DEVELOPMENT OF REFERENCE MATERIALS FOR GMO DETECTION

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DEVELOPMENT OF REFERENCE MATERIALS FOR GMO DETECTION

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ABSTRACT

DEVELOPMENT OF REFERENCE MATERIALS FOR GMO DETECTION

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The reliable detection and quantification of Genetically Modified Organisms (GMO) is strongly dependent on validated methods as well as calibration systems. Today, Certified Reference Materials are used as reliable source of template DNA in quantitative real-time PCR assays constructing validated methods for routine analysis of GMOs. In addition to that, obtaining and assessing plasmids for use as real-time PCR standards and positive PCR controls are increasingly used methodologies. To enforce the labeling regulations of GMOs, the application of DNA plasmids as calibrants is becoming essential for the practical quantification of GMOs. This study reports the construction of plasmids for qualitative screening assay for 35S promoter and NOS terminator as GMO elements, and relative quantification assays in Maize and Soya events, Bt11 and GTS 40-3-2 respectively.

Reference GM plasmids provided convenient and reliable positive controls for GM PCR tests. Soya GM event GTS 40-3-2 and endogenous control plasmids assessed within-laboratory assays were resulted to be acceptable in terms of reproducibility standard deviation and repeatability relative standard deviation. As a result of verification and measurement uncertainty data based on single laboratory data, constructed plasmids provided an excellent and economic alternative to plant DNA extractions for positive control material. However, further study is needed showing enhanced amplification efficiency and inter-laboratory verification data for using constructed plasmids as template DNA in validated methods.

Key words: GMO detection, plasmid DNA calibrants, real-time PCR, reference materials.

ÖZ

GDO TESPİTİ AMACIYLA REFERANS MATERYALLERİN GELİŞTİRİLMESİ

KESKİN, Batuhan Birol
Yüksek Lisans, Biyokimya Bölümü
Tez Yöneticisi: Prof. Dr. Hüseyin Avni ÖKTEM

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Genetiği değiştirilmiş organizmaların (GDO) tespiti ve kantitasyonunun güvenirliği için valide edilmiş metotların kullanımı kalibrasyon sistemlerinin oluşturulması esastır. Günümüzde bu amaçla yoğunlukla kullanılan gerçek zamanlı kantitatif PCR tabanlı rutin analizler için bu valide metotların oluşturulması sertifikalı referans materyallerin DNA'sı kullanılarak yapılmaktadır. Gerçek zamanlı PCR analizlerinde genomik DNA'nın yerine plazmit DNA'nın kalibrasyon standardı ve pozitif kontrol olarak kullanımı giderek artmaktadır. Bu çalışmada, kalitatif tespit için 35S promotör ve NOS terminatör gen bölgeleri; kantitatif analiz için mısırda Bt11 ve soyada GTS 40-3-2 GDO çeşidine yönelik plazmitler oluşturulmuştur.

Oluşturulan plazmitlerle elde edilen sonuçlar, bu plazmitlerin PCR analizleri için kullanımı konusunda bazı avantaj ve dezavantajlarını göstermektedir. Referans GDO plazmitlerinin GDO'ya yönelik PCR analizleri için kullanışlı ve güvenilir birer pozitif kontrol DNA olduğu ortaya konmuştur. Çalışmada GTS 40-3-2 soya türü ve soyaya özgü Le1 için oluşturulan plazmitlerin kantitasyon amaçlı ölçüm belirsizliği açısından kabul edilebilir değerlerde olduğu görülmektedir.

Sonuç olarak, laboratuvar içi ölçüm belirsizliği verilerine göre referans plazmitler bitki genomik DNA muadillerine nazaran ekonomik ve hatta kullanılabilirlik açısından da daha kolay çözüm sunabileceğine dair gösterge niteliğindedir. Yapılan çalışmanın

valide olabilmesi açısından ileriki aşamalarda amplifikasyon verimliliğinin geliştirilmesi ve laboratuvarlar arası karşılaştırmalı testlerin gerçekleştirilmesine ihtiyaç duyulmaktadır.

Anahtar kelimeler: GDO tespiti, plazmit DNA referanslar, gerçek-zamanlı PCR, referans materyaller.

To My Family..

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EQUATIONS

LIST OF ABBREVIATIONS

SOC- Super Optimal broth with Catabolite repression

adh1- Alcohol Dehydrogenase 1 gene

Le1- Soybean Lectin 1 gene

CHAPTER 1

INTRODUCTION

1.1 Genetically Modified Organisms (GMOs)

Genetic engineering can be defined as the science in which the properties of an organism modified intentionally by interference of genetic material and transformation of genes for creating new living organism variations (Uzogara 2000). Genetically modified organisms (GMOs) can be described as organisms which have arisen through alteration of their genetic materials (DNA), however has not been altered in a natural way like by mating or natural recombination. Genetic modification, as an application of modern biotechnology, allows transfer of selected individual genes to be transferred from one organism to another, between both of related and non-related species. Genetically modified soya, maize, cotton and oil-seed rape are the crops are the most common types of GMOs, as they have been a few of the highly consumed and commercialized crop varieties in the world since modern agriculture techniques were acquired (http://ec.europa.eu/food/food/biotechnology/qanda/h1_en.print.htm 2009). Those varieties developed for having insect resistance, herbicide resistance, as well as pathogen resistance which are regarded as most common construct groups. However, the range of transgenic organisms now comprise a wide variety of crops and constructs currently (Hails 2000).

1.2 History

Genetically modified (GM) crops are products which have acquired novel genetic characteristics, thanks to opportunity of late advances in molecular biotechnology. Crop genetic engineering is a technology developed in the early 1980s that reached its

first commercial launch in the mid-1990s and relies on the ability to transfer novel genes to crop plants by nonsexual means (Bennett, Chi-Ham et al. 2013).

By the late 1980s, GM crops which are virus resistant tobacco and tomato were on sale in China, but they did not become widespread until 1994. Flavr Savr™ tomato was the first GM crop, which has been modified to increase its quality and shelf-life, likewise the first generation GM crops were engineered to improve resistant to insects, virus (disease) resistance and tolerance to herbicides, referred as production traits (Bennett, Chi-Ham et al. 2013). The GM crop practice now also includes second-generation traits. These traits include improved composition and product quality, abiotic stress tolerance, nutrient-use and photosynthetic efficiency, and also nutritional improvement, compared to others.

According to International Service for the Acquisition of Agri-Biotech Applications (ISAAA), in 2013 175.2 million hectares of biotech crops reported to be grown in the world, with a growth rate of 3%, 5 million bigger compared to 170 million hectares, which was reported in 2012. Assuming 1996 as first, the 2013 was the 18th year of commercialization, in 12 of the 17 years growth rates were more than 10% as the growth was continuing after 17 consecutive years of increases remarkably. In total, the global area of biotech crop grown have increased from 1.7 million hectares to more than 175 million hectares from 1996 to 2013. This corresponds to more than 100-fold increase, making biotech crop technology the fastest crop technology application adopted in near past. It is clear that this rapid adoption rate is proving of its applicability and the substantial advantages it delivers to producers and consumers (James 2013).

In addition to these, as of 2012, in developing countries more biotech crops were grown than industrial countries for the first time. (Figure 1.1)

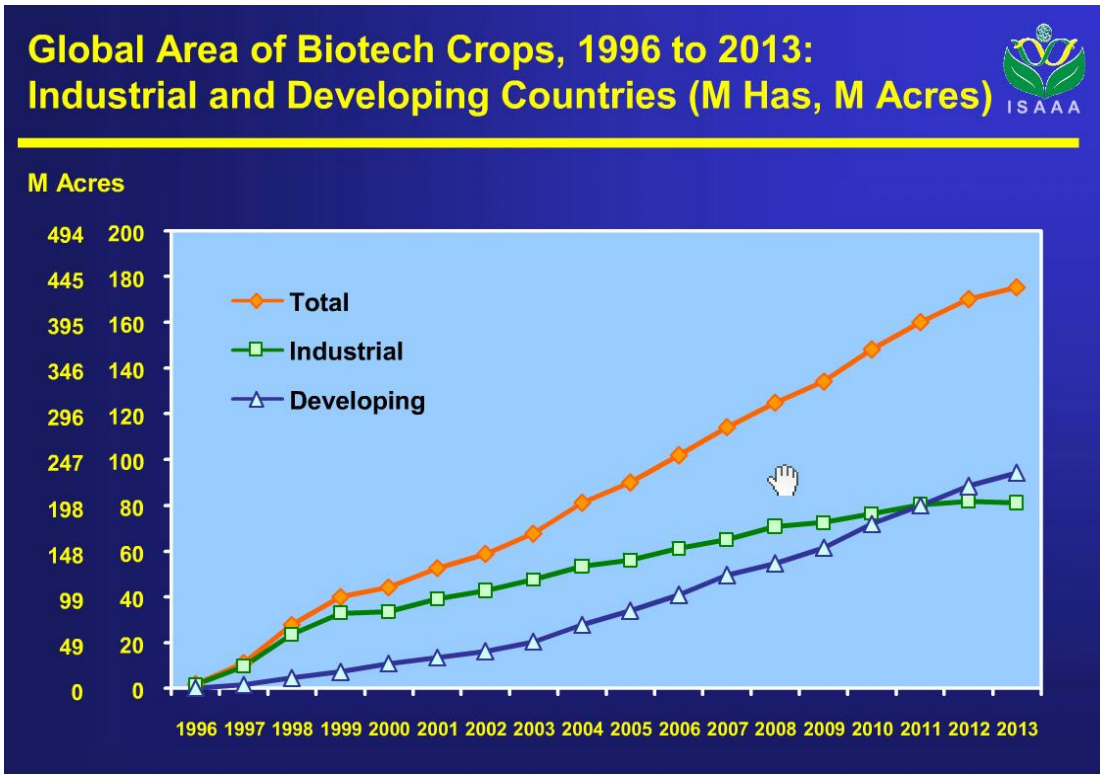


Figure 1.1: Global Area of Biotech Crops in 2013 (James 2013) Source: International Service for the Acquisition of Agri-Biotech Applications

1.3 Applications

Genetic modifications, especially for crops, are considered for some enhancement of traits which are generally developed for commercial purposes. These traits are focused productive qualities e.g. increasing starch or sugar content, control of ripening, resistance to diseases, resistance to pests, herbicide resistance for weed control purposes, drought tolerance, tolerance for freezing, salt tolerance, plant structure and production of specialty substances like vitamins as well as pharmaceuticals.

As Brookes stated in his report; "In 2011 GM herbicide tolerant (HT) soybeans dominate, accounting for 38% of the total, followed by insect resistant (IR: largely Bt) maize, HT maize and IR cotton with respective shares of 25%, 19% and 12%. In total,

HT crops account for 63%, and insect resistant crops account for 37% of global plantings" (Brookes G 2013).

1.4 Methodology

The technique of plant genetic engineering by DNA delivery to obtain GM Crops is generally called plant transformation. Although a there are various methods for the transformation of the DNA, both *Agrobacterium*- and biolistic-mediated DNA delivery methods considered as the two commonly adopted approaches (Barampuram and Zhang 2011).

Agrobacterium tumefaciens is a soil bacterium which induces tumor formation by infecting many types of plant species. The bacterium does not enter the plant cell, but transfers part of the Ti plasmid to the plant nucleus. The transferred part of the Ti genome is called T-DNA. It becomes integrated into the plant genome, where it expresses the functions needed to synthesize opines and to transform the plant cell. Transferring genes into the T-DNA region by co-transferring Ti plasmids facilitates integration of desired sequences into the host genome. So far, 80% of the produced transgenic plants have been modified with *Agrobacterium tumefaciens* based plant genetic engineering methods. (Nester 2008).

Other methods that are being used for plant transformation are Electroporation-mediated transformation, Polyethylene glycol (PEG)-mediated transformation, Silicon Carbide-mediated transformation (SCMT), microinjection and chloroplast-mediated transformation.

Generally, ensuring that the transformation is carried out to introduce the gene of interest with the methods above requires the use of selectable marker genes. These marker genes allow resistance to certain conditions like antibiotic concentration, so there are some concerns about eliminating the need for selectable marker genes in the future.

1.5 Concerns

Genetically Modified Crops have always been problematic since their first appearance for several reasons. Environmental and human health concerns were the primarily debated subjects. Moreover, DNA modification of living organisms, copyrighting biological innovations and commercial opportunity equality concerns are heavily debated.

Although recombinant microbes have been used in many industrial applications for a long time, when crops are concerned, GM crops draw big reaction as they are consumed directly. And also ideological point of subject, intellectual properties, regulation of food security have been discussed in ethical aspects. Although GMO technology is seriously criticized, while these concerns are hold on, GM Crop engineering has been the fastest adopted agricultural technology in the history of this field (Chassy 2007).

1.6 GMO Regulations and Validations

1.6.1 GMO in Turkey

The Turkish Biosafety Law (Law No. 5977) was put into force on September 26, 2010 and with two relevant regulations at the same time: one of them is regarded as the "New GMO Regulations" which consists of GMOs and their products and the other one was related to procedures and rules of the "Biosafety Council" which reorganizes the operation procedures and authority of the committee. Both of the regulations were published at August 13, 2010 in the Official Gazette No. 27671 (http://www.resmigazete.gov.tr/eskiler/2010/08/20100813-4.htm). There are still Biosafety Council and related scientific subcommittees in Turkey for evaluation of the risk and potential socio-economic impact of the genetically modified organisms. Three soybean GM events (MON-04032-6, A2704-12, and MON89788) and 13 maize events

(Bt11, DAS59122, DAS1507, NK603, DAS1507 x NK603, NK603 x MON810, GA21, MON89034, MON89034 x NK603, Bt11 x GA21, 59122 x 1507 x NK603, 1507 x 59122 and MON88017 x MON810) were approved to be used only in feed (http://www.tbbdm.gov.tr/en/Home/BioSafetyCouncilHome).

As summarized in GMO-Compass' evaluation, British consultant Graham Brookes (PG Economics) stated that "Turkey's biosafety law has had a substantial negative economic impact on the food manufacturing and livestock production sectors. Turkey has approved 16 GM crop plants for feed use and introduced a zero tolerance threshold for the presence of unapproved GMOs. This number stands in stark contrast to the 56 GM crops which are marketed globally for food and animal feed production. Total separation of different GM crops along the entire global production and transport chain is practically impossible to achieve, meaning that even slight traces of unapproved GMOs can make many agricultural imports unmarketable in Turkey".

According to this study, Turkey's biosafety policy caused a remarkable market and trade loss, corresponding to $0.8 billion as of 2009. When compared to Turkish food and drink sector's annual net profitability, this value is corresponding between 33% and 50% of the sector in total (GMO-Compass 2012).

1.6.2 GMO in Other Countries

Similar to Turkey's policy, there is a strict biosafety regulations and applications on GMOs worldwide, indeed multidisciplinary councils and advisory committees are organized for the evaluation of technical and scientific concerns regarding to the GMOs. However, the strictness of policies vary among countries.

For instance, in the European Union the Genetically Modified crop applications are strictly regulated and a framework has been structured extensively since early 90s for EU legislation on GMOs. The EU has the probably strictest regulations for GMO levels as low as 0.9%, requiring the exceeding contents of food and feed to be labelled. And a zero tolerance policy is practiced for GMOs which have not been approved

meaning that freights of GMOs over threshold and unapproved GM crop products will be rejected or destroyed (Davison 2010). Primarily, impact of GMOs on human health and protection of the environment have been the concern of EU legislations. A detailed approval process based on scientific risk assessment for health and environment is implemented for the authorization of a GMO or a GMO derived food or feed product. Moreover, legislation of EU aims to inform consumers about GM food and feed in market by labelling, as the labelling is mandatory when genetic modification of crops is in question. In 1998, Regulation (EC) No. 1139/98 was brought into force, which made the GM food and feed labeling mandatory in food ingredients. However, these labeling could only be shown on products of which DNA and protein detection could be applied. These labeling requirements which take the detection of DNA or protein into consideration led to the necessities related to GMO detection activities in the EU.

After this regulation, EU amended (EC) No. 1139/98 with (EC) No. 49/2000 to update some concepts like 1% GMO threshold and adventitious presence. After that, quantitative GMO detection and labelling became important issues.

In 2003, "Regulation (EC) No. 1829/2003 concerning genetically modified food and feed" and "Regulation (EC) No. 1830/2003 concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms" have been taken into force. Food and feed consisting of, containing, or produced from GMOs have been regulated by "Regulation (EC) No 1829/2003 on genetically modified food and feed" to be marketed or produced in European Union. Shortly, according to regulations only authorized GM food and feed can be placed on the EU market. European Community and member states, and also the European Food Safety Authority (EFSA) are involved in processing single-risk assessment for this authorization (Žel, Milavec et al. 2012).

For the evaluation of GMO specific issues in technical and scientific view, many countries have structured specific multidisciplinary advisory groups e.g Canadian Food Inspection Agency (CFIA), Australia and New Zealand Food Authority (ANZFA), Animal and Plant Health Protection Inspection Service (APHIS) (USA),

The Environmental Protection Agency (EPA) (USA), Food and Drug Administration (FDA) (USA) as in EU. These authorization bodies are responsible for assessment of GM crops and products based on experimental data (Kamle and Ali 2013).

1.7 Products and Services

GMO detection analysis is the fastest growing food testing segment globally and by 2018, food safety analysis sector is predicted to reach $19.7 billion according to a recent report from Global Industry Analysts (GIA) (GIA 2013).

In all chains of production steps for food and feed as well as seed lots, traceability and labelling requirements should properly be met, and detection of GMO presence, identification and quantification of the GM content should be carried out. The basis for every type of GMO detection technology is to determine the content discrimination between the unmodified plant and the transgenic variety.

Principally, validated biological methods or test kits are used in testing. Along with institutional laboratories, commercial laboratories provide GMO testing services.

1.8 GMO Detection Technologies

Genetic modifications result in change of DNA, RNA or protein molecules, so detection can be done associated corresponding molecule prior to modification (Miraglia, Berdal et al. 2004). Modified DNA or expressed protein regarding to modified DNA could be identified in raw or processed food and feed derived from GMOs (Ahmed 2002). For detection validity, it is important to obtain reference food or feed matrix and also statistically acceptable parameters in sampling and detection. As DNA is more stable molecule than protein, detection strategies are generally centered in DNA based methods.

1.8.1 Protein-based methods

Immunoassay techniques based on antibodies are the methods of choice for laboratory and field use, and it allows qualitative and quantitative approaches for known target analytes (Brett, Chambers et al. 1999). Depending of the detection approach, both monoclonal and polyclonal antibodies can be used (Kamle and Ali 2013). Protein immunoassay can detect 1% threshold level for the recombinant protein presence in sample matrix to be tested (Stave 2002).

1.8.1.1 ELISA

ELISA (enzyme-linked immunosorbent assay) tests are suitable for detection of recombinant proteins resulting from genetic modification of DNA. For detection of GMO, various ELISA methods have been developed and used. Practicability, rapidity and cost effectiveness of the method are the main advantages of ELISA test strips (Thomison and Loux 2001). However, their application is limited to protein molecules which are undenaturated, as the heat processes cause denaturation the proteins. This cause detection of proteins challenging.

In addition to that, for sensitive assays which are also specific to single epitope, monoclonal and polyclonal antibodies are being used for the development of immunoassays targeting recombinant protein like CP4EPSPS as used in detection of genetically modified soybean (Park 2004).

1.8.1.2 Immuno-strip

As a variation of ELISA format, immune-strips are designed under sandwich ELISA principles. In method, extracted sample is soaked to a filter membrane, then it was let to be combined with a complex particle composed of dye and antibody. Recombinant protein representing the GM in the sample combines with this complex particle. Then this complex compound is run on the filter membrane having two distinct zones; for

test and control. The first one is for capturing protein-complex compound, it allows the compound molecules to be aligned intensely, and to be visible by an observer. If the sample is to give positive result, test zone will show a line to be seen with naked eye. In contrast, this zone will be blank as the negative sample is applied on the membrane. However, a visible mark line will be observed on control zone no matter the sample is positive or negative, confirming the test is working flawlessly. (Miraglia, Berdal et al. 2004).

1.8.1.3 Immuno-PCR

"Immuno-PCR" is a combined method, which is sensitive and specific for detecting the antigen, by using a DNA sequence specific to the target protein. This DNA marker is used as a PCR template, allowing sensitivity of PCR and protein specificity at the same time (Liang, Cordova et al. 2003). This method was reported to be used for Cry1Ac, a Bt toxin protein (Allen, Rogelj et al. 2006).

1.8.2 Nucleic acid-based methods

A GMO construct is typically a composition of these elements: The promoter component which is functioning as an on/off switch for target gene to be expressed; the inserted/altered gene which has been is coding for intended trait; the terminator element for initiation of the inserted/altered gene to stop its activity. In addition to these elements, several different components can be present within inserted gene sequence, which functions as controlling and stabilizing agents of the inserted gene, demonstrating the inserted sequence as a marker, or facilitating organization of order different elements in the construct (Figure 1.2). Inheriting the integrated gene construct is also an important issue, because the trait should be inherited stably. Hence, the genome organism in which the genome modified is also an important factor. The recombination event which occurs in single cell uniquely, used to generate entire transgenic plants is called as GMO "event".

Nucleic acid-based GMO detection is a DNA targeted approach and look for transformed sequences. As PCR is capable of detection of sequences which are endogenous to taxa as well as transformed sequence, it allows to track relative amplification parameters and it is regarded as "gold standard" for GMO testing in Europe and Asia (Fagan 2007).

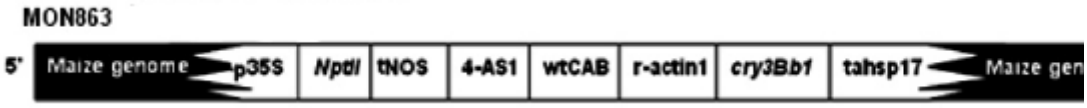


Figure 1.2: MON863 GMO event resulting from transformation of synthetic constructs into maize genome. Source: GMO Detection method Database (GMDD) (http://gmdd.shgmo.org/event/view/84)

Target DNA is amplified in vitro, when suitable buffer solution, DNA polymerase enzyme, dNTP mixture; oligonucleotide primers are present in PCR tube. It is important that there are no PCR inhibitors in reaction in solution. It selectively amplifies specific sequences of DNA sequence from variety of sources (i.e. bacteria, virus, human, plant) millions of times logarithmically in a short time. Also microsatellite analysis, RFLP and sequencing are examples of PCR technique based genetic applications.

This sensitivity and availability of amplification parameters allows PCR methods to give quantitative analysis results. The first method validated by European Union Reference Laboratory is PCR based, and may be used for the analysis of many authorized GMOs that could be placed on the market (Lipp, Brodmann et al. 1999). This method screens for 35S promoter and NOS terminator sequence (Pietsch, Waiblinger et al. 1997). Method validation is coordinated by The Institute for Reference Materials and Measurements (IRMM), which is one of the seven institutes of the Joint Research Centre (JRC) of European Commission.

PCR assays are intended to amplify specific regions of DNA therefore it allows also the detection of a specific sequence of the recombinant DNA. The sequence which is used will help to adjust the selectivity of the test. For example, designing a method for detecting promoter or a terminator sequence would be used for screening purposes as many of the GMO constructs have these promoter and terminator sequences. These elements are originally obtained from bacteria or viruses therefore detecting these elements may not be the exact confirmation of GMO presence in the test sample. Additionally, to be more certain for the presence GM material, more selective test which is specific for analyte would need to be applied to sample. If the sample contains bacteria or virus which are having screen specific sequences, the test would result as false negative. Indeed there are many factors that could cause false positive results, therefore, validated and standardized methods are needed to be applied in laboratory testing.

Event-specific PCR methods, unlike construct-specific assays (Figure 1.3), are designed to amplify the junction region of plant genome and inserted DNA sequence. Each GM event contains this unique junction region, therefore it allows specific detection of the event. Construct-specific methods involve regions inside the construct of the recombinant DNA. Sometimes event developer use multiple constructs or multiple events for GM trait. As construct-specific methods would be specific to amplify more than one GM event, they are less specific compared to event-specific approaches evidently.

1.8.2.1 End-point PCR

End-point PCR is a qualitative detection method which is referring to final PCR products obtained at the end of PCR reaction cycles to be detected. Generally in this method, single-target approach is applied and amplifications are carried out in separate tubes. So, if there is multiple elements to be detected, a series of PCR tests may need to be applied on the sample.

End-point PCR assay results are evaluated by observing presence of the amplification, therefore semi-quantitative assay may only be applied by comparing relative amplification intensity of known template DNA concentrations (Tozzini, Martínez et al. 2000). Although it is possible, it is generally not practical to design quantitative assays with End-point PCR.

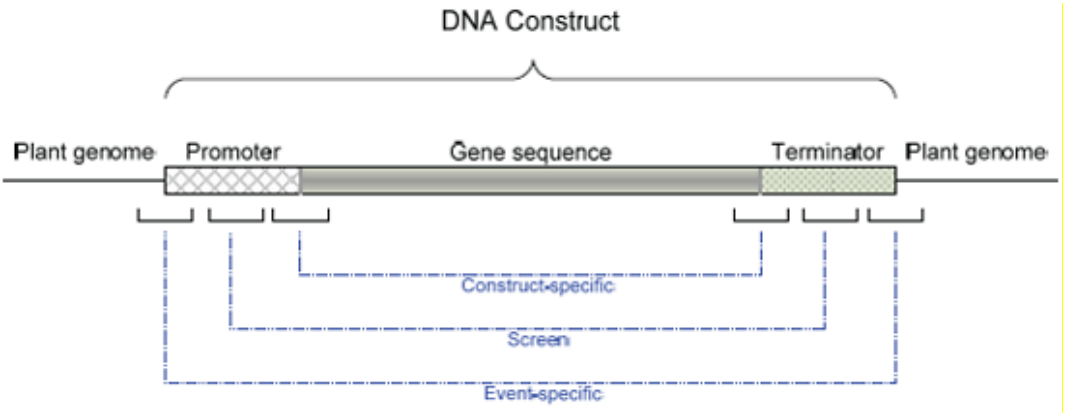


Figure 1.3: Simplified diagram of transformed DNA construct and targeting approaches. Source: Australian Government Department of Agriculture (http://www.daff.gov.au/agriculture-food/biotechnology/reports/maintaining_product_integrity_in_the_australian_seed_and_grain_supply_chain/section_1_introduction/chapter_4_an_introduction_to_sampling_and_testing)

1.8.2.2 Real-time PCR

Real time PCR is used for quantification of a target DNA sequence. For the products to be detected, amplification of DNA templates are carried out with sequence specific oligonucleotides labeled with a fluorescent reporter therefore the detection of the amplified product can be done as reaction goes on. Real-time PCR has superior efficiency in all other PCR assays in validating and estimating the number of copies of inserted genes into the host genome (Holst-Jensen, Ronning et al. 2003). With Real-

time PCR, PCR product is detected during the amplification process by monitoring an increase in fluorescence throughout the PCR therefore it can be used efficiently and fast as a quantitative detection method. Fluorescent dye or probes allow detection of hybridization as fluorescent signal. In both case, the intensity of the fluorescence is directly proportional to the amount of amplified product. This technique is increasingly used for end-point analysis and for qualitative detection purposes. This novel feature of RT-PCR is due to its increased intrinsic specificity and the fact that it allows extrapolation of results directly from the instrument software allows skipping over gel electrophoresis analysis of PCR products, a step that causes the main laboratory contamination risk (Querci, Foti et al. 2009).

1.8.2.3 Microarray techniques

Microarray advances concurrent recognition of various DNA sequences simultaneously, theoretically, are very suited for utilization as a screening technique for GMO detection. In late 2007, the first commercial microarray tool for GMO screening was approved in the EU through a inter-laboratory study which was facilitated by the Joint Research Center of the European Commission (HAMELS, LEIMANIS et al. 2007). The effectiveness of the Dualchip® GMO test (Dualchip is an enlisted trademark of Eppendorf Array Technologies) was evaluated as a qualitative technique for screening for GMO authorized in the European Union. In the collaborative study, detection of target DNA elements was possible at 0.1% GM concentration with a precision rate of 95% utilizing blind DNA sample references.

1.8.2.4 DNA fingerprinting techniques

DNA fingerprinting can also be considered as a detection approach for GMOs. A DNA fingerprinting methodology has been developed by the Canadian Food Inspection Agency (CFIA) for qualitative detection of approved and unapproved GM crops. The method targets basic genetic components, for example, promoters and terminators, and gives a unique "fingerprint" pattern focused around the gene sequence adjoining the promoter or terminator (the coding region of introduced trait gene). Limit of Detection (LOD) value of 0.5% GM seed or grain could be obtained with DNA fingerprinting patterns developed by the CFIA. Although DNA fingerprinting is not a quantitative technique for interpreting GMO levels in a sample, it could be used to screen wide variety of GM events simultaneously. NMI, in Australia is working with CFIA for validation of these methods and development of new fingerprint patterns for Australia (Raymond, Gendron et al. 2010).

1.9 GMO Reference Materials

Reference materials which have been used as positive controls for qualitative and quantitative analysis are described in GMO Detection Technologies section. Usually, a certified reference material is preferred over a reference material without a certificate or where the certificate is lacking essential information (Žel, Mazzara et al. 2008). If fair trade, environment protection, food reliability and consumer protection issues are in question, measurement results become very important for public confidence in modern societies. Hence, certified reference materials (CRMs) are critical requirements of modern analytical quality assurance as they allow calibration of instruments, method validations, and quality control of methods and laboratories based on traceability and comparability of measurement results. Today, both the American Oil Chemists' Society (AOCS) and the Institute for Reference Materials and Measurements (IRMM) and are the two major developers of CRMs for GMO detection purposes. There have been many different CRMs for different GM events have been developed and introduced by IRMM and AOCS, which includes over 30 dried powder

CRMs, 10 genomic DNA CRMs, and 3 plasmid DNA CRMs. Certificates carry a validated certified value with its uncertainty values for measurement which is traceable either to an internationally accepted reference or a SI unit. The intended use of those parameters for each CRM is stated on their certificates. However, there are still many GMO events that are not available for corresponding CRMs. Dried powder CRMs and plasmid DNA calibrants are the two types of CRM DNAs which is intended to be used for GMO analysis. Preferably CRMs should be used for the presence of the analyte in question, when they are not available, a positive control sample (e.g., in-house or collaboratively verified) can be used as reference sample. It is important to be aware that CRMs are certified for the presence of a given event and not for the absence of other events. However, trace contaminations of CRMs by other GM events may be detected on a regular basis.

One important issue that needs to be emphasized is the biological factors related with the sample tested in relation to reference materials. There are some important factors which is mostly plant related, that can have an impact on GMO quantification like tissue ploidy, parental origin and zygosity of the GM plant (Zhang, Corlet et al. 2008). One of examples is that of seeds which are composed of different endosperm, tissues, pericarp and embryo. Each of these tissues has a different ploidy levels and has a different ratio of maternal/ paternal origins. Therefore, the correlation between mass and DNA copy number is complex and may vary from sample to sample. Variable ratios of different tissues can be present influencing the final result of tests in analyzed samples.

1.10 Plasmid DNA Calibrants as GMO Reference Materials

The CRMs prepared as dried powder have been used since early days as a benchmark for quantity traceability analysis and GM amounts due to their properties of similarity with blind samples and easy traceability to the International System of Units (SI) of mass (gram). However, some obstacles like limited quantification range, inconvenient preparation procedures, high cost and difficulty to get homogeneous candidate samples may cause some limitations with the dried powder CRMs for GMO analysis. To

overcome these problems, plasmid calibrants was started to be used as CRMs was and have gained popularity (Taverniers, Windels et al. 2005). The main advantage of plasmid calibrants which are recombinant plasmids containing genetically modified sequence and endogenous reference gene sequence is having a known copy/copy ratio.

In contrast to conventional CRMs whose genomic DNA molecules are latter extracted from their plant matrix, plasmid calibrants have been demonstrated to be a good alternative to those genomic DNAs in GMO quantification. Besides, when compared plasmid DNA to genomic DNA as standards for quantification of GM elements, their results showed that the standard curve obtained with plasmid calibrant gave a better determination regarding to the true GM percentage in blind samples (Burns, Corbisier et al. 2006). Furthermore, if certain precaution measures are taken to prevent contamination, plasmid calibrants have several important advantages over dried powder CRMs, such as lower cost, higher stability and easier production procedures. Despite the plasmid calibrants have some contamination problems during experimental procedures, those problems may be easily overcome (Borst, Box et al. 2004). When their convenience and low cost are considered, plasmid calibrants have been evaluated as a good substitute for CRMs obtained using raw plant matrices. International collaborative validation is required for proposing a CRM for a GMO and up to now, there have been some examples of plasmids which had been validated as CRM DNAs. For instance, a plasmid reference have been validated for the detection of Roundup Ready soybean event GTS-40-3-2 by inter-laboratory ring trial (Lievens, Bellocchi et al. 2010). Four other plasmid reference DNAs, ERM-AD413, ERM-AD415, ERM-AD425 and ERM-AD427, have also been certified and commercialized worldwide by IRMM.

1.11 Aim of the Study

In the world, dried powders and plasmid calibrants are now basically two types of CRMs used for GMO detection and quantification. Dried powder CRMs are produced by mixing GM and non-GM seed powders at known ratios of mass/mass in terms of

grams. In our approach, plasmid calibrants which are recombinant plasmids containing specific sequences for detection of the GM event and the endogenous reference (35S promoter, NOS terminator, Bt11 event-specific, maize endogenous, GTS 40-3-2 event-specific and soya endogenous fragments) were mixed with a copy/copy ratio. Constructed plasmid DNAs were developed to overcome some limitations liberated by the dried plant matrix powder CRMs and have been demonstrated to be an alternative to genomic DNA (gDNA) extracted from conventional dried powder CRMs.

In this study, construction of plasmid DNAs was carried out which of them could be used as positive control templates in end-point and real-time PCR reactions for qualitative and quantitative GMO detection purposes. For this purpose, pCAMBIA 1304 derived single-target plasmids were constructed for targeting 35S promoter, soybean event GTS 40-3-2 and endogenous soybean gene, maize event Bt11 and endogenous maize gene. Apart from this, untouched pCAMBIA 1304 plasmid contains NOS terminator sequence and it was verified with tNOS screening method by PCR. Consequently, 35S target plasmid could be used as a double-target (35S & NOS) template. For the convenience and adaptability, target fragments were chosen from the methods that are found in EU Database of Reference Methods for GMO Analysis, and cloned into the plasmid. The cloning of fragments was carried out by double digestion of the fragments and sticky-end ligation. Presence of 35S and NOS fragments in plasmid constructs are verified qualitatively by PCR. The usability of reference plasmids for relative quantification of event GTS 40-3-2 and Bt11 was shown by using SYBR Green real-time PCR assays. For testing the reliability of the approach, verification and measurement uncertainty based on within (single) laboratory results was estimated for plasmid constructs composed of Roundup Ready Soya relative quantification components; GTS 40-3-2 event-specific PCR and Soya endogenous Le1 PCR amplifications.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals, Reagents and Kits

All of chemicals in this study were purchased from Sigma-Aldrich Chemical Company, AppliChem Chemical Company, and Merck Chemical Company. Double distilled water was used for each solution prepared to be used in experiments. PCR grade water is used for plasmid dilutions and PCR mixtures. Chemicals, enzymes, and molecular biology kits for methods such as electrophoresis, polymerase chain reaction (PCR), nucleic acid purification and handling and enzymatic digestion were purchased mainly from Fermentas (Thermo Fisher Scientific Inc; Ontario, Canada), NANObiz (Turkey) and Roche Applied Science (IN, USA).

2.1.2 Plasmid, Bacterial Host Strain and Media

pCAMBIA 1304 plasmid was purchased from Cambia (Australia) and *E.coli* strain TOP10 bacteria was purchased from Invitrogen (CA, USA). Luria Bertani (LB) medium which is prepared with appropriate antibiotics is used for culturing TOP10 cells. SOC medium was used to improve recovery of transformants during growing period after introduction of plasmids into competent TOP10 cells.

2.1.3 Oligonucleotides

Oligonucleotides were purchased from biomers.net GmbH (Germany) and İontek (Turkey) in lyophilized tubes.

2.2 Methods

2.2.1 DNA Isolation Methods

Bacterial Plasmid DNA isolations, plant genomic DNA isolations from dried powders and recovery of DNA from agarose gel were carried out in the study. DNA amounts obtained were determined by measuring UV absorption by using an ND-2000 spectrophotometer at 260 nm (NanoDrop Products, Wilmington, DE). And the absorbance ratio of 260/280 nm wavelength of the extracted DNA was evaluated to measure purity of DNA samples (accepted ratios are between 1.7 and 2.0).

2.2.1.1 Plasmid DNA Isolation

pCAMBIA 1304 plasmid and modified pCAMBIA1304 constructs were isolated from the host TOP10 *E.coli* by DNA4U Plasmid Isolation Kit (NANObiz Nano-Biotechnological Systems, Ankara, TR). Plasmids were eluted by PCR-grade water in last step of isolations. A single colony of TOP10 was cultured in 5 mL of liquid LB with continuous shaking at 180 rpm overnight at 37°C.1.5 mL of culture was starting material for each isolation and isolation procedure is applied according to procedure of the provider. At the end of the procedure retained plasmid DNA in the spin column was eluted with 50 μL of dH_2O provided within the kit.

2.2.1.2 DNA Isolation from Plant Materials

Plant genomic DNA purifications were done from GMO Certified Reference Materials of Joint Research Center Institute for Reference Materials and Measurements (JRC-IRMM) 10% Roundup Ready™ Soya Bean Powder, certified Reference Material ERM-BF410 and 5% Bt11 Maize GMO Standard ERM-BF412 were purchased from Fluka (Sigma–Aldrich, Buchs, Switzerland). Purified DNAs were used as templates

for cloning of GMO bearing and endogenous fragments, and also for positive control amplifications in PCR.

NANObiz DNA4U Plant Genomic Isolation Kit was used to isolate genomic DNA. According to kit procedure, 20 mg of seed flour is used as starting material. At the end of the procedure, the genomic DNA which retained in the dried spin column was eluted with 50 μL of pre-heated dH_2O provided within the kit.

2.2.1.3 Recovery of DNA from Agarose Gel and PCR Products

Digested and linearized plasmid fragments and PCR amplified sequences for cloning were recovered from agarose gel by DNA4U Gel Extraction Kit. Bands of DNA fragments were excised from gel using a clean lancet blade under black light UV (long-wavelength). The gel-slices was placed in 2 mL-tubes and weighed for applying kit procedure.

Before restriction enzyme digestion of PCR amplified fragments, PCR products are purified with DNA4U PCR Purification Kit (NANObiz) according to the instructions of the provider. The kit procedure employs usage of spin-columns, buffers and centrifugations at room temperature. At the end of the procedure, the DNA retained on the dried silica column was eluted with 30 μL of dH_2O provided with both the gel extraction and PCR clean-up kit.

2.2.2 Construction of Single-Target Plasmids

2.2.2.1 Preparation of Competent *E.coli* TOP10 Cells for Transformation

Rubidium chloride (RbCl) based method was used to prepare competent TOP10 *E. coli* cells. A single chosen colony of TOP10 was inoculated in 5 mL of liquid LB with continuous shaking at 180 rpm at 37°C overnight. The prepared pre-culture was used to culture liquid LB medium with a volume of 200 mL. Culture was orbitally shaken at 180 rpm and 37°C until the bacterial suspension reached to an OD at 600 nm around 0.5. Then cells were chilled on ice and temperature was maintained with ice during

procedure. Solution of medium with grown bacteria was incubated on ice for 15 min, then centrifuged at 4000x g to obtain pellet for 10 minutes in a cooled centrifuge at 4°C. Re-suspension of centrifuged pellet was carried out with filter sterilized pre-chilled 40 mL of re-suspension buffer at pH 5.8. This buffer contains 100 mM RbCl, 50 mM Manganese (II) chloride, 10 mM Calcium chloride, 30 mM Potassium acetate and 15% (v/v) glycerol. In latter step, an ice incubation for 15 min and centrifugation for obtaining pellet were carried out one more time. Then a second re-suspension buffer of 8 mL used which is again filter sterilized and cooled on ice to re-suspend centrifuged pellet. This buffer contains 10 mM RbCl, 10 mM MOPS, 75 mM Calcium chloride and 15% (v/v) glycerol with pH 6.8. Finally, re-suspended solution was dispensed as 100 µL volumes directly into tubes as they were cooled with liquid nitrogen After a final incubation on ice for 15 min, aliquots of 100 µL were dispensed into clean 1.5 mL-tubes and immediately frozen in liquid nitrogen. For further use, they were stored at -80 freezer. (See Appendix B for medium compositions)

2.2.2.2 Heat-Shock Transformation of pCAMBIA1304 Plasmid into *E.coli* TOP10 strain

Heat-shock method was used to transform competent TOP10 *E. coli* cells. Plasmid DNA between 10 and 50 ng was introduced on thawed competent cells by pipetting on ice while the cell solution was still icy. Then tube was mixed by finger tapping gently, then incubated on ice for 30 minutes. Then heat-shocked was done on cells by incubating tube in heat block at 42°C for 45 seconds. After the heat-shock, tubes were rapidly taken on ice for 1 minute. 500 µL SOC medium was added on immediately after 1 min incubation, then the tubes were taken into 37°C incubator and orbitally shaken for 1 hour at 180 rpm for the recovery of the cells. This cell mixture were then spread on pre-warmed and kanamycin supplemented LB plates with 100 µg/ml concentration. Verification of transformations was done by checking colonies with colony PCR; randomly chosen colonies were labelled and taken as PCR templates. Amplifications having expected product size were regarded as verification of transformation.

2.2.2.3 Digestion Conditions for Linearization of pCAMBIA1304

Depending on the known sequence of plasmid (GenBank Accesion No: AF234300.1, Appendix A) *XhoI* and *NcoI* restriction enzymes are ordered for linearization of pCAMBIA1304. Restriction enzymes were purchased from Fermentas (Thermo Scientific Company), and a double digestion method was optimized with 2X Tango Buffer. Double Digestion of plasmid DNA is carried out according to following digestion conditions and incubated at 37^0C for 2 hours. Listing of components can be shown as in table 2.1.

Table 2.1 Double Digestion Reaction Components Used for pCAMBIA 1304

Component	**Amount of component (20 µl)**
DNA	500-1000ng
Fermentas 10X Tango Buffer	4 µl
Fermentas NcoI	2 µl
Fermentas XhoI	2 µl
ddH_2O	Up to final volume

2.2.2.4 Primer Design for PCR Cloning

For the compatibility of target plasmids with pre-validated methods, cloning fragments were chosen from the sequences which include target sequences to be detected in EU Database of Reference Methods for GMO Analysis. Chosen detection primers and corresponding cloning primers are listed in Table 2.2.

Table 2.2 Chosen Primer Oligonucleotides for GM Detection PCR Assays

Method	Target	Method Primers	Amplicon Length
Method Qualitative PCR method for detection of Cauliflower Mosaic Virus 35S promoter (Lipp et al.,2001).	35S (Element-Specific)	Primer Forward: 5'-CCACGTCTTCAAAGCAAGTGG-3'	123 bp
		Primer Reverse: 5'-TCCTCTCCAAATGAAATGAACTTCC-3'	
Qualitative PCR method for detection of nopaline synthase terminator (L 00.00-31, 1998).	NOS (Element-Specific)	Primer Forward: 5'-GAATCCTGTTGCCGGTCTTG-3'	180 bp
		Primer Reverse: 5'-TTATCCTAGTTTGCGCGCTA-3'	
Quantitative PCR method for detection of maize event Bt11 (Mazzara et al., 2005).	Bt11 (Maize Event-specific)	Primer Forward: 5'-GCGGAACCCCTATTTGTTTA-3'	70 bp
		Primer Reverse: 5'-TCCAAGAATCCCTCCATGAG-3'	
	adh1 (Maize taxon-specific)	Primer Forward: 5'-CGTCGTTTCCCATCTCTTCCTCC-3'	135 bp
		Primer Reverse: 5'-CCACTCCGAGACCCTCAGTC-3'	
Quantitative PCR method for detection of soybean event GTS-40-3-2 (Mazzara et al., 2007).	GTS 40-3-2 (Soybean Event-specific)	Primer Forward: 5'-TTCATTCAAAATAAGATCATACATACAGGTT-3'	84 bp
		Primer Reverse: 5'-GGCATTTGTAGGAGCCACCTT-3'	
	le1 (Soybean taxon-specific)	Primer Forward: 5'-CCAGCTTCGCCGCTTCCTTC-3'	74 bp
		Primer Reverse: 5'-GAAGGCAAGCCCATCTGCAAGCC-3'	

For cloning fragments, primers were designed by adding restriction site and random nucleotide bases for efficient cleavage of restriction enzymes, XhoI and NcoI.(Figure

2.1) The main approach was adding restriction site bases to actual detection primers, however, isolation of fragments shorter than 100bp (70 bp for Bt11 amplicon) from the agarose gels were challenging. Therefore, for the convenience of agarose gel isolation, longer amplicon producing primer pairs were designed for GTS 40-3-2 and le1 fragment cloning. (Table 2.3)

XhoI Site:

NcoI Site:

Figure 2.1: Visual representation of restriction site sequences and their cleavage positions by restriction enzymes. As shown in table 2.3, four additional non-complementary nucleotide bases were added for restriction digestion enzymes to work functionally.

Table 2.3 Chosen Primer Oligonucleotides for Amplifying Target Sequences in PCR Cloning

Cloning Targets	Cloning Primers	Amplicon Length
35S (Element-Specific)	Primer Forward: 5'-CTAACTCGAGCCACGTCTTCAAAGCAAGTGG -3'	143 bp
	Primer Reverse: 5'-AGTCCCATGGTCCTCTCCAAATGAAATGAACTTCC -3'	
Bt11 (Maize Event-specific)	Primer Forward: 5'-CTAACTCGAGGCGGAACCCCTATTTGTTTA -3'	90 bp
	Primer Reverse: 5'-AGTCCCATGGTCCAAGAATCCCTCCATGAG -3'	
adh1 (Maize taxon-specific)	Primer Forward: 5'-CTAACTCGAGCGTCGTTTCCCATCTCTTCCTCC -3'	155 bp
	Primer Reverse: 5'-AGTCCCATGGCCACTCCGAGACCCTCAGTC -3'	
GTS 40-3-2 (Soybean Event-specific)	Primer Forward: 5'-CTAACTCGAGCCTTCAATTTAACCGATGC -3'	380 bp
	Primer Reverse: 5'-AGTCCCATGGGATAGTGGGATTGTGCGTCA -3'	
le1 (Soybean taxon-specific)	Primer Forward: 5'-CTAACTCGAGCCAGCTTCGCCGCTTCCTTC -3'	635 bp
	Primer Reverse: 5'-AGTCCCATGGGCGATCGAGTAGTGAGAGTCG -3'	

As pCAMBIA1304 plasmid has two XhoI and an NcoI restriction sites, double digestion with these two enzymes resulted in three linear fragments. The longest linear fragment having XhoI and NcoI restriction sites which had 9424 bp length was isolated for ligation.

2.2.2.5 PCR Conditions for Amplification of Target Sequences

The target sequences which are listed in Table 2.3, were amplified with PCR prior to the ligation with the linearized plasmid DNA. PCR primers were designed with added sequences of restriction sites and additional 4 bases for restriction enzymes to be functional. 1X Taq Buffer, 2mM dNTP mix, 2,5mM $MgCl_2$, 0,5 μM primer oligo each and 2UI Taq polymerase enzyme in 25μl is used for cloning PCR amplifications. Thermal cycling conditions for PCR amplifications are listed in Appendix C.

2.2.2.6 Restriction Enzyme Digestion of PCR Amplified Target Fragments

Double Digestion of restriction sites of amplicons are carried out according to following digestion conditions and incubated at 37^0C for 2 hours.

Table 2.4 Double Digestion Reaction Components Used for Cutting Sticky-end Target Sequences in PCR Cloning

DNA (100-500ng)	10uL
2X Fermentas Tango Buffer	6uL (10X Buffer)
Fermentas NcoI	2uL
Fermentas XhoI	2uL
ddH_2O	10uL

2.2.2.7 Conditions for Ligation of Amplified Fragments into Plasmid

Ligation is carried out according to following ligation conditions and incubated at 22^0C for 60 minutes in thermal cycler. Table 2.5 shows reaction parameters.

Table 2.5 Ligation Conditions for Integration of Target Sequences in PCR Cloning

Vector DNA	100ng
Insert	1:1 to 5:1 molar ratio of the vector
Fermentas 10X Ligase Buffer	2uL
T4 DNA Ligase	1uL
ddH_2O	up to 20uL

2.2.2.8 Colony PCR for Verification of Cloned Target Fragments

To confirm cloning of GMO bearing fragments into entry vector, colony PCR was done. The primer sets which have been used for amplification of cloning fragments were also used for colony PCR. The grown bacteria on LB agar containing 100 μg/ ml kanamycin was used as a template and replica plate was done for each used bacteria. The PCR products were analyzed by agarose electrophoresis. Verified clones were stocked in 40% glycerol stock and stored in -80°C.

2.2.3 Verification and Measurement Uncertainty Based on Single Laboratory Results of Plasmid Constructs as Positive Control Templates in GMO Detection Methods

2.2.3.1 Determination of 35S and NOS Fragments by Conventional PCR

Colony PCR verified plasmid bearing TOP10 cells were grown overnight and plasmid purified. Purified plasmids were used as PCR template in qualitative detection method. (Appendix E) PCR products were observed on 2.5% agarose gel. Presence of 123bp amplicon fragment for 35S detection and 180bp amplicon fragment were checked for verification.

2.2.3.2 Determination of Bt11, Maize Taxon Specific, GTS 40-3-2 and Soybean Taxon Fragments by Conventional PCR

Colony PCR verified plasmid bearing TOP10 cells were grown overnight and plasmid purified. Purified plasmids were used as PCR template in qualitative detection method. (Appendix D) PCR products were observed on 2.5% agarose gel. Presence of 70bp and 135bp amplicon fragments was screened for Bt11 and *adh*1 respectively for checking fragments' integration to plasmid. Presence of 84bp and 74bp amplicon fragments was observed for GTS 40-3-2 and le1 representing fragments respectively for the verification of sticky end ligation of the fragments.

2.2.4 Verification and Measurement Uncertainty of Plasmid Constructs as Calibrator Plasmid in Quantitative RT-PCR Assays

2.2.4.1 Determination of Event Specific and Taxon Specific Fragments by RT-PCR for Bt11 Maize and GTS 40-3-2 Soya

Plasmids which are intended to be used as RT-PCR calibrants were prepared from overnight grown cultures and purified by plasmid purification. DNA concentration were measured by nanodrop and diluted to be used in PCR protocol. PCR protocols were prepared according to Roche LightCycler® FastStart DNA Master^{PLUS} SYBR Green I manual, as the kit was planned to be used in RealTime-PCR amplification verifications for our constructed plasmids. Annealing temperatures of reactions were retrieved from Quantitative PCR method for detection of maize event Bt11 (Mazzara et al., 2005) and Quantitative PCR method for detection of soybean event GTS-40-3-2 (Mazzara et al., 2007) (Appendix E). Qualitative detection with respect to negative control samples were observed using LightCycler Software v4.1

2.2.4.2 Preparation of Plasmid Dilutions for Constructing Ready-to-Use Template Standards

For constructing a Quantitative PCR method which measures the DNA content of GMO in a sample, the results should be expressed as GM copy numbers in relation to the DNA copy numbers of a taxon-specific target, determined in terms of the haploid genome. Therefore we needed a conversion approach between plasmid mass and copy number. Assuming that the average mass of 1bp dsDNA is 660g/mole, and using Avogadro's constant (6.0221×10^{23}) following equation is carried out to make the conversion.

Equation 2.1 Calculation of Copy Number of Plasmids with Molecular Weight Conversion

$$\text{number of copies (molecules)} = \frac{X \text{ ng} * 6.0221 \times 10^{23} \text{ molecules/mole}}{(N * 660 \text{ g/mole})^{\dagger} * 1 \times 10^{9} \text{ ng/g}}$$

Where:

X = amount of amplicon (ng)

N = length of dsDNA amplicon

660 g/mole = average mass of 1 bp dsDNA

6.0221×10^{23} = Avogadro's constant

By applying this conversion equation, it becomes possible to estimate copy number of plasmid calibrators having GMO and taxon-specific fragments to be used as PCR templates. Therefore, serial dilutions were obtained from known copy number of purified plasmids by reading DNA concentrations with nanodrop and diluting plasmids from 10^6 to 10 copy numbers for constructing standard curves resulting from PCR amplifications.

2.2.4.3 Construction of Standard Curves for Reference Plasmids for Quantification of Event GTS 40-3-2 in Soya

Applicability of reference plasmids for quantification was intended to be shown for GMO event GTS 40-3-2 in Soya, therefore purified DNA of DNA ERM - BF410gk Certified Reference Material from JRC (100g of event GTS 40-3-2/1000g of Soya Powder) was used as positive control samples for verification purposes and 10 ng of isolated CRM DNA was used as positive controls.

Purified plasmid DNA samples of constructed plasmids having GTS 40-3-2 and Lectin event specific fragments were diluted according to final volume of the sample in reaction (5µl). Therefore several dilutions were carried out to meet 200000 copy of each plasmid per µl of undiluted sample of the setup by applying the conversion between copy numbers and mass of pDNA. That corresponds to 10^6 copy of each modified plasmid per 5µl sample. After that, seven dilutions in water were made to obtain following samples as known samples in PCR runs: 10^6– 10^5 – 10^4 – 10^3 – 10^2 – 50 – 25 – 10^1 copies per sample.

During PCR reaction, each sample generates a fluorescence regarding to hybridized DNA according to the amplification approach which is measured by RT-PCR cycler's sensing filters. It could be regarded as signal values in each cycle and can be plotted against cycle numbers. Simply, the higher copy number of the template found in reaction, the earlier amplicon signal generated and exceeds threshold earlier. A logarithmic correlation occurs between cycle numbers and template concentrations, as the PCR reaction results exponential numbers of products. This allows plotting amplification curves for each sample along the cycle numbers in the reaction. The fractional cycle number is referred to as the crossing point (Cp) on the LightCycler. Standard curves with calibrator plasmids were constructed for both GMO event GTS 40-3-2 and taxon-specific lectin (Le1) amplifying Real-Time PCR. Crossing Point (Cp) / logarithm of DNA copy number curves were drawn. Event specific and endogenous PCR amplifications were carried out in triple duplicates.

2.2.4.4 Statistical Analysis for the Applicability of Plasmid Calibrants in GMO Quantification

Each measurement or test has an error of estimation. When repeated, a test or estimation occasionally gives an alternate result, despite the fact that it normally is very much alike to the first result. Hence, a test or estimation gives just a rough guess of the true value of the quantity to be measured approximately. Therefore, measurement uncertainty is required when a measurement is in question.

As stated in "Guidance Document on Measurement Uncertainty for GMO Testing Laboratories" of JRC, "Measurement Uncertainty (MU), which should take account of all effects on a measurement process, is the most important single parameter that describes the quality of measurement" (S. Trapmann 2009). And also, analytical methods and their measurement uncertainties (MUs) are enforced in EU legislation and should be considered when a measurement is taken based on analytical methods. Therefore, we implemented evaluation of MU within the Q-PCR data of calibrator plasmids.

After the data of PCR amplifications for both event-specific and endogenous sequences were obtained, within-laboratory reproducibility (S_{RL}) and within-laboratory reproducibility relative standard deviation (RSD_R) are calculated for bias control to obtain verification and measurement uncertainty reports based on single laboratory results (Appendix F). Then the estimation of the uncertainty component associated with bias (u_{biasr}) and absolute bias ($bias_a$) was carried out. Relative Standard Uncertainty (RSU) was evaluated by combining Relative bias ($bias_r$) and within-laboratory reproducibility relative standard deviation (RSD_R). Finally, data on Limit of Detection (LOD), Limit of Quantification (LOQ) and Linearity were estimated (Appendix G-H).

CHAPTER 3

RESULTS AND DISCUSSION

In this study, constructed plasmids having 35S promoter, NOS terminator, Bt11 event-specific, Maize endogenous, GTS 40-3-2 event-specific and Soya endogenous fragments which have been used in PCR assays regarding to GMO detection methods were verified to be cloned and multiplied, used as calibrator DNA templates.

Moreover, usability of plasmids bearing GTS 40-3-2 event-specific and Soya endogenous fragments as calibrator template in qPCR assays were shown. Some statistical considerations using to obtain verification and measurement uncertainty reports based on single laboratory results were examined to show their reliability as reference materials in GMO quantification.

3.1 DNA Isolation from Certified Reference Materials

Column purification based method used for genomic DNA isolation from dried powder CRMs. For this purpose, NANObiz Plant DNA Isolation Kit was used. Eluted DNA content was highly concentrated (<200ng/µl) and the 260/280 nm absorption of eluents were over 1.8 threshold value as a purity indication. This method was successfully provided enough DNA for cloning and suitable enough for further PCR amplifications.

3.2 Confirmation of High-Copy pCAMBIA 1304 plasmids in Host *E. coli* TOP10 Cells and Plasmid DNA Isolation

First of all unmodified pCAMBIA 1304 plasmid transformed via heat-shock transformation and colony PCR was performed with NOS test primers to verify transformation. After confirmation, plasmid isolation with plasmid isolation kit was

applied on overnight grown transformed TOP10 *E.coli* culture. Then to ensure that the intact (12361 bp) plasmid was transformed, plasmid was cut with EcoRI restriction enzyme and was run on agarose gel (Figure 3.1). After linearized plasmid length was confirmed, additional modifications were performed on isolated plasmids to construct calibration templates.

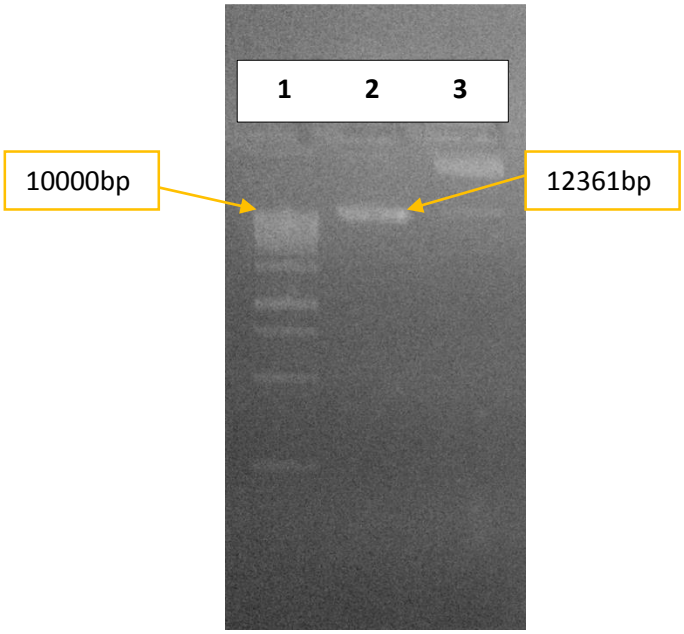


Figure 3.1: Length verification of unmodified plasmid. Lane 1- 250-10000bp ladder, lane 2 - Linear DNA band over 10000bp (EcoRI treated plasmid), lane 3- Untreated plasmid.

3.3 Amplification and Double-Digestion of Target Sequences to be cloned into plasmid

Target sequences were intended to be cloned from their original source; therefore CRM DNA's were used as template for cloning primer pairs. Oligonucleotide pairs in table 2.3 were used as primer sequences. Amplification resulted in additional sequences which allow XhoI and NcoI double digestion at the ends of target sequences. 35S, Bt11 and adh1 cloning amplifications were carried out with DNA template

isolated from 5% Bt11 Maize GMO Standard ERM-BF412, GTS 40-3-2 and Lectin cloning amplifications were carried out with DNA template isolated from 10% Roundup Ready™ Soya Bean Powder ERM-BF410 CRMs. PCR amplifications of cloning fragment was performed, then PCR purifications were carried out for each amplicon set.

Double-digestion of fragments were carried out with 2X Tango Buffer which is suitable for XhoI and NcoI double-digestion reactions. For this step, PCR purified fragments used in digestion reactions, then the reaction samples were run on agarose gels to obtain fragments free from enzyme buffers and small DNA fragments resulting from digestion reactions. In these steps, fragments which are less than 100 bp were significantly lost. Therefore, pooling multiple product samples were required for obtaining visible bands. Then visible fragments were excised from agarose gels and purified with gel extraction kit. Although the eluted DNA contents were below 250ng, they were quite enough for ligation reactions.

3.4 Preparation of Linearized pCAMBIA 1304 Prior to Ligation of Target Sequences

As pCAMBIA 1304 plasmid has single NcoI and double XhoI restriction sites, double digestion resulted in various size of fragments. This could be the result of fragment between two XhoI sites, and fragment between two of the XhoI sites and NcoI site. As it could be seen in lane 5 (Figure 3.2), the longest fragment corresponding to 9424 bp fragment between XhoI and NcoI sites was intended to be ligated with our target fragments. Therefore, the band at the top of the gel was excised and extracted with gel extraction kit.

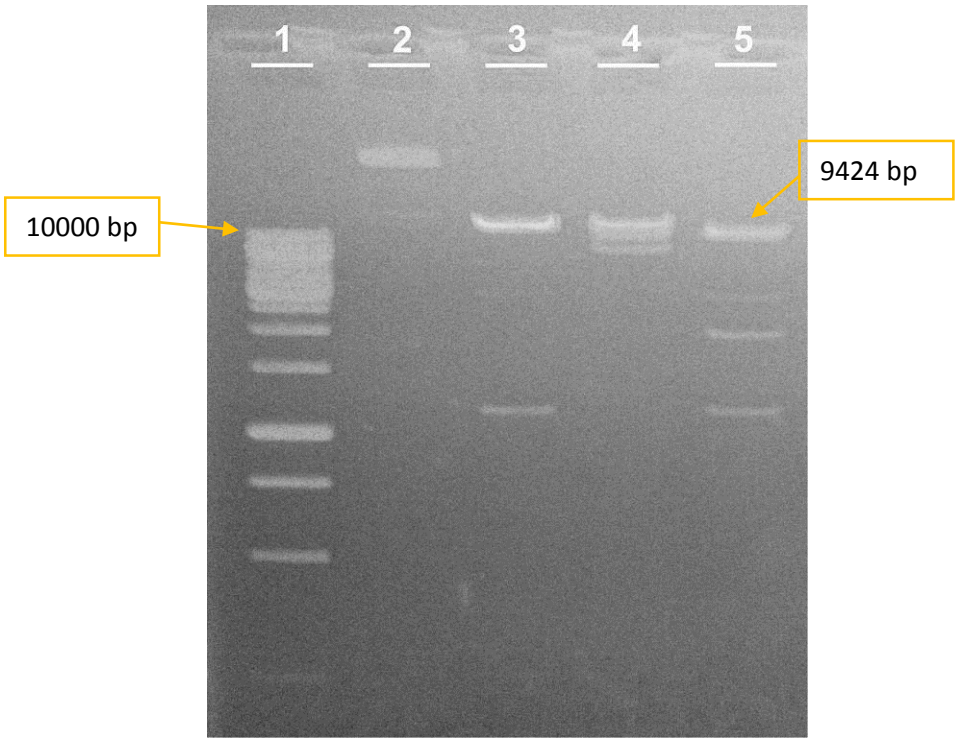


Figure 3.2: Verification of pCAMBIA 1304 plasmid digestion by restriction enzymes on 1% agarose gel. Lane 1: 10.000 bp DNA ladder; lane 2: Uncut plasmid; lane 3: XhoI digestion; lane 4: NcoI digestion; lane 5: double digestion with XhoI ve NcoI restriction enzymes.

Eluted extraction from single band was not containing enough DNA content due to the previous purification procedures; however, multiple extractions were pooled for obtaining satisfactory DNA for subsequent ligation reactions.

3.5 Ligation of Single-Target Fragments to pCAMBIA 1304 Vector and Transformation of Plasmid Constructs

A sequential path was followed during the construction of plasmids. Colony PCR was applied with the cloning primers to chosen random colonies grown on Kanamycin LB agar plates, one of the confirmed colonies was grown overnight, plasmid purified and they were checked qualitatively with test method primer pairs and PCR protocols.

First, Bt11 event-specific and maize taxon-specific PCR amplifications which are intended to be used in Bt11 detection and quantification (Figure 3.3a,b)

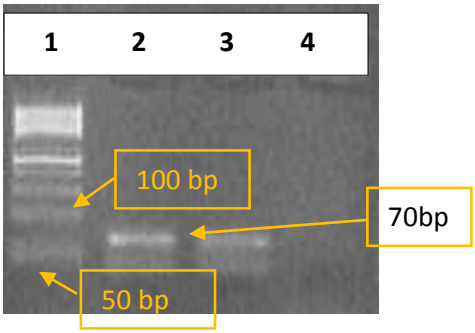


Figure 3.3a: Verified Bt11 event-specific fragment amplification for cloning on 2.5% Agarose Gel. Lane 1: 50bp DNA ladder. Lane 2-3: 70bp amplicon band. Lane 4: No-template control.

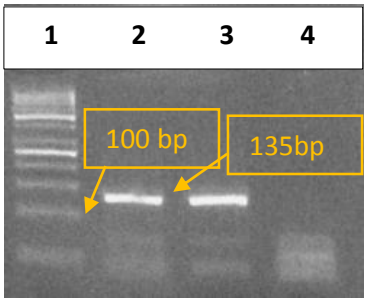


Figure 3.3b: Verified *adh1* endogenous maize fragment amplification for cloning on 2.5% Agarose Gel, Lane 1: 50bp DNA ladder. Lane 2-3: 135bp amplicon band. Lane 4: No-template control.

Then 35S target fragment was cloned into the vector and verified with 35S and NOS detection methods. (Figure 3.4)

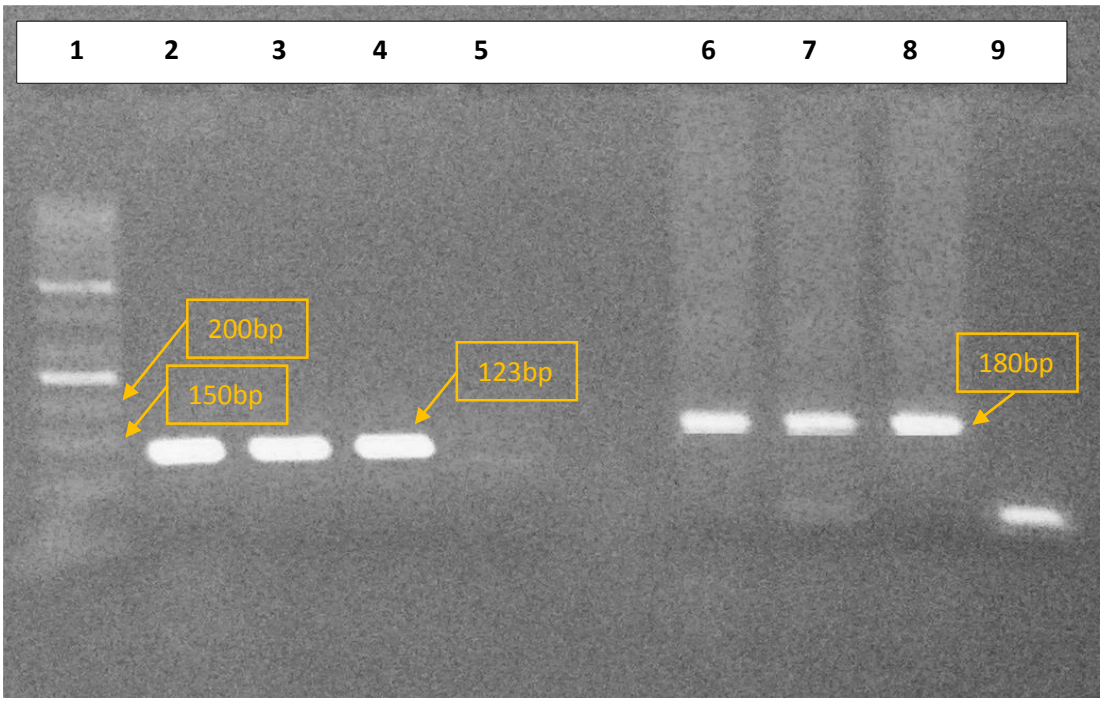


Figure 3.4: Electrophoretic analysis of 35S promoter and NOS terminator test method amplification check of constructed plasmids with Certified Reference Material DNA (5% GMO Bt11) in 2.5% Agarose gel electrophoresis. Plasmid and maize genomic DNA was amplified with 123 bp and 180 bp length of size, respectively. Line 1: DNA Ladder (50bp) Line 2: The amplification of 35S fragment done with CRM (50ng Bt11 5% DNA) Line 3, 4: The amplification was done with 10ng 35S-NOS bearing plasmid Line 5: No-template negative control Line 6: The amplification of NOS fragment done with CRM (50ng Bt11 5% DNA) Line 7, 8: The amplification was done with 10ng 35S-NOS bearing plasmid Line 9: No-template negative control.

The gel extraction procedures with amplified fragments were challenging when the fragment length was around 100 bp, because silica column DNA purification was not efficient enough with small fragments. Therefore, for remaining constructs which were planned to be used as reference DNA in Roundup Ready Soya detection, longer fragment amplifying cloning primers were designed. Primer pairs designed for 635 bp

fragment including 74 bp soya taxon-specific region, and 380 bp fragment including 84bp GTS 40-3-2 event-specific region. Both cloning and test method amplification were verified for plasmid templates. (Figure 3.5a,b)

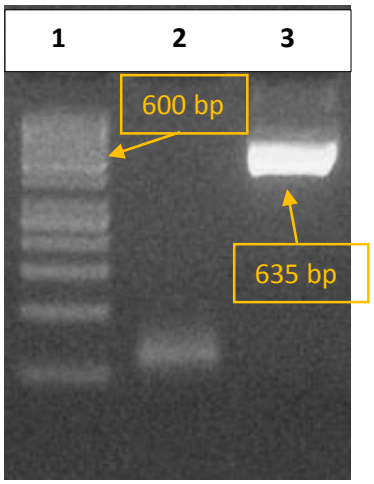


Figure 3.5a: Verification of amplified 635 bp cloning fragment for Le1 on 2% Agarose Gel. Lane 1: 50bp DNA Ladder, Lane 2: No-template control, Lane 3: 635 bp cloning fragment.

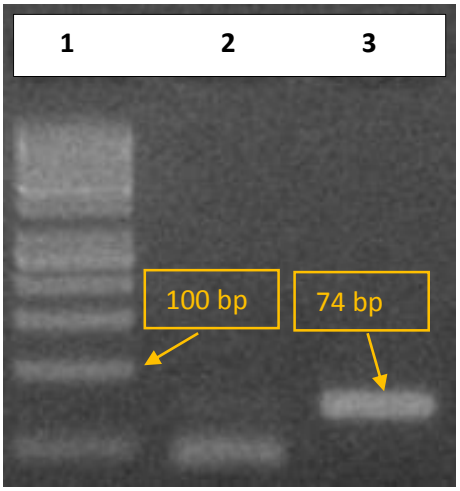
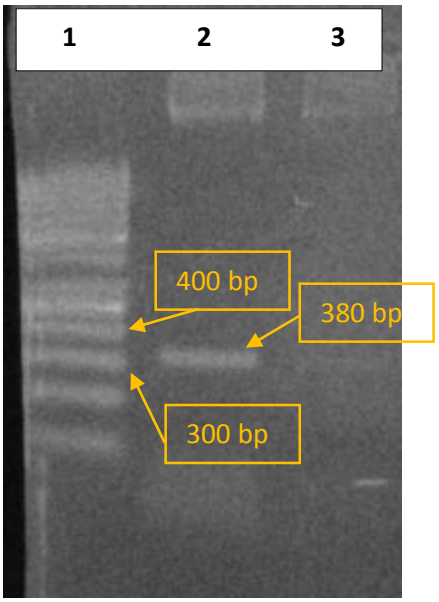


Figure 3.5b: Verification of amplified 74 bp event-specific detection fragment for Le1 on 2.5% Agarose Gel. Lane 1: 50bp DNA Ladder, Lane 2: No-template control, Lane 3: 74 bp event-specific fragment.



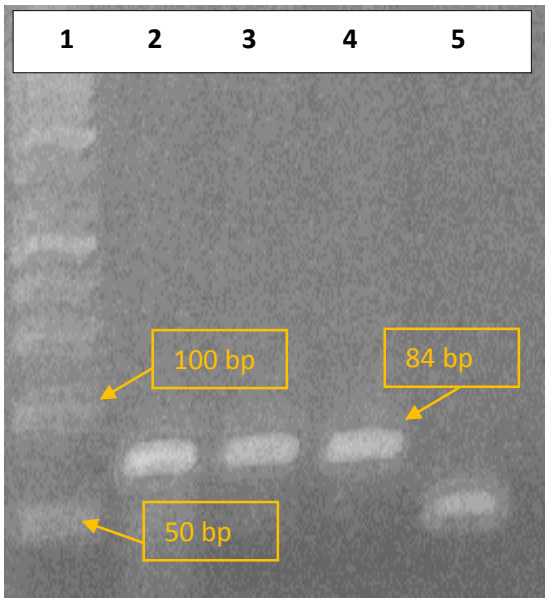


Figure 3.6a: Verification of amplified 380 bp cloning fragment for GTS-40-3-2 on 2% Agarose Gel. Lane 1: 100bp DNA Ladder, Lane 2: 380 bp cloning fragment. Lane 3: No-template control

Figure 3.6b: Verification of amplified 84 bp event-specific fragment for GTS-40-3-2 on 2.5% Agarose Gel. Lane 1: 50bp DNA Ladder, Lane 2-3-4: 84 bp event-specific amplicon fragment. Lane 3: No-template control

3.6 Verification of Bt-11 and *adh1* Targeted Amplifications via SYBR Green I Real-time PCR

Amplifications of constructed Bt11 and adh1 target plasmids and verification of SYBR Green I based fluorescence detection of PCR products have been investigated. Plasmids were mixed to obtain $5x10^5$ to 50 copy number, No-Template control samples were prepared and 10ng of DNA samples of 1% and 5% GMO Bt11 CRMs are also used as positive control templates. PCR amplification conditions were designed according to kit recommendations (Appendix E). Qualitative verification of dilution amplifications were inspected and specificity of amplifications were confirmed with melting curve analysis within LightCycler Software v4.1 (Figure 3.7).

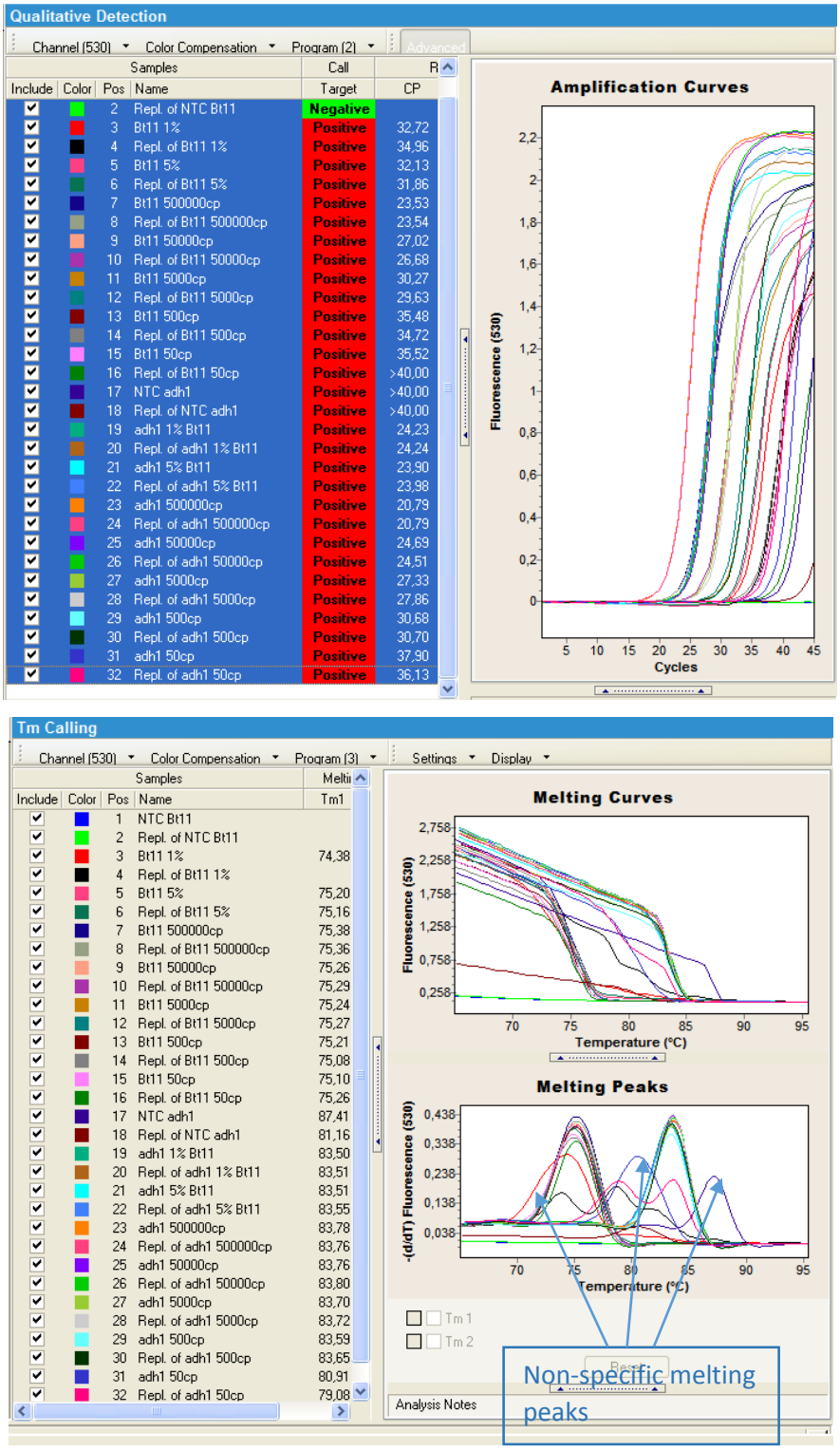


Figure 3.7: SYBR Green I Assay Based Real-time PCR amplification for qualitative detection of event bt11 and maize *adh1* target templates. Amplification curves are

obtained with each dilution of plasmid templates and CRM DNAs (above). No-template controls of Bt11 assay gave negative results as expected. Melting-curve analysis was performed (below). Positive results are interpreted as non-specific amplifications due to different T_ms from the expected product T_ms as a result of melting-curve analysis.

Amplification conditions for thermal cycler program would let running for two PCR sets to be performed simultaneously, therefore they could be evaluated in same run. Bt11 and *adh1* amplifications resulted in qualitative SYBR Green I signal. Only *adh1* negative controls gave amplification at late cycle stages (>40), however, they seemed to be non-specific and different T_m values from amplicon T_ms.

3.7 Measuring Uncertainty of GTS 40-3-2 and Le1 Target Plasmids as Calibrants in Roundup Ready Soya Quantitation

Quantitation based usage of calibrator plasmid dilutions were used as templates for Roundup Ready Soya quantification; GTS 40-3-2 target assays for Roundup Ready event and Le1 target assays for soya endogenous Lectin were performed. PCR conditions were designed according to T_m and amplicon length of products regarding to SYBR Green kit recommendations (Appendix E). Calibration curves were plotted and Cp values were extrapolated (Appendix G).

Measurement of uncertainty estimations were performed mainly in few steps; within-laboratory reproducibility standard deviation and repeatability relative standard deviation were calculated (Appendix G). Bias was controlled then expanded uncertainty was calculated from combined uncertainty. Then Estimation of Uncertainty Component Associated with Bias and Relative Standard Uncertainty calculation was performed. Limit of Detection (LOD) and Limit of Quantification (LOQ) measures was obtained at the end (Appendix H). In Appendix F, detailed equations are explained for the proper measurement uncertainty calculations for analytical results. And in Appendix G and Appendix H, calculation results were tabulated.

In the light of these estimations overall evaluation results were tabulated (Table 3.1 and Table 3.2). From these data it can be inferred that measurement uncertainties meet the requirements of analytical testing for GMO. However, apart from measurement uncertainty, there are properties of methods that is critical for quantification of GMOs like Amplification Efficiency. This property does not meet the Minimum Performance Requirements of European Network of GMO Laboratories for Analytical Methods of GMO Testing (ENGL 2009) This can be due to the improper dilution of plasmids, as a small shift of pipetting error can manipulate all concentrations from 10^6 to 10 copy number per microliter. Pico Green based measurement of plasmid DNA concentration allows more accurate copy number calculations, and it may be used to overcome this problem.

Table 3.1 Overall Quantitative Evaluation of Three Duplicate Real-time PCR Measurement of GTS 40-3-2 Plasmid Dilution Sets

RR Soya (GTS 40-3-2)	**Set 1**	**Set 2**	**Set 3**
Calibration Curve Equation	y = -1.96x + 35.47	y = -1.5x + 33.11	y = -2.6x + 41.42
LOD (Limit of Detection)	0.04	0.41	0.30
RSU (Relative standard Uncertainty) (Genome Copy Number)	89.50	56.80	2.20
UΔ (Combined Uncertainty, 95%, k=2)	$10^6 \rightarrow 0.03$	$10^6 \rightarrow 0.04$	$10^6 \rightarrow 0.0$
	$10^5 \rightarrow 0.01$	Not determined (ND)	$10^5 \rightarrow 0.02$
	$10^4 \rightarrow 0.03$	$10^4 \rightarrow 0.04$	$10^4 \rightarrow 0.34$
	$10^2 \rightarrow 0.14$	$10^2 \rightarrow 0.18$	ND

Table 3.2 Overall Quantitative Evaluation of Three Duplicate Real-time PCR Measurement of Le1 Plasmid Dilution Sets

Lectin (Le1)	**Set 1**	**Set 2**	**Set 3**
Calibration Curve Equation	y = -3,13x + 38,78	y = -2,96x + 40,95	y = -3,64x + 43,48
LOD (Limit of Detection)	$1.94x10^{-10}$	$2.91x10^{-2}$	$1.23x10^{-4}$
RSU (Relative standard Uncertainty) (Genome Copy Number)	4.73	46.41	18.53
UΔ (Combined Uncertainty, 95%, k=2)	$10^6 \rightarrow 0.01$	$10^6 \rightarrow 0.05$	ND
	$10^5 \rightarrow 0.03$	$10^5 \rightarrow 0.06$	$10^5 \rightarrow 0.03$
	$10^4 \rightarrow 0.04$	$10^4 \rightarrow 0.03$	$10^4 \rightarrow 0.04$
	$10^3 \rightarrow 0.01$	$10^3 \rightarrow 0.04$	$10^3 \rightarrow 0.01$
	$10^2 \rightarrow 0.002$	ND	ND

CHAPTER 4

CONCLUSION

Proper reference materials are essential for estimation of qualitative and quantitative PCR assays for GM events. Our work has shown that plasmids can be produced conveniently, where reference plant matrix derived reference material DNAs are not present, commercially available or impractical to be used for this purpose frequently. The aim of this study was developing synthetic DNA constructs, by modifying circular plasmid DNA, which of them could be used as novel template in conventional and real-time PCR assays in place of dried powder Certified Reference Materials prepared for GMO detection and quantification. There have been various studies and products on calibrator plasmids regarding to GMO detection. Also, in here, plasmid calibrants have been demonstrated to be an alternative to genomic DNA purified from conventional dried powder CRMs.

By integrating screening specific 35S fragment to pCAMBIA 1304 plasmid, new construct could be able to use for screening PCR assays. As pCAMBIA 1304 plasmid have a NOS terminator region, constructed plasmid was available to be used as double target positive controls in PCR assays for 35S promoter and NOS terminator.

Likewise, event-specific and taxon-specific fragments for Bt11 event in Maize and GTS 40-3-2 event in soya have been cloned to pCAMBIA 1304. These fragments were intended to be used in real-time PCR based quantification methods and amplifications were verified with SYBR Green I based real-time PCR.

Usability of constructed plasmids for GMO quantification purposed real-time PCR assays on Roundup Ready Soya were shown with by assaying by its event-specific GTS 40-3-2 and taxon specific Le1 target plasmids. For this purpose, both of these plasmids were diluted, calibration curves were plotted, and measurement of uncertainty estimations were calculated for each reference sample concentrations through amplification readings. Relative standard deviation uncertainty obtained by bias control, limit of detection and limit of quantification values were in guided limits which have been mentioned in EU Joint Research Center Guidance Document for GMO Laboratories. Also amplification Efficiency and R^2 Coefficient parameters were suitable for Le1, as defined in Analytical Methods of GMO Testing defined as Minimum Performance Requirements. For GTS 40-3-2, this can be enhanced by proper handling and dilution of plasmid DNA solutions in microliter level.

From verification and measurement uncertainty data based on single laboratory data, it can be interpreted that plasmids may provide economic and excellent replacement to plant matrix derived DNA extractions for positive control material. Their use as positive controls is likely to increase for method validation, quality control and method development: in particular plasmid targets provide a convenient means to produce solutions to test detection limits and error rates of qualitative and quantitative PCRs.

Based on within (single) laboratory results, when using plasmids for GM quantification caution must be applied and several measures undertaken to increase accuracy of the measurements. The data presented here have shown that plasmids, with proper treatment and in many cases, can provide standards equivalent or more accurate than genomic DNA extractions. Moreover, the data should be investigated with inter-laboratory comparisons in future work; which can validate the reliability of these synthetic constructs as reference materials.

REFERENCES

Ahmed, F. E. (2002). "Detection of genetically modified organisms in foods." Trends in biotechnology **20**(5): 215-223.

Allen, R. C., S. Rogelj, S. E. Cordova and T. L. Kieft (2006). "An immuno-PCR method for detecting Bacillus thuringiensis Cry1Ac toxin." Journal of Immunological Methods **308**(1–2): 109-115.

Barampuram, S. and Z. Zhang (2011). Recent Advances in Plant Transformation. Plant Chromosome Engineering. J. A. Birchler, Humana Press. **701:** 1-35.

Bennett, A. B., C. Chi-Ham, G. Barrows, S. Sexton and D. Zilberman (2013). "Agricultural Biotechnology: Economics, Environment, Ethics, and the Future." Annual Review of Environment and Resources **38**(1): 249-279.

Borst, A., A. T. A. Box and A. C. Fluit (2004). "False-Positive Results and Contamination in Nucleic Acid Amplification Assays: Suggestions for a Prevent and Destroy Strategy." European Journal of Clinical Microbiology and Infectious Diseases **23**(4): 289-299.

Brett, G. M., S. J. Chambers, L. Huang and M. R. A. Morgan (1999). "Design and development of immunoassays for detection of proteins." Food Control **10**(6): 401-406.

Brookes G, B. P. (2013). GM crops: global socio-economic and environmental impacts 1996-2011.

Burns, M., P. Corbisier, G. Wiseman, H. Valdivia, P. McDonald, P. Bowler, K. Ohara, H. Schimmel, D. Charels, A. Damant and N. Harris (2006). "Comparison of plasmid

and genomic DNA calibrants for the quantification of genetically modified ingredients." European Food Research and Technology **224**(2): 249-258.

Chassy, B. M. (2007). "The history and future of GMOs in food and agriculture." Cereal foods world: 169-172.

Davison, J. (2010). "GM plants: Science, politics and EC regulations." Plant Science **178**(2): 94-98.

ENGL (2009). Definition of Minimum Performance Requirements for Analytical Methods for Gmo Testing.

Fagan, J. (2007). Monitoring GMOs Released into the Env. and the Food Production System. Biosafety First. T. Traavik and L. L. Ching, Tapir Academic Publishers.

GIA (2013). Food Safety Testing—A Global Strategic Business Report.

GMO-Compass. (2012). "Turkey's biosafety law causes heavy losses to agri-food chain." from http://www.gmo-compass.org/eng/news/552.turkeys_biosafety_law_causes_heavy_losses_agri_food_chain.html.

Hails, R. S. (2000). "Genetically modified plants – the debate continues." Trends in Ecology & Evolution **15**(1): 14-18.

HAMELS, S., S. LEIMANIS, M. MAZZARA, G. BELLOCCHI, N. FOTI, W. MOENS, J. REMACLE and G. V. D. EEDE (2007) "Microarray Method for the Screening of EU Approved GMOs by Identification of their Genetic Elements." DOI: 10.2788/32983.

Holst-Jensen, A., S. B. Ronning, A. Lovseth and K. G. Berdal (2003). "PCR technology for screening and quantification of genetically modified organisms (GMOs)." Anal Bioanal Chem **375**(8): 985-993.

http://ec.europa.eu/food/food/biotechnology/qanda/h1_en.print.htm. (2009). "GMOs in a nutshell." from http://ec.europa.eu/food/food/biotechnology/gmo_nutshell_annexes_en.htm#annex.

James, C. (2013). Global Status of Commercialized Biotech/GM Crops: 2013. Ithaca, NY, ISAAA.

Kamle, S. and S. Ali (2013). "Genetically modified crops: Detection strategies and biosafety issues." Gene **522**(2): 123-132.

Liang, H., S. E. Cordova, T. L. Kieft and S. Rogelj (2003). "A highly sensitive immuno-PCR assay for detecting Group A Streptococcus." Journal of Immunological Methods **279**(1–2): 101-110.

Lievens, A., G. Bellocchi, D. De Bernardi, W. Moens, C. Savini, M. Mazzara, G. Van den Eede and M. Van den Bulcke (2010). "Use of pJANUS-02-001 as a calibrator plasmid for Roundup Ready soybean event GTS-40-3-2 detection: an interlaboratory trial assessment." Anal Bioanal Chem **396**(6): 2165-2173.

Lipp, M., P. Brodmann, K. Pietsch, J. Pauwels, E. Anklam, T. Börchers, G. Braunschweiger, U. Busch, E. Eklund and F. Eriksen (1999). "IUPAC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder." Journal of AOAC International **82**(4): 923.

Miraglia, M., K. G. Berdal, C. Brera, P. Corbisier, A. Holst-Jensen, E. J. Kok, H. J. P. Marvin, H. Schimmel, J. Rentsch, J. P. P. F. van Rie and J. Zagon (2004). "Detection and traceability of genetically modified organisms in the food production chain." Food and Chemical Toxicology **42**(7): 1157-1180.

Nester, E. (2008). "Agrobacterium: The Natural Genetic Engineer 100 Years Later." APSnet.

Park, H. (2004). "Detection of genetically modified soybean by monoclonal and polyclonal antibodies." Journal of Allergy and Clinical Immunology **113**(2, Supplement): S151.

Pietsch, K., H. Waiblinger, B. U., P. and A. Wurz (1997). "Screeningverfahren zur Identifizierung gentechnisch veraanderter pflanzlicher Lebensmittel." Deutsche Lebensm.-Rundsch.(93): 35-38.

Querci, M., N. Foti, A. Bogni, L. Kluga, H. Broll and G. Eede (2009). "Real-Time PCR-Based Ready-to-Use Multi-Target Analytical System for GMO Detection." Food Analytical Methods **2**(4): 325-336.

Raymond, P., L. Gendron, M. Khalf, S. Paul, K. Dibley, S. Bhat, V. D. Xie, L. Partis, M.-E. Moreau, C. Dollard, M.-J. Coté, S. Laberge and K. Emslie (2010). "Detection and identification of multiple genetically modified events using DNA insert fingerprinting." Analytical and Bioanalytical Chemistry **396**(6): 2091-2102.

S. Trapmann, M. B., H. Broll (2009). Guidance document on measurement uncertainty for GMO testing laboratories. Luxembourg, European Commision Joint Research Center.

Stave, J. W. (2002). "Protein immunoassay methods for detection of biotech crops: applications, limitations, and practical considerations." J AOAC Int **85**(3): 780-786.

Taverniers, I., P. Windels, M. Vaitilingom, A. Milcamps, E. Van Bockstaele, G. Van den Eede and M. De Loose (2005). "Event-Specific Plasmid Standards and Real-Time PCR Methods for Transgenic Bt11, Bt176, and GA21 Maize and Transgenic GT73 Canola." Journal of Agricultural and Food Chemistry **53**(8): 3041-3052.

Thomison, P. R. and M. M. Loux. (2001). "Commonly Used Methods for Detecting GMOs in Grain Crops." from http://ohioline.osu.edu/agf-fact/0149.html.

Tozzini, A. C., M. Martínez, M. F. Lucca, C. Vázquez Rovere, A. J. Distéfano, M. del Vas and E. Hopp (2000). "Semi-quantitative detection of genetically modified grains

based on CaMV 35S promoter amplification." Electronic Journal of Biotechnology **3**(2): 25-34.

Uzogara, S. G. (2000). "The impact of genetic modification of human foods in the 21st century: a review." Biotechnol Adv **18**(3): 179-206.

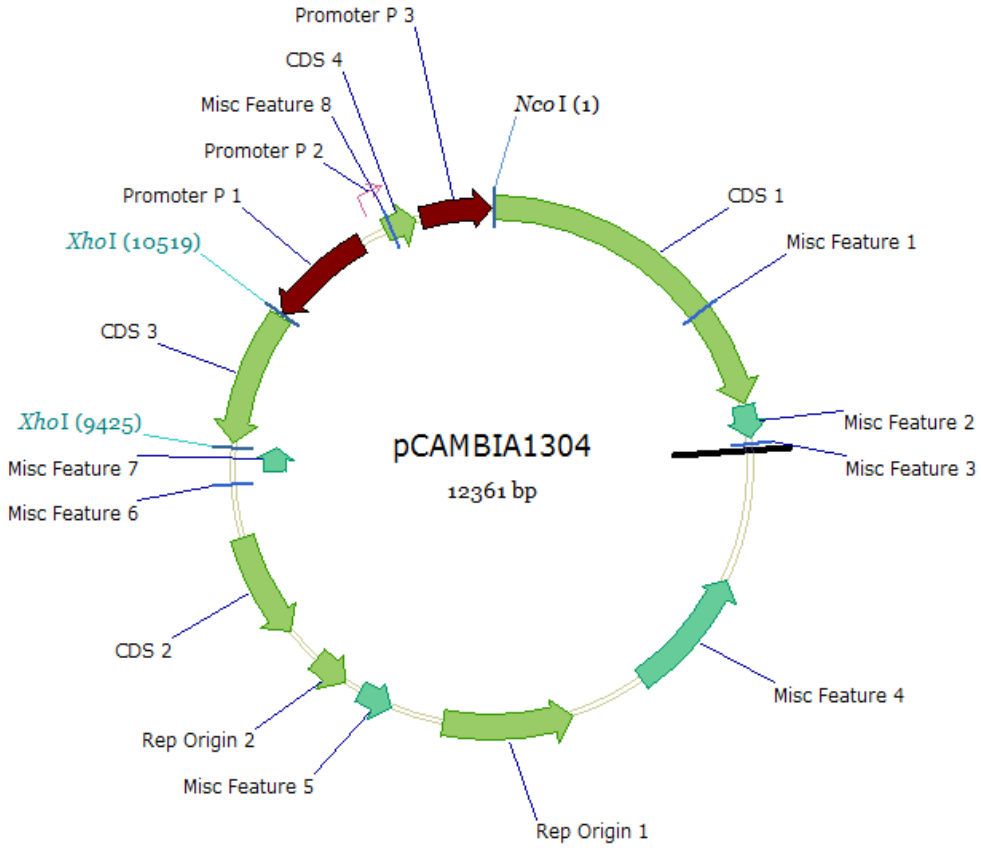
Žel, J., M. Mazzara, C. Savini, S. Cordeil, M. Camloh, D. Štebih, K. Cankar, K. Gruden, D. Morisset and G. Eede (2008). "Method Validation and Quality Management in the Flexible Scope of Accreditation: An Example of Laboratories Testing for Genetically Modified Organisms." Food Analytical Methods **1**(2): 61-72.

Žel, J., M. Milavec, D. Morisset, D. Plan, G. Eede and K. Gruden (2012). How to Reliably Test for GMOs. How to Reliably Test for GMOs, Springer US**:** 1-95.

Zhang, D., A. Corlet and S. Fouilloux (2008). "Impact of genetic structures on haploid genome-based quantification of genetically modified DNA: theoretical considerations, experimental data in MON 810 maize kernels (Zea mays L.) and some practical applications." Transgenic Res **17**(3): 393-402.

APPENDIX A

MAP, FEATURES AND FULL SEQUENCE OF pCAMBIA 1304



A.1 pCAMBIA 1304 Plasmid Sequence

```
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2161 tttggaaacg gcagagaagg tactggaaaa agaacttctg gcctggcagg agaaactgca
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3661 accccggcac agatcgcgca cgcccgcgag ctgatcgacc aggaaggccg caccgtgaaa
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4021 tggccggttt gtctgatgcc aagctggcgg cctggccggc cagcttggcc gctgaagaaa
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4621 caaaggcacg cgcatcggcg gtgaggttgc cgaggcgctg gccgggtacg agctgcccat
4681 tcttgagtcc cgtatcacgc agcgcgtgag ctacccaggc actgccgccg ccggcacaac
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4861 acgctaagtg ccggccgtcc gagcgcacgc agcagcaagg ctgcaacgtt ggccagcctg
4921 gcagacacgc cagccatgaa gcgggtcaac tttcagttgc cggcggagga tcacaccaag
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5041 gcgcagctac cagagtaaat gagcaaatga ataaatgagt agatgaattt tagcggctaa
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5161 tggaggaacg ggcggttggc caggcgtaag cggctgggtt gtctgccggc cctgcaatgg
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5281 aaatcggcgc ggcgctgggt gatgacctgg tggagaagtt gaaggccgcg caggccgccc
5341 agcggcaacg catcgaggca gaagcacgcc ccggtgaatc gtggcaagcg gccgctgatc
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5641 gcatggccag tgtgtgggat tacgacctgg tactgatggc ggtttcccat ctaaccgaat

5701 ccatgaaccg ataccgggaa gggaagggag acaagcccgg ccgcgtgttc cgtccacacg
5761 ttgcggacgt actcaagttc tgccggcgag ccgatggcgg aaagcagaaa gacgacctgg
5821 tagaaacctg cattcggtta aacaccacgc acgttgccat gcagcgtacg aagaaggcca
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6001 gcgagatcac agaaggcaag aacccggacg tgctgacggt tcaccccgat tacttttttga
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6241 aggaggaggc ggggcaggct ggcccgatcc tagtcatgcg ctaccgcaac ctgatcgagg
6301 gcgaagcatc cgccggttcc taatgtacgg agcagatgct agggcaaatt gccctagcag
6361 gggaaaaagg tcgaaaaggt ctctttcctg tggatagcac gtacattggg aacccaaagc
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6481 cacacatgta agtgactgat ataaaagaga aaaaaggcga tttttccgcc taaaactctt
6541 taaaacttat taaaactctt aaaacccgcc tggcctgtgc ataactgtct ggccagcgca
6601 cagccgaaga gctgcaaaaa gcgcctaccc ttcggtcgct gcgctcccta cgccccgccg
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7021 ggtgtgaaat accgcacaga tgcgtaagga gaaaataccg catcaggcgc tcttccgctt
7081 cctcgctcac tgactcgctg cgctcggtcg ttcggctgcg gcgagcggta tcagctcact
7141 caaaggcggt aatacggtta tccacagaat caggggataa cgcaggaaag aacatgtgag
7201 caaaaggcca gcaaaaggcc aggaaccgta aaaaggccgc gttgctggcg tttttccata
7261 ggctccgccc ccctgacgag catcacaaaa atcgacgctc aagtcagagg tggcgaaacc
7321 cgacaggact ataaagatac caggcgtttc cccctggaag ctccctcgtg cgctctcctg
7381 ttccgaccct gccgcttacc ggatacctgt ccgcctttct cccttcggga agcgtggcgc
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7501 gctgtgtgca cgaacccccc gttcagcccg accgctgcgc cttatccggt aactatcgtc
7561 ttgagtccaa cccggtaaga cacgacttat cgccactggc agcagccact ggtaacagga
7621 ttagcagagc gaggtatgta ggcggtgcta cagagttctt gaagtggtgg cctaactacg
7681 gctacactag aaggacagta tttggtatct gcgctctgct gaagccagtt accttcggaa
7741 aaagagttgg tagctcttga tccggcaaac aaaccaccgc tggtagcggt ggttttttttg
7801 tttgcaagca gcagattacg cgcagaaaaa aaggatctca agaagatcct ttgatctttt
7861 ctacggggtc tgacgctcag tggaacgaaa actcacgtta agggattttg gtcatgcatt
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7981 tgatccccag taagtcaaaa aatagctcga catactgttc ttccccgata tcctccctga
8041 tcgaccggac gcagaaggca atgtcatacc acttgtccgc cctgccgctt ctcccaagat
8101 caataaagcc acttactttg ccatctttca caaagatgtt gctgtctccc aggtcgccgt
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8281 tcagtaagta atccaattcg gctaagcggc tgtctaagct attcgtatag ggacaatccg
8341 atatgtcgat ggagtgaaag agcctgatgc actccgcata cagctcgata atcttttcag
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8761 accccaagaa gctaattata acaagacgaa ctccaattca ctgttccttg cattctaaaa
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8881 atcgacggag ccgattttga aaccgcggtg atcacaggca gcaacgctct gtcatcgtta
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9061 ctgacgccgt cccggactga tgggctgcct gtatcgagtg gtgattttgt gccgagctgc
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9241 attcgggggga tctggatttt agtactggat tttggtttta ggaattagaa attttattga
9301 tagaagtatt ttacaaatac aaatacatac taagggtttc ttatatgctc aacacatgag
9361 cgaaacccta taggaaccct aattccctta tctgggaact actcacacat tattatggag
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9781 cctccagaag aagatgttgg cgacctcgta ttgggaatcc ccgaacatcg cctcgctcca
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10321 accatcggcg cagctattta cccgcaggac atatccacgc cctcctacat cgaagctgaa
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10621 ggattgtgcg tcatcccttа cgtcagtgga gatatcacat caatccactt gctttgaaga
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10741 accactgtcg gcagaggcat cttgaacgat agcctttcct ttatcgcaat gatggcattt
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10861 gaatccgagg aggtttcccg atattaccct ttgttgaaaa gtctcaatag ccctttggtc
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10981 atcacatcaa tccacttgct ttgaagacgt ggttggaacg tcttcttttt ccacgatgct
11041 cctcgtgggt gggggtccat ctttgggacc actgtcggca gaggcatctt gaacgatagc
11101 ctttccttta tcgcaatgat ggcatttgta ggtgccacct tccttttcta ctgtcctttt
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11221 ttgaaaagtc tcaatagccc tttggtcttc tgagactgta tctttgatat tcttggagta
11281 gacgagagtg tcgtgctcca ccatgttggc aagctgctct agccaatacg caaaccgcct
11341 ctccccgcgc gttggccgat tcattaatgc agctggcacg acaggtttcc cgactggaaa
11401 gcgggcagtg agcgcaacgc aattaatgtg agttagctca ctcattaggc accccaggct
11461 ttacacttta tgcttccggc tcgtatgttg tgtggaattg tgagcggata acaatttcac
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11761 atgctagagc agcttgagct tggatcagat tgtcgtttcc cgccttcagt ttagcttcat
11821 ggagtcaaag attcaaatag aggacctaac agaactcgcc gtaaagactg gcgaacagtt
11881 catacagagt ctcttacgac tcaatgacaa gaagaaaatc ttcgtcaaca tggtggagca
11941 cgacacactt gtctactcca aaaatatcaa agatacagtc tcagaagacc aaagggcaat
12001 tgagactttt caacaaaggg taatatccgg aaacctcctc ggattccatt gcccagctat
12061 ctgtcacttt attgtgaaga tagtggaaaa ggaaggtggc tcctacaaat gccatcattg
12121 cgataaagga aaggccatcg ttgaagatgc ctctgccgac agtggtccca aagatggacc
12181 cccacccacg aggagcatcg tggaaaaaga agacgttcca accacgtctt caaagcaagt
12241 ggattgatgt gatatctcca ctgacgtaag ggatgacgca caatcccact atccttcgca
12301 agacccttcc tctatataag gaagttcatt tcatttggag agaacacggg ggactcttga
12361 c

APPENDIX B

COMPOSITIONS OF MEDIA

B.1 Luria Bertani (LB) Medium

Table B.1 LB Medium Components

Component	**Amount of component (1000 mL)**
Yeast Extract	5 g
Tryptone	10 g
NaCl	10 g

Final pH should be 7.0. If solid LB is needed for plates, 15g of Bacteriological Agar should be added for each 1000 mL of LB Medium.

B.2 SOC Medium

Table B 2. SOC Medium Components

Component	**Amount of component (1000 mL)**
Bacto Tryptone	20 g
Bacto Yeast Extract	5 g
5M NaCl	2 ml
1M KCl	2.5 ml
1M $MgCl_2$	10 ml
1M $MgSO_4$	10 ml
1M glucose	20 ml

Glucose should only be added after autoclaving the solution with the remaining ingredients and letting it cool down. Sterilize the glucose solution by passing it through a 0.2 μm filter.

APPENDIX C

PCR CYCLING CONDITIONS FOR AMPLIFICATION OF FRAGMENTS TO BE CLONED

C.1 Bt11 Cloning Amplification PCR Program and Amplicon Sequence

Table C.1 Bt11 Cloning PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	30 s/95 °C
	30 s/60 °C
	15 s/72 °C
Number of cycles	40
Final extension	6 min/72 °C

5'-
CTAACTCGAGGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAAT
ATGTATCCGCTCATGGAGGGATTCTTGGACCATGGGACT-3'

C.2 Maize Endogenous adh1 Cloning Amplification PCR Program and Amplicon Sequence

Table C.2 adh1 Cloning PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	30 s/95 °C
	30 s/62 °C
	60 s/72 °C
Number of cycles	45
Final extension	7 min/72 °C

5'-
CTAACTCGAGCGTCGTTTCCCATCTCTTCCTCCTTTAGAGCTACCACTATA

TAAATCAGGGCTCATTTTCTCGCTCCTCACAGGCTCATCTCGCTTTGGATC
GATTGGTTTCGTAACTGGTGAGGGACTGAGGGTCTCGGAGTGGCCATGG
GACT-3’

C.3 35S Promoter Cloning Amplification PCR Program and Amplicon Sequence

Table C.3 35S Cloning PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	25 s/95 °C
	30 s/58 °C
	45 s/72 °C
Number of cycles	50
Final extension	7 min/72 °C

5’-
CTAACTCGAGCCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCAC
TGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCT
CTATATAAGGAAGTTCATTTCATTTGGAGAGGACCATGGGACT-3’

C.4 GTS 40-3-2 Cloning Amplification PCR Program and Amplicon Sequence

Table C.4 GTS 40-3-2 Cloning PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	30 s/95 °C
	45 s/58 °C
	45 s/72 °C
Number of cycles	35
Final extension	7 min/72 °C

5’-
CTAACTCGAGCCTTCAATTTAACCGATGCTAATGAGTTATTTTTGCATGCT
TTAATTTGTTTCTATCAAATGTTTATTTTTTTTTACTAGAAATAACTTATTG
CATTTCATTCAAAATAAGATCATACATACAGGTTAAAATAAACATAGGG
AACCCAAATGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGAT

AAAGGAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAG
ATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAAC
CACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGG
ATGACGCACAATCCCACTATCCCATGGGACT-3’

C.5 Soya Endogenous Lectin (Le1) Cloning PCR Amplification and Amplicon sequence

Table C.5 Le1 Cloning PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	30 s/95 °C
	45 s/61 °C
	60 s/72 °C
Number of cycles	35
Final extension	7 min/72 °C

5’-
CTAACTCGAGCCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGCCCCT
GACACAAAAAGGCTTGCAGATGGGCTTGCCTTCTTTCTCGCACCAATTGA
CACTAAGCCACAAACACATGCAGGTTATCTTGGTCTTTTCAACGAAAACG
AGTCTGGTGATCAAGTCGTCGCTGTTGAGTTTGACACTTTCCGGAACTCTT
GGGATCCACCAAATCCACACATCGGAATTAACGTCAATTCTATCAGATCC
ATCAAAACGACGTCTTGGGATTTGGCCAACAATAAAGTAGCCAAGGTTCT
CATTACCTATGATGCCTCCACCAGCCTCTTGGTTGCTTCTTTGGTCTACCC
TTCACAGAGAACCAGCAATATCCTCTCCGATGTGGTCGATTTGAAGACTT
CTCTTCCCGAGTGGGTGAGGATAGGGTTCTCTGCTGCCACGGGACTCGAC
ATACCTGGGGAATCGCATGACGTGCTTTCTTGGTCTTTTGCTTCCAATTTG
CCACACGCTAGCAGTAACATTGATCCTTTGGATCTTACAAGCTTTGTGTT
GCATGAGGCCATCTAAATGTGACAGATCGAAGGAAGAAAGTGTAATAAG
ACGACTCTCACTACTCGATCGC CCATGGGACT-3’

APPENDIX D

PCR CYCLING CONDITIONS FOR QUALITATIVE VERIFICATION OF CONSTRUCTED PLASMIDS

D.1 Bt11 Event-specific Test Method PCR Program and Amplicon sequence

Table D.1 Bt11 Event-Specific PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	30 s/95 °C
	40 s/60 °C
	90 s/72 °C
Number of cycles	35
Final extension	6 min/72 °C

5'-
GCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGC
TCATGGAGGGATTCTTGGA-3'

D.2 Maize Endogenous *adh1* Test Method PCR Program and Amplicon sequence

Table D.2 *adh1* Maize-specific PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	30 s/95 °C
	30 s/62 °C
	60 s/72 °C
Number of cycles	45
Final extension	7 min/72 °C

5'-
CGTCGTTTCCCATCTCTTCCTCCTTTAGAGCTACCACTATATAAATCAGGG
CTCATTTTCTCGCTCCTCACAGGCTCATCTCGCTTTGGATCGATTGGTTTC
GTAACTGGTGAGGGACTGAGGGTCTCGGAGTGG-3'

D.3 35S Promoter Test Method PCR Program and Amplicon sequence

Table D.3 35S Screening PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	25 s/95 °C
	30 s/58 °C
	45 s/72 °C
Number of cycles	50
Final extension	7 min/72 °C

5'-
CCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGG
GATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGG
AAGTTCATTTCATTTGGAGAGGA-3'

D.4 NOS Terminator Test Method PCR Program and Amplicon sequence

Table D.4 NOS Screening PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	20 s/95 °C
	40 s/54 °C
	40 s/72 °C
Number of cycles	35
Final extension	6 min/72 °C

5'-
GAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTA
CGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGAT
GGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAA
AACAAAATATAGCGCGCAAACTAGGATAA-3'

D.5 GTS 40-3-2 Event-specific Test Method PCR Program and Amplicon sequence

Table D.5 GTS 40-3-2 Event-Specific PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	30 s/95 °C
	30 s/55 °C
	15 s/72 °C
Number of cycles	45
Final extension	7 min/72 °C

5'-
TTCATTCAAAATAAGATCATACATACAGGTTAAAATAAACATAGGGAAC
CCAAATGGAAAAGGAAGGTGGCTCCTACAAATGCC -3'

D.6 Soya Endogenous Lectin (Le1) Test Method PCR Program and Amplicon sequence

Table D.6 GTS 40-3-2 Event-Specific PCR Cycling Parameters

Activation/initial denaturation	3 min/95 °C
Amplification	30 s/95 °C
	30 s/60 °C
	15 s/72 °C
Number of cycles	45
Final extension	7 min/72 °C

5'-
CCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGCCCCTGACACAAAAA
GGCTTGCAGATGGGCTTGCCTTC-3'

APPENDIX E

SYBR Green I BASED REAL-TIME PCR PROGRAMS FOR TESTING PLASMIDS CONSTRUCTED PLASMIDS BY CLONING OF BT11/*adh1* AND GTS 40-3-2-Le1 DETECTION FRAGMENTS

Table E.1 PCR components of SYBR Green I Based RT-PCR

PCR Grade Water	**9 µl**
Primer Fw (10µM)	**1 µl**
Primer Rev (10µM)	**1 µl**
Master Mix	**4 µl**
Total Volume	**15 µl**

Template Volume: 5 µl

E.1 Bt11 Event-specific and adh1 Endogenous Test Method PCR Program

Bt11 and adh1 primer pairs have very close annealing temperatures, therefore they could be incorporated in the same run.

Table E.2 Bt11 Event-Specific and *adh1* taxon-specific PCR Cycling Parameters

Program Name	**Activation/Initial Denaturation**		
Cycles	**1**	**Analysis Mode**	**None**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
95	00:10:00	20	0	0	0	None

Program Name	**Quantification**		
Cycles	**45**	**Analysis Mode**	**Quantification**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
95	00:00:10	20	0	0	0	None
60	00:00:10	20	0	0	0	None
72	00:00:05	20	0	0	0	Single

Program Name	**Melting Curves**		
Cycles	**1**	**Analysis Mode**	**None**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
95	00:00:00	20	0	0	0	None
65	00:01:00	20	0	0	0	None
95	00:00:00	0.1	0	0	0	Continuous

Program Name	**Cooling**		
Cycles	**1**	**Analysis Mode**	**None**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
40	00:00:30	20	0	0	0	None

E.2 GTS 40-3-2 Event-specific Test Method PCR Program

Table E.3 GTS 40-3-2 Event-Specific PCR Cycling Parameters

Program Name	**Activation/Initial Denaturation**		
Cycles	**1**	**Analysis Mode**	**None**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
95	00:10:00	20	0	0	0	None

Program Name	**Quantification**		
Cycles	**45**	**Analysis Mode**	**Quantification**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
95	00:00:10	20	0	0	0	None
55	00:00:05	20	0	0	0	None
72	00:00:04	20	0	0	0	Single

Program Name	**Melting Curves**		
Cycles	**1**	**Analysis Mode**	**None**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
95	00:00:00	20	0	0	0	None
65	00:01:00	20	0	0	0	None
95	00:00:00	0.1	0	0	0	Continuous

Program Name	**Cooling**		
Cycles	**1**	**Analysis Mode**	**None**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
40	00:00:30	20	0	0	0	None

E.3 Le1 Endogenous Test Method PCR Program

Table E.4 *adh1* taxon-specific PCR Cycling Parameters

Program Name	**Activation/Initial Denaturation**		
Cycles	**1**	**Analysis Mode**	**None**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
95	00:10:00	20	0	0	0	None

Program Name	**Quantification**		
Cycles	**45**	**Analysis Mode**	**Quantification**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
95	00:00:10	20	0	0	0	None
60	00:00:10	20	0	0	0	None
72	00:00:05	20	0	0	0	Single

Program Name	**Melting Curves**		
Cycles	**1**	**Analysis Mode**	**None**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
95	00:00:00	20	0	0	0	None
65	00:01:00	20	0	0	0	None
95	00:00:00	0.1	0	0	0	Continuous

Program Name	**Cooling**		
Cycles	**1**	**Analysis Mode**	**None**

Target (°C)	**Hold (hh:mm:ss)**	**Slope (°C /s)**	**Sec Target (°C)**	**Step size (°C)**	**Step Delay (cycles)**	**Acquisition Mode**
40	00:00:30	20	0	0	0	None

APPENDIX F

DATA ANALYSIS ON MEASUREMENT UNCERTAINTY OF CALIBRATION PLASMIDS

F.1 Considerations on In-House Repeatability of Analytical Results

Cp values regarding to amplifications were extrapolated from standard curve are referred as analytical result (c_1, c_2, …)

c_i: Mean of the analytical results

$$c_i = \frac{c_1 + c_2}{2}$$

d_i: absolute difference between two analytical results

$$d_i = |c_1 - c_2|$$

rad_i: relative difference between analyses

$$rad_i = \frac{d_i}{c_i} \times 100$$

S_{RL}: within-laboratory reproducibility standard deviation

$$S_{RL} = \frac{d}{d2}$$

Where d is the average difference and d2 is the constant regarding to independent measurement number.

RSD_R: The (within-laboratory) repeatability relative standard deviation

$$RSD_R = \frac{\overline{rad}}{1.13}$$

where $\overline{\text{rad}}$ corresponds to average relative differences and 1.13 is constant when 2 independent measurements were made.

F.2 Bias Control and Estimation of Uncertainty Component Associated with Bias

We assume our certificate values are equal to $\log_{10}$ of plasmid copy number per reaction; and accuracy is calculated by estimating standard deviation of two independent analytical results.

U_m: Uncertainty of Measurements for Each Concentration Value

$$u_m = \frac{stdev\ of\ measurement}{\sqrt{\#\ of\ measurements\ stdev\ involved\ (2)}}$$

Δ_m: absolute difference between measurement and certified value

$$\Delta_m = |c_m - c_{RM}|$$

c_m: measured value mean

c_{CRM}: certified value (refers to predicted plasmid copy number)

u_Δ: combined uncertainty of measurement and certified value

$$u_\Delta = \sqrt{u_m^2 + u_{CRM}^2}$$

where u_{CRM} is measurement uncertainty of certified value. u_{CRM} was not applicable for our study, therefore it was not involved in estimation.

U_{Δ}: Expanded uncertainty, ($2*u_m$) obtained by multiplication of coverage factor k=2 in a confidence level about 95%.

If **$\Delta_m < U_{\Delta}$,**

It could be concluded that there was no bias.

The relative standard uncertainty associated with the bias called **u_{biasr},** can be calculated with following formula:

$$U_{biasr} = \sqrt{\frac{{RSD_R}^2}{n} + \left(\frac{u_{CRM} \times 100}{c_{CRM}}\right)^2}$$

$bias_r$: relative bias

$$bias_r = \frac{c}{c_{CRM}}$$

$bias_a$: absolute bias

$$bias_a = c - c_{CRM}$$

u_{biasa}: absolute bias uncertainty

$$u_{biasa} = \sqrt{\frac{S_{RL}^2 + (c + RSD_{RL})^2}{n} + u_{CRM}^2}$$

u_0: absolute standard uncertainty

$$u_0 = \sqrt{S_{RL}^2 + u_{biasa}^2}$$

RSU: relative standard uncertainty

$$RSU = \sqrt{{RSD_R}^2 + u_{biasr}^2}$$

F.3 Limit of Detection (LOD)

The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single-laboratory validation.

$$\text{LOD} = \sqrt{\frac{u_0 \times 4}{1 - (4 \times RSU^2)}}$$

u_0: absolute standard uncertainty

RSU: relative standard uncertainty

F.4 Limit of Quantitation (LOQ)

The limit of quantification is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

$$\text{LOQ} = \sqrt{\frac{u_0^2}{RSU_{max}^2 - RSU^2}}$$

u_0: absolute standard uncertainty

RSU: relative standard uncertainty

RSUMAX: .largest acceptable relative standard uncertainty

APPENDIX G

TABULATED DATA FOR MEASUREMENT UNCERTAINTY ESTIMATIONS FOR CONSTRUCTED GTS 40-3-2 REFERENCE PLASMIDS

G.1 In-House Repeatability Calculations of Three Independent Duplicate Readings of Calibration Plasmid Dilutions

Table G.1. Tabulated values for within-laboratory reproducibility standard deviation and the (within-single laboratory) repeatability relative standard deviation estimations for three independent measurements

Reading Set 1									
Dilutions	**x1**	**x2**	**Ci**	**(c1-c2)**	**Di**	**radi**	**d**	**SRL**	**RSDR**
10^6	6,3783	6,4751	6,4267	-0,0967	0,0967	1,5053	-0,0484	ND	1,5936
10^5	4,8050	4,8406	4,8228	-0,0356	0,0356	0,7390	-0,0178	ND	ND
10^4	3,4557	3,3691	3,4124	0,0866	0,0866	2,5366	0,0433	ND	ND
10^2	4,3773	4,6828	4,5300	-0,3055	0,3055	6,7438	-0,1528	-0,1354	ND
Reading Set 2									
Dilutions	**x1**	**x2**	**Ci**	**(c1-c2)**	**Di**	**radi**	**d**	**SRL**	**RSDR**
10^6	6,5827	6,6414	6,6121	-0,0587	0,0587	0,8882	-0,0294	ND	1,4324
10^4	2,8339	2,7785	2,8062	0,0555	0,0555	1,9765	0,0277	ND	ND
10^2	2,5827	2,3347	2,4587	0,2480	0,2480	10,0849	0,1240	0,1099	ND
Reading Set 3									
Dilutions	**x1**	**x2**	**Ci**	**(c1-c2)**	**Di**	**radi**	**d**	**SRL**	**RSDR**
10^6	5,9578	5,9578	5,9578	0,0000	0,0000	0,0000	0,0000	ND	0,2764
10^5	4,9372	4,9646	4,9509	-0,0274	0,0274	0,5528	-0,0137	ND	ND
10^4	3,7212	4,1943	3,9578	-0,4731	0,4731	11,9542	-0,2366	-0,2097	ND

Table G.2 In-House Repeatability Calculations of Three Independent Duplicate Readings of Calibration Plasmid Dilutions

		Trueness		
		Reading 1	**Reading 2**	**Reading 3**
Concentration	**Certificate Values**	**Standard Deviation**	**Standard Deviation**	**Standard Deviation**
10^6	6	0,0484	0,0293	0,0484
10^5	5	0,0178	ND	0,0178
10^4	4	0,0433	0,0277	0,0433
10^2	2	0,1527	0,1240	ND

G.2 In-House Bias Control and Estimation of Uncertainty Component Associated with Bias of Three Independent Duplicate Readings of Calibration Plasmid Dilutions

Table G.3. Uncertainty of Measurements for Each Concentration Value of Three Readings

Um Uncertainty of Measurements for Each Concentration Value			
Concentration	**Reading 1**	**Reading 2**	**Reading 3**
10^6	0,0342	0,0207	0
10^5	0,0126	ND	0,0097
10^4	0,0306	0,0196	0,1673
10^2	0,1080	0,0877	ND

Table G.4 Expanded Uncertainties* of Measurements

Concentration	Reading 1	Reading 2	Reading 3
10^6	0,0684	0,0415	0,0000
10^5	0,0252	ND	0,0194
10^4	0,0612	0,0392	0,3346
10^2	0,2160	0,1754	ND

* Expanded uncertainty, ($2*u_m$) obtained by multiplication of coverage factor k=2 in a confidence level about 95%.

Table G.5 The relative standard uncertainties associated with the bias and Relative Bias of Measurements

	Reading 1	Reading 2	Reading 3
Ubiasr	1,1269	1,0128	0,1955
Biasr (10^6)	1,0711	1,1020	0,9929
Biasr (10^5)	0,9646	ND	0,9902
Biasr (10^4)	0,8531	0,7016	0,9894
Biasr (10^3)	ND	ND	ND
Biasr (10^3)	2,2650	1,2294	ND

Table G.6 Tabulated Values for the Calculation of Absolute Bias of Measurements for Different Plasmid Concentrations

	Reading 1	**Reading 2**	**Reading 3**
$Bias_a$ (10^6)	0,4267	0,6121	-0,0422
$Bias_a$ (10^5)	-0,1772	ND	-0,0491
$Bias_a$ (10^4)	-0,5876	-1,1938	-0,0422
$Bias_a$ (10^3)	ND	ND	ND
$Bias_a$ (10^3)	2,5300	0,4587	ND

Table G.7 Tabulated Values for the Calculation of Absolute Bias Uncertaintyof Measurements for Different Plasmid Concentrations

	Reading 1	**Reading 2**	**Reading 3**
u_{biasa} (10^6)	5,6720	7,9820	4,4148
u_{biasa} (10^5)	4,5381	ND	3,7000
u_{biasa} (10^4)	3,5411	5,2911	3,0010
u_{biasa} (10^3)	ND	ND	ND
u_{biasa} (10^2)	4,3312	5,0454	ND

Table G.8 Tabulated Values for the Calculation of Absolute Standard Uncertainty of Measurements for Different Plasmid Concentrations

	Reading 1	**Reading 2**	**Reading 3**
u_0 (10^6)	5,6736	7,9832	4,1400
u_0 (10^5)	4,5401	ND	3,7014
u_0 (10^4)	3,5437	5,2929	3,0027
u_0 (10^3)	ND	ND	ND
u_0 (10^2)	4,3333	5,0473	ND

Table G.9 Tabulated Values for the Calculation of Relative Standard Uncertainty of Measurements for Readings

Reading 1 RSU		**Reading 2 RSU**		**Reading 3 RSU**	
89,4928	**8.95%**	56,7889	**5,68%**	0,3385	**0.03%**

*If RSU ≤ 0.30 (%30) LOQ is calculated.

Table G.10 Calculated Values for Limit of Detection and Limit of Quantification

	Reading 1	**Reading 2**	**Reading 3**
LOD	0,0360	0,4080	0,2970
LOQ	1435,0920	27667,00	12,1700

APPENDIX H

TABULATED DATA FOR MEASUREMENT UNCERTAINTY ESTIMATIONS FOR CONSTRUCTED Le1 REFERENCE PLASMIDS

H.1 In-House Repeatability Calculations of Three Independent Duplicate Readings of Calibration Plasmid Dilutions

Table H.1. Tabulated values for within-laboratory reproducibility standard deviation and the (within-laboratory) repeatability relative standard deviation estimations for three independent measurements

Reading Set 1									
Dilutions	**x1**	**x2**	**Ci**	**(c1-c2)**	**Di**	**radi**	**d**	**SRL**	**RSDR**
10^6	6,0096	6,0192	6,0144	-0,0096	0,0096	0,1594	-0,0048	ND	0,5512
10^5	5,0096	5,0511	5,0304	-0,0415	0,0415	0,8257	-0,0208	ND	ND
10^4	3,9425	3,9936	3,9681	-0,0511	0,0511	1,2882	-0,0256	ND	ND
10^3	2,9201	2,9105	2,9153	0,0096	0,0096	0,3288	0,0048	ND	ND
10^2	2,0735	2,0703	2,0719	0,0032	0,0032	0,1542	0,0016	0,0014	ND
Reading Set 2									
Dilutions	**x1**	**x2**	**Ci**	**(c1-c2)**	**Di**	**radi**	**d**	**SRL**	**RSDR**
10^6	6,0078	6,0719	6,0398	-0,0641	0,0641	1,0619	-0,0321	ND	1,3608
10^5	4,9411	5,0255	4,9833	-0,0844	0,0844	1,6934	-0,0422	ND	ND
10^4	3,8947	3,9352	3,9149	-0,0405	0,0405	1,0347	-0,0203	ND	ND
10^3	3,0879	3,0373	3,0626	0,0506	0,0506	1,6533	0,0253	0,0224	ND
Reading Set 3									
Dilutions	**x1**	**x2**	**Ci**	**(c1-c2)**	**Di**	**radi**	**d**	**SRL**	**RSDR**
10^5	5,1016	5,1030	5,1023	-0,0014	0,0014	0,0269	-0,0007	ND	1,0353
10^4	3,7967	3,8201	3,8084	-0,0234	0,0234	0,6132	-0,0117	ND	ND
10^3	3,1016	3,0261	3,0639	0,0755	0,0755	2,4658	0,0378	0,0335	ND

Table H.2 In-House Repeatability Calculations of Three Independent Duplicate Readings of Calibration Plasmid Dilutions

		Trueness		
		Reading 1	**Reading 2**	**Reading 3**
Concentration	**Certificate Values**	**Standard Deviation**	**Standard Deviation**	**Standard Deviation**
10^6	6	0,0048	0,0321	ND
10^5	5	0,0208	0,0422	0,0007
10^4	4	0,0256	0,0203	0,0117
10^3	3	0,0048	0,0253	0,0378
10^2	2	0,0016	ND	ND

H.2 In-House Bias Control and Estimation of Uncertainty Component Associated with Bias of Three Independent Duplicate Readings of Calibration Plasmid Dilutions

Table H.3 Uncertainty of Measurements for Each Concentration Value of Three Readings

U_m Uncertainty of Measurements for Each Concentration Value			
Concentration	**Reading 1**	**Reading 2**	**Reading 3**
10^6	0,0034	0,0227	-
10^5	0,0147	0,0298	0,0005
10^4	0,0181	0,0143	0,0083
10^3	0,0034	0,0179	0,0267
10^2	0,0011	ND	ND

Table H.4 Expanded Uncertainties* of Measurements

Concentration	Reading 1	Reading 2	Reading 3
10^6	0,0068	0,0454	0
10^5	0,0294	0,0597	0,0010
10^4	0,0362	0,0286	0,0165
10^3	0,0068	0,0358	0,0534
10^2	0,0023	ND	ND

* Expanded uncertainty, ($2*u_m$) obtained by multiplication of coverage factor k=2 in a confidence level about 95%.

Table H.5 The relative standard uncertainties associated with the bias and Relative Bias of Measurements

	Reading 1	Reading 2	Reading 3
Ubiasr	0,3898	0,9622	0,7321
Biasr (10^6)	1,0024	1,0066	ND
Biasr (10^5)	1,0061	0,9967	1,0205
Biasr (10^4)	0,9920	0,9787	0,9521
Biasr (10^3)	0,9718	1,0209	1,0213
Biasr (10^2)	1,0359	ND	ND

Table H.6 Tabulated Values for the Calculation of Absolute Bias of Measurements for Different Plasmid Concentrations

	Reading 1	**Reading 2**	**Reading 3**
$Bias_a$ (10^6)	0,0144	0,0398	-
$Bias_a$ (10^5)	0,0304	-0,0167	0,1023
$Bias_a$ (10^4)	-0,0319	-0,0851	-0,1916
$Bias_a$ (10^3)	-0,0847	0,0626	0,0639
$Bias_a$ (10^2)	0,0719	ND	ND

Table H.7 Tabulated Values for the Calculation of Absolute Bias Uncertainty of Measurements for Different Plasmid Copy Numbers per Reaction

	Reading 1	**Reading 2**	**Reading 3**
u_{biasa} (10^6)	4,6426	5,2332	ND
u_{biasa} (10^5)	3,9468	4,4861	4,3398
u_{biasa} (10^4)	3,1956	3,7307	3,4249
u_{biasa} (10^3)	2,4512	3,1280	2,8984
u_{biasa} (10^2)	1,8548	ND	ND

Table H.8 Tabulated Values for the Calculation of Absolute Standard Uncertainty of Measurements for Different Plasmid Copy Numbers per Reaction

	Reading 1	**Reading 2**	**Reading 3**
u_0(10^6)	4,6426	5,2333	ND
u_0(10^5)	3,9468	4,4862	4,3399
u_0(10^4)	3,1956	3,7308	3,4250
u_0(10^3)	2,4512	3,1281	2,8986
u_0(10^2)	1,8548	ND	ND

Table H.9 Tabulated Values for the Calculation of Relative Standard Uncertainty of Measurements for Readings

Reading 1 RSU		**Reading 2 RSU**		**Reading 3 RSU**	
4,7330	**0.47%**	1,6666	**0.17%**	**18,5344**	**1.85%**

*If RSU ≤ 0.30 (%30) LOQ is calculated.

Table H.10 Calculated Values for Limit of Detection and Limit of Quantification

	Reading 1	**Reading 2**	**Reading 3**
LOD	1,94707E-10	0,0291	0,00012275
LOQ	7524874,1600	1380,6248	15602,1275