POSITION PAPER

Applicability of PCR Detection Methods to Detect Stacked Trait Products

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The intention of this document is to provide information regarding the use of PCR-based detection methods to analyze samples of seed or grain containing traits from multiple genetically modified (GM; transgenic or genetically engineered) events. For the purpose of this paper, such multiple-event combinations will be referred to as 'stacks' and will be limited to stacked products produced intentionally through conventional breeding (crossing of plants carrying individual events), those that occur adventitiously by crossing of two plants containing different events during cultivation, or due to natural segregation processes in the field resulting in lower-order stacks different from the initial seed planted.

- To date, there is no PCR or other molecular approach that can distinguish, without statistical analysis, between the presence of stacked events in a bulk sample, and the presence of a seed mixture of two or more individual events that comprise the stack.
- There are no existing molecular methods developed for the detection and identification of stacks, whether originating from the planted breeding stack or from lower-order stacks produced during the growing season containing combinations of the same events. It is not possible to use PCR or any other molecular approach to differentiate the presence of stacks in a bulk seed sample beyond detecting the presence of the individual single events.
- There are no methods to distinguish whether a bulk seed lot contains a low-level mixture of single events or stacked events.
- PCR methods developed for the detection of single events should continue to be the method of choice for GM detection.
- The examination of single seeds/grains or an approach where the conditions of the International Seed Testing Association (ISTA) statistical techniques are met can be used to determine whether stacks are present. Even when a stack of two or more events is present, it cannot be determined in grain whether this stack is an intentional combination, or one due to adventitious pollination in the field.

Detection and identification of individual events

To detect specific GM events, the standard method is presently event-specific Polymerase Chain Reaction (PCR). The event-specific PCR relies on the unique sequences created as a consequence of the transformation event. In real-time quantitative PCR, a probe is also defined, which is located between the PCR primers. When properly validated, the method amplifies DNA from only the specific event to the exclusion of all other GM events and non-transformed plant lines.

Detection and identification of breeding stacks
Breeding stacks are the combination of single GM events that are brought together via the conventional breeding process. Since plant breeding combines the genes of the single events and their flanking sequences, the same PCR method can be used. As there is no change in what is being detected, the single and breeding stack cannot be differentiated in bulk samples since a positive detection of multiple GM events would arise from a bulk sample of a stack of those events or a bulk mixture of seed of the individual single events.

The characterization of the single GM event typically describes the location of the inserted DNA within the plant genome. During the conventional breeding process used to stack GM events, the location of the insert and the flanking sequences does not change. Each of the inserted genes and their flanks of a breeding stack will be located on different chromosomes or at genetic distance that make it impossible to cover with single PCR assay, since qPCR amplicons are typically <300 base pairs. Consequently, due to the technological limitations of PCR, it is impossible to design a single PCR methodology to specifically detect together the multiple GM events of a stacked trait product.

Analysis of individual seed, plants or grain, using multiple, event-specific PCR assays can determine if multiple events are present in that sample. However, if several seeds (or more commonly, grains from a bulk shipment) are subjected to PCR as a bulk sample results will indicate the presence of multiple events in the sample but cannot distinguish between stacks or a mixture of individual events.

Positive event-specific PCR results indicating the presence of more than one event in a bulk sample may arise via:

1. A sample containing multiple GM events and/or non-transgenic seeds;
2. A sample containing non-transgenic, individual GM events and/or breeding stacks;
3. A sample containing breeding stacks, lower-order stacks (segregants) and/or multiple GM events.

Furthermore, this analysis cannot differentiate between the planted breeding stack and lower-order stacks produced through segregation during the growing season containing combinations of the same events since, on a molecular level, these two types of stacks appear identical.

The results can be especially difficult to interpret if they indicate a low-level of GM events in the sample. This can be illustrated by an example of bulk samples each containing 1000 seeds/grains (assuming event-specific PCR assays were properly validated and have a limit of detection of less than 0.1%) as shown in Tables 1 and 2.

Table 1: Expected results of qualitative, event-specific PCR tests on individual 1000-seed/grain samples containing single and stacked events.

Sample #	Number of each type of seed in the sample					
	Event 1	Event 2	Event 1 × Event 2 stack	Non-transgenic	Theoretical Result PCR 1**	Theoretical Result PCR 2**
1	1	0	0	999	Positive	Negative
2	0	1	0	999	Negative	Positive
3	0	0	1	999	Positive	Positive
4	1	1	0	998	Positive	Positive
5	0	1	1	998	Positive	Positive
6	1	0	1	998	Positive	Positive
7	1	1	1	997	Positive	Positive

**PCR 1 targets Event 1; PCR 2 targets Event 2.

Samples 3 through 7 are indistinguishable as they all yield positive signals for both events, even though sample 4 contains no stacked seeds, samples 5 through 7 contain a mixture of stacked and individual events, and sample 3 contains only the stack. In this case, the only conclusions possible are the presence of event 1 in sample 1, of event 2 in sample 2 and of both events in samples 3 through 7. It is not possible to determine the source of the two events in samples 3 through 7, that is, whether the signal is from single or stacked events.

The next question is whether breeding stacks can be detected using real-time PCR in bulk samples. Table 2 shows an example of the quantitative results that might be expected from the same types of samples as were examined in Table 1.

Table 2: Expected results of event-specific PCR tests on individual 1000-seed/grain samples containing single and stacked events.

Sample #	Number of each type of seed in the sample					
	Event 1	Event 2	Event 1 + Event 2 stack	Non-transgenic	Theoretical Result PCR1	Theoretical Result PCR2
1	2	0	0	998	Positive (~0.2%)	Negative
2	0	2	0	998	Negative	Positive (~0.2%)
3	0	0	2	998	Positive (~0.2%)	Positive (~0.2%)
4	2	2	0	996	Positive (~0.2%)	Positive (~0.2%)
5	1	2	1	996	Positive (~0.2%)	Positive (~0.3%)
6	2	1	1	996	Positive (~0.3%)	Positive (~0.2%)
7	1	1	2	996	Positive (~0.3%)	Positive (~0.3%)
8	2	3	0	995	Positive (~0.2%)	Positive (~0.3%)
9	3	2	0	995	Positive (~0.3%)	Positive (~0.2%)
10	2	2	1	995	Positive (~0.3%)	Positive (~0.3%)

The first observation is that samples 3 and 4 give the same results; the stack sample is not distinguishable from the single-event seed mixture. In samples 5 and 6, there appear to be small differences that might be interpreted as a combination of single-event seed mixtures and stacked events. However, there are other mixtures which can give the same

results without stacks being present (samples 8 and 9). In addition, samples with a different proportion of stacks present in the sample can give the same result (samples 7 and 10). Moreover, because of sampling and measurement uncertainty, real-time PCR is not accurate enough to distinguish between, for example, 0.2% and 0.3% of an event in a sample.

Statistical approaches

The International Seed Testing Association (ISTA) has proposed a statistical approach (implemented in SeedcalcStack9, www.seedtest.org) to quantify up to three events in seeds in a conventional seed lot and give an estimate of what percentage of the seeds comprise stacked events. The approach is a pooled-seed testing approach and involves the examination of a recommended minimum of 20 to 30 small pools.

Akiyama (2005) proposed the analysis of individual grains of maize as a means of determining whether stacks were present in grain samples. In addition, Mano et al. published a paper describing a testing method that relies on testing multiple sub-samples to evaluate weight/weight GMO content in maize grains. These approaches are only feasible where the concentration of stacks in the grain sample is significant (e.g., in a situation where the concentration is approaching 5%).

Though these approaches may be feasible, they need to be considered in terms of applicability for customs agencies wanting to test bulk shipments of many tons of grain. Such tests, at that scale, would likely be laborious and inefficient, adding time and expense to the import process and likely hindering trade.

Reference materials (RM)
Reference materials for commercialized events are available for the single events. As breeding stacks consist of combinations of these individual events, the single-event reference materials have been proven to be suitable for calibration or validation of methods used to analyse the stacked events. Stacked trait reference materials are therefore not necessary or useful.

Conclusions
There are no existing methods, nor methods that can be developed, for the detection, differentiation, and identification of stacks, regardless of whether these originate from the planted breeding stack or from lower-order stacks produced during the growing season containing different combinations of the same events. Additionally, there are no methods to determine whether a bulk seed lot contains a low-level mixture of single events or stacked events. PCR methods developed for the single events are the methods of choice for GM detection.

Literature
Akiyama et. al. 2005. Quantitative Detection System for Maize Sample Containing Combined-Trait Genetically Modified Maize. Anal. Chem., 2005, 77 (22), pp 7421–7428. doi: 10.1021/ac051236u.

Mano et. al. 2011. Practicable Group Testing Method to Evaluate Weight/Weight GMO Content in Maize Grains J Agric Food Chem. 2011, 59(13), pp 6856-6863. doi: 10.1021/jf200212v. Epub 2011 Jun 7.