CO-EXTRA

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RE Restricted to a group specified by the consortium (including the Commission Services)	
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1 SUMMARY

In the EU, the production and commercialization of GMOs and GMO-containing or GMO-derived food and feed products are regulated by the 'Food and Feed Regulation' (EC) No 1829/2003 and the 'Labeling and Traceability Regulation' (EC) No 1830/2003. Labeling is mandatory above a 0.9% threshold GMO percentage, relative per ingredient (and translated as plant taxa for analytical purposes). Analytical methods for detection and accurate quantification of GMO contents in derived food and feed products require adequate reference materials as positive and negative controls and as control for the correct quantification.

This report reviews the cloning and application of plasmid DNA markers usable as positive controls. Provided the suitability of plasmids as calibrants in GMO analysis can be demonstrated, plasmids would represent an ideal solution to overcome the current lack of independent calibrants.

This report presents the state-of-the-art today in the EU, on the use of such calibrants for GMO detection and quantification. The origin and history of plasmid DNA markers are discussed and the types of 'GMO reference plasmids' are described. The introduction of plasmid DNA markers in the field of GMO control, and the initiation of a European database containing different types of taxa-specific and GMO-specific plasmid markers, have some structural and theoretical implications, which are also discussed. Besides a literature overview on the use of plasmids in GMO analysis, the theoretical benefits and limits of the use of plasmids as reference material are summarized.

It should be noted that a detailed discussion on the technical difficulties and quality control aspects of the production of plasmids as (certified) reference materials is out of the scope of this document.

2 INTRODUCTION

The detection, authentication and quantification of GM plants and of their derived products used in the food/feed-processing area are a major challenge. In the European Union, according to GM Food/Feed Regulations, the EU has decided to label GM food/feedstuffs containing GM plants or GMO-derived ingredients and additives. The present analytical threshold for labeling is set to 0.9 %. Such detection and quantification require analysis tools in order to be able to comply with the European legislation requirements. Recently, recommendation 2004/787/EC of the European Commission suggested that 'the results of quantitative analysis should be expressed as the number of target DNA sequences per target taxon specific sequences calculated in terms of haploid genomes'.

By definition, analytical methods require reference materials as external or internal positive and negative controls (ISO/DIS 24276). Such material is also necessary for laboratory accreditation. To date, matrix reference materials have been produced from ground seeds to resemble as close as possible a food and feed sample. Certified Reference Materials (CRMs) are available for all GM events authorized in Europe under (EC) No 1829/2003 and for some other GM events (for an overview reference is made to e.g. http://www.irmm.jrc.be and http://www.aocs.org). Pure CRM seed powder standards are difficult to obtain in due time, are expensive and time-consuming to produce and remain prone to adventitious contamination or natural variations, inherently linked to seed production practices, and finally subject to GM plant withdrawal from the market while their derived products being still in the supply chains.

Instead of seed powder as a source of reference DNA analyte, plasmid DNA could represent a very elegant alternative analyte. Plasmids are small, relatively easy to produce at low cost and represent a suitable substrate for efficient amplification of a certain DNA sequence with high accuracy. Plasmids are very versatile and are capable of maintaining in a reproducible manner any particular DNA sequence that does not interfere with fundamental processes of either plasmid or host stability. However, regardless which type of reference material is preferred, homogeneity, transport and long-term stability of the material will need to be proven, before this material fulfils the requirements of a reference material.

Plasmids should satisfy with the requests of continuity and stability of provisions of the reference material, even for GMO already withdrawn of the market. In certain cases plasmids might also represent a solution for GMO events that are not yet authorized in Europe or that are lacking CRM, provided that reliable sequence

information allowing to unambiguously detect the GMO event, is available.

The objective of this report is to set out the state-of-the-art on the development, availability, and applicability of plasmid DNA calibrants for GMOs. First, the history and evolution of plasmid DNA calibrators for GMO analysis are outlined. An overview of available literature is provided. Different types of plasmid calibrants will be described, together with their applications in the frame of Belgian and European initiatives taken towards harmonization of development and use of plasmids, in the form of a database compilation of the plasmid DNA markers and real-time PCR assays for GMOs.

This document does not survey the interest or limits of other types of calibrants (e.g. genomic DNA (gDNA), nor any aspects of commutability of plasmids with other calibrants.

3 DEFINITIONS: REFERENCE MATERIALS (RMS) AND CERTIFIED REFERENCE MATERIALS (CRMS)

The quality of analytical methods and their results depends to a great extent on the use of reference materials (RMs). RMs or standards play a role in the achievement of traceability, the calibration of laboratory equipment and methods, the monitoring of laboratory performance (internal quality control, IQC), method validation and method comparison between laboratories (Segura et al., 2004; Walker and Lumley, 1999). A laboratory should always demonstrate traceability of all produced results to reliable measurement standards, particularly for accreditation purposes (Fig. 1).

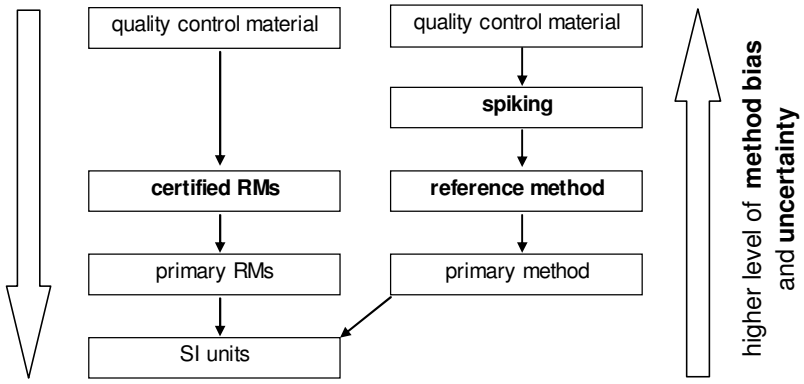

Figure 1: The traceability chain (materials on the left, methods on the right) and the relationship between traceability and uncertainty of measurement (Pan, 1996; Walsh, 1999)

The official ISO definition of a RM is "material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process. RM is a generic term; a RM can only be used for a single purpose in a given measurement. A CRM is defined as a 'reference material, characterized by a metrologically valid procedure for one or more specified properties'. Any CRM is to be accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability (ISO Guides 31, 34 and 35).

The term 'certified' distinguishes a CRM from a RM. Certification is defined as the whole process of obtaining the 'property values' and their uncertainty arising from homogeneity testing, stability testing and characterization. It should be noted that reference material need to be tested for homogeneity and stability to the same extend as a CRM. Characterization of a RM can be translated as the establishment of its property value. The assignment of the proper value must be accompanied by a statement of uncertainty, including the uncertainty components coming from heterogeneity and/or instability of the material (van der Veen et al., 2001c). Official guidelines (ISO Guides 31, 34, 35) and literature references exist on the production and certification of RMs in general, and from material preparation, homogeneity testing (van der Veen et al., 2001b), stability testing (Linsinger et al., 2001; van der Veen et al., 2001a) and characterization (van der

Veen and Pauwels, 2000; van der Veen et al., 2001c), in particular.

Analyte homogeneity, stability during transport, stability during storage, traceable property values and their uncertainties, represent the most important product characteristics, together with the certificate (in the case of CRMs) and the 'commutability' of the material (Emons et al., 2004). Commutability describes the 'similarity of analytical response obtained for a given material to the response obtained from routine samples' (ISO 17511, 2003). Commutability defines the match between the RM and routine samples and can be considered at different levels: (food/feed) matrix match, analyte level match, uncertainty match and format match. First, the matrix of the RM should be similar to the matrix of the unknown sample. Second, the compounds and their concentrations should be similar (analyte level match). Third, the same source and size of error should be encountered when analyzing reference samples and unknown samples (Huber, 1998a). In summary this means, that a commutable material has to behave the same way as the sample under investigation using a specific measurement procedure.

The above-described characteristics form prerequisites for RMs and apply to both matrix RMs and pure analyte or pure substance RMs. 'Matrix RMs' allow the evaluation of effects on the product context comprising the analyte and all effects arising from the processing of the material (Emons et al., 2004). Matrix RMs are generally introduced at the beginning of the analytical procedure. They can be used to evaluate the quality of the whole procedure, including sample homogenisation, analyte extraction and the analyte determination step. Pure analyte RMs apply to the analytical determination step only and can be used e.g. for instrument calibration at that level (Walker and Lumley, 1999).

Nor a detailed comparison of the different uses nor the limitations of their use in the validation of an analytical process are within the scope of this document.

4 ORIGIN AND HISTORY OF PLASMID DNA MARKERS FOR GMOS

The first commercially available CRMs for GMO analysis were matrix RMs, consisting of a mass fraction of GM powder, prepared from GM seeds or beans, present in an amount (mass) of non-GM powder, prepared from non-GM seeds or beans (Trapmann et al., 2002; 2003a; 2003b; 2004). These CRMs, produced by the EC JRC's Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and available in the range from 0 to 5 % weight/weight (w/w) GMO, have proven to be useful in complying with the European legislative stipulations. At that time, until the publication of Recommendation 2004/787/EC, the GMO threshold stipulated in the EC GMO legislation was understood as to express a mass percentage (Trapmann and Emons, 2005). To date, no CRMs are available which are certified for their GM copy number ratios, but efforts are made to certify the existing matrix CRMs for their copy number ratio (Trapmann, personal communication).

Due to the increasing number of commercial GMO products and applications worldwide, the production of matrix CRMs has become unmanageable. Also, the production process is very expensive, cumbersome and prone to the presence of impurities. The availability of one matrix material per GMO event, suitable for the DNA extraction control, and additionally reference material suitable for calibration is to be considered as a more realistic situation.

Provided the commutability of plasmids can be proven, plasmids would form an ideal basis for the development of the required calibrants.

As said above, legal thresholds for mandatory labeling of GMO products have been recently proposed as genome copy percentages, although other units can be kept for specific purposes. This means that the limits for mandatory labelling are recommended to be expressed as analytical limits, i.e. numbers of haploid genome copies or equivalents (Commission Recommendation 2004/787/EC). The modularity of the analytical procedure for GMO analysis (Holst-Jensen and Berdal, 2004) has been recognized by the ENGL (ENGL, 2004). The modular validation approach suggests that each step in the procedure is seen as an independent method or 'module', existing on its own and therefore requiring an adapted, separate validation process. For the validation of the different steps - sampling, sample preparation, DNA extraction and DNA determination - RMs are needed that are representative for the input of the considered step. For the PCR step, this means that pure DNA solutions may be used as calibration RMs (Fig. 2).

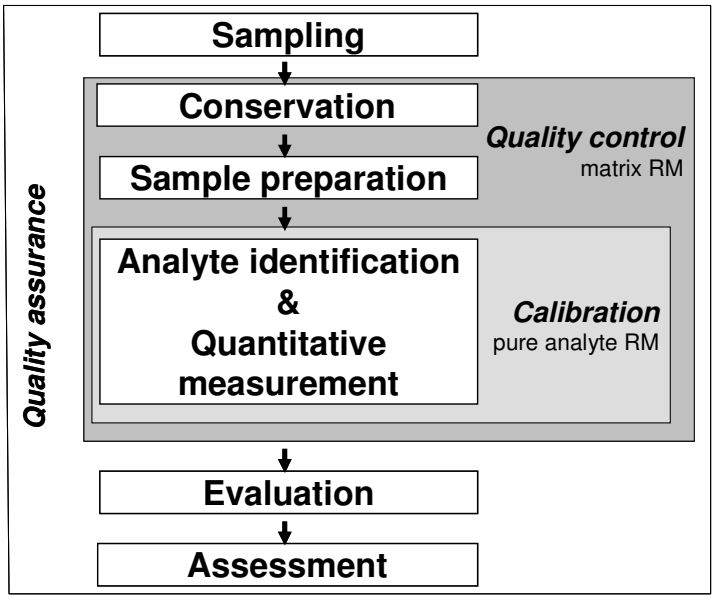

Figure 2: Analytical process and use of reference materials

As pure analyte RMs, we have the choice between genomic and plasmid DNA, provided the commutability of both could be proven. Different types of genomic and plasmid DNA standards have been used as calibrators for quantitative real-time PCR in other applications, as reviewed by several authors (Bustin, 2000; Ferr, 1992; Ferr et al., 1994; Giulietti et al., 2001). Genomic DNA would need to be extracted from a matrix first and thus is liable to matrix availability (which is thus highly dependant on commercial crops / seeds production) and effects and processing influences such as degradation. Plasmid DNA vectors, containing the sequence of interest, would be preferred because of their relatively simple and cheap production process. It is likely that the stability of plasmids can be achieved easily (at least for a limited time period). Caution needs however to be taken for possible dissemination of small-sized plasmid DNA molecules, considering their relatively higher capacity to be transmitted by aerosols compared to large genomic DNA fragments.

Within the frame of the Belgian OSTC (Office for Scientific, Technical and Cultural Affairs) project 'Detection and identification of commercialized genetically modified plants (GMPs)' (SPSD I, 1998-2001), under co-ordination of Dr. W. Moens (Scientific Institute Public Health, Belgium), the GMO research group at CLO-DvP (now ILVO-T&V), coordinated by Dr. Marc De Loose, has been investigating the usefulness and applicability of plasmid DNA standards in GMO analysis. In 1999, long before the genome-based measurement and expression units for GMOs were recommended (Comm. Rec. 2004/787/EC), they initiated the cloning of a soybean specific sequence and a GTS 40-3-2 (Roundup Ready, RR) soybean specific T-DNA/plant DNA junction fragment. The plasmids pAS104 and pAS106, containing a 359 bp fragment of the RRS-specific p-35S/plant junction and a 118 bp sequence of the soybean-specific lectin (Le1) gene respectively, were constructed as a model system to investigate the usefulness of synthetic DNA markers. Both plasmids were deposited at the Belgian Coordinated Collection of Micro-organisms (BCCM^{TM}/LMBP) at the University of Ghent (Belgium). Molecular information on both clones, including a circular map of the plasmid vectors, can be consulted online, via the website of the BCCM^{TM}/LMBP collection (http://www.belspo.be/bccm/db/).

Within the frame of the same project, it was demonstrated that a dilution series of both plasmid molecules, expressed in absolute copy numbers, can be used as calibrators in real-time PCR, allowing the accurate and sensitive quantification of RRS (Taverniers et al., 2001). In the follow-up Belgian OSTC project 'Tracing and authentifying GMOs and derived products in the agro-food sector" (SPSD II, 2002-2004), the production of taxa- and transgenic event-specific plasmid DNA RMs for all commercial GMOs was initiated and all resulted plasmids were deposited at the BCCM and fully documented by scientific reports. In addition to the partners of both SPSD/OSTC projects (ISP/IPH; ILVO; CRA-W), also the IHCP (EC-JRC), as official representative of the Community Reference Laboratory (CRL) at the European Community, and INRA, a participant in the GMOchips and QPCRGMOFOOD FP5 European research programs, provided the BCCM/LMBP collection with plasmids issued from their experimental GMO activities (including the scientific

reports).

5 TYPES AND APPLICATIONS OF PLASMID DNA MARKERS

Different types of DNA sequences may be considered as a valuable analyte in the detection of GMOs: I) taxon specific (plant taxa and donor organisms), II) generic recombinant genetic elements (such as common 5' promoter or 3' terminator elements), III) trait-specific elements, IV) construct-specific elements, and V) event-specific elements. The use of such DNA sequences within the detection and/or identification and quantification of GM material is listed in Table 1. A GMO reference plasmid may as such contain any DNA fragment enabling the detection and / or quantification of GM material in a sample as a GM marker.

Table 1 Different types of DNA sequences used as targets in GMO detection, identification and quantification

TYPE	DETECTION	IDENTIFICATION	QUANTIFICATION
ENDOGENOUS	+		+
GENERIC ELEMENTS	+		
TRAITS	+	+	
CONSTRUCT	+	+	+
EVENT		+	+

5.1 5.1 First level GMO reference plasmids: the single amplicon plasmids

The concept of plasmid DNA standards for GMOs was introduced with the successful construction and deposition of two plasmid DNA markers in an existing database, as described above. This research work led to the first publication on this topic in 2001 (Taverniers et al., 2001). The idea then grew to extend this initiative, i.e. to construct plasmid calibrators for all commercialized GMOs and to initiate a database of DNA markers for GMOs. For this purpose, different partners of a Belgian OSTC project (2002-2004, see above) and partners of the QPCRGMOFOOD and GMOchips FP5 European research programs, started to clone event-specific and endogenous, species-specific sequences, including donor organisms controls such as CaMV. Besides transformation event-specific DNA segments, also screening elements such as promoters and terminators, fragments of inserted genes and other parts from the T-DNA construct e.g. junctions between different genes in one cassette or junctions between two adjacent transgene cassettes, have been targeted for cloning.

The first generation of plasmids contains only one DNA analyte marker that is either transformation event-specific or construct-specific or endogenous species/cultivar-specific. This choice was made to avoid as far as possible cross-contaminations by multiple target plasmids. Also generic markers such as promoter or terminator sequences which are frequently used for mass screening could be considered, e.g. in differential quantitative PCR to detect unknown GMOs. Finally, plasmids harboring large fragments developed during the course of the "consensus approach" of GMOchips by INRA, were also deposited at the BCCM by the Belgian IHP laboratory.

The respective steps in generating this type of plasmid vectors are the following:

- Define target amplicon by bioinformatics analysis
- Perform PCR and clone amplicon into a vector (e.g. “pGEM" or “TOPO" type)
- Characterize cloned insert (restriction analysis, sequencing)
- Subclone amplicon as an EcoRI fragment into pUC18

5.2 Second level GMO reference plasmids: the dual amplicon plasmids

The second generation GMO reference plasmids contain two markers, e.g. an event-specific target sequence on the one hand, and a taxon/cultivar-specific sequence on the other hand. Such plasmids can be

used for relative quantification of GM material according to the recommendation 2004/787/EC .

A cloning scheme for dual amplicon plasmids consists of the following steps:

- Define target amplicons by bioinformatics analysis
- First PCR on each amplicon using overlapping primers at one end of each amplicon
- Second PCR using end primers to the combinatory amplicon
- Clone the combinatory amplicon into e.g. pUC18
- Characterize insert (restriction analysis, sequencing)

The co-cloning of both DNA analyte sequences ensures a perfect 1:1 ratio over the different calibration points of a standard curve in RT-PCR. These dual amplicon plasmids can serve as calibrant in the relative quantitative determination of a certain genetic element in a sample.

Dual amplicon plasmids have first been constructed by Weighardt et al. (2004) and were called pJanus™ plasmids. Similar kinds of plasmids have already been described and their use in screening for the quantitative detection of GM material in a sample has been documented (Weighardt et al., 2004; Weighardt, 2005; Mattarucchi et al., 2005; Moreano et al., 2005). This type of plasmids is being used preferentially as a calibrant for quantitative detection for the presence of one single GM-event in a sample.

5.3 Third level GMO reference plasmids: the multitarget amplicon plasmids

Finally, pyramiding in subsequent co-cloning steps several genetic elements into one plasmid could lead to multitarget copy plasmids. This strategy has been followed in a number of cases especially focused on markers for the detection of GM maize (Kuribara et al., 2002; Yoshimura et al., 2005a). Multitarget amplicon plasmids have been demonstrated to be useful in different applications of GMO quantification by several authors (Shindo et al., 2002; Taverniers et al., 2004; Taverniers, 2005; Yoshimura et al., 2005a).

Although advantageous with respect to equivalence in relative copy number of each marker in the controls, the need of comparable amplification efficiencies for each marker in e.g. real-time PCR analyses, may limit the applicability of this type of complex plasmid as a reference material.

Also, as for the dual plasmid system, the taxon reference system might become useless when contamination with the plasmid has occurred somewhere along the analytical process.

6 GMO REFERENCE PLASMIDS: SYSTEM FOR DOCUMENTATION AND DEPOSIT

In the establishment of a pENGL™ plasmid database, an important step was to introduce a minimum level of harmonization or standardization for the different constructed plasmids. An important step in this achievement has been the obligation to document the construction of each pENGL plasmid in a scientific dossier, drafted in coordination with the group of William Moens from the Institute of Public Health (IPH, Brussels).

Each scientific dossier corresponds to a single plasmid, which is encoded by a unique identification tag 'pENGL-nn-xxx', were 'nn' represents the number of the laboratory and 'xxx' is the number of the plasmid given by the laboratory.

Based on the information within the respective scientific dossiers, a Filemaker-Pro database has been developed. Each record of the database contains general information on the plasmid clone, applicability of the plasmid marker (set of primers and TaqMan(r) probe), information on host strain properties, cloning vector features, and data on the cloned DNA segment. In addition, a detailed protocol of the cloning procedure, including quality assessment and authentication measures carried out on the resulting plasmid,

is documented.

In order to standardize as much as possible, all plasmids to be deposited have to be introduced into a pUC-vector background. This means that any intermediate (e.g. a PCR vector used in commercial kits for cloning) need to be sub-cloned into a pUC background. The final construct needs to be fully sequenced before submission.

The process of subcloning to pUC-vectors and the production of first-, as well as second- and third-generation GMO reference plasmids is currently in progress. Scientific dossiers are completed for each single plasmid. These scientific dossiers can in due time be made available, together with the GMO reference plasmids, to the Enforcement Laboratories within the ENGL, while conditions of availability for private laboratories are to be defined.

Such project of building a European Community bank of GMO markers has been approved along the "European Network of GMO Laboratories" (ENGL). Already, the Trademarks "pENGL'" for single amplicon plasmids and "pJanus'" for dual amplicon plasmids have been registered. To date, already forty four single or dual plasmids have been filed as "Safe Deposit" at the 'Belgian Coordinated Collection of Microorganisms' (BCCM) in Ghent (Belgium), a world master plasmid depository under the Budapest treaty.

It is considered opportune however that in the future the co-ordination and maintenance of such a GMO reference plasmid bank is being supervised and financed by the official centralized services of the European Commission (e.g. the Directorate General of the Joint Research Centre). Furthermore, an interface between the Co-Extra participating laboratories and the "European Community Bank of GMO markers" should be developed in order to facilitate the exchange of RMs and fully support the further development of plasmids as validated and normalized RMs for GMO detection and quantification.

7 GMO REFERENCE PLASMIDS: STRUCTURAL AND THEORETICAL IMPLICATIONS

In line with the stipulations of the international standards ISO/DIS 24276, ISO/DIS 21569, and ISO/DIS 21570, GMO reference plasmids should have characteristics as stipulated in the norms.

Reference material should preferentially represent an unambiguous equivalent of the analyte as present in a certain sample.. Applied to DNA as a substrate, and taking into account the requirements set out by the international standards ISO/DIS 21569 and ISO/DIS 21570, 'equivalence' in GMO reference plasmids should pertain to the DNA sequence as present in the analytical sample.

Among experts, the type of RMs to be used for the calibration of GMO analysis has been debated for years now. An important point of discussion is the certification of suitable materials and the implementation of official ISO definitions of RMs and CRMs. The value of the existing matrix CRMs resides in the fact that a certificate of their property accompanies the (C)RM. The basis of the certification lies in the characterization or property value assessment (see also above). A certified value is always accompanied by a measurement uncertainty at a stated level of confidence (ISO-VIM, 1993). The current lack of certification is the main weakness for the use of plasmids as a (C)RM.

Real-time PCR measures a fluorescent signal, which is converted to a number of copies of a certain sequence through the use of calibrators. With the Commission Recommendation 2004/787/EC, GMO units of PCR based measurements and expression have been proposed to be equated. Pure DNA calibrators such as plasmid markers may match very well the scope of the current EC recommendation.

The choice of the DNA sequence for the endogenous marker is critical and should at least fulfill some general features in terms of specificity, stability, and physico-chemical properties for analytical purposes as described in the ISO CEN standards. The choice of the GMO-specific DNA targets is mostly dependent on the specificity linked to the purpose of use (e.g. event-specificity is linked to one particular transformation event). The physico-chemical properties of the target DNA sequence, like size, sequence content (e.g. AT-rich), difference in resistance to degradation, etc. can be very important in determining the efficiency and the accuracy of the detection of the analyte in a sample (LOD, LOQ).

The target fragment is optimally cloned into a vector free of any commercial constraints, such as a pUC vector. The reference plasmids can then be made available to the different stakeholders through a legal distribution network according to the stipulations set out by the Treaty of Budapest.

8 BENEFITS AND LIMITATIONS OF GMO REFERENCE PLASMIDS

The development of the GM reference plasmids is a relatively simple, straightforward, two-steps cloning process, consisting of a ligation and a transformation. Not only are GMO reference plasmids easily processed and stored, a major strength of plasmid DNA standards is found also in their universal applicability. The target specificity of the plasmids makes them very useful as positive control sample or as calibrators in target-specific PCR reactions. Opponents to plasmid DNA claim that this point exactly is the main drawback: plasmid DNA solutions have a limited applicability as they can only be used for one single PCR. However, target-specific, pure DNA RMs are needed in order to allow calibration according to ISO Guide 35, namely independent from the matrix materials used for quality assurance.

Plasmid solutions are pure or single substance RMs, which according to Walker and Lumley (1999), cannot fulfill the function of a matrix RM used for quality control, but can only be used for instrument calibration. As described above, several research groups have proven the value of single amplicon, dual amplicon, as well as multitarget amplicon plasmids as positive control samples and calibrators for GMO quantification. Pure DNA solutions, containing the target sequence at a known amount or concentration, can serve for the assignment of values to materials. In the case described here, this is the quantification of transgenic DNA, relative to the total genomic DNA, through real-time PCR. Multiple literature references from the last years have shown that the property values of the plasmids, which are the copy numbers of the specific target DNA sequence, are sufficiently stable and well established, in order to be used for quantifying the target in other samples. Stability can be additionally improved by adding stabilizing agents. Consequently, pure DNA solutions can be of high value also in method performance assessment or validation (ISO-VIM, 1993).

The expensive processing and certification process of matrix CRMs, including extensive blending, homogenizing, difficult sample extraction and other production steps, implies that the cost price for matrix CRMs produced by national and official institutes is very high (Huber, 1998b; Maier et al., 1997).

As a first step in evaluating the possiblity to produce plasmid (C)RMs, JRC-IRMM has initiated the first copy number certification studies, with the objective of certifying existing matrix CRMs for their copy number ratio (Trapmann, personal communication).

9 CONCLUSIONS

THE INTRODUCTION OF PLASMID DNA AS A NEW TYPE OF RMS FOR GMO ANALYSIS COULD BE AN IMPORTANT STEP FORWARDS IN THE STANDARDIZATION OF METHODS FOR GMO ANALYSIS. UP TILL NOW, HARMONIZATION OF ANALYTICAL METHODOLOGIES WAS HAMPERED TO A GREAT EXTENT BY THE LACK OF SUITABLE RMS FOR THE CALIBRATION.

Several types of plasmids have been developed in a standardized way and documented by scientifc dossiers. An official European database collection of these (possible) plasmid RMs for GMOs has been developed. Plasmids containing either species-specific gene sequences, or a fragment from the inserted T-DNA, such as an event-specific T-DNA/plant-DNA border junction or a specific rearrangement within the T-DNA, have been and are still being collected and documented. This ENGL plasmid library and database have the potential to serve as the basis of harmonizing GMO analysis in the future worldwide.

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