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Detection of food and feed obtained by new plant mutagenesis techniques

European Network of GMO Laboratories (ENGL)

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1 **Executive summary**

2 The European Network of GMO Laboratories (ENGL) has reviewed the possibilities and
3 challenges for the detection of food and feed obtained by new plant mutagenesis
4 techniques based on genome editing.

5 The procedures for the validation of detection methods as part of the *market authorisation*
6 of genome-edited plant products will in principle be similar as for the current conventional
7 GMOs. It is, however, questionable if an event-specific quantitative detection method can
8 be developed for such GMOs, particularly when they are characterised by a short DNA
9 alteration consisting of one or a few base pairs. Such detection methods will probably lack
10 the specificity required to target the unique DNA alteration in the genome-edited plant and
11 quantification of the presence of the specific product in a complex food or feed material
12 may not be possible. Detection methods may therefore fail to fulfil the method performance
13 requirements and, as a consequence, may result in the rejection of the application. For
14 genome-edited plant products with a large DNA alteration an event-specific quantitative
15 detection method may be developed and could pass the validation process in case the DNA
16 alteration does not also occur naturally; this will need to be demonstrated.

17 The ENGL will need to review the minimum performance requirements that are applied for
18 GMO method validations in view of the specific characteristics of genome-edited plants.
19 This should provide further guidance to applicants for market authorisation and to the EU
20 Reference Laboratory for GM Food and Feed (EURL GMFF) for validation of the event-
21 specific methods. *E.g.* it is currently unclear how to demonstrate or assess the specificity
22 of the method against all existing marketed varieties. Furthermore, it needs to be
23 evaluated under which conditions an event-specific detection method would be required
24 for all DNA alterations in a multi-edited plant and how to quantify such products based on
25 measurement results for the individual genome edits.

26 For *market control*, considering the current knowledge and state of the art of DNA analysis,
27 it is not possible for enforcement laboratories to detect the presence of unauthorised
28 genome-edited GMOs in food or feed entering the EU market without prior information on
29 the altered DNA sequences. The same kind of screening methods that are commonly used
30 to detect conventional GMOs cannot be applied nor could be developed for genome-edited
31 GMOs as there are often no common sequences that could be targets for such screening
32 methods. Targeted or non-targeted DNA sequencing may be able to detect specific DNA
33 alterations in a product, however, this does not necessarily confirm the presence of a
34 particular genome-edited GMO since the same DNA alteration could occur naturally in other
35 plants obtained by conventional breeding or mutagenesis techniques which are exempted
36 from the GMO regulations.

37 Therefore, it is concluded that validation of an event-specific detection method and its
38 implementation for market control will be feasible for genome-edited GMOs carrying a
39 known DNA alteration that has been shown to be unique and can only be obtained by
40 application of a genome editing technique. With the current technological capabilities,
41 market control will, however, not be possible in the absence of an event-specific detection
42 method or for unknown genome-edited food or feed products.

43

44

45 **1 Introduction**

46 In the European Union the authorisation system for the introduction of GMOs in the agro-
47 food chain is governed by stringent legislation to ensure:

- 48 • the safety of food and feed for health and the environment;
- 49 • consumers' choice between GM, organic and conventionally-produced food;
- 50 • the functioning of the internal market, i.e. once authorised, GM products can be placed
51 on the market anywhere in the EU¹.

52 The EU policy on GMOs is inclusive as it addresses the development of GMOs, the stepwise
53 release into the environment, the general cultivation and seed production, marketing,
54 labelling and the whole agro-food chain, up to the consumption by humans and animals.

55 The EU Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF), hosted
56 by the Joint Research Centre (JRC) of the European Commission, is legally mandated to
57 assess and validate the detection methods submitted by the applicants (GMO producers)
58 for authorisation of GMOs². For this task, the EURL GMFF is assisted by a consortium of
59 national reference and enforcement laboratories, known as the European Network of GMO
60 Laboratories (ENGL), who has issued a guidance document explaining the minimum
61 performance requirements (MPR) for analytical methods of GMO testing³. Since the
62 labelling legislation² is based on the GMO content present in the food or feed product, one
63 of the requirements refers to the accurate quantification of the "GM fraction" in such
64 products. GMOs or GM food and feed products that do not meet the requirements of the
65 legislation should not be present on the market or, in specific cases⁴, should not exceed
66 the Minimum Required Performance Limit (MRPL) that has been set at 0.1 % in mass
67 fraction (see Text box 1).

68 The EURL GMFF also has a legal mandate under the "Official Controls Regulation"⁵, which
69 defines harmonised rules on official controls and, among others, activities performed to
70 ensure compliance to the food and feed laws related to the presence of GMOs. In that
71 context, official controls should control the implementation of the labelling requirements
72 and prevent infringement of the legislation due to the presence of unauthorised GMOs on
73 the market. To implement this Regulation, Member States have appointed National
74 Reference Laboratories (NRLs) and official laboratories to perform analyses on food, feed
75 and seed⁶ products in their national markets; this is performed by applying – when
76 available – first-line screening methods and the detection methods validated for the
77 authorised and known unauthorised GMOs.

¹ In line with Directive (EU) 2015/412 Member States may, however, restrict or prohibit the cultivation of an authorised GMO on all or part of their territory.

² Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Union* L268:1-23.

³ European Network of GMO Laboratories (2015) Definition of minimum performance requirements for methods of GMO testing (http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf).

⁴ Commission Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired. *Off. J. Eur. Union* L166: 9-15.

⁵ Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products (Official Controls Regulation). *Off. J. Eur. Union* L95:1-142.

⁶ In contrast to food and feed (and seed used as or in food or feed), there is a zero tolerance for GMOs present in seed lots used for cultivation.

Text box 1

**Different authorisation statuses of GMOs
from an EU perspective**

Authorised

Authorised GM material is allowed on the EU market. Authorisation mostly concerns the import of GMOs and their use in food and feed. Few authorisations have been submitted for cultivation of GM crops and currently one GM maize is authorised for cultivation.

GMOs in this category can be present on the market in food and feed material. Validated identification and quantification methods and reference materials are available for these GMOs. According to Directive 2001/18/EC, Regulation (EC) No 1829/2003 and (EC) No 1830/2003, the presence of such authorised GMOs in food and feed shall be indicated on the label of the product. Labelling requirements do not apply for GMOs intended for food, feed or direct processing when the presence is below 0.9% and provided that these traces are adventitious or technically unavoidable.

Non-authorised

- Pending authorisation: a GMO has been authorised for commercial use in one or more third countries, while a submitted application for authorisation in the EU is pending.
- Authorisation expired: a GMO of which the authorisation has expired and no renewal application has been submitted.

Only for feed materials and feed additives, GMOs in these two categories above may be present at a level below 0.1% related to mass fraction, under the conditions of Commission Regulation (EU) No 619/2011. A validated quantification method and certified reference material are available for these GMOs.

- GMOs that have been authorised for any other purpose than for placing on the market, under Part B of the Directive 2001/18. The authorisation for these purposes (e.g. experimental uses and field trials) is granted and applied at national level.
- GMOs that have not been authorised for placing on the market, as or in products, under Directive Part C of 2001/18 or Regulation 1829/2003.

GMOs in these two categories are not allowed on the EU market. Zero-tolerance applies.

78

79 During the past years, several new plant breeding techniques, including targeted
80 mutagenesis techniques generically called "genome editing", have been employed to create
81 diversity for exploitation in plant breeding (reviewed in ⁷). Instead of the random mutation
82 of many genes at the same time (as in conventional mutation breeding techniques) or the
83 random insertion of new genes (as in conventional GMOs), genome editing allows the site-
84 specific alteration of the DNA sequence of one or a few selected genes, resulting in single
85 nucleotide variants (SNV) or short or large insertions or deletions (InDels). These DNA
86 alterations may be present either in a homozygous or heterozygous state in the genome,
87 i.e. all or only part of the chromosome copies (called the alleles of a gene) may carry the
88 alteration (e.g. in a tetraploid (4n) crop between one and 4 DNA copies may contain the
89 DNA alteration).

90 Upon request of DG SANTE, the JRC reviewed in 2011 the state-of-the-art of some of the
91 emerging new plant breeding technologies, their level of development and adoption by the
92 breeding sector and the prospects for a future commercialisation of crops based on them⁸.
93 Additionally, challenges for the detection of organisms developed through these techniques
94 were evaluated⁹. The topic has since been discussed during meetings of the ENGL. In the
95 past few years, a novel innovative technique for genome editing, CRISPR-Cas, with wider

⁷ Scientific Advice Mechanism (2017) New techniques in Agricultural Biotechnology. European Commission (https://ec.europa.eu/research/sam/pdf/topics/explanatory_note_new_techniques_agricultural_biotechnology.pdf#view=fit&pagemode=none).

⁸ Lusser, M., Parisi, C., Plan, D., Rodríguez-Cerezo, E. (2011) New plant breeding techniques. State-of-the-art and prospects for commercial development. Luxembourg, Publications Off. Eur. Union, 184 p. (<https://publications.europa.eu/en/publication-detail/-/publication/12988d6d-c6a4-41b2-8dbd-760eeac044a7/language-en>).

⁹ Lusser, M., Parisi, C., Plan, D., Rodríguez-Cerezo, E. (2012) Deployment of new biotechnologies in plant breeding. *Nature Biotechnology* 30:231–239 (doi:10.1038/nbt.2142).

96 potential and easier applicability, has rapidly advanced plant biology research and the
97 development of applications for plant breeding^{7,10}.

98 In 2018, the European Court of Justice ruled that organisms obtained by new mutagenesis
99 techniques, i.e. genome editing, in contrast to conventional mutagenesis techniques that
100 have a long history of safety, are not exempted from the GMO legislation¹¹. The JRC
101 received a mandate from DG SANTE to elaborate, together with the ENGL, on the
102 implications of this ruling for the detection of such organisms.

103 This document addresses questions related to the new analytical challenges for the
104 detection, identification and quantification of genome-edited food and feed products of
105 plant origin. Those may relate (1) to the compliance with the GM food and feed legislation²,
106 including the requirements for method validation as part of the GMO authorisation
107 procedures, and (2) to the provisions of the official controls regulation⁵ on the routine
108 testing of food and feed by the enforcement laboratories.

109 This document has been endorsed and released for publication by the Steering Committee
110 of the ENGL.

111 The ENGL experts consulted have an in-depth expertise with respect to GMO analysis but
112 the viewpoints mentioned here are not based on extensive experimental work on genome-
113 edited food or feed.

114

¹⁰ Khatodia, S., Bhatotia, K., Passricha, N., Khurana, S.M.P., Tuteja, N. (2016) The CRISPR/Cas genome-editing tool: Application in improvement of crops. *Front. Plant Sci.* 7:506 (doi: 10.3389/fpls.2016.00506).

¹¹ European Court of Justice, C-528/16 - Judgement of 25 July 2018. See:
<http://curia.europa.eu/juris/document/document.jsf?docid=204387&mode=req&pageIndex=1&dir=&occ=first&part=1&text=&doclang=EN&cid=515140>

115 **2 Terminology used in this document**

116 The term **conventional GMOs** will be used throughout this report to refer to GMOs
117 obtained by recombinant DNA technology and characterised by the presence of introduced
118 DNA sequences from the same or other species in the final plant. GMOs require an
119 authorisation before entering the EU market, otherwise they are called unauthorised and
120 their presence is restricted; this is further clarified in Text box 1.

121 **Genome editing**, also called gene editing, is a group of site-directed mutagenesis
122 techniques that allows adding, removing, or altering DNA sequences at a specific location
123 in the genome. It is mostly achieved with the aid of the cell's natural DNA
124 recombination/repair system activated with the use of a site-directed nuclease (SDN),
125 creating a double-strand DNA break at a defined location, a repair template sequence
126 consisting of an added nucleic acid molecule (e.g. an oligonucleotide or longer nucleic acid
127 sequence with partial sequence similarity to the target site), or the combination of both
128 (modified from ⁷). The techniques require the presence of the SDN in the recipient host
129 cell (in this document we will refer only to plant cells, but other organisms can be targets
130 of genome editing), either following stable integration of recombinant DNA into the plant
131 genome, or by transient expression or delivery of a protein/nucleic acid complex into the
132 cell. When recombinant DNA has been used, it can be selected against in subsequent
133 generations resulting in genome-edited plants that no longer contain any recombinant
134 DNA^{12,13}. In the frame of this report, plants obtained with genome editing techniques that
135 contain inserted foreign DNA are excluded, as these will be similar to the current
136 conventional GMOs.

137 Early but limited success was first achieved with protein-directed SDNs such as
138 meganucleases, zinc finger nucleases (ZFNs) and transcription activator-like effector
139 nucleases (TALENs). The techniques of genome editing have advanced rapidly following
140 the development of RNA-directed SDNs based on the bacterial CRISPR (clustered regularly
141 interspaced short palindromic repeats) system and CRISPR-associated (Cas) nucleases⁹.
142 Editing of single nucleotides can also be achieved using a specific set of enzymes referred
143 to as "base editors", which aim at modifying DNA at specific sites without involving double-
144 strand breaks¹⁴.

145 The DNA sequence alterations introduced through any of the genome editing techniques
146 may be single nucleotide variants (SNV), short or large insertions or deletions (called
147 InDels), or, less frequently, gene duplications, inversions and translocations¹⁵. "Short" DNA
148 alterations, as mentioned in this report, are referring to changes in one or a few base pairs,
149 while "large" alterations refer to alterations of several dozen base pairs. However, there is
150 a grey zone between "short" and "large" sequence alterations. When talking about the
151 specificity of a detection method, the criterion to be assessed is not the sequence length
152 itself, but whether or not a given DNA alteration occurs already in any plant species or
153 potentially could occur and whether or not it can be unequivocally attributed to the
154 application of a genome editing technique. This may need to be assessed on a case-by-
155 case basis using approaches which should be defined by the ENGL.

156 By analogy to the term "transformation event" used in GMO legislation², we propose here
157 to use "**genome-edited event**" to refer to the plant product that contains the altered DNA
158 sequence, as indicated above, at a specific site in the genome as a result of the genome
159 editing technique applied. A prerequisite is that no foreign DNA remained in the genome
160 of the final plant (from vector backbone or other 'unwanted' integrations), which was not
161 removed by segregation. Furthermore, as genome editing may result in several intended
162 DNA alterations in the genome, each of these multi-edits, when segregating independently,

¹² Zhang, Y., Liang, Z., Zong, Y., Wang, Y., Liu, J., Chen, K. *et al.* (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat. Commun.* 7:12617.

¹³ Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q. *et al.* (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8:14261.

¹⁴ Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., Qiu, J.-L., Wang, D., Gao, C. (2017) Precise base editing in rice, wheat and maize with a Cas9- cytidine deaminase fusion. *Nat. Biotechnol.* 35:438-440.

¹⁵ Zhu, C., Bortesi, L., Baysal, C., Twyman, R.M., Fischer, R., Capell, T., Schillberg, S., Christou, P. (2016) Characteristics of genome editing mutations in cereal crops. *Trends Plant Sci.* 22:38-52.

163 should be regarded as a separate genome-edited event and, for the enforcement, would
164 require a specific detection method.

165 The term "**detection**" as referred to in this report encompasses different aspects: (1) the
166 "finding" of a target sequence, *i.e.* detection *sensu stricto*, without necessarily being
167 specific for the genome-edited event; (2) the identification of the detected sequence as a
168 specific genome-edited event; (3) and the quantification of the genome-edited event,
169 expressed in mass fraction per total mass of the ingredient or plant species (m/m %). For
170 marketing authorisation under the GMO regulations, all three aspects of the broader
171 interpretation of "detection", *i.e.* including quantification, need to be fulfilled as the
172 detection method needs to be able to quantify the presence of the genome edited event at
173 the GMO labelling threshold for authorised events (0.9 m/m %). The same applies to GMOs
174 with pending or expired authorisation status that are detected in feed⁴, where it needs to
175 be assessed if their mass fraction is below the minimum required performance limit (0.1
176 m/m %). Methods for the detection of unauthorised GMOs, however, do not, in principle,
177 need to be quantitative as detection *sensu stricto* is sufficient for assessing non-compliance
178 of the product (cf. zero tolerance policy).

179

180

181 **3 Validation of detection methods for genome-edited events**
182 **under an EU authorisation request**

183 **3.1 Possibilities and challenges for analytical methods**

184 In an authorisation context, the GMO producer wanting to apply for market authorisation
185 of a GMO has to submit a complete dossier for risk assessment. This dossier shall include
186 a detection, identification and quantification method, with supporting method performance
187 data, and the reference material should be made available. GMO producers (applicants)
188 should follow the Guidelines publicly available to prepare the 'method validation dossier'
189 (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>). In the EU authorisation and control
190 context, it is required that analytical methods are specific to unambiguously identify the
191 GMO, that they provide a dynamic range around the labelling threshold (i.e. 0.9 m/m %),
192 and that they reach the desired level of sensitivity, robustness, ease of use and accuracy
193 of quantification.

194 At the time of writing, more than 150 applications for authorisation of mostly plant GMOs
195 for food or feed uses have been submitted in the EU since the GMO legislation came into
196 force².

197 In most of these cases, the GMOs contained one or more foreign DNA sequences of up to
198 several thousand nucleotides long. The genetic transformation procedures employed for
199 their generation have resulted in an "event" of insertion of new DNA sequence(s). For each
200 insertion, two unique insert-to-plant junctions are generated at both ends of the integration
201 site. The unique junctions created during a transformation event can be exploited as a
202 unique identification marker for developing methods of detection specific for each
203 conventional GMO (often referred to as "event-specific" detection).

204 Although genetic modifications may affect other classes of molecules such as RNA and
205 proteins and gradually down to metabolites, which can all be targets of analytical methods,
206 the benchmark technology for analytical detection, identification and quantification of
207 GMOs is typically based on real-time PCR (also called quantitative PCR or qPCR), targeting
208 the DNA molecule. This technology provides a million-fold amplification of a selected target
209 DNA sequence of typically 70-150 base pairs, located across one of the insert-to-plant
210 junctions. qPCR can provide high sensitivity and robustness for the precise relative
211 quantification of GM-material, even at low levels, in food and feed products. When qPCR is
212 targeting the unique sequences of transformation events, it ensures the required level of
213 specificity to be in compliance with the legal requirements.

214 The EURL GMFF validates the detection methods provided by applicants for market
215 authorisation in an interlaboratory validation trial involving qualified ENGL laboratories.
216 The validated quantitative method and certified reference materials (CRMs) for calibration
217 and quality control of the method constitute a complete "toolkit" for the unequivocal
218 identification and quantification of a GMO^{16,17}.

219 In order to evaluate whether PCR-based methods may fulfil the ENGL performance criteria
220 when applied to detection and quantification of genome-edited products, the experience
221 from other fields of diagnosis has been reviewed. The analysis was focused on the detection
222 and quantification of SNVs and small InDels since large insertions/deletions may constitute
223 a likely unique sequence (to be demonstrated) with suitable unique junction regions that
224 can be targeted by PCR.

225 The specificity of qPCR methods may be enhanced by use of particular probes (Minor
226 Groove Binding (MGB) probes) as shown in other fields, e.g. to genotype SNVs in peripheral

¹⁶ Trapman, S., Corbisier, P., Schimmel, H., Emons, H. (2009) Towards future reference systems for GM analysis. *Anal. Bioanal. Chem.* 396:1969-1975.

¹⁷ Corbisier, P., Emons, H. (2019) Towards metrologically traceable and comparable results in GM quantification. *Anal Bioanal. Chem.* 411:7-11.

227 blood or in bulk raw milk^{18,19}. Assessment of the performance of a SNV allelic discrimination
228 assay described in those reports indicated that quantitative parameters such as PCR
229 efficiency, slope and linearity could be in line with those established by the ENGL. However,
230 in those studies the target relative concentration tested (50%) was much higher than the
231 levels required for the quantification of GMOs (i.e. 0.1%-5%). Not only the sensitivity of
232 the method but also its specificity clearly is an issue, particularly when applied to market
233 products with a complex composition.

234 Systematic studies on effects of mismatches for primers and probes^{20,21} with qPCR methods
235 have shown that non-specific amplification from non-target sequences may occur and
236 induce quantification errors ranging between 33% and 63% or even a 658-fold
237 underestimation of the initial copy number showing the difficulties to properly quantify
238 SNVs. A probe-free quantitative PCR method has been shown to detect minor mutant
239 alleles with a frequency as low as 0.1% and high specificity was obtained by adding to the
240 reaction mix a 'T-blocker' oligonucleotide designed to prevent amplification of the wild type
241 sequence²².

242 This and other strategies would require however significant level of method optimisation
243 to ensure successful transferability for reliable use and reproducible response across
244 laboratories.

245 Droplet digital PCR (ddPCR) methodology used for the detection of off-target DNA
246 alterations (generally InDel mutations) generated by sequence-specific nucleases (SSNs)
247 could be similarly applied to the detection and quantification of genome-edited events.
248 ddPCR methods have been used for the screening and confirmation of particular mutations
249 in induced pluripotent stem cells or primary cells at very low concentrations (0.01% and
250 0.2% for SNV or InDel mutations^{23,24}); these are levels comparable to those required by
251 the labelling provisions of the EU legislation framework on GMOs. An internal reference
252 probe specific for a sequence not affected by the mutation but included in the target PCR
253 product has been used in those assays to assess the total amount of wild-type alleles
254 present in the sample. In those ddPCR assays the simultaneous quantification of both wild-
255 type and mutated sequences from the same PCR amplicon facilitated the precise relative
256 quantification independently from potentially interfering parameters such as DNA quality
257 and amplification efficiency. The correct design of two probes each binding to the mutated
258 and wild-type sequence is therefore crucial for ensuring the required specificity of the
259 method; this substitutes the use of taxon-specific genes for relative quantification of the
260 GM events as currently proposed in the ENGL document on Minimum Performance
261 Requirements.

¹⁸ de Andrade, C.P., de Almeida, L.L., de Castro, L.A., Driemeier, D., da Silva, S.C. (2103) Development of a real-time polymerase chain reaction assay for single nucleotide polymorphism genotyping codons 136, 154, and 171 of the *prnp* gene and application to Brazilian sheep herds. *J Vet Diagn Invest.* 25:120-124 (doi: 10.1177/1040638712471343).

¹⁹ Feligini, M., Bongioni, G., Brambati, E., Amadesi, A., Cambuli, C., Panelli, S., Bonacina, C., Galli, A. (2014) Real-time qPCR is a powerful assay to estimate the 171 R/Q alleles at the *PrP* locus directly in a flock's raw milk: a comparison with the targeted next-generation sequencing. *J. Virol. Meth.* 207:210-4 (doi: 10.1016/j.jviromet.2014.07.017).

²⁰ Süß, B., Flekna, G., Wagner, M., Hein, I. (2009) Studying the effect of single mismatches in primer and probe binding regions on amplification curves and quantification in real-time PCR. *J Microbiol Meth.* 76:316-319 (doi:10.1016/j.mimet.2008.12.003).

²¹ Stadhouders, R., Pas, S.D., Anber, J., Voermans, J., Mes, T.H., Schutten, M. (2010) The effect of primer-template mismatches on the detection and quantification of nucleic acids using the 5' nuclease assay. *J. Mol. Diagn.* 12:109-117 (doi:10.2353/jmoldx.2010.090035).

²² Kim, H., Ruby, A.E., Shandilya, H.G., Virmani, A.K., Rahman, N., Strange, C.M., Huuskonen, J. (2018) T-blocker: a simple and robust probe-free quantitative PCR assay to detect somatic mutations down to 0.1% frequency. *BioTechniques* 65:205–210.

²³ Miyaoka, Y., Berman, J.R., Cooper, S.B., Mayerl, S.J., Chan, A.H., Zhang, B., Karlin-Neumann, G.A., Conklin, B.R. (2016) Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. *Sci. Rep.* 6:23549 (doi:10.1038/srep23549).

²⁴ Mock, U., Hauber, I., Fehse, B. (2016) Digital PCR to assess gene-editing frequencies (GEF-dPCR) mediated by designer nucleases. *Nat. Protoc.* 11:598-615 (doi: 10.1038/nprot.2016.027).

262 Other authors²⁵ have compared the relative specificity and sensitivity of real-time qPCR
263 versus ddPCR assays in detecting and quantifying SNVs or small InDels in individual
264 founder transgenic mice generated by CRISPR/Cas9 mutagenesis; they observed a lower
265 rate of false-positive unedited events when using a ddPCR platform. Significant higher
266 sensitivity to all single and small InDel mutations and better accuracy were obtained also
267 by using a locked nucleic acid probe for the real-time or ddPCR reactions that increases
268 the discrimination of mismatch sequences and destabilises non-specific binding. In
269 summary, the ddPCR methods seem to be preferred above qPCR methods, however the
270 accuracy, trueness and specificity of the methods have not been systematically evaluated
271 for genome-edited products.

272 Moreover, the development of event-specific PCR methods as described above may not
273 lead to sufficiently specific quantification methods for short altered sequences (one or a
274 few nucleotides long).

275 Alternatively, in biomedicine, special modelling methods using next-generation sequencing
276 (NGS) data have been developed and validated to detect low abundant variants in cancer
277 samples²⁶. A similar framework could potentially be applied to food and feed samples.
278 Briefly, in such approaches, high-throughput sequencing (NGS) methods are applied either
279 to sequence entire genomes (WGS, whole genome sequencing) or multiplexed barcoded
280 amplicons. The variant's detection specificity and selectivity are then statistically estimated
281 in relationship to the crop natural variation. The main issue with this methodology is to
282 avoid or limit false positives. As far as we know, such methodologies have never been
283 formally applied on food and feed products and would require proper validation and
284 benchmarking. As the first step in NGS is done by PCR, the same possible limitation is the
285 inappropriate quality of the extracted DNA; however, other factors such as the variation in
286 crop genome size and ploidy (e.g. hexaploid wheat or tetraploid potato) and the level of
287 knowledge of expected variability for the entire genepool for each crop also complicate the
288 interpretation of the results. Another issue is the sensitivity of these NGS approaches for
289 detecting variants present at low frequency in the analytical sample. The sensitivity of NGS
290 to detect somatic mutations as a function of sequencing depth and allelic fraction has been
291 investigated in tumour samples²⁷. Simulations show that a theoretical sensitivity of 0.58 is
292 expected for a mutation allele fraction of 0.05 (5%) with tumor sequencing depth of 60x.
293 These sensitivity values are not in line with the acceptance performance requirements
294 established by the ENGL for GMO analysis.

295 **3.2 The event-specificity requirement of detection methods**

296 Specificity is the property of a detection method to respond exclusively to the target of
297 interest. Annex III to Regulation (EU) No 503/2013²⁷ states that '*the method shall be
298 specific to the transformation event (hereafter referred to as 'event-specific') and thus
299 shall only be functional with the genetically modified organism or genetically modified
300 based product considered and shall not be functional if applied to other transformation
301 events already authorised; otherwise the method cannot be applied for unequivocal
302 detection/identification/quantification.*'

303 For current transformation events, the method specificity is ensured by targeting the
304 junction between the inserted transgene sequences and the plant DNA, which is a unique
305 identification marker created *de novo* upon the randomly inserted transgene sequence.
306 Moreover, as it will be highly unlikely that exactly the same transgenic genome sequence
307 will be created *de novo* a second time, this unique marker is also ensuring traceability to

²⁵Falabella, M., Sun, L., Barr, J., Pena, A.Z., Kershaw, E.E., Gingras, S., Goncharova, E.A., Kaufman, B.A. (2017) Single-step qPCR and dPCR detection of diverse CRISPR-Cas9 gene editing events in vivo. *G3: Genes/Genomes/Genetics* 7:3533-3542 (doi: <https://doi.org/10.1534/g3.117.300123>).

²⁶Cibulskis, K., Lawrence, M.S., Carter, S.L., Sivachenko, A., Jaffe, D., Sougnez, C., Gabriel, S., Meyerson, M., Lander, E.S., Getz, G. (2013) Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat. Biotechnol.* 31:213-219.

²⁷ Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. *Off. J. Eur. Union* L157: 1-47.

308 the process that generated the GMO, independent of further breeding activity to cross the
309 GM event into different genetic backgrounds.

310 The situation is more complex for genome-edited plants. First, in the absence of foreign
311 DNA in the genome-edited plant, the altered sequence, whether short or long, may not
312 necessarily be unique, i.e. the same DNA alteration may already exist in other varieties or
313 in wild plants of the same or other species (see examples in Text box 2). Secondly, as a
314 result of the ease of use and site-specificity of the genome-editing techniques, exactly the
315 same DNA alteration may be created by different operators (companies, researchers)
316 independently, in order to create plants with a desired phenotype such as disease
317 resistance. This would make it impossible to trace the genome-edited event to a unique
318 identification marker, developed by a specific company in a specific genome-editing
319 experiment by current state-of-the-art technologies. The ownership of and liability for a
320 genome-edited plant may therefore be unclear.

321 Text box 2

322 **Examples of genome editing applications in plants**
323 **with similarity to existing commercial varieties**

324 The Canadian company Cibus Inc. used Oligonucleotide Directed Mutagenesis (ODM, a form of genome
325 editing) on single cells (protoplasts) followed by regeneration in tissue culture to create a mutated
326 *Brassica napus* plant (canola) with a single nucleotide change in the *BnAHAS1* gene leading to an amino
327 acid substitution of the corresponding protein; the mutated protein confers resistance to certain 'group
328 2' herbicides (e.g. imidazolinones and sulfonylureas). Although the mutated plant was isolated after
329 ODM treatment, the company reported that the single nucleotide mutation was the result of a
330 spontaneous somaclonal variation that occurred during the tissue culture process, and not due to the
331 specific oligonucleotide used as template in the ODM protocol. The mutated plant line was subsequently
332 crossed with a conventionally-bred imidazolinone-tolerant canola line (CLEARFIELD canola variety SP
333 Cougar CL), which contains the same mutation in its *BnAHAS3* gene, to produce canola line 5715,
334 homozygous for the mutation in both *BnAHAS1* and *BnAHAS3* genes²⁸; these genes, residing on the C
335 and A subgenome of *B. napus*, respectively, encode protein subunits of the acetohydroxyacid synthase
336 (AHAS) enzyme. Another company, Pioneer Hi-Bred, developed a similar imidazolinone-tolerant canola
337 by chemically inducing variation in the *BnAHAS2* gene using microspores. Besides canola, there are
338 many other agriculturally-important crops, including maize, wheat and rice, as well as weeds, showing
339 the same trait as a result of similar sequence alterations in their AHAS genes^{29,30}.

340 In tomato, genome editing has been conducted in a key gene (*psy1*) responsible for the red colour of
341 the tomato fruit. A range of sequence alterations (from SNVs to short InDels) were produced through
342 CRISPR-Cas9, all resulting in the yellow fruit phenotype³¹. Yellow tomato varieties also occur already
343 on the market, either produced by conventional breeding or as a result of chemical mutagenesis. All
344 these varieties contain mutations in the same gene but at different positions.

345 In maize, a high amylopectin variety was produced by CRISPR-Cas targeted deletion of the waxy (*Wx1*)
346 gene directly in elite inbred lines³². *Wx1* is one of the most studied "classical" maize genes, with over
347 200 spontaneous or induced mutations (deletions, insertions, translocations of various length) known
348 to lead to the waxy phenotype³³. A commercial natural waxy maize variety cultivated since the mid-
349 1980s contains a sequence deletion in the middle of the gene³⁴.

350 For market authorisation, applicants have to submit an event-specific detection method
351 and demonstrate that the method is specific for the GMO. This would require full knowledge
352 of all existing sequence variations for all varieties and wild plants of all species used for

²⁸ Hu, M., Pu, H., Kong, L., Gao, J. Long, W., Chen, S., Zhang, J., Qi, C. (2015) Molecular characterization and detection of a spontaneous mutation conferring imidazolinone resistance in rapeseed and its application in hybrid rapeseed production. *Mol Breed.* 35:46, <https://doi.org/10.1007/s11032-015-0227-3>.

²⁹ Tan, S., Evans, R.R., Dahmer, M.L., Singh, B.K., Shaner, D.L. (2005) Imidazolinone-tolerant crops: history, current status and future. *Pest Manag. Sci.* 61:246–257.

³⁰ Svitashv, S., Schwartz, C., Lenderts, B., Young, J.K., Cigan, A.M. (2016) Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. *Nature Comm.* 7:13274 (DOI: 10.1038/ncomms13274).

³¹ D'Ambrosio, C., Stigliani, A.L., Giorio, G. (2018) CRISPR/Cas9 editing of carotenoid genes in tomato. *Transg. Res.* 27:367–378.

³² Chilcoat, D., Liu, Z.B., Sander, J. (2017) Use of CRISPR/Cas9 for crop improvement in maize and soybean. *Prog. Mol. Biol. Transl. Sci.* 149, 27–46 (doi: 10.1016/bs.pmbts.2017.04.005).

³³ MaizeGDB (2017) Gene Record: GRMZM2G024993/ZEAMMB73_617956 (wx1 - waxy1) [Classical Gene List] Maize Genetics and Genomics Database. Available online at: http://www.maizegdb.org/gene_center/gene/12768.

³⁴ Fan, L., Bao, J., Wang, Y., Yao, J., Gui, Y., Hu, W., et al. (2009) Post domestication selection in the maize starch pathway. *PLoS ONE* 4:e7612 (doi: 10.1371/journal.pone.0007612).

353 food or feed production, which would serve as reference basis^{35,36,37,38} (see Text box 3). In
354 case of single nucleotide alterations it will be difficult to guarantee that the same SNV does
355 not exist in other varieties/populations, or will be created spontaneously in future plants.
356 The same problem may exist in case of more than a single nucleotide alteration, and even
357 for larger gene deletions or duplications which may exist already in conventional varieties³⁹.
358 Without access to continuously updated pan-genome databases for all plant species, it is
359 probably not possible for applicants to provide this information or for the EURL GMFF to
360 verify this information and to conclude that the method submitted is event-specific.

361 Text box 3

362 **Variability of plant genomes**

363 Advances in whole genome sequencing in recent years have revealed that the genome sequences of
364 crop species are diverse and dynamic. Dispensable genes may constitute a significant proportion of
365 the pan-genome, e.g. around 20% in soybean⁴⁰. A comparison between two maize inbred lines showed
366 that their genomes contained respectively 3,408 and 3,298 unique insertions and deletions (InDels),
367 with an average size of approximately 20 kbp (20,000 base pairs) and a range covering 1 kbp to over
368 1 Mbp⁴¹. Currently, comprehensive knowledge on the genomic variability among commercial plant
369 varieties of agricultural crops is not available. Moreover, it remains unclear to what extent such
370 information would provide a substantial contribution to the detection of genome-edited events,
371 especially against the background of the high dynamics of plant genome sequences.

372 Spontaneous natural mutations are expected to change the genome at each reproduction cycle. For
373 instance, there is a seven in 1 billion chance in the model plant *Arabidopsis thaliana* that
374 any given base pair will mutate in a generation⁴², meaning that 175 new variants (SNVs) would arise
375 per 100 individual plants per generation. This natural mutation rate may be increased as much as 250-
376 fold as a result of the stresses induced by *in vitro* culture conditions that are commonly used for the
377 breeding of many commercial crops, e.g. in rice, more than 54,000 novel DNA sequence variants were
378 identified in a line that went through *in vitro* culture (and 8 cycles of self-fertilisation), compared to
379 the wild-type line, without showing any different phenotype under normal growing conditions⁴³. The
380 relatively slow rate of natural mutation has also been increased by several orders of magnitude by
381 conventional mutagenesis, such as irradiation or chemical treatment of seeds or pollen, which have
382 been applied in plant breeding for several decades⁴⁴. Such mutant plants, which are exempted from
383 the GMO regulations, have been incorporated in traditional breeding programmes and have contributed
384 to the current crop diversity.

385 Consequently, it is expected that applicants will not be able to develop an event-specific
386 detection method for a genome-edited plant carrying a DNA alteration that may not be
387 unique. It will need to be assessed on a case by case basis if a given DNA alteration
388 corresponds to a specific genome-editing event that can be targeted by a detection method

³⁵ Hirsch, C.N., Foerster, J.M., Johnson, J.M., Sekhon, R.S., Muttoni, G., Vaillancourt, B., Penagaricano, F. (2014) Insights into the maize pangenome and pan-transcriptome. *Plant Cell Online* 26:121–135; Lu *et al.* (2015) High-resolution genetic mapping of maize pan-genome sequence anchors. *Nat. Comm.* 6:1-8.

³⁶ Li, Y.-H., Zhou, G., Ma, J., *et al.* (2014) *De novo* assembly of soybean wild relatives for pan-genome analysis of diversity and agronomic traits. *Nat. Biotechnol.* 52:1045-1054.

³⁷ Alaux, M., Rogers, J., Letellier, T., *et al.* (2018) Linking the International Wheat Genome Sequencing Consortium bread wheat reference genome sequence to wheat genetic and phenomic data. *Genome Biol.* 19:1-10.

³⁸ The 3,000 rice genomes project (2014) *Gigascience* 3:7; Zhao, Q., Feng, Q., Lu, H., *et al.* (2018) Pan-genome analysis highlights the extent of genomic variation in cultivated and wild rice. *Nat. Genet.* 50:278–284.

³⁹ Custers, R., Casacuberta, J.M., Eriksson, D., Sagi, L., Schiemann, J. (2019) Genetic alterations that do or do not occur naturally; consequences for genome edited organisms in the context of regulatory oversight. *Front. Bioen & Biotech.* 6:213.

⁴⁰ Li, Y. H., Zhou, G., Ma, *et al.* (2014) *De novo* assembly of soybean wild relatives for pan-genome analysis of diversity and agronomic traits. *Nat. Biotechnol.* 32:1045-1052.

⁴¹ Jiao, Y., Peluso, P., Shi, J., *et al.* (2017) Improved maize reference genome with single-molecule technologies. *Nature* 546: 524-527.

⁴² Ossowski, S., Schneeberger, K., Lucas-Lledó, J.I., Warthmann, N., Clark, R.M., Shaw, R.G., Weigel, D., Lynch, M. (2010) The rate and molecular spectrum of spontaneous mutations in *Arabidopsis thaliana*. *Science* 327:92-94.

⁴³ Zhang, D., Wang, Z., Wang, N., Gao, Y., Liu, Y., Ying, W., Yan, B., Zhibin, Z., Xiuyun, L., Yuzhu, D., Xiufang, O., Chunming, X., Bao, L. (2014) Tissue culture-induced heritable genomic variation in rice, and their phenotypic implications. *PLoS ONE* 9:e96879 (doi:10.1371/journal.pone.0096879).

⁴⁴ Jankowicz-Cieslak, J., Tai, T.H., Kumlehn, J., Till, B.J. (2016) *Biotechnologies for Plant Mutation Breeding*. SpringerLink ISBN 978-3-319-45019-3; Anderson, J.A., Michno, J.-M., Kono, T.J.Y., Stec, A.O., Campbell, B.J., Curtin, S.J., Stupar, R.M. (2016) Genomic variation and DNA repair associated with soybean transgenesis: a comparison to cultivars and mutagenized plants. *BMC Biotechnol.* 16:41.

389 fulfilling all minimum performance requirements, including regarding specificity. It is
 390 currently unclear how this specificity can be assessed, both *in silico* and experimentally.

391 In conclusion, whereas the detection *sensu stricto* of genome-edited events may in a
 392 limited number of cases be technically feasible, the same level of specificity for
 393 identification as currently applicable to conventional GM event-specific methods most likely
 394 will not be achieved for methods targeting most genome-edited plants. This will have
 395 important consequences for enforcement of the GMO legislation.

396 **3.3 The minimum performance requirements for analytical** 397 **methods of GMO testing**

398 The European Network of GMO Laboratories (ENGL) elaborated in 2015 the third version
 399 of the guidance document on minimum performance requirements for analytical methods
 400 of GMO testing⁴⁵. The document is addressed to applicants submitting detection methods
 401 according to Regulation (EC) No 1829/2003 and it provides criteria upon which methods
 402 for GMO analysis are assessed and validated by the EURL GMFF. The ENGL document takes
 403 into account all the requirements of the relevant international standards (ISO 24276, ISO
 404 21570, ISO 21571) and recommendations of the Codex Alimentarius⁴⁶.

405 Method validation is an essential component of the measures that a laboratory, operating
 406 its methods under accreditation to ISO/IEC 17025, shall implement before releasing
 407 analytical data. The standard requires that the analysis of a sample is performed by using
 408 "fully validated" methods. 'Full' validation of an analytical method usually includes an
 409 examination of the characteristics of the method in an inter-laboratory method
 410 performance study.

411 It is important to underline that the ENGL document refers to polymerase chain reaction
 412 (PCR) based methods since those are generally applied across applicants and control
 413 laboratories for GMO analysis. It details the acceptance criteria and performance
 414 requirements for 1) DNA extraction and purification methods, 2) PCR methods for the
 415 purpose of quantification and, 3) PCR methods for the purpose of qualitative detection
 416 (Table 1).

417 **Table 1.** Acceptance criteria and method performance parameters considered in the
 418 ENGL document on minimum performance requirements for methods of GMO
 419 testing (version 2015).

Criteria	DNA extraction	Quantitative PCR	Qualitative PCR
Method acceptance criteria	Applicability Practicability DNA concentration DNA yield DNA structural integrity Purity of DNA extracts	Applicability Practicability Specificity Limit of Detection (LOD) Robustness Dynamic Range Trueness Amplification Efficiency R ² Coefficient Precision Limit of Quantification (LOQ)	Applicability Practicability Specificity Limit of Detection (LOD) Robustness
Method performance requirements		Trueness Precision	False positive rate False negative rate Probability of detection

420 It should thus be considered to which extent the analytical methods proposed for genome-
 421 edited plants would (1) comply with the current provisions of the ENGL document as it is,
 422 and (2) if additional explanatory notes or amendments need to be made in order to provide

⁴⁵ European Network of GMO Laboratories (2015) Definition of minimum performance requirements for analytical methods of GMO testing (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>).

⁴⁶ Codex Alimentarius Commission (2009) Foods derived from modern biotechnology. FAO/WHO, Rome, Italy.

423 a quality and compliance framework for analytical approaches not yet covered. The most
424 critical aspects for consideration include the following elements:

- 425 - Applicability/Practicability of the method, e.g. for new technologies, e.g. next-
426 generation sequencing, the equipment may not be widely available, the quality
427 assurance parameters and uncertainty estimation are still under development, and
428 training may be required in the enforcement laboratories to make sure the methods
429 can be applied in a reliable way.
- 430 - Specificity to be demonstrated in silico and experimentally: small nucleotide changes
431 may not be significant enough to generate a unique sequence that can be exploited
432 to develop a detection method that is specific for identification of the genome-edited
433 event; which databases and plant samples need to be used for demonstration of the
434 event-specificity of the method?
- 435 - Robustness of the method: it needs to be assessed if methods targeting a SNV would
436 be sufficiently robust against small modifications to the testing conditions.
- 437 - Sensitivity (Limit of Detection/Limit of Quantification): what is the proof of evidence
438 required to demonstrate that a method has an acceptable specificity and limit of
439 detection also in complex food or feed samples?

440 Further consideration is necessary in order to provide guidance on the requirements for
441 detection methods for genome-edited products containing multiple DNA alterations. A
442 characteristic of genome editing techniques such as CRISPR-Cas and TALEN is the
443 possibility to simultaneously modify all alleles of a gene or different genes
444 simultaneously^{47,48,49,50,51,52}. This may lead to plants having multiple alterations in their
445 genome at one or more loci, which may be present in a homozygous or heterozygous state
446 (i.e. all copies of the gene may have the same alteration or different alterations). Event-
447 specific detection methods would be required to target all different alterations in the
448 genome in case they may segregate in subsequent generations. Analysing the performance
449 of multiple methods on a single genome edited plant makes it more laborious for the EURL
450 GMFF to perform the method validation in an interlaboratory trial and for the enforcement
451 laboratories to carry out the verification of these methods when they are implemented in
452 the laboratory. The case of multiple genome-editing events is to some extent similar as
453 that experienced for stacked transformation events (which also cannot be specifically
454 detected in food and feed), with the difference that in the latter case, the regulatory
455 approach demands the validation of a detection method for each of the single
456 transformation events composing the stack, before the validation of the same methods on
457 the stacked product can be started. For genome-edited plants, the "single events" may not
458 exist independently when multiple alterations have been created at once. Furthermore, the
459 quantification of multi-edited plants cannot be performed using the current state of the
460 art, and only the single events can be measured, similarly as for conventional GMO stacks.
461 Therefore, when two or more single genome-edited events belonging to the same

⁴⁷ Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., Qiu, J.-L. (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32: 947-952.

⁴⁸ Wang, Z.P., Xing, H.L., Dong, L., Zhang, H.Y., Han, C.Y., Wang, X.C., Chen, Q.J. (2015) Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in *Arabidopsis* in a single generation. *Genome Biol.* 16:144.

⁴⁹ Miao, C., Xiao, L., Hua, K., Zou, C., Zhao, Y., Bressan, R.A., Zhu, J.-K. (2018) Mutations in a subfamily of abscisic acid receptor genes promote rice growth and productivity. *PNAS* 115:6058-6063.

⁵⁰ Yu, Z., Chen, Q., Chen, W., Zhang, X., Mei, F., Zhang, P., Zhao, M., Wang, X., Shi, N., Jackson, S., Hong, Y. (2018) Multigene editing via CRISPR/Cas9 guided by a single-sgRNA seed in *Arabidopsis*. *J. Integr. Plant Biol.* 60:376-381, <https://doi.org/10.1111/jipb.12622>.

⁵¹ Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., ... Gao, C. (2017). Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nature Publishing Group*, 8, 1-5. <http://doi.org/10.1038/ncomms14261>

⁵² Peterson, B. A., Haak, D. C., Nishimura, M. T., Teixeira, P. J. P. L., James, S. R., Dangl, J. L., & Nimchuk, Z. L. (2016). Genome-wide assessment of efficiency and specificity in CRISPR/Cas9 mediated multiple site targeting in *Arabidopsis*. *PLoS ONE*, 11(9), 1-11. <http://doi.org/10.1371/journal.pone.0162169>

462 ingredient are found in a food or feed sample, it cannot be concluded if these originate
463 from a multi-edited plant or from separate single-event plants.

464

465

466 **4 Detection of genome-edited events in the context of** 467 **market control**

468 Every day, shipments of thousands of tons are arriving at EU harbours where they await
469 clearance for unloading the commodity. Verification of compliance with the EU food and
470 feed legislation is achieved through a mixed system of document traceability and
471 laboratory testing. According to EU legislation, accompanying documentation is provided
472 with the indication on whether the lot contains GMOs or not. Moreover, custom inspectors
473 collect and prepare a sample for laboratory analyses (controlling for GMOs, mycotoxins,
474 heavy metals, pesticides, etc.) according to the applicable sampling schemes and
475 recommendations.

476 Bulk grain that arrives in a harbour, and similarly any food or feed product produced from
477 it, is a compound product composed of different source materials, including crop varieties
478 with different genetic backgrounds, cultivated by various farmers in various regions of the
479 world and present in different proportions. Samples taken from these products are
480 analysed by the official control laboratories of the EU Member States for the presence of
481 GMOs. Real-time PCR-based methods are well-established analytical techniques adopted
482 by all control laboratories in the EU. Methods for detection need to be robust and applicable
483 to the typical heterogeneous nature of food and feed samples tested by enforcement
484 laboratories.

485 The current first-line approach employed by enforcement laboratories to analyse samples
486 for the presence of GMOs is based on an analytical screening strategy for DNA sequences,
487 such as gene promoters (*e.g. CaMV p35S*), gene terminators (*e.g. t-nos*), or protein coding
488 sequences (*e.g. cp4 epsps, pat or cry1Ab*) that are commonly found in authorised as well
489 as in unauthorised conventional GMOs. These methods will react positively for all GMOs
490 that contain the element-specific sequences and further testing will need to identify the
491 specific GMO(s) present in the sample.

492 Based on the outcome of the initial screening, the second step will be to test for the
493 presence of authorised GMOs using event-specific methods, or for known unauthorised
494 GMOs for which construct- or event-specific methods are available. This strategy may lead
495 to the direct identification of an unauthorised GMO (in the case of known unauthorised
496 GMOs that may have been detected earlier), but it may also lead to the conclusion that
497 some of the detected GMO screening targets could not be explained in this way. These
498 unexplained elements may point indirectly at the presence of (additional) unauthorised
499 GMOs in the sample; additional research, for example using targeted sequencing⁵³, is
500 required to elucidate the background of the identified GMO elements. In this way GMOs
501 without an EU authorisation request, with or without prior information on the modification,
502 may be detected⁵⁴.

503 For genome-edited crops, such screening methods generally are not possible, as such crops
504 do not contain any transgene sequence nor any other common element that can be
505 screened for. In the absence of targets that are common and therefore specific for a large
506 group of genome-edited plants no general screening approach is applicable or can be
507 developed.

508 Alternative approaches for the detection of unauthorised GMOs have been developed in
509 recent years. Screening of market samples using NGS has been proposed by a few EU

⁵³ Košir, A.B., Arulandhu, A.J., Voorhuijzen, M.M., Xiao, H., Hagelaar, R., Staats, M., Costessi, A., Žel, J., Kok, E.J., van Dijk, J.P. (2014) ALF: a strategy for identification of unauthorized GMOs in complex mixtures by a GW-NGS method and dedicated bioinformatics analysis. *Sci. Rep.* 7:14155 (DOI:10.1038/s41598-017-14469-8).

⁵⁴ ENGL (2012) Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials. Guidance document of the ENGL. (<http://gmo-crl.jrc.ec.europa.eu/doc/2011-12-12%20ENGL%20UGM%20WG%20Publication.pdf>).

510 control laboratories for the detection of unauthorised GMOs^{55,56,57}. It uses the known
511 sequences of conventional GMOs (common elements or coding sequences of transgenes)
512 as a "bait" to detect both authorised and unauthorised GMOs in a market sample. This
513 screening approach is dependent on the presence of combinations of foreign DNA
514 sequences and cannot detect genome edits (of any length of insertion or deletion). As a
515 consequence there are no robust laboratory methods to assure that unauthorised genome-
516 edited products could be prevented from entering the market.

517 While unlikely to occur in marketed crops, unwanted transgenic sequences may potentially
518 have remained in the genome in case the genome editing technique employed involved
519 integration of the construct into the plant genome and it was not carefully segregated out
520 in subsequent crosses. This will require developing additional screening methods for the
521 detection and as well the identification of such unintentionally remaining recombinant DNA
522 sequences.

523 The implementation of methods for the detection of genome-edited crops depends strongly
524 on the prior knowledge of the sequence alteration and on the availability of reference
525 material. Only if the analytical procedure for detection, identification and quantification of
526 a genome-edited product had been found fit for the intended purpose by the EURL GMFF,
527 then the validated method may be generally applied for control purposes.

528 In the absence of a market authorisation request in the EU, some genome-edited plants
529 may have been authorised in other markets, or information could have been published in
530 scientific journals. When the DNA alteration in such plants is known, and would be
531 sufficiently informative to be targeted by a detection method, the application of such
532 method, already published or to be developed, may allow detection of the genome-edited
533 product. However, at the current state no assessment has been carried out for any method
534 for the detection of any genome-edited plant product by the EURL.

535 The detection of very small sequence 'signatures' by bioinformatics and of genetic or
536 methylation 'scars', as hypothesised recently⁵⁸, does not provide realistic evidence and
537 proof that a new breeding technique was applied and has caused a detected DNA alteration.
538 Signatures like the PAM sequence (PAM- Protospacer adjacent motif - a 2-6 bp DNA
539 sequence immediately following the DNA sequence targeted by the Cas nuclease) are
540 relevant only for the CRISPR technique and vary depending on the type of Cas protein
541 used. "Scars" are potentially created in cells that have been directly treated by any
542 mutagenesis technique or passed through tissue culture and are not exclusively induced
543 by genome editing. Moreover, it is not clear to what extent epigenetic changes are stable
544 across breeding generations.

545 The identification of DNA alterations from genome editing, therefore, remains extremely
546 difficult as the altered sequences may mimic naturally occurring sequence variants, or they
547 cannot be distinguished from those alterations obtained with conventional mutagenesis.

548 An alternative approach for the detection of unauthorised GMOs has been proposed in
549 2010, using documentation-based screening for products that potentially contain
550 unauthorised GMOs using web crawling and text mining technologies using descriptive
551 keywords, to be followed by analytical confirmation⁵⁹. Such laborious approach, if

⁵⁵ Fraiture, M.A., Saltykova, A., Hoffman, S., Winand, R., Deforce, D., Vanneste, K., De Keersmaecker, S.C.J., Roosens, N.H.C. (2018) Nanopore sequencing technology: a new route for the fast detection of unauthorized GMO. *Sci. Rep.* 8:7903.

⁵⁶ Košir, A.B., Arulandhu, A.J., Voorhuijzen, M.M., Xiao, H., Hagelaar, R., Staats, M., Costessi, A., Žel, J., Kok, E.J., van Dijk, J.P. (2017) ALF: a strategy for identification of unauthorized GMOs in complex mixtures by a GW-NGS method and dedicated bioinformatics analysis. *Sci. Rep.* 7:14155.

⁵⁷ Arulandhu, A.J., van Dijk, J., Staats, M., Hagelaar, R., Voorhuijzen, M., Molenaar, B., van Hoof, R., Li, R., Yang, L., Shi, J., Scholtens, I., Kok, E. (2018) NGS-based amplicon sequencing approach; towards a new era in GMO screening and detection. *Food Control* 93:201-210.

⁵⁸ Bertheau, Y. (2019) New Breeding Techniques: Detection and Identification of the Techniques and Derived Products. In: *Reference Module in Food Science, Encyclopedia of Food Chemistry*, pp. 320-336 (doi.org/10.1016/B978-0-08-100596-5.21834-9)

⁵⁹ Ruttink, T., Morisset, D., Van Droogenbroeck, B., Lavrac, N., Van Den Eede, G.L.M., Zel, J., De Loose, M. (2010) Knowledge-technology-based discovery of unauthorized genetically modified organisms. *Anal. Bioanal. Chem.* 396:1951-1959.

552 implemented by all actors in the field, could be considered as a way to collect world-wide
553 information on the development and marketing of genome-edited crops, but it remains to
554 be evaluated to what extent such an approach would be practical as it relies on open
555 international collaboration, communication and voluntary exchange of information.
556 Moreover, analytical confirmation would still be very challenging to enforce the regulations.

557

558 **5 Conclusions**

559 This report highlights analytical challenges and limitations related to the detection,
560 identification and quantification of genome-edited food and feed products of plant origin.

561 Similarly to current conventional GMOs, products of genome editing can only be readily
562 detected and quantified in imports of commodity products by enforcement laboratories
563 when prior knowledge on the altered genome sequence, a validated detection method and
564 certified reference materials are available.

565 The ENGL has issued guidelines specifying the minimum performance requirements of
566 methods for GMO testing. The document is informative for applicants submitting an event-
567 specific detection method for a GMO as part of a request for market authorisation and
568 provides the acceptance criteria for the EURL GMFF when validating the detection method.
569 While this document will need to be reviewed to clarify the implications for methods for
570 genome-edited GMOs, it is on the basis of the current knowledge and technical capabilities
571 unlikely that a method for a genome-edited GMO would fulfil the performance
572 requirements. It would need to be demonstrated that such methods provide the level of
573 applicability, selectivity and specificity needed for the enforcement of legislation. The
574 largest bottleneck relates to providing proof for the origin of a detected DNA alteration, i.e.
575 to be able to demonstrate that it was created by genome editing and refers to a unique
576 genome-edited event that can be traced back to a single developer. This may be possible
577 for large DNA alterations, *e.g.* a large sequence deletion, not mimicked by identical
578 alterations that have been detected already in the (natural) plant pan-genome. According
579 to the current state of the art, for small DNA alterations affecting one or a few DNA base
580 pairs, the specificity of a detection method cannot be ensured. In all cases, it will be
581 challenging to demonstrate the specificity of a detection method for a genome-edited GMO,
582 as this would require access to a substantial proportion of the genetic variation in the
583 germplasm of all plant species that are used for food and feed production at any time.

584 In the absence of prior knowledge on the potential genome-edited alterations in a crop,
585 their detection and identification by the enforcement laboratories does not seem to be
586 feasible. A general analytical screening strategy, as employed for conventional GMOs,
587 cannot be developed for most genome-edited GMOs. When a DNA alteration has been
588 detected, there are also no procedures that allow to unambiguously conclude that genome
589 editing has created the alteration. At the same time, it may be possible to set up screening
590 strategies for particular sequences of interest, for instance when the sequences are unique
591 and known to result from genome-editing.

592 Therefore, many products obtained by genome editing may enter the market undetected.
593 Moreover, if a suspicious product would be detected at the EU market, it is unclear how to
594 provide legal proof on whether or not a modified sequence originated from a genome
595 editing technique.

596 In conclusion, implementation of the GMO legislation will be possible for authorised
597 genome-edited plants for which an event-specific detection method could be validated.
598 With the current technical capabilities, market control will, however, not be possible in the
599 absence of an event-specific detection method or for unknown genome-edited food or feed
600 products.

601



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