universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Characterization of NK603 transgene in a stacked maize variety"

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2015 / Vienna 2015

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 838
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Ernährungswissenschaften
Betreut von / Supervisor:	Univ. Doz. Dr. Alexander Haslberger

Danksagung

An dieser Stelle möchte ich mich ganz herzlich bei Dr. Christian Brandes für die Ermöglichung und die unermüdliche Unterstützung meiner Masterarbeit bedanken. Zusammen mit Sina Ben Ali haben sie mir die Methoden der Molekularbiologie nahegelegt und mir bei dem Schreiben meiner Arbeit immer wieder neue Impulse gegeben. Ein besonderer Dank geht auch an Sina Ben-Ali für die Einführung in die praktische Laborarbeit. Ihre Unterstützung und Hilfsbereitschaft konnten mir bei jedem aufkommenden Problem zur Lösung verhelfen. Außerdem möchte ich mich bei Mag. Rupert Hochegger und der gesamten AGES für die Ermöglichung und Genehmigung meines Masterprojektes bedanken.

Ein besonderer Dank geht auch an Univ. Doz. Dr. Alexander Haslberger für die offizielle Betreuung meiner Masterarbeit.

Des Weiteren bedanke ich mich ganz herzlich bei Miriam Macke und Lea Ranacher für das Korrekturlesen meiner Arbeit. Ihr habt mir damit sehr geholfen.

Zuletzt möchte ich mich bei meiner Familie, meinem Freund und bei all meinen Freunden bedanken, die mir neben gutem Zureden und tatkräftiger Hilfe, allein durch ihr Dasein eine große Unterstützung waren und sind.

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List of abbreviations

bp	base pair
C.	Confidence
Ct	Cycle threshold
CV	coefficient of variation
ddH_2O	double destilated water
ddNTP	Dideoxynucleotide triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ERA	Environmental risk assessment
f or fwd	forward
GM	Genetically modified
HCl	Hydrochloric acid
HRM	High resolution melting
kb	kilobase
$MgCl_2$	Magnesium chloride
MM	Master mix

nm	nanometre
PCR	Polymerase Chain Reaction
r or rev	reverse
rpm	rounds per minute
SNP	Single nucleotide polymorphism
ssDNA	single stranded DNA
T-DNA	Transfer DNA

1. Introduction

In 2014, 30% of cultivated maize (184 million hectares) was genetically modified (GM) maize and around 135 different events in GM maize were authorized worldwide [Transgen, 2015a]. Since the introduction of genetically modified organisms (GMOs) in the European Union (EU) in 1997, several GM maize varieties are authorized for the import and use in food and feed based on regulation (EC) 1829/2003. Only one maize event (MON810, responsible for insect resistance) is released for the commercial cultivation in the EU [Umweltbundesamt, 2015]. However, there are many other GM maize varieties in the pipeline for release in the EU. For the use of GM products in food and feed different GM maize varieties are authorized (e.g. NK603 x MON810 and NK603). In addition, there is an increasing trend to make use of stacked events (GMOs including several transgenes). The genetic stability of GMOs required by the "Guidance for risk assessment of food and feed from genetically modified plants" [EFSA, 2011] and by the directive 2001/18/EC is an important parameter for the approval of GMOs in the EU. For identification and quantification of GMOs using real-time Polymerase Chain Reaction (PCR), the stability of the transgene sequence and its border regions are of great importance. However, the post-transformational stability of commercial DNA inserts and their flanking regions has not been studied in detail. Usually, in the course of risk assessments, the genetic stability of GM plants is checked through methods (e.g. Southern blots), which are only detecting major changes. Small changes like single nucleotide polymorphisms (SNPs) cannot be detected. Nevertheless, one nucleotide change, deletion or insertion may have unintended effects and should not be underestimated.

In this study, the NK603 transgene of a stacked maize event (NK603 x MON810) including its genomic border regions was characterized and checked for its genetic stability in several individual maize grains. The NK603 construct, which is responsible for tolerance toward the herbicide glyphosate, is of popular use. For this investigation, real-time PCR with High Resolution Melt (HRM) analysis and subsequent Sanger sequencing, which are suitable for the detection of even minor DNA changes like SNPs, were used.

2. Literature review

2.1. Regulatory context

2.1.1. EU directive about the intentional release of GMOs into the environment

The directive 2001/18/EC passed in the European Parliament and approved by the Council on March 12, 2001 repeals the directive 90/220/EEC and deals with the intentional release of GMOs into the environment. It is the cornerstone of European GMO legislation and regulates the release of GMOs for experimental purposes (field trials), their placing on the market by cultivation, the import of GMOs and the transformation of GMOs into industrial products. Since it is a directive, it had to be transposed into national law until October 15, 2002 by EU member states. The aim of the directive is the approximation and harmonization of laws and regulations in all member states. Nevertheless, the main purpose is to ensure the protection of human health and the environment in accordance with the precautionary principle. Additionally, the efficiency and transparency of the approval process is another important objective. The new legal framework contributes to establishing a common procedure for the risk assessment of GMOs [EC, 2001; Transgen, 2015c].

Before its market release, every GMO needs to be notified to the national authority of the concerning member state. Aside from other required information this notification has to include an environmental risk assessment (ERA). The national authority has to report all data to the European Commission and to the responsible national authorities of the respective member states. After this procedure, the assessment can begin and only after all these authorities have approved the GMO, its release is permitted. The approval is valid in all EU member states for a maximum of 10 years. Then, a new risk assessment has to be conducted seeking for a renewed approval [EC, 2001; Transgen, 2015c].

The ERA has to be performed according to the principles described in Annex II and the required basic information listed in Annex III of EU directive 2001/18/EC must be included. Additionally, the ERA has to be carried out on a case-by-case basis followed

by a step-by-step assessment approach. The aim of the assessment is to identify and evaluate possible adverse effects of GMOs on human health and the environment. These effects can have a direct or indirect impact on human health and the environment by various mechanisms. One of these mechanisms is the genetic instability of a plant. Every pertinent mechanism, which leads to an adverse effect, should be well investigated in an ERA. Hence, the control of genetic stability is an essential part of the ERA and the genetic stability of the insert is listed as basic requirement in Annex III [EC, 2001; Transgen, 2015c].

An innovation in the directive 2001/18/EC is the post-market environmental monitoring plan of the GM plant. Unintended long-term and indirect adverse effects on humans, animals and environment must be included into the risk assessment. The regulation 1829/2003/EC on GM food and feed prescribes the obligation of applicants to implement a monitoring-plan of the GMO corresponding to Annex VII of the directive 2001/18/EC [EFSA, 2010]. The post-market monitoring plan is built upon the results of the ERA and is an essential part of the pre-market notification given to the national authority of the corresponding EU Member State. Usually, the monitoring plan can be divided into case-specific monitoring and general surveillance [Umweltbundesamt, 2011]. The reason for the establishment of a post-market monitoring mechanism is not a lack of reliability of the ERA, but helps to increase the protection level through the investigation of long-term and indirect effects of GM plants on humans, animals and the environment [EFSA, 2010].

Since each post-market monitoring is defined depending on the event, the plant and ERA results, genetic stability is not necessarily included as a monitored factor.

2.1.1.1. Directive (EU) 2015/412

This directive amends the directive 2001/18/EC by allowing European member states to choose restriction or prohibition of the GMO cultivation in their territory. Product approvals to market them for cultivation in the EU are regulated with a standard procedure, which is anchored in European law. So far, some EU member states, including Austria, have applied the safeguard clause in article 23 of directive

2001/18/EC by which the GMO marketing for the purpose of cultivation could be prohibited. The cultivation ban must be justified by an existing risk for human health or the environment. However, the legal instrument previously used is not appropriate to ensure the long-term prohibition of GMOs for commercial crop cultivation. The newly created directive (EU) 2015/412 allows each EU Member State already in the framework of the authorization procedure to restrict or prohibit the cultivation of particular GMOs in its territory [EC, 2001; EU, 2015].

2.1.2. EU regulation about genetically modified food and feed

The procedure, as well as the conditions under which an authorization of genetically modified food and feed may be approved, is determined by regulation (EC) no. 1829/2003. Since its establishment in 2004, it has replaced the Novel Food regulation (258/97) regarding GM food as well as the directive 2001/18/EC related to GM feed. In addition, this regulation expands regulation (EC) No. 1830/2003 about traceability and labeling of GMOs. In short, GMOs in food and feed have got their own regulation with more stringent security requirements, enhanced labeling and increased information rights of the public. In contrast to the Novel Food regulation, the notification procedure was extended and ingredients, additives and flavors of food and feed, as well as feed itself made out of GMOs were included. This also applies to those in which the GMO is no more traceable [Spök et al., 2004; Transgen, 2015b]. This regulation excludes foods, ingredients and additives, which are not made out of, but with help of a GMO, e.g. milk, meat or eggs of animals feed with genetically modified plants. Due to this regulation there is a uniform procedure in the EU for the authorization of all food and feed covered by the regulation. The procedure entails two essential steps. First, a scientific assessment by the European Food Safety Authority (EFSA) is performed based on documents, including data and investigations conducted and provided by the applicant. Subsequently, the European Commission and the Standing Committee on the Food Chain and Animal Health make a decision about the authorization of the food or feed product [Transgen, 2015b].

In contrast to a directive, a regulation comes into force in all member states automatically and has not to be transformed into national law.

2.2. Zea mays

Although the discovery of America maize was brought to Europe in the 15th century, its large-scale use as a crop was not until couples of centuries later. Therefore, the corn plant is a relatively young crop in Europe. In other cultures maize has been cultivated since 5000 years B.C. Therefore, maize has a considerable diversity of shape. The origin of corn is most likely in Central America and Mexico [Maiskomitee, 2015]. Together with wheat and rice, corn belongs to the major food crops of the world. In South-America, Africa and eastern Indonesia corn is the main grain used in human consumption. However, globally the majority of corn is used as animal feed. In the United States, for example, 80% of the maize crop is fed to livestock as grain or as silage [FAO, 2015].

The use of corn is manifold. Especially the production of biofuels (bioethanol) or its use for the production of heat and electricity in biogas plants is becoming increasingly important. There are new maize varieties, which are optimized for high yields in biomass. Such corn plants are significantly larger, but the energy maize varieties that are currently available are not genetically modified [Transgen, 2015a].

All cultivated maize forms belong to the same botanical species *Zea mays* L., which is mapped to the root corn (*Tripsaceae*) of large plant family grasses (*Gramineae*). It is a wind-pollinated and monoecious plant, to which the male and female flowers are arranged spatially separated. The male flowers are at the top of the main shoot, while the female flowers are formed in the leaf axillas [Maiskomitee, 2015].

Particularly important for this paper, focusing on the genetic stability of GM maize, is the consideration of the natural mutation rate, which is also applied for the foreign inserted DNA. The natural mutation rate of maize is considered as high as $3x10^{-8}$ substitutions per site per generation. However, for a maize hyper-variable microsatellite sequence a mutation rate of $8x10^{-4}$ is assumed [la Paz et al., 2010].

2.3. Stacked events

In recent years an increasing number of GM plants with stacked events have been registered in the EU for authorization under directive 2001/18/EC and regulation (EC) No. 1829/2003 [Spök et al., 2007].

A “stacked event” is defined as a line, which has more than one inserted transgene (event). There are several methods for the production of stacked events, which can be divided into direct simultaneous introduction of transgenes in a genome, and into iterative processes. Iterative processes again include retransformation of single-event plants with new transgenes and conventional cross breeding with single-event plants [Taverniers et al., 2008]. The natural crossing of two GMO lines produces hybrids with stacked events, whereby the hybrid has the properties of both parental lines. Most of the authorized and commercially used stacked events are produced by conventional breeding and not by a genetic intervention. Therefore, these stacked events display a special case in the risk assessment.

After controversial discussions about the handling of stacked events regarding the risk assessment, a revised version of the EFSA Guidance Document was published 2007. The risk assessment of plants with stacked events has to be carried out in accordance with the EFSA Guidance Document. Each inserted event has to be assessed. If the event has already been assessed as a single event, all information on the potential risks must be made available. Nevertheless, applicants have to assess the intactness and stability, the expression pattern and the potential interactions between the events. The applicants must prove that the properties and characteristics of a transgene are equal in a stacked and in a single event. Furthermore, they have to verify that there is no impact on human health or on the environment through different expression patterns. Due to different genetic backgrounds, altered expression patterns of a range of proteins in a stacked event compared with a single event are expected by the GMO panel [EFSA, 2007].

For the assessment of the intactness of the inserted event, the EFSA Guidance proposes Southern blots and PCR analyses as suitable methods. However, minor

changes like point mutations, small deletions or other small rearrangements cannot be detected by Southern blot [Spök et al., 2007].

The quantification analytics of stacked events turned out to be problematic in homogeneous corn products. This is because there is no possibility to make a differentiation analytically between a single and a stacked event in processed corn due to the lack of a independent detection method. Consequentially, in a product such as cornmeal with a determined content of 0.7% for MON810 and 0.7% for NK603 the individual values are added. Provided the company cannot prove that it is indeed a matter of a stacked event, the added values (1.4%) are exceeding the threshold of 0.9% for GMOs in food in the EU.

2.4. Genetic stability

Genetic stability is one of the conditions for the admission of GM crops defined in the EU directive 2001/18/EC. The insertion of the transgene DNA construct should occur without any genomic disruption and the insert has to be stable within the population as well as within generations. This is also important for the guaranteed coexistence of GM plants and non-GM plants anchored in the European law [EC, 2001]. To determine the genetic stability in GM plants poses a challenge for the risk assessment.

The main factors influencing stability of the transgene are the position effect and the structure of the loci. The position effect means, that depending on the position of the transgene, the DNA surrounding the transgene may have an influence. The structure of the locus includes the number of transgene copies, their intactness and their relative arrangement. They can have an impact on the likelihood of physical interactions, on further recombination within the locus and on epigenetic mechanism like DNA methylation, which may result in gene silencing. At least, it can lead to the expression of aberrant RNA species from the locus [Kohli et al., 2010].

Generally, during the production of a transgenic plant it is desired, that there is only one copy of the transgene inserted, which encodes for the intended trait. This means, only specific and known genotypic and phenotypic changes to the engineered plant

should occur. Consequently, all progeny plants of the same parents carrying the same transgene should have the same phenotype among themselves and as their parents. Comparing the phenotypes of the progenies and their non-transgenic parents, they should have the same phenotype except in the trait encoded by the transgene. However, in practice this does not correspond to reality. The new developed transgenic plant population from the same experiment shows phenotypic variations. This leads to the selection of the plants, which have the desired property [Wilson et al., 2006].

Recent studies show minor rearrangements in inserts of transgenic plants. However, low-resolution detection methods as used in routine analysis for risk assessment like Southern blot and Fluorescent in situ hybridization (FISH) are not suitable to detect these small rearrangements. A change in gene expression is often seen as a result of changes in epigenetic patterns, even though a minor rearrangement can be the cause. Therefore, the effect of minor changes is underestimated and should be better included in future risk assessments [Kohli et al., 2010].

A distinction is made between transformational DNA modifications and post-transformational DNA modifications. In this paper I focus on the post-transformational DNA modification. Nevertheless, it is important to address the first issue before we deal with the post-transformational DNA modification, which is available in the context of genetic stability.

2.4.1. Transformational DNA modification

In the last few decades, the assumption that the insertion of a transgene into a plant can be precise has been debilitated. So far, the fact that the transformation process itself may already pose a risk for unintended effect has been scarcely considered in the risk assessment. However, until now the transgene has been seen as the major risk source of the transgenic plant [Wilson et al., 2006].

Disorders like multiple insertions, duplications, translocations or deletions of the insert can occur as transformational DNA modification. Even rearrangements within the

transgene or the surrounding genomic DNA can appear. The prevalence and type of the modification depends on the way of the method with which the transgene construct is brought into the engineered plant [Neumann et al., 2011]. The most common used methods for the production of transgenic plants are particle bombardment and *Agrobacterium*-mediated transformation [Wilson et al., 2006]. Due to particle bombardment multiple copies of the transgene in tandem or inverted repeat structures are often observed [Neumann et al., 2011; Wilson et al., 2006]. Whereas in *Agrobacterium*-mediated transformation the detection of tandem repeats, incomplete DNA integration, rearrangements or the insertion of plasmid backbone sequences is predominant [Wilson et al., 2006].

The following studies are a few of the many studies, which detected undesired DNA changes caused by the production of transgenic plants. Hernandez et al. (2003) established a truncation of the Cry1A(b) gene at the 3´-end of the MON810 transgene in maize, which was produced by particle bombardment. The truncation results in a complete loss of the NOS-terminator element [Hernandez et al., 2003]. Windels et al. (2001) found various rearrangements at the 3´Nos junction of the soy bean event 40-3-2 produced by particle bombardment. Furthermore, the pre-integration site may have been rearranged [Windels et al., 2001]. In addition, in transgenic rice and oat, which were produced by particle bombardment in two studies it was shown that the intact transgene is often associated with rearranged and truncated transgene fragments [Kohli et al., 1998; Pawlowski and Somers, 1998].

2.4.2. Post-transformational DNA modification

So far, post-transformational changes of inserts and their flanking regions received low consideration in risk assessment, despite the huge effects they may have on the stability of the GMO construct. Since genetic instability may lead to differences within plant populations or within plant generations, future investigations in this issue are important for risk assessment, traceability and labeling of GM plants [Neumann et al., 2011].

Already existing studies on this issue are mainly performed with non-commercial GMOs. In contrast, commercially used GMOs are much less inspected and there exist only a few studies [Neumann et al., 2011]. Genetic stability is commonly tested after five generations by Southern blotting, which helps to give a comprehensive overview on the stability of a large genomic region comprising the transgene. However, the method is not suitable for the detection of small changes like SNPs, which too are not desired. Depending on the regions in which the small changes appear, they may lead to unintentional changes in GM plants. These alterations, for example, can affect the ingredient composition and the morphology [Neumann et al., 2011]. Despite the possible serious effects of SNPs there is no guidance given by the EFSA GMO Panel for the assessment of the genetic stability over several generations [Spök et al., 2007].

Factors affecting the genetic stability are the number and structure of the transgene integration locus, variability in the nucleotide sequence and epigenetic changes. Also discussed are viral sequences as factors causing unpredictable instabilities [Neumann et al., 2011]. Number and structure of the transgene integration locus in a genome play an important role for the genetic stability. Transgene stability can be affected due to multiple insertions, which increases the probability of homologous recombination between different transgene. Multiple insertions are particularly present if particle bombardment was used for transformation. Moreover, repetitive sequences located near the transgene or within the transgene may in theory promote homologous recombination and chromosomal rearrangements [Pla, 2012]. Further, transgenes inserted in a region with high transposition activity leads to a higher likelihood of rearrangements by active scattering of the insert to different parts of the genome [Aguilera et al., 2008; Pla, 2012]. The variability in the nucleotide sequence of the transgene is another factor influencing the genetic stability. Regarding this matter, it should be taken into account that there is a natural mutation rate, which also includes the inserted DNA. Therefore it is important for the research in this area to investigate whether the mutation frequencies in transgenes are higher than in the genomic DNA [Pla, 2012]. Due to the third factor, epigenetic changes can influence transgene expression. Even though the nucleotide sequence remains constant, the transgene

stability is impaired. For example, by increased cytosine methylation of a promoter region from a transgene, the transgene expression is inhibited. This phenomenon can vary within lines of the same species [Pla, 2012], which can be confirmed by the study of La Paz et al. (2010). In this study significant differences in asymmetrical DNA methylation between the 5´ flanking regions of different commercial MON810 varieties were verified by bisulfite sequencing PCR [la Paz et al., 2010].

The first study reporting transgene instability at the genomic level in plants transformed by particle bombardment was performed by Choffnes et al. (2001). In 300 progenies of the soybean line (*Glycine max*) containing four copies of the bovine ß-casein transgene in a single locus, transgene inheritance was investigated by Southern blotting. It was examined, that in the progenies (T1 and T2 generation) the number of transgene copies were shrinking, which speaks for an instable transgene inheritance. Stable inheritance is a condition for genetic stability. In addition a high frequency of rearrangement in the T1 and T2 generation was observed. None of the plant progenies showed gene silencing of the transgene, although they contained multiple transgene copies [Choffnes et al., 2001].

Tizaoui et al. (2012) investigated the number of functional inserts, the transgene inheritance and recombination frequencies between linked inserts of transgenic tobacco lines over three generations. The transgene inheritance behaved in accordance with to Mendelian law. In contrast, due to transgene instability Mendelian segregation was only confirmed in five out of eleven lines. This transgene instability may be caused by complex rearrangements. In addition, it was shown that the recombination frequency was increased between linked inserts. Interesting was the unstable and increasing transgene expression in nearly all investigated lines across generations [Tizaoui and Kchouk, 2012], which is in contrast to the results of Choffnes (2001) [Choffnes et al., 2001]. This can be explained by possible amplification or duplication of the insertion site [Tizaoui and Kchouk, 2012].

Aguilera et al. performed the first study about the post-marketing stability of GM commercial seed varieties. This was implemented due to the analysis of the intactness

of the MON810 transgene in all maize varieties available on the market at this time. For this purpose a combined qualitative approach with DNA and protein-based analytical methods were used. 24 out of 26 tested varieties showed genetic stability, whereas two varieties exhibited genetic instability. One of the varieties showed an absence of the MON810 construct [Aguilera et al., 2008]. This could be an example for a GMO variety that has lost its insert during lifetime.

In a study of Rosati et al. (2008) the transcriptional activity of the 3´ junction region of MON810 maize was investigated. After Hernandez et al. (2003) found a truncation at this site [Hernandez et al., 2003], Rosati et al. (2008) examined if there is an impact due to the truncation on the read-through transcription downstream the truncation site. Genomic instability and protein differences from different regions were revealed. Further, rearrangements were detected at the 3´ end of MON810. In addition, the loss of parts of the Cry1A(b) gene (including the stop codon) as well as of the NOS terminator could be demonstrated. These changes result in the expression of only a partial Cry1A(b) toxin, a new read-through transcript and new proteins with no homology to other known proteins [Rosati et al., 2008].

Ogasawara et al. (2005) examined the mutation rates of the *epsps* transgene and the endogenous gene *B-conglycinin* in Roundup-Ready® GM soybean. The resulting high mutation rates of 1 mutation per 1144 bp (*epsps* transgene) and 1 mutation per 1079 bp (*B-conglycinin* gene) demonstrated similarity. Accordingly to that, in this case the transgene is also subjected to the natural variability. Nevertheless, on the proteomic level significant differences were revealed. Only four mutations in the transgene lead to a change of amino acid, whereas in the *B-conglycinin* gene 25 amino acid substitutions were identified [Ogasawara et al., 2005]. If there is - despite a mutation - an absence of amino acid change, we are talking about a silent mutation, which is in turn resulting by substitution of the third base in a nucleotide codon. This study indicates stability of the transgene in Roundup-Ready soy lines.

La Paz et al. (2010) examined the genetic stability of MON810 maize varieties by Southern blot analysis and DNA mismatch endonuclease assays. Due to Southern

analysis the absence of any rearrangement was demonstrated. Beyond that, the more precise DNA mismatch endonuclease assays showed a lack of polymorphism within the transgene. However, 6 SNPs were detected in the 5´ flanking region 500 bp upstream from the transgene locus. Nevertheless, the mutation rate of about $1.6x10^{-5}$ substitutions per nucleotide per generation ranges within the natural mutation frequency of $8x10^{-4}$ for a maize hypervariable sequence [la Paz et al., 2010].

Papazova et al. (2006) tested the genetic stability of junction regions flanking the T-DNA of transgenic *Arabisdopsis thaliana* L. model plants. This was performed due to exposition to tissue culture stress and subsequent amplification and screening of the junction regions. With this method even small nucleotide changes can be identified. However, no changes were detected, hence the junction regions showed genetic stability [Papazova et al., 2006]. In a similar study of Papazova et al. (2008) the plants were exposed to oxidative stress and the impact of gene stacking was examined. The transgene junction regions remained stable [Papazova et al., 2008].

In a study of Neumann et al. (2011), the border regions of MON810 in transgenic maize seeds were screened by real-time PCR with Scorpion primers and subsequent Sanger sequencing for small nucleotide changes. Also in this study genetic stability was shown [Neumann et al., 2011]. The same method was used in a study of Madi et al. (2013), where the 3´ end of the insert in Roundup Ready (RR 40-3-2) soybeans was examined for small nucleotide changes. Even though, a large number of samples were screened, no mutation was detected [Madi et al., 2013].

Ben Ali et al. (2014) studied the genetic stability in a single event in oilseed rape (GT73) and in a stacked event in maize (MON88017 x MON810). As method real-time PCR and HRM with subsequent Sanger sequencing was used. The transgene of the oilseed rape and the 5´ flanking region of the maize insert showed genetic stability. In contrast, in 2 out of 100 stacked maize samples in the 3´ flanking region a heterozygous point a mutation was detected. This result is in contrast with recent studies showing genetic stability in MON810 single events [la Paz et al., 2010; Neumann et al., 2011], whereby

the hypothesis of the higher susceptibility of stacked events can be established [Ben Ali et al., 2014].

As reported, there are different study results regarding the genetic stability in transgenic plants, which show that every new GMO should be assessed individually and case by case.

2.5. NK603 transgene

NK603 is the name for the glyphosate-tolerant corn event. The gene responsible for the glyphosate tolerance is CP4 EPSPS encoding for the enzyme 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS (EC number 2.4.1.19)). EPSPS is normally present in all plants, bacteria and fungi and is involved in the synthesis of the aromatic amino acids tryptophan, tyrosin and phenylalanine. These amino acids are essential for the survival of plants. Usually, the enzyme can be inactivated by glyphosate, which leads to the death of the organism. However, the gene CP4 EPSPS from soil bacterium *Agrobacterium tumefaciens* strain CP4 encodes a glyphosate tolerant form of the enzyme EPSPS. This leads to the ability of the plant to tolerate the herbicide glyphosate. Therefore this gene was isolated and used to develop maize and other plants with glyphosate-tolerance through particle bombardment with the plasmid vector PV-ZMGT32 containing the transgene. As host organism for Roundup Ready® maize served *Zea mays* L.. In July 2004, it was authorized for food and feed by the European Commission, but not for the release into the environment. In April 2015, the European Commission renewed the authorization. Since October 2007, the stacked maize event NK603 x MON810 (object of this investigation) is authorized for food and feed in the EU according to regulation (EC) No. 1829/2001. The genetic stability of the NK603 insert was tested by Southern blot analysis. For this, genomic DNA was isolated from the plant material of over six generations of crossing and three generations of self-pollination. It could be shown that the single insert was inherited stable and after Mendelian segregation. In addition, the stable expression of the EPSPS gene could be confirmed over generations by a bioassay and an enzyme linked immunosorbent assay (ELISA) [CERA, 2015b].

2.5.1. Description of the NK603 construct

The 7229 bp NK603 transgene is constructed of the following elements:

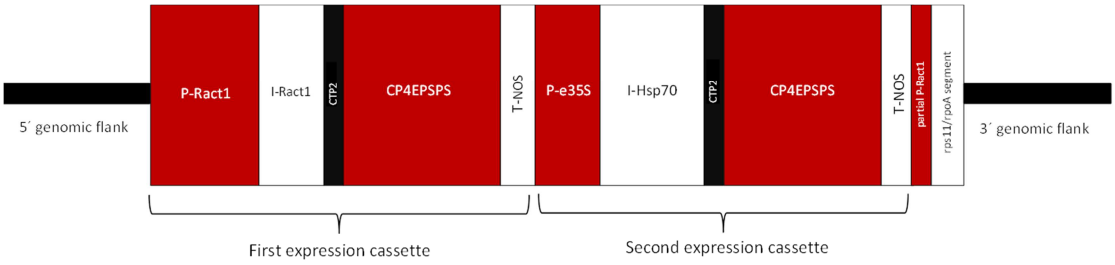


Figure 1*: Transgene construct NK603 modified after Heck et al. (2005) [Heck et al., 2005]*

In order to increase the expression rate of a glyphosate tolerant enzyme the construct consists of two transgene expression cassettes. Nevertheless, two different promoters are used; the P-ract1 and P-e35S (see Fig. 1). The first expression cassette begins with the constitutive promotor (P-Ract1) and the transcription increase intron (I-Ract1) of the rice (*Oryza Sativa*) Actin1 gene. These two elements consist of 1.4 kb and are operably connected to CTP2 with 0.2 kb isolated from *Arabidopsis thaliana*. The according sequence encodes for a chloroplast transit peptide, which is responsible for the transfer of the CP4 EPSPS protein to the chloroplast, where the aromatic amino acid synthesis occurs. CTP2, in turn, is operably connected to the CP4 EPSPS gene isolated from *Agrobacterium sp.* strain CP4 and with a length of 1.4 kb. Expression of this gene leads to a protein with the desired tolerance to glyphosate. CP4 EPSPS again is operably connected to T-NOS, which is isolated from *Agrobacterium tumefaciens* T-DNA and exists in the 3´ nontranslated region of the nopaline synthase gene. Its function is the termination of transcription and the direction of polyadenylation of the mRNA. Afterwards the first expression cassette is operably connected to the second one, which only differs in its promoter region. The promoter (P-e35S) for the second expression cassette, with 0.6 kb, is isolated from the *Cauliflower mosaic virus* 35S gene. I-Hsp70 is the following transcription increase intron, which has a length of 0.8 kb and is an intron of the heat shock protein 70 from *Zea mays*. As well as in the first expression cassette, the promoter region is operably connected to CTP2, than to CP4 EPSPS and T-NOS. At the 3´ end of the transgene an inversely linked 217 bp partial

sequence of P-Ract1 and a segment of rps11/rpoA finish the transgene construct [BiosafetyScanner, 2015; Heck et al., 2005; Monsanto, 2002].

Heck et al. figured out that the CP4 EPSPS gene of the first and second expression cassette differs in two nucleotides. There are two changes of bases in the second CP4 EPSPS gene. One of these changes leads to a silent codon substitution. This means that the translated amino acid remains the same, because the new codon encodes for the same amino acid. The second alteration leads to a substitution of a leucine codon to a proline codon. As a result, two CP4 EPSPS polypeptides are expressed with minor differences. However, the change does not affect the active site of the protein [Heck et al., 2005].

3. Materials and Methods

3.1. Materials

3.1.1. Object of investigation

3.1.1.1. Stacked event NK603 x MON810

The examined stacked event of NK603 and MON810 with the unique identifier MON-ØØ6Ø3-6 x MON-ØØ81Ø-6 is a product of traditional breeding resulting from the hybridization of the respective inbred lines [CERA, 2015a]. To meet the requirements of the directive (EC) No. 1829/2003 unique identifiers were developed. This helps the explicit identification and correct labeling of GMO products [Aguilera et al., 2008]. In this investigation, progenies (F2 generation) of the corn grains from Canada with the trade name DKC 26-79 were used. The crops were grown on loamy soil in Nova Scotia in Canada and the harvested corn was shipped on treated pallets. MON810 contains the gene Cry1A(b) encoding a toxin responsible for an insect resistance (European corn borer and other lepidopterans) and NK603 is responsible for the tolerance to the nonselective herbicide glyphosate (N-phosphonomethyl-glycin). Conventional breeding of the parental inbred lines produced the hybrid, whereas each parental inbred line was genetically modified with the particle acceleration method [CERA, 2015a].

Emphasis of this project was on the genetic stability and characterization of the NK603 construct in the stacked event of DKC 26-79.

Maize grains of the variation 6831RHXT were used as positive controls. 6831RHXT contains the transgenes NK603, HerculesXtra and Liberty Link T25.

3.1.2. Primer

3.1.2.1 Primer for verification of the transgenes NK603 and MON810

Event	Primer	Sequence	Reference
MON810	VW01_fwd	5´-TCGAAGGACGAAGGACTCTAACG-3´	[JRC, 2005a]
MON810	VW03_rev	5´-TCCATCTTTGGGACCACTGTCG-3´	
NK603	24f	5´-ATGAATGACCTCGAGTAAGCTTGTTAA-3´	[JRC, 2005a]
NK603	24r	5´-AAGAGATAACAGGATCCACTCAAACACT-3´	

Table 1*: Primer for verification of the transgenes NK603 and MON810*

3.1.2.2. Primer for zygosity testing

Primer	Sequence	Reference
5´GP forward	5'-GTCAAAGGATGCGGAACTGTT-3'	[Nan and Huabang, 2010]
5´TP reverse	5'-AAAGAACAAGTTGGATGCCGC-3'	
3´GP reverse	5'-GAGTAAGCTTGTTAACGCGG-3'	

Table 2*: Primer for zygosity testing*

3.1.2.3. Primer for PCR efficiency

Primer	Sequence	Reference
Adh1 forward	5´-CGTCGTTTCCCATCTCTTCCTCC-3´	[JRC, 2010]
Adh1 reverse	5´-CCACTCCGAGACCCTCAGTC-3´	

Table 3*: Primer for PCR efficiency*

3.1.2.4. Primer for screening of NK603 with real-time PCR and HRM analysis

Primer design was performed with the following procedure. The assumption of the NK603 elements published by Heck et al. (see Fig. 1) and by Biosafety Scanner served as a starting point [BiosafetyScanner, 2015; Heck et al., 2005]. Previous results of the laboratory provided blast documents, which show a high homology to the NK603 sequence. Additionally, some fragments of the NK603 transgene were already published. The detailed source of each primer is given in the table below.

Screening Section	Primer	Sequence (5´→ 3´)	Amplicon length	Target region	Source	
					Accession N°/Patent	Author
1	**1f**	AGAGCCTCACGTTTCCAGGG	294 bp	5´ genomic flank → NK603 insert	US PATENT 8273959 B2	[Behr et al., 2012]
	1r	GCCGCCCTAGGGATATCAAG				
2	**2f**	AGAAGAGAGTCGGGATAGTCCA	115 bp	P-ract1/I-ract1		
	2r/3r	TTGGGCCACCTTTTATTACCG				
3	**3f**	TATGCTTGAGAAGAGAGTCGGG	121 bp			
	2r/3r	TTGGGCCACCTTTTATTACCG				
4	**4f**	TCGGTAATAAAAGGTGGCCCAA	410 bp			
	4r/5r	AGCACTTTGGGCTTTAGGAACT			ACCESSION EU155408	[Shen et al., submitted 2007]
5	**5f**	TAAAATAGCTTTCCCCCGTTGC	118 bp			
	4r/5r	AGCACTTTGGGCTTTAGGAACT				
6	**6f**	CGTTGCAGCGCATGGGTATT	367 bp			
	6r	GCGTTTCTTTGGAAGCGGAG				
7	**7f**	GAATGGGGCTCTCGGATGTAGA	> 329 bp	P-ract1/I-ract1 → CTP2	ACCESSION EU155408	[Shen et al., submitted 2007]
	7r	TTCTGCACACCATTGCAGATTC			ACCESSION JN400386	[Preuss et al., 2012]
8	**8f**	TGACAAATGCAGCCTCGTGC	> 331 bp	P-ract1/I-ract1 → CP4 EPSPS	ACCESSION EU155408	[Shen et al., submitted 2007]
	8r	TGGGAGATCGACTTGTCGCC			ACCESSION AY125353	[Son et al., 2004]
9	**9f**	CGTCGTCGTGGGGATTGAAG	389 bp	CTP2 → CP4 EPSPS	ACCESSION JN400386	[Preuss et al., 2012]
	9r	GGCATTGCCGAAATCGAGCG			ACCESSION AY125353	[Son et al., 2004]
10	**10f**	AGGCGACACCTGGATCATCG	367 bp	CP4 EPSPS		
	10r	CTCGATGACCGTCGTGATGC				
11	**11f**	TCTACGATTTCGACAGCACCT	350 bp			
	11r	CAGGCGGATGGTGCGCACGC				
12	**12f**	CTCCGCACAGGTGAAGTCC	367 bp			
	12r	GCGCGGGTTGATGACTTCG				
13	**13f**	CGTCGAGACGGATGCGGACGGCG	383 bp			
	13r	CGGTCGCCCCTTCCGCGAAGGCG				
14	**14f**	ATATCCGATTCTCGCTGTCGCC	369 bp			
	14r	GAGAGTTCGATCTTCGCGCC				

Table 4*: Primer for the NK603 Screening*

Screening Section	Primer	Sequence (5´→ 3´)	Amplicon length	Target region	Source	
					Accession N°/Patent	Author
15	**15f**	TCGCCACCCATCTCGATCAC	281 bp	CP4 EPSPS → T-NOS	ACCESSION AY125353	[Son et al., 2004]
	15r	TAATCATCGCAAGACCGGCA				
16	**16f**	GTTGCCGGTCTTGCGATGAT	499 bp	T-NOS → P-e35S		
	16r	GTCTCAATCGGACCATCACATC			ACCESSION KJ608140	[Wu et al., 2014]
17	**17f**	AAGTGGATTGATGTGATGGTCCG	432 bp	P-e35S → *Zm*hsp70		
	17r	AGGCAGAGGGCGGAGTGAGCGCG			US PATENT 5424412	[Brown and Santino, 1995]
18	**18f**	ACGCGCTCACTCCGCCCTCTGCC	386 bp	*Zm*hsp70		
	18r	AATAAGCTCTGCAGACGAACAA				
19	**19f**	TAATTTGTTCGTCTGCAGAGCTT	365 bp			
	19r	AGAAGGCATCGAGCAAGATACG				
20	**20f**	GAGTTTCCTTTTTGTTGCTCTC	444 bp	*Zm*hsp70 → CP4 EPSPS		
	20r	GCTGCTTGCACCGTGAAG			ACCESSION AY125353	[Son et al., 2004]
21	**21f**	ACGAGCTTCCCGGAGTTCA	402 bp	CP4 EPSPS → T-NOS/partial P-ract1		
	21r	AAGCTTGGTACCGAATTCCCCG			US PATENT 8273959 B2	[Behr et al., 2012]
22	**22f**	AAATTATCGCGCGCGGTGTC	306 bp	T-NOS → 3´ genomic flank		
	22r	CACTAGAGTGGAAGTGTGTCGC				
23	**23f**	ATGAATGACCTCGAGTAAGCTTGTTAA	108 bp	partial P-ract1 → 3´ genomic flank		[Behr et al., 2012] [JRC, 2005b]
	23r	AAGAGATAACAGGATCCACTCAAACACT				
24	**24f**	ACACACTTCCACTCTAGTGTTTGAGTGG	201 bp	3´ genomic flank		[Behr et al., 2012]
	24r	AAGTGGTGTACGGTTAAGTTGTATACG				
25	**25f**	TTAGCAATGGCTCGTAATGCGGC	200 bp			
	25r	AACCCCATCTTCGGCGTCGCTCCG				

Table 4*: Primer for the NK603 screening*

3.1.3. Reference sequence and primer location

The employed reference sequences are listed below. Each primer location is highlighted with a specific color given below the respective sequence together with their exact location in brackets.

US PATENT 8273959 B2 - Sequence 7

1-304 Zea maize genomic DNA

305-349 construct vector DNA

350-498 rice actin 1 promoter DNA

```
“1 aatcgatcca aaatcgcgac tgaaatggtg gaagaaagag agaacagaga gcctcacgtt
 61 tccagggtga agtatcagag gatttaccgc ccatgccttt tatggagaca agaaggggag
121 gaggtaaaca gatcagcatc agcgctcgaa agtttcgtca aaggatgcgg aactgtttcc
181 agccgccgtc gccattcggc cagactcctc ctctctcggc atgagccgat cttttctctg
241 gcatttccaa ccctagagac gtgcgtccct ggtgggctgc tcggccagca agccttgtag
301 cggcccacgc gtggtaccaa gcttgatatc cctagggcgg ccgcgttaac aagcttactc
361 gaggtcattc atatgcttga gaagagagtc gggatagtcc aaaataaaac aaaggtaaga
421 ttaccggtca aaagtgaaaa catcagttaa aaggtgtata aagtaaaata tcggtaataa
481 aaggtggccc aaagtgaa”                                    [Behr et al., 2012]
```

Primer 1f Primer 1r (48-341)

Primer 2f Primer 2r/3r (380-492)

Primer 3f Primer 2r/3r (372-492)

Primer 4f (471-536)

*Sequence in bold (308-498) is repeated in US Patent 8273959 B2 - Sequence 8 with 2 more bases between cg (426 T inserted) and tg (456 G inserted)

ACCESSSION EU 155408

Oryza sativa (japonica cultivar-group) actin (Act2) gene

```
  “1 tagctagcat actcgaggtc attcatatgc ttgagaagag agtcgggata gtccaaaata
 61 aaacaaaggt aagattacct ggtcaaaagt gaaaacatca gttaaaaggt ggtataaagt
121 aaaatatcgg taataaaagg tggcccaaag tgaaatttac tcttttctac tattataaaa
181 attgaggatg tttttgtcgg tactttgata cgtcattttt gtatgaattg gtttttaagt
241 ttattcgctt ttggaaatgc atatctgtat ttgagtcggg ttttaagttc gtttgctttt
301 gtaaatacag agggatttgt ataagaaata tctttaaaaa aacccatatg ctaatttgac
361 ataatttttg agaaaaatat atattcaggc gaattctcac aatgaacaat aataagatta
```

```
 421 aaatagcttt ccccgttgc agcgcatggg tatttttct agtaaaaata aaagataaac
 481 ttagactcaa aacatttaca aaaacaaccc ctaaagttcc taaagcccaa agtgctatcc
 541 acgatccata gcaagcccag cccaacccaa cccaacccaa cccaccccag tccagccaac
 601 tggacaatag tctccacacc cccccactat caccgtgagt tgtccgcacg caccgcacgt
 661 ctcgcagcca aaaaaaaaaa aagaaagaaa aaaaagaaaa agaaaaaaca gcaggtgggt
 721 ccgggtcgtg ggggccggaa acgcgaggag gatcgcgagc cagcgacgag gccggccctc
 781 cctccgcttc caaagaaacg ccccccatcg ccactatata catacccccc cctctcctcc
 841 catcccccca accctaccac caccaccacc accacctcca cctcctcccc cctcgctgcc
 901 ggacgacgag ctcctccccc ctcccctcc gccgccgccg cgccggtaac caccccgccc
 961 ctctcctctt tctttctccg ttttttttc cgtctcggtc tcgatctttg gccttggtag
1021 tttgggtggg cgagaggcgg cttcgtgcgc gcccagatcg gtgcgcggga ggggcgggat
1081 ctcgcggctg gggctctcgc cggcgtggat ccggcccgga tctcgcgggg aatggggctc
1141 tcggatgtag atctgcgatc cgccgttgtt gggggagatg atgggggtt taaaatttcc
1201 gccatgctaa acaagatcag gaagagggga aaagggcact atggtttata tttttatata
1261 tttctgctgc ttcgtcaggc ttagatgtgc tagatctttc tttcttcttt ttgtgggtag
1321 aatttgaatc cctcagcatt gttcatcggt agtttttctt ttcatgattt gtgacaaatg
1381 cagcctcgtg cggagctttt ttgtaggtag aagatggct"
```

[Shen et al., submitted 2007]

Primer 4f (127-536)

Primer 4r/5r (127/419-536)

Primer 5f (419-536)

Primer 6f Primer 6r (435-801)

Primer 7f (1130-39)

Primer 8f (1372-235)

ACCESSION JN400386

```
"1 catggcgcaa gttagcagaa tctgcaatgg tgtgcagaac ccatctctta tctccaatct
  61 ctcgaaatcc agtcaacgca aatctccctt atcggtttct ctgaagacgc agcagcatcc
 121 acgagcttat ccgatttcgt cgtcgtgggg attgaagaag agtgggatga cgttaattgg
 181 ctctgagctt cgtcctctta aggtcatgtc ttctgtttcc acggcgtgca tgcttcatgg"
```

[Preuss et al., 2012]

Primer 7r (1130-39)

Primer 9f (138-437)

ACCESSION AY125353

1-159 = CTP

160-1530 = CP4 EPSPS

1531-1831 = NOS

1832-1946 = repeated fragment of CP4 EPSPS gene

```
"1 cacataaaac cccaagttcc taaatcttca agttttcttg tttttggatc taaaaaactg
61 aaaaattcag caaattctat gttggttttg aaaaaagatt caatttttat gcaaaagttt
121 tgttccttta ggatttcagc atcagtggct acagcctgca tgcttcacgg tgcaagcagc
181 cggcccgcaa ccgcccgcaa atcctctggc ctttccggaa ccgtccgcat tcccggcgac
241 aagtcgatct cccaccggtc cttcatgttc ggcggtctcg cgagcggtga aacgcgcatc
301 accggccttc tggaaggcga ggacgtcatc aatacgggca aggccatgca ggccatgggc
361 gccaggatcc gtaaggaagg cgacacctgg atcatcgatg gcgtcggcaa tggcggcctc
421 ctggcgcctg aggcgccgct cgatttcggc aatgccgcca cgggctgccg gctgaccatg
481 ggcctcgtcg gggtctacga tttcgacagc accttcatcg gcgacgcctc gctcacaaag
541 cgcccgatgg gccgcgtgtt gaacccgctg cgcgaaatgg gcgtgcaggt gaaatcggaa
601 gacggtgacc gtcttcccgt taccttgcgc gggccgaaga cgccgacgcc gatcacctac
661 cgcgtgccga tggcctccgc acaggtgaag tccgccgtgc tgctcgccgg cctcaacacg
721 cccggcatca cgacggtcat cgagccgatc atgacgcgcg atcatacgga aaagatgctg
781 cagggctttg gcgccaacct taccgtcgag acggatgcgg acggcgtgcg caccatccgc
841 ctggaaggcc gcggcaagct caccggccaa gtcatcgacg tgccgggcga cccgtcctcg
901 acggccttcc cgctggttgc ggccctgctt gttccgggct ccgacgtcac catcctcaac
961 gtgctgatga accccacccg caccggcctc atcctgacgc tgcaggaaat gggcgccgac
1021 atcgaagtca tcaacccgcg ccttgccggc ggcgaagacg tggcggacct gcgcgttcgc
1081 tcctccacgc tgaagggcgt cacggtgccg gaagaccgcg cgccttcgat gatcgacgaa
1141 tatccgattc tcgctgtcgc cgccgccttc gcggaagggg cgaccgtgat gaacggtctg
1201 gaagaactcc gcgtcaagga aagcgaccgc ctctcggccg tcgccaatgg cctcaagctc
1261 aatggcgtgg attgcgatga gggcgagacg tcgctcgtcg tgcgtggccg ccctgacggc
1321 aaggggctcg gcaacgcctc gggcgccgcc gtcgccaccc atctcgatca ccgcatcgcc
1381 atgagcttcc tcgtcatggg cctcgtgtcg gaaaaccctg tcacggtgga cgatgccacg
1441 atgatcgcca cgagcttccc ggagttcatg gacctgatgg ccgggctggg cgcgaagatc
1501 gaactctccg atacgaaggc tgcctgatga gctcgaattc gagctcggta ccggatccaa
1561 ttcccgatcg ttcaaacatt tggcaataaa gtttcttaag attgaatcct gttgccggtc
1621 ttgcgatgat tatcatataa tttctgttga attacgttaa gcatgtaata attaacatgt
1681 aatgcatgac gttatttatg agatgggttt ttatgattag agtcccgcaa ttatacattt
1741 aatacgcgat agaaaacaaa atatagcgcg caaactagga taaattatcg cgcgcggtgt
1801 catctatgtt actagatcgg ggatcgatcc cccaccggtc cttcatgttc ggcggtctcg
1861 cgagcggtga aacgcgcatc accggccttc tggaaggcga ggacgtcatc aatacgggca
1921 aggccatgca ggccatgggc gccagg"
```

[Son et al., 2004]

Primer 8r (1372-254)

Primer 9r (138-456)

Primer 10f Primer 10r (378-744)

Primer 11f Primer 11r (494-843)

Primer 12f Primer 12r (675-1041)

Primer 13f Primer 13r (804-1186)

Primer 14f Primer 14r (1140-1508)

Primer 15f Primer 15r (1352-1632)

Primer 16f (1611-1630)

Primer 20r (661-180)

Primer 21f (1450-164)

*Sequence in bold (1688-1832) is covered by US PATENT 8273959 B2 and differs in 1825 and 1826 (Insertion of CG)

ACCESSION KJ608140

1-551 = CaMV35S promoter; regulates cp4-epsps gene

```
"1 attgagactt ttcaacaaag ggtaatatcc ggaaacctcc tcggattcca ttgcccagct
 61 atctgtcact ttattgtgaa gatagtggaa aaggaaggtg gctcctacaa atgccatcat
121 tgcgataaag gaaaggccat cgttgaagat gcctctgccg acagtggtcc caaagatgga
181 cccccaccca cgaggagcat cgtggaaaaa gaagacgttc caaccacgtc ttcaaagcaa
241 gtggattgat gtgatggtcc gattgagact tttcaacaaa gggtaatatc cggaaacctc
301 ctaggattcc attgcccagc tatctgtcac tttattgtga agatagtgga aaaggaaggt
361 ggctcctaca aatgccatca ttgcgataaa ggaaaggcca tcgttgaaga tgcctctgcc
421 gacagtggtc ccaaagatgg acccccaccc acgaggagca tcgtggaaaa agaagacgtt
481 ccaaccacgt ctcaaagcaa gtgattgatg tgatatctcc actgacgtaa gggatgacgc
541 acaatcatac t"                                          [Wu et al., 2014]
```

Primer 16r (1611-269)

Primer 17f (239-42)

US Patent 5424412

I-*Zm*hsp70

```
 "1 agatctaccg tcttcggtac gcgctcactc cgccctctgc cttgttact gccacgtttc
61 tctgaatgct ctcttgtgtg gtgattgctg agagtggttt agctggatct agaattacac
121 tctgaaatcg tgttctgcct gtgctgatta cttgccgtcc tttgtagcag caaaatatag
181 ggacatggta gtacgaaacg aagatagaac ctacacagca atacgagaaa tgtgtaattt
241 ggtgcttagc ggtatttatt taagcacatg ttggtgttat agggcacttg gattcagaag
301 tttgctgtta atttaggcac aggcttcata ctacatgggt caatagtata gggattcata
361 ttataggcga tactataata atttgttcgt ctgcagagct tattatttgc caaaattaga
421 tattcctatt ctgtttttgt ttgtgtgctg ttaaattgtt aacgcctgaa ggaataaata
481 taaatgacga aattttgatg tttatctctg ctcctttatt gtgaccataa gtcaagatca
541 gatgcacttg ttttaaatat tgttgtctga agaaataagt actgacagta ttttgatgca
601 ttgatctgct tgtttgttgt aacaaaattt aaaaataaag agtttccttt ttgttgctct
661 ccttacctcc tgatggtatc tagtatctac caactgacac tatattgctt ctctttacat
721 acgtatcttg ctcgatgcct tctccctagt gttgaccagt gttactcaca tagtctttgc
781 tcatttcatt gtaatgcaga taccaagcgg ccatgg"
```

[Brown and Santino, 1995]

Primer17r (239-42)

Primer 18f Primer 18r (19-404)

Primer 19f Primer 19r (379-743)

Primer 20f (640-180)

US Patent 8273959 B2 – Sequence 8

1-164 = *Agrobacterium tumefaciens* nos 3 'terminator

165-381 = construct vector DNA

382-686 = *Zea maize* plastid genes, rps11 and rpoA

687-1183 = *Zea maize* genomic DNA

```
 "1 gacgttattt atgagatggg tttttatgat tagagtcccg caattataca tttaatacgc
61 gatagaaaac aaaatatagc gcgcaaacta ggataaatta tcgcgcgcgg tgtcatctat
121 gttactagat cggggatatc ccggggaat tcggtaccaa gcttttataa tagtagaaaa
181 gagtaaattt cactttgggc caccttttat taccgatatt ttactttata ccacctttta
241 actgatgttt tcacttttga ccaggtaatc ttacctttgt tttatttgg actatcccga
301 ctctcttctc aagcatatga atgacctcga gtaagcttgt taacgcggcc gccctaggga
361 tatcaagctt ggtaccacgc gacacacttc cactctagtg tttgagtgga tcctgttatc
421 tcttctcgaa ccataacaga ctagtattat ttgatcattg aatcgtttat ttctcttgaa
```

```
 481 agcggtttca ttttttttta cagacgtctt tttttaggag gtcgacatcc attatgcggc
 541 ataggtgtta catcgcgtat acaacttaac cgtacaccac ttttagcaat ggctcgtaat
 601 gcggcatctc ttccgctacc agcacctttt accataactt ctgctcgttg caaacccact
 661 gtacgaatag catctactgc tgttctgctg actttatttt ttttaataaa gtgaaaaacc
 721 ataaaatgga caacaacacc ctgcccttca ctaccggtcg gagcgacgcc gaagatgggg
 781 ttcaacacgg tcgcgacacg gatgcaacgg accctccaag ccaatactcg aggccggacc
 841 gacgacgtag gcaggggtgg ccataacgac ggtggcggca tccaacttgt tctttccctt
 901 tctctgtctt caacttgcgc cggcagtctg ctagacccag gggatgctgt gtggaggaga
 961 ggtcgcgggg cccgattttt atagcctggg cgaggacgag cttggccgaa ccgatccaga
1021 gctctgcgca aatcacgaag aaccagtggg gccgctcgcg cctagcccac cgccaggagc
1081 ggggcttgtt gcgagccgta gcgtcgggaa ggggacgacc cgctaggggg gcccatgctc
1141 cagcgcccag agagaaaaaa agaaaggaag gcgcgagatg atg"
```

[Behr et al., 2012]

Primer 21r (1450-164)

Primer 22f Primer 22r (95-400)

Primer 23f Primer 23r (317-424)

Primer 24f Primer 24r (382-582)

Primer 25f Primer 25r (583-782)

Primer 2f Primer 2r/3r (195-309)

Primer 3f Primer 2r/3r (280-309)

*Sequence in bold (189-381) is repeated in US Patent 8273959 B2 - Sequence 7 with 2 less bases (232 C deleted) and (263 A deleted)

3.1.4. Kits

- Wizard® DNA Clean-Up System (Promega)
- Go Taq® Polymerase (Promega)
- 5x Go Taq® buffer green (Promega)
- Type-it HRM PCR Kit (Qiagen)
- BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)
- Antartic Phosphatase Reaction Buffer (New England Biolabs® Inc.)
- Exonuclease I (New England Biolabs® Inc.)
- Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase Kit (Thermo Scientific)
- Performa® V3 96-Well Short Plate (Edge Bio)
- Qubit® dsDNA BR Assay Kit (Life Technologies)

3.1.5. Equipment list

- Bio-Rad Chemi XRS Gel Documentation system
- Centrifuge 5415 D (Eppendorf)
- Freezer apparatus (Constructa Serva, -20°C)
- 3500 Dx Genetic Analyzer (Applied Biosystems)
- Mastercycler ep (Eppendorf)
- Nanophotometer™ from IMPLEN
- Qubit 2.0 Fluorometer (Life Technologies)
- Rotor Gene Q with specific software package Rotor-Gene 2.0.2.4 (Qiagen)
- Refrigerator (Liebherr, +4°C)
- Thermomixer comfort (Eppendorf)
- Vac-Man® Laboratory Vacuum Manifold (Promega)

3.2. Methods

3.2.1. Sample preparation

3.2.1.1. DNA purification and extraction

Each maize grain was crushed separately by using a household garlic squeezer and a mortar. Then, from each sample approximately 150 mg were placed in a 1.5 mL tube. For purification and extraction of DNA the Wizard® DNA Clean-Up System from Promega was used.

First 820 µL TNE buffer, 150 µL guanidine HCl and 30 µL Proteinase K were added to each sample and incubated over night at 60 °C in a thermomixer under constant shaking. Next day, the samples were centrifuged with 13200 rpm for five minutes with the centrifuge 5415 D (Eppendorf). 600 µL of the supernatant were transferred into a 1.5 mL tube. Then, 300 µL Chloroform (<99 %) was added and the samples were vortexed for 20 seconds. After an 8 minute centrifugation at 13200 rpm, 500 µL of the watery supernatant were transferred into a new 1.5 mL tube. Afterwards, 2 µL RNAse/H_2O mixture in a relation of 1:4 was added and incubated for 30 minutes at 60°C in a thermomixer. Thereafter, the samples were centrifuged for a few seconds. Each sample was resuspended with 1 mL of Wizard cleanup resin and the resin/DNA mix was pipetted into a Syringe Barrel of the Vac-Man® Laboratory Vacuum Manifold (Promega). Prior to that, the Syringe Barrel was attached at the Luer-Lok®, extension of each Wizard® minicolumn and its tip was inserted into the Vacuum Manifold. Then, the vacuum was applied immediately to draw the solution through the minicolumn. As soon as the solution had been pulled through the minicolumn, the vacuum was broken to avoid drying. The washing step was performed after the DNA binding step by drawing 1 mL Isopropanol (80%) twice through the minicolumn under applied vacuum. To get rid of the isopropanol residues in the minicolumn, the vacuum was re-applied for 1 minute. The syringe Barrel was removed and the minicolumn was transferred into a new lidless 1.5 mL tube. In order to purify the mixture, 20 µL of nuclease-free water was given in the center of each minicolumn, which then was centrifuged shortly at 13200 rpm. Finally, for the elution step the minicolumns were transferred into a

new 1.5 mL tube. The extracted genomic DNA was eluted applying 60 µL of Tris buffer (pH 7.4, 10 mM, 70 °C) in the center of each minicolumn an incubation of 10 minutes was conducted at room temperature. In the end, the samples were centrifuged for 1 minute at 13200 rpm to finally elute the DNA into the tubes. The minicolumns were discarded. Lastly, the DNA samples were stored at -20°C.

3.2.1.2. Photometer

To determine qualitative and quantitative parameters of the purified genomic DNA samples the Nanophotometer™ (IMPLEN) was used. DNA and proteins have different maxima of light absorbance. The specific absorbance of DNA is 260 nm and the one of proteins is 280 nm. Consequently, measured extinction at 260 nm is proportional to DNA concentration and the DNA concentration can be determined due to the Lambert-Beer Law.

In addition to the concentration of the samples, the 260/280 ratio of absorbance was determined. It helps to assess the purity of the DNA sample. The ratio for pure DNA is 1.8. Any deviations of 1.8, point out contamination of the sample caused by proteins or other contaminants. Only clean samples with a ratio between 1.8 and 1.9 were used for further investigations.

3.2.1.3. Fluorometer

In addition to the use of the Photometer for the determination of quantity and quality of the DNA in the genomic samples, the "Qubit® 2.0 Fluorometer" was used as well. The instrument uses fluorescent dyes that only bind to specific target molecules to determine the concentration of nucleic acids. Therefore, quantitation by fluorometer is more sensitive than the UV absorbance method [LifeTechnologies, 2015]. The measures were performed with the Qubit® dsDNA BR Assay Kit. Firts the working solution was prepared with 199 µL dilution buffer and 1 µL concentrated assay reagent per sample. Then, the genomic samples were diluted 1:50 (196 µL working solution and 4 µL genomic sample) with the previous prepared working solution in 500 µL tubes. This ensured that the final volume of each sample was 200 µL. The Qubit® dsDNA BR Assay Kit contains two standards (0 µg/mL DNA and 100 µg/mL

DNA), which were diluted 1:20 with the working solution to a volume of 200 µL (10 µL standard and 190 µL working solution). After vortexing and centrifugation, the diluted samples and the standards were incubated for 10 minutes. By measuring the two standards, a standard straight line was created and the DNA concentration of each sample could be determined.

3.2.2. PCR

3.2.2.1 PCR to verify the presence of MON810 and NK603

To examine whether the samples contain both events, NK603 and MON810, for every sample a qualitative PCR with subsequent 2.5% agarose gel evaluation was performed. For characterisation of the MON810 transgene, the primer pair VW01_fwd and VW03_rev and for the NK603 transgene the primer pair 23f and 23r was used (see 3.1.2.1.). The primer pair for section 23 is listed in the protocol for NK603 quantification from the Joint Research Centre (JRC) Biotechnology & GMOs unit from the European Commission and amplifies a 108 bp fragment of the 3´-flanking region [JRC, 2005b]. The primer pair VW01_fwd and VW_03_rev for the MON810 detection is listed in the protocol for MON810 quantification [JRC, 2005a].

The reaction mixture with a reaction volume of 20 µL for a qualitative PCR was as follows:

Component	Final Volume	Final Concentration
Go Taq® buffer green (5x)	4 µL	1x
dNTP (40 mM)	0.5 µL	1 mM
each Primer (100 µM)	0.5 µL	2.5 µM
Go Taq® DNA Polymerase (5u/µL)	0.3 µL	0,075 u
ddH_2O to 20 µL	12.8 µL	-
Genomic DNA sample (~ 150 ng/µL)	1 µL	~ 0,75 ng

Table 5*: Reaction mixture for qualitative PCR to verify the presence of NK603 and MON810 transgenes*

The temperature program was chosen as follows:

Initialization step:	94 °C - 10 min	
Denaturation step:	94 °C - 30 sec	40 cycles
Annealing step:	58 °C - 30 sec	
Extension step:	72 °C - 30 sec	
Final extension:	72 °C - 1 min	
Final hold:	16 °C	

Afterwards, 5 µL of each PCR product was loaded on a 2.5 % agarose gel (see 3.2.3.2.).

3.2.2.2. PCR for zygosity testing

To determine the degree of NK603 zygosity a PCR-based testing method of Nan and Huabang was applied. For this purpose the primers listed in Table 2 (3.1.2.2.) were taken. 5´GP (forward primer) binds to the 5´ genomic region located next to the transgene insertion side, whereas the reverse primer 3´GP binds to the 3´ genomic region of the insertion side. One precondition for the amplification is the absence of the transgene in at least one allele. As a result, wild type or hemizygous samples show amplicons with a length of 365 bp. On the contrary, in homozygous samples the amplification fails. However, hemizygous samples are also positive for wild type as well as for NK603 [Nan and Huabang, 2010].

The reaction mixture contained following components:

Component	Final volume	Final concentration
5x Phusion *HF or GC* Buffer	4 µL	1x
dNTP (10 mM each)	0.4 µL	0,2 mM each
each Primer (100 µM)	1 µL	2.5 µM
Phusion Hot Start II High-Fidelity DNA Polymerase	0.2 µL	0,075 u
Template DNA (~ 150 ng/µL)	2 µL	~ 300 ng
ddH_2O	to 20 µL	-

Table 6*: Reaction mixture for qualitative PCR to test zygosity*

The temperature program chosen was as follows:

Initialization step:	98 °C - 2 min	
Denaturation step:	98 °C - 10 sec	Touchdown: 18x -0,5°C/cycle, then 22x57 °C
Annealing step:	66 °C - 30 sec	
Extension step:	72 °C - 30 sec	
Final extension:	72 °C - 7 min	
Final hold:	16 °C	

3.2.2.3. PCR for primer testing

For the characterization of NK603 different primers were designed and tested in various combinations. The Phusion Hot Start II High-Fidelity DNA Polymerase Kit (Thermo Scientific) was used with following reaction mixture:

Component	Final volume	Final concentration
5x Phusion HF or GC Buffer	4 µL	1x
dNTP (10 mM each)	0.4 µL	0,2 mM each
each Primer (100 µM)	1 µL	2.5 µM
Phusion Hot Start II	0.2 µL	0,075 u
High-Fidelity DNA Polymerase		
optional Mg2+ (25mM)	*e.g. 0,8 µL*	*(+ 1mM à 2,5 mM)*
optional DMSO	*(0,6 µL)*	*(3%)*
Template DNA	1 µL	~ 150 ng
(~ 150 ng/µL)		
ddH_2O	to 20 µL	-

Table 7*: Reaction mixture for qualitative PCR to test primer pairs*

Depending on the complexity of the target DNA, the buffer can be varied (HF or GC) and Mg^{2+} or DMSO can be added. The HF buffer was used as default, because of a lower error rate. Only for difficult templates, such as GC-rich templates, the GC buffer was used. With the addition of $MgCl_2$ as cofactor of the polymerase, the product yield can be improved, but at the same time the specificity is reduced. The reduction of

specificity leads to an increase of the error rate.

The temperature program chosen was as follows:

Initialization step:	98 °C - 2 min	
Denaturation step:	98 °C - 10 sec	
Annealing step:	66 °C - 30 sec	Touchdown: 18x -0.5°C/cycle, then 22x57 °C
Extension step:	72 °C - 30 sec	
Final extension:	72 °C - 7 min	
Final hold:	16 °C	

To lower the amplification of unspecific DNA sequences the touchdown PCR was chosen as default method. The annealing step began with an annealing temperature of 66 °C. In the first 18 cycles, the temperature was gradually decreased by 0.5 °C. Then 22 cycles followed with 57 °C. The higher the temperature the more specific is the binding of primers and with it also the amplicon. Consequently, in the next cycles the unspecific binding of primers is prevented due to the increased number of specific amplicons. The extension time can be chosen in dependence of the amplicon length (30 s/500bp).

3.2.3. Gel electrophoresis

3.2.3.1. 1% Agarose gel for genomic DNA

For this research, agarose gel electrophoresis was used. After purification and extraction of DNA from the different samples a 1% agarose gel was applied to assess the DNA degradation and quality. 1.5 g of agarose powder was weighed and dissolved in 150 mL TAE buffer by cooking until the solution cleared up. Then 4 µL of ethidium bromide were added and the whole mix was given into a gel chamber. 20 minutes later the gel was polymerized and transferred into an electrophoresis chamber. 5 µL of the Quantitas Fast DNA Marker (M, 200 bp-10 kb) from Biozym® was applied in the first gel lane. 8 µL of each sample was pipetted into a gel lane together with 2 µL of DNA loading buffer (5x). The gel ran for 23 minutes at 140 V, 400 mA and 100 W. After

that, the gel bounds were evaluated under UV light with Bio-Rad Chemi XRS Gel documentation system.

3.2.3.2. 2.5% Agarose gel for PCR products

For the 2.5 % agarose gel, 3.75 g of agarose were weighed in 150 mL TAE buffer. After heating this solution, 4 µL ethidium bromide were added. Some PCR products already contain the loading buffer property (when amplified with Go Taq Buffer green). In this case, 5 µL of the PCR product were directly loaded onto the gel. If a loading buffer was needed (when HF-/GC buffer was used), 5 µL of the PCR product were applied together with 4 µL loading buffer. As marker 5 µL of the Quantitas Fast DNA Marker M (Biozym®) with bounds from 100 bp to 2 kb was used.

3.2.4. Real-time PCR and HRM analysis

3.2.4.1. PCR efficiency

To prove the reliability of the following real-time PCR analysis the PCR efficiency was determined. For this, two samples with high quality (sample 11 and 36) and two with low quality (sample 2 and 9) were chosen. First, each sample was diluted to 40 ng/µL. With this dilution a dilution series was generated (1:4, 1:16, 1:64 and 1:256) for each sample. For the performance, a maize-specific primer pair for a sequence of the house keeping gene alcohol dehydrogenase-1 (Adh1) was used. Each sample was measured in duplicate. Due to the resulting Ct-values and the known concentrations a standard straight line could be developed by the program Rotor-GeneQ Series. The PCR efficiency could be calculated with the slope (m) of the standard straight line as follows: $E(\%)=(10^{(-1/m)}-1)*100$. For a striven efficiency between 110% and 90%, the slope should be between -3.1 and -3.6.

3.2.4.2. Performance

The real-time PCR and HRM analysis was performed with a Rotor-GeneQ instrument from Qiagen and the software used afterwards for representation and evaluation of results is called Rotor-GeneQ Series (version 2.0.2.4, Qiagen). For PCR the Type-it HRM PCR Kit from Qiagen was used with following reaction mixture:

Component	Volume per 16 µL reaction	Final concentration
HRM PCR Mastermix (2x)	8 µL	1x
each Primer (10 µM)	1,12 µL	0,7 µM
DNA template (~40 ng/µL)	1,6 µL	~4 ng/µL
ddH2O	to 16 µL	-

Table 8*: Reaction mixture for quantitative PCR and HRM analysis of each screening section*

The temperature program was chosen as follows:

Initialization step:	95 °C - 5 min	
Denaturation step:	95 °C - 10 sec	x 55 cycles
Annealing step:	55 °C - 30 sec	
Extension step:	72 °C - 30 sec	
HRM:	70 - 95 °C	

The Type-it HRM PCR Kit contains the fluorescent dye EvaGreen®, which spectral properties are similar to those of the fluorescent dye SYBR green. Therefore, the channel for SYBR green was chosen by means of the software.

To evaluate the differences in the amplification of each sample the Ct-values were determined, respectively. For this purpose a threshold was defined at the beginning of the exponential grow. The Ct-value is the cycle number of a sample where the exponential grow of the target sequence begins. Comparing the Ct-values of different samples helps to make relative statements about the amount of the target sequence and the efficiency of the amplification.

The post PCR melting analysis was performed by gradually increasing temperature after the last PCR cycle. Consequently, it leads to the melting of the dsDNA amplicons and therefore the concentration of the intercalating dye is diminishing. A melting

curve can be generated for each sample by plotting the fluorescence intensity against the temperature. The melting curves were normalized and temp-shifted to make them comparable. A sample from the middle of the pile was chosen as reference and the software showed HRM scores (confidence values). The reference has a confidence value of 100% and the sample with the biggest deviation has the lowest confidence value. These two samples of each screening run were used for sequencing.

3.2.5. Sequencing

The prerequisite for sequencing is the PCR product of the corresponding sample. For the research at hand direct sequencing was applied, which means that the PCR product was directly sequenced without a previous cloning of the fragment.

Sequencing was performed using the Sanger sequencing method that includes the dideoxy chain termination method. The preparation of the samples can be divided into 3 steps:

1) PCR product clean up (enzymatic)
2) Cycle sequencing reaction
3) Formamide and filter step for purification

The PCR product clean up mix was prepared from following components:

Compounds	Final Volume
ddH_2O	1,55 µL
Buffer for Exonuclease I	0,1 µL
Buffer for Antartic Phosphatase	0,1 µL
Alkaline Phosphatase	0,2 µL
Exonuclease I (*E.Coli*)	0,05 µL
Final volume per sample	2 µL

Table 9*: Reaction mixture for PCR product clean up (preparatory step of sequencing)*

The phosphatase degrades all dNTPs to dNMP and the exonuclease degrades all ssDNA. They prevent any reaction interference from preparative PCR. Hence, for purification 2 µL of the mix were added to 5 µL of each sample in a 96-Well microplate. To activate the enzymes, the reaction mixture must be incubated for 15 minutes at

37 °C. After that the enzymes need to be inactivated by degradation at 80 °C for 15 minutes. After a short centrifugation for 6 seconds, these two temperature steps have been performed in a thermocycler.

In contrast to a normal PCR, the sequencing PCR requires separate master mixes for the forward and reverse primer (see Table 10). In addition to the usual dNTPs, the sequencing PCR reaction mix contains also fluorescent ddNTPs. Every ddNTP (ddATP, ddTTP, ddCTP, ddGTP) has a different color of fluorescence. The absence of the 3'-hydroxyl group in ddNTPs prevents the binding of further dNTPs during chain elongation by DNA polymerase. Consequently, it leads to the production of fragments with different lengths of which each ends with one of the four ddNTPs.

The two master mixes for the sequencing PCR consists of following components per sample:

Component	MM forward primer (final volume)	MM reverse primer (final volume)
ddH_2O	4,5 µL	4,5 µL
BigDye® Buffer	2 µL	2 µL
Primer forward (5 µM)	1 µL	-
Primer reverse (5 µM)	-	1µL
BigDye®(contains dNTPs and ddNTPs)	0,5 µL	0,5 µL
Final volume per sample	8 µL	8 µL

Table 10*: Reaction mixture for sequencing PCR*

For each sample 8 µL of the MM forward Primer and 8 µL of the MM reverse primer were given into two different 96-well microplates and 2 µL of the purified sample was added, respectively. Subsequently, the sequencing PCR was performed in a thermocycler with following stepped elongation time sequencing protocol by Platt et al. (2007):

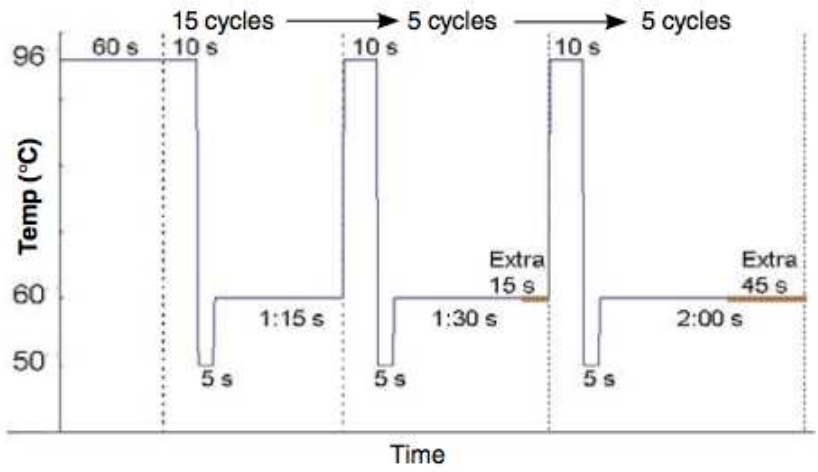


Figure 2*: Sequencing protocol with stepped elongation time [Platt et al., 2007]*

This was followed by the purification of the products with the Performa® V3 96-Well Short Plate Kit (Edge Bio) and 5 µL formamide were added to each sample. Finally, the samples were transferred into the Sequencer 3500 Dx Genetic Analyzer (Applied Biosystems).

4. Results

4.1. General aim and approach of the experiments

HRM analysis and Sanger sequencing were applied to analyze the genetic stability of the NK603 transgene and its border regions in transgenic maize. For this purpose, the transgene and its flanking regions were divided into 25 sections, whereas each section was analyzed separately. Montgomery (2007) showed that for SNP detection the amplicon length should be less than 400 bp [Montgomery et al., 2007]. Therefore the amplicon lengths of the different sections ranged between 108 bp to 498 bp. 24 of the 25 associating primer pairs were newly designed (see Table 4). The remaining primer pair (23f and 23r) employed is normally used for the official detection of NK603 [JRC, 2005b].

HRM analysis was used to identify possible mutations within the transgene NK603. 20 DNA samples were used to analyze each of the 25 sections. Two out of these 20 samples were subjected to Sanger sequencing. For this, the sample showing the highest deviation (low confidence value) and the reference sample (confidence value of 100%) was chosen.

An analysis of the "border regions" ranging from the GMO insert DNA to the flanking genomic DNA is important in quantitative analysis. Thus, characterization of the border regions (section 1 and 23) was included into the screening and focus was placed on these two sections containing the upstream 5´ and downstream 3´ border region. For this, 140 samples were screened for each border region in duplicate. In contrast to the other screening sections, at least ten samples showing low confidence values and one reference sample were sequenced.

The resulting sequences were compared (blasted) with the reference sequences given in 3.1.3. By using such an analysis, mutations can be confirmed and the transgene can be characterized.

4.2. Sample characteristics

4.2.1. Sample quality

Quality and quantity of extracted DNA may significantly affect subsequent analysis and the final results. Therefore, a careful DNA extraction and purification, as described in 3.2.1.1., is crucial. To assess the purity of DNA, the ratio 260/280 was determined by a photometer (3.2.1.2.). In addition to the purity, the DNA concentration was ascertained by photometer and by fluorometer. The concentrations measured by photometer ranged from 50 to 247 ng/µL resulting in an average value of 146 ng/µL (n=153). However, the DNA concentrations measured by fluorometer (3.2.1.3.) ranged from 15 to 285 ng/µL and the average value was 121 ng/µL (n=148). By applying the samples on a 1% agarose gel, the DNA degradation could be examined (3.2.3.1.).

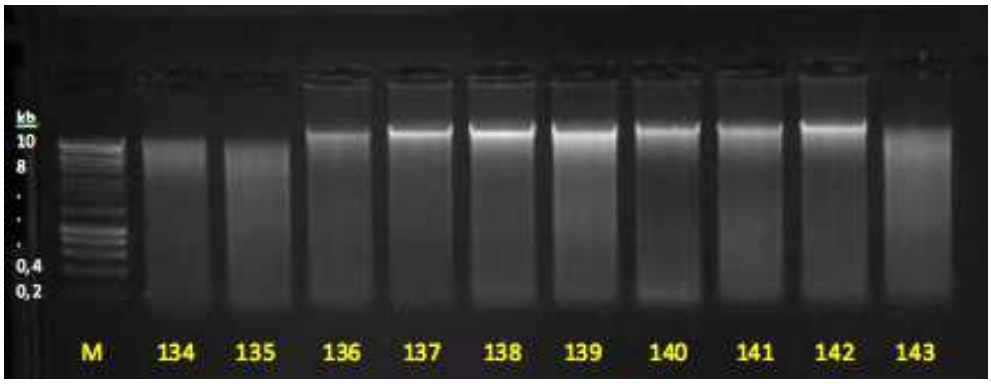


Figure 3*: 1% Agarose gel loaded with genomic DNA samples*

An example of ten DNA samples depicted in Figure 3, shows that most of the samples 136 to 142 contain very minor degraded genomic DNA. A brighter band means that the sample contains a higher concentration of genomic DNA. Every sample has a tail after the band, which is indicating a low DNA degradation. The samples 134, 135 and 143 show an absence of a distinct band at 10 kb. The same case appeared in 33 samples. Nevertheless, in 27 of the 33 samples the two events MON810 and NK603 have been verified. Hence, an absence of a clearly bound at 10 kb does not necessarily mean an absence of genomic DNA. For this reason, these 27 samples were still included in further experiments.

4.2.2. Verification of MON810 and NK603

In addition to the DNA quality, the presence of the two transgenes NK603 and MON810 have been checked. The results are essential for further investigations. The validation was performed by qualitative PCR with event specific primers and subsequent applying of the PCR products on a 2.5 % agarose gel (for further descriptions see 3.2.2.1. and 3.2.3.2.).

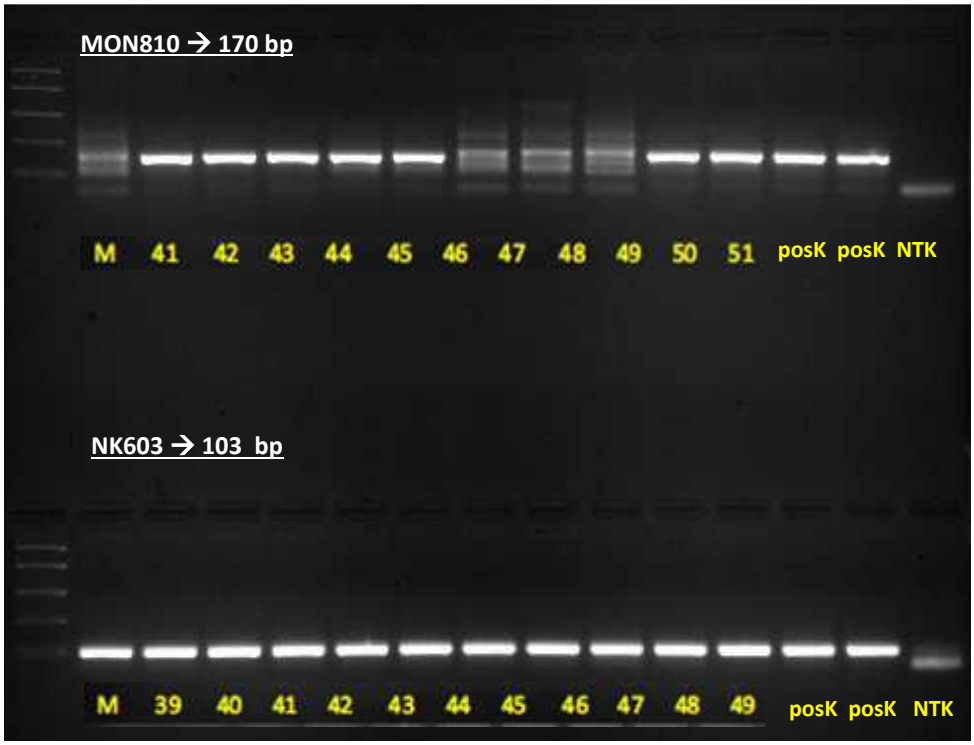


Figure 4*: Verification of MON810 and NK603 on a 2.5% agarose gel*

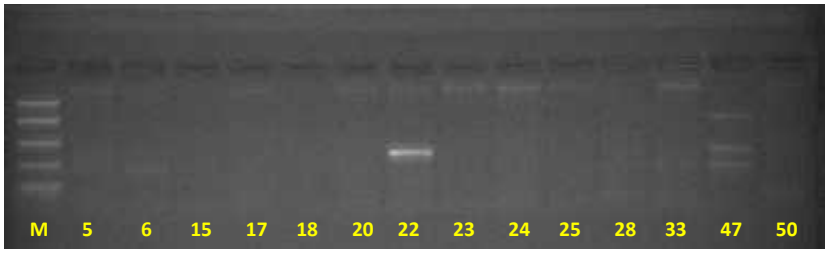
In Figure 4 the upper row represents the PCR products obtained with a primer pair for the transgene MON810 (see 3.1.2.1.). The amplicons should have a length of 170 bp, and therefore, the bands should be between the marker bands for 100 and 250 bp. Except for the samples 41, 47, 48 and 49, every sample showed a bright band at 170 bp. It is assumed that these samples, including the samples with a weak band, contain the transgene MON810. The lower row represents the amplicons of the transgene NK603. They have a length of 108 bp. Therefore, their bands should be located at around the marker band for 100 bp. Every sample from 39 to 49 showed a band in this area. Thus, the transgene NK603 was confirmed in each sample. Two samples from a maize variety with stacked events were used as a positive control. One of the samples

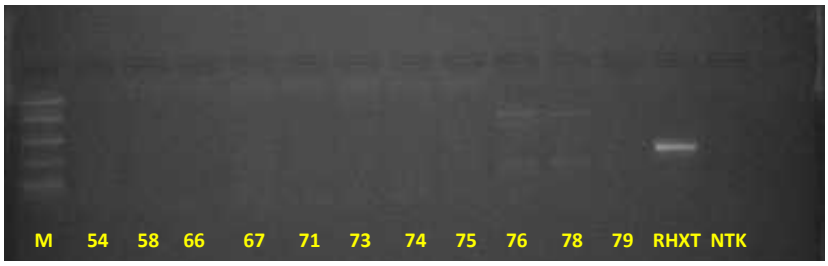
carries the transgene MON810 (Fig. 4, upper row) and the other sample carries NK603 (Fig.4, bottom row). The non-template control (NTK) includes only the master mix without a sample.

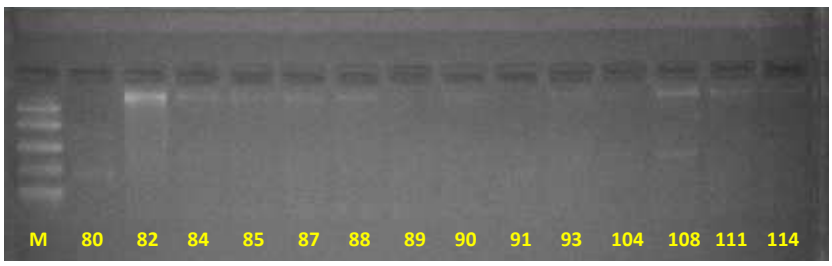
In this way, each sample could be used to determine the presence of the transgenes NK603 and MON810, respectively. At least, in each sample the transgene NK603 could be confirmed, whereas in 15 samples an absence of MON810 was observed.

4.2.3. Zygosity

For zygosity testing a PCR-based method from Nan and Huabang was performed (see 3.2.2.2.) with a subsequent gel electrophoresis on a 2.5% agarose gel [Nan and Huabang, 2010]. The absence of the transgene in, at least, one allele of a sample (wild type or hemizygous) results in an amplicon length of 365 bp. 50 samples were tested for wild type using the mentioned zygosity test.







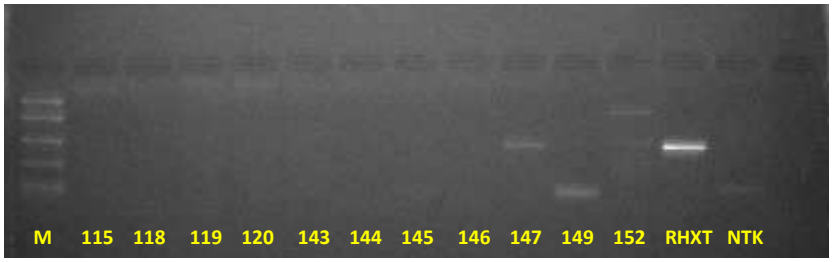


Figure 5*: 2.5% Agarose gel for wild type checking*

On the gel picture (Fig. 5) it is visible that only two samples (22, 147) out of the 50 tested samples were positive for the wild type, which does mean that we are dealing with a pure wild type or a hemizygous (contains wild type and NK603) sample. Indeed, the two samples are hemizygous, which was confirmed by the PCR based verification of NK603 (see 4.2.2), whereby in every sample the transgene NK603 could be detected. Accordingly, the other 48 samples must be homozygous in relation to the transgene.

4.3. Screening by real-time PCR and HRM analysis

By means of real-time PCR and HRM analysis quantitation curves and melting curves were obtained, respectively. The Ct-value of each sample could be determined by the quantitation curve. Mean values, standard deviations and variation coefficients of each run were calculated. Ct-values greater than 42 were evaluated as not detectable and samples determined as significant outliers ($P<0.05$) regarding their Ct-value after the Grubbs´ test were excluded of further analysis. In addition to a Ct-value in a defined range, the quantitation curve of each sample should have the shape of a sigmoidal curve. Samples without a sigmoidal shape were excluded as well. These two criteria are prerequisite for each sample for the reliability of the subsequent melting curve. Thus, the real-time PCR serves as a quality check of the amplicon of each sample. After real-time PCR, an HRM analysis was performed. The output of the HRM analysis is the melting curve, which is specific for different DNA templates. Only one single change of base can lead to a deviation of the melting curve. Therefore, the HRM analysis is suitable to identify SNPs. A complete overlap of the melting curves from two samples is necessary to conclude that the amplicons have the same sequence. Nevertheless, a specific and efficient amplification of the target DNA (verified by quantitation curve) is a prerequisite for reliable and reproducible melting curves. However, for an efficient amplification, quality and quantity of the DNA-samples have a significant importance [Druml and Cichna-Markl, 2014].

4.3.1. PCR efficiency

As already described in 3.2.4.1., the PCR efficiency is a measure for the reliability of the following real-time PCR analysis.

Sample	PCR efficiency
11 (high quality)	99.92%
36 (high quality)	98.97%
2 (low quality)	108.87%
9 (low quality)	103.46%

Table 11*: Samples used for testing PCR efficiency and their resulting PCR efficiency*

The resulting PCR efficiency values of these samples were located between 90% and 110% (see Table 11). Therefore, the subsequent real-time PCR results are reliable.

4.3.2. Screening of the whole NK603 transgene

For each screened fragment, the same 20 samples with high DNA quality were chosen from 148 prepared samples. Based on the DNA concentration that was previously obtained by fluorometer (see 3.2.1.3.), the 20 samples had been diluted to 40 ng/µL. Intercalation of the fluorescent saturation dye EvaGreen® between the bases of the minor groove of dsDNA and simultaneously detection of fluorescence signal during PCR run is the precondition for the output of an amplification curve for each sample. As control, a sample of the maize variety 6831 RHXT, which also contains the transgene NK603 was tested as well in each run.

4.3.2.1. Evaluation of the screening – one example

To picture every single screening evaluation would be beyond capacity. Hence, only one evaluation of a screening section is presented herein. For this example, the section 19 was chosen. The results of the other screening sections are summarized in Table 13, whereas the evaluations are given in the appendix in detail.

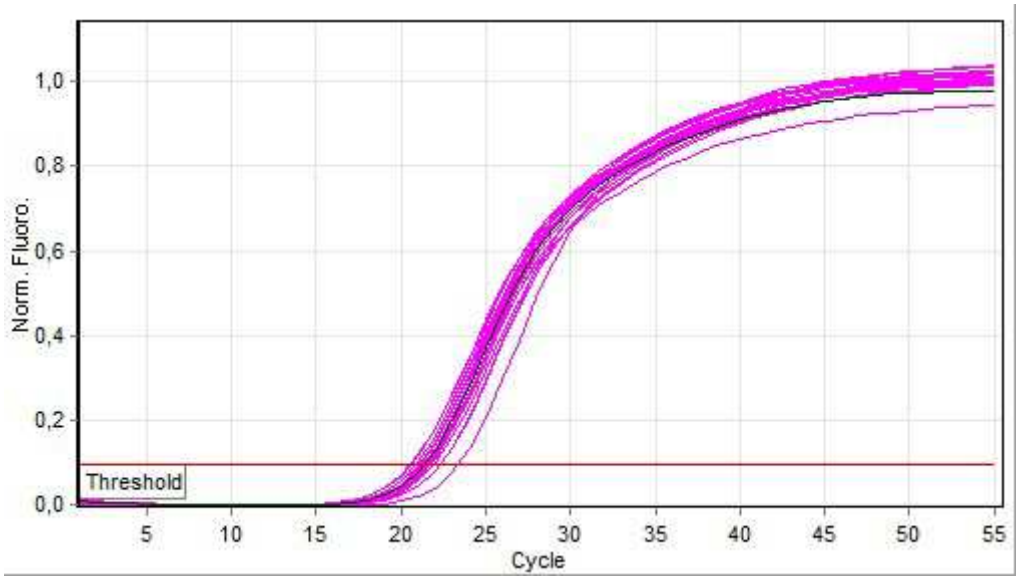


Figure 6*: Amplification curve obtained with EvaGreen and primer pair 19f and 19r for section 19*

In this experiment, the amplification curves shown in Figure 6 demonstrate the same shape and there is no marked difference between each sample. This suggests an efficient amplification. For the confirmation of this suggestion the following calculations with the obtained Ct-values were performed.

Sample	Ct-value	Confidence %
55	21.3	97.5
56	21.2	95.9
59	21.1	99.4
61	21.2	99.4
63	21.2	93.5
98**	22.2	69.8
99	21.2	88.5
100	20.8	92.4
101	21.4	87.7
103	21.8	88.3
127	20.5	99.9
128	22.2	92.8
129	21.5	98.8
130	20.4	79.0
132**	21.0	100.0
133	21.6	93.0
137	20.7	98.9
139	21.6	75.7
140	21.2	97.3
141	23.3	79.6
RHXT Pool	21.2	48.6

Table 12*: Ct-values and HRM confidence values obtained by screening of section 19*

***Samples selected for sequencing*

The obtained Ct-values are listed in Table 12. They range between 20.4 (sample 130) and 23.3 (sample 141). The resulting mean of the Ct-values is 21.4. Consequently, the standard deviation is 0.7 Ct-units and the variation coefficient is 3.0%. The low Ct-values (< 43) and the amplification curves reaching the plateau plead for an efficient amplification. In contrast to a quantitation curve (see Fig. 6) differences in melting

curves have DNA sequence variations as a cause, while differences in quantitation curves results from varying DNA concentrations.

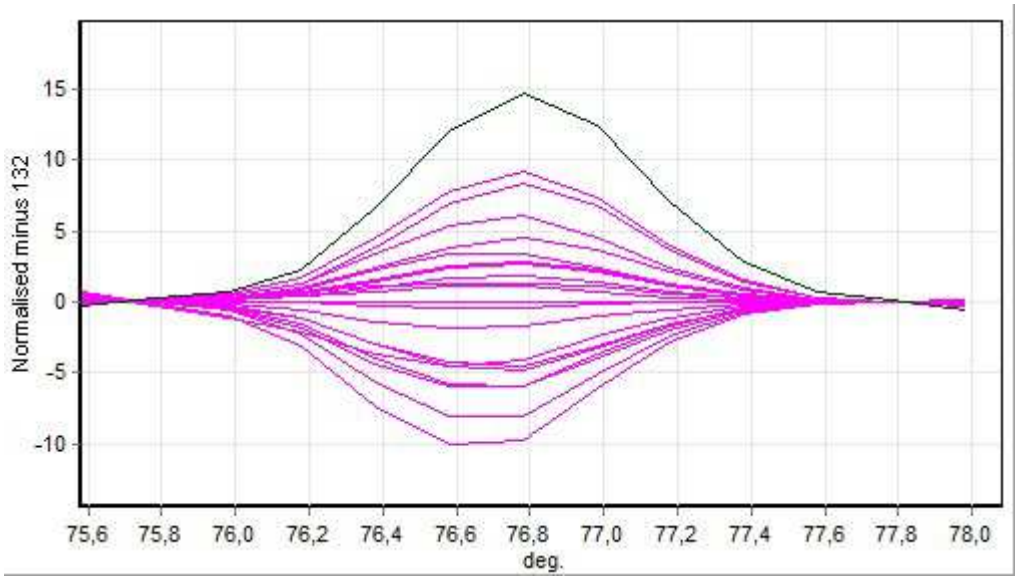


Figure 7*: Difference graph for HRM obtained with EvaGreen and primer pair 19 f and 19 r*

In this run, sample 132, in the middle of the pile of the difference graph shown in Figure 7, was chosen as reference and, therefore, has a confidence value of 100%. The obtained confidence values are listed above in Table 12. The sample with the lowest confidence value is 98** with 69.8%. This sample and the reference sample 132** were subjected to direct sequencing of the PCR products.

4.3.2.2. Screening results of all screening sections

All in all 25 primer pairs were used to screen the whole transgene.

Screening Section	Mean Ct-value	Standard deviation	CV %	Lowest confindence value (%)	Reference sample (confidence value of 100%)
1	19.5	0.6	3.3	93.3 (sample 103)	132
2	19.6	0.7	3.5	88.1 (sample 103)	127
3	19.7	0.7	3.8	20.6 (sample 103)	140
4	20.3	0.7	3.3	49.1 (sample 103)	59
5	22.0	0.9	3.9	88.8 (sample 98)	56
6	25.7	1,7	6.4	89.4 (sample 139)	55
7	20.8	0.7	3.4	77.5 (sample 103)	61
8	19.5	0.6	3.3	74.6 (sample 98)	133
9	20.4	0.7	3.2	73.5 (sample 100)	56
10	20.8	0.8	3.7	81.5 (sample56)	129
11	19.6	0.7	3.3	81.4 (sample 141)	56
12	19.4	0.7	3.4	79.8 (sample 139)	132
13	19.4	0.7	3.6	62.1 (sample 56)	132
14	20.3	0.7	3.7	86.5 (sample 141)	132
15	19.6	0.7	3.5	69.4 (sample 100)	133
16	21.3	0.7	3.2	3.7 (sample 130)	133
17	20.1	0.8	4.2	27.7 (sample 100)	61
18	20.5	0.6	2.8	94.7 (sample 137)	101
19	21.4	0.6	3.0	69.8 (sample 98)	132

Table continues on the next page

Screening Section	Mean Ct-value	Standard deviation	CV %	Lowest confindence value (%)	Reference sample (confidence value of 100%)
20	20.2	0.7	3.3	49.1 (sample 141)	59
21	19.6	0.7	3.7	79.6 (sample 139)	99
22	20.6	0.7	3.4	94.5 (sample 100)	99
23	21.8	0.6	2.9	79.3 (sample 103)	132
24	17.6	0.5	3.1	86.5 (sample 61)	63
25	20.4	0.6	3.0	84.8 (sample 103)	132

Table 13*: Screening results of each screening section*

4.3.3. Screening of the border regions

For screening of the flanking regions of the transgene NK603, 140 samples were screened in duplicate. 140 samples exceed the capacity of the Rotor Gene for one run. Hence, for each flanking region two sets of two runs were needed to screen all 140 samples in duplicate. For the respective flanking regions the screening was conducted with the primer pair 1f and 1r (for 5´-border region) and the primer pair 23f and 23r (for 3´-border region). The resulting amplicons had a length of 294 bp and 108 bp each. Since both amplicons have less than 400 bp, SNPs should be detectable through this method [Montgomery et al., 2007].

The temperature program was chosen as follows:

Initialization step: 95 °C - 5 min

Denaturation step: 95 °C - 10 sec
Annealing step: 55 °C - 30 sec
Extension step: 72 °C - 30 sec
} x 45 cycles

HRM: 83 - 90 °C

4.3.3.1. Screening of the 5´ border region of NK603

From the first screening set (70 samples measured in duplicate), the samples revealing a confidence value lower than ten percent in both runs were submitted for sequencing. However, in contrast to the first screening set, the second run of the second screening set, containing the second 70 samples, was conducted with samples diluted to 40 ng/µL. As a result, the second run of the second screening set showed up lower deviations in confidence values. Thus, from the second set, only samples with similar low (< 70 %) confidence values in both runs were sequenced. A table with the results containing the mean Ct-values and confidence values for each sample is given in the appendix (Table 17).

The Ct-values given in Table 17 (appendix) are expressed as the mean value of both runs and ranged between 17.7 (sample 158) and 21.2 (sample 55). The mean value of all Ct-values is 19.6, the standard deviation is 0.9 and the variation coefficient is 4.5%. Samples with strongly divergent Ct-values (P<0.05) were excluded of the calculations and are highlighted with *. The confidence values of both runs are given in Table 17. The ten samples showing the lowest confidence values in both runs (34, 38, 59, 100, 183, 69, 72, 110, 112, 170) and the reference sample (105) are highlighted with ** and were sequenced directly.

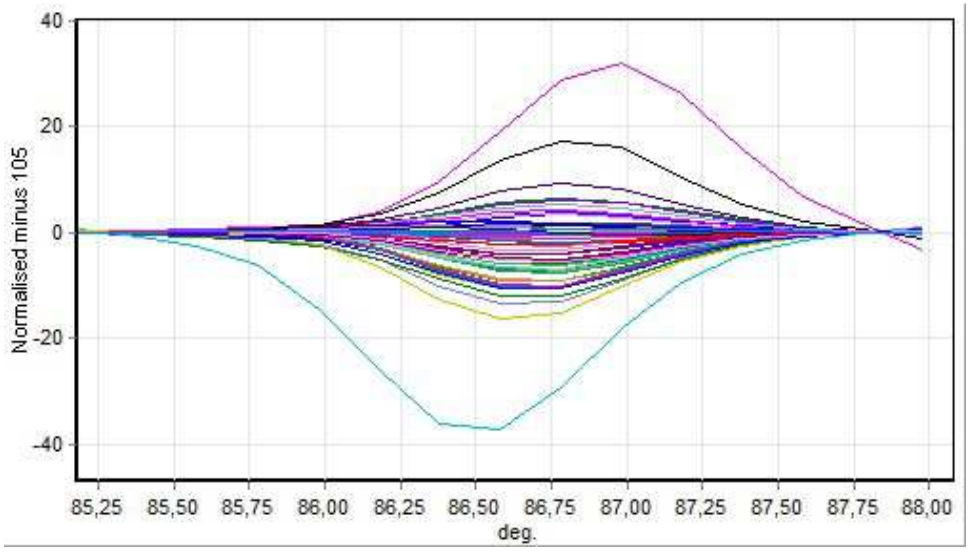


Figure 8*: Difference graph of the second screening from the second 70 samples, obtained with HRM analysis by using the HRM kit*

The difference graph normalised with reference 105 is illustrated in Figure 8 and represents the second 70 samples in the second run. According to the cited values in Table 17, the five most deviating samples no. 69, 72, 110, 112 and 170, are also depicted in Figure 8.

4.3.3.2. Screening of the 3´ border region of NK603

The first 70 samples were screened undiluted and the second 70 samples were diluted to 40 ng/µL. From the first 70 samples, the samples with a confidence value lower than 20 were sequenced. In contrast, the second 70 diluted samples had much lower deviations in confidence values. Five samples with the lowest confidence values of the first 70 (undiluted) and second 70 samples (diluted) were chosen for sequencing, respectively.

The Ct values given in Table 40 in the appendix as the mean value of both runs range between 20.76 (sample 19) and 24.12 (sample 97). The mean value of all Ct values is 20.00, the standard deviation 0.76 and the variation coefficient is 3.45%. Samples with strongly divergent Ct values were excluded of the calculations and are labelled with *. The confidence values of both runs are given in Table 40. Five samples showing the lowest confidence values in both runs of the first 70 samples (38, 100, 180, 182, 183) and five samples of the second 70 samples (69, 72, 110, 112, 171) labelled with **, were sequenced directly.

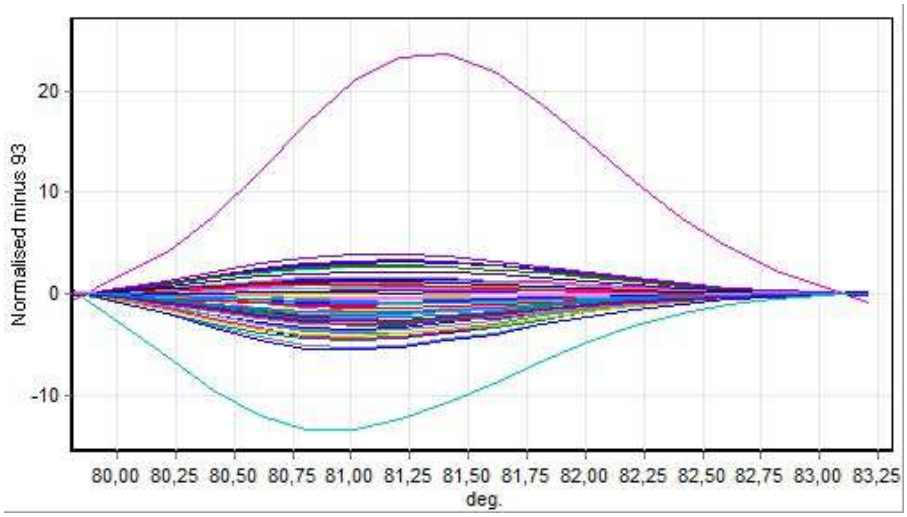


Figure 9*: Difference graph of the second screening of the 3´end from the second 70 samples, obtained with HRM analysis by using the HRM kit*

The difference graph normalised with reference 93 illustrated in Figure 9 represents the second 70 samples of the second run. According to the cited values in Table 40, the two most deviating samples 72 (purple - 3.5 C%) and 112 (cyan – 33.8 C%) are also depicted in Figure 9.

4.4. Sequencing results

All samples with low confidence values from the 25 different screenings listed in Table 13 (4.3.2.2.) were sequenced. From the results, it is assumed, that the samples are genetically stable, which means they have no differences in their sequence. This is also the case with the samples from the screening of the flanking regions, highlighted with ** in Table 17 and 40 (see appendix). However, this applies only to the studied regions.

For the forward and reverse Primer one sequence respectively is received. This allows sequencing errors to be avoided and the reliability of the results to be increased. Each sequence was blasted at [NCBI(BLAST), 2015] with the corresponding reference sequence as query sequence.

The sequencing output from the amplicons of the sample 63, which were obtained by PCR with the primer pair 24f and 24r, served as an example. This primer pair binds at the 3´end of the NK603 transgene on the *Zea mays* plastid genes, rps11 and rpoA. The sample 63 served as reference sample and therefore had a confidence value of 100%.

```
LOCUS       Sample63_Section24_fwd     180 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 CTGTTATCTC TTCTCGAACC ATAACAGACT AGTATTATTT GATCATTGAA TCGTTTATTT
       61 CTCTTGAAAG CGGTTTCATT TTTTTTTACA GACGTCTTTT TTTAGGAGGT CGACATCCAT
      121 TATGCGGCAT AGGTGTTACA TCGCGTATAC AACTTAACCG TACACCACTT GGTCATAGCT
```

Figure 10*: Output - forward sequence from sample 63, section 24*

```
LOCUS       Sample63_Section24_rev       178 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 ACACCTATGC CGCATAATGG ATGTCGACCT CCTAAAAAAA GACGTCTGTA AAAAAAAATG
       61 AAACCGCTTT CAAGAGAAAT AAACGATTCA ATGATCAAAT AATACTAGTC TGTTATGGTT
      121 CGAGAAGAGA TAACAGGATC CMCTCAAACA CTAGAGTGGA AGTGTGTACK GGCCGTCG
```

Figure 11*: Output - reverse sequence from sample 63, section 24*

The amplicons had a length of 201 bp. At the beginning and at the end of sequencing, the signals are poor. Therefore, the resulting usable sequences consist of less than 200 bp.

```
Score         Expect  Identities   Gaps      Strand    Frame
313 bits(346) 1e-89() 174/175(99%) 0/175(0%) Plus/Plus
Features:
Query  408  GGATCCTGTTATCTCTTCTCGAACCATAACAGACTAGTATTATTTGATCATTGAATCGTT  467
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGATCCTGTTATCTCTTCTCGAACCATAACAGACTAGTATTATTTGATCATTGAATCGTT  60

Query  468  TATTTCTCTTGAAAGCGGTTTCATTTTTTTTTACAGACGTCTTTTTTTAGGAGGTCGACA  527
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TATTTCTCTTGAAAGCGGTTTCATTTTTTTTTACAGACGTCTTTTTTTAGGAGGTCGACA  120

Query  528  TCCATTATGCGGCATAGGTGTTACATCGCGTATACAACTTAACCGTACACCACTT  582
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TCCATTATGCGGSATAGGTGTTACATCGCGTATACAACTTAACCGTACACCACTT  175
```

Figure 12*: Blasted forward sequence of sample 63, section 24 (Sbjct) against the Query sequence for the Zea maize plastid genes, rps11 and rpoA (US Patent 8273959 B2) [Behr et al., 2012]*

```
Score         Expect  Identities   Gaps      Strand     Frame
298 bits(330) 2e-85() 166/167(99%) 0/167(0%) Plus/Minus
Features:
Query  382  ACACACTTCCACTCTAGTGTTTGAGTGGATCCTGTTATCTCTTCTCGAACCATAACAGAC  441
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  167  ACACACTTCCACTCTAGTGTTTGAGKGGATCCTGTTATCTCTTCTCGAACCATAACAGAC  108

Query  442  TAGTATTATTTGATCATTGAATCGTTTATTTCTCTTGAAAGCGGTTTCAttttttttAC  501
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  107  TAGTATTATTTGATCATTGAATCGTTTATTTCTCTTGAAAGCGGTTTCATTTTTTTTTAC  48

Query  502  AGACGTCttttttAGGAGGTCGACATCCATTATGCGGCATAGGTGT  548
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47   AGACGTCTTTTTTTAGGAGGTCGACATCCATTATGCGGCATAGGTGT  1
```

Figure 13*: Blasted reverse sequence of sample 63, section 24 (Sbjct) against the Query sequence for the Zea maize plastid genes, rps11 and rpoA (US Patent 8273959 B2)[Behr et al., 2012]*

By blasting the amplicons with the corresponding reference sequence US Patent 8273959 B2 [Behr et al., 2012], it is verified that each base of the forward amplicon matches that of the reference sequence (see Fig. 12). The same result can be observed for the reverse amplicon with the exception that there is a single discrepancy at the locus 540 labelled in yellow in Figure 13. Instead of a C for Cystein, an S was detected. S means that the result of the sequencing is G or C. If we take a look at the same locus of the blasted reverse sequence, highlighted in green in Fig. 13, the discrepancy cannot be confirmed. Thus, it is concluded that there is no evidence for a mutation.

At the locus 407 of the blasted reverse sequence, highlighted in yellow in Fig. 13, instead of a thymine, a K was detected, which means the result of the sequencing is thymine or guanine. Unfortunately, it is not possible to compare this locus with the

one of the forward amplicon to confirm the detection, because this section is missing. However, the locus is at the beginning of the amplicon and the signal is not succinct for one of the four bases. Thus, the finding is negligible.

The analysis of each sequenced sample was performed in this manner. In the following table for each screening section (including the screening of the flanking regions) the results of the sequenced samples blasted with their corresponding query sequence are given.

Screening Section	Sequenced Samples	Confidence value %	Amplicon length	Target region	Query sequence	Region of query sequence	Results
1	103 132 34 38 59 100 105 183 69 72 110 112 170	93.3 100 3.0 0.0 2,9 6.0 100 1,5 59.5 20,8 17,4 0.3 31	294 bp	5´ genomic flank → NK603 insert	US PATENT 8273959 B2 Sequence 7	48-341	**No differences**
2	103 127 139	88.1 100 89.5	115 bp	P-ract1/I-ract1		380-494	**Insertion at 426 (T) and 456 (G)**
3	103 140	83.6 100	121 bp			372-494	**Insertion at 426 (T) and 456 (G)**
4	59 103 139	100 49.1 61.7	410 bp		ACCESSION EU155408	127-536	**Mutation at 337** (4x G instead of A; 1x R instead of A, 1x K instead of A)
5	56 98	100 88.8	118 bp			419-536	**No differences**
6	55 130 139 12 21	100 97.7 89.4	367 bp			435-801	**Deletion of AA at 670 and 671**
7	61 103	100 77.5	>329 bp	P-ract1/I-ract1 → CTP2	ACCESSION EU155408 ACCESSION JN400386	1130-1419 1-39	**Mutation at 1204 (G instead of A)**

Table 14: *All sections, their region, their amplicon length, their query sequence and their screening/sequencing results (section 1-7)*

Screening Section	Sequenced Samples	Confidence value %	Amplicon length	Target region	Query sequence	Region of query sequence	Results
8	98 133	74.6 100	>331 bp	P-ract1/l-ract1 → CP4 EPSPS	ACCESSION EU155408 ACCESSION JN400386 ACCESSION AY125353	1372-1419 1-240 151-254	**Mutation at 238 (C instead of T)** of the JN400386 query sequence **Mutation at 153 (G instead of A) and 156 (G instead of C)** of the AY125353 query sequence
9	56 100	100 73.5	389 bp	CTP2 → CP4 EPSPS	ACCESSION JN400386 ACCESSION AY125353	138-240 151-456	**Mutation at 238 (C instead of T)** of the JN400386 query sequence **Mutation at 153 (G instead of A), 156 (G instead of C**), **354 (G instead of C), 364 (C instead of A), 366 (C instead of G)** of the AY125353 query sequence
10	56 101 129	81.5 83.3 100	367 bp	CP4 EPSPS	ACCESSION AY125353	378-744	**Mutation at 471** (C instead of G)**, 477** (G instead of C) **and 615** (Y instead of T).
11	56 127 100	100 85.8 88.2	350 bp			494-843	**Mutation at 615** (Y instead of T) **Mutation at 800** (Y instead of T) Only in sample 127: **Mutation at 622** (R/M instead of A) **Mutation at 648** (R/S instead of G)
12	132 139	100 79.8	367 bp			675-1041	**Mutation at 800** (3xY, 1xC instead of T)
13	56 127 132 142	62.1 99.8 100 65.8	383 bp			804-1186	**No differences**
14	132 139 141	100 86.6 86.5	369 bp			1140-1508	**No differences**
15	100 133	69.4 100	281 bp	CP4 EPSPS → T-NOS		1352-1632	**Deletion of GAGCTCGGTACCGGATCCAATT at 1541-1562**
16	4 9 63 130 133	58.5 3,7 100	499 bp	T-NOS → P-e35S	ACCESSION AY125353 ACCESSION KJ608140	1611-1824 1-269	**No differences**

Table 15*: All sections, their region, their amplicon length, their query sequence and their screening/sequencing results (section 8-16)*

Screening Section	Sequenced Samples	Confidence value %	Amplicon length	Target region	Query sequence	Region of query sequence	Results
17	61 99 100	100 50.8 27,7	432 bp	P-e35S → *Zm*hsp70	ACCESSION KJ608140 US PATENT 5924412	239-551 1-42	**Insertion of T at 491 and of G at 502**
18	101 137	100 94.7	386 bp	*Zm*hsp70	US PATENT 5424412	19-404	**No differences**
19	98 132	69.8 100	365 bp		US PATENT 5424412	379-743	**No differences**
20	81 141	49.1	444 bp	*Zm*hsp70 → CP4 EPSPS	US PATENT 5424412 ACCESSION AY125353	640-812 162-180	**No differences**
21	99 139	100 79.6	402 bp	CP4 EPSPS → T-NOS/partial P-ract1	ACCESSION AY125353 US PATENT 8273959 B2 Sequence 8	1450-1687 1-164	**Deletion of GAGCTCGGTACCGGATCCAATT at 1541-1562**
22	98 137	99.6 99.5	306 bp	T-NOS → 3´ genomic flank	US PATENT 8273959 B2 Sequence 8	95-400	**No differences**
23	103 132 38 100 180 182 183 69 72 110 112 171	42.9 100 0.0 9,6 9,2 11,6 0.3 84.6 2,5 14,6 22,4 81.3	108 bp	partial P-ract1 → 3´ genomic flank		317-424	**No differences**
24	61 63	86.5 100	201 bp	3´ genomic flank		382-582	**No differences**
25	103 132	84.8 100	200 bp			583-782	**No differences**

Table 16*: All sections, their region, their amplicon length, their query sequence and their screening/sequencing results (section 17-25)*

The query sequences highlighted in grey are published as the official sequence fragments of the NK603 transgene. Consequently, the sequence of non-mutated NK603 samples should exactly match to the concerning query sequence of the sections 1, 2, 3, 18, 19, 22, 23, 24 and 25. However, in contrast to these sections, the

comparison of the sequenced samples with the query sequence of the sections 4, 6, 7, 8, 9, 10, 15, 17 and 21 may result in small differences. In this case, mutations cannot be detected by a blast analysis between the resulting sequence and the appropriate query sequence, but instead by a comparison within several samples. If there are the same “alterations” in different samples, it can be assumed that there is no mutation, and the sequence of the corresponding NK603 region differs from the query sequence. Therefore, in these sections the results are labelled in green, which means there are no differences within the sequences of the samples.

In section two and three, which overlap, the same two insertions can be found at the same loci in each sample by blasting with the according query sequence. The reason for this alteration is that the primer pairs for these two 5´ sections also bind at the 3´-end of the transgene (189-381 of sequence 8 from US PATENT 8273959 B2). Accordingly, the amplification with these primer pairs leads to two different amplicons, one of the 5´-end and one of the 3´-end. The amplicon sequence of the 3´-end has two additional bases in exactly these two loci. This is also confirmed by its query sequence (US Patent 8273959 B2 – Sequence 8). As a result, we obtained two additional signals for these two bases by sequencing this amplicon mixture. Thus, it can be assumed that sections two and three are not changed at the 5´-end.

Sections eight and nine were blasted against different query sequences. Two of these query sequences overlap. The mutations found in the overlapping area do not match, and therefore, they canceled each other out.

The CP4 EPSPS gene exists twice within the transgene. However, they differ in two nucleotides [Heck et al., 2005]. In sections ten, eleven and twelve, which target regions contain the CP4 EPSPS gene, two of the five different alterations (615 and 800 of the query sequence) can be attributable to the differences between the two gene-cassettes. The other changes occurring always at the same loci of different samples, seem to be peculiar to the genuine NK603 sequence.

However, sample 127 of section 11 constitutes an exception. The resulting sequence differed in two loci (highlighted in red in Fig. 14 and 15) compared to the query sequence (622 and 648).

```
Query   602   ACGGTGACCGTCTTCCCGTTACCTTGCGCGGGCCGAAGACGCCGACGCCGATCACCTACC   661
              ||||||||||||| |||||| ||||| |||||||||||||||||||| || ||||||||||
Sbjct   94    ACGGTGACCGTCTYCCCGTTRCCTTGSGCGGGCCGAAGACGCCGACRCCKATCACCTACC   153
```

Figure 14*: Blasted forward sequence of sample 127 (Sbjct) of section 11 against query sequence*

```
Query   614   TTCCCGTTACCTTGCGCGGGCCGAAGACGCCGACGCCGATCACCTACCGCGTGCCGATGG   673
              | |||||| ||||||| |||| |||||||||||| ||||||||||||||||||||||||
Sbjct   218   TYCCCGTTMCCTTGCGSGGGCSGAAGACGCCGACSCCGATCACCTACCGCGTGCCGATGG   159
```

Figure 15*: Blasted reverse sequence of sample 127 (Sbjct) of section 11 against query sequence*

As already mentioned, the first variation, highlighted in green, at locus 615 of the query sequence is attributable to the difference between the two gene cassettes. Figure 14 shows the forward sequence (Sbjct) blasted against the query sequence and in figure 15 the reverse sequence (Sbjct) blasted against the query sequence is shown. In the first locus instead of an A, an R (which stands for A or G) was detected in the forward sequence (see Fig. 14) and an M (which stands for A or C) was detected in the reverse sequence (Fig.15). Furthermore, in the second locus instead of a G, an R (G or A) respectively an S (G or C) was detected in the forward (Fig.14) and reverse (Fig. 15) sequence. In such a case, we have a close look at the sequencing chromatogram.

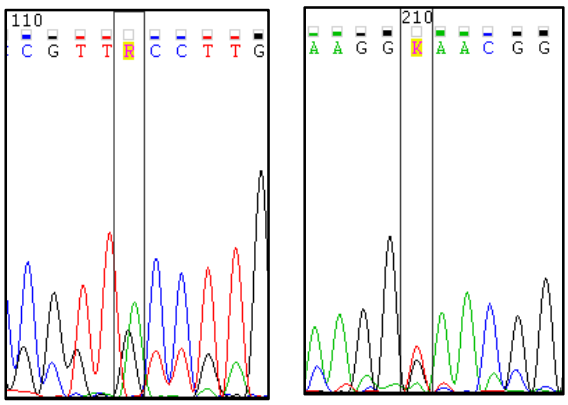


Figure 16*: Chromatogram of sample 127 (forward and reverse) from section 11, locus 622 of the query sequence*

These two figures, regarding the first deviation in locus 622 of the query sequence, show the chromatogram of the forward (left) and reverse (right) sequence in this locus. The resulting reverse sequence refers to the complementary strand. In both sequences the signal for A (in the complementary sequence for T) is clear. In addition,

the signal is larger than the signal of the second detected base, which leads to the unclear result. However the second detected base differs in the forward and reverse sequence.

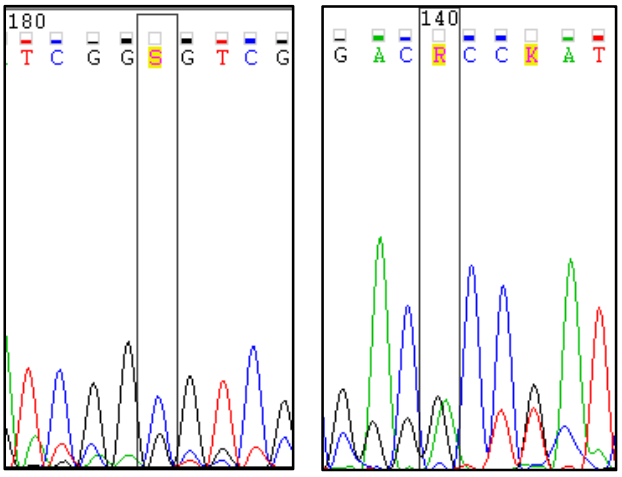


Figure 17: *Chromatogram of sample 127 (forward and reverse) from section 11, locus 648 of the query sequence*

The same is true for the second variation examined at locus 648 of the query sequence. If the second detected base would be the same one at the same locus, it might be an obvious SNP occurring heterozygous. Therefore, in this case it would be tempting to assume that there may be a mutation, but it has to be clarified by further investigations.

5. Discussion

Due to advisory reports concerning the principles for the GMO safety assessment published by the Food Agriculture Organization/World Health Organization (FAO/WHO) [FAO/WHO, 1996, 2000], the safety assessment of GM plants was globally harmonized and applied in GMO regulation. Even though, regarding the assessment of stacked events, differences in GMO regulations can still be observed between the EU and other parts of the world: In the EU, a stacked event is seen as a new GMO, which has to be newly assessed, despite its production by traditional breeding [EFSA, 2011]. Therefore, the applicants have to provide the same documents for a new stacked event as well as for a new single GM event. However, if the underlying single events had already been assessed in their parental lines, some of the required data for the stacked event are not as relevant. The Commission implementation regulation (EU) No 503/2013 on applications for authorization of GM food and feed in accordance with regulation (EC) 1829/2003, established in June 2013, demands the assessment of the insert stability, the transgene expression including the expressed product, and the potential synergistic or antagonistic effects in a stacked GM event [Kok et al., 2014].

Since 2007, the stacked event NK603 x MON810 is authorized in the EU for food and feed according to the regulation (EC) No 1829/2003. For the insert detection the authorisation holder Monsanto is obligated to provide a method, which is an event specific real-time quantitative PCR based method. This method is evaluated and validated by the Community reference laboratory, which is established under Regulation (EC) No 1829/2003 and published by the European Union Reference Laboratory for GM Food and Feed [EC, 2015]. The evaluation and validation is based on recommendations and requirements for GMO testing elaborated by the European Network of GMO Laboratories (ENGL) [JRC, 2015]. The according primer pairs bind at the border region of the respective event, which explains the importance of particularly these regions. In addition, the successful amplification of these regions, including genomic and transgene DNA, provides a proof of the insert integrity.

However, the applicants performed the investigation for possible post-transformational DNA modifications for the validation. An independent verification is

not regulated by law. In addition, in risk assessment, stacked events are often considered as safe, when their parent lines were already favourably assessed. Nevertheless, different inserts in a stacked event may affect each other and should not be seen as comparable with the single event in their parental lines. Stacked GM plants are mainly produced by natural crossing of two GM lines, containing one or more inserts. Kok et al. (2013) assumed that the stability of the transgene is not at greater risk if two GM lines are crossed compared with a crossing of two conventional varieties [Kok et al., 2014].

The genetic stability in GM plants is mostly tested by Southern blots. Likewise, genetic stability of the F1 generation from NK603 x MON810 maize was tested by Monsanto [Monsanto, 2005]. With this method it is possible to detect major rearrangements. Nevertheless, investigations on the genomic level in risk assessment are lacking and minor changes like SNPs cannot be detected [Kohli et al., 2003; Spök et al., 2007; Wilson et al., 2006]. SNPs occur more frequently than major changes [Madi et al., 2013] and they may, depending on their location in the genome, have a huge impact on the plant organism. Therefore, the effects of SNPs should not be underestimated. Many SNPs can lead to a change of phenotype of the plant [Madi et al., 2013].

The companies producing GM crop seeds assume that the F1 generation of a crop is harvested and commercialized and is not intended for further breeding. Therefore, the genetic stability over generations is not seen as important. However, since the harvested seeds are used for human and animal nutrition, the genetic stability of the F2 generation is important as well. Furthermore, despite advice against, some farmers favor to use the F2 generation or classical seeds for further breeding, e.g. because of financial troubles.

The aim of this study was to analyze the genetic stability of NK603 and its border regions in progenies (F2 generation) of the NK603 x MON810 stacked GM maize hybrid DKC 26-79. This was performed using HRM analysis and Sanger sequencing. The transgene and its border regions (including genomic and transgene DNA) were divided into 25 sections with lengths ranging from 108 bp to 498 bp. 24 of the

25 corresponding primer pairs were newly designed. It was assumed that samples with mutations could be detected by HRM analysis and verified by subsequent sequencing. The resulting sequences were compared to their query sequences. Due to the sequencing results, the transgene could also be characterized. From the results, it is concluded, that the samples are genetically stable (expect two possible mutations), which means they have no differences in their sequence.

According to La Paz et al. (2010) one mutation occurs naturally in $3x10^8$ tested nucleotides of the maize genome [la Paz et al., 2014]. For my experiments it is valid to make the following calculations. If it is assumed that each nucleotide has the same mutation rate, (a) one nucleotide has to be tested in $3x10^8$ samples or (b) $3x10^8$ nucleotides must be tested in one sample, to detect a natural occurring mutation. The following can be calculated: excluding the investigated 5´ and 3´ border regions 7531 bp of the transgene were tested in 20 replications (20 samples). Thus, we screened 150,620 nucleotides (20 samples x 7531 bp). 150,620 nucleotides are just a few compared with the natural mutation rate, and, thus, below the requirements for making a reliable conclusion. However, testing $3x10^8$ nucleotides would be outside the demands for a thesis. Moreover, the aim of the study was to investigate if there are deviations from the natural mutation rate and this can be analyzed with a moderate number of nucleotides. Particularly, if the results of Ben Ali et al., (2014) are taken: two mutations were found in 16,000 nucleotides [Ben Ali et al., 2014]. Therefore, it was concluded that if there are mutations below the natural mutation rate, 150, 620 tested nucleotides should be an appropriate number for their detection. However, the fact that particular regions (hot spots) of the genome are more susceptible to mutations than others must be considered. Especially, the insertion of foreign DNA must be considered as a special case. According to Papazova et al. (2008) the T-DNA and its surrounding DNA is more sensitive to mutations [Papazova et al., 2008]. Additionally, it is assumed by Ho et al. (1999) and Kohli et al. (1999) that the transgenic CaMV 35S promoters may trigger rearrangements [Ho et al., 1999; Kohli et al., 1999]. The transgenic maize line of this study has a CaMV 35S promoter in each of its transgenes. Hence, and regarding the assumed higher susceptibility of a stacked event,

it seems that there is a strong difference between the natural mutation rate and the actual mutation rate in a GM plant.

The 3´ and 5´ border regions are more important for quantitative analysis than the remaining part of a GMO construct. A rearrangement in these regions may lead to errors in quantitative results (Ben Ali 2014). Moreover, these regions are more susceptible to mutations [Papazova et al., 2008]. Hence, we focused on the examination of these loci and tested 56,280 nucleotides (140 samples x 402 bp).

A precondition for the investigations in this study was that each sample contains the transgenes NK603 and MON810. For this, a PCR with primer pairs binding at the border regions of the transgenes with a subsequent 2.5% agarose gel evaluation was performed. The transgene can be present in a homozygous state (in both alleles) or in a hemizygous state (only in one allele), which is defined as degree of zygosity. In each sample, the NK603 transgene could be confirmed, whereas in 10% of the samples an absence of MON810 was detected. A zygosity test of the NK603 transgene in 50 samples revealed two hemizygous and 48 homozygous samples. The investigated variety of this study belongs to the F2 generation. Since it is assumed that the F1 generation was hemizygous for the transgene, it should be inherited after the Mendelian segregation law. This would mean that the F2 generation would consist of 25% homozygous, 50% hemizygous and 25% wild type samples. However, this is not the case. The 0% wild type and only 1% hemizygous for NK603 can be explained by the preferential selection for transgene-positive gametes in herbicide resistant plants treated with the appropriate herbicide, which is even patented [Conner, 1997]. In contrast, the 10% wild types for MON810 range within the realms of possibility.

The high suitability of HRM analysis for the detection of single nucleotide changes is described by several studies [Druml and Cichna-Markl, 2014; Montgomery et al., 2007]. This is based on the fact that a melting profile depends on the GC-content, length, sequence and strand complementary of the amplicon. Just one nucleotide change leads to variation in the melting profile. However, two amplicons differing in one nucleotide can have the same melting behavior even though the sequence is not

exactly identical. This can be the case if for instance in one sample two mutations are occurring, which in total cancel each other out (e.g. A to C and C to A) [Druml and Cichna-Markl, 2014]. In this case, which might be rare, it can happen to detect a sample as a false negative. In this study, for each section a reference sample with a confidence value of 100% was sequenced. By this means, the number of false negative samples was reduced.

Since the quality and quantity of isolated DNA samples may significantly affect the final results, a careful DNA extraction and purification was performed. For the assessment of the DNA concentration of the samples a photometer as well as a fluorometer was used. The resulting mean concentration by photometer was 146 ng/µL (n=175) and the mean concentration measured by fluorometer was 121 ng/µL. On the one hand, quantitation by fluorometer is more sensitive than the UV absorbance method and on the other hand the UV spectrophotometer makes it possible to reveal the presence of impurities (260/280ratio) in addition to the DNA concentration. As it is visible by comparing the resulting mean values of the photometer and the fluorometer measures, the measured concentrations by fluorometer were significantly lower than the ones of the photometer. This suggests that the presence of impurities may disturb the DNA measurement by photometer. The DNA degradation could be examined by applying the samples on a 1% agarose gel. Using the above described methods, in addition to the 1% agarose gel, allowed to sort out and discard samples with bad quality and to obtain reliable DNA concentration values. All in all, 30 of 183 samples were discarded.

Except for the screening of the border regions for each of the 23 screening sections, 20 chosen samples were screened. The sample showing the highest deviation (low confidence value) and the reference sample (confidence value of 100%) was subjected to Sanger sequencing. For the border regions 140 samples were screened in duplicate, respectively and twelve samples (5´ border region) or rather eleven samples (3´ border region) were chosen for sequencing. In addition for each border region a reference sample (confidence value of 100%) was sequenced. The range of deviation or rather

the confidence values were strongly associated with variations in DNA concentration. This can be particularly shown with the screening of the 5´ border regions. In contrast to the first 70 samples (set 1), the second 70 samples (set 2) of the second run were diluted to 40 ng/µL. As a result the second 70 samples with nearly equal DNA concentrations showed up lower deviations in confidence values. This is illustrated by comparing the mean confidence values, standard deviations and variation coefficients of the undiluted and diluted samples of the second set. The first run with the undiluted samples had a mean confidence value of 64.8, a standard variation of 30.3 and a variation coefficient of 46.8%. In contrast the second run with the diluted samples had a mean confidence value of 84.4%, a standard variation of 21.4 and a variation coefficient of 25.4%. This shows the importance of equal sample DNA concentrations for an appropriate comparison of the confidence values. The impact of the unequal sample DNA concentrations may be the reason for some false positive samples, which were chosen for sequencing.

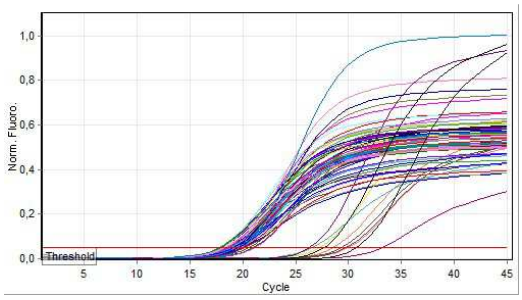


Figure 18*: Amplification curve of section 1, set 2, first run with undiluted samples*

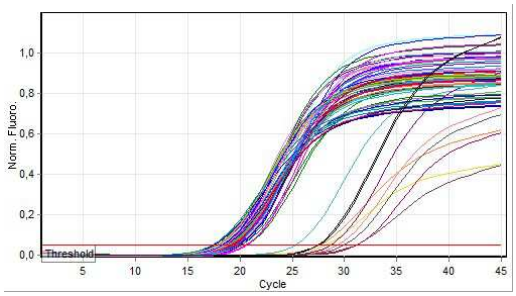


Figure 19*: Amplification curve of section 1, set 2, second run with diluted samples*

Oddly enough, comparing the Ct-values exhibits no significant difference. Nevertheless, there is a prominent variation comparing the shapes of the amplification curves illustrated in Figure 18 and 19. These results are in contrast to the fact that, particularly, the sample DNA concentration has an impact on the Ct-value. This may be because the sample DNA concentrations differ not significantly among themselves. However, despite the lack of impact by the sample DNA concentrations on the

Ct-value, there is a huge impact on the comparability of the confidence values. Therefore, it is still import to have equal DNA concentrations in all samples.

Only fragments of the NK603 construct are published or rather freely accessible. Therefore, query sequences with high homology to the genuine NK603 sequence were taken as a query sequence. In addition, the resulting sequences of different samples from one section were compared among themselves. If a deviation from the query sequence was demonstrated in each sample for one section, it was assumed that the query sequence in these loci is different compared to the actual NK603 sequence, since the occurrence of the same mutation in each sample is unlikely. Due to comparing all resulting sequences with their corresponding query sequence, no unambiguous mutation or rearrangement was found. In section eleven, which concerns the CP4 EPSPS gene, in one sample a single nucleotide change was detected in the forward and reverse sequence. However, at the according locus in both strands two bases were detected, (a) the origin base and (b) the new additional base (see also Fig. 16 and 17 in 4.4.). Two bases can be detected for one locus if a mutation occurs heterozygous. Nevertheless, for an obvious heterozygous mutation the second base (see b) would be the same in the forward and reverse strand. Indeed, the second base differed in the forward and reverse strands. Consequently, for clarification, if a mutation exists in this sample, further investigations are needed. This is especially necessary regarding the fact that the possible mutation concerns a region expressing the CP4 EPSPS protein.

As mentioned before, the object of this investigation contains the two transgenes NK603 and MON810. Both of the transgenes include the CaMV 35S promoter and the maize Hsp70 intron [CERA, 2015a]. Therefore, between sections 16 and 20, it is possible that the origin of the amplified and examined DNA segments is, in addition to the NK603, also the MON810 transgene. As a result, a part of the MON810 transgene had been screened for mutations as well. However, no mutations were found in these areas. If a mutation had been found, further investigations would be needed to assign the mutation to a specific transgene. In short, we could conclude that neither in

MON810 nor in NK603 a mutation was found in the CaMV 35S promoter and the maize Hsp70 intron.

Also mentioned above, the 35S CaMV promoter may trigger rearrangements [Ho et al., 1999; Kohli et al., 1999]. In this study, except of one possible mutation, the screened regions of the NK603 transgene showed genetic stability. This would collide with the hypothesis that the CaMV promoter triggers rearrangements. However, a mutation was detected in the 3´ MON810 region of the same variety by Ben Ali et al. (unpublished), which in turn would confirm the hypothesis. If the 35S CaMV promoter affects the genetic stability should be clarified by further studies. Insert instabilities in maize with MON810 events were detected [Aguilera et al., 2008; Ben Ali et al., 2014]. In studies about transformational as well as in studies about post-transformational DNA modifications in MON810, instabilities were exclusively found at the 3´ end of MON810 [Ben Ali et al., 2014; Hernandez et al., 2003; Rosati et al., 2008]. In contrast to MON810, studies about the NK603 transgene on the genomic level are rare and show genetic stability [Heck et al., 2005; Nielsen et al., 2004]. The MON810 transgene, especially the 3´ end, seems to be more susceptible to modifications compared to the NK603 transgene. It has to be considered, that in this study only fragments of the NK603 transgene were screened and, therefore, it cannot be concluded that the whole transgene is stable. In consideration of latter and the few studies regarding the stability of NK603 on the genomic level, further studies should be conducted in future. In addition, only few studies about the genetic stability in stacked events exist. With the advent of new methods like next generation sequencing (NGS) it would be possible to perform whole genome analyses. This would allow determining differences in mutation rates by comparing single and stacked events. Moreover, changes in gene expression could be studied more efficiently by NGS. Hence, at least, there are many more factors influencing the genetic output than the DNA sequence, future investigations should also focus on the transcriptomic and proteomic level.

6. Conclusion

There are only few studies examining the post-transformational stability on the genomic level. In risk assessment, genetic stability is mainly investigated with methods (e.g. Southern blots), which are not suitable for the detection of small nucleotide changes. In addition, studying the genetic stability of stacked events is insufficient. Therefore, in this study real-time PCR with HRM analysis was used, which is suitable to detect even single nucleotide changes. The object of investigation was the NK603 transgene and its border regions in a stacked maize event (NK603 X MON810). Samples with possible mutations, detected by HRM, were subjected to Sanger sequencing. The NK603 transgene including its border regions was divided into 25 screening sections. The two sections containing the 3´ and 5´ border regions were investigated in a higher sample number, because these regions are important for quantification in GMO analytics. Therefore, mutations in these loci may produce incorrect results. For each screening section (excluding screening section 23) primer pairs were newly developed and tested. The screened regions, which did not cover the whole transgene, showed genetic stability. Only in one sample two possible mutations were detected in the region expressing the CP4 EPSPS gene. However, the presence of these mutations has to be clarified in further investigations. Due to this study, new NK603 event specific primers were designed and previously unknown sequences of the NK603 transgene were characterized. These two study outputs can be used in further investigations of the NK603 insert in different plant organisms. In addition to these results, Ben Ali et al. (unpublished) found two mutations located in the MON810 transgene of the same variety. Put into the context of the existing literature, the MON810 transgene seems to be more susceptible to mutations than the NK603 transgene.

Regarding the increasing number of commercial GMOs containing an expanding number of stacked events, it is necessary to clarify if there is a positive association between the number of stacked events and the mutation rate. Usually, only the insert and its border regions are analyzed. However, instabilities triggered by a genetic modification may also occur in other genomic areas. By using powerful methods like

Next Generation Sequencing (NGS) it will be possible in future investigations to (a) compare the mutation rates of single and stacked events and (b) to perform whole genome analysis in a simplistic way. Furthermore, studies performed on the transcriptomic and proteomic level will be also more attractive for future investigations.

7. Abstract

7.1. Abstract (english version)

The use of genetically modified seeds in the EU has been substantially increasing since the introduction of genetically modified organisms (GMOs) in 1996. Thus, the controls of food, feed and seed are gaining importance. The genetic stability of GMOs required by the directive 2001/18/EC is an substantial parameter for the approval of GMOs in the EU.

In this study the transgene NK603 of maize with a stacked event (NK603 x MON810) was characterized with its genomic border regions and checked for genetic stability. For the identification and quantification of GMOs with real-time PCR methods, the stability of the GMO sequence and the 5' and 3' border regions is of great importance. Genetic instabilities can lead to incorrect results. PCR with High Resolution Melting (HRM) analysis and Sanger sequencing has been applied as a screening method to examine the DNA for mutations. This method makes it possible to detect small changes in DNA sequence. The NK603 transgene with a size of about 7 kb and the adjacent genomic regions were divided into different sections for screening. Potentially positive samples were sequenced and compared to the reference sequence. Thus, single nucleotide polymorphisms (SNPs) were investigated. In the examined regions of the NK603 construct with the corresponding border regions no genetic instabilities were observed.

In this study developed primers can be used for further verification of the NK603 construct by HRM analysis. In this connection, the sequenced construct from this study can serve as a reference sequence.

7.2. Abstract (german version)

Der Einsatz von gentechnisch verändertem Saatgut in der EU steigt seit der Einführung von gentechnisch veränderten Organismen (GVOs) 1996 erheblich an. Dadurch gewinnen die Kontrollen von Lebensmittel, Futtermittel und Saatgut immer mehr an Bedeutung. Die genetische Stabilität eines GVOs ist laut der Richtlinie 2001/18/EC ein wichtiger Parameter für die Zulassung von GVOs in der EU.

In dieser Studie wurde bei Mais mit einem „Stacked Event“ (NK603 x MON810) das Transgen NK603 mit seinen genomischen Grenzregionen am 5´- und 3´-Ende charakterisiert und auf genetische Stabilität überprüft. Für die Identifizierung und Quantifizierung von GVOs mit Real-time PCR-Methoden ist die Stabilität der GVO Sequenz und der 5´- und 3´- Grenzregionen von großer Bedeutung, da Instabilitäten zu falschen Ergebnissen führen können. PCR mit anschließender High Resolution Melting (HRM) Analyse sowie Sanger-Sequenzierung wurde als Screening-Methode angewendet, um die DNA auf Mutationen zu untersuchen. Dieses Verfahren ermöglicht es geringe Veränderungen der DNA-Sequenz zu erkennen. Das NK603 Transgen mit einer Größe über 7 kb und die angrenzenden genomischen Regionen wurden für das Screening in verschiedene Abschnitte unterteilt. Potentiell positive Proben wurden sequenziert, mit der Referenzsequenz verglichen und damit auf Einzel-Nukleotid Polymorphismen (SNPs) untersucht. Bei den untersuchten Regionen des NK603-Konstrukts mit den dazugehörigen Grenzregionen konnten keine genetischen Instabilitäten festgestellt werden.

Eine weitere Bedeutung der Studie besteht darin, dass die entwickelten Primer für weitere Überprüfungen des NK603-Konstrukts mittels HRM-Analyse genutzt werden können, wobei das in dieser Arbeit sequenzierte Konstrukt als Referenzsequenz verwendet werden kann.

8. Appendix

8.1. Literature index

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8.2. Confirmation

Hiermit erkläre ich, Magali Castan, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Des Weiteren versichere ich, die aus fremden Quellen direkt oder indirekt übernommenen Gedanken als solche kenntlich gemacht zu haben.

Die Arbeit habe ich bisher keinem anderen Prüfungsamt in gleicher oder vergleichbarer Form vorgelegt. Sie wurde bisher nicht veröffentlicht.

_____

Datum/Unterschrift

Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

8.3. Curriculum vitae

M A G A L I C A S T A N

Voraussichtlich MSc. im November 2015

Geboren am 5. Dezember 1985 in Freiburg im Breisgau

Deutsche und französische Staatsbürgerschaft

A U S B I L D U N G

Okt. 2012 - laufend	**Universität Wien** Masterstudium Ernährungswissenschaften mit Spezialisierung in: Molekulare Ernährung
Okt. 2007 – Nov. 2011	**Universität Wien** Bachelorstudium Ernährungswissenschaften Bachelorarbeit zum Thema *Einfluss der Ernährung auf die DNA-Methylierung*, Prof. Goldenberg
Juni 2006	**Goethe Gymnasium** Freiburg (D) Abitur im naturwissenschaftlichen Zweig

B E R U F S E R F A H R U N G

Okt. 2015 - März 2016	**Agentur für Gesundheit und Ernährungssicherheit (AGES)**, Wien FemTech-Praktikantin in der Abteilung Lebensmittelsicherheit/Molekularbiologie; Aufgaben: Transkriptomanalyse von gentechnisch veränderten Mais

Okt. 2014 – Feb. 2015	**Agentur für Gesundheit und Ernährungssicherheit (AGES)**, Wien FemTech-Praktikantin in der Abteilung Lebensmittelsicherheit/ Molekularbiologie; Aufgaben: Optimierung von RNA-Extraktionsmethoden aus Maiskörnern und -blättern
März 2014 – Juli 2014	**Agentur für Gesundheit und Ernährungssicherheit (AGES)**, Wien Masterarbeit in der Abteilung Lebensmittelsicherheit/ Molekularbiologie zum Thema genetische Stabilität in gentechnisch veränderten Mais
Juli 2013 – 8. Aug. 2013	**Institut für Umweltanalytik,** Möhrendorf (D) Praktikantin für Routineuntersuchungen der örtlichen Trinkwasserversorgung und für Lebensmittelanalytik

S P R A C H E N U N D E D V K E N N T N I S S E

Deutsch	Muttersprache
Englisch	gute Kenntnisse
Französisch	gute Kenntnisse
PC	MS Office (Word, Excel, Power Point)

8.4. Screening results

Table 17*: Screening results of the 5´ border region (section 1)*

Results of the first screening section are shown in Table 15. Samples of set 1 (samples 1 to 183) and set 2 (samples 5 to 176) were analyzed separately (in two different runs, which were repeated on a different day).

Set 1				Set 2			
Sample	**Mean Ct-value**	**Confidence % (1st run)**	**Confidence % (2nd run)**	**Sample**	**Mean Ct-value**	**Confidence % (1st run)**	**Confidence % (2nd run)**
1	19.5	35.6	39.0	5	18	92.0	90.3
3	19.8	51.2	61.2	9	20.8	77.8	87.7
4	19.6	88.4	80.7	10*	29.7		
6	19.2	38.1	43.5	15	18.9	61.3	83.2
7*	28.3			20*	30.5		
8	19.1	43.1	71.9	22*	28.1		
11	20.8	60.9	9.1	57	19.4	54.2	95.0
12	20.3	73.8	71.9	64	19.5	99.0	99.4
13	19.4	47.8	81.3	65	18.8	43.8	90.1
14	20.1	49.7	55.8	67	19.6	98.3	89.3
16	19.5	92.1	98.9	68	18.4	48.0	96.3
17	20.1	84.4	78.7	69**	19	67.6	51.4
18	19.7	98.8	86.5	70	19.2	54.5	91.2
19	20.2	98.6	89.3	71	19.1	96.0	99.9
21	20.3	99.4	90.3	72**	20.8	0.0	2.7
23	20.8	17.3	37.8	73	19.3	73.0	99.9
24	20.9	46.1	52.7	74	18.2	95.0	94.1
25	20.3	99.6	99.4	75*	25.9		
26	20.2	45.1	44.8	77	18.9	91.2	87.2
27	19	98.7	98.9	78	20	72.7	94.7
29	20.5	14.6	10.2	79	20.4	51.5	57.8
31	19.4	59.9	82.9	80	19.2	88.7	99.9

Set 1				Set 2			
Sample	Mean Ct-value	Confidence % (1st run)	Confidence % (2nd run)	Sample	Mean Ct-value	Confidence % (1st run)	Confidence % (2nd run)
32	20.5	99.6	92.3	81	18.5	98.1	99.1
33	20.8	38.7	28.2	82	17.9	87.5	84.4
34**	20.9	1.0	5.1	83*	27.8		
35	21	16.2	26.9	84	19.2	85.3	99.5
36	21.2	45.9	57.7	85	18.5	92.0	85.5
37	19.1	29.7	22.0	86	18.3	37.4	99.5
38**	20.8	0.0	0.0	87	18.3	84.8	90.0
39	21.1	99.6	92.2	89	19.4	73.9	86.9
40	20.7	84.1	93.9	90	19.3	85.3	93.5
41	20	35.5	61.9	91	19.5	69.1	73.8
42	20.1	64.4	30.8	92	19.3	76.4	99.7
43	19.6	63.4	61.3	93	18.6	83.6	65.7
44	20.5	80.6	88.3	95	20	91.2	96.2
45	20.1	11.7	10.5	97*	27.5		
46	20.4	92.0	70.7	104	19.4	98.0	95.7
49	19.8	81.2	68.7	105**	19.3	100.0	100.0
50	20.7	89.4	75.5	106	18	62.5	82.6
51	19.6	96.7	100.0	107	18.2	11.0	73.6
52	20.6	91.6	74.1	108	17.8	56.7	73.4
53	20.3	85.3	93.5	109	19.6	61.0	99.7
54	19.8	92.7	71.7	110**	19.5	0.1	34.7
55	21.2	90.4	3.0	112**	19.3	0.0	0.5
58	20	99.5	94.6	113	19.2	30.4	73.0
59**	20.6	2.9	2.9	114	19.4	97.1	81.0
60*	29.9			115	19.1	45.2	82.9
61	19.7	11.7	3.7	116	19.5	83.0	89.8
62	20.2	7.2	8.0	117	19.4	95.7	98.9
63	19.3	8.5	11.2	118	18.2	74.4	99.2
96	19.6	100.0	97.3	119	19.3	51.9	98.4

Set 1				Set 2			
Sample	**Mean Ct-value**	**Confidence % (1st run)**	**Confidence % (2nd run)**	**Sample**	**Mean Ct-value**	**Confidence % (1st run)**	**Confidence % (2nd run)**
98*	25.1			138	19	97.9	98.3
99	19.9	98.5	98.6	155	19	24.7	99.2
100**	19.2	3.1	8.8	156	18.1	99.9	98.0
101	19.6	58.4	27.9	157	18.8	58.9	90.1
102*	28.3			158	17.7	88.1	99.9
103	20.7	22.9	21.1	159	17.8	69.7	66.5
127	19.4	96.1	30.3	160	19.4	97.0	89.6
128	20.9	26.8	7.4	161*	32.2		
129	20.9	98.5	98.1	162	18.7	25.1	68.1
130	19.2	41.0	40.9	163	18.8	99.6	99.4
131	20.7	99.4	85.2	164	18.7	9.0	68.9
133	20.9	10.8	6.4	165	18.5	56.2	98.9
177	20.6	14.5	15.1	166*	29.7		
178	20.9	35.1	24.6	167	20	43.4	98.1
179	21	54.8	18.0	170**	18	23.0	39.0
180	19.7	12.9	6.8	171	18.7	18.8	65.9
181	19.5	77.3	35.7	174	19.9	76.6	94.1
182	20.7	34.5	38.2	175	18.4	14.4	89.7
183**	21	2.8	0.2	176	19.5	18.6	99.8

** Outlier; ** Selected for sequencing*

Table 18: Screening results of section 1 with 20 samples

Results of the first screening section are shown in Table 16. The Ct-values ranged from 18.6 to 20.5 with an average Ct-value of 19.5 and a standard deviation of 0.6 (CV=3.3%).

Sample	Ct-value	Confidence %
55	20.3	98.8
56	20.2	99.5
59	20.3	97.7
61	19.0	99.1
63	18.7	99.8
98	20.5	96.8
99	19.4	99.3
100	19.2	96.5
101	19.4	99.9
103**	20.2	93.3
127	18.8	99.8
128	20.1	95.0
129	20.1	95.7
130	18.9	99.2
132**	19.1	100.0
133	19.9	99.9
137	18.8	99.4
139	20.0	96.8
140	18.6	98.9
141	19.4	99.5

*** Selected for sequencing*

Table 19: Screening results of section 2

Results of the second screening section are given in Table 17. The Ct values ranged from 20.5 to 22.6 with an average Ct-value of 21.6 and a standard deviation of 0.8 (CV=3.9%).

Sample	Ct-value	Confidence %
55	22.3	97.1
56	22.3	99.2
59	22.1	99.4
61	20.9	99.9
63	20.6	100.0
98	22.6	98.7
99	20.8	97.6
100	20.9	99.7
103**	22.4	88.1
127**	20.9	100.0
128	20.8	99.0
129	23.5	98.0
130	21.2	99.2
132	22.2	99.6
133	22.3	93.5
137	20.5	95.6
139**	21.6	89.5
140	21.1	91.4
141	22.0	91.8

*** Selected for sequencing*

Table 20: Screening results of section 3

Results of third screening section are shown in Table 18. The Ct values ranged from 18.7 to 20.8 (two outliers excluded) with an average Ct-value of 19.7 and a standard deviation of 0.7 (CV = 3.8%).

Sample	Ct-value	Confidence %
55	20.7	99.9
56*	27.6	-
59	20.8	99.2
61*	25.5	-
63	19.2	98.1
98	20.7	99.9
99	19.1	98.2
100	19.2	97.5
101	19.3	99.6
103**	20.6	83.6
127	19.0	99.9
128	19.3	100.0
129	20.4	99.2
130	19.2	99.8
132	19.0	95.3
133	20.3	91.6
137	18.7	99.6
139	20.1	99.9
140**	19.0	100.0
141	20.5	94.8

** Outlier; ** Selected for sequencing*

Table 21: Screening results of section 4

Results of the fourth screening section are shown in Table 19. The Ct values ranged from 19.3 to 21.2 with an average Ct value of 20.3 and a standard deviation of 0.7 (CV = 3.3%).

Samples	Ct-value	Confidence %
55	21	83.1
56	21	93.5
59**	20.8	100
61	20.1	97.7
63	19.4	89.5
98	21.2	87.9
99	19.8	91.7
100	19.8	76.7
101	20.1	79.1
103**	21.1	49.1
127	19.7	99.7
128	21.1	97.4
129	20.5	95
130	19.8	97.5
132	19.3	96.7
133	20.7	95.7
137	19.3	99.5
139**	20.7	61.7
140	19.5	95.3
141	20.9	77.3

*** Selected for sequencing*

Table 22: Screening results of section 5

Results of the fifth screening are shown in Table 20. The Ct values ranged from 21.2 to 24.6 with an average Ct value of 22.0 and a standard deviation of 0.9 (CV = 3.9%).

Samples	Ct-value	Confidence %
55	22.8	98.5
56**	22.8	100
59	22.6	99.9
61	21.4	99.3
63	21.3	100
98**	24.6	88.8
99	21.6	99
100	21.5	94.9
101	21.4	99.2
103	22.7	94.1
127	21.2	99.4
128	21.4	99
129	22.5	99.2
130	21.6	96.1
132	21.3	98
133	22.3	99.9
137	21.3	96.5
139	22.4	97.5
140	21.4	90.8
141	22.8	96.3

*** Selected for sequencing*

Table 23: Screening results of section 7

Results of the sixth screening section are shown in Table 21. The Ct-values ranged from 22.9 to 30.1 with an average Ct value of 25.7 and a standard deviation of 1.7 (CV = 6.4%).

Samples	Ct-value	Confidence %
55**	26.5	100
56	29.1	96
59	26	99.3
61	25.1	99.8
63	25.1	98
98	26.7	99.1
99	24.5	99.9
100	24.3	99.6
101	25.1	98.4
103	26.4	99.7
127	24.6	99.7
128	26.1	98.4
129	26	99.5
130**	24.4	97.7
132	26.9	99.6
133	25.6	99.7
139**	24.7	89.4
140	24.6	99.4
141	30.1	98.7
142	22.9	97.4

*** Selected for sequencing*

Table 24: Screening results of section 7

Results of the seventh screening section are shown in Table 22. The Ct values ranged from 19.5 to 22.0 with an average Ct value of 20.8 and a standard deviation of 0.7 (CV = 3.4%).

Samples	Ct-value	Confidence %
55	21.3	99.8
56	22	98.9
59	21.4	99.9
61**	20.5	100
63	20.5	98.7
98	21.9	97.2
99	20.3	99.9
100	20.4	92.1
101	20.5	98.2
103**	21.3	77.5
127	19.8	98.5
128	21.1	93.4
129	21.1	100
130	20.3	98.5
132	19.8	96.9
133	21	94.6
137	19.5	99.6
139	20.9	91
140	20.3	99.7
141	21.8	96.5

*** Selected for sequencing*

Table 25: Screening results of section 8

Results of the eighth screening section are shown in Table 23. The Ct values ranged from 18.6 to 20.6 with an average Ct value of 19.5 and a standard deviation of 0.6 (CV = 3.3%).

Samples	Ct-value	Confidence %
55	19.9	85.1
56	20.2	91.2
59	19.9	81.3
61	19.0	91.2
63	18.6	90.6
98 **	19.7	74.6
99	19.3	91.5
100	18.8	77.9
101	19.1	90.9
103	19.9	77.9
127	19.1	99.3
128	19.8	98.5
129	19.8	99.9
130	18.7	99.8
132	19.6	99.8
133**	20.5	100.0
137	18.8	99.1
139	20.0	90.2
140	20.6	97.0
141	18.6	96.0

*** Selected for sequencing*

Table 26: Screening results of section 9

Results of ninth screening section are shown in Table 24. The Ct values ranged from 19.5 to 21.7 with an average Ct value of 20.4 and a standard deviation of 0.7 (CV = 3.2%).

Samples	Ct-value	Confidence %
55	20.9	99.3
56**	20.9	100
59	20.9	99.1
61	20.1	95.3
63	19.8	98.9
98	21.1	99.7
99	19.9	97.4
100**	19.7	73.5
101	19.9	95.9
103	20.9	87.6
127	19.6	99.3
128	21.1	97.3
129	20.5	96.1
130	19.6	98.9
132	19.5	94
133	20.6	98.7
137	19.5	89.6
139	20.9	74.6
140	20.3	90.5
141	21.7	74.9

*** Selected for sequencing*

Table 27: Screening results of section 10

Results of the tenth screening section are shown in table 25. The Ct values ranged from 19.2 to 22.0 with an average Ct value of 20.8 and a standard deviation of 0.8 (CV = 3.6%).

Samples	Ct-value	Confidence %
55	21.9	95.1
56**	22	81.5
59	21.7	93.2
61	20.5	99.7
63	20.6	99.6
98	21.8	99.7
99	20.6	97.7
100	20.4	85.1
101**	20.5	83.3
103	21.6	89.4
127	20.3	94.8
128	21.5	99.7
129**	21.3	100
130	20.1	94.7
132	20.3	96.6
133	21.4	99.6
137	20.2	98.3
138	20.1	93.7
140	20.8	86.6
142**	19.2	55

*** Selected for sequencing*

Table 28: Screening results of section 11

Results of the eleventh screening section are shown in table 26. The Ct values ranged from 18.6 to 21.0 with an average Ct value of 19.6 and a standard deviation of 0.7 (CV = 3.3%).

Samples	Ct-value	Confidence %
55	20	99.8
56**	20.3	100
59	20	97.6
61	19	96.3
63	19	99.8
98	20.3	97.8
99	19	97.6
100**	19	89.1
101	19.1	93.1
103	20	90.9
127**	19.1	86.9
128	20.2	99.3
129	19.9	99.5
130	18.9	99.8
132	18.8	97.6
133	19.7	99.1
137	18.6	88.9
139	20	87.4
140	19.4	96.6
141**	21	81.4

*** Selected for sequencing*

Table 29: Screening results of section 12

Results of the twelfth screening section are shown in Table 27. The Ct values ranged from 18.6 to 20.4 with an average Ct value of 19.4 and a standard deviation of 0.7 (CV = 3.4%).

Samples	Ct-value	Confidence %
55	19.9	99.3
56	20.4	98.6
59	20	94.8
61	19.1	91.4
63	18.9	100
98	20.4	98.5
99	18.9	99.5
100	18.9	99.3
101	19	97.9
103	20.2	87.7
127	18.7	97.9
128	20.3	99.9
129	19.7	99.3
130	18.7	99
132**	18.6	100
133	19.7	97.3
137	18.6	96.1
139**	19.9	79.8
140	18.7	98.4
141	19.9	89.7

*** Selected for sequencing*

Table 30: Screening results of section 13

Results of the 13th screening section are shown in Table 27. The Ct-values ranged from 17.9 to 20.4 with an average Ct value of 19.4 and a standard deviation of 0.7 (CV = 3.6%).

Samples	Ct-value	Confidence %
55	20.2	90.6
56**	20.4	62.1
59	20.1	92.9
61	19	99.4
63	19	93
98	20	99.5
99	19.1	99.7
100	18.9	80.6
101	19.1	90.9
103	20.3	99.7
127**	18.8	99.8
128	20.3	94.6
129	20	95.8
130	18.8	83.9
132**	18.8	100
133	19.7	99.4
137	18.7	91.2
138	19.7	98.3
140	19.1	89.6
142**	17.9	65.8

*** Selected for sequencing*

Table 31: Screening results of section 14

Results of the 14th screening section are shown in Table 29. The Ct values ranged from 19.2 to 21.8 with an average Ct value of 20.3 and a standard deviation of 0.7 (CV = 3.7%).

Samples	Ct-value	Confidence %
55	21.1	99.3
56	20.8	98.8
59	20.9	99.9
61	19.9	99.9
63	19.8	99.7
98	21.1	98.7
99	20	98.5
100	19.7	92.9
101	19.9	98.9
103	20.9	95.8
127	19.2	99.9
128	21	97.6
129	20.7	99.6
130	19.4	99.9
132**	19.3	100
133	20.7	97.2
137	19.4	95.7
139**	20.9	86.6
140	20	98.2
141**	21.8	86.5

*** Selected for sequencing*

Table 32: Screening results of section 15

Results of the 15th screening section are shown in Table 30. The Ct-values ranged from 18.6 to 21.3 with an average Ct-value of 19.6 and a standard deviation of 0.7 (CV = 3.5%).

Sample	Ct-value	Confidence %
55	20.3	99.8
56	20.5	91.1
59	20.2	99.9
61	19.1	80.9
63	18.9	94.0
98	19.8	89.9
99	19.1	99.9
100**	19.0	69.4
101	19.3	92.5
103	20.2	94.8
127	19.0	96.4
128	19.7	90.5
129	20.0	95.1
130	18.9	72.6
132	18.9	93.5
133**	19.9	100.0
137	18.6	94.3
139	20.1	70.0
140	19.6	70.0
141	21.3	77.2

***Selected for sequencing*

Table 33: Screening results of section 16

Results of the 16th screening section are shown in Table 31. The Ct-values ranged from 20.1 to 22.4 with an average Ct-value of 21.3 and a standard deviation of 0.7 (CV = 3.2%).

Samples	Ct-value	Confidence %
55	22	90.3
56	21.9	94.7
59	21.7	67.5
61	20.8	71.3
63**	20.5	58.5
98	22.2	95.9
99	20.9	97.9
100	20.8	64.1
101	21.1	83.4
103	22	94.5
127	20.5	97.5
128	22.4	97.5
129	21.8	94.1
130**	21.2	3.7
132	20.4	99.1
133**	21.5	100
137	20.1	74.2
139	21.6	75.3
140	20.4	99.1
141	21.7	76

*** Selected for sequencing*

Table 34: Screening results of section 17

Results of the 17th screening section are shown in Table 32. The Ct-values ranged from 19.0 to 22.3 with an average Ct-value of 20.1 and a standard deviation of 0.8 (CV = 4.2%).

Sample	Ct-value	Confidence %
55	20.7	77.5
56	20.8	86.4
59	20.5	99.4
61**	19.5	100.0
63	19.5	92.3
98	21.1	74.3
99**	19.6	50.8
100**	19.4	27.7
101	19.6	70.8
103	20.5	64.3
127	19.3	84.3
128	20.8	70.2
129	20.5	74.6
130	19.4	55.4
132	19.4	89.7
133	20.2	64.0
137	19.0	82.1
139	20.6	99.6
140	19.3	49.5
141	22.3	59.9

*** Selected for sequencing*

Table 35: Screening results of section 18

Results of the 18th screening section are shown in Table 33. The Ct-values ranged from 19.5 to 21.8 with an average Ct-value of 20.5 and a standard deviation of 0.6 (CV = 2.8%).

Samples	Ct-value	Confidence %
55	20.3	99.4
56	20.3	98.5
59	20.2	99.8
61	20.4	95.5
63	20.3	95.3
98	21.4	97.5
99	20.4	97.4
100	20	99.9
101**	20.4	100
103	20.7	97.6
127	19.5	99.6
128	21.5	98.4
129	20.7	99.8
130	19.7	99.9
132	20.2	100
133	20.6	98.6
137**	19.9	94.7
139	20.6	96.5
140	20.4	97.3
141	21.8	95.1

*** Selected for sequencing*

Table 36: Screening results of section 19

Results of the 19th screening section are shown in Table 34. The Ct-values ranged from 20.4 to 23.3 with an average Ct-value of 21.4 and a standard deviation of 0.7 (CV = 3.0%).

Sample	Ct-value	Confidence %
55	21.3	97.5
56	21.2	95.9
59	21.1	99.4
61	21.2	99.4
63	21.2	93.5
98**	22.2	69.8
99	21.2	88.5
100	20.8	92.4
101	21.4	87.7
103	21.8	88.3
127	20.5	99.9
128	22.2	92.8
129	21.5	98.8
130	20.4	79.0
132**	21.0	100.0
133	21.6	93.0
137	20.7	98.9
139	21.6	75.7
140	21.2	97.3
141	23.3	79.6

*** Selected for sequencing*

Table 37: Screening results of section 20

Results of the 20th screening section are shown in Table 35. The Ct-values ranged from 19.3 to 21.3 with an average Ct-value of 20.2 and a standard deviation of 0.7 (CV = 3.3%).

Samples	Ct-value	Confidence %
55	20.8	91.3
56	21	88.4
59**	20.8	100
61	19.8	98.7
63	19.3	99.8
98	21	92.7
99	19.9	93.5
100	19.7	72.1
101	20	87.4
103	20.9	54.6
127	19.6	91.7
128	21.3	91.9
129	20.6	99.5
130	19.7	82.6
132	19.5	97.4
133	20.5	96.6
137	19.3	98.5
139	20.5	67
140	19.5	95.6
141**	20.9	49.1

*** Selected for sequencing*

Table 38: Screening results of section 21

Results of 21th screening section are shown in Table 36. The Ct-values ranged from 18.7 to 20.8 with an average Ct-value of 19.6 and a standard deviation of 0.7 (CV = 3.7%).

Samples	Ct-value	Confidence %
55	20.2	98.6
56	20.3	99.7
59	20.2	99.9
61	19.3	99.9
63	19	98.3
98	20.5	99.8
99**	19.3	100
100	18.8	96.1
101	19.3	99.5
103	20.2	95.2
127	18.8	95.5
128	20.3	97.8
129	20.2	93.7
130	18.8	96.9
132	18.8	92.3
133	20	94
137	18.7	86.3
139**	20.2	79.6
140	18.8	93.5
141	20.8	80.7

*** Selected for sequencing*

Table 39: Screening results of section 22

Results of the 22th screening section are shown in table 37. The Ct-values ranged from 19.6 to 21.9 with an average Ct-value of 20.6 and a standard deviation of 0.7 (CV = 3.4%).

Samples	Ct-value	Confidence %
55	21.3	99.5
56	21.4	98.9
59	21.2	99.8
61	20.2	99.8
63	19.9	98
98**	21.4	99.6
99	20.1	100
100	19.9	94.5
101	20.2	99.5
103	21.2	97.7
127	19.8	99.8
128	20.9	98.8
129	20.8	99.2
130	19.7	99.2
132	19.7	99.9
133	20.7	98.2
137**	19.6	99.5
139	21	97.4
140	20.2	99.4
141	21.9	94.9

*** Selected for sequencing*

Table 40: Screening results of the 3´ border region (section 23)

Results of the 3´ border region screening (section 23) are shown in Table 38. Samples of set 1 (samples 1 to 183) and set 2 (samples 5 to 176) were analyzed separately (in two different runs which were repeated on a different day).

Set 1				Set 2			
Sample	**Mean Ct-value**	**Confidence % (1.Run)**	**Confidence % (2. Run)**	**Sample**	**Mean Ct-value**	**Confidence % (1.Run)**	**Confidence % (2. Run)**
1	21.1	76.5	89.3	5	21.1	94.6	93.4
3	21.1	85.5	99.5	9	22.7	86.4	95.5
4	20.8	80.9	96.4	10*	30.4		
6	20.9	94.8	98.1	15	22.4	99.9	99.6
7*	28.8			20*	30.5		
8	20.8	93.6	96.3	22*	29.9		
11	23.2	45.1	40.6	57	22.4	96.2	98.9
12	22.1	94.0	97.7	64	22.6	99.9	98.3
13	21	86.5	89.9	65	23.1	83.5	95.3
14	22.2	87.2	91.5	67	22.5	99.9	97.4
16	20.9	89.1	92.1	68	21.3	92.1	99.6
17	21.2	87.5	90.4	69**	21.9	84.5	84.8
18	20.8	81.4	89.7	70	22.1	99.9	95.7
19	20.8	91.0	89.9	71	22.4	98.6	98.0
21	22.1	95.9	99.9	72**	22.9	1.4	3.5
23	22.6	45.9	52.5	73	23	99.9	99.3
24	22.1	56.9	48.0	74	21.1	98.4	98.5
25	22.1	99.5	98.5	75*	27.5		
26	22	69.6	65.7	77	22	93.5	99.6
27	20.8	98.9	79.4	78	23.7	97.8	93.6
29	23.3	22.1	32.0	79	22.1	73.6	95.1
31	20.8	84.5	64.7	80	21.9	96.8	99.6
32	21.2	99.8	89.6	81	21.4	97.1	94.7
33	22.4	20.8	19.5	82	20.9	99.4	99.6

Set 1				Set 2			
Sample	Mean Ct-value	Confidence % (1.Run)	Confidence % (2. Run)	Sample	Mean Ct-value	Confidence % (1.Run)	Confidence % (2. Run)
34	22.4	20.7	11.3	83*	29.5		
35	22.3	58.2	84.6	84	21.9	96.9	99.8
36	22.3	79.7	93.8	85	21.8	94.8	95.0
37	20.9	65.7	59.2	86	21.4	99.7	99.8
38**	22.4	0.0	0.0	87	21.7	89.6	93.1
39	22.6	88.9	76.3	89	22.7	86.4	90.7
40	21.6	97.0	99.7	90	22.9	93.7	98.5
41	21	46.2	55.8	91	23.6	90.0	96.8
42	22.1	68.9	53.5	92	22.4	99.6	99.5
43	21	90.7	78.4	93	21.3	100.0	100.0
44	22.1	81.2	85.3	95	22.6	95.3	98.7
45	21.8	42.6	33.9	97	24.1	95.7	99.6
46	22.3	98.9	89.5	104	22.6	99.1	100.0
49	21.1	85.6	82.6	105	22.8	99.3	99.6
50	22.2	96.5	99.9	106	21	99.7	87.8
51	21	92.1	99.8	107	20.9	93.2	95.8
52	22	93.5	99.0	108	21	98.6	95.4
53	21.4	79.2	54.9	109	23.1	99.9	99.5
54	21	98.6	99.5	110**	22	14.6	-
55	22.6	32.6	43.9	112**	22.4	11.0	33.8
58	21	87.2	84.9	113	22.3	81.6	97.7
59	22.4	20.5	31.3	114	22.6	89.0	91.3
60*	28.3			115	22.3	92.3	99.4
61	22.5	25.7	30.1	116	22.7	99.3	99.7
62	22	43.1	63.1	117	22.4	99.4	98.3
63	21.3	35.8	53.3	118	21.6	99.5	99.1
96	21.1	100.0	100.0	119	22.7	98.4	98.8
98*	28.5			138	22.4	99.5	99.6
99	21.2	88.7	87.7	155	22.2	99.7	100.0
100**	21	8.4	10.8	156	21	87.8	92.3
101	21.2	51.1	53.6	157	22.5	88.6	94.5
102*	28.9			158	21.2	98.8	97.5
103	22.7	48.7	37.1	159	21.2	94.7	89.1
127	21.3	43.2	41.0	160	22.3	97.3	94.9

Set 1				Set 2			
Sample	**Mean Ct-value**	**Confidence % (1.Run)**	**Confidence % (2. Run)**	**Sample**	**Mean Ct-value**	**Confidence % (1.Run)**	**Confidence % (2. Run)**
128	22.4	22.6	17.6	161*	32		
129	23.2	95.1	98.2	162	21.3	99.8	98.1
130	21.3	48.9	28.9	163	22.6	77.5	99.0
131	22.4	92.0	99.3	164	22.6	99.2	89.3
133	23.1	35.6	28.1	165	22.1	86.2	96.5
177	22.3	55.9	26.3	166*	31.1		
178	22.5	22.4	21.9	167	22.7	97.6	99.5
179	22.6	26.6	16.2	170	21	86.5	89.8
180**	21.1	10.1	8.2	171**	21.2	79.4	83.1
181	21.1	35.8	30.5	174	22.6	96.0	95.9
182**	22.5	16.6	6.7	175	21.5	98.8	99.8
183**	22.5	0.2	0.4	176	22.7	98.4	99.5

** Outlier; ** Selected for sequencing*

Table 41: Screening results of section 23 with 20 samples

Results of the 23th screening section are shown in table 39. The Ct-values ranged from 21.0 to 23.0 with an average Ct-value of 21.8 and a standard deviation of 0.6 (CV = 2.9%).

Sample	Ct-value	Confidence %
55	22.6	95.3
56	22.7	96.4
59	22.5	96.0
61	21.4	92.3
63	21.1	96.7
98	23.0	89.8
99	21.4	94.6
100	21.4	82.3
101	21.5	95.9
103**	22.3	79.3
127	21.4	97.2
128	21.5	99.9
129	22.6	99.6
130	21.1	100.0
132**	21.3	100.0
133	22.3	99.4
137	21.0	97.4
139	22.1	91.3
140	21.4	96.4
141	22.6	93.5

*** Selected for sequencing*

Table 42: Screening results of section 24

Results of the 24th screening section are shown in Table 40. The Ct-values ranged from 16.9 to 19.0 with an average Ct-value of 17.6 and a standard deviation of 0.5 (CV = 3.1%).

Sample	Ct-value	Confidence %
55	18.1	98.6
56	17.8	99.7
59	17.2	98.0
61**	19.0	86.5
63**	17.7	100.0
98	17.7	90.4
99	17.2	99.6
100	17.4	97.9
101	17.5	98.7
103	16.9	92.8
127	17.2	96.3
128	17.2	99.0
129	17.4	97.3
130	18.2	99.9
132	18.7	98.1
133	17.9	97.2
137	17.5	92.0
139	17.5	87.9
140	17.3	91.5
141	17.1	92.8

*** Selected for sequencing*

Table 43: Screening results of section 25

Results of the 25th screening section are shown in Table 41. The Ct-values ranged from 19.4 to 21.2 with an average Ct value of 20.3 and a standard deviation of 0.6 (CV = 3.0%).

Samples	Ct-value	Confidence %
55	21.2	96.0
56	21.0	100.0
59	21.1	89.1
61	20.1	99.8
63	19.6	99.9
98	21.2	98.8
99	20.0	99.7
100	20.0	96.6
101	20.1	100.0
103**	21.2	84.8
127	19.8	98.0
128	20.3	97.4
129	20.8	98.3
130	19.7	99.5
132**	19.7	100.0
133	20.8	99.9
137	19.4	97.0
139	20.7	89.7
140	19.8	93.1
141	20.5	94.9

*** Selected for sequencing*