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Detection of microorganisms, obtained by new genomic techniques, in food and feed products

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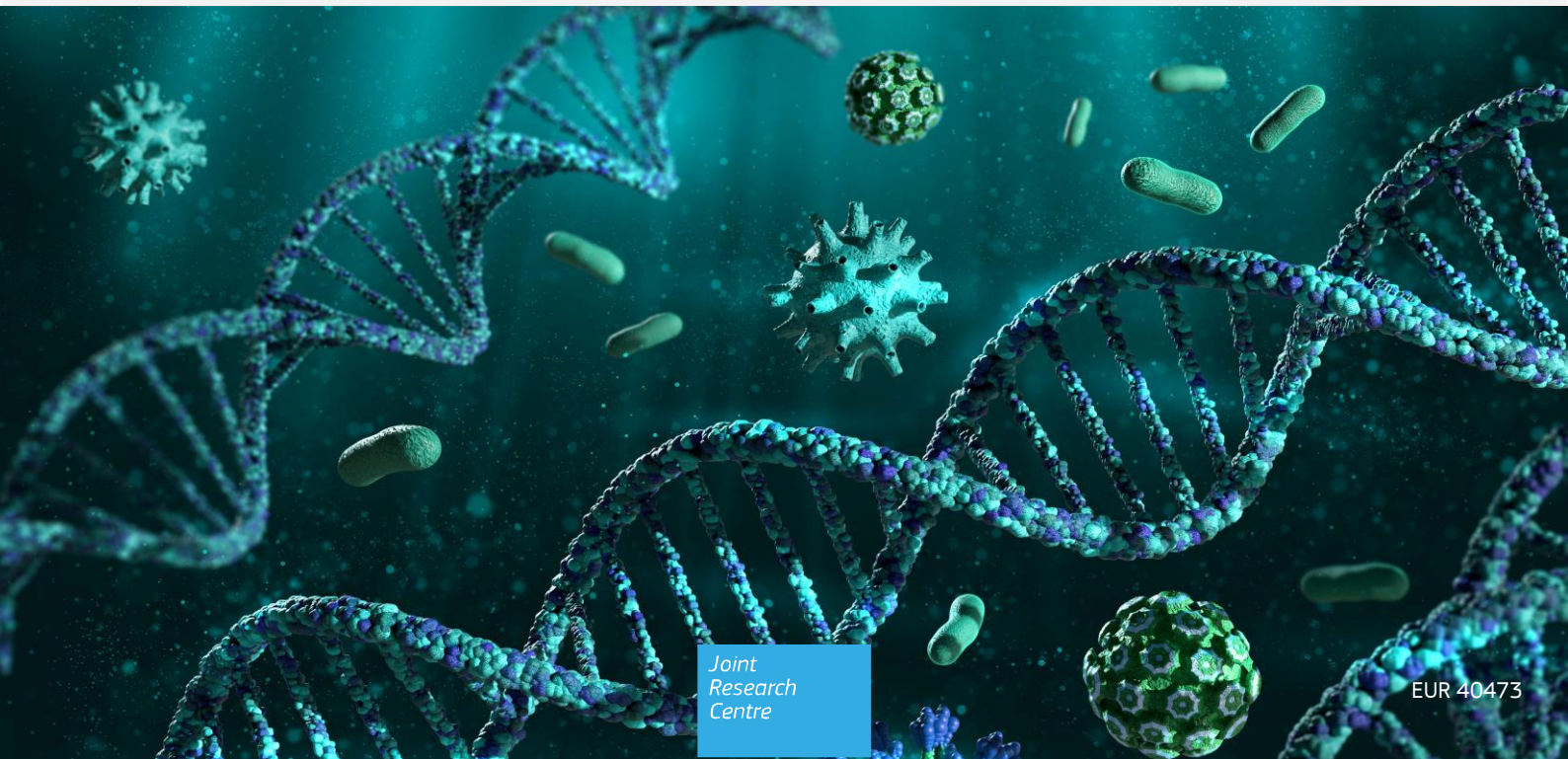

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Contents

Abstract

New genomic techniques (NGTs) can be used for the targeted modification of the genome, not only of plants but also of microorganisms. Traceability of genetically modified microorganisms obtained with an NGT (NGT microorganisms) is currently a regulatory requirement under the EU GMO legislation. This report highlights the possibilities and challenges to detect NGT microorganisms (NGT-Ms) in food and feed using analytical technologies. The document builds upon the conclusions of the previously published report on the detection of plant products developed by targeted mutagenesis and cisgenesis (ENGL, 2023a) and adds issues specific for the detection of NGT-Ms.

Authors

The European Network of GMO Laboratories (ENGL) Working Group (WG) on New Mutagenesis Techniques was established based on a mandate adopted at the 43rd meeting of the ENGL Steering Committee on 09 June 2021. The WG accepted an extension of its mandate in 2023 to develop a report on the possibilities and challenges for the detection of microorganisms obtained by an NGT in or as food and feed.

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Executive summary

This report, compiled by the European Network of GMO Laboratories (ENGL), investigates the analytical possibilities and challenges related to the detection of microorganisms modified using new genomic techniques (NGT-Ms). Extending the previous report on the opportunities and challenges for the detection of plant-based food and feed resulting from targeted mutagenesis and cisgenesis (ENGL, 2023a), this report focuses on NGT-Ms, excluding those with insertion of foreign recombinant DNA.

New genomic techniques, such as CRISPR-Cas and other techniques of genome editing, have become instrumental in the modification of microbial strains for use in food and feed. Microorganisms obtained through NGTs are also used in other sectors, including enzyme production, probiotics, biofertilizers with many products being developed outside the EU. These microorganisms are subject to traceability and labelling requirements under the current EU GMO legislation. This implies the necessity for accurate and reliable detection methods. However, placing on the market, traceability and labelling of GM food and feed within the EU requires not only precise detection but also analytical identification and quantification methods.

The report classifies NGT-M products into four categories and focusses on Categories 3 and 4, characterised by residual DNA or viable organisms which can theoretically be monitored using DNA-based detection techniques. Nevertheless, detecting NGT-Ms poses considerable analytical challenges. Certain modifications made by NGTs — such as single nucleotide variants (SNVs) or small insertions/deletions — are indistinguishable from natural mutations or those resulting from conventional mutagenesis techniques. This makes it difficult to trace the origins of genetic modifications and to ensure compliance with current GMO legislation.

Polymerase Chain Reaction (PCR)-based approaches, including digital PCR (dPCR), continue to play a key role in regulatory testing by official control laboratories, although they may struggle to identify very small genetic changes with required precision. High-throughput sequencing (HTS) methods, such as whole genome sequencing (WGS), provide higher resolution and can accurately determine specific strains or different genetic modifications, especially in single-ingredient microbial samples. Nevertheless, HTS still faces obstacles in routine testing of complex food or feed mixtures and in accurate quantification, due to technical limitations and problems with data interpretation. Additional challenges relate to the ability of microorganisms to exchange DNA material through horizontal gene transfer, coupled to their natural genomic mutability. In the absence of reference databases recording such genetic diversity, differentiating between natural and engineered mutations becomes highly difficult.

Overall, the authors acknowledge the technological advancement offered by targeted (*e.g.*, PCR) and untargeted (*e.g.*, sequencing) methods to detect uniquely modified NGT-Ms. With the growing use of NGTs, forthcoming traceability will require further developments in sequencing detection techniques, flanked by comprehensive databases and supported by standardised approaches. However, in alignment with the conclusions drawn on NGT plants (ENGL, 2023a), this report highlights the inability of these methods to detect and unambiguously identify unknown or small mutations in microorganisms, especially in the context of routine laboratory control.

From a regulatory perspective, persistent research gaps regarding the minimum performance parameters of detection and identification methods in both plants and microorganisms pose challenges for the consistent evaluation and oversight of NGT-derived products. These gaps may

affect the ability to establish harmonised standards for method validation, comparability, and traceability, which are essential for regulatory compliance.

Scope

In 2023, the ENGL revised its previous report (ENGL, 2019) on the possibilities and challenges for the detection of food and feed plant products obtained by targeted mutagenesis and cisgenesis (ENGL, 2023a). Similar techniques, and NGTs in general, are also used for the targeted modification of microorganisms.

The current report focusses on the possibilities and challenges for the detection of microorganisms obtained through NGTs (NGT-Ms) in or as food and feed. The major aim is to evaluate to what extent the conclusions of the corresponding report on plant products could be extended to the detection of NGT-Ms. The presence of an NGT-M could result either from the intentional inclusion of the NGT-M, or the product it produced, as an authorised ingredient in the food or feed or from the unintended presence of an (un)authorised NGT-M used as processing aid during the production of a food or feed additive. In line with the mandate of the WG, the focus of this report is on NGT-Ms that do not contain inserted recombinant foreign DNA.

1 Introduction

There are many conventional techniques for the genetic modification of microorganisms with a long history of use (basic microbial genetics). Unlike in plants, homologous recombination has been widely used in microorganisms for the targeted introduction of new genes or the replacement of endogenous genes with copies from a related organism. To facilitate such genetic modifications, microorganisms have historically been manipulated using mechanisms such as conjugative plasmid transfer and cell fusion, which allow the introduction of foreign genetic material into the host (Shitut *et al.*, 2022; Vasileva *et al.*, 2022). Newer techniques, including genome editing, create additional opportunities, not only for producing small mutations and knockouts, but also for inserting longer DNA fragments by homologous recombination or transposition. Such new genomic techniques (NGTs) have become standard tools for microbial strain modification along with established techniques including conventional mutagenesis and recombinant DNA technology (Broothaerts *et al.*, 2021). In some cases, the analytical differentiation between genetically modified microorganisms (GMMs) produced by established or new genomic techniques may be challenging or impossible, and the occurrence of natural mutations and horizontal gene transfer (HGT) may add to the complexity of analytical detection and identification. It needs to be stressed that insertion of foreign DNA with the use of NGTs would lead to the generation of conventional GMMs, for which event-specific methods can usually be developed for enforcement analysis. However, because of asexual reproduction and HGT, *i.e.* the inheritance of genetic material by transfer between different species without reproduction, the term foreign DNA is more difficult to define in microorganisms compared to plants1. For instance, bacteria can take up DNA from the environment (transformation), through direct cell-to-cell contact (conjugation) or through the involvement of bacteriophages mediating gene transfer between donor and recipient cells (transduction) (Arnold *et al.*, 2021). HGT is not limited to bacteria but also occurs in other microorganisms and is considered a fundamental evolutionary molecular mechanism in microbial genetics (Li *et al.*, 2023; Del Duca *et al.*, 2022). Therefore, identifying whether the origin of a specific gene in a microbial species was the result of a natural process or was induced by an NGT can be challenging.

The terminology used in this document is compliant with the terminology used in the report on NGT plants (ENGL, 2019; ENGL, 2023a).

In 2011 EFSA published a guidance document on the risk assessment of GMMs and their products intended for food and feed use (EFSA GMO Panel, 2011). Depending on the type of product and the degree of purification, GMM-derived products were classified according to four categories. The same classification could be applied to NGT-Ms.

Category 1: Chemically defined purified compounds and their mixtures in which both NGT-Ms and their DNA have been removed (*e.g.*, amino acids, vitamins);

Category 2: Complex products in which both NGT-Ms and their DNA are no longer present (*e.g.*, cell extracts, most enzyme preparations);

[1] In the Commission's NGT proposal, *foreign DNA* is defined as "DNA produced by whatever means outside a recipient organism from a donor organism that is sexually incompatible (non-crossable)"

Category 3: Products derived from NGT-Ms in which NGT-Ms capable of multiplication or of transferring genes are not present, but in which their DNA is still present (*e.g.*, heat-inactivated starter cultures, biomasses single-cell protein preparations, and cell extracts);

Category 4: Products consisting of or containing NGT-Ms capable of multiplication or of transferring DNA (*e.g.*, alive starter cultures for fermented foods and feed).

These categories are suitable for differentiating NGT-Ms-derived products for the purpose of conducting the risk assessment required for authorisation of GM food and feed in the EU. From the perspective of GMO traceability and official control, DNA-based analytical methods are mainly applicable to products from Category 3 and Category 4 NGT-Ms as they may contain DNA from NGT-Ms. These methods can also be applied to verify if living microorganisms and their DNA have been removed from Category 1 and Category 2 products. Living NGT-Ms present in Category 4 products may also be detected through *in vitro* culture techniques on suitable media, provided that they harbour detectable phenotypic characteristics. Growing NGT-Ms may then be identified through a variety of techniques, including DNA genotyping by PCR or sequencing. However, this is beyond the scope of the current report, which focusses on identification of the NGT-induced modifications rather than the type of microorganism.

2 Genetic modification techniques applied to microorganisms

Microorganisms have been utilised for food and feed production for thousands of years, long before their role in these processes was fully understood. Traditional products such as bread, beer, wine, vinegar, yogurt, and cheese were developed without knowledge of the microorganisms driving the fermentation. For decades, bacteria, filamentous fungi and yeasts have been harnessed for the production of food and feed components, including additives, enzymes, and other valuable biomolecules such as vitamins and amino acids, and in nature, microbes also play a crucial role in biomass recycling.

Microorganisms evolve naturally through both mutations and horizontal gene transfer, where genetic material is exchanged through mechanisms such as transformation - the uptake of DNA from the environment (Dalia *et al.*, 2014). This DNA may integrate into the recipient's genome or persist as an independent plasmid, contributing to genetic diversity. Advancements in microbial research have enabled the cultivation of pure strains for breeding and mutagenesis to enhance desirable production traits. Species used in industrial production often undergo rounds of spontaneous or induced mutagenesis followed by selection and may be further bred or genetically engineered to optimise production yield.

Before the advent of recombinant DNA technology (discussed in the upcoming ENGL GMM report) and NGTs, microbial strain improvement relied on spontaneous random mutagenesis, directed evolution under selective pressure, or exposure to mutagens such as chemicals and ionizing radiations to increase mutation rates. These early methods laid the foundation for modern genetic engineering approaches, which offer more precise and efficient microbial strain development strategies.

In recent years, genome editing tools have been widely applied in microorganisms (for a recent review, see Lv *et al.*, 2023). Early techniques such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) required complex customisation, whereas techniques based on clustered regularly interspaced short palindromic repeats (CRISPR), particularly CRISPR-Cas9, offer a simpler and more efficient approach (Yang *et al.*, 2025). CRISPR-Cas9 enables precise DNA modifications via double-stranded breaks (DSBs), repaired through non-homologous end joining or homologous recombination.

New methods of targeted mutagenesis are being continuously developed *e.g.*, base-editing tools that allow targeted mutations without DSBs or prime editing that enables all possible nucleotide conversions along with targeted insertions and deletions with higher accuracy. These tools have been established in industrially relevant fungi such as Saccharomyces cerevisiae (Lv *et al.*, 2023), *Yarrowia lipolytica* (Bae *et al.*, 2019), and Aspergillus species (Huang *et al.*, 2019). Multiple approaches were also developed for the introduction of the CRISPR-Cas9 tools into the cells of an organism of interest, based on stable DNA integration systems, or transient plasmid expression vectors or ribonucleoprotein complexes. Genome editing in microorganisms enables gene insertions, deletions, and multiplex modifications, for instance to enhance biomolecule production. Furthermore, unlike plants, many microorganisms can be efficiently edited without the use of methods classified as NGTs (*e.g.* using homologous recombination, transposon mutagenesis, etc.), though CRISPR remains valuable for engineering certain strains.

Overall, the genetic modifications found in NGT-Ms are similar to those in plants obtained by targeted mutagenesis and cisgenesis, and the analytical challenges, therefore, will also be similar as well. A key challenge is detecting unauthorised genome-edited microorganisms, particularly

those with small, marker-free nucleotide changes, as these modifications are indistinguishable from natural mutations, complicating PCR-or sequencing-based detection methods.

3 Applications of NGTs in microorganisms

In September 2023, EFSA published a horizon scanning report (Ballester *et al.*, 2023), which provided an overview of microorganisms and their products obtained by NGTs. In 2024, this report was instrumental for publication of a scientific opinion by EFSA on the risk assessment of NGT-Ms and their products under Category 3 and 4 when these are placed on the market as or in food or feed or in the environment (EFSA, 2024).

This horizon scanning report provided a systematic review based on key terms used in search engines and other online resources. Selection criteria included use of genomic techniques developed after 2001, Category 3 products derived from GMMs, Category 4 products consisting of or containing GMMs and usage for release into the environment or placing on the market as or in food and feed. In total, over 17,000 references were identified, of which only 35 cases met the selection criteria above.

The majority of NGT-Ms were developed or were commercialised in China and in the USA. These applications involved CRISPR-based methods, sometimes in combination with homologous recombination. The main purpose of these techniques is to knock out genes encoding non-desirable characteristics or to introduce genes encoding desirable traits. However, newer techniques, based on such site-directed nucleases (SDN), were also used for introducing targeted mutations in microorganisms.

Data from the report shows that the only CRISPR-Cas based SDN-1 mediated modification (introduction of a small mutation at a targeted location) was associated with a microalgae case. Of the remaining cases, approximately 50 % exhibited introduction of foreign genetic material (classified by the report as SDN-3 type changes), and 25 % exhibited introduction of small changes using a DNA template (classified by the report as SDN-2 type changes). The remaining 25 % of cases were predominantly associated with plasmid-mediated (Cas9) homologous recombination in yeast and were classified by the report as combinations of SDN-2 and/or SDN-3 type changes (no further information was provided on this grouping). Other NGTs, including phage-based approaches, oligonucleotide-directed mutagenesis, meganucleases, ZFNs and TALENS, were not identified as part of the study.

From the 35 NGT-Ms, 22 of these were yeast (Saccharomyces cerevisiae), 11 were bacteria, and one each of a fungal endophyte and microalgae. Fifteen cases were classified as Category 3 and 20 cases as Category 4 GMMs. Cited usage of the NGT-Ms were mainly as (or as a source of) food or food additives (30 cases) but also for (or as a source of) feed or feed additives (3 cases) or for use in agriculture as bacterial biofertilizer (2 cases). Products were either commercialised (8 cases), in pre-commercialisation status (9 cases) or under development (18 cases). Six of the cases had patents granted, whilst five others were undergoing a patent application process. We can expect that with the technological and scientific progress the number of NGT-Ms on the global market will increase in the near future.

Unlike most NGT plants or animals, NGT-Ms may also be used as cellular factories to produce certain compounds. The end products are generally purified to remove the microorganism and the residual DNA as much as technically possible. However, this is not always the case. For instance, probiotics and brewing starter cultures may intentionally contain viable or unviable cells and traces of residual DNA may be detectable in certain end products.

4 Potential detection strategies

It must be emphasised that analytical methods based on DNA detection are designed for the monitoring of Category 3 and Category 4 NGT-Ms. Such methods can, however, also be used to examine whether Category 1 and Category 2 products do not contain detectable DNA remaining from the NGT-Ms. This is similar to plant products that should not contain DNA, *e.g.*, sugar or oil extracted from NGT plants.

The challenges to detect and identify the NGT-Ms are similar to the challenges that were described for NGT plants. The term NGTs is an umbrella term to describe a variety of techniques that can alter the genetic material of a microorganism. As in the case of plants, applying genome editing, including targeted mutagenesis and cisgenesis, to modify microbial genomes can result in single nucleotide variants (SNVs), sequence substitutions, insertions or deletions (InDels) of various sizes and insertion of partial or whole genes from other microorganisms. Similar to NGT plants, if the same genomic modifications may also result from natural processes or conventional mutagenesis, identification of an NGT-M as the result of the application of an NGT may not be possible (Broothaerts *et al.*, 2021; Guertler *et al.*, 2023).

In general, approaches for the detection and identification of NGT-Ms may be based on PCR or high-throughput sequencing (HTS) or a combination of both.

PCR-based methods

Like for NGT plants (ENGL, 2019), the use of PCR-based methods, including real-time PCR and dPCR is relevant at the technical level to target the genetic modifications introduced by NGTs in microorganisms. Such techniques are widely used and mastered by the EU GMO enforcement laboratories. The development and validation workflow of such PCR-based methods to comply with the minimum performance requirements (MPR) for methods of GMO testing in the EU is harmonised and described in guidance documents (ENGL, 2015, 2023b).

The use of such PCR-based methods is expected to be satisfactory for the detection and identification of NGT-Ms containing sufficiently long and unique genetic modifications (ENGL, 2019). However, in case of NGT-Ms with SNVs or short mutations of a few nucleotides, the analytical methods may not always meet the requirements of the current MPR guidance (ENGL, 2015, 2023b) in terms of specificity and sensitivity. Additionally, in case the genetic modifications of interest are either small, arose spontaneously, or resulted from conventional mutagenesis techniques, their detection does not necessarily represent proof that they were introduced by an NGT. Consequently, such PCR-based detection methods cannot always be used to unambiguously identify a particular NGT-M (ENGL, 2019).

PCR-based methods may be applied as first-line screening tools, complemented with further investigations using HTS in case of positive samples. Screening in this context may target species-specific markers for bacteria, yeasts or microalgae (Deckers *et al.*, 2020a, b) and common screening elements like antimicrobial resistance (AMR) genes (Berbers *et al.*, 2020). AMR genes can be used for selection during the development of the GMMs, but nowadays companies mostly use alternative selection approaches. We must, however, note that AMR genes can occur naturally in bacteria found in food and feed products (Ojha *et al.*, 2023), as well as in the plasmids they harbour (Florez *et al.*, 2021) since the AMR genes originate from bacteria. For detection of potential NGT-induced modifications, unique PCR screening methods would, therefore, usually need to be applied for each

potential genetic modification. This will make such screening approaches for routine practices both time- and resource-intensive in view of the increasing number of genetic targets.

Sequencing-based methods

Although their use is less widespread than PCR-based methods among the EU GMO enforcement laboratories, the interest for sequencing-based methods, such as HTS, in the GMO detection field is growing (Fraiture *et al.*, 2017, 2019). The first considerations for quality criteria to assess sequencing data have been described in ENGL guidance documents (De Keersmaecker *et al.*, 2025; ISO 20397-2:2021). HTS represents an attractive option for identifying a specific NGT-M through whole genome sequencing (WGS) and simultaneous detection of several key targets composing a unique genetic fingerprint associated to the specific NGT-M. Unlike for plants and animals, the WGS approach could be applied for microorganisms with a small genome size, such as viruses, bacteria and some fungi like yeast (genome size ~ 12 Mb) (Collins *et al.*, 2021, Table 1). WGS is already commonly used on bacterial and viral genomes in the context of outbreak source-tracking, allowing specific identification of the line responsible for an outbreak and its origin (Barretto *et al.*, 2021; Nouws *et al.*, 2021; Vashisht *et al.*, 2023; Berggreen *et al.*, 2023). Similar WGS analysis and subsequent bioinformatics approaches may be applied for the identification of known and characterised NGT-Ms and may allow recognition of a specific line in which the NGT modifications were introduced.

At the present time, the use of sequencing-based methods is still limited to pure products consisting of isolated lines on which WGS can be performed. Application of HTS to identify NGT-Ms is currently not feasible for food or feed samples composed of different species. Indeed, despite promising metagenomic studies, the analysis of mixed samples containing several microorganisms is still very complex, especially if the mixture is composed of different closely related species (Buytaers *et al.*, 2020; Ahlinder *et al.*, 2022; Imanian *et al.*, 2022; Delikanli-Kiyak *et al.*, 2025; Xiao *et al.*, 2025).

Table 1. Genome size ranges for different organisms

Type of organism	Genome size ranges	Reference
Virus	~ 2 kb to ~ 2 Mb	Chaitanya (2019)
Bacterium	~ 100 kb to ~ 15 Mb	Ochman and Caro-Quintero (2016)
Fungus	~ 8 Mb to ~ 180 Mb	Mohanta and Bae (2015)
Animal	~ 20 Mb to ~ 130 Gb	Gregory (2005)
Plant	~ 60 Mb to ~ 150 Gb	Pellicer *et al.* (2018); Cranage (2022)

Source: ENGL

For screening purposes, targeted sequencing methods or enrichment procedures based on hybridisation capture could potentially be applied to detect many NGT-Ms with known modifications. Repeatability and transferability of sequencing-based methods has been demonstrated for an oilseed rape feed product spiked with genome-edited oilseed rape (Pallarz *et al.*, 2023). However, research gaps persist regarding the minimum performance parameters of such methods in both plants and microorganisms, particularly in terms of their reliability, sensitivity, and applicability across diverse contexts.

While quantification is not necessary for unauthorised NGT-Ms, the current sequencing-based methods, including those based on HTS, are not suitable for quantification of (authorised) NGT-Ms in food and feed. Most of the currently used HTS-based methods include a PCR step which has a direct impact on the quantification of the target sequences (Arulandhu *et al.*, 2018; Debode *et al.*,

2019, Fraiture *et al.*, 2023). Therefore, it is expected that sequencing-based methods may be used for detection, but not for the quantification of NGT-Ms in or as food or feed in the near future.

For PCR and sequencing-based methods, the above considerations are applicable only in case the NGT-Ms and the NGT-induced genetic modifications are known. Given the natural mutation rate in microorganisms, mutations that are detected in a microorganism cannot necessarily be explained as derived from application of an NGT. In addition, the whole genome sequence of the conventional microorganism should be available as reference genome during bioinformatics analysis (Lopera *et al.*, 2023). If the NGT-M line is unknown, the same challenges for detection and identification exist as for unknown NGT plants.

5 Challenges and additional considerations associated with detection of NGT microorganisms

Because analytical methods in the field of GMO analysis are based on detection, identification and quantification of specific target DNA sequences, similar challenges will apply for NGT plants and NGT-Ms. In case of NGT-Ms with SNVs and short InDels, the required specificity and sensitivity of the detection, identification and/or quantification method may not be achieved in every case. Despite individual successes, as described in the previous chapter, several challenges limit the general applicability of analytical methods targeting short mutations for enforcement purposes.

Similar to NGT plants, the mutation size highly restricts the flexibility (in terms of positioning the primers and probe along the genetic sequence) needed to design a suitable PCR method targeting mutations in NGT-Ms. The design of a PCR-based method to distinguish sequences that differ by one or a few nucleotides from a natural sequence may not in all cases be sufficient to deliver the required specificity of the method (Shillito *et al.*, 2021; Hommelsheim *et al.*, 2014). In addition, while the specificity of a PCR method can be increased by Locked Nucleic Acid modified primers or Minor Groove Binder probes, this does not resolve this problem in case the mutation occurs in repetitive DNA or in DNA made up mostly of certain bases (*e.g.* either G and C or A and T bases). Furthermore, the development of a fit for purpose detection method would require prior knowledge of the mutations to detect.

Whilst NGT-Ms present similar detection challenges compared to both NGT plants and conventional GMMs, these challenges are further amplified in NGT-Ms. Some of these challenges, applicable also to conventional GMMs, are linked to their higher propensity to mutate compared to higher organisms. Indeed, due to microbes' fast evolving rate, pan-genomes, representing the genetic variation in the genome found within a species (Medini *et al.*, 2005; Aggarwal *et al.*, 2022), will continuously expand with newly identified mutations. The speed and diversity at which mutations emerge in microorganisms, paired with the ability of NGTs to introduce small mutations, can result in two specific challenges. First, constantly expanding microbial pangenomes lead to increased difficulties in identifying the origin of a mutation (natural or targeted), compared to NGT plants. Second, the possibility that living GM microorganisms, once they are authorised and introduced into the food or feed chain, may accumulate further mutations, creates detectability and interpretability issues. Furthermore, as most microorganisms do not rely on sexual compatibility, but instead on horizontal gene transfer to exchange genetic material, the boundary between cisgenesis and transgenesis becomes less clear.

In microorganisms with relatively small genomes, some of these constraints could be resolved by sequencing-based approaches, such as targeted sequencing or WGS. However, identification of NGT-Ms could be achieved only in a simple product consisting of or containing a specific NGT-M strain. Furthermore, as previously explained, HTS cannot always unambiguously identify whether a mutation occurred spontaneously or is a result of conventional mutagenesis techniques. For the time being, application of HTS for food/feed composed of many ingredients including microorganisms and plants would not be feasible in routine market control. Moreover, up to now methods based on HTS do not allow for precise quantification of NGT-Ms in food/feed products.

Application of HTS, which is still a new technology for many official laboratories, would require both investment in laboratory equipment and training not only on the analytical procedure and bioinformatics pipeline, but also on the interpretation of the results. Additionally, a laboratory must be equipped with the relevant infrastructure and knowledge base appropriate for the handling and

analysis of living microorganisms, which can present further challenges when these may be genetically modified (*e.g.,* biosafety requirements according to Directive 2009/41/EC). For this purpose, also wildtype reference microbial genome sequence databases would need to be developed for bioinformatic sequence comparisons (Hurel *et al.*, 2020; Lopera *et al.*, 2023). However, various initiatives have already demonstrated that WGS can be routinely applied to isolated living microorganisms for monitoring in the food industry, particularly to determine whether contamination by a pathogen is due to the presence of a new strain or the persistence of an existing strain within the facility (Allard *et al.*, 2016; Gomes *et al.*, 2025).

Sequencing approaches afford further developmental opportunities in order to provide demonstratable evidence of their applicability for routine detection of NGT-Ms. While progress is being made in standardising laboratory protocols, there is a lack of consensus on standardised bioinformatic methods for data analysis and results interpretation, highlighting the need for guidelines and consensus among institutions (Barretto *et al.*, 2021). In addition, the current deficit in the provision of proficiency tests for the identification (and quantification) of GMMs (and NGT-Ms) provides a barrier to demonstrating competency in this area.

Under the current regulatory framework, applicants have to provide an event-specific detection method for the NGT-M for which authorisation is requested. Such method will be validated by the EURL GMFF and made available for the NRLs and routine laboratories. Currently, there are no minimum performance requirements established for methods based on HTS and it is not clear if such methods will ever fulfil the basic requirements under the GMO regulations, *e.g.,* with regard to quantification of the NGT-Ms. It is rather foreseen that HTS methods may in the future be used by more experienced enforcement laboratories for a first-line screening of products that may contain NGT-Ms, thereby targeting many potential targets at once.

6 Conclusions

This report aims to inform the European Commission on analytical aspects related to the detection, identification and quantification of NGT-Ms and food/feed products derived thereof. This report builds upon an ENGL report from 2023 on the detection of food and feed plant products obtained by targeted mutagenesis and cisgenesis (ENGL, 2023a). The major challenges for the detection of various NGT-Ms in or as food or feed that were identified by the working group are similar to the challenges described for NGT plants. The same new genomic techniques, introducing different types of genetic modifications, can be used in both NGT-Ms and NGT plants. Application of NGTs in microorganisms can result in deletions, insertions or substitutions of diverse sizes. Therefore, the analytical challenges for the development and validation of methods for detection, identification and quantification of NGT plants summarised in Table 2 of the ENGL (2023a) report still remain valid for NGT-Ms (Annex 1).

Like in NGT plants, the shortest DNA sequence alterations (SNVs) are the most challenging when developing a detection method that is specific for an NGT-M. This is partly related to technical difficulties for targeting short mutations within an unmodified DNA sequence context, and partly to the fact that similar short mutations may occur naturally in the target microorganisms. Methods developed for some SNVs or short mutations may, therefore, fail to meet the current minimum performance requirements for analytical methods of GMO testing. In case of known, larger modifications that do not already exist in the microbial species, detection, identification and quantification would normally be feasible using event-specific PCR-based methods.

Enforcement laboratories usually apply screening methods in a first phase when analysing a sample for the presence of GM plants. Such screening methods target common foreign genetic elements present in various conventional GM plants. This approach cannot be applied to NGT-products (plants or microorganisms) containing only alterations of endogenous DNA or sequences present in the pan-genome of the species. Instead, specific methods for every possible mutation will need to be applied, increasing the complexity of the analytical work. Overall, detection of unknown products of targeted mutagenesis or cisgenesis, without prior knowledge of the modified DNA sequences, will not be possible with the current technology used by most of the enforcement laboratories, especially in complex samples.

Some NGT-Ms with small genomes could be identified by HTS if pure microbial samples would be available. However, even applying HTS techniques would not easily allow for the detection of unknown and unauthorised NGT-Ms (especially in case of small mutations). Targeted sequencing could be used to detect and identify multiple targets of known larger sequences specific to particular NGT-Ms. This could be combined in the next step with selected PCR-based techniques for quantification purposes.

Theoretically, HTS-based approaches may also be developed for a semi-targeted approach on genes that are known to be a potential target for NGT-induced modifications. However, this has not been investigated so far. It would require access to a knowledge database containing information on all potential endogenous genomic targets in the various microbial families that, when modified by an NGT, could create beneficial characteristics. Similarly, a database of all described NGT-Ms with their modified sequences, and one containing the reference (pan-)genomes, would be required to compare any given putatively mutated sequence to. If analytical detection of large amounts of diverse NGT-Ms would become an important enforcement requirement in the future, a dataspace linking all these databases, and (informatics or machine learning-based) tools to interrogate them,

may be useful to develop. Currently, identification of a few specific NGT-Ms is mostly achieved in the form of research studies by some enforcement laboratories.

More likely than with plants, applications of NGTs to microorganisms may result in a genomic sequence already present in the pan-genome of a specific species. Such a DNA sequence can also occur naturally in the future as microorganisms can multiply very quickly, so new genetic diversity in microorganisms is developed much faster when compared to plants. Therefore, it may be impossible to analytically evaluate whether a particular genetic modification is produced by targeted mutagenesis or cisgenesis rather than resulting from conventional breeding or natural mutagenesis.

Importantly, as some microorganisms are capable of natural transformation and exchanges of genetic material through horizontal gene transfer, defining NGT-Ms as microorganisms that do not contain inserted foreign DNA might not sufficiently describe the nature of these organisms. This would provide additional complexity to the detection and identification of both NGT-Ms and conventional GMMs compared to GM plants.

It is concluded that analytical testing may support traceability and labelling of NGT-Ms with known and unique sequence modifications. Further developments at both the level of the available technologies and their validation, may further expand the enforcement actions to more known NGT-Ms in the future.

Technical advancements will, however, not resolve the problem of distinguishing identical DNA sequences generated naturally, by mutation or homologous recombination, or by an NGT. Furthermore, unknown modifications created by an NGT will remain extremely difficult to detect and identify as the product of an NGT. In case of small mutations and any modification that is not unique in the microbial gene pool, it is expected that analytical tools will not lead to a solution supporting enforcement. An integrated data-driven approach, including comprehensive sources of knowledge from literature, company websites and patent databases, as well as genome sequencing data for various species and strains, may be needed to allow enforcement for a broad range of NGT-Ms.

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List of abbreviations and definitions

Abbreviations	Definitions
AMR	Antimicrobial Resistance
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9
DNA	Deoxyribonucleic acid
DSB	Double-Stranded Breaks
dPCR	digital Polymerase Chain Reaction
EFSA	European Food Safety Authority
ENGL	European Network of GMO Laboratories
EU	European Union
EURL GMFF	European Union Reference Laboratory for GM Food and Feed
GM	Genetically Modified
GMM	Genetically Modified Microorganism
GMO	Genetically Modified Organism
HGT	Horizontal Gene Transfer
HTS	High-Throughput Sequencing
InDels	Insertions and Deletions
JRC	Joint Research Centre
MPR	Minimum Performance Requirements
NGT	New Genomic Technique
NGT-M	NGT microorganism (microorganism that is genetically modified through an NGT)
PCR	Polymerase Chain Reaction
SDN	Site-Directed Nucleases
SNV	Single Nucleotide Variant
TALENs	Transcription Activator-Like Effector Nucleases
WG	Working Group
WGS	Whole Genome Sequencing
ZFNs	Zinc-Finger Nucleases

List of tables

Annex 1. Summary of analytical challenges for NGT plants.

Table copied from the ENGL (2023a) report on the "Detection of food and feed plant products obtained by targeted mutagenesis and cisgenesis"

Table 2. Analytical challenges for the development and validation of methods for detection, identification and quantification of plant products developed by targeted mutagenesis or cisgenesis.

Genomic technique	Type of modification	Method development and validation	Method implementation for enforcement
Targeted mutagenesis	SNV	Technical feasibility depends on sequence context (case-by-case), but event-specificity, robustness and quantitative use of the method generally difficult to demonstrate	Difficult/impossible to implement such methods for analytical enforcement, when the results are not reliable
	Short mutation	Technical feasibility depends on sequence context (case-by-case), but event-specificity, robustness and quantitative use of the method may in some cases be difficult to demonstrate	Difficult/impossible to implement such methods for analytical enforcement, when the results are not reliable
	Long insertion or deletion	Technically feasible, but event-specificity depends on whether the modification created a new, unique sequence junction or not	Feasible, when event-specificity is demonstrated
Cisgenesis	Targeted sequence insertion or substitution	Technically feasible, but event-specificity of the method depends on whether the altered new sequence is different (and by how many nucleotides) from similar sequences already existing in the species	Feasible when event-specificity is demonstrated. Feasible, but implementation issues may occur in case the new sequence only differs from existing sequences by a SNV or short mutation
	Random whole gene insertion	Feasible	Feasible
Intragenesis	Random whole gene insertion or targeted sequence insertion or substitution	Feasible	Feasible
Various techniques	Multiple modifications	Requires development and validation of one method per modified site, significantly increasing the workload; technical feasibility depends on the types of modifications (see above)	Analysis of each plant product with all individual methods known for the species present in the product will enormously increase the analytical workload and will rapidly make analytical enforcement impossible to continue in the same way. Also difficult to evaluate product quantity in case of multiple modifications derived from a single plant, some of which may also segregate out.

Source: ENGL, 2023a

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