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Verification of the Bayer CropScience Method for the Detection of LL601 in Rice Using Real-time PCR

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Executive Summary

The Technical Services Division of USDA's Grain Inspection, Packers and Stockyards Administration conducted a study to verify the performance of an event-specific method to detect the LL601 event in rice. Bayer CropScience provided the 100% LL601 rice in the form of whole-grain, rough rice as well as the written protocol for performing the PCR reactions.

Results from this study confirm that the method can successfully detect the LL601 event in long-grain rice samples at a level as low as 0.01% without interference from the LL62 event.

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1. Background

The purpose of this study is to provide independent verification of the performance of the Bayer CropScience method for the event-specific detection of the genetically-engineered rice variety, Liberty Link (LL) 601. Key parts of this verification include determination of the limit of detection and to determine if the method is selective for LL601 in the presence of LL62.

The protocol is a real-time quantitative TaqMan® PCR procedure for determination of the relative content of LL601 DNA to total rice species DNA in a sample. The target-specific protocol uses two specific primers for LL601 and amplifies a 66-bp PCR fragment, measured during each cycle using a target-specific oligonucleotide FAM/TAM probe. Relative quantitation of LL601 is achieved by amplification of a rice-specific 68-bp fragment of *Phospholipase D* (PLD). This is a rice-specific endogenous gene and the protocol employs gene-specific primers and a sequence-specific VIC/TAM probe. The PCR reactions for the target and reference genes are performed in separate wells (simplex).

Bayer CropScience provided GIPSA with long-grain (rough) rice containing 100% LL601 and the event-specific method for LL601 entitled "Real-time PCR for selected herbicide tolerant rice (HTR) for rice seed / grain samples." Certified reference material for LL62 was obtained through the American Oil Chemists Society (AOCS) in the form of rice genomic DNA at 10 µg/vial (catalog number 0306-I). GIPSA Non-Transgenic rice 13/16 (CA13/16) DNA samples, derived from long-grain rice, was obtained from GIPSA file samples and used as the control blank.

2. Experimental Plan

Initial work using the "Dellaporta-derived Method for DNA Extraction..." provided by Bayer CropScience did not provide amplifiable rice genomic DNA using PCR amplification. Therefore, a publicly available CTAB extraction method including an additional RNAase digestion step at the end of the extraction was used in this study.

Specificity studies were performed on long-grain rice samples fortified with either 1% LL62 or 1% LL601 or non-fortified. In addition, Bayer CropScience provided samples (P1, P2, P3, P 4) fortified between 0.02 to 0.1 levels of LL62 and/or LL601 which were also analyzed using the method. Each sample was amplified as a simplex format using: (1) event-specific LL601 primers/probe and (2) endogenous control PLD primers/probe. Sensitivity studies were performed on DNA extracts of LL601 diluted with blank rice DNA to maintain a constant rice DNA level of 200 ng per reaction. All PCR reactions were run in triplicate except reference standards were run in duplicate.

Table 1. Fortification levels of GIPSA and Bayer Crop Science prepared samples

Sample	**Level of LL 62 (%)**	**Level of 601 (%)**	**PCR Result (LL601 Specific Method)**
CA13/16 Non-Transgenic (GIPSA)	0.0	0.0	Negative
1% LL62 (GIPSA)	1.0	0.0	Negative
1% LL601 (GIPSA)	0.0	**1.0**	**Positive**
P1 (Bayer Crop Science)	0.0	**0.02**	**Positive**
P2 (Bayer Crop Science)	0.01	0.0	Negative
P3 (Bayer Crop Science)	0.1	0.0	Negative
P4 (Bayer Crop Science)	0.0	**0.03**	**Positive**

3. Experimental Results

3.1 DNA Extraction and Quantification

Rice genomic DNA was extracted using minor modifications of a publicly available CTAB extraction protocol. A second RNAase incubation at 65°C for 30 minutes was required upon isolation of rice genomic DNA, followed by phenol/chloroform extraction and ethanol precipitation. The DNA was quantified using a pico-green reagent kit from Molecular Probes (catalog # P7589). Upon quantification of individual samples, each concentration was adjusted to 40ng/µl using 0.5 x TE (Tris-EDTA, pH 8.0) buffer. The integrity of the isolated rice DNA was characterized using agarose gel electrophoresis by size separation on a 0.8% agarose gel set at 100 volts for 30 minutes. A total of 200 ng DNA (5µl) was loaded into individual wells and onto the gel. Intact, high molecular weight rice genomic DNA was observed in all samples but frequently contained some RNA contamination. The low levels of RNA contamination did not seem to affect the PCR efficiency.

3.2 PCR Reaction Protocol

Table 2. Reagents and concentrations per reaction well for the LL601 specific system

Reagent	**Concentration Stock**	**Final Concentration**	**ml per 1 rxn**
H_2O	-	-	5
SHA040	10 µM	400nM/rxn	1
SHA041	10 µM	400nM/rxn	1
TM098	10 µM	200nM/rxn	0.5
Taq polymerase, ABI master mix	2x concentrate	1x concentrate	12.5
DNA	40 ng/ µl	200 ng/rxn	5
Total Volume			25

Table 3. Reagents and concentrations per reaction well for the PLD reference system

Reagent	Concentration Stock	Final Concentration	ml per 1 rxn
H_2O	-	-	6
KVM159	10 µM	200nM/rxn	0.5
KVM160	10 µM	200nM/rxn	0.5
TM013	10 µM	200nM/rxn	0.5
Taq polymerase, ABI master mix	2x concentrate	1x concentrate	12.5
DNA	40 ng/ µl	200 ng/rxn	5
Total Volume			25

The PCR reactions were performed with ABI universal PCR master mix reagent, a commercially available Taq DNA polymerase enzyme purchased from Applied Biosystems, Inc. Ten µg of rice Certified Reference Material (100% LL62) was purchased from the American Oil Chemists Society (AOCS) catalog number 0306-I. Primers and probes for both the reference specific PLD and target specific (LL601) genes are shown below:

LL601 Target Reaction		
Name	*Description*	*5'– 3' sequence*
SHA040	Forward Primer	TCT AGG ATC CGA AGC AGA TCG T
SHA041	Reverse Primer	GGA GGG CGC GGA GTG T
TM098	Probe	FAM-CCA CCT CCC AAC AAT AAA AGC GCC TG-TAMRA

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

Table 4. Two-step PCR amplification reaction conditions

Stage	Temp	Time	Cycles
UNG	50 °C	2 min	1
Initial denaturation	95 °C	10 min	1
Amplification	95 °C	15 sec	45
	60 °C	1 min	

Negative control reactions:
(1) Reagent blank sample that went through the entire purification procedure
(2) GIPSA file sample- Long-grain rice (repeated testing demonstrated to be negative)
(3) LL62- gravimetrically fortified at 1% (w/w%)

Positive control reactions:
(1) LL601- gravimetrically fortified at 1% (w/w%).
(2) 200ng, 100ng, 10ng, 1ng, 0.1ng, 0.01ng rice genomic DNA derived from 100% LL601 (40ng/µl) serially diluted into 0.5x TE buffer and amplified with primers/probes specific for LL601. The LL601 transgene was amplified using PCR reaction conditions as described in this report.
(3) 200ng, 100ng, 10ng, 1ng, 0.1ng, 0.01ng rice genomic DNA derived from 100% LL601 (40ng/µl) serially diluted into 0.5x TE buffer and amplified with primers/probes specific for PLD. The endogenous control gene was amplified using PCR reaction conditions as described in this report.

Test Samples:
DNA was extracted and quantified for rice samples containing 100% LL601, 1% LL601 (prepared by GIPSA), 1% LL62 (prepared by GIPSA), and samples fortified with 0.02% LL601 (P1, prepared by Bayer Crop Science) and 0.03% LL601 (P4, prepared by Bayer Crop Science) using the CTAB extraction and isolation procedures given in this report.

Specificity testing resulted in suitable amplification of both the PLD endogenous control and taxon-specific (LL601) PCR products for the 1% LL601 gravimetrically fortified, P1 and P4 (containing LL601) samples. As expected, 1% LL62, P2 and P3 samples exhibited suitable amplification of the PLD endogenous control, but no amplification for the taxon-specific product was detected. (**See Table 5**.)

Sensitivity testing was performed by diluting the 100% LL601 DNA with non-transgenic, long-grain rice blank DNA at the levels of 1%, 0.1%, 0.05%, 0.01%, and 0.005%. The total rice DNA content was maintained at 200 ng for this experiment. All PCR reactions amplified for both the reference samples and target down to 0.01%. At 0.005%, one target reaction out of three showed successful amplification (**Table 6**).

4. Conclusions

Based on the experiments conducted, the Bayer CropScience real-time event-specific method for LL601 in rice showed a limit of detection of 0.01% in long-grain rice samples using the CTAB extraction protocol. The method also shows specificity towards LL601 with no observed cross-reactivity with LL62. The data presented in this report confirms that the method performs as documented and is applicable for the event-specific detection of LL601 in long-grain rice.

Table 5. LL601 Specificity Results

Well	Sample Name	Detector Name	Reporter	Task	Ct	Quantity	Qty Mean	Qty StdDev
1	LL601 200ng	TM013	FAM	Standard	21.838137	200		
2	LL601 200ng	TM013	FAM	Standard	21.799809	200		
3	LL601 100ng	TM013	FAM	Standard	22.706297	100		
4	LL601 100ng	TM013	FAM	Standard	22.725533	100		
5	LL601 10ng	TM013	FAM	Standard	26.114038	10		
6	LL601 10ng	TM013	FAM	Standard	26.205755	10		
7	LL601 1.0ng	TM013	FAM	Standard	29.206018	1		
8	LL601 1.0ng	TM013	FAM	Standard	29.191494	1		
9	LL601 0.1ng	TM013	FAM	Standard	32.610916	0.1		
10	LL601 0.1ng	TM013	FAM	Standard	32.74916	0.1		
11	LL601 0.01ng	TM013	FAM	Standard	36.46204	0.01		
12	LL601 0.01ng	TM013	FAM	Standard	35.94841	0.01		
13	CA13/16	TM013	FAM	Unknown	21.878515	181.84546	192.20491	8.561852
14	CA13/16	TM013	FAM	Unknown	21.826063	188.55801	192.20491	8.561852
15	CA13/16	TM013	FAM	Unknown	21.752216	198.43059	192.20491	8.561852
16	CA13/16	TM013	FAM	Unknown	21.740921	199.98558	192.20491	8.561852
17	CA13/16	TM013	FAM	Unknown	22.168541	148.81862	148.66023	1.2787298
18	CA13/16	TM013	FAM	Unknown	22.179617	147.68384	148.66023	1.2787298
19	CA13/16	TM013	FAM	Unknown	22.179174	147.729	148.66023	1.2787298
20	CA13/16	TM013	FAM	Unknown	22.153154	150.40947	148.66023	1.2787298
21	P1	TM013	FAM	Unknown	20.941181	347.55573	343.20538	5.791174
22	P1	TM013	FAM	Unknown	20.940289	347.77026	343.20538	5.791174
23	P1	TM013	FAM	Unknown	20.964514	341.99646	343.20538	5.791174
24	P1	TM013	FAM	Unknown	20.99227	335.49902	343.20538	5.791174
25	P2	TM013	FAM	Unknown	21.460371	242.7728	249.89194	8.0546875
26	P2	TM013	FAM	Unknown	21.437475	246.64467	249.89194	8.0546875
27	P2	TM013	FAM	Unknown	21.425098	248.76343	249.89194	8.0546875
28	P2	TM013	FAM	Unknown	21.353472	261.38687	249.89194	8.0546875
29	P3	TM013	FAM	Unknown	20.863361	366.7587	364.6253	6.170798
30	P3	TM013	FAM	Unknown	20.90666	355.94687	364.6253	6.170798
31	P3	TM013	FAM	Unknown	20.8489	370.4425	364.6253	6.170798
32	P3	TM013	FAM	Unknown	20.868917	365.35318	364.6253	6.170798
33	P4	TM013	FAM	Unknown	20.73948	399.54095	385.05414	13.713575
34	P4	TM013	FAM	Unknown	20.761114	393.6117	385.05414	13.713575
35	P4	TM013	FAM	Unknown	20.847729	370.7424	385.05414	13.713575
36	P4	TM013	FAM	Unknown	20.826117	376.32138	385.05414	13.713575
37	1% LL601	TM013	FAM	Unknown	22.78595	97.130226	100.24658	2.3331678
38	1% LL601	TM013	FAM	Unknown	22.745052	99.91459	100.24658	2.3331678
39	1% LL601	TM013	FAM	Unknown	22.708063	102.501564	100.24658	2.3331678
40	1% LL601	TM013	FAM	Unknown	22.723127	101.43997	100.24658	2.3331678
41	1% LL62	TM013	FAM	Unknown	21.159492	298.88403	306.94302	13.043012
42	1% LL62	TM013	FAM	Unknown	21.156023	299.60144	306.94302	13.043012
43	1% LL62	TM013	FAM	Unknown	21.139885	302.9616	306.94302	13.043012
44	1% LL62	TM013	FAM	Unknown	21.032389	326.32504	306.94302	13.043012
45	RB	TM013	FAM	Unknown	Undetermined	0		
46	RB	TM013	FAM	Unknown	41.40914	2.50E-04		
Slope	-3.3318913	cycles/log decade						
Y-Intercept		29.407598						
R^2		0.9990486						

Well	Sample Name	Detector Name	Reporter	Task	Ct	Quantity	Qty Mean	Qty StdDev
49	LL601 200ng	TM098	FAM	Standard	20.181898	200		
50	LL601 200ng	TM098	FAM	Standard	20.244946	200		
51	LL601 100ng	TM098	FAM	Standard	21.288744	100		
52	LL601 100ng	TM098	FAM	Standard	21.069363	100		
53	LL601 10ng	TM098	FAM	Standard	24.79294	10		

54	LL601 10ng	TM098	FAM	Standard	24.985949	10		
55	LL601 1.0ng	TM098	FAM	Standard	27.595165	1		
56	LL601 1.0ng	TM098	FAM	Standard	27.607046	1		
57	LL601 0.1ng	TM098	FAM	Standard	30.791887	0.1		
58	LL601 0.1ng	TM098	FAM	Standard	31.048513	0.1		
59	LL601 0.01ng	TM098	FAM	Standard	33.828064	0.01		
60	LL601 0.01ng	TM098	FAM	Standard	33.95806	0.01		
61	CA13/16	TM098	FAM	Unknown	Undetermined	0		
62	CA13/16	TM098	FAM	Unknown	Undetermined	0		
63	CA13/16	TM098	FAM	Unknown	Undetermined	0		
64	CA13/16	TM098	FAM	Unknown	Undetermined	0		
65	CA13/16	TM098	FAM	Unknown	Undetermined	0		
66	CA13/16	TM098	FAM	Unknown	Undetermined	0		
67	CA13/16	TM098	FAM	Unknown	Undetermined	0		
68	CA13/16	TM098	FAM	Unknown	Undetermined	0		
69	P1	TM098	FAM	Unknown	31.796951	0.05023758	0.055723045	0.007057682
70	P1	TM098	FAM	Unknown	31.830473	0.04903227	0.055723045	0.007057682
71	P1	TM098	FAM	Unknown	31.500275	0.06228313	0.055723045	0.007057682
72	P1	TM098	FAM	Unknown	31.521355	0.061339196	0.055723045	0.007057682
73	P2	TM098	FAM	Unknown	Undetermined	0		
74	P2	TM098	FAM	Unknown	Undetermined	0		
75	P2	TM098	FAM	Unknown	Undetermined	0		
76	P2	TM098	FAM	Unknown	Undetermined	0		
77	P3	TM098	FAM	Unknown	Undetermined	0		
78	P3	TM098	FAM	Unknown	Undetermined	0		
79	P3	TM098	FAM	Unknown	Undetermined	0		
80	P3	TM098	FAM	Unknown	Undetermined	0		
81	P4	TM098	FAM	Unknown	30.737068	0.10826643	0.11343257	0.012917823
82	P4	TM098	FAM	Unknown	30.521404	0.12657471	0.11343257	0.012917823
83	P4	TM098	FAM	Unknown	30.583002	0.121050544	0.11343257	0.012917823
84	P4	TM098	FAM	Unknown	30.876865	0.097838596	0.11343257	0.012917823
85	1% LL601	TM098	FAM	Unknown	26.614464	2.1456842	2.3661737	0.15828949
86	1% LL601	TM098	FAM	Unknown	26.406042	2.4954011	2.3661737	0.15828949
87	1% LL601	TM098	FAM	Unknown	26.483938	2.3584807	2.3661737	0.15828949
88	1% LL601	TM098	FAM	Unknown	26.42289	2.4651291	2.3661737	0.15828949
89	1% LL62	TM098	FAM	Unknown	Undetermined	0		
90	1% LL62	TM098	FAM	Unknown	Undetermined	0		
91	1% LL62	TM098	FAM	Unknown	Undetermined	0		
92	1% LL62	TM098	FAM	Unknown	Undetermined	0		
93	RB	TM098	FAM	Unknown	Undetermined	0		
94	RB	TM098	FAM	Unknown	Undetermined	0		
Slope	-3.178393	cycles/log decade						
Y-Intercept		27.66831						
R^2		0.9981657						

Table 6. LL601 Sensitivity Results

Well	Sample Name	Detector Name	Reporter	Task	Ct	Quantity	Qty Mean	Qty StdDev
1	CA13/16 200ng	TM013	FAM	Standard	22.183905	200		
2	CA13/16 200ng	TM013	FAM	Standard	22.28848	200		
3	CA13/16 100ng	TM013	FAM	Standard	23.190878	100		
4	CA13/16 100ng	TM013	FAM	Standard	23.220287	100		
5	CA13/16 10ng	TM013	FAM	Standard	26.41397	10		
6	CA13/16 10ng	TM013	FAM	Standard	26.352617	10		
7	CA13/16 1.0ng	TM013	FAM	Standard	29.483068	1		
8	CA13/16 1.0ng	TM013	FAM	Standard	29.568401	1		
9	CA13/16 0.1ng	TM013	FAM	Standard	32.89839	0.1		
10	CA13/16 0.1ng	TM013	FAM	Standard	32.816597	0.1		
11	CA13/16 0.01ng	TM013	FAM	Standard	36.036976	0.01		
12	CA13/16 0.01ng	TM013	FAM	Standard	36.07204	0.01		
13	1% LL601 serial dil.	TM013	FAM	Unknown	22.412102	173.86034	184.88673	12.942059
14	1% LL601 serial dil.	TM013	FAM	Unknown	22.350838	181.66435	184.88673	12.942059
15	1% LL601 serial dil.	TM013	FAM	Unknown	22.222717	199.13554	184.88673	12.942059
16	1% LL601	TM013	FAM	Unknown	24.755922	32.40821	32.594845	0.5417915
17	1% LL601	TM013	FAM	Unknown	24.722021	33.205284	32.594845	0.5417915
18	1% LL601	TM013	FAM	Unknown	24.76617	32.171043	32.594845	0.5417915
19	0.1% LL601	TM013	FAM	Unknown	22.302418	188.07935	192.90227	6.706524
20	0.1% LL601	TM013	FAM	Unknown	22.287752	190.06662	192.90227	6.706524
21	0.1% LL601	TM013	FAM	Unknown	22.212767	200.56082	192.90227	6.706524
22	0.01% LL601	TM013	FAM	Unknown	22.415726	173.40927	169.25058	9.862262
23	0.01% LL601	TM013	FAM	Unknown	22.392244	176.35236	169.25058	9.862262
24	0.01% LL601	TM013	FAM	Unknown	22.545656	157.9901	169.25058	9.862262
25	0.005% LL601	TM013	FAM	Unknown	22.542868	158.30612	164.32713	7.026415
26	0.005% LL601	TM013	FAM	Unknown	22.426727	172.04732	164.32713	7.026415
27	0.005% LL601	TM013	FAM	Unknown	22.505287	162.62796	164.32713	7.026415
28	CA13/16	TM013	FAM	Unknown	22.32793	184.6715	180.21925	4.360922
29	CA13/16	TM013	FAM	Unknown	22.395386	175.95578	180.21925	4.360922
30	CA13/16	TM013	FAM	Unknown	22.363443	180.03049	180.21925	4.360922
Slope	-3.2127137	cycles/log decade						
Y-Intercept		29.609224						
R^2		0.9998833						

Well	Sample Name	Detector Name	Reporter	Task	Ct	Quantity	Qty Mean	Qty StdDev
49	LL601 10 ng	TM098	FAM	Standard	22.920479	10		
50	LL601 10 ng	TM098	FAM	Standard	23.140324	10		
51	LL601 1ng	TM098	FAM	Standard	26.22856	1		
52	LL601 1ng	TM098	FAM	Standard	26.257214	1		
53	LL601 0.1ng	TM098	FAM	Standard	29.250816	0.1		
54	LL601 0.1ng	TM098	FAM	Standard	28.886545	0.1		
55	LL601 0.01ng	TM098	FAM	Standard	32.983067	0.01		
56	LL601 0.01ng	TM098	FAM	Standard	32.358826	0.01		
61	1% LL601 serial dil.	TM098	FAM	Unknown	26.496851	0.78657657	0.82736975	0.035986688
62	1% LL601 serial dil.	TM098	FAM	Unknown	26.404753	0.8409122	0.82736975	0.035986688
63	1% LL601 serial dil.	TM098	FAM	Unknown	26.382458	0.85462046	0.82736975	0.035986688
64	1% LL601	TM098	FAM	Unknown	27.070871	0.5187195	0.49685565	0.0489029
65	1% LL601	TM098	FAM	Unknown	27.295187	0.44083524	0.49685565	0.0489029
66	1% LL601	TM098	FAM	Unknown	27.038578	0.53101224	0.49685565	0.0489029
67	0.1% LL601	TM098	FAM	Unknown	29.643213	0.080293566	0.0733278	0.022371048
68	0.1% LL601	TM098	FAM	Unknown	29.464777	0.09138726	0.0733278	0.022371048
69	0.1% LL601	TM098	FAM	Unknown	30.343912	0.04830258	0.0733278	0.022371048
70	0.01% LL601	TM098	FAM	Unknown	32.950085	0.007295623	0.006903211	7.77E-04
71	0.01% LL601	TM098	FAM	Unknown	33.217857	0.006007832	0.006903211	7.77E-04
72	0.01% LL601	TM098	FAM	Unknown	32.929348	0.007406177	0.006903211	7.77E-04
73	0.005% LL601	TM098	FAM	Unknown	Undetermined	0		
74	0.005% LL601	TM098	FAM	Unknown	Undetermined	0		
75	0.005% LL601	TM098	FAM	Unknown	35.002	0.001647168		
76	CA13/16	TM098	FAM	Unknown	Undetermined	0		
77	CA13/16	TM098	FAM	Unknown	Undetermined	0		
78	CA13/16	TM098	FAM	Unknown	Undetermined	0		
Slope	-3.1747437	cycles/log decade						
Y-Intercept		26.165855						
R^2		0.9950929						