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DATUM	27 february 2013
KENMERK	CGM/130227-05
ONDERWERP	Advice: Molecular characterisation of GMOs for use in clinical and veterinary studies

Dear Mrs Mansveld,
Please find attached our advice ‘Criteria for the molecular characterisation of GMOs for medical and veterinary applications’.

Summary
In recent years in the Netherlands clinical and veterinary studies involving genetically modified organisms (GMOs) have been regularly carried out. This ‘deliberate release into the environment’ of GMOs can pose risks to humans and the environment. To assess the possible environmental risks, these GMOs must be characterised in sufficient detail. In this advisory report COGEM explains how and in what detail the genome of a GMO for a medical or veterinary application should be described in order to carry out the risk assessment.

COGEM differentiates between three different aspects of genetic characterisation. The first is the characterisation of the parental organism. The characteristics of the parental organism form the basis for the environmental risk assessment, an important requirement for which is the confirmation of the identity of the organism. The second aspect is the molecular characterisation of the intended modification. To correctly determine how the modification will influence the environmental risks it is necessary to know that the actual modification made in the GMO is the same as the intended modification. The third aspect of the molecular characterisation is the possible presence of unintended modifications. Unintended modifications to the genome of a GMO may be made during its production as a result of natural processes. These modifications may affect the fitness of the GMO and therefore the outcome of the environmental risk assessment.

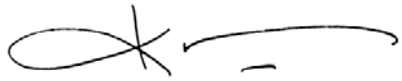
To obtain a better understanding of the aspects mentioned above, COGEM is in favour of a sequence analysis of the whole genome of the GMO. However, the Commission notes that the nucleotide sequence does not necessarily have to be submitted with the application for consent. In

principle, it is only necessary to provide the results of the comparison between the expected and actual order of sequences and the impact of any possible deviations. The advice also suggests other methods which can be used to investigate these aspects.

If the above-mentioned requirements are met, COGEM is of the opinion that the genetic characterisation of GMOs will be sufficient to support a sound assessment of the environmental risks.

The grounds on which COGEM has reached its conclusions and the resulting criteria for the molecular characterisation of GMOs for medical and veterinary applications are set out in the enclosed report.

Yours sincerely,



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Criteria for the Molecular Characterisation of GMOs for Medical and Veterinary Applications

COGEM advice CGM/130227-05

1. Introduction

Before a genetically modified organism (GMO) can be released into the environment it must first be characterised in sufficient detail. This characterisation must give an accurate picture of the characteristics of the GMO and is therefore of fundamental importance for the environmental risk assessment. In practice, it includes both a genetic and a phenotypic description. These descriptions are complementary and both are necessary to obtain a complete picture of the GMO's characteristics.

Only in the last few years have clinical and veterinary studies involving GMOs been carried out with any regularity. In the Netherlands these studies require an environmental permit for 'deliberate release into the environment'. In the last five years COGEM has advised on 22 applications for these environmental permits, the majority of which were for the use of a viral vector, but some were for GM bacteria and naked DNA.[1,2,3,4,5,6,7,8] In 6 of the 22 cases, COGEM was not in a position to give a positive recommendation on the application.[7,9,10,11,12,13] In most cases this was due, entirely or in part, to an inadequate genetic characterisation of the GMO to be used.

If the data provided are insufficient to permit a verification of the genetic organisation of the GMO, COGEM is unable to draw any conclusions about the environmental risks. In such cases, the only remaining option is to take the 'worst case approach', which for a deliberate release into the environment means a negative recommendation.

This advisory report has been prepared to inform applicants, the Ministry of Infrastructure and the Environment and other stakeholders about the level of detail required when providing the necessary genetic information. It concentrates on the information COGEM requires in the submitted genetic description, or molecular characterisation, of GMOs to be used in clinical and veterinary studies. In the interests of clarity, and because the applications give no reason for it, this report does not discuss the requirements for a phenotypic description of GMOs in detail. In exceptional cases, however, interpreting the molecular characterisation will be more dependent on the phenotypic characterisation of the GMO than normal. For these situations the requirements for the phenotypic characterisation are briefly discussed in addition to the requirements for the molecular characterisation. The reason for the different sets of requirements and the ways in which applicants can meet them is explained further in this report.

2. Similarities with the molecular characterisation of GM crops

COGEM has considerable experience with applications for deliberate release of GM crops into the environment and how to obtain a good picture of the genetic composition of these GMOs. Based on this experience, COGEM has set out and explained its requirements for the molecular

characterisation of GM crops in two different publications.[14,15] The advice presented here is based on the requirements for the molecular characterisation of GM crops and guidelines derived from previous advisory reports on the deliberate release into the environment of GMOs for medical and veterinary applications.

COGEM considers the identification of the parental organism to be the primary requirement for the proper assessment of the environmental risks of a GMO. In addition, it is important to know which genetic elements have been inserted into or removed from the GMO and what the functions of these elements are. For this reason the characterisation should include an inventory of the intended modifications. It is important that the actual modifications made to the genome of the GMO are validated and that the validation process is easy to follow in an imitable/ comprehensible way. A detailed description of the way in which the GMO has been constructed can provide a useful reference for this process.

Depending on the parental organism and the way in which the modification is carried out, unintended modifications may also occur in addition to the planned modifications. This is the last part of the characterisation and covers possible rearrangements, deletions and point mutations in the genome of the GMO. At the same time, it should be determined whether any other pieces of DNA present during the construction of the GMO have unintentionally been inserted into the genome of the GMO.

COGEM considers it important that in the molecular characterisation of GM crops the GMO is analysed for new open reading frames (fusion ORFs) created as a result of the intended modification.[15] A fusion ORF consists partly of a coding sequence from the parental organism and partly of a sequence from the insert. The fusion ORF may lie within a reading frame other than that used by the inserted transgene and, in a double-stranded genome, may lie on the opposing strand. In theory, no more than 12 fusion ORFs can be created.

In principle, fusion ORFs can also be created as a result of a deletion. In such cases, the fusion ORF will consist of sequences from the parental organism on either side of the deleted region. A deletion can lead to no more than six fusion ORFs. COGEM notes that in many viruses the ORFs are only transcribed in one direction along the genome. This halves the total number of fusion ORFs that can actually give rise to new proteins.

In theory, fusion ORFs can lead to the synthesis of proteins with unknown properties. The likelihood of such fusion ORFs leading to the creation of a functional protein is considered to be very small. COGEM points out that a large number of defective ribosomal products (DRiPs) are formed during protein synthesis.[16,17] These DRiPs are abnormal or non-functional polypeptide chains, which are rapidly broken down again and used as antigenic determinants. COGEM considers it likely that the fusion ORFs, as abnormal polypeptides, will suffer the same fate as these DRiPS. COGEM also notes that the GMOs for medical or veterinary applications are usually biologically contained. Even if a fusion ORF could have a negative effect, because of the biological containment this effect would in most cases be limited to the volunteer or laboratory animal concerned. These risks fall under other legislation than the environmental legislation and therefore fall outside the scope of this advice. The risks to laboratory animals and volunteers are

assessed by medical/ethical review committees and other bodies. If a biologically non-contained system is involved, however, COGEM cannot rule out the possibility that a fusion ORF could give rise to an environmental risk.

Other than in the development of GM crops, the modifications made to bacteria and viruses are often highly specific, the site in the genome where the modification is to be made, is being determined in advance. This means that the fusion ORF can be taken into account when designing the modification. If biologically non-contained GMOs are used, COGEM advises identifying possible fusion ORFs during the molecular characterisation and carrying out a bioinformatic analysis to anticipate whether these fusion ORFs could be harmful.

All planned and unplanned modifications for which COGEM considers it necessary to be able to establish with certainty whether the GMO matches its theoretical description are described in the next chapter.

3. Criteria for the molecular characterisation of GMOs for medical and veterinary applications

Due in part to past experience with applications for consent to release GM viruses and GM bacteria into the environment, the following criteria apply to all GM viruses and GM bacteria unless otherwise stated. COGEM notes that criteria have not yet been established for studies that use a GMO based on another microorganism, such as a yeast. In such situations COGEM's advice is to use the criteria given below as guidelines until further notice. The same goes for medical or veterinary studies in which use is made of naked DNA.

3.1 Identification of the parental organism

The organism in which the intended modifications are to be made forms the basis of a GMO. Knowledge of the characteristics of this parental organism (or acceptor organism) is therefore an essential starting point for the environmental risk assessment of a GMO, and it is evident that relevant scientific information about these characteristics must not be omitted from the application.

To obtain an understanding of the impact of the parental organism on the intended GMO, COGEM is of the opinion that the identity of the parental organism must be defined in detail. This is the first part of the molecular characterisation.

In the case of viruses, COGEM argues that the identity of the parental organism must be verified from a sequence analysis of its genome, and the applicant should state which distinguishing features have been used. This puts the emphasis on the result of the bioinformatic analysis and so there is no need to submit the complete nucleotide sequence.

The same approach can be taken for bacteria. Given the way bacteria are usually phenotypically typed, COGEM considers that a sequence analysis limited to the characteristic part or parts of the genome of the relevant bacterial strain will provide sufficient evidence. In this case, besides the molecular characterisation, an additional phenotypic characterisation will be necessary in order to fully verify the identity of the parental organism.

3.2 Intended modification of the parental organism

Based on the GMOs on which COGEM has issued advice in recent years, various sorts of modifications can be distinguished. These include the insertion of a heterologous or homologous sequence into the genome of the parental organism, the deletion of parts of the genome of the parental organism, or a combination of both of these. The modification can also comprise one or more mutations or point mutations in existing sequences. To assess how these modifications may affect the potential environmental risks, it is evidently essential to know what function the insert has in the donor organism or what the function is of the relevant sequence in the acceptor organism. This information is needed to determine the impact of the modification on the characteristics of the GMO and the resulting environmental risks.

In the following sections the criteria for the molecular characterisation are set out and discussed for each type of modification. For GMOs with a combination of different types of modifications, the molecular characterisation will of course have to meet the criteria for all modifications. It should be noted that an accurate description of the way in which the GMO has been constructed provides an important frame of reference for the molecular characterisation. Information that can be very useful for this includes a description of the plasmid DNA used to make the modification, as well as a description of the coding sequence used to make the insertion, deletion or point mutation and, if applicable, the regulatory sequences they contain.

3.2.1 Addition of heterologous or homologous sequences

Genetic modification is often used to add new characteristics to a microorganism. In most cases a complete transgene, with or without regulatory elements, is inserted into the genome of a parental organism.[3,9,10,11,12,13,18,19,20,21,22] However, it is also possible to insert coding elements into an existing gene to create a fusion protein. This was the case during a clinical study with a conditionally replicating adenoviral vector for brain tumours.[23,24]

To be able to assess the environmental risks of the GMO, the genes or sequences that have been inserted into the genome of the parental organism must be known. Depending on the way in which the GMO is constructed, it is possible that multiple copies of that sequence will have been inserted into the genome of the GMO. These copies may be inserted in tandem at the same insertion site or distributed throughout the genome. As this has an influence on the expression pattern of the sequence, information about the distribution of these copies, along with the function of the sequence, is important for obtaining a good assessment of the environmental risks.

COGEM therefore considers that the insertion site, the orientation of the insert and the number of copies of the insert should be established. In COGEM's opinion, this information should be obtained from a full sequence of the insert. This sequence analysis should cover the insert and any recombination sequences used, and should extend over a few dozen nucleotides flanking the insert in the genome of the acceptor organism. To support the sequence analysis, use may also be made of a Southern blot analysis in order to satisfy the stated criteria. The application should state the nucleotide sequence of the insert and the flanking regions. If the nucleotide sequence deviates

from the expected sequence, COGEM expects the impact of these deviations on the final environmental risks to be discussed in the application.

3.2.2 Deletion or disruption of genes from the genome of the parental organism

In practice, the synthesis of a particular protein in an organism can be prevented by removing all or part of the gene that codes for that protein or breaking the reading frame of this gene by inserting a new piece of genetic material.[6,7,8,23,24,25] If no additional sequence remains in the final GMO, this is referred to as a 'clean deletion mutant'.

To verify the deletion or disruption of the target gene and the way this has been achieved in practice, COGEM recommends that an analysis is made of the sequence in the relevant region of the genome of the GMO. The sequence to be analysed, including any recombinant sequences used, should include the flanking regions unique to the parental organism, which are therefore not present in the transfer vector used for the modification. A description of the function of the inactivated gene is needed in order to assess how the modification influences the potential environmental risks. As in the previous section, the nucleotide sequence of the region containing the deletion or disruption should be submitted by the applicant, and the possible effects of any deviations must be discussed.

3.2.3 Mutations and point mutation of genes of the parental organism

The intended modification may also consist of one or more point mutations in a target gene. To assess the impact of the modification on the potential environmental risks, the original and new functions of the altered gene must be described.

To verify the mutations in the target gene and the way these have been made in practice, COGEM recommends that an analysis is made of the sequence in the relevant region of the genome of the GMO. As for a deletion, the sequence to be analysed, including any recombinant sequences used, should include the flanking regions unique to the parental organism, which are therefore not present in the transfer vector used for the modification. For this type of modification, too, the nucleotide sequence of the region containing the relevant mutation or mutations should be submitted, and the possible effects of these mutations must be discussed.

3.3 Unintended modifications in the genome of the GMO

During the construction and production of the GMO it is possible that, in addition to the planned genetic modification, naturally occurring processes may lead to unintended alterations to the genetic material. Depending on the type of modification, the fitness of the GMO may be affected, which in turn will also affect the outcome of the environmental risk assessment. COGEM is therefore of the opinion that the molecular characterisation of a GMO must also provide a clear picture of the presence of any unintended modifications. COGEM makes a distinction between two different types of unintended modifications: 1. *the introduction of unintended heterologous DNA sequences*, and 2. *genetic variation relative to the parental organism*. The second type of modification comprises naturally occurring rearrangements, deletions and point mutations. The

way each type of modification can be identified and described and their potential impacts on the environmental risk assessment are discussed below.

3.3.1 Unintended heterologous DNA sequences

When constructing GMOs use is often made of a transfer vector, which consists of the sequence needed to make the intended modification as well as 'backbone DNA'. This backbone DNA contains things like the elements needed for the production of the transfer vector in bacteria, such as an 'origin of replication' (ori) and an antibiotic resistance gene. Depending, among other things, on the way in which the GMO is made, it is possible that all or part of the backbone DNA will be transferred to the genome of the GMO. Moreover, as a result of the production process in *E. coli*, the transfer vector may become contaminated with *E. coli* DNA fragments. It can therefore not be ruled out that the GMO will contain fragments of *E. coli* DNA. In a similar fashion, a GMO may be contaminated if during the process use is made of carrier DNA, such as salmon sperm DNA.

These and similar unintended contaminations can affect the characteristics of the GMO. To determine whether these characteristics should be included in the environmental risk assessment it is important to know whether any sequences have been transferred to the GMO, and if so, which ones and what their functions are in the donor organism.

COGEM therefore argues that the molecular characterisation of a GMO must also show whether any unintended heterologous sequences are present in the GMO. This can be done by analysing the restriction pattern with a number of restriction enzymes and/or several Southern blot analyses, but it is also possible to map the sequence of the entire genome of the GMO and examine it for the presence of additional sequences. The application must include a description of how the alignment with the expected sequence was performed and give the results of this analysis, as well as the possible impact of the additional sequences on the characteristics of the GMO. When assessing the environmental risks in such cases, COGEM is interested in the function and the impact of unintentionally inserted heterologous DNA sequences and not in the sequences themselves, and so the complete sequence does not have to be provided with the information on the molecular characterisation.

3.3.2 Genetic variation in GM viruses

Depending on the type of virus, modifications to the genome occur naturally to a greater or lesser degree, and most point mutations are the result of natural sequence variation. This natural variation is already included in the biological characterisation of a virus species. COGEM is of the opinion that the point mutations that could arise during the construction and production of GM viruses are highly likely to fall within the scope of this natural variation. However, the possibility of a point mutation or a combination of point mutations affecting the characteristics of the GMO cannot be ruled out.

Current scientific knowledge is insufficient to make a reliable prediction of the effect of each point mutation. COGEM therefore argues that mapping point mutations as part of the molecular characterisation will provide no additional useful information for the environmental risk

assessment, while pointing out that the existence of any point mutations that alter relevant characteristics in the GMO will be picked up by the phenotypic characterisation, for example during the extensive pre-clinical research phase, and will thus be included in the environmental risk assessment already.

It is also possible that the genetic variation will affect large parts of the genome as a result of the exchange of genome segments or through recombination. Host cells are used in the construction and production of GM viruses. If these cells contain relevant related viruses or parts of a viral genome, either unintentionally or by design, exchange of viral sequences during the production of the GM virus cannot always be ruled out. Any such exchange would affect the characteristics of the virus and should therefore be included in the environmental risk assessment.

Given the possible influence on the characteristics of the GM virus, COGEM recommends that its genome should be analysed for genetic exchanges, rearrangements and/or deletions. This can be done by carrying out Southern blot analyses with a sufficient resolution to allow identification of any modifications. This can also be achieved by sequencing the genome of the GMO and comparing this sequence with the expected nucleotide sequence of the GM virus. Here too, COGEM considers it relevant to have information on how the alignment was performed, for example in the form of a graphic presentation, and an estimate of the impacts of any modifications. If all these requirements are met, COGEM will not need to have the nucleotide sequence for the environmental risk assessment, and so this will not have to be submitted with the application. COGEM also draws attention to the value of a supportive phenotypic characterisation.

3.3.3 Genetic variation in GM bacteria

Genetic information is exchanged frequently in bacteria. In this process, new DNA is incorporated into the genome of a bacterium and existing DNA is discarded. These processes can also occur during the production of GM bacteria. The production of GM bacteria usually involves the isolation of a pure bacterial culture. In the production facility any form of contact with other bacterial strains is avoided, minimising the chances of new (bacterial) DNA being introduced into the GMO and restricting any modifications to the genomic organisation to removal of parts of the genome or rearrangement of the GMO's own genetic material.

The use of GM bacteria in medical studies has so far been limited to non-pathogenic bacteria or pathogenic bacteria from which the virulence factors have been removed. If the GM bacteria fall into these categories, they will not possess pathogenic characteristics, in which case COGEM considers the likelihood that any rearrangements or deletions will lead to a gain of function to be negligible. COGEM is therefore of the opinion that the environmental risk assessment for this category of GM bacteria requires no further information about any rearrangements or deletions in the entire genome of GM bacteria. A thorough analysis of the genome for deletions and rearrangements is therefore not considered to be necessary for the molecular characterisation of these GM bacteria.

However, use could be made of GM bacteria in which the function of the virulence factors has been altered by a disruption of the corresponding coding sequence, for example by the insertion of a short DNA sequence. COGEM does not rule out the possibility that rearrangements and/or

deletions could reverse such alterations. For medical applications with genetically modified pathogenic bacteria in which the virulence factors have been rendered harmless in this way, COGEM therefore considers it necessary, in addition to a phenotypic characterisation, to carry out a molecular inventory of rearrangements and deletions in the location of the disabled genes.

This can be done with a Southern blot analysis with a sufficient resolution to allow identification of any modifications. The rearrangements or deletions can also be identified by carrying out a sequence analysis of the relevant region. In this case, COGEM considers it sufficient to give the result of the comparison between the sequence obtained and the expected nucleotide sequence and, if applicable, an estimate of the impact of any modifications.

3.4 Conclusion

COGEM considers a thorough molecular characterisation essential for the environmental risk assessment of the deliberate release into the environment of GMOs. This report has concentrated on the most important aspects of this molecular characterisation and how the applicant can obtain and present the relevant information. It should be noted that the suggested methods given in this report are specific for each individual aspect of the molecular characterisation.

COGEM draws attention to the fact that the nucleotide sequence of the whole genome of the GMO can provide answers to satisfy the criteria for all the aspects considered necessary for the molecular characterisation. Given the state of the art of the current sequence analysis techniques, the strength of the evidence and the cost-effective way in which the nucleotide sequence of an organism can now be obtained, COGEM recommends analysis of the nucleotide sequence of the whole genome as the preferred method.

COGEM stresses the importance of comparing the obtained nucleotide sequence with the expected sequence in relation to the possible unintended modifications mentioned above, and the need for a description of the potential impact of any deviations on the characteristics of the GMO. It is not necessary to submit the entire nucleotide sequence with the application; the sequence of the region in which the intended modifications have been made is sufficient. However, the applicant remains free to hand over other parts of the sequence as well if these support certain conclusions.

4. Criteria for the molecular characterisation of yet to be constructed GMOs

A clinical or veterinary study can be carried out with a limited number of GMOs that have already been constructed and characterised. Legally, however, it is also possible for consent to be given for a series of GMOs, of which several variants have yet to be created or characterised. In such cases, the applicant is bound to the GMOs described in the permit and may only use those GMOs whose molecular characterisation satisfies the specifications stated in the permit.

COGEM can also carry out its environmental risk assessment on the basis of a detailed theoretical description of the genome of the GMO. In these cases, of course, each GMO must be characterised before being used in a clinical or veterinary study and the molecular structure of its genome must match the genetic description in the permit. In this case, too, COGEM recommends

that the requirements mentioned above should serve as a reference standard for the molecular characterisation.

The Commission notes that it is essential to be able to assess how the applicant will satisfy the stated requirements. COGEM therefore recommends carrying out the molecular characterisation, in accordance with the criteria, of at least one GMO which is representative of the group of GMOs to be created. The resulting data should be included in the application. COGEM can use these data to assess whether the molecular characterisation of the yet to be created and produced GMOs will satisfy the requirements and provide sufficient information on which to draw conclusions about their genetic composition. If the requirements are met and the GMO is the same as the theoretical description, it can be used in the study.

This means that any deviations from the intended modifications, or any unintended modifications that may arise in practice settings, will not have been included in the environmental risk assessment. The consent does not therefore include the use of a GMO that resembles the described GMO but is not genetically identical to it, and at the very least the permit will have to be amended or another attempt will have to be made to construct the GMO correctly.

If the above requirements are met, COGEM contends that human and environmental safety can still be guaranteed even if a permit is issued for the use of GMOs of which some have not yet been characterised molecularly. In such cases, COGEM will hold the applicant responsible for the correct analysis and assessment of the molecular composition of the other GMOs.

5. Summary

To summarise, COGEM is of the opinion that the molecular characterisation of a GMO to be deliberately released into the environment for a clinical or veterinary study must meet the following criteria:

Identification of the parental organism

- The identity of the parental organism must be established. This can be derived from several specific characteristics in the sequence of the genome of the parental organism.

Characterisation of the intended modification

- If sequences are inserted into the genome of a microorganism, the elements to be inserted into the GMO must be known, as well as the number of copies of the insertion cassette and the exact location and function of those elements.
- If one or more genes in the genome of the microorganism are removed or the ORF of these genes is interrupted, the elements that are no longer expressed, the exact location of the modification and the function of these elements must all be known.
- If the modification consists of one or more point mutations, the exact mutations to be introduced need to be known and both the original and the new characteristics of the gene involved must be given.
- The above-mentioned modifications must be fully characterised by means of a sequence determination. This sequence determination must cover the region of the intended

modification, including any recombination sites used, and must extend across the flanking sequences of the genome of the parental organism.
- If the GMOs used are not biologically contained, as an extra safety measure the fusion ORFs must be mapped and, where possible, their functions described.

Characterisation of unintended modifications in the genome of the GMO

- The GMO must be analysed for the presence of any unintended heterologous sequences. This can be done by determining the restriction pattern or by carrying out a full Southern blot analysis or a sequence analysis of the entire genome of the GMO.
- The genomes of GM viruses should be tested for deletions and/or rearrangements and the impact of unexpected modifications must be explained. The genomic organisation can be sufficiently described by carrying out a number of Southern blot analyses or by comparing the theoretically expected nucleotide sequence of the GMO with the sequence obtained in practice.
- As for GM viruses, the genomes of GM bacteria should be tested for deletions and/or rearrangements and the impact of any unexpected modifications must be explained. However, this is not necessary for GM bacteria based on non-pathogenic bacterial strains or pathogenic bacterial strains from which the coding sequences for the virulence factors have been removed.

For applications that also include GMOs that have not yet been constructed or produced, COGEM makes the following additional requirements.

- The application must include a molecular characterisation that satisfies the above criteria of at least one GMO representative of the group of GMOs to be created.
- The way in which the other GMOs will be constructed must be described in detail.
- The exact genetic organisation or molecular blueprint of the GMOs yet to be constructed must be supplied.
- The application must contain a thorough description of the methods to be used for the molecular characterisation of the GMO, including the technical specifications, sensitivity and robustness of the methods.
- The specifications the GMO must meet in these tests must be clearly described.
- The methods and specifications must be the same as those used for the molecular characterisation of the representative GMO mentioned above.

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