CHAPTER 8

GMO Detection and Identification Using Next-generation Sequencing

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8.1 The Current Landscape of GMO Detection

Both within the European Union (EU) and other countries, legislation related to genetically modified organisms (GMOs) has been established to guarantee their traceability in the food and feed chain, helping facilitate the consumer's right to freedom of choice.[1–4] To ensure the implementation of this legislation, competent authorities are performing, with the help of national enforcement laboratories, official controls on food, feed and seed products available on the market. Analogously to the foodborne pathogen field, most enforcement laboratories are currently chiefly using molecular techniques, such as real-time polymerase chain reaction (qPCR).[5–8] For routine GMO analysis, the presence of GMOs (*i.e.* detection) is usually assessed

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in an initial step using qPCR screening markers targeting transgenic elements. A first common set of markers, such as p35S and tNOS, often covers the majority of GMOs, irrespective of whether the GMOs are authorized in the EU or not. Application of a second set of markers allows discrimination of certain GMOs, such as t35S pCAMBIA which targets approximately 30% of EU unauthorized GMOs, or gat-tpinII which is found only in the genetically modified (GM) events 356043, 98140 and 73496. On the basis of the results of this qPCR screening analysis, a list of potential EU-authorized GM events present in the sample under investigation is drawn up. The corresponding event-specific qPCR methods are applied afterwards for their subsequent identification and quantification.[5,6,9–11]

However, as this system was originally developed to target more specifically GM events that have officially entered the authorisation procedure in the EU, some issues regarding the detection of EU-unauthorized GMOs may be encountered. Firstly, GMOs containing none of the transgenic elements specifically targeted during the qPCR screening analysis will fail to be detected. Recent estimates indicate, however, that this occurs in less than 10% of EU-unauthorized GMOs when the screening markers p35S and tNOS are both used.[10,12,13] Secondly, in case of EU-unauthorized GMOs containing at least one transgenic element targeted by the qPCR screening analysis, two situations can occur. On the one hand, when a sample is composed of both EU-authorized and unauthorized GMOs that share some common transgenic elements, such as p35S and tNOS, the positive results of the qPCR screening assay by the identification of EU-authorized GMOs do not guarantee the absence of EU-unauthorized GMOs. On the other hand, if no link can be established between the observed qPCR screening signals and the list of EU-authorized GMOs, the presence of EU-unauthorized GMOs may only be suspected. Moreover, qPCR event-specific methods are usually not available for EU-unauthorized GMOs.[4,10]

To overcome these issues, either the specific sequences of the transgene flanking regions that are unique for each GM event, or alternatively the sequences of unnatural associations of elements from the transgenic cassette, constitute the indubitable proof of the presence of GMOs. Therefore, to reinforce the current GMO detection system, analogously to the foodborne pathogen field, the application of next-generation sequencing (NGS) technologies is increasingly being investigated and advised by means of either the whole- genome sequencing (WGS) or the targeted approach.[4,8,14–17]

8.2 Applying NGS to GMO Detection: Current Approaches

NGS offers the potential to simultaneously sequence multiple samples discriminated on the basis of unique barcodes individually added upstream during the library preparation step. NGS approaches are of particular interest for the detection of GMOs because they allow characterization of crucial

sequence information, including unnatural associations of transgenic elements as well as transgene flanking regions, facilitating unambiguous detection of their presence as well as identification. Two main NGS approaches can be used towards this end, highly dependent on the precise composition of the GMO sample(s) (Figure 8.1).

When an isolated single GMO is available, the WGS approach has been shown to be suitable for its molecular characterization, including its transgenic cassette(s) and related transgene flanking region(s) (Table 8.1). WGS is currently often applied for GM microorganisms (GMMs), which are generally easy to isolate and typically only have a small genome, ranging in size from several Kbp to a few Mbp. This approach is also increasingly used for GM plants and animals. The WGS approach requires the sequencing of a DNA library composed of sheared genomic DNA extracted from a sample without any *a priori* knowledge of its nucleotide composition.[4,15,17,18]

However, the success of the WGS approach is dependent upon the availability of good reference plasmids and genomes for specific varieties and organisms, which is crucial for performing the reference-based assembly step, whereby generated reads are mapped against a reference sequence that serves as a template to reconstruct the specific sequence within the sample. This necessitates, however, that high-quality reference sequences closely related to the specific host under investigation are available, which is often not the case. In such instances, a *de novo* assembly step is required, whereby all reads are compared against each other to find regions of overlap

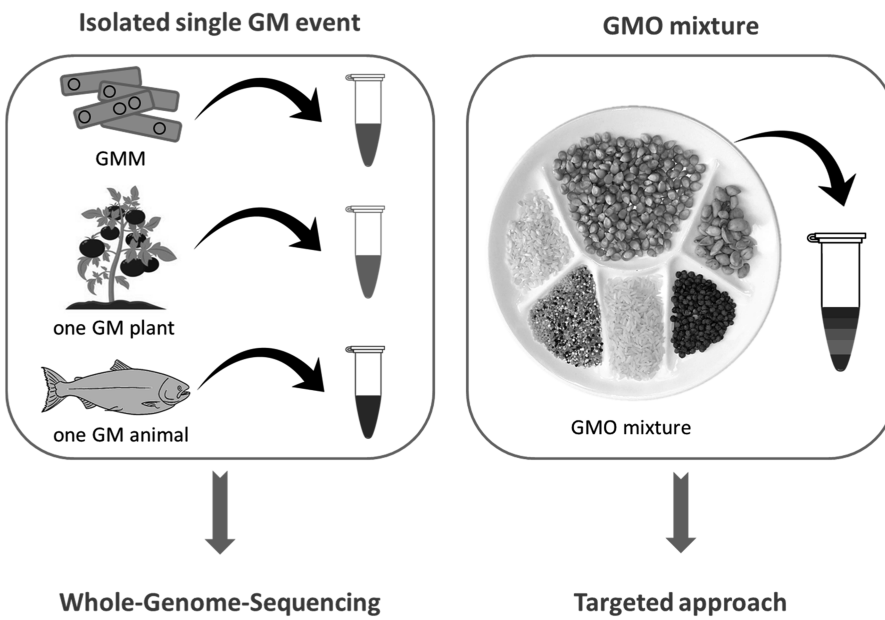

Figure 8.1 Appropriate NGS approaches to detect GMOs based on sample composition.

Table 8.1 Non-exhaustive list of GMOs characterized by means of WGS.

Biological kingdom	Species	Genome size	GM events	NGS platforms	Reference
Bacteria	*Bacillus subtilis*	4 Mbp	GM riboflavin-overproducing *B. subtilis*	HiSeq (Illumina); MiSeq (Illumina); MinION (Oxford Nanopore Technologies)	21 and 25
			GM riboflavin-overproducing *B. subtilis*	MiSeq (Illumina); HiSeq (Illumina); GS Junior System (454 Life Sciences, Roche)	26
Plantae	Papaya	372 Mbp	PRSV-YK	MiSeq (Illumina)	27
	Flax	373 Mbp	FP967	HiSeq (Illumina)	28
	Rice	385 Mbp	LLRICE62	HiSeq (Illumina)	29
			TT51-1	HiSeq (Illumina)	30
			Bt rice	HiSeq (Illumina)	31
			SNU-Bt9	HiSeq (Illumina)	32
	Soybean	1115 Mbp	MON17903	HiSeq (Illumina)	33
			MON87704	HiSeq (Illumina)	33
			GE-J16	HiSeq (Illumina)	34
			ZH10-6	HiSeq (Illumina)	34
Animalia	Pig	2700 Mbp	162_1	HiSeq (Illumina)	35
	Cattle	2700 Mbp	hLF	HiSeq (Illumina)	36

and thereby construct the genome sequence based on deducing the correct path through the overlapping reads, which is both a tediously complex and computationally demanding process. In addition, the specific abilities of different NGS platforms could also be combined, such as aligning high-quality short reads generated by the Illumina® technology to substitutes for reference sequences created by the long reads generated by the Pacific Biosciences® and/or Oxford Nanopore® technologies. Lastly, based on the data generated by the WGS applied on a single GMO, new event- or construct-specific qPCR methods may be developed.[4,5,15,19–24]

In the case of samples composed of several different GMOs (GMO mixture) at trace levels that share similar transgenic elements, their detection and identification are not currently achievable reasonably by means of the WGS approach, as statistically demonstrated by Willems *et al.*, 2016.[31] For such samples, the DNA library should first be enriched with the sequences of interest, which, therefore, requires a minimum of prior knowledge of the sequence in question, and is therefore considered as a targeted approach. The generated reads can be analysed afterwards with both the reference-based and *de novo* assembly-based methods, which will be less prone to the aforementioned bottlenecks because the proportion of background reads that interfere with the analysis, will have been substantially reduced. Given that the majority of samples encountered in GMO routine

analysis are GMO mixtures, this targeted approach provides crucial support to the enforcement laboratories. Note that the targeted approach can be used for isolated single GMOs as well. Even though enrichment of sequences of interest can also be carried out by hybridization methods (*i.e.* magnetic beads or microarrays that are associated with specific probes), this step has until now only been performed by PCR amplification methods that target GMO for which the sequence information is only partially known. More precisely, DNA walking methods, permitting isolation of the unknown sequences flanking the known sequences, were successfully investigated[4,5,14] (Table 8.2).

Regarding the selection of the known sequence(s) to anchor, the vip3Aa2 element was used by Liang *et al.*,[37] on the Mir162 maize event. In order to propose an integrated strategy, DNA walking methods anchoring key elements previously detected in qPCR screening analysis, such as p35S, tNOS and t35S pCAMBIA, have been suggested.[4,9,38,39,41,42] This strategy ensures that the majority of GMOs, irrespective of whether they are EU-authorized or not, are covered by using p35S and tNOS, while only EU-unauthorized GMOs are targeted by t35S pCAMBIA. The validity

Table 8.2 Non-exhaustive list of GMOs identified by means of DNA walking followed by NGS. The samples tested were composed of either one single GM event, or several GM events (also referred to as a GMO mixture).

Types of samples	GM events	Anchoring sites	NGS platforms	Reference
Single GM event	Mir162 maize	vip3Aa2	HiSeq (Illumina); PacBio RS (Pacific Biosciences)	37
	Bt rice	p35S, tNOS, t35S pCAMBIA	PacBio RSII (Pacific Biosciences)	38
	Bt rice	p35S, tNOS, t35S pCAMBIA	MinION (Oxford Nanopore Technologies)	39
GMO mixture	MON863 maize, Bt rice	p35S, tNOS, t35S pCAMBIA	PacBio RSII (Pacific Biosciences)	38
	MON863 maize, Bt rice, RRS soybean	p35S, tNOS, t35S pCAMBIA	PacBio RSII (Pacific Biosciences)	38
	MON810 maize, NK603 maize, TC1507 maize, Bt11 maize	p35S, tNOS	PacBio RSII (Pacific Biosciences)	38
	MON810, MON89034 MON88017 maize, MON15985 maize	p35S, tNOS	PacBio RSII (Pacific Biosciences)	40

of this integrated strategy was successfully tested on unprocessed and processed food matrices containing a single GM event (Bt rice) at high and low concentrations, as well as several GMO mixtures of different GM events at trace levels (Bt rice, MON863, RRS, MON810, NK603, TC1507 and Bt11).[10,38,39,43] Using the same philosophy, a DNA walking method starting from the p35S and tNOS elements was applied by Košir *et al.*, on a GMO mixture composed of one GM event (MON810) at high concentration combined with three other GM events (MON89034, MON88017 and MON15985) at low concentrations.[40]

By employing these DNA walking methods to enrich the sample, the DNA libraries obtained contain several amplicons with a size ranging typically from 100 bp to 4 Kbp. To safeguard the entire representativeness of all GM events potentially present in the tested samples, all these amplicons need to be sequenced, which is experimentally less tedious when using NGS technologies compared with classical Sanger sequencing technologies since the latter require an additional purification step for each of the individual amplicons. Even though Liang *et al.*[37] obtained similar results when sequencing an enriched DNA library with a short-read and long-read NGS platform (see Table 8.2), current long-read technologies, such as Pacific Biosciences® and Oxford Nanopore®, offer the advantage of being able to deal with heterogenic libraries and to attain read lengths of up to 60 and 200 Kbp, respectively, in contrast to the short-read technologies, such as Illumina® technology. Such long reads mean that performing either reference-based or *de novo* assembly is no longer necessary, thus reducing the complexity of the bioinformatics analysis, particularly when samples comprise multiple GMOs that share some identical transgenic elements.[4,37–40,44] The feasibility of using the MinION platform from Oxford Nanopore technologies was recently demonstrated for the first time, to our knowledge, on a GMO sample by Fraiture *et al.*, representing a significant advance in order to help enforcement laboratories to provide a timely and cost-effective answer in times of crisis. This successful demonstration was further enhanced by the small size of the device, its relatively affordable price for an NGS instrument, and its ability to generate (raw) data in 'real-time'.[39]

Ideally, data generated would first be compared against a comprehensive database containing at least all sequences known to correspond to the transgenic cassettes and the transgene flanking regions from EU-authorized GMOs, allowing determination of the potential presence of EU-unauthorized GMOs. In this manner, the presence of EU-unauthorized GMOs cannot be masked through the positive identification of EU-authorized GMOs, as in the current qPCR GMO detection system. To the best of our knowledge, no such databases are, however, currently publicly available. The recently created JRC GMO-Amplicon database, containing GMO sequences generated by qPCR screening, represents a significant step in this direction.[4,38,39,45]

8.3 Challenges for the Detection and Characterization of GMOs Using NGS Related to the Host

According to EU legislation, GMOs are defined as organisms "in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination" in order to provide new properties. GMOs falling under the scope of this process-based definition have classically been created using the biolistic or *Agrobacterium* approaches. The resulting GM event is typically characterized by a unique insertion of the transgenic cassette, containing the gene of interest, into a specific location on the host genome.[1–4] The possibility of detecting the sequence(s) of interest providing proof of the presence of GMOs by means of NGS may be subject to different challenges depending on the specific host, which could be a microorganism, plant or animal. For microorganisms, one of the main bottlenecks is related to the sequencing of their plasmids. These often harbour different genes of interest and selection markers, such as antimicrobial resistance (AMR) genes, which are essential for the construction and subsequent selection of the GMM. This renders the accurate characterization of GMM of paramount importance, but requires that several challenges are addressed at both the wet- and dry-lab levels, including, amongst others: their specific extraction, which may be especially difficult for large plasmids; the assembly of short reads, which is hampered by the high frequency of repetitive regions and the lack of specific plasmid databases; and the proper 'closing' or circularization of their sequences by means of detecting their complementary ends. In specific relation to the assembly step for microorganisms, this process is computationally less demanding compared with animals and plants, but remains nevertheless challenging. This is due to the aforementioned bottleneck of the plasmids, as it is difficult to separate the genomic and plasmid reads during the *de novo* assembly stage, often resulting in an assembled sequence erroneously containing mixed genomic and plasmid parts. To mitigate these plasmid-derived issues, the construction of comprehensive host and plasmid databases containing high-quality host and plasmid reference genomes, respectively, would be expected to aid enable reference-based assembly methods for GMO detection. When no such information is available, plasmids can still be conjugated to other hosts such as *Escherichia coli* with well-characterized genomes in order to facilitate the process of *de novo* plasmid assembly, by first subtracting the genomic content through read mapping against the host reference genome.[22–24,46–49] For plants and animals, the bioinformatics analysis is equally or even more challenging due to their large and complex genomes. Plants can be particularly problematic due to their high levels of heterozygosity and large number of repeated regions. Additionally, most crop species of interest for genetic modification have genomes of higher ploidy.[4,17,50,51]

8.4 Conclusion and Perspectives

The successful implementation of NGS technologies on GMO samples has great potential in strengthening the successful detection of EU-unauthorized GMOs as part of the current GMO detection system. However, since this approach requires substantial know-how and expertise in very specific niche areas of molecular biology and bioinformatics, one recommendation would be to introduce these novel methodologies only in certain well-equipped enforcement laboratories that would be considered as sentinels. The success of the proposed NGS approaches lies thus in their appropriate use, in terms of both the wet- (*i.e.*, types of samples and enrichment) and dry-lab (*i.e.*, adapted analysis workflows) aspects, highlighting the compelling need to clearly delineate the specific questions and desired purposes to ensure that these are properly incorporated into the routine GMO detection system at all levels from sample analysis to generation of the result.[4] Moreover, as NGS allows the characterization of GMOs at the nucleotide level, the data generated will help facilitate development of new event- or construct-specific qPCR methods.

Additionally, new challenges are expected to arise as biotech companies are increasingly turning towards genome editing techniques, including in particular the CRISPR/Cas9 system, for the creation of biotech organisms. In principle, in terms of detection and characterization, both the WGS and targeted approaches could be applied to these biotech organisms in agreement with recommendations described in the above sections. Detection and characterization can be difficult but still remain possible for biotech organisms containing only highly characterised and limited genetic modifications, such as single nucleotide polymorphisms (SNP). However, due to the high similarity of these genetic modifications with the ones induced by conventional breeding or natural processes, the determination of the origin of these mutations may present a considerable challenge.[52–57]

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