characteristics”.

Official Verification Status

The Real-time PCR method modules (protocols PGS0494 and PGS0476) that are part of the procedure described in this document have been officially verified by the United States Department of Agriculture (USDA), Grain Inspection Packers and Stockyards Administration (GIPSA): http://www.gipsa.usda.gov

Rice grain DNA extraction

The detection method described in this document has been developed, tested and validated following the application of rice grain DNA extraction protocol PGS0426. This protocol has been validated and published by the European Union (EU), Joint Research Centre (JRC), Community Reference Laboratory in the context of the validation of the detection method for the LLRICE62 event. The rice grain DNA extraction protocol, including the validation results, are available at http://gmo-crl.jrc.it/summaries/LLRICE62_DNAExtr_sampl.pdf.

Performance characteristics

Applicability

This method was optimized for analysis of 200 ng DNA extracted from grain samples containing mixtures of Rice harboring the *P35S::bar* sequence and conventional Rice, according to DNA extraction protocol PGS0426, which was validated and published by the JRC-CRL:
http://gmo-crl.jrc.it/summaries/LLRICE62_DNAExtr_sampl.pdf.
Other DNA extraction procedures may be used, but performance characteristics must be verified.

Specificity

The Real-time PCR system PGS0494 for detection of the *P35S::bar* Target was tested on a number of non-Target DNA samples and was shown to be Target-specific. The Real-time PCR system PGS0476 for detection of the Rice Reference system was tested on a number of DNA samples derived from other crop taxa and was shown to be taxon-specific.

Stability

The Real-time PCR method quantifies the relative DNA amount of the *P35S::bar* Target relative to total Rice DNA through the *phospholipase D* endogenous gene used as Reference system, which should be quantitatively stable in different genetic backgrounds in order to allow stable testing results. It was experimentally demonstrated that the Reference system was sufficiently quantitatively stable in the different genetic backgrounds tested in order to allow stable testing results.

Amplification efficiency

Note that it has been observed that the Target reaction shows differential amplification efficiencies for different targets. When *LLRICE601* event DNA is used as template DNA lower amplification efficiencies are achieved compared to when *LLRICE62* event DNA is used.

Limit of Detection (LOD)

This method was validated using a number of Rice grain bulk sizes spiked with different fractions of grain harboring *P35S::bar sequence*-containing material. To detect one single homozygous *P35S::bar*-containing grain, the largest Test sample size validated is 6,000 conventional grains spiked with one single homozygous grain containing the *LLRICE62* event, and 1,500 conventional grains spiked with one single homozygous grain of the *LLRICE601* event.

Real-Time PCR for semi-quantitative analysis

The PCR set-up for the Reference system (i.e. Endogenous taxon control) and for the Target sequence should be carried out separately (Simplex). Duplex PCR, i.e. using different fluorescent labels for the probes in a single reaction, has not been tested, nor validated, and is not recommended.

For each PCR, following control samples must be included in the analysis:

1. "Positive Sample Control" (PSC): 200 ng of DNA extracted from a 3,000 conventional grains bulk sample containing one single Target-containing grain of the *LLRICE62* event.

2. "Negative Sample Control" (NSC): 200 ng of DNA extracted from conventional rice grain.

3. "Sensitivity Sample Control" (SSC): 200 ng of DNA extracted from a 6,000 conventional grains bulk sample containing one single homozygous Target-containing grain of the *LLRICE62* event.

4. "No Template Control" sample (NTC): A sample of the PCR matrix containing no template DNA, e.g. water instead of DNA.

For the analysis of the PSC and NSC samples, *two* DNA extractions are performed, followed by *duplicate* Real-time PCR analysis per DNA extract.

For the analysis of the SSC sample, *one* DNA extraction is performed, followed by *duplicate* Real-time PCR reactions per DNA extract

For each of the three Test samples, per laboratory sample analyzed, *one* DNA extraction is performed, followed by *duplicate* Real-time PCR reactions per DNA extract.

Separate amplification curves for each primer / probe system (Target and Reference system) are preferably generated in the same analytical amplification run.

Real-Time PCR set-up

1. Mix gently and centrifuge the required amount of components needed for the run. Keep thawed reagents cooled below 4°C.
2. To prepare the master mixes, while keeping all tubes cooled below 4°C, add apart from the DNA samples, the reagents in the order pointed out in Table 1a and 1b in two separate reaction tubes, one for the Target run and one for the Reference system run. Always prepare sufficient amounts of Master Mix by including an extra number of reactions.
3. Mix gently and centrifuge briefly
4. Add **10 µL** of the NTC, each of the control DNA samples (20 ng/µl genomic DNA) and test DNA samples (20 ng/µl genomic DNA)
5. Dispense **15 µL** per reaction of the PCR mix for the *P35S::bar* Target reaction
6. Dispense **15 µL** per reaction of the PCR mix for the *Phospholipase D* Reference system reaction
7. Place an optical seal on the reaction plate and briefly centrifuge the plate
8. Run the PCR with cycling conditions described in Table 2

Table 1a. PCR mix for one *P35S::bar* Target reaction (PGS0494).

Components	Final concentration	Volume per reaction
1. Universal Master Mix (2X)	1x	12.5 µL
2. MDB498 (10 µM)	400 nM	1 µL
3. DPA143 (10 µM)	400 nM	1 µL
4. TM099 (10 µM)	200 nM	0.5 µL
PCR Mix volume		**15 µL**
200 ng control DNA / unknown DNA		10 µL
Total volume		**25 µL**

Table 1b. PCR mix for one *Phospholipase D* Reference system reaction (PGS0476).

Components	Final concentration	Volume per reaction
1. Nuclease-free water	-	1 µL
2. Universal Master Mix (2X)	1x	12.5 µL
3. KVM159 (10 µM)	200 nM	0.5 µL
4. KVM160 (10 µM)	200 nM	0.5 µL
5. TM013 (10 µM)	200 nM	0.5 µL
PCR Mix volume		**15 µL**
200 ng control DNA / unknown DNA		10 µL
Total volume		**25 µL**

Table 2. Cycling conditions.

	Stage	Temp.	Time	Data Collection	Cycles
1	UNG	50°C	2 min	No	1
2	Initial denaturation	95°C	10 min	No	1
3	Amplification	95°C	15 sec	No	45
		60°C	1 min	Yes	

Data analysis

Subsequent to the Real-time PCR, analyze the run following the procedure below:

1. Use the default baseline range from cycles 3 to 15.
2. Set the threshold: display the amplification curves of one system (e.g. Target) using the ΔRn-axe displayed in the logarithmic mode. Locate the threshold line in the centre of the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the update button to ensure changes affect the Ct values.
3. Repeat the procedure described under 1) and 2) on the amplification plots of the other system (e.g. Reference system).

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument’s software calculates the Ct-values for each reaction.

Save the settings and export all the data into spreadsheet software, e.g. Microsoft Excel for further calculations.

Materials

Equipment

1. 1.5 mL and 2.0 mL microcentrifuge tubes
2. Vortex
3. Micropipettes
4. Microcentrifuge
5. MicroAmp Optical 96-well Reaction Plate (ABI Product number 4306737) and Optical seals (ABI Product number 4311971) or equivalent
6. Block-type thermal cycler real-time PCR system

Reagents

1. TaqMan® Universal PCR Master Mix (ABI Product 4304437) or equivalent
2. Primers and probes (HPLC-purified) described in table 3

Table 3: Primer and probe sequences and fluorescence labels.

***P35S::bar* Target Reaction**		
Name	**Description**	**5' – 3' sequence**
MDB498	Forward primer	TAT.CCT.TCg.CAA.gAC.CCT.TCC
DPA143	Reverse primer	ATg.TCg.gCC.ggg.CgT.CgT.TCT.g
TM099	Probe	6FAM 5'- TCT.ATA.TAA.ggA.AgT.TCA.TTT.CAT.T-3' MGBNFQ

***Phospholipase D* Reference system Reaction**		
Name	**Description**	**5' – 3' sequence**
KVM159	Forward primer	Tgg.TgA.gCg.TTT.TgC.AgT.CT
KVM160	Reverse primer	CTg.ATC.CAC.TAg.CAg.gAg.gTC.C
TM013	Probe	VIC-TgT.TgT.gCT.gCC.AAT.gTg.gCC.Tg-TAMRA