**Implications of Using PCR Detection Methods with Stacked Products**

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October, 2014

Scope

The scope of this document is the use of PCR-based detection methods to analyse bulk samples of seed or grain containing multiple genetically modified (GM; transgenic or genetically engineered) events in different loci in the plant genome. For the purpose of this discussion, such multiple-event combinations will be referred to as ‘stacks’.

This discussion is limited to stacked products (e.g., plant or seed products comprised of stacks) produced intentionally through conventional breeding (crossing of plants carrying individual events), or adventitiously by crossing of two plants containing different events during cultivation.

What is an event?

In plant biotechnology, the term event refers to a genetic construct inserted into a specific site in a plant’s genome for the purpose of conferring a phenotypic characteristic.

What is a stack?

A stack contains two or more independently-created events brought together in the same plant, seed or grain by conventional plant breeding. An example is the stacking of an event expressing herbicide resistance and an event expressing insect resistance.

Seed stacks, field stacks and mixtures

“Seed stacks” (stacked traits products or breeding stacks) are those plant products produced through conventional breeding between plants carrying independent GM events (also refers to as breeding stack). The stacks therefore express the traits of the multiple individual events. A “Field stack” is an adventitious combination of events arising from open pollination in the field. “Mixtures” refer to the event combinations that may occur in the commodity chain when grain is mixed that contains multiple events, seed stacks, or field stacks. Commodity grain potentially contains mixtures of commercial events and stacks.

Detection and identification of individual events

The events may be detected by a number of methods. However, if the intention is to detect an event specifically, the standard method is presently event-specific Polymerase Chain Reaction (PCR).

Event-specific PCR relies on sequences specific to the insertion and its flanking sequences. These are most often derived from the anchoring of one PCR primer in the genetic construct inserted into the plant during the transformation process, and the other PCR primer anchored in the host genomic DNA adjacent to the insertion site (flanking sequences). In real-time PCR, a probe will also be defined, which is located between the PCR primers. The event-specific PCR, when properly validated, will amplify DNA from only the specific event to the exclusion of all other events and non-transformed plant lines.

Detection and identification of stacks

In most, if not all cases, the inserted DNA from different events in a stack is located on different chromosomes or different members of a homologous pair. Even if the insertions are on the same DNA molecule, the physical distance between the insertions may not be short enough to cover with a single PCR assay due to the technological limitations of PCR. Thus the components of a stack cannot be detected by a single PCR assay method. Therefore, a PCR methodology cannot be designed to specifically establish multiple events as components of a stack.

Analysis of individual seed, plants or grain, using multiple, event-specific PCR assays can determine if stacks are present in that sample. However, if a number of 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 individual events.

Positive event-specific PCR results indicating more than one event can arise via one of several possibilities:

1. a mix of seeds containing non-transgenic and multiple individual events;
2. a mix of seeds containing non-transgenic and stacks; or
3. a mix of seeds containing non-transgenic, individual events and stacks.

Analysis cannot differentiate between intentional seed stacks and adventitious field stacks, because 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 events in the sample. This can be illustrated by an example of samples each containing 1000 seeds/grains (assuming that 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 question then arises as to whether stacks can be detected using real-time quantitative PCR. 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 quantitative, 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 exactly 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, quantitative PCR is not accurate enough to distinguish between, for example, 0.2% and 0.3% of an event in a sample.

Statistical approaches

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 the two or more individual events that comprise the stack.

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%).

Conclusions

Currently, there are no molecular methods that are or can be developed for the detection and identification of stacks, whether originating via intentional or adventitious crossing, and 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. However, due to the nature of stacks where two or more events are present, it is not possible to use PCR or any other molecular approach to determine the presence of stacked seed or grain in a bulk seed sample beyond detecting the presence of the two or more individual single events. The examination of single seeds/grains or an approach where the conditions of the 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.

Reference materials (RM):

Reference materials for commercialized events are available for the single events. As stacked seeds and plants consist of combinations of these individual events, the single-event reference materials are suitable for calibration or validation of methods used to analyse the stacked events.

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 6856-63. doi: 10.1021/jf200212v. Epub 2011 Jun 7.