EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials
Food & Feed Compliance

# Explanatory notes to ANNEX III of Commission Regulation (EU) No 503/2013 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

*The current guidelines to the applicant include explanatory notes on Annex III of Regulation (EU) 503/2013 as well as reference to forms and templates that have to be filled in upon submission of an application. It is recommended that the applicant uses these templates available at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm. In case of requests regarding the preparation of an application, please do not hesitate to contact the EURL GMFF at* jrc-eurl-gmff@ec.europa.eu

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| **Annex III**<br><br>**Validation of methods for the detection, identification and quantification of the transformation event, and requirements for control samples and the certified reference material** | The text is self- explanatory. |
| **1. INTRODUCTION**<br>1. For the purposes of implementing Article 5(3)(i) and (j) and Article 17(3)(i) and (j) of Regulation (EC) No 1829/2003, this Annex sets out requirements on:<br>(a) the performance characteristics of the submitted method(s);<br>(b) technical requirements regarding the type of information that the applicant must submit so as to verify that those requirements are met;<br>(c) samples of the food and feed and their control samples;<br>(d) certified reference material. | |
| 2. The applicant must include information on the method as such and on the method testing carried out by the applicant.<br><br>3. The applicant shall also consider further guidance and information about the operational procedures of the validation process that is made available by the EU Reference Laboratory (EURL) as referred to in Article 32 of Regulation (EC) No 1829/2003, assisted by the European Network of GMO Laboratories. | The event-specific method for detection, identification and quantification of a GMO, the control samples and the sample of food and feed are part of the application for authorisation (Reg. (EC) No 1829/2003 Article 5(3)(i) and (j) and Article 17(3)(i) and (j)). The applicant is invited to submit a copy of the method and of the testing results together with the control samples and the sample of food and feed to the EURL GMFF. |

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| **2. DEFINITIONS**<br><br>For the purpose of this Annex, the following definitions shall apply:<br>(a) 'certified reference material' means reference material as referred to in Articles 5(3)(j) and 17(3)(j) of Regulation (EC) No 1829/2003 and corresponds to any material or substance, one or more of whose property values are certified for calibration or quality control of methods. It is accompanied by a certificate that provides value of the specified property, its associated uncertainty and a statement of metrological traceability; | |
| (b) 'method performance requirements' means the minimum performance criteria that the method shall demonstrate upon completion of the validation study carried out by the EURL, according to internationally accepted technical provisions. | For a detailed description of the "Definition of minimum performance requirements for analytical methods of GMO testing" released on 20/04/2015, please refer to http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf.<br>Hereinafter the document is referred to as 'MPR_2015'. |
| **3. METHOD VALIDATION** | |
| **3.1. Information about the method** | |
| A. The method(s) shall refer to all the methodological steps needed to analyse the relevant food and feed material in accordance with Articles 5(3)(i) and 17(3)(i) of Regulation (EC) No 1829/2003.<br><br>For a particular food or feed material, the methodological steps shall include the methods for DNA extraction and the subsequent quantification in a real-time Polymerase Chain Reaction (PCR) System. In such a case, the whole process from extraction up to the PCR-technique shall constitute a method. The applicant shall provide information about the whole method. | For more details, refer to page 4 and Annex 1, page 15, of MPR_2015.<br><br>The information regarding the method should be duly reported in the fields 1, 2 and 3 of the form named "Format to provide information on GM detection methods and related samples" available at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm. This form constitutes an integral part of the dossier to be submitted to the EURL GMFF. |

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| B. The applicant shall be allowed to refer to validated protocols, if available and appropriate, for method modules used in the analytical procedure such as a DNA extraction protocol from a certain matrix.<br><br>In that case, the applicant shall provide experimental data from an in-house validation in which the method module has been successfully applied in the context of the application for authorisation. | MPR_2015, page 4 and Annex 1 page 15, referring to the concept of module.<br>*('Format to Provide information on GM detection methods and related samples', item 1.5).* |
| C. The applicant shall demonstrate that the method(s) fulfils the following requirements: | |
| 1. The method(s) shall be specific to the transformation event (hereafter referred to as 'event-specific') and thus shall only be functional with the genetically modified organism or genetically modified based product considered and shall not be functional if applied to other transformation events already authorised; otherwise the method cannot be applied for unequivocal detection/identification/quantification. This shall be demonstrated with a selection of non-target transgenic authorised transformation events and conventional counterparts. This testing shall include closely related transformation events. | An event-specific method targets the unique insert-to host organism junction originated in the transformation event.<br><br>The EURL GMFF will run *in-silico* specificity analyses of a proposed method against available databases and the Central Core Sequence Information System (CCSIS).<br>*('Format to Provide information on GM detection methods and related samples', item 2.1).* |
| 2. The method(s) shall be applicable to samples of the food or feed, to the control samples and to the certified reference material. | A DNA extraction and purification protocol has to be submitted with details of testing and evidence that the method allows to obtain genomic DNA of a quantity and quality acceptable with reference to the MPR_2015. For further details on the samples of food and feed and their control samples, please refer to explanatory notes given in 3.3 below.<br>If the use of commercial kits is proposed, the applicant has to provide justification and clear experimental evidence supporting the choice (e.g. significant improvement of the quality of the DNA, significant gain of time or cost reduction or replacement of hazardous chemicals). Should a method refer to a commercial kit or to a reagent that is |

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| | at a later stage withdrawn from the market, the applicant has to provide an alternative extraction/purification procedure and bridging evidence to show that the revised procedure extracts DNA of acceptable quality and quantity from the same matrix and/or detects, identifies and quantifies the transformation event, according to the MPR_2015 requirements.<br>*('Format to Provide information GM detection methods and related samples', item 2.2).* |
| 3. The applicant shall take into consideration the following documents for the development of the detection method:<br>(a) Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions: ISO 24276;<br>(b) Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction: ISO 21571;<br>(c) Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods: ISO 21570;<br>(d) Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods: draft European standard ISO 21569. | The text is self-explanatory. |
| 4. The method shall also take into consideration the more detailed requirements set out in the common criteria set by the EURL and the ENGL for minimum performance requirements for analytical methods for GMO testing. These criteria are part of the guidance provided by the EURL. | Please refer to MPR_2015. |
| D. For the purpose of implementing Articles 5(3)(i) and 17(3)(i) of Regulation (EC) No 1829/2003, the applicant shall provide the event-specific quantitative detection method(s) of the genetically modified material. | "quantitative detection method(s)" means a method or methods consisting of the different modules, with the capacity to detect, identify, and quantify the presence of a transformation event in a food or feed product. In order to meet also the requirements of Commission Regulation (EU) No 619/2011, this method has to provide quantification of 0.1 % in mass fractions of GM-material in feed. |

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| The applicant shall discuss the validity and limitations of the detection methods in the various types of foods and feeds (the various matrixes) that are expected to be placed on the market. | It is recognised that the DNA extraction module is essential to deliver good quality DNA to the downstream qPCR modules. Procedures to extract DNA from raw commodities (grain/seeds) are well established. In discussing the validity and limitations of the method, the applicant may make reference to scientific literature to elaborate on the appropriateness of a DNA extraction procedures applicable to the matrix(es) corresponding to the next steps of processing, expected to be placed on the market.<br>*('Format to Provide information GM detection methods and related samples', item 2.2).* |
| E. The applicant shall provide a complete and detailed description of the method.<br><br>The following points shall be clearly addressed by the applicant: | *('Format to Provide information GM detection methods and related samples', item 2.3).* |
| 1. Scientific basis: the applicant shall provide an overview of the principles of how the method works. This overview shall include references to relevant scientific publications. | The text is self-explanatory. |
| 2. Scope of the method: the applicant shall indicate the matrix(es) (for example, processed food, and raw materials), the type of samples and the percentage range to which the method may be applied. | In addition to discussing the scope of the method, the applicant has to indicate the matrix(es) on which the DNA extraction/purification procedures and real-time PCR assays were performed and report the method performance parameters (MPR_2015, point 2.2, pages 5-6. |
| 3. Operational characteristics of the method: the required equipment for the application of the method shall be specified, with regard to the analysis as such and the sample preparation. Further information of any specific aspects crucial for the application of the method shall also be included. | The type of equipment needed for running the method, both for the sample preparation and for all modules of the method has to be indicated. Similarly, the equipment(s) on which the method was developed and optimized have to be clearly indicated. All data have to be included in the application and crucial aspects addressed. *Inter alia*, the number of PCR runs, the number of samples and replicates and whether the modules (GM- and taxon-specific reference module) for the relative quantification are run on the same or on separate runs. |
| 4. Protocol: the applicant shall provide a complete optimised protocol of the method. The protocol shall present all the details as required to transfer and apply the method independently in other laboratories. | The protocol should be written in the form of a standard operating procedure and should comply with the requirements of the MPR_2015.<br>The "Template protocol format for submission of a GMO specific real-time PCR system" should be used (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). |

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| 5. A prediction model (or a similar tool) needed to interpret results and to make inferences shall be described in full details. Instructions for the correct application of the model shall be provided by the applicant. | The unit of measurement used to express the concentration of the GM-event has to be clearly indicated as either mass fraction of GM DNA or GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes. If the latter is chosen, it has to be indicated how the copy numbers of the transformation event and the taxon specific DNA have been calculated.<br><br>Note: if the measurement is made in copy numbers, the mass/mass value has to be also provided, taking due account of the zygosity of the event in the positive control sample. |
| 6. Breeding schemes that are to be applied for the production of genetically modified food and feed and their impact on the interpretation of results shall be provided by the applicant. | The information should be filled in at "EURL format to provide information" form under point 4.5 on the genetic background of positive and negative control samples (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). The applicant has to provide a schematic representation and description of the breeding tree of the event and indicate the zygosity (homo- or hemizygosity of the transformation event in the positive control sample) and the maternal/paternal contribution to the transformation event in the positive control sample. The relation between the positive and negative control sample has to be clearly described. |
| **3.2. Information about the method testing carried out by the applicant** | |
| A. The applicant shall provide all the available and relevant data of the method optimisation and testing carried out. These data and results shall be presented, where possible and appropriate, by using the performance parameters as referred to under point 3.1.C.4. | The performance parameters are described in the MPR_2015.<br>When methods are already fully validated and should be verified for the ability to detect/identify/quantify the events in a stacked GMO material, Annex 2 of the MPR_2015 applies. |
| The applicant shall also provide a summary of the testing carried out and the main results as well as all the data including the outliers. | Summary of the testing is intended as the result of the quantification of the individual replicates of the test samples (including outliers) as well as the individual values of slopes, efficiency and $R^2$ of the calibration curves, in addition to the averages of the relevant parameters, to assess compliance with the MPR_2015. Results of testing the Limit of Detection should be provided as Cq values and not as positive and negative scores.<br>In case of need, the EURL GMFF can request to receive raw data. |

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| B. The applicant shall ensure that the provided information demonstrates the robustness of the method for inter- laboratory transferability. For this purpose, the applicant shall provide the results of the testing of the method by at least one laboratory that is different from the laboratory which has developed the method. | Robustness of the PCR module, please refer to MPR_2015, page 10-11.<br><br>All results of participants to the inter-laboratory transferability study have to be clearly reported by the applicant; in case more than one laboratory is involved, each laboratory should be independent from the other(s) and its set of data clearly identified in the report. The aim of the transferability study is to document that the method protocol is fully optimised and properly described to allow inter-laboratory transfer and to increase the chance of success of a collaborative study. Therefore, the results of each laboratory are considered individually and not gathered to produce an estimate of reproducibility<br>*('Format to Provide information GM detection methods and related samples', item 3.1).* |
| C. The applicant shall provide the following information about the method development and the method optimisation: | |
| 1. primer pairs tested and probe, if appropriate, including a justification as to how and why the proposed primer pair has been selected; | Primers and probes (the amplification system) have to respect the acceptance criteria on the specificity of the method MPR_2015, pages 7-8.<br>*('Format to Provide information GM detection methods and related samples', item 3.2).* |
| 2. stability testing, which shall be established through the submission of experimental results from testing the method with different plant varieties; | A taxon-specific module intended for quantitative analysis should target a single-copy DNA sequence per allele within the taxon. The absence of copy number variation should be demonstrated. Please refer to MPR_2015, pages 7 for details.<br>*('Format to Provide information GM detection methods and related samples', item 3.3).* |
| 3. specificity, which shall be established through the submission of the full sequence of the insert(s) in a standardised electronic format, together with the base pairs of the host flanking sequences so as to enable the EURL to assess the specificity of the proposed method by running homology searches in a molecular database; | Specificity, please refer to MPR_2015, pages 7-8<br>Sequences have to be submitted according to the guidance "Guideline for the submission of DNA sequences and associated annotations within the framework of Directive 2001/18/EC and Regulation (EC) No 1829/2003", Section V. and following the "Form for the submission of DNA sequences and associated annotations to the European Union Reference Laboratory for GM Food and Feed or EFSA, according to appropriate existing EU legislation". All files containing sequence information will be stored into the Central DNA Core Sequence Information System (CCSIS) database, to ensure confidentiality.<br>*('Format to Provide information GM detection methods and related samples', item 3.4).* |
| 4. precision, the relative repeatability standard deviation shall be less than or equal to 25 % related to mass fraction over the whole dynamic range of the method. | Precision, please refer to MPR_2015, page 9.<br>*('Format to Provide information GM detection methods and related samples', item 3.5).* |

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| D. The applicant shall, in addition to the information required under Sections A, B and C provide the following information regarding the testing:<br><br>1. participating laboratories, time of the analysis and outline of the experimental design, including the details about the number of runs, samples, replicates, etc.;<br>2. description of the laboratory samples (such as size, quality, date of sampling), positive and negative controls as well as certified reference material, plasmids and alike used;<br>3. description of the approaches that have been used to analyse the test results and outliers;<br>4. any particular points observed during the testing;<br>5. references to relevant literature or technical provisions used in the testing. | 1. Laboratories participating in the inter-laboratory transferability study (see above 3.2.B): outline = SOP;<br>2. Description has to be complete;<br>3. If several approaches were used, these have to be reported and the reason for the chosen approach should be given;<br>4. All unexpected observations are to be reported, together with the explanation found;<br>5. References should be complete and allow accessing the information. If that is difficult, copies should be provided.<br>*('Format to Provide information GM detection methods and related samples', item 3.5).* |
| **3.3. Samples of the food and feed and their control samples** | |
| For the purpose of implementing Articles 5(3)(j) and 17(3)(j) of Regulation (EC) No 1829/2003, the applicant shall, together with the information required under Sections 1, 2 and 3 of this Annex, also provide samples of the food and feed and their control samples of a type and amount to be specified by the EURL for the specific application for authorisation.<br>The information accompanying the control samples shall include information on the breeding of the plant which has been used for the production of the control samples and on the zygosity of the insert(s).<br>The applicant may use the same raw material for the production of certified reference material and for the production of control samples. | **Control samples**<br>Please consult the guidance document 'Note to the applicants on the type and nature of control samples according to Reg. (EC) No 1829/2003' at:<br>http://gmo-crl.jrc.ec.europa.eu/doc/CRLGMFF_Note_control_samples.pdf<br>The EURL GMFF appreciates the provision of genomic DNA as positive and negative control samples. Intact seeds are also acceptable, in the range of 1-3 kg.<br>DNA quality and quantity should be in compliance with the MPR_2015. Absence of contamination from other GMOs and common or related species should be demonstrated. The amount of DNA required by the EURL GMFF is described in Appendix I to this document. For details on DNA acceptance criteria, please refer to MPR_2015, pages 5-6.<br><br>**Positive control sample**<br>To avoid heterogeneity in the preparation of the analytical sample, the positive control |

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| | sample has to derive from (plant) material that contains 100% (or as close as possible) of the transformation event(s) that is the subject of the application. Summary of testing and results should accompany the purity statement. The positive control sample has to contain only the event(s) relevant to the application and not others. In case of stacks, it has to be derived from the stack that is subject of the application, not from a mixture of samples containing the single events or sub-combinations of events that are present in the stack.<br>In case genomic DNA is provided as positive control sample, the zygosity of the positive control sample should reflect the zygosity of the event(s) in the marketed material. If the material on the market is represented by seeds/grains homozygous for the transformation event(s), the positive control sample should be homozygous for the transformation event(s); if the material on the market will mostly be hemizygous for the transformation event(s), also the positive control sample should be hemizygous for the transformation event(s).<br><br>**Negative control sample**<br>The negative control sample should have the same genetic background of the positive control sample, but does not contain the insertion region of the transformation event(s). It is required that the positive and negative control samples have the same ploidy level.<br><br>**Samples of Food and Feed.**<br>The samples of food and feed are used to validate the DNA extraction module. For the purposes of the EURL GMFF, one type of matrix which is relevant in the context of the application is sufficient as food and feed sample. The food and feed sample should contain the transformation event(s) subject of the application at a minimum content of 0.9% (or other relevant legal labelling threshold). If, due to processing the amount of the transformation event is not quantifiable at the 0.9% level, the first material upstream in the processing that allows the extraction and quantification of the appropriate amount of the transformation event should be submitted as food and feed sample. The applicant should justify such a selection.<br>Seeds/grains often represent the first product in the food/feed chain hosting the |

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| | transformation event, hence they are acceptable as food and feed sample. Depending on the application, this can be for instance seeds, grain or flour.<br>If available, details of analytical tests on the food and feed sample to detect presence of other species or other transformation events should be communicated to the EURL GMFF.<br>Details of the food and feed samples should be provided in field 4 of the 'Format to provide information on GM detection methods and related samples' form (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).<br>In case of stacked-transformation events, the food and feed sample should consist of the harvest (grains).<br><br>In case of questions concerning the control samples or the sample of food and feed, please consult the EURL GMFF at jrc-eurl-gmff@ec.europa.eu.<br>It is the responsibility of the applicant to verify that the material amounts are sufficient for the EURL GMFF to do the in-house verification and the full validation when necessary. The minimal amounts required are presented in Appendix I. The material should be listed in an accompanying letter, including instructions for storage conditions and specific handling of the material, where appropriate. The EURL GMFF reserves the right to request additional material (positive and negative controls) as needed.<br>*('Format to Provide information GM detection methods and related samples', item 4 and subsequent).* |

**Appendix I.** Table indicating the requested amount of DNA to be submitted by the applicant for single and stacked events

| **Type** | **Positive control sample** | **Negative control sample** |
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| Single event (full validation) | 350 µg | 3.5 mg |
| Single event* | 80 µg | 0.8 mg |
| Stack of 2 single events A X B* | 160 µg | 1.6 mg |
| Stack of 3 single events A X B X C* | 240 µg | 2.4 mg |
| Stack of 4 single events A X B X C X D* | 320 µg | 3.2 mg |
| Stack of 5 single events A X B X C X D X E* | 400 µg | 4.0 mg |

* EURL verification for validated method (no full validation)

The above calculations are indicative and refer to methods based on two standard curves, whose calibration curves are built in serial dilutions, with no addition of background DNA and for a maximum of 250 ng of DNA in reaction. These amounts are applicable to maize, soy, cotton and oilseed rape. Notwithstanding the above information, depending on the validation protocol submitted and on the need for further testing, the EURL GMFF may request additional positive and negative control samples as purified genomic DNA or viable seeds.