VS0136; A Combined Protocol for PCR Detection of GM in Seed

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Executive Summary

In the near future, EU thresholds for adventitious presence of GM in certified seed will be set and suitable regulatory testing protocols must be adopted. The EC working group, 'Seed Legislation, Sampling and Detection', on which Defra is represented, has identified and addressed several potential problems in the measurement and reporting of GM presence in seeds. In brief, different testing methods can give results that differ enough to alter the accept/reject decision for a seed lot where the actual GM content is near the threshold value. These discrepancies are due to the complex genotype of some seeds, copy number of GM events, and whether qualitative or quantitative molecular methods are used. The potential for misinterpretation of results is compounded by the units used for thresholds in different legislation. To date, thresholds for GMO labelling in food (e.g. EC1830/2003) have been expressed as % GMO, which for seeds or grain is most likely to be interpreted as % GMO seeds in total seed. However, EC technical guidance documents (European Commission, 2004) and future legislation specific to seeds, is likely to define units of thresholds as % GM genomes. The latter is also called % GM DNA.

In its simplest terms, in a diploid crop (having two copies of its genome) this means that if a GM presence has come from cross-pollination, the GM seeds will have one genome from the GM parent and one from the non-GM parent. When this 'F1' seed is measured as % GM seeds, each GM - containing seed will give a signal and therefore count as a 'whole' GM. However, using % GM DNA as the units, each GM containing seed only contains 50% GM, because one genome is from the non-GM parent. The % GM DNA test result would therefore be half the result from %GM seed measurements.

In order to alleviate this problem of % GM seed and % GM DNA interpretation we have developed and tested a combined protocol for PCR testing of seed (COPs) based on the framework described below. This protocol uses initial qualitative tests on seed pools followed by quantitative real-time PCR on the positive pools. When initial seed pool screening has suggested that the result is near the GM threshold in question, and that the uncertainty of seed pool testing alone could result in an incorrect labelling/rejection decision, real-time PCR on the positive pools is used to provide better resolution. This method is cheaper and more accurate than using real-time PCR on a large single analytical sample, and provides more data such as the ability to detect differences between admixtures of more than one GM event and GM event 'stacks' (events crossed into the same genome).

This work initially involved growing and genotyping with molecular methods oilseed rape (OSR) plants with different and complex GM genotypes in order to test the protocol performance. The plant lines were successfully made and sufficient seed for the tests was generated.

Preliminary statistical analysis provided a testing plan with reasonable seed pool size and numbers of pools. Qualitative and quantitative (TaqMan) PCR tests were optimised and applied to two seed mixtures (0.9% and 0.3% GM seed) for three types of assay - two event specific and one GM gene specific.

Results showed that, as expected, homozygous GM seed gave the same result for % GM seed and % GM DNA. Whereas heterozygous GM seed gave approximately half the result for % GM seed compared to %GM DNA in most cases. In general, the real-time PCR assays always gave the expected result which indicated %GM DNA was either lower, higher or the same as the % GM seed result but the resolution of the result varied between assays. Some results showed significant deviations from those expected. Seed pool methods in general exhibited a large uncertainty and some real-time PCR results were consistently lower than expected. Likely causes for problems in this testing system were found to be: under-mixing of the analytical seed sample prior to DNA extraction; maintaining efficient DNA extraction over many seed pools; and level/cost of replication necessary to obtain accurate results. All of these factors can be improved upon for future COPs - type testing.

In conclusion, we found that COPs would provide useful information when test results are close to GM thresholds and could inform more reliable GM labelling decisions for thresholds based on % GM DNA than seed pool - type testing alone but COPS also provides more information about the genotypes of GM seed than real-time PCR alone can.

Introduction

Impurity testing in seeds has traditionally been done using either single or sequential seed-pool tests. From an analytical seed sample (e.g. 3000 seeds) equal seed pools are made and analysed individually for the trait of interest. Using binomial statistics, the number of seed pools positive for the trait can be used to estimate the total number of positive seeds in the analytical sample. The accuracy of the method increases with increasing number of pools (each with smaller numbers of seed) but this also increases expense of the whole test. Therefore a sequential plan, in which an initial screen is done on a small number of pools, followed by larger number of pools if a clear answer is still not obtained, is often used to reduce cost. These semi-quantitative tests work well because given a known (and low) false positive and negative rate the binomial statistics give well-defined risks for the seed lot being falsely accepted (consumer's risk, β) or falsely rejected (producer's risk, α). Testing plans can be designed to conform to a specified threshold and α and β risk.

Seed pool testing can only work when the units for the threshold in question is the seed. When the tested unit is a variable fraction of one seed, then the associated statistics and results are no longer valid. EC guidance for testing of GM presence in food (European Commission, 2004) specifies the use of units as % GM DNA. In order to maintain comparable results throughout the food chain, it seems likely that any future EU thresholds for adventitious presence of GM in seeds will also be specified in terms of % GM DNA, determined by methods such as real-time PCR (see review in

Lipp *et al.*, 2005). For seeds which are always homozygous because of their species' mating system (such as soybeans), this is not a problem, because seeds with 100% GM DNA (both haploid genomes GM) will also be 100% GM seeds. However, for heterozygous GM seeds and seeds with more complex genomes (e.g. maize) results will be inconsistent using different units. Figure 1 shows how these inconsistencies can arise in a simple system. In practice, more complicated mixtures of genotypes may result in a continuously variable ratio of % GM DNA to % GM seed in a sample. The most likely current outcome of these problems, if seed pools alone were used, is that % GM would be overestimated by a factor of two for heterozygous species, increasing the α risk of the test. β risk could also be increased, but this would be dependent on the definition of the GM events under question. For example if the test used was not sufficiently specific to an individual GM event and more than one event were present in the tested seed, either as a mixture or a 'stack' (i.e. more than one GM event in the same genome), then the % GM seed result could be considerably lower than a real-time PCR % GM DNA result (increased β risk). Although cheap and effective in some circumstances, it would therefore appear prudent to not rely entirely on seed-pool testing for GM threshold enforcement and to employ real-time PCR when initial screens indicate that results are close enough to the threshold for the unit used (% GM seed or % GM DNA) to alter any decision based on the result. Real-time PCR alone could be used for GM testing of seed, however many labs still lack the expensive equipment necessary to carry it out routinely. Also, real-time PCR has a more variable uncertainty associated with its results than seed-pool testing, especially when performed on large single seed bulks and there is some question of the limit of detection being sufficiently low with real-time PCR in standard seed samples (often 3000 seeds or more).

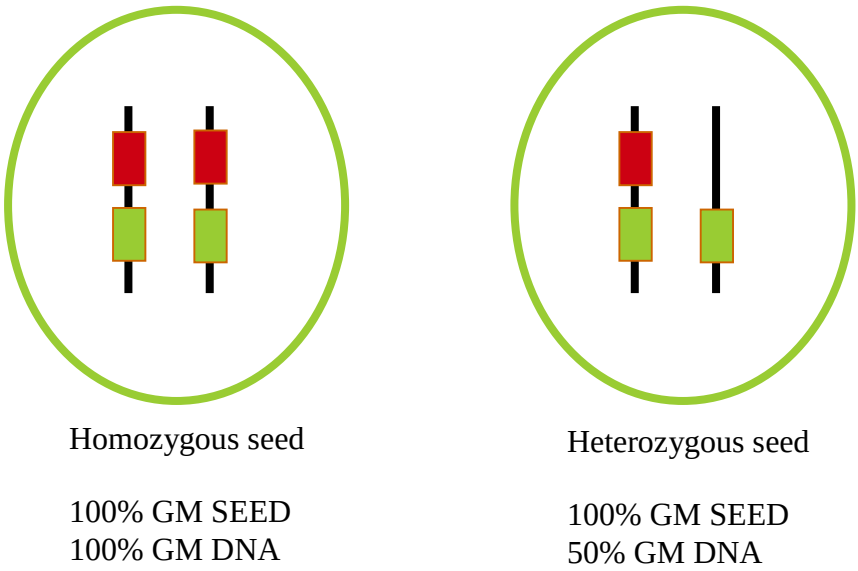
The objective of the COPs design presented here is to provide more information with which to inform GM threshold enforcement decisions than either seed pool or real-time PCR tests alone, by providing an estimate of the average GM genotype of the tested seed. In brief, an initial seed pool test is performed by standard, qualitative PCR on DNA extracted from each pool. If the result is within half to twice the threshold (as % GM seed) then real-time PCR is carried out on the seed pools that were positive. A significant deviation between the % GM seed and % GM DNA result indicates the genotype of the GM event may affect the test decision. If more than one event is tested by COPs then the co-occurrence of the events in the positive pools can distinguish between event mixtures (which under current interpretation of EU guidelines, would be summed to give the total % GM DNA) and event stacks (which may not be authorised). COPs is intended to be used following GM event identification screens so the number of GM events quantified by COPs would be determined by such screens beforehand.

Oilseed rape (OSR) was chosen for this work because it is an important economic UK crop and it is an opportunistic outcrosser and therefore capable of different GM genotypes. OSR is an allotetraploid but effectively behaves as a diploid species (the genotype of both parent species remaining stable in intra-specific crosses) and it is treated as a simple diploid in this study.

It is not the scope of this report to review seed pool and real-time PCR methods for GM testing, but rather to examine the specific situations which may benefit from the COPs type of protocol. Testing statistics have been applied elsewhere specifically to

seed tests (Redmund, *et al.*, 2001) and real-time PCR (Laffont *et al.*, 2005). Here we examine only the empirical statistics of the COPs tests performed.

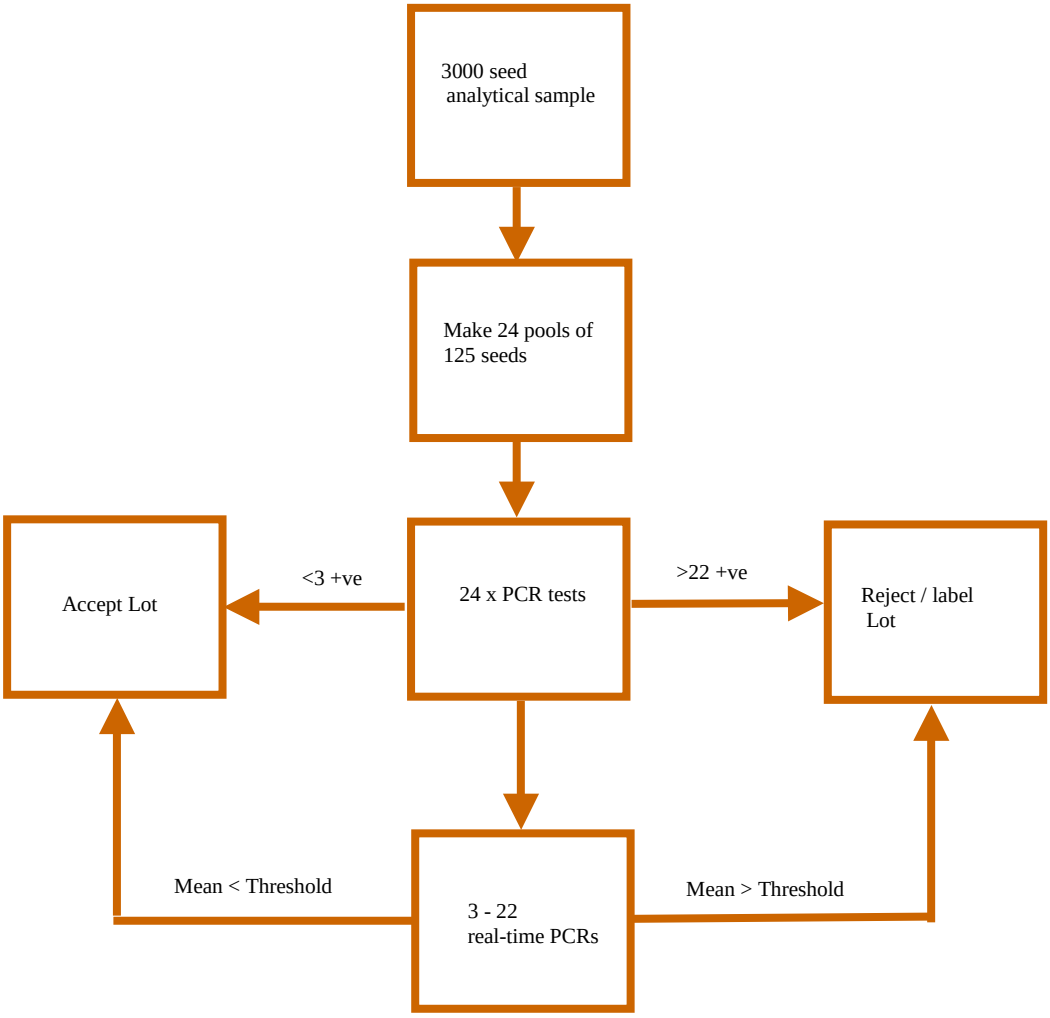
Figure 1. Illustrating the inconsistency between % GM DNA and % GM seed. Each line represents a haploid genome in a single seed. The green boxes represent targets of real-time PCR control genes i.e. quantifying total target taxon DNA. The red boxes represent PCR (seed pool and real-time PCR) GM targets.



Methods

The basis of COPs is the ability of the seed pool test and real-time PCR test to give different results for heterozygous GM seed down to concentrations approximately half of a specified GM threshold. For our purposes 0.9% and 0.3% (as % GM seed) were chosen as upper an lower limits for a threshold value. The limiting factors in the COPs testing plan design were: (i) an analytical sample capable of 95% probability of detection of 0.1% GM seeds (= 3000 seeds); (ii) a cost effective number of seed pools, allowing replicate tests in standard 96-well PCR plate format; and (iii) the expected relative standard deviation (RSD) of event-specific real-time PCR methods. Note that (ii) and (iii) are interdependent because as the number of seed pools increases, the number of seeds in each decreases and therefore the RSD of the real-time PCR on each pool decreases. Preliminary statistical analyses showed that for 24 seed pools with 125 seeds in each, the 'overlap' of a real-time PCR uncertainty and the seed pool test uncertainty should be less than 5% for a real-time PCR method with RSD <15% with heterozygous GM seeds. The test plan thus devised is show in Figure 2. Although 15% RSD is lower than expected for many real-time PCR methods, we have found it achievable with the methods and seed pool size used here.

Figure 2. Diagram of COPs plan.



In order to evaluate the COPs plan shown in Figure 2, OSR plants containing two GM events, MS8 and RF3, as used in the UK Farm Scale Evaluations, were grown in glasshouse conditions and crossed to produce a seed progeny which consisted solely of a single genotype (Figure 3). This genotype was expected to give different COPs results, depending on the PCR tests used.

Figure 3. Generation of test GM OSR seed. N.B. The female parent is male sterile and therefore all seed produced must be from cross with male parent.

Female parent × Male parent
MMrr MMRR

↓

F1 progeny
MMRr

MMRr = 100% GM DNA, 100% GM seed MS8
MMRr = 50% GM DNA, 100% GM seed RF3
MMRr = 150% GM DNA, 100% GM seed BAR

PCR and real-time (TaqMan) PCR used in this work are described in detail elsewhere. The event specific tests, RF3 and MS8 are described in Allnutt *et al.*, 2005a; and the BAR PCR test, which detects both MS8 and RF3 is described in Weekes *et al*., 2005. The MMRr genotype seed used was therefore expected to give equivalent results for MS8 seed pool and real-time PCR, whereas RF3 results for seed pools would be double those for real-time PCR and BAR results for real-time PCR would be 1.5 times those of BAR for seed pools. This single seed resource therefore provided several seed testing scenarios depending on the test used.

Two analytical samples of 0.9% and 0.3% MMRr seed were made by counting 27 and 9 seeds into 2973 and 2991 non-GM seeds (Acrobat variety) respectively. These were mixed by shaking and dispensed into 24 seed pools of 125 seeds each in 5ml nalgene tubes. DNA was extracted from each pool using the method for OSR seeds and leaf and standard PCR performed as described in Allnutt *et al*., 2005b. PCR products from seed pool tests were run on agarose gels, stained by ethidium bromide and scored as positive or negative for the GM target. Normally we would expect the COPs to operate by testing only the positive PCR seed-pools with real-time PCR, but at this developmental stage all pools were tested with real-time PCR. Each standard and real-time PCR was performed in duplicate. Because the real-time PCR methods two assays, an endogenous control and the GM target, duplication of each gives four potential quantification results. These four results were used to calculate the RSD and mean for each real-time test overall result.

Results and Discussion

The real-time PCR tests had variable RSDs which were calculated from four replicates and the distribution of which was assumed to be normal. Results from all tests are summarised in Table 1. The uncertainty of the seed pool tests was determined by the binomial distribution and the number of positive pools observed. Real-time PCR RSD varied from 4% to 30%, this emphasises the importance of empirical determination of the uncertainty by replicate tests, only by doing this could the power of any comparison between the real-time quantification and the seed pool quantification be determined. One real-time PCR result exhibited a downward bias. The 0.9% GM seed sample gave 0.66% for MS8 which was expected to be 0.9%. The reason for this is not known, although in general the accuracy of the real-time PCRs was good. A more significant problem was evident in the number of positive seed pools obtained from the 0.9% analytical sample. The detection limit of the qualitative PCR test used is well below the minimum GM seed content per pool (0.4% GM DNA) however, the observed number of positive pools was 11 compared to the expected 16. A possible explanation for this is under-mixing of the analytical sample and subsequent clumping of GM seed in the pools. This is evident from individual pools' real-time PCR results, some of which were higher than expected for a random distribution of seeds (not shown). This problem did not seem to occur for the 0.3% analytical sample where 7 or 8 positive pools were observed and 7 were expected.

Seed pool tests in the first phase of the COPs plan showed that the RF3, MS8 and BAR positive pools did not segregate, i.e. the same pools were always positive. As expected, this demonstrates that the events were stacked (crossed into the same genome) with a high statistical power (the probability of this occurring by chance is very small, $P << 0.001$). In 'real' situations a single pool in which the MS8 and RF3 events did not occur together would prove that a mixture of events was present (although stacks could also be present as a mixture).

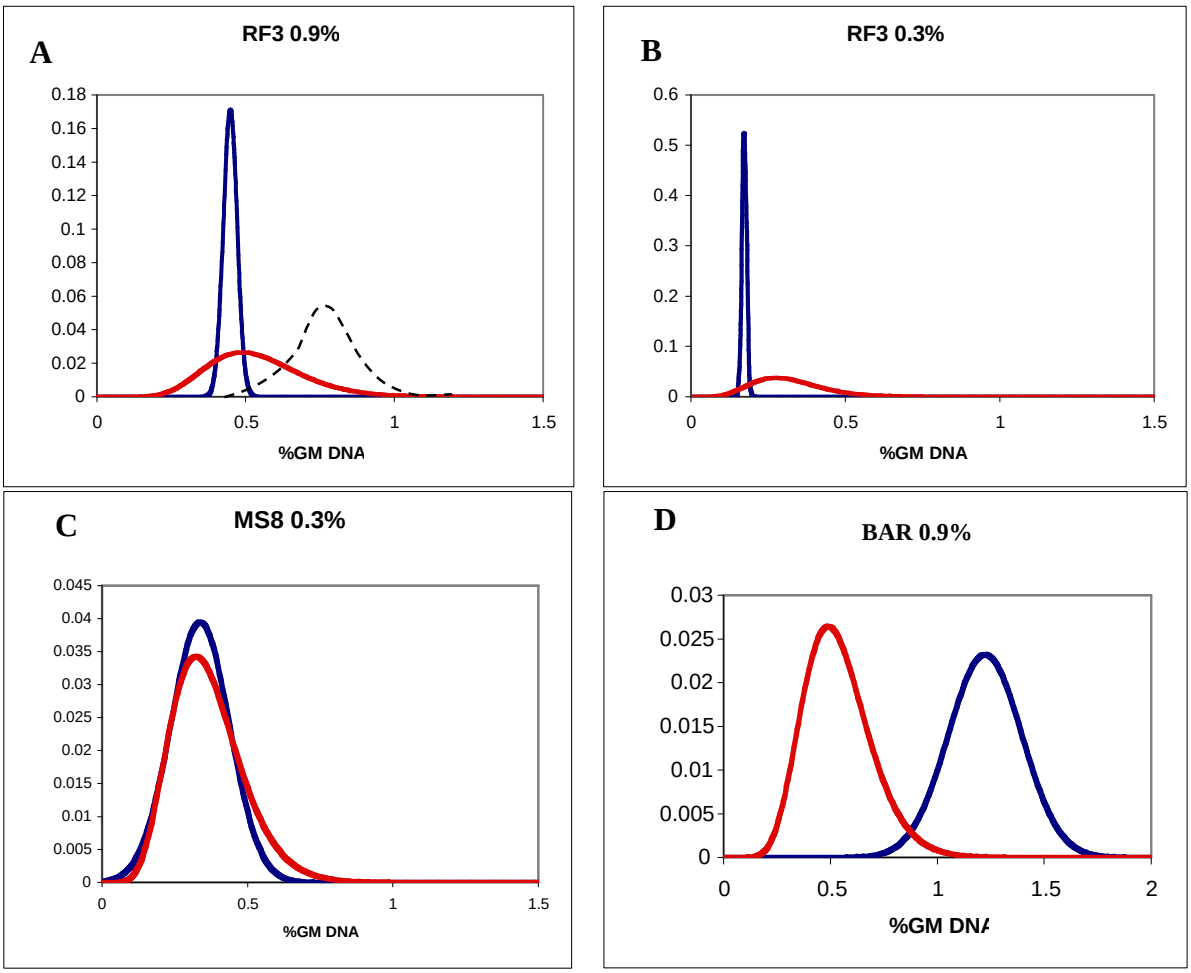
Table 1. COPs results for 0.9% and 0.3% GM seed analytical samples. P = probability of actual % GM being the equal given the observed distributions from real-time and seed pool tests. Z = observed zygosity: expected zygosity

		Real-time PCR		Seed Pools			
Test	% GM seed	Mean % GM DNA	RSD %	Tests +ve/ 24	% GM seeds	Z	P
RF3	0.9	0.45	5.20	11	0.54	0.8:0.5	0.27
RF3	0.3	0.17	4.10	7	0.32	0.53:0.5	0.08
MS8	0.9	0.66	9.41	11	0.54	1.2:1	0.40
MS8	0.3	0.34	30.0	8	0.37	0.91:1	0.91
BAR	0.9	1.22	14.0	11	0.54	2.23:1.5	0.05
BAR	0.3	0.47	14.0	7	0.32	1.47:1.5	0.34

Despite these problems, the disparity between real-time and seed pool PCRs was consistently in the direction expected, i.e. for RF3, % GM DNA was lower than % GM seed; for BAR, % GM DNA was higher than % GM seed; and for MS8, % GM

DNA was very similar to % GM seed. Figures 4, A to D illustrate the disparity of % GM DNA and % GM seed revealed by COPs. In all the cases examined COPs would have successfully shown-up potential errors in % GM determination by seed-pool testing alone due to the genotype of the GM seed, but with varying confidence (see P values in Table 1).

Figure 4. Probability distributions of COPs results for: A = 0.9% GM seed, RF3; B = 0.3% GM seed, RF3; C = 0.3% GM seed, MS8; and D = 0.9% GM seed, BAR. P = probability of observation. The blue line is the normal distribution for observed real-time PCR means. The red line is the binomial distribution for the expected % GM seeds. In panel A, the dashed line is the expected binomial distribution (with 16 positive tests, rather than the 11 actually observed).



The tests for RF3 with the 0.9% GM seed analytical sample (Figure 4A) gave a 27% probability that the % GM seed and % GM DNA were the same, but as shown, a lower 'overlap' would have been expected if the under-mixing of seed had not occurred (dashed line). Figure 4B illustrates the potentially very different probability characteristics of the real-time and seed pool methods, the result for real-time PCR having a very low standard deviation, and therefore a sharp curve, compared to the seed-pool binomial distribution which is more dispersed - despite this a good degree of confidence (P = 8%) could be placed on the results not being the same and a

zygosity of 0.53 being estimated for the GM seed in the sample. Figure 4C illustrates the situation where the GM is homozygous and % GM DNA and % GM seed are expected to be the same: both distributions almost completely overlap (P = 91%). Figure 4D shows the effect of a stacked GM and non-event specific tests (MS8 plus RF3 detected by the BAR assay), where the % GM DNA is significantly greater than the % GM seeds (P = 5%).

Some pools which were scored as negative in the first COPs stage were subsequently found to have levels of GM equivalent to less than one heterozygous seed (<<0.4%) but significantly above zero, by the real-time PCR assay. These pools would not normally be tested in COPs and they would not contribute a significant proportion of GM to the overall real-time PCR mean (including positive pools). However, they do indicate a potential problem with seed pool quantification of GM, that broken seeds, or chaff, can give a positive GM signal. This would lead to over-estimated GM from the seed pool tests. This did not occur to a problematic level in this study because only lower numbers than expected of positive seed pools were observed. However, we also suspect that under-mixing of the 0.9% GM seed analytical sample had occurred. If more vigorous mixing had been used it is possible that chaff and broken seed could have been a bigger problem. Further experiments would be required to investigate this problem - which would vary between species, variety and age/condition of seed.

This development work has shown that the combined seed testing protocol presented here (and other protocols which use a similar approach) can provide invaluable information on the genotypic make-up of adventitious GM presence in a seed sample. The protocol could prevent GM labelling mistakes in instances close to thresholds of 0.15 to 1.35% GM DNA. Further optimisation and development work is required to determine the limits of such a protocol, not least its application to other species with more complex genotypes than OSR.

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