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European Network of GMO Laboratories

Working Group “Seed Testing” (WG-ST)

Working Group Report

The working group on "Seed Testing" was established on the basis of a mandate adopted at the 26th ENGL Steering Committee meeting of 25-26 March 2014. The working group was chaired by *Rupert Hochegger*, Austrian Agency for Health and Food Safety (AGES), Vienna, Austria.

The other members of the working group were: *Niccolo' Bassani*, European Commission' Joint Research Centre (JRC), *Anke Belter*, Land Office for Environmental Protection Agency of Saxony-Anhalt; FG13 Monitoring Laboratory for Genetic Engineering, Halle, Germany, *Ottmar Goerlich*, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim, Germany, *Lutz Grohmann*, Federal Office of Consumer Protection and Food Safety, Berlin, Germany, *Joachim Kreysa*, European Commission' Joint Research Centre (JRC), *Marc De Loose*, Institute for Agricultural and Fisheries Research (ILVO), Merelbeke Belgium, *Marco Mazzara*, European Commission' Joint Research Centre (JRC), *Roy Macarthur*, the Food and Environment Research Agency, Sand Hutton, York, United Kingdom, *Elena Perri*, Consiglio per la ricerca in agricoltura e l’analisi dell’economia agraria, Centro di Sperimentazione e Certificazione delle Sementi (CRA-SCS), Tavazzano, Italy, *Bojan Rajcevic*, European Commission' Joint Research Centre (JRC), *Mathieu Rolland*, GEVES, Beaucouzé cedex, France, *Christian Savini*, European Commission' Joint Research Centre (JRC), *Slawomir Sowa*, Plant Breeding and Acclimatization Institute, National Research Institute, Blonie, Poland, *Brigitte Speck*, Center for Agricultural Technology Augustenberg, Karlsruhe, Germany, *Catelijne Van Beekvelt*, Ministerie van Infrastructuur en Milieu, Den Haag, the Netherlands, *Daniela Villa*, Consiglio per la ricerca in agricoltura e l’analisi dell’economia agraria, Centro di Sperimentazione e Certificazione delle Sementi (CRA-SCS), Tavazzano, Italy.

The mandate of the working group was the following:

The WG-ST shall prepare an ENGL/EURL advice to the Commission concerning the testing of seed samples for the adventitious presence of GM seeds.

In preparing this advice the WG-ST shall take due account of, and describe the current practice of GMO seed testing in the Member States of the European Union.

As the practical detection limit for GM seed in non-GM seed lots can be lowered by means of sub-sampling and increased efforts of testing, the WG-ST is asked to estimate the relation between effort (cost) and theoretical detection limit for the most relevant crops.

Executive summary

Testing seed lots for the unintended presence of genetically modified (GM) seeds is carried out in European Union Member States (MS). The aim of the testing of seeds for genetically modified organisms (GMOs) is to test whether GMOs are present in non-GM seed lots. Splitting samples of seeds taken from lots into subsamples, testing for the presence of GM seeds in each subsample, and counting the number of positive subsamples is a suitable method for estimating the proportion of GM seeds impurities with a specified probability. The detection of lower proportions of GM seeds in lots requires the analysis of larger seed samples and larger amounts of DNA. This entails more effort and cost to detect lower quantities of GM seed.

A decision to form a Working Group (WG) for "seed testing" (WGST) was taken during the 26th ENGL Steering Committee meeting. The WGST was formed to study the relation between the impurity of GM seed that could be detected and the cost of the analyses required to detect the unintended presence of GM seed in conventional seed lots. The WGST was tasked with producing a report on the issue for the approval of the ENGL Steering Committee. The report would then form the basis of ENGL/EURL advice to the Commission on testing seed samples for the unintended presence of GM seeds for the most important crops.

The WG elaborated a statistical model to describe the relation between the impurity level of GMO seeds in seed lots that will, with a high probability, be reliably detected by test plans (the limit of detection) and the cost of the test plans needed to achieve this and effort devoted to the plan.

Lowest-cost test plans were estimated for crops with test plan limits of detection at 5%, 0.9%, 0.5%, 0.1%, 0.05%, 0.01% and 0.005% GM seed.

As the limit of detection for a plan is reduced, an increasing number of subsamples are required and the change in estimated cost becomes inversely proportional to the change in the estimated limit of detection. A halving from a low limit of detection to a lower limit of detection approximately doubles the estimated cost of the laboratory analysis. For high test plan limits of detection this has no effect on laboratory costs because there is a certain minimum effort required to test working samples of any size. The analysis showed that the rate at which cost increases is determined by the properties of the seed being tested: specifically, the size of the seed and the number of genome copies per mass of DNA.

In the European Union, maize seed lots are among the most commonly tested commodities for GMO presence. For maize, it is estimated that the same effort (analysis of two subsamples with a single grind for each subsample) and same cost applies for any plans' limit of detection higher than 0.11% GM seeds in a lot. Reducing the limit of detection for a plan to a value below 0.11% GM seeds in maize seed lots, requires that the higher number of seeds be split into more subsamples so that GM DNA can be reliably detected if it is present in any of the seeds in the working sample.

Contents

1. Introduction

1.1. Global seed market: relevance of the issue

There has been a marked growth of the international seed trade in the period between the mid '80s and 2010; the value of the market has seen a 10 fold increase[1]. In 2012, the European Union (EU) commercial seed market had a value of approximately EUR 7 billion, representing more than 20% of the total worldwide market for commercial seed, ranking slightly behind the U.S.A. (EUR 9.2 billion) and China (EUR 7.6 billion). In 2002/2003, the EU became a net exporter of planting seeds. Currently, the EU plant reproductive material (PRM) sector is highly competitive at a global level. It is the largest exporter worldwide with an export value of EUR 4.4 billion - more than 60% of exports worldwide. The sector is highly concentrated (the largest 10 companies represent nearly 67% of the global seed market), but small and medium enterprises (SME) and micro-enterprises still play an important role in the EU internal market, notably in niche markets such as organic crops[2].

The production of seeds in the EU occupied over 2 million ha in 2013 (increase by 7%). The highest rate of growth can be observed in maize (+16%), pulses (+9 %), and cereals (+6%). Small-grain cereals are the first seed production with almost 1.2 million ha in 2013, what corresponds to a production of more than 4.1 million tons in 2012. It is followed by grasses / forage species (307 985 ha), maize (196 110 ha), oilseeds (134 860 ha), potato (106 600 ha) and pulses (60 000 ha)[3].

Seeds are produced in almost all Member States. France is the largest producer of seeds with 335 230 ha before Germany (195 365 ha) and Italy (185 570 ha). Seeds are generally classified in groups of species. The groups defined in current EU legislation (see Paragraph 4, "EU legal basis on seed material") are:

1. fodder plant seed
2. cereal seed
3. material for the vegetative propagation of the vine
4. propagating material of ornamental plants
5. forest reproductive material
6. agricultural plant species
7. beet seed
8. vegetable seed
9. seed potatoes
10. oil and fibre plants
11. vegetable propagating and planting material, other than seed
12. fruit propagating material and fruit plants intended for fruit production

Genetically modified (GM) seed may be produced for the EU or non-EU markets. In addition, seeds are imported into the EU from countries where genetically modified organisms (GMO) of the same species are planted. Hence, the possibility of the unintended presence of GM

seeds in a conventional seed lot cannot, *a priori,* be excluded. Testing conventional seed lots for the presence of GM seeds can provide assurance that surveillance programs are working.

1.2. Testing of seed lots for the unintended presence of GM seeds

Testing seed lots for the unintended presence of GM seeds is practised in EU Member States. No minimum threshold is set for labelling of seed lots if any authorised GM seeds are found to be present. In most EU countries sampling seems to be based on the ISTA seed testing rules (International Rules for Seed Testing) but hitherto no GM-focussed, practical guidance exists neither for seed sampling nor for the analytical methods to be applied.

In the EU, food and feed products that consist of or contain materials produced from GMO may be placed on the market, provided that the GMO is authorised for food or feed purposes and the product is appropriately labelled. The unintended technically unavoidable presence of an authorised GMO product does not trigger the labelling requirement provided that the GM content is in a proportion no higher than 0.9% (the labelling threshold) of the food or feed ingredient[4]. No similar labelling threshold for the presence of GM seed in conventional agricultural seed lots has been adopted at the EU level, although Article 21 (2) of the European Union (EU) Directive 2001/18/EC does provide this option[5]. However, competent authorities in some Member States have applied a requirement of labelling seed lots if the content of GM seeds authorized for cultivation exceeds a particular impurity level.

According to EU legislation, official methods for detecting and quantifying the GM presence and content in food and feed are event-specific (real-time) PCR-based procedures[4,6,7]. The GM content is expressed in percentage of mass fraction or in percentage of the haploid genome equivalent as the ratio in copy numbers between the GM event target and an endogenous species- (or taxon-) specific target that represents the haploid genome of the considered plant. The event-specific methods are validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) [8,9].

In contrast to food and feed, the quantity of GM seeds in conventional seed lots is defined as the proportion of deviant seeds in the lot. This cannot necessarily be estimated from the proportion of GM DNA copy numbers in the sample. Seeds are composed of different tissues such as endosperm, embryo and pericarp. These may have, with different ploidy levels, different ratios between maternal and paternal origins and different DNA contents. In addition, the ratio between tissue mass and DNA copy number varies between seed varieties bred from a specific GMO. These biological factors have considerable impact on real time PCR based GM quantification results particularly for heterozygous maize seeds[10-12]. Therefore, measurements of the proportion of GM DNA cannot be expected to represent an estimate of the proportion of GM seeds. If the mass/mass reference material used for

quantification is genetically the same as the GM sample the outcome of the quantification will represent an estimate of the proportion of GM seeds.

Hence, a different approach for testing the presence of GM seed in seed lots is required compared to what is prepared in food and feed. Previous studies have shown that splitting samples of seeds taken from lots into subsamples, testing for the presence of GM seeds in each subsample, and counting the number of positive subsamples can be a suitable method to estimate the impurity level in conventional seed lots[13-14].

For instance German seed testing laboratories organized two ring trials, one with maize seeds in 2011 (20 laboratories) and another one with oilseed rape in 2012 (11 laboratories) to assess the performance of test methods[15]. A testing plan was designed to detect the presence of GM seeds at a test value of 0.1% for a laboratory sample at a confidence level of 95%. By applying this testing plan, up to six subsamples of 1,000 seeds each were analysed by a qualitative event-specific PCR targeting maize MON810 or oilseed rape GT73. Seedcalc[16] was used to interpret the observed numbers of positive subsamples to successfully discriminate between samples that contained 0.1% GM seeds and those that contained 0.017%. No false positive or false negative results were observed. The quantitative real time PCR analyses of the same samples for the maize target provided estimates of the proportion of GM content that were much more variable.

Testing for the presence of GM seeds using the qualitative sub-sampling approach consists of sampling and detecting the presence of discrete particles (seeds and DNA molecules). Hence, the detection of lower proportions of GM seeds in lots requires the analysis of larger seed samples and larger amounts of DNA. This entails an increase in effort and cost. The rate at which cost increases is determined by the properties of the seed being tested: specifically the size of the seed and the number of genome copies per mass of DNA[17].

Hence, in order to consider options for detecting unintended presence of GM seeds in seed lots we need to estimate the relation between the level of GM seeds impurity that will be detected and the effort and cost of achieving the detection. The aim is to provide competent authorities with information that can be used to come to a decision about what can reasonably be achieved by testing and the main factors that affect the performance of testing.

Test plan performances can be modelled statistically, by for example using Seedcalc or similar approaches or by undertaking validation studies with testing in laboratories.

1.3. Review of the FVO reports of audits carried out in EU member states on the official control of genetically modified organisms (2009 – 2013)

As a Commission service, the Food and Veterinary Office (FVO) performed a number of audits, inspections and related activities in Member States (MS). Focus of the review was on audits that evaluated the system of official controls for genetically modified organisms including their deliberate release into the environment. The audits were carried out in Portugal, Spain, Germany, France, The Netherlands, Poland and Slovakia between 2009 and 2013.

The main findings of the audits are the following:

- The plans for seed controls by competent authorities (CA) vary between MS. In some MS the International Seed Testing Association (ISTA) rules for seed testing are implemented. Other MS implement national resolutions or general principles. However, in some cases the inspectors could not find out what criteria are implemented for sampling of seeds lots.
- Some MS apply zero tolerance, while some have set thresholds that may differ according to the authorization status of the event.

Final reports of the audits carried out in EU Member States by the Food and Veterinary Office are available on DG-SANCO's website (http://ec.europa.eu/food/fvo/ir_search_en.cfm).

1.4. Review of the questionnaire on GMO seed testing practice in EU Member States

In order to collect information concerning the practice related to testing for the presence of GMOs in seeds within the EU member states the ENGL Working Group on Seed Testing circulated a questionnaire to national competent authorities. A few questions were asked: a) the average percentage of seed lots tested for GMOs in 2012/13; and b) the number of seed lots which were positive for GMO(s) in 2012/13.

Additionally, competent authorities were asked to provide information related to following items: plant species, maximum number of kernels tested per seed lot, number and size of subsamples tested separately and detection method used (PCR, ELISA, Bioassay, etc.).

Thirteen questionnaires were received. It appears that in some Member States no regular testing for the presence of GMOs in seed lots is performed, while in others the average proportion of seed lots tested for that purpose may reach 80% for some plant species. The number of seed lots tested positive for GMO presence during the years 2012 and 2013 differed among Member States and ranged from 0 to 52.

2. Mandate

A decision to form a Working Group (WG) for "seed testing" (WGST) was taken during the 26th ENGL Steering Committee meeting. Task was given to the WGST to prepare an ENGL/EURL advice to the Commission concerning the testing of seed samples for the unintended presence of GM seeds. As the practical detection limit for GM seed in non-GM seed lots can be lowered by means of subsampling and increased efforts of testing, the WGST was given the task to estimate the relation between effort (cost and practicability) and theoretical detection limit for the most relevant crops. The WGST was tasked with producing a report on the issue for the approval of the ENGL Steering Committee to be held on 15-16 September 2014.

3. Definitions

Composite sample. The composite sample is formed by combining and mixing all the primary samples taken from the seed lot.

Limit of detection (LOD) of the test plan. The LOD of the test plan is the lowest level of impurity in the seed lot that will be detected with the probability of 95%.

Plant Reproductive Material (PRM). Propagating material including seeds and any other propagating plant material. It comprises seeds for crop plants, tubers, rhizomes, propagating materials originating from vines or other crops, forest and shrubs seeds.

Primary sample. A primary sample is a portion taken from the seed lot during one single sampling action.

Seed. Botanical definition: results of the fertilization of an ovule. Morphologically consists of i) a plant embryo that may develop into a seedling during germination, ii) stored nutrients and iii) a protective seed coat (Testa). In the framework of EU legislation (or OECD standards) seed may comprise both "True seeds" (botanical definition) and units retaining additional structures (e.g. pericarps or residues of the floral structures). In the context of this report seed refers to agricultural (with cereals, fodder, beets, oil and fibre plants), vegetable species and seed potatoes. (From ISTA Handbook on seedling evaluation III edition)

Seed lot. A seed lot is a specified quantity of seed that is physically and uniquely identifiable.

Submitted sample. A submitted sample is a sample that is to be submitted to the testing laboratory and may comprise either the whole of the composite sample or a subsample thereof.

Subsample. A subsample is a portion of a sample obtained by reducing a sample.

Working sample. The working sample is the whole of the submitted sample or a subsample thereof, on which one of the quality tests described in the ISTA Rules is made and must be at least the weight prescribed by the ISTA Rules for the particular test.

4. EU legal basis on seed materials

The current EU legislation for plant reproductive material (PRM) has been developed since the 1960s. Today, the framework consists of 12 basic EU Directives covering variety listing as an authorisation for marketing and specific marketing requirements for different species (fodder plant seed, cereal seed, sugar beet seed, seed of oil and fibre plants and vegetable seed, vine propagating material, seed potatoes, vegetable reproductive material other than seed, fruit plant propagating material, ornamental plants, forest reproductive material). The Council Directive 2002/53/EC[18] concern the acceptance for inclusion in the common catalogue of varieties of agricultural plant species. Once the variety is listed in the catalogue, the seed can be marketed in line with the directives on the marketing of seeds.

As mentioned before, GMOs are dealt in a separate legislation involving Directive 2001/18/EC[5], Regulation (EC) No 1829/2003[4] and Regulation (EC) No 1830/2003[19]. GM seed varieties are included in the current seed certification legal framework by the Council Directive 98/95/EC[20]. This contains amendments to the directives on the marketing of seeds including the labelling of genetically modified lots. To complete the framework, the Commission Recommendation of 13 July 2010[21] give guidelines for the development of national co-existence measures to avoid the unintended presence of GMOs in conventional and organic crops.

On the topic of the GM testing the Commission Recommendation 2004/787/EC[22] defines technical guidance for sampling and detection of GMOs and material produced from GMOs as or in products in the context of Regulation (EC) No 1830/2003.

5. The International Seed Testing Association and its Rules

The International Seed Testing Association (ISTA)[14] has the aim to develop, adapt and publish standard, reliable and reproducible procedures for sampling and testing seeds, and to promote uniform application of these procedures for evaluation of seeds moving in international trade.

Methods included in the ISTA Rules are validated with the aim of providing test procedures that give reliable and reproducible results. ISTA has a number of Technical Committees to provide scientific and technical advice on test procedures. Since 2001, a GMO Technical Committee exists and organises training courses and proficiency tests on GMO testing for ISTA accredited laboratories.

The ISTA Rules are designed for the principal crop species, including agricultural and vegetable species, tree and shrubs, flower, spices, herbs and medical plants (almost 1000 species). In 19 chapters, many of the internationally accepted sampling or testing methods for seed quality evaluation are provided.

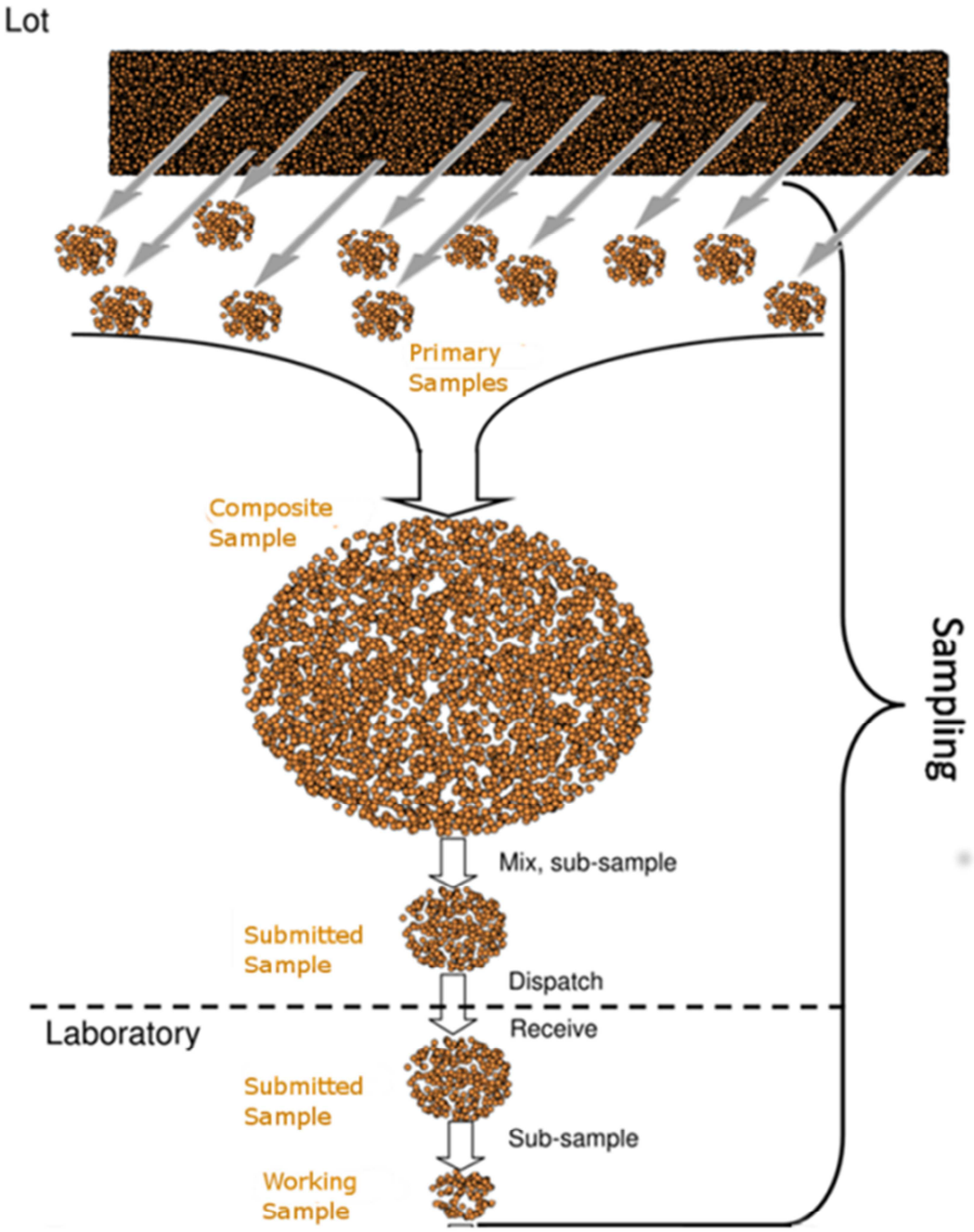
For the scope of this document, the most relevant chapters are chapter 2 “Sampling” and chapter 19 “Testing for seeds of genetically modified organisms”. Chapter 19 gives guidelines to detect, quantify or confirm the presence of GMO seeds in seed lots. In the field of GMO testing, ISTA has developed a system based on a performance approach targeting the uniformity in GMO testing results rather than on a harmonisation of GMO testing methodology.

5.1. ISTA rules for sampling

Methods for sampling seed lots are described in Chapter 2 of the ISTA rules.

The objective of sampling is to obtain a sample of a size suitable for testing and representative of the seed lot. Here “representative” means that the probability of a constituent being present in a sample is determined only by its level of occurrence in the seed lot. Figure 1 shows how the working sample to be analysed is obtained from the seed lot.

Figure 1. The sampling scheme from a seed lot according to ISTA rules



The composite sample is built from the seed lot by taking primary samples of appropriate and equal size from different positions of the whole lot and combining them. From this composite sample, subsamples are obtained by sample reduction procedures at one or more stages forming the submitted sample and finally the working sample for testing. Sampling and sample reduction must be performed using appropriate techniques and equipment. If the composite sample is of appropriate size or is difficult to mix and to reduce under warehouse conditions, it can be submitted as it is and reduced in the laboratory.

At the time of sampling, the seed lot must be as uniform as possible. If there is documentary or other evidence of heterogeneity, or the seed lot is found to be obviously heterogeneous, sampling must be refused or stopped.

A list of definitions related to the different steps of the sampling procedure according to ISTA rules are provided under Paragraph Definitions. A comparison of the sampling definitions between ISTA rules and CEN standards can be found in Annex 1 for completeness purposes.

6. Seed testing workflow

A correct sampling process requires that:

1. Each primary sample is a representative sample of the location in the lot from which it is taken;
2. The locations from which subsamples are taken, while not necessarily strictly random, are at least not generally correlated with high or low-prevalence parts of the lot (i.e. they are "functionally random");
3. The processes by which the composite, submitted and working samples are formed do not lead to bias in the composition of these samples.

If we assume that sampling is undertaken in a technically correct way then the variables that define a plan for detecting GMOs and how it will perform are:

A. The number of primary samples,
B. The size of the working sample,
C. The false negative rate associated with the method of detection or identification. This is driven by factors such as: representativeness of DNA extracted and delivered to the PCR, and sensitivity of the detection method.

6.1 Sampling

Samples are taken from the seed lot according to the ISTA sampling rules (chapter 2) to produce a submitted sample.

6.2 Setting up a test plan

A test plan for the submitted sample is produced. The test plan specifies the size of the working sample, and the number of subsamples into which the working sample is split for testing. The limit of detection of a test plan can be estimated at this stage to check if it addresses the goal of the test.

The previously mentioned Seedcalc software, in its 'Qual Design Plan' tab, provides a tool for estimating the probability of detecting a given proportion of GM seeds in a lot and for producing plans that meet targets for limit of detection.

6.3 Working sample/preparation and processing of subsamples

According to the test plan, the working sample is prepared from the laboratory sample. Defined numbers of seeds are collected manually or using a seed counting machine. Alternatively the 1000 seed weight (TSW) can be calculated and samples of defined seed numbers can be generated by weighing.

Optionally the seeds may be washed with water and dried before grinding to exclude dust that might give false positive results.

6.4 Grinding

The seeds are ground using a mill which is suitable for the sample size. If the laboratory sample is split in more than one subsample the subsamples should be treated as independent samples. The efforts corresponding to the grinding step increase proportionally to the number of subsamples to be tested.

6.5 DNA-Extraction

DNA is extracted from a test portion of each ground subsample. Each ground subsample should be sufficiently homogeneous and the test portion should contain enough particles so that this sample step introduces no additional uncertainty into the plan.

To reduce the work, alternatively equal amounts of the flour from several subsamples can be combined to one test portion, and the DNA can be extracted from the homogenized mixture. This method can only be employed if from this combined test portion, the laboratory is able to detect a single GM seed in the combined subsample. If the combined test portion is positive, the subsamples should be tested individually according to the chosen testing plan.

6.6 Qualitative analysis by PCR

The DNA-extracts are tested by qualitative PCR for the presence of GM-sequences. In general, at European level, real time PCR methods are used for GM testing. In the case of testing subsamples from seed lots for unintended presence of GM, these methods are used to provide qualitative results (presence/absence).

7. Test plan performance and related costs

The primary parameter that describes the performance of a test plan is the proportion of GM seed that will be detected with a high probability. A seed testing plan may be designed taking this primary parameter and the costs of the plan taking into account. This can be achieved by combining a suitable sample size with a number a subsamples that are tested. Table 1 gives test plans for GM seeds in maize which are estimated (Annex 2) to provide the

specified limits of detection of the test plan (between 5% and 0.005% GM seeds in a lot) for a minimum cost. Testing costs have been normalised to 100% for the minimum cost plan that provides a test plan limit of detection of 0.1% GM maize seed (3 subsamples with a single grind per sub sample). This reflects a currently commonly applied limit of detection in the most commonly tested products. Costs are expressed this way because the effect on cost of changing targets for the test plans limit of detection is more clearly expressed on this scale.

Figure 2 also shows in more detail the relation between the cost and limit of detection for test plans aiming to detect GM seed in maize seed lots.

Table 1. Relationship between LOD of the lowest estimated cost test plan for GM seeds in maize and effort devoted to the plan

Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
5%	68	2	2	69%
0.9%	378	2	2	69%
0.5%	680	2	2	69%
0.1%	3279	3	3	100%
0.05%	6435	5	5	162%
0.01%	31668	21	21	654%
0.005%	63210	42	42	1300%

Figure 2: Relationship between LOD of the lowest estimated cost test plan for GM seeds in maize and cost of the plan

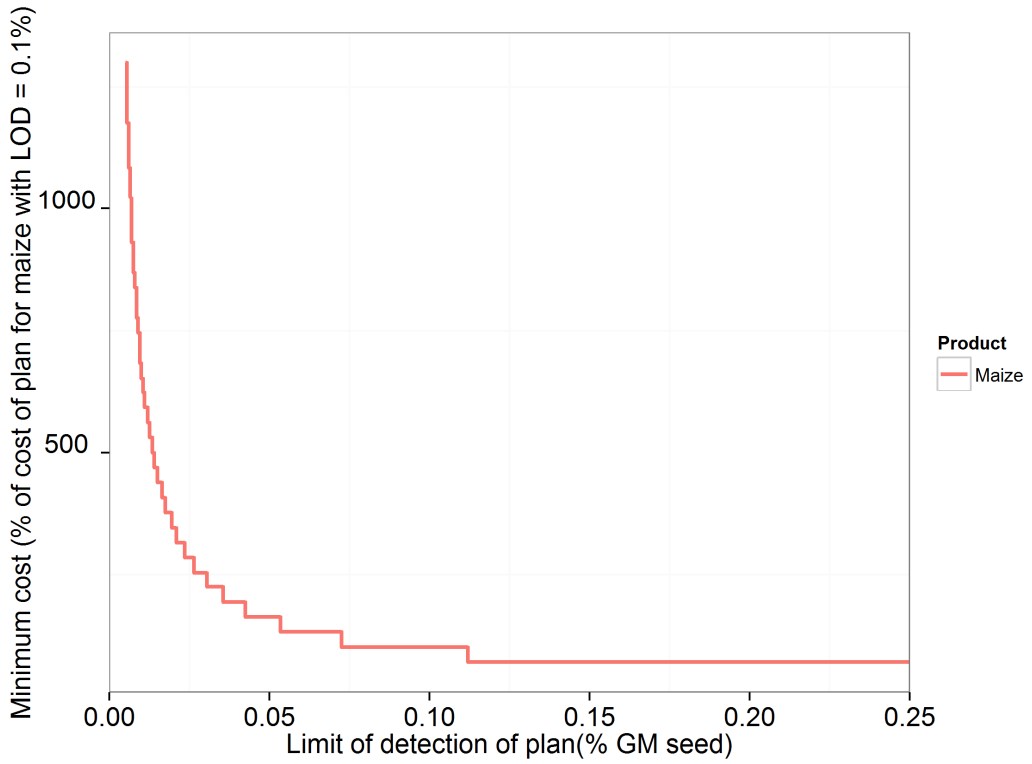


Table 1 shows the relationship between the estimates of cost and limit of detection of a test plan: The number of seeds that must be tested to meet a target for the limit of detection for a plan is approximately inversely proportional to the limit of detection; a halving in the limit of detection for a plan requires testing of approximately the double the number of seeds to achieve the set purpose. For high limits of detection this has no effect on laboratory costs because there is a certain minimum effort required to test working samples. For maize it is estimated that the same effort (analysis of two subsamples with a single grind for each subsample) and same cost applies for any limit of detection higher than 0.1% GM seeds in a lot (Table 1, Figure 2).

Reducing the limit of detection for a plan to a value below 0.1% GM seeds in maize seed lots requires that the higher number of seeds be split into more subsamples so that GM DNA can be reliably detected if it is present in any of the seeds in the working sample. Hence, the estimated relative cost increases from 69%[a] to 100% when the number of subsamples analysed in the laboratory increases from two to three (Table 1, Figure 2).

In reducing further the limit of detection for a plan an increasing number of subsamples are required and the change in estimated cost becomes inversely proportional to the change in

[a] All costs are expressed as a proportion of the cost of the cheapest plan that provides a limit of detection of 0.1% GM seed in a lot of maize seed (3 subsamples each with a single grind)

the estimated limit of detection. A halving from a low limit of detection to a lower limit of detection approximately doubles the estimated cost of the laboratory analysis (Figure 2). For example, halving the limit of detection from 0.01% to 0.005% GM seed in a maize seed lot doubles the estimated laboratory cost from 654% to 1300% (Table 1).

This general pattern also applies to the other seed types considered in this study (Annex 2) with the change in cost tending to an inversely proportional relation with the limit of detection for plans with low limits of detection.

For test plans based on common current practice (cost=100%), minimum achievable limits of detection are estimated to be in the range 0.06 to 0.09% GM seed in lots for sunflower, maize, pea and barley; in the range 0.01 to 0.04% GM seed in lots for sugar beet, tomato, Oilseed rape soybean, alfalfa, and cotton; and less than 0.01% for rice and papaya. The limit of detection for GM seeds in wheat seed lots is estimated to be much higher: 0.3% GM seed (Annex 2). Between-species variation in the minimum achievable limits of detection for a fixed cost is caused by differences in the genome size. Wheat has a particularly large genome; papaya and rice have a particularly small genome (see Table A.1 in Annex 2).

Increasing limits of detection above 0.1% is not estimated to lead to proportional cost savings (Annex 2).

These estimates apply to the initial detection of the presence of GMO seed. Further analyses undertaken on submitted samples following an initial positive result to demonstrate presence above a particular level or other further confirmatory analysis may require considerably more resources than the initial detection. However, if the proportion of samples that produce an initial positive result is small (e.g. 1 or 2%) than the effect on the long term average cost per working sample tested will be a small proportion of the total cost of analyses.

The analysis of seeds which are particularly difficult to homogenise: for example particularly oily seeds may incur a larger cost than the analysis of maize seeds.

The estimates have been made by making a fairly conservative assumption about the false negative rate that is associated with the detection by PCR. Qualitative detection methods are for some applications acceptable, if they have a false negative rate of no higher than 5% at the limit of detection (10 copies of GM target DNA), as assessed through a collaborative study [ref http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf]. This means that the false negative rate may be close to zero for higher target DNA concentrations. We decided to accept test plans that will be robust also against a false negative rate at the upper limit of 5%. The false negative rate observed within a laboratory may be lower. Other test plans can be used if the false negative rate associated with the detection by PCR is known to be much less than 5% (see example and Annex 2).

Example: Plans to detect the presence of GMO seeds in a maize seed lot with a limit of detection of 0.1%

Testing maize seed lots with a target limit of detection of 0.1% is a common scenario in Europe and is therefore taken as a detailed example:

"A limit of detection of 0.1%" means that we want to have a 95% chance of detecting presence where 0.1% of the seeds are GM.

The first test plan is based on Seedcalc "Qual Plan Design" using a method that has a zero false negative rate. On this basis, the lowest cost test plan is:

> *Take a working sample of 2995 seeds. Divide the seeds into two portions for grinding (see Annex 2, effect of seed size and upper limit of the volume of seeds that can be ground in a single run). Combine the two flours. The **screening test** is positive if the sample gives a positive result.*

*Here, for the test plan with a target limit of detection of 0.1% we require that the analytical method has a **zero** false negative rate if there is one GM seed in the 2995 seed sample. If 200ng of DNA is taken for PCR analysis then a total of 39058 haploid maize genomes are analysed. (Annex 2, Table A.1). If at least one GM seed is present in the 2995 seeds working sample then 13 haploid GM genomes are expected to be present in the PCR (based on the simple calculation: number of haploid maize genomes divided by number of seeds). Hence, under this scenario the analytical performance that we would require of the PCR method used to test the extracted DNA is a zero false negative rate where 13 haploid GM genomes are expected to be present in 200 ng of DNA.*

Grohmann et al.[15] report, according to results of the validation of a test plan using 0.1% GM maize and rapeseed samples, it can be expected that "during routine analysis a true 0.1% GMO content will not fail detection by qualitative PCR, even if composite samples of 3000 seeds are tested": On the basis that this equates to a false negative rate of zero at this GM level, the following test plan (Annex 2, Equation 3) would have a limit of detection of a little under 0.1% (0.0998%):

> *Take a working sample of 3000 seeds. Prepare three flours by grinding subsamples of 1000 seeds each. Take a representative portion of flour from each subsample to form a composite flour sample representing the 3000 seeds. Take two test portions for DNA extraction. Test each DNA extract (200 ng DNA) using a PCR method that has a false negative rate of zero where 13 haploid GM genomes per 200 ng of DNA are present.*
>
> *The **screening test** is positive if at least one of the subsamples gives a positive result (further PCR tests of separate test portions of the three flours prepared from the 1000 seeds subsamples should be undertaken to confirm GM presence).*

PCR methods for the detection of GM DNA that may have false negative rates that are greater than zero are used. In order to maintain a limit of detection at 0.1% for the test plan larger working samples are required (Annex 2, Equation 3).

Dividing the working sample into separate subsamples (before testing) can be used to maintain the concentration of GM DNA at a sufficiently high level for reliable detection (at least 13 genomes per 200 ng of DNA in this case) if a single GM seed is present in this larger working sample. Dividing the working sample into subsamples also reduces the effect of the false negative rate for detecting GM flour on the probability of detecting the presence of GM seeds. For example, if the detection of 13 or more haploid GM genomes per 200ng DNA in flour has a false negative rate of 5% then the detection method can be applied in the following test plan to provide a limit of detection of 0.1%:

> *Take a working sample of 3279 seeds. Divide the seeds into three equal subsamples. Grind each subsample. Test each of the three flours for GM presence with a method that has a false negative rate that is no higher than 5% where 13 haploid GM genomes per 200 ng of DNA are present. This is similar to the three-subsample plan presented in Table 1.*
>
> *The **screening test** is positive if at least one of the subsamples gives a positive result. (Further testing of subsamples may be undertaken to confirm GM presence).*

Note that the plans presented in the report (Table 1 and Annex 2) are based on the target false negative rates being achieved where expected number of haploid GM genomes per 200 ng of extract is 16.7. The expected number of 16.7 haploid GM genomes provides a higher probability that at least 10 GM genomes per 200 ng are supplied to PCR. 10 haploid GM genomes per 200 ng was assessed by the working group as being a reasonable estimate for a limit of detection for PCR based detection in general (Annex 2).

Potato is a specific case and therefore not covered in this document. The potato tubers official sampling procedure has been studied for a long time and it has been drafted in the UNECE Guide on Seed Potato Field Inspection Recommended practices GE.6/BUR/2014/5. The representativeness of the sampling is badly influenced by the size of the tubers and the deterioration (due to high water content) leads to problem of storage and movement of the tubers. The suggested quantity to sample in the document is at least 20 kg on a seed lot of 10000 kg.

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Annex 1. Comparison of sampling definitions between CEN standards and ISTA rules

CEN/TS 15568 "Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Sampling strategies"	**Definitions from chapters 2 and 19 of the ISTA Rules** (ISTA Handbook on seed sampling II edition)
3 Terms and definitions For the purposes of this document, the following terms and definitions apply.	NOTE These definitions are used in the framework of the seed certification schemes (EU, OECD).
3.1 Consignment Quantity of some commodity delivered at one time and covered by one set of documents. The consignment may consist of one or more lots or part(s) of lots ISO 7002:1986].	**Consignment:** A consignment is a quantity of seed dispatched and received at one time and covered by a particular contract or shipping document. The size of the consignment is not limited (ISTA Handbook on seed sampling II edition).
3.2 Lot Stated portion of the consignment to be tested for presence of GMO.	**Seed lot**. A seed lot is a specified quantity of seed that is physically and uniquely identifiable.
3.3 Increment Quantity of material taken at one time from a larger body of material. (NOTE Increments may be tested individually aiming at estimation of the variation of any characteristic throughout a lot (or between lots)). [ISO 7002:1986]	**Primary sample**. A primary sample is a portion taken from the seed lot during one single sampling action. Primary samples must be of approximately equal size.
3.4 Item Actual or conventional object (a defined quantity) on which a set of observations may be made.	*No corresponding definition*

[ISO 7002:1986]	
3.5 Sample One or more items (or a portion of material) selected in a prescribed or systematic manner from a lot. NOTE It is intended to provide information representative of the lot, and, possibly, to serve as a basis for decision on the lot.	**Sample**. *A generic definition of sample is not provided in ISTA Rules.*
3.6 File increment sample Increment that is retained for a specific period of time for further analysis.	*In the seed sampling all the primary samples forms the composite sample. No file increment is retained for further analysis.*
3.7 Bulk sample Composite of the increments taken from a lot.	**Composite sample**. The composite sample is formed by combining and mixing all the primary samples taken from the seed lot.
No corresponding definition	**Submitted sample**. A submitted sample is a sample that is to be submitted to the testing laboratory and may comprise either the whole of the composite sample or a reduced sample thereof.
3.8 Laboratory sample Sample as prepared for sending to the laboratory and intended for inspection or testing [ISO 7002:1986]	**Working sample**. The working sample is the whole of the submitted sample or a reduced sample thereof, on which one of the quality tests described in the ISTA Rules is made. It must be at least the weight prescribed by the ISTA Rules for the particular test.
No corresponding definition	**Seed bulk** (From ISTA Rules chapter 19) The seed bulk is the whole working sample that is prepared at one time (e.g. grinding, DNA or protein extraction) and analysed (e.g. end-point PCR, ELISA, real-time PCR).

3.9 Test portion Sample, as prepared for testing or analysis, the whole quantity being used for analysis or testing at one time. [ISO 3534-1:2006]	*The term "test portion" is not used in seed testing* **(Seed group, from ISTA Rules chapter 19).**
3.10 Lot size Number of items or quantity of material constituting the lot. [ISO 7002:1986]	**Lot size**. It is the weight of the seed lot. In seed lots the lot size should not exceed defined weights as stated in chapter 2 of the ISTA Rules.
3.11 Sample size Number of items or quantity of material constituting the sample. [ISO 7002:1986]	*Procedures for obtaining the submitted and working sample. Reported the sample reduction methods. Minimum sizes of working samples are prescribed in the appropriate chapter of each tes*t.
3.12 Sample division Process of selecting one or more representative subsamples from a sample by such means as riffling or mechanical dividing.	**Subsample (seed group).** A subsample is a portion of a working sample obtained by reducing the working sample. Reduction methods are listed in ISTA Rules chapter 2. **Seed group from ISTA Rules Chapter 19.A** seed group is one of the portions of the working sample that is separately prepared (e.g. grinding, DNA or protein extraction) and analysed (e.g. end-point PCR, ELISA, real-time PCR) when using the group testing approach.
3.13 Sampling uncertainty Part of the total estimation uncertainty due to one or several of the following: - the failing of a sample to accurately represent the lot;	In ISTA Rules Sampling uncertainty is not addressed as it is taken into account in the tolerance tables used for the expression of the results.

- the random nature of sampling; - the known and accepted characteristics of the sampling strategy	
3.14 Sampling strategy predetermined procedure for the selection, withdrawal and preparation of samples from a lot to yield the required information so that a decision can be made regarding the acceptance of the lot.	Strategies used to sample a seed lot, procedures for sampling seed lots: They comprise sampling intensity, taking primary samples, obtaining the primary and the submitted sample. It is made in order to take a sample representative of the seed lot.
No corresponding definition	**Duplicate sample**. A duplicate sample is another sample obtained for submission from the same composite sample. All requirements for submitted sample concerning size, marking and sealing are also valid for the duplicate sample.

Annex 2. Estimation of limits of detection for test plans to detect GMO seeds in seed lots

The following text describes the statistical analysis of the testing of seed lots for the presence of GMO. This analysis was focused on estimating a limit of detection for a range of possible test plans for detecting the presence of GM seeds in seed lots. This was done with the aim of helping competent authorities to understand the relation between the proportion of GM seeds in seed lots that will, with a high probability, be reliably detected by test plans and the cost of the test plans needed to achieve this.

We assume that the aim of the testing of seeds for GMOs is to test whether GMOs are present in non-GM seed lots. We assume that there is no threshold at which GMOs are allowed in non-GM seed lots. Hence, any reliable[b] positive result in a sample taken from a non-GM lot indicates that the lot is likely to be non-compliant. There are also a number of technical assumptions and estimates used in the analysis:

1. We are applying the common assumption that the number and the size of primary samples was sufficiently large such that the seed lot is "functionally homogenous"[c] with respect to the number of primary samples that are used to form the working sample.

2. Sampling seeds is unbiased with respect to the presence of GMOs.

3. The DNA extraction is unbiased with respect to the presence of GMO.

4. DNA is uniformly dispersed in the DNA extract.

5. The false positive probability associated with the PCR test is low, and the false negative probability associated with the PCR test is no higher than 5% at the LOD [ENGL guidance on method verification http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf] if at least the target number of DNA copies from each seed in the working sample is delivered to the PCR.

6. DNA is extracted from each subsample independently. 200 ng of each extract DNA is delivered to each PCR.

7. There is an upper limit to the volume of seeds that can be ground in a single run of 800 ml.

[b] With a sufficiently low false positive probability

[c] A lot is functionally homogenous if the variation in the lot has only a small effect on the GMO proportion that we are estimating

8. The relative costs of analysis, additional subsamples, and grinds are as described in this document. Costs for the seeds are not included.

9. Unintended presence may include the presence of GMOs in seed by outcrossing i.e. homozygosity with respect to the presence of the GM event cannot be assumed. Hence test plans are based on an assumption of no more than one copy of target GM DNA per genome.

The form of the test plan

The test plans examined here are those where:

1. The working sample is divided into one or more subsamples. Each subsample is homogenised (ground). If the mass of the subsample is large then it might be necessary to split the subsample before grinding and then to recombine the ground seeds before DNA extraction.
2. DNA is then extracted from each subsample and tested independently by PCR.

The cost of the test plan depends on the number of subsamples that must be analysed and the amount of effort required grinding the seeds.

Estimation of the limit of detection of the test plan

A working sample of n seeds is taken. It is divided into r subsamples which are analysed independently using a method with a false negative rate (due to for example genome size and sample volume and random sporadic blunders) f_N and a low false positive rate. If one or more subsamples produce a positive result then the presence of GMOs in the lot is indicated.

For a subsample from a working sample taken from a lot that contains a proportion L GM seeds the probability of at least one positive result p_D is

$$p_D = 1 - \left[1 - (1 - f_N)(1 - (1 - L)^{\frac{n}{r}}) \right]^r \qquad (1)$$

The limit of detection of the test plan L_D is the value of L for which p_D=0.95.

Then the working sample size needed to achieve a limit of detection L_D (or limit of detection for a test plan) can be easily directly estimated using,

$$n = r \frac{log\left(1 - \frac{1 - 0.05^{1/r}}{1 - f_N}\right)}{log(1 - L_D)} \qquad (2)$$

$$L_D = 1 - \left(1 - \frac{1-0.05^{1/r}}{1-f_N}\right)^{r/n} \qquad (3)$$

This approach is similar to that implemented on the "Qual Plan Design" tab in Seedcalc.

The effect of seed properties on analytical effort and cost

The drivers for testing more subsamples are:

- Where, in order to maintain a sufficiently high probability of getting a GM-positive seed in the working sample given a target LOD of the test plan, more seeds need to be analysed than can be homogenised in a single run.
- Where, in order to maintain a 95% probability of detection of the presence of target DNA in an extract given a single GM seed in the working sample, the number of seeds per DNA extraction needs to be limited.

The effect of genome size on the number of analytical subsamples

PCR methods tend to reliably (at least 95% of the time) give a positive response where a few copies of target DNA are present in a reaction. A fixed mass M of DNA is delivered to each PCR. If we consider that we need to be confident of at least 10 copies being present in each PCR from each single seed in the subsample for our PCR method to provide a probability of detection of at least 95% for a GM seed, then, from the Poisson distribution, we require that the DNA contains an expected average of 16.7 genomes from each seed, i.e. that the number of subsamples r must be at least

$$r \geq \frac{n \times 16.7}{g \times M} \qquad (4)$$

Where n is the number of seeds in the working sample and g is the number of genome copies per ng of DNA, and M is the mass of DNA delivered to each PCR.

Usual practice is to deliver 200ng of DNA to each PCR reaction: M=200 ng.

The effect of seed size on sample preparation costs

In order to extract the DNA seeds must be finely ground. In addition to the effect of genome size, if the *volume v* of the subsample is larger than the capacity of the grinder V then each subsample must be split into h_{ss} grinding samples prior to being ground, where

$$h_{ss} \geq \frac{n/r \times v}{V} \qquad (5)$$

Where n is the number of seeds in the working sample, r is the number of sub samples and v is the volume of a seed in ml and V is the capacity of the grinder.

Grinders with a volume of 1 litre are commonly used. These provide a usable capacity of approximately 800 ml: V=800 ml

Table A.1 gives the expected number of copies of DNA in an extract containing 200 ng of DNA and the estimated volume per seed for a number of products.

Table A.1: Expected size of seeds and number of DNA copies per extract

Common name	Scientific Name	1000-seed mass (g)	Bulk density ($kg.m^{-3}$)	Average volume per seed (ml)	Genome copies in 200 ng DNA
Papaya	*Carica papaya*	15[I]	500[VIII]	0.0300	262903
Rice	*Oryza sativa*	25[II]	560.65[IX]	0.0446	221769
Sugar beet	*Beta vulgaris ssp. saccharifera*	5[III]	500[VIII]	0.0100	129024
Tomato	*Lycopersicon esculentum*	3.5[IV]	500[VIII]	0.0070	102569
Soybean	*Glycine max*	150[V]	760.9[IX]	0.1971	87713
Oilseed rape	*Brassica napus*	4[VI]	669[X]	0.0060	82741
Alfalfa	*Medicago sativa*	2[VI]	769[XI]	0.0026	64768
Cotton	*Gossypium hirsutum*	120[VII]	560.6[IX]	0.2141	43544
Maize	*Zea mays*	380[VI]	720.8[IX]	0.5272	39058

Sunflower	*Helianthus annuus*	175[VI]	480.6[IX]	0.3641	32277
Pea	*Pisum sativum*	200[VI]	609[IX]	0.3284	23442
Barley	*Hordeum vulgare*	40[VI]	720.8[IX]	0.0555	20070
Wheat	*Triticum aestivum*	40[VI]	744.8[IX]	0.0537	6126

[I] http://dx.doi.org/10.1590/S2317-15372013000200008
[II] American-Eurasian Journal of Agronomy 2 (3): 130-137, 2009
[III] Euphytica09-1998, Volume 103, Issue 2, pp 259-263
[IV] http://hazerainc.com/essential-information/1000-seed-weight/
[V] http://www.montana.edu/cpa/news/wwwpb-archives/ag/baudr182.html
[VI] http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/agdex81/$file/100_22-1.pdf?OpenElement
[VII] https://www.icac.org/tis/regional_networks/asian_network/meeting_5/documents/papers/PapAvtonomovV.pdf
[VIII] Conservative estimate made by assuming density is similar to low density seeds
[IX] http://www.tapcoinc.com/content/product_data/Tapco_Catalog_09_p88-94.pdf
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[XI] http://www.caes.uga.edu/departments/bae/extension/handbook/documents/Density%20of%20Agricultural%20Products.pdf

Estimate of total cost of the test plan

The total cost of the test plan is assumed to be built up of a base cost (cost of analysis of a single subsample which requires a single grind). To this is added a cost for each additional subsample which includes the cost of a single grind per subsample. Finally an additional cost for grinding split subsamples is added. Given the analysis of r subsamples requiring a total of h grinds the cost c is estimated to be

$$c = f + c_r(r-1) + c_h(h-r) \qquad (6)$$

Where f is the cost of carrying out a test on a single subsample, c_r is the cost of adding an additional subsample to the analysis and c_h is the cost of adding an additional homogenisation to a subsample.

The costs of a test plan were expressed on a scale relative to the costs of a plan which is based on the analyses of single subsample with a single grind. This has a cost of one. The costs of additional subsamples and the cost of additional grindings per subsample were estimated by the working group on this scale.

The costs are estimated to be

Cost of a single analysis using a single subsample 1

Cost of adding an additional subsample to the analysis	0.8
Cost of adding an additional grind to a subsample	0.6

The total cost of the plan is calculated using equation 6 and then costs for each plan are normalised to 100% based on the cost of the least cost plan for a target limit detection of 0.1% for maize. Hence the costs expressed in this way describe how changing of the target limit of detection for the test plan may change the expected cost of testing within a laboratory. The analyses of commodities with larger genome and/or seeds size will result in higher costs because of the increased number of subsamples to be analysed.

This approach was taken, while between-laboratory variation in absolute costs and the way that costs are expressed varies considerably between laboratories, because it was considered by the WG that the relative costs of the components of the test plan would be more stable.

The procedure followed to find the lowest cost plans

For each target limit of detection and product (Table 1), the working sample size required for plans between 1 and 200 subsamples was calculated (Equation 2). Plans with an insufficient number of subsamples to deliver enough DNA to the PCR (Equation 4) were excluded. The total number of grinds needed for each plan was calculated (Equation 5). The cost of each plan was calculated (Equation 6). Finally, the lowest cost plan within each combination of target limit of detection and product was selected.

Table A.2 shows lowest cost test plans estimated for 13 species (or crops) for test plan limits of detection at 5%, 0.9%, 0.5%, 0.1%, 0.05%, 0.01% and 0.005% GM seed. Figure A.2 shows the relation between the cost and limit of detection of plans. Figure A.3 shows the relation excluding wheat.

Table A.3 shows the minimum limit of detection that can be achieved for plans costing no more than 100% and plans costing no more than 200%. These are plans where the working sample size is maximised with respect to the number of subsamples in the plan so that Equation 4 is *only just* satisfied.

If the false negative rate of the PCR test is estimated to be much lower than 5% then other test plans can be used (Table A.4 and Table A.5) For example, if the false negative rate associated with the PCR detection method is 0.1 % then (Equation 4: f_N=0.001, r=1, n=3000)

a limit of detection for a test plan of 0.1% GM seed in a lot can be achieved by employing the analysis of a single subsample of 3000 seeds, assuming the number of seeds can be ground in a single grinding step.

Table A.2: Lowest cost plans (PCR false negative probability ≤ 5% where at least 10 copies are present)

Crop	Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
Maize	5%	68	2	2	69%
Maize	0.9%	378	2	2	69%
Maize	0.5%	680	2	2	69%
Maize	0.1%	3279	3	3	100%
Maize	0.05%	6435	5	5	162%
Maize	0.01%	31668	21	21	654%
Maize	0.005%	63210	42	42	1300%
Alfalfa	5%	68	2	2	69%
Alfalfa	0.9%	378	2	2	69%
Alfalfa	0.5%	680	2	2	69%
Alfalfa	0.1%	3398	2	2	69%
Alfalfa	0.05%	6798	2	2	69%
Alfalfa	0.01%	31851	9	9	285%
Alfalfa	0.005%	63393	17	17	531%
Barley	5%	68	2	2	69%
Barley	0.9%	378	2	2	69%
Barley	0.5%	680	2	2	69%
Barley	0.1%	3279	3	3	100%
Barley	0.05%	6408	6	6	192%
Barley	0.01%	31644	27	27	838%
Barley	0.005%	63180	54	54	1669%
Cotton	5%	68	2	2	69%
Cotton	0.9%	378	2	2	69%
Cotton	0.5%	680	2	2	69%
Cotton	0.1%	3398	2	2	69%
Cotton	0.05%	6558	3	3	100%
Cotton	0.01%	31746	13	13	408%
Cotton	0.005%	63275	25	25	777%
OilseedRape	5%	68	2	2	69%
OSR	0.9%	378	2	2	69%
OSR	0.5%	680	2	2	69%
OSR	0.1%	3398	2	2	69%
OSR	0.05%	6798	2	2	69%
OSR	0.01%	31955	7	7	223%
OSR	0.005%	63462	14	14	438%

Table A.2: (continued) Lowest cost plans (PCR false negative probability ≤ 5% where at least 10 copies are present)

Product	Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
Papaya	5%	68	2	2	69%
Papaya	0.9%	378	2	2	69%
Papaya	0.5%	680	2	2	69%
Papaya	0.1%	3398	2	2	69%
Papaya	0.05%	6798	2	2	69%
Papaya	0.01%	32793	3	3	100%
Papaya	0.005%	64330	5	5	162%
Pea	5%	68	2	2	69%
Pea	0.9%	378	2	2	69%
Pea	0.5%	680	2	2	69%
Pea	0.1%	3279	3	3	100%
Pea	0.05%	6435	5	5	162%
Pea	0.01%	31648	23	23	715%
Pea	0.005%	63204	46	46	1423%
Rice	5%	68	2	2	69%
Rice	0.9%	378	2	2	69%
Rice	0.5%	680	2	2	69%
Rice	0.1%	3398	2	2	69%
Rice	0.05%	6798	2	2	69%
Rice	0.01%	32793	3	3	100%
Rice	0.005%	64330	5	5	162%
Soybean	5%	68	2	2	69%
Soybean	0.9%	378	2	2	69%
Soybean	0.5%	680	2	2	69%
Soybean	0.1%	3398	2	2	69%
Soybean	0.05%	6798	2	2	69%
Soybean	0.01%	31896	8	8	254%
Soybean	0.005%	63408	16	16	500%
Sugarbeet	5%	68	2	2	69%
Sugarbeet	0.9%	378	2	2	69%
Sugarbeet	0.5%	680	2	2	69%
Sugarbeet	0.1%	3398	2	2	69%
Sugarbeet	0.05%	6798	2	2	69%
Sugarbeet	0.01%	32165	5	5	162%
Sugarbeet	0.005%	63702	9	9	285%

Table A.2: (continued) Lowest cost plans (PCR false negative probability ≤ 5% where at least 10 copies are present)

Crop	Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
Sunflower	5%	68	2	2	69%
Sunflower	0.9%	378	2	2	69%
Sunflower	0.5%	680	2	2	69%
Sunflower	0.1%	3398	2	2	69%
Sunflower	0.05%	6476	4	4	131%
Sunflower	0.01%	31705	17	17	531%
Sunflower	0.005%	63240	34	34	1054%
Tomato	5%	68	2	2	69%
Tomato	0.9%	378	2	2	69%
Tomato	0.5%	680	2	2	69%
Tomato	0.1%	3398	2	2	69%
Tomato	0.05%	6798	2	2	69%
Tomato	0.01%	32040	6	6	192%
Tomato	0.005%	63569	11	11	346%
Wheat	5%	68	2	2	69%
Wheat	0.9%	378	2	2	69%
Wheat	0.5%	680	2	2	69%
Wheat	0.1%	3186	9	9	285%
Wheat	0.05%	6336	18	18	562%
Wheat	0.01%	31592	88	88	2715%
Wheat	0.005%	63175	175	175	5392%

Figure A.2: Relation between cost and limit of detection of test plans

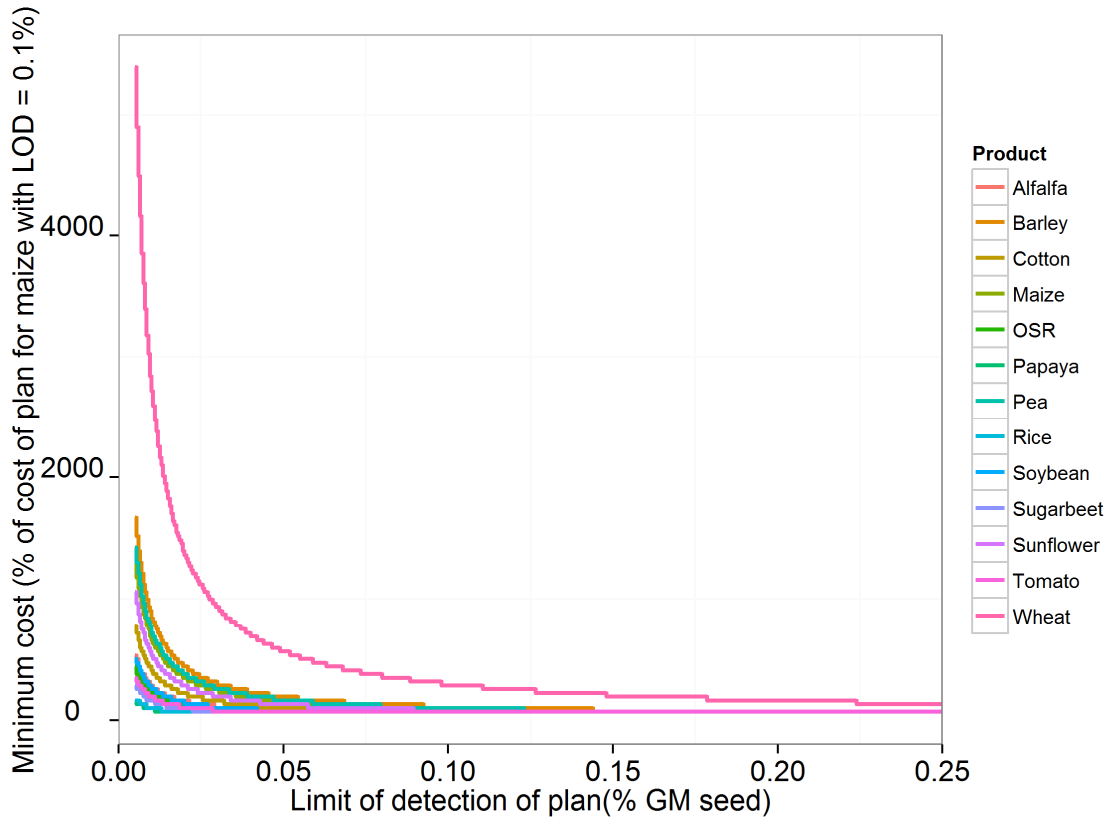

Figure A.3: Relation between cost and limit of detection of test plans (excluding wheat)

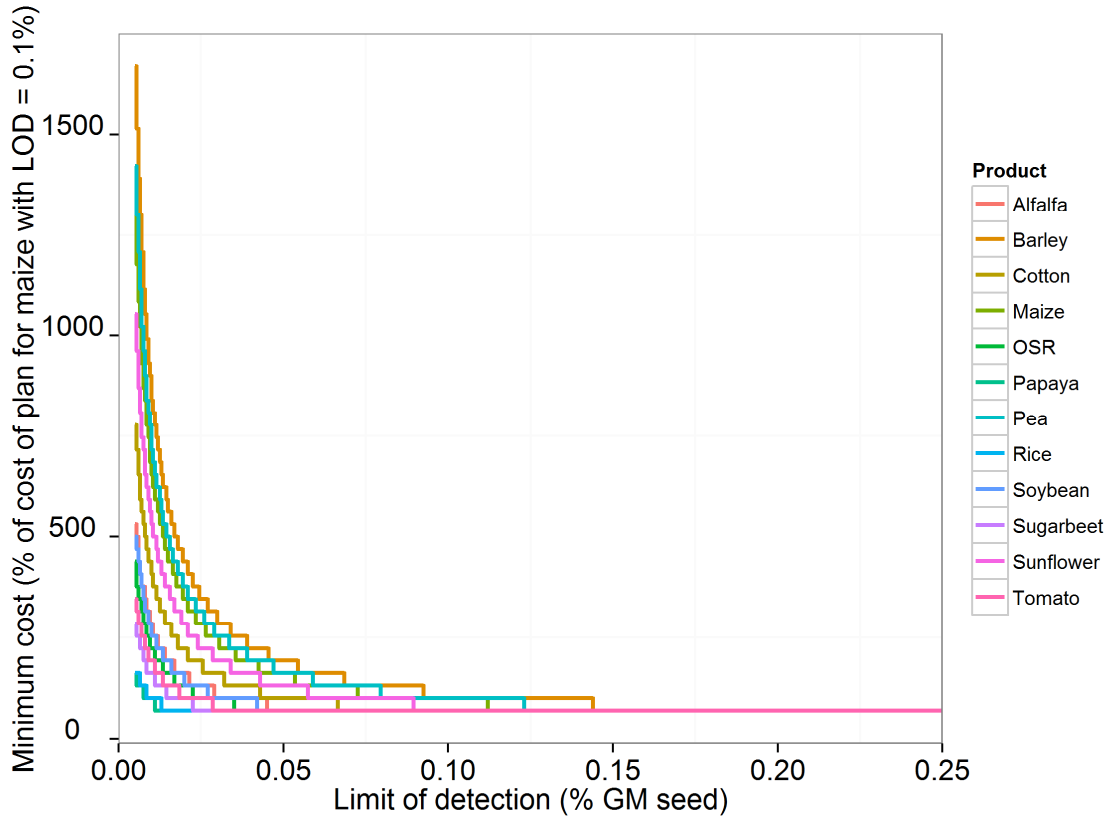

Table A.3: minimum limits of detection achievable with plans costing 100% and 200%

Product	Minimum LOD (% GM seed in lot) cost=100%	cost=200%
Papaya	0.0075	<0.005
Rice	0.0085	<0.005
Sugar beet	0.0145	0.0075
Tomato	0.0185	0.0090
OSR	0.0225	0.0110
Soybean	0.0270	0.0135
Alfalfa	0.0290	0.0140
Cotton	0.0430	0.0210
Sunflower	0.0575	0.0285
Maize	0.0725	0.0355
Pea	0.0795	0.0390
Barley	0.0925	0.0455
Wheat	0.3025	0.1480

Table A.4: Lowest cost plans (PCR false negative probability ≤ 1% where at least 10 copies are present)

Crop	Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
Maize	5%	63	1	1	38%
Maize	0.9%	355	1	1	38%
Maize	0.5%	641	1	1	38%
Maize	0.1%	3048	3	3	100%
Maize	0.05%	6075	5	5	162%
Maize	0.01%	30300	20	20	623%
Maize	0.005%	60560	40	40	1238%
Alfalfa	5%	63	1	1	38%
Alfalfa	0.9%	355	1	1	38%
Alfalfa	0.5%	641	1	1	38%
Alfalfa	0.1%	3208	1	1	38%
Alfalfa	0.05%	6134	2	2	69%
Alfalfa	0.01%	30328	8	8	254%
Alfalfa	0.005%	60592	16	16	500%
Barley	5%	63	1	1	38%
Barley	0.9%	355	1	1	38%
Barley	0.5%	641	1	1	38%
Barley	0.1%	3048	3	3	100%
Barley	0.05%	6072	6	6	192%
Barley	0.01%	30290	26	26	808%
Barley	0.005%	60580	52	52	1608%
Cotton	5%	63	1	1	38%
Cotton	0.9%	355	1	1	38%
Cotton	0.5%	641	1	1	38%
Cotton	0.1%	3066	2	2	69%
Cotton	0.05%	6096	3	3	100%
Cotton	0.01%	30312	12	12	377%
Cotton	0.005%	60576	24	24	746%
OSR	5%	63	1	1	38%
OSR	0.9%	355	1	1	38%
OSR	0.5%	641	1	1	38%
OSR	0.1%	3208	1	1	38%
OSR	0.05%	6134	2	2	69%
OSR	0.01%	30338	7	7	223%
OSR	0.005%	60606	13	13	408%

Table A.4: (continued) Lowest cost plans (PCR false negative probability ≤ 1% where at least 10 copies are present)

Crop	Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
Papaya	5%	63	1	1	38%
Papaya	0.9%	355	1	1	38%
Papaya	0.5%	641	1	1	38%
Papaya	0.1%	3208	1	1	38%
Papaya	0.05%	6417	1	1	38%
Papaya	0.01%	30670	2	2	69%
Papaya	0.005%	60820	4	4	131%
Pea	5%	63	1	1	38%
Pea	0.9%	355	1	1	38%
Pea	0.5%	641	1	1	38%
Pea	0.1%	3048	3	3	100%
Pea	0.05%	6075	5	5	162%
Pea	0.01%	30294	22	22	685%
Pea	0.005%	60544	44	44	1362%
Rice	5%	63	1	1	38%
Rice	0.9%	355	1	1	38%
Rice	0.5%	641	1	1	38%
Rice	0.1%	3208	1	1	38%
Rice	0.05%	6417	1	1	38%
Rice	0.01%	30480	3	3	100%
Rice	0.005%	60750	5	5	162%
Soybean	5%	63	1	1	38%
Soybean	0.9%	355	1	1	38%
Soybean	0.5%	641	1	1	38%
Soybean	0.1%	3208	1	1	38%
Soybean	0.05%	6134	2	2	69%
Soybean	0.01%	30328	8	8	254%
Soybean	0.005%	60585	15	15	469%
Sugarbeet	5%	63	1	1	38%
Sugarbeet	0.9%	355	1	1	38%
Sugarbeet	0.5%	641	1	1	38%
Sugarbeet	0.1%	3208	1	1	38%
Sugarbeet	0.05%	6417	1	1	38%
Sugarbeet	0.01%	30412	4	4	131%
Sugarbeet	0.005%	60656	8	8	254%

Table A.4: (continued) Lowest cost plans (PCR false negative probability ≤ 1% where at least 10 copies are present)

Crop	Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
Sunflower	5%	63	1	1	38%
Sunflower	0.9%	355	1	1	38%
Sunflower	0.5%	641	1	1	38%
Sunflower	0.1%	3066	2	2	69%
Sunflower	0.05%	6084	4	4	131%
Sunflower	0.01%	30304	16	16	500%
Sunflower	0.005%	60576	32	32	992%
Tomato	5%	63	1	1	38%
Tomato	0.9%	355	1	1	38%
Tomato	0.5%	641	1	1	38%
Tomato	0.1%	3208	1	1	38%
Tomato	0.05%	6134	2	2	69%
Tomato	0.01%	30354	6	6	192%
Tomato	0.005%	60610	11	11	346%
Wheat	5%	63	1	1	38%
Wheat	0.9%	355	1	1	38%
Wheat	0.5%	612	2	2	69%
Wheat	0.1%	3033	9	9	285%
Wheat	0.05%	6069	17	17	531%
Wheat	0.01%	30324	84	84	2592%
Wheat	0.005%	60648	168	168	5177%

Table A.5: Lowest cost plans (PCR false negative probability ≤ 0.1% where at least 10 copies are present)

Crop	Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
Maize	5%	59	1	1	38%
Maize	0.9%	334	1	1	38%
Maize	0.5%	602	1	1	38%
Maize	0.1%	3002	2	2	69%
Maize	0.05%	6000	4	4	131%
Maize	0.01%	30000	20	20	631%
Maize	0.005%	60000	40	40	1256%
Alfalfa	5%	59	1	1	38%
Alfalfa	0.9%	334	1	1	38%
Alfalfa	0.5%	602	1	1	38%
Alfalfa	0.1%	3014	1	1	38%
Alfalfa	0.05%	6004	2	2	69%
Alfalfa	0.01%	30000	8	8	256%
Alfalfa	0.005%	59984	16	16	506%
Barley	5%	59	1	1	38%
Barley	0.9%	334	1	1	38%
Barley	0.5%	602	1	1	38%
Barley	0.1%	3000	3	3	100%
Barley	0.05%	6000	6	6	194%
Barley	0.01%	30004	26	26	819%
Barley	0.005%	59976	51	51	1600%
Cotton	5%	59	1	1	38%
Cotton	0.9%	334	1	1	38%
Cotton	0.5%	602	1	1	38%
Cotton	0.1%	3002	2	2	69%
Cotton	0.05%	6003	3	3	100%
Cotton	0.01%	30000	12	12	381%
Cotton	0.005%	60000	24	24	756%
OSR	5%	59	1	1	38%
OSR	0.9%	334	1	1	38%
OSR	0.5%	602	1	1	38%
OSR	0.1%	3014	1	1	38%
OSR	0.05%	6004	2	2	69%
OSR	0.01%	29995	7	7	225%
OSR	0.005%	59982	13	13	413%

Table A.5: (continued) Lowest cost plans (PCR false negative probability ≤ 0.1% where at least 10 copies are present)

Crop	Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
Papaya	5%	59	1	1	38%
Papaya	0.9%	334	1	1	38%
Papaya	0.5%	602	1	1	38%
Papaya	0.1%	3014	1	1	38%
Papaya	0.05%	6029	1	1	38%
Papaya	0.01%	30026	2	2	69%
Papaya	0.005%	60004	4	4	131%
Pea	5%	59	1	1	38%
Pea	0.9%	334	1	1	38%
Pea	0.5%	602	1	1	38%
Pea	0.1%	3000	3	3	100%
Pea	0.05%	6000	5	5	163%
Pea	0.01%	30008	22	22	694%
Pea	0.005%	60016	44	44	1381%
Rice	5%	59	1	1	38%
Rice	0.9%	334	1	1	38%
Rice	0.5%	602	1	1	38%
Rice	0.1%	3014	1	1	38%
Rice	0.05%	6029	1	1	38%
Rice	0.01%	30009	3	3	100%
Rice	0.005%	60000	5	5	163%
Soybean	5%	59	1	1	38%
Soybean	0.9%	334	1	1	38%
Soybean	0.5%	602	1	1	38%
Soybean	0.1%	3014	1	1	38%
Soybean	0.05%	6004	2	2	69%
Soybean	0.01%	30000	8	8	256%
Soybean	0.005%	59985	15	15	475%
Sugarbeet	5%	59	1	1	38%
Sugarbeet	0.9%	334	1	1	38%
Sugarbeet	0.5%	602	1	1	38%
Sugarbeet	0.1%	3014	1	1	38%
Sugarbeet	0.05%	6029	1	1	38%
Sugarbeet	0.01%	30004	4	4	131%
Sugarbeet	0.005%	59992	8	8	256%

Table A.5: (continued) Lowest cost plans (PCR false negative probability ≤ 0.1% where at least 10 copies are present)

Crop	Target for LOD of test plan	Working sample size (seeds)	Subsamples	Grinds	Cost
Sunflower	5%	59	1	1	38%
Sunflower	0.9%	334	1	1	38%
Sunflower	0.5%	602	1	1	38%
Sunflower	0.1%	3002	2	2	69%
Sunflower	0.05%	6000	4	4	131%
Sunflower	0.01%	30000	16	16	506%
Sunflower	0.005%	60000	32	32	1006%
Tomato	5%	59	1	1	38%
Tomato	0.9%	334	1	1	38%
Tomato	0.5%	602	1	1	38%
Tomato	0.1%	3014	1	1	38%
Tomato	0.05%	6029	1	1	38%
Tomato	0.01%	30000	5	5	163%
Tomato	0.005%	59990	10	10	319%
Wheat	5%	59	1	1	38%
Wheat	0.9%	334	1	1	38%
Wheat	0.5%	600	2	2	69%
Wheat	0.1%	3006	9	9	288%
Wheat	0.05%	6001	17	17	538%
Wheat	0.01%	29988	84	84	2631%
Wheat	0.005%	60120	167	167	5225%

The effect of the violation of assumptions on the estimates produced in this study

The main assumptions underlying this analysis are:

1. Sampling (seeds and DNA) is unbiased with respect to the presence of GMOs.
2. The number and the weight of primary samples were sufficiently large such that the seed lot is functionally homogenous.
3. The DNA extraction is unbiased with respect to the presence of GMO.
4. DNA is uniformly dispersed in the DNA extract

If any of the first four assumptions are not met, then Equation 2 is no longer valid; the true limit of detection may be higher than the estimated limit of detection of the test plans. A new statistical model will be needed if these are not reasonable assumptions.

5. The PCR applied to the DNA extract will provide a positive response with a probability of at least 95% if at least 10 GMO DNA copies are present.
6. 800 ml of seeds can be homogenised in a single run.
7. 200ng of DNA can be analysed in a single PCR.
8. The cost of the first subsample, additional subsamples, and additional grinds is in the ratio 1:0.8:0.6
9. The false positive probability associated with individual PCR tests is low

These five assumptions define how much effort is required to achieve a target for a test plan limit of detection and the cost associated with the effort. Changes in the volume that can be homogenised or mass of DNA that can be tested or number of DNA copies needed will change the effort required to achieve a particular test plan limit of detection. Different values can be accommodated by changing constants in Equations 4 and 5. A change in the ratio of fixed to variable costs would change the rate at which costs were estimated to increase, but not the estimated target limit of detection for a test plan at which costs begin to increase. Changes in the estimated costs can be accommodated by changing the constants in Equation 6. A higher false positive rate would increase the number of replicates necessary to achieve a given test plan limit of detection reliably and may make very low limits of detection (much less than 0.1%) practically unachievable.

10. The GMO events may be present as single copies on only one haploid from a multiploid genome.

This is a decision to not make an assumption: we are *not* assuming that GMO events may be present only as the result of the mixing of GM with non-GM seeds; we are allowing for the possibility of presence due to outcrossing.

11. The seed lot is large and the cost of seeds is low cost compared to the cost of the analysis. Testing of lots that are small[d] and/or containing high value seeds[e] is outside of the scope of this study.

[d] less than 10 times the working sample size

[e] In this context seeds are 'high value' if the value of the seeds in the working sample is similar to or larger than the cost of testing the sample

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