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International Ring Trial for the Validation of an Event-Specific Golden Rice 2 Quantitative Real-Time Polymerase Chain Reaction Method

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Supporting Information

ABSTRACT: This article describes the international validation of the quantitative real-time polymerase chain reaction (PCR) detection method for Golden Rice 2. The method consists of a taxon-specific assay amplifying a fragment of rice Phospholipase D α2 gene, and an event-specific assay designed on the 3′ junction between transgenic insert and plant DNA. We validated the two assays independently, with absolute quantification, and in combination, with relative quantification, on DNA samples prepared in haploid genome equivalents. We assessed trueness, precision, efficiency, and linearity of the two assays, and the results demonstrate that both the assays independently assessed and the entire method fulfill European and international requirements for methods for genetically modified organism (GMO) testing, within the dynamic range tested. The homogeneity of the results of the collaborative trial between Europe and Asia is a good indicator of the robustness of the method.

KEYWORDS: *international validation, Golden Rice 2 (GR2), quantitative real-time PCR (qPCR), genetically modified organism (GMO), endogenous taxon-specific gene, event-specific method*

INTRODUCTION

In rice the biosynthetic pathway of β-carotene, a precursor of vitamin A, is normally active in leaves but not in the eaten part of the plant, the grain, mainly constituted by endosperm. Golden Rice 2 (GR2) produces and accumulates β-carotene in the endosperm and was engineered with the aim of fighting vitamin A deficiency, which affects the life of millions of people who do not have access to enough fresh vegetables, dairy product, eggs and meat, but rely on rice as a staple food.[1−5] GR2 belongs to the second generation of Golden Rice plants and accumulates significantly more β-carotene in the endosperm than the first generation of Golden Rice. The International Rice Research Institute in the Philippines (IRRI) is coordinating the introgression (i.e., introduction by conventional breeding) of the GR2 trait into local varieties widely used by farmers and fit for the target growing conditions.[6] Cultivation in the target countries will require authorization by the responsible authorities, which in most cases is guaranteed only if a method for the detection and, in case of labeling requirements, quantification of the GM event is available.

The use of DNA-based methods, and in particular polymerase chain reaction (PCR), is preferred to other analytical approaches for genetically modified organisms (GMOs) screening and quantification because PCR has been proven to be very accurate, both when applied to raw ingredients and to processed food/feed samples.[7−11] PCR and real-time PCR are internationally recognized and recommended for GMO analysis.[12,13] For the development of a method for the detection of GR2, IRRI asked for the collaboration of the Molecular Biology and Genomics (MBG) Unit of the Institute for Health and Consumer Protection of the European Commission Joint Research Centre. The MBG Unit hosts the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF), which has 10 years of experience in validation of quantitative real-time PCR (qPCR) methods and has fully validated about 60 methods for GMO detection.[14−17] The EU-RL GMFF, together with the European Network of GMO Laboratories (ENGL), defined globally accepted performance requirements for analytical methods of GMO testing.[18] These requirements are also in line with the Codex Alimentarius guidelines for methods for detection, identification, and quantification of specific DNA sequences and proteins in food.[19] The developed method is a simplex TaqMan event-specific method for the determination of the relative content of GR2 DNA to total rice DNA and is composed of two qPCR assays: a new rice taxon-specific assay, PLD-GR, which amplifies a 124 bp fragment of rice Phospholipase D α2 (PLD) gene, and an event-specific assay, GR2, which amplifies a 121 bp fragment across the 3′ junction between transgenic insert and plant DNA. Detailed information on the development and single laboratory validation of the method, including assessment of the copy number and stability of the taxon-specific assay, and of the specificity, limit of detection (LOD), limit of quantification (LOQ), dynamic range, and robustness of the PLD-GR and GR2 assays, can be found in the dedicated article.[20]

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This paper reports on the subsequent international validation of the method through a collaborative trial. Such a validation trial is a fundamental step to ensure that the method performance measured during single laboratory validation is reproducible when the method is run by different laboratories, operators, instruments, and conditions, and in this study notably in different geographic regions. It verifies the robustness and fitness for purpose of the method, by testing that its performance matches pre-established criteria when it is used in a population of laboratories that is representative of the target users.[21−23] The immediate purpose of the development of the detection method for GR2 is the authorization for cultivation and for food use of the GM rice in the Philippines, where labeling is mandatory for foods containing GM ingredients above a 5% threshold,[24] and at later stages in other Asian countries (e.g., Indonesia and Bangladesh). However, the method could in the future be needed by European control laboratories to check for the presence of GR2 in rice imports from Asia.[25−29] Thus, for the validation of the GR2 detection method, we selected eight laboratories in Europe, of which seven are European national reference laboratories and one operates in Switzerland,[30] and eight laboratories in Asia, belonging to the official GMO control networks including universities, national institutes, and food safety authorities. The 16 laboratories were selected among those potentially going to use the method on the basis of their geographic location, with the aim of obtaining a good distribution of test results across the European and Asian territory.

The analytical procedures for the detection of GMOs, from sampling to determination of GM content, are composed of distinct successive or parallel steps, termed modules. Different modules can be validated separately and combined together afterward, provided that the pre-established performance criteria are respected for the preceding or parallel module.[23,31−33] This approach allows higher flexibility than the validation of the full method because independently validated modules (e.g., DNA extraction and qPCR) can be combined together according to their availability or appropriateness for the target material. In many cases it allows reducing the costs of method validation: when one or more modules that constitute a method are already validated there is no need to include them in the validation study. We considered that the taxon- and the event-specific PCR-assays that constitute a qPCR method can also be regarded as modules and, thus, chose to validate them independently.[31−34] The validation samples were prepared in haploid genome equivalents (hge), calculated by taking into account a rice haploid genome size of 0.5 pg.[35] The rice material used for this study was homozygous, with a ratio between the GM event and the taxon-specific gene of 1;[20] the GM percentages expressed in hge percentages are thus equivalent to mass GM percentages.

The experimental design and results of the validation of the qPCR detection method for GR2 are presented and discussed in the following paragraphs.

MATERIALS AND METHODS

Material and ***DNA extraction*** are described in the Supporting Information.

Quantitative Real-Time PCR. The qPCR reactions for the PLD-GR taxon-specific assay and the GR2 event-specific assay are optimized to be performed in a volume of 25 μL including 5 μL of DNA sample; the primers and probe sequences for each assay are reported in Table S1, Supporting Information. The qPCR reaction mixtures of PLD-GR and GR2 assays contained the following reagents: 1× TaqMan universal master mix (Applied Biosystems, Warrington, United Kingdom), 300 nM primer forward, 300 nM primer reverse (Eurofins Genomics, Ebesberg, Germany), 150 nM probe (Applied Biosystems, Warrington, United Kingdom), 5 μL DNA solution (between 0.02 ng and 200 ng of template DNA per reaction), and nuclease free water (Promega, Madison, USA) to reach the volume of 25 μL. The thermal profile was as follows: 50 °C for 2 min; 95 °C for 10 min; 45 cycles of 95 °C for 15 s, and 60 °C for 60 s; fluorescence data were acquired at the end of the elongation step of each cycle.

Sample Preparation. Two sets of samples were prepared, one for the validation of the taxon-specific assay, labeled PLD, and one for the validation of the GR2 event-specific assay and for a test of the performance of the entire method, labeled GR2. The two sets of samples, consisting of five standard curve samples and 20 blind samples each, were prepared and shipped to all laboratories. PLD and GR2 standard curve samples were prepared from non-GM and GM DNA, respectively, by serial dilution and were labeled S1−S5 PLD/GR2. The hge content and DNA concentration of the standard curve samples are described in Tables S2 and S4, Supporting Information. PLD and GR2 blind samples were prepared independently and consisted of five samples at different DNA concentrations labeled B1−B5 PLD/GR2, which were then subdivided into four samples each and randomly labeled from U1 to U20 PLD/GR2. PLD blind samples were prepared with non-GM DNA, diluted to obtain the desired hge content; GR2 blind samples were prepared with GM DNA, diluted in a background of non-GM DNA to obtain the desired GM hge content in the defined total hge content. The hge content and DNA concentration of the blind samples are described in Tables S3 and S5, Supporting Information. All dilutions were prepared with 0.1× TE and all samples were thoroughly homogenized before any subsequent use. After preparation the samples were tested with a qPCR experiment and divided into 21 aliquots each. The validation kits were prepared from those aliquots and one randomly chosen kit was tested in accordance to the validation protocol prior to the shipment of kits to participating laboratories.

International Collaborative Trial. The collaborative trial was organized by the EU-RL GMFF: 16 laboratories evenly distributed over European and Asian countries were selected to participate to the study (see list of participating laboratories in Table S6, Supporting Information). Each laboratory received a kit for the validation study that included all reagents and samples needed for the validation, with the exception of the no-template control sample, that had to be prepared. Kits were shipped in dry ice to all laboratories with the exception of those located in China and India, for which blue ice had to be used due to the impossibility to ship in dry ice to those countries. All participating laboratories also received a detailed protocol to follow and an excel file for reporting results and were requested to report any deviation from the protocol which may have occurred during the preparation and execution of the qPCR experiments. The laboratories had to run three qPCR plates: one loaded with PLD standard curve samples and PLD blind samples (plate A), for the quantification of PLD blind samples with the PLD-GR assay; one for the quantification of GR2 blind samples with the GR2 assay, loaded with GR2 standard curve samples and GR2 blind samples (plate B); and one for the quantification of the amount of rice taxon-specific PLD target in GR2 blind samples with the PLD-GR assay, loaded with PLD standard curve samples and GR2 blind samples (plate C). Each sample was pipetted in three adjacent wells and the three resulting Cq values were averaged. A no template control was included in the plate, also pipetted in triplicate. The four blind samples with the same hge content were considered as independent replicates, yielding a total of four replicated measurements per laboratory for each blind sample. The layout of the plate setup is available in Figures S1−S3, Supporting Information.

Performance Parameters. In accordance with ISO 5725-1[21] we evaluated two measures of accuracy: trueness and precision. Trueness was expressed as mean percent bias (difference between the mean value of the test results and the accepted reference value divided by the

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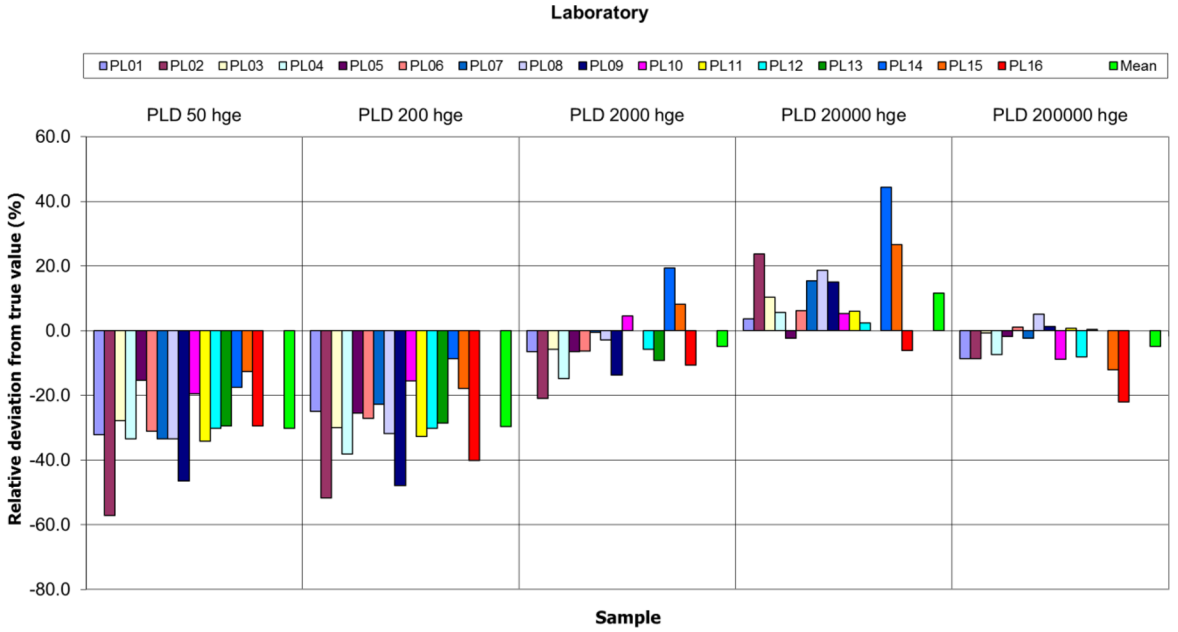

Figure 1. Relative percent deviation from the target hge content for PLD-GR taxon-specific assay. Colored bars represent the difference between the hge measured by each laboratory and the true hge content in percentage of the latter; the green bar on the right represents the mean of the relative deviations of all laboratories for each level of hge content. PL01-PL16: codes assigned to the 16 testing laboratories; PL11 at 2000 hge, PL13 at 20 000 hge, and PL14 at 200 000 hge do not show up in the graph because their results for those levels were excluded from the analysis as outliers.

Table 1. Summary of Validation Results for the PLD-GR Taxon-Specific Assay (16 Laboratories)

	test sample haploid genome equivalents				
	level 5 50	level 4 200	level 3 2000	level 2 20000	level 1 200000
number of outliers	0	0	1	1	1
reason for exclusion[a]			C	C	S/D
measured hge content	**35**	**141**	**1905**	**22335**	**190438**
(95% CI)	(32; 38)	(129; 153)	(1795; 2014)	(20911; 23758)	(182734; 198143)
relative repeatability standard deviation, RSD_r %	19.6	13.9	9.0	7.1	8.1
relative reproducibility standard deviation, RSD_R %	23.2	20.0	13.0	13.0	10.1
bias %	−30.2	−29.6	−4.8	11.7	−4.8
(95% CI)	(−36.1; −24.3)	(−35.6; −23.6)	(−10.2; 0.7)	(4.6; 18.8)	(−8.6; −0.9)

[a]C, Cochran test; S, single Grubbs test; D, double Grubbs test.

accepted reference value). Precision was expressed as relative repeatability standard deviation (RSD_r) in percent relative to the accepted reference value, and as relative reproducibility standard deviation (RSD_R) in percent relative to the accepted reference value. We evaluated also two other performance parameters associated with each assay: the amplification efficiency, in percent, and the linearity, expressed as coefficient of determination, R^2. The amplification efficiency was calculated from the slope of the standard calibration curve using the formula: efficiency = $((10^{(-1/\text{slope})}) - 1) \times 100$. Linearity was calculated as coefficient of determination of the standard curve obtained by linear regression analysis. The ENGL minimum performance requirements for analytical methods of GMO testing (MPR) set the criteria that a method must meet to be accepted for validation, and the criteria to be respected to declare the method valid after the international validation. These criteria are in line with Codex Alimentarius guidelines.[18,19] We referred to the criteria set in the MPR and Codex documents to evaluate the performance of the qPCR assays and method. According to them the percent bias should be within ±25% of the accepted reference value over the whole dynamic range assessed; the RSD_r should be below or equal to 25% over the whole dynamic range and the RSD_R should be below 35% over the whole dynamic range, or below 50% at relative concentrations minor than 0.2% or hge lower than 100; the average slope values of the standard curves should be in the range between −3.6 and −3.1, corresponding to amplification efficiencies between 90% and 110%; average R^2 values of the standard curves should be equal to or above 0.98.

Data Analysis. The results of the collaborative trial were analyzed following the steps described in ISO 5725-2.[36] The identified outlying results and/or laboratories were excluded from the analysis, in accordance with ISO 5725-2, and were evidenced throughout the text. For the PLD-GR assay, results from plate A and C were compared by evaluating differences in slope, linearity, and efficiency of the standard curves through Bland−Altman plots for agreement. Limits of agreement were obtained as the average difference between paired values from plates A and C plus/minus twice the standard deviation of all paired differences, following the Bland−Altman approach.[37] Homogeneity of results between European and Asian laboratories was assessed by comparing method performance parameters such as mean measured hge/GM % content, RSD_r, RSD_R, and bias. Mean measured GM content and bias were compared by means of two-tailed Student's 95% confidence intervals, whereas variability was evaluated by estimating the coefficient of variation of the measured GM content alongside the 95% confidence interval using the modified McKay method.[38]

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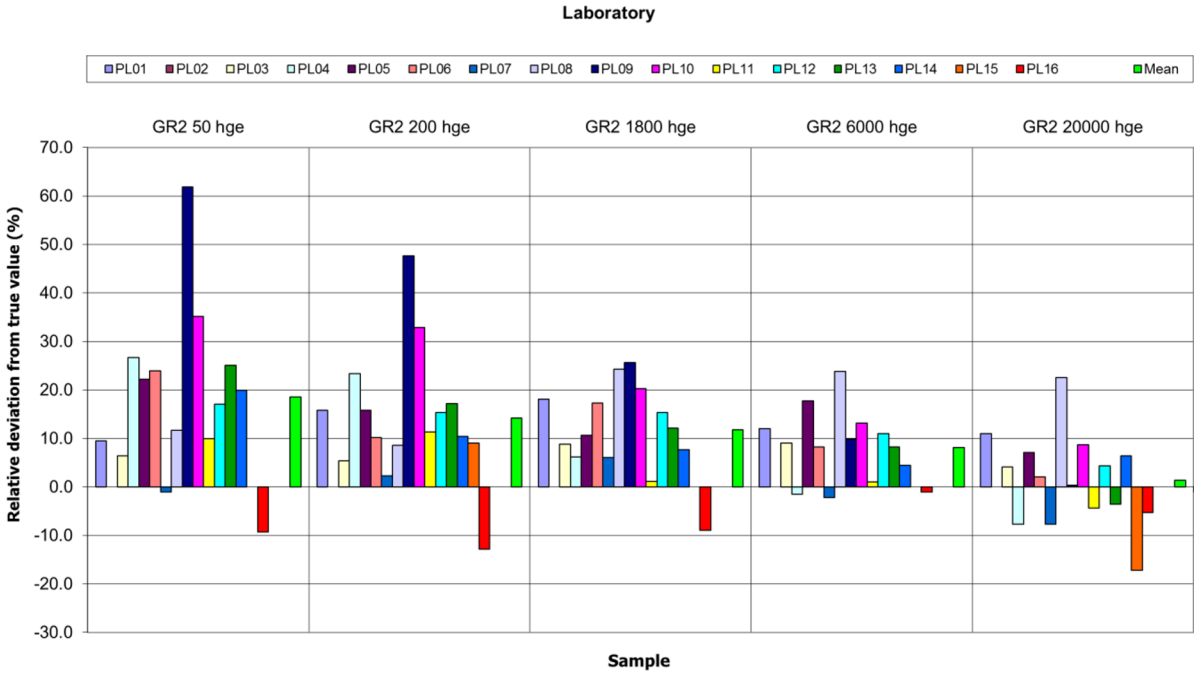

Figure 2. Relative percent deviation from the target hge content for GR2 event-specific assay. Colored bars represent the difference between the hge content measured by each laboratory and the true hge content in percentage of the latter; the green bar on the right represents the mean of the relative deviations for each level of hge content over the analyzed laboratories. PL01−PL16: codes assigned to the 16 testing laboratories. The following values do not show up in the graph: PL2 results for the five levels because they were excluded from the analysis as outliers; PL15 results at 50, 1800, and 6000 hge because they were excluded from the analysis as outliers.

RESULTS

Validation of PLD-GR Taxon-Specific Assay. Results submitted by one laboratory for level 3, and from a second laboratory for level 2, were identified as outliers by the Cochran test at 1% significance level and discarded; results from a third laboratory for level 1 were identified as outliers from the single/double Grubbs test at 1% significance level and discarded from the analysis. The relative deviation from the target hge content (accepted reference value) for each blind sample tested for each laboratory is shown in Figure 1. Mean measured hge content, RSD_r, RSD_R, and bias are reported in Table 1. The measured RSD_r, RSD_R, and the bias measured for three out of five samples meet the criteria, but the two blind samples with lower hge content showed a bias of −30.2% and −29.6%, thus above the limit of −25% set by MPR and Codex guidelines.[18,19] The 95% confidence intervals calculated around the measured hge content include the accepted reference value for level 3, while the accepted reference value for level 2 is higher than the calculated 95% confidence interval and those for levels 1, 4, and 5 are lower. The 95% confidence intervals calculated around the measured bias overlap with the range of values considered acceptable for all tested levels (see Table 1). The full set of quantification results submitted by laboratories is available in Tables S7 and S8, Supporting Information. In addition to the experiment described above (plate A), PLD standard curve samples were tested in a second plate for the relative quantification experiment (plate C) that is described in the paragraph Relative Quantification below. The values obtained by participating laboratories for the slope, amplification efficiency and linearity (R^2) of PLD-GR standard curves are reported in Table S9, Supporting Information. One laboratory could not run plate C, thus standard curve parameters were not reported for plate C for that laboratory. Standard curve parameter values were within the established criteria, ranging from −3.52 to −3.28, with an average of −3.43 for slope, from 92.4 to 101.8, with an average of 95.7% for PCR efficiency and from 0.995 to 0.999, with an average of 0.998 for linearity. Additionally, since plate A and C were performed independently, we compared the slope, PCR efficiency and linearity of the PLD-GR standard curve measured for plate A and C for each laboratory, to identify possible differences between the two plates, yet none was identified. Bland−Altman plots are presented in Figure S4, Supporting Information, and suggest a satisfactory level of concordance between the plates having narrow limits of agreement.

Validation of GR2 Event-Specific Assay. One laboratory was identified as outlier at all hge levels for both Cochran and single/double Grubbs tests at a 1% significance level, thus results from this laboratory were excluded from the analysis. Additionally, results submitted by one laboratory for level 5 were identified as outlier by all outlier tests considered at a 1% significance level and discarded; results from the same laboratory for levels 2 and 3 were identified as outliers by the Cochran test considered at a 1% significance level and discarded. The relative deviation from the target hge content for each blind sample tested for each retained laboratory is shown in Figure 2. In Table 2 the mean hge content, the percentage RSD_r, RSD_R, and bias, all meeting the established criteria, are reported. The accepted reference value for level 1 is included in the 95% confidence interval calculated around the measured hge content, while for levels 2, 3, 4, and 5 the accepted reference value is lower than the calculated 95% confidence interval. The 95% confidence intervals calculated around the measured bias overlap with the range of values considered acceptable for all tested levels (see Table 2). The full set of quantification results submitted by laboratories is available in Tables S10 and S11, Supporting Information. Table

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Table 2. Summary of Validation Results for the GR2 Event-Specific Assay (15 Laboratories)

	test sample haploid genome equivalents				
	level 5 50	level 4 200	level 3 1800	level 2 6000	level 1 20000
number of outliers	1	0	1	1	0
reason for exclusion[a]	all tests		C	C	
measured hge content	**59**	**228**	**2012**	**6489**	**20277**
(95% CI)	(54; 64)	(213; 243)	(1915; 2109)	(6228; 6750)	(19218; 21336)
relative repeatability standard deviation, RSD_r %	10.5	5.3	4.1	4.6	5.1
relative reproducibility standard deviation, RSD_R %	17.1	12.8	9.1	8.0	10.4
bias %	18.5	14.2	11.8	8.2	1.4
(95% CI)	(8.6; 28.4)	(6.6; 21.7)	(6.4; 17.2)	(3.8; 12.5)	(−3.9; 6.7)

[a]C, Cochran test; S, single Grubbs test; D, double Grubbs test.

S12, Supporting Information, reports the slope, amplification efficiency, and linearity values obtained by the participating laboratories for the GR2 standard curve with the GR2 event-specific assay (plate B). Slope values ranged from −3.50 to −3.93, with an average of −3.65; the PCR efficiency ranged from 79.8 to 93.1%, with an average of 88.0%. These values of slope and efficiency slightly exceed the MPR and Codex guidelines.[18,19] However, one laboratory presented outstanding deviation in terms of slope and PCR efficiency for the GR2 assay, very likely due to fact that it received the validation samples with some delay. If we exclude the values of slope and PCR efficiency for that laboratory we obtain an average slope of −3.63 and an average efficiency of 89% for the GR2 assay. Measured linearity, which ranged from 0.993 to 1.000 with an average of 0.997, indicates that the GR2 assay has a linear response over the dynamic range tested.

Relative Quantification. GR2 blind samples were designed to have a variable GM content in a fixed total amount of rice DNA, and the relative GM % content of these samples was calculated by dividing the measured GR2 hge content by the measured PLD taxon-specific gene hge content and multiplying by 100 (GM % = GR2/PLD × 100). The measured GR2 hge content of GR2 blind samples was obtained from the plate run for GR2 event-specific assay validation (plate B); the measured PLD taxon-specific gene hge content was obtained from the plate for the quantification of GR2 blind samples with PLD-GR taxon-specific assay (plate C, see Figures S2 and S3, Supporting Information). Out of 16 laboratories, 14 were retained for the analysis. One laboratory could not run plate C and did not present results for the relative quantification of GR2 samples. Additionally, since the relative quantification of the GM % content of GR2 samples relied on the results of both plates B and C, the outliers discarded during the analysis of the results of plate B were discarded also from this analysis. We thus discarded all results from one laboratory and the results submitted by another laboratory for levels 2, 3, and 5. The relative deviation from the target GM % content for each blind sample tested for each laboratory is shown in Figure 3. Table 3 reports the mean GM % content measured, the percentage RSD_r, RSD_R, and bias calculated for the laboratories retained for the analysis. The 95% confidence intervals calculated around

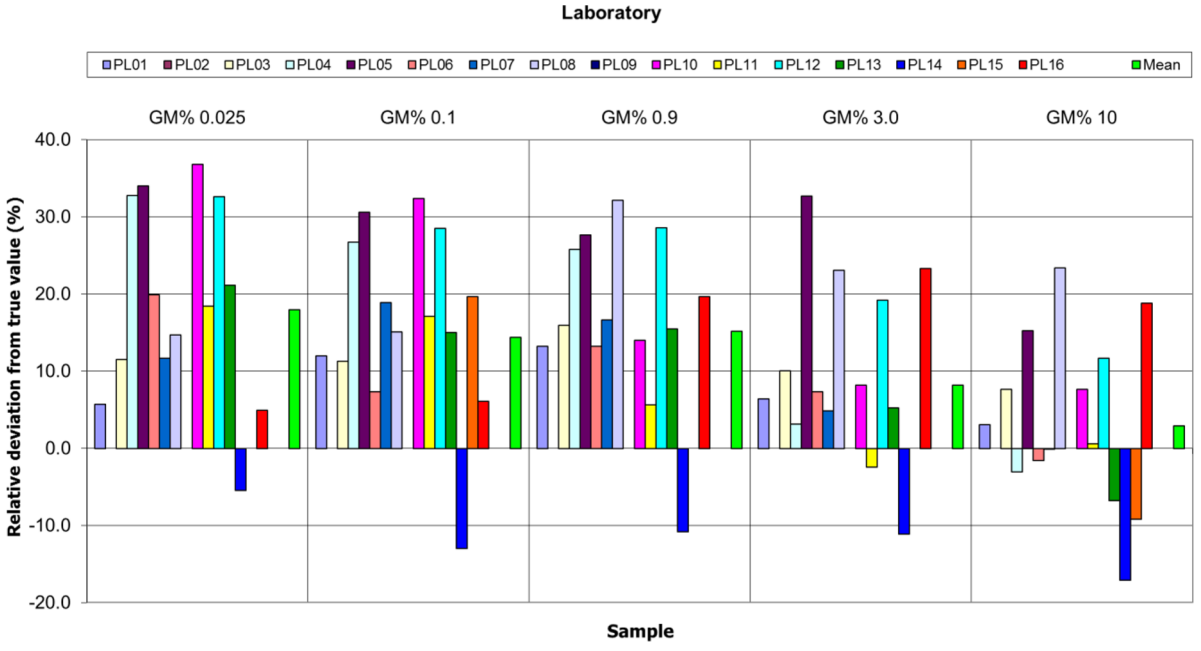

Figure 3. Relative percent deviation from the target value of GM percentage (% hge/hge, equivalent to mass/mass) for all laboratories. Colored bars represent the difference between the GM % content measured by each laboratory and the true GM % content in percentage of the latter; the green bar on the right represents the mean of the relative deviations over the analyzed laboratories for each level of GM % content. PL01-PL16: codes assigned to the 16 testing laboratories; the following values do not show up in the graph: PL2 results for the five levels because they were excluded from the analysis as outliers; PL9 results for the five levels because they were not available for this analysis; PL07 results at GM level 10% because they have very small relative deviation from the target value and the corresponding histogram is not evident in the graph, PL15 results at GM percentages 0.025%, 0.9%, and 3.0% because they were excluded from the analysis as outliers.

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Table 3. Summary of Validation Results for the Entire Method Relative Content Quantification (14 Laboratories)

	test sample GM %[a]				
	level 5 0.025	level 4 0.1	level 3 0.9	level 2 3.0	level 1 10
number of outliers	1	0	1	1	0
reason for exclusion	all tests		C	C	
measured GM % content	**0.0296**	**0.12**	**1.05**	**3.3**	**10.4**
(95% CI)	(0.0276; 0.0316)	(0.11; 0.12)	(0.99; 1.11)	(3.1; 3.5)	(9.7; 11.0)
relative repeatability standard deviation, RSD_r %	11.5	7.6	5.6	6.7	7.0
relative reproducibility standard deviation, RSD_R %	14.8	12.1	10.7	12.2	12.4
bias %	18.4	16.3	16.7	10.0	3.6
(95% CI)	(10.5; 26.2)	(9.4; 23.1)	(9.9; 23.5)	(2.9; 17.1)	(−2.9; 10.1)

[a]GM % expressed as hge/hge, equivalent to mass/mass. C, Cochran test; S, single Grubbs test; D, double Grubbs test.

the measured GM % content include the accepted reference value for level 1, while the accepted reference values for levels 2, 3, 4, and 5 are lower than the calculated 95% confidence interval. The 95% confidence intervals calculated around the bias measured overlap with the range of values considered acceptable for all levels tested (see Table 3). The full set of GM % contents calculated on the basis of data submitted by all laboratories is available in Tables S13 and S14, Supporting Information.

Homogeneity of Validation Results. The two geographical areas included in the validation study, Europe and Asia, present differences in terms of climate (e.g., average temperature and relative humidity), length of samples traveling time, and quality certification requirements for GMO testing laboratories. We hypothesized that these differences might have influenced the results presented by laboratories, introducing a factor of nonuniformity that would limit the significance of the results of the validation study. To verify the homogeneity of the results received we compared data presented by the laboratories located in Europe (laboratories from Austria, Belgium, France, Germany, Italy, Slovenia, Spain, and Switzerland; see Table S6, Supporting Information) with the results of the laboratories located in Asia (two laboratories from China, one from India, Japan, Korea, Malaysia, Philippines, and Singapore). The results that were identified as outliers and removed from the analysis of the full set of validation data presented above were removed also from this analysis. Detailed results for the two assays and the complete method are reported in Tables 4−6 and in Figure 4.

PLD-GR. For PLD-GR taxon-specific assay the results presented in Table 4 and Figure 4a are based on eight laboratories for both groups. Consistently with the overall analysis, results submitted by two European laboratories, one for level 3 and another for level 2 and from one Asian laboratory for level 1 were discarded from the analysis. No significant difference was identified between the two groups for the measured hge content, bias and coefficient of variation of the measured PLD hge content, at a 95% confidence level, for any of the levels tested. As observable in Figure 4a, for the taxon-specific assay the coefficient of variation of the measured hge content is generally higher for the Asian group than for the European group, and it tends to decrease with the increase of hge content for both groups. The RSD_r and RSD_R are within MPR and Codex guidelines for both groups, the percent bias is slightly higher than recommended for the two levels with a lower DNA content for both groups, in line with the results of the overall analysis.[18,19]

GR2. For GR2 event-specific assay the results presented in Table 5 and Figure 4b are based on eight laboratories for the European group and on seven laboratories for the Asian group. Consistently with the overall analysis, results submitted by one Asian laboratory for levels 2, 3, and 5 were discarded from the analysis. No significant difference was identified between the two groups for the measured hge content, for bias, and for the coefficient of variation of the measured hge content, at a 95% confidence level, for any of the levels tested. For the GR2 assay the coefficient of variation of the measured hge content is generally higher for the Asian group than for the European

Table 4. Summary of Validation Results for the PLD-GR Taxon-Specific Assay Grouped by Geographical Area (8 Laboratories for Each Group)

	European laboratories test sample hge					Asian laboratories test sample hge				
	level 5 50	level 4 200	level 3 2000	level 2 20000	level 1 200000	level 5 50	level 4 200	level 3 2000	level 2 20000	level 1 200000
number of outliers	0	0	1	1	0	0	0	0	0	1
reason for exclusion[a]			C	C						S/D
measured mean	**36**	**144**	**1900**	**21689**	**196354**	**34**	**138**	**1909**	**22900**	**183678**
(95% CI)	(33; 39)	(132; 155)	(1784; 2015)	(20387; 22991)	(188833; 203875)	(28; 40)	(113; 163)	(1697; 2122)	(20160; 25639)	(168845; 198511)
coefficient of variation	0.100	0.096	0.066	0.065	0.046	0.211	0.214	0.133	0.143	0.087
(95% CI)	(0.066; 0.206)	(0.063; 0.197)	(0.042; 0.146)	(0.042; 0.144)	(0.030; 0.093)	(0.138; 0.452)	(0.140; 0.460)	(0.088; 0.277)	(0.094; 0.298)	(0.056; 0.194)
$RSD_{r\ \%}$	19.9	11.4	8.1	7.5	9.0	19.2	16.2	9.7	6.8	6.9
$RSD_{R\ \%}$	19.9	13.8	9.6	9.2	9.0	26.8	25.6	15.7	15.5	10.6
bias %	−28.3	−28.1	−5.0	8.4	−1.8	−32.0	−31.1	−4.5	14.5	−8.2
(95% CI)	(−34.3; −22.3)	(−33.9; −22.3)	(−10.8; 0.8)	(1.9; 15.0)	(−5.6; 1.9)	(−44.0; −20.1)	(−43.4; −18.7)	(−15.2; 6.1)	(0.8; 28.2)	(−15.6; −0.7)

[a]C, Cochran test; S, single Grubbs test; D, double Grubbs test.

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J. Agric. Food Chem. 2015, 63, 4954−4965

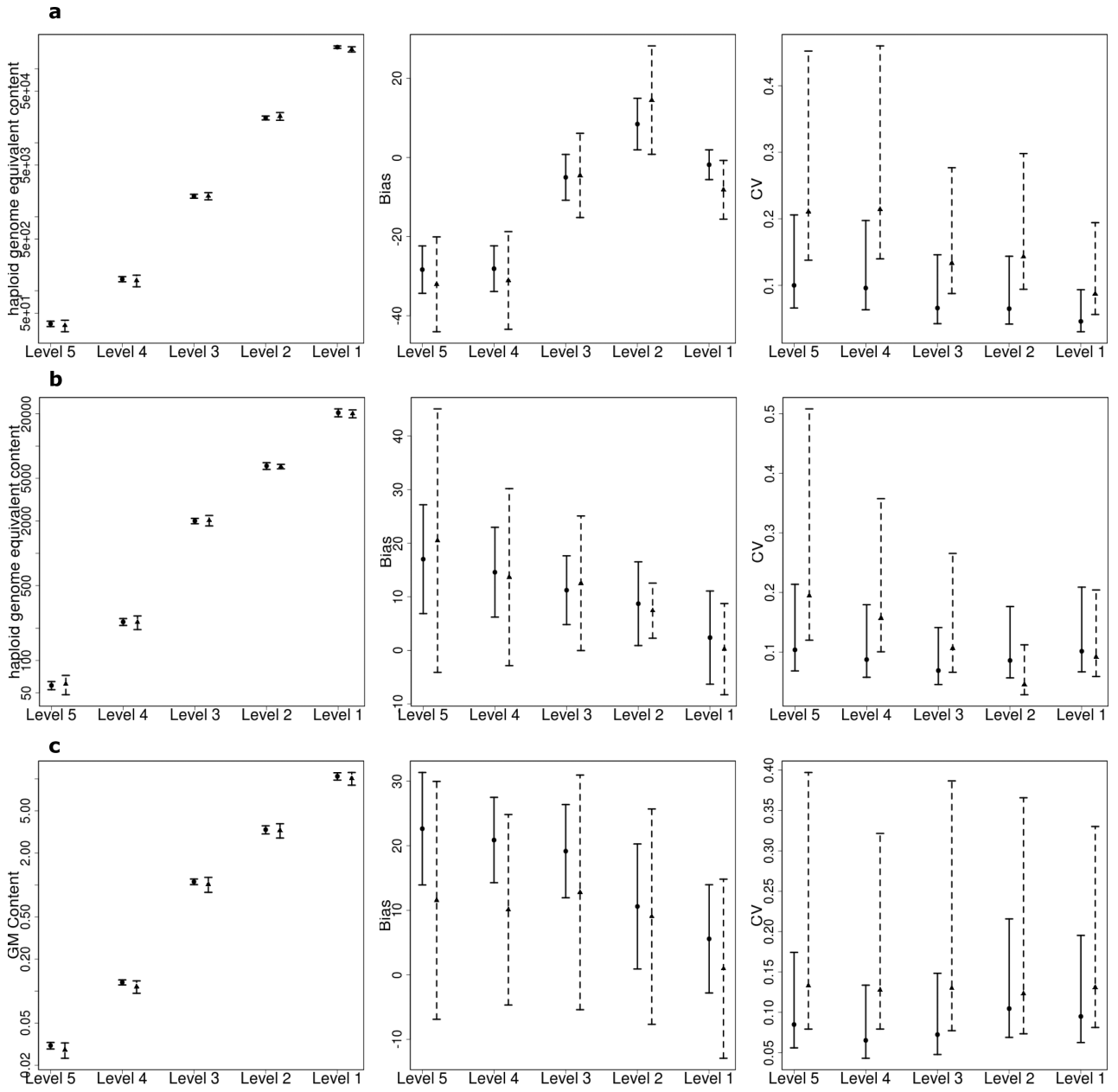

Figure 4. Scatterplots of the measured hge/GM content, bias and coefficient of variation of the measured hge/GM content with 95% confidence intervals for European (dot) and Asian (triangle) laboratories. (a) PLD-GR taxon-specific assay; (b) GR2 event-specific assay; (c) GR2 complete method. The *y* axis of the measured hge/GM content scatterplots is in logarithmic scale.

group only for levels 3, 4, and 5, and it remains constant across the levels for European laboratories, while it decreases with the increase of GR2 hge content for Asian laboratories (Figure 4b). The RSD_r, RSD_R, and percent bias are within the acceptance criteria for both groups.

GM %. For the entire method the results presented in Table 6 and Figure 4c for the European group are based on eight laboratories, those for the Asian group on six laboratories. Results submitted by one Asian laboratory for levels 2, 3, and 5 were discarded from the analysis. No significant difference was identified between the two groups for the measured GM % content, bias, and coefficient of variation of the measured GM % content, at a 95% confidence level, for any of the levels tested. For the entire method the coefficient of variation of the measured GM % content is generally higher for Asian laboratories and rather constant across the GM % levels for both groups (Figure 4c). The RSD_r, RSD_R, and percent bias are in line with the requirements for both Asian and European laboratories.

DISCUSSION

We presented the results of the international validation of the two modules that constitute a qPCR detection method for GR2, the PLD-GR taxon-specific assay and the GR2 event specific assay. These results show that the two assays have a good performance in terms of RSD_r, RSD_R, and bias when assessed with an international collaborative trial, both as independent modules, with absolute quantification, and when combined together, with relative quantification. The dynamic range assessed goes from 50 to 200 000 hge for the PLD-GR taxon-specific assay, from 50 to 20 000 hge for the GR2 event-specific assay and from 0.025% to 10% relative hge/hge GM %

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J. Agric. Food Chem. 2015, 63, 4954−4965

Table 5. Summary of Validation Results for the GR2 Event-Specific Assay Grouped by Geographical Area (8 Laboratories for Europe and 7 Laboratories for Asia)

	European laboratories test sample hge					Asian laboratories test sample hge				
	level 5 50	level 4 200	level 3 1800	level 2 6000	level 1 20000	level 5 50	level 4 200	level 3 1800	level 2 6000	level 1 20000
number of outliers	0	0	0	0	0	1	0	1	1	0
reason for exclusion[a]						all tests		C	C	
measured mean	**59**	**229**	**2002**	**6523**	**20478**	**60**	**227**	**2025**	**6445**	**20047**
(95% CI)	(53; 64)	(212; 246)	(1887; 2118)	(6054; 6992)	(18741; 22215)	(48; 73)	(194; 260)	(1799; 2251)	(6136; 6753)	(18346; 21749)
coefficient of variation	0.104	0.087	0.069	0.086	0.101	0.194	0.157	0.106	0.046	0.092
(95% CI)	(0.068; 0.214)	(0.058; 0.180)	(0.046; 0.141)	(0.057; 0.176)	(0.067; 0.209)	(0.120; 0.508)	(0.101; 0.358)	(0.066; 0.266)	(0.028; 0.112)	(0.059; 0.204)
$RSD_{r\ \%}$	8.4	4.9	4.4	2.9	3.4	12.7	5.7	3.8	6.2	6.6
$RSD_{R\ \%}$	12.7	9.7	7.9	9.0	10.6	22.3	16.5	11.1	7.0	10.8
bias %	17.0	14.6	11.2	8.7	2.4	20.5	13.7	12.5	7.4	0.2
(95% CI)	(6.9; 27.2)	(6.2; 23.0)	(4.8; 17.6)	(0.9; 16.5)	(−6.3; 11.1)	(−4.1; 45.1)	(−2.8; 30.2)	(0.0; 25.1)	(2.3; 12.6)	(−8.3; 8.7)

[a]C, Cochran test; S, single Grubbs test; D, double Grubbs test.

Table 6. Summary of Validation Results for the Entire Method Relative Content Quantification Grouped by Geographical Area (8 Laboratories for Europe and 6 Laboratories for Asia)

	European laboratories test sample GM %[a]					Asian laboratories test sample GM %[a]				
	level 5 0.025	level 4 0.1	level 3 0.9	level 2 3.0	level 1 10	level 5 0.025	level 4 0.1	level 3 0.9	level 2 3.0	level 1 10
number of outliers	0	0	0	0	0	1	0	1	1	0
reason for exclusion						all tests		C	C	
measured GM %	**0.031**	**0.12**	**1.07**	**3.3**	**10.6**	**0.028**	**0.11**	**1.01**	**3.3**	**10.1**
(95% CI)	(0.028; 0.033)	(0.11; 0.13)	(1.01; 1.14)	(3.0; 3.6)	(9.7; 11.4)	(0.023; 0.032)	(0.10; 0.12)	(0.85; 1.18)	(2.8; 3.8)	(8.7; 11.5)
coefficient of variation	0.085	0.065	0.072	0.105	0.095	0.133	0.128	0.130	0.123	0.131
(95% CI)	(0.056; 0.174)	(0.043; 0.134)	(0.048; 0.148)	(0.069; 0.216)	(0.063; 0.195)	(0.079; 0.397)	(0.079; 0.322)	(0.077; 0.387)	(0.073; 0.366)	(0.081; 0.330)
$RSD_{r\ \%}$	10.8	8.0	6.2	7.4	7.1	12.8	7.0	4.2	5.5	6.8
$RSD_{R\ \%}$	12.6	9.5	9.0	12.3	11.3	17.3	14.1	13.5	13.2	14.4
bias %	22.6	20.9	19.2	10.6	5.6	11.5	10.1	12.8	9.0	1.0
(95% CI)	(13.9; 31.3)	(14.3; 27.5)	(11.9; 26.4)	(0.9; 20.3)	(−2.8; 14.0)	(−6.9; 30.0)	(−4.7; 24.8)	(−5.4; 30.9)	(−7.7; 25.7)	(−12.9; 14.8)

[a]GM % expressed as hge/hge, equivalent to mass/mass. C, Cochran test; S, single Grubbs test; D, double Grubbs test.

content for the combined method. The RSD_r, RSD_R, and bias measured meet the MPR and Codex guidelines for all tested levels and assays/methods, with the exception of the samples at 50 and 200 hge of the PLD-GR taxon-specific assay. For those levels the percentage bias measured was of −30.2% and −29.6%, respectively. However, the 95% confidence interval calculated for the bias of these two levels includes the limit of −25% suggested for bias.[18,19] Thus, from a purely statistical point of view it cannot be excluded that their measured bias lies inside the accepted range. It is also important to note that 50 and 200 rice hge correspond to 0.025 and 0.1 ng of rice DNA, respectively, and that while it is important that the event-specific method is able to reliably quantify very small amounts of DNA, it is in practice very unlikely that the taxon-specific assay is used to quantify such small amounts of rice DNA. Therefore, the limitation mentioned above for the PLD-GR assay is of negligible relevance for the practical application of the method. In fact, hypothesizing a 5% GM labeling threshold, 200 rice hge per reaction would correspond to 10 GM event hge per reaction, far below the LOQ of the GR2 assay (50 hge). At 2000 rice hge per reaction the number of GM hge would instead correspond to 100, thus reliably quantifiable with the GR2 assay, and generally with any GM event-specific assay. The RSD_r measured with this validation study for PLD-GR taxon-specific and GR2 event-specific assays is in line with the RSD_r measured during the single laboratory validation of two assays, whose data is presented in the article on the development and single laboratory validation of GR2 qPCR detection method.[20] In fact, for the PLD-GR assay the RSD_r for samples at 50 and 200 000 hge was 19.8% and 4.2%, respectively, in the single laboratory validation and 19.6% and 8.1%, respectively, in this collaborative trial (Table 1). The RSD_r for the GR2 assay was 13.5% and 11.7%, respectively, in the single laboratory validation for samples containing 50 and 20 000 hge of the GR2 event and 10.5% and 5.1%, respectively, in this collaborative study (Table 2). The efficiency and linearity of

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the PLD-GR taxon-specific assay and the linearity of the GR2 event-specific assay were fully within the established performance criteria, while the efficiency of the GR2 assay was slightly lower than recommended, with an average slope of −3.65 and an average efficiency of 88.0%.[18,19] Given that GR2 blind samples were quantified correctly with the event-specific assay, both with absolute and relative quantification, this slight underperformance in terms of efficiency does not constitute a serious problem. In addition to this, if we exclude from the calculation of the GR2 assay average slope and efficiency the values measured by Lab 9, which presented deviations for those parameters, we obtain an average slope of −3.63 and an average efficiency of 89%, even nearer to the recommended value range.

In this validation study, we adopted the principle of modularity, according to which the different assays that constitute a detection method can be validated separately, provided that the pre-established performance criteria are respected for the preceding or parallel assay,[31−34] and we validated the taxon-specific and the event-specific assays that constitute the GR2 detection method independently. Validating the taxon-specific and event-specific assays independently has different advantages. Establishing the dynamic range of the two assays separately allows the users of the method complete freedom in the choice of GM percentages and DNA concentrations to test and establishes clearer limits on the highest and lower DNA concentrations that can be used in function of the target GM percentage. This is important when countries with different labeling regulations regarding GMO presence in the food or feed chain and different labeling thresholds set for unintended GMO presence are going to use the method.[7,11,24,39−41] The independent validation of the taxon-specific and event-specific assays also allows the users of the method to decide whether to use a delta Ct method or a two-standard curves method, depending on sample availability and quality, screening strategies or the experience of laboratory personnel. For example, a delta Ct method normally requires more DNA than a two-standard curves method for the preparation of standard curve samples, but it may be preferred for other reasons. The taxon-specific assay can be immediately used for other rice GM event-specific detection methods, without the need to revalidate both assays; in this case only the event-specific qPCR method would need to be developed and validated. On the other hand, if a GMO testing laboratory has already implemented the use of one rice taxon-specific assay, it may adopt only the event-specific assay and use it in combination with the pre-existent taxon-specific assay. Among the taxon-specific assays previously developed for rice GM quantification[42−48] only the qPCR assay developed on the sucrose phosphate synthase gene (SPS) was internationally validated independently from an event-specific GM detection method.[45,46] However, to our knowledge this is the first time that both the assays that constitute a GM event-specific qPCR detection method have been developed and validated independently, in absolute hge, and that a relative quantification experiment was performed to confirm the results obtained with the independent assays. The relative quantification experiment was performed by running the taxon-specific and the event-specific assays on two different plates with two independently prepared standard curves; some laboratories run the two plates on different days. Despite these factors are potential sources of variability, the results showed that when the two assays were combined to quantify GR2 DNA in relation to total rice DNA content, their performance in terms of accuracy was comparable, if not better, to the one of the single assays and in line with MPR and Codex guidelines,[18,19] confirming the robustness of the two assays. The samples tested were designed to comply with the European legislation on GMOs,[25−30] so that this qPCR detection method will be available in case of need.

According to ISO 5725-1 and to the IUPAC protocol for the design, conduct and interpretation of method-performance studies,[21,22] if a method is intended for international use, the laboratories participating to the collaborative trial should be from different countries. We chose to include laboratories from the two main areas where the method was developed and is likely to be used: Asia and Europe. These two geographical areas, however, present differences in terms of climate, distance from the organizing laboratory and quality certification requirements for GMO testing laboratories. The assumption behind a collaborative study is that the measured repeatability should be homogeneous between participating laboratories, in order to be able to consider the repeatability standard deviation (RSD_R) obtained with the study applicable to any laboratory.[21] We thus considered important to verify the homogeneity of the results of the collaborative study, by comparing the two subgroups that presented possible sources of variability, Europe and Asia. This analysis did not identify any significant difference between the results of the two groups for what concerns the measured hge/GM percentage content, bias and coefficient of variation, thus confirming the homogeneity of the results received. However, the coefficient of variation of the measured hge/GM percentage content was generally higher for Asian laboratories, a factor that does not compromise the homogeneity of results and that was expected, given that the group covers an area that is wider and more heterogeneous than Europe. To our knowledge, this is the first time that the homogeneity of the results of the international validation of a qPCR detection method was verified.

In conclusion, with the cooperation of the laboratories that participated to this collaborative trial, we have validated the taxon-specific and the event-specific assays that constitute the qPCR detection method for the GR2 event. The two assays were validated independently, a factor that will allow the users of the GR2 detection method to test a wide range of GM concentrations with flexibility on starting sample DNA concentration. This method, developed in collaboration with IRRI, optimized, single laboratory-tested[1] and validated by the EU-RL GMFF can be reliably used for the quantification of GR2 DNA in a sample, over the validated dynamic range.

■ ASSOCIATED CONTENT

Supporting Information

Description of the samples for the validation of the PLD-GR taxon-specific assay, of the GR2 event-specific assay, and of the combined GR2 detection method (Tables S2−S5); list of the laboratories that participated to the collaborative trial (Table S6); the full set of quantification results for the PLD-GR assay (Tables S7−S8), for the GR2 assay (Tables S10−S11), and for the relative quantification (Tables S13−S14); standard curve parameters values obtained for the PLD-GR assay (Table S9) and the GR2 assay (Table S12); layout of plate set up for the validation (Figures S1−S3); scatterplots of the comparison of PLD-GR standard curve parameters measured for plate A and C (Figure S4). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b00951.

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J. Agric. Food Chem. 2015, 63, 4954−4965

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Notes

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ABBREVIATIONS USED

ENGL, European Network of GMO Laboratories; EU-RL GMFF, European Union Reference Laboratory for Genetically Modified Food and Feed; GMO, genetically modified organism; GR2, Golden Rice 2; hge, haploid genome equivalents; IRRI, International Rice Research Institute; LOD, limit of detection; LOQ, limit of quantification; MBG, Molecular Biology and Genomics; MPR, ENGL minimum performance requirements for analytical methods of GMO testing; PCR, polymerase chain reaction; qPCR, quantitative real time PCR; PLD, Phospholipase D α2; RSD_r, relative repeatability standard deviation; RSD_R, relative reproducibility standard deviation

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