Opinion of the Belgian NRL-GMO on the consequences of ECJ ruling of 25 of July on enforcement and more specifically on the analytical tools needed for this

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Terms of reference : the following questions have been formulated by the FOD Public Health and send to stakeholders involved, one of them being the NRL GMO.

> *« Following the European Court of Justice's ruling on mutagenesis, the Member States informed the Commission that the application of the GM framework to NBT products posed problems.*
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> *The Commission requests Member States to provide specific information in order to possibly organize a working group and a joint meeting of the Contained use, Deliberate release and GM Food Feed committees.*
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> *- Difficulties (including the impact on resources) encountered during inspections and / or for analytical methods of detection during official controls;*
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> *- Experience feedback in confined use;*
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> *- Examples of specific products or situations for which the application of the GMO legislation (as clarified by the judgment of the European Court of Justice of 25 July) is problematic;*
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> *- Specific products (patented?) Already available in third countries;*
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> *- Ongoing research and needs related to NBT;*
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> *- Feedback on the uses of products using these new techniques;*
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> *The Belgian responsible authority could seize the opportunity to show COM the problems we also encounter in cases where the GMO is not obtained via an NBT. For example, discrepancies between legislations contained use / deliberate release / medicinal products / enzymes etc .... »*

The Belgian NRL GMO (CRA-W, Sciensano and ILVO) formulated the following opinions and answers on the topics as described above.

In the first part we address the following questions :

1. Difficulties (including the impact on resources for analytical methods of detection during official controls)
2. Examples of specific products or situations for which the application of the GMO legislation (as clarified by the judgment of the European Court of Justice of 25 July) is problematic;
3. Ongoing research and needs related to New Breeding Techniques (NBT)
4. Problems we also encounter in cases where the GMO is not obtained via an NBT. For example, discrepancies between legislations contained use / deliberate release / medicinal products / enzymes etc.....

As the type of products being the results of the use of Site Directed Nuclease mutagenesis type 1 (SDN), from the genetic/molecular point of view are very divers, we will describe only the most simple case and its impact on the activities of the official testing and the work of the National Reference Laboratories (i.e. the GMO analysis in the context of enforcement). This means that we will focus on addressing the question to products that are obtained by an SDN technology and resulting in one mutation in a gene in one locus of the genome. In this case we consider a small indel (basepair change, small insertion or small deletion), being the result of NHEJ repair.

Further in the document we describe other types of changes in the genome being as well the result of NHEJ, but are from the analytical point of view at least more complex, such as mutation in all genes of a gene family at different loci in the genome. In such case also more fundamental questions need to be addressed : e.g. what is the «event» in this case. Are multiple mutations in different loci to be dealt with in a similar way as stacked events, as we know them today in the context of the "conventional GMO's"?

1. **Difficulties (including the impact on resources for analytical methods of detection during official controls)**

Detection of GMO is currently based on the amplification of GMO specific DNA fragments. These fragments can be either fragments for screening such as fragments of promotors, terminators that are often used for the development of GMO or DNA fragments that comprise the junction of the inserted fragment and the locus where the insertion took place (event-specific detection). Typically fragments of a length between 100 to 200 bp are amplified in the PCR. PCR methods can be either qualitative or quantitative. An integrated approach of different steps in the analysis is used, which is in line with the legislation (starting with a screening, followed by identification and quantification). The quantitative result has an output in % GMO per species.

The official control laboratories are accredited, according to ISO17025, for the overall procedure and the individual analysis.

For the detection of mutants the currently three step approach will not be possible to be used for several reasons:

- We will need dedicated methods for each individual mutant, because there are no general screening elements in this case.
- Secondly the currently applied PCR methods will not be suitable, as one needs to identify only a one base pair difference.
- Thirdly in the current approach we make use of endogenous gene to determine the quantity. It needs to be evaluated whether this approach will work for the mutants. Of course alternatives could be thought on, but need to be developed. One could potentially find inspiration how this mutant detection is done in the clinical context. However, one needs to realize that the analysis is less complex as the sample is only coming from one individual. In the case of plants or food/feed samples the situation will be much more complicated. Products will at least contain more than one individual of a particular species, but most likely even more species. This means that specificity of the locus to be analyzed needs to be studied in detail in a broad range of species that are likely to be present in the product to be analyzed.
- Finally, if a method allows to detect a mutant, it cannot be excluded that exactly the same mutant already exists is nature or is obtained by applying mutagenesis tools of which the products are excluded from the application of the legislation. Of course, if other type of information (traceability) is available the legal value could be stronger in this case.

The current approach to monitor the presence of non-authorized GMO is not applicable for the detection of non-authorized SDN mutants, such as CRISPR-Cas9 mutant, as they will not contain common elements that can be tested for in a screening approach. This means that a direct detection method needs to be developed, which is impossible if no information is available on what to look for (see next question)

2. **<u>Question 2: Examples of specific products or situations for which the application of the GMO legislation (as clarified by the judgment of the European Court of Justice of 25 July) is problematic</u>**

Enforcement in function of the detection of unauthorized SDN mutants will be highly problematic or even impossible. First, because products that are developed and commercialized outside Europe are developed and commercialized in a different legal context. As a consequence, in the most cases molecular information on the mutation (genetic changes) will not be available. Secondly, because this SDN technology (e.g. CRISPR-Cas9) is rather easy to apply and as a consequence one can expect a fast growing number of plant varieties developed by applying it. Moreover the application of the technology will not be restricted to the major commodity crops, but is applicable in all plant species. For enforcement, this means that more plant species need to be monitored.

There is also a scientific and technical issue, which make detection of unknown mutants impossible even in the simplest case where the target of the monitoring is a pure variety. To demonstrate the dimension of the complexity, we consider a variety in which all plants have the same genotype (e.g. hybrid maize).

A maize genome consist of approximately 2,3 Giga base pairs. The question will be : Where and how to look for a one base pair change? But if one wants to demonstrate a change, being the result of a mutagenesis approach (in this case SDN) one need to be able to compare the “suspect” locus and the mutation in that locus, to a reference sequence. What will be the reference sequence , knowing that genomes in plants and all organisms are dynamic? Genome changes occur naturally, as they are the basis of variation, essential in the process of survival of the fittest. In other words a universal comparator for a species does not exist.

As stated above an analysis of a pure hybrid or a vegetative propagated crop would be the most simple case. Of course in real live most products, and especially commodities, will consist of a mixture of varieties. This means that one can expect a lot of polymorphisms between these varieties and as consequence “dilution” of the signal coming from the mutant, resulted from applying the SDN technology. Testing for the presence of this kind of mutants in real life food and feed end products will be completely impossible because the background noise signal.

In summary detection of unknown mutants is not possible today for several reasons:

1. A universal comparator genome does not exist. This means that, if differences between genomes (maize 2,3 Giga base pairs) are found, it is impossible to identify a candidate that could be the result of SDN.
2. In theory, it would be possible to demonstrate the presence of a mutation in a genome that is not existing in a wide range of varieties. This could be an indication that this mutant is obtained by SDN mutagenesis. However, not a proof, because the owner, responsible for the development of the product can always claim, that this genotype has been found in nature or is obtained by classical mutagenesis. One can argue that this indication can be sufficient to start a court case, but this would result in a long procedure, which is far away from the time that is currently given to the laboratories (2 weeks) to report result allowing the enforcement body to take legal and undisputable actions.

3. In contrast with the detection of transgenic plants, the detection of a mutant does not give any information on the process that has been used to obtain this mutant. In transgenic plants the junction fragment is not only identifying the genotype, but contains us such also a direct proof that the process of transgenesis that has been used to obtain this genotype. (statistically the occurrence of the inserted fragment, joint to that specific plant sequence is in nature not existing). It can only be explained by the fact that it is obtained via gene transfer (Agrobacterium mediated or direct gene transfer)

Based on literature and reports, in general, it is expected that first products developed outside Europe will soon be ready for commercialization. At this moment, the EU member states are not in a position to enforce the implementation of the legislation as it is interpreted by the ECJ.

3. Question 3: Ongoing research and needs related to NBT

A lot of research is going on to improve detection methods such as droplet digital PCR, which might be more suitable for detection of known mutants. This could be a potential solution in the case a mutant is authorized within Europe and information on the mutant is available.

There is also enormous progress in the domain of DNA sequencing e.g. Next Generation Sequencing and linked to this the bioinformatics tools to interpret the obtained data. By doing this there is in the long run, a shift possible from dedicated searching for a particular target towards collecting large datasets and looking for correlations between parameters within these datasets. But this will not bring solutions for the enforcement laboratories at this moment. The major bottlenecks will always be: 1/ What will be the reference to compare with and 2/ finding a mutant is not a proof that SDN approach has been the process used to obtain this mutant

4. Question 4: Problems we also encounter in cases where the GMO is not obtained via an NBT. For example, discrepancies between legislations contained use / deliberate release / medicinal products / enzymes etc ....

In the context of enforcement EC/2001/18 and EC/1829/2003, the analytical tools that we have available are also not 100% perfect. For instance, the indirect approach used to detect unknown GMO is limited in a sense that when the presence of screening element is explained by the presence of authorized GMO, the analytical procedure is stopped. This means that even if in that sample an non-authorized and unknown GMO is present, it will be "masked" by the authorized GM events and as a consequence no further analysis to search for non-authorized GMO is "triggered".

The second case is the situation of stacked events. In reality it is impossible in a food/feed sample to distinguish the situation where the product contain the two single events, only the stacked event or the stacked and the single events. On the basis of the quantitative data in some specific cases indications that suggest a certain composition could be observed, but these data will highly probable not stand in front of a court case.

General Comment: Data collection and data management

New analytical tools are currently available and/or are under development. They open new prospective. However, it will always be necessary to make a scientific evaluation, whether they will able to generate data that are useful to answer the specific question, addressed (in this case do they allow to detect a mutant obtained via SDN). High Resolution Mass Spectrometry and Next Generation Sequencing (NGS) are examples of these new tools.

It should be realized that these methods generate a huge number of data that need to be interpreted. Currently, also in this domain a lot of progress is being done. This makes possible to evolve from a

targeted analytical approach towards looking for correlations within and between datasets and in this way to identify potential problems in the food chain. There is a huge potential for exploring these possibilities and they may be integrated in concepts such as data sharing and block chain.

This new methods may lead to new strategies in food safety control. This will need investment in equipment and expertise. Case by case, we will have to evaluate the possibilities whether this new technologies will also effectively may lead to solving the problems. In the case of detection of mutants obtained via SDN, these tools will not allow to prove whether a mutant is the result of the use of SDN or not. This fact is not due to the limitations of the technology as such, but are explained by the nature of this difference. Mutations as such do not differ on the basis of the way they are obtained. However, finding the mutant as such may be of importance to trigger further investigation.

NGS analysis will need to be combined with bioinformatics analysis, data searching, database comparisons. In order to implement these new methods and strategies in the context of enforcement, substantial investments in infrastructure and expertise will need to be made.